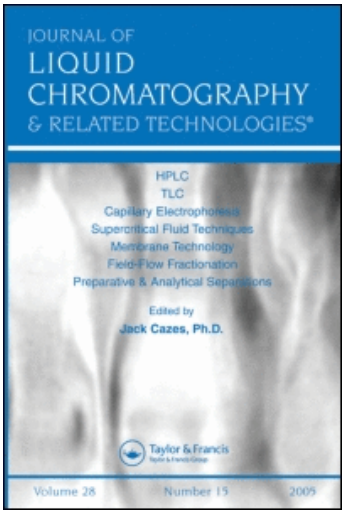

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Two Dimensional Quantitative Analysis in Planar Chromatography

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TWO DIMENSIONAL QUANTITATIVE ANALYSIS IN PLANAR CHROMATOGRAPHY

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ABSTRACT

The quantitative analysis of biological materials presents serious problems for the biochemist. The procedures to produce gels and separate phospholipids are time consuming and tedious. In the past, interpretation of the data was qualitative at best and quite subjective. With the introduction of sophisticated instrumentation and software, accurate and reliable data can be obtained.

In this paper, the methodology and techniques of two dimensional planar chromatography are explored. We will show that parameter selections can greatly influence the data. Comparisons of actual to calculated concentrations can show a 10% - 55% variation depending on the sample, methodology and techniques used.

INTRODUCTION

Electrophoresis and thin layer chromatography are used to analyze proteins and lipids. The spotting, developing, and staining procedures are time and effort

intensive. Often, the results are variable from one gel/plate to another. These two dimensional media provide a large quantity of information. It is critical that the instrumentation used to determine the results can accurately read and quantitate the data. Otherwise, all the time and effort put forth in the production of the sample is wasted. The Shimadzu CS9000U scanning densitometer and the QuantaScan 2D Analysis software were used. We will discuss the important parameters necessary to optimize data accuracy.

MATERIALS

Phospholipids spotted on HPTLC plates were used. The soybean phospholipids used were: Phosphatidylcholine (PC), Phosphatidyl Ethanolamine (PE), Phosphatidyl Inositol (PI), and Phosphatidic Acid Sodium Salt(PA). The standards were made in chloroform with the following concentrations: 0.25, 0.50, 1.0, 1.5, 2.0, and 3.0μg/μL. An additional PC solution, 5μg/μl, was used for the internal standard. The standards and samples were spotted in 1 μL aliquots spotted on a silica gel 60 HPTLC plate (Merck). The plates were developed in chloroform:methanol:7N aqueous ammonia (65:30:4) first. The plates were then rotated 90 degrees and developed with chloroform:methanol:acetic acid:water (170:25:25:6). After drying, the plates were sprayed with a copper sulfate phosphoric acid solution (25g copper sulfate in 8% phosphoric acid). The plates were heated at 160 degrees C for 20 minutes to fix the stain. The gel sample was a photograph of a 2D silver stained gel scanned in two sections. A Shimadzu CS9000U was used in addition to a software package called QuantaScan 2D

Analysis. This software runs on an external IBM AT or compatible and collects and manipulates the data. A dot matrix printer, Epson FX 80, was used for the hard copy.

METHOD

The sample analysis was performed in the following order. The steps will be described in more detail later.

Determine the mode of operation - Reflection, Transmission, or Fluorescence

Determine the appropriate wavelength for the analysis

Collect the data on the external computer

Compensate for background inconsistencies or interference

Perform necessary signal processing functions such as smoothing

Produce a spot table

Select spot for normalization

Construct the calibration curve

Analyze the unknowns

Performing the steps in this order will ensure accurate and reproducible data. It also allows

comparison of several different gels or plates even when there is some variation in sample development. The error in the quantitative data is much less than that associated with the production of the sample.

Determine the Mode of Operation

For translucent gels, either transmission or reflectance can be used. The thickness of the gel will affect how much light penetrates the gel. Opaque media, such as a TLC plate, require reflectance mode. Increased sensitivity can be obtained by using a fluorescent label.

