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A rapid method for relative quantitation of lipid classes separated by thin-layer chromatography

No adequate procedures have been available for rapid and reproducible quantitation of large numbers of lipid samples. For this reason many areas amenable to lipid studies remain unexplored and the role of lipids in many biochemical and physiological responses is not well understood.

Methods for extracting and purifying lipid samples[1,2], and for separating these samples by thin-layer chromatography (TLC) into the individual classes of neutral and phospholipids have existed for some time[3,4]. Recent developments in this area have produced somewhat more efficient methods for individual lipid class separations[5-7]. One of the limiting steps in lipid analysis is quantitation of the lipid classes after TLC separation. Numerous analytical procedures have been developed in an attempt to accomplish this task[8-17]. Most of these procedures are both slow and tedious as they require the isolation of the lipid class from the separation media for subsequent analysis. Of the procedures developed for quantitation of lipids on intact chromatograms, the char-densitometric technique appears to be applicable to quantitation of multiple samples. Other char techniques attempted in our laboratory were either not useful for phospholipids[15] or useful for lower quantities of material[16]. The char-densitometric procedure, described below, was designed to analyze changes in per cent distribution of serum lipid classes. This procedure allows for rapid and reliable analysis of multiple samples on a thin-layer chromatogram with good reproducibility between different chromatograms.

Experimental

Materials and methods. Individual lipid standards and artificial mixes were obtained from Supelco, Inc., Bellefonte, Penn., U.S.A. Chromatographic spray bottles were obtained from Arthur H. Thomas Company, Philadelphia, Penn., U.S.A. A Model SD-3000 dual-beam spectrodensitometer, Model SDC-300 density computer, was obtained from Schoeffel Instrument Company, Westwood, N.J., U.S.A. A Model CRS-100 digital electronic integrator and associated paper readout was obtained from Infotronics Corp., Houston, Texas, U.S.A.

Thin-layer chromatograms were prepared and developed as previously reported[4,5]. Visualization of the developed chromatograms was accomplished by spraying the plates with an aqueous 20% ammonium bisulfate solution[14] until they appeared translucent. The translucent chromatograms were transferred to a hot plate and charred at 170°. Even distribution of heat on the glass plates was accomplished by placing a 25 × 25 × 1 cm aluminum sheet between the hot plate surface and the thin-layer chromatogram, by maintaining the charring plates in a draft free enclosure, and by placing the chromatogram on the metal sheet at ambient temperature and heating to char temperature at 5°/min. Chromatograms were held at char-temperature for 1 h before the hot plates were turned off and the entire system slowly cooled to ambient temperature over a 30-min time period. All charred plates are photographed for reference purposes.

The charred lipid spots on the chromatograms were scanned on the dual-beam

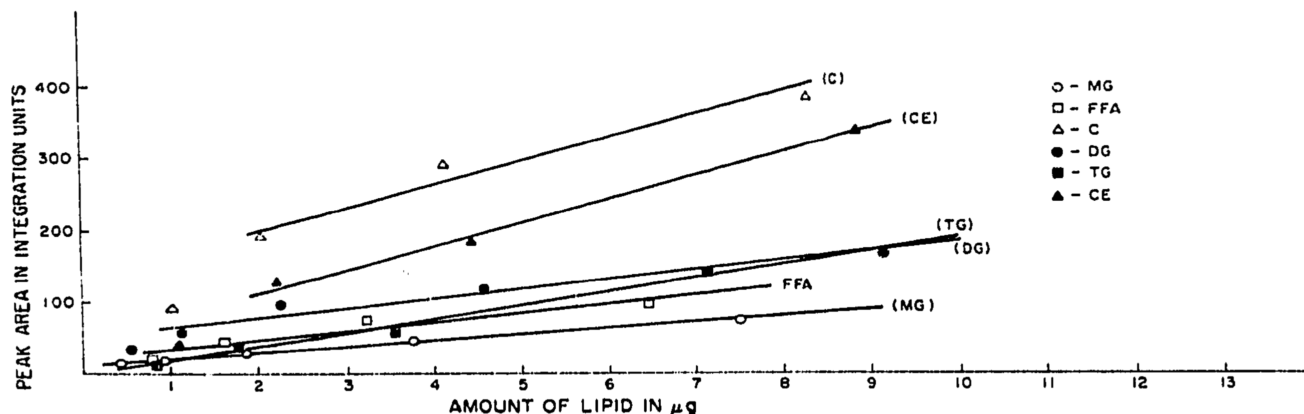

Fig. 1. Lower range (0–10 μg) for neutral lipids. Six neutral lipids classes with μgrams lipid spotted *vs.* peak area in integration units. The fatty acid on all neutral lipids was palmitic acid. MG, monopalmitin; FFA, palmitic acid; C, cholesterol; DG, dipalmitin; TG, tripalmitin; CE, cholesterol palmitate.

