# Oxidized Phosphatidylcholines from Defatted Soybean Flakes Taste Bitter

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Three phosphatidylcholines differing in chromatographic mobility on thin-layer plates were isolated from residual lipids of hexane-defatted soy flakes. These soy phosphatidylcholines (SPC) were designated SPC-A, SPC-B, and lyso-SPC. All three possessed keto and hydroxy fatty acid esters but no aldehydic esters. A seven-member panel rated 0.05% suspensions of these phospholipids for intensity of bitter taste, based on the scoring system 0 = none to 3 = strong. Suspensions of SPC-A scored 0.9. Both SPC-B and lyso-SPC at the 0.05% level rated 3.0 and upon reevaluation at 0.01% scored about 1.0. Based on recovered weights, these phosphatidylcholines represent a minimum of 0.08% in defatted flakes. They may well contribute to the bitter taste in soy flakes.

Flavor is one of the major deterrents to greater use of soy protein products in foods (Hammonds and Call, 1972). Beany and bitter flavors, predominant in raw, full-fat, and defatted soy flours (Moser et al., 1967), remain detectable in all commercially manufactured soy flours, concentrates, and isolates (Kalbrener et al., 1971).

Identifying flavor constituents in soybeans will help find methods to either prevent their formation or remove them. Cowan et al. (1973) covered these aspects in a review on soy flavor components. Generally, inactivation of enzymes prevents formation of additional off-flavors. Aqueous ethanol and solvents containing alcohol are useful in extracting residual lipids and flavor components from defatted soy flakes (Honig et al., 1969) and soy proteins (Eldridge et al., 1963). Usually no efforts are made to prevent lipid oxidation either during processing of beans into flakes and protein isolates or during storage.

Sessa et al. (1974) have established that a strong bitter taste arises from autoxidation of unsaturated fatty acid constituents on soybean phosphatidylcholine (SPC) because none developed with hydrogenated SPC. In this latest investigation, we isolated and identified the residual choline-containing phospholipids in defatted soy flakes, and a taste panel determined their flavor characteristics.

## EXPERIMENTAL SECTION

**Materials.** The steps followed to isolate phosphatidylcholines from dehulled, defatted soy flakes are outlined in Figure 1.

**Extraction of Residual Lipids.** In step 1, dehulled, hexane-defatted flakes prepared from certified, seed-grade soybeans (Amsoy variety) according to the previously described procedure of Sessa et al. (1969) were extracted with 15 vol of 80% ethanol for 4 hr at 38°C. The extractions were performed under nitrogen with mechanical stirring by a high-speed, high-torque, nonaerating stirrer. The ethanol solubles, after filtration through a Soxhlet thimble, were concentrated to a syrup on a rotary evaporator, below 40°C. The syrup was diluted with enough water to freeze in a dry ice–alcohol mixture and then freeze-dried to yield 16% ethanol solubles. This extract containing the residual lipids was refrigerated under nitrogen in a sealed container.

**Separation and Hydrogenation of Phospholipids.** In steps 2 to 4, 5 g of ethanol solubles dispersed in 20 ml of chloroform–methanol–water (42.5:42.5:15, v/v) (Sjövall et al., 1968) was chromatographed on a column (3.2 × 65 cm) packed with 100 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Absorbed material was eluted with 1 l. of the same solvent combination. Effluent fractions of 10 ml were desolventized at 40°C on a rotary evaporator and then analyzed for organic phosphorus by thin-layer chromatography (TLC) (see Chromatographic Methods section). Crude phospholipids, representing a yield of approximately 2.6% of the defatted flakes, were recovered from effluent fractions 20 through 26. Column chromatography was repeated several times until about 10 g was collected.

To remove small amounts of saponins and sugars that remained, 10 g of crude phospholipids, dissolved in 100 ml of benzene–95% ethanol (9:1, v/v), was treated with 1 g of acid-washed, activated Darco G-60 (Atlas Chemical Industries, Inc., Wilmington, Del.). The suspension was stirred and filtered through a sintered glass funnel.

Since purified phospholipids in the filtrate readily deteriorated after solvent removal, giving rise to hydroperoxides, short-chain aldehydes, and strong rancid odors, the filtrate was hydrogenated. In 100 ml of benzene–95% ethanol (9:1, v/v) the purified phospholipid was dissolved and hydrogenated with 1 g of 10% palladium-on-carbon catalyst (Matheson Coleman and Bell, Norwood, Ohio) at 45 psi and room temperature for 1.5 hr. This step prevented formation of rancid odors, preserved whatever oxidative deterioration had occurred endogenously, and minimized further artifact formation during isolation of the choline-containing phospholipids.