Determine the Appropriate Wavelength

A wavelength scan must be done on both the background and the sample spot. To do this, the densitometer used must have a monochromator. Figure 1 shows a wavelength scan of the background of the sample gel overlaid on the sample scan. The solid line shows the sample scan while the dashed line shows the background. This sample should use a wavelength with a maximum absorbance for the sample and minimum absorbance for the background. Scanning was done at 540nm. Figure 2 shows the scan at 540nm. Figure 3 shows a 400nm scan. Many spots were not detected.

Collect the Data on the PC

The instrument and computer used a parallel GPIB board. The QuantaScan software uses a Capitol Equipment Corporation PC<>488 board in the computer. The board for the CS9000U is from Shimadzu. The instrument is controlled directly from the PC. The data can also be converted into ASCII or Lotus format for further manipulation.

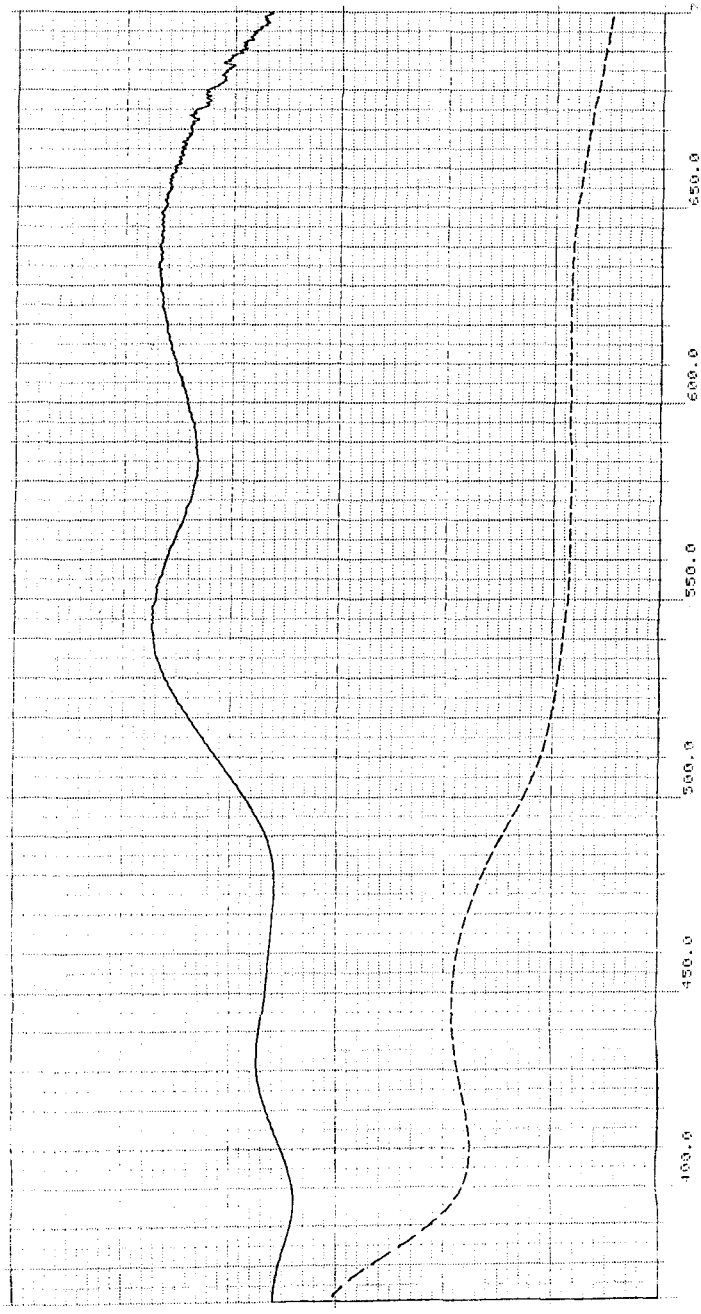

Figure 1. Wavelength scan of sample and background. Sample: Solid line; Background: Dashed line.

