

## EVALUATION OF DURUM WHEAT AND DURUM PRODUCTS

### II. Separation and Identification of the Sitosterol Esters of Semolina<sup>1</sup>

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#### ABSTRACT

A fundamental study of the composition of the lipids of *Triticum durum* and *T. vulgare* wheats is described. For comparative purposes, the study was expanded to include the ground endosperm of several types of seeds. Qualitative analysis, which was facilitated by means of column, thin-layer, and gas-liquid chromatography, indicated major differences in the sitosterol ester fractions. Sitosteryl palmitate was found to be substantially absent in the milled products of *T. durum*. A method of quantitative chemical analysis was developed which employed thin-layer chromatography in conjunction with a photo photometer. The quantitative technique has been successfully employed not only for the analysis of sitosteryl esters, but in addition, it has been found useful for the detection and estimation of the presence of other cereal grains in semolina.

Gortner (1) in 1908 published a note in the *Journal of the American Chemical Society* which reported a "supposedly new compound" in wheat oil. It was a crystalline substance, isolated from ether extracts, which contained nitrogen and melted at 96.5°C. This is one of the early reports of the probable presence of a steryl ester in wheat.

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In 1926, Ball (2) described a series of collective crystallizations from flour extracts. One of these fractions melted at 96°–97°C. and was probably a sitosterol ester.

Walde and Mangels (3) in 1932 found a material in flour which was relatively insoluble in cold acetone and melted at about 93°C. They stated that this was probably an ester of sitosterol and that durum semolina did not give this particular crystal.

In 1933, Spielman (4) isolated and identified sitosteryl palmitate from wheat flour oil. He obtained the steryl ester by cold-temperature crystallization, and performed a carbon-hydrogen analysis of the hydrolysis products, namely sitosterol and palmitic acid. His work also included optical rotation studies using his own data and data from others. From this work he concluded that the sterol portion should contain approximately 28% dihydrositosterol and 72% sitosterol which he believed to be rich in alpha and beta modifications.

Not much is recorded in the literature from 1933 until 1952, when Matveef (5) attempted to utilize the recorded difference in steryl ester content between durum and other wheats in establishing a test whereby one could determine the amount of farina present in semolina by measuring sitosteryl palmitate. Several papers followed, giving modifications of the test. Included in these papers was one by Guilbot (6), who summarized many of these modifications, cited work of his own, and stated that the Matveef test was not satisfactory because of lack of reproducibility and lack of accuracy.

Nelson *et al.* (7) in 1963 used column chromatography to isolate a material from wheat which melted at 93°C. This was "probably" the sitosteryl palmitate described by Spielman (4).

The purpose of this research was to isolate and identify the sitosteryl esters present in durum and hard wheat, and to compare the relative amounts present in the different varieties.

### Materials and Methods

Semolinas from pure varieties of Sentry, Wells, Lakota, and Min-dum durums, as well as farina from Selkirk, a hard red spring wheat, were prepared on an experimental Allis mill and purified on a modified Minneapolis purifier to give a yield of about 55% on a wheat basis. The different varieties were individually extracted with petroleum ether (b.p. 30°–60°C.) in a large Soxhlet extractor for 48 hr. The solvent from the extracted lipids was removed under vacuum on a roto-evaporator, and the lipids were stored at -18°C. under nitrogen.

Rapid extractions of the semolina and the farina were also employed. Ten grams of the material was placed in a 200-ml. Erlenmeyer

flask and to this was added 30 ml. of petroleum ether (b.p. 30°–60°C.). The flask was placed on a Burrell shaker for 15 min. Following this, the solids were removed from the solvent by vacuum filtration and washed twice with approximately 10-ml. portions of petroleum ether. The lipid solution was evaporated slowly on a steam bath to a volume of 5–10 ml., whereupon this solution was transferred to a smaller container and reduced to 2 ml. by passing nitrogen over the surface of the liquid. The ether extracts obtained by this method were spotted directly on thin-layer plates.

