THE SCALE UP AND MODELLING OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

A thesis submitted to the University of London

for the degree of

DOCTOR OF PHILOSOPHY

by

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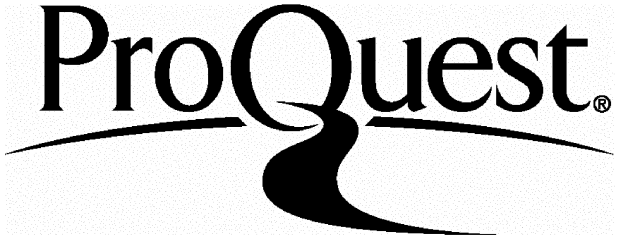

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ABSTRACT.

This thesis concerns the scale up and modelling of High Performance Liquid Chromatography. It aims to fulfil the need for a good empirical model which describes and predicts chromatographic performance but without recourse to a mathematical procedure which requires many assumptions and is mainly theoretical.
The project therefore attempted to predict large-scale separations from small-scale analyses on the basis of fractionation diagrams. The results show that the target was met in part i.e. the model was developed but could not be verified on a large scale. The reason was the unexpectedly good separation on a large scale which equalled that achieved on the analytical scale.

The project also aimed to show the sensitivity of the large scale separation to process variables, and to devise a practical protocol for running large scale chromatographic separations. The thesis contains reports of experimental optimisation of chromatography conditions which will be of interest to other chromatographic researchers. The large scale column can be packed and operated satisfactorily provided certain rules are observed. The detailed points are summarised in the conclusion to the thesis (Chapter 5).

Its claims to originality and novelty are :

- the use of fractionation diagrams as a modelling tool for chromatographic processes.

- a detailed examination of 35 micron particles of polymeric origin in a large scale HPLC column (silica is widely used and majority of experiments are carried out on analytical scale)

- comparisons of the behaviour of samples of different diffusion coefficient on a large scale column at various flow rates (h/ν curves and the related implications)

- a novel method for the determination of column band broadening and extra-column band broadening

The thesis is laid out as follows :

1. The introduction section contains an overview of the history and development of HPLC, a literature survey of erythromycin analytical separation techniques, all the chromatographic theory relevant to this project including band broadening, column supports, etc., and a brief review of models used in mathematical modelling.

2. The results section describes the analytical separation of aromatic compounds where familiarisation with the HPLC system and the theory is outlined; this is followed by the analytical separation of erythromycin where the method for the separation of erythromycin is described and several aspects of the separation are investigated.
   The semi-preparative stage mainly describes the treatment of the packing material and the packing of the columns for the scale up studies. The natural progression to a large scale Prochrom column (180 x 60 mm) is then shown together with an explanation of the system, its operation and its results, with particular regard to efficiency and band broadening. Finally, the principles of modelling using fractionation diagrams are explained and the theory supported by results.

3. Discussion of the results obtained:

a) Comparisons of the performance of large and small scale chromatography columns.

b) The role of extra-column band broadening in large scale chromatography with regard to velocities and Reynolds number.

c) Discussions and conclusions of findings of modelling studies based on fractionation diagrams.

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1. Aims and Objectives

Aim 1. To commission and operate a large scale Prochrom chromatography system in order to purify erythromycin. To troubleshoot analytical and large scale chromatography systems.

Aim 2. To determine the effect of mobile phase variations on the separation of erythromycin. This is because the large scale system requires the preparation of substantial amounts of mobile phase and operation of the system is susceptible to operator error.

Aim 3. To determine how overload conditions and scale would affect the separation.

Aim 4. To establish the extent of extra-column band broadening on a large scale column.

Aim 5. To determine the role of h/ν curves with different compounds on the Prochrom system and the factors influencing the minimum value of reduced plate height.

2. Introduction

2.1 The history and development of HPLC

Summary

This chapter deals with the development of chromatography from its early stages at the beginning of the 20th century, progressing through some of its milestones, up to the first signs of the emergence of the state of the art HPLC technique in the late 1960s.

As Tswett is regarded as the father of chromatography, a substantial section is devoted to his revolutionary first paper which laid the foundations for a whole range of separation techniques.

2.1.1 The early development of chromatography

Although the first signs of chromatography began to appear in the 19th century it was not until 1903 when the Russian botanist M. S. Tswett published a paper (1903) on the separation of xanthophylls and chlorophyll on an inulin (polysaccharide originally obtained from the tubers of the dahlia) column that chromatography was officially recognised as a new separation technique. The author tested 110 different column packing materials and emphasised the importance of adsorption in achieving good separation.

2.1.1.1 Tswett and his revolutionary paper

Tswett in his paper (1903) tried to explain the phenomenon called adsorption which at that time was not very well known and understood. He conducted experiments on the insolubility of chlorophyll in petrol and ligroin (a petroleum fraction comprised largely of heptane and hexane with a boiling point in the range of 90 to 120°C). Green leaves contain chlorophyll which comprises five pigments. When these leaves are soaked in ligroin, only carotene pigment can be extracted from them. However, the addition of only a small amount of another solvent (e.g. alcohol) enables the extraction of all the remaining pigments. He ascribed this phenomenon to adsorption. In this case the adsorption tissues were green leaves. He correctly predicted from this outcome that

other substances could be used as adsorbents. He then went on to test more than a hundred different substances for use as adsorbents. Amongst them were elements such as sulphur, magnesium, zinc, iron, and lead; oxides and hydroxides; acids; phosphates; nitrates; aldehydes; amides; alkaloids and others. Bone charcoal appeared to be the best adsorbent as it adsorbed the pigments completely. This was due to "the outstanding porosity of this substance" (Quincke 1859). Solvents which he used included: alcohol, acetone, ether, chloroform, and ligroin.

The author described the process of adsorption as a physical one. He correctly pinpointed the need for a carefully chosen solvent whose properties were known. It had to be of a definite chemical composition, easily obtainable in a pure state, and of easily determinable solubility. This paper, which was published in 1903 in Russian and only translated later (1906) into German, laid the basis for chromatography. Tswett showed here great scientific thinking: a person who was capable of analysing and interpreting data with great precision and able to draw very sensible conclusions from them, bearing in mind that he was a botanist rather than a chemist.

2.1.1.2 Tswett's two additional papers

In his second publication Tswett (1906*) described some of the aspects of chromatography which remain critical today in achieving a satisfactory separation. Some of them are: the importance of regular packing (fine particles giving better separations than coarse particles); purity of packing material; the importance of the top of the column not being disturbed when applying the sample or the solvent; and the possibility of chemical change on the columns because of adsorption phenomena.

The third paper (1906**) was devoted to the utilization of columns with a larger diameter (2-3 cm) - preparatory columns.

Tswett was undoubtedly far ahead of his time. Unfortunately his work was not appreciated for several reasons: firstly his results were difficult to reproduce as they required considerable technical expertise; Willstätter and Stoll (1913) expressed doubts about the feasibility of this separation. Williams (1947) claimed that this new technique was not needed at that time although Mikes (1966) stated that the method could indeed be useful.

These factors, together with Tswett's final thesis being written in Russian and therefore hard to access, postponed further development in this area of analytical research.

2.1.2 Development of chromatography in the 1930s

Some twenty years after Tswett's breakthrough, during which time chromatography had lain dormant, Kuhn (1931*) and Lederer (1931*) together with Winterstein (1931) used preparative chromatography to separate alpha and beta carotene and also egg-yolk pigments.

2.1.2.1 Manhattan project and API project

The use of chromatography really took off in the 1940s when two main projects were undertaken: the Manhattan project and the API (American Petroleum Institute) project.

The Manhattan project (Spedding et al. 1947) dealt with the purification of rare earth elements by ion-exchange chromatography.
The API project developed in the late 1940s and early 1950s used preparative liquid chromatography to fractionate samples of virgin crude oil and petroleum distillates to determine their contents of paraffins, naphthalenes, olefins and aromatics (Camin and Raymond 1973).

2.1.2.2 Preparative nature of early chromatography

Initially chromatography was carried out for quantitative, qualitative and preparative analysis using columns of larger diameters and larger particle sizes (100μm and more). The flow of the mobile phase along the column was dependent on gravity, sometimes accelerated by hydrostatic pressure. Because of the large particle sizes used the efficiency of these columns was quite low (Engelhardt 1979).

2.1.3 Other separation techniques

Although to begin with chromatography had a preparative character, it shortly developed into an analytical method. Kuhn, Lederer and Winterstein's first important chromatography separations gave rise to the development of other analytical separation techniques. 1938 marked the beginning of thin layer chromatography (Izmailov and Shraiber 1938).

In 1941 Martin and Synge introduced partition chromatography. Paper chromatography emerged thanks to Consden, Gordon and Martin in 1944. Ion exchange chromatography was developed in 1949 by Spedding followed by gas chromatography in 1952 (James and Martin), capillary GC in 1958 (Golay) and size exclusion chromatography in 1959 (Porath and Flodin). The first signs of a new method of separation appeared in the publications of several workers: Hamilton (1966) and particularly Giddings (1965) who gave an account of a fundamental theory of HPLC.

Liquid chromatography can be devided into two main classes: classical or conventional LC, and modern or high performance liquid chromatography also known as high pressure LC (HPLC). The latter is distinguished from conventional LC by its use of sophisticated instruments, high efficiency columns, and extremely sensitive detectors.

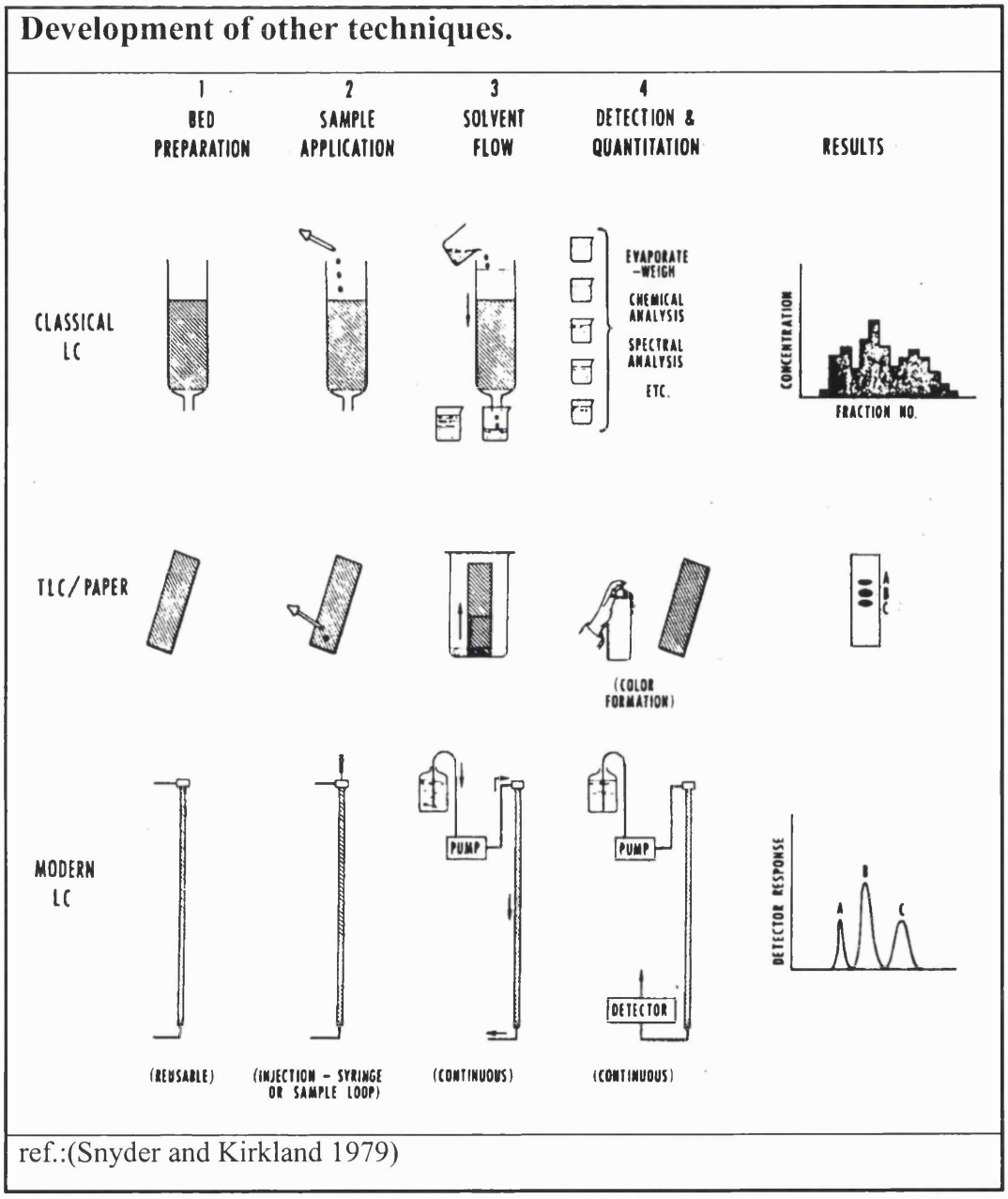


Figure 2.1-1 Examples of equipment used for classical, TLC/paper and modern LC.

2.1.3.1 Gas chromatography versus liquid chromatography

Liquid chromatography arose in the late 1960s and is a complementary method to gas chromatography. In fact gas chromatography gave the theoretical base for liquid chromatography. The latter developed as a result of a limitation of GC: liquid

chromatography can easily separate substances which cannot be vaporised readily. HPLC is particularly suitable for the separation of compounds with:

a) high polarity

b) high molecular weight

c) a tendency to ionise in solution

d) thermal instability.

As there are differences between LC and GC, allowances had to be made for LC. As liquids are 20 to 100 times more viscous than gases, higher pressures are experienced in LC.

As the diffusion rates in LC are between 3000 to 30000 times lower than in GC, particles of much lower diameter have to be used (5-10μm). The columns used in LC are shorter: about 100 to 250 mm long, 5mm in diameter, and with operating pressures of 300 to 3000 psi. The equipment must withstand high pressures and the use of eluents of various compositions and pH range: for this reason stainless steel and PTFE are the materials most commonly used by manufacturers (Knox 1978).

2.1.4 Classical chromatography

Classical chromatography was performed on glass columns roughly 100cm x 5cm. The column had to be emptied after the analysis and repacked for the next separation. As many of the stationary phases were not very efficient, long columns (with consequent long retention times) had to be used to accomplish the analysis. The consumption of solvent was consequently high. Additionally, the individual samples had to be collected manually. A typical separation therefore required several hours which was very time-consuming. The results were recorded as a chromatogram, a bar graph of sample concentration against fraction number (Snyder and Kirkland 1979).

The packing material used in these separations included aluminium oxide, calcium carbonate, magnesium oxide, and fuller's earth (an earthy hydrous aluminium silicate, capable of absorbing grease): there was no mention of silica gel which is the most popular packing material today (Verzele and Dewaele 1985).

2.1.4.1 Classical LC versus HPLC

MODULE	TRADITIONAL **Liquid Chromatography.**	HPLC
COLUMNS	used once then discarded	closed reusable columns which enable the running of many separations
SAMPLE APPLICATION	requires skill and time on the part of the operator	precise sample injection using syringe injection or sample valve
SOLVENT FLOW	by gravity feeding of the column	by high pressure pumps which result in controlled flow of solvent
FRACTIONS	collected manually	fraction collector
DETECTION	manual analysis of fractions	on - line detectors
SEPARATION TIME	time - consuming, long hours	several minutes (less than one hour)
CHROMATOGRAM	a bar chromatogram of sample concentration vs. fraction number	sharp peaks (plot of concentration vs. detector response)

Table 2.1-1 A comparison of traditional and HPLC techniques.

The "birth" of HPLC was a result of an immense improvement in instrumentation, packing material, columns and, above all, in the understanding of chromatographic theory.

2.1.5 HPLC - High Performance Liquid Chromatography

2.1.5.1 Historical development of HPLC

HPLC plays an important role as a fast, sensitive and accurate analytical method. The development of HPLC was triggered by the discovery of DNA. The more recent technique of separating subnanolitre quantities of nucleotides and nucleosides from hydrolysates of DNA and RNA was not available when HPLC was first being developed. The results achieved using the columns available were not satisfactory: retention times were still long and other characteristics were not reproducible even when applying modules from the same company; pump flowrates were inconsistent and a sensitive universal detector for HPLC which could be as good as the flame ionization detector for GC did not exist (Brown 1990).

In 1958 the first liquid chromatograph was produced (Spackman et al. 1958).

Some years later Horvath and Lipsky (1966)(1967) pioneered the development of HPLC. The authors constructed and published a complete system for separations.

The technique was later developed (1968-1973) into modern HPLC by Huber, Kirkland, Majors, Snyder, Unger, Karger, Knox and other scientists. By the end of this period, HPLC developed using 5 μm silica gel and 10 μm reversed phase bonded silica (Majors 1973).

The development of microparticle chemically bonded packing was a major breakthrough which made HPLC a valuable separation technique for the life sciences (Kirkland 1971, 1972). The reputation and convenience of this technique was enhanced by the introduction of reversed phase packing which made it a more universal tool (Halasz and Sebastian, 1969). Subsequent to 1975 the microprocessor began to play a major part in the wide range of chromatography data processing and data acquisition (Scott 1985). By the late 1970s HPLC became an accepted technique in any laboratory requiring a good separation (Snyder and Kirkland 1979).

As HPLC has developed the size of particles used has decreased. Today use is made of micro-particulate column packing consisting of uniform silica particles of spherical shape, usually sized between 3 and 10 μm. Seventy-five percent of separations are now performed on chemically bonded phases. Typically the column length is 100 to 250 mm

and the diameter 4.6 mm. The mobile phase is pumped through the column at 1 to 5 ml/min. (Lindsay, 1992).

HPLC serves as an analytical tool and its speed, high resolving power, versatility and predictability facilitate the performance of highly satisfactory qualitative and quantitative analyses with samples of different origins and structure. These analyses give a high number of theoretical plates coupled with good resolution and capacity.

Taken together these factors make HPLC a good candidate for scale-up and production-scale LC for the high value-added products which are becoming so important.

HPLC uses narrow columns of small diameter (2-8mm). The speed of separations is increased by pumps with high pressures: 10-400 bar. (Engelhardt, 1979).

The revival of LC in 1970, leading to the development of HPLC, induced the study of preparative separations. Preparative LC has generated wide appreciation in the pharmaceutical industry. With the rapid development of new pharmaceuticals arising from the recent remarkable progress in genetic engineering (recombinant products), the need has emerged for a good preparative technique which can fulfil the current requirements of speed, purity, quantity, quality and cost effectiveness.

2.1.5.2 Main features of HPLC

- small particles (3-5 μm)
- regular (uniform) particles
- spherical particles

These three features in particular helped to minimise diffusion and enabled a rapid interaction of the solute with the surface. Having these aspects under control leads to an improved column efficiency, i.e. increase in plate number.

Great progress in the field of instrumentation brought about the following characteristics of modern HPLC technique:

- powerful and versatile technique

- more convenient and less operator dependent
- minimum sample preparation
- on-line (continuous) detection - increased speed of data capture and analysis (advances in microprocessor development- computer controlled instrumentation)
- highly sensitive detectors - with the use of spectrophotometers operating down to 190nm, it is possible to detect almost any compound type
- more reproducible operation
- greater accuracy and precision
- better & faster separation.

2.2 Chromatographic theory

2.2.1 Chromatography characteristics

2.2.1.1 Capacity factor (k')

In partition chromatography, which is based on the partitioning of a solute between a stationary and a mobile phase, the principle is governed by Nernst's law:

$$K = Cs/Cm \qquad (2.2.1)$$

where K is the distribution constant and Cs and Cm are concentrations of the solute in the stationary and mobile phases, respectively. This can be expressed in relation to the capacity factor as:

$$K = k' \, Vm/Vs \qquad (2.2.2)$$

where Vm and Vs are the volumes of a sample in the mobile and stationary phases, respectively.

The capacity ratio in liquid chromatography is the ratio of the amounts of the compound in the stationary and mobile phases.

$$k' = (t_R - t_o)/t_o \text{ or } k' = (V_r - V_o)/V_o \qquad (2.2.3)$$

where t_R is the retention time of a compound - which is the time that a peak spends in the column

t_o is the hold-up time - the time of an unretained, unadsorbed compound; alternatively, the retention time of the mobile phase

V_r retention volume

V_o mobile phase volume of a column

2.2.1.2 Selectivity factor (α)

It is expressed by the separation factor α and is a measure of the ease of the separation.

$$\alpha = k_2' / k_1' \qquad (2.2.4)$$

where k_1' and k_2' are capacity factors of compounds 1 and 2, respectively.
This means that high values of α can give a better separation. It follows that if $\alpha = 1$ the separation does not take place. The α values are much more important in preparative chromatography than they are in analytical chromatography. There is a desire to achieve the highest possible value of selectivity factor that will allow greater loads to be used on the column and consequently an increased rate of production.

2.2.1.3 Resolution (R_s)

Is a degree of disengagement of two bands. Small values of R_s usually appear as overlapped bands (Snyder, Glajch and Kirkland 1988). To calculate R_s from the band, the following equation can be used:

$$R_S = \left(t_2 - t_1\right) / \left[(1/2)\left(W_1 + W_2\right)\right] \qquad (2.2.5)$$

t_1 and t_2 refer to the retention times of two adjacent bands and W_1 and W_2 are their baseline bandwidth. This equation is used when bands 1 and 2 are completely separated. ($R_s \geq 1.5$).
However, when the peaks overlap, baseline measurements become difficult. In these circumstances the widths of the peaks are measured at their half height.

$$R_s = 1.18\ (t_2 - t_1) / (w_1 + w_2) \qquad (2.2.6)$$

where w_1 and w_2 are peak widths of compounds 1 and 2 at half height.
For another way of calculating resolution see the end of Section 2.2.2.3.

2.2.1.4 Symmetry (S)

The symmetry factor determines how symmetrical the peak is. This is measured at 10% of the height of a peak where the peak is devided into two parts by the perpendicular running from the peak crest to its base. This gives us two widths of the peak w_{p1} and w_{p2}. Thus symmetry can be calculated as:

$$S = \frac{w_{p2}}{w_{p1}} \qquad (2.2.7)$$

Symmetry S=1 is ideal but small variations from this value are acceptable.

2.2.2 Efficiency

2.2.2.1 Number of theoretical plates (N)

The efficiency of a chromatographic system can be expressed by the number of theoretical plates:

$$N = 5.54(V_R/w_{1/2})^2 = 5.54\ (t_r / t_{w1/2})^2 \qquad (2.2.8)$$

where $w_{1/2}$ is the peak width at half height

t_r is the retention time of a compound

$t_{w1/2}$ is the half width at half height

2.2.2.2 Height equivalent to a theoretical plate (H)

Column performance can also be expressed by the plate height or height equivalent of one theoretical plate H and it is equal to:

$$H = L/N \qquad (2.2.9)$$

where L is a column length and N is a number of theoretical plates.

2.2.2.3 Derivation of the number of theoretical plates

Because sample zones in HPLC are nearly Gaussian in shape, they can be defined by the equation for the standard error function. As a result the measure of band width of a chromatographic peak is the same as the standard deviation of the distribution σ. Height Equivalent to a Theoretical Plate (HETP) was defined by Martin and Synge (1941) as

$$H = \frac{\sigma^2}{L} \qquad \text{or:} \qquad (2.2.10)$$

$$N = \frac{L^2}{\sigma^2} \qquad (2.2.11)$$

where σ is a band width and L is a column length.

If we assume that zone width w is approximately 4σ the equation for the theoretical plates can look like:

$$N = \frac{L^2}{\left(\frac{w}{4}\right)^2} = 16\left(\frac{L}{w}\right)^2 \qquad (2.2.12)$$

When N is calculated from a chromatogram, L and w must be converted to time based units which gives:

$$N = 16\left(\frac{t_R}{W}\right)^2 \text{ or } N = 5.54\left(\frac{t_R}{w}\right) \qquad (2.2.13)$$

where W is the peak width at the baseline of a chromatogram

w is the peak width at half height.

The factors 16 and 5.54 come from statistical mathematics related to the Gaussian shape of the peak.

Plate height is usually calculated from N:

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

and this formula is useful when comparing the efficiencies of columns of different lengths (Bidlingmeyer 1992).

To determine how the resolution is related to other separation parameters and how it can be controlled by choosing the best experimental conditions, the following equation (also known as Purcell's equation) is used:

$$R_s = (1/4)(\alpha - 1)N^{0.5}[k'/(1+k')]$$

2.2.3 Porosity.

One of the routine checks done on a column is to calculate the bed porosity. This determines how good the column is. Bed porosity is determined from a void volume-volume of an unretained peak such as uracil. If that is not possible, the t_0 value is taken from the first baseline "blip" on the chromatogram.

2.2.3.1 Total Porosity (ε_t)

$$\varepsilon_T = \frac{V_0}{V_C} = \frac{Ft_0}{\pi r^2 L} \qquad (2.2.14)$$

where r is the column radius

F is a volumetric flowrate

V_C is the column volume

A well-packed column would have a porosity in the range of 0.6-0.9. Porosity which exceeds 0.9 means that the compound used for the test was retained.

2.2.3.2 Internal and external porosity

Total porosity consists of two types of porosity:

a) porosity within the particles or intraparticle porosity (internal porosity) and

b) porosity between the particles or interparticle porosity (external porosity)

$$\varepsilon_T = \varepsilon_i + \varepsilon_e \qquad (2.2.15)$$

where

ε_e is external porosity

ε_i is an internal porosity

The interparticle (or external) porosity has a value between 0.35 and 0.5. The value of the overall porosity is between 0.6 and 0.8.

2.2.4 Darcy's law and Carman-Kozeny equation

In order to calculate a pressure drop across the column Darcy's law equation is used. It is a permeability equation which relates the flow of fluid through a porous medium to the pressure gradient causing the flow. Darcy's law was essentially derived from Ohm's law of fluid flow..

2.2.4.1 Ohm's law

Ohm's law states that the flux of a current is proportional to the pressure drop per unit length

$$q = -K_p \frac{dp}{dz} \qquad (2.2.16)$$

where K_p is a constant of proportionality (empirical parameter) and $\frac{dp}{dz}$ is the pressure drop per unit length.

2.2.4.2 Darcy's law

Darcy's law enables the calculation of a total flow through a known medium under the given hydrostatic conditions.

$$K_F = \frac{F\eta L}{A\Delta p} \qquad (2.2.17)$$

where

K_F is a specific column permeability

A is the surface area of the column opening

μ is a mobile phase viscosity

Δp is a pressure drop across the column

The disadvantages of Darcy's law are its limited applicability to laminar flow and the fact that it does not reveal much about the flow pattern. There was therefore a need for a more developed equation. This came in the form of the Carman-Kozeny equation (Coulson and Richardson 1978). This equation extends to turbulent flow and also includes a porosity parameter.

2.2.4.3 Carman-Kozeny law

The Carman-Kozeny equation relates permeability and porosity. This equation is also used to calculate a particle size. The size of particles in a packing is not normally uniform but usually falls within a given range. Obviously, the narrower the given range the better.

2.2.4.3.1 Permeability for non-porous particles

Carman-Kozeny derived the equation to calculate permeability from particle size for columns filled with regular non-porous spherical particles.

$$K_{CK} = (d_p^2\ \varepsilon_e^3)/[k(1-\varepsilon_e)^2] \qquad (2.2.18)$$

where K_{CK} is the Carman - Kozeny permeability

d_p the particle size

ε_e the interstitial (external) porosity and

k a constant related to the form of the particles ($150<k<200$)

2.2.4.3.2 Permeability for porous particles

For porous packings the chromatographic permeability is:

$$K_{TO} = \frac{u\eta L}{\Delta p} \qquad (2.2.19)$$

where u is linear velocity

K_{TO} can be related to K_F as follows:

$$K_F = \varepsilon_t K_{TO} \qquad (2.2.20)$$

where K_F is specific permeability (Darcy's law)

Linear velocity

The velocity used in the equation is a linear velocity of the mobile phase.

This can be calculated in two ways:

a) when t_0 is known:

$$u = \frac{L}{t_0} \qquad (2.2.21)$$

[cm/sec]

where t_0 is a hold-up time (the time when mobile phase is eluted of the column)

b) when t_0 is not known, it is calculated from volumetric flowrate F

$$u = \frac{F}{A\varepsilon_t} \qquad (2.2.22)$$

where F is a volumetric flowrate [ml/min]

A is a geometric cross-section of the column [cm^2]

2.2.5 H/u curve

The plate theory of chromatography was developed by Martin and Synge (1941).

This theory managed to describe the results but does not explain how and why the chromatography works. Therefore a number of researchers have attempted to describe

band spreading in the column. Two of these theories have become most important: Van Deemeter equations and the Knox equation.

2.2.5.1 Van Deemter equation

The best known approach (theory and equation) is that by van Deemter, Zuderweig and Klinkerberg (1958) which is based on the mass balance model.
This equation expresses the sum of all the contributions to the H value:

$$H = A_H + B_H/u + C_H u \qquad (2.2.23)$$

where A_H is the eddy diffusion

B_H is the longitudinal (axial) diffusion

C_H is the mass transfer resistance

u is the linear solvent velocity

Peak dispersion against mobile phase velocity is often expressed in a van Deemter plot or H/u curve. This curve can tell us about the quality of the packing material used, whether the packing procedure was correct, and whether this was the right chromatographic system for the compounds of interest to be separated.

2.2.5.2 Knox equation

The above van Deemter equation is now, however, obsolete. Knox and Parcher (1969) and Bristow and Knox (1977) later discovered that the A-term exhibited dependency on velocity for columns packed both with non-porous glass beads and porous non-spherical particles of Chromosorb. This fact led to the so-called Knox equation which is now widely used amongst researchers.

$$h = A\nu^{1/3} + \frac{B}{\nu} + C\nu \qquad (2.2.24)$$

where ν is a reduced velocity, defined on the next page.

In the early days of chromatography, B term and A term were neglected as it was not possible to measure the former and the latter was negligible because the columns were not very well packed at that time. Therefore the plate height was determined by the C term or H = Cu. However, with the advent of the use of small particles, the B and particularly the A terms became vitally important (Verzele and Dewaele 1985)

A, B and C terms are defined as follows:

$A = 2\lambda d_p$ (eddy diffusion term) (2.2.25)

$B = 2\gamma D_m$ (longitidinal diffusion term) (2.2.26)

$C = \omega d_p^2/D_m$ (mass transfer term) (2.2.27)

where

λ is a packing factor which value equals 0.7

γ is a geometrical factor with a value of 0.5

ω is a geometry factor

other terms are defined elsewhere.

The dominant factors which control plate height are therefore: flow velocity, particle diameter, packing structure, packing geometry and diffusion coefficient. They are commonly referred to as kinetic parameters.

The longitudinal diffusion or B-term becomes significant at very low flowrates particularly as the diffusion coefficient is large. The mass transfer or C- term is directly proportional to reduced velocity. It represents the right side of the h/v curve. It is directly proportional to the particle size and indirectly proportional to the diffusion coefficient of the solute. This means that the slope of the right hand side of the curve will be steeper the larger the particle size and the minimum reduced velocity will be reached at lower velocities. This is because the mass transfer contributions would lead to a larger band broadening. However, the tendency in chromatography is to use higher velocities in order to reduce the separation time. It is therefore obvious that, to enable the chromatographer to operate at low plate heights (large column efficiencies) and larger velocities, a small particle diameter needs to be employed. Although the C-term is affected by the diffusion coefficient it is affected to a much greater extent by the particle diameter because the C-term increases according to the square of the latter. Solutes with

larger diffusion coefficients are preferred at high velocities as large diffusion coefficients in this case reduce the value of C-term (unlike in the case of the B-term) as they speed up the diffusion process.

2.2.5.3 Reduced velocity and reduced plate height

The lowest value on the H/u curve is directly related to the size of the particles. In order to compare columns with different particle sizes reduced parameters of plate height and velocity can be used. The equations for reduced plate height and reduced velocity follow:

Reduced velocity:

$$\nu = \frac{d_p u}{D_m} \qquad (2.2.28)$$

where:

ν is reduced velocity, also known as particle Peclet number

d_p is a particle size

D_m is a diffusion coefficient of a solvent

u is linear flowrate

Reduced velocity tells whether an average solute molecule is moved primarily by the flow or by diffusion. If the value is >1, the solute movement is governed by the flow. On the other hand, a value <1 would indicate that diffusion has priority over flow in the movement (Giddings 1965).

Reduced plate height:

$$h = \frac{H}{d_p} \qquad (2.2.29)$$

H is the height equivalent to a theoretical plate

For a moderately well-packed column h=3 (Subramanian 1991)

2.2.5.4 The optimum flowrate

The ideal reduced velocity occurs when ν is between 3 and 5. To calculate the volumetric flowrate which corresponds to this value, the diffusion coefficient D_m needs to be determined. This comes from Wilke-Chang equation (1955):

$$D_m = \frac{7.4.10^{-8}\left(\Psi_2 M_2\right)^{0.5} T}{\eta V_1^{0.6}} \text{ [cm}^2\text{.s}^{-1}\text{]} \qquad (2.2.30)$$

where

M_2 is the molecular weight of the solvent [g]

Ψ_2 is the solvent association factor which accounts for solute-solvent interactions (1 for non-polar solvents, 1.9 for methanol, 1.5 for ethanol and 2.6 for water)

T temperature in [°K]

η is viscosity [cP]

V_1 molar volume of the liquid solute at its normal boiling point (molecular weight of solute/density) [$cm^3.mol^{-1}$]

The optimum flowrate is then calculated:

$$u = \frac{\nu D_m}{d_p} \qquad (2.2.31)$$

$$u = \frac{F}{A\varepsilon_T} \qquad (2.2.32)$$

2.2.6 Reynolds number

Giddings (1965) in "Dynamics of chromatography" analysed the laminar and turbulent flow in straight tubes and tubes containing granular materials.

2.2.6.1 Reynolds number for straight tubes

Reynolds number is an indication of laminar or turbulent flow. The standard equation used for the calculation of Reynolds number is applicable to straight tubes.

$$\mathrm{Re} = \frac{\rho u d_t}{\eta} \qquad (2.2.33)$$

where ρ is density of the mobile phase [kg/m^3]

u is linear flowrate [m/sec]

d_t is the internal diameter of the tubing [m]

η is a dynamic viscosity [cpoise= 10^{-3} Nsm^{-2}=10^{-3}kgms^{-2}*m^{-2}s]

The linear flowrate is calculated:

$$u = \frac{F}{A60} \qquad (2.2.34)$$

where A is the cross section area of the tube used.

In straight tubes, the flow is regarded as laminar up to the value of Re 2100. When the Re reaches the value of 4000, the flow is turbulent. The region in between is a transition region.

2.2.6.2 Reynolds number for packed columns

For columns packed with granular material, the Reynolds number is calculated as follows:

$$\mathrm{Re} = \frac{\rho d_p \nu}{\eta} \qquad (2.2.35)$$

where

d_p is a particle diameter

ρ is a mobile phase density

ν is a linear velocity

η is a viscosity

The linear flowrate is calculated:

$$u = \frac{F}{A\varepsilon_T 60} \qquad (2.2.36)$$

where A is a cross section area of the column ($=\pi * r_c^2$)

In tubes packed with granular material, on the other hand, turbulent flow already starts at Re=1 and develops into the fully turbulent flow at Re=100.

The turbulent flow, with its flattening profile, equalises the velocities in the tubing.

The importance of equations is particularly relevant when the contribution of band broadening is studied in preparative chromatography with and without the column.

2.2.7 Statistics

2.2.7.1 The arithmetic mean

$$\overline{X} = \frac{\Sigma X}{N} \qquad (2.2.37)$$

where X is a measurement value

ΣX is the sum of the measured values

N is number of measurements

2.2.7.2 The standard deviation

Can be calculated

$$s = \sqrt{\frac{\Sigma\left(X - \overline{X}\right)^2}{N-1}} \qquad (2.2.38a)$$

or

$$s = \sqrt{\left(\frac{\sum X^2 - \frac{\left(\sum X\right)^2}{N}}{N-1}\right)} \qquad (2.2.38b)$$

where

$\sum X^2$ is the sum of the squares of all values of X taken singly

$(\sum X)^2$ is the square of the sum of all values of X

2.2.7.3 The standard error

$$S.E. = \frac{s}{\sqrt{N}} \qquad (2.2.39)$$

where s is standard deviation

2.2.7.4 Confidence limits

$$95\%.CL = \overline{X} \pm t_{0.05} S.E. \qquad (2.2.40)$$

where $\overline{X}$ is the arithmetic mean

$t_{0.05}$ is "Student" constant for 95% confidence limits

2.2.7.5 Degrees of freedom

$$D.F. = N-1 \qquad (2.2.41)$$

The equations were taken from Parker (1979) and Caswell (1982)

2.3 Column band broadening and extra-column band broadening

Summary

This section deals with dispersion of the sample during a separation. The final band width of a peak is the sum of band broadening in the column and outside the column. Here both of these aspects are explained.

2.3.1 The separation process

A chromatographic separation is characterised by two phenomena: differential migration of the sample compounds and diffusion both taking part concurrently.

2.3.1.1 Differential migration

Chromatographic separation is possible due to differential migration of the compounds of a sample. The separation takes place because the molecules of different compounds are more or less adsorbed to the stationary phase and thus are eluted from the column earlier or later. Differential migration depends on the equilibrium distribution of each compound between the stationary and mobile phases.

The interaction of the sample with the stationary phase is a thermodynamic property. The factors which affect the rate of differential migration are: the composition of the mobile phase, the composition of the stationary phase and the temperature.

2.3.1.2 Dispersion

Dispersion, on the other hand, is governed by kinetics. Excessive dispersion leads to a decrease in the quality of the separation, making the peaks wide and consequently overlapping.

Column band broadening

As the solute band passes through the column, the width of the band increases and the solute is diluted by the mobile phase.

There are three main contributors to mobile phase band broadening: *eddy diffusion, longitudinal diffusion, and mass transfer.*

- *Eddy diffusion*

Eddy diffusion - also known as *multiple flowpaths* - is a result of the different microscopic flowpaths followed by the solvent. Thus sample molecules take different directions according to which solvent molecule they follow. Some of the molecules will move faster along the column (e.g. in wide paths) and some will move slower (e.g. in narrower paths). As a result, all this contributes to the to the spreading of molecules along the column.

- *Longitudinal diffusion*

Longitudinal diffusion is related to a sample. Whether the mobile phase is moving or steady, the sample molecules spread in all directions. This becomes significant particularly at low mobile phase flowrates (Snyder and Kirkland 1979).
Axial dispersion includes, in addition to molecular diffusion, the tortuosity of the flow and non-homogeneity of the column bed.

- *Mass transfer*

Mass transfer is divided into two groups: mobile and stationary phase mass transfer.

* *Mobile phase mass transfer*

Mobile phase mass transfer refers to the solvent molecules moving between two particles. Molecules in the middle of the stream are carried away by the flow faster than those close to the particle walls. As a result some of the molecules are eluted from the column faster than others.

* *Stagnant mobile phase mass transfer*

Stagnant mobile phase mass transfer is linked to the solvent molecules entering the pores of the particle. Some of the molecules will enter further into the pore than others. The result is band broadening.

* *Stationary phase mass transfer*

Once the molecules have entered the pores there will be an interaction between the particle and the molecules of solvent and the sample. The interaction is stronger if the penetration of the solvent or sample molecule is deeper. The deeper the penetration, the longer the time spent in the stationary phase and thus the longer it takes for that molecule to be eluted from the column. This action is called stationary phase mass transfer.

Extra-column band broadening

Extra-column band broadening is a result of band broadening caused by a source other than the column. It takes place in the parts of the equipment through which the sample travels: i.e. anywhere between the injector and the detector inclusive.
In most cases the sample is diluted by using wider bore tubing between the injector and detector or by simply injecting a larger amount of sample using larger loops (Snyder and Kirkland 1979)

Therefore the contributions to band broadening (Dolan and Snyder 1989) result from:

1. volume of injected sample
2. detector time constant
3. connecting tubing
4. fittings
5. detector volumes.

⇒ *Volume of injecting sample*

If the sample is dissolved in the mobile phase the extra-column band broadening value is twice the sample volume. If, however, the sample is dissolved in a weaker solvent, it is possible to use larger injection volumes without the impact of band broadening.

⇒ *Detector time constant*

A slow response of the detector can also contribute to band broadening of the sample. The time constant is usually adjustable to fast or slow response. Fast response gives a small value of band broadening but a noisy baseline.

The contribution of the detector time constant to band broadening W_{tc} equals:

$$W_{tc} = 4t_c F$$

The time constant should not be more than 1/12 the baseline width of the first band of interest. A value of 0.25 sec is usually acceptable.

⇒ *Connecting tubing.*

The width of the tubing also contributes to band broadening: the wider the tubing the more diluted the sample and therefore more axial spreading occurs. Usually 0.01-in. tubing is used anywhere between the injector and the detector. Although even thinner tubing would be preferred its use can result in blockages and thus high pressures.

⇒ *Detector volume*

The volume of the flowcell contributes to band broadening and many flowcells have a capacity of approximately 8 µl. Band broadening in the detector is roughly equal to eight times the volume of the flowcell, or approximately 64 µl. However, this value can be higher as additional factors play a role such as the tubing connecting the inlet and outlet ports, and the shape of the flowcell. To minimise the broadening of early eluting peaks, the volume of the detector cell should be less than about 1/10 the volume of the peak of interest V_p: $V_c < 0.1\ V_p$ (Sternberg 1966).

2.4 Wall effect and infinite diameter columns

Knox and Parcher (1969) in their paper on "infinite diameter columns" investigated the ratio of column to particle diameter and its influence on plate height.

According to this theory, the wall effect is caused by inequalities in the flow pattern through the cross section of the column. The column consists of a central circular core with a random bed structure. That core is surrounded by a layer of about 3 particle diameters from the column wall where the packing structure is different from the core and is dominated by a smooth wall. The experiments showed that the flowrate of the mobile phase in this layer was much greater than in the rest of the column.

According to the authors, h (reduced plate height) is a function of ν (reduced velocity) for columns with the same ratio of column length to column diameter and particle size. This function also depends on the ratio of column to particle diameter.

They arrived at a general equation:

$$\frac{d_c^2}{L} = 2.4 d_p \qquad (2.3.1)$$

where d_c is the column internal diameter

d_p is the particle diameter

The ratio of column to particle diameter is:

$$\rho = \frac{d_c}{d_p} \qquad (2.3.2)$$

Their experiments at different velocities using different ratios of ρ showed that there is a region of ρ between 6 and 8 where the reduced plate height - h - suddenly increases. They therefore plotted log(h) vs. log(ρ) at a particular reduced velocity ν=300. The sigmoidal profile revealed that there is a region at ρ =7 where the gradient of the curve sharply increases and then it levels off.

To avoid working in the region of a rapid increase in h the concept of infinite diameter column was introduced.

When an infinite diameter column is used the sample injected at the head of the column would never reach the column walls by the time it reached the foot of the column. In these circumstances the sample will not be affected by the wall effect and the disturbances connected with it.

Golay (1961) first noticed the boundary layer effect ("wall effect") when experimenting in gas chromatography. He concluded that the increase in plate height "h" due to the wall effect was independent of the column diameter when the column diameter was much larger than the particle diameter. According to Golay the thickness of the layer is about $0.5d_p$.
He also suggested that the wall effect can be eliminated by roughening the inner column wall to slow the fast moving boundary layer.

2.5 Laminar and turbulent flow

2.5.1 Inertial and viscous forces

Laminar and turbulent flow are expressed in terms of Reynolds number. The Reynolds number for a flow of liquid through a granular material is the ratio of inertial forces to viscous forces (Giddings 1965).

- *Inertial force (IF):*

$$IF = \rho v^2 \qquad (2.3.3)$$

where

ρ is a density

v is a velocity

- *Viscous force (VF):*

$$VF = \frac{\eta v}{d_p} \qquad (2.3.4)$$

where

η is a viscosity

d_p is a particle diameter

2.5.1.1 Reynolds number

A ratio of the two gives us the Reynolds number for a flow of liquid through a granular material.

$$\mathrm{Re} = \frac{IF}{VF} = \frac{\rho v^2}{\frac{\eta v}{d_p}} = \frac{\rho v d_p}{\eta} \qquad (2.3.5)$$

From the equation it is obvious that velocity is a critical factor whether the flow is laminar or turbulent.

2.5.2 Laminar flow

In the pre-HPLC era the velocities at which chromatography was performed were generally low. Chromatography was performed at laminar flow which is characterised by a flow in which the fluid layers do not generally mix but slide over each other. Mixing only takes place on the molecular level. The velocities vary according to the distance from the centre of the tube. In a cross-section of a chromatography column the velocity is greatest in the middle. As a consequence the overall profile of liquid flow through a column is that of a bullet. Thus sample molecules travelling at different velocities along the column contribute to band broadening (sample diffusion). In laminar flow the viscous force is the driving force.

2.5.3 Turbulent flow

It follows therefore that by increasing the fluid velocity the flow gradually becomes turbulent. It is important to emphasise that this transfer is gradual.
Turbulent flow can be defined as tortuous movements of fluid in different directions at any one time. Turbulent flow is predominantly governed by inertial forces.
In the light of the theory about laminar flow, one would expect the profile of turbulent flow to be similar to that of laminar but perhaps more accentuated. As a result significant diffusion effects would be expected leading to a decrease in column efficiency. However this is not the case.

2.5.3.1 The equalisation effect

Because of the "velocity equalisation effect" - a term first coined by Giddings in 'Dynamics of Chromatography' (1965) - the turbulent flow profile appears as a flattened velocity profile.
According to this theory the velocity of the fluid is lower than expected because of tortuosity. In a transition region there are areas with higher or lower velocities. Turbulence will develop first in the regions of high velocity gradually moving to other regions as the velocity increases. High velocity channels lead to an increase in flow

resistance (represented by an increase in pressure drop in Darcy's law equation). As a consequence the flowrate decreases. This phenomenon appears in transition flow regions as well as in fully turbulent regions.

The consequences of this phenomenon are:

- a decrease in zone spreading (better column efficiency)
- an increase in mass transfer due to "turbulent diffusivity"

Turbulent diffusion also leads to an enhancement of longitudinal and lateral diffusion. Longitudinal diffusion governs over lateral diffusion nearer the walls whilst in the centre of the channel the two are approximately equal.

Turbulent diffusivity is directly proportional to the mean flow velocity. In fully developed turbulent flow the contribution of the mobile phase to the plate height is expressed by a constant. According to Giddings the value of this constant is larger than one particle diameter $H_t > d_p$.

2.6 Silica and polymers

Currently the most commonly used reversed phase packing materials are chemically modified silica and polymers.

2.6.1 Silica

Silica is used in the form of gel (porous glasses). It contains silanol groups (Si-OH) which when chemically, physically or thermally changed can influence the properties of the material. Hydroxyl groups of silanols adsorb water - they are hydrophylic. By chemical modification these groups can become hydrophobic. As silica gel is slightly acidic, alkaline substances are more strongly retained than substances which are neutral or acidic.

2.6.1.1 Physically bonded stationary phases

There are some disadvantages in using physically coated stationary phases: the mobile phase has to be presaturated prior to the analysis otherwise stripping off of the stationary phase is experienced. On the other hand the stationary phase can be "lost" by the shear force of the fast movement of the mobile phase. All this results in a decrease in retention times and thus poorer resolution (Hamilton and Sewell 1978).

2.6.1.2 Chemically bonded stationary phases

To address some of these shortcomings chemically bonded stationary phases have been introduced. These have a number of advantages to physically bonded stationary phases: they can be used for polar and ionic compounds freely; and gradient elution can be applied without worrying about the stationary phase being stripped off.

Chemically bonded stationary phases are prepared by creating covalent bonds in two ways: (a) by esterification - reaction with alcohols creating Si-O-C forms (hydrolytically unstable); or (b) by reaction with organosilanes forming Si-O-Si bonds monomers or polymers under anhydrous conditions or by reaction with hydrolysis.

The important thing is that all silanol groups should be substituted otherwise the analysis could be affected. As the maximum number of OH- groups which will enter the bonding reaction is approximately half, the remaining unreacted OH- groups have to be "end-capped" by applying a silylating agent such as trimethylchlorosilane (Engelhardt 1979). If end-capping is not realised extensive retention, tailing and poor resolution will be experienced.

Preparation of chemically bonded stationary phases.

The advantage of a chemically coated phase is that there is no stripping of the phase as it is covalently bonded to the solid. As a result the mobile phase does not have to be pre-saturated with the stationary phase.

There are several pathways for the preparation of chemically bonded stationary phases. The principle is to "involve" all the silanol groups (-Si-OH) in the chemical reaction. However this is not entirely possible and as a result the behaviour of the stationary phase is affected by the extent to which this task has been achieved. Coating with a thin layer can yield either a monomer or a polymer. Polymers tend to be more stable than monomers. The final product is hydrophobic.

Silanol groups can thus react with primary alcohols in the reaction called esterification to produce silicate esters.

Materials with covalent Si-C and Si-N bonds are prepared by a two step reaction. First the silanol groups are chlorinated by thionyl chloride. Chlorinated silica produced in this way then reacts with Grignard reagent which results in Si-C bonds or with amines giving Si-N bonds.

However the most useful bonded-phase packing is that based on siloxanes. In this case the final product can be either in a monomer or a polymer form. The bonds formed in this way (Si-O-Si-C) are achieved by reaction with organochlorosilanes or organoalkoxysilanes.

If the reaction takes place in the absence of water molecules the formation of polysiloxanes is suppressed. This coating, usually referred to as "brushes", is hydrolytically unstable and therefore not very suitable for LC.

On the other hand, when water is used during the reaction, bonded polymers are formed.

To test the degree to which the surface silanol groups are shielded, a methyl red test is carried out. In this case, the silica gel is shaken together with a methyl red solution and afterwards washed with pure benzene (benzene is carcinogenic and therefore COSHH rules need to be observed). If, after this procedure, the silica gel retains a red violet colour, some free silanol groups are present. (Engelhardt, 1979)

2.6.2 Polymeric stationary phases

The early polymeric packing material introduced by Porath and Flodin in 1959 was limited to low pressure chromatography and therefore not suitable for use in HPLC.
It was only in 1964 when Moore introduced a new type of synthetic polymer - polystyrene - that the further enrichment of the utilisation of polymers in chromatographic separation was achieved. Moore managed to prepare poly (styrene-divinylbenzene) copolymer, commonly known as PSDVB, synthetically. This was of a macroporous nature. The properties of this copolymer enabled it to be used in liquid chromatography without pressure limitations.

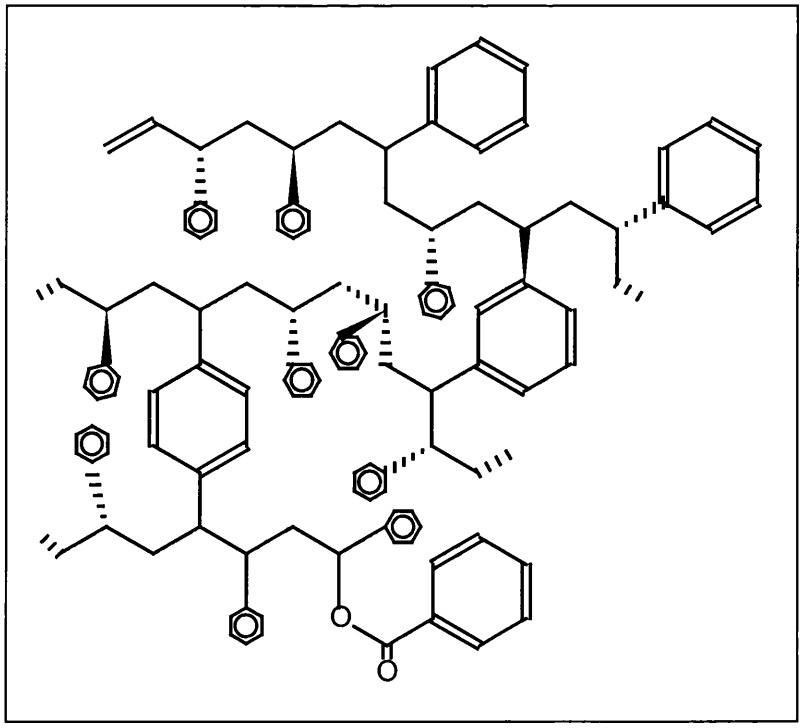


Figure 2.6-1 The structure of polystyrene-divinylbenzene

Here the structure of polystyrene-divinylbenzene copolymer is presented consisting of benzene rings intertwined with methylene chains. Divinylbenzene (predominantly 1,4-isomer) functions as a cross linker the amount of which determines the rigidity of the material. The structure, which is racemic, is not simple and varies according to the type of porogen used, the polymerisation initiator (e.g. the presence of esters when peroxide initiators such as perbenzoic acid is used), the type of emulsifier, the degree of purity of the monomers entering the reaction (e.g. the presence of 1,3-divinyl benzene isomer as an impurity), etc., as can be demonstrated on the structure. A good reference on polymers, their chemistry and their properties can be found in Stevens (1999).

2.6.2.1 Natural and synthetic polymers

Thus there are two kinds of polymeric packing material which differ firstly in their origin: *natural polymers* (agarose, cellulose and dextran); and *synthetic polymers* such as P(SDVB), polyacrylamide and polymethylmethacrylate. If categorised by the morphology of the particles they can be devided into microporous and macroporous particles. This categorisation is derived from the size of the pores which is dependent on the degree of cross-linking.

2.6.2.2 Microporous and macroporous particles

Microporous particles with a low degree of cross linking (less than 10% of the total monomer content) contain larger pores. This tends to cause the particles to swell and compress more easily.

On the other hand, macroporous particles provide a high degree of cross-linking with large pores as a result of the presence of a porogen. A porogen can be an organic solvent, a non-solvent, or a linear polymer. These polymer particles tend to swell less and are much more rigid and stable. In this form they can be used for chromatographic separations at much higher pressures.

2.6.2.3 The preparation of polymeric packing material

In the past polymers were prepared by crushing the bulk polymeric packing material in ball mills. (Svec and Frechet 1996). This however resulted in the production of material of a wide range of sizes and non-spherical irregularly shaped particles. These particles tended to create voids and clog up the column during packing. The columns were therefore not very efficient. Spherical particles give better efficiencies than irregular particles.

Emulsion, dispersion and suspension polymerisation

There are three ways of producing spherical beads of a given diameter with a predetermined pore size: *emulsion, dispersion and suspension polymerisation.* The choice of technique usually determines the size of the beads.

Emulsion polymerization consists of a hydrophobic monomer which is rendered soluble by micelles when dispersed into the aqueous phase. This technique produces beads of a size less than 1 μm.

In *dispersion polymerization* monomers are dispersed in an organic liquid medium mixed with a polymeric steric stabilizer. The end of polymerisation is indicated by the precipitation of polymer coated with stabilizer. This polymerization yields beads sized between 1 and 10 μm.

These first two techniques are not very suitable for the polymerization of macroporous packing material as they produce beads of a wide range of sizes. Therefore the only current suitable method is *suspension polymerisation.* This method requires a water insoluble monomer which contains a free radical initiator which is stirred into the water. This process forms droplets of a given size which can be controlled by the stirring speed and the amount of suspending agent used. The porosity of the particle is determined by the porogen. The porogen is soluble in the monomer mixture. It works in such a way that it remains inside the newly formed beads and is removed after polymerization has finished. The size of the pores is also controlled by the amount of cross-linking monomer, the composition of the porogen, and the polymerization temperature. The amount of divinylbenzene used determines the pore structure and properties of the

polymer. A high degree of cross-linking forms a rigid gel which is pressure and solvent resistant (does not swell).

Solutes are eluted in order of polarity which in reversed phase means the more polar samples are eluted first followed by the less polar samples. Solute retention can be manipulated by changing the composition of the mobile phase (using more organic solvent or more water) or by changing the structure of the stationary phase according to the polarity of the chemically bonded groups on silanols with the decreasing polarity of the groups phenyl, C_8, C_{18} (Lindsay 1992).

2.6.3 Normal and reversed phase

There are two kinds of stationary phases according to their polarity: traditional polar stationary phase, referred to as normal phase; and non-polar stationary phase, also called reversed phase. Although normal phase liquid chromatography is used for solutes of a wide range of polarity, very polar solutes which are strongly retained on the stationary phase are better separated using reversed-phase mode. This mode employs a mobile phase usually consisting of a mixture of water and an organic solvent such as methanol or acetonitrile. If a highly polar compound is bound to water which is more polar the use of more organic solvent will help to elute the solute from the column. Reversed phase is preferred for bio-analysis because a fermentation broth is an aqueous solution (Knox 1978).

2.7 Erythromycin

Summary.

Erythromycin is a model sample used in this project therefore the background to this antibiotic is outlined, and its physical and chemical properties stated. Its industrial production is briefly mentioned.

