The Phosphatides of Safflower Seeds Involved in Color Formation Occurring in Extracted and Heated Crude Oils[1]

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Abstract

Studies on crude safflower oils have established that three phosphatides extracted with the oil from the kernel of the seed are responsible for color formation. The phosphatides have been isolated and identified as phosphatidylethanolamine (PE), phosphatidylmyoinositol (PI) and phosphatidylcholine (PC). Phosphatidylethanolamine was the most potent contributor to color formation, followed by phosphatidylinositol and phosphatidylcholine. The color-forming compounds were separated from the crude oil by precipitation with water and obtained in pure form by column chromatography on DEAE cellulose. The components were identified by comparison of R_f values of intact and deacylated phosphatides with those of known reference compounds, by determination of molar ratios of ester, glycerol, choline, inositol, nitrogen and phosphorus, and by qualitative and quantitative gas chromatographic analysis of the fatty acid residues.

Introduction

It is well known that crude vegetable oils contain various phosphatides (1). The phosphatides may cause darkening of the oils upon heating but they can easily be removed from the oil by refining or degumming to prevent this color formation (2). Industrial processing of an experimental crop of a newly developed high oil-yield safflower variety, however, yielded unacceptably dark crude and refined oils which led in part to the rejection of this variety by oil processors. It was our objective to explore the causes for this unusually dark color and to find ways for its elimination (3).

Our investigations led to the conclusion that the major part of dark color in the industrially processed oil must have been caused by phosphatides which are usually present at much higher concentrations in the oil of this variety and apparently formed during periods of heating prior to refining. This presentation is concerned with a complete qualitative and quantitative analysis of the individual phosphatides involved in color formation and compares their color-forming potentials. We are at present investigating the chemical changes that lead to color formation caused by these phosphatides and a manuscript is in preparation which discusses the color problem of brown-striped safflower oil and its solution in detail.

Experimental Procedures

Materials

A new brown-striped safflower variety, Arizona Brown Stripe No. 12417, has been used in this study. The seed was obtained from Western Cotton Products Company, Phoenix, Arizona and was grown in 1965. Commercial seed, preponderantly Gila, was used for reference purposes.

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Extraction of Seeds

Whole clean seeds (100 g) were cracked in a laboratory disc mill and extracted at room temperature with hexane containing 3.5% methanol (500 ml). The solvent thoroughly contacted the cracked seed through high speed mechanical mixing for 2 min in a Sorvall Omnimixer. Three further extractions of the solid residue remaining after filtration and washing were necessary to remove 98% of the extractable color-forming material from the seed. The solvent from the combined extracts was then removed in an atmosphere of nitrogen under reduced pressure (~1 mm Hg) and a temperature not exceeding 25 C. A clear yellow oil (47 g) was obtained.

Separation of Precursor From the Oil

Crude oil (47 g) was thoroughly mixed with water (0.9 g) and centrifuged for 10 min at 35,000 *g*; the supernatant oil was decanted, and the walls of the centrifuge tubes were cleaned from adhering oil with acetone saturated wipers. The solid residue was repeatedly washed with cold acetone (3 × 10 ml), using a vibrator for mixing and centrifugation for separation. The washed residue was then dried over $CaCl_2$ in an atmosphere of nitrogen under reduced pressure leaving a dry white powder (705 mg).

Thin Layer Chromatography

Precoated silica gel plates, 0.25 and 1.0 mm thick, from Brinkman Instruments, Inc. were used for analytical and preparative separations. The plates were developed in lined chambers with chloroform-methanol-water (60:50:4) or butanol-acetic acid-water (6:2:2) as solvent systems. The components were made visible after spraying with an anisaldehyde-sulfuric acid spray (4), the molybdenum blue spray for detecting phosphate containing compounds (5) and a ninhydrin spray for the detection of components with free amino groups (ninhydrin spray reagent 0.1%, Brinkmann Instruments, Inc. No. 8908108). Quantitative data on separated components were obtained after their elution from silica with chloroform-methanol-water (10:5:1).

