Assay of Cyclopropenoid Lipids by Nuclear Magnetic Resonance

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ABSTRACT

Nuclear magnetic resonance (NMR) method for the quantitative analysis of cyclopropenoid fatty acids (CPFA) in lipids is described. Good accuracy is obtained at CPFA concentrations of 1% to 100%. At a CPFA concentration of 10%, NMR is accurate to 0.5%. The position of absorption of the two ring methylene hydrogens is solvent dependent.

INTRODUCTION

The cyclopropene function occurs in the fatty acid chain of seed lipids from many plants of the order Malvales. Lipids possessing this small ring are held responsible for certain physiological disorders in farm and laboratory animals (1) and have demonstrated some co-carcinogenic properties (2). Three cyclopropenoid fatty acids, sterculic acid, $CH_3(CH_2)_7\overset{CH_2}{\diagdown\!\!\diagup}C{=}C(CH_2)_7CO_2H$, malvalic acid, $CH_3(CH_2)_7\overset{CH_2}{\diagdown\!\!\diagup}C{=}C(CH_2)_6CO_2H$, and sterculynic acid, $HC{\equiv}C(CH_2)_7\overset{CH_2}{\diagdown\!\!\diagup}C{=}C(CH_2)_6CO_2H$ (3,4), have been isolated from natural sources. A fourth acid has been suggested (5) and is believed to be C_{17} homolog of malvalic acid.

Quantitative analysis for cyclopropenoid lipids has long been a problem. Their methyl esters cannot be gas chromatographed as can other fatty acids because of instability on the column (6). To use gas liquid chromatography (GLC) for the analysis of cyclopropenoid fatty acids (CPFA), a thermally stable derivative of the cyclopropene ring must be formed. Schneider and coworkers (6) report a method involving reaction of transesterified lipids with silver nitrate in methanol to form ether and ketone derivatives which are analyzed by gas chromatography. The procedure is somewhat complicated and oils containing 5% or less CPFA must have the reaction products further separated from the oil. Coleman (7), in a review of five methods for the determination of cyclopropenoid fatty acids, recommended this method to analyze standards for the simpler Halphen reaction.

Raju and Reiser (8) have added methanethiol across the

TABLE I

Precision of Analysis by NMR[a] for Cyclopropene in One Part *S. foetida* Oil[b] Diluted With Four Parts Methyl Oleate

CPFA,[c,d] %	Sample concentration,[e] %	Sweep width, cps
9.4	9	100
9.6	9	100
9.8	9	100
10.8	9	100
10.0	9	250
9.6	9	250
9.7	17[f]	250
9.8	5	250

9.8 ± 0.28, average

[a]Each determination performed on a different sample, some on different days, using a Varian HA-100.

[b]Sterculia *foetida* seed oil assayed 49.2% CPFA by NMR before dilution and 51 and 55% via Halphen Reaction (12) using purified methyl sterculate as a standard.

[c]CPFA = cyclopropenoid fatty acids.

[d]Halphen determination of this oil gave 7.9%, 7.2% and 8.8%. Standard for these Halphen assays was purified methyl sterculate.

[e]Sample diluted with 80% carbontetrachloride-20% methanol.

[f]Inferior resolution.

TABLE II

Analysis by NMR[a] for Cyclopropene in Various Dilutions of S. *foetida* Oil Methyl Esters in Methyl Oleate

SFO:oleate[b]	CPFA, %
1:0	50.0
1:4	9.1
1:9	5.05
1:24	1.67
1:49	1.0 ± 0.2

[a]Instrument used is Varian HA-100, sweep width 250 cps.

[b]SFO = sterculia *foetida* seed oil; CPFA = cyclopropenoid fatty acid.

TABLE III

Comparison of Analyses for Cyclopropene in Rat Depot Fat

Sample no.	CPFA by Halphen assay,[a] %	CPFA by NMR assay, %
1	2.03 ± 0.09	2.76
2	1.80 ± 0.02	2.87
3	2.47 ± 0.12	2.92
4	2.33 ± 0.09	2.36
5	15.9 ± 0.9	14.0
6	17.4 ± 0.07	13.5[b]
7	20.3 ± 0.9[c]	14.0[c]
8	0.7 ± 0.06[d]	<0.5[d]

[a]According to Bailey (12) except sealed reaction tubes were used.

[b]Triplicate determinations on separate days.

[c]The same oil listed immediately above, but transesterified. $AgNO_3$-methanol method (6) produced a value of 15.4 ± 0.8%.

[d]Liver fat.

