Cottonseed Flavonoids as Lipid Antioxidants

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ABSTRACT

Methanolic extracts of cottonseeds were found to possess antioxidant activity. Separation of antioxidant components was achieved by paper, thin layer (TLC) and gas liquid chromatography (GLC) and spectral analysis. Chromatographic technique indicated that the flavonoids quercetin and rutin were the major flavonoids present. GLC analysis of the cottonseed extract eluted from TLC plates, developed in n-butanol/acetic acid/water (4:1:5, v/v/v) and ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:1:1, v/v/v) solvents, corresponded in retention times to the rutin standard (10^{-4} M). TMS derivatives of the aglycone fraction, prepared by acid hydrolysis, correlated to the retention time of quercetin. Spectral analysis of the aglycone fraction also indicated quercetin derivatives to be present in cottonseed. The glycosyl substitution of the flavonoids were identified by the 3 chromatographic procedures to be glucose and rhamnose. Chromogenic spray reagents were used to further characterize the cottonseed flavonoids and flavonoid components on TLC and paper chromatography. The investigation, therefore, demonstrated that quercetin derivatives appear to be the main flavonoid species in cottonseed. Rutin was found to be one of the major quercetin glycosides. Quercetin and rutin are shown to possess potent antioxidant activity.

INTRODUCTION

The commonly used antioxidants today are synthetic compounds and consumers tend to reject them (1). Furthermore, the possible toxicity of synthetics used as antioxidant has been a subject of study in recent years. All this leads to interest in preparing antioxidants from natural food stuffs by extraction, purification and fractionation. Certainly no assurance whatsoever can be given that a fraction or a compound isolated from natural sources is safe. Nevertheless, such an antioxidant would be a natural component of foods that we have consumed for many years (1).

The flavonoids, a widely distributed group of plant phenolics, are of considerable interest as potential natural antioxidants that are already present in many foods and might perhaps be used as additives for others (1,3). Among naturally occurring substances of potential antioxidant activity, flavonoids are of particular interest. They are widely distributed in the plant kingdom.

The present study was initiated to identify natural antioxidants of cottonseed and to consider their potential use as substitutes for presently used antioxidants in food or other products.

MATERIALS AND METHODS

Preparation of Extracts

Cottonseeds *(Gossypium hirsutum)* were delinted using concentrated sulfuric acid and then were repeatedly washed with ice-cold water to remove the acid. The seeds were dried in an oven at 105 C for 48 hr and ground (1 mm screen) in a Wiley mill. One hundred fifty g of ground cottonseed was defatted in a Soxhlet apparatus with petroleum ether for 24 hr. The residue was air dried at room temperature for 12 hr, then extracted with methanol in a Soxhlet apparatus for 48 hr. The methanolic extract was concentrated in vacuo on a rotary evaporator at 40 C to a volume of 200 mL.

Test of Antioxidant Activity

Antioxidant activities of test samples were determined using the spectrophotometric method of Marco (4) as modified by Miller (5) and Pratt (6). This technique has been reported in detail by Taga (7).

A β-carotene spray solution for detecting antioxidants was prepared using a modification of the method of Philip (8). β-Carotene (12 mg) was dissolved in 30 mL chloroform; 2 drops of purified linoleic acid and 60 mL ethanol were added to the carotene-chloroform solution. TLC plates were sprayed and the plates were then exposed to daylight until background color was bleached (2-3 hr). Spots in which yellow color persisted were judged to have antioxidant activity with the intensity of color related to the amount of activity.

Acid Hydrolysis of the Extract

Acid hydrolysis was conducted as reported by Mabry et al. (9) in which 4.0 mL of the hot methanol extract was placed in a 100 mL pear-shaped flask and evaporated to dryness under reduced pressure on a rotary evaporator at 45 C. The residue was redissolved in one drop of methanol (spectral grade) and 1.5 mL of 1.67 N HCl was added. The resulting solution was placed in a 3 mL vial, capped and heated in a boiling water bath for 45 min. After heating, 2.0 mL diethyl ether and 1.0 mL water were added with vigorous shaking. The ether (upper) phase was removed and divided into 2 vials; the contents of 1 vial were evaporated to dryness under nitrogen for gas liquid chromatographic (GLC) analysis and the contents of the other concentrated for spectral, paper and thin layer chromatographic (TLC) analysis. The aqueous phase, containing the sugars, was also divided into 2 vials. One was evaporated to dryness for GLC analysis; the other concentrated for spectral and TLC analysis.