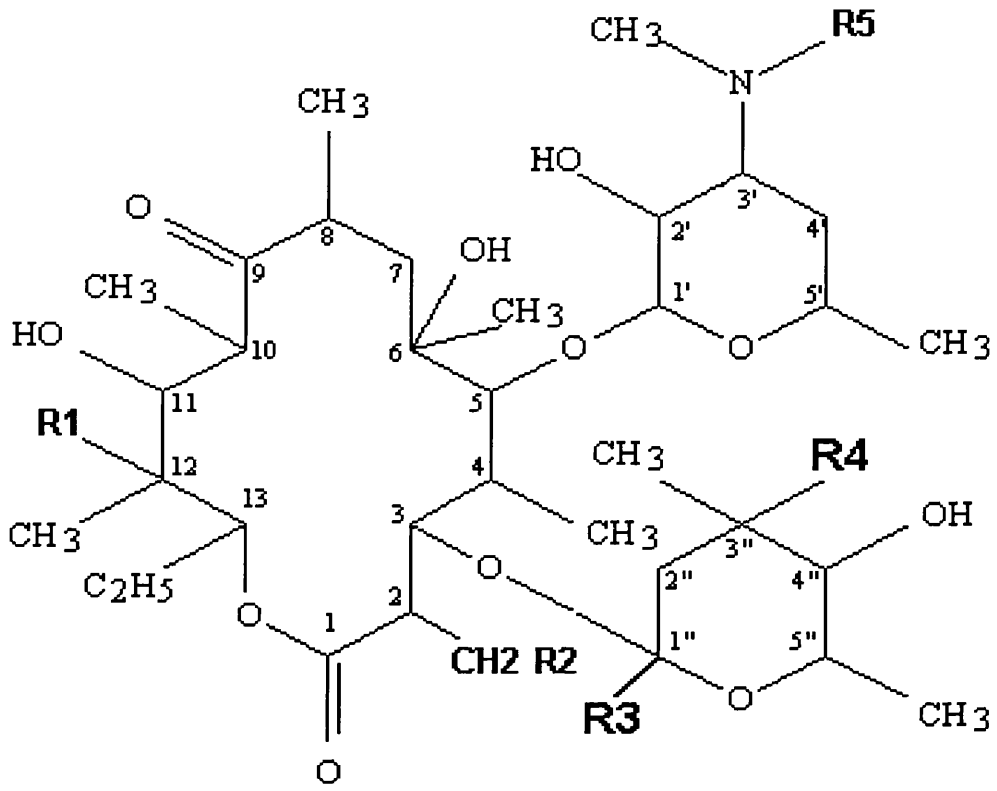
This chapter also presents the results of a literature survey of the separation of erythromycin on two types of packing material: bonded silica and polymeric stationary phase.

Overview.

Erythromycin was selected as the model for this PhD study as it is intensively utilised in our Centre for research into up stream and down-stream processing of antibiotics.

In order to scale up the process, a method for the separation of a crude erythromycin has to be established. Therefore this chapter focuses on the separation of erythromycin by HPLC technique as presented by other researchers.

2.7.1 Erythromycin characteristics



Erythromycin

	R_1	R_2	R_3	R_4	R_5
erythromycin A (EA)	OH	H	H	OCH_3	CH_3
erythromycin B (EB)	H	H	H	OCH_3	CH_3
erythromycin C (EC)	OH	H	H	OH	CH_3
erythromycin D (ED)	H	H	H	OH	CH_3
erythromycin E (EE)	OH	OH	H	OCH_3	CH_3
erythromycin F (EF)	OH	-O-		OCH_3	CH_3

Figure 2.7-1 The structure and legend of general erythromycin.

Erythromycin is an antibiotic used to treat infectious diseases. It acts by interfering with ribosomal protein synthesis within the bacterial cell. Erythromycin first described as Ilotycin was isolated in the Lilly Research Laboratories in 1952. It was produced from a

sample of soil collected in the Philippine Archipelago. The sample contained a streptomycetes which was named "erythraeus" by Waksman and Henrici (Florey 1960). Erythromycin is a macrolide antibiotic (see section 3.2.1.2) with a broad and essentially bacteriostatic action against many gram positive bacteria. It is used in conditions such as diphtheria, pertussis, Legionnaire's disease and other respiratory tract infections but also as an alternative to penicillin in penicillin-allergic patients (Dale 1993).

Empirical formula: $C_{37}H_{67}NO_{13,}$ molecular weight: 733.4772, UV max. at 278nm Erythromycin forms 14-membered ring macrolide. It decomposes under acidic conditions. It is poorly soluble in water as a free base and it is soluble in some organic solvents such as amyl acetate and chloroform. It decomposes rapidly at 25°C. Maximum stability is in the range of pH = 6 - 9.5 (Kavanagh and Dennin 1963).

Crude erythromycin sample contains erythromycin A as the main product and usually B, C, D, E and F forms as minor components. All types of erythromycin have a desosamine ring but they vary in four R groups (see the general structure of erythromycin). Erythromycin A and B have cladinose sugar whereas erythromycin C has a mycarose sugar. They have very similar properties.
Other products which can appear in the crude sample of erythromycin are anhydroerythromycin A, enol ether of erythromycin A and N-demethyl of erythromycin A.

As a consequence of erythromycin acidic liability which generates erythromycin 8,9-anhydro-6,9-hemiketal and erythromycin 6,9,9,12-spiroketal, when administered orally erythromycin comes in the form of film or enteric -coated preparations. It can also be used as a salt or ester as in these forms it is much more acid stable (Weinstein and Wagman 1978).

2.7.2 Industrial production and isolation

Erythromycin is produced extra-cellularly from a strain of streptomyces erythreus by fermentation. It is removed from a fermentation broth by centrifugation or filtration and

extracted with a water-immiscible organic solvent such as methyl isobutyl ketone or ethyl acetate and transferred to acidic water. This concentrates and purifies the antibiotic which is afterwards precipitated by adding diethyl ether or n-hexane (Vandamme 1984). This is followed by purification on silica gel or on an aluminium column. the adsorbed erythromycin is eluted by a mixture of an organic solvent and water with pH between 3 and 8 (Suzuki et al. 1973).

COMPANY	COUNTRY
Dista products Ltd.	Liverpool, ENGLAND
Eli Lilly company	Indianapolis, Indiana, USA
Abbott Laboratories	North Chicago, Illinois, USA
Asia Pharmaceut. Ltd.	Hong Kong
Chemobiotic Ltd.	Innishannon, IRELAND
China National Chemical Import & Export Corporation	Peking, CHINA
Compania Industrial Produtora de Antibioticos S.A.R.L. (CIPAN)	Lisbon, PORTUGAL
Compania Espanola de Penicillina y Antibioticos S.A.	Madrid-Arenjulz, SPAIN
Dainippon Pharmaceutical co. Ltd.,	Tokyo, JAPAN
Fermic S.A. de C.V.	Ixapalapa, MEXICO
Instituto Biochemico Italiano	Milano, ITALY
Pfizer, Korea Company Ltd.	Seoul, SOUTH KOREA
Pliva Pharmaceutical & Chemical Works,	Zagreb, YUGOSLAVIA
Roussel-Uclaf	Romainville, FRANCE
Silom Medical Company	Bangkok, THAILAND
Soho Industri Pharmasi	Djakarta, INDONESIA
Tarchomin Pharmaceutical Works (POLFA)	Warsaw, POLAND

Source: *Vandamme (1984)*

Figure 2.7-2 Examples of erythromycin production companies world-wide.

2.7.3 Analytical separation of erythromycin

Two different kinds of packing material were utilised when analysing erythromycin: derivatized silica packing and poly(styrene-divinylbenzene) packing material. Compounds of interest were detected by using UV detector or electrochemically.

2.7.3.1 Analytical separation of erythromycin on silica

Tsuji and Goetz (1978) researching HPLC of erythromycin on a C_{18} column concluded that the pH of the mobile phase for relatively pure samples was between 6 and 6.5 while for a complex biological matrix 7.8 was suitable. The ratio of acetonitrile and methanol of 45% to 10% in the mobile phase tends to give the optimum separation. With regard to the stability of the sample the ideal pH should be 7.5-8.8. At higher pH values erythromycin degrades to dihydroerythromycin, at lower pH values it degrades to erythromycin A enol ether.

From the previous research it is clear that columns with derivatized silica reversed-phase packing material are unstable above pH=7. But analyses carried out at higher pH give better separations. Mobile phase at lower pH cannot be used as erythromycin is labile under mild acidic conditions. Therefore when using derivatized silica packing material the compromised value of pH should be between 7 and 8.

Cachet et al (1987) found out that TMA (tetramethylammonium) salts improve the shape of the erythromycin peak and reduces retention time. As far as the mobile phase is concerned, results showed that methanol cannot be used on its own because it is not sufficiently miscible with other compounds. This is not the case for acetonitrile.
Cachet et al. (1987) studied the influence of temperature and pH on the peak shape and retention times of erythromycins. It turned out that elevated temperature reduced retention times and improved the shape of the peaks. On the other hand, increased temperature in the long run has a detrimental effect on column life. The addition of TMA into the mobile phase improved peak shapes by eliminating the tailing. Also the performance of the column was tested over a period of about two months. The research shows that older columns gave better results than a brand new one. Ageing apparently eliminates metal impurities from the silica backbone and thus affects silanol activity.
The addition of TBA to the mobile phase resulted in a decrease of retention times. It also improved the peak symmetry and the efficiency.

Croteau et al (1987) used electrochemical detection for HPLC assay of erythromycin and its esters. They preferred this detection method because a UV detector is not

sensitive enough and molar absorption of erythromycin at 220 nm is too low. In order to assess the relative concentration of erythromycin esters, they had to adopt the measures of their continuous hydrolysis in biological fluids and during sample preparation.

Cachet et al. (1991) attempted to separate erythromycin A from its impurities. The separation between erythromycin A and erythromycin E appeared to be the most critical. By substituting TMA (tetramethylammonium) for TBA (tetrabuthylammonium) and decreasing the concentration of acetonitrile in the mobile phase (less than 25%), it was possible to separate erythromycin A from erythromycin E. The authors also concluded that the length of the column has an affect on the separation: the retention time of the sample compounds increases with the length of the column. In order to decrease the overall retention time of all the sample compounds a column switching technique was employed. This works on the principle that the compounds which are eluted from the column slowly go through a short column (7.5cm) whereas those which are retained for a very short period of time are passed through a column of greater length. Thus a compromised retention time is achieved. In this particular method, the retention time was reduced from 110 mins to 60 mins.
Since erythromycin lacks a significant chromophore, UV detection can be performed only at a low wavelength which is 215nm.

Polish authors Gwozdz, Holska and Kulinska (1991) tried to look into the way $NaClO_4$ in mobile phase affects the separation. They presented two different graphs: one when 0.1mol/L $NaClO_4$ was used in the mobile phase and one without $NaClO_4$. It is noticeable that the presence of $NaClO_4$ remarkably improves the separation and the symmetry of the peak while the pH of the mobile phase remains the same (pH=10.5). In order to do this, the mobile phase had to be saturated with silica.
The authors also pointed out that their columns were used for over two years and no deterioration of packing was detected.

As there are certain disadvantages in using derivatized silica as a packing material due to the interaction of metal impurities with the silica backbone, Paesen et al. (1993) tried to solve the problem. They added TBA (tetrabutylamonium hydrogensulfate) or TMA

(tetramethylamonium phosphate) into the mobile phase. TBA acts as a shielding agent for the silanols. TBA or TMA is used to weaken the interactions of basic substances (erythromycin) with residual silanol groups on the silica backbone.
Other experiments were done by heating a packing material conditioned with a mobile phase containing phosphoric acid which removes metal impurities from a silica backbone and thus influences the silanol activity. At the same time it is important to maintain low pH as a residual silanol activity still exists when pH=7 and low pH helps to avoid ionization of silanols (Paesen 1993).

2.7.3.2 Analytical separation of erythromycin on PSDVB

More research has been done on the influence of particle pore size on separation in poly(styrene-divinylbenzene) packing material. Paesen et al. (1991) tested narrow pore particles (100Å) but satisfactory results were only achieved on tetracyclines. However, the use of wide pore particles (300Å and 1000Å) enabled them to separate erythromycin E from erythromycin A with detection at 215nm.
The influence of buffer pH in the mobile phase was investigated, with the increase of pH values for k' increased. Below pH=8, the capacity factor and selectivity factor decreased rapidly. The use of other organic modifiers such as methanol or tetrahydrofuran did not yield any improvement in the separation.

Cachet et al (1991) stated in another paper that columns packed with P(SDVB) were used successfully for doxycycline, oxytetracycline but were not suitable for erythromycin because no baseline separation could be obtained.

Kibwage et al. (1985) tested the impact of temperature on columns packed with poly(styrene-divinylbenzene). They observed that the temperature of the column is directly proportional to the retention time. This is in contrast to bonded silica reversed phase columns where the retention of erythromycin is inversely proportional to the temperature. They also noticed that by adding methanol into the mobile phase k' values decrease. By adding tetramethylamonium phosphate to the mobile phase the number of theoretical plates increased. At higher temperatures the peak shapes improved. Another

factor under investigation was the pH of the mobile phase which influences the stability of erythromycin. This was most obvious when pH dropped below pH=6.5 With increased pH the retention times of compounds increased until pH reached 8 when the retention time levelled off and did not exhibit any further increase. As for the concentration of the buffer, this gives decreased values of k' as it rises (Kibwage et al. 1985).

Nilsson et al. (1987) concentrated on the changes of flowrate in connection with the number of theoretical plates on polymer columns. They observed that an increase in flowrate has a negative influence on the number of theoretical plates. The same relationship was observed when increasing the temperature of the column from 25° to 70°C.
The authors also examined the effect of pH on the separation of erythromycin using a polymeric column with amperometric detection. It turned out that an alkaline environment had a beneficial effect on the separation by significantly improving N and k'. However, it was found that above pH=9 the retention time unexpectedly decreased.

A collaborative study conducted by Paesen et al. (1993), was carried out in six different laboratories using seven polymeric columns. The problems they encountered were linked to a high flow rate and the way the column was heated. In the former case the baseline noise experienced in some laboratories was eliminated by simply changing the detector, using longer tubing between the column and detector or by reducing the flowrate. As for the column heating, the best results were achieved with immersion baths. Heating by means of a hot air oven resulted in a loss of resolution due to uneven heating.

Janecek et al. (1993) compared the separation of erythromycins using silica based and polymer columns. The separation on the polymer column was performed at pH=9.6. However, very low column efficiency was achieved (N=730). As for the silica column, the separation was better as sterically shielded octyldiisopropylsilica was used with a neutral mobile phase.

Conclusion

With these developments and improvements in instrumentation erythromycin can now be separated using state-of-the-art HPLC technology.

The literature survey on the HPLC separation of erythromycin predominantly focuses on the utilization of two kinds of packing material: bonded phase silica and polymer packing material. Each of these methods has its advantages and disadvantages.

It seems that there is not yet a method developed which can separate all the erythromycins and its degradation products which form during pH changes.

Silica packing material is known to be less stable (it dissolves) at pH values above 7. However, mobile phase pH at values above 7 gives better separation. Acidic pH cannot be used for the separation of erythromycins as it is acid labile. Therefore many experiments on silica had to be carried out using a compromised pH value of between 7 and 8.

2.8 Axial and radial compression.

Introduction.

In order to perform large-scale liquid chromatography specialised equipment has to be used such as columns designed to withstand high pressures and pumps producing high flowrates. To avoid any dead volumes in the column and to carry out the desired separation the packing material can be compressed axially or radially. For axial compression the method of slurry packing is used (Hostettman et.al. 1986).

2.8.1 Types of compression

2.8.1.1 Static compression

Compression serves to eliminate any voids in the bed. *Static compression* is a temporary process where the bed is compressed during the packing of a column and then the process is stopped. The disadvantage of this process is that after the bed is packed and during the bed settling further voids can be created which have a detrimental effect on the chromatography and the efficiency of the column.

Radial

The example of the static procedure is radial compression. A cartridge with flexible walls is placed in a stainless steel container with a fluid. Pressure is applied to the liquid which radially presses on the side of the walls and compresses the packing.

2.8.1.2 Dynamic compression

Axial

Dynamic compression is a continuous process whereby the resin is compressed during column packing as well as during the normal running operation.

Dynamic axial compression (DAC) is the most popular column technology today. (Hostettmann et.al. 1986).

2.8.2 Packing pressure

Freitag et al. (1994) tested methods of column packing to find out how they influenced permeability, packing density and total porosity at different pressures. The packing material used here was giga-porous highly cross-linked styrenic particles.

The researchers looked at the influence of inlet pressure on the flowrate when different packing pressures were used.

According to their findings there is a non-linear relationship between the column inlet pressure and flowrate when the column is packed at different pressures. The curves are linear initially but later, as the pressure increases, they exhibit deviation from linearity. This deviation is shown well before the inlet pressure reaches the packing pressure. This, as the authors explain, is due to the swelling properties of polymeric packing. An attempt to prevent the swelling was made by rinsing the packing material with water for 20 minutes at the packing pressure to remove most of the methanol from the packing but this did not lead to success as the subsequent results were virtually the same.

However, conditioning the column with water made a difference to the permeability of the packing which is higher when the column is conditioned with water rather than packed with pure methanol.

Generally porosity attains the minimum value when density reaches the maximum value. Permeability, on the other hand, exhibits a steady decline during packing. When looking at porosity and permeability, up to a pressure of about 6000 psi both curves show a declining tendency. However, going beyond that value results in a much gentler decrease in permeability and increase in porosity. The density increases steadily until the pressure reaches 6000 psi and then there follows a rapid decrease. When the packing was examined afterwards by scanning through an electron microscope there was no evidence of particle breakage. This implies that beyond about 6 500 psi the column bed structure is different from that of a column packed at lower pressures.

The efficiency of the column increases with the increase in packing pressure. However, reaching pressures beyond 6000 psi results in a decrease in efficiency. These experiments were also carried out on columns which were water conditioned. These columns showed generally lower efficiencies than the columns which were not water conditioned.

2.8.3 Packing techniques - dry, wet and slurry

Sarker and Guiochon (1995) tested consolidation of the packing material in chromatographic columns under dynamic axial compression. The columns were packed by three different techniques: dry packing, wet packing and slurry packing. The study shows that slurry packing and to a certain extent wet packing are more efficient than dry packing where consolidation is smaller.

2.9 Mass and volume overload

2.9.1 Loadability

Loadability is the amount of sample (in volume or mass) which should be injected on the column to work under optimal conditions (Godbille and Devaux 1976).

Unlike in analytical chromatography where the sole goal is to achieve a good separation, in preparative chromatography this goal can be compromised, the emphasis being on achieving a large amount of highly pure material. This requirement cannot be met without overloading a column. The sample for analytical purposes is small and highly diluted. In preparative separations large concentrated samples are called for. (Bidlingmeyer 1987).

Gotsick and Schmidt (1992) measured the peak width at half height of compounds being separated on packing material of three different sizes (3,5 and 8 μm) at an increased load. The results show that the peak widths increased insignificantly until the load reached a value of ≈100 μg/g packing. Beyond that load the peak widths increased rapidly, i.e. became independent of the particle size.

Colin (1987) claims that higher flowrates allow larger loads. Although the maximum flowrate should be used in preparative chromatography the pumping system imposes restrictions. The derivation follows:

Throughput can be expressed as:

$$TP = \frac{Q_{INJ}}{t_R} \qquad (2.9.1)$$

where

Q_{INJ} is the injected quantity of a sample and

t_R is the analysis time.

$$Q_{INJ} = q_0 \rho V_C \qquad (2.9.2)$$

where

q_0 specific mass loadability (quantity of sample per unit mass of a stationary phase)

ρ_p is the packing density and

V_c is the column volume

The analysis time can be expressed:

$$t_R = (1 + k')V_C\varepsilon_T / F \qquad (2.9.3)$$

where

ε_T is the column total porosity

F is the solvent flowrate.

The combination of the last two equations gives us:

$$TP = \frac{Fq_0\rho}{(1 + k')\varepsilon_T} \qquad (2.9.4)$$

This equation shows that TP is proportional to the flowrate and does not depend on the column design. This result implies that maximum flowrates should be used for separations. Although one should make the most of expensive equipment there are restrictions in using maximum flowrates and these come either from pressure limitations on the column or on the rest of the equipment.

Done (1976) claims that larger volumes of diluted samples are better than smaller volumes of more concentrated compounds.

THE EFFECT OF SAMPLE VOLUME ON HETP AT LOW AND HIGH LOADINGS OF PHENETOLE ON TWO DIFFERENT COLUMNS					
WLCU - 6 (sample 1.77μg)			SPHERISORB 20 (sample, 120μg)		
VOLUME (μl)	CONCENTRATION (mg/ml)	HETP (mm)	VOLUME (μl)	CONCENTRATION (mg/ml)	HETP (mm)
0.25	7.08	0.0147	1	120	0.265
0.5	3.54	0.0136	3	40	0.228
1.0	1.77	0.0141	10	12	0.199
2.5	0.71	0.0140	25	4.8	0.180
6.2	0.29	0.0152	-	-	-
25	0.07	0.0203	-	-	-
Done(1976)					

Table 2.9-1 The impact of sample volume on HETP.

This table shows that smaller volumes combined with a larger concentration give better efficiencies and as the volume increases the concentration of a sample has to decrease to maintain good efficiencies. This view is also shared by Guiochon et al. (1994) who claim that concentration overload is the more economical approach.

2.9.1.1 Maximum amount of the sample loaded on silica

The rule of thumb for predicting the maximum amount of the sample which can be loaded on the column isl gram of sample mixture to be separated per 30 g of silica gel (Verzele et al. 1992). This assumption is used for fairly easy separations. For more difficult separations the amount of silica is increased to 100 g of silica per gram of mixture. The column was gradually loaded by larger amounts of sample. Up to 128-mg samples the chromatograms looked acceptable. With a 250-mg sample the results of the analysis of collected fractions show very good separation with only a minor overlap. However, when a 750-mg sample was loaded peaks were overlapped and displacement effects took place (Verzele et al. 1982).

2.9.2 Mass and volume overload

The column can be overloaded by mass (concentration) and by volume.

2.9.2.1 Volume overload profile

The volume of the sample which can be introduced onto the column depends on the column diameter and length, sample solubility, mobile and stationary phases, and resolution. In order to achieve maximum resolution the volume should not be greater than one-third of the volume of the earliest peak of interest. However, much greater volumes can be introduced if the values of α are large (Snyder and Kirkland 1979)

Wehrli and Hermann (1976) were interested in the influence of the feed volume on peak broadening. Their study shows that when small volumes of the sample are loaded on the column the peak broadening is constant. After the sample load reaches a certain value every increase in the sample load initiated an increase in peak broadening. The peak widths remained constant until the feed volume reached the value of the volume standard deviation. A further increase in injection volume caused a deviation from a Gaussian to a rectangular peak shape.

Volume overload will cause the peaks to develop square tops whilst the front of a peak remains in the same position and rear tailing is detected. Thus the "rears" of the first peaks get closer to the "fronts" of adjacent peaks as the volume increases. Increased sample volume leads to a transition from elution to frontal analysis (Scott and Kucera 1976).

2.9.2.2 Mass overload profile

The situation is quite different when mass overload is applied. There are three effects participating in mass overload. As the sample is injected it starts spreading along the column resulting in band spreading. This is similar to volume overload and is a dispersive effect. However, the deactivating effect also plays a role here. The high mass of a sample causes deactivation of the adsorbent and the polarity of the mobile phase increases. This results in a decrease in the retention times of all the components of the sample. The solute which is present in the largest amount exhibits the greatest reduction in the retention distance. All these factors result from non-linear adsorption taking place where isotherms exhibit a pronounced tailing (Klinkerberg 1960).

This is similar to the principle of displacement chromatography (Horvath et al. 1981). Here the column is first equilibrated with a carrier solvent. Then a feed is introduced

which has previously been dissolved in the carrier solvent and which has a strong affinity to the stationary phase. Next the displacer substance is introduced which has a stronger affinity to the stationary phase than any of the components of the sample mixture. As the displacer moves along the column the "displacement train of zones" - in the order of increasing affinity to the stationary phase - moves with it. The product is then collected in the form of square-wave concentration pulses. After this action is completed the column has to be rid of the displacer and regenerated (re-equilibrated) with the carrier. It is important to note here that the concentration of the sample components collected is high and they travel at the same velocity.

2.9.2.3 Combination of mass and volume overload.

Knox and Pyper (1986) investigated the combined method of overloading the column-by volume and by mass simultaneously to achieve higher throughput.
However, they found that applying large sample volumes in conjunction with high sample concentrations led to the volume overload eventually being completely absorbed by the concentration overload.

Elution, Frontal Analysis And Displacement Chromatography.

In *elution chromatography* a small amount of the sample solution is injected. The separation of the components is in the form of "bands" or "peaks". It is the most used mode in chromatography.

Frontal analysis is widely used to determine breakthrough curves in protein separations. In principle, the sample is continuously fed onto the column until the breakthrough occurs. This is the point at which the maximum capacity of the column is reached. Loading is stopped and superseded with the elution or desorption step to separate or recover the desired components.

In *displacement chromatography* a small sample which has a large affinity to the stationary phase is fed into the column. In the next step a solvent which has an even

higher affinity to the adsorbent than the solute is introduced. This solvent displaces the solute attached to the stationary phase.

2.10 Modelling

The chromatographic separation process is the result of a combination of differential migration rates (thermodynamics) and dispersion (kinetics).
The thermodynamic contribution to the chromatographic process is related to adsorption on the stationary phase and the mutual interactions of the components of the injected mixture under overloaded conditions. Thermodynamics controls band position.
Kinetic processes encompass the mobile and stationary phase effects and their interaction with the solute molecules. Kinetics controls band profiles.
As a result there are two main models on which the majority of the modelling of chromatographic processes are based: equilibrium models and kinetic models.

2.10.1 Equilibrium model

This theory assumes a direct local equilibrium between the mobile and stationary phases, neglecting axial dispersion and mass transfer resistance. The advantage of this theory is that it predicts experimental retention times for chromatographic columns with fast mass transfer rates (Ruthven and Ching, 1993).

The equilibrium model concerns the equilibrium composition of two phases and the influence of various parameters (e.g. pressure and temperature). It is expressed in terms of an equilibrium isotherm which is related to a distribution coefficient (DC). DC is the ratio of the concentration of the sample in the adsorbed phase (solid phase) to the concentration of the sample in the unadsorbed phase at equilibrium.
Under the conditions of analytical chromatography the samples are very dilute. It is therefore assumed that the DC at equilibrium is constant. The profile of such isotherms is linear. On a preparative scale, however, large concentrations of samples are used and the DC is no longer constant. The isotherms in this case deviate from linearity (non-linear isotherms).

Examples of non-linear isotherms are Langmuir (also the most popular), bi-Langmuir, Fowler and Freundlich isotherms.

In single component isotherms, the model is straightforward, consisting of the mass balance equation for one component. The model explains the kinetics of equilibration between two phases of the system and permits the calculation of a band profile for a pure component.

With multi-component competitive isotherms, on the other hand, the interaction between the components of the mixture have to be taken into account. The amount of a component in a solution decreases with an increased concentration of another adsorbed compound. This means that the amount of each component analysed is less than if that component were analysed alone.

isotherm	purpose
linear	small sample concentrations
Langmuir (convex shape)	large sample concentrations; single component adsorption on a homogenous surface
Anti-Langmuir(concave shape)	quadratic isotherm
Fowler	designed to correct for 1st order deviations from Langmuir isotherm
Freundlich	adsorption of polar compounds on polar adsorbents

Table 2.10-1 The various types of isotherms and their use.

The equilibrium theory of multi-component isothermal adsorption was first developed by Glueckauf (1949). It also provides general location or retention times of elution peaks. On the other hand if mass transfer is significant it does not describe the peak shapes accurately. Equilibrium theory has been used for the study of multi-component interference effects by Helfferich and Klein (1970) and the ideal displacement development by Rhee and Amundson (1982).

2.10.2 The equilibrium-dispersive model (equilibrium theory plus)

This model assumes that all the contributions deriving from the non-equilibrium can be aggregated into an apparent axial dispersion term. Axial dispersion is a result of axial molecular diffusion, the tortuosity and the non-homogeneity of the column packing (Giddings 1965). It is used when mass transfer kinetics are fast but not infinitely fast. It was first studied by Van Deemter et al. (1956) and Giddings (1965). The model is correct only if mass transfer in the column is controlled by molecular diffusion across the mobile phase flowing around the packing particles and if the exchange of sample components between the mobile and stationary phase is very fast.

2.10.3 Kinetic models

The models combine the mass balance equation with a kinetic equation. Although kinetic models are more exact than equilibrium-dispersive models the differences become negligible when the rate constants are reasonably high i.e. when column efficiencies are above 100 theoretical plates. (Shirazi and Guiochon 1992). The different kinetic models summarised by Ruthven (1984) are the Langmuir kinetic model, the linear kinetic model and the general rate model.

2.10.4 Plate models

Plate models are hypothetical models of band retention and band broadening and they introduce a powerful tool to measure the efficiency of the column through HETP.
There are two kinds of plate model theories: the Martin and Synge model (1941) and the Craig model (1944).

2.10.4.1 Martin and Synge model.

In the model, the column is devided into a series of small artificial cells, each with complete mixing (Ruthven 1984). This gives a set of first order ordinary differential equations (ODE) that describe the adsorption and interfacial mass transfer. The drawback of this theory is that it is not suitable for multi-component chromatography as the equilibrium stages may not be assumed equal for different components.

Martin and Synge (1941) based their plate model on the notion that the column is devided into a number of theoretical plates within each of which the equilibrium between the mobile and stationary phases occurs. One theoretical plate represents one layer. A column consists of a number of layers the height of which is given by the height equivalent to a theoretical plate (HETP). HETP is a constant.
In order to study non-linear effects Martin and Synge assumed a linear isotherm and the influence of the mass transfer kinetics and axial dispersion. In order to achieve a quick equilibrium the HETP should be as small as possible.

2.10.4.2 Craig distribution model.

The *second* kind of plate model is based on the distribution factors that determine the equilibrium of each component in each artificial stage. The model is solved by recursive iterations rather than ODE. The description of the Craig model was given by Eble et al. (1987) and Sheshadri and Deming (1984). The Craig model was used for the study of the column overload problem by Ernst (1987). Recently Velayudhan and Ladisch (1993) used a Craig model with a corrected plate count to simulate elution and frontal adsorption.
The peak dispersion in the Craig model is less than in the Martin and Synge model. If the same results are to be achieved the number of stages in the Craig model has to be reduced. When three models were compared (the Gaussian, Martin and Synge, and Craig models) for 25 and 100 theoretical plates the difference between these three profiles was negligible.

3. RESULTS

3.1 Analytical separation of aromatic compounds: nitrobenzene, naphthalene, fluorene and fluoranthene

Summary.

The aim of this chapter is to introduce a technique of HPLC used initially for the separation of aromatic compounds such as nitrobenzene, naphthalene, fluorene and fluoranthene as standard compounds.

Standard chromatographic calculations were carried out to evaluate the chromatograms and aspects of the performance of the chromatographic system such as: retention volume V_R, capacity factor k', selectivity factor α, resolution R_S and the number of theoretical plates N. The number of data points collected was investigated. The troubleshooting table is also presented at the end of this chapter to illustrate the problems associated with this technique.

3.1.1 High performance liquid chromatography - reversed phase.

One of the major and widely used techniques for the separation of various pharmaceuticals is reversed- phase high performance liquid chromatography (RP-HPLC).

HPLC is a very powerful separation technique which enables the effective separation of compounds of a very similar chemical structure in the minimum of time. This technique uses on-line monitoring, sensitive detectors, and columns packed with small particles. Efficient pumps increase the rate of separation. Higher pressures are generated as a result of the small particle size used to pack the column, but this is balanced by the high performance achieved and the reduction in the time needed to separate the compounds.

3.1.1.1 Principles of separation

The chromatographic separation of the sample compounds is the result of an interaction between the molecules of the sample, the stationary phase and the mobile phase. It is based on the difference in distribution coefficients between the mobile and stationary

phases. Separation takes place in the chromatography column which contains two phases: stationary and mobile. In the case of liquid chromatography the mobile phase is liquid and the stationary phase can be either liquid (partition chromatography) or solid (adsorption chromatography). The stationary phase in reversed-phase is non-polar. The samples are eluted from the column in order of polarity with the most polar samples being eluted first followed by the less polar which are more strongly retained on the stationary phase.

In order to elute the sample compounds from the column more quickly a less polar and more organic solvent has to be used. Thus if pure water was used the separation might take several hours but by introducing solvents like methanol, acetonitrile and tetrahydrofuran the separation time reduces significantly.

3.1.1.2 Factors affecting separation performance

There are various factors which influence the performance of the run. For example: solvent concentration, solvent chemistry (whether the solvent is a proton donor, a proton acceptor, or exhibits dipole moment), the presence of a buffer, its concentration and pH, flowrate, the temperature at which the method is run, and the nature of the stationary phase.

3.1.1.3 Stationary phase

Silica

Silica packing material comes in the form of a gel. In such a state it is regarded as polar, that is hydrophilic. It contains silanol groups (Si-OH) which adsorb water - they are hydrophilic. By modifying the surface of the stationary phase physically or chemically the characteristics of the stationary phase change (see section 2.6). By chemical reaction with compounds such as organosilanes or alkoxysilanes they become hydrophobic. As silica gel is slightly acidic, alkaline substances are more strongly retained than are substances which are neutral or acidic. Packing materials based on silica can only be used below pH 8 because they react with alkali to form soluble silicates.

CHARACTERISTICS OF SILICA.	
particle characteristics	rigid, porous
particle diameter	3-5 μm
stability	excellent mechanical stability under pressure
column efficiencies	high
reproducibility	good
pH range	2-7.5
pore diameter	more than 5nm
specific surface area (SSA)	the smaller the SSA the lower are the k' values; SSA is inversely proportional to pore diameter
true density of silica gel	2.2 g/cm^3
packing density	0.3 to 0.6 g/cm^3
surface reaction	silica can react as an acid, base or neutral

(Meyer, 1994)

Table 3.1-1 Characteristics of silica.

3.1.1.4 Mobile phase in reverse phase LC

The distribution of a solute between two phases is determined by the interaction of the solute species with each phase. The relative strength of these interactions is determined by the polarity of the sample and that of the mobile and stationary phases.

In chromatography based on adsorption the forces existing between the stationary phase and the solute (adsorbent-adsorbate forces) are: dispersion forces (van der Waals forces), induction forces (based on dipole moment of the molecules or ions), electrostatic forces (contributing to the adsorption of polar molecules on polar adsorbents), hydrogen-bonding (exists amongst molecules which are proton donors or proton acceptors) and charge transfer (plays a role in the bonding of many molecular complexes) (Snyder 1968).

Polarity is a term used as an index of the ability of compounds to interact with one another through mechanisms mentioned above (Lindsay 1992). The more polar the molecule the more strongly it will interact with other molecules. The interaction of solute molecules with mobile and stationary phases of similar polarity can lead to unsatisfactory separation. This fact explains why the mobile and stationary phases in

reversed-phase chromatography should be polar and non-polar respectively (i.e. the polarities of the two phases are very different).

Polarity can be expressed in various ways, e.g. as the solubility parameter or as solvatochromic polarity (Lindsay 1992). However, the most common way of measuring the polarity is by using a polarity index. In reversed-phase chromatography the four most common used solvents in the order of increasing polarity are water, methanol, acetonitrile and tetrahydrofuran. Here their relative polarity is measured in terms of how quickly they can elute the sample off the column provided the separation is run in all cases under the same conditions. Water being the weakest solvent in reversed-phase chromatography (with polarity similar to the non-polar stationary phase) it will retain the sample on the stationary phase for a very long time as it is not strong enough to elute it off the column. If however tetrahydrofuran is introduced, the interaction of the solute molecules and the mobile phase is stronger than that of the solute and the stationary phase (much stronger) and the solute is eluted off the column faster.

The most polar "solvent" in RP-HPLC is water. As more organic solvent is introduced to the column the sample components are eluted from the column more quickly. The most used solvents in RP are methanol, ethanol, propan-2-ol, acetonitrile and tetrahydrofuran. Propan-2-ol seems to be one of the best solvents in reversed-phase chromatography. However, the very high viscosity of this solvent limits its use. Depending on the nature of the sample other agents often have to be introduced to enable separation to take place (Snyder, Glajch and Kirkland, 1988).

The stronger the solvent used, the shorter the retention time of the compound of interest. For RP-LC chromatography the strength of solvents increases as the polarity decreases. This means that water is the most polar, least organic, and weakest solvent. It is followed by methanol, acetonitrile, ethanol and tetrahydrofuran, in increasing order of strength, as the most commonly used solvents in RP - HPLC.

Properties of solvents in LC

SOLVENT	SOLVENT STRENGTH	VISCOSITY [cPoise]	UV CUT OFF [nm]	BOILING POINT [°C]
WATER	very large	1.00	180	100
METHANOL	0.95	0.6	200	65
ETHANOL	0.88	1.2	200	78
i-PROPANOL	0.82	1.9	205	82
ACETONITRILE	0.65	0.37	210	82
TETRAHYDROFURAN	0.45	0.46	220	66

source: (Snyder 1978), (Snyder 1968).

Table 3.1-2 Properties of some reversed-phase solvents.

Characteristics of most common solvents in reversed phase HPLC are presented here.

3.1.2 Materials and Methods

The column used was 250 x 4.6 mm; volume: 4.2ml; Hypersil C_{18} silica, reversed-phase; 5μm particles; flowrates: 0.75 mL/min or 1ml/min. Sample loads were within the ranges 2-20μl or 0.2-2μl. The temperature used was ambient. Compressed air at 80 psi was used for injection.

The mobile phase used was acetonitrile or methanol (Hipersolv™, far UV grade for HPLC, BDH Laboratory Supplies, Poole, UK) in different concentrations. The water was de-ionised using a Purite water purification system. The mobile phase was filtered using 0.2 μm Nylon 66 filters (Phenomenex, Macclesfield, UK) and degassed using compressed helium (BOC) before introduction into the HPLC system.

Sample preparation

The samples were prepared using standard chemicals:

nitrobenzene: ρ = 1.1985 g/ml; BDH Ltd. Poole England, FW($C_6H_5.NO_2$) = 123.11

naphthalene: Scintran, BDH Chemicals Ltd Poole England, FW($C_{10}H_8$)= 128.17

fluorene: Sigma, FW ($C_{13}H_{10}$) = 166.2

fluoranthene: 98%, Aldrich - Chemie, West Germany, FW($C_{16}H_{10}$) = 202.26

Samples of different concentration were dissolved in methanol or acetonitrile and then stored in volumetric flasks of 10 or 25 ml volume.

For calibration the samples were subsequently diluted in acetonitrile or methanol (depending on the mobile phase used). The samples were loaded onto the column via, initially, a Beckman manual injector with a 20 µl loop and the samples were introduced using a Hamilton syringe (50 µl). Later, a pneumatically-operated autosampler (Beckman System Gold 507) was used fitted with variable loop size.

Modular chromatographic system.

All the experiments were carried out on a Beckman System Gold chromatographic system (Beckman Instruments, Inc., Fullerton, CA, USA).

The system consists of a solvent module 126, autosampler 507, diode array detector used in a dual wavelength mode (model 168) with a flowcell capacity of 10 µl.

The autosampler was fitted with a 10, 20, 50 or 100 µl sample loop. The 126 solvent module comprises two pumps, each of which controls up to four solvents. The modular system was controlled by the System Gold IBM PS/2.

System Gold enables the data to be translated into different formats which are directly compatible with spreadsheets such as Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) or Microcal Origin.

UV detection.

UV detection is used for the detection of compounds which contain chromofore (the solutes absorb UV or visible radiation).

Absorption of the radiation is governed by the Beer-Lambert law (Lindsay 1992):

$$A = e\,c\,b \qquad (3.1.1)$$

where A is absorbance

e is molar absorptivity (dm^3/mol.cm)

c is the concentration of the solute (mol/dm^3)

b is the path length of the cell (cm)

UV detectors come either as fixed wavelength or variable wavelength detectors. The fixed wavelength detectors can usually only work with one wavelength which the researcher chooses himself. Variable wavelength detectors, on the other hand, enable use to be made of the whole range of wavelengths. This feature is utilised by diode array detector which enables the scanning of each component of the sample for its impurities; and also the determination of the wavelength at which the compound of interest absorbs at its maximum. The diode array detector has been used in this project.

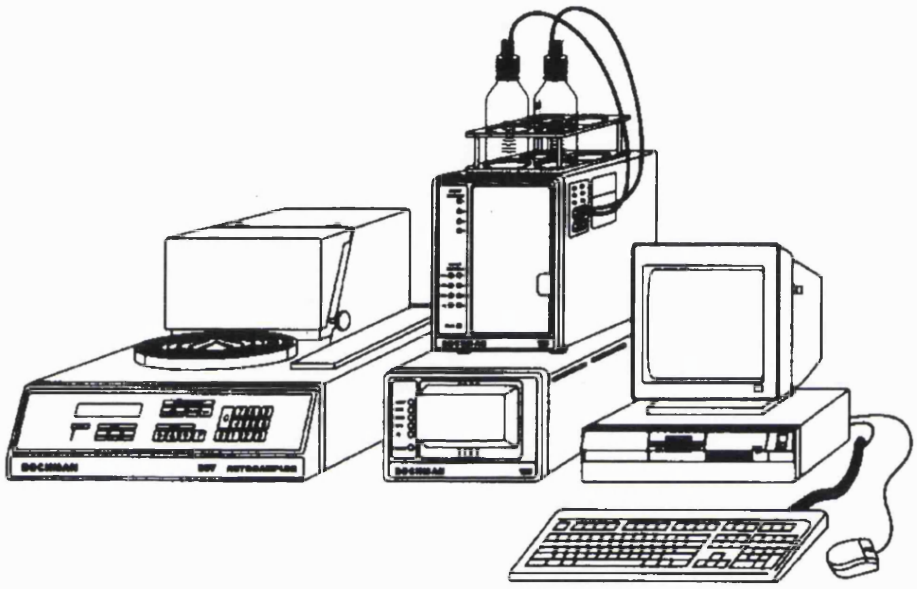


Figure 3.1-1 Beckman HPLC system.

3.1.3 Aims and objectives.

The aim of this chapter is:

- to check the automated system
- to ensure the system will deliver efficient separations under ideal conditions
- to check the method of calculation.

3.1.4 Data collection and results

A set of aromatic compounds was used to evaluate the capacity, selectivity, resolution and efficiency of the chromatographic column. Several different conditions were used to

to separate the aromatic compounds. A comparison between the graphical measurements from the chromatogram (i.e. ruler and pencil technique) and electronic measurements (computer measurements) was made.

Here a typical chromatogram of nitrobenzene, naphthalene, fluorene and fluoranthene, respectively is presented. All four compounds were separated with 90% acetonitrile or 90% methanol. The differences are immediately obvious. The sample was dissolved in 90% methanol in both cases, therefore the peaks separated under the same conditions led to nice symmetrical peaks. With 90% acetonitrile the "clashes" between the mobile phases led to the peaks becoming asymmetrical. The term "clashes" refers here to discrepancies existing between two mobile phases of different viscosity (the solute mobile phase different from that of the principal running mobile phase) which meet when the sample is introduced onto the column. This leads to increased band broadening as viscous fingering - also known as Saffmann and Taylor instability - is experienced (Shalliker et al. 1999). The column efficiencies for all the compounds were calculated and all the data needed for this are in Appendix 2. Generally the efficiencies for all compounds run at the same conditions (as is the case here) should lead to the same "N". However there are variations in the number of theoretical plates.

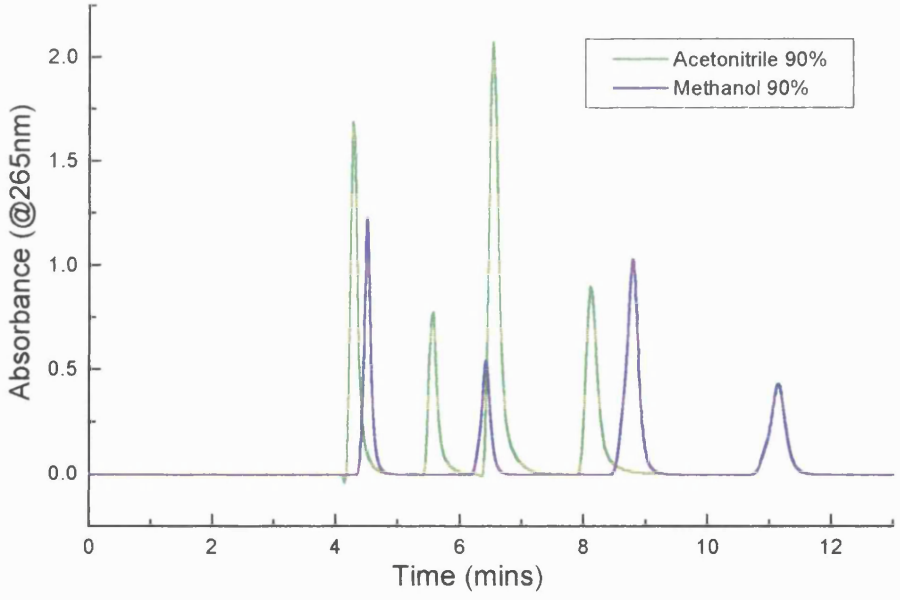


Figure 3.1-2: A chromatogram of four aromatic compounds at 90% methanol/water and 90%acetonitrile/water conditions

The number of theoretical plates can be found in Appendix 2 Section 8.2.2.

3.1.4.1 Manual calculations.

Flow rate versus retention volume.

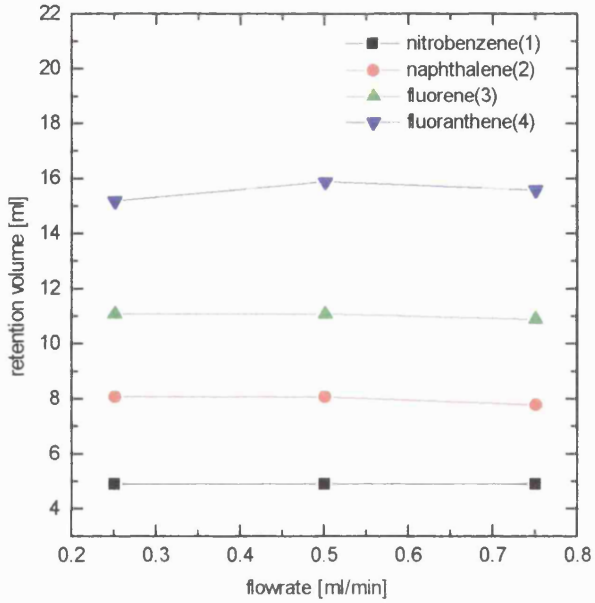


Figure 3.1-3 Flowrate/retention volume relationship of four aromatic compounds.

Conditions:

70% acetonitrile/water, sample concentration: 0.02g/l; detection: 245 nm; injection: 20µl.

The graph shows how each of the aromatic standards has its characteristic retention volume irrespective of flowrate (fig.3.1.3).

Number of theoretical plates or column efficiency.

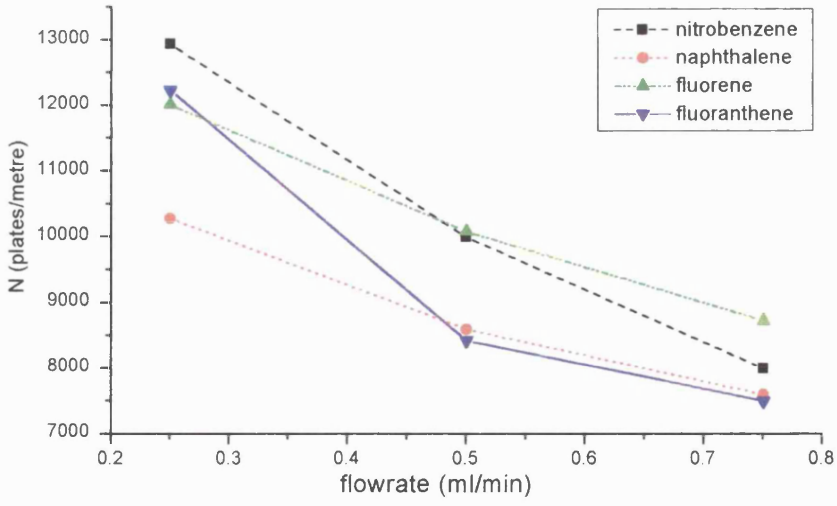


Figure 3.1-4 The effect of flowrate on the column efficiency.

Conditions:

70% acetonitrile/water; concentration: 0.02g/l; detection: 245nm; injection: 20µl

Here the measurements needed for the calculations of N were done with a ruler from the charts, rather than electronically from the actual data. As a result, the data values are lower than expected (see section 3.1.4.2.2).

The graph shows the change in the number of theoretical plates with changes in the flowrate. The curve for each compound shows how N decreases with increased flowrate. The data used for the construction of this graph were then used in calculations to plot the H/u curve (see figure 3.1-5). H was calculated using the equations in sections 2.2.2.1 and 2.2.2.2.

H/u curve.

	H=L/N (cm)			
u (cm/sec)	nitrobenzene	naphthalene	fluorene	fluoranthene
0.025	0.008	0.001	0.008	0.008
0.05	0.01	0.012	0.01	0.012
0.075	0.013	0.013	0.01	0.013

Table 3.1-3 H values for nitrobenzene, naphthalene, fluorene and fluoranthene at different flowrates.

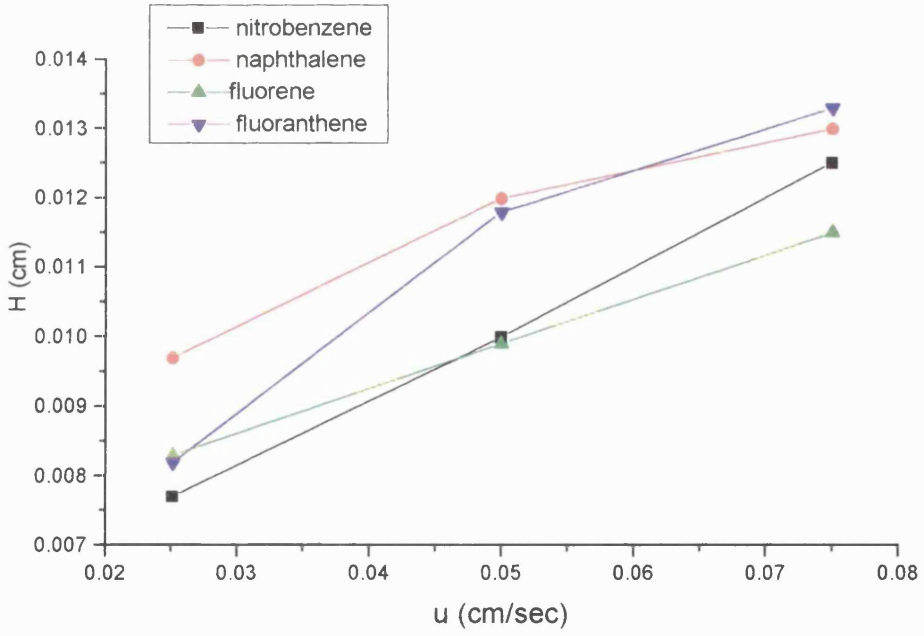


Figure 3.1-5 H/u curve of aromatic compounds.

Conditions:

70% acetonitrile/water; concentration: 0.02g/l; detection: 245nm; injection: 20µl

The H/u graph contains three points for each compound: nitrobenzene, naphthalene, fluorene and fluoranthene. The values of H increase with increasing values of the linear flow rate "u". The shape of the H/u curve is usually perceived as an asymmetrical U-shaped curve. Although here the number of data points is less than optimum they are sufficient to show a tendency towards the lowest point on the curve. At very low flow rates the values of H should sharply increase. The smallest value of H is for nitrobenzene followed by fluoranthene, fluorene and finally naphthalene. The values of H are perhaps too high indicating rather low values of N. This is due to inaccuracies associated with the manual measuring of peak widths.

It is obvious that to achieve the minim value of H one has to work at very low velocities. In our case the minimum was not quite reached for lack of time.

The H/u curve, also known as a van Deemter plot, expresses how well the column is packed and what the ideal flow rate for particular conditions should be. In essence it determines whether a column is working properly. It is the sum of a number of different aspects which contribute to band broadening. The lowest point on the graph (the lowest value of H) indicates the flow rate which should be used to give the best performance of the column.

3.1.4.2 Electronic calculations

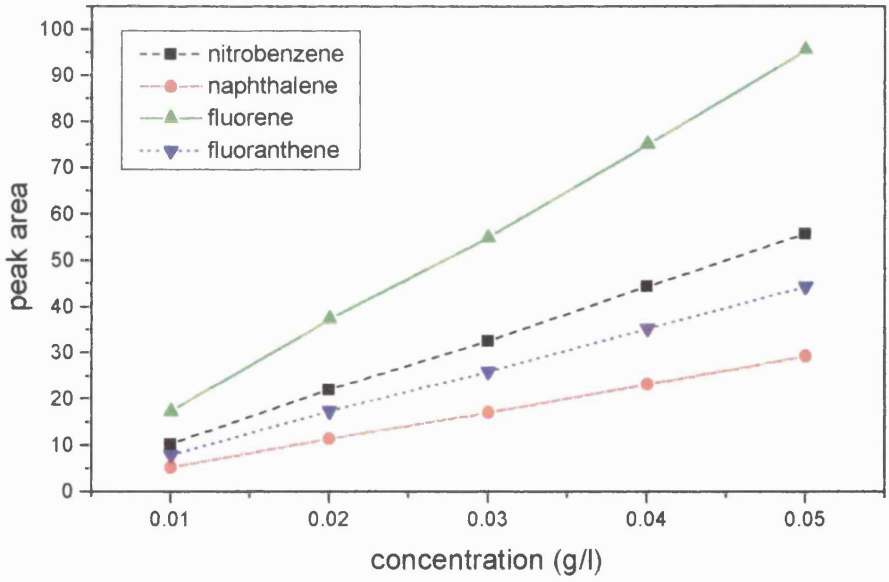


Figure 3.1-6 Concentration vs. peak area for all four compounds.

Conditions:

90%acetonitrile/water; injection:10µl; F:0.75ml/min; detection: 265nm; column: Hypersil 5micron ODS.

Calibration curves serve to determine the concentration of the samples as well as to test the precision and accuracy of the system.

Here the calibration curves of nitrobenzene, naphthalene, fluorene and fluoranthene were constructed by running samples at increasing concentrations between 0.01-0.05g/l. At the maximum concentration the detector response is linear with the coefficients of determination being very close to figure 1.

CONCENTRATION	PEAK AREA			
(g/l)	nitrobenzene	naphthalene	fluorene	fluoranthene
0.01	10.22	5.31	17.36	7.95
0.02	21.98	11.48	37.38	17.44
0.03	32.55	17.11	54.97	26.01
0.04	44.31	23.19	75.08	35.27
0.05	55.67	29.32	95.56	44.41
coefficient of determination	1	1	1	1

Table 3.1-4 Concentration and peak area values for the four compounds.

How many data points to use?

The number of data points collected in chromatography is vital as they have an impact on the outcome of subsequent calculations of the number of theoretical plates (i.e. on column efficiency).

As can be seen from the previous results, the number of theoretical plates calculated from graphical measurements (manual measurements of parameters like half width and retention time) led to small values. When the data was compared with measurements calculated electronically the results were immediately obvious. This reinforces the fact that the data should be calculated electronically as it is the most accurate method to date. This section explains the basis on which this assumption was made, the relevance of this for early and late eluted peaks, gives comparisons of different data points and corresponding "half widths", and draws conclusions from the findings.

It was calculated electronically using Microcal origin software where the peak of interest is integrated. This enables the software to calculate the half width at half height and other factors such as peak height and peak area. The data for t_r and $t_{w1/2}$ obtained from the Microcal Origin calculations are provided in Appendix 2. When inetegrating the peak Microcal Origin software determines from the number of data points available the crest of the peak, its start and its finish. This obviously brings another problem: if the number of data points is insufficient the peak crest will not be determined correctly and this consequently leads to inaccuracies in calculating the half widths.

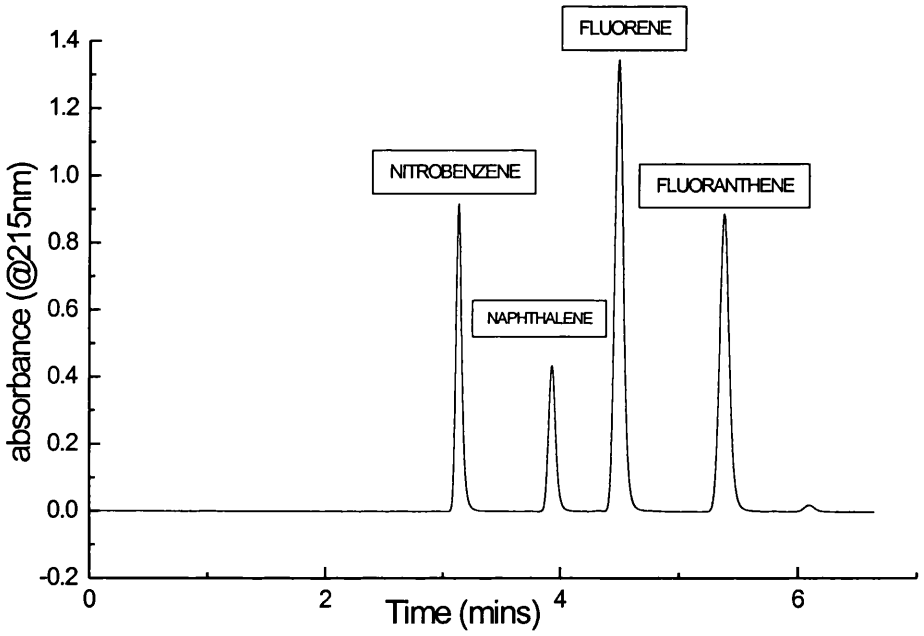


Figure 3.1-7 The standard chromatogram of aromatic compounds.

