# Homogeneously Catalyzed Hydrogenation and Gas-Liquid Chromatographic Analysis of the Methyl Esters of Cyclopropene Fatty Acids

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## ABSTRACT

Various concentrations of cyclopropene fatty acids have been determined down to 0.2% by the use of gas liquid chromatographic (GLC) analysis of the methyl esters of fatty acids that have been quantitatively hydrogenated using a homogeneous transition metal complex catalyst. The effectiveness of the use of bromotris(triphenylphosphine)-rhodium(I), $Br(P(C_6H_5)_3)_3Rh$, as a homogeneous hydrogenation catalyst to convert the cyclopropene ring to a cyclopropane ring has been evaluated and compared with the analogous chloro- and iodo-complexes. The hydrogenation/GLC method of analysis has been compared with the method of titration with hydrogen bromide in benzene and with the method involving the use of high resolution nuclear magnetic resonance (NMR).

## INTRODUCTION

The adverse physiological effects of cyclopropene fatty acids (CPFA) are now well documented (1-7). These effects can arise even with low levels of CPFA and because some seed oils notably *Gossyppium hirsutum* (cottonseed) and *Ceiba pętrandra* (kapok) are used to provide food products both for animals and for man (8), much research has been directed toward developing a satisfactory analytical method for the CPFA content of seed oils.

The procedures that have been found to be most useful are: (a) a colorimetric method based on the Halphen test (9), (b) titrations involving the use of hydrogen bromide (10-12), (c) nuclear magnetic resonance (NMR) (13,14), (d) GLC following the formation of derivatives (15,16).

The methods and their limitations are well documented in the literature. Coleman (17) reviewed 5 methods of quantitative analysis and concluded that "the Halphen test calibrated with a reasonably pure cyclopropenoid fatty acid that has been analyzed by the GLC/silver nitrate/methanol method (15) or the modified HBr in benzene method (10) be used for determining low levels (5% or less) of cyclopropenoid fatty acid in fats and oils." Hammonds et al. later suggested that the Halphen reaction could best be practiced on the methyl esters of cyclopropenoid material because the results obtained when using oils were different from those obtained when using the methyl esters (9). The latter results gave a better correlation with determinations made by titrimetry using a 0.1 M solution of hydrogen bromide in benzene/acetic acid. Some uncertainty exists about the accuracy of these results because acetic acid can react with the cyclopropene ring to give spurious results (10), and more than monobromination may occur when solutions 0.1 M in hydrogen bromide are used (10,17).

The NMR spectrum of cyclopropene fatty acids in oils has also been well documented (13,14,18). The method of analysis employing 60 MHz or 100 MHz NMR instruments for the analysis of *Sterculia foetida* oil gave very low results for CPFA content in our experience. Better results were obtained when the sample used consisted of methyl esters separated from the other products of the esterification reaction and a 220 MHz instrument was used, allowing baseline resolution of the important peaks caused by cyclopropene methylene and $\omega$-methyl protons.

The chromatograms obtained from the GLC separation of the methyl esters of *S. foetida* fat using conventional columns normally consist of a series of broad overlapping peaks. An early claim was made that the results obtained for the CPFA content from such traces gave a good correlation with results obtained from a HBr titration (19), but this method of CPFA analysis has not gained acceptance although there are some recent reports (27,28) of the use of direct GLC methods and these are discussed later. Currently derivatization is usually accepted as necessary because of the instability of the cyclopropene ring under GLC conditions. The GLC analysis of methyl mercaptyl derivatives of CPFA was suggested (16) but was unsuitable for low levels of CPFA and was later shown not to be quantitative (15).

The GLC analysis of ether and ketone derivatives produced from the reaction of transesterified lipids with silver nitrate in methanol (15) was criticized because of its complicated nature and the necessity of carrying out further separation during analysis when the sample contains less than 5% CPFA content (14). Nonetheless, this method has been widely used and was recommended by Coleman (17).

