Gas Chromatographic Analysis of Cyclopropenoid Acids in Cottonseed Oils

G.S. FISHER and W.H. SCHULLER[1], Southern Regional Research Center[2], New Orleans, LA 70179

ABSTRACT

A gas chromatographic method of analysis for cyclopropenoid acids in cottonseed oil, as the methyl esters, is described. With a glass column packed with a methyl silicone substrate on an inert support, methyl sterculate and methyl malvalate can be chromatographed without decomposition. Although methyl malvalate was not well resolved from methyl linoleate, it could be quantitated accurately at concentrations as low as 0.03% by a peak-height method. Quantitation can be done manually with an internal standard, or with a data system without an internal standard. The method does not require calibration with a cyclopropenoid acid standard, i.e., it is a primary method. Results compare favorably with those obtained by HBr titration. Less than 1 mg of oil is required for an analysis.

INTRODUCTION

At present, some form of hydrogen bromide (HBr) titration is generally accepted as the best primary method for determining the cyclopropenoid acid (CPA) content of cottonseed oils (1-5). There is no general agreement on a single variation of the method. These methods can yield accurate results, but all variations of the basic method involve potential sources of error. These errors include loss of HBr during storage or titration, interference by monoglycerides and epoxides, overbromination, loss of CPA during sample cleanup, fractionation of the oil or esters during cleanup, and reaction of CPA with acetic acid during titration in this solvent (1). Another primary method is needed. Selective derivatizations of CPA followed by gas chromatographic (GC) analysis of the products have been proposed as primary methods (6,7). However, Coleman (5) found that the method of Raju and Reiser (6) was unsatisfactory and that the method of Schneider et al. (7) was satisfactory at high CPA levels but not at the levels found in cottonseed oils.

Although Recourt et al. (8) used a direct GC method for analysis of methyl esters of several oils that contained 8-58% CPA, others (6,7) have concluded that CPA esters are too unstable for analysis by GC methods. Since those studies, however, major advances have been made in gas chromatography of unstable compounds. At our request, a column manufacturer chromatographed samples of *Sterculia foetida* and cottonseed methyl esters on an inert glass column of the type used for sterol alcohols and found that peaks for malvalate and sterculate could be detected in the cottonseed esters. There was no obvious decomposition of CPA esters in either sample. Encouraged by these results, we reinvestigated gas chromatography of CPA methyl esters and developed the method reported in this paper.

EXPERIMENTAL PROCEDURES

Materials

Distilled-in-glass solvents (Burdick & Jackson), tetramethylammonium hydroxide solution (TMAH) (Aldrich Chemical, 20% in methanol), methyl nonadecanoate (Supelco Inc.), and AOAC standard cottonseed esters (Supelco Inc., 0.72% CPA) were used as received. Samples of refined, bleached, and deodorized cottonseed, corn, and peanut oils were purchased from local groceries. Crude cottonseed and *S. foetida* oils were extracted from seed with hexane. Methyl malvalate and methyl sterculate were obtained by reworking small samples prepared about 10 years ago in this laboratory. Oxidation products were removed by chromatography on alumina and small amounts of other fatty acid esters were removed by low temperature crystallization. Final products were about 98% pure by gas chromatography.

Esterification

Methyl esters were prepared by interesterifying about 10 mg oil with 0.3 mL methanol/benzene (1:2) with 25 μL TMAH solution as the catalyst. Reaction time was 30 min at 50 C or 16 hr at room temperature. Upon completion, hexane (0.2 mL) and water (0.1 mL) were added. After the mixture was shaken and allowed to settle, the lower layer was transferred to another tube and extracted with about 0.2 mL hexane. The extract was combined with the original upper layer and washed twice with 0.1-mL portions of methanol/water (1:1). The final hexane solution was chromatographed without further purification.

When the internal standard was to be used, the esterification was scaled up to about 0.1 g oil. The final solution was dried, transferred to a tared vial, and evaporated to constant weight at 60 C in a stream of nitrogen. An equal weight of a 1% solution of methyl nonadecanoate was added, followed by about 2 mL decane.

Whenever esters or solutions of esters were to be stored before use, 0.1% propylgallate was added. Such samples were stored under nitrogen at 10 C.