Column chromatography was employed to separate the nonpolar fraction from the crude lipids obtained from Soxhlet extraction. A column containing approximately 15 g. of 325-mesh silicic acid was prepared and eluted discontinuously according to the procedure of Hirsch and Ahrens (8). Petroleum ether (b.p. 30°–60°C.) was used as the solvent to elute the nonpolar fraction from the crude lipids. This solvent appeared to give the same results as the more commonly used solvent, 1% diethyl ether in 99% petroleum ether (b.p. 60°–70°C.). This fraction was then separated further on thin-layer plates.

Thin-layer chromatography (TLC) was used to isolate the sitosteryl esters. The plates were coated with silica gel G, 0.25 mm. thick. When preparative plates were used, the thickness was 0.75 mm. The most successful solvent system employed was 100% carbon tetrachloride, one of the several solvent systems described by Kaufmann *et al.* (9) for separation of the esters of cholesterol. Both the crude lipid from the rapid extraction and the nonpolar fraction from the silicic acid column gave good separation of steryl esters. To produce better separation, all plates were removed from the tank and dried, and the development process was repeated a second time. Visualization of the spots was accomplished by spraying with 50% aqueous sulfuric acid and heating at approximately 170°C. for 10 min.

Since sitosteryl ester standards were not commercially available, they were synthesized according to the method of Mahadevan and Lundberg (10) for preparation of cholesteryl esters. This method involves the interesterification of the steryl acetate and the methyl ester of a fatty acid in the presence of sodium ethoxide, under reduced pressure.

Gas-liquid chromatography was employed to compare retention times of the steryl esters. The instrument used was a Beckman GC-2A, equipped with a thermal conductivity detector and a Brown recorder. An aluminum column, 0.25-in. diameter, 6 ft. long, was packed with 100/120-mesh Gas Chrom Z, coated with 1% SE-30, and maintained at 230°C. The inlet temperature was 350°C., and the detector tempera-

ture 240°C. A flow rate of about 90 ml. of helium per min. and a current of 200 ma. were used.

To determine the presence of the palmitate in sitosteryl palmitate, an 8-ft. aluminum column containing 100/120-mesh Gas Chrom Z coated with 25% diethylene glycol succinate was maintained at 199°C., with inlet temperature 250°C. and detector temperature 205°C. Other conditions were similar to those previously described. The methyl ester of palmitic acid was prepared by the boron trifluoride method described by Metcalfe and Schmitz (11).

The Koeffler Hot Stage was used to determine the melting point of sitosteryl palmitate.

Quantitative data were obtained by two means, the Beckman Analytrol and the Photovolt densitometer. The Beckman Analytrol Model RB was used with the B-5 cam, which is designed for electrophoretic separation of proteins stained with bromphenol blue. However, by photographing the spots obtained by thin-layer separation, making a 10 by 1.2-in. enlargement of the negative, and analyzing the density of the spots on the enlarged print, quite good results were obtained.

A Photovolt densitometer Model 530, equipped with a Varicord recorder, was also used. This instrument permitted direct analysis of thin-layer plates. As the spots were scanned, the change in density was recorded on the Varicord recorder (response setting of 5) in the form of triangles. The area of the triangles was proportional to densities of the spots on the thin-layer plates. The densitometer was modified by use of a slit of approximately 0.2 by 28 mm. instead of the much smaller one furnished by the manufacturer. This modification enabled the entire spot to be scanned.

### Results and Discussion

Column chromatography was used initially to separate the lipids into seven principal fractions. These fractions are arranged in Table I according to their relative polarity. The nonpolar hydrocarbons and steryl esters appear at the top of the fraction column and the highly polar phospholipid fraction appears at the bottom. Repeated fractionation of the lipids indicated that the values in Table I are typical for the semolina and farina prepared from whole wheat and for their corresponding purified endosperm products. The nonpolar lipid fraction of semolina represented less of the total lipid fraction than did the corresponding nonpolar lipid fraction of farina.