spectrodensitometer at a wavelength high enough to avoid absorbance by glass. Alternate lanes were spotted providing an adjacent empty lane for background correction by the densitometer. The plates were automatically scanned using transmittance techniques available with the densitometer. As the scan progresses, peaks corresponding to the optical density of the particular spot are recorded on a linear output chart and the peak areas simultaneously integrated by an electronic integrator. Comparison of the chromatogram picture, the strip chart peak record, and

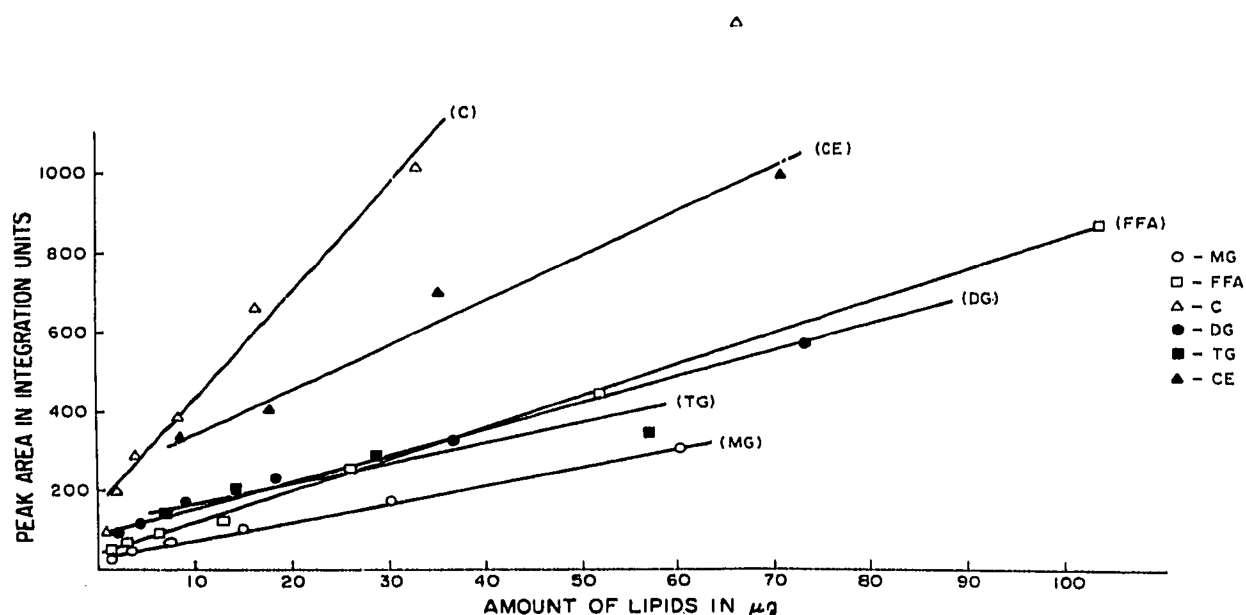

Fig. 2. Upper range (10–100 μg) for neutral lipids. Six neutral lipid classes with μgrams lipid spotted *vs.* peak area in integration units. The fatty acid on all neutral lipids was palmitic acid. MG, monopalmitin; FFA, palmitic acid; C, cholesterol; DG, dipalmitin; TG, tripalmitin; CE, cholesterol palmitate.

