CHROM. 3511

AN IMPROVED METHOD FOR THE QUANTITATIVE ANALYSIS OF LIPID CLASSES VIA THIN-LAYER CHROMATOGRAPHY EMPLOYING CHARRING AND DENSITOMETRY*

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(Received March 15th, 1968)

SUMMARY

A procedure for the quantitative analysis of lipid classes by thin-layer chromatography (TLC) via charring and densitometry in which the effect of unsaturation is eliminated by hydrogenation is described The sample is hydrogenated in ethanol at room temperature with the Adams catalyst. Quantitative analysis is carried out by fractionation of the sample on thin-layer plates and analysis of charred spots via densitometry. Elimination of unsaturation provides more uniform charring properties and simplifies the selection of reference standards when required. The method is demonstrated on standard mixtures of reference compounds.

INTRODUCTION

Although thin-layer chromatography (TLC) using densitometry and other techniques of charred spot measurement[1–14] offers a simple, fast micromethod for lipid class analysis, a completely satisfactory method for the quantitative analysis of these compounds has not been developed. The basic problem is these techniques is in the charring process. No single technique can be expected to give quantitative carbonization of all compounds found in natural lipids. Thus the method, perforce, must remain empirical and considerable reliance must be placed on the use of standards. However, because of the complexity of natural lipids, identical standards are not generally available and something less than ideal must be used. Some factors are more important than others with respect to the yield of carbon in the charring process. With lipid classes, most of which are relatively nonvolatile, the degree of unsaturation of the constituent fatty acids is highly important[10,11,12]. Hydrogenation may be used to nullify this factor and simplify selection of standards when they are required.

* This investigation was supported in part by PHS Research Grants No. HE-05735 and HE-08214 from the National Institutes of Health, Public Health Service.

EXPERIMENTAL

Standards

Highly purified (> 99 %) stearic acid, tristearin, linoleic acid, methyl linoleate, cholesteryl linoeate and trilinolein were purchased from the Lipids Preparation Laboratory of The Hormel Institute, Austin, Minn.

Polar lipids were prepared from natural sources by conventional techniques or obtained from commercial sources and purified by TLC. Cerebrosides (CERE) were isolated from milk phospholipids and chromatographed as two separate spots, ceramidemonohexoside and ceramidedihexoside[15]. Otherwise all standards were chromatographically homogeneous as shown in the results. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SPH) were obtained from egg lipid; phosphatidylinositol (PI), as well as PC and PE were also obtained from crude soybean lecithin. Phosphatidylserine (PS) was obtained from several sources, particularly beef brain and bovine milk phospholipids. Samples of PS and PE were also purchased from Pierce Chemical Co., Rockford, Ill.

PROCEDURE

Hydrogenation

A sample of 1 to 2 mg is hydrogenated in 2 ml of 95 % ethanol with approximately 1 mg of platinum oxide catalyst (Adams catalyst, Matheson, Coleman and Bell, East Rutherford, N.J.) in a small, flat-bottom glass-stoppered cylindrical flask, 20 mm in diameter × 80 mm high, with a small side arm for introduction of hydrogen. The reaction flask is flushed thoroughly with hydrogen which is then passed over the sample with just sufficient pressure to keep the glass stopper ajar on a small piece of metal wire. The solution containing the dispersed catalyst is stirred vigorously by means of a magnetic stirrer. A period of two hours' stirring at room temperature was found to give complete hydrogenation of common polar lipids. This reaction period was established by determination of the constituent fatty acids (gas–liquid chromatography as methyl esters) of samples of the reference polar lipids. After reduction of the sample, the catalyst is removed by filtration through a fine porosity sintered glass funnel containing a pad of about 2 cm of anhydrous sodium sulfate. The catalyst is washed four or five times with small amounts of chloroform–methanol (2:1) in order to recover the entire sample. Recoveries of the order of 95 % are readily obtained by this technique with no apparent preferential loss of components on the catalyst.

Thin-layer chromatography

Chromatoplates (20 × 20 cm) with a 0.25 mm layer of Silica Gel G (Merck, Darmstadt, Germany) activated at 110° for 1 h were used for all separations. Just prior to use the plates were developed in anhydrous ethyl ether to remove most of the organic impurities in the adsorbent to the top of the plate.