**Purification of Choline-Containing Phospholipids.** In steps 5 to 7, the partially hydrogenated phospholipids were chromatographed on acid-treated Florisil (Supelcosil ATF-061, Supelco Inc., Bellefonte, Pa.) as described by Sessa et al. (1974). Fractions containing choline (see Chromatographic Methods section) were eluted from the column with chloroform–methanol (1:1, v/v) and methanol. These were pooled, stripped of solvent on a rotary evaporator, and chromatographed on columns of DEAE-cellulose (0.89 mequiv/g; Supelco Inc., Bellefonte, Pa.) according to the procedure of Rouser et al. (1967). Effluents were monitored at 274 nm. Fractions comprising a peak were pooled, stripped of solvent, and analyzed for choline by TLC (see Chromatographic Methods section). All column fractions were stored in a refrigerator under a blanket of nitrogen gas. TLC samples were dissolved in mixtures of benzene–ethanol.

Those column fractions containing choline phospholipids were each fractionated on preparative silica gel plates (see Chromatographic Methods section). After chromatography, the separated bands were quantitatively eluted (Privett et al., 1965). The eluted material dissolved in chloroform–methanol (1:1, v/v) was then passed through a 1-cm column containing 5 g of Sephadex LH-20 to re-

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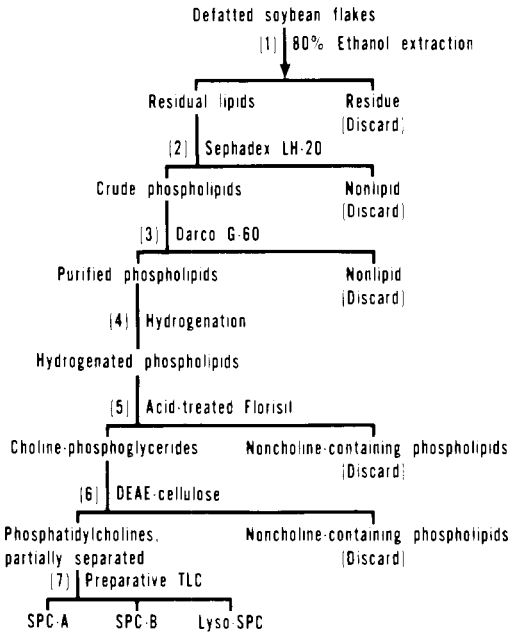


Figure 1. Flow sheet for fractionation, isolation, and purification of choline-containing phospholipids from dehulled, defatted soybean flakes: SPC, soy phosphatidylcholine; TLC, thin-layer chromatography.

move silica gel residue and inorganic salts.

**Chromatographic Methods.** To monitor effluents from the various chromatographic columns, one-dimensional TLC was done on either 0.25 mm precoated or 2 mm preparative silica gel F-254 plates (E. Merck, Darmstadt, Germany, distributed by Brinkman Instruments, Inc., Waterbury, N.Y.) and developed with chloroform–methanol–water (75:25:4.2) (Oette, 1965). To purify choline phospholipids by preparative TLC, up to 50 mg was chromatographed on each 20 × 20 cm preparative plate. Fractions from DEAE-cellulose columns were also analyzed by two-dimensional TLC with solvent combinations proposed by Singh and Privett (1970). Developed chromatograms were sprayed with molybdenum blue reagent (Dittmer and Lester, 1964) for visualizing phospholipids, Dragendorff reagent (Wagner et al., 1961) for choline, and 0.5% potassium dichromate in 50% sulfuric acid followed by heating 30 min at 190°C for organic material.

**Fatty Acid Analyses.** Phosphatidylcholines, designated SPC-A and SPC-B, were each saponified. The free fatty acids were extracted with diethyl ether and were esterified with diazomethane in ether solution. These esters were chromatographed on thin-layer plates developed with *n*-hexane–diethyl ether (1:1, v/v). Spots containing esters of fatty acids were located with 5% phosphomolybdic acid in 96% ethanol followed by heating at 110°C for 10 min (Garssen et al., 1971), hydroperoxides with 5% potassium iodide followed by 1% starch in 1% acetic acid, carbonyl compounds with 0.4% 2,4-dinitrophenylhydrazine in 2 *M* hydrochloric acid, and aldehydes with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (Rahn and Schlenk, 1973).

**Chemical Analyses.** Soy phosphatidylcholines, SPC-A, SPC-B, and lyso-SPC, were analyzed for carbon, hydrogen, nitrogen (Dumas), choline nitrogen (Böttcher et al., 1961), phosphorus, and fatty acid ester groups (Antonis, 1960).

**Spectral Analyses.** A Beckman DK2A spectrophotometer recorded ultraviolet (uv) absorption spectra over the wavelength region 220–360 nm; 80% ethanol was used. Absorbances at 274 nm of effluents from DEAE-cellulose columns were read on a Beckman DU spectrophotometer.

