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STUDIES IN THE CHROMATOGRAPHIC ANALYSIS
OF SOME ANTHRAQUINONE DERIVATIVES

by

ROBERT KENNETH HARLE, M.Sc., A.R.I.C.

A Doctoral Thesis

submitted in partial fulfilment of the requirements for the
award of Doctor of Philosophy of the
Loughborough University of Technology.

September 1975.

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The author certifies that the work contained in this thesis is his original work, and that it has not been submitted, in full, or in part, to this or any other institution for a higher award.

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I. INTRODUCTION.

1. General.

Hydrogen peroxide is made commercially by the cyclic reduction and oxidation of solutions containing alkyl-substituted anthraquinones. Such solutions contain not only the alkyl anthraquinone, but also certain oxidation and reduction products which do not revert to the active compounds in the plant working solution, and which thus reduce the efficiency of the plant for the production of hydrogen peroxide. Section 2 examines the chemistry involved in the hydrogen peroxide manufacturing process and degradation routes of the various active materials. The identification and quantitation of the components of the working solution is thus of considerable significance in the economic production of, industrially important, hydrogen peroxide.

2. The Hydrogen Peroxide Manufacturing Process and Probable Components of Working Solution.

2.1 Starting Materials.

The active starting material is generally 2-ethyl-9,10-anthraquinone (EAQ) (I), dissolved in a 1:1 mixture of 'Sextate' and 'Aromasol H'. 'Sextate' is predominantly methylcyclohexyl acetate, while 'Aromasol H' is a mixture of alkyl polysubstituted benzenes.

2.2 The Process.

The cyclic process is in two stages^{1,9}, the reduction of Working Solution with hydrogen in the presence of a catalyst, followed by oxidation by air and extraction of the hydrogen peroxide with deionised water, (Fig.II). The Working Solution is cycled constantly in a continuous process, with additional materials added as necessary to maintain the solution volume and concentration.

EAQ is reduced by hydrogen to the corresponding quinol, 2-ethyl-9,10-dihydroxyanthracene (EAQH₂) (II), but may be, and usually is, reduced further. After the primary reduction the next major reduction step is the reduction of one of the aromatic rings, with the formation of 2-ethyl-5,6,7,8-tetrahydro-9,10-dihydroxyanthracene (H₄EAQH₂) (III). This compound may be reduced further to 2-ethyl-1,2,3,4,5,6,7,8-octahydro-9,10-dihydroxyanthracene (H₈EAQH₂) (IV). Both of these last-named compounds will react with oxygen to give quinones, these being respectively 2-ethyl-5,6,7,8-tetrahydro-9,10-anthraquinone (H₄EAQ) (V), and 2-ethyl-1,2,3,4,5,6,7,8-octahydro-9,10-anthraquinone (H₈EAQ) (VI). These compounds (I - VI) are main constituents of the Working Solution, but do not occur in solution at the same point in the cycle. In the stable, oxidised, Working Solution normally used for analyses, the quinols do not appear, having been converted to the corresponding stable quinones. Compounds I - VI are well-characterised main constituents of the Working

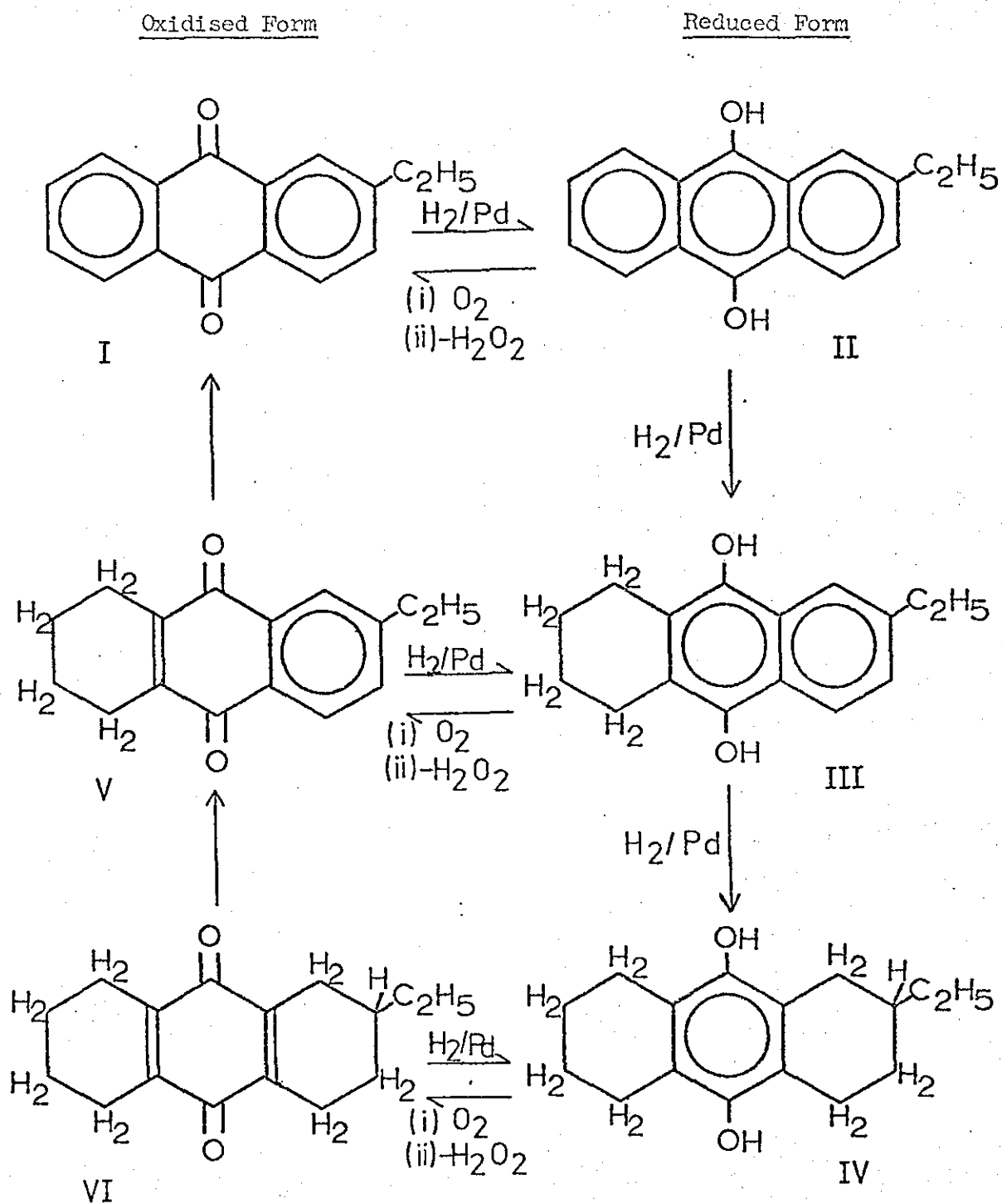


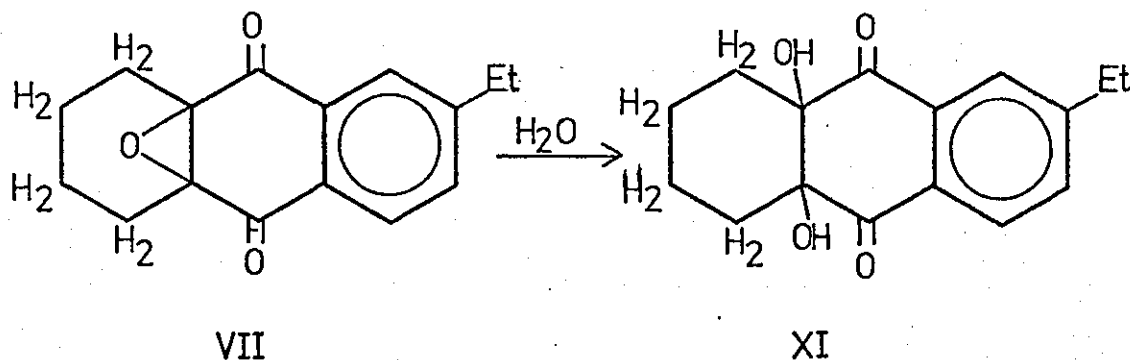
Fig.I.1. Main Oxidation/Reduction Cycle.

Solution, usually kept in an efficient production equilibrium with each other by careful control of plant operating conditions.

2.3 Possible Side-Reactions and By-Products.

Reactions other than catalysed hydrogenation and aerial oxidation also occur. These reactions may involve the species OH^- , H^+ , H_2O_2 and others, for example in epoxidations, (Fig. I.2). This gives rise to series of compounds derived from H_4EAQ and H_8EAQ by epoxidation followed by reduction, firstly of the epoxy group and subsequently of the two 'quinone' carbonyl groups. From H_4EAQ the series would be:-

H_4EAQ (V) epoxidised to H_4EAQ Epoxide (VII), then reduction to hydroxy-pentahydro H_4EAQ (VIII) and subsequently to dihydroxy-hexahydro-, (IX), and trihydroxy-heptahydro-, (X), derivatives, (Fig. I.2). A different reaction, namely hydrolysis, would give from H_4EAQ Epoxide a dihydroxy compound (XI):



Another type of side-reaction involves the reaction of, or loss of, a quinonoid oxygen atom, e.g. (Fig. I.3).

Some of these compounds have been synthesised, (Appendix A) or isolated and have been found by polarography to exist in Working Solution. Any compounds which are not polarographically active are not directly recognisable by this technique, so that a method of

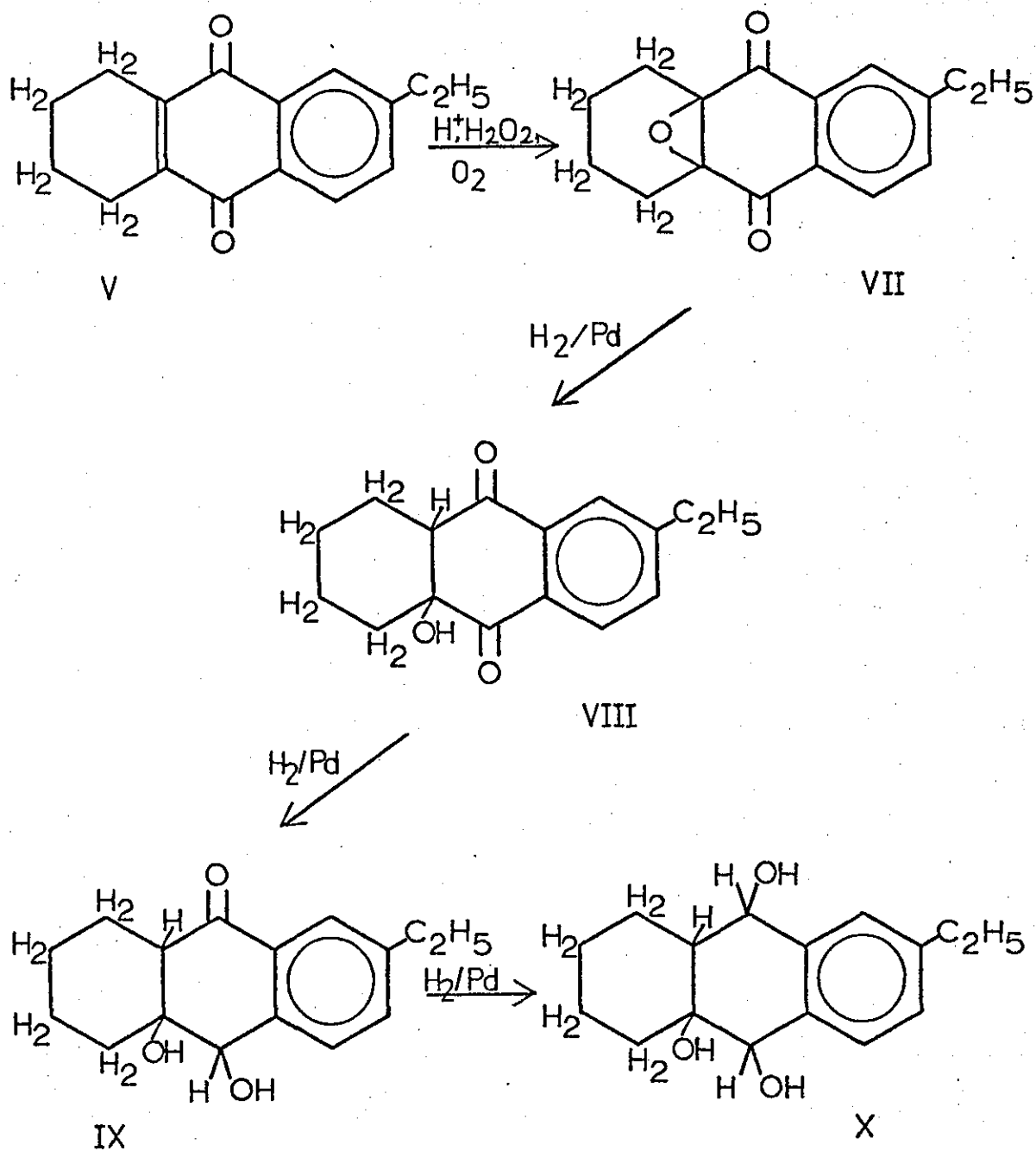


Fig.I.2. Epoxidation and Reduction of H₁₂EAQ.

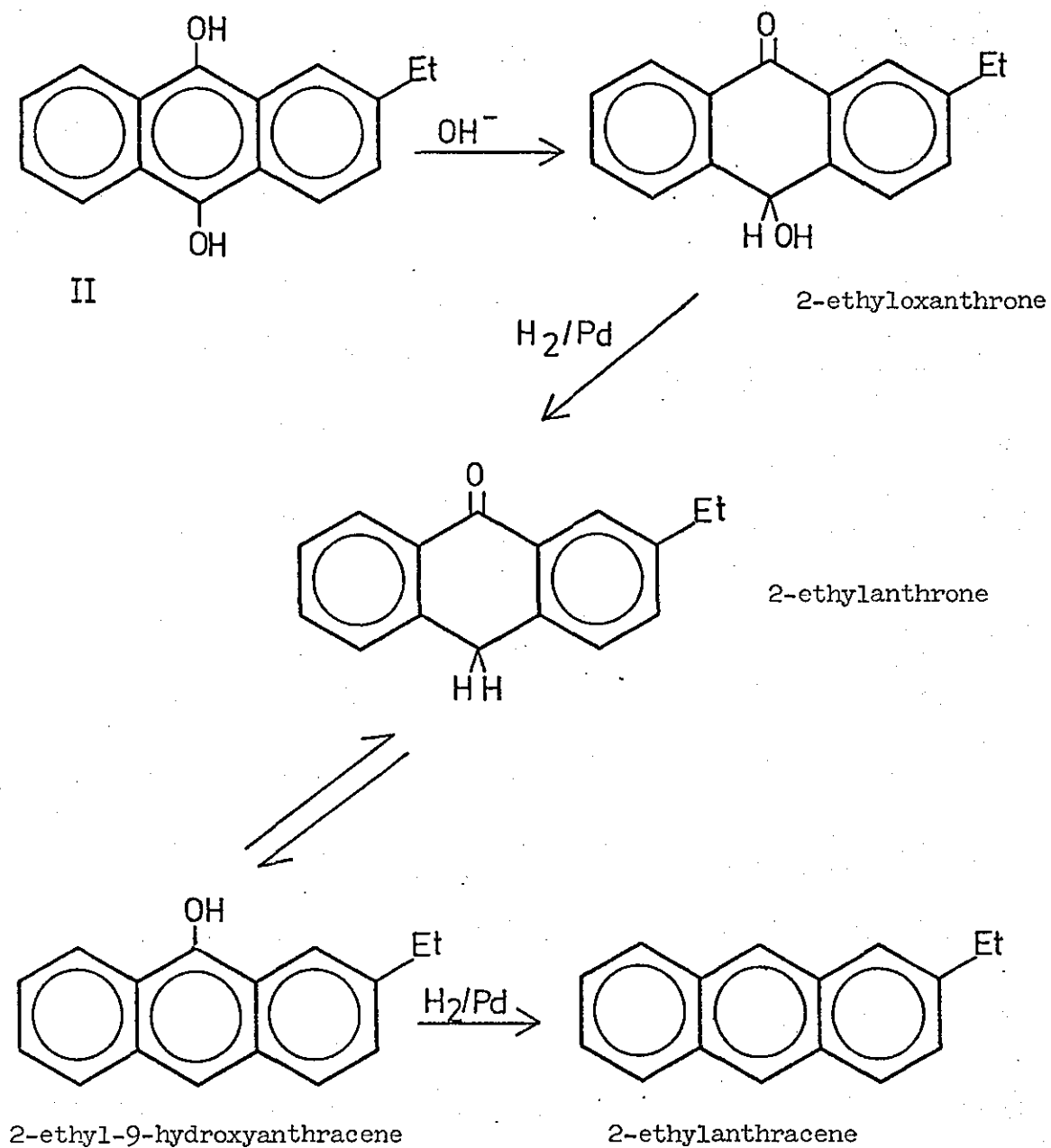
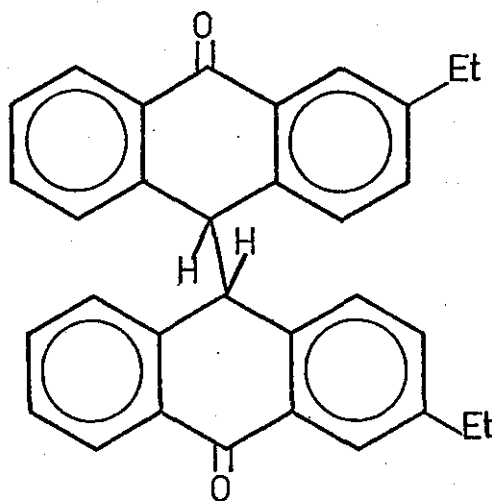


Fig.I.3. Reactions of Quinonoid Oxygen Atom.

detecting and determining such compounds would be advantageous in a complete investigation of Working Solution composition.

Many other reactions, which occur only at very low probabilities and therefore in small amounts in a single production cycle, could nevertheless have made a significant contribution to the overall composition of the Working Solution over several years of cycling, many times a day. One would expect small amounts of compounds with completely saturated ring systems, or ring-opened compounds, together with compounds of the dianthryl type, for example:-



Products from these types of reaction and others as yet unknown contribute to the composition of the Working Solution, making the separation and quantitation of even the major components a very complex problem.

3. Possible Approaches to the Analysis of Working Solution.

3.1 General.

A method suitable for this analysis should be rapid and easy to carry out in plant conditions. It should be both qualitative and quantitative for the required components so that an equilibrium might

be maintained between them by plant adjustments. The method is also required to be adaptable for use in trace component identification as might be necessary for detailed studies of plant solutions.

Two approaches to the overall problem are possible:

- (a) Analysis of the Working Solution without prior separation into its components.
- (b) Analysis by separation of the components and quantitative measurement of each component.

Methods using both of these approaches will be discussed individually.

3.2 Polarography.

Many of the compounds in Working Solution are amenable to polarographic oxidation and reduction, as they contain quinone, carbonyl or epoxy groupings.

Polarography has been used in the estimation of the main components of Working Solution^{2,3}, and in studies of Working Solution with particular reference to compounds other than the main constituents.⁴ The technique has been used in quantitative estimation of Working Solution composition, but suffers from certain major disadvantages. Chief of these is the difficulty of resolution between the half-wave potentials of the various components. The differential cathode ray polarographic technique⁵ gives overlapping peaks in the analysis of Working Solution, and, depending on the half-wave potential separation, each peak may contain more than one compound. Where these potentials are closer than about 25mv the usefulness of the results would thus be in considerable doubt.

Another difficulty is that certain of the Working Solution components may be electrochemically inactive, e.g. fully reduced or oxidised stable compounds. These would be undetectable and could form an unidentified and chemically significant part of the solution.

Polarography is thus a usable technique in connection with the analysis required, but, with the limitations of resolution and requirement of electroactive compounds or ready conversion to such activity, would not be the technique of first choice.

A partial answer to the resolution problem was proposed by Russian workers⁶ who used a T.L.C. separation, (see Section 3.3) followed by polarographic estimation of the separated H_4 EAQ and EAQ. This procedure has some application, but in investigation of Working Solutions could not give enough separation of the many compounds on the plate to enable quantitative recovery and measurement to be made. The technique is also not appropriate for quality control working under plant conditions.

3.3 Thin-Layer Chromatography, (TLC).

The Russian paper⁶ previously cited describes a TLC system to separate EAQ from H_4 EAQ using a ternary methanol/n-butanol/water system as eluant. Little other work seems to have been published on the compounds of interest, however, possibly because quantitative analysis of mixtures of these compounds in mixtures would require high resolution between spots, which is difficult to achieve.

Although the development of a method for analysis of the main components of Working Solution by TLC should be possible, it is not likely that TLC could resolve all the components. This is because of the relatively low number of theoretical plates used in TLC separations^{7,8} and because of the small size of the plates used. Quantitation is also a difficulty with TLC and is not usually of the required accuracy or reproducibility. This method was considered as unsuitable for the present purposes.

3.4 Gas-Liquid Chromatography, (GLC).

To be successfully chromatographed by the technique of GLC

the compounds to be examined must be thermally stable and relatively volatile. If these conditions are not met, the compounds will either stay at the injection point or will pyrolyse and give complicated patterns of breakdown products at the detector. This type of behaviour is of use in the identification of unknown single substances and some mixtures, but would not be applicable to Working Solution analysis, as many of the components are relatively involatile and decompose to tars on heating, while others, for example the components of 'Aromasol H', chromatograph readily.

The technique has been examined⁴, but was found to be unsatisfactory because of the above difficulties. Derivatisation of the main components might be used to determine them in Working Solution, but unknown compounds could not readily be investigated using GLC. It is thus considered an unsuitable technique for the purposes of the work herein.

3.5 High Performance Liquid Chromatography, (HPLC).

Modern high efficiency liquid chromatography is well suited to the analysis of thermally unstable or involatile compounds. The technique is very flexible in use, as many parameters of the separation process may be changed to give optimum running conditions. The separations obtained are usually rapid and highly efficient, and, once optimised, are relatively easily applied to routine analytical use. The detectors now available permit both qualitative and quantitative analysis of compounds at low concentration, while the various types of chromatography have been developed to high efficiencies by using modern column packings and packing techniques.

The limitations on use of HPLC for specific compounds are few, that is, the compound must be soluble in a solvent, must be able to interact with a stationary phase of some sort and must be detectable by one of the many types of detector now available. The compounds in

Working Solution fulfil all of these conditions, since they are already in solution in the sample and, for example, all possess some absorption in the U.V. region, permitting the use of a low-volume flow-through U.V. detector.

This was thus chosen as the technique most suitable for the analysis of Working Solution.

II. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

1.1. General.

It is not intended to give exhaustive coverage of HPLC technique and apparatus here, since the modern texts^{10,11,12} cover the subject in detail. General techniques will be described if specifically relevant to the thesis subject but will otherwise be only mentioned as necessary to complete coverage of various points.

1.2 Definition of High Performance Liquid Chromatography.

HPLC may be defined as that branch of liquid chromatography using closed columns of high-efficiency materials and requiring the use of pumps to attain high flow-rates. This practical definition indicates the main differences between the traditional type of column chromatography, using large diameter columns of low efficiency and gravity feeding of the eluant, and HPLC with its smaller diameter, high efficiency, re-usable columns requiring many atmospheres pressure to obtain fast analysis times. These fast analyses, often of the order of a few

minutes, may be attained with good resolutions because of the efficiency of the columns and column technology used.

1.3 History.

High Performance Liquid Chromatography (HPLC), also variously known as High Speed, High Efficiency or High Pressure Liquid Chromatography, may be considered to have started from 1969, when a special session on liquid chromatography at the Fifth International Symposium on Advances in Chromatography¹⁵ brought the technique to the attention of a wide audience. The basic technique dates back to the work of Tswett¹⁶, however, and while recent advances have increased the efficiency, nevertheless the basic work dating before 1969 is still valid.

The rapid increase in interest in HPLC and the great effort and investment applied to its development are largely the result of the urgent need to separate, identify and quantitate complex biological and bioactive materials, in which field HPLC is outstandingly successful¹⁴. The technique may be said to have had a long gestation period, a very rapid labour and to have been born fully grown in concept and practice. This came about because the basic theory is essentially the same for GLC and HPLC, and this theory had been available before the 1969 Symposium focussed attention on this 'new' technique. The work of Martin and Synge¹⁷ in 1941, for which they received the Nobel Prize, led to the development of GLC and other forms of chromatography. The corresponding development of a high-efficiency form of liquid column chromatography came some 15 to 20 years later, when suitable low-volume high-sensitivity flow-through detectors and other instrumentation were developed. The growth of HPLC has been rapid, with a proliferation of papers and reviews now available on a wide range of aspects and applications. It has gone in 6 years from a specialist research technique using home-made apparatus to a sophisticated branch of chromatography, with instruments readily available for most purposes from quality

control to the most demanding of research facilities. It is expected this popularity will continue to increase since this is a technique with a great potential application in fields of biology, biochemistry, pharmacy and many other branches of science.

2. Types of Chromatography Available to HPLC.

2.1 Liquid/Liquid Chromatography.

In LLC the separating mechanism is that of partition of the solute between two liquid phases. One phase is the eluant while the other is attached or coated onto the support material. The two liquid phases are of different polarities so that the distribution coefficient of the solute between them is not unity. If the two phases were identical in polarity, no normal liquid/liquid chromatography could occur. It follows that there are two cases of interest, namely those of the stationary phase (on the support) being either more polar or less polar than the mobile phase (the eluant).

If the eluant is less polar than the stationary phase, then the more polar the solute molecule is the more it will interact with the stationary phase and be retarded relative to the eluant flow. This is the normal mode of LLC.

If, however, the eluant is more polar than the stationary phase, the reverse of the normal mode follows and more polar solute molecules are eluted first. This is termed the 'reversed phase' mode of operation. It is apparent that the two modes are complementary in that the full range of solutes may be chromatographed by one mode or the other. The support material in coated columns does not, in theory, take part in chromatography.

2.2 Liquid/Solid Chromatography.

The mechanism of separation in this mode is that of reversible

adsorption onto the surface of the solid packing material. Interactions of a solute with different surface groupings are possible, leading to a certain measure of specificity of some adsorbents. The degree of interaction between the adsorbent and solute molecules is dependent to a large extent on the amount of competition between solute and solvent molecules for the active surface sites. The solvent is thus an important variable in LSC separations. LSC is also the basis of Thin Layer Chromatography, (TLC), another technique of considerable importance in organic chemistry.

2.3 Ion-Exchange Chromatography.

Ion-exchange processes occurring between solutes and stationary phases holding suitable exchange groups may be used to separate compounds according to their affinities for the exchanger. The process is limited to compounds possessing groups which are ionic or potentially ionic in character, and is also largely limited to compounds in aqueous solution. The analysis of amino-acids and nucleic acids has been effected on a routine basis using this type of chromatography for some time¹⁸.

2.4 Exclusion Chromatography.

The separation of compounds by molecular size and shape may be effected using exclusion chromatography. In this the molecules of different sizes are passed through a bed containing pores of a uniform size, which would generally be of the same order of size as the molecules being separated. Entry of solute molecules into these pores is then dependent on size and shape of the solute. The smaller solute molecules enter the pores easily, thus, in diffusion into and out of the pores, they have a long and relatively tortuous path through the column. Large molecules will not diffuse into the pores and have correspondingly short flow-paths. They will thus elute before smaller

molecules, giving an order of elution of compounds which is different from the other modes, as the larger molecular-weight compounds in the other types of chromatography tend to be retained longer than low molecular-weight species.

The process may be carried out in both aqueous and non-aqueous solvents, with a variety of bed-materials of different physical and chromatographic characteristics. It is used to a large extent in analysis and characterisation of polymers by molecular-weight distribution. When it is required to use soft gels, e.g. Sephadex, the technique is not usable with HPLC as the gels would disintegrate under pressure.

2.5 Processes Suitable for Analysis of Working Solution.

The compounds forming Working Solution are essentially insoluble in water. Ion-exchange, which requires that the compounds investigated be soluble in water and polar if not ionic, is thus contraindicated.

Most of the constituents, certainly those forming the largest amount by weight in solution, are derived by relatively small chemical changes from the initial alkyl-anthraquinone. They thus possess the same carbon ring system in the same configuration, i.e. they are the same size, shape and approximate molecular weight (except for the solvents and the possible compounds of the dianthryl group). Exclusion chromatography may thus be discounted from the possible modes of chromatography, except in investigation of compounds of the dianthryl group.

The remaining two types, namely LSC and the two variations of LLC, are both suitable for W.S. analysis, since both utilise differences in behaviour of compounds which should be found in Working Solution. The LLC mode should be able to separate EAQ and H_4 EAQ, for example, because of the enhanced solubility of H_4 EAQ in non-polar phases arising

from the reduction of its benzenoid ring. Similarly, LSC should be usable in the same case because the reduction causes a loss of planarity in the molecule and loss of one aromatic ring, both affecting the adsorption process.

The modes of chromatography used in the investigation are thus those of LSC and LLC for the majority of the work.

3. Chromatographic Materials and General Procedures.

3.1 Column Packing Materials and Supports.

3.1.1 General.

Column packing materials in LSC and support materials in LLC may be divided into low-efficiency and high-efficiency packings. The high-efficiency group may be further divided into small particle-size totally porous materials and pellicular materials. These will be discussed in general terms, but for greater detail and current information reference may be made to the texts already quoted^{10,11,12}, review articles^{19,20,21}, and manufacturer's literature. In the last group the Dupont, Waters Associates and Varian literature is possibly the more informative in the amount of general technical and practical detail given as well as in specific product information.

3.1.2 Low-Efficiency Packings.

Low-efficiency packings in HPLC are usually large particle-size, totally-porous materials, (see Fig. II.1). They have poor mass-transfer characteristics, that is to say a solute compound will diffuse into and out of the particle slowly and thus be slow in equilibrating its concentration at the surface with that in solution under flow conditions.

This type of packing finds use in preparative chromatography, where its ability to take a high solute loading and its relative

cheapness are particularly useful. In LLC the support would be coated with the liquid stationary phase, and here again the high loading capacity for stationary phase allows relatively large amounts of solute to be chromatographed.

The relatively inexpensive low-efficiency packings may also be used in an exploratory function to find an appropriate packing type before going on to use more expensive high-efficiency materials.

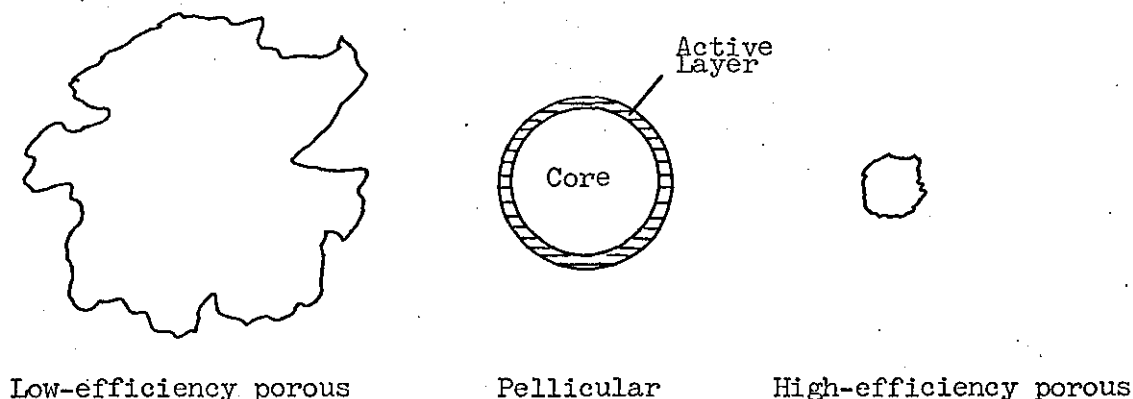


Fig.II.1 Types of Column Packing Materials.

3.1.3 High-Efficiency Pellicular Packings.

A pellicular packing consists of particles with a solid inert core coated with a thin layer of porous active material, (see Fig. II.1). The thin layer of porous material ensures rapid mass-transfer characteristics, rapid equilibration of the solute with the surface and consequently small amounts of band-spreading.

These packings, although very efficient, have a small volume of active material per unit volume of packed column and thus have small capacities for solutes. Preparative work with these materials must thus be on a smaller scale than with totally porous types, typically of the order of one tenth of the amount which could be used with the porous varieties.

3.1.4 High-Efficiency Totally Porous Packings.

Packings of this type have small particle size, usually of the order of 5-10 microns, (see Fig. II.1) and combine the advantages of the two previous types of packing, namely high capacity and rapid mass-transfer. The particles must be of a very small size range for best efficiency, so that the mean path of all the solute molecules is the same. Differences in particle size lead to different path lengths around the outside of the particles and also into the pores of the solid. Different path lengths result in band broadening and loss of resolution and efficiency, e.g. Ref.12, p.3-5.

These materials cannot be easily packed by simple tap-and-fill techniques because the surface effects arising from small particle size, such as electrostatic repulsion and aggregation, do not allow a good reproducible packing to be obtained. Special techniques are necessary to pack these materials, notably slurry packing.

3.2 Column Packing Techniques.

3.2.1. Tap-Fill.

This simple packing procedure involves a step-wise addition of the packing material followed by a vertical, and sometimes also horizontal, tapping or vibration of the column. Small amounts of material are bedded down reproducibly in thin layers, leading to good packing, for suitable materials.

Pellicular and large diameter totally-porous materials may be successfully packed in this way, but for particles of less than 20 microns diameter reproducible efficient packing is not generally obtainable because of the surface effects mentioned in section 3.1.4.

Tamping the packing material with a rod may be used in conjunction with tap-and-fill, but has the disadvantage that particles of the packing may be crushed and so contribute to column blocking and loss of efficiency by increasing the range of particle size.

3.2.2 Slurry-Packing Techniques.

These pressure assisted techniques use a slurried form (a thick suspension) of the packing material which is pumped at high speed and high pressure into the column, thus cancelling out effects of surface energy, electrostatic repulsion and aggregation^{22,23}.

The preparation of the slurry is of considerable importance in the production of efficient columns, as the final characteristics of the column depend on the quality of the slurry. Packing of the column should be fast and smooth, with no time allowed for the particles to interact with each other until they reach their final position in the column.

Two types of slurry are in general use, namely the balanced-density and the stabilised aqueous types, 'stabilised' here referring to the reduced tendency of the particles in the slurry to form aggregates and thus precipitate. The balanced-density method employs a slurry of the column packing in a suspension medium of the same density as the particles suspended. The appropriate density is usually obtainable by mixing tetrabromo- or tetrachloroethylene with other suitable organic solvents, such that when the predried packing is added to the degassed solvent and is mixed in and dispersed by ultrasonic agitation it will remain in suspension with no visible settling or flotation for at least one hour²².

The column to be packed is then fitted with a high-pressure reservoir at the top and is filled with the balanced-density solvent to the bottom of the reservoir. Sufficient of the slurry to pack the column, with some excess to ensure complete filling at one operation, is then added to the reservoir, followed by a thin layer of water, then the reservoir is connected to the high-pressure pumping system. A high pressure of an organic solvent is then suddenly applied to the reservoir and the contents are forced at high flowrate into the column.

The stabilised aqueous slurry would be made in a similar way,

but with a dilute ammonia solution as the suspending solvent. This effectively prevents the production of electrostatic charges. The procedure is otherwise similar to that used in the balanced-density method²⁶.

After packing by the slurry techniques it is generally necessary to remove the slurry solvent and to equilibrate the column with the chromatographic solvent to be used²². This would generally require the passage of several hundred column volumes of various solvents going from the most polar, often water, through in stages to the polarity of the solvent to be used in analysis. It is generally recommended that the column should be equilibrated for up to 12 hours by pumping the analytical solvent before it is fully in equilibrium, (e.g. see ref. 11, p.268).

3.3 Elution Techniques.

3.3.1 General.

Of the parameters, which affect the elution time or elution volume in HPLC, the ones which are most readily changed in the course of an analysis are flowrate, solvent strength and temperature. To effect the rapid analysis of a mixture of a number of compounds it may be necessary to change these characteristics in a regular manner to alter the retention of some compounds which would otherwise elute too rapidly or too slowly. This is termed solvent, flow or temperature programming whichever parameter is changed. Elution with a solvent of fixed composition is termed isocratic elution and is the mode of elution most usually used.

3.3.2 Isocratic Elution.

This mode of operation is the easiest to use with the majority of lower-cost instrumentation and has no significant disadvantages when used to separate a simple mixture with few components. The solvent/column packing system may be optimised to give adequate separations and

then gives reproducible and reliable results. For a complex multi-component mixture it becomes more difficult to optimise separations for all components, and it is then advisable to alter one of the parameters mentioned above to optimise successive regions of the chromatogram during the analysis.

3.3.3 Temperature Programming.

Temperature change may be used to reduce solvent viscosity and increase solubility of difficultly soluble compounds, but temperature programming as such is rarely used in HPLC. This is partly because a change of temperature sufficient to markedly affect the chromatographic behaviour of a compound, (ref 11, p.272), would normally be enough to cause the solvent to boil, or in LLC would cause stripping of an applied liquid phase. The results obtainable by temperature programming are usually more easily obtained by one of the other programming techniques.

3.3.4 Flow Programming.

This simple means of increasing resolution is applicable to those mixtures where the separation of the initially-eluted peaks is incomplete, but where the last-eluted compounds are spread over long periods and are well-separated.

By starting the elution at low flow-rate the number of effective plates, N , for the first peaks is increased, (see section IV.2). The separation of these peaks, at a higher efficiency, is thus improved relative to simple isocratic elution. As the separation proceeds, the flow-rate is increased in a regular manner, thus reducing the time required to elute the last compounds but also reducing the efficiency of the separation of these compounds. With many late-eluting compounds, however, separation is usually so great that some efficiency may be sacrificed in order to make the elution quicker and the peaks sharper, and thus more easily quantitated.

Flow programming cannot change the fundamental parameters of the chromatographic system, such as the resolution, but can make better use of systems optimised for resolution of the middle cut of compounds, and thus may be a very useful additional technique with relatively simple types of apparatus.

3.3.5 Solvent Programming, (Gradient Elution).

The stepwise or continuous change of solvent strength during elution of a mixture results in changes in the capacity factor, k' , (see section IV.2), for each component such that all components may be eluted with an optimum k' of about 1.5 to 4.0²⁵. This results in the peaks being better separated, as earlier peaks will be spread through a larger volume of eluant just as later ones are eluted more quickly in a smaller volume. The early peaks lose little of their sharpness but are better separated, so that resolution is increased. Late-eluting peaks are considerably sharper, depending on the gradient used, and may because of this give more information than isocratic elution, such as small concentrations of compounds which may be seen under gradient elution but would otherwise be low, wide peaks, so becoming lost in baseline drift.

Solvent strength in LSC and LLC is usually changed in gradient elution by the mixing together of two solvents, one of which is more polar than the other, in such a way that the type of gradient produced at the column is reproducible. Reproducibility of the gradient is important in the qualitative analysis of samples with a broad range of compounds, as gradient elution has a major use in exploratory work in this type of problem. The compounds of interest in a mixture may be found by a gradient elution study, followed by the design of a simpler elution system, preferably isocratic, for routine analyses. Gradient elution devices for producing the gradient at the entry port to a reciprocating pump, thence through the pump to the column, are simply

made, (see fig. II.2), but are not as reproducible as those gradients generated by two high-pressure pumps delivering the two solvents directly to the high-pressure system. The associated extra expense of another pump and the necessary control circuitry for pumping rates is often high, tending to approximate to the cost of a single isocratic routine analysis instrument. The tendency is thus to use gradient elution as a research or exploratory tool and aim for isocratic elution methods in routine work. Having said this, however, there will be some cases ²⁴ where a mixture cannot be resolved without gradient elution and in this case the high cost of the instrumentation would have to be accepted.

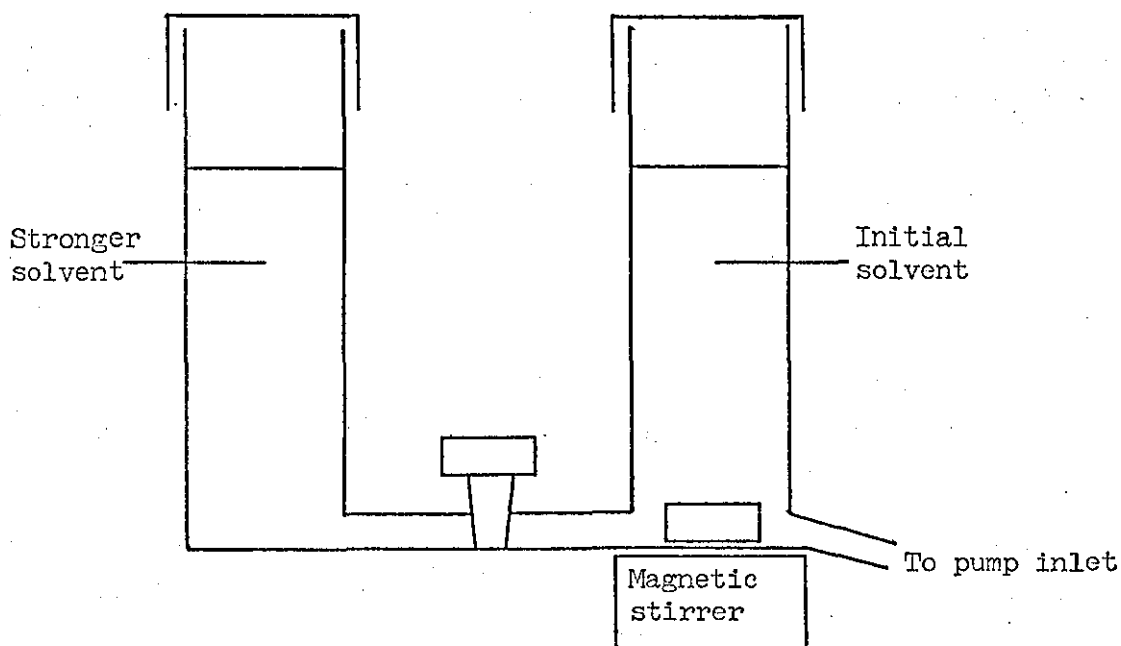


Fig.II.2. Two-Chamber Gradient Elution Device.

Solvent programming is possibly the most powerful tool available to completely resolve broad range mixtures in HPLC. It is the expense of the necessary equipment which tends to limit its general use. Such effects as baseline drift due to change of solvent composition, resulting in change in U.V. absorption and viscosity, may be largely

eliminated with some detectors by choice of solvents and do not detract to any extent from its uses. The newer chemically-bonded column packings, (see Chapter IV), may even be used in gradient LLC, a combination not usable in conventional LC. In summary therefore it may be said that gradient elution has many advantages over isocratic elution in speed of operation and ability to handle a wide range of compounds in a single elution, but that the expense required to produce the necessary closely controlled mixtures is often not justifiable in many analytical situations.

III INSTRUMENT DESIGN, ASSEMBLY AND MODIFICATIONS.

Introduction.

A discussion follows of the merits and disadvantages of the various general components in HPLC systems; this is not intended to be a detailed or exhaustive discussion, as such treatment is available elsewhere^{10,11,12}. General articles and reviews of HPLC apparatus are now frequent in journals such as Analytical Chemistry reviews and the Journal of Chromatographic Science. Items cited herein will thus be selected examples. A brief description of the reasons for choosing the eventual form and components of the operative machine is given so that an appreciation may be obtained of its uses and limitations, relative to other instruments.

Developmental aspects will be covered in the sections giving the details of the apparatus and its assembly.

The detector and its electronics are dealt with in a separate section, as the factors and considerations involved in choosing, designing and building a suitable detector are complex. It is to be noted

that the type and construction of the detector unit is of considerable importance to the quality of results produced.

1. Performance Required and Design Considerations.

1.1 Introduction and General Background.

At the time of starting the study described herein most HPLC separations were being made with relatively low pressures, about 1,000 p.s.i.; a capacity for 5,000p.s.i., whilst available with certain manufacturers' instruments, was not considered essential by most workers.

Developments in column technology have ensured that, for most separations, even at high speeds, pressures in excess of 2,000p.s.i. are not necessary. The maximum pressure capability decided upon at the design stage was 2,500p.s.i.. The maximum pressure selected is a most important parameter in the design stage, as this controls the type of valves, joint fittings, pump, etcetera, selected.

The apparatus used in HPLC may be represented in a block diagram, (fig. III.1). The generally available instrumentation^{27,28} conforms to this scheme with certain variations and additions as necessary to fulfil more advanced functions, (shown in broken lines in fig. III.1), such as gradient elution.

1.2 Specific Components.

1.2.1 Pump.

There are three types of pump in general use in HPLC. These are:-

Pneumatic, (simple or amplifying)

Reciprocating, (plunger and diaphragm)

Syringe Type.

Pneumatic.

This type is operated by pressure from a gas cylinder, usually

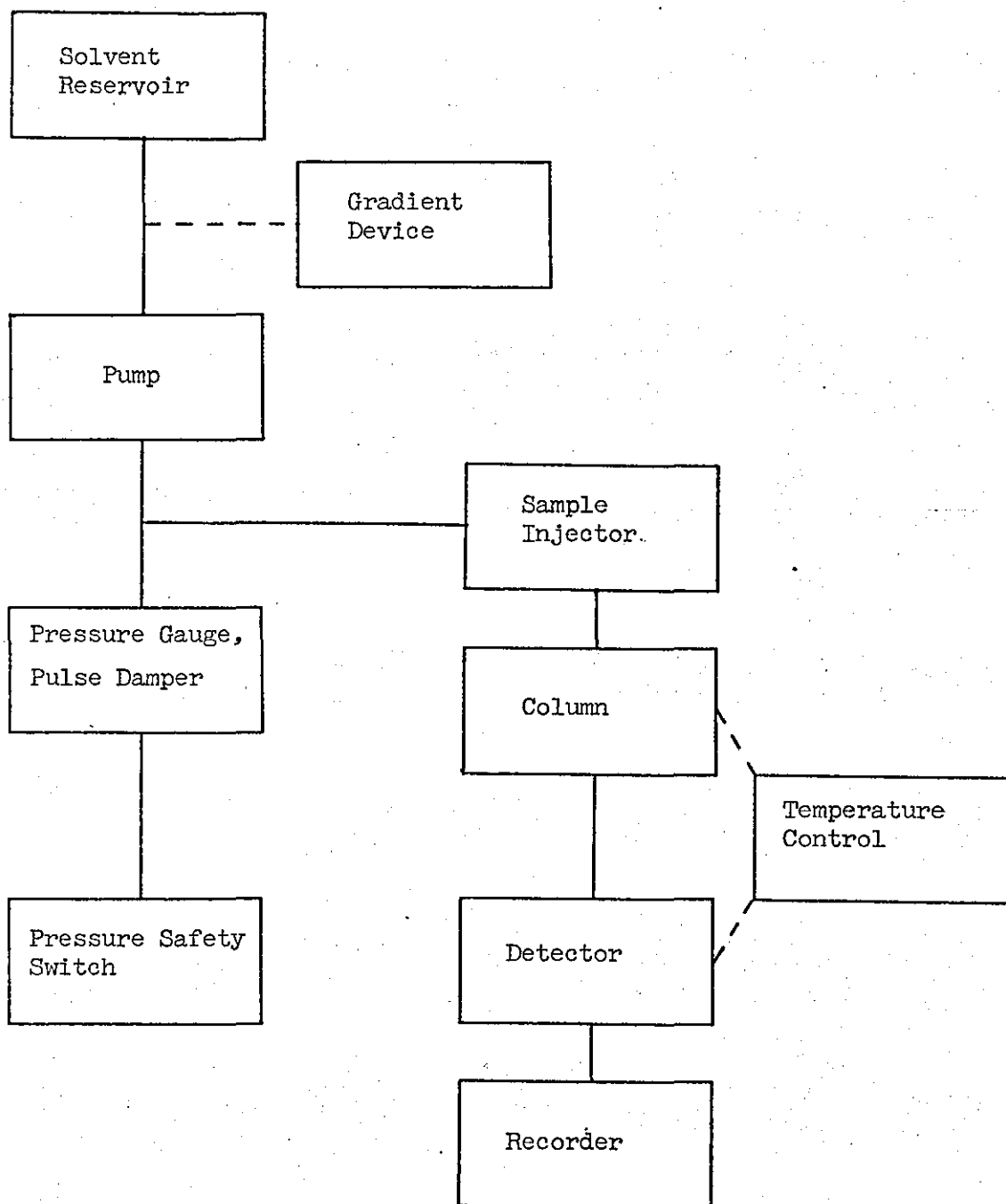


Fig. III.1. Block Diagram of an HPLC Instrument.

up to 500p.s.i. A higher pressure type is the pneumatic amplifier pump, the eventual maximum pressure developed by it being dependent on the amplification factor, which depends on the surface areas of the two plungers in the pump. Both types have the limitation that they can deliver only a limited volume of mobile phase before having to be refilled. This refill can be very rapid for the amplifier pump, often only a few seconds. Solvent delivery is usually pulse-free with this type of pump.

Reciprocating, (Plunger and Diaphragm Types).

These pumps generate pressure by the reciprocating action of a piston or diaphragm driving a small volume of solvent at a time out of the pump chamber. Non-return valves then allow more solvent to enter the chamber from a reservoir at atmospheric pressure for the next stroke. Solvent delivery is thus pulsed, which may be a disadvantage when using a high-sensitivity detection system. Solvents can be rapidly changed, however, so that this type of pump lends itself more readily for use in gradient elution than other types. This factor, and its relatively low cost, makes it a viable choice for low-cost systems.

Syringe Pumps.

The syringe pump is usually a vertical cylinder of stainless steel holding a stainless steel, screw-driven, plunger to displace the cylinder's contents. The screw-drive is powered by a stepping motor which drives through gears. The stepping rate, hence the rotational speed, of the motor is controlled by complex electronic circuits. Although these pumps deliver an essentially pulse-free constant flow of solvent they are relatively very expensive. The cylinder containing solvent is often inconvenient to clean when changing from one solvent to another, and gradient elution requires two such pumps and associated electronic control mechanisms. Thus for a gradient elution facility the cost is currently about ten times the cost of a simple reciprocating pump.

Pump Chosen.

On grounds of cost, flexibility of use for gradient and isocratic

modes, and availability, a reciprocating plunger pump was chosen. A description of the pump and other components will be given later in the 'Equipment Assembly' sections.

1.2.2 Pulse Damping Devices and Pressure Gauges.

The use of the reciprocating pump made it necessary to incorporate means of reducing the size of pressure pulses to acceptable levels. A convenient way of achieving this is by the use of Bourdon-type pressure gauges, which act as pressure-storage systems on the pumping stroke and thus help to maintain a steady pressure at the column inlet. It was also considered desirable to incorporate a safety cut-out switch so that, if a pressure build-up occurred in the system, the maximum safe pressure of the various components would not be exceeded. This safety cut-out was also based on the Bourdon gauge principle, and thus would act as a second pulse-damper in the high-pressure line. The pressure gauge, for monitoring the column pressure and detection of pressure build-up in case of a blockage in the system, was chosen with a 0-4,000p.s.i. scale to allow for momentary overshoot of the maximum safe pressure without damage to the Bourdon tubes.

1.2.3 Valve-Gear.

Several different valves were examined in the initial studies. The final choice of valves included three types:

- (a) 'bonnet' stop-valves
- (b) metering needle valve
- (c) ball valve, on/off.

(a) 'Bonnet' Stop-Valves.

These screw-action valves were selected to be able to hold a pressure differential of 3,000p.s.i. across the valve without leaking. It was found that Simplifix valves leaked through the valve seat above 500p.s.i. and were not suitable. Valves marketed by Techmation Ltd.,

from the Hoke and from the Whitey companies were satisfactory up to 3,500p.s.i. for short periods and up to 2,500p.s.i. for continuous running.

(b) Metering Needle Valve.

A valve was needed to maintain a constant but low flow of solvent to the reference side of the detector. A metering needle valve was chosen as it is inherently more accurate in controlling flow reproducibility than is a screw-action stop valve, which would be a crude alternative. Needle valves are not designed to act as stop valves, hence to make it possible to stop the reference flow a 'bonnet' stop-valve was incorporated before the needle valve.

(c) Ball Valve, On/Off.

A quick-action on/off valve is required in the use of the stop-flow injection technique to ensure a minimum disturbance to the detector baseline. The rotary ball-valve provides this action and may be made pressure-tight to 3,000p.s.i.. The 'bonnet' valve, while being somewhat cheaper, is not quick in action. Operation of the ball-valve used has been satisfactory over twelve months at pressures up to 3,000 p.s.i. for short periods.

1.2.4 Pressure Fittings.

The fittings used to connect the different components and tubing were variously, 'Kromlok', 'Gyrollok' and 'Swagelok', all in stainless steel. Although the various makes of fittings differ in detail it was found that good joints could be made, if necessary, on interchanging properly made joints of the three above types. This is not recommended by the manufacturers, but did allow some intermixing of ready-made joints to allow greater flexibility in assembly and subsequent reassembly when changing the arrangement of components for various reasons, (e.g. see section III.3.2.4).

1.2.5 Injection Devices.

The method of introduction of the sample in HPLC is a particularly critical factor in determining the performance of the chromatographic system²⁹. There are three commonly used methods,

- (a) Septum injection
- (b) Stopped-flow injection
- (c) Micro-sampling valves

Many variations on these are used^{11,29}, but the basic principle of all methods is to apply the sample to the column in an infinitely thin band or spot or as near this ideal condition as possible. The emphasis on injection technique in HPLC is because of the relatively small ratio of eluant volume (often less than 10ml) compared with the sample size in this technique (typically 1 μ l). In GLC, for example, the mobile phase has a very much larger volume than the injected sample (e.g. 100-200ml), so that any deficiency in injection technique leading to imperfect sample application is normally less critical with respect to peak shapes and resolutions. In HPLC, however, the very high sensitivity detectors now available have detection cells which may approximate to the applied sample size, hence any extra-column peak broadening due to bad sample application, or excessive dead-volume within the overall system, is unacceptable.

(a) Septum Injection.

Septum injection devices are usually variations on the theme of a T-joint connected directly to the end of the column, (fig. III.2). The solvent flow is through the side-arm of the 'T' and past the injection needle, so that a sample injected through a septum on the other arm of the 'T' is placed at the nearest possible point to the column and is immediately swept on to the column, with no possibility of back-diffusion and dead-space band-broadening. In practice the limitations of this method are those of the injection syringe and the septum material. If the syringe is not able to withstand pressures up to about 5,000p.s.i.

without leakage past the plunger, then accurate injections will not be possible. The type of septum material is critical with regard to its behaviour in contact with organic solvents. If the septum swells, softens or dissolves in the eluant system, then leakage of solvent and loss of pressure will result, leading to poor reproducibility of peak retention times and peak areas. For low pressure systems and with suitable syringes and septa this technique is probably the most simple and convenient.

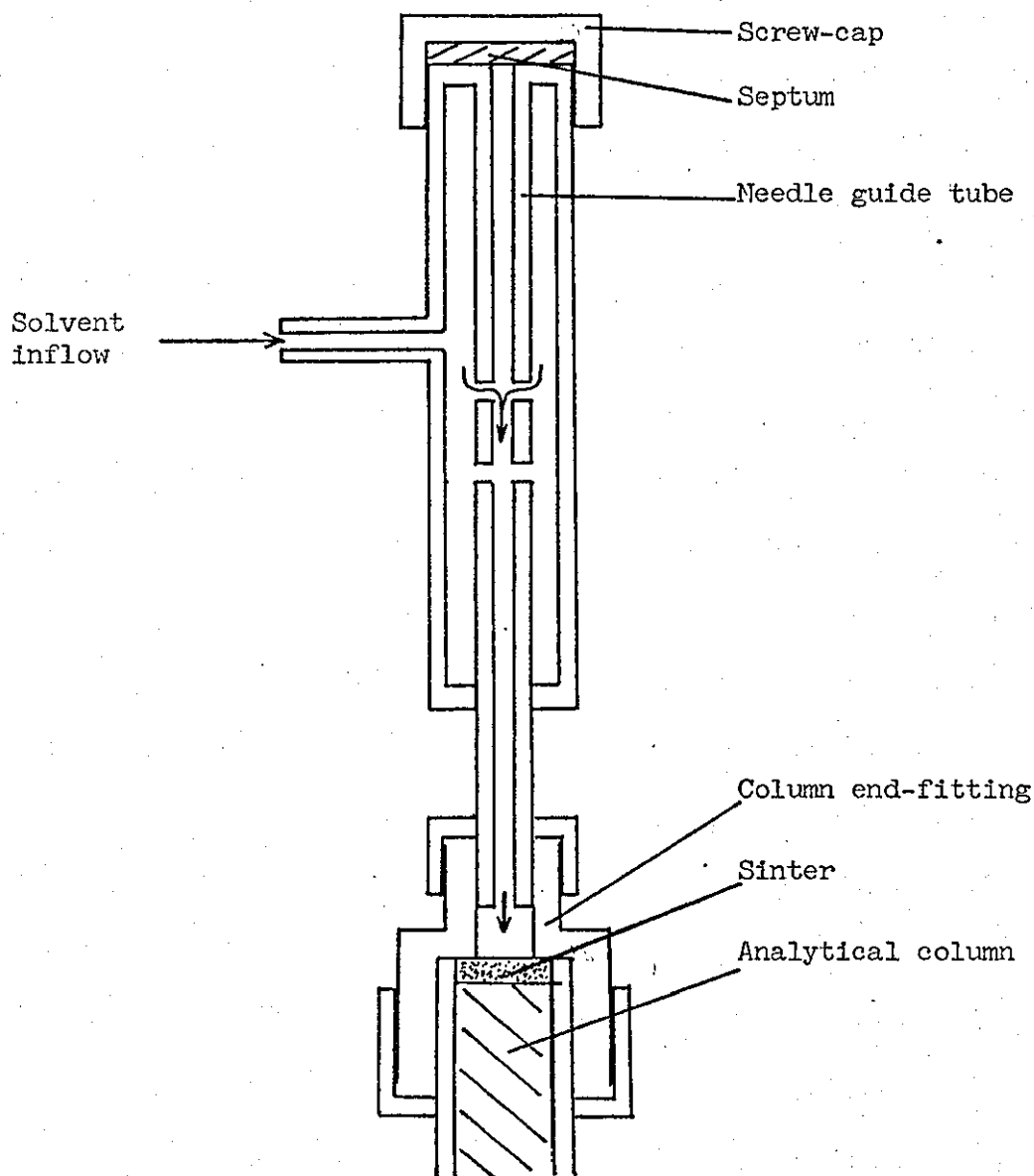


Fig. III.2. Septum or Stopped-Flow Injector Fitting.

(b) Stopped-Flow Injection.

The physical appearance of this type of device is often very similar to the previous type, with the addition of a quick-action on/off valve in the solvent line before the injector. The difference in use is that, when injecting a sample, the on/off valve is turned to 'off', the pump is switched off, and the septum or other seal is removed from the injector head. Injection is then carried out at atmospheric pressure, the seal is replaced and tightened and the pump is started. The on/off valve may then be turned 'on', instantaneously applying the normal working pressure to the injector and sweeping the sample onto the column. The advantages of this technique are that the syringe may be a standard type, since it is used at atmospheric pressure, and that the septum is not perforated, thus ensuring its longer life and greater leak resistance.

(c) Micro-Sampling Valves.

The sample is held in an internal cavity in the valve, or in an external loop of tubing for larger volumes, and is introduced onto the column by rotating the valve rotor to a position where the cavity is then in the eluant line. The injection is limited to a single sample size unless it is modified by removing the external loop and replacing it with another loop of different capacity. The valve is invariably expensive because of the great accuracy of machining required to ensure pressure-tight operation while simultaneously providing a constant internal volume. The advantages of this type of injection device are that it is used at the system operating pressure, thus reducing baseline fluctuations due to the injection, and that it is the type most easily adapted to automation, to allow many samples from, say, industrial plant pipelines to be sequentially injected into a monitoring HPLC machine. Sample application should in theory be as sharp as with the other techniques, but, in many valves, excessive dead-volume between the valve and column head may cause broadening of the injected sample band.