Paper Chromatography

The water soluble portions of the deacylated lipids were chromatographed in descending fashion on Whatman No. 1 paper with isopropyl alcohol-30% ammonia-water (7:2:1) and made visible with a molybdate spray (6). The lipids were deacylated with methanolic sodium hydroxide, a method which simultaneously produces deacylated lipid and fatty acid methyl esters (7).

Test for Color Precursor

Spectrophotometric Method. Safflower oils (1.9 ml) containing color-precursor or precursor-free oils to which precursor was added, were placed in ½ dram vials without cap and heated in an aluminum heating block at 160 C. After maximum color formation was reached (about 20 hr for a precursor-phosphatide level of 0.5 mg phosphorus per gram of oil), ab-

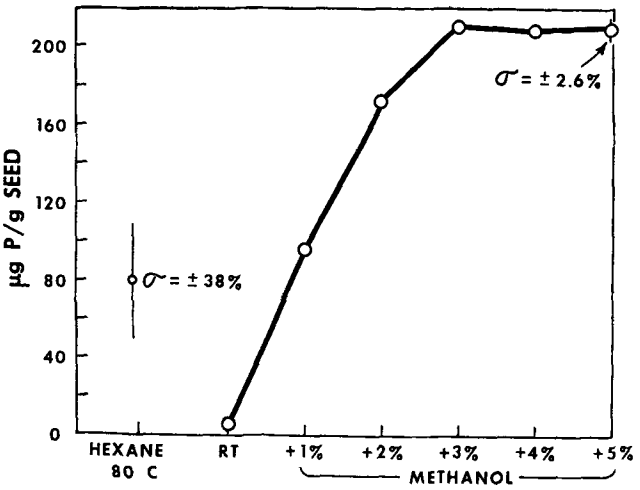

FIG. 1. Extraction of color precursor from cracked safflower seeds with hexane and hexane-methanol mixtures.

sorbance was measured at 550 mμ with a pathlength of 1,2,5 or 10 mm. When partially or fully purified precursors were tested for their color-forming potential, they were dissolved in water-saturated chloroform or chloroform-methanol (19:1), added to precursor-free oil, and the solvent was evaporated at reduced pressure and room temperature atmosphere of nitrogen. Commercial refined, bleached and deodorized safflower oil without additives was used as precursor-free oil.

Determination of Phosphorus Content. The method of Bartlett (8) was used to obtain values for oil samples, for compounds eluted from analytical and preparative TLC plates and for column eluates. For oil samples very low in phosphorus (after removal of phosphatides) and for compounds eluted from analytical TLC plates, the following micro method was used in the range of 0.02 to 0.2 μg phosphorus per sample: The sample was placed in a 18 × 150 mm test tube with 10 N H_2SO_4 (0.25 ml) and heated at 180 C for 30 min in an aluminum block; hydrogen peroxide was then added dropwise until the solution was completely decolorized (2–20 drops depending on the amount of organic material present in the sample) while heating continued for another 30 min. After cooling to room temperature, 0.22% ammonium molybdate solution (0.10 ml), water (0.90 ml) and the Fiske-Subbarow reagent (0.10 ml) were added with agitation, and the test tubes heated in a boiling water bath for 7 min. Absorbance was then mea-

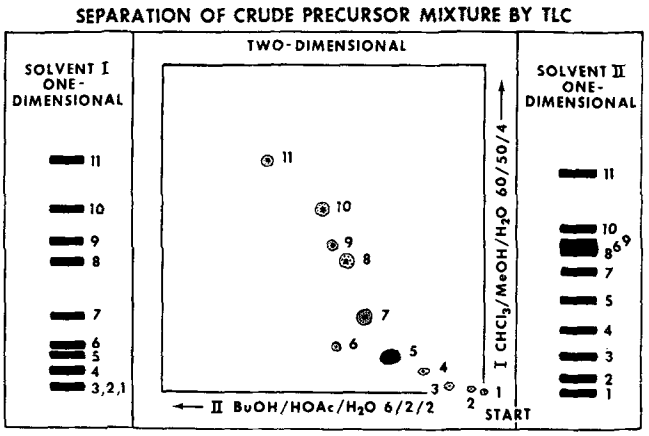

FIG. 2. Separation of crude precursor mixture by one-dimensional and two-dimensional TLC.

