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liquid chromatography detectors

second, completely revised edition

R.P.W. Scott

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Volume 33 **Liquid Chromatography Detectors.** Second, Completely Revised Edition by R.P.W. Scott

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PREFACE TO THE SECOND EDITION

The renaissance of liquid chromatography, provoked by the spectacular development of gas chromatography, took place in the late 1960's and early 1970's. The first edition of this book published in 1977 described the detectors that were available at that time and which provided a performance matching that of the contemporary equipment with which they were associated. It is interesting to note that the most popular detectors then, the UV detector, the refractometer detector, the fluorescence detector and the electrical conductivity detector are still the most commonly used detectors today, nearly a decade later. Detector design, however, has changed very significantly over the intervening years. Modern-high efficiency columns provide very narrow peaks and very fast separations, and thus the physical design of the detectors had to change to meet these new challenges. In 1977, there was little real understanding of the important role played by the detector in the overall function of the chromatographic system and although some of the factors were pointed out in the first edition of this book, in retrospect they appeared to be little understood.

This book is an entirely new presentation of the subject of liquid chromatography detectors. It now contains sections dealing with the fundamental aspects of the interaction between columns and detectors and the interaction between ancillary equipment and the detector. It brings the reader up to date with new designs and novel detecting systems that have been developed since 1977 and extends significantly the subject of the association of the liquid chromatography detector with spectroscopic techniques. In particular, the book now explores the association of liquid chromatography with nuclear magnetic resonance spectroscopy, infrared spectroscopy and atomic absorption spectroscopy.

The author would like to thank his colleagues, Mrs. Elena Katz, Dr. Kenneth Ogan and Mr. Gary Schmidt for their many helpful suggestions in the preparation of this book, his wife Barbara Scott for her help in editing the manuscript and Mrs. Carol Nash for her untiring efforts in the preparation of the camera-ready copy the credit for which is solely hers.

December 1985 R.P.W. Scott

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Chapter 3

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CHAPTER 1

HISTORY, FUNCTION AND CLASSIFICATION OF DETECTORS

History and Function

A liquid chromatographic detector is a device that locates, in the dimension of space or time, the positions of the components of a mixture that has been subjected to a chromatographic process and thus permits the senses to appreciate the nature of the separation that has been obtained. This definition, by necessity, has to be broad as it needs to encompass all detecting systems ranging from the elaborate electronic devices presently available, to the human eye or even the sense of smell. Tswett in his pioneering chromatographic separation of plant pigments used the human eye to determine the nature of the separation he obtained and even today, as one of the more common separation techniques employed is thin-layer chromatography, the human eye is still one of the more frequently used detectors. The human eye, as a liquid chromatography (LC) detector, however, has severe limitations. The majority of substances that are chromatographed are colorless and thus have to be chemically changed to render them visible; furthermore the retinic response of the eye is not linear which, when coupled with the variation of the iris to light intensity, makes the eye a poor detecting system for quantitative estimation. The human eye, in fact can only be used for quantitative work as a null sensing device where closely similar light intensities are being matched as in the use of a comparator.

The lack of a satisfactory detector was probably the greatest single impediment to the development of LC over the years past. The rapid development of gas chromatography (GC) arose solely from the availability of sensitive detecting systems and it is interesting to note, that LC had to await the introduction of sensitive LC detectors, before the rapid advances in the technique that have occurred over the past few years, could take place. High sensitivity detectors have provided accurate concentration profiles of eluted solutes and allowed the magnitude of the solute dispersion (band spreading) that occurs in the column to be determined. The data produced has permitted the development and confirmation of theories that describe the various factors contributing to band dispersion which in turn have improved column technology. Thus, the impressive separations that are achieved today in both GC and LC can be directly attributed to the introduction of sensitive on-line detecting systems; that is detectors

that can be directly connected to the column outlet.

Returning to the initial development of LC detectors, the first attempt to develop an alternative to visual detection was to collect the column eluent as a large number of fractions and to subsequently analyze each fraction by appropriate techniques such as colorimetry or titration. The concentration of solute in each fraction was then plotted against fraction number and a chromatographic histogram obtained. This procedure was extremely laborious and time consuming and was only effective for well resolved mixtures. Furthermore, a chromatographic histogram did little to aid in the improvement of column technology. The concept of on-line detection, where an appropriate sensing device is connected directly to the column outlet, was established in the late thirties and early forties. Such a device provides a suitable signal that is directly related to the concentration of solute in the eluent. Examples of two of the early on-line detectors are given by the electrical conductivity detector described by Martin and Randall (1) and the refractive index detector described by Tiselius and Claesson (2). This book is concerned solely with on-line detection and, as the original definition of LC detectors embraced all kinds of detecting methods, it would be appropriate to specifically define on-line detectors. *An on-line LC detector is a device through which the column eluent flows and which provides a continuous output, usually electrical in nature, that is some function of the mass of solute or concentration of solute in the mobile phase passing through it.* The output from such a system can be passed directly, or by way of some suitable electronic device, to a strip chart recorder which will then provide a continuous record of the mass of solute eluted, or concentration of solute in the eluent with respect to time. In this way a graphical representation of the separation is obtained. Such systems can provide accurate concentration profiles of eluted solute bands and thus a quantitative analysis of the original mixture can also be achieved.

The major progress in the development of LC has occurred over the past two decades and this has been due in part to the impetus provided by the rapid advances that have taken place in the technique of GC. GC detection principles have been directly applied to the development of LC detectors. Furthermore, many of the scientists involved in the development of GC detectors, on exhausting the challenges of GC, turned their attention to the field of LC . Between 1956 and 1960 at least six GC detectors were invented and developed to their full potential; the progress in the development of LC detectors has been, by way of contrast, slow and arduous.

The problem of detection in LC is far greater than in GC because low concentrations of a solute in a liquid do not modify the overall physical characteristics of the liquid (e.g. density,

dielectric constant etc.) to the same extent that low concentrations of vapor in a gas modify the physical properties of the gas. It follows that LC detectors which function on the principle of measuring some overall physical property of the eluent will provide signals from low concentrations of solute that may be only equivalent to signals arising from small changes in ambient conditions or fluctuations in column flow rate. The detector signal will therefore be commensurate with that of the detector noise and will consequently exhibit very limited sensitivity. Nevertheless steady progress in detector development has been made and there are a number of effective LC detectors available. However, there is at present no LC detector that possesses all the necessary attributes that are required for a completely versatile liquid chromatograph. There is no universal detector in LC that compares with the flame ionization detector in GC. The most sensitive general detector is the UV detector and the most versatile probably the now obsolete wire transport detector. If more specific detectors are considered, the most sensitive is probably the fluorescence detector, although the electrochemical detector could be a close competitor.

Over the past decade there has been a continuous interaction between improved detector performance and improved column performance; each advance being mutually dependent on the other. Initially high sensitivity detectors permitted a precise column theory to be developed which resulted in the production of columns of much greater efficiency. The improved efficiency, however, produced peaks of very small volume, small that is, compared with the dispersion that occurred in the connecting tubes and sensing volume of the detectors themselves. At this point, the ultimate efficiency obtainable from the column was determined by the geometry of the detector, not its sensitivity. As a result the design of the detector was altered; the dimensions and geometric shape of the connecting tubes were changed and the volume of the sensing cell were greatly reduced which permitted the use of very small diameter particles for column packing. The small diameter particles resulted in even higher efficiencies (3) and even smaller peak volumes. The smaller diameter particles also provided columns that would produce much faster separations (4) which at the extreme permitted solute mixtures of moderate complexity to be separated in a few seconds (5). In such separations, individual peaks often had widths of only a few milliseconds. The standard deviation of such peaks was of the same order as the time constant of the detector and its electronics and thus the ultimate speed of a separation was again limited by detector design. As a consequence the time constant of detector electronics has also been significantly reduced. An example of the high speed separation of a simple mixture is shown in Figure 1. The analysis is complete in less than four seconds.

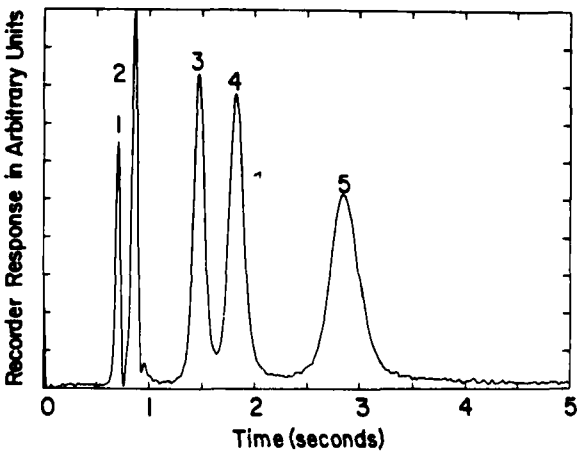

Figure 1. High-speed isocratic separation of a five-component synthetic mixture. Packing: Hypersil 3-μm, Column I.D.: 0.26 cm, Column length: 2.50 cm, Mobile phase: 2.2% methyl acetate in n-pentane, Linear velocity: 3.3 cm/sec. 1 p-xylene, 2 anisole, 3 nitrobenzene, 4 acetophenone, 5 dipropyl phthalate.

The introduction of small bore columns (6) producing peaks of a few microliters in volume placed an even greater strain on detector design and at the time of writing this book the specifications of commercially available detectors are again the limiting factors that control column performance. Column technology is now well understood and columns of far better performance than presently employed can be constructed, but they will have to await improvement in detector design before their full advantages can be realized.

Classification of Detectors

Detectors can be broadly classified into two types. *Bulk Property* detectors which function by measuring some bulk physical property of the column eluent (e.g. dielectric constant or refractive index) and *Solute Property* detectors which function by measuring a physical and/or chemical property that is characteristic of the solute only (e.g. UV absorption). This classification is not completely precise, for example, the UV detector, which is usually classed as a solute property detector, when used with an ethyl acetate-heptane solvent mixture as the mobile phase will give a constant background signal due to UV absorption by the ethyl acetate. Furthermore, any fluctuation in ethyl acetate content of the mobile phase will appear as noise on the detector output. It follows that the UV detector, although a solute property detector, behaves as a hybrid between a bulk property detector and a solute property detector under some conditions of use.

There are two other possible ways of classifying detectors. The first is to define them as either concentration sensitive detectors or mass sensitive detectors. Concentration sensitive detectors provide an output that is directly related to the concentration of the solute in the eluent whereas a mass sensitive detector give a response that is proportional to the mass of solute eluted per unit time and consequently is independent of the volume flow of the solvent. This type of classification has been used satisfactorily for GC detectors, but as there is only one detector in LC that responds to the mass of solute eluted per unit time (7) and furthermore, it is very insensitive and not commercially available, this classification is not, at present, appropriate for LC detectors.

The second alternative classification is to define detectors as specific and non-specific detectors. In this sense a specific detector would be exemplified by the fluorescence detector as it detects only those substances that fluoresce. An example of a non-specific detector would be the refractive index detector that detects all substances that have a refractive index different from that of the mobile phase. The classification of detectors as specific and non-specific is acceptable, but in this book detectors will be classified as bulk property detectors and solute property detectors as it more closely associates the detector with its basic method of measurement.

Irrespective of its class, a LC detector should ideally have the same characteristics as the ideal GC detector with a sensitivity of about 10^{-12}-10^{-11} g/ml and a linear dynamic range of five or six orders. The LC detector should also be completely versatile and detect all classes of solutes while at the same time being independent of the characteristics of the mobile phase and consequently the mobile phase composition. Under such circumstances changes can be made in the composition of the mobile phase during chromatographic development, by gradient elution for example, without affecting the output of the detector. Unfortunately, no LC detector so far devised nearly approaches these specifications. However, if taken together as a group, the present LC detectors do embrace most of the important characteristics of the ideal detector. For this reason, work in the field of LC often requires the availability of a number of detectors of different types, so that the one appropriate can be chosen for any particular application.

The properties of the detection system employed for a chromatographic analysis can be as critical as the properties of the column itself and it is now well understood that column performance and detector performance are interactive. The properties of the detector controls the mass sensitivity and the concentration sensitivity of the whole of the chromatographic system. Detector characteristics also determine the minimum

diameter of the column that is to be employed (and consequently solvent economy) and indirectly controls the maximum volume of sample that can be placed on the column. It follows that to design the optimum column for a particular separation and to determine the correct operating conditions it is necessary to have available accurate values for the various pertinent detector specifications that control the overall performance of the total chromatographic system.

It is now necessary to identify those properties of the detector that are critical for the effective performance of a chromatograph, to define them, describe how to measure them and determine the most useful units in which they should be reported.

Synopsis

The slow development of LC from the time of Tswett, to the late 1950's was entirely due to the lack of high sensitivity on-line detectors. Since, the inception of effective LC detectors there has been a continuous synergistic interaction between column development and detector development which has resulted in the present highly sophisticated LC systems of today. There are a number of ways of classifying LC detectors, *specific* and *non-specific* detectors, *mass* and *concentration sensitive* detectors and finally *bulk property* and *solute property* detectors. The classification of detectors as bulk property and solute property detectors is recommended. Bulk property detectors respond to a change in some overall property of the eluent such as refractive index or dielectric constant whereas solute property detectors respond to some property that is unique to the solute alone. In practice solute property detectors are rarely ideal and many respond, at least weakly, to the same property of the mobile phase as well as the solute.

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CHAPTER 2

PERFORMANCE CRITERIA OF LIQUID CHROMATOGRAPHY DETECTORS

In order to evaluate a detector for use in liquid chromatography (LC), accurate performance criteria or specifications must be provided by the manufacturers. This is necessary to assess the pertinence of a given detector for a particular chromatographic separation, and/or to permit a rational comparison with other detectors. More important, such performance criteria will allow the optimum column to be designed to achieve a particular separation for which the detector is to be used. It follows that the performance data provided for each detector must be presented in a standard form and given in standard units which will be consistent between detectors that function on widely different principles. The principal characteristics of a detector that will fulfill these requirements are given as follows.

Principal Detector Characteristics

- Dynamic range
- Response index or linearity
- Linear dynamic range
- Detector response
- Detector noise level
- Detector sensitivity or minimum detectable concentration
- Total detecting system dispersion (detector cell and connecting tube)
- Connecting tube form and dimensions
- Cell dimensions, cell volume
- Overall detector time constant (sensor and electronics)
- Pressure sensitivity
- Flow rate sensitivity
- Temperature range

In the past, there has been much confusion in the field of LC, not only with respect to the units in which the above specifications should be given, but even in the exact definition of these criteria. This confusion has resulted partly from the use of criteria developed for other instrumental devices which are not applicable to LC detectors and partly due to some manufacturers selecting ambiguous criteria in order to present their products in the best possible light. It follows that the various criteria and specifications of a given detector must be unambiguously defined and very carefully described.

As already stated, a detector can be either a mass sensitive or concentration sensitive device, that is to say, the detector

output can be some function of mass of solute passing through it per unit time or mass of solute per unit volume of mobile phase. The flame ionization detector used in gas chromatography (GC) is a mass sensitive detector in that, within limits, the response is constant for a given mass flow and independent of the volume flow of hydrogen or carrier gas that passes through it. LC detectors that are at present commercially available are entirely concentration sensitive devices, thus, the parameter measured by the detector will be assumed to be mass/unit vol. or g/ml. Before dealing with each detector characteristic, it would be useful to consider the nature of the detector output and to discuss the units in which the detector characteristics are defined.

The Nature of the Detector Output

There are three common types of detector output, proportional, integral and differential. A proportional detector, as its name implies, is one that provides a signal that tends to be directly related to the concentration of solute in the mobile phase passing through it. Thus

$$y = Ac$$

where y is the output of the detector in appropriate units
c is the concentration of solute in the mobile phase in g/ml
A is a constant.

All proportional detectors are designed to give as near a linear response as possible to minimize calibration and calculation procedures when used for quantitative analysis. In many instances the output from the *sensing device* of the detector may not be linear, in fact, it is often logarithmic or exponential and thus, the associated electronics have to be designed to render the output linear. For example, a sensing device with an exponential output when used with a logarithmic amplifier would provide a linear response.

Theoretically, there is no reason why a detector should not have a logarithmic, exponential or any other functional output provided the function could be explicitly defined. However, this would render chromatograms from the detector unfamiliar, difficult to interpret, and quantitative analysis more involved and time consuming. For this reason detectors that do not have a linear output are not readily acceptable by chromatographers generally.

The profile of an eluted peak closely resembles the Gaussian or error function curve where the independent variable is volume of mobile phase and the dependent variable is solute concentration. If the flow rate through the column is kept constant, then the independent variable can be replaced by time. Thus, a linear detector will provide an accurate representation of the Gaussian

profile of the peak as shown in Figure 1.

If a detector with a linear output is connected to an integrating amplifier, an integral output can be simulated, an example of which is also given in Figure 1.

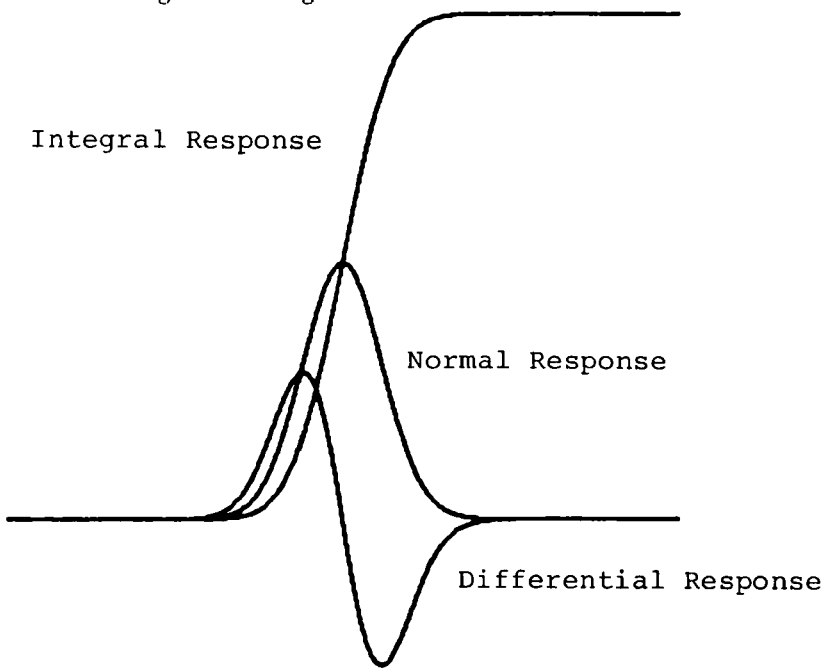

Figure 1. Types of detector response to a Gaussian elution curve.

For a concentration sensitive detector to directly provide an integral response:

$$y = {}_{o}\int^{v} cdv = cv = m$$

where y is the detector output
c is the concentration of solute/unit volume
v is the total volume of mobile phase passed through the detector
m is the total mass of solute.

It is seen that the integral curve is directly related to mass of solute, and that the step height of the integral curve is proportional to the total mass of solute eluted.

To the best of the author's knowledge only one detector in normal operation provides an integral output and that is the mass detector which is not commercially available. However, all detectors provided with flow-through cells or the equivalent can, if

desired, be used to provide an integral output and this will be discussed later under "Special Detector Techniques". The integral chromatogram has advantages for quantitative analysis providing the peaks are well resolved; the integral chromatogram, however, is *not* popular among most chromatographers as, where separation is not complete, and for multicomponent mixtures, the individual solute bands are less easily located and identified than in the normal chromatogram.

In a like manner, a differential output can be simulated if the output from a linear detector is connected to a differential amplifier and an example of such a curve is also included in Figure 1.

For a concentration sensitive detector which provides directly differential output

$$y = \frac{dc}{dv}$$

The value of the differential curve is very limited as the chromatogram having unresolved peaks becomes very complex and extremely difficult to interpret. Furthermore, differentiation of the detector output can, under some circumstances, increase the noise by as much as an order of magnitude and consequently, reduce the detector sensitivity by the same amount. It is seen from Figure 1 that at the peak maximum of the *normal* curve the differential curve goes from a positive value, through zero to a negative value and thus, the electrical analogue of this signal can be made to activate a suitable timing device for the automatic recording of retention times. In normal operation the only detector that gives a differential response directly is the heat of adsorption detector and, moreover, only under very specific conditions. Under most working conditions the heat of adsorption detector response only tends towards the differential form, in that, the output for a normal Gaussian peak will be sigmoid in shape, the front being a positive peak which is followed by a negative tailing peak.

Units Employed in Detector Specifications

Detector specifications involving length, time and mass are normally given in centimeters, seconds, and grams, respectively and consequently, there is little disagreement in the literature over such measurements. The units employed in specifying detector sensitivity, and to a lesser extent dynamic range, however, has been the subject of considerable controversy. This disagreement arises from the diverse physical properties to which the different detectors respond. The UV detector responds to changes in UV absorption, the refractive index detector responds to changes in refrac-

tive index. As a consequence, the UV detector sensitivity is often given in absorption units and the sensitivity of the refractive index detector given in refractive index units. Such a procedure allows the performance of UV detectors to be compared with each other and the performance of refractive index detectors to be also compared, but does not permit a rational comparison between UV detectors and refractive index detectors. In a similar manner, defining the sensitivity of the electrical conductivity detector in mho's (reciprocal ohms) permits no comparison with either the UV detector or the refractive index detector.

It will be seen later that detector sensitivity is extremely important as it controls both the overall chromatographic mass sensitivity and concentration sensitivity and even limits the maximum k' (capacity factor) at which a solute can be eluted from a given chromatographic column. Such characteristics, however, require the sensitivity of the detector to be given in concentration units, e.g. g/ml, and furthermore, detector sensitivities given in concentration units will allow the performance of detectors functioning on quite different principals to be rationally compared. The problem arises, however, in defining the appropriate solute with which to measure sensitivity in these units. Obviously, toluene could be employed in defining the sensitivity of both the UV detector and the refractive index detector. However, it would not be suitable for measuring the sensitivity of the electrical conductivity detector as it does not ionize. Furthermore, if a solute of higher refractive index and lower absorption was chosen, the refractive index might be made to look more sensitive then the UV detector. Conversely, by a different choice of solute, the UV detector could be made to appear to have a higher sensitivity than the refractive index detector. The situation is further complicated by the fact that if the UV detector is a multiwavelength detector, then the sensitivity will change with the wavelength chosen.

It is therefore not surprising that some confusion has arisen over the units in which detector sensitivity is measured. Instrument manufacturers have avoided the problem by *not* employing concentration as a unit of sensitivity, leaving the chromatographer to express sensitivity given in absorption units or refractive index units, etc. in terms of concentration. This could be considered unreasonable from the point of view of the chromatographer who can not easily calculate the chromatographic mass and concentration sensitivity of his/her equipment, often does not know the extinction coefficient, refractive index or electrical conductivity, etc. of the solutes that are being chromatographed. Furthermore, from the point of view of quantitative analysis the chromatographer is only interested in the mass or concentration of the solutes in the sample anyway.

The manufacturer, in specifying detector performance, has a

responsibility to provide the chromatographer with the information that is needed. It will therefore be recommended that, as well as giving sensitivity in units characteristic of the method of measurement, a solution of quinine sulphate in ethanol should be used as the solute for sensitivity measurement in terms of concentration. The choice of this solute is not an ideal solution to the problem. It was chosen as it has a significant refractive index difference to that of most solvents, has a significant absorption in the UV, is ionized and consequently, its solution is conductive and, furthermore, has a significant fluorescence over a range of UV wavelengths and in particular at 254 nm. It must again be emphasized that this procedure may not be an ideal solution to the problem, but goes some way in helping the chromatographer by providing sensitivity in terms of minimum detectable concentration. This will permit the pertinent chromatographic properties of the system to be calculated and provides a common basis for the comparison of the sensitivities of different detectors. When dealing with specific types of detectors, alternative solutes may be more appropriate for sensitivity measurement than quinine sulphate. At the very least, however, it is recommended that minimum detectable concentration of this standard solute should be given for the most common detectors such as refractive index detectors, UV detectors, electrical conductivity detectors, and fluorescence detectors.

The Dynamic Range of a Detector

There are two quite different detector response ranges of which the operator should be aware, the Dynamic Range of the detector and the Linear Dynamic Range of the detector, and in almost all instances the two ranges are *not* synonymous. The dynamic range of the detector is that solute concentration range over which the detector will provide a concentration dependent output. The minimum of this range will be at the solute concentration level where the detector output is twice the value of the noise (which will be discussed later), and the maximum where the output of the detector fails to respond to an increase in solute concentration and becomes saturated. The dynamic range is usually quoted in orders of magnitude, is dimensionless, and is given the symbol D_R; e.g., $D_R = 3 \times 10^3$.

Detector Linearity

The linear dynamic range of a detector is that range of solute concentration over which the response of the detector is linear.

As stated, a linear detector is one with an output that is described by the following equation:

$$y = Ac$$

where y, A and c have the meaning previously ascribed to them.

Due to the imperfections of electrical and mechanical devices, no detector is truly linear and designers of detectors can only try to approach this ideal performance. Most manufacturers claim that their detectors are linear, but the extent to which their instruments approach true linearity varies significantly between one and another.

It is therefore important for a chromatographer to have some measure of the linearity of the detector in use and this requires some means of providing a numerical value that indicates the extent to which the function deviates from true linearity.

Fowlis and Scott (1) suggested a way in which a measure of the detector linearity could be determined. They put forward the following power function that could accurately describe the output of all detectors that were approximately linear.

$$y = Ac^r \qquad (1)$$

where r is the Response Index of the detector and the other symbols have the meaning previously ascribed to them.

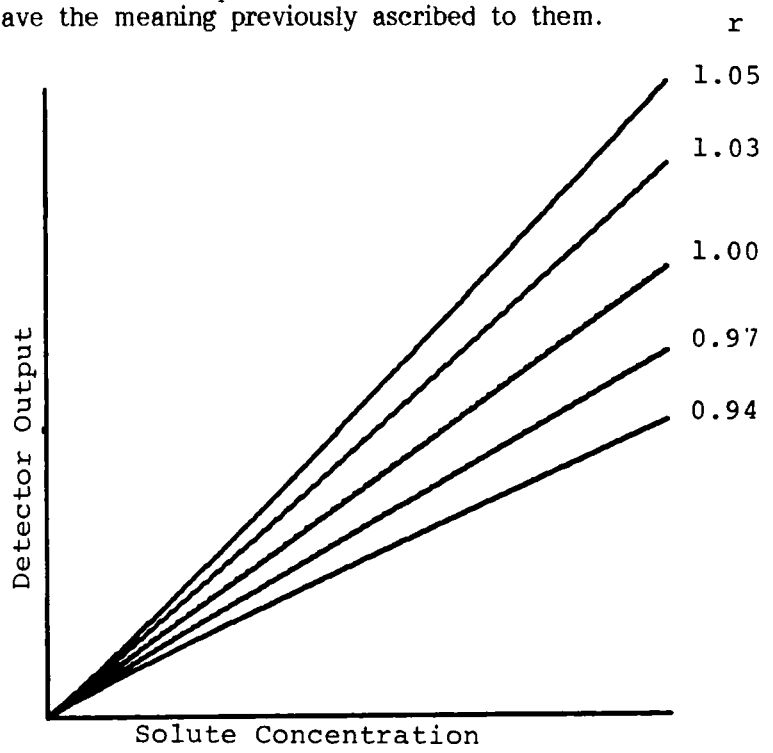

Figure 2. Graphs of detector output against solute concentration for detectors having different response indices.

It follows that for a truly linear detector, r = 1 and the proximity of *r* to unity would indicate the extent to which the detector's performance deviates from true linearity. Figure 2 shows curves relating detector output to solute concentration for different values of *r*. It is seen that all the curves appear to approximate closely to a straight line, but the errors involved in assuming that they are linear are shown in Table 1.

TABLE 1

THE ANALYSIS OF A TWO COMPONENT MIXTURE USING DETECTORS HAVING DIFFERENT RESPONSE INDICES

Solute	r = 0.94	r = 0.97	r = 1.0	r = 1.03	r = 1.05
1	11.25%	10.60%	10%	9.42%	9.05%
2	88.75%	89.40%	90%	90.58%	90.95%

It is seen from Table 1 that errors in the lower level component can be as much as 12.5% (1.25% absolute) for r = 0.94 and 9.5% (0.95% absolute) for r = 1.05. For most practical purposes r should lie between 0.98 and 1.02 if reasonable linearity is to be assumed. However, it should be pointed out that if *r* is known, then a correction can be applied, and thus take into account any non-linearity that may exist. This is the basic advantage of expressing linearity in terms of a response index. It should be emphasized that for the most accurate work the response index of the detector should be determined and applied where appropriate.

The E19 committee of the ASTM (2) which was founded to provide recommendations for specifying LC detectors suggested an alternative procedure for defining detector linearity. According to the E19 committee linearity should be defined as follows: "*the linear range* of a PD (photometric detector) is that range of concentration of a test substance over which the response of the detector is constant to within 5% as determined from a linearity plot". "The linear range should be expressed as the ratio of the highest concentration to the minimum detectable concentration". This procedure for defining detector linearity is perfectly satisfactory and ensures a minimum linearity performance and consequently, also ensures reasonable quantitative accuracy. However, the response is not explicitly defined as in the use of the response index and thus, quantitative measurements cannot be corrected for any slight error in linearity that may exist to obtain the absolute maximum quantitative accuracy from the detector.

The Determination of the Response Index of a Detector

There are two methods that can be used to measure the response index of a detector, the Incremental Method and the

Logarithmic Dilution Method. The former requires no special apparatus other than the chromatograph itself, while the latter requires special apparatus which fortunately is very simple to fabricate.

The Incremental Methods of Linearity Measurement

The incremental method is based on the generation of a curve relating detector output to solute concentration and employs the detector, together with its ancillary electronic apparatus and recorder, connected to a suitable column. The column can be the one that is employed for the analysis or one packed with glass beads to ensure that sufficient band dispersion is produced to allow a peak of measurable width to be eluted. Depending on the absolute sensitivity of the detector, samples of the chosen solute of appropriate size are dissolved in the mobile phase, injected onto the column and the peak recorded. Duplicate injections are recommended for each solute concentration. The sample size for successive calibrations should be increased by a factor of three until the linearity range of the detector is covered. If no data acquisition and processing equipment is available, the width of each peak is measured at 0.607 of the peak height, and from the chart speed and the mobile phase flow rate, the peak width in ml of mobile phase is calculated. It should be noted that the peak width at 0.607 of the peak height is equal to twice the standard deviation of the peak which is assumed to be Gaussian. The distance measured is thus equivalent to half the peak width at the base. It follows that from the knowledge of the mass of solute injected and the width of the base of the peak in ml of mobile phase, the average concentration of the solute in the total peak can be calculated. Now the concentration at the peak maximum can be taken as twice the average concentration so that the maximum concentration of the peak can be calculated from the following equation:

$$c = \frac{ms}{wQ}$$

where c is the concentration of solute in the mobile phase at the peak maximum in g/ml.
m is the mass of solute injected in g
w is the peak width at 0.0607 of the peak height in cm
s is the chart speed of the recorder in cm/min
Q is the flow rate in ml/min.

The logarithm of the peak height y is then plotted against the logarithm of the solute concentration at the peak maximum c as given by equation (2).

From equation (1):

$$\text{Log } y = \text{Log } A + r \text{ Log } c \qquad (2)$$

Thus, the slope of the Log/Log curve will give the response index *r*. If the detector is truly linear, r = 1, and the slope of the curve will be $\pi/4$.

The same data can be employed to determine the linear range as defined by the ASTM E19 committee (2). In this case, however, a linear plot of detector output to solute concentration as opposed to the logarithmic plot is recommended and the concentration at which the detector linearity deviates as much as 5% is identified.

The Logarithmic Dilution Method Of Linearity Measurement

The logarithmic dilution method for detector calibration was introduced by Lovelock (3) for GC detectors and was further modified by Fowlis and Scott (1). The system provides a continuous flow of solvent containing solute, the concentration of which decreases logarithmically with time.

A known mass of solute is introduced into a well-stirred reservoir through which a flow of pure solvent continuously passes. The solution is thus continuously diluted and the concentration of the solute in the exit flow from the reservoir is monitored by the detector under examination. A diagram of the dilution system is shown in Figure 3.

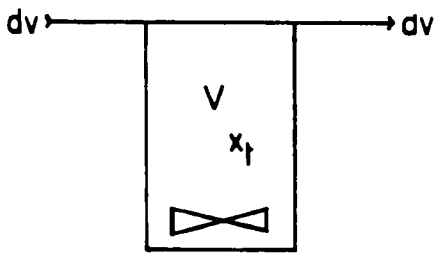

Figure 3. Diagram of logarithmic dilution vessel.

Let the vessel have a volume V, and let the concentration of solute in the vessel be c_t after time *t*. Let a volume dv of pure solvent *enter* the vessel displacing a volume dv *from* the vessel. The mass of solute removed will be $dm = c_t\, dv$. Now the change in mass dm in the dilution vessel will result in a change of solute concentration dc_t, thus

$$V dc_t + c_t dv = 0$$

and $$\frac{dc_t}{c_t} = \frac{-dv}{V}$$

integrating, $$\text{Log } c_t = \frac{-v}{V} + k$$

$$= \frac{-Qt}{V} + k$$

where Q is the flow rate
v is the volume passed through the system after time t
k is the constant of integration.

Now when $t = 0$, $c_t = c_o$

where c_o is the initial concentration of solute.

Thus $$k = \text{Log } c_o$$

Therefore $$\text{Log } c_t = \frac{-Qt}{V} + \text{Log } c_o$$

or $$c_t = c_o e^{\frac{-Qt}{V}}$$

Thus, if the logarithm of the detector output is plotted against time, then for a truly linear detector a straight line will be produced having a slope of -Q/V. If the detector has a response index of r and the slope of the line is ϕ, then

$$\phi = \frac{-Qr}{V} \text{ or } r = -\phi \frac{V}{Q} \qquad (3)$$

Thus, the response index of the detector can be determined, but the accuracy of this determination will depend upon the constant nature of Q, the flow rate, and consequently, a good quality, constant flow pump should be employed. Manufacturers generally do not give the response index of their detectors, and, therefore, for highly accurate work its value needs to be determined.

It should be pointed out that the logarithmic dilution method should not be used to determine linearity if the linearity measurement recommended by the E19 committee of the ASTM is to be employed

The Linear Dynamic Range of a Detector

The linear dynamic range of a detector is not the same as its dynamic range, as already stated, because the linearity of most detectors deteriorates at high concentrations of solute and in some instances, also at very low concentrations of solute. The linear dynamic range of a detector is also quoted in orders of magnitude of concentration and is given the symbol D_L; e.g.

$$D_L = 5 \times 10^{-8} - 2 \times 10^{-5} \text{ g/ml } (0.98<r<1.02).$$

The first reported concentration is usually that which will provide a signal equivalent to twice the noise level and the second reported concentration is the limit at which the response factor was determined. At present, manufacturers do not usually differentiate between D_R and D_L and do not quote a range for the response index r, however, it is hoped that in the future such data will be made available. Some manufacturers do mark the least sensitive setting of the detector as non-linear (N/L), which is a step towards a more rational approach to specifying linear dynamic range.

Detector Response

The Detector Response can be defined in two ways, either as the millivolt output from unit change in solute concentration (in which case, as in dynamic range and detector sensitivity, the solute has to be specified) or as the output in millivolts that would result from unit change in property of measurement, e.g. refractive index units. Detector Response can have different dimensions depending on the basic property the detector is measuring. However, if solute concentration is employed as the basic property of measurement, then the dimensions will be the same for all detectors and will permit a rational comparison between different detectors. The dimensions of Detector Response based on the measurement of solute concentration will be millivolts per gram per millilitre. The Detector Response has been designated the symbol R_c. It can be determined by injecting a known mass of solute on a column and by measuring the response from the dimensions of the eluted peak. Assuming the concentration in mass/per unit volume of solute eluted at the peak maximum is twice the average concentration then,

$$R_c = \frac{hwQ}{sm} \qquad (4)$$

where h is the peak height in mV
w is the peak width at 0.607 of the peak height in cm
s is the chart speed in cm/min
Q is the flow rate of mobile phase in ml/min
m is the mass injected.

The response for a given detector will be different for different solutes; in the case of the UV detector, the response will be a function of the extinction coefficient of the solute and for a refractive index detector, the refractive index of the solute. For this reason the response of two detectors of the same type and geometry can only be compared if the same solute and mobile phase are employed. When comparing the response of detectors of the same type but different geometry, then other factors have also to

be considered. For example, in comparing the response of two UV detectors from different manufacturers, the path lengths of the respective detecting cells must be taken into account which will be discussed later.

Detector Noise

Detector noise is the term given to any perturbation on the detector output that is not related to an eluted solute. Detector noise is an extremely important characteristic of a detector as it determines among other properties, the ultimate sensitivity of the device. Detector noise has been arbitrarily divided into three types which are depicted in Figure 4. The first type is *short term noise* or high frequency noise, sometimes called "grass", and consists of base line perturbations that have a frequency that is significantly higher than that of the eluted peak. *Short term* noise is not usually a serious problem as it often can be entirely eliminated by an appropriate noise filter, and further, does not seriously obscure the presence of a solute peak as shown in the upper curve of Figure 4.

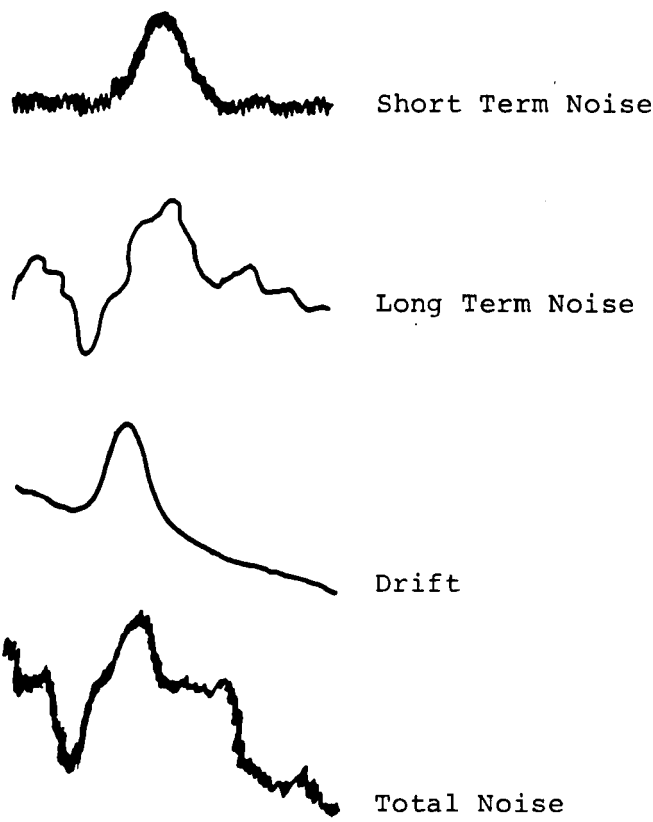

Figure 4. Different types of detector noise.

The second form of noise is called *long term noise* and consists of base line perturbations that have a lower frequency similar to that of the eluted peaks. This type of noise is shown in the second curve in Figure 4. This is the most serious type of detector noise as it cannot be differentiated from an eluted peak of the same amplitude. The eluted peak that can be easily identified in the first curve cannot be identified from the *long term noise* in the second curve. Furthermore, any filter that would eliminate *long term noise* would virtually remove the solute peak as well. *Long term noise arises* largely from the sensing system of the detector itself not from the detector electronics and is frequently caused by component instability or small changes in ambient conditions.

In some instances *long term noise* may be specific to a particular type of detector. For example, the refractive index detector is sensitive to both temperature changes and pressure changes as the refractive index of liquids varies significantly with both temperature and pressure. It follows that the fluctuation of temperature or pressure in the refractometer cell resulting from ambient changes or changes in column flow rate will be the cause of any long term noise and determine the magnitude. Such noise can only be reduced by improved design of the detector.

Drift

Perturbation of the detector output having a frequency significantly less than the frequency of the eluted peak is called *drift* and an example is shown in the third curve in Figure 4. It is seen that the *drift* does not obscure the eluted peak but detectors operating with significant drift will require frequent adjustment of the base line. Drift can result from slowly changing output from the power supply to the detector, lamp aging, changes in ambient temperature, but more often is due to changes in the composition of the column eluent. This often results from incomplete equilibrium being attained on changing the mobile phase. When detectors are operated at or near maximum sensitivity, all three types of noise are usually present and the detector output resembles the fourth curve in Figure 4.

Measurement of Detector Noise

The noise associated with a particular detector is defined as the maximum amplitude of the combined short and long term noise measured over a period of about 10 min (the E19 committee recommends a period of 15 min). The detector must be connected to the column and the mobile phase passed through it over the period of measurement. For a 4-5 mm I.D. column, a flow rate of 1 ml/min is appropriate and the maximum amplitude of the combined short and long term noise is measured in mV and corrected to an attenuation of unity as shown in Figure 5. The detector noise will be given the symbol N_D.

$$N_D = v_1 A = v_1 / S \qquad (5)$$

where v_1 is in millivolts as measured on the recorder scale
A is the attenuation factor
S is the amplification factor

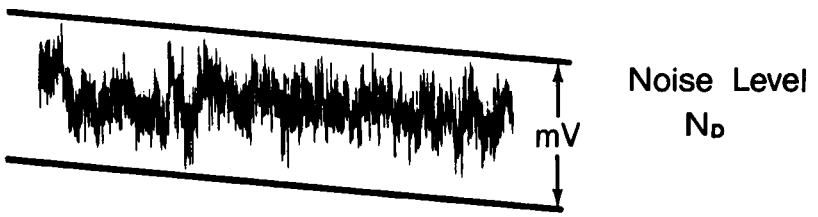

Figure 5. Measurement of detector noise level.

It should be noted that attenuation is the reciprocal of amplification and individual manufacturers may use either method to designate the sensitivity setting.

Minimum	Amplification	1	2	4	8	16	32	Maximum
Sensitivity	Attenuation	32	16	8	4	2	1	Sensitivity

Where detectors are sensitive to pressure changes and variation in column flow rate, the noise level under static conditions is sometimes quoted. This is occasionally the method for specifying noise level for refractometer detectors which are very sensitive to pressure changes within the cell resulting from variations in flow rate. Although useful, such specifications of noise level are not really valid as the chromatographer can never utilize the detector without a flow of mobile phase passing through it. It could be argued that the manufacturer of detectors cannot be responsible for the pump providing constant pressure or a constant flow rate. However, all solvent delivery systems provide mobile phase flow rates that have some variation and it is really the responsibility of the detector manufacturer to design the detector such that the effects of pressure and flow variations are minimized.

It should be noted that at the high sensitivity ranges of some detectors, filter circuits are automatically introduced to reduce noise. Under these circumstances the noise level should be determined at the lowest attenuation (or highest amplification) that does not include noise-filtering devices (or at best the lowest attenuation with the fastest response time) and then corrected to an attenuation of unity.

Detector Sensitivity

Detector sensitivity is the minimum concentration of solute in mass/unit volume passing through the detector that can be discerned from the noise. The size of the signal relative to the noise that can allow the solute signal to be discerned from the noise has to be arbitrarily defined and it is generally accepted that a signal-to-noise ratio of two will permit unambiguous identification of a signal. It follows that for a concentration sensitive detector, the sensitivity X_D is given by

$$X_D = \frac{2N_D}{R_c} \quad (g/ml) \tag{6}$$

R_c and N_D being determined in the manner previously described.

It should be emphasized that the sensitivity of a detector is *not* the minimum *mass* of solute that can be detected. This would be the *system* mass sensitivity and would depend on the column employed. During the development of a chromatogram the peaks become broader as the retention volume increases. Thus, a given mass of solute may be detected at low retention where peaks are sharp and high, but if the chromatographic conditions are changed so that the same mass of solute is eluted later in the chromatogram, the peak will be broad and low and may not be discernible from the noise. Thus, any sensitivity quoted for the detector as the minimum mass detectable must be carefully examined and, if the data is available, the sensitivity must be calculated in the manner given above. Some manufacturers have taken the minimum detectable concentration and multiplied its value by the cell volume of the detector and given the product as the minimum detectable mass of solute. This method of specifying detector sensitivity is *particularly* misleading. For example, a concentration sensitive detector having a true sensitivity of 10^{-6} g/ml and a cell volume of 10 µl will be attributed a sensitivity of 10^{-8} g. This of course is grossly incorrect as it is the peak volume that has the major effect on the minimum detectable mass and not the detector volume. Thus, even when arranging the chromatographic conditions to provide the maximum apparent mass sensitivity, the values quoted on the basis of the product of the true sensitivity and cell volume can be very different from the the real value for the mass sensitivity. Specifications for detectors given by manufactures are becoming more rational, but misleading values for detector sensitivity are still given and chromatographers need to carefully assess their significance if not given in one of the forms discussed here.

The sensitivity as defined here is sometimes called the minimum detectable concentration (MDC). This definition is a valid alternative, but it is more appropriate to maintain the use of the

term sensitivity as it is complimentary to system mass sensitivity and system concentration sensitivity which of course is calculated from detector sensitivity.

The Mass Sensitivity of a Chromatographic System

The mass sensitivity of a chromatographic system, which includes column, sample valve, connecting tubes and detector, is defined as that mass of solute *m* that will provide a peak height that is twice the noise level. Consider a chromatographic peak, with a height equivalent to twice the noise level, being sensed by a detector with a maximum sensitivity of X_D (g/ml). The peak volume can be taken as $4\sigma_c$(σ_c is the volume standard deviation of the peak as it is eluted from the column) and the concentration at the peak maximum as twice the mean concentration.

Thus $$\frac{2m}{4\sigma_c} = X_D$$

or $$m = 2\sigma_c X_D \qquad (7)$$

Now from the plate theory $$\sigma_c = \frac{V_r}{\sqrt{n}}$$

where V_r is the retention volume of the solute
and n is the column efficiency.

Thus $$m = \frac{2V_r X_D}{\sqrt{n}} \qquad (8)$$

now $$V_r = V_o(1+k') \qquad (9)$$

where V_o is the column dead volume
and k' is the capacity ratio of the solute.

and $$V_o = \varepsilon\pi r^2 \ell \qquad (10)$$

where ℓ is the column length
r is the column radius
ε is the fraction of the column volume that is occupied by the mobile phase.

Consequently, substituting in equation (8) for V_r from (9) and V_o from (10)

$$m = \frac{2\varepsilon\pi r^2 \ell(1+k')X_D}{\sqrt{n}} \qquad (11)$$

Thus, *m*, the mass sensitivity of the chromatographic system depends exclusively on the detector sensitivity, column

dimensions, column efficiency and the capacity factor k' of the eluted solute. However, irrespective of the column properties, the mass sensitivity is directly related to the detector sensitivity. It will also be seen later that the radius of the column for an optimized system will depend on the extra column dispersion of the system which is largely due to that taking place in the detector itself. Consequently, the detector has a major influence on mass sensitivity as a result of both its concentration sensitivity and its dispersion. Later, when the instrument dispersion is discussed together with its effect on column radius, another expression for the system mass sensitivity will be derived.

The Concentration Sensitivity of a Chromatographic System

The concentration sensitivity of a chromatographic system is defined as that concentration of solute that will provide a peak height equivalent to twice the noise level. The concentration sensitivity of a chromatographic system can be derived directly from the system *mass* sensitivity. If the minimum detectable mass is dissolved in the maximum permissible sample volume (that sample volume that will limit the increase in peak variance to a maximum of 10%) (4) then this concentration of the solute will constitute the minimum detectable sample concentration.

Now the maximum sample volume is $1.1\sigma_c$, (i.e. V_s), and the mass sensitivity given by equation (7) is $2\sigma_c X_D$.

Thus, the concentration sensitivity X_c is given by

$$X_c = \frac{2\sigma_c X_D}{1.1\sigma_c} = 1.8X_D \qquad (12)$$

It is seen from equation (12) that the system concentration sensitivity is also directly (and this time solely) dependent on the detector sensitivity and is *entirely independent* of the column dimensions and efficiency. Consequently, in contrast to the system mass sensitivity, the system concentration sensitivity is not dependent on the detector dispersion. It should be noted, however, *that the maximum permissible sample volume has to be employed to realize the maximum concentration sensitivity.*

The Maximum Capacity Factors of an Eluted Peak

The maximum k' at which a solute is eluted is also controlled by the detector sensitivity. As the k' of an eluted solute increases, the peak becomes more disperse. As a result, the peak becomes smaller and ultimately, the peak will disappear into the detector noise. If it is merely necessary to unambiguously identify the existence of a peak, then the peak maximum would probably have to be five times the noise level. This is also an

arbitrary assumption based on experience but in fact any preferred value for the signal to noise level can be substituted if desired. Now the minimum detectable concentration is twice the noise level by definition and thus, at the maximum k' the concentration must be $2.5X_D$. The concentration at the peak maximum can be taken as twice the average concentration, so if there is a mass *m* contained in a peak of base width $4\sigma_c$ which from the plate theory is equal to $4\sqrt{n}(v_m+Kv_s)$

where v_m is the volume of mobile phase in a theoretical plate
v_s is the volume of stationary phase in a theoretical plate
K is the partition coefficient of the solute,

$$\text{then} \quad \frac{2m}{4\sqrt{n}(v_m+Kv_s)} = 2.5X_D$$

$$\text{or} \quad m = 5X_D\sqrt{n}(v_m+Kv_s) \tag{13}$$

Now as previously stated the maximum permissible sample volume V_s is given by

$$V_s = 1.1\sqrt{n}(v_m+Kv_s) \tag{14}$$

The effect of the sample volume on peak width will be greatest for the early peaks and will progressively decrease for subsequent wider peaks. Now the resolution of both early and later peaks must be given equal emphasis. The sample volume should therefore be chosen such that the increase in width of the first unretained peak is restricted to a maximum of 5%. Thus, K in equation (14) must be made zero.

$$\text{Thus} \quad V_s = 1.1\sqrt{n}(v_m) \tag{15}$$

Consequently, if the solute concentration in the sample is X_S,

$$\text{then} \quad m = X_sV_s = 1.1\ X_s\ \sqrt{n}(v_m) \tag{16}$$

Equating equations (13) and (16)

$$1.1X_s\sqrt{(v_m)} = 5X_D\sqrt{n}(v_m + Kv_s)$$

Now as $Kv_s/v_m = k'$

$$\text{then} \quad \frac{1.1X_s}{5X_D} = 1+k' \tag{17}$$

$$\text{or} \quad k' = 0.22X_s/X_D - 1 \tag{18}$$

It follows that the maximum value of k' depends strongly on

the detector sensitivity (or the minimum detectable concentration) and the sample concentration. Consequently, the detector sensitivity also sets an ultimate limit to the peak capacity that can be provided by the column. This limit of k', however, is relatively high as shown in Table 2 for the injection of a sample at a concentration of 0.1% $^{w}/_{v}$.

TABLE 2

LIMITING VALUES OF k' FOR DETECTORS OF DIFFERENT SENSITIVITY AND A SAMPLE CONCENTRATION OF 0.1%

Detector Sensitivity (g/ml)	Maximum k'
10^{-5}	21
10^{-6}	219
10^{-7}	2199
10^{-8}	22,000

Nevertheless, at a given sample concentration it is the detector sensitivity that ultimately limits the maximum k' value at which a solute can be eluted and not the characteristics of the column. This, of course, is subject to the caveat that the sample is placed on the column contained in the maximum permissible volume and there is sufficient volume of sample available.

The Total Detecting System Dispersion

Separations are achieved in LC by employing a mobile and stationary phase system that will move the individual solute bands apart during development and by designing the column to keep the individual solute bands narrow. Obviously, the more narrow the bands and the further they are moved apart, the better the separation. The detector and its connecting tubes cannot affect the degree to which the solute bands are moved apart, as this depends solely on the characteristics of the two phases, but they can affect the width of the solute bands. Band spreading that takes place in the connecting tubes or cell volume itself results from poor radial transfer of the solute in the liquid and the parabolic velocity profile of the mobile phase that exists in all tubular conduits through which the mobile phase passes. As LC techniques develop, columns will provide higher and higher efficiencies, which means the solute bands will become more narrow and provide improved resolution. At present, the base width of the peak eluted from a high efficiency column in terms of volume of mobile phase may only be about 2 to 10 µl. It follows, therefore, that a detector having a cell volume of 8 µl will contribute significantly to the band width and thus, the real efficiency and resolving power of the column may not be realized. Peaks already separated in the column will be merged together due to the solute bands being broadened in the detector. In a similar way, the time

constant of the amplifier and the recorder can contribute to the apparent band width as recorded on the chart. It is therefore necessary to consider in some detail the relative contribution of the chromatographic apparatus to the total solute dispersion.

Extra Column Dispersion

Extra column dispersion is that contribution to the total peak dispersion which takes place outside the column and may significantly degenerate the separation that has been previously obtained in the column itself. There are four major sources of extra column dispersion which are as follows:

1. Dispersion due to the sample volume which can be considered to contribute a variance σ_s^2.
2. Dispersion in sample valve/column and column/detector connecting tubes that can be considered to contribute a variance σ_t^2.
3. Dispersion in the detector cell which can be considered to contribute a variance σ_d^2.
4. Dispersion due to the detector response time which can be considered to contribute a variance σ_r^2.

Consequently, by summing the variances from each source the final extra column variance σ_e^2 is given by

$$\sigma_e^2 = \sigma_s^2 + \sigma_t^2 + \sigma_d^2 + \sigma_r^2$$

It follows that the total permitted extra column variance, i.e. 10% of the column variance (σ_c^2) as suggested by Klinkenberg (4), has to be shared between each source of dispersion.

Thus

$$\sigma_e^2 = 0.1\sigma_c^2 = \sigma_s^2 + \sigma_t^2 + \sigma_d^2 + \sigma_r^2$$

Unfortunately, the magnitude of the deleterious effect of each source of extra column dispersion on the overall chromatographic performance is different so that equipartition of the allowable dispersion over the four sources is not optimal (5). Ideally, the whole of the extra column dispersion should be allotted solely to the sample volume which would then provide maximum chromatographic mass and concentration sensitivity. However, this is not possible as the detector has to be allotted a significant proportion of the extra column dispersion to allow it to achieve reasonably high sensitivity. This is particularly so for those detectors that respond to a change in some optical property of the eluent as a detector cell of significant volume is necessary for adequate light measurement. In practice, the dispersion resulting from connecting tubes and detector response time should, by careful

design, be kept to an absolute minimum so that the major portion of the extra column dispersion can be shared equally between the sample volume and the detector cell. It is *vital*, however, that the variance allotted to the detector is used *as exclusively as possible in the sensing volume of the detector* and not in the internal connecting tubes as, only in the sensor, can extra column dispersion be tolerated to provide the necessary cell dimensions for high sensitivity.

However, in whatever manner the total value of the variance σ_e^2 due to extra column dispersion is distributed, it must not exceed 10% of the column variance, i.e.

$$\sigma_e^2 = 0.1\sigma_c^2 = \frac{0.1V_r^2}{n}$$

or

$$\sigma_e = \frac{0.32V_r}{\sqrt{n}}$$

Now for the whole of the chromatogram to be effectively useful, the most narrow peaks (i.e., those peaks eluted close to the column dead volume) must not be broadened by the extra column dispersion. That is to say, in any chromatographic separation peaks eluted close to the dead volume should be considered equally important as those eluted elsewhere in the chromatogram.

Thus

$$\sigma_e = 0.32V_o/\sqrt{n}$$

Now

$$V_o = \pi r^2 \ell \varepsilon$$

where V_o is the column dead volume
r is the column radius
ℓ is the column length
ε is that fraction of the column occupied by the mobile phase

Thus

$$\sigma_e = 0.32\varepsilon\pi r^2\ell/\sqrt{n}$$

Now as the efficiency n for a well packed column is independent of the column radius r, an explicit equation for r can be obtained, i.e.

$$r = \left(\frac{\sigma_e\sqrt{n}}{0.32\ \pi\ell\varepsilon} \right)^{1/2} \qquad (19)$$

Equation (19) allows the minimum column radius to be calculated for a column of given length and efficiency. Consequently, as the volume flow of mobile phase through a column is directly

related to the square of the column radius, from equation (19) the solvent economy (i.e., the amount of solvent consumed in a given chromatographic analysis) can be shown to be directly related to the extra column dispersion and consequently, to the total detector dispersion.

It is interesting to consider the implication of equation (19) on the mass sensitivity of the chromatography system.

Reiterating equation (11), the minimum mass detectable is given by

$$m = 2\varepsilon\pi r^2 \ell(1+k')X_V/\sqrt{n}$$

Substituting for r from equation (19)

$$m = 2\varepsilon\pi\left(\frac{\sigma_e\sqrt{n}}{0.32\pi\ell\varepsilon}\right)\ell(1+k')X_D/\sqrt{n}$$

$$= 6.25\sigma_e(1+k')X_D \qquad (20)$$

It is seen from equation (20) that the minimum detectable mass, or mass sensitivity of a chromatographic system, where the column has been designed to have the optimum radius for the detector employed, is directly proportional to the extra column dispersion *and* the detector concentration sensitivity. It follows that detector dispersion is *as important as* detector sensitivity in its influence on the overall chromatographic mass sensitivity where the chromatographic system has been optimized with respect to the radius of the column. The effect of extra column dispersion and in particular, detector dispersion on the overall mass sensitivity of the chromatogaphic system is not generally appreciated or completely understood. As the total extra column dispersion is the integral of a variety of sources, the distribution and nature of the various sources of dispersion will now be considered in some detail.

Connecting Tube Form, Dimensions and Volume

There are two parts to the mobile phase conduit system of a LC detector that can individually contribute to detector dispersion; the conduit connecting the column to the detector cell and the detector cell itself. Unfortunately, *both* have to exist in the modern LC detector, the cell itself to provide the actual detection, and the conduit from the column to the cell, because in almost all instances, the sensing cell can not be associated directly with the column. In most cases, the connecting tube takes the form of a simple open tube which is often the major source of dispersion in the detector. Band dispersion in open tubes was examined theoretically by Golay (6),

Atwood and Golay (7), and experimentally by Scott and Kucera (8), and Lochmuller and Sumner (9). The variance per unit length of an open tube according to Golay is given by

$$H = 2D_m/u + r^2u/24D_m \qquad (21)$$

where H is the variance per unit length
u is the linear velocity of the solvent through the tube
r is the radius of the tube
D_m is the diffusivity of the solute in the solvent.

At relatively high linear velocities $2D_m/u << r^2u/24D_m$

Consequently,

$$H = r^2u/24D_m$$

Furthermore, as

$$Q = \pi r^2u$$

where Q is the flow rate through the tube

$$H = Q/24\pi D_m \qquad (22)$$

H is the variance per unit length and to obtain the volume variance (σ_V^2) per unit length (a more useful value for the chromatographer) the variance per unit length has to be multiplied by the square of the cross sectional area.

$$\text{i.e. } \sigma_V^2 = H(\pi r^2)^2 = \pi Q r^4/24D_m$$

$$\text{or} \quad \sigma_V = (\pi Q/24D_m)^{\frac{1}{2}} r^2 \qquad (23)$$

It is seen that the dispersion, in terms of the standard deviation of the solute band, increases directly with the square root of the flow rate and inversely as the square root of the solute diffusivity in the mobile phase. The overriding factor, however, that controls the dispersion is the tube radius and it would appear that the problem of connecting-tube dispersion could be easily eliminated by reducing the tube diameter to an appropriately small value. Unfortunately, there is a practical limit to the extent the radius can be reduced due to the liability of blockage. In practice, it is found that a connecting tube diameter of less than 0.007 in. (0.18 mm) is too liable to frequent blockage to be acceptable. In Figure 6, curves relating σ_V and r calculated from equation (23) for a tube 10 cm long and for three different flow rates are shown together with the tube dispersion that can be tolerated if two types of modern LC columns are to be used with a given connecting tube. The values for the permissible band dispersion for the columns in Figure 6 represent a 5% increase in

band width suggested as acceptable by Klinkenberg (4). The first column, 3 cm long, 4.6 mm I.D. and packed with 3 μm particles, giving an efficiency of about 5000 theoretical plates at the optimum velocity, represents a high speed column that would be employed for the fast separation of a relatively simple mixture. The second column, a small bore column, 1 mm I.D., 25 cm long and packed with 5 μm particles, represents a relatively high-performance column having ca. 25,000 theoretical plates when operated at the optimum velocity and which would be employed for more difficult separations. The column efficiency is calculated for the most narrow peak (a dead volume peak) as all solutes in a mixture must be given equal emphasis. The diffusivity was taken as that equivalent to benzyl acetate in n-heptane at 25°C *viz.* 3.1 x 10^{-5} cm^2/sec.

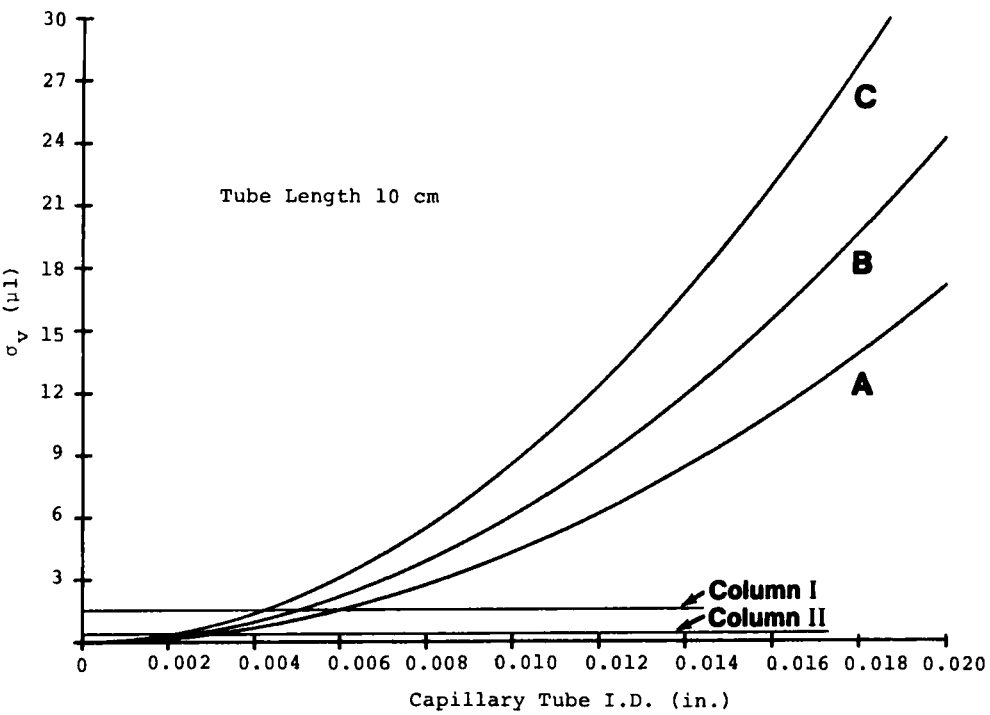

Figure 6. Dispersion (standard deviations of eluted peaks assumed Gaussian) plotted against tube diameter. A: 1 ml/min, B: 2 ml/min, C: 4 ml/min.

It is seen that only a connecting tube 0.005 in. I.D.could be employed with the high speed column and a tube of 0.002 in. I.D. would have to be used with the small bore column. In practice tubes having an I.D. of less than 0.007 in. are not practical due to their liability to blockage. It follows that either the minimum length of connecting tube must be used or the column connected directly to the detector cell, or alternatively, a different form of connecting tubes must be employed.

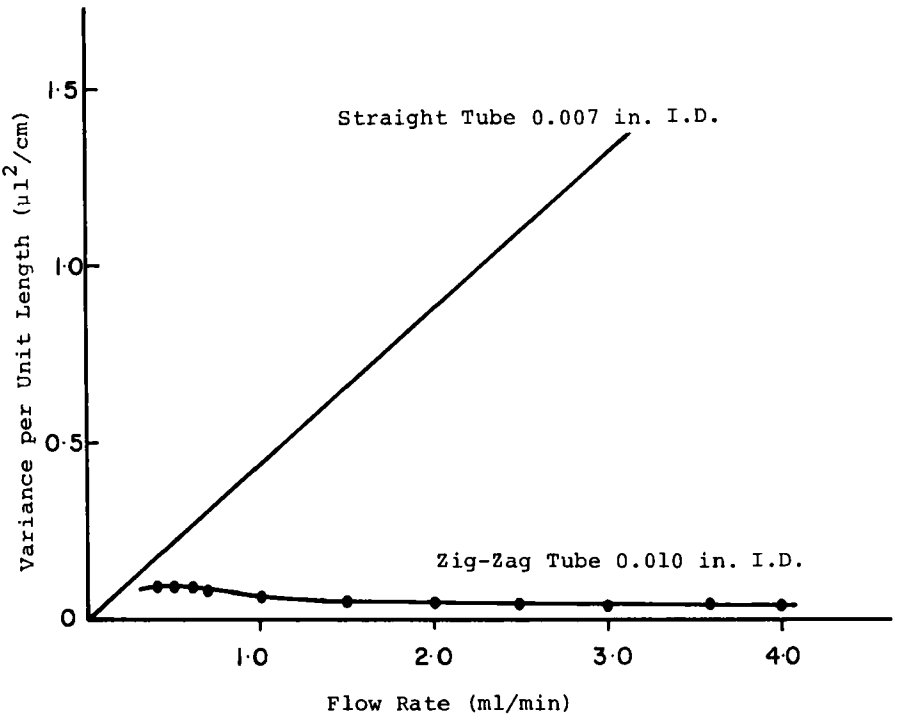

Figure 7. Graphs of peak variance against flow rate for straight and zig-zag tubes.

The dispersion in an open tube results from the parabolic velocity profile that exits under conditions of Newtonian flow. A number of attempts have been made to change the geometry of the connecting tube in order to introduce radial flow and thus break up the parabolic velocity profile and consequently, reduce dispersion. One of the pioneers of this approach was Halasz et al. (10) who crimped and bent the tube into different shapes. Although reduced dispersion was achieved, the intermittent restricted cross sectional area that occurred at each constriction became a serious source of tube blockage. More recently, Tijssen (11) developed a theory to describe radial flow that was introduced into small diameter open tubes by very tight coiling. Tightly coiled open tubes exhibit very significantly reduced band dispersion, particularly at high flow rates. Furthermore, employing tubes, for example, with an I.D. of 0.010 in. (0.254 mm) would ensure that they were very unlikely to block. Another way of introducing radial flow in connecting tubes was developed by Katz and Scott (12) where the tube was formed into a serpentine shape which introduced radial flow in much the same way as the coiled tubes of Tijssen (11). However, as the direction of flow was continually reversed in the serpentine form, the consequently induced radial flow was even greater than in the coiled tube and

the band dispersion further reduced. The advantage of the serpentine tube besides its decreased dispersion lies in the fact that the radial flow is initially induced at much lower flow rates and thus, columns of smaller diameters can be employed with such connecting tubes. Curves relating the variance per unit length for a straight tube calculated from the Golay equation (6) and a serpentine tube are shown in Figure 7. It is seen that at high flow rates the dispersion is greatly reduced by the radial flow induced in the serpentine tube.

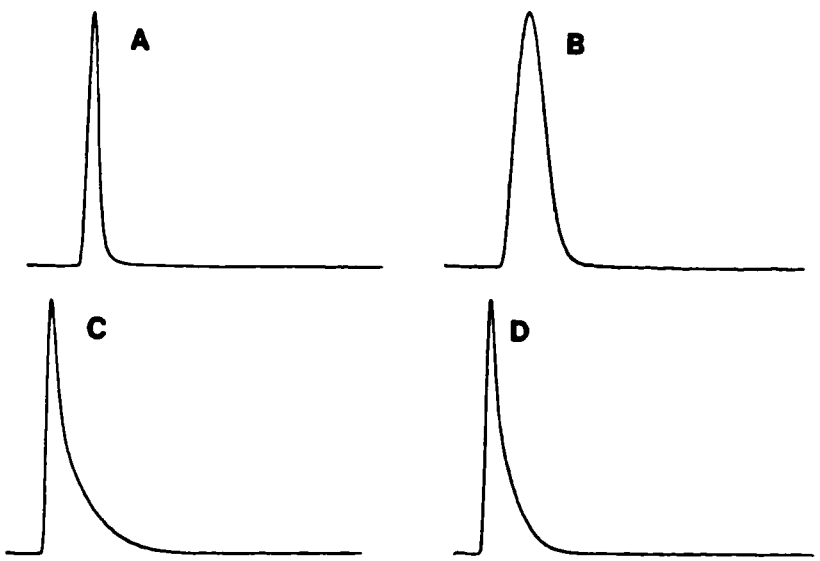

Figure 8. Dispersion from different forms of connecting tubes.

Examples of the dispersion that can occur in tubes of different geometric shape and different internal diameters are shown in Figure 8. These dispersion curves were obtained using a Perkin-Elmer LC-85B low dispersion detector (flow cell volume 1.4 µl) in conjunction with a Valco valve having a 1 µl internal loop. The data was recorded on a Bascom-Turner recorder with a data acquisition rate of 10 msec per point. The flow rate employed was 2 ml/min, the solvent was methanol, and 0.11 ng of magnesium nitrate was used as the solute. The dispersion curve for the serpentine tube is shown in Figure 8A. The I.D. of the tube was 0.25 mm and the amplitude of the serpentine form, peak to peak, was 1 mm. The dispersion due to this tube was calculated to be 0.09 $\mu l^2/cm$. The dispersion curve shown in 8B was obtained from the same length of tube but was coiled as opposed to the serpentine form. It was also 0.25 mm I.D. and the amplitude of the coil was also 1 mm. It is seen that the dispersion of this tube was 1.01 $\mu l^2/cm$, more then 10 times greater than that of the serpentine tube. Curve C is obtained from a straight tube which was also 0.25 mm I.D. and which gave a variance of 0.17 $\mu l^2/cm$, twice as great as the ser-

pentine tube. It should be pointed out, however, that the dispersion was measured at 0.607 of the peak height and consequently, does not take into account the serious tailing effect from the open tube. In Figure 8D the effect of a straight tube of the same length, but only 0.18 mm I.D., shows a further reduced dispersion having a variance of 0.12 $\mu l^2/cm$, about the same dispersion as that of the serpentine tube, but with a very serious tail that is not taken into account in the variance measurement. Furthermore, straight tubes 0.18 mm I.D. would have a much greater chance of blockage and much higher impedance to flow.

The curves in Figure 8 clearly demonstrate the relative performance of tubes of different geometry. Although the coiled tube has a high variance, the peak is almost symmetrical, but obviously, the curve from the serpentine form is both better in shape and contributes less variance. Nevertheless, the dispersion is still very large when compared with the dispersion from the small bore column previously discussed. *Ipso facto* connecting tubes should be made as short as possible whatever their geometric form.

Cell Dimensions and Cell Volume

The dispersion in the detecting cell also results from the parabolic velocity profile that occurs under Newtonian flow, but it is more complicated due to the fact that the aspect ratio of the tube is small (length/radius) and consequently, the simple relationship of Golay (6) does not apply. Atwood and Golay (7) theoretically examined the dispersion that takes place in short tubes of small aspect ratios and some examples of the dispersion profile obtained from such tubes are shown in Figure 9. It is seen that from a short tube a broad asymmetric dispersion profile is obtained which, as the diameter of the tube decreases, becomes sharper in the front but with an extended tail. It should be noted that the diameter of the tube has to be significantly reduced to provide an efficiency of about 20-30 theoretical plates before a symmetrical peak is obtained. The equation for these curves was developed by the same authors and experimentally verified. The dispersion curves shown in Figure 9, however,are rarely exhibited in practice by actual detector cells as the profile is further modified by the manner of entrance and exit of the mobile phase to and from the cell.

