One-Dimensional and Two-Dimensional ^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR) Analysis of Vitamin E Raw Materials or Analytical Reference Standards

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Two-dimensional spectral analysis (COSY, HETCOR) was utilized to make the complete ^{13}C- and ^{1}H-NMR assignments for α-, β-, γ-, and δ-tocopherol as well as for the acetate and succinate esters of α-tocopherol. ^{13}C-NMR was found to be especially useful in distinguishing between the various tocopherols and distinguishing between the *d*-isomer and the *d,l*-racemic mixture. HETCOR spectra were also found to be useful for the qualitative identification of mixtures of the tocopherols and sesame oil. Using a procedure designed to minimize errors arising from spin relaxation and nuclear Overhauser effects, ^{13}C-NMR peak integrals were used to quantitate α-tocopherol and δ-tocopherol in the presence of sesame oil using benzoic acid as the standard for calibration of the quantitation. The NMR results were compared to a capillary column gas chromatographic analysis of the individual α-tocopherol and δ-tocopherol reference materials.

KEY WORDS: α-tocopherol determination; nuclear Overhauser effect; spin lattice relaxation; heteronuclear; gated decoupling; capillary gas chromatographic analysis; primary standards; vegetable oil; sesame oil; stereoisomers.

INTRODUCTION

Vitamin E USP consists of either *d*-,α-tocopherol or *d,l*-α-tocopherol as the free phenol, the acetate ester, or the succinate ester (1). The pure compounds are viscous liquids at room temperature and they have been prepared by synthesis or by isolation from plant sources (e.g., wheat germ oil, sunflower oil, and similar oils). The articles of commerce may also contain small amounts of β-, γ-, or δ-tocopherol as well as organic solvents or vegetable oils. The USP assay of either pure vitamin E or vitamin E preparations is a gas chromatographic analysis in which the method is calibrated by a reference sample supplied by the USP organization (1). In this procedure a column packed with a 2–5% dimethylpolysiloxane liquid phase, operated isothermally (245–265°C), gives very good resolution of α-tocopherol from the other tocopherols, but β-tocopherol and γ-tocopherol may be poorly resolved from each other. Newer capillary column gas chromatographic methods have been shown to give baseline resolution of α-, β-, γ-, and δ-tocopherol (2). A high-performance liquid chromatographic method for the analysis of tocopherols in vegetable oils and fats has been standardized (IUPAC method 2.432), and multisite, collaborative studies have shown that this method gives good precision and the chromatograms show baseline resolution of α-, β-, γ-, and δ-tocopherol (Fig. 1) (3).

While both the gas chromatographic and the liquid chromatographic methods are generally satisfactory, they both require an authentic reference standard of known purity with which to calibrate the assay. The major objective of the present study was to determine the utility of ^{1}H- and ^{13}C-NMR methods in establishing the identity and purity of tocopherol samples that might ultimately be used as "reference standards" for the chromatographic methods.

MATERIALS AND METHODS

d,l-α-Tocopherol (labeled as approx. 95%), *d*-γ-tocopherol, *d*-δ-tocopherol (labeled as approx. 90%), *d*-α-tocopherol acetate, and *d*-α-tocopherol succinate were obtained from the Sigma Chemical Company (St. Louis, MO). A sample of *d*-β-tocopherol (labeled as containing 1.3% α, 96.9% β, 2.9% γ, and 0% δ isomers) was obtained as a gift from the Henkel Corporation (La Grange, IL). The reference standard of benzoic acid (catalog No. 24,238-1, Aldrich Chemical Co., Milwaukee, WI) and the sample of sesame oil (catalog No. O-154, Fisher Scientific Co., Springfield, NJ) were used as received.

All NMR spectra were obtained using a Varian VXR-300 spectrometer using a 5-mm switchable, broadband probe (300 MHz for ^{1}H and 75 MHz for ^{13}C). All spectra were obtained in deuterochloroform (total sample volume, ≅0.6 ml) at ambient temperature (≅24°C in the probe). ^{1}H-NMR spectra were obtained using a spectral width of 2213 Hz, an acquisition time of 3.99 sec, a relaxation delay of 2.0 sec (unless otherwise specified), and a pulse angle of 45°. ^{13}C-NMR spectra were obtained using a spectral width of 16,500 Hz, an acquisition time of 0.9 sec, a relaxation delay of 3.0 sec (unless otherwise specified), either continuous (for qualitative) or gated (for quantitative analysis) waltz-modulated proton decoupling, and a pulse angle of 45°. Two-dimensional proton–proton correlated spectra (COSY) and proton–carbon heteronuclear correlated spectra (HETCOR) were acquired with essentially the same parameters, with 256 increments in the second frequency domain for COSY and 256 increments (64 increments for narrowed spectra of only aliphatics) in the second frequency domain for HETCOR spectra.