Infrared (ir) absorption spectra were recorded on a Perkin-Elmer Model 621 grating ir spectrophotometer. Spectra of phospholipids were obtained with 0.1-mm potassium bromide cells containing 8–10% solutions in carbon tetrachloride or chloroform and those of fatty acid methyl esters were run in both carbon tetrachloride and carbon disulfide.

High-resolution nuclear magnetic resonance (NMR) spectra were taken in deuteriochloroform with a Varian HA-100 spectrometer with tetramethylsilane as the internal standard.

**Taste Evaluation.** Seven trained tasters rated aqueous suspensions of partially hydrogenated and unhydrogenated SPC-A, SPC-B, and lyso-SPC for intensity of bitter taste, based on the scoring system of 0, none; 1, weak; 2, moderate; 3, strong. The average score of all responses was then expressed as a bitter intensity value (BIV). A 0.05% aqueous solution of caffeine served as the intensity standard for a moderately bitter taste (BIV = 2.0). Hydrogenated SPC prepared from soybean oil (Sessa et al., 1974), stored for 1 year in a dry state at 4°C, served as an internal control. It was dispersed in nitrogen-saturated water by sonication for 10 min at 100 W with a Branson Model S125 sonifier equipped with a 1-cm diameter probe set 1 cm below the surface of liquid. Sonication of 50 ml was performed in a 4.5-cm sample jar placed in an ice bath. Precautions to minimize chemical degradation were observed (Hauser, 1971). Both hydrogenated and unhydrogenated SPC-A, SPC-B, and lyso-SPC were readily dispersed in water by gentle swirling of the flasks.

Samples containing 0.05% SPC dispersed in carbon-filtered tap water were submitted to the panel. A randomized presentation of four 7-ml samples consisted of: 0.05% caffeine as the standard for BIV, carbon-filtered tap water, hydrogenated SPC from oil as internal control, and either the hydrogenated or unhydrogenated SPC from defatted flakes. If BIV = 3.0, higher dilutions of SPC were tasted.

## RESULTS AND DISCUSSION

**Identification of Choline–Phosphoglycerides.** DEAE-cellulose column chromatography (step 6, Figure 1) removed additional impurities and separated the various forms of phosphatidylcholines eluted from the acid-treated Florisil column. The elution pattern of uv-absorbing lipids (Figure 2) shows three major peaks designated 1, 2, and 3 and two minor, partially separated peaks that were combined and designated fraction 2A. Effluents from peak 1, making up fraction 1, contained neutral lipids and no detectable phosphorus or choline when analyzed by TLC and were discarded. Fraction 2 from peak 2 appeared to contain a single spot (Figure 3) with an $R_f$ value equal to that of L-$\alpha$-phosphatidylcholine ($\beta,\gamma$-dipalmitoyl-) (synthetic, A grade, Calbiochem, San Diego, Calif.).

Chromatography of fraction 2A showed the presence of two major spots (Figure 4): one with an $R_f$ value equal to that of egg lysophosphatidylcholine and the other faster moving component corresponded to that of bovine brain sphingomyelin in the acidic solvent of the second dimension. The chromatographic standards were purchased from Pierce Chemical Co., Rockford, Ill. Both spots in fraction 2A gave positive color reactions for phosphorus and choline. Fraction 3, not illustrated, possessed a component with the mobility of egg lysophosphatidylcholine along with several unidentified phospholipid spots that trailed extensively when chromatographed in two dimensions. Attempts to isolate chromatographically pure lysophosphatidylcholine from fraction 3 were unsuccessful.

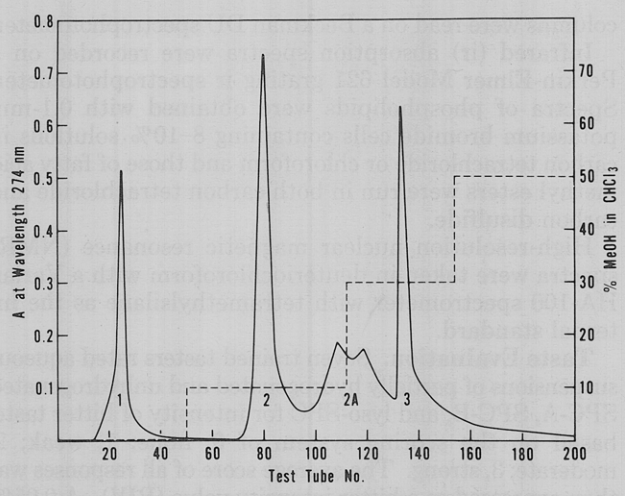


Figure 2. Elution pattern of uv-absorbing constituents (180 mg of partially purified choline phosphoglycerides from defatted soy flakes) on DEAE-cellulose column (dimensions 2.1 × 3.7 cm). Stepwise elution performed with chloroform and methanol, v/v; solvents indicated. Flow rate 100 ml/hr with ~6 ml collected in each tube. Absorbance of tube effluent monitored at 274 nm.

