THIN-LAYER CHROMATOGRAPHIC SEPARATION AND COLORIMETRIC ANALYSIS OF BARLEY OR MALT LIPID CLASSES AND THEIR FATTY ACIDS*

D. E. WALSH**, O. J. BANASIK*** AND K. A. GILLES***

Department of Cereal Technology, North Dakota State University of Agriculture and Applied Science, Fargo, N.D. (U.S.A.)

(Received May 25th, 1964)

In the past few years, techniques of thin-layer chromatography have been developed which enable the researcher to separate components of complex lipid mixtures. The technique employs a powdered adsorbent (silica gel "G") affixed rigidly to a glass plate, 15 cm × 15 cm × 0.5 cm[1].

For use in lipid research, two types of thin-layer techniques, adsorption and reverse phase chromatography, have been developed[2]. In adsorption chromatography, a sample is adsorbed on a solid phase of silica acid and a suitable solvent allowed to elute and separate the components. In reverse phase chromatography, a sample is adsorbed in a liquid phase of silicone supported on a silicic acid medium and a suitable solvent is allowed to elute and separate the components. The technique of thin-layer adsorption chromatography has been applied to the fractionation of lipids into their general lipid classes; the technique of reverse phase thin-layer chromatography has been employed for the separation of fatty acids[2]. Used together, adsorption and reverse phase chromatography on thin-layers facilitated detailed analysis of lipid materials.

This paper describes colorimetric procedures for the quantitative estimation of barley or malt lipid classes and their fatty acid composition separated by adsorption and reverse phase thin-layer chromatography.

EXPERIMENTAL

Apparatus and colorimetric procedures

Preparation of lipid extracts

Finely ground barley or malt was extracted with petroleum ether (30–60° b.p.) in a Soxhlet apparatus for a 48 h period. After extraction the petroleum ether solution of lipids was removed and reduced in volume to 10 ml under an atmosphere of nitrogen. The last traces of solvent were removed in a vacuum oven at 20°. All

---

* Published with the approval of the Director of the Agricultural Experiment Station, North Dakota State University, Fargo, N.D. as Journal Series No. 48. The research described is a partial fulfilment for a M. S. degree by D. E. WALSH.

** Research Chemist, Beech-Nut Co., Canajoharie, N.Y.

*** Associate Professor and Professor, respectively, Department of Cereal Technology, North Dakota State University, Fargo, N.D.

samples were then stored under an atmosphere of nitrogen at a temperature of —10° until they were needed for analysis.

Preparation of the plates

Three different types of chromatoplates were used to achieve the separation: thin- and thick-layer adsorption plates, and reverse phase partition plates.

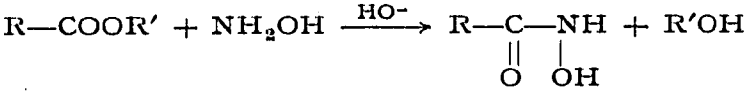
Thin-layer adsorption, used for lipid fractionation, consisted of glass plates (20 cm × 20 cm × 0.4 cm) coated with a thin-layer (250 μ) of silica gel "G". The layer was applied as a slurry consisting of 60 g of Merck silica gel "G" suspended in 100 ml of water and dried in a forced air oven for 2 h at 130°. Thick-layer chromatograms, used for preparative purposes, consisted of glass plates coated with 1000 μ of a silica gel "G".

Reverse phase partition chromatoplates which were used for separation of methyl esters of saturated fatty acids, consisted of glass plates coated with a layer (500 μ) of 10 % siliconized silica gel "G". The gel was applied as a slurry containing silica gel "G" (60 g) suspended in a mixture of diethyl ether (10 ml) and Dow Corning 200 fluid (6 ml). The plate was dried in a forced air oven for 2 h at 130°.

Hydroxamic acid method

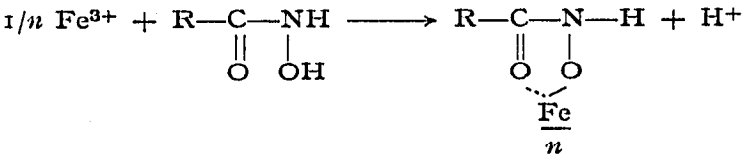
Two colorimetric assay procedures were modified to provide quantitative methods for determination of lipids separated on thin-layer chromatoplates. The hydroxamic acid and the dichromic acid color tests were used.