In the quantitative analysis of the tocopherols by ^{1}H-NMR, quantities of the reference standard of benzoic acid (typically 13 mg) and the tocopherols (typically 30–35 mg) were weighed directly in a small test tube, then the final volume was brought to approximately 0.6 ml with deuterochloroform. The proton spectra were obtained using the parameters as described above and an acquisition delay of 3.0 sec was used in the standard assay procedure (the acquisition delay was varied from 0 to 10 sec to study the effect of this parameter). The area of one of the tocopherol peaks (H-4, H-5, or H-7 as appropriate) was measured by electronic integration in triplicate and the integrals of the *ortho*

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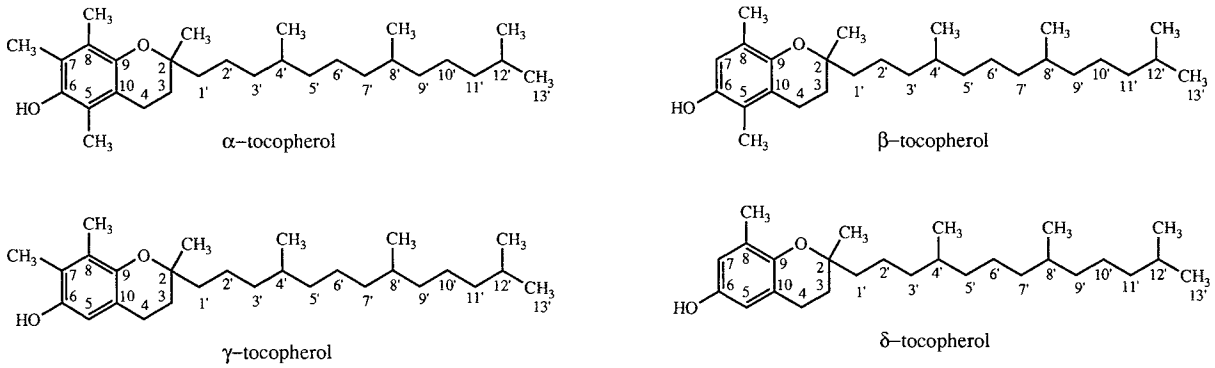

Fig. 1. Structure of α-, β-, γ-, and δ-tocopherol and numbering used for spectral assignments.

protons of benzoic acid (8.12 ppm) were also measured in triplicate. The apparent milligrams of tocopherol (T) was then calculated using Eq. (1) from the milligrams of benzoic acid standard (B), in integrals of the tocopherol peak (I_T), the integral of the *ortho* protons of benzoic acid (I_B), the molecular weights of the two compounds, and the number of protons for the tocopherol group being integrated (N_T = 1 for H-5 and H-7, N_T = 2 for H-4).

$$T = B * \frac{I_T}{I_B} * \frac{MW\ T}{MW\ B} * \frac{2}{N_T} \quad (1)$$

In the quantitative analysis of the tocopherols by ^{13}C-NMR, a known quantity of the reference standard of benzoic acid and the tocopherol were prepared in the same manner as for the proton NMR assay. The gated decoupled carbon spectrum of the sample was obtained using the acquisition parameters as described above and an acquisition delay of 12 sec. The areas of the signals were measured in triplicate by electronic integration (C-5, C-7, C-8, or C-10 for the tocopherols; C-4 of benzoic acid at δ = 133.7). The apparent milligrams of tocopherol present (T) was calculated using Eq. (2).

$$T = B * \frac{I_T}{I_B} * \frac{\text{mol. wt. T}}{\text{mol. wt. B}} \quad (2)$$

For the gas chromatographic analysis of the composition of the tocopherols, a 2-mg/ml sample of each specific tocopherol was prepared in carbon tetrachloride. A 4-μl portion was injected (1:20 split) onto a 15-m, DB-1 bonded phase fused quartz capillary column (J & W Scientific, Folsom, CA) that was operated at 4.0 lb/in.^{2} at an initial temperature of 80°C. Immediately after the injection, the column temperature was programmed at 16°C/min to a final temperature of 260°C and it was observed that the four tocopherols eluted after the column had reached the end of the program. After the solvent front, the integrals of the flame ionization detector peaks for the α-, β-, γ-, and δ-tocopherols and other unknown contaminates were electronically measured. The four tocopherols gave baseline resolution of each peak (typical retentions times: α = 17.6 min, β = 14.5 min, γ = 14.8 min, δ = 11.8 min). The purity of the individual tocopherol was expressed as a percentage of integrals for all peaks (the three other tocopherols and the minor unknown components) exclusive of the solvent front.

RESULTS AND DISCUSSION

Though the ^{13}C-NMR chemical shifts of the side-chain carbons were closely grouped, all of the carbons were well resolved. The chemical shift assignments (Table I) were made from the proton decoupled spectra, the attached proton spectra (APT), proton–proton correlated spectra (COSY), and proton–carbon correlated spectra (HETCOR) for α-, β-, γ-, and δ-tocopherol and the two esters of α-tocopherol. The assignments for α-tocopherol have been made by Brownstein *et al.* (4) and Matsuo *et al.* (5,6), but the assignments made by these two groups were not completely in agreement with each other. Brownstein and co-workers' studies had focused primarily on the side-chain carbons and our assignments (Table I) are in agreement, but our assignments are not in agreement with their aromatic carbon assignments. The studies by Matsuo *et al.* had focused primarily on the assignments of the aromatic carbons using europium chemical shift reagents and our assignments for the aromatic carbons are in agreement. While the chemical shifts of the two oxygenated carbons (C-6, C-9) should be the two most downfield signals, Brownstein had incorrectly assigned C-10 and C-9 as the most downfield peaks. Of the closely spaced peaks in the 122- to 117-ppm region, Matsuo *et al.* appeared to have assigned them correctly in the sequence of C-8, C-7, C-5, and C-10, while Brownstein *et al.* incorrectly assigned them to C-7, C-8, C-6, and C-5. To help resolve this difference in assignments by Brownstein *et al.* and Matsuo *et al.*, the 2-D long-range (J = 5 Hz) HETCOR spectrum of α-tocopherol was obtained and it was found to show a ^{3}J cross peak between C-9 and H-4 and ^{3}J cross peaks between C-6 and its two flanking methyl groups. Thus both these long-range three-bond coupling observations and simple chemical shift theory would be consistent with the assignments of Matsuo *et al.* and the values shown in Table I. In the standard HETCOR spectra of β-tocopherol a ^{1}J cross peak was observed between H-7 and C-7, and in the long-range HETCOR (J = 10 Hz) the anticipated ^{3}J coupling between H-7 and both C-9 and C-5 was observed. As an additional test of the self-consistency of the aromatic carbon assignments, it was observed (Table I) that the addition of a methyl group to β-, δ-, or γ-tocopherol produced a shift of ~+6.0 ppm in the carbon to which it was attached and a ~−3.0-ppm shift in the carbon *para* to the point of attachment [generalized literature values = +9.3 and −2.9 for the methyl-group substituent effect (10)].

