test tubes, a mixer and an 80 C oven or water bath. The shortened preparation time allows large numbers of samples to be processed in a batch mode. The results generally agree with an accepted method within the limitations of the GC procedure.

The method should prove useful in studying the effect of environmental factors on and plant breeding for wax composition where the available sample may be small and a large number of treatments and replicates must be processed. If automatic injection of the GC is used, large numbers of samples can be processed on a continuous basis by a single operator. This makes the method particularly suitable for industrial use in product development and quality control.

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A Flavonoid Antioxidant in Spanish Peanuts *(Arachia hypogoea)*

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ABSTRACT

Hot methanol extracts of Spanish peanuts were found to possess antioxidant activity. Thin layer (TLC) and paper chromatography of the methanolic peanut extracts yielded 6 fluorescent bands of which one exhibited potent antioxidant activity. Further separation by TLC showed this band to be a complex mixture of 3 components that were tested for antioxidant activity. One component demonstrated all of the antioxidant activity associated with the parent band. Analysis of this antioxidant by paper chromatography and TLC, chromatographic spray reagents and spectral analysis demonstrated that the compound was dihydroquercetin.

INTRODUCTION

Most varieties of oilseeds contain phenolics that retard oxidation in the native seed as well as in extracted oil. Activities of these compounds are as primary antioxidants, synergists and chelators (1). Among naturally occurring substances of potential antioxidant activity, flavones and flavonones are of particular interest (1). Flavonoids, a major group of plant phenols, are widely distributed in nature and occur as glycosides that, on hydrolysis with acid and heat, produce aglycone and sugar moities (2). Flavonoids are also found in association with proteins and tannins (3). Flavonoids possess both chain-breaking and metal-deactivating functions as antioxidants (1). The flavonoids consist of 2 hydroxylated or methoxylated aromatic nuclei linked by a condensed 3-carbon chain (2).

The present study was initiated to investigate aqueous and methanolic extracts of Spanish peanuts.

PROCEDURES AND METHODS

Extraction of Samples

Fifty g of peanuts *(Arachia hypogoea)* were homogenized in a Waring blender with 250 mL of water containing 2 drops of antifoam A, then boiled for 5 min. The mixture was filtered and washed with 200 mL hot water and the insoluble material was discarded. The filtrate was freeze-dried and the residue was brought to a volume of 50 mL with water. Thus, 1 mL of extract contained the water-soluble components from 1 g of peanuts. Alcohol-soluble components were extracted by a similar technique except absolute methanol was substituted for water and antifoam A was omitted.

Paper Chromatography

Methanolic extracts were repeatedly streaked on Whatman #3MM chromatographic paper (23 x 47 cm) until a total of 3 mL had been applied. The papers were equilibrated for 12 hr and developed in the upper phase of n-butanol/acetic acid/water (4:1:5, v/v/v) (BAW). The chromatograms were dried and examined under short (250 nm) and long (360 nm) ultraviolet (UV) wave lengths before and after exposure to ammonia fumes. Major fluorescent bands were eluted with 80% aqueous methanol (AR grade).

The eluted components were further separated by a 2-dimensional technique using 21 cm square Whatman #3MM paper. Chromatograms were developed ascendingly using tertiary butanol/acetic acid/water (3:1:1, v/v/v) (tBAW) as the solvent, air-dried and redeveloped in the second dimension in 15% acetic acid.

Thin Layer Chromatograph (TLC)

TLC plates precoated with silica gel were also used to separate constituents of the methanolic extract. The plates were activated at 100 C for 15 min and streaked with 0.25 mL extract. The plates were developed using the upper phase of an ethyl acetate/formic acid/water (10:2:3, v/v/v) (EFW) mixture. Bands of interest were scraped from TLC plates, soaked for 30 min in 50 mL spectral grade methanol, filtered and evaporated in vacuo to near dryness on a rotary evaporator at 45 C. The residue was redissolved in 1.0 mL methanol (spectral grade) and filtered through glass wool to remove any residual silica gel. The components were further separated by streaking on silica gel TLC plates (0.5 mL/plate) and developing in BAW.

Tests of Antioxidant Activity

Antioxidants on TLC plates were detected using the carotene spray method of Philip (4). Nine mg β-carotene were dissolved in 30 mL chloroform. Two drops of purified

linoleic acid and 60 mL of ethanol were added to the β-carotene-chloroform solution. This solution was sprayed on chromatograms streaked with the antioxidant solution. After spraying, the chromatograms were exposed to daylight for 2-3 hr or until background color was bleached. Bands in which yellow color persisted possessed antioxidant activity and were subjected to further testing. Antioxidant activities of eluted components were determined by the method proposed by Marco (5) and modified by Miller (6) and Pratt (1). This technique has been reported by Taga et al. (7). All tests were run in triplicate.