Cyclopropane fatty acids are thermally stable on GLC columns and consequently hydrogenation of transesterified lipids followed by GLC analysis has been suggested (20-22), but the use of the usual heterogeneous hydrogenation catalysts can result in incomplete hydrogenation and/or decomposition of the cyclopropene ring (23). A wide variety of heterogeneous hydrogenation catalysts (Re, Rh, Ir, Pd, Pt) of varying activity were found to be unsatisfactory (11). The reaction of cyclopropene esters with some hydrogenation catalysts in the absence of hydrogen has been shown to cause cyclopropene ring cleavage (24). Complexes of the type $X(P(C_6H_5)_3)_3Rh$ (X = Cl, Br, I) are known to be highly effective and specific homogeneous catalysts for the hydrogenation of alkenes to alkanes (25) and thus we have investigated the use of such catalysts to stabilize the methyl esters of CPFA before analysis by GLC.

## EXPERIMENTAL

### Reagents

Chloro-, bromo- and iodotris(triphenylphosphine)-rhodium (I) were prepared by literature methods (25). All solvents were dried and redistilled. NMR spectra were recorded on a Perkin Elmer R32 (90MHz) and on a Varian HA 220 (220MHz). GLC was carried out using a Pye 204 instrument and GLC/mass spectrometry using a Pye 104/Kratos MS30 system. Hydrogenations carried out at pressures above 1 atmosphere of hydrogen were performed using a Low Pressure Catalyst Hydrogenation Apparatus supplied by Chas. W. Cook of Birmingham, England.

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### Esterification of *Sterculia foetida*

Typically, *S. foetida* nuts (100g) were macerated in an MSE top drive macerator with hexane (400 mL), centrifuged, filtered and rotary evaporated under reduced pressure.

*S. foetida* fat (250 mg) was transesterified with 1% sodium methoxide in methanol (50 mL) under reflux for 30 min, diluted with an equal volume of water and extracted 3 times with 20 mL aliquots of petroleum spirit (b.p. 40-60 C). The combined extracts were dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure using a rotary evaporator.

The methyl esters were separated from other components when required by eluting them through a 10 cm silica column with petroleum spirit (b.p. 40-60 C). An ultraviolet detector was used to detect the desired methyl esters.

### Hydrogenation of the Methyl Esters of *S. foetida*

*Using a vacuum line (1 atmosphere pressure of hydrogen).* Typically 1:1 (v/v) mixture of benzene/ethanol (25 mL) was placed in a flask fitted with a stirrer that was mounted in ptfe bearings and contained an iron bar so that it could be driven at 3,000 rpm by an external horseshoe magnet mounted in a mechanical stirrer. The efficiency of this stirring system had previously been evaluated in the hydrogenation of concentrated solution of cyclohexene where the rates of hydrogen uptake are much more rapid than for a typical hydrogenation of the methyl esters of *S. foetida* oil. Stirring speeds of 1,500 or 3,000 rpm did not alter the rate of uptake, indicating that gaseous diffusion is not a rate-controlling factor. The flask was connected to a vacuum line. The bromo-catalyst (0.097g, $10^{-4}$ mol) was contained in a hollowed out side arm that could be rotated to allow the catalyst to drop into the solvent mixture once it had been degassed. The degassing was carried out by alternately freezing the solvent mixture in liquid nitrogen, pumping off excess gas and thawing the solvent. The freeze, pump, thaw cycle was repeated twice more. Hydrogen was admitted to the flask at atmospheric pressure and after formation of a yellow solution of the dihydride complex, the methyl esters of *S. foetida* fat (200 mg) in degassed benzene/ethanol (5 mL) were admitted to the flask and hydrogenation was left to proceed overnight.

The reaction mixture was taken to dryness under reduced pressure in a rotary evaporator and the residue extracted with 5 mL of petroleum spirit (b.p. 40-60 C). The reaction mixture was then eluted through a 10 cm silica column with 25% diethyl ether/petroleum spirit (b.p. 40-60 C), leaving a colored band at the top of the column because of the catalyst. The volume of the sample was reduced to ca. 5 mL under reduced pressure using a rotary evaporator.