Table I. ^{13}C-NMR Chemical Shift Assignments for the Tocopherols[a]

Position	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	α-Acetate	α-Succinate
C-2	74.5	74.5	75.5	75.6	75.0	75.1
C-3	31.5	31.4	31.4	31.4	~31.1 Br[b]	31.1 Br
C-4	20.8	20.8	22.3	22.5	20.6	20.6
C-5	118.5	119.2	112.1	112.6	124.9	124.9
C-6	144.5	145.7	146.2	147.7	140.5	140.5
C-7	121.0	115.3	121.6	115.7	126.6	126.7
C-8	122.6	124.0	125.8	127.3	123.0	123.1
C-9	145.6	146.0	145.8	146.1	149.4	149.5
C-10	117.3	120.3	118.3	121.3	117.3	117.4
C-1′	39.8	39.7	40.1	40.0	40.1 Br	40.1 Br
C-2′	21.1	21.0	21.0	21.0	21.0	21.0
C-3′	37.6[a]	37.5[a]	37.5[a]	~37.5	~37.5	~37.5
C-4′	32.7	32.7	32.7	32.7	32.7	32.7
C-5′	37.4[a]	37.4[a]	37.3[a]	~37.5	~37.5	~37.5
C-6′	24.5	24.5	24.5	24.5	24.5	24.5
C-7′	37.3	37.3	37.3	37.3	37.3	37.3
C-8′	32.8	32.8	32.8	32.8	32.8	32.8
C-9′	37.5[a]	37.5[a]	37.4[a]	~37.5	~37.5	~37.5
C-10′	24.8	24.8	24.8	24.8	24.8	24.8
C-11′	39.4	39.4	39.4	39.4	39.4	39.4
C-12′	28.0	28.0	28.0	28.0	28.0	28.0
C-13′	22.6[c]	22.6[c]	22.6[c]	22.6[c]	22.6[c]	22.6[c]
C2–CH_3	23.8	23.8	24.1	24.1	24.0 Br	24.0 Br
C5–CH_3	11.3	11.0	—	—	11.8	11.8
C7–CH_3	12.2	—	11.9	—	12.9	12.9
C8–CH_3	11.8	15.8	11.9	16.0	12.1	12.0
C4′–CH_3	~19.7	19.7[b]	19.7[b]	19.7[b]	19.7[b]	19.7[b]
C8′–CH_3	~19.7	19.8[b]	19.8[b]	19.8[b]	19.8[b]	19.8[b]
C12′–CH_3	22.7[c]	22.7[c]	22.7[c]	22.7[c]	22.7[c]	22.7[c]
$\underline{CH_3}$–COO	—	—	—	—	20.5	—
$CH_3\underline{C}OO$	—	—	—	—	169.7	—
$\underline{CH_2}$–COO	—	—	—	—	—	29.0, 28.6
CH_2–$\underline{C}OO$	—	—	—	—	—	170.8, 178.1

[a] Superscripts a, b, and c denote interchangeable chemical shift assignments.
[b] Broad linewidth.

While the assignments of the ^{13}C-NMR signals of the side-chain carbons made by Matsuo and Urano (5) were not in error, they were incomplete. The side-chain assignments made by Brownstein *et al.* (4) were complete and they are consistent with the APT, COSY, and HETCOR spectra obtained in these laboratories. In general there were very few differences observed for the side chain of the α-, β-, γ-, and δ-tocopherol series, but there were some unusual aspects of the acetate and succinate ester of α-tocopherol. In the latter two compounds, the signal for C-1′ was broadened to the point that it was barely detectable and the signals for C-3 and C2-CH_3 were also broadened (Table I). All three of these groups were bonded to the same atom (C-2) and the line broadening that was observed could possibly be the result of slow equilibrium of two conformations in solution.

Vitamin E and the other tocopherols are frequently utilized as either the *d*-isomer or the *d,l*-racemic mixture. The present studies of the tocopherols show little difference in the ^{1}H-NMR spectra of the *d*-isomer and the *d,l*-racemic mixture. In contrast, the ^{13}C-NMR spectra of *d*-isomer and the *d,l*-racemic mixture were fairly easy to distinguish. As previously reported (4), *d,l*-α-tocopherol is a mixture of four diastereoisomers that can have essentially identical aromatic carbon chemical shifts, but many of the individual side-chain carbons will appear to be a complex of four spectral lines. In our studies with *d,l*-α-tocopherol, this difference between the two materials was observed in many of the side-chain spectral lines, but the effect was most easily distinguished using carbons 1′, the (3′,5′,9′)-complex, 3,4′-CH_3, and 8′-CH_3 as markers.