Acid Hydrolysis of Antioxidant Solutions

One mL of eluant was evaporated, in vacuo, to dryness on a rotary evaporator at 45 C and the residue redissolved in 1 drop of methanol (spectral grade). This was placed with 1.5 mL 2N HCl in a small vial, capped and heated in a boiling water bath for 45 min. Two mL diethyl ether were added with vigorous shaking. The ether (upper) phase was removed, placed in another vial and evaporated to dryness. The residue was dissolved in 0.5 mL spectral grade methanol. The water phase was evaporated to near dryness on a rotary evaporator, the residue was redissolved in 0.5 mL water and chromatographed to test for sugars.

Spectral Identification of Flavonols

Spectra (200-360 nm) were obtained using a Beckman Spectrophotometer Model 25. For sodium acetate (NaOAc) spectra, coarsely powdered, anhydrous reagent-grade sodium acetate was added to a microcuvette containing the antioxidant solution until a 2 mm layer of NaOAc remained on the bottom of the cuvette. The spectra were recorded 2 min after addition of NaOAc. A second spectrum was run 5-10 min later. The sodium methoxide spectrum was measured immediately after 3 drops of stock solution (2.5 g/100 mL methanol) were added to the test solution. The $AlCl_3$ spectra were recorded immediately after the addition of 6 drops $AlCl_3$ stock solution (5 g $AlCl_3$ added carefully to 100 mL spectral grade methanol) to a cuvette containing the antioxidant solution. Another spectrum was run after 1 min. Immediately after the addition of 3 drops stock HCl solution (50 mL concentrated HCl and 100 mL distilled water) to the cuvette containing the $AlCl_3$-antioxidant solution an $AlCl_3$/HCl spectrum was recorded.

Chromatographic Spray Reagents

Ferric chloride-potassium ferricyanide ($FeCl_3$-$K_3Fe(CN)_6$). Equal volumes of aqueous 1% solutions of each salt were mixed together, making an orange-brown solution with no blue color, and sprayed. Phenols give a immediate blue color (8).

Ferric chloride ($FeCl_3$). Two g $FeCl_3$ in 100 mL ethanol were sprayed immediately after mixing. Phenolics with trihydroxy and dihydroxy grouping give distinct blues and greens colors, respectively. Other phenolics give red or brown colors (9).

Ammonical silver nitrate. Thirty mL of NH_4OH and 70 mL water were added to 3.4 g of $AgNO_3$ in 100 mL of water until the solution became clear. After spraying, chromatograms were heated in an oven 5-10 min at 105 C. Brown, black or gray spots indicated reducing compounds (10).

Vanillin-p-toluenesulphonic acid (Van-pts). Chromatograms were sprayed with a solution of 2 g vanillin and 1 g p-toluenesulphonic acid in 100 mL absolute ethanol and heated for 5-10 min at 100 C. A strong red-violet color is produced by flavonoids with the phlorogluccinol nucleus whereas a weak pink color after prolonged heating is given by those containing a resorcinol nucleus (11).

Diazotized p-nitroanilne (DPNA). Five mL 0.5% p-nitroaniline in 2N HCl were mixed with 0.5 mL 0.5% $NaNO_2$, and 15 mL 20% sodium acetate solution. After drying, the chromatograms were sprayed with 20% Na_2CO_3. Phenolic compounds with free ortho- and para-hydryoxyl groups yield a brown color (12).

Twenty percent Na_2CO_3. Phenolics undergo visible and fluorescent characteristic color changes caused by the ionization of hydroxyl groups and subsequent increases in conjugation when exposed to Na_2CO_3 (13).

Iodine vapor (I_2). Chromatograms were exposed to iodine fumes for 15 min. Brown spots indicate sugar mercaptals, alcohols, hexonic acids, glycerides, N-acylamino sugars, neutral and acid polysaccharides (14).

Aniline oxalate. Equal volumes of 1.8% oxalic acid in water and 1.8% aniline in ethanol were mixed. Sprayed chromatograms were then heated at 100 C for 10 min. Hexoses produce green-brown colors, pentoses brilliant red and uronic acids give yellow colors (15).

p-Anisidine. One g p-anisidine HCl in 10 mL absolute methanol was added to 90 mL n-butanol. After 0.1 g sodium hydrosulfite was added, the chromatograms were sprayed. Ketohexoses react to give yellow colors; deoxysugars and aldohexoses, light brown; aldopentoses, brown; methyl pentoses, green; uronic acids, red-pink colors (16).

p-Anisidine HCl. Chromatograms sprayed with 3% p-anisidine HCl in n-butanol were heated at 100 C for 10 min. Ketohexoses react to give yellow colors; deoxysugars and aldohexoses, light brown; aldopentoses, brown; methyl pentoses, green; uronic acids, red-pink colors (17).

