Hydrazine Reduction in the Gas Liquid Chromatographic Analysis of the Methyl Esters of Cyclopropenoic Fatty Acids

J. CONWAY, W.M.N. RATNAYAKE and **R.G. ACKMAN***, Canadian Institute of Fisheries Technology, Technical University of Nova Scotia, P.O. Box 1000, Halifax, N.S., Canada B3J 2X4

ABSTRACT

Hydrazine was used to hydrogenate the double bond of cyclopropenoic fatty acids (CPEFA) without cleaving the cyclic system. Using gas liquid chromatography (GLC), quantitation studies showed that it was possible to accurately determine CPEFA at concentrations as low as 0.1%. Studies with kapok seed oil methyl esters illustrated the potential application of such a technique to selectively hydrogenate the CPEFA to cyclopropanoic fatty acids (CPAFA), rendering them stable on the column, with a minimum effect on the degree of unsaturation of the remaining double bonds. Hydrazine reduction was found to be a simple and accurate method of measuring CPEFA in seed oils. Decomposition of pure methyl sterculate (methyl 9,10-methylene-9-octadecenoate) on GLC columns of various polarities also was examined.

INTRODUCTION

Cyclopropenoic fatty acids (CPEFA), especially malvalic and sterculic, have been studied widely in the last 20 years due to their physiological effects on animals (1-3). Various plant families such as Malvaceae, Sterculiaceae and Bombacaceae have been found to contain CPEFA (4,5). Cottonseed (*Gossypium hirsutum*) oil contains small amounts (0.4-1.3%) of CPEFA (6-8), while greater amounts (8.7-14.4%) have been found in kapok (*Ceiba pentandra*) seed oils (6,7). Since these oils are of importance in developing countries, it is essential to have an unsophisticated, simple technique to accurately determine their CPEFA content.

The principal methods currently being used for the analysis of CPEFA are: (a) a colorimetric method based on the original Halphen test (9); (b) titrations involving hydrogen bromide (10,11); (c) nuclear magnetic resonance (NMR) (12); (d) gas-liquid chromatography (GLC) analysis of silver nitrate/methanol (13) or methyl mercaptyl (14) derivatives; (e) direct GLC analysis of methyl esters (6,15); (f) a potentiometric titration (16), and (g) homogeneously catalyzed hydrogenation followed by GLC (17). The limitations and drawbacks of some of these methods have been investigated by Coleman (18). A modified Halphen test (19) in combination with the GLC silver nitrate method has been recommended for low levels of CPEFA in fats and oils. However, the GLC analysis of ether and ketone derivatives produced from the reaction of transesterified lipids with silver nitrate in methanol involves the summation of the areas of four peaks from each of the CPEFAs. In addition, the separation of the enonic isomers of the CPEFA derivatives has been found to be inadequate in some columns (6).

Since CPEFA can polymerize or partially decompose at high temperatures (20), direct GLC analysis without prior CPEFA derivatization has not been widely used. Nevertheless, it has been reported that direct GLC analysis of methyl esters on glass capillary columns coated with Carbowax 20M gave results very near to those obtained after silver nitrate derivatization (6,21). It was suggested that the direct GLC method was an accurate and rapid method to determine simultaneously cyclopropenoic and cyclopropanoic fatty acids. However, there was slight decomposition of cyclopropenoic acids at high column temperatures (190 C), and 170 C was found to be the most suitable temperature. Fisher and Schuller (15) reported that with an on-column injection on a glass column packed with methyl silicone on an "inert" support, methyl malvalate and sterculate could be chromatographed without decomposition. It was claimed that, although methyl malvalate was not well resolved from methyl linoleate and was underestimated, it could be quantitated accurately at concentrations as low as 0.03% by a peak-height method. In addition, slight decomposition of CPEFA was observed with column deterioration.

In a recent study, homogeneous hydrogenation using a rhodium complex catalyst proved useful in stabilizing the methyl esters of CPEFA before GLC analysis (16). In contrast to cyclopropenoic acids, the corresponding cyclopropanoic acids (CPAFA) are thermally stable. The reproducibility of the method was greater than that obtained using HBr or NMR methods. One major drawback of this method is the complex manipulation and elaborate equipment involved.