CHROMATOGRAPHIC PROCEDURES

Paper Chromatography

Whatman Chromatographic paper No. 3MM (46 × 57 cm) was used initially to separate components of the extract. Ca. 3 mL of the cottonseed extract was streaked evenly onto the filter paper 8 cm from the top edge. The upper phase of n-butanol/acetic acid/water (4:1:5, v/v/v) (BAW) mixture provided the solvent system and chromatograms were developed in descending order. The chromatographic cabinet, containing the undeveloped chromatograms, was equilibrated overnight with ca. 100 mL of the solvent before development. After the chromatograms were developed (ca. 14 hr), they were dried and examined under ultraviolet (UV) radiation using a long wavelength (366 nm). Major fluorescent bands were eluted by refluxing in methanol (spectral grade). Components of extracts were further characterized by paper chromatography using BAW, phenol saturated with water (80 g phenol/20 mL water) and tertiary butanol/acetic acid/water (3:1:1, v/v/v) as solvents.

Thin Layer Chromatography

TLC was used to separate the components of the methanolic extracts. A volume of 75 μL of concentrated extract was streaked on each precoated TLC Silica Gel G plate, which had been activated for 30 min at 100 C. Several solvents were used to develop the TLC plates (20 × 20 cm). The solvents that gave the best separation were BAW and ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:1:1, v/v/v/v) (EMFW). Bands were noted by UV light

(366 nm) after the TLC plates had dried. The fractions of interest were scraped from the plates and soaked in methanol (spectral grade) for 2 hr. The mixture was filtered through Whatman #42 filter paper, the residue washed with methanol and the filtrate evaporated in vacuo to near dryness at 40 C on a rotary evaporator.

Gas Liquid Chromatography

GLC was used to separate and identify trimethylsilyl ethers of flavonoids and sugar fractions. The reagent used for silylation was Sylon-HTP (Supelco, Inc., Bellefonte, PA) and consisted of a mixture of hexamethyldisilazane, trimethylchlorosilane and pyridine (3:1:9). Samples for silylation were evaporated to dryness in a nitrogen atmosphere and 0.5 mL of silylating reagent was added. The mixtures were tightly capped and heated for 30 min in a boiling water bath to facilitate derivatization.

Separation was accomplished using a Varian Aerograph Model 2440-10 GLC equipped with a flame ionization detector (FID) and a 6 ft × 1/4 in (2.0 mm i.d.) glass column coated with 2% SE 30 and 1% SP-2401 (supelco). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The injector and detector temperatures were operated at 230 C for the sugar determination and 310 C for the flavonoid determination.

The fractions were run isothermally at 180 C for the sugar and 260 C for the flavonoid aglycone fractions. The eluted glycosides from TLC were run isothermally at 220 C for 6 min and then programmed until a maximum temperature of 260 C was obtained with a rate of change of 6 C/min. The temperature was maintained at 260 C until the development was complete.

Spray Reagents

Ferric chloride-potassium ferricyanide ($FeCl_3$-$K_3Fe(CN)_6$). Equal volumes of aqueous 1% solutions of each salt were freshly mixed together, making an orange-brown solution. Phenols give blue colors immediately (Barton et al., 1952).

Ferric chloride 2% in alcohol ($FeCl_3$). Phenols with 2 or more neighboring hydroxyl groups or ortho or para hydroxy-carbonyl groups give colored chelates. Dihydroxy compounds are usually green, trihydroxy blue, and others brown or red (Reio, 1958, 1960).

RESULTS AND DISCUSSION

In Figure 1, the results demonstrate that the unhydrolyzed extract possessed marked antioxidant activity. Descending paper chromatography of methanolic extracts of unhydrolyzed cottonseed using BAW as the solvent developed 3 distinct fluorescent bands. These bands corresponded to Rf values of 0.20, 0.33 and 0.98; labeled A, B and C. These separated fractions each possessed antioxidant activity (Fig. 2). Fraction C was found the most effective in preventing oxidation of β-carotene. Activity of bands A and B were considerably lower, however, the concentration of components in fractions shown in Figure 2 are not known. Thus quantitative comparisons among trials are difficult to assess. Ferric chloride-potassium ferricyanide spray reagent indicated that phenolic compounds were present in each fraction.

The glycoside, aglycone and hydrolyzed sugar fractions were characterized by TLC, GLC and paper chromatography. Various TLC solvents were used to separate the fractions. The data in Table I indicate the best solvents to be BAW and EMFW. These solvents were able to separate quercetin, dihydroquercetin and rutin standards (10^{-4} M). The plates were sprayed with ferric chloride-potassium ferricyanide to detect phenols.