Dispersion of solute bands in cells of different dimensions have been experimentally measured with concentric inlet and outlet connections (13), but this data although pertinent to some detectors (e.g. conductivity detectors) is not generally useful for optical detectors where inlet and outlet tubes normally have to be radially oriented. Radial entry and exit flow from the detector cell introduce significant radial mixing and thus reduces the extent of the dispersion that would be expected to result from low

aspect ratio cells. The dispersion contribution of a connecting tube-cell combination is given in Table 3. The connecting tube is serpentine in form, 30 cm long and 0.010 in (0.254 mm) I.D. and the cell 3 mm long and 0.85 mm I.D.

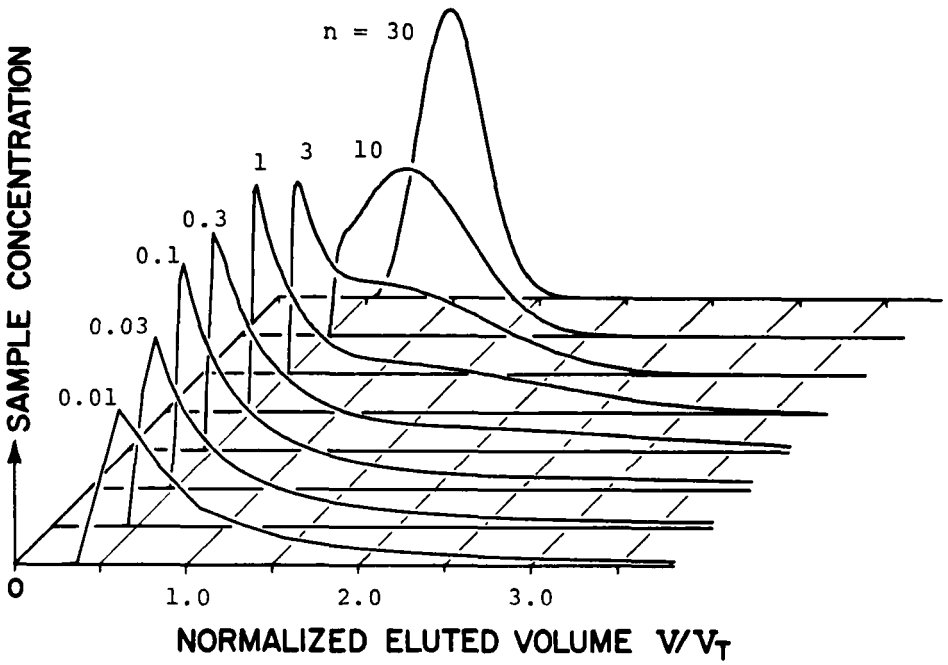

Figure 9. Elution peak shapes as a function of normalized tube length, *n*, in plates.

TABLE 3

DISPERSION IN DIFFERENT PARTS OF THE DETECTOR SYSTEM

Flow Rate (ml/min)	Cell and Serpentine (μl^2)	Serpentine (μl^2)	Cell (μl^2)
1	9.51	5.70	3.81
2	7.78	2.64	5.14
4	17.64	1.98	15.66

It is seen that the dispersion varies significantly with flow rate but in a rather complicated way. The dispersion of the serpentine connecting tube is fairly high at low flow rates but is reduced to a relatively small value at high flow rates. This would be expected from the curves shown in Figure 7. The cell on the other hand has relatively small dispersion at low flow rates but increases very significantly at high flow rates which would

also be expected from a short cylindrical tube. To some extent the two systems tend to compensate the resulting overall dispersion increasing relatively slowly with increasing flow rate. Nevertheless, a significant dispersion occurs and, as shown earlier, column radius would need to be adjusted if the maximum mass sensitivity and column efficiency is to be realized.

Overall Detector Time Constant (Sensor and Electronics)

The band width as depicted by the recorder may be significantly broader than that actually sensed by the detector due to dispersion effects that arise not only from the detector conduit and the cell, but also from the finite nature of the response time of the detector sensor, the amplifier, and even the recorder. In general, the response of the sensor is relatively fast, but the correct sensor has to be chosen to ensure that it is not the limiting factor in the overall detector response. An example of a slow and fast response sensor is provided in the work of Scott et al. (14), who examined the time constants of a cadmium sulphide sensor and compared it with that of an I.P.28 photomultiplier sensor. The output from transient changes of high light intensity was monitored by means of a Bascom-Turner high speed recorder for each sensor and the records obtained are shown in Figure 10A and 10B. The actual curve recorded for the cadmium sulphide photoconductor, given in Figure 10A, shows a very significant a.c. noise which was smoothed by a five point smoothing routine available in the Bascom-Turner software. The smooth curve is shown superimposed on the signal curve and also normalized to full scale. The logarithm of the expanded curve is also included and from this it can be seen that, from the slope of the log curve, the time constant of the sensor is about 2.5 sec. A time constant of this magnitude is completely unacceptable in modern high-speed chromatography systems where a complete separation can be obtained in a period commensurate with the time constant of the sensor. In Figure 10B the response of the photomultiplier sensor operated under similiar experimental conditions is shown together with the smoothed normalized curve and the normalized logarithmic response. The UV lamp was operated from a d.c. supply and thus, little a.c. noise was present on the unsmoothed output. From the slope of the Log curve, the time constant of the photomultiplier sensor was found to be 40 msec. Such a sensor response time is quite suitable for the majority of modern high-speed chromatography applications.

Amplifiers and recorders have an inherent time constant that arises from their respective circuit design, but a further time constant is often purposely introduced to remove high frequency noise. If this time constant is of commensurate period to the time standard deviation of the eluted peak, then the peak will suffer significant broadening. The effect of amplifier time constant on the shape of detected chromatographic peaks has been elegantly treated by Vandenheuvel (15), Schmauch (16), and

Sternberg (17). For those readers wishing to study the effect of time constants on peak distortion in detectors, it is recommended to read the work of Sternberg.

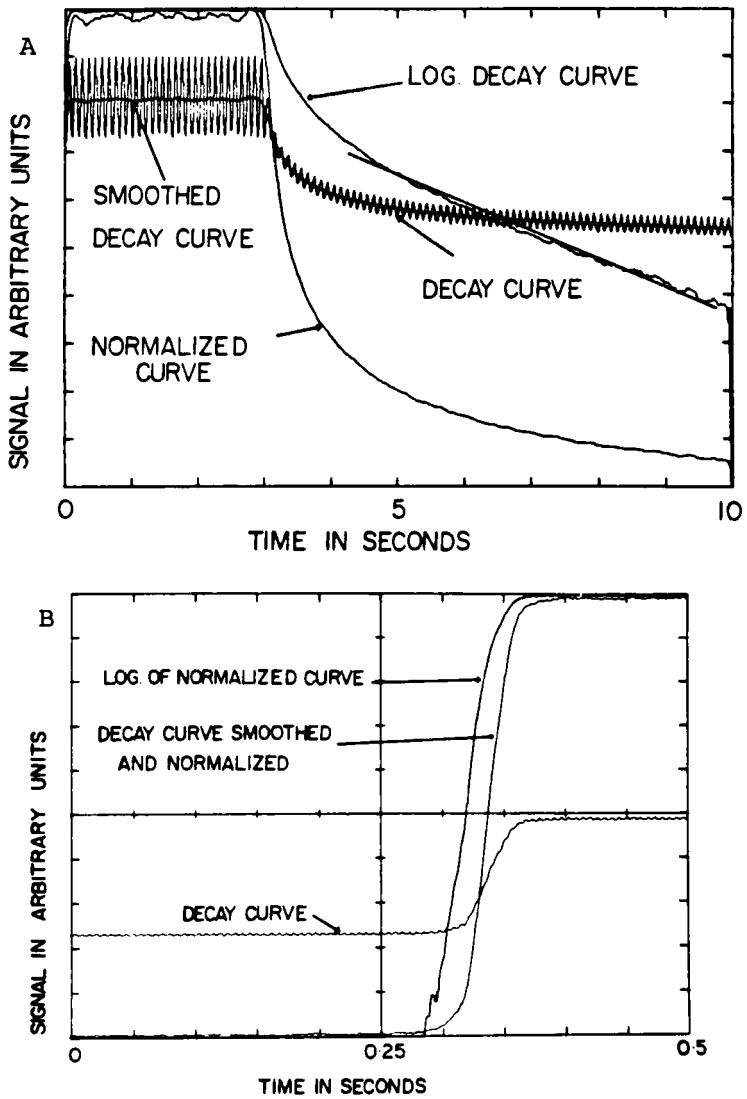

Figure 10. Response curves for different photosensors.

Sternberg developed the following equation to describe the peak shape after being distorted by an amplifier with a time constant of T'.

$$X = \frac{X_o e^{-t/T'}}{T'} \int e^{t/T' - (t-t_o)/2T_t^2} dt \qquad (24)$$

where X is the voltage output of the amplifier to the recorder
X_o is a constant
t_o is the time at the peak maximum
T_t is the time standard deviation of the eluted peak
t is the elapsed time.

The explicit solution of the above equation is rather complicated,but the distortion of a normal Gaussian peak can be clearly illustrated from curves obtained from the above equation by the use of a computer. Consider a column, 25 cm long and 4.6 mm in diameter having a dead volume (V_o) of 3 ml, an efficiency n of 12,000 theoretical plates and operated at a flow rate of 1 ml/min (Q). From the plate theory the standard deviation of the dead volume peak in ml of mobile phase will be,

$$\sigma_v = V_o/n^{1/2} = 3/(12{,}000)^{1/2} = 0.0274 \text{ ml}$$

Thus, the time standard deviation σ_t which is obtained by dividing the volume standard deviation by the flow rate is given by

$$T_t = \sigma_t = \sigma_v/Q = 0.0274/Q = 0.27 \times 60 = 1.64 \text{ sec}$$

Thus, taking the values for the time constant of the amplifier of 0.6 and 1.5 sec and replacing T_t in equation (24) by the calculated value of 1.64 sec, the elution profiles can be obtained. The shape of the resultant elution profiles is shown in Figure 11. Data for the curves was calculated over the range t = 4.9 to t = 8.2 sec.

It is seen that the effect of a 1.5 sec time constant is to seriously distort the peak, both by increasing the peak width and reducing the peak height. This would result in a reduction of the resolving power of the column and the sensitivity of the system.

The effect of the amplifier time constant is to combine two functions, an exponential function and a Gaussian function. As these two functions describe physical phenomena that are not interacting in the sense that they proceed independently of one another, the variance of the combined function is equivalent to the sum of the variances of each individual function.

The time variances of an exponential function of the form

$$e^{-t/T'} \text{ is } T'^2 \qquad (25)$$

and that of a Gaussian function

$$e^{-t^2/2T_t^2} \text{ is } T_t^2 \qquad (26)$$

Consequently, $T'^2 + T_t^2 = T^2$

where T^2 is the time variance of the resulting peak as described by the recorder. Now T_t^2 is the time variance of the solute band leaving the column and thus, if a band width increase of 5% is considered acceptable ($T = 1.05\ T_t$).

$$\text{then} \quad T_t^2 + T'^2 = (1.05\ T_t)^2 = 1.103\ T_t^2$$
$$\text{thus} \quad T'^2 = 0.103\ T_t^2$$
$$\text{and} \quad T' = 0.32\ T_t$$

Thus, the maximum value of T' that can be tolerated to maintain column resolution will be 32% of the time standard deviation of the eluted peak.

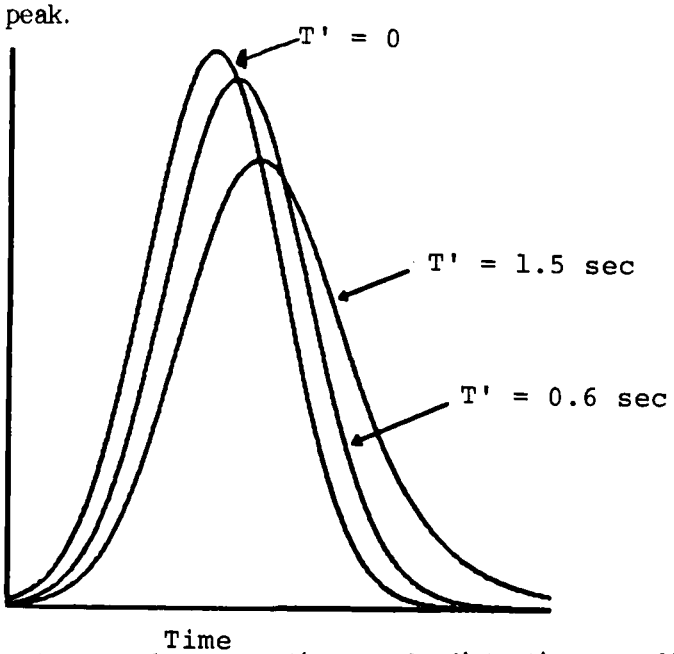

Figure 11. Curves demonstrating peak distortion resulting from significant amplifier time constant.

This, however, assumes that the whole of the permitted extra column dispersion is allotted to the response time of the detector which, as already discussed, is not possible; the major proportion of the allowable extra column dispersion must be available to the sample volume and cell dispersion to provide adequate mass and concentration sensitivity. If only 1/10 of the extra column dispersion is allotted to detector response time, then

$$T_t^2 + T'^2 = (1.005\ T_t)^2 = 1.0100\ T_t^2$$
$$\text{thus} \quad T'^2 = 0.0100\ T_t^2$$
$$\text{and} \quad T' = 0.1\ T_t$$

It follows that the maximum permissible time constant will be only 10% of the peak standard deviation if sufficient extra column dispersion is retained for sample volume and detector cell volume

to ensure adequate mass and concentration sensitivity. This can place severe demands on the electronic system of a detector if high speed columns are employed. A typical high-speed column might be 3 cm long, 4.6 mm I.D. and packed with 3 μm particles. Such a column would have a dead volume of about 350 μl, an efficiency of about 5000 theoretical plates and would be operated at a flow rate of about 5 ml/min. Consequently, the standard deviation of the dead volume peak would be 5 μl and its time standard deviation, the ratio of the volume standard deviation to the flow rate, *viz.* 60 msec. The overall time constant of the detecting system must therefore be one tenth of this, i.e. 6 msec, a very small value compared with those that are characteristic of contemporary LC detectors.

The Time Constant of the Recorder

The potentiometric recorder does not have a time constant of the form normally associated with the amplifier which, in general, results from a capacity resistance network intrinsic in the amplifier circuit. The response of an amplifier to an instantaneously applied constant voltage is normally an exponential function of time, whereas for a potentiometric recorder the response is usually linearly related to time. The linear response results from the feed back circuitry incorporated in the sensor system which is necessary for stability. In Figure

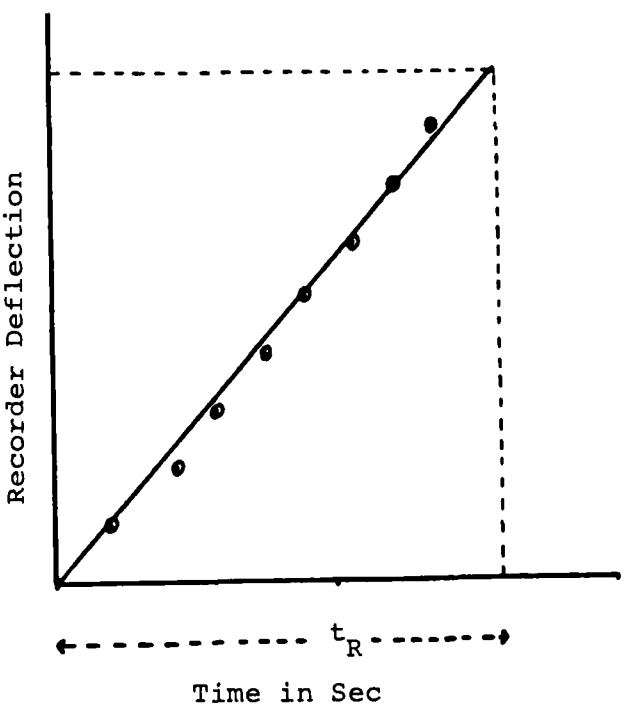

Figure 12. Response curve of a potentiometric recorder.

12 the recorded reading is plotted against time for an instantaneous applied constant 9 mV signal, the points being taken directly from the chart. The recorder was the Honeywell Electronik 196, 10 mV potentiometric recorder operated with a chart speed of 1 cm/sec and having a specified balancing time of 0.5 sec. It is seen that the response is approximately linear.

Now for a linear function,the time variance T^2, is given by

$$T^2 = \frac{t_R^2}{18} \qquad (27)$$

where t_R is the time taken for the recorder to reach the applied voltage.

In the example given

$$T^2 = \frac{(0.79)^2}{18} = 0.035 \text{ sec}^2$$

and thus the time standard deviation, T = 0.19 sec.

It is seen that if a recorder is employed having a balancing time of about 0.5 sec, the contribution in time variance to the eluted peak is very significant and could seriously effect the chromatographic performance. If a high-speed column is again considered, the time standard deviation permitted was 6 msec.

Consequently, from equation (27)

$$(6)^2 = t_R^2/18$$

or

$$t_R = 6\sqrt{18}$$

$$= 25.5$$

It follows that for contemporary high-speed LC , the balancing time of the recorder needs to be about 25 msec. A balancing time of 25 msec is about an order of magnitude less than that of the majority of fast response potentiometric recorders presently available. It would appear that for high-speed chromatography a computor data system or digital recorder such as the Bascom-Turner recorder would be distinctly preferable to a potentiometric recorder.

Pressure Sensitivity

The pressure sensitivity of a detector can be very important as it can control the long term noise of the detector. In many instances, pressure effects on detector output are promoted by flow changes and consequently, pressure sensitivity and flow sensitivity can be strongly interactive. Pressure sensitivity, how-

ever, can depend on mobile phase viscosity changes during gradient elution development whereas flow sensitivity can be considered independent of solvent viscosity. Pressure sensitivity can be important if there is any significant impedance subsequent to the detectors that would result from exit tubes of a too small diameter and switching devices for fraction collection or multidimensional chromatography.

Bulk property detectors such as the refractive index detector or the dielectric constant detector are often particularly pressure sensitive and for that reason wide diameter exit tubes are strongly recommended, however, such detectors are not useful for multidimensional analyses. It follows that the pressure sensitivity of the detector should be specified by the manufacturer. The pressure response D_p should be given as the output in mV for unit pressure change in the detector cell. The pressure response should be given in both mV/psi and $mV/kg/m^2$. It is also recommended that the pressure noise is given in terms of that pressure change which would provide a signal equivalent to the noise, i.e.

$$N_p = N_D/D_p$$

where N_p is the pressure that would provide a signal equivalent the detector noise level.

With such information, the effect of pressure fluctuations on detector noise could be directly assessed. In order to allow the chromatographer to determine whether a detector could be used in multidimensional analyses, the absolute pressure tolerance of the detector should also be given, which can be defined as the maximum working pressure of the detector where there is no loss of mobile phase due to cell leakage. The maximum working pressure of a detector P_D should be given in both psi and kg/m^2. For a normal operation the detector P_D should lie between 30 and 100 psi. For a detector to be employed for multidimensional analyses, however, pressures at least 2000 to 3000 psi are often necessary.

Flow Sensitivity

The flow sensitivity of a detector is, as stated previously, closely associated with its pressure sensitivity, but can be due solely to flow changes and independent of the pressure in the detector cell. Experimentally, however, it can be extremely difficult to determine the flow sensitivity of a detector under conditions where there is no accompanying pressure change in the cell. Nevertheless, the flow sensitivity of a detector needs to be known particularly where flow programming development is likely to be employed. The flow sensitivity of the detector also determines the flow control that is necessary with respect to the pump that is to be employed with the chromatograph. The conditions for

measurement of flow sensitivity must, by necessity, be arbitrary, but it should be measured using a pure solvent (not a mixture) and with no constriction in the detector exit conduit. The flow sensitivity D_Q should be given in mV/ml/min. In a similar manner to pressure, the noise level N_Q equivalent to unit change in flow rate should also be included, i.e.

$$N_Q = N_D/D_Q$$

where N_Q is the noise equivalent to a change in flow rate of 1 ml/min.

Temperature Sensitivity

Chromatographic systems often operate under conditions where the ambient temperature can change significantly over a relatively short period of time. If the detector sensor or the detector electronics respond to temperature changes then this will result in baseline drift. Temperature effects can be important in laboratories where there is no air conditioning and where relatively high ambient temperatures are achieved during the day time and conversely significantly lower temperatures during the evening and night. The overall sensitivity of the detector system to changes in temperature should therefore be given and is best defined as the output in millivolts per unit change in ambient temperature. The detector temperature sensitivity has been given the symbol D_t and is reported in millivolt/°C.

Detectors also have a limited temperature range over which they can operate satisfactorily. The maximum/minimum operating temperatures which the detector can satisfactorily function should also be given.

Summary of Detector Criteria

1. The Dynamic Range - The dynamic range of a detector is that concentration range over which the detector will give a concentration dependent output. It has been assigned the symbol D_R and is given as the ratio of the solute concentration, at which the detector fails to respond to a further concentration change, to the minimum detectable concentration or detector sensitivity. The units are dimensionless. A value for the dynamic range is particularly useful when the detector is being employed for preparative chromatography.

2. The Response Index - The response index of a detector is a measure of its linearity and has been given the symbol r. It is a dimensionless constant and for a truly linear detector should take the value of unity. In practice the value of r should be between 0.98-1.02 and a knowledge of its value is very important where accurate quantitative analyses are required. If r is known,

accurate quantitative results can be obtained even if the detector is significantly non-linear.

3. The Linear Dynamic Range - The linear dynamic range of a detector is that concentration range over which the detector response is linear within the restraints defined by the Response Index. It has been assigned the symbol D_L. The numerical value would be equal to the ratio of the maximum solute concentration, at which the response factor was determined, to the minimum detectable concentration or the detector sensitivity, the units of which will be dimensionless. It is useful to know the value of D_L when analyzing a sample where the individual solutes present in the mixture cover a wide concentration range.

4. Detector Response - The detector response can be defined in two ways; either as detector output per unit change in concentration of a specified solute or as the detector output per unit change in the physical property measured by the detector (e.g. refractive index). In the former case the units of measurement will be in mV/g/ml. The detector response has been given the symbol R_c. In conjunction with the detector noise level, it allows the detector sensitivity, or minimum detectable concentration to be calculated.

5. Noise Level - The noise level of a detector is measured in mV and is taken as the maximum amplitude of the combined short and long term noise taken over a period of about 10 min. It has been given the symbol N_D and is used in determining the detector sensitivity. It can not be too strongly emphasized that detectors must *not* be compared on the basis of the *magnitude of their noise or response.* They can only be compared on the basis of their relative *signal-to-noise* ratio at a *specific solute concentration.*

6. Detector Sensitivity - The sensitivity of the detector, or as it is sometimes termed the minimum detectable concentration, can also be defined in two ways. It can be defined as that solute concentration that will provide a signal equivalent to twice the noise level (in which case the solute must be specified) or as that change in the physical property measured by the detector that will provide a signal equivalent to twice the noise. In the first case the sensitivity is given in units of g/ml and in the second case the units will depend on the detector involved. Defining the sensitivity in units of g/ml is more valuable to the chromatographer as it allows the direct calculation of the overall mass and concentration sensitivity of the chromatographic system and even the maximum k' value of the last eluted peak. If sensitivity is specified in terms of concentration, then it has been given the symbol X_D.

7. Overall Detector Dispersion - The overall detector dispersion

is extremely important as it directly governs the magnitude of the column radius with which the detector can be used and consequently, the solvent consumption together with the mass and concentration sensitivity of the total chromatographic system. The detector dispersion also indirectly controls the maximum k' value at which a solute can be eluted. It is specified either as the variance σ_d^2 (in μl^2) or as the standard deviation σ_d (in μl) resulting from the total detecting system. The following information should also be made available; the length, internal diameter, and geometric form of the connecting cell and volume of the sensing tube.

8. Detector Time Constant - The detector time constant which includes the response of the detector sensor as well as that of the electronics has been given the symbol T_D, and is specified in msec. The value of T_D also indirectly controls the solvent consumption, mass and concentration sensitivity of the overall chromatographic system.

9. Maximum Working Pressure - The maximum working pressure of the detector determines the maximum impedance that can be permitted from post detector valves or columns as used in multidimensional analysis. It has been given the symbol P_d and should be reported in both psi and kg/m^2.

10. Pressure Sensitivity - The pressure sensitivity of a detector is the output in millivolts that results from unit pressure change at the detector inlet. It has been given the symbol D_p and should be specified in both mV/psi and in $mV/kg/m^2$.

11. The Detector Flow Sensitivity - The detector flow sensitivity is the output in mV that corresponds to unit change in flow rate; it is reported in mV/ml/min and has been designated the symbol D_Q. The flow sensitivity of the detector ultimately determines the flow rate control required by the chromatographic pump.

12. Temperature Sensitivity - The temperature sensitivity of the detector is defined as the millivolt output that corresponds to 1°C ambient temperature change. It has been given the symbol D_t.

Synopsis

There are three common types of detector response normally achieved by electronic signal modification. These are normal, integral and differential responses. The most common and, of course, the most popular, is the normal response. Integral response can be useful for measurement of peak area and the differential response in useful for identifying retention times. The units of detector specification are, wherever possible, length, time and mass, but under certain circumstances other units such as refractive index, conductivity, (mho's) etc. are used where the

nature of the sensing system is not directly related to concentration. There are two response ranges for a detector, the *dynamic range* and the *linear dynamic range*. The *dynamic range* is that concentration range over which the detector will provide a concentration dependent output. The minimum will be the detection limit and the maximum will be at the level where the output from the detector fails to respond to an increase in solute concentration. The *linear dynamic range* is that range of a solute concentration over which the response of the detector is linear. The linearity can be defined in a number of ways. The one recommended is the use of the *response index* which for a truly linear detector is equivalent to unity.

The proximity of the value of the index to unity is thus a measure of its linearity. The linearity of a detector can be determined experimentally by the *incremental method* or the *logarithmic dilution method* of *calibration*. The *detector response* is defined as the millivolt output resulting from unit change in solute concentration. It is an important specification as, in conjunction with the noise level, provides the ultimate *detector sensitivity* or *minimum detectable concentration*. There are three types of *noise* that can be exhibited on the output of detector, *short term noise*, *long term noise* and *drift*. The actual *noise level* specified for a detector is measured in millivolts and is the combination of all three forms of noise measured over a period of 10 min. The detector *sensitivity* or *minimum detectable concentration* can be defined as that concentration change that provides a signal equivalent to twice the noise. It is sometimes given in units that the detector actually measures. For example, the *sensitivity* of a UV detector maybe given as the minimum change in absorption units that provides a signal equivalent to twice the noise.

The chromatographic system as a whole including the detector also has a defined sensitivity. The *mass sensitivity of the chromatographic system* depends not only on the detector sensitivity, but also on the column dimensions. The *concentration sensitivity of the chromatographic system,* on the other hand, depends solely on the *detector sensitivity* providing the sample is placed on the chromatographic system in the maximum permissible sample volume. The *detector sensitivity* also controls the maximum capacity factor at which a solute can be eluted.

Another important detector specification is the total *detector system dispersion*. It is made up of the dispersion in the connecting tube and that in the sensing cell. The *dispersion* inherent in the detector controls the *minimum column radius* that can be used and consequently, *the solvent consumption. Detector dispersion* also indirectly controls the *mass sensitivity*. Connecting tubes can take various forms: straight, coiled or serpentine. Serpentine connecting tubes are recommended, as they provide the

smallest dispersion. Serpentine and coiled tubes induce a radial mixing which renders the peak shape symmetrical. For optimum performance, the *major portion* of extra column dispersion must be alloted to the *cell volume* to provide adequate detector sensitivity and the *injection volume* to provide system sensitivity. Dispersion resulting from connecting tubes and the time constant of the system must be *minimum.* The *time constant* of both the amplifier system and the potentiometric recorder should be of the order of *2 or 3 msec* and certainly less than 50 msec. This is likely to necessitate the use of a digital recorder for high-speed separations as opposed to a potentiometric recorder.

The *pressure sensitivity* defined as that pressure equivalent to the noise level is very important as it determines both the limits of pressure and flow variation that can be tolerated from the pump. The specification of the *maximum working pressure* is also important where multidimensional column systems are required to be employed since there is a significant flow impedance subsequent to the detector cell.

A similar specification, the *flow sensitivity* should also be defined in terms of that flow change which will provide a signal equivalent to twice the noise level. The value of the *flow sensitivity* is also important in the design of the pump to be used with the detector.

The sensitivity of the detector to changes in ambient temperature should be defined and given as the millivolt output that will result from unit change in ambient temperature. This is useful where, due to lack of ambient temperature control, such as adequate air conditioning, significant temperature changes can take place and thus effect the detector output.

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CHAPTER 3

BULK PROPERTY DETECTORS

One of the first on-line liquid chromatography detectors to be developed in the early forties was, in fact, a bulk property detector, the refractive index detector (1). Bulk property detectors continuously monitor some physical property of the column eluent and by the use of a suitable transducer provide a voltage - time output that is either proportional to the physical property being measured, or made proportional to the concentrations of the solute eluted. The properties of the mobile phase that are most commonly monitored in commercially available bulk property detectors are refractive index, electrical conductivity, and dielectric constant, the dielectric constant detector being the least popular of the three.

Any eluted solute will be detected by a bulk property detector providing the magnitude of the physical property of the solute being measured differs sufficiently from that of the mobile phase. The sensing device usually consists of a small volume cell of appropriate geometry that is either made a part of an optical measuring system or is fitted with suitable electrodes to measure some electrical property of the cell contents. In some instances a reference cell is also provided that is filled with pure mobile phase and the output from the measuring cell and reference cell are compared, the difference provides the output to the recorder. Such reference cells are almost always included in the refractive index detector as they help to compensate for changes in the light intensity emitted from the light source and to some extent changes in ambient temperature. In effect, reference cells help to reduce the noise level of the detector and thus increase its sensitivity.

Bulk property detectors have a somewhat restricted sensitivity that is directly due to the measuring principle on which they function. Consider an hypothetical bulk property detector that monitors, for example, the density of the eluent leaving the column. Assume that it is required to detect the concentration of a dense material, such as carbon tetrachloride (e.g. specific gravity 1.595), at a level of 1 μg/ml in heptane (e.g. specific gravity 0.684). This situation is typical for a bulk property detector and will be favorable for *this* hypothetical detector, as the solute to be detected exhibits a large difference in density from that of the mobile phase.

Let the change in density resulting from the presence of the solute at a concentration of 10^{-6} g/ml be Δd.

It follows that (to a first approximation) $\Delta d = (d_1 - d_2)X_s/d_1$

where d_1 is the density of the solute, carbon tetrachloride
d_2 is the density of the mobile phase, heptane
X_s is the concentration of the solute to be detected.

Thus, for the example given,

$$\Delta d = (1.595 - 0.684) \times 10^{-6}/1.595$$
$$= 5.71 \times 10^{-7}$$

Now the coefficient of cubical expansion of heptane is approximately 1.6×10^{-3} per ^0C . It is therefore possible to calculate the change in temperature $\Delta\theta$ that would produce a change in density of the mobile phase that would be equivalent to the presence of carbon tetrachloride at a concentration of 10^{-6} g/ml.

Thus,
$$\Delta\theta = \frac{5.71 \times 10^{-7}}{1.6 \times 10^{-3}} \ ^\circ C$$
$$= 3.6 \times 10^{-4} \ ^\circ C$$

Now if it is assumed that a concentration of one part per million of carbon tetrachloride is just detectable, i.e., the thermal noise must be half the detectable signal, then the thermal fluctuations must be maintained at a level below 1.8×10^{-4} °C. Such temperature stability can be extremely difficult to maintain in practice and thus, the temperature control can place a severe limit on the sensitivity that can be obtained from such a detector. Even the heat of adsorption and desorption of the solute from a column packed with silica gel can easily result in temperature changes of the order of 1.8×10^{-4} °C.

In a similar manner the density of the contents of the detector cell will change with pressure. It follows that the detector would respond to fluctuations in pump pressure or indirectly to fluctuations in mobile phase flow rate if there was a significant flow resistance subsequent to the detector as in the case of multidimensional column systems . It follows that a bulk property detector, functioning on the measurement of density could only be employed with pumps having very constant flow rates and pressure control. The above argument applies equally to other bulk property detectors which monitor refractive index or dielectric constant and it can therefore be concluded that all bulk property detectors will have a limited sensitivity and have to be employed with very well controlled mobile phase supply systems.

As a result of the limited sensitivity of bulk property detectors, they have also a very limited *linear dynamic* range, usually about three orders of magnitude and sometimes considerably

less. Their potential *dynamic* range, however, is relatively large, perhaps, four or five orders of magnitude, but this extends into the very low sensitivity (high concentration) areas of normal detector performance and consequently, would only be useful in large scale liquid chromatography (LC). Unfortunately, the detector sensitivity settings on commercially available bulk property detectors usually do not permit the device to be operated at very low sensitivities. This is a disadvantage, as bulk property detectors are very appropriate for preparative LC where solute concentrations are high and out of the range of most of the detectors presently available. A low sensitivity, bulk property detector would be a very useful addition to the present range of commercially available detectors.

Due to the general susceptibility of bulk property detectors to changes in ambient conditions such as column flow rate, and mobile phase composition (other than the presence of an eluted solute) they cannot be used with certain chromatographic development techniques such as gradient elution, temperature programming or flow programming. It follows that bulk property detectors are almost solely used under isothermal and isocratic conditions of development. The major areas of application of bulk property detectors are for detecting solutes that do not absorb in the UV wavelength range, or do not fluoresce. They are also employed where relatively low sensitivities can be tolerated or are required. Bulk property detectors are frequently employed in gel permeation chromatography, where in many cases, the solutes do not contain UV chromophores and, as already stated, in preparative chromatography. Bulk property detectors are used in perhaps less than 20% of the total field of LC applications. Most manufacturers of chromatographic equipment supply at least one type of bulk property detector, usually the refractive index detector, a second often being the electrical conductivity detector. They are usually available separately, in modular form, but may also be incorporated as an integral part of the complete liquid chromatograph.

When entering the field of liquid chromatography, the scientist is always faced with the problem of detector selection. The subject of detector choice will be dealt with later in this book but at this point, it should *again* be emphasized, that there is no ideal LC detector. Consequently, the practicing liquid chromatographer needs to have at least two, if not more, different types of detector available, or the full versatility of the technique will not be realized. It is therefore recommended that one of the detectors available should be a bulk property detector, which should probably be the refractive index detector. This would be a particularly appropriate detector if preparative or semi-preparative chromatography is likely to be required. If the separation and quantitative analysis of ionic materials are contemplated, then the refractive index detector might be replaced

by the electrical conductivity detector. Nevertheless, considering bulk property detectors as a whole and despite their relatively low sensitivity, the refractive index detector might be considered as the closest approach to a *non-destructive universal* detector.

The Refractive Index Detector

The refractive index detector was one of the first on-line detectors to be developed and was described by Tiselius and Claesson (1) in 1942. It was also the first detector to be made commercially and was at one time the only on-line detector available for general use. It is universal, in the sense that it detects all solutes that have a refractive index different from that of the mobile phase and is probably the *next* most popular to the UV detector.

Since the original model of Tiselius there have been many papers published that describe different methods of refractive index monitoring. From these publications, four general methods emerge for measuring refractive index; the angle of deviation method, the critical angle method, the Fresnel method and the Christiansen method. The theory behind each of these methods will now be discussed.

The Angle of Deviation Method

When a monochromatic ray of light passes from one isotropic medium, A, into another, B, it changes its wave velocity and direction. The change in direction is called refraction and the relationship between the angle of incidence and the angle of refraction is expressed in Snell's law of refraction

$$n'_B = \frac{n_B}{n_A} = \frac{\sin P}{\sin Q}$$

where P and Q are the angle of incident light in medium A, and the angle of refraction in medium B, respectively, measured from the normal,
n_A and n_B are the refractive indices of medium A and medium B, respectively,
n'_B is the refractive index of medium B relative to medium A.

Refractive index is a dimensionless constant that normally decreases with increasing temperature; values given in the literature are usually measured at ambient temperature using the mean value for the two sodium lines. The refractive indices of some common solvents used as a mobile phase are given in the Table of Physical Properties of Different Solvents given in the Appendix.

If a cell is constructed in the form of a hollow prism through which the mobile phase flows, a ray of light passing through the prism will be deviated from its original path and this can be focused onto a photocell. As the refractive index of a mobile phase passing through the detector changes due to the presence of solute, the angle of deviation of the transmitted light will also be altered; thus, the amount of light falling on the photocell will be changed and modify its output. The angle of deviation method for refractive index monitoring has been commonly employed in many commercial refractometers.

The modern refractive index detector is the result of considerable research and development carried out over many years probably starting with the work of Zaukelies and Frost (2) and Vandenheuvel and Sipas (3) and now carried on largely by the effort of scientists in the research and development laboratories of the major instrument companies. An example of a modern refractive index detector is the R-410 manufactured by Waters Associates. A schematic diagram that illustrates the principle of operation of such an instrument is shown in Figure 1.

The differential refractometer measures the deflection of a light beam due to the difference in refractive index between the sample and reference liquids in a single compact sample cell. A beam of light from the incandescent lamp passes through the optical mask which confines the beam within the region of the cell. The lens collimates the light beam and the parallel beam passes through the cells (containing the sample and reference liquids) to the mirror. The mirror reflects the beam back through the sample and reference cell to the lens, which focuses it onto a photocell.

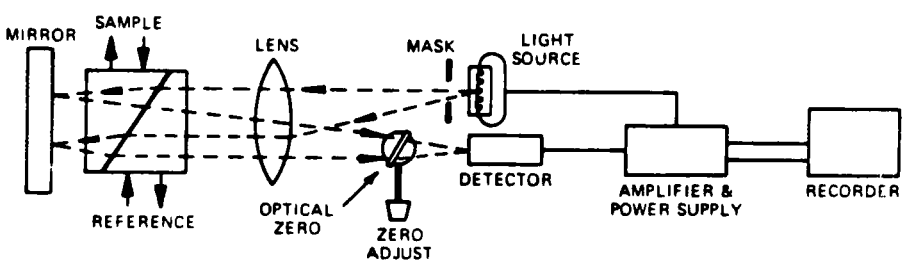


Figure 1. A schematic diagram of the Waters refractometer detector.

The location of the focused beam rather than its intensity is determined by the angle of deflection resulting from the difference in refractive index between the two parts of the cell. As the beam changes location on the photocell, an output signal is

generated. This signal is amplified and provides an output to a meter or recorder. The optical zero glass deflects the beam from side to side to adjust for zero output signal.

By using the deflection principle of refractometry, it is possible to use one sample cell throughout the entire refractive index range from 1.00 to 1.75. An optical block and heat exchanger are provided to bring the liquid temperature to the temperature of the cell at all flow rates normally encountered. An example of a chromatogram obtained from this type of detector is shown in Figure 2.

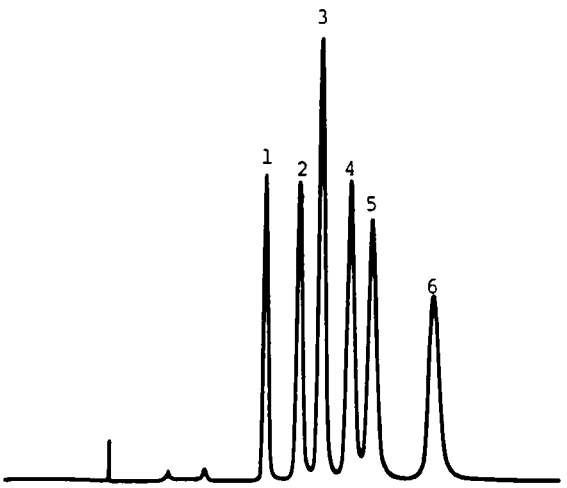


Figure 2. Chromatogram of sugars monitored by a refractometer detector. Samples: 1=xylose, 2=glucose, 3=sucrose, 4=maltose, 5=lactose, 6=maltotriose.

The solutes separated in Figure 2 are carbohydrates which do not absorb in a normal UV range and would be difficult to detect by other detectors. The column was a type of reversed phase and the mobile phase an acetonitrile/water mixture. The above example is a good illustration of the advantage of having a refractive index detector available for special applications. The only practical alternative methods of detection for these components would be to use derivatives or employ a UV detector and operate at very low wavelengths (190-200 nm). Unfortunately, at these wavelengths, baseline stability is difficult to maintain.

The Critical Angle Method

When a ray of light passes from a medium of low refractive index to one of high refractive index, the refracted ray is bent away from the normal in the second medium. If the angle of the incident ray is increased, the angle of the refracted ray increases until the refracted ray is parallel to the boundary

surface between the two media. At this point the incident ray is totally reflected and the angle of incidence is called the critical angle. Thus, if the second medium is made the mobile phase, the angle of incidence is arranged to be close to the critical angle and the reflected ray focused onto a photocell, any change in the refractive index of the mobile phase will change the amount of light reflected onto the photocell. Due to the angle of incident light being close to the critical angle, slight changes in refractive index will result in significant changes in the intensity of the reflected light and thus in the output of the photocell. It appears at this time, there is no commercially available refractive index detector that is based *explicitly* on this method of detection.

The Fresnel Method

This method utilizes the relationship between reflectance from an interface between two transparent media and their refractive indices as given by the Fresnel equation:

$$R = 1/2 \left[\frac{\sin^2(i-r)}{\sin^2(i+r)} + \frac{\tan^2(i-r)}{\tan^2(i+r)} \right]$$

where R is the ratio of reflected light intensity to incident intensity,
i is the angle of incidence,
r is the angle of refraction.

Now

$$\frac{\sin i}{\sin r} = \frac{n_1}{n_2}$$

where n_1 is the refractive index of medium 1,
n_2 is the refractive index of medium 2.

Thus, if the medium 2 represents the liquid eluted from the column, then any change in n_2 will result in a change in R and thus, a measurement of R could determine changes in the value of n_2 due to solute being present in the eluent.

Conlon (4) utilized the principle to develop a practical refractive index detector. His device is now obsolete and cannot be used with present day high efficiency columns, however, it will be described as it illustrates the principle of the Fresnel method and also demonstrates the general arrangement of a refractive index detector. A diagram of his detector is shown in Figure 3. The sensing element consists of a rod prism sealed into a tube through which the column eluent flows. The rod prism was made from a glass rod 6.8 mm in diameter, 8 to 10 cm long, bent to the correct optical angle and an optically clear flat ground on the

apex of the bend as shown in Figure 3. The optical flat was then sealed into a window of a suitable tube which acted as a flow-through cell.

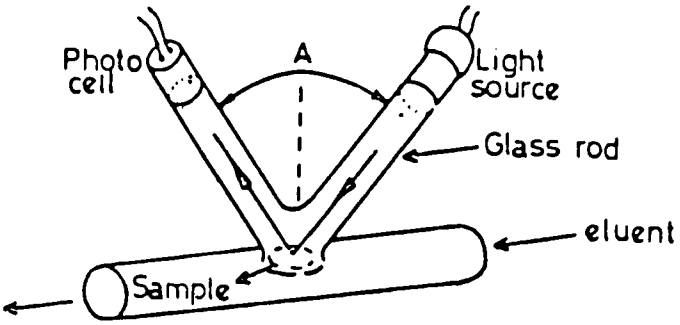


Figure 3. Detector cell utilizing the Fresnel principle.

The photocell is arranged to be one arm of a Wheatstone bridge and the out of balance signal fed to a suitable recorder. Another photocell can be placed so as to receive light directly from the light source and if this is situated in the reference arm of a Wheatstone bridge, it can help compensate for slight variations in the light source due to voltage fluctuations.

The disadvantages of this system are that it does not contain a reference cell to compensate for temperature changes or changes in solvent composition, and the relatively large cell volume would not permit the high efficiencies obtained from present day columns to be realized.

An example of a commercially available detector based on the Fresnel principle is the Perkin-Elmer LC-25 Differential Refractometer and a diagram of the optical system of the detector is shown in Figure 4. A tungsten source supply provides the operating voltage to the tungsten lamp, which is the energy source to the flow cell. The flow cell assembly is mounted on a heat exchanger to maintain constant temperature. The tungsten lamp energy is directed through an infrared filter to prevent heating the cell contents and then into a magnifier assembly which splits the single beam into two. The two beams are then focused on both the sample and reference cells contained in the flow cell. The prism assembly emits the refracted beams through two focusing lenses to the detector diodes, which are part of a pre-amplifier assembly. The prism assembly also reflects a view of the sample and reference cells to a user view port employing two flat mirrors to reflect the image.

The sample and reference signals from the detector diodes are converted to one voltage output which represents the difference between the two signals. This voltage is then fed to a

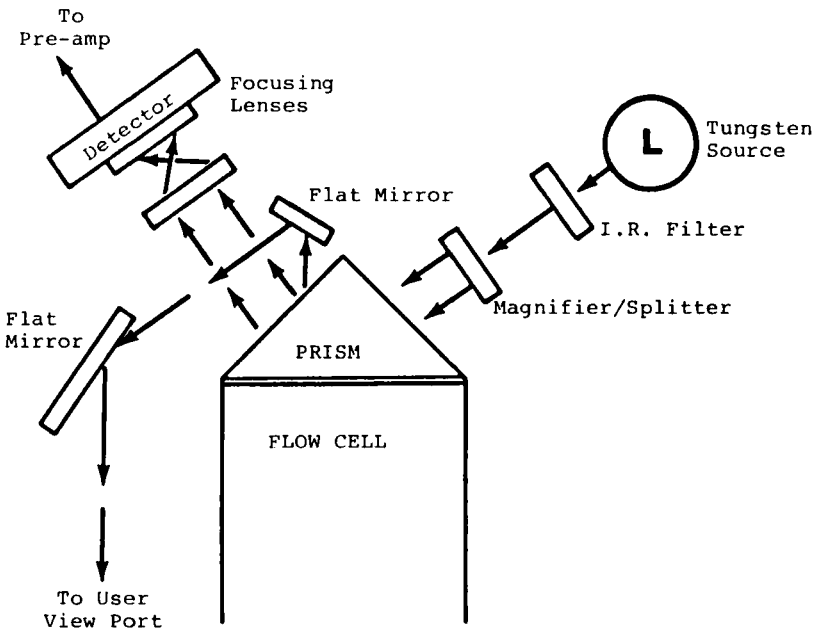


Figure 4. A schematic diagram of the optical system of the LC-25 differential refractometer.

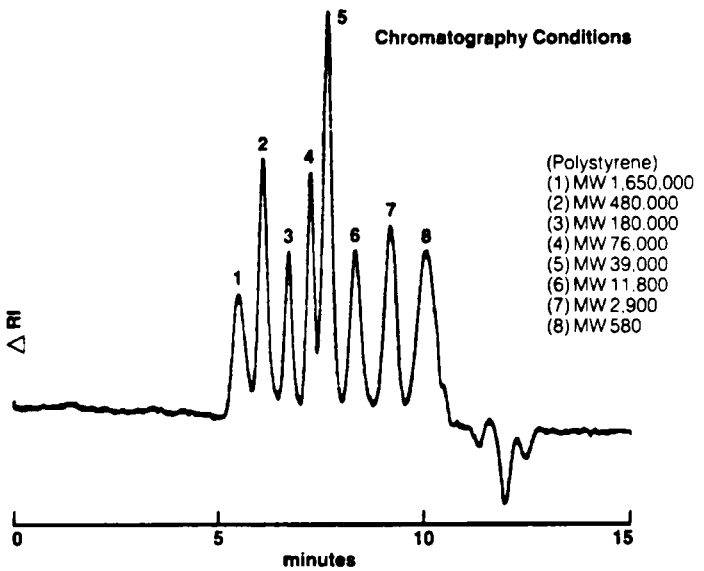


Figure 5. The detection of a polystryene standard mixture by the LC-25 differential refractometer.

linearizing processor and zero adjust amplifier before being attenuated by a range switch, and thence to a recorder.

The range of refractive index covered by this instrument for a given prism is limited and consequently, three prisms are made available covering the refractive index range of 1.25-1.40, 1.31-1.44, and 1.40-1.55, respectively. An example of a separation monitored by this detector is shown in Figure 5. The chromatogram depicts the separation of some polystyrene standards for molecular weight determination. The column is packed with 5 μm particles and a flow rate of 0.8 ml/min was employed. The separation was achieved in less than 15 min. This is a typical application for a refractive index detector. In this particular sample, the polystyrene polymers would absorb in the UV but the separation of similar polymers not having a UV chromophore would require the unique properties of a refractive index detector.

The Christiansen Effect Detector

This method of refractive index monitoring arose from the work of Christiansen on crystal filters (5,6). If a cell is packed with particulate material having the same refractive index as the mobile phase passing through it, light will pass through the cell with little or no refraction or scattering. If, however, the refractive index of the mobile phase changes, there will be a refractive index difference between the mobile phase and that of the granular packing. This difference in the refractive index results in light being refracted away from the incident beam and thus, the intensity of the transmitted beam is reduced. If the transmitted beam is focused onto a photocell and the refractive indices of the mobile phase and packing initially matched, then changes in refractive index resulting from a solute in the mobile phase will cause scattering and reduction in the output from the photocell.

In practice as the optical dispersion of the two media is likely to differ, the refractive indices will only match at one particular wavelength and thus, the *fully* transmitted light will be largely monochromatic. It follows that a change in refractive index of the mobile phase will change both the intensity and the wavelength of the transmitted light falling onto the photocell.

This device has been manufactured as a commercial detector by the Gow-Mac Company, who claimed it has a sensitivity of 10^{-6} refractive index units at a signal-to-noise ratio of two. This is equivalent to a sensitivity of about 9 x 10^{-6} g/ml benzene (refractive index 1.501) in heptane (refractive index 1.388). The cell volume was kept to 8 μl and as the cell was packed with particulate material, the band dispersion in the cell was minimal.

Different cells packed with appropriate materials were necessary to cover the refractive index range of 1.31 to 1.60. A diagram of a Christiansen effect detector is shown in Figure 6.

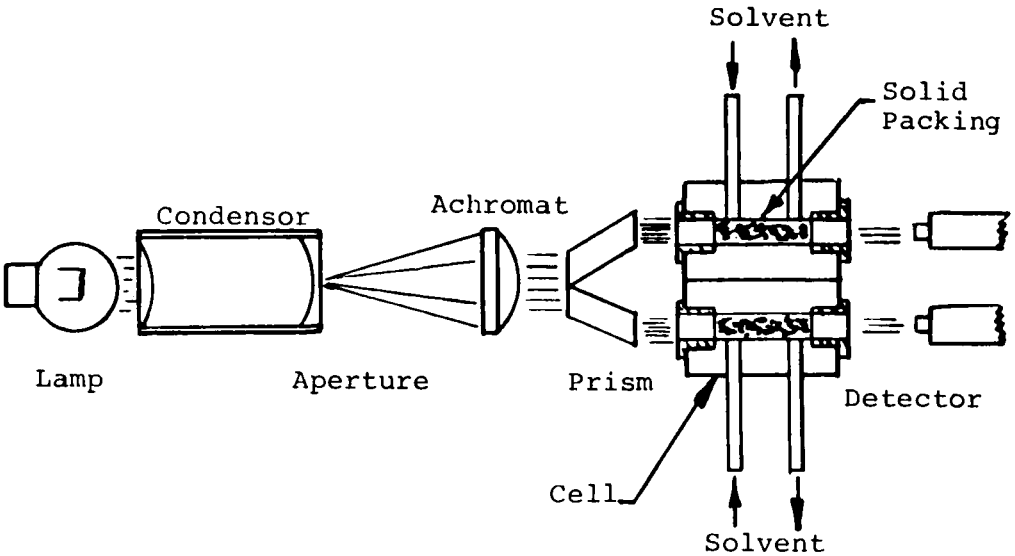


Figure 6. The Christiansen effect detector.

The optical module contains the prefocused lamp, the voltage of which is adjustable to permit operation at low energy when maximum sensitivity is not required. The condensing lenses, aperture, achromat, and beam splitting prisms are mounted in a single tube to prevent contamination from dust and permit easy alignment of optics. The system uses two cells, identical and interchangeable.

This detector is relatively inexpensive but suffers from the disadvantage that cells with packings of the appropriate refractive index must be used for each type of mobile phase employed. Close matching of the refractive indices of the cell packing and the mobile phase can be achieved by the use of mixed solvents and by adjustment of the cell temperature.

Applications of the Refractometer Detector

The refractive index detector is fairly simple to use and is sometimes employed in the preliminary scanning of samples. It is frequently used as the detector in gel permeation or exclusion chromatography. This detector is particularly valuable in the size separation of polymers where, providing the polymer has more than ten monomer units, the refractive index is directly proportional to the concentration of polymer and practically independent of the molecular weight. In a LC laboratory that has to handle large numbers of different samples, it is desirable to

have a refractive index detector available for special applications although it is likely to be employed for only a relatively small proportion of the total samples analyzed.

The Electrical Conductivity Detector

An acid, base or salt, when dissolved in water, ionizes into charged ions. If the acid, base and salt are represented by HA, BOH and BA, respectively, the ionization can be described by the following equations

$$HA \rightarrow H^+ + A^-$$

$$BOH \rightarrow B^+ + OH^-$$

and

$$BA \rightarrow B^+ + A^-$$

If the acid or base is weak, ionization is incomplete and an equilibrium condition occurs where

$$K_A\ (HA) \leftrightarrow (H^+) + (A^-)$$

$$K_B\ (BOH) \leftrightarrow (B^+) + (OH)$$

and K_A and K_B are the dissociation constants of the acid and base, respectively.

In any event, some ions are produced, their concentrations being dependent on both the original concentration of the acid or base, their respective dissociation constants and the physical properties of the solvent. Under the influence of a potential gradient, the ions in solution can carry an electric charge and thus, if a voltage is applied across two electrodes situated in the solution, a current will flow between the electrodes and the solution is said to be conducting. It is fairly obvious that such a system could be used as a method of detecting ionic species in LC, but before discussing the design of electrical conductivity detectors, it would be advantageous to consider in more detail the properties of ions in solution.

Early investigations into the electrical properties of solutions containing ions were somewhat frustrating as DC voltages were employed which resulted in the production of hydrogen and oxygen at the electrodes. This effect, called polarization, changed the electrical resistance of the system at the electrodes and produced confused results. It was not until AC potentials were employed at the electrodes, which rendered polarization effects insignificant, that the true electrical properties of solutions could be identified. It follows that if the measurement

of the electrical properties of ionic solutions is to be used effectively in a LC detecting system, AC potentials must be applied to any electrode configuration employed.

Experiments using AC potentials across the electrode system demonstrated that ionic solutions obeyed Ohms Law i.e.

$$V = RI$$

where V is the applied potential in volts,
I is the current flowing between the electrodes in amps,
R is the electrical resistance between the electrodes in ohms.

Generally, in physical chemistry the conductivity of the solution is of greater interest and is equivalent to the reciprocal of the specific resistance of a solution. The specific resistance of a solute is numerically equivalent to the potential in volts across the faces of a centimeter cube of the solution when carrying unit current. In electrical conductivity detectors it is the resistance of the solution that is actually monitored and it is the change in electrical resistance of the mobile phase in the presence of a solute that provides the output from the detector. For this reason, despite the term electrical conductivity detector, the functioning of the detector will be considered in term of resistance measurement.

The chromatographer is so familiar with water as the medium in which the ionization of solutes takes place that he tends to overlook the possibility of other solvents. Liquid ammonia, liquid sulphur dioxide, hydrogen fluoride, hydrogen sulphide and hydrogen cyanide are some typical examples of alternative ionizing media. Compared with water these substances ionize in the following manner

Substance	Cation	Anion
$2H_2O$	$(H_3O)^+$	OH^-
$2NH_3$	$(NH_4)^+$	NH_2^-
$2SO_2$	$(SO)^{2+}$	SO_3^{2-}
$2HF$	$(H_2F)^+$	F^-
$2H_2S$	$(H_3S)^+$	SH^-
$2HCN$	$(H_2CN)^+$	CN^-

Liquid ammonia and liquid sulphur dioxide appear to offer attractive possibilities for chromatographic purposes as both these substances, particular liquid sulphur dioxide, are very good solvents for organic substances. Such liquids would also lead to a unique form of supercritical chromatography (7). Furthermore, they are likely to ionize specific organic materials that otherwise do not normally ionize in water or are insoluble in

water. Those substances hitherto not ammenable to separation by aqueous ion-exchange chromatography might be efficiently separated by ion-exchange chromatography using liquid sulphur dioxide as the mobile phase. Employing liquid sulphur dioxide as an ionizing mobile phase also opens up possibilities for the design of entirely new forms of ion-exchange media.

The liquid chromatographer, at the first thought of such a system, may well be dismayed at the problems of toxicity and mechanical handling that appears to accompany the use of such materials. However, in the early days of refrigeration, entirely sealed systems were manufactured successfully for use in domestic environments employing both ammonia and liquid sulphur dioxide as the refrigerating liquid. It follows that the development of sealed LC systems incorporating such materials for use by scientists in a laboratory environment appears distinctly possible.

The first effective conductivity detector to be described was that of Martin and Randall (8). Improved cell designs have been described by Harlan (9), Sjoberg (10) and more stable and sensitive electronic circuits for use with conductivity detectors have been discussed by Avinzonis and Fritz (11) and Berger (12). Scott et al. (13) inserted electrodes in the walls of a column to monitor changes in band dispersion along a chromatographic column by conductivity measurement. More recently, Keller (14) described a bipolar electrical conductivity detector and Kourilova et al. (15) described a conductivity system with a detecting cell of only 0.1 μl volume.

An electrical conductivity detector cell consists of a small chamber containing two electrodes, across which an AC potential is applied for the reasons already discussed. The volume of the cell should be as small as possible to minimize dispersion. The electrodes should be constructed of an inert conducting material such as stainless steel, gold or platinum and they should be well insulated from one another so that only the resistance of the liquid between them is measured. The electrical capacity of the cell should also be kept to a minimum by ensuring that the surface area of the electrodes is as small as possible. If the cell has significant electrical capacity and a bridge circuit is used, then as an AC potential has to be employed to eliminate polarization, electrical balance will be difficult to achieve due to the out of phase capacity current across the bridge. The resistance of an ionic solution can change significantly with temperature and it may be necessary to thermostat the cells.

As a bulk property detector, the conductivity detector has limited sensitivity as a result of trace impurties, ionic in nature, in the mobile phase. Disolved carbon dioxide and ammonia can be a particular problem. The detector is not, however, very

sensitive to changes in flow rate and pressure and can only be used with gradient elution if the *ion* concentration remains constant.

A block diagram of a simple form of an electrical conductivity detector system is shown in Figure 7. The basic system usually consists of a frequency generator which may be in the range of 1-10 kHz depending upon the design, but should not be less than 1 kHz to eliminate polarization of the electrodes. The AC voltage is applied across the electrode system in a resistance network which can take various forms. The most common form employed is that of the Wheatstone Bridge, the electrodes take the place of one resistance arm of the bridge and the complimentary arm being an adjustable resistance to permit zero control. The out of balance signal is then passed to a precision rectifier which provides an output proportional to the change in resistance of the conductivity cell. As the concentration of ions is inversely proportional to the cell resistance, a linearizing amplifier is necessary to provide an output that is proportional to ion concentration. The output from the linearization amplifier is then passed to an appropriate attenuator.

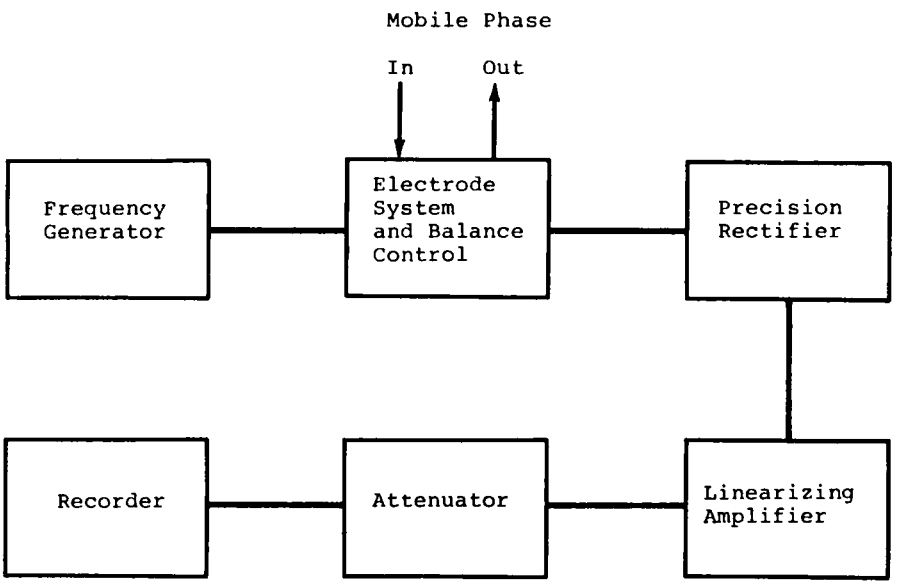


Figure 7. An electrical conductivity detector system.

An example of a simple conductivity cell is shown in Figure 8. The electrodes consist of two stainless-steel discs, the mobile phase entering through the center of one electrode and out through the center of the other.

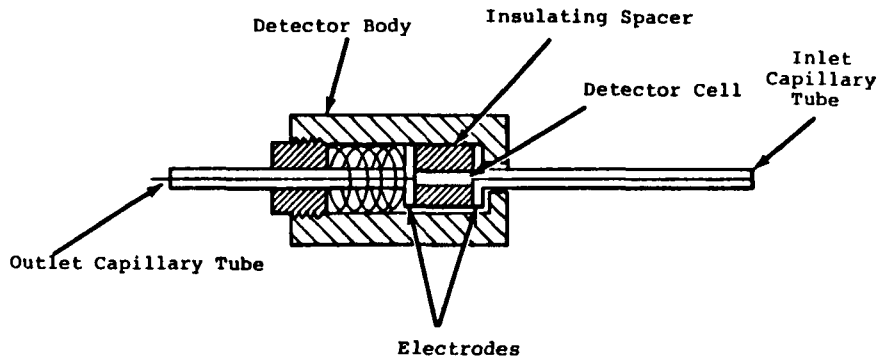


Figure 8. An example of an electrical conductivity cell.

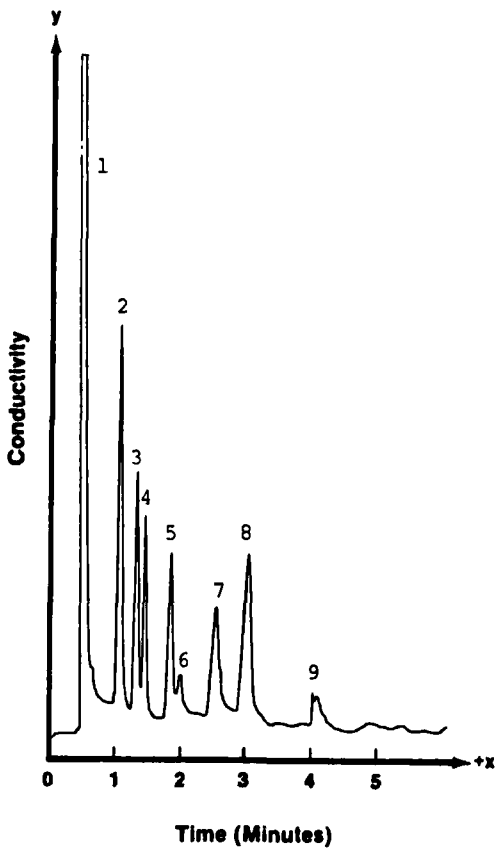


Figure 9. A chromatogram of the separation of some common anions monitored by an electrical conductivity detector. Anions: 1=pseudo-peak, 2=chloride, 3=nitrate, 4=bromide, 5=nitrate, 6=phosphate, 7=phosphite, 8=sulfate, and 9=iodide.

The disc electrodes are separated by an appropriate spacer which can be made of glass or some other insulating material, but must be inert to the mobile phase to be used and, in particular, buffer solutions. Leak proof seals are achieved by the use of appropriate washers, e.g.PTFE. The whole electrode system is housed in a detector body made from a suitable insulating material and the leak proof seal maintained by an appropriate spring compressed by a threaded plug. An example of a typical chromatogram obtained from such a detector is shown in Figure 9. The chromatogram shows the separation of 8 anions in less than 5 min, employing a C_{18} reversed-phase column and a mobile phase consisting of 1 mM tetrabutylammonium hydroxide adjusted to pH 8.0 and saturated with a solution of potassium hydrogen phthalate. The separation was developed at a flow rate of 1.5 ml/min and the sample contained 50 ppm of all the anions.

Applications of the Electrical Conductivity Detector

Conductivity detection has been particularly valuable in the analysis of water, soap products and detergents, soft drinks and other materials containing acids, bases or salts. It is used extensively in water analysis including drinking water and boiler feed water. The food industry, both human and animal, also utilizes it in routine analytical procedures. The conductivity detector is used as a monitor in the separation and identification of anions by ion-exchange chromatography in the wine and beer industry and is used extensively in environmental analysis. It is employed in the analyses of plating baths, nuclear fuel reprocessing streams and in clinical laboratories in the analysis of blood, spinal fluids, etc. Conductivity detectors can,under very carefully controlled conditions, achieve high sensitivities and, for example, detect concentrations of sodium chloride down to 10^{-9} - 10^{-8} g/ml. However, as already stated, it is very susceptable to variation in ionic materials in the mobile phase. This type of detector competes directly with the refractive index detector as the second most important bulk property detecting system in LC.

The Dielectric Constant Detector

Under the influence of small fields, electrons move quite freely through conductors, whereas in insulators or dielectrics these fields displace the electrons only slightly from their equilibrium positions. As an electric field acting on a dielectric causes a separation of positive and negative charges, the field is said to polarize the dielectric. The polarization can occur as a result of two effects: the induction effect and the orientation effect. An electric field always induces dipoles in molecules on which it is acting, whether or not they contain dipoles to begin with. If the dielectric does contain molecules that are permanent dipoles, the field tends to align these dipoles along its own direction. As a result of the induction or

orientation, it is found experimentally that when a dielectric is introduced between the plates of a capacitor, the capacitance is increased by a factor ε, called the dielectric constant. Thus, if C_0 is the capacitance with a vacuum between its plates, the capacitance with a dielectric is $C = \varepsilon' C_O$. In this way the dielectric constant of a substance can be defined. Due to the electromagnetic nature of light, it's transmission is also affected by the dielectric constant of the medium it passes through. It follows that the refractive index of a substance is a complementary property to dielectric constant and in some circumstances is a direct function of it. For example, for non-polar substances, or mixtures of non-polar substances, the relationship between the dielectric constant ε' and the refractive index of the substance or mixture, n, is given by

$$\varepsilon' = n^2$$

For semipolar substances or mixtures of semipolar substances and non-polar substances the above equation has to be modified to the following form

$$\frac{\varepsilon'-1}{\varepsilon'+2} = \frac{n^2-1}{n^2+2}$$

For polar substances or mixtures of polar substances and semi polar substances, however, the relationship breaks down and there is no simple function that describes refractive index in terms of dielectric constant.

In general, the more polar the substance the larger is its dielectric constant. This is always true for substances having monofunctional groups and generally true for substances having more than one functional group but there are exceptions. For example, dioxane with two ether groups has a fairly low dielectric constant although it is a very polar solvent. The low value for the dielectric constant results from the fact that the two dipoles are electrically in opposition and thus partially neutralize the effect of each others charge. This effect is worth considering when choosing the mobile phase for use with dielectric constant detector.

In normal chromatography the mobile phase is usually *less polar than the solutes being eluted* as they need to be retained on the column to achieve a separation. Thus, the presence of a solute in the mobile phase will, in general, increase the dielectric constant of the mobile phase. In reversed-phase chromatography the converse often applies. The *solute is usually less polar than the mobile phase* and, consequently, the dielectric constant of the mobile phase is *decreased* by the presence of a

solute. If a device is situated at the end of the column which responds to changes in dielectric constant, such a device can act as a chromatographic detector.

In practice the sensory element often takes the form of a cylindrical or parallel plate condenser. To maintain column efficiency, the volume of the condenser has to be very small and, as the sensitivity of the device is directly related to capacity of the condenser, the plates have to be very close together.

The capacity (C) of a parallel plate condenser is given by:

$$C = \frac{0.080\ (N-1)A\varepsilon'}{d}$$

where ε' is the dielectric constant,
N is the total number of plates,
A is the area of each plate in cm^2,
d is the distance between the plates in cm.

The capacity of a cylindrical condenser is given by

$$C = \frac{0.2416\varepsilon l}{\text{Log } r_1/r_2}$$

where l is the length of the cylinder in cm,
r_1 is the radius of the outer cylinder in cm,
r_2 is the radius of the inner cylinder in cm.

The impedance (i) of a condenser, which is in effect its resistance to an alternating electrical supply is given by

$$i = 1/2\pi fC$$

where f is the frequency of the applied AC potential.

It follows that as the capacity *C* is directly proportional to the dielectric constant between the electrodes of the cell, then the electrical impedance of the cell will also vary inversely with the dielectric constant. Furthermore, the change in impedance with dielectric constant will increase with the frequency of the applied potential. Consequently, if the cell impedance is measured by a suitable detecting system, the sensitivity of the detector will be improved by operating at a higher frequency.

The most appropriate circuit to use in dielectric constant measurement is an AC bridge, the detector condenser being situated in one arm of the bridge; however, the type of bridge that is appropriate depends on the design of the detector cell. If the capacity of the cell is reasonably large, (100 pF or more) a Wein

Bridge can be used, if small, (1-10 pF) then it is more appropriate to use the Schering Bridge; both these bridge systems will be described but before this is done, some discussion on the balancing of an AC bridge is appropriate. No capacitor is ideal insomuch that there is always some resistance component associated with it. If the plates of the detector cell are not insulated from the mobile phase and the mobile phase is conducting, then the resistance component can obviously be very large. Now the voltage developed across the resistive component of the condenser is out of phase with the voltage developed across the capacity component. It follows that to balance the bridge the two components have to be balanced separately or no null balance point will be found. It should also be noted that if the eluted solute changes both the dielectric constant and the resistance of the mobile phase, as in the case of ionized solutes, then both the resistance and the capacity of the detecting cell will be changed and thus could provide a greater response than the effect of either alone. Thus, a dielectric constant detector that responds to both dielectric constant and conductivity of the mobile phase would be very sensitive to ionic solutes and this could be achieved by using uninsulated plates in the detector capacitor. The theory of the combined effects of electrical conductivity and dielectric constant on detector output has been discussed in detail by Haderka (16) and workers interested in this aspect of dielectric constant detection are recommended to read his original paper.