Hydrazine is a versatile, mild reducing agent for olefinic bonds and can be used to selectively hydrogenate certain double bonds without affecting the configuration and position of the remaining double bonds in unsaturated fatty acids (22,23). The diimide generated in situ by the oxidation of hydrazine by oxygen in the presence of a very weak acid (the solvent itself acts as a proton source) is the actual reducing agent (22). Thus, hydrogenation also can be carried out with other diimide producing agents such as hydroxylamine and ethyl acetate (24), hydrolysis of potassium azodiformate, and decomposition of p-toluene-sulfonyl hydrazide (25). The most common and convenient way of producing diimide involves the oxidation of hydrazine with oxygen. Considering the strained nature of the cyclopropene ring system, it was thought that a selective hydrogenation of the double bonds of CPEFA using hydrazine-oxygen could be achieved easily without a modification of the remaining unsaturated fatty acids. Then the resulting cyclopropane fatty acids, known to be stable for GLC analysis, could be quantitated accurately.

This paper reports our studies on (i) the stability of methyl sterculate (19:CE) on several GLC liquid phases; (ii) the selective hydrogenation of methyl sterculate with hydrazine and limits of quantitation, and (iii) the application of the hydrazine reduction technique for the analysis of cyclopropenoic acid (malvalic acid 18:CE and sterculic acid 19:CE) in kapok seed oil. For convenience, the corresponding cyclopropanoid acids will be designated 18:CA and 19:CA.

MATERIALS AND METHODS

Samples

A pure sample of methyl sterculate was obtained from the Southern Regional Research Center, New Orleans, Louisiana, and authentic kapok seeds were obtained from Sri Lanka.

Oil Extraction

The kapok seeds were crushed into coarse particles, and the oil was extracted under reflux with hexane. The seeds were found to contain about 27% oil.

*To whom correspondence should be addressed.

Preparation of the Methyl Esters

Kapok seed oil (250 mg) was transesterified with 0.5% sodium methoxide in methanol (50 ml) under reflux. After 45 min, the solution was diluted with distilled water (25 ml) and extracted with hexane (3 × 20 ml). The combined extracts were washed with water (20 ml), dried over anhydrous sodium sulphate and the solvent removed under reduced pressure using a rotary evaporator. The methyl esters were diluted in hexane to obtain a solution for further analysis.

Hydrazine Reduction

A typical hydrazine reduction is described below. About 1.0-1.5 mg of fatty acid methyl esters was taken to dryness under nitrogen in a flat-bottomed (125 ml) flask. Twenty-five ml of 96% ethanol and 0.5 ml of 95% hydrazine (Eastman Kodak Co., Rochester, New York) were added and the flask placed in a water bath held at 35-37 C. The reaction proceeded under an atmosphere of oxygen with constant stirring (magnetic stirrer). After 60 min, distilled water (25 ml) was added and the methyl esters were extracted with hexane (3 × 25 ml). The combined hexane extracts were washed with distilled water (20 ml), dried over anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator. A hexane solution of this product was analyzed by GLC.

Thin-Layer Chromatography (TLC)

Preparative TLC was carried out on Prekote Absorbosil-5 silica gel plates (Applied Science Laboratories Inc., State College, Pennsylvania), 20 cm × 20 cm. The plates were cleaned by developing in ethyl acetate and activated by heating at 110 C for 30 min. Silver nitrate-TLC ($AgNO_3$-TLC) plates were prepared by dipping the plates horizontally in a 10% solution of silver nitrate in acetonitrile for 30 min and drying at 110 C for 60 min. A hexane solution of the sample (15 mg/100 μl hexane) was streaked on the plate with development in pure benzene. Following drying, application of a 2′,7′-dichlorofluorescein spray and examination under UV light (254 nm) showed three bands (R_f=0.72, 0.51 and 0.29), corresponding to saturated, monounsaturated and diunsaturated fatty acid methyl esters, respectively. The bands were scraped off and extracted with chloroform: hexane (1:1, v/v, 3 × 20 ml) for examination by GLC.

Gas Liquid Chromatography

Initially, three different Perkin-Elmer gas chromatographs were used for the decomposition studies. A Perkin-Elmer 3920 chromatograph equipped with a flame ionization detector and a stainless steel wall-coated open-tubular column, 47 m long × 0.25 mm i.d., coated with SILAR-5 CP. The operating parameters were: column temperature 180 C, detector and injector port temperature 250 C, carrier gas (He) pressure - 60 p.s.i.g. A Perkin-Elmer 900 equipped with a flame ionization detector and a fused silica capillary column, 50 m × 0.24 mm i.d., coated with methyl silicone OV-101 (Quadrex Corporation, New Haven, Connecticut). The operating parameters were: column temperature - 180 C, detector and injector port temperature 250 C. The carrier gas (He) pressure was 60 p.s.i.g. A Perkin-Elmer Sigma 4 equipped with a flame ionization detector and a fused silica capillary column, 30 m long, 0.32 mm i.d., coated with bonded Carbowax 20 M in the form of Supelcowax-10 (Supelco Inc., Bellefonte, Pennsylvania). The operating parameters were: column temperature 170 C, detector and injector port temperatures 200 C. The carrier gas (H_2) pressure was 20 p.s.i.g. An Oxisorb purifying cartridge was used in the carrier gas line.