The ^{1}H-NMR spectral assignments of only a very few protons of α-tocopherol have appeared in the literature (7–9). Even at 300 MHz, most of the side-chain multiplets overlap badly and singlets for the aromatic protons have very similar chemical shifts. For *d*-δ-tocopherol the doublet for H-7 (6.47 ppm) and the doublet for H-5 (6.37 ppm) were easily recognized and the corresponding COSY spectra showed coupling of H-7 with H-5 and with its "*ortho*" methyl group at C-8 (2.11 ppm). In addition to being coupled with H-7, H-5 also showed a small coupling with its *ortho* alkyl-group protons at C-4 (2.67 ppm). The COSY spectrum also clearly showed the coupling between H-4 (2.67 ppm) and H-3 (1.8 ppm). In a similar manner, using the 1-D ^{1}H-NMR and 2-D COSY spectra, the protons of the two rings of each tocopherol were assigned (Table II).

The ^{1}H-NMR signals of many of the side-chain methine

Table II. ^{1}H-NMR Chemical Shift Assignments for the Tocopherols[a]

Position	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	α-Acetate	α-Succinate
C3–H's	~1.8[b]	~1.8	~1.8	~1.8	~1.8	~1.8
C4–H's	2.60	2.60	2.67	2.67	2.59	2.58
C5–H	—	—	6.37	6.37	—	—
C7–H	—	6.46	—	6.47	—	—
C1′–H's	~1.5	~1.5	~1.5	~1.5	~1.5	~1.5
C2′–H's	~1.3	~1.3	~1.3	~1.3	~1.3	~1.3
C3′–H's	~1.4	~1.4	~1.4	~1.4	~1.4	~1.4
C4′–H	~1.4	~1.4	~1.4	~1.4	~1.4	~1.4
C5′–H's	~1.4	~1.4	~1.4	~1.4	~1.4	~1.4
C6′–H's	~1.3	~1.3	~1.3	~1.3	~1.3	~1.3
C7′–H's	~1.1	~1.1	~1.1	~1.1	~1.1	~1.1
C8′–H	~1.4	~1.4	~1.4	~1.4	~1.4	~1.4
C9′–H's	~1.4	~1.4	~1.4	~1.4	~1.4	~1.4
C10′–H's	~1.3	~1.3	~1.3	~1.3	~1.3	~1.3
C11′–H's	~1.2	~1.2	~1.2	~1.2	~1.2	~1.2
C12′–H	~1.5	~1.5	~1.5	~1.5	~1.5	~1.5
C2–CH_3	1.22	1.22	1.24	1.24	1.23	1.23
C5–CH_3	2.11	2.08	—	—	1.98[c]	1.96[c]
C7–CH_3	2.15	—	2.13	—	2.02[c]	2.01[c]
C8–CH_3	2.11	2.09	2.11	2.11	2.09[c]	2.08[c]
C4′–CH_3	0.84[a]	0.84[a]	0.84[a]	0.84[a]	0.84[a]	0.84[a]
C8′–CH_3	0.83[a]	0.83[a]	0.83[a]	0.83[a]	0.83[a]	0.83[a]
C12′–CH_3	0.88[b]	0.88[b]	0.88[b]	0.88[b]	0.88[b]	0.88[b]
C13′–H's	0.85[b]	0.85[b]	0.85[b]	0.85[b]	0.85[b]	0.85[b]
C6–OH	4.18	4.35	4.25	4.51	—	—
$\underline{CH_2}$–COO	—	—	—	—	—	2.8, 2.9
$\underline{CH_3}$–COO	—	—	—	—	2.32	—

[a] Superscripts a, b, and c denote interchangeable chemical shift assignments.
[b] ~ denotes approximate proton chemical shifts obtained from the HETCOR experiments.

and methylene protons could not be determined from the normal 1-D spectra. However, the approximate chemical shifts of the methine and methylene protons could be estimated from the HETCOR spectra (values denoted with the "~" symbol in Table II).

^{13}C-NMR is not frequently used for quantitative analysis because some carbon signals may be much larger than expected because of the nuclear Overhauser effect and some carbon signals may be much smaller than expected because of spin lattice relaxation time effects. A major objective of this study was directed toward the minimization of these two sources of error for the analysis of the tocopherols. Most ^{13}C-NMR spectra are obtained by continually irradiating the proton resonances while observing the Fourier transform free induction decay signals of the carbon nuclei. Under these conditions each carbon nuclei appears as a single line, which greatly simplifies the spectra for doing quantitations. However, the nuclear Overhauser effect causes the integral of the carbons attached to a proton to be much larger than the integral of carbons that do not have an attached proton. One approach to decoupling the protons from the carbon signals, while minimizing the nuclear Overhauser effect, is to use "gated proton decoupling experiments," where the proton transmitter is turned on only when the carbon free induction decay is being observed (Fig. 2). If the proton transmitter is on only during the acquisition time (0.9 sec in this study) and is turned off the remainder of the time (referred to as the "acquisition delay" in the following graphs), the nuclear Overhauser effect will be reduced.

In addition to having an impact on the nuclear Overhauser effect, the acquisition delay parameter will also have an effect on the extent of the relaxation of the spin system. If these spin relaxation effects predominate (i.e., for carbons with no protons), the peak intensity will grow larger as the acquisition delay increases (as seen for C-8 of δ-tocopherol in Fig. 3). If the nuclear Overhauser effect predominates (i.e., carbons with protons attached), the peak intensity will decrease as the acquisition delay increases (as seen for C-5 and C-7 in Fig. 3). For α-tocopherol, the C-5, C-8, and C-10 carbons do not have attached protons, therefore the integral intensity increases as the acquisition delay increases (Fig. 5).