TABLE IV

Effect of Solvent on Position of 3,3-Cyclopropenyl Hydrogens

Solvent	Differences between methyl center (B) and cyclopropene peak (A) in ppm[a]
CCl_4	.16 (δ = 0.72)
CCl_4-20% methanol	.16
CCl_4-20% Acetonitrile	.15
CCl_4-50% $CHCl_3$	.13
$CHCl_3$-20% methanol	.12
CCl_4-20% benzene	.08
CCl_4-60% pyridine	0 (δ = 0.88)
None	.11[b]

[a]Data for CCl_4 obtained on both a Varian A-60 and HA-100 using 1,2-dipropyl-; 1,2-dipentyl-; 1,2-diheptyl-; 1,2-dioctylcyclopropene, methyl malvalate and sterculate. All other data obtained on HA-100 using methyl sterculate, except for neat solution.

[b]Data obtained on a Varian A-60 using 1,2-dipropyl- and 1,2-dipentylcyclopropene.

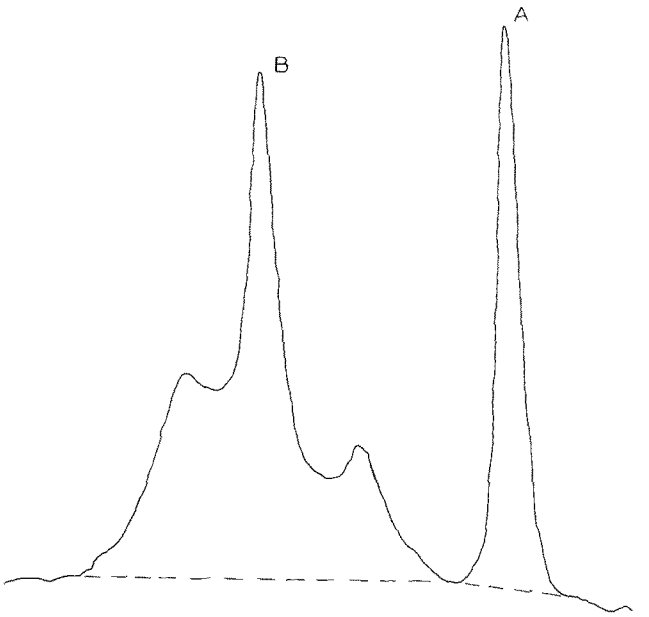


FIG. 1. Upfield portion of the NMR spectrum of Sterculia *foetida* oil (SF oil). The peak labeled A is from the hydrogens on the cyclopropene ring; the triplet B is from the terminal methyl group. The sample is 49.2% cyclopropenoid fatty acid (CPFA). Sweep width is 100 cycles.

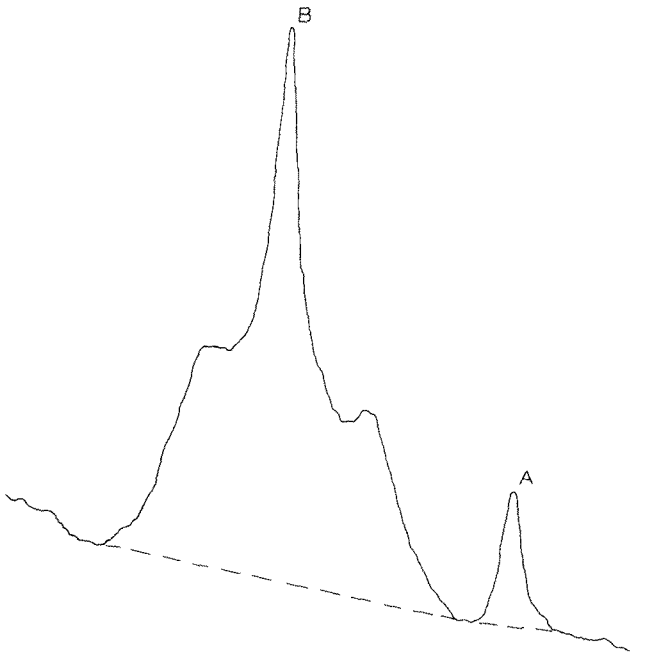


FIG. 2. Upfield portion of the NMR spectrum of SF oil diluted 4:1 in methyl oleate. Peaks A and B are from the cyclopropenoid and methyl hydrogens, respectively. The spectrum measures 9.6% CPFA.