*Hydrogenation at pressures above 1 atmosphere.* A 250-mL glass reaction vessel containing bromocatalyst (0.097 g, $10^{-4}$ mol) was placed in the hydrogenation apparatus, purged with nitrogen and hydrogen then charged with a solution of methyl esters of *S. foetida* fat (200 mg) in benzene/ethanol (1:1 v/v, 30 mL) that had been previously separately degassed on a vacuum line as described above. For pressures greater than 60 psi, a stainless-steel vessel was used. The apparatus was charged to the required pressure and left to rock overnight. The products were usually yellow solutions which darkened to a red color on exposure to the atmosphere. The samples were prepared for analysis as described above.

*Reaction between X ($P(C_6H_5)_3)_3Rh$ (X = Cl, Br) and the methyl esters of* S. foetida *oil.* The procedure outlined for hydrogenation was carried out except that the mixture was kept under nitrogen rather than hydrogen, and in one case 0.6g, $6.2 \times 10^{-4}$ mol of bromocatalyst was used. Samples were taken periodically through a narrow port with a hypodermic syringe with a long needle while maintaining a slight positive pressure of nitrogen. The samples were cooled to −78 C and immediately analyzed by GLC. Hydrogenation was carried out after leaving the mixture to stand overnight, but only occurred where an excess of catalyst had been used.

*HBr titrations.* Method I, described by Rosie and Shone (10), was used with modifications using a totally enclosed apparatus to minimize losses of HBr and using chlorophenol red as an indicator to improve detection of the endpoint of the titration.

## RESULTS AND DISCUSSION

The effectiveness of $X(P(C_6H_5)_3)_3Rh$ to function as homogeneous hydrogenation catalysts for alkenes is in the order:

$$Cl < Br < I.$$

However, the iodocomplex is more susceptible to oxidation, so the more stable chloro- and bromocomplexes were mainly used in this study. The highly specific nature of the catalytic action under very mild conditions led to the expectation of quantitative hydrogenation of the methyl esters of CPFA. A typical chromatogram obtained from a GLC separation of the hydrogenated methyl esters of *S. foetida* oil carried out on an Apiezon L column is shown in Figure 1. In contrast to the large number of overlapping peaks that are observed in chromatograms of unhydrogenated samples, the chromatogram in Figure 1 contains 4 clearly resolved major peaks caused by methyl palmitate (ECL = 16.0), methyl dihydromalvalate (ECL = 17.7), methyl stearate (ECL = 18.0) and methyl dihydrosterculate (ECL = 18.7). In addition, a small peak is at ECL = 18.5. GLC/mass spectrometry analysis allowed unequivocal identification of the 4 major components and demonstrated that the minor peak is because of the presence of free triphenylphosphine arising from the known dissociation of the catalyst during hydrogenation (25). In some chromatograms, an even smaller broad peak was observed at ECL = 21.6. This was identified as being triphenylphosphine oxide and presumably arises from aerial oxidation of free triphenylphosphine during the preparation of the hydrogenated sample for analysis. The sterculate and malvalate are easily determined as their dihydro-derivatives (Fig. 1). Other unsaturated acid residues—oleate and linoleate—are also converted to the saturated compound, methyl stearate. This conversion is advantageous to the determination of the CPFA content as less likelihood exists of overlapping of peaks because of CPFA and other unsaturated acids (23).

The use of $Cl(P(C_6H_5)_3)_3Rh$ as the hydrogenation catalyst for the methyl esters of *S. foetida* fat gave lower than expected values for the CPFA content with low precision. In order to ascertain whether CPFA was being lost by reaction with the catalyst (24), the nature of the catalyst and the conditions of the hydrogenation particularly the pressure of hydrogen were varied (Table I). For the chlorocomplex, the apparent CPFA content rises significantly when pressures greater than 1 atm of hydrogen are used. For the bromocomplex, the observed CPFA content increases, but slightly with increase in hydrogen pressure from 1-3.3 atm and is unaltered when changing from 3.3-13.3 atm. Similarly the use of the iodocomplex gives higher results for the CPFA content when pressure greater than 1 atmosphere is used. The observation that within the limits of experimental error the values for the CPFA content given when using the bromocomplex at 1 atm,