Of interest also is the difference shown between the polar fractions of the semolina and farina. The increase in the polar phospholipid

TABLE I  
 PRINCIPAL LIPID FRACTIONS ISOLATED FROM SEMOLINA AND FARINA BY  
 COLUMN CHROMATOGRAPHY  
 (Percent of total lipids)

FRACTION	WHOLE WHEAT <sup>a</sup>		PURIFIED <sup>b</sup>	
	Semolina	Farina	Semolina	Farina
Nonpolar (hydrocarbons, steryl esters, etc.)	1.8	4.1	1.3	4.7
Triglycerides	69.8	66.1	66.7	53.1
Free fatty acids	4.0	2.5	5.1	5.5
Sterols	6.5	6.6	8.1	4.9
Unknown	5.1	6.3	2.8	2.7
Pigments	4.1	4.4	5.2	8.7
Polar (phospholipids, etc.)	8.4	9.9	10.7	21.3

<sup>a</sup> Entire kernel ground by means of roller. The bran was reduced further by a hammer mill to pass a U.S. No. 20 sieve.

<sup>b</sup> Roller-milled endosperm purified by density in air and particle size separation.

fraction of Selkirk endosperm when compared with Selkirk whole wheat has been shown previously by Nelson *et al.* (7), who employed a different solvent. In the corresponding semolina products derived from the durum wheats, the apparent difference in phospholipid content is much less.

*Identification of Sitosteryl Palmitate.* Figure 1 is a photograph of a thin-layer plate, the series of spots on the left showing the separation obtained when the nonpolar fraction of Selkirk farina lipids was further separated by thin-layer chromatography, using silica gel G, and carbon tetrachloride as the solvent. The top spot was identified as the saturated sitosteryl esters and contained principally sitosteryl palmitate.

The second and third spots on the plate are prepared standards of sitosteryl palmitate. Note the similar  $R_F$  values as compared with the top spot of the Selkirk fraction.

The top spot of the Selkirk fraction appeared very slowly when exposed to iodine vapors, indicating little or no unsaturation. It showed typical saturated steryl ester colors after heating when sprayed with sulfuric, phosphomolybdic, or phosphotungstic acid solutions.

A gas-chromatographic study using 1% SE-30 as liquid phase on the solid support Gas Chrom Z showed that the synthesized fatty acid esters of sitosterol all produced peaks having the same retention time, although they differed considerably from similar cholesteryl esters. The top spot of the Selkirk nonpolar lipid fraction, when eluted from several heavily spotted thick-layer (0.75 mm.) plates and injected into an SE-30 column, gave a peak corresponding in retention time to the sitosteryl esters.

To verify the presence of the palmitate in the ester, spots eluted

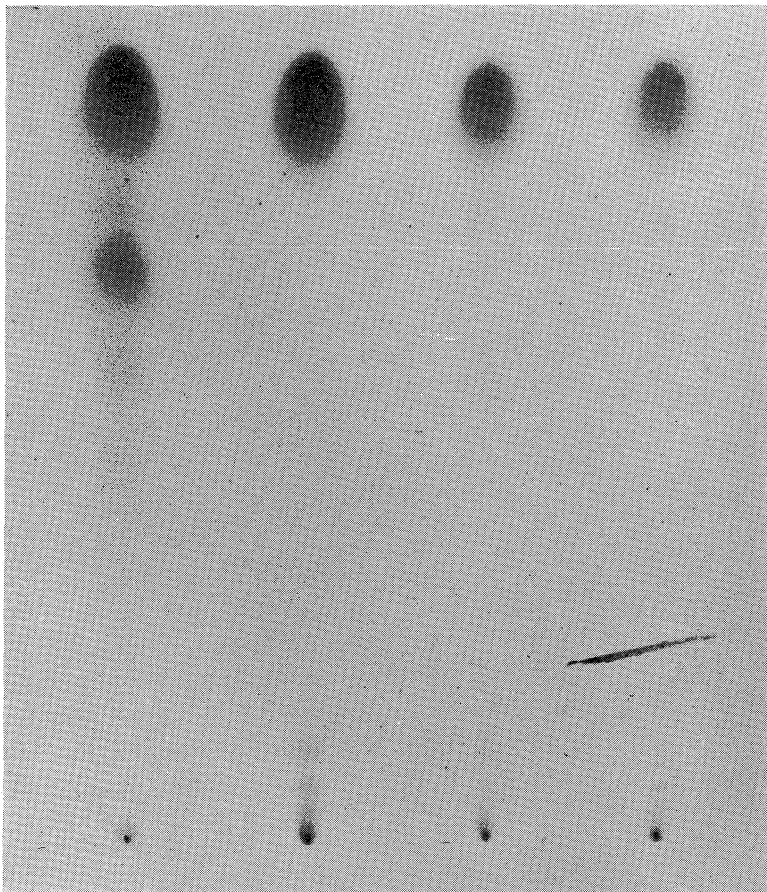


Fig. 1. Thin-layer chromatoplate. Left, nonpolar lipids obtained from Selkirk farina by column chromatography. The middle two spots are synthesized sitosteryl palmitate. Spot on right is recrystallized sitosteryl palmitate obtained from Selkirk farina.

