Journal of Chromatography, 104 (1975) 123–133

CHROM. 7799

RELATIONSHIP BETWEEN DENSITOMETRIC PEAK AREA AND CONCENTRATION OF LIPID IN THE QUANTITATIVE ANALYSIS OF A LIPID MIXTURE SEPARATED BY THIN-LAYER CHROMATOGRAPHY

TERESA CASTELLANI, POUL KJELGAARD-NIELSEN and JACOB WOLFF-JENSEN
Department of Dermatology and Central Laboratory, The Finsen Institute, Copenhagen (Denmark)
(First received May 3rd, 1974; revised manuscript received July 5th, 1974)

SUMMARY

A mixture of the neutral lipids squalene, cetyl stearate, triolein, oleic acid and cholesterol was separated by thin-layer chromatography and the components were measured quantitatively by photodensitometry.

Several empirical expressions relating densitometric peak area (A) and lipid concentration (C) were subjected to statistical analysis, and the relationship $A = k \cdot C^B$ is linear in the logarithmic form $\log A = B \cdot \log C + \log k$ within 95% confidence limits on the observed results.

Exponent B, representing the proportionality factor in the expression relating the relative differential changes in peak area and concentration, is constant for lipids studied on the same chromatoplate, whereas the constant k is lipid dependent. All standard measurements per plate are therefore utilized for estimating a common B value, which gives greater accuracy than in the individual cases.

The plate capacity for measurements of unknown lipid concentrations may consequently be increased, as a common B value, based on a reduced number of standard measurements, may be estimated with the same accuracy as an individual B value, based upon the non-reduced number of standard measurements.

Equations are given for the calculation of B and k and for the determination of unknown concentrations with appropriate confidence intervals.

INTRODUCTION

Thin-layer chromatography (TLC) is a widely used method for separating complex biological mixtures, including lipids[1,2]. Methods for the quantitative determination of the individual separated lipid components fall into two categories:

(1) removal of the components from the adsorbent and their measurement by spectroscopy[3] or gravimetry.

(2) keeping the components in the adsorbent, and determination of the components in the spots either by direct measurement[4] or by photodensitometry after they have been located[5].

The former method has several drawbacks: it is difficult to remove the com-

ponents completely from the adsorbent; recovery rates as low as 50% have been reported[6]; there is a risk of extracting impurities; gravimetric determination is not much used, as the amounts of the substances involved are often so small that special balances are required.

The second method is widely used, as it eliminates the above problems. It is rapid and sensitive, provided that the examiner exerts the utmost care in applying lipid to the chromatoplate, centering the spots in relation to the light source, and finally in determining the areas[7]. Factors such as the thickness and degree of moisture of the adsorbent[7], the method used in light adsorption[8] and the process required to locate the components on the chromatoplate (charring in the case of lipids) are of decisive importance to the accuracy of the analysis[9–11]. Lastly, the reliability of the analysis is also influenced by the shape and demarcation of the spots as well as the distribution of components within the spot[12].

In any indirect measuring method, a linear calibration curve affords marked advantages:

(1) It can be plotted graphically with far greater accuracy than a non-linear curve.

(2) Conversion of ordinate values by means of the curve into estimated abscissa values can be achieved with the same accuracy throughout the range.

(3) If statistical methods, *e.g.*, regression analysis, are used for deriving the parameters of the calibration curve, estimations such as those mentioned under item (2) can be performed far more rapidly with the linear model. Furthermore, methods for testing the validity of the model and of assessing the uncertainty of the estimates are standard practice[13,14].

The present study, therefore, was designed to achieve the transformation of peak areas, determined from a densitometric curve, and lipid concentrations that gives a linear calibration graph.

