INTRODUCTION
RESEARCH ON SOYBEAN flavor has been extensive, and many patents for improving or reducing flavor of soybean products have been granted. Still, this factor adversely affects the acceptance, and in many ways limits the use, of soy products in foods. Kalbrener et al. (1971) reported that the predominant heavy and bitter smells of mature soybeans are still present in commercial soy flours, concentrates and isolates. Oxidative degradation of lipids, which occurs either during storage or processing, is a common cause of objectionable flavors in foods.

Bitter taste is not usually associated with lipid degradation products. However, Weiss and Diermar (1939) established that bitterness can arise from oxidation of phospholipids (from lupine and rape). Sessa et al. (1969) demonstrated that oil-free phosphatides from soy become intensely bitter after irradiation with ultraviolet light. Sessa et al. (1969), Rackis et al. (1970) and Maga and Johnson (1972) have reported on lipid oxidation and changes in lipid composition of soy products as affected by processing and storage.

We have isolated an intensely bitter fraction containing a modified phosphatidylcholine as the major constituent from autoxidized, defatted soybean flakes. The bitter taste of soybeans may involve oxidation of phospholipids. The purpose of this investigation was to purify soy phosphatidylcholine (SPC) and to determine by organoleptic evaluation whether a bitter taste develops during its autoxidation. Hydrogenated SPC was prepared for a flavor reference. Chemical and physical analyses were used to follow the course of oxidation of these compounds.

EXPERIMENTAL
Preparation of SPC and hydrogenated SPC

SPC was purified by column chromatography of 1-g portions of oil-free refined soy lecithin (Nutritional Biochemicals Corp., Cleveland, Ohio). A 500-ml column of Astrol (Supelcosil ATT-061, Supelco Inc., Bellefonte, Pa.) was used to elute the material containing SPC and phosphatidylcholine by using hexane:ether (9:1) as the elution solvent. The column was eluted with 200 ml hexane and then 200 ml of a 1:1 mixture of hexane:ether and 200 ml of a 1:1 mixture of ether:ethanol.

The material eluting with chloroform:methanol (1:1) was stripped and then rechromatographed on a column of DEAE cellulose (Selectael type 49 with capacity 1.14 meq/g from Brown Co., Berlin, N.H.) according to the procedure of Rouzer et al. (1967).

To prepare hydrogenated SPC, refined soy lecithin was freed of residual oil and phosphatidylethanolamine by the procedure of Aneja et al. (1971) and then hydrogenated according to example 22 of a U.S. patent by Davis (1962). This material was subjected to the same chromatographic procedures used to purify SPC.

Analytical methods
The materials that eluted from the DEAE cellulose columns with chloroform:methanol (9:1) were identified as phosphatidylcholines by their infrared spectra recorded on a Perkin-Elmer Model 621 grating infrared spectrophotometer. These spectra, obtained by the procedure of Marrett and Stott (1954), showed strong bands at the frequencies: 2916; 2850; 1730; 1460; 1375; 1240; 1080, broad doublet; and 970 cm⁻¹. Absorption bands were identical with those of synthetic phosphatidylcholine (1,2-dimyristoyl, C₁₆:₀-dipalmitoyl, Calbiochem, San Diego, Calif.).

Thin-layer chromatography of 300-μg amounts of our SPC and hydrogenated SPC and 99% pure SPC (The Hormel Institute, Austin, Minn.) was carried out on either 0.25 mm or preparative precoated silica gel 1–224 plates. The derivatizations were performed by Brinkman Instruments, Inc., Waterbury, N.Y. (used for lipids). The material was developed with the following solvent systems: chloroform:methanol:water, 75:25:4.2, Oette, (1965); chloroform:methanol:conc ammonium hydroxide, 140:50:7, Chapman, (1972); chloroform:methanol:acetic acid:water, 176:25:22:4, Nichols and James, (1964). Spots were visualized by spraying with 0.5% potassium dichromate in 50% sulfuric acid followed by heating 30 min at 150°C. With each sample only one spot appeared which gave positive color reactions with phosphatase, moloney reagent (Dittmer and Lesher, 1964) and for choline with Dragendorff reagent (Warner et al., 1961). With the three solvent systems the Rf values of our samples were equal to that of the purchased SPC (Fig. 1).

SPC and hydrogenated SPC were separated by refluxing with 0.5M potassium hydroxide in chloroform:methanol (1:1) for 4

Fig. 1—Thin-layer chromatography of 1-mg amounts of soy phosphatidylcholines (SPC) on silica gel 0.25 mm precoated plates. Development: chloroform:methanol:water 75:25:4.2, O., A, SPC, B, hydrogenated SPC, C, SPC from The Hormel Institute.
hr. After acidification and extraction with ether, the combined ether extracts were back-extracted with water and treated with diazomethane to convert the fatty acids to methyl esters. Fatty acid composition of both were determined by gas-liquid chromatography (Table 1). Hydrogenation of SPC reduced the amount of 18:2 and 18:3 fatty acids by about 90%.