CPA-Free Cottonseed Esters

These esters were prepared by reacting cottonseed esters with silver nitrate (7) or salicylic acid (N.L. Lovegren et al., unpublished data). The derivatives formed from the CPA esters were removed from the product by adsorption on alumina. Both methods gave Halphen-negative esters that had the same composition as the starting esters except for the absence of sterculate and malvalate.

Gas Chromatography

The GC analyses were done with a Hewlett-Packard 5710A dual-column, flame-ionization chromatograph interfaced with a Hewlett-Packard 3350 laboratory automation system. It was operated in the single column mode. Any similar chromatograph should be satisfactory, provided it has a heated on-column injection inlet that will accept a 0.25-in. glass column. The data system is convenient, but not essential, if an internal standard is used. Injection-port temperature was 200-250 C. Detector temperature was 300 C. These temperatures are not critical. The carrier gas was nitrogen at 20-30 mL/min. Column temperature was 180-210 C. Flow and temperature near the upper limit were used for most samples to minimize the time required for an analysis, but samples containing low concentrations of

[1] Retired.

[2] The Southern Region, Science and Education Administration, U.S. Department of Agriculture.

CPA required low flow or temperature, or both.

The critical part of the method is the column. It must be inert and it must provide at least 4000 effective theoretical plates. The original column was a 6-ft by 0.25-in. od (2 mm id) glass tube, Hewlett-Packard configuration 5 (heated on-column injection), specially packed with 5% SP2100 on 100/120 Supelcoport. It gave 5400 plates when received, but platage decreased to about 4500 plates after several months' use. Most of the results reported here were obtained with ordinary commercial columns of the same dimensions, one packed with the same packing, the other with 5% SF-96 on 100/120 Supelcoport. The stock SP2100 column gave about 4300 plates when received and has not changed. The SF-96 column gave only about 4000 plates as received, but after 2 or 3 weeks it gave about 4800 plates and has maintained this platage for several months.

RESULTS AND DISCUSSION

Stability

Stability of the CPA esters during GC analysis was established with simple mixtures of methyl stearate, sterculate and nonadecanoate. With methyl silicone-packed glass columns, peak-area normalization gave quantitative recoveries of the sterculate as a single peak. As shown in Figure 1, a single peak also was found for methyl sterculate in typical cottonseed esters. This figure also illustrates the need for a very efficient column to get usable resolution of malvalate from linoleate.

In contrast to glass columns, a stainless-steel column packed with the same SP-2100 on Supelcoport gave a second peak from sterculate. The 2 peaks combined did not account for all the sterculate added. Nickel and aluminum columns gave little or no decomposition of sterculate but were much less efficient than glass columns. Even in glass, diethyleneglycol succinate, the classic substrate for GC analysis of fatty acid esters, decomposed sterculate. With Apolane-87, which is less polar than SP-2100, sterculate and stearate emerged as a single peak.

As shown in Figure 2, decomposition of sterculate to give peak 11a can occur even with glass columns. Presumably, malvalate was also decomposed, but its second peak was under the linoleate peak. Peak 11a does not always indicate a bad column. In the chromatogram shown in Figure 3, peak 11a came from cottonseed oil used to prepare the CPA-free esters, not from the added sterculate. Periodically, and when more than about 0.02% of peak 11a is found in a sample, column condition should be checked by running a sample, such as the AOAC CPA-standard esters, that contains little or none of this peak.

When a column begins to deteriorate, it can often be restored by injection of diethylethylenediamine or hexamethyldisilazine. The column used to get Figure 2 could not be regenerated, but the decomposition of sterculate was