Conditions:

90%methanol/water; concentration: 0.5g/l; injection: 5μL; flowrate: 0.75ml/min; detection: 265nm

This is a chromatogram of standard compounds nitrobenzene, naphthalene, fluorene and fluoranthene in that order. It is an example of a good separation with narrow and symmetrical peaks well resolved with no overlapping taking place. Detection of the peaks is well within the linear capacity of the detector.

The width of the peaks increases gradually. This is due to dispersion of the sample compounds as they travel along the column. To determine how well the column is

packed, the efficiency of the column should be calculated. This can be done from the retention time and the width of the peak at half height. These two values are then used to calculate the number of theoretical plates "N" as an indicator of column efficiency. Ideally, N should be the same for all the peaks in the chromatogram. Failure to achieve this could be also due to the way the data is collected.

Here the number of theoretical plates - N - was calculated when various numbers of data points were collected that is 6400, 3200, 1600, 1000, 300 and 100.

	N (plates/metre)			
# OF POINTS COLLECTED	nitrobenzene	naphthalene	fluorene	fluoranthene
6400	71 680	79 480	75 190	79 930
3200	69 050	82 070	75 190	79 930
1600	63 810	77 000	71 280	83 780
1000	69 050	87 620	79 420	83 780
300	50 420	79 870	77 000	83 780
100	48 840	19 250	24 910	36 350

Table 3.1-5 N calculated using electronic method when different number of data points was collected

Looking at the nitrobenzene column, the N values drop steadily as fewer and fewer points are collected. This is symptomatic of fast eluted peaks where the absence of sufficient number of data points collected would make the peak appear wider therefore giving lower N values which can be misleading. This dependence of N on the number of data points collected gradually ceases for the peaks which are eluted off the column later. The table also shows the variations in N values from one compound to another. Ideally, the values of N should be the same for all the peaks (under the linear conditions). Although certain deviations from average values are acceptable, large differences indicate a problem. This is the case when 300 points were collected: the nitrobenzene peak gives N=50 420p/m whereas fluoranthene gives a much higher value of N=83 780.

# of points collected	nitrobenzene		naphthalene		fluorene		fluoranthene	
	t_R	$t_{w1/2}$	t_R	$t_{w1/2}$	t_R	$t_{w1/2}$	t_R	$t_{w1/2}$
6400	3.14	0.055	3.93	0.066	4.49	0.077	5.38	0.09
3200	3.14	0.056	3.93	0.065	4.49	0.077	5.38	0.09
1600	3.13	0.058	3.93	0.067	4.49	0.079	5.38	0.088
1000	3.14	0.056	3.93	0.063	4.49	0.075	5.38	0.088
300	3.13	0.066	3.94	0.066	4.48	0.076	5.38	0.088
100	3.13	0.067	3.93	0.133	4.47	0.133	5.4	0.133

Table 3.1-6 Values of retention time and half width for all four compounds.

Parameters used for the calculations of N (data gained electronically), the table is related to a Table 3.5. Please note that the column length used in the calculations of N was 25cm.

In the table above t_R corresponds to the retention time of a compound and $t_{w1/2}$ is its width at half height. The retention time of each individual peak with regard to the data points collected does not change significantly.

On the other hand, the width at half height has a profound effect on the calculation of N (number of theoretical plates) and the $t_{w1/2}$ value varies according to the number of data points collected. As fewer data points are collected the value of $t_{w1/2}$ becomes larger and the inaccuracy of measurement increases.

This is most obvious in the case of early eluted peaks. In the case of late eluted peaks the differences in the values of $t_{w1/2}$ are not very great. However, when a very small number of data points is collected (e.g. 100 data points) the peak width value for all the peaks becomes extremely inaccurate and the resulting N value is unreliable. The matter is further compounded when calculations of N are done manually.

MANUAL CALCULATIONS FROM CHROMATOGRAM				
	N (plates/metre)			
	nitrobenzene	naphthalene	fluorene	fluoranthene
t_R(cm)	3.95	8.9	12.5	17.9
$t_{w1/2}$ (cm)	0.35	0.4	0.5	0.55
N(plates/metre)	2 820	10 850	13 740	23 470

Table 3.1-7: N values calculated from the parameters obtained manually from the chromatogram.

Conditions:

Column: ODS silica C_{18}, F=1ml/min, detection: 265nm, mobile phase: 90%acetonitrile/water.

Here the values of N were calculated from the retention time and width of each peak as measured from the chromatogram (i.e. manually using pencil and ruler).

Although the number of data points collected was maximum i.e. 6400 the values of N are far from the values obtained electronically. The values of N are increasing with each peak in succession but numbers are very low. The accuracy of the measurement decreases as the peak elutes from the column faster. Those peaks are very narrow and the measurements of $t_{w1/2}$ manually are subject to a large statistical error.

This shows that the manual technique is not reliable and the data gained are misleading. Therefore the technique should be abandoned and only the electronic technique of calculating N should be used.

Half width of fast- and slowly-eluted peaks

In this section the issue of early and late eluted peaks is discussed in more detail. Here four different sets of data points were collected and the half width corresponding to this data was compared.

If the separation data was collected for 13 minutes, 1560 points were collected at a detector frequency of 2 Hz (the number of points collected per second). The detector was capable of collecting data at higher frequencies: 1, 2, 4, 8 and 16 Hz but only the 2 Hz frequency was used. During the translation of the sample from System Gold software into a form which can easily be imported into the standard spreadsheet

packages used currently to analyse the data (e.g. Microcal Origin) the data points were further reduced to avoid large data files and to speed the importing process. This resulted in 300, 520 and 800 data points being obtained. The two tables presented here with a highlighted "half width" data column (which corresponds to a half width measured at half height) also show other factors calculated such as area under the peak, peak retention time and peak height. The tables thus enable direct comparisons of the following values: number of data points, and half width. The tables are presented for two "extreme" peaks: the first (nitrobenzene) and the last (fluoranthene).

nitrobenzene				
number of points	peak area	retention time t_R	peak height	half width
300	0.1606	4.5	1.22	0.1
520	0.1615	4.5	1.08	0.13
800	0.1617	4.5	1.18	0.12
1560	0.1611	4.5	1.23	0.12

Table 3.1-8 The dependence of half width on the number of points collected for nitrobenzene as the fast eluted peak.

Nitrobenzene, as the first compound in this sample mixture, was eluted off the column as a fast peak with a very narrow band. In order to calculate N, the half width of the peak is critical. This table shows how the number of data points collected (300, 520, 800 and 1560) affects the accuracy of the data further calculated. Clearly in the case of nitrobenzene, the higher the number of data points, the more accurate the calculation of the peak width. Unlike other peaks in the chromatogram, peaks which elute very fast require the highest number of data points to calculate the peak width at half height.

fluoranthene				
number of points	peak area	retention time t_R (mins)	peak height	half width
300	0.135	11.15	0.43	0.25
520	0.136	11.15	0.43	0.28
800	0.136	11.15	0.43	0.27
1560	0.394	10.98	1.30	0.28

Table 3.1-9 The low impact of data points collected on the half width for fluoranthene as a late eluted peak.

Fluoranthene is the last peak to be eluted off the column in this sample mixture. It appears that the minimum threshold for the data points needed is much lower than in the case of nitrobenzene. When comparing the retention times and widths of both peaks, fluoranthene was travelling along the column for more than twice the time of nitrobenzene. Consequently due to the dispersion taking place the fluoranthene peak widened and the peak width reached a value more than double that of nitrobenzene.

This peak thus appears much wider than the first one, the elution time also taking longer.

Consequently a lower number of data points is acceptable in order to calculate accurately the half width for wide- and late-eluted peaks.

Whilst wide peaks are not a typical feature of analytical chromatography in its true sense, they are ever present in large scale chromatography because of the much higher load typically used in such systems.

Number of theoretical plates and half width

The following table shows how the number of theoretical plates calculated varies with the number of data points collected.

# of points collected	N (plates/metre)			
	nitrobenzene	naphthalene	fluorene	fluoranthene
1560	33 100	40 600	39 400	38 700
300	44 900	40 300	27 500	44 100

Table 3.1-10 The differences in N values depending on the number of points collected for all four aromatic compounds.

points	mean	standard error (yEr+-)	Student's **t** for 3 degrees of freedom	range of 95% confidence
1560	37 900	1 658	3.182	32 650-43 200
300	39 200	4 032	3.182	26 360-52 020

Table 3.1-11 Statistical interpretation of data collection at high and low frequencies.

reference:

(Parker 1979) (Casswell, 1982)

This table shows how discrepancies can develop in calculations if insufficient data points are collected. N should be the same or similar for every peak of the chromatogram. The discrepancies in N are greater at 300 points and fewer at 1560 points.

Note that, however many data points are collected, N is always greater than when calculated graphically. This is due to inaccuracies in measurement.

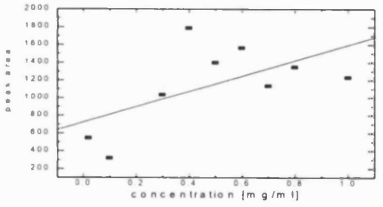
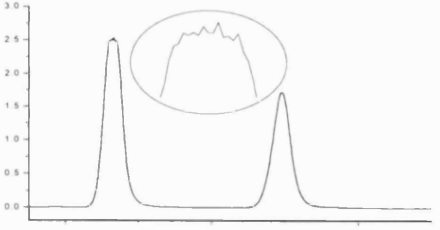
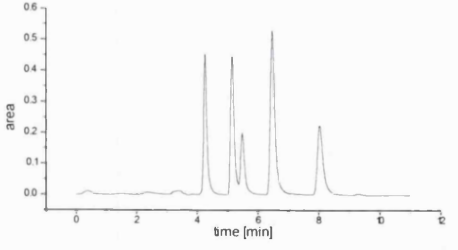
Conclusion

The number of data points collected is vital particularly for fast eluted peaks where insufficient data points can seriously affect further calculations of column efficiency. In preparative chromatography, where wide peaks are experienced, a lower number of data points is satisfactory as the half width can still be calculated accurately.

3.1.4.3 Troubleshooting

The following table is presented to pinpoint some common mistakes which are experienced when performing chromatography. These are gradually eliminated as the

researcher becomes more skilful and knowledgeable in chromatographic separations. This table is based on the experiences derived from our own work.

TROUBLESHOOTING OF HPLC SYSTEMS				
NUMBER	**PROBLEM**	**SYMPTOM**	**PICTURE**	**SOLUTION**
1	preparation of the sample not accurate	calibration curves are not linear		prepare samples accurately (weighing out and diluting correctly is crucial)
2	samples of very high concentrations loaded on the column	jagged peaks due to limitations of detector		use lower concentrations of the samples (dilution) do not go beyond 2.0 AUFS
3	samples stored in plastic bottles (the plastisizer - thalide- reacted with the sample)	double - peaks appeared		samples should be stored in glass bottles

RESULTS. HPLC Troubleshooting table.

4	samples dissolved in a solvent different from the one in the mobile phase (different viscosities)	band broadening shown here as back tailing due to viscous fingering		samples should preferably be dissolved in the mobile phase or, when further dilution follows, diluted in the mobile phase
5	pressure fluctuations	timings of the peaks are not accurate due to an air-bubble in the pump		remove the air bubble by increasing the flowrate (drain open)
6	blocked frits on the column	shoulders appearing on the peaks		clean the frits (sonication)

3.2 Analytical separation of erythromycin

Aim.

The aim of this chapter is to present the development of an analytical method for the separation of erythromycin A as a main component of erythromycin. Erythromycin was used as a model on which all the studies on modelling and scale-up were performed.

The reasons for the choice of a particular column and running conditions are explained.

In preparative chromatography a large amount of mobile phase is utilised. Precision during the preparation can be compromised. Therefore various experiments were conducted with changes in mobile phase composition to see what impact they had on the separation.

3.2.1 Erythromycin

3.2.1.1 Brief description

Erythromycin is a macrolide antibiotic. It is slightly soluble in water, methanol (1:5) and chloroform. It is unstable in highly acidic and highly alkaline conditions. Maximum stability is between pH 6 and 9.5. It forms salts with weak and strong acids (Kavanagh and Dennin 1963)

3.2.1.2 Structure of erythromycin

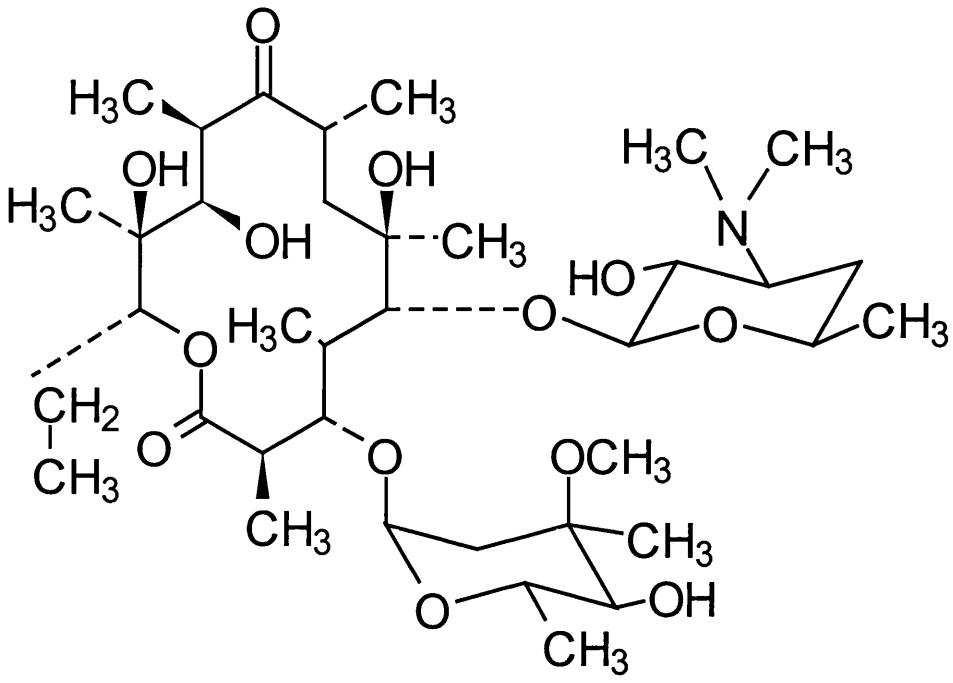


Figure 3.2-1 A structure of erythromycin A.

3.2.2 Materials and methods

3.2.2.1 Erythromycin and its impurities

The sample used in these experiments was a crude erythromycin (Abbott Laboratories, Chicago, Illinois) with a purity of erythromycin A of about 70%.

Experiments were conducted to identify other impurities in the sample. These were done by running the standards available and then comparing the results with the separation of crude erythromycin. A graph of the results is given below:

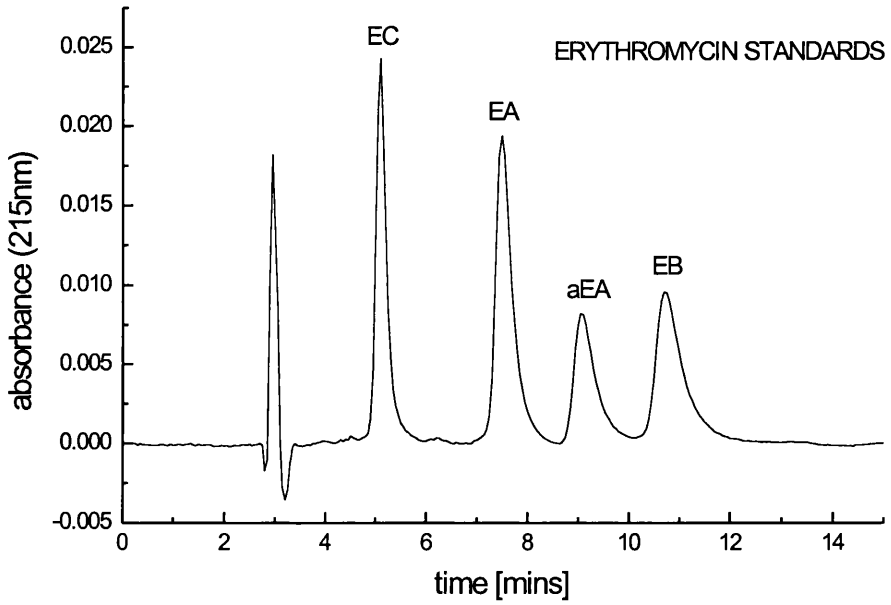


Figure 3.2-2 Erythromycin standards.

Here three erythromycin impurities were identified as erythromycin C (EC), anhydroerythromycin A (aEA) and erythromycin B(EB) shown here in relation to the main peak of erythromycin A (EA).

Sample preparation.

A sample of erythromycin was dissolved in a methanol/buffer mixture (1:1) and injected automatically using a Beckman System Gold 507 autosampler.

Solubility.

The solubility of the sample was tested. The sample should be dissolved in equal parts of methanol and phosphate buffer (pH=7; 0.2 M). The sample takes a long time to dissolve in this mixture but it is easily soluble in methanol. Therefore, the sample was first dissolved in methanol and then diluted with the buffer. The maximum concentration achieved was 50mg/ml. Attempts to achieve a higher concentration resulted in precipitation of erythromycin from the solution.

3.2.2.2 Mobile phase.

The mobile phase composition was: 45:5:50= acetonitrile: buffer: water. Acetonitrile (Hipersolv^{TM}, far UV grade for HPLC, BDH Laboratory Supplies, Poole, UK) was mixed with de-ionised water (filtered through Purite water purification system) and then

potassium phosphate was added. Initially two potassium phosphate buffers - potassium dihydrogen orthophosphate (KH_2PO_4), (Fisons Scientific Equipment, Loughborough, Leics., UK) and potassium monohydrogen phosphate (K_2HPO_4), (Sigma Chemicals, St. Louis, MO, USA) of 0.2M concentration were mixed together to reach pH 9.
The mobile phase was filtered using 0.2 μm Nylon 66 filters (Phenomenex, Macclesfield, UK) and degassed using compressed helium (BOC) before introduction into the HPLC system.

3.2.2.3 Column specifications and packing material

The column used in these experiments was PLRP-S (Polymer Labs.; Church Stretton, Shropshire, UK) with a pore size of 1000Å and a particle diameter of 8 μm. It is a reversed phase column with polymeric (PSDVB) packing material. The dimensions of the column were 250 x 4.6mm. The porosity of the column was calculated as 0.61.

3.2.2.4 Packing material

Polymeric stationary phases versus silica

The method of separation of erythromycin A employs a column with polymeric packing material. Over recent decades polymers have become more and more popular because of their rigidity, their ability to withstand higher pressures (which was not the case when polymers first appeared in the form of gel) and their ability to be used across the whole range of pH. This last feature in particular gives polymer a distinct advantage over silica which can only be used over a relatively narrow pH range (see section 2.6).

3.2.2.4.1 Properties of polymer columns

	PSDVB (POLYMER LABS)
Column dimensions (cm)	25 x 0.46
Chemistry of packing	styrenic
Particle size (μm)	8
Particle shape	spherical
Specific surface area (m^2/g)	500
Average pore diameter (Å)	1000
Plates per meter for EA	11 756
Mobile phase:	45:5:50 = acetonitrile: phosphate buffer (pH=7 or 9, 0.2M): water
CHEMICAL SPECIFICATIONS	1. pH= 1-13 2. clean-in-place regeneration (using acids, bases or solvents; e.g. 0.5M NaOH) 3. broader mobile phase range selectivity 4. improved resolution and capabilities in analysis of polar compounds
PHYSICAL SPECIFICATIONS	5. reproducible performance 6. high flow rates - enables flowrates 1 500cm/hour 7. High capacity + excellent adsorption kinetics for a broad range of molecules 8. high loads - can be loaded until performance degrades (can later be restored) 9. reduced solvent cost - (suitable for scale-up)
DISADVANTAGES	10.less pressure tolerance 11.swelling/shrinkage changes (occur with a change of organic modifier content in the mobile phase)

Table 3.2-1 Physical and chemical properties of polymer columns. (Polymer Labs.)

A commonly used polymeric packing material is polystyrene - divinylbenzene. It is made by the copolymerization of styrene and divinylbenzene. The latter functions as a

cross linker. PSDVB is a highly cross-linked copolymer with excellent physical and chemical properties. (see also Polymeric stationary phases in section 2.6.2. of Introduction). Here the separation is based on the adsorption of a sample on the stationary phase.

3.2.2.5 Cleaning the column

A column which is used on a daily basis needs to be cleaned regularly to yield reproducible and reliable results. This is a part of the method validation.
There were two standard procedures used in cleaning.

3.2.2.5.1 Routine cleaning

This method was used on a weekly basis. The column was first flushed with 45% acetonitrile/water for a sufficient time to eliminate any buffer residues (at 1ml/min for approximately 30-45 mins). This was followed by a gradient of 45-100% acetonitrile/water over 20 min. The column was then flushed with 100% acetonitrile as a strong solvent to eliminate any impurities which may have adhered to the stationary phase.

3.2.2.5.2 Cleaning with acid/base

The scale up studies required the column to be loaded with large amounts of the sample. The separation studies were performed at 35 % acetonitrile as a running mobile phase. After this was completed 55% acetonitrile was introduced without the sample being injected onto the column and the use of stronger mobile phase led to elution of some impurities which had not eluted previously. However some material remains, and after a while this leads to clogging of the pores of the stationary phase due to an alkaline sample being flushed out with mobile phase which is also of an alkaline nature. Over a period of time the accumulation of residues on the column led to distortion of peaks and deterioration of column performance. The above cleaning routine was therefore insufficient and there was a need for a more rigorous cleaning procedure.
A new cleaning solution was used which contained methanol and 0.5% phosphoric acid (in a ratio of 1:1) as the sample was of a basic nature. The alkaline nature of

erythromycin pairs with the acidic nature of phosphoric acid to yield a salt which can easily be flushed off the column. The column was flushed with this solution at 0.5ml/min for several hours. This was repeated in both directions to clean the front frit. This cleaning process proved essential to maintain reproducibility.

This method was developed to provide a satisfactory separation of erythromycin for our purposes. It is possible that some sample is lost when run under the standard conditions.

3.2.3 Method development

3.2.3.1 Original method for the separation of erythromycin

The method developed for the separation of erythromycin with polymeric column was based on the paper by Paesen et al. (1993)

The degree of selectivity depended on the pore size of the packing material polystyrene divinylbenzene (PSDVB) which in this case was 1000Å. This polymeric packing is stable for pH values between 1 and 13; it also gives a better peak shape and a longer column life for the separations than is the case with silica packing.

Other researchers, Paesen, Roets and Hoogmartens (1991), tested this separation method using three pore sizes to determine the best selectivity: 100, 300 and 1000Å. The experiments showed that the best results were achieved with a larger pore size, that is 300 or optimally, 1000Å.

Organic modifiers such as methanol, acetonitrile and THF, control band spacing in reversed phase chromatography. Another type of solvent which plays a role in the separation is 2-methyl-2-propanol. The use of this solvent has an impact on the separation of erythromycin impurities.This results in a better resolution of the sample components namely: AEA, psEAEN and EB: and means that the omission of (2-methyl-2-propanol) results in an unsatisfactory separation of a crude erythromycin sample.

The figure below presents profiles of erythromycin separation at 1ml/min with a 100 µl injection volume and 2ml/min with a 500 µl loop injection. The concentration of the sample in both cases was 8g/l and the composition of the mobile phase was the same as in the original method.

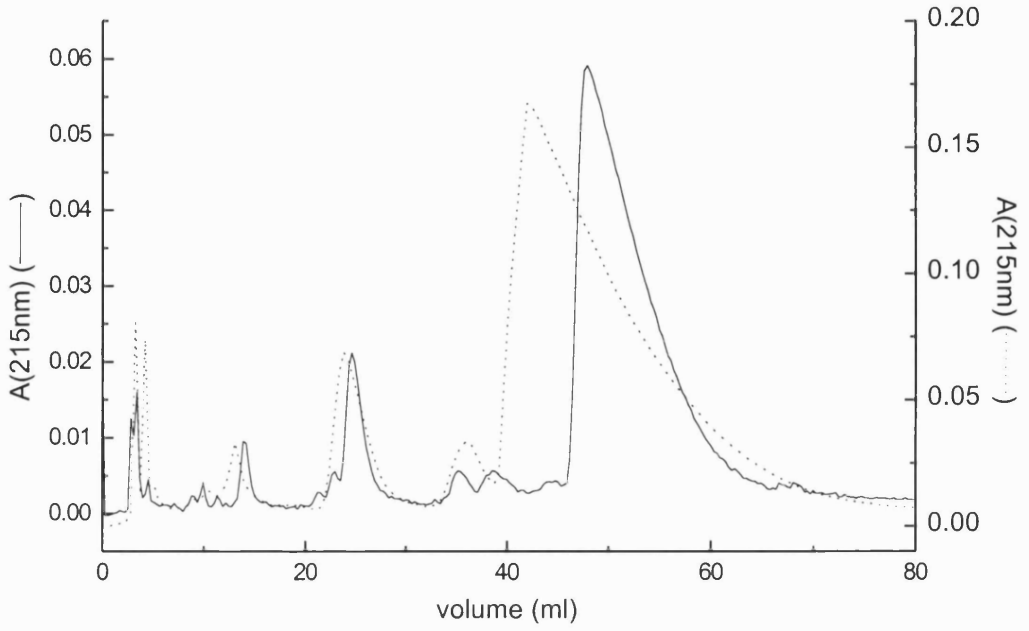


Figure 3.2-3 A profile of erythromycin separation at 1ml/min. (---) and at 2ml/min (...).

3.2.3.1.1 Conditions of original method.

The research was originally carried out using the same conditions as stated in the method: this required acetonitrile, 2- methyl-2-propanol and water together with a phosphate buffer (K_2HPO_4 0.2M, pH=9).

The method adopted was derived from the collaborative study of Paesen et al. (1993). The mobile phase was acetonitrile : 2-methyl-2-propanol : 0.2M potassium phosphate buffer pH=9 : water (3:16.5:5:75.5). The column was operated at a temperature of 70°C, with a flowrate of 1ml/min and the UV detection at 215nm.

3.2.3.1.2 Reasons for using modified method

The original method proved capable of separating erythromycin compounds quite successfully. However, it is not very suitable for large scale application for several reasons. Firstly, 2-methyl-2-propanol is solid at room temperature and needs to be heated to be used. When in a liquid state it is too volatile to be handled in large quantities. Secondly, the method consumed a high volume of solvent which could be quite costly in the long run; and thirdly, the rather high viscosity of the solvent generated high pressures on the HPLC system. Consequently we were forced to modify

the method and refrain from the use of this solvent (2-methyl-2-propanol) in our further experiments in order to meet the needs of scaling up.

We therefore resorted to using a single solvent, i.e. acetonitrile. Experiments with this solvent proved that separation using the modified method was possible although not completely satisfactory: the method does not enable us to separate all the components of the sample to the desired extent. But this was not the aim, anyway.

3.2.3.2 Modification of the method

The column used in this method was PLRP 1000Å 250 x 4.6mm employed also by Paesen, Roets and Hoogmartens (1991).

It was necessary to establish a method which would be based on acetonitrile as the main solvent, with buffer and water. A gradient was run from 10-90% acetonitrile/water/buffer to find the optimum concentration of solvent. This was then determined by extrapolation of time and the concentration gradient curve (when the main peak of EA was detected). The experiments with acetonitrile and a phosphate buffer showed that the main peak (EA) came off at about 45% of acetonitrile/water concentration in the mobile phase. This concentration was then used as the basis for all the other experiments.

3.2.3.2.1 Modified (final) version.

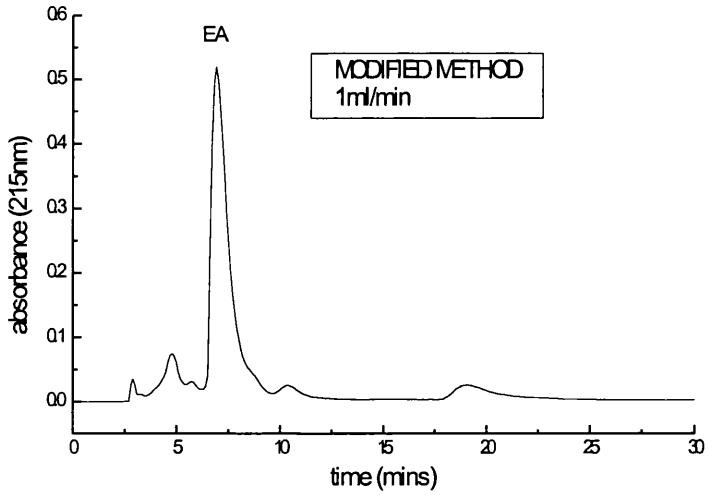


Figure 3.2-4:Modified version of the original method without 2-methyl-propan-2-ol.

Conditions: column: PLRP-S 1000Å, 8 μ 4.6 x 250mm; sample: 8mg/ml; temperature: 70 ℃; loop: 100μl; mobile phase: 45:5:50 = ACN: phosphate buffer (pH=9; 0.2M): water

This method gave a reasonable separation of the main compound erythromycin A and its impurities. It is an isocratic method which is convenient for modelling chromatographic processes as well as scaling up.

3.2.4 Results - Changes in the mobile phase composition

In order to scale up it is important to know how changes in the method can influence the separation.

The standard mobile phase composition was 45:5:50(% v/v)=acetonitrile: buffer: water. The buffer: K_2HPO_4 concentration: 0.2M and pH=9 (before added into the mobile phase). Temperature: 70°C. Flowrate: 1ml/min.

The following factors were examined:

- buffer concentrations
- buffer pH
- the use of different phosphate buffers [Na_2HPO_4, $(NH_4)_2HPO_4$].
- temperature (various levels up to 70°C)
- sample concentration
- flowrate
- a change in the acetonitrile concentration (35%, 55%).

Unless otherwise stated samples were loaded at a concentration of 8g/l using the loop capacity of 20μl (0.16mg) with erythromycin A being detected at 215nm.

3.2.4.1 Buffer concentration

The values of buffer concentration investigated were the values higher and lower than the 0.2M concentration which was the standard buffer concentration used for the

separation of erythromycin. Therefore the values at which the separation was carried out were 0.1; 0.15; 0.25; 0.3M.

3.2.4.1.1 Graphs of the profiles at 0.1M and 0.3M.

Here graphs of two extremes of buffer concentration (0.1M and 0.3M) are presented to compare the differences.

In all cases other values were calculated such as the number of theoretical plates, capacity factor, selectivity factor and resolution factor. That data is in the attached Appendix. 3.

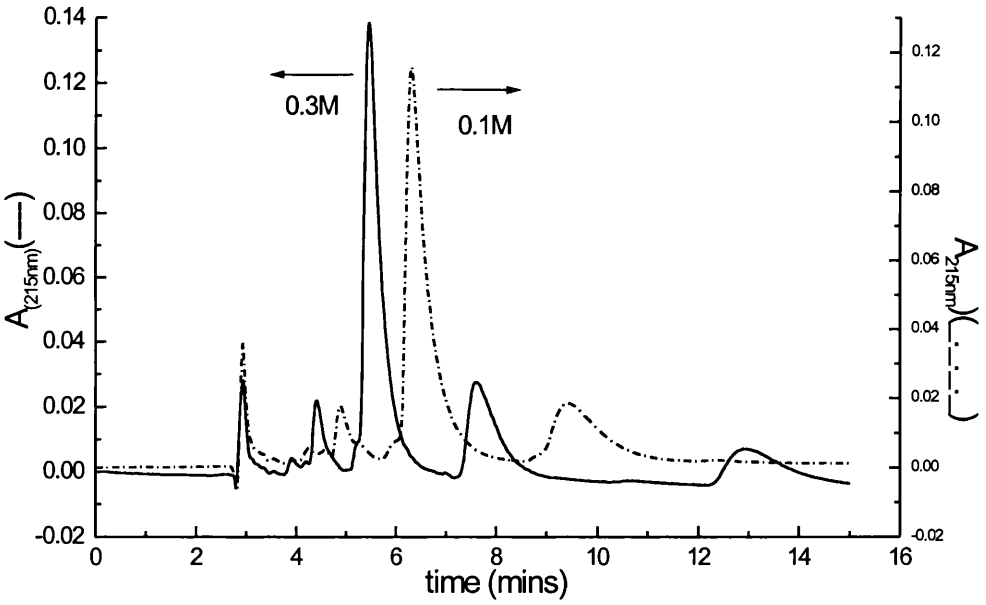


Figure 3.2-5: Profiles of erythromycin at 0.1M and 0.3M buffer concentration.

Conditions: column: PLRP-S 1000Å, 8 μ 4.6 x 250mm; sample: 8mg/ml; temperature: 70 °C; loop: 20μl; mobile phase: 45:5:50 = ACN: phosphate buffer : water; flowrate: 1ml/min

$N_{(0.1M)}$ = 1343p/c (5373p/m)

$N_{(0.3M)}$ = 4330p/c (17 320p/m)

The two graphs show that the EA peak is eluted faster at higher buffer concentration. This has an impact on other particularly late eluting impurity peaks.

At 0.3M buffer concentration the main peak of EA together with two late eluting impurities appear on the chromatogram. At 0.1M buffer concentration the second late eluting impurity is not seen as it eluted from the column much later.

Resolution in both cases is quite good although the efficiency is much better at the higher ionic strength (0.3M). It is also of interest that while the time delay between the peaks of EA is quite small, in the case of the late eluting impurities the time delay is significantly greater.

3.2.4.1.2 Buffer concentration vs. Retention time

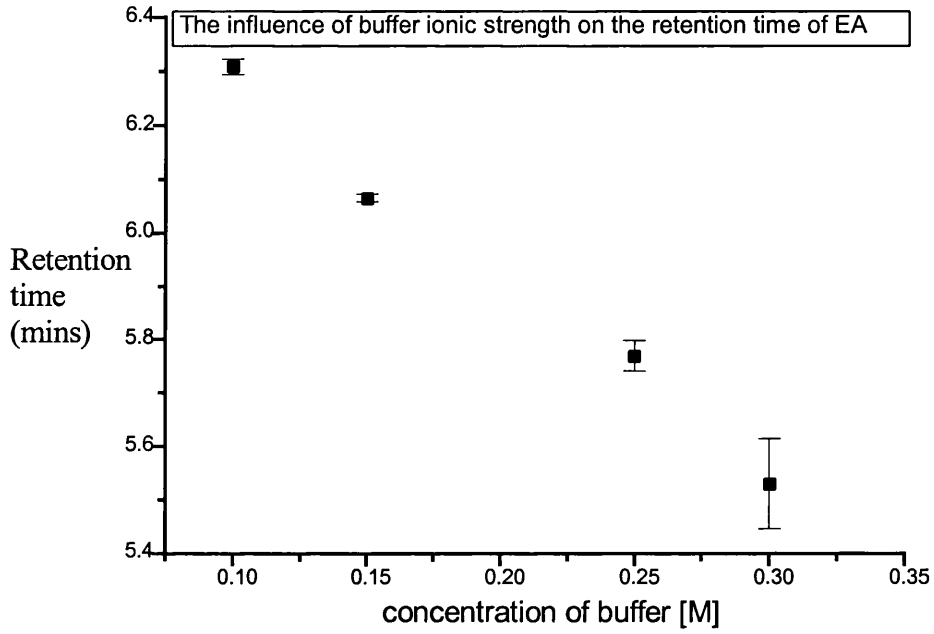


Figure 3.2-6: The influence of buffer concentration on the retention time of erythromycin A.

The increased concentration of the buffer decreases the retention time of erythromycin A. Although the last value of retention time at the 0.3M concentration presents a large error the trend is clearly decreasing. It is believed that this is because the erythromycin base ions compete with the buffer ions for the stationary phase. The stronger the buffer concentration the more saturated the stationary phase is with buffer ions. As a result, EA is "expelled" from the column faster the more concentrated the buffer is.

3.2.4.1.3 Buffer concentration vs. N - plate number.

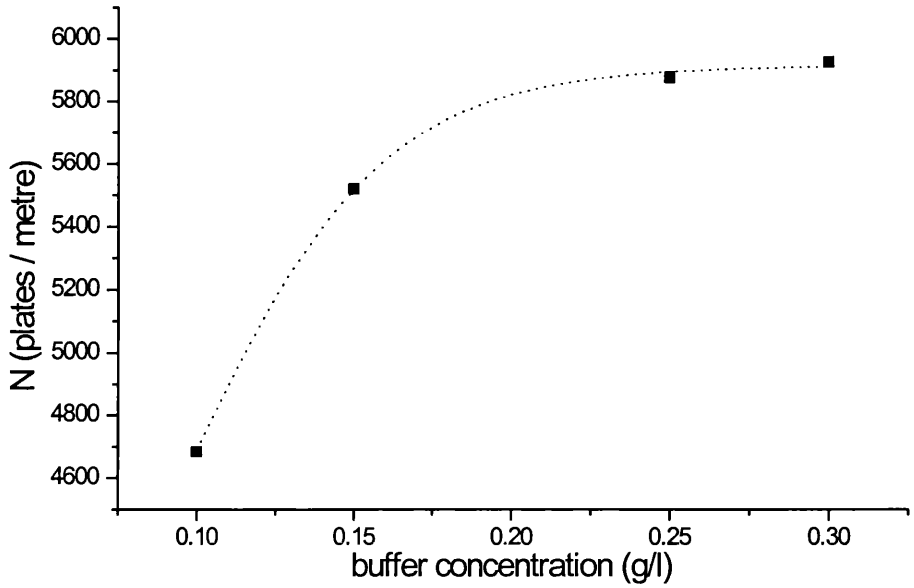


Figure 3.2-7: Buffer concentration versus number of theoretical plates N.

This graph shows the influence of buffer concentration on the efficiency of the column. It is clearly seen that the efficiency increases as the buffer concentration increases. However, at higher concentrations the differences in N are becoming minor. That is indicated on the curve as a plateau. If there is a tendency to use the column at a higher buffer concentration, going much beyond 0.3M will not contribute to improved separation significantly.

When no buffer was used in the mobile phase no peaks appeared in the chromatogram after 15 mins. However, when mobile phase with 0.1M concentration of buffer was introduced some compounds of the erythromycin sample were eluted off the column within 15 mins. As more concentrated buffer was added to the mobile phase the retention time reduced. This proves that buffer concentration can thus also be used to improve selectivity.

It seems that an increase in the ionic strength of the buffer encourages the dissociation of hydrogen ions from the protonated form of the sample molecule leading to a decreased retention time of erythromycin A.

From the graph we can also observe that the retention times of other peaks namely erythromycin B and erythromycin C are reduced as a result of elevated ionic strength. As the method was run for 15 mins no EB peak was detected at 0.1M concentration of buffer. However, already at 0.15M concentration the start of the peak was clearly

visible (not shown on the graph) and, by the time a 0.3M concentration of buffer was obtained, the whole peak was eluted within the method running time.

A similar observation could be made on the EC peak. At lower values of buffer concentration the EC peak exhibits a shoulder which diminishes as the concentration increases.

3.2.4.2 Buffer pH.

Another factor investigated was buffer pH. The compound changes from ionized to non-ionized form according to the buffer pH. This consequently has an impact on the retention time of EA and also on N.

3.2.4.2.1 Buffer pH vs. retention time

The plot of buffer pH against retention time depends very much on the nature of the sample. That is, whether the sample is an acidic or basic compound and what its pK_A value is.

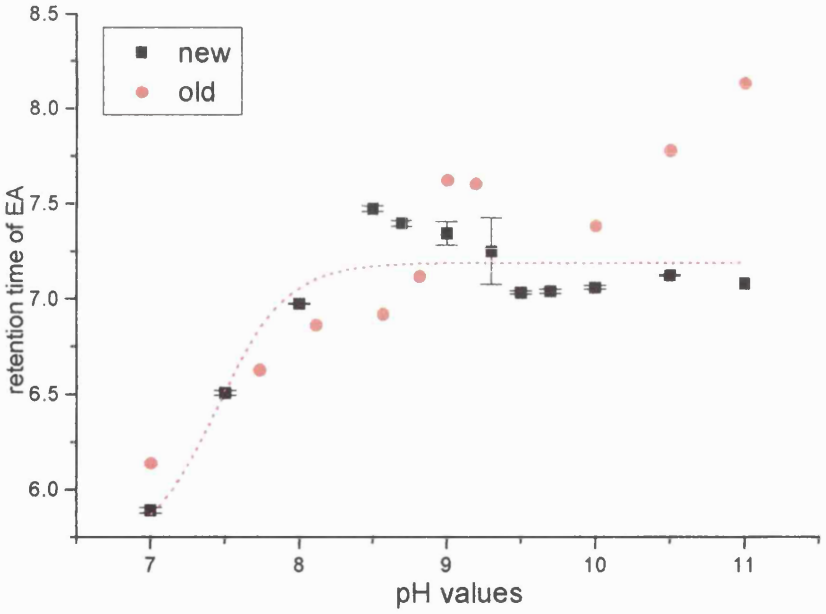


Figure 3.2-8 The influence of buffer pH on retention time of EA.

Conditions: column: PLRP-S 1000Å, 8 μ 4.6 x 250mm; sample: 8mg/ml; temperature: 70 ℃; loop: 100μl; mobile phase: 45:5:50 = ACN: potassium phosphate buffer (0.2M): water; flowrate: 1ml/min

This graph shows how the increase in buffer pH changes the retention time of erythromycin. The retention time increases rapidly as the pH approaches the value of 9. After that the retention time values reach a plateau. This same profile was observed by Kibwage et al. (1985).

Here two graphs are presented:

the graph with round points contains the data gained earlier (old data) and the graph with squares contains comparatively new measurements (new data).

At the time of the old data measurements, the practical skills of the researcher were still developing and, as a result, the data appears to be very chaotic and not following any pattern. Therefore the old data is subject to a large statistical error.

Subsequently the method was repeated and these later results are presented as "new data". To be able to derive any conclusion from the practical studies of pH on the retention time of EA, the old data ought to be discounted and the experiments had to be repeated. These later experiments are included here under "new" data. Only the new data was used for the fitting of a sigmoidal curve. (figure 3.2-8)

Discussion.

The value pK_A (EA)= 8.8 indicates that erythromycin is a moderately strong base. Provided that the ionized form is less retained on the stationary phase than the non-ionized form (reversed phase chromatography) the variation of retention time with pH can be explained as follows:

At a lower pH the molecule of the base dissociates. The ionized form is less attracted to the stationary phase (which is non-polar) and therefore it is eluted from the column earlier. As the pH value becomes greater dissociation is increasingly discouraged and the molecules show a decreasing tendency to become ionized. This results in an increasing retention of EA as the non-ionized form is more attracted to the stationary phase. At the same time it appears that the shape of the peak improves as a result of the elevated pH value.

3.2.4.2.2 Buffer pH vs. N.

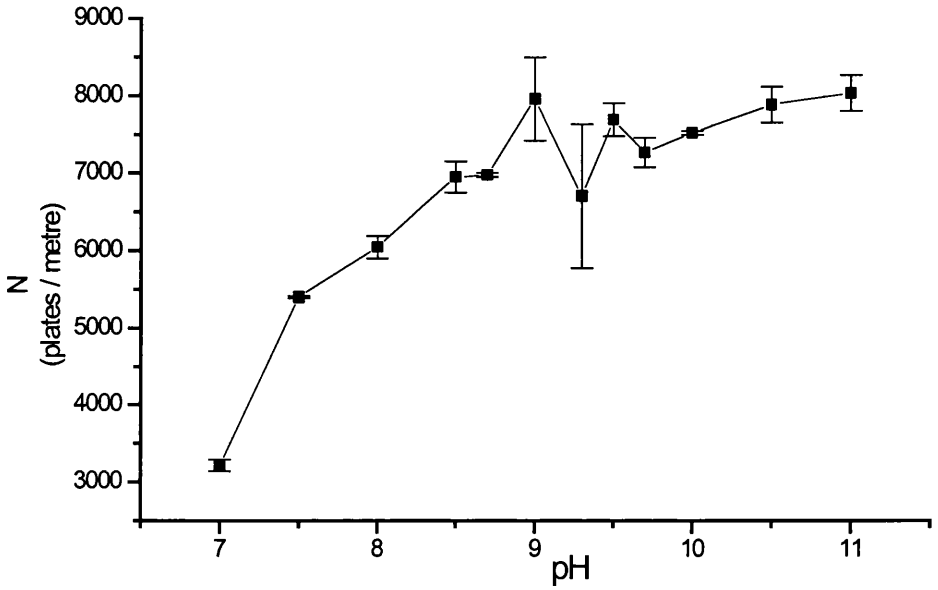


Figure 3.2-9: Buffer pH with regards to column efficiency.

This is a plot of buffer pH and its impact on N - a number of theoretical plates. The profile shows that column efficiency increases steadily with the increase in buffer pH. It is believed that this is because erythromycin is more stable at higher pH than at lower pH. At low pH erythromycin becomes increasingly unstable and decomposes.

3.2.4.3 Sodium and ammonium phosphate buffer

The standard buffer used in the experiments was potassium phosphate. In this section other buffers were used namely sodium and ammonium phosphates and their impact on the separation was investigated.

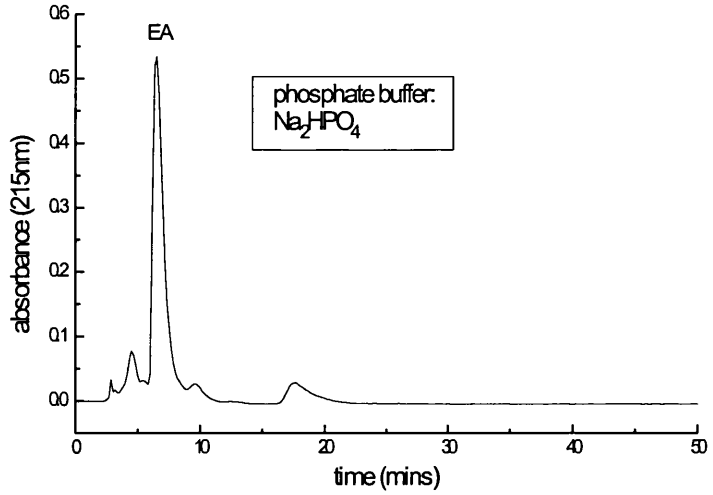


Figure 3.2-10: Profile of erythromycin with sodium phosphate buffer in mobile phase.

N = 337 plates/column (1350 plates/metre)

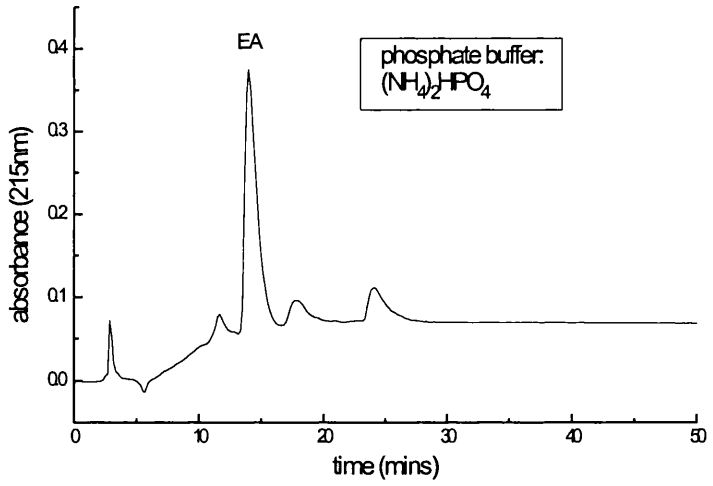


Figure 3.2-11: A profile of erythromycin with ammonium phosphate buffer.

N = 483 p/c (1930 p/m)

Conditions:

column: PLRP-S 1000Å, 8 μ 4.6 x 250mm; sample: 8mg/ml; temperature: 70 °C; loop: 100μl; mobile phase: 45:5:50 = ACN: phosphate buffer (0.2M): water; flowrate: 1ml/min

The graphs indicate how a change in buffer, i.e. sodium or ammonium, affect the overall separation of erythromycin A.

In terms of column efficiency, the best results are achieved with ammonium buffer (1930p/m) followed by sodium buffer (1350p/m) followed closely by potassium buffer (1300p/m).

The elution time of EA with sodium phosphate is 6.5minutes; with ammonium phosphate it is 14minutes; and with potassium phosphate it is 6.9minutes.

The graph with ammonium phosphate shows a sudden drift in the baseline. This does not appear to be satisfactory. One of the reasons could be that the column was not equilibrated for a sufficient time. Under the circumstances, though, the prolonged retention time of EA rules out the use of ammonium phosphate as a potential buffer.

When potassium or sodium phosphate are used, the resolution of the early eluted impurity peaks (those which come off before the main peak of EA) is better. These are the peaks such as EC and EE. However, in the case of ammonium phosphate, EE is almost a part of EC. The resolution in this case is quite poor. Later eluted impurity peaks such as those of anhydroerythromycin A (aEA) and pseudo Erythromycin A Enol Ether (psEEA) are resolved quite satisfactorily with all three buffers. On the other hand, comparing the efficiencies of the columns (N) with different buffers, ammonium phosphate appears to give the best efficiency. A repeated experiment with ammonium phosphate buffer would shed more light on this problem. If the repeated experiment with ammonium phosphate still gives the highest efficiency but at a reduced retention time, then ammonium phosphate should be given preference as a buffer over sodium phosphate or potassium phosphate. Unfortunately the experiment was not repeated.

3.2.4.4 Column temperature

Temperature vs. retention time

The original method mentioned previously required the column to be heated to 70°C. The main reason for this was the high density of 2-methyl propan-2-ol.

However, this method does not use 2-methyl propan-2-ol.

Here the influence of temperature on the separation of erythromycin was investigated.

The temperatures investigated were 30° to 70°C in 10 degree increments.

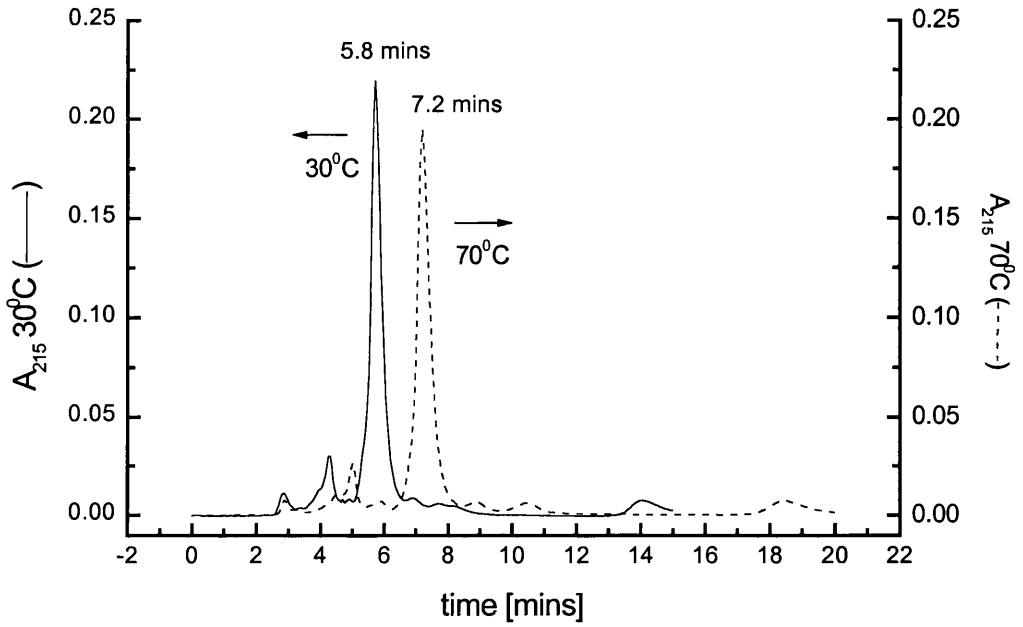


Figure 3.2-12: Profiles of erythromycin at 30°C and 70°C.

This graph shows how temperature influences the separation of erythromycin.
The main peak of EA comes off later when the temperature is increased. This also leads to a better resolution of the peaks.

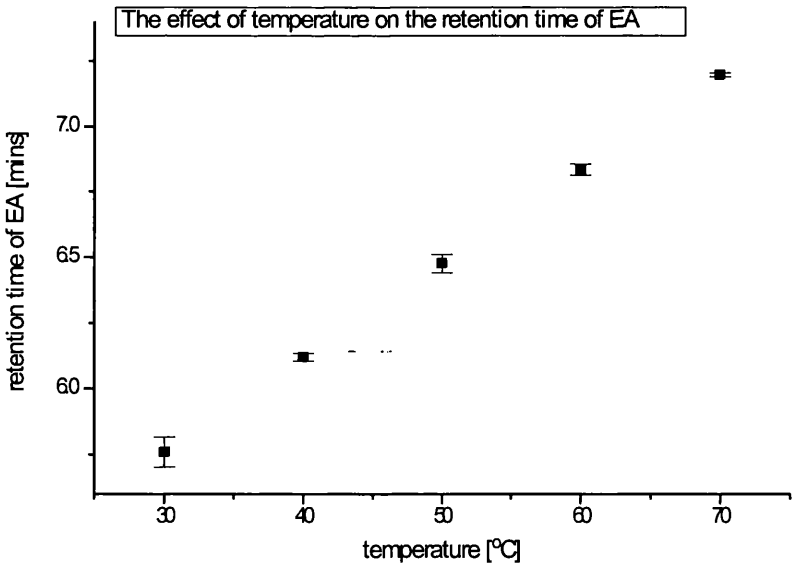


Figure 3.2-13: The influence of column temperature on the retention time of erythromycin A.

The influence of column temperature on the retention time of erythromycin A. This graph shows how with increased temperature the retention time of erythromycin A increases.

This feature can be of benefit on an analytical scale. However, heating the column to 70°C on a large scale can prove to be economically unfeasible due to high electricity consumption. It also imposes a risk as the boiling point of acetonitrile is 81°C.
Therefore, when experiments on a large scale were conducted (section 3.4), the column was not heated and ambient temperature was used throughout.

3.2.4.4.1 Temperature vs. column efficiency N

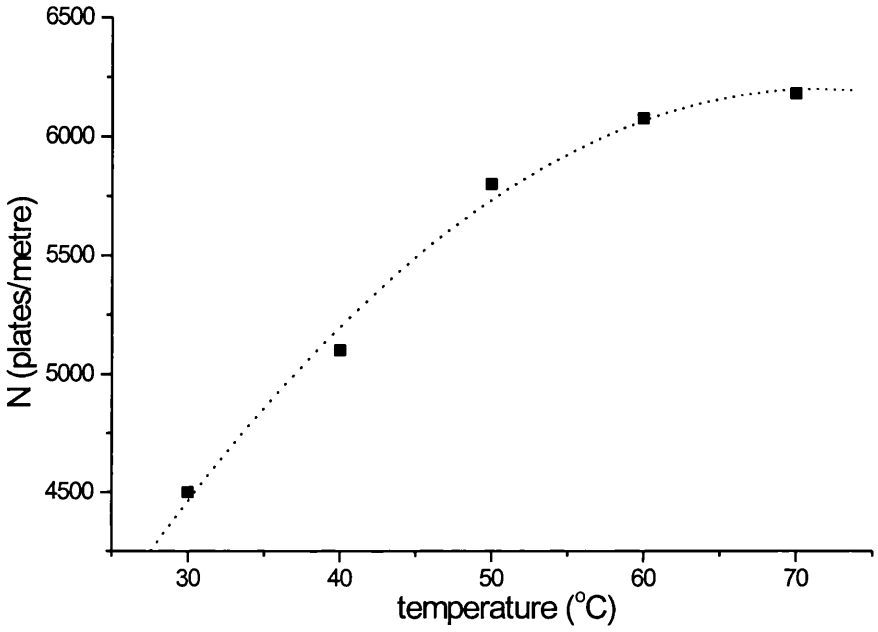


Figure 3.2-14: Effect of temperature on column efficiency.

The graph shows the temperature of the column vs. the number of theoretical plates. The profile has an increasing tendency which suggests that increased temperature improves the efficiency of erythromycin A separation. This was also confirmed by Kibwage et al. (1985). Increasing temperature improves N because the elevated temperature increases the rate of the chemical adsorption but also decreases the viscosity of the solvent.