The freeze-dried aqueous layers of the separation mixture of each SPC were analyzed for amine compounds by ascending paper chromatography on Whatman No. 1 paper developed with n-propanol:ammonium hydroxide:water (60:30:10). Chromatograms showed only one spot that gave a positive color reaction for choline and no reaction with ninhydrin reagent. The Rf value and color reaction of this component were identical to those of choline chloride (Calbiochem, San Diego, Calif.).

Microanalyses for carbon, hydrogen, nitrogen (Dumas), phosphorus and choline (Kushlan) of phospholipid dispersions during the 4-wk storage were observed by thin-layer chromatography. A Beckman DK2A recording spectrophotometer recorded ultraviolet absorption spectra over the wavelength region 220-260 nm. Absorbance at 232 nm (diene conjugation) was read on a Gilford Model 240 spectrophotometer. When necessary, suspensions were diluted with absolute ethanol. Absorbance readings per mg/ml are expressed as EB ecm. In this way, results were compared on an equal weight basis.

The extent of oxidation during storage was assayed with TBA. To measure relative amounts of TBA-reactive substances, 1.0 ml of the phospholipid suspension or a dilution of it was assayed with TBA. To measure relative amounts of TBA-reactive substances, 1.0 ml of the phospholipid suspension or a dilution of it was assayed with TBA.

The bitter detection threshold of autoxidized SPC was determined by 10 experienced tasters. More tasters were used in these tests to increase the reliability of the threshold determination. Solutions ranging in concentration from 0.001 to 0.025% were presented along with one water sample. Each taster was instructed to record which samples were bitter. The bitter detection threshold is defined as that concentration at which 50% of the panel gave a positive response.

RESULTS & DISCUSSION

Compositional changes during autoxidation

A 0.3% suspension of SPC without added Cu++ was turbid and gave a pH of 3.8. During 4 wk at 25°C, turbidity gradually cleared and pH dropped to 3.0. Fatty acid analysis showed decreases in diene content from 62.0 to 48.3% and in triene content from 6.5 to 3.2%. Hydroperoxides were detected on thin-layer chromatograms of unhydrogenated SPC suspensions stored at 25°C by spraying

Table 1—Fatty acid compositiona of soy phosphatidylcholine (SPC) and hydrogenated SPC

<table>
<thead>
<tr>
<th>Fatty acidb</th>
<th>SPC</th>
<th>Hydrogenated SPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>16.8</td>
<td>13.4</td>
</tr>
<tr>
<td>18:0</td>
<td>5.1</td>
<td>67.4</td>
</tr>
<tr>
<td>18:1</td>
<td>9.4</td>
<td>11.7</td>
</tr>
<tr>
<td>18:2</td>
<td>62.0</td>
<td>7.0</td>
</tr>
<tr>
<td>18:3</td>
<td>6.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a Calculated as percentage of total gas-liquid chromatographic peak area, as methyl esters.

b Glass column (6 ft X 3/8 in. o.d.) packed with 15% Carbowax 20M on 80-100 mesh Chromosorb W/AW. Carrier gas-helium 40 ml/min; oven-temperature programmed 150°-220°C at 1.5°/min. Inlet, 210°C; detector 210°C.

Table 2—Analysis of SPC and hydrogenated SPC

<table>
<thead>
<tr>
<th>Constituent (%)</th>
<th>SPC Calculated</th>
<th>SPC Found</th>
<th>Hydrogenated SPC Calculated</th>
<th>Hydrogenated SPC Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>65.80</td>
<td>62.68</td>
<td>65.40</td>
<td>64.42</td>
</tr>
<tr>
<td>H</td>
<td>10.29</td>
<td>10.40</td>
<td>10.87</td>
<td>11.11</td>
</tr>
<tr>
<td>N</td>
<td>1.77</td>
<td>1.88</td>
<td>1.76</td>
<td>1.79</td>
</tr>
<tr>
<td>P</td>
<td>3.92</td>
<td>3.73</td>
<td>3.89</td>
<td>3.69</td>
</tr>
<tr>
<td>Choline</td>
<td>15.32</td>
<td>14.15</td>
<td>15.20</td>
<td>14.67</td>
</tr>
</tbody>
</table>

a C₁₈:₂H₃₄NO₄P, molecular weight 790.3. Molecular weight calculation was based on fatty acid analysis.

b C₁₈:₄H₃₄NO₄P, molecular weight 769.9.
the developed plates with starch-potassium iodide reagent (Oette, 1965). Therefore, part of the disappearance of diene and triene fatty acids may be attributed to autoxidation. The decrease in percentage of dienes and trienes was proportional to an increase in saturated and monoenic fatty acids: 16:0 from 16.8 to 10.5%; 18:1 from 7.8% to 5.1% and 18:2 from 9.4 to 6.0%. Two additional unidentified peaks with retention times for methyl esters of lauric and myristic acids appeared on the chromatogram.