Lipids that contained ester groups will react quantitatively with hydroxylamine to form hydroxamic acids which form a dark blue chelated complex in the presence of ferric ion. GODDU, LEBLANC AND WRIGHT[3] proposed the following equation for the hydroxamic color reaction:



(1)

Ester + Hydroxylamine ⟶ Hydroxamic acid



(2)

(Blue chelated complex)

The following stock solutions, used in the hydroxamic acid reaction, were stored at 5° to minimize degradation:

1. Ferric perchlorate solution. Five grams ferric perchlorate were dissolved in 10 ml of 70 % perchloric acid and 10 ml of water. The solution was diluted to 100 ml with cold 95 % ethanol.

2. Alcoholic NaOH solution. 6 % NaOH in 95 % ethanol.

3. Hydroxylamine solution, 4 % hydroxylamine hydrochloride in 95 % ethanol.

The ferric perchlorate reagent and the hydroxylamine reagent were prepared immediately prior to use. The ferric reagent was prepared by adding 4 ml of the stock ferric perchlorate solution to 3 ml of the 70 % perchloric acid and diluting to 100 ml with 95 % ethanol.

The hydroxylamine reagent was prepared by adding 1 volume of 4 % hydroxylamine hydrochloride in ethanol to 2 volumes of 6 % sodium hydroxide, centrifuging and decanting to remove the precipitated reactant, NaCl.

Lipid samples to be evaluated were removed from thin-layer chromatograms by scraping the marked area of silica gel "G" into a test tube. The silica gel did not interfere with the color reactions, but had to be removed by centrifugation before colorimetric readings could be taken. The colorimeter was adjusted to zero with a blank which contained a similar amount of silica gel "G" that had been scraped from the same chromatogram and treated in the same manner as the sample.

One ml of the hydroxylamine reagent was added to a test tube which contained the lipid to be evaluated. The reagent and sample were mixed thoroughly, heated in a boiling water bath for 1 min, removed from the bath and allowed to cool for several minutes. Three ml of the ferric reagent was added; to remove the silicagel, the reaction mixture was shaken vigorously, centrifuged for 2 min at 3600 r.p.m., decanted into a colorimeter tube, and centrifuged again for 2 min at 3600 r.p.m. The optical density of the mixture was read at 532 mμ in a Spectronic 20 colorimeter. Care was taken to assure that each reading was taken 15 min from the time of the addition of the ferric reagent. The optical density values were then converted to micrograms by the use of standard curves.

Dichromic acid method

The hydroxamic acid colorimetric procedure, specific for ester groups, was not suitable for use with lipids that contained no ester linkages. For these lipids, the dichromic acid colorimetric procedure of JOHNSON[4] was modified and used with thin-layer chromatography.

The dichromic acid reagent, which was stored in a glass stoppered bottle, was prepared by dissolving 5 g of $Na_2Cr_2O_7 \cdot H_2O$ in 20 ml of water and diluting to 1 l with 95 % H_2SO_4.

Lipid samples to be evaluated were scraped from thin-layer chromatograms and contained silica gel "G". The silica gel did not interfere with the color reaction but had to be removed by centrifugation before colorimetric reading could be taken.

One ml of the dichromic acid reagent and 1 ml of water were added to a test tube which contained the silica gel and lipid to be evaluated. The reagent and sample were mixed thoroughly, heated in a boiling water bath for 30 min, removed from the bath and allowed to cool to room temperature. Five ml of water were added; to remove the silica gel, a procedure identical to that used for the hydroxylamine acid test was followed. The optical density of the reaction mixture was read at 440 mμ in a Spectronic 20. The colorimeter was adjusted to an arbitrary optical density of 0.7 with a blank which contained a similar amount of silica gel that had been scraped from the same chromatoplate and treated in the same manner as the sample. The optical density was converted to micrograms by the use of standard curves.

Chromatographic procedures

Lipid fraction preparation

Samples of each lipid fraction were prepared by the use of thick-layer chromatography. The method of MALINS AND MANGOLD[5] was modified for use with 1000 μ thick-layer chromatoplates. The application of lipids to the preparative plate used twenty closely spaced points of application as shown in Fig. 1. About 2.5 mg of lipids were

applied to each of the twenty points; thus, a total of 50 mg was fractionated on each plate.

