AN ABSTRACT OF THE THESIS OF

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Title: Glass Capillary Gas Chromatographic Analysis For Trace Amounts of Cyclopropenoid Fatty Acids

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Norman E. Pawlowski

Dietary cyclopropenoid fatty acids (CPFA) have long been known to cause pronounced physiological disorders in both farm and laboratory animals. Past work has shown CPFA to be a powerful promoter of carcinogenesis in trout, rats and mice. Sterculic and malvalic acids (CPFA's) are found in seed lipids of plants from the order Malvales. Two members of this order are cotton and kapok, both of which are used extensively as cooking oils for human consumption.

Present chemical and instrumental methods of analysis for CPFA are effective only at CPFA levels above 0.1%, and accurate only at levels above 1%. A more sensitive method of analysis was developed exploiting recent technological advances in glass capillary gas chromatography (GCGC). By the use of cold on-column injection, and positioning of the column at the base of the detector flame, this method eliminates two problem areas found in other GC methods. The other common component involved in CPFA decomposition, during GC analysis, is the column. Vitrious silica

columns with an inert stationary phase, SE-30, were shown to separate the highly reactive CPFA without decomposition. Proof of stability during analysis was obtained by changing the variables of relative time on column and column temperature. This method not only allows individual determination of sterculic and malvalic acid concentrations, it is rapid, accurate (to the 70 parts per million range), and is superior to other instrumental and chemical methods.

The CPFA concentration for okra, hollyhock, cheese weed, seashore mallow, kapok, and white cap cottonseed oil are as follows: 0.3% to 0.92%, 0.33%, 2.6%, 2.6%, 12.8%, and 75ppm, respectively. The two different values for okra were found because seeds from two different growing seasons were analyzed. No CPFA could be detected in Diet Imperial Margarine, raw cocoa beans, cocoa butter or Lucca's winterized cottonseed oil.

Glass Capillary Gas Chromatographic Analysis for Trace Amounts of Cyclopropenoid Fatty Acids

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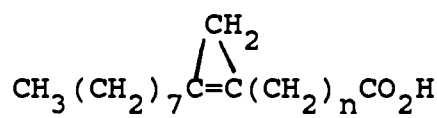
INTRODUCTION

DISCOVERY

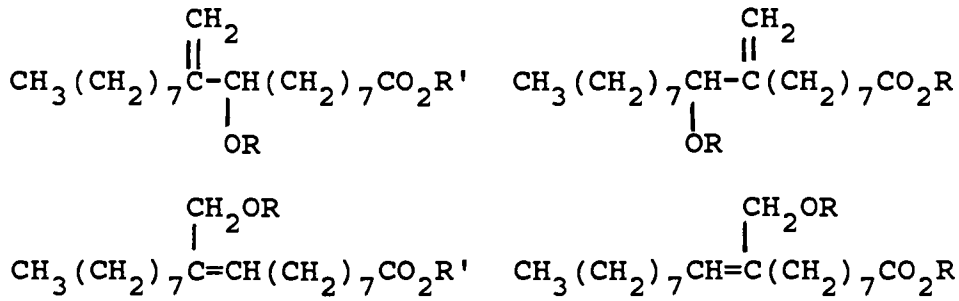
Many chemical and instrumental methods of analysis for cyclopropenoid fatty acids (CPFA) have been developed over the years. An empirical test was developed by G. Halphen in 1897 to detect adulteration of edible oils by the addition of less expensive cottonseed oil (Halphen, 1898). Once the Halphen test was derived all oils were tested and classified as Halphen active or inactive. Most oils from the plant order Malvales were found to be active while most oil sources outside this order were inactive. It was not until 1952 that the responsible reactive component was discovered and isolated by J. R. Nunn (1952). He named this compound sterculic acid, since it was isolated from Sterculia foetida oil (SFO), and assigned it the structure 9,10-methyleneoctadecenoic acid. In 1957 another Halphen active species was isolated from cottonseed oil by J. J. MacFarline and assigned the structure 8,9-methyleneheptadecenoic acid (MacFarline et al., 1957). This compound was given the common name malvalic acid. Further analysis showed both of these

species to be significant components of oils from the seeds (as well as tissues) of both cotton and Sterculia foetida. The chemical formulation for these two cyclopropenoid fatty acids can be represented as

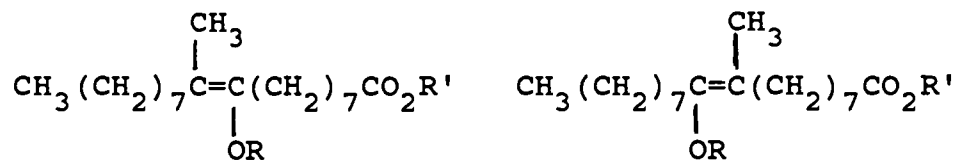


where n = 6 for malvalic acid and n = 7 for sterculic acid.

The cyclopropene ring, one of the most strained structures found in Organic chemistry, is a surprising unit to find occurring in nature. As can be predicted from such a strained structure the ring is very reactive. This is due to a 27 kcal/mole reduction in strain energy upon conversion to the cyclopropane via addition reactions (Carter and Frampton, 1964). The ring also has a tendency to complex with metals and readily undergoes ring-opening reactions. Nunn (1952) discovered that sterculic acid polymerizes at room temperature via a ring-opening reaction, in contrast to the situation for simple cyclopropenes with substituents at the 3-position which undergo ene reactions while maintaining the three member ring. The fatty acid free carboxyl group apparently induces ring opening. Major products include allylic derivatives of the type shown below



where R and R' are sterculic acid residues (Rinehart et al., 1961). The occurrence of these products is readily explained in terms of the intermediacy of cyclopropyl cations which ring-open to their allyl isomers. Two other products of the type shown below were also observed.



These products could not be accounted for by Rinehart, but Kircher (1964) demonstrated that, under the conditions used, the olefinic linkages are not degraded. He postulated protonation of the cyclopropene ring at its 3-position followed by ring-opening to vinyl cations. This is in agreement with the findings of York et al. (1973) that vinyl carbenes are thermally accessible from cyclopropenes. Greenberg and Harris (1982) reviews many reactions of the cyclopropenes and suggests mechanisms involved which could account for biological activity of the cyclopropenoid fatty acids.

Nuclear magnetic resonance (NMR) can be used to verify the structure of CPFA. Sterculic and malvalic acid both have two distinct absorptions between delta = 1.1 and 0.6 where delta represents the chemical shift due to a change in the applied magnetic field (Pawlowski et al., 1972). A single sharp peak at delta = 0.76 is due to the unsplit pair of protons on the cyclopropene ring bridge. Protons in three member and strained rings typically show shifts towards higher field absorption. The absorption at delta = 0.76 for a 1,2-disubstituted cycloproprene ring is consistent with shift constants observed in numerous other cyclopropane and substituted cyclopropene systems, and matches exactly the spectra of synthetic sterculic and malvalic acids. The other absorption at delta = 0.92 is typical of fatty acids and alkyl compounds with an unshifted, terminal methyl group.

The plant order Malvales includes four families: Malvaceae, Sterculiaceae, Tiliaceae, and Bombacaceae. Some commonly recognized members of the order are cotton, kapok, okra, durian, china chestnut, hibiscus, cocoa, hollyhock, marsh mallow, and china jute (Carter and Frampton, 1964). Although several of these are not consumed directly by man, they may enter the food chain through ingestion by animals which can be eaten by man. In the United States, as well as western European countries, cottonseed (Gossypium hirsutum) oil is used in the preparation or production of many food products, while

kapok (Eridandran anfractosum) serves a similar role in Asian countries. Cottonseed meal has long been used as a major protein supplement for farm animals including chickens, pigs, cattle and fish. Cottonseed flour is receiving increased attention as a source of protein for human consumption and roasted glandless cottonseed kernels are a snack item available in the United States. It has also been suggested that glandless cottonseed has potential as a commercial source of edible lecithin (Cherry and Gray, 1981). Peanut butter, another popular food item, is sometimes extended with cottonseed oil. Durian is a highly prized fruit cultivated throughout Southeast Asia (Berry, 1980b).

EFFECTS

Farm animals consuming cottonseed products show numerous physiological disorders. As early as 1892 it was discovered (Harrington and Adrianee, 1893) that cottonseed and cottonseed meal, which contained large amounts of liquid oil, produced hardening of agricultural products such as butter, sheep suet, lard and beef tallow. This is just the opposite of what is expected since cottonseed oil is highly unsaturated. In 1928 chickens fed cottonseed oil, as part of their diet, developed pink egg whites and bronze yolks which had a pasty texture [Sherwood, 1928]; oils which caused this effect also

produced a positive Halphen reaction. In addition it was found that when dairy cows ingested these oils their milk quality decreased appreciably.

In 1965 Phelps et al. (1965) found rats fed a diet containing CPFA exhibited several deleterious effects among which were growth retardation, increase in organ to body weight ratio, increase in saturation of tissue lipids, delayed sexual development in females and altered fatty acid metabolism. Miller et al. (1969) found a high incidence of prenatal and postnatal mortality in progeny of rats fed CPFA. Various workers have reported CPFA inhibited fatty acid desaturase in chickens (Allen et al., 1967, Johnson et al., 1969), rats (Raju and Reiser, 1966), pigs, cows (Phelps et al., 1965), and trout (Roehm et al., 1970) causing the stearate to oleate ratio to rise. This rise in stearate to oleate ratio is responsible for the hardening of fat observed in farm animals. Nixon et al. (1974) did a comprehensive study with rats comparing a diet containing 1% CPFA (source SFO), 1% hydrogenated SFO, and 1% corn oil (control). The rats fed the diet containing 1% CPFA, when compared with the control group, exhibited 50% growth retardation, two-fold increase of organ to body weight (due to enlarged liver and kidney), more than two-fold increase in saturation of tissue lipids, as well as abnormal histopathology when compared with rats on both the control (1% corn oil) and the 1% hydrogenated SFO diets. It was further found that rats on

the diet containing the 1% hydrogenated SFO exhibited no adverse effects and behaved the same as those on the control diet. Nixon, like Phelps, concluded all detrimental effects could be associated with alteration of the normal lipid metabolism and membrane function. In 1976 Ferguson (1976) found rabbits fed 0.27% CPFA developed liver cells not only with altered morphology but, often, with extensive damage.