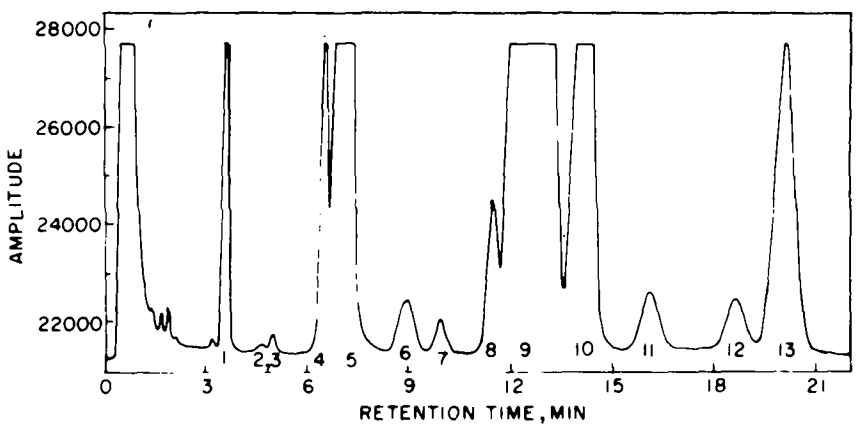

FIG. 1. Cottonseed methyl esters plus methyl dodecanoate: 4800 plate column; 200 C; 30 mL/min nitrogen; peaks 1. myristate, 2. pentadecadienoate + pentadecenoate, 3. pentadecanoate, 4. palmitoleate, 5. palmitate, 6. heptadecadienoate + heptadecenoate, 7. heptadecanoate, 8. malvalate, 9. linoleate + oleate, 10. stearate, 11. sterculate, 12. dihydrosterculate, 13. nonadecanoate.

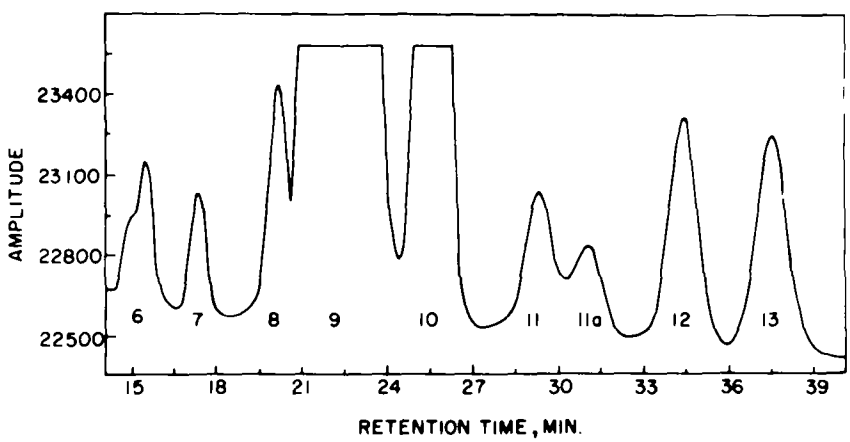

FIG. 2. Cottonseed esters plus methyl nonadecanoate: bad 4500 plate column; 185 C; peak 11a, sterculate decomposition product, rest as in Fig. 1.

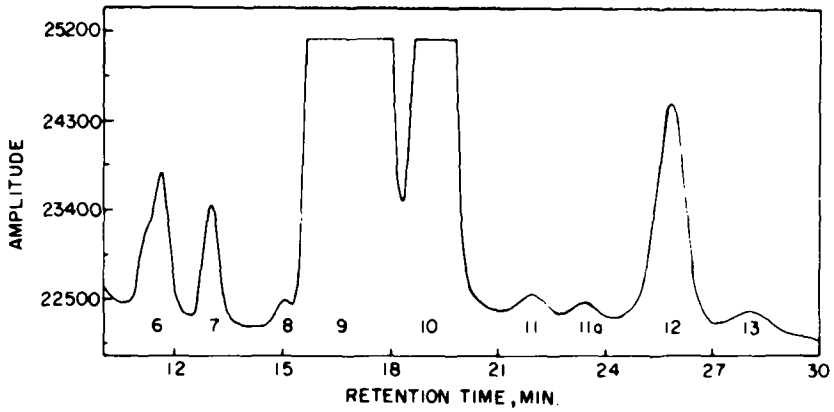

FIG. 3. CPA-free cottonseed esters plus malvalate, sterculate, and nonadecanoate: 4200 plate column; 185 C; peaks as in Fig. 2.

eliminated by repacking the portion of the column that was in the injection port. Deterioration of the column can be delayed by injecting .1 or .2 of 1 μL of a 1% solution of the amine in hexane with each sample.