Thick-layer chromatograms were developed with a solvent system of petroleum ether (60–70° b.p.), diethyl ether, and acetic acid in a volume ratio of 90:10:1.5, respectively[5]. After development, the plates were removed from the chromatographic tanks and dried. The location of the four lipid fractions was determined with short wave ultraviolet light and the areas of the chromatograms which contained the lipid fractions were scraped into sintered glass filters of medium porosity. The lipid fractions were eluted from the silica gel into weighing flasks with 5:1 mixture of chloroform and methanol. Weights of each lipid fraction were determined gravimetrically after removal of the solvent by evaporation in a stream of nitrogen. These lipid fractions were used then to determine colorimetric standard curves.

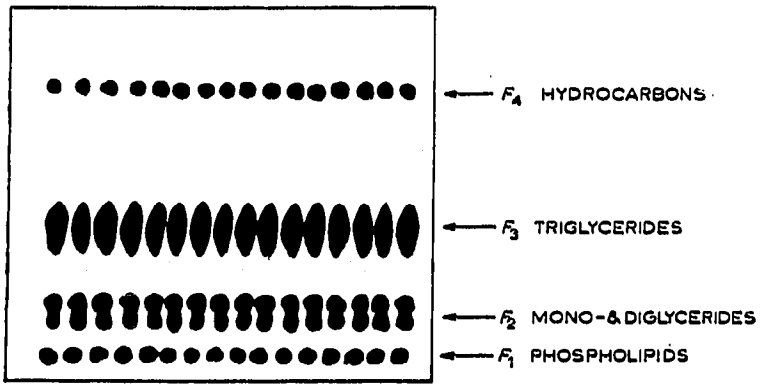


Fig. 1. Thick-layer preparative chromatogram for lipid fractions.

Standard curves

To prepare the standard curves each lipid fraction that was obtained from the thick-layer procedure was rechromatographed by the thin-layer technique in 20 different concentrations (10 μg to 300 μg). After development, the spots were located with short wave ultraviolet light, removed, and placed in test tubes.

The hydroxamic colorimetric method was applied to fractions F_1, F_2, and F_3 while the dichromic acid colorimetric method was applied to fraction F_4, the hydrocarbons. Four standard curves, one for each fraction, were prepared by graphing optical density *versus* micrograms of lipids, one of which is shown in Fig. 2.

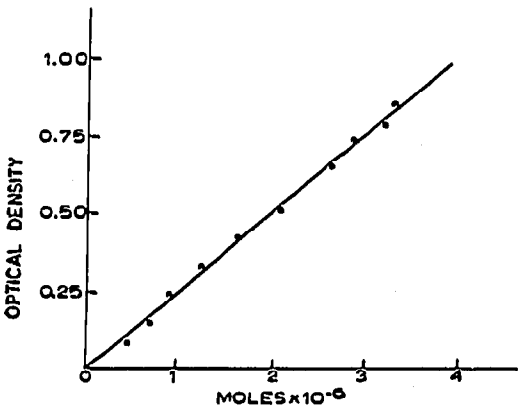


Fig. 2. Standard curves for colorimetric analysis of fatty acid methyl esters.

Inasmuch as all methyl esters or their mercuric acetate adducts gave the same color reaction per micromole[6], a standard curve for methyl esters was determined with methyl oleate (Fig. 2). Pertinent data for all standard curves are contained in Table I.

TABLE I

STANDARD CURVE CORRELATIONS AND REGRESSION EQUATIONS

Standard curve	*Correlation coefficient*	*Regression equation**
F_1 = phospholipids	0.980	$Y = 3.55 \cdot 10^{-3} + 5.84 \cdot 10^{-4} X'$
F_2 = mono, diglycerides	0.982	$Y = 1.61 \cdot 10^{-2} + 3.89 \cdot 10^{-4} X$
F_3 = triglycerides	0.996	$Y = 4.47 \cdot 10^{-4} + 4.2 \cdot 10^{-4} X$
F_4 = hydrocarbons	0.990	$Y = 0.69 - 2.14 \cdot 10^{-3} X$
Methyl oleate	0.985	$Y = 1.80 \cdot 10^{-3} + 0.327 X'$

* Y = optical density; X = micrograms of lipid; X' = micromoles of ester.

Lipid fraction estimation

The extractable lipids of barley and malt were chromatographically fractionated by the method of MALINS AND MANGOLD[5]. This method, as shown in Fig. 3, fractionated the lipids into four broad classes of compounds: the phospholipids (F_1), the mono- and diglycerides (F_2), the triglycerides (F_3), and the hydrocarbons (F_4).