For the quantitative analysis of tocopherols, the method was based on a comparison of the carbon integrals of the individual tocopherols to the integrals of a known quantity of benzoic acid as the reference standard. After adjusting the

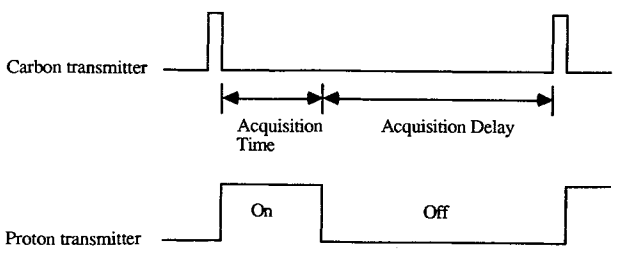

Fig. 2. Gated decoupling used to reduce the nuclear Overhauser effect while obtaining proton decoupled ^{13}C-NMR spectra.

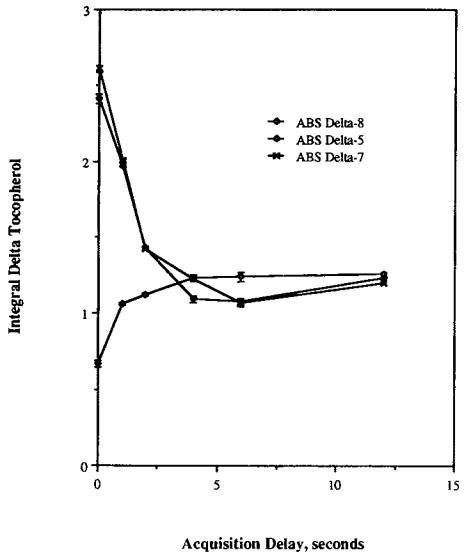

Fig. 3. ^{13}C-NMR integrals of δ-tocopherol in the mixture of α-tocopherol, δ-tocopherol, benzoic acid, and sesame oil in deuterochloroform. Integrals are in absolute intensity units.

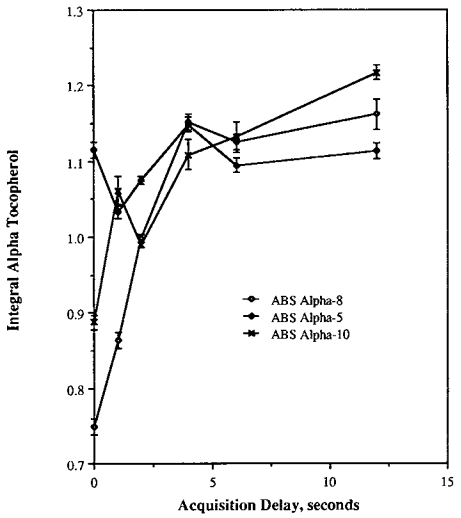

Fig. 5. ^{13}C-NMR integrals of α-tocopherol in the mixture of α-tocopherol, δ-tocopherol, benzoic acid, and sesame oil in deuterochloroform. Integrals are in absolute intensity units.

relative tocopherol/benzoic acid integrals for differences in their molecular weights, one can then calculate the apparent amount of tocopherol that was present [*via* Eq. (2)]. This procedure was used for δ-tocopherol (Fig. 4) and for α-tocopherol (Fig. 6), and it was found that calculated values at short acquisition delays were too high for carbons with attached protons and too low for carbons with no attached proton. If an acquisition delay of at least 6 sec was used, very good results were obtained.

The effect of the variation of the acquisition delay parameter on the ^{1}H-integrals was found to be much less than its effect on ^{13}C-integrals. As the acquisition delay parameter was increased, the peak integrals for H-4, H-5, and H-7 for δ-tocopherol increased as theory would predict, but the effect was very small (Fig. 7). If the total pulse cycle time (acquisition delay + data acquisition time) was equal to or smaller than the spin lattice relaxation time of the ^{1}H nuclei, the peak integrals would be very small because of saturation effects, then the integrals would become larger as the acquisition delay was increased. In the present study, the data acquisition time was held constant at 3.99 sec, therefore the total pulse cycle time used in Fig. 7 ranged from 3.99 to 13.99 sec. Thus, it would appear that the spin lattice relaxation times for H-4, H-5, and H-7 were approximately 1.0 sec or less. When the peak integrals of δ-tocopherol and benzoic acid were used to calculate the amount of tocopherol present

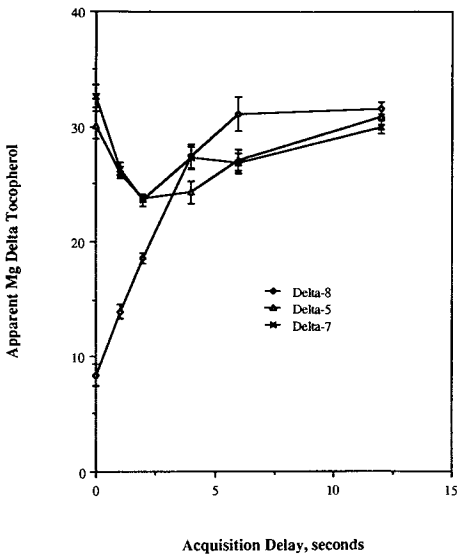

Fig. 4. Apparent milligrams of δ-tocopherol present based on carbon integrals in Fig. 3. The sample contained 33.0 mg α-tocopherol, 35.1 mg δ-tocopherol, 47.9 mg sesame oil, and 13.5 mg benzoic acid dissolved in deuterochloroform.