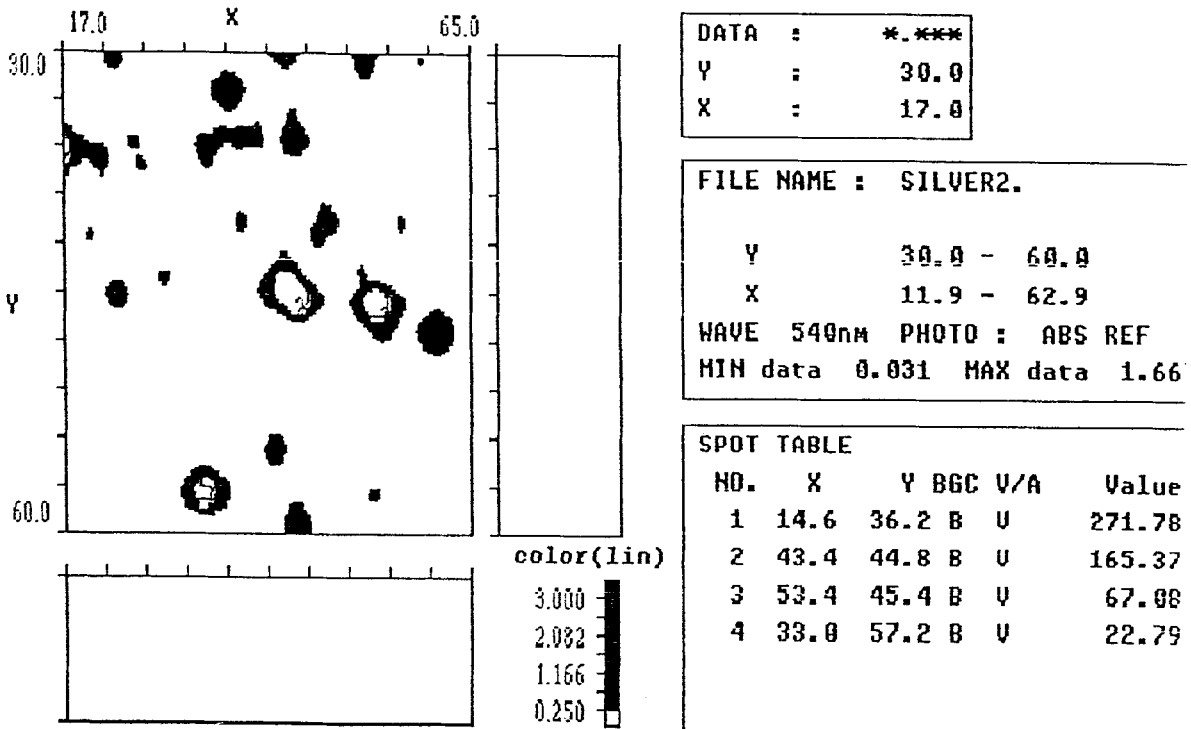

Figure 2. Sample scan at 540 nm; Y range 30 - 60 mm.