METHOD

Preparation of chromatoplates

A solution containing 9 g of 99.5% ammonium sulphate, 7 ml of 99.5% methanol and 93 ml of distilled water was prepared, and to 71 ml of this solution 30 g of Kieselgel H (Merck, Darmstadt, G.F.R.) were added. The mixture was allowed to stand for 1 h, smeared on cleaned glass plates (20 × 20 cm) to a thickness of 0.3 mm. Five plates were prepared at a time using a Shandon Unoplan spreader, dried at room temperature for 45 min and then at 120° for 30 min, cooled and stored in a Scheibler desiccator over silica gel blue indicator. Before use, the coated plates were placed in chambers containing diethyl ether for 12 h so as to remove organic impurities. After air drying, the adsorbent layer was divided into 12 lanes, each 13 mm wide. Between these lanes, the gel was scraped off in 2-mm wide channels so as to prevent sideways movement of the components in the course of separation.

Immediately before applying the samples, the plates were reactivated by heating them at 90° for 30 min.

Standard solutions

A standard solution was prepared for each individual pure reference lipid,

using squalene (98%), cetyl stearate (95–99%), triolein (*ca.* 99%), oleic acid (*ca.* 99%), cholesterol (99%), and toluene for analysis (minimum 99.5%) (Merck). Suitable mixtures of these lipids were obtained by accurate measurement of the standard solutions.

From these mixtures, four solutions were prepared for constructing the calibration graphs.

The concentrations of the final standard solutions were as follows: squalene, 0.04–0.16 g per 100 ml; cetyl stearate, 0.06–0.24 g per 100 ml; triolein, 0.04–0.16 g per 100 ml; oleic acid, 0.02–0.08 g per 100 ml; and cholesterol, 0.01–0.04 g per 100 ml.

The standard solutions were stored in a refrigerator at 0–1°.

Technique of application

The standard solutions were applied with a 20-μl capillary pipette (Carlsberg pipette), 1½ cm from the lower edge of the plate in successive, densely placed, small spots ($N = 5$), forming a narrow band through the entire cross-section of the lane parallel to the lower edge of the plate. By this technique, the width of the developed spots was kept constant, and this afforded a constant ratio between the slit length of the scanner and the width of the spot. This technique gives greater precision than application in circular spots by the photodensitometric determination[15]. Care should be taken not to damage the gel during the application. After application, the plates were dried at room temperature for half an hour so as to avoid washing out during the development.

All standard solutions were applied to the same chromatoplate, and the determination on each solution was carried out in triplicate (12 lanes on each plate).

Development

A system of multiple solvents was used consisting of:

(1) *n*-hexane (separation to a limit of 17 cm above the site of application);

(2) benzene (separation to a limit of 15.5 cm above the site of application);

(3) *n*-hexane–diethyl ether–acetic acid (70:30:1) (separation to a limit of 9 cm above the site of application).

Between each separation, the plates were dried at room temperature for 10 min. Into solvent chambers, filter-paper was placed so as to attain a quicker equilibrium, which is considered to have been established after half an hour. There should be fresh mixtures in the chambers before each experiment.

Charring

The chromatoplates were charred in an incubator (Pye Series 104 Chromatograph) at 250° for 1 h and then gradually cooled. Most workers used a corrosive agent, such as sulphuric or chromic acid, but owing to the difficulties in applying these reagents (evenly distributed over the entire plate) and the health hazards in using highly corrosive agents in a freely dispersed form, we used the technique advocated by Walker[16]. In his technique, a solution of ammonium sulphate is incorporated in the silica gel such that on heating sulphuric acid is generated, evenly dispersed throughout the layer.

Scanning

The chromatoplates were scanned with a Chromoscan photodensitometer

(Thin-Layer Scanner, Type A), slit size 10 × 0.5 mm. The apparatus should be adjusted so as to afford a light transmission of 100% when the light passes through the chromatoplate in an area outside the developed spots.

The area beneath the densitometric curve was determined by two methods, partly by planimetry (OTT Compensation Planimeter No. 19 for measuring unit) and partly by triangulation.