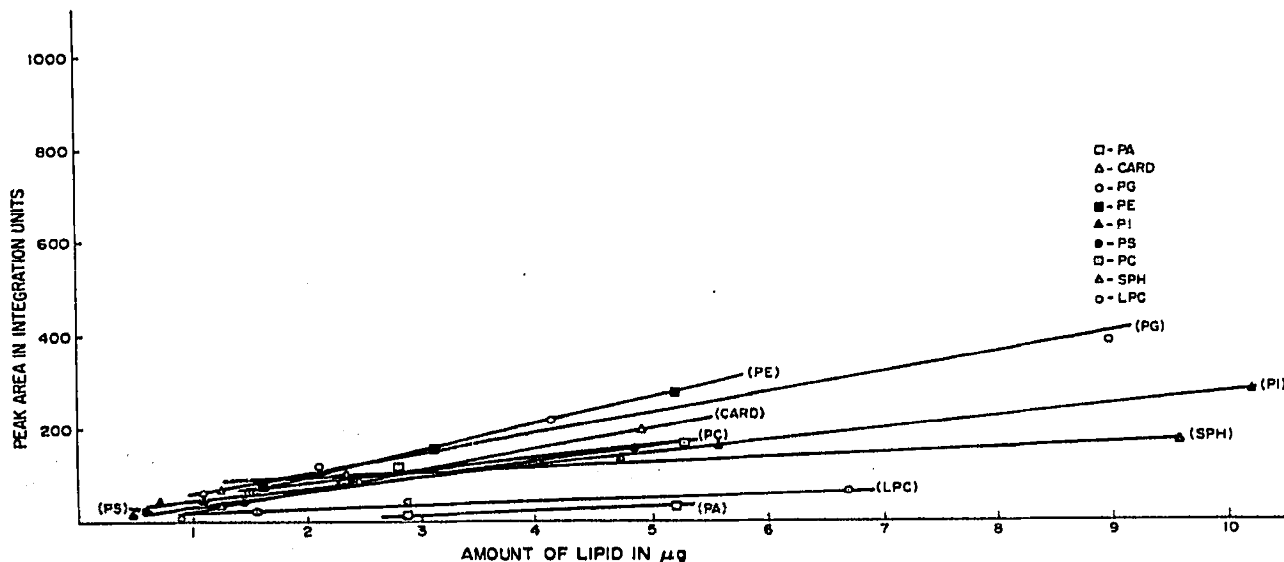

Fig. 3. Lower range (0–10 μg) for phospholipids. Nine phospholipid classes with μg lipid spotted *vs.* peak area in integration units. PA, phosphatidic acid; CARD, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