In conclusion, the benefits of operating at a higher temperature are:

- better resolution of the peaks
- greater column efficiencies (higher number of theoretical plates)

3.2.4.5 Sample concentration

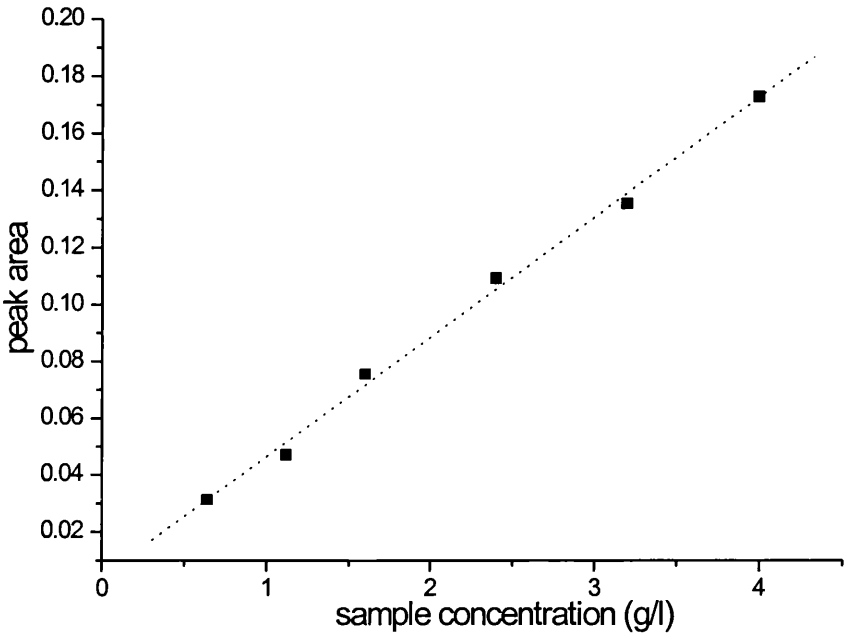


Figure 3.2-15: The amount of erythromycin loaded onto the column versus peak area.

conditions:

45:5:50=ACN:phosphate buffer (0.2M):water; F=1ml/min; loop: 100µl; detection: 215nm; sample: 8g/l; T=70℃; column: PLRP-S

The graph shows how, with the increased load of erythromycin sample, the peak area increases. The relationship is within linear limits as small concentrations of the sample were loaded.

3.2.4.6 Flowrate study

The Van Deemter equation expressed in terms of H/u curve serves to determine the ideal flowrate at which the column should be used. It also gives information about how well the column is packed.

3.2.4.6.1 Flowrate / N relationship.

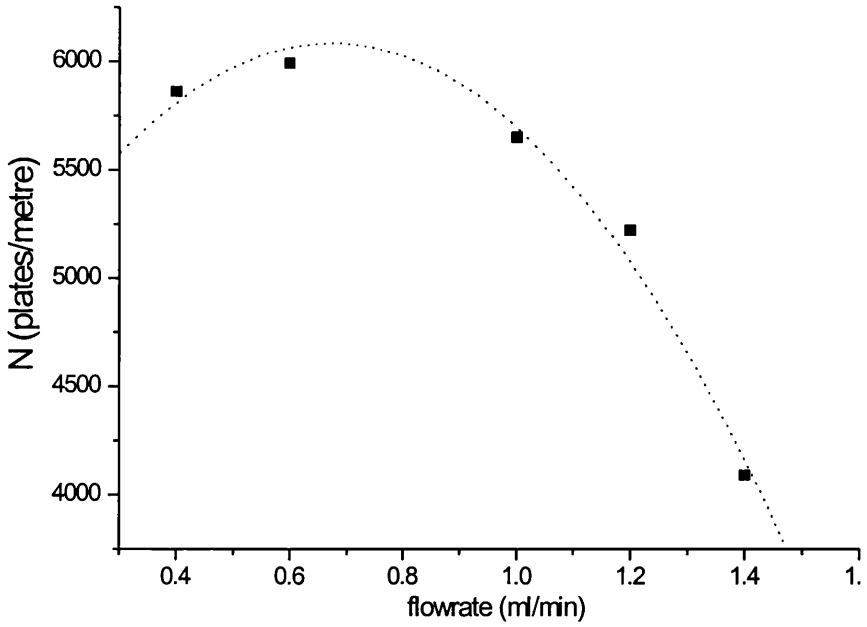


Figure 3.2-16: The impact of flowrate on column efficiency.

This graph shows the relationship between the flowrate and the number of theoretical plates i.e. column efficiency.

This polynomial fit used for the scatter of the points indicates that there is already some maximum value of N which appears at approximately F= 0.7ml/min. This is supposed to be the ideal flowrate to be used for the type of column and the conditions such as mobile phase.

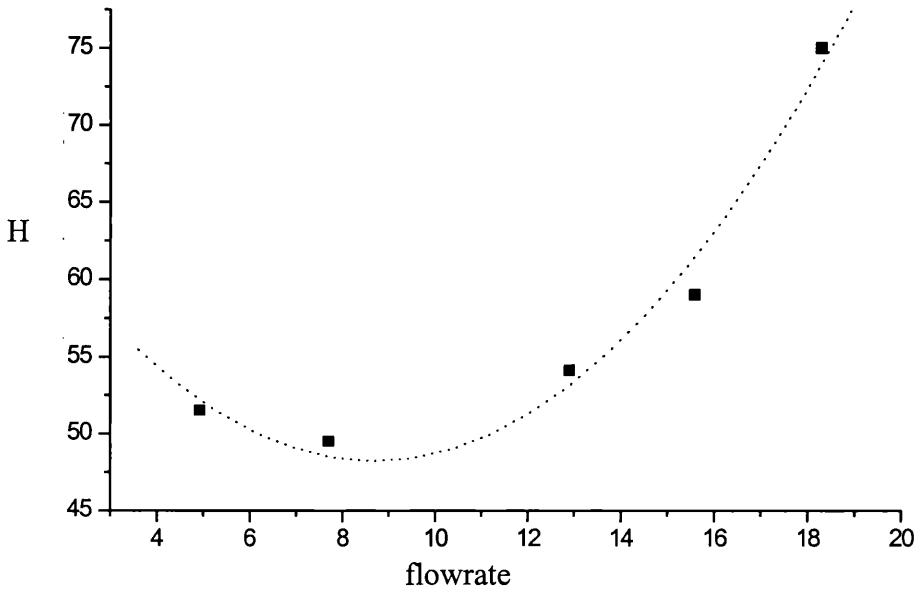


Figure 3.2-17: The impact of flowrate "u" on plate height H.

The H/u curve serves the purpose of determining how well the column was packed and determines what the ideal flowrate should be for the particular running conditions. Operating at a higher flowrate has a detrimental effect on efficiency and should therefore be avoided.

3.2.4.7 Acetonitrile concentration.

The mobile phase composition used throughout all the experiments on the scale up and development of EA separation was 45% acetonitrile/buffer/water. Here the experiments were conducted to investigate the change in acetonitrile concentration.

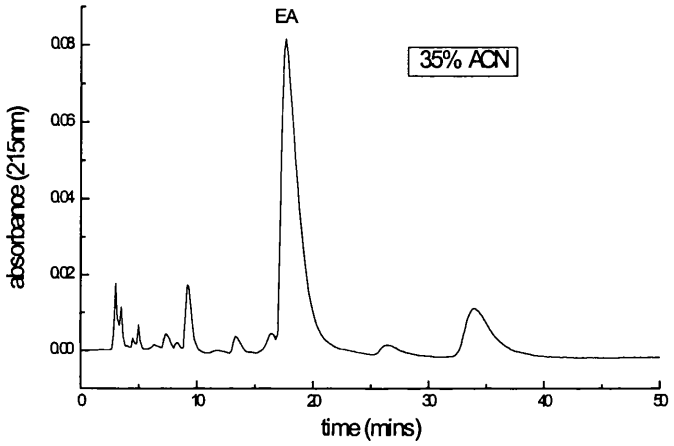


Figure 3.2-18: A profile of erythromycin at 35% acetonitrile concentration.

N= 622 plates/column = (2500 plates/metre)

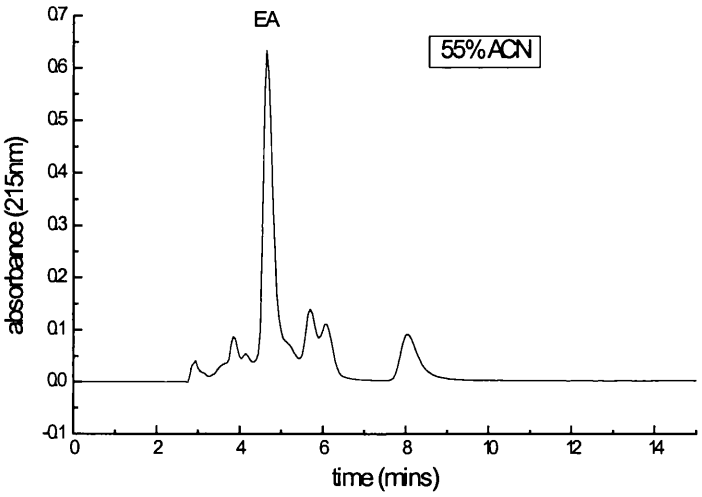


Figure 3.2-19: A profile of erythromycin at 55% acetonitrile concentration.

N= 1930 plates/column (7720 plates/metre)

Conditions:

column: PLRP-S 1000Å, 8 μ 4.6 x 250mm; sample: 8mg/ml; temperature: 70 °C; loop: 20μl; mobile phase: ACN: potassium phosphate buffer (0.2M): water; flowrate: 1ml/min

Here two different concentrations were used: 35% and 55% acetonitrile, respectively.

The best results were achieved using a concentration of 35% in terms of the separation of EA from all the other constituents. However, the disadvantage is the resulting longer retention times of peaks. This can be a drawback when a larger column is used. The main peak of EA in this case comes off at 17.6 mins. The whole separation takes approximately 40 mins. Column efficiency is approximately 2500p/m which is very low.

At a 55% acetonitrile concentration, the peak identified as aEA, coming just after EA peak, appeared to be splitting into two peaks. These peaks were eluted closely at 5.7mins. and 6.1 mins respectively. This was followed by the peak of ps EA enol ether. The whole separation seemed to take less than 10 minutes. The column efficiency was much higher than in the case of 35% acetonitrile (7720p/m)however the peak shape is not very satisfactory.

3.2.5 Conclusion.

The method used for the separation of erythromycin was presented here. The method used acetonitrile as the main solvent in the mobile phase and omitted the use of 2-methyl-propan-2-ol.

Variations in mobile phase composition were investigated including buffer concentration; buffer pH; the use of different phosphate buffers; acetonitrile concentration; column temperature and H/u curve.

The buffer concentration at higher values (> 0.2M) gives better column efficiencies than at lower values. However going much beyond 0.25-0.3M does not yield much better results.

Out of sodium, potassium and ammonium buffers, a potassium buffer appears to give the best results in terms of resolution and retention time.

As for buffer pH, the resolution and column efficiency improve with increased pH values. This is also due to better stability of EA at higher pH.

Elevated temperature has a positive effect on the separation of erythromycin as it improves column efficiency although at the same time it increases the retention time of EA (see also discussion regarding this issue).

The study of flowrate so far shows that the ideal flowrate which should be used on this column falls within the range of 0.6 to 0.8ml/min.

With a 55% acetonitrile concentration the peak of EA is eluted much faster and resolution is still acceptable but on a large scale with large loads this could create a problem of overlapping peaks.

3.3 Separation of erythromycin on a semi-preparative column.

Summary

This chapter deals with an investigation into the packing material most suitable for scale-up. The aim was first to compare the performance of columns packed with beads of spherical and irregular shapes. This was then followed by comparisons of columns containing different types of reversed phase support - styrenic or acrylic. Finally, three columns (one analytical and two semi-preparative) were packed with 35 micron particles and the separations on those columns proved successful. Based on these results, this packing material was used to pack a large scale PROCHROM chromatography column.

Introduction

Using a semi-preparative column is the next step in the scaling up of the erythromycin production process. As the separation of erythromycin on the analytical column was performed using polymeric packing material it was necessary to continue with this type of support when scaling up.

As the dimensions of the semipreparative column change (particularly as the internal diameter increases) there is a resulting increase in column volume: this has an impact on various components used in the column. For example, using beads of the same size as were used for the analytical separation (8 μm) can lead to an increased pressure in the column. To prevent this particles of a larger diameter were employed (35 μm or 40μm). However this usually leads to deterioration in the separation.

At this stage it was decided to test various packing materials to determine which was the most suitable for our separation.

Two kinds of packing material were compared: Amberlite XAD-4 (supplied by Sigma Aldrich) and Amberchrom CG-161 beads (manufactured by Toso Haas). The intention was to test these types of packing material and the efficiency of the separation which resulted.

3.3.1 Irregular versus spherical shape particles

In order to find a suitable packing material at a reasonable price which gave a satisfactory yield and purity of final product two types of packing material were used. Both were polymeric purchased from different suppliers (Sigma Aldrich and Toso Haas). This packing was used to pack a stainless steel semi-preparative column (100x10mm) purchased from Jones Chromatography.

Two types of packing material were utilised here to compare the chromatographic performance of spherical and non-spherical particles.

3.3.1.1 Amberlite XAD-4.

3.3.1.1.1 Physical and chemical description.

Amberlite XAD4 (Sigma Aldrich) is a non-ionic cross-linked polystyrene packing material. It comes in the form of white translucent insoluble beads of a macro-reticular structure with a high surface area (≥750 m^2/g). The size of the particles was in the range 300 to 1 200 μm.

The size of the particles was excessively large for chromatography purposes. We therefore decided to crush the particles. Initially the particles were of regular spherical shape. After crushing they became of irregular shape of roughly 40μm size.

3.3.1.1.2 Pre-treatment of the beads

The packing was shipped as a water-wet product imbibed with sodium chloride and sodium carbonate salts to retard bacterial growth. These salts had to be washed from the adsorbent prior to use.

The packing beads were washed, crushed and sieved and in such a state were ready to be packed. The washing stage, consisting of several sub-stages, is tabulated below.

3.3.1.1.2.1 Washing of XAD-4 packing material

1.	wash with methanol
2.	vacuum filtration
3.	wash with methanol/water (1:1)
4.	filtration
5.	wash with 0.1M NaOH /methanol (1:1)
6.	UV absorbance measured on the spectrophotometer at 250nm. Repeat step "5" until no significant changes in the absorbance appears - i.e. the UV stabilizes
7.	wash the beads with 100% acetone
8.	wash with water/acetone (1:1)
9.	wash with 0.1M HCl /methanol (1:1)
10.	measure the UV absorbance and repeat step "9" if necessary
11.	wash with methanol/water (1:1)
12.	measure the pH of the solution containing the beads (will be around 2.9) and should be adjusted to pH of approximately 4-5
13.	rinse with "high millipore" water
14.	store in 20% methanol/water

Table 3.3-1: The procedure for washing XAD-4 packing.

3.3.1.1.2.2 Crushing of the beads

Crushing of the beads was required to decrease the size of the particles available and at the same time to obtain irregularly shaped particles.

This was performed using two methods:

- a mortar and pestle;
- a mechanical grinder.

3.3.1.1.2.2.1 Mortar and pestle method

The required amount of beads was poured into the mortar and using rotary movements, the beads were crushed under applied pressure to the smaller irregular size. This method

was very laborious and time-consuming. This was an uneconomical way of preparing the material.

3.3.1.1.2.2.2 Mechanical grinder method

A mechanical grinder was tried as an alternative. The packing material was poured into a large electrically powered grinder where it was ground by a means of a static pestle and a revolving mortar.

This method required a lot of packing material. Experience showed us that if the speed at which the mortar revolved was higher than desired a lot of packing material was crushed to fines. On the other hand a slower speed yielded particles too large to be used for packing. The experiment produced only a very small quantity of beads of a satisfactory size.

In conclusion, it proved difficult to produce fruitful results with this experiment. The most difficult aspect was to find a trade-off between the pressure and speed applied. As a consequence, we refrained from further use of this technique and reverted to using mortar and pestle. Although laborious and inefficient this method eventually produced a sufficient quantity of particles to enable wet sieving to be carried out.

3.3.1.1.2.3 Wet sieving.

The ground resin produced by mortar and pestle was a slurry containing a large distribution of particle sizes. It was therefore necessary to isolate a fraction of the crushed resin which was more homogenous in size. This was achieved by wet-sieving through a set of three sieves (Endecotts Ltd.).

The system consisted of a series of sieve-plates mounted on top of each other with decreasing mesh size. The sieves were arranged in descending order of mesh size: 90μm on top; 50μm in the middle; and 40μm on the bottom. Wet packing material was poured on the top sieve with the coarsest grid (90μm). Then high purity water was applied from the top to wash the beads down to the lower level sieves of 50μm and 40μm mesh. Water eventually trickled from the bottom of the set of sieves but the arrangement did not work very well. When water was applied to the sieves they had to be shaken for some time to obtain even a minimal rate of throughput of material. The end result was a

high proportion of material which was either too small in diameter (and was washed away) or too large (which remained on the larger grid sieves).

3.3.1.1.3 Column packing

Despite all the difficulties experienced during the treatment process sufficient material was eventually collected to enable the column to be packed. The packing was carried out using a Shandon column packer at an applied pressure of 6000 psi (McLaughlin 1994). This value was taken from the thesis produced by a previous student who used the same packing material and column packer. When however, the packing pressure of 6000 psi was applied, the particles were crushed as the pressure was too high to be used for this type of packing.

A detailed description of the packing procedure using the Shandon column packer is in section 3.3.3.1.5. on CG-300 packing material.

Conclusion.

Although use of the mortar and pestle method to crush the beads was far from ideal as it was both very time consuming and inefficient it still enabled the required amount of material to be obtained. However this method of obtaining irregularly shaped particles would not be recommended for use on a large scale.

The packing of the column was unsuccessful for the following reason: the pressure applied during packing was too high for this particular type of packing material. When such a high pressure was applied to the irregularly-shaped particles their sharp edges were easily broken off and crushed to create fines. This resulted in column blockages generating high pressures which rendered the column unusable.

3.3.1.2 Amberchrom CG-161.

Amberchrom ® chromatographic resin purchased from Toso Haas (Linton, Cambridgeshire, UK) was used to pack a semipreparative column of 100x10 mm (Jones chromatography, Hengoed, Mid. Glamorgan, UK).

Amberchrom chromatographic resin belongs to the group of macroporous synthetic polymeric packing materials. It is widely used for the separation of peptides, proteins,

nucleic acid, antibiotics, phytopharmacologicals and vitamins. It is available in two structures: as a methacrylate or a styrenic.

3.3.1.2.1 Pre-treatment of Amberchrom ® chromatographic resin.

As the resin was supplied dry it had to be hydrated and the fines removed prior to use.

3.3.1.2.1.1 Hydration of the resin.

For the hydration experiments, the solvent used was 20% acetonitrile in water.
A given amount of resin (corresponding to the volume of the column) was weighed. This was mixed with three times the volume of hydration solvent (20% acetonitrile/water). The solution was stirred with a mechanical stirrer and the resin left to hydrate for 24 hours. This ensured that the resin was thoroughly hydrated and free of trapped air. Then the supernatant was decanted and fresh hydration solvent was added.

3.3.1.2.1.2 Removal of fines.

To ensure smooth packing it was imperative to remove any fines which might clog the column. This was done using a vacuum pump to take off the supernatant from the settled resin. The resin was then resuspended, left to settle and suctioned off. This procedure was repeated twice more. The settling time was 1-2 hours.
The last step before packing the column was to adjust the concentration of the slurry That is defined as the volume of settled gel to the total volume of the slurry.
The slurry was transferred to a measuring cylinder and left to settle for 24 hours. Then the slurry concentration was adjusted to 70-80% by pouring out the excess solvent.

3.3.1.2.2 Packing the column.

The column was packed in a similar way to the XAD-4 packing the only difference being the pressure applied. This time effort was concentrated on applying the lowest possible pressure. However the pressure recommended by the manufacturer was far too low to be detected on the pressure gauge of the column packer. The column packer used was designed for packing at comparatively high pressures.

As a result the packing pressure limit was unintentionally exceeded and once again the column became unusable.

After this pre-packed columns CG-161 and CG-71 were purchased.

3.3.2 Styrene versus methacrylate support.

Two columns CG-161 and CG-71 (supplied by Toso Haas) were used to test different polymeric packing materials, in this case styrenic or acrylic. The columns, made of Teflon, were prepacked with 35μm diameter packing material.

The specifications of both columns are given below.

3.3.2.1 CG-161 and CG-71.

	CG-161 (TOSO HAAS)	CG-71 (TOSO HAAS)
Column dimensions (cm)	15 x 0.75	15 x 0.75
Chemistry of packing	styrenic	methacrylate
Particle size (μm)	35	35
Particle shape	spherical	spherical
Specific surface area (m^2/g)	900	500
Average pore diameter (Å)	150	250
porosity(ε_T)	not determined	0.76
Mobile phase:	45:5:50= ACN: buffer: water	45:5:50=ACN :buffer: water
column volume (ml)	6.6	6.6
flowrate (ml/min)	1	1

Table 3.3-2 Properties of CG-161 and CG-71 columns.

The columns were tested initially with erythromycin to determine the quality of the separation. The results showed that the CG-71 column with methacrylate support gave overall better separation than CG-161 column. Therefore the CG-71 column was used for a further set of experiments.

3.3.2.2 Results- CG-71

Volume and concentration overload have different profiles. In the case of volume overload the retention time of a compound of interest does not change with load. The peaks become progressively wider developing front tailing. At high volume overload the peaks exhibit flat top features.

In the case of concentration overload sample of increased concentration loaded onto a column show a decrease in retention times with the development of rear tailing - the peaks become progressively asymmetrical.

Scale-up experiments were carried out to investigate the impact of column overload on the separation of erythromycin A. The columns were overloaded by volume and concentration. With volume overload the sample concentration was kept constant at 50g/l and the column was loaded with 100, 200, 500 and 1000µl sample volumes. The profiles of these runs are shown below.

3.3.2.2.1 Volume overload.

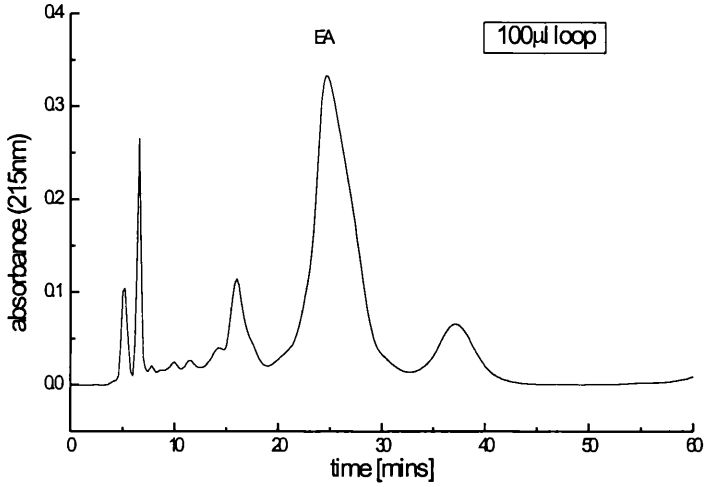


Figure 3.3-1: Volume overload with 100µl loop.

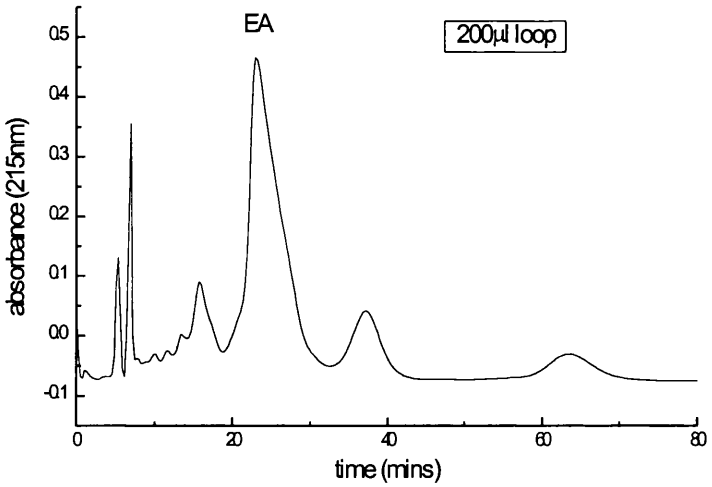


Figure 3.3-2: Volume overload with 200µl loop.

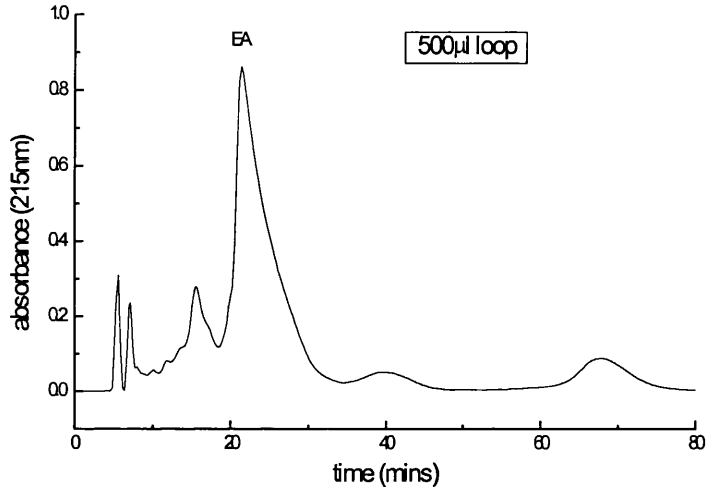


Figure 3.3-3: Volume overload with 500µl loop.

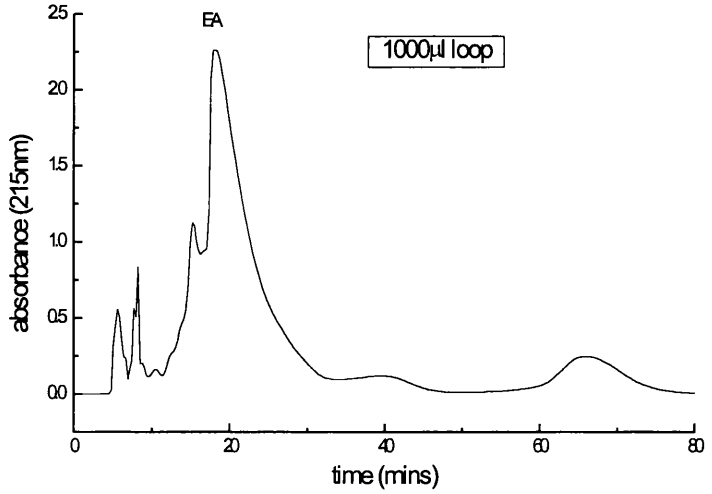


Figure 3.3-4: Volume overload with 1000µl loop.

Conditions: column: Toso Haas CG-71; flowrate: 1ml/min; temperature: 60°C; mobile phase: 45:5:50 = acetonitrile: phosphate buffer (pH=9; 0.2M): water; sample: 50mg/ml

The above four chromatograms present profiles of peaks from studies on volume overload. However, because the initial concentration of the sample was high, the profiles show that the governing force here is concentration overload.

From the above four chromatograms it can be seen how volume overload influences the profile of the main peak of erythromycin A.

While at small loads (100μl loop) the peak of EA appears symmetrical, as the sample volume loaded on the column increases the EA peak is shifted to the left as it is eluted earlier from the column. At the same time the EA peak is becoming less and less symmetrical exhibiting back tailing. As the column is becoming more and more saturated with the sample, the active sites of the stationary phase are blocked resulting in "premature" elution of the sample peak front.

The EA shift towards lower retention times at large volumes eventually results in joining with the earlier eluting impurity EC. At that stage, the separation of EA deteriorates significantly to such an extent that the EA is no longer separated from its impurities satisfactorily. The profile of such a peak has a sharp front with a pronounced back tailing. The resolution of EA from early eluting impurities decreases.

The results also indicate that the possible isotherm resulting from these profiles will be of a convex shape (Langmuirian) as the sample retention time decreases with an increasing load.

A typical feature experienced with volume overload is the "flat top" which is about to form on the peak injected with the 1000μl loop. This is typical of volume overload where eluting of all the sample from the loop takes a considerable time (especially with large sample volume injection) and that is shown as a flat top on the profile of a peak.

This feature is however much more common in preparative chromatography than in analytical chromatography. Another reason is a limited sample solubility.

3.3.2.2.1.1 Volume overload versus column efficiency.

The following table shows how the overload by volume decreases the efficiency of the column.

loop (µl)	N (plates /column)
100	6 900
200	6 140
500	5 160
1000	2 850

Table 3.3-3 A table of injection volume and corresponding column efficiencies.

With the increased sample volume loaded the efficiency decreases.

Conclusion.

The increased sample load has an effect on the shape of the peak and on the efficiency of the column. Larger loads shift the main peak of EA to the left. The profiles of the peaks change according to the isotherm they follow. With increasing loads the peaks become less symmetrical and in this case shift towards the lower retention times typical of concentration overload profiles.

3.3.2.2.2 Mass overload.

Mass overload occurs when the volume of the sample injected is kept constant and the concentration of the sample varies. Here the constant volume injected throughout was 50µl and the concentration of the sample was gradually increased from 30g/l through 40 and 50g/l to 60g/l, respectively.

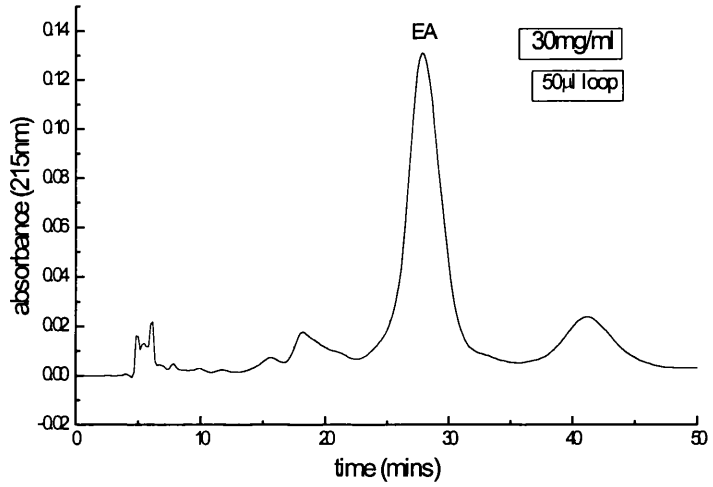


Figure 3.3-5 Concentration overload at 30g/l.

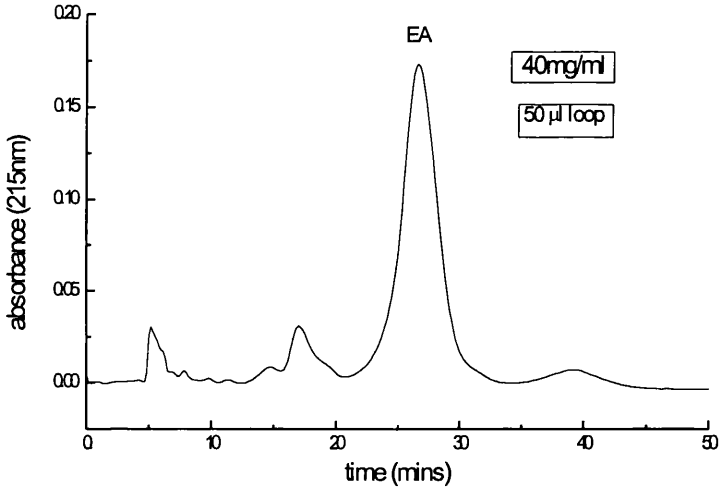


Figure 3.3-6: Concentration overload at 40g/l.

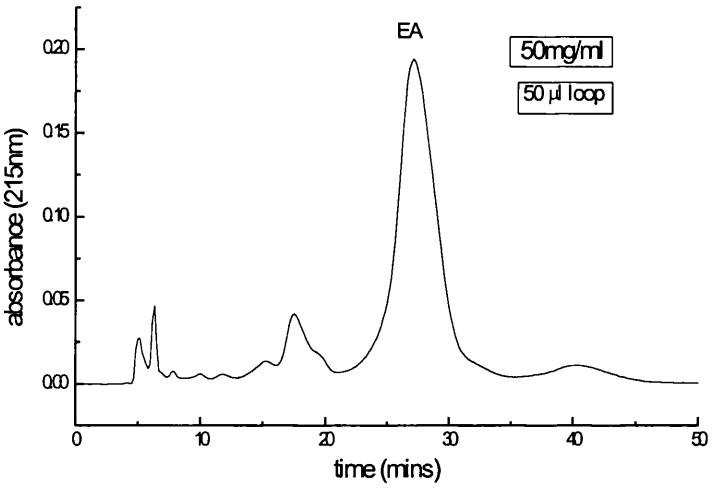


Figure 3.3-7: Concentration overload at 50g/l.

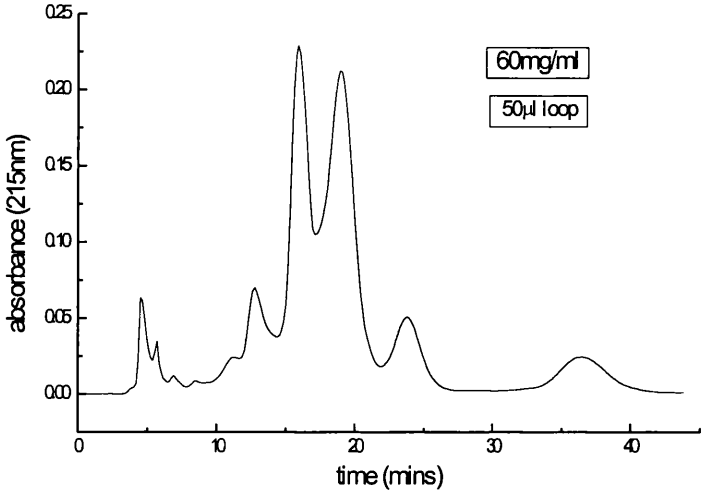


Figure 3.3-8: Concentration overload at 60g/l

Conditions: column: Toso Haas CG-71; flowrate: 1ml/min; temperature: 60°C; mobile phase: 45:5:50 = acetonitrile: phosphate buffer (pH=9; 0.2M): water

Here an attempt was made to investigate the impact of concentration overload on peak profiles. However, such a small volume of the sample was injected (50 µl) that the true nature of concentration overload could not be observed. The peak profiles hardly changed with load until 60g/l was loaded.

The sample concentration of 60g/l is beyond the solubility of EA at given conditions. The fact that this indeed is the case is illustrated by two peaks appearing instead of one when the sample of 60g/l is injected. Both peaks appear much earlier than EA should elute. It is believed that the sample precipitated or decomposed. One of the peaks is probably a result of sample precipitation. This clearly indicates the importance of knowing the sample solubility prior to scale up. Obviously this concentration should not be used in further studies.

To verify our speculations the separation needs to be repeated. The current explanation is also supported by the fact that the sample of this concentration (60g/l) started to solidify soon after being prepared thus suggesting that this must be the limit of the sample's solubility.

3.3.2.2.3 Acetonitrile concentration.

This study was already carried out on an analytical scale (see section 3.2.4.7.)

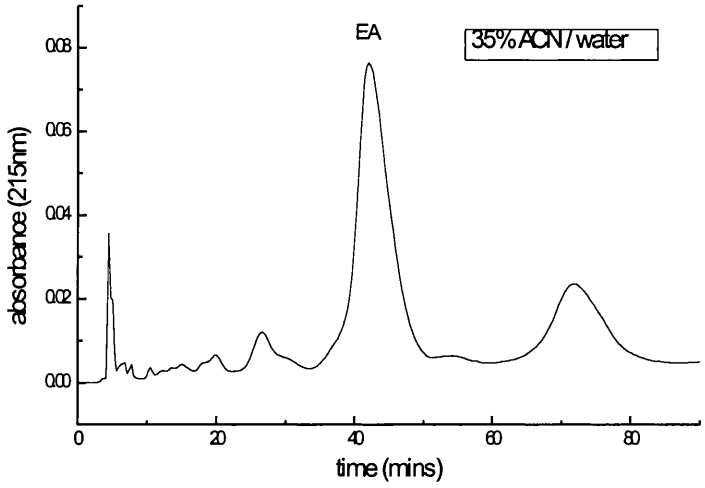


Figure 3.3-9: A profile of erythromycin at 35% acetonitrile concentration.

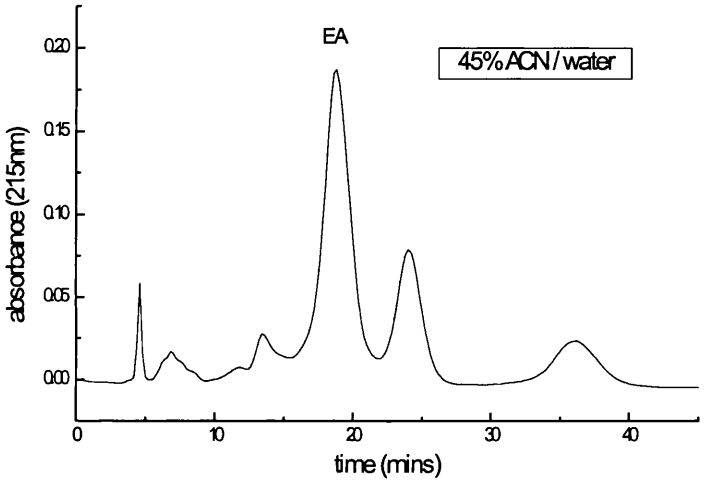


Figure 3.3-10 A profile of erythromycin at 45% acetonitrile concentration.

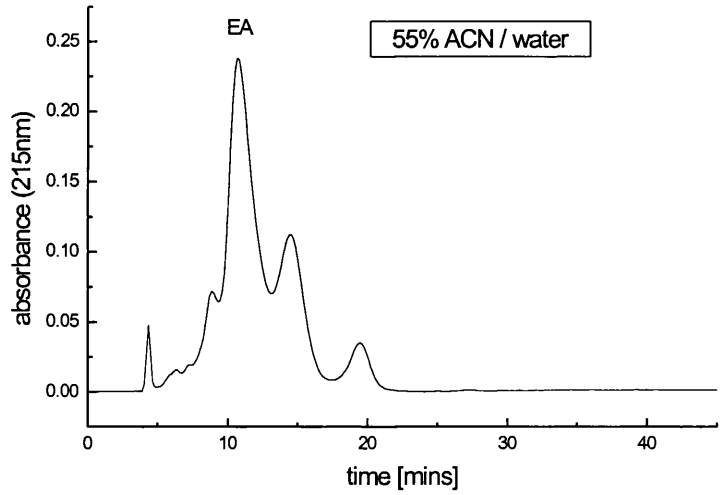


Figure 3.3-11 A profile of erythromycin at 55% acetonitrile concentration.

Conditions: column: TOSO HAAS CG-71, methacrylate; sample : 20mg/ml; loop: 50μl; temperature: 60°C; flowrate: 1ml/min; mobile phase: ACN :phosphate buffer (pH=9; 0.2M) : water

These graphs show how a different concentration of acetonitrile influences the resolution of erythromycin components.

In the case of 35 % ACN /water the elution time is very long: even after 90 mins one of the components (the last impurity) remained on the column. Although the resolution is very satisfactory, scale up at these conditions can have a huge impact on the solvent cost.

Increased concentration of acetonitrile (to 55% ACN /water) results in the whole sample being eluted within 20 mins. This, however, was at the cost of lost resolution where all the peaks were "squeezed" together. The separation is very poor.

Chromatograms prove that accuracy during the preparation of the mobile phase is crucial. Inaccuracy can result in an unsatisfactory separation. At the same time, the retention time of the peak of interest is difficult to predict. However, that is important to know in order to collect the right component from the sample.

3.3.3 CG-300 packing

This packing material was obtained from the Toso Haas company and it was supplied pre-hydrated. The particle size was 35micron and this stationary phase was used to pack analytical, semipreparative and preparative columns as part of the scale up study. The columns were packed and tested with acetone and then erythromycin. The profiles of the resulting chromatograms are in the results section of this chapter.

3.3.3.1 Materials and methods

3.3.3.1.1 Packing material

CG-300 is a polymer packing material with a pore size of 300°A. This packing material was made available to us courtesy of the Toso Haas company (Linton, Cambridgeshire,

UK). Accordingly we chose this packing material to pack the following columns, including the Prochrom large scale chromatography column.

3.3.3.1.2 Column assemblies

The empty column assemblies were obtained from Jones Chromatography (Hengoed, Mid. Glamorgan, UK). The construction material is stainless steel and they are available in different sizes.

For the purposes of scale up the length of the columns remained the same only the internal diameter increased.

The dimensions of the columns were:

150 x 4.6mm

150 x 7.8mm

150 x 10mm.

The same packing material was then used to pack a PROCHROM preparative column.

3.3.3.1.3 Column specifications.

number	Column name	support	dimensions (mm)	particle size (μm)	pore size (°A)	column volume (ml)	flowrate (ml)
1	CG-300	styrene	150 x 4.6	35	300	2.5	1
2	CG-300	styrene	150 x 7.8	35	300	7.2	2.88
3	CG-300	styrene	150 x 10	35	300	11.8	4.73
4	CG-300	styrene	180 x 60 Prochrom	35	300	509	170(210)*

Table 3.3-4 Specifications of the columns packed with CG-300 packing material.

Please note that 170ml/min was a scaled up flowrate calculated for the Prochrom column

with a 15cm long bed. However, the column bed was 18cm and therefore the modified flowrate to accommodate this expansion was 210ml/min.

3.3.3.1.4 Scale up equations.

When scaling up it is important to use two equations to increase flowrate and a sample load to reproduce a chromatogram. (Bidlingmeyer 1992)

The following equations were used to scale up the flowrate and volume of the sample injected onto the columns.

For the volumetric flowrate:

$$F_2 = F_1 \frac{A_2}{A_1} = F_1 \frac{r_2^2}{r_1^2}$$

where

F_1 and F_2 are flowrates for small and large column, respectively. A_1 and A_2 are cross sections of column 1 and 2 and r_1 and r_2 are their corresponding internal radii.

For the volume injected:

$$V_2 = V_1 \frac{Vc_2}{Vc_1} = V_1 \frac{r_2^2 L_2}{r_1^2 L_1}$$

where V_1 and V_2 are sample volumes injected on column 1 and 2,

Vc_1 and Vc_2 are column volumes, L_1 and L_2 are column lengths.

3.3.3.1.5 Packing of columns

Prior to packing the slurry had to be rid of fines. This procedure proved to be crucial for satisfactory column packing. Failure to remove fines resulted in column blockages and high pressures. Removal of fines was described earlier in the section 3.3.1.2.1.2. on the pre-treatment of Amberchrom chromatographic resin. Once this procedure was accomplished the packing material was ready to be packed.

3.3.3.1.5.1 Description and operation of the packer.

Columns were packed following the guidelines laid down by the manufacturers of the packing material (Toso Haas) and the Shandon column packer. All three columns were packed at UCL using a Shandon HPLC column packer (Shandon Southern Products Ltd., Astmoor, Runcorn, Cheshire). The Shandon column packer is designed for the rapid and efficient packing of stainless steel HPLC columns from analytical to semi-preparative scale. The unit consists of a piston pressure intensifier pump, a stainless steel column reservoir and 4 solvent vessels.

The flow for the packing procedure is triggered using two pistons. A large piston draws nitrogen gas (supplied via nylon tubing) and pushes on a smaller piston which draws the

packing solvent from a reservoir. There is a relationship between the gas pressure and the solvent pressure. For this particular column packer the relationship is:

Solvent pressure = 60 x Gas Pressure

Therefore the gas pressure is set for the desired packing solvent pressure. The maximum pressure allowed is 10 bar gas pressure (for a maximum solvent pressure of 600 bar).

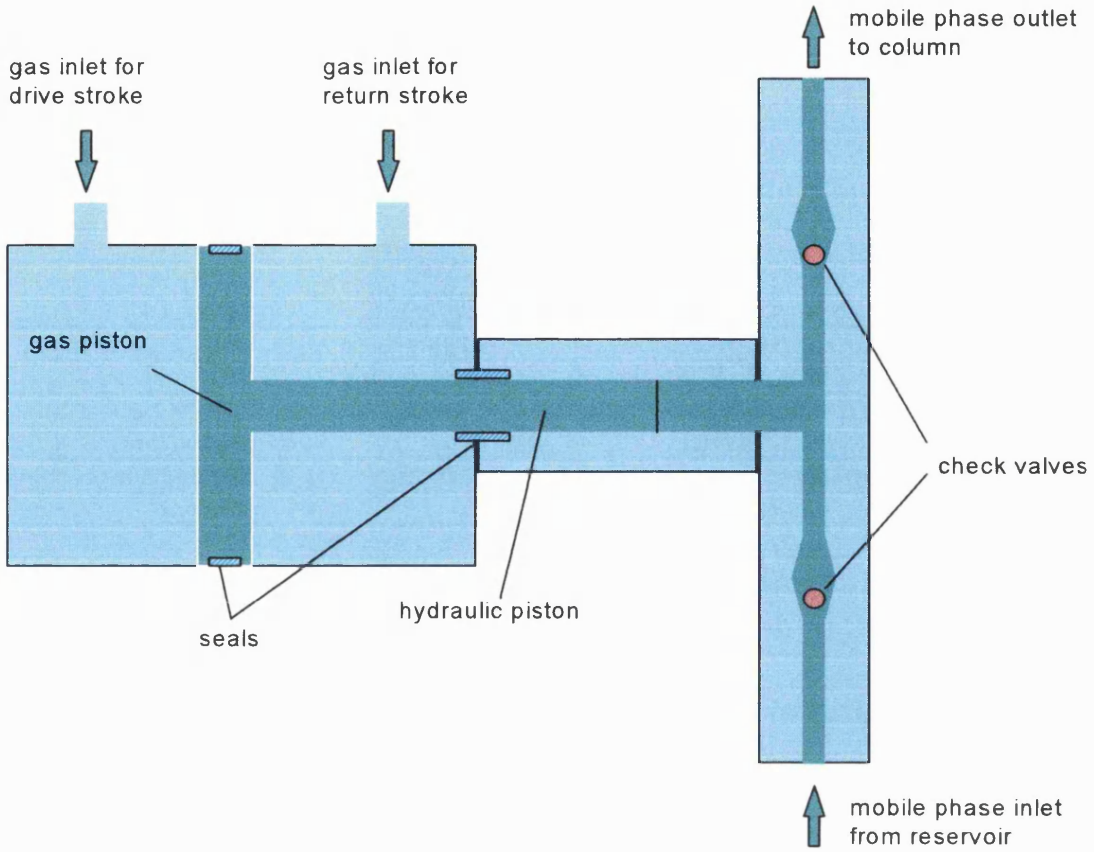


Figure 3.3-12 A diagram of a single piston pump used in Shandon column packer.

This type of single piston pump also known as pneumatic amplifier pump is can operate at high pressures and flow rates but are renowned for providing inaccurate constant flow of mobile phase. But for their earlier mentioned attributes, they are ideal for packing columns where variations of the flow rates through the column do not matter.

The ratio of liquid pressure to gas pressure is 60 which is also the ratio of the area of gas piston to the area of hydraulic piston. Gas piston with a large surface area is attached to hydraulic piston with much smaller surface area.

On the drive stroke, the gas pumped into the "gas chamber" pushes the piston to the right. The inlet for the mobile phase is closed and the outlet is open to the column. at the end of the drive stroke, the gas in the chamber is vented and the gas enters on the other side of the gas piston to start the return stroke.

On the return stroke, the outlet valve closes, the inlet valve closes and the pump head refills with mobile phase. The pump can be started and stopped by operation of a valve fitted between the cylinder regulator and the pump.

The piston displacement of the Shandon packer is 2.3ml. Multiple strokes are necessary to fill semipreparative columns with the chromatographic support.

3.3.3.1.5.2 Packing procedure.

Before the packing procedure commences the whole system is cleaned with methanol. The empty column is then attached to the column reservoir. The packing material is made into a homogeneous slurry before being poured into the column reservoir. The volume of the slurry is usually about 1.5 times the volume of the empty column to make sure the column is packed efficiently i.e. with no voids.

The pressure of nitrogen gas is then applied in compliance with the desired solvent packing pressure. For the CG-300 packing material the manufacturers recommended a solvent pressure of 25 bar.

Once the gas pressure has been applied the packing procedure commences with loud pump pulsations which gradually slow down before finally stopping altogether. The pulsations only last a matter of a few seconds and when they stop the packing is complete. For safety reasons it is necessary to turn all switches off. The column is then disconnected and the end fittings screwed in. The surplus packing material is then pushed out of the reservoir with water.

3.3.3.1.5.3 The settling of the particles after packing.

It is good practice to run the mobile phase at low flow rates through the newly-packed columns for several hours after packing. This ensures a proper consolidation of the freshly packed resin.

3.3.3.2 Results

Three columns were packed with the aforementioned packing material. Each column was then tested for its efficiency with acetone and then the scale up study was carried out using a model sample of erythromycin.

Each column was gradually loaded with increasing amounts of the sample. The volumes of the samples used on all three columns were calculated following the rules of scale up: i.e. the volumes injected were proportional to the column volume. In all three cases the erythromycin concentration was 40g/l and the exact volumes injected together with column efficiencies are stated in the tables of Appendix 4. Each sample (where possible) was injected twice and chromatograms evaluated in terms of column efficiency. The profiles follow:

3.3.3.2.1 Column 150 x 4.6mm.

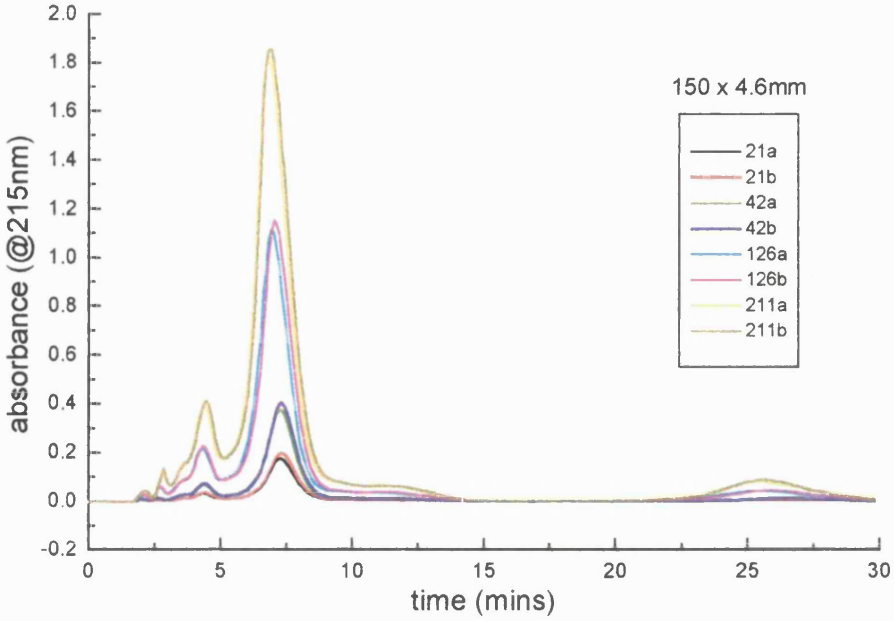


Figure 3.3-13 Gradual overloading of the 4.6mmID column with erythromycin.

The traces show a progressive overloading of the column. Although not immediately obvious, the trace suggests that the CG-300 gives rise to a convex isotherm (Langmuir) as the retention times are slowly shifting towards lower values with increased load. The graph shows how the baseline separation of erythromycin A from its impurities becomes affected with increasing load until the peaks overlap.

3.3.3.2.2 Column 150 x 7.8mm

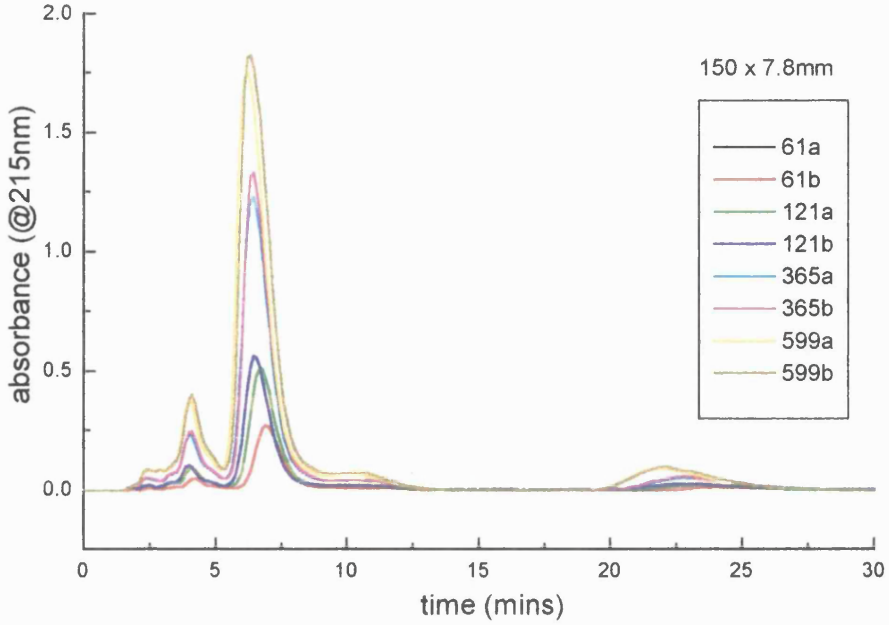


Figure 3.3-14 Gradual overloading of the 7.8mmID column with erythromycin

3.3.3.2.3 Column 150 x 10mm.

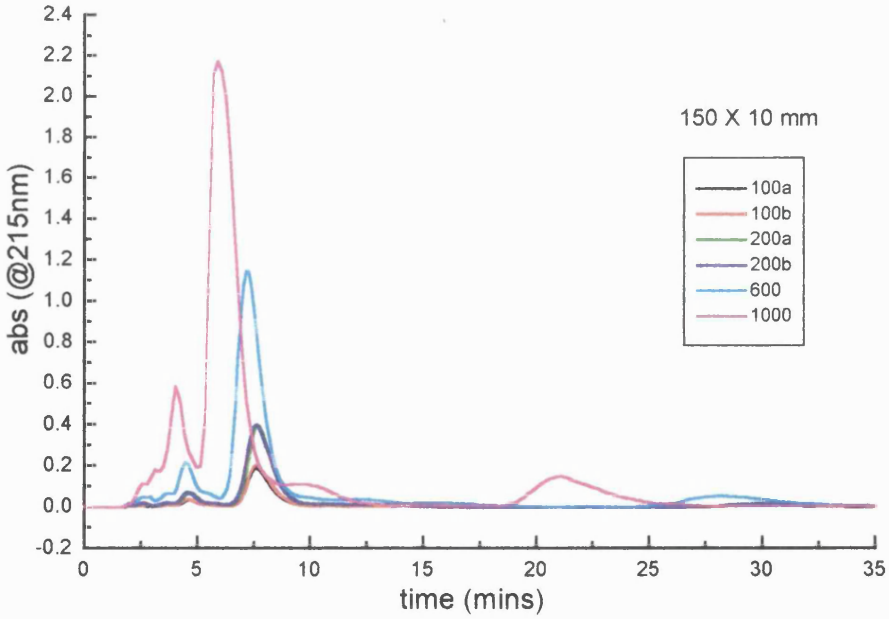


Figure 3.3-15 Gradual overloading of the 10mmID column with erythromycin

Here the column was overloaded in the same way as in the previous two cases. The shift to the left is significantly more profound here. Although that is a general trend here it is also possible that inaccuracies linked to operator's error could have contributed to

greater time differences. The numbers next to the figure indicate the amount of sample injected in μl.

Here only the average column efficiencies for each injection volume are shown. For each load the data seem to be very similar. A general trend of a decrease in column efficiency with an increase in load becomes obvious at larger loads. The data is also evaluated in statistical terms. The average column efficiencies decrease with load as follows: 1387, 1355, 1135 and 844 plates per metre.

	Column efficiency (plates/metre)					
% of the column volume	150 x 4.6mm	150 x 7.8mm	150 x 10mm	mean on rows	se(Er±)	sd(Er±)
0.85	1370	1500	1290	1386.7	105.99	61.19
1.7	1350	1290	1425	1355	67.64	39.05
5.1	1145	1120	1140	1135	13.23	7.64
8.5	884	875	772	843.7	62.23	35.93

Table 3.3-5 Statistical evaluation: standard deviation and standard error on average sample volumes injected.

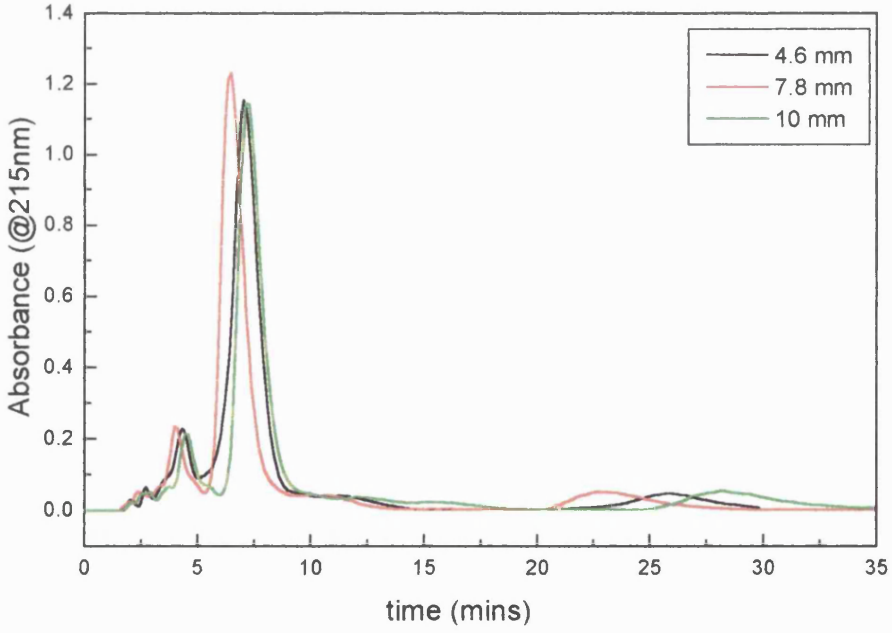


Figure 3.3-16 Superimposition of erythromycin traces on all three columns.