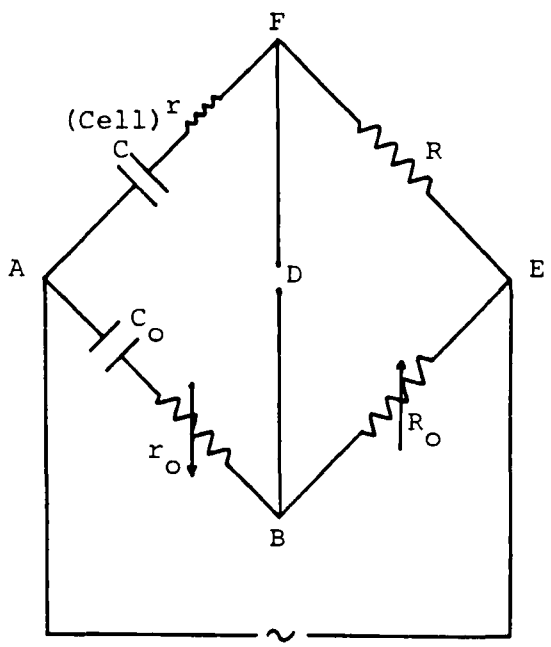


Figure 10. The Wein Bridge.

A diagram of the Wein Bridge used for detectors having capacities of 100 pF or more is shown in Figure 10. ABEF is the bridge with an AC potential applied across A and E. The out of balance signal is sensed across B and F by D which can be an AC amplifier and rectifier feeding a potentiometric recorder. C and r represent the capacity and resistance of the detector cell, respectively. C_O is a standard reference capacitor or can be the capacity of the reference cell if one is employed; R is a fixed resistor and R_O and r_O are variable resistances for obtaining balance.

Balancing is achieved by iterating the adjustments of R_O and r_O for zero output across FB. The conditions of balance are

$$\frac{R}{R_o} = \frac{r}{r_o}$$

and

$$\frac{C}{C_o} = \frac{R_o}{R}$$

Any change in C or r due to the presence of solute in the detecting cell will result in an off balance signal across FB.

For small capacity cells the Schering Bridge can be used which was originally designed for testing cables. A diagram of the Schering Bridge is shown in Figure 11. The alternating supply is applied across AE and the out of balance signal taken across FB in a similar manner to the Wein Bridge.

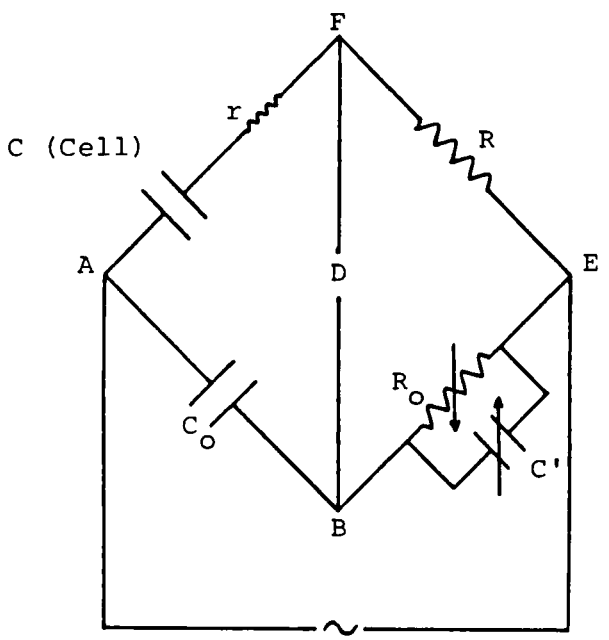


Figure 11. Schering Bridge.

In this figure C and r represent the capacity and resistance of the detecting cell, respectively, R and C_O are the reference resistance and capacitor and if a reference cell is employed, C_O can be the capacity of the reference cell. R_O and C' are a variable resistance and capacity, respectively, for obtaining balance. Balancing is achieved by the iterative adjustment of R_O and C', when balanced, any subsequent change in C or r resulting from solute in the detector cell will produce an off balance signal across FB. The conditions for balance are as follows

$$\frac{C}{R_o} = \frac{C_o}{R}$$

and

$$\frac{r}{R} = \frac{C'}{C_o}$$

It should be emphasized that the resistance component of the cell across the detector capacity reduces the bridge sensitivity to changes in dielectric constant or makes the initial balancing procedure tedious. To reduce this effect, and to ensure that system responds to changes in dielectric constant *only*, the plates of the detecting condenser are often well insulated from the mobile phase, thus eliminating the resistive component of current.

Another method of measuring changes in dielectric constant is to make the condenser containing the dielectric part of an oscillator circuit. For example, if the capacitor is connected in parallel with an inductor and made the frequency source of an oscillator, the frequency will depend, among other things, on the capacity of the condenser. If the output is heterodyned, with the output of a stable reference oscillator and balanced, then the different frequency resulting from a change in capacity due to the presence of a solute can be passed to a discriminator, rectified and the DC output fed to a recorder. This alternative method of measuring changes in dielectric constant can be extremely sensitive. However, it is not the electrical measuring system that determines the overall detector sensitivity, but the limitations inherent in bulk property detectors that have already been discussed.

One of the early dielectric constant detectors to be described was that by Grant (17) but the detector cell had a volume of 2-3 ml. Poppe and Kunysten (18) designed a dielectric constant detector which included a reference cell for temperature compensation. The cell consisted of two stainless-steel plates 2 cm x 1 cm x 1 mm separated by a gasket - 50 μm thick, the two cells, the reference cell and the detecting cell were identical and clamped back to back, thus, sharing a common electrode. The stated sensitivity was 10^{-6} g/ml for chloroform ($\varepsilon' = 4.81$) in isoctane, the cell system, however, was found to be very sensitive

to pressure changes even when constant flow pumps were employed. Fluctuations in the outlet pressure of the column were thought to deform the plates and thus produce sporadic noise. Another detector described by Ooguri employed a capacitance cell, cylindrical in form with a central concentric electrode. The cell was used as the capacitor of a tuned circuit that was heterodyned with 9 MHz oscillator, the difference frequency being rectified and fed to a suitable recorder. The volume of the cell was stated to be 10 µl, and 10 µg of material was detectable. However, the sensitivity in terms of concentration was not given.

It was not until 1979 that the first dielectric constant detector for LC became commerically available (19). Beninngfield and Mowrey (20) described this detector in detail including several applications and Bade et al. (21) also reported a number of applications for this particular device. A diagram of the detecting cell is shown in Figure 12.

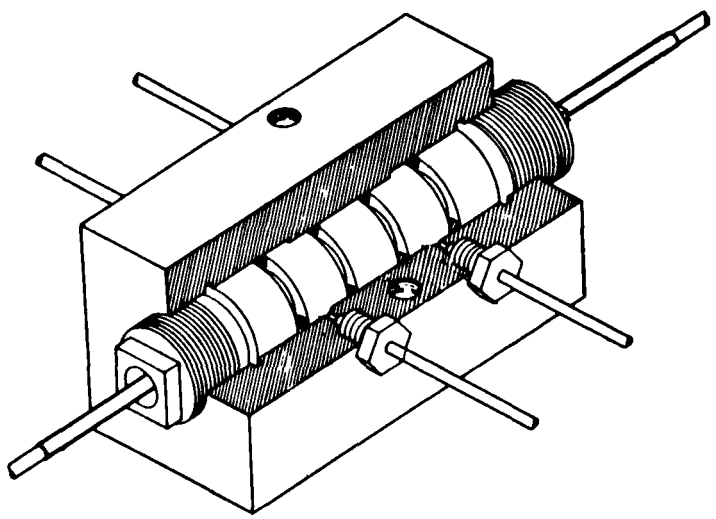


Figure 12. A dielectric constant detector cell.

The dielectric constant detector is a differential-type detector with both a reference and a sensor cell. Figure 12 is a cut-away diagram of the detector cell. Each cell consists of a concentric cylinder (inner electrode) inside a larger diameter cylinder (outer electrode) which forms the outer wall of the cell. The two cylinders are electrically isolated with a cylindrical flow path. In operation, the mobile phase flows through the wall of the outer cylinder, divides around the small inner cylinder, and exits 180 degrees from the entrance port. The inner cylindrical electrodes are 1.26 cm in diameter and 0.625 cm in length. The outer cylinder of each cell has a dual diameter which provides each cell with two different spacings ("d" spaces)

between the electrodes. Therefore, the dielectric constant as measured in each cell is the result of both "d" spacings with the cell constant adjustable and dependent upon the position of the inner electrode. The 0.625 cm length of the inner electrode can be moved in or out to provide fine adjustment of the cell calibration constants in order to precisely match the reference and sense cells' capacities. This arrangement provides a nominal separation distance of approximately 0.009 cm between the two electrodes with a cell volume of approximately 22 µl.

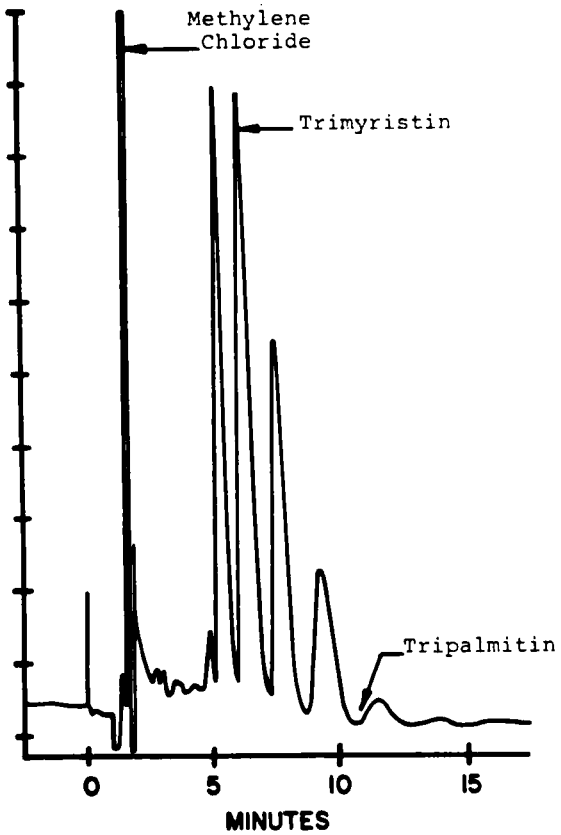


Figure 13. The separation of triglycerides employing the dielectric constant detector.

The exact volume and clearance depend upon the position of the inner electrode with respect to the outer electrode and its two "d" spacings.

The cell electrodes are constructed from stainless steel for corrosion resistance, strength, and thermal stability. The outer electrode is maintained at ground potential and common to both cells. This design also provides a common thermally controlled environment for the two cells and simplifies the construction of the inlet and outlet tubing from the chromatograph, since

electrical isolation of the tubing is not required. As shown in Figure 12, PTFE O-rings seal the ends of the cells and center the inner electrodes. A Kel-F® spacer is used at each end to insulate and aid the PTFE O-rings in sealing the two cells. Two adjustment screws provide a means of adjusting the inner electrodes for matching the two cell constants.

This adjustment procedure is accomplished by simultaneously turning both of the adjustment screws, one in and the other one out, until the cell constants are matched. Each matched cell forms part of the capacitance in parallel with an inductor producing a resonant circuit. The cells are made to control the frequency of two digital oscillators and the output from each fed to a mixer and the difference frequency passed to a frequency/voltage convertor. The output from the convertor with suitable modification is then fed to the recorder. An example of the use of the detector in separation of some triglycerides contained in cottonseed is shown in Figure 13. The reversed-phase column was 15 cm packed with 10 μm particles and operated at a flow rate of 1.5 ml/min. The mobile phase was 15% methylene chloride, 15% tetrahydrofuran, 30% acetonitrile and 40% water.

The linear dynamic range of this detector is quoted as 3.5×10^4, but the range will depend upon the relative difference between the dielectric constant of the solute and that of the mobile phase employed. The maximum sensitivity was quoted as 0.1×10^{-6} g/ml. The volume of the cell is approximately 22 μl, which is rather large for modern LC detectors and certainly too large for use in conjunction with modern high speed or small bore columns. Where the volume of the cell is not important as in preparative LC, it does provide a practical alternative for the refractive index detector. The sensitivity is very similar to that of the refractive index detector, but the linear dynamic range is almost an order of magnitude greater. However, this apparent wider dynamic range is a result of its linear response extending into relatively high concentrations of solute (e.g. 1% w/v), concentrations that are above the practical limit in analytical LC.

Additional Bulk Property Detecting Systems

The bulk property detectors previously described employ well established detecting methods that have been investigated by numerous scientists and are, for the most part, used in practice in the field of chromatography. There are, however, a number of bulk property detecting systems that have been described in the literature and that have either not achieved popularity with chromatographers generally, not been developed to their full potential or have a limited performance. Some of these detecting systems will be discussed in this chapter to give the reader an idea of the range of physical properties that have been examined

as a possible basis for chromatographic detection. Hopefully, it may also stimulate the reader to develop some of them to a greater level of sophistication and reliability.

The Thermal Lens Detector

When lasers are focused onto absorbing material, the refractive index may be effected in such a way that the medium behaves as a lens. This effect was first reported by Gorden et al. (22,23) in 1964 and since then has been investigated by a number of workers. Thermal lens formation results from the absorption of laser light, which may be extremely weak. The excited-state molecules subsequently decay back to ground states and as a result localized temperature increases occur in the sample. Since the refractive index of the medium depends on the temperature, there is a resulting spatial variation of refractive index, which is equivalent to the formation of a lens within the medium.

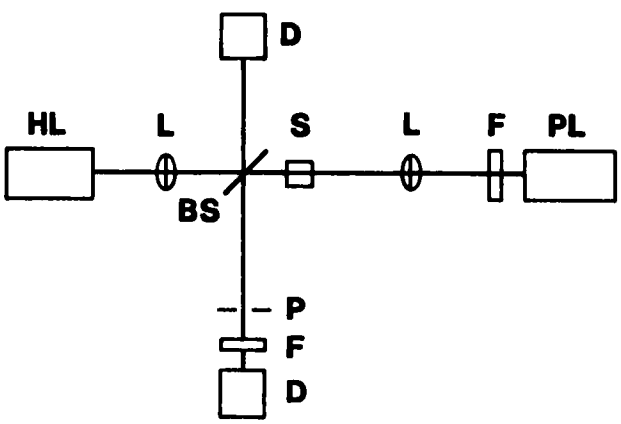


Figure 14. Thermal lens detector.

The temperature coefficient of refractive index for most liquids is negative, consequently, the insertion of a liquid in the laser beam produces a concave lens that results in beam divergance.

The thermal lens effect has been utilized by Buffett and Momis (24) to develop an interesting small volume LC detector. A diagram of their system is shown in Figure 14. It consists of a heating laser HL, the light from which is passed directly through the sample (S) via two lens (L) and a half mirror (BS). Another laser, the probe laser (PL), passes light in the opposite direction through one lens (L), through the sample to a half mirror where the light is reflected onto a photocell (D). Between the mirror and the photocell is a filter to remove the heating

laser light and a pinhole (P). When an absorbing solute arrives in the cell a thermal lens is produced which causes the probe light to diverge and consequently reduces the intensity of the light passing through the pinhole onto the photocell.

The cell can be made a few microlitres in volume and thus would be suitable for use with small bore columns. A sensitivity of 10^{-6} AU was claimed for the detector and a linear dynamic range of about three orders of magnitude (although by now this may have been increased). As with other bulk property detectors, the thermal lens detector would not be suitable for use with gradient elution. The use of lasers make the detector extremely expensive, however, the availability of a UV laser, should it be developed, might make the device more useful.

Basically, the thermal lens detector is a special form of refractive index detector and, as such, has potential as a type of universal detector. There is also the possibility that it could be developed into a more sensitive detecting system than the conventional refractive index detector.

Differential Viscometer Detector

The presence of the solute in the mobile phase eluting from a column will change the viscosity of the mobile phase either decreasing or increasing it. This change in viscosity will be particularly significant where polymers are being separated, by for example, gel permeation. High-molecular-weight polymers can greatly increase the viscosity of the mobile phase even at very low concentrations.

A commercial detector based on the measurement of viscosity has been developed by Viscotek Corporation. The differential measurement is based on the fluid analog of the Wheatstone Bridge, a diagram of which is shown in Figure 15, and the detector is a modified form of this bridge.

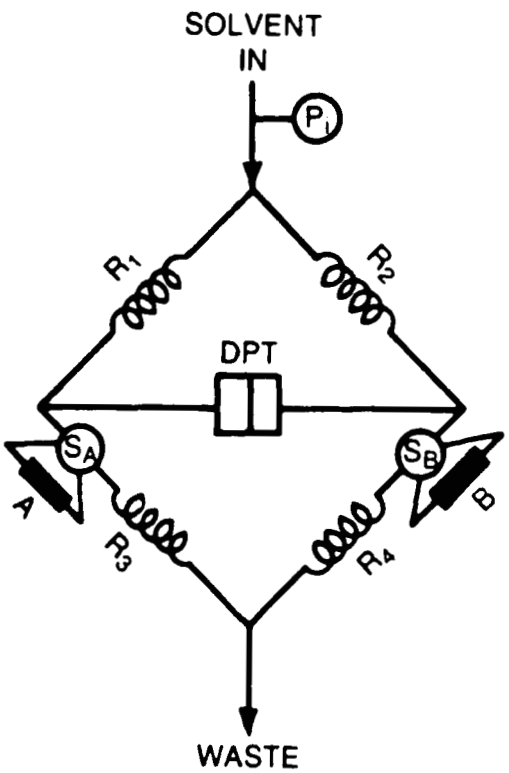
Solvent flows continuously through a bridge network which consists of four matched stainless-steel capillaries. The differential pressure across the bridge is zero until the sample contained in the column eluent passes into capillary R_3.

As a consequence,the differential pressure begins to rise until it reaches a steady state value of ΔP proportional to the specific viscosity of the solution. The differential pressure is monitored continuously on a strip chart recorder. The equation relating ΔP to specific viscosity is:

$$\frac{\Delta P}{P_i} = \frac{\eta}{2\eta + 4} \approx \frac{\eta}{4}$$

where P_i is inlet pressure of solvent to the bridge and held constant.
η is the viscosity difference between the solvent and that of the solute carrying the solvent.

A form of the viscosity detector has been used for exclusion chromatography, and prior experiments employing a single capillary have demonstrated the feasibility and usefulness of a continuous on-line viscosity detector in conjunction with a concentration detector (RI or UV). The sensitivity of the device, however, is rather poor, the minimum detectable concentration being about 10^{-4} g/ml. To date, the instrument has only been used in a recent application in polymer chromatography.



R_1, R_2, R_3 R_4 = Matched SS Capillary Tubing
A, B = Solution Holdup Reservoirs
S_A, S_B = Switching Valve
P_i = Solvent Inlet Pressure Gauge
DPT = Differential Pressure Transducer

Figure 15. The Viscotek differential viscometer.

The Interferometer Detector

The interferometer detector was developed by Bakken and Stenberg (25) in 1971. The principle of this detector is based on the change in effective pathlength of a beam of light passing through a cell when a solute is present in the mobile phase. If the light from the cell focused onto a photocell is coincident with a reference light beam from the same light source, interference fringes will be produced; the fringes will change as the pathlength of one light beam changes (from the detector cell) and thus as the concentration of solute increases in the detector cell, a series of electrical pulses will be generated by the photocell as each fringe passes over it.

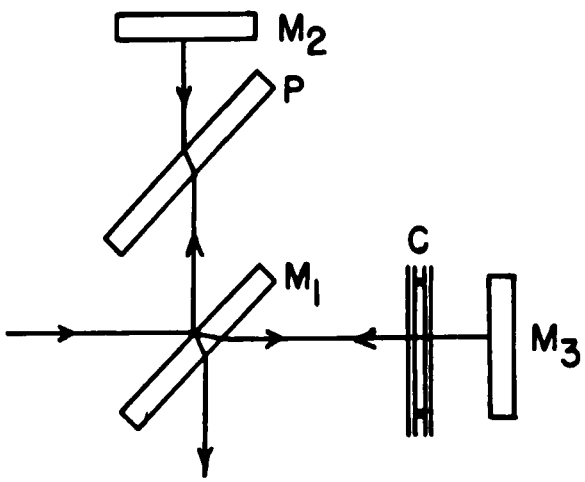


Figure 16. The interferometer detector

The change in fringe pattern is also an indirect way of measuring the change in refractive index. In fact, this detector is a form of refractometer detector: the pulses from the changing interference fringes also constitute an integration procedure, the total number of pulses being directly related to the total mass of solute present in the eluted peak.

The effective optical pathlength, d, when the light-filled sample cell is placed in the interferometer, depends on the change of refractive index of the liquid, Δn, and pathlength, ℓ, of the cell according to the following equation

$$d = \Delta n \ell$$

Further, it is possible to relate a number of fringes (N) (sensitivity), which move past a given point (or the number of cyclic changes of the central portion of the fringe pattern), to the refractive index change by the equation

$$N = 2\Delta n \ell / \Lambda$$

where Λ is the wavelength of the light used.

The larger N is for a given Δn, the more sensitive the detector becomes. Therefore ℓ must be large and Λ as small as practical. The practical limit of the length of cell is determined by the maximum dead volume allowable for efficient flow through the cell and minimum dispersion.

A diagram of the optical system employed by the authors is shown in Figure 16. Light from the source strikes a half silvered mirror M_1 and is divided into two paths. Part of the beam is reflected onto a plain mirror M_2 and back through M_1 onto the photocell. P is a compensating glass plate. The other beam passes through the cell C to a plain mirror M_3 and thence back through the cell C to the half silvered mirror M_1 where it is also reflected onto the photocell.

The trace representing the elution of 8 µl of dioxane is shown in Figure 17. Each peak in Figure 17 shows the passage of a fringe across the photocell and the combination of the four peaks represents the single *chromatographic* peak. The number of fringe peaks will be directly proportional to the total refractive index change and this will be proportional to the total mass of solute present. Detection by this method of incompletely resolved peaks could be very confusing and difficult to interpret, but this system is certainly one of the more novel methods for LC detection. In common with all bulk property detectors, this detecting system is only applicable to isocratic and isothermal methods of development.

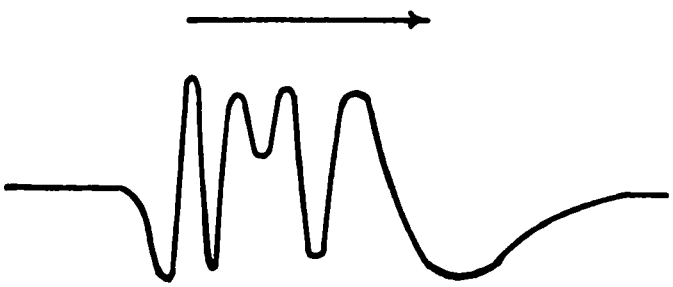


Figure 17. Chromatogram from interferometer detector.

An interferometer detector that is commercially available has been manufactured by Optilab which is illustrated in Figure 18 (26).

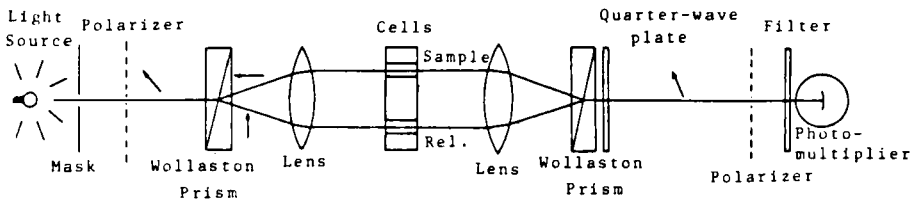


Figure 18. The Optilab interference refractometer, light path scheme.

The light source beam is linearly polarized at -45° to the horizontal plane. Horizontal and vertical polarized light are produced by splitting the beam by a Wollaston prism. The beams are focused on the sample and reference cells. The beam passing through the sample cell is horizontally polarized, while the reference beam is polarized vertically. After passing the cells, the beams are focused on a second Wollaston prism, followed by a quarter-wave plate which has its fast axis at -45° to the horizontal plane. A beam that is linearly polarized in the fast axis plane will, after passing the plate, lead another linearly polarized but orthogonal beam by a quarter of a wavelength. The phase difference results in a circularly polarized beam.

It can be assumed that each of the beams focused on the Wollaston prism consists of two such perpendicular beams which, after the quarter-wave plate, result in two circularly polarized beams of opposite rotation. These beams will interfere with each other to yield the original linearly polarized beam. A second polarizer is placed at an angle (90-β) to the first one, allowing 35% of the signal to reach the photomulitiplier. A filter transmitting light at 546 nm precedes the photomultiplier.

If the sample cell contains a higher concentration of solute than the reference cell, the refractive index in general will be higher and the interfering beams will be out of phase. The refractive index difference, Δn, and the phase difference, Δp, are related by

$$\Delta p = \Delta n b \, (360) \, \lambda - 1 \text{ degrees}$$

where b is the cell length
λ is the wavelength of the light source

The circularly polarized beams, therefore, will interfere to yield a linearly polarized beam which is rotated $\Delta p/2$ degrees,and the amplitude striking the photomultiplier, A_p, will then be:

$$A_p = A_o \cos(90 - \beta - \Delta p/2) = A_o \sin(\beta + \Delta p/2)$$

The high sensitivity possible employing this system makes the usual extreme electronic amplification unnecessary. The sensitivity is due to the very high primary optical signal; as a result, an excellent signal-to-noise ratio can be achieved. An example of the use of this detector to monitor the separation of a mixture of triglycerides from coconut oil is shown in Figure 19.

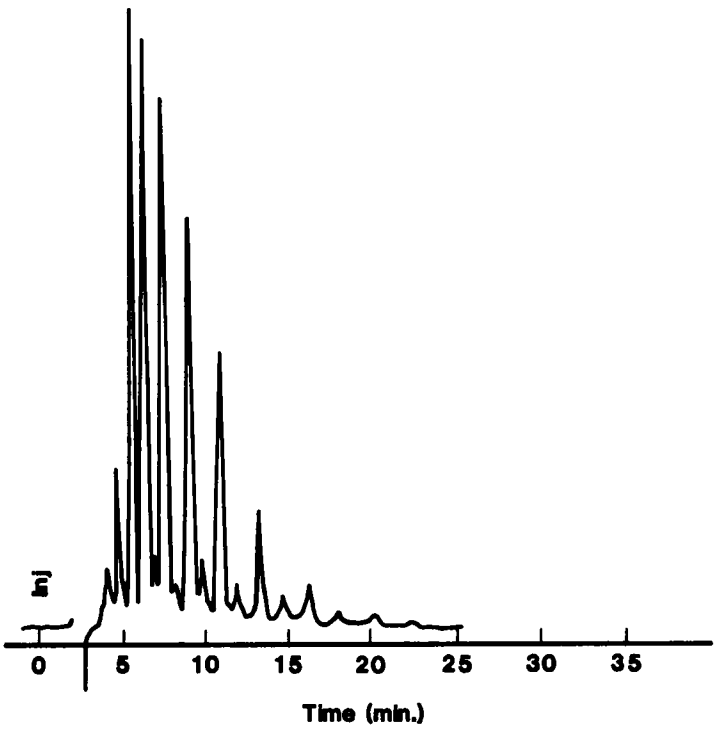


Figure 19. Separation of triglycerides in coconut oil.

The separation was carried out on a reversed-phase column employing a acetonitrile/water mixture containing silver nitrate as the mobile phase.

The Density Detector

The density of the mobile phase leaving a column will be changed when a dissolved solute is present, and thus, the continuous measurement of solvent density can become an effective method of detection. Such a method has been described by Fornstedt and Porath (27) and a diagram of their detecting cell is shown in Figure 20. It consists of a spherical glass plumb that is supported from the arm of an automatic, continuously recording electrobalance (Cahn) and is totally immersed in the eluent leaving the column. The plumb is 5 mm in diameter and about 0.4 g in weight. The solvent flows around the plumb and then into the overflow tube as shown in Figure 20. In order to maintain stability, the chamber containing the float is situated in a

thermostat and the mobile phase brought to the temperature of the cell prior to entering by passage through a metal coil situated between the column and the detector chamber.

Owing to its nature, the arrangement is inherently very sensitive to mechanical and electrical noise. Mechanical vibrations can be avoided by mounting the balance on a very heavy support. Electrical disturbances must be minimized by careful earthing. Temperature gradients within or around the apparatus can seriously disturb the system and therefore should also be minimized.

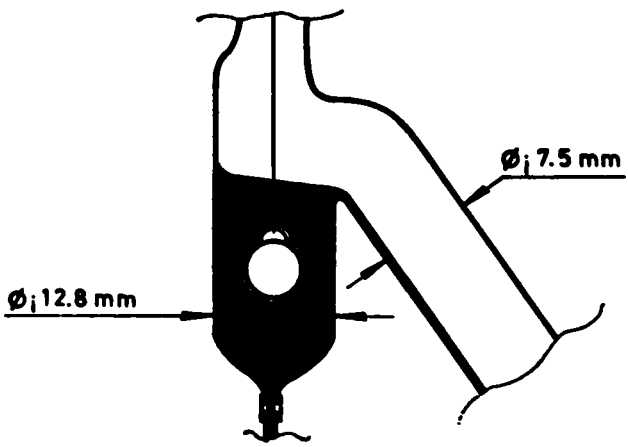


Figure 20. The density detector.

The entire liquid system should be thermally insulated so that the maximum range of temperature variation is one degree above or below the prevailing ambient temperature. A further disadvantage of the system is the large dead volume of mobile phase in the detector chamber itself that is necessary to contain the glass plumb. The relatively large volume would cause very serious dispersion of the detector if employed with modern high efficiency columns. An example of a chromatogram of a mixture of tert.-Boc-valine and phenylalanine methyl ester separated on Sephadex LH-20 and obtained from this detector is shown in Figure 21. Each peak represents 50 mg of solute and so it is seen that the detector exhibits very poor sensitivity. It will, however, detect any solute having a density different from that of the solvent, but again it has to be used under conditions of isocratic development.

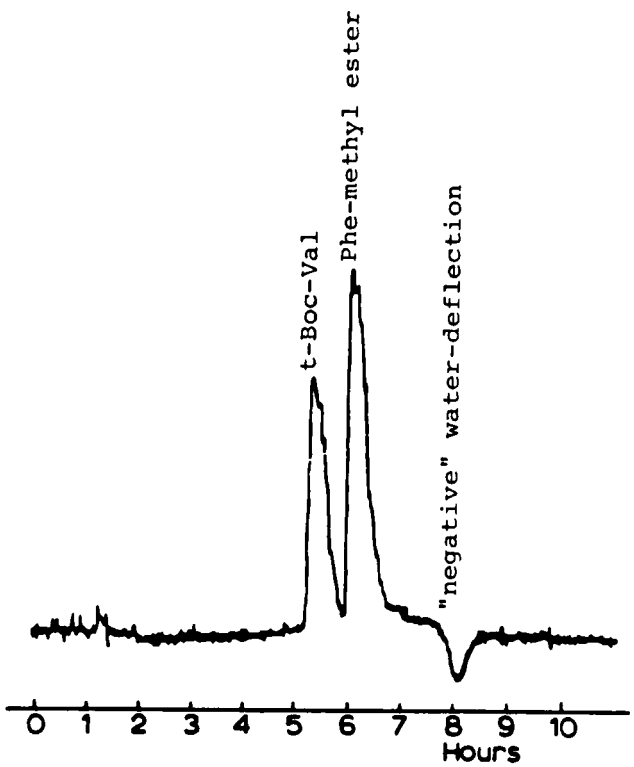


Figure 21. Chromatogram from density detector.

The Density Balance Detector

The density balance detector is a novel application of the Gow-Mac Gas Density Balance and has been described by Quillet (28). The gas density balance detector functions by measuring the differential flow across the base of two columns of gas, one containing pure carrier gas, the other containing the solute vapor as it is eluted from the column. The flow of gas resulting from the pressure difference at the base of each column is sensed by appropriately positioned thermistors. A diagram of a gas density bridge is shown in Figure 22. Quillet situated the density balance detector in an appropriate thermostat and passed a flow of liquid through the reference cell and the column eluent through the sample cell. The normal ancillary detector electronics were employed, a chromatogram obtained from his apparatus demonstrating the detection of 10 µl of cetane and decaline is shown in Figure 23. According to Quillet, the sensitivity of the detector was about 10^{-4}-10^{-5} g/ml and had a linear dynamic range of about one order. In the opinion of the author, it is highly unlikely that the detector is responding to difference in density between the column eluent and the reference liquid but is more likely to be responding to changes in the thermal properties of the mobile phase such as thermal conductivity or specific heat.

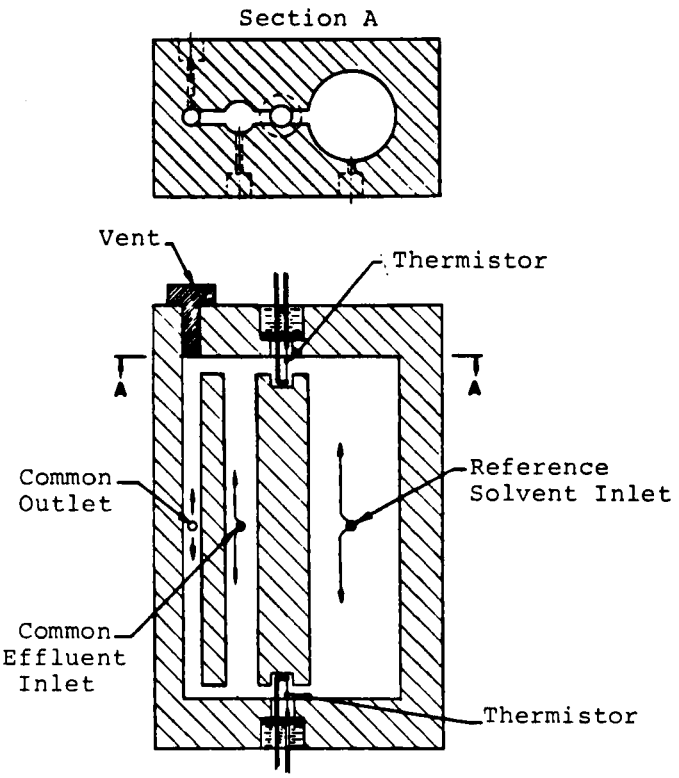


Figure 22. The density bridge detector

These properties of the mobile phase will be changed in the presence of the solute and could affect the output of the thermistor in much the same way as a thermal conductivity detector. The detector would have to be used under isocratic conditions of development but if investigated further might provide the basis of a very useful detector for preparative LC where high sensitivities are not required.

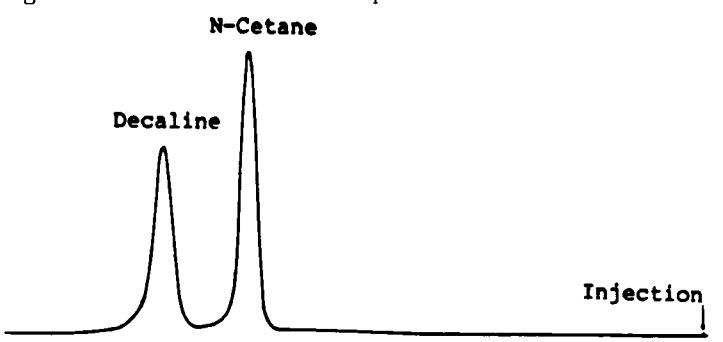


Figure 23. Chromatogram from the density bridge detector.

The Thermal Conductivity Detector

The presence of a solute in the mobile phase leaving a chromatographic column will change the thermal properties of the mobile phase, and thus, a device that continuously monitors the thermal properties of the eluent could act as an effective detector; the katharometer employed as a gas chromatographic (GC) detector functions on this principle. The thermal properties that are changed significantly when a solute is present in the mobile phase are thermal conductivity and specific heat. Ohzeki et al. (29) have described, what they term, a thermal conductivity detector for use in LC.

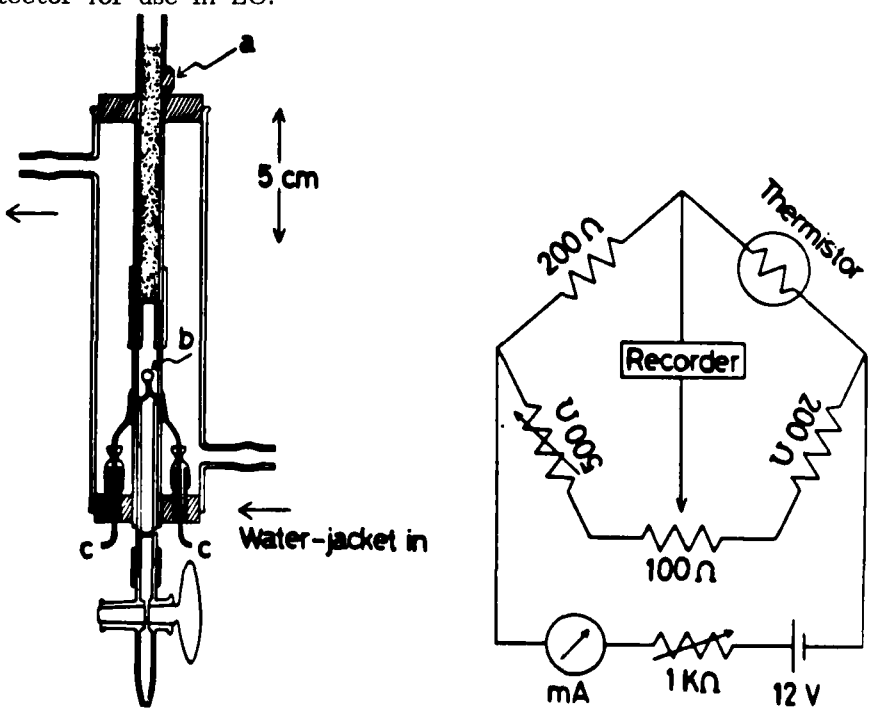


Figure 24. The thermal conductivity detector.

It appears, however, on reading their paper that the detector is probably responding more to changes in the specific heat of the mobile phase than to thermal conductivity. A diagram of their detector together with the bridge circuit employed with it is shown in Figure 24. A thermistor is situated in the mobile phase leaving the column and is heated by a current passing through it. The thermistor comes to an equilibrium temperature and thus a constant resistance when the heat generated by the current passing through is equal to the heat lost to the mobile phase. In the presence of a solute the heat lost to the mobile phase will differ resulting in a change in temperature of the thermistor. The resistance of the thermistor will also be changed which will unbalance the bridge and provide an output to the recorder. An

example of some elution curves obtained from the detector for blue dextran separated on a Sephadex G-25 column is shown in Figure 25. The peaks in Figure (23) indicate that a very useful level of sensitivity can be obtained from this detector and further, the dead volume of this device could be made relatively small, and thus, it could be used with high efficiency columns. It would, however, have to be employed under isocratic conditions of development and as the detecting system depends on temperature measurement, careful thermostating of the total system would be necessary.

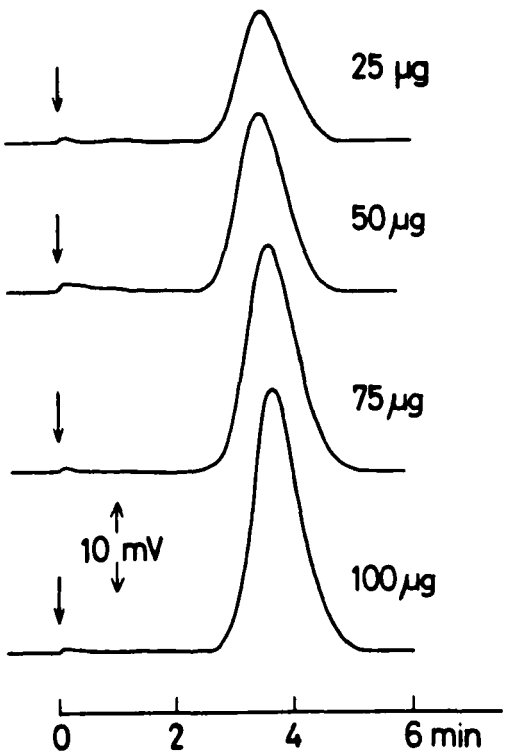


Figure 25. Chromatograms obtained from the thermal conductivity detector.

Synopsis

Bulk property detectors continuously monitor some physical property of the column eluent. The presence of a solute modifies that property and provides an output that can be recorded. Bulk property detectors have *limited sensitivity* due to the fact that changes in ambient conditions, temperature, pressure, etc. provide signals commensurate to that from the presence of a solute.

The three most common bulk property detectors are the *refractive index detector*, the *electrical conductivity detector*, and the *dielectric constant detector*, the latter being rarely used. There are four general methods for measuring refractive index; the *angle of deviation method*, the *critical angle method*, the *Fresnel method* and the *Christiansen method*. Except for the critical angle method, all have been employed in commercial instruments. The refractive index detector is probably the most popular detector next to the UV detector and is *used extensively in polymer analyses* where, provided the polymer has more then 10 monomer units, the response of the detector is independent of the molecular weight. The electrical conductivity detector measures the change in resistance of the mobile phase between two electrodes situated in line with the column eluent. It is very useful for *detecting ionic materials* and is used in the *analyses of foods both animal and human, beer and wine, and to some extent, in clinical laboratories in the analysis of blood and spinal fluids*. The dielectric constant detector functions similarly to a refractive index detector in that for non-polar and semi-polar substances, the dielectric constant is a function of the refractive index. The cell consists of a capacitor and the mobile phase flows between the insulated plates. Detection is achieved by measuring the impedance of the cell as the dielectric constant changes.

A large number of additional bulk property detector systems have been examined, which for the most part are not at present commercially available. The *thermal lens* detector, which is a form of a refractive index detector, functions on the absorption of laser light by the solute resulting in a local temperature increase that changes the refractive index of the medium. The *differential viscometer detector* functions on the measurement of changes in the viscosity of the eluent in the presence of a solute. The *interferometer detector* measures the change in interference patterns as the pathlength of a beam of light with respect to a reference beam changes in the presence of a solute contained in the mobile phase. There is an interferometer detector made commercially by Optilab utilizing interferometric principles in a slightly different way. The *density detector* measures the change in density of a solute as it is eluted from the column by the change in weight of a plumb situated in the eluent stream. Although the density detector has very poor sensitivity in a sense, it is a *universal detector*. The *density balance detector* is an adaptation of the gas density balance detector used in GC, but used with a liquid mobile phase instead of gas. Its sensitivity appears to be poor. Another application of a GC detector to LC is based on the gas *thermal conductivity detector*. Again the system is relatively insensitive.

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CHAPTER 4

SOLUTE PROPERTY DETECTORS

The ideal solute property detector measures some property that is exclusive to the solute only, as opposed to bulk property detectors that measure some overall property of the column eluent and which is modified by the presence of a solute. Solute property detectors are the most widely used type of detector in liquid chromatography (LC) today. In this class of detectors is found the most sensitive, the most specific and those with the widest linear dynamic range. Unfortunately, not all these attributes are contained in one single detector. Two popular examples of solute property detectors are the UV detector and the fluorescence detector. Solute property detectors, however, often have limited versatility as they restrict the choice of mobile phase to those solvents that do not possess the property that they measure. In practice solute property detectors are often used in a compromise situation where they perform as a hybrid between a solute property detector and a bulk property detector. An example of this would be the use of a fixed wavelength (254 nm) UV detector with a mobile phase consisting of a small percentage of ethyl acetate in a hydrocarbon solvent. Ethyl acetate itself absorbs UV light at 254 nm, although not strongly, and the effect of this absorbance is eliminated by use of the same solvent mixture in the reference cell. However as the mobile phase is now also providing a signal to the detector it is, in effect, partially a bulk property detector.

Solute property detectors can place stringent demands on the purity of the solvent employed as the mobile phase. For example the UV detector often operates at sensitivities of 10^{-8} to 10^{-7} g/ml and thus the mobile phase must be free from UV absorbing materials at these concentration levels. This is necessary because trace impurities often have significantly differing polarities from that of the bulk solvent and thus it may take a considerable time for the solvent to attain equilibrium with the stationary phase. During this period, which in practice may be many hours, the base line from the detector continually drifts as the trace components 'break through' the column. This drift is often misinterpreted as instability in the detector or detector electronics as opposed to a change in mobile phase composition. In general, it is advisable to eliminate the column when determining the properties of the detector. The detector does, however, have to be tested under dynamic flow conditions and under such circumstances, the column should be replaced by a length of capillary tubing that has approximately the same flow impedance as the column.

In the author's experience, if precise retention measurements and quantitative accuracy are required all solvents should be cleaned prior to use. A necessary procedure that must also apply to spectral grade and distilled in glass solvents. Solvents can be cleaned by passing them through packed beds of an appropriate adsorbent such as activated alumina, silica gel or reversed-phase material depending on the phase system to be employed. When cleaning solvents by passage through a bed of adsorbent it is advisable to limit the volume of mobile phase passed through the system and frequently replace the bed with fresh adsorbent. If excess of solvent is passed through the bed, impurities adsorbed on the media are liable to be eventually eluted and recontaminate the purified solvent. For most solute property detectors the solvent or solvents used as the mobile phase have to be chosen to be compatible with the detector employed.

The UV Absorption Detector

UV absorption detectors respond only to those substances that absorb UV light at the wavelength of the source light. A great many compounds absorb light in the UV range (180-350 nm) including all substances having one or more double bonds (π electrons) and substances having unshared (non bonded) electrons, e.g., all olefins, all aromatics and compounds for example containing >C=O, >C=S, -N=O, -N=N- groups. The detector usually consists of a short cylindrical cell through which passes the column eluent and through which a beam of UV light is focussed onto a photo electric cell. Alternatively the light passing through the cell is allowed to be dispersed by a defraction grating or quartz prism and then focussed onto a photo electric cell. When UV absorbing solutes are present in the mobile phase, light is absorbed and thus the intensity of light falling on the photo cell is reduced producing an electrical output which can be amplified and fed to a recorder. The relationship between the intensity of UV light transmitted through the cell and solute concentration is given by Beer's Law

$$I_T = I_0 e^{-klc} \qquad (1)$$

or

$$\ln I_T = \ln I_0 - klc$$

where I_0 is the intensity of the light entering the cell
I_T is the light transmitted through the cell
l is the path length of the cell
c is the concentration of solute in the cell
k is the molar absorption coefficient of the solute for the specific wavelength of the UV light.

If equation (1) is put in the form

$$I_T = I_0 10^{-k'lc} \qquad (2)$$

then k' is termed the molar extinction coefficient

Differentiating equation (1) with respect to solute concentration:

$$\frac{\partial (\text{Ln } I_T/I_o)}{\partial c} = -kl$$

It is seen that the sensitivity of the detector as measured by the transmitted light will be directly proportional to the value of the extinction coefficient *k* and the path length of the cell, *l*. It follows that to increase the sensitivity of the system, *l* should be increased; however there is a limit to which the path length can be increased as the total volume of the cell and, in particular, the length of the cell must be restricted to ensure minimum peak dispersion in the detector. To restrict band dispersion in the cell to a reasonable level, the radius of the cell must be reduced as the path length is increased. This results in less light falling on the photocell which in turn may reduce the signal-to-noise ratio. The two effects oppose one another and consequently this process of increasing sensitivity is limited unless photocells of significantly inherent lower noise level become available. Most modern UV cells have a path length lying between 1 mm and 10 mm and internal diameters of about 1 mm or less.

From equation (2) $\text{Log } \frac{I_T}{I_o} = k'lc = A$ (3)

where A is the absorbance

ΔA is commonly employed to define the detector sensitivity where the value of ΔA is the change in absorbance that provides a signal-to-noise ratio of 2.

Thus $\Delta A = k'l\Delta c$ (4)

Where Δc is the detector concentration sensitivity or the "Minimum Detectable Concentration" which is the parameter of importance to chromatographers.

Thus $\Delta c = \Delta A/k'l$ (5)

It is clear that two detectors having the same sensitivity defined as the minimum detectable change in absorbance will not necessarily have the same sensitivity with respect to solute concentration. Only if the path lengths of the two cells are identical will they also exhibit the same concentration sensitivity. This can cause some confusion to the chromatographer who would expect instruments defined as having the same spectroscopic sensitivity to have equivalent chromatographic sensitivity. To compare the sensitivity of two detectors given in units of absorbance the pathlengths of the cells in each instrument must be taken into account.

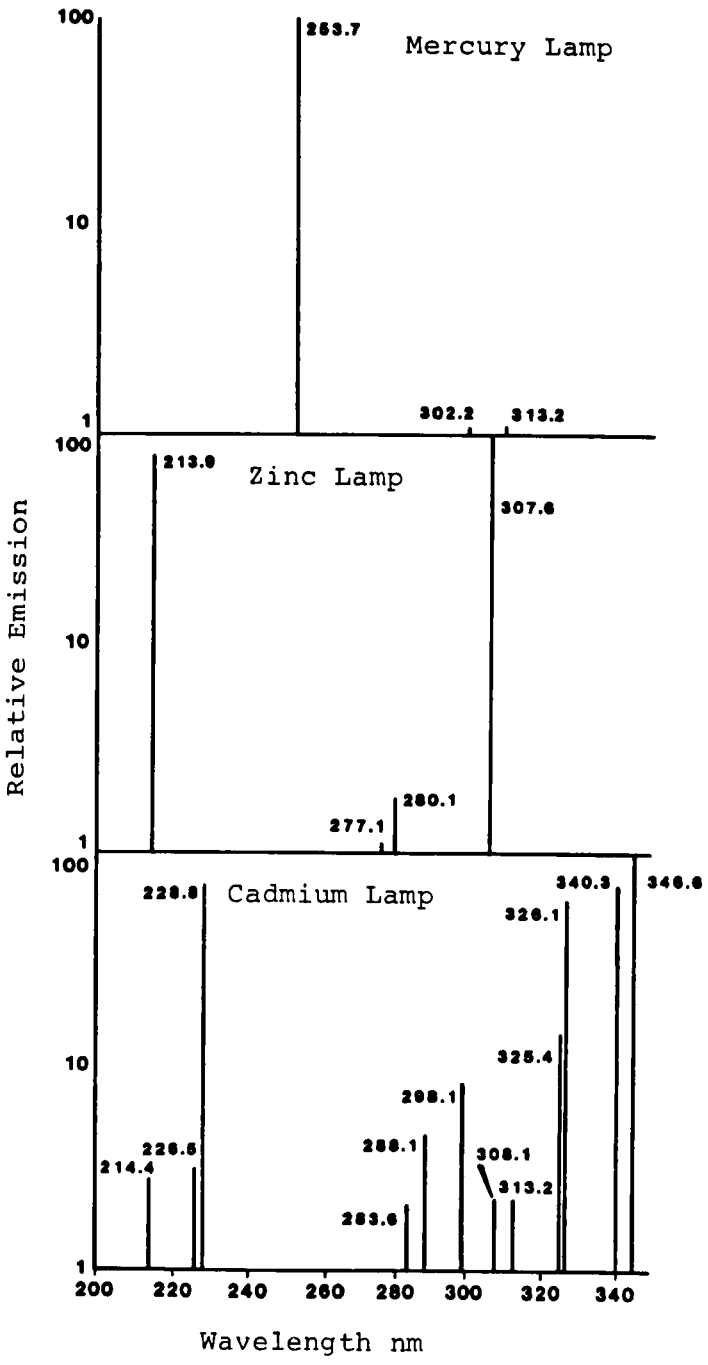


Figure 1. The emission spectra of three different discharge lamps.

There are two common types of UV detector, the fixed wavelength detector and the variable wavelength detector, both of which are commercially available. The two types differ greatly in design and function and therefore will be described separately.

The Fixed Wavelength UV Detector

The fixed wavelength detector, as its name implies, operates with light of a single wavelength (or nearly so) which is generated by a specific type of electric discharge lamp. For example, the use of the low-pressure mercury arc discharge lamp provides UV light predominately at 254 nm. There are a number of other lamps employed as UV light sources for LC detectors such as, the low-pressure cadmium lamp (225 nm) and the low-pressure zinc lamp (212 nm). Each type of lamp requires its own unique power supply for the most efficient operation which involves different striking voltages and operating currents. The light emission from *none* of the lamps is strictly monochromatic, light of other discrete wavelengths but often of significantly lower intensity, is always present. The emission spectra of the mercury, cadmium, and zinc lamps are shown in Figure 1.

It is seen that if a completely monochromatic light source is required then an appropriate filter would have to be employed to eliminate light of different wavelengths. The low-pressure mercury light source (wavelength 253.7 nm) is by far the most commonly used in fixed wavelength detectors and is the nearest to monochromatic of the three lamps. However, it does provide light of significant intensity below 200 nm. Light of such wavelengths is usually absorbed by the mobile phase and is not transmitted through the cell. The zinc lamp has a major emission line of interest at 213.9 nm, but the emission line at 307.6 nm is also of comparable intensity and under such circumstances may well have to be removed by an appropriate filter. The cadmium lamp has a major emission line of interest at 228.8 nm. The cadmium emission spectrum, however, has large numbers of emission lines of similar intensity to the line of interest at 228.8 nm and consequently, the use of an appropriate filter to eliminate light emitted at higher wavelengths would seem to be highly advisable. Both lamps are becoming increasingly popular in the biotechnology field where many of the substances of interest have higher extinction coefficients at the lower wavelengths.

A diagram of a typical optical system employed with the fixed wavelength detector is shown in Figure 2. Light from the UV source is passed through an aperture and collimated by an appropriate lens, or lens system. It then travels through two absorption cells, one the sample cell and the other the reference cell. Light having passed through each cell falls onto a photo electric sensor. The output from each photo cell is then processed by a

suitable electronic system where initially the output from each cell is compared to eliminate variations in light intensity from the source.

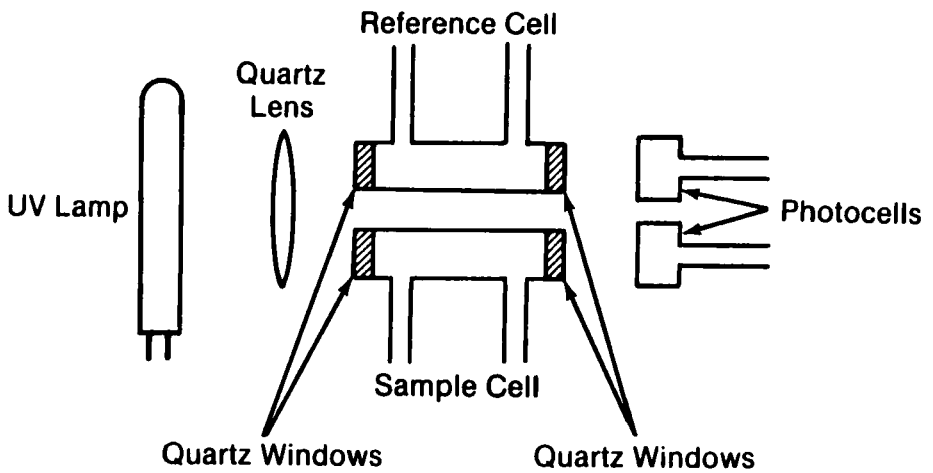


Figure 2. A typical optical system for a fixed wavelength UV detector.

The difference signal is then fed to an appropriate amplifier to render the output linearly related to solute concentration and then to a recorder.

In some early models of UV detectors cadmium sulfide photo cells were employed. These cells did not provide a response to the incident light intensity that could be described by a simple algebraic function and so linearity between voltage output and solute concentration over a significant concentration range had to be obtained by employing an amplifier with a somewhat complex amplifying function. Modern detectors, however, use silicon photo diodes either as single diodes or as diode arrays that provide a linear output with light intensity and, thus, by using a simple logarithmic amplifier the output can be related directly to solute concentration.

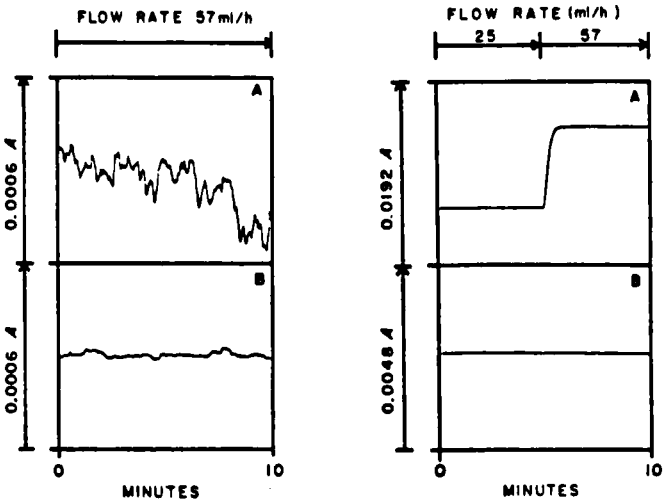
One of the first effective small volume UV detectors suitable for use with high efficiency columns was that described by Horvath and Lipsky in 1966 (1). In 1968 Kirkland (2) described in detail another small volume detector which subsequently, in a slightly modified form, provided the basic design for the first DuPont UV detector. The cell was cylindrical in shape, 1 mm in diameter and had a path length of 1 cm giving a total cell volume 7.5 µl. Kirkland also used the 254 nm UV light emitted from a low-pressure mercury vapor lamp as the light source and claimed a noise level of ± 0.0002 absorbance at a full scale sensitivity of 0.01

absorbance units. The upper limit of the linear dynamic range was about 1.2 absorbance units equivalent to a concentration of about 10^{-4} g/ml. A cell volume of 7.5 µl would, today, be considered too large as the peak dispersion from such a cell would be too great if it were to be used with an optimized column of small diameter. Well designed contemporary UV detectors have cell volumes no larger than 2 µl and often closer to 1 µl in volume. Today manufacturers have designed their own unique UV detectors often with propriety cell design and associated with unique electronics. While each is novel in detail, they all function on more or less the same principle first used by Horvath and Kirkland.

UV cells can be very sensitive to both flow rate and temperature changes unless they are carefully thermostatted. The effect of temperature control on the noise level of UV detectors was examined in the very early days of LC development by Brooker (3). Essentially the eluent from the column must be brought to the same temperature as the detector cell assembly by the use of an appropriate heat exchanger if detector stability at high sensitivities is to be achieved. The heat exchanger and the detector cell is thermostatted by the same coolant stream. In Brooker's apparatus the heat exchanger consisted of a metal coil situated between the column and the detector and immersed in a cooling bath. If this apparatus were to be used with the high-efficiency columns employed today, the heat exchanger would have to be carefully designed, so that the band dispersion was kept at a level that would not impair the column performance. However, from the point of view of demonstrating the importance of temperature control, the system provided essential information. The effect of thermostatting the detector on its stability is shown in Figure 3. It is seen that the noise level of the thermostatted system has been very significantly reduced by ensuring that the detector and column eluent were at the same temperature. It is also seen that under isothermal conditions of operation, the detector has become virtually *insensitive* to *changes in flow rate.*

The fixed wavelength UV detector is the detector most commonly employed today. It has been found useful in the whole gamut of LC applications and has a number of significant advantages over the most popular bulk property detector, e.g. the refractive index detector. The UV detector has a relatively high sensitivity, is relatively stable and, (probably its most important feature) it can be used readily with gradient elution development. Operating the detector under gradient elution conditions of development would require the selection of solvents that are transparent at the wavelength of the UV light employed. This can be achieved providing the wavelength of the UV light is

not too low. The fixed wavelength UV detector is very suitable for quantitative analysis as it can provide a linear dynamic range of at least three orders of magnitude.

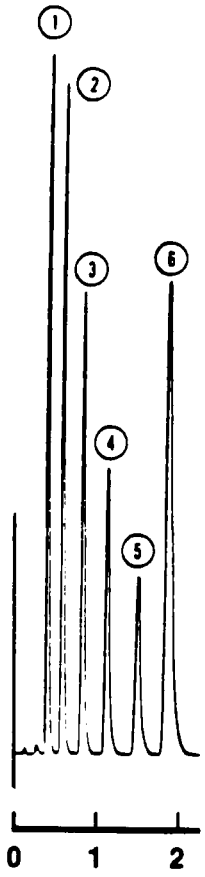


Noise Level Effect of Change of Flow

Figure 3. Effect of temperature control on the stability of the UV detector. A = No temperature control of cell or column eluent. B = Cell and column eluent measured at the same temperature.

The use of a fixed wavelength detector operating at wavelengths other than 254 nm is relatively uncommon. However, LC applications arising from the increased activity that is taking place in the biotechnology field, may, in the future, provide a significantly greater demand for detectors operating at 212 nm and 225 nm. Substances of biological interest frequently absorb more strongly at the lower UV wavelengths and consequently detectors operating at 212 nm can selectively provide increased sensitivity for many substances of biological origin. A typical chromatogram obtained from a UV detector operated at 254 nm is shown in Figure 4.

A novel type of UV detector designed specifically for use in preparative chromatography was described by Miller and Strusz (4). The basic design is shown in Figure 5. The column is positioned above the delivery tube shown at the top of the figure. The column eluent passes through the tube and over a supporting plate. There is no cell of fixed path length, the liquid simply flows over the plate and the effective path length assumes the thickness characteristic of the flowing film. A UV lamp is situated below the plate and a photo cell normal to the plate and on the opposite side to that of the lamp. A reference photo cell is also mounted close to the lamp to compensate for changes in light emission. The



Column: Pecosphere™ 3x3 C_{18}
Mobile Phase: methanol-water(75:25)
Flow Rate: 2.0 ml/min.
Detection: UV, 254 nm

Sample:
1. Benzene
2. Toluene
3. Ethylbenzene
4. Isopropylbenzene
5. tert-Butylbenzene
6. Anthracene

Figure 4. Chromatogram obtained from a fixed wavelength UV detector.

change in intensity of the light falling on the photo cell due to absorption by the liquid film provides an electrical output that is amplified and fed to a suitable recorder. The film thickness depends, among other factors, on flow rate and consequently the detector is flow sensitive. Flow sensitivity is, however, not very important in preparative scale chromatography. It is more important that the detector responds linearly to concentrations as high as 10^{-2} g/ml, a valuable feature for a preparative detector. A further advantage of this detector is the very low flow impedance of the device which allows it to accept the extremely high flow rates employed in preparative chromatography without

placing an excessive back pressure on the column or the detector itself. This detector is commercially available and is manufactured by Gow Mac Inc.

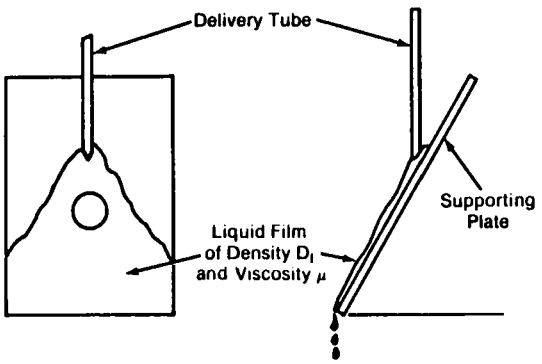


Figure 5. UV detector for preparative chromatography.

The Variable Wavelength UV Detector

The variable wavelength detector differs from the fixed wavelength detector in that the light source provides a continuous spectrum over a wide range of wavelengths and consequently with the aid of an appropriate optical system allows any particular wavelength to be selected for detection. The development was provoked by the need to increase sensitivity for particular solute types by choosing a wavelength at which the extinction coefficient of the solute of interest was relatively large. A further desirable feature was the possibility of obtaining UV spectra of eluted solutes directly during chromatographic development.

There are two basic types of variable wavelength detector, the dispersion detector and the diode array detector. Both types require similar broad emission light sources such as the deuterium discharge lamp or the xenon discharge lamp the former being the more common. Both the dispersion type of variable wavelength detector and the diode array detector can provide *complete absorption spectra* of an eluted solute; the former by stopping the solvent flow and scanning the solute while stationary in the cell, the latter by direct "on the fly" scanning.

There is a very important difference between the two basic variable wavelength detectors. In the dispersive instrument the light is dispersed prior to entering the detector cell so that the light passing through it is virtually monochromatic. However, if the incident light can cause fluorescence at another wavelength, then the light falling on the photo sensor will contain both the transmitted light and the fluorescent light. Consequently, the

integrity of the spectrum may be impaired and the linear response of the detector degenerated. In general, due to the monochromatic nature of the incident light, the fluorescent effect is small and normally the spectra obtained from a dispersion instrument are reasonably exact and quantitative results reliable. The diode array detector, however, operates in quite a different manner. In this detector, light of all the wavelengths generated by the light source passes through the cell and the transmitted light is then dispersed over the array of diodes so that each diode detects light of a discrete wavelength. However, the light falling on a particular diode, again, may not originate solely from the light generated by the source and may include some fluorescent light. In this case, however, the situation is exacerbated by the fact that the incident light is polychromatic, which both increases the probability of fluorescence and the level of its intensity. This means that, under some circumstances, measurement of the 'transmitted light' would not give a true measure of absorbance and, furthermore, a true absorption spectrum could not be obtained.

In Figure 6, the UV absorption spectra of benzpyrene are shown; one taken on a dispersive variable wavelength detector and the other on a diode array detector. The third is the emission spectrum of benzpyrene taken on a fluorescence spectrometer at an excitation wavelength of 292 nm. The difference between the two absorption spectra is clearly seen and the difference can be identified as coinciding with the fluorescence emission spectrum although it must be remembered that the emission spectrum is for only one excitation wavelength and the effect of many others will also be present in the cell of a diode array detector. It follows that although the diode array detector is a very fast response multi-wavelength detector and, as such, is extremely useful, any absorption measurements, absorption ratios or absorption spectra that are obtained from it to be used for identification purposes, must be treated with a certain amount of circumspection. It should also be noted that the presence of fluorescent light may significantly effect the linearity of the detector and its linear dynamic range. This situation is difficult to predict and thus calibration curves should always be obtained if a diode array detector is to be employed for quantitative analysis.

The ideal multi-wavelength detector would be a combination of both the dispersion instrument and the diode array system. A diffraction grating would provide monochromatic incident light which, after passing through the cell, would then be dispersed by a second grating onto a diode array. In this way, the light measured would only be incident light or, conversely, exclusively the fluorescence light. The integrity of any spectrum obtained in this way would be maintained together with the linear response of the detector.

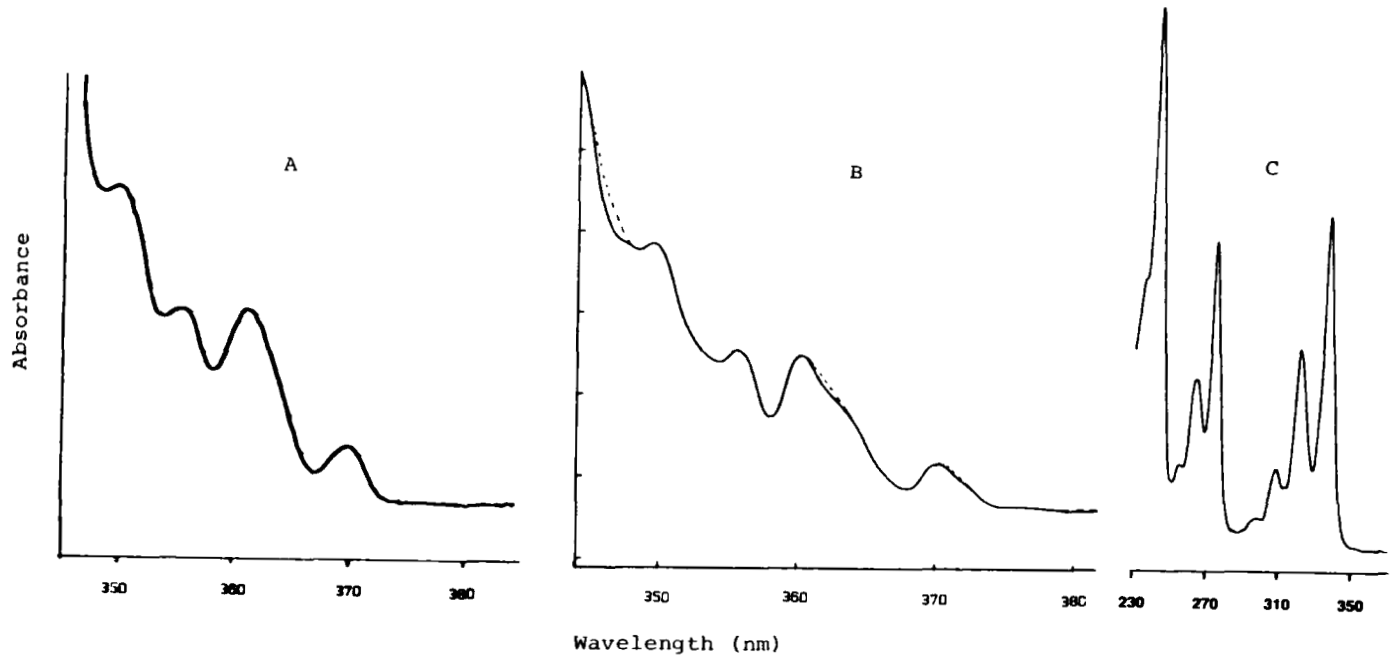


Figure 6. UV absorption spectra and fluorescence emission spectra of benzpyrene. A = absorption spectra obtained from a dispersion spectrometer; B = absorption spectra obtained from a diode array spectrometer; C = fluorescence spectrum. Note modification of absorption spectrum from diode array spectrometer by fluorescence (broken line) particularly at 340 to 350 nm that corresponds to the fluorescence at the same wavelength shown in C. (Absorption spectra provided by Prof. Charles Lochmüller of Duke University).

The Variable Wavelength Dispersive UV Detector

A diagram of the optical system of the Perkin-Elmer LC 95 dispersive, variable wavelength detector is shown in Figure 7. Light from a UV source, a deuterium lamp, falls onto a concave mirror that collimates the beam onto a diffraction grating. The dispersed beam is then focussed by means of another concave mirror, through an aperture in a plane mirror, onto another plane mirror, through the sample cell and then onto a photo cell.

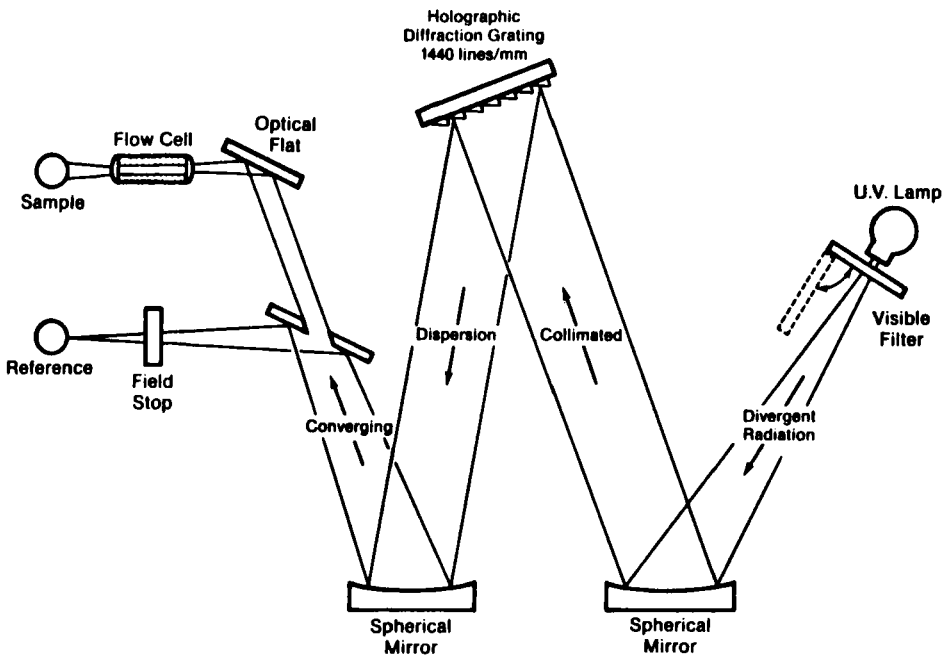


Figure 7. Optical system of the Perkin-Elmer LC 95 dispersive, variable wavelength detector.