RESULTS AND DISCUSSION

Observation of the chromatograms of acid-hydrolyzed methanolic extracts of Spanish peanuts with visible and UV light before and after exposure to ammonia fumes showed identical fluorescent bands. Positive reactions with ferric chloride-potassium ferricyanide indicated these compounds to be phenolics.

Descending paper chromatography of the methanol-extracted peanuts developed in BAW yielded 4 fluorescent bands. Bands corresponding to Rf values of 0.91, 0.70, 0.47 and 0.08 fluoresced yellow, blue, blue and light blue, respectively, under UV (360 nm) radiation. All bands became brighter when exposed to ammonia fumes. A positive response to β-carotene spray indicated that only the band of Rf 0.91 possessed antioxidant activity. The reaction to ferric chloride-potassium ferricyanide spray indicated the activity was caused by a phenolic compound. The band was eluted into 80% aqueous methanol. Figure 1 shows the antioxidant activity of the compound(s) of the band.

Ascending TLC on silica gel plates developed in EFW yielded 6 fluorescent bands with Rf values of 0.08, 0.49, 0.64, 0.73, 0.95 and 0.97. Excellent resolution of the methanolic extract was obtained in this system. The fluorescent band, Rf 0.97, gave positive reactions to β-carotene and ferric chloride-potassium ferricyanide sprays. The antioxidant activity of the components of this band is shown in Figure 2.

Further separation of the band (Rf 0.97) in BAW yielded 3 components with Rf values of 0.94, 0.48 and 0.31. The fluorescent band at Rf 0.94 exhibited antioxidant activity with β-carotene spray. Figure 3 shows antioxidant activity of the eluted and separated compounds.

Two-dimensional cochromatography on paper was used to help characterize the compound with an Rf 0.97 in EFW and Rf 0.94 in BAW. A single spot fluoresced at Rf 0.86 (tBAW) and Rf 0.67 (15% acetic acid). Cochromatography in 3 solvent systems (BAW, tBAW and EFW) with standards

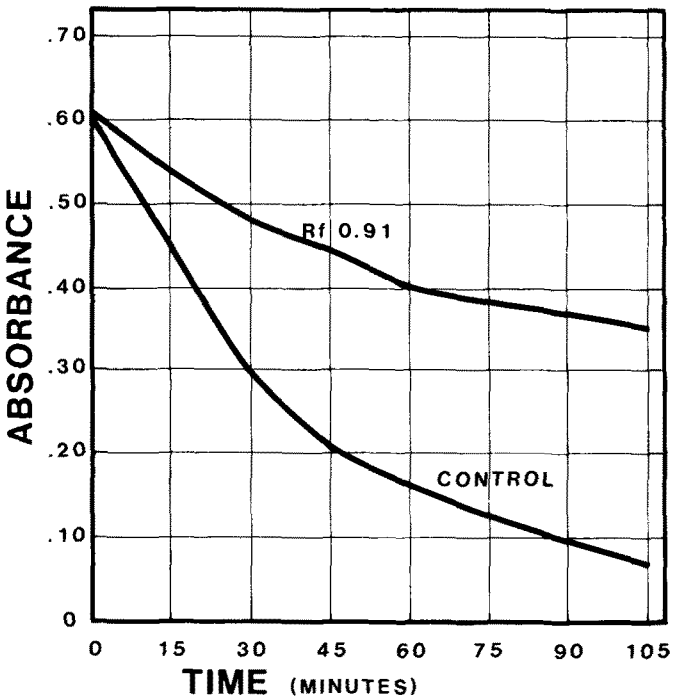

FIG. 1. Antioxidant activity of components with Rf of 0.91 when developed in BAW on paper.

