HIGH PRESSURE LIQUID CHROMATOGRAPHY

Biochemical and Biomedical Applications

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CHAPTER 5

QUANTITATION

In order to perform quantitative analyses or kinetic studies by high pressure liquid chromatography, it is necessary to be able to determine accurately the concentration of one or more components present. With the measurement and calculation methods available, high pressure liquid chromatography is a highly accurate analytical technique, comparable in accuracy to gas chromatography. The quantitative results obtained depend on the complete process of analysis from preparation of the sample to interpretation of the results. As in a chain, the analysis is no better than the weakest link. Therefore, each step must be considered in developing techniques and interpreting the final results.

There are four basic steps in quantitative liquid chromatography: (1) chromatography, (2) integration, (3) determination of sample composition, and (4) statistical analysis of the data. In the chromatography step, an analog signal is generated by the detector and recorded in the form of chromatographic peaks. The area under the peak is converted to digital data in the integration process. Peak areas can be integrated either by manual methods or by using integrating devices. The calculation step consists of relating these data to the composition of the sample. In the final step, the data are expressed through statistics.

I. Chromatography

A. Sample Preparation

Using high pressure liquid chromatography in biochemical and biomedical research, one of the most important parts of the analytical procedure is the preparation of the sample. If the samples are not properly and quickly handled, compounds such as nucleotides can be easily degraded by the enzymes present in tissues. Pipetting precision is essential because large errors in accuracy can be caused by relatively small pipetting errors. In preparing cell extracts using the diethyl ether extraction of TCA from a sample, volume changes can cause as much as 10% difference in reproducibility. The presence of a large concentration of salts also causes differences in chromatograms which result in quantitative errors. In making up solutions of samples or standards, the careful calculation of molarity or concentration is necessary. Too concentrated a solution may cause overloading of the column; thus poor peak shapes are obtained, or there will be problems in the subsequent chromatograms. Also errors in the final data can result from faulty weighings or dilutions of standards, reagents, or samples. Not only is sample preparation important, but care must be taken in sample storage because problems such as changes in concentration due to evaporation, contamination, and salting-out effects can prevent accurate analyses. In addition, some nucleotides may catalyze their own decomposition if not stored under appropriate pH conditions.

It is of the utmost importance that not only the procedure for preparation and handling of the sample be standardized, but also the standard or references should be handled and stored in the very same way as the samples. The probability of accurate results will be increased if extreme care is taken in the standardization procedure.

B. Sample Introduction

Since the sample volume used in high pressure liquid chromatography is so small, it is critically important that the sample be carefully and quantitatively introduced into the column. The syringe must be absolutely clean and not blocked, no air bubbles must be present, and the sample volume must be measured precisely. If possible, the syringe should be rinsed several times with the sample solution so that there is no dilution effect. The needle must be wiped dry to prevent extra drops from being injected. For this purpose, Kimwipes should be used to prevent contamination by a used

cloth. In most instruments, the needle fits right into the column so that no sample is lost during the injection process. If the stopped-flow technique of injection is used, the pressure is stopped for the injection process. The injection technique is not as critical using this technique as it is when the sample is injected into the flowing stream through a septum, i.e., pressure is not stopped. It is also important to make sure the syringe is emptied completely. The sample must be injected slowly so that all the liquid is introduced evenly in the column, thus preventing the formation of a pool of sample at the injection port.

C. Separation of Peaks

In the chromatography process, in which the compounds migrate differentially, there may be decomposition of components during the chromatographic process. Care must be taken to avoid thermal or chemical degradation of any of the compounds present. Decomposition may lead to the presence of new peaks, the absence of expected peaks, or the masking of peaks normally in the chromatogram. If there is peak overlap or tailing, different eluents, column packings, or change in temperature should be tried. If, however, separations which were previously good deteriorate, it is possible that the column packing has changed. If the problem is due to an accumulation of impurities on the column, the column should be washed well. If, however, washing does not help, it is possible that the packing material has decomposed irreversibly and a new column should be tried. For an example of the poor separation caused by column deterioration see Fig. 3–23. In the upper chromatogram there is excellent separation of ATP and GTP in a cell extract of rat liver. However, these peaks in the same sample are poorly resolved in the lower chromatogram which had been run a few days before on a column that had been used for $2\frac{1}{2}$ years.