from several thick-layer plates were transesterified with methanol in the presence of boron trifluoride (11). The methyl ester was recovered with petroleum ether and analyzed by gas chromatography with diethylene glycol succinate as liquid phase on Gas Chrom Z. A single major peak was obtained, with retention time equal to that of methyl palmitate.

A melting point was determined on this eluted material, after recrystallization in ethanol, and was found to be  $91^{\circ}\text{C}.$ , the same as the standard. A mixed melting point showed no difference.

*Identification of Other Sitosteryl Esters.* A comparison of  $R_F$  values

of other prepared sitosteryl ester standards with durum and hard red spring wheat lipids is shown in Fig. 2. The first two spots are not standards, but are a methyl ester of a fatty acid and sitosteryl acetate, respectively, both used in preparing the standards (10). These materials

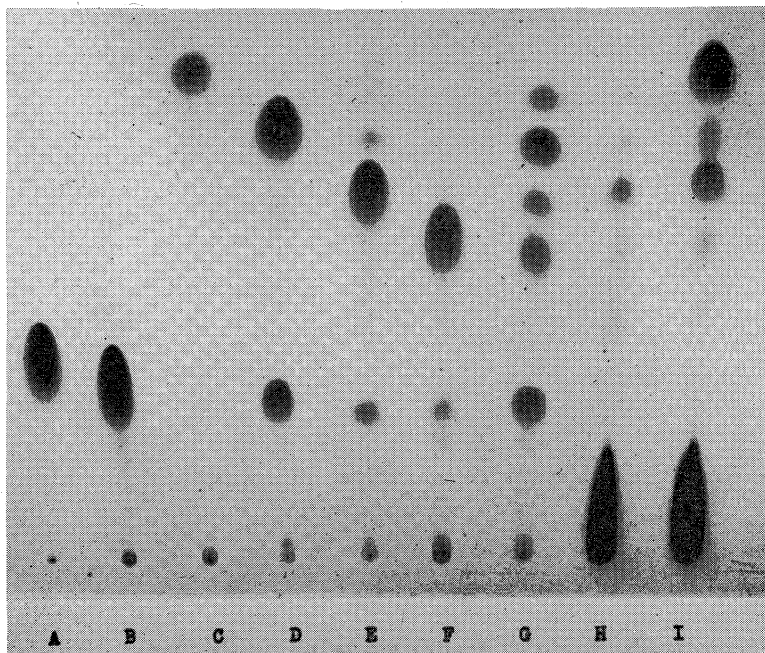


Fig. 2. Thin-layer chromatoplate. A, methyl oleate; B, sitosteryl acetate; C, sitosteryl palmitate; D, sitosteryl oleate; E, sitosteryl linoleate; F, sitosteryl linolenate; G, a mixture of A to F; H, Wells semolina lipids; and I, Selkirk farina lipids.

show as impurities in all the standards except sitosteryl palmitate, which was recrystallized several times. In comparing  $R_F$  values of the standards with those of the spots obtained from Lakota and Selkirk lipids, a close similarity is observed. No steps were taken to identify the very faint spot which appears below the sitosteryl linolenate in the wheat separations. However, on the basis of cholesteryl ester separations performed by Mahadevan and Lundberg (10), who used a solvent system of petroleum ether (b.p. 60°–80°C.) and benzene, 60:40 (v/v), one would predict this spot to be sitosteryl arachidonate. The great difference between the amount of sitosteryl palmitate present in the hard red spring wheat and in the durum is also evident in Fig. 2.

Further evidence to identify the other sitosteryl esters included the rapid formation of color on exposure to iodine vapors. This reaction

was an indication of the degree of unsaturation; when sprayed with sulfuric acid and heated, the TLC spots gave colored products identical with the reference standards.