Diets containing CPFA have also shown adverse effects on cholesterol levels. Tennet (1959) and Goodnight and Kemmerer (1967) both found cockerels, on diets containing cottonseed oil or SFO (200 mg/day), produced significantly higher levels of plasma cholesterol and aortic atherosclerosis than birds fed diets containing an equivalent amount of non-CPFA containing oils. Ferguson et al. (1976) found similar higher plasma levels in rabbits when fed diets containing 0.27% CPFA, 0.5% cholesterol, or a combination of the two. They also exhibited a higher incidence of aortic atherosclerosis. Eisele et al. (1982) found serum cholesterol levels increased by 40%, over the control, when rabbits were fed 0.5% CPFA for 28 days. When Matlock (1985) compared mice fed diets of either 0.5% CPFA for six weeks or 0.7% CPFA for eight weeks with mice on a control diet those on the diet rich in CPFA exhibited hyperlipidemia, hypercholesteremia, and increased levels of free serum cholesterol. He further found the percentage of saturated

fatty acid residues increased at the expense of the monosaturated fatty acids in the high density lipoproteins and cholesterol esters. There was also an increase in linoleic acid at the expense of arachidonic acid in all the serum lipid fractions.

The effects of CPFA's have been tested in trout, rats, and mice. Sterculic acid has been found to be carcinogenic in rainbow trout (Salmo gairdneri) (Sinnhuber et al., 1976) and, when fed at 50 parts per million CPFA to 1 part per billion aflatoxin B_1, cocarcinogenic, causing an increase in hepatomes (Lee et al., 1968, Sinnhuber et al., 1968 and 1974). Sinnhuber et al. (1976) concluded CPFA's are powerful promoters in fish, acting upon spontaneous tumors which might not otherwise be detected. Fan et al. (1982) did a short term dietary study using cottonseed oil, known to be richer in malvalic acid than sterculic acid. This study found no significant depression of several MFO enzymes in rats, which led the workers to conclude there were no deleterious effects associated with cottonseed oil. The length of time used for the test diet was not only much shorter than comparable studies, but the dietary level was 0.3% while most comparable studies use 0.5% or higher levels. It is not clear as to how these particular enzymes might effect the biological activity of CPFA, if they effect the activity at all. Large variations in control levels may have contributed to the lack of observed enzyme depression

in this study. Malvalate, which has been shown to be only about one-third as active as sterculate [Pawlowski et al., 1985], is the principle CPFA in cottonseed oil. The objectivity of Fan et al. is further obscured by their financial interest in the continued use of low cost cottonseed oil in commercial processing of corn chips. Tinsley (1981 and 1982) observed promotional activity of mouse mammary tumors by cottonseed oil, when compared to twelve other lipid sources, at the 0.5% dietary level.

Glandless cottonseed kernels, as purchased for human consumption, added to the diet of rainbow trout (20% level), as a protein source, produced a 75% tumor incidence as compared with 0% for the control group (Hendricks et al., 1980).

Nature may have had a reason for CPFA in plants as was suggested by Binder and Chan (1982). The CPFA found in cotton plants (approximately 0.5%) has a moderate ability to inhibit lepidopterous larval growth. This effect is greatest in the young immature plants, the point of entry for the larvae and coincidental with the highest CPFA content. As a plant matures the CPFA concentration decreases (Yano et al., 1972, Pandey and Subrahmanyam, 1986, Fisher and Cherry, 1983).

Cyclopropenoid fatty acids not only cause numerous deleterious effects on farm animals, but accumulate in their body tissues and lipids. Data on trout, rabbits, chickens, chicken eggs, mice, and rats suggest CPFA is

incorporated into body lipids at near the dietary level (Roehm et al., 1971, Shenstone and Burley, 1975, Fan et al., 1982). Hawkins et al. (1985) found CPFA concentration in milk proportional to the level in the feed of dairy cattle. These could serve as an additional source of CPFA for humans consuming the animals, or animal products. Man can easily avoid plants known to contain the CPFA, but how is one to know if the chicken or fish you are about to eat has been fed cottonseed meal or some other CPFA source. This emphasizes the need for a fast, simple, reliable quantitative test for determining the CPFA content at the typical levels found in possible food sources. Before discussing this work a review of the known methods of analysis is in order.

METHODS

The first chemical test became known as the Halphen test (Halphen, 1898). It was developed to detect the presence of cottonseed oil as an adulterant in more expensive edible oils. Halphen's original test involved the addition of equal volumes (1 to 3 ml) of oil, amyl alcohol and carbon disulfide containing 1% free sulfur to an open tube which was heated slowly in a boiling bath of aqueous sodium chloride for 10 to 15 minutes. This heating period was sufficient to allow some loss of carbon disulfide. The presence of cottonseed oil was indicated

by the formation of a pink to red solution. The variation in color was found to be due to the formation of a number of pigments in various proportions (Bailey et al., 1965). Unfortunately the proportions and amounts of these pigments are also affected by impurities in the sample.

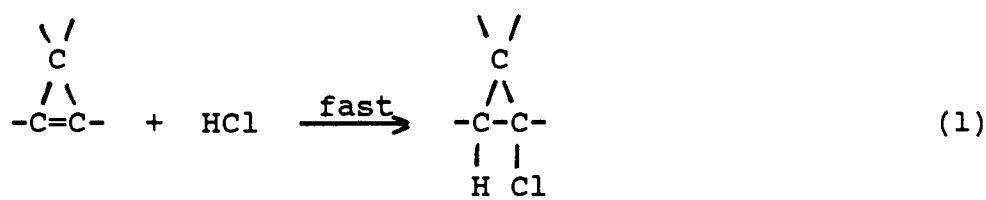
Since the original Halphen test was developed many modifications have been tried (Nunn, 1952, Shenstone and Vickery, 1961, Bailey et al., 1965). Those tired included variation of heating times, changing the temperature of the bath, addition of excess sulfur, omission of amyl alcohol, the addition of an additional reagent such as pyridine, and the use of sealed tubes in place of the condensation tube. All of these modifications had one thing in common and that was the varying end point. Deutschman and Klaus (1960) improved on the conventional qualitative methods by comparing spectrophotometrically determined end point readings with a standard absorbance curve created by the addition of known concentration pure sterculic acid to corn oil. This method has a reported accuracy of $\pm$10% with a 95% confidence at levels of 0.03-0.04% sterculic acid according to Magne (1965). Bailey et al. (1961 & 1965) further refined Deutschman's method with the use of standards whose concentrations are established by an HBr-titration. Bailey et al. (1965) also introduced some color stabilizing techniques. All of these are considered as the Halphen method and, as such, have several disadvantages. They are based upon the use of

standards, which, at best are not of exactly known concentrations (usually determined by the Halphen method itself), only the total CPFA concentration can be determined, and results are dependent upon the source and purity of the oils used. It has also been found CPFA can undergo dimerization reactions in the standard thus changing the apparent concentration of the standard, which is a common reaction in concentrated solutions containing this labile, reactive, strained ring (Lee et al., 1968). The Halphen test does have an advantage over other chemical methods in that it is quick, simple, and does not require expensive instrumentation. Coleman (1970) concluded the Halphen test was the best general method, when calibrated, with a reasonable pure CPFA standard analyzed by the silver nitrate-methanol/GLC method or the HBr-benzene back titration method for determining levels below 2%. Coleman's finding is based upon his evaluation of five quantitative methods as they existed in 1970. This laboratory concluded the Halphen test can still serve as a good qualitative, and sometimes semiquantitative, method for detection of CPFA, but only when the concentration is above 1%, unlike Magne (1965) who claimed a detection level of 0.03% sterculic acid.

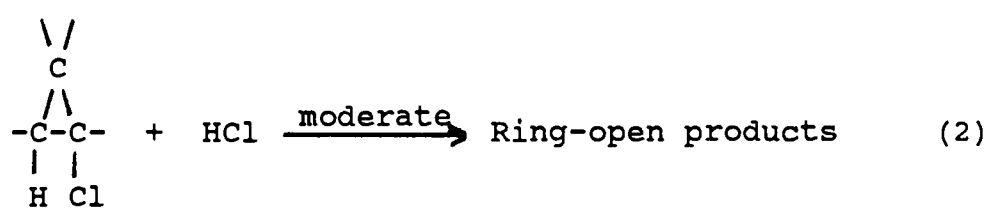
Hammonds et al. (1971) developed what became known as the Modified Halphen Test. In this method the methyl esters of the oils are used in place of the oils. Hammonds found little correlation between HBr-titration

and his Modified Halphen method of analysis unless oil was first converted to its methyl ester. Evidently color development is influenced by the presence of cyclopropenoid glycerides and the use of methyl esters instead of the parent CPFA containing oil results in improved absorbance correlation. In 1972 Coleman and Firestone (1972) made one further change in this method when they found butanol to be a better solvent for the methyl esters.

Another basic analytical method exploited the reaction of an hydrohalic acid with the cyclopropene moiety. It was believed that concentrated HCl reacted only with the cyclopropene group; however, 2 moles of HCl may react with each mole of cyclopropene as follows:



(1)



(2)

As can be seen, the hydrohalic acid also reacts with CPFA hydrohalide addition product, a halocyclopropane (shown in reaction 2). Hydrohalic acid can also react with the olefinic bonds in normal fatty acids, epoxy fatty acids,

and with autoxidation intermediates, present in all natural oils. All these reactions contribute to the rapidly fading end point common in the hydrohalic acid titrations of cyclopropenes. In order to remove interfering species, pretreatment of the oils with lithium hydride (Smith et al., 1960), mild acetalysis (Smith et al., 1961), or activated alumina (Harris et al., 1963) has been used to partically eliminate the problem.