For quantitative densitometry, ideally each component should be separated completely with R_F values of between about 0.3 and 0.8. This condition can be readily attained with neutral lipids as previously demonstrated[4]. It is more difficult to achieve complete separations of complex mixtures of polar lipids but by judicious manipulations of combinations of chloroform, methanol, water and acetic acid or

ammonia most of the common polar lipids can be resolved in one-dimensional systems within this range, as illustrated in the results.

Charring and densitometry

The spots are charred by spraying the plates with concentrated sulfuric acid diluted to 70 % (v/v) with a saturated aqueous solution of potassium dichromate[5,11] and heating them for 45 min in a forced-draft oven at 200°. Densitometry is carried out with a Photovolt Densitometer Model 52-C (Photovolt Corp., New York, N.Y.) modified as previously described for TLC[4]. Any commercial equipment designed especially for densitometry of chromatoplates would, of course, be satisfactory. Quantification is made on the basis of the areas of peaks obtained with a strip chart recorder operated in conjunction with the densitometer.

RESULTS

The separation of a number of polar lipids by one-dimensional TLC satisfactory for quantitative analysis via densitometry is illustrated on reference compounds in Fig. 1. Other solvent systems may be required for particular separations, but in general those illustrated in Fig. 1 are applicable to a wide variety of lipids. For highly complex mixtures, reference may be made to the comprehensive work of SKIPSKI and his coworkers[16,17], who have developed solvent systems for the fractionation of virtually all known polar lipids by combinations of one dimensional solvent systems.

Standard curves for a wide variety of hydrogenated neutral and polar lipids are shown in Figs. 2 and 3, respectively. The values used to construct standard curves are expressed in terms of carbon content in each case. These results show that up to a

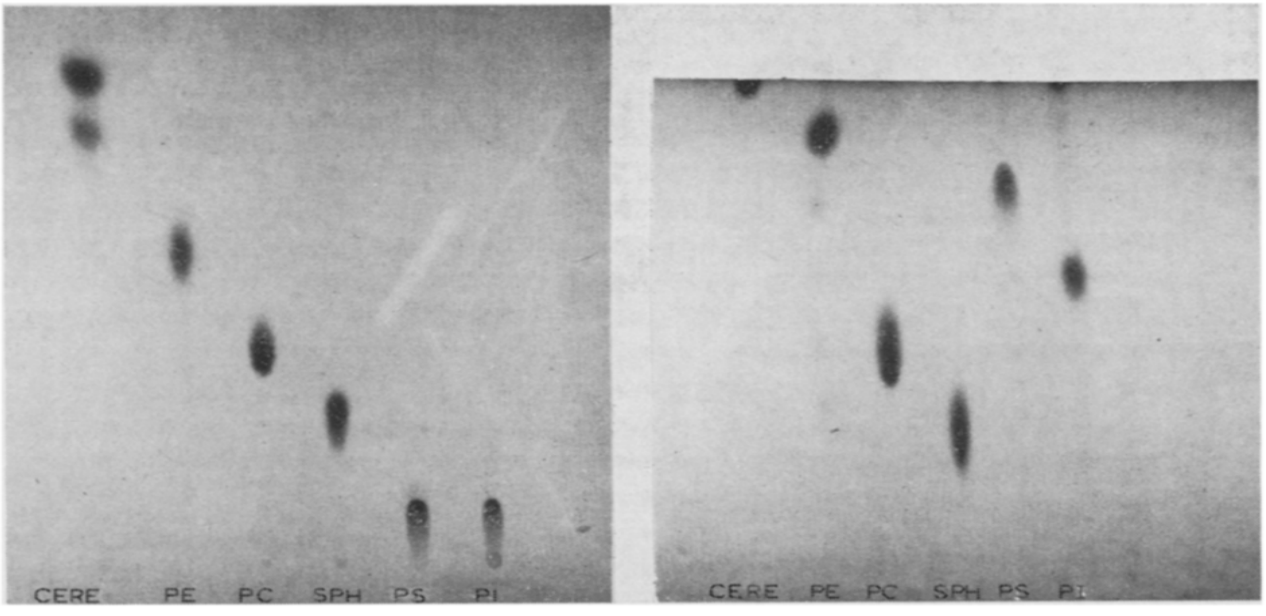

Fig. 1. Separation of reference polar lipids by TLC on plates coated with silica gel. Plate 1 was developed with chloroform–methanol–ammonium hydroxide–water (75:30:4:0.5) on Silica Gel G. Plate 2 was developed with chloroform–methanol–acetic acid–water (50:30:8:4) on Silica Gel H. CERE = Cerebrosides; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SPH = Sphingomyelin; PI = phosphatidylinositol; PS = phosphatidylserine.