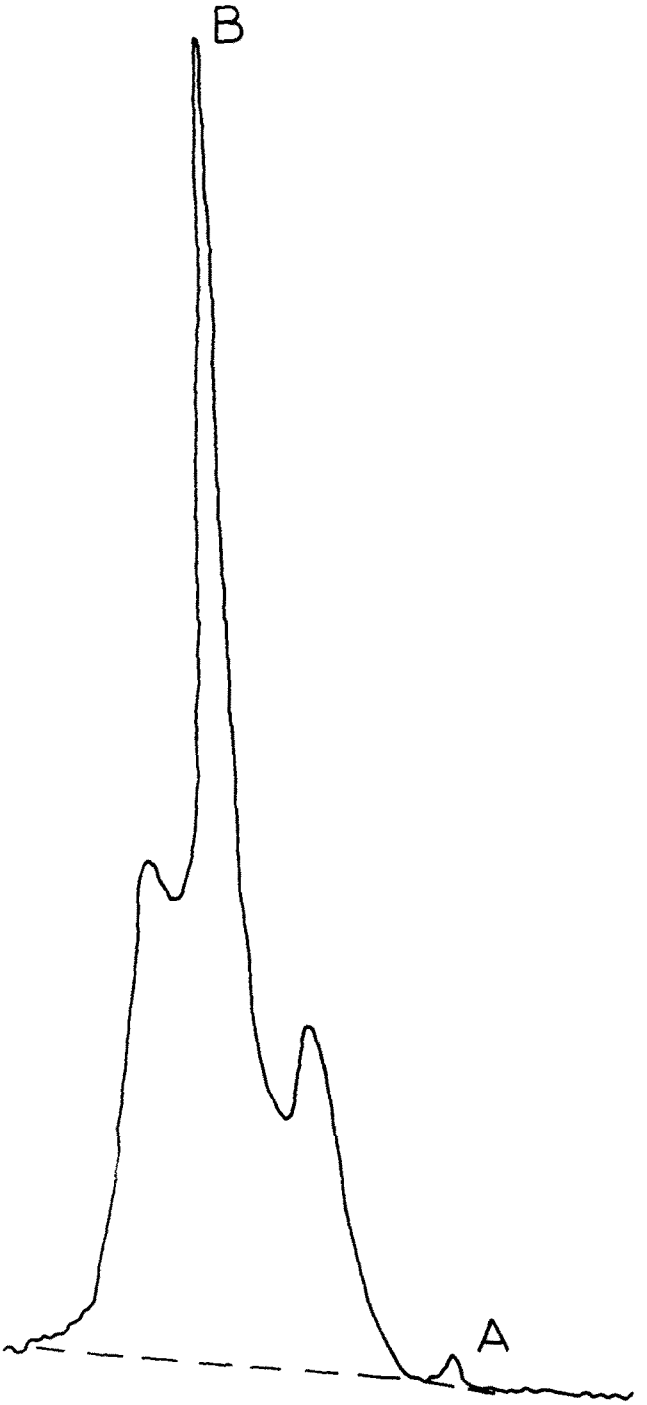


FIG. 3. Upfield portion of the NMR spectrum of SF oil diluted 49:1 in methyl oleate using a sweep width of 250 cycles. Areas A and B are from the cyclopropenoid and methyl hydrogens. The spectrum measures 1.0% CPFA.

double bond of the cyclopropene ring, forming a thio ether which can be gas chromatographed. Coleman (7) found this method to be neither reproducible nor linear. Our study of the reaction indicates that methanethiol adds across the double bond of the cyclopropene ring via a free-radical initiated mechanism. The reaction appears to be quantitative, but we have not studied it as a method of analysis. See References 9 and 10 for a review on the addition of thiols to olefins.

Strong acid preferentially adds across the reactive double bond of the cyclopropene ring. Coleman (7) has reviewed several methods for the quantitative titration of the cyclopropenoid function using hydrobromic acid. Results from the HBr-tolune titration were consistently low, and this method was judged unsuitable. The HBr-acetic acid titration was plagued with acetic acid-cyclopropene interactions. A HBr-benzene variation (11) gave improved results, but some interaction persisted with cottonseed oil samples. Coleman (7) recommends the HBr-benzene method as well as the previously mentioned silver nitrate-methanol method to calibrate standards for the Halphen reaction. However we find the HBr-benzene method gives poorly defined endpoints, which renders it neither precise nor accurate.

The literature on the Halphen reaction is too voluminous to review here. It is generally accepted that the absorbance of the Halphen reaction lacks precision and that the background varies with the type and source of lipids (12). As a method of analysis, it is quicker, simpler and requires

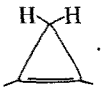
less sample material than any other method reported to date, but, unfortunately, requires an accurately known standard. Coleman (7) recommends the Halphen test of Bailey et al. (12) as the best general method for routine analysis of cyclopropenes. Hammonds and coworkers (13) have since reported a modification of the Bailey method which employs pressurized reaction capsules and transmethylation of natural oils. The pressurized reaction capsules stabilized developing colors and transmethylation of oils produces better correlations between the Halphen reaction and hydrogenbromide titration. Hammonds' method appears to be the best routine procedure for the Halphen assay we have found to date. The precision between multiple determinations on a given day is generally within 5%, but variations in the Halphen value of the same oil sample do occur from day to day. Also, values obtained from transmethylated oils are sometimes double the natural oils. The discrepancy between oils and transesterified oils is baffling.

Analysis by NMR

We wish to present here the use of nuclear magnetic resonance spectroscopy (NMR) as a rapid, simple and quantitative method of analysis for the cyclopropenoid function in lipids. By this method, natural lipid triglycerides need not be transesterified or cleaned up before analysis. A 5 mg sample is sufficient, although 30 mg is more desirable, and lipids with as low as 1% cyclopropenoid can be measured. NMR is a direct method since the 1,2-disubstituted cyclopropene function itself is seen and measured in the spectrum. Interfering substances, should they be present, can also be seen, allowing one to immediately judge the accuracy of a measurement.