Harris et al. (1964) developed an analogous, but faster hydrohalogenation method. This method is based upon the use of a reagent consisting of anhydrous HBr in glacial acetic acid (0.1 N HBr) which is titrated into the oil in question. Even though the reaction is more stoichiometric than HCl it is very slow at room temperature. Again epoxides, peroxides, hydroperoxides (autoxidation intermediates), normal olefins (unsaturated fatty acids), and the cyclopropane intermediate interfere with the reaction and cause the results to be high. For this reason the samples must be pretreated to remove some of these interfering substances, but even this does not ensure complete reaction according to Gaylord (1956). Harris et al. (1963) improved upon this method by doing a two step titration. The first step was done at 3 C (to remove epoxides) and the second at 55 C. This provides a much more rapid reaction, plus, has the advantage of a sharper end point. If, however, peroxides or conjugated hydroxydienes are present this method will not work.

Harris et al. (1964) later believed that the oils tend to develop oxidation products at 55 C which can also interfere with the reaction results causing him to concluded it would be best to pretreat all samples; the net results is a method which is very time consuming. Fisher and Schuller (1981) further discovered there is a loss of HBr during storage (reagent deteriorates to 90% its original concentration within five hours) and/or during titration as well as some loss of CPFA during sample pretreatment. The CPFA can also react with the acetic acid used as solvent thus lowering its apparent concentration. Feuge et al. (1969) developed an alternate HBr-titration method based upon the use of toluene, rather than acetic acid, as the solvent. This method also demonstrated the loss of reagent strength as well as even lower recoveries of CPFA as compared to those found by the HBr-acetic acid titration (Fisher and Schuller, 1981).

The disadvantages of any hydrohalogenation method is the numerous manipulations required to ensure absence of the interfering components, fading end point, difficulty of determining the quantity of hydrohalogen required to reach the end point, low reliability of the method, and that individual CPFA concentrations can not be determined.

Crystal violet, used as an indicator in all the hydrohalogenation reactions, creates a major problem. This is a very difficult indicator to "see" and the results will vary depending upon the experimentor.

Zeringue and Feuge (1981) found crystal violet to be only slightly soluble in the solvent, which added to the already existing problem of determining an exact, consistent end point. In order to avoid the use of an indicator Zeringue and Feuge (1981) published a variation on the HBr titration method involving the use of a potentiometrically determined end point. He used a silver-silver nitrate indicating electrode with a Calomel reference and a saturated KCl salt bridge. The reaction chamber was a closed system in order to exclude air from the sample and prevent evaporation of HBr. The HBr was introduced into the system below the surface of the sample to minimize any evaporation and chance of reaction with the atmosphere. There are several disadvantages to Zeringue's potentiometrically determined end point. The major one is the requirement for standardization of the HBr solution immediately prior to the titration. Another is HBr's reaction with interfering components, as previously discussed. Although this method requires fairly inexpensive equipment it is time consuming, requires pretreatment of samples, standardization of reagent, a special reaction chamber, only total CPFA can be measured, and since the titration has an inherent inaccuracy of several percent, levels of CPFA below 5% cannot be determined, although the author claimed reproducible results at the 1% level when compared to results obtained from the HBr-toluene titration method.

This is not significant since the HBr-toluene titration method consistently gives low results.

In the mid 1960's the use of instrumental methods were developed for analysis of CPFA containing oils. According to Magne (1965) one of the first studied was infrared absorption. Although IR is a primary method, a standard must be used as the extention coefficient of absorption is unknown. In order to determine an extention coefficient for absorption a pure sample of CPFA is needed. Another reason a standard must be used is to calibrate the instrument for the particular cell to be used since cells of sufficient quality for quantitative work are generally not available. One serious problem with the IR method, as in various chemical methods, is its determination of total CPFA concentration rather than the concentration of malvalic acid and sterculic acid separately.

Since the cyclopropene moiety is a very strained structure it thermally degrades when heated and the use of Gas Liquid Chromatographic (GLC) methods of analysis, requiring elevated temperatures, result in decomposition of the CPFA. The fact that derivatives of a thermally labile substance often produce products with better thermal stability led to the development of three methods based upon preparation of derivatives of the CPFA containing oils prior to GLC analysis. The first method involved hydrogenation of the cyclopropene ring to

cyclopropane. Wilson et al. (1961), as well as others (Cornelius and Shone, 1963, Cornelius et al., 1965), tried a hydrogenation method but found several peaks for each CPFA would appear unless hydrogenation conditions were very carefully controlled. Another major error with this system developed because all fatty acids of the same length, regardless of number of multiple bonds, would yield the same product thus preventing usable results when unsaturated fatty acids might be present. The cyclopropene group will react more rapidly than any other group, but slow hydrogenation of cyclopropane and olefinic bonds cause a problem in determining the amount of time for reaction since there is a break in the rate of hydrogen uptake. Bland and fellow workers' (1984) method of hydrogenation by a homogenous transition metal complex did not overcome this problem. Although hydrogenation prevents decomposition of the CPFA on the column, the problems already discussed prevent this method from being a valid solution.

Kircher (1965) developed a method based upon GLC analysis of CPFA derivatives formed by reacting the methyl esters of the oil with silver nitrate in methanol. If only low levels of CPFA (below 5%) are present, in order to prevent column overload, the derivatives must be concentrated, after the reaction, via separation on a bench top chromatographic column packed with alumina and eluted with hexane. At higher levels of CPFA this

concentration step was not necessary. This method also required an internal standard. Although this method requires large amounts of time for sample preparation the results are comparable to those obtained from HBr titration, as well as other methods, and has a variation of only 3% between samples. This system does have several advantages over the HBr-titration. The first is the analysis for individual CPFA concentrations, reported as a ratio of malvalate to sterculate, and the second is a lower detection level for the CPFA, reported to be reliable down to 0.1%, but found to be useful only at 1% and above when used in this laboratory. Schneider et al. (1968) found this method gave results that were high if the CPFA concentration was above 2%, but this may be due to the method used to determine the peak area.

The third method, developed by Raju and Reiser (1966), was based upon formation of mercaptan derivatives of the CPFA-containing oil prior to analysis by GLC, with a thin layer chromatographic (TLC) technique used to isolate these derivatives. Their resulting chromatograms contained several unidentifiable peaks along with the expected peaks. Coleman (1970), trying to repeat their work, discovered the mercaptan formation reaction was not quantitative under the specified conditions since the products, after the alloted reaction time, still gave a positive Halphen reaction thus indicating the presence of unreacted CPFA. Pawlowski found the addition of the RSH

group across the cyclopropene double bond proceeds via free radicals (Pawlowski et al., 1972) thus increasing the possibility of many side reactions. The mercaptan derivative method also requires the use of an internal standard.

Pawlowski et al. (1972) developed a technique involving the use of the NMR. Protons in chemically different environments undergo resonance at different values (chemical shift) in the NMR spectrum. When the spectrum between delta = 1.1 to 0.6 is observed for a cyclopropenoid fatty acid two distinct absorptions are observed. One absorption is due to the two hydrogens on the cyclopropene and the other absorption, seen as three fused peaks, is due to the terminal methyl group on the fatty acid. Unusual impurities and total unsaturation can be observed in the baseline of the total NMR spectrum. This technique is rapid, simple, and quantitative. The analysis is done directly upon the natural lipids and does not depend upon a standard or derivative. It was found that the method is quantitative only at CPFA concentrations greater than 1%, although concentrations as low as 0.1% can be estimated; however, only total CPFA concentration can be determined. One major disadvantage of this method, other than expense and availability of equipment, is the possible interference of impurities and/or solvents in the sample. When Pawlowsk et al. tried to compare their results to other methods, they found only

the NMR determination produced consistent results. According to Boudreaux et al. (1972) high resolution NMR could be an excellent rapid, nondestructive method for assaying purity of methyl sterculate and methyl malvalate concentrates.

In 1981 Kint et al. (1981) developed a technique based upon the presence of a strong band at 1870 cm^{-1} in the Raman Spectrum for the cyclopropene double bond. This method gives the total CPFA concentration, requires expensive equipment and does not give a total lipid profile even though it can detect, according to Kint, CPFA down to the 0.03% level. Our laboratory was not able to detect CPFA below 1%. Since this method, like the NMR method, depends upon Fourier Transform of a very weak signal using nearly identical programs, it is likely that both methods have similar lower limits of 0.1%. Since neither of these methods require much sample preparation, crude raw oils, and even whole seeds, can be analyzed.

Mass spectroscopy has been suggested as a method for determining ring position in a carbon chain (Eisele et al., 1974), using the silver nitrate-methanol derivative form of the methyl esters. The cost of equipment, when compared with the Halphen test, make this an unreasonable qualitative technique for determination of CPFA content.

Another instrumental method which has been developed is Reverse Phase High Pressure Liquid Chromatography (RP-HPLC). Bianachini et al. (1982), after determining the

best acetonitrile water ratio to be 85:15 v/v, quantitatively determined the limit of CPFA concentration to be dependent upon the detector UV wavelength chosen. Gaydou et al. (1983a) by combining this method with GLC found a lower detection limit of 0.15% CPFA. It is necessary to purify the samples after methylation as well as filtering them just prior to analysis on the HPLC. An internal standard is also required.

Loveland et al. (1983) developed an HPLC technique of analysis using a differential refractive index detector which is not as sensitive as a GC detector, but better than the UV detector used in RP-HPLC. The differential refractive index detector gives comparable results to both NMR and silver nitrate-methanol derivative GLC as well as Gaydou's combination HPLC and GLC method. This method has the advantage of being fairly simple, (although requiring expensive equipment and an internal standard) accurate, gives individual concentrations for malvalate and sterculate and can provide a partial fatty acid profile. Like any other method there are several shortcomings. One is the detectors sensitivity and the other is the sterculate peak is not completely resolved from the linoleate peak. Sterculate appears as a small shoulder after linoleate emerges.

In 1967 Recourt et al. (1967) demonstrated isomerization or partial/total decomposition of CPFA during direct GLC analysis of their methyl esters.