The unhydrolyzed and hydrolyzed cottonseed extract components possessed Rf values that were nearly identical to the standards of quercetin and rutin in 5 solvent systems (Table I). A quarter of the plate was sprayed with ferric chloride-potassium ferricyanide so the phenolic bands could be detected. Seven bands were eluted from the plates by scraping and extracting as previously described.

The results obtained from the GLC analysis indicated that rutin was present. From the BAW solvent plates, the band eluted at Rf value of 0.56 corresponding to the Rf value for the rutin standard, 0.55. When this band was run on the GLC, a peak appeared at a retention time of 7.5 min and the peak for the standard appeared at 7.5 min. The band correlating to rutin on the EMFW solvent plate had an Rf value of 0.56 and the standard had an Rf value of 0.56. The GLC analysis results gave a retention time of 7.5 min for the band. These results strongly indicate that rutin is present in cottonseed.

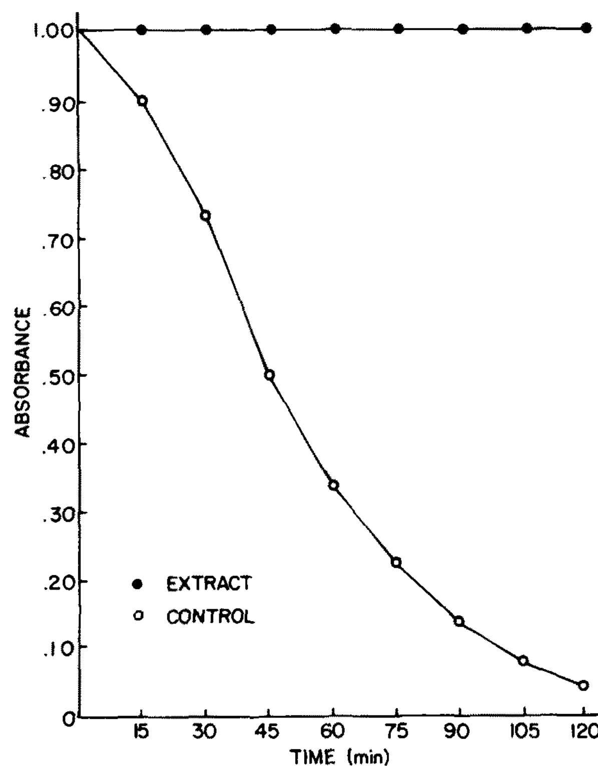

FIG. 1. Antioxidant activities of unhydrolyzed cottonseed extract as determined by spectrophotometric test.

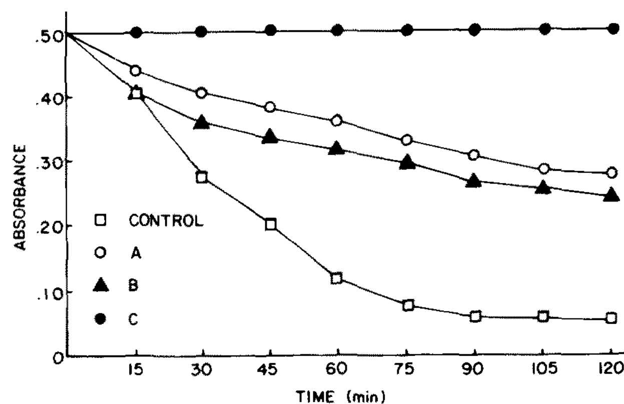

FIG. 2. Antioxidant activities of paper chromatography fractions A, B and C.

TABLE I

Rf Values of Phenolic Compounds[a] on Thin Layer Chromatography

Solvent	Unhydrolyzed cottonseed extract	Aglycone fraction	Dihydroquercetin[b]	Quercetin[b]	Rutin[b]
Butanol/acetic acid/ water (4:1:5, v/v/v)	0.0; 0.26; 0.44; 0.56; 0.68; 0.80; 0.94	0.84	0.98	0.84	0.55
Ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:1:1, v/v/v/v)	0.0; 0.13; 0.23; 0.56; 0.70; 0.83; 0.95	0.60; 0.95	0.98	0.84	0.55
Chloroform/ethanol/ methyl ethyl ketone (12:2:1, v/v/v)	0.0; 0.66; 0.69; 0.80; 0.88	0.98	0.42	0.98	0.0
Ethanol/chloroform (1:3, v/v)	0.0; 0.46; 0.72	0.91	0.75	0.91	0.0
Ethyl acetate/petroleum ether (3:1, v/v)	0.0; 0.21; 0.56; 0.63	0.50; 0.63	0.83 0.77	0.50	0.0

[a]Determined by spraying with ferric chloride-potassium ferricyanide.
[b]Standard solution, 10^{-4} M.