To illustrate truly the similarities in the performance of all three columns, the traces of erythromycin separation on all three columns were superimposed here. The profiles are very similar.

3.3.3.2.4 Column load

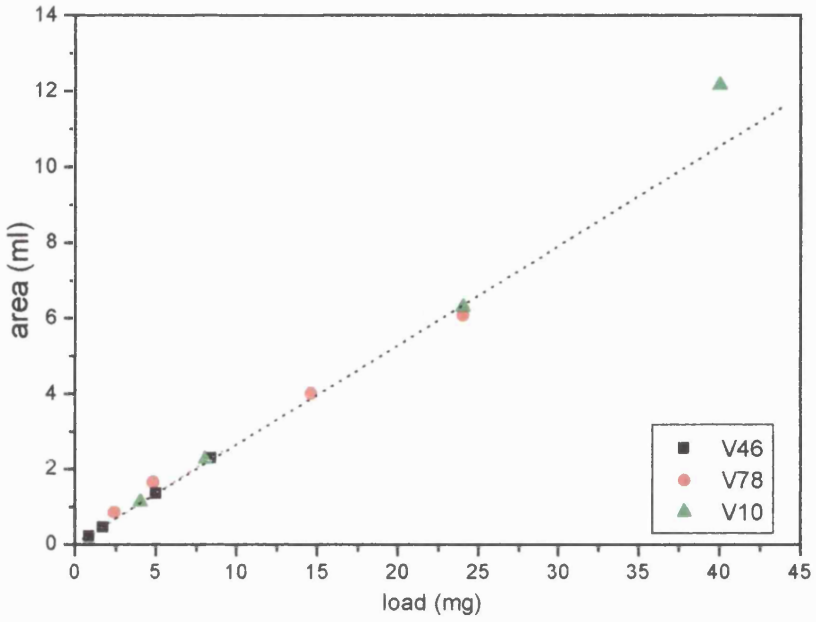


Figure 3.3-17 The profile of load against peak area on columns of different sizes.

This graph plots the amounts of the sample loaded on all three columns (as shown in the previous three graphs) and their corresponding peak areas. The plot shows a linear correlation between these two factors. At a very large load (approximately 40mg on 10mm ID column) the value deviates from linearity.

3.3.3.2.5 Conclusion.

The profiles show that the columns are well packed and that the packing procedure was successful. The porosity values for all three columns were established as 0.77 (for t_0=1.92 mins taken as the first blip on the chromatogram). The column efficiencies and asymmetry factors were very similar indicating that the columns were equally well packed and for the type of solute used here it is probably the best separation which could be had for this type of packing and chemistry involved.

3.3.4 The CG-300 columns: comparisons of their performance

Summary

This chapter presents the performance of four columns of different internal diameter packed with CG-300 polymeric packing material. The performance is assessed in terms of column efficiency, and asymmetry factor, with two compounds. The intention was to look at the impact the compounds have on the efficiency of the separation and how column performance changes with use.

3.3.4.1 Materials and methods.

Two compounds were used to verify the performance of four different columns: erythromycin and nitrobenzene.

With one exception - the large scale Prochrom column - the columns complied with the rules of scaling up, i.e. the bed length remained the same together with the type of packing material, its chemical properties and its particle size. The sample concentrations varied according to the sample used. The columns are all described in a previous section (paragraph 3.3.3.1) on semipreparative columns. Nitrobenzene is only slightly attracted to the stationary phase whereas erythromycin is bound to the stationary phase to a considerable extent.

number	Column name	bed length (cm)	support	column internal diameter (mm)	particle size (μm)	pore size (°A)
1	CG-300	15	styrene	4.6	35	300
2	CG-300	15	styrene	7.8	35	300
3	CG-300	15	styrene	10	35	300
4	Prochrom	18	styrene	60	35	300

Table 3.3-6 Specifications of four columns packed with CG-300 packing.

3.3.4.2 Results.

3.3.4.2.1 Erythromycin.

CG-300	N (plates/column)	N (plates/metre)	α (Asymmetry factor)
150 x 4.6 mm	172	1145	0.88
150 x 7.8 mm	168	1120	1.1
150 x 10 mm	171	1140	1.1
180 x 60 mm	205	1140	0.92

Table 3.3-7 Plate numbers and asymmetry factors for columns tested with erythromycin.

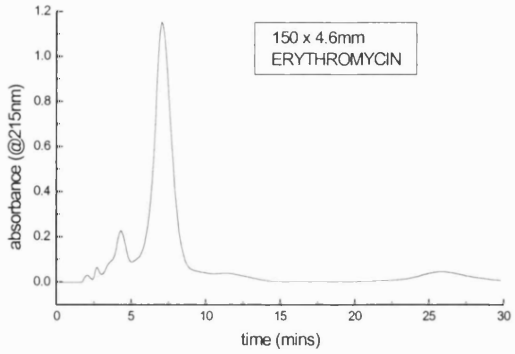
Here the results of the separation of erythromycin on the aforementioned columns are presented. The concentration of the sample in all cases was 40g/l.

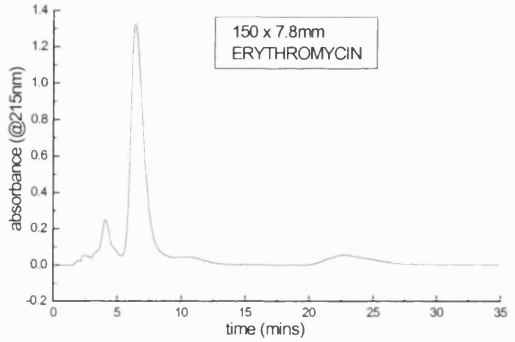
The efficiencies of all the columns tested with erythromycin are fairly low due to the chemical interaction of the sample with the stationary phase.

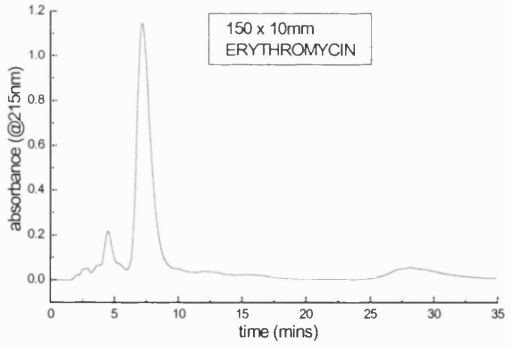
The efficiencies of all four columns are strikingly similar suggesting that the column efficiencies do not change with scale. This indicates that large scale columns can be as efficient as smaller scale columns.

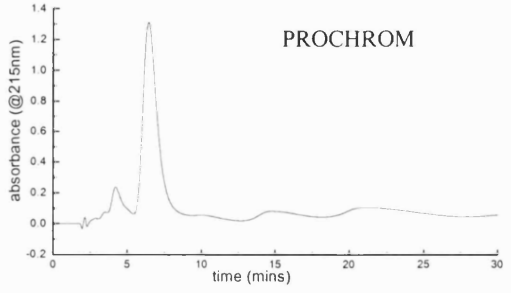
The asymmetry factor, in all cases lying within the range 0.9 to 1.1, indicates that the peaks are quite symmetrical. The profiles of the peaks are presented on the following page:

CG-300 ERYTHROMYCIN.









3.3.4.2.2 Nitrobenzene.

CG-300	N (plates/column)	N (plates/metre)	α (Asymmetry factor)
150 x 4.6 mm	570	3800	0.55
150 x 7.8 mm	205	1370	1.25
150 x 10 mm	626	4180	1.23
175 x 60 mm	603	3550	1.55

Table 3.3-8 Plate numbers and asymmetry factors for columns tested with nitrobenzene.

The results in the table reflect the latest status of the columns.

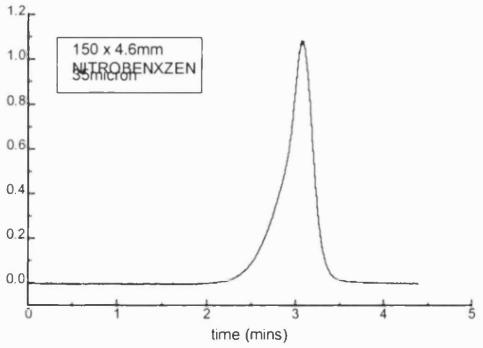
The 4.6mm ID column had not been used for several months and it appears that its performance regarding symmetry had further deteriorated. It appears that the column bed was the subject of further channelling. However its efficiency (3800 plates/metre) is almost as good as that of the best column examined here, i.e. 10mm ID (4180plates/metre).

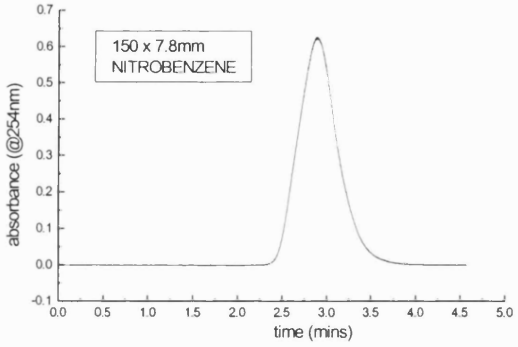
The 7.8mm ID column, which displays the lowest efficiency (1370plates/metre), deteriorated dramatically. This is attributable to the fact that the 7.8mm ID column had been heavily used.

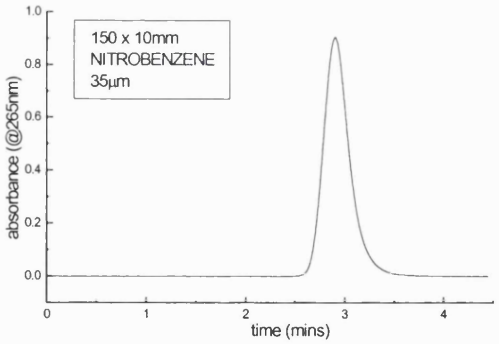
The 10mm ID column's frequency of usage was the same as the column of 4.6mm ID yet does not show any signs of deterioration. This indicates that the column was well packed ranking it as the best column in this set of experiments.

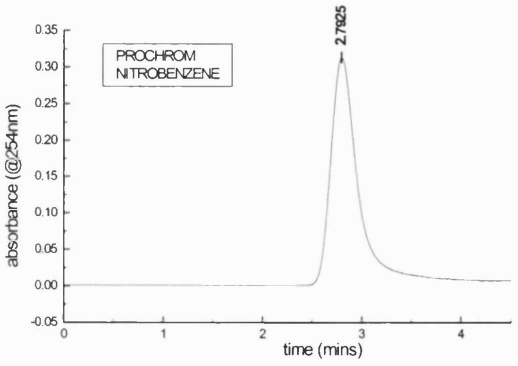
The Prochrom column shows the second best efficiency (3550plates/metre) for the peak of nitrobenzene out of all the columns examined. However the symmetry of the peak was affected by a further increase in back tailing.

CG-300 NITROBENZENE









3.3.4.3 Conclusion

The prediction of the columns' performance with different samples is not a simple task. Different scales can give different results in terms of efficiency and symmetry. The best advice is to test them out.

Two issues were addressed:

1. Comparisons of efficiencies and symmetries tested with one compound on columns of different scales.
2. Comparisons of columns' efficiencies and symmetries with different compounds (erythromycin and nitrobenzene).

1) Higher efficiencies are achieved with nitrobenzene followed by erythromycin. Nitrobenzene interacts with stationary phase very little, consequently it is subject to band broadening to a lesser extent. As a result, column efficiencies with this compound are larger than with erythromycin.

It is expected that the columns' performance with erythromycin would result in lower number of theoretical plates. This is because the interaction of erythromycin with the stationary phase is stronger than all the other compounds used here.

In terms of symmetries, more symmetrical peaks were achieved with erythromycin than with nitrobenzene.

N.B. The experiments with erythromycin were carried out just after the columns had been packed whereas the nitrobenzene experiments were conducted after the columns had been used for a number of other separations. Two of the columns (those of 4.6mm and 10mm ID) were barely used during that period of time whilst the other column (of 7.8mm ID) was used heavily throughout.

2a) Erythromycin.

It appears that the chemical interaction has a double effect on the chromatographic performance. On the one hand it improves the shape of the peaks whilst on the other it

decreases the efficiency. The evaluation of the column was carried out before its deterioration. The results with erythromycin on different scales show remarkable consistency. The average efficiency value is 1140p/m, slightly lower for the 7.8mm column (only 1120p/m). Symmetries are very good, close to 1. The 4.6mm and the Prochrom columns exhibit slight front tailing whilst the remaining columns exhibit back tailing (however these symptoms are barely visible).

2b) Nitrobenzene.

The experiments with nitrobenzene were conducted last and reflect the performance of the columns at this later date.

The column (4.6mm ID) was only used, when first packed, for a few test runs with acetone and erythromycin. After that the column was stored and not used for about a year. During that period the bed packing structure had disintegrated leading to a collapse of the column bed rendering it unusable.

The 4.6mm ID column had a problem with front tailing when it was first packed and tested with acetone. This data is not presented here because it lacked consistency. This front tailing was further exacerbated (for undetermined reasons) when the column was tested later with nitrobenzene. Despite the peak tailing, the column's efficiency seems very good reaching 3800p/m.

Although the N value is not entirely valid here because of the highly asymmetrical shape of the peak it can be used as a rough indicator of the column efficiency. (The equation for calculating the number of theoretical plates should only be applied to Gaussian peaks i.e. peaks which are symmetrical).

The 7.8mm ID column shows that the performance of the column had deteriorated. The column had been used intensively over a long period of time having been loaded with large samples, both in terms of concentration and volume. Therefore the value of N is very low (only 1370p/m) in comparison to other columns.

The best packed column seems to be the 10mm ID column which reaches an efficiency of 4180p/m and also the best symmetry (1.23).

The Prochrom column has a slightly lower efficiency but one which is still fairly good.

The symmetry of the peaks improves as the internal diameter of the columns increases. However, in the case of the Prochrom column the symmetry of the peak actually deteriorates.

3.4 Large scale chromatography using a PROCHROM column

Summary

This chapter describes a Prochrom preparative chromatography column. It further outlines the operating, packing, and unpacking procedures. An example of separation using the Prochrom column is presented.

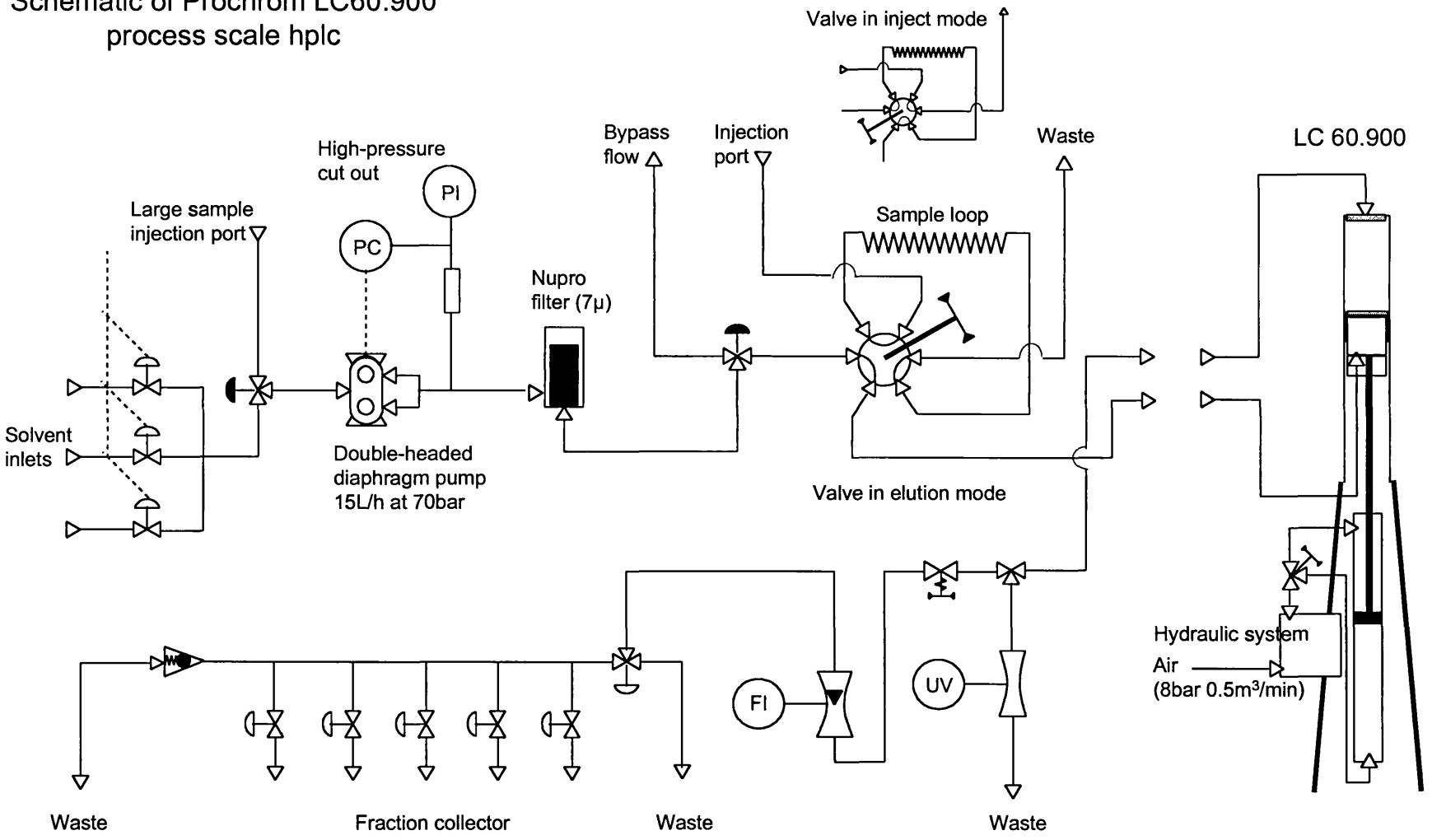
3.4.1 Introduction

The Prochrom unit (Champigneulles, France) is a large scale chromatography unit suitable for the purification of proteins and antibiotics. It is a highly sophisticated system based on the Dynamic Axial Compression (DAC) principle (see section 2.8.1.2.). The unit operates at flowrates of up to 300 ml/min (18 litres/hour) and can separate up to 23g of material per injection.

3.4.2 Description.

The Prochrom unit consists of several modules. They are a solvent module, the column unit, a personal computer and a UV detector. Large scale chromatography units are associated with handling large amounts of solvents. This can be potentially dangerous. To comply with safety requirements the Prochrom solvent module and the column unit are constructed of stainless steel and are placed in an explosion-proof area. The personal computer and the UV detector are based in a control room.

The system can be operated manually or automatically. Manual operation is used for isocratic separations. For gradient elution the system needs to be used in automatic mode. This is done by means of a dedicated personal computer (100MHz with 16MB RAM; Hewlett Packard) which controls the opening and closing of the solenoid valves. The software for this operation was written in LabView (National Instruments, Austin, Texas, USA).



Prochrom large scale chromatographic unit.

Figure 3.4-1 Large scale prochrom unit

3.4.2.1 Risk Assesment (COSHH) and Prochrom.

Handling large quantities of organic solvents imposes a risk, common in large scale set-ups, to the health and safety of the researcher and his co-workers. Consequently the Control of Substances Hazardous to Health (COSHH) rules and regulations had to be complied with when installing the Prochrom unit. The solvents used present a risk of explosion. To avoid this happening at least one of the sources which can create the explosion risk has to be eliminated.

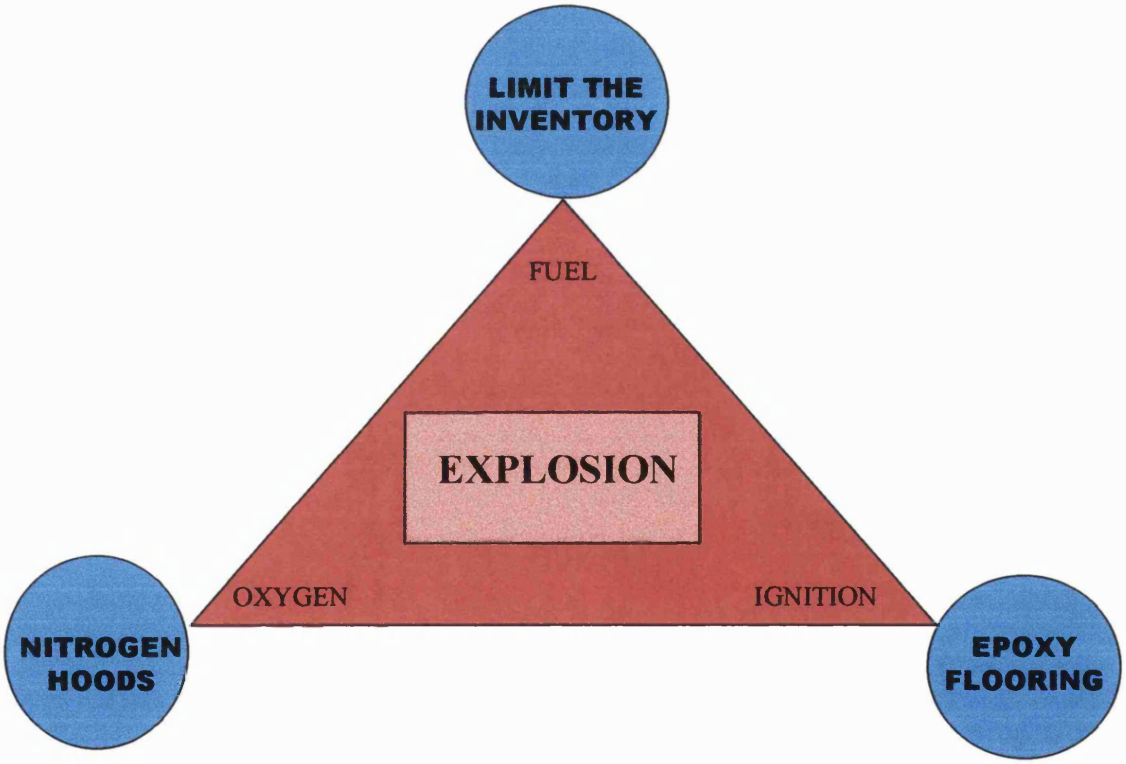


Figure 3.4-2 Three sources of explosion in working environment.

The diagram above presents the three factors oxygen, ignition and fuel which, if all present together may result in an explosion. The elimination of one of these sources minimizes the risk of a potential disaster occurring.

All these features were taken into account when designing the room for the operation of large scale Prochrom chromatography unit.

Ignition

To prevent sparks generated from static electricity and from metallic impact the floor in the solvent suite was formed from a layer of epoxy resin covered with conducting paint. The build-up of static electricity was also prevented through the use of conducting pipe-work which was bonded to a separate electrical earth. Finally all electrical equipment in the solvent suite was either intrinsically safe or flameproof.

Oxygen.

Nitrogen hoods were installed above the vessels which contained mobile phase to drive away "surplus" oxygen.

Fuel.

In the event of a fire, by limiting the inventory and using only the essential equipment in the room (which is constructed mostly of stainless steel) the progress of fire can be stifled. Any used solvent was deposited in waste drums which were regularly emptied.

Other safety features were:

- use of anti-static lab coats (to eliminate sparking)
- valves on the solvent module and the column skid were operated pneumatically instead of electrically to prevent sparking
- all the equipment was flame-proof (made of stainless steel)
- sockets and plugs in the room were flame-proof
- presence of ventilators to ensure exchange of air

In case of emergency, all the electrical appliances could be switched off using the main safety switch. A specially designed shower was also installed adjacent to the working area to flush chemicals from any operator accidentally exposed to them or to quench flames quickly in case of an accident.

All the people working in the area have to wear safety goggles, safety gloves and laboratory coats.

To prevent an unauthorised person entering the solvent suite the Centre is working on introducing a swipe card system to access the room. Thus only people directly involved in the research in the area will be able enter.

3.4.2.2 Solvent module.

The solvent flow is controlled by a volume duplex diaphragm pump (LEWA, Leonberg, Germany) for normal, corrosive or toxic chemicals and light slurries with viscosity up to 40 cPs. This type of pump minimises pressure pulsation and helps clean the pumping system. The accuracy of the pump delivery was high unless very low flowrates were used (less than 10ml/min). Accordingly the use of very low flowrates is not recommended with this pump.

The solvent is drawn from up to three different lines with the aforementioned pump. It then passes through a 7micron NUPRO filter which traps any impurities which might contribute to the creation of a column blockage. The pressure is monitored by a pressure gauge. The system also contains a pressure transducer which functions as a safety valve.

The chromatograph has provision for syringe injection (for small samples) or pump injection (for large samples). Using small sample injection, the material is injected into the column by means of a 6-way-valve (SWAGELOK, London Valve and Fitting Company, West Sussex, UK) mounted on top of the module immediately before the column.

The solvent module has facilities to collect fractions from several fraction outlets. The switches on this module are operated pneumatically by means of compressed air.

The important feature is a valve which enables a column by-pass operation. This facility helps to remove any air bubbles trapped in the lines before they enter the column. Running the system in by-pass mode also helps in determining the back pressure on the system.

3.4.2.3 UV Detector.

A UV detector from Beckman Instruments Inc. (CA., USA) was used for the detection of the sample and transfer of data to a PC for storage.

3.4.2.4 Column

The large scale chromatography column is mounted on a skid which keeps it in place and also contains the facilities for column packing.

The monitoring of pressure is possible via three different pressure gauges which are linked. Compressed air is directed into the hydraulic pump (Haskel, Sunderland, England) which pumps oil. The oil pushes onto the hydraulic jack which drives the piston. Accordingly the choice of air pressure affects the pressure at which the column is compressed.

The ratio between the jack and column pressure can be calculated as follows:

oil pressure x 0.28 = piston pressure

The column is 90 cm long and its internal diameter is 6 cm. The length of the bed can be varied enabling a researcher to use any bed length up to 60cm (to allow a proper compression). The construction is of 316 stainless steel (SS) and the maximum working pressure on the column is 70 bar. The column also has a flow distributor incorporated in the SS top flange to create a plug flow (an even distribution of the mobile phase and the sample).

3.4.3 Column operation

Although the column has a facility to be operated in an upwards or downwards direction of flow generally the column should be operated in the same direction as it was packed.

Prior to the starting operation of the column the mobile phase should be degassed and free of any particles. The by-pass valve should be in the 'ON' position initially to bleed any trapped air into the drain. The pumps are then set to the required flowrate and after the flow is stabilised and free of any potential air the valve is switched back to the "column mode" to allow the flow to pass through the column. After about one column volume of mobile phase has passed through the column the effluent can be directed from the drain into the bottle which contains fresh mobile phase. In this way the mobile phase can be recycled. After several runs (depending on the concentration of the sample utilised) the mobile phase becomes contaminated and is no longer suitable for use. At

this stage therefore it needs to be replaced with fresh stock. Fractions can be collected or the flow can be directed to the drain.

At the end of the day's work the column should be flushed with mobile phase which does not contain any buffer. For longer term storage the column should be stored with a high percentage of organic solvent to prevent any bacterial growth in the column.

3.4.4 Column packing

The design of the LC.60 VE 900 column is based on the Dynamic Axial Compression (DAC) principle. This means that compression takes place during packing as well as during column operation.

The column is packed vertically - upwards. The piston is ramped up to the appropriate pre-calculated height on the column. This is dependent on the desired bed length.

The preparation and pre-treatment of the slurry prior to packing is described in the section on the Pre-treatment of Amberchrom chromatographic resin (section 3.3.1.2.1.). The slurry was made up of 100% methanol which was replaced with 45% acetonitrile/water just after the column had been packed. These solvents seemed to work well for our column packing. Other solvents had been tested for the packing of large scale chromatography columns with mixed success. Sarker and Guiochon (1996) tested the performance of silica columns packed with different solvents. The columns were rated in increasing order of efficiency: n-heptane, acetonitrile, acetone, methanol and iso-propanol.

Slurry of an appropriate concentration is poured into the column. The column is then closed with the top flange and the piston moved up to a pre-set pressure value which should not be exceeded. As the hydraulic jack moves up compressing the packing material the pump can be heard cycling. This noise gradually ceases indicating that the packing process is nearing completion. Once the packing process is finished the mobile phase can be introduced to equilibrate the column. Care should be taken not to use a flowrate which generates a pressure higher than the one at which the column was packed. Failure to do so results in deformation of the packing. The column should be equilibrated over several hours to enable the packing to settle.

3.4.4.1 Column packing - points to remember:

- Column packing is a skill which comes with experience. Factors which play a major role in packing can be omitted due to inexperience on the part of the researcher. Therefore it helps to familiarise oneself with the ins and outs of the system as well as reading the manual.
- It was proved that one of the most important things when packing the column is proper preparation. This refers mainly to pre-treatment of the slurry - the elimination of fine particles present in the packing. It is imperative that this is done thoroughly. Failure to do so results in unsuccessful packing of the column due to fines blocking the flowpath of the fluid.
- The other vital issue is to use the correct pressure for the packing. The pressure should not exceed the manufacturer's recommendation under any circumstances. With particles like CG-300 which are polymer based, particles under pressure can deform but do not break. However this deformation is reversible. Once the pressure is lifted (when the column is unpacked) the particles revert to their previous shape.(Colin and Briand 1998).
- The other crucial issue in the column packing procedure is to clean the in-line filter. A blocked filter generates unnecessarily high back pressures and also wears out the pump.
- Once the column had been packed successfully it was necessary to test it. This was done with a selection of samples.

3.4.4.2 Packing pressure

The issue of the pressure at which the column should be packed is a tricky one. Although

the manufacturer quotes a maximum pressure which can be imposed on the particles this is not necessarily the recommended pressure to be used.

Colin and Briand (1998) conducted several experiments with Amberchrom CG-300 packing of about 20 micron particles on a Prochrom column to investigate the efficiency of columns packed at different pressures.

Their conclusions were as follows:

The higher the pressure at which the column is packed the lower the efficiency. Also a column which is packed at a lower pressure allows operation at a wider range of

flowrates. If the column is packed at higher pressures the operating flowrate range is limited by the more rapid increase in operating pressure which occurs.

Logically it follows that one would opt for the lowest possible pressure to give the best efficiency and also the widest range of flowrates. In reality, however, the matter is not so simple. Experience tells us that packing columns at lower pressures results in voids as the packing particles are not compressed sufficiently. If the column is packed at too high a pressure the particles are compressed and the permeability decreases as does the rate of flow of the mobile phase through the column.

To achieve accurate results one should use a pressure at which the velocity is such that the piston and solvent pressures are the same. This means the column bed length does not increase with flowrate.

The determination of an ideal solvent velocity was achieved by simple curve fitting over the points of piston pressure vs. solvent velocity. The paper claims it to be at a pressure of about 12bar and a solvent velocity of 0.013cm/sec.

3.4.5 Column unpacking

When the packing is no longer capable of providing a good separation the column needs to be repacked. The procedure for unpacking is very straightforward. The metallic clamp and the top flange are removed from the top of the column and the packing is pushed out by the piston in one solid piece as a single plug.

Prior to re-packing the top flange and the NUPRO filter should be sonicated.

3.4.6 Results

3.4.6.1 Prochrom separation

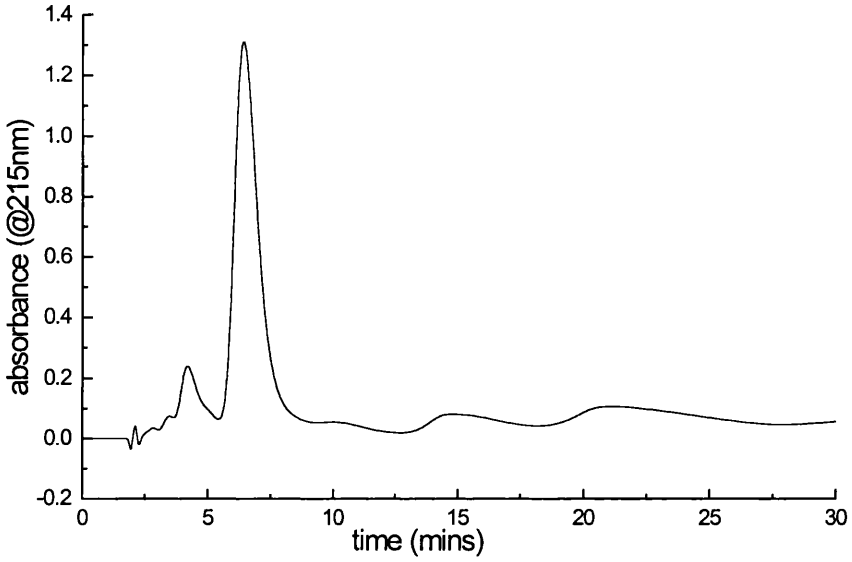


Figure 3.4-3 The separation of erythromycin on large scale Prochrom chromatography column.

Conditions:

Column: Prochrom 18cm x 6cm, injection loop: 20ml, sample concentration: 40g/l, (0.8g of the sample) flowrate: 210ml/min; running pressure: 5 bar; mobile phase: 45:5:50=ACN: buffer (pH=9; 0.2M): water

this is an example of an erythromycin sample separated on the Prochrom column. The column efficiency was 1140p/m and the symmetry factor 0.92. Impurities eluted after EA are widely spread but do not interfere with the main peak.

3.4.6.2 Particle size distribution.

Although the technology of particle manufacture has advanced immensely the particles purchased are still sold as a range (mesh size) rather than a single size. Here the particle size of one batch was investigated.

Three particle sizes are presented here, all from the same batch:

35 micron is the particle size quoted by the manufacture

27 micron is the particle size obtained by measuring the volume distribution of the particle size on a Malvern particle sizer

8.5 micron is the hydrodynamic particle size calculated using Darcy's law equation.

3.4.6.2.1 Manufacturer's quote

The particles come in a range of sizes and although the manufacturer's quote was 35micron this was not necessarily the average size. On the other hand the particles were subject to several column packing/unpacking activities. As every compression of the packing generates some fines, these fines were essentially generated from the larger particles. This could have reduced the average particle size.

3.4.6.2.2 Malvern particle sizer

The Malvern particle sizer determines the volume percentage distribution of the particle size of the packing material. Specific surface area: $0.25m^2/cm^3$.

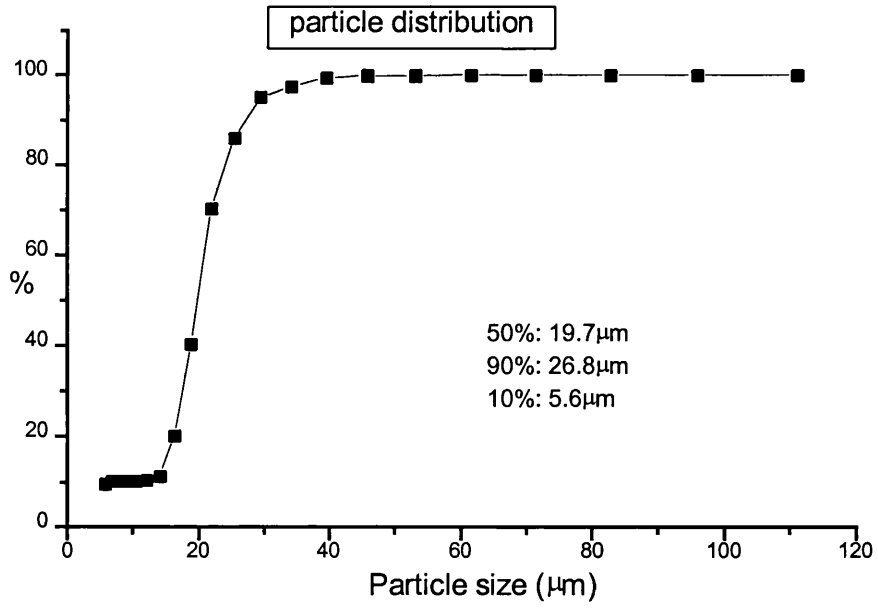


Figure 3.4-4 Particle size distribution by volume.

Here the particle sizer determined that:

10% of particles are 6micron size or less;

50% of particles are 20micron size or less; and

90% of particles are 27micron in size or less.

Ideally the ratio of d_{p90} / d_{p10} should be equal or less than 1.5. This value is particularly related to an analytical chromatography where small particles are used. Generally, the largest particles in the chromatographic bed determine the efficiency of the column and smallest its permeability.

In our case the value achieved was approximately 4.5 thus indicating that particle size distribution is large. This is however common when working with large particles as in our case is 35 micron.

3.4.6.2.3 Hydrodynamic particle size.

However, the particle size calculated using Darcy's law equation is very low. The pressure is inversely proportional to the particle size. This could possibly explain why the particles are so small. In order to achieve, by calculation, a particle size of 35 micron the pressure would have to be 17 times lower than is indicated.

3.4.6.2.4 Calculations using Darcy's law equation

The values entered in the Darcy's law equation to calculate the hydrodynamic particle size.

F (ml/min)	ID (cm)	column porosity (ε)	bed length (cm)	viscosity (poise)	permeability factor (K)	pressure 10^6 (mg/cm^2)
69	6	0.75	17.5	0.0089	600	7

Table 3.4-1 The values entered for Darcy's equation which yielded 8.5 micron hydrodynamic particle size.

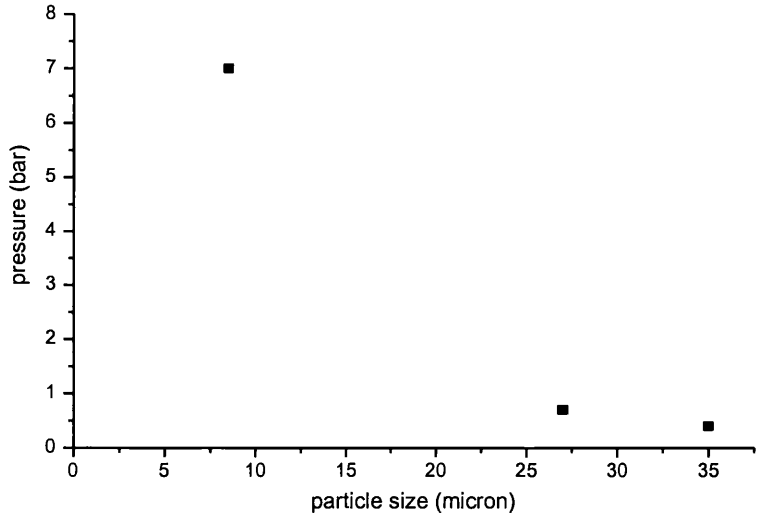


Figure 3.4-5 Graph of dependence of particle size on column pressure.

d_p (μm)	8.5	27	35
Δp (bar)	7	0.7	0.41

Table 3.4-2 Table with particle size and corresponding column pressure.

The above graph and table show the size of particles and their corresponding pressures calculated from Darcy's equation. This graph is consistent with the findings of Colin and Briand (1998).

The "extra large" pressure detected and used for the calculations could be due to a "hidden" pressure generated as the mobile phase flowed through the column frits. The size of the inlet column frit openings was 15microns. The pressure was measured before the injector and this could explain the increased pressure values.

To sum up, the particle size quoted by the manufacturer should only be taken as a rough estimate. The hydrodynamic particle size cannot be entirely taken at face value as there are other factors such as pressure which can substantially distort the particles.

For the purposes of this research, the measurement of particles with a particle sizer was found to be the best and most accurate method.

3.4.6.3 Pressure flowrate relationship

Large scale chromatography uses powerful pumps which can deliver litres of mobile phase per hour. To determine the pressure generated by the system at a particular flowrate and thus identify any irregularities one should construct a pressure-flow curve. It should be stressed that one should work in the linear part of the curve to be able to explain any irregularities on the part of the system

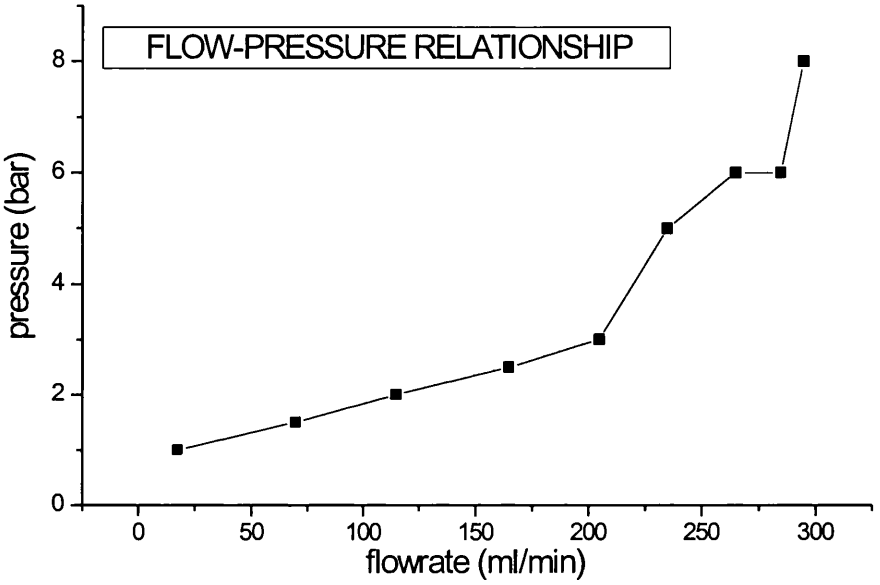


Figure 3.4-6 Flow/pressure relationship on Prochrom column.

This graph represents the calibration of the Prochrom pump. The relationship appears to be linear up to about 200 ml/min. A further increase in the flowrate increases the pressure on the system and the relationship becomes non-linear until the pressure seems to level off.

Yet further flowrate increases generate high pressures as the system struggles to deliver mobile phase at the set value. This leads to irregularities in the flow pattern because of pump fluctuations.

3.4.6.4 Volume overload on a large scale column

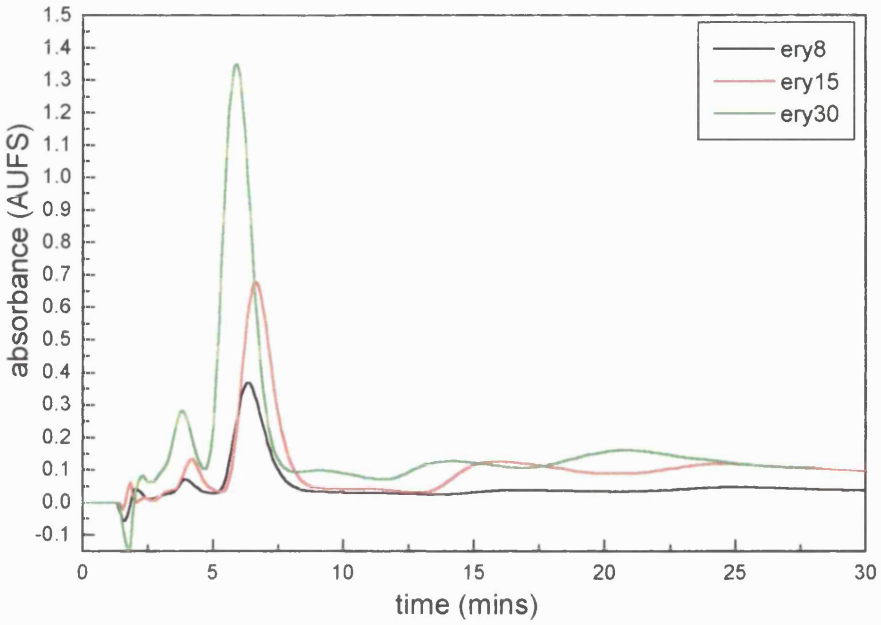


Figure 3.4-7 Volume overload on Prochrom column with loops of 8, 15 and 30ml.

The column becomes progressively overloaded with volume. Also visible is a decrease in retention time as the load increases suggesting a convex isotherm. This feature was shown on a smaller scale with 15 cm columns packed with CG-300. Although not shown on this graph, the use of a 30 ml load eventually leads to a trace with flat top peaks, a typical feature of the limitation on the sample solubility (see figure 3-48).

3.4.6.5 Column ruggedness and reproducibility

The reproducibility of the method is crucial particularly in large scale chromatography where the same product of the same purity should be achieved. This means the peak should have the same retention volume in order to be captured with the same accuracy.

The column reproducibility was tested with three different loop sizes. Samples of erythromycin were injected five times and the profiles from each loop were superimposed.

The results of all the runs with the three different loops are shown here (see figure 3-46, 3-47 and 3-48 and also Appendix 5). From the observed peaks it can be seen that the best reproducibility was achieved with the 15 ml loop. The next best reproducibility was obtained with the 8ml loop with the lowest reproducibility being obtained with the 30ml loop.

The efficiency achieved with the 8ml loop was 650p/m, with the 15ml loop it was 730p/m, and with the 30ml loop it was 632p/m.

The expectation had been that the best efficiency would have been achieved with the 8ml loop followed by the 15 and the 30ml loop. However this was not the case.

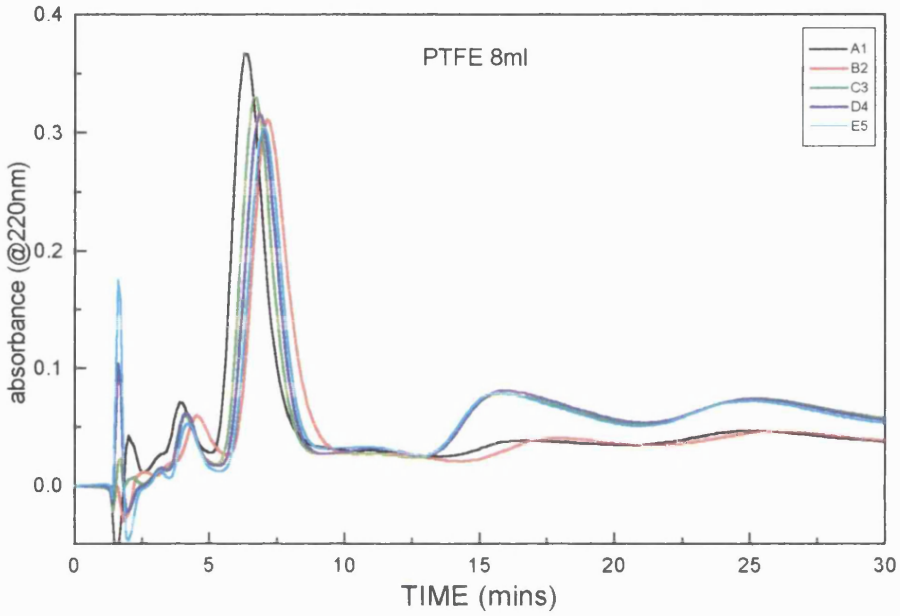


Figure 3.4-8 Test of reproducibility on Prochrom column with 8ml loop.

Here as in all the following cases the runs are shown in the order of the injection with A1 marked as the first sample injected and E5 as the last. The legends on all these profiles are numbered 1-5 according to the order in which the samples were injected.

The same amount of erythromycin was loaded onto the column 5 times in succession to test the reproducibility or robustness of the system. The slight shifts in the retention time are due to inaccuracies relating to the way the Prochrom column was operated. The processes of recording the data and injecting the sample should both start at the same time. However, because these two tasks had to be initiated in different rooms with both operations being undertaken by a single operator, inconsistencies were bound to occur. The increase in the absorbance of later eluted impurities noticeable for some later repetitions is due to recycling of the mobile phase. The repeated re-use of recycled mobile phase leads to an increase in the offset between the pure and recycled (contaminated or impure) mobile phase. This phenomenon is also seen on the front of the chromatogram as the first blip which appears as a ghost peak gradually becoming a more positive peak with increased absorbance.

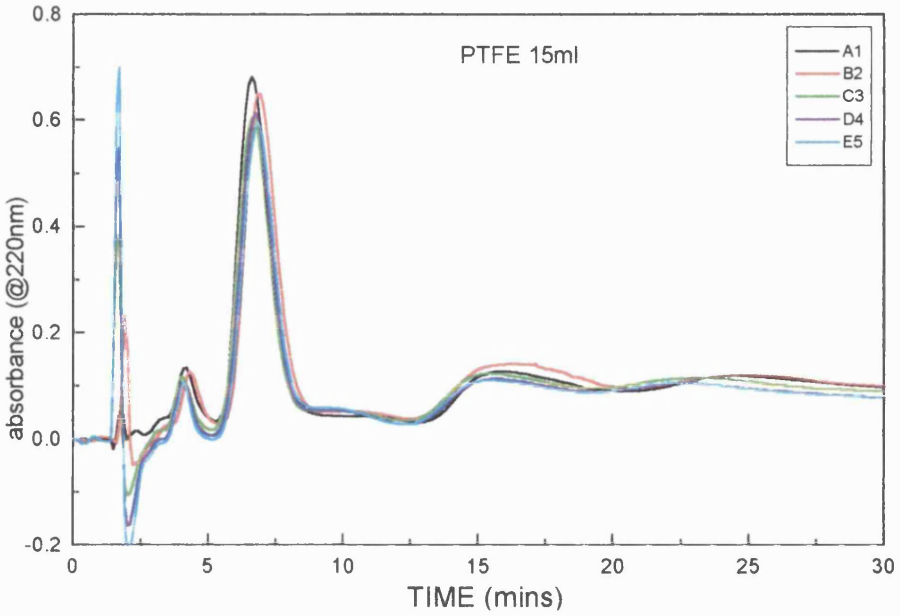


Figure 3.4-9 Test of reproducibility on Prochrom column with15ml loop.

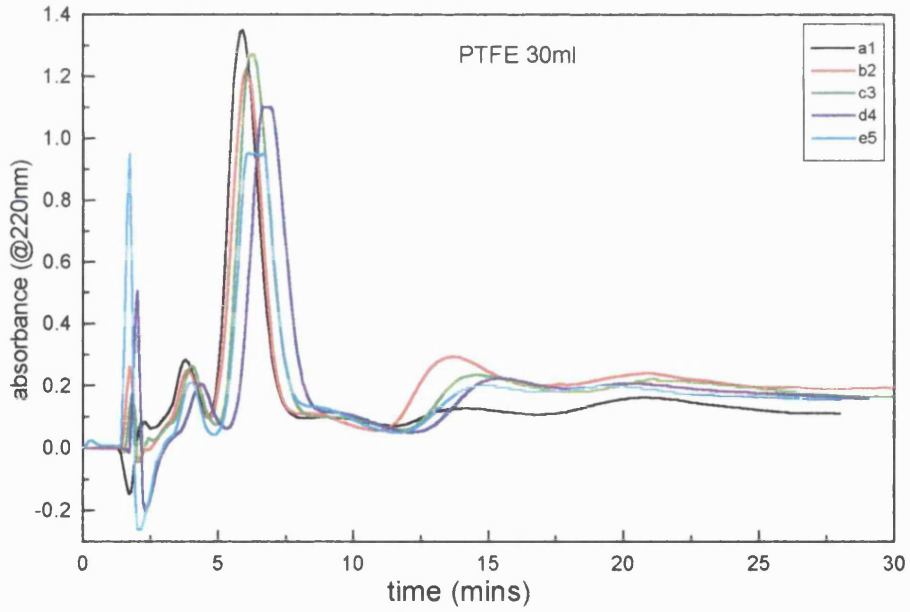


Figure 3.4-10 Test of reproducibility on Prochrom column with 30ml loop

With the 30 ml loop satisfactory reproducibility was not guaranteed. This was largely due to reaching the limit of detector capacity as more and more sample of large volume and high concentration was loaded onto the column. Every time another sample injection was made the detector offset value increased. As a result with later injections (d4 and e5) the sample of erythromycin A is eluted off the column with a flat top. This

becomes more and more pronounced as the sample is repeatedly injected. The evaluation of the chromatograms including this feature thus becomes difficult. Consequently the injection of large sample volumes should be avoided. Although acetonitrile was used originally to determine the erythromycin's solubility this solvent is less suitable as erythromycin is even less soluble in it than in methanol. The sample of erythromycin is first dissolved in methanol (we managed to reach a concentration of 120g/l without any problem). It is only when further dilution with buffer takes place that precipitation happens.

Average values for column efficiencies with 8, 15 and 30 ml loops are 650p/m, 730p/m and 630p/m respectively (see also appendix 5). When calculations of standard deviation and standard error were made (66 and 30, respectively) the overall "winner" in reproducibility studies appears to be the profiles injected with the 15ml loop. With only three values taken into account in the case of the 30 ml loop, the overall standard deviation and standard error values are the lowest at 25 and 15, respectively. It has to be emphasised however, that all the runs with the 30 ml loop are equally important for the calculations of standard deviation and error and the profiles reflect the real data. With this in mind, it is obvious that reproducibility results are the worst in the case of the 30 ml loop.

These figures are presented here only to point out the differences in efficiencies with volume injected. It is important to note that these values were acquired from some of the older runs on the Prochrom system when the column was not at its best. This explains its low efficiency in comparison to some other data on the Prochrom system.

3.4.7 Troubleshooting the PROCHROM column

Summary

This section illustrates "the trials and tribulations" of packing a large scale chromatography column. It gives examples of some problems commonly encountered and how they can be tackled.

3.4.7.1 The history of the Prochrom column used

The Prochrom column had been purchased in the early 1990s and had lain dormant for several years before being brought into operation for this project. The column had to be commissioned and placed in a flame-proof area to comply with the safety guidelines regarding the handling of large quantities of organic solvents.

Here the Prochrom column was packed on several occasions. The profiles of the tested peaks are shown and related problems outlined.

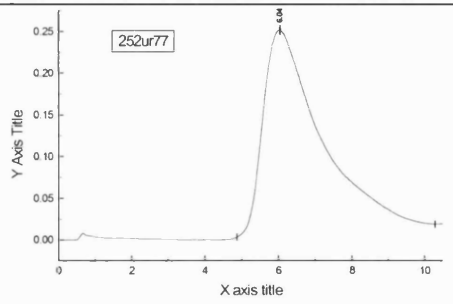
Symptom	Cause	Solution	Consequences / Examples (where applicable)
High pressure detected within the system	fines in the system	unpack the column and remove the fines from the packing material by decantation (settling)	only very low flowrates can be used and an increase in flowrates leads to a decrease in packing permeability
Bed length gradually increases	hydrodynamic pressure higher than the piston pressure	compress the bed prior to the run	the retention times are not consistent and thus difficult to compare
Irregularities in the flow (later detected by the peak back tailing)	channelling in the column bed	re-pack the column	

Table 3.4-3 Troubleshooting table for the Prochrom system.

In the following section the symptoms, causes and solutions outlined in the table 3.4-3 are analysed in detail.

3.4.7.2 Factors to observe

Several factors have to be observed when packing the column to make sure that it is well packed:

Symmetry.

Here a sample of acetone was used to determine the quality of column packing. Judging by the appearance of the peak (its symmetry) one would assume the column is well packed.

However, looking at other factors - e.g. column efficiency and back-pressure - more about the actual quality of column packing procedure can be revealed.

Operating pressure.

The flowrates which we intended to use were about 210ml/min but here only flowrates of a maximum of 55ml/min could be used. Initially, the pressure generated at this flowrate was acceptable but later increased dramatically until it was the maximum pressure allowed on the column (the operating pressure should not exceed the packing pressure). The increase in pressure causes deformation of the particles and a decrease in the column permeability. The fact that we could not increase the flowrate indicates that there was a problem on the column.

Column efficiency.

The column efficiency values with acetone were much lower than those obtained on the well packed column (about 6000 plates/metre). The efficiency obtained was up to 1400 plates/metre. Such a low efficiency for acetone also indicates that the column needs attention.

3.4.7.3 Symptoms

3.4.7.3.1 The presence of fines

At this stage it was found that the problems with the packing were due to the in-line filter becoming blocked. The blockage was caused by the resin which had not been thoroughly pre-treated. The resin contained fines which entered the system causing blockages and generating high pressures. Upon determining this the filter was removed, cleaned and sonicated. The resin was settled prior to column packing until the supernatant was completely clear (the indicator that there are no fines present).

3.4.7.3.2 Back pressure difficulties

We also made small adjustments to the system so that the back pressure could also be monitored when by-passing the column. This arrangement proved to be invaluable as regular monitoring of the back pressure can give an early indication of a blocked filter.
It was accordingly found that the filter has to be sonicated regularly. Although no fines from the resin were found again, small impurities did appear and were trapped on the filter particularly as the mobile phase was recycled.