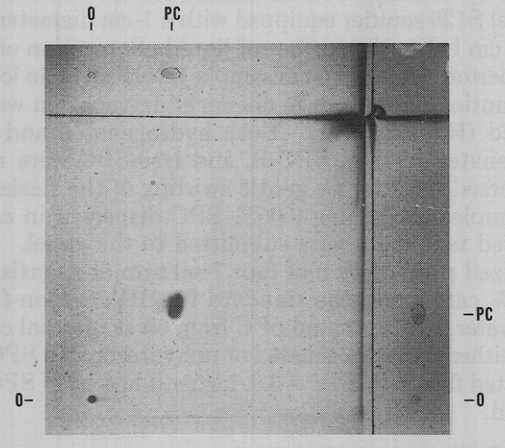


Figure 3. Two-dimensional TLC of DEAE column fraction 2 from peak 2 (see Figure 2) on silica gel plate with chloroform–methanol–ammonium hydroxide (65:35:5, v/v) in first dimension (vertical dimension) followed by drying for 10 min in nitrogen and then development in second dimension (from left to right) with chloroform–acetone–methanol–acetic acid–water (100:40:20:20:10, v/v); origin, 0. Synthetic phosphatidylcholine, PC, was chromatographed as a reference standard by single development in both the vertical and horizontal dimensions.

Phospholipids in fractions 2 and 2A were separated and further purified by preparative TLC (step 7, Figure 1). The isolated phospholipids–designated SPC-A, SPC-B, and lyso-SPC– were each desalted by passage through a Sephadex LH-20 column. Cochromatography with various phospholipid standards in one dimension on TLC plates developed with chloroform–methanol–water (75:25:4.2) indicated that SPC-A possessed an $R_f$ value equal to that of synthetic phosphatidylcholine, SPC-B with an $R_f$ of sphingomyelin, and lyso-SPC with that of lysophosphatidylcholine.

Even though sphingolipids have been found in higher plants (Carter et al., 1954; Van Handel, 1953), none contained a choline base. The decreased mobility of SPC-B on thin layers may therefore be due to oxidation known to affect the chromatographic behavior of phospholipids. May and McCay (1968), Sessa et al. (1974), and also Pont and Holloway (1967) reported decreases in the mobility of oxidized phospholipids on TLC. According to Pont and Holloway, the mobility of both oxidized phosphatidylcholine and phosphatidylethanolamine compared to the $R_f$ value of sphingomyelin in chloroform–methanol–water (65:25:4).

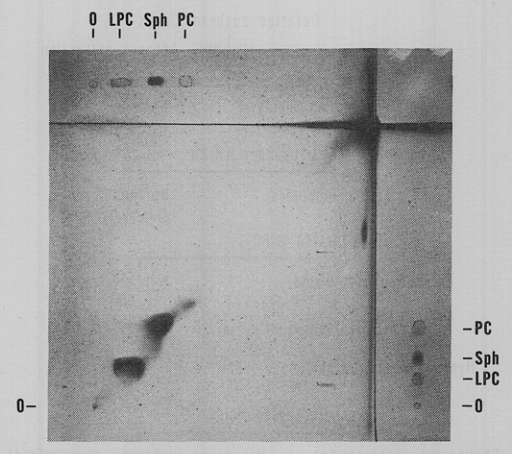


Figure 4. Two-dimensional TLC of DEAE column fraction 2A from peak 2A (see Figure 2) on silica gel plate with chloroform–methanol–ammonium hydroxide (65:35:5, v/v) in first dimension (vertical dimension) followed by drying for 10 min in nitrogen and then development in second dimension (from left to right) with chloroform–acetone–methanol–acetic acid–water (100:40:20:20:10, v/v); origin, 0. Synthetic phosphatidylcholine (PC), bovine brain sphingomyelin (Sph), and egg lysophosphatidylcholine (LPC) were chromatographed as reference standards by single development in both the vertical and horizontal dimensions.

Table I. Analysis of Partially Hydrogenated Soy Phosphatidylcholines (SPC)

| Constituent | SPC[a] | SPC-A | SPC-B | Lyso-SPC |
|---|---|---|---|---|
| C, % | 64.49 | 61.41 | 60.03 | 54.82 |
| H, % | 10.96 | 9.83 | 9.71 | 9.53 |
| N (Dumas), % | 1.74 | 1.68 | 1.36 | 2.34 |
| N (choline), % | 2.00 | 1.60 | 1.41 | 2.51 |
| P, % | 3.77 | 3.60 | 3.76 | 5.28 |
| Ester $\epsilon_p$[b] | 2325 | 2336 | 2021 | 944 |

[a] From soybean oil (Sessa et al., 1974). [b] Calculation based on P content (Rapport and Alonzo, 1955).