RESULTS AND DISCUSSION

The following models were investigated:

Model 1: $A = B_1 \cdot C + k_1$
Model 2: $A^2 = B_2 \cdot C + k_2$
Model 3: $H \cdot L = B_3 \cdot C + k_3$
Model 4: $A^2/(H \cdot L) = B_4 \cdot C + k_4$
Model 5: $A^2/H = B_5 \cdot C + k_5$
Model 6: $A^{1.5} = B_6 \cdot C + k_6$
Model 7: $A = k_7 \cdot C^{B_7}$

where

A = area between densitometric curve and baseline determined by planimetry (peak area).

H = maximum ordinate of densitometric curve.

L = basic length of densitometric curve.

C = lipid concentration.

B_i, k_i = function parameters, $i = 1,2 \ldots 7$.

Model 1 is the simple linear relation, model 2 the relation demonstrated by Frei[8] and model 3 corresponds to model 1, differing only in that the area is calculated by triangulation, not by planimetry. Models 4–6 are attempts at relating the spot volume and lipid concentration. Model 4 presupposes that the axis of the lipid spot at right-angles to the direction of scanning is constant, whereas model 5 presupposes constancy of the ratio between the axis of the lipid spot at right-angles to and in the direction of the scanning, respectively. In model 6, the quadratic dimension is converted into a cubic dimension.

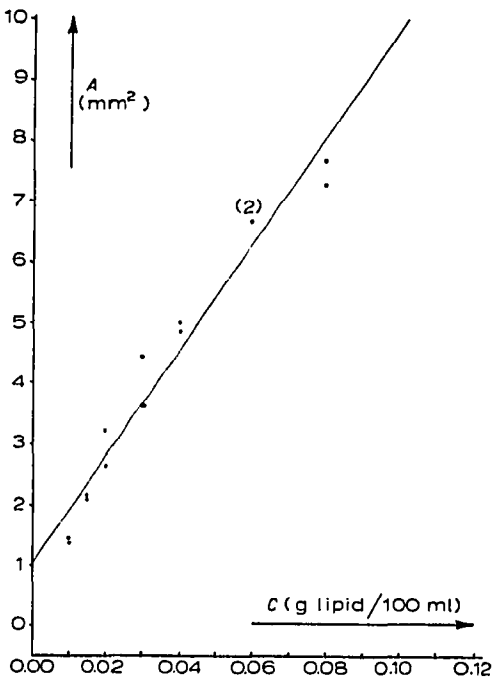
In experiments using the lipids squalene, cetyl stearate, triolein, oleic acid and cholesterol, it was soon apparent that models 3–5 were not acceptable, whereas models 1, 2 and 6 were acceptable in several, but not all, cases. In most cases, moreover, the constant k_1 did not differ significantly from zero.

Thereafter, model 7 was formulated, being a generalization of models 1, 2 and 6 for $k_1 = k_2 = k_6 = 0$, these models arising from model 7, provided that B_7 assumes values of 1, 1/2 and 2/3, respectively. Model 7 does not presuppose a pre-fixed value for the exponent of A (corresponding to the reciprocal of B_7). Accordingly, it must be assumed that this model will be of wider validity. The investigations confirmed this assumption, which was tested after logarithmic transformation on both sides of the equation:

$$A = k \cdot C^B$$

which gives the simple linear model

$$\log A = B \cdot \log C + \log k$$



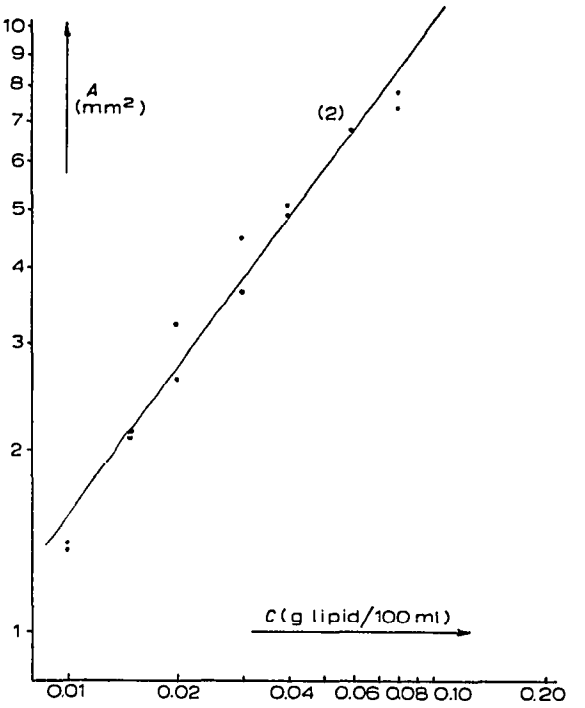


Fig. 1. Peak area *versus* concentration (linear scale) for squalene. Regression line: $A = 86.54 \cdot C + 1.03$.

Fig. 2. Peak area *versus* concentration (logarithmic scale) for squalene. Regression curve: $A = 63.60 \cdot C^{0.81}$.

If there is linear dependence between dependent and independent variable, the correlation between the two is, of course, highly significant. Also, the means of the dependent variable for the different levels of the independent variable are permitted to vary only at random around the regression line.

The use of an objective criterion is necessary in many cases, as a graphical depiction is usually not sufficiently varied. An example is presented in Figs. 1 and 2, where the measured areas for squalene are traced as a function of known concentrations (grams of lipid per 100 ml). Fig. 1 is plotted on a linear scale, and the empirical regression line

$$A = 86.54 \cdot C + 1.03$$

is shown. In Fig. 2 the same data are plotted on a logarithmic scale, and the empirical regression line corresponding to the equation

$$A = 63.60 \cdot C^{0.81}$$

is shown.

The result of the linearity test in this case is that the data in Fig. 1 cannot be

TABLE I

LIPID CONSTANTS AND *B* VALUES

Lipid	*Regression lines*			
	Individual		*Parallel*	
	k	*B*	*k*	*B*
Squalene	44.97	0.691	43.57	0.678
Cetyl stearate	20.48	0.651	21.62	
Triolein	34.81	0.644	37.84	
Oleic acid	41.14	0.714	36.81	
Cholesterol	51.76	0.691	49.34	

represented by the empirical regression line, whereas those in Fig. 2 can be so represented in statistically acceptable terms.

A statistical analysis such as the one performed cannot prove that a postulated relationship between peak area and lipid concentration is true, but it can indicate the

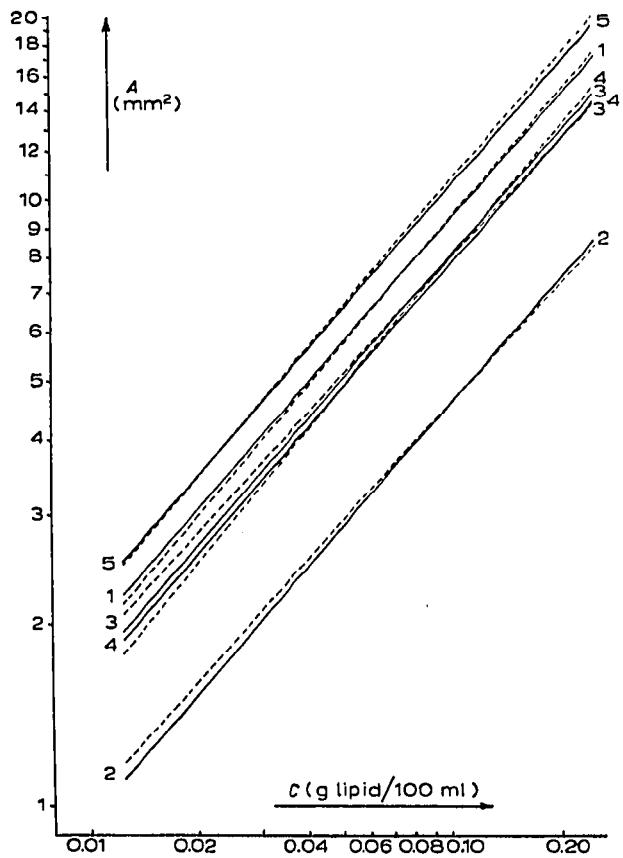


Fig. 3. Peak area *versus* concentration (logarithmic scale). 1, Squalene; 2, cetyl stearate; 3, triolein; 4, oleic acid; 5, cholesterol. Full lines: parallel regression curves. Broken lines: individual regression curves.

likelihood that it holds. On the basis of a 95% significance level, the postulated relationship

$$A = k \cdot C^B$$

could not be rejected, unlike all other postulated relations.