TABLE I

Analyses of Crude Color Precursor Mixture Separated by Thin Layer Chromatography

Component	Abundance, %	Rf × 100 1—solvent—2		Spot test for Phosphorus	Spot test for Nitrogen (ninhydrin)	Color-forming potential[a]
1	2–7	0.0	0.0	+	−	<2
2	<1	0.7	4.3	−	−	<1
3	3	1.7	11	−	−	<1
4	2	6.5	19	−	−	<1
5	32	11	28	+	−	32
6	2	14	45	+	+	<2
7	23	23	37	+	−	62
8	16	40	43	+	+	66
9	2	46	46	−	−	<1
10	5	56	50	−	−	<1
11	6	71	67	−	−	<1

[a] Color-forming potential represents a comparative absorptive value based on total material isolated.

sured at 830 mμ with 50 mm pathlength in a microcell.

Column Chromatographic Separations

Microgranular DEAE-cellulose (Whatman, DE52) was suspended in 10% acetic acid and agitated for 10 min after which the resin was washed with water until the filtrate was neutral. The resin was then washed with methanol followed by chloroform-methanol (2:1). One part settled resin and one part deaerated solvent mixture were poured into a 2.5 × 100 cm column (Chromatronix Corporation, Berkeley, Calif.). The column was provided with an extension of equal length and diameter and packed to a height of 94 cm under 10 psi of constant pressure obtained by flow rate regulation. A gradient was then applied with 500 ml chloroform-methanol (2:1) in the mixing chamber and chloroform (500 ml) in the solvent reservoir. The column was equilibrated with chloroform (1000 ml) before the sample (1 g, precursors separated from the oil) dissolved in 5 ml of water-saturated chloroform-pentane (9:1) was injected onto the column. Elution with a flow rate of 60 ml/hr was then started with a gradient obtained by constant volume mixing (mixing chamber not vented) from a mixing chamber containing chloroform (4.0 l) and a reservoir containing 4.0 l of methanol-water (95:5). After collection of 200 fractions (20 ml each), elution was briefly interrupted. One half (2.0 l) of the solvent in the mixing chamber (chloroform-methanol-water, 33:64:3) was transferred to the empty reservoir and ammonium acetate (46.2 g) added. A linear gradient elution (mixing chamber vented) was then started from 0 to 0.30 molar ammonium acetate, and another 200 fractions were collected. Aliquots (100 μl) were removed from each fraction and assayed for phosphorus (Fig. 3). The pooled fractions 32–52 and 70–82 were then evaporated to dryness under reduced pressure in an atmosphere of nitrogen and at a temperature not exceeding 20 C. Remaining in the respective fractions were 320 mg of compound 5 (PC) and 162 mg of compound 8 (PE) which were immediately made up to solutions containing 20 mg/ml with chloroform-methanol (19:1) and stored in a freezer. Pooled fractions 290–304 were evaporated to about 100 ml volume and mixed with the same amount of water; remaining organic solvents were removed by evaporation. The aqueous emulsion was then dialyzed against running distilled water and lyophilized, yielding cpd 7 (PI) as a white fluffy powder (230 mg). Compound 7 was dissolved in 11.5 ml chloroform-methanol (19:1) and stored as were compounds 5 and 8.