Because of the decomposition of the CPFA various methods of analysis were done using derivatives. With the advent of capillary columns the direct analysis of methyl esters of oils was again tried (Bianchini et al., 1981). The resulting chromatogram showed incomplete peak resolution between methyl stearate and methyl malvalate when low levels of CPFA are present. When the chromatogram is repeated, after lowering the column temperature from 190 to 170 C, the peaks are resolved. These results are found to be similar to those obtained using the same conditions only derivatizing the sample first.

Fisher and Schuller (1981) observed the decomposition problem, but solved the problem by repacking the column whenever an additional peak appeared in the chromatogram. Their results are comparable with HBr-titration . The GC has the advantage of determining the individual CPFA concentrations plus requiring a smaller sample (less than 1 mg), and requiring only minimal sample preparation. The even greater significance of this method is the total lipid profile obtained from the analysis. The one disadvantage is equipment cost.

Table 1 summarizes these various methods giving their detection limits and accuracy levels. As can be seen neither chemical nor instrumental methods can detect CPFA concentrations at the levels found to cause physiological disorders in animals.

TABLE 1

COMPARISIONS OF VARIOUS CHEMICAL AND INSTRUMENTAL METHODS USED TO DETERMINE CYCLOPROPENOID FATTY ACID CONTENT

METHOD	DETECTION LEVEL (%)	ACCURACY LEVEL (%)
Halphen[a]	1	1
Hydrohalogen	2	5
NMR	0.1	1
Raman	0.1	1
HPLC	1	1
Gas Chromatography	Decomposition	---
CH_3SH/GC	Unreliable	---
$AgNO_3$/GC	1	3-6
Hydrogenation/GC	Unreliable	---
HPLC/GC	0.15	1

a levels below 2%

Although past experience has shown CPFA to be unstable during GLC analysis the development of Fused Silica glass capillary columns and on-column glass injectors should solve this problem since the primary source for sample thermal decomposition occurs on the surface of some instrumental component, for example the injector, column material, column support, and/or detector. Fused silica columns have the advantage of being able to avoid sample contact with many of these common GC components. The columns are flexible and can be threaded through the detector up to the base of the flame eliminating contact with the detector. This technique, combined with a cold on-column injection technique, avoids the sample contacting any instrumental component except for the column which is made from the lowest reactive material possible. Since the liquid phase is coated on the column wall, no support material is involved. If non-volatile materials or polymers are in the sample, they most likely are concentrated at the head of the column. Should these impurities contaminate the column, they can be removed by removing a few centimeters from the column head and repositioning the slightly shorter column into the injector, thus removing the contaminate with the short piece of column. The loss of a few centimeters will not influence the efficiency of a 30 meter column. The glass capillary gas chromatographic (GCGC) method has several advantages over other

instrumental methods among which are equipment availability, sample preparation, speed of analysis, results in the form of a total lipid profile, and separate concentration values for each cyclopropenoid fatty acid, making it a viable primary intrumental method for low level CPFA analysis.

This project developed a quantitative analytical technique for analysis of methyl ester fatty acids for low levels of methyl malvalate and/or methyl sterculate utilizing a fused silica glass capillary gas chromatographic system. Once developed, this technique was applied to the analysis of several plants from the Malvales order, and selected food items believed to contain cottonseed oil.

EXPERIMENTAL

EQUIPMENT

All analyses are carried out on a Varian Aerograph Model 3700 Gas Chromatograph equipped with a flame ionization detector, converted for use with a glass capillary column, and fitted with a septumless, pneumatic head, on-column glass lined injector (Scientific Glass Engineers). The Model 3700 was used in conjunction with a Hewlett-Packard 3380A Integrator. The analytical column, a J & W fused silica capillary column, (30 m long X 0.32 mm ID) coated with a liquid phase of DB-1, cross-linked SE-30, (film thickness 0.25μm) is employed with H_2 as the carrier gas (30 cc/min), N_2 the make up gas (30 cc/min), and compressed air (300 cc/min). ALLTECH Gas Purifier, OXY-Trap, and Indicating OXY-Trap units are placed in the carrier gas line to ensure the absence of trace quantities of oxygen, water and other impurities in the hydrogen. Since a cold on-column injection technique is used, the column temperature is initially at 40 C then, after injection, raised to 120 C to flash vaporize the sample. After ten minutes a programmed temperature rise of 2 degrees per minute increases the column temperature to a final temperature of 190 C. The detector is maintained at 210 C through out the analysis.

The data from the HP 3380A Integrator is normalized before the various peak areas are determined and results reported as per cent total area for each methylated fatty acid.

CHEMICALS

Solvents are either glass distilled or purchased HPLC quality.

PREPARATION OF OILS FROM SEEDS

Seeds are dried overnight at a maximum temperature of 85 C, ground using a mortar and pestile, and placed in a Soxhlet apparatus with anhydrous ether for the lipid extraction. After 48 hours the lipids are removed from the excess ether by low pressure evaporation and stored at -20 C.

TRANSESTERIFICATION

The lipid sample is heated with a 0.1% methanolic sodium methoxide solution in the ratio of 1:25 until solvation takes place, maximum time of one hour, at a temperature not to exceed 70 C. The reaction is then allowed to stand over night at room temperature. Caution must be taken to ensure the absence of any moisture during

transesterification. This includes the use of oven dried glassware (just prior to use), as well as using a drying tube, along with a condensation tube, as part of the reaction apparatus.

The methylated fatty acids are then separated via a water/hexane extraction. Fifty milliters of hexane and five times as much water as methanolic sodium methoxide are placed in a separatory funnel with the transesterification reaction mixture. After sufficient time is allowed for phase separation the aqueous layer is removed, an equivalent amount of water added to the funnel and the process is repeated. After the third washing, the hexane layer is removed from the separatory funnel and placed in a rotovac system for evaporation of the hexane, under reduced pressure, from the methylated fatty acids. The sample is then stored at -20 C until needed. Since it has been observed that analytical results for natural products vary with storage time, all natural samples are analyzed as soon as possible after extraction and transesterification.

SAMPLE PREPARATION

Methylated fatty acid samples are dissolved in hexane at a concentration resulting in 0.6 μl injected sample producing a usable chromatogram. Samples can range from 2 μg to 30 μg per milliter hexane depending upon initial

lipid concentrations. For many natural products 30 μg, or more, of sample are required. Trial volume/volume dilutions are made and a chromatogram is prepared in order to determine the correct dilution range. This trial can then be diluted or concentrated (by evaporating part of the solvent off using nitrogen gas) until a good chromatogram is obtained. Once the desired dilution is found a new sample can be prepared using analytical techniques. A weighed sample (to the nearest 0.1 mg) rather than a volumetrically measured sample is used for the first dilution in hexane. If further dilution is required then serial dilutions, using a volumetric pipet and volumetric flask, can be made.

STANDARDIZATION

Initially, a commercial standard, RM-2 (Supelco, Inc. AOCS Animal and Vegetable Oil Reference Mixtures), can be used to determine sequence and retention times for the various methylated fatty acids. SFO can then be analyzed, using the same experimental conditions, and the various peaks can be identified from their respective retention times. The two peaks not found in the standard are methyl malvalate and methyl sterculate. These peaks can be identified through analysis of known samples containing methyl malvalate and methyl sterculate. This analysis also gives their respective retention times. Corn oil,

when analyzed, is found to contain the same fatty acids, except in different proportions, as SFO with the exception of malvalate and sterculate. Since SE-30 type columns separate on the basis of molecular weight, retention times for each fatty acid methyl ester are consistent with the molecular weight range of the sample. Once all peak retention times are determined for SFO it can be used to determine optimum conditions such as flow rates, temperature increase rate for the column, and column temperature range. SFO can also be used to determine the best injection technique.

CONDITIONS

An on-column, cold injection technique can be developed with the initial column temperature (at time of injection) set at 40 C. After one minute the column temperature is increased to 120 C and held there for 10 minutes, to flash vaporize the sample, before starting a program temperature rise of 2 degree/minute. The final temperature reached by the column is 190 C with the FID detector maintained at 210 C. These conditions, along with previously stated flow rates, are found to give the best separation and most consistent results.

SOLVENT

HPLC quality hexane is the solvent used for all methylated sample dilutions.

LOWER LIMITS

In order to determine a lower detection limit for CPFA, various dilutions of SFO in corn oil are prepared from the methylated oils. Both the SFO and corn oil samples are weighed on an analytical balance, accurate to 0.1 mg, in the desired ratio and dissolved to 1.00 ml volume in hexane. When very low concentrations of SFO are needed the same procedure can be used with the initial dilution being further diluted (serial dilution technique), since it is not possible to weigh samples in the microgram range with standard laboratory equipment. An example of this procedure is as follows: 0.10 ml of a solution, prepared from a 1.00 ml dilution of 0.0014 g SFO and 0.3041 g corn oil is further diluted to 5.00 ml (in hexane) to obtain a usable chromatogram from a 0.6 μl injection. This solution is 0.46% SFO in corn oil.

RESULTS AND DISCUSSION

PEAK IDENTIFICATION

Methylated fatty acid standards, purchased from Supelco, are used to determine both sequence and retention times for some common fatty acids known to be present in corn oil. The methylated form is needed since it is not possible to analyze oils, using gas chromatographic methods, when the lipids are present in their triglyceride form. These standards can also be used to determine the best chromatographic conditions for good separation. Once the chromatogram from the commercial standard is completed, the peaks in both methylated corn oil and SFO (Sterculia foetida oil) can be identified from their respective chromatograms and retention times. The only difference between the SFO and corn oil is the presence of the methyl malvalate and methyl sterculate peaks in the SFO. These two peaks can be identified, as to their retention times, by analysis of a sample known to contain methyl malvalate and methyl sterculate. As can be seen in Figure 1 the peak identification for SFO is as follows: Palmitate (16:0), Malvalate (18:CE), Linolenate (18:3), Linoleate (18:2), Oleate (18:1), Stearate (18:0), and Sterculate (19:CE). Any reference to GC (gas chromatographic) analysis implies the fatty acids are in

their methylated ester form and, as such, the "methyl" label will not be repeated, but implied by the "-ate" ending on the name of the specific ester. The retention times indicated are valid for the specified set of conditions (for example carrier gas flow rate, programmed temperature rise, column length, and stationary phase thickness) and will vary proportionally with a change in any of these conditions. As can be seen from Figure 1 good separation of the malvalate and sterculate peaks from the other peaks is obtained. This has not been the case in earlier work. Fisher and Schuller (1981) were unable to obtain complete separation of methyl malvalate from methyl linolenate or methyl sterculate from methyl stearate when low levels of CPFA (cyclopropenoid fatty acids), such as found in cottonseed oil, were present. Similar problems were encountered by Bianchini et al. (1981) and Gaydou et al. (1983b and 1984).