Chromagenic spray reagents were used to further characterize the flavonoids and flavonoid components on TLC plates. Chromatograms were subjected to ammonia vapor to aid in visualizing flavonoid compounds in visible and UV light (9,13,14). The glycoside bands corresponding to rutin gave reactions identical with rutin standards. The aglycone corresponding to quercetin gave reactions identical with authentic quercetin.

Pratt and Wender (15) reported that cottonseed extracts possess isoquercetin (quercetin-3-glucoside) and rutin (quercetin-3-rhamno-glucoside). Thus, when the extracts were acid hydrolyzed, quercetin resulted as the aglycone. According to Struck and Kirk (16) methylated derivatives of quercetin and kaempferol are found in cottonseed flower petals, but in much lower concentrations than nonmethylated flavonols. Struck and Kirk (13) report the 4 flavonols —quercetin, gossypetin, herbacetin and kaempferol—are found in *Gossypium hirsutum* species.

GLC analysis of hydrolyzed extracts (TMS derivatives) gave a peak with a retention time of 8.3 min. The retention time of the quercetin standard was also 8.3 min. Other distinct peaks occurred at 9.9 min and 11.9 min, indicating other aglycone, gossypetin, kaempferol, herbacetin and methylated derivative of quercetin. Standards of these aglycones were not available for analysis.

TMS derivatives of sugar fractions gave GLC peaks with retention times of 2.3 min and 6.5 min. The retention time for standards of L-rhamnose and D-glucose were 2.3 min and 6.5 min. The sugar fraction gave several other peaks. Components of sugar fractions gave Rf values of 0.21 and 0.61 in BAW and 0.23 and 0.50 in EMFW solvents. Rf values for glucose and rhamnose were 0.21 and 0.61 in BAW and 0.23 and 0.50 in EMFW solvents. Sugar chromogenic sprays gave reactions identical to the standards of glucose and rhamnose. Glucose is the sugar molecule that forms the glycoside, isoquercetin. Glucose and rhamnose form the disaccharide glycosyl substitution of rutin.

The band from the aglycone fraction that correlated with the quercetin standard when observed under UV light (366 nm), was eluted from the paper chromatogram in methanol (spectral grade). The band was then used for spectral analysis. The results, Table II, confirm that the band eluted was quercetin. The TMS derivatives of the aglycone band eluted from paper chromatography gave a retention time of 8.3 min; this is identical to the retention times of TMS derivatives of quercetin.

TABLE II

Comparison of Unknown Aglycone Fraction with Quercetin Standard[a] on Paper Chromatography

Compound	Paper chromatographic Rf values[b] Solvents 1	2	3	Spectral Data λ max, nm
Quercetin	0.70	0.13	0.93	253, 370
Unknown	0.70	0.13	0.93	253,[c] 369[c]

[a]Quercetin standard, 10^{-4} M.
[b]Solvent 1 is BAW, solvent 2 is phenol saturated with water, solvent 3 is teritary butanol/acetic acid/water (3:1:1, v/v/v).
[c]λ max same for all 3 solvents.

The spectrophotometric test for antioxidant activity was performed on the 7 bands eluted from the BAW solvent TLC plates previously discussed. The bands corresponded to Rf values of 0.0, 0.26, 0.44, 0.56, 0.68, 0.80 and 0.94. Compounds in all bands, except the band at the origin, showed antioxidant activity. The band at the origin was prooxidant. The activity of compounds with Rf values of 0.56 and 0.80 are shown in Figure 3. The band corresponding to Rf 0.68 had essentially the same activity as the band with Rf 0.56. The bands representing Rf 0.26, 0.44 and 0.94 had antioxidant activity to a lesser degree than the ones shown in Figure 3. On hydrolysis, all the antioxidant activity appeared to be caused by derivatives of quercetin. Quercetin standard (10^{-4} M) had a greater antioxidant activity than the rutin standard (10^{-4} M). According to Pratt (6), rutin, a glycosyl substitution with a disaccharide, has reduced antioxidant activity. Using commercial preparations of quercetin and rutin, the antioxidant activity of rutin was found to be approximately half that of quercetin (6).

