Magic Angle Sample Spinning NMR Spectroscopy of Liquids as a Nondestructive Method for Studies of Plant Seeds

Venceslav Rutar

Since magic angle sample spinning eliminates line broadening arising from differences in magnetic susceptibility, it significantly improves NMR spectroscopy of liquids that are found in an inhomogeneous environment. The use of this technique facilitates nondestructive measurements of oil composition in viable plant seeds, studies of germination processes, and detection of dissolved carbohydrates in small animals.

Nuclear magnetic resonance (NMR) has found widespread applications in various fields of science. Since it is essentially *nondestructive*, studies of those samples that should be left intact appear especially tempting. This paper confirms that adoption of magic angle sample spinning (MASS) improves resolution in liquidlike spectra of plant seeds, thus making other versions of NMR obsolete. The technique will have practical significance as illustrated by nondestructive measurements of oil composition in single seeds. Subsequent plant-breeding decisions can be made on the basis of individual properties and MASS NMR will certainly become the method of choice in development of new varieties with better oil quality.

BACKGROUND

MASS utilizes fast mechanical rotation about an axis, making the magic angle (54°44′) with the external magnetic field. This technique successfully reduces the broadening arising from the chemical shift anisotropy or magnetic dipole–dipole interactions between nuclei and—in combination with heteronuclear decoupling and polarization transfer—it gives rise to high-resolution spectra of dilute spins (such as ^{13}C) in solids.

NMR of liquids is "easier", since internal molecular motions already eliminate effects of the chemical shift anisotropy and magnetic dipole–dipole interactions. For many samples the resolution is limited only by the inhomogenity of the external magnetic field; therefore, development of better instruments is continuously improving the quality of NMR spectra. On the other hand, there is an important exception: If the liquid is found in an inhomogeneous environment, differences of the magnetic susceptibility broaden resonances and mediocre resolution is achieved even with the best magnets. Plant seeds represent a typical example. They contain a large amount of oil (up to 50%) existing in a liquidlike form. Broad-line NMR provides a sufficient distinction between the liquid and solid part, so that intensities of ^1H signals quickly determine oil content (Bauman et al., 1963; Alexander et al., 1967; Zupančič et al., 1967). Since the measurements are nondestructive, they became indispensable for the development of new varieties. High-resolution proton NMR has been tried to characterize oil composition (Conway and Johnson, 1969), but individual differences in magnetic susceptibility give rise to severe line broadening and they seem to prevent widespread applications. The use of ^{13}C NMR appeared more promising, because 20 times larger chemical shift dispersion makes characteristic responses sufficiently resolved (Schaefer and Stejskal, 1974), but inherently low carbon sensitivity requires long measuring times that are not very attractive for large-scale plant-breeding programs.

These problems can be solved by MASS, which eliminates line broadening arising from differences in magnetic susceptibility (Chapman et al., 1972; Doskočilová et al., 1975; Van der Hart et al., 1981; Garroway, 1982). Since any reduction of the line width makes NMR resonances higher, better resolution and increased sensitivity are obtained simultaneously.

Department of Chemistry, Iowa State University, Ames, Iowa 50011.

Table I. Assignment and Relaxation Times (T_1) of ^1H Resonances in the MASS Spectrum of Liquidlike Oil in a Single Sunflower Seed

chem shift,[a] ppm	assignment	T_1,[b] s
0.92	$-CH_3$	1.02
1.33	bulk-CH_2-	0.46
1.61	$-COCH_2CH_2-$	0.37
2.07	$=CHCH_2CH_2$	0.47
2.27	$-COCH_2-$	0.39
2.78	$=CHCH_2CH=$	0.45
4.12, 4.31	CH_2OCO-	0.37, 0.35
5.30	$CHOCO-$	0.64
5.34	$-CH=$	0.83

[a] Measured from external TMS. Accuracy about ±0.05 ppm. [b] Accuracy about ±0.01 s.