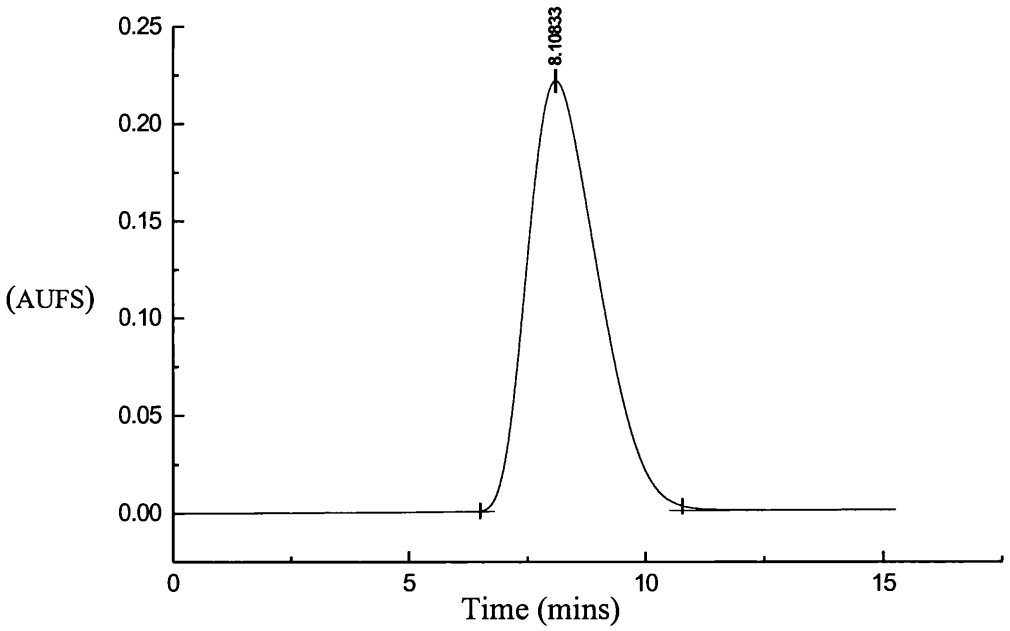


Figure 3.4-11 The acetone sample on the Prochrom system experiencing high pressures at 54ml/min.

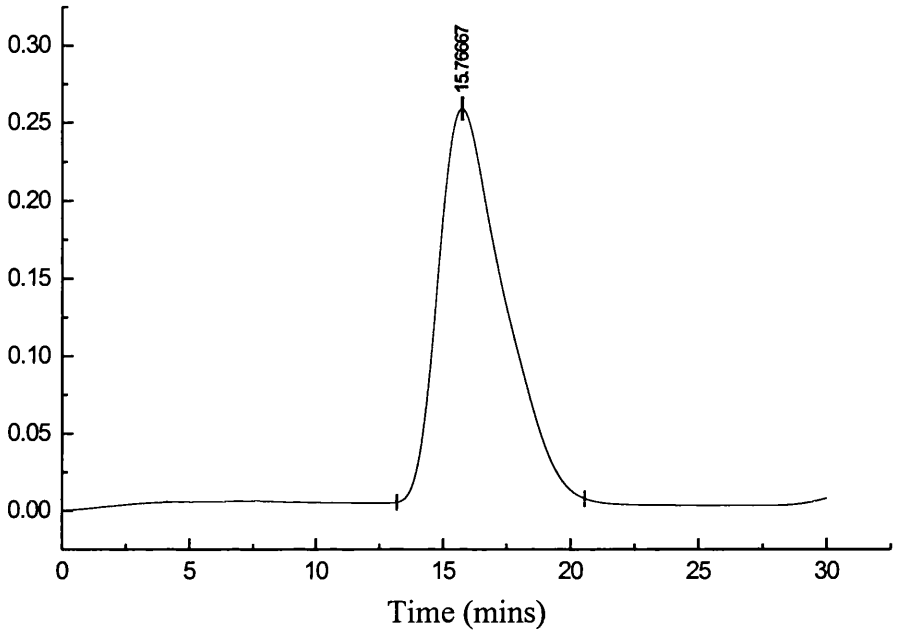


Figure 3.4-12 Elution profile of acetone at 28 ml/min on the Prochrom system.

Above are examples of two peaks of acetone at two different flowrates. In both cases the column was packed at the normal pressure recommended by the manufacturer (25-30 bar piston pressure). However the pressure gradually increased to unacceptable levels even at low flowrates such as 28ml/min. These symptoms indicated that there was a blockage present. Upon unpacking the column and settling the packing in a glass cylinder with a solvent it was revealed that the column contained a substantial amount of fines and also that the in-line filter was blocked.

3.4.7.3.3 Example of a combination of factors

3.4.7.3.3.1 Poor resolution.

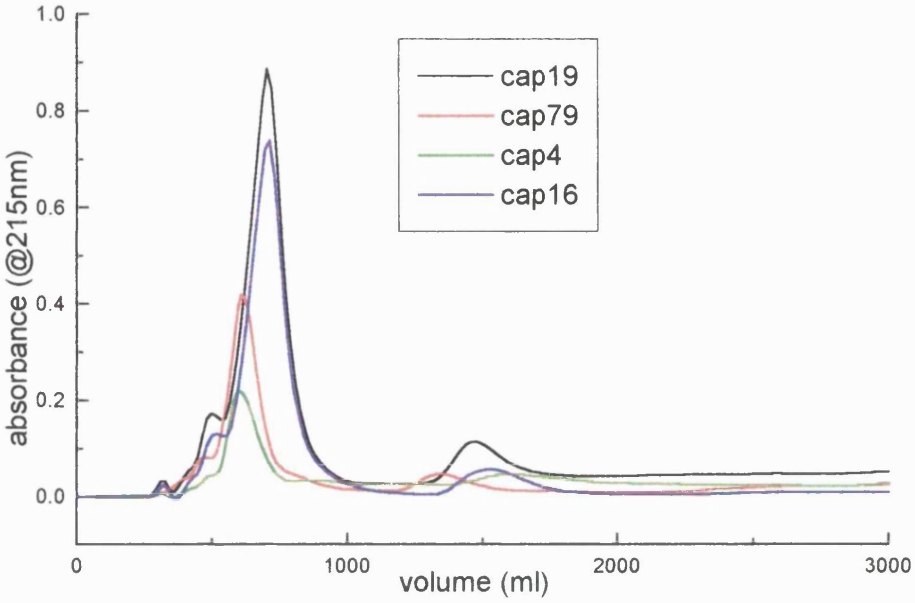


Figure 3.4-13 Profiles of erythromycin when injected with loops of various capacities: 4, 7.9, 16 and 19ml.

Here the different erythromycin profiles are shown when injected with different loops. The peaks are so wide that even at this flowrate at low injections the peaks are hardly resolved (front impurities and EA are merging together). Samples of 20g/l concentrations were used in this set of experiments. The legends on these following profiles refer to the capacities of the loops used, i.e. 19ml, 7.9ml, 4ml and 16ml.

The column was packed in the normal way applying the pressure (approximately 25 bar) recommended by the manufacturer of the particles.

The flowrate here was 71ml/min which was the maximum flowrate possible with regard to the pressure limit allowed by the packing. The intended flowrate according to the scale up rules for a 15cm column should be 170ml/min. At a flowrate of 71ml/min one would expect a complete separation of the sample compounds, the resolution probably being more than one. However, that is not the case here.

The peaks are rather broad and injections of large volumes of concentrated sample resulted in a flat top on the profile of the erythromycin A peak.

The profiles also show how increasing amounts of sample loaded on the column resulted in the merging of the peaks. This is visible with early eluting impurities which

merge with erythromycin A at higher loads. The concentration of the sample used was 20g/l except in the case of the 30ml loop when the sample concentration was 40g/l.

3.4.7.3.3.2 Flat top peaks.

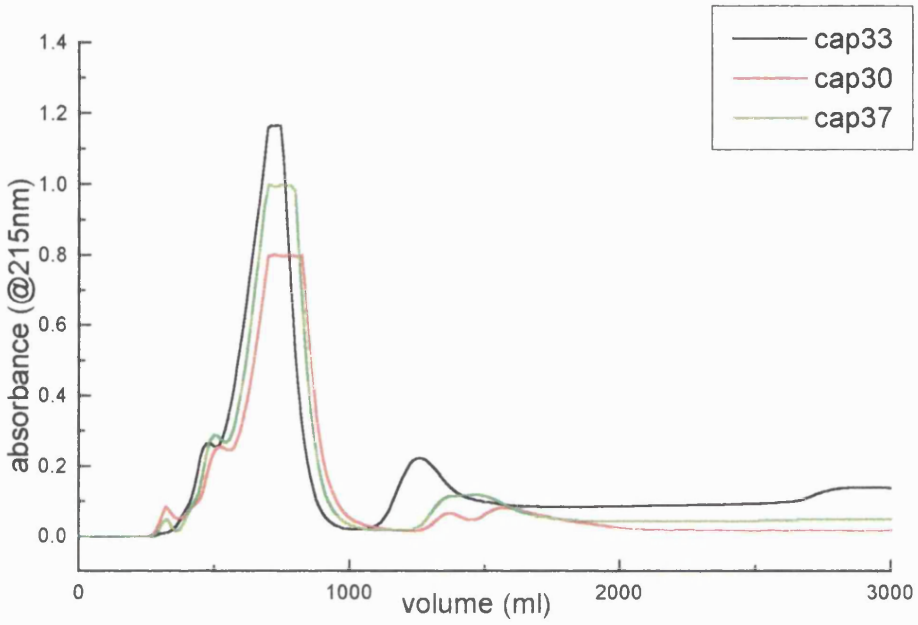


Figure 3.4-14 Volume overload with large capacity loops (30, 33 and 37ml) on the Prochrom system.

The problem is further compounded by the limited solubility of the sample.
This is clearly indicated in the form of flat top peaks. The higher sample concentrations show this effect in an even more pronounced way. The mechanism of the formation of the flat top peaks are explained in the section 3.4.6.5 on the column reproducibility. The concentrations and volumes used in this set of experiments follow:

loop capacity (ml)	concentration of the sample injected	amount of the sample loaded (mg)
30	40	1200
33	20	660
37	20	740

Table 3.4-1 Loop capacities and calculated corresponding sample loads.

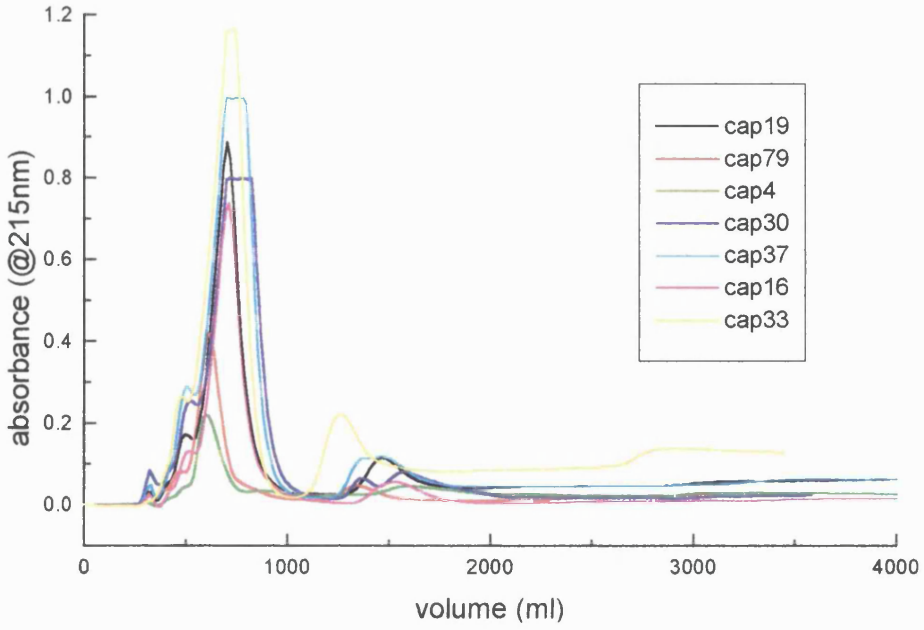


Figure 3.4-15: The overall picture of all the traces of erythromycin with all the loops.

This graph presents a combination of traces from figures 3.4-13 and 3.4-14.

As mentioned earlier these traces were obtained at a fairly low flowrate and the pressures were high. It comes as a surprise that under the prevailing conditions we managed to obtain relatively good separation profiles from this column. However, due to the pressure limitations encountered, proper scale up studies could not be carried out.

The loop specifications and sample concentrations are stated in the following table:

STAINLESS STEEL LOOP				
sample concentration (g/l)	loop internal diameter (mm)	loop length (m)	loop capacity (ml)	amount of erythromycin loaded (mg)
20	2.88	0.614	4	80
20	2.88	1.23	7.9	158
20	2.88	2.4	15.6	312
20	2.88	3.02	19.6	392
40	2.88	4.6	30	1200
20	2.88	5.06	33	660
20	2.88	5.7	37	740

Table 3.4-2: Loop specifications and sample amounts injected onto the column.

3.4.7.3.4 Increase in column bed length.

It was observed during the experiments that the column bed length increased at higher flowrates. The column bed should remain the same length throughout or decrease slightly (due to dynamic axial compression (DAC)) to eliminate the formation of any channels.

However, it was found that the bed length consistently increased at higher flowrates (above about 150ml/min). The column bed can increase by almost 33% more than its original length.

However, the column manufacturer later supplied a report in which these problems were explained (Colin and Briand 1998) and (Colin et al. 1998).

The explanation.

The column was packed upwards. Therefore the column should be operated in the same direction. However, operating the column in an upward mode gives rise to certain difficulties. The problem stems from two forces which operate in opposite directions. There is a force which pushes the piston downwards due to the flow of the mobile phase through the column (solvent pressure) and a force which pushes the piston upwards due to a dynamic axial compression (piston pressure). The two forces compete. The force which dominates the process will cause the bed either to expand or contract.

When the flowrate is low the pressure at which the piston is pushed up is greater than the solvent pressure tending to push the piston down. The hydrodynamic force becomes dominant at higher flowrates when large volumes of the fluid are forced through the column. This causes the expansion of the column bed and the piston is pushed downwards.

Under the normal operating conditions used in our laboratory (flowrates of around 250ml/min), the hydrodynamic force ruled over the piston pressure (column packing pressure). The practical consequence of operating under such conditions is that the column bed continuously expands. As a result the sample compounds separated on such a column are eluted later and later as the bed length increases.

One way to circumvent this problem is to stop the flow prior to sample injection to allow the chromatographic bed to be compress and to return to its original length.

This solution works when samples are eluted off the column quickly. However, for late eluted compounds the flow cannot be stopped and consequently the retention times of those peaks might not be correct.

3.4.7.3.5 Irregularities in the flow

Here another attempt to pack the column was made. On first inspection, the column appeared to be well packed in terms of symmetry of the peaks and of the efficiency of the column. However, when the sample was run over a range of flowrates the irregularities became obvious.

Two compounds were used, acetone and uracil, and both were run over a range of flowrates.

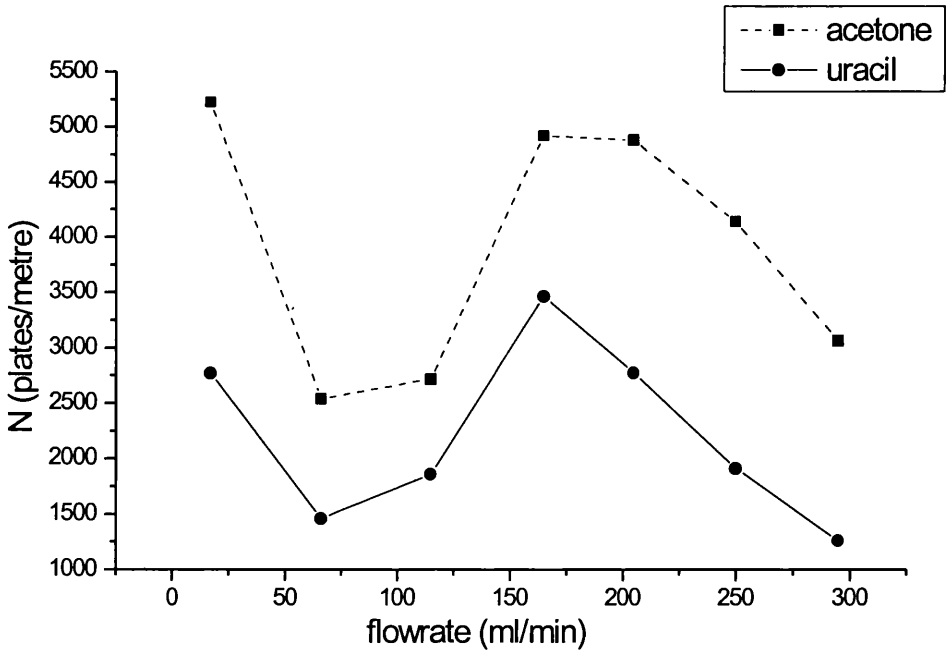


Figure 3.4-16 Example of irregular flow pattern on the Prochrom system.

	ACETONE		URACIL	
flowrate (ml/min)	N (plates/column)	N (plates/metre)	N (plates/column)	N (plates/metre)
17	862	5224	456	2768
66	420	2541	240	1460
115	448	2715	307	1860
165	817	4920	571	3462
205	810	4880	458	2775
250	682	4137	315	1912
295	505	3062	208	1262

Table 3.4-6 The values of column efficiencies with acetone and uracil.

The variations in column efficiency with different flowrates indicate the development of column channelling. This, of course, could not be seen with a sample run at one flowrate only. The channels in the column eventually led to a collapse of the column bed.

The results of these runs as shown on the graph (figure 3.4-16) surprised us but nevertheless we ran the erythromycin sample on the column (figure 3.4-17). The separation appeared satisfactory but after this sample the column bed collapsed suddenly. When the column was tested afterwards with uracil the peaks developed a severe tailing which could not be remedied by any means (figure 3.4-18).

The possible reason for this could be that the column might have been packed at a lower pressure than perhaps it should have been thus not allowing the bed to compress sufficiently.

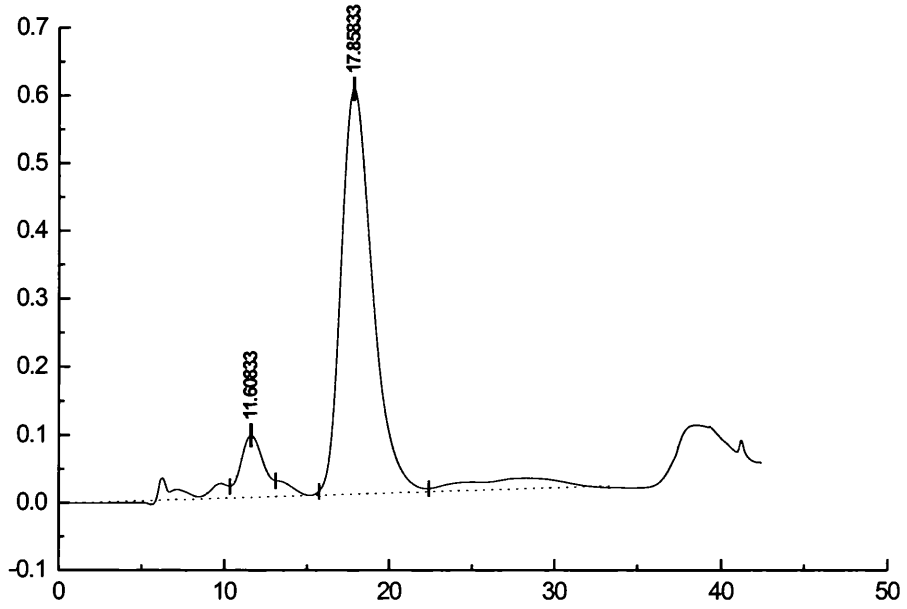


Figure 3.4-17: The trace of erythromycin just before the collapse of the chromatographic bed.

The sample of erythromycin was separated at 66ml/min on this column. The separation results are as good as those on our last column. The concentration of the sample: 20g/l. The injection volume: 4ml.

After this separation the column's performance deteriorated suddenly. This is indicated here by a severe back tailing on the uracil sample.

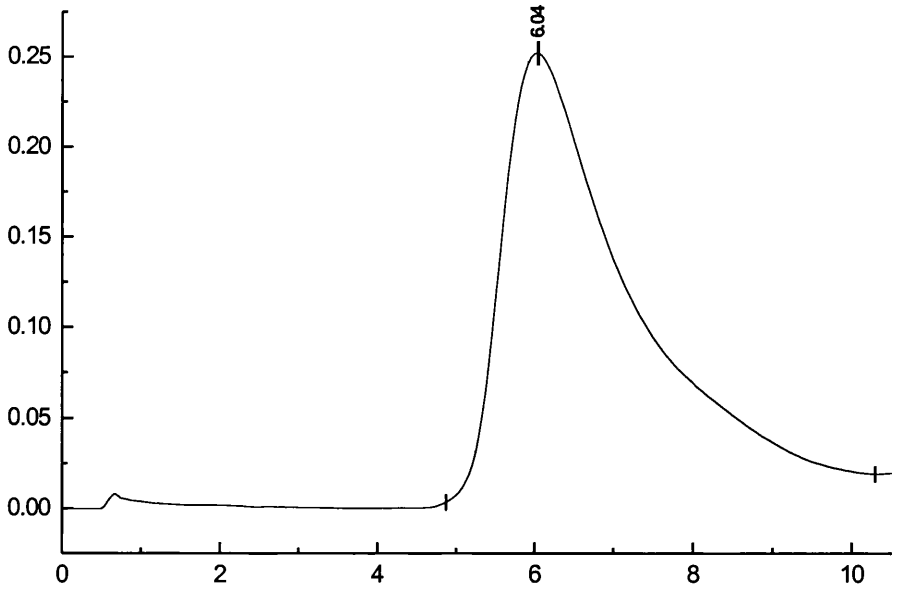


Figure 3.4-18 Back tailing on the uracil sample symptomatic of bed channelling.

3.4.7.4 The lessons learned

One should become thoroughly familiar with the system and how it works with the help of a manual, particularly the system's pipe-work diagram. Training given by an experienced user is invaluable.

Proper preparation of the packing material prior to column packing is of paramount importance, particularly the removal of fine particles which can block the system and render the column unusable.

The construction of a diagram of efficiency versus flowrate can give a quick indication of the quality at which the column was packed. From this diagram any irregularities can be identified.

Every column has its own "foibles" or "quirks". These are rarely mentioned in the manual and the earlier one acquaints oneself with them and finds a solution the better. In this case one of the problems was the column bed gradually increasing its length. The more information that is available to the user of the column the faster a solution can be found. This refers to a report by the manufacturer of the column who pointed out that indeed this was one of the features of the system.

To sum up, it had been thought that a better design would have been one allowing the column to be packed in a downwards direction where additional expansion of the bed is less likely to occur. However, subsequent information received from Prochrom indicates that the problems also exist with columns packed downwards.

3.5 Column efficiency vs. Flowrate

Summary.
The aim of this section is to look at the effect of flowrate change on the separation of three compounds: acetone, uracil and erythromycin. The performance of these solutes on a large scale Prochrom chromatography column was compared from the point of view of column efficiency, and the volume and concentration of the sample injected. h/ν curves were also constructed for each of the compounds and conclusions drawn.

3.5.1 Introduction

Preparative columns generate column variances (i.e. column band broadening) so large that extra-column band broadening is usually insignificant (Verzele and Dewaele 1985). To the best of our knowledge, so far, direct comparisons of the data obtained on large scale columns with extra-column separations have not been carried out. Here, therefore, an attempt was made to do just that.
To establish column band broadening the samples of acetone, uracil and erythromycin were run on a Prochrom column and the data was correlated (compared) with separations of these compounds on the system without the column.

3.5.2 Materials and methods.

The column was packed as stated in the chapter on the column and its operation. The mobile phase used was 45:5:50 (v/v%) acetonitrile: phosphate buffer (pH=9, 0.2M): water for erythromycin, and 45% acetonitrile/water for the acetone and uracil samples. The mobile phase was degassed with helium prior to use. The standard wavelength for erythromycin was 215nm and for uracil and acetone 265nm.

3.5.2.1 Sample concentration

For the experiments on the column, the following concentrations were used:

acetone:	0.5% (87.1mM)
uracil:	0.25mM

erythromycin: 20g/l (28.3mM)

3.5.2.2 Column efficiency calculations

Calculations of the number of theoretical plates were made using the standard equation for N (see section 2.2.2.1. on Chromatography theory).

3.5.3 Results

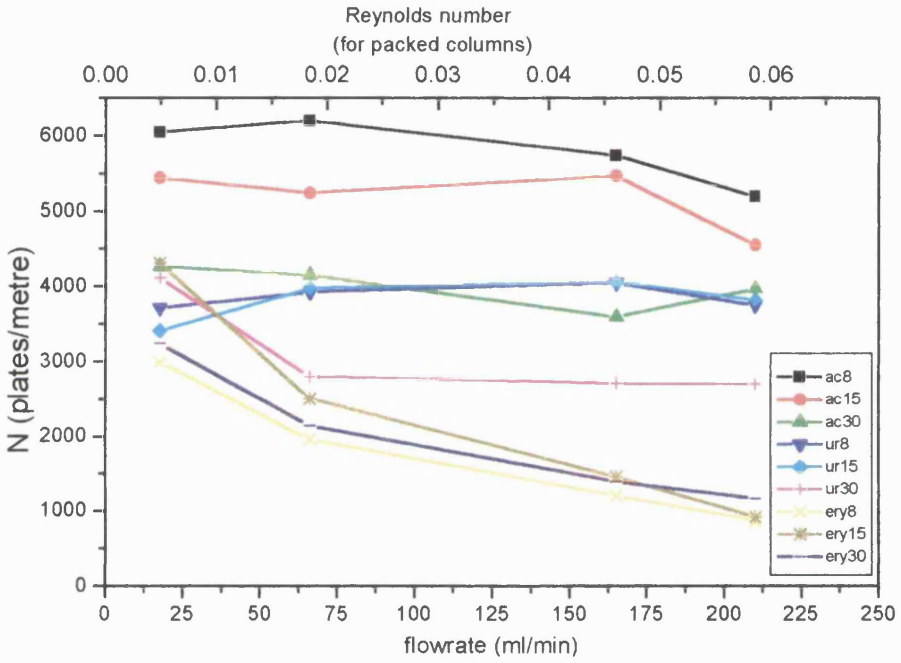


Figure 3.5-1 Column efficiencies with acetone, uracil and erythromycin on Prochrom column.

This plot shows the effect of mobile phase velocity on the column efficiency calculated in terms of the number of theoretical plates. The study was carried out with three different solutes: acetone, uracil and erythromycin. Three loops of different capacities were employed: 8, 15 and 30ml for each solute.

3.5.3.1 Acetone and uracil

The curves of *acetone* (black, red and green) injected with three different loops show a decline in efficiency every time the volume injected increases. At higher flowrates the efficiency also decreases with the increase in the flowrate used. With 30ml loop injection the profile is slightly different but this is attributed to a data variation and

essentially should have the same trend as in the previous two cases, i.e. a flat line which starts to decrease with high flowrates.

As for *uracil* (blue, light blue and pink) the curves obtained when 8 and 15ml of the solute were injected are intertwined and generally show very similar values of N. The values at the lowest flowrates were low, then increased as the flowrate rose and then became steady before dropping again. With the 30 ml loop the profile is similar to acetone: the N values drop dramatically to approximately 2800p/m.

3.5.3.2 Erythromycin

With erythromycin, all three curves show a steady decline in the values of N as the flowrate increases. The values of N are not dependent to the same extent on the volume injected as was the case with acetone and uracil. If anything, the curve actually shows the opposite trend. The profile with the 8ml loop gives the lowest value of all three curves. This is followed by the data with the 30 ml loop and the highest values are achieved with the 15 ml loop particularly at low flowrates. At high flowrates, the N values with the 15ml loop drop to the same level as the data for the 8 ml loop. However, at high flowrates, the differences between the values of N obtained from the three different loops is minimal.

However, if one chooses to use very low flowrates, the 15 ml loop gives much higher efficiencies in comparison to the others. Overall however, as high flowrates are preferred in chromatography, for the separation of erythromycin the amount of the sample injected does not have such a large impact on the efficiency of the column and therefore the use of the sample loops is interchangeable.

3.5.4 Packed columns and laminar flow

Reynolds numbers were calculated using the standard equation for calculating the Reynolds number for packed columns (see Chromatographic theory section 2.2.6.2.).

Although the range of flow rates used here is similar to those used for the study of extra-column band broadening (17.5ml/min-210ml/min) the Reynolds numbers indicate that, unlike in extra-column separations, here the flow is laminar only.

3.5.5 The solutes and the number of theoretical plates

3.5.5.1 Acetone and uracil

Acetone and uracil are compounds which have weak attraction to the stationary phase. (They are often used as test solutes for determining the quality of column packing technique, deterioration of the column efficiency over a period of time, and for calculating column void volume). These compounds are therefore eluted off the column faster than erythromycin which is eluted slowly because of a stronger attraction to the stationary phase. As acetone and uracil "reside" in the column for a relatively short time they are subject to band broadening to a lesser extent than erythromycin. This results in greater values of N for these two compounds. The efficiency does not decrease so dramatically with increasing velocity as is the case with compounds more attracted to the stationary phase such as erythromycin (C term in the van Deemter equation).

Acetone and uracil are compounds with $k'_{acetone} > k'_{uracil}$. This leads us to believe that the peaks of uracil would generate a greater number of theoretical plates than acetone. However, the experimental results reveal that the opposite is the case. Acetone gives higher values of N than uracil. Acetone with its greater retention times than uracil gives larger values of N. Interestingly, the peak width for acetone in comparison to uracil is almost the same.

3.5.5.2 Erythromycin

The sample of erythromycin is subjected to adsorption-desorption due to chemical interaction between the stationary phase and the sample. This leads to a decrease in the number of theoretical plates with an increase in flowrate. The slope of the curve representing the C term in the van Deemter equation increases with the greater interaction with the stationary phase as the velocity in the column increases. Calculations of the ideal flowrate were also made. (see h/v curve, section3.6).

Erythromycin is eluted off the column later than acetone and uracil due to the kinetics of adsorption-desorption, and therefore is subject to band broadening on the column to a greater degree. This consequently leads to a reduction in efficiency. The slope of the curve of band broadening with regard to mobile phase velocity (C term) increases more sharply with an increase in flowrate than in the case of uracil and acetone. This is due to

the chemical interaction between the sample and the stationary phase and it means that the efficiency drop is greater than for compounds less attracted to the stationary phase.

3.5.6 Column efficiency and mobile phase velocity

The curves of uracil and acetone when measured with 8 and 15ml loops show a stable horizontal line.

Generally the maximum efficiency is achieved at low flowrates and where the solutes are eluted off the column at a flowrate close to the ideal. This corresponds the velocity on the h/v curve at which the overall band broadening is lowest.

This can be seen in the case of acetone and uracil. With uracil, slight variations are seen with the drop in efficiency at low flowrates. This drop can perhaps be attributed to fluctuations in flowrate.

3.5.7 Column efficiency and sample volume injected

3.5.7.1 Acetone and uracil

The graph (see figure 3.5-1) shows the change in column efficiency with the sample volume injected. The samples were injected with three different loops of capacities: 8ml, 15ml and 30ml.

For uracil and acetone (having low attraction to the stationary phase) an increase in the sample volume injected results in a decrease in column efficiency.

Acetone was injected at a higher molar concentration in comparison to uracil. The concentration of acetone was so high that every time the greater volume of the sample was injected, the efficiency dropped visibly. With greater volumes injected the column was progressively more overloaded and this contributed to greater band broadening.

The situation appears to be slightly different for uracil. When the sample was injected through 8 and 15 ml loops the efficiency hardly changed. However, injection with the 30ml loop resulted in a visible drop in the efficiency.

The sample of uracil was injected at a relatively low molar concentration (0.25mM) in comparison to acetone (87.1mM). This low concentration enabled N to sustain the same level even though a greater volume of the sample was injected. However, a further

increase in the sample volume injected eventually led to a decrease in the value of N as the column became progressively overloaded.

3.5.7.2 Erythromycin

The results with erythromycin are not affected by the sample volume injected to such an extent as was the case with acetone and uracil. This is because with acetone and uracil the hydrodynamic flow properties dominate, whereas with erythromycin the chemical interaction plays a major role.

3.5.8 Reynolds number in tubes with granuiar material

The laminar flow in packed columns is expressed in terms of Reynolds number. For a Reynolds number of 1 or less the flow is regarded as laminar. A Reynolds number above 100 indicates a turbulent flow. Intermediate values indicate that the flow is in the transition region between laminar and turbulent.

Reynolds numbers for a packed column with an internal diameter 6cm								
F(ml/min)	25	50	100	150	200	250	300	350
u(m/s)	0.0002	0.0004	0.0008	0.0012	0.0016	0.002	0.0024	0.0028
Re	0.007	0.014	0.028	0.042	0.056	0.07	0.084	0.098

Table 3.5-1 A table of Reynolds numbers for a flow through a packed column calculated for the conditions taken from figure 3.5-1.

From the theory (Chapter 2.2) and the calculations summed up in the table it is seen that the flow is laminar throughout the whole flowrate range.

3.5.9 Conclusion.

Three different solutes acetone, uracil and erythromycin were employed as test samples on a large scale Prochrom chromatography column.

Acetone and uracil, having a small interaction with the stationary phase, are eluted off the column quickly. As a result they are subject to column band broadening to a lesser extent and consequently generate greater column efficiencies. Acetone gives better efficiencies (up to 6000p/m) than uracil (only 4000p/m).

As uracil elutes off the column before acetone, the column efficiency with uracil would be expected to be greater than with acetone. The results on the column show that $N_{(acetone)}$ is larger than $N_{(uracil)}$. This means that band broadening on the column is greatest for uracil because its retention times are lower than those of acetone.

On the other hand the column efficiency with erythromycin is affected by adsorption to the stationary phase which contributes to band broadening. Greater band broadening leads to lower efficiencies.

The peaks of the samples eluted off the column early are affected by the amount injected i.e. concentration and volume of the sample. With a sample of such a low concentration such as uracil greater volumes can be injected with no detriment to column efficiency. With large sample concentrations the injection of greater volumes will inevitably lead to a decrease in efficiency.

In the case of samples more attracted to the stationary phase such as erythromycin the volume of the sample injected, particularly at high flowrates, does not have an impact on column efficiency. The efficiency is only governed by the strength of interaction with the stationary phase. The drop in efficiency becomes more and more prominent as the flowrate increases (C term). The efficiency curve is a line ever decreasing with the increasing flowrate.

3.6 h / v curves

Summary

Here h/v curves for acetone, uracil and erythromycin generated from the data on the large scale Prochrom column are presented. The data is evaluated with regard to an ideal flowrate and the general shape of the curves.

3.6.1 Methods

The values required for the calculations of reduced velocity and reduced plate height were taken from the graph of column efficiency against flowrate. The diffusion coefficients were calculated using a Wilke-Chang equation. (see section 2.2.5.4.).

3.6.1.1 Porosity.

sample	hold-up time (t_0)	column porosity
uracil	1.83	0.76
acetone	2.4	0.99
erythromycin	6.76	2.79

Table 3.6-1 Prochrom column porosity calculated with different solutes: uracil, acetone and erythromycin.

Please note that the flow rate taken into account was 210ml/min and the Prochrom column volume was 509 ml.

3.6.2 Results

3.6.2.1 Acetone

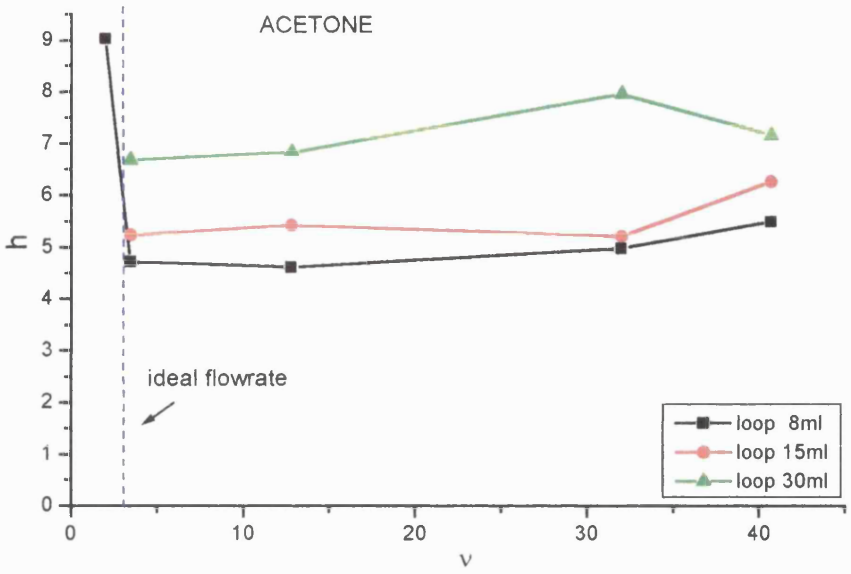


Figure 3.6-1 h/ν curves of acetone.

The graph is almost a mirror image of the plot of column efficiency against flowrate for acetone. Here, however, it is expressed in terms of reduced flowrate and reduced plate height. This way of plotting a graph appears to be convenient as the curves can tell us more about how well the column was packed; it enables an assessment to be made of the flowrate one can use to make the most of the column; and it can also assist in predicting the continuation of the curve trend.

Well packed columns have minimum values of h between 2 and 4. The ideal flowrate in all cases is when $\nu=3$.

What we can see here is that the left hand part of the curve almost reached this ideal flowrate. The diffusion coefficient for acetone is $1.42 .10^{-5}$ $cm^2/sec.$ in 45% acetonitrile/water and the ideal flowrate was calculated as at 15.5ml/min ($\nu=3$). So the value of h is likely to be very similar to the value reached at 17.5ml/min. i.e. h=4.7. The value of h is close to 4 which is regarded as a well packed column.

A separation was also conducted at a flowrate lower than the ideal. The results show a sharp increase in band broadening as the "B" term increases with the decrease in velocity.

The h/ν curves also show how the values of h increase with volume injected. The implication of volume injected on column efficiency was explained previously in section 3.5.

3.6.2.2 Uracil

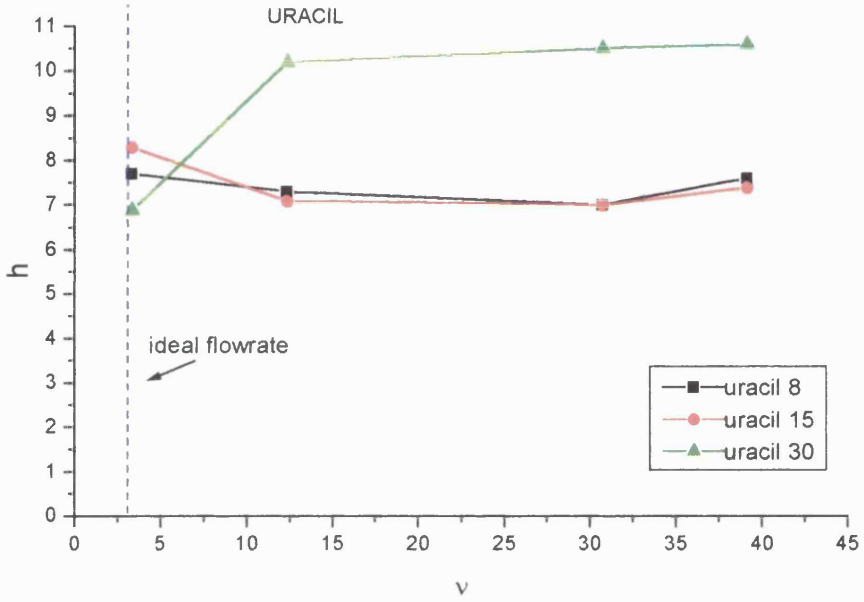


Figure 3.6-12 h/ν curves of uracil at different loop volumes.

The ideal flowrate was calculated to be 16.1ml/min giving h=6.9. The reduced flowrates range from about 3 to approximately 40.

It is believed that the larger values of h for uracil in comparison to acetone are due to greater extra-column band broadening for uracil and its lower retention times in comparison to acetone.

3.6.2.3 Erythromycin

The ideal flowrate was calculated to be 4.3ml/min for erythromycin when ν=3. It is therefore assumed that at this flowrate the efficiency of the column would be the greatest and therefore the value of h the lowest.

The diffusion coefficient of erythromycin in 45% acetonitrile /water is $3.94.10^{-6}$ cm^2/sec.

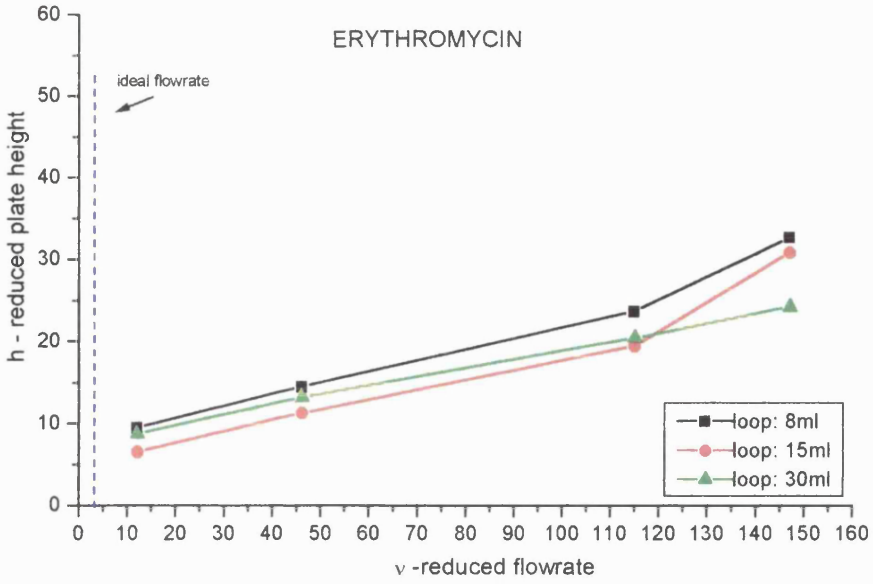


Figure 3.6-3 h/ν curves of erythromycin.

The figure shows how the reduced plate height increases with reduced velocity. The profiles with all three loops are shown. The ideal flowrate is shown as a dashed blue line. The graph covers a wide range of reduced flowrates (10-145). This is due to the low diffusion coefficient of erythromycin in comparison to uracil and acetone. The slope of the right hand part of the curve is steep due to the adsorption-desorption process which slows the process of diffusion and increases band broadening. The best results at larger velocities are achieved with the 30ml loop, followed by the 15 and 8ml loops. However, at lower flowrates, the order of the 30 and 15ml loops is reversed, i.e. injection with the 15 ml loop gives better results than the 30ml loop.

The values of reduced flowrate, also known as particle Peclet number, depend on a diffusion coefficient. The reduced velocity ν is inversely proportional to D_m. As D_m for erythromycin is very small the ν term is very large. The graph also shows how dramatically the slope of the curve of erythromycin is affected by the increased value of ν. As the flowrate increases (ν>120) the slope of the curve becomes sharper. This means that band broadening increases faster and the efficiency decreases faster. The minimum flowrate used for the measurements is very close to the ideal flowrate. The top value of the curve (top right corner of the graph) with the maximum value of h~ 33 suggests that, if possible, lower flowrates should be employed in the separation of erythromycin as they would lead to greater efficiencies and therefore the column's properties could be better utilised. The intercept of the curve with the dashed line of an

ideal flowrate would give an approximate value of $h_{(erythromycin)}$~7. This value is close to the value of uracil $h_{(uracil)}$= 6.9 which is higher than for acetone $h_{(acetone)}$= 4.7.

3.6.2.4 h / ν curve of acetone, uracil and erythromycin together

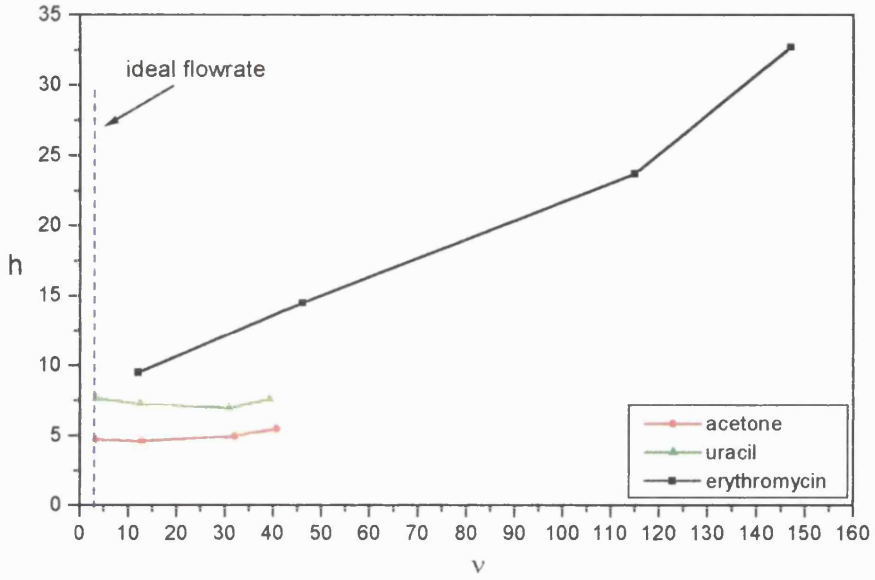


Figure 3.6-4 h/ν curves for acetone, uracil and erythromycin with 8ml loop injection.

This graph shows h/ν curves for acetone, uracil and erythromycin. The curves reveal how the solutes are affected by the flowrate. For the purposes of clarity only one curve for each solute is presented: acetone (8ml lop), uracil (8ml loop) and erythromycin (8ml loop). The dashed curve is that of an ideal flowrate (ν=3) for all three compounds.

3.6.3 Column efficiency and h/ν curve

The calculations of N to be expected on the Prochrom column.

h	particle size (micron)	bed length (cm)	N (plates/column)
2	35	17.5	2500
4	35	17.5	1250

Table 3.6-2 Column efficiency calculated from the "h" values of well-packed column.

The maximum values of N on the large scale Prochrom column for acetone, uracil and erythromycin were 1060, 720 and 760 plates/column; respectively.

If the top value of "h" is taken into account, it can be seen that with acetone that figure has almost been reached. One can say that the column was well packed when tested with acetone. The figure for uracil is higher but, as mentioned earlier, this is due to a lower retention time. The erythromycin curve if intercepted with the ideal flowrate curve would have a value of h~7. All three compounds show that the column is packed very satisfactorily and probably cannot be packed any better.

solute	diffusion coefficient (cm^2/sec)	ideal flowrate (ml/min)	reduced plate height "h" at ν=3.
acetone	$1.42.10^{-5}$	15.5	4.7
uracil	$1.48.10^{-5}$	16.1	6.9
erythromycin	$3.94.10^{-6}$	4.3	~7

Table 3.6-3 The values of diffusion coefficients, calculated ideal flowrates, and approximate corresponding reduced plate heights for acetone, uracil and erythromycin.

3.6.4 Conclusion

The h/ν curves of three compounds acetone, uracil and erythromycin were presented here. The curves of acetone and uracil are very similar and the range of reduced flowrates is much smaller than in the case of erythromycin. This is due to a larger diffusion coefficient. In both cases the curves are flat. This presents itself as a great advantage as the use of high flowrates to speed up the process of elution would be at no expense to the efficiency of the column.

The erythromycin curve, on the other hand, stretches over a range of reduced flowrates which far exceed the range used by acetone and uracil. This is because of the much smaller value of erythromycin's diffusion coefficient.

The curve has a rather steep trend and any increase in flowrate has essentially a detrimental effect on the column efficiency. Consequently lower flowrates should be employed as they can improve the efficiency substantially.

Another aspect here is the proximity of the curves to an ideal flowrate. In the case of acetone and uracil the curve has almost reached a point and the corresponding "h"

values at those flowrates indicate that acetone is close to the value of 4 which is in the "well packed" region.

The situation is different with erythromycin where the ideal flowrate occurs at 4.3ml/min. This value is not quite reached but when extrapolated the corresponding h value is believed to be approximately 7.

The strong interaction of the sample with the stationary phase also leads to a rapid increase in the "h" value.

3.6.5 Discussion

The results show that it is advisable to determine h/v curves to be able to run samples efficiently and make the most of the column and packing material available.

At the same time, as each compound behaves differently on the column, the sample intended for the separation should be tested over a range of flowrates. It is important that as much information as possible is obtained about the behaviour of the sample on the column and its interaction with the stationary phase. This can help to establish the best possible running conditions.

It may seem that choosing the correct conditions for the separation can be time consuming but it is time well spent. It also seems that the optimum theoretical conditions might not necessarily be the ideal conditions in practice, but a trade-off may be necessary between the best and what can be afforded in terms of time and money. Nevertheless the knowledge gained from the h/v curve is extremely valuable and should certainly be taken into account prior to any serious separations being considered.

From the point of view of the expected efficiency on the column if we assume that reduced efficiency is h=<2,4> then the efficiency would be in the range 1250-2500 plates/column. The efficiency achieved with acetone was $N_{(acetone)}$= 1050 plates/column (with 8ml loop) and the maximum value for $N_{(uracil)}$ is about 700 plates/column (with 8ml loop). For erythromycin the best results are achieved with an 8 ml loop at lower flowrates where the value of $N_{(erythromycin)}$ =760 plates/column giving, surprisingly, a slightly greater value than for uracil.

3.7 Extra-column band broadening (ECBB)

3.7.1 Approach 1

Summary.

Extra-column band broadening on a large scale chromatography system was investigated with regard to Reynolds numbers and flowrates. The results were compared with the performance of acetone, uracil and erythromycin on the Prochrom column.

3.7.1.1 Materials and methods

Here we dealt with extra-column band broadening resulting from the connecting tubing as well as from the sample volumes injected.

The impact of extra-column band broadening on the separation of several compounds on a large scale Prochrom system was investigated. The band broadening study was conducted by running through the system separate samples of acetone and uracil injected with different loops at different flowrates without the column in place.

Here an attempt was made to calculate the ECBB using the standard equation for the height equivalent to a theoretical plate. Whereas in the original equation for columns H=L/N where L is the length of the column bed, here the length of the bed was replaced by the length of all the tubing carrying the sample. This therefore included a sum of all the extra-column band broadening contributions: viz. the injector, the detector, and the tubing which carries the sample along the system.

Loops employed in the study of ECBB.

loop capacity (ml)	internal diameter (cm)	loop length (m)
8 (PTFE)	0.159 (1/16")	4.04
15 (PTFE)	0.159 (1/16")	7.6
30 (PTFE)	0.159 (1/16")	15.2
30 (SS)	0.288 (0.113")	4.6

Table 3.7-1 Dimensions of loops used in the experiments.

part of the system	material	internal diameter (in)	length (cm)	tube volume (ml)
from injector to column tubing	SS	1/16	78	1.47
tubing leading to the column	PEEK	1/16	200	3.77
column out to the split	PTFE	1/16	235	4.65
split to the wall	SS	1/16	146	0.67
wall to the detector	SS	1/16	126	0.57

Table 3.7-2 Dimensions of the tubing in the Prochrom system.

The loops used for acetone and uracil.

Loops (ml)	acetone	uracil
PTFE 8	√	-
PTFE 15	√	-
PTFE 30	√	√
SS 30	√	-

Table 3.7-3 Different usage of loops for acetone and uracil.

3.7.1.1.1 Reynolds number

A good indicator of the flow characteristics - laminar or turbulent flow - in a tube is the Reynolds number. Here the Reynolds number was calculated for a temperature of 35°C. Calculations of Reynolds for temperatures of 25° and 30°C are included in Appendix 6. The viscosity values were taken from a paper by Colin et. al. (1978) for 45% acetonitrile/water at different temperatures.

Reynolds number for a straight tube with an internal diameter 1/16"(0.159cm)									
F(ml/min)	25	50	75	100	150	200	250	300	350
u(cm/s)	21	42	63	84	126	169	211	253	295
$Re_{@35°C)}$	431	863	1294	1726	2590	3452	4315	5178	6041

Table 3.7-4 A table of Reynolds numbers and linear flowrates for a flow through a straight tube at 35°C.

It appears that the best "fit" (although not perfect) with the data of ECBB against flowrate was achieved with an elevated temperature of 35°C. The Reynolds number is only an approximate indicator of the changes in the flow pattern which can be due to temperature but can also be due to the presence of loops in the system. Coiled tubing speeds up the onset of turbulent flow.

As for comparisons between PTFE and stainless steel (SS) tubing performance, it appears that the start of turbulence appears earlier with SS tubing than PTFE. This can be attributed to the rougher internal surface of the SS tubing which encourages radial convection and leads to turbulent flow faster than in the case of PTFE which has smoother walls.

3.7.1.1.2 Sample concentration

For the purposes of the experiments conducted without the column the following concentrations were used:

acetone: 0.5%, (87.1mM)

uracil: 0.5mM.

3.7.1.2 Extra-column band broadening.

Extra-column band broadening on an analytical scale is a well-researched subject (see the Discussion).

However there seems to be a scarcity of scientific data available on extra-column band broadening on large scale systems.

We attempted to fill this gap and measured the extra-column band broadening by simply disconnecting the column from the system and running the samples of acetone and uracil through it.

Band broadening in analytical systems

In analytical chromatography columns of high efficiencies are used. Research results so far conclude that the more efficient the column the greater is the impact of extra-column band broadening on overall band dispersion (Colin et al.1979).

Band broadening in preparative systems

The general assumption is that analytical columns are approximately three times more efficient than preparative columns (Herbert 1991).

The dispersion on preparative columns is therefore more significant in comparison to extra-column band broadening. Consequently the accepted view is that extra-column band broadening plays a marginal role in the overall peak dispersion.

3.7.2 Results

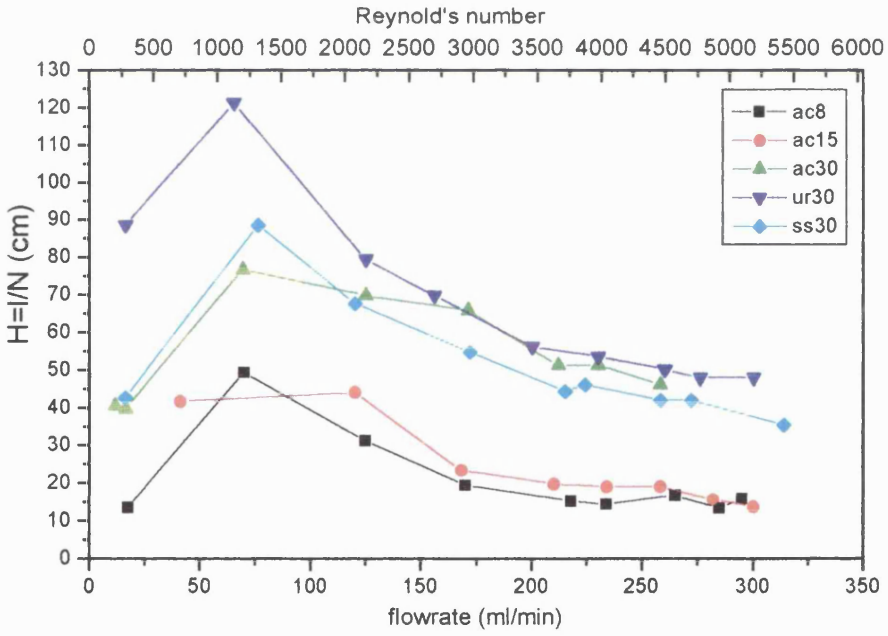


Figure 3.7-1 Extra-column band broadening on a large scale chromatography system with acetone and uracil.

This is the profile of extra-column band broadening on the Prochrom large scale chromatography system measured without the column. The samples of uracil and acetone were injected with different loop volumes. The loop capacities and internal diameters are detailed in the Materials and Methods section.

3.7.2.1.1 Profile of extra-column band broadening with regard to flowrate

Laminar flow

Low velocities give a laminar profile. As the velocity increases (0-70 ml/min) but remains within the limits of laminar flow, the flow profile becomes an elongated bullet. In our graph this is indicated by an increasing value of H with flowrate. This profile reaches its maximum value when the Reynolds number reaches the maximum value for the laminar flow (Re=2100). From the point of view of chromatography, band broadening at this mobile phase velocity (75-80ml/min) appears to be the greatest: therefore this flowrate is not suitable to operate in.

Transition region

A further increase in velocity introduces turbulent flow. This process is gradual and a fully developed turbulent flow is only reached when Re = 4000. From the onset of turbulent flow (here F>80 ml/min) the profile starts to change as the velocity equalisation effect takes place. The term, first coined by Giddings in 'Dynamics of Chromatography' (1965), is explained by the author as the gradual reduction in speed due to the tortuosity of flow and an increase in flow resistance with increased velocity. The result of this is a gradual reduction in band-broadening. Here it is represented by a gradual decrease in H as the radial flow takes place.

Turbulent flow

When the full turbulent flow is reached (above about 250ml/min here) the velocities are all reduced, brought to a similar value, and thus give a flattened bullet profile. This marks the end of the decrease in the band broadening, i.e. any further increase in velocity will not lead to a further reduction in band broadening. In chromatography terms, this velocity region would be the most favourable in which to operate (Re>4000).