**Microanalyses.** Both SPC-A and SPC-B have phosphorus and nitrogen contents comparable to those of hydrogenated SPC from soybean oil (Table I). Phospholipid diesters reported by Rapport and Alonzo (1955) all had values of $\epsilon_p$ around 2000. The synthetic phosphatidylcholine standard possessed an $\epsilon_p$ of 2306; therefore, oil SPC and meal SPC-A and SPC-B are all fatty acid diesters. SPC-B is similar to SPC-A in both phosphorus and fatty acid ester content. SPC-B has a lower nitrogen content and an N/P atomic weight ratio of 0.8 in comparison to 1.0 for SPC-A. These data indicate that SPC-B is an altered form of SPC-A. Lyso-SPC is about 88% pure based on phosphorus analysis of egg lysophosphatidylcholine ($P$ = 6.0% and an $\epsilon_p$ of 1008).

**Spectral Analyses.** Both ir and NMR spectra were used to establish the identity of the three phosphatidylcholines further. The major ir absorption bands of the isolated phosphatidylcholines (Table II) were identical with those of synthetic phosphatidylcholine. The lyso species showed more absorption than that of SPC-B at approximately 3400 $cm^{-1}$ owing to a secondary hydroxyl group. Any absorption due to C–O stretch of secondary alcohol should appear at approximately 1090 $cm^{-1}$ but would be obliterated by absorption bands caused by $CH_2$–O–P. The spectra of SPC-B differed from that of SPC-A. SPC-B possessed two additional absorption bands appearing at

Table II. Major Ir Absorption Bands ($cm^{-1}$) of Phosphatidylcholines

| Synthetic PC[a] | SPC-A[b] | SPC-B[b] | Lyso-SPC[b] | Assignment |
|---|---|---|---|---|
| | | 3400 br | 3400 br | Intermol. bonded OH |
| 2910 | 2925 | 2925 | 2920 | C—$CH_3$ asym. str. |
| 2850 | 2860 | 2855 | 2855 | $CH_2$ sym. str. |
| 1730 | 1735 | 1735 | 1735 | C=O ester str. |
| | | 1585 | | O—C—O carboxylate ion asym. str. |
| 1460 | 1465 | 1465 | 1465 | $CH_2$ scissor |
| | | 1415 | | O—C—O carboxylate ion sym. str. |
| 1375 | 1380 | 1380 | 1380 | C—$CH_3$ sym. $CH_3$ deform. |
| 1240 | 1250 | 1245 | 1245 | P=O ester str. |
| 1170 | 1170 | 1170 | 1170 | C=O ester str. |
| 1080; 1065 | 1095; 1070 | 1095; 1070 | 1090; 1060 | $CH_2$—O—P doublet |
| 970 | 970 | 970 | 970 | P—OH str. |

[a] L-α-Phosphatidylcholine (β,γ-dipalmitoyl-). [b] From defatted flakes.

Table III. NMR Proton Absorptions of Phosphatidylcholines

| τ value | | | |
|---|---|---|---|
| Synthetic PC[a] | SPC-A[b] | Lyso-SPC[b] | Assignment |
| 9.14 | 9.13 | 9.14 | $CH_3$ |
| 8.76 | 8.76 | 8.76 | $CH_2$ |
| | 8.00 | 8.00 | $CH_2$—CH= |
| 7.74 | 7.74 | 7.72 | $CH_2$—CO |
| | 4.69 | 4.70 | CH=CH |
| 6.12 | 6.10 | 6.10 | $CH_2N^+(CH_3)_3$ |
| 6.67 | 6.66 | 6.70 | $N^+(CH_3)_3$ |
| 6.20 | 6.17 | 6.17 | $CH_2OCO$ |
| | | 5.98 | CHOH |
| 5.74 | 5.68 | ~5.7 | $CH_2OPO$ |
| 4.86 | 4.85 | | CHOCO |

[a] L-α-Phosphatidylcholine (β,γ-dipalmitoyl-). [b] From defatted flakes.

1585 and 1415 $cm^{-1}$. Band assignments at these wavelengths may be the asymmetric and symmetric stretching modes of a carboxylate ion, which possibly contaminates our preparations.

NMR absorptions of a synthetic phosphatidylcholine, SPC-A, and lyso-SPC and their assignments (Table III) confirm the identity from defatted flakes. At the instrument temperature, SPC-B was too insoluble in deuteriochloroform to give a satisfactory spectrum. Lyso-SPC is a 1-acyl lyso-SPC because absorption of methine proton on CHOH was apparent in its spectrum.