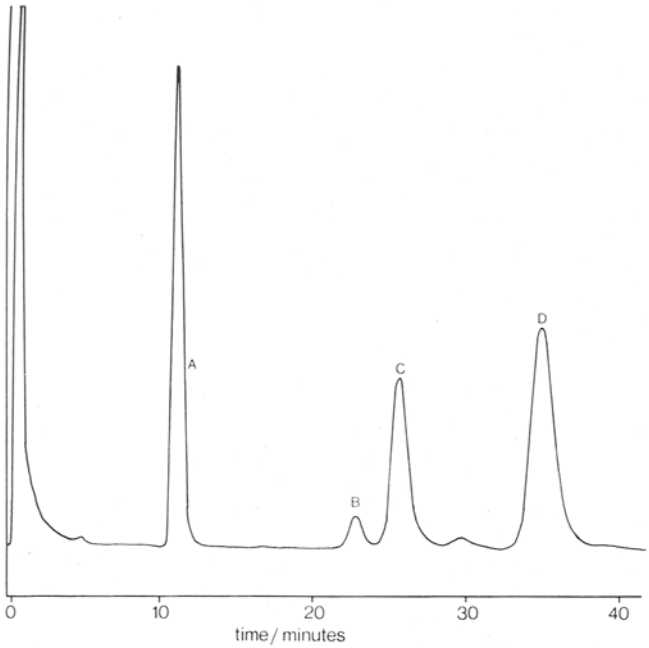


**FIG. 1. GLC analysis of hydrogenated methyl esters of *S. foetida* oil. A = methyl palmitate; B = methyl dihydromalvalate; C = methyl stearate; D = methyl dihydrosterculate; 1.5% Apiezon L column; 3m; 190 C; carrier gas, $N_2$, flow 20 $cm^3$ $min^{-1}$.**

**TABLE I**

**Percentage of CPFA Content of Methyl Esters of *S. foetida* Oil Determined with Different Catalysts and Different Pressures**

| Catalyst | CPFA content (%) | | |
|---|---|---|---|
| | P = 1 atm | P = 3.3 atm | P = 13.3 atm |
| $Cl(P(C_6H_5)_3)_3Rh^I$ | 54.00 | 60.00 | 61.00 |
| $Br(P(C_6H_5)_3)_3Rh^I$ | 62.45 | 63.45 | 63.44 |
| $I(P(C_6H_5)_3)_3Rh^I$ | 56.90 | 62.39 | 63.09 |

Each figure is obtained from a hydrogenated sample of a different batch of methyl esters from the same sample of *S. foetida* seeds and is the mean of 5 GLC results.

3.3 atm or 13.3 atm pressure of hydrogen and by the iodocomplex at 3.3 atm or 13.3 atm pressure of hydrogen suggests that the use of the bromocomplex gives a true CPFA content of the sample, whereas in the case of the chlorocomplex a significant loss of CPFA may be caused by reaction with the catalyst.

The influence of catalyst on the degradation of CPFA was investigated by periodically sampling a benzene/ethanol (1:1) solution of the methyl esters of *S. foetida* and the chloro- or bromocomplex under nitrogen. When a 2:1 mol ratio of complex to methyl esters of CPFA was used, a rapid degradation occurred such that the first sample taken after 5 min gave a chromatogram in which no peaks attributable to CPFA were present. Subsequent admission of hydrogen caused hydrogenation in the usual way and the GLC analysis gave a chloromatogram in which only methyl palmitate and methyl stearate were present. The ratio of palmitate to stearate was unaltered compared with samples that had only been hydrogenated, illustrating that no loss of fatty acid material other than CPFA occurs in samples that have been exposed to chloro- or bromocomplex before hydrogenation.

In cases where the molar ratio of methyl esters of CPFA to chloro- or bromocomplex was 3.76:1 (as used typically