Devices Used.

A micro-sampling valve, by Specac Ltd., was used initially, but was found to be unreliable with respect to the volume injected and to leak under operating conditions.

The majority of work described herein was with stopped-flow injection, which was found to be satisfactory on all counts, as well as allowing different sample volumes to be applied.

1.2.6 Column Temperature Control.

Much of the work was carried out at ambient temperature, but some work at elevated temperatures necessitated the use of an oven which was fitted for the purpose with a sensitive temperature controller and circulating fan. The temperature control obtained was not as good as that obtained with commercially available ovens, but was to within $\pm 1^{\circ}\text{C}$ at the temperature selected.

1.2.7 Solvent Reservoir.

A stainless steel or glass reservoir is usually used in HPLC. Some reservoirs may be equipped for the degassing of solvents, but it was found convenient to degas small quantities (ca. 200ml.) of the required solvent and then add it to a simple stainless steel reservoir connected to the pump inlet. This avoided the production of gas bubbles in the detector flow-cell.

Gradient elution with external generation of the gradient requires a specialised solvent reservoir system, such that two solvents can be mixed in exact proportions and delivered to the pump. Several devices have been described^{30,31} and reviewed¹³.

The gradient device used herein was a simple one, of two cylinders joined at the base by a narrow tube, (see fig. II.1). The pump was fed from one of the cylinders which was equipped with a stirrer. The outflow from this cylinder was consequently made up from the second

cylinder, usually holding a stronger solvent. This general type of device, that is of two connecting containers, may be used in various forms to generate different gradients³¹.

1.2.8 Columns.

A variety of sizes of column were used. All were made of stainless steel, with stainless steel end-fittings appropriate to the size of column. Accounts of column fittings, of other relevant column design factors and of performance are numerous^{23,28,32}. It is sufficient to note that minimum-dead-volume fittings with stainless steel or PTFE sinters were used at the column outlet, and that similar fittings with sinters or a quartz yarn plug were used at the injection end of the column. Any special or unusual features of columns are noted as appropriate in later sections, dealing with the practical work.

2. Detector Selection.

2.1 General Requirements of a Suitable Detector.

The detector should have as many as possible of the following characteristics.

- (a) Sensitivity to all compounds to be investigated
- (b) Sensitivity to small amounts of solutes
- (c) Low baseline noise level and high baseline stability
- (d) Wide linear range, for use in both analytical and preparative work
- (e) Low dead-volume of cell and connections
- (f) Low sensitivity to changes in flow-rate and ambient temperature.
- (g) Compatibility with gradient elution
- (h) Preferably non-destructive so that further investigations of separated compounds might be made.
- (i) Preferably low-cost.

No single detector as yet possesses all of these attributes. An examination of available detectors was thus required so that an optimum choice could be made.

The subject has been treated in considerable detail in books^{10,11,12} and in various reviews, that by Veening³³ being particularly useful. These reviews cover a number of types of detector based on different detection principles. Some of these detectors are at an experimental or development stage and are thus not suitable for the present work. These developing types include those using ultrasonic⁴⁹, streaming potential⁵¹, vapour pressure⁵², interferometric^{53,60}, electron capture^{55,61}, mass spectrometry^{56,57}, spray impact⁵⁸ and permittivity⁵⁹ principles. Some other detectors were unsuitable for obvious reasons, for example the radioactivity detector was not applicable because there is no radioactivity in the Working Solution, nor can any be easily induced in it. After consideration of the more suitable types, discarding those above, the following remained:

- 1 The micro-adsorption detector
- 2 The refractive-index detector
- 3 The polarographic detector
- 4 The ultra-violet absorption detector
- 5 The fluorescence detector
- and 6 The mass-transfer detector.

These will now be briefly described.

2.1.1 The Micro-Adsorption Detector, (M.A.D).

The principle of this is the measurement of heat produced on the adsorption of compounds from the elution solvent onto an appropriate adsorbent in contact with sensitive temperature sensors. The characteristic response of the detector is shown in fig. III.3. This indicates one of the limitations of this detector, namely the desorption (negative) peak after the initial adsorption peak. This makes the detector of very

limited use in systems where peaks might overlap and otherwise be closely spaced. Another limitation in the present study would be that baseline drift due to permanently absorbed impurities, and consequent loss of performance, might be high when passing through it the very complex mixture that is the Working Solution. It may be noted that the MAD is not compatible with gradient elution, as the changing composition of the solvent would cause artefact peaks and baseline drift to an unacceptable degree.

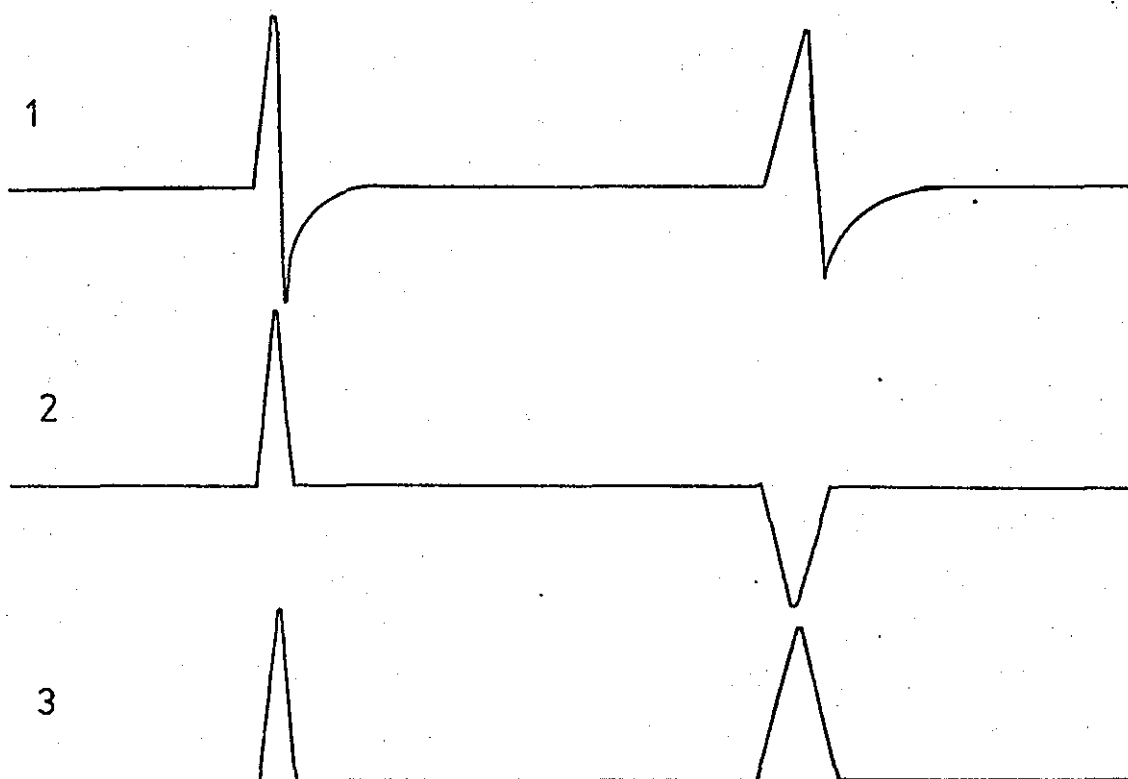


Fig. III.3. Typical Detector Responses.

1. M.A. Detector.
2. R.I. Detector.
3. U.V. and Mass Transfer Detectors.

2.1.2 The Refractive Index Detector, (R.I.).

The R.I. detector is one of the two most widely-used types and is the nearest to being a universal detector, as it relies on a bulk property of the eluted compounds and not on specific chemical or physico-chemical properties. The requirement for detection of a compound is that the compound and the eluting solvent should have different Refractive Indices. Sensitivity to specific compounds depends on this difference, as the response of the detector is a measure of the refractive index of the eluted solution compared to the refractive index of the pure reference eluant. The difficulty of matching the reference side with the analytical side of the detector precludes the use of this detector for gradient elution, except at low sensitivity. For low concentrations of eluted compounds, or when otherwise working with high detector sensitivity, it is necessary to control the temperature of the detector to within $\pm 0.001^{\circ}\text{C}$ ^{10, p117}. This is because the change of Refractive Index of solvents commonly used in chromatography is of the order of 10^{-4} R.I. units per degree C.; it is thus imperative that the temperature of both eluate and reference streams should be closely matched by using heat exchanger coils between the analytical column and the detector. This increases the dead-volume of the system and contributes to peak-broadening.

The two principles used in the two available types of R.I. detector, namely the Fresnel and deflection types, are described and illustrated in the texts already cited^{10,11,12}, and in Veening's review³³. The characteristic response of the detector is shown in fig. III.3. Further discussion of this detector will be limited to the summary and discussion in section 2.1.7.

2.1.3 The Polarographic Detector.

This detector was included because of the obvious relevance of such a type of detector in relating chromatographic results to the

present polarographic analytical methods used by Laporte Industries. The information obtained by a suitable polarographic detector, possibly in conjunction with another type of detector, would correlate known active compounds with certain peaks and show non-polarographically-active compounds clearly, allowing a possible quicker identification of unknowns by elimination of some of the main peaks.

The key to the use of such a combination of detectors lies in the word 'suitable'. For a polarographic detector to be usable it requires that the substances to be investigated are in a conducting medium. This 'supporting electrolyte' is generally a salt solution in an aqueous medium. This limits the use of such a detector to the ion-exchange, gel-permeation and reverse-phase chromatographic modes, of which only the last is potentially suitable in the present analyses. To use a polarographic detector in conjunction with a non-aqueous solvent system would require a complex interfacing system to remove the non-aqueous solvent and redissolve any eluted components in a suitable polarographic solvent. This would undoubtedly affect resolution and sensitivity adversely, and this type of detector is thus unsuitable, despite its good sensitivity and other features. Another factor against its use was that, although some small-volume detectors had been built in connection with various researches^{34,35,36}, there was not a commercially available detector at the time of commencing the study.

2.1.4 Ultra-Violet/Visible Absorption Detector.

The most widely used HPLC detector is the Ultra-Violet absorption (U.V.) detector, usually operating at fixed wavelengths. A fast-scanning U.V. spectrophotometric detector has recently been developed³⁷, but the majority of commercially available detectors³³ use a single fixed wavelength of 254nm, the strongest emission line of the low-pressure mercury lamp spectrum. This system is convenient for three main reasons. Firstly many compounds have an absorption maximum in the

ultra-violet at or near this wavelength, thus allowing a large number of compounds to be detected. Secondly the 254nm emission is a sharp line source, strictly at 253.7nm, and this spectral homogeneity lends itself more readily to the linear quantitation of compounds. Finally the low-pressure mercury lamp is an established standard lamp used in the calibration of spectrophotometric equipment and consequently is available as a stable, consistent and miniaturised form suitable for incorporating into small detectors. Its high-intensity light at 254nm is also suitable for exciting phosphors which then emit at higher wavelengths, thus permitting the detector to operate at the higher wavelength and giving a wider range of specified wavelengths, if necessary.

A slightly different type of U.V. detector is now coming into increasing use, this being the variable wavelength detector. It is similar to a standard double-beam U.V./visible spectrophotometer but with narrow beam optics, suited to the use of small capacity flow-through detection cells. The ability to change the wavelength used for monitoring through a continuous spectrum allows the selection of a wavelength which will give maximum absorption by a specific compound, thus maximising the sensitivity for that compound and minimising interference from other compounds. This type of detector has undoubted advantages in the analysis for known compounds on a routine basis, and has some advantages in research where it may be possible to monitor the column for absorption by a specific chromophore and thus be more specific in identification of eluted components of, say, an unknown reaction product.

In the present work it is notable that all of the known compounds in Working Solution have some absorption in the region of 254nm, some having very high absorption, e.g. EAQ with $\epsilon \approx 103,000$. The simple U.V. detector is thus particularly suitable in this respect.

In general it is accepted^{10,11} that the U.V. detector is versatile, has high sensitivity, good baseline stability, low flow dependence and is relatively free from interference by temperature fluctuations.

A comparison of photometric detectors³⁸ indicates that the fixed wavelength 254nm detector is a good general-purpose detector, although it is not as specific as some of the other detectors because of its general applicability. The characteristic response of this detector is shown in fig. III.3, in comparison with the R.I. and M.A.D. responses.

The fixed-wavelength U.V. detector may readily be used with all forms of elution programming, and this, with its simplicity in use and the other points mentioned above, makes it a particularly outstanding type for the present work.

2.1.5 The Fluorescence Detector.

Although few of the compounds of Working Solution have been recorded as having fluorescence this is mainly because the pure compounds have not in general been tested for fluorescence. It is possible that many of the compounds, being planar and having large conjugated ring systems, are fluorescent.

The use of fluorescence in the present work as a primary or main detector was not considered to be worthwhile, because of the unknown extent of application of such a detector and the necessity to use aromatic-free eluants in order to get a stable reference response without interferences. It was not considered reasonable to build a detector to operate specifically in the fluorescence mode. The incorporation of a fluorescence facility in a U.V. absorption detector has been found to be feasible³⁸ and might be of value in extensions of present work. Knowledge of which compounds fluoresce should give information on their structure, thus making identification of unknowns simpler. The great sensitivity of fluorescence detection with suitable compounds is documented, particularly where otherwise unsuitable compounds have been derivatised with a fluorescent reagent such as dansyl chloride³⁹ or dansyl hydrazine^{39,40}. Every fluorescent compound has a corresponding U.V. absorption peak, often giving as great a sensitivity for that

compound as does fluorescent emission. No advantage would thus be obtained by using fluorescence, except in the gaining of information as already indicated.

2.1.6 The Mass-Transfer Detector.

The principle of this detector is to take a sample of the column eluate on a moving transport system (often a wire) and to then remove the solvent by careful heating; the solute thus deposited on the wire is then pyrolysed in an oven and the pyrolysis products determined using a gas-chromatography flame-ionisation detector, (F.I.D.). The detector is thus not dependent at all on the elution solvent used and is independent of column operating temperature and temperature fluctuations around the detector itself. These desirable features are offset by the variations in sensitivity to various types of compounds, for example hydrocarbons and carbohydrates. An alternative to the pyrolysis/F.I.D. method has been described by Scott and Lawrence⁴¹, and involved the conversion of the organic compound into carbon dioxide and water, followed by catalytic hydrogenation of carbon dioxide to methane. The methane so produced is then determined by the F.I. Detector. This method gives sensitivities related to the carbon content in the compounds investigated and gives a wider range of linear response.

The basic disadvantage, however, is still that the mass-transfer type of detector is large, relatively complicated to operate (wire travel rate, gas flows and heating all require careful control) and is relatively insensitive because the wire only picks up a small proportion of eluate from the column. Problems of poor solution distribution on the surface of the wire can lead to serious noise production at the F.I.D. The mass-transfer detector was thus not considered suitable for this work.

2.1.7 Summary of Detectors and Conclusions.

A tabular form of summary for the main detector types, (Table III.1) illustrates relevant points. This table summarises data given by Snyder and Kirkland¹¹ and Varian Aerograph¹², and gives the main points of comparison. Munk⁴² compares the U.V., R.I. and M.A. detectors and concludes that the U.V. detector is the best detector to use if the compounds to be investigated absorb in the ultraviolet. Later, Scott⁴³ concludes from both theoretical and practical considerations that the M.A. detector is not viable as a detection system in HPLC.

Consideration of the available relevant facts led to the choice of the fixed wavelength U.V. detector as the detector to be used in the present work, for the reasons detailed above (section 2.1.4.).

On grounds of cost, it was decided to build a detector and its associated electronics and optics. The basic machining and instrumental requirements were within the departmental facilities available, for a relatively simple detector, and a suitable design basis was thus sought.

2.2 Detector Design.

At the time of commencing this study the most comprehensive construction details for a U.V. photometric detector were published by Thacker and others^{44,45,46} of the Oak Ridge National Laboratory in the U.S.A. They gave a reasonable amount of information on the components and construction of a two-wavelength dual-beam U.V. photometric detector of suitable characteristics for use in the present work. The device of Pourcel⁴⁷, was of a similar but less sophisticated detector and described less detail.

The Oak Ridge papers were thus used as the basis for building the detector. Later in the research programme it was possible to obtain complete blueprints, circuit and machining diagrams for the detector.⁴⁸ These fabrication drawings arrived after the detector had been built. The version built from the published data, while being much simpler in

Parameter (Units)	UV (Absorbance, A)	RI (RI Units, RIU)	Transport (Detector current, amp)	Polarography (Diffusion current, μ amp)	Fluorimeter	M.A.D. ($^{\circ}$ C)
Selectivity	Selective	General	General	Selective	Selective	General
Gradient compatibility	Yes	No	Yes	No information	Yes	No
Upper limit of linear dynamic range	2.56A	10^{-3} RIU	10^{-8} A	2×10^{-5}	---	10^{-10} $^{\circ}$ C
Linear range (a) (b)	5×10^4 3000	10^4 3000	10^5 1700	10^4 ---	10^3 ---	--- 2×10^2
Sensitivity to favourable sample	5×10^{-10} g/ml	5×10^{-7} g/ml	5×10^{-7} g/ml	10^{-10} g/ml	5×10^{-10} g/ml	10^{-9} g/sec
Flow sensitivity	No	No	Yes	Yes	No	Yes
Temperature sensitivity	Low	10^{-4} RIU/ $^{\circ}$ C	No	1.5%/ $^{\circ}$ C	Low	Yes
Cell volume (μ l)	8	3-6	---	10	10	9
Peak shape	Positive only	Positive or negative	Positive only	Positive only	Positive only	Differential

N.B.: (a) Ref. 11
(b) Ref. 12

Table III.1.

Summary of Detector Performances.

construction, was similar in dimensions and optical layout to the original Oak Ridge detectors.

The detector consisted of two parts, connected by multicore electrical cable, these parts being the detector head and the associated electronics. This follows the 'Mark I' ORNL layout. The essential differences between the present device and the Marks I and II ORNL devices are in the simplified construction of the detector head, the design of the flow-through cell, the modified phosphor/filter system in the optical system and in an improved method of zeroing the electrical output. These points, the development of the system and the overall construction of the detector will be described in section 4, and in Appendix C.

2.3 Chart Recorder.

In order to accurately correlate the two sets of chromatograms produced by the two-wavelength detector, it was necessary to use a two-channel chart recorder. The instrument chosen, a Telsec T-700, had variable ranges from 10mv to 1 volt f.s.d., and had six chart speeds. It was found to be reliable and completely satisfactory over a period of $2\frac{1}{2}$ years.

3. Instrument Assembly.

3.1 Initial Assembly.

When first assembled, the parts were built into a cabinet which had previously formed part of a GLC instrument, (the Griffin & George D6). The cabinet was floor-mounted and, although it had an operational constant temperature oven, was found to lack the required flexibility of use since it only allowed the use of 1 metre or 80cm columns. It was not feasible to modify the original cabinet to allow for very short or very long columns, and it became necessary to rebuild the

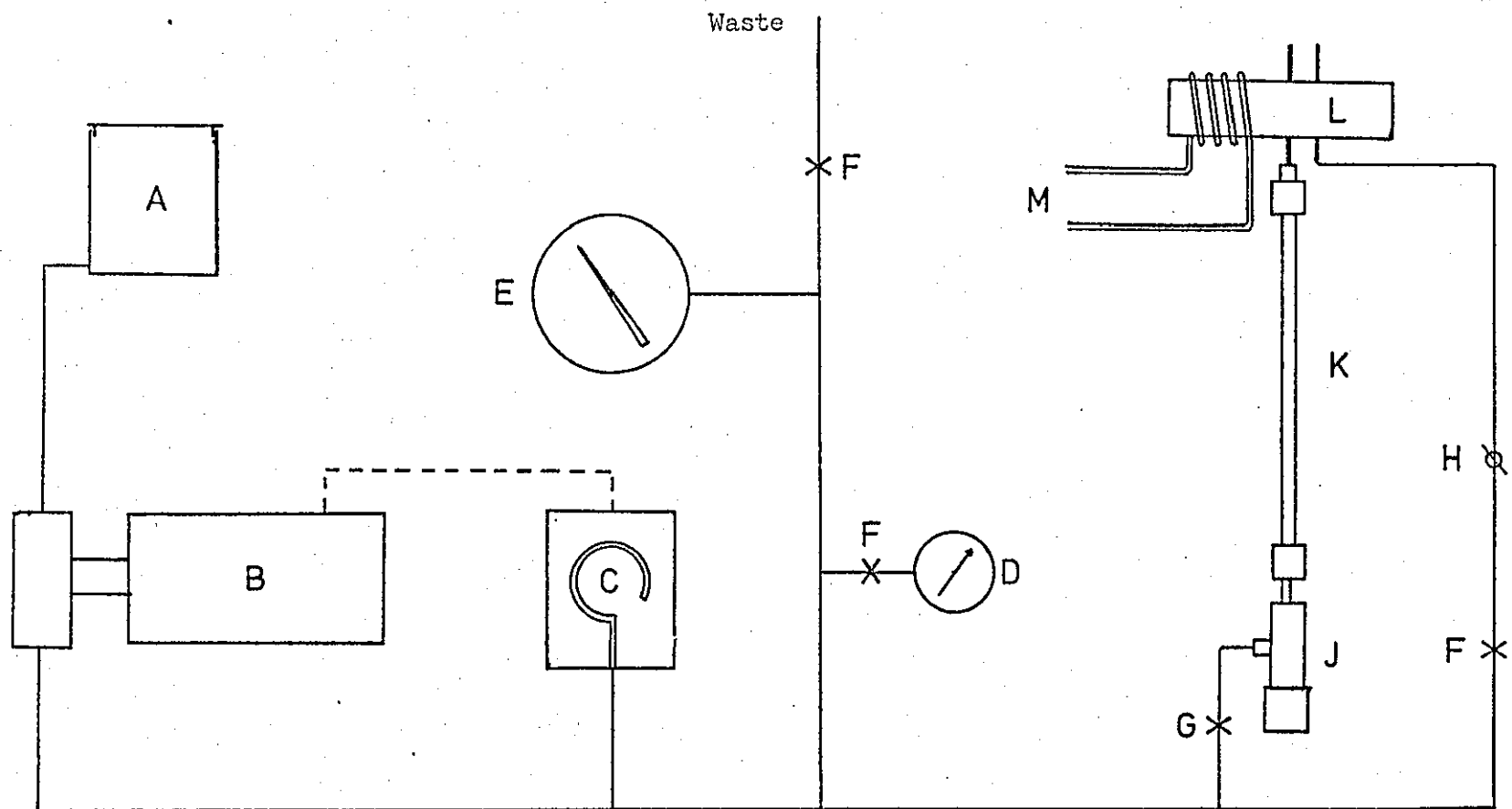


Fig. III.4 Assembly Diagram of HPLC Chromatograph Constructed.

Key to Fig. III.4.

- A. Reservoir, 1-litre, 304 Stainless Steel.
- B. Pump, plunger type, with variable stroke.
- C. Safety switch, pressure operated, up to 3000p.s.i.
- D. Low-pressure gauge, 0-400p.s.i.
- E. High-pressure gauge, 0-4,000p.s.i.
- F. Bonnet shut-off valves.
- G. Rotary ball valve, for stopped-flow injection.
- H. Metering needle valve, for reference flow control.
- J. Injector assembly, for stopped-flow injection.
- K. Analytical column.
- L. Detector head.
- M. Detector cooling coils.

machine into a smaller, more compact bench-mounted cabinet.

3.2 Final Assembly.

The final assembly is shown diagrammatically in fig. III.4. A full list of components and their suppliers is given in Appendix B, a brief description of the construction and components is given here for continuity.

3.2.1 The Pump and Fittings.

The plunger type reciprocating pump chosen was a model HM/SS2C by Metering Pumps Ltd. The flow-rate is controlled in this pump by adjusting the length of stroke with a micrometer stop. The pump is rated to 2,400p.s.i. continuous working pressure and has been satisfactorily operated at pressures up to 2,500p.s.i., with occasional short periods up to 3,000p.s.i., for two years without significant problems.

The fittings required, to enable the pump to be used with $\frac{1}{8}$ " o.d. tubing, could not be obtained in stainless steel, the material of choice, and brass fittings were used in this one situation in the initial studies. After some months of running with this arrangement the pump began to lose efficiency intermittently, and required dismantling. It was found that the stainless steel ball-valve assemblies had become coated with a white deposit which was causing them to seat badly and thus lose pressure. The white deposit, which was found to be a zinc compound, probably the hydroxide, was found to have come from the brass fittings which, by an electro-chemical couple effect between the brass and the stainless steel, had become corroded. This effect was eliminated by bright nickel plating the brass components before re-assembly of the pump-head. No further corrosion of this type has taken place.

Pulsations from the pump were damped by the pressure gauges and the safety switch (fig. III.4, C, D, E) and were not of significance

at normal sensitivities, (see section 4, Detector Performance).

3.2.2 Gauges.

The two pressure gauges and the pressure-operated safety switch were of the Bourdon type, and also served as pulse-dampers. The safety-switch could be set at any desired value, but was generally at 2,500p.s.i. A micro-switch operated by one end of the Bourdon tube was in series with the power-supply to the pump and cut off power when the set pressure was exceeded.

The performance of the pump and the pressure-holding ability of the hydraulic system could be usefully monitored by observation of the gauge behaviour; thus it was possible, for example, to tell by a long-term pressure drift that the pump non-return valve gear needed attention before performance seriously deteriorated, and this allowed suitable maintenance to be planned with minimum disruption of the research programme.

3.2.3 Injector Assembly and Ball-Valve.

The device originally used was a four-port rotary injection valve, made by Specac Ltd., of 2 microlitre nominal capacity. This was unsatisfactory, despite various repairs, because of leakage past the rotor and incorrect sample size on injection.

The device used for the majority of this work was a Pye injector as used on the Pye LCM2 instrument. It has been used in stopped-flow injection mode and has proved satisfactory in most respects. The two factors which have caused some problems are the necessity of using long-needle syringes, (at least 10cm), and the difficulty of ensuring a long-lasting septum seal at the injection point.

The use of long-needle syringes requires more care and a more precise injection technique than would otherwise be needed. This is because the long needle is very easily bent if any sideways force is

applied to it, and, when so distorted, becomes very stiff in operation. A shorter version of the Pye injector would be better in this respect, allowing the use of shorter, more robust, needles and allowing slightly faster injections; this is not a significant factor herein.

The standard septum material normally used in the Pye injector screw cap was not resistant to organic solvents. The septum material softened, swelled and gradually extruded into the small needle guide tube, with the consequent appearance of small particles of rubber at the injection point at the column end-sinter. The use of a PTFE coated rubber septum material, from Pye Ltd., reduced the rate of attack significantly, but to date no ideal septum has been found. This is a potentially serious problem, as it is possible that the quality of the injection may be reduced by accumulation of rubber particles at the column entry. This effect has been avoided by changing septa frequently, before they show signs of breaking up.

The ball-valve used in conjunction with the injector has proved trouble-free and pressure-tight in operation over a period of one year.

3.2.4 Tube and Tube Fittings.

The majority of fittings used were 'Swagelok'. Some of the valves required 'Gyrollok' fittings to adapt them to receive $\frac{1}{8}$ " o.d. tubing and these fittings were used where necessary. Some 'Kromlok' fittings were used and were found to be satisfactory in performance. It was found that it was possible to mix certain of the makes of fittings when necessary and still obtain leak-free joints. This is sometimes expedient in assembling apparatus in different configurations. It would obviously be preferable to use one make of fitting throughout so that joints would be completely interchangeable.

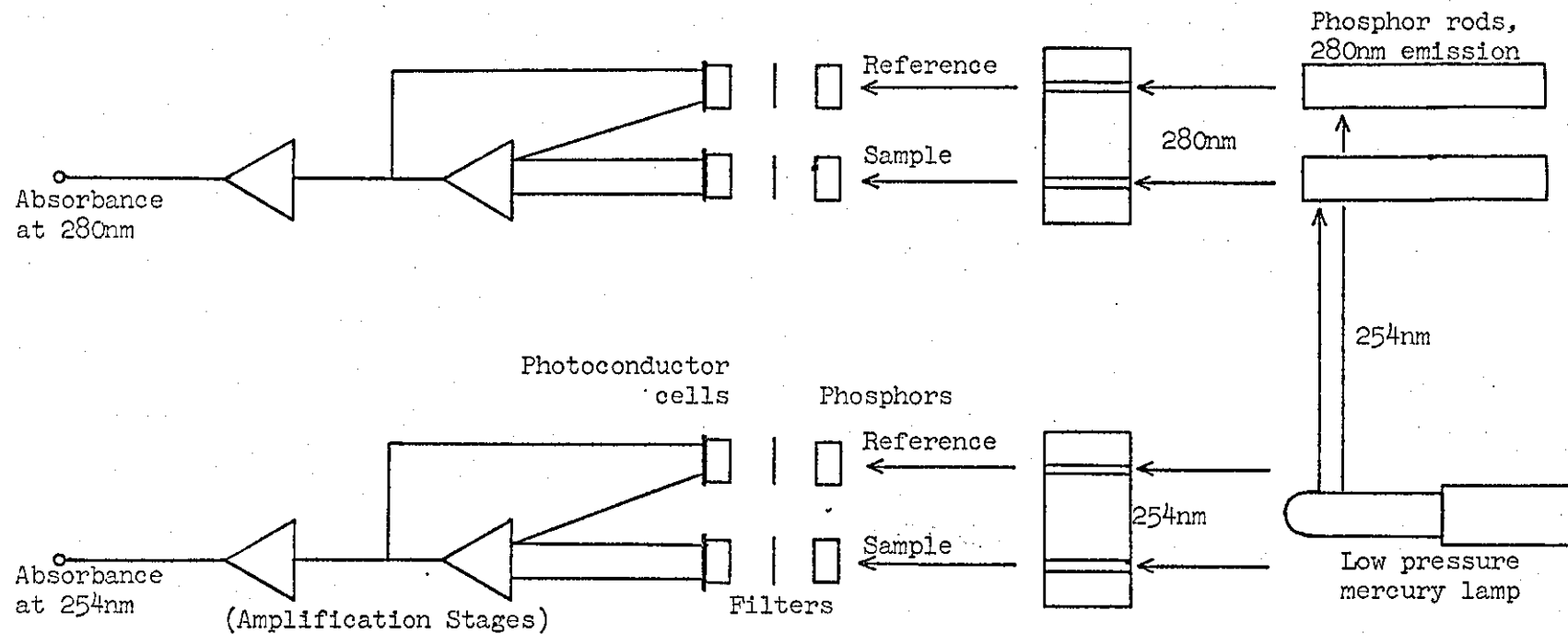


Fig. III.5 Schematic Diagram of Detector Head and Electronics.

4. Detector Construction and Development.

4.1 General Principles.

As indicated in section 2.2 the basic design was that published by Thacker et al^{44,45,46}. A schematic diagram of the detector head and electronics is shown in fig. III.5. The principles used are relatively simple, there are no moving parts, and the head is thus compact and robust.

The operating principle for the 254nm channel is the passage of light of 254nm wavelength from a low-pressure mercury lamp through the sample and reference sides of the flow-cell, followed by the conversion of this light by a phosphor to green light and subsequent detection of this by a green-sensitive photo-conductor cell. The amount of ultra-violet light falling on the phosphor, and the response of the photo-conductor, is thus proportional to the amount of absorbing material in the light-path.

The principle of the 280nm channel is essentially identical to the 254nm channel except that the 280nm light is produced by excitation of two phosphor rods at 254nm by light from the mercury lamp. The detector head and physical arrangement therein is shown in fig. III.6.

The electronic circuitry is arranged so that the resistance of the reference photo-conductor controls the gain of the first-stage amplifier. This ensures that fluctuations in light intensity from the source will be offset by a similar variation in the gain, giving a more stable output and thus a better baseline with respect to short-term noise and fluctuation.

The arrangement for zeroing the detector output on a chart recorder in the ORNL detector was a variable resistor which acted as a variable attenuator of the output signal. Further adjustment of the output magnitude was by a four-step voltage-splitting attenuator at the output, and by application of an opposing voltage at the input of the

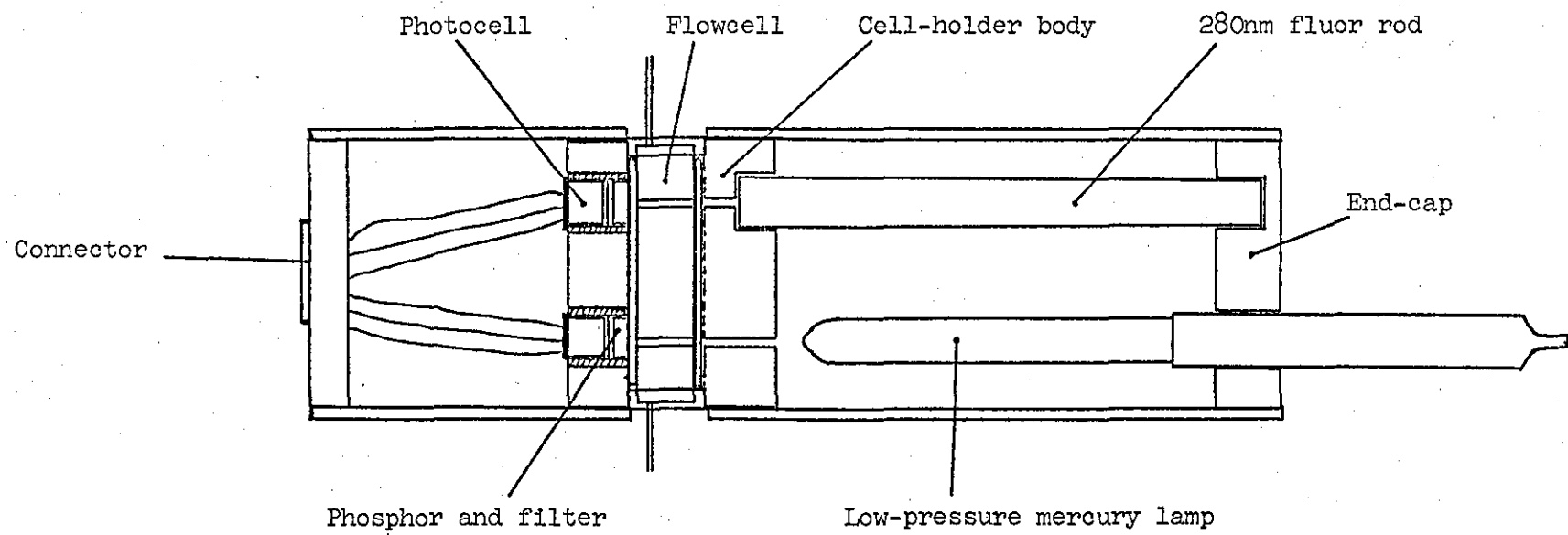


Fig. III.6.

Sectional Diagram of Detector Head.

second-stage amplifier. This had the effect of shifting the baseline by discrete steps, so that the zeroing adjuster on the chart-recorder could then be used to obtain a satisfactory zero or baseline position. These three adjustments were simplified in the detector built for the work herein, (see section 4.5).

The parts of the detector which differ from those of the ORNL detector will be described here. Further diagrams and information are in Appendix C and in the working diagrams of CAPE 2002⁴⁸.

4.2 Construction of the Detector Head.

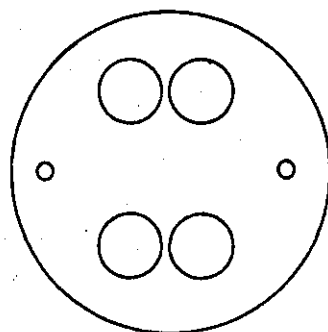
The CAPE 2002 diagrams give details of some fifteen components to be machined and assembled to form the detector head assembly. The detector made from the information in Thacker's papers required seven components and less machining operations. The two end-caps were substantially identical with the ORNL specifications, as were the two parts of the tubular body. The main difference was in the design and construction of the detector cell and the cell-holder assembly.

The cell-holder of the Loughborough detector was made in two parts, (see fig. III.7). This greatly simplified the assembly and alignment of components.

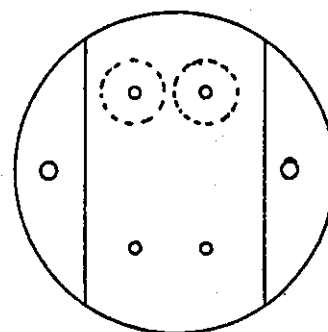
The flow-cell of the ORNL detector was made in four parts, consisting of a block of rigid PVC in which were machined appropriate light-paths and transfer passages, with two stainless steel caps holding connector tubes, the whole assembly being held together by a screw and retained in a circular PVC holder, (fig. III.8). The Loughborough cell (fig. III.9) was machined in brass with stainless steel connecting tubes silver soldered into the block. The mating surfaces of the block were ground and polished to a mirror finish with progressively finer grades of diamond powder to give a good seal, and the whole assembly was bright nickel-plated to give a corrosion-resistant bright finish. The inside surfaces of the flow-cell were plated by using a fine platinum



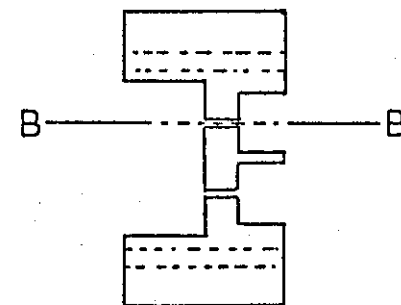
Vertical
section



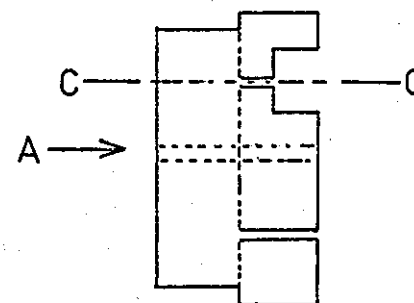
Face view



View from A



Section through C-C



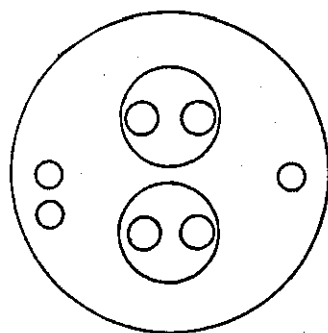
Vertical section through B-B

Photocell holder

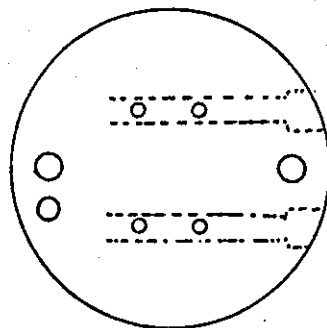
Cell-holder body

Figure III.7.

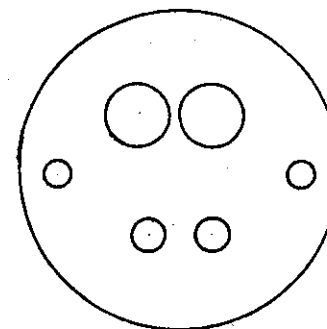
Diagram of Cell-Holder of Loughborough Detector.



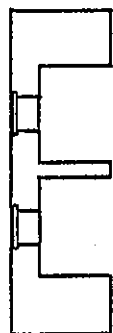
Face view



Face view



Face view



Side section view
Detector & filter holder



Side section view
Aperture plate



Side section view
Fluor support

Fig. III.8 Components of Cell-Holder of ORNL Detector.

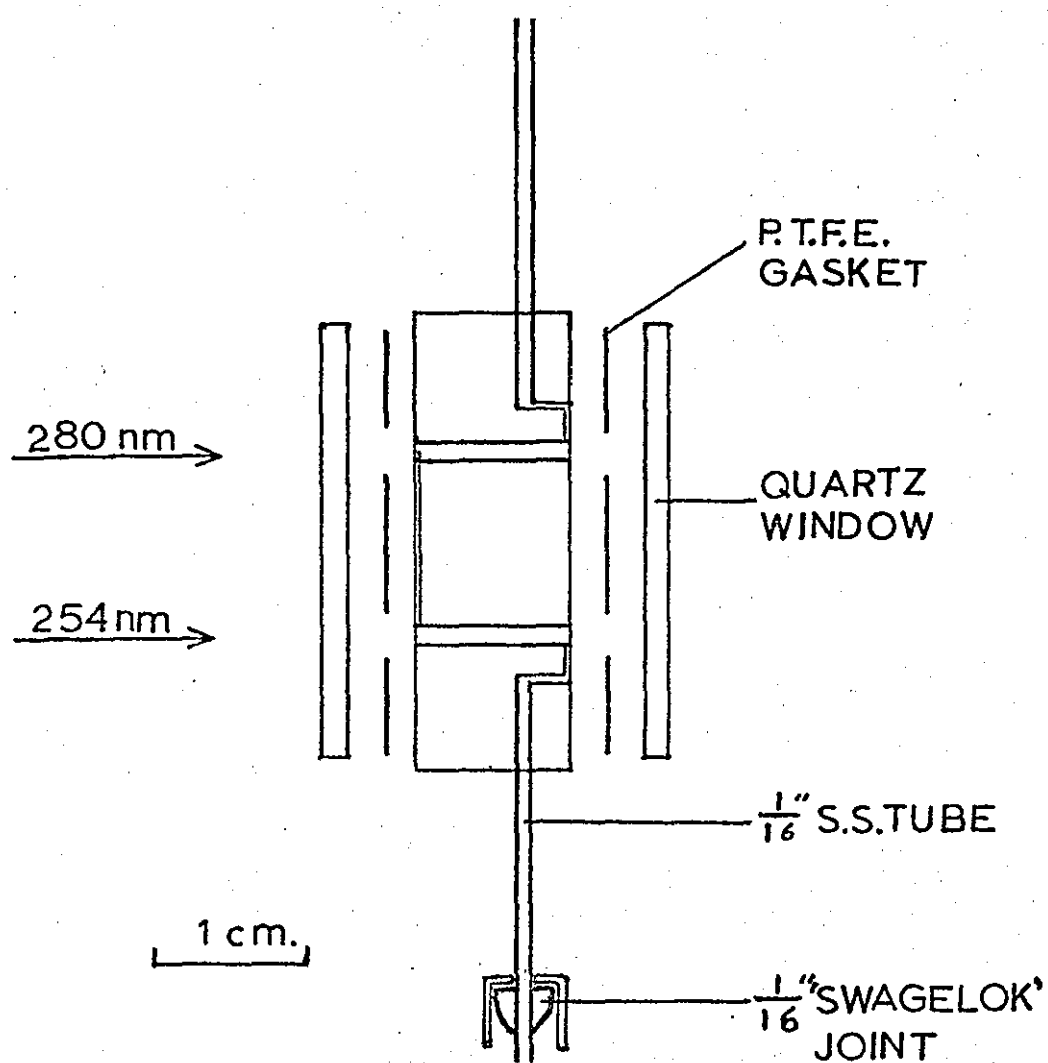


Fig. III.9.

Diagram of Loughborough Cell.

wire electrode through the centre of the hole. This did not give a mirror finish to the sides of the holes, but the finish was improved with polishing them by repeatedly drawing a length of soft string through the holes. It is probable that flow-dependence in similar detectors is due to turbulence induced by a poor finish, so it is considered that this detail is of some importance with regard to the performance of the detector.

A cell-design similar to the ORNL cell (fig. III.8) using a PTFE machined block, but with the connecting tubes fitted into the block, was found to be unsuccessful because of the difficulty of sealing the tubes into the block. The solvents used in these studies, mainly heptane and water/methanol, are 'searching', that is they tend to leak freely through relatively small cracks and holes. It is considered that, for trouble-free operation, joints in contact with solvent should be kept to a minimum, and thus the Loughborough design is superior to the ORNL original in this respect. The Loughborough design also has a shorter flow-path within the cell-block and is probably slightly more efficient in the transferring of eluted compounds through the cell without mixing.

4.3 The Phosphor and Colour Filter Assembly.

The phosphor used by ORNL was type 2382 made by Sylvania in the U.S.A. It was not possible to obtain this specific phosphor, but the information given by Thacker⁴⁶ on the absorption spectrum of this and the type 2301 phosphor enabled a suitable alternative phosphor to be obtained.

A suitable phosphor was that used in the preparation of TLC fluorescent plates, namely the Merck product known as F₂₅₄. This material was found to absorb light in the region between 250nm and 300nm and emitted light of wavelengths between 500nm and 550nm (see fig. III.10), and was thus suitable for use with the photoconductors and would respond

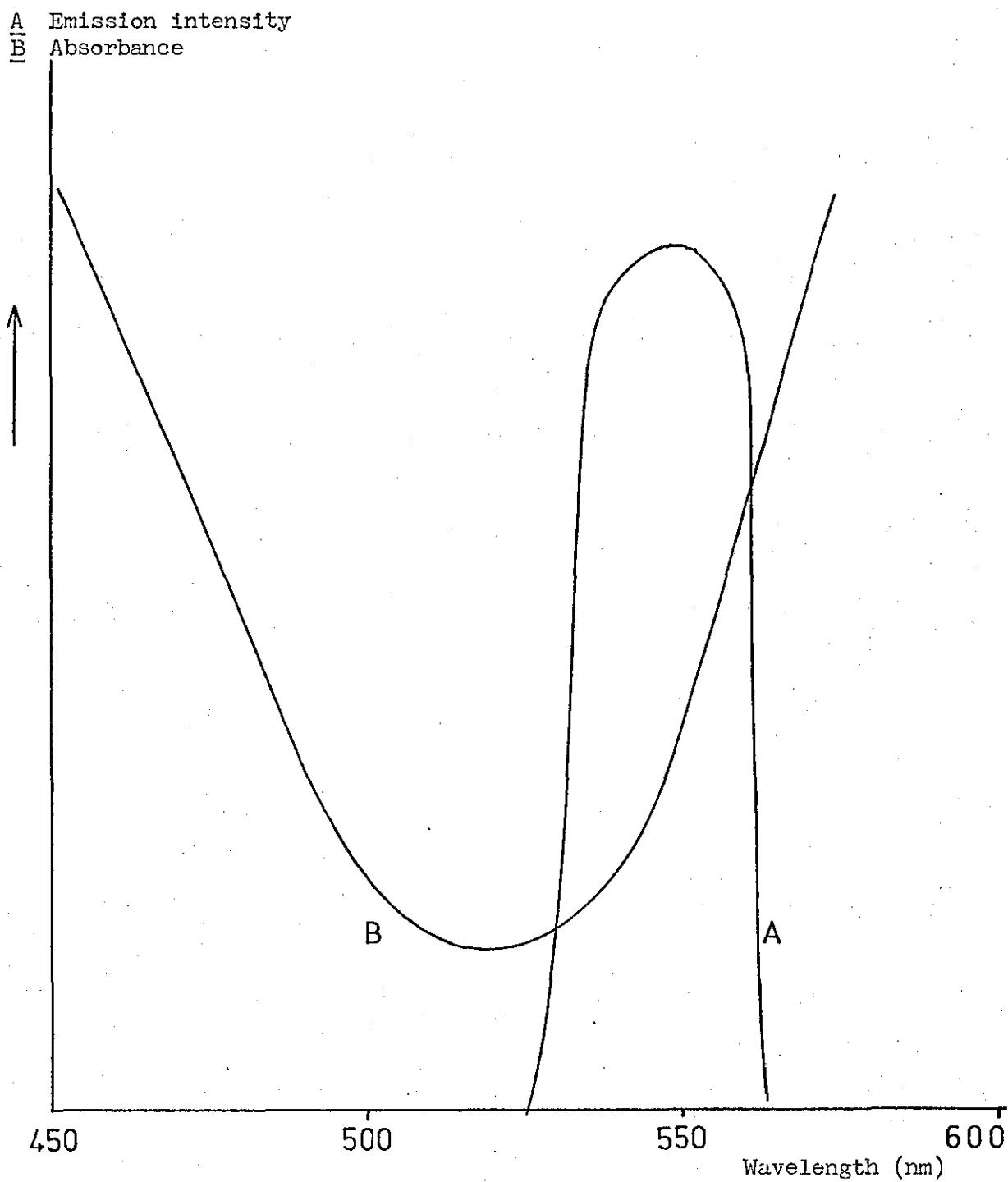


Fig. III.10 Optical Characteristics of the Phosphor and Filter.

Curve A Emission of phosphor; $\lambda_{\text{excitation}} 254\text{nm}$.

Curve B Absorbance curve of green filter.

to both 254nm and 280nm radiation.

This phosphor was made into a suspension (ca. 20%) in chloroform containing a small amount of polystyrene as binder. This suspension was applied as a thin layer to one side of discs of cellulose acetate film, ca. 1mm thick, which had been cut to the same diameter as the active surface of the photo-cells. After drying the resultant layer of phosphor was found to be responsive to U.V. light and was relatively robust. In later use, when the detector cell developed a leak and the solvent contaminated the optics, it proved possible to decontaminate the phosphor surface with a jet of ethanol with no subsequent deterioration in performance. The critical factors in the production of a suitable phosphor were found to be the concentration of polystyrene (which is U.V. opaque) and the thickness of the phosphor layer (it is necessary to produce as much as possible of the emitted light without stopping its transmission through the phosphor layer. The phosphors produced in the above way have been reliable in use for about eighteen months, and no deterioration in their performance has been evident.

The green filter, necessary to remove wavelengths other than those generated by emission from the phosphor, was initially obtained in the form of glass discs. These were too large to fit in the final detector configuration, could not be cut to size, and so another filter material was sought. A dark green cellulose acetate sheet was chosen, as the transmission characteristics were satisfactory (fig. III.10), and it could easily be cut into discs of the same size as the phosphor discs.

The phosphor, filter and photoconductor cell were then assembled in a short length of polythene tube of the same diameter; the whole of one such assembly was about 8mm in diameter and 8mm long. These assemblies were relatively easy to fit into their appropriate holes in the back-plate of the cell-holder assembly, (see fig. III.7).

4.4 Improved Baseline Adjustment.

The baseline and range adjustment on the ORNL instrument was by two principles, namely, by voltage attenuation across various resistors and by application of fixed voltages to the second-stage amplifier in opposition to the signal from the first amplifier.

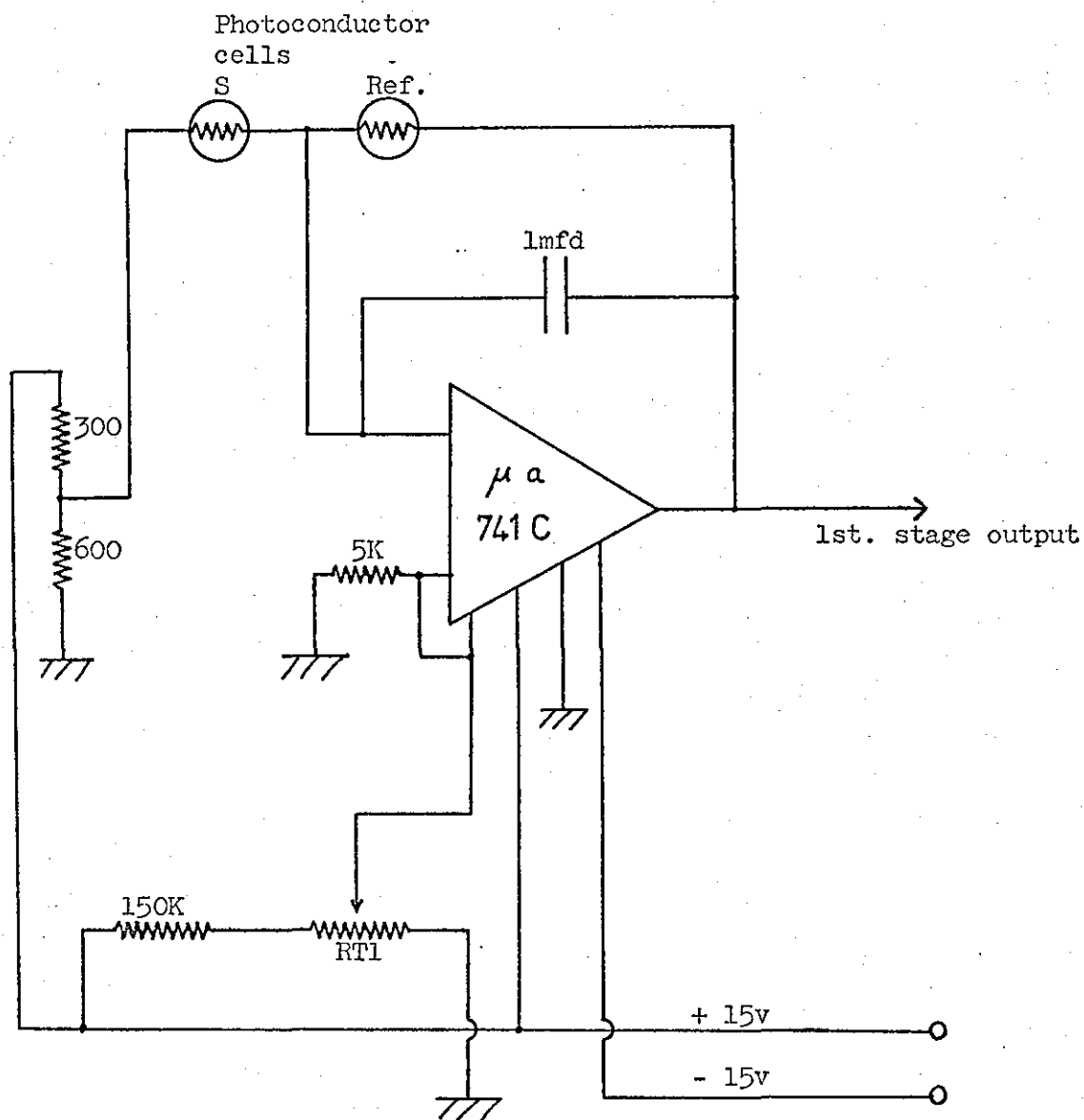
By applying an opposing voltage to the first-stage amplifier it was possible to adjust the first-stage output, throughout the range of the instrument, so that a suitable baseline could always be established without adjusting the recorder zeropoint. This was achieved by balancing the standing voltage generated across the measuring photoresistor with a voltage generated across a ten-turn variable potentiometer, (fig. III.11). The output of the first-stage amplifier could, by this means, be made zero.

The use of this modification and a multi-range two-channel recorder made the other ORNL adjusting circuits unnecessary. The modified circuit, when used with the recorder as described above, has been successfully used over a period of two years.

5. Detector Performance.

5.1 Calibration.

The detector was calibrated by filling the measuring cell with solutions of known amounts of methyl iso-butyl ketone in n-heptane. The reference side was filled with pure n-heptane and the chart recorder deflections for each solution were recorded in cm deflection from a zero point, set by filling both sides of the cell with pure n-heptane. Absorbances of the MIBK solutions were determined with a Unicam SP500 spectrophotometer. The resulting values, plotted for both channels, are shown in figs. III.12 and III.13. The first figure, with both axes linear, shows that both channels have non-linear responses above a value of about 0.3 Absorbance units. Figure III.13, with the Absorbance axis



RT1 10-turn 500 Ω for photocell back-off

Fig. III.11.

Part of Detector Circuit Diagram, Showing Zeroing Arrangement.

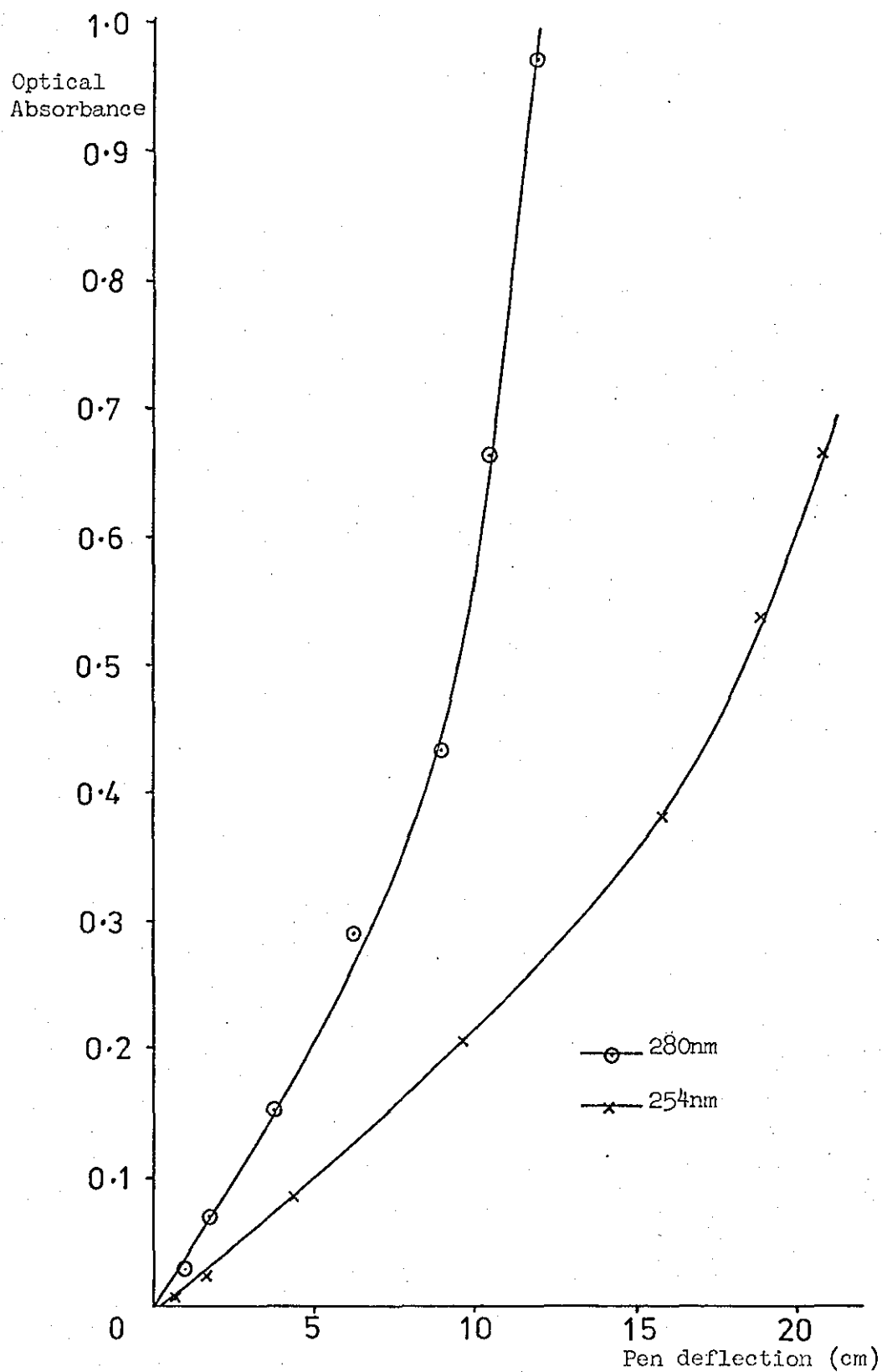


Fig. III.12 Calibration of Detector with Linear Absorbance Scale.