**Spectral Evidence of Oxidation.** SPC-A, as well as SPC-B and lyso-SPC, absorbs uv light at wavelengths 227 and 274 nm, whereas hydrogenated SPC isolated from soybean oil shows nonspecific absorbance over the entire wavelength region 220–360 nm (Figure 5). Since hydrogenation of the oxidized fatty acids was incomplete, absorbance at 227 nm could be due to a mixture of dienols that absorb at 233 nm and α,β-unsaturated carbonyls that absorb at 220 nm. As shown in Table IV, the absorptivity at 274 nm of SPC-A isolated from defatted flakes stored for 1 year at 4°C is 1.5 times that of SPC-A from freshly prepared defatted flakes. Values for SPC-B and lyso-SPC were nearly 3 times that of SPC-A. Evidently, this increase in absorbance at 274 nm is associated with the presence of oxidation products, most likely conjugated diene ketones

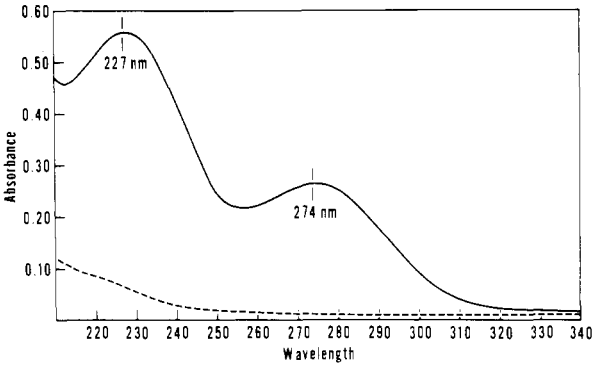


Figure 5. Uv absorbance spectrum of SPC isolated from defatted soy flakes stored 1 year at 4°C (solid line) and a partially hydrogenated sample of SPC from soybean oil which was stored 1 year at 4°C (dotted line).

Table IV. Uv Absorptivity[a] of Partially Hydrogenated SPC's

| Compd | Absorptivity λ 227 nm | Absorptivity λ 274 nm |
|---|---|---|
| SPC-A | 2.14 | 0.66 |
| SPC-A[b] | 2.31 | 1.08 |
| SPC-B | 2.83 | 1.67 |
| Lyso-SPC | 3.83 | 1.92 |

[a] Absorbance divided by the product of sample path length (in centimeters) and the concentration of substance (in grams per liter). [b] From defatted flakes stored 1 year at 4°C.

resulting from decomposition of conjugated hydroperoxides. About a twofold increase in absorbance at 274 nm, noted when spectra were rerun in alkali, is further evidence for enolizable substances (Holman et al., 1945) in the SPC samples from defatted flakes.

Robertson et al. (1973) evaluated oxidative stability of oil derived from field- and storage-damaged soybeans based on several parameters, including the ratio of absorptivities at 228 vs. 274 nm. This ratio, $R$, was 11.0 for oil from undamaged soybeans. $R$ values for oils from stored beans moderately to badly damaged ranged from 3.2 to 1.4. In our study, respective $R$ values were 3.2, 2.1, 2.0, and 1.7 for SPC-A from freshly prepared flakes, SPC-A from flakes stored 1 year, lyso-SPC, and SPC-B. Apparently, some oxidation occurs during storage of soy flakes, SPC-B oxidizes more than SPC-A, and lyso-SPC retains oxidized fatty acid. However, these phosphatidylcholines contained little or no aldehydes that absorb at either 450 or 532 nm when assayed with thiobarbituric acid according to Sessa et al. (1974).

Fatty acid constituents from both SPC-A and SPC-B, converted to their respective methyl esters, had absorption maxima at wavelengths 227 and 274 nm. Based on a molar extinction of 22,040 for a $C_{18}$-oxodiene methyl ester (Vioque and Holman, 1962), the fatty acids present amounted to 4.2% conjugated ketodiene.

A qualitative ir spectral scan of these fatty acid methyl esters showed secondary hydroxyl absorptions at 3460 and 1095 $cm^{-1}$; some weak absorptions, due to a mixture of α,β-unsaturated ketones and conjugated unsaturated ketones, appeared at 1700, 1660, 1640, and 1590 $cm^{-1}$, isolated cis absorption at 3005 $cm^{-1}$, and absorption due to diene conjugation at 990 and 950 $cm^{-1}$. These absorption bands were in addition to those at frequencies 2920, 2860, 1740, 1440, 1360, 1170, and 720 $cm^{-1}$ in the spectrum of unoxidized fatty acid methyl esters derived from SPC isolated from soybean oil. Based on these spectral analyses, some of the unsaturated fatty acids in