Method

Protons in chemically different environments undergo resonance at different values (chemical shift) in a NMR spectrum. Figures 1-8 show only the up-field portion (area between δ=1.1-0.6) of some typical spectra of lipids containing cyclopropenoid fatty acids. The peak labeled A is due to the two hydrogens on the cyclopropene functions,



. The three fused peaks labeled B are due to methyl groups, $-CH_3$, at the ends of the fatty acid chains. The terminal methyl groups of all fatty acids containing an adjacent methylene, $-CH_2-CH_3$, are split into a triplet for reasons which are discussed in introductory books on NMR (14,15). To the left of the methyl peak appears a strong peak, due to the numerous methylene hydrogens present in lipids (peak C, shown only in Figure 5).

The area under a peak is proportional to the number of hydrogens in solution giving rise to said peak. Thus, if the sample shown in Figure 1 were 100% cyclopropenoid fatty acid, the area under B, representing the three methyl hydrogens, should be 1.5 times larger than the area under peak A from the two cyclopropenyl hydrogens. Obviously, as the per cent cyclopropene decreases in a mixture of fatty acids, A will proportionally decrease compared to B. If one assumes that each fatty acid has only one terminal methyl, i.e., no branching in the aliphatic chain, then the ratio A/B (times 150) is the molar per cent of cyclopropenoid fatty acid (CPFA) present. If one approximates the average fatty acid molecular weight of a lipid as 294, the mole per cent of CPFA is very nearly the weight per cent expressed as sterculate.

EXPERIMENTAL PROCEDURES

Oil samples should be diluted with an inert solvent. Carbon tetrachloride is an excellent solvent; it is inert and does not absorb in the NMR. For instruments which need an internal lock, carbon tetrachloride-methanol is a good choice.

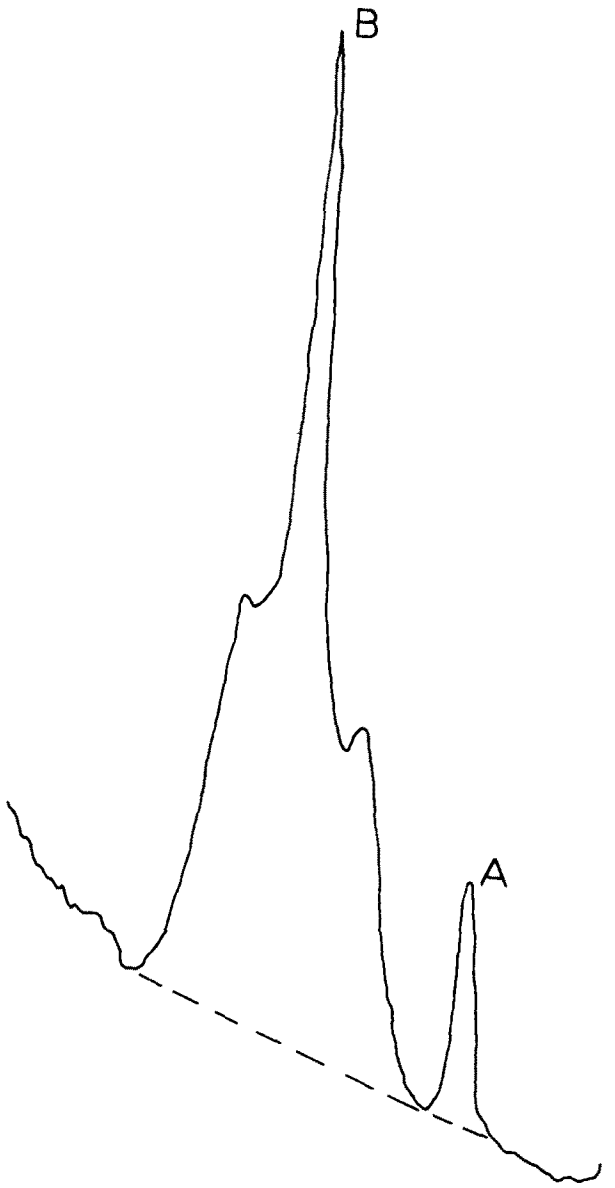


FIG. 4. Upfield portion of an NMR spectrum of SF oil diluted 4:1 in methyl oleate. This figure shows the methyl and cyclopropenoid peaks on the side of an unusually large tail from the methylene peak. This spectrum measures 9.55% cyclopropene.