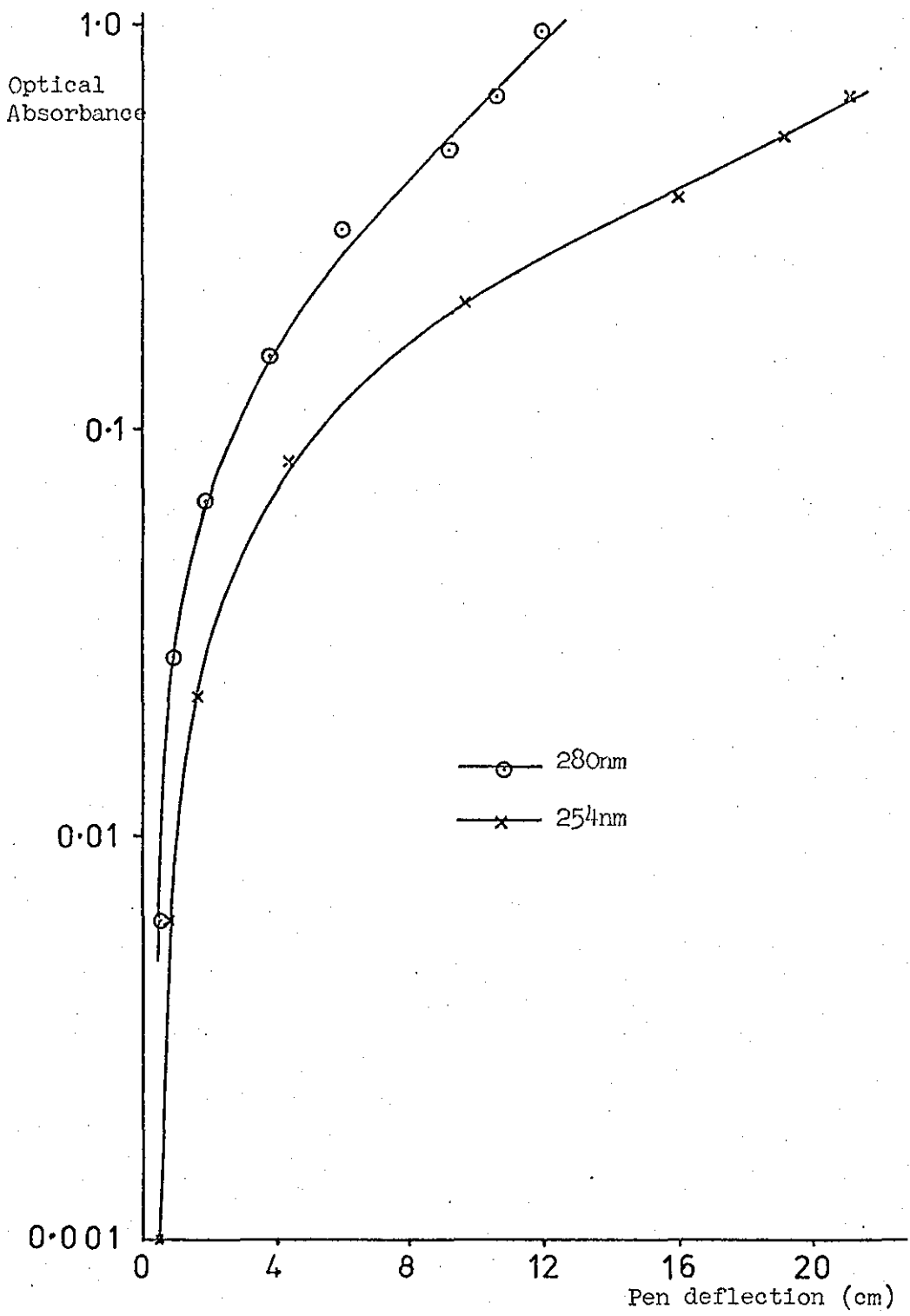


Fig. III.13.

Calibration of Detector with Logarithmic Absorbance Scale.

logarithmic, shows that the response above the 0.3 Absorbance unit level is essentially logarithmic (i.e. the log-lin plot has a linear portion above 0.3A).

This behaviour does not preclude the use of the region above 0.3 A.U., but does mean that concentration of solutes is not linearly related to peak height in such cases. Since most analytical procedures make use of calibration graphs determined experimentally, from measurement of detector response to known solutions, it follows that the non-linear detector response is not a significant difficulty. The response for both channels at levels below about 0.2 A.U. is essentially linear, within experimental error.

The 254nm channel uses essentially monochromatic light as explained earlier. For this reason the quantitative measurements to be made in these studies were made using this channel, to avoid the difficulties imposed by the wider spectral band-width of the 280nm channel. The 254nm channel also has a better response per unit deflection than the 280nm channel and thus gives greater sensitivity. Values obtained from fig. III.12 for this response are 0.03 A.U/cm and 0.06 A.U/cm for the 254nm and the 280nm channels respectively, showing that the 254nm channel has twice the response of the other channel.

5.2 Noise and Baseline Stability.

The noise level and baseline stability of a detector are influenced by several factors. Some of these factors are inherent in the detector design while others depend on the operational situation in which the detector is used.

The first set of factors, those inherent in the design and manufacture of the detector, may be divided into physical and electronic effects. The electronic effects may arise from instability in the power-supply, poor instrument connections, instability of the mercury lamp and both short- and long-term fluctuations in the responses of

individual photo-conductor cells. In this detector the first three causes are largely eliminated by choice of suitable components and due care in running the machine. The stability of the photo-conductor cells is good in controlled conditions but does change with rise in temperature. This is because the cells are photoresistors and resistance is related to the temperature of the material. There is also a thermal effect on the production of electrons in the photoresistor, which tends to give rise to random noise and make short-term stability worse.

Physical factors mainly arise from the running conditions of the chromatograph. The flow-rate and temperature of the eluate stream can be critical, especially at high detector sensitivity, mainly because of optical effects in the flow-cell arising from a velocity gradient or from a temperature (and hence Refractive Index) gradient across the cell.

Temperature is thus an important parameter in the operation of this type of detector. Brooker⁶² described the modification of a commercial detector to give better temperature control and describes the considerable decrease in noise level obtained. Although he attributes this improvement to reduction of the physical, i.e. optical, factors, it would appear from his data that a large contribution to the improvement was made by cooling the photoresistors. It has been found in practice that, in this type of detector, the heating effect of the mercury lamp is considerable, and that heating of the ingoing tube, carrying eluate, by convection from the lamp housing can lead to a very unstable baseline at high sensitivity. The detector should be cooled in such circumstances.

Cooling was achieved by making a tight-fitting four-turn coil of $\frac{1}{8}$ " o.d. copper tubing to fit the outside tubing of the detector-head. This tubing was connected to a similar coil immersed in a water-bath, and tap-water was passed through these coils. The coil around the detector-head also acted as a clamp to hold the head in position.

Brooker used a heat-exchanger arrangement between the column

and the detector, but this was considered to add too much dead-volume to the detector and was thus not used. The short length of tubing between the column and the detector was insulated with polystyrene foam so that no convective heating or cooling could occur at that point.

Results of this cooling arrangement are shown in fig. III.14. The decrease in noise, particularly the short-term noise associated with pump pulsations (frequency 70 cycles/min.), is very significant, and detector cooling was thus used as a routine procedure. The principle was extended by using ice-water cooling to reduce the detector temperature to about 10°C. This gave a further slight improvement in baseline stability.

5.3 Summary of Detector Design and Performance.

The detector as built is similar in design to the ORNL detector with differences noted in section III.4. The noise-level under best running conditions, (see fig. III.14), is of the order of 0.001 Absorbance units, which gives a linear operational range of about 300 (i.e. 0.001 to 0.3 A.U.). This is considerably smaller than that quoted by Snyder and Kirkland¹¹ for commercial detectors, (see Table III.1), but is considered adequate for the present work; it is in better agreement with the data given by Varian Aerograph¹², and, if one takes the figures for upper limit of dynamic range (2.56A) as the basis for claiming a linear operating range of 3,000, then the Loughborough detector performs as well in the higher sensitivities as commercial instruments.

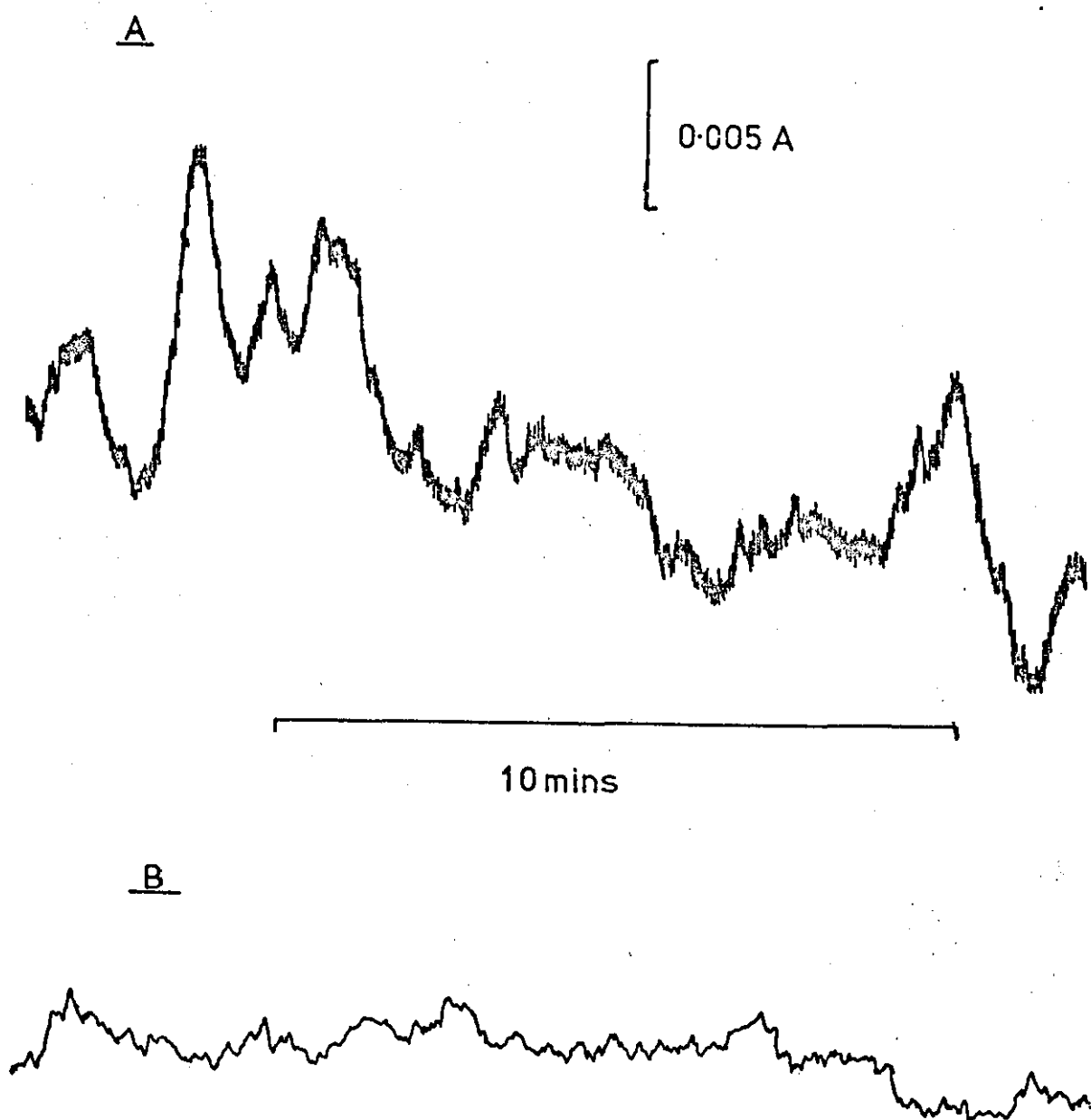


Fig. III.14. Detector Baseline Noise Level at Maximum Sensitivity.

A Without detector cooling.

B With ice-water cooling.

IV QUALITATIVE CHROMATOGRAPHY.

1. Introduction.

Studies on a number of chromatographic systems will subsequently be described, particularly directed to the development of a system which would resolve adequately the main components of Working Solution for later quantitative evaluation. The two main components of interest, EAQ and H_4 EAQ, were used as the main test compounds in the investigations since resolution of these two is a pre-requisite for a suitable system.

In order to present the data obtained from qualitative work in a form suitable for comparative study it is necessary to define and explain various parameters and properties of column systems. The theory of column performance is briefly discussed, with related practical considerations, in section 2. A more complete treatment may be found in standard texts^{10,11,12}.

Similarly a brief account of the properties of solvents, with respect to chromatography, is given in section 3. These properties are

relevant to all the types of chromatography used.

The properties of the respective column packing materials will be summarised in the appropriate chapters.

2. Column Theory.

2.1 Expressions of Measured Column Performance.

The measurement of chromatographic peaks, and the examination of the data so obtained using certain recognised expressions, gives the chromatographer quantitative information on the effect of changes in variables in the chromatographic system. Optimisation, for separation or for speed, may often be carried out expeditiously by using data from exploratory runs, in conjunction with known behaviour of similar column systems, to predict the performance of a particular column under a range of operating conditions. These expressions and the type of information derived from them will be briefly described, proofs and derivations of these relationships are omitted but may be found in the standard texts.

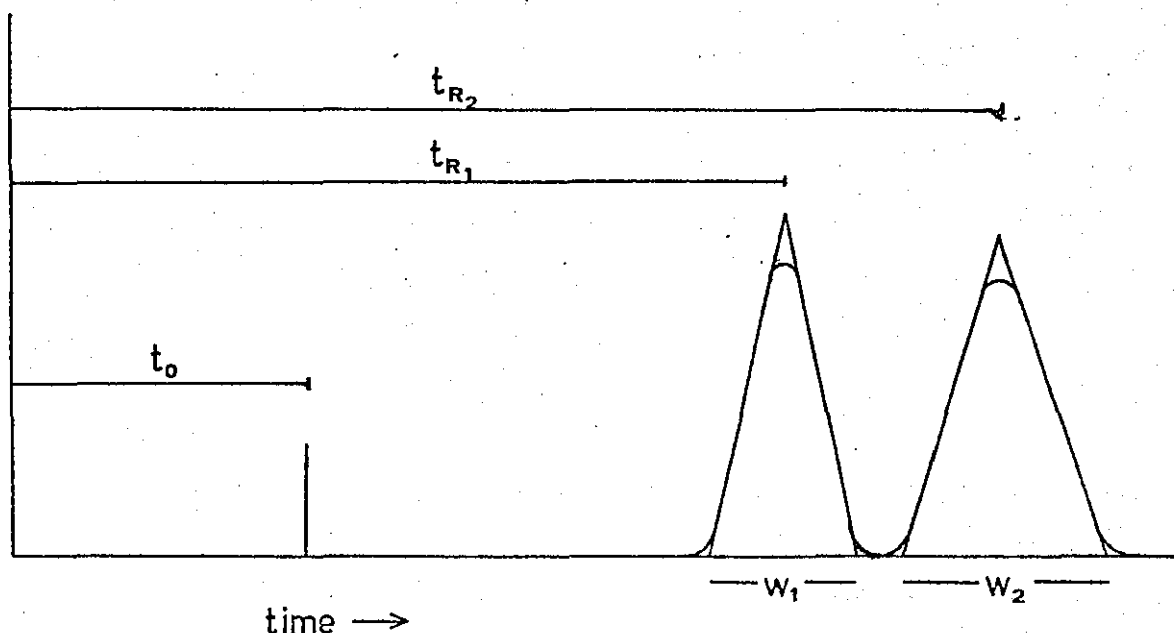


Fig. IV.1. Peak Measurement Parameters.

2.1.1 Capacity Factor, k' , and Separation Factor, α .

The capacity factor, k' , is defined as the ratio of the molar concentrations of the solute in the stationary and in the moving phases. From this relationship, essentially the distribution coefficient for the solute, a practical method of determination of k' may be derived, and this is expressed as:

$$k' = \frac{t_R - t_0}{t_0}$$

where the terms are those shown in fig. IV.1. In this figure t_0 represents the time required to elute a non-retained solute, t_R the retention time of a peak, and w the width, in time units, of the peak.

It is apparent that in order to separate two peaks, their respective k' values must be significantly different. The necessary difference will depend to some extent on peak width, and thus on the column efficiency. A measure of the separation may be given by the separation factor, α , where:

$$\alpha = \frac{k'_2}{k'_1}$$

The value of k' is optimum between 1.5 and 4.0²⁵. Values below this imply very little interaction with the column packing, hence little chromatography occurs. Values of k' much above 4 imply excessive interaction, with attendant band-spreading and the production of wide, flattened, peaks.

2.1.2 Resolution, R .

The amount of separation as expressed by the separation factor, α , gives some indication of the selectivity of the chromatographic system with regard to the compounds under study. The degree of resolution between peaks, that is, the amount of overlap or interference of the adjacent peaks, is also dependent on the peak width. Wide peaks will obviously need greater separation from adjacent peaks than narrow peaks

to achieve good resolution. The resolution, R , of a pair of chromatographic bands is defined as being equal to the distance between band centres, divided by the average band width. In the terms used in fig. IV.1:

$$R = \frac{(t_{R2} - t_{R1})}{(\frac{1}{2})(w_1 + w_2)}$$

This expression is useful for determining the resolution achieved by a system. Another expression for R , in terms of α , N and k' , used in prediction of resolution with respect to other column parameters, is discussed in section 2.2.

2.1.3 Column Efficiency, N , and Plate Height, H .

Band width of eluted compounds is dependent on the efficiency of the column. The efficiency is usually given as N , the theoretical plate number, where:

$$N = 16 \left[\frac{t_R}{w} \right]^2$$

The number N relates only to a specific column and column arrangement, since N is dependent on several band-broadening processes and these will differ from column to column. It is usually found that N is approximately constant for different peaks in a chromatogram.

It follows that if a given column of length L cm has an efficiency of N plates, then the height of a single theoretical plate, H , is given by:

$$H = \frac{L}{N}$$

The height H is also termed the 'height equivalent to a theoretical plate', HETP. The band-broadening processes previously mentioned may be described in terms of the sum of the individual contributions of each process to the overall HETP:

$$H = H_P + H_D + H_S + H_M$$

The terms represent the contributions from packing inequalities (H_p), longitudinal molecular diffusion of the solute in the mobile phase (H_D), mass-transfer in the stationary phase (H_S) and mass transfer in the mobile phase (H_M). Each of these will now be briefly considered.

The physical characteristics of the packing material, that is the size and shape of the particles, and the regularity of their packing in the column are the two factors controlling H_p . This term may be minimised by using small-diameter particles of very uniform size and shape in very well-packed, homogeneous, columns.

Longitudinal molecular diffusion depends on the velocity of the solvent flow, hence the solute residence time in the column, and also on the degree to which diffusion is limited by the particles of packing. The H_D term may be minimised by using high flow-rates and small diameter particles.

The mass-transfer of the solute between the mobile and stationary phases, and also mass-transfer within these phases, will contribute to the H_S and H_M terms. In a situation where the solute is continuously equilibrating between the mobile and stationary phases, that is, in chromatography, it is necessary to have as rapid an equilibration as possible in order to avoid band-broadening. It follows that, if flow through a column is very rapid, the equilibration will not be complete and solute on the stationary phase will still be desorbing when the main solute band has passed further down the column. The term H_S may thus be minimised by operating at low flow-rates to allow full equilibration to take place. The plate height contribution of H_M , that is of mass transfer in the mobile phase, is dependent on the particle diameter, packing structure, diffusion coefficient of the solute in the mobile phase, and on the flow velocity of the solvent. The dependence on particle diameter is as a squared power and is of particular importance. H_M decreases for regularly packed, tight-packed columns of small-diameter particles, thus producing a larger surface area in more intimate

contact with the whole of the solute band.

The conditions required to optimise H conflict for the various terms involved; a compromise for the rate of flow has to be chosen so that a satisfactory efficiency can be obtained. The need for the column packing to be as small, uniform and regularly-packed particles is common to all terms, and is a prerequisite of virtually all high efficiency columns. The pellicular type of packing is, at first sight, an exception to this generalisation but does conform to the same type of behaviour as small particles because a large proportion of the relatively large diameter is inactive and thus contributes nothing to the packing and flow behaviour. The thin pellicle of active material then approximates to the small diameter particles with respect to contributions to H .

To summarise, therefore, we may say that highest efficiency is attained by columns which have been packed tightly, carefully and in a very homogeneous manner, with a packing material consisting of particles with a small diameter and with a narrow range of particle diameters. Such a column should be operated at a relatively low flow-rate to obtain the maximum N value, but the flow-rate used must also be suitable with regard to the length of time allowed for each analysis. The solvent should ideally be of low viscosity to allow better mobile-phase mass-transfer of the solutes.

A source of losses of efficiency not covered in the above discussion is the contribution of extra-column band-broadening in connecting tubing and column end-fittings. This is greatly minimised by correct design of apparatus, using minimum-dead-volume fittings and by keeping connecting tubes, for example between the column and detector, as short and as straight as possible.

2.2 Expressions Used in Prediction and Control of Performance.

A full account of the various expressions is given by Snyder

and Kirkland¹¹, chapter 3. Expressions of the dependence of pressure on various column parameters and also of the relationship of analysis time to other parameters are given, but the main predictive aid for qualitative separation work is the equation for resolution, R.

This is given as:

$$R = \frac{1}{4} (\alpha-1) \sqrt{N} \frac{k'}{k'+1}$$

(i) (ii) (iii)

and may be sub-divided into sections representative of different properties of a column. The terms as shown above are essentially independent of each other and can thus be considered, and optimised, separately.

Term (i) represents separation selectivity and is dependent on the chemistry of the interactions between solutes and the chromatographic system. It may be altered by changing the composition of the mobile or the stationary phase.

The second term, proportional to the efficiency of the column, is dependent on the factors described in section 2.1.3. This term is usually altered in practice by changing the column length, L, or the solvent flow-rate.

The final term may be varied by a change of solvent strength or solvent functionality so that appropriate k' values may be obtained.

It may be seen that the composition of the mobile phase is of considerable importance in the determination of the chromatographic characteristics of the separation system; a detailed understanding of these effects is necessary in order to obtain best results.

3. Mobile Phase Properties.

3.1 Solvent strength.

The solvent strength parameter, ϵ^0 , has been defined as 'the adsorption energy of the solvent per unit area of the standard activity surface'. This definition was presumably intended for use in adsorption

chromatography however, if solvents are arranged in order of ϵ^0 , a generally applicable series of solvent strengths appears. This series differs for different adsorbents and purposes, but only in minor detail. The respective positions of solvents in the series is a measure of their ability to displace other solvents from a specific adsorbent, and consequently is a guide to their polarity and to their use in chromatography. The series obtained by use of the parameter, ϵ^0 , is termed the eluotropic series. A selection of the solvents used in these studies is given in Table IV.1, together with some other common solvents. The viscosity and UV cutoff values are included as being further important factors in the choice of suitable solvents.

If a suitable pure solvent cannot be found it is necessary to use mixtures of solvents to obtain appropriate characteristics. The calculation of an ϵ^0 value for a mixture may be complex, but rule-of-thumb procedures, and others⁶³ based on tables previously calculated for several possible mixtures, may be used to quickly find a suitable solvent system.

3.2 Secondary Solvent Effects.

These may be due to specific interactions of the solvent with certain functional groups on the solute or on the chromatographic support, as for example in hydrogen-bond formation. These types of interaction are discussed in detail by Snyder⁶³ and need only be mentioned herein.

The specific interactions possible between solvent and other species may be a valuable aid in altering the specificity of adsorption of some species relative to others from which they must be separated. By use of a suitable solvent system, of a similar ϵ^0 value but different selectivity characteristics, it is sometimes possible to effect otherwise difficult separations.

The solvent system selected must also be compatible with the

Table IV.1

Properties of Some Chromatographic Solvents (a).

Solvent	$\epsilon^{\circ}(\text{Al}_2\text{O}_3)$	Viscosity (cP, 20°C)	UV cutoff (m μ)
n-Pentane	0.00	0.23	210
iso-Octane	0.01	---	210
n-Heptane	0.01	---	210
Carbon Tetrachloride	0.18	0.97	265
iso-Propyl Ether	0.28	0.37	220
Toluene	0.29	0.59	285
Methylene Chloride	0.42	0.44	245
Dioxane	0.56	1.54	220
Ethyl Acetate	0.58	0.45	260
Methyl Acetate	0.60	0.37	260
Amyl Alcohol	0.61	4.10	210
Diglyme (b)(c)	---	---	220
iso-Propanol	0.82	2.30	210
Ethanol	0.88	1.20	210
Methanol	0.95	0.60	210
Water	large	1.00	190

(a) Largely from ref. 63, pp 194-195

(b) Bis(2-methoxyethyl) ether

(c) Inferred from ref. 64, and from experimental observations.

compounds to be separated, should allow the detector to function at high enough efficiency and sensitivity, and preferably should be easily removed to permit recovery of eluted compounds.

The function of the solvent is not simple, as indicated above, and is probably the most important factor in attaining good chromatography in LLC and LSC. Solvent properties are not so important in gel chromatography, as interactions are minimised in this chromatographic mode.

V. QUALITATIVE REVERSED-PHASE LIQUID-LIQUID CHROMATOGRAPHY.

1. Introduction.

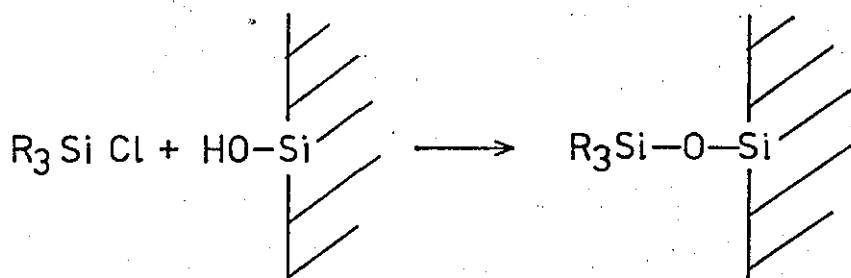
The use of reversed-phase liquid-liquid chromatography in this study was limited to the use of the bonded-phase material known as Zipax ODS Permaphase.

1.1 Bonded-Phase Packing Materials.

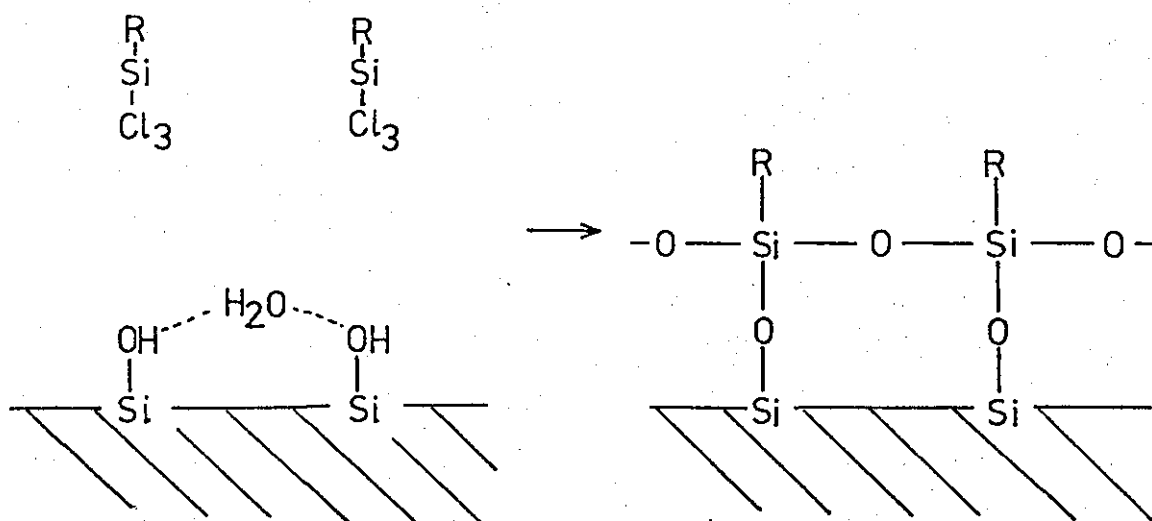
The difficulty of maintaining a stable surface coating concentration of the commonly used liquid-liquid chromatography stationary phases while in contact with chromatographic solvents has led to the investigation of methods of bonding various organic molecules to solid support materials. The large amount of work published on these topics has been summarised⁶⁵ and reviewed^{66,67}. The main methods of forming the bonded surface layers are by esterification, by Grignard reaction or by silicone formation. The packing used in the present studies was of the silicone type.

The reaction of surface hydroxyl groups of silica with chloro-

organosilane compounds leads to the hydrolysis of the chlorosilane and the formation of a silicone compound with the original organosilane group bound to the surface:



If the reagent used is a dichloro- or trichloro-silane, there is in addition the formation of cross-linking bonds between adjacent organo-silane groups, leading to formation of a surface-film of silicone polymer under suitable conditions:



This latter type of reaction is most commonly used for the preparation of the silicone types of packings.

1.2 Zipax ODS Permaphase.

This packing is a siliconised version of the Dupont product Zipax, which is a pellicular silica packing material with a chromatographically inert surface of silica in the form of 'microspheres' which are fused together and to the surface of the core. This surface is

siliconised by a treatment with octadecyltrichlorosilane, which gives a surface-bonded polymer coating of octadecylsilane groups. The octadecyl group then normally acts as a paraffinic surface coating in LLC.

As the packing has a particle size of approximately 20 to 37 microns, it may be packed by simple tap-fill methods.

1.3 Mode of Operation.

The mechanism involved in reversed-phase liquid-liquid chromatography is the partitioning by solubility of a solute between two liquid phases, where the less polar liquid is the stationary phase. The most common solvent systems used with ODS Permaphase are water-methanol mixtures, in appropriate proportions.

A Du Pont publication⁶⁸ indicated that EAQ could be readily separated from the 2-tert-butyl and 2-methyl analogues using an ODS Permaphase column and a solvent consisting of a 1:1 mixture of methanol and water. Although the various reaction products of EAQ in Working Solution do not differ from EAQ in the same respect as the alkyl analogues it was felt that this system was a good starting-point for this investigation.

2. Experimental.

2.1 Reagents.

Zipax ODS Permaphase (E.I. Du Pont, Instruments Division) was used as received.

Methanol, laboratory reagent grade, was not treated further.

Water, double-distilled, was taken as required from the laboratory supply.

Working Solution components as listed in Appendix A.

2.2 Column Preparation.

Two columns, of 1.2m and 80cm respectively, were prepared by the normal tap-fill method. Columns were of $\frac{1}{8}$ " o.d. stainless steel tubing with a stainless-steel sintered fitting at the outlet end and a quartz-yarn plug at the inlet. The columns were eluted after packing with pure methanol to remove bubbles and ensure removal of any residual contaminants prior to chromatography.

2.3 Chromatography.

2.3.1 Chronological Order of Studies.

It is necessary, in order to understand the pattern of the studies described in this chapter, to have an idea of the chronological order of the experiments. At the beginning of the experimental work, when the instrument had been constructed and shown to be operational, it was decided to begin the chromatographic studies on ODS Permaphase. The studies on the 1.2m column date from this period, when 'teething troubles' were still being encountered in the instrument.

Much later, at the period coincident with work described in Chapters VII and VIII, it became necessary to re-examine the ODS Permaphase system. This was done using the 80cm column, for technical reasons which will be given in section 2.3.3.

2.3.2 Chromatography Using the 1.2m Column.

The solvent composed of 50% methanol/50% water as indicated in section 1.3 was made. It was found that large amounts of dissolved gases were present in this and in other methanol/water mixtures, so that it was necessary to apply a low vacuum to the solvent in a closed flask for up to 5 minutes in order to stop bubble formation in the detector cell.

When samples of a mixture of H_4 EAQ and EAQ were applied to the column they were eluted as a single unresolved band some distance from

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the void volume position. Samples of Working Solution gave a similar broad peak, with some small peaks closer to the void volume position.

Other concentrations of methanol, from 75% to 25% methanol in water, were used with similar results. The peaks obtained for a mixture of H_4 EAQ and EAQ merely changed in width and position, but were not resolved. At no point in these experiments was even partial resolution of the mixture of the two principal components observed.

These results, obtained over a wide range of solvent characteristics, showed that the system was not capable of giving the resolution required, although the injections of a mixture of anthraquinone, 2-methylantraquinone, 2-ethylantraquinone and 2-tert-amylantraquinone were readily resolved using the conditions given by Dupont⁶⁸. This indicated that the problem of resolution of H_4 EAQ and EAQ was considerably more difficult than had been anticipated.

The negative results obtained here led to the direction of research being altered to a different type of chromatography, namely liquid-solid chromatography, the work on which is reported in Chapters VI to X.

2.3.3 Chromatography Using the 80cm Column.

Later during the liquid-solid chromatography studies information obtained from Laporte⁶⁹, intimating that separations of major W.S. components could be obtained using ODS Permaphase, led to a re-examination of this system.

The original 1.2m ODS column was re-installed in the machine but gave results identical to those originally obtained. In order to ensure that the column used was well-packed it was decided to pack another column with great care. An 80cm Pye-Unicam column was prepared and cleaned in the usual manner and was very carefully packed with ODS Permaphase.

The resulting column gave similar results to those obtained with

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the 1.2m column, except that the peaks obtained were slightly narrower, indicating a better packing of the column. The system still gave no indication that separations of H_4 EAQ and EAQ could be obtained.

Further information from Laporte⁶⁹ showed that some separations had been obtained using a Varian HPLC system equipped with a constant-temperature oven and operated at elevated temperatures. All the studies so far reported had been at room temperature, and it thus appeared that the increase in temperature was a particularly critical factor.

To test the effect of temperature it was decided to instal the 80cm column in an oven and operate it at higher temperatures. A Gallenkamp drying oven was converted to give a finer temperature control by switching the heater with an electronic relay operated by a contact thermometer. A motor-driven fan was also installed in the roof of the oven to provide a greater measure of air-circulation. The column was installed, in a U-shape, inside the oven, with the injector and detector units just outside the oven.

The effect of temperature on the chromatography was found to be marked. At 40°C, indications of some separation of H_4 EAQ and EAQ were obtained using a 50% methanol/water solvent. Raising the temperature to 60°C reduced the separation slightly compared with that which had been obtained at 40°C.

Reduction of the methanol content to 45% gave slightly better separation than with the 50% solvent, and it was also found that H_8 EAQ eluted later than H_4 EAQ, as a well-separated rounded peak, at about 60°C.

A further reduction of methanol content, to 40% methanol, gave a significant separation (R about 0.5) of the H_4 EAQ and EAQ peaks at 65°C, whilst H_8 EAQ was completely separated from this double peak.

A 35% methanol solution gave a resolution of R approximately 0.6 between H_4 EAQ and EAQ at 45°C, but the peaks obtained were rounded. A similar separation was obtained at 55°C but with sharper peaks.

Throughout these studies involving the oven it was found that

the temperature fluctuations of the oven, and hence of the column eluate flowing through the detector, caused relatively large cyclic fluctuations in the baseline, making the interpretation of the chromatograms difficult, in some cases. It was not possible to further modify the oven to reduce the fluctuations, although various measures, such as wrapping the column in metal foil, were examined with limited success. It was not feasible to purchase a constant-temperature oven of the performance required. In view of the difficulties involved, and considering the relative success of the LSC studies running concurrently with the LLC studies here described, it was decided not to proceed with reversed-phase liquid-liquid chromatography.

3. Conclusions.

The ODS Permaphase/aqueous methanol system was shown to be incapable of resolving the two main components of Working Solution at room temperature.

The same system at elevated temperatures gave some resolution of H_4 EAQ and EAQ, and completely resolved H_8 EAQ. It is considered that this system might be improved to give a performance suitable for the purposes of the investigation, but that such improvement and optimisation would take a considerable time. In order to avoid probable duplication of this work at Laporte and Loughborough it was decided that the liquid-liquid chromatography studies should be discontinued at Loughborough in favour of liquid-solid chromatography.

VI QUALITATIVE ADSORPTION CHROMATOGRAPHY

ON ALUMINA AND MODIFIED ALUMINA

1. Introduction.

Alumina is widely used as an adsorption chromatographic material both in column and in thin layer chromatography. A considerable body of knowledge exists on the various aspects of the chromatographic use of alumina; a comprehensive account of this and other adsorption chromatographic subjects has been given by Snyder⁶³. A brief account only will be given of the chromatographic characteristics and their modification for alumina.

1.1 Properties of Alumina as an Adsorbent.

Of the various crystalline forms of alumina which are stable or metastable at Room Temperature, that which is generally used in chromatography is an impure form of γ -alumina. The surface structure is complex, due to various lattice defects and varying amounts of strongly and weakly adsorbed water. The degree of hydration of the alumina used in chromatographic studies is of great importance to the

type of separation obtainable. Maintaining a stable hydrated surface, or a reproducibly deactivated surface, may be considered as one of the chief difficulties when working with alumina, and is usually overcome by either using water-saturated solvents or a solvent containing an alcohol which plays a similar role to that of water on the active surface.

It is known that several types of reactions⁷¹ may be catalysed by basic alumina packings, so that samples containing susceptible materials, when applied to such a column, may react during the separation to form completely different compounds. A typical example of this behaviour is found in the simple aliphatic ketones, such as acetone, which undergo a base-catalysed aldol condensation and thus should not be used as solvents in chromatography on alumina.

The initial activity of the alumina is generally controlled by pre-drying the powder, at about 150-200°C, and then adding a known amount of water. The water thus added rapidly equilibrates over the whole surface, which then has typical polar adsorbent properties, and is particularly useful in the separation of compounds according to the number of olefinic double bonds or aromatic rings in the sample molecules.^{73,71}

1.2 Modifications to Alumina.

The ability of certain aromatic nitro-compounds to form molecular complexes with aromatic compounds possessing an extended ring system was used for the preparation of derivatives for qualitative identification purposes, e.g. picric acid complexes with anthracene, phenanthrene, etc. More recently the compound 2,4,7-trinitrofluorenone (TNF) has been used in this context by Orchin and Woolfolk⁷², who also described in their paper how an anthracene-TNF complex, when chromatographed on an alumina column, was quantitatively dissociated into its components, leaving the TNF adsorbed on the column.

Recently this phenomenon has been applied in the field of HPLC by Karger et al⁷⁵, using TNF-impregnated Corasil I columns. Corasil

I is a pellicular silica adsorbent, and Karger found that, using dry heptane as eluant, a useful separation of several polycyclic aromatic hydrocarbons could be achieved. A similar type of chromatography involving molecular-complex formation with a silver-impregnated 'Zipax' column gave separations of several aza-heterocyclic compounds.⁷⁶ The potential of this type of chromatography is thus not limited to simple aromatic hydrocarbons.

It was considered that the ability of alumina to adsorb TNF could be used to prepare a stable phase which could undergo reversible molecular-complex formation with planar ring systems, such as EAQ, H₄EAQ and H₈EAQ, and that these compounds might be separated according to the number of aromatic or aromatic/quinonoid rings present in the compound. This idea was examined as described herein.

2. Experimental.

2.1 Reagents and Adsorbents.

Heptane, as 'n-Heptane to I.P. specification', was obtained from B.D.H. Ltd., and was dried over sodium.

2,4,7-Trinitro-9-fluorenone was obtained from the Aldrich Chemical Co.Ltd., and was used without further treatment.

Several types of alumina were used, being of different sieve fractions, activities or basicities, as indicated in each experiment.

2.2 Preparation of Adsorbents and Columns.

2.2.1 Alumina Packings.

A suitable amount of alumina was dried at 150°C overnight, (16 to 20hrs), allowed to cool in a stoppered conical flask. Samples were deactivated, if necessary, by addition of a measured amount of water, followed by vigorous shaking for 5 minutes and finally being allowed to stand for one hour. The alumina was then packed in straight,

dry stainless-steel columns which had been previously cleaned with various organic solvents. The tap-fill method (Ch. II, 3.2.1) was used with each added portion being sufficient to fill about 5mm of the column. The column was fitted at the inlet with a stainless-steel sinter or quartz-yarn plug, and was fitted at the top end with a short extension piece and funnel. This allowed column packing to be carried on past the end of the column so that when it was packed to the bottom of the funnel the extension could be carefully removed and a suitable column end-fitting could be attached.

2.2.2 TNF-Alumina Packings.

The alumina to be used was first dried as in 2.2.1, without water-deactivation. When cool, the required amount of TNF was added to the flask, followed by enough acetone (dried over molecular sieve 3A) to form a thin slurry. The flask containing the slurry was then fixed to a Buchi 'Rotavapor' unit and was rotated for a few minutes prior to application of vacuum. The vacuum was then applied and the acetone was removed at room temperature, leaving a free-flowing powder. This powder was then extracted in a Soxhlet apparatus with sodium-dry n-heptane for 24 hours to ensure that no readily extractable material remained and was dried in the Rotavapor unit, again, to a free-flowing maroon powder.

This powder was packed into columns in a similar manner to that used for the unmodified alumina.

2.3 Chromatography on TNF-Impregnated Alumina.

2.3.1 Chromatography on 0.2% TNF-Alumina.

Karger⁷⁵ used a loading of 0.1% TNF on Corasil I columns.

Since the alumina used herein has a higher surface area than Corasil I a higher loading of 0.2% TNF was chosen to attempt to achieve similar results.

A 0.2% packing was made as described in section 2.2.2, using 10g of 'Camag' neutral alumina, Brockmann activity 1, sieve range 100-200. It was packed in an 80cm Pye-Unicam column, of $\frac{1}{8}$ " o.d., and was conditioned by running with dry heptane for one hour. Samples of Working Solution components were then injected.

The results obtained for an injection of 0.25 μ l of 1% H₄EAQ and 1% EAQ, at a flow-rate of 0.15ml/min. of dry n-heptane, are shown in Table VI.1.

Table VI.1.

0.2% TNF-Alumina, Column 80cm x 2mm i.d.

Compound	t_R (mins)	k'	R	N
H ₄ EAQ	11.25	0.91	1.00	268
EAQ	13.85	1.35		158

These results indicated good separative potential for the system. A second column was made using a finer sieved fraction, of 120 to 150 mesh size, of the same alumina used above, impregnated as before. This column, of length 5 feet (1.5m) was packed and then was connected by a straight adapter to the above 80cm column. This increase in length to 2.3m was made to obtain greater efficiency and thus better separations.

An investigation was made of the effect of solvent flow-rate on the efficiency, N, and on the resolution, R, for this column. Samples of 1 μ l of a mixture of 1% H₄EAQ and 1% EAQ in Sextate solution were injected and were eluted with dry n-heptane. The results are shown in Table VI.2.

Table VI.2

0.2% TNF-Alumina, 2.3m Column. Effect of Flow-Rate.

Flow-Rate (ml/min)	t_R^{EAQ} (min)	$N_{\text{H}_4\text{EAQ}}$	N_{EAQ}	R^b
1.7	6.3	150 ^a	125 ^a	0.45 ^a
1.1	10.3	262	177	0.70
0.55	20.8	611	356	1.05
0.30	51.0	688	514	1.70

a Separation poor, results estimated

b Separation factor for $\text{H}_4\text{EAQ}/\text{EAQ}$

These results indicated that good separations of H_4EAQ and EAQ could be obtained at low flow-rates, i.e. less than 0.5ml/min, but that analysis times would be long. This behaviour is typical of large-diameter porous packings, where mass-transfer is slow and good equilibration is only obtained at slow flow-rates. At this stage, however, a fast analysis was not considered as important as good separation, and the lower flow-rate was used in further investigations.

Samples of H_8EAQ , H_4EAQ and EAQ were applied, separately and as a mixture, to the column, later followed by a Working Solution (WS) sample. A good separation of the WS sample into four large peaks was correlated with peaks for the main components as shown in Table VI.3.

The k' values of peaks obtained on injection of WS correspond almost exactly with those of peaks obtained on injection of pure compounds expected to be present, which indicates that the peaks obtained from WS contained these compounds. Thus peak B contains any H_8EAQ in the WS, but is not necessarily wholly due to H_8EAQ . Similarly, the other WS peaks may be correlated with H_4EAQ , EAQ and Aromasol solvent.

Peak A, that is due to Aromasol, is effectively un-retained by the column, and as a non-interacting band gives a measure of the maximum efficiency possible for the column system. This is about 1180

plates, which gives a minimum plate height of 1.95mm, which indicates the relatively low efficiency of the packing material. Additional information is obtainable from the data in Table VI.3. The values of N and R for the peaks obtained in the WS experiment were wider than for pure compounds, which suggests that the separation mechanism is of two parts, namely a main separation by number of aromatic or quinonoid rings in the solute, and a further, smaller, separation based on other chemical differences between solutes. This might be expected with TNF-alumina under these conditions, where the predominant mechanism of both TNF and alumina should lead to separation mainly by double-bond character of the solute. Such residual effects as would affect trace components of WS, with other chemical structural features from the main compounds, would lead to slight band-broadening within the main 'group' band.

Table VI.3.

Comparison of Major WS Peaks with Pure Compounds.

Compound	k'	N	R
Aromasol	0.05	1180	
H ₈ EAQ	0.37	773	1.91
H ₄ EAQ	0.64	625	1.18
EAQ	1.14	493	1.52
A ^a	0.06	1150	
B ^a	0.39	660	1.92
C ^a	0.65	385	0.95
D ^a	1.14	472	1.33

a Letters assigned to WS peaks in order of elution.

The preliminary results obtained with these columns were promising, but for more detailed investigations it was thought necessary to reduce analysis times and maintain or increase efficiencies and

resolution. It was considered that alteration of the TNF loading might achieve these requirements.

2.3.2 Chromatography on Other TNF-Alumina Adsorbents.

A sample of 150-200 mesh alumina, (Camag neutral) was loaded to 0.5% with TNF as in section 2.2.2 without Soxhlet extraction. This was because extraction of the 0.2% packings did not remove any material. This packing was packed into a 1.25m column of $\frac{1}{8}$ " o.d., 2mm i.d.

Samples of WS were then injected, but it was found that relatively large (ca. 1 μ l) injections of concentrated solutions were necessary to obtain any response from the detector. Samples of 1% EAQ and 1% H₄EAQ were not eluted at all, which suggested that the components had been strongly adsorbed onto the packing material.

In an attempt to clarify the mode of chromatography that had been operating in the case of the 0.2% TNF-alumina it was decided to saturate a packing with TNF. Such a packing should have given very good chromatograms over long times, due to strong interaction of solutes with the column, if the TNF was the active principle of the adsorbent.

A saturated adsorbent was prepared by a procedure similar to that of section 2.2.2, but with the exception that 1.0g TNF was used to treat 10g of neutral alumina (BDH active alumina, Brockmann activity 1, sieve fraction 53-63 μ) in acetone (25ml), followed by washing, by decanting, of the adsorbent with acetone (3 x 25ml). A 1.25m column, 2mm i.d., was prepared and run with dry heptane.

Injections of a mixture of H₄EAQ and EAQ were not resolved on this column at normal flow-rates and were eluted rapidly as a single, sharp, peak. Only at extremely low flow-rates could any resolution be obtained.

In order to further investigate the role of TNF, a further column of a new 0.2% TNF-alumina was prepared. Injections of EAQ and H₄EAQ were not eluted from this column when run under the same conditions

as the original 0.2% columns. Injections of WS gave a group of partially-resolved peaks which corresponded to similar peaks obtained by injecting Aromasol.

2.3.3 Conclusions on TNF-Alumina.

The behaviour shown in sections 2.3.1 and 2.3.2 is incompatible with the concept of molecular -complex formation between TNF and solutes as the chromatographic principle. An alternative possibility is that chromatography was between alumina and the various solute compounds. This does agree with the results obtained, which may best be interpreted in terms of varying degrees of deactivation of the alumina surface. The adsorbents which strongly adsorbed injected samples would thus be highly active, whereas the TNF-saturated adsorbent, with virtually all surface sites occupied by TNF molecules, would have virtually no activity in chromatography.

It was concluded that, despite the initial good results obtained with 0.2% TNF-alumina, it was not feasible to use molecular-complex formation with TNF on alumina as a basis for separations; further, it was decided to investigate unmodified alumina as an adsorbent. Further conclusions are discussed in section 3.1.

2.4 Chromatography on Alumina.

A 10 micron particle size alumina packing (Alox T, ex Merck) was obtained and was packed into a 30cm column, i.d. 1.6mm, with stainless steel sinter end-fittings. The material, which was supplied as a very narrow particle-size-range powder, was not treated prior to packing the column. The column was conditioned with dry n-heptane prior to use.

Injections of pure Aromasol and undiluted WS gave identical chromatograms, that is to say the quinone derivatives were not eluted and the aromatic mixture which comprises Aromasol was partly resolved. The column was thus too active.

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Reduction of the activity of the column was achieved by pumping through it water-saturated n-heptane. The activity of the packing was reduced by this means until injections of EAQ were eluted as peaks. While running with water-saturated heptane it was found that eluted bands were wider than would be expected for such a small-particle-size packing. Because of these wide peaks the resolution obtained between H_4 EAQ and EAQ was of the order of $R = 0.9$ even at low flow-rates; analysis times of about two hours would thus be required for separation using these conditions. On achieving some separation of the two main components it was necessary to stabilise the column, to ensure that the wide peaks were not being generated by an inhomogeneous deactivation of the adsorbent. This stabilisation was achieved by pumping half-saturated heptane, made by mixing equal amounts of water-saturated and dry n-heptane, for several hours.

After a period of stabilisation, samples of WS were injected at flow-rate 0.2ml/min. Five peaks were obtained, one of which was between 'A' and 'B', at $t_R = 55$ mins., but which was too small to be measured. The data on the four main peaks are given in Table VI.4.

Table VI.4.

Working Solution Separation on Alox T.

Band	t_R (min)	k'	N	R
A	20.0	0.08	788	5.4
B	87.5	3.73	253	
C	120.0	5.49	212	1.25
D	167.5	8.05	288	1.31

The total analysis time, of 2 hours, is unacceptable for routine applications. There is an obvious discrepancy between the efficiency values, N, for peak A, probably Aromasol, and the other peaks

which strongly suggests the operation of a band-broadening effect involving slow adsorption-desorption kinetics. This should be overcome by a change in the solvent/adsorbent system, since the packing efficiency, indicated by the efficiency value for peak A, was satisfactory.

It is sometimes more appropriate to deactivate alumina by adding a small proportion of an alcohol to the solvent, as mentioned in section 1.1, in which case the alcohol acts in place of water at the adsorbent surface. This procedure was examined by using 1% dry ethanol in dry n-heptane as solvent. It was found that this concentration was too high, as a mixture of H_4 EAQ and EAQ was eluted at the void volume, t_0 , with no separation.

In order to ensure that the hydration condition of the column packing was known and could be reproduced, it was decided to make an accurately deactivated Alox T packing. The packing was made as in section 2.2.1, with 4% added water, and was packed into a 50cm column of 1.6mm i.d. It was then equilibrated with a solvent consisting of 0.1% iso-propanol and 0.2% ethyl acetate in n-heptane, this being considerably less polar than 1% ethanol in heptane.

Separate samples of H_4 EAQ and EAQ were injected and were found to elute after the same time on chromatograms. A mixture of them was not resolved, and the peak containing them was eluted very soon after the void volume.

3. Conclusions.

3.1 TNF-Alumina.

The adsorbent did not act in the way it was expected to, namely by forming molecular complexes with solute molecules. This was probably because the very strong adsorption of TNF onto the alumina resulted in a reduction of the delocalised π -electron system responsible for formation of molecular complexes between TNF and aromatic ring

systems in solution. Such an effect would lead to TNF merely blocking active alumina sites, as appears to have happened.

The TNF-alumina system shows no advantages over alumina alone in the chromatography of Working Solution; the initially favourable results obtained on 0.2 TNF-alumina may be explained by a deactivation of the alumina by some unknown means, possibly moisture from the air, to a level of activity suitable for separations to occur. This particular level of activity was difficult to reproduce.

3.2 Alumina.

Results obtained with the Alox T column (Table VI.4) showed that separations similar to those obtained with 0.2% TNF-alumina could be obtained, but that optimisation of the solvent system would be required. Indications of the difficulty in obtaining suitably reproducible results, together with the favourable results obtained in concurrent work with silica adsorbents, led to the work on alumina being discontinued in favour of other adsorbents which presented less problems in connection with surface activity.

VII QUALITATIVE CHROMATOGRAPHY ON MEDIUM EFFICIENCY SILICA ADSORBENTS.

1. Introduction.

Silica is the most popular chromatographic adsorbent in current use for column chromatography and TLC. Snyder⁶³ has described the chromatographic properties of silica in relation to other adsorbents, in considerable detail. A summary of the main features will thus be given here; since medium and high efficiency packings differ mainly in physical respects, this account applies to both groups with regard to their chemical activity.

The term 'medium efficiency' refers herein to two types of adsorbent, Corasil II and non-professionally-packed ten-micron silica. This division into medium- and high-efficiency is arbitrary, but, as will be shown, is valid in practical usage. It should be pointed out that there is a considerable difference in performance between the 'medium' efficiency packings so defined and the low-efficiency materials used in 'classical' column chromatography.

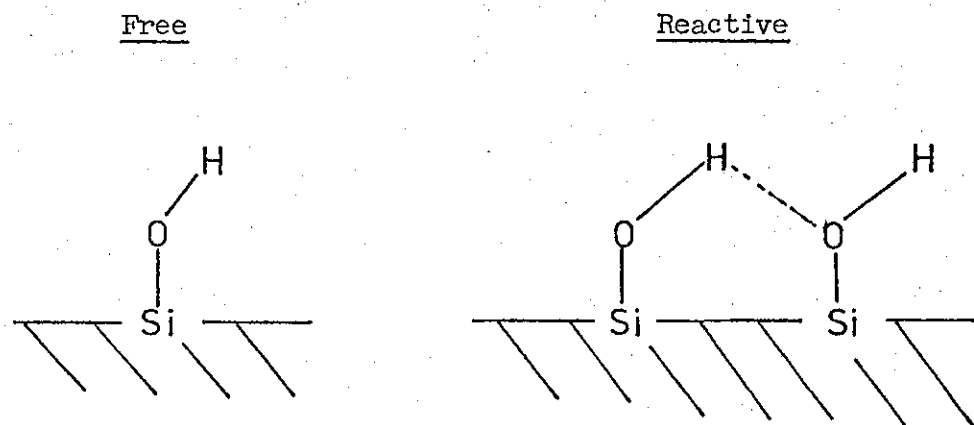
1.1 Surface Structure and Adsorption Sites.

Chromatographic silica is an amorphous solid, usually totally porous and with a wide range of pore diameters and specific surface areas. The specific surface area of a particular sample is dependent on the pH of the sodium silicate solution hydrolysed to give the gel, and also on the subsequent heat treatment of the washed gel. At temperatures above 200°C considerable loss of water occurs because of formation of siloxane groups at the surface, and a loss of surface area then occurs by irreversible bonding of adjacent surfaces to one another.

The chemical structure of the silica surface is mainly that of hydroxyl groups attached to silicon atoms forming part of the siloxane structure of the solid. Other groups may be present at the surface, such as siloxane linkages formed by condensation of two adjacent hydroxyl groups, but the main groups with respect to chromatographic activity are the two recognised types of surface hydroxyl groups. These are the 'free' and the 'reactive' types, shown in fig. VII.1, which differ in their ability to form hydrogen bonds with neighbouring hydroxyl groups, this ability depending largely on the distance between the surface hydroxyl groups.

Fig. VII.1.

Types of Surface Hydroxyl Groups.



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The surface hydroxyl groups are regarded as acidic sites, which interact with solutes by hydrogen bonding. Interaction of the surface with polycyclic aromatic compounds is probably by the reactive groups⁶³, whereas interaction with mono-functional solutes is probably by the free groups. There is still some uncertainty about these functions, but it is now generally accepted that the 'reactive' groups are the most important in chromatography of the majority of solutes, since they provide the possibility of the formation of multiple hydrogen bonds between the surface and poly-functional solutes, and these constitute the majority of compounds investigated by adsorption chromatography.

1.2 Practical Considerations.

Silica has similar chromatographic characteristics to alumina, but is less likely to cause changes in sensitive and labile compounds. It also has a higher linear capacity, i.e. more linear adsorption isotherms, than alumina and can thus be used with larger amounts of solutes. Silicas of different specifications are available with good batch-to-batch reproducibility. The ability to separate solutes by olefinic or aromatic double-bond character is less marked in silica than in alumina, but chromatography is otherwise very similar to alumina, and the eluotropic solvent series for the two adsorbents are almost identical.

Deactivation of silica is achieved in a similar way to that of alumina, namely by drying at 150° - 200°C followed by addition of known amounts of water and then allowing the surface to equilibrate overnight. It is often found that, as with alumina, it is more convenient to add a small proportion of an alcohol to the eluant, thus displacing some of the surface water with alcohol molecules. This is considerably more convenient in practical use than trying to maintain a constant partial-water-saturation of a solvent.

2. Experimental.

2.1 Adsorbents and Reagents.

Corasil II (Waters Associates (Inst.) Ltd.) was used as received. Corasil II is a pellicular packing with a double thickness layer of porous silica on the surface. The surface area of the active material is $14\text{m}^2/\text{g}$ ⁷⁷; this compares with a surface area of between 500 and $600\text{m}^2/\text{g}$ ⁶³ for totally porous silicas, linear capacity is thus much less than for totally porous packings.

Merckosorb SI 60 (obtained through Anderman & Co. from E. Merck, A.G., Darmstadt) is a totally porous silica packing available in particle sizes of 5, 10, 20 or $30\mu\text{m}$, and is supplied with a sieve analysis for each batch. The range of particle size is small, and surface area is $500\text{m}^2/\text{g}$. This packing is identical with that sold in the Varian 'Micropak' columns. The particle size used in the studies herein was $10\mu\text{m}$.

Heptane was 'n-heptane to I.P. Specification', dried over sodium.

Ethyl acetate was laboratory reagent grade (BDH), dried over dry sodium carbonate.

Di-iso-propyl ether (BDH) was laboratory reagent grade, used with no further treatment.

Ethanol (absolute) and tert. amyl alcohol (BDH) were used with no further treatment.

2.2 Preparation of Columns.

2.2.1 Corasil II.

As Corasil II is a relatively large ($37\text{--}50\mu$) diameter material it may be readily packed by tap-fill methods. The single column used in these investigations ($1\text{m} \times \frac{1}{8}"$ o.d. straight stainless steel column, with stainless steel sinter end-fittings) was cleaned and dried before

packing and was then filled, using the tap-fill method, in about 5mm steps.

The column was initially conditioned (i.e. de-aerated and thoroughly wetted) with dry n-heptane, and was then eluted with 1% ethanol in n-heptane.

2.2.2 Merckosorb SI 60.

The 10-micron grade of silica used here could not be readily packed by a tap-fill method, and slurry-packing similar to that described in Chapter III.3.2.1 was used.

The silica was dried at ca. 140-150°C overnight, and was then suspended in pure tetrachloroethylene. A vacuum was applied for a short time to the flask holding the slurry in order to remove gases from within the porous solid. Sufficient tetrabromoethane was then added to obtain a balanced-density slurry and the whole mixture was ultrasonically homogenised, giving a slurry which did not noticeably separate over 24 hours.

An appropriately cleaned and prepared column was connected to a high pressure reservoir and the column was filled, to the bottom of the reservoir, with the balanced-density solvent. The silica slurry was then added to fill the reservoir and the top of the reservoir was connected to the chromatographic pump by a stop-valve. A solvent (n-heptane here) was then pumped to high pressure (ca. 3,000p.s.i.) at a high flow-rate (ca. 5ml/min) on the pump side of the valve and was then quickly released to the reservoir. This rapid application of pressure forced the slurry quickly down onto the lower column sinter, packing the column firmly.

After packing in this way the column was eluted with solvents changing by steps from polar to less-polar solvents until the chosen chromatographic eluant was being used. This procedure ensured that no strongly-adsorbed compounds would elute when chromatography was started.

2.3 Chromatography on Corasil II.

2.3.1 Preliminary Investigation.

In order to find a suitable solvent system for separation of H_4 EAQ and EAQ it was necessary to define the limits of solvent strength corresponding to (a) rapid elution with no separation and (b) complete retention of samples.

It was found that both 1% and 0.2% ethanol in n-heptane gave no separation, with elution of both compounds at the void volume. Elution with pure n-heptane resulted in complete retention of the compounds. The solvent strength range suitable for chromatography is thus small, and it was considered that optimum conditions would probably be best obtained by using a less polar solvent than ethanol in n-heptane.

2.3.2 Use of Ethyl Acetate in n-Heptane as Eluant.

As may be seen from Table IV.1 ethanol has a high ϵ^0 value of 0.88. Ethyl acetate, by its suitability as a solvent and its lower polarity, was appropriate to replace ethanol. A level of 1% ethyl acetate in n-heptane was found to give some separation of H_4 EAQ and EAQ, and moreover separated samples of Working Solution into some 5 or 6 peaks.

In order to further investigate the behaviour of this system an injection of 0.5 μ l undiluted Working Solution was applied to the column and was eluted with pure n-heptane. This resulted in the elution of Aromasol only. The solvent was then changed in steps to 0.1% ethyl acetate and later to 0.2% ethyl acetate; this resulted in the elution of a further 7 peaks.