Since these samples of semolina and farina were extracted by shaking 15 min. in petroleum ether, and the entire ether extract, after being reduced in volume, was spotted directly on the plate (Fig. 2), dark-colored areas appeared near the origin. Doubtless, these dark areas include portions of the lipid fraction other than the nonpolar fraction.

*Effect of Location and Crop Year on Steryl Ester Content.* Lakota, Wells, Sentry, and Mindum semolinas, when treated by the quick extraction method and spotted on thin-layer plates, all showed only very small quantities of sitosteryl palmitate. The similarity of lipids extracted from semolinas prepared from durum grown in three foreign countries is shown in Fig. 3. Wells durum grown in nine different loca-

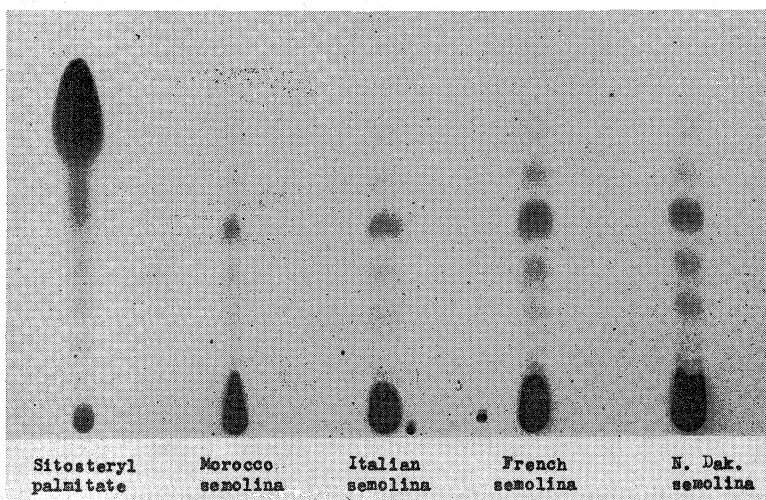


Fig. 3. Thin-layer chromatoplate showing similarity of composition of lipids extracted from semolinas prepared from durum grown in Morocco; Italy, France, and North Dakota (respectively, left to right).

tions in North Dakota, the principal durum-producing region of the United States, was extracted and spotted on thin-layer plates. Two of these locations included both dry-farmed and irrigated crops. Little change in the steryl ester content was noted. Similarly, the sitosteryl palmitate present in lipids extracted from Wells durum grown during the 1960, 1961, 1962, and 1963 crop years did not change appreciably.

*Analysis of Different Crops for Steryl Esters.* The lipids from several



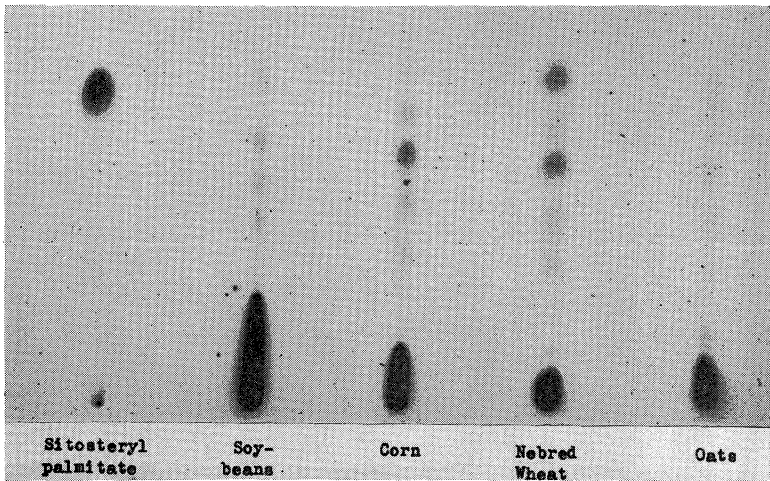


Fig. 4. Thin-layer chromatoplate. Left to right, sitosteryl palmitate and lipids derived from soybeans, corn, Nebred wheat, and oats.