Dilution serves two purposes. First, it decreases the viscosity of oil samples, effecting better resolution. Second, the two hydrogens on the 3 position of the cyclopropene ring are solvent dependent; carbon tetrachloride will shift them up field (to the right) from the methyl group. The solvent dependence of these hydrogens in the NMR will be discussed later. A dilution of ten parts solvent to one part oil is ideal. Exact dilution is not important in that the ratio of the areas under two peaks is dilution independent. A dilution of 20:1 achieves little increased resolution and solvent shift while increasing baseline noise. Dilutions much less than 10:1 should be avoided.

Any high resolution NMR is satisfactory for this type of analysis. We have used the Varian A-60 and HA-100 instruments with excellent results. However the two peaks of interest are too close together to allow satisfactory use of the instrument's integrator. A base line must be drawn under each absorption and the area measured precisely with a planimeter. Drawing in the base line is facilitated by experience; several examples are shown by the dotted lines in Figures 1-8. Figure 7 shows both the highest and lowest

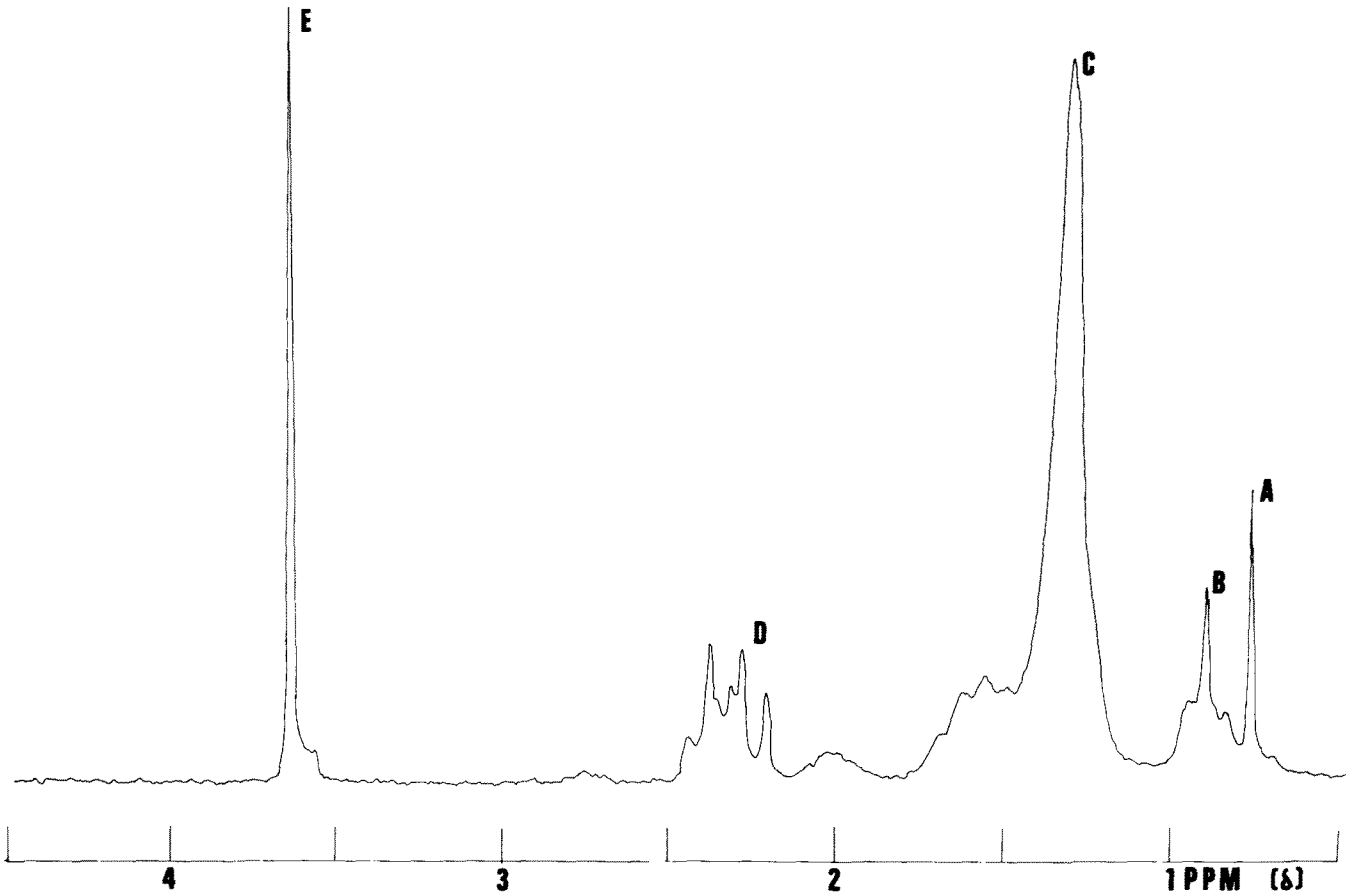


FIG. 5. NMR spectrum of SF oil distilled after transesterification with methanol. Assignments lettered are: A, cyclopropene; B, terminal methyl; C, methylene; D, mixture of O=C-CH_2 and C=C-CH_2-C=C; E, methyl ester. Expansion of the scale and integration with a planimeter shows 64.8% cyclopropene in this sample.

possible base lines drawn on a spectrum where the methyl triplet is not distinctly separated from other absorptions. The difference between per cent cyclopropenoid fatty acid (CPFA) from these two base lines is only 3%, or an 8% overall range for the sample.