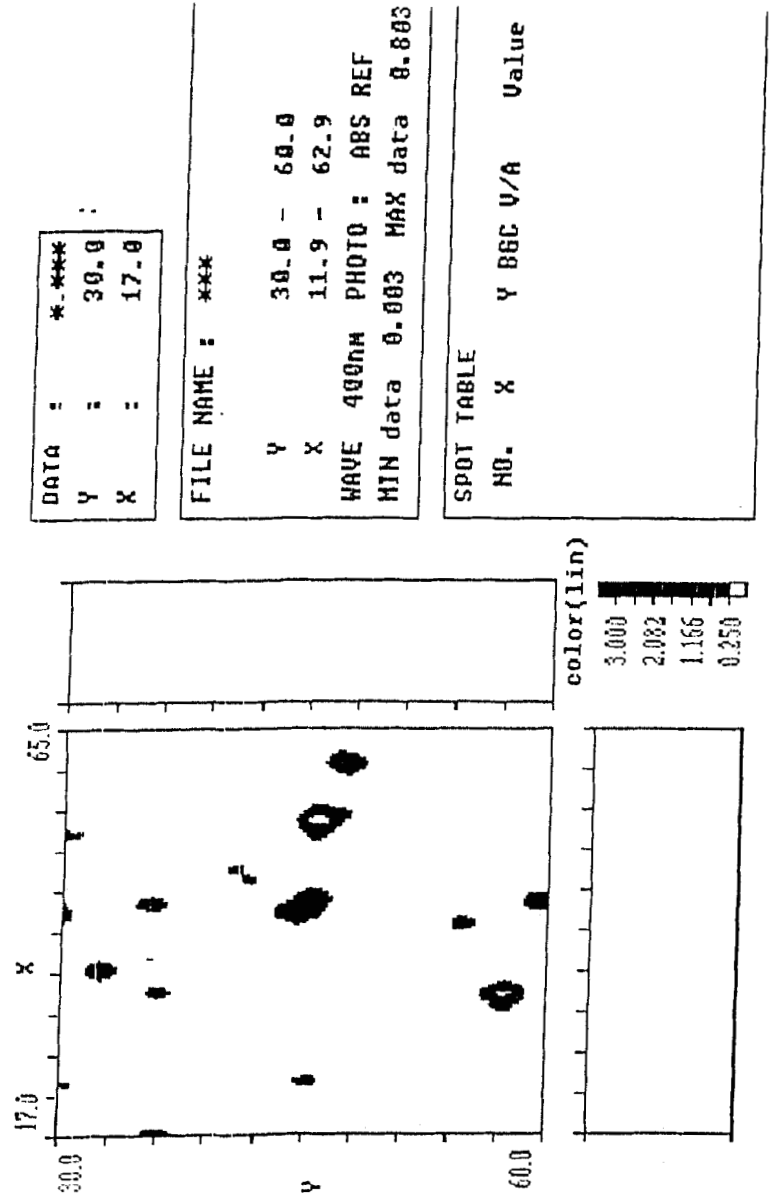

Figure 3. Sample scan at 400 nm; Y range 30 - 60 mm.

Compensate for the Background

There are several ways to do this. One way to compensate for background is to use a dual wavelength measurement. The sample is scanned at two wavelengths and one is subtracted from the other. This is a very powerful feature that corrects for surface imperfections and scattering.

For practical considerations, the light will scatter by the same amount despite the wavelength used. The scattered light can then be subtracted from the sample scanned at the analytical wavelength. Based on the wavelength scan of the sample spot and background, 540nm and 430nm were chosen. Figure 4 shows the 540nm measurement and Figure 5 shows the same area scanned at 430nm. The two scans are subtracted in Figure 6. Another method subtracts the background by using a software selection labelled "Background by Level Slice." The user types an absorbance value and this absorbance is subtracted directly from the data points. The background absorbance value is clearly visible from a color scale on the CRT. Another approach uses background subtraction along either an X or Y line cursor.

Signal Processing

Irregularities in the gel or plate surface can lead to a somewhat noisy image. The peak table generation then takes longer and many small, undesirable spots are detected. To reduce this inconvenience, smoothing can be done before the spot table is generated. An average value is calculated for each pixel in the image based on surrounding pixels.