RESULTS AND DISCUSSION

^1H is the most abundant spin in all biological materials, and NMR spectra are obtained within 1 min. Resonances of the solid part are generally too broad to be observed by Fourier transform spectroscopy with typical bandwidth of 5 kHz and delay 30 μs between the excitation pulse and signal acquisition. Figure 1a shows the MASS spectrum of a single sunflower seed as recorded by a spectrometer operating at proton frequency 300 MHz. Although the line width (about 15 Hz) is still 2 orders of magnitude larger than in spectra of extracted liquids or solutions, the results allow determination of chemical shifts for all characteristic peaks (Table I). Further digital apodization using Gaussian multiplication improved apparent resolution (Figure 2) and vicinal homonuclear couplings ($^3J_{HH}$) became resolved. The signal at 2.27 ppm represents protons attached to C-2, and they are coupled through three bonds to two nearest neighbors, thus giving rise to a typical triplet. The quartet at 2.07 ppm reveals couplings with three protons (see Table I), etc. The above conclusions merely confirm that ^1H MASS detected signals of oil as the only liquidlike component in sunflower seeds and any further discussion would repeat well-known facts about NMR.

Relaxation times T_1 were determined by the standard inversion–recovery technique, and they additionally illustrate the state of oil. It behaves as a liquid with relatively fast molecular motions contributing to spin–lattice relaxation under the "extreme narrowing" condition, and

0021-8561/89/1437-0067$01.50/0

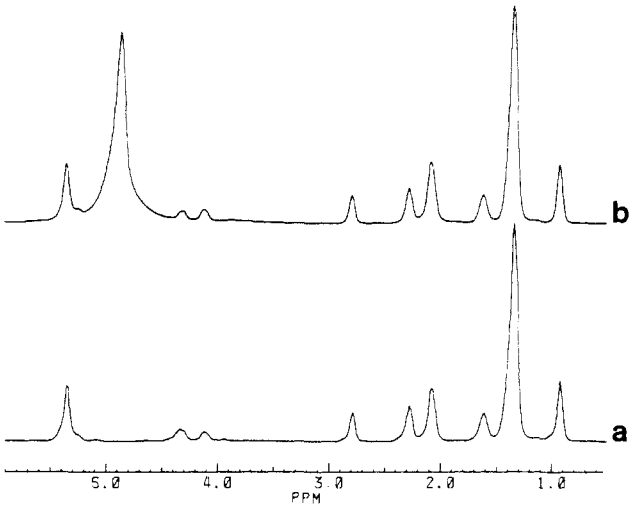

Figure 1. (a) Typical ^1H MASS spectrum of a single sunflower seed. All resonance peaks are assigned to liquidlike oil, and they facilitate nondestructive characterization of oil composition. The signal at 2.78 ppm determines the amount of linoleic fatty acid. (b) Spectrum of the same seed after soaking in water for 30 min.

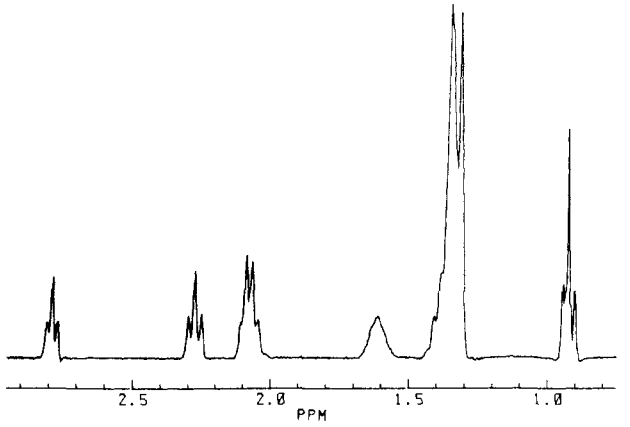

Figure 2. Part of the ^1H MASS spectrum of a single sunflower seed. Gaussian multiplication has been used to enhance resolution and to show coupling patterns arising from vicinal homonuclear couplings.

typical correlation times τ_c must be shorter than approximately 10^{-9} s. The values of T_1 that were measured separately on all resolved lines (see Table I) therefore reflect differences in proton–proton distances and segmental mobility. CH_2 groups have shortest relaxation times since proton pairs are close and their mutual magnetic dipole interaction provides an efficient relaxation mechanism. The central part of the molecule is more rigid, thus leading to values $T_1 < 0.4$ s, while segmental mobility increases along the fatty acid chain, thus contributing to less effective relaxation, and bulk CH_2 groups (resonating around 1.33 ppm) have $T_1 = 0.46$ s. The terminal methyl protons are rotating even faster, and their relaxation time is 1.02 s. On the other hand, CH protons are not relaxed as quickly, because they interact only with remote partners. Reported values 0.83 and 0.64 s again reflect the fact that –CH= groups in the chain undergo faster motion than the glyceride proton CHOCO in the central part of the molecule.