TABLE II
Characterization of DEAE-Cellulose Column Fractions[a]

Component	Assigned structure	Analyses					
		Phosphorus	Glycerol	Ester	Choline	Inositol	Nitrogen
5	Phosphatidylcholine	1.00	1.07	2.02	1.02		1.01
7	Phosphatidylmyoinositol	1.00	1.06	1.98		0.95	
8	Phosphatidylethanolamine	1.00	1.11	1.89			0.99

[a] Values are given as molar ratio relative to phosphorus.

Group Analyses

Glycerol was obtained after hydrolysis of the intact phosphatides with 2 N HCl according to Renkonen (9) and assayed using the technique of Hannahan and Olley (10). Phosphorus was determined by the method of Bartlett (8), choline as reported by Wheeldon and Collins (11), the fatty acid esters by the method of Rapport and Alonzo (12) and myo-inositol according to a microbiological assay using *Saccharomyces carlsbergiensis* (13).

Gas Liquid Chromatography

The fatty acid methyl esters were obtained by deacylation of the intact phosphatides as described by Hendrickson and Ballou (7) and analyzed on a Varian Aerograph Model 1520 B gas chromatograph equipped with a hydrogen flame ionization detector. Two columns were used: 9 ft × ½ in. stainless steel, packed with 10% FFAP coated on 60–80 mesh DMCS treated Chromosorb W. The column was operated at 233 C with a helium flow rate of 50 ml per min. 20 ft × 1/16 in. stainless steel, packed with 3% Apiezon L coated on 100–120 mesh Aeropak 30. The column was operated at 210 C with a helium flow rate of 19 ml per min.

Peaks were identified by comparing the relative retention times with a semilogarithmic plot of relative retention time versus chain length and degree of unsaturation of known standards and by comparing hydrogenated and nonhydrogenated samples. For hydrogenation, the fatty acid methyl esters (1 mg) were dissolved in methanol (5 ml), platinum oxide (10 mg) was added, and the mixture was exposed to a pure hydrogen atmosphere and shaken for 15 hr (14). Peak areas were measured by planimetry and were corrected for detector response differences before area normalization.

Results and Discussion

Isolation of Color-Forming Compounds

We obtained reproducible extraction of color precursors from safflower seed with hexane containing 3–5% methanol at room temperature. However, under standard conditions of oil extraction, with boiling hexane as solvent, only part (25–50%) of the color-forming compounds were removed from the kernel (Fig. 1). This result is explained by the fact that the color precursors are phosphatides, usually bound to proteins via a bonding in which water plays an important role (15).

The color precursors were separated from the bulk of the oil by precipitation with water. When 2% of water was added to the oil a semisolid precipitate resulted after mixing and high speed centrifugation. This material contained more than 96% of the total phosphorus originally present in the oil, and the supernatant oil did not form color upon heating.

The crude precursor material was separated by TLC for analysis of its composition. A summary of the analytical data is given in Figure 2 and Table I. Solvent system 1 was specially developed and resolved the mixture by two dimensional TLC in combination with a standard second system into 11 components. Preparative one dimensional TLC and elution of each separated compound were used to obtain quantitative data and the color-forming potential of each component. To obtain comparative values on color formation, the eluted compounds were added to oil free of color precursor, and absorbance was measured and compared after heating of the samples. It was found that three of the 11 components were responsible for more than 95% of the total color-forming potential of the mixture. They were compounds 5, 7 and 8, the major components, and all three contained phosphorus.

Figure 3 illustrates the final separation of the three color precursors from each other and the remaining eight contaminants by column chromatography on DEAE-cellulose. A chloroform-methanol-water and chloroform-methanol-ammonium acetate gradient in series were used for elution. From 1000