If this expression is differentiated with respect to C and both sides of the equation are divided by A, the result is

$$dA/A = B \cdot (dC/C)$$

The relation

$$A = k \cdot C^B$$

on the other hand, indicates that the relative differential change in the peak area is directly proportional to the relative differential change in the concentration, with B as the proportionality factor. Under special circumstances, B may assume a value of unity, and in that case model 1,

$$A = k \cdot C$$

will apply, but in most cases B will be less than unity.

The investigations revealed, moreover, that the exponent B is constant for the lipids used on the same chromatoplate, whereas the constant k is lipid dependent. Owing to this fact, the regression lines for the individual lipids are depicted logarithmically as parallel lines.

The above statement is based upon the fact that a variance homogeneity exists within the individual lipids (Bartlett test) and that the regression coefficients (B) do not differ significantly from each other (F-test).

The advantage of formulating a model in which B is lipid independent is that the calculation of B as well as of the accidental experimental error is then based upon all observations, which gives considerably better estimates.

As an example, Table I gives the lipid constants and B values calculated for each lipid separately and those calculated for all lipids together. The corresponding regression lines are plotted in Fig. 3.

Calculation of k and B for the individual lipids is carried out by simple linear regression analysis with one dependent variable y (= log A) and one independent variable x (= log C). In the case where the B value is to be common to all lipids (parallel regression lines), a multiple regression analysis is carried out:

$$y = \bar{y} + B(x - \bar{x}) + \sum_{j=1}^{n} L_j (P_j - PM) \qquad (1)$$

As before, y corresponds to the logarithm of the peak area and x to the logarithm of the lipid concentration. $\bar{y}$ is the mean of all $y = \left(\sum_{j=1}^{N} y_j\right)/N$ and $\bar{x}$ is the

mean of all $x = \left(\sum_{j=1}^{N} x_j\right)/N$, where N is the number of observations for all lipids together. $P_1, P_2, \ldots P_n$ are a set of non-stochastic variables (n = number of lipids -1) which can only assume a value of 0 or 1. For measurements made for lipid number 1, P_1 will be 1, whereas all other P values are 0, and *vice versa*. $L_1, L_2, \ldots L_n$ are the regression coefficients, and PM is the common mean of the P values (= 1/number of lipids).

In eqn. 1, it is presupposed that the number of observations per lipid (NS_j) is constant. Provided that this is not so, PM is replaced with $\bar{P}_j = NS_j/N$.

The above multiple regression model will result in a common B value, and the k value for the jth lipid is obtained by inserting into the model the values $x = 0$, $P_j = 1$ and all other P values = 0, and lastly by taking the antilogarithm of the resulting value.

TABLE II

CONCENTRATIONS CALCULATED ON THE BASIS OF THE INDIVIDUAL REGRESSION LINES

Lipid	*Concentration (g lipid/100 ml)*		
	Known	*Estimated*	*90% confidence interval*
Squalene	0.160	0.151	0.136 –0.168
	0.120	0.123	0.111 –0.136
	0.080	0.0858	0.0799–0.0944
	0.040	0.0384	0.0342–0.0432
Cetyl stearate	0.240	0.235	0.194 –0.284
	0.180	0.179	0.150 –0.214
	0.120	0.126	0.107 –0.150
	0.060	0.0585	0.0475–0.0721
Triolein	0.160	0.149	0.130 –0.170
	0.120	0.126	0.111 –0.143
	0.080	0.0854	0.0755–0.0967
	0.040	0.0383	0.0329–0.0446
Oleic acid	0.080	0.0770	0.0698–0.0849
	0.060	0.0631	0.0575–0.0692
	0.040	0.0400	0.0365–0.0437
	0.020	0.0198	0.0178–0.0221
Cholesterol	0.040	0.0380	0.0345–0.0420
	0.030	0.0320	0.0291–0.0351
	0.020	0.0200	0.0183–0.0219
	0.010	0.0099	0.0089–0.0110