It appeared from this experiment that separations might be achieved with 0.1% ethyl acetate as solvent, but it was found that the use of this solvent strength resulted in long chromatograms and unacceptably wide peaks. The 0.1% level was thus too low.

Following the separations obtained by stepwise gradient elution

it was decided to attempt gradient elution using the inter-connected reservoir device described in Chapter II. Several combinations of initial and final concentrations of ethyl acetate were investigated, and one of the resulting chromatograms, which is typical of the separations obtained, is shown in fig. VII.1. The complex nature of the Solution is indicated by the number of peaks found.

Since the choice of ethyl acetate as the polar component of the eluant showed promise, it was decided to investigate the properties of the system using 1% ethyl acetate as eluant. The data for different solvent flow-rates for injections of 0.2 μ l of 1% solutions of H_8 EAQ, H_4 EAQ and EAQ in Sextate are shown in Tables VII.1, VII.2 and VII.3. The effect of flow-rate on values for N is marked, and is not linear inasmuch as that for an increase by a factor of 4 in flow-rate the values of EAQ and H_4 EAQ were reduced by about half. The values for k' were constant, and the values for resolution were only slightly improved by running at the slowest flow-rate. These results indicated that the column was well-packed and was suitable for use in further investigations, but was not, in its present system, suitable for quantitative analyses, due to lack of resolution. In order to attempt to increase the resolutions obtained while still retaining the other column characteristics it was decided to alter the solvent character, by using a mixture of solvents containing different active groups, while at the same time maintaining the same solvent strength for the overall solvent mixture. This was attempted by reducing the level of ethyl acetate (ϵ° 0.58) to 0.5% from 1% and substituting for it di-iso-propyl ether (ϵ° 0.28) at 1%. The mixture was thus: ethyl acetate, 0.5%; di-iso-propyl ether 1.0%; and n-heptane, 98.5%.

Chromatograms obtained using this solvent mixture showed no advantage over the 1% ethyl acetate system. Further work was thus carried out using the 1% ethyl acetate system, as this simple solvent mixture had given reliable results in the earlier studies.

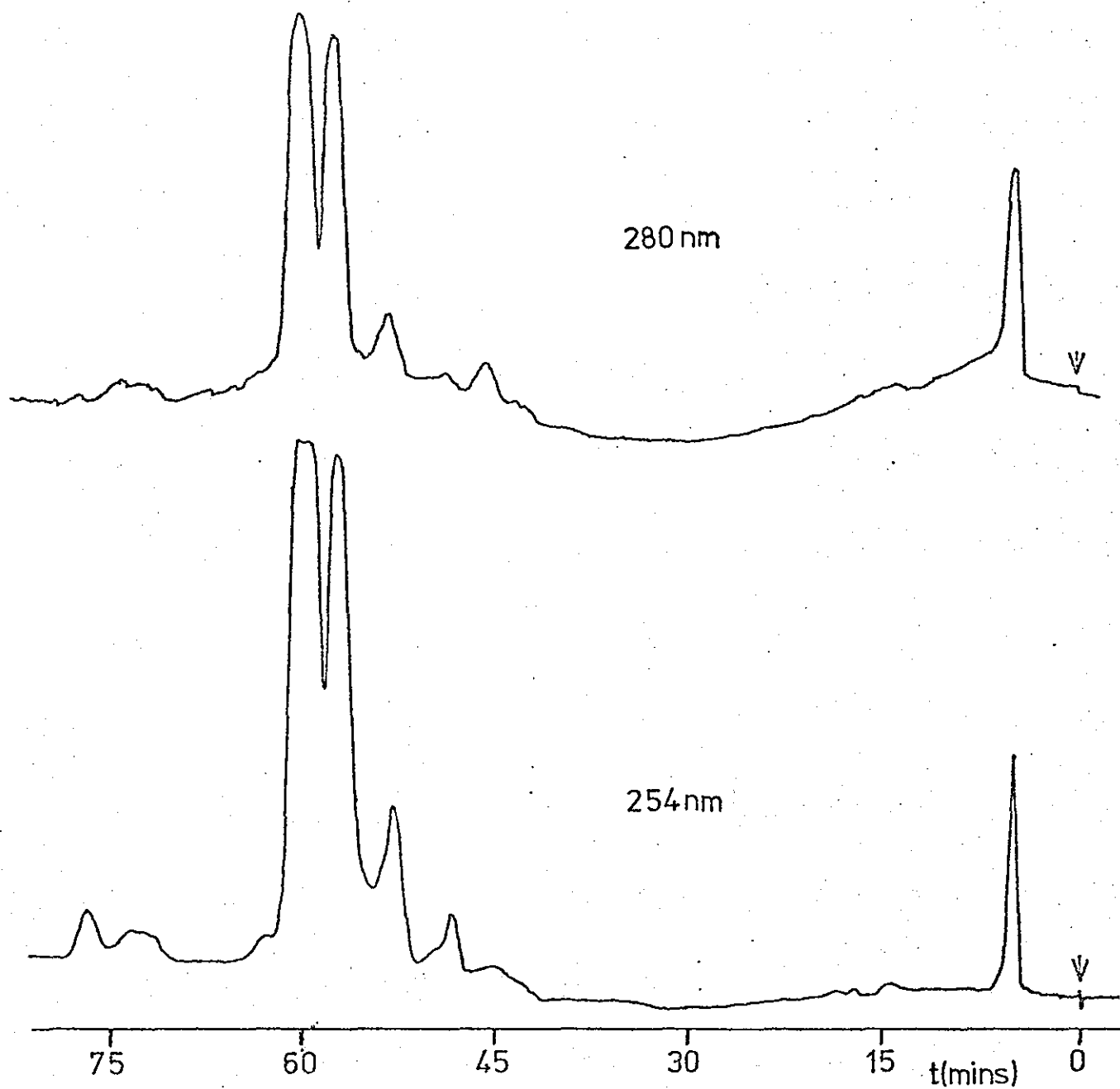


Fig. VII.1a. Gradient Elution of Working Solution.

Gradient from 0% to 2% ethyl acetate in n-heptane; flow-rate 1ml/min.

Absorption scale non-linear.

Table VII.1.

Separation of H₈EAQ, H₄EAQ and EAQ at Flow-Rate 1.8ml/min.

Compound	t _R (min)	N	k'	R
H ₈ EAQ	2.33	602	1.81	0.85
H ₄ EAQ	2.70	486	2.25	
EAQ	3.03	452	2.65	0.62

Table VII.2.

Separation of H₈EAQ, H₄EAQ and EAQ at Flow-Rate 0.85ml/min.

Compound	t _R (min)	N	k'	R
H ₈ EAQ	4.55	1095	1.83	0.82
H ₄ EAQ	5.1	650	2.19	
EAQ	5.7	642	2.57	0.71

Table VII.3.

Separation of H₈EAQ, H₄EAQ and EAQ at Flow-Rate 0.45ml/min.

Compound	t _R (min)	N	k'	R
H ₈ EAQ	9.7	1244	1.77	1.00
H ₄ EAQ	11.0	860	2.14	0.76
EAQ	12.2	874	2.49	

Following the earlier success using gradient elution the other common mode of programming, that is flow programming, was examined in the elution of Working Solution aliquots on a semi-preparative basis. Samples of undiluted Working Solution, typically $1\mu\text{l}$ to $10\mu\text{l}$, were injected onto the column and were then eluted at a very slow flow-rate. The flow-rate was then slowly and regularly increased by driving the micrometer pump-stroke-length limiter with a very slow electric motor, so that the final flow-rate after about one hour would be typically about ten times faster than the initial rate. A chromatogram of such a flow-programmed run is shown in fig. VII.2, where the solvent was 1% ethyl acetate in n-heptane and the sample was $6\mu\text{l}$ of Working Solution. The major components, EAQ, H_4EAQ and H_8EAQ , elute as a large flat-topped multiple peak in the 254nm trace because their high absorbance at this wavelength overloaded the detector amplifiers and resulted in a non-linear output. The 280nm channel gives a more realistic impression of the separation obtained.

The semi-preparative aspect of these runs was in the collection of three fractions of the eluted materials. These fractions were, approximately, as follows:

Fraction A, from injection to the trailing edge of the H_8EAQ peak,

Fraction B, from the trailing edge of the H_8EAQ peak to the trailing edge of the EAQ peak,

and Fraction C, from the trailing edge of the EAQ peak to the end of the run, i.e. until nothing more apparently eluted.

These fractions were collected from a number of such runs and were combined and evaporated down under reduced pressure. The resulting mixtures were then investigated by HPLC, this work will be described in Chapter XI. The 1% ethyl acetate was particularly suitable for use in this exercise because both the ethyl acetate and the n-heptane could

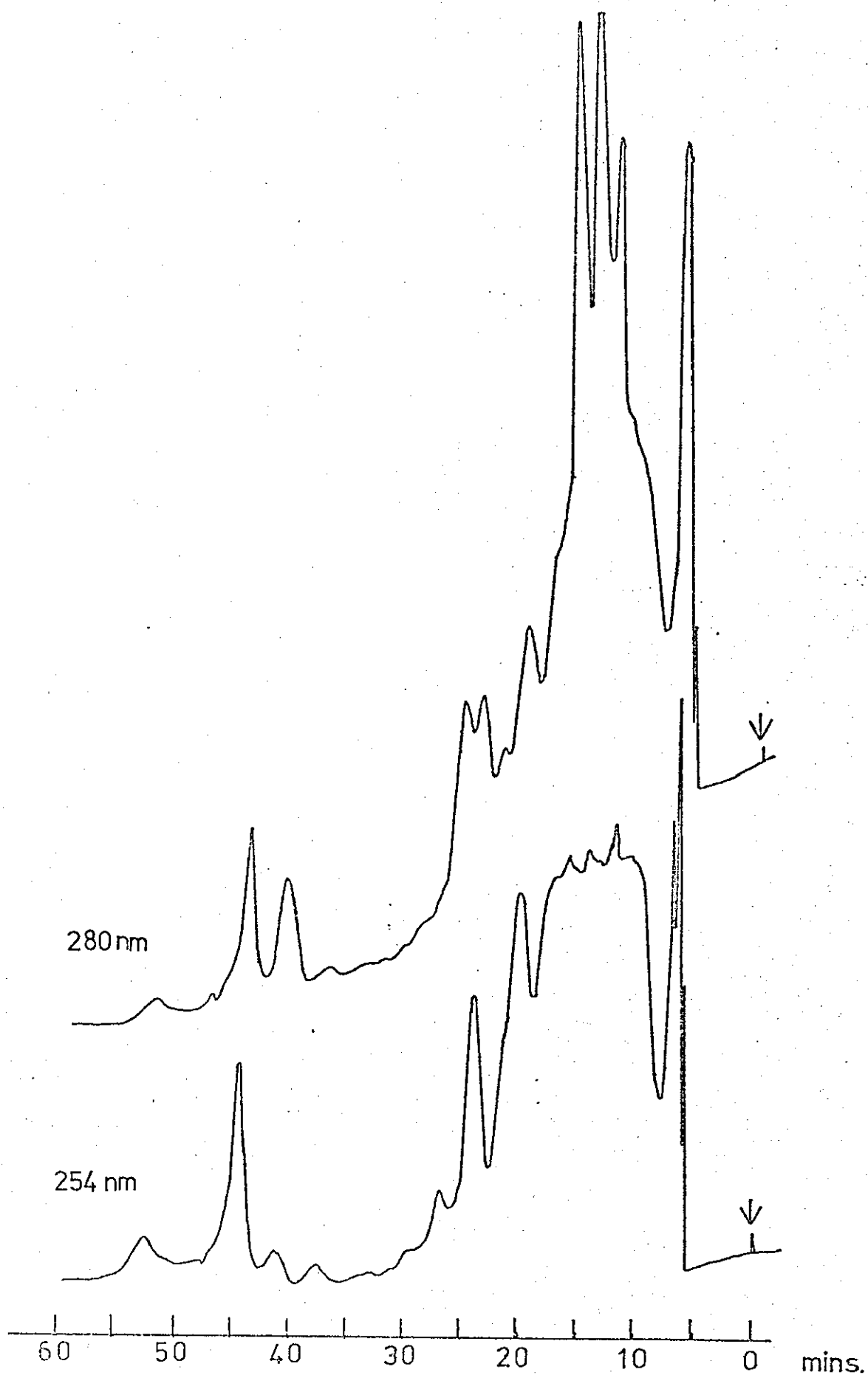


Fig. VII.2. Flow-Programming of Elution of Working Solution
with 1% ethyl acetate in n-heptane.

Flow-programme from 0.2ml/min to 2.5ml/min. Absorption scale non-linear.

be readily removed to leave dry residues for further investigation.

Throughout this work using ethyl acetate solvent mixtures it became apparent that the resolution obtained between the major component peaks, together with the efficiency values obtained, were not satisfactory for quantitative analytical studies, where ideally one would require base-line separations of the components. The resolution values obtained, for example those of Tables VII.2 and VII.3, were not high enough to enable quantitation of the peaks to be carried out with sufficient precision and accuracy, because overlap of the peaks would introduce considerable inaccuracies. A further attempt was made, by changing the solvent composition and hence its group-specificity, to alter the k' values of the main peaks and thus obtain better separations.

2.3.3 Use of Alcohols in n-heptane as Eluants.

The use of alcohols instead of ethyl acetate in the solvent should in theory change the specificity of the chromatography system to certain functional groups. Interactions between the ester carbonyl group and the adsorbent should be weaker, and of different character, to those between alcoholic hydroxyl groups and adsorbent.

The two alcohols which were used in these studies were isopropanol and tertiary-amyl alcohol, with ϵ^0 values (alumina) of 0.82 and 0.61 respectively.

It was found that concentrations of more than 0.1% t-amyl alcohol gave no separation of EAQ from H_4 EAQ or H_8 EAQ, and that after a reduction of the alcohol level it took between a half-hour and one hour to stabilise at the lower level. A level of 0.075% was chosen for further investigations.

At this level some separation ($R \approx 0.7$) of EAQ and H_8 EAQ was obtained. EAQ and H_4 EAQ were not resolved. In the chromatography of solutions of 'pure' H_8 EAQ, and mixtures containing this compound, using the t-amyl alcohol/n-heptane it was found that a compound peak of,

apparently, some three or four different peaks was produced in the chromatogram, (see fig. VII.3). This multiplicity of peaks was not present for EAQ, which gave a single peak of good efficiency and with only a slight tailing at the rear. This shows that the chromatographic role of this alcohol is probably not the straightforward one for a polar solvent but is more complicated, as, with other simpler polar solvents, the H_8 EAQ solutions used gave only single peaks. Further discussion of this phenomenon is given in section 3.1.

Further studies using the t-amyl alcohol system in an investigation of the relationship of H_8 quinone to H_8 quinol will be described in Chapter XI. For the purposes of the present studies it was decided that the use of t-amyl alcohol would introduce difficulties, because of the multiple peaks obtained for H_8 EAQ and because of peak tailing (see Chapter XI), and that a simpler secondary solvent should be used.

By using various concentrations of iso-propanol it was possible to obtain chromatograms of Working Solution in the isocratic mode (fig. VII.4) which, whilst being overloaded as far as the main components were concerned, showed a number of components eluting after the EAQ- H_4 EAQ- H_8 EAQ block. This showed that there were many (>15) minor components in Working Solution which were more polar (i.e. have longer retention times) than EAQ.

When the level of iso-propanol was reduced, to 0.1%, 0.08% and finally to 0.06%, it was found that separations of EAQ, H_4 EAQ and H_8 EAQ could be obtained. Further, it was found that injections of H_8 EAQ gave rise to a single peak, in contrast to the behaviour of the tert-amyl alcohol system. The results obtained with a 0.06% iso-propanol solvent at flow-rate 1ml/min are shown in Table VII.4.

The values in Table VII.4 show that the system has relatively high efficiency ($N > 1000$ on average), satisfactory k' values (between 1.5 and 4) and resolution that could, possibly, be usable in quantitative investigations. The length of analysis time (ca. 30 mins. to

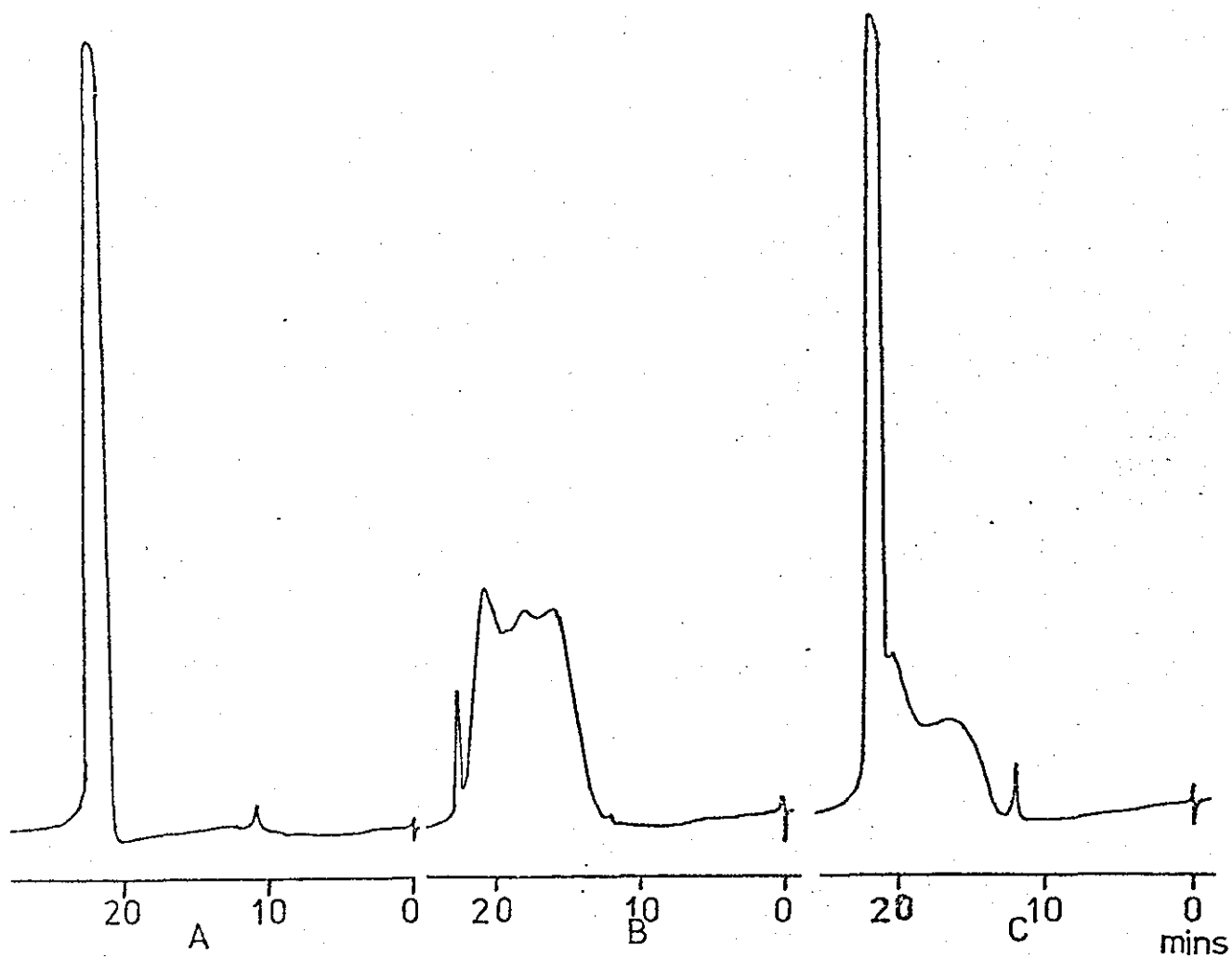


Fig. VII.3. Isocratic Elution with 0.075% tert-Amyl Alcohol in n-Heptane.

Flow-rate 0.5ml/min.

- | | | |
|----------|-------------|--|
| <u>A</u> | 0.5 μ l | 1% EAQ |
| <u>B</u> | 1 μ l | 1% H ₈ EAQ |
| <u>C</u> | 0.5 μ l | 1% EAQ + 0.5 μ l 1% H ₈ EAQ |

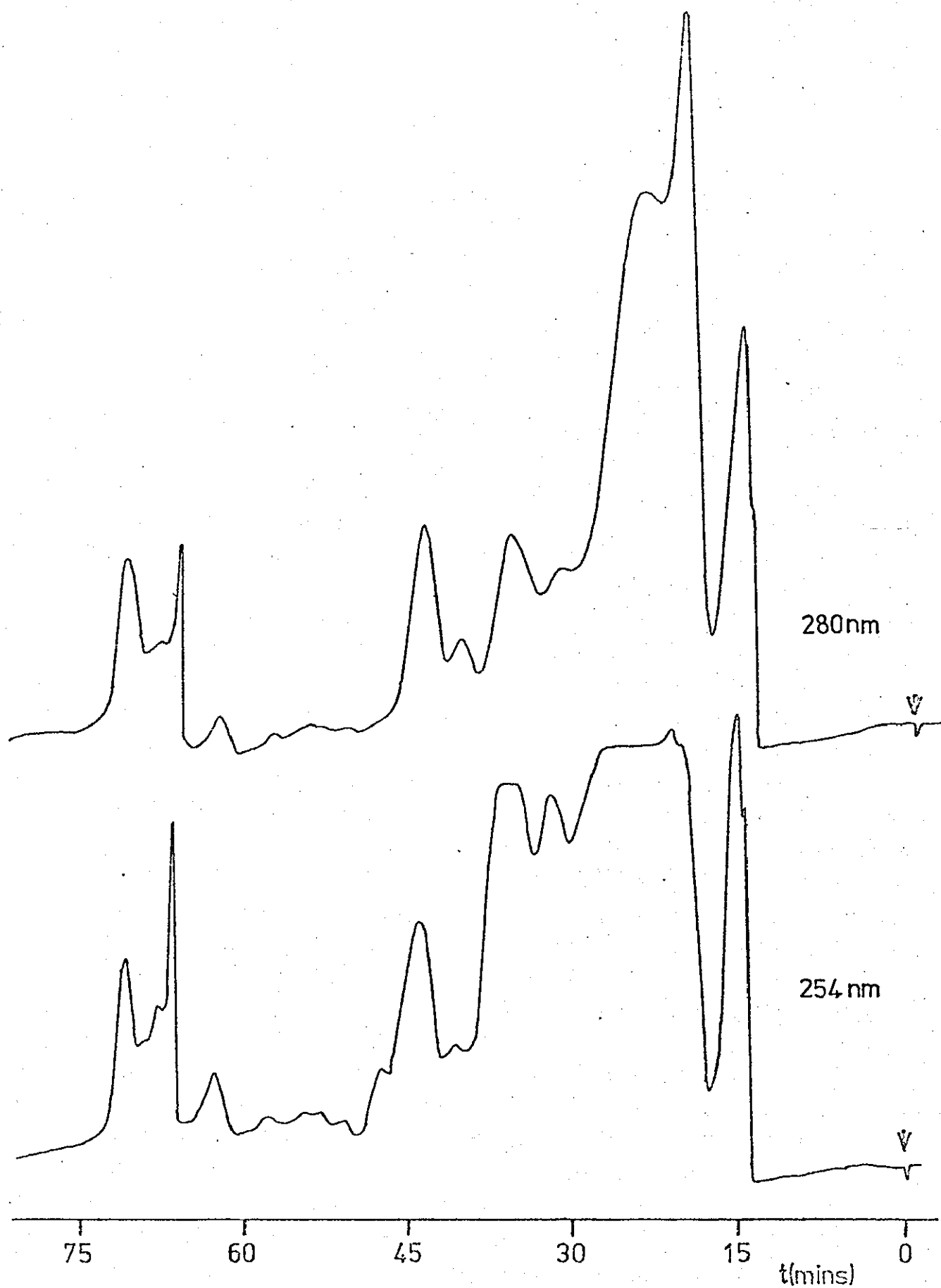


Fig. VII.4. Isocratic Elution of Working Solution

with 0.2% iso-propanol in n-heptane at 1ml/min. Sample size 1 μ l.

Absorbance range non-linear.

completely clear EAQ) and the difficulty of stabilising the solvent-adsorbent system imposed difficulties, however, since the very low level of 0.06% iso-propanol would make the equilibrium of adsorbed/desorbed alcohol very sensitive to small changes in solvent composition and temperature. Thus, although this system was considered capable of further development in the quantitative direction, the practical difficulties made such work inadvisable.

Table VII.4.

Separation of Main Components Using 0.06% iso-Propanol.

Compound	t_R (min)	N	k'	R
H ₈ EAQ	18.5	912	1.96	1.06
H ₄ EAQ	21.0	1393	2.36	0.87
EAQ	23.0	1532	2.68	

2.4 Chromatography on Merckosorb SI-60.

2.4.1 Materials Used.

Two columns of Merckosorb SI-60, of 10 μ particle size, were packed as described in section 2.2.2. These columns were about 2mm i.d. and were respectively 1 metre and 0.5 metre in length. The 1 metre column was the first to be prepared using a balanced-density slurry, and during the packing of it the reservoir had to be re-filled twice to provide sufficient packing material. The 0.5 metre column was packed in one stage, and was expected to give a better performance.

The 0.5 metre column was used in series with the Varian Micro-pak column in the studies which form part of Chapter VIII. The joined columns constitute a high-efficiency system, so that the work using the Merckosorb/Varian coupled columns is described with the other high efficiency systems.

2.4.2 Chromatography on the 1 Metre Column.

The one-metre column was run with a 2% ethyl acetate in n-heptane solvent, as this had given good results with Corasil II and in many respects was considered a suitable starting choice.

With a flow-rate of 1.4ml/min separations of H_8 EAQ, H_4 EAQ and EAQ were obtained; in addition H_4 EAQ was separated from H_4 EAQ Epoxide. The H_4 EAQ Epoxide peak was so close to the EAQ peak when injected as a mixture, however, that no separation of these compounds could be seen. The results shown in Table VII.5 show this behaviour, and also give an indication of the relatively poor efficiency of the column when compared with professionally packed columns (cf. Table VIII.13).

Table VII.5.

Separation of Main Components of Working Solution on a 1m Merckosorb SI-60 Column.

Compound	t_R (min)	k'	N	R
H_8 EAQ	6.2	2.1	558	0.71
H_4 EAQ	7.0	2.5	544	0.97
H_4 EAQ Epoxide	8.4	3.2	391	0.11
EAQ	8.6	3.3	346	

From these results it may be seen that the efficiency advantage of the 10μ packing over Corasil II had not been realised. It was probable that this was due to the poor packing technique in this first column, and also to the length of the column used, which made the packing more difficult. The normal length for 10μ columns from instrument manufacturers is 30cm, or sometimes 50cm, as it is much more difficult to slurry-pack a one-metre column. Accordingly a 50cm column was packed as described above.

2.4.3 Chromatography on the 0.5 Metre Column.

A 2% ethyl acetate in n-heptane solvent was used with this column. Results obtained were slightly better than those with the 1 metre column as shown in Table VII.6, but were not of the high efficiency expected, such as those obtainable with packed columns of similar silica prepared by instrument manufacturers. Although good separations of EAQ, H_4 EAQ and H_8 EAQ were obtained it was found that H_4 EAQ and H_4 EAQ Epoxide eluted at the same retention time, and hence were not resolved (see Table VII.7).

Table VII.6.

Elution of Main Components with 2% Ethyl Acetate
at ca. 0.5ml/min Flow-Rate.

Compound	k'	N	R
H_8 EAQ	4.5	604	1.41
H_4 EAQ	5.7	718	1.54
EAQ	7.6	529	

Table VII.7.

Elution with 2% Ethyl Acetate at ca. 1ml/min Flow-Rate.

Compound	t_R (min)	N	R
H_4 EAQ	8.0	455	0.06
H_4 EAQ Epoxide	8.1	437	1.08
EAQ	9.75	497	

Although this performance was reasonably encouraging it was decided that further work with silica adsorbents should be on high-efficiency pre-packed columns, as described in Chapter VIII. Further work using the laboratory-packed columns was limited to the use of the

50cm column to extend the Varian Micropak column, as also described in Chapter VIII.

3. Conclusions.

3.1 Corasil II.

The one-metre Corasil II column was shown to have uses for semi-preparative work, and gave moderate resolution of the three main quinone peaks. Efficiencies were higher when iso-propanol was used at low concentrations in n-heptane in place of ethyl acetate.

The behaviour of tert-amyl alcohol, with regard to the formation of multiple peaks for H_8EAQ , is at first sight an anomaly, but may be explained if one considers two factors pertaining to this behaviour. Firstly, the aliphatic group of the alcohol, consisting of the tertiary amyl group, is very bulky and would tend to obstruct access to the silica surface if the alcohol were hydrogen-bonded to that surface. Interactions might thus occur mainly between solute molecules and the surface coating of paraffinic groups, leading to what is essentially liquid-liquid rather than liquid-solid chromatography.

Secondly, in the formation of H_8EAQ by catalytic hydrogenation it is possible to produce several isomers, for example by hydrogenation at the 2-position leading to the ethyl group being axial or equatorial to the reduced ring. Other isomers would arise if the two reduced rings adopted the cyclohexene twisted ring structure, with the structures at opposite ends of the molecule either identical or inverted. Such isomers need not differ significantly in chemical properties but might well differ in their solubilities, leading to the formation of a single peak in LSC but several peaks in the 'pseudo' LLC postulated to occur with tert-amyl alcohol.

Considerably more work would need to be carried out to confirm or deny these postulates, and this was not possible at the time. A

point in favour of the LLC mechanism is that equilibrium of t-amyl alcohol with the adsorbent took longer than for iso-propanol. This suggests a strong interaction of the alcohol with the surface and difficulty in displacing it from its adsorption sites, possibly because of limited access.

The Corasil II column was not considered to be efficient enough to resolve the expected number of major and minor component peaks at the level of performance needed for quantitative work, but the results obtained gave very strong grounds for continuing this work with higher-efficiency silica columns.

3.2 Merckosorb SI-60.

Although the attempts to produce high-efficiency columns with this packing met only partial success, nevertheless the columns obtained gave good separations of the main quinones. A quantitative method of analysis for these quinones could have been developed with the 50cm column, but since it was intended primarily to develop such a method to include simultaneous estimation of the H_4 EAQ Epoxide the lack of resolution of this compound was a difficulty which might have proved time-consuming to overcome. The development of such a method was thus left to the high-efficiency columns purchased from Varian and Waters Associates, as described in Chapter VIII.

In conclusion it may be said that the lack of efficiency of the laboratory-packed columns compared to purchased columns was probably due to the lack of appropriate facilities, notably a high-pressure, high-output pump, when these studies were carried out. The large pulsations due to the pumping action of the HM/SS2C were unavoidable if a high throughput and pressure were to be reached, and these pulsations would contribute to packing inequalities.

3.3 General Conclusions.

The medium-efficiency systems investigated here gave satisfactory results, promising enough for preliminary work to be extended into similar systems with higher efficiency. This increased efficiency should result in narrower peaks, better resolutions and thus allow more flexibility in choice of solvent and running conditions.

It appears that silica is much less variable in activity than alumina in the context of this study, and is thus more suitable. No evidence was found of adverse reactions during chromatography.

The advantages of gradient elution and flow programming in the qualitative analysis of multi-component mixtures were shown to be considerable in comparison to normal isocratic elution. Furthermore, the use of solvents containing different functional groups resulted in significant differences in the chromatograms obtained.

VIII. QUALITATIVE CHROMATOGRAPHY ON HIGH-EFFICIENCY SILICA ADSORBENTS.

1. Introduction.

The characteristics of silica adsorbents, described in section 1, Chapter VII, apply equally to the high-efficiency adsorbents used in the studies comprising this chapter. The principal differences between low-, medium- and high-efficiency packings are in the particle-size distribution of the adsorbent and in the efficiency of the packing process.

The majority of work described here was carried out on two commercially packed columns, one purchased from Varian Associates and the other from Waters Associates.

The Varian column was packed, using a balanced-density technique, with a ten-micron grade of Merckosorb SI-60; this material is also sold in the U.S.A. under the trade-name of Lichrosorb Si-60. The column was fitted with porous 1mm thick PTFE sinters in the end-fittings. The injection technique recommended by Varian for use with such columns is to inject the sample directly into the sinter, this requires a very

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accurate placing of the syringe needle in order to ensure that the end-sinter should not be punctured and the packing disturbed. Majors⁷⁸ describes the packing technique and gives representative results obtained with similar columns.

The Waters column was similarly packed by a balanced-density technique with the Waters adsorbent ' μ -Porasil', a totally-porous ten-micron silica. The end-fittings held five-micron stainless-steel sinters, and the column was of internal diameter 4mm to allow higher loading. The major difference between the two columns, apart from internal diameter, was that the Waters column had been drilled out slightly, giving an improved inside surface to the tube and leading to better packing efficiencies and performance. This technique was first described by Asshauer and Halasz²³, and, more recently, by Waters Associates⁷⁹ who give representative values for N of about 3,400 to 5,000 plates. These values are approximately twice those quoted for the Varian column.

2. Chromatography on the Varian SI-10 Column and the Linked SI-10/ Merckosorb SI.60 Column.

2.1 Materials.

2.1.1 Columns.

When first used the Varian SI-10 column gave sharp peaks of good efficiency for about 50 injections, using the recommended technique of injecting into the PTFE sinter. After this initial period artifact peaks were observed in the form of 'ghost' peaks of smaller size than, and later than, the true peaks. This effect was found to be due to the complete penetration of the sinter by the syringe needle, the sample being injected up to several millimetres down the column. The subsequent disturbance of the bed material on syringe removal it is thought accounts for the double peaks, by the splitting of the applied sample into two parts.

The damage to the sinter and to the end of the column could not be quickly repaired because of difficulty in obtaining suitable sintered PTFE. As an interim measure it was decided to couple the 50cm Merckosorb SI-60 column described in Chapter VII to the injection end of the Varian column, using a straight adapter filled with Merckosorb SI-60 powder. It was hoped that this would allow satisfactory injections whilst retaining much of the performance of the Varian column, although some efficiency would be unavoidably lost.

When the sintered PTFE was obtained the Varian column was repaired by removing the perforated sinter and 5mm of packing, followed by careful hand-packing of this 5mm with new adsorbent and replacement of a new sinter. Subsequent injections were made by using a spacer on the syringe needle to give injections precisely halfway through the sinter, and this proved satisfactory in practice.

2.1.2 Solvents.

Heptane, 'n-heptane to I.P. Specification', (BDH).

2,2,4-Trimethylpentane, to I.P. Specification, (BDH).

n-Hexane, laboratory reagent grade, (BDH).

Ethyl acetate, laboratory reagent grade, (BDH), dried over sodium carbonate.

iso-Propanol and di-isopropyl ether were of laboratory reagent grade, (BDH).

1,4-Dioxan was double-distilled and dried over molecular sieve, 3A, before use.

Dimethyl digol, (Fisons S.L.R.), was passed through a column of 10cm dry silica and 5cm dry alumina before use and was stored in a tightly sealed dark bottle.

All solvent compositions are given in percentage v/v.

2.2 Chromatography on the Linked Varian/Merckosorb SI-60 Column.

The first solvent system used was 2% ethyl acetate in n-heptane, as earlier results (Chapter VII) indicated that this would be a reasonable starting point. A good separation of the quinones of Working Solution was obtained at a flow-rate of about 1.8ml/minute, as indicated in Table VIII.1.

Table VIII.1.

Separation of Quinones Using 2% Ethyl Acetate.

Compound	t_R (min)	N	k'	R
H_8 EAQ	7.05	1100	2.2	1.9
H_4 EAQ	8.75	1357	3.0	
EAQ	11.0	1062	4.0	1.96

The purchase of this higher efficiency column was well justified by the good separations (R circa 2) so obtained.

Having obtained good separation of the quinone compounds it was next necessary to enlarge the scope of the separation to include H_4 EAQ Epoxide. This compound is of considerable importance in the efficient running of the industrial plant. As the epoxide is usually present in relatively small amounts and has less U.V. absorbance than, for example, EAQ, the small H_4 EAQ Epoxide peaks must be particularly well resolved if quantitation is to be achieved. It was found that ethyl acetate solvent mixtures did not give a resolution of H_4 EAQ Epoxide from other main components. Thus a different solvent system had to be developed in order to obtain the required separation.

Working on the rule-of-thumb principle of 'like interacts with like' it seemed possible that an ether would interact preferentially with the epoxide, which may be classed as a cyclic ether. Di-isopropyl ether in conjunction with ethyl acetate showed no advantage in

chromatography of the main quinones (Chapter VII, 2.3.2), and it was considered that this ether had only limited similarity to the epoxide. A cyclic ether, 1,4-dioxan which more closely approaches the character of the epoxide structure, was used in a series of solvent mixtures.

The use of 2% Dioxan in n-heptane resulted in the resolution, from a Working Solution sample, of a peak between H_4 EAQ and EAQ. An increase in Dioxane concentration to 3% produced a double peak between H_4 EAQ and EAQ, of which one peak was definitely ascribed to H_4 EAQ Epoxide. Further increases, to 5% and 10%, and a reduction to 1% Dioxan, gave a series of results represented graphically in figure VIII.1, which is a plot of the capacity factor, k' , against concentration of Dioxan in the solvent for the four main compounds. The values of other parameters, obtained with 10% Dioxan, are given in Table VIII.2, where flow-rate was about 0.4ml/min. and the compounds were injected separately.

The curves for the quinones in figure VIII.1 are approximately parallel, but the curve for H_4 EAQ Epoxide may be seen to approach that of EAQ for more dilute dioxan solutions. It was, in fact, found that a 1% dioxan solvent mixture did not resolve H_4 EAQ Epoxide from EAQ, and the curves reflect this behaviour.

Table VIII.2.

Elution With a 10% Solution of 1,4-Dioxan in n-Heptane.

Compound	t_R (min)	N	k'	R
H_8 EAQ	14.5	1164	0.28	1.60
H_4 EAQ	17.5	1166	0.55	1.73
H_4 Epoxide	21.0	1764	0.86	0.90
EAQ	23.0	1410	1.04	

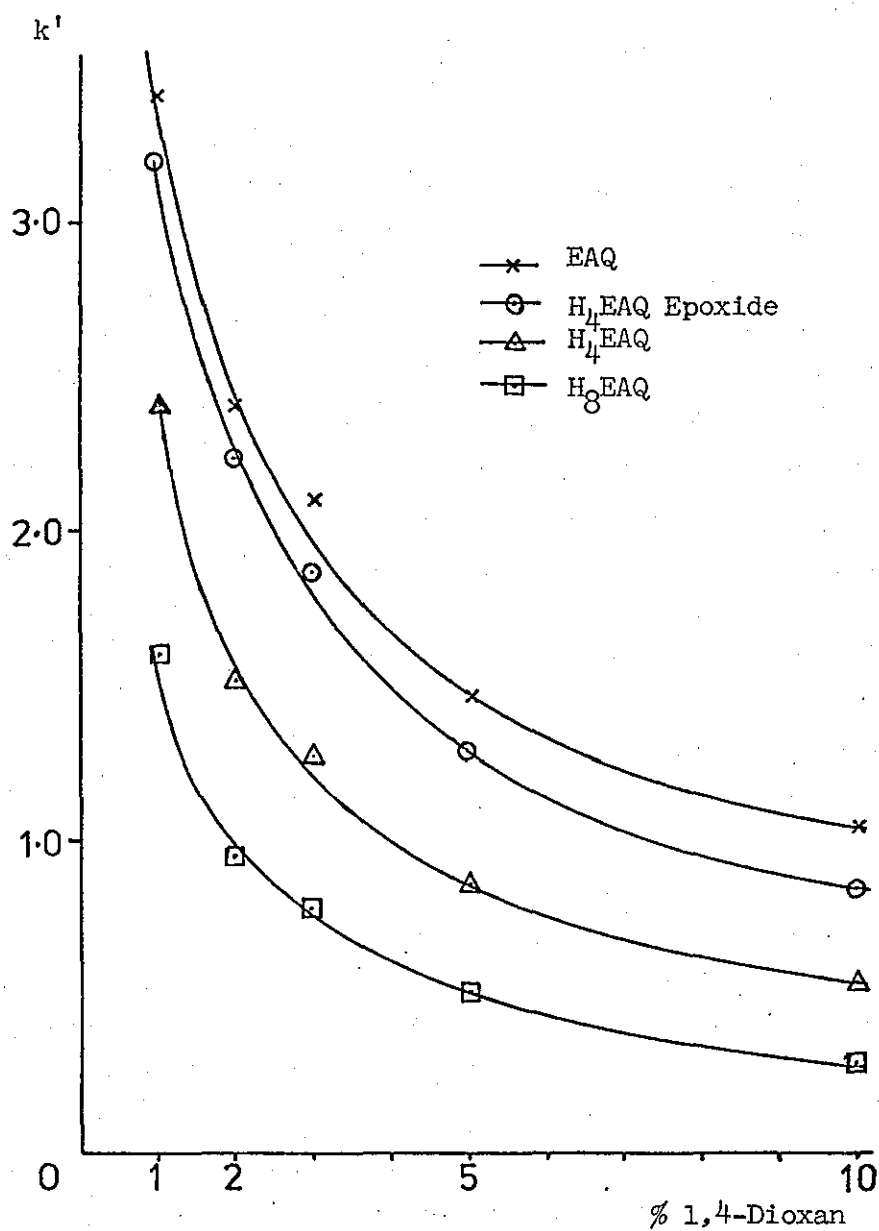


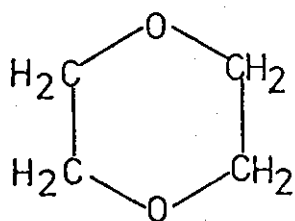
Fig. VIII.1 Capacity Factor Curves for Dioxan.

Conditions as in text.

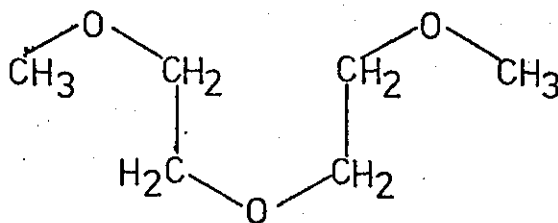
These results had an implication regarding the selection of a suitable solvent system so that best separation could be obtained. When a solvent is found that gives such convergent plots of k' against concentration of solvent it is often possible to select a concentration which gives a maximum separation of one component by taking a vertical line from the concentration axis. The intercept points of the curves on this line then give the relative positions of peaks eluted with the solvent of the chosen concentration values. In the present case a 10% solution of dioxan gave the best separation of the epoxide from EAQ and H_4 EAQ.

Although 1,4-dioxan thus showed promise as a subsidiary modifying solvent, other properties of the system must be considered. The known hazard of toxic effects of dioxan made it desirable that another, potentially less hazardous, solvent should be found.

The compound bis(2-methoxyethyl) ether, also known as dimethyl digol and as diglyme, was examined as an alternative to 1,4-dioxan. The structures of the two compounds are similar in an important respect, each ether group forms part of an ethoxyethyl or methoxyethyl group:



1,4-Dioxan



'Diglyme'

The published data^{86,87} on diglyme indicates that it and similar compounds are less hazardous and less volatile than 1,4-dioxan. It is an industrial solvent and is thus generally available. The information available about its use in chromatography is sparse. The

approximate placing of diglyme in Table IV.1 was made on the basis of a reference to a similar compound and also by observations during chromatography.

5% diglyme in n-heptane gave a good separation of a Working Solution sample into H_8 EAQ, H_4 EAQ and EAQ, as shown in Table VIII.3, with a small double peak, too small to be accurately measured, between H_4 EAQ and EAQ. This result was similar to that obtained with the 1,4-dioxan solvent system, and indicated that overall similar results might be expected.

Table VIII.3.

Elution of Working Solution at 0.5ml/min with 5% Diglyme in n-Heptane.

Compound	t_R (min)	N	k'	R
H_8 EAQ	10.05	1122	0.34	1.28
H_4 EAQ	12.10	586	0.61	
EAQ	16.75	748	1.23	2.09

An attempt to investigate this system with higher and lower concentrations of diglyme gave anomalous results, however, as the k' values did not change as expected with concentration. The k' values obtained for H_4 EAQ and EAQ are shown in Table VIII.4.

Table VIII.4.

Values of k' and Diglyme Concentration for H_4 EAQ and EAQ.

Diglyme concentration (% v/v)	10	5	2	1.5	1
k' H_4 EAQ	0.47	0.61	0.62	0.64	0.68
k' EAQ	1.02	1.23	1.18	1.31	1.24

These observations suggested that the various concentrations below 10% either had not equilibrated with the column packing, or were in a region where a complete layer of solvent molecules covered the adsorbent sites and the excess solvent made little difference, except at higher concentration (about 10%) where the higher solvent strength would begin to be more important. Either explanation requires the assumption of strong adsorption of solvent molecules onto the adsorbent, which implies that there is a certain critical concentration of diglyme in solution at which all the surface active sites are blocked. Above this solution concentration any desorption of diglyme from the surface would be rapidly replaced from solution. Below this critical concentration an adsorption-desorption equilibrium would operate, and in this range of concentration the k' values of solute compounds would be affected significantly by change in solvent composition. This type of behaviour would be expected from a strongly-adsorbed solvent component, and might result in the formation of a functional-group-selective layer on the adsorbent. The chromatographic mode of operation would then not necessarily be solely LSC, but might involve contributions from LLC.

It is apparent from the similarity of the k' values in Table VIII.4 that all of the diglyme concentrations were above the critical concentration proposed above. Further studies on this linked-column system were terminated upon the repair of the Varian SI-10 column; the studies were continued on the SI-10 column alone.

2.3 Chromatography on the Varian SI-10 Column.

The Varian column, with dimensions of 25cm length and 2.2mm internal diameter, was expected to give high numerical efficiencies as indicated in section 2.1.1. Such high efficiencies from a narrow column result in narrow, low volume, bands being eluted from the column and thus require greater care in the selection and use of low-volume tubing and fittings to ensure that these high efficiencies are not lost by

band-broadening effects between column and detector. These considerations would be more critical for the Varian column alone than for the lower-efficiency linked column used above.

After repair the column was eluted with a diglyme solvent, of 1.5% diglyme in n-heptane, and a separation of Working Solution similar to that obtained with the linked columns was obtained. As a check of the chromatographic behaviour of the column it was decided to change to ethyl acetate, which did not give separation of H_4 EAQ Epoxide in previous studies. It was found that a separation of the epoxide was obtained, at 1%, 2% and 3% ethyl acetate. It is suggested that the strong adsorption of diglyme onto the silica had resulted in a layer of adsorbed diglyme which was not removed by ethyl acetate solutions.

On changing back to 1.5% diglyme in n-heptane the separations of Working Solution components were again good, with a small double peak between H_4 EAQ and EAQ as before. The k' values at a flow-rate of 0.84ml/min are shown in Table VIII.5 for a sample of 0.05 μ l of undiluted Working Solution.

Table VIII.5.

Values of k' for Working Solution Components.

Component	Solvent 1	Solvent 2	H_8 EAQ	H_4 EAQ	H_4 EAQEpox	Unknown	EAQ
k'	0.1	0.25	0.45	0.85	1.20	1.40	1.70

The first two peaks were due to the aromatic solvent, which forms a major part of the Working Solution.

Reduction of the diglyme concentration to 1.0% gave slightly larger k' values, which are shown together with other parameters in Table VIII.6. The running conditions used were a flow-rate of 0.50ml/min with 0.1 μ l of 1% solutions of each compound injected. Included in the table are parameters for benzil, which was chosen from several other

compounds for use as an internal standard for the quantitative investigations described in Chapter X.

Table VIII.6.

Working Solution Components Eluted with 1% Diglyme in n-Heptane.

Compound	t_R (min)	k'	N	R
H ₈ EAQ	2.40	0.65	673	2.0
H ₄ EAQ	3.30	1.28	620	0.45
H ₄ EAQ Epoxide	3.50	1.59	1512	1.34
EAQ	4.05	2.1	1240	3.4
Benzil	5.80	3.4	1716	

Table VIII.6 shows that the quinones and benzil were well separated, but that H₄EAQ Epoxide was eluting closer to H₄EAQ than to EAQ. This was considered to be undesirable for quantitative work because the overlap of adjacent peaks would make peak area measurement difficult. Attempts were made to improve the resolutions and efficiencies obtained by changes in the solvent system.

Changing the main solvent from n-heptane to n-hexane and to 2,2,4-trimethylpentane did not improve the separations, so n-heptane was retained as the main solvent.

The concentration of diglyme was changed from 1.0% to 0.7% and then to 0.5%, each with a long period (about 2 hours) of elution to equilibrate the column with the solvent before making injections. These experiments showed that the k' values changed significantly at concentrations below 1%, and it was thus desirable to investigate this range of concentrations in detail.

In order to remove adsorbed diglyme from the column the eluant was changed from 1% diglyme to pure 2,2,4-trimethylpentane, and this solvent was passed until the adsorbed diglyme had been removed.

The amount of diglyme activity left on the column was monitored by making injections of a mixture of H_4 EAQ, EAQ and H_4 EAQ Epoxide at intervals of time and observing the elution characteristics. If the column had desorbed all of the adsorbed diglyme, then the injected compounds would not be eluted but would adsorb onto the column. The results of this experiment, at a flow-rate of 0.4ml/min, are shown in Table VIII.7, which shows retention times of the compounds eluted from an injection at a time, T_c , after the changeover from 1% diglyme to the pure 2,2,4-trimethylpentane. The same information is shown graphically in figure VIII.2.

Table VIII.7.

Elution Times of Compounds from a Diglyme-Coated Silica Column.

T_c (mins)	t_R H_4 EAQ (mins)	t_R H_4 Epoxide (mins)	t_R EAQ (mins)
Before T_c	5.35	6.2	8.3
5	6.4	7.5	9.1
18	7.9	9.2	11.1
44	12.1	14.1	16.4
85	20.4	22.9	26.0
117	23.0	26.3	29.3
185	26.75	28.8	30.75

These results showed that diglyme strongly adsorbs onto the column packing, to the extent that it is very difficult to remove without using a stronger solvent than diglyme. The graphical data indicate that the retention times for all three of the components tend to stabilise after about three hours elution of the pure hydrocarbon; this observation indicates that the diglyme does not completely desorb under the mild solvent strength conditions used. This corroborates the earlier results obtained with ethyl acetate, after changing over from a diglyme

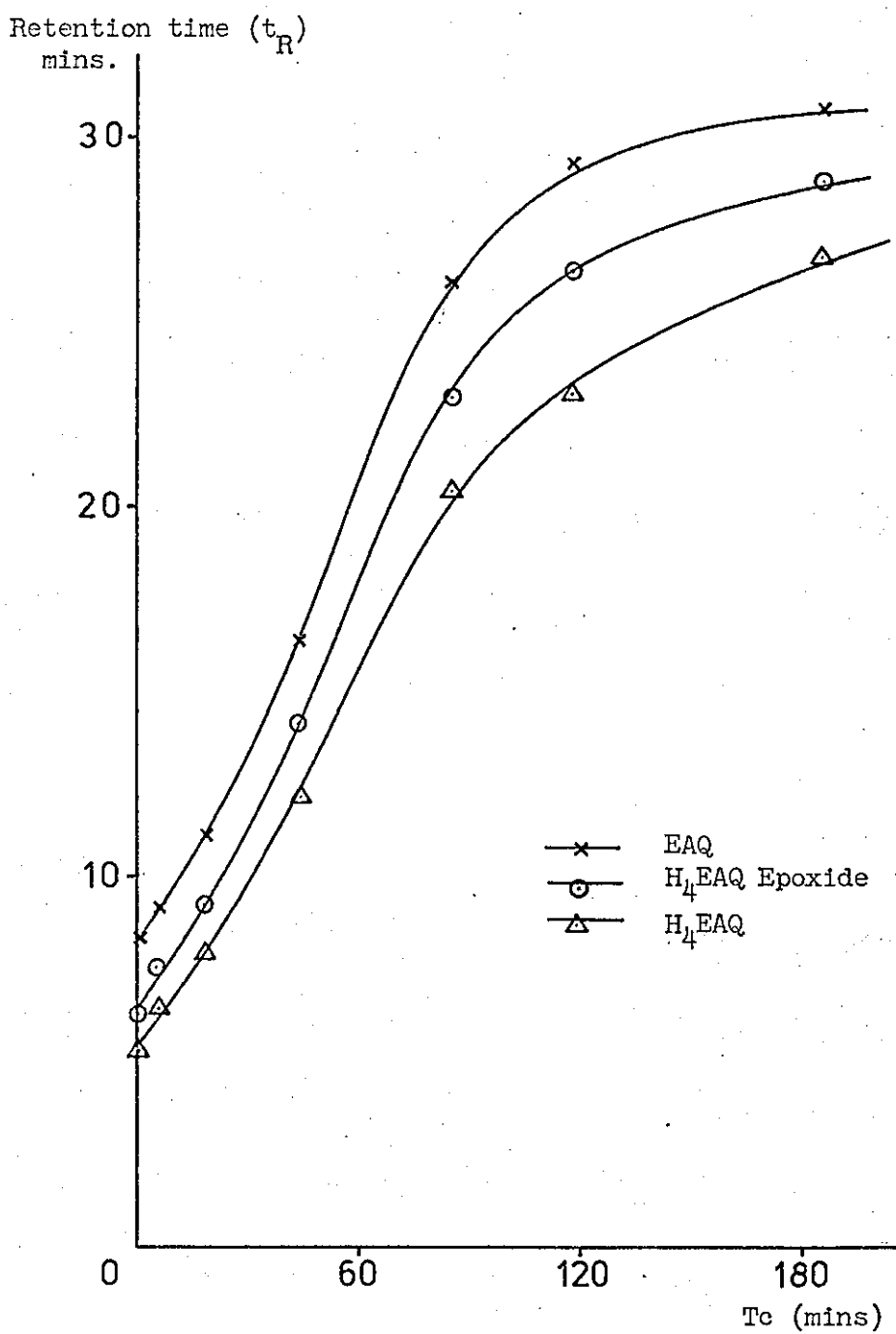


Fig. VIII.2 Variation of t_R with T_c

(see text, p.131)

solvent, since 1% ethyl acetate in n-heptane is also not a strong solvent and would not easily displace diglyme.

After about four hours elution with pure iso-octane a change to a low concentration of diglyme in n-heptane was effected. The k' values for these lower solvent concentrations and those values obtained earlier with 1%, 0.7% and 0.5% are shown in Table VIII.8, and are shown graphically in figure VIII.3.

Table VIII.8.

Values of k' and R Obtained with Low Diglyme Concentrations.

% Diglyme	k'_{H_4EAQ}	R	k'_{Epox}	R	k'_{EAQ}
1.0	1.25		1.68		2.24
		1.27		1.61	
0.70	1.44		1.96		2.92
		1.38		2.22	
0.50	1.88		2.38		3.45
		1.34		2.51	
0.25	2.67		3.42		4.68
		1.50		2.12	
0.10	3.71		4.71		5.43
		1.93		1.23	

The curves for H_4EAQ and H_4EAQ Epoxide are seen to be parallel and differ from EAQ in their degree of curvature. From these curves it may be seen that a diglyme concentration of about 0.2% should give a separation of these compounds with H_4EAQ Epoxide eluting midway between H_4EAQ and EAQ , with all of these peaks eluting at k' values between 3 and 5. This corresponds to an analysis time of about 12 minutes for a t_0 value of 2 minutes. Such a system should be most suitable for quantitative analytical work, particularly so if the peak widths could be further reduced by increasing the column efficiency.

The strong adsorption of diglyme, and the inability of a weaker solvent to readily displace it, suggested a way of altering the analysis times required without significantly changing the resolution. A relatively weaker solvent than diglyme, if used in a solvent mixture containing

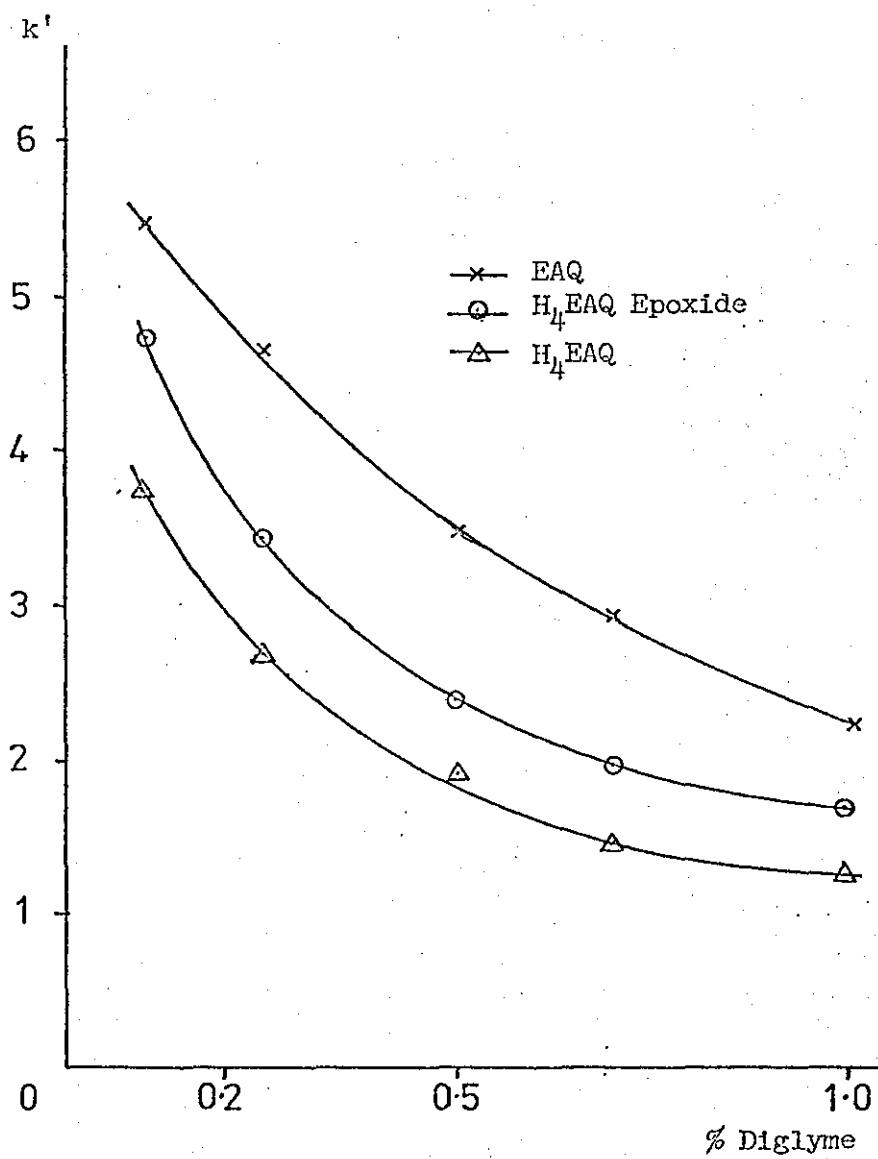


Fig. VIII.3 Capacity Factor Curves for Diglyme.

Conditions as in text.

a low concentration of diglyme, would have the effect of increasing the overall strength of the mixture whilst retaining the selectivity achieved by the use of the diglyme/silica system. Such an increase in solvent strength should result in a uniform decrease in retention times for all of the eluted components, and hence in shorter analysis times. The third solvent in this modified diglyme system would need to have rapid adsorption/desorption characteristics, unlike diglyme and t-amyl alcohol (Chapter VII), so that the chromatography would continue to occur essentially between a diglyme system and the solute.

The first solvent investigated in this respect was ethyl acetate, since its behaviour was known from the preceding studies. Solutions containing 0.2% diglyme with 0.5%, 1%, 2.5%, 5% and 10% ethyl acetate respectively were prepared. After equilibrating the column with each solvent in turn, samples of a solution containing H_8 EAQ, H_4 EAQ, H_4 EAQ Epoxide and EAQ were injected. Results derived from these chromatograms, using a flow-rate of 0.7ml/min, are shown graphically in figure VIII.4. The data for a solvent system giving good resolutions and efficiencies are shown in Table VIII.9. It should be noted that the values for N are particularly high, when compared with those of Table VIII.6, for example, because of the low flow-rate used.