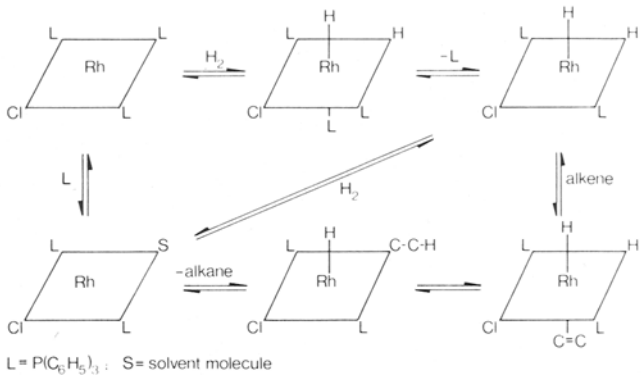


**FIG. 2. Hydrogenation of alkenes using $Cl(P(C_6H_5)_3)_3Rh$ as catalyst.**

in the analytical technique), the CPFA content fell rapidly so that after 15 min in both cases the catalyst had degraded ca. 2.5-3 mol of CPFA/mol of catalyst. The variation in number of moles degraded suggests that more than one mode of degradation may exist but the result does show that the degradation is stoicheiometric rather than catalytic. The mixture could not subsequently be hydrogenated, indicating that the complex is changed and can no longer function as a hydrogenation catalyst. The products of this decomposition are currently being investigated.

The mechanism of the hydrogenation of alkenes catalyzed by $Cl(P(C_6H_5)_3)_3Rh$ has been much studied (26). Cyclopropenes would be expected to follow the accepted catalytic cycle shown in Figure 2. In addition to the steps shown in the catalytic cycle, the active species, $Cl(P(C_6H_5)_3)_2SRh$ (S = solvent), can react with an alkene to produce an alkene complex, which, in the case of ethene, has been shown to be easily isolated (25).

$$Cl(P(C_6H_5)_3)_2SRh + \text{alkene} \rightleftharpoons Cl(P(C_6H_5)_3)_2\,(\text{alkene})Rh + S.$$

Two competing factors might be identified in the attempted quantitative hydrogenation of CPFA. First, hydrogenation of the cyclopropene will occur once the rhodium dihydride complex has been formed and, as previously observed (25), the use of higher pressures of hydrogen will enhance the hydrogenation reaction; second, the coordinatively unsaturated species, $RhCl(P(C_6H_5)_3)_2S$, may form an alkene complex with the cyclopropene that could be followed by degradation of the cyclopropene ring. This degradation will result in apparent loss of CPFA content in the hydrogenation/GLC method because the products of this degradation are not observed in the chromatogram.

The bromocomplex, $RhBr(P(C_6H_5)_3)_3$, is superior to the chlorocomplex in effecting quantitative hydrogenation of the cyclopropene because it is a better hydrogenation catalyst for alkenes.

Other parameters of the hydrogenation/GLC method were investigated. In Table II is shown the CPFA content of a number of hydrogenated samples of methyl esters of *S. foetida* fat. Results are quoted for 1 nonpolar column (1.5% Apiezon L) and 1 polar column (10% SP 2330, a cyanosilicone). The good agreement of results for CPFA content shown in Table II indicates that once the sample has been hydrogenated, it does not undergo degradation during analysis. The results demonstrate the precision that can be obtained with a standard deviation of 0.40 for the results given.

The homogeneous catalyst can be separated from the hydrogenated methyl esters simply by eluting the sample

**TABLE II**

**CPFA Content of Three Different Samples of Hydrogenated Methyl Esters of *S. foetida* Fat Using Two Different GLC Columns**

| Sample[a]/column | CPFA content (%)[b] 1.5% Apiezon L | 10% SP 2330 |
|---|---|---|
| 1 | 62.63 ± 0.09 | 62.62 ± 0.23 |
| 2 | 62.50 ± 0.14 | 62.26 ± 0.30 |
| 3 | 62.10 ± 0.22 | 63.25 ± 0.27 |

[a]Each sample is a different batch of methyl esters from the same original sample of *S. foetida* fat and each was hydrogenated using $Br(P(C_6H_5)_3)_3Rh$ as catalyst.

[b]Each value given is the mean of 6 GLC obtained using identical conditions for the 2 columns (both of which were 3 m), i.e., temperature, 180 C; carrier gas flow rate, 20 $cm^3$ $m^{-1}$; sample, 0.5 μL.

**TABLE IV**

**The CPFA Content of *S. foetida* Obtained Using Different Analytical Techniques**

| Method | CPFA (%) |
|---|---|
| Hydrogenation/GLC on methyl esters of *S. foetida* oil[a] | 62.6 ± 0.7 |
| HBr titration on *S. foetida* oil | 57.8 ± 2.1 |
| 220 MHz proton NMR on separated methyl esters of *S. foetida* oil | 55.6 ± 1.9 |
| Unhydrogenated methyl esters/glass capillary column[b] | 62.5 |

[a]1.5% Apiezon L column.