An impression of the reproducibility can be gained from Tables II and III. For each of the five lipids used, these tables give the measured area value for four different, known concentration levels of each lipid (three measurements at each level). Thereafter, the concentration was calculated partly on the basis of the five individual regression lines (Table II) and partly on the basis of the five parallel regression lines

TABLE III

CONCENTRATIONS CALCULATED ON THE BASIS OF THE PARALLEL REGRESSION LINES

Lipid	*Concentration (g lipid/100 ml)*		
	Known	*Estimated*	*90% confidence interval*
Squalene	0.160	0.153	0.137 –0.170
	0.120	0.124	0.111 –0.138
	0.080	0.0857	0.0771–0.0953
	0.040	0.0378	0.0338–0.0423
Cetyl stearate	0.240	0.229	0.206 –0.256
	0.180	0.177	0.159 –0.197
	0.120	0.127	0.114 –0.141
	0.060	0.0604	0.0541–0.0675
Triolein	0.160	0.145	0.130 –0.161
	0.120	0.124	0.111 –0.138
	0.080	0.0856	0.0770–0.0951
	0.040	0.0400	0.0358–0.0446
Oleic acid	0.080	0.0792	0.0711–0.0883
	0.060	0.0642	0.0577–0.0715
	0.040	0.0397	0.0358–0.0442
	0.020	0.0190	0.0170–0.0212
Cholesterol	0.040	0.0384	0.0345–0.0428
	0.030	0.0322	0.0289–0.0358
	0.020	0.0199	0.0179–0.0222
	0.010	0.0097	0.0087–0.0109

(Table III). The results in Tables II and III were derived as follows:

(1) *Individual regression lines*

$y = B \cdot x + k = \bar{y} + B(x - \bar{x})$

where $k = \bar{y} - B \cdot \bar{x}$. The equation is solved with respect to x:

$$x = \frac{y - \bar{y}}{B} + \bar{x}$$

whereupon the "unknown" lipid concentration is obtained by taking the antilogarithm of x. If y is determined as the mean of the logarithms of M of mutually independent measurements of the peak area for the same solution, the following equation for the variance of x is obtained, a differentiation of the first order being considered sufficient:

$$V(x) = \frac{1}{B^2}\left[V(y) \cdot \left(\frac{1}{M} + \frac{1}{NS}\right) + (x - \bar{x})^2 \cdot V(B)\right]$$

$V(y)$ and $V(B)$ resulted from the simple linear regression analysis comprising NS observations per lipid. Thereafter, the standard deviation on x was calculated as $\sqrt{V(x)}$, and the confidence limits can be calculated on the basis of NS less 2 degrees of freedom (t-distribution).

(2) *Parallel regression lines.*

$$y = \bar{y} + B(x - \bar{x}) + \sum_{i=1}^{n} L_i (P_i - PM)$$

where n = number of lipids -1. If the equation is solved with respect to x, the result is

$$x = \frac{y - \bar{y} - \sum_{i=1}^{n} L_i (P_i - PM)}{B} + \bar{x}$$

If y is determined as stated above, the result is the following variance on x:

$$V(x) = \frac{1}{B^2}\left\{\left(V(y) \cdot \left(\frac{1}{M} + \frac{1}{N}\right) + (x - \bar{x})^2 \cdot V(B) + \right.\right.$$

$$+ \sum_{i=1}^{n} (P_i - PM)^2 \cdot V(L_i) + 2(x - \bar{x}) \sum_{i=1}^{n} (P_i - PM) \cdot CV(BL_i) +$$

$$\left.\left. + \sum_{i=1}^{n} \left[(P_i - PM)\left[\sum_{j=1}^{n} (P_j - PM) \cdot CV(L_iL_j)\right]\right]\right\}$$

where N is the total number of observations for all lipids together, n the number of lipids -1 and $i \neq j$. The variances $V(y)$, $V(B)$, and $V(L_i)$ as well as the co-variances $CV(BL_i)$ and $CV(L_iL_j)$ are known from the multiple regression analysis.