Suspensions of hydrogenated SPC without added Cu++ gave a pH of 4.0. The homogenized suspension was not stable and a gel-like mass settled on standing. However, this material was readily redispersed by swirling the flask before each test. After storage for 4 wk, the pH of this suspension dropped to 3.4.

Thin-layer chromatography (Fig. 2) showed degradation of SPC at 1, 2, and 4 wk and degradation of hydrogenated SPC only at 4 wk of storage at 25°C. SPC with Rf = 0.25 (chromatogram A) and the components with lower mobility gave positive color reactions for phosphorus. In B, C and D, the component with mobility greater than that of SPC gave a positive color reaction for carbonyl compounds with 2% 2,4-dinitrophenylhydrazine in hydrochloric acid. Extensive trailing of this spot in chromatogram D indicated decomposition of this carbonyl compound. In E, hydrogenated SPC degraded to a slower moving component that gave positive color reactions for phosphorus and choline and possessed an Rf value equal to that of lysophosphatidylcholine (Rf = 0.15) run as a standard for comparison. The faster moving component gave no color reaction with 2,4-dinitrophenylhydrazine and showed no trailing on charring.

Diene conjugation and TBA-reactive substances

Little ultraviolet absorbance, except for minor peaks at 232 and 274 nm, was observed in freshly prepared suspensions (without added Cu++) of SPC in air-saturated water; SPC in nitrogen-saturated water; and hydrogenated SPC in air-saturated water. Since the last two showed no increases on storage, the increase in absorbance at 232 nm of SPC dispersed in air-saturated water results from autoxidation of the unsaturated fatty acids. As shown in Figure 3, conjugated dienes formed rapidly during the first 240 hr. After 240 hr, absorbance values at 232 nm declined and the peak at this wavelength gradually shifted to 222 nm. Schauenstein (1967) reported that autoxidation of polyunsaturated esters in water gives rise to water-soluble substances that absorb at 222 nm. Whether we are observing compounds of similar nature has yet to be determined.

The formation of TBA-reactive substances from SPC paralleled the formation of conjugated dienes during the initial stages of oxidation. When the absorbance values at 232 nm declined, those at 532 nm continued to increase until a maximum was reached at 580 hr of storage. The TBA assay measures not only the amount of hydroperoxides that are degraded to TBA-reactive substances, but also the amount of secondary oxidation products that result from the hydroperoxide degradation. Suspensions of hydrogenated SPC showed no increase in amounts of TBA-reactive substances.

### Copper-catalyzed oxidation

With some of our SPC preparations, the rate of oxidation varied greatly from that recorded in Figure 3. Morita and Fujimaki (1972) demonstrated that Cu++ initiates oxidation of SPC and also catalyzes the breakdown of the hydroperoxides. Therefore, Cu++ was used in our systems to overcome some of the inherent but unknown factors that affect rate of oxidation.

Cu++ added to suspensions of SPC at the 0.25 ppm level had little effect (Fig. 4). Absorbance values denoting formation

<table>
<thead>
<tr>
<th>Table 3—Effect of storage on bitterness intensity values (BIV) of SPC * and hydrogenated SPC*&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E&lt;sub&gt;1%1cm&lt;/sub&gt; of SPC</th>
<th>BIV&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Hydrogenated SPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hr at 25°C)</td>
<td>232 nm</td>
<td>532 nm</td>
<td>SPC</td>
</tr>
<tr>
<td>Cu++</td>
<td>No Cu++</td>
<td>1.0 ppm Cu++</td>
<td>1.0 ppm Cu++</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>13.7</td>
<td>2.5</td>
</tr>
<tr>
<td>240</td>
<td>–</td>
<td>14.0</td>
<td>2.5</td>
</tr>
<tr>
<td>–</td>
<td>432</td>
<td>5.1</td>
<td>2.9</td>
</tr>
<tr>
<td>–</td>
<td>672</td>
<td>7.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\* Tasted at 0.1% concentration
\* Defined in text
\* Standard error of BIV mean over range 0.4 to 2.0 = 0.3
of conjugated dienes and TBA-reactive substances are similar to those reported in Figure 2.