One problem that always faces chromatographers is whether there is any retention of solutes on the column that will bleed off in subsequent runs. The process is referred to as "ghosting." In order to determine whether or not there is "ghosting," it is possible to run an experiment using isotopes. An experiment such as this was carried out by Brown (1970). A standard solution of adenine nucleotides was run followed by a cell extract containing ^{14}C adenine and guanine nucleotides. Fractions were collected for both the samples and the low concentrate wash after each sample. Another standard solution of adenine nucleotides was run and fractions of this sample and the subsequent wash were also collected. The radioactivity of all the fractions from each solution was counted and it was found that peaks of the radioactive sample as plotted corresponded to the chromatogram from the analyzer (see Fig. 4–4). The wash solutions and the standard solution

run both before and after the ^{14}C samples were found to have only background levels of radioactivity. Therefore, it was concluded that the nucleotides were eluted completely.

D. Detection and Amplification

Important detector specifications are sensitivity, linearity, and specificity. Errors in detection and amplification can be due to lack of familiarity with electronics. Knowledge of detector specificity is important in quantitative analysis. For example, all compounds do not absorb in the UV. Therefore, some compounds will not have peaks in a chromatogram obtained using a UV detector. Also, as was discussed in the section on detectors, column flow rate, detector electronics, and temperature all affect the quantitative results. It is important to know which parameters are critical with the detector being used so that proper precautions can be taken. It is necessary to know if the concentration of the compound to be quantitated is linear with the area of the peak. Modified Beer's laws plots should be determined for each component in a sample. An example of the plot of concentration of AMP (in nanomoles) vs the area (in square centimeters) of the AMP is shown in Fig. 5–1.

II. Integration

The integration step consists of converting the detector signal into numerical data. Since the detector signal is usually in the form of peaks in a chromatogram from a strip chart recorder, it is necessary to convert the

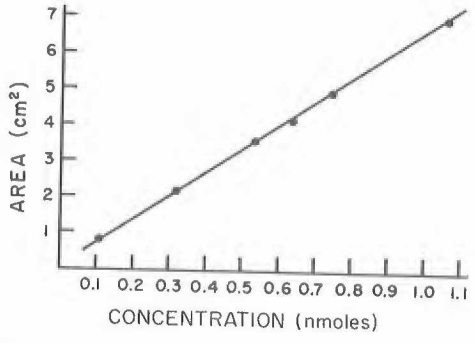

Fig. 5–1. Plot of concentration (in nanomoles) of AMP vs area (cm^2) of AMP peak. [Reproduced from Brown (1970).]

peak area or heights to numbers. This can be done manually or by electronic techniques. If electronic techniques are used, neither a recorder nor an operator may be necessary for the conversion step. However, it is useful to have the chromatogram as it is sometimes possible to spot differences in peak areas or shapes that might not be detectable as easily with numbers alone. These differences could be due to actual reactions in the cell or cell extracts or could be symptoms of trouble in the chromatographic system. When a strip chart recorder is used, characteristics that can affect the accuracy of the results are the dead band, linear range, pen speed, shifting of the balance point, chart paper degree of filtering, or irregular pen movements. The first two characteristics are fully described in Varian's "Basic Liquid Chromatography."

A. Manual Methods

The manual methods for converting detector signals into numerical data are: (1) planimetry; (2) measurement of peak height; (3) measurement of height times width at half-height; (4) triangulation; and (5) cut and weigh.