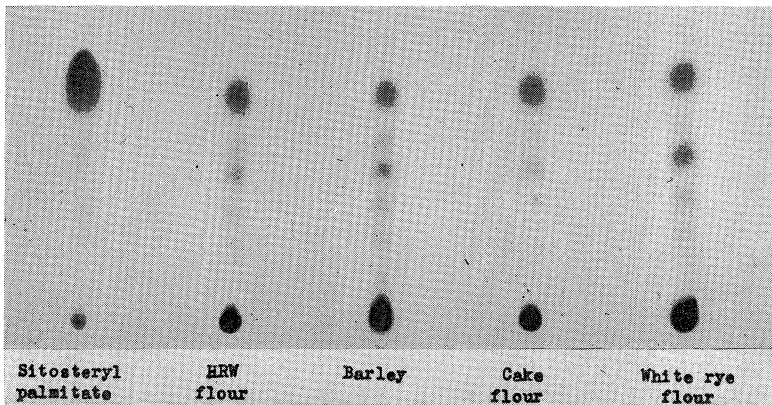


Fig. 5. Thin-layer chromatoplate. Left to right, sitosteryl palmitate and the lipids derived from a commercial hard red winter wheat flour, barley flour, a commercial cake flour, and a commercial white rye flour.

other crops were extracted and spotted on thin-layer plates and developed with carbon tetrachloride to determine qualitatively their steryl ester content. These are shown in Figs. 4 and 5. Again, the top major spot is sitosteryl palmitate; this is followed by the oleate, linoleate, and linolenate esters. It is of interest that flours from the winter wheats, including Nebred wheat, show strong sitosteryl palmitate spots.

*Quantitative Determination of Sitosteryl Palmitate.* Over a number of years, attempts have been made to detect the presence of non-

durum materials in semolina. The work described above suggested the possibility that the difference in sitosteryl palmitate content between durum and hard red spring wheat could be used as a means of detecting blends.

Mixtures of Wells semolina and Selkirk farina were prepared by mixing the proper weighed amounts, giving a total weight of 10 g., and extracting in petroleum ether with shaking for 15 min. The ether solution was filtered by suction from each mixture and washed with petroleum ether, and all ether solutions were reduced to a similar volume; approximately 2 ml. TLC of these ethereal solutions is shown in Fig. 6. Note the change in intensity of the top spots as the percent-

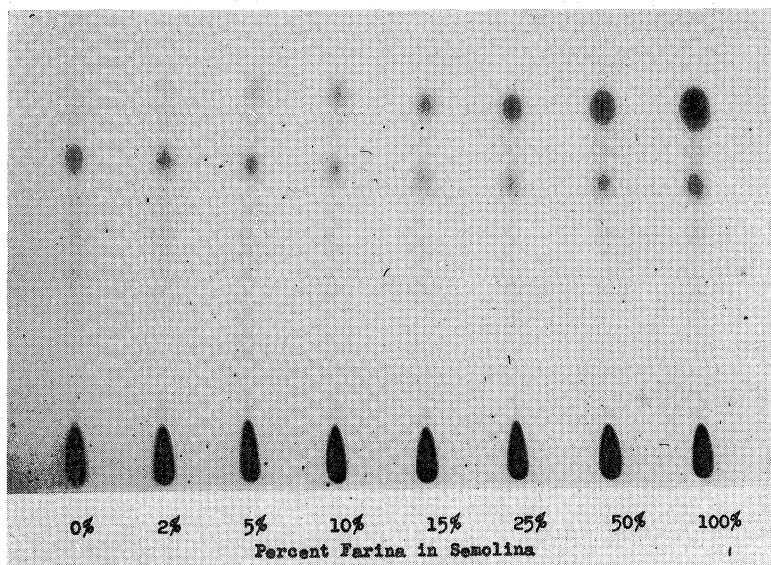


Fig. 6. Thin-layer chromatoplate showing increase in sitosteryl palmitate (upper spot) with increase in amount of farina in mixtures containing farina and semolina. Amount of farina in the mixtures shown, left to right: 0, 2, 5, 10, 15, 25, 50, and 100%.

age of farina increases in the mixtures. The density of these spots was measured by means of the Beckman Analytrol. It was necessary, however, to photograph the thin-layer plate and make an enlarged print to obtain sufficient spot area to give triangles large enough to measure. The areas of the triangles were plotted against percentage farina in semolina, and a straight-line relationship resulted.