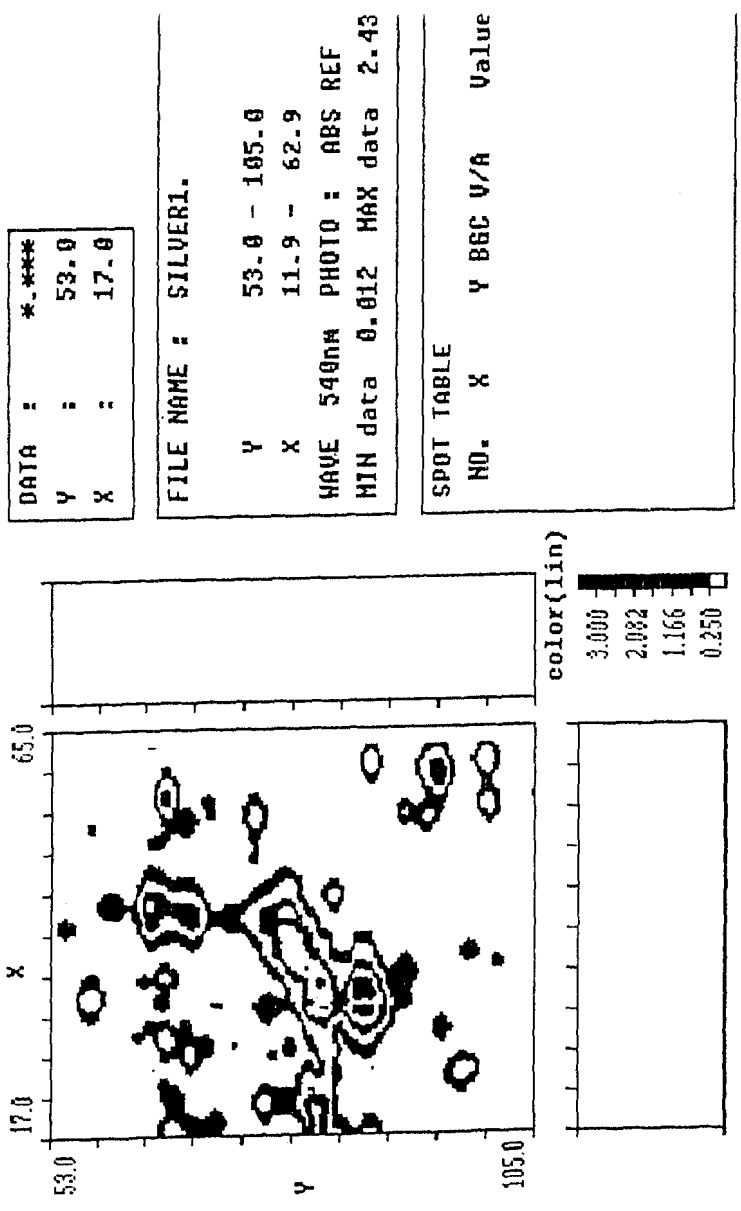

Figure 4. Scan at the sample maximum (540 nm); Y range 53 - 105 mm.

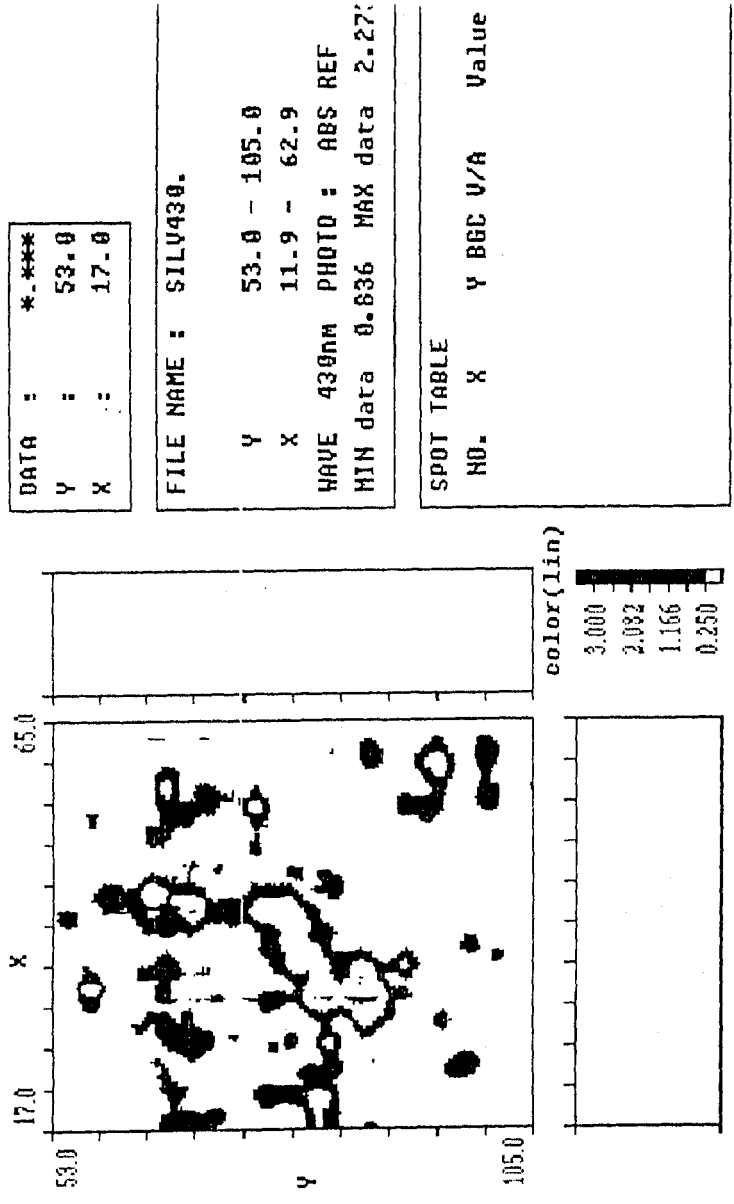

Figure 5. Scan at the background maximum (430 nm); Y range 53 - 105 mm.