Plug flow

When very low velocities are used (less than 25ml/min) all the velocity layers in the bullet profile are flattened as the velocities across the whole cross section are the same. A plug flow is achieved. This results in the minimum possible band broadening. From

the point of view of chromatography, operation at these flowrates would be ideal. However, from a practical point of view, operating at such low flowrates is not economically viable.

3.7.2.1.2 Extra-column band broadening and the sample volume injected

Here the sample of acetone was injected with three loops: 8, 15 and 30ml. The uracil sample was injected with a 30 ml loop.

The graph shows how an increase in the volume of acetone injected gradually increases band broadening and gives the maximum band broadening with the 30 ml loop. For the sample of uracil, the band broadening value is largest of all.

The increase in H with the increased sample volume is due to an axial diffusion. The larger the sample injected (in volume terms) the greater the axial dispersion.

It would be expected that acetone and uracil would give similar values of extra-column band broadening due to their close diffusion coefficients. However this is not the case.

3.7.2.1.3 Extra-column band broadening and loops of different internal diameter

Comparisons were made between loops of the same capacity but different internal diameters. A PTFE loop with an internal diameter (ID) of 1/16"=0.159cm was compared to a stainless steel (SS) loop with an ID of 0.288cm. Both loops were coiled with the coiled diameter for both loops being approximately 12cm.

3.7.2.2 Main findings

Shape

ECBB has a particular profile when related to the flowrate, that of a parabola. At very low velocities the ECBB is low due to a plug flow, at high velocities low ECBB is attributed to radial convection due to turbulent flow. The region in between is characterised by first an increase in H value (ECBB) until it reaches the maximum at which the flow is still laminar and then it decreases as turbulent flow starts to form. The point of inflexion gives the highest value of ECBB and therefore the flowrate

corresponding to it should not be used in operation. Here we found the inflexion point to be at approximately 75ml/min (63cm/sec).

Volume injected

It was found that the injection of larger volumes of acetone led to greater extra-column band broadening. This is due to axial dispersion taking place in the tubing.

Uracil versus acetone

The results with uracil injected with a 30 ml loop lead to greater ECBB than is the case with acetone.

PTFE versus stainless steel (SS) loop

ECBB measured when acetone was injected through a 30 ml stainless steel loop appeared greater than when a 30 ml PTFE loop was used.

3.7.2.3 Band broadening in analytical and large scale chromatography

3.7.2.3.1 Analytical scale

The normal profile of *extra-column band broadening* (ECBB) in straight tubes on an analytical scale is a straight line, band broadening increasing with the increase in linear flowrate. The whole range of flowrates employed on an analytical scale covers strictly the laminar flow. *Column band broadening* (CBB) on an analytical scale is also in the laminar flow region only. As a result the data on extra-column band broadening and total band broadening can be superimposed and band broadening on the column can be determined.

3.7.2.3.2 Large scale

On a large scale, the profile of *extra-column band broadening* with regard to flowrate is different from that on the analytical scale. It is that of a parabola. Reynolds numbers reveal that both laminar and turbulent flow are present.

However, the profiles of *column band broadening* experiments on a large scale employing flowrates up to 250ml/min. cover only laminar flow.

Total band broadening (TBB) on a large scale which consists of CBB and ECBB covers only laminar flow. Therein lies the problem. Like should be compared with like. Total band broadening at laminar flow should be compared with extra-column band broadening at laminar flow. This requires measurements at very low linear velocities which perhaps can only be determined from the current experimental data by extrapolation. ECBB is calculated in the same way as H (plate height) for the efficiency on the column: that is the value 1/N is multiplied by L which is the length of the tubing. However as the tubing used on the large scale is very long the resulting values of ECBB are high - higher than the values for total band broadening. This makes it rather difficult to compare the band broadening on the column with the data measured without the column.

3.7.2.3.3 Extra-column band broadening and total band broadening

The equations used for the calculations of extra-column band broadening are not applicable. Or at least they cannot be used when comparing the data generated on the column.

There are several reasons for this:

- the length of the tubing on the system which carries the sample is far too long to be incorporated in the equation used for the calculations of ECBB
- extra-column band broadening (ECBB) appears to be greater than TBB including all ECBB + column contributions (CBB)
- if the calculations are correct and the numbers are real then the ECBB (which was measured by us) is far too large and currently cannot be interpreted.

All these statements lead to one conclusion: that the data on the column and without the column cannot be compared directly on a large scale. It seems the equations used so far for the calculation of ECBB are applicable to an analytical scale only.

The interpretation of the data on ECBB currently available in the literature does not relate it to the data on the column: in other words there is very little on the comparison of data found on the column and without the column.

3.7.2.3.4 Calculations

These can be done by calculating the extra-column band broadening at a flowrate which corresponds to the same Re number on the column. The preliminary results showed that this can lead to much smaller values of ECBB. The problem can be further reduced by not taking into account the tubing as the total volume of the tubing is so small that at the flowrates commonly employed in preparative chromatography the time it takes for the sample to travel from the injector to the detector would be several seconds. This time is far too short to generate any substantial extra-column band broadening.

The conclusion however remains that the extra-column band broadening values still cannot be subtracted from the total band broadening as the values are too large. This is under the assumption that the calculations of H_{extra} are correct.

A Reynolds number of 0.07 on the column corresponds to a flowrate of 250ml/min. In order to achieve the same laminar flow without the column the Reynolds number would have to be about Re=147 to give us the band-broadening we should use in our calculations. That would correspond to a maximum flowrate of about 10.45 ml/min in the tubing without the column. In fact it seems sensible that the data should be superimposed at a point where laminar flow is the same.

3.7.3 Approach 2

Summary.
Here the second approach to an interpretation of extra-column band broadening is presented. The data acquired from extra-column band broadening measurements are calculated in a different way from the first approach.

3.7.3.1 Calculations of band broadening

For the sake of brevity, the theory of the dynamics of flow with regard to Reynolds number in the tubing is not repeated here as it was covered above in Approach 1 on extra-column band broadening.
The assumption was made that the length of tubing through which the sample travels, excluding the column, is negligible. This is because it was calculated that it would take only several seconds for the sample to leave the system from the point of injection. The extent of this contribution is minimal (see the table of tubing dimensions in Approach 1).

3.7.3.1.1 Calculations of extra-column band broadening

The extra-column band broadening calculated in terms of height equivalent to a theoretical plate was calculated using following the equation:

$$H_{ECBB} = \frac{1}{N_{ECBB}} \qquad (3.7.1)$$

$$N_{ECBB} = 5.54\left(\frac{t_{rc}}{t_{w1/2}}\right)^2 \qquad (3.7.2)$$

where t_{rc} is the retention time of the sample on the column at that particular flowrate

$t_{w1/2}$ is the width at half height of a peak measured under the same conditions but without the column

3.7.3.1.2 Calculations of total band broadening

The data were then compared to the total band-broadening on the system with the Prochrom column present.

Total band broadening consists of column band broadening and extra-column band broadening. This is expressed as an equation:

$$H_{total} = \frac{1}{N_{total}} \qquad (3.7.3)$$

$$N_{total} = 5.54\left(\frac{t_{rc}}{t_{wc1/2}}\right)^2 \qquad (3.7.4)$$

where $t_{wc1/2}$ is a width of the peak at half height on the column.

3.7.3.1.3 Calculations of column-band broadening

Consequently, column band broadening can therefore be calculated as:

$$H_{COLUMN} = H_{TOTAL} - H_{ECBB} \qquad (3.7.5)$$

3.7.4 Results.

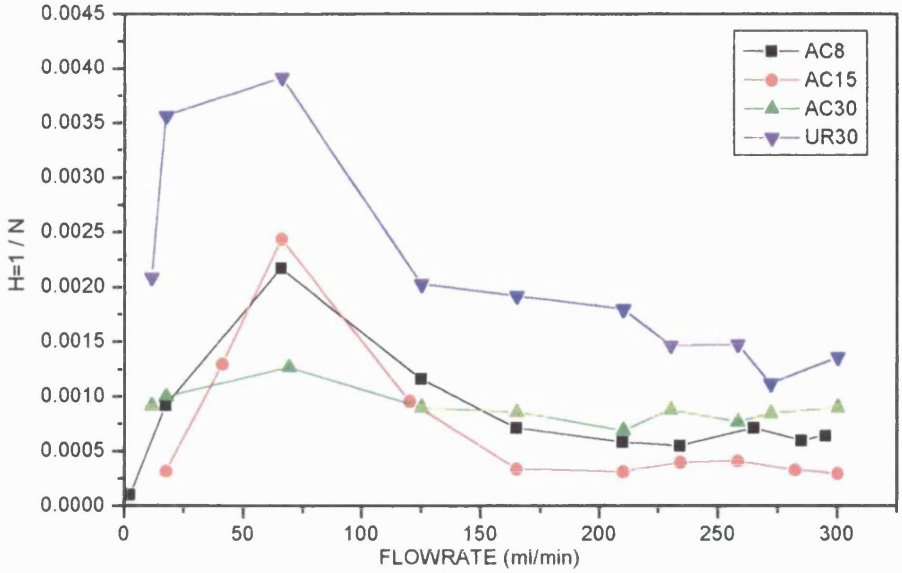


Figure 3.7-2 Extra-column band broadening on the Prochrom system.

Here the extra-column band broadening on the Prochrom system without the column is presented. The minimum band broadening is seen with the 15ml loop, particularly at greater flowrates. This is followed by the 8ml loop and 30 ml loop for acetone. Uracil shows the greatest extra-column band broadening of all. The profile is similar to the one for extra-column band broadening interpreted in the first approach. Although the calculations of H values are different the profile remains the same. The explanation of the graph's profile will therefore not be repeated here: only differences will be pointed out. Here the ECBB for acetone with the 15ml loop is lower than with the 8ml loop.

3.7.4.1.1 Total band broadening

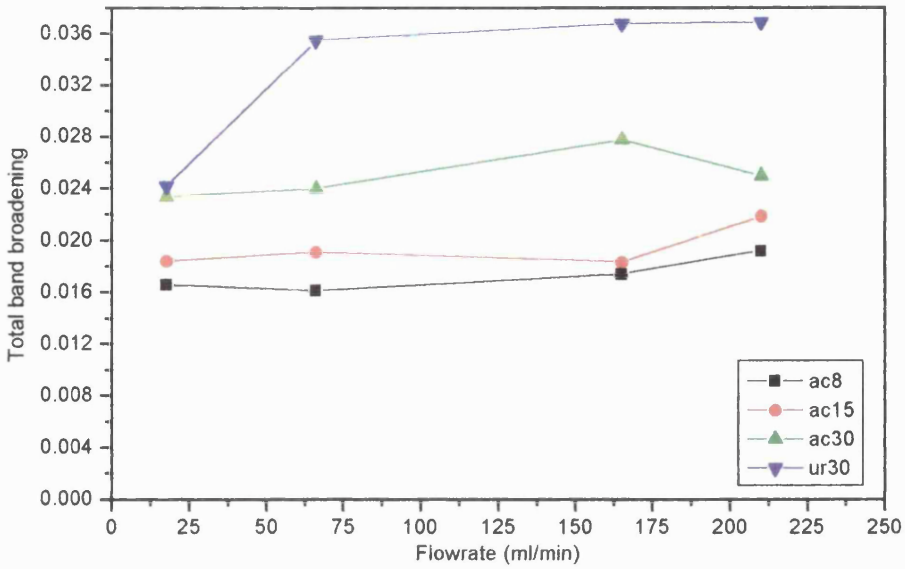


Figure 3.7-3 Total band broadening.

This figure shows the total band broadening (TBB). The data were collected for four different flowrates: 17.5, 66, 165 and 210ml/min.

In the profile of total band broadening against flowrate TBB increases with the increased volume of the sample injected on the column. Therefore band broadening of acetone increases in the order of 8, 15 and 30 ml. Uracil (30ml) gives the greatest total band broadening of all.

3.7.4.1.2 Column band broadening

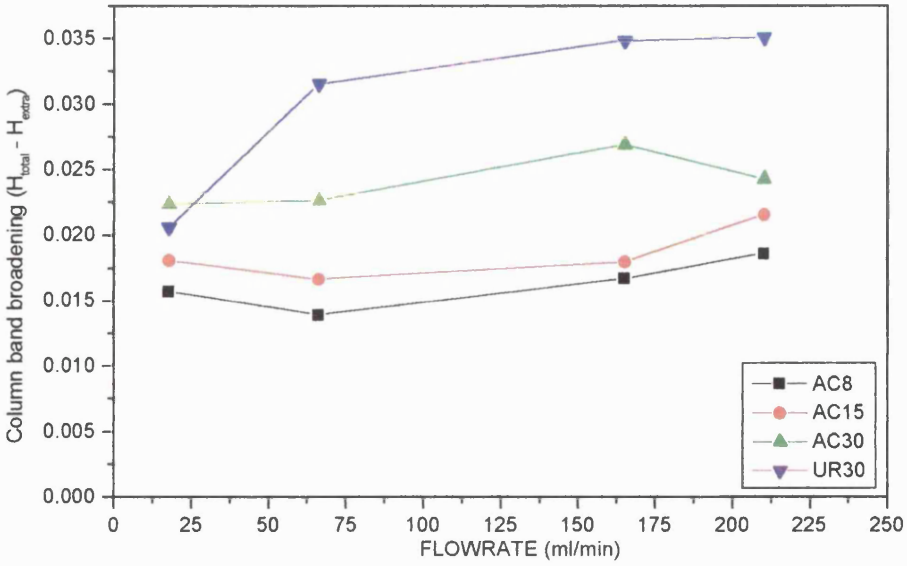


Figure 3.7-4 Column band broadening.

The graph of column band broadening (CBB) is almost identical to that of total band broadening. This is due to the fact that the extra-column band broadening values which were extracted from the total BB values are very small. Consequently the resulting differences are very small.
It can therefore be deduced that column band broadening is a major contributor to total band broadening. It is believed that the greatest column band broadening achieved with uracil is due to its lower retention time than acetone.

In conclusion three profiles were presented here: extra-column band broadening; total band broadening; and the difference between these two, i.e. column band broadening.
Whilst the profiles of TBB and CBB are very similar the profile of ECBB is different. This is because, as explained earlier, studies have revealed that extra-column band broadening encompasses both laminar and turbulent flow whereas total band broadening covers only laminar flow.

The extra-column band broadening values are minute in comparison to those of column band broadening. This means that the main contributor to band broadening is the column rather than the external tubing. When looking at the graph of extra-column band broadening, as mentioned earlier, the minimum values are achieved with a 15ml loop and acetone. The predictions are that this is probably due to the coil diameter of the 15 ml loop being slightly smaller than that of the 8ml loop. According to Tijssen (1978) more tightly coiled loops lead to an earlier onset of radial mixing and, as a consequence, an earlier reduction in band broadening.

3.7.5 Conclusions from both approaches

Two approaches were presented here for the interpretation of extra-column band broadening.
The first approach has its advantages and disadvantages. However, it leads to a conclusion that the extrapolation of extra-and total-band broadening cannot be directly related due to significantly large extra-column band broadening values. And this is even

after the assumption is made that the length of the tubing will not be taken into account. This makes it difficult to assess the column contribution. Although this was an interesting attempt it was fruitless and did not yield any solution.

The second approach appears much more satisfactory. The data can be directly superimposed and also match the reality better because the bulk of the total band broadening comes from the column.

It is believed that column band broadening also includes a contribution from frits. Although the design of current large scale chromatography columns and, particularly, their inlet and outlet ports minimise any dead space this cannot be eliminated entirely. Consequently it is believed that the design of the sample distribution at the top of the column is responsible to some extent - to a degree which is difficult to assess - for the column band broadening.

The second approach to the interpretation of ECBB can be applicable to large scale chromatography where extra-column band broadening is minimal. However, in the case of analytical chromatography, the length of the tubing has to be taken into account as it can contribute to a large extent to a successful separation. But band broadening on a small scale has already been tackled by other researchers and calculation methods are well established.

3.8 Fractionation diagrams as a chromatographic modelling tool

Summary.

In this chapter a new method is presented for interpreting chromatographic data using fractionation diagrams as a tool to determine the efficiency of a separation process.

Attempts were made to model the purification of crude erythromycin by means of fractionation diagrams. The experiments were carried out on a semi-preparative scale and the results then compared to large scale separations. The process is evaluated in terms of fractionation diagrams and purification factor (PF)/yield diagrams.

3.8.1 Introduction

The idea of a fractionation diagram as a modelling tool was derived from the work done by Richardson et. al. (1990) and Richardson (1987) to obtain pure protein by protein precipitation. The results were plotted in terms of fractionation diagrams which represented the purification of a product protein relative to total contaminated protein.

We believed that this technique could be applied to the purification of erythromycin by reversed-phase chromatography.

3.8.2 Materials and methods

Semi-preparative studies.

For the semi-preparative experiments the standard HPLC Beckman System Gold (Beckman Instruments, Inc., Fullerton, CA, USA) was used.

The column employed was one from a series of columns packed for the scale up studies with the dimensions 150 x 7.8mm (supplied by Jones Chromatography, Hengoed, Mid. Glamorgan, UK). The packing material was CG-300 35micron (PS-DVB) supplied by Toso Haas (Linton, Cambridgeshire, UK). The fractions collected from the semi-preparative runs were analysed off-line on the PLRP column (250 x 4.6mm) with 8 micron particles (Polymer Labs.; Church Stretton, Shropshire, UK). The flowrates used for the semi-preparative and analytical columns were 2.88ml/min and 1ml/min., respectively.

The sample of crude erythromycin was supplied by Abbott (Abbott Laboratories, Chicago, USA). The initial purity of the sample was 70%. The sample concentration was 40mg/ml. On the semi-preparative scale a volume of 0.5 ml of erythromycin (20mg) solution was injected onto the column.

Large scale studies.

For the large scale studies the Prochrom column (Champigneulles, France) was used packed with the same type of particles as the semi-preparative column, i.e. 35μm CG-300 polymer packing. On the large scale a sample of erythromycin with a concentration of 40mg/ml and a volume of 30ml (1200mg) was loaded onto the column.

3.8.3 Construction of fractionation diagrams

The fractionation diagrams utilised experimental data obtained from the semi-preparative and analytical separations.

The procedure was as follows:

- *Separation on semi-preparative column.*

The separation on a semi-preparative column was performed and fractions of the same time interval (15 sec) were collected.

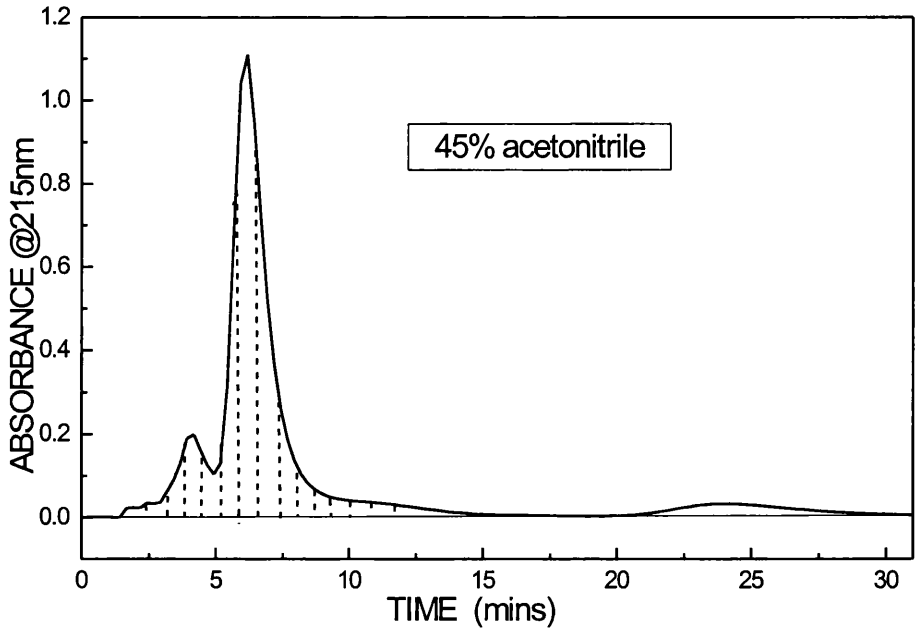


Figure 3.8-1 An example of a chromatographic trace of erythromycin on a semi-preparative column with 45% acetonitrile concentration.

- *Fraction analysis.*

The fractions were analysed on the analytical column.

- *Calculation of erythromycin A to impurities ratio.*

Peak elution profiles of the product component and neighbouring impurities were integrated to determine the percentage ratio of erythromycin A to impurities in each fraction.

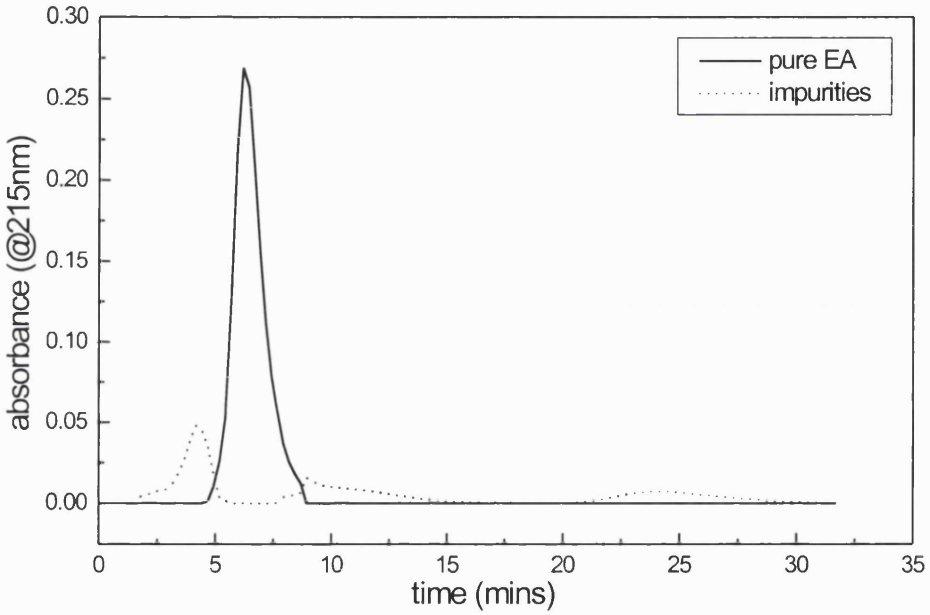


Figure 3.8-2 An example of a ratio of pure erythromycin A and its impurities after all the collected fractions from the first trace had been analysed.

- *Construction of fractionation diagrams from cumulative values.*

This data was then used to construct the fractionation diagram.

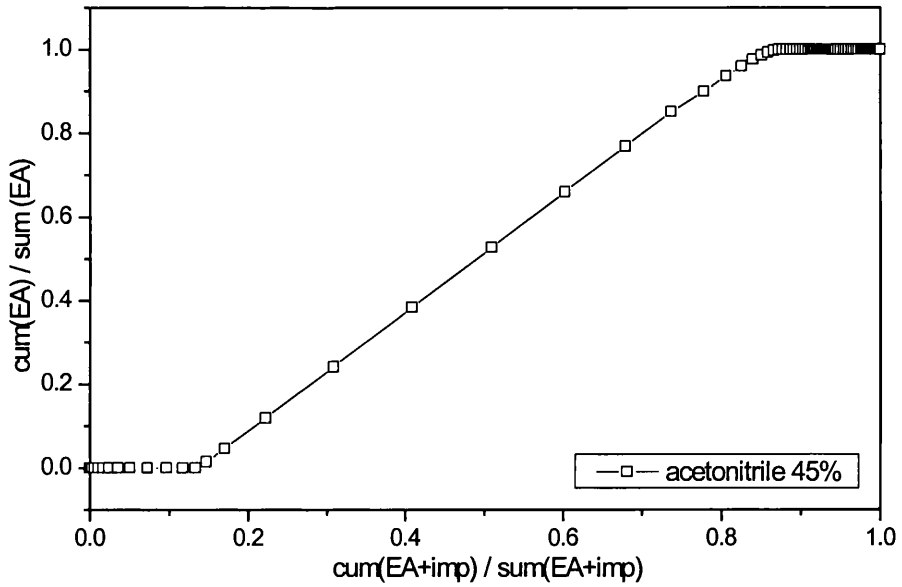


Figure 3.8-3: Fractionation diagrams from a trace run at 45% acetonitrile concentration.

- *Purification factor / yield diagram construction.*

By double-cut fractionation the purification factor and yield could be determined for each fraction and data plotted.

The resulting selection of purification factor/yield diagrams enables a choice to be made of the optimum conditions at which the sample should be run according to predetermined parameters.

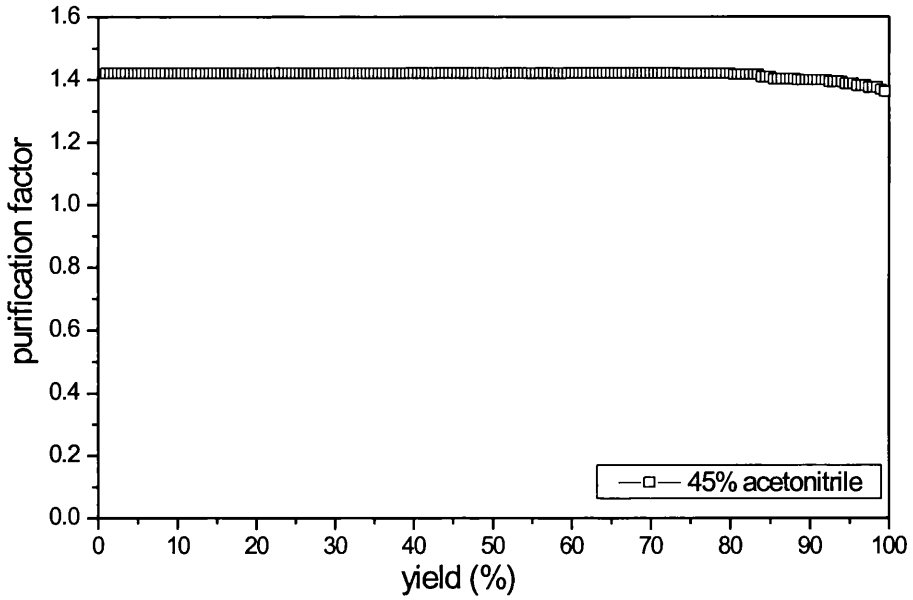


Figure 3.8-4: Purification factor/yield diagram at 45% acetonitrile concentration.

FRACTIONATION DIAGRAM - DETAILS.

The fractionation diagram curve has a sigmoidal shape. The slope of this curve corresponds to the purity of the fraction. The steeper the slope the purer the fraction. The horizontal lines correspond to the fractions containing impurities only.

The fractionation diagram reflects the change in the composition of each fraction as the sample compounds are eluted off the column. The terms on the x-axis and the y-axis are cumulative and divided by the total (maximum) value so that the maximum values of x and y achieved is 1.

The *fractionation diagram* is a plot with x- and y- axis from 0-1.

The x-axis: was calculated from the sum of the cumulative increments of erythromycin A and the impurities (as they were eluted off the column) divided by the total EA and impurities.

The y-axis: equals the sum of the cumulative increments of erythromycin A divided by the total erythromycin A.

An example of the calculations used for the construction of a fractionation diagram:

fraction number	EA	Impurities	cum(EA)	cum(imp)	cum(EA)/ tot(EA)	cum(EA+imp)/ total (EA+ imp)
1	2	3	2	3	2/20	(2+3)/(20+6)
2	3	2	5	5	5/20	(5+5)/(20+6)
3	4	1	9	6	9/20	(9+6)/(20+6)
4	5	0	14	6	14/20	(14+6)/(20+6)
5	6	0	20	6	20/20	(20+6)/(20+6)
Σ (total)	20	6	-			

Table 3.8-1 An example of calculations to construct a fractionation diagram.

CALCULATION OF PURIFICATION FACTOR AND YIELD.

Purification factor is defined as a ratio between the final and initial conditions and is an indicator of the efficiency of a process.

$$PF = \frac{OUTPUT}{INPUT} = \frac{SLOPE}{0.7} = \frac{\frac{EA}{EA + imp}}{0.7} \qquad (3.8.1)$$

Output corresponds to the slope of the fractionation diagram curve and input is the initial purity of the sample.

$$YIELD = \frac{EA}{\sum EA} \qquad (3.8.2)$$

The purification factor and yield are defined from double-cut fractionation. This means that two points on the fractionation diagram need to be selected to determine the purification factor and yield.
Therefore for a double-cut fractionation it follows that:

$$PF = \frac{Y_i - Y_j}{X_i - X_j} \qquad (3.8.3)$$

And yield is calculated:

$$YIELD = Y_i - Y_j \qquad (3.8.4)$$

where Y_i and Y_j are two different points on the fractionation curve whose values are read from the y-axis and X_i and X_j are those same points whose values are read from the x-axis.

For the separation of erythromycin A the purification factor was therefore calculated as follows:

$$PF = \frac{\frac{cum_i(EA)}{\Sigma EA}}{\frac{cum_i(EA+imp)}{\Sigma(EA+imp)}} - \frac{\frac{cum_j(EA)}{\Sigma EA}}{\frac{cum_j(EA+imp)}{\Sigma(EA+imp)}} \qquad (3.8.5)$$

where $cum_{i,j}(EA)$ are cumulative terms of pure erythromycin and ΣEA is the total amount of erythromycin A in the sample.

The term $cum_{i,j}(EA+imp)$ is a cumulative term of erythromycin A and impurities and Σ(EA+imp) is the total amount of erythromycin A and impurities present in the sample.

And yield:

$$YIELD = \frac{cum_i(EA)}{\Sigma EA} - \frac{cum_j(EA)}{\Sigma EA} \qquad (3.8.6)$$

3.8.4 Results

By changing the running conditions several PF/yield diagrams can be constructed and appropriate conditions chosen to achieve a desired yield and purification factor.

Here we have shown only a few examples to illustrate points such as the change in the concentration of acetonitrile, the impact of different buffer types, and the effect of temperature.

3.8.4.1 Change of acetonitrile concentration in the mobile phase

Changes in solvent concentration were used as a model example to show how the whole procedure leading to PF/yield diagram was carried out. The subsequent two examples show only the final PF/yield diagram.

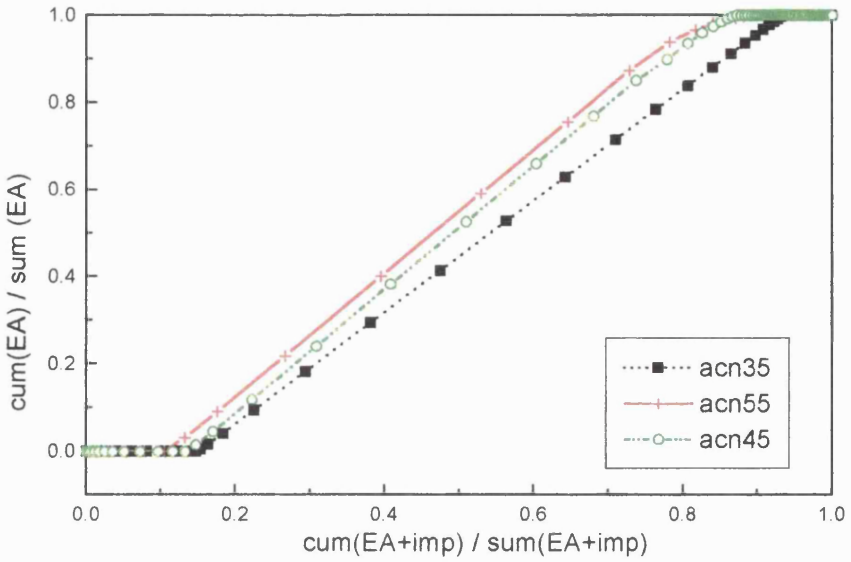


Figure 3.8-5 Fractionation diagrams from three different traces run at 35%, 45% and 55% acetonitrile concentration.

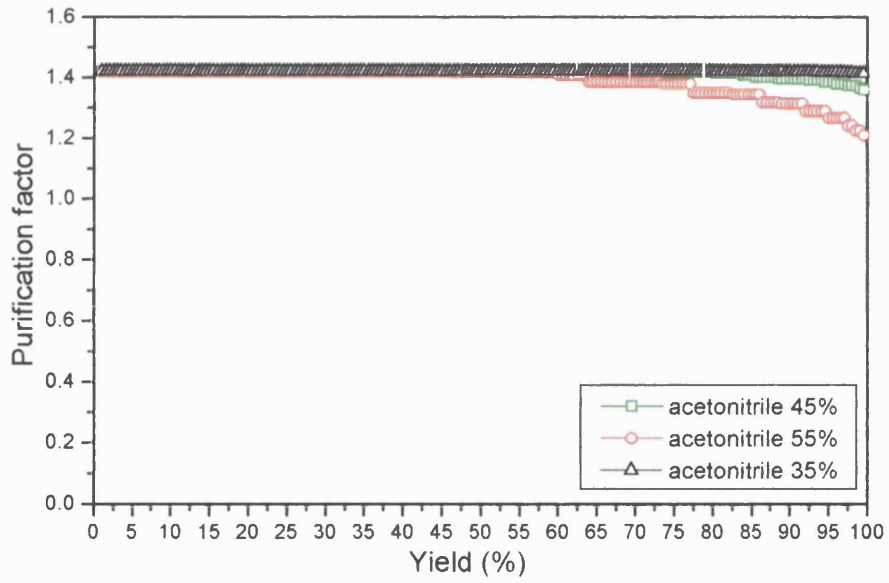


Figure 3.8-6 The final result of maximum purification factor/yield diagram.

3.8.4.2 Change in the buffer type used in mobile phase.

Other effects

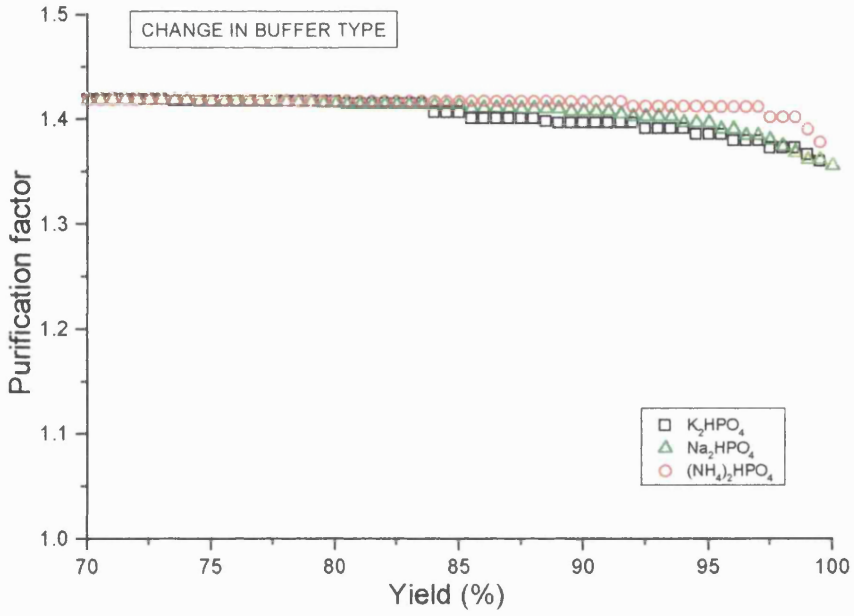


Figure 3.8-7 Purification factor/yield diagram of erythromycin separation employing three different buffers

The graph shows how the purification factor depends on the yield. Up to approximately 83% yield the purification factor remains virtually the same for all three buffers. The situation changes when a higher yield is required. The purification factor starts decreasing depending on the buffer used. It is obvious from the graph that the most favourable buffer would be ammonium mono-hydrogen phosphate which even at 95% yield can offer a purification factor value of approximately 1.41 whereas other buffers drop below the value of 1.39.

As large yields are favourable in purification one should aim for the highest possible yields combined with the highest possible purification factors. With the criteria set in this way ammonium phosphate buffer would be preferred for the purification of erythromycin.

3.8.4.3 Effect of temperature on purification factor/yield.

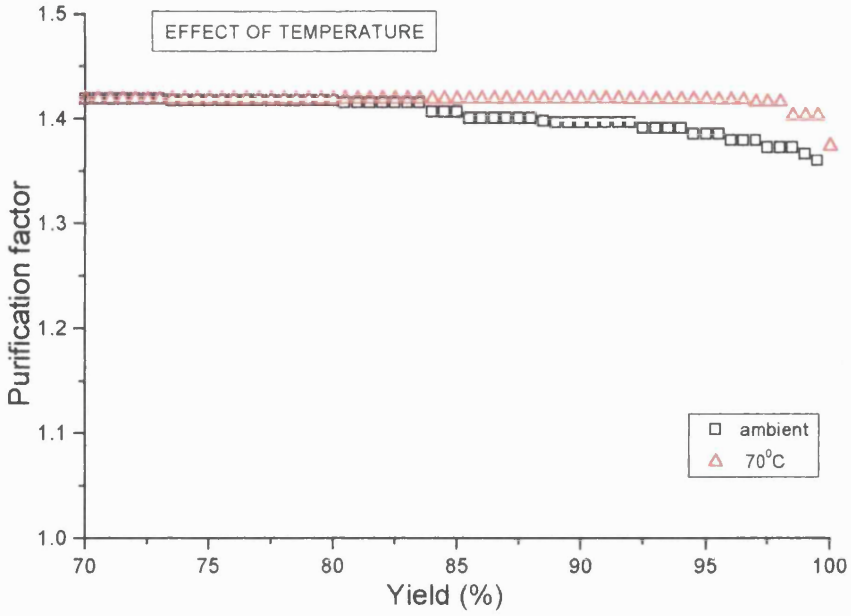


Figure 3.8-8 Effect of temperature on purification factor/yield diagram.

Temperature is an important factor in chromatography. Its effect however has to be weighed against cost. Heating the column can be affordable on an analytical scale but may be rather costly on a large scale particularly as the cross-section which has to be heated can be quite large. Also the greater amounts of solvents commonly employed in large scale chromatography can cause a considerable safety hazard if heated to a temperature of 70°C (particularly as the boiling point of acetonitrile is 81°C).

Nevertheless the effect of temperature was investigated here. The results show that heating the column can lead to an improvement in the process leading to higher purification factors at higher yields.

3.8.5 Large scale separation

When similar experiments were intended to be carried out on a large scale to verify the model, it was found that the profiles of erythromycin trace generated on the 150 x 7.8mm column and on the large scale column could not be superimposed.

3.8.6 Conclusion

The method proved not to be of value because the large scale Prochrom chromatography column gave a better separation than the semipreparative column. If the results are so fundamentally different comparisons between them and any extrapolation from them

become impossible. It is important to emphasise that this fact was not known to us initially as it has always been assumed that a separation on a large scale would be inferior to that on a small scale. Here, however, the results point out to the reverse finding.

The example shows that this technique of analysing the data cannot be used as it is impossible to perform extrapolation between the scales. This is because the efficiency of a large scale column is far better than that of a small scale one. It is believed that this is attributed to a "wall effect" which is virtually non-existent on a large scale but responsible for the deterioration of column performance on an analytical scale.

4. DISCUSSION

4.1 Mathematical modelling, PCA and fractionation diagrams.

Mathematical Modelling

The modelling of chromatographic processes can be done in several ways. One of the most frequently used modelling tools is mathematical modelling. However, although widely used, there are some problems associated with this tool.

Firstly, when used many assumptions have to be made. According to how accurately these assumptions are made the model can be closer to or further from reality. Secondly, whilst easy to carry out with one component, when several components are to be separated (as is usually the case) the profiles of a multi-component mixture under overloaded conditions are difficult to predict as the components are competing for the active sites on the stationary phase. Thirdly, the isotherms which are part and parcel of mathematical modelling are based on pure compounds. If one can separate the compound of interest for the construction of isotherms, there is little point in constructing the isotherm in the first place. Lastly, but probably most importantly, in order to embark on the route of mathematical modelling a thorough knowledge of computer programming is required because complicated mathematical equations need to be solved. If not equipped with this skill the targets will not be met.

Bearing all this in mind, from the practical point of view mathematical modelling was a non-starter for the current project.

PCA

Another way of predicting the performance of the column on a large scale is by means of Principle Component Analysis (PCA) (Pate 1998). PCA is a multivariate statistical technique for modelling the chromatographic process using matrix algebra. This technique can be applied to the modelling of non-linear chromatography in overload conditions.

The advantage of PCA over conventional mathematical modelling is that it requires no underlying assumptions about the mechanism of the chromatography, nor any data defining the isotherms or the mass transport of the individual compounds. PCA offers

considerable potential for the rapid and reliable modelling of chromatographic processes and can achieve greater accuracy than the traditional mathematical approaches.
This statistical method is believed to be very powerful as it can decrease substantially the number of experiments one has to perform in order to predict the profiles of chromatographic separations. However, the main drawback of this method that it is an inter-polative rather than extra-polative method. This means that some experiments still have to be performed on a large scale to predict profiles under overloaded conditions rather than predicting the large scale profiles from small scale experiments.

Fractionation Diagrams

The modelling method employed by us has never been applied to chromatography. It was previously developed for the precipitation of proteins (Richardson 1987) (Richardson et al. 1990). It therefore required some adjustments in calculations to construct fractionation diagrams. This method enabled us to model chromatographic process and examine how changes in running conditions can affect the separation from the point of view of the efficiency of the process. The efficiency of the process was defined as the ratio of output to input purity. The model was based on double cut fractionation. The final product of this modelling produced purification factor/yield profiles. These are important when one wants to produce erythromycin of a given purity and yield. These results are clearly indicated on the graphs (figures 3.8-6 & 3.8-7) where the acetonitrile concentration in the mobile phase was changed, or different buffers were used. Striking results were found in the studies with temperature changes (figure 3.8.8). The PF/yield profile points out the advantage of opting for higher temperature during separation. Performing erythromycin separations at higher temperatures enables higher yields to be obtained at no expense to the purification factor. From the results obtained by studying the impact of temperature on column efficiency (figure 3.2-14) it is also seen that increased temperature is a positive factor in chromatographic separation. Increased temperature increases the column efficiency. This is therefore certainly a factor which should be scrutinised further (see Main recommendations).
To sum up, the model was constructed based on fractionation diagrams which can help to determine the optimal conditions for the separation of erythromycin. The model was developed to facilitate the transition from small scale to large scale experiments bearing

in mind that large scale separations are usually inferior to analytical ones. It was however discovered that large scale separations were as good as small scale separations if not better. This fact made us realise that we cannot verify our model. Furthermore there is little point in modelling a system if large scale separations prove to be better and consequently lead to the acquisition of a purer compound.

Much work was put into the construction of fractionation diagrams particularly associated with the laborious analysis of numerous collected fractions. When discovered that the model could not be applied to a large scale (which was not until large scale separations were conducted) the focus of the thesis had to be redefined.

4.2 New direction.

The research interests were redirected to studies of large scale separations and their sensitivity to process variables. This is particularly important as analytical chromatography is now well understood but there is certainly room for further exploration and improvement in regard to large scale HPLC.

In order to do this a thorough understanding of HPLC technique in both its theoretical and practical aspects had to be mastered. In this project the basics of chromatography were practised on aromatic compounds. This part of the work is documented in the first chapter of the Results section where several aspects of chromatography were investigated on the standard compounds nitrobenzene, naphthalene, fluorene and fluoranthene used as training materials.

Once confidence was acquired the preliminary studies on erythromycin were conducted. The method of separating erythromycin was developed and the separation performance tested on columns of different internal diameter. This was part of the scale up studies. These results are presented in the chapters on the analytical and semi-preparative separation of erythromycin. Only then could we concentrate on work on a large scale which was our second objective.

The work on a large scale required that the column first be commissioned. To set up the column was a challenging task which required help from the electrical and mechanical workshop. There was a need for strict observance of the rules and regulations regarding safety (COSHH).

In the following section the reasons for the choice of the sample and the packing material are given. This is followed by the preparation of the Prochrom system prior to packing the column bed. The latter section therefore provides a written protocol for the successful packing of a large scale DAC (dynamic axial compression) column. References from the work of other researchers are also provided.

Erythromycin sample and CG-300 packing

In this project erythromycin was used as a model sample. Erythromycin was chosen for this project as it is a convenient mixture of related components which are not easily separated by any other method. It can provide a wealth of material to study and from which to derive lessons from a chromatographic point of view. Erythromycin is also widely used throughout our research centre for studies of upstream and downstream processing.

As for the chromatographic support, although either silica or polymer based stationary phases would be equally suitable to perform the task, we decided to give priority to a polymeric packing material rather than C_{18} silica for several reasons. Firstly, silica based reversed-phase materials have been in use for a long time - their properties are well known and consequently they have been employed extensively for the separation of many small molecules. Secondly, the use of silica is limited to a narrow range of pH - from 2 to 7. High pH leads to dissolution of silica whereas with acidic eluents ligand cleavage is a problem. This is however unfortunate for erythromycin where preferably a higher pH (close to or higher to its p_{KA} value of 8.9) should be used because low pH leads to erythromycin decomposition (Janecek et al. 1993). This is however a strong point for polymers as they are almost completely pH resistant.

These reasons led us to opt for polymeric stationary phases for our chromatographic separation of erythromycin.

The method was established on a small scale using PLRP packing material with 8 micron particles. As this particle size is not suitable for use on a large scale it had to be decided what particle size to use. The particle size commonly used in analytical chromatography (5-8 micron) is totally inconvenient for large scale chromatography. In preparative chromatography the efficiency (although important) might have to be compromised at the expense of the flow rate and pressure restrictions as well as

loadability (the amount of the sample which can be loaded onto the column). Colin (1987) derived a direct correlation between the size of the particles and loadability. The particle size is directly proportional to the loadability of the sample but at the expense of column efficiency (which decreases with an increase in particle size).

Apart from particle size, attempts were made to verify other aspects of chromatographic support such as particle shape (irregular or spherical) with XAD-4 and CG-161 particles; and different polymeric materials, polystyrene or methacrylate (CG-300 and CG-71). The results of these findings can be found in sections 3.3.1, 3.3.2 and 3.3.3. In the end polystyrene spherical particles were chosen in the form of CG-300 material in 35 micron size (to comply with the pressure restrictions and load limitations). This material was made available to us free courtesy of TOSO HAAS.

4.3 Prochrom column packing

Initially, packing the Prochrom column proved to be a challenging task for the inexperienced researcher but once the ropes were learned the column could be packed quite successfully without too much difficulty. The problems encountered during packing were associated with pre-treatment of the resin, the pressure applied, and general dexterity with the operation. It also proved crucial to clean the frits prior to packing and to clean the in-line filters every time the back pressure appeared to increase. Just as with other activities in life, preparation is everything. (The rule of the six "P's": proper planning and preparation prevent poor performance.)

Prior to packing, sufficient time should be devoted to the preparation of the resin and the system, including the column.

The resin should be prepared in advance as this task can take several days. The ideal technique with which to pack resin in preparative chromatography is slurry packing (Sarker and Guiochon 1995). Smaller packing can usually be packed dry but larger particles need to be slurry packed to ensure better consolidation.

Prior to use the slurry has to be cleared of any fines. At the same time the resin must be allowed to swell. This can be done as follows:

The slurry should be mixed with a large amount of solvent (ratio of slurry to solvent 1:5), poured into a large glass cylinder (approximately 2 litres) and left to settle for

several hours. If, after this, fine particles are found the solvent has to be poured out and replenished with fresh solvent. The whole mixture must then be resuspended and allowed to settle again. The procedure has to be repeated until one is confident that there are no fines present. After this the final concentration of the slurry needs to be adjusted to 80% (i.e. 80% resin and 20% solvent) and the whole mixture poured into a beaker in which the resin can be easily resuspended. Resuspending the slurry should only be done with a spatula, never with a magnetic stirrer because the latter causes breakage of the particles.

Solvent utilisation.

The different solvents employed for the packing of a preparative column can give different results. Sarker and Guiochon (1996) tested several solvents on alkyl bonded silica. Initially the column was tested with methanol and later with n-heptane. Although in both cases the columns were packed at the same pressure the resulting column beds were of a different length. The column packed with n-heptane had a shorter bed packed with a higher packing density, i.e. lower permeability. The authors concluded that heptane wetted the modified silica surface better than methanol and better dispersed the particles of chemically bonded alkyl silica. This was also confirmed by Poole and Poole (1993). On the other hand, the efficiency of the "n-heptane" column was worse than in the case of methanol. The best results in terms of efficiency were achieved in decreasing order with iso-propanol followed by acetone, methanol, acetonitrile and finally n-heptane (Sarker and Guiochon 1996).

These results are helpful but it is believed that the outcome of these solvent studies would be markedly different if polymeric support was used instead of silica. This is because of the swelling properties associated with the former type of packing.

Our packing experiments were carried out only with methanol. It was a convenient solvent to use as it is cheap, easy to handle, and less toxic than acetonitrile. All these features are important when dealing with large scale packing. The properties of methanol also enabled the resin to swell properly which cannot be achieved if, for example, it is mixed with a comparatively greater proportion of water. The maximum swellability for this type of particle is achieved with pure solvent. Methanol enabled us to pack the column satisfactorily. Initially concerns were expressed over the ideal

solvent which could enable the particles to float in the solvent prior to compression. This concern stemmed from the fact that, if the packing settles too fast before it is compressed, the compression will not yield a homogenous bed. However experience taught us that if one proceeds quickly from the point when the slurry is poured into the column and compression follows shortly afterwards, the homogeneity of the bed will not be compromised and the bed will be packed satisfactorily. This fact was also confirmed by Sarker and Guiochon (1995) and Koh and Guiochon (1998) This is in contrast to some previous works where it was suggested that the column should be packed slowly in increments.

Frits

The issue of frits was mentioned in the chapter on the Prochrom system. Clogged up frits generate high pressures and can distort the chromatographic trace (see also section 3.4.4.). The importance of clean frits therefore cannot be over emphasised. Frits allow uniform flow and are very sensitive to clogging. They should be cleaned regularly either by sonicator or chemically (Sarker and Guiochon 1995). The frit on the top flange can be sonicated separately or flushed with methanol. When the inlet frit is blocked the chemical treatment suggested by Sarker and Guiochon (1995) can be followed: the top flange and piston head are connected to an Erlenmeyer flask and that is connected to the vacuum pump. The frit is then flushed with water, methanol, ammonium hydrogenfluoride and nitric acid. The authors also reported that provided the frits are clean the column can be used for up to 200 hours. They also claim that repeated unpacking and re-packing of the column does not have any deleterious effect on the separation.

Chevron seals

The role of the Chevron seals which are sandwiched between the internal column wall and the packing material is to keep the resin in place. This can only be achieved when the nuts which are responsible for the proper functioning of Chevron seals are tightened snugly (i.e. finger tight). This will prevent any leakage of the packing on the side of the column wall. Overdoing this procedure, however, leads to the seals being pushed up by the pressure and damaged.

Column packing

The piston is lowered to a predetermined level which allows the entire volume of the resin slurry to be poured into the column. The tubing leading from the top of the column into the system should be disconnected and directed into an empty vessel. This is used for the surplus liquid expelled during the compression of the bed. The compressed air pressure which is related to piston pressure should be set to a value which does not exceed the maximum recommended packing pressure stipulated by the manufacturer of the resin.

Resuspended slurry is poured in, the column is promptly closed with a metallic clump, and the pressure is applied. Once the packing procedure is complete the tubing linking the column and the system should be reconnected. At this stage the packing material in the column should be allowed to consolidate for several hours by pumping mobile phase through it. The column is left under compression throughout the whole procedure as well as during column operation (principle of Dynamic Axial Compression) (Hostettmann et al. 1986) (Verzele et al. 1988).

Another author suggests a procedure for packing a large scale column with polymeric packing. It should be packed first at a pressure not exceeding the maximum pressure recommended by the manufacturer. Once this is accomplished the flow rate/pressure curve should be established. Then the column should be unpacked and repacked at 90% of the maximum pressure determined from the pressure/flow rate relationship. The running conditions should not exceed 80% of the pressure determined from the pressure/flow rate curve. Once the column is packed it should be tested initially with fast eluting samples to establish the quality of the packing procedure. If the tests are satisfactory the column should be subjected to stabilisation and settling. These are performed by pumping the mobile phase through the column at 70% of the packing flow rate for several hours (e.g. overnight). After this the column should once again be tested for consolidation of the packing.

Allowing the packing to settle for several hours after compression by pumping solvent through it proved to be important. Failure to allow the bed to consolidate can give a false impression that the column might not be well packed when all it needs is to be

flushed for a sufficient time with solvent. Only then can the true nature of the packing quality be revealed.

Packing pressure

In our experiments we used a packing pressure of approximately 25 bar. Reports of studies by Colin et al. (1998) and Colin and Briand (1998) on the Prochrom system revealed that one should preferably opt for a lower rather than a higher packing pressure to allow a wide range of flow rates to be used. The results of the company's study showed that the higher the pressure at which the column is packed the more limited is the choice of higher flow rates at which the column can be operated without exceeding the column's operating limits. The study also revealed that the efficiencies achieved with the column packed at comparatively lower pressures were greater than when the columns were packed at maximum pressure.

Packing the preparative column so that the desired bed length is obtained can be a tricky business. Ideally the length of the preparative column bed should be the same as that of the analytical column used for the scale up studies. Although the volume of the settled resin is known, it can be difficult to take a "compression factor" into account.

Therefore packing the column to achieve a required bed length can be a process of trial and error. For example, in our case we used approximately 600ml of the settled resin and the final volume we achieved after compression was about 510ml. Our aim was to achieve a bed length of about 15 cm. Instead, a bed length of 18 cm resulted which seemed to be near enough to our target. Obviously the bed length depends on several factors such as the compression pressure and the solvent used. If the final bed length is close to the value desired one can settle for this length. Alternatively, the column might need to be repacked.

Although Colin (1987) debates over an ideal length of column and specifies that the column should be between 10 and 20 cm long, we believe that the column should be as long or as short as is necessary for the separation. Secondly, the column length in large scale chromatography is dependent on, and dictated by, the length of the analytical column on which all the ground work was done in order to comply with the rules for scale up. Of course, a column with a bed longer than really needed results in significant expense on the part of the operator as packing material can be very costly.

Once the column has been packed satisfactorily its performance can be tested with several compounds. This is discussed in the following section dedicated to the testing of the sensitivity of large scale separations to process variables.

4.4 Key factors: h/v curves, wall effect and extra-column band broadening

h/v curves

A well packed column should have h (reduced plate height) values in the range of 2 to 4. In our case the h/v curves for acetone, uracil and erythromycin yielded values higher than 4 although acetone came very close to this value ($h_{ACETONE} = 4.6$).

Several factors contributed to this low value such as the concentration of the sample, the temperature of the column, and the flow rate. In the next section the reasons behind the following findings are explained.

Acetone, uracil and erythromycin

In our experiments we tested the Prochrom preparative column with acetone, uracil and erythromycin over a wide range of flow rates. The h/v curves were constructed in each case for three different injection volumes. For acetone the values of h throughout the whole range of flow rates varied from 4.6 to approximately 8. For uracil the values were higher (i.e. indicating lower efficiency) with h between 6.9 and 11.5. And finally for erythromycin the values of h were between 6 and 32. It can be seen the best (highest) efficiency was achieved with acetone, followed by uracil and finally erythromycin (see figure 3.5-1). The efficiency achieved is expected to be better with samples like acetone and uracil where little physicochemical interaction is taking place and these samples are little subjected to band broadening. The efficiency with acetone reached almost 6000 plates per metre. The efficiency with uracil was lower - about 4000 plates per metre - we believe owing to the extra-column band broadening to which very early eluted peaks are subjected.

Volume injected and sample concentration

These samples also show visible differences in efficiencies with the sample volume injected. Whilst with acetone every increase in volume injected led to a visible decrease in efficiency with uracil 8 ml and 15 ml injections led to very similar efficiencies. This is attributed to concentration differences where the concentration of acetone was

87.1mM as opposed to 0.25mM for uracil. Greater band spreading due to a greater concentration is consistent with the rules of Fick's law of diffusion where the rate of diffusion is directly proportional to the concentration.

This fact also explains that if a lower concentration of the sample (e.g. acetone) was used the minimum values of reduced plate height h at minimum reduced velocity (also known as particle Peclet number) ν=3 would be lower.

For erythromycin the lowest values of efficiency achieved ranged from 4000 plates per metre dropping down to 1500 plates per metre as the flow rate increased. The efficiency decreased steadily with flow rate. This is symptomatic of samples which interact with the stationary phase through adsorption. It appears that efficiency, in the case of erythromycin, is not affected by the sample volume injected. It is believed that this is due to adsorption-desorption kinetics and slow equilibration.

Reduced velocity and diffusion coefficient

To see this in perspective the h/ν curves for all three compounds were constructed. h/ν curves can help us determine the optimum condition of operating the column and also how well the column is packed. For example, although the operating flow rates for all three samples were the same, the h/ν curves can tell us how close we were working in relation to an ideal operating flow rate, i.e. ν=3, when the reduced flow rate value reaches its absolute minimum. This is clearly visible from the graph (figure 3.6.1). Whilst uracil and acetone are very close to this ideal flow rate this is not the case with erythromycin which has not quite reached this value. It is also obvious that a further increase in the flow rate when working with acetone and uracil will not compromise the efficiency to any significant extent and one can indeed use fairly high flow rates (pump capacity permitting) without worrying about efficiency. With erythromycin the lowest possible flow rates would be the best option as any increase in flow rate results in a substantial decrease in efficiency. The differences in the calculated reduced flow rates amongst acetone, uracil and erythromycin stem from the different values of diffusion coefficients which are relatively larger for acetone and uracil: $1.42\ 10^{-5}$ cm^2/sec and $1.48.10^{-5}$ cm^2/sec, respectively. For erythromycin the diffusion coefficient is $3.94.10^{-6}$ cm^2/sec.