Table VIII.9.

Elution with 0.5% Ethyl Acetate and 0.2% Diglyme at Flow-Rate 0.3ml/min.

Compound	t_R (min)	k'	N	R
H_8 EAQ	6.5	1.65	1878	2.52
H_4 EAQ	8.2	2.35	1912	2.37
H_4 EAQ Epoxide	10.0	3.08	2699	1.86
EAQ	11.55	3.71	2635	

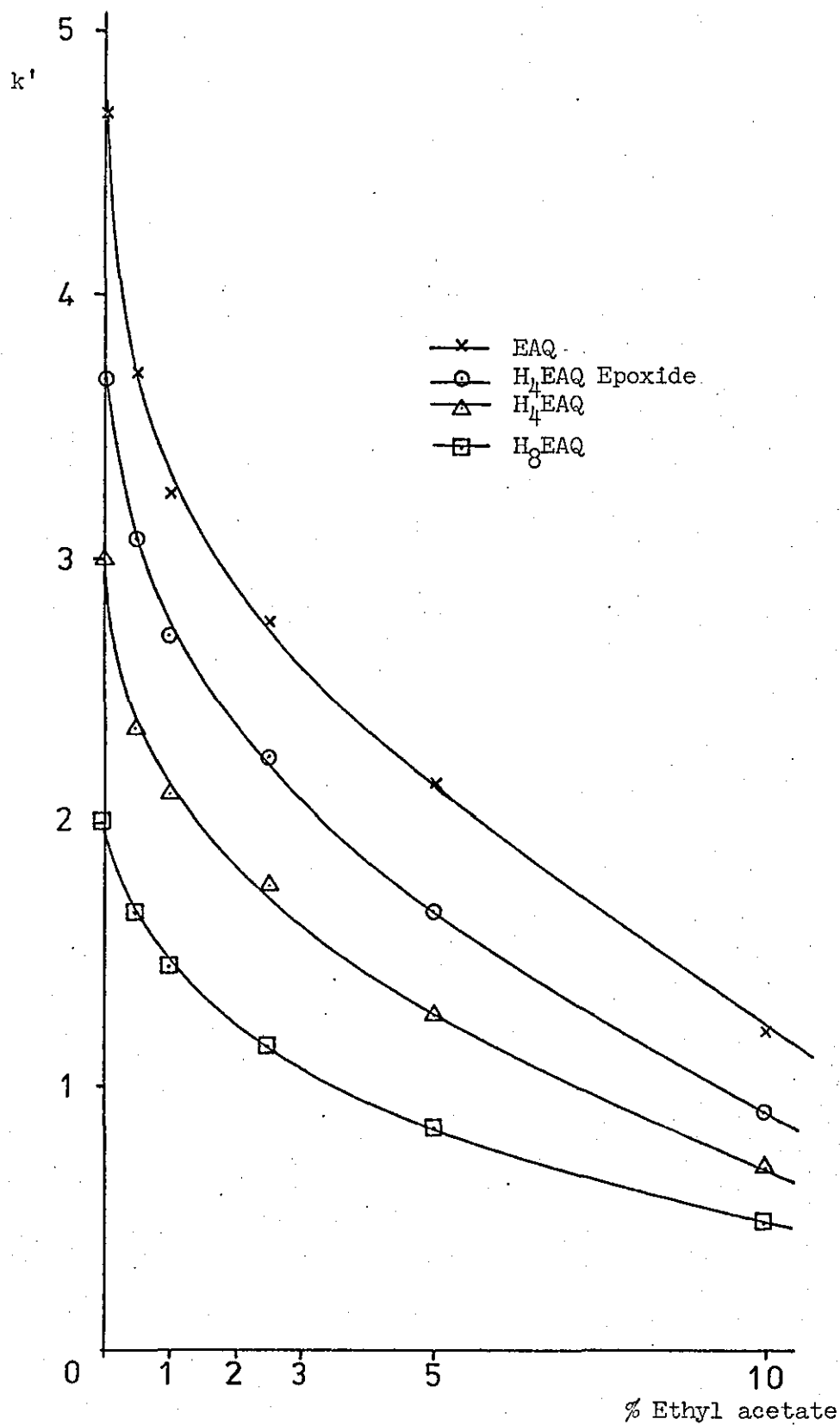


Fig. VIII.4 Capacity Factor Curves for Mixtures Containing
Ethyl Acetate with 0.2% Diglyme.

In figure VIII.4 the values of k' for 0% ethyl acetate were taken from figure VIII.3. The curves of figure VIII.4 show that the separation of H_4 EAQ Epoxide from its neighbouring compounds remained similar between 0% and 10% ethyl acetate content, but that k' values, and hence analysis times, were reduced to one quarter of their 0% values by the addition of 10% ethyl acetate. The use of the principle of adding a different solvent purely to increase solvent strength, while retaining the benefits of the chromatography on the diglyme/silica surface was thus demonstrated and was shown to be effective in reducing analysis times without prejudice to the separations obtained.

Although ethyl acetate has a very low U.V. absorption at concentrations of about 1%, it would be necessary to replace it with a non-absorbing species if it were desired to reduce the detector noise-level of the system to a minimum. For this reason, and to further investigate the behaviour of the ternary type of solvent system used above, it was decided to use iso-propanol and later di-iso-propyl ether as ternary solvents, with diglyme and n-heptane to obtain data on the behaviour of diglyme in solution with three modifying solvents of different functionality.

At levels indicated by previous work it was found that iso-propanol had the effect of causing the curves obtained to rise at the higher isopropanol concentration, as shown in figure VIII.5. This was when the iso-propanol was mixed in solution with 0.2% diglyme. This effect may be explained on the basis that as the concentration of iso-propanol increases, the stronger solvent strength of the alcohol displaces the diglyme from the column, thus causing chromatography to occur between the solute and the more active surface remaining after desorption of diglyme. This increase would result in a greater solute-solid interaction and hence larger k' values.

To check the validity of this explanation two solutions containing 0.5% diglyme with 0.1% and 0.2% iso-propanol respectively were

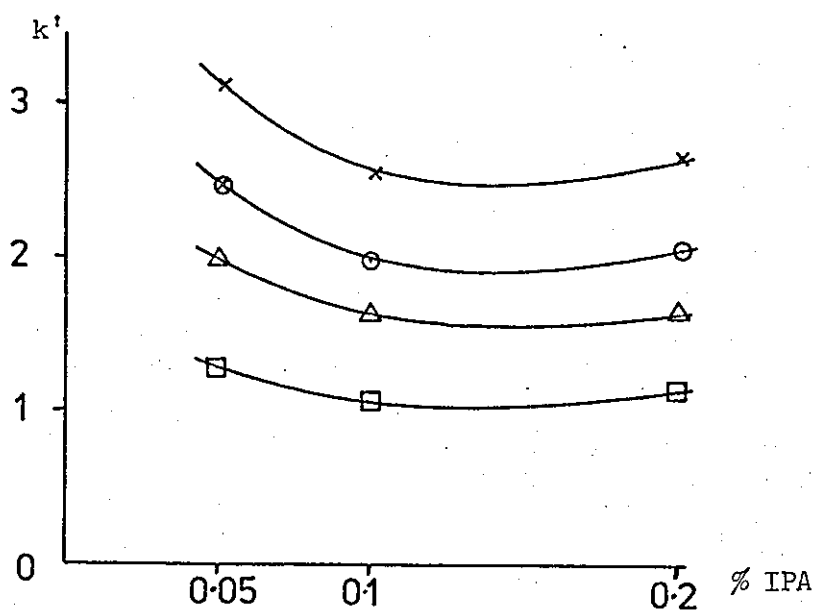


Figure VIII.5.

Capacity Factors for Mixtures Containing iso-Propanol with 0.2% Diglyme.

(Symbols as in Fig. VIII.4.)

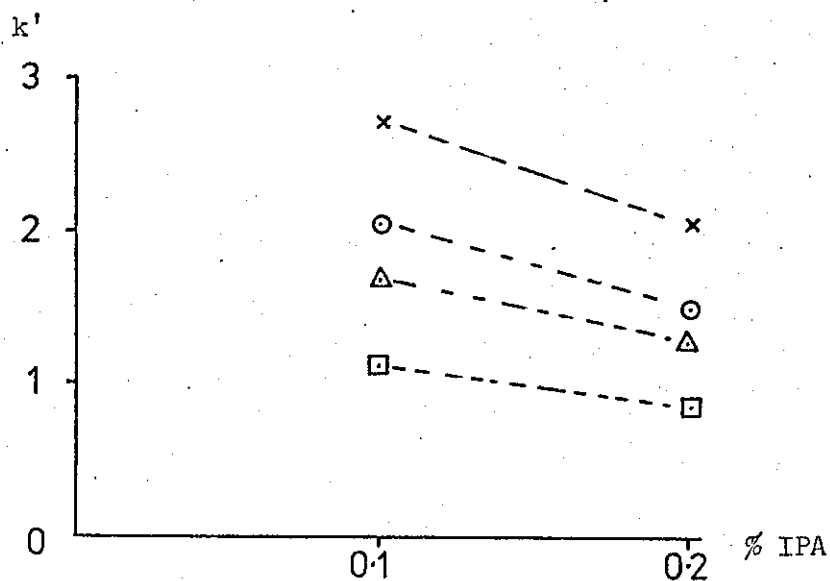


Figure VIII.6.

Capacity Factors for Mixtures Containing iso-Propanol with 0.5% Diglyme.

(Symbols as in Fig. VIII.4.)

prepared, to ensure a higher concentration of diglyme at the active surface and thus to maintain saturation of the surface. It is interesting to note that the 0.1% iso-propanol, 0.5% diglyme, data points coincide with points at the same iso-propanol level with 0.2% diglyme, but that with 0.2% iso-propanol, 0.5% diglyme, the values are lower. This is shown in figure VIII.6, which also shows that the relative position of the H_4 EAQ Epoxide band was shifted towards H_4 EAQ when the 0.2% iso-propanol, 0.5% diglyme solvent was used. These results support the suggestion that iso-propanol removes diglyme from the adsorbent; in order to reduce the k' values with iso-propanol it would be necessary to increase the diglyme concentration significantly above the 0.2% concentration.

Di-iso-propyl ether (IPE) has a similar functional group to that of diglyme; moreover it has a lower solvent strength than ethyl acetate (ϵ 0.28 compared with ϵ 0.58 for ethyl acetate). In order to offset an increase in the amount of 'ether-group-function' in solvents containing both diglyme and IPE it was decided to reduce the diglyme concentration to 0.1%. The diglyme surface layer should be maintained at this lower level because the lower solvent strength of IPE would not displace it from the surface as might ethyl acetate. The solvent system used contained 0.1% diglyme and 2% IPE.

The results obtained using this solvent system are shown in Table VIII.10, for a flow-rate of 0.7ml/min and injection of 0.2 μ l of a synthetic Working Solution.

The data in Table VIII.10 shows that the values of N are high, over 1,000 plates for all components. This factor, with good resolutions and short analysis times, suggested that this was an optimum chromatography system for the main quinone compounds and the H_4 EAQ Epoxide. At this stage a column of even higher efficiency was acquired, and subsequent work was done using the Waters ' μ -Porasil' column.

Table VIII.10.

Elution with 0.1% Diglyme, 2% IPE in n-Heptane.

Compound	t_R (min)	k'	N	R
H ₈ EAQ	2.55	2.0	1664	2.28
H ₄ EAQ	3.2	2.76	1600	1.69
H ₄ Epoxide	3.85	3.53	1171	1.07
EAQ	4.35	4.1	1314	

2.4 Chromatography on the Waters ' μ -Porasil' Column.

The Waters column, of length 30cm and internal diameter 4mm, had been claimed⁷⁹ to have efficiency values of about twice those attained with the Varian SI-10 column, and, because of its larger dimensions, was said to be much less sensitive to extra-column band-broadening effects. When first obtained it was conditioned with 2% IPE in n-heptane.

It was decided to take advantage of the uncontaminated new column in order to assess the performance of some non-diglyme-containing solvent systems, before using diglyme systems to continue quantitative studies.

Samples of some of the H₄EAQ Epoxide degradation products, namely the 'mono-hydroxy' and 'di-hydroxy' reduction products (Chapter I, compounds VIII and IX respectively) and 'hydrolysed H₄EAQ Epoxide' (Chapter I, compound XI), became available from Laporte Industries. Trial injections of solutions of some of these compounds with some of the previously investigated solvent systems indicated that a fairly strong solvent would be required to chromatograph them quickly. It was decided to use an iso-propanol in n-heptane solvent to investigate the separation of these compounds.

A solution containing approximately equal amounts of the above-

mentioned compounds and of H_4 EAQ Epoxide was made up, and aliquots of this were eluted with 2% iso-propanol in n-heptane at a flow-rate of 1.8ml/minute. Although separations were adequate, the efficiency values obtained from these runs ranged from $N = 459$ to $N = 1156$, a disappointing range of efficiencies, lower than had been expected. It was thought that the column might not be homogeneous with respect to surface hydroxyl activity, which might make the adsorption-desorption process for the hydroxyl compounds slow and reduce efficiency. In order to ensure a uniformly active column a small amount (about 10ml) of a 1:3 mixture of water and ethanol was pumped through the column, and this was followed by elution with 100ml each of ethanol, pure iso-propanol, 25% iso-propanol in n-heptane, 5% iso-propanol and then 2% iso-propanol. After a suitable stabilisation period with this latter solvent the mixture of hydroxyl compounds was again injected. Results similar to the earlier injections were obtained with regard to k' values, but N values for the hydroxy-compounds were between 1547 and 1975, which at a flow-rate of 1.8ml/min was considered good.

Later a series of quantitative studies was made on these hydroxy-compounds using this column and a 3% iso-propanol in n-heptane solvent at 1ml/min flow-rate. The detailed results of the qualitative aspects of these studies are shown in Table VIII.11. It is interesting to note that two, overlapping, peaks were obtained for the 'di-hydroxy' compound with most of the separation systems used. This phenomenon will be discussed in section 3. A typical chromatogram is shown in figure VIII.7.

On changing from 2% iso-propanol to 2% di-iso-propyl ether it was found that H_4 EAQ epoxide eluted at the same position as EAQ. At a flow-rate of 2ml/minute the EAQ peak was eluted at $k' 7.55$ with an efficiency $N = 2450$ plates; the epoxide had a similar k' value. This solvent was used here to try to clarify the action of diglyme in an attempt to explain the results in terms of deactivation of the adsorbent

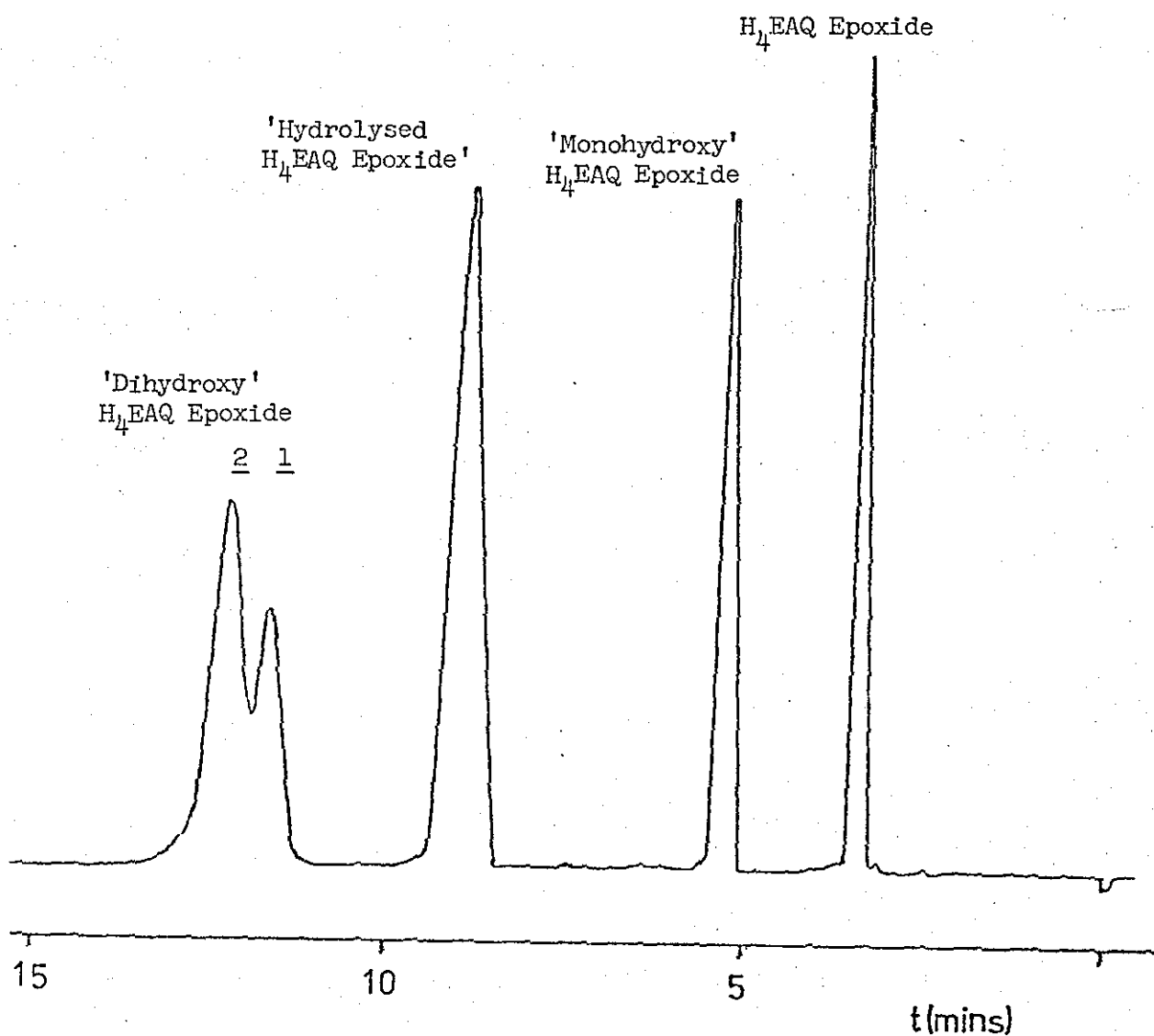


Fig. VIII.7 Separation of Degradation Compounds of H_4 EAQ Epoxide.

Elution with 3% iso-propanol in n-heptane at flow-rate 1ml/minute.

or formation of a selective surface phase. Results obtained using the IPE/n-heptane solvent were considered as a 'blank' value, from which to gauge the effect of added diglyme.

Table VIII.11.

Elution of H₄EAQ Epoxide Degradation Products with 3% iso-Propanol.

Compound or Number	k'	R	N	N/t (N/second)	H (mm)
H ₄ EAQ Epoxide	0.11		4147	21.5	0.072
VIII	0.76	7.23	4064	13.3	0.074
XI	2.03	8.13	3559	6.7	0.084
IX peak 1	3.02	4.04	3229	4.6	0.093
IX peak 2	3.22	0.71	3246	4.4	0.092

When the previously developed solvent of 2% IPE and 0.1% diglyme in n-heptane was used it was found that the k' values for EAQ and H₄EAQ Epoxide respectively were 2.09 and 1.89. Table VIII.12 shows results obtained with solvents containing 2% IPE, with 0%, 0.1% and 0.2% diglyme respectively.

Table VIII.12.

Effect of Diglyme Concentration on EAQ and H₄EAQ Epoxide Capacity Factors Using 2% IPE in n-Heptane Solvent.

% Diglyme	k' H ₄ EAQ Epoxide	k' EAQ
0	7.6	7.55
0.1	1.89	2.09
0.2	1.37	1.59

From the data in Table VIII.12 it is seen that the addition of a small amount of diglyme has a marked effect on k' , this effect being slightly differential with respect to the epoxide group, i.e. the presence of the epoxide group causes the molecule to be eluted earlier than if the group were absent. If the mechanism of chromatography in the presence of diglyme were the formation of a selective surface coating which interacted particularly strongly with the epoxide, then one would expect the epoxide to become later-eluting on the formation of such a surface-layer. This was not the case, so the mechanism is presumably one of selective deactivation of sites able to interact with the epoxide group. The epoxide would then have less interaction with the column than would EAQ, whose group-selective adsorption sites on the silica are less deactivated by diglyme. This factor will be discussed in section 3.

The 2% IPE, 0.1% diglyme solvent was further investigated, at different flow-rates, and these studies showed that individual efficiency values of over 6,000 plates could occasionally be obtained at low flow-rates, 0.5ml/min or less. Representative N values at flow-rate 0.5ml/minute were about 5,300, but these dropped to about 3,000 plates at about 2ml/minute or higher. This loss did not present a problem, as separations at 3,000 plates are found to be quite satisfactory.

Before attempting a detailed quantitative study of this system an injection was made of a mixture of EAQ and benzil, a previously examined potential internal standard. The benzil was not well resolved from EAQ ($R = 0.54$), and because of this poor resolution could not be used as an internal standard with this particular solvent system.

This finding posed a considerable problem, as benzil was otherwise an eminently suitable internal standard material and, in previous studies using 1% diglyme in n-heptane, had eluted in a clear space in the chromatogram. It was therefore necessary to either change the internal standard or to change the solvent system. The latter was, in

practice, simpler and quicker to effect. The solvent was thus changed, from 2% IPE with 0.1% diglyme, to 2% di-iso-propyl ether with 0.2% diglyme in n-heptane. This relatively small change in composition was effective in increasing the resolution between EAQ and benzil to $R = 1.5$, which was satisfactory for quantitative use.

This change in solvent composition altered the parameters of other separations slightly. Quantitative studies were later carried out and are reported in Chapter X. The data relating to the qualitative aspects of these studies are shown in Table VIII.13. This data may be compared with the data of Table VIII.10, which applies to the Varian column at a lower flow-rate, but which indicates the parameters obtained using a 2% IPE, 0.1% diglyme system. Table VIII.13 was compiled from chromatograms run at a flow-rate of 1.0ml/minute, using an injection of 0.2 μ l of a synthetic Working Solution containing EAQ, 0.4%; H₄EAQ, 1%; H₈EAQ, 0.5%; H₄EAQ Epoxide, 0.5%; and benzil, 0.75%, all in 'Sextate'. The chromatogram shown in figure VIII.8 is representative of the separations achieved.

Table VIII.13.

Elution of a Synthetic Working Solution with 2% IPE, 0.2% Diglyme.

Compound	k'	R	N	N/t (N/sec)	H (mm)
H ₈ EAQ	0.65	2.94	2012	6.36	0.149
H ₄ EAQ	1.08		3203	8.03	0.093
H ₄ EAQ Epoxide	1.37	2.04	4367	9.58	0.069
EAQ	1.59	1.36	3643	7.31	0.082
Benzil	1.92	1.84	4305	7.67	0.069

It is apparent by simple comparison of the N values that the Varian column is about one-half or one-third as efficient as the Waters column using similar solvents, allowing for difference in solvent flow-rate.

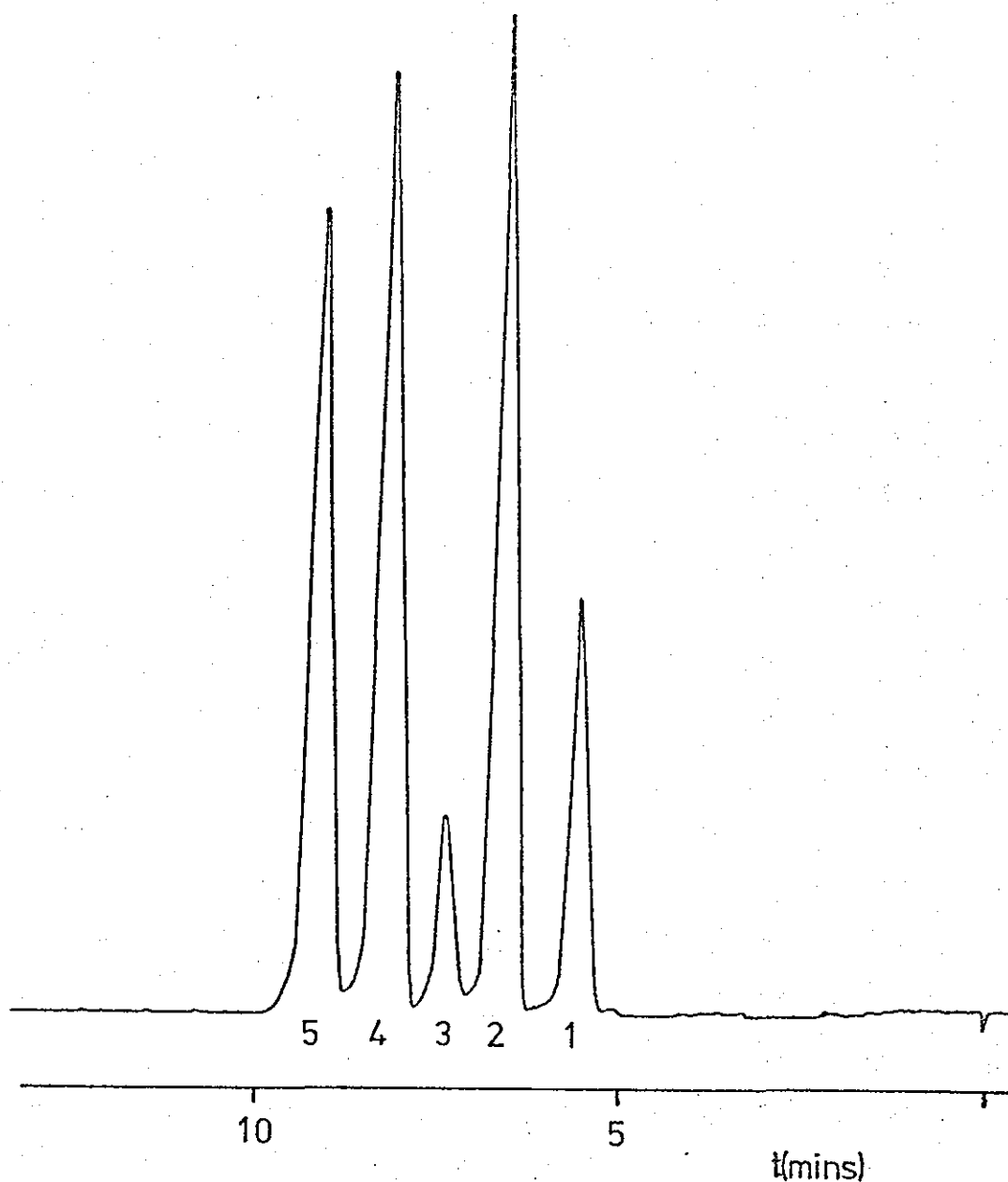


Fig. VIII.8 Separation of Synthetic Working Solution.

Eluted with 2% IPE, 0.2% diglyme in n-heptane at flow-rate 2ml/minute.

Peak identities:

- 1 H_8 EAQ
- 2 H_4 EAQ
- 3 H_4 EAQ Epoxide
- 4 EAQ
- 5 Benzil (Internal Standard)

The solvent system of 2% v/v di-iso-propyl ether, 0.2% diglyme and 97.8% n-heptane was, on the basis of these results, considered to be the most appropriate solvent system for the separation of the major components of Working Solution. Further studies were carried out to determine the optimum conditions for separations.

2.5 Determination of Optimum Machine Running Conditions.

Good quantitation of chromatograms, obtained using the optimised solvent system, is dependent on the reproducibility of the column in operation. The running conditions should be chosen to give the best consistency while at the same time giving the fast analysis times and high efficiencies needed for routine industrial applications.

Two factors are particularly important in the choice of running conditions, these being solvent flow-rate and sample size injected.

2.5.1 Effect of Solvent Flow-Rate.

The solvent flow-rate affects directly the efficiency of the column and the analysis time. High flow-rates produce fast analyses but low efficiencies, a compromise usually has to be made between both parameters.

The effect of flow-rate was investigated by injecting 0.2 μ l samples of synthetic Working Solution at different solvent flow-rates. The data obtained from these chromatograms was then processed to give various relationships used to find the best running conditions. Table VIII.14 shows the effect of flow-rate on N, H and N/t (plates generated per second) for EAQ and H₄EAQ. These values are also shown graphically in figures VIII.9 and VIII.10, for N and N/t to flow-rate respectively.

From the data in Table VIII.14 and figures VIII.9 and VIII.10 it is seen that N increases with decreasing flow-rate, as was expected. There is a definite inflexion point in the curves for both H₄EAQ and EAQ between about 1ml/minute and 2ml/minute flow-rates, at about 3,200

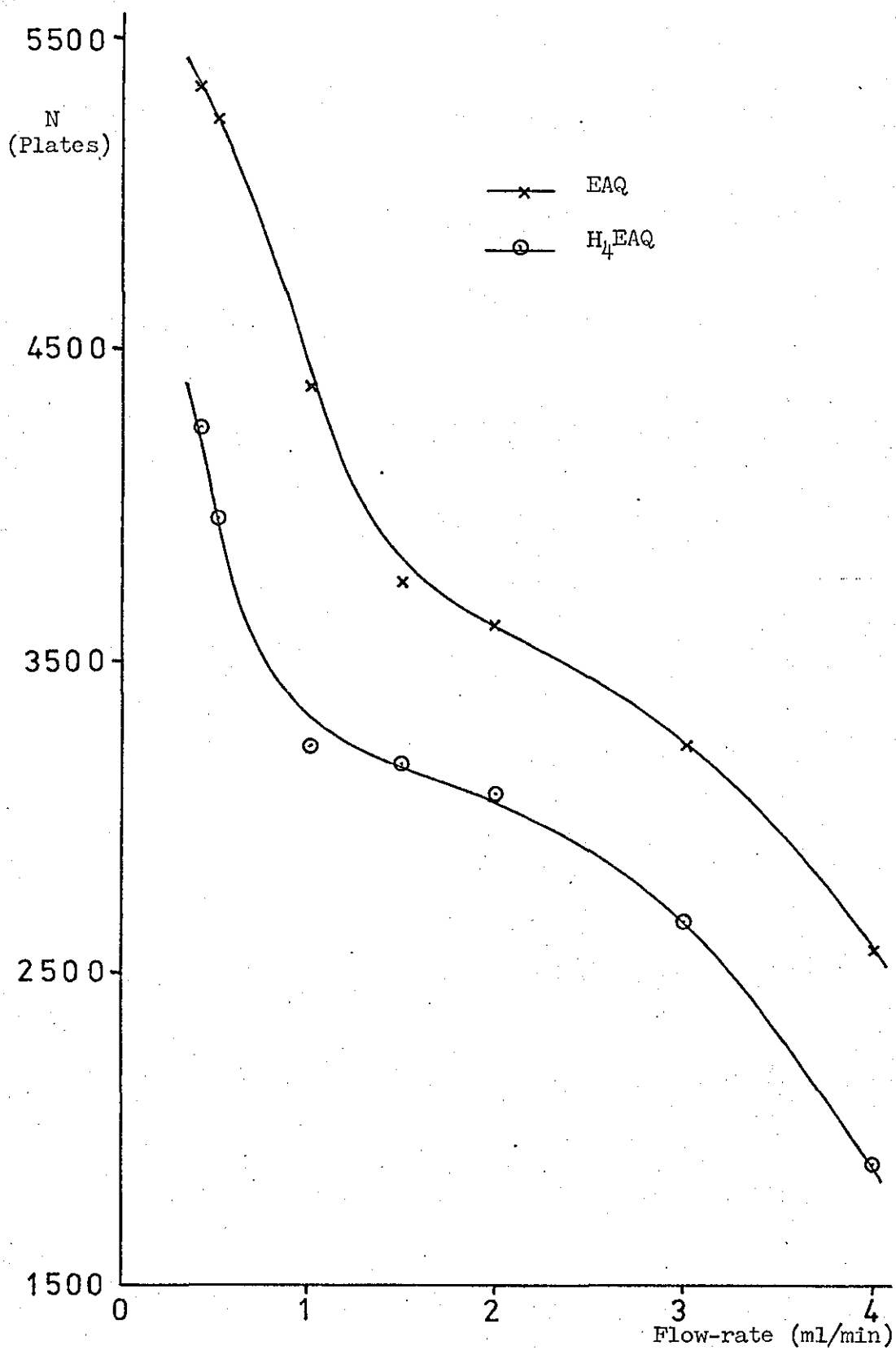


Fig. VIII.9 Dependence of Efficiency (N) on Flow-Rate.

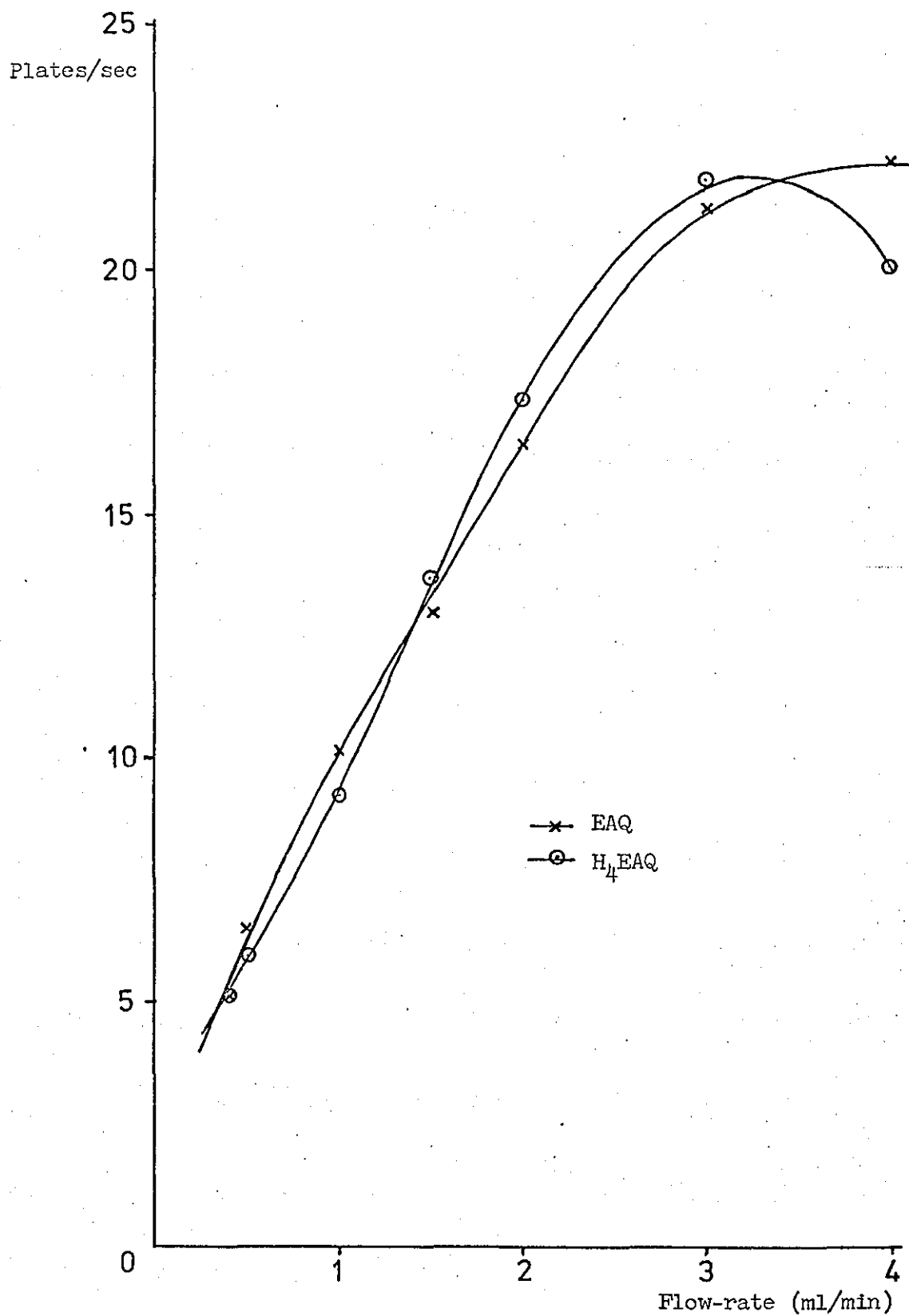


Figure VIII.10.

Variation of Number of Plates Generated per Second with Flow-Rate.

plates, and the values for plates generated per second rises to 22 at about 3 to 4ml/minute. The required best running conditions are thus at 3 to 4ml/min for fastest analyses or at 2ml/minute for better efficiency and slightly less rapid analyses. Below 2ml/minute there is little gain in efficiency until flow-rates of about 0.5ml/min are reached, whereas above 3ml/minute the efficiency drops relatively rapidly with increase in flow-rate, and resolution of peaks suffers both by loss of column efficiency and by the rapid elution of peaks which are too fast for the detector and recorder to fully register.

A compromise value for flow-rate was chosen of 2ml/minute, so that good efficiencies coupled with acceptable analysis times were obtained.

Table VIII.14.

Relationships Between Flow-Rate and N, H and N/t.

Flow-rate (ml/min)	H_4 EAQ			EAQ		
	N	H (mm)	N/t (N/sec)	N	H (mm)	N/t (N/sec)
0.40	4240	0.071	5.1	5337	0.056	5.1
0.50	3953	0.076	5.9	5237	0.057	6.5
1.00	3224	0.093	9.2	4380	0.068	10.1
1.5	3170	0.095	13.6	3750	0.080	13.0
2.0	3080	0.097	17.3	3634	0.083	16.4
3.0	2666	0.113	21.8	3223	0.093	21.2
4.0	1890	0.159	20.0	2573	0.116	22.1

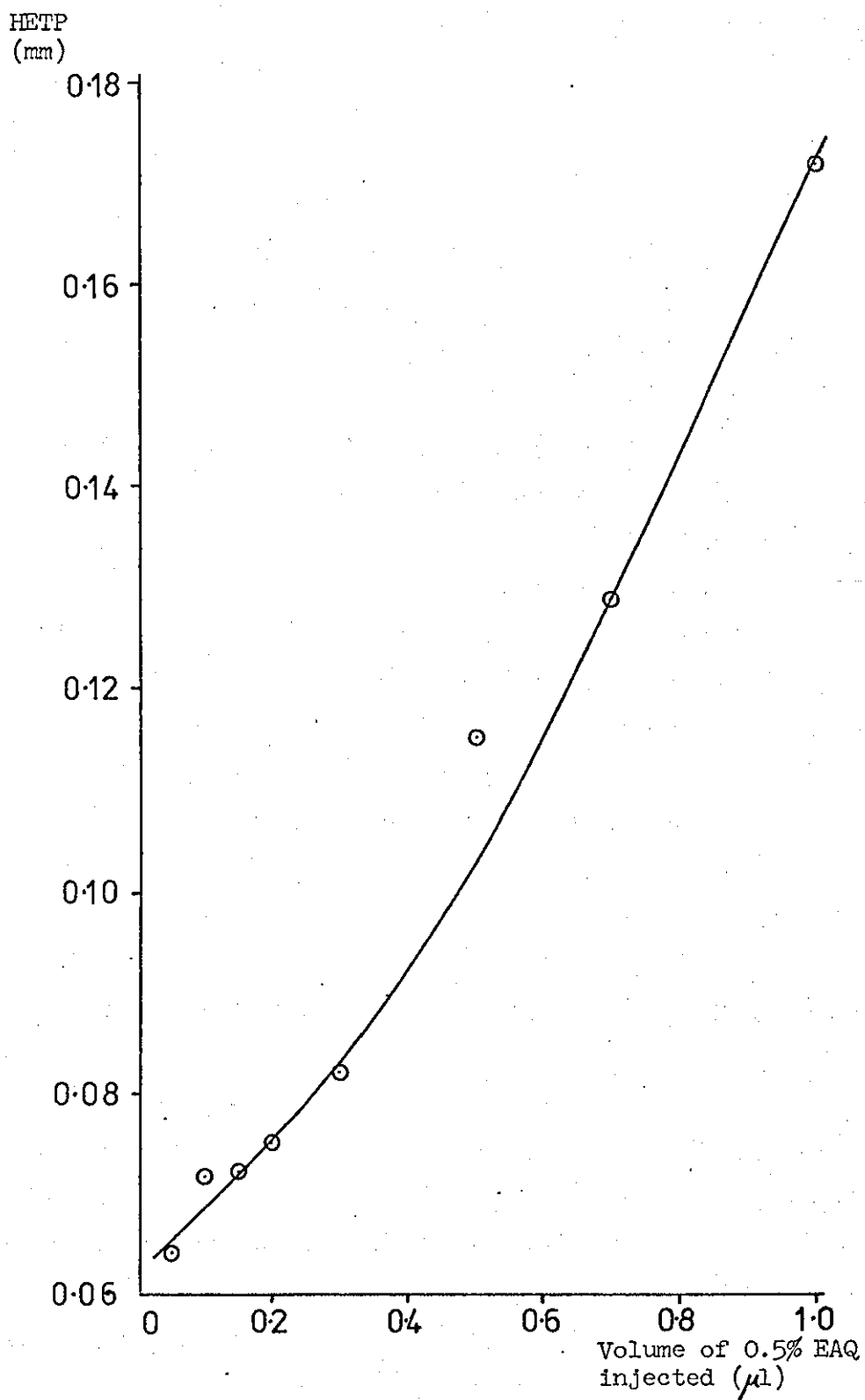


Figure VIII.11.

Variation of Plate Height (HETP) with Volume of Solution Injected.

2.5.2 Effect of Injected Sample Volume.

The effect of sample volume on column efficiency was determined by injecting different volumes of a 0.5% w/v EAQ solution at a solvent flow-rate of 2.0ml/minute. The peaks so obtained were measured and their N and H values were calculated and plotted as shown in figure VIII.11, H versus volume of 0.5% EAQ injected.

The data, as might be expected, shows a definite dependence of H on volume injected. The injection of $1\mu\text{l}$ reduces the N value (increases the plate height, H) by a factor of about three compared with the injection of $0.1\mu\text{l}$. In order to use a sample large enough to reduce syringe inaccuracies and still maintain a high column efficiency it was decided to inject volumes of $0.2\mu\text{l}$ for the subsequent quantitative work. The inaccuracy involved in using such a small volume should be reduced by the use of an internal standard. A study of this forms part of Chapter X.

3. Conclusions.

3.1 Use of Binary Solvent Mixtures.

The use of ethyl acetate, isopropanol and di-iso-propyl ether as components in binary solvent mixtures was found to give poor separation of H_4EAQ Epoxide from the quinone compounds. Dioxan and diglyme gave acceptable separations of the epoxide, but were superseded by ternary systems containing diglyme.

Iso-propanol in n-heptane gave satisfactory resolutions and efficiencies for the three H_4EAQ Epoxide degradation products. A quantitative study using this system is reported in Chapter X.

3.2 Use of Ternary Solvent Mixtures.

The modification of the behaviour of diglyme-containing solvents by using ethyl acetate, iso-propanol and di-iso-propyl ether in

admixture resulted in the development of an optimum solvent system for quantitative analysis. This solvent contained 2% di-iso-propyl ether and 0.2% diglyme in n-heptane. Quantitative studies with this system are described in Chapter X.

3.3 Mode of Action of Diglyme as a Chromatographic Solvent.

Diglyme was found to adsorb strongly onto the silica surface and was not readily displaced by n-heptane or by di-iso-propyl ether or ethyl acetate solutions. Solutions containing iso-propanol showed evidence of displacement of diglyme from the surface, which could be countered by increasing the concentration of diglyme in the solvent.

When added to 2% di-iso-propyl ether in n-heptane it was found that diglyme markedly reduced the k' values of all of the Working Solution components, but preferentially reduced that of H_4 EAQ Epoxide relative to EAQ. This may be accounted for by the preferential adsorption of diglyme onto silica sites which show selective adsorption of ether functional groups. By blocking the ether-adsorbing sites more than other sites the epoxide would be eluted earlier as observed.

No chromatographic evidence was found of the formation of a liquid phase at the silica surface. It is concluded that diglyme acts in the manner described above, by preferentially blocking adsorbent sites, and is more successful for this purpose than simple ethers, such as di-iso-propyl ether, possibly because the tri-ether structure of diglyme allows an effect to operate which is analogous to the 'chelate' effect in inorganic chemistry. Any one ether group of diglyme, when adsorbed at the surface, would ensure that the other two groups were close at hand, and thus there would be much less chance of all three groups desorbing at the same time. It would be difficult to remove such an adsorbed species except by using a solvent which could form much stronger hydrogen-bonds with the surface.

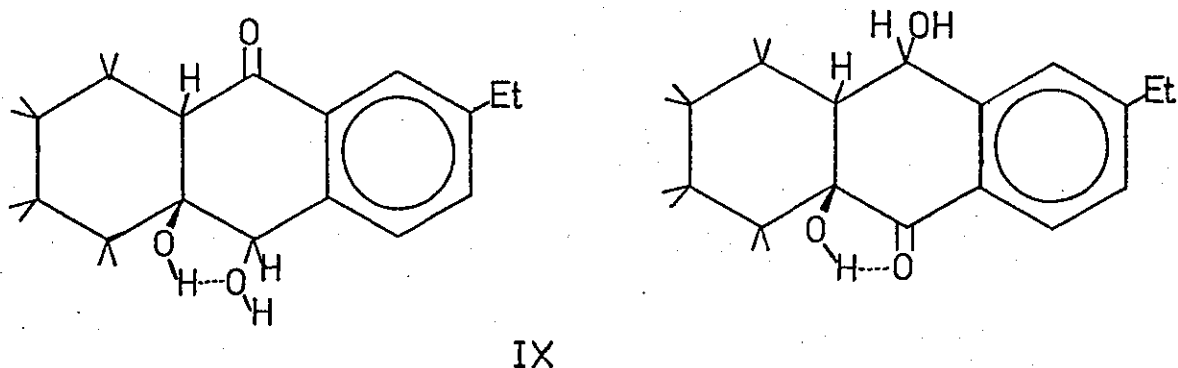
The use of diglyme, and of 1,4-dioxan, are specific examples

of a general case, that is, of the use of di- or tri-functional compounds to selectively and strongly block certain adsorption sites and thus reversibly alter the chromatographic characteristics of an adsorbent. One might thus expect similar effects from, for example, thio-diethers, glycol di-esters, glycerol tri-esters and other similar compounds. One of the attractive features of the use of diglyme is that it can be removed by a strong solvent, e.g. ethanol, and the column can then be changed in its group-selectivity by application of another solvent system, thus ensuring the maximum usage of the often expensive pre-packed column.

3.4 Separation of H_4 EAQ Epoxide Degradation Products.

These compounds were well-separated by a 3% iso-propanol in n-heptane solvent; quantitative studies are reported in Chapter X. The order of elution seems, at first sight, illogical. Since the 'mono-hydroxy' compound has one hydroxyl and two carbonyl groups, the 'di-hydroxy' compound has two hydroxyls and one carbonyl, and the 'hydrolysed H_4 EAQ Epoxide' has two hydroxyls and two carbonyls, one would expect them to elute in that order. On inspecting the structures (Chapter I, compounds VIII, IX and XI respectively) it is seen that the groups attached to the ring-junction carbon atoms are trans to each other, and thus will only interact with the surface if the hydroxyl is on the side of the molecule which is adsorbing onto the surface. When viewed in this manner the observed elution order is explained. The 'mono-hydroxy' compound has two carbonyls in the plane of the centre ring and only one axial hydroxyl at the ring-junction. The 'hydrolysed H_4 EAQ Epoxide' has a trans pair of hydroxyls on ring-junctions and two carbonyl groups in the plane of the ring, but only one of the hydroxyls can interact with the surface at any one time; however, since the compound has this arrangement on each side of the planar molecule it interacts in this manner each time it adsorbs, whereas the 'mono-hydroxy' compound can only

adsorb in this way, statistically speaking, on every other adsorption. The 'di-hydroxy' compound is of particular interest here, as there is the possibility of two isomeric forms as shown:



These two forms will derive from a difference in the position of reduction of the 'mono-hydroxy' compound. The structures differ sufficiently to cause two separate peaks under the conditions used in these studies, and the position of elution is explained by the probability of two hydroxyl groups being on the same side of the molecule. This would make a much stronger adsorption than for either of the other compounds. It is thus suggested that the above compounds are those labelled 'IX, peak 1' and 'IX, peak 2' in Table VIII.11.

3.5 General Conclusions.

A successful solvent/adsorbent system was developed and optimised, and the application of a principle of selective deactivation was studied and elucidated.

The superiority of the small-particle-diameter high-efficiency columns over the medium-efficiency columns (Chapter VII) was clearly shown by the great increases in plate numbers obtained.

The chromatographic behaviour of eight of the Working Solution components (including the solvents) have been investigated.

It is considered that with the development of these systems, and with the information implied above, the purely qualitative studies were complete. The above systems were then used to quantitate and further characterise the compounds making up Working Solution.

IX QUANTITATIVE CHROMATOGRAPHY THEORY.

1. Introduction.

In the application of liquid chromatography to quantitative analysis, the analyst has to optimise and manipulate his chromatographic system and instrumentation to obtain the best accuracy and precision. To achieve this end it is necessary to understand and be able to minimise the sources of error likely to arise in carrying out analyses.

Similarly, it is important to carefully select the method of quantitation to ensure that it is of an accuracy, precision and speed that will not introduce a larger source of error than the chromatographic process itself.

Lastly, the statistical interpretation of the data obtained by the chosen methods must give relevant information, and this in turn requires that the analyst should be conversant with the statistical processes used and the degree of confidence he can expect to have in his results.

These general areas will now be briefly discussed^{10,11,12}.

2. Sources of Error.

2.1 Sources of Error in Sampling and Sample Preparation.

The problem of obtaining a satisfactory and truly representative sample of a bulk material is greater than is generally appreciated. Considerable care is necessary, for example, when sampling large storage facilities, to ensure that the material sampled has not segregated, been locally contaminated by air, water or metal salts, or separated into layers. Even on a small scale similar problems may arise, and suitable precautions should be taken, by thoroughly mixing the material to be tested, to ensure that the sample taken corresponds to the bulk material.

When the sample is subsequently prepared for analysis it is possible for further errors to arise. These arise, for example, in the weighing or pipetting of solutions, making up of solutions to a certain volume and in ensuring that any dilutions performed are accurate. It is also possible that some component of a sample may be insoluble or only slightly soluble in a diluting solvent, in which case the results obtained would give low values for such a component.

It is apparent that the production of high-quality results requires care from the first stages of sample acquisition throughout the analysis, and also implies the existence of a minimum, irreducible, level of error applying to all quantitative analysis. This arises from the accumulation of small errors from all of the above steps and from tolerances in the instrumentation and apparatus used.

2.2 Sources of Error in Chromatography.

One major source of error arises from the introduction of the sample into the chromatographic system. In the present work, using stopped-flow injection with a one-microlitre syringe, the reproducibility of the volume delivered by the syringe is one of the limiting factors.

Since the long injection device requires that the sample be held in the needle, which has to be in contact with the chromatographic solvent while the needle is being inserted, the possibility arises of the diffusion of the sample into the solvent in the body of the injection device.

The injection technique, including the preliminary thorough washing of the syringe with sample solution, must be standardised so that the possibly sizeable errors inherent in this operator-dependent step may be minimised to reduce bias in results or large variations in results.

The processes involved in chromatography require some interaction between the solutes and stationary phases. It is not unusual to find that such interactions result in chemical changes in the solutes (e.g. the case of alumina, Chapter VI), or in distortion or tailing of peaks, or even in irreversible adsorption of some components of a mixture. Such behaviour requires selection of the optimum chromatographic system to overcome it and to give sharp, well-separated peaks, since these are the type most easily quantitated. Similarly, changes in the instrument running conditions and in the solvent can largely eliminate peak tailing, which is fortunately not as much of a problem in HPLC as it is in GLC.

2.3 Sources of Error in Detection and Display of Eluted Compound Peaks.

Some of the sources of error, such as temperature and flow-rate dependence, have already been described in Chapter III, in relation to the particular detector used herein. Other factors, such as faulty connections between instruments, poor earthing and voltage stabilisation, contribute very little to a well-designed and well-operated instrument, but may sometimes cause problems.

The effect of sample size may be critical in determining the limitations of use of a method (see Chapter VIII, 2.5.2). If a large

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sample causes the detector response to go into a non-linear part of its response range, then the results obtained would have to be discounted, unless a standardisation method could be used to provide calibration.

It is usually found more accurate and precise to use standardisation and calibration techniques rather than to try to establish a method based on fundamental relationships such as the U.V. absorbance of compounds. Standardisation techniques will be described in section 4 of this Chapter.

The errors introduced by the opto-electronic and electronic components of the detector, such as baseline drift and short- and long-term noise, were minimised as described in Chapter III. The errors inherent to parts within the recorder, in the potentiometer balancing circuits and in the pen and chart drive systems, had presumably been minimised by the recorder manufacturers, but nevertheless must be recognised as being present. The chart-drive, for example, was by a stepping motor, which at very low speeds gave a perceptible stepping of the forward movement of the paper, which might have an effect on measurement of peak widths. Any slack, or 'backlash', in the pen-drive, contributing to the recorder dead-band, would lead to loss of accuracy in measuring peak heights.

3. Methods of Quantitation of Results.

There are three types of quantitation in general use. These are based on:

1. Peak height measurement.
2. Manual peak area measurement.
3. Electronic and mechanical peak area integration.

3.1 Peak Height Measurement.

When using a chart recorder, this is the simplest quantitation

method, consisting simply of drawing a baseline for the peak concerned and measuring the height from the peak maximum to the point on the interpolated baseline directly below it. The accuracy of the method depends on the reproducibility of running conditions and on the proper measurements being made by the operator. It is the simplest and fastest manual method for reducing chromatograms to usable figures. The alternative methods, measuring peak area, are, however, fundamentally more accurate unless considerable calibration of the peak height system is carried out.

3.2 Manual Peak Area Measurement.

3.2.1. Height Times Half-Height-Width.

Since most chromatographic peaks from optimised systems approximate to triangles, the area of the triangle may be calculated simply from the formula:

$$A = \frac{HW}{2}$$

where H is the triangle height and W is the base width. A simplification of this method, by substituting the peak height for H and approximating the peak-width at half-height to $\frac{W}{2}$, gives results proportionate to the peak area but slightly less than the actual peak area. This difference is not important if calibration curves of the compounds to be tested are available. The use of the half-height-width avoids the difficulty of deciding exactly what the base width should be in cases of tailing or overlapping peaks. Fairly accurate widths, even for fast peaks, can usually be obtained by an increase in the chart-drive speed.

3.2.2 Triangulation.

Carrying the previous principle further, it is usually possible to construct a triangle on the peak by drawing tangents to the peak at

the half-height and interpolating a suitable baseline. The point of intersection of the two tangents is then the apex of the triangle and the normal triangle area formula would be used.

A major limitation with this technique is with the construction of tangents to the peak, for a small difference in the angles of the tangents, particularly on narrow, sharp, peaks, can lead to very large errors in the area calculation. Similarly, some peaks approximate to the Gaussian shape, and determining the correct tangent might thus be difficult.

3.2.3 Cut and Weigh.

As the name suggests, this involves the cutting out of the peak, followed by an accurate weighing of the paper so obtained. The paper is one of the limiting factors, since it has to be very homogeneous to give good reproducibility. The cutting-out part of the procedure is subject to operator error in deciding exactly what constitutes the peak. On the whole, the method is, with care, superior to the previous two methods, particularly for irregular peaks, since it does measure the whole peak and is not an approximation.

3.2.4 Planimetry.

Whereas the first two area measurement methods only approximated to the exact peak area, the cut-and-weigh and planimetry methods follow the actual peak outline and thus give a better representation of the true peak area.

The planimeter is a mechanical device by means of which a skilful operator can get results of similar accuracy to the cut-and-weigh method without destroying the chromatogram. The pointer of the planimeter is used to follow the peak outline and the peak area is shown as a number, convertible to peak area in various units by using different factors, this number being derived from the distance travelled by the

planimeter drive wheel in contact with the chart paper. It is necessary to have experience and skill in the use of the planimeter in order to obtain good results, and the measurements are time-consuming.

3.3 Electronic and Mechanical Peak Area Integration.

3.3.1 Mechanical Integration.

The most widely-used mechanical integrator, in use with a wide range of chart recorders, is the DISC integrator. This device may be adapted for use in conjunction with a number of different manufacturers' recorders or may be built in as an optional extra. The device operates by a mechanical linkage between the recorder pen-drive and a mechanism which converts the pen deflection to vertical movement of the integrator pen. The integrator pen then draws a zig-zag trace, with each leg of the zig-zag representing a certain peak area. The number of such lines drawn under a peak is directly proportional to the area of the peak. The system and its mechanical principle are described in several texts and in a DISC publication⁸⁰.

This device is relatively simple to use, is cheap compared to electronic integrators, and has been in wide use in GLC for a number of years. It gives good accuracy with well-separated peaks but is slightly more difficult to use with partly-resolved peaks, and allowance has to be made for baseline drift.

3.3.2 Electronic Integration.

Electronic integrators generally take their input signal directly from the detector as a voltage, and convert this to a series of pulses of frequency proportional to the voltage. The pulses thus generated may be counted electronically or electro-mechanically, and triggering circuits working on rate of change of the pulse-rate enable prints-out of counts after peaks. The printout may be simply as several numbers, or in more advanced instruments may be further processed to yield data such as the proportion of each peak to the sum of all peaks

in a chromatogram. This approach leads inevitably to the concept of using small dedicated computers to completely analyse results without any operator intervention, but this is relatively costly and is normally only justifiable for laboratories with a large routine sample throughput.

The problems encountered with electronic integrators are mainly concerned with their ability or inability to allow for baseline drift and to discriminate between partially resolved peaks or inflection points. The more expensive instruments, currently about £2,000 to £3,000, have facilities for coping with these difficulties, but the simpler instruments require constant adjustment of the baseline level in order to obtain reproducible results.

3.4 Methods of Quantitation Used in These Studies.

Two methods were used; these were peak height measurement, because of its speed and simplicity, and electronic integration because of the smaller subjective error likely to occur. The peak heights of the studied chromatograms were taken as previously described. The integrator used was a Kent Chromalog Mark I.

This integrator, one of the first types to be used routinely in GLC, was a relatively simple instrument in that it had no means of allowing for baseline drift, and its function was to integrate peaks and record and display the count by an electro-mechanical mechanism printing onto paper roll. No further treatment of the data so obtained was possible with this instrument. It was possible to set the machine to print out at certain levels, e.g. 1% of f.s.d., above the set baseline, and there were also two circuits to trigger printout when peaks had been sensed and measured.

4. Conversion of Numerical Data to Sample Concentration.

Having obtained numerical data it is necessary to correlate

these with certain concentrations or amounts of the substances analysed.

There are three methods of making such correlations, these are

1. Internal standardisation
2. Calibration by external standards
3. Area normalisation.

Of these, the first two are the more widely used in HPLC.

4.1 Internal Standardisation.

By the addition of accurately known amounts of an internal standard compound to sample solutions and subsequent chromatography of the mixtures it is possible to eliminate errors associated with apparatus, injection technique and some other factors. The height or area of the internal standard peak is used as a reference-point; by comparing the other peaks to the internal standard, which represents an accurately known amount of material, the relative amounts in the other peaks may be found. The results are often given graphically by plotting the ratio of area (or height) of sample peak divided by the area (or height) of the internal standard peak against the percentage or weight of the sample.

It follows that the internal standard peak must be well separated from, but close to, the peaks it is required to measure. Also, it must be similar in size to the measured peak or peaks and should not interact with it chemically. This method gives good results with respect to accuracy and precision, since the effects of errors of injection and differences in flow-rates from run to run are minimised.

4.2 Calibration by External Standards.

If the running conditions and injection techniques are sufficiently well stabilised and controlled it is possible to use external rather than internal standards. With this technique a series of samples of different known concentrations of the substance to be determined and injected and the peak parameter (area or height) is plotted against the

amount of sample in the injection. By this means a calibration graph is obtained, which is valid under those running conditions only, but which can be used to correlate a value of area or height with an amount of sample.

This is slightly simpler to use than internal standardisation, particularly when a large number of samples must be examined, but the chromatographic conditions have to be rigorously controlled in order to get reliable results.