A planimeter is a device which measures area by tracing the perimeter of the peak. (Fig. 5–2). The area is presented digitally on a dial. Since the precision and accuracy of this method depends not only on the device itself but largely on the steadiness and skill of the operator, the peaks should be traced several times and the results averaged. This method is not as accurate as the other methods. It is tedious and time-consuming as well. The second manual method is the measurement of peak height (Fig. 5–3). Although this procedure is simpler than the measurement of peak areas, it is not as accurate since peak height does not always change linearly with sample size. This is especially true if operating conditions vary or when the column is overloaded. In the third method, the area is approximated by multiplying the height of the peak by the width at half-height (Fig. 5–4). The accuracy of this method is affected by the measurement of the width since a narrow peak can adversely affect the precision. Rather than using the base, the width at half-height is used to reduce the errors due to tailing of the peaks and any baseline irregularities. The fourth method is that of triangulation in which a triangle is constructed by drawing tangents to the slope of the peak (Fig. 5–5). The area is calculated by the triangle formula $A = \frac{1}{2}BH$, where the base is taken as the distance between the intersection of the two tangents with baseline and the height is measured by the shortest distance between the baseline and the intersection of the two tangents. With this method, it is assumed that the peak is symmetrical. The accuracy and precision of this method is dependent on the skill of constructing the triangle and the complexity of drawing the tangent lines. A slight error in the placement of the tangents can have a large

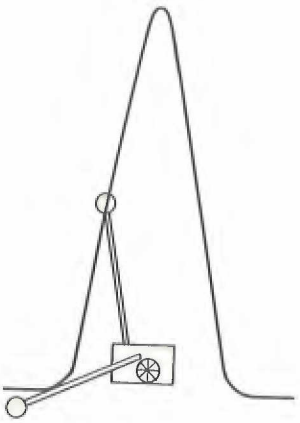

Fig. 5–2. Use of planimeter.

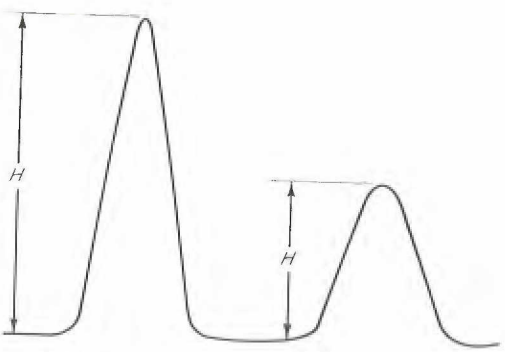

Fig. 5–3. Measurement of peak height.

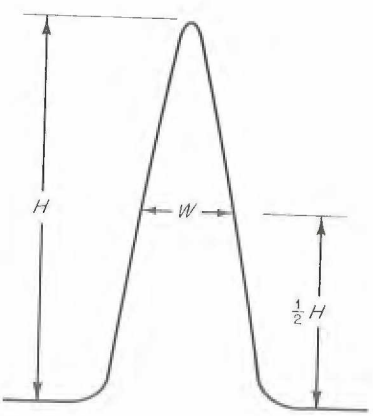

Fig. 5–4. Measurement of area using peak height and width at half-height.

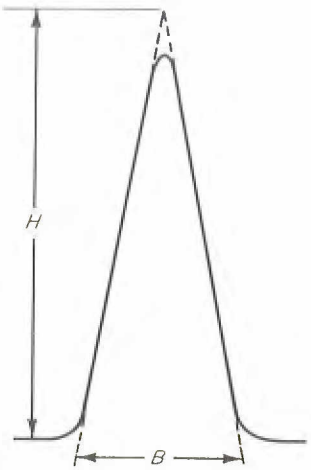

Fig. 5–5. Triangulation.

effect on the results. The fifth manual method used is the cut-and-weigh method; the peak is cut out of the chromatogram and weighed on an analytical balance. The constancy, weight and moisture content of the chart paper, and the skill in cutting are factors that affect the accuracy. Moreover, the chromatogram is destroyed; this can be a serious disadvantage. It has been found that cutting and weighing of a xerox copy of the chromatogram will minimize these disadvantages.