The whole lipids of barley and malt were applied with a micropipet to the thin-layer adsorption chromatoplate about 2 cm from the bottom of the plate. Each chromatoplate was developed with the solvent mixture of petroleum ether (60–70° b.p.), diethyl ether, and acetic acid in a volume ratio of 90:10:1.5, respectively[5].

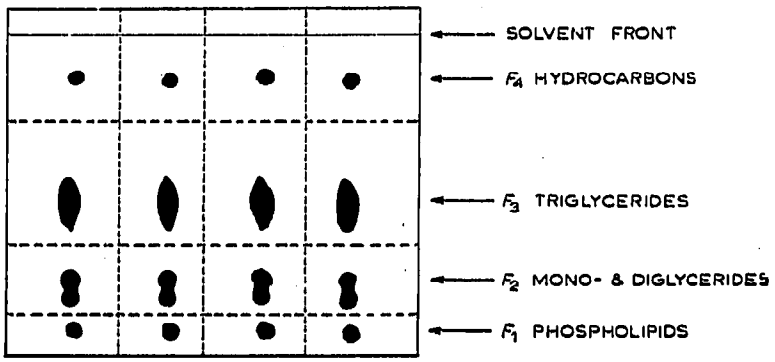


Fig. 3. Thin-layer chromatogram for quantitative analysis of lipid fractions.

The location of the components on the chromatogram was determined by the use of ultraviolet light. No indicator was necessary because each lipid class showed a small amount of fluorescence.

The lipids were removed from the plate by simply scraping into a test tube that area of the chromatogram which contained the desired component. Fractions F_1, F_2,

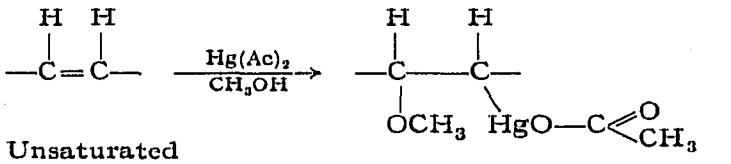
and F_3 were assayed quantitatively by the use of the hydroxamic acid color test for esters[3]. Fraction F_4, containing mostly hydrocarbons, was assayed quantitatively by the use of the dichromic acid color test for organic compounds[4].

Fatty acid analysis

Fifty μg of crude lipids of barley and malt were fractionated by the thick-layer procedure. Each lipid fraction was removed from plates by scraping, placed under 10 ml of methanol, and transesterified to methyl esters by the boron trifluoride method described by METCALFE AND SCHMITZ[7]. After cooling, the methyl esters were removed from the alcohol solution by petroleum ether (30–60° b.p.) extraction and dried with a nitrogen stream.

Due to their polarity, the critical pairs, methyl oleate and methyl palmitate, methyl myristate and methyl linolate, had identical R_F values and could not be separated in this form chromatographically. However, by the use of mercuric acetate addition compounds, the unsaturated esters were separated from the saturated esters[2] in a two step chromatographic procedure to give complete separation of all methyl esters for subsequent colorimetric analysis.

The unsaturated methyl esters were converted to their mercuric acetate addition compounds by heating the ester with 15 % mercuric acetate in methanol for 5 min according to the following equation[8]:



Unsaturated ester

Mercuric acetate adduct

The mercuric acetate adducts and saturated methyl ester were removed from the methanol solution by chloroform and water extraction and applied to a thin-layer absorption chromatoplate. At the center and at both edges of the plate, an indicator mixture of mercuric acetate adducts of oleic, linoleic, and linolenic methyl esters was applied. The plate, as shown in Fig. 4, was developed with a solvent system of diethyl ether and petroleum ether (30–60° b.p.) in a ratio of 1:4 v/v to separate the saturated esters from the mercuric adducts. The saturated esters