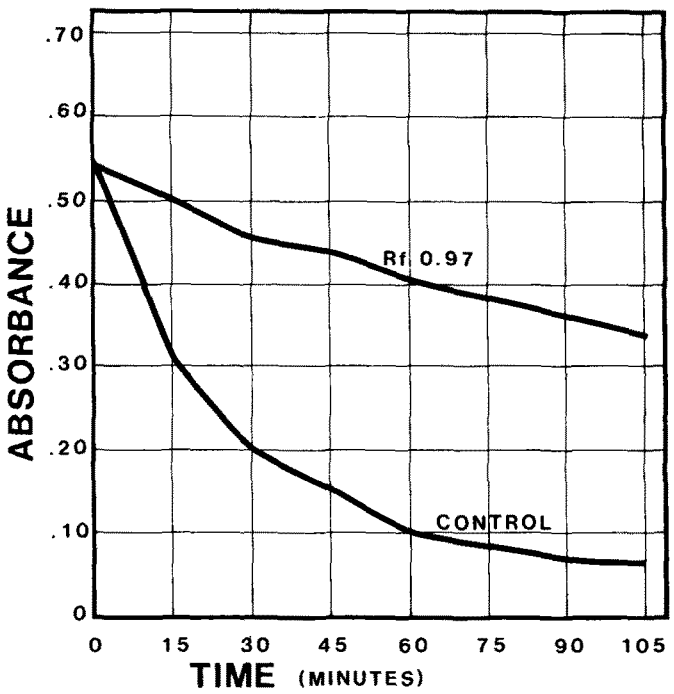

FIG. 2. Antioxidant activity of component with Rf of 0.97 when developed in EFW on paper.

indicated that the hydrolyzed compound was dihydroquercetin (taxifolin). Reactions of the chromatographic spray reagents before acid hydrolysis are shown in Table I. Positive color reaction to ferric chloride-potassium ferricyanide indicated the compound to be a phenol. Blue coloration observed with ferric chloride suggested a phenolic compound with 2 or more adjacent hydroxyl groups. Appearance of a nonfading brown spot indicated a glycoside following exposure to I_2 vapors.

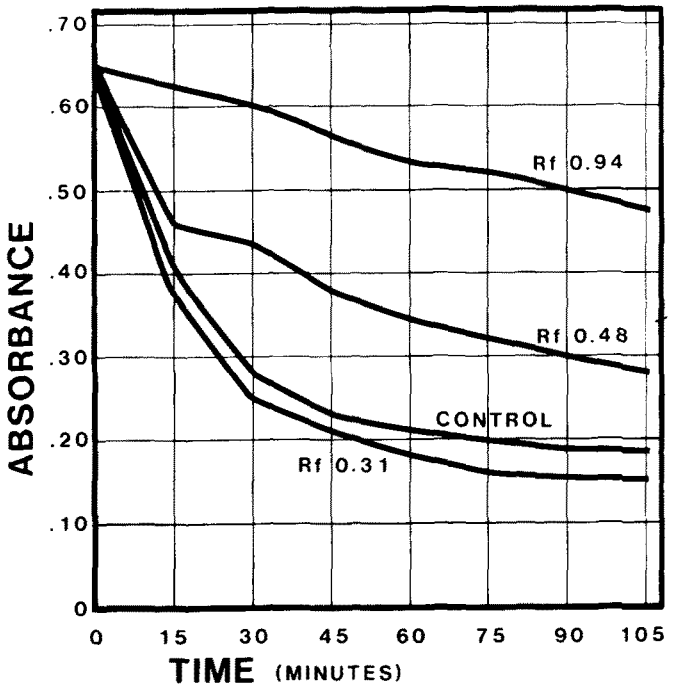

FIG. 3. Antioxidant activity of component of parent band Rf 0.97 (in EFW) developed in BAW on TLC.

TABLE I

Summary of Spray Reagents on Band Rf 0.94 Resolved by BAW

Spray reagent		Color reaction in visible light
Unhydrolyzed		
β-carotene		Yellow
Ferric chloride		Blue
Ferric chloride-potassium ferricyanide		Blue
I_2 vapor		Brown—not faded
Hydrolyzed		
β-carotene		Yellow
Ammonical silver nitrate		Dark brown
Vanillin p-toluenesulphonic acid		Slight red
20% Na_2CO_3	*Visible Light*	*UV (long)*
Before spray	Pale yellow	Yellow
After spray	Tan	Dark tan
Diazotized p-nitroaniline	*Visible Light*	*With Na_2CO_3*
	Tan	Brown

Table I shows reactions after hydrolysis. A dark-brown spot produced by ammonical silver nitrate was characteristic of reducing compounds. After spraying with 20% Na_2CO_3, visible and fluorescent color changes indicate a compound containing phenolic hydroxyl groups with color changes caused by the ionization of the hydroxyl groups (13). A positive reaction to diazotized p-nitroaniline is indicative of phenolic substances with free ortho- or para-hydroxy groups (12). The slight red color produced by the vanillin p-toluenesulphonic acid spray suggested a possible flavonoid with the phloroglucinol nucleus (11).