B. Integrating Devices

A number of integrating devices have been designed for direct attachment to recorders so that the peak areas are recorded and integrated simultaneously. Examples of these are electronic ball and disc, and analog and voltage integrating devices. One of the most widely used integrators in gas chromatography is the ball and disc type, manufactured by Disc Instruments Inc., because it provides automation relatively inexpensively. The accuracy and precision obtained is dependent on careful adjustment of the operation of the recorder and is limited by the mechanical performance of the recorder. However, although the results are excellent when the baseline of the chromatogram is steady and the separation of the peaks is good, the disc recorder is difficult to use under other conditions. The electronic digital integrator is one in which the chromatographic input signal is fed into a voltage to frequency converter and an output pulse rate, proportion to the peak area, is generated. The pulses from the voltage to frequency converter are accumulated and printed out as a measure of peak area when the

slope detector senses a peak. The major advantages of this integrator are the wide linear range, high count rate, and sensitive power detection. Although they are expensive, the cost may be worthwhile because of their high precision, sensitivity, and the rapidity with which they work.

Computers are being used to integrate peak areas. Although digital integrators are accurate and sensitive devices for measuring the peak areas, they do not handle the calculations or interpret the data. Computer systems that have been used include off-line, hybrid, dedicated computers, or multichannel dedicated, or time- shared computers. In the off-line, batch-processing approach, the data on printed or punched paper tape from the digital integrator is manually transferred to a computer for processing. The hybrid has an integrator on-line to a computer. In the dedicated computer, a single computer is attached to a liquid chromatograph. This type of system is used mainly for research studies. In the multichannel dedicated computer many chromatographs are on-line to one computer dedicated to this work. In the time-shared computer, chromatographs, as well as other analytical instruments, are on-line to one large computer. Time-shared services have made off-line computation available to small laboratories and can provide good computorial powers and storage of such data as compound name, retention time, and response factor.

Burtis and Gere (1970) investigated six integration techniques and tabulated the precision of the results obtained by the various integration methods. The time required to perform the integration varied over a wide range. Ten chromatograms were picked at random for evaluation of these techniques and operators skilled in the various techniques performed the integration. The results (Table 5–1) show that the manual methods are expensive in terms of time expended on the integration. The precision of the digital integration was far better than the majority of the manual methods. The disc ® integrator, although not as fast or as precise as the digital integrator, was more rapid and more precise than manual methods. The digital integrator can correct the peak area for baseline drift and offset, sense and separate shoulders and overlapped peaks, and identify peaks by relative retention time. The on-line computer also has many advantages. This system not only detects peaks by relative retention times, but it also calculates area percentage of each peak, calculates composition results in desired units, and types an output of the quantitative result. Although the time of integration and precision are about as good as the digital integrators, its main advantage is that it has greater manipulative power.

When quantitating nucleotide peaks, at least 10 samples of a standard solution of each nucleotide should be run. Because absorbance is directly related to molar extinction coefficients at the wavelength used, separate calibrations are required for each nucleotide. The concentration of a

TABLE 5–1

COMPARISON OF INTEGRATION METHODS[a]

	Planimeter		Triangulation		$H \times W$ at $\frac{1}{2}H$		Cut and weigh		Disc		Digital	
	Avg.	σ rel (%)	Avg.	σ rel (%)	Avg.	σ rel (%)	Avg.	σ rel (%)	Avg.	σ rel (%)	Avg.	σ rel (%)
Peak 1	0.04	20	0.05	14	0.04	12	0.005	14	0.04	18	0.03	0.10
Peak 2	4.84	1.65	4.79	8.77	4.52	3.76	5.04	1.98	4.57	0.44	4.56	0.88
Peak 3	14.01	5.64	13.70	4.53	13.56	1.62	14.99	2.80	14.05	1.00	14.06	0.36
Peak 4	18.52	3.29	18.52	4.75	18.66	3.05	18.40	1.22	18.73	2.46	18.73	0.27
Peak 5	8.12	5.67	8.55	3.74	8.56	2.22	8.01	2.12	8.07	2.60	8.24	0.49
Peak 6	20.18	6.49	20.32	3.25	20.07	2.04	20.09	1.39	20.21	1.14	20.16	0.20
Peak 7	16.11	3.66	15.83	1.58	16.41	3.71	15.85	1.45	16.06	0.68	15.95	0.38
Peak 8	18.19	2.03	18.25	1.81	18.17	1.65	17.58	1.19	18.28	0.71	18.27	0.49
Time to trace (min)	45–50		45–60		50–60		100–120		15–30		5–10	
Precision (σ rel)[b]	4.06%		4.06%		2.58%		1.74%		1.29%		0.44%	

[a]From Burtis and Gere (1970).
[b]Excludes peak 1.