The area under the cyclopropene peak is critical in samples with a low CPFA content. The per cent CPFA is calculated by dividing the area from the methyl absorption, a large value, into the area from the cyclopropene absorption, a low value (and multiplying by 150). Errors in drawing the base line under the cyclopropene peak in samples with a low CPFA or errors in measurement of its area will produce magnified errors in the resultant per cent CPFA. For example the area under peak A in Figure 3, measured by a planimeter, is between 0.02-0.03 sq. in. Using these values to calculate per cent CPFA produces 0.8% and 1.2%, respectively, whereas an error of 0.01 sq. in. in Figure 1 produces only an infinitesimal difference in the per cent CPFA. It is difficult to quantitatively measure less than 1% CPFA by NMR, although we were able to discern a mislabeled bottle of cottonseed oil. The oil in question was labeled 0.6% CPFA, and by NMR we were able to say it was less than half the labeled value. Halphen analysis yielded a value of 0.2%.

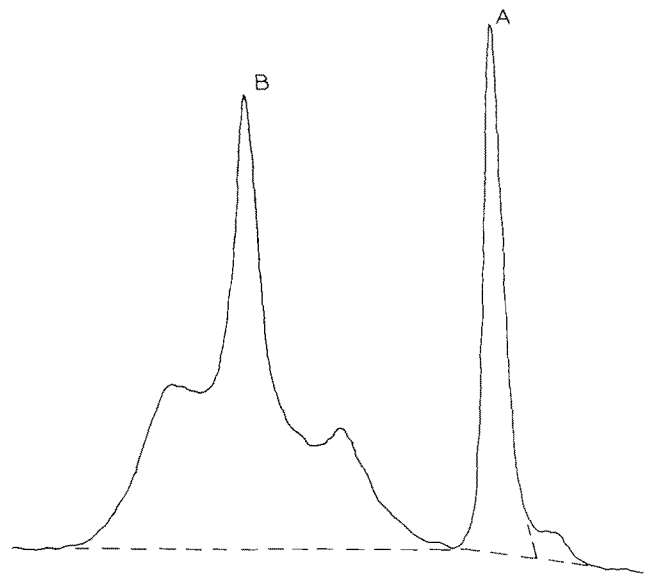


FIG. 6. Upfield portion of a NMR spectrum of aged methyl esters of SF oil. The shoulder on the right side of the cyclopropene peak, A, is believed to be cyclopropene hydrogens from polymerized cyclopropene. Measurement within the dotted lines gives 47.4% CPFA.

RESULTS

Table I shows the precision of this method on successive determinations. Repeated scanning of a sample in the instrument will give an exact trace over previous scans. The values in Table I were obtained from different samples and from adjusting (tuning) the instrument between each determination. How the instrument is tuned will affect peak shape and size but not the relative areas.

Table II shows the agreement of this method with dilution over a range of different CPFA percentages. The sweep width, listed in these tables, is simply an expression of the scale over which the instrument draws the peaks. With a sweep width of 100 cps one has larger areas to measure so that area measurement is not as critical (provided one has a large enough planimeter).

How will impurities influence the analysis by NMR?

Hexane has two methyl groups with absorption identical to the terminal methyl groups of fatty acids (at 0.88δ). Therefore hexane will cause the CPFA content to appear low without spectral evidence of its presence. In a 100 g oil sample adulterated with 1 g hexane, a 10% CPFA content would appear as 9.5% and a 50% CPFA content would appear as 47.5% CPFA. An impurity without methyl absorption in the 0.9δ region would cause the CPFA value to appear high. The extent of the error here is the result of not taking the weight per cent of the impurity into account. There are many compounds and solvents with methyl groups that do not absorb in the 0.88δ region. The absorption of ethyl ether is close enough to cause interference, and an example of such interference is shown in Figure 7.

It is difficult to find an exact measure of CPFA content to compare to NMR analysis. Footnotes b and d in Table I compare the results from NMR analysis to the Halphen analysis. The purified methyl sterculate referred to as a standard for the Halphen reaction was determined to be 96-98% by catalytic hydrogenation to the cyclopropane ester followed by GLC. NMR analysis of this oil demonstrated to be ca. 89% cyclopropenoid with another 8% in the cyclopropane form (presumably as a result of cyclopropene polymerization, and this polymer would not be seen on a gas chromatograph).