The light, not passing through the aperture in the first plane mirror, is reflected onto a second reference photo cell that compensates for any changes in energy from the light source. The electronic system associated with the detector is similar to that of the fixed wavelength detector. The output from the sample cell is balanced against that from the reference cell, the difference signal amplified logarithmically to provide an output that is linear with solute concentration, then passed to an attenuator and finally to a recorder.

In order to eliminate thermal noise and flow sensitivity, the inlet flow of solvent from the column must be brought to the same temperature as the detector cell before it actually enters the cell. The heat exchanger used for this is shown in Figure 8 which embodies many of the standard features of modern LC detector heat exchangers. The inlet tube is first coiled tightly round the exit tube to make good thermal contact and then wrapped round an

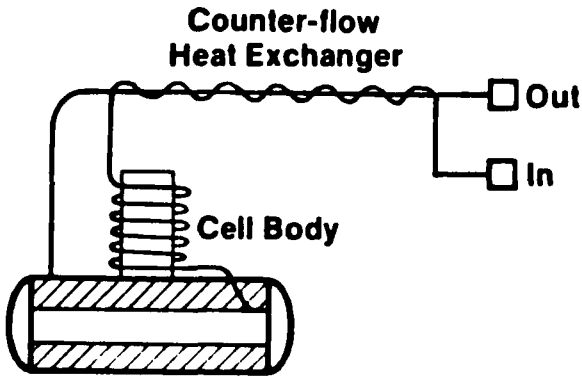


Figure 8. Heat exchanger.

extension of the detector cell body itself before entering the detector cell. This ensures effective countercurrent heat exchange. The coiling of the tube induces radial flow across the tube and thus reduces peak dispersion while at the same time ensuring good thermal contact as previously discussed.

Two chromatograms obtained from a variable wavelength dispersive UV detector are shown in Figure 9.

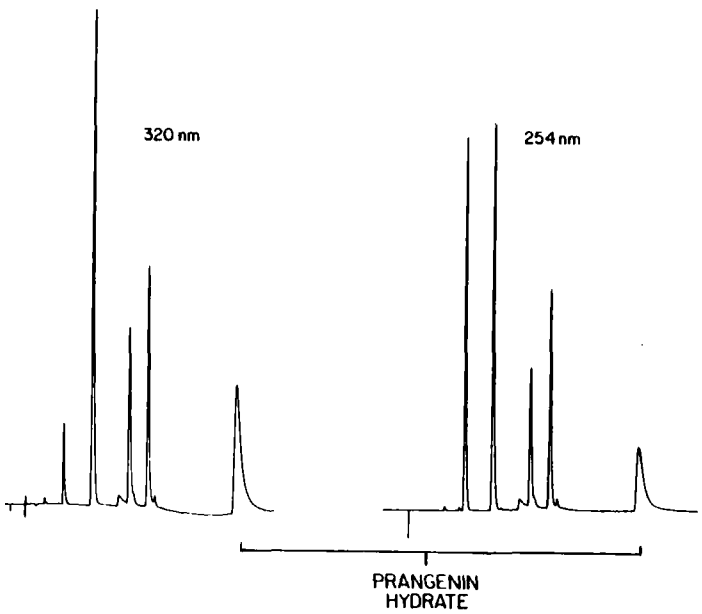


Figure 9. An example of the use of a multi-wavelength detector.

The two chromatograms were monitored at 254 nm and 320 nm respectively. The advantage that results from the use of two different wavelengths is clearly seen. The response of the first major peak is significantly enhanced by monitoring the eluent at 254 nm. Conversely the second and third major peaks exhibit a greater response when monitored at 320 nm.

The Diode Array Detector

The use of a diode array as an alternative to the mechanical dispersive system just described is one of the more recent developments in commercial LC detectors. One of the first workers to describe the use of diode array systems was Talmi (5,6) in 1975 and the technique was further developed by Yates and Kuwana (7) and Milano et al. (8) in 1976. Since that time a considerable amount of development work has been carried out on diode array detectors and there are now a number of systems commercially available. The *mechanical dispersive* system is basically a monochromator that selects a particular wavelength with which to monitor the column eluent by the angular adjustment of an appropriate prism or grating. The *diode array system*, in contrast, utilizes a polychromator which disperses the spectrum *after* passing through the detector cell across an array of diodes and consequently, allows the whole of the spectral transmission to be monitored simultaneously. Thus by monitoring the output from one individual diode the column eluent can be monitored at one particular wavelength. Alternatively by scanning all the diodes the UV absorption spectrum of the eluent can be obtained almost instantaneously. The value of this type of detector for research purposes is immediately obvious and the elimination of the mechanical moving parts renders the detector much more reliable albeit, at present, significantly more expensive.

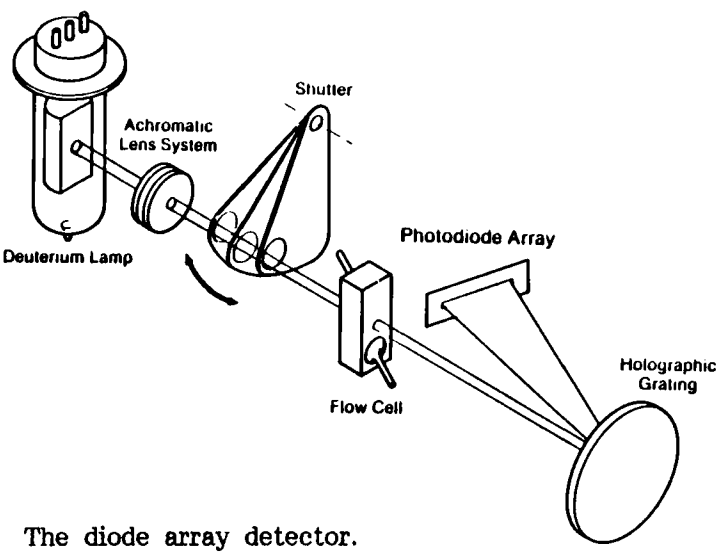


Figure 10. The diode array detector.

The main disadvantage of the system lies in the fact that light of all wavelengths is present in the cell simultaneously and consequently, there is a real possibility that there will also be fluorescent light present at the wavelength being monitored.

A diagram of the Hewlett-Packard Model HP1040A diode array system is shown in Figure 10. Light from a deuterium lamp is focussed by an achromatic lens system through a flow cell onto a holographic grating. The dispersed light then falls onto a photodiode array. As a consequence light of all wavelengths generated by the lamp that has passed through the cell is simultaneously monitored by the array of diodes. Thus by selecting a particular diode the absorption of light of a specific wavelength can be continuously recorded. Conversely the output of all the diodes can be simultaneously presented on a suitable display thus providing instantly an absorption spectrum of the contents of the cell.

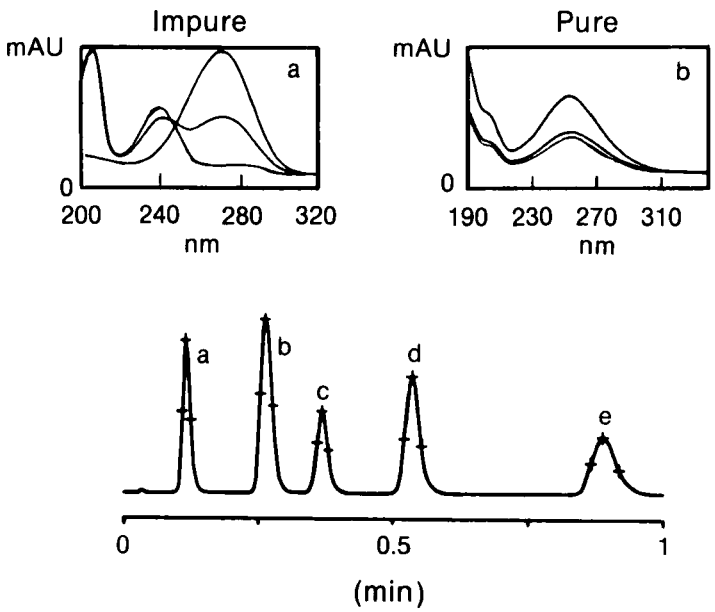


Figure 11. Chromatogram monitored by the diode array detector.

The diode array detector can be used in a number of ways other than as a multi-wavelength detector or for identifying solutes from their UV spectra. Figure 11 shows a chromatogram containing five peaks. Spectra have been taken of peaks *a* and *b* halfway up the rising side of each peak, at the top of each peak and halfway down the trailing side of each peak. The spectra are also included in the Figure 11. It is seen that the shape of the spectra change throughout the elution of peak *a* indicating that it is impure and is in fact a complex peak containing more than one

solute. The spectra for peak *b* however exhibit a consistent shape throughout the whole peak confirming that the peak is pure and uncontaminated with other solutes.

The Fluorometric Detector

Fluorescence is a specific type of luminescence. When molecules are excited by electromagnetic radiation to produce luminescence the phenomena is termed photoluminescence. If the release of electromagnetic energy is immediate or stops on the removal of the exciting radiation the substance is said to be fluorescent. If, however, the release of energy is delayed or persists after the removal of the existing radiation then the substance is said to be phosphorescent. Due to its persistence, or even short, but significant, lifetime, phosphorescence is of little use as a process for LC detection. Fluorescence, however, has been shown to be extremely effective and detectors based on fluorescence measurement have provided some of the highest sensitivities available.

When light is absorbed by a molecule, a transition to a higher electronic state takes place and this absorption is highly specific to the molecule concerned; radiation of a particular energy or wavelength is only absorbed by a specific molecular structure. If electrons are raised, due to absorption of energy, to an upper excited single state then such transitions are responsible for the characteristic visible or UV absorption spectra observed for such compounds. If the excess energy is not dissipated rapidly by collisions with other molecules, or by other means, the electron will return to the ground state with the emission of energy in the form of electromagnetic radiation. This results in fluorescence. As some energy is always lost before emission occurs, the emitted fluorescent energy is always of longer wavelength than the absorbed or exciting radiation. Excellent discussions on the theoretical basis of fluorescence have been given by Guilbault (9) Udenfriend (10) and Rhys Williams (11) and readers interested in learning more of the theory and use of fluorescence as a means of detection are recommended to refer to these books.

In comparison with other detection techniques fluorescence affords greater sensitivity to sample concentration but less sensitivity to instrument instability and such macroscopic properties as temperature and pressure. In part this may be attributed to the nature of the measured experimental parameter, which in fluorescence detection is a signal super-imposed upon a very low background. In contrast, for example, to optical absorbance measurements, the signal is superimposed upon a high and sometimes unstable background. The major disadvantage of fluorescence detection is that not all compounds fluoresce under normal high-performance liquid chromatography (HPLC) conditions. However, the large number of fluorescent materials, including

biochemicals in, foods, drugs, dye intermediates, etc. recommend the detection technique to specific areas of application. Further the scope of the technique can be extended by the use of fluorescent reagents such as Fluoropa (o-phthalaldehyde) and Fluorescamine (4-phenylspiro(furan-2-(3H),1'-phthalan)-3',3'-dione). These reagents react with primary amines such as amino acids to produce derivatives that fluoresce when irradiated with UV light. The use of such reagents requires a post column reactor of the correct design to restrict band dispersion and loss of resolution. The design of post column reactors will be discussed later.

Most fluorescence detectors are configured in such a manner that the fluorescent light is viewed at an angle (usually at right angles) to the exciting incident light beam. This geometric arrangement, minimizes any of the incident light that could provide a background signal to the fluorescence sensor. As a consequence the fluorescence signal is detected against a dark background and provides a maximum signal to noise ratio. This is often achieved by inserting a filter, opaque to the incident light, between the cell and fluorescence sensor. The fluorescence signal I_f is given by

$$I_f = \phi\ I_o\ (1-e^{-kcl}) \qquad (6)$$

where ϕ is the quantum yield (the ratio of the number of photons emitted to the number of photons adsorbed)
I_o is the intensity of the incident light
c is the concentration of the solute
k is the molar absorbance
l is the path length of the cell.

Fluorescence detectors vary widely in complexity. The simplest consisting of a single wavelength excitation source and a sensor that monitors fluorescent light of all wavelengths. For selected applications this simple form of detector can be very sensitive but it is obviously not very versatile with the excitation light available at only one wavelength and no means of selecting the emission wavelength on which to monitor. At the other extreme there is the fluorescence spectrometer that has been fitted with a flow cell of appropriate dimensions. Such a system is of course a highly complex and versatile device that allows the excitation wavelength to be selected, the emission wavelength to be chosen and if the chromatographic development is stopped, excitation and/or emission spectra to be obtained. The optical system of such a device is given in a simplified form in Figure 12.

Light from a broad emission source is focussed by means of a ellipsoidal mirror onto a toroidal mirror and hence onto a grating. Monochromatic light, selected by the angular position of the grating, is then focussed by a spherical mirror and then by an ellipsoidal mirror onto the sample contained in a sample cell.

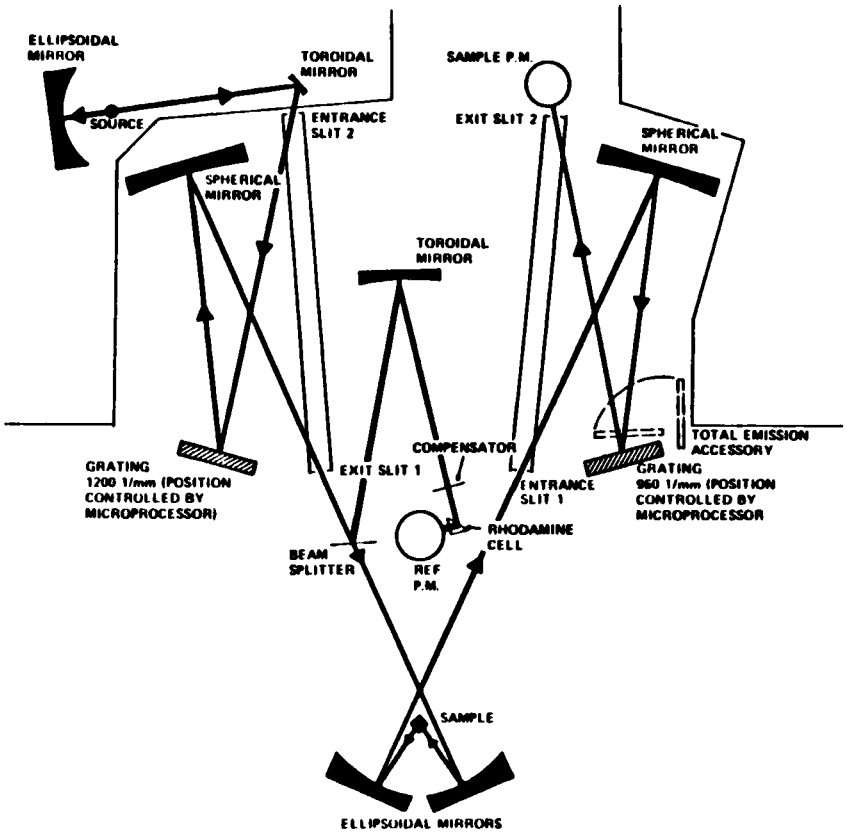


Figure 12. Diagram of a fluorescence spectrometer.

Between the spherical mirror and the ellipsoidal mirror the excitation light passes through a beam splitter where a portion of the light is focussed by a toroidal mirror through a compensating cell filled with a solution of rhodamine which provides a reference fluorescence and then onto a reference photo cell. The reference cell compensates for any change in fluorescence due to changes in incident light intensity. The fluorescent light from the sample is focussed by means of another ellipsoidal mirror and a spherical mirror onto another grating. The emitted light is thus dispersed and light of a particular wavelength selected by the positional angle of the second grating then falls on a second photo cell. The somewhat complex optical system allows both the excitation wavelength and the wavelength of the fluorescent light to be selected and also allows both excitation and emission spectra to be obtained.

The wavelength of the excitation light and the wavelength of the emission light that is to be monitored can with some instruments, be programmed and consequently can be changed at predetermined intervals throughout the development of a chromatogram. An example of a chromatogram obtained in this manner is shown in Figure 13. The mixture contained 16 EPA

priority pollutants. There are three wavelength changes and all 16 components are clearly detected, albeit some being present at very small concentrations. The best single compromise wavelength, (305/430 nm) will detect only 13 of these compounds.

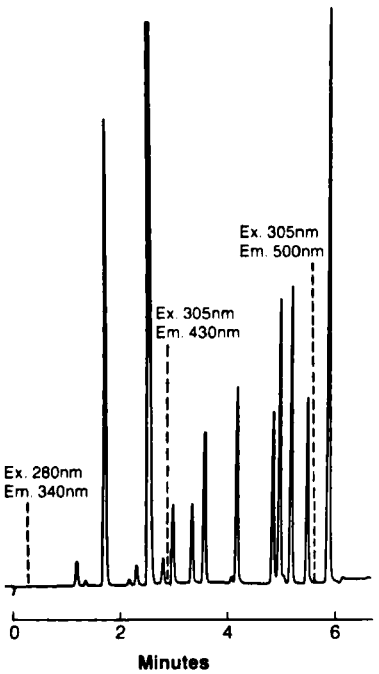


Figure 13. Chromatogram of 16 EPA priority pollutants run on a 5 µm HCODS column with three wavelength changes. Column: P-E HS-3 C_{18}; Mobile Phase: 60% - 100%, methanol in water in 5.0 min; Flow rate: 3.0 ml/min.

Transport Detectors

Transport detectors are a unique type of solute property detector. In most solute property detectors, the sensing system monitors some property of the solute that is not shared by the solvent or that the solvent has to markedly less extent. It follows that such detecting systems are,to some extent, selective in their detecting capabilities and further restricts the choice of solvents to those that do not possess the property being measured. Thus, the choice of mobile phase is limited, and this can be particularly disadvantageous when employing gradient elution development. The transport type of detector was developed to overcome these limitations.

A transport detector consists of a carrier that can be for example, a metal chain, wire or disc that continuously passes through the column eluent taking a sample with it as a thin film of mobile phase adhering to its surface. The mobile phase is then

removed, usually by evaporation, leaving any solute contained in the mobile phase as a coating on the carrier. The carrier is then examined by a suitable detecting procedure to monitor the solute alone. If a flame ionization detector (FID) is employed to monitor the solute, it will detect any substance containing carbon and permit the use of any solvent providing it is reasonably volatile. The only limitations to this system is that the solute must be involatile, or it would be lost during the evaporation of the mobile phase. The system appears ideal, but there are some disadvantages, the main one being a relatively low sensitivity (ca. 10^{-6} g/ml), but it appears with improved design, this sensitivity might be significantly improved.

The Moving Wire Detector

The first wire transport detector was the detecting system originally developed by James et al. (12), in 1964 and subsequently was manufactured by Pye Unicam and became commercially available.

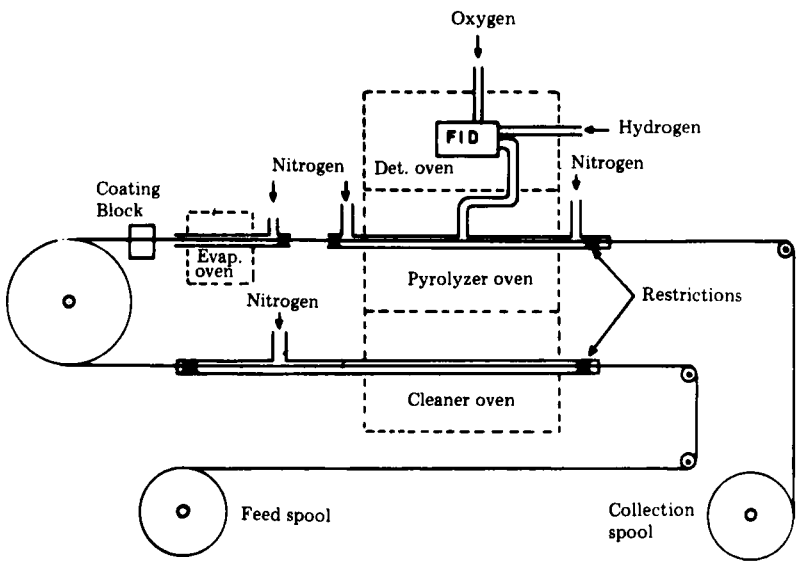


Figure 14. The moving wire detector.

A diagram of the Pye Unicam moving wire transport detector is shown in Figure 14. Wire from a spool passes through a cleaning furnace maintained at 750°C, then round a pulley and through the effluent stream of mobile phase from the chromatographic column. After being coated with mobile phase, the wire passes into an evaporator oven at a temperature of about 105 °C and then into a pyrolysis furnace. A nitrogen stream sweeps the solvent vapor out of the evaporator oven and when the wire enters the pyrolysis

furnace, two nitrogen streams from either end of the pyrolysis furnace sweep the pyrolysis products from the solute on the wire directly into a FID. The output from the detector is fed to a suitable amplifier and then to a recorder. The sensitivity of the detector was about 5×10^{-6} g/ml but varied with the nature of the substances detected. Furthermore, the linear dynamic range was less than two orders of magnitude. Although establishing the viability of the detecting system, the performance left a lot to be desired as an effective LC detector.

About the same time as the development of the wire transport detector, Haahti and Nikkari (13) described a similar device, but more simple in design, employing a chain loop in place of wire. A diagram of their apparatus is shown in Figure 15. A gold chain, driven by a synchronous motor passes over a coating block where the chain is wetted with the column eluent and then into an evaporator. The mobile phase is removed by evaporation, and the chain then passes directly through the flame of a FID.

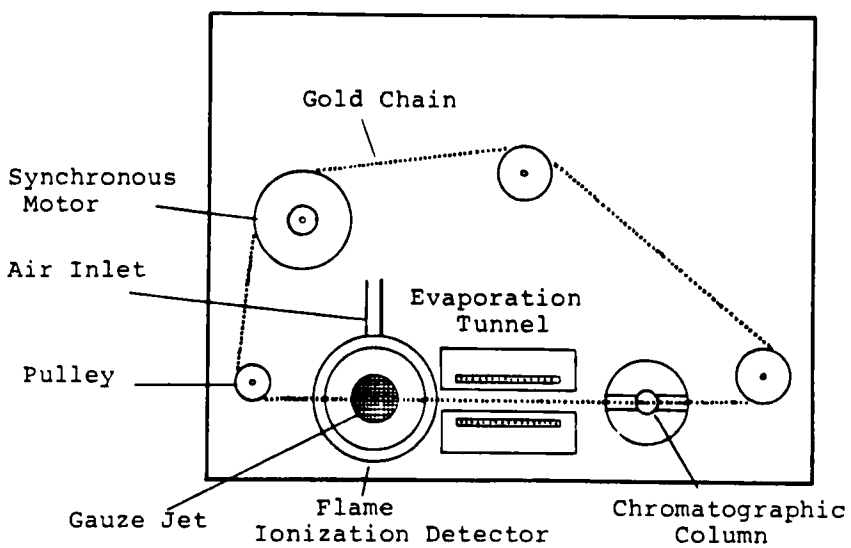


Figure 15. The chain detector.

During combustion of the solute in the hydrogen flame, ions are produced in the normal way, and the resulting ionic current amplified and fed to a suitable recorder. Due to the occlusion of local, high concentrations of solute between the links of the chain, the detector was extremely noisy and, thus, provided relatively low sensitivity. A chromatogram obtained from the moving chain detector is shown in Figure 16. The noise spikes on the peak are clearly seen, which besides affecting the overall sensitivity of the detector, also rendered quantitative analysis difficult. The advantage of the moving chain detector was, in its simplicity, relative to the moving wire detector.

In the early sixties, many workers in the field attempted to improve the performance of the transport detectors and, in 1966, Karmen (14) introduced an aspirating system to draw the pyrolysis products into the hydrogen flame detector. In 1970, Scott and Lawrence (15) developed the system of Karmen further and introduced a modified form of detector in conjunction with the FID. The full sensitivity of the moving wire detector employing a pyrolysis system is only realized for certain compounds, for example, high boiling hydrocarbons, such as squalene or long chain fatty acids, such as stearic acid. For highly oxygenated compounds, such as carbohydrates, polyglycols, etc., the sensitivity of the detector may be reduced by as much as one order of magnitude.

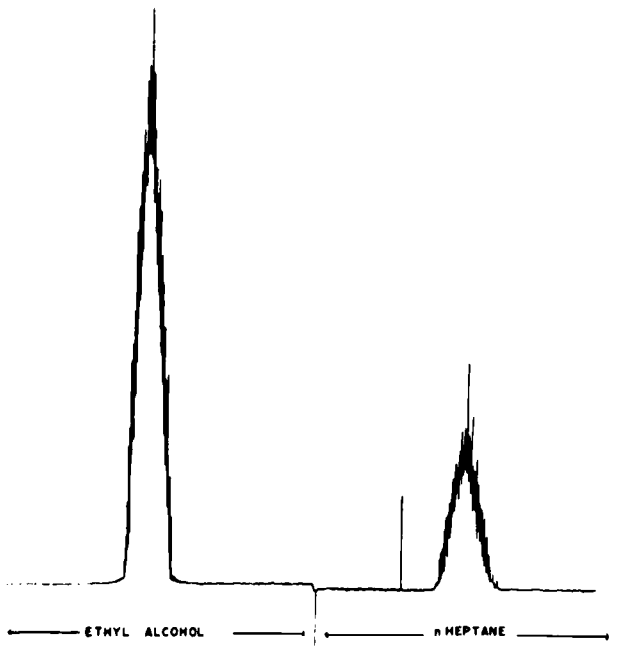


Figure 16. Chromatogram obtained from the moving chain detector. Sample: mineral oil and surfactant, solvent: n-heptane, ethyl alcohol, column: 2 x 300 mm, column packing: silica gel, flow rate: 0.7 ml/min, chart speed: 24 cm/min, evaporator temperature: 150°C, N_2 flow rate: 30 ml/min, H_2 flow rate: 25 ml/min, O_2 flow rate: 30 ml/min.

The sensitivity of the wire transport detector, besides being dependent on the noise level of the system, also depends on the quantity of volatile pyrolysis products produced from the solute. Excluding synthetic polymers, which often quantitatively produce monomers on pyrolysis, many compounds only yield a few percent of their mass as volatile combustible pyrolysis products. Thus, the FID may only, in effect, detect a few percent of the solute

carried into the pyrolyzer by the moving wire. If, however, instead of pyrolyzing the solutes, they were completely combusted in an oxygen or air stream, then all the carbon in the solute would be converted to carbon dioxide. Further, if the carbon dioxide was then reduced to methane by mixing with excess hydrogen and passing over a nickel catalyst, all of the carbon in the solute would be detected as methane by the FID. Such a system could increase the sensitivity of the detector to compounds that give low yields of volatiles on pyrolysis. Furthermore, potentially this detecting system should have a wide linear dynamic range and a predictable response.

A diagram of the moving wire detector modified in this way is shown in Figure 17. The FID was modified by enlarging the hydrogen lines in the detector body which was necessary to reduce the flow impedance of the detector system and permit the satisfactory operation of an aspirator. The detector was connected to a 2 in. length of 1/2 in. diameter thin-walled stainless-steel tube and then to the aspirator. The 1/2 in. tube was closed with a loose wad of quartz wool and filled with about 2 g of nickel catalyst. The nickel catalyst was prepared by adsorbing a saturated solution of nickel nitrate on 20/40 BS mesh brick dust, decomposing the nitrate to the oxide by heating to 500°C for 3 h and reducing the oxide to metallic nickel in a stream of hydrogen at 250°C. The aspirator consisted of a jet and a venturi and was placed in line with the hydrogen flow to the detector.

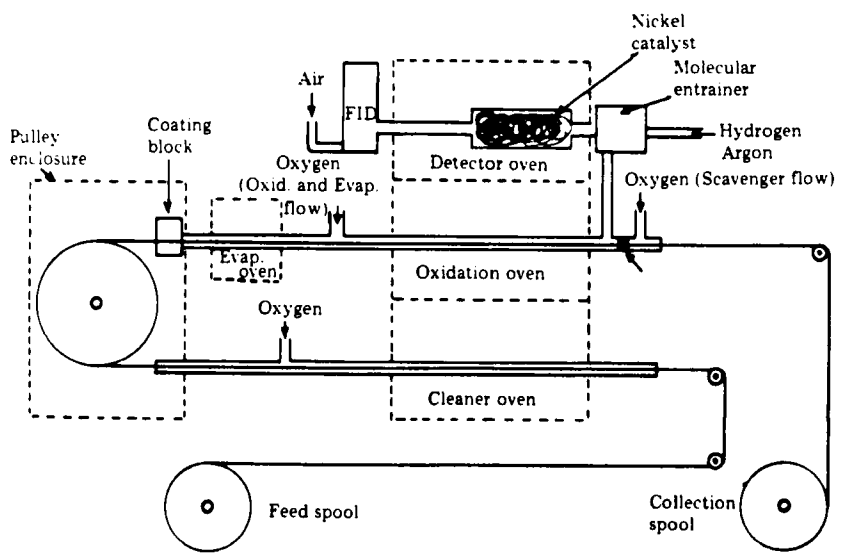


Figure 17. Modified moving wire detector.

The passage of hydrogen from the jet to the venturi resulted in a pressure drop around the venturi, and thus, allowed other gases to be sucked continuously into the hydrogen stream.

The reduced pressure side of the aspirator was connected to the side limb of the oxidation tube by means of a silicon rubber sleeve. It is seen from Figure 17 that the two-tube system in the normal wire director has been replaced by a single tube. The oxygen or air is fed in at the center of this tube, providing both the evaporator flow and the oxidation flow. The oxidation and cleaning tubes were constructed from quartz.

The linear dynamic range of the system was shown to be about four orders of magnitude as indicated by the curve in Figure 18. The response index determined for a series of compounds of different chemical types was found to be between 0.96 and 1.04.

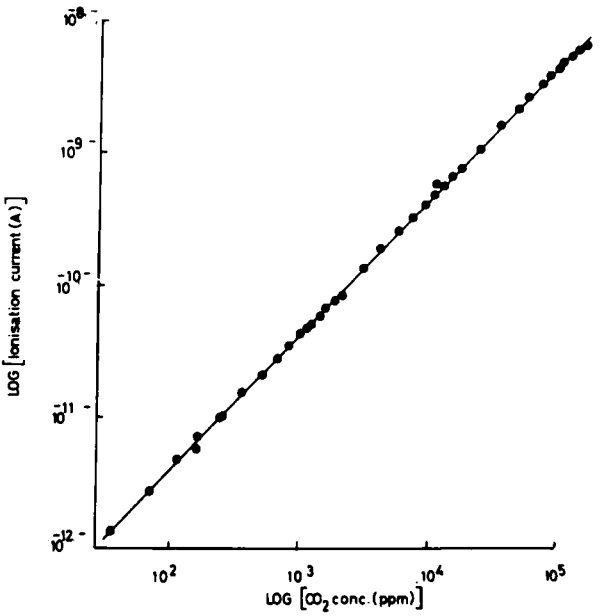


Figure 18. Linearity curves for the moving wire detector.

This instrument originally made commercially by Pye Unicam, is no longer available although still extensively used. It was found by Scott and Lawrence (15) that the response of the detector was proportional to the carbon content of the solute and, thus, if the percentage of carbon in the solute was known prior calibration was not necessary. The response of the detector to carbon content, however, was only tested with a limited number of compounds and thus, this relationship should only be assumed with caution. A chromatogram of blood lipids obtained from the Pye Unicam instrument employing incremental gradient elution development is shown in Figure 19.

Van Dijk (16) developed a spray procedure for coating the wire in an attempt to improve the sensitivity of the detector. The column effluent passed directly to an atomizer, the nozzle of

which was situated directly above the wire and one to two millimeters from it. The effect of the spray coating the wire was firstly, to concentrate the solute in the mobile phase, due to partial evaporation of the solute during atomization and, secondly, to increase the load on the wire by the formation of droplets. A linear dynamic range of about 3×10^{3} was obtained from this system. The author also claimed a sensitivity increase of about 50 over the conventional pyrolysis moving wire detector. It is difficult to determine from the publication the exact sensitivity that was obtained but from the calibration curves that were given, the sensitivity appeared to be about 3×10^{-6} g/ml.

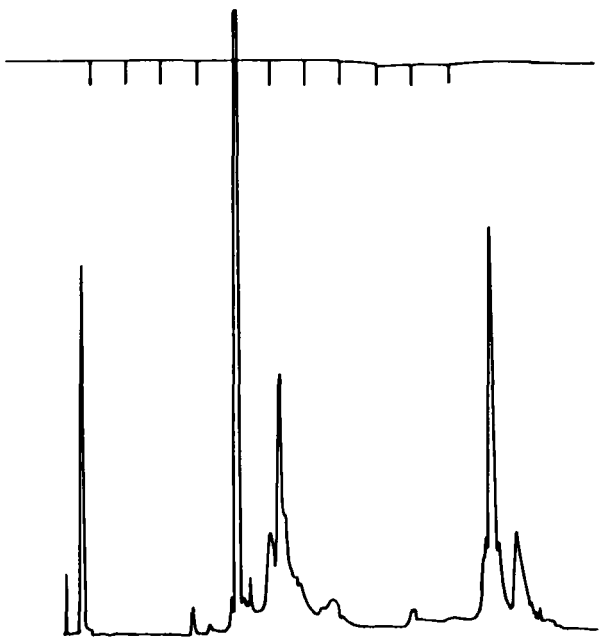


Figure 19. Chromatogram of blood lipids employing incremental gradient elution.

Yang et al. (17) also developed a thermal spray procedure for coating the wire and claimed an increased sensitivity. The authors employed a heated chamber situated above a moving stainless-steel belt through which the conduit from the column passed. The solvent was rapidly brought to its boiling point resulting in a spray leaving the exit of the conduit and coating the belt. The authors also employed a photoionization detector and an electron capture detector as alternatives to the FID.

Compton and Purdy (18) modified the FID of a Pye Unicam detector by inserting a rubidium silicate glass bead above the flame and thus changed it into a thermal ionic detector. The detector was then selectively sensitive to nitrogen and phosphorus compounds.

Stolyhwo et al. (19) attempted to improve the sensitivity of the detector by using metal spirals wound on wire and also stranded wire to increase the surface area of the carrier and, thus, increase the proportion of the column eluent taken into the detector. The authors claim a sensitivity limit for triolein of 100 nanograms. Again, the exact volume of mobile phase in which this mass of solute was contained was not clear from this publication. If the 100 nanograms of triolein was eluted in a peak 1 ml wide at the base, the concentration at the peak maximum would be twice the average concentration, i.e. 2×10^{-7} g/ml, which, for a transport detector, would be a great improvement on sensitivity. If, however, the same mass was eluted as an early peak in the chromatogram with a band width of only 50 µl, then the sensitivity would only be 4×10^{-6} g/ml, which would be no better than the previously developed transport systems. This uncertainty emphasizes the importance of specifying sensitivity in terms of minimum detectable concentration, which allows the direct comparison of the sensitivity of one detector with another.

Pretorius and Van Rensburg (20) attempted to increase the quantity of column eluent taken on to the carrier by coating the wire with sodium silicate, kaolin and copper kaolin. Sensitivity was again not quoted in terms of minimum detectable concentration, so the precise change in sensitivity that resulted from the coated carrier is not clear. From this publication, however, it would appear that a significant improvement in sensitivity was realized. The introduction of the wire coating procedure, however, further complicates an already complex instrument. It would seem that this approach might lead to serious instrumental problems arising from the dust produced by the disruption of the coating from abrasion as the wire passed round the pulleys and on rewinding the wire on the spool.

Slais and Krejci (21) replaced the normal FID with the alkali FID to selectively detect chlorine compounds. These workers used a combustion technique as opposed to pyrolysis, mixed the products of combustions with hydrogen, and then passed the mixture directly to the alkali FID. At a column flow rate of 0.37 ml/min, the sensitivity of the detector was stated to be 3×10^{-7} g/sec, which is equivalent to a sensitivity of about 1.6×10^{-6} g/ml. The moving wire detector has also been modified to provide radioactivity detection by Dugger (22) for monitoring tritium or carbon-14 labelled compounds. To detect carbon-14 compounds, the solute on the wire was oxidized to carbon dioxide, and the radioactive gas passed to a Geiger-Muller tube. To detect tritium, the tritiated water produced during oxidation of the solute was then passed over heated iron to reduce it to hydrogen, which was then also passed through a Geiger-Muller tube. Specifications and performance characteristics of the apparatus were not given.

The wire transport system has many attractive characteristics as a LC detector, but for general use, its sensitivity needs to be increased by at least an order of magnitude. Further, the overall system needs to be simplified to render it more reliable, less expensive and easier to operate.

The Disc Detector

The disc detector originally described and developed by Dubsky (23) employed a rotating gauze disc as the carrier. A diagram of this apparatus is shown in Figure 20.

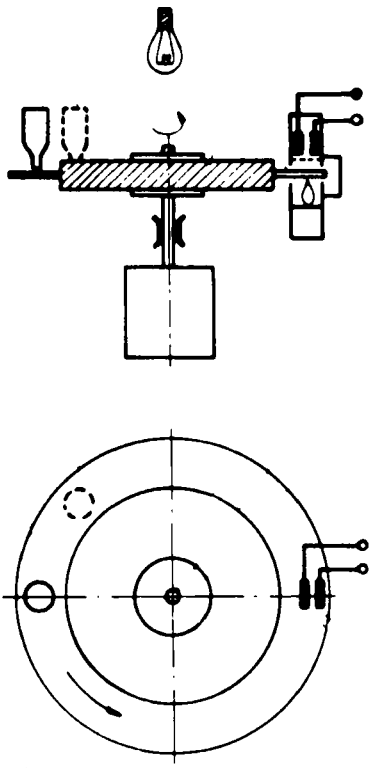


Figure 20. The disc detector.

It consists of a rotating disc, the perimeter of which is made of wire gauze. The column exit is situated just above the gauze, and the effluent flows through the gauze, the excess being collected below the gauze. A little ahead of the point of coating, in the direction of rotation of the disc, is situated an infrared lamp, which evaporates the solvent, leaving the solute coated on the gauze. Diametrically opposite to the point of coating is situated the FID. The flame jet is situated beneath the gauze, such that the flame itself is in contact with the gauze. The electrodes of the FID are placed above the gauze directly over the flame. The ions collected by the electrodes provide a current, which is fed to an amplifier and thence to a recorder. The system has the advantage of being simple, compared with the conventional wire

detector, but although the author claims a tenfold increase in sensitivity, this is difficult to confirm, as the specific sensitivity in terms that permit comparisons with other detectors was not given. Szakusito and Robinson (24) claimed that the metal gauze disc carrier produced excessive noise and, in particular, "spikes", resulting from local concentrations of solute accumulating during evaporation at the intersections of the wire mesh. The wire disc was replaced by an alumina disc 4.5 in. in diameter and with the edge tapered to 0.25 mm thick. This thin edge was used for coating and detection. It was claimed that a significant reduction in noise was achieved but, again the sensitivities obtained were not specified in terms that would allow comparison with other detectors. The life of the alumina disc also seems questionable, as it would seem that, in continued use, the pores of the alumina would eventually become blocked by incompletely combusted solutes, or mobile phase components, such as 'buffers' or inorganic substances, which would not burn. The disc does appear to be a more simple transport system than the wire or chain, but its reliability and sensitivity still need to be established.

The Electrochemical Detector

The electrochemical detector is one of the most sensitive and the most specific LC detectors available. It will respond to substances that are either oxidizable or reducible. The output from the detector results from the electron flow caused by an electrochemical reaction that takes place at the surface of an electrode. The reaction can be either oxidation or reduction and if the reaction proceeds to completion, exhausting all the reactant, then the current flow becomes zero and the total charge passed will be proportional to the total mass of material that has been reacted. This procedure is, for obvious reasons, termed coulometric detection. If, however, the electrolyte is flowing past the electrodes, e.g. the electrodes are situated in the eluent from a column, the solute, which constitutes the reactant, will be continuously replaced throughout the elution of a peak. Thus, while there is solute present between the electrodes a current will be maintained, albeit, varying in magnitude. This method of electrochemical detection, is termed amperometric detection and is, at present, virtually the only method employed in LC. It is used almost to the complete exclusion of coulometric detection.

The various forms of electrode configuration will be discussed later but at this stage it can be said that normally, three electrodes are employed, the working electrode, the auxiliary electrode and the reference electrode. The electrochemical process is made to occur at the working electrode and the reference electrode compensates for any change in eluent

conductivity. The reactions that take place in the electrode compartment can be complex, however, the major effect can be described as follows.

The reaction at the surface of the electrode is extremely rapid and proceeds almost to completion. This results in the layer close to the electrode being virtually depleted of reactant. Consequently a concentration gradient is established between the electrode surface and the bulk of the solution. This concentration gradient results in the solute diffusing into the depleted zone at a rate determined by the concentration in the bulk solution. As the current generated at the electrode surface depends on the rate at which solute reaches the electrode, the detector exhibits a linear response with respect to solute concentration.

The response of the detector, i, can be described by the following equation:

$$i = nFAK_T cu^a \qquad (7)$$

where n is the number of electrons per molecular involved in the reaction
F is Faraday's Constant
A is the area of the working electrode
K_T is the limiting mass transfer coefficient
c is the solute concentration
u is the linear velocity of the mobile phase above the electrode
a is a constant and usually takes a value between 1/3 and 1/2.

It is seen from equation (7) that the current i and consequently the sensitivity of the detector can be increased by increasing the area of the electrodes A, the transfer coefficient K_T or the velocity of the mobile phase past the electrodes u. n and F are constants depending on the nature of the electrochemical reaction.

It would appear that increasing the electrode area would be an attractive means of increasing detector sensitivity. Unfortunately increasing the electrode area also results in increasing noise and in fact usually results in an overall reduction in sensitivity. Weber and Purdy (25) and Hanekamp et al. (26) showed that under certain circumstances a reduction in detector size provides a significant increase in signal-to-noise ratio and hence improved sensitivity. Operating the detector at higher flow velocities will also increase the rate of solute transfer and improve sensitivity and this will be a secondary advantages of reducing the dimensions of the detector. However, because of the effect of flow rate on detector response, as shown by equation (7), it follows that the detector will be very flow

sensitive and thus can only be used under constant flow conditions and certainly not with flow programming methods of development. In general, miniaturization of the cell will also reduce band dispersion and render the detector more suitable for use with modern high efficiency and small bore columns.

Electrode Configuration

The electrodes can take a number of different geometric forms which have been described in some detail by Poppe (27). The most popular are the thin-layer cells. Some examples of different electrode configurations are shown in Figure 21.

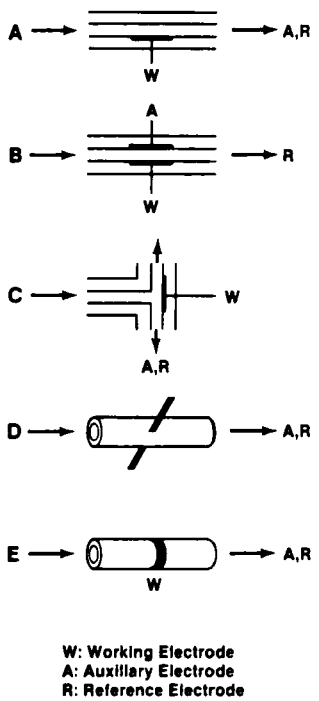


Figure 21. Examples of different electrode configurations.

A and *B* are two common forms of thin-layer cells. *A* has the working electrode sealed into the wall of the cell with the reference electrode and auxillary electrode situated downstream to the working electrode. *B* is a similar cell but with the auxillary electrode also situated in the wall of the cell but opposite the working electrode. In this case only the reference electrode is placed downstream. *C* is an example of a wall jet electrode where the column eluent is allowed to impinge directly onto the working electrode situated directly opposite the jet. This arrangement increases the linear velocity of the solute passing the electrode u and by the scrubbing action of the jet increases the transfer coefficient K_T, both effects increasing the overall sensitivity of the detector. *D* and *E* are two examples of cylindrical electrodes;

in *D* the working electrode takes the form of a rod stretching across the diameter of the cell and in *E* the working electrode is an annular ring set in the wall of the tubular cell. In both cases the auxiliary and reference electrodes are situated downstream to the working electrode.

Electrode Construction

The choice of material that can be used for the construction of the working electrode is somewhat restricted owing to the mechanical ruggedness and long term stability that is required. The most common material is carbon paste made from a mixture of graphite and some suitable dielectric material. The disadvantage of this material is its solubility in some solvents unless special waxes or polymers are used as dielectric binders to contain the graphite. Vitreous or "Glassy" carbon is also an excellent material for electrode construction particularly if it is to be used with organic solvents. This material is manufactured by slowly carbonizing a suitable resin at elevated temperatures and then heating it to a very high temperature to cause vitriation. Vitreous carbon is relatively pure, is mechanically strong and has good electrical properties. It can be readily cleaned mechanically and performs particularly well, relative to other electrode materials, when used at negative potentials. In general, "Glassy" carbon electrodes are to be preferred over carbon paste electrodes due to their resistance to solvents but a number of other carbon type electrodes have been described (28, 29) which may in the future hold good possibilities for particular applications. Mercury was the material first used by Kermula (30) in 1952 for electrochemical detection and is still occasionally used in the form of amalgamated gold discs, amalgamated platinum wire (31) and as pools in capillary tubes. Hanekamp et al. (32) discussed the various merits of different detector designs based on the principle of the dropping mercury electrode. They concluded that the linear dynamic range and response times of an optimally designed dropping electrode detector was far better than that of most UV detectors. Joynes and Maggs (33) employed carbon impregnated silicone rubber membranes as working electrode material and Takata and Muto (34) examined the use of platinum and silver gauze.

Electrochemical detection imposes certain restrictions on both the type of chromatography that can be employed and the mobile phases that can be used. Because, in most cases, the detecting process requires the mobile phase to be conducting, it must contain water, which means that "normal phase" chromatography is not a practical chromatographic system. Furthermore, very high solvent concentrations may render the mobile phase insufficiently conducting for some applications. It follows that reversed-phase chromatography is the ideal system for electrochemical detection.

Certain stringent precautions have to be taken, however, for the effective use of these detectors. The mobile phase must be completely free of oxygen which can be removed by bubbling helium continuously through it while contained in its reservoir. It follows that it is also extremely important to remove oxygen from the sample before an injection is made. The solvents must also be free of metal ions otherwise a very unstable base line will result. Sometimes the mobile phase can be cleaned prior to entering the pump by pre-electrolysis. Under some circumstances pure non-aqueous solvents such as acetonitrile can be used as the mobile phase but certain salts such as tetrabutyl-ammonium hexafluorophosphate have to be added to render the solvent conducting.

Basic Electrochemical Detector Electronics

The basic circuit for an electrochemical detector is shown in Figure 22.

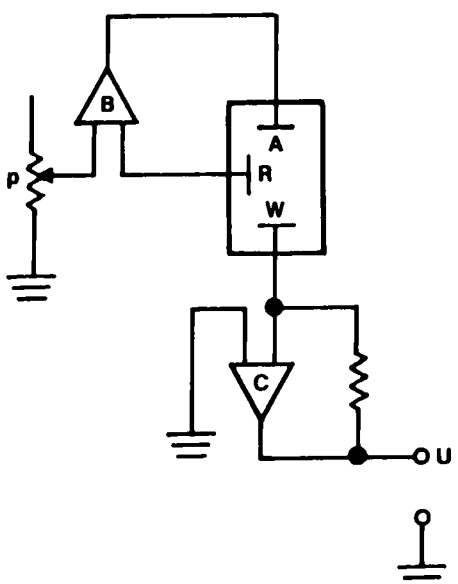


Figure 22. Basic circuitry for electrochemical detector.

The auxiliary. electrode *A* is held at a fixed potential by amplifier *B* the voltage being selected by potentiometer *P*. The potential near the auxillary electrode is sensed by the reference electrode *R*. As stated before, the reference electrode allows the voltage to be compensated for changes in the mobile phase conductivity. On arrival of a solute at the surface of the working electrode that can be either oxidized or reduced, a

current is developed which is converted by amplifier *C* to a voltage output *V*. This output can either be fed directly to a suitable recorder or through an appropriate attenuator system, a filter circuit and then to the recorder. Sometimes only two electrodes are used in which one electrode acts as both a reference and auxiliary electrode. However, under these circumstances, the potential difference between the electrodes which, in fact, controls the oxidation reduction, can change as the conductivity of the solution changes as a consequence, for example, of a change in ionic strength of the mobile phase.

The electrochemical detector is extremely sensitive, but suffers from two main drawbacks. Firstly, the mobile phase has to be extremely pure, in particular, free of oxygen and metal ions. Secondly, by-products of the oxidation or reduction processes are often absorbed on the surface of the electrodes and thus, if quantitative activity is required, frequent calibration is necessary. Ultimately the electrodes have to be cleaned usually by mechanical abrasion and replaced in the cell. Electrochemical detection is particularly suitable for small bore columns and possibly, in the future, LC capillary columns, due to the fact that the detector can be made extremely small in size. The detector has had a fairly wide area of application. It has been used under oxidizing conditions for the detection of phenols, hydroquinone and catechols and in particular for many compounds of biological interest including, catacholamines (35). It has been used to determine substances of industrial interest, and agricultural chemicals (36). In its oxidation form, it has been used to detect amines of various types together with phenols and thiols. It has also been used in the analysis of ascorbic acid in food and biological materials, and in the pharmaceutical industry, for the analysis of multivitamin products.

In the reduction mode, electrochemical detectors can be used for detecting quinones, nitro compounds and consequently for the analysis of various forms of explosives (37). With the use of reagents containing aromatic nitro groups, derivatives of amines, ketones, and acids can also be detected by electrochemical detection.

Haroon et al. (38) developed a dual electrode system for the detection of vitamin K compounds. The device utilized two sequential generator/detector electrodes. Vitamin K was electrolyzed at the first electrode and the reaction products were then detected electrochemically at a second electrode. The minimum mass of vitamin K_1 that they claim could be detected was 100 pg. They applied their system to the analysis of rat liver extracts and claimed it was superior in both sensitivity and specificity to the UV detector.

An example of the detection of some catacholamines and related compounds (39) using the electrochemical detector is shown in Figure 23. The chromatogram shows eighteen compounds important in central nervous system physiology. The mixture was injected into a buffered solution and separated by reversed-phase chromatography.

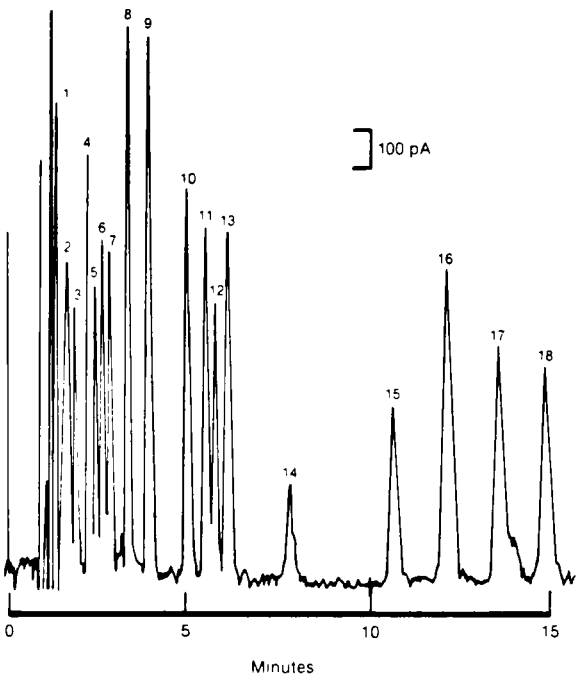


Figure 23. Chromatogram of some catacholamines and related compounds using the electrochemical detector. Chromatographic Conditions: column: HC-3 C_{18} (100 x 4.6 mm); mobile phase: aqueous solution of 100 mM formic acid, 0.35 mM octane sulfonic acid, 1.0 mM citric acid, 0.10 mM EDTA, 5.0% actonitrile, 0.25% (v/v) diethylamine, pH to 3.10 with KOH; flow rate: 1 ml/min; detection: oxidative amperometric with glassy carbon electrode at +800 mV potential vs. Ag/AgCl. Identification: 1 = 3,4-dihydroxymandelic acid (200 pg); 2 = L-dopa (600 pg); 3 = vanillymandelic acid (400 pg); 4 = norepinephrine (200 pg); 5 = α-methyldopa (600 pg); 6 = 3-methoxy-4-hydroxyphenylglycol (400 pg); 7 = epinephrine (200 pg); 8 = 3,4-dihydroxybenzylamine (200 pg); 9 = normetanephrine (400 pg); 10 = dopamine (200 pg); 11 = metanephrine (400 pg); 12 = 3,4-dihydroxyphenylacetic acid (200 pg); 13 = N-methyldopamine (400 pg); 14 = tyramine (1 ng) 15 = 5-hydroxyindole-3-acetic acid (200 pg); 16 = 3-methoxytyramine (400 pg); 17 = 5-hydroxytryptamine (200 pg); 18 = homovanillic acid (400 pg).

The Atomic Spectroscopic System As An Element Specific Detector

The association of a spectrometer with the liquid chromatograph is usually for the purpose of structure elucidation of the eluted solute, a procedure that will be discussed in a later chapter. The association of an atomic spectrometer with the liquid chromatograph, in contrast, is almost exclusively for the specific detection of the metalic and semi-metalic elements. The atomic spectrometer is a highly specific detector, and for element detection perhaps more so than the electrochemical detector. However, in general, a flame atomic absorption spectrometric (AAS) system is not as sensitive. If an atomic emission spectrometer or an atomic fluorescence spectrometer is employed then multi-element detection is possible. The inductively coupled plasma spectrometer can also, under some circumstances, provide multi-element detection but all three instruments are extremely expensive particularly in terms of an LC detector. It follows that most LC/AAS combinations employ a flame atomic absorption spectrometer or occasionally an atomic spectrometer fitted with a graphite furnace. Furthermore the spectrometer is usually set to monitor one element only, throughout the development of any given separation.

The main application of the LC/AAS system is to help determine metal speciation in samples, not merely to identify the presence of a particular element. It is not enough to detect the presence of lead, mercury or chromium, but to be able to identify the form in which they are present. Depending upon the chemical form of a mercury compound it may or may not be toxic. Similarly if chromium is present in the tertiary form it is not particularly dangerous; conversely, in its sixth valency state, it is strongly carcinogenic. It follows that the liquid chromatograph can be employed to separate the different species and the atomic spectrometer can identify and confirm the presence of a specific element in the appropriate peaks.

There have been a number of reviews in the literature on the identification of metal species by LC/AAS (40-42) but to successfully utilize the combination, both the LC and the spectrometer system have to be optimized and this has also been the subject of a number of publications (43-45). It has been claimed (44) that the poor sensitivity obtained from the LC/AAS system relative, to that obtained from the atomic spectrometer alone, was due to the dispersion that takes place in the column. Although substantially true, this misunderstanding arises from the fact that the spectroscopist views the chromatograph as just another sampling device and not as a separation system. The point of interfacing a liquid chromatograph with an atomic spectrometer is to achieve a separation before detection and consequently, the important dispersion characteristics are not those that occur in the column but those that occur in the interfaces between the detector and the spectrometer and in the spectrometer itself.

Dispersion in these two sources does not merely reduce element sensitivity, but can also destroy the separation originally obtained in the column. The magnitude of the extra column dispersion is particularly important if high-speed chromatography columns, packed with particles of very small diameters, are to be employed, since these columns produce very narrow peaks a few microliters in volume. High-speed columns seem to be ideally suited for use with AAS as they can be operated at the high flow rates necessary for efficient solvent aspiration into the spectrometer nebulizer. Unfortunately due to the basic design of most AAS instruments a significant length of tubing is necessary for the interface, if the normal operation of the spectrometer is not to be impeded and the controls are to be maintained readily accessible.

Katz and Scott (46) solved this problem by the use of low dispersion serpentine tubing as the interface between the exit from the UV detector of the liquid chromatograph and the spectrometer. A diagram of the interface employed is shown in Figure 24. The tube was 49 cm long 0.25 m I.D. and the serpentine form had a peak to peak amplitude of 1 mm. The efficacy of the interface is demonstrated by the dispersion curves shown in Figure 25.

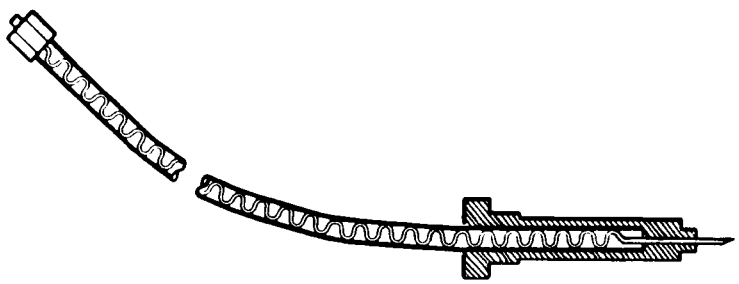


Figure 24. Diagram of serpentine tubing.

All the tubes are the same length; the center peak being obtained from an exactly similar tube to the serpentine interface but straight. The advantage of the serpentine form is clearly seen. The peak on the right is for a plastic tube similar to that normally employed for an LC/AAS interface and the serious dispersion that results is also clearly demonstrated. An example of the chromatograms from a blood sample monitored by both a UV detector and an AAS detector employing the serpentine interface is shown in Figure 26; the AAS chromatogram clearly shows the peak containing iron.

It would appear that the iron peak from the AAS was significantly more narrow than that from the UV detector. This apparent anomaly arises from the fact that the UV peak is a multi-component peak containing a number of unresolved substances only one of which contains iron.

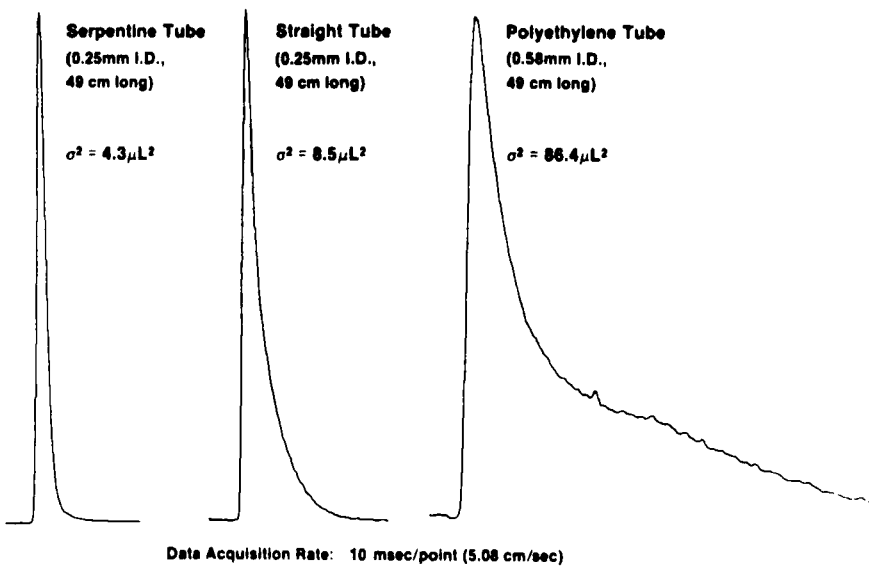


Figure 25. Peak dispersion in connecting tube. Mobile phase: methanol; flow-rate: 2 ml/min; solute: $Mg(NO_3)_2$.

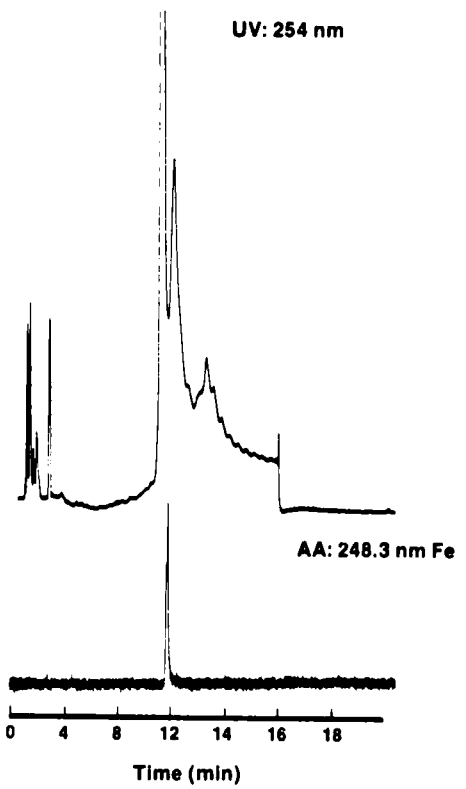


Figure 26. Chromatograms of a blood sample using both the UV detector and AAS detector employing the serpentine interface.

The LC/AAS has been employed in a number of extremely interesting applications. Holak (47) has used the LC/AAS system to monitor the separation of a number of mercury containing drugs; the compounds separated were mersalyl, thimerosal and phenyl mercuric borate. Suzuki et al. (48) used the technique to identify the metals bound to isoproteins extracted from liver tissue. Robinson and Boothe (49) used the selectivity of the LC/AAS system to monitor the alkyl leads in sea water and Messman and Rains (50) separated four alkyl leads, tetramethyl lead (TML), trimethylethyl lead (TMEL), dimehyldiethyl lead (DMDEL), methyl triethyl lead (METL) and triethyl lead (TEL) in gasoline and an example of their separation is shown in Figure 27. The use of the combination of LC and AAS is relatively new and it is likely that the use of the dual system will be developed significantly further in the near future.

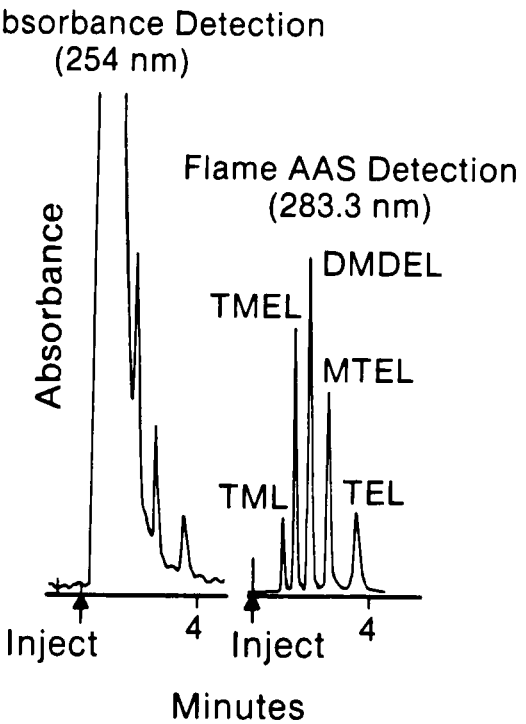


Figure 27. An example of a separation employing the selectivity of the LC/AAS system.

The Radioactivity Detector

The use of radioactive tracers in the study of reaction mechanisms has been steadily increasing over the last two decades. Radioactive tracers have been used to elucidate the mechanism of complex laboratory reactions, in photosynthetic chemistry and, in particular, to follow metabolic pathways of substances, synthetic and natural, in both plants and animals. In fact, the first reported use of an in-line radioactivity detector fitted directly to a gas chromatograph was in a paper by James and Piper (51) who developed the detector to study the synthesis of lipids, glycerides and fatty acids in plant tissue. James and his co-workers designed a small volume radioactivity counter that was placed in line with the column eluent but subsequent to an oxidizing furnace. The solute from the column was combusted to CO_2 and water and the tritiated water and radioactive carbon dioxide from the carbon fourteen tracer reduced in a second furnace containing iron and was then counted during passage through a Geiger-Muller tube. By integrating the output from the Geiger counter a measure of the total activity of the peak could be obtained. In LC, however, as the mobile phase is a liquid, the same procedure cannot be used, and an alternative method of counting has to be resorted to.

In 1961, Schram and Lombaert (52) designed a flow through cell for the continuous radioactive monitoring of liquid streams by scintillation counting utilizing anthracene powder as the scintillation agent. The counting cell was made from a 60 cm length of polyethylene tubing 2.2 mm I.D. and 3.2 mm O.D., which was filled with anthracene powder by a slurry packing technique employing a suspending solvent composed of 30% v/v of water in ethanol. The packed tube was then coiled into a spiral, which was placed in a flat Lucite vial containing silicone oil. The size of the anthracene particles affected both the pressure drop across the tube and its counting efficiency. The authors compromised on the particle size, using particle diameters of 300 μm for carbon fourteen counting and 150 μm for tritium counting. The cell volume was about 1 ml which, although the inherent band dispersion was significantly reduced due to the presence of the anthracence packing, would be far too great for modern high-efficiency small bore columns. (Due to interparticulate radial flow, dispersion in a packed bed is much less than in an open tube.) The detector tube was situated in a light sealed box and counting was achieved using one or more refrigerated photomultipliers. The counting efficiency obtained was about 2% for tritium and about 55% for carbon-14. The background gave about 60 counts/min. At a signal-to-noise ratio of two, 1.5 nC/ml of tritium and 0.05 nC/ml of carbon-14 could be detected.

In 1964, Sjoberg and Agren (53) described a dual in-line UV absorption and radioactivity detecting system. This type of system is of great practical value as the output from the

absorption detector discloses the position and relative proportion of all the UV absorbing solutes present in the mixture whereas the radioactivity detector monitors only those solutes that are radioactive. The radioactivity detector cell of Sjoberg and Agren consisted of a commercially available plastic scintillation tube in the form of a spiral, 1 3/4 in. diameter composed of a single tube 2 ft. long, 0.7 mm I.D. and 1.5 mm O.D. having a volume of 0.3 ml. Such a cell would cause very serious band dispersion if employed with high efficiency columns. In the original paper by Sjoberg and Agren, an excellent example is given of the use of the two simultaneous detecting systems in the separation of some acid soluble nucleotides extracted from diploid tumor ascillis cells 30 min after incubation with radioactive inorganic phosphate.

The radioactive detecting systems devised by Schram and Lombaert (52) and Sjoberg and Agren (53) were reasonably efficient for counting carbon-14 and isotopes having β-particle emission of higher energy but their efficiency for counting tritium was relatively poor. Furthermore, some compounds could be adsorbed by anthracene, thus causing a build-up of background noise and loss of such compounds. The cell could also only be used with solvents which do not affect the material of the cell, anthracene or the scintillator. Scharpenseel and Menke (54) attempted to improve the efficiency of the tritium count by employing a toluene based liquid scintillation system. The column eluent or a portion thereof is mixed with the reagent and then passed to the scintillation cell. Counting efficiency for tritium was increased to 2-5%, but the system was extremely sensitive to the presence of salts even when the effluent to scintillator ratios were very low. Hunt (55) attempted to improve this system by replacing the toluene based scintillator with a solution of napthalene 2,5-diphenyloxazole and (1,4-bis-2(4-methyl-5-phenyloxazole)benzene) in carefully purified dioxane. The cell Hunt used was a coiled tube similar to that of Schram and Lombaert (52) so that band dispersion would again be a serious problem if high column efficiencies were required. Hunt, however, achieved a counting efficiency for tritium of about 14% and about 70% for carbon fourteen.

Schutte (56) examined the methods of Hunt and Sjoberg and developed a dual system of absorption and radioactivity detection. Schutte also introduced a heterogeneous scintillation system employing cerium activated lithium glass beads as the scintillation agent. A diagram of the cell employed by Schutte is shown in Figure 28. The U tube at the base is packed with the scintillator beads and is connected directly in line with the column eluent. The counting efficiency of this system is relatively poor, 0.2% for tritium and 17% for carbon-14. The advantage of this method is that it lends itself to the design of cell and connecting tubes that would provide minimum band

dispersion due to the fact that the counting cell is packed and therefore could possibly be used with high-efficiency micro-particulate columns.

The moving wire detector has also been modified by Dugger (22) to provide radioactivity detection for tritium and carbon-14 labelled compounds. In order to detect carbon-14 the solute on the wire was oxidized to carbon dioxide and water and the radioactive combustion products passed to a Geiger counter and detected in much the same manner as James and Piper (51) detected radioactive carbon dioxide in the eluent from a gas chromatograph. To detect tritium, the tritiated water produced during oxidation was passed over heated iron to reduce it to hydrogen and tritium and then passed through a Geiger counter. Specifications and performance characteristics were not given.

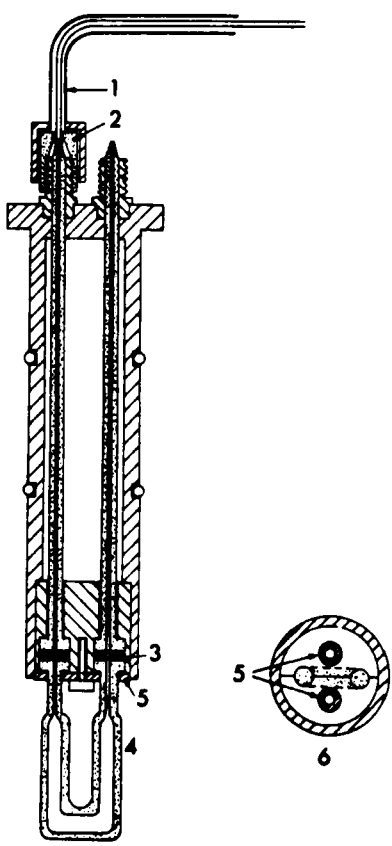


Figure 28. Flow cell for heterogeneous radioactivity counting. 1 = metal cap and bent sleeve for light-proofing; 2 = Swagelok fitting; 3 = Viton O-rings; 4 = interchangeable borosilicate glass cell; 5 = semi-circular collar disks; 6 = bottom view of cell and probe.

Continuous flow monitoring of radiation in LC column eluents, which usually involves a scintillation counting technique, can be classed as either homogeneous or heterogeneous counting systems. In homogeneous counting techniques the column eluent is mixed with a liquid scintillation reagent before passing through a cell situated between the photomultiplier tubes of a scintillation counter. In heterogeneous counting systems the column eluent is passed directly through a flow cell packed with a suitable scintillation powder such as anthracene or cerium activated lithium glass beads. Heterogeneous counting systems are free of chemical quenching effects and the sample can be easily recovered. However, the counting efficiency is generally low and the solubility of the scintillator can effect the choice of mobile phase. As previously stated solutes can absorb on the surface of the scintillator with the result that its efficiency is impaired or it exhibits a continually increasing level of background activity. Heterogeneous counting systems are best employed in preparative chromatography where the concentration of the solutes is high and consequently the level of radioactivity is also relatively high.

Homogeneous radioactivity detectors are to be preferred in analytical LC where the recovery of the sample is not important but sensitivity and versatility is essential. Nevertheless the coupling of a homogeneous radioactivity monitor to a liquid chromatograph will still require some compromise between the sensitivity of the monitor and the speed and resolution obtained from the liquid chromatograph.