Although the results provide only "trivial" information, they demonstrate that adoption of MASS allows high-resolution spectroscopy *without* destroying the objects. This unique advantage should stimulate important further applications. Development of new plant varieties will certainly benefit from nondestructive measurements of oil composition that are possible by the following procedure.

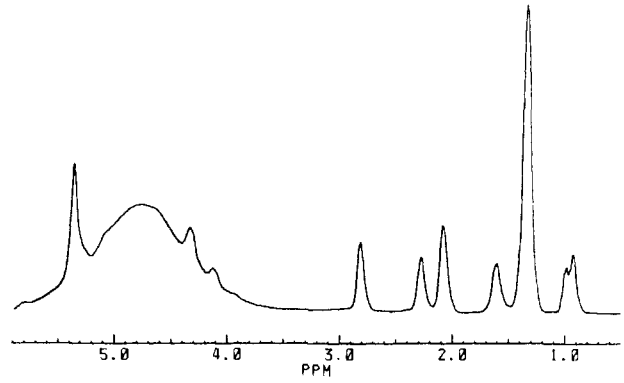

Figure 3. ^1H MASS spectrum of a single bean (variety black turtle) showing substantial amounts of adsorbed moisture giving rise to a broad resonance centered around 4.8 ppm. Oil contains linolenic acid contributing to characteristic peak at 0.98 ppm.

Sunflower oil is composed mostly of triglycerides of linoleic (18:2), oleic (18:1), and some saturated fatty acids. The peak at 2.78 ppm, which is clearly distinguished in MASS ^1H spectra (Figures 1 and 2), represents two protons attached to C-11 in the linoleic acid (=CHCH_2CH=). On the other hand, methyl groups terminate all fatty acid chains, and the integral of their resonance at 0.92 ppm is normalized to 300. The peak at 2.78 ppm arises from two protons found only in the linoleic acid, and this integral (i.e., 152 in Figure 1a) is divided by 2 to find the molar percentage of linoleic acid (76% in this particular seed). The signal around 5.34 ppm (see Table I) results from four olefinic protons in linoleic and from two olefinic protons in oleic acid, as well as from the C*H*OCO– response. When relative contributions are taken into account, oil in this seed is found to contain 11% oleic acid. Proton NMR is unable to determine accurately the length of the fatty acid chain, since most –CH_2– groups contribute to the peak at 1.33 ppm; therefore, detailed composition of saturated fatty acids (abundance 13%) is not measurable by this approach.

The same experimental technique has been applied to corn and peanut seeds. As long as linoleic and oleic are the only unsaturated fatty acids, measurements do not require any complicated procedures. The presence of the linolenic fatty acid (18:3) can be detected quite easily (Figure 3), since it contributes to the characteristic peak at 0.98 ppm. Quantitative determinations, however, would require deconvolution of overlapping methyl peaks and precise integration of other responses, thus hampering accuracy of final results. It was not attempted in this study.

Analytical application of ^1H NMR clearly has some drawbacks, and it may become unreliable for certain types of seeds. On the other hand, it must be stressed again that valuable genetic material is not destroyed by the measurement that appears ideal for plant-breeding programs.

MASS will also improve studies of water in biological systems. Traditional broad-line approaches detected all observable ^1H signals, and subsequent separation of responses relied mostly on differences in relaxation times (T_1, T_2, $T_{1\rho}$, etc.). Mixtures of several liquidlike components are better characterized, if chemical shifts unambiguously distinguish and assign all resonances. The water peak at about 4.8 ppm can be sharp (Figure 1b), broadened (Figure 3), or even unobservable (Figure 1a) depending on the amount and state of water.

Low natural abundance (1.1%) of the only magnetically active carbon isotope ^{13}C makes detection less attractive, since data accumulation times may become prohibitively long. Adoption of MASS gives rise to excellent resolution

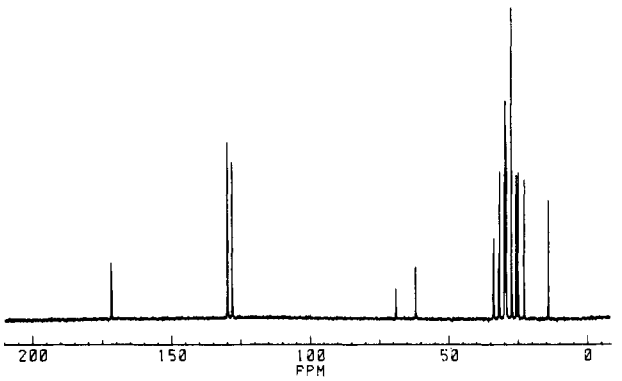

Figure 4. ^{13}C spectrum of a single sunflower seed as obtained within 15 min. Since magic angle sample spinning reduced inhomogeneous line broadening, the resonances show remarkable resolution and sensitivity.