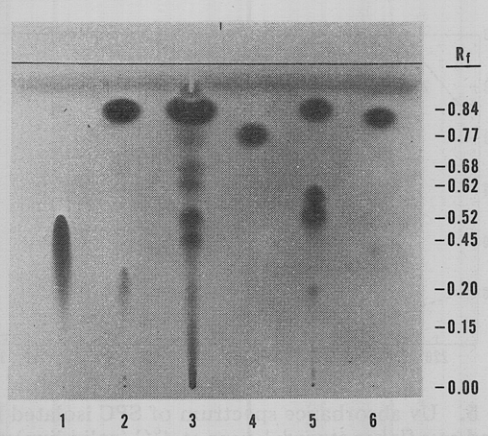


Figure 6. TLC of fatty acid methyl esters and related substances on silica gel. Development with *n*-hexane–diethyl ether (1:1, v/v): (lane 1) oleic acid; (2) fatty acid methyl esters from oil SPC; (3) fatty acid methyl esters from SPC-B; (4) 12,13-epoxystearate methyl ester; (5) fatty acid methyl esters from *Lesquerella stonensis*; and (6) 1-octadecanal.

SPC's from soy flakes were oxidized to hydroxyl and conjugated keto groups. No oxidized fatty acids were detected in SPC from soybean oil.

**Chromatographic Evidence of Oxidation.** Fatty acid methyl esters from oil SPC and SPC-B from defatted flakes were separated by TLC on silica gel plates with the solvent system *n*-hexane–diethyl ether (1:1, v/v). Spots were revealed by spraying with phosphomolybdic acid followed by heating. SPC-B possessed seven spots plus unresolved components that trailed from $R_f$ 0.15 to the base line (Figure 6), whereas fatty acid methyl esters derived from oil SPC gave only one spot, $R_f$ 0.84. The $R_f$ values of 0.77, 0.68, and 0.62 indicated that SPC-B contained keto groups based on a positive color reaction with 2,4-dinitrophenylhydrazine reagent and the absence of color formation with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol, the aldehyde-specific spray reagent of Rahn and Schlenk (1973). Although the reference compound 12,-13-epoxystearate possessed an $R_f$ of 0.77 similar to one of the spots in lane 3, it gave no color reaction with 2,4-dinitrophenylhydrazine reagent. The two methyl ester spots with $R_f$ values of 0.52 and 0.45 may indicate the presence of hydroxy fatty acid methyl esters since they possessed chromatographic mobilities, comparable to those of hydroxy fatty acid methyl esters from *Lesquerella stonensis*.

The nature of the unresolved components with $R_f$ values between 0 and 0.20 is unknown. No hydroperoxides were evident when plates were sprayed with potassium iodide–starch reagent (Oette, 1965). Presumably, partial hydrogenation incorporated into the isolation scheme reduced preformed hydroperoxides to corresponding hydroxy compounds, and no other hydroperoxides were formed during subsequent isolation. Although the content of oxidized fatty acids and unresolved components was not quantitatively determined, their content in SPC-A appeared to be much lower than in SPC-B.

**Bitter Taste.** BIV of partially hydrogenated phosphatidylcholines isolated from soybean oil and defatted flakes is given in Table V. Both SPC isolated from soybean oil and freshly prepared flakes were weakly bitter. A weak bitter taste must be an inherent character of these SPC's, since SPC from soybean oil contained no detectable oxidized fatty acids and SPC-A from freshly prepared defatted flakes contained approximately 1.2% keto fatty acids (calculated from uv absorbance of its fatty acid methyl esters at λ 274 nm). However, BIV of SPC-A isolated from defatted flakes stored for 1 year at 4°C increased nearly threefold. Increased absorbance at λ 274 nm for this SPC (Table IV) showed that appreciable oxidation to ketodienes had occurred during storage. SPC-B and lyso-SPC, both showing strong uv absorbance at 227 and 274 nm, were even more extensively oxidized than SPC-A from defatted flakes stored for 1 year. They were strongly bitter (BIV = 3.0) when tasted at the 0.05% level, and at the 0.01% concentration level they were weakly bitter. Indeed, partially hydrogenated phosphatidylcholines isolated from defatted soy flakes are bitter when tasted at low concentrations and a strong bitter taste is in some way related to oxidation of the unsaturated fatty acid constituents of these phospholipids.

Table V. Bitter Intensity Value (BIV) of Partially Hydrogenated SPC's

| Compd | Concn tasted | BIV[a] |
|---|---|---|
| SPC[b] | 0.05 | 0.7 |
| SPC-A | 0.05 | 0.9 |
| SPC-A[c] | 0.05 | 2.4 |
| SPC-B | 0.05 | 3.0 |
| | 0.01 | 1.2 |
| Lyso-SPC | 0.05 | 3.0 |
| | 0.025 | 1.8 |
| | 0.01 | 0.8 |

[a] Standard error of BIV mean over range 0.7 to 2.4 = 0.3. [b] From soybean oil (Sessa et al., 1974). [c] From defatted flakes stored 1 year at 4°C.