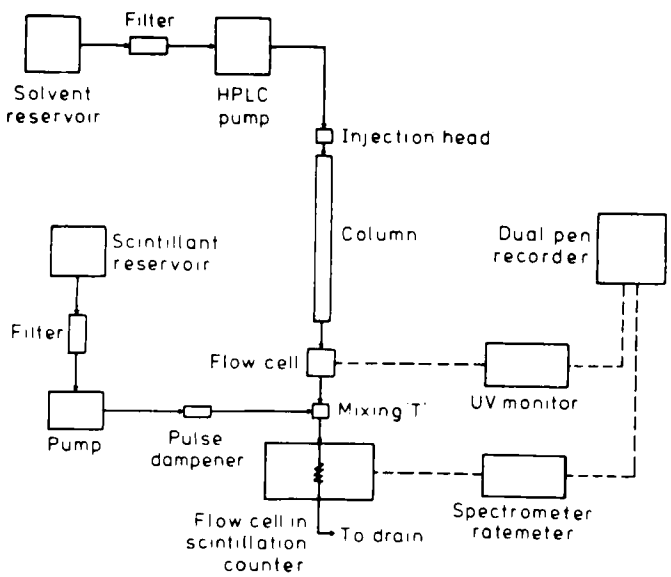


Figure 29. Diagram of an HPLC fitted with a continuous-flow radioactivity detector.

Reeve and Crozier (57) theoretically investigated the optimization of the combination of a homogeneous radioactivity detecting system with a liquid chromatograph and a diagram of the apparatus they designed, on a basis of their findings, is shown in Figure 29. The column eluent passes through the cell of a UV detector, then to a small volume mixing T where it is mixed with the scintillation reagent. The reagent is delivered by a micrometering pump fitted with an appropriate pulse dampener. The authors recommended dioxane naphthalene mixture as scintillation reagents for "forward phase" chromatography (i.e. silica gel columns and phase systems) but the optimum amount of napthalene and the best reagent/column flow ratios had to be arrived at by experiment. The counting efficiency for aqueous solvents was low and reagent/eluent flow ratios had to be in excess of 10. However, by adding a detergent based emulsifier it was found that the counting efficiency could be significantly improved.

Mackey et al. (58) described an optimized heterogeneous radioactivity detector cell based on a packed tube similar in principle to that of Schutte (56), employing cerium-impregnated glass powder. A diagram of their cell is shown in Figure 30. The glass they used was NE901 and NE913 supplied by Nuclear Enterprises Edinburgh, U.K. They claimed that the optimized detector provided a counting efficiency for carbon-14 radiation of greater than 70% which is an efficiency normally expected from standard liquid scintillation techniques. In order to achieve this performance the electronics also had to be optimized.

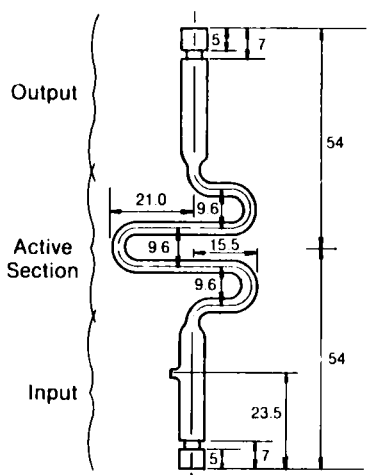


Figure 30. Diagram of flow-through cell. Dimensions in mm. Output: 6 mm O.D., 2 mm I.D. Input 6 mm O.D., 0.9 mm I.D.

Radioactivity detectors are, at present, not frequently used in LC separations but may well find increasing use in the expanding fields of biotechnology. To date its most common application appears to be for monitoring biosynthetic pathways in

both animal and plant metabolism. It is likely that if and when demand for this type of detector increases more efficient and less costly instruments will become available.

An example of the use of the radioactivity detector to monitor some alkylethoxylate urinary metabolites by Mackey et al. (58) is shown in Figure 31. The separation was achieved by the use of a reversed-phase column and an acetonitrile-acetate buffered-water mobile phase. The radioactive metabolites are clearly seen. Although the metabolites can be separated by LC and with the aid of the radioactivity detector, the peaks can be monitored there could remain a serious problem of identification.

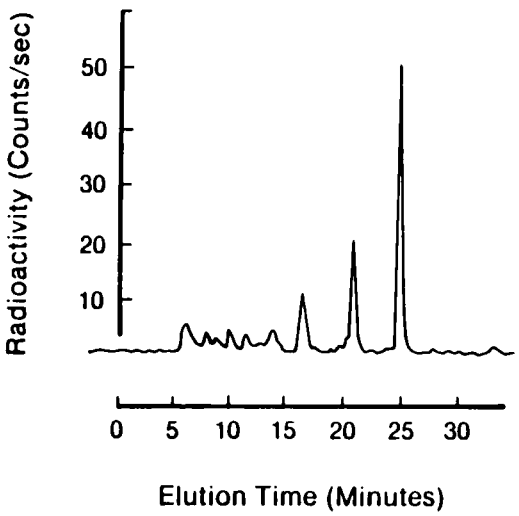


Figure 31. Example of the use of the radioactivity detector to monitor some alkylethoxylate urinary metabolites.

Additional Solute Property Detectors

The solute property detectors described so far are those more commonly used or, have at least at some time or other formed the basis of a frequently used, commercially available detector. There are, however, a number of detectors that have been developed that have either, not been developed into a commercial product or, have found a very limited area of application. Many of these detectors have, in fact, useful potential for specific chromatographic analyses. Some of these detectors will now be described to give the reader a broader view of solute property detectors in general, and to illustrate the wide range of solute properties that have been investigated with a view to the development of viable LC detecting systems.

The Heat of Adsorption Detector

During the passage of a solute peak through a chromatographic column the solute is continuously adsorbed onto the stationary phase in the front portion of the peak and continuously desorbed in the rear portion of the peak. The adsorption-desorption effect results from the continuously increasing solute concentration in the front of the peak causing more and more solute to be driven onto the stationary phase to maintain solute equilibrium between the two phases; at the same time in the rear portion of the peak the reverse process occurs. As the solute concentration falls, so more solute is desorbed from the stationary phase to maintain solute equilibrium between the two phases. The adsorption of a solute by the stationary phase is accompanied by the evolution of heat which results from the heat of solution of the solute in the stationary phase if it is a liquid and the heat of adsorption of the solute on the adsorbent if the stationary phase is a solid. Thus if the temperature is monitored at any point in the column, during the passing of a solute band, the temperature will rise above the column temperature as the front part of the peak passes the point of measurement and will fall below the column temperature as the rear part of the peak passes; subsequently the temperature will return to the original column temperature. This effect was employed by Claxton (59) in 1959 as a detecting system to monitor LC eluents.

The heat of adsorption detector, devised by Claxton, consists of a small plug of adsorbent, usually silica gel, through which the chromatographic eluent passes subsequent to leaving the column. Embedded in the silica gel is either a thermocouple or a thermistor that continuously measures the temperature of the adsorbent and mobile phase. When an eluted solute comes into contact with the silica gel, the heat evolved causes the temperature to rise. As the solute is subsequently desorbed from the silica gel, heat is absorbed and the temperature falls. The output from the temperature measuring device thus records an increase in temperature and then a decrease in temperature relative to its surroundings and an S-shaped curve results.

The heat of adsorption detector has been investigated by a number of workers (60-62), but, although once commercially available, has not been extensively used as an LC detector. One reason for this is the curious and unpredictable shape of the temperatures-time curve that results from the detection of the usually Gaussian or Poisson concentration profile of a peak eluted from the column. The shape of the curve changes with the operating conditions of the chromatograph and with the retention volume of the solute. Thus, for closely eluted peaks, it produces a complex curve that is extremely difficult to interpret. A diagram of the heat of adsorption detector cell designed by Cashaw et al. (62) is shown in Figure 32.

The detector is composed of multi-thermocouples (thermopile) with a suitable adsorbent embedded at one end which becomes the hot junction, with the opposite end serving as the cold junction. Iron and constantan, insulated with PTFE, were used as the thermoelectric base wires. The wires were held parallel and wound around a plexiglass board, so that the iron and constantan wires alternated. The wires were permanently located by covering them with a sodium silicate solution and allowing the solution to dry. After drying, the PTFE was removed from the wire, the wires were cut along the thickness of the board so that a little of wire was exposed at each end. The exposed ends were welded together to form a group of thermocouples which comprised the thermopile. Two thermopiles were obtained from each operation and the number of thermocouples in each thermopile was equal to the number of loops made around the plexiglass board.

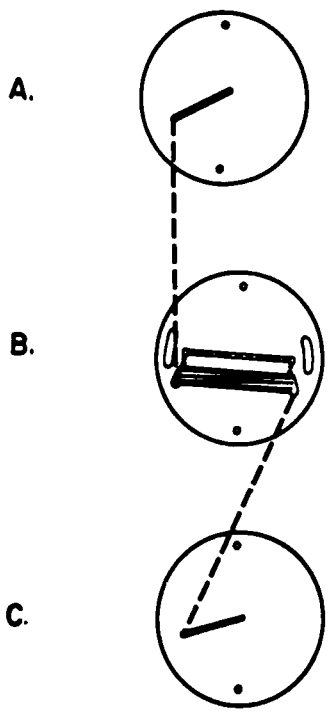


Figure 32. The heat of adsorption detector.

The thermopile was mounted in a PTFE disc, and soldered to PTFE-insulated leads which were connected to the input terminals of the amplifier. The thermopile was thus contained between two further discs each having a groove that allows the mobile phase to flow over one set of junctions. The three PTFE columns were situated between two steel flanges containing cavities for centering the columns.

A chromatogram obtained from this detector is shown in Figure 33. It is seen that the output from the detector is somewhat confusing and difficult to interpret. The response of the detector has been examined by computer simulation by Smuts et al. (63) and an explicit equation describing the temperature change as a function of the concentration profile of the eluted peak and the thermal properties of the detector cell and column has been derived by Scott (64).

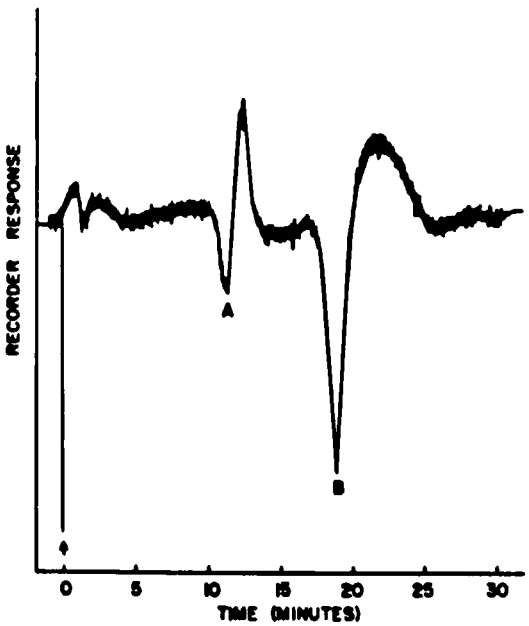


Figure 33. Chromatogram obtained from a heat of adsorption detector.

The equation derived by Scott is as follows

$$\theta_v = \psi\, e^{-\phi v} \int_0^v e^{\phi v} \left[x_o \frac{e^{-v_v n}}{n!} - X_o \frac{e^{-v/C_a}}{C_a} \int_0^v {}^{-v/C_a} \frac{e^{-v_v n}}{n!}\, dv \right] dv$$

where θ_v is the temperature of the detecting cell,
ψ is a constant,
ϕ is a constant representing the heat loss factor,
C_a is the detector plate capacity-column plate capacity ratio,
v is the flow of mobile phase through the detector in plate volumes of the attached column,
n is the efficiency of the attached column.

With the aid of the computer in a manner similar to that of of Smuts et al. (63) , the relative values of θ for v = 74 to 160 were calculated for a column having an efficiency of 100 theoretical plates, and for C_a taking values of 0.25, 0.5, 1, 2

and 4, and for ϕ taking values of 0.01, 0.05, 0.25 and 1.25 respectively. The 20 curves shown in Figure 34 represent the shape of the θ_v versus v_α curves and the integral of θ_v versus v_α curves for the different values of C_a and ϕ and are all normalized to the same peak height. The curves shown cover the practical range of heat loss factors for the detector cell and demonstrate the effect of changes in detector capacity/plate capacity ratios that would result from different detection cell designs detecting a peak of constant width.

The curves for different values of C_a would also represent the effect on peak shape of solutes of different retention, and thus different peak widths passing through a detecting cell of fixed dimension, but having an adsorbent different from the stationary phase of the column. Examination of Figure 34 shows that the major effect on peak shape is the ratio of cell capacity to column plate capacity C_a. It is seen that when the capacity of the detector cell is less then the plate capacity of the column (C_a<1), the negative part of the signal is dominant, whereas when the detector cell capacity exceeds that of the column (C_a>1), the positive part of the signal dominates. For this reason, the integral of the detector signal for C_a>1 rises to a peak but does not return to the baseline.

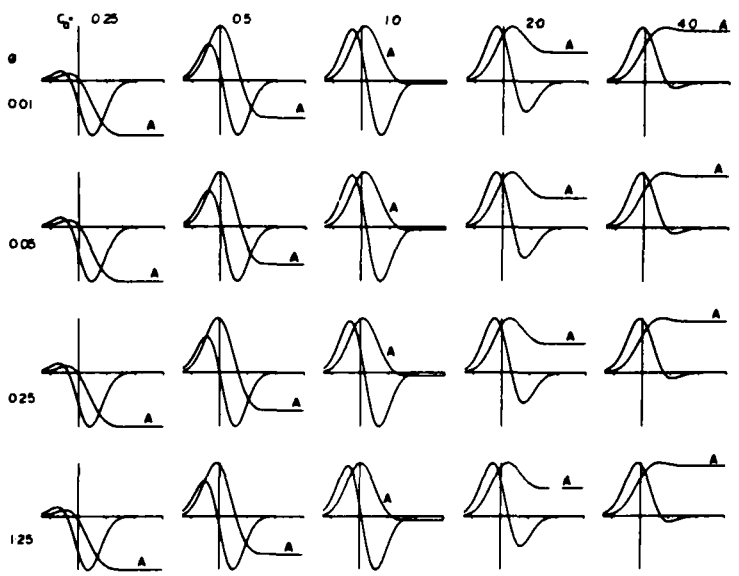


Figure 34. Temperature curves and integral temperature curves from the heat of adsorption detector (theoretical). A = Integral curves, C_a = detector-column, φ = detector heat loss factor.

For C_a<1 the integral curve first rises and then falls below the baseline and does not return. Only when C_a=1 does the detector signal simulate the differential form of the Gaussian curve, and its integral describe the true elution curve.

It follows that for the detector to be effective and useful, C_a must at all times be unity, and thus the detector must have the same plate capacity as the column for all solutes. This means that the detector must employ the same adsorbent, the same geometry, and be packed to have the same plate height as the column. A situation that is obviously very difficult to meet in practice.

Bearing in mind other limitations of the detector, such as its inability to cope with gradient elution and temperature program methods of development, and that the adsorbent has to be changed from time to time, the practical difficulties of construction make the heat of the adsorption detector one of the least attractive solute property detectors for LC. One possible area of application of this detector would be as a detector for the small bore LC column (65). Such columns, constructed of stainless-steel might have sufficient radial heat transfer if situated in a thermostating liquid, that a thermocouple placed at the radial center and close to the column exit might give a true Gaussian differential curve for an eluted solute. Used in conjunction with an integrating amplifier an effective heat of adsorption detecting system providing a true representation of the elution curve might be feasible.

The Spray Impact Detector

The production of electrical charges during the disruption of a liquid to produce an aerosol has been known for many years. As far back as 1892, Lenard (66) noted the ionization of air at the base of a waterfall where the water splashed against rocks. Christianson (67) investigated the electric charges produced when water was sprayed onto a solid plate and more recently Loeb (68) examined the effect in greater detail and termed the phenomenon "spray electrification". The charges are thought to be generated by the rapid break-up of a liquid surface either by a gas stream or by impact with a solid surface. Various mechanisms have been suggested to explain the production of electrical charge, but that generally accepted at this time is the one given by Mattison (69). Mattison suggests that any ions present in the liquid reside just below the surface and are not situated in the surface layer. If a new surface is formed by the sudden break-up of the original surface, the ions in the new surface are pulled inward by attraction from the bulk liquid, the force of which will depend on both the field associated with the ion and its mobility. The surface thus becomes rich in the slower, larger species of ion. For example, with water, the new surface would become richer in hydroxyl ions than hydrogen ions and thus would attain a net negative charge. In the author's opinion, there is still some uncertainty as to the exact mechanism of charge formation and for those readers interested in this subject they are recommended to refer to Mattison's original paper.

Mowery and Juvet (70) have employed the spray electrification effect in a novel form as a LC detector, primarily for use with reversed-phase liquid chromatography. A stream of the eluent from the column is allowed to strike a conducting target to form a spray and the potential of the electrode is monitored. A diagram of their detector is shown in Figure 35. The target electrode is an electrically isolated conducting rod which is connected to a suitable electrometer that permits both the measurement of electrode potential and electrode current.

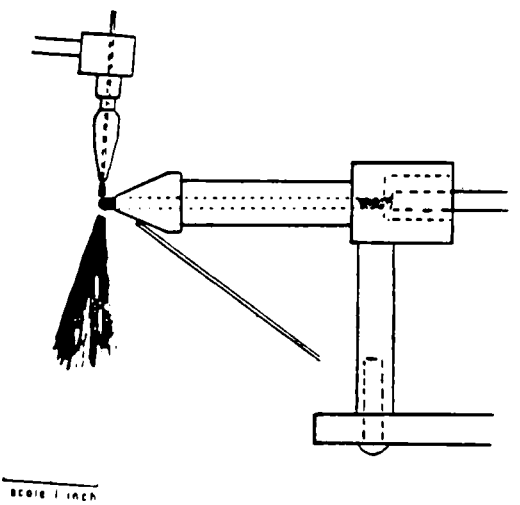


Figure 35. The spray impact detector.

Two types of electrodes were investigated, one composed of glassy carbon and the other of gold-plated platinum. A coaxial cable connected the electrode to the electrometer, via a soldered connection to the electrode which was supported on a high resistance laminated plastic mounting. On impact between the eluent and the target, the target becomes negatively charged and the resulting spray becomes positively charge. Small quantities of solute in the mobile phase changes this potential drastically and as an example, at a standing voltage of 2,000 V, 1.5 x 10^{-7} g of nitrophenol present in the eluent was found to produce a voltage change of 400 V. The detector system was situated in an earthed metal box through which filtered laboratory air was passed. This was necessary to reduce noise from air contaminants and reduce the effect of charged droplets that tended to accumulate in the neighborhood of the electrode. The performance characteristics of the system investigated by Mowery and Juvet (70) are shown in Tables 1-3. In Table 1, the detection limit and linear dynamic range of the detector for a series of organic compounds employing water as the mobile phase is given for the two different electrode types. Generally, it was found that the

TABLE 1

PERFORMANCE CHARACTERISTICS OF THE SPRAY IMPACT DETECTOR MOBILE PHASE, BOILED DISTILLED WATER

Compound	Detection Limit, g/sec	Linear Dynamic Range log (base 10)	Target Material
n-Octanoic acid	7×10^{-11}	4.0	gold
n-Nonanoic acid	8×10^{-11}	4.0	gold
n-Decanoic acid	5×10^{-11}	4.0	gold
Trifluoroacetylacetone[a]	3×10^{-10}	4.0	gold
Trifluoroacetylacetone[b]	2×10^{-9}	3.0	gold
Ammonium 8-anilino-1 naphthalene sulfonate	9×10^{-10}	3.5	gold
o-Nitrophenol	9×10^{-10}	3.5	gold
o-Nitrophenol	2×10^{-10}	3.5	carbon
Sodium dodecylsulfate	5×10^{-10}	3.5	gold
Sodium dodecylsulfate	2×10^{-10}	3.5	carbon
Sodium sulphonate	2×10^{-10}	-	carbon
Sodium tridecylsulfate	5×10^{-10}	3.3	carbon
Ethylamine	2×10^{-9}	2.5[c]	carbon
n-Octanol	8×10^{-8}	-	carbon
n-Nonanol	8×10^{-8}	-	carbon
Ethyl butyrate	1×10^{-7}	>2	carbon
2-Heptanone	2×10^{-7}	-	carbon

[a]Effluent flow rate, 3.1 ml/min.
[b]Effluent flow rate 8.6 ml/min.
[c]Includes positive and negative areas

TABLE 2

PERFORMANCE CHARACTERISTICS OF THE SPRAY IMPACT DETECTOR FOR SOME INORGANIC COMPOUNDS. MOBILE PHASE, BOILED DISTILLED WATER. GOLD TARGET ELECTRODE

Compound	Detection Limit g/sec	Linear Dynamic Range log (base 10)
Lithium nitrate	1×10^{-9}	2.7 + 1.7[a]
Potassium nitrate	9×10^{-10}	2.8 + 1.5[a]
Lanthanum nitrate	4×10^{-10}	3.0 + 1.5[a]
Thorium nitrate	5×10^{-10}	3.0 + 1.5[a]

[a]Two linear regions of different slope with discontinuity occuring at zero current.

TABLE 3

PERFORMANCE CHARACTERISTICS OF THE SPRAY IMPACT DETECTOR USING ORGANIC AND MIXED ORGANIC/WATER MOBILE PHASES. CARBON TARGET ELECTRODE

Compound	Detection Limit g/sec	Peak Direction	Mobile Phase
Glycine	5×10^{-11}	+	acetonitrile[a]
Stearic acid	7×10^{-10}	+	acetonitrile[a]
Sodium tridecylsulfonate	3×10^{-10}	+	12.5% acetonitrile/ water
Sodium tridecylsulfonate	8×10^{-11}	+	25% acetonitrile/ water
o-Nitrophenol	2×10^{-7}	+	acetonitrile[b]
o-Nitrophenol	1×10^{-10}	+	15% acetonitrile/water
Sodium cholate	3×10^{-11}	+	15% acetonitrile/water
p-Ethylphenol	1×10^{-7}	+	15% acetonitrile/water
n-Heptanol	3×10^{-7}	+	15% acetonitrile/water
Thorium nitrate	4×10^{-8}	-	1 mg/ml sodium dodecylsulfate/water
Water	3×10^{-6}	+	methyl ethyl ketone
Dimethylformamide	1×10^{-6}	+	methyl ethyl ketone
Ethyl acetate	4×10^{-5}	-	methyl ethyl ketone
n-Heptane	7×10^{-6}	-	methyl ethyl ketone
Toluene	3×10^{-5}	-	methyl ethyl ketone
n-Octylaldehyde	1×10^{-6}	-	methyl ethyl ketone
n-Octanol	2×10^{-8}	-	methyl ethyl ketone
o-Nitrophenol	2×10^{-7}	+	methyl ethyl ketone
Tetrachloroethylene	7×10^{-6}	-	methyl ethyl ketone
2-Heptanone	1×10^{-5}	-	methyl ethyl ketone

[a] Target first conditioned by wetting with water.
[b] No water conditioning of target electrode.

"glassy carbon" electrode gave a higher sensitivity and, furthermore, required no prior conditioning before use with aqueous mobile phases.

It is seen from Table 2 that very high sensitivities were attainable with a linear dynamic range for most substances of 3 to 4 orders of magnitude. In Table 2, the sensitivity and linear dynamic range of the detector is given for a number of inorganic compounds again employing water as the solvent. Very high sensitivities are again realized and it is interesting to note the detector has two linear response ranges for each solute.

In Table 3, the sensitivity is given for a number of substances employing a range of different mobile phases. it appears that the solvent employed can have a profound effect on

the detector sensitivity. Employing methyl ethyl ketone as an alternative to acetonitrile or an acetonitrile/water mixture as a solvent reduces the detector sensitivity by two or three orders of magnitude. In a similar manner the sensitivity varies significantly with the type of solute. For example, sensitivities of 10^{-10} g/sec are realized for o-nitrophenol contained in a 15% v/v solution of acetonitrile in water, but in the same solvent the sensitivity of the detector to p-ethylphenol is only 10^{-7} g/sec. A chromatogram obtained with this detector for a mixture n-C_8, n-C_9 and n-C_{10} fatty acids is shown in Figure 36. The high sensitivity of the detector to these type of substances is clearly indicated.

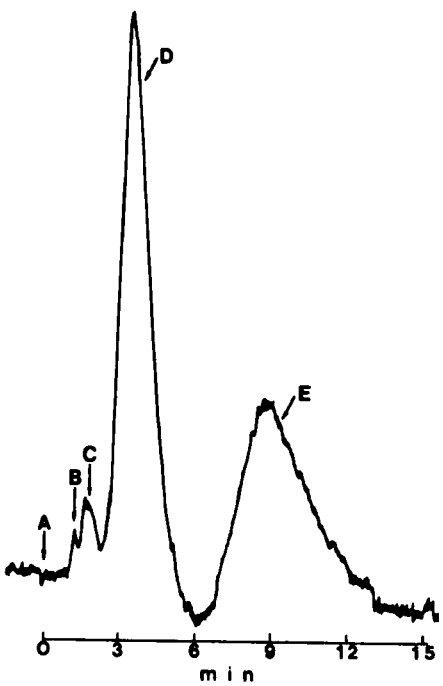


Figure 36. Chromatogram from the spray impact detector. Separation of the n-C_8, C_9, and C_{10} fatty acids. Column 60 x 0.6 cm μBondapak C_{18}/Corasil; target, gold; mobile phase, boiled distilled water at 5.9 ml/min air flow, 4550 ml/min. A, injection point; B, inorganic impurities; C, 7.2 ng octanoic acid; D, 120 ng nonanoic acid; E, 80 ng decanoic acid.

This detector has still not been developed extensively and no commercial model is available at present. Some further work on it will be necessary before it might become a routine instrument for LC purposes. However, it does appear to hold exciting prospects and future work may demonstrate its application to ion exchange chromatography. The extra column dispersion that is produced from

this type of detector is, at this time, uncertain but it would appear that it should be possible to reduce or maintain any dispersion to an acceptably low level.

The Electron Capture Detector

The electron capture detector only gives a significant response to electron capturing substances and, therefore, even when used as a gas chromatography (GC) detector it will only detect certain classes of compounds. A significant proportion of the solvents used in LC do not give a response with electron capture detection (ECD) and, therefore, such solvents can be volatilized along with any solute that is present, passed through the detector which will then respond to the solute only, providing it has electron capturing properties. This principle was developed by Nota and Palombari (71) to provide an effective LC detector. Such a detector, however, is very selective but nevertheless, as a number of important classes of compounds (e.g., pesticides, carcinogens, etc.) are electron capturing in nature, this detector could be very valuable aid in pollution studies and food analysis.

A diagram of the apparatus developed by Nota and Palombari is shown in Figure 37.

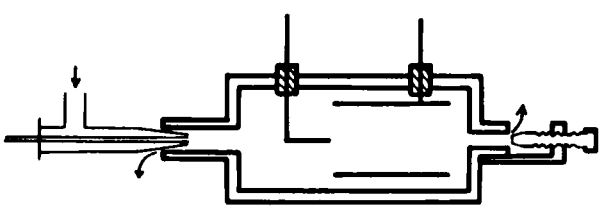


Figure 37. The electron capture detector designed by Nota and Palombari (71).

The eluent from the column passes directly into a nebulizer, the outlet tube from the column terminating at the nozzle of the nebulizer. A portion of the atomized eluent passes directly into an ECD and then out to waste. The ECD is operated under the same conditions as those that would be used if it were employed as a GC detector. The work of Nota and Palombari established the system as a viable LC detector and showed that the solvents benzene, hexane, cyclohexane, pyridine, methanol, ethanol, diethyl ether and acetone could all be employed as components of the mobile phase without significantly affecting the standing current of the detector. The disadvantage of this system was that by employing a

nebulizer, it was necessary to use relatively large volumes of nitrogen and further the interface between column and detector introduced significant band dispersion.

Willmott and Dolphin (72) developed an improved form of the detector and a diagram of their detecting system is shown in Figure 38. It is seen that the eluent is vaporized directly into the detector in an atomized form by means of a heated transfer tube situated in an oven.

On leaving the column, the eluent passes into a stainless-steel transfer tube (1000 mm x 1.59 mm O.D. x 0.25 mm I.D.), enclosed in the oven, the temperature of which (300°C) is such that the liquid is completely vaporized. The increase in volume involved in this transition, forces the vapor into a ^{63}Ni ECD, which is maintained at 300°C in the same oven as the transfer tube. A purge of 30 ml/min of nitrogen sweeps the vapor through the detector into a coil of stainless-steel tubing (2000 mm x 2.3 I.D.) from which it is collected as a liquid.

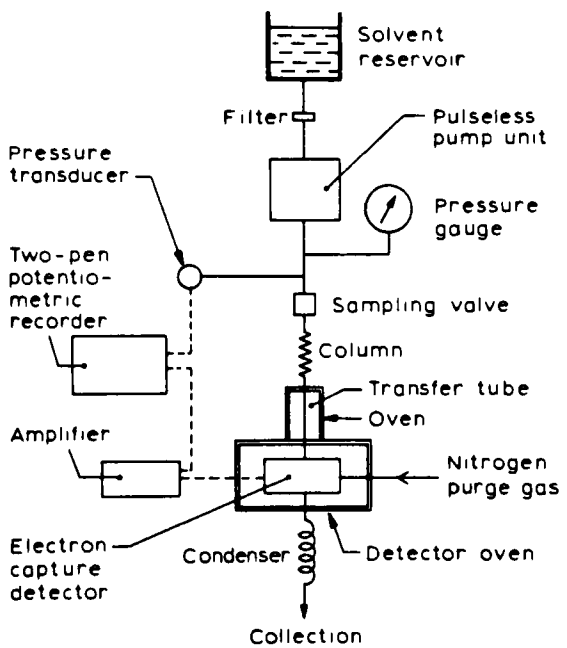


Figure 38. The Pye Unicam LC electron capture detector.

The ECD signal is amplified using either pulse mode amplifier or a constant current amplifier. The latter can operate with 60 V pulses having a nominal width of 1 μsec over a frequency range 0-130 kHZ. The standing current could be varied between zero and 5 x 10^{-9} A. These conditions are normal for its use as a GC detector.

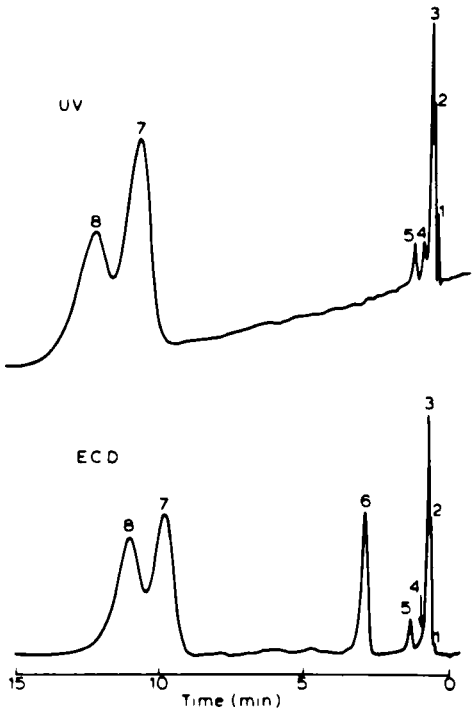


Figure 39. The comparative performance of the UV and ECD. 1 = heptachlor impurity, 2 = aldrin, 3 = heptachlor, 4 = heptachlor impurity, 5 = heptachlor impurity, 6 = lindane, 7 = endrin, 8 = dieldrin. UV Chromatogram: detector range 0.05 a.u., wavelength 230 nm. Sample composition: aldrin 1.4×10^{-7} g, heptachlor 2.9×10^{-7} g, lindane 4.0×10^{-7} g, endrin 13.5×10^{-7}, dieldrin 12.7×10^{-7} g. ECD Chromatogram: attenuation x512, standing current 0.73×10^{-9} A, purge 30 ml/min N_2. Sample composition: aldrin 1.1×10^{-8}, heptachlor 2.3×10^{-8} g, lindane 3.2×10^{-8} g, endrin 10.8×10^{-8} g, dieldrin 10.1×10^{-8} g.

The detector described by Willmott and Dolphin (72) is now manufactured commercially by Pye Unicam. The sensitivity to electron capturing substances it extremely high (1.2×10^{-10} g/ml) but the linear dynamic range is only about 100.

A chromatogram of pesticides from the ECD by Wilmott and Dolphin is shown in Figure 39. The chromatographs shown are obtained from the ECD and a UV detector placed in series with the column eluent. It is seen that 3.2×10^{-8} g of lindane is clearly detected by the ECD whereas the UV detector exhibits no response at all. This combination, again, demonstrates the advantages that can result from the use of two detectors to simultaneously monitor the column eluent. Using two or more detectors in this way, however, requires that the interconnecting tube does not contribute significantly to band dispersion. Further, the dynamic range of the detector is only about one order of magnitude greater

than its linear dynamic range. However, the high sensitivity of the detector, could make it an extremely valuable aid in the trace analysis of pesticides and other similar substances.

Synopsis

Solute property detectors measure some property that is exclusive to the solute only and not to the column eluent. Among them are the *most sensitive, most specific* and those having the *widest linear dynamic range.* In general, they require to be used with very pure mobile phases or solvents free from substances that possess the property being measured.

The most popular solute property detector is the *UV detector.* There are two types of UV detector, the *fixed wavelength detector* and the *variable wavelength detector.* The most common fixed wavelength detector operates at 254 nm, and has a linear dynamic range of over three orders of magnitude, a sensitivity of about 3 x 10^{-8} g/ml and a cell volume of 2 to 5 µl. *Unless carefully thermostatted it can be very sensitive to changes in flow rate.* Detectors operating at 212 nm and 224 nm are becoming increasingly useful for applications in the biotechnology field.

There are two forms of *variable wavelength detector* the *dispersive instrument* and the *diode array instrument.* In the former, polychromatic light is dispersed either by a prism or more commonly by a diffraction grating and light of a narrow range of wavelengths selected for absorption measurement. Thus the light used for detection is essentially monochromatic. The variable wavelength dispersive UV detector has a sensitivity of about 1 x 10^{-7} g/ml about an order of magnitude less than the fixed wavelength detector, a linear dynamic range of about three orders of magnitude or a little less and a cell volume of 2-5 µl. Within the limits of its resolution *it can provide an accurate spectrum of an eluted solute* by stopping the column flow and scanning.

In the *diode array detector* polychromatic light is passed through the cell and then dispersed by a prism or grating. The dispersed light then falls across a diode array and the absorption of the solute at a particular wavelength obtained by selecting the output from a specific diode. The sensitivity, linear dynamic range and cell volume is similar to that of the dispersion instrument. Spectra can be obtained by scanning the diodes "on the fly" without the need to stop the flow. However, due to the fact that the light through the cell is polychromatic the light sensed at a given wavelength may contain fluorescent light as well as that contained in the incident light and consequently, *the integrity of the spectra obtained must only be assumed with some caution.*

The fluorescence detector is one of the two *most sensitive* and *specific detectors available*, the other being the electrochemical detector. The detector can vary widely in complexity from one with a single excitation wavelength and a polychromatic sensor to the equivalent of a fluorescence spectrometer. The latter permits the selection of both the wavelength of the excitation light and the wavelength of the fluorescent light to be monitored. Such a complex instrument can also provide emission and fluorescence spectra if so desired. The sensitivity of the detector varies widely with the substance being detected and will depend on the quantum efficiency of the solute. Sensitivities of 1 x 10^{-9} g/ml are frequently achieved although the linear dynamic range can often be less than two orders of magnitude. There are *two disadvantages;* the *solvents* employed for the mobile phase *must be completely free of fluorescing substances* and *only those substances that fluoresce will be detected.* The latter problem, can be mitigated to some extent by the use of fluorescent derivatizing reagents.

The *transport system* for LC detection was developed to render the detector independent of the choice of mobile phase and allow any solvent to be used without compromise. The column eluent flows over the transporter, which may be a *moving wire, chain or disc* which takes up all, or a portion of the column eluent. The solvent is then evaporated from the transporter, usually by heating, and the solute is left as a coating on the surface. The transporter then carries the solute into a detection area, where it is sensed by suitable means, such as pyrolysis and subsequently detected by passing the pyrolysis products to a flame ionization detector. The *transport detectors, by and large, are not very sensitive*, 1 x 10^{-6} g/ml being about the best that can be achieved, although they do have potential for being significantly improved. They can have a *linear dynamic range of about three to four orders* and a response that is proportional to the carbon content of the solute being detected. They have found application in areas where the solutes do not have unique optical or electrical properties relative to those of the mobile phase.

The *electrochemical detector* competes in *sensitivity* and *specificity* with the fluorescence detector although generally for quite different types of solutes. The electrode system is relatively simple but the *materials from which the electrodes are fabricated need to be carefully chosen.* Three electrode systems are favored and the current produced by the electrochemical reactions that are made to take place at the working electrode constitutes the sensing signal. The electrochemical detector *suffers from two disadvantages*; the mobile phase must be *scrupulously free of metal ions* and the working electrode tend to become *contaminated* with use and needs to be *frequently cleaned.* The selectivity of the detector can be controlled, in part, by adjustment of the electrode voltage or by the use of specially configured electrodes. The sensitivity of the detector varies

considerably with the electrode geometry and operating conditions; a sensitivity of 1 x 10^{-9} g/ml is usually readily obtained. Its *linear dynamic range is about three orders of magnitude* and it is *significantly flow sensitive.*

The *atomic absorption spectrometer*, albeit an *expensive* detector can be used for *element specific detection.* It is usually employed with a conventional detector such as the UV detector. Due to the physical arrangement of the AAS and LC detectors, a fairly long interface is necessary between the two systems which must be *carefully designed to reduce extra column dispersion.* The combination is valuable for the *identification of metal speciation* such as different forms of chromium or lead. The chromatographic system separates the species of the element and the AAS selectivity identifies only those peaks that contain the element. Due to the inherent dispersion that must take place in the chromatographic column the *sensitivity is not particularly exciting* (about 5 x 10^{-8} at best) and the *linear dynamic range*, due to the mode of operation, may only be about *2 orders of magnitude* or perhaps a little more. Nevertheless, the combination is becoming increasing useful in environmental studies.

The increasing interest in the biotechnology field, in turn, has promoted a renewed interest in the *radioactivity detector.* Metabolic pathways in both animal and vegetable systems are often followed by means of radioactive markers. Consequently, the metabolites are separated by LC and those solutes that are radioactive identified by the radioactivity detector. At present the radioactivity detector *is a somewhat clumsy and expensive device* that even in its present state of development cannot be used to advantage with modern high-efficiency columns. *Scintillation methods of detection* are almost exclusively employed by either mixing the column eluent with a scintillation agent prior to entering the scintillation counter or by passing the eluent through a bed of insoluble scintillating material situated in the counter itself. The most recent radioactivity detectors have a *counting efficiency for carbon-14 of about 70%* and for *tritium about 2 - 15%.* This efficiency is comparable to liquid scintillation counting.

There are a number of detectors that have either *not found favour* for various reasons, *not become commerically available*, or both. An example of these is the *heat of adsorption detector.* The detector cell contains a small quantity of adsorbant in which is embedded a temperature sensor such as thermocouple or thermopile. As the solute is eluted from the column it is adsorbed onto the cell contents and the heat of adsorption is liberated and the temperature rises. When the solute is eluted out of the cell the solute is desorbed, the heat of adsorption absorbed and the temperature falls. This results in an *S shaped elution curve being traced by the detector which is extremely difficult to interpret* particularly if two peaks are only partially resolved.

The differential form of the elution curve accounts among other factors for its unpopularity; it *can, however, be very sensitive.* Another interesting, but unpopular detector is the *spray impact detector.* It functions on the charge built up on a metal plate when the column eluent is sprayed onto it from a jet and solute is present in the eluent. The response is *linear over several orders* of magnitude and is *extremely sensitive* to specific compounds. The *difficulties involved in disposing of the spray and vapor*, coupled with the need for *large volumes of air or nitrogen* to form the spray has inhibited the development of the detector. A similar type of detector, the *electron capture detector*, operates on a similar principal and has failed in popularity for the same reasons, although, not to the same extent, as this detector has been made commercially available. The eluent from the column is again vaporized, but by means of a thermal vaporizer and the gas and vapor passed directly through a GC electron capture detector. It is *highly specific, highly sensitive* but only has linear *dynamic range of about two orders* of magnitude. It has also not achieved popularity as a result of its *gas consumption and the solvent vapor disposal problem.*

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CHAPTER 5

SPECIAL DETECTOR TECHNIQUES

Multi-functional Detectors

Today liquid chromatography (LC) has been developed to a high level of sophistication and columns can be designed to give very fast analyses (1-3) or if required, extremely high resolution (2,4,5) and as we have already seen there is also a veritable plethora of different detectors available. However, there remains a serious need for increased versatility in detection devices and, further, some inexpensive means to confirm solute identification, particularly when there are forensic aspects to the LC analysis. The latter is often attempted by associating the liquid chromatograph with an appropriate spectroscopic technique such as mass spectrometry (MS) as in LC/MS. However, as will be discussed in a subsequent chapter, there still remains serious interfacing problems involved with the LC/MS combination and both interface and mass spectrometer become a very expensive detecting system to be associated with a relatively inexpensive chromatograph. A less sophisticated alternative is to monitor the LC eluent by means of UV absorption simultaneously at different wavelengths, employing a diode array detector as already discussed, or with a number of detectors, each operating on different principles. Such a combination would conveniently provide different responses from different solutes (6) and thus aid in confirmation of solute identity. However, in the past, this has necessitated the use of a number of different detectors connected either in series or parallel. This procedure, besides rendering the system very expensive, also either reduces sensitivity or causes serious solute band broadening which results from the multiplicity of dispersion processes that occur in each detector. Consequently, the disadvantages that arise from the use of separate detectors in series or parallel make the system unsatisfactory for multi-functional detection.

Some years ago, DuPont developed a bifunctional detector, Model No. 386 that monitored LC eluents simultaneously by UV and fluorescence detection. Although no longer available, the design of the UV/fluorescence detector (UV/F) will be described as it illustrates the basic principles of bifunctional detectors. A diagram of the basic DuPont bifunctional detection system is shown in Figure 1. Light from an appropriate UV source is collimated by mean of a quartz lens, through a cylindrical cell and the transmitted light focussed by means of another quartz lens onto a photo diode. The output is processed by suitable electronic

circuitry to provide a signal, derived from the absorption of UV light, that is proportional to the concentration of solute in the cell.

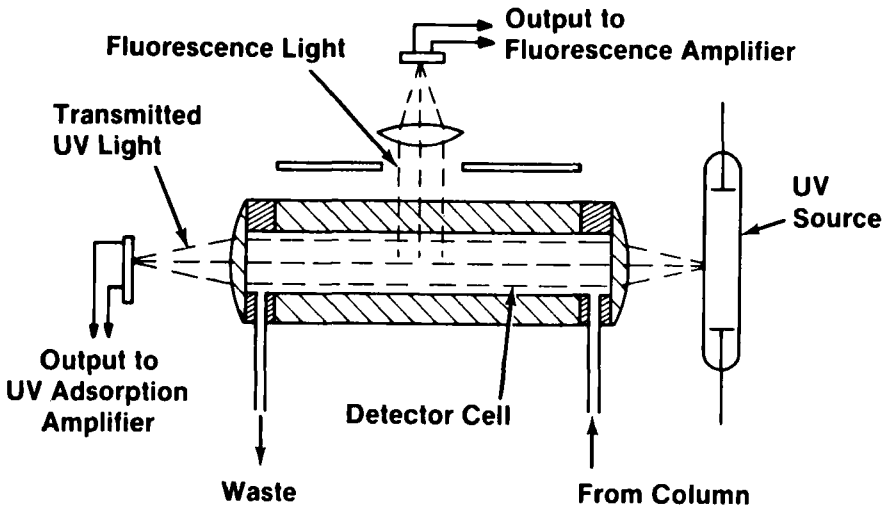


Figure 1. The DuPont bifunctional UV/fluorescence detector.

The body of the cell is made of an appropriate transparent material and fluorescent light, resulting from excitation by the incident UV light and emitted normal to it, is focussed by means of another lens onto a second photo diode. The output from the diode is processed by a second amplifier and provides a signal derived from the fluorescent light also linearly related to solute concentration. By means of a two pen recorder the chromatograms derived from both UV absorption and fluorescence can be simultaneously plotted.

The bifunctional system can thus confirm the presence of those substances that both absorb UV light and fluoresce, helping to confirm the identity of certain types of solutes. The DuPont instrument was the pioneer of multi-functional detection but, due to a number of problems that arose with its operation, in particular the rather serious dispersion that took place in the cell and eluent conduits and, perhaps, also because it was a little ahead of its time, it did not prove to be either a technical or commercial success.

More recently (1984), Baba and Housako (7) described another bifunctional detector but this time based on the UV absorption detector combined with the electrical conductivity detector. A diagram of their detector is shown in Figure 2. The UV absorption system is very similar to that of the DuPont bifunctional detector. UV light is collimated through the cell and focussed by a second quartz lens onto a photo diode, the output from which, is processed by suitable electronic circuitry in the usual manner.

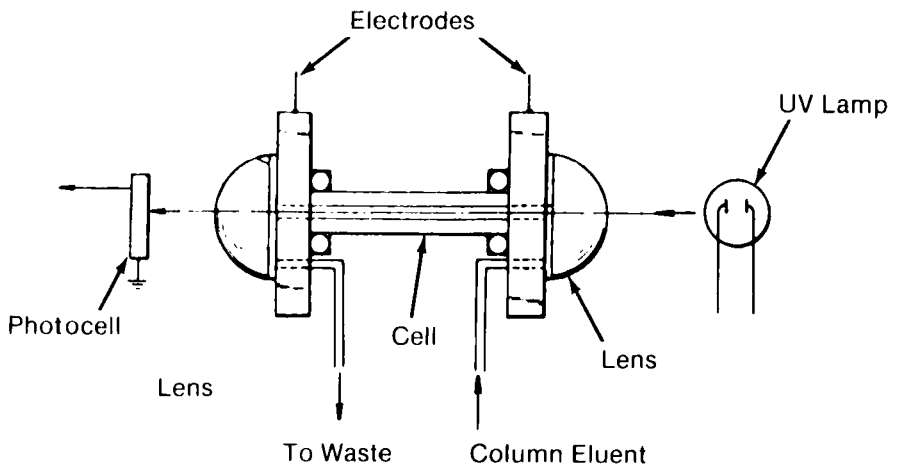


Figure 2. UV/conductivity bifunctional detector.

The ends of the cell, however, where the column eluent enters and leaves the cell, are made of stainless steel or other suitably inert metal and serve as the electrodes of an electrical conductivity system. An AC potential is applied across the electrodes that can form one of the arms of an AC bridge, such as the Wein bridge. As the impedance of the cell changes due to the presence of an ionic solute, the out of balance signal is processed to provide an output linearly related to solute concentration. Consequently, the bifunctional detector can provide simultaneously chromatograms that represent both the change in UV absorption and the change in electrical conductivity of the cell due to the presence of eluted solutes. Thus ionic and UV absorbing substances are selectively monitored. This device is a fairly recent innovation and the technical and commercial success of it remains to be established.

Another bifunctional detector was recently developed by Knauer and has been made commercially available in Europe. This bifunctional detector operates on the basis of UV absorption and changes in refractive index. As such, it is particularly interesting as it combines a solute property detector with a bulk property detector. Consequently, the combination will cover a very wide field of applications. A diagram of the instrument is shown is Figure 3.

There are two light sources. One a low pressure mercury vapor lamp from which light passes through a filter, through the cuvette and onto a photo electric cell. This photo cell provides the UV signal which is processed by suitable electronics to provide an output linearity related to solute concentration. Light from the filament lamp passes through a half silvered mirror which reflects the light back through the cuvette onto a concave mirror. The reflected light from the concave mirror passes back through

the cell, through the 1/2 silvered mirror, through a slab of glass which is adjustable for zero output, and onto a prism. Light is reflected from the prism onto two photo cells and as the refractive index of the liquid in the cuvette changes, the beam is deflected away from the apex of the prism increasing the light on one photocell and decreasing it on the other. The differential output is also processed by to an appropriate electronic system to provide a signal proportional to changes in refractive index. Specifications for the detector were not very explicit.

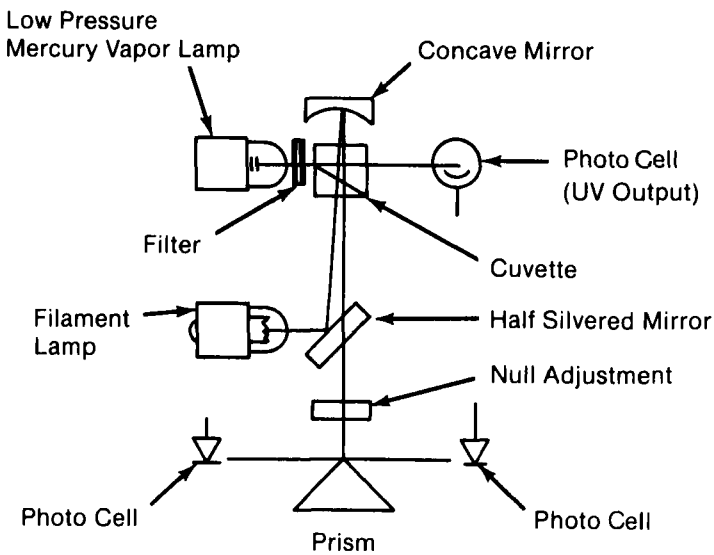


Figure 3. UV/refractive index bifunctional detector.

It would appear that the sensitivity of the refractive index function was about 10^{-6} refractive index units and that of the UV absorption function about 0.001 absorption units. The cell volume was 12 µl which is rather large to be used for modern high-performance liquid chromatographic (HPLC) columns. Nevertheless, the detector system is an interesting combination due to the fact that, together, the combined performance of the UV and refractive index detectors approach that of the universal detector.

In 1985 Schmidt and Scott (8, 9) introduced the trifunctional detector, which was in fact, a combination of the bifunctional detectors of DuPont, and Baba and Housako (7) but with a re-designed cell and eluent conduits to minimize cell dispersion. A diagram of their detector, now manufactured by Perkin-Elmer Corporation, is shown in Figure 4.

The UV absorption system is again very similar to the bifunctional detectors and consisted of a low pressure mercury discharge lamp and a solid state photocell fitted with a quartz

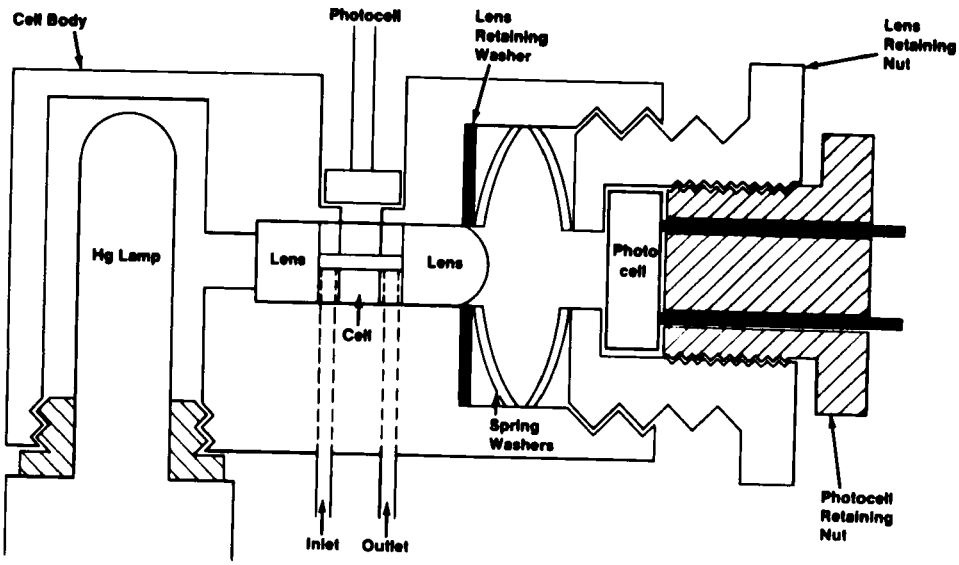


Figure 4. Diagram of the multi-functional detector.

window to permit light transmission at 254 nm. The cell was 3 mm long and terminated at the light source end by a cylindrical quartz window and at the other end by a plano-convex quartz lens that dispersed the light passing through the cell over an extended area of the photo sensor. Situated next to the lens and window were stainless-steel discs having a central hole 0.8 mm I.D. Two stainless-steel tubes, the inlet 0.010 in. I.D. and 0.020 in. O.D., and the outlet 0.020 in. I.D. and 0.030 in. O.D., were connected at right angles through the walls of the stainless-steel discs to the central aperture. The two discs were separated by a 1.5 mm length of quartz tubing 0.8 mm I.D. and the same O.D. as the stainless-steel discs. The quartz window, lens, stainless discs, and the central quartz cell were all sealed by means of Kaptan gaskets 0.005 in. thick. The cell was maintained leak proof by a retaining washer and four spring washers as shown in Figure 4. A second solid state photo cell was situated at right angles to the quartz spacer to receive fluorescent light emitted normal to the incident light. A filter, opaque to the incident light at 254 nm, (not shown in the diagram), was situated between the quartz spacer and the second photo cell to eliminate the effect of scattered incident light. The stainless-steel inlet tube was connected directly to the reducing union of the column by a low dispersion serpentine (10), connecting tube. This length of connecting tubing provided considerable versatility with respect to column detector connection and permitted the detector to be situated a significant distance from the column and injection system. Furthermore, the use of serpentine tubing for the conduit

allowed this versatility without contributing significant extra column dispersion. The inlet tube to the cell was electrically connected directly to the column and consequently was at earth potential, but the outlet tube was insulated by means of a short length of teflon tubing from the exit tube. The outlet was thus electrically isolated and the two stainless-steel discs would, therefore, constitute the electrodes of an electrical conductivity cell.

The output of the axial photo sensor was fed to the UV amplifier, the output of the photo sensor normal to the cell axis was connected to the fluorescence amplifier and the two stainless-steel discs were connected to the conductivity measuring electronic system. A block diagram of the electronic system is shown in Figure 5.

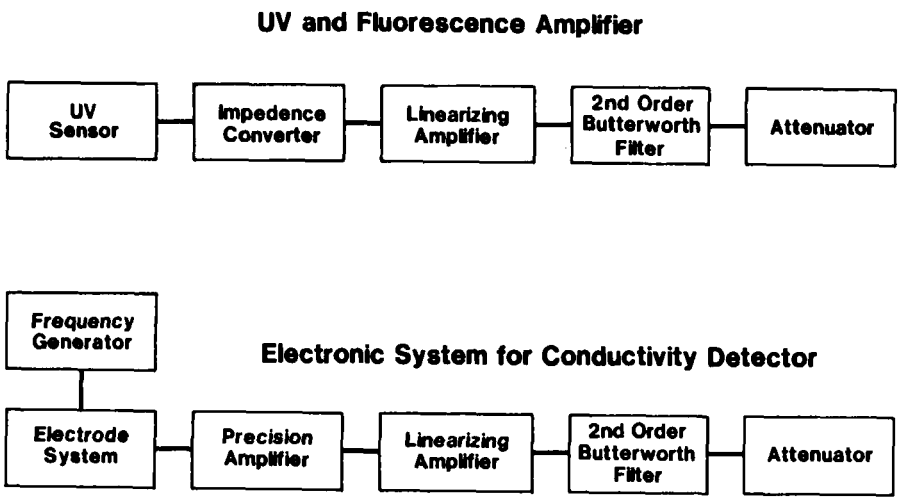


Figure 5. Schematic diagram of the UV and fluorescence amplifier; and the electronic system for the conductivity detector.

The UV and the fluorescence amplifier were very similar and consisted of an impedance converter as the first stage which was balanced to provide zero output by appropriate zero controls. The next stage consisted of an appropriate amplifier to render the electrical output linearly related to solute concentration which was followed by an electronic filter to eliminate high frequency noise. The final stage was a simple six-step binary attenuator with a maximum attenuation of 32 to provide a suitable output to a recorder. The only difference between the UV and fluorescent amplifier was in the linearizing amplifier which had to have an appropriate response for the respective function that it modifies. The electronic system for the conductivity detector comprised of a 1 kHz frequency generator the output of which was fed to the detector electrodes and then passed to a precision rectifier to provide a DC signal that was related to the change in conductivity of the cell. The DC output was then modified by another

appropriate amplifier to provide an output that was linearly related to ion concentration. Finally, the output from the linearizing amplifier was fed to another filter and attenuator for direct connection to a 10 mV recorder.

The sensitivity of the UV absorption function was 1.7×10^{-7} g/ml of toluene with a linear dynamic range of about 1.5×10^{3}. These specifications compare well with those of the standard fixed wavelength UV detector. The fluorescence function provided a sensitivity of 2.5×10^{-8} g/ml for dansyl iso-leucine and a linear dynamic range of 1.2×10^{3}. Finally, the sensitivity of the conductivity function to sodium chloride was shown to be 5×10^{-8} g/ml with a linear dynamic range of 3×10^{3}. The response indices were 0.975, 0.95, 1.042 for the UV function, the fluorescence function and the conductivity function respectively. The dispersion of the cell was small, equivalent to a standard deviation of about 2.8 µl.

An example of the use of the multi-functional detector is given in Figure 6.

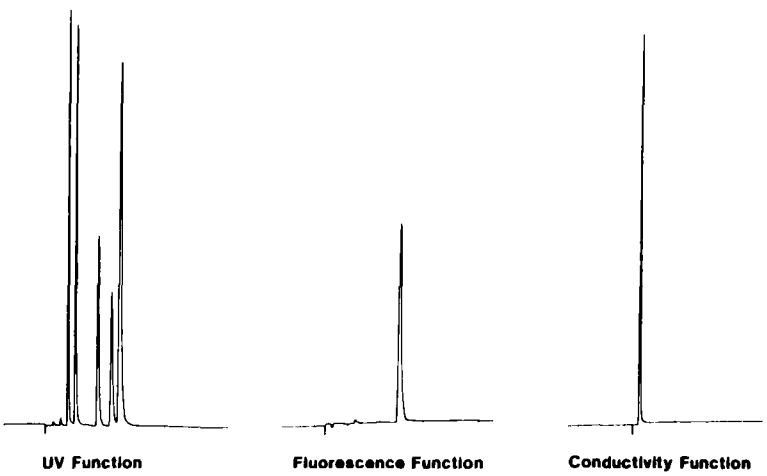


Figure 6. Chromatograms showing the use of all three detection systems. Solvent: 65% acetonitrile/water; flow rate: 2 ml/min; column: 3 cm long 4.6 mm I.D.; packing: 3 µm C_{18} reverse-phase.

The left-hand chromatogram is for a synthetic mixture that contains anthracene, a number of other aromatic hydrocarbons and sodium chloride. It is seen that a complete separation is obtained, but, of course, there will be no indication of sodium chloride as it does not absorb in the UV. The center chromatogram shows a single peak from the fluorescence detector illustrating the position of anthracene and the absence of other fluorescing materials. The third chromatogram from the electrical conductivity function clearly shows a sodium chloride peak which

was not apparent in either of the previous chromatograms. On the other hand, it does not show the anthracene or aromatic hydrocarbons as they do not conduct.

Such a system would automatically and quickly give information as to whether a sample had been exposed to seawater, ionic materials or even possibly blood serum. Multifunctional detection is relatively new and holds exciting possibilities for future use. It is likely that we can look forward further innovative developments of this concept. It has also been demonstrated that multi-functional detection can be achieved without significant band dispersion and without compromising sensitivity or linear dynamic range. Another advantage that has yet to be fully realized, is that multi-functional detection also provides the availability of a number of single functions in a single instrument that are always available at choice.

Chemical Derivatization As A Sensitivity Enhancement Technique

Chemical derivatization has become a very popular technique for increasing the sensitivity of a specific type of detector to compounds for which it normally exhibits little or no response. An examples of this procedure would be the reaction of an aliphatic alcohol, which contains no UV chromophore, with benzoyl chloride to form the benzyl ester which could then be detected by the UV detector. Derivatization can also be used to permit the use of an alternative type of detector to increase sensitivity. For example an amino acid may exhibit only weak absorption in the UV, but when reacted with a suitable fluorescing reagent, could be detected by means of the fluorescence detector at concentration levels one or two orders of magnitude lower than with the UV detector. It is clear that derivatization procedures can increase significantly the versatility of many detectors.

Most derivatization procedures are carried out in order to obtain a quantitative analysis of the sample. It follows that the derivatization reactions must be highly repeatable and must proceed to completion. The method must also be carefully chosen to suit the general nature of the sample; for example the use of benzoyl chloride to form the benzoic ester of methanol in aqueous solution would obviously be completely inappropriate. Furthermore, in this particular example, the methanol could not easily be quantitatively removed from its aqueous environment for subsequent reaction.

Chemical derivatization can be carried out in two ways. The sample can be derivatized and then injected onto the column which has been termed *precolumn derivatization*. Conversely, the column eluent can be mixed with the derivatizing reagent prior to the detector, the reaction allowed to complete by the use of a suitable reactor and the products then passed through the

detector. The latter procedure has been termed *post column derivatization*. These two procedures will be discussed separately.

Precolumn Derivatization

An excellent and detailed discussion of pre-column and post column derivatization has been given by Lawrence (11) and includes a comprehensive list of available reagents, procedures and application examples. One of the more popular procedures is fluorescence derivatization and probably the most popular fluorescent reagent is dansyl chloride (12). 5-Dimethylamino-naphthalene-1-sulphonyl chloride (dansyl chloride, DNS-chloride or DNS-Cl) reacts with phenols and primary and secondary amines under slightly basic conditions to form a fluorescent sulphonate ester or sulphonamide. The quantum efficiency of dansyl derivatives is high whereas the reagent itself does not fluoresce. Unfortunately, the hydrolysis product, dansylic acid is strongly fluorescent and can cause interferences with water-soluble derivatives. These derivatives, however, are often removed by the subsequent chromatographic process. The detection limits of the dansyl derivatives are often in the low nanogram range (ca. 1 x 10^{-9} g/ml) and the excitation and emission maxima can vary between 350-370 nm excitation and 490-540 nm emission. Some examples of the application of this reagent are in the analysis of amino acids (13), alkaloids (14), barbiturates (15) and pesticides (16).

NBD-Chloride (4-chloro-7-nitrobenz-2,1,3-oxadiazole) reacts with aliphatic primary and secondary amines to form highly fluorescent derivatives. Aromatic amines, phenols and thiols yield weakly or non-fluorescent derivatives, consequently the reagent is specific for aliphatic amines. The reaction is carried out under basic conditions and the products are extractable from aqueous mixtures by solvents such as benzene or ethyl acetate. The fluorescence can be significantly reduced by the presence of water and so the solutions should be dry and the reagent can obviously not be used to form derivatives for reversed-phase chromatography. Detection limits are again in the fraction of a nanogram range (2-5 x 19^{-10} g/ml). The advantages of this reagent over dansyl chloride is that both the reagent an its hydrolysis products are non-fluorescent. The excitation and emission wavelengths are also higher (480 nm excitation and 530 nm emission). NBD-chloride derivatives have been used for the analysis of amino acids (17), amphetamines (18), alkaloids (19) and nitrosamines (20).

Fluorescamine (4-phenylspiro(furan-2-(3H),1'-phthalan)3,3'-dione) is also a commonly used fluorescence reagent. It reacts almost instantly and selectively with primary amines while the excess of the reagent is hydrolyzed to a non-fluorescent product. The reagent itself is also non-fluorescent. The reaction is carried out in aqueous acetone at a pH of about 8-9 and the derivatives can be chromatographed directly. The excitation and

emission wavelengths are 390 nm and 475 nm respectively. Two disadvantages of this reagent are its cost and the fact that the products are less stable, can not be stored and should be injected on the column immediately after formation. Fluorescamine has been employed in the analysis of polyamines (21), catecholamines (22) and amino acids (23).

A less costly alternative to fluorescamine is o-phthaldehyde (OPT) the derivatives of which are more stable and consequently can be stored overnight if necessary. It is used in a similar manner to fluorescamine the detection limits being about 0.1 nanogram (ca. 4 x 10^{-10} g/ml). OPT has been used in the analysis of dopamine (24), catecholamines (25) and histamines (26). Other fluorescence reagents sometimes used are 4-bromomethyl-7-methoxycoumarin, diphenylindenonesulfonyl chloride, dansyl-hydrazine and a number of fluorescent isocyanates for details of which the reader is recommended to read the review by Lawrence (11).

Another group of popular derivatizing reagents are those that enhance the UV and visable absorption of a solute. Such reagents permit solutes with no inherent UV or visable chromophore to be detected by the fixed wavelength UV detector or the multi-wavelength detector. Two of the common reagents of this type are the phenyl and methylisothiocyanates. These reagents react with amino acids to form thiodantoins. The mechanism involves the initial formation of a substituted thiourea which then suffers ring closure to from the thiohydantoin. The derivatives are stable under acid conditions and can therefore be used for amino acid sequencing (27, 28) in peptide hydrolysates. The phenyl derivatives are about two orders of magnitude more sensitive than the methyl analogues and are detected at 260 nm down to levels of about 10^{-9} g/ml.

Another popular reagent in this class is benzoyl chloride which has been used extensively in the detection of alcohols and phenols. Similar reagents, p-nitrobenzoyl chloride, p-methoxy-benzoyl chloride and 3,5-dinitrobenzoyl chloride, due to their ring substitution, give significantly greater UV absorption and consequently provide even higher sensitivities. These reagents have been employed in the analysis of hydroxysteroids (29), digitalis glycosides (30), carbohydrates (31) and amphetamines (32) among many other applications. 2,4-Dinitrofluorobenzene (DNFB) or Sangers Reagent has also been used for similar applications such as the analysis of amino acids and amines (32). It reacts with both primary and secondary amines and phenols but not with aliphatic alcohols consequently it is useful for determining phenols in the presence of aliphatic hydroxy compounds. The sensitivity realized from this reagent is similar to that obtained from the dinitrobenzoyl derivatives.

There have been a number of reagents employed for the esterification of carboxylic acids. 2-Naphthacyl bromide has been used for the esterification of fatty acids and barbiturates (33), the absorption provided by the naphthalene ring giving sensitivity levels of detection of about 4 x 10^{-9} g/ml. The benzyl esters of fatty acids have been prepared with the use of 1-benzyl-3-p-tolytriazine (34) and the p-methoxyanilides of fatty acids prepared by means of triphenylphosphine (35). The triphenylphosphine is used firstly to convert the fatty acid to its acid chloride form which, in turn, is reacted with p-methoxyaniline to form the p-methoxyanilide. These derivatives have an absorption maxima at about 254 nm and provide a sensitivity of about 5 x 10^{-9} g/ml for the original fatty acid. Probably the most common acetylating agent is acetic anhydride which has been satisfactorily used for the formation of the acetamide derivatives of biogenic amines (36). However, these derivatives are also more easily separated chromatographically and the purpose of derivatization was more to aid in the separation than increase the detectability of the solutes. 2,4-Dinitrophenylhydrazine appears to be the reagent of choice for ketones and aldehydes and has been used in the analysis of ketosteroids (37,38) and ketoacids (39,40). Detection limits for these derivatives are about 2 x 10^{-9} g/ml if monitored at either 254 nm or 336 nm.

Derivatization procedures have been used to enhance the sensitivity of different solutes to a variety of other detecting systems. Reagents have been suggested for rendering solutes amenable to electrochemical detection, radioactivity detection and even atomic absorption spectrometers when used as detectors. However, further discussion on precolumn derivatization techniques is outside the scope of this book and those interested are recommended to read the book by Frei and Lawrence (41) that deals exclusively with derivatization procedures.

Post Column Derivatization

Post column derivatization does not merely require the selection of the most appropriate reagent to react with the solute to render it detectable, but also involves the modification of the chromatographic system to allow reaction to take place. This necessitates the insertion of a post column reactor between the column and the detector.

The reactor is required to:

1. Provide a source of reagent and a means of mixing it efficiently with the column eluent.

2. Ensure reaction is complete before the derivatization product enters the detector.

3. Minimize the dispersion that takes place in the reactor so that the integrity of the separation that takes place in the column is maintained.

It follows that the post column reactor has to be very carefully designed and a diagram showing the principle of a post column reactor system is shown in Figure 7.

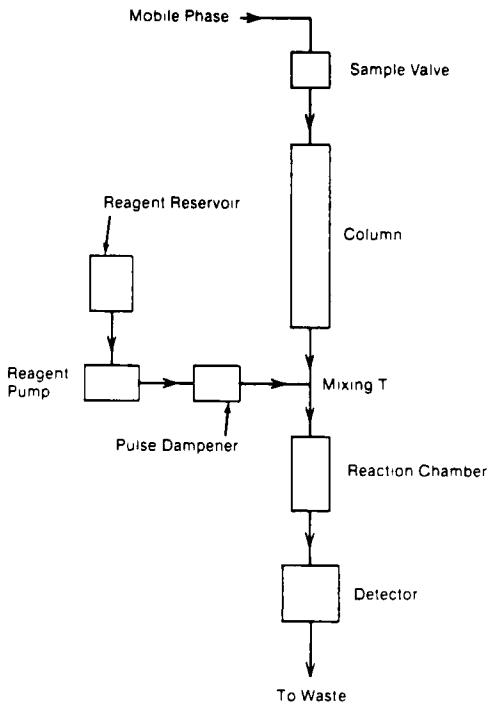


Figure 7. Chromatographic system incorporating a post column reactor.

It consists basically of three parts, a reagent reservoir and pump, mixing T and a reaction chamber.

The pump should provide a pulse-free flow of reagent as there is little resistance down stream to the detector and many detectors are more or less flow sensitive. Thus either a syringe type reagent pump should be used or if a reciprocating pump is employed then it should be fitted with a very efficient pulse dampener. The former is probably preferable but syringe pumps can be clumsy to refill.

The design of the mixing T is critical and different kinds of mixing systems have been the subject of study by Scholten et al. (42). The mixing T has to ensure that the column eluent is intimately mixed so that there are no local portions of column eluent, however small, that do not contain a sufficient quantity of reagent to allow complete derivatization. Furthermore the T

has to be designed so that the mixing process does not disperse the solute bands after they have left the column. An example of a small volume mixing T is furnished by the design of Schmidt and Scott (43), a diagram of which is shown in Figure 8.

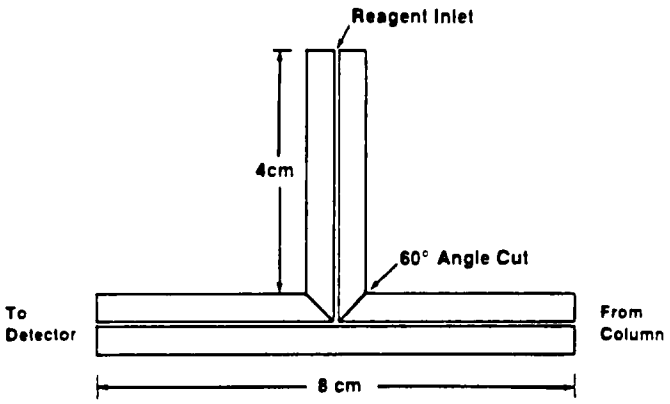


Figure 8. Mixing Tee

Their mixer consisted of two pieces of 1/16 in. O.D. 0.007 in. I.D. tube, 8 and 4 cm long. The 8-cm length of tube, that was used to connect the column to the detector, was radially bored in the center with a 60° angle conical cut that just intersected the central conduit. The end of the 4-cm length of 0.007 in. I.D. tube, which was used for reagent delivery, was formed with a 60° cone permitting the two pieces of tube to be interlocked at right angles. The two tubes were housed in a drilled out Swagelok T. As seen in Figure 8 this method of fabrication ensured virtually no dead volume associated with the actual mixer. A chromatogram illustrating the performance of the mixer is shown in Figure 9. Chromatogram A shows a peak that results from an injection of 1 μg of copper. The mobile phase consisted of an aqueous solution of 45 mM of sodium tartrate and 10 mM of sodium hexane sulphonate adjusted to a pH of 3.1 with phosphoric acid. The reagent that was passed through the side arm of the T was an aqueous solution of 4-(2-pyridylazo)resorcinol, ammonium acetate and free ammonia. The reagent produces an intensely colored complex, with an absorption maximum at 500 nm, for a number of different metals including copper. Although fairly good mixing is achieved by the mixing T, there is residual noise clearly seen on the base line of chromatogram A. The remaining noise was virtually eliminated by the insertion of a 5-cm length of serpentine tube (10) between the mixer and the detector as shown by the baseline in chromatogram B. The low dispersion serpentine tube introduces violent radial mixing and thus eliminates any heterogenuity in the liquid entering the detector.