A sample of *S. foetida* oil, transesterified in methanol and distilled under high vacuum, was reacted with silver nitrate in methanol according to the method of Schneider and coworkers (6) and the products analyzed via GLC. Nine determinations averaged 55.7% CPFA with a range of 3.4% and an average deviation of 1.1%. NMR determinations of the same oil gave five identical traces measuring 50.3% CPFA. Halphen analysis (13), using as a standard 89% methyl sterculate as determined by NMR, yielded a value of 54% CPFA. The presence of materials which would not pass through a GLC would be a source of error in the silver nitrate-methanol method, but in this instance we used distilled methyl esters, and the NMR did not show a significant amount of polymer in the sample.

Table III shows the comparison between the NMR and Halphen methods applied to animal fat. Because of the discrepancy between the two methods in sample number six, it was transesterified with methanol to produce sample number seven. Applying the silver nitrate-methanol method (6) to this oil yielded a value of 15.4 ± 0.8% CPFA, compared to 20.3% for the Halphen assay and 14% from the NMR assay. The value from the silver nitrate-methanol assay (6) might also be high, depending on the amount of material that does not pass through a GLC. NMR analysis of the liver sample (last value in Table III) is not as precise as the other samples because of the small amount of cyclopropene present.

Many attempts were made to compare the various hydrobromic acid titration (7,15) methods to our results. Nebulous endpoints render these methods, in our opinion, unsatisfactory as a quantitative measure.

Solvent Shifts

As mentioned earlier the chemical shift of the two ring hydrogens on 1,2-disubstituted cyclopropenes is solvent dependent. It is necessary to take advantage of this solvent shift to remove the overlap of absorption bands and thus aid in the interpretation of the spectrum. Magne (16) used NMR as a method of analysis for cyclopropenoids in lipids but had difficulty in clearly resolving the ring methylene proton signal from that of the terminal methyl group. Table IV shows the difference between the cyclopropene band and the center of the methyl peak with changes in solvent. The methyl peak and other peaks remain unshifted with respect to tetramethylsilane (TMS).

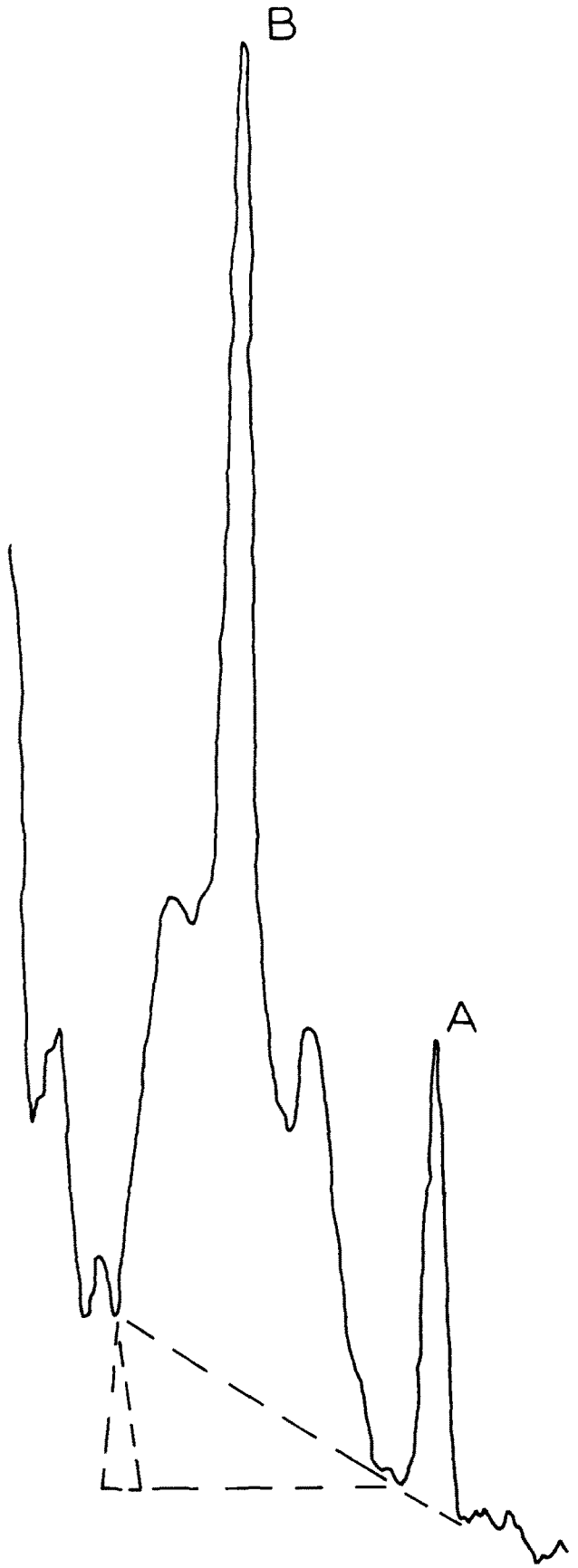


FIG. 7. Upfield portion of a NMR spectrum with the methyl absorption not clearly resolved from the methylene absorption in a sample of rat fat. Using top base line this spectrum gives a value of 15.5% CPFA. The bottom and outside base lines produce a value of 12.4%CPFA.A possible interfering substance is believed to be ethyl ether. Applying heat and vacuum to this sample removed the interfering substance(s), and the spectrum gave a distinct value of 13.5% CPFA.