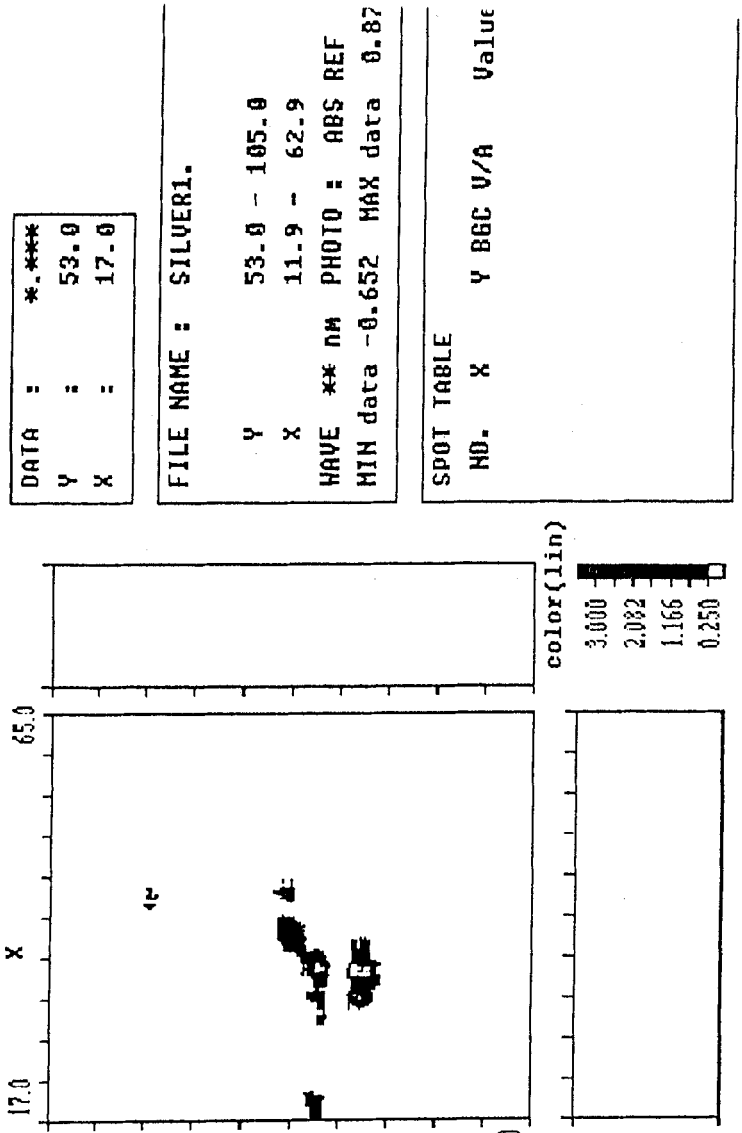

Figure 6. Dual wavelength Background subtraction.

SPOT File List

NO.	X.POS	Y.POS	V/A	BGC	AREA (mmxmm)	Volume
1	14.6	36.2	V	B	9.60	271.784
2	43.4	44.8	V	B	6.80	165.378
3	53.4	45.4	V	B	3.12	67.087
4	33.0	57.2	V	B	1.12	22.795

Figure 7. Spot Table output.

Produce a Spot Table

The spot table can be generated manually or automatically. The manual operation allows you to draw around the spots of interest. Up to 40 spots can be integrated in this way. For the integration of several spots, the automatic mode is preferred because the spot edge will be consistently detected. This ability increases reproducibility. Figure 7 shows the spot table produced automatically from the gel scanned in Figure 2.

The parameters for automatic spot determination include:

Volume or Area Output
Base Surface Correction
Smoothing Count
Sensitivity
Absolute Spot Level

These parameters will be discussed in detail since they are quite important in finding the spots.

Volume or Area Output

When deciding whether to use volume or area output, think of the spot in this way. The spot has

outlying edges that have a lower absorbance than the concentrated center. As the spot is scanned, a gaussian type peak is generally produced.
If you choose the area output, a single x-y slice will be integrated. If you choose volume output, the entire cone will be integrated. You will get more accuracy from the volume integration.

Base Surface Correction

The base surface correction gives a spot volume based on the average spot periphery, or the bottom level as zero, on a determined zero level. Usually, the average or zero level base surface correction works best.