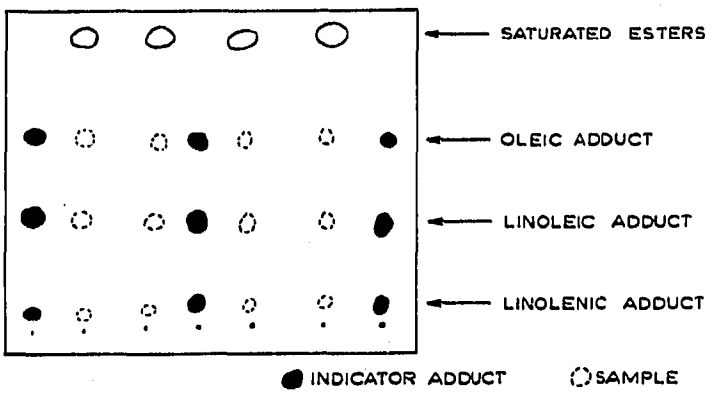
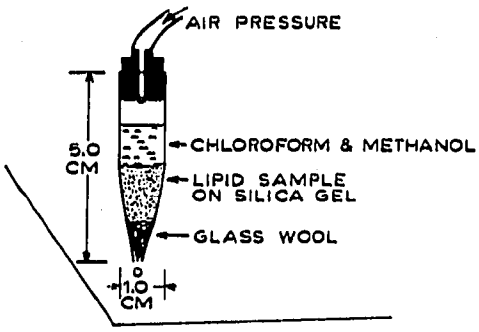


Fig. 4. Thin-layer chromatographic separation of mercuric acetate adducts of unsaturated fatty acids.

were scraped from the solvent front and were reserved for separation on another chromatoplate.

The adsorption chromatogram, which now contained only the mercuric acetate adducts of unsaturated fatty esters, was redeveloped with a solvent system of *n*-propanol and acetic acid (100:1, v/v). The position of each adduct was determined by spraying only the indicator areas with 2,7-dichlorofluorescein, which caused mercuric adducts to become visible as purple spots. Using this as a means of locating the separated adducts of the lipid fractions, the identified areas were scraped into test tubes for colorimetric analysis.

The saturated esters that were removed from the solvent front were placed in an applicator tube (Fig. 5). A methanol:chloroform (1:5, v/v) solution was used to elute the saturated esters from the applicator tube onto a reverse phase siliconized chromatoplate which was fitted subsequently with a 3 inch paper wick (Whatman No. 1) as shown in Fig. 5. The wick provided a means of moving the solvent front beyond



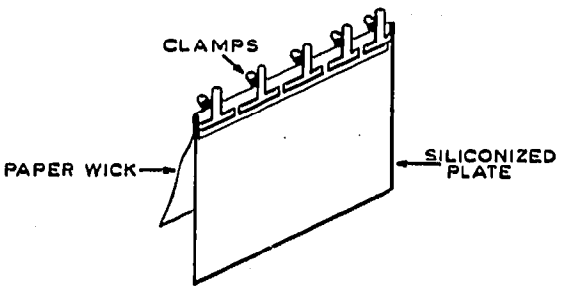


Fig. 5. Applicator tube for transferring saturated esters and siliconized chromatoplate with a paper wick.

silica gel layer thus effecting a better separation of the saturated fatty acid esters. In addition, indicator samples of the unsaturated critical pairs were applied at both edges and the center of the plate and developed with a solvent system of acetonitrile, acetic acid, and water (70:10:25, v/v/v). When the solvent reached the end of the 3 inch wick, the plate was removed and placed in an iodine chamber to indicate the location of the saturated esters by iodine adsorption of the critical pairs (Fig. 6). Each saturated ester was scraped into individual test tubes and quantitatively evaluated by the hydroxamic acid colorimetric procedure.

Optical density readings, taken from the hydroxamic colorimetric procedure,

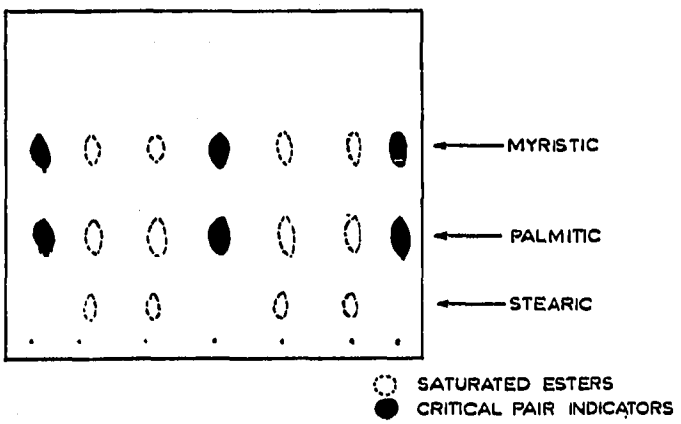


Fig. 6. Reverse phase chromatogram of saturated fatty acid esters.

were converted to micromoles of ester by use of the standard curve determined for methyl oleate (Fig. 2).