Quantitation

Like all conventional electronic integrators, our data system uses peak-area normalization to calculate composition. Areas of small peaks like malvalate on the front of large peaks like linoleate are estimated by dropping a perpendicular from the valley to the baseline. This partitioning of the overlapping area assumes that the overlap is symmetrical. This method of quantitation gave reproducible concentrations for malvalate and sterculate in cottonseed esters. However, careful examination of the chromatograms indicated that the overlap was not symmetrical. Hence, the accuracy of the quantitation was checked by analyzing simulated cottonseed esters prepared by adding known amounts of a mixture of methyl-malvalate, methyl-sterculate, and methyl-nonadecanoate (internal standard) to CPA-free cottonseed esters. As shown in the last column of Table I, the computer method was reasonably accurate for all but the lowest level of sterculate, but underestimated malvalate at all levels. Figure 3 is one of the best chromatograms of the lowest-level sample. At 200 C, with faster carrier-gas flow, with a less efficient column, or with much lower concentration of malvalate, the malvalate peak becomes a shoulder that cannot be quantitated by the computer.

Because the chromatogram of the CPA-free esters was still on the baseline at the emergence time of malvalate, the height reported by the data system for the malvalic peak should be correct, even though the area is wrong. Hence, we tried quantitation from peak heights. Comparison of peak height and area data for the mixtures of pure esters that had been analyzed revealed that the ratio of area or concentration (C) of a peak to its height (H) was a linear function of its retention time (T). That is, C=kHT for malvalate, stearate, sterculate, and nonadecanoate. This relationship was also valid for the heptadecanoate and dihydrosterculate peaks in cottonseed esters. Hence, malvalate and sterculate concentrations can be calculated from the concentration of nonadecanoate added as an internal standard or from the concentration found by peak-area normalization for heptadecanoate, stearate, or dihydrosterculate, by the equation: $C_x = (C_r H_x T_x)/H_r T_r)$, where C, H, and T are concentration, height and retention time, the subscript x denotes malvalate or sterculate, and the subscript r is one of the 4 reference esters.

This method of quantitation gave acceptable accuracy for both malvalate and sterculate with all 4 reference esters (see Table I). By manual measurement of peak heights and retention times, the lowest malvalate concentration could be determined in all 5 GC runs. Within experimental error, concentrations calculated from manual measurements for several other samples agreed with those calculated from heights and retention times supplied by the data system. Hence, the method can be used with any good electronic integrator. In fact, when the internal standard is used, malvalate and sterculate concentrations can be calculated without an integrator. As would be expected, the accuracy of the determinations decreases with decreasing concentrations of CPA; thus the method is only semiquantitative for samples containing much less than 0.05% CPA.

As shown in Tables II and III, reproducibility as well as accuracy was similar for all reference peaks. Hence, we use all 3 reference peaks for quantitation and average the results. Malvalate concentration can be calculated from the peak-area normalization value for sterculate concentration, but we prefer to use this value as a cross-check on the values calculated by the equation. Occasionally, one of the reference peaks gives values that are substantially higher than those calculated from the other 2 reference peaks. Inspection of the chromatogram usually reveals that the

TABLE I

Recovery of Added CPA Esters[a]

Added (%)	Recovered (% of added) based on[b] C17	C18	C19Δ	C19	COMP
	Sterculate				
0.429	104.2	99.3	102.1	99.3	100.9
0.137	98.5	100.0	100.0	100.0	95.6
0.072	95.8	95.8	97.2	100.0	91.7
0.034	85.2	88.2	88.2	91.2	73.5
	Malvalate				
0.368	101.4	98.9	98.9	98.9	88.9
0.118	95.8	97.5	97.5	97.5	83.9
0.062	93.5	91.9	93.5	95.2	75.8
0.030	103.3	100.0	103.3	103.3	75.0[c]

[a]Mixture of methyl malvalate, methyl sterculate and methyl nonadecanoate added to CPA-free cottonseed methyl esters.

[b]C17 = Methyl heptadecanoate found; C18 = methyl stearate found; C19Δ = dihydrosterculate found; C19 = methyl nonadecanoate added; COMP = peak-area normalization by computer.

[c]Average of 3 runs. None found in 2 others.