With the Perkin-Elmer 3920 and 900 chromatographs, Fisher Recordall series 5000 recorders with stepping integrators were utilized. For the Sigma 4 model, a Perkin-Elmer LCI-100 laboratory computing integrator was used. The Supelcowax-10 was found to be the best column for the analysis of methyl sterculate and hence detailed studies of the fatty acid methyl esters of kapok seed oil were carried out on this column.

RESULTS AND DISCUSSION

Even though direct GLC analysis of methyl esters has been used (6), the question of what percentage of the CPEFA decomposes on the column remains. In the present study, the decomposition of authentic methyl sterculate (19:CE) was investigated on three capillary GLC columns of different chemistries and polarities, viz., medium polarity (SILAR-5CP, stainless steel column), moderate polarity (Supelcowax-10, fused silica column) and non-polar (OV-101, fused silica column). A mixture of 19:CE and methyl heptadecanoate (internal standard) in a 1:1 ratio was used for these studies. The SILAR-5CP column gave three peaks instead of one for 19:CE, of which the sum total area accounted for only 39% of the injected amount (Table I). This would indicate that substantial decomposition and/or polymerization had occurred on the polar column. In a similar manner, the OV-101 column also showed three peaks, instead of one, with a 78% area recovery. On the other hand, the recovery of 19:CE on the Supelcowax-10 column was slightly greater than 100%.

However, the chromatograms showed four peaks; the major peak accounted for approximately 92% of the recovered material. Thus, with polyglycol columns similar to Supelcowax-10, one must be aware that if direct GLC analysis of normal methyl esters is performed, these products of decomposition could be hidden under the peaks for other fatty acids normally encountered in seed oils. The decomposition of CPEFA on the SILAR-5CP could be due partly to the metal injection port and stainless steel column as noted by Fisher and Schuller (15). Similarly, decomposition on the OV-101 fused silica column, which is likely to be due to the substrate, could be due partly to the metal injection port. Careful study shows that with a fused silica column (Supelcowax-10) and a glass-lined injection port, 8% decomposition of sterculate occurred. Our findings are confirmed by Bianchini et al. (6), who stated that even at 170 C on a Carbowax-20M fused silica column with a glass injection port, "slight" decomposition occurred. The instability of the cyclopropene ring at higher temperatures is the main cause for the loss of CPEFA on GLC. The type of liquid phase, column material and the injection port may have a substantial effect on the total decomposition of CPEFAs.

The hydrazine reduction of CPEFA must be such that the hydrogenation is complete without any development of oxidation products. In order to determine the optimum conditions, a mixture of 15% 19:CE with a pure internal standard (19:0) was subjected to hydrazine reduction. The reaction products were recovered and analyzed by GLC on the Supelcowax-10 fused silica column. Optimum conditions, leading to 100% reduction with no oxidation products, were found to be one hr with an atmosphere of oxygen. A longer reaction time than one hr resulted in the development of oxidation products. To ascertain the accuracy of the quantitation of CPEFA using hydrazine reduction, known ratios of 19:CE and an internal standard (19:0) were subjected to complete hydrazine reduction. As shown in Table II, there was a good agreement between the experimentally obtained recovery percentages and the actual per-

TABLE I

Percent Recovery[a] of Methyl Sterculate on GLC Columns of Different Polarities

	SILAR-5CP	OV-101	Supelcowax-10
Mean[b] (%)	39.0	78.3	102.0
Standard deviation	2.8	3.4	1.2
Coefficient of variation (%) (C.V.)	7.2	4.3	1.2

[a]The percent recovery was calculated on an area basis with respect to the internal standard 17:0.
[b]Number of injections = 5.

TABLE II

Quantitation on Supelcowax-10 at 172 C of Various Amounts of CPAFA after Hydrazine Reduction

	Experimental recovery percentages after reduction[a]		
Actual percentage of methyl sterculate[a]	Mean[b]	Standard deviation	Coefficient of variation
9.94	9.37	0.54	5.7
3.55	3.32	0.16	4.8
1.27	1.28	0.07	5.4
0.66	0.66	0.09	13.2
0.13	0.10	0.02	19.0

[a]Percent by weight with respect to the internal standard (19:0).
[b]Number of injections = 5.

centages of 19:CE. Thus, it is possible to quantitate CPEFA accurately at levels of 0.1% with the hydrazine reduction technique. Bianchini et al. (6), using silver nitrate methanol derivatization and direct GLC analysis of adulterated peanut oil, were unable to quantify CPEFA at the 0.3% level. However, the hydrazine reduction technique with two GLC analyses may be less efficient in some respects than the direct analysis with an on-column GLC injection technique, claimed by Fisher and Schuller (15) to be accurate in determining CPEFA concentrations as low as 0.03%.