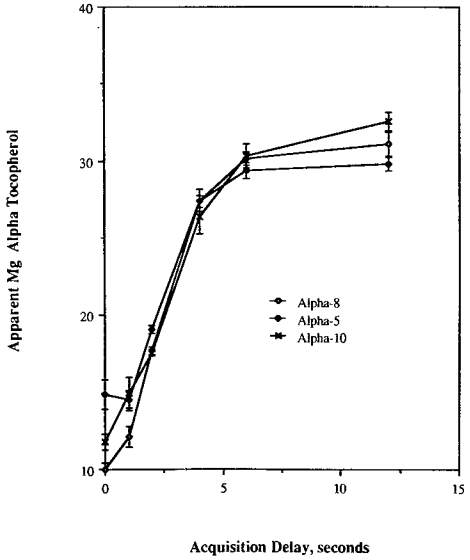

Fig. 6. Apparent milligrams of α-tocopherol present based on carbon integrals in Fig. 5. The sample contained 33.0 mg α-tocopherol, 35.1 mg δ-tocopherol, 47.9 mg sesame oil, and 13.5 mg benzoic acid dissolved in deuterochloroform.

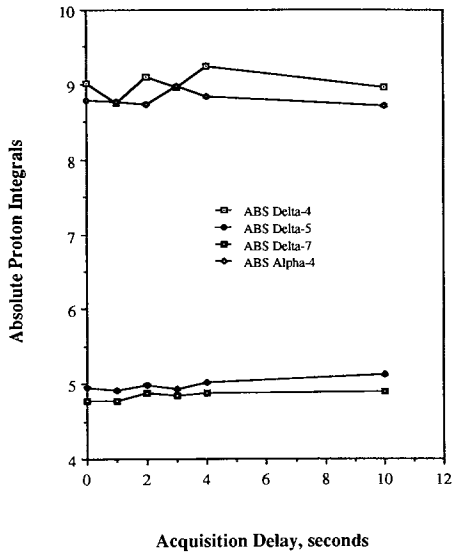

Fig. 7. ^{1}H-NMR integrals of α- and δ-tocopherol in the mixture of α-tocopherol, δ-tocopherol, benzoic acid, and sesame oil in deuterochloroform. Integrals are in absolute intensity units.

[*via* Eq. (1)], the apparent amount of tocopherol present (Fig. 8) appears higher at very short acquisition delays, perhaps because of some spin saturation of the benzoic acid, which would be expected to have a longer relaxation time than the tocopherols.

The use of ^{1}H-NMR for the quantitative analysis of α-tocopherol in the presence of other tocopherols and vegetable oils was more difficult because the spectrum of α-tocopherol does not provide an aromatic proton and its aromatic methyl groups overlap the methyl groups of the other tocopherols. Nonetheless, the triplet for H-4 of α-tocopherol was resolved from the peaks of sesame oil and δ-tocopherol. It was also found that the intensities of the H-4

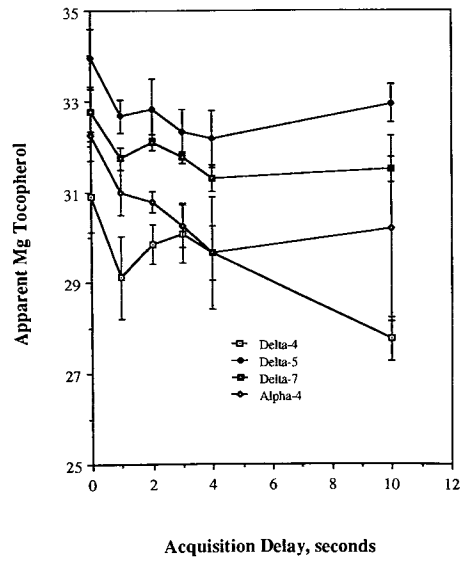

Fig. 8. Apparent milligrams of α- and δ-tocopherol present based on proton integrals in Fig. 7. The sample contained 33.0 mg α-tocopherol, 35.1 mg δ-tocopherol, 47.9 mg sesame oil, and 13.5 mg benzoic acid dissolved in deuterochloroform.

integrals were relatively unaffected by variations in the acquisition delay (Fig. 7). At very short acquisition delays, the apparent amount of α-tocopherol present (Fig. 8) calculated from the relative tocopherol/benzoic acid integrals was higher, perhaps because of some spin saturation of the benzoic acid.

With regard to the qualitative analysis of tocopherols, these materials are viscous oils and the "pure" commercial preparations may also contain other members of the tocopherol family and small amounts of vegetable oils if they were obtained from natural sources. While ^{1}H-NMR can identify the individual tocopherols in an impure sample to some extent, ^{13}C-NMR was found to be much more useful in this respect. In order to evaluate the ability of ^{1}H- and ^{13}C-NMR to identify and to quantitate these types of samples, 33.0 mg α-tocopherol, 35.1 mg δ-tocopherol, 47.9 mg sesame oil, and 13.5 mg benzoic acid were dissolved in deuterochloroform, then analyzed. The ^{1}H-NMR spectrum of this mixture gave sufficient resolution of the H-4, H-5, and H-7 peaks to allow for the identification and quantitative analysis (Table III) of the mixture, but the ^{13}C-NMR spectrum of the same sample gave more data with which to characterize uniquely each tocopherol (Fig. 9). In particular, the 110- to 150-ppm region of the spectrum afforded baseline resolution of 12 tocopherol peaks in this area and the C-2 signals in the 74- to 77-ppm region adjacent to the chloroform triplet were also well resolved. The signals in the 10- to 40-ppm area of the spectrum were much more difficult to assign uniquely because the side-chain carbons of the different tocopherols have very similar chemical shifts and the sesame oil itself has 20 peaks in the 10- to 40-ppm region. Though the 10- to 40-ppm region of the mixture might appear to be hopelessly complex, the 2-D HETCOR spectrum (Fig. 10) of this region of the same mixture allowed for some identifications to be made. The following cross peaks in the HETCOR spectrum could be used to differentiate α-tocopherol from δ-tocopherol: α C-3, δ C-3, α C-4, δ C-4, α C-5CH_3, α C-7CH_3, α C-8CH_3, and δ C-8CH_3.