DISCUSSION

When used together, the lipid fractionation and fatty acid analytical procedures gave detailed quantitative analysis of lipids. The standard error, based on the colorimetric standard curves for the lipid fractionation method, was ± 0.35 %. The standard error, based on the colorimetric standard curves for the fatty acid analytical procedure, was ± 0.24 %.

These standard errors indicated that this method was valuable as a tool for quantitative investigation of complex lipid mixtures. Table II shows colorimetric analysis of separated barley lipid fractions. By employing a series of different concentrations, the data show that an application of 1400 to 2200 μg was the most efficient range for evaluation. The lower concentrations were less effective because

TABLE II

RECOVERY OF BARLEY LIPID FRACTIONS DETERMINED COLORIMETRICALLY

Lipid applied (μg)	*Fraction weight*					*Recovery* (%)
	F_1 (μg)	F_2 (μg)	F_3 (μg)	F_4 (μg)	*Total wt. recovered* (μg)	
800	62.44	35.74	522.57	41.99	662.74	82.8
1000	79.57	35.74	713.22	74.62	903.15	90.3
1200	113.83	61.43	832.27	88.61	1096.14	91.3
1400	130.95	87.12	1094.18	111.92	1424.17	101.7
1600	130.95	112.82	1237.03	125.90	1606.70	100.4
1800	148.08	138.51	1427.51	177.18	1891.28	105.1
2000	165.21	164.20	1546.56	191.17	2067.14	103.4
2200	182.34	189.89	1594.18	233.13	2199.54	100.0
					Average	98.8

of the variability caused by lower amounts of lipid contained in fractions 1, 2 and 4. This caused small changes in optical density at the low end of the scale which were difficult to estimate.

Table III further illustrates the precision of the colorimetric determination

TABLE III

RECOVERY OF METHYL OLEATE DETERMINED COLORIMETRICALLY

Methyl oleate applied (μg)	*Methyl oleate determined* (μg)	% *Recovered*
0.6	0.570	95.0
0.8	0.723	90.4
1.0	1.012	101.2
1.2	1.285	107.1
1.4	1.320	94.3
1.6	1.694	105.9
1.8	1.694	94.1
2.0	1.797	89.9
3.0	2.921	97.4
4.0	4.045	101.1
	Average	98.1

for the six fatty acids found in the hydrolyzed lipid fractions. A concentration range of 0.6 to 4.0 μg produced the most accurate results for all six fatty acids. Beyond these limits, the results were more erratic and less dependable.

ACKNOWLEDGEMENTS

The authors wish to thank the Malting Barley Improvement Association of Milwaukee, Wisc. for grants towards the support of this research.

SUMMARY

A method for detailed quantitative analysis of barley and malt lipids was developed. Extractable lipids were fractionated into four broad classes of compounds: phospholipids, mono- and diglycerides, triglycerides and hydrocarbons. The hydroxamic acid colorimetric test for ester groups was modified to quantitatively measure lipids that contained ester groups and the dichromic acid test for organic compounds was modified to quantitatively measure lipids, such as hydrocarbons, which contained no ester group. Standard curves were prepared for the four lipid fractions by the use of a thick-layer preparative procedure. Each lipid fraction can be analyzed for fatty acid composition by a thin-layer chromatographic method which separates fatty acids in the form of saturated fatty acid methyl esters and mercuric acetate adducts of unsaturated fatty acid methyl ester. The method gave quantitative results for both fatty acid analysis and lipid fraction analysis.

REFERENCES

1 J. E. MEINHARD AND N. F. HALL, *Anal. Chem.*, 21 (1949) 185.
2 H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
3 R. F. GODDU, N. F. LEBLANC AND C. M. WRIGHT, *Anal. Chem.*, 27 (1955) 1251.
4 J. JOHNSON, *J. Biol. Chem.*, 181 (1941) 707.
5 D. C. MALINS AND H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
6 F. SNYDER AND N. STEPHENS, *Biochim. Biophys. Acta*, 34 (1959) 244.
7 L. D. METCALFE AND A. A. SCHMITZ, *Anal. Chem.*, 33 (1961) 363.
8 E. JANTZEN AND H. ANDREAS, *Chem. Ber.*, 92 (1959) 1427.