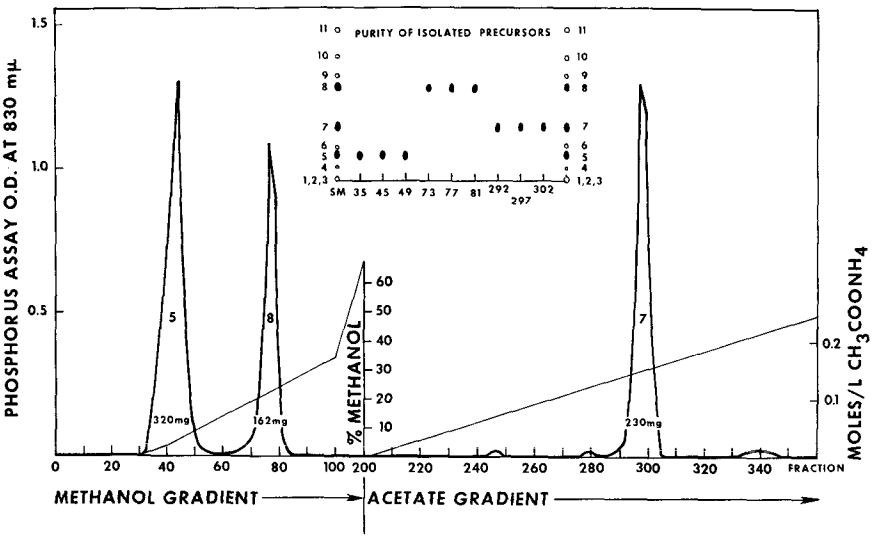

FIG. 3. Separation of color precursor (1 g) on DEAE-cellulose.

TABLE III
Fatty Acid Composition of Safflower Oil Phosphatides[a]

Phospholipid	Fatty acids[b]									Other	Total unsaturated	Color-forming potential (PC = 1.0)
	14:0	16:0	18:0	18:1	18:2	18:3	20:0	22:0	22:1			
Phosphatidylcholine	0.2	13.8	4.1	12.1	68.1	0.1	0.4	0.5	0.4	0.3	80.7	1.0
Phosphatidylethanolamine	0.2	19.5	2.9	8.3	69.1	....	....	....	....	....	77.4	4.1
Phosphatidylmyoinositol	0.1	29.0	4.8	4.4	59.7	....	0.7	0.7	....	0.6	64.1	2.7

[a] Composition expressed as corrected area percentage.
[b] Ratio of carbon length to degree of unsaturation.

mg of mixture applied to the column, 320 mg of component 5, 162 mg of component 8 and 230 mg of component 7 were obtained in pure form. The methanol gradient up to 66%, the relatively large amounts of eluate and the slow flow rate were all necessary to obtain the reported separation.

Identification of Color-Forming Compounds

The purified color precursors were identified as phosphatidylethanolamine (PE), phosphatidylmyoinositol and phosphatidylcholine. They were characterized (a) by comparison of R_f values of the intact and deacylated phosphatides with those of known reference compounds; (b) by determination of molar ratios of ester, glycerol, choline, inositol, nitrogen and phosphorus; and (c) by qualitative and quantitative gas chromatographic analysis of the fatty acid residues.

Comparison of R_f values of intact and deacylated unknowns with PE, PI and PC showed chromatographic identity of component 5 with PC, component 7 with PI and component 8 with PE for both the intact and deacylated unknowns. The deacylated phosphatides were separated by descending paper chromatography and the intact molecules by ascending TLC.

Analytical data for components 5, 7 and 8 obtained through group analyses are given in Table II. The molar ratios found for compounds 5, 7 and 8 were in good agreement with those for PC, PI and PE.

The fatty acid residues of each individual phosphatide were analyzed as methyl esters by GLC to determine the qualitative and quantitative composition. Table III summarizes the results of these analyses for the three color-forming phosphatides. All peaks identified as unsaturated fatty acid methyl esters disappeared after hydrogenation of the samples and resulted in proportional increases of the corresponding saturated esters. The composition of fatty acids in the three isolated phosphatides were similar; all have linoleic acid as the major acid followed by palmitic, oleic and stearic acids. Degree of unsaturation and color-forming potential do not seem to be directly proportional. Phosphatidylethanolamine had a color-forming potential of 4.1 compared to 1.0 for PC, yet total unsaturation was nearly equal for PC and PE.

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