TABLE 5–2

TABULATION OF AREAS UNDER THE PEAKS OF A STANDARD SOLUTION OF AMP[a,b]

Spectrum no.	Area/nmole
163	6.42
171	6.81
173	6,74
174	6.87
175	6.92
176	6.49
180	6.76
181	6.81
182	6.52
183	6.69
Mean	6.71
Standard deviation	0.18
Coefficient of variation	2.8

[a]From Brown (1970).

[b]The AMP solution was 6.08×10^{-5} *M*. The volume of sample solution injected ranged from 6 to 8 μl. *Instrument*: Varian LCS 1000. *Starting volume*: 50 ml. *Flow rates*: 12 and 6 ml/hr. *Eluents*: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. *UV output*: 0.08.

nucleotide in a cell extract is then determined by relating the peak area to the peak area of 1 nmole of the standard nucleotide. In all cases, the same method of calculation should be used for standards as for samples. An example of the use of this method of calculating the peak area by multiplying the height times the width at half-height, is the AMP run by Brown (1970) (Table 5–2). The standard deviation of the 10 samples was 0.18 and the coefficient of variation 2.8%. The concentration of all adenine nucleotides and cell extracts can be calculated by relating peak areas to that of the standard AMP since all adenine nucleotides have the same molar extinction coefficient. A comparison of values of total adenine nucleotide content as determined by high pressure liquid chromatography and by an enzymic assay in four different blood samples showed close agreement. These values are shown in Table 5–3 (Brown, 1970).

TABLE 5–3

TOTAL ADENINE NUCLEOTIDES (AMP + ADP + ATP) (nmoles/μl)[a,b]

	Sample			
	H	P	A	S
Varian LCS 1000	0.387	0.344	0.354	0.229
Enzymic analysis	0.393	0.332	0.348	0.230

[a]From Brown (1970).

[b]*Instrument*: Varian LCS 1000. *Column*: 1 mm × 3 m; packed with pellicular anion exchange resin. *Eluents*: 0.015 *M* KH_2PO_4; 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. *Flow rates*: 12 ml/hr, 6 ml/hr. *Starting volume of low concentration eluent*: 50 ml. *UV output*: 0.08.

III. Calculation

In liquid chromatography, as in other chromatographic methods, the composition of the solute must be calculated. There are three principal methods used for relating the digital data to the composition of the sample: normalization, internal standardization, and calibration techniques.

A. NORMALIZATION

In the normalization method it is assumed that the entire sample is eluted and detected. The percentage of peak x, in Fig. 5–6, is given by the formula:

$$\%x = \frac{A_x}{A_x + A_y + A_z} \times 100$$

A_y, A_x, and A_z represent the individual peak areas. This method can be useful when analyzing complex mixtures. Since the relative peak areas obtained in liquid chromatography are not always related to composition, because the response of a given detector may be different for each molecular type or class of compound, it is necessary to use response factors. This will

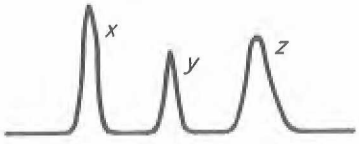

Fig. 5–6. Normalization.

correct not only for the response of the detector, but also can serve as a factor for band-broadening with time. Since response factors are not easy to estimate, they are best obtained by analyzing standard samples. Response factors relative to a reference compounds, are given by the formula:

$$F_x = \frac{F_r A_r W_x}{A_x W_r}$$

A_x and A_r are the areas of the solute and reference peaks, respectively, W_x and W_r are the amount of the solute and reference compounds, respectively, and F_r is the response factor assigned to the reference compound. Corrected areas are obtained by multiplying the area by the relative response factor. Since different detectors operate on different principles, different factors must be calculated for different detectors.