Baseline resolution can be changed by adjusting the attenuation setting on the integrator/recorder. Above 0.1% CPFA the attenuation is set so all peaks are on scale and completely resolved, as seen in Figure 1. Even though the peaks of linolenate, linoleate and oleate are very close together they are completely resolved. When the concentration of one component becomes very low, compared to the other components, the attenuation can be increased (expanding the baseline) to make components of lower concentration more apparent. This often places higher

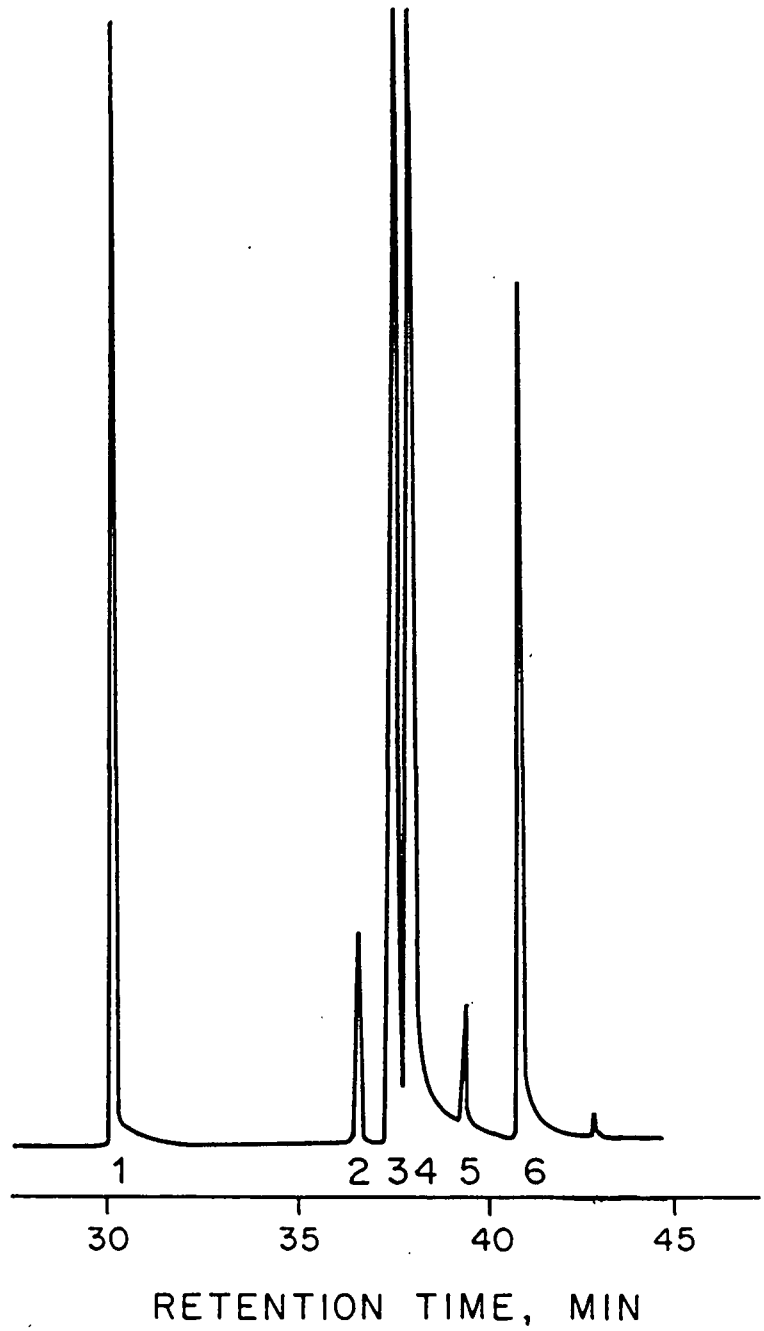


FIGURE 1. 33% Sterculia foetida oil methyl esters diluted in corn oil analyzed on DB-1 capillary column. 1-palmitate, 2-malvalate, 3-linolenate and linoleate, 4-oleate, 5-stearate, and 6-sterculate.

concentration components off scale on the chart paper. Since the integrator will still determine the respective peak areas when this happens, all that is effected is the appearance of the chromatogram. This type of change in attenuation is often required during analysis when one wishes to determine the concentration of minor components in the sample. When the baseline is expanded for low levels of CPFA the linolenate, linoleate, and oleate peaks appear to merge (with peak tops off scale). This merging baseline, as shown in Figure 2, is best considered as "one" large peak and, as such, can be calculated as a total area rather than trying to calculate three separate areas. To simplify comparisons, even when separate values are obtained, these three components are considered as one and calculated as such. No attenuation adjustment is required for the integrator during analysis since it is capable of handling signals over the range of -0.1 mv to 1.0 mv; however, the recorder attenuation is adjusted to give the best presentation for the chromatogram.

THERMAL STABILITY OF CPFA

In the past, attempts to analyze cyclopropenoid fatty acids by gas chromatography have been unsuccessful due to the decomposition of the CPFA during analysis. Typically, a substance fails to survive gas chromatography because of decomposition occurring due to either unimolecular thermal

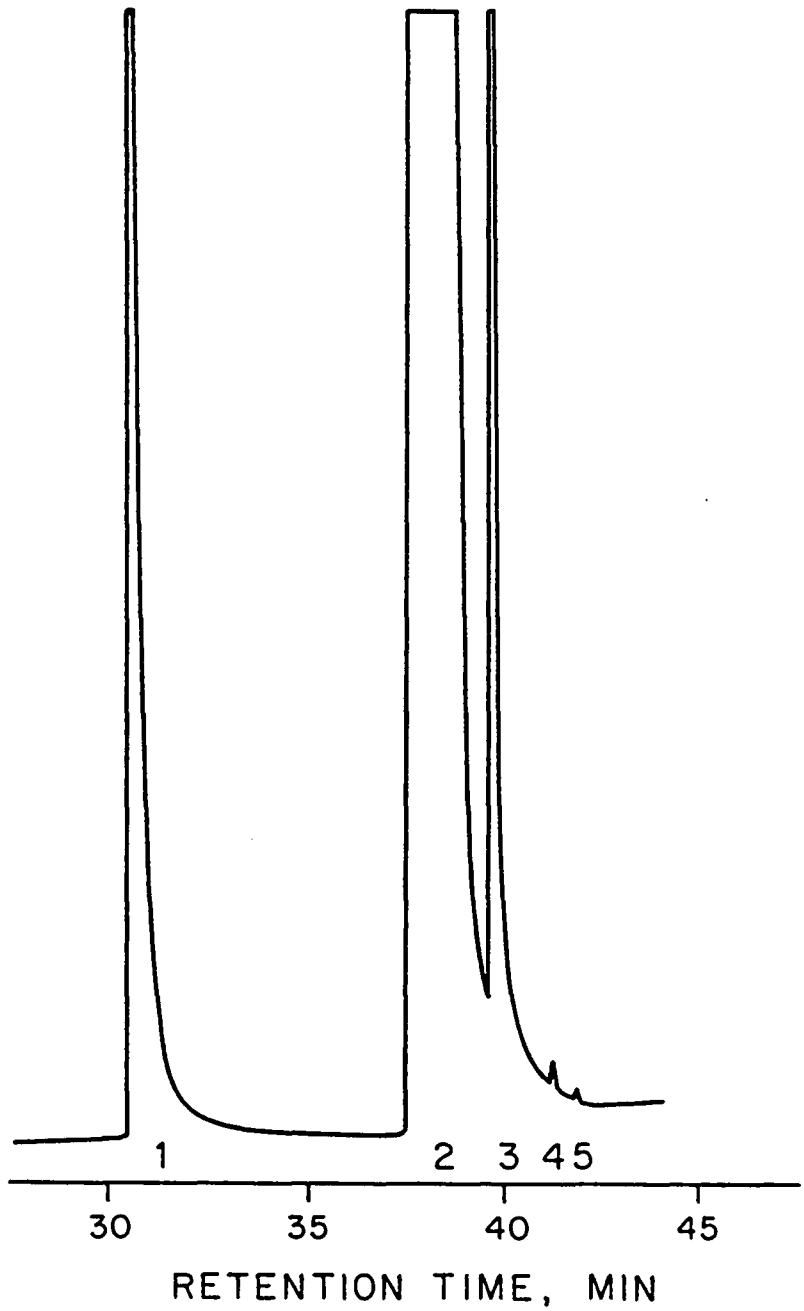


FIGURE 2. 0.5% Sterculia foetida oil methyl esters diluted in corn oil analyzed on DB-1 capillary column. 1-palmitate, 2-malvalate, 3-linolenate, linoleate, and oleate, 4-stearate, and 5-sterculate.

decomposition or a reaction with some component of the instrument. This could be a reaction with the column, or some component of the column, the walls of the injector or detector, or some impurity in the coating on the walls of these instrumental components. Reactions with a column component might involve the material used to construct the column walls, the solid support, or the liquid phase. When considering kinetics, unimolecular thermal decomposition commonly occurs by a first order rate of reaction. If the surface of another material is involved, the reaction would be expected to be of higher order, even though surfaces and stationary phases involved may appear to be zero order in their contribution to the kinetic rate equation. Before considering this specific problem a general discussion of the underlying theories is needed.