The primary objective of this study was to develop NMR procedures to identify and to quantitate tocopherol "raw materials" or materials that would ultimately be used as standards for the calibration of chromatographic assays. As means of evaluating the ^{1}H- and ^{13}C-NMR quantitative methods, a sample containing approximately equal amounts of α-tocopherol, δ-tocopherol, and sesame oil was analyzed using the acquisition delay of 3.0 sec for proton spectra and 12.0 sec for carbon spectra. Using this procedure, the δ-tocopherol was found to have a purity of 87.7% by carbon analysis and 89.5% by proton analysis (Table III), but the difference between the two values was within the standard deviation. Though there were differences between the measurements *via* C-5, C-7, and C-8 for δ-tocopherol, the largest difference was very small (within 1.5 times the standard deviation) and it did not appear that there were any errors associated with residuals of the nuclear Overhauser effect.

For α-tocopherol, the purity was found to be 94.3% by carbon and 91.7% by proton analysis (Table III), but the difference between the two methods was smaller than the standard deviation of the carbon analysis. Though the results were satisfactory in the present situation, the use of ^{1}H-NMR alone for the assay of α-tocopherol in such mixtures

Table III. Comparison of the Various Analytical Methods in the Determination of the Apparent Purity of Tocopherols[a]

Analytical method	δ-Tocopherol		α-Tocopherol	
	mg	%	mg	%
C-5	30.83 ± 0.29	87.8 ± 0.8	29.76 ± 0.43	90.2 ± 1.3
C-7	29.98 ± 0.57	85.4 ± 1.6		
C-8	31.49 ± 0.58	89.7 ± 1.7	31.09 ± 0.78	94.2 ± 2.4
C-10			32.55 ± 0.57	98.6 ± 1.7
Avg.	30.77 ± 0.76[b]	87.7 ± 2.2	31.13 ± 1.40[b]	94.3 ± 4.2
H-4	30.10 ± 0.66	85.8 ± 1.9	30.26 ± 0.47[b]	91.7 ± 1.4
H-5	32.33 ± 0.48	92.1 ± 1.4		
H-7	31.77 ± 0.13	90.5 ± 0.4		
Avg.	31.40 ± 1.16[b]	89.5 ± 3.3		
GC		93.0 ± 0.9[c]		96.5 ± 0.6[d]

[a] Acquisition delay was 3.0 sec for proton and 12.0 sec for carbon NMR assays.
[b] The sample contained a mixture of 35.1 mg δ-tocopherol, 33.0 mg α-tocopherol, and 47.9 mg sesame oil.
[c] GC assay was on δ-tocopherol alone, but from the same source as the NMR assays.
[d] GC assay was on α-tocopherol alone, but from the same source as the NMR assays.

might be less reliable in general because one has only the H-4 signal for the basis of the quantitation.

Both the ^{1}H- and the ^{13}C-NMR quantitative results for δ-tocopherol and α-tocopherol gave slightly lower values than the GC analysis (Table III). The apparently higher purities obtained by GC analysis could possibly be attributed to the presence of low molecular weight organic solvent impurities in the tocopherols that merged with the solvent peak and would not have been included in the purity calculation. For the δ-tocopherol, the difference between the GC and the proton analysis was not statistically significant and the difference between the GC and the carbon analysis was very small (two times the carbon standard deviation). For α-tocopherol, the difference between the GC and the carbon method was not statistically significant.

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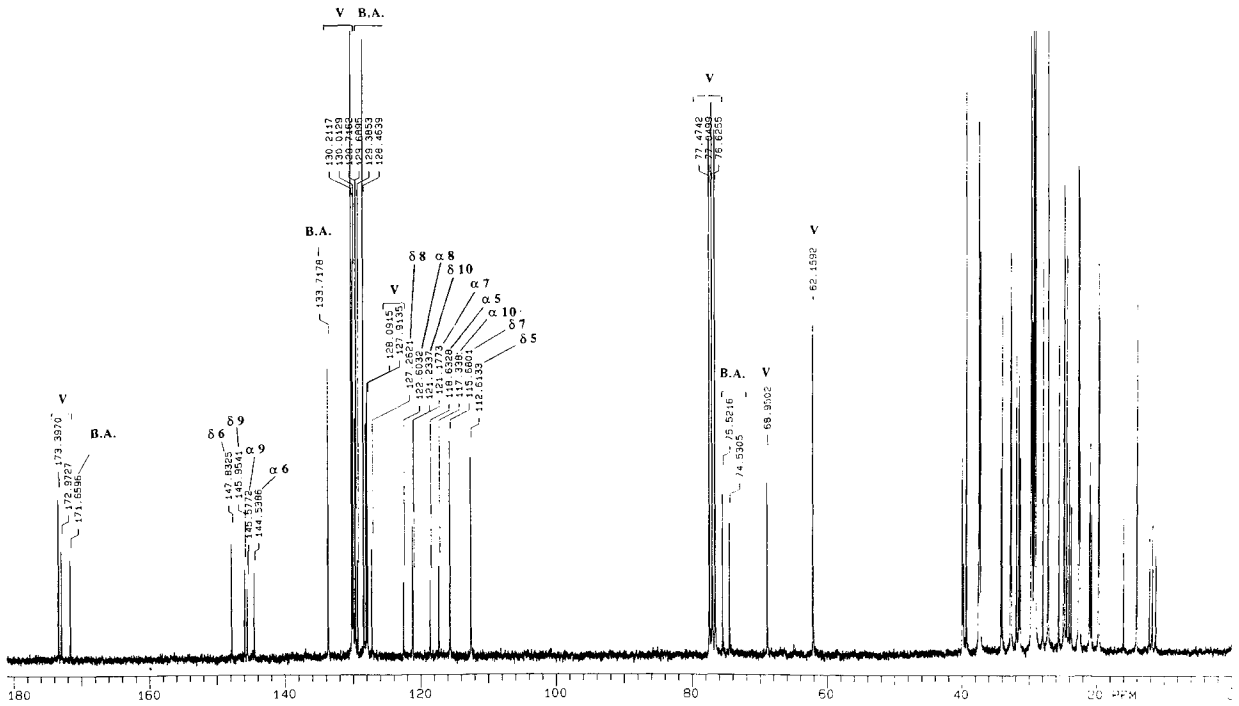