Thereafter, the standard deviation on x can be calculated as $\sqrt{V(x)}$, and the confidence limits can be calculated on the basis of $(N-n-2)$ degrees of freedom (t-distribution). If the number of observations per lipid is constant $= NS$, the result will be:

$$N-n-2 = NS \cdot (n + 1) - n - 2.$$

Thus, the ratio between the number of degrees of freedom for the multiple and the simple case exceeds $n + 1$ = number of lipids, provided that $NS > 2$. This tendency counteracts to some extent the parallelism of the regression lines, so that the confidence intervals are largely of the same magnitude in both cases.

It was mentioned above that exponent B is constant for the five lipids on the same chromatoplate. On the other hand, it is not permissible to use a B value calculated for observations for one plate in connection with observations for another plate, as B has proved plate dependent. Also, the lipid constants and their mutual sizes vary from plate to plate. Accordingly, one cannot neglect to include standard mixtures for each chromatoplate, but the existence of a constant B value for the individual plate means that B can be fixed with the same accuracy as the individual (lipid dependent) B values for considerably fewer total observations. This permits an increased number of experimental measurements per chromatoplate to be made. If the number of standard observations is preserved, the inaccuracy in B will be reduced.

ACKNOWLEDGEMENTS

Thanks are due to Mrs. Lene Pedersen and Mr. Ole Ellermann, laboratory technicians, for technical assistance and to Mrs. Liselotte Lærkeborg for typing the manuscript.

REFERENCES

1 H. K. Mangold and D. C. Malins, *J. Amer. Oil Chem. Soc.*, 37 (1960) 383.
2 D. C. Malins and H. K. Mangold, *J. Amer. Oil Chem. Soc.*, 37 (1960) 576.
3 H. Gänshirt and K. Morianz, *Arch. Pharm. (Weinheim)*, 293 (1960) 1065.
4 S. J. Purdy and E. V. Truter, *Analyst (London)*, 87 (1962) 802.
5 M. L. Blank, J. A. Schmit and O. S. Privett, *J. Amer. Oil Chem. Soc.*, 41 (1964) 371.
6 R. Paquin and M. Lepage, *J. Chromatogr.*, 12 (1963) 57.
7 M. S. J. Dallas, *J. Chromatogr.*, 33 (1968) 337.
8 R. W. Frei, *J. Chromatogr.*, 64 (1972) 285.
9 O. S. Privett and M. L. Blank, *J. Amer. Oil Chem. Soc.*, 39 (1962) 520.
10 R. T. Louis-Ferdinand, D. G. Therriault, W. F. Blatt and M. Mayer, *Clin. Chem.*, 13 (1967) 773.
11 D. T. Downing, *J. Chromatogr.*, 38 (1968) 91.
12 H. Rasmussen, *J. Chromatogr.*, 27 (1967) 142.
13 A. Hald, *Statistiske Metoder*, Det Private Ingeniørfond, Copenhagen, 1948, p. 411.
14 G. E. P. Box, W. R. Cousins, O. L. Davies, F. R. Himsworth, H. Kenney, M. Milbourn, W. Spendley and W. L. Stevens, in O. L. Davies (Editor), *Statistical Methods in Research and Production*, Oliver and Boyd, Edingburgh, London, 3rd ed., 1967, p. 170.
15 B. Wiklund and R. Eliasson, *J. Chromatogr.*, 68 (1972) 153.
16 B. L. Walker, *J. Chromatogr.*, 56 (1971) 320.