Table II. Assignment of ^{13}C Resonances in the MASS Spectrum of Liquidlike Oil in a Single Sunflower Seed

chem shift,[a] ppm	assignment[b]
13.95	$(18)_{lo}$
22.61, 22.73	$(17)_{lo}$
24.83	$(3)_{lo}$
25.58	$(11)_{l}$
27.17	$(8, 14)_{l}$, $(8, 11)_{o}$
29.21–29.84	$(4\text{–}7, 15)_{l}$, $(4\text{–}7, 12\text{–}15)_{o}$
31.56	$(16)_{l}$
32.01	$(16)_{o}$
33.64, 33.81	$(2)_{lo}$
61.83	CH_2O–
68.96	CHO–
127.90, 127.97	$(10, 12)_{l}$
129.61, 129.69	$(9, 13)_{l}$, $(9, 10)_{o}$
171.40, 171.68	–OCO–

[a] Measured from external TMS. Accuracy about ±0.05 ppm. [b] Assignment given by Wenkert et al. (1976). Numbers denote carbon position. Abbreviations used: l = linoleic, o = oleic.

(Shoolery, 1985), and at the same time it significantly improves the signal to noise ratio. Both favorable effects are accomplished simultaneously; therefore, ^{13}C NMR will complement ^{1}H spectroscopy with two main advantages: (i) Larger chemical shift dispersion allows better assignment; (ii) Signals of water (if present) do not obscure other resonances.

Figure 4 shows a typical MASS ^{13}C spectrum of a single sunflower seed. Since oil is the only liquidlike component, assignment of peaks (Table II) utilizes characteristic chemical shifts (Wenkert et al., 1976). Double bonds have a pronounced effect shifting resonances of $-CH=$ groups to 128–131 ppm, and carbon spectroscopy unambiguously identifies unsaturated fatty acids. On the other hand, CH_2 groups more than three bonds from the methyl or carboxyl end in the saturated fatty acid chain give rise to the peak at 29.84 ppm; therefore, distinction between palmitic (16:0) and stearic (18:0) fatty acid becomes practically impossible in complex mixtures.

The amount of linoleic acid is measured by integrating exclusive contributions at 25.58, 31.56, 127.90, and 127.97 ppm (Table II). In the next step the integral of the peaks at 127.9 ppm is compared with the difference between the integrals at 129.6 and 127.9 ppm to obtain the ratio between the linoleic and oleic acid: $l/o = I(127.9)/[I(129.6) - I(127.9)]$. This particular seed was found to contain 75% linoleic, 15% oleic, and 10% saturated fatty acids. The presence of linolenic fatty acid (18:3) was not detected within the accuracy of this experiment (about 2%), because characteristic resonances at 20.47, 127.14, 127.75, and 131.47 ppm did not appear in the spectrum.

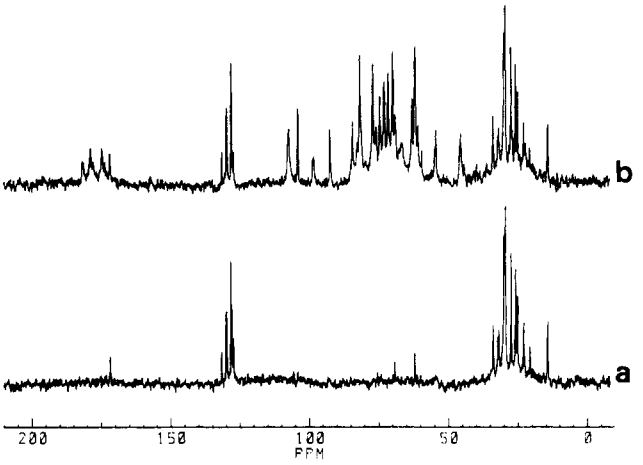

Figure 5. (a) MASS ^{13}C spectrum of a single bean showing oil as the only liquidlike component. (b) Spectrum after soaking in water, which starts germination process and makes signals of dissolved carbohydrates and proteins observable.