value of about 20 μg of carbon the same standard curve may be used for all common polar and neutral lipids. (The standard curves in Figs. 2 and 3 are identical.) It is apparent from these results that hydrogenation has two effects, *viz.* (1) it provides the same degree of char for all the common lipid classes, at least, and (2) extrapolation of the curve passes through the origin. The significance of these observations is that peak areas of spots with R_F values between approximately 0.3 and 0.8 may be compared directly, that is, for compounds of known carbon content. This feature of

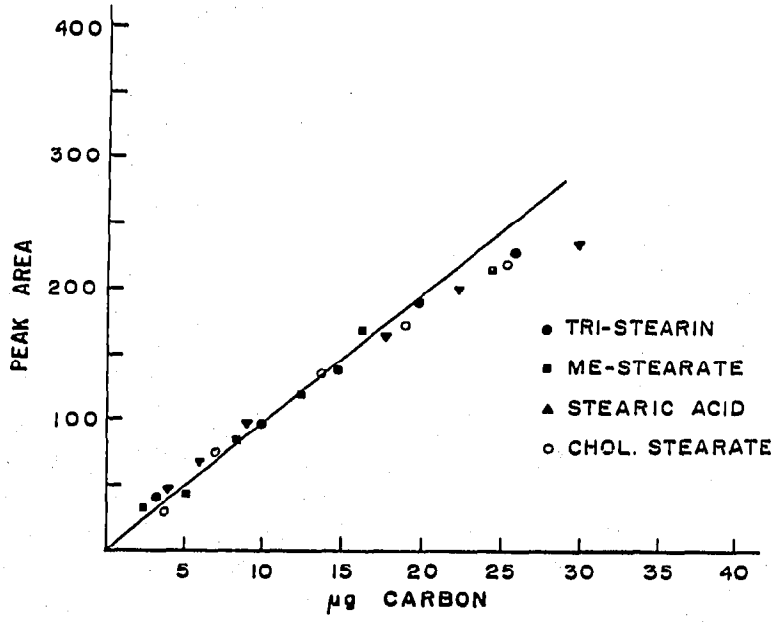

Fig. 2. TLC analysis of charred neutral lipids. Standard curve for fully saturated neutral lipid classes.

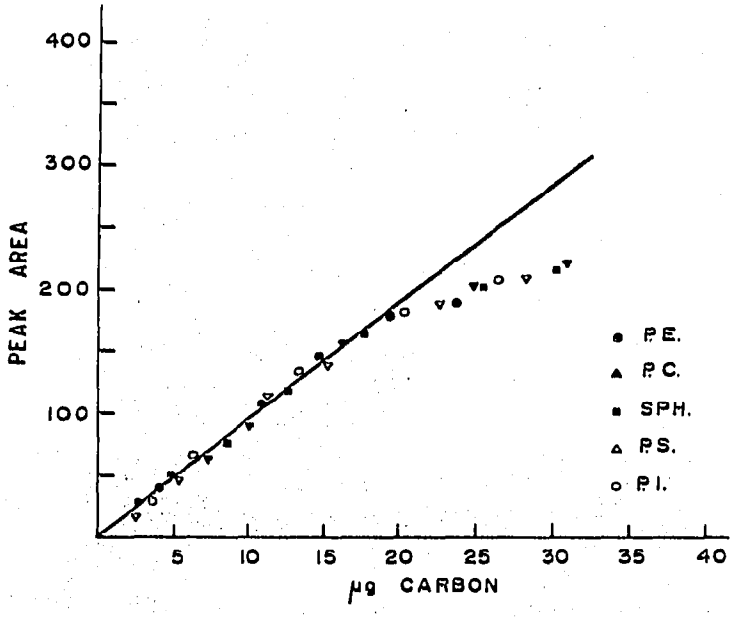

Fig. 3. TLC analysis of charred hydrogenated phospholipids. Standard curve for fully saturated polar lipid classes. PE = Phosphatidylethanolamine; PC = phosphatidylcholine; SPH = sphingomyelin; PS = phosphatidylserine; PI = phosphatidylinositol.