Hydrazine reduction was used to measure the CPEFA in kapok seed oil. About 1.5 mg of kapok seed oil fatty acid methyl esters were reduced with 0.5 ml of hydrazine under an atmosphere of oxygen for one hr. The final product was analyzed on the Supelcowax fused silica column. The equivalent chain length (ECL) values on Supelcowax-10 (Table III) are quite similar to other studies of CPEFA using a Carbowax-20M column. The chromatograms of the methyl esters of kapok seed oil fatty acids, before and after hydrogenation, are given in Figure 1.

In order to determine the percent by weight of 18:CE and 19:CE in the kapok seed oil, 16:0 already present in the oil was chosen as the internal standard. It is a valid standard because 16:1 is present only in trace amounts and is slightly affected by hydrazine reduction. It is possible to use the standard peak-area normalization method already built into most of the modern computerized integrators to calculate the concentrations. Because there may be errors in accurately weighing and injecting small amounts of samples, it was felt that it would result in more variation than the internal standard method used in this study. An appropriate external standard, such as iso-22, also could be used. The normalized peak areas of kapok seed oil methyl esters with respect to 16:0 before and after hydrazine reduction are shown in Table IV.

As noted by other workers (6) *cis* vaccenic acid (18:1n7) and dihydromalvalate (18:CA) are not always resolved on

TABLE III

Equivalent Chain Length (ECL) Values of Methyl Esters of Kapok Seed Oil Fatty Acids on Supelcowax-10

	ECL values	
Fatty acid	Present work	Literature (6)[a]
14:0	14.0	14.0
16:0	16.0	16.0
16:1n7	16.30	16.29
17:0	17.0	17.0
17:1	17.20	17.21
17:2	17.65	17.66
18:CE	17.93	17.92
18:0	18.0	18.0
18:1n9	18.19	18.21
18:1n7 + 18:CA	18.27	18.27
18:1n7	18.36	–
18:2n6	18.63	18.66
19:CE	18.90	18.92
19:CA	19.23	19.26
20:0	20.0	20.0
20:1n9	20.15	20.13
22:0	22.0	22.0

[a]Carbowax-20M in glass.

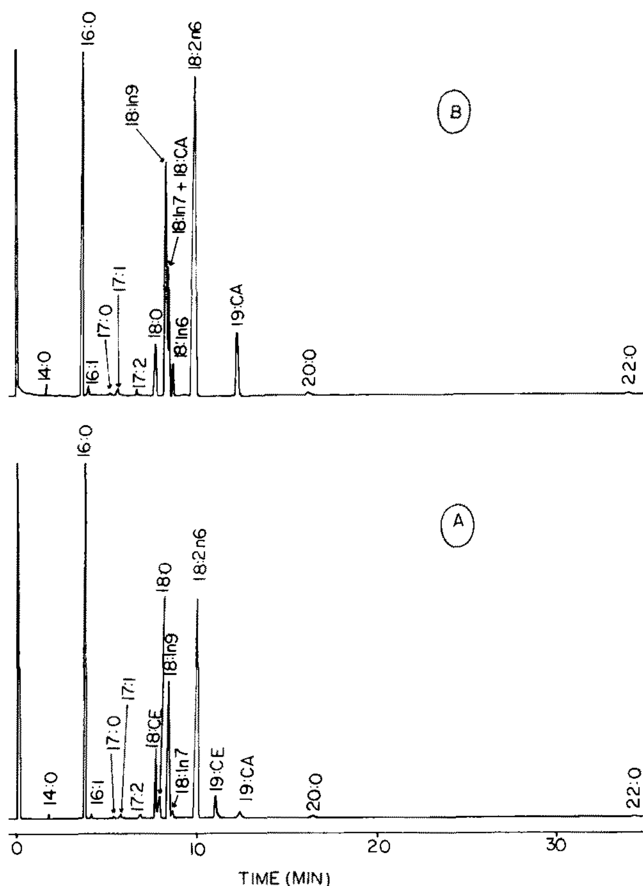

FIG. 1. GLC analysis of kapok seed oil fatty acid methyl esters on a Supelcowax-10 fused silica capillary column at 172 C. A, before hydrazine reduction, and B, after hydrazine reduction.