TABLE II

Reproducibility with Esters[a]

	Concentration (%) calculated from[b]		
	C17	C18	C19Δ
	Range		
Malvalic	0.340-0.361	0.335-0.371	0.345-0.374
Sterculic	0.196-0.211	0.202-0.212	0.199-0.220
	Mean (standard deviation)		
Malvalic	0.349(0.007)	0.356(0.012)	0.359(0.011)
Sterculic	0.203(0.005)	0.207(0.004)	0.209(0.008)
Total CPA[c]		0.561(0.016)	

[a]Seven replicates on a single batch of methyl esters.
[b]See Table I.
[c]Mean of all calculations.

TABLE III

Reproducibility with Cottonseed Oil[a]

	Mean (standard deviation), %, calculated from[b]		
	C17	C18	C19Δ
Malvalic	0.455(0.027)	0.443(0.018)	0.431(0.014)
Sterculic	0.244(0.008)	0.238(0.011)	0.232(0.005)
Total CPA[c]		0.680(0.030)	

[a]Four batches of esters from a cottonseed oil.
[b]See Table I.
[c]Mean of all calculations.

peak that gives the odd results is broad or has an unresolved shoulder or tail. The odd values are therefore rejected, and the remaining 2 values are averaged. If no 2 of the sterculate concentrations agree with each other or with the computer value, the run is discarded. For Table II, GC parameters were kept constant in an attempt to make quantitation the only source of variation. In contrast, for Table III, esterification and chromatographic parameters were all varied within the ranges given in the experimental section. These deliberate variations of conditions only increased the coefficient of variation of the CPA content from 3 to 4%. This variation is similar to that reported by Coleman (5) for HBr titration.

HBr titration data were available for 3 of the samples that we analyzed by gas chromatography. Results are compared in Table IV. The excellent agreement between the results by HBr titration in toluene and by peak-height quantitation of GC data indicates that both methods give

TABLE IV

Comparison with HBr Titration

Oil	HBr (%)	GC (%)	GC/HBr
E0114[a]	0.570	0.561	0.98
SUP[b]	0.72	0.673	0.93
NVI[c]	0.518	0.473	0.91

[a]Esters from a commercial refined cottonseed oil, titrated in toluene (4).
[b]Official AOAC esters, titrated in acetic acid.
[c]Esters from a commercial cottonseed salad oil, titrated in acetic acid.

accurate CPA concentrations. The excellent recovery of added malvalate and sterculate at these levels (Table I) supports the accuracy of the GC method. Hence, the poor agreement between HBr-acetic acid titration and gas chromatography suggests that Brown's method (2) gives high values, perhaps because his correction factor, which was determined by titrating pure CPA esters, is not applicable at the low concentration of CPA present in cottonseed esters.

Our GC method has some advantages over the HBr methods. It yields individual concentrations for malvalate and sterculate, which cannot be obtained by titration. It requires less than 1 mg oil and only minimal sample preparation; CPA content of a single cottonseed thus can be determined easily. Its major disadvantage is that it requires a gas chromatograph and a strip-chart recorder that are much more expensive than a buret and a stirrer.

ACKNOWLEDGMENTS

The authors thank D.M. Ottenstein, Supelco, Inc., for his assistance in the early phases of this work. They also thank R.O. Feuge, L.P. Codifer, Jr., H.J. Zeringue and C.J. Fernandez of this laboratory for supplying samples and HBr titration data.

REFERENCES

1. Harris, J.A., F.C. Magne and E.L. Skau, JAOCS 40:718 (1963).
2. Brown, L.E., Ibid. 46:654 (1969).
3. Rosie, D.A., and G.G. Shone, Analyst 94:477 (1969).
4. Feuge, R.O., Z. Zarins, J.L. White and R.L. Holmes, JAOCS 46:185 (1959).
5. Coleman, E.C., J. Assoc. Off. Anal. Chem. 53:1209 (1970).
6. Raju, P.K., and R. Reiser, Lipids 1:10 (1966).
7. Schneider, E.L., S.P. Loke and D.T. Hopkins, JAOCS 45:585 (1968).
8. Recourt, J.H., G. Jurriens and W. Schmitz, J. Chromatogr. 30:35 (1967).

[Received March 30, 1981]