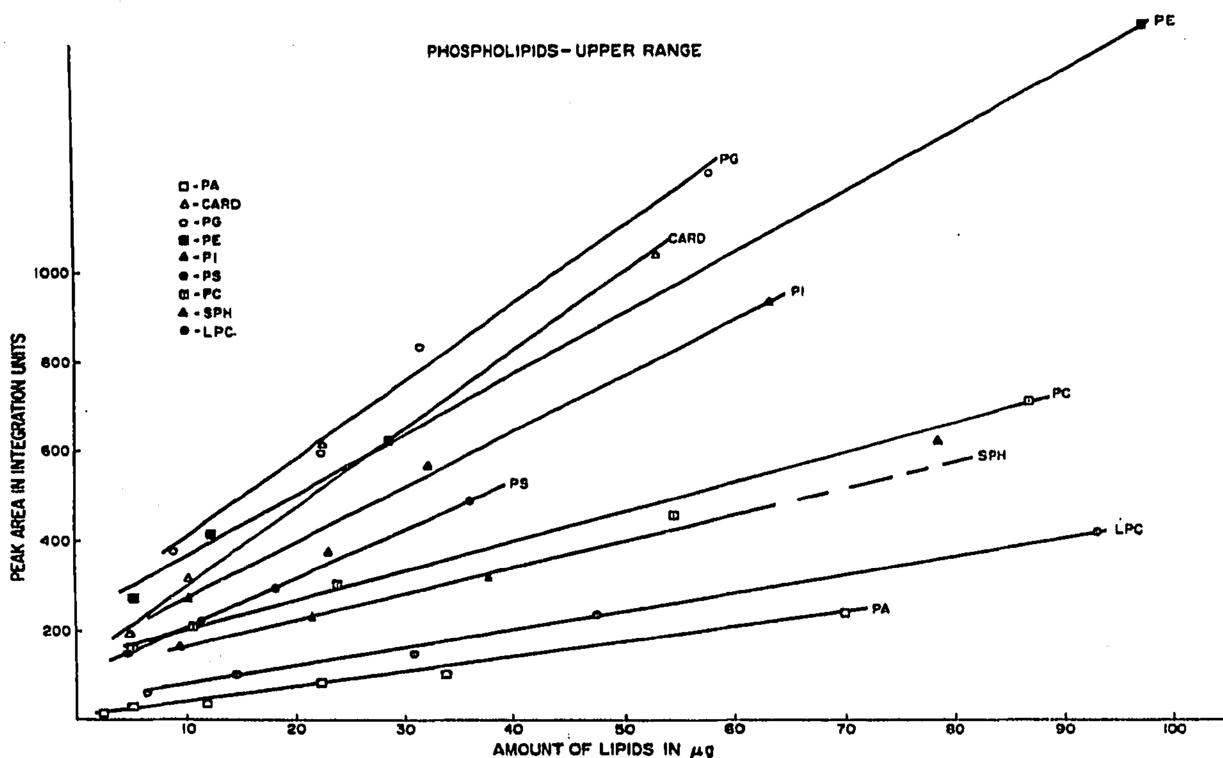

Fig. 4. Upper range (10–100 μg) for phospholipids. Nine phospholipid classes with μg lipid spotted *vs.* peak area in integration units. PA, phosphatidic acid; CARD, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

the integrator output provides the data required for the relative quantitation. The results are expressed as a relative per cent distribution within the spotted lane.

Results and discussion. Figs. 1–4 demonstrate the response of this procedure to increasing amounts of spotted and developed lipid material for each lipid class investigated. The demonstrated linear response region is the same from plate to plate although slight differences in the slope of the responses may occur between different plates. For most lipids the entire region tested was linear, in comparison to a rather restricted range as reported by FEWSTER *et al.*[16]. Table I shows the reproducibility, from lane to lane on the same plate and from plate to plate obtained by this method with artificial mixtures of both neutral and phospholipids. These data demonstrate the overall precision and reliability of this procedure.

This procedure provides a method for determining the relative per cent distribution of a complex lipid sample. While this procedure, in itself, does not determine

TABLE I

VARIABILITY IN QUANTITATION OF ARTIFICIAL LIPID MIXTURES BY THE CHAR-DENSITOMETRIC TECHNIQUE

	Phospholipids								
	LPC	*SPH*	*PC*	*PS*	*PI*	*PE*	*PG*	*CARD*	*PA*
	Lane to lane								
Relative % composition	9.5	22.4	28.8	3.4	9.5	12.9	6.3	3.0	3.2
S.D.	0.62	0.81	1.72	0.76	0.78	0.14	0.19	0.47	0.53
	Plate to plate								
Relative % composition	10.5	24.8	31.3	2.7	7.4	11.9	5.8	2.3	2.2
S.D.	0.80	1.79	1.52	0.99	1.08	0.53	0.41	0.58	0.59
	Neutral lipids								
	MG	*FFA*	*C*	*DG*	*TG*	*CE*			
	Lane to lane								
Relative % composition	3.7	12.0	29.0	3.4	18.3	33.4			
S.D.	0.36	1.25	1.51	0.25	1.41	1.15			
	Plate to plate								
Relative % composition	3.5	11.7	28.1	3.3	19.0	34.1			
S.D.	0.92	3.12	2.08	0.69	2.51	3.13			

the absolute amount of material present, it is readily applicable in instances where comparisons of lipid class distribution between samples derived from the same or similar origins are desired. Using this procedure eight samples can be analyzed on one chromatogram and 10–15 chromatograms can be quantitated per day with relative ease. Samples are generally analyzed in duplicate or triplicate in our laboratory and standard mixes spotted in the two outside lanes. The use of a dual-beam densitometer compensates for variation in the thickness of the plate coating. As reported by FEWSTER *et al.*, it is best to quantitate plates within a few hours after charring[16].

The char technique used in this procedure, as described by BOROWSKI AND ZIMIŃSKI[18] appears to give better results than those using sulfuric acid or sulfuric

acid plus an oxidizing agent. One advantage is that a much cleaner plate background is obtained which is more applicable to densitometric quantitation. It also gives good results for phospholipids in contrast to the method of BIEZENSKI *et al.*[15]. Finally, the range of response is wider than that reported by FEWSTER *et al.*[16].

As several reports suggest, the char technique yields different slopes of integration units plotted versus the amount of lipid applied in μmole or μg. The use of relative per cent distribution rather than absolute quantitation is useful for screening samples obtained from similar sources, for example, serum or tissue. The procedure can be used for determining large changes in a particular lipid class with reliability. Absolute quantitation could be achieved by comparison to known amounts of lipid applied to the same plate as suggested by FEWSTER *et al.*[16]. Relative per cent distribution measurement of lipid classes appears to provide useful data in assessing major changes in lipid class distribution.

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