The Kinetic Theory requires several things to occur before a reaction can take place. The first requirement is for the molecules to colloid with each other and be correctly oriented when the collision occurs. The second requirement is for the reacting species to have sufficient energy when they collide to react. Equation (3) can be used to represent the general reaction between several species (R_1, R_2, ...) forming several products (P_1, P_2 ,...)

$$R_1 + R_2 + \ldots \underset{k_r}{\overset{k_f}{\rightleftharpoons}} P_1 + P_2 + \ldots \qquad (3)$$

where k_f and k_r are the specific rate constants for the forward and reverse reactions, respectively. The specific rate constants are completely dependent upon the conditions under which the reaction is occurring. In general the actual rate of a reaction can be specified as

$$\text{Rate} = k[A]^x[B]^y \qquad (4)$$

where [A] and [B] represent the molar concentrations of the reactants and k is a specific rate constant for the defined reaction conditions, including temperature, pressure, the nature of the reacting species, and any other factor which might influence the rate of the reaction. The x and y power must be determined experimentally as the concentration of each component may or may not change the rate of the reaction. If there is a one to one effect (that is, doubling the concentration of reactant A doubles the rate of the reaction) then x will equal one and the reaction will be first order with respect to component A. If, however, as the concentration doubles the rate increases by a factor of four then x will equal two in the rate equation and the reaction will be second order. In some instances a change in concentration of a component does not effect the rate and is indicated as x equals zero. When this happens the reaction is said to be zero order with respect to component A. The y value can be determined in a similar manner and the sum of x and

y will give the overall rate order for the reaction. For the purpose of this discussion we will let the reaction be first order in A and B, respectively. If k_f is substituted for k and A and B represent the reactants in Equation (3) then Equation (4) represents the rate for the general reaction shown.

According to Arrhenius a specific rate constant can be represented as

$$k = A\ e^{-(E_a/RT)} \qquad (5)$$

where A is defined as a frequency factor, E_a the activation energy for the reaction, R the Universal Gas constant, and T the absolute temperature. From Equation (5) it can be seen the rate constant, k, of any reaction will increase with increasing temperature as long as the activation energy is positive. The "A" term is related to both the number of collisions and the orientation of those collisions. As the temperature increases the number of collisions will increase which, in turn, increases the probability that the molecules will be correctly oriented. The exponential term in Equation (5) represents the probability that the reacting molecules are actually oriented correctly.

Activation energy can be defined as the minimum energy necessary to form an activated complex during a collision between two reactants. The activation complex,

according to Transition State Theory, is an intermediate in any reaction which could be represented by the relationship

$$\text{Reactants} \rightleftharpoons \text{Complex} \rightleftharpoons \text{Products} \qquad (6)$$

When the energy of the reaction is much larger than the energy of the products the reaction will be essentially a non-reversible reaction. This is the case in any decomposition reaction involving a CPFA. In a slow reaction, the activation energy is much larger than the average energy content of the molecules. One way to increase this average energy content is to increase the temperature of the reaction system.

For a given reaction, activation energy is related to several factors (often referred to as "nature of the reactants") including the bond energies involved in the reactants and products. Strain energy is an important contributor to bond energy. The higher the strain energy of a bond the easier the bond can be broken. Reactions proceed when energy is released from breaking a strained bond to form a new product of lower energy. In the case of three member rings strain is due to the bending or deformation of bond angles causing normal sp^3 bonds to assume more "p" character. For this reason a three member ring represents a higher energy state than a straight chain or even a larger ring. If a double bond,

which normally assumes a 120^{o} bond angle, is added to a three member ring even more strain will be put into the system as the bonds are bent from their preferred 120^{o} angle to the 60^{o} angle of the three member ring. This is the case for the structure of the cyclopropene moiety. Carter and Frampton (1964) suggest that the extra strain energy for cyclopropene over cyclopropane is 27 kcal/mole. This value is calculated from experimenally determined values for the heats of combustion and formation of the cyclopropene, the heat of hydrogenation to cyclopropane and isomerization of the cyclopropene to methylacetylene.

When considering the decomposition for a CPFA, Kinetic Theory suggests the rate of decomposition will be a function of time on the column (exposing the reactant to a higher probability of meeting the various criteria required for reaction) and the temperature of the column. Changing these variables experimentally should change the rate of decomposition of the CPFA. Normally, changing variables on two different reactants would result in different rates of decomposition for each one, however, this is not the case for CPFA. Both methyl malvalate and methyl sterculate have long hydrocarbon chains on the 1- and 2-positions of the thermally unstable cyclopropene moiety. Since both have the same chain substituted on the 1-position and similar chains (differing by a CH_2 group) on the 2-position, both should decompose at identical

rates. This fact suggests a test to determine whether or not CPFA is thermally decomposing on the capillary column.

Actually, three different tests for thermal decomposition of CPFA can be considered. The first compares the absolute analysis of CPFA by GC with another quantitative method. This technique is impractical since no other good analytical method, especially for low levels of CPFA, is known. The second test involves analyzing the same sample using several different column temperatures and comparing the results. Since reactions, particularly thermal decomposition, increase their rate by a factor of approximately three for every ten degree increase in temperature a substance, decomposing during GC analysis, should show a decrease in concentration at a higher temperature. Although this method sounds reasonable, it could easily yield misleading results in a gas chromatographic method since residence time on the column is decreased with increasing temperature and the two effects might compensate for each other. The third method of testing for decomposition during analysis involves changing to a different relative time each component is on the column. This is done by increasing the time on the column of each methyl ester and comparing the area ratios of methyl malvalate to the other methylated fatty acids. If the flow rate of the carrier gas is decreased slightly during the run the amount of time each fatty acid remains on the column will increasing thus theoretically

increasing the proportional amount of decomposition for each CPFA which would, in turn, be indicated by a decrease in the ratio of the CPFA areas. Since a program temperature increase is also used, each CPFA will be eluting at a higher temperature, again increasing the possibility of decomposition. If the area ratio remains constant for malvalate to sterculate and/or to the other fatty acids then the cyclopropenes are either not decomposing or they are decomposing at the same rate. To eliminate the possibility of equivalent rates of decomposition the inlet pressure (flow rate of carrier gas) is slightly decreased after emergence of the malvalate peak. This causes sterculate to remain on column longer thus increasing its residence time and the chance for decomposition to occur. If the area ratios are again compared and they remain the same then one may conclude the two CPFA are not decomposing on the column, at a detectable rate, under the conditions employed.

Three fatty acid methyl esters are compared at two different inlet pressure values. As can be seen from Table 2 the ratios are consistent for both pressures (flow rates). The methyl malvalate to methyl sterculate was 0.05 for all runs while malvalate to linoleate and malvalate to stearate changed slightly from 0.33 to 0.36 and 1.33 to 1.40 respectively. Although there is some variance, especially with the malvalate to stearate, the ratios can be considered well within the limit of

TABLE 2

THERMAL STABILITY OF CYCLOPROPENOID FATTY ACIDS

ON DB-1 CAPILLARY COLUMN

AT MAXIMUM COLUMN TEMPEATURE 190 C

Inlet Pressure[a]	Malvalate Sterculate	Malvalate Linoleate	Malvalate Stearate
8	0.05	0.36	1.33
8	0.05	0.35	1.33
10	0.05	0.35	1.39
10	0.05	0.33	1.40
10	0.05	0.33	1.40
10→8[b]	0.06	0.36	1.36

a H_2 flow rate gauge value (proportional to time on column)

b pressure lowered after emergence of malvalate peak

reproducibility for the GC and, as such, indicates no decomposition or, if there is decomposition, an equivalent rate of decomposition for the two CPFA. When the inlet pressure is decreased after the emergence of the malvalate peak the ratios remain constant. This indicates no detectable decomposition of the CPFA while on the column. From these results it can be concluded that methyl malvalate and methyl sterculate are both thermally stable on this particular column at these specified conditions.

COMPARISON OF VARIOUS METHODS

Various methods for analysis of CPFA content in a SFO fraction were compared. Table 3 indicates the results of the comparison of the different methods. In the case of NMR, the most reliable method to date, and HPLC the total CPFA concentration is 35% for both, while it is 34.9% for silver nitrate/methanol derivative GLC method. The GCGC method indicates a total CPFA concentration of 34.5% which, although lower is certainly comparable. Attempts were made to compare the various hydrobromic acid titration (Coleman, 1970) methods to these results, but nebulous endpoints render these methods, in our opinion, unsatisfactory as a quantitative method.

The GCGC method is a much faster method of analysis, since it is a primary method, and does not require a derivative of the methyl esters for analysis as the silver

TABLE 3

ANALYSIS OF *STERCULIA FOETIDA* OIL METHYL ESTERS

BY VARIOUS METHODS FOR CYCLOPROPENOID FATTY ACID CONTENT

METHOD OF ANALYSIS		TOTAL CPFA	MALVALATE	STERCULATE
HPLC	(n=4)[a]	35 ±1	8.3±0.4	26 ±2
$AgNO_3$/GC[b]	(n=4)	34.9± 1.1	7.9±0.4	27.0±0.7
NMR	(n=1)	35.0±0.1	---	---
GCGC[c]	(n=8)	34.4±1.2	7.6±0.27	26.8±0.9

a n represents the number of replications
b $AgNO_3$/methanol derivatives analyzed by GC
c Glass Column Gas Chromatography

nitrate/methanol GLC method does. It is also faster than the HPLC method as sample preparation time is less. The NMR method, although also a primary method, has the disadvantage of only giving the total CPFA concentration while HPLC, GCGC, and $AgNO_3$/methanol derivative GLC methods all give concentration of the individual fatty acids.