In the post column derivatization used by Schmidt and Scott (43) the reaction was virtually instantaneous and consequently, as

long as the mixing was complete, the reactants could pass directly into the detector. Unfortunately, this somewhat ideal situation does not always occur and in many cases provision has to be made to allow slower reactions to proceed to completion before the reaction mixture is permitted to enter the detector. This requires a volume of suitable geometric form to be interposed between the mixer and the detector. This will ensure that the solvent/reagent mixture takes a finite time to pass from the mixer to the detector, during which the reaction can proceed to completion. However, this volume interposed between mixer and detector, must not contribute significantly to the dispersion of the eluted peak or the separation achieved by the column would be impaired. Thus the "delay volume" or reaction volume must be carefully designed.

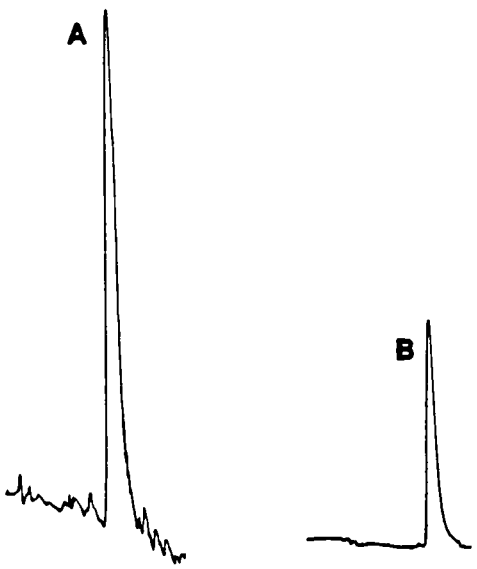


Figure 9. Improved mixing performance using a low dispersion serpentine connecting tube. A = 1 μg of Cu without serpentine tube; B = 0.5 μg of Cu with a 5-cm length of serpentine tube.

There are a number of geometric forms that "delay volumes" can take and the design of post column reaction systems have been discussed by Scholten et al. (42), Frei and Scholten (44) and Stewart (45). There are four common forms, simple tubes, long narrow tubes, long serpentine tubes and packed tubes. Simple tubes are usually employed with segmented flow and this procedure has been discussed in some detail by Gfeller et al. (46). This technique reduces dispersion by segmenting the flow between mixer and detector with bubbles of gas. The bubbles have to be removed before the flow enters the detector which can be inconvenient and itself cause some dispersion. Nevertheless, the technique has been employed successfully in post column derivatization and is a common procedure in many automatic analyzers. The long narrow

bore tube is frequently employed, particularly if the reaction mixture has to be heated to ensure complete reaction, as heat exchange can be quite rapid and easily accomplished. An example of post column derivatization, where it is necessary to heat the mixture to ensure complete reaction is in the use of the reagent ninhydrin for the detection of amino acids. The serpentine tube should provide even better heat exchange and less dispersion. However, due to its relatively recent introduction, its use has, as yet, not been reported other than by Schmidt and Scott (43). The packed tube is also fairly popular as it can provide a significant volume in a relatively short length of tube and still maintain limited peak dispersion. The dispersion is restrained due to the rapid and continuous change in the direction of flow velocity that occurs between particles. This breaks up the parabolic velocity profile of the interparticulate flow which is responsible for peak dispersion. The packed bed is not as effective as the serpentine tube as it contains a dispersion contribution (the multipath term) that the serpentine tube does not possess. However, packed beds are frequently employed in post column reactors.

The reagents used in post column derivatization will be specific for the particular solutes to be detected but will often be the same or similar to those used in precolumn derivatization. Reagents that produce derivatives that absorb visible light are popular as simple, relatively inexpensive detecting systems can be employed (43). In general, derivatization, particularly post column derivatization, is only used as a last resort to provide sensitivity or selectivity which is difficult or impossible to obtain by other means. Post column reactors complicate an already complex instrument, render it more difficult to operate, make it more expensive and unfortunately, however well designed, tends to denegrate the separation that has been achieved (often with difficulty) by the column.

The Differential Detector

Commercial detectors are designed to give a linear output that is directly related to the concentration of solute passing through them. The curve described by the recorder is thus a true representation of the Gaussian profile of the eluted peak as discussed in the first chapter. In the vast majority of LC separations this type of output is necessary but there are a limited number of applications where an alternative form is desirable. All detectors that employ reference cells for comparison purposes can be connected to the column system in such a way as to provide alternative functional outputs. By appropriate but simple modifications, detectors can be made to provide differential and integral outputs or display only changes in sample composition relative to that of a standard. It should

be emphasized that these techniques will only be useful in certain limited areas of application and will only be resorted to when the standard LC techniques are inadequate or unsuccessful.

The shape of the differential form of the Gaussian function has already been described. It is sigmoid in shape with a positive maxima at the first point of inflexion of the Gaussian curved and a minimum at the second point of inflexion. If the peaks are completely resolved on the chromatogram then they can be clearly and easily identified in their differential form. if the peaks are not completely resolved, however, then the differential curve of the unresolved peaks are confused and extremely difficult to interpret. For this reason the differential form of the Guassian function is very rarely used. If the elution profile of the solutes from the column are not Gaussian in form, however, then the differential form can be extremely valuable.

The concentration of a solute X_V eluted after v volumes of mobile phase have passed through the column will be some function of v, (f(v)).

Thus $$X_v = f(v)$$

and $$\frac{dX}{dv} = \frac{d(f(v))}{dv} = f'(v)$$

For finite values of dv, i.e. Δv, where Δv is small

then $$\frac{\Delta X_v}{\Delta v} = f'(v)$$

and $$\frac{X_{v+\Delta v} - X_v}{\Delta v} = f'(v)$$

or $$X_{v+\Delta v} - X_v = \Delta v f'(v) \qquad (1)$$

Further if Δv is constant then $X_{V+\Delta V} - X_V$ will be directly proportional to f'(v) the differential function of the eluted peak.

The value of $X_{V+\Delta V} - X_V$ can easily be measured in practice when using the UV detector or refractive index detector providing the detectors have reference cells. The column is connected to the detector in the manner shown in Figure 10. This procedure was first employed in gas chromatography (GC) by Boeke (47) and was introduced to LC by Essigmann and Catsimpoolas (48). The column eluent passes directly to the sample cell and thence to the reference cell by way of a short length of tubing, the volume of which is the volume Δv.

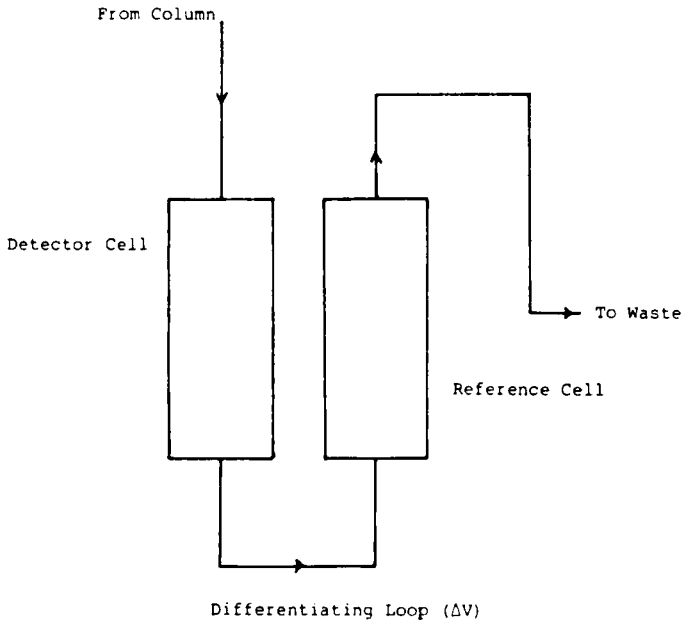


Figure 10. Detector cells connected to provide a differential output.

As the signal from the reference cell acts in opposition to the signal from the sample cell, the output from the detector is $X_{V-\Delta V} - X_V$ as given in equation (1) and will always provide the differential of the function that describes the concentration of solute in the mobile phase.

An application of the differential mode of the UV detector is shown in the chromatogram at the top of Figure 11. The chromatogram shows the separation of a mixture of different tetracyclines and it is seen that there is very serious peak tailing. This tailing results from the poor desorption characteristics of the solute from the stationary phase, silica gel. The quantitative results from such a chromatogram would give an extremely poor precision particularly if peak area measurements were employed. Seeking an alternative phase system that would affect a satisfactory separation could be a very time consuming operation. However, it should be noted that the front of each peak is relatively sharp and if the tails of the peaks could be made equally sharp then the separation could produce satisfactory analytical results. In the center chromatogram shown in Figure 11 the mixture is separated by frontal analysis (49). This is achieved by employing a sample valve with a large volume loop (16 ml). The use of large volume sample loops for preparative chromatography and frontal analysis has been discussed by Scott and Kucera (50) and the resulting chromatogram shows, firstly a series of rising steps as each solute breaks through the column and finally a series of descending steps as each solute is eluted from the column. It should be noted that the rising steps have

sharp fronts like the fronts of the peaks in the upper chromatogram, whereas the descending steps are broad and diffuse similar to the tails of the peaks eluted normally.

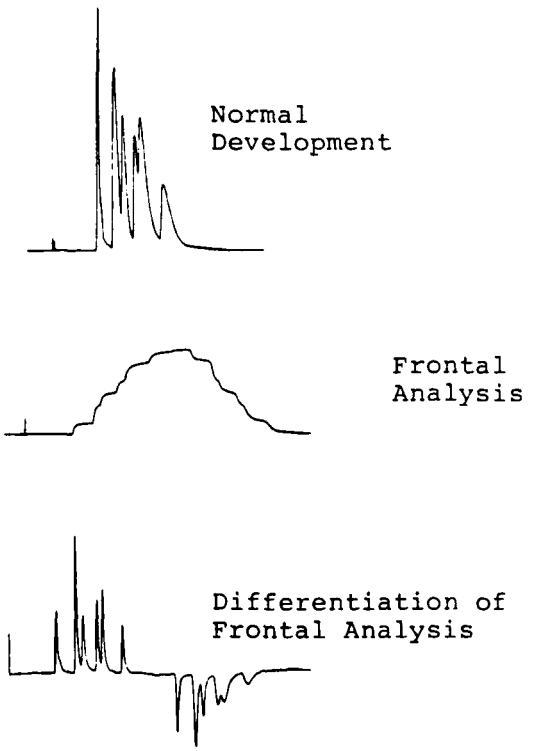


Figure 11. The separation of a mixture of tetracyclines.

In the lower chromatogram the effect of differentiating the ascending steps of the chromatogram obtained by frontal analysis is shown. The same column and phase system was employed as that used to obtain the upper chromatogram but the sample cell and reference cell were connected by a piece of stainless-steel tubing 3 cm long 0.03 in. I.D. having a volume of 19 μl (Δv). The total sample volume employed was 16 ml. It is seen from the lower chromatogram a greatly improved separation is obtained that is quite satisfactory for quantitative analysis. It should be pointed out, however, that only the differential curves from the ascending steps of the frontal analysis curves should be used. The chromatogram obtained from differentiating the elution steps will be even worse than those from the original elution chromatogram. The peak heights of the differential chromatogram are linearly related to sample mass as shown by the calibration curves given in Figure 12 and thus can be used for quantitative analysis.

In Figure 12 the peaks labeled one to six respectively refer to those substances shown separated in Figure 11. Another example of the advantages of the differential mode of detector operation is shown in Figure 13.

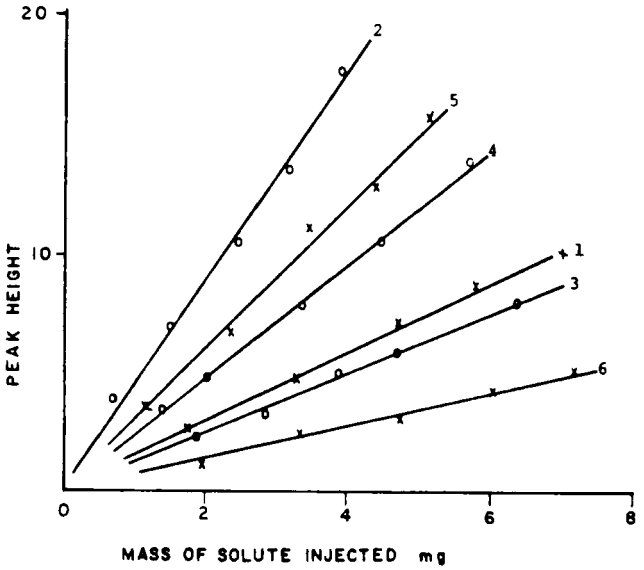
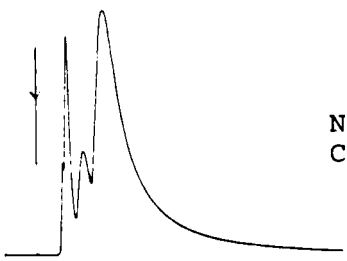


Figure 12. Calibration curves relating peak height to mass of sample from the differentiation of frontal analysis curves.



Normal Elution Chromatogram

Differential Chromatogram from Frontal Analysis

Figure 13. Chromatograms demonstrating the use of the differential display in the separation of a fermentation extract.

In the upper chromatogram of Figure 13 is shown the separation of an extract from a fermentation broth. It is seen that a very poor separation indeed, is obtained. In the lower chromatogram of Figure 13 is shown the result of differentiating a frontal analysis of the same mixture. A good separation is obtained and again the chromatogram is quite suitable for quantitative analysis.

The use of the differential mode of detector operation can be extremely useful in cases where the normal chromatographic development gives very poor separation resulting from peak tailing. However, the technique does require significantly more sample for frontal analysis than with normal elution development so that sufficient sample must be available. Furthermore, the response of the detector operation in its differential mode is two orders of magnitude less sensitive than when operated normally and thus high detector sensitivities have to be employed.

Integral Detection

Any detector can be made to give an integral response if desired and for this purpose no special use of a reference cell is necessary. In practice, the need for a detector having an integral response is very rare as electronic or digital integrators can carry out the same function more efficiently and with greater precision. However, for those who, for some reason or another, cannot utilize the more conventional methods of integration the alternative use of the detector as an integrator will be described.

If n solutes are completely eluted from the column in volume v

$$\text{then } \int X_1 dv + \int X_2 dv + \ldots \int X_n dv = M_1 + M_2 \ldots M_n$$

where X_1, $X_2 .. X_n$ are the concentrations of the solutes $1,2..n$ after a passage of mobile phase of volume v and $M_1, M_2 ... M_n$ are the masses of individual solutes in the mixture. Thus on the elution of solutes $1,2..n$ the resulting integral curve will be in the form of a series of steps, the height of the step for each solute being proportional to the mass present. It should be pointed out that analysis by normalization of each solute step can only be carried out if the detector response for each solute is the same. Alternatively the product of the step height and the response factor for each solute can be normalized to provide the required quantitative analysis.

The system used to provide an integral of the output from a detector is shown in Figure 14. Mobile phase from a reservoir is pumped continuously through the sample cell of the detector and

back again to the reservoir. The column eluent is allowed to flow directly into the reservoir and ideally the volume of mobile phase in the reservoir should be maintained constant.

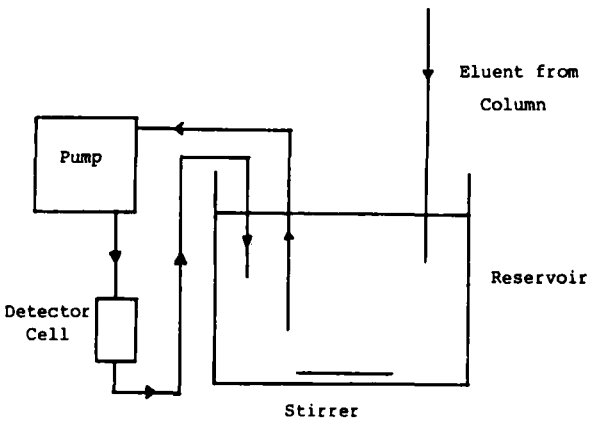


Figure 14. Experimental system to provide chromatographic integration.

The volume of mobile phase in the reservoir should therefore be at least fifty times the volume of mobile phase through the column to completely elute all the solutes. If all the solutes are eluted in 10 ml of mobile phase then the volume of mobile phase in the reservoir should be at least 500 ml. The reservoir should be continuously stirred by suitable magnetic stirrer and the volume of mobile phase circulated through the detector and back to the reservoir should be maintained at about 1% of the total volume of mobile phase/min. In the above example a flow rate of 5 ml/min would be appropriate. An example of an integral chromatogram obtained by the above procedure is shown in Figure 15. It is seen that a typical integral chromatogram is obtained that is quite suitable for quantitative analysis. As the total mass of each solute eluted from the column is, in effect, diluted by the 500 ml of mobile phase in the reservoir, high detector sensitivities, again, need to be employed as in differential detection. Furthermore, it is advisable to use the maximum charge that can be placed on the column that will not impair the separation or the column performance. The reservoir can also act as a post column reactor. The appropriate reagent dissolved in the mobile phase in the reservoir will react immediately with any solute eluted from the column to provide a derivative that can be detected by the particular detector being employed. Using the integral method of detection in this way can only be successful if the derivative of the solute is not labile. Many fluorescing reagents used in the detection of amino acids and peptides provide labile fluorescent products and so have to be used with some caution. In some instances the reaction conditions can be adjusted to give derivatives having reasonably permanent fluorescence.

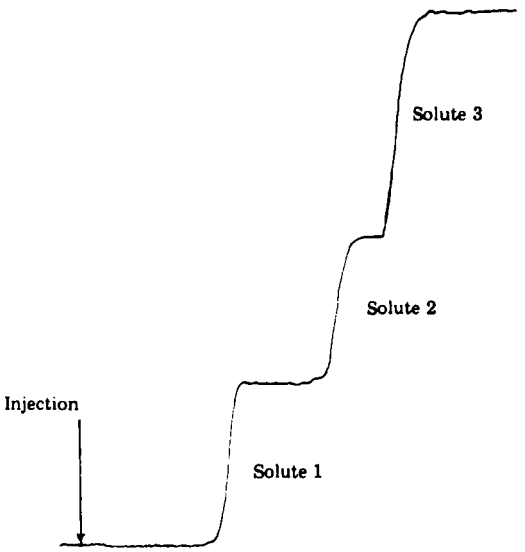


Figure 15. Elution curve obtained by integral detection.

Vacancy Chromatography

The term "vacancy chromatography" was introduced by Zhukhovitski and Turkel'taub (51) and is used to describe the chromatographic technique in which the mobile phase consists of a solvent containing a mixture of components which are maintained at constant concentration. Upon injection of a "sample", the resulting chromatogram may show negative and/or positive peaks depending on whether the components detected in the sample are present at a lesser or greater concentration than in the mobile phase.

If the mobile phase (carrying a constant concentration of solute X_o) is fed continuously onto a chromatographic column and equilibrium is allowed to become established, the eluent from the column will also contain the solute at a concentration X_o. If a sample of the same solute dissolved in the mobile phase at a concentration X_1 is now injected onto the column where $X_1 \neq X_o$ then this will produce a perturbation on the concentration X_o and from the plate theory, the equation for this perturbation when sensed by the detector at the end of the column will be

$$X_N = (X_1 - X_o) \frac{e^{-W^2/2N}}{\sqrt{2\pi N}} \qquad (2)$$

where X_N is the concentration of solute in the mobile phase in the Nth plate (i.e. that concentration sensed by the detector)

v is the volume flow of mobile phase measured in plate volumes,
N is the number of theoretical plates and W=v-N.

It is obvious from equation (2) that if $X_1 > X_0$, a positive peak will be produced and if $X_1 < X_0$, a negative peak will be produced.

In the vast majority of chromatographic separations the sample is injected onto the column containing mobile phase only and thus the eluted peak is always positive representing an increase in solute concentrations in the detector. However, the chromatographic process will operate in exactly the same way if the mobile phase contains a given concentration of the solute that is contained by the sample but the concentration of solute in the sample is less than that in the mobile phase. In this case, on injection, a negative perturbation of solute in the mobile phase will be eluted through the column resulting in a negative peak being produced by the detector at the same retention time as a positive peak for the same solute would have been eluted. Such a system can be extremely useful for monitoring the composition of complex mixtures in process control. The apparatus used is shown in Figure 16.

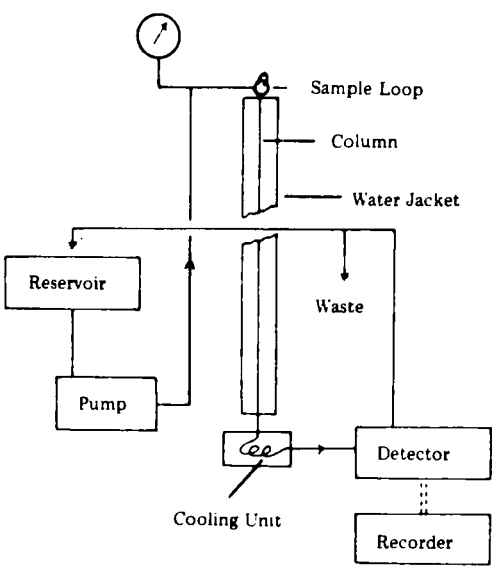


Figure 16. Diagram of apparatus for vacancy chromatography.

A sample of the mixture of substances is chosen that has the composition that is required to be maintained in the process and dissolved in the mobile phase to a known concentration that is appropriate for the detector employed. The mobile phase is then pumped continuously through the sample valve, column, detector and back to the reservoir. To monitor the process streams a sample is taken, diluted in the mobile phase to the same concentration as

the standard mixture in the mobile phase and injected onto the column. If the sample has the correct composition no peaks will be recorded by the detector. However, any substance present in excess of that in the standard mixture will show as a positive peak and any substance present below that in the standard mixture will be shown as a negative peak. The area of the positive or negative peak will be proportional to the excess or deficiency of the respective substance relative to the the standard. Scott et al. (52) examined the technique of vacancy chromatography in the separation of nucleic acid bases and an example of the use of the technique is shown in Figure 17.

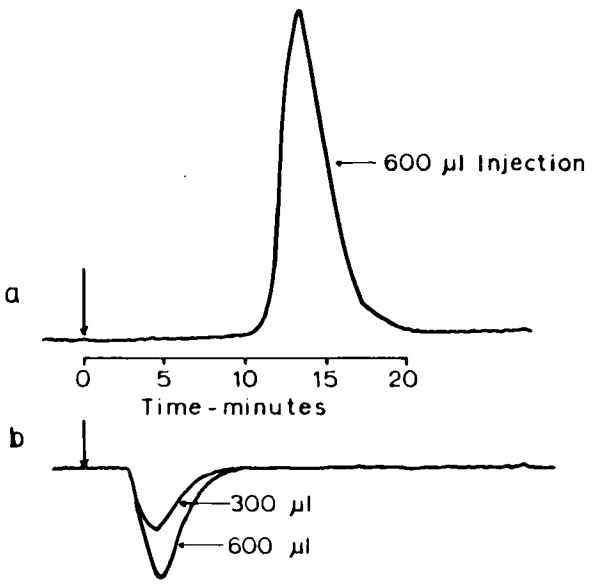


Figure 17. Chromatograms obtained by vacancy development. a = Positive peak obtained from 600 µl injection of uracil and hypoxanthine. b = Negative peaks obtained from 300- and 600-µl injections of uracil only.

Figure 17a shows the positive peak obtained for a 600-µl injection of sample containing concentrations of uracil and hypoxanthine at the same levels as in the mobile phase but with the concentration of cytosine increased from 4 µg/ml to 4.4 µg/ml. There is no perturbation of the base line for the first three compounds because the mobile phase/stationary phase equilibrium is not disturbed by the injection. There is, however, a positive peak corresponding to the difference in concentration of 0.4 µg/ml of cytosine. Figure 17b shows negative peaks obtained from 300-and 600-µl injections of sample equivalent to the mobile phase except for the omission of hypoxanthine. In this case a negative peak corresponding to the difference in concentration of hypoxanthine (0.5 µg/ml) is obtained. Under these conditions, differences in concentration of hypoxanthine can be determined without any interference from uracil from which, under normal

elution conditions, it is not completely resolved.

Vacancy chromatography can be an extremely effective technique for identifying and determining changes in the composition of a complex mixture including the appearance of unknown impurities. This form of chromatography has, to date, not been exploited to any significant extent but it remains a very useful procedure for product or process control and is likely to become more popular as LC increases its field of application.

Synopsis

Multi-functional detectors monitor the column eluent by the measurement of more than one physical or chemical property simultaneously, employing a single sensing cell. To date, *three bifunctional detectors and one trifunctional detector* have been described. The three bifunctional detectors have combined *UV absorption and fluorescent detection, UV absorption and electrical conductivity detection* and *UV absorption and refractive index detection.* The latter uniquely combines *a bulk property detector* with a *solute property detector* producing, at least in theory, the nearest approach to a *universal detector.* The trifunctional detector incorporates UV absorption, electrical conductivity and fluorescence functions. *Multi-functional detection* provides detector *versatility* and a means of *confirming solute identity.* Such detectors have to be designed, so that the performance specifications are not seriously compromised, and the cell and eluent conduits do not contribute significantly to peak dispersion.

Chemical derivatization is used largely as *a sensitivity enhancing technique* but can also be used to improve solute selectivity. In the former case a solute, to which the detector does not respond, is reacted with suitable reagent to provide a derivative to which the detector is sensitive. An example of this would be the use of a *fluorescence reagent to render a solute, such as an amine, sensitive* to fluorescence detection. There are a large number of reagents available that can introduce fluorophores, UV and visible chromophores, electrochemical sensitive groups etc. into a wide range of different solute types. Chemical derivatization can be carried out in two ways. The derivative can be prepared before chromatographic separation *-precolumn derivatization-* or the reagent can be added to the column eluent and derivatization allowed to take place just prior to detection *-post column derivatization.* Post column derivatization requires the design of *special apparatus* that must provide a *pulse free supply of reagent,* a device to ensure *complete mixing* and an appropriate delay volume to provide sufficient time for the *reaction to proceed to completion.*

Furthermore, the post column reaction system must not contribute significantly to peak dispersion. Chemical derivatization *is often an essential procedure in order to obtain adequate detector sensitivity*, however, because it adds to the complexity of the analysis and can also reduce the resolving power of the column, it should *only be resorted to when absolutely necessary.*

Many detectors fitted with reference cells can be used to provide *differential chromatograms.* This is achieved by passing the eluent in series through the two cells. If frontal analysis is employed, the differential detector will provide a normal chromatogram. *Differential detection* can be *used to advantage where*, due to adsorption effects on the column, *normal development results in unresolved, tailing peaks.* This technique is not commonly known and consequently not frequently used. The *integral detector* functions by allowing the eluent to flow into a vessel containing a significant volume of mobile phase and circulating the contents continuously through the detector during the development of the chromatogram. So far the integral detector *has proved to be of academic interest only. Vacancy chromatography* is a technique that has been little used but *could be very useful in quality control.* The mobile phase is made up to contain the solutes of interest at the required relative composition and passed continuously through the column and the detector. The sample, containing the same solutes, is diluted to the same overall composition of the mobile phase and injected onto the column. Those *solutes* in the sample that are present *at lower concentration than those in the mobile phase* will give *negative peaks* and those *present in excess* give *positive peak.* The size of the peak will be proportional to the difference between the content of the sample and that of the standard in the mobile phase.

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CHAPTER 6

SPECTROSCOPY IN CONJUNCTION WITH LIQUID CHROMATOGRAPHY TO IDENTIFY SOLUTE STRUCTURE

Modern liquid chromatography (LC) can separate very complex mixtures, but having achieved the required separation, there remains the problem of elucidating the structure of the eluted components. In the past, the solutes eluted from the column were collected as fractions, concentrated and then examined by suitable spectroscopic methods. An alternative procedure is to pass the column eluent directly into a spectrometer and obtain the required spectroscopic data concurrent with the separation process. There are two ways to do this and the method used depends on the speed at which the spectroscopic data can be acquired and, secondly, on the type of spectroscopic data that is to be obtained. If the spectroscopic data can be obtained rapidly, as in the case of the rapid scanning mass spectrometer (LC/MS) or the Fourier Transform infrared spectrometer (LC/FTIR), the column eluent can be monitored continuously by passing the eluted solute, via an appropriate interface device, directly into the spectrometer. If the scanning rate of the spectrometer is relatively slow, and some form of sample flow cell is employed, as in the case of some nuclear magnetic resonance (NMR) and IR spectrometers, then the interrupted elution procedure can be used. The flow of mobile phase is stopped when the peak maximum has reached the absorption cell of the spectrometer, the scan is initiated, and when the spectrum has been obtained, the flow of mobile phase is started again. Little or no loss in resolution is sustained by this procedure, as the only band dispersion that occurs, during the scan time results from longitudinal diffusion, which in liquids is extremely small. If the sample, however, has relatively few components or, spectra are only required for one or two solutes then the stop-flow procedure may well not be worthwhile. Under these circumstances an on-line chromatograph/spectrometer combination might be an unnecessary luxury and spectra could be obtained more economically, and just as rapidly, by trapping the sample and taking the spectrum in the normal manner.

The five most common spectroscopic techniques employed for the elucidation of molecular structure are UV spectroscopy, IR spectroscopy, Raman spectroscopy, MS, and NMR, the latter probably being the most informative. However, before discussing the different chromatography/spectroscopy systems, the basic difference between the *spectrometer-chromatograph combination* and the *spectroscopic detector* should be discussed. The *spectroscopic detector* is designed and constructed *specifically as an LC detector.* It will contain a flow-through cell of appropriately small volume to suit modern LC columns, and a means of obtaining

an output that is proportional to the absorbance of the cell contents that can be fed to a chart recorder. On the other hand, in a chromatograph/spectrometer combination, the spectrometer is normally a standard instrument that can be employed simply as a spectrometer if required. However, with the use of an appropriate interface, the spectrometer can be directly associated with a liquid chromatograph, but in doing so, the spectrometer remains virtually unchanged.

The diode array detector is usually considered a special type of spectroscopic detector detector, fitted to a liquid chromatograph. Conversely, in a chromatograph spectrometer combination, the chromatograph is often viewed as a unique sampling system associated with the spectrometer.

The Combination of the Liquid Chromatograph with the Nuclear Magnetic Resonance Spectrometer

NMR is the only spectroscopic technique that can, under many circumstances, and without the aid of supplementary spectroscopic information, unambiguously identify the structure of an unknown compound. It follows that the association of the liquid chromatograph with the NMR spectrometer would be a very powerful analytical system for the separation and identification of unknown mixtures. There are, however, some serious difficulties involved with the association of these two techniques. The four main problems that must be solved, were outlined by Bayer et al. (1) who, in 1979, were one of the first research groups to successfully combine the liquid chromatograph on-line with the NMR spectrometer. Firstly, the intensity of an NMR signal is dependent on the flow rate of the solvent and, as the flow rate increases, the signal decreases. However, according to Bayer, at flow rates between 0.5 ml and 2 ml/min, this decrease in signal can be restricted to a reasonable level by the correct design of the flow cell. Secondly, in order to realize high NMR resolution, the magnetic field must be extremely homogeneous and, to achieve this, the sample tube is usually spun at fairly high speeds. This, so far, has been impossible to achieve in flow-through NMR cells and, consequently, some spectral resolution is lost on the association of the spectrometer with the chromatograph. Thirdly, the solvent itself, if it contains protons, can interfere with the spectra of the solute. Under some circumstances, solvents may be chosen that do not contain protons, e.g. carbon tetrachloride or deuterated solvents. Restriction of the solvent choice to non-proton containing compounds impairs the chromatographic efficiency, while the use of deuterated solvents can be very expensive. The use of small bore columns (2) would significantly reduce solvent consumption and render the use of deuterated solvents more economically viable, but such columns would demand very small cell volumes and minimal extra column dispersion. Interface connecting tubes would need to be serpentine in form (3) and the cell volume significantly reduced below those presently

employed. Finally, the sensitivity of the NMR spectrometer is not nearly as great as that of the UV spectrometer or the mass spectrometer, and consequently, much larger charges have to be placed on the column. Nevertheless, practical LC/NMR systems have been designed, albeit not yet with optimum performance. A diagram of the cell designed by Bayer et al. (1) is shown in Figure 1.

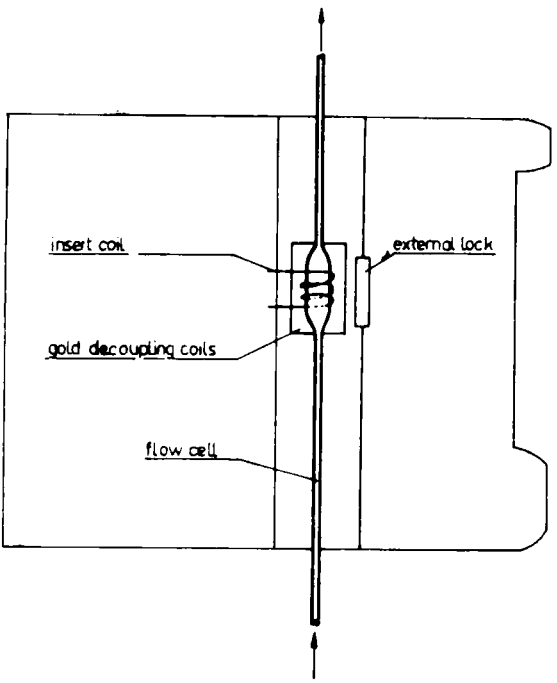

Figure 1. Schematic diagram of ^{1}H-NMR flow-probehead.

The volume of the cell was kept as small as possible to minimize band dispersion, but at the same time, the geometry of the flow cell had to provide optimum synchronization with the NMR sensing coil. Consequently, the walls of the cell had to be straight and parallel to the axis of the coil. the volume of the cell was about 420 µl (very large for high-performance liquid chromatographic (HPLC) columns) and thus, at a flow rate of 1 ml/min allowed a residence time of about 25 sec. To achieve adequate sensitivity, Fourier Transform techniques were employed. The layout of the system used by Bayer et al (1). is shown in Figure 2 and is fairly typical of all LC/NMR combinations.

In 1980, Haw et al. (4) developed the LC/NMR system further, employing a superconducting magnet and a novel probe cell. The work was undertaken to improve the analysis of petroleum-based and synthetic fuels. A diagram of their flow cell is shown in Figure 3A. The receiver coil is wound on a 5-mm NMR tube, which is

connected at the base to a 1.5-mm I.D. tube, through which enters the eluent from the column. The spectrometer was operated with an external lock.

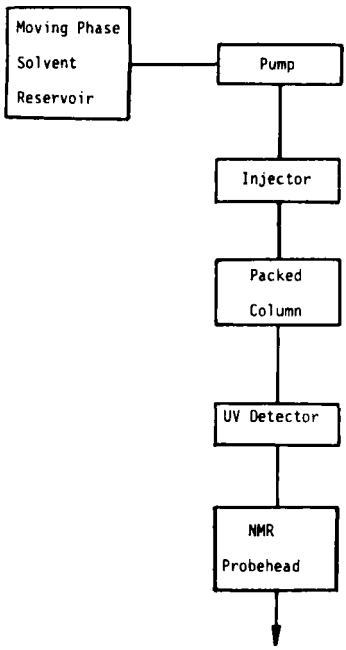

Figure 2. Experimental arrangement for on-line HPLC/NMR coupling.

The NMR resolution was about 5 Hz, which was about the same as that achieved by Bayer et al. (1). The data system that was used required 400 msec for each spectral file, with a data transfer time of 110 msec and thus, if spectrum integrals were used to provide chromatograms, then the data points taken for each peak would be at a rate of approximately 2 per sec. A chromatogram obtained from the system of Haw et al. (4) presented as proton spectrum integrals against file number is shown in Figure 4. It should be noted that there are only 5 to 10 points per peak for the early peaks, so that peak definition would be poor. However, the refractive index detector that was placed in series with the NMR instrument, would provide an additional elution curve which would have high integrity. Unfortunately, the cell volume, although not stated, was obviously rather large for use with modern high-efficiency columns. Furthermore, the system was insensitive, requiring a sample volume of 500 µl and consequently semi-preparative chromatographic equipment and large elution volumes.

These weaknesses were recognized by the authors and in 1981 Haw et al. (5) described an improved flow cell, a diagram of which is shown in Figure 3B. Numerous horizontally designed flow through cells were examined but it was found that a vertical

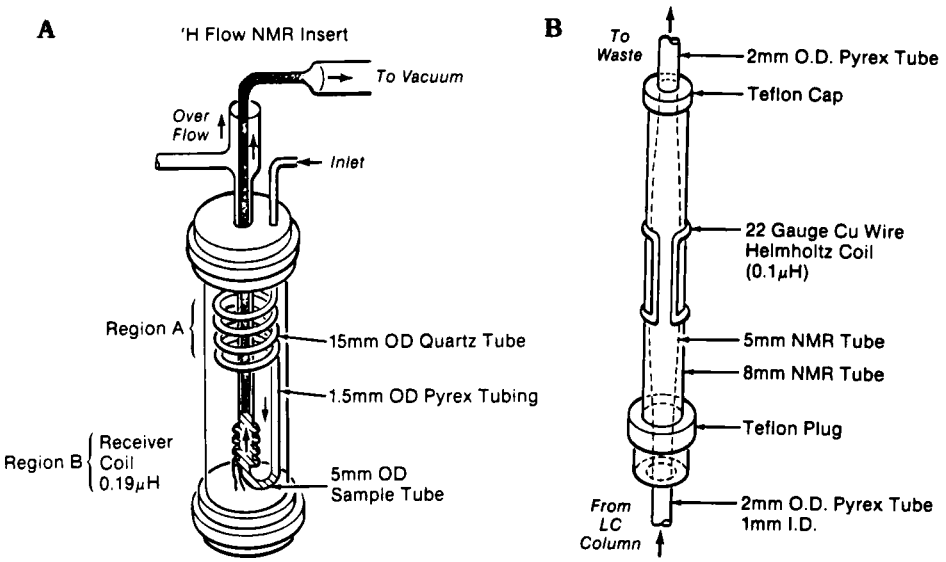

Figure 3. (**A**) Diagram of LC/^{1}H NMR insert. The sample is polarized in region A (within magnetic field) and free induction decays are monitored in region B. (**B**) LC/^{1}H NMR flow insert for 200 MHz operation.

configuration gave the better NMR line widths (more narrow NMR peaks and thus higher NMR resolution). Equally important, the vertically aligned tube did not trap air bubbles in the cell.

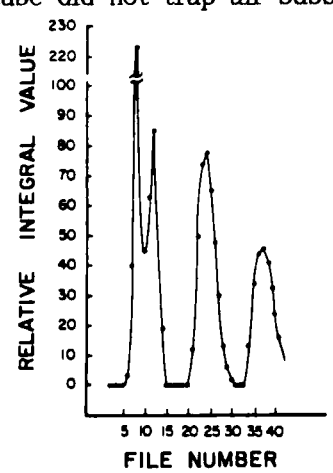

Figure 4. Plot of proton integrals vs. file number for model mixture.

The inlet and outlet sections of the cell were tapered to prevent eddies which was not only important from the point of view of minimizing chromatographic peak dispersion but also to maintain satisfactory NMR resolution. The volume of the coil region of the tube was 120 µl which, as seen from Figure 3B, only constitutes part of the total cell volume and consequently is far too great to realize the high resolving power of modern LC columns.

This cell provided an increased NMR resolution of 2-4 Hz and reduced the sample volume to 10-25 µl which is still very large in comparison with those normally employed in analytical LC. A set of NMR spectra obtained from the cell is shown in Figure 5. The flow rate was 1 ml/min and the first 15 spectra were taken over a period of 15 sec each (250-µl windows). Spectra 17 - 32 were taken over 30 sec intervals (500-µl windows) and spectra 36 - 40 over 60 sec intervals (1-ml windows). It is clearly seen that even with the improved cell the chromatographic resolution is seriously compromised to obtain NMR sensitivity. Multi-component mixtures that are only just resolved would result in a number of solutes being contained in the sample cell at one time and consequently impair the integrity of the NMR spectrum. Nevertheless, the work of Haw et al. (5), at the time, represented a significant advance in the LC/NMR technique.

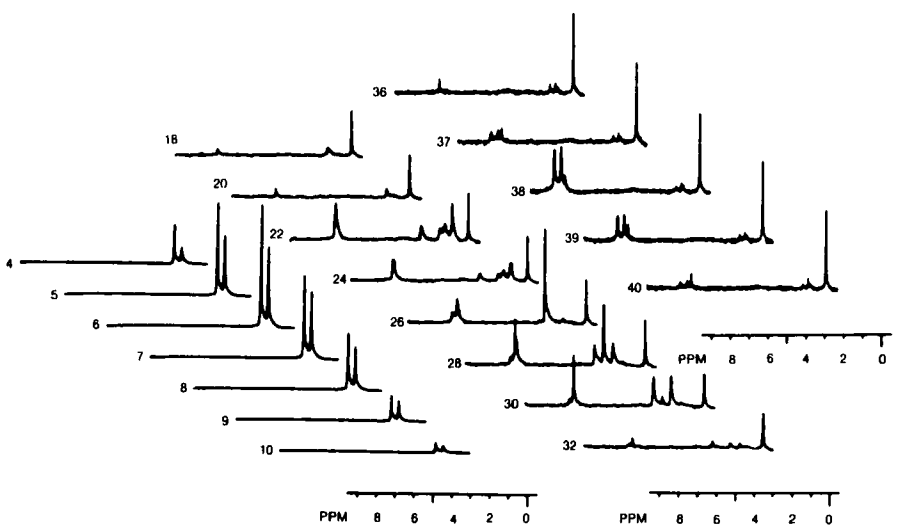

Figure 5. LC/^{1}H NMR profile for model mixture. A 25-µl sample was injected and 1K data points were taken per file.

Bayer and his colleagues (6), developed further their original LC/NMR system (1) to provide less band dispersion and greater NMR sensitivity and resolution. An NMR resolution of 0.5

Hz was claimed but, again, this advantage appeared to be achieved at some significant loss in chromatographic resolution. Nevertheless, a resolution of 0.5 Hz was also a significant step forward. A diagram of the cell employed is shown in Figure 6. It could be used either in the continuous flow mode or the stop flow mode. The cell consisted of an inverted U tube 2 mm I.D., the inlet connection being 0.2 mm I.D. 2 m long and the exit tube 1 mm I.D. The long length of connecting tube between the chromatograph and the sample tube was necessary to isolate the NMR spectrometer from the chromatograph to prevent interference with the homogeneity of the magnetic field. Nevertheless, this long length of connecting tube between chromatograph and spectrometer must inevitably cause significant band dispersion; it follows that if close association of the two instruments is prohibitive, then the use of low dispersion connecting tubing may again be essential. Bayer et al. (1), also demonstrated that very useful NMR spectra could be obtained from aqueous solvent mixtures.

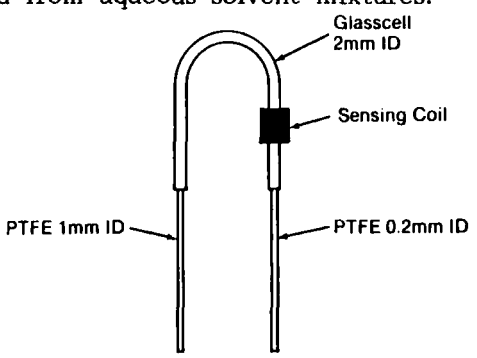

Figure 6. Schematic diagram of ^{1}H NMR flow probehead suitable for cryomagnets.

The next step forward in the development of the LC/NMR combination was reported by Laude and Wilkens (7). These authors tackled the two primary problems associated with the LC/NMR combination, namely the limited sensitivity of the NMR spectrometer and the need to ensure sample pre-magnetization before measurement. Up to this time the LC/NMR combination required the use of preparative or at least semi-preparative columns to provide adequate sample to the spectrometer. The use of analytical columns would require on-column injection in the μg range, while, at the same time, maintaining NMR resolution of better than 1 Hz in a flowing system. Furthermore, if the full efficiency of the analytical column was to be realized then dispersion in the column-sample tube connection must be kept to a minimum. The major improvement introduced by Laude and Wilkins (7) was remarkably simple and that was to place a commercial stainless-steel LC column directly in the magnet bore without loss of resolution or sensitivity. This procedure not only ensured complete premagnetization of the eluent, but also virtually eliminated the connecting tube. A block diagram of their apparatus is shown in Figure 7A. The mobile phase supply system

was a Varian Model 5060 which was placed 3 m away from the NMR spectrometer to eliminate any interaction with the magnetic field. The column was situated inside a Nicolet 300 MHz wide bore superconducting NMR spectrometer and the connection to the pump was made with stainless-steel tubing. A 3-m length of tubing prior to the column was not detrimental to the chromatographic system as it was situated prior to the injection valve. A diagram of the probe sample cell is shown in Figure 7B.

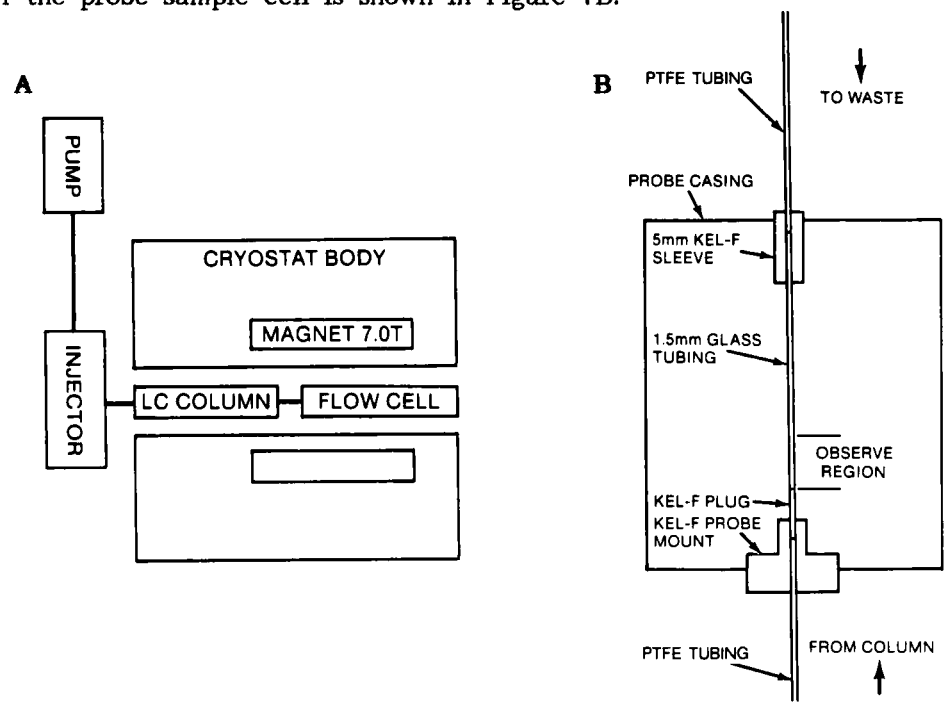

Figure 7. (**A**) Block diagram of the on-line HPLC/NMR system. (**B**) Schematic diagram of the flow cell with a 20-µl sensing volume.

It was necessary to employ glass tubing in the observation region to ensure adequate NMR resolution. This sample tubing, however, only had a volume of about 20 µl, a volume very much less than any previously reported. The PTFE plug acted as a union between the PTFE connecting tube (0.25 mm I.D.) and the sample tube. Due to the column being much closer to the sample tube the length of connecting tube was only a few centimeters long and thus provided only a small contribution to peak dispersion. The on-line spectra obtained for 50-µg samples of n-heptane and naphthalene are shown in Figure 8, clearly demonstrating the improved sensitivity that was obtained.

The performance obtainable from the LC/NMR combination has improved rapidly over the last five years. For really effective on line use as an aid in the elucidation of the structure of solutes eluted from a chromatograph, however, further improvement

is desirable. The peak dispersion that takes place between the column and the sample cell and, indeed, that which takes place in the cell itself, needs to be further reduced. The standard deviation of the dispersion that takes place in the connecting tube and cell should be less than 10 μl if the resolution obtainable from modern high-efficiency columns is to be realized. The attachment of the observation tube directly to a small bore column is also an attractive possibility. It would also be interesting to explore the advantages of using sample tubes packed with glass beads to reduce the peak dispersion. Dispersion in packed beds is very much smaller than that in open tubes of the radius employed for NMR sample tubes. Furthermore, glass beads should not have an adverse effect on the NMR resolution and the radial flow that takes place between the beads would partially simulate the effect of spinning the tube.

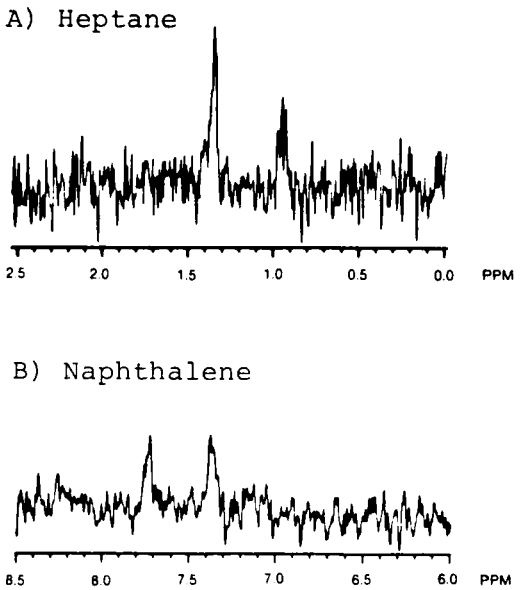

Figure 8. ^{1}H NMR spectra for heptane and naphthalene, sample size, 50 μg injected onto the column. The spectra are for eight coded scans in a system flowing at 0.5 ml/min.

The sensitivity of the LC/NMR combination, although greatly increased over the last three years, still needs to be increased by an order of magnitude for effective use of the system. At the present state of development it is questionable whether on-line or off-line measurement of NMR spectra is to be preferred. It still might be more efficient to trap the solutes as they are eluted and run the spectra off-line. However, the situation is borderline at this time and if the same relative improvement is realized over the next three years of development to that which has occurred over the previous three years then a practical, efficient on-line LC/NMR combination should become available in the not to distant future.

The Combination of the Liquid Chromatograph with the Mass Spectrometer

The LC/MS combination is another valuable aid in the structure elucidation of eluted solutes but the system is not as comprehensive as the LC/NMR combination. The successful interpretation of a mass spectrum for structure identification can occasionally require other spectroscopic information such as the IR spectrum of the substance to indicate the presence of specific functional groups. Nevertheless the LC/MS combination can be a very useful analytical technique.

Unfortunately, the combination of the liquid chromatograph with the mass spectrometer is fraught with difficulties. The difficulties arise from the inherent incompatibility of the two systems. This incompatibility stems from the fact that while the solute is eluted from the chromatograph slowly at a low concentration in the liquid eluent, the mass spectrometer responds to the mass of solute entering it per unit time. Consequently, a solute feed velocity change is desirable between the two systems to achieve a satisfactorily high sensitivity on the part of the mass spectrometer. Such a feed velocity change could be developed by employing the already established 'chromatographic memory' (8). In this procedure the eluting solvent is allowed to flow over a moving wire, the solvent evaporated and the wire subsequently wound on a reel. On completion of the chromatogram the wire would then be fed rapidly into the mass spectrometer at an appropriately increased speed to provide the fast sampling rate demanded by the mass spectrometer. This could be but one solution to the LC/MS interface problem, but in any event there is a real need for an efficient LC/MS interface to help in the identification of the components of complex mixtures of biochemical and biological origin.

MS, however, also has certain *advantages* over other spectroscopic methods that in some ways makes it an ideal technique to combine with LC. Mass spectra can be obtained rapidly, only sub-μg amounts of material are required to provide satisfactory spectra and the data produced is highly informative with respect to molecular structure. There are two well established methods that can be used to interface a liquid chromatograph with a mass spectrometer. Firstly, the direct inlet system developed by McLafferty and co-workers (9-11) and secondly, the wire transport system developed by Scott et al. (12,13). The former takes a proportion of the column eluent and passes it directly into a conventional mass spectrometer volatilizing both solvent and solute into the ion source. The latter employs the wire transport system in the normal way and the solvent is evaporated from the wire after passage through the column eluent stream. The wire, coated with the residual solute,

then passes through a suitable interface directly into the ion source of a quadruple mass spectrometer, where it is volatilized into the electron beam. Both methods have been established as viable methods for LC/MS operation and the system involving the wire transport system has been commercially available for some time. A third method reported by Horning et al. (14) is also a possible system for sample introduction from a liquid chromatograph into a mass spectrometer but has not yet been developed to is fullest capability. The method developed by Horning et al. (14) involves the vaporization of a portion of the column eluent and both the solvent and solute vapors pass directly into a chamber containing a radioactive source. Positive ions are produced by a complex series of ion molecular reactions which then pass through a micropore aperture directly into the ion source of a quadruple mass spectrometer. The authors claim a sensitivity of 5-10 ng per spectrum but it would appear that the *solute* must be reasonably volatile for the system to function. Furthermore, as the solvent is vaporized with the solute and both enter the mass spectrometer, the effect of gradient elution development on the resulting spectra is uncertain as the character and composition of the solvent is continuously changing.

The Direct Inlet System

The direct inlet system for introducing a portion of the column eluent directly into a mass spectrometer was first devised by McLafferty and co-workers (9-11) and a diagram of their interface is shown in Figure 9.

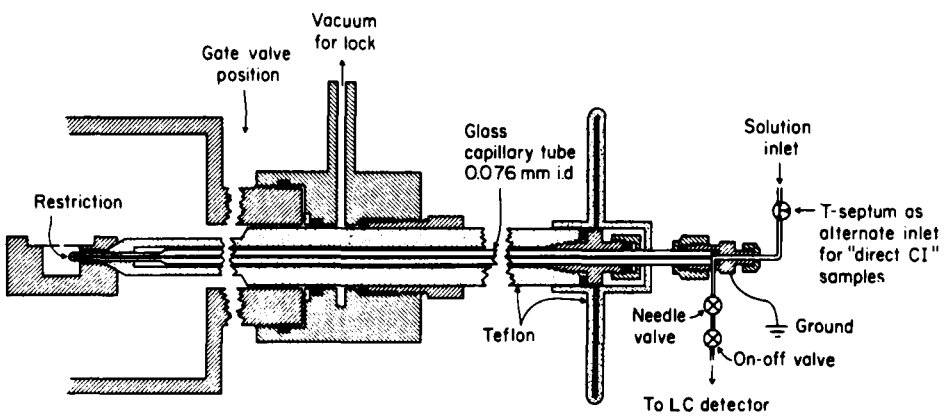

Figure 9. LC/MS direct inlet system.

The Hitachi RMH-2 mass spectrometer was employed, appropriately modified to provide chemical ionization spectra. A differential pump (pumping rate 250 l/sec) was added to the ion source region to provide adequate removal of the solvent vapor. The column eluent passed into the ion source via a glass capillary (0.076 mm I.D.) which passed through the center of a PTFE rod. The PTFE rod was inserted through the vacuum lock provided for probe injection

and a vacuum tight seal was maintained by the use of O-rings. The required restriction at the tip of the glass tube, to control the quantity of sample entering the mass spectrometer, was achieved by drawing out the tip in a small flame. At a flow rate of 10 µl/min through the glass tube the delay time was about 6 sec. Flow rates in excess of 10 µl/min could be employed but the chance of electrical breakdown in the ion source was greatly increased. The authors state that if mobile phases having significant conductivity, employed electrical breakdown can be avoided by employing a quadruple mass spectrometer as an alternative, where voltages at the ion source are closer to earth potential.

In the McLafferty system the solvent serves as the ionizing agent in the mass spectrometer and thus elution with mixed solvents or by gradient development can confuse the interpretation of the spectra produced. For example, 3-hexanone, benzene and methyl palmitate provide the protonated molecular ion $M+H^+$ in methanol whereas the major peaks produced with pentane as the ionizing agent for 3-hexanone and androstanone result from hydride abstraction and are of the form $M-H^+$. The authors noted that compounds that have insufficient vapor pressure for normal inlet sampling procedures are not detected by this system, which unfortunately excludes a significant range of substances that are separated by LC. An example of chromatograms obtained by the McLafferty system is shown in Figure 10.

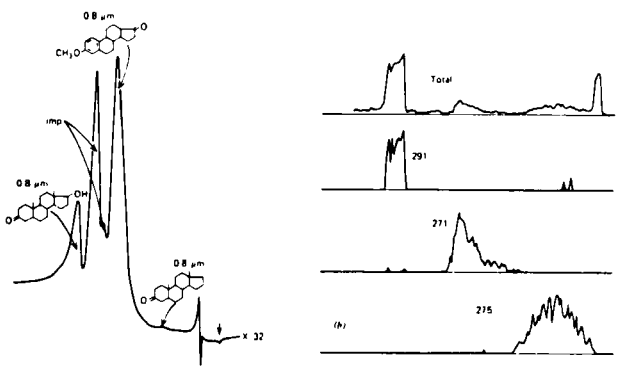

Figure 10. Chromatograms from the LC/MS direct inlet system.

The chromatograms shown in Figure 10, compared with more recent results, may appear inferior but it must be remembered that they illustrate the first successful LC/MS system to be developed. The liquid chromatograph was the Waters Assoc. ALC202 fitted with the M6000 pump for gradient development. The chromatograms represent the separation of a mixture of S-α-3-androstanone, estrone methyl ether and androstanalone present at levels of approximately 200-250 µg. A linear gradient was employed over a period of 10 min, the initial solvent concentration being 40% acetonitrile and 60% water and the final solvent being 100% pure acetonitrile at a

flow rate of 1 ml/min. The mass spectrometer was operated at a resolution of 1500, the source temperature was 200°C, the ionizing electron energy 500 eV and the emission current 0.17 mA. The mass spectrometer scan speed was 10 sec/decade from mass 600 to mass 120 with a fly back time of 2 sec. The chromatogram on the left of Figure 10 was obtained by a UV detector and the top chromatogram on the right obtained from the total ion current of the mass spectrometer. The three lower chromatograms on the right of Figure 10 are reconstructed mass spectra using the ion masses of the respective quasi molecular ions of each components. It should be noted that the androstanone peak was hardly detected by the UV monitor but gave a significant peak on the chromatogram from the mass spectrometer. However the large peak shown by the UV monitor was not shown by the mass spectrometer. The authors claimed that 1 μg of cholesterol could be easily detected and 0.2 μg of tert.-butyl anthraquinone could be readily identified by monitoring solely on the peak mass of 265. The direct inlet system was obviously demonstrated to be viable but had the disadvantage that it would only monitor substances that had significant volatility or vapor pressure at 200°C and furthermore the character of the spectrum produced would vary with the composition of the solvent employed.

Voyksner et al. (15) reported the results from a systematic examination of the effects of solvent composition, source pressure and source temperature on the LC/MS performance. In order to do so, they had to develop an appropriate apparatus that would allow them to change the source pressure in a controlled manner and determine the nature of the ionization that resulted. The apparatus they used is shown in Figure 11.

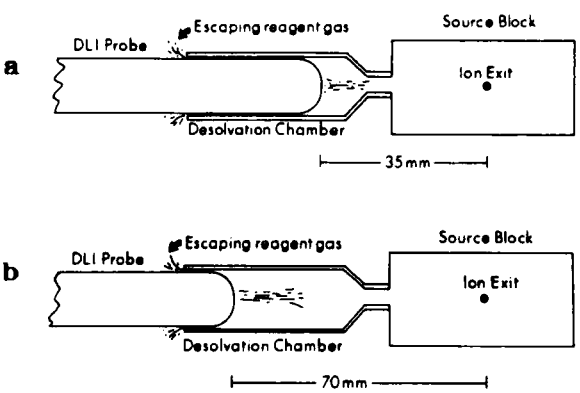

Figure 11. The effects of probe position on source conductance: (a) probe in desolvation chamber, reducing the conductance of the reagent gas out of the desolvation chamber resulting in a *higher* source pressure; (b) probe mostly retracted from the desolvation chamber, enabling easy loss of gas out of the desolvation chamber, resulting in *lower* source pressure (DLI = direct liquid inlet).

They employed a probe that slid inside a sleeve which constituted the desolvation chamber. By moving the position of the probe inside the tube, the impedance to the flow of escaping reagent vapor could be varied. The reagent vapor was removed by a secondary pumping procedure. The results they obtained showed that the sample response varied significantly with pressure and unfortunately the optimum source pressure varied with solvent composition. As a consequence, this effect caused considerable difficulty in maintaining optimal operating conditions in the ion source when gradient elution development was employed. It was found that varying the solvent composition had little or no effect on protenated molecular ion sensitivity, but it did change the amount of fragmentation observed. The extent of fragmentation also appeared to depend very significantly on the source temperature. The authors concluded that using direct inlet introduction of sample to the mass spectrometer required careful optimization of conditions to obtain repeatable results.

In 1982 Krien et al. (16) examined the use of microbore columns in LC/MS systems. The advantages of microbore columns are very apparent when the amount of sample is limited, for example, for those samples of biological or biochemical origin, as the small bore column allows relatively high concentrations of solute to be eluted even when small masses of the individual solutes are available.

Krien et al. (16) developed a probe system that was attached to the end of the microbore column which itself penetrated the mass spectrometer such that its exit was close to the ion source. A diagram of their probe system is shown in Figure 12.

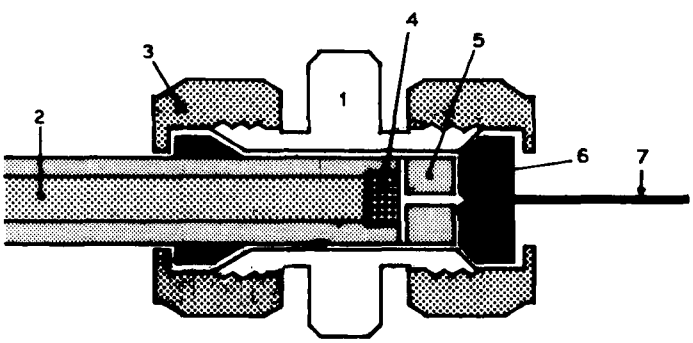

Figure 12. Schematic drawing of the connection between the microbore column and the capillary tubing. 1 = Modified Swagelock union (1/16 in.); 2 = microbore-packed column (1/16 in. O.D., 1 mm I.D.); 3 = Vespel ferrule; 4 = 0.5-μm porosity filter; 5 = PTFE tubing (1/16 in. O.D., 0.2 mm I.D., length 5 mm); 6 = 1/16 in. Vespel ferrule; 7 = capillary tubing (0.3 mm O.D., 50 μm I.D.).

The column was terminated by a 1/2 μm porosity filter and a short piece of PTFE tubing. Subsequent to the tubing, was a Vespel ferrule carrying a length of quartz tubing 0.3 mm O.D., 50 μm I.D. that projected into the ion source of a quadrupole mass spectrometer.

In Figure 13A the separation of a number of juvenile hormones is demonstrated and a spectrum taken for one of these is shown in Figure 13B. The mass ranges of the solutes are not particularly high (molecular weight 300), but nevertheless the interface employing the probe system was demonstrated to be effective and useful.

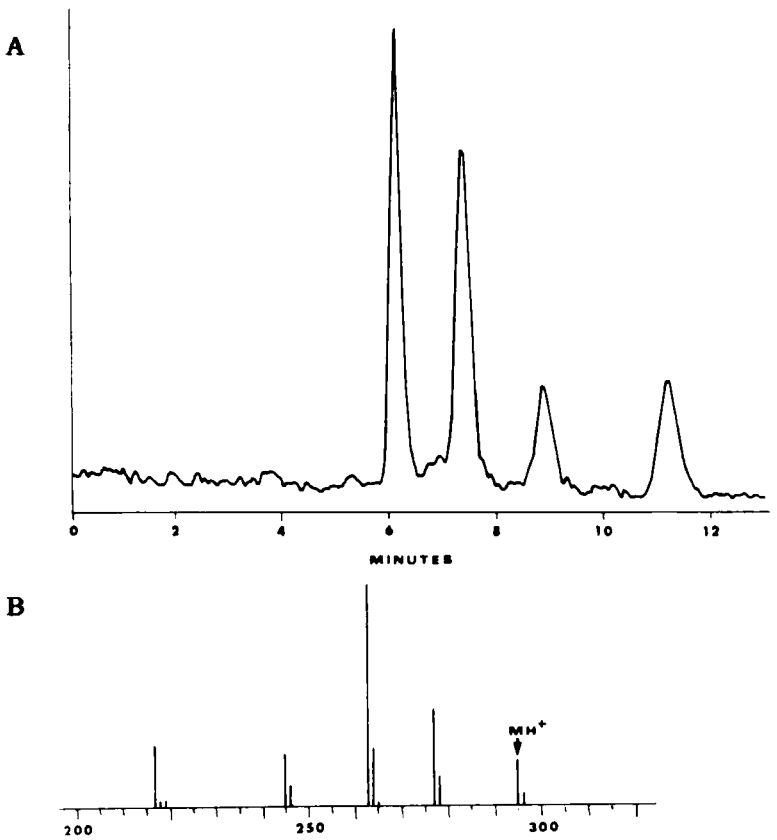

Figure 13. (**A**) Analysis of four juvenile hormones separated on a reversed-phase column. Mobile phase: acetonitrile-water mixture (75:25). Flow rate: 50 μl/min. (**B**) Influence of the mobile phase on the chemical-ionization mass spectra of juvenile hormone (m/e = 294).

The volatilization of the mobile phase from a quartz capillary was examined by Bruins and Drenth (17) who used an apparatus very similar to that of Krien et al. (16). It was shown that significant heat transfer was necessary to support the evaporation of

the liquid. It was also shown that the liquid vaporized inside the capillary and the distance between the liquid front and the end of the capillary was strongly dependent on the temperature. The heat conduction to the capillary was achieved by resting it in a heated copper block. Relatively insoluble substances tended to block the capillary.

Niessen and Poppe (18) extended the LC/MS system to the use of open tubular LC columns. A diagram of their interface is shown in Figure 14. Due to the very small flow from the open tubular column makeup liquid had to be used which carried the eluent through a 4-μm nickel diaphragm enclosed between PTFE spacers. In fact, this resulted in the dilution of the eluent, but as the mass spectrometer responds to the mass of solute entering the ionization chamber per unit time and, furthermore, as the makeup solvent necessary for volatilization also acted as the chemical ionizing agent, no significant loss of sensitivity was noted. The capillary column used was 5 μm I.D. and a few meters long and the authors reported detection limits of 1-10 pg. However, the sensitivity would have to be significantly increased if the use of open tubular columns was to become effective for general use in LC/MS.

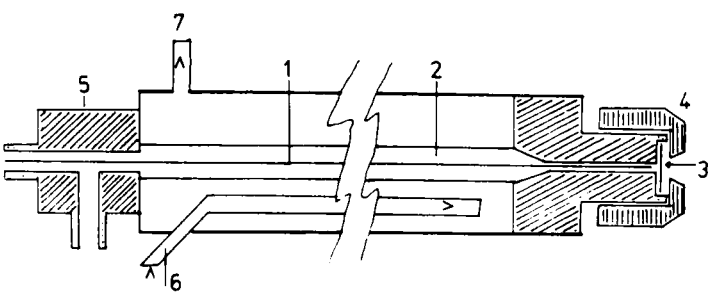

Figure 14. Schematic diagram of the laboratory-built DLI probe (10 mm O.D.). 1 = Fused-silica open-tubular column; 2 = makeup liquid; 3 = 4-μm nickel diaphragm enclosed between two PTFE spacers; 4 = stainless-steel nut; 5 = modified Swagelock 1/16 in. T-Piece; 6 = cooling water in; 7 = cooling water out.

Another approach to the volatilization of solvents by direct inlet coupling was the *electrospray* system reported by Whitehouse et al. (19). Electrically spraying the eluent into a dry "bath-gas" creates a dispersion of charged droplets which rapidly evaporate. As the droplets grow smaller, the increase in surface charge density and the accompanying decrease in radius of curvature, results in electric fields being formed that are strong enough to desorb solute ions. Part of the resulting dispersion of ions in the "bath-gas" passes through a small orifice or channel into an evacuated region to produce a supersonic free jet. The core of this jet then passes through a conical skimmer orifice and the ions are then transported to the inlet of mass analyzer. The authors suggested that the flexibility, convenience, sensitivity,

cleanliness and ease of maintenance of this type of interface made the electrospray inlet system the basis of a very effective and practical LC/MS interface.

An alternative direct introduction interface was reported by Covey and Henion (20) which they termed the *thermospray* interface. It differed from those previously described in that it had a dual probe interface which is introduced into the mass spectrometer via the standard direct insertion probe inlet. The dual purpose LC/MS interface could provide the conventional direct liquid inlet system or, alternatively, a copper vaporizer situated at the end of the probe could be heated electrically to produce thermospray ionization. A diagram of the probe system is shown in Figure 15.

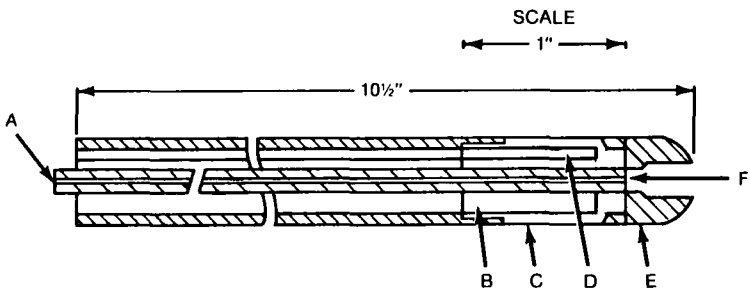

Figure 15. Dual purpose direct liquid inlet/thermospray LC/MS probe interface: A = central microbore conduit (0.004 in. I.D.) through-put; B = two 50-W 120-V ac cartridge heaters; C = heated copper vaporizer; D = thermocouple; E = removable end cap; F = stainless-steel pinhole diaphragm.

It consisted of a central stainless-steel tube 0.004 in I.D. that passed through a heated copper block which acted as the vaporizer and was heated by two 50 W cartridge heaters. A thermocouple was also included in the block to monitor the temperature. At the end of the tube, prior to a removable cap was a stainless-steel pin hole diaphragm. The detection limits of the system was about 100 ng when employed in the thermospray mode for involatile labile compounds and when used with 2 mm I.D. small bore LC columns operated at a flow rate of 150 µl/min. Practical detection limits obtained in general use were reported to be 250 ng in the direct liquid inlet mode and about 100 ng in the thermospray mode.