Accuracy of quantitative results obtained by ^{13}C NMR can be remarkably good, if the solution is doped with $Cr(acac)_3$ (Wehrli and Wirthlin, 1980). The paramagnetic additive quenches NOE, and it also makes relaxation times short; therefore, intensities of resonances exactly reflect composition of the sample. Since plant seeds should be left intact, a large spread of T_1 values ranging from 0.2 to 2.3 s (Rutar et al., 1977) and possible differences of NOE shed some doubt on the reliability of results. It is theoretically possible to get around this difficulty by using gated decoupling and long relaxation delays, but the unavoidable increase of the measuring time does not appear very tempting. If one really wants to use ^{13}C NMR as a nondestructive analytical method for plant-breeding purposes, the signal to noise ratio should be maximized by INEPT (Burum and Ernst, 1980) or DEPT (Pegg et al., 1982), while the influence of systematic errors is reduced in the selection process which *compares* compositions of different seeds measured under identical conditions.

Increased sensitivity and resolution of the MASS ^{13}C NMR facilitates better studies of seed germination already initiated by Colnago and Seidl (1983). Spectra of sunflower and corn seeds confirm the presence of soluble sucrose, which is activated immediately after soaking in water. On the other hand, a study of a bean (Figure 5) reveals a more complex germination process. The dry seed contains oil as the only liquidlike component, and Figure 5a shows its high-resolution spectrum. Various fatty acids (including linolenic) can be identified. Soaking in water for 2 h starts germination without affecting oil (Figure 5b), while substantial amounts of dissolved carbohydrates (mostly sucrose) become observable. In addition, some proteins or their fragments are also released into the solution, since the peaks between 174 and 182 ppm can arise only from carbonyl carbons in amino acids.

NMR spectroscopy of other living organisms (small animals, bacteria, etc.) also benefits from MASS. Suggested observable spins are mostly ^{1}H, ^{13}C, and ^{31}P in their natural abundance, but possible experiments may include injection of ^{15}N- or ^{19}F-labeled substances, which would serve as probes to follow processes in vivo.

EXPERIMENTAL NOTES

MASS requires rotation of irregularly shaped objects that may impose some mechanical problems. Frequencies up to 1 kHz appear sufficient for most liquidlike samples, because spinning must average out only broadenings ar-

ising from differences in magnetic susceptibility. Spinning side bands sometimes appear (especially in ^1H spectra), but they can be pushed out of the displayed spectral window by readjusting the rotation speed. Addition of proton-free powder (Al_2O_3, sulfur, etc.) is a helpful experimental hint for better balancing of the spinner and faster rotation.

The use of the high-field instrument is certainly helpful, if the signal to noise ratio must be increased; however, it appears superficial for determination of linoleic acid using ^1H NMR. Since the important peak at 2.78 ppm can be resolved also in lower magnetic fields, it is possible to develop cheaper instruments for large-scale routine measurements.

Registry No. l, 60-33-3; o, 112-80-1; linolenic acid, 463-40-1.

LITERATURE CITED

Alexander, D. E.; Silvela, L. S.; Collins, F. I.; Rodgers, R. C. Analysis of Oil Content of Maize by Wide-Line NMR. *J. Am. Oil Chem. Soc.* **1967**, *44*, 555–558.

Bauman, L. F.; Conway, T. F.; Watson, S. A. Heritability of Variations in Oil Content of Individual Corn Kernels. *Science (Washington, D.C.)* **1963**, *139*, 498–499.

Breitmaier, E.; Voelter, W. *^{13}C NMR Spectroscopy*; Verlag Chemie: Weinheim, New York, 1978.

Burum, D. P.; Ernst, R. R. Net Polarization Transfer via a J-Ordered State for Signal Enhancement of Low-Sensitivity Nuclei. *J. Magn. Reson.* **1980**, *39*, 163–168.

Chapman, D.; Oldfield, E.; Doskočilová, D.; Schneider, B. NMR of Gel and Liquid Crystalline Phospholipids Spinning at the Magic Angle. *FEBS Lett.* **1972**, *25*, 261–264.

Colnago, L. A.; Seidl, P. R. Application of Carbon-13 Nuclear Magnetic Resonance to the Germination of Soybean Seeds in Vivo. *J. Agric. Food Chem.* **1983**, *31*, 459–461.

Conway, T. F.; Johnson, L. F. Nuclear Magnetic Resonance Measurement of Oil "Unsaturation" in Single Viable Corn Kernels. *Science (Washington, D.C.)* **1969**, *164*, 827–828.

Doskočilová, D.; Tao, D. D.; Schneider, B. Effects of Macroscopic Spinning upon Linewidth of NMR Signals of Liquid in Magnetically Inhomogeneous Systems. *Czech. J. Phys.* **1975**, *B25*, 202–209.