the method was illustrated further in another experiment, in which three standard mixtures were analyzed in triplicate. The chromatoplates for these analyses are shown in Fig. 4. These mixtures were selected so that one (mixture A) represented a sample containing both neutral and polar lipids. Mixtures B and C could feasibly represent mixtures of neutral and polar lipids, respectively, that may be used as a standard representing compounds that have R_F values in the optimum region of the plate for comparison with mixture A. However, since all compounds give the same degree of char after hydrogenation (Figs. 2 and 3) it is evident that only one compound need be used as a standard for all compounds separating in the optimum region of the plate. This is one of the advantages of hydrogenation.

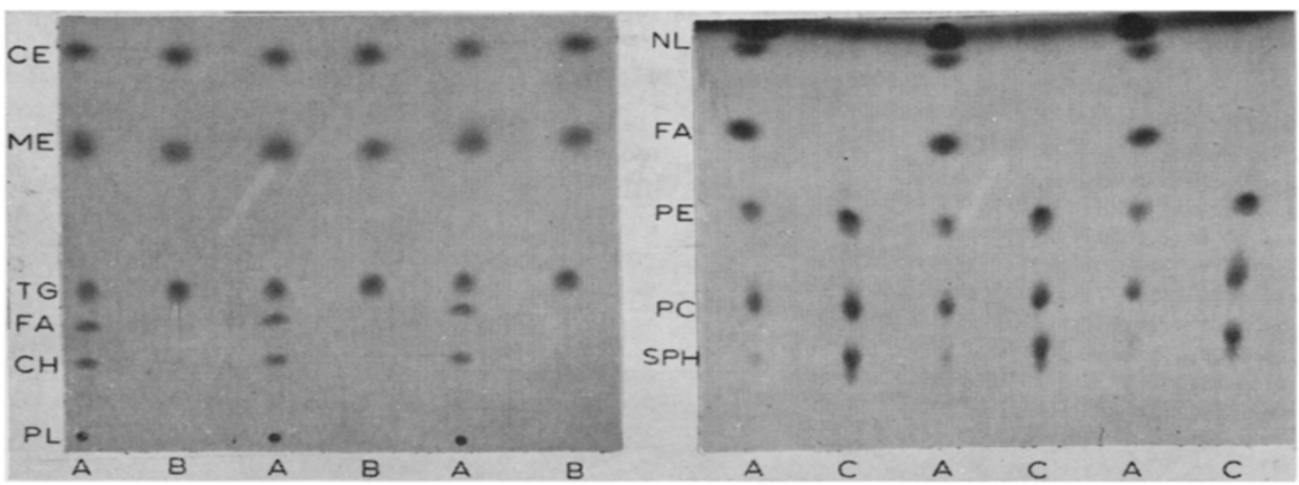

Fig. 4. TLC plates showing the separation of three standard mixtures after hydrogenation. Original composition: mixture A = cholesteryl linoleate (CE), methyl linoleate (ME), trilinolein (TG), linoleic acid (FA), cholesterol (CH), polar lipids (PL), neutral lipids (NL); mixture B = CE, ME and TG; mixture C = PE, PC and SPH. Plate 1 was developed with petroleum ether–ethyl ether–acetic acid (85:15:1). Plate 2 was developed with chloroform–methanol–water–acetic acid (65:25:4:1).

Standard mixture A, containing both neutral and polar lipids, was chromatographed first (along with mixture B) in a solvent system designed for the separation of neutral lipids (Plate A, Fig. 4). The polar lipids of this mixture were then separated in a solvent system designed specifically for these compounds (Plate B, Fig. 4). For more complex mixtures additional solvent systems may be employed and spots common to the different plates used to normalize all values. The results of these analyses were calculated on the basis of the direct proportionalities of the peak areas (Table I). The high reproducibility of the method may be noted first from the low values for the standard error of the mean for the three determinations. In spite of the fact that the cholesteryl esters and cholesterol classes are outside the optimum region of the plate, the values are in remarkably good agreement with the known composition. Even closer agreement could be obtained through the use of standards as evidenced by the excellent agreement between the known composition and the values obtained with the simpler mixtures which are in the optimum region of the plate.