DILUTION STUDY

The lowest detection level for cyclopropenoid fatty acids can be determined by running chromatograms on a series of solutions prepared by diluting decreasing amounts of SFO in a known quantity of corn oil until neither CPFA peak can be seen on the chromatogram, regardless of the attenuation setting on the recorder. Once this point is reached the percentage of SFO in corn oil can be increased until a detectable level for methyl sterculate (the higher concentration of the two CPFA in SFO) is observed on the chromatogram. The concentration for the 100% SFO, as determined by GCGC, is used to calculate concentration of the various dilutions . As can be seen from Table 4, these predicted (calculated) values and the experimental values are very similar down to approximately the 1% SFO in corn oil level. Below the 1% level the measured value decreases more rapidly than the

TABLE 4

DILUTION EFFECT AS DETERMINED BY

GLASS CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS

OF STERCULIA FOETIDA OIL METHYL ESTERS IN CORN OIL

Percent SFO[a] in Corn Oil	n	MALVALATE EXP[b]	MALVALATE Pred[d]	STERCULATE EXP[b]	STERCULATE Pred[d]	TOTAL CPFA[c]
100	12	7.45	---	35.15	---	42.6
50	4	3.9	3.7	18.18	17.6	22.1
10	9	0.66	0.68	3.01	3.2	3.7
5	4	0.3	0.35	1.5	1.67	1.8
2.5	8	0.1	0.18	0.8	0.86	0.9
0.82	2	---	0.06	0.12	0.28	0.1
0.50	6	---	0.04	0.08	0.17	0.1
0.46	4	---	0.03	0.088	0.12	0.1
0.32	3	---	0.02	0.017	0.11	T
0.28	5	---	0.02	T	0.10	T
0.27	5	---	0.02	0.008	0.096	T

a SFO was fractionally distilled
b exp percentage determined by average of n replications
c sum of exp values to nearest 0.1%
d predicted percentage calculated from concentration 100% SFO

predicted value. Several explanations for this discrepancy can be given with the following being the most plausible.

It is well known that CPFA solutions have a limited stability and decompose with time regardless of storage temperature. This suggests the decomposition is due to an autoxidation process. The autoxidation reactions, involving singlet oxygen, have been found to be zero order with respect to the substrate (Firmer and Antebi, 1980). This suggests that the amount of CPFA undergoing autoxidation will be constant and, as such, will be more noticeable in dilute solutions. If the transesterified oils are stored under N_2 (or some other inert atmosphere) the rate of decomposition should decrease, but it would not stop as sources of autoxidation, other than singlet oxygen, are present in the form of peroxides and hydroperoxides naturally present in all oils.

Lee et al. (1968) found dimerization occurs, due to intermolecular association, with methyl malvalate, methyl sterculate, or any 1,2-dialkylcyclopropene when stored. If the samples are diluted with an inert solvent the intermolecular forces will be decreased thus extending the shelf life stability of the compounds. It would be thought that other fatty acid esters would help decrease interactions but, since they can bind with atmospheric oxygen, they will actually increase the rate of reaction due to autoxidation reactions. In the case of the solutions

used in this work various storage times were involved so calculations based upon the original concentration of SFO would not be accurate for solutions prepared much later. Since the dilution study did take place over several months this could account for part of the difference between measured concentration and predicted.

The key question in this work concerns whether or not the cyclopropene compounds are thermally decomposing on the column during GCGC analysis. As previously discussed, kinetic studies of thermal decomposition of a substrate have always found decomposition to be dependent upon the concentration of the substrate. That is, the rate of the decomposition reaction is always first order or greater with respect to the substrate. In the case of CPFA the low values in Table 4 are better correlated with storage of the cyclopropene containing compound in the oils diluting the CPFA. The cyclopropene moiety is much more reactive towards the peroxides present in the oil then the other fatty acids and, as such, will readily decrease in concentration.

Even though interactions are occurring in all samples those of higher concentrations will be less obviously effected. For example, if 50 ppm concentration is lost from a component, due to this interaction, originally present at the 10% level the observed decrease is much less obvious than if the component was present at the 0.010% level originally.

The indicated lower limit of quantitation appears to be 0.007% CPFA or 70 part per million (ppm). This solution was made from a solution containing 0.27% SFO in corn oil. Even though this value is not in good agreement with the predicted value the experimental value may be closer than it looks to the actual concentration. These discrepancies between predicted and experimental maybe due to one or more of the factors already discussed.

The practical lower limits of detection are dependent upon resolution of the baseline without overloading the column or the broadening of major peaks to the extent that they interfere with the CPFA peaks. Trace impurities can also interfere or mask the CPFA peaks. Another limiting factor is the sensitivity of the detector and the ability of the integrator to differentiate very small peaks. Often it is obvious some CPFA is present from the chromatogram, but it is not possible for the HP3380A Integrator to differentiate between noise and the desired peaks. The GCGC method would be of value qualitatively even after the "lower quantitative limit" is reached. Samples which are considered inactive to the Halphen reaction would be shown to be active by GCGC.

The Hewlett-Packard 3380A Integrator used in this work has several variables which, depending upon how they are adjusted, can cause the results to vary. The area reject was set at 100 integrator units for the reason previously discussed. Although all peaks are stored only

those having an area greater than this preset value will be evaluated when the percentage of total area is determined for each peak. When a peak having an area of 112 integration units (attenuation 2) was included its integrated area was 0.0096% of the total area. If the attenuation was changed to 8 this same peak area would be equivalent to 0.025% of the total area. Slope sensitivity can be adjusted in such a way to trigger acceptance or rejection of certain peaks. If a peak does not elute rapidly, resulting in a wide flat peak, the sensitivity setting determines whether the peak is considered a peak (integrated) or noise (discarded). Most analyses are carried out with the slope sensitivity set on automatic. Just prior to sample injection a test of background noise is made and the integrator automatically sets a sensitivity value which results in the exclusion of most of the noise resulting in a less cluttered baseline. After the run, the slope sensitivity value is indicated on the chromatogram. When the attentuation is changed in order to expand the baseline, the noise level is magnified and the slope sensitivity must be adjusted to remove as much of this noise as possible from the integration. If it is not changed too many peaks will be falsely included resulting in some later peaks being ignored, since the HP 3380A can only evaluated 54 peaks. Attenuation values range from 32 for concentrated samples down to 2 for the

most dilute samples with the sensitivity adjusted accordingly at the lower attenuation values.

Conventional electronic integrators use peak-area normalization to calculate composition. Areas of small peaks on the front of large peaks are estimated by dropping a perpendicular from the valley to an artificial baseline drawn by the integrator. This partitioning of the overlapping area assumes that the overlap is symmetrical. Although this method gives consistent results, careful examination of chromatograms indicate overlap is not always symmetrical. When accuracy was checked quantitatively, the method was reasonably accurate for all but the lowest levels of CPFA (Fisher and Schuller, 1981). It was found the malvalate area was consistently underestimated due to this overlap. Fisher and Schuller found when comparing peak height and area data for the mixture of pure esters the ratio of area by concentration of a peak to its height was a linear function of its retention time. This relationship is given by Equation (7)

$$C = k\ H\ T \qquad (7)$$

where C is the concentration, k is a constant, H the height, and T the retention time. Using internal standards this method was quantitative above the 0.05% CPFA level and semiquantitative below this level. This

method of integrator analysis is referred to as peak height quantitation. In the case of the dilution studies using SFO good peak separation is observed so this method of integration is not needed. When natural products are analyzed however, many more minor peaks appear in the baseline making this a useful technique.

EQUILIBRIUM STUDY

The reaction used to convert a triglyceride to its respective methyl esters is referred to as transesterification. It is accomplished by dissolving the triglyceride in an excess of dry methanol with a trace of sodium as a catalyst. The catalyst must be in either an acidic or basic media, but, in this case, since cyclopropenes are sensitive to an acid, only a basic media may be used. Anhydrous methanol, and sodium methoxide, react with the triglycerides in an equilibrium reaction to form a mixture of methyl esters and glycerol as shown in Equation (8).

$$\text{RCOO-glyceride} + CH_3OH \overset{\text{catalyst}}{\rightleftharpoons} \text{RCOO-}CH_3 + \text{glycerol} \qquad (8)$$

In the presence of excess $NaOCH_3$ the methyl esters can further react to form their respective sodium salts as shown in Equation (9)

$$\text{RCOOR'} + NaOCH_3 \rightleftharpoons \text{RCOO}^-Na^+ + \text{R'}OCH_3 \qquad (9)$$

where R and R' can be hydrogen, an alkyl, or glycerine. Literature references to this procedure use an excess of sodium methoxide which would lead one to believe that there might be a loss of yield of fatty acid methyl esters due to this second reaction. In order to determine if the loss of methylated fatty acid occurs due to the formation of the sodium salt of the fatty acid, an experiment was designed. The experiment involved the addition of excess sodium methoxide to a weighed quantity of methyl oleate. This should shift the equilibrium towards the formation of sodium oleate according to Le Chatelier Principle. The reaction was allowed to proceed under normal transesterification reaction conditions. After sufficient time elapsed the mixture was washed with a water/hexane rinse several times. The hexane soluble product was removed, excess solvent evaporated off via low pressure evaporation, and the resulting product weighed. This weighed product was equal to the original quantity of methyl oleate. The product was analyzed on the GCGC and identified by its retention time to be methyl oleate. Any soluble salt of oleic acid formed would have been partially soluble in the water layer as well as in the hexane layer thus forming an emulsion between the two layers. Since good separation between the two layers was observed, along with the weight and identification of

product being as stated, it can be concluded there was no significant loss of methyl oleate due to the formation of its sodium salt. The $NaOCH_3$ catalyst has not significantly altered the equilibrium of the reaction due to the very large excess of methanol. The excess methanol shifted the equilibrium in Equation (8) to the right.

APPLICATION

Once column stability was assured for oils containing CPFA the GCGC method was applied to various plants and food items. The criteria for analysis were: that the plant be in the order Malvales, or that the food label indicated the use of an oil from a plant in this order. Originally, only plants found growing in Oregon were to be analyzed, but several plant sources not commonly found in Oregon were included. Plants consumed directly by man, such as okra and cocoa, were analyzed along with those consumed by grazing animals which might later be eaten by man, such as hollyhock and cheese weed. Along with food items containing cottonseed oil, or some other suspected source of CPFA, several cottonseed and kapok oil samples were analyzed. A possible new protein source, seashore mallow was also included along with several fractionally distilled oil samples of Sterculia foetida and Hibiscus syriacus.