B. Internal Standards

In order to eliminate apparatus and procedure errors, internal standards can be used. The requirements for an internal standard are as follows:

1. It must be completely resolved from all the unknowns
2. It must elute near the peak of interest
3. It must be similar in concentration to the peak of interest
4. It must be chemically similar but not present in the original sample
5. It must be chemically inert

The percentage of one compound can be calculated by using the formula:

$$\%x = \frac{A_x}{A_{is}} \times \frac{F_x}{F_{is}} \times \frac{W_{is}}{W_s} \times 100$$

The areas are represented by A_x and by A_{is}, the correction action by F_x and F_{is} of the unknown peak and internal standard, respectively, and the weights by W_s and W_{is} of the sample and internal standard, respectively. This procedure is used when the sample is not completely eluted, when it is necessary to measure one or two peaks accurately, or to compensate for errors made during the preparation of the sample. A graphical interpretation of results are shown in Fig. 5–7.

C. Calibration

In calibration, one external standard is used so that there can be direct comparison of the peak area of the sample to that of a standard which has

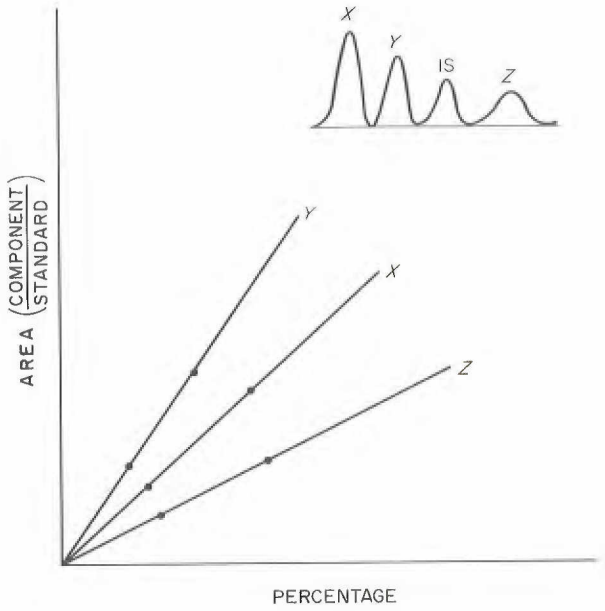

Fig. 5–7. Use of internal standard.

been injected directly. A calibration factor K is determined by injecting the standard solutions and the results are expressed by the formula:

$$\%x = A_x K$$

This technique depends upon the precise injection of the standards. It is particularly useful in analyzing simple mixtures or for trace analysis. If the peak areas are plotted versus the known weight of the compound, this is known as absolute calibration (Fig. 5–8). If, however, the areas of the sample divided by the standard are plotted against the weight of the sample divided by the standard, this is known as a relative calibration curve (Fig. 5–9). In practice, an accurately known amount of an internal standard is added to an unknown sample and the mixture is chromatographed. The area ratios are measured and from the calibration graph, the weight ratios of sample to standard are obtained. Since a known amount of standard was added, the amount of unknown sample can be calculated. The advantages of this technique are that the quantities injected need not be accurately measured and the detector response does not have to be known nor remain constant. The major disadvantage, especially in dealing with nucleotide pools of cell extracts, is the difficulty in finding a standard that does not interfere with the components in the sample.

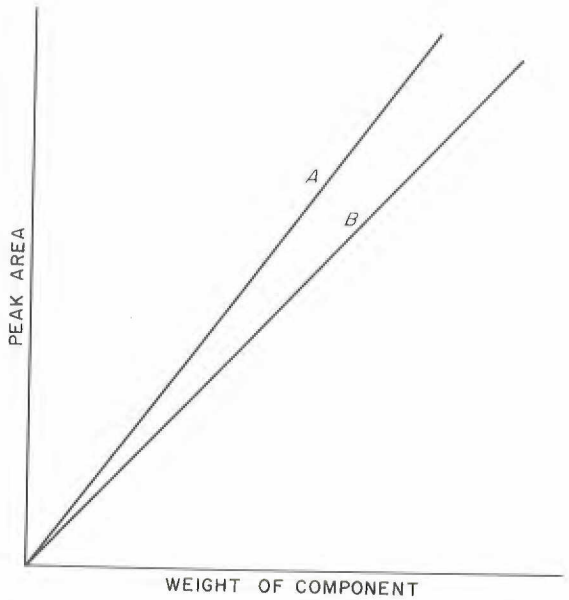

Fig. 5–8. Absolute calibration curve.

