Improved Halphen Method for Measuring Cyclopropenoid Fatty Acids Using Monoclinic Sulfur

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An improved method is described for the quantitative determination of cyclopropene fatty acids (CPFA) in lipid extracts of plant or animal tissues, based on the formation of a pink-colored complex by the reaction of the cyclopropene ring with sulfur dissolved in carbon disulfide and pyridine (Halphen reaction). Assay parameters such as time, temperature, solvents, and reagents were evaluated to select the best reagents and procedure. Crystalline monoclinic sulfur and spectroquality pyridine are essential for reproducibility of the method. A typical standard curve for methyl sterculate is linear over the range 0 to 100 μg CPFA.

Since the cyclopropenoid fatty acids (CPFA) have been shown to cause many deleterious biological effects (Phelps *et al.*, 1965; Reiser and Raju, 1964; Schneider *et al.*, 1961; Sheehan and Vavich, 1965), a satisfactory analytical procedure for measuring their concentration in both vegetable oils and animal tissue lipids would be most useful and necessary for future research. However, the unstable characteristics of these fatty acids have made development of a simple analytical procedure somewhat difficult. Three basic methods have been used for assay of CPFA activity as follows: the Halphen reaction in which sulfur dissolved in carbon disulfide reacts with the cyclopropene ring to produce a colorimetrically measurable chromogen (Deutschman and Klaus, 1960; Halphen, 1897); the hydrohalogen acid titration in which halogen acids are added to the double bond of the cyclopropene ring (Feuge *et al.*, 1969; Harris *et al.*, 1963, 1964; Magne *et al.*, 1966); and methods of gas–liquid chromatography of cyclopropenoid derivatives (Kircher, 1965; Raju and Reiser, 1966; Schneider *et al.*, 1968). Infrared spectroscopy and nuclear magnetic resonance methods have also been investigated (Baily *et al.*, 1965; Magne, 1965).

This paper reports an improved Halphen method which provides a simple, reproducible quantitative assay for determining the CPFA concentration in vegetable oils and animal tissue lipids. Improvements were based on choice of a more soluble form of sulfur, quality of pyridine, time, and temperature of reaction studies, and preparation of methyl sterculate standard. The spectrophotometric method of Deutschman and Klaus (1960) is generally unsatisfactory at low levels of CPFA. The method is not reproducible, partially because of the less soluble amorphous sulfur used. The form of sulfur used in the present paper was monoclinic sulfur, which is completely soluble in carbon disulfide at the level described. The quality of the pyridine is also an important factor and spectroquality pyridine is required for reproducibility of the order presented in this paper. Using the conditions and reagents set forth in this paper, a reliable and reproducible method that is simple and quick is available for determination of CPFA concentrations in various oils and lipid extracts.

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MATERIALS AND METHODS

Assay parameters such as time, temperature, solvents, and reagents were evaluated to select the best reagents and procedure as follows.

Reagents. METHYL STERCULATE. Prepared by the urea adduct method of Kircher (1964) from *Sterculia foetida* oil. COLOR REAGENT. 4% Monoclinic crystalline sulfur dissolved in A.R. grade carbon disulfide (w/v). MONOCLINIC CRYSTALLINE SULFUR. Monoclinic sulfur was prepared according to Sommer (1940) as follows. Reagent-grade pyridine was heated to 85° C and sufficient A.R. or USP grade flowers of sulfur (amorphous) was added to saturate the solution. The solution was allowed to cool to room temperature and then refrigerated overnight at 4° C to precipitate the monoclinic crystals. The crystals were separated by filtration, allowed to air dry for 24 hr to remove the pyridine, ground to a powder, and stored in a brown bottle at room temperature.

Procedure. A stock standard of methyl sterculate was made by dissolving a known quantity of freshly prepared methyl sterculate in diethyl ether; aliquots of the stock standard were diluted with diethyl ether to provide working standards which contained 25, 50, 75, and 100 μg of methyl sterculate per ml. An oil or lipid sample to be analyzed for CPFA was dissolved in diethyl ether and diluted with ether to contain approximately 25 to 100 μg of CPFA/ml of diethyl ether.

One milliliter of unknown or standard was placed in a 22 × 175 mm test tube (permanently marked for 10-ml volume) and 1 ml of color reagent and 8 ml of *spectroquality* pyridine were added. A reagent blank was prepared in the same manner using 1 ml of diethyl ether. Each tube was mixed by swirling, capped with a 25-mm glass marble, and placed in a glycerin bath at 48° C for 15 min. The temperature of the bath was increased to 95° C (required approximately 25 min), held at 95° C for 5 min, and then the temperature of the bath was increased to 105° C. After holding at 105° C for 1 hr, the