Fig. 9. ^{13}C-NMR spectrum of the mixture of α-tocopherol, δ-tocopherol, benzoic acid, and sesame oil in deuterochloroform. The tocopherol peaks that could easily be identified are labeled as either α-or δ-tocopherol, while unresolved peaks are labeled only with the hydrogen position. The sesame oil peaks are labeled V, and the benzoic acid peaks B.A.

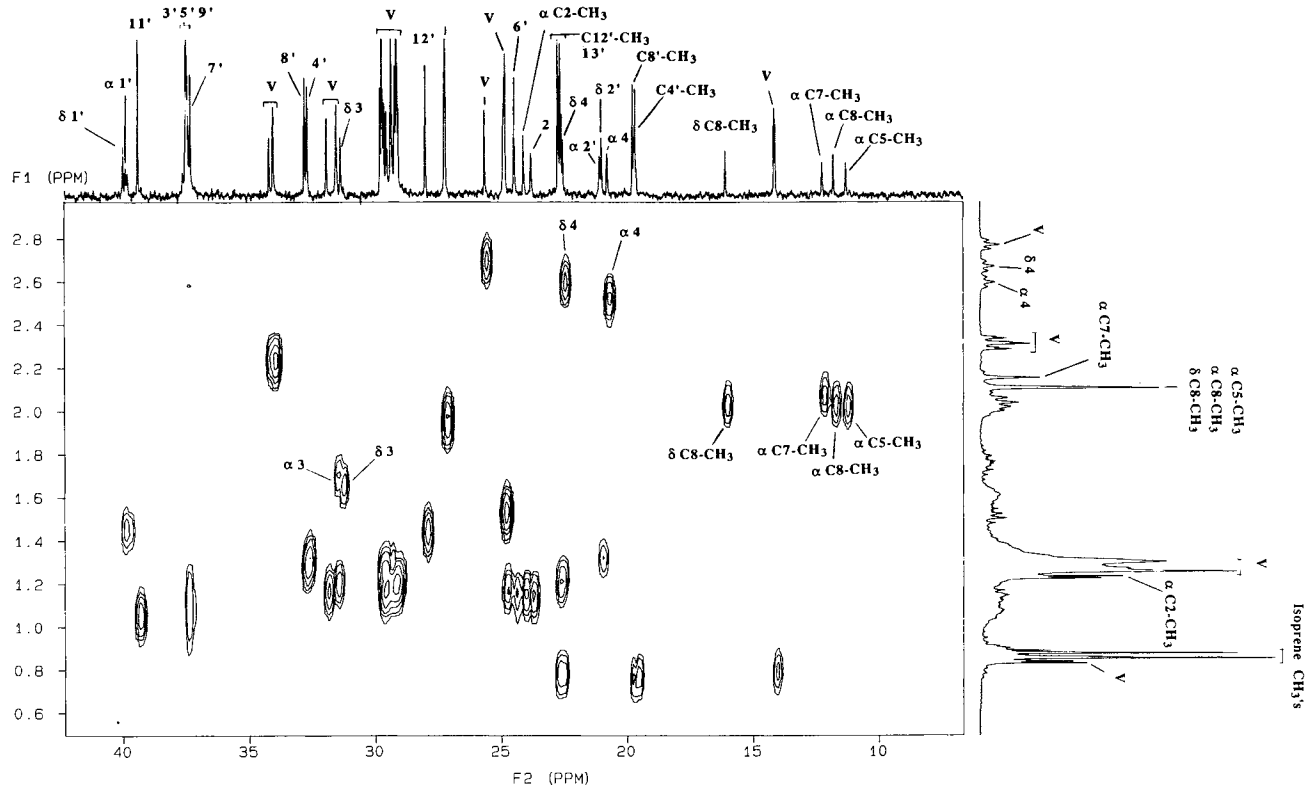

Fig. 10. HETCOR of the aliphatic region of the mixture of α-tocopherol, δ-tocopherol, and sesame oil in deuterochloroform. The sample contained 28.0 mg α-tocopherol, 26.0 mg δ-tocopherol, and 51.1 mg sesame oil in deuterochloroform. The tocopherol cross peaks that could easily be identified are labeled as either α- or δ-tocopherol, while unresolved peaks are labeled only with the position number on either the 1-D proton or the carbon spectra. The sesame oil peaks are labeled V.

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