columns of polarity similar to Carbowax-20M. Thus, in order to determine if any 18:CA is present in the original unhydrogenated kapok seed oil, preparative $AgNO_3$-TLC was used to separate the saturated, the monounsaturated and diunsaturated fatty acid methyl esters. Silver nitrate destroys the cyclopropenoic acids, but 18:CA, which is

TABLE IV

Normalized Peak Areas[a] of Kapok Seed Oil Methyl Esters Before and After Hydrazine Reduction and the Fatty Acid Composition

Fatty acid	Before hydrogenation	After hydrogenation	Corrected normalized peak area	Fatty acid composition[b]
14:0	7	7	7	0.2
15:0	tr	tr	tr	
16:0	1000	1000	1000	23.8
16:1	15	14	15	0.3
17:0	7	7	7	0.2
17:1	23	22	23	0.5
17:2	23	23	23	0.5
18:CE	328	–	348	8.3
18:0	104	146	104	2.5
18:1n9	787	865	787	18.8
18:1n7	47	47	47	1.1
18:CA	–	348	–	–
18:1n6	–	82	–	–
18:2n6	1524	1413	1524	36.4
19:CE	168	–	195	4.7
19:CA	71	266	71	1.7
20:0	21	19	21	0.5
20:1	8	6	8	0.2
22:0	11	13	11	0.3

[a]Peak areas were normalized with respect to 16:0.

[b]Fatty acid composition was calculated using corrected normalized peak areas.

stable in the presence of $AgNO_3$, will migrate with the saturates. GLC analysis of the extracted saturated band on the Supelcowax-10 column did not reveal the presence of 18:CA in the original oil. If 18:CA is thought to be present in the unhydrogenated oil samples, TLC analysis must be done to quantitate it. This is the only drawback of this technique.

As shown in Table IV, the amount of 18:1n7 with respect to 16:0 remains constant before and after hydrazine reduction. Thus, by subtracting the normalized peak area by direct GLC analysis of 18:1n7 from the combined normalized peak area of 18:CA+18:1n7 obtained after reduction, the normalized peak area of 18:CA with respect to 17:0 can be determined. From the peak areas of 18:CE (before hydrogenation) and its hydrogenated products, viz 18:CA, it can be deduced that the percentage decomposition of 18:CE on the Supelcowax-10 column is 6%. Similarly, due to the decomposition of 19:CE, the sum of peak areas of 19:CE and dehydrosterculate (19:CA) before hydrogenation is slightly less than the normalized value of 19:CA after hydrogenation. Thus, the difference in the peak areas of 18:1n9 occurred. From Table IV, it is clear that hydrogenation of the other unsaturated fatty acids is minimal and in original 19:CE on the Supelcowax-10 column was calculated to be 10%, which is in good agreement with the decomposition studies of authentic methyl sterculate described earlier.

In addition to the hydrogenation of the cyclopropene ring, slight hydrogenation of 18:2n6 to form 18:1n6 and 19:1n9 occurred. From Table IV, it is clear that hydrogenation of the other unsaturated fatty acids is minimal and in no way interferes with the accurate determination of the cyclopropenoic acids.

To calculate the fatty acid composition of kapok seed oil, the normalized peak area of 18:CA after hydrogenation replaced the value of 18:CE from the direct GLC analysis. In addition, the modified value of 19:CE replaced the value of 19:CE before hydrogenation. All values for the remaining fatty acids were taken from the direct GLC analysis. The most important fatty acids in kapok seed oil, as illustrated in Table IV, are palmitic (24%), oleic (19%) and linoleic (36%). The percentages of 18:CE, 19:CE and 19:CA were found to be 8.3, 4.7 and 1.7, respectively. These results are quite similar to kapok seed oil analyses recently reported by other workers (6,7).

Previous workers (6) compared different methods of CPEFA quantitation in various oil samples. It was found that NMR was unsuitable where CPEFA content was low; direct GLC analysis of CPEFA content varied with temperature and column, and with HBr titration the end point was poorly defined. Hydrazine reduction, on the other hand, does not rupture the cyclic structure, selectively hydrogenates the CPEFA without modifying the remaining fatty acids except for a slight effect on 18:2n6, and is easy to carry out. In addition, with this technique quantitation of CPEFA down to 0.1% is possible. Thus, hydrazine reduction prior to GLC analysis is an accurate and relatively rapid method to measure CPEFA quantitatively in oil samples.

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