The rate of oxidation leading to decreased absorbance at 323 nm is accelerated when suspensions of SPC contain 1.0 ppm Cu++. In Figure 5, formation of conjugated dienes reached a maximum (Ε₂₂ / ε cm = 13.7) in 180 hr rather than 240 hr. In both suspensions the extent of oxidation is about the same. The 232-nm absorbing peak shifted to one absorbing at 252 nm within 432 hr rather than 672 hr. The suspension cleared and pH dropped to 2.9. The TBA-reactive substances absorbing at 532 nm continued to form after absorbance due to diene-conjugation reached its maximum. In this suspension the amounts of TBA-reactive substances were at a maximum (Ε₂₂ / ε cm = 3.6) at about 340 hr. Since little decrease in absorbance of TBA-reactive substances was observed in the other two systems over the entire 4 wk, the additional Cu++ must have caused further degradation of the secondary oxidation products. New preparations of SPC treated according to the same procedure with 1.0 ppm Cu++ yielded reproducible results. On the basis of four replicates for SPC with 1.0 ppm Cu++, the relative standard deviation for each point on the plots (Fig. 5) is 6.4% for measurements at wavelength 232 nm and 9.8% for measurements at wavelength 353 nm.

Addition of Cu++ to hydrogenated SPC dispersed in water had virtually no effect either on absorbance at 232 nm or 532 nm.

Bitter taste

Our samples were evaluated only for bitter taste even though rancid flavors also developed with SPC during storage. In Table 3, 0.1% suspensions of SPC and hydrogenated SPC were both rated weakly bitter at zero time. However, synthetic phosphatidylcholine, containing saturated palmitic fatty acids, when tasted at this level, gave a BIV of 0.3. Since both SPC and hydrogenated SPC broke down when dispersed in water (see Fig. 2), their suspensions may contain some lysophosphatidylcholine and choline. These, when tasted at the 0.1% level, possessed only a trace of bitterness: lysophosphatidylcholine BIV = 0.4 and choline BIV = 0.3. Therefore, they do not cause the weak bitter taste. The initial weak bitter taste in suspensions of SPC and hydrogenated SPC must be due to some oxidation of the constituent unsaturated fatty acids that occurred either during handling or sample preparation.

Since the relationship between our chemical and physical tests versus flavor scores is not known, these tests were used solely to establish a common time for tasting the samples. For example, when the conjugated diene content of a suspension of SPC either with 1.0 ppm Cu++ or none reached a maximum (Ε₂₂ / ε cm = 14), the BIV of both suspensions was 1.6 and 2.0 respectively. The scores of these two systems did not vary significantly. BIV of SPC increased almost threefold from the initial level of 0.8.

Upon further oxidation, the conjugated diene content of SPC decreased. Suspensions of SPC were next evaluated when they became clear and dropped to about pH 3.0. This drop occurred at 432 hr for SPC system with 1.0 ppm Cu++ and at 672 hr for an SPC system without Cu++. Extent of oxidation as measured by TBA assay was the same in both. The SPC system strongly bitter. However, hydrogenated SPC, tasted after storage for 672 hr, gave a BIV of 0.8, identical to that of the freshly dispersed sample.

From data in Table 4, the average threshold from three trials for detecting bitterness in autoxidized SPC was at the 0.006% concentration.

Table 4—Bitter response to SPC autoxidized
<table>
<thead>
<tr>
<th>Concentration (ppm Cu++)</th>
<th>Bitter response (%a)</th>
<th>Bitter response (%b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0025</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.010</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>0.005</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>0.003</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>0.001</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Percent by weight in carbon-filtered tap water
b Percentage of panelists giving positive response

According to our results, SPC definitely becomes bitter during storage. Autoxidation of the unsaturated fatty acids attached to SPC is indicated because the hydrogenated SPC treated similarly did not increase in BIV. Weiss and Diemair (1939), Evans et al. (1960) and Kalbrenner et al. (1973) reported that bitterness can arise from autoxidation of unsaturated fatty acids or their methyl esters. Our present studies show that the bitter factor of SPC, oxidized for 4 wk under the conditions used in this investigation, resides with the components in chromatogram D (Fig. 2) that possess Rr less than 0.25 and give positive color reactions for phosphorus. Therefore, a modified form of SPC may be a source of bitterness in defatted soybean meal, especially since concentrates of the bitter principle from this meal showed chromatographic equivalence to the autoxidized SPC. Future work will include further characterization of the soy bitter principle as it is related to autoxidized SPC and evaluation of the effect that soy protein has on oxidation of SPC. We found that oxidation is catalyzed by a crude soybean lipoxygenase. Rackis et al. (1972) demonstrated that the three- to fourfold increase in bitterness of soybeans during maturation correlated with an increase in lipoxygenase activity. Whether a bitter taste develops in enzymatically oxidized SPC is yet to be determined.

REFERENCES


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