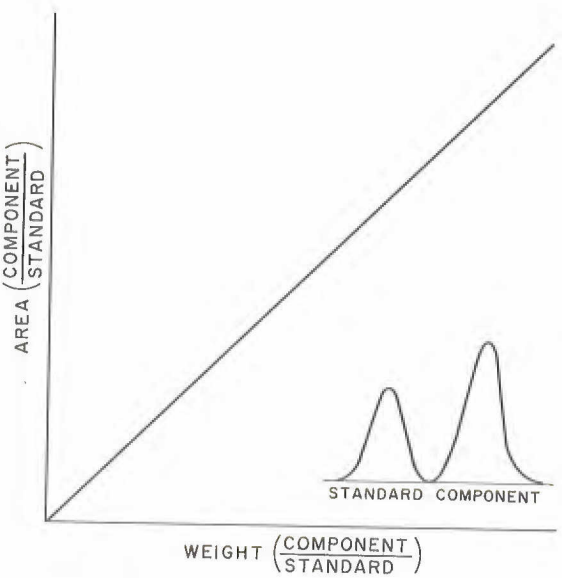

Fig. 5–9. Relative calibration curve.

IV. Statistical Treatment of Data

A. Definition of Terms

In quantitative analysis, the terms accuracy, precision, repeatability, and reproducibility are frequently used. Accuracy is defined as the measurement of difference between a trial value and the true value. Precision is an expression of exactness or a measure of how well replicate values agree. Accuracy is difficult without precision but precision does not insure accuracy. However, precision with proper calibration gives accuracy. When a single operator repeats an analysis on the same apparatus, repeatability is the term used to define the precision. If the same analysis is run by different operators on different instruments, possibly in different laboratories, the term reproducibility is used to define the precision.

B. Sources of Error

In all measurements, there are errors. Errors can be divided into two categories: indeterminate and determinate. Indeterminate errors are random errors which cannot be eliminated and are inherent in the analytical technique. If minimized, they give high precision. Determinate errors are those errors whose cause and magnitude can be determined. If the determinate errors can be minimized, high accuracy is achieved. However, they can never be completely eliminated. In high pressure liquid chromatography, some determinate errors are: (1) poor sampling techniques; (2) decomposition on the column; (3) change in detector response; (4) recorder performance; (5) calculation errors; and (6) operator prejudice and carelessness.

Errors in poor sampling techniques can result from poor preparation of the sample or improper storage which may cause decomposition or change in concentration. It is possible that all the sample is not injected completely into the column or that there is inaccurate measurement in the syringe. The sample may decompose on the column, causing inaccurate results, or there may be contamination of the sample before injection. It is even possible that the wrong sample may be taken for analysis or the wrong solution may be prepared. The incorrect calculation of the concentration may cause large errors. Overloading of the column cannot only cause poor peak shape and masking of peaks in that particular chromatogram, but in many chromatograms thereafter. As in all analytical techniques, complete and accurate labeling of samples is absolutely necessary. Changes in detector responses may be caused by changes in temperature or flow rates. Also it is important to realize that different detectors give different responses to the same

compound and there are different responses by one detector for different compounds. For example, when a UV detector is used, the absorbance is proportional to the molar extinction coefficient. Therefore, for each compound separate calibrations are required. It is also possible that the recorder performance is defective. Calculation and calibration errors are possible sources of inaccuracies. Calculation errors are especially common when there is peak overlap or a drifting baseline. There may be errors in the integrating operation. Another possible source of determinate errors is operator prejudice; the results may be predetermined in the mind of the operator. As was pointed out in Chapter 3, one of the most important factors in obtaining the best performance from a liquid chromatograph is an alert, careful, well-trained technician or operator who has the responsibility for the overall operation.