Garroway, A. N. Magic-Angle Sample Spinning of Liquids. *J. Magn. Reson.* **1982**, *49*, 168–171.

Pegg, D. T.; Doddrell, D. M.; Bendall, M. R. Proton-Polarization Transfer Enhancement of a Heteronuclear Spin Multiplet with Preservation of Phase Coherency and Relative Component Intensities. *J. Chem. Phys.* **1982**, *77*, 2745–2756.

Rutar, V.; Burgar, M.; Blinc, R.; Ehrenberg, L. ^{13}C NMR Determination of the Oil Composition in Individual Plant Seeds. *J. Magn. Reson.* **1977**, *27*, 83–90.

Schaefer, J.; Stejskal, E. O. Carbon-13 Nuclear Magnetic Resonance Measurement of Oil Composition in Single Viable Soybeans. *J. Am. Oil Chem. Soc.* **1974**, *51*, 210–213.

Shoolery, J. N., private communications, 1985.

Van der Hart, D. L.; Earl, W. L.; Garroway, A. N. Resolution in ^{13}C NMR of Organic Solids Using High-Power Proton Decoupling and Magic-Angle Sample Spinning. *J. Magn. Reson.* **1981**, *44*, 361–401.

Wehrli, F. W.; Wirthlin, T. *Interpretation of Carbon-13 NMR Spectra*; Heyden & Son: London, Philadelphia, Rheine, 1980.

Wenkert, E.; Buckwalter, B. L.; Burfitt, I. R.; Gašić, M. J.; Gottlieb, H. E.; Hagaman, E. W.; Schell, F. M.; Wovkulich, P. M.; Zheleva, A. Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Naturally Occuring Substances. In *Topics in Carbon-13 NMR Spectroscopy*; Levy, G. C., Ed.; Wiley: New York, London, Sydney, Toronto, 1976.

Zupančič, I.; Vrščaj, S.; Porok, J.; Levstek, I.; Eržen, V.; Blinc, R.; Paušak, S.; Ehrenberg, L.; Dumanović, J. Transient NMR Selection Method in Plant Breeding. *Acta Chem. Scand.* **1967**, *21*, 1664–1665.

Received for review November 3, 1987. Revised manuscript received June 28, 1988. Accepted August 3, 1988.

Determination of Fenvalerate in Seawater and Sediment Utilizing Isotopic Dilution and GC/MS

Jerry R. Hale, Michael D. Walla, and Thomas A. Bryson*

Fenvalerate, a pyrethroid insecticide commonly applied to many South Carolina agricultural products, is a challenging subject for analytical determination. Exhibiting an exceptional tendency to adsorb onto many surfaces, this analyte resists reproducible trace analysis. This research demonstrates the advantages of using isotopic dilution in a gas chromatography/mass spectrometry (GC/MS) method. Quantitative determinations of fenvalerate in seawater and estuarine sediment represent practical applications of this procedure. Analysis of fenvalerate in seawater at 8 μg/L was performed with an RSD (n = 8) of 3.32%. Concurrently, the syntheses of two deuterium-labeled analogues of fenvalerate are presented.

The controversy encountered in the last 20 years regarding the use of DDT and other chlorinated insecticides has prompted a search for alternative methods of insect control (Carson, 1962; Sanders, 1975; Georghiou and Saito, 1983). There have been many innovative approaches including the use of insect hormones, naturally occurring insecticidal agents, audio disrupters, and electronic insect killers (Quraishi, 1977; Janes, 1985; Mandara, 1985). One class of natural insecticides was determined to be particularly attractive for agricultural development. Pyrethrins I and II (1, 2), first isolated from *Chrysanthemum cinerariaefolium* in 1924, were found to exhibit exceptional potency (Elliott, 1974). These compounds rapidly penetrate insect cuticle and immobilize the pest. This property coupled with efficient toxicity makes these natural products desirable models for a new breed of pesticide. Several synthetic derivatives have been constructed that introduce requisite commercial features such as low volatility, photostability, and even greater toxicity. Fenvalerate (3), a first-generation synthetic analogue, has been proposed for use in South Carolina as a substitute for the banned pesticide toxaphene.

A substantial amount of information regarding the environmental fate of fenvalerate has been reported (Shell, 1984; Reed et al., 1983). Photolytic degradation is the principal vehicle by which the pesticide is depleted after

Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208.

0021-8561/89/1437-0070$01.50/0