Due to the nature of plant cycles, all seeds were gathered when possible and stored at -20 C until they could be analyzed. Yano et al. (1972) found the maturity of the seeds directly affected the concentration of CPFA so immature seeds should be analyzed whenever possible. Since low levels of CPFA are found in the leaves and stems, they can be added to the seeds during the extraction process. This was the case for both hollyhocks and cheese weed. Since it was difficult to separate cheese weed seeds from the rest of the plant, the whole plant was used in the extraction process. With hollyhock a sample containing only seeds and one containing seeds, stems, and leaves was analyzed with only a slight differences in the CPFA level noted. This may be due to the low levels of lipid in stems and leaves. All plants gathered for this work had their lipids extracted from the seeds and were transesterified immediately prior to the GCGC analysis. Food items were handled similar to plants, in that the lipids were extracted, transesterified and analyzed with as little storage as possible between extraction and analysis.

The results of the plant and food analyses is reported in Table 5. Although complete free fatty acid profiles are determined, only the sterculate and malvalate percentages are shown with the percentage of oil extracted from the seeds.

TABLE 5

GLASS CAPILLARY GAS CHROMATOGRAPHIC
ANALYSIS OF SELECTED PLANTS AND FOOD PRODUCTS
FOR CYCLOPROPENOID FATTY ACID CONTENT

TABLE 5

Product	Oil Content of Seeds (%)	n	Malvalate (%)	Sterculate (%)	Total CPFA
Okra	1.8	4	P	0.034±0.014	0.03
		6	0.35±0.04	0.57 ±0.28	0.92
Hollyhock	2.5	6	0.23±0.04	0.10 ±0.04	0.33
Cheese weed	1.5	4	---	2.6 ±0.2	2.6
Raw Cocoa Beans	20	3	A	A	A
Cocoa Butter	80	1	A	A	A
Kapok	10-14	5	8.96±0.60	3.81 ±0.04	12.8
Margarine*	--	3	A	A	A
Seashore mallow	14	3	2.03±0.95	0.54 ±0.21	2.6
White Cap Cottonseed Oil	--	4	---	0.0075±0.0007	(75ppm)
Winterized cottonseed oil (Lucca)	__	3	A	A	A

* Diet Imperial, label included cottonseed oil
P peak indicated, but too small to evaluate
A no peak indicated

Two different okra (Hibiscus esculentus) samples upon analysis had a total CPFA content of 0.03% and 0.92% respectively. The sample having the lower level CPFA was purchased very late in a hot, dry growing season. It appeared to be more mature as the variety had large, well developed seed pods and, when dried, had a much stronger odor than the second sample. The second sample, purchased early the following summer (wet growing season) was less mature looking (small seed pods), had little smell when being dried, and was found to contain more CPFA. These two samples may also have been different varieties as Fisher and Cherry (1983) found, when comparing twelve varieties of cottonseed grown in India, a mean concentration of 0.83 % with a range of 0.33% which suggests varietal differences can cause variance in CPFA content. Growing conditions (Pandey and Suri, 1982) can also effect the CPFA content along with the maturity of the seed. Raju and Reiser (1966), using a mercaptan derivative/GLC method found total CPFA concentration in okra to be 0.5% which is somewhat similar in that it is between the values found in the two different samples.

The first sample of okra seeds were actually analyzed twice in that the transesterified esters were stored for a number of months before the analysis was repeated. The resulting chromatogram contains numerous new peaks indicating the presence of new products. The peaks are the results of decomposition/autoxidation of the esters

during storage. This phenomenon was also observed in analysis of other transesterified oils which were stored over a period of time. Although identification of all the peaks on the chromatogram was not made, the malvalate and sterculate concentrations had decreased.

Hollyhock seeds (Althaea rosea) were collected in the fall and stored at -20 C until the following spring before lipid extraction and transesterification. Analysis indicated a 0.33% CPFA level with the malvalate being the dominate form of the CPFA. Raju and Reiser (1966) found a much higher level of malvalate at approximately 4.5% with just a trace of sterculate. Since his analysis was done using a mercaptan derivative some error, as previously discussed, could make his value a little high. He may also have analyzed less mature seeds or a different variety. The seeds used in this study were gathered late in the season so they would have been very mature which might result in a lower concentration of CPFA then if seeds would have been gathered earlier in the growing season. When analysis was attempted later with this same sample the chromatogram indicated many new peaks similar to the okra sample. If, however, seeds were stored for a period of months then the oil was extracted and transesterified similar CPFA levels to those obtained the first time were found. This observation suggests storage in the seed form is preferred as the triglycerides in the seeds are relatively resistant to air or autoxidation.

The lipids were extracted from the leaves, stem and seeds of the cheese weed (Malva parviflora) plant, commonly found growing in Oregon. Analysis showed a 2.6% sterculate level with no malvalate indicated. The chromatogram did show several peaks, equivalent to approximately 0.5% area, over the same chromatographic region observed for malvalate. These peaks may have masked the malvalate peak. It is difficult to get low level results for cheese weed since the large number of small peaks overload the integrator. These small peaks may have prevented identification of the malvalate peak, if malvalate concentration was less than 54 other peaks overloading the integrator. This seemed to be a common problem for some natural samples as there were many trace fatty acids present along with the major fatty acids at significantly higher concentrations.

Guittard Chocolate Company (Burlingame, California) and Ambrosia Chocolate (Milwaukee, Wisconsin) supplied raw cocoa beans which, upon analysis, indicated an absence of CPFA at the low level of detection for this method. This is not too surprising as the bean would have been harvested at maturity rather than when immature. It would be interesting to analyze the immature seeds since cocoa is a member of the plant order Malvales. Cocoa butter (supplied by Guittard and Amborsia Chocolate Company) was also analyzed with the same results as would be expected

since the cocoa butter would be a later product in the processing of the cocoa bean.

Dr. Mir Isham, University of Delaware, supplied seashore mallow (Kosteletzkya virginica) seeds for analysis. Seashore mallow is being considered as a new protein source and animal feed making the levels of CPFA present important. The results indicated 2% malvalate and 0.5% sterculate. Gaydou and Ramanoelina (1984) found the total CPFA content to be almost 5% by GLC analysis. Again growing conditions, seed maturity, and variety may account for this large difference. From either set of results it is obvious seashore mallow would not be a good source of protein until the CPFA is removed via some type of processing. Standard refining processes are not enough according to Sarojini et al's. (1985) findings which indicated refining of cottonseed oil decreased the CPFA concentration some, but did not decrease it to zero. Berry (1982), working with China chestnuts found boiling the nuts for an extended time did not decrease the CPFA level. Later studies with Durian (popular fruit grown in Southeast Asia) indicated cooking at temperatures above 200 C decreases the biological activity of the CPFA, but quantitative studies indicated a higher concentration of the CPFA in the cooked product than in the raw (Berry, 1980a). Cooking the seashore mallow seeds at temperature above the 200 C value might lead to autoxidation of off

flavor products. An alternative would be removal of the oil from the seeds.

Kapok (Ceiba pentandra) contains 9% malvalate and 4% sterculate according to the GCGC method. Gaydou et al. (1983a) found a range for the total CPFA concentration in kapok to be between 11.6 and 14.4% when he used an HBr method, GLC method, and HPLC-GLC combined method. Bianchini et al. (1981), using a silver nitrate-methanol derivative/GLC method found an average of 12% for the total CPFA content. All of these values are certainly comparable. It is surprising that the GCGC analysis showed this close an agreement as the oil was extracted from the seed in 1976 and stored at -20 C until it was transesterified just prior to analysis.

An oil sample, labeled white cap cottonseed, was found and analyzed by GCGC with the results indicating 0.0075% sterculate and 0% malvalate. When this sample was analyzed by this laboratory, using NMR, in 1976 the CPFA content was much higher at 0.3% indicating definite deterioration of the oil.

Several samples of products were analyzed and found to be free of any CPFA, at the detection level possible by the GCGC method. One product, produced by Lucca, labeled "Winterized Cottonseed Oil" was found to have 0% CPFA. Another product, Diet Imperial Margarine, had a label stating that one source of vegetable oil used in the margarine was cottonseed oil so it was also analyzed with

similar results. In this type of analysis the absence of sterculate and/or malvalate is important in that the food item being analyzed can be considered safe, at least from causing any harmful effects due to CPFA.

Both Sterculia foetida and Hibiscus syriacus oil underwent fractional distillation to concentrate the CPFA for use in various animal test diets. Different fractions were analyzed using the GCGC method. Since both of these samples represented fractions rather than total CPFA content in the seeds the results are not shown on Table 5, but are included here for reference purposes. Much higher results for both malvalate and sterculate would be found when analysis is carried out on the oil extracted from fresh seeds.

The SFO fraction was analyzed and found to contain a total CPFA concentration of 34.6% which is in close agreement with both NMR (35.0%) and HPLC (35%) results carried out at the same time. Malvalate concentration was found to be approximately 7.6% while sterculate was 27%. When the results are compared with NMR and HPLC work done on the same SFO samples several years earlier the GCGC results are lower indicating some deterioration of the oil during storage. The original oil was extracted in 1976 so this is not too surprising. Fresh SFO contains 10% malvalate and 50% sterculate.

Several fractions of Hibiscus syriacus oil were analyzed with difficulty in that the chromatogram

indicated the presence of many fatty acid esters. Malvalate was found to be at the 14% level while sterculate was absent. Again, this oil is a fraction distilled in the mid 1970's so this does not reflect the actual concentration found in the seeds and could account for the number of peaks observed in the chromatogram. If a fresh sample, either feshly distilled or extracted from the seeds, was analyzed some sterculate would have been found as demonstrated by Bohannon and Kleiman's (1978) work indicating 13.4% malvalate and 3% sterculate when they analyzed the methylated seed oil directly. When the oil was originally analyzed using NMR the sterculate concentration was between 2 and 3% while the malvalate concentration was 20%.

The purpose of this work was to find a fast, simple, reliable quantitative method for determining cyclopropenoid fatty acid content at typical levels found in possible food sources. The glass capillary gas chromatographic method is just such a method. It is accurate down to the 70 ppm range and has the advantage of using readily available equipment.

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