Subsequently, the procedure was simplified by use of a Photovolt densitometer equipped with a Varicord recorder. This device per-

mitted direct measurement of the density of the TLC spots. The results are shown in Fig. 7. A straight-line relationship here confirmed the

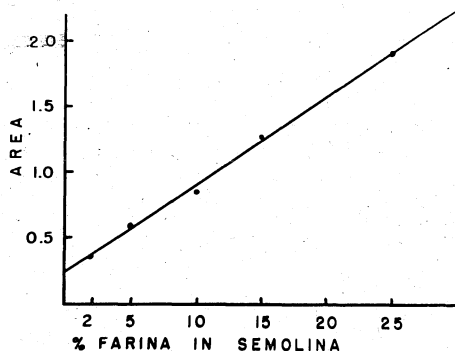


Fig. 7. Linear relationship between area and density of the sitosteryl palmitate TLC spot and percent of farina contained in a mixture of farina and semolina.

previous findings and indicated the potential usefulness of this phase of the research as an analytical technique.

### Conclusions

Sitosteryl palmitate has been isolated from several cereals by thin-layer chromatography and identified by gas chromatography, color change, melting point, mixed melting point, and  $R_F$  comparisons.

Sitosteryl oleate, sitosteryl linoleate, and sitosteryl linolenate have been separated also by thin-layer chromatography and identified by reaction with iodine, color changes, and  $R_F$  comparisons.

Several different wheats and other crops were qualitatively analyzed by thin-layer chromatography to show whether or not sitosteryl esters were present.

Very little sitosteryl palmitate was noted in the durum varieties tested. Moreover, the trace of material that did occur on the spot possessing the same  $R_F$  value as sitosteryl palmitate appeared to be of about the same intensity in all cases.

A mixture of farina and semolina, when extracted by shaking for 15 min. in petroleum ether (b.p.  $30^{\circ}$ – $60^{\circ}$ C.), gave spot densities of sitosteryl palmitate on thin-layer chromatoplates which were proportional to the known percent of farina in the mixture. This technique may provide the basis for convenient detection of other cereal grains in durum semolina.

### Literature Cited

1. GORTNER, R. A. A supposedly new compound from wheat oil. *J. Am. Chem. Soc.* 30: 617 (1908).

2. BALL, C. D., JR. A study of wheat oil. *Cereal Chem.* **3**: 19-39 (1926).
3. WALDE, A. W., and MANGELS, C. E. Variation in properties of acetone extracts of common and durum wheat flours. A preliminary report. *Cereal Chem.* **7**: 480-486 (1930).
4. SPIELMAN, M. A. The sitosterol esters in wheat flour oil. *Cereal Chem.* **10**: 239-242 (1933).
5. MATVEEF, M. Detection des farines de ble tendre dans les semoules et pates alimentaires. *Compt. Rend. Acad. Agr. France* **39**: 658 (1952).
6. GUILBOT, A. Untersuchungen über Sterolester im Getreide und ihre bedeutung für die Unterscheidung von Durum- und Vulgare-Weizen. *Berichte auf der Durum- und Teigwaren-Tagung vom 24-25 Februar 1959 in Detmold.*
7. NELSON, J. H., GLASS, R. L., and GEDDES, W. F. Silicic acid chromatography of wheat lipids. *Cereal Chem.* **40**: 337-343 (1963).
8. HIRSCH, J., and AHRENS, E. H. The separation of complex lipid mixtures by the use of silicic acid chromatography. *J. Biol. Chem.* **233**: 311-320 (1958).
9. KAUFMANN, H. P., MAKUS, Z., and DEICKE, A. F. Die Dünnschicht-Chromatographie auf dem Fettgebiet. II. Trennung der Cholesterin-Fettsäureester. *Fette Seifen Anstrichmittel.* **63**: 235-238 (1961).
10. MAHADEVAN, V., and LUNDBERG, W. O. Preparation of cholesterol esters of long chain fatty acids and characterization of archidonate. *J. Lipid Research* **3**: 106-110 (1962).
11. METCALFE, L. D., and SCHMITZ, A. A. A rapid preparation of fatty acid esters. *Anal. Chem.* **33**: 363-364 (1961).