The use of the thermospray system was further developed by Voyksner and Haney (21). They noted that the LC/MS thermal spray system frequently produced molecular weight information (parent ions) and exhibited lower detection limits then the other forms of LC/MS interfaces. They found that when used with a, 0.1 M ammonia acetate buffer in a high percent water solvent, optimum sensitivities were realized. The optimum interface-temperature varied with solvent composition and could be determined by maximizing the solvent-buffer ion intensities. Thermospray

ionization resembles chemical ionization using ammonia as the ionized agent. The system produces protonation, ammonium addition and proton-bound solvent molecule clusters. The ionization procedure with this system was reported to be very "soft" resulting in very few fragment ions being formed. The system was used successfully for the analysis of triazine herbicides and organo-phosphorus pesticides, excellent specificity and sensitivity was observed.

Blakely and Vestal (22) employed the thermospray system with the quadrupole mass spectrometer and demonstrated that it could provide stable vaporization and ionization at flow rates up to 2 ml/min with an aqueous mobile phase. If the mobile phase contained a significant concentration of ions in solution (ca. 10^{-4} to 1 M) no extra thermal ionization source is required to achieve detection of many non-volatile solutes at the sub-nanogram level. They found that with weakly ionized mobile phases a conventional electron beam needs to be used to provide gas-phase reagent ions for the chemical ionization of the solute. The thermospray system has been effectively used by Voyksner et al. (23) in the analyses of anti-cancer drugs.

The Wire Transport LC/MS System

This system, as already stated, utilizes the wire transport method of sampling the column eluent, the solvent is removed by evaporation and the solute, coated on the wire, passed directly through the ion source of a quadrupole mass spectrometer where it is volatilized directly into the electron beam. The quadruple mass spectrometer is particularly applicable to this method of sample introduction, as the ion source is only a few volts above earth potential and the presence of an earthed wire passing through it does not interfere with the electron, or ion optics, of the electrode configuration. The success of the system hinges on the design of a suitable interface that can permit the passage of the wire from an environment at atmospheric pressure through the ion source and out again while maintaining a pressure of 10^{-6} mmHg in the ion source.

A diagram of the overall system is shown in Figure 16. The wire employed was that supplied for the wire transport detector, 0.005 in. O.D. and made of stainless steel. The wire from the drive system passes over an electrically insulated pulley, over a coating block (where the column eluent wets the wire), into the left hand interface of the mass spectrometer and thence through the ion source. It then exits through another identical interface, round another pulley and back to the drive system. In the lower portion of Figure .16 is shown the location of the interface with respect to the ion source it is seen that the wire leaves the interface about 2 mm from the ion source and less than a centimeter from the electron beam. A potential is applied across the two pulleys causing a current of about 200 mA to pass

through the wire. The heat generated by the current is rapidly conducted and convected from the wire to the air and thus the temperature of the wire only rises a few degrees above ambient temperature. On entering the ion source, however, where the pressure is about 10^{-6} mmHg, heat can only be lost by radiation and the wire heats rapidly up to a temperature of 200-300 °C which volatilizes the adhering solute directly into the electron beam of the ion source.

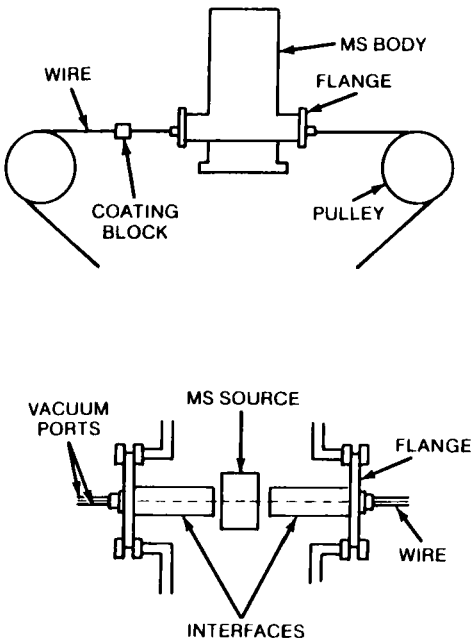

Figure 16. The wire transport LC/MS system.

In this way substances having very low vapor pressure can be volatilized without decomposition and the system, in effect, acts as a continuous probe sampling device.

A diagram of the interface is shown in Figure 17. The main body of the interface is constructed of stainless steel and is fitted to the side flanges of the Finnigan mass spectrometer, such that the interfaces are re-entrant to the ion source and terminate a few millimeters from the electron beam. The interface itself consists of two chambers separated and terminated by ruby jewels 1/10 in. in diameter and 0.018 in. thick. The jewels in the left-hand interface have central apertures 0.010 in. in diameter where the sample is introduced into the mass spectrometer. The jewels in the right-hand interface where the wire leaves the mass spectrometer to the winding spool have central apertures 0.007 in. in diameter. The larger diameter apertures on the feed side of the spectrometer are employed to reduce 'scuffing' of the wire and possibly loss of solute.

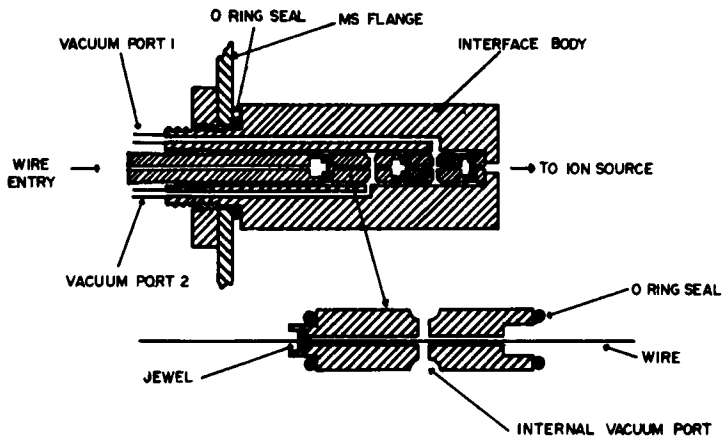

Figure 17. The LC/ wire transport interface.

It was necessary to employ ruby jewels in the interface to prevent frictional erosion of the apertures by the stainless-steel wire which would enlarge them and increase the leakage of air into the MS. The first chamber of each interface was connected directly to a 150 l/min rotary pump, which reduced the pressure in the first chamber to about 0.1 mmHg. The second chamber of each interface was connected to an oil diffusion pump backed by a 150 l/min rotary pump. The pressure in the second chamber of each interface was reduced by the system to about 5 to 10 μm Hg. The entrance and the exit apertures were fitted with an helium purge T junction. Helium passed through the T junction replaced the air entering the mass spectrometer through the interfaces. In this way background spectra from air contaminants were reduced. The T junction also afforded a method of introducing methane or other suitable gases if chemical ionization spectra were required. A photograph of the apparatus is shown in Figure 18.

The mass spectrometer can be operated under normal conditions except that the filament current employed should be about 3 mA. The instrument can be used with the standard data handling system supplied for the Finnigan mass spectrometer and a typical set of operating conditions is given in Table I.

The sensitivity of the instrument for diazepam was found to be about 4 x 10^{-6} g/ml. The wire samples the eluent at 10 μl/min at the maximum wire speed and thus as the scan speed of the mass spectrometer was 1 scan/sec this sensitivity corresponded to ca. 7 x 10^{-10} g of diazepam per spectrum. The pressure in the source could be maintained at 1 x 10^{-6} mmHg. The use of helium as a purge gas in the interface reduces the noise level of the mass spectrometer by a factor of three with a commensurate increase in sensitivity.

In Figure 19 a chromatogram obtained from a fermentation extract is shown and this sample contains solutes that cover a wide range of polarity. The mixture was first chromatographed using the incremental gradient elution technique (24). A charge of 2 mg was injected onto the column and the detector used was the wire transport detector. The chromatogram produced is shown at the top of Figure 19.

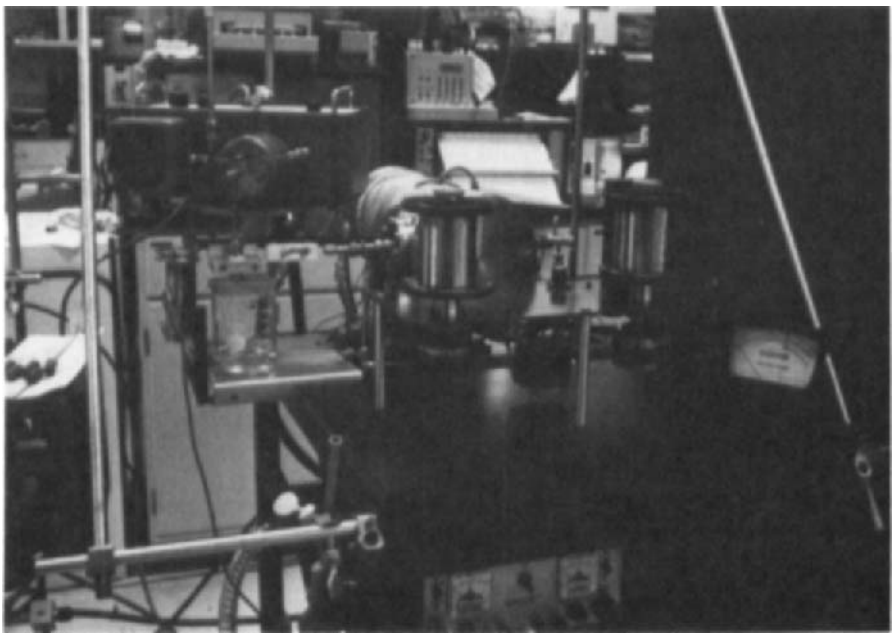

Figure 18. The wire transport LC/MS instrument.

TABLE 1

MASS SPECTROMETER OPERATING CONDITIONS

Filament current	3 mA
Electron energy	60 V
Source pressure	3 x 10^{-6} mm Hg
Mass range	60-199; 200-399; 400-650
Integration time	3; 5; 8
Sample/AMU	1; 1; 1
Threshold	1
Attenuation	5
Mass range setting	high
Mass run time	400 min
Delay between scans	3 sec

During the chromatographic development fractions were collected for each peak and numbered as shown in the Figure 19. These were concentrated in a current of nitrogen to about 0.1 ml and each fraction examined using the LC/MS system as a solid probe injection device. A drop of each sample was spotted onto the wire for a few seconds and the total ion-current traces of these probe samples are shown on the right-hand side of Figure 19. All the fractions were placed on the moving wire over a period of 8.5 min. Thus the spectrum for an individual probe sample was obtained in approximately 25 sec:

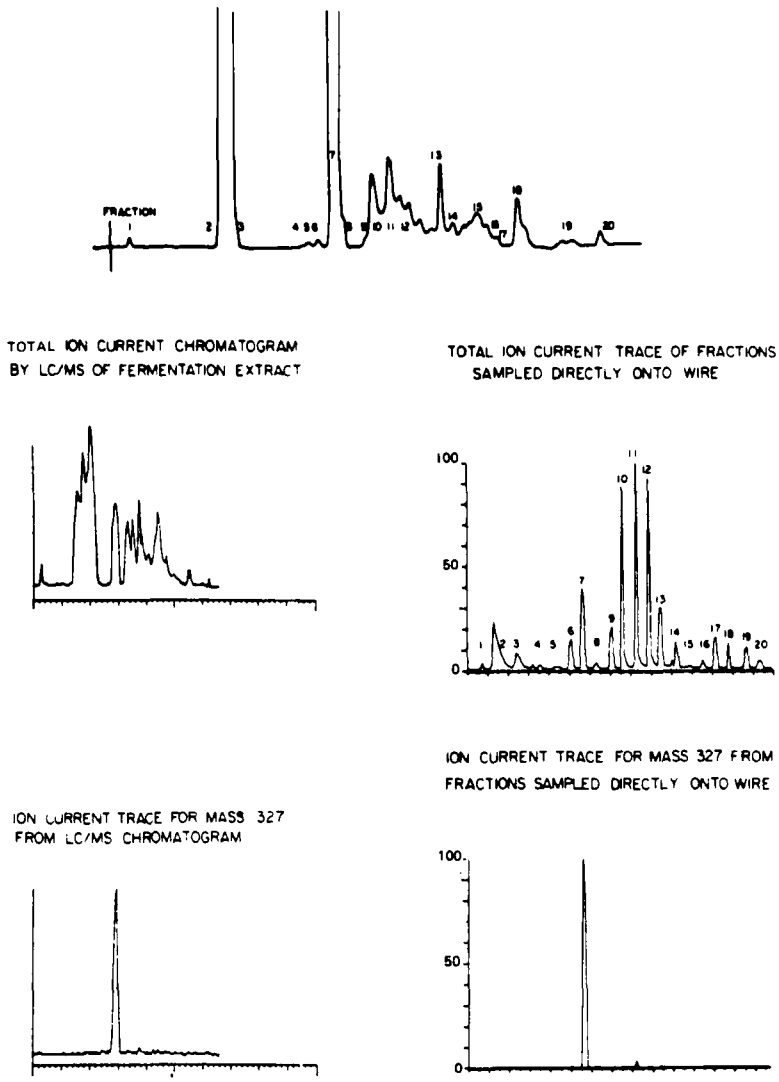

Figure 19. Chromatogram of a fermentation extract by incremental gradient elution.

The same sample was then chromatographed on the LC/MS system using a 6 mg charge and the same operating conditions. The chromatogram obtained from the total ion-current monitor is shown as a hard copy on the left-hand side of Figure 19. It is seen that due to the nature of the hard copy presentation, the chromatogram is somewhat compressed in size relative to the original using the wire transport detector. However, it is seen that the same pattern of peaks is obtained although owing to the larger charge size employed, the resolution is not as good.

A spectrum obtained from the probe injection sample number 7 is shown as the lower spectrum in Figure 20.

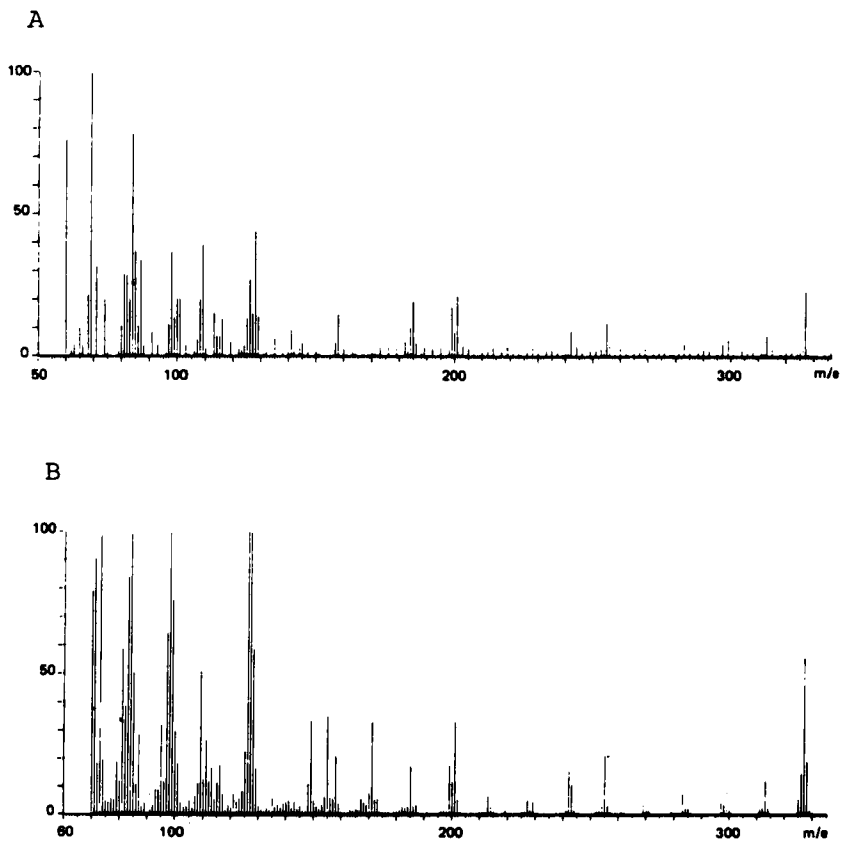

Figure 20. (**A**) Spectrum 285-262 from LC/MS chromatogram. (**B**) Spectrum from fraction 7.

It is seen that the last, most significant ion has a mass of 327; thus a reconstructed chromatogram from the ion current of mass 327 was obtained for the probe samples. This is seen as the lower chromatogram on the right-hand side of Figure 19. Peak 7 is clearly selected and using the same mass 327 a reconstructed chromatogram was obtained from the original ion-current chromatogram from the LC/MS run. This is shown at the bottom of the right-hand side of Figure 19 and again it is seen that the peak is clearly and unambiguously selected from the mixture. A spectrum taken from the respective peak in the LC/MS chromatogram is shown at the top of Figure 20 and can be compared with the spectrum from fraction 7. It is seen that basically the two spectra are identical although the spectrum from fraction 7 obviously contains traces of a contaminating material.

The wire transport system for LC/MS has several distinct advantages. Firstly, as a transport system is employed, its performance is completely independent of the solvent used in the chromatographic system provided it is reasonably volatile. Thus gradient elution development using solvents of any polarity can be employed without affecting the quality of the mass spectra. Secondly, because the transport system is, in effect, a continuous solid probe injection device, spectra of substances of very low volatility, such as those normally met in LC separations, can be readily obtained. Thirdly, the spectra obtained can be electron impact spectra, which, in general, are far more informative for structure elucidation than spectra obtained by chemical ionization. It should be pointed out, however, that the sensitivity of the wire transport LC/MS system is only moderate but with some development, sensitivities of 10^{-7}-10^{-8} g/ml should be obtainable.

The Finnigan LC/MS Transport System

An LC/MS system based on the device developed by Scott et al. (13) was modified by McFadden et al. (25) and is now commercially available and manufactured by Finnigan Inc. The Finnigan instrument, however, utilizes a continuous band or ribbon as the transport medium to increase the quantity of eluent sampled from the column and thus increase the overall sensitivity. Chemical ionization is also employed as an alternative to electron impact ionization to improve sensitivity, but does so at the expense of limiting the structural information obtained from the spectra relative to that obtained from electron impact spectra. The design and performance of the instrument was described in detail by McFadden et al. (25) and a diagram of their system is shown in Figure 21. LC effluent is taken up on the stainless-steel or high temperature plastic ribbon (3.2 mm wide, 0.05 mm thick) and transported to the vac-locks. Partial evaporation which occurs prior to passage through vac-lock No. 1 can be aided by a combination of heat, gas flow or vacuum as desired. Removal of solvent is completed in vac-locks No. 1 and No. 2 so that less

than 10^{-7} g/sec of solvent enters the mass spectrometer. Vac-lock No. 1 is pumped with a 500 l/min forepump equipped with an oil mist eliminator and vac-lock No. 2 is pumped with a 300 l/min forepump. Depending on the tolerances set for the vac-lock interface pieces, these chambers are maintained in the pressure ranges 1-20 Torr (vac-lock No. 1) and 0.1-0.5 Torr (vac-lock No. 2) so that the mass spectrometer analyzer section can be pumped to a satisfactory level around 10^{-6} Torr.

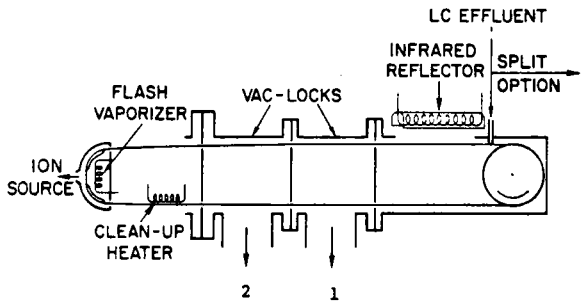

Figure 21. The Finnigan LC/MS transport system.

Flash vaporization of the sample occurs by radiant heating in a small chamber butted directly to the solid probe entrance of the mass spectrometer ion source. Heat input is provided by a Nichrome heater contained in a quartz tube. The belt travel distance through the chamber is 6.5 cm, so that for most belt speeds (2-4 cm/sec), point residence time is 2-3 sec. By comparison with the temperature of vaporization for solid probe samples, it appears that the belt temperature rises to within 20-30°C of the chamber temperature.

The slot in the interfaces for passage of the belt is formed by two "L"-shaped sapphire pieces which are attached to the stainless-steel flange or vacuum closure bar by epoxy cement. The belts used are either 0.05 or 0.075 mm thick and the slot tolerance is set to be 0.075 mm greater than the belt thickness (i.e., either 0.125 or 0.15 mm). The belt width is 0.317 cm and the slot width is 0.325 cm. A ribbon 0.32 cm wide travelling at a speed of 2.5 cm/sec will carry away a liquid film 0.2 mm thick from a solvent flow of 1 ml/min and if the solvent film can be evaporated without loss of solute, then the ribbon will transport virtually 100% of the solute into the mass spectrometer. Sample utilization will then depend only on the efficiency of the flash vaporization step. In practice, some sample is lost by spray processes and the flash vaporization cannot be fully efficient for all compounds. Nevertheless, yields in the range of 25-40% have been attained with an LC/MS ribbon interface system. It follows that the quantity of column eluent taken from the ribbon will be twenty times greater than that taken by the wire and provide significantly improved sensitivity.

Efficiency of solvent removal through the two vacuum locks is very high, particularly for lower boiling solvents such as hexane. With approximately 10^{-2} g/sec (1 ml/min) of hexane flowing on to the belt, the hexane MS spectral background indicates that about 10^{-7} g/sec is entering the ion source. The enrichment of sample/solvent is therefore in the range of 10^{5}. Lower boiling solvents such as pentane or methylene dichloride give higher enrichment. Higher boiling solvents such as toluene, dioxane, or isooctane should be avoided since they require heat input for efficient solvent evaporation. With the current apparatus, optimum performance could be attained with a solvent flow rate of up to 0.85 ml/min. At a higher solvent flow rate, sample is lost due to spray evaporation at the first vacuum lock.

A photograph of the belt interface system and ion source is shown in Figure 22. An example of the use of the belt interface system when used to monitor the separation of a pesticide mixture is shown in Figure 23. In chromatogram (a) the separation depicts the total ion current monitored by the mass spectrometer during development and chromatogram (b) was obtained by simultaneous monitoring with a UV detector at 254 nm.

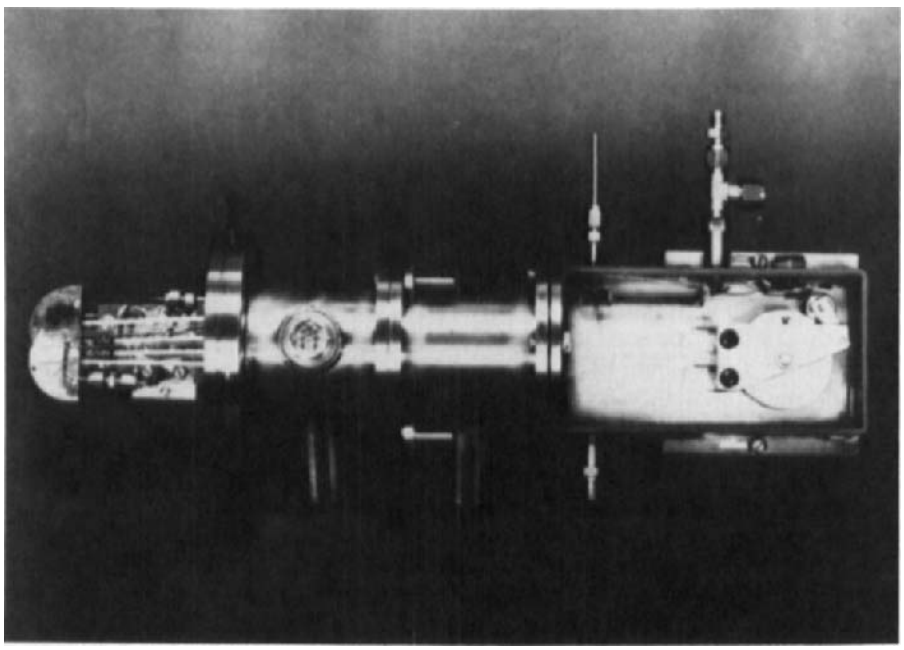

Figure 22. The Finnigan LC/MS interface and ion source.

Reasonable chromatographic fidelity is maintained during MS detection and very little cross contamination occurs between one compound and its adjacent neighbors. The mass spectra obtained at the top of each peak are also shown in Figure 23. (Oscillographic traces rather than computer-generated bar graphs are presented here in order to show more realistically the actual quality of spectra produced in the LC/MS mode.) The sensitivity of the system for carbaryl obtained by monitoring the peak mass of 144 was claimed to be 1 ng but insufficient data was given to convert this to the more significant units of g/ml in the column eluent.

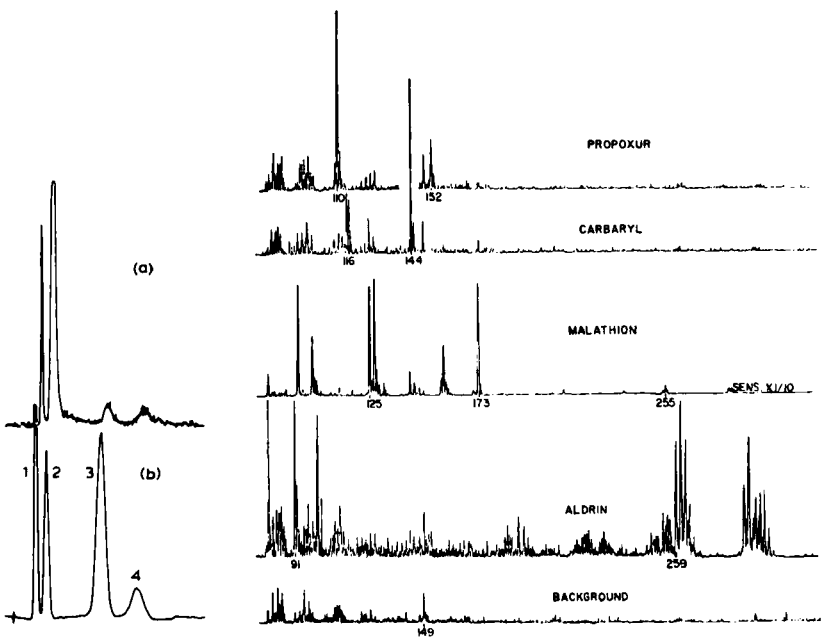

Figure 23. Chromatogram and mass spectra from the Finnigan LC/MS instrument.

Alcock et al. (26), has reviewed the various types of transport interfaces employed in LC/MS with particular reference to their use with small bore columns. Games et al. (27) examined the peak dispersion that could take place in the transport interface itself together with the electronics associated with the mass spectrometer. They concluded that the LC/MS system incorporating the transport interface behaved as a low dispersion LC detector and consequently could be used very effectively with small bore columns.

Hayes et al. (28), conducted a systematic study of the effect of the method of solvent deposition on the transport medium, on the overall performance of the LC/MS system, they designed a

nebulizer to spray the column eluent on to the moving belt, a diagram of which is shown in Figure 24. It consists of a 1/16 in. O.D. 0.007 in I.D. tube situated concentrically inside a pyrex tube which carries the nebulizing gas to form the spray. The pyrex tube was held inside an outer steel tube by means of a screw cap and an O-ring seal as shown in Figure 24. The role of nebulizer temperature and gas flow rate on chromatographic performance was measured and the influence of belt speed on peak variance also determined. On the basis of the results from their studies the authors claimed that HPLC columns could be satisfactorily used with the transport interface and at the same time produce good quality mass spectra. They claimed that their method of deposition was an important factor in the successful operation of the system. They also claimed detection limits of 40 pg for polynuclear aromatic hydrocarbons and a linear dynamic range of over four orders of magnitude.

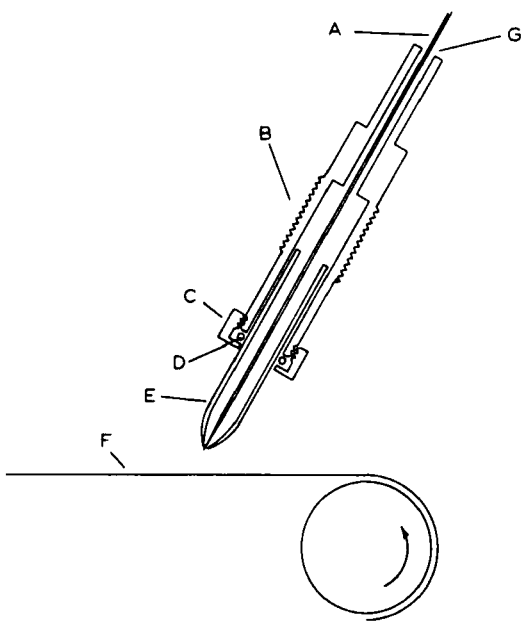

Figure 24. Diagram of spray deposition device: A = transfer line from LC and spray orifice 1/16 in. O.D., 0.007 in. I.D. stainless-steel; B = stainless-steel body of spray device; C = retaining cap; D = O-ring seal; E = Pyrex tip to form gas spray; F = moving belt; G = gas inlet.

Stout and daCunha (29), developed a simplified form of the belt interface which could be inserted into the conventional probe inlet system of the mass spectrometer. The approach taken was to apply the column eluent to the moving belt while situated inside a single evacuated chamber pumped by a single 300 l/min mechanical vacuum pump. The belt then passed into the ion source of the mass spectrometer through a length of 1/2 in. O.D. tubing that was inserted through the normal probe inlet sampling system. The major

features claimed by the authors were (1) simple insertion of the interface through the probe inlet, (2) the use of only one pumping stage, (3) the sample is volatilized in the 'ion volume', (4) the interface can accommodate flow rates as large as 200 µl/min and thus is suitable for use with small bore columns.

There are alternative methods of ionization for MS purposes other than electron impact ionization and chemical ionization. Two techniques that have recently found much interest for the molecular weight determination of non-volatile, thermally labile biomolecules (those of pertinence to liquid chromatography) are secondary ion mass spectrometry (SIMS) and laser desorption mass spectrometry (LDMS). In 1976 Benninghoven et al. (30) demonstrated that keV primary ions at low current densities could be used to desorb intact molecular ions from organic compounds adsorbed on metal surfaces and at the same time provide good sensitivity. Closely related to SIMS is fast atom bombardment (FAB) which used a neutral primary atom beam and a liquid sample matrix (31, 32). High-performance magnetic instruments can be employed with FAB and impressive results obtained from a wide variety of difficult samples. The important difference between SIMS and FAB does not appear to be the different type of ionizing beam but the use of a liquid sample matrix for ionization. The LDMS of organic compounds (33) did not achieve wide-spread interest until 1978 when Kistemaker and co-workers (34), demonstrated that sub-microsecond laser pulses could be used to desorb and ionize pseudomolecular ions from many molecules. Bulk solids (34, 35) and thin films on inert (36) and metal (37) substrates have been used to produce pseudomolecular ion by LDMS.

Fan et al. (38) developed a belt interface system for LC/MS that could provide both SIMS and LDMS methods of ionization. It consisted of a quadrupole mass spectrometer with a moving belt and a Finnigan/INCOS data system. Samples were deposited on the belt by means of the thermospray technique. A diagram of the dual ionization source is shown in Figure 25. Both ion beam and laser were aligned to strike the belt at 45° and at right angles to reach other. Xenon gas, ionized by electron impact, was used as the source of primary ions which were focussed by an Einzel lens and steering plates through an aperture on to the belt forming an oval spot having an area about 5 mm^2. The beam strikes the belt with an energy of about 3 keV. The laser light could be focussed on the same spot as an alternative by a suitable set of mirrors and lenses. Ultimately the authors employed a carbon steel belt which had a blackened surface and consequently absorbed more energy from the laser beam. Providing laser energies were kept reasonably low there was no degradation of the surface. Comparing the results from the two methods of ionization, laser desorption had the advantages that it provided more reliable molecular weight information. Furthermore, by varying the power of the laser, different degrees of fragmentation could be achieved and consequently more structural information could be obtained.

However, as it was difficult to control the laser energy absorbed, the mass spectra were less reproducible. In contrast, continuous ionization by primary ion bombardment provided more characteristic fragment peaks without losing molecular weight information and both the mass spectra and the chromatograms were more stable and reproducible.

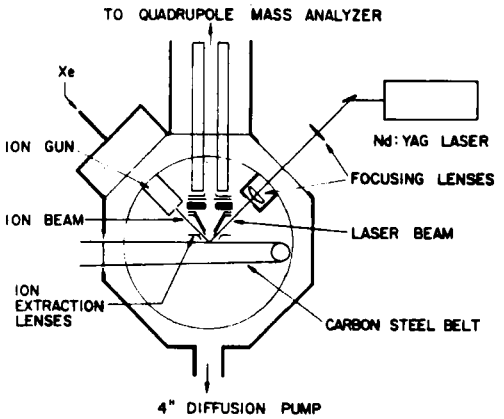

Figure 25. Schematic of the ionization source chamber.

It is obvious that the LC/MS system has been developed to a far greater extent than LC/NMR and there are at present a number of commercial systems available. The LC/MS is also becoming more versatile with a number of choices of interface and even choices of ionization techniques available. There is a desperate need for methods to elucidate structures of compounds of biological origin. Although, LC/MS will help a little in this area, the vast majority of compounds of interest have molecular weights far in excess of those that can be measured by the mass spectrometer. Active LC/MS development is not enough, a strong, parallel, development must take place to extend the molecular weight range of the mass spectrometer itself. This is essential if the MS is to cope with the structure elucidation of the high molecular weight compounds.

The Combination of the Infrared Spectrometer with the Liquid Chromatograph

The problems associated with the combination of the IR spectrometer with the liquid chromatograph (LC/IR) are very similar to those met in the LC/NMR combination. The sensitivity of the IR spectrometer is much less than that of the UV spectrometer or the mass spectrometer and, furthermore, solvent interference is much greater. Moreover, if solvents were chosen that would exhibit minimum spectra interference then the limitation placed on the chromatographic development would be completely unacceptable. As a result, prior to about 1975, LC/IR was carried out off-line, the peak was collected, the chromatography solvent removed and the solute then dissolved in a suitable IR solvent and the spectra taken in the normal way. In

fact, this is not an unacceptable procedure today as the need for IR spectra in structure elucidation is much less than that of MS or NMR. The IR spectrum is far less informative for unknown compounds and in the majority of examples, provides functional group information only.

The situation changed a little with the introduction of Fourier Transform IR (FTIR) as, in the data processing, the spectra were stored in computer memory. Consequently the spectra of the solute could be obtained (at least in theory) by subtracting the spectra of the solvent from the spectra of the solute plus solvent.

In 1975 Kizer et al. (39) demonstrated that solute spectra could be obtained from an on-line LC/FTIR combination by subtracting the spectra of the solvent from the combined spectra of the solute and solvent. The sensitivity, however, appeared to be extremely poor. In 1979 Kuehl and Griffiths (40) approached the problem in another way employing a rather crude but nevertheless effective transport system. Ribbon transport devices were tried together with pre-concentration techniques which were not very successful. Finally, they used a carousel of cups containing potassium chloride (KCl) a diagram of which is shown in Figure 26. The carousel resembles a fraction collector and consequently the device is more like an off-line system than an on-line system. The carousel had 32 cups fitted with a fine mesh screen each containing KCl powder. The position of the carousel was controlled automatically and only three positions were actively used. In the first position the sample was deposited on the KCl, in the second position a stream of air was drawn through the KCl to remove the solvent and in position three the spectrum was taken. The use of the carousel containing KCl powder certainly increased the sensitivity of the LC/IR combination significantly but the finite increments of sample made the system unsuitable for modern high-resolution columns. Jinno and co-workers (41,42) employed a potassium bromide (KBr) plate as a transport system. The eluent from a small bore column (flow rate 5 µl/min) was allowed to fall on the plate and evaporate, the plate being moved as each new peak was eluted. The eluent was also monitored by a UV detector to identify when the plate should be moved. After the chromatogram had been developed the plate was scanned by a IR spectrometer and the spectra of each solute obtained. Good sensitivity was reported but the procedure and for that matter, the method of Kuehl and Griffiths (40) was little more than novel methods of fraction collection and, in fact, were really off-line procedures. In 1983, Brown and Taylor (43) introduced a micro IR cell, 3.2 µl in volume, that they employed with a small bore column and claimed an overall increase in mass sensitivity of about 20 orders of magnitude relative to a standard 4.6 mm I.D. column. They also employed an FTIR spectrometer but

the actual improvement was obscured by the fact that the column length of the small bore column was significantly different from that of the standard column.

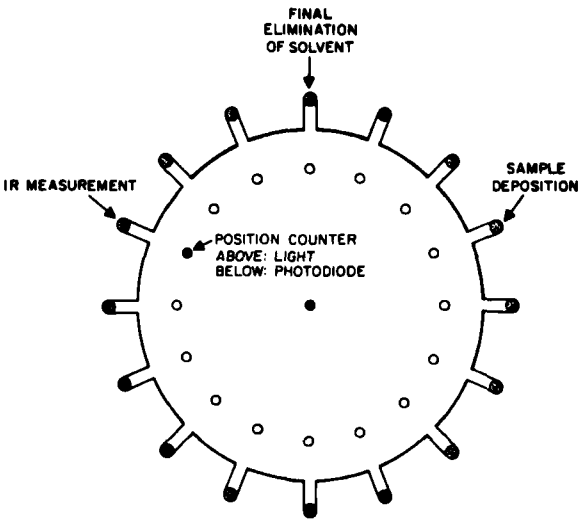

Figure 26. Carousel used for transporting the KCl sample cups used in HPLC/FTIR between the positions used for sample deposition, solvent elimination, and spectral measurement.

An alternative interface for the association of microbore high-performance columns with FTIR was described by Johnson and Taylor (44). They used their system to reduce the detection limits to below 50 ng. A diagram of their flow cell is shown in Figure 27. It consisted of a crystal element block of either calcium fluoride or potassium bromide, approximately 10 x 10 x 6 mm with a 0.75-mm cylindrical hole drilled through it. The crystal was sealed in place by the use of EM Science (Gibbstown, NJ, U.S.A.) microbore end fittings, which compressed the PTFE gaskets situated at either end of the crystal. The IR beam intercepts the flow path at right angles; the shape of the spectrometric viewing region is consequently circular in the cross section. Since the focal diameter of the Nicolet 6000C was 3 mm and the hole in the cell was 0.75 mm in diameter, a Barnes Model 600 4x beam condenser (Spectra-Tech, Stamford, CT, U.S.A) was used to reduce the focal diameter to approximately 0.75 mm. An aluminum cell holder, compatible with the beam condenser, was made to provide adjustment and cell alignment. From their studies of the cell the authors came to several conclusions. Firstly, it became apparent that although a maximum IR signal could be obtained by measurement at the chromatographic peak maximum, the maximum signal-to-noise was achieved by integrating spectra taken across $\pm$ 1.53 σ from either side of peak maximum. Secondly, they noted it was of greater benefit to modify the IR beam to fit a suitable chromatographic cell than to try to force fit the chromatographic system to an existing spectrometric system.

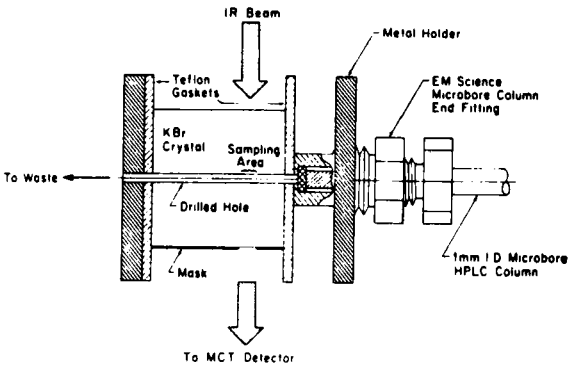

Figure 27. Zero dead volume micro-HPLC flow cell.

Conroy and Griffiths (45) developed a solvent extraction device that could be employed with a LC/FTIR combination. The device involved an extraction procedure that took the column eluent and continuously extracted the desolved solute into dichlormethane. The dichlormethane was then concentrated and finally dispersed onto a plug of potassium chloride powder. This device appeared to be a little clumsy and in fact, was really an off-line fraction collecting procedure.

Sabo et al. (46) developed an LC/FTIR system for both normal and aqueous reversed-phase chromatography using an attentuated total reflectance flow cell. The cylindrical internal reflectance flow cell contained a ZnSe crystal, had a nominal volume of 24 µl and an equivalent transmission cell path length of ca. 4-22 µm over the usable range. On-the-fly FTIR spectra of the components separated from 100-µl injections of mixtures 2% acetophenone and ethyl benzoate and 1% nitrobenzene gave clearly identifiable spectra. However, relative to other LC/FTIR systems this was not a very sensitive device.

Johnson et al. (47) developed a rather unique extraction cell for a LC/IR system. They used the segmented flow extraction of an aqueous effluent from a reversed-phase system and separated the extraction solvent by means of a hydrophobic membrane. A diagram of the layout of their apparatus is seen in Figure 28A. The system involved two pumps. One pump provided the solvent for chromatographic development and the second the extraction solvent system which could be either chloroform or carbon tetrachloride. After passage through the column, the two solvents met at a T junction and formed a segmented flow consisting of mobile phase and solvent. The membrane separator is shown in Figure 28B and was made from stainless steel. The connections were made to accommodate standard PTFE connectors. The volume on either side of the membrane was about 16-µl and the membrane itself was expanded with 0.2-µm pores. The membrane when compressed between the body of the extractor provided a seal between the two chambers.

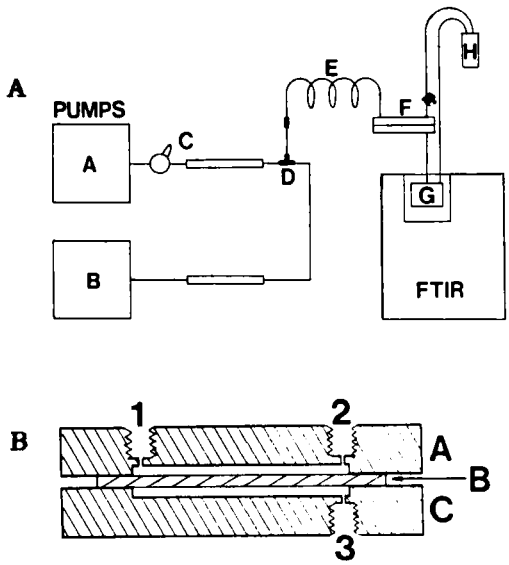

Figure 28. (**A**) Schematic diagram of RP-HPLC/FTIR instrument: A = pump delivering methanol/water solution; B = pump delivering extraction solvent; C = HPLC injector and C_{18} column; D = segmenting tee; E = extraction coil; F = membrane phase separator; G = zero dead volume flow cell interface; H = waste. (**B**) Cross sectional view of membrane separator: A and C = upper and lower stainless-steel membrane holders; B = 0.2-μm Gore-Tex membrane; 1 = segmented stream inlet; 2 = aqueous waste outlet; 3 = organic-phase outlet.

By regulating the differential pressure across the membrane by means of tubing clamps on the segmented stream outlet, the amount of organic solvent that was squeezed through the membrane could be controlled permitting the *exclusion* of the methanol/water phase. From the outlet of the extractor a 45-cm length and 0.3-mm I.D. PTFE tubing connected the outlet of the separator to the FTIR. The device obviously had very serious peak dispersion and could not be used with modern high resolving power columns. On-line spectra were obtained with less than 300 μg of injected sample. This, when compared with other devices, is a relatively poor sensitivity. Consequently, when the poor sensitivity is considered with the serious band dispersion of the system, its usefulness appear a little dubious. However, it does represent another novel system for obtaining IR spectra from LC column eluents.

In general, the situation with respect to LC/IR is very similar to that of LC/NMR. There is still inadequate sensitivity coupled with extensive peak dispersion in the interface systems so far described. Furthermore, whereas the LC/NMR combination can provide very valuable structure information for an eluted solute, in contrast the information provided from LC/IR is more limited. Nevertheless, interfaces with very significant improvement in both sensitivity and dispersion have been realized over the past few years. However, at present, IR spectra may well be more easily

obtained by off-line procedures involving the collection of fractions containing the peaks of interest and running the spectra off-line in the normal way.

Synopsis

Spectrometers are combined with *liquid chromatographs* to help *elucidate* the *structure* of the eluted *solutes*. The need for the spectrometer chromatographic combination has increased recently due to the improved resolution that can be obtained from modern LC columns and the consequent capability of handling extremely complex mixtures. There are four common chromatographic spectrometer combinations. *LC/UV, LC/FTIR, LC/MS and LC/NMR. LC/NMR* provides the *maximum structural information* and is the *least sensitive*, whereas LC/UV provides *minimum structural information* but it is the *most sensitive*.

The development of LC/NMR was only started in the late 1970's. There are a number of *problems* associated with the development of this combination, the major factor being the *low sensitivity* of the NMR instrument followed in importance by *its sensitivity to flow rate changes*, the need for the magnetization of the solvent system prior to measurement and the *interference of the solute spectra* with that of *the solvent*. Improvements have been slow and arduous and at present a resolution of about *0.5 Hz is possible* but, at best, *at least 50 µg* of material is required to provide an adequate spectrum. *The best system so far* developed resulted from the *insertion of a stainless-steel chromatographic column into the NMR spectrometer* itself without seriously disturbing the homogeneity of the magnetic field and consequently the resolution that was obtained. At the *present time*, the *value of an on-line LC/NMR system is questionable*. The alternative *off-line method* which involves collecting the peaks as they are eluted and running the spectra after concentration in a *more appropriate* solvent seems the *most practical*.

LC/MS is another *valuable system* for *structural elucidation* of eluted solutes. *LC/MS is not as comprehensive* in its structural information as NMR, but has a great advantage in that it is *far more sensitive*. There is some *incompatability between LC and MS* due to the fact that the sample is *eluted slowly* at a low concentration in the *column eluent*, whereas the *MS requires rapid sampling* at *high concentration*. There are two basic systems for LC/MS interfacing. The *direct inlet system*, where the eluent from the column passes directly into the mass spectrometer and the *transport system* where the solute is carried into the mass spectrometer by means of a moving wire or belt. The direct inlet system can be aided by various devices such as, *electrospray* and *thermospray*. In general, the *solvent is used as an ionizing agent* producing *chemical ionization spectra*. *Small bore columns* have been used with *success* with the direct inlet system and even the use of *open tubular LC columns* have been explored.

The transport system initially employed a wire as the transport medium but the commercial model employs *a belt* sometimes *made of plastic* and sometimes *made of metal. Solvent is evaporated* from the transport medium and the *resultant coating of solute* passes through appropriate vacuum locks into the *ion source* of *the mass spectrometer* where the *solute is vaporized. Alternative methods of coating* the transport medium has been investigated, *including spraying.* Alternative ionizing methods such as *secondary ion MS* and *laser desorption MS* has also been used with the *transport LC interface* and in one interface *both methods of ionization* could be *made available* as required. Both the direct inlet LC/MS system and transport interface system are commercially available and function well, albeit, with perhaps limited sensitivity. The *value of LC/MS is limited by the MS range of the spectrometer* itself, particularly when applied to biotechnology problems where the molecular weight of the solutes are often beyond the range of the MS.

The *combination of the IR spectrometer with the LC* is susceptable to *similar problems* to that of the *LC/NMR system.* However, *structural information* from an IR spectrum is *far less useful* than the NMR or MS spectra, in fact, for completely unknown substances *only functional group information can be obtained.* The IR system is *far less sensitive* then the MS and also suffers *interference* from *the solvent employed.* Choosing non-interfering solvents can improve the IR spectra but places far too great a limit on the efficient use of the LC. *Flow through cells* and *crude transport devices* have been tried and exhibit *varying degrees of efficacy. In general, LC/IR systems* developed at this time *leave a lot to be desired* and considering the limited information that the IR spectra provides, *collection of the peak in fractions,* removal of the solvent and re-desolving in an appropriate IR solvent *appears to be the preferential method for obtaining IR spectra.* Further developments in sensitivity and interfacing systems may make the on-line LC/IR devices more useful.

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CHAPTER 7

LIQUID CHROMATOGRAPHIC DATA ACQUISITION AND COMPUTER PROCESSING

The use of the computer to acquire and process the output from a liquid chromatography (LC) detector is now almost universal. Sometimes the computer takes the form of a simple integrater more often it involves the use of a microcomputer. The procedure can conveniently be divided into two parts: the process of data acquisition; and subsequently, the manipulation of the data to provide an analytical report presented in an appropriate form. Some LC instruments have a dedicated computer that is used solely for its own operational control, data acquisition and processing while others share a central computer. Central computers usually time share with five, ten, or even twenty different chromatographs and, as well as handling chromatographic data, also provide a laboratory housekeeping function, monitoring the progress of samples through the laboratory from admission to the final report. The housekeeping function of the computer will not be discussed as it falls outside the scope of this book and the subject of this chapter will be confined to the process of data acquisition, processing and reporting.

Data Acquisition

There are several basic components of a laboratory data system which are outlined in a simplified form in Figure 1. The detector provides an analogue output that is passed to a scaling amplifier and then on to an analogue to digital (A/D) convertor which delivers a digital equivalent to the analogue signal. The digital signal is then passed through a suitable interface to the computer which usually places the data in store.

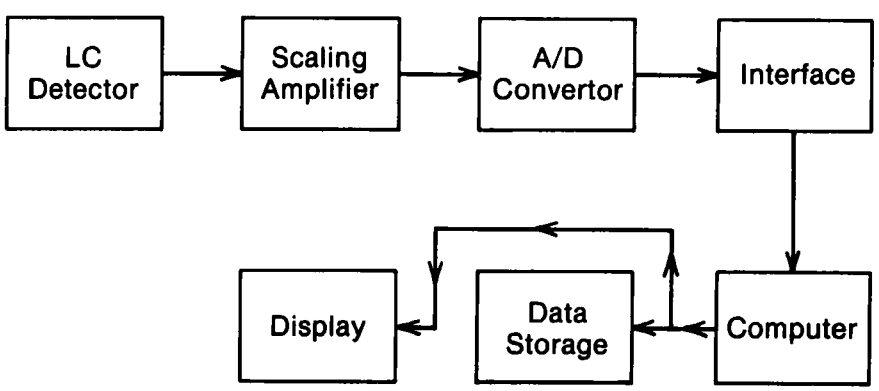


Figure 1. Block diagram of a typical data acquisition system.

The store can be a disc or the random access memory (RAM) of the computer itself. The computer will, at appropriate intervals, take the data, process it and then display it on a CRT screen or printer as required. The data can be processed "on the fly" in which case the intervals may be only a few milliseconds or, at the end of the chromatogram, in which case the interval may be several minutes or perhaps more than an hour.

The acquisition of the data and its manipulation prior to it being stored by the computer will now be discussed. In detail, the acquisition system must access the analogue output from the detector, which will usually be in millivolts, scale the output to a suitable voltage range (usually in volts), convert this analogue voltage to a digital equivalent and then transfer the digital value to a defined location in the computer memory. The scaling operation, which is necessary to ensure the voltage has a magnitude suitable for digital conversion, is usually achieved by the use of an operational amplifier (OP-AMP) or in some instances an autoranging amplifier. An autoranging amplifier is one that automatically adjusts its amplification factor to keep the output voltage within certain limits while simultaneously providing an output code that identifies the amplification factor being used. The disadvantages of such an amplifier over the simple OP-AMP lie in its expense and the need for a somewhat more complex program to handle the data satisfactorily. The advantages of the autoranging amplifier is that it can cope with a signal having a wider dynamic range with the same resolution for small signals as it has for large signals. Consequently, it is more often found in the more sophisticated data acquisition systems. Due to the fact that LC detectors generally do not have a linear dynamic range of much more than three or four orders of magnitude the less expensive approach is to take the recorder output and use the OP-AMP as the scaling amplifier. Such a system would probably be adequate for over 95% of all LC analyses.

The A/D Convertor

After the signal has been scaled to an appropriate value it must be converted to digital form. There are a number of ways of achieving this A/D conversion. These include the sample and hold/successive approximation convertor, the dual slope integrating convertor, the single slope integrating convertor and the voltage to frequency (V/F) convertor. A detailed discussion of the advantages and disadvantages of different A/D convertors is also not germane to this book and consequently only the single V/F convertor will be described as an example. Readers wishing to know more on the subject of A/D convertors are recommended to read the book, Data Acquisition and Conversion Handbook (1). A diagram of an A/D convertor is shown in Figure 2.

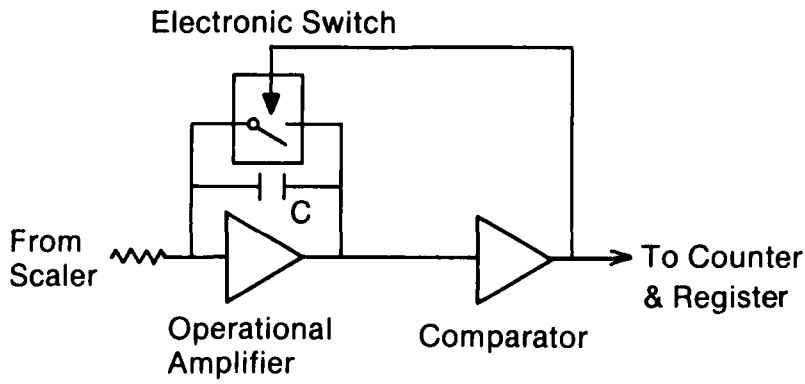


Figure 2. The basic A/D convertor.

It consists of an integrator that could be constructed from an OP-AMP with a feedback condenser which is charged by the input voltage from the scaler amplifier. The output from the integrator passes to a comparator which activates when the potential across the condenser (C) reaches a preset voltage. The activation of the comparator causes a pulse to pass to a counter and at the same time, activates an electronic switch that discharges the condenser and thus cause the process to start again. Obviously the time taken to charge the condenser will be *inversely* proportional to the applied voltage and consequently the frequency of the pulses from the comparator will be *directly* proportional to the applied voltage. The frequency generated by the voltage controlled oscillator is sampled at regular intervals by a counter which then transfers the number of counts in binary form to a register. The overall system is shown diagrammatically in Figure 3. The output from the detector of 1.7 mV (10 mV maximum output) is scaled by an appropriate amplifier to 0.17 V (1 V maximum output). The analog signal is converted to its digital equivalent, sampled by a counter, and then fed to an 8-bit register. The 8-bit register has a maximum decimal count of 255 and thus the decimal equivalent of the register output of 0.17 V would be 43 (i.e. 43/255 x 1 V ≃ 0.170 V). The data contained by the register must now be regularly sampled and passed to the computer.

Transmission of the Data to the Computer

After the analog signal has been converted to a digital form, the next step is to make the digital value available to the computer. There are two major modes of signal transmission: *serial* transmission and *parallel* transmission. The first to be considered is the serial transmission mode.

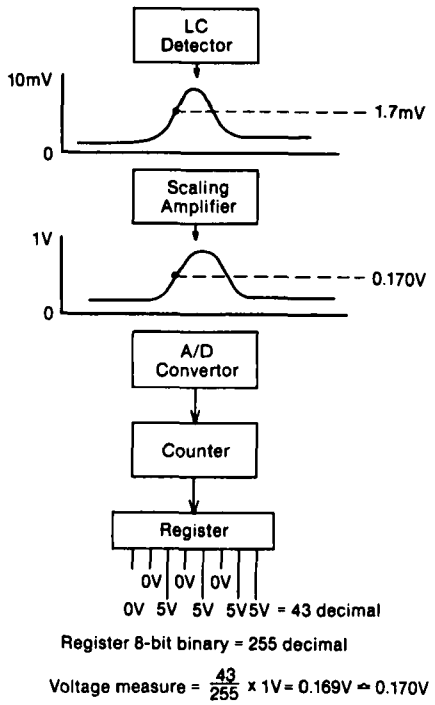


Figure 3. The digital conversion of an LC detector output.

Serial Transmission

As the name *serial* implies, in this mode the digital word is sent to the computer one bit at a time. The output of a binary counter is a parallel output since each of the output bits has its own data output line and the output value for each bit is simultaneously available. To use a serial transmission scheme, this parallel output must be converted to a serial form. One way to accomplish this transformation is to use a Universal Asynchronous Receiver Transmitter (UAR/T). The heart of the UAR/T is a shift register. The detailed operation of the UAR/T will also not be given here and those interested in pursuing the subject further are referred to a paper by Reese (2) on data acquisition and processing. It will be sufficient to say that the shift register is strobed by a signal from the computer that displaces the binary number bit by bit sequentially from the register to the computer.

The serial data transmission mode finds its greatest use in multiple detector/convertor systems where data must be sent over moderate to long distances. It is easy to implement, has good noise immunity, and is quite reliable. Its main disadvantage is its moderate speed of transmission, around 3000 bytes/sec maximum, which might limit its use in some very high-speed applications.

Parallel Transmission

The second mode of data transmission to be considered is the parallel mode. In this type of data transmission the outputs of the counter are directly connected to a peripheral interface adapter (PIA) and thence to the computer data bus. The computer data bus is the parallel system of conductors by which the binary data is transferred between the central processor, memory, and peripheral circuits. As the data bus is used for all data transfer and each transfer involves different voltage levels, the data bus cannot be connected continuously to the register of the A/D output. Isolation is achieved by a series of dual input and-gates with tristate outputs, one gate for each data bit. This system only allows the data on the input to appear at the output on reception of a signal from the computer. An and-gate is a device, the output of which will be identical with the logic state of one input on applying a logic 1 voltage to the second input. The operation of the and-gate system is shown in Figure 4.

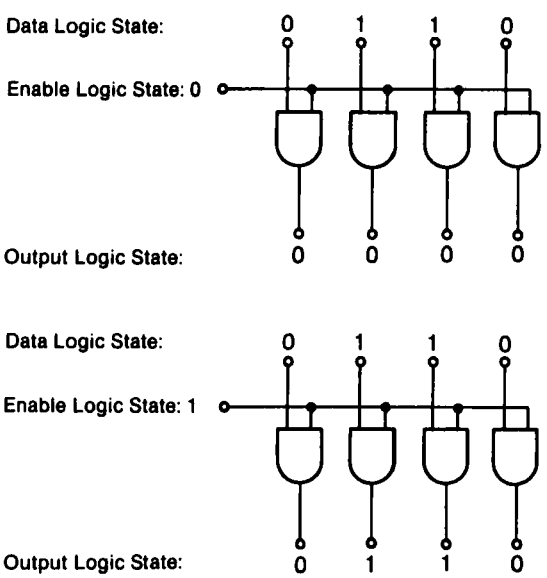


Figure 4. The function of the and-gates in the PIA.

In the upper diagram the digital output from the A/D convertor appears on one of the inputs of each and-gate, and as the second inputs are inactive the output of all gates is logical zero. On activating the second input of each gate with a logical 1 voltage (signal sent by the computer when requiring data), the logic state of the output of the gate is identical with the logic state of the first input. As soon as the data has been read into the computer accumulator, the activating voltage (enabling pulse) is removed and the output of all the gates returns to zero. The data now located in the accumulator is then transferred to either the

computer random access store or to a buffer store and then to a tape or disc.

The parallel data transfer mode is simple to use and allows for greater flexibility than the serial mode. However, it requires numerous connections between the PIA and the A/D convertor, and is, therefore, limited to those cases where the convertor and computer are very close. This type of transmission is very popular in instruments which have a built-in dedicated microprocessor.

At this point the data acquisition function has been completed. The information concerning the nature of the separation and the quantity of material separated resides in the computer memory as a series of binary words. Each binary word represents the detector output at a given time and each consecutive word represents the passage of the same interval of time. Typically, one chromatographic data point will occupy 2 words of memory (16 bits) and thus a chromatogram, 20 min long, acquired at one data point per second, will occupy 2400 words of memory. Consequently, by appropriate programming the chromatogram can be reconstructed from the data, in its original or modified form and thus permits different aspects of the separation to be examined in detail. For those readers who wish to pursue the subject further, the book by Wilkins et al. (3) and Malmstadt and Enke (4) are recommended. Those who wish to design their own data acquisition system would find the book by Klingman (5) helpful. The complete circuitry and software necessary to employ a Commodore 64 computer to acquire and process chromatographic data has been described by Lyne and Scott (6) which provides a very low cost data acquisition system for any existing chromatograph.

Data Processing and Reporting

Data from the chromatograph can be manipulated in two ways. The data can either be processed on arrival at the computer and only the processed data such as retention time or peak area stored in the computer memory. Under these conditions the data is said to be processed "on the fly" and has the dubious advantage of providing results immediately a peak is eluted. This procedure is certainly less expensive as it requires little computer memory and is often employed where the mircoprocessor has very limited RAM associated with it. It has the very significant disadvantage of not allowing the data to be processed by alternative methods to provide more accurate results. This is because the raw data (the actual detector output data) is discarded immediately after processing and its not available for further manipulation.

The alternative form of data processing is to store each detector output value as it is received by the computer in RAM or, if desired, on disc and to process the data after the chromatogram

has been completed. This procedure, that permanently stores each raw data point, allows any number of different method of reducing the data to be used for retention time calculation and peak area or peak height measurement and further allows the chromatogram to be reconstructed. Moreover, not only can the chromatogram be reconstructed, specific parts of the chromatogram can be selected for display, the time axis can be contracted or expanded to suit particular reporting formats and the peak heights can be amplified to emphasize the presence of trace impurities. Some data processing software provides both options although, if total raw data storage is one option, the alternative seems hardly worth choosing.

Data Processing

The first and most important processing function that all software has to perform is peak detection. The start of a peak can be identified by a significant change in signal (the change having a magnitude that can be defined by the user) or by a previously defined increase in rate of change of signal. The start of the peak having been identified, the integration of the peak can be commenced, and in the next step the peak maximum has to be identified. The peak maximum is identified as that point where the difference between two consecutive data points is negative (the first derivative of the signal is negative) or some refinement of this condition. The number of this data point (not its magnitude) is taken as representing the retention time of the peak. The end of the peak is identified as that point where the difference between consecutive points is less than a previously defined value (the magnitude of the first derivative of the signal has reached a minimum negative value) and at this point the integration is arrested and the value of the integral stored.

Such a procedure provides the times of the peak start, maximum, and end of the peak, together with the peak height and the area. The simple process outlined has many obvious shortcomings; for instance it does not compensate for baseline drift on the area measured. Baseline drift corrections can be accomplished in the following manner and can be understood by referring to Figure 5. The raw, uncorrected, digital values representing the detector output are summed from the point where the peak was first sensed to the peak end. This is equivalent to the sum of all the Y values over X from X_1 to X_n in Figure 5. The average value of the baseline (Y_B) is obtained by adding Y_1 to Y_n and dividing by 2. This average value (Y_B) of the baseline is multiplied by the number of values accumulated as the peak integral, n, and this produce ($n\ Y_B$) is substracted from the raw peak area to give the corrected area.

The data processing techniques so far discussed have been basic and relatively elementary with respect to handling chromatographic data.

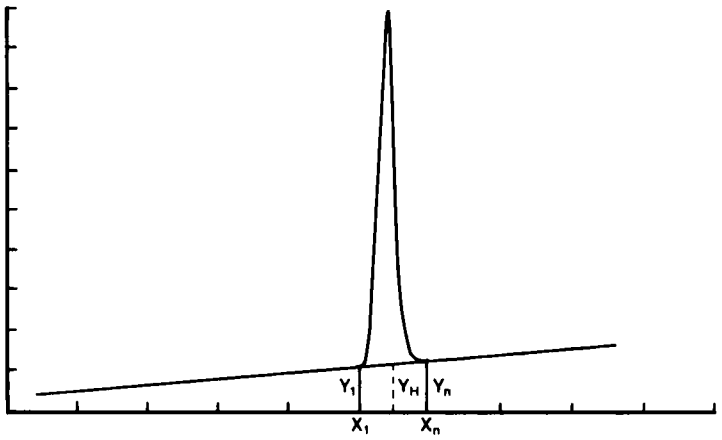


Figure 5. Baseline drift correction.

A more detailed treatment of the programming process is not relevant to this book but one or two of the more important and sophisticated techniques should be mentioned. The processing program should be able to identify peak valleys between unresolved peaks (where the first derivative of the signal becomes positive before the baseline is reached), peak skimming should also be possible where peaks are detected on the tail of a major peak and their height and area must be assessed. Correction procedures for irregular baseline drift that results from temperature programming or solvent programming development techniques should also be available. It should be borne in mind, that however clever and sophisticated the data processing program may be, the results can never be better than that provided by the chromatographic process itself or the specifications that the detector will allow. If the detector has only a linearity of three orders of magnitude, the accurate assessment of a component four orders smaller than the reference standard must always be suspect, even though the data processing program itself will appear to provide a precise result.

Data Acquisition Parameters and Chromatograph Control

In most chromatographic systems that have a dedicated computer associated with it, the computer will also control the operation of the chromatograph and therefore the pertinent operating conditions have to be entered, via the computer keyboard in an appropriate manner. Furthermore, when the data is reported, these operating conditions should also to be reported.

Data that should be entered would be, mobile phase flow rate, initial solvent composition, final solvent composition, program period, program function and detector sensitivity. At the same time, the data acquisition conditions also have to be entered and

these would include data acquisition rate, start time, end time and possibly other information depending on the nature of the data acquisition system. There should also be provision for such basic information as date, time, sample name, sample source and the name of chemist submitting the sample to be entered. A print out of the operating conditions might take the form of that shown in Table 1.

TABLE 1

CHROMATOGRAPHIC OPERATING CONDITIONS

Date: 11/2/83 Time: 14:23
Sample: Chlorestrol: No. 1335 - Chemist P.D. Smith: Laboratory 72
Detector Sensitivity x 1 Data Acquisition Rate: 5 dp/sec

SOLVENT PROGRAM

Solvent A	Methanol
Solvent B	Water
Solvent C	-
Solvent D	Acetonitrile

METHOD 10

PUMP SECTION

Step	Time	Flow	%A	%B	%C	%D	Curve
0	10.0	2.00	20.0	70.0	0.0	10.0	
1	15.0	2.00	70.0	10.0	0.0	20.0	2.0
2	5.0	2.00	70.0	10.0	0.0	20.0	
3	3.0	2.00	20.0	70.0	0.0	10.0	2.0
4	2.0	2.00	20.0	70.0	0.0	10.0	
5	2.0	2.00	20.0	70.0	0.0	10.0	

Maximum Pressure 5,000 psi

The first part of the table is self explanatory. The second part headed Method 10 is a printout from the Perkin-Elmer Series 410 LC pump. The pump can accommodate 5 steps in its program operation and program four different solvents A, B, C, and D. In the table the initial solvent composition was 20% A, 70% B and 10% D and was allowed to pass through the system at a flow rate of 2-ml/min for 10 min. After 10 min the composition is changed to 70% A, 10% B and 20% D. This new solvent eventually passes through the system after a period of 15 min and the function of the solvent program during this period is defined by the number 2 which may permit either linear, logarithmic or exponential change in solvent composition with time as required by the specific separation

being carried out. The second step for 5 mins maintains the same solvent composition and represents an isocratic development period. In steps 3, 4 and 5, the solvent is returned to its initial composition to ensure that the column is re-equilibrated in readiness for the next analysis. It is seen that the program given is relatively simple, but could, if required, be extremely complex involving multiple stages of development and several different program functions.

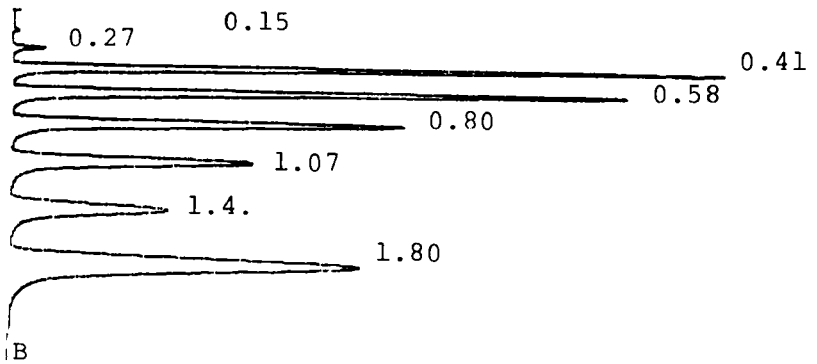
Having operated the chromatograph under defined conditions; and achieved the required separation, the data in store can now be processed in a suitable manner. The method used for the data processing varies greatly from one data system to another. For example, the magnitude of the signal that is necessary to discriminate it from the background noise and thus indicate the start of a peak must be defined; the choice of either peak areas or peak heights for quantitative evaluation must also be selected; the response factors for the individual components to be determined may have to be entered; the conditions for measuring areas of unresolved peaks under specific conditions, for example where the peak is skimmed from the tail of a much larger peak must be defined in order to extract the area of the unresolved peak. An example of a print out of an analytical report is shown in Table 2. The example is taken from the Perkin-Elmer LC 100 integrator.

The file number and the run number is printed at the top of the report to identify the sample being analyzed. The date, and time the analysis was started, is also reported and on the second line the method used and the date and time the method was last edited is included. This information is followed by a display of the chromatogram. At the top of each peak a label giving the retention time is printed. The analysis is then reported and, in order that the analysis can be separately filed apart from the chromatogram, the re-integration method is again printed out together with details of sample, and times and dates of the analysis. The basic data are reported in tabular form. On the left hand side is the retention time of each peak given in increasing order of elution. The next column gives the area of each peak and the third column the height of each peak. By the side of the peak height data are codes describing how the baseline was constructed. In the given example, for the first peak the symbol V means that the baseline was drawn valley to valley between the peaks. The symbol T means that the baseline was projected to the right from the last baseline reference point to the next baseline point. Various other codes are available such as BC, which is short for baseline correction and allows heights and areas to be measured when the peak is eluted as a small component on the tail of a major component. Various program systems use different codes. In the next column, the area percentage is reported which is the result of a normalization

TABLE 2

REINTEGRATION WITH METHOD 3

FILE 26 RUN 1 STARTED 15:00.9 85/11/14
% METHOD 3 LASTEDITED 15:00.3 85/11/14



REINTEGRATION WITH METHOD 3
FILE 26 RUN 1 STARTED 15:00.9 85/11/14
% METHOD 3 LAST EDITED 15:00.3 85/11/14

RT	AREA	HEIGHT	BC	AREA PERCENT	HEIGHT PERCENT
0.15	4822	3.5002	V	0.1154	0.2923
0.27	29276	15.8534	T	0.7009	1.3239
0.41	628873	339.2357	T	15.0560	28.3283
0.58	732895	292.9185	T	17.5464	24.4606
0.80	625354	187.3305	T	14.9718	15.6433
1.07	506062	115.8603	T	12.1158	9.6751
1.41	427806	75.2895	T	10.2422	6.2872
1.80	1221801	167.5254		29.2515	13.9894

8 PEAKS > AREA REJECT 4176890 TOTAL AREA
8 PEAKS > HEIGHT REJECT 1197.5136 TOTAL HEIGHT

procedure, each area being reported as a percentage of the total area.