Acid hydrolysis was used to determine if the antioxidant was a glycoside. After hydrolysis, the water layer (bottom) was evaporated to dryness, leaving behind a white residue. The residue was redissolved in 0.5 mL water, rechromatographed using the solvent systems of isopropanol/acetic acid/water (3:1:1, v/v/v), ethyl acetate/acetic acid/water

(3:3:1, v/v/v), n-butanol/acetic acid/water (5:1:2, v/v/v) and n-propanol/ethyl acetate/water (65:10:25, v/v/v) and tested for sugars by spray reagents. Ascending chromatography was performed on hydrolyzed and unhydrolyzed antioxidant solutions. Only the hydrolyzed sample contained a free sugar when sprayed with aniline oxalate. The sugar from the antioxidant appeared in the same area as a galactose standard and produced a brown color with aniline oxalate, suggesting that the unknown was a hexose. Reaction toward p-anisidine and p-anisidine HCl, light brown, indicated the sugar to be an aldohexose. Separation did not appear when the unknown was cochromatographed with the galactose standard.

Spectral analysis was employed for further identification of the antioxidant. The methanolic spectrum showed a peak at 290 nm. Spectral data resembled that of dihydroquercetin (taxifolin) (Table I). Spectrophotometric and chromatographic analysis demonstrated that the antioxidant was dihydroquercetin (taxifolin). Quantitative comparison of spectral curves for the antioxidant with an authentic sample demonstrated that the peanuts contained 3.4×10^{-4} mol/kg of dihydroquercetin.

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Lipid-Lipase Interactions. I. Fat Splitting with Lipase from *Candida rugosa*

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ABSTRACT

Commercial dry lipase from *Candida rugosa* (formerly *C. cylindracea*) was used to catalyze hydrolysis of tallow, coconut oil and olive oil at 26-40 C. A methodology was developed to yield results reproducible within ±10% and to achieve essentially complete hydrolysis. From the hydrolysis data, an empirical relationship was developed that shows that the percentage of free fatty acid formed is almost a linear function of the logarithm of reaction time and the logarithm of enzyme concentration. A 95-98% hydrolysis of the 3 substrates was achieved experimentally in 72 hr, requiring 15 units lipase per milliequivalent (U/meq) of coconut oil or tallow and 6 U/meq of olive oil. The kinetics of lipolysis were determined for all 3 substrates and were found to approximate first order. Lipolysis rate was higher for olive oil than for tallow and coconut oil; no significant differences were observed between the latter 2 substrates. No statistically significant change in overall reaction rate was found when the hydrolysis was run at 26 C, 36 C or 46 C. Although the literature cites calcium or sodium ions and albumin as beneficial adjuvants to enzymatic lipolysis, these additives appeared to have no significant beneficial effect on the reaction. On the other hand, hydrocarbon solvents and nonionic surfactants showed an adverse effect.

INTRODUCTION

The present industrial process for fat and oil hydrolysis involves pressures of ca. 700 psi and temperatures of 480 F or higher (1) for a period of ca. 2 hr to achieve 96-99% hydrolysis. The resulting products are extremely dark fatty acids and discolored dilute (10%) aqueous solutions of glycerol. The fatty acids are unusable as obtained and need to be redistilled to remove color and by-products. The glycerol layer, after concentration, usually is distilled to remove color and impurities. These processes are energy intensive and give rise to a variety of undesirable side reactions. For example, highly unsaturated fatty acids can polymerize, and, if the temperature rises above 450 F, anhydrides form that, if heating continues, decompose to yield ketones and hydrocarbons (1).

In the interest of conserving energy and to minimize thermal degradation, we set out to study enzymatic hydrolysis of triglycerides. This approach would lead to little or no additional color development, cause no chemical degradation and a more concentrated glycerol solution might also be achieved. A variety of lipases of different origins have been studied in the biochemical literature. Pancreatic lipase was the first fat-splitting enzyme to be thoroughly investigated. However, it is not useful for rapid total fat splitting because it is specific only for the α-positions of fatty triglycerides (2,3).

A 1965 patent (4) discloses the purification of a highly active lipase obtained from a newly found species of the yeast *Candida cylindracea*, now named *Candida rugosa* (5). Benzonana and Esposito (6) later showed that this lipase catalyzed complete hydrolysis of several natural oils to free fatty acids and glycerol in 8-20 hr. Another patent (7) describes the use of this same lipase in the hydrolysis of olive, soybean and linseed oils.

[1] Presented in part at the 73rd National Meeting of the American Oil Chemists' Society, May 1982, Toronto, Ontario, Canada.