4.3 Area Normalisation.

This technique, used much more in GLC than in HPLC, requires that all of the applied sample should be eluted as peaks. The areas of all the peaks are then summed, and the area of a component peak divided by the sum gives a measure of the proportion of the component in the applied sample.

The main reason why this is not more useful in HPLC is because many detectors have a response which varies considerably depending on the type of compound being eluted, unlike the F.I. Detector in GLC, which is dependent for size of response on the carbon content of the eluted compound. The U.V. detector, for example, shows very little response with aliphatic hydrocarbons but gives a large response to fused-ring aromatics. The summing of peak areas as they are obtained by an integrator is thus not valid as a means of summing concentrations. A weighting factor, for the detector's specific response to each component, would have to be established and used to convert each area to a common scale of values where such normalisation would be valid. This procedure is tedious and often, for unknown or poorly characterised compounds, is not possible.

5. Statistical Interpretation of Results.

While it is possible to use statistical methods of considerable complexity in investigation of errors, in inter- and intra-method variations, and in assignment of degrees of confidence to results obtained, it is only intended here to describe two simple functions, in addition to the mean or average, in common use. These are the standard deviation, σ , and the coefficient of variation, C.

5.1 Standard Deviation, σ .

In order to determine whether a series of results is significantly more precise, that is more closely reproducible, than another and different series, it is necessary to apply some operation to the sets of figures to bring them to a comparable state. A simple method is to take the difference between the highest and lowest values of the series, this being termed the range, and to compare the ranges of the two sets. It is, however, possible that one or two 'wild' readings might artificially expand the range and so distort the conclusions obtained by such a comparison. In order to allow for such 'wild' readings, and to allow for less drastic deviations from the mean, a method is used of weighting the values depending on their distance from the mean. This gives rise to the standard deviation, σ , which is mathematically defined as:

$$\sigma = \frac{\sqrt{\sum (X_i - \bar{X})^2}}{N - 1} \quad \text{for small values of } N$$

where

X_i = each measured value

\bar{X} = mean value

N = number of measurements (preferably 5 or more)

The normal Gaussian distribution curve of probability indicates that 68% of all results will lie within $\pm \sigma$ of the mean value and that

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95% of all results will lie within $\pm 2\sigma$ of the mean value.

Values of σ would normally be quoted with the mean, thus giving information about the precision of a particular determination; however, in comparison of peaks of different sizes it is still difficult to compare precision without a further function, the coefficient of variation, C.

5.2 Coefficient of Variation, C.

The coefficient of variation is a simple and logical way of converting values of σ for specific values of a mean to a general measure of precision which can be used regardless of peak size.

This conversion is simply carried out by dividing the value of σ by the value of the mean and then multiplying by 100 to bring it to a percentage:-

$$C = \frac{\sigma}{X} \times 100$$

The value for C gives a more rapid way of appreciating which of many series of figures gives the better precision.

X QUANTITATIVE CHROMATOGRAPHIC STUDIES.

1. Introduction.

1.1 General.

The accuracy and precision of quantitative data obtained using the systems described in Chapter VIII were investigated in order to find the most suitable conditions and procedures for routine analyses.

Results from the two methods, i.e. peak area and peak height measurements, were first compared with respect to precision. The precision of results is more important in most cases than accuracy because, with the use of appropriate standardisation, precise but inaccurate (i.e. biased) data can be corrected. If the technique is very imprecise, standardisation is in general a waste of time.

After determining the most suitable quantitation method, this was used to construct external standard calibration curves for individual components of Working Solution. These curves were then used in the analysis of a synthetic Working Solution of known composition, and

subsequently for the examination of samples of Working Solutions. The external standardisation method was chosen for routine work because of the relatively simple operations involved. Internal standardisation was also investigated in an attempt to reduce errors not affected by the external standardisation procedures.

Factors connected with improving precision will also be discussed.

1.2 Operating Conditions.

The quantitative studies were made mainly using the 2% IPE, 0.2% diglyme in n-heptane solvent with the Waters column. Some earlier data, obtained using 1% diglyme in n-heptane with the Varian SI-10 column, has been included. To avoid repetition of descriptions of specific systems, the systems and conditions shown in Table X.1 have been denoted as A and B. Any departure from these is described as necessary.

Table X.1.

Chromatographic Systems and Operating Conditions.

Parameter	System A	System B
Solvent	1% diglyme in n-heptane	0.2% diglyme, 2% IPE in n-heptane
Column	Varian SI-10	Waters μ -Porasil
Flow-rate	0.5ml/min	2.0ml/min
Sample size	0.1 μ l	0.2 μ l
Detector Wavelength	254nm	254nm
Recorder f.s.d.	200mV	100mV

The syringe used was a 1 microlitre Hamilton series 7000 model with a 12.5cm needle. The tip of the needle was slightly modified by careful grinding so that the tip was flattened; this was to

reduce sample size variations and losses by diffusion into the body of the injector device. A spacer was fitted, as described in Chapter VIII, to ensure the accurate positioning of the syringe needle.

2. Comparison of Precisions Obtained Using Peak Area and Peak Height Measurements.

2.1 Method.

The 'Kent Chromalog' Mark I integrator input was connected, through a voltage divider (a ten-turn, ten kilohm, calibrated potentiometer), to the recorder 254nm-channel input. The integrator had a fixed range of from 0 to 10mV f.s.d., which necessitated the use of the voltage divider when using the 100mV and 200mV ranges on the recorder.

The integrator and voltage divider were adjusted so that a full scale deflection on the recorder gave a similar full scale deflection on the integrator meter. A check of the accuracy of the matching of these was made by adjusting the recorder pen to various deflections and noting the corresponding integrator meter deflection.

Having adjusted the range, the integrator zero and triggering controls were set to print out readings at appropriate points in the chromatograms. It was found that the baseline of the integrator was unstable, and that it was necessary to reset the integrator zero before each injection.

When the chromatograph and integrator were appropriately adjusted injections were made by a set procedure. This required the shutting of the injector ball-valve and switching off the pump, followed by injection of the sample, release of the injector ball-valve, and re-starting the pump. This series of operations was completed, typically, in 15 to 20 seconds.

The chromatograms were then measured and, together with the integrator data, were processed as described in Chapter IX to obtain

the mean, standard deviation and coefficient of variation for each parameter of each peak.

2.2 Results Using System A.

A series of ten injections of a 1% H_4 EAQ solution using system A, with 1.5% diglyme in n-heptane solvent, gave a coefficient of variation of 14.0% for peak area measurement and 2.9% for peak height measurement. The discrepancy between these values may have been partly due to slight variations in integrator baseline adjustment, but cannot be wholly ascribed to this effect.

Later work with the standard system A, using six injections of a 10% dilution of Working Solution sample, gave the results shown in Table X.2.

Table X.2.

Injections of a 10% Dilution of Working Solution.

<u>Peak</u>	<u>Peak area</u>			<u>Peak height</u>		
	mean area (counts)	σ	C(%)	mean height (mm)	σ	C(%)
Solvent 1	41.3	1.97	4.8	22.9	1.16	5.1
Solvent 2	30.2	4.36	14.4	16.7	0.75	4.5
H_8 EAQ	14.5	4.85	33.4	7.3	0.27	3.7
H_4 EAQ	497.3	15.24	3.1	149.8	4.24	2.8
H_4 EAQ Epoxide	85.0	8.37	9.8	22.8	1.03	4.5
Unknown	13.0	3.41	26.2	8.3	1.08	13.0
EAQ	614.0	33.11	5.4	158.6	4.74	3.0

These results show that for large peaks, e.g. H_4 EAQ and EAQ, the two methods give similar values for coefficients of variation, but that for small and medium peaks the peak height measurement has typically twice the precision of the integration.

2.3 Results Using System B.

In obtaining values for the work on external standardisation, described in section X.3, a series of solutions of different concentrations of two pure components were injected using system B. Coefficients of variation for two of these series of solutions are shown in Table X.3.

Table X.3.

Coefficients of Variation for H_8 EAQ and H_4 EAQ at Several Concentrations.

Concentration (% w/v)	C for H_8 EAQ (%)		C for H_4 EAQ (%)	
	Area	Height	Area	Height
1.0	3.5	2.2	6.2	3.8
0.8	3.8	1.5	4.7	1.8
0.6	4.1	1.4	9.5	3.7
0.4	5.7	2.6	6.2	1.9
0.2	7.5	1.4	11.7	3.9
0.1	25.3	8.6	10.0	2.2

The peak height measurements are again seen to be more precise than area measurement, particularly so for smaller peaks. For the majority of the solutions shown in Table X.3 a coefficient of variation of about 2 to 3 might be expected from peak height measurements. The value for peak area measurement would also be much more dependent on peak size than that for height, since small peaks require a very precise control of integrator baseline to obtain precise results.

In order to determine whether the precision obtainable by the two methods was changed significantly in measurements of components resolved from a mixture it was necessary to inject aliquots of such mixtures. This was carried out as part of other studies described in section X.3. A synthetic Working Solution, containing 0.4% EAQ, 1%

H_8 EAQ, 0.5% H_8 EAQ and 0.5% H_4 EAQ Epoxide, was made and six aliquots were injected. The coefficients of variation derived from these data are shown in Table X.4.

Table X.4.

Coefficients of Variation for a Synthetic Working Solution.

Component	C for Peak area (%)	C for Peak height (%)
H_8 EAQ	2.7	1.4
H_4 EAQ	3.1	1.3
H_4 EAQ Epoxide	11.8	1.4
EAQ	5.9	1.9

In view of the consistently superior precision of the peak height measurement method over the use of the Kent Chromalog integrator peak area measurements it was decided to use solely peak height measurements in further quantitation studies.

3. External Standardisation by Peak Height.

3.1 Construction of Calibration Graphs for the Main Components.

Solutions were made up of H_8 EAQ, H_4 EAQ, EAQ and H_4 EAQ Epoxide in Sextate solvent. The solutions contained 1%, 0.8%, 0.6%, 0.4%, 0.2% and 0.1% w/v of the solute respectively, except for EAQ solutions which contained 0.5%, 0.4%, 0.3%, 0.2%, 0.1% and 0.05% w/v.

Six injections of each dilution of each component were made using the standard system B. The peak heights were measured and standard deviations were calculated for each set of injections. The four sets of data obtained are given in Tables X.5 to X.8 inclusive. The data are also shown graphically in figures X.1 to X.4. Each datum point is plotted together with a line representing $\pm 1\sigma$.

Table X.5.

Peak Heights, Standard Deviations and Coefficients of Variation

for H₈EAQ.

Conc ⁿ ·H ₈ EAQ (%)	1.0	0.8	0.6	0.4	0.2	0.1
Mean peak height (mm)	118.6	97.3	76.8	51.7	27.6	14.0
σ	2.56	1.44	1.04	1.33	0.38	1.21
C (%)	2.2	1.5	1.4	2.6	1.4	8.6

Table X.6.

Peak Heights, Standard Deviations and Coefficients of Variation

for H₄EAQ.

Conc ⁿ ·H ₄ EAQ (%)	1.0	0.8	0.6	0.4	0.2	0.1
Mean peak height (mm)	146.8	123.3	97.7	69.8	37.5	18.8
σ	5.50	2.25	3.60	1.29	1.45	0.42
C (%)	3.8	1.8	3.7	1.9	3.9	2.2

Table X.7.

Peak Heights, Standard Deviations and Coefficients of Variation

for H₄EAQ Epoxide.

Conc ⁿ ·H ₄ EAQ Epoxide (%)	1.0	0.8	0.6	0.4	0.2	0.1
Mean peak height (mm)	57.3	45.7	36.3	23.5	12.0	6.1
σ	2.88	2.58	1.92	1.41	0.63	0.43
C (%)	5.0	5.7	5.3	6.0	5.3	7.1

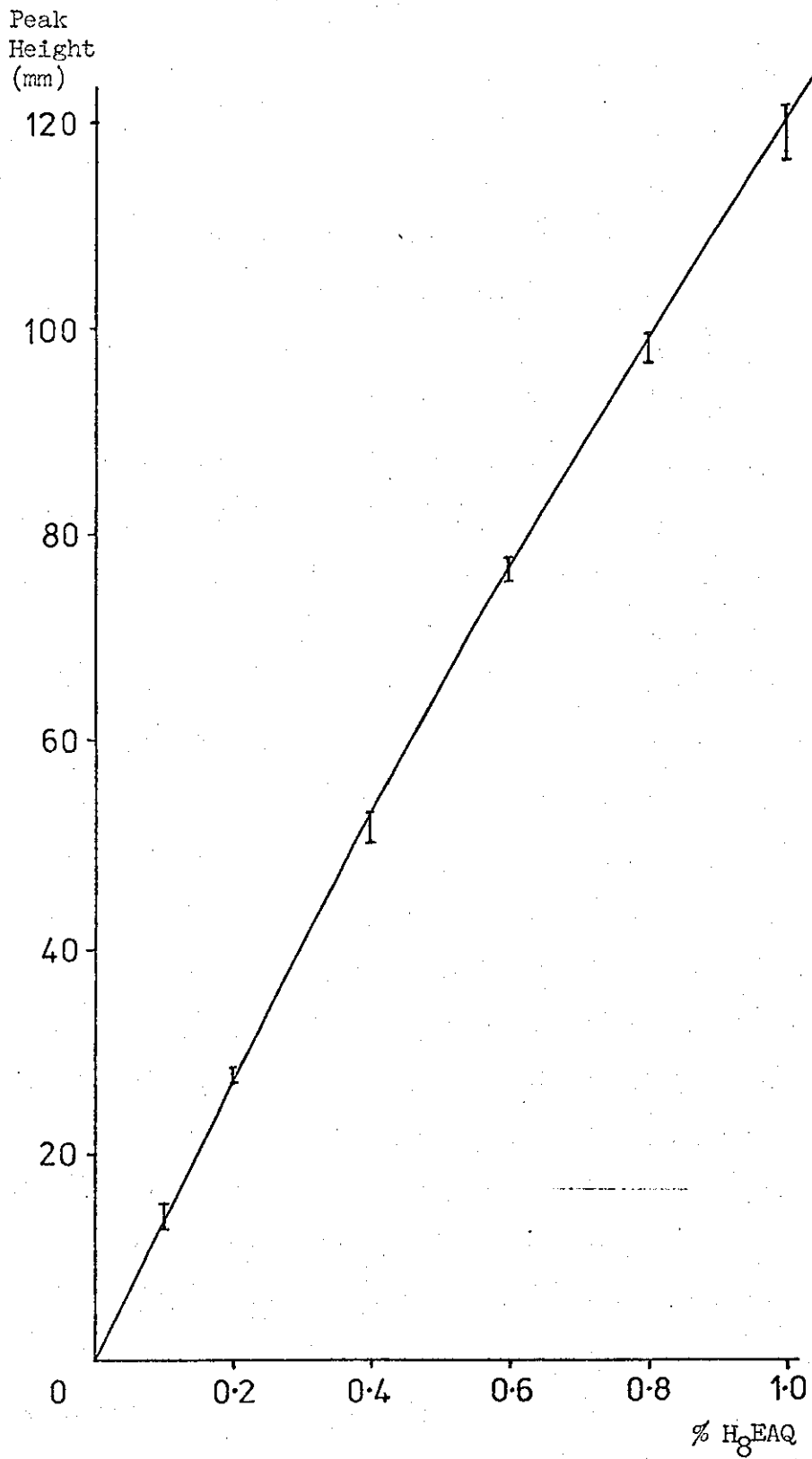


Figure X.1.

Variation of Peak Height with Concentration for H₈EAQ

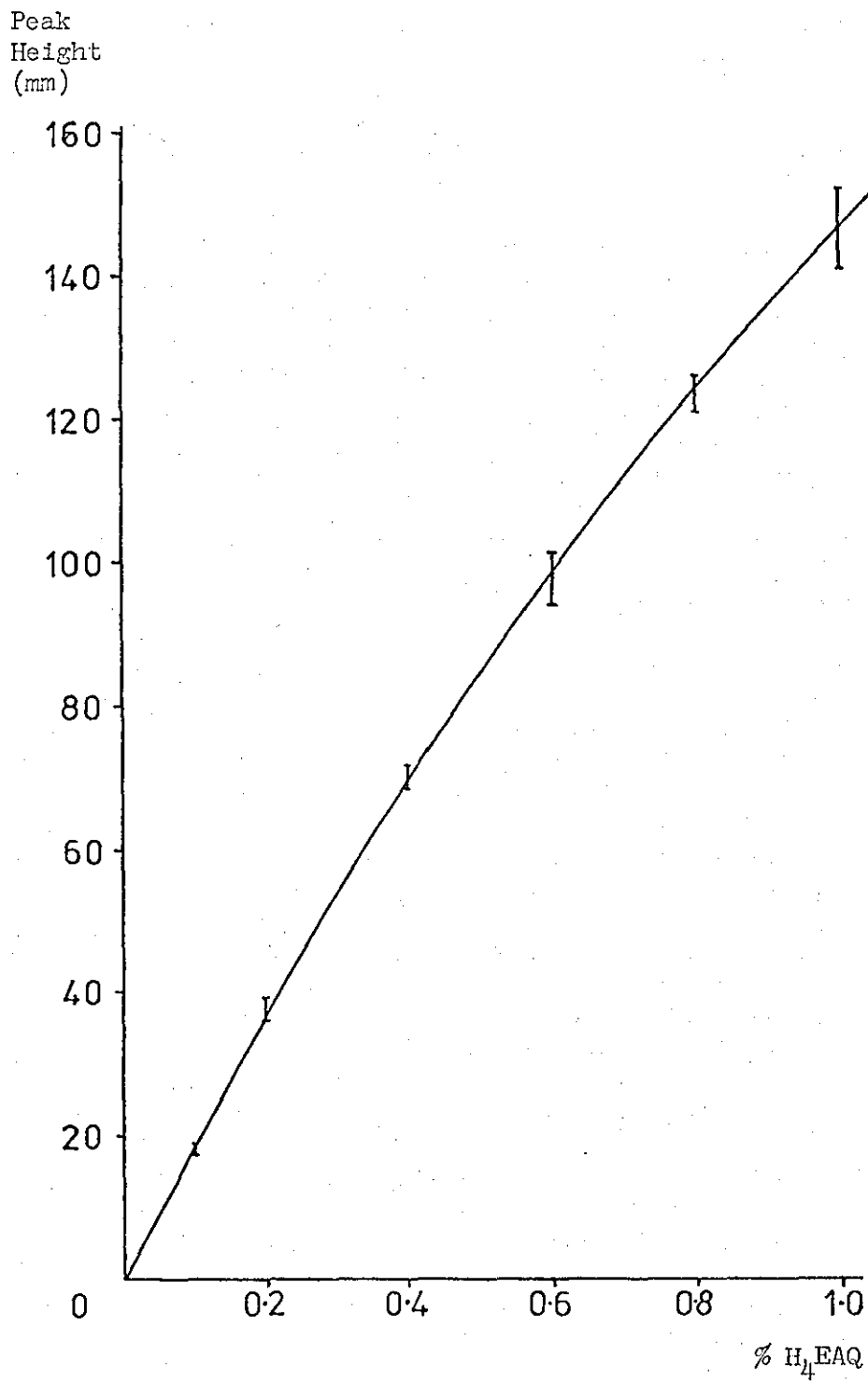


Figure X.2.

Variation of Peak Height with Concentration of H₄EAQ.

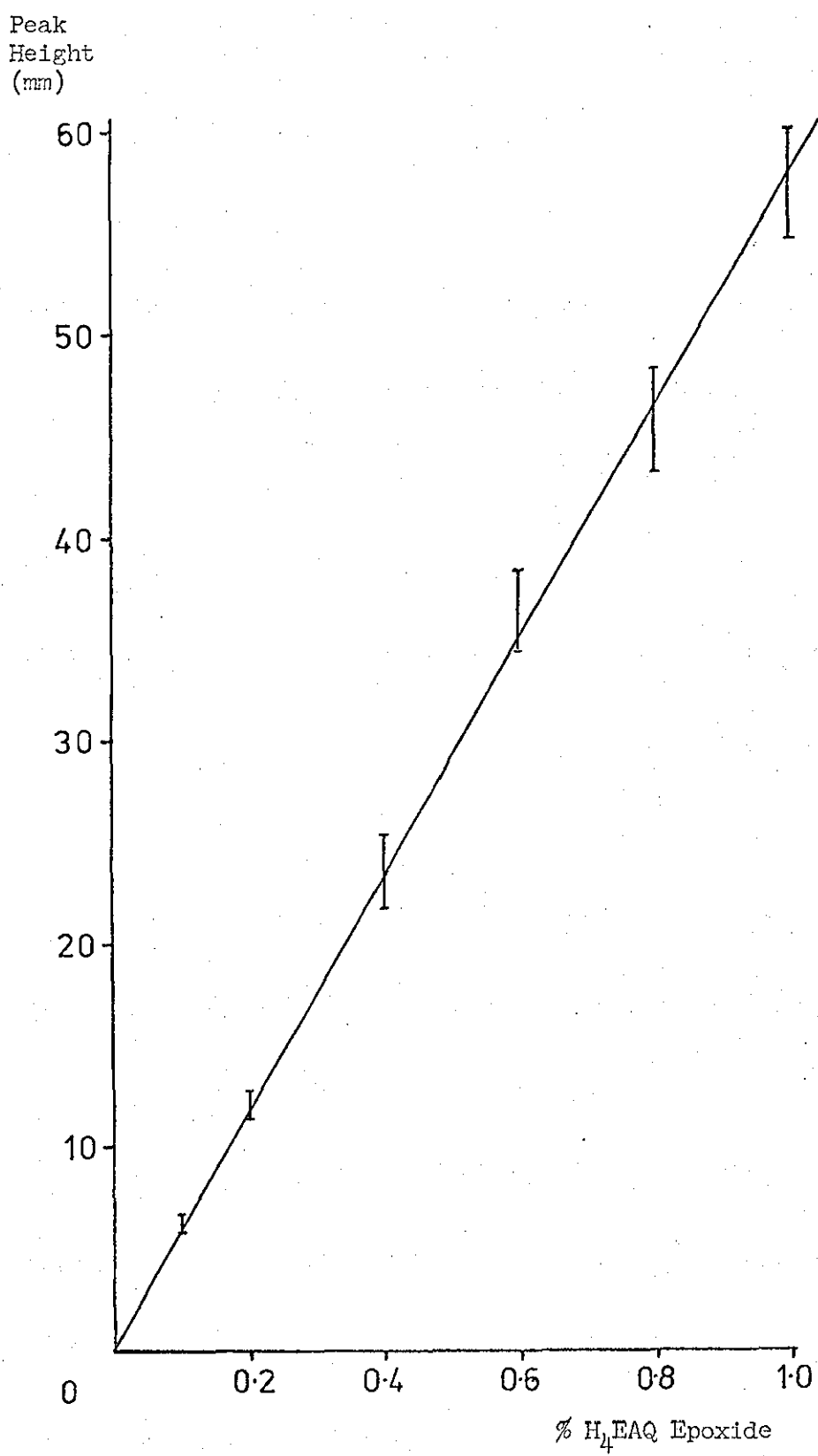


Figure X.3.

Variation of Peak Height with Concentration of H_4EAQ Epoxide.

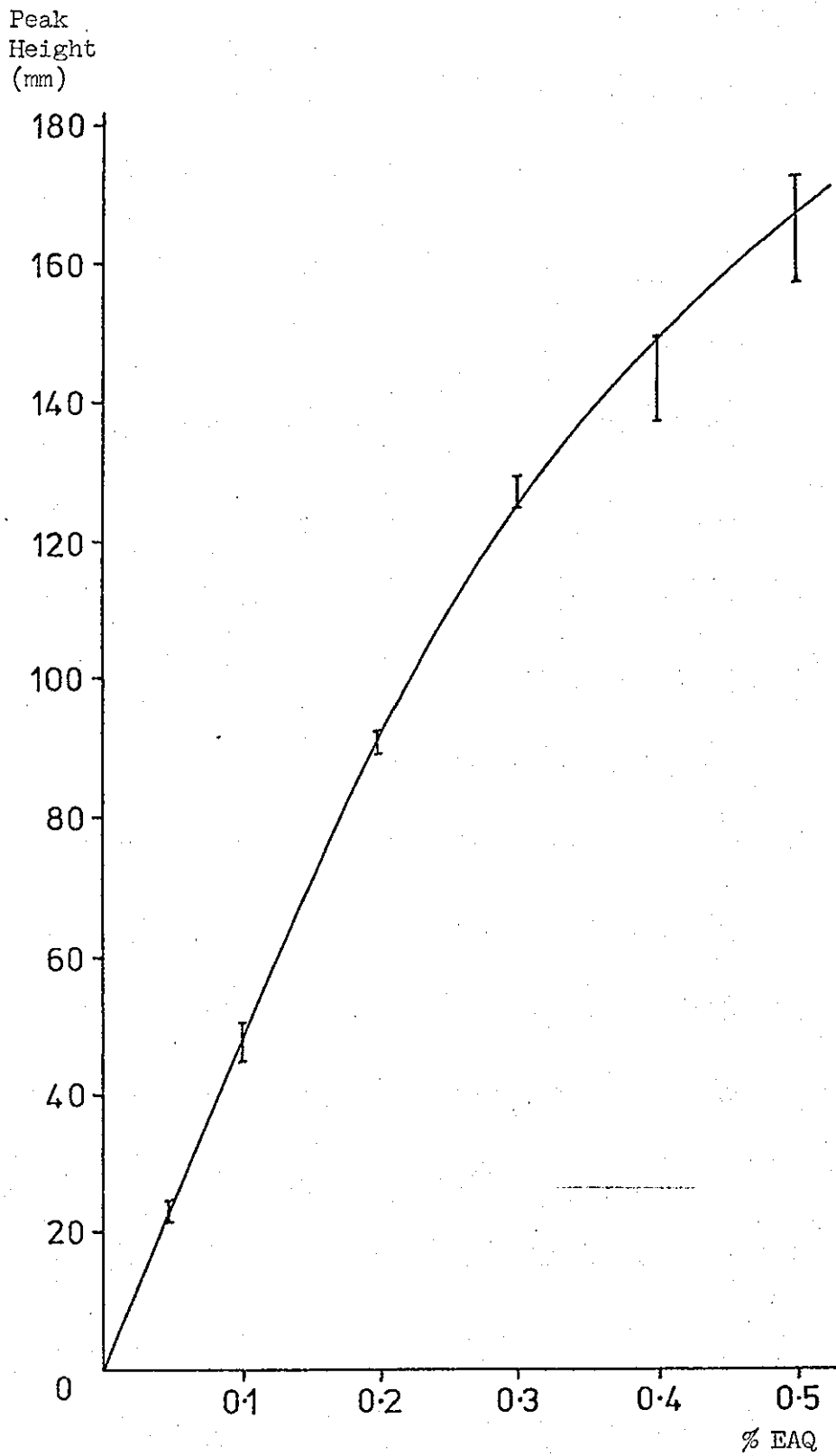


Figure X.4.

Variation of Peak Height with Concentration of EAQ.

Table X.8.

Peak Heights, Standard Deviations and Coefficients of Variation
for EAQ.

Conc ⁿ . EAQ (%)	0.5	0.4	0.3	0.2	0.1	0.05
Mean peak height (mm)	164.9	143.4	127.1	90.4	46.9	22.5
σ	7.41	5.98	1.96	1.24	2.89	1.58
C (%)	4.5	4.2	1.5	1.37	6.2	7.0

The calibration curves obtained (figs. X.1 to X.4) may be seen to be approximately linear below about 120mm height, and curve more above this peak height. This curvature might be expected, as the detector calibration (Chapter III) showed that the detector response became more non-linear as absorbance, equivalent to peak height, increased. Apart from this curvature the calibration was acceptable.

3.2 Construction of Calibration Graphs for H₇EAQ Epoxide and its Degradation Products.

For this group of components a 3% iso-propanol in n-heptane solvent was used to elute the Waters column at 1.0ml/minute, as described in Chapter VIII, section 2.4. A representative chromatogram of this separation is shown in fig. VIII.7.

This group of compounds is usually present at low concentrations in Working Solution. In addition the individual components have considerably lower U.V. absorbances than, for example, EAQ, so that the peaks one might expect to find in Working Solution would typically be small at the dilutions usually used for the quinone components' determination. In order to measure appropriately low concentrations of these compounds two methods were used to increase the peak sizes of the eluted components. Firstly, the recorder f.s.d. was changed from 100mV to 50mV, which doubled the peak height but also increased the baseline noise. Secondly, for the two lowest concentrations, injections

of 0.2 μ l and of 1 μ l were made. The peak-heights of the 1 μ l injections, when adjusted to the equivalent of a 0.2 μ l injection, were very similar to those obtained with the 0.2 μ l injections. It was possible to obtain measurable peaks down to a concentration of 0.02% for H₄EAQ Epoxide and each of its degradation products. The expected loss of efficiency resulting from using a larger aliquot (see Chapter VIII) made no significant differences to the resolutions obtained. The data derived from these chromatograms are given in Table X.9 to X.12 inclusive and are shown graphically in figures X.5 to X.8. The data do not include accurate standard deviation and coefficient of variation values because only three injections of each solution were made. The calculation of standard deviation from three points is not statistically valid. The range of values obtained can, however, be used to give an estimate of standard deviation. In this case, with three readings, the standard deviation was obtained by multiplying the range by 0.591¹². The estimated standard deviation values are included in the Tables, but are not shown graphically. The values shown in Tables X.9 to X.12 have been adjusted to be equivalent to a 0.2 μ l injection with the recorder at 100mV full-scale deflection.

Table X.9.

Values of Peak Heights and Standard Deviation for H₄EAQ Epoxide.

Conc ⁿ . H ₄ EAQ Epoxide (%)	1.0	0.75	0.5	0.25	0.1	0.02
Mean peak height (mm)	119.0	99.2	74.2	38.1	16.0	4.25
Estimated σ	5.9	4.7	1.8	0.4	1.2	---

Table X.10.Values of Peak Heights and Standard Deviation for'Monohydroxy' H₁₁EAQ Epoxide.

Conc ⁿ . 'Monohydroxy' (%)	1.0	0.75	0.5	0.25	0.1	0.02
Mean peak height (mm)	96.8	78.3	57.0	28.4	12.0	2.45
Estimated σ	5.0	3.8	1.5	0.4	1.4	---

Table X.11.Values of Peak Heights and Standard Deviation for'Hydrolysed H₁₁EAQ Epoxide'.

Conc ⁿ . 'Hydrolysed' (%)	2.0	1.5	1.0	0.5	0.2	0.04
Mean peak height (mm)	97.5	78.3	56.2	27.2	11.4	2.2
Estimated σ	5.3	3.5	0.3	0.6	2.0	---

Table X.12.Values of Peak Heights and Standard Deviation for'Dihydroxy' H₁₁EAQ Epoxide.

Conc ⁿ . 'Dihydroxy' (%)	2.0	1.5	1.0	0.5	0.2	0.04
Mean peak height (1) (mm)	37.8	29.0	21.0	10.5	3.8	0.8
Estimated σ (1)	2.0	2.0	0.6	0.6	1.8	---
Mean peak height (2) (mm)	53.5	40.3	30.8	15.5	5.9	1.2
Estimated σ (2)	2.4	2.0	1.2	0.7	0.9	---

The results indicate a similar range of standard deviation values to those found in section 3.1. The calibration curves shown in figures X.5 to X.8 are satisfactory for routine analytical use, but that of the 'Dihydroxy' compound poses a particular problem. The

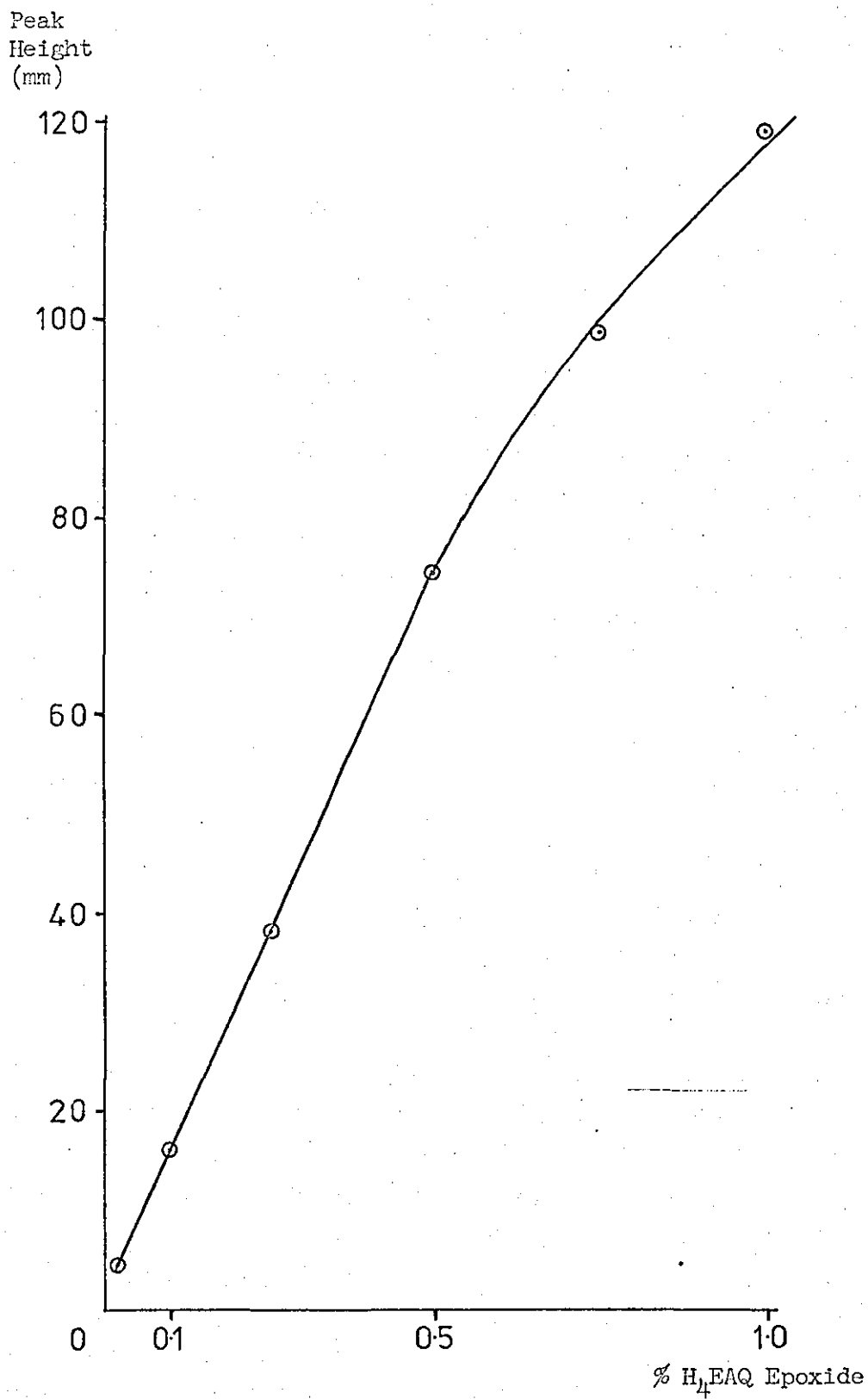


Figure X.5.

Variation of Peak Height with Concentration of H₄EAQ Epoxide.

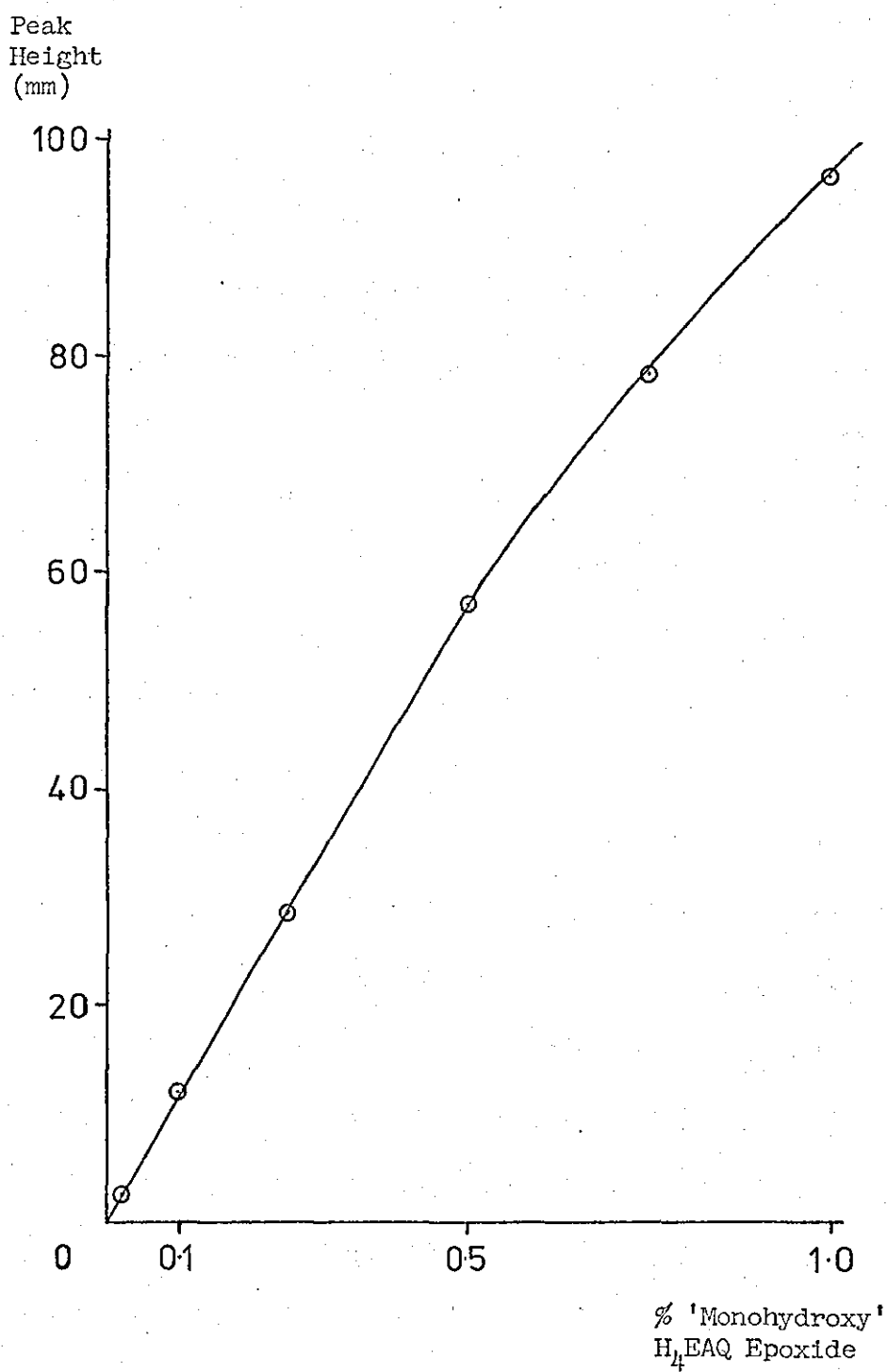


Figure X.6.

Variation of Peak Height with Concentration of
'Monohydroxy' H_4 EAQ Epoxide.

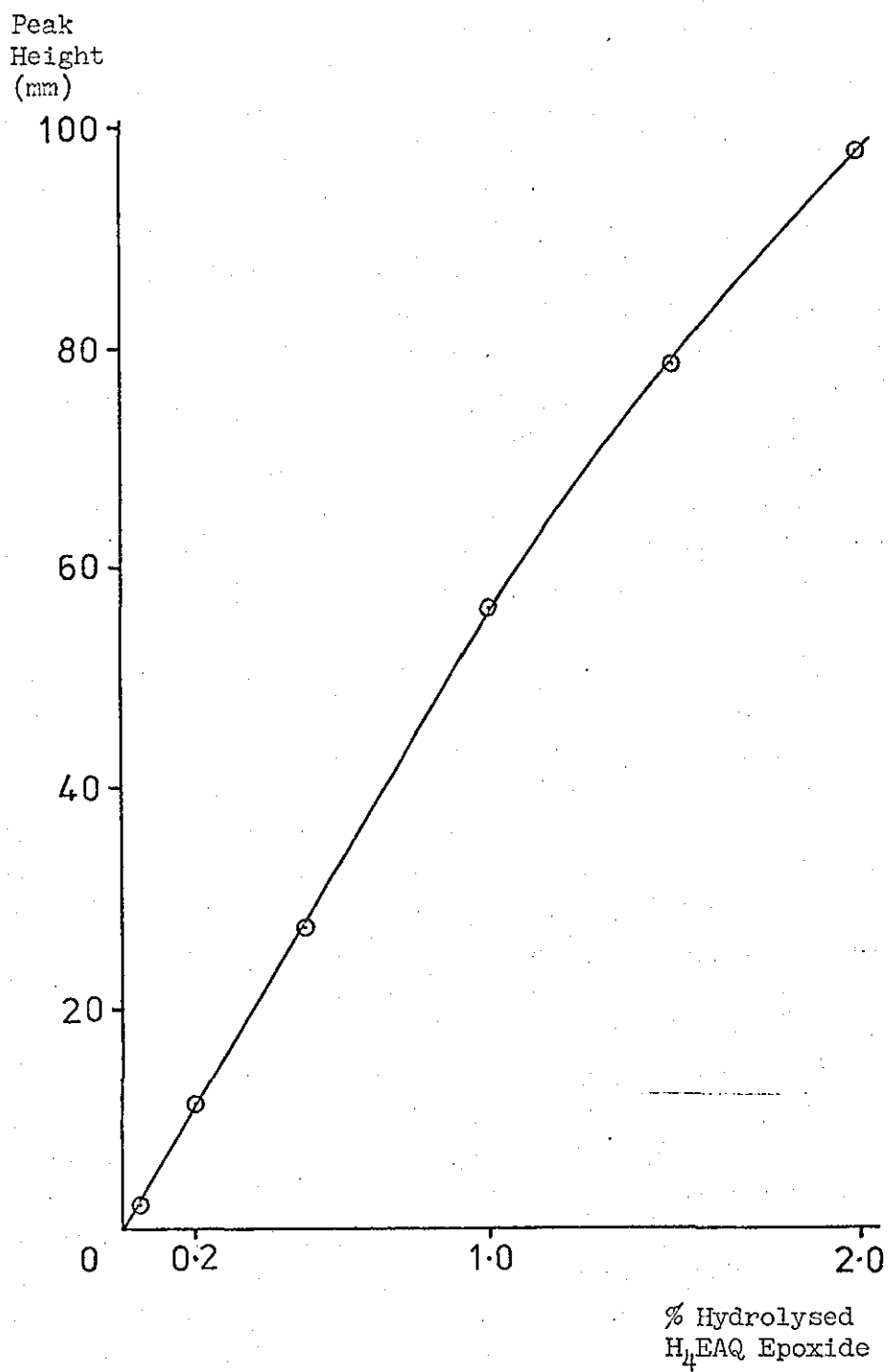


Figure X.7. Variation of Peak Height with Concentration of
'Hydrolysed H_4 EAQ Epoxide.'

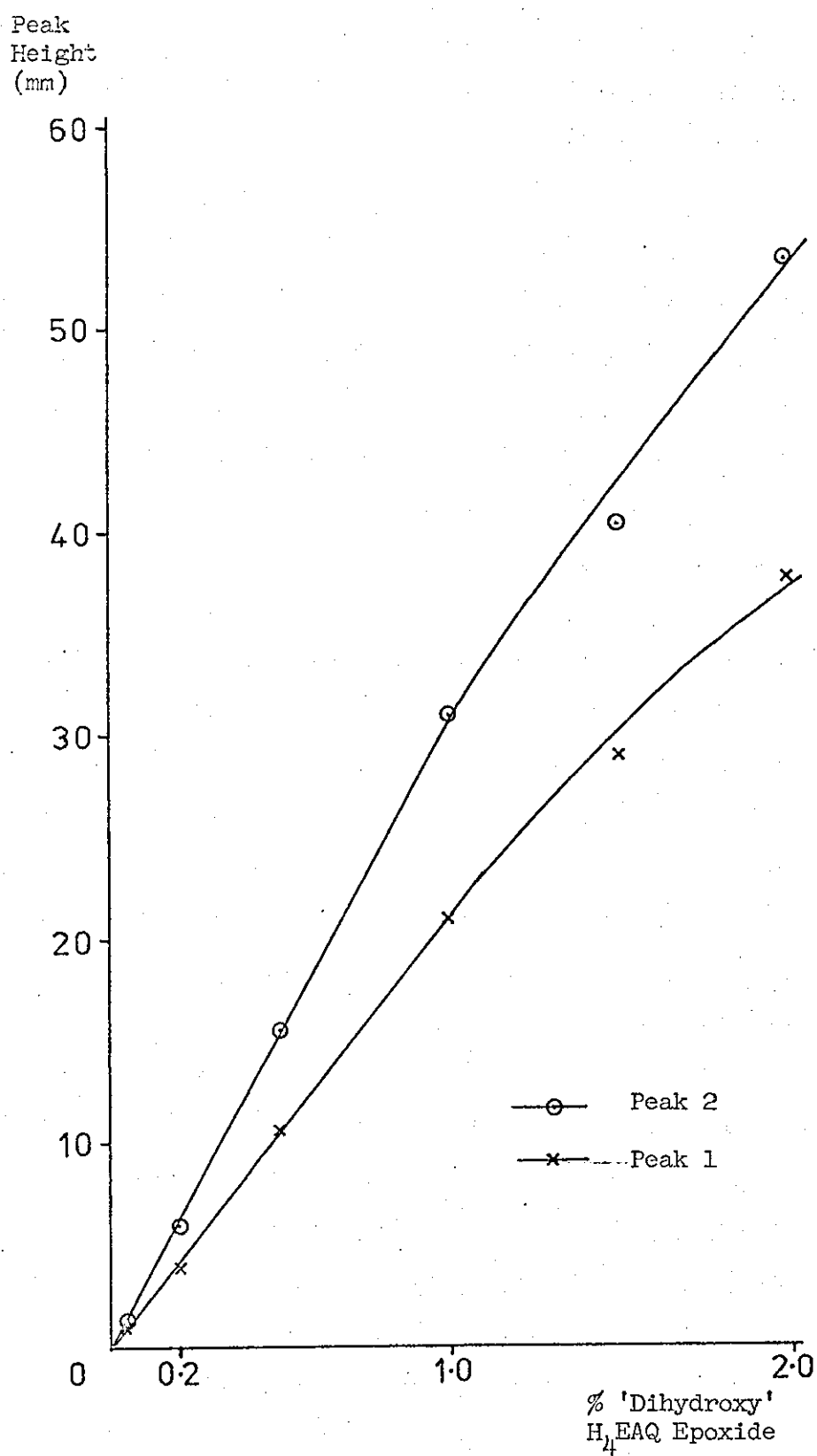


Figure X.8. Variation of Peak Height of the Two Peaks of 'Dihydroxy'

H_4 EAQ Epoxide with Overall Concentration.

solution to this would be to isolate pure samples from each peak, so that accurate calibration could be carried out for each component, but this was not possible in these studies.

3.3 Analysis of a Synthetic Working Solution.

In order to test whether the calibration graphs obtained in Section 3.1 were applicable to the analysis of components of a mixture, a synthetic Working Solution was examined.

Two solutions, representing appropriate dilutions of a synthetic Working Solution, were made up, one containing EAQ, 0.4%; H_4 EAQ, 1%; H_8 EAQ, 0.5% and H_4 EAQ Epoxide, 0.5%, dissolved and made up in Sextate as sole solvent, and the other solution being a 1:1 dilution of the first solution with Sextate. By using two dilutions it was hoped that the peak heights for individual components would lie in the middle of the height range for one or other of the solutions, thus allowing greater overall precision. The values obtained for composition for one solution could also be used as an internal check of the data from the other.

Only a small amount of H_8 EAQ was available at the time, and this limited the maximum volume of solution which could be made up. Weighings were made on a Stanton 'Unimatic' single-pan balance, reading to four places (i.e. to 0.1mg). This seemed satisfactory at the time, since the components to be weighed were of the order of 10mg, to be made up to 2.0ml in Sextate, and an error of \pm 0.1mg on this represents only 1%. Subsequent results indicated that these weighings were less accurate than had been expected (see later, this section) and it would have been better, in retrospect, to have used a five-figure balance for these weighings.

The 1:1 dilution was made using a Hamilton 100 microlitre syringe to transfer 500 μ l each of the above synthetic Working Solution and of Sextate to a 2ml stoppered vial, where they were thoroughly

mixed. It was expected that the accuracy of the dilution would be determinable by comparison of the results from the diluted and the undiluted solutions, since the ratios of each of the four components in the two solutions should give the same numerical value, thus also acting as an internal check on the validity of individual results.

Replicates of each solution were injected six times. The peak heights were measured and standard deviation and coefficient of variation were evaluated. The mean peak height was then used in conjunction with the appropriate calibration curve given in section 3.1 to estimate the amount of the component in the sample solution. The range of percentage composition corresponding to \pm one standard deviation at the estimated composition was also obtained.

Values obtained for peak heights, standard deviation and coefficient of variation values, are shown in Table X.13 for both solutions. The derived values of percentage composition are shown in Table X.14, together with the ratio of these values. The ratio of the two sets of results gave the dilution ratio actually achieved, as shown.

The data in Tables X.13 and X.14 show that results of acceptable accuracy and precision were obtained for the four components, when the previously mentioned weighing errors are taken into account. The ratio of the two sets of results shows that a dilution of about 1.07 (mean) to 1 was achieved. The concentrations shown in the analysis results were of the same order, within expected levels of error, as those aimed for in making the solutions. It is considered that they represent the actual concentrations in solution. Further work, using more accurately prepared solutions, would be advisable but sufficient of all the necessary components was not available at the time.

Table X.13.

Analysis of Synthetic Working Solution Using System B.

Component	H ₈ EAQ	H ₄ EAQ	H ₄ EAQ Epoxide	EAQ
Working Solution:				
Mean peak height (mm)	70.4	151.2	30.4	140.4
σ	0.96	1.92	0.42	2.68
C(%)	1.4	1.3	1.4	1.9
1:1 dilution:				
Mean peak height (mm)	37.8	95.4	16.5	85.1
σ	1.29	1.74	0.45	1.88
C (%)	3.4	1.8	2.9	2.2

Table X.14.

Quantitation of Synthetic Working Solution Analyses.

Component (Nominal %)	H ₈ EAQ (0.50%)	H ₄ EAQ (1.00%)	H ₄ EAQ Epoxide (0.50%)	EAQ (0.40%)
Working Solution:				
Concentration (%w/v)	0.57	1.040	0.535	0.365
$\pm 1\sigma$ (%w/v)	0.008	0.030	0.010	0.012
1:1 Dilution:				
Concentration (%w/v)	0.302	0.59	0.285	0.190
$\pm 1\sigma$ (%w/v)	0.011	0.013	0.008	0.005
Calculated Ratio of Dilution	1.06	1.12	1.07	1.04

3.4 Analysis of Plant Working Solutions.

Three samples of Working Solutions from different sources were provided for analysis. For two of these, (solutions X and Y) analysis results were available derived from the polarographic procedures routinely employed by Laporte. The three samples are shown herein as Working Solutions X, Y and Z.

3.4.1 Analyses for Main Components.

The procedure adopted was to prepare an accurately diluted solution of Working Solution and inject $0.2\mu\text{l}$ aliquots, using chromatographic system B. The chromatogram peaks were measured and percentage compositions of the four main components were found as before. The peak measurements are shown in Table X.15; percentage compositions of the solutions are shown in Table X.16.

Table X.15.

Peak Height Measurements for Working Solutions X, Y and Z.

Solution	Dilution	H ₈ EAQ (mm)	H ₄ EAQ (mm)	H ₄ EAQ Epoxide (mm)	EAQ (mm)
X	5%	1.5	70.0	4.5	77.0
Y	10%	2.5	122.0	7.0	127.0
Z(i)	5%	10.0	49.0	0.5	82.0
Z(ii)	10%	18.5	94.0	1.0	142.0

Table X.16.

Percentage Composition of Working Solutions X, Y and Z.

Solution	H ₈ EAQ (%w/v)	H ₄ EAQ (%w/v)	H ₄ EAQ Epoxide (%w/v)	EAQ (%w/v)
X ^(a)	0.24 (0.40)	8.10 (7.19)	1.6 (2.0)	3.44 (3.30)
Y ^(a)	0.20 (0.20)	7.9 (6.85)	1.2 (0.95)	3.03 (3.53)
Z(i)	1.50	5.60	trace	3.66
Z(ii)	1.45	5.80	0.20	3.62

(a): Values in parentheses were supplied by Laporte, from analyses using polarographic techniques.

The data shown in Table X.16 indicate that the two methods do give similar results. The precision of the polarographic method was not available, and thus a statistical test to compare the results of the two methods could not be used. The results obtained are, however, within acceptable limits of agreement, except for H₄EAQ.

The concentrations obtained for H₄EAQ by HPLC differed by 14%, in both solutions, from those obtained by polarography. If the polarographic result is taken as being accurate, there are two possible explanations of the higher values obtained by HPLC; either the composition of the solutions changed between the polarographic and chromatographic analyses or there was an interfering compound in both Working Solutions which increased the size of their H₄EAQ peaks by the same proportional amounts in their chromatograms.

The first of these alternatives would require the presence in both Working Solutions of a sizeable amount of a compound other than those examined herein, and this would have to elute in the same position as H₄EAQ. The presence or absence of such a compound could be ascertained only by using a different chromatography system.

The second alternative, of a change in the composition of the solutions, is much more probable, however, since a period of almost

a year had elapsed between the polarographic and the chromatographic analyses of the samples. Polarographic analyses performed by Laporte have shown that the composition of Working Solution changes with time after removal from the circulating plant solution. It is suggested that the high H_4 EAQ results are due to such changes, and further, that the increase in H_4 EAQ concentration is largely due to the conversion of residual H_4 EAQuinol, present in a quinhydrone complex, to H_4 EAQ by aerial oxidation. The quinhydrone type of complex is discussed further in Chapter XI in connection with studies on H_8 EAQ, but may be invoked here in the similar case of H_4 EAQ, since both H_8 EAQ and H_4 EAQ should be capable of forming the quinhydrone.

The polarographic and chromatographic methods would have to be applied simultaneously on the same bulk sample of plant solution if it were required to compare the results obtained. Comparison of results of analyses under these conditions would be more meaningful than the comparisons considered here.

3.4.2 Analyses for Degradation Products of H_4 EAQ Epoxide.

The system described in section 3.2 was used to elute diluted samples of Working Solutions. It was found that peaks eluting at positions corresponding to those of the H_4 EAQ Epoxide degradation products were too small to be quantitated from these diluted solutions. Injections of $0.5\mu l$ aliquots of the undiluted Working Solutions gave larger peaks, but the complexity of the chromatograms was such that quantitation was not feasible. Further work on these later-eluting compounds and on the chromatographic system would be required to obtain a reliable qualitative and quantitative method capable of discriminating the unresolved peaks presently obtained. The chromatograms obtained for Working Solutions X, Y and Z are shown in figures X.9, X.10 and X.11, respectively. The running conditions were a flow-rate of 1ml/minute of 3% iso-propanol in n-heptane. Only the 254nm channel record is shown, with a non-linear

absorbance range for the first group of main components. The range was changed at two points in the chromatogram to obtain higher sensitivity for the later peaks; these changes are marked by a break in the trace and an indication of the amount by which the sensitivity was increased. The peaks corresponding to the compounds of interest, as previously identified in Chapter VIII, are arrowed and are numbered as below.

<u>Peak Number</u>	<u>Compound</u>
<u>1</u>	'Monohydroxy' H_4 EAQ Epoxide
<u>2</u>	'Hydrolysed H_4 EAQ Epoxide'
<u>3</u>	'Dihydroxy' H_4 EAQ Epoxide, peak 1.
<u>4</u>	'Dihydroxy' H_4 EAQ Epoxide, peak 2.

The chromatograms may be seen to be dissimilar in many respects. The peaks 1, 3 and 4, which arise by reduction of H_4 EAQ Epoxide, are large in solution X and small in both Y and Z, whereas peak 2, arising from hydrolysis of the epoxide, is more prominent in sample Y and small in X and Z. These observations, together with the values for H_4 EAQ Epoxide content obtained in section 3.4.1, indicate that there are three, alternative, processes occurring in the different solutions. In solution X the reduction of a relatively high level of H_4 EAQ Epoxide resulted in the formation of significant amounts of the hydroxylated compounds of peaks 1, 3 and 4, whereas a similar level of the epoxide in solution Y gave rise mainly to the hydrolysed epoxide, peak 2. In solution Z the low initial epoxide level resulted in very little formation of any of the breakdown products. Further, it is suggested that the large peak eluting after peak 1 in figure X.10 is due to further compounds derived from or involved in the hydrolysis of H_4 EAQ Epoxide, or in similar hydrolysis reactions not occurring in the other two solutions.

3.5 Possible Improvement to the Injection and Other Parts of the Procedure.

The basic operations already discussed, such as weighings and

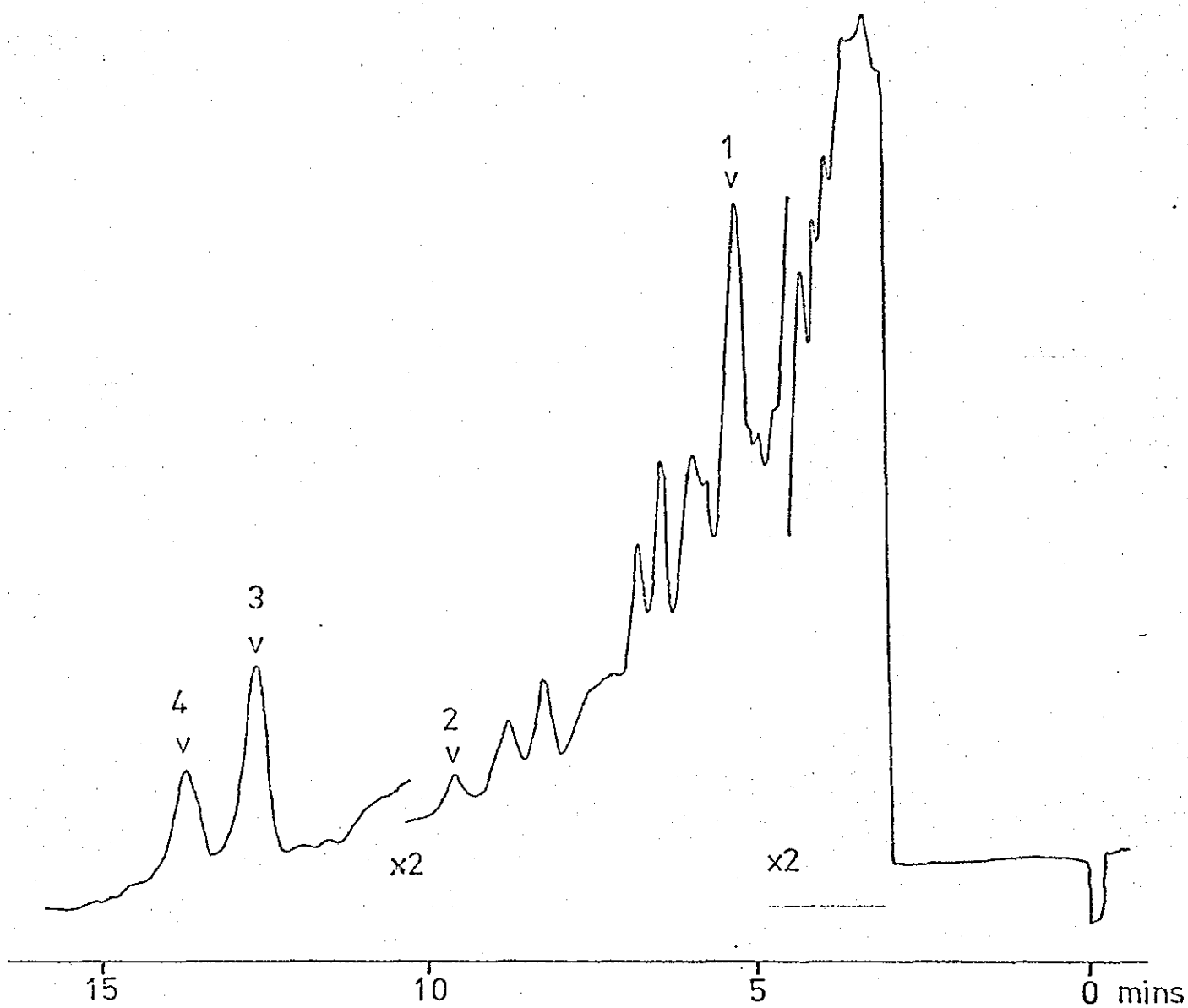


Fig. X.9. Chromatogram of Working Solution X.