Elimination of errors due to unsaturation is well illustrated in Table II, which shows the results of analyses made before and after hydrogenation. The above anal-

TABLE I

ANALYSES OF STANDARD MIXTURES OF REFERENCE COMPOUNDS AFTER HYDROGENATION (DIRECT PROPORTIONALITIES OF PEAK AREAS)

Abbreviations: CE = cholesteryl linoleate; ME = methyl linoleate; TG = trilinolein; FA = linoleic acid; CH = cholesterol; PE = natural phosphatidylethanolamine; PC = natural phosphatidylcholine; SPH = natural sphingomyelin.

	A		*B*		*C*	
	Known	*Found*	*Known*	*Found*	*Known*	*Found*
CE	10.6	11.1 ± 0.4[a]	35.5	35.9 ± 0.4		
ME	30.4	30.6 ± 0.4	31.1	30.9 ± 0.3		
TG	18.4	18.3 ± 0.1	33.4	33.2 ± 0.1		
FA	14.0	13.8 ± 0.3				
CH	8.9	8.3 ± 0.4				
PE	5.8	6.2 ± 0.4			35.6	36.1 ± 0.4
PC	9.7	9.6 ± 0.4			32.4	32.2 ± 0.2
SPH	2.2	2.1 ± 0.3			32.0	31.7 ± 0.3

[a] Mean ± standard error of three determinations.

TABLE II

EFFECT OF HYDROGENATION ON ANALYSES VIA DIRECT PROPORTIONALITIES OF PEAK AREAS (EQUAL WT. % OF COMPONENTS)

	Before hydrogenation		*After hydrogenation*	
	Corrected peak area[a]	*Wt. %*	*Corrected peak area*	*Wt. %*
CE	167 ± 2.7[b]	18.5	149 ± 2.5	16.7
ME	162 ± 2.6	17.8	149 ± 2.4	16.7
TG	153 ± 3.0	16.9	148 ± 2.6	16.6
PE	148 ± 2.7	16.3	148 ± 2.7	16.6
PC	141 ± 2.6	15.6	149 ± 2.4	16.7
SPH	135 ± 3.0	14.9	148 ± 2.8	16.6

[a] Mean ± standard error of three determinations.
[b] Area corrected on the basis of percent carbon in each component.

yses (Table II) were made without standards, that is, by direct comparison of peak areas. The errors, of course, could be completely eliminated in this case by the use of standards because identical compounds are available for use as standards. The peak areas for some of the compounds were in fairly close agreement before and after hydrogenation, but in mixtures errors in even one compound are reflected in all components.

Charring of spots may be carried out by a number of different reagents and the yield of carbon will vary accordingly. The yield of carbon in any charring procedure depends on the relative rate of oxidation and evaporation of the sample (spot). Even with high molecular weight lipid classes appreciable evaporation will occur when the chromatoplate is heated above 200° (ref. 11). Strong oxidizing reagents should be used to complete the oxidation to carbon as quickly as possible, but the oxidizing agent and the heating conditions should not be so drastic as to convert the sample

to carbon dioxide. The charring conditions employed here appear to be as nearly optimum as can be expected inasmuch as all compounds gave the same degree of char. Quantitative conversion of the compounds to carbon, of course, is not claimed; it is doubtful that any single procedure could be developed that would give a quantitative conversion of lipids to carbon in view of the large differences in structures of these compounds.

Since most lipid classes exist as complex mixtures of molecular species varying in type and amounts of constituent fatty acids it is virtually impossible to obtain identical standards and to predict the error that will be given by charring properties as a result of differences in degree of unsaturation. Furthermore since close to identical standard curves can be obtained via hydrogenation, it is apparent that the effect of other structural differences in lipid classes has less influence on their charring properties than the type and amount of unsaturation. Thus, simple saturation of the unsaturated linkages not only provides more uniform charring characteristics, it simplifies the standards that may be used when they are required.

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