Smoothing Count

The smoothing count is used for noisy data previously smoothed. The higher the number chosen, the more points used in the smoothing procedure.

Sensitivity

An arbitrary scale from 1 - 100 sets the sensitivity level for detecting spots. The least sensitive setting is 100 and the most sensitive setting is 1. A low value (1 - 25) means smaller spots will be detected while a higher value (75 - 100) detects only large spots.

Absolute Spot Level

This parameter provides spot detection based solely on the absorbance value of an individual pixel when paired with the Base Surface Correction setting of Level.

When these parameters are appropriately set, the QuantaScan software can automatically find the desired spots.

Select Spot for Normalization

To compare two gels or plates to each other, it is imperative that the same amount of one substance be applied to both as an internal standard. This spot will then provide a basis for comparison between the two samples. If the samples develop slightly differently, or there is variation between media, each sample can still be quantitated accurately. All areas from within the same gel or plate will be ratioed to the material selected for normalization. The phospholipid plates had individual internal standard spots on each plate.

Construct the Calibration Curve

The spot table and the concentrations for the spots of interest are used to construct the calibration curve. A first, second, third order, or point-to-point fit are available. A log function may be performed on the y axis. As many as 10 points can be used for the calibration curve. Once the curve is made, it can be displayed with the equation and coefficients. The curve is then stored and recalled for the unknown analysis.

Standard curves were constructed for both raw and normalized volume data. Table 1 shows the data from four phospholipids at six concentrations spotted on HPTLC plates. Table 2 shows the data normalized to an internal standard. The correlation coefficients for

TABLE I

Data Used for Phospholipid Standard Curve Based on Volumes

Lipid	0.25 μg/μL	0.50 μg/μL	1.00 μg/μL	1.50 μg/μL	2.00 μg/μL	3.00 μg/μL
PE	34.6	54.4	57.7	133.9	158.4	129.6
PC	32.9	50.3	57.5	113.1	135.7	116.7
PI	19.9	28.9	34.8	75.5	99.2	61.6
PA	35.7	63.2	53.4	127.6	169.5	189.4

TABLE II

Data Used for Phospholipid Standard Curve Based on Normalized Volumes

Lipid	0.25 μg/μL	0.50 μg/μL	1.00 μg/μL	1.50 μg/μL	2.00 μg/μL	3.00 μg/μL
PE	13.0	16.8	29.9	41.7	49.9	72.2
PC	12.3	15.5	27.8	35.2	42.8	64.9
PI	7.4	8.9	18.1	23.5	31.3	45.3
PA	13.4	19.5	30.0	39.7	53.5	77.5

TABLE III

Correlation Coefficient Comparisons
for Linear Regression Data

Lipid	Raw Volume	Normalized Volume
PE	0.824	0.999
PC	0.905	0.997
PI	0.706	0.998
PA	0.949	0.999

each set were calculated and are shown in Table 3. The correlation coefficients were vastly improved after normalization. Figure 8 shows the calibration curve using the raw volume data. Figure 9 shows the same phospholipid after the data was normalized.

Analyze the Unknowns

The calibration curve is selected from a directory listing. The data in RAM is used with the selected calibration curve. The internal standard spot number and sample spot number are entered. A concentration value appears for each spot entered.

RESULTS

The sample data shown in Table 4 are from the calibration curves in Table 1 and 2. The column labelled "CONC VOLUME" in Table 4 gives the concentrations calculated from the raw volumes. The differences range from 9 to 55 percent. When the volumes were normalized to the internal standard spot, the percent difference diminished dramatically. Each plate was spotted, developed and sprayed.