Unhydrogenated SPC-A and SPC-B, and lyso-SPC isolated from defatted soybean flakes, possessed rancid flavors in addition to bitter tastes. Since rancid flavor was not noted with the partially hydrogenated SPC-A, SPC-B, and lyso-SPC, the hydrogenation step effectively minimized artifact formation during their isolation.

Unhydrogenated SPC oxidized in the presence of $Cu^{2+}$ catalyst (Sessa et al., 1974) exhibited little absorbance at 274 nm compared to SPC's isolated from defatted flakes. The previously unhydrogenated, oxidized SPC and those under study here all contained aldehydic components that gave positive color reaction with thiobarbituric acid, whereas hydrogenated SPC's from defatted flakes did not. Despite these differences, they all tasted strongly bitter when evaluated at low concentrations. We noted a relationship between the SPC's isolated in this study and SPC's oxidized with $Cu^{2+}$ catalyst: turbidity of the SPC's dispersed in water changed upon oxidation. Oxidation of unsaturated fatty acid constituents gives the SPC a more hydrophilic character. All dispersions that appeared milky white tasted weakly bitter at the 0.05–0.1% concentration levels. With more extensive oxidation, dispersions of SPC's were more easily formed and appeared more transparent. Dispersions of the most bitter tasting SPC's in both the previous model system and this one, i.e., SPC-B and lyso-SPC, were optically clear. The physical behavior of the oxidized phospholipid in water may affect intensity of the bitter taste.

Unhydrogenated SPC and lyso-SPC were isolated from a bitter-tasting commercial sample of a double-bleached, hydroxylated lecithin. Chemical and physical analyses of these two phospholipids indicated that they both contained oxidized fatty acids. Dispersions of each phospholipid at the 0.05% concentration level were optically clear, and when tasted by panel members at this concentration, SPC rated a BIV = 2.8 and lyso-SPC scored 3.0. On the basis of isolated yields, these two phosphatidylcholines account for the bitterness of this particular lecithin sample. Dehulled, defatted soy flakes we used contained at least 0.08% phosphatidylcholines. At this level, these con-

stituents could contribute to their bitter taste.

Isolation, identification, and quantitation of oxidized fatty acid constituents of SPC's partially characterized are underway. Hopefully, the distinguishing properties of SPC-A and SPC-B will be characterized.

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# Bitterness Prevention in Citrus Juices. Comparative Activities and Stabilities of the Limonoate Dehydrogenases from *Pseudomonas* and *Arthrobacter*

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A comparison of the limonoate dehydrogenases of *Pseudomonas*-sp. 321-18 and *Arthrobacter globiformis* in orange juices and model systems has demonstrated the wide differences in activity and stability of the two enzymes. Both enzymes have been shown to function in freshly prepared navel orange juice. A comparison of the effectiveness of the two enzymes in reducing the eventual limonin content of freshly prepared navel orange juice revealed that 200 munits/ml of juice of the limonoate dehydrogenase of *Pseudomonas* reduced the eventual limonin content of a 21-ppm juice to 3 ppm, a level below the general bitterness threshold, whereas comparable activity levels of the enzyme from *Arthrobacter* caused substantially smaller decreases in eventual limonin content. This wide difference in activity at low pH is explained by the instability of the limonoate dehydrogenase of *Arthrobacter* at pH 3.5 and the relative stability of the *Pseudomonas* enzyme.

Limonin bitterness in citrus juices continues to be an important economic problem for the citrus industry, especially in California. As citrus production and processing increase, the problem becomes more acute. Recently, two methods were reported which reduced limonin-caused bitterness in citrus products. One is the metabolic debittering method of Maier et al. (1973) which reduces the amount of limonin precursor (Maier and Margileth, 1969) in the intact fruit. The other is an enzymatic juice treatment which converts the limonin precursor to a nonbitter product (Hasegawa et al., 1973). In the latter method, the enzyme limonoate dehydrogenase of *Arthrobacter globiformis* (LD-Ag), a limonoate:$NAD^+$ oxidoreductase, is used to convert the limonin precursor present in the juice, limonoate A-ring lactone (LARL), to nonbitter 17-dehydrolimonoate A-ring lactone (17-DLARL), and thereby prevent the formation of bitter limonin.

Further work to develop a more efficient enzyme system led to the isolation of another limonoate dehydrogenase, from *Pseudomonas*-sp. 321-18 (LD-Ps) (Hasegawa et al., 1974c). This enzyme, a limonate:$NAD(P)^+$ oxidoreductase, also catalyzes the conversion of LARL to 17-DLARL and

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