The flavonoids of cottonseed have been found to possess antioxidant activity. Quercetin derivatives appear to be the major flavonoid species in cottonseed. Rutin and isoquercetin were found to be the major quercetin glycosides.

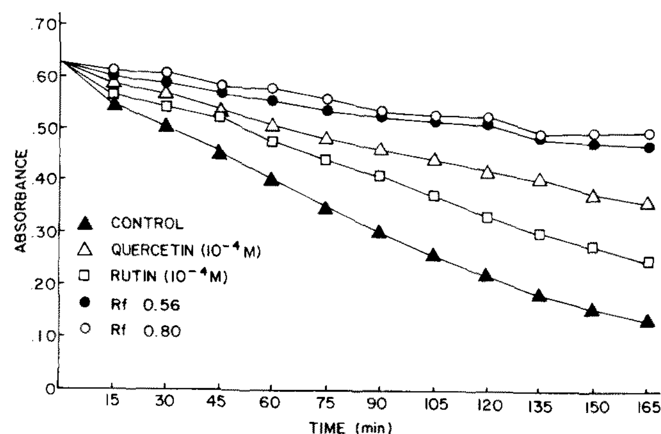

FIG. 3. Antioxidant activity of 2 unhydrolyzed bands eluted from TLC plates plus quercetin and rutin standards.

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☘Synthesis and Polymorphism of 1,2-Dipalmitoyl-3-acyl-*sn*-glycerols[1]

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ABSTRACT

Stereospecific 1,2-dipalmitoyl-*sn*-glycerol and 1,2-dipalmitoyl-3-acyl-*sn*-glycerols with even-carbon saturated fatty acyl chains of 2-16 carbons in length were synthesized. The polymorphic behavior and packing arrangements of the most stable crystal form obtained, from the solvent of crystallization, were studied by differential scanning calorimetry and powder X-ray diffraction. Three different layered packing modes were identified: (a) double-layer diglyceride-type; (b) triple-layer triglyceride-type; (c) double-layer triglyceride-type. The first type of packing was represented by 1,2-dipalmitoyl-3-unsubstituted, 3-acetyl and 3-butyryl-*sn*-glycerols packed in a bilayer with their long hydrocarbon chains in a parallel arrangement. In the second type of packing, shown by 1,2-dipalmitoyl 3-hexanoyl and 3-octanoyl-*sn*-glycerols, the shorter acyl chains formed a middle layer interposed between 2 layers of the 1,2-palmitoyl chains of *sn*-glycerol. The third type of crystal packing was exhibited by 1,2-dipalmitoyl-3-dodecanoyl and 3-tetradecanoyl-*sn*-glycerols and tripalmitin, was analogous to trilaurin in which the acyl chains at the 1 and 2 positions of glycerol formed an extended, nearly straight line and the 3-acyl chain was folded to lie parallel and alongside the acyl chain at the 1 position. The intermediate member of the series, 1,2-dipalmitoyl-3-decanoyl-*sn*-glycerol, exhibited both the second and the third type of chain packings when obtained from different solvents of crystallization.

INTRODUCTION

In triglycerides, substitution with different fatty acids at the 1 and 3 positions of glycerol produces a chiral center at the glycerol 2-carbon, and thus, optical activity. Most naturally occurring triglycerides show optical activity because of specific distributions of different fatty acids at the 1, 2 and 3 positions of glycerol (1). Specific triacylglycerol structure has important implications for the physical properties of triglycerides and perhaps for physiological characteristics, e.g. enzymatic hydrolysis (2), and subsequent absorption or metabolism (3). Fats with 1 and 2 long-chain and 3 short-chain saturated fatty acyl *sn*-glycerols are common in nature, e.g., butter oil contains ca. 10% of 1,2-(long chain) diacyl-3-butyryl-*sn*-glycerol (4).

A characteristic feature of triglycerides in the solid state is their polymorphism, because of the possibility of different packing arrangements of similar lattice energy. This polymorphism has been the subject of numerous studies by different workers extending over almost a century (5). However in many earlier studies racemic mixtures were studied and their properties may not be the same as specific isomers (6).

In the work reported here a homologous series of optically active triglycerides was synthesized. The 1 and 2 position of *sn*-glycerol was palmitate, whereas the substitution at the 3 position was varied with the fatty acyl chain containing 2-16 carbons. Systematic thermal and X-ray diffraction studies of these compounds have been used to gain information about their complex polymorphic behavior.

MATERIALS AND METHODS

Chemicals

Tripalmitin was obtained from the Hormel Institute, Austin, MN. Fatty acids used were purchased from Sigma

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