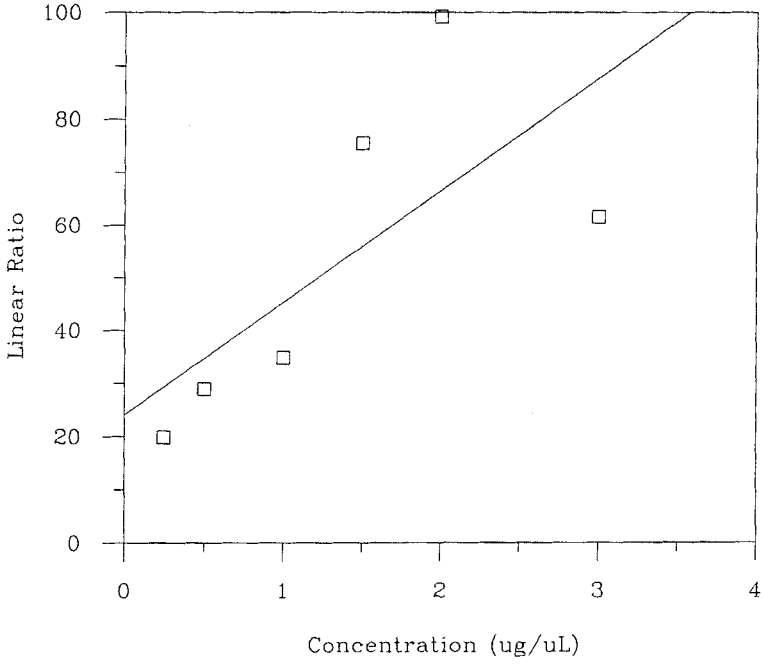

Figure 8. Calibration curve from raw volumes - phosphatidyl inositol.

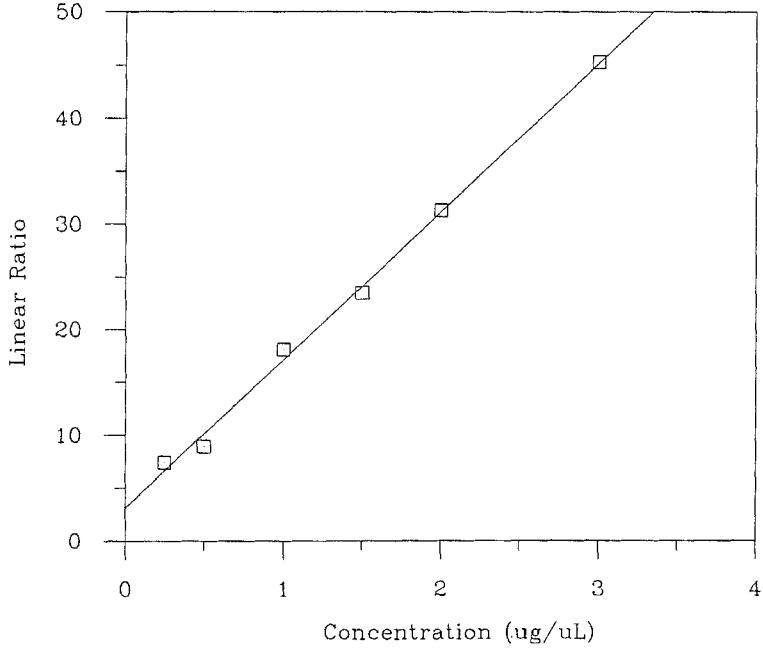

Figure 9. Calibration curve from normalized volumes - phosphatidyl inositol.

TABLE IV

Phospholipid Analysis Actual vs. Calculated Concentrations

		ACTUAL CONC μG/μL	CONC VOLUME μG/μL	REL %DIFF	CONC NORM μG/μL	REL %DIFF
SAMPLE 1						
	PE	3.00	3.28	9.3	3.09	3.0
	PC	2.00	1.63	18.5	1.81	9.5
	PI	2.00	2.25	12.5	1.97	2.5
	PA	1.00	0.71	29.0	0.92	8.0
SAMPLE 2						
	PE	2.00	1.47	26.5	1.93	3.5
	PC	1.00	0.45	55.0	0.95	5.0
	PI	2.00	1.61	19.5	1.85	7.5
	PA	3.00	2.22	26.0	3.08	2.6

There were significant variations among plates due to the spraying reagent. However, only a 10% difference is seen in the results. The comparison is dramatic and clearly indicates the importance of ratioing the volume to an internal standard spot.

DISCUSSION

Accurate quantitative analysis requires careful standard preparation, spotting technique, development, and spraying. Electrophoretic gels also require careful attention to application of sample, development and staining. We have shown that difficult two dimensional media can be quantitated accurately.

Besides careful laboratory technique, the densitometer used must have a high degree of accuracy. The CS9000U with its QuantaScan software can provide the biochemist with reliable quantitative data without guesswork.

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