Conditions as in text.

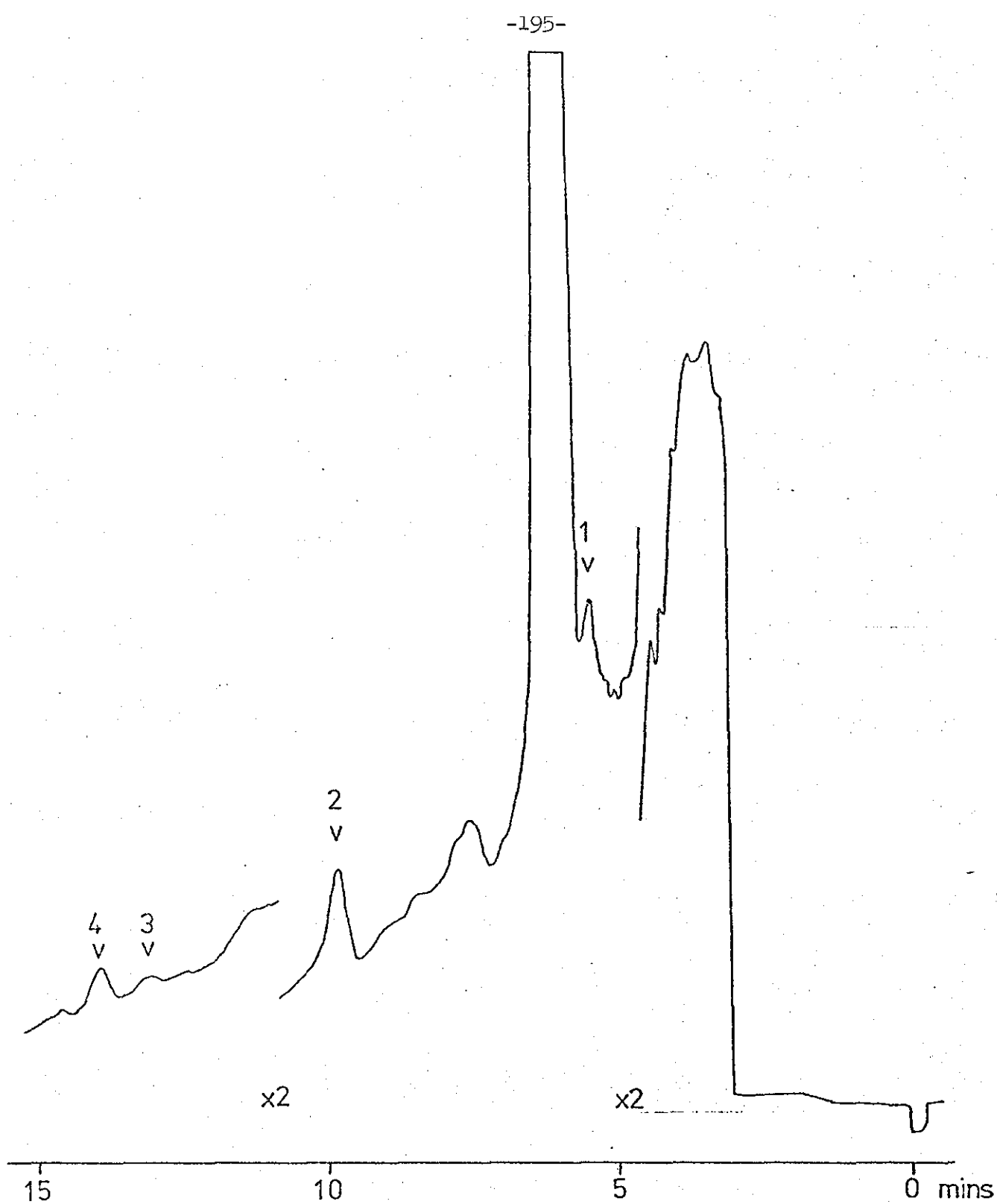


Fig. X.10 Chromatogram of Working Solution Y.

Conditions as in text.

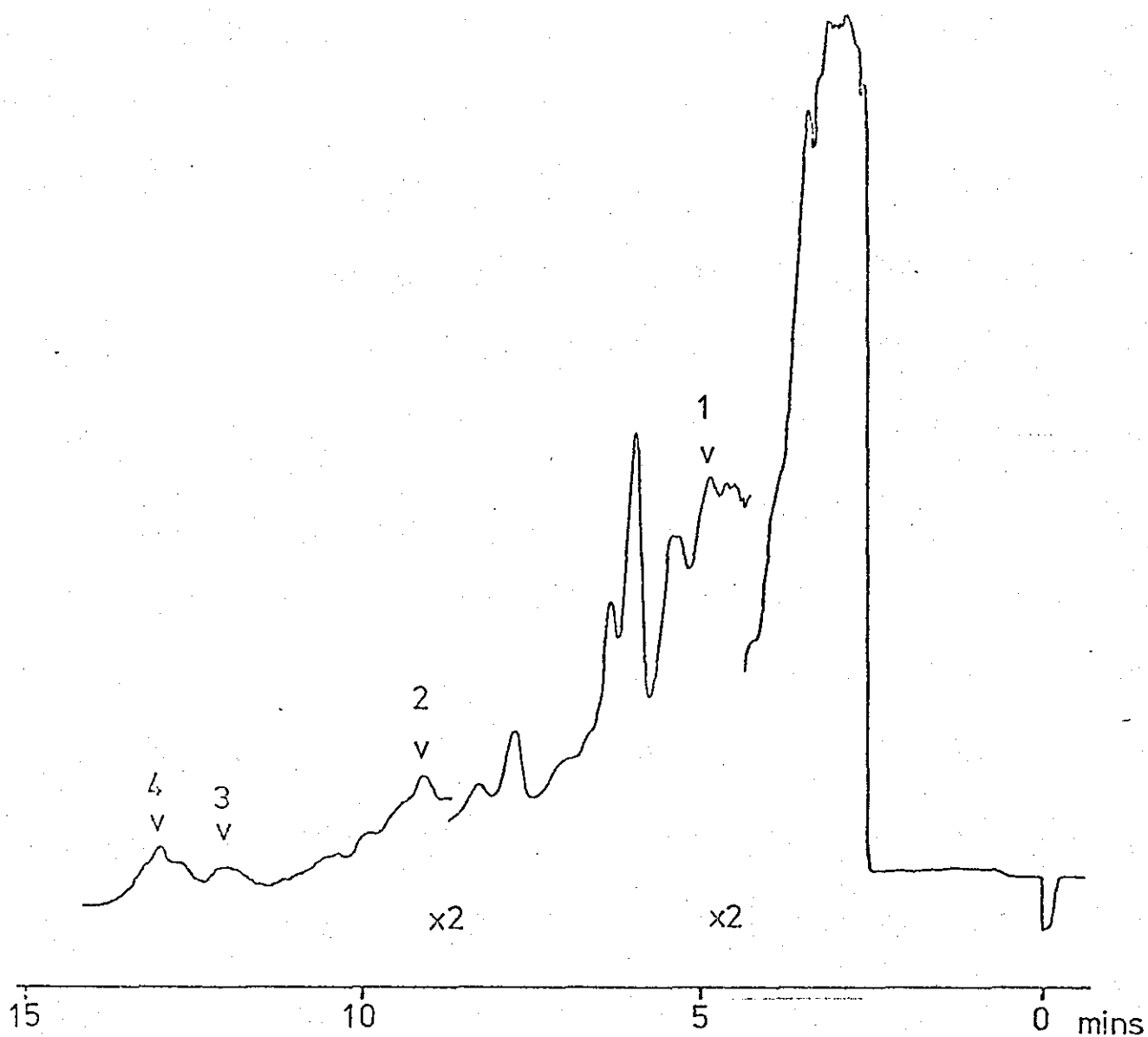


Fig. X.11 Chromatogram of Working Solution Z.

Conditions as in text.

dilutions, would be easier and more accurate using larger amounts of standard compounds. The relatively small amounts available at this stage of the investigations could be increased considerably for use in routine analyses.

A significant increase in precision should follow from the use of syringes fitted with a constant-volume-injection device, such as the Cheney adapter. The syringe used in these studies did not have this type of device, but a similar syringe with a Cheney adapter was available for a short time. A study of the precision of injections using the Cheney device, compared to injections using the same syringe but without using the Cheney device, showed that replicate injections with it were between $1\frac{1}{2}$ and 2 times as precise as those without it. These results are shown in Table X.17, where the volume of solution injected was $0.1\mu\text{l}$ in each of seven injections.

Table X.17.

Comparative Study of Precision of Injections Using the Cheney Device.

		H_8EAQ	H_4EAQ	H_4EAQ Epoxide	EAQ
<hr/>					
with Cheney:					
	C (%)	5.7	4.5	3.6	3.6
without Cheney:					
	C (%)	11.1	6.6	6.6	5.8

The small injected volume of $0.1\mu\text{l}$ gave higher values for coefficients of variation, so magnifying the difference in precision between the two sets of data. An advantage of this device is that it is quickly adjustable to give different injection volumes.

4. Effect of Internal Standardisation on Precision.

The use of the internal standard here was limited to determining the increase in the precision of peak height measurements when the standard was used as described in Chapter IX. Solutions were made from a Working Solution (solution X of the preceding section) and a synthetic Working Solution, both also containing known concentrations of benzil, the substance chosen as internal standard.

Benzil has a high UV absorption ($\epsilon = 20,500$ at 259nm in methanol), is relatively stable in solution and elutes in an appropriate position using chromatographic systems A or B. The sample of benzil used herein was part of a batch of 99.2% purity which had been specially purified, by zone-refining, by EDH Ltd.

4.1 Use of Internal Standardisation with Chromatographic System A.

This study attempted to test the applicability of the internal standardisation principle in the present context by deliberately injecting volumes of larger or smaller size than were normally used with chromatographic system A. The size of these injections ranged from 0.08 to 0.12 microlitres. A 10% dilution of Working Solution X in Sextate, containing 0.50% benzil, was injected twelve times using different volumes within the above range. The peak heights for each component were measured and the standard deviations and coefficients of variation were calculated. Each peak height was then 'normalised' by dividing it by the benzil peak height of the same injection and multiplying by 100 to convert it to a percentage. The standard deviations and coefficients of variation of these normalised values were calculated and were compared with the first, uncorrected, set of data as shown in Table X.18.

Table X.18.

Internal Standard Normalisation of Varied Injection Volumes.

Peak	1	2	H ₈ EAQ	H ₄ EAQ	H ₄ EAQ Epoxide	6	EAQ
C, uncorrected (%)	19.6	18.0	15.1	11.5	16.2	14.9	11.6
C, normalised (%)	4.7	4.8	9.1	5.0	9.8	13.6	5.0

The peaks obtained for H₈EAQ, H₄EAQ Epoxide and peak 6 were small, and were not improved as much as the larger peaks because of the difficulty of measuring them accurately. The H₄EAQ Epoxide peak and peak 6 were also partially overlapped by H₄EAQ and EAQ, which led to variations in their sizes not attributable to sample size variation. Apart from these cases it may be seen that, despite the large variation in sample size (+ 20% on 0.1 microlitre), the use of internal standard normalisation was effective in increasing the precision obtained.

The data shown in Tables X.17 and X.18 show that internal standard normalisation particularly improved the precision of the larger and better-separated peaks, whereas careful control of the sample volume injected increased the precision of all of the four main peaks in similar proportions. While the results in these tables are not from identical experiments, (i.e. one set of injections was deliberately varied, the other was replicated), the conclusion must be drawn that the use of the constant-volume injector device with internal standardisation would result in significantly improved precision of results over the use of either one singly.

4.2 Use of Internal Standardisation with Chromatographic System B.

A study similar to that detailed in the preceding section was carried out using two synthetic diluted Working Solutions, numbered 1 and 2. Number 1 contained H₈EAQ, 0.5% w/v; H₄EAQ, 1% w/v; EAQ, 0.4% w/v;

-200-

H₄EAQ Epoxide, 0.5% w/v, all in Sextate solvent. Solution number 2 contained half the concentrations of the first solution. Benzil was added to both solutions to make its concentration 0.75% w/v in both. The size of injections was again varied, over the range of 0.19 to 0.21 microlitres, and six injections were made of each solution. A treatment of the results similar to that in section 4.1 gave the data shown in Table X.19. Also included for comparison in this table are values obtained for solution 1 when six replicate 0.20 microlitre injections were made, without normalisation.

Table X.19.

Internal Standard Normalisation in Varied Injections Using
Chromatographic System A.

Solution	H ₈ EAQ	H ₄ EAQ	H ₄ EAQ Epoxide	EAQ
<u>1</u> C, uncorrected (%)	7.4	7.0	8.2	6.8
C, normalised (%)	2.6	0.9	4.5	0.7
C, replicate (%)	1.4	1.3	1.4	1.9
<u>2</u> C, uncorrected (%)	7.2	6.1	11.1	6.2
C, normalised (%)	1.6	2.0	4.3	1.3

Comparison of the uncorrected values with the replicate values shows that the loss of precision expected from the variation of sample size had in fact occurred, and that by normalisation on the internal standard the loss of precision was largely corrected. Use of internal standard normalisation in conjunction with careful sample size control would thus be expected to give even lower values for coefficients of variation than those shown in the Table. It is generally acceptable for routine analytical procedures to give coefficients of variation of 1% or less, and this level was bettered by two of the four normalised results despite the deliberate variation in sample volumes. With the

use of carefully-controlled sample volumes and of internal standard techniques it is reasonable to expect coefficients of variation consistently of the order of 1%.

5. Conclusions.

5.1 Method of Quantitation.

The peak height method was shown to be consistently more precise than the electronic integrator used. Other methods such as planimetry and cut-and-weigh, were not examined.

The peak height measurement can accumulate errors from two main factors. These are the fixing of the position of the baseline for a peak, and the peak height measurement itself, which is affected by the thickness of pen trace and by the measuring instrument used. It is considered that, while this method gave acceptable precisions, it could be improved upon by area measurement methods using more sophisticated electronic integrators which are now more readily available.

5.2 Use of External Standardisation.

The use of external standards was shown to give satisfactory calibration graphs for the four components analysed using chromatographic system B. The H_4 EAQ Epoxide degradation products also gave satisfactory calibration curves, but the standardisation of the 'di-hydroxy' compound was not completely satisfactory because the original very small sample consisted of two components, probably isomers. Both components gave similar calibration curves, and it is expected that, after the isolation of each compound in a pure state, it should be possible to obtain acceptable calibration curves for each.

The use of the external standardisation curves in analyses of a synthetic "Working Solution" gave similar percentage composition values to those expected, within the estimated limits of experimental

error. These results, with acceptable coefficients of variation, are regarded as representing the true compositions of the solutions examined. The small deviations from expected concentration values were attributable to errors accrued from solution preparation.

Analyses of Working Solutions gave results similar to those obtained by polarographic techniques. The gradual changes in Solution composition known to occur made close comparison of the two methods somewhat unrealistic. A meaningful comparison would have to be carried out simultaneously using the same bulk sample solution, by both techniques, to be valid. Concentration values for H_4 EAQ were found to be some 14% higher than those found by polarography, and this is most readily explained by postulating the existence of a quinhydrone compound of H_4 EAQ and H_4 EAQuinol in Working Solutions.

The H_4 EAQ Epoxide degradation products were identified in three Working Solution samples. Quantitative measurements were not feasible because of the great congestion of unresolved peaks, making it impossible to determine a baseline for height measurements. Some semi-quantitative comparisons and conclusions were made, however, based on the proportions of hydrolysis and reduction products of H_4 EAQ Epoxide in the various Solutions.

5.3 Increase of Precision by Internal Standardisation.

Benzil was found to be a satisfactory internal standard, and the precision obtained by using normalisation was significantly better than by the uncorrected peak height measurement procedure.

The use of internal standardisation in routine analytical situations may require more time and care in sample preparation than external standardisation, but would lead to higher precision if operational factors were a significant source of errors. Reduction of sample injection errors by using the Cheney device was briefly investigated.

It was found to give better precisions than the standard syringe, and is recommended for use with both internal and external standardisation procedures.

XI APPLICATION OF HPLC IN THE INVESTIGATION OF COMPOUNDS OTHER THAN
THE MAIN COMPONENTS OF WORKING SOLUTION.

1. Possible Approaches to the Problem.

As in other investigations of this type, there are two possible approaches, namely the analytical and the synthetic. Using the analytical method the mixture is separated into its components and each is identified by techniques such as nuclear magnetic resonance spectroscopy, mass spectroscopy and ultra-violet and infra-red spectroscopy. The synthetic approach requires an understanding of what might be present in the mixture, so that individual compounds thought to be present might be synthesised. They may then be shown to be present, or absent, in the mixture by separative techniques such as GLC, TLC and HPLC, coupled with a confirmation of identity using chromatographically identical compounds,

It is often found that a mixture of both approaches is necessary to get the best of either. An analyst may, for example, be able to give the synthesist a good indication of what type or types of compound are present by simple preliminary experimental separations, thus allowing

a more rapid approach to be made by the exclusion of compounds unlikely to be present.

The chromatograms shown in Figures VII.2 and VII.4, and discussed in Chapter VII, show clearly that Working Solution is far from being a simple mixture of a few components. Over thirty peaks, plus inflexion points, may be seen in certain of the chromatograms on Corasil II using 1% ethyl acetate in n-heptane, and it is known that this solvent does not elute all of the applied sample (see section 2). The identification of all of these compounds would thus require extensive studies and facilities.

Both analytical and synthetic approaches were used. These studies were subordinated to the main objective of devising qualitative and quantitative analytical methods for the main components of Working Solution. Information and experimental data were collected whenever possible. The separation systems used and data may thus appear somewhat unsuitable because of this piecemeal approach.

2. Analytical Investigation of Working Solution.

2.1 Semi-Preparative Chromatography on Corasil II.

The semi-preparative chromatography described in Chapter VII (section 2.3.2), and illustrated by the chromatogram shown in figure VII.2, gave three fractions of the eluted components. In order to obtain enough of single components to be identifiable, it was necessary to process about 200 microlitres of Working Solution by HPLC on Corasil II. The size of injections used, about 10 μ l, overloaded the detector response at 254nm, but it was seen from the 280nm trace that the column did not appear to be seriously overloaded.

The fractions obtained were all an orange-brown colour, fractions A and C being viscous liquids whilst B was solid. Dilutions of each of these fractions were made and the solutions were further

examined by HPLC. It became apparent that the initial fractions did not have the sharply defined ranges of compounds that had been expected. This was probably due to the difficulty of estimating exactly when a component, which had just passed through the detector, was actually leaving the outlet tube from the detector. The original aim, to cut the Working Solution into sharply-defined fractions and thus eliminate the over-loading of the column by the main components in subsequent studies, was achieved to some extent, but it is apparent from the second set of chromatograms that both EAQ and H_4 EAQ occur, to differing extents, in all three main fractions.

The chromatograms of fractions A and B, shown in figures XI.1 and XI.2, were obtained with the 2% di-iso-propyl ether, 0.2% diglyme in n-heptane solvent and the Waters μ -Porasil column; the chromatogram of fraction C, shown in figure XI.3 was also obtained using the Waters column, but using 3% isopropanol in n-heptane.

The large peaks at 6.5 and 8 minutes in figures XI.1 and XI.2 were found, by injection of standard samples, to be due to H_4 EAQ and EAQ respectively. A smaller peak (arrowed) at 5.7 minutes in fig. XI.1 was due to H_8 EAQ. Of the three peaks between H_4 EAQ and EAQ in fig. XI.1 it is thought that the middle one was due to H_4 EAQ Epoxide because of the similarity of its position to that of a standard sample. The sharp peak at 3 minutes was due to less-volatile Aromasol components, which had not evaporated off from fraction A with time. There are thus some nine components of fraction A not firmly or even tentatively assigned.

Figure XI.2, showing the chromatogram of fraction B, shows that much of the H_4 EAQ and EAQ in the original Working Solution was concentrated in this fraction. No other major peaks were present; the baseline irregularities before and after EAQ probably correspond to certain of the peaks observed in the chromatogram of fraction A.

Figure XI.3, a chromatogram of fraction C, was obtained at a higher sensitivity than the first two chromatograms. The off-scale

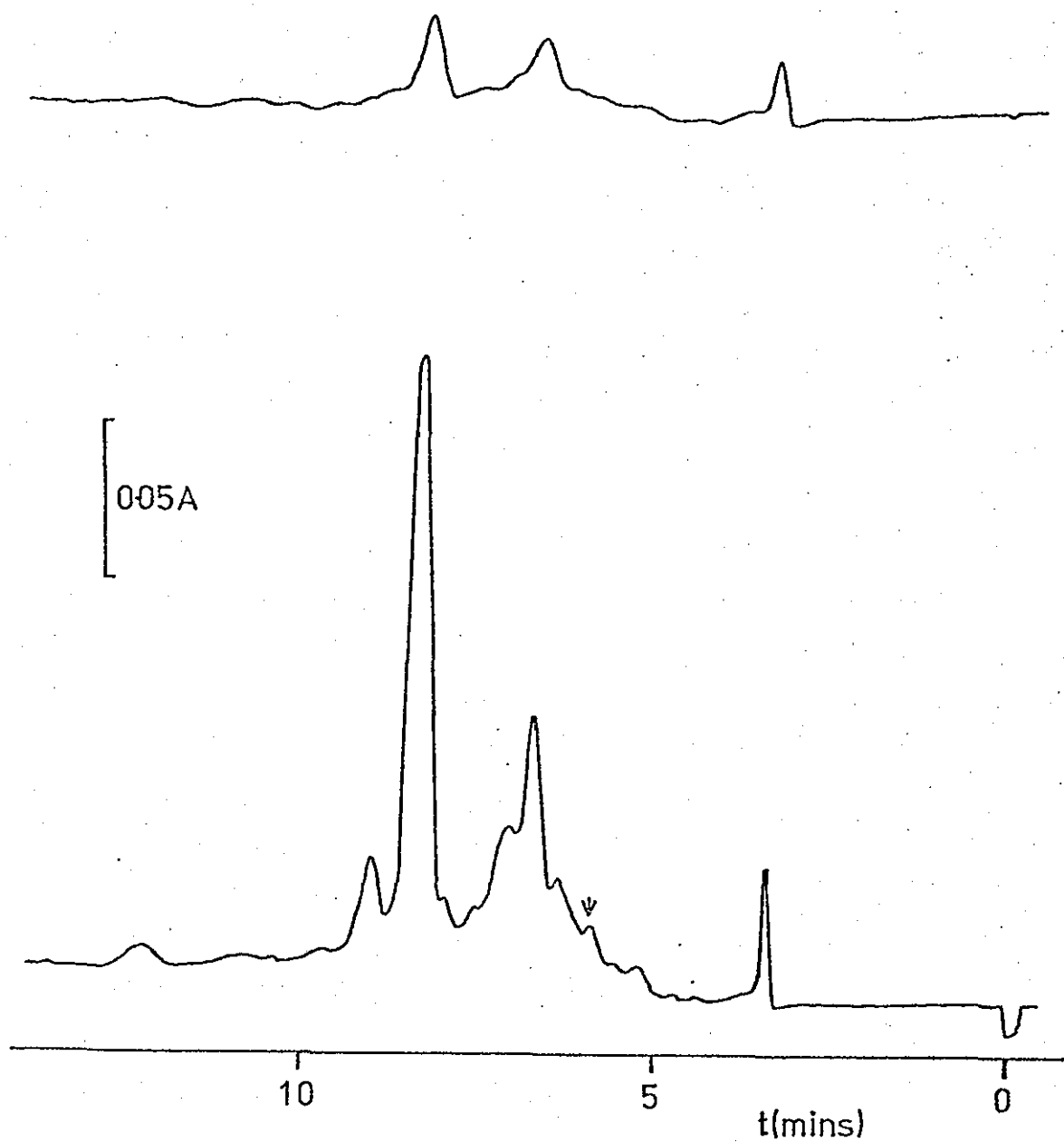


Fig. XI.1 Chromatogram of Fraction A from Semi-Preparative Runs.

Solvent 0.2% diglyme, 2% IPE in n-heptane, at flow-rate 2ml/min.

Lower trace, 254nm, upper trace 280nm.

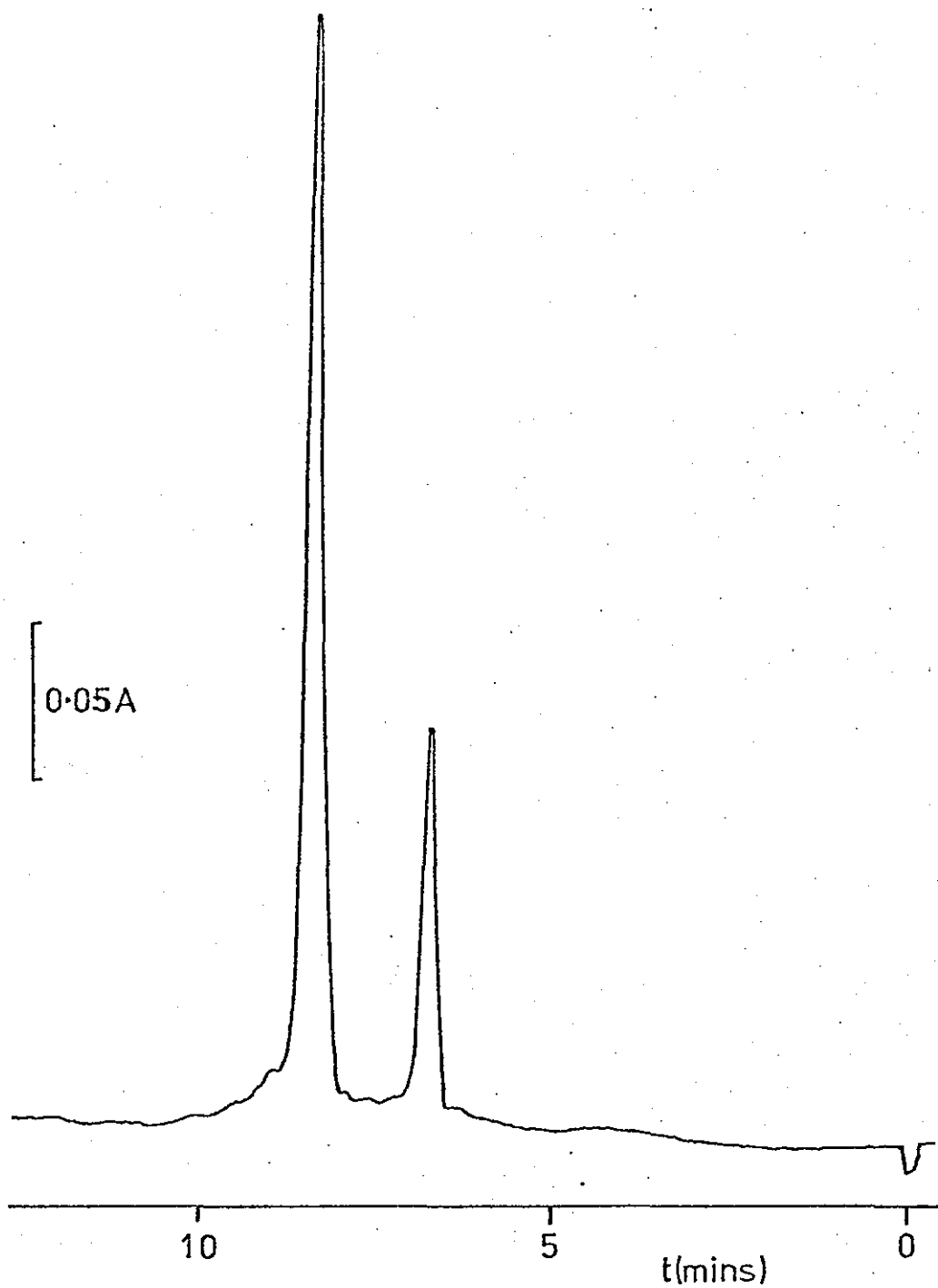


Fig. XI.2 Chromatogram of Fraction B from Semi-Preparative Runs.

Solvent 0.2% diglyme, 2% IPE in n-heptane, at flow-rate 2ml/min.

254nm trace only.

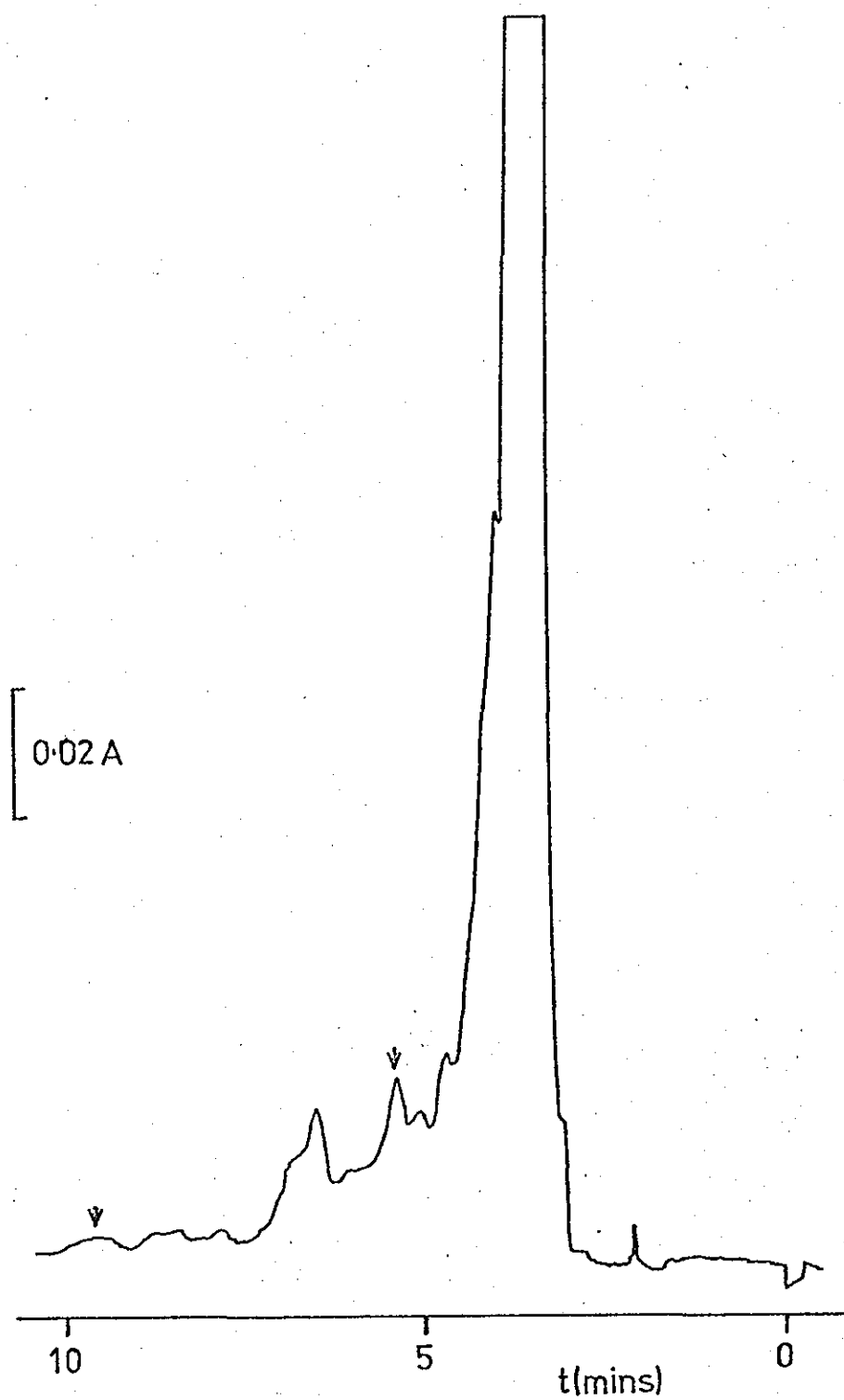


Fig. XI.3. Chromatogram of Fraction C from Semi-Preparative Runs.

Solvent 3% iso-propanol in n-heptane, at flow-rate 1ml/min.

254nm trace only.

complex peak at about 4 minutes contains EAQ, H_4 EAQ and compounds eluting with them. The peak at 5.3 minutes (arrowed) and the peak at 9.5 minutes (arrowed) corresponded to the peaks of standard solutions of the 'monohydroxy' H_4 EAQ Epoxide degradation product and 'hydrolysed H_4 EAQ Epoxide' respectively. There are at least eight compounds unassigned in this chromatogram. It is notable that the 'di-hydroxy' compound derived from H_4 EAQ Epoxide was apparently not present (no peak was assigned to it), which indicates that the 1% ethyl acetate solution used in the original fractionation was not strong enough to elute this and compounds of similar behaviour.

In order to estimate the amount of material not eluted by 1% ethyl acetate a model TLC experiment was carried out. Four solvents containing 1%, 2%, 10% and 25% ethyl acetate in n-heptane, were used to develop TLC plates (10cm, Merck 'Kieselgel' F₂₅₄) which had been spotted with 1 μ l and 2 μ l amounts of Working Solution. It was found that a dark-brown base-line spot was not eluted with either 1% or 2% ethyl acetate, and that some material was left at the base-line even with 10% ethyl acetate. The 25% ethyl acetate solvent was the only one to leave an apparently clean baseline after elution. A 'tail' of unresolved bands was continuous on all of the plates up to the elution front, although there were recognisable spots for EAQ and H_4 EAQ as assigned by running reference compounds. It was concluded from this study that a considerable number of components would be left near the injection point in HPLC using 1% ethyl acetate in n-heptane.

Further semi-preparative chromatography of the fractions A, B and C was not attempted because of lack of time. In the light of subsequent information it seems that future preparative separations of Working Solution components would be best eluted using an iso-propanol gradient, probably from 0% to 10% iso-propanol in n-heptane, using the Waters μ -Porasil column and a large applied sample, e.g. 10-50 μ l. This type of elution would tend to give better resolution of earlier and

later peaks, and would be more rapid than the original isocratic 1% ethyl acetate runs.

2.2 Gel-Filtration Chromatography.

Although the majority of Working Solution components, having about the same molecular size and shape, would not be resolvable by presently-available gel-permeation or gel-filtration chromatography (GPC), there is a possibility (Chapter I, p.7) that compounds of the dianthryl group might be present. These compounds, with a molecular-weight twice that of the EAQ group, might be resolvable by GPC using the smaller pore-size column packing materials.

A Waters Associates instrument, the ALC/GPC 501 with R.I. detector, was used with a tetrahydrofuran solvent to elute Working Solution samples and thus to attempt to obtain direct evidence of the presence of compounds with molecular-weights greater than the EAQ group. The available column was a composite of three columns, each of 4 foot length and 6mm internal diameter, connected end-to-end. Two columns contained 'Styragel' of pore-size 10^4 \AA and the third contained 'Styragel' of $2 \times 10^2 \text{ \AA}$. The resolution lower limit for this composite column was of the order of molecular-weight 1,000. A sample of Working Solution was diluted with THF and 2ml aliquots were injected at a THF flow-rate of 2.5ml/minute. A broad Gaussian-type peak started eluting after 50 minutes, taking 15 minutes to clear the column. The leading edge of this peak is shown in figure XI.4.

The evidence for the existence of the dianthryl type of compounds from this chromatogram is not definite, as may be seen from the figure. The presence of the inflexion points (arrowed), where a smooth curve would normally be obtained for a single compound or group of similar compounds, may indicate that the dianthryl group, or similar dimers or trimers, are present. The columns used were not, however, of an optimum pore-size for the samples examined, as they were normally

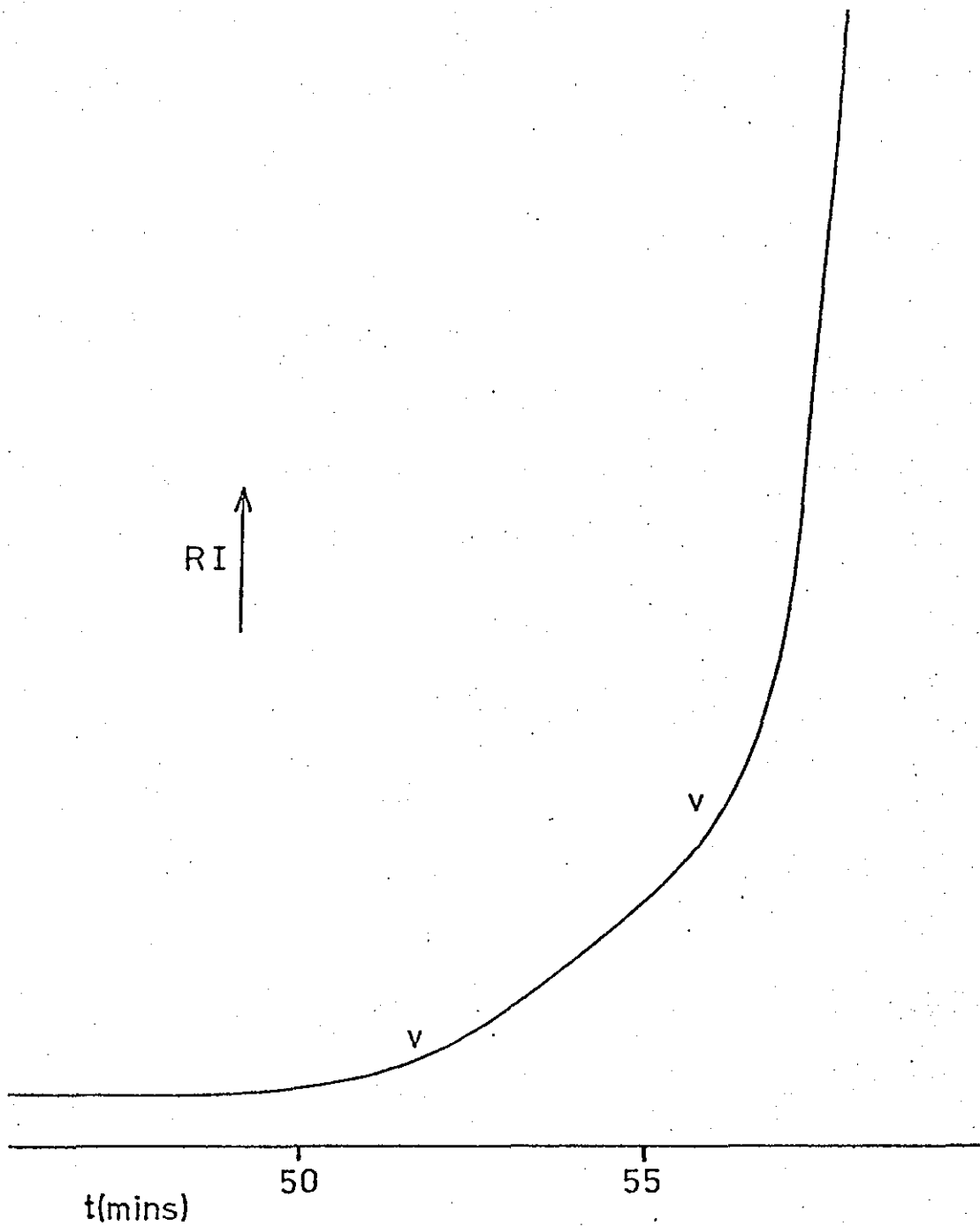


Figure XI.4.

Gel Filtration Chromatogram of Working Solution.

Only leading edge of peak is shown.

used for characterisation of higher molecular weight organic polymers. A smaller pore-size column would be able to definitely confirm or deny the tentative indications, but such a column was not available within the department. The work in this direction was thus terminated.

3. Synthetic Investigation of Working Solution.

3.1 The 'Anthrone' Degradation Route.

One of the degradation routes possible for EAQ involves the conversion of the reduced EAQ to 2-ethyloxanthrone and thence to 2-ethylanthrone. Both of these latter compounds have been demonstrated polarographically to exist in Working Solutions under some plant running conditions. It is known^{70,81}, however, that anthrone exists in solution in tautomeric equilibrium with anthranol, and that the ratio of anthrone to anthranol is dependent on the solvent and on substituents in the anthrone rings. It is thus probable that EAQ can in one reduction cycle form 2-ethyl-9-anthranol. A further reduction would lead to the formation of an unstable dihydro-ethylanthranol which, on exposure to air, as happens in the A.O. process, would be converted to 2-ethylanthracene. This series of reactions is described in Chapter I, fig. I.3, except for the unstable dihydro-ethylanthranol. This series of reactions is well summarised by Rodd⁸¹, who states that the hydrogenation of anthranols and subsequent formation of anthracenes is "a general method for reducing anthraquinones to the corresponding anthracenes."

The probability of 2-ethylanthracene being formed thus seemed high, and, since it would be relatively inactive when formed, the product would increase in concentration with time in the plant Solution. Polarography would not readily detect 2-ethylanthracene, since it has no reducible groups, but since the anthracenes have very high U.V. absorbances it should be detectable at high sensitivity by the HPLC U.V. detector.

The investigation of this degradation route was made in two ways. Firstly, a study of 2-ethylanthrone was to be made to characterise it chromatographically and to find whether the tautomerism to the anthranol was detectable. Secondly, a synthesis and characterisation of 2-ethylanthracene was attempted to determine whether or not the anthrone route went to completion in this direction.

3.1.1 Preparation and Characterisation of 2-Ethylanthrone.

Two methods were used in attempts to prepare 2-ethylanthrone. The method of Siavtsillo et al.⁸² involved reduction of 2-EAQ with tin and hydrochloric acid in glacial acetic acid. The second method was by the reduction of 2-EAQ using alkaline dithionite, as described by Battegay and Hueber⁸³. Both experimental procedures are described in Appendix A.

Procedure A.

The sample obtained using the tin/hydrochloric acid reduction, sample A, had a melting-point of 60-62°C, in agreement with the published value⁸² of 60-62°C, and was examined by U.V. and I.R. spectroscopy. The UV spectrum showed two peaks, at λ_{max} 262nm and 273nm, with ϵ_{max} values of 2×10^4 and 1.76×10^4 respectively. The IR spectral data is shown in Table XI.1 in comparison with similar data for anthrone. Relative band absorbances are included in parentheses.

The close similarity for all of the main peaks between anthrone and sample A is very strong evidence that sample A was in fact 2-ethylanthrone. The five peaks observed for sample A and not for anthrone may be accounted as due to the alkyl group characteristic frequencies (1394cm⁻¹, CH₃; 1460cm⁻¹, -CH₂- or -CH₃; 2830cm⁻¹, -CH₂-; 2920cm⁻¹, -CH₃) or due to the substitution by the ethyl group in one aromatic ring (835cm⁻¹, corresponds to 2 adjacent aromatic protons, as in the 2-substituted ring). Furthermore, there was no evidence of the presence of a hydroxyl group at around 3000cm⁻¹, which indicated that the compound

was 2-ethylanthrone and not 2-ethylanthranol.

Table XI.1.
I.R. Data for 2-Ethylanthrone^(a) and for Anthrone^(b).

Wavenumber anthrone	Wavenumber 2-ethylanthrone	Wavenumber anthrone	Wavenumber 2-ethylanthrone
713 (10)	717 (10)	----	1460 (6)
----	835 (9)	1603 (10)	1608 (10)
928 (9)	937 (8)	1658 (10)	1650 (10) 1660
1155 (6)	1150 (4)	----	2830 (2)
1174 (8)	1175 (6)	2860 (1)	2890 (3)
1302 (10)	1311 (10)		
1321 (10)	1320 (10)	----	2920 (8)
1346 (4)	1348 (4)	3000 (2)	2990 (1) 3010
----	1394 (5)		

(a) The 2-ethyl-anthrone spectrum was obtained using a film of the solid evaporated from a carbon tetrachloride solution onto a sodium chloride disc.

(b) Ref. 88.

The sample was also subjected to mass spectrometry, the main peaks of which are detailed in Table XI.2 together with values obtained by Hall and Oliver⁸⁴ for anthrone. Metastable peaks were also found at m/e values of 193, 167.8, 155, 130, 178 and 141.5, in descending order of intensity. An interpretation of the mass spectrum on the basis of 2-ethylanthrone is given in figure XI.5. This interpretation was partly based on the paper of Hall and Oliver with regard to the ring expansion and acetylene loss steps. The molecular ion was at m/e 222, as would be required for 2-ethylanthrone.

An interesting point from this interpretation is in the

Table XI.2.

Comparison of Mass Spectrometric Data for Anthrone and 2-Ethylanthrone.

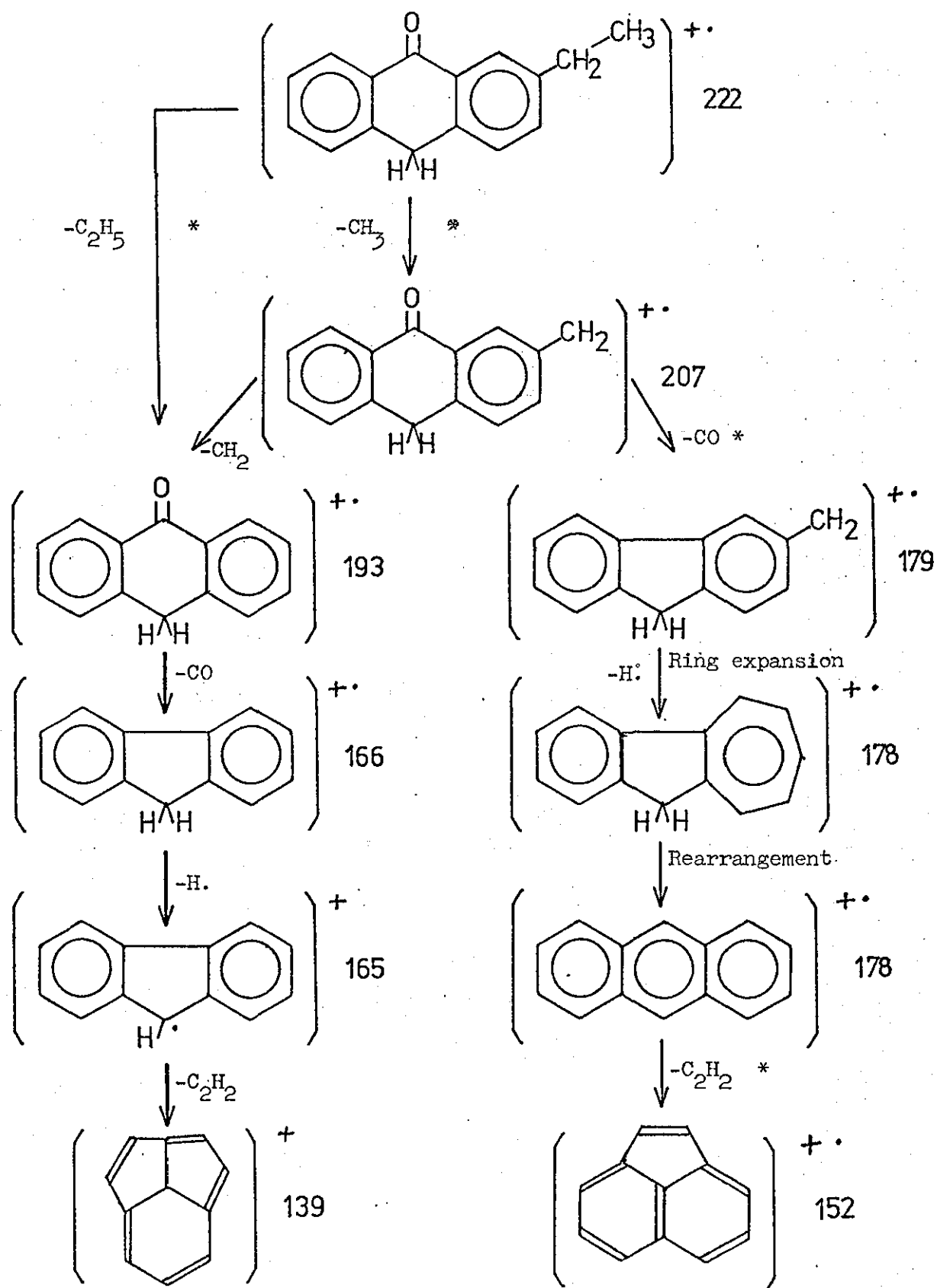
m/e	50	51	62	63	69.5	74	75	76	81.5	82	82.5	83
R.I. Anthrone (a)	12	9	6	15	6	8	6	5	5	12	14	6
R.I. 2-Ethylanthrone (b)	2	3	2	5	1	2	3	6	1.5	2	3	2
m/e	83.5	87	88	89	115	139	150	151	152	153	163	164
R.I. Anthrone (a)	---	6	---	6	6	9	---	---	---	---	12	10
R.I. 2-Ethylanthrone (b)	3	2	4	10	3	4	2	4	6	2	5	3
m/e	165	166	176	177	178	179	193	194	207	208	222(M)	223
R.I. Anthrone (a)	88	17	---	---	---	---	10	100	---	---	---	---
R.I. 2-Ethylanthrone (b)	30	4	5	5	21	11	51	10	25	5	100	18

(a) Relative Intensity taking m/e 194 = 100

(b) Relative Intensity taking m/e 222 = 100

Figure XI.5.

Structural Interpretation of 2-Ethylanthrone M.S. Data.



* Indicates presence of supporting metastable peaks.

formation of the 194 m/e fragment. There is a strong metastable peak at m/e 167.8 which corresponds, using the relationship $m^* = \frac{b^2}{a}$ for the two fragments, to the loss by the molecular ion of a $-C_2H_5$ group. This route would thus seem to be the main route to the m/e 193 fragment, as no metastable peak could be found for the other route by loss of $-CH_2$ from the m/e 207 fragment. Such a metastable peak would be expected at m/e 179.9 .

The combined spectral evidence showed that this compound, sample A, was 2-ethylanthrone and not 2-ethyl-anthranol or 2-ethyl-anthraquinone. This confirmation of the structure of sample A is of importance in view of the HPLC results obtained herein.

Procedure B.

The reduction using alkaline dithionite gave a product of doubtful purity. The Kofler melting-point of most of the sample B material was 45° - 47° C, with some crystals melting at 59° - 65° C. Since this preparation was carried out to give an alternative sample of 2-ethylanthrone, later in the course of these studies, the relative impurity was tolerable, since the difficulty of purifying this low-melting compound after this preparation is considerable.

3.1.2 Chromatographic Studies on 2-Ethylanthrone.

For these studies, unless otherwise stated, the sample used was 2% sample A, in Sextate.

When freshly-prepared, the 2-ethylanthrone solution gave a single peak, at a k' value of 3.7 compared with EAQ at a k' of 3.3 , when eluted with 3% ethyl acetate from the diglyme-coated Varian SI-10 column. The 2-ethylanthrone peak, with an efficiency of $N = 720$, had a noticeably larger tail than other components examined in this solvent system and was partly-resolved from EAQ ($R \approx 0.6$).

On changing the solvent to 2% diglyme in n-heptane with the same column the k' values changed to 1.94 and 2.29 for EAQ and 2-ethyl-anthrone respectively, with a resolution of about $R = 0.5$. The

tailing of the 2-ethylanthrone peak was even more marked. When injected after 24 hours storage the same solution gave rise to two peaks, with k' values of 1.94 and 2.33, and with the same pronounced tailing of the second peak. This phenomenon of the double peak was checked by injecting other well-characterised compounds in case it was a 'ghost' peak, and was found to be a true chromatogram. A later further injection, of the 2-ethylanthrone plus EAQ, gave a similar two-peak chromatogram with the k' 1.94 peak enlarged relative to the later peak. It appeared from these results that the 2-ethylanthrone, when left to stand for some time in solution, formed considerable amounts of EAQ. This was not in accord with Siavtsillo's findings⁸² that 2-ethylanthrone was stable for several months, albeit in benzene solution.

In the preparation of sample B several chromatograms were run of solutions produced during the extraction and purification stages. A sample of the ether solution of the recrystallization stage gave three peaks, one due to a trace of 2-ethylanthracene (see section 3.2) and the other two in the same positions as were occupied by samples of EAQ and 2-ethylanthrone. A solution from earlier in the purification gave 6 peaks, of which the two corresponding to EAQ and 2-ethylanthrone had the same peak height ratio as the later, recrystallised material. It is possible that the EAQ and 2-ethylanthrone have exactly the same behaviour through the recrystallisation process, but this is considered unlikely.

The tentative conclusion from this relatively small amount of evidence is that 2-ethylanthrone relatively quickly converts to EAQ on standing in suitable solvents; this conversion might proceed through 2-ethylanthranol, but no direct evidence of this (i.e. a definite peak) could be found, indirect evidence might be the excessive tailing noted for the 2-ethylanthrone peak. Alternatively this effect could be the result of tautomerism taking place during chromatography, leading to later elution of molecules which were injected as 2-ethylanthrone and

eluted as 2-ethylanthranol. The results given in the Russian paper⁸² indicated that 2-ethylanthrone is readily changed by tautomerism to 2-ethylanthranol in alkaline solution, with subsequent rapid oxidation to EAQ. It seems that 2-ethylanthrone may be so labile that it will not go further in the degradation route to 2-ethylanthracene, but goes to other compounds instead.

3.2 Preparation and Chromatographic Study of 2-Ethylanthracene.

The final, stable, compound in the 2-ethylanthrone degradation pathway is 2-ethylanthracene. A preparation involving the reduction of EAQ by boron hydrides⁸⁵, generated in situ by reaction between boron trifluoride and sodium borohydride, gave an off-white solid of melting-point 150° - 154°C by Kofler hot-stage, compared with the published value of 155° - 156°C ⁸². On melting and allowing to cool, crystallisation occurred as flat plates, which had a blue fluorescence under long-wavelength UV light.

A UV spectrum of a very dilute solution ($0.75 \times 10^{-5}\text{M}$) of this material in n-hexane gave a spectrum very similar to that of anthracene. Absorbance maxima were observed at 252nm, 253.8nm, 255.5nm, 275nm, 340nm, 357nm and 366nm. Of these, only the first three peaks were of high intensity, with ϵ values of 5.1×10^4 , 1×10^5 and 9.5×10^4 respectively.

A fluorescence spectrum at a concentration of about $5 \times 10^{-4}\text{M}$ gave a spectrum similar to that of anthracene. The spectrum obtained is shown in figure XI.6.

The evidence of the melting-point, UV spectrum and fluorescence spectrum was considered to be proof that the compound was 2-ethylanthracene, and the compound was thus used in chromatographic experiments searching for evidence of 2-ethylanthracene in samples of Working Solution.

The high absorbance of 2-ethylanthracene at the exact wave-

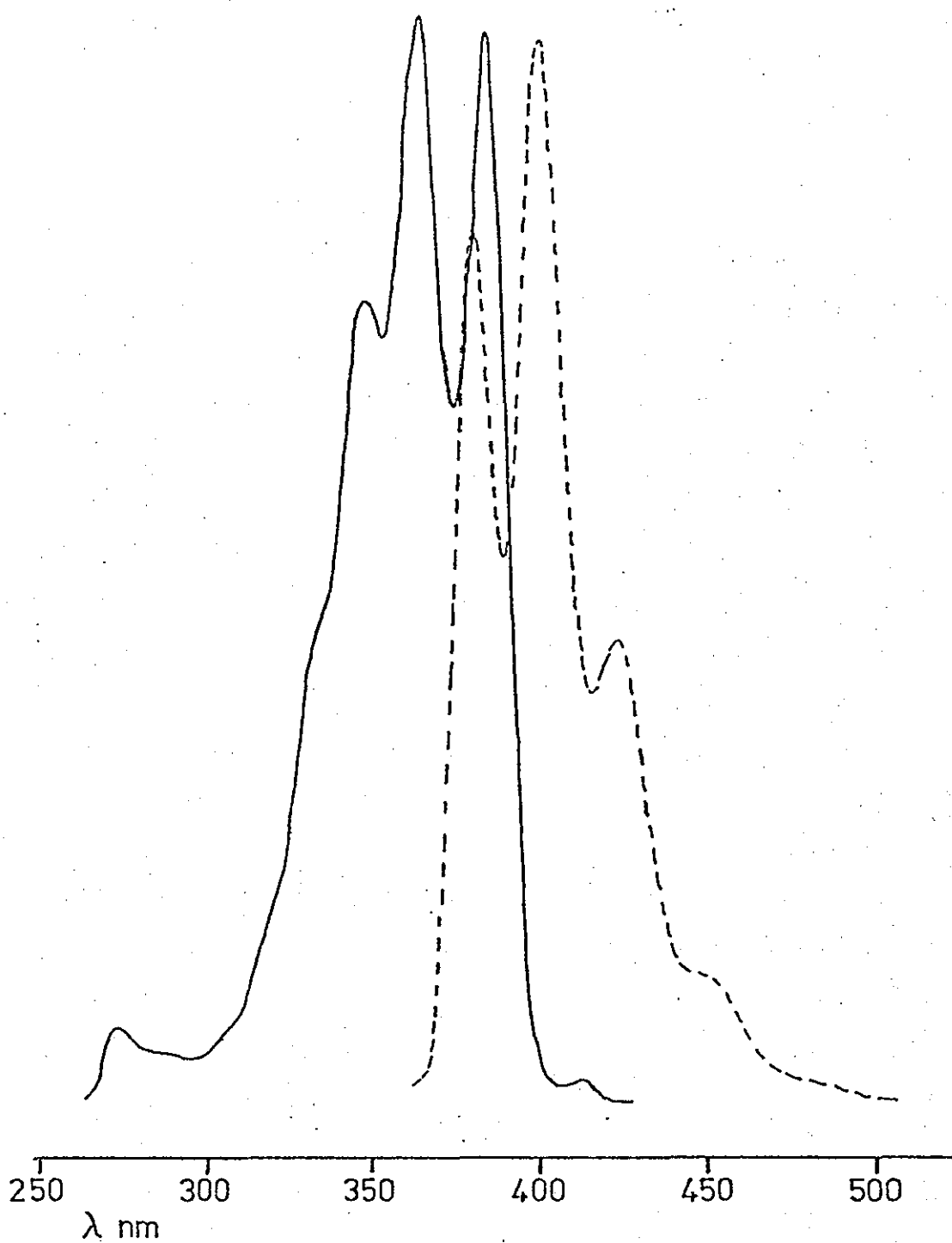


Figure XI.6.

Fluorescence Spectrum of 2-Ethylanthracene in n-Hexane.

Concentration 5×10^{-4} M.

emission = 400nm.

excitation = 360nm.

length of the 254nm detector channel gave a very high sensitivity for this compound. The absorbance at 280nm, being virtually negligible, gave a good identification for 2-ethylanthracene in chromatography. Any peak strong at 254nm but not registered at 280nm would in the sample in question be most likely to be due to this compound. Further, it was expected that 2-ethylanthracene would elute between the Aromasol and the quinone peaks, since it has no strongly-adsorbing functional group.

Solutions containing 2-ethylanthracene were injected onto the Waters column and were eluted with 2% IPE and 0.2% diglyme in n-heptane. Single peaks were obtained, occurring about midway between the Aromasol and H_8 EAQ peak positions. The k' values obtained for meta-xylene, naphthalene and 2-ethylanthracene peaks are given with that of H_8 EAQ in Table XI.3.

Table XI.3.

Capacity Factor Values for Aromatic Hydrocarbons and H_8 EAQ.

Compound:	m-xylene	naphthalene	2-ethylanthracene	H_8 EAQ
k'	0.11	0.29	0.41	0.77

The 2-ethylanthracene peak is well-separated from adjacent peaks and would thus be readily observed, if present, in a Working Solution.