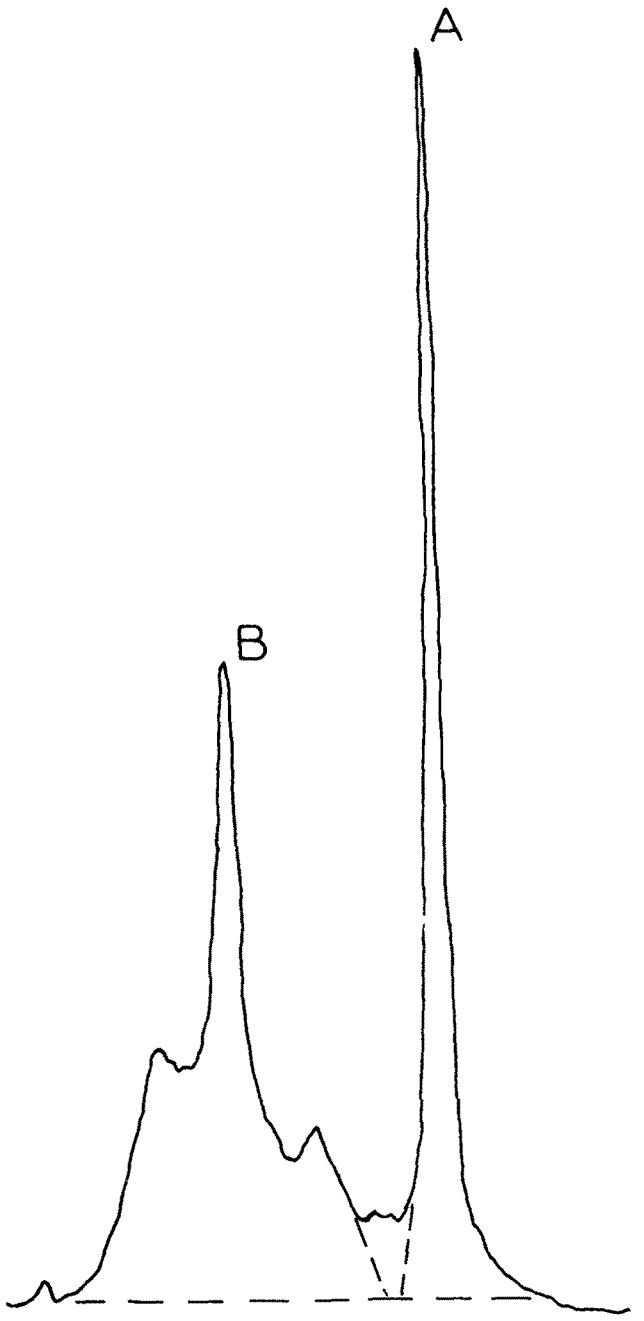


FIG. 8. The upfield portion of a NMR spectrum of methyl sterculate showing an interfering absorption between the methyl and cyclopropene peaks. Dotted lines show extrapolation of these peaks to the base line. Spectrum measures 94% CPFA.

Jackman and Sternhell (17) theorize that solvent shifts arise principally from "collision complexes." These collision complexes must involve the formation and breaking of a bond, e.g., charge transfer or hydrogen bond, and are associated with a specific geometry. Since aromatic solvents shift the ring hydrogens down field, one concludes that the two rings must be situated so that the cyclopropenyl hydrogens are in the dishielding cone of the aromatic ring. The attractive force is presumably a charge transfer type complex involving the orbitals of the two rings. Surprisingly the cyclopropene rings appear to form a "collision complex" with themselves as evidenced by the shift of neat solutions (Table IV).

The"collision complex"between two cyclopropene rings must be weak since dilution with an inert solvent shifts the two ring hydrogens up field. Heat is also reported to diminish the extent of "collision complexes" in other systems (17). This may partially explain the ease with which 1,2-disubstituted cyclopropenes polymerized at low temperatures. The lower molecular weight cyclopropenes, which are sterically less hindered for forming "collision complexes," polymerize more easily than the heavier 1,2-substituted rings. Methyl sterculate polymerizes more easily than *S. foetida* oil, and 1-methylcyclopropene is reported to spontaneously polymerize at -20 C (18). A high free energy ground state is no doubt responsible for the facile polymerization, with "collision complexes" contributing a favorable entropy. The entropy effect will be reflected in the rate of polymerization at various temperatures. We have not measured the rates, but have emperically observed that 1,2-disubstituted cyclopropenes do not store well at -30 C, yet are surprisingly stable at room and higher temperatures.

ACKNOWLEDGMENTS

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