[b]OV 101; length, 25 m; i.d., 0.2 mm; temperature 190 C.

through a 10 cm silica column with 25% diethyl ether/petroleum spirit (b.p. 40-60 C). The catalyst remains on the column. This procedure was not regarded as essential, but possibly advisable in case of deleterious effects that might arise if rhodium was deposited in the GLC column.

The results in Table III are for a hydrogenated sample that has been analyzed before separation of the catalyst (precolumn) and after separation of the catalyst (postcolumn). The agreement of these results demonstrates that this separation of the catalyst does not result in loss of CPFA content.

The results obtained from the hydrogenation/GLC method have been compared with those obtained using the HBr method and the NMR method. We have found that the hydrogenation/GLC method gives more reproducible results than either of the other 2 methods. A difference was found between the values obtained for the 3 methods, but the hydrogenation/GLC method identifies the acids and, by internal normalization, provides a direct comparison of the CPFA and the other fatty acids present. In addition to this work, a preliminary study was carried out using a glass capillary GLC column into which unhydrogenated methyl esters were injected and although some decomposition of the CPFA occurred, the enhanced resolution of the column allowed a calculation of CPFA content based on summing the area caused by all peaks arising from CPFA. This result is shown in Table IV and is in excellent agreement with the value obtained from the hydrogenation/GLC method. Two recent reports have been made of the direct GLC analysis of methyl esters of CPFA but both sets of workers still indicate that some decomposition of the CPFA can occur on their columns (27,28).

The lack of precision of the HBr titration method, even after we had modified it to reduce losses of HBr because of its volatility and to improve detection of the endpoint is in agreement with the results of other workers. Pawlowski et al. found all HBr titration methods to be unsatisfactory as a quantitative measure of CPFA content (14). Nixon, in a personal communication, quoted a value of 63.1 ± 8.8% for the CPFA content of methyl esters of *S. foetida* oil when using the original HBr titration method of Rosie and Shone (10). A recent report claims higher precision for a modified HBr method (6). Schneider et al. (15) found a mean value of 55.93% for total CPFA in *S. foetida* oil using their GLC method but a value of 52% using HBr titration. The lower values found with HBr titration of 3.9% for Schneider et al. and 4.8% in our work appears to indicate a common observation.

In order to investigate typical samples of seed oils containing low levels of CPFA, a batch of methyl esters of *S. foetida* fat that contained methyl nonadecanoate as internal standard was diluted with methyl esters of corn oil. Solutions calculated to contain 0.7% and 0.21% of CPFA were found by hydrogenation/GLC to contain 0.75% and 0.18%, respectively, illustrating the ability of the method to be used to analyze for low levels of CPFA.

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**TABLE III**

**The CPFA Content of Methyl Esters of *S. foetida* Oil Obtained for Samples Taken Before and After Separation of the Catalyst by Column Chromatography**

| | Methyl dihydrosterculate (%) | Methyl stearate (%) | Methyl dihydromalvalate (%) | Methyl palmitate (%) | Total methyl CPFA (%) |
|---|---|---|---|---|---|
| Precolumn | 56.5 | 17.5 | 5.6 | 19.8 | 62.1 |
| Postcolumn | 56.2 | 17.6 | 5.7 | 20.0 | 62.1 |

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# Chia Seeds as a Source of Natural Lipid Antioxidants

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## ABSTRACT

Chia (*Salvia* sp) seeds were investigated as a source of natural lipid antioxidants. Methanolic and aqueous extracts of defatted chia seeds possessed potent antioxidant activity. Analysis of 2 batches of chia-seed oils demonstrated marked difference in the fatty acid composition of the oils. In both batches, the oils had high concentrations of polyunsaturated fatty acids. The major antioxidant activity in the nonhydrolyzed extract was caused by flavonol glycosides, chlorogenic acid ($7.1 \times 10^{-4}$ mol/kg of seed) and caffeic acid ($6.6 \times 10^{-3}$ m/kg). Major antioxidants of the hydrolyzed extracts were flavonol aglycones/kaempferol ($1.1 \times 10^{-3}$ m/kg), quercetin ($2.0 \times 10^{-4}$ m/kg) and myricetin ($3.1 \times 10^{-3}$ m/kg); and caffeic acid ($1.35 \times 10^{-2}$ m/kg). Two methods were used to measure antioxidant activities. Both were based on measuring bleaching of β-carotene in the coupled oxidation of β-carotene and linoleic acid in the presence of added antioxidants.