The chromatogram of fraction A of the Corasil semi-preparative runs (fig. XI.1) shows no significant peak in the position expected for 2-ethylanthracene. It is not present in this fraction to any appreciable extent; it may, however, be argued that 1% ethyl acetate may not have eluted 2-ethylanthracene from the original Corasil II column for some reason. This is highly improbable but not impossible.

In order to obtain definite data on the presence of 2-ethylanthracene in Working Solutions a series of injections was made of Working Solutions obtained from different sources. No evidence of the

presence of 2-ethylanthracene could be found in the chromatograms of Laporte Warrington Working Solution at anything above trace level. Slightly larger peaks in samples of Finnish and Spanish Working Solutions could most readily be accounted for by the different aromatic solvent used by these plants. This solvent contains noticeable amounts of naphthalene and traces of higher fused-ring aromatics which would tend to build up in solution as more volatile compounds were lost by attrition.

3.3 Alternative Degradation Routes for EAQ.

The gel chromatography reported in section 2.2 may offer a possible degradation route leading to the formation of dimerisation products. Rodd⁸¹ cites a number of compounds simply derived, by reduction and oxidation processes, from anthraquinone, anthrone or anthrahydroquinone; it is reasonable to assume that such reactions will not be greatly affected by the presence of an alkyl side-chain in one aromatic ring. A series of dimerised compounds is possible, similar in some ways to the series of compounds in the 'anthrone' degradation route. The compound 2,2'-diethyl-10,10'-dihydrodianthrone, shown in Chapter I, (p.7), is of particular interest, since Siavtsillo⁸² reported that this was the main product of the oxidation of 2-ethylanthrone exposed to hydrogen peroxide, in benzene solution, at 60°C. This type of oxidation condition is, of course, similar to that in the A.O. hydrogen peroxide process, although at a lower temperature.

An attempt was made to convert 2-ethylanthrone to this dimeric compound, using relatively mild conditions, following the report that the dimer could be obtained by aerial oxidation of a solution of an anthranol.

A small amount, (about 200mgs), of 2-ethylanthrone was dissolved in 1M sodium hydroxide solution, forming a yellow solution, and residues were removed by filtration through glass wool. The yellow solution gradually became opaque on standing; the yellow opacity formed

from the air interface downwards. It was thought that this was due to the oxidation to EAQ in alkaline solution mentioned by Siavtsillo, and in order to form the dimer rather than EAQ a large volume of air was drawn through the solution over a period of one hour. The resulting cloudy solution was extracted with ether. The ether extract was allowed to evaporate very slowly. After several days a few small needle-like crystals formed; these were removed from the solution and washed by a short immersion on the tip of a micro-spatula in warm ether vapour. The product had a melting point of 198° - 199.5°C on a Kofler hot-stage, (cf. the published value of 190° - 192°C ⁸²).

There was not, unfortunately, sufficient of the material for a mass spectrum to obtain a molecular ion weight and thus confirm the identity of the compound. It was not EAQ, 2-ethylanthrone or 2-ethyl-oxanthrone, since these compounds have considerably lower melting-points.

4. The Quinol-Quinone Relationship in H_8EAQ .

The acquisition of a sample designated ' H_8EAQ Quinol' from Laporte enabled experiments to be made on the stability and behaviour of the quinol in solution.

Chromatography was carried out on a Corasil II column with two solvent systems, of 0.1% tert-amyl alcohol and of 1% ethyl acetate in n-heptane respectively. These solvents were also used in the studies described in Chapter VII. The conclusions reached at that stage regarding the mode of action of tert-amyl alcohol are relevant to these chromatographic studies and were further investigated.

4.1 Chromatography Using a 0.1% t-Amyl Alcohol Solvent.

In order to determine which type of chromatography was pre-dominant in this system, injections of solutions of H_8EAQ and of EAQ in

Sextate were made. The elution order of these compounds should show whether a weak reversed-phase liquid-liquid chromatography (H_8 EAQ eluting after EAQ) or liquid-solid chromatography (H_8 EAQ eluting before EAQ) was the dominant mode of chromatography. It was found that EAQ eluted after H_8 EAQ (k' values 1.39 and 0.50 respectively) and thus a reversed-phase mode of chromatography was not occurring. The efficiency values found for these peaks ($N_{EAQ} = 1,400$ and $N_{H_8EAQ} = 155$) are, however, so much different that the H_8 EAQ must have been giving an unresolved group of peaks as noted earlier (Chapter VII).

When a solution containing ' H_8 EAQ Quinol' was carefully made up, under a nitrogen gas cover, and injected, it was found that two main peaks eluted, at k' values 0.03 and 2.13 respectively. Neither of these corresponded to H_8 EAQ at k' 0.50, which would be expected to be present arising from oxidation of the quinol. A series of injections of this solution, after exposure to air, all gave similar chromatograms, even after exposure to air overnight. An equilibrium condition thus formed rapidly on exposure to air. Figure XI.7 shows chromatograms of the initial solution (A) and of the same solution after standing in contact with air for 1.75 hours (B). The main features of the chromatogram of interest are as follows:

- (a) A sharp-fronted band, eluting at the void volume with a very long tail extending back to:-
- (b) A rounded peak with no significant tailing.
- (c) There is no discrete peak visible at k' 0.5 corresponding to H_8 EAQ.

It may be deduced from the long, curving tail of the first peak, and the fact that in all similar chromatograms the tail ended at the rounded peak, that the first and last peaks contained two compounds which were in a dynamic equilibrium. Further, that the concentration of H_8 EAQ (the expected oxidation product of H_8 EAQ Quinol) was not at any time large enough to yield a peak attributable to it.

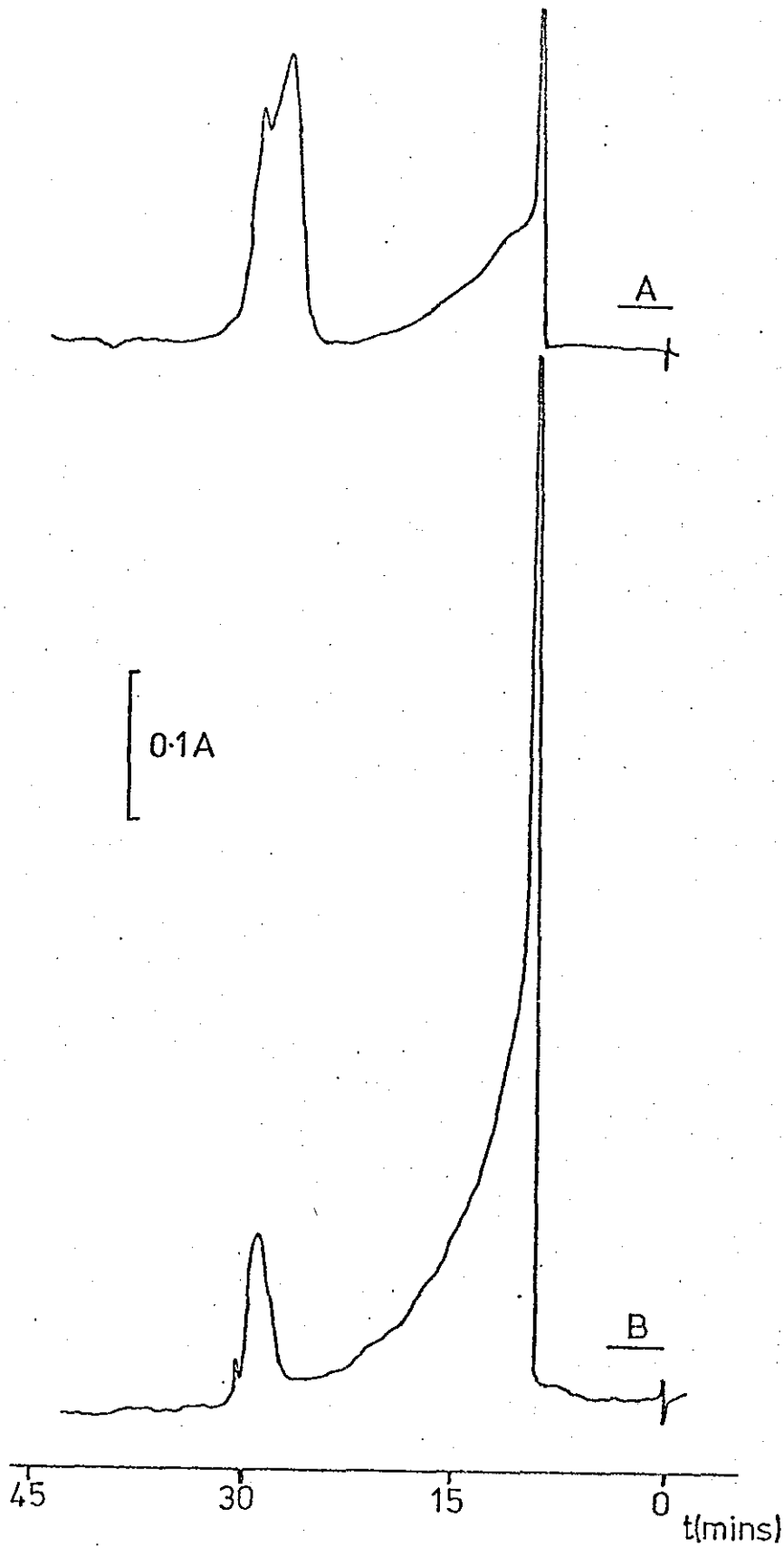


Figure XI.7.

Elution of ' H_8 EAQuinol' with 0.1% tert-Amyl Alcohol in n-Heptane.

Flow-rate 0.5ml/minute.

A Initial ' H_8 EAQuinol' solution. B Same solution after 1.75 hours.

The results pose the following questions to be answered:

1. Why is H_8EA Quinol not converted totally to H_8EAQ , particularly since the process occurring between the first and second injections appeared to be rapid?
2. Why is the product of the aerial oxidation of H_8EA Quinol eluted at the void volume, which position is normally occupied by completely non-retained compounds? and
3. What is the nature of the apparent equilibrium between the first and the last peaks?

The answers may be obtained by considering the chromatograms as being of a quinhydrone system.

The initial sample, containing mostly H_8EA Quinol, would give a larger quinol peak than subsequent samples of the oxidised solution. Later samples, after exposure of the solution to air, would contain both the quinol and the quinone. The quinone would not be in free solution as such, but as a quinhydrone, a relatively stable molecular complex formed by hydrogen-bonding between a quinol and a quinone. The formation of the stable quinhydrone would effectively stabilise the quinol with respect to further aerial oxidation, and the formation would also tend to stabilise at an equi-molar level of H_8EAQ to H_8EA Quinol, since further production of the quinone would be effectively stopped by the quinhydrone stabilisation. The long tail between the first- and last-eluted peaks could be due to a rapid dynamic equilibrium occurring during chromatography, resulting in the chromatographing of the quinhydrone components as separate components for varying periods of time in the column and thus resulting in variable retention times.

This explanation would answer questions 1 and 3 posed above. To answer the second point it is necessary to reconsider the nature of the chromatographic solvent, as in Chapter VII.3.1. If, as earlier proposed, there was on the silica a layer of tert-amyl groups, the effect of exclusion of large molecules from the surface would arise,

since molecules above a certain size would not be able to fit in between the bulky amyl groups. It is possible that the quinhydrone complex was above this size, and because of exclusion from interactions with the surface it passed through the column with the solvent front.

4.2 Chromatography Using a 1% Ethyl Acetate Solvent.

A freshly-made solution of 'H₈EAQuinol', when injected and eluted with 1% ethyl acetate, gave a sharp peak corresponding to H₈EA⁻Quinone followed by two, flatter and wider, peaks at k' values of 2.06, 3.71 and 5.71 respectively. This chromatogram is shown as A in figure XI.8; chromatogram B is of the same solution injected after 1.5 hours exposure to aerial oxidation.

In this system it would be expected that H₈EAQuinol and the quinhydrone would elute after H₈EAQ, since unrestricted liquid-solid chromatography occurs with the chosen solvent. It might be expected, moreover, that the quinol would elute later than the quinhydrone, since the hydrogen-bonding ability of the quinhydrone, and hence its ability to interact with the adsorbent, had been largely utilised in holding the two components together. If we tentatively identify the second eluted peak as the quinhydrone and the final peak as H₈EAQuinol it may be seen that, within 1.5 hours of exposure to the atmosphere, the H₈EAQ level had risen three-fold and that the levels of quinol and quinhydrone had fallen similarly. The presence of three peaks, particularly that attributable to H₈EAQ, may be explained by the ability of the unhindered silica surface to interact with quinhydrone and separate its components, whereas the tert-amyl alcohol precluded this possibility.

5. Conclusions.

Although the studies described in this Chapter were carried out principally to explore and to define further areas for study, the

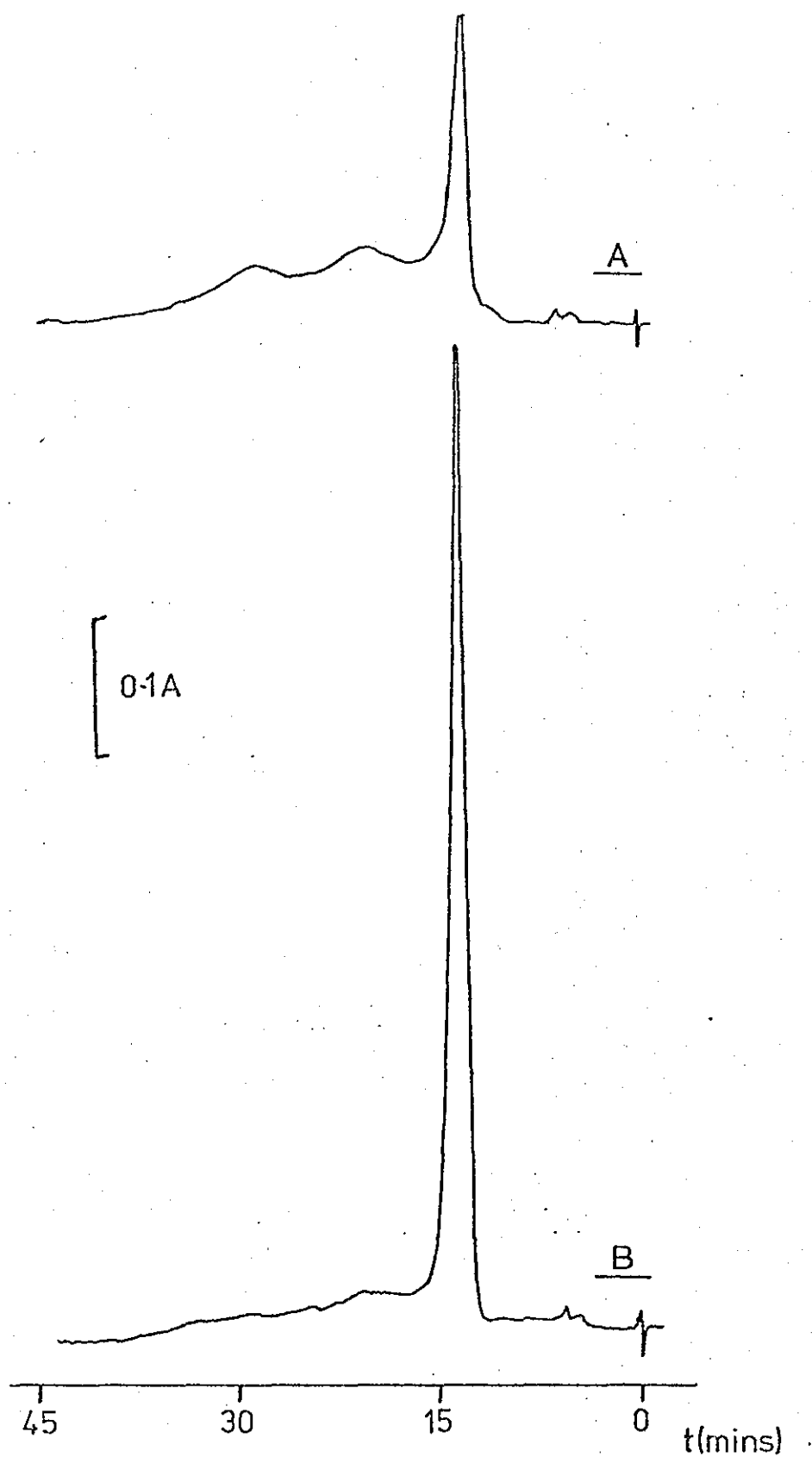


Figure XI.8.

Elution of ' $\text{H}_8\text{EAQuinol}$ ' Solution with 1% Ethyl Acetate in n-Heptane.

Flow-rate 1ml/minute.

A Initial ' $\text{H}_8\text{EAQuinol}$ ' solution. B Same solution after 1.5 hours.

conclusions and information obtained, both on the subject compounds and on the chromatography systems used, have significance with regard to understanding the involved chemistry of 'Working Solution'.

5.1 Semi-Preparative Chromatography of Working Solution.

The semi-preparative runs on Corasil II with 1% ethyl acetate in n-heptane had only limited success due to the low linear capacity of Corasil II, the dead-volume of detector outflow tubes and the amounts of sample required to be injected.

For future, semi-preparative work a more optimal system would probably use a totally-porous high-efficiency column, eluting samples of, say, 10 to 50 microlitres by gradient elution between 0% and 5% or 10% iso-propanol.

It should be possible, using such a system, to obtain milligram quantities of many components and thus be able to identify them by M.S. and I.R. spectroscopy and other instrumental techniques.

5.2 Gel-Filtration Chromatography..

The gel-filtration mode of chromatography would be applicable to studies of dimer products arising from EAQ degradation. The pore-sizes of the column used herein were too large for a definite resolution of dimer from monomer species, but smaller pore size materials are now available and should furnish more definite proof than the tentative identification of the existence herein of dimers in Working Solution.

5.3 The 'Anthrone' Degradation Pathway.

Although 2-ethylanthrone has been found, using polarography, in Working Solution, there is no evidence that the degradation finally yields 2-ethylanthracene. No 2-ethylanthracene was found in Warrington Working Solution samples; traces of a peak of similar retention in other Working Solution samples were probably due to impurities in their

aromatic hydrocarbon solvent.

Another possible route, from 2-ethylanthrone to a dimer species has been shown here, and by Siavtsillo⁸², to be relatively easily capable of producing dimer species. This is also circumstantial evidence to support the tentative identification of dimers in Working Solution by gel-filtration. The paper by Siavtsillo appears to be of particular value in these studies.

The characteristics of the mass spectrum of 2-ethylanthrone and of other appropriate spectra of 2-ethylanthrone and 2-ethylanthracene were recorded and used for identifications.

Some evidence indicated that 2-ethylanthrone was converted over a period of hours to EAQ. This should be further investigated by sample injections over a suitable period.

5.4 The Quinol-Quinone Relationship in H_8 EAQ.

It was concluded that a solution of H_8 EAQuinol exposed to the air will rapidly oxidise, within an hour, to give an equilibrium mixture containing both the quinol and the quinone, in combination as a quinhydrone. As a consequence of this it might be possible for H_8 EAQuinol to occur in the oxidised form of Working Solution in the quinhydrone complex. The presence of traces of hydrogen peroxide in the solution would, however, militate against this possibility, since the peroxide would tend to gradually oxidise the quinol to quinone. The gradual changes known to occur in Working Solution on standing may be due to this and other types of slow reaction. The quinol form of H_4 EAQ might also be expected to form a quinhydrone in solution, since both H_4 EAQ and H_8 EAQ have quinone character. EAQ, on the other hand, has the character of a 1,4-diketone rather than a quinone and thus might less readily form the quinhydrone.

5.5 Chromatographic Information.

The tert-amyl alcohol system was shown to behave as a liquid-solid chromatography system for certain species. It is proposed that this solvent forms a layer of alcohol at the active surface in such a way that a steric exclusion takes place of large molecules from the surface, thus causing them to elute at the void volume.

This effect would have to be confirmed by further studies, but does offer a plausible explanation for the chromatographic behaviour of certain compounds examined herein.

XII GENERAL DISCUSSION AND CONCLUSIONS.

1. Construction and Operation of the Instrument.

At the time of the commencement of these studies it was commonly held that it was a viable proposition to build H.P.L. chromatographic instruments. Recent developments in commercial instruments and in pricing policies have made this viability less certain, but it should still be possible to build an instrument equivalent or superior to commercial cost-equivalents, particularly if the labour costs can be discounted. Previous experience of similar instrument construction is a great advantage in the expeditious assembly of components and subsequent 'trouble-shooting' of the overall system.

The construction of the two-channel ultraviolet absorption detector is, when modifications to the original specification are made, within the capabilities and facilities of many small establishments. The advantages of building the detector, in preference to buying one, are that the physical configuration of the detector head and that the optical and electronic components may be chosen for

specific situations and types of chromatography, e.g. preparative, automated, recycling and so on. The apparent cost advantage is also very significant.

The performance of the instrument constructed and described herein was satisfactory for the purpose of this programme of analytical studies. The chromatograph was somewhat less satisfactory for operations using gradient elution because of a relatively large 'dead-volume' of tubing, about 5ml, between the gradient device and the column inlet. This could be reduced if necessary by using small-diameter tubing throughout; a low dead-volume would allow a gradient to be more accurately and precisely applied to the column. Flow-programming was, however, successfully carried out, and proved useful qualitatively.

The detector performance was not as good as that claimed for commercially-available detectors (Chapter III), but was none-the-less adequate. Non-linearity of the calibration above 0.3A units was allowed for by calibration graphs of standard compound concentrations in quantitative studies. It is probable that the Absorbance range of the detector could be increased by small changes in the design of the head and electronics, but such design optimisation was beyond the terms of reference of these studies. Baseline stability of the detector was considerably improved by cooling the detector head with ice-water cooling coils.

2. Qualitative Chromatography.

Reversed-phase liquid-liquid chromatography on Zipax ODS Permaphase was found to give some separation of the main components of Working Solution at elevated temperatures; no separation was observed, however, when the system was operated at room-temperature. A reversed-phase mode was thought to occur in chromatography using a tert-amyl alcohol in n-heptane eluant on silica (Chapters VII and XI) but this postulate was not confirmed on further study.

Liquid-solid (adsorption) chromatography was shown to be capable of separating the main components of Working Solution, using several chromatography systems.

Chromatography using alumina and modified alumina stationary phases was found to be less satisfactory than that on silica because of the strong dependence of the surface activity of alumina on moisture-content of the system. This moisture-sensitivity has been controlled by other workers by adding small concentrations of water or an alcohol to the chromatographic solvent, but it was found, for the separations under study, to be more expedient to change to silica adsorbents.

A range of different particle-size silica adsorbents were investigated. The smaller particles were found to give considerably higher efficiencies than larger sizes or a pellicular adsorbent, namely Corasil II. The packing procedure and column technology were found to be critical to the efficiency values obtained; the columns marketed by instrument manufacturers were found to have the highest efficiencies because of the advanced packing techniques and column preparation employed in their preparation. A Waters μ -Porasil column, for example, gave up to 6,000 plates under optimum conditions, compared with values of 1,000 plates or less for tap-fill and similar techniques.

The silica adsorbents were generally much less sensitive to changes in moisture-content of solvents. It was found that small amounts of moderately polar compounds such as ethyl acetate in a n-heptane solvent were sufficient to maintain a constant level of surface activity on all of the samples of the chromatographic grade silicas examined. The surface activity was found to be considerably modified by tert-amyl alcohol and also by diglyme, both compounds being strongly adsorbed onto the surface. The surface modification by diglyme was used to obtain advantageous separations of H_4 EAQ Epoxide from other main components of a Working Solution. This strongly-adsorbing type of behaviour may be a general property of di- or tri-functional compounds, and

it is suggested that similar advantageous reversible modification of the silica surface might be obtained by using other compounds, such as di-thioethers for separation of certain sulphur compounds. Control of the degree of separation was obtained in these studies by selecting the appropriate concentration of diglyme in the solvent; the speed of elution was controlled by addition to the eluant of a suitable concentration of a solvent of weaker solvent strength, which did not affect the separations obtained but allowed the compounds to be eluted more quickly. This principle, of modifying the surface activity of an adsorbent with one component of a solvent and controlling speed of elution with another component, should be capable of further extension and application.

Elution of the four main components, namely H_8 EAQ, H_4 EAQ, EAQ and H_4 EAQ Epoxide, was found to occur in order of the number of aromatic rings present in the eluted compounds. H_4 EAQ Epoxide, having an epoxide ring in addition to the H_4 EAQ structure, eluted between H_4 EAQ and EAQ. The elution order of the H_4 EAQ Epoxide degradation products has been explained elsewhere (Chapter VIII). Other components, many as yet unidentified, were noted in chromatograms of Working Solution.

The application of gel-filtration chromatography gave some indication that dimers of the EAQ-derived compounds were present in Working Solution. This observation could well reward further investigation, from the industrial point of view, since it is possible by relatively simple means to convert some of the anthraquinone-derived dimers back to monomers. If significantly large amounts of suitable dimer compounds were present in Working Solution it might be economically feasible to devise treatments to recover and convert them back to productive components such as H_4 EAQ and EAQ.

3. Quantitative Chromatography.

The development of efficient qualitative separation systems

allowed quantitative studies to be undertaken. The Waters -Porasil column, the most efficient of those examined, was employed for these studies.

It was found that the peak-height measurement method of quantitation gave significantly less variation than the integrated peak area obtained using a relatively simple electronic integrator. This was largely due to the inability of the integrator to allow for variations in baseline level. A more sophisticated integrator would be expected to be at least as precise as peak height measurement.

An improvement in precision of repetitive injections was found when using a Cheney device to give reproducible injection volumes. This reduction of the error arising from variability of volume injected resulted in a halving, approximately, of the overall coefficient of variation for these injections.

The use of internal standard normalisation to increase precision was investigated and was found to substantially improve the precision of results obtained from a series of varied volume injections. The use of internal standardisation is thus recommended wherever feasible.

External standardisation using peak height measurement was found to be a valid analytical method for the main components of Working Solution. Results obtained from analyses, using this method, of synthetic and Working Solution samples, were substantially in agreement with the values expected. The precision of results for individual components was normally within the range 1-2%. This precision should be significantly improved upon by using the Cheney device or a similar adaptation. Higher precision would also be obtained by employing internal standardisation.

The H_4 EAQ degradation products were investigated and gave satisfactory calibration graphs for use in the external standardisation method. When Working Solution samples were chromatographed, however, these compounds were not readily quantifiable because of the lack of a

suitable baseline, arising from the very large number of overlapping peaks present in chromatograms of Working Solutions. Better resolution of these bands, probably using a less concentrated solvent than the 3% iso-propanol in n-heptane here used, might allow external standardisation to be applied. It is unlikely that internal standardisation could be applied in the case of these compounds because there is no elution position in the chromatograms which is definitely unoccupied and into which an internal standard material could be fitted.

4. Elucidation of the Chemistry of Working Solution.

It was found that the 2-ethylanthrone degradation pathway postulated earlier (Chapter I) did not go to completion. No evidence was found of peaks attributable to 2-ethylanthracene in three Working Solution samples examined.

An indication of the presence of compounds of the dimer type, obtainable by oxidation of 2-ethylanthrone, was noted in Working Solution. It is possible that some of any 2-ethylanthrone formed in the process may be converted to dimeric compounds.

Evidence was found indicating that 2-ethylanthrone becomes aerially oxidised to EAQ on standing in solution. Such ready oxidation, to the dimer or to EAQ, would be one explanation why no 2-ethylanthracene was found; there would simply not be enough of the necessary precursors for 2-ethylanthracene formation.

Further investigation of the possible presence of dimers is recommended, particularly since this group of compounds might be more readily isolated than the products of other degradation routes, by preparative gel filtration, for example.

The investigation of the H_8 EAQuinol-Quinone relationship in solution led to the proposal that a quinhydrone type of compound is formed in solution. If this suggestion is correct it ~~might~~ imply that

the present cyclic process for hydrogen peroxide is working at less than peak efficiency, because the quinol form of H_8EAQ , (and presumably of H_4EAQ) would be stabilised to aerial oxidation by quinhydrone formation before the quinol was totally oxidised to the quinone. Not all of the quinol would yield hydrogen peroxide in such a case. The extent of quinhydrone formation in solution, the question of whether H_4EAQ forms a quinhydrone, and the degree of oxidation of the quinol in peroxide production are thus of some significance. It is suggested that, from the degree of 'quinone character' exhibited by each quinone, the degree of quinhydrone formation would be $H_8EAQ > H_4EAQ > EAQ$. This order is based on the similarity of the quinone structures to substituted benzoquinone, naphthoquinone, and anthraquinone respectively.

The examination of degradation products of H_4EAQ Epoxide in three samples of Working Solution allowed tentative conclusions to be reached on the conditions giving rise to these products. If there is a very low level of H_4EAQ Epoxide in solution, it is considered unlikely that there will be very significant amounts of hydrolysed or reduced compounds. If H_4EAQ Epoxide is present in significant amounts, then either the hydrolysis or the reduction process will be favoured. It appears that both reactions do not occur to great extents simultaneously. The type of degradation product most suitable for conversion back to an active compound might therefore be found and plant running conditions adjusted to convert all H_4EAQ Epoxide to that degradation product, prior to reclaiming an active compound from it.

It may be seen that the chemistry of the mixtures termed Working Solution is extremely complex and that reactions interlock in various ways. It is considered that the somewhat 'rule-of-thumb' approach to the investigations of Working Solution may be greatly helped and systematised by further application of HPLC to this problem, and that day-to-day running of the manufacturing plant could be more closely

controlled by the use of routine HPLC analyses, both for the main quinone components and as a 'fingerprint' technique to identify significant changes in the proportions of various peaks in qualitative chromatograms.

Appendix A.

Sources of Standard Samples of Components of Working Solution.

The compounds used in these studies as standard or reference materials derived from two sources. Laporte Industries donated samples of several of the compounds; 2-ethylanthrone and 2-ethylanthracene were synthesised.

1. Donated Samples.

- (a) 2-ethylanthraquinone: The main raw material for the 'A.O.' process, this was a sample from bulk stock, twice recrystallised.

Pale yellow needle crystals, melting point 103° - 104° C.
- (b) H_4 EAQ: The sample used herein was purchased from BASF, Germany, and was twice recrystallised from methanol.

Orange needle crystals, melting point 155° - 159° C.
- (c) H_8 EAQ: Obtained by passing air through a methanol solution of H_8 EAQuinol, followed by recrystallisation from methanol.

It is an orange crystalline solid.
- (d) H_8 EAQuinol: Obtained by hydrogenation of EAQ at 150° C and 2,000p.s.i. over a Palladium catalyst. The product is then isolated and recrystallised from methanol in absence of air.

It is a yellow-orange solid.
- (e) H_4 EAQ Epoxide: Prepared by an exothermic reaction between H_4 EAQ and concentrated (60%) hydrogen peroxide solution in alcoholic solution. The reaction product crystallises from solution on cooling and may be recrystallised from methanol.

It forms small white needle crystals.
- (f) 'Monohydroxy' and 'Dihydroxy' H_4 EAQ Epoxide: The hydrogenation of H_4 EAQ Epoxide at atmospheric pressure and temperature

results in the absorption of 1 mole or 2 moles of hydrogen, resulting in the 'monohydroxy' or 'dihydroxy' compounds respectively.

Both are white crystalline solids.

- (g) 'Hydrolysed H_4 EAQ Epoxide': This results from the acid hydrolysis of H_4 EAQ Epoxide.

It is a white crystalline solid.

2. Synthesised Samples.

- (a) Preparation of 2-ethylanthrone after Siavtsillo et al.⁸²

A 1-litre flask was equipped with a reflux condenser, dropping funnel and electric heating mantle. In the flask were placed granulated tin, AR, (39.0g), 2-ethylanthraquinone (33.6g) and glacial acetic acid (240ml).

The flask was heated to reflux, and to it was added, by dropping funnel, concentrated hydrochloric acid (108mls). During the addition of this acid the colour of the solution lightened from a very dark grey-brown to become yellow. The solution was refluxed for a further 30 minutes and was filtered hot through a glass sinter.

This solution was added to 1 litre of distilled water, when a yellow oil separated out. The whole solution was extracted with diethyl ether (3 x 100ml) and the aqueous layer was discarded. The ether layer was washed with water until acid-free, then was filtered and dried over sodium sulphate. This solution was mixed with 60°-80° petroleum ether in equal amounts and was cooled gradually with liquid nitrogen. The yellow solid which separated was filtered on a pre-cooled Buchner filter.

Crude yield = 13g. Melting point 52°-56°C.

This crude product was recrystallised from n-hexane, giving a creamy-white solid, 'Sample A', of melting point 60°-62°C.

(b) Preparation of 2-ethylanthrone after Battegay & Hueber⁸³.

To a 2-litre flask were added 2-ethylanthraquinone, (7.0g), sodium hydroxide (25g) and water (600ml). This suspension was brought to boiling under reflux, when sodium dithionite (about 35g) was added in small portions through the condenser. Each addition resulted in a strong foaming and transient red colouration in solution. The solution was then filtered hot and the deep-red filtrate was acidified, whereupon it changed to yellow. It was then cooled in ice, when a brown solid separated out. This solid was filtered off, dried on the filter and dissolved in diethyl ether (40ml). The solution was dried over sodium sulphate and to it was added n-hexane (100ml). It was cooled in liquid nitrogen, giving a precipitate. The ether was decanted off and a further 50ml of n-hexane was added to the precipitate, allowed to warm until the precipitate redissolved and then cooled again.

This left a substance, under hexane, which melted at just above room temperature. On standing some solid was obtained by partial evaporation of n-hexane, this solid having a melting point of 45° - 47° C for the main bulk of it, but with some crystals melting at 59° - 65° C.

This method gave considerably more difficulty in recovering the product than the first described procedure.

(c) Preparation of 2-ethylanthracene after Bapat et al.⁸⁵

To a cooled solution of sodium borohydride (10g), in diglyme, (25ml), was added a suspension of EAQ, (5.0g), in diglyme, (25ml). This ice-cooled solution was stirred slowly, in a fume-cupboard, while a solution of boron trifluoride-etherate (3.55g) in diglyme (12.5ml) was added in small portions. The solution was then allowed to warm up to room temperature and was then warmed to 30° C for 1 hour with stirring.

The solution was poured into about 400ml distilled water, giving a brown oil and an emulsion which fluoresced under long-wave-length U.V. light. This was left to separate overnight and was extracted

with n-hexane (2 x 100ml), leaving a brown tarry solid insoluble in the solvent. The hexane phase was then washed with water and passed down an alumina column. The eluate was collected and evaporated to dryness, followed by recrystallisation from n-hexane. The white solid had a strong blue fluorescence and melting point 150° - 154° C.

Yield of pure sample = 40mg.

Yield of impure sample from supernatant evaporation
= 0.3g approx.

Most of the product was probably lost in the brown oil, insoluble in hexane.

Appendix B.Instrument Parts and Suppliers.1. Chromatograph Components.

The components shown in fig. III.4 are here described and lettered as in the figure.

<u>Component.</u>	<u>Letter.</u>	<u>Supplier.</u>
1 litre stainless steel reservoir.	A	Catering suppliers.
Reciprocating pump, type HM/92/SS2C, rated to 2,400p.s.i. with PTFE gland and flameproof motor.	B	Metering Pumps Ltd., Uxbridge Road, Ealing, London.
Pressure safety switch, variable setting up to 3,000p.s.i. Model TS 10 MC	C	K.D.G. Instruments Ltd., Crawley, Sussex.
Pressure gauge, 0-400p.s.i.	D	Budenberg Gauge Co. Ltd., Broadheath, Manchester.
Pressure gauge, 0-4,000p.s.i. with steel Bourdon tube and 6" dial, designated by "Fig.11/13".	E	
Bonnet stop valves, rated to 2,500p.s.i.	F	Hoke International Ltd., Barnet, London.
Rotary ball valve, Whitey type 42F2.	G	Techmation Ltd., Edgware Way, Edgware, Middlesex.
Micro-metering valve, Whitey type 21RS2-316	H	
Injection device, Cat. No. 538479	J	Pye-Unicam Ltd., Cambridge.
Simplifix tubing couplings, various sizes, 316 stainless steel.	-	Techmation Ltd., Edgware Way, Edgware, Middlesex.

2. Detector Components.

Low-pressure mercury-neon 'PEN-RAY' lamp, made by Ultra-Violet Products Inc., California; Model 11 SC-1	Shandon Southern, (agents), Camberley, Surrey.
Fluorescent rod, 254 + 280nm, part no. 8390 - 02	LKB Instrumtents Ltd., Selsdon, South Croydon, Surrey.

Photoconductor cells,
Clairex, type CL 905 HLL-T

Walmore Electronics Ltd.,
11 Batterton Street,
London W.C.2.

Fluorescent phosphor, Merck,
type F₂₅₄,
254nm and 280nm to 540nm

Anderman & Co. Ltd.,
East Molesey,
Surrey.

Other components, such as the silica flow-cell windows and the metal machining bar-stock, were obtained from common laboratory or engineering sources.

Appendix C.Detector Construction Details.1. Machining Diagrams.

The material of the main parts of the detector was aluminium tube and round bar of appropriate dimensions.

End-pieces and cell-holder components were machined to fit the tubular body with a clearance of about .005".

2. Circuit Diagram.

The diagram shown represents Channel One; Channel Two is identical but may have a different response. This may be corrected for, if required, by adjusting the output with RT2 and RX.

All components except the photocells were obtained from Radiospares Ltd.

3. Assembly.

Order of assembly is as follows:-

3.1 Assemble detector cell with quartz windows and PTFE seals into the cutaway in the cell-holder body.

3.2 Carefully position the photocell holder on the other side of the cell, put in 4BA bolts and nuts and tighten gradually and evenly to obtain leak-free seal. CAUTION: the quartz windows may crack if over-tightened or tightened unevenly.

3.3 Slide the cell-holder assembly into the main body as far as

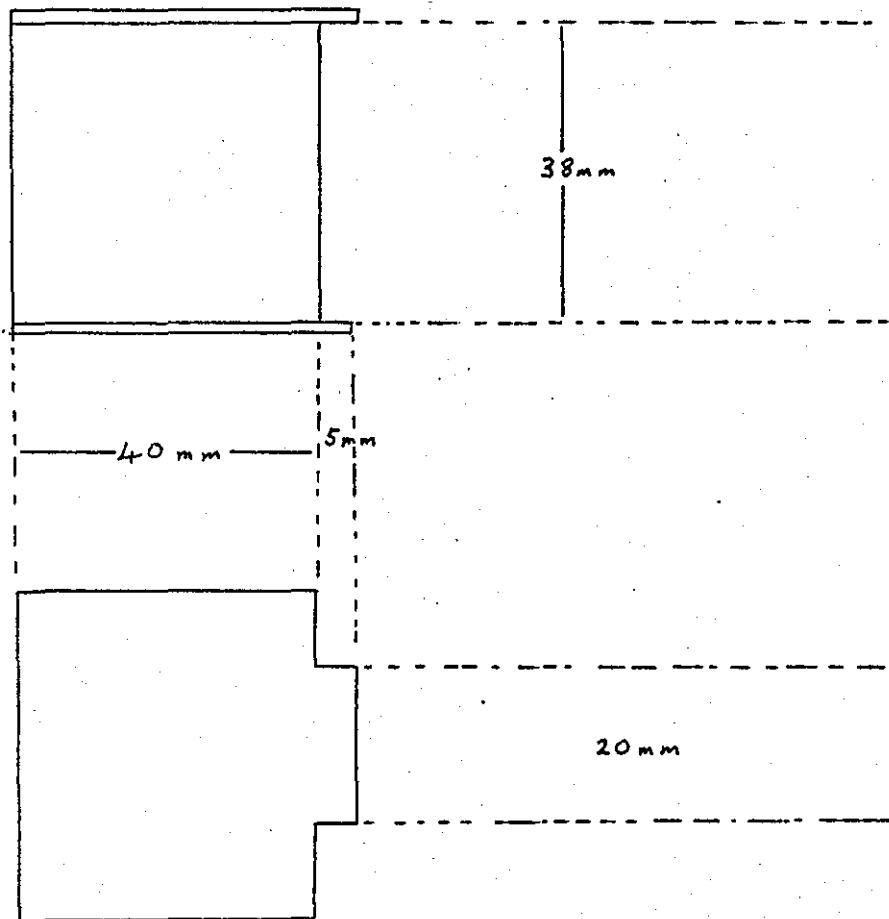
it will go and fit into it the two quartz phosphor rods; then fit the lamp-holder onto the other end of the rods and slide the cell-holder assembly, rods and lamp-holder back into the main body to their final positions. Tighten the two grub-screws to fix the positions.

3.4 Fit the photocell/filter/phosphor assemblies into their appropriate positions in the photocell holder and fit the end-cap complete with electrical connector. If the cutaway in the main body and the tongues on the end-cap are well machined the resulting interference fit should hold the parts firmly together.

3.5 Fit the cooling coils to the detector, then fit the mercury lamp. Ensure that no light can enter at the top or bottom of the flow-cell assembly (use black PVC tape or rubber strip to seal it).

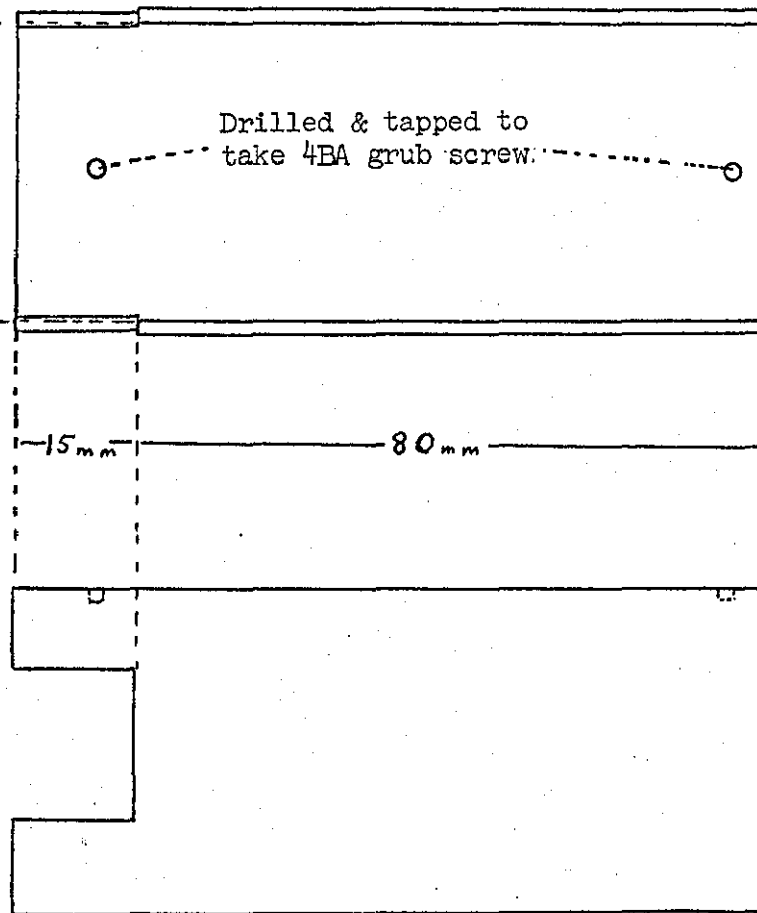
3.6 Connect to electronics by suitable connector cable.

Vertical section



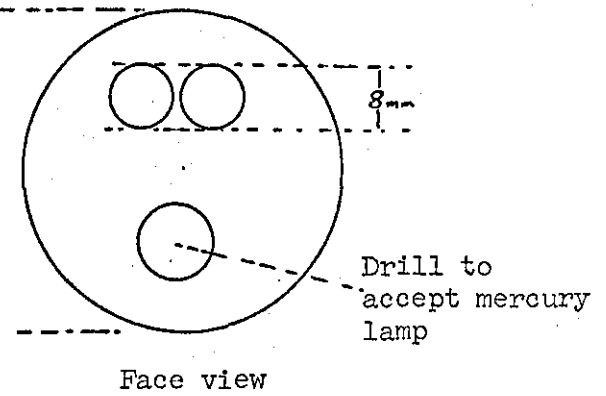
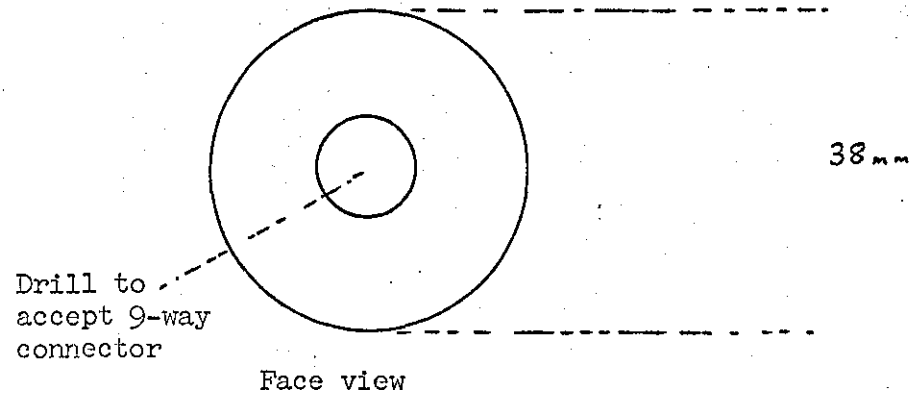
End cap, downward view

Vertical section

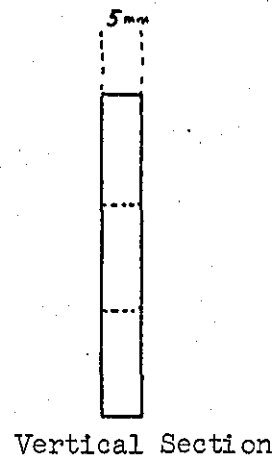


Main body, downward view

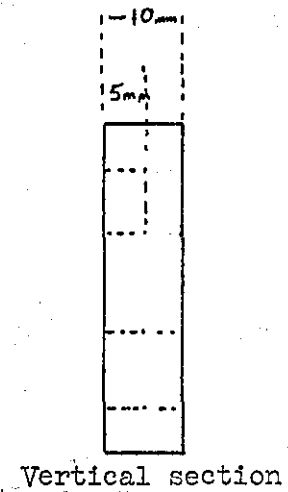
Detector Body.



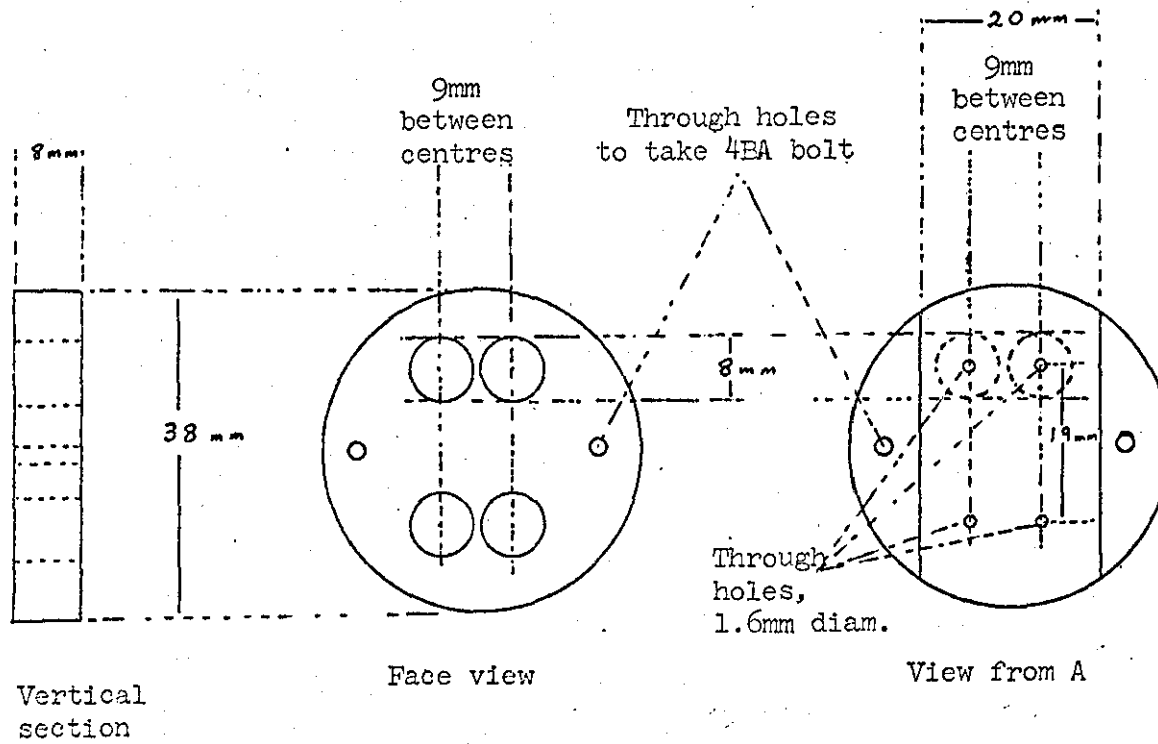
Material:
Black PVC
or Bakelite



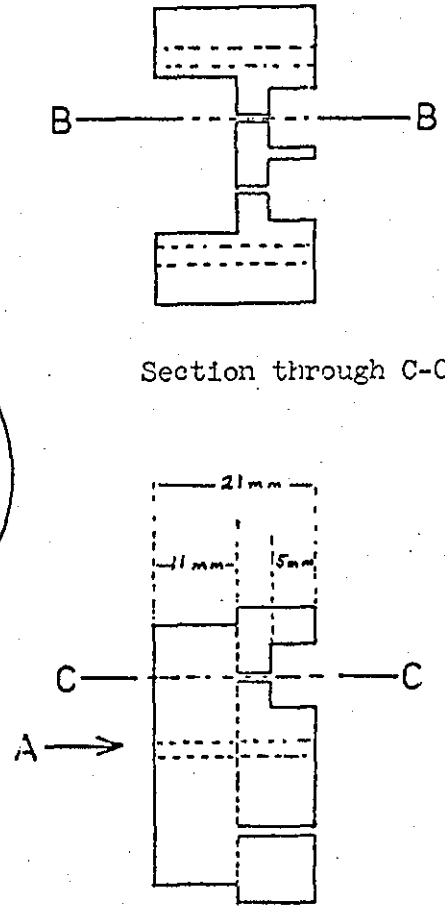
Photocell Connector Holder



Lamp Holder

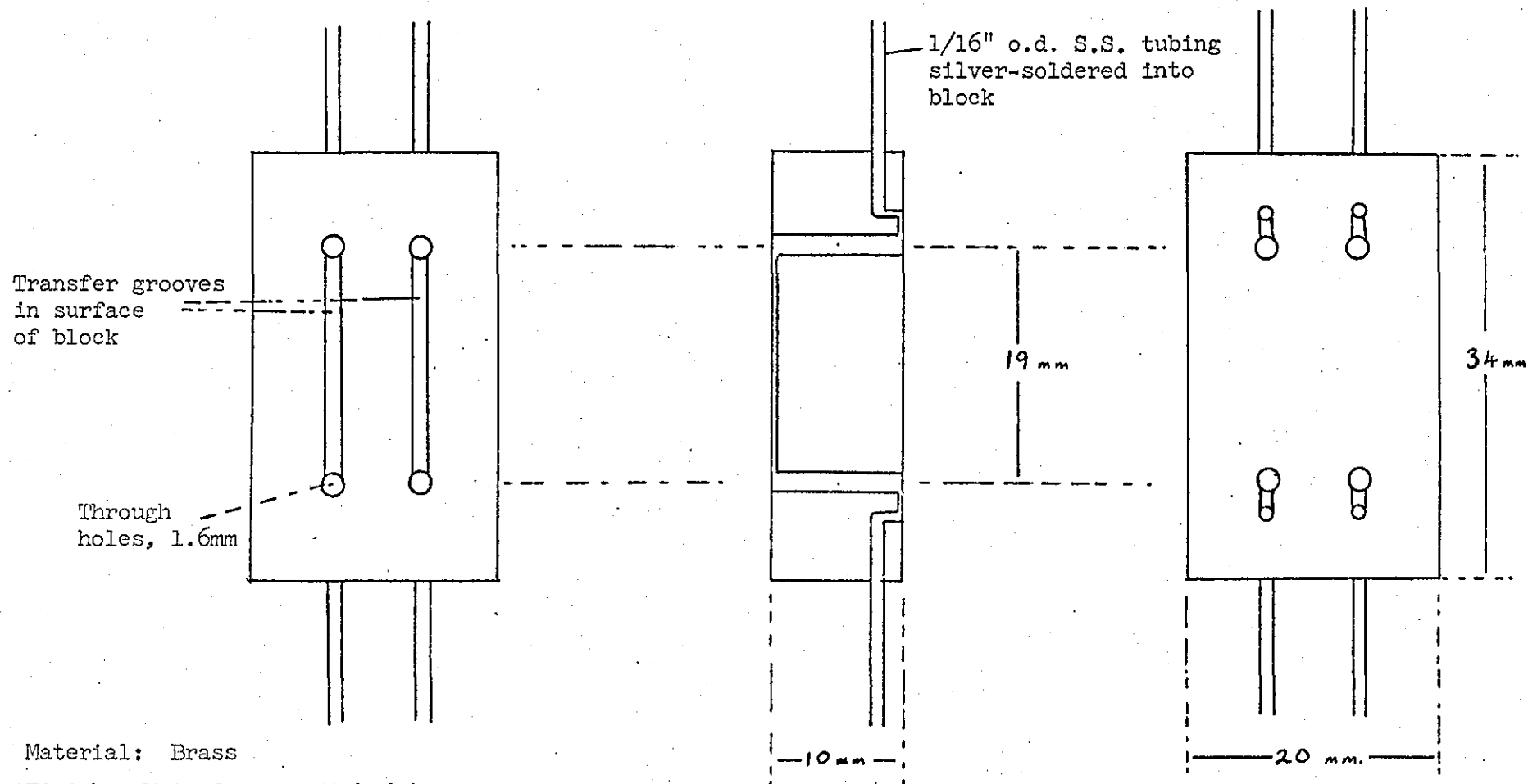


Photocell holder



Cell-holder body

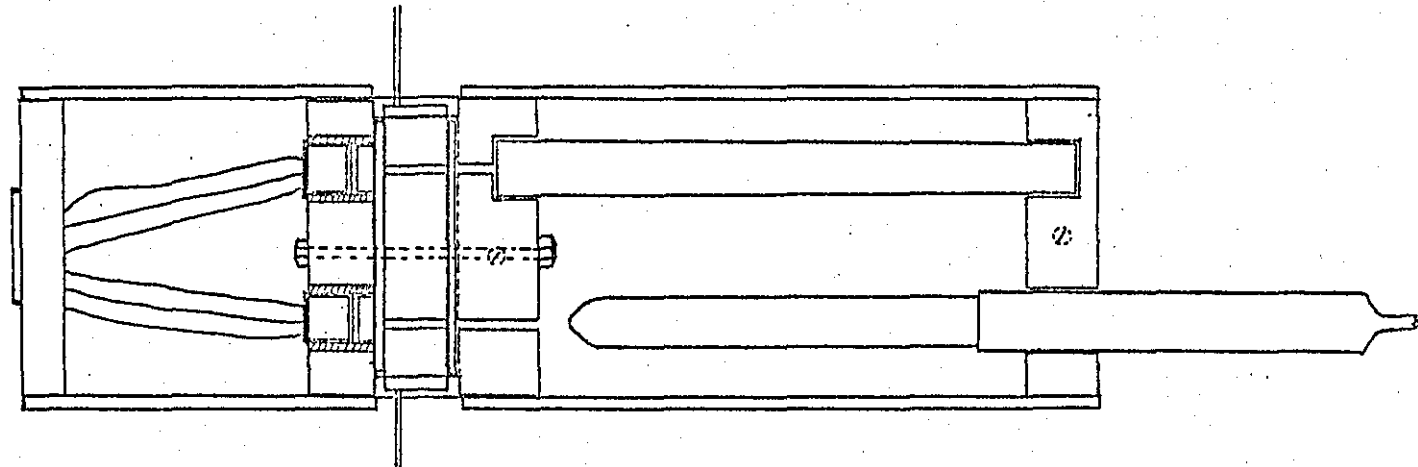
Cell-Holder Assembly.



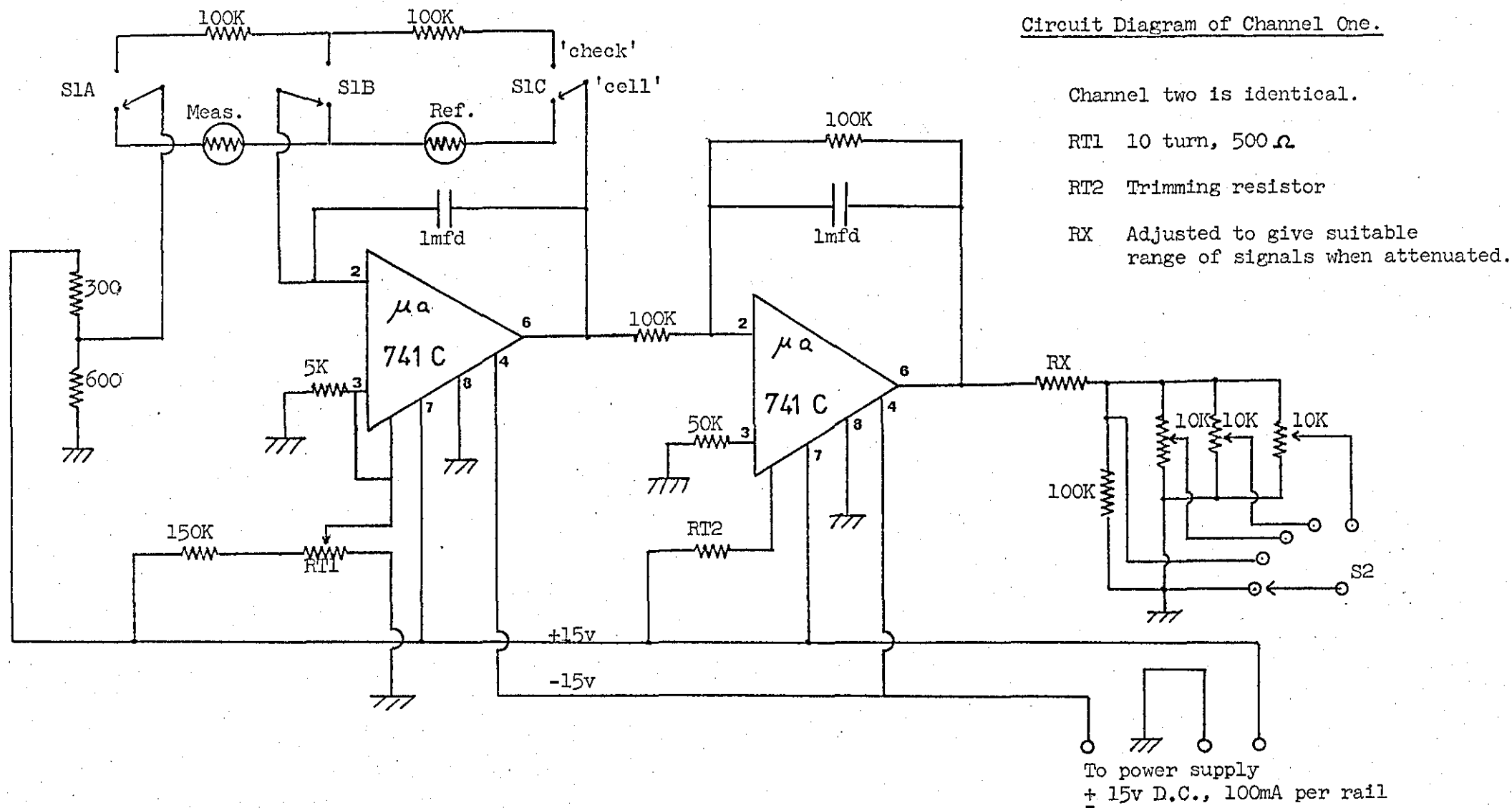
Material: Brass

Finish: Main faces polished to
mirror finish, then
bright nickel plated.

Detector Cell.



Sectional Diagram of Detector Head.



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