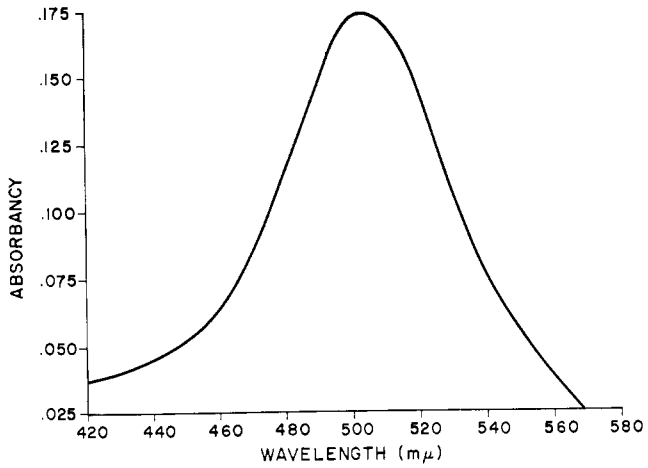

Figure 1. Absorbance of pure methyl sterculate

Table I. Absorbance of Freshly Prepared Methyl Sterculate

Methyl sterculate, μg	Absorbance ± S.D.
25.4	0.085 ± 0.003[a]
50.7	0.163 ± 0.004
76.1	0.246 ± 0.006
101.4	0.332 ± 0.007

[a] Each point represents 16 observations ± standard deviation.

Table II. Recovery of Cyclopropenoid Fatty Acids in *Sterculia foetida* Oil

Sterculia foetida oil, mg	Calculated CPFA,[a] μg	Analyzed, CPFA, μg	Percent recovery, μg analyzed/ μg calculated × 100
25	8.0	7.0	87.5
50	6.0	15.0	93.7
75	24.0	25.0	104.1
100	32.0	32.0	100.0
150	48.0	47.0	97.9

[a] Based on 32.5% CPFA in *Sterculia foetida* oil.

tubes were allowed to cool to room temperature, made to 10 ml volume with spectroquality pyridine, mixed, transferred to colorimeter cuvettes, and read at 505 mμ in a spectrophotometer (Coleman Jr. or equivalent) exactly 1 hr after removal from the bath.

A standard curve was plotted and the concentration of CPFA of the unknown was calculated from the standard curve as methyl sterculate.

RESULTS AND DISCUSSION

The characteristic absorption curve of methyl sterculate obtained under the assay conditions described above is shown in Figure 1. Maximum absorption occurs in the region 500 to 510 mμ with the peak at 505 mμ. In some samples masking pigments or reaction by-products result in an orange reaction product instead of the salmon pink of the Halphen reaction. Lipids of control animals (not supplemented with CPFA) often showed this off-color orange reaction product. The absorption of these orange reaction products shows no maximum in the range 500 to 510 mμ but tailing from a peak around 450 mμ could introduce some error. In our laboratories the degree of this false reaction color was usually less than 0.05% of the total lipid extract.

Table III. Recovery of Cyclopropenoid Fatty Acids from Cottonseed, Corn, and Safflower Oils after the Addition of Methyl Sterculate or *Sterculia foetida* Oil

Oil with added methyl sterculate	Calculated % CPFA	Analyzed % CPFA	Recovery %
Cottonseed oil	1.94	1.98	102
Corn oil	1.26	1.24	98
Safflower oil	1.33	1.31	98
Oil with added *Sterculia foetida* oil			
Cottonseed oil	1.36	1.42	105
Corn oil	0.79	0.75	95
Safflower oil	0.79	0.74	94

Table IV. Cyclopropenoid Fatty Acid (CPFA) Content of Lipid Extracts of Liver, Blood Plasma, and Body Fat of Rats Fed *Sterculia foetida* Oil

Lipid extract of	Concentration of *S. foetida* oil in the diet, %	CPFA in lipid extract, %
Liver	1	1.29
	2	3.25
	3	6.88
Body fat	1	1.04
	2	3.43
	3	4.75
Blood plasma	3	3.76

A typical standard curve is listed in Table I where each value represents 16 observations. The linear equation of the line is represented by the equation

$$Y = 306.654X + 0.109 \quad (1)$$

and the correlation for the line is 0.998.

The data in Table II show that this method recovered from 87.5 to 104.1% of CPFA in the range of 8 to 48 μg CPFA as *Sterculia foetida* oil.

Recovery experiments with known concentrations of methyl sterculate or *Sterculia foetida* oil added to various oils are presented in Table III. The calculated CPFA activity of the various oils with the cyclopropenoid fatty acids added was compared to the analyzed values and expressed as percent recovery. Addition of methyl sterculate or *Sterculia foetida* oil resulted in good quantitative recovery. Additional experiments have been carried out with a wide range of oils and biological lipid extracts with similar quantitative recoveries.

The CPFA concentrations of lipid extracts of rats fed *Sterculia foetida* oil are reported in Table IV. The method of lipid extract used in our laboratory was a combination of Folch *et al.* (1951, 1956) and Bligh and Dyer (1959) using chloroform:methanol (2:1) and magnesium chloride (0.003 *M*) as the extraction solvents. Analyses of the various organs of chickens fed *Sterculia foetida* oil have also been done.

These data show that considerable amounts of CPFA can be absorbed and stored by the rat (or chicken) and that the total accumulated CPFA in liver and body fat is roughly proportional to the dietary intake.

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