## INTRODUCTION

Many vegetable oils are protected by natural, endogenous polyphenolic antioxidants. These antioxidants exert a marked protective effect on plant lipids even though the natural oils contain high concentrations of polyunsaturated fatty acids (PUFA) (1) that are usually very susceptable to oxidation. Polyphenols responsible for this protective action are most commonly flavonoids and cinnamic acid derivatives that occur abundantly throughout the plant kingdom (2,3).

Seeds of the chia plant (*Salvia hispanica L.*, and other *Salvia* members of the family Labiatae) have long been components of foods of American Indians and Mexicans (4). The seeds yield between 25% (5) and 35% (6) extractable oil, which contain high concentrations of PUFAs. In fact, the fatty acid composition is such that the oil may range from a salad oil of excellent composition to a commercial drying oil (7). Because of the highly unsaturated nature of the oil, the seeds probably contain potent lipid antioxidants.

The current investigation was initiated to identify the major antioxidant principals of chia seeds and to ascertain their potential as food antioxidants.

## MATERIAL AND METHODS

### Extraction of Phenolic Compounds

Two different samples of chia seeds, purchased from a local health food store, were air dried at 40 C for 48 hr, then finely ground (1 mm screen) in a Wiley mill. One thousand grams were then extracted in a Soxhlet extractor with 3 L of petroleum ether (b.p., 35-60 C) for 24 hr, to remove the fats and other petroleum ether soluble components. The residue was air dried at room temperature for 12 hr and then reextracted with 5,000 mL methanol in a Soxhlet for 48 hr. The methanolic extract was concentrated in vacuo on a rotary evaporator at 40 C.

The dried methanolic extract was suspended in 1,000 mL of 1-butanol and transferred to a 4,000 mL separatory funnel. Water (ca. 100 mL) was added and the mixture was shaken vigorously. With the addition of 5 L of petroleum ether, the phenolic components (and other compounds of intermediate polarity) precipitated into the aqueous phase. The organic phase was discarded. The aqueous phase was freeze-dried and suspended in methanol. This fraction is referred to as the crude extract.

### Total Phenolic Compounds

Total concentration of phenolics in the crude extract was determined by a modification of the method of Bray and Thorpe (8). Dried samples and standards were prepared in 60:40 acidified methanol/water (0.3% HCl). Test solutions (samples or standards) of 100 μL were added to 2.0 mL of 2% $Na_2CO_3$. After 2 min, 100 μL of 50% Folin-Ciocalteau reagent were added and allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm on a Beckman spectrophotometer, model 25. The blank consisted of all reagents and solvents without test compounds or standard. The standard was caffeic acid prepared in concentrations of 0.001 mg/mL to 1.0 mg/mL. The phenolic concentrations were determined by comparison with the standard calibration curve.

### Hydrolytic Cleavage of Flavonol Glycosides and Cinnamic Acid Esters

Aliquots of the crude extract in HCl-methanol (2M) were heated in tightly capped 5 mL vials for 45 min at 100 C. After heating, 3 mL of water were added to the vials. The contents were transferred to a separatory funnel and extracted 3 times with 2 mL diethyl ether. The aqueous phase was discarded. The ether phases were combined and evaporated to dryness. The residue was redissolved in 0.5 mL spectral-grade methanol.

Completeness of hydrolysis was determined by spotting hydrolyzed and nonhydrolyzed extracts on 20 cm × 20 cm cellulose thin layer chromatographic (TLC) plates and developed in 3 different solvent systems: (a) 15% acetic acid, (b) chloroform/formic acid/water (10:9:1 v/v/v) and