In the next column the same is carried out for peak heights, the height of each peak being reported as a percentage of the total peak height. At the bottom of the table the number of peaks that are greater then the area reject signal is reported. This means that a peak area has to exceed a given value before it can be identified unambiguously as a peak or, alternatively, the signal-to-noise ratio of a peak area must exceed a given magnitude. The total peak area is also reported. The same is also

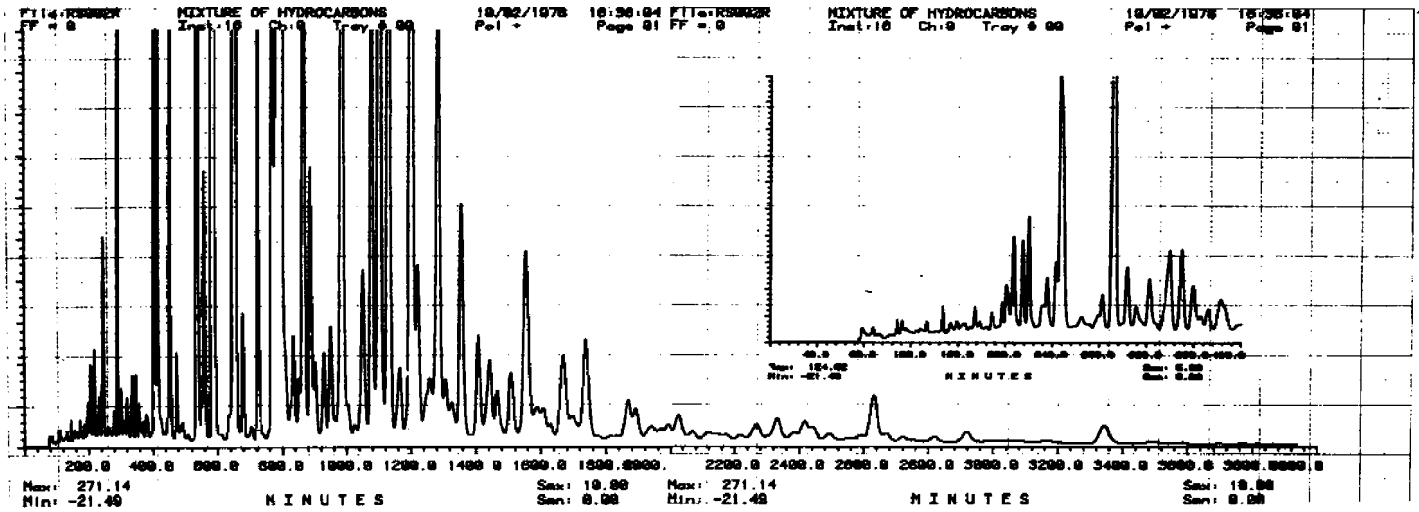


Figure 6. Chromatogram of aromatic hydrocarbons separated from coal.

carried out for the peak height measurement. Unless the height of a peak has a magnitude greater than a predefined value greater then the noise level, it is not identified as a peak. The two methods of confirming the identity of a peak are used to ensure that no small peaks are ignored and conversely, long term noise is not interpreted as a peak. The total peak height is also reported. Should a calibration factor be used to relate the area of two peaks to an absolute percentage value then this is entered into the system before processing.

Presentation of Chromatograms

The LC-100 is a relatively simple data processing system and with more sophisticated equipment and software, chromatograms can be presented in a more complex way. An example of a chromatogram presented from a CIS system is shown in Figure 6. Figure 6 depicts a chromatogram of the aromatic hydrocarbons that can be obtained by the solvent extraction of coal. The chromatogram illustrates several interesting programming features. First of all it represents a 2 1/2 day chromatogram employing a column with an extremely high resolving power and consequently requires the concatenation of a number of chromatograms into a single file. It should be noted that there are 150 individual substances identifiable from the chromatogram. Examination of the major chromatogram reveals that it is very difficult to identify any of the individual peaks eluted early in the chromatogram. They are drawn so closely together that they appear as noise and not individual peaks. One of the attractive features of the software is that the first 300 min can be expanded to demonstrate the integrity of the separation and confirm the identification of the peaks. This is shown as an insert in the chromatogram. The advantages of being able to expand part of the chromatogram with a greater scaling factor is very obvious. Another example of the same system is shown in Figure 7 and this is a chromatogram of cinnamon bark oil separated on a 10 m x 1 mm I.D. microbore column, the detector was a fixed wavelength UV detector with a very small cell volume to accommodate the characteristics of the microbore column. However, in the major chromatogram there are only two peaks of a size that permits confirmation of their unique resolution. It is seen from the insert that two portions of the chromatogram have been expanded, both with respect to the retention time axis and also the detector output axis where in the original chromatogram the jumble of peaks appear at the noise level of the detector. The expanded section clearly demonstrates that excellent resolution was obtained for the early components and that they have solute profiles of high integrity. The last peak marked B in the chromatogram was eluted at a k' of about 50 and would normally be hardly discernable from the baseline due to its inherent dispersion. However, again expanded on both the time axis and the detector output axis, the peak can be examined and it is interesting to note the peak is very symmetrical even though it was eluted at a k' of about 50.

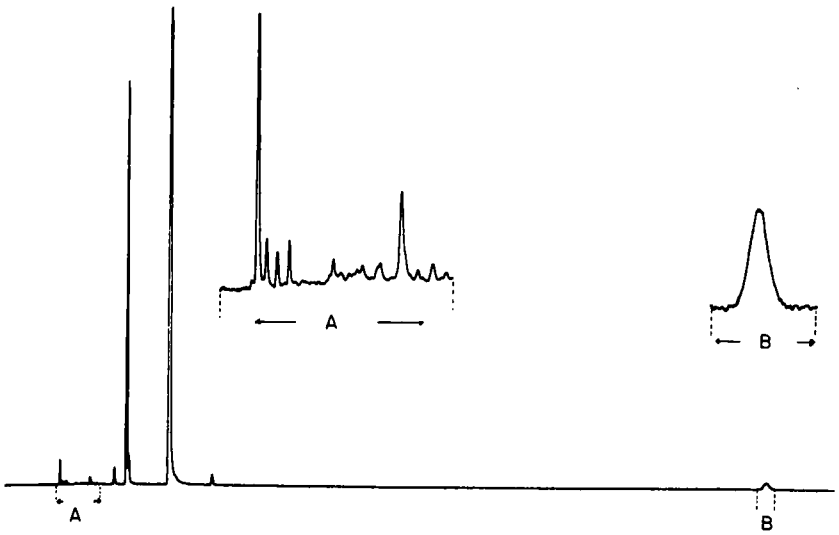


Figure 7. Chromatogram of cinnamon bark oil.

One further example of the value of more sophisticated presentation software is shown in Figure 8. The upper chromatogram is the same as that shown in Figure 6 and again the confirmation of the early peaks relative to the noise is extremely difficult. In the lower chromatogram the time axis is modified by the power function of 1/4. This in effect expands the early part of the chromatogram and contracts the latter part. The lower chromatogram gives an excellent illustration of the total separation across the whole chromatogram where the upper chromatogram does not. The individual peaks in the early part of the chromatogram are clearly identifiable when expanded by the fractional power function. It should be borne in mind, however, that a chromatogram of this kind can not provide quantitative information on a peak area basis unless the area obtained is corrected for the retention time modification by the power of one quarter.

It is clear that data acquisition and data processing does not only provide information more rapidly but also more precisely and, furthermore, can present a chromatogram in a far more meaningful way. With the advent of the modern inexpensive microprocessers, sophisticated data acquisition and processing systems will become readily available at very low cost and consequently will become a feature of virtually all liquid chromatographs. However, it should again be emphasized that the accuracy and precision of the results from computer data acquisition and processing systems can only be as good, and *not better*, than that of the raw data produced by the chromatograph system and the detector.

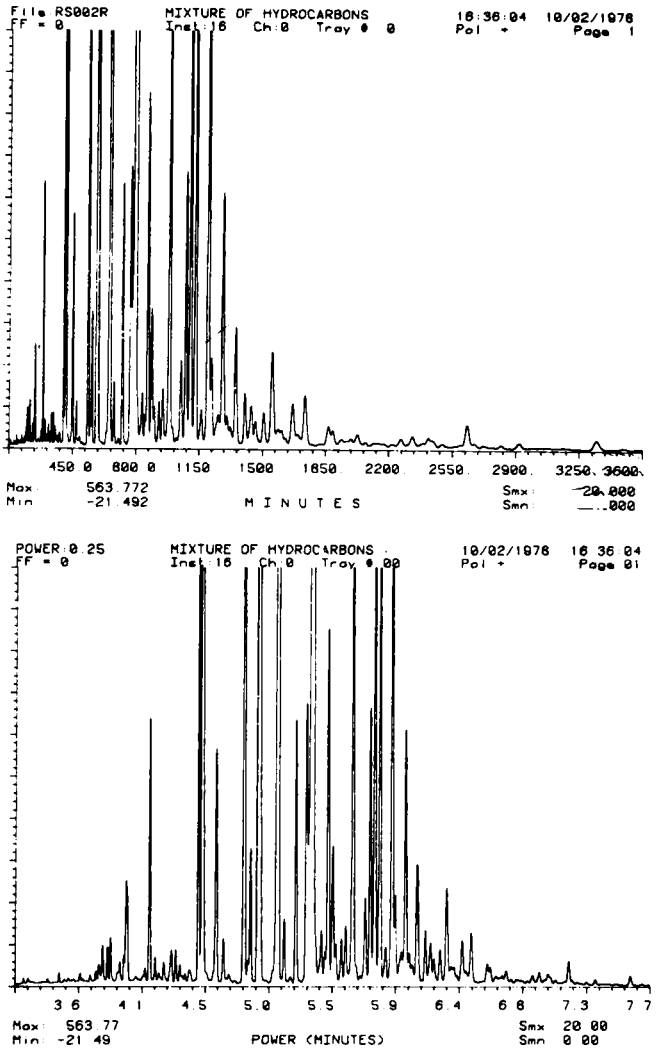


Figure 8. Chromatogram with modified time base.

Synopsis

The total *computer processing* of *liquid chromatography data* consists of *two parts*: *Data acquisition* and *data processing*. Data acquisition requires firstly that the output from the computer is *scaled to a suitable voltage* that will permit *analog to digital conversion*. There are a number of different types of A/D convertors. The *simplest* being the *voltage to frequency convertor*. The signal converted to its digital form *finally resides in a register*. The register has a parallel output of binary data that is transmitted to the computer by serial transmission using a *asynchronous receiver transmitter* or by

parallel transmission using a *peripheral interface device* that isolates the register from the data bus of the computer. The data can be stored either in the RAM of the computer or on a tape or a disc peripheral store. The *computer* sometimes also *acts* as a *controlling system* for the chromatograph and thus appropriate instructions must be fed into the computer as to the manner in which the chromatograph is to be operated. In the reporting of the data the operating conditions are first printed out together with such sample information as the name of the analyst, the date and the time. When the data is processed it is used firstly to *provide the chromatogram* and subsequently the *area and peak height analysis*, either by *normalization* or by area and peak height *comparison with a standard*. There are a number of software methods of presenting the chromatogram that can include the expansion or the contraction of either the time axis of the chromatogram, or the detector output axis. Either the detector output axis can be *amplified* to *emphasize the presence of minor components* or alternatively the *time axis* can be *extended* to show the resolution of *closely eluted peaks*. An interesting alternative is to *modify the time axis* by *a power function* of less than unity so that the *beginning of the chromatogram is expanded* and the latter part of the chromatogram *is contracted*. Such a procedure allows the whole of the chromatogram to be displayed with equivalent discrimination along the entire length of the chromatogram.

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CHAPTER 8

THE SELECTION OF THE APPROPRIATE DETECTOR

There are four commonly used commercially available detectors from which the liquid chromatographer can choose for any particular application. They are, namely, the refractive index detector, the conductivity detector, the UV detector and the fluorescence detector. The most commonly used detector is the UV detector followed in popularity by the refractive index detector. These two detectors are the "work horse" detectors of liquid chromatography (LC), any others being chosen for very specific applications. No single detector can perform as a universal detector capable of handling all types of separations problems, but in the author's experience, the UV detector has the greatest versatility, which, combined with high sensitivity and wide linear dynamic range, should, therefore, be the liquid chromatographers first choice in the selection of his armory of detectors.

The UV Detector

The UV detector can only be employed to detect those substances that absorb in the UV range of wavelengths but, as already discussed, the majority of substances do absorb to some extent in this range. However, if the liquid chromatographer does have some specific application in mind, he should check that the substances of interest absorb UV light at an appropriate wavelength. The UV detector has a fairly high sensitivity and, thus, can be used for the detection of trace components in a mixture and because it has a relatively wide linear dynamic range, it can be used for both qualitative and quantitative analysis. Its relatively high sensitivity in general makes it unsuitable for detection in preparative chromatography, but if a variable wavelength UV detector is available, a wavelength can often be chosen where the solutes exhibit relatively little adsorption, thus reducing its sensitivity and making it more appropriate for detection in preparative work. The UV detector, however, does not lend itself to solvent gradient elution with silica gel columns as those solvents that are convenient for use often adsorb to different extents in the UV and, thus, during gradient development, the base line is irregular or exhibits significant drift. Rabel (1) suggested a series of solvents for gradient development, that would be used with the UV detector that minimized the drift and base line irregularities but unfortunately did not eliminate them. Although in general, the UV detector is at a disadvantage when used with gradient elution development for silica gel columns it can be used very effectively for reversed-phase separations where the solvents used (methanol, acetonitrile, tetrahydrofuran and water) do not absorb at the UV

wavelength normally employed. In ion exchange chromatography, changes in buffer concentration frequently do not affect the base signal of the UV detector anything like the extent that non-polar solvents tend to do. Thus, the UV detector can also be used effectively under gradient elution development in ion exchange chromatography. Modern UV detectors have small cell volumes and small diameter connecting tubes, and so they can be effectively used with most modern high-efficiency microparticulate columns without impairing the resolution obtained. UV detectors having high sensitivity, operating at one or perhaps two fixed wavelengths are available at a range of prices that make them available to most laboratories interested in LC. Unless the chromatographic analysis is to be restricted to very specific applications, the UV detector is recommended as the first choice for general chromatographic work.

In the vast majority of straight forward analytical applications the performance of the fixed wavelength detector will be found to be perfectly adequate and its suitability should always be carefully examined before the purchase of more complex UV detectors is considered. If a variable wavelength detector is deemed necessary then either the dispersion instrument or the diode array detector could be the possible choice. In the remote case where UV spectra are required 'on the fly' then the diode array detector should be considered. However, the chromatographer should avoid choosing expensive detectors which provides many facilities that *might* be required but, in fact, in practice will *never be used.*

The Refractive Index Detector

The refractive index detector, being a bulk property detector, has a significantly lower sensitivity than the UV detector but more nearly approaches the universal detector in the sense that it will detect any solute that has a refractive index different from that of the mobile phase. The presence of substances that do have the same refractive index as the mobile phase can easily be checked by changing the refractive index of the mobile phase without changing significantly the polarity characteristics of the mobile phase. Thus the sequence of elution of the solutes will remain the same. For example, if the mobile phase consists of a specific mixture of hexane and ethyl acetate, the hydrocarbon component can be replaced by heptane or octane, and the mixture re-chromatographed. The solvent mixture will now have a different refractive index, and any solute that had the same refractive index as the hexane/ethyl acetate mixture will be detected without significantly changing the elution order of the solutes. The refractive index detector, as well as being less sensitive than the UV detector, has a smaller linear dynamic range, perhaps only two to three orders of magnitude and is thus less suitable for the quantitative analysis of a mixture having solutes present over a wide concentration range. Due to its

relatively low sensitivity it is commonly used as the detector in preparative LC and can be recommended for such use. It also finds a major application in the detection of solutes that do not have UV chromophores. It is often used as the detector in gel permeation or, more correctly, exclusion chromatography, particularly in the separation of hydrocarbon polymers, carbohydrates and polypeptides, although polypeptides can be detected with the UV detector. The refractive index detector is even less suitable for gradient elution development than the UV detector and is not even suitable for use with gradient elution in ion exchange chromatography. The refractive index detector is a robust detector and at low and intermediate sensitivities is generally simple to operate and is relatively inexpensive. It is recommended as the second choice in LC detectors but this of course can not be an arbitrary choice. Any detector chosen must be appropriate for the solutes in the mixtures to be analyzed. The refractive index detector suffers from three major drawbacks in practice. It is very pressure sensitive and flow sensitive and therefore, at maximum sensitivity must be used with a very stable pulse free pump. The detector is also very sensitive to ambient temperature changes and therefore must be well thermostated and when operated at maximum sensitivity preferably be situated in a temperature controlled room. Finally, the refractive index detector must be operated under isocratic conditions and then only after adequate time has been allowed for the column to come into equilibrium with the mobile phase. Despite these limitations, however, the refractive index detector has areas of application where no other detector can be effectively used.

The Fluorescence Detector

The remaining two popular, commercially available, LC detectors will now be discussed, but an order or preference will not be given. The choice of these detectors will depend solely on the nature of the mixture the chromatographer is required to separate. The fluorescence detector is very specific, detecting only those substances that fluoresce, but, as a result of its specificity, it can have an extremely high sensitivity. However, although it has a very high sensitivity, it has a relatively poor linear response and has a linear dynamic range which may be less than two orders of magnitude. For quantitative work, it is strongly recommended that the detector is calibrated against known standards over the complete concentration range that it is to be used. Its major area of application, (besides the detection of those substances that naturally fluoresce) is for the detection of primary amines, in particular, amino acids and peptides by employing an appropriate fluorescing reagent such as Fluoropa or Fluorescamine. This may necessitate the use of a post column reactor which must be carefully designed to minimize band dispersion. Fluorescence detectors are available at a range of prices, but an effective and reliable detector operating at a fixed excitation wavelength can be obtained at a relatively low

cost. If the fluorescence detector purchased, is combined with a UV detector and/or other functions, the cost of the dual or ternary detecting system will be more, but still considerably less than that of two or three individual detectors. The fluorescence detector is recommended for use where high sensitivity is required and where solutes naturally exhibit fluorescence or, as already stated in conjunction with an appropriate fluorescing reagent. The main disadvantage of the fluorescence detector, besides its restricted linear dynamic range, is its sensitivity to trace contaminents in the mobile phase that fluoresce. Such mobile phase impurities can produce both long term noise and baseline drift. Nevertheless, the fluorescence detector is one of the most sensitive LC detectors available.

The Electrical Conductivity Detector

The electrical conductivity detector is another sensitive bulk property detector that has special areas of application. The detector can only be used to detect ionic substances and thus, the mobile phase must be an ionizing medium, and this usually takes the form of aqueous solutions sometimes containing a polar solvent, together with an appropriate buffer. The mobile phase itself can have a reasonably high conductivity, as the detector can function at reasonably high sensitivities even in the presence of a significant base signal but is unsuitable for gradient elution involving changes in buffer concentration or changes in any other ionic content of the mobile phase. The detector can be made very small in volume and thus, band dispersion in the conductivity cell can be made minimal and not impair the performance of high-efficiency microparticulate columns. This detector can be recommended for use in detecting all ionic materials and is probably the most inexpensive detector available either as a mono-functional detector or as a multi-functional detector. The disadvantages of the conductivity detector become apparent under conditions where the mobile phase exhibits very little conductivity. Under these circumstances, traces of dissolved carbon dioxide or ammonia can provide a significant background signal and thus effect the magnitude of both the long term noise and the drift. This, however, is not so much a detector problem but that of chromatographic hygiene. Carbon dioxide can be removed by displacement with helium gas whereas ammonia can be removed by adsorption on an appropriate ion exchange resin. Nevertheless, despite these disadvantages, the conductivity detector provides the maximum sensitivity to ionic compounds generally that do not posses unique solute properties such as fluorescence or oxidizing or reducing properties.

Summary

In choosing an appropriate LC detector, the UV detector and the refractive index detector can be recommended for general work, where a wide range of different solute mixtures are required to be

separated and analyzed. Other detectors should only be chosen because their functional characteristics specifically suit the type of samples that are to be analyzed. A versatile LC laboratory would employ both UV and refractive index detectors and have at least two other specific detectors available if required.

Practical Hints on Detector Operation

The basic functions and operational procedures for using LC detectors are normally given in the manual supplied with the equipment. However, in the operation of the apparatus for practical LC analyses, the instructions given by the manufacturers are sometimes inadequate. The following points may be of value to the practicing chromatographer to help him realize the full potential of his equipment.

Detector Sensitivity or Attenuation

The maximum sensitivity or minimum attenuation of a detector should never be used unless called for by the nature of the sample. The maximum sensitivity is only necessary where the amount of sample is limited, the sample concentration is very small, or the column capacity is, for some reason or other, seriously restricted. Such conditions are rare in the extreme. Consequently, the lowest (not the highest) sensitivity should be chosen that will provide the necessary separation without overloading the column. There is absolutely no advantage, *per se*, in operating a detector at its maximum sensitivity. In contrast there is every advantage in not doing so. At low sensitivity, the operation of the detector will be more stable, it will exhibit less noise, be less suceptible to changes in ambient conditions and consequently, if operated within its linear dynamic range, will give more precise quantitative results. There is often an irrational desire on the part of the inexperienced chromatographer to operate, at all times, at maximum detector sensitivity. This is quite contrary to the efficient use of an LC detector.

Detector Cells

The refractive index detector and occasionally the UV detector are provided with sample cells and reference cells to obtain a balanced output. The two cells must provide similar signals to the photocells and amplifier, therefore the reference cell must be filled with the mobile phase being used with the column. If the column eluent is changed, then the reference cell must be filled with the new mobile phase. Some workers when using non-absorbing mobile phases such as heptane in conjunction with a UV detector, do not fill the reference cell with the mobile phase but obtain a balance when it is empty and contains air only. Although a satisfactory balance can often be obtained in this way, this can be an undesirable procedure as the offset signal due to the heptane in the cell, which is balanced off by the zero

adjustment, can (depending on the electronic circuit employed) encroach on the linear range of the amplifier and may reduce it significantly.

If the two cells, when filled with the same mobile phase still do not produce a balance, then it is likely that the cell windows are contaminated and require to be cleaned. If the cells do become contaminated or dirty, they can usually be cleaned by passing acetone or chloroform through the cell. If solvents fail to clean the cell then 10% nitric acid solution can be used. However, after cleaning, the acid must be rapidly washed from the cell with pure water to prevent it corroding the stainless steel fittings from which the cell is fabricated. If the cell still remains contaminated it must be dissembled and the windows cleaned as directed by the manufacturer. It is extremely important to follow the manufacturer's instructions closely when dissembling and reassembling the cell, otherwise the cell may be severely damaged. In particular when reassembling the cell after cleaning, care should be taken not to tighten the cell locking screws too tightly otherwise the cell windows or lenses will be damaged. However, the cells must be tightened sufficiently so as not to permit leaks.

Bubbles - Their Removal and Prevention

Bubbles can occur in the cell producing a violent off scale deflection that can be either negative or positive. If the problem is in the reference cell, the deflection is usually negative and conversely in the sample cell the deflection is positive. Bubbles can form from air dissolved in the mobile phase particularly when aqueous mixtures such as methanol and water are used. It is a good practice to degas all solvents prior to use by shaking in a flask under vacuum before filling the solvent reservoir or by bubbling helium gas continuously through the solvent reservoir. To aid in the elimination of bubbles, should they form, the column should be connected to the *lower* connecting tube entering the detector and exit from the upper connecting tube via another tube bent downwards in the form of an inverted U. This permits a slight negative pressure to be applied to the cell aiding in the removal of bubbles and, as any bubble entering the bottom of the cell will rise, it will pass readily through the cell and exit tube. Connecting the column to the upper connecting tube of the detecting cell has the opposite effect and can make bubbles extremely difficult to remove.

If a bubble remains in the cell, then the mobile phase flow rate should be increased and a slight back pressure applied to the exit of the cell by restricting the flow. This reduces the size of the bubble and as result of the increased pressure, often permits its free exit. If the bubble is particularly recalcitrant, then the union between the column and the detector should be loosened and a flow of polar solvent such as acetone (or

some other polar solvent miscible with the mobile phase) forced back through the cell causing the bubble to be removed via the loose union. One note of warning should be added. When applying back pressure to the cell to remove bubbles, the pressure should not be allowed to become so great as to cause leaks between the cell window and the gaskets.

Spurious Peaks

During the development of a chromatogram, unexpected and foreign peaks are sometimes observed. There can be a number of causes for these peaks.

(1) Elution of Sample Solvent

In some cases it is necessary to dissolve a sample prior to injection in a solvent that does not have the same composition as the mobile phase. Under such circumstances the solvent employed in the sample may be eluted as a peak, sometimes in the same chromatogram, or sometimes as a broad peak in a subsequent chromatogram. It should be remembered that any substance, not common to the mobile phase will appear as a *sample solute* even though it may only be a foreign component of the *sample solvent.* The only solution to this problem is to use the mobile phase as a solvent for the sample.

(2) Contaminated Syringe

If the syringe or sample valve has not been cleaned adequately from the previous sample, then small contaminating peaks from the original sample will be produced interfering with the chromatogram of the fresh sample. Considerable care, therefore, should be taken to clean syringes and valves between samples by washing with the mobile phase and then at least once with the sample itself. Contamination is particularly likely when using 1-μl syringes or small volume sample valves (0.1-1 μl).

(3) Air Dissolved in the Sample

Air dissolved in the sample will usually be eluted close to the dead volume and will change the refractive index of the mobile phase. This change in refractive index will produce a peak on both the refractive index detector and the UV detector, particularly when using high detector sensitivities. To eliminate this effect the sample should also be degassed provided the components of interest used are involatile. As already stated desolved air will be a particular problem when using the electrochemical detector.

(4) Detector Displacement Effects

When using a polar solvent in the mobile phase, the quartz windows of some detector cells or other materials of construction can adsorb a thin layer of the polar solvent on the surface. If a more polar solute is eluted from column, in some cases it will displace the polar solvent and this will result in a change of light absorption and produce a spurious peak at the beginning of the eluted peak. Subsequent to the solute passing from the cell, the polar solvent again replaces the absorbed solute producing a positive or negative tail to the peak. This effect can be reduced or eliminated by choosing a more polar solvent for the mobile phase but at the same time reducing its concentration so the elution sequence of the solutes remain sensibly constant.

Baseline Instability

Detector noise in its various forms has already been discussed but, in practice, measures can often be taken to reduce or eliminate them.

Short Term Noise

Short term noise appears as "grass" on the base line and this type of noise can often be completely eliminated. If the short term noise persists at low detector sensitivities, the source is often in the recorder and can be eliminated by either slightly reducing sensitivity or increasing the degree of damping. If the short term noise only occurs at high sensitivity, then this is arising from the detector amplifier and can be eliminated by interposing an active or passive filter between the amplifier output and recorder. Another source of short term noise arises from the pulsation from a reciprocating pump. This can be usually identified by the fact that the frequency of the noise matches the frequency of the pump stroke. Pump noise can be reduced by incorporating a pulse damping device between the pump and the column and also by thermostating the detector cells. The latter can be extremely important when using the UV detector.

Long Term Noise

Long term noise as already defined has a frequency of the same order as the eluted peaks and thus is far more difficult to reduce than short term noise or drift. In fact, it is the long term noise that ultimately limits the sensitivity of the detector. Long term noise can increase progressively during the lifetime of a chromatographic column as it becomes contaminated by trace materials from the samples analyzed which accumulate on the stationary phase and eventually elute irregularly. A silica column can often be cleaned by the following method: six column dead volumes of heptane, dichloromethane, ethyl acetate, acetone, ethanol and water are passed sequentially through the column. This

procedure completely deactivates the column and in the process may wash out the majority of the contaminating impurities. The column then has to be reactivated and this can be achieved using the same solvents and the same volumes of each solvent and passing them through the column in the reverse order. This procedure should significantly reduce any long term noise that has developed and if it does not, then the only alternative is to replace the column with a new one. Reversed-phase columns are cleaned by the opposite procedure. Pure methanol or acetonitrile is passed through the column followed by acetone, methylene dichloride and n-heptane. After pumping about three dead volumes of n-heptane through the column, the solvent series is reversed. About five dead volumes of methylene dichloride, five dead volumes of acetone and finally five dead volumes of methanol or acetonitrile are passed through the column and the solvent chosen for the mobile phase can then be used. This procedure will clean the column from contaminants in almost all instances; if it does not, however, and the long term noise persists, then again, the column must be replaced.

Drift

There are two main sources of drift both due to non-equilibrium conditions in the column and detector. If the detector, column and mobile phase are not in thermal equilibrium, then serious drift can result. This can be eliminated by thermostating the detector, column and mobile phase supply. Another source of drift results from incomplete mobile phase equilibrium with the stationary phase or incomplete mixing in the mobile phase preparation. Such drift always occurs on changing the composition of the mobile phase and to eliminate this drift, mobile phase should be pumped through the column-detector system until a stable base line is obtained. This problem can be exacerbated by the presence of trace impurities in the mobile phase. Such impurities break through the column continuously and irregularly over a long period of time and can result in persistent drift. The only solution to this problem is to ensure the purity of the solvents used in the mobile phase by careful cleaning and purification. Distilled in glass solvents may not necessarily be sufficiently pure to ensure drift-free detector operation.

The Conductivity Detector

The conductivity detector measures the conductivity of the mobile phase by determining the impedance between two electrodes situated appropriately in the column eluent. This is achieved by making the conductivity cell one arm of a Wheatstone Bridge across which voltage is applied. It follows that only one, or in some cases neither, of the electrodes can be at earth potential. As the mobile phase is conducting there will be electrical continuity from the electrodes via the mobile phase to the column. There will

also be electrical continuity between the column (usually made of stainless steel) by way of the connecting tubes to the pump which will be earthed. As a result of this indirect earth continuity to the electrodes an "earth loop" can be formed which can cause serious detector instability. This instability can take the form of violent electronic noise at one extreme, and at the other, very sluggish movement of the recorder pen. This problem can be very difficult to deal with. The "earth loop" can interfere with either the operation of the recorder or the detector electronics. On some recorders there is a removable earth connection to the recorder input. Removing this connection and "floating the recorder" may help to reduce this problem. In a similar manner some amplifiers can be floated with respect to earth. This should also be carried out wherever possible. The situation is exacerbated when using mobile phases of high conductivity. Mobile phases of high conductivity results from using buffers of high ionic strength. The earth loop can be reduced by employing significantly lower buffer concentrations and achieve approximately the same retention characteristics of the mobile phase by also adjusting the pH. In general, changing the pH of the buffer has relatively little effect on the conductivity of the mobile phase whereas changes in buffer concentration can have a very significant effect on its conductivity. Manufacturers of more recently designed instruments attempt to reduce the "earth loop" condition by interposing a length of insulating tubing such as PTFE between the column and the detector cell. This in effect increases the path length of the mobile phase and helps reduce "earth loop" effects. This arrangement is satisfactory providing the insulated connecting tube has dimensions such that any band dispersion resulting from it is kept to a satisfactory minimum. As already stated the major problem associated with the use of a well designed conductivity detector is the drift that arises, when operating at maximum sensitivity and low conductivity, from mobile phase contaminants. It is extremely important to remove ionic materials from the mobile phase, such as carbon dioxide and ammonia, if a stable baseline and the maximum sensitivity is to be realized.

The Refractive Index Detector

The refractive index detector is very sensitive to fluctuations in cell pressure, flow rate and the temperature of the mobile phase. If the pump employed is reciprocating in action, this often produces short term noise on the detector output and may in fact be the limiting factor with respect to sensitivity. If the ambient temperature changes during a chromatogram then significant drift will almost surely result. The effect of pump pulses can be reduced by employing a good quality pump or an effective pulse dampener. Thermostating the detector cell will significantly reduce thermal drift and may also help reduce the

detector susceptibility to changes in pressure. When using a refractive index detector the best results are obtained by thermostating the cell and employing a syringe type pump.

In some commercial refractive index detectors the optical system is designed to cover a specific range of refractive indices. Manufacturers of such instruments usually provide a number of cell or optical systems that are interchangeable and cover the practical range of refractive indices normally met in LC. Where such instruments are used the refractive index of the mobile phase being employed should be checked and the appropriate cell system used. If the incorrect optical system is used by mistake, it will usually be found that a balance point cannot be obtained with the mobile phase in both cells or, if balance is obtained, the sensitivity of the system is extremely low. The refractive index detector although relatively simple to operate is probably the most difficult instrument to use *at maximum sensitivity* due to is general instability under these conditions.

Most commercial refractometers have a very restricted linear dynamic range, sometimes less than three orders of magnitude. When using the refractive index detector for quantitative analysis it is advisable to check the linear range of the instrument.

Quantitative and Qualitative Analysis

The technique of LC has developed rapidly over the past decade and, because of the introduction of high sensitivity, linear detectors, is now being used both as a separation technique and also for quantitative and qualitative analysis. The chromatographic data provided by the detector output gives the retention time or retention volume of a peak, by which the solute may be identified or its identity confirmed. From the relative peak height or peak areas, the proportion of the solute originally present in the mixture can also be determined. However, the precision of the chromatographic data obtained depends not only on the performance of the detector, but on the control of the chromatographic conditions and also the method of measuring the data. Scott and Reese (2) investigated the overall precision that could be obtained from LC data and determined the necessary control over the chromatographic variables to obtain retention data with a precision of 0.1%. Some of the factors that control the precision of chromatography measurements will now be considered.

Solvent Composition

The composition of the solvent used as the mobile phase can have a profound effect on solute retention and is used as an operating variable to control the retention of the solutes in a given mixture and consequently the chromatographic selectivity. It follows that if retention times are required to be measured with a

precision of 0.1%, then the solvent composition must be maintained sufficiently constant to permit this precision to be achieved. Curves relating the corrected retention times of three solutes to solvent composition are shown in Figure 1.

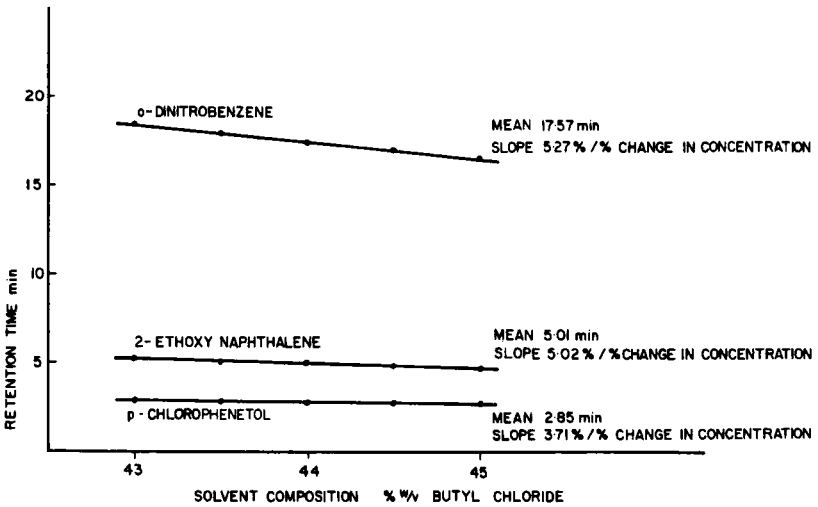

Figure 1. Graphs of corrected retention time against solvent composition for three solutes.

Solvent composition is taken as the % w/v of the polar solvent in the dispersing solvent. In fact, it has been shown (3) that the *reciprocal* of the retention volume is linearly related to the solvent composition, but over a small solvent composition range, the relationship between solvent composition and retention can be taken as linear. Thus the points in Figure 1 are force-fitted to a linear function and the results are summarized in Table 1.

TABLE 1

SOLVENT CONCENTRATION TOLERANCES FOR RETENTION TIME PRECISION

Solvent	Tolerance for Retention Time Precision		
	Retention Time at 44% of Butyl Chloride in Heptane (min)	Concentration Tolerance for 1% Precision (% v/v)	Concentration Tolerance for 0.1% Precision (% v/v)
p-Chlorophenatol	2.85	±0.14	±0.015
2-Methoxynaphthalene	5.01	±0.10	±0.010
o-Dinitrobenzene	17.27	±0.10	±0.010

It is seen that, to achieve a precision of 0.1%, the solvent concentration must be maintained to within 0.01% w/v. This level of control of solvent composition is fairly easy to maintain providing a closed solvent system is employed, but it is extremely difficult, if not impossible, to make up a solution to this accuracy using volatile solvents. It is, therefore, recommended that large bulks of solvent are made up if precise results are required, and each new solvent checked by chromatographing a standard solute. Appropriate correction factors can then be calculated and employed where retention times are to be compared with those obtained from previously used solvents. It follows that retention time measurements made with a precision of 0.1% may well not be possible in routine LC analytical labs and probably must be carried out in laboratories specializing in highly accurate retention measurements.

The Solvent Pump

Scott and Reese employed the Waters 6000M pump for their work, which was found to give a flow of mobile phase over a period of about 12 h, with a standard deviation of 0.07%. This performance was amazingly good but such precision can only be maintained if the pump is operated with the necessary precautions. The majority of chromatographers treat precision LC pumps as just another piece of plumbing hardware, whereas, in fact, they should be treated with the care and respect given to an analytical balance. It was found that, to maintain the precision, of 0.1% the following procedures needed to be taken. The pump should never be allowed to run dry, otherwise abrasion between piston and cylinder produces small leaks. Any mobile phase used should be filtered through a 0.2 μm Millipore filter, particularly if the solvent had been dried over activated silica gel or alumina. The usual method of filtering by a filter paper was found to be inadequate. The filter contained in the pump should be regularly changed, and the inlet tubes to the pump should have as large a bore as possible (2 mm I.D) to prevent the pump from being starved of solvent. The pump should never be subjected to a back pressure greater than the rated maximum.

The mobile phase must be brought to a constant and fixed temperature (and thus constant density) prior to entering the column and, if the volume flow rate through the column is to be maintained constant, then the pump must deliver a constant mass flow-rate to the column. As the pump is designed to provide a constant volume flow rate, then it must be supplied with solvent at a constant density and its displaced volume must also remain constant. If a precision of 0.10% is required, then the displaced volume of the pump and the solvent density must also be maintained constant to within this level of precision. It is almost impossible to thermostat the pump so the ambient temperature of the pump and solvent reservoir must be controlled, and this means that the temperature of the room in which the apparatus is

situated must be precisely controlled. Most pumps are made of stainless steel, which has a coefficient of cubical expansion of about 1.3 x 10^{-5}/°C i.e., 0.0013%/°C and thus, the effect of ambient temperature changes of the pump volume will be negligible. The cubical expansion of solvents, however, is much higher and for heptane is 1.25 x 10^{-3}/°C i.e. 0.125%/°C. Thus, to maintain the solvent density to a precision of 0.1%, the ambient temperature must be maintained constant to 0.4°C. This control of ambient temperature is not unreasonable in normal heat-controlled and air-conditioned laboratories, but has to be maintained if the required precision is to be achieved. This control may well be difficult to achieve in some environments.

Column Temperature

It it well known that the retention volume and retention time of a solute varies considerably with temperature, and in Figure 2 the retention volume of the three solvents determined over a narrow temperature range is shown plotted against temperature.

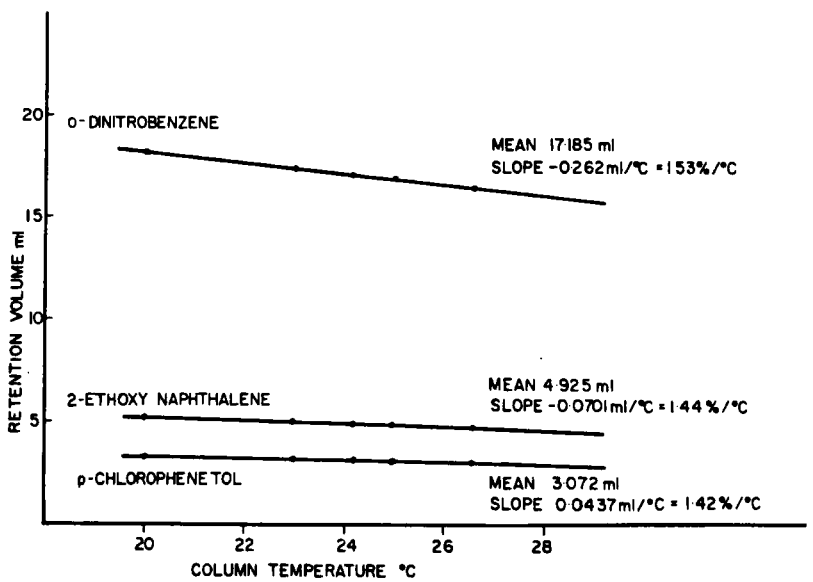

Figure 2. Graphs of corrected retention volume against column temperature for three solutes.

The relationship between retention volume and temperature is, in fact, logarithmic, but over the small range of temperatures concerned, it is approximately linear, so a linear function is force-fitted to the results for the three solvents. A summary of the results obtained from the regression analysis of the data used in Figure 2 is shown in Table 2. It is seen that, to attain a precision of 0.1%, the temperature of the solvent and column must be maintained to within 0.04°C. It is not difficult to maintain this level of temperature control on the thermostat bath, but it can be extremely difficult to return to a given temperature to

within 0.04% (after prior change). It should also be pointed out that column temperature control of 0.04% would be extremely difficult, if not impossible, to obtain if an air bath was employed. Due to the relatively low thermal capacity and specific heat of air, local variations within an oven of 1°C can usually be found in gas chromatograph hot air ovens. Thus, for precise work, liquids are recommended as the thermostating medium of LC columns.

TABLE 2

TEMPERATURE TOLERANCES FOR RETENTION TIME PRECISION

Solute	V' at 23.8°C (ml)	k'	Temperature Control for 1% Precision (°C)	Temperature Control for 0.1% Precision (°C)
p-Chlorophenatol	3.072	0.945	±0.35	±0.04
2-Methylnapthalene	4.925	1.519	±0.35	±0.04
o-Dinitrobenzene	17.185	5.301	±0.33	±0.03

Sample Load

The mass of the sample injected onto a LC column can significantly affect both the solute retention time and column efficiency (3,4). In Figure 3, the retention time obtained from a column for the solutes o-dinitrobenzene and 2-ethoxy naphthalene are shown, plotted against the mass of solute injected into the column. It is seen that, for precise comparative work, either the mass of sample injected must be kept constant, or the total mass of each solute maintained at a level below 1.0 µg. Thus, irrespective of the specifications of the detector and its performance, for precise results, the operating conditions of the chromatograph as a whole must be carefully controlled. However, the method of measuring the data can also affect the precision of the measurement obtained. There are generally two methods of data measurement - manual and automatic data processing involving, at one extreme, a simple electronic integrator, and at the other, computer processing.

Manual Measurement of Chromatographic Data

Retention distances can be measured manually on the recorder chart with a good quality rule. All distances should be estimated to the nearest 0.1 mm. The base line under each peak is constructed, using a sharply tipped pencil and the peak height taken as the distance between the constructed base line and the center of the recorder trace at the peak maximum.

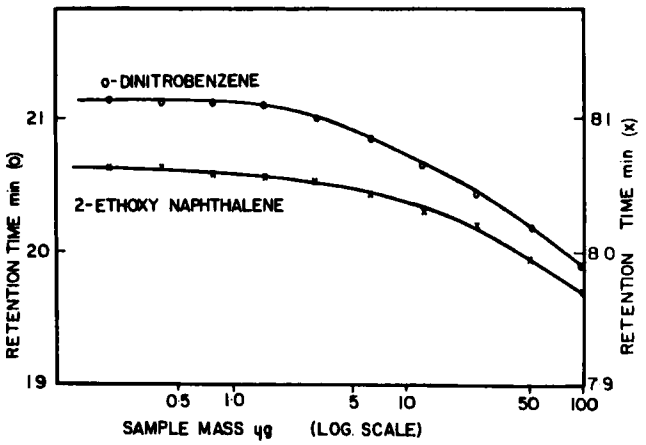

Figure 3. Graphs of retention time against sample mass for two different solutes.

The peak width is taken as the distance between the *inside* edge of the recorder trace and the *outside* of the trailing edge of the peak at 0.6065 of the peak height. The peak widths are best measured by means of a 3X comparitor and graticule which should be calibrated in units of 0.2 mm or less. Peak widths can then be estimated to the nearest 0.1 mm. The peak area can be taken as proportional to the product of the peak height and the peak width.

Computer Data Processing

There are two general methods of data acquisition by a computer; in the first, the output from the detector is sampled, a limited amount of data is temporarily stored; the data is processed, and the processed data in the form of retention times, efficiencies, peak areas, etc. is permanently stored. In the second method, the data is sampled, and each data point in the chromatogram is permanently stored, the chromatographic data required is obtained by subsequently processing the stored data. The former method is inflexible and, as the data is discarded, reprocessing by an alternative procedure is not possible, and a reconstructed chromatogram cannot be obtained. The second method is far more flexible, the data can be processed by any chosen method or by a number of different methods, if required and as the raw chromatographic data is permanently available a reconstructed chromatogram, in whole or in part, can be obtained on any chosen scale and presented on a cathode ray tube screen or plotter. The stored data can also be statistically tested, and any points representing outliers can be rejected.

There are two main factors in the acquisition of data that can affect the precision of the chromatographic results obtained; they are the data sampling rate and the signal-to-noise level of the detector. Mini-computers that are employed for chromatographic data acquisition have in general (unless of an exceptional design), a maximum sampling rate of 240 samples/sec, which has to be shared between the number of stations involved in the time-sharing system, and there will be a maximum sampling rate for any one station of 60 samples/sec. This would not be true for the more expensive 16 and 32 bit computers. However, in this instance, the 8 bit computers only, will be considered as it represents the data processing system that will be in the economic range of most chromatographers. The limit of 240 samples/sec is imposed on the computer by the auto ranging amplifiers associated with the A/D convertor that is necessary to utilize the complete linear dynamic range of the detector. If a disc storage system is used in conjunction with the computer, the time of data transfer to the disc will also limit the sampling rate of the computer but not reduce it below 240 samples/sec. The 60 sample/sec limit for each station results from the fact that a sampling rate in excess of the 60 Hz frequency of the main electricity supply could result in unacceptable noise unless very expensive equipment was used . Thus, if there are 10 stations from which data is to be acquired, then the maximum sampling rate for each station will be 24 data points/sec. The fact that a finite data acquisition rate exists causes a discrimination limit to be imposed on any chromatographic results obtained, unless the data is processed from the disc using special software. Consider a peak having a time width of 10.3 sec sampled at a rate of 5 samples/sec, then a value of either 10.2 or 10.4 will be taken by the computer as the peak width. Thus, assuming the peak is eluted at a retention time of 360 sec, and employing the standard equation for column efficiency:

$$N_1 = 4x(360/10.2)^2 = 4893$$
$$N_2 = 4x(360x\ 10.4)^2 = 4793$$

Thus, the sampling rate will permit a discrimination of 100 theoretical plates in 4983, equivalent to 2.0% and this precision of measurement due to the sample acquisition rate being 5 samples/sec, cannot be improved. It can be shown in a similar way that, irrespective of the control over chromatographic conditions, a precision of 1% in column efficiency cannot be realized unless the data acquisition rate is greater than 10 samples/sec.

The noise level of the signal that is digitized can also significantly affect the overall precision of the chromatographic data obtained. An example of this is shown by the crests of the two replicate peaks, shown in Figure 4, taken from computer data and expanded on an oscilloscope screen. It is seen that, although the differences in retention time between the two peaks is only 2.1 sec, as a result of a noise spike on the front of the crest of the first peak and noise spike on the back of the peak crest from

the second peak, the measured difference in retention times is 4.4 sec. It follows that, to attain the highest precision, the noise has to be significantly reduced or, if possible, eliminated from the detector signal.

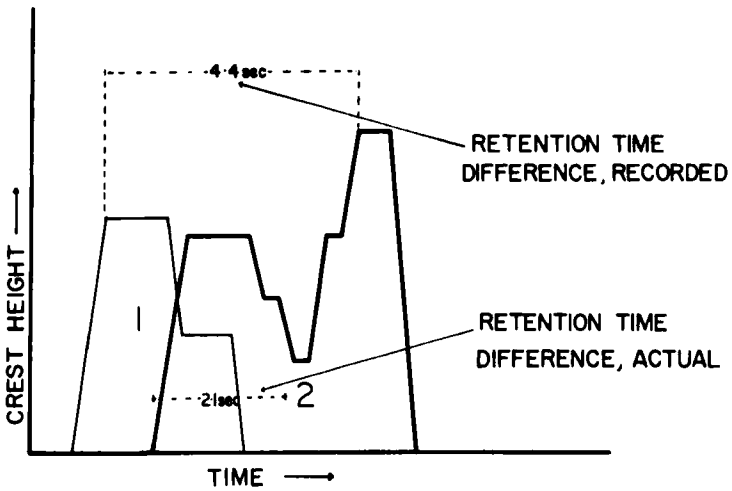

Figure 4. Peak crests reconstructed by the computer (99.9-100% peak height).

There are several ways of reducing noise. The first and obvious method is to average a number of points and take the average value after data acquisition. This procedure is frequently used and is sometimes called the "slice" method of data acquisition. This method has the advantage of reducing noise without distorting the peak, but at the same time may reduce the data acquisition rate which is also undesirable. Another method of reducing noise is to use an "on the fly", exponential smoothing procedure. This procedure is very effective, does not reduce the data acquisition rate, but tends to distort the peak. If all data points are stored on disc, very sophisticated smoothing procedures are possible, including the rejection of outliers; furthermore, this procedure in no way affects the rate of data acquisition. An alternative procedure is to interpose a filter circuit between the detector output and the A/D convertor. This procedure has already been discussed and, again, does not affect the rate of data acquisition but, if not designed correctly, can produce peak dispersion and asymmetry. Further, an active filter would be more effective than a passive filter, as the active filter would have a much sharper frequency cutoff and, thus, provide more efficient noise rejection.

Qualitative Analysis

The basic chromatographic measurements for qualitative

analysis are retention times (T_R) and retention volumes (V_R). This requires the accurate location in time of the peak maximum which makes small demands on the detector. The response of the detector need not be linear, although as previously discussed, the signal should be as free from noise as possible. Although T_R and V_R are the basic chromatographic measurement, they are not normally employed for solute identification purposes.

From the theory of chromatography, the retention volume V_R for a liquid solid system is given by:

$$V_{R(A)} = V_o + K_A A_S$$

where V_o is the void or dead volume of the column measured as the retention volume of a fully permeating but unretained peak
K_A is the distribution coefficient of the solute A
A_S is the surface area of the stationary phase.

It should be noted that K_A is the characteristic that will permit the identification of the solute. Now both V_o and A_S will vary from column to column, depending on its packing density and, thus, the variability of V_o is eliminated by chromatographing a completely non-adsorbed solute N, which will have a a retention volume equivalent to V_o. Thus, the corrected retention volume V'_R is given by

$$V'_{R(A)} = V_{R(A)} - V_o = V_{R(A)} - V_N = K_A A_S$$

The only variable left to eliminate is A_S, and this can be eliminated by chromatographing another standard solute S added to the original mixture containing solute A,

then, $$V'_{R(S)} = V_{R(S)} - V_o = V_{R(S)} - V_N = K_S A_S$$

thus, $$V'_{R(A)}/V'_{R(S)} = (V_{R(A)} - V_o)/(V_{R(S)} - V_o) = (V_{R(A)} - V_N)/(V_{R(S)} - V_N)$$

$$= K_A A_S / K_S A_S = K_A / K_s$$

The function $V'_{R(A)}/V'_{R(S)}$ is called the retention ratio of solute A to the standard solute S and is the value that permits the identification of solute A to be confirmed as its value depends only on K_A and K_S both of which are solely characteristic of the solute and phase system and not dependent on the packing characteristics of the column.

At a constant flow rate, all retention volume measurements can be replaced by retention times.

Thus, $T'_{R(A)}/T'_{R(S)} = (T_{R(A)}-T_o)/(T_{R(S)}-T_o)$

$= (T_{R(A)} -T_N)/(T_{R(S)}-T_N) = K_AA_S/K_SA_S = K_A/K_S$

Retention times can be measured extremely precisely under carefully controlled chromatographic conditions. In Table 3, the precision of retention time and retention ratio measurements determined by Scott and Reese (2) are given, and it is seen that the standard deviations of retention times are about 0.1% or less. The precision of retention ratios are about the same, but the retention ratio data can be used for different columns having different packing characteristics provided the same stationary phase is used in conjunction with the same solvent system. It is not advisable to rely on retention ratios obtained from one phase system to unambiguously identify an unknown substance. The retention ratios of the known and unknown solute at a given standard should be compared on at least two phase systems. If the known and unknown substances give the same retention ratios to the standard respectively on each phase system, then more confidence can be placed in the identification of the unknown solute.

TABLE 3

PRECISION OF RETENTION TIME AND RETENTION RATIO MEASUREMENTS

(T_1, T_2 and T_3 are the retention times of solutes 1, 2 and 3 respectively.)

	Solute 1 (k'= 0.94)	Solute 2 (k'= 1.50)	Solute 3 (k'= 5.21)
Mean Retention Time (min)	6.283	8.119	20.421
Standard Deviation (sec)	0.38	0.20	0.46
Standard Deviation (%)	0.10	0.04	0.04
		T_2/T_1	T_3/T_2
Retention Ratios Mean		1.2922	2.5153
Standard Deviation		0.00119	0.00111
Standard Deviation		0.092	0.044

Precision as an Alternative to Resolution

Providing retention times can be measured with high precision then retention times can be used to determine the composition of a mixture of two substances that, although having finite differences in retention times, are eluted as a single peak by the column

employed. This can only be achieved if the S.D. of the measured retention time is small compared with the retention time difference of the two solutes.

Consider two solutes eluted close together such that a single composite peak is produced. From the plate theory, the concentration profile of such a peak can be described by the following equation:

$$X_{AB}=\frac{X_A}{\sqrt{(2\pi n_A)}}\cdot\exp[-(v_A-n_A)^2/2n_A]+\frac{X_B}{\sqrt{(2\pi n_B)}}\cdot\exp[-(v_B-n_B)^2/2n_B] \quad (1)$$

Where X_{AB} is the concentration of solutes A and B at any point in the composite peak,
X_A is the initial concentration of solute A,
X_B is the initial concentration of solute B,
n_A is the column efficiency for solute A,
n_B is the column efficiency for solute B,
v_A is the volume of mobile phase passed through the column in units of plate volumes of solute A,
v_B is the volume of mobile phase passed through the column in units of plate volumes of solute B.

If T_A and T_B are the retention times of solutes A and B equation (1) can be transformed into:

$$X_{AB}=\frac{X_A}{\sqrt{(2\pi n_A)}}\cdot\exp[-(n_A/2)(t/t_A-1)^2]+\frac{X_B}{\sqrt{(2\pi n_B)}}\cdot\exp[-(n_B/2)(t/t_B-1)^2] \quad (2)$$

where the variable v is now replaced by variable t, the elapsed time. It is seen from equation (2), that when only solute A is present, the function will exhibit a maximum at $t = t_A$ and , if only solute B is present, it will exhibit a maximum at $t = t_B$. It follows that the composite curve will give a range of maxima between $t = t_A$ and $t = t_B$ for different ratios of X_A to X_B and thus from the value of t at the maxima of the composite peak X_A/X_B can be determined.

For closely eluted peaks $n_A = n_B$ and thus, as the function $2n_A$ is in effect an average dilution factor resulting from the dispersion, they can be replaced by a constant. The efficiencies n_A and n_B in the exponent function, however, can only be considered equal if the peak is symmetrical as, in the part of the composite peak that determines its maximum, the rear part of the first peak is combined with the front part of the second peak. In liquid-solid chromatography, the concentration profiles of eluted peaks are rarely symmetrical, and, thus, n_A must represent the efficiency of the rear half of the peak for solute A. Similarly, n_B must represent the efficiency of the front half of solute B. Further, the detector response to solutes A and B must be taken into account. Thus, if D is the detector signal then equation (2) can be put into the form

$$D = C\{\alpha X_A \cdot \exp[-(n/2)(t/t_A-1)^2+\beta X_B \cdot \exp[-(n/2)(t/t_B-1)^2]\} \quad (3)$$

where C is a constant,
α is the response factor of the detector to solute A,
β is the response factor of the detector to solute B.

The system was examined by Scott and Reese (2) employing nitrobenzene and fully deuterated nitrobenzene as the solutes. Their elution times were 8.927 and 9.061 min, respectively, thus having a retention difference of 8.04 sec. The separation ratio of the two solutes was 1.023, and the efficiencies of the front and rear portions of the peaks were 5908 and 3670 theoretical plates, respectively. The detector was found to have the same response for both solutes, i.e. $\alpha = \beta$. Thus, inserting these values in equation (3)

$$D = C\{(X_A \cdot \exp[(-3670/2)(t/8.927 - 1)^2] + X_B \cdot \exp[(-5908/2)(t/9.06\ 1-1)^2]\} \quad (4)$$

Employing a range of values for X_A/X_B the retention time of the composite peak was calculated from equation (4) by means of a computer. The curve relating the composition of the mixture to retention time is shown in Figure 5.

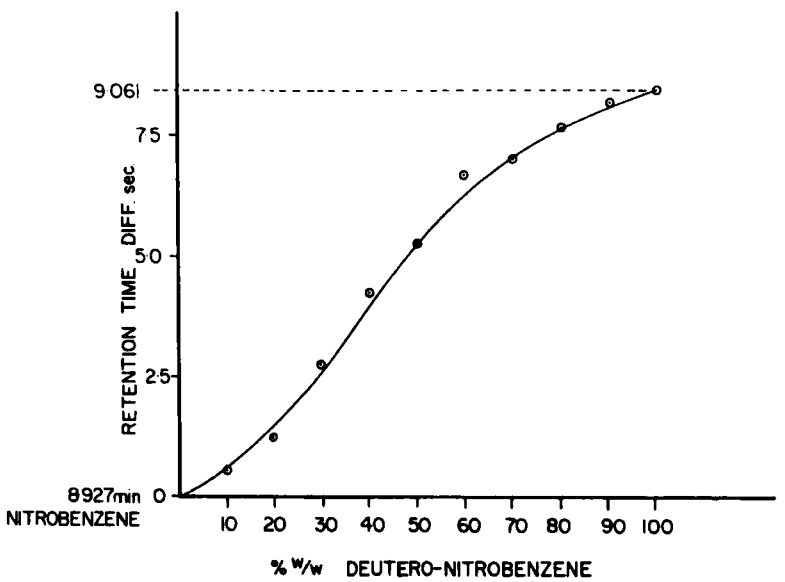

Figure 5. Graph of retention time difference against sample composition for different mixtures of nitrobenzene and deutero-nitrobenzene.

A series of mixtures of nitrobenzene and deuterated nitrobenzene were made to a known concentration ratio and the retention time of the composite peak was determined experimentally. The retention time of each mixture was determined in triplicate, and the average for each mixture is represented as plotted points in Figure 5. It is seen that close agreement is obtained between the experimental points and the theoretical curve.

Employing very precise methods of measuring retention times as a means of determining the composition of unresolvable binary solute mixtures would be extremely valuable in the analyses of configurational isomers. Providing the retention time of a known mixture of the two components is available (in most instances one pure isomer and a 50% (w/v) mixture of one isomer in the other can be obtained) a calibration curve can be calculated theoretically. The asymmetry ratio of the peak for one pure component and the column efficiency for that component is usually the only further information required as the detector response factors for configurational isomers are generally identical.

Quantitative Analysis

Quantitative analysis by LC, as opposed to qualitative analysis imposes stringent demands on the performance of the detector. Consequently, for accurate quantitative analysis the detector must have a linear response (or a known response index) and must be operated within its linear dynamic range; further, the baseline noise must be minimal if peak area measurements are to be employed. The basic measurements employed for quantitative analysis are peak heights or peak areas. In general analysis, peak heights tends to give more precise results than peak areas.

The Internal Standard Method

A simple form of the *internal* standard analytical procedure is as follows. A known weight (W_A) of the solute to be determined (A) is chromatographed with a known weight (W_S) of standard S. Let the peaks for solute A and standard S have peak heights of h_A and h_S respectively.

Then,
$$\frac{W_A}{W_S} = \alpha \frac{h_A}{h_S} \text{ or } \alpha = \frac{W_A h_S}{W_S h_A} \qquad (5)$$

where α is known as the calibration constant.

A known weight W'_S of the standard is now added to the mixture containing an unknown weight W'_A of solute A and a sample of the mixture chromatographed. If in the resulting chromatogram, the solute peak and the peak for the standard have peak heights of h'_A and h'_S respectively, then:

$$\frac{W'_A}{W'_S} = \alpha \frac{h'_A}{h'_S} \qquad (6)$$

After substituting α from equation (6), the unknown weight of solute A in the mixture is given by:

$$W'_A = \frac{W_A h_S}{W_S h_A} \times \frac{W'_S h'_A}{h'_S} \qquad (7)$$

This procedure can be used employing peak area instead of peak heights by substituting values for peak areas for peak heights in equations (5), (6) and (7).

Equation (6) is quite general and can be used for any number of different substances in the mixture (providing they are adequately resolved) using only one standard, and the calibration factor is determined for each different solute relative to the standard.

If the detector response is the same for all solutes, which is sometimes the case, particularly when separating polymers using a refractive index detector, then a normalization procedure can be used. Under these circumstances, if the peak heights are h_1, h_2. . .h_n for solutes 1,2,3...n, then the percentage of any solute P is given by

$$\%P = \frac{h_p}{h_1 + h_2 + h_3 . \; . \; .h_n} \times 100 \qquad (8)$$

Again, peak areas can be substituted for peak heights in equation (8) if desired. The precision of quantitative analytical data employing peak heights and peak areas as determined by Scott and Reese (2) and obtained under carefully controlled chromatographic conditions is given in Table 4. It is seen, except for peaks eluted late in the chromatogram, that peak height analysis gives more precise results than peak area analysis. The standard deviations for components determined at levels of 2.0%, 10% and 82% are 0.05%, 0.12%, and 0.15% respectively, demonstrating that precise results can be obtained from LC analysis providing sensitive, linear and stable detectors are employed. It should also be emphasized that the precision given above is not solely dependent on the characteristics of the detector but are equally dependent on the careful control of the chromatographic conditions.

The Use of an External Standard

An alternative, but less accurate method of quantitative analysis by LC is to employ an *external* standard. This method obviates the necessity of finding a standard that is separated from the other components of the mixture and allows the actual

substance that is to be assayed to be used as the reference solute. Prior to analysis, a calibration curve is obtained relating mass of solute against peak height or peak area.

TABLE 4

PRECISION FOR THE ANALYSES OF A MIXTURE EMPLOYING PEAK HEIGHTS AND PEAK AREAS NORMALIZATION MEASURED BY COMPUTER WITH A 0.5-SEC INPUT-TIME CONSTANT

	Peak 1 (k'= 0.94)	Peak 2 (k' = 1.50)	Peak 3 (k'=5.21)
Analysis by Peak Heights			
Mean	1.935	16.491	81.574
Standard Deviation	0.0465	0.121	0.148
Standard Deviation (% of the mean)	2.46	0.736	0.18
Analysis by Peak Area			
Mean	0.633	7.483	91.884
Standard Deviation	0.032	0.072	0.0823
Standard Deviation (% of the mean)	5.071	0.97	0.09

This is achieved by injecting known masses of solute onto the column (by means of a sample valve of appropriate volume) and measuring the respective peak height or area of each eluted peak. It is advisable to measure each calibration point in duplicate and if possible in triplicate; the average of the replicate measurements being used for the calibration curve. The unknown sample is then chromatographed and the area or height of the peak of interest in the sample measured. The mass of the solute in the sample is then read off from the calibration curve.

The external calibration procedure is less precise than the internal standard method due to the fact that peak areas are compared from separate chromatographic analysis. Consequently, any changes in the apparatus from run to run and any changes in the ambient conditions will reduce the overall precision of measurement. One method of reducing this error is to alternatively run sample and calibration standards. In this way the effect on precision of any systematic changes in chromatographic performance or ambient conditions can be minimized.

Synopsis

The *four most commonly used* LC detectors are the *UV detector*, the *refractive index detector*, the *fluorescence detector* and the

electrical conductivity detector. The *UV detector is the most popular* providing the *highest sensitivity, widest linear dynamic* range and the most stable performance. It can be used effectively with *gradient elution in reversed-phase systems* but is generally *unsuitable* for *gradient elution with silica gel columns* unless very special solvents are employed which restricts the versatility of the chromatography. The *fixed wavelength* detector can be used for the *majority of chromatographic applications* that require a UV detector. Multi-wavelength detectors such as the dispersion or diode array detectors can be used to allow wavelength selection to increase detector selectivity or provide "on the fly" UV spectra of an eluted peak.

The *refractive index detector* is the *most popular bulk property detector* and is employed where the solutes of interest do not possess a UV chromophore and consequently can not be detected by the UV detector. The refractive index detector is the *nearest approach to a universal detector*, has a *restricted dynamic range* and can *not be used with gradient elution.* The refractive index detector is the second choice of detector next to the UV detector. The *fluorescence detector*, the third most popular detector, is one of the *most sensitive*, but has a *restricted linear dynamic range.* It can be used with gradient elution providing the solvents are free of fluorescing materials. It is, without doubt, the *most commonly used high sensitivity detector.* The *electrical conductivity detector* is employed for the *detection of ionic substance*, sometimes in the presence of unresolved non-ionic material. It *is sensitive*, has a *reasonably wide linear dynamic range* and can be employed with gradient elution providing the ionic strength of the solvent does not change during development. *It can be a relatively inexpensive detector* and is the fourth most commonly used detector.

Detectors should be operated at the lowest sensitivity that will still avoid column overload. Under these conditions the *maximum stability* and *quantitative accuracy* will be achieved. Unless the nature of the sample determines that high sensitivity must be employed then intermediate or low sensitivities should always be used. To avoid bubbles being formed in the detector cell, the *mobile phase should always be degassed.* Bubbles in the cell can often be removed by solvent wash or applied back pressure to the detector cell. To avoid spurious peaks, the *sample should be dissolved in the mobile phase* if at all possible, the sample should be degassed and syringes and sample valve should be washed well with mobile phase between samples.

Short term noise can sometimes be *reduced by adjustment of the recorder dampening or sensitivity* but is usually derived from the detector electronics and consequently has to be tolerated unless appropriate electronic filters are provided. Any increase in long term noise is usually due to impurities being eluted from the column and does not normally originate from the detector

itself. *Appropriate solvent washes or column replacement will usually reduce the long term noise to its original level.* Drift can also result from column contamination that will also be reduced by a solvent wash or column replacement. However, *drift can also result* from *poor temperature equilibrium* and thus, may be removed by improved thermostating. The *stability* of the *conductivity detector depends on the absence of trace ionic materials* such as carbon dioxide or ammonia in the mobile phase which should be removed by careful cleaning. The *stability of the refractive index detector*, on the other hand, *depends* on *ambient conditions* such as *temperature*, *flow rate* and *cell pressure* and consequently for stable operation these variables have to be carefully controlled.

To achieve high precision in qualitative analysis by retention measurement *temperature, flow-rate, solvent composition and sample size have to be carefully controlled.* If highly precise retention measurements can be achieved then retention measurement can be usefully employed to determine the relative quantitative composition of an unresolved peak containing two solutes. If *quantitative analysis* is carried out *by computer data acquisition* and processing, *the accuracy will depend* on the *rate of data acquisition.* In *quantitative analysis peak heights* or *peak areas can be employed*, the choice depending on the characteristics of the chromatograph that is used. *Internal or external standard* are the *techniques normally selected* although under *certain conditions total peak normalization* can be used. *Internal standards*, where the standard is added directly to the sample, *give the more precise quantitative results* but demand greater chromatographic resolution or longer analysis times. *The external standard procedure* allows *the solute* of interest itself to be used as the standard but *requires that standard and sample are run alternately to reduce the effect of performance changes from run to run.*

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APPENDIX

SOME PHYSICAL PROPERTIES OF SOLVENTS IN COMMON USE IN LIQUID CHROMATOGRAPHY

Solvent	Cut Off (nm)	Refractive Index	Density (20°C) (g/ml)	Boiling Point (°C)	Dielectric Constant
n-Pentane	205	1.358	0.6214	35.4	1.844
n-Heptane	197	1.388	0.6795	98.4	1.924
Cyclohexane	200	1.427	0.7739	80.7	2.023
Carbon Tetrachloride	265	1.466	1.5844	76.7	2.238
n-Butyl Chloride	220	1.402	0.8809	78.5	7.39
Chloroform	295	1.443	1.4799	61.1	4.806
Benzene	280	1.501	0.8737	80.1	2.284
Toluene	285	1.496	0.8623	110.6	2.379
Dichloro-ethane	232	1.424	1.3168	39.8	9.08
Tetra-chloroethylene	280	1.938	1.3292	87.2	3.42
1,2-Dichloro-ethane	225	1.445	1.2458	83.5	10.65
2-Nitro-propane	380	1.394	0.9829	120.3	25.52
Nitromethane	380	1.394	1.1313	101.2	35.87
n-Propyl Ether	200	1.381	0.7419	89.6	3.39
Ethyl acetate	260	1.370	0.8946	77.1	6.02
Ether	215	1.353	0.7076	34.5	4.34
Methyl acetate	260	1.362	0.9280	56.3	6.68
Acetone	330	1.359	0.7844	56.5	20.70
Tetrahydro-furan	225	1.408	0.8842	66.0	7.58
Acetonitrile	190	1.344	0.7822	81.6	37.5
n-Propanol	205	1.380	0.7998	97.2	20.3
Ethanol	205	1.361	0.7850	78.3	24.6
Methanol	205	1.329	0.7866	64.7	33.6
Water	180	1.333	0.9971	100.0	80.3
Acetic acid	210	1.329	1.0437	118.0	6.15

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LIST OF SYMBOLS
(Units Defined in Text)

A	area
A_s	total surface area of adsorbent in the column
C	electrical capacity
C_a	detector 'plate' capacity
c	solute concentration
c_o	initial concentration of solute
c_t	solute concentration after time t
D_m	diffusivity of solute in the mobile phase
D_p	detector pressure response
D_Q	detector flow response
d	density
F	Faraday constant
f	frequency
H	variance per unit length or 'height of a theoretical plate'
h	peak height
I	current in amperes
I_o	incident light intensity
I_T	transmitted light intensity
i	current
K	distribution coefficient
K_T	limiting transfer coefficient
k	molar absorption coefficient
k'	capacity factor
l	length
m	mass of solute
N_D	detector noise level
N_p	detector pressure sensitivity
N_Q	detector flow rate sensitivity
n(N)	column efficiency
n_A	refractive index of medium A
P	pressure
Q	flow rate
R	electrical resistance
R_d	detector response
r	radius
r	response index
S	amplifier factor
s	chart speed
T'	amplifier time constant
T_t	time standard deviation of peak
t	elapsed time
t_A	retention time of solute A
u	linear velocity
V	electrical potential
V_o	column dead volume
V_r	retention volume
v	volume

v_l electrical potential
v_m volume of mobile phase per plate
v_s volume of stationary phase per plate
w peak width
X_c system concentration sensitivity
X_D detector concentration sensitivity
X_o initial concentration of solute in the mobile phase on the column
X_N concentration of solute in the Nth plate
X_s concentration of solute in the sample
y detector output

ε fraction of column volume occupied by the mobile phase
ε' dielectric constant
θ temperature
η viscosity
ϕ quantum yield
σ_c S.D. of peak from column
σ_d S.D. due to dispersion in the detector cell
σ_e S.D. of total extra column dispersion
σ_r S.D. of dispersion due to detector time constant
σ_s S.D. resulting from finite sample volume
σ_t S.D. due to dispersion in tubing

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