Ideal flow rate

As for proximity to an ideal flow rate, with acetone and uracil reaching the point at roughly 15.5ml/min and 16.1 ml/min, respectively, their corresponding values of reduced plate height were 4.7 and 6.9, respectively. With erythromycin, by extending the line it is believed the value reached could be about the same as for uracil, i.e. about 7. This is quite good for a sample which strongly interacts with the stationary phase to achieve results the same as for a sample, such as uracil, which is hardly bound to the column.

Throughput as the amount of sample purified per time unit is one of the most important factors in process chromatography. As demands are made to produce large amounts of pure compounds in as short a time as possible the process needs to be efficient. This requirement applies also to the column efficiency which should be high if large amounts of sample are loaded onto the column. The use of efficient columns can save substantial amounts of time and money in a purification process. However, according to the h/ν curve, the highest efficiency is achieved at a very low flow rate. This suggests that researchers should work in the region of low velocities. To achieve both high throughput and high efficiency is not feasible. Therefore there will have to be a trade off between throughput and efficiency with a possible sacrifice in efficiency in order to achieve acceptably higher throughputs.

Reduced plate heights measured with erythromycin increase with an increase in flow rate. It also showed that although samples of different volumes were injected the volume did not seem to affect the efficiency very much. Calculation of the h/ν curves revealed that unlike acetone and uracil, which have large diffusion coefficients, erythromycin has a small diffusion coefficient reflecting slower diffusion. The situation is further compounded by the strong interaction of erythromycin with the stationary phase. The h/ν curve is an ideal way of determining the working conditions and the solute's interaction with the stationary phase.

This, however, reinforces the fact that the evaluation of a column needs to be performed on the sample for which the column is intended. It also emphasises the importance of the h/ν curve as a tool for evaluating the column and for selecting the right conditions at which to operate.

Temperature

Another factor which has an impact on the efficiency and thus the minimum h value is temperature. Although controversial results have been published regarding this issue - temperature either had a beneficial effect (Tsuji and Goetz 1978) (Kibwage et al. (1985) or a negative effect (Poppe and Kraak 1983) (Nilsson et al. 1987) - it is believed that the effect of temperature should generally be positive.

This view stems from the fact that increased temperature leads to a decrease in the viscosity of the solvent which directly affects diffusion (Poole and Schuette 1984).

Secondly, the theoretical model of van Deemter plot (H/u curve) at different temperatures constructed by Warren and Bidlingmeyer (1988) reveals that the optimal linear velocity is shifted to higher values with increased temperature and the slope of the right hand side of the h/ν curve (C term) decreases with temperature. In other words the values of H improve with temperature for higher velocities. Most researchers operate at higher rather than lower velocities and therefore it is believed that a higher temperature should help to improve column efficiency.

The negative effect of temperature on H is attributed to radial thermal gradients across the column when the eluent temperature is different from the temperature of the column wall (Warren and Bidlingmeyer 1988). Other researchers claim that axial thermal gradients can also develop due to resistive heating where the inlet and outlet of the column have different temperatures (Halasz et al. 1975). Our research results with erythromycin on an analytical scale show an increase in the column efficiency (see figure 3.19) with an increase in temperature, i.e. a positive effect of temperature. At the same time the retention time increases with temperature. From a production point of view, the costs of heating a large scale column will need to be considered.

Based on the theoretical van Deemter plot, it is believed that if the h/u curves were to be constructed for this type of separation at different temperatures the general trend of the curves would comply with the model derived by Warren and Bidlingmeyer (1988). However, this claim needs to be supported by experimental results (see section on future work).

In conclusion, the current h/ν curves generated from the data obtained on the Prochrom column would, in the case of erythromycin, lead to lower values of h if the separations were conducted at elevated temperatures. This would consequently lead to a lower

minimal "h" value at an ideal reduced flow rate (ν=3) which might fall within the range of h from 2 to 4.

Temperature control is a particularly crucial factor in a separation as it can reveal the driving force behind a particular separation. It appears that a different separation mechanism is responsible for the separation of erythromycin on modified silica and on polymers.

Column temperature and silica-based stationary phases

The effect of temperature on the separation of erythromycin on silica based phases was reported by researchers such as Cachet et al. (1987). It was found that an increase in temperature on silica leads to a decrease in the retention time of erythromycin A. Several researchers studied the phenomenon and revealed that the impact of elevated temperature on the separations on silica generally resulted in a decreased retention time of a compound of interest. This was attributed to enthalpy which was the driving force (Antia and Horvath (1988), Grushka et al. (1982), Morel and Serpinet (1980), Morel and Serpinet (1982), Cole and Dorsey (1992)).

Temperature and polymeric stationary phases

The situation is different when polymeric stationary phases are used. Our research results for erythromycin on polymeric stationary phases exhibited the opposite effect, i.e. with increased temperature the erythromycin retention time increased (see figure 3-18). This is consistent with the results of Kibwage et al. (1985) who conducted similar experiments on erythromycin. Addo-Yobo et al. (1988) found the same correlation between elevated temperature and the retention time of compounds on polymeric stationary phases. These researchers studied the heat of adsorption of cephalosporin-C on XAD-2 resin. The information on the heat of adsorption assists in predicting how the retention of the adsorbate might vary with temperature and whether entropy or enthalpy governs the separation. Their results conclude that the driving force for this type of separation on a polymeric stationary phase is entropy.

To sum up, the driving forces of the erythromycin separation at elevated temperature appear to be dependent on the type of stationary support used: ODS silica or polymer. With a silica bonded stationary phase the separation is driven by enthalpy and results in

decreased retention time with increased temperature. The driving force behind the separation on a polymeric support is entropy resulting in increased retention time with increased temperature.

h/ν curves and ideal flow rate

h/ν curves are a great help when determining the most suitable flow rate to be used with regard to column efficiency. Their shapes reveal the more and less favourable regions of flow rate in which to operate.

Obviously the general shape of h/ν curves has its minimum h value which corresponds to a minimum reduced velocity of 3. The minimum h value corresponds to the maximum column efficiency. The h/ν curve for erythromycin revealed that minimum h would be approximately 7. The intercept with the y axis at $\nu=3$ would yield the value of $h = 7$. If one operated in this region one would be working at the highest possible efficiency for this separation, i.e. essentially operating at plug flow. If one opts for even lower flow rates the reduced plate height would increase sharply. This is associated with the B term in Van Deemter plot which accounts for axial diffusion and column tortuosity. This term rapidly increases with the decrease in flow rate. This region is not normally operated in. If one decides to operate at greater flow rates the reduced plate height increases again but less sharply than in the case of very low flow rates. The drop in efficiency here is associated with non-equilibrium effects, such as mass transfer resistance which is responsible for the slow mass transfer kinetics. In the case of erythromycin the slow chemistry is responsible for the decrease in efficiency.

From this description it is clearly seen that the construction of h/ν curves in the early stages of analytical separation is crucial and would aid in deciding at which flow rate to operate. As the flow rate is than scaled up proportionally to the column volume (the same linear velocity) one can be certain of working in the same region of the h/ν curve across the scales. However, since on a preparative scale the flow rates utilised are usually higher, a compromise has to be made between column efficiency and flow rate.

Reynolds number and column efficiency

If one wants to decrease further the minimum value of h another option of interpreting the data can shed more light on how to improve the efficiency of the column. This

option is by relating them to Reynolds number (see also section 2.2.6.2). This can reveal whether one is working in the laminar, turbulent or transition flow region to obtain an even more complete picture.

Striking results have been found when data generated on the Prochrom column were related to Reynolds number.

From the point of view of band broadening in chromatography, laminar flow is unfavourable. Turbulent flow leads to a decrease in band broadening which is why there is a desire to operate in the turbulent region.

When working on an analytical scale, because of the low flow rates (1-10ml/min) and small column internal diameters (4.6mm ID), one expects to be working in the laminar region. When working at higher flow rates (17.5 to 250ml/min) on a larger scale one would expect to encounter Reynolds numbers in the transition or turbulent regions. However, this has proved not to be the case. Although a higher flow rate range was employed the Reynolds numbers calculated for this larger column all fell within the laminar flow range. In order to reach higher Reynolds numbers the flow rates would have to increase substantially. Alternatively, the particle size would have to be increased to make the flow more tortuous thus contributing to an increase in turbulence. Another way of increasing the Reynolds number is by decreasing the viscosity of the solvent. This could be done by increasing the temperature of the column. If that was possible the minimum h value could be shifted towards lower values (h=2-4).

Wall effect

Another effect which explains such good performance by the preparative column is the wall effect. Golay (1961) first described the concept of the wall effect and later Knox and Parcher (1969) coined the term "infinite diameter column" (see section 2.4). The wall effect is associated with analytical columns where it causes a decrease in column efficiency partly because of their narrow internal diameter. Knox and Parcher (1969) introduced the concept of an infinite diameter column where the sample never reaches the column wall between the point of injection and the point of leaving the column. This avoids the deleterious effects associated with a thin layer near the wall.

This phenomenon is not applicable to a preparative column because the column walls are wide apart. This is "good news" for large scale chromatography because the

efficiencies are not affected in the same way as on a small scale. This also means that the efficiencies of preparative columns can be on a par with those of analytical columns.

Column efficiency and scale

Comparisons between the scales with nitrobenzene and erythromycin reflect precisely that (see section 3.3.3.3). Although the number of theoretical plates achieved with nitrobenzene on the Prochrom column was slightly lower (3550p/m) in comparison to the results on smaller diameter columns (up to 4100p/m), the experiments with erythromycin showed a close match.

The results show the average value of N to be 1140 plates/metre. Although Colin et al. (1987) claim that the efficiencies of large scale columns have N values about one third of the N values on an analytical scale we believe that, provided the column is well packed, the efficiencies of preparative columns can rival those of analytical ones. The conclusion therefore is that column efficiency essentially does not change with scale.

Analytical scale and Extra-column band broadening

Research studies of the extra-column band broadening phenomenon were attempted by several workers, e.g. by Scott and Kucera (1971), Martin et al. (1975), and Knox and Gilbert (1979). However, their research was geared to ECBB on a small scale. They developed equations to calculate ECBB in laminar flow. We found that operating on an analytical scale means working in laminar flow (low Reynolds numbers). This was consistent with the mathematical predictions of Golay (1958) who derived an equation to calculate extra-column band broadening on an analytical system. The equation revealed that ECBB is directly proportional to the radius of the tube, its length and the flow rate used, and inversely proportional to the diffusion coefficient. This means that any increase in flow rate would always lead to an increase in ECBB.

The onset of turbulent flow introduces radial mixing and consequently reduces ECBB. Turbulent flow is therefore a positive phenomenon which by reducing extra-column band broadening decreases total band broadening and leads to greater column efficiencies.

Some researchers therefore looked into ways of physically altering the tubing to trigger the onset of turbulent flow and thus decrease extra-column band broadening.

Tijssen (1978) found that geometrical deformation of the tube - making it into a tight coil - can reduce ECBB. This deformation introduces secondary flow or radial convection which disturbs laminar flow. This is exacerbated by a further increase in flow rate. The final profile of ECBB against flow rate is that of a parabola. The point of maximum ECBB (the inflexion point) is where laminar flow starts to change into turbulent flow. Tijssen therefore correctly identified that the dominant factors for the calculation of ECBB in laminar flow (i.e. flow rate and tube diameter) are not dominant in turbulent flow. At high velocities (turbulent flow) band broadening is mainly affected by a dispersion coefficient and it is indirectly proportional to the *coil aspect ratio*. This is the ratio of the diameter of the tube to the diameter of the coil.

Consequently two separate equations were derived to calculate extra-column band broadening , one for laminar and one for turbulent flow.

The dispersion in geometrically deformed tubes (squeezed, twisted and coiled) was also extensively studied by Hofmann and Halasz (1979, 1980) and Halasz (1979). According to the authors mass transfer in a straight tube with a small internal diameter can be increased by roughening the internal wall of the tube thus encouraging the turbulent flow to start earlier than would otherwise be possible.

The effect of secondary flow resulting from the use of serpentine shaped tubes was achieved by Katz and Scott (1983). The reduction of ECBB in these zig-zag shaped tubes is substantial. The reduction derives from the principle that at each serpentine bend the direction of flow reverses. This increases secondary flow which leads to radial convection. The maximum point on the parabola occurs at a much lower flow rate than is the case with coiled tubing. This means that the start of the ECBB reduction process is pushed towards the lower flow rates. This makes serpentine tubing better than coiled tubing in terms of the reduction in extra-column band broadening.

The general conclusion from these studies therefore is that extra-column band broadening which plays an important part in analytical chromatography can be substantially reduced by "deforming" the tubing in various ways.

<u>Large scale chromatography and extra-column band broadening</u>

However, when it comes to information on extra-column band broadening on a large scale very little is available. This made us decide to research this field. Our extra-

column band broadening (ECBB) studies were conducted by measuring the band widths of the peak without the column in place (by-passing the column).
Two approaches were presented.

Approach 1

The measurements for the first approach were similar to that for calculating the height equivalent to a theoretical plate, i.e. H=L/N where L was the length of the tubing through which the sample passed.
The profile of the ECBB measured against flow rate yielded a parabola reaching its maximum at approximately 75ml/min (63cm/s) which corresponds to a Reynolds number of approximately 1295. Extra-column band broadening measured over a wide range of flow rates on the large scale Prochrom chromatography system achieved a parabolic profile with a maximum corresponding to a point where laminar flow reaches its maximum. This is also the point at which the transition region starts, i.e. the region in which a laminar flow is "converting" gradually into a turbulent flow. A fully turbulent flow is developed when Re reaches a value of 4000 (for equation see section 2.2.6.1.). Here this is indicated by the levelling of the curve.
At low flow rates, small values of extra column band broadening are due to plug flow. The decrease in ECBB at high flow rates is due to turbulent flow as explained earlier.
This clearly indicates the system operating conditions which should be used to avoid excessive band broadening. In this case the highest possible band broadening was yielded at about Re=1295 which corresponded to a flow rate of approximately 75ml/min. This is a clear "no-go" area. Before and after this point the ECBB values drop. This suggests that one should work at higher flow rates (225ml/min and Re~4000) to avoid high degrees of ECBB. The results of our research also show the effect of increased injection volume on ECBB. As reflected on the graph (figure 3.63), the greater the injected volume the greater is the extent of ECBB. When comparing the ECBB measured with acetone against that of uracil, the ECBB of acetone was lower than that of uracil when injected with the same loop volume. This would explain why, when referring to the efficiency graph (figure 3.57), uracil samples have lower efficiencies than acetone.

When looking at Reynolds numbers across the range of flow rates it seems that the values do not correspond to the "classic" values for laminar flow (Re=2100). In the graph (figure 3.63) the start of the turbulent flow appeared much earlier than anticipated. It is believed that this could be due to temperature changes or the coiled loops which were used for the experiments. Coiled tubing speeds up the point at which turbulent flow begins, as observed by Tijssen (1978).

All these results are perfectly valid and credible until one attempts to superimpose an ECBB plot on the data obtained from the measurements made through the column.

This problem also stems from the fact that flow patterns with regard to Reynolds number are different when measurements are made on the column and without the column. What this means is that measurements made with the column encompassed the laminar region only. Measurements carried out without the column cover both laminar and turbulent flow. In the light of this it is difficult to superimpose the data.

If one attempts to apply the same calculations for total band broadening (in order to arrive eventually at column band broadening) one finds that that is not possible as ECBB gives much greater values than total band broadening.

Approach 2

To avoid this problem we employed the second approach where the calculations enabled us to compare extra-column band broadening with column band broadening (see section 3.7.2.). The outcome of these results is that ECBB is very small in comparison to column band broadening. This basically means that when attempting to eliminate band broadening one should predominantly concentrate on the column although the contribution of other parts of the system which carry the sample should not be underestimated. This is related particularly to improvements in the column inlet to enable an even distribution of the sample. Although progress has been made in this field the real contribution which this factor makes to band broadening is difficult to estimate.

Aim	*Achievements*	*Sections/Figures*
To commission the Prochrom system and troubleshoot the analytical and Prochrom systems	The Prochrom column was successfully commissioned. However, the efficiency of the large scale column was the same as that of the analytical column. This made the verification of the fractionation diagrams as a modelling tool on a large scale not only difficult but impossible. Troubleshooting remains a crucial part of successful separation. It identified the mistakes and problems which can be encountered on both scales. The critical steps are the elimination of fines from the packing slurry and the proper preparation of the large column prior to packing.	Section:3.4 figures 3.4-1 to 3.4-8 see also: section: 3.8 figures: 3.83-3.88 Analytical and Prochrom troubleshooting sections: 3.1.4.3; 3.4.7.3.1 to 3.4.7.3.3 & 4.1
To determine the effect of mobile phase variations on mobile phase separation	**Factors examined:** buffer concentration (its increase leads to a decrease in the retention time of erythromycin and an increase in column efficiency) buffer pH (its increase leads to an increase in column efficiency and an increase in retention time until it reaches a plateau)	section 3.2.4.1 section 3.2.4.2

Aim	*Achievements*	*Figures/sections*
To determine the effect of mobile phase variations on mobile phase separation (continued)	the use of different buffers (ammonium buffer gives the best efficiency when compared to sodium or potassium buffers)	section 3.2.4.3
	temperature (elevated values leads to an increase in column efficiency and the retention time of erythromycin when tested on polymeric stationary phases due to entropy being a driving force; in fractionation diagram studies elevated temperature led to a higher purification factor)	section 3.2.4.4 & section 4.4
	sample concentration	section 3.2.4.5
	flowrate (from the H/u curve the efficiency decreases rapidly with flowrate)	section 3.2.4.6
	acetonitrile concentration (a higher concentration of acetonitrile gives higher efficiency)	section 3.2.4.7
To determine the effect of overload conditions and scale on the separation	The increase in load decreases the column efficiency.	section 3.3.3.2
	However, the results of comparisons of different scales reveal that an increase in scale does not lead to a decrease in efficiency - the efficiency remains the same. This is due to the "wall effect" which is absent on the large scale.	section 3.3.4 & section 4.4 section 2.4 (theory)

Aim	*Achievements*	*Sections/figures*
To establish extra-column band broadening on the Prochrom system	Extra-column band broadening (has a profile of a parabola and depends on the Reynolds number in the tubing between the injection loop and the column. For flow in the pipes the Reynolds number ranges from 60 to 6000. The minimum band broadening is found either in the region of very low velocities due to a plug flow or very large velocities occurring in the turbulent region)	section 3.7.2 &section 3.7.2
To determine the sensitivity of process variables	Sample solubility (should be as high as possible to allow the highest possible throughput; low sample solubility is a great limitation in large scale production; maximum erythromycin solubility was 50g/l in methanol and buffer, a higher concentration led to sample precipitation)	section 3.3.2.2.2
	Sample volume injection (large volume injections do not necessarily lead to a decrease in column efficiency provided the sample concentration is low; large volume injections with samples of high concentration lead to a drastic decrease in column efficiency; efficiency with samples more attracted to the stationary phase is less affected by the volume injected)	section 3.5.7 figure 3.5-1

Aim	*Achievements*	*Sections/figures*
To determine the sensitivity of process variables (continued)	Mobile phase velocity (the minimum plate height for acetone and uracil is reached at 15.5ml/min and 16.1ml/min, respectively. For erythromycin the value was established by extrapolation to be 4.3ml/min. The efficiency is hardly affected by velocity unlike with erythromycin where efficiency decreases rapidly with increased velocity)	section 3.6 (figures 3.6-1 to 3.6-4)
	Particle size distribution (should be the lowest possible but greater distribution on a large scale is unavoidable due to large particles being used, d_{p90}/d_{p10} on CG-300 was 4.5)	section 3.4.6.2
	Sample diffusion coefficient (erythromycin with a comparatively low diffusion coefficient leads to slow diffusion and consequently a large particle Peclet number unlike with acetone and uracil where the results are reversed)	section 3.6.3 & section 4.4
	Packing pressure (it is believed that lower packing pressures can lead to higher column efficiencies)	section 3.4.4.2 and section 6

6. FUTURE WORK AND MAIN RECOMMENDATIONS

1. h/v curves

A) Run the separation of erythromycin at different flow rates and plot the h/v curves for different temperatures. Do this on ODS silica stationary phase and PLRP stationary phase.

B) Determine a relative ratio of entropy to enthalpy for both cases.
For this pure samples are needed and the method used can be taken from the paper by Addo-Yobo et al. (1988) for the determination of heat of adsorption.

2. Measure the isotherm for erythromycin and determine the capacity of the column.

A) To do so isolate erythromycin A from a crude sample and use one of the methods to determine isotherms described by Guiochon et al.(1994)

B) Determine the dynamic capacity of the CG-300 packing (see Cox 1995)

3. Pack the Prochrom column at lower pressures than packed originally (i.e. 25 bar) and measure the efficiency of the column at different flow rates. This set of experiments is designed to verify the theory suggested by the Prochrom company that large columns packed at lower flow rates could give greater efficiency and allow for higher flow rates.

4. The preparation of pure impurities by separation on the Prochrom column.

4. To facilitate the operation of the Prochrom column full automation of the operation is suggested. This would be of particular help when injecting the sample and collecting the fraction at which times it is also essential to be consulting the PC screen.

7. REFERENCES

References.

Addo-Yobo, F.Y.A.A., Slater, N.K.H. and Kenney, C.N.; The Chem. Eng. J. 39(1988)B9

Bidlingmeyer, B.A., Practical HPLC methodology and applications, John Wiley & Sons, Inc., New York - Chichester - Brisbane - Toronto - Singapore, 1992

Bristow, P.A. and Knox, J.H.;Chromatographia 10 (1977) 279

Brown, P.R.; Anal. Chemistry 62(1990)995A

Cachet, Th., Kibwage, I.O., Roets, E., Hoogmartens, J. and Vanderhaeghe, H.; J.Chromatogr., 409(1987) 91

Cachet, Th.,. De Turck, K., Roets, E. and Hoogmartens, J.; J. Pharm. and Biomed. Anal.; 9(1991) 547

Camin, D.L. and Raymond, A.J.; J. Chromatogr. Sci. 11 (1973) 625

Caswell,F.; Success in statistics, John Murray (publishers) Ltd, London, UK 1982

Colin, H. and Briand, V.; Report on the packing of Amberchrom CG-300 10-20μm material in a DAC column (Prochrom LC.50). (Company report) 1998

Colin, H., Briand, V. and Martin; K.; Report on the packing of the Pharmacia source RPC 15 μm material in a DAC column (Prochrom LC.50). (Company report) 1998

Colin, H., Diez-Masa, J.C. and Guiochon, G.; J. Chromatogr. 167 (1978) 41

Colin, H.; Sep. Sci. Technol.; 22(1987) 1851

Colin, H.; Martin, M. and Guiochon, G.; J. Chromatogr. 185 (1979) 79

Consden, R., Gordon, A. and Martin, A.J.P.; Biochem. J., 38 (1944) 224

Coulson, J.M. and Richardson, J.F. Chemical Engineering (vol 2) 3rd edition, Unit Operations; Pergamon Press, Oxford, 1978

Cox, G.B. in Process scale Liquid Chromatography, Subramanian, G. (ed.); WCH, Weinheim, 1991, Federal republic of Germany (p. 64)

Craig, L.C.; J. Biol. Chem., 155 (1944) 519

Cretier, G. and Rocca, J.L.; Sep. Sci. Technol., 22 (1987) 1981

Croteau, d., Valee, F., Bergeron, M.G. and Le Bel, M.; J. Chromatogr., 419(1987)205

Dale, M.; The Extra Pharmacopeia (30th edition)edited by J.F. Reynolds, London, The Pharmaceutical Press, 1993

Dolan, J.W. and Snyder, L.R.; Troubleshooting LC systems, Humana Press, Totowa, New Jersey, 1989

Done, J.N.; J. Chromatogr.,125 (1976) 43

Eble, J.E., Grob, R.L., Antle, P.E. and Snyder, L.R. J.Chromatogr. 405 (1987) 1

Engelhardt, H., High Performance Liquid Chromatography, Springer-Verlag, Berlin, Heidelberg, New York 1979

Ernst, P.; Aust. J. Biotechnol., 1 (1987) 22

Florey, M.E.; The clinical applications of antibiotics, Vol IV. Erythromycin and other antibiotics, Oxford University Press, 1960

Freitag, R., Frey, D. and Horvath, C.; J. Chromatogr. 686(1994)165

Giddings, J.C.; Dynamics of chromatography, Part I, Principles and Theory.; Marcel Dekker, New York, USA, 1965

Glueckauf, E.; Discuss Faraday Soc. 7 (1949) 12

Godbille, E. and Devaux, P.; J.Chromatogr., 122(1976)317

Golay, M.J.E. in Gas Chromatography (eds.; H.J.Noebels, R.F.Walls and N.Brenner), Academic Press, London-New York, 1961

Golay, M.J.E.; in "Gas Chromatography 1958" (ed. D.M.Desty), Butterworths, London, (1958) p.36

Gotsick, J.T. and Schmidt, D.E. Jr.; J. Chromatogr., 590(1992) 77

Guiochon, G., Shirazi, S.G. and Katti, A.M.; Fundamentals of preparative and non-linear chromatography, Academic Press, London, UK, 1994

Gwozdz, E., Holska, W. and Kulinska, A.; Chemia Analyticzna, 36 (1991) 215

Halasz, I., Endele, R. and Asshauer, J.; J. Chromatogr., 112 (1975) 37

Halasz, I.; J.Chromatogr., 173 (1979) 229

Halasz,I. and Sebastian,I.; Anglu. Chem., 8 (1969) 453

Hamilton, P.B.; Adv. Chromatogr., 2 (1966) 3

Hamilton,R.J. and Sewell,P.A., Introduction to high performance liquid chromatography, Chapman and Hall, London,, UK, (1978)

Helfferich, F. and Klein, G.; Multicomponent chromatography - theory of interference, Marcel Dekker, New York, (1970)

Herbert, N.R.; "Comparison between analytical and preparative liquid chromatography" in Preparative and Process scale Liquid Chromatography, (ed. G. Subramanian), Ellis Horwood Ltd., 1991, Chichester, England

Hofmann, K. and Halász, I. J. Chromatogr., 173 (1979) 211

Hofmann, K. and Halasz, I.; J.Chromatogr., 199 (1980) 3

Horvath, Cs. and Lipsky, S.R.; Nature (London) 211 (1966) 748

Horvath, Cs., Preiss, B.A. and Lipsky, S.R.; Anal. Chem.39 (1967) 1422

Horvath, Cs., Nahum, A. and Frenz, J.H.; J. Chromatogr., 218 (1981) 365

Hostettmann, K., Hostettmann, M. and Marston, A.; Preparative chromatographic techniques; Springer-Verlag; Berlin, Heidelberg, 1986

Izmailov,N.A. and Shraiber, M.S.; Farmatsiya nr. 3 (1938)

James, A.t. and Martin, A.J.P., Biochem. J., 50 (1952) 679

Janecek, M., Quilliam, M.A., Bailey, M.R. and North, D.H.; J. Chromatogr., 619(1993)63

Katz, E.D. and Scott, R.P.W.; J.Chromatogr. 268 (1983) 169

Kavanagh, F.and Dennin, L.J.; Analytical microbiology, Academic Press, New York, London, p.289, (1963)

Kibwage, I.O., Roets, E., Hoogmartens, J. and Vanderhaeghe, H.; J. Chromatogr., 330(1985)275

Kirkland,J.J.; J. Chromatogr. Sci., 9 (1971) 206

Kirkland,J.J.; J. Chromatogr., 10 (1972) 593

Klinkenberg,A. in Gas Chromatography (ed. by R.P.W.Scott), Butterworths, London, p.182, (1960)

Knox, J.H. and Gilbert, M.T.; J.Chromatogr., 186 (1979) 405

Knox, J.H. and Parcher, J.F.; Anal. Chem., 41 (1969) 1599

Knox, J.H. and Pyper, H.M. ;J.Chromatogr., 363 (1986) 1

Knox, J.H.; HPLC; Edinburgh University Press, Edinburgh, UK, 1978

Koh, J-H. and Guiochon, G.; J.Chromatogr. 796 (1998) 41

Kuhn, R. and Lederer, E., Ber. Deut. Chem. Ges., 64 (1931) 1349

Kuhn, R. and Lederer, E., Naturwissenschaften, 19 (1931) 306

Kuhn, R., Winterstein, A. and Lederer, E., Hoppe-Seyler's Z. Physiol. Chem., 197 (1931) 141-60

Lindsay,S., High Performance Liquid Chromatography, second ed., John Wiley & Sons, Chichester ,UK (1992)

Majors, R.E.; Anal. Chem., 45 (1973) 755

Martin, M., Eon, C. and Guiochon; G.; J. Chromatogr., 108 (1975)229

Martin,A.J.P. and Synge,R.; Biochem. J., 35 (1941) 1358-68

McLaughlin, D.J.; MPhil thesis, UCL, 1995

Meyer, V.R.; Practical High Performance Liquid Chromatography, Wiley, Chichester, 1994

Mikes, O.; Laboratory Handbook of Chromatographic Methods, van Nostrand Company Ltd., London, 1966

Moore, J.C., J. Polym. Sci.1, part A, 2 (1964) 835

Nilsson, L-G. Walldorf, B. and Paulsen, O.; J.Chromatogr., 423(1987), 189

Paesen, J., Calam, D.H., Miller, J.H.M.B., Raiola, G., Rozanski, A., Silver, B. and Hoogmartens, J.; J. Liquid Chromatogr., 16 (1993) 1529

Paesen, J., Claeys, P., Roets, E. and Hoogmartens, J.; J. Chromatogr., 630(1993)117

Paesen, J., Roets, and Hoogmartens, J.; Chromatographia. 32 (1991) 162

Parker, R.E.; Introductory Statistics for biology; E.Arnold (Publishers) Ltd.; 1979

Parry, G.S.; Acta Crystallog., 7 (1954) 313

Pate, M., PhD thesis: A practical investigation into the use of PCA for the modelling and scale-up of HPLC, University College London, 1998

Poole, C.F. and Poole, S.K.; Chromatography Today, Elsevier, Amsterdam, 1993

Poole, C.F. and Schuette, S.A.; Contemporary practice of chromatography, Chapter 4, Elsevier, Amsterdam, 1984

Poppe, H. and Kraak, J.C.; J. Chromatogr., 282(1983)399

Porath, J. and Flodin, P.; Nature, 183 (1959) 1657

Quincke, G., Pogg. Ann., 18 (1859) 326

Rhee, H-K. and Amundson, N.R., AIChE, 28 (1982) 423

Richardson,P, Hoare,M. and Dunnill,P.; Biotech. and Bioeng., 36, (1990) 354

Richardson,P.; PhD thesis, University College London, London, 1987

Ruthven, D.M. and Ching, C.B., in Preparative and Production scale chromatography, (ed. by Ganetsos and Barker), Chromatographic science series, vol. 61; Marcel Dekker, Inc., New York - Basel - Hong Kong, 1993

Ruthven, D.M.; Principles of adsorption and adsorption processes, Wiley, New York, 1984

Sarker, M. and Guiochon, G. J. Chromatogr., 702 (1995) 27

Sarker, m. and Guiochon, G.; J. Chromatogr.,741 (1996) 165

Scott, R.P.W. and Kucera, P.; J. Chromatogr., 119 (1976) 467

Scott, R.P.W. and Kucera, P.; J.Chromatogr. Sci., 9 (1971) 641

Shalliker, R.A., Broyles, B.S. and Guiochon, G.; J. Chromatogr., 865 (1999)73

Sheshadri, S. and Deming, S.N. Anal. Chem., 56 (1984) 1567

Shirazi, S.G. and Guiochon,G.; J. Chromatogr., 603 (1992) 1

Snyder,L.R., Glajch and Kirkland, J.J.; Practical HPLC method development, John Wiley &Sons, Chichester, 1988

Snyder, L.R.; Principles of adsorption chromatography, New York, Marcel Dekker 1968

Snyder, L.R.; Techniques of chemistry, 2nd.ed., Vol. III, Part I, (A. Weissberger and E.S.Perry, eds.), Wiley-Interscience, New York, 1978, Chap. 2

Snyder,L.R. and Kirkland,J.J. Introduction to Modern Liquid Chromatography, 2nd edition, Wiley Interscience; New York, 1979

Spackman, D.H., Stein, W.H. and Moore, S.; Anal. Chem., 30(1958)1190

Spedding, F.H.; Discussions Faraday Soc., 7 (1949) 214

Spedding, F.H., Fulmer, E.I., Butler, T.A., Gladrow, E.M., Porter, P.E., Powel, J.E., and Wright, J.M.; J. Am. Chem. Soc., 69 (1947) 2812

Stephenson, G.A., Stowell, J.H., Toma, P.H., Pfeiffer, R.P. and Byrn, S.R.; J. Pharmaceut. Sci., 86 (11) (1997) 1239

Sternberg, J.C. in Advances in Chromatography, Vol. 2, (J.C.Giddings and R.A. Keller, eds.), Dekker, New York, (p.205) 1966

Stevens, M.P., Polymer chemistry. An Introduction. 3rd edition, Oxford University Press, Oxford, 1999

Subramanian, G. (ed.); Preparative and Process scale Liquid Chromatography, Ellis Horwood Ltd., 1991, Chichester, England

Suzuki, T., Asai, M. and Sugita, N.; Jpn. Tokkyo Koho 73 76 (1973) 880

Svec, F. and Frechet, J.M.J.; Science, 273 July (1996)

Tijssen, R.; Sep. Sci. Technol., 134 (1978) 681

Tompkins, E.; Discussions Faraday Soc., 7 (1949) 232

TOSO HAAS catalogue (1999)

Tsuji, K. and Goetz, J.F.; J. Chromatogr., 157 (1978)185

Tsuji, K. and Goetz, J.F.; J. Chromatogr., 147(1978) 359

Tswett, M., Ber. Deut. Bot. Ges., 24 (1906) 316-23 (*)

Tswett, M., Ber. Deut. Bot. Ges., 24 (1906) 384-93(**)

Van Deemter,J.J. , Zuiderweg,F.J. and Klinkerberg, A.; Chem. Eng. Sci., 5 (1956) 271

Vandamme, E.J. (editor); Biotechnology of industrial antibiotics; Drugs and The Pharmaceutical Sciences; vol. 22, Marcel Dekker, New York, 1984

Velayudhan, A. and Ladisch, M. R.; Biotechnology, 49 (1993) 123

Verzele, M. and Dewaele, C.; Preparative high performance liquid chromatography. A practical guideline; Gent, Belgium, Spring 1985

Verzele,M., Dewaele,C., Van Dijck, J. and Van Haver, D.; J. Chromatogr., 249(1982)231

Warren, F.V. Jr. and Bidlingmeyer, B.A.; Anal. Chem., 60 (1988) 2824

Wehrli, A. and Hermann, U.; J. Chromatogr., 125(1976)59

Weinstein, M.J. and Wagman, G.H. (editors); Journal of chromatography; Antibiotics, Isolation, Separation and Purification, Elsevier Scientific publishing company (1978)

Wilke, C.R. and Chang, P.; AIChE J., 1 (1955) 264

Williams, T.I., An Introduction To Chromatography, Blackie & Sons Ltd., London and Glasgow, 1947

Willstätter, R. and Stoll, A.; Untersuchungen über Chlorphyl. Methoden und Ergebnisse J.; Springer, Berlin, 1913

8. APPENDICES

8.1 Appendix 1 - Nomenclature

A	surface area (geometric cross section) of the column
aEA	anhydroerythromycin A
A_H	eddy diffusion constant
a_S	surface area of adsorbent per plate
B_H	longitudinal (axial) diffusion coefficient
CBB	column band broadening
C_H	mass transfer resistance coefficient
C_m	concentration of solute in mobile phase
C_S	concentration of solute in stationary phase
DAC	dynamic axial compression
D_m	diffusion coefficient of a solute
d_p	particle diameter
$\frac{dp}{dz}$	pressure drop per unit length
d_t	internal diameter of the tubing
EA	erythromycin A
EB	erythromycin B
EC	erythromycin C
ECBB	extra-column band broadening
ED	erythromycin D
EE	erythromycin E
F	mobile phase volumetric flowrate
H	height equivalent to the theoretical plate
h	reduced plate height
k	constant related to the form of the particles
K	distribution constant
k_1', k_2'	capacity factor of compounds 1 and 2
K_{CK}	Carman Kozeny permeability

K_F	specific column permeability
K_P	constant of proportionality
L	column bed length
M	limiting mass of sample
M_2	molecular weight of the solvent
N	number of theoretical plates (efficiency of the column)
PLRP	polymeric reversed phase
PSDVB	polystyrene divinylbenzene
psEEA	pseudo Erythromycin A Enol Ether
q	flux of the current
r	column radius
RP	reversed-phase
R_S	resolution factor
S	symmetry factor
sd	standard deviation
se	standard error
t_1, t_2	retention time of two adjacent bands
TBA	tetrabutylammonium ion
TBB	total band broadening
THF	tetrahydrofuran
TMA	tetramethylammonium ion (salt)
TP	throughput
t_c	detector time constant
t_0	the hold-up time or a dead time
t_R	retention time of peak of interest
$t_{w1/2}$	half width and half height
u	linear velocity
V	sample volume injected onto the column
V_1	molar volume of the liquid solute
V_c	column volume
V_o	mobile phase volume of a column
Vr	retention volume

V_s	volume of compound in stationary phase
V_m	volume of compound in mobile phase
W	peak width
w or $w_{1/2}$	peak width at half height
W_1, W_2	baseline bandwidths of two adjacent bands
w_{p1}, w_{p2}	peak widths of compound 1 and 2 at 10% height

Greek characters

α	selectivity factor or a separation factor
ε_T	total porosity of the column
ε_e	external porosity of the column
ε_i	internal porosity of the column
η	mobile phase viscosity
ν	reduced velocity (particle Peclet number)
ρ	solvent density
σ	peak standard deviation
ψ_2	solvent association factor

8.2 Appendix 2 - Aromatic compounds

8.2.1 Loop size and the number of theoretical plates.

LOOP SIZE (μL)	N (plates/metre)			
	nitrobenzene	naphthalene	fluorene	fluoranthene
5	33 215	36 680	36 900	34 940
10	39 812	42 146	40 975	42 840
20	32 600	35 740	38 570	37 820

Table 8.2-1 Calculations of a number of theoretical plates for the four compounds when injected with different loop sizes.

Conditions:

90%methanol/water; concentration: 0.1g/l; detection: 265nm; flow rate: 0.75ml/min; column: 250 x 4.6mm C_{18} Phenomenex

This table shows the influence on N of the amount of sample injected.

When comparing 5, 10 and 20μL injections, the optimum result of N appears to be given with a 10μL injection, both in terms of consistency and efficiency. The data deviates from the mean when a 5 μL injection is used and even more so when a 20μL injection is employed. Generally, however, the N data for nitrobenzene deviate significantly from the data for other compounds. If the data are so different for early eluted peaks, the frequency may need to be increased further to collect additional data.

loop	mean	sd (yEr+-)	se (yEr+-)	95% confidence	sum
5,10,20μL	37 686.5	3 322.1929	959.03449	35 580-39 800	452 238

Table 8.2-2 Statistics calculated on all the values from the table 8.2-1.

The table here shows statistical characteristics of data analysis such as the mean, standard deviation, standard error of the mean, and the sum. From this data the statistical range was calculated for one SD, two SD and for 95% probability. Although this can give some indication of the statistical distribution of the data, an alternative

evaluation would be to examine each row separately, i.e. every volume injected on its own.

	mean	sd(yEr+-)	se(yEr+-)	95% confidence	sum
N(5)	35433	1719	859	32700-38170	141735
N(10)	41443	1332	666	39323-43560	165773
N(20)	36182	2671	1335	31930-40430	144730

Table 8.2-3 Statistics on values from each loop separately

For 2 SDs and for 95% probability all the data for all three loops fall within the range. However, this is not the case for 1SD. This is simply to illustrate how the range can vary with SD. Therefore we can assume that all the data are valid and that there are no big discrepancies.

8.2.2 Wavelength and "N"

WAVELENGTH (nm)	N (plates/metre)			
	nitrobenzene	naphthalene	fluorene	fluoranthene
265	19 650	32 170	26 790	35 350
280	28 300	32 170	38 350	33 300

Table 8.2-4 Wavelength and number of theoretical plates

Conditions:

90% methanol/water; injection: 20μL; flowrate:0.75ml/min; concentration: 0.3g/l

Wavelength	mean	se(yEr+-)	sum
265nm	28 490	3 436	113 960
280nm	32 985	2 033	131 940

Table 8.2-5 Mean, standard error and sum on N values at two different wavelengths

wavelength (nm)	1 SD (range)	2 SD (range)	95% confidence (range)
265	21 620-35 360	14 750-42 230	17 560-39 423
280	28 915-37 055	24 845-41 125	26 515-39 455

Table 8.2-6 Statistics on wavelength data

265nm				
compound	peak area	t_R (mins)	height	half width
nitrobenzene	0.42922	4.46667	2.484145	0.15
naphthalene	0.28229	6.35	1.49951	0.16667
fluorene	0.65493	8.69167	2.38316	0.25
fluoranthene	0.39446	10.98333	1.30226	0.275

Table 8.2-7 Peaks details for aromatic compounds at 265nm.

280nm				
compound	peak area	t_R	height	half width
nitrobenzene	0.26519	4.46667	1.77279	0.125
naphthalene	0.21434	6.35	1.15282	0.16667
fluorene	0.20436	8.66667	0.85218	0.20833
fluoranthene	0.63643	10.98333	2.00732	0.28333

Table 8.2-8 Peaks details for aromatic compounds at 280nm

Here, a number of theoretical plates was calculated for the compounds for which data were collected at two detector wavelengths: 265nm and 280nm. The frequency at which the data points were collected was 2 Hz in both cases.

From an initial examination of the table it would seem that the data vary significantly for every compound and for both wavelengths. On closer inspection, however, and after applying statistical analysis for 95% probability, all the data fall within the range shown. This indicates that statistically the variation in terms of N is acceptable.

So far all the data presented were obtained in conditions of 90% methanol/water. The following set of data were carried out at 90% acetonitrile.

8.2.3 Mobile phase change and "N"

8.2.3.1 Methanol / water and 90% Acetonitrile / water

	N(plates/metre)			
mobile phase	nitrobenzene	naphthalene	fluorene	fluoranthene
90% methanol	33 090	40 550	39 390	38 680
90% acetonitrile	34 510	38 400	34 050	39 660

Table 8.2-9 Column efficiency at 90% methanol/water and 90% acetonitrile/water

90% methanol/water				
compound	peak area	t_R (mins)	height	half width
nitrobenzene	0.1611	4.50833	1.22549	0.11667
naphthalene	0.09552	6.41667	0.54055	0.15
fluorene	0.25025	8.7833	1.02853	0.20833
fluoranthene	0.13564	11.14167	0.43296	0.26667

Table 8.2-10 : Peak details for aromatic compounds with 90% methanol/water

90% acetonitrile/water				
compound	peak area	t_R (mins)	height	half width
nitrobenzene	0.25115	4.275	1.88444	0.10833
naphthalene	0.13322	5.55	0.83285	0.13333
fluorene	0.41797	6.5333	2.08945	0.16667
fluoranthene	0.20862	8.10833	0.91608	0.19167

Table 8.2-11 Peak details for aromatic compounds with 90% acetonitrile/water

	mean	sd(yEr+-)	se(yEr+-)	sum
N (90%methanol & 90%acetonitrile)	37291.25	2919.99	1032.37	298330

Table 8.2-12 Statistics on all the data with 90 % acetonitrile/water and 90% acetonitrile/water.

solvent	1 SD (range)	2 SD (range)	95% confidence (range)
90%methanol & 90% acetonitrile	34371-41211	31450-43131	34850-39732

Table 8.2-13 Standard deviations and 95% confidence values for the data above

When looking through the chromatogram and N for 90% acetonitrile/water and comparing it to N gained for each compound from 90% methanol/water, it might be assumed that acetonitrile would give better results than methanol. Initial inspection suggests that this is not the case. However statistical analysis for both solvents eliminates data which are out of range and thus for 95% probability with a confidence limit ±1658x2.365 for seven degrees of freedom, certain values are automatically excluded (such as the values for nitrobenzene and the odd value for fluorene at 90% acetonitrile/water). The remaining values are within the range and therefore acceptable.

solvent	mean	sd(yEr+-)	se(yEr+-)	sum
90%methanol	37 928	3316	1658	151 710
90% acetonitrile	36 655	2797	1398	146 620

Table 8.2-14 Statistics on 90%methanol and 90%acetonitrile separately

solvent	1 SD (range)	2 SD (range)	95% confidence (range)
90% methanol	34612-37928	31296-44560	32652-43204
90% acetonitrile	33858-39452	31061-42249	32207-41103

Table 8.2-15 Standard deviations and 95% confidence values for the data 90%methanol and 90%acetonitrile separately

If the data for each mobile phase are evaluated separately, the data for all compounds fall into the range for 95% probability.

8.2.4 Influence of sample concentration on the number of theoretical plates

SAMPLE concentration (g/L)	N (plates/metre)			
	nitrobenzene	naphthalene	fluorene	fluoranthene
0.01	33 440	36 240	34 900	36 230
0.02	33 440	36 350	34 990	36 310
0.03	33 575	32 100	34 990	36 310
0.04	28 830	36 240	34 900	36 230
0.05	28 830	32 100	34 900	36 230

Table 8.2-16 Column efficiency data for samples of different concentrations

Conditions:

90% acetonitrile/water; injection:20μL; detection: 265nm; flowrate: 0.75ml/min

N(load)	mean	sd(yEr+-)	se(yEr+-)	sum
nitrobenzene	31 623	2550.2441	1140.50384	158 115
naphthalene	34 606	2288.09528	1023.26732	173 030
fluorene	34 936	49.29503	22.04541	174 680
fluoranthene	36 262	43.8178	19.59592	181 310
all	34 356.75	2344.97238	524.35176	687 135

Table 8.2-17 Statistics on the data from table 8.2-18

Examining the data for nitrobenzene, naphthalene, fluorene and fluoranthene, it appears that certain inaccuracies are introduced in early eluted peaks which are gradually eliminated as the evaluation is moved to the later eluted peaks. As mentioned earlier, this is linked to the inaccurate calculations relating to the number of data points collected. For later peaks on the column this phenomenon becomes less profound and the data for fluorene and fluoranthene are almost identical.

This is further reflected in the standard deviation and standard error of the mean which values decrease drastically as a transition is made from faster to more slowly eluted peaks.

compound	95% confidence range
NITROBENZENE	27992-35254
NAPHTHALENE	31351-37861
FLUORENE	34866-35010
FLUORANTHENE	36200-36324

Table 8.2-19 95% confidence values on the data from table 8.2-20

All the data for each compound fall comfortably within the range of 95% probability.

8.2.5 Evaluation of capacity factor, selectivity factor and resolution

In this section the capacity factors k', selectivity factors α and resolution factors R_S (calculated in two different ways) are compared.

The above-mentioned factors are standard indicators of column performance. They serve the purpose of evaluating chromatograms

8.2.5.1 Sample concentration

concentration (g/l)	k'-capacity factor			
	nitrobenzene	naphthalene	fluorene	fluoranthene
0.01	1.471	2.175	2.696	3.559
0.02	1.48	2.176	2.7	3.56
0.03	1.48	2.17	2.7	3.56
0.04	1.48	2.17	2.696	3.559
0.05	1.48	2.17	2.696	3.559

Table 8.2-21 Capacity factors for the data from different sample concentrations

	α_{12}	α_{23}	α_{34}	R_{S12}	R_{S23}	R_{S34}	$R_S(N)_{12}$	$R_S(N)_{23}$	$R_S(N)_{34}$
0.01	1.48	1.24	1.32	5.78	3.61	4.95	13	7.8	10.9
0.02	1.47	1.24	1.32	5.82	3.61	4.94	12.82	7.84	10.92
0.03	1.46	1.24	1.32	5.55	3.54	4.94	12.58	7.36	10.92
0.04	1.47	1.24	1.32	5.59	3.61	4.95	11.97	7.82	10.91
0.05	1.47	1.24	1.34	5.41	3.51	4.95	11.97	7.36	10.91

Table 8.2-22 Selectivity factor and resolution values for the data from different sample concentrations

8.2.5.2 Wavelength

	k'			
wavelength(nm)	nitrobenzene	naphthalene	fluorene	fluoranthene
265	1.63	2.74	4.12	5.46
280	1.63	2.74	4.098	5.46

Table 8.2-23 Capacity factors for the data recorded at different wavelengths

	α_{12}	α_{23}	α_{34}	R_{S12}	R_{S23}	R_{S34}	$R_S(N)_{12}$	$R_S(N)_{23}$	$R_S(N)_{34}$
265nm	1.68	1.5	1.33	7.02	6.63	5.15	14.77	16.42	10.87
280nm	1.68	1.5	1.33	7.62	7.29	5.56	17.67	16.29	12.99

Table 8.2-24 Selectivity factor and resolution values for the data at different wavelengths

8.2.5.3 Mobile phase

	k'			
SOLVENT	nitrobenzene	naphthalene	fluorene	fluoranthene
90% METHANOL	1.647	2.77	4.166	5.55
90% ACETONITRILE	1.51	2.26	2.84	3.77

Table 8.2-25 Capacity factors for the data recorded with different mobile phases

	α_{12}	α_{23}	α_{34}	R_{S12}	R_{S23}	R_{S34}	$R_S(N)_{12}$	$R_S(N)_{23}$	$R_S(N)_{34}$
METHANOL	1.68	1.5	1.3	8.45	7.79	5.86	19.24	18.49	13.2
ACETONITRILE	1.5	1.3	1.3	6.23	3.87	5.19	13.86	8.83	11.26

Table 8.2-26 Selectivity factor and resolution values for the data recorded with different mobile phases

8.2.5.4 Loop size

	k'			
LOOP(μL)	nitrobenzene	naphthalene	fluorene	fluoranthene
5	1.66	2.79	4.2	5.62
10	1.7	2.848	4.27	5.68
20	1.63	2.74	4.11	5.48

Table 8.2-27 Capacity factors for the data recorded with different loop volumes

LOOP (μl)	α_{12}	α_{23}	α_{34}	R_{S12}	R_{S23}	R_{S34}	$R_S(N)_{12}$	$R_S(N)_{23}$	$R_S(N)_{34}$
5	1.68	1.51	1.34	8.26	7.552	5.68	19.33	17.98	13.11
10	1.69	1.49	1.33	8.91	7.96	6.069	21.67	18.95	13.53
20	1.68	1.5	1.3	8.088	7.51	5.78	19.02	17.31	13.03

Table 8.2-28 Selectivity factor and resolution values for the data recorded with different loop volumes

In relation to these indicators, different requirements exist for analytical and preparative chromatography. Whilst in analytical chromatography these factors can be changed by altering, e.g. the composition of the mobile phase, in preparative chromatography small values for the critical peaks will be ever present due to the size of the sample load being used. (Snyder and Kirkland 1979)

For example the concentration of the sample loaded on the column increases linearly. k'-values are almost identical for each of the compounds separately. This is further reflected on selectivity factors which vary only slightly.

The selectivity factor α changes its value according to the composition of the mobile phase.

The resolution factors have been calculated in two different ways and therefore should not be directly compared.

Resolution factor R_S is based strictly on the resolution between two adjacent peaks using only their retention times and peak widths at half height to calculate its values. A resolution factor of 1 indicates a good separation between the peaks. Obviously, values > 1 point to further improvement in the separation whereas values <1 indicate that the separation has deteriorated and the peaks are merging.

To evaluate chromatograms when N, α or k' changes it is advisable to use $R_S(N)$. This is particularly helpful during method development. As can be seen, the resolution

factors calculated in different ways are not the same and hence are not directly comparable.

The data for all the factors calculated for concentration show little difference which is in agreement with what would be expected in analytical chromatography. As for the evaluation of injection size, small variations in values were detected. This applies to all the indicators mentioned.

8.3 Appendix 3 - Analytical separation of erythromycin on PLRP column

8.3.1 Temperature

temperature (°C)	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^N{}_{S\,EXA}$	N(p/c) p/m	t_R (mins)
30	0.7	1.28	1.83	2.14	9.53	(1125) 4 500	5.7
40	0.79	1.43	1.81	1.89	8.51	(1275) 5 097	6.67
50	0.87	1.59	1.83	1.99	9.69	(1448) 5 792	6.47
60	0.92	1.75	1.9	2.29	9.91	(1500) 6 000	6.87
70	1	1.88	1.88	3.25	11.28	(1543) 6 172	7.2

Table 8.3-1 Capacity factors, selectivity factors, resolutions, column efficiencies and retention times for the data recorded at different temperatures on PLRP-1000 column.

N.B. The void volume was the same for all these runs: V_0=2.5mins.*1ml/min =2.5ml

8.3.2 Flowrate

flowrate (ml/min)	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^N{}_{S\,EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
0.4	0.95	1.81	1.91	3.73	11.21	(1460) 5860	18.97	6.74
0.6	0.96	1.85	1.93	3.57	11.68	(1498) 5992	12.33	4.33
0.8	0.86	1.69	1.97	3.2	10.8	(1467) 5867	9.03	3.36
1	0.93	1.77	1.9	3.15	10.8	(1411) 5650	7.18	2.59
1.2	0.92	1.75	1.9	3.21	10.34	(1305) 5220	5.88	2.14
1.4	0.93	1.74	1.87	2.41	8.83	(1023) 4090	4.98	1.82

Table 8.3-2 Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded at different flowrates on PLRP-1000 column.

8.3.3 Acetonitrile concentration

acetonitrile concentration (%)	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^{N}_{S\,EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
35	2.62	5.98	2.28	4.3	13.68	(622) 2490	17.67	2.53
55	0.399	0.7	1.75	1.07	6.78	(1930) 7721		

Table 8.3-3 Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded at different acetonitrile concentrations on PLRP-1000 column.

8.3.4 Buffer concentration

buffer concentration (g/l)	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^{N}_{S\,EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
0.1	0.71	1.1	1.54	1.97	6.42	(1343) 5373	5.45	2.6
0.15	0.83	1.33	1.6	3.07	6.97	(1655) 6621	6.05	2.6
0.25	0.73	1.21	1.66	2.46	7.05	(1522) 6085	5.8	2.63
0.3	0.66	1.03	1.57	1.82	5.3	(2343) 5373	5.45	2.68

Table 8.3-4 Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded with different buffer concentrations on PLRP-1000 column.

8.3.5 Buffer pH

Buffer pH	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\ EXA}$	$R^{N}{}_{S\ EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
7A	0.61	1.04	1.71	1.56	5.2	830 3320	5.92	2.9
7B	0.6	1.02	1.7	1.49	5.05	816 3265	5.87	
7C	0.61	1.03	1.69	1.46	4.85	767 3070	5.88	
7.5A	0.67	1.24	1.85	2.51	8.62	1341 5370	6.48	
7.5B	0.68	1.25	1.84	2.59	8.59	1355 5420	6.52	
7.5C	0.68	1.25	1.84	2.6	8.59	1355 5420	6.52	
8A	0.72	1.41	1.96	3.05	10.65	1440 5755	6.98	
8B	1.11	1.4	1.26	1.06	2.98	1550 6195	6.97	
8C	0.724	1.4	1.93	3.32	10.67	1550 6195	6.97	

Table 8.3-5 Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded at different buffer pH on PLRP-1000 column.

Buffer pH	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^{N}_{S\,EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
8.5A	1.14	1.57	1.38	1.32	4.69	1638 6550	7.45	
8.5B	1.16	1.59	1.37	1.34	4.81	1795 7180	7.5	
8.5C	1.15	1.57	1.37	1.14	4.77	1780 7120	7.47	
8.7A	1.11	1.54	1.39	1.77	4.92	1732 6930	7.37	
8.7B	1.12	1.55	1.38	1.58	4.83	1750 6990	7.4	
8.7C	1.13	1.56	1.38	1.67	4.85	1755 7020	7.42	
9A	1.13	1.57	1.39	1.24	5.45	2093 8370	7.45	
9B	1.06	1.49	1.41	2.39	5.7	2156 8624	7.23	
9C	1.09	1.53	1.4	1.38	5.02	1724 6895	7.35	
9.3A	0.74	1.38	1.86	2.7	8.68	1211 4844	6.9	
9.3B	1.11	1.56	1.41	3.45	5.46	1913 7653	7.43	
9.3C	1.1	1.56	1.42	1.55	5.59	1905 7620	7.42	
9.5A	1	1.42	1.42	1.35	5.55	2030 8115	7.02	
9.5B	1	1.43	1.43	1.33	5.46	1865 7460	7.03	
9.5C	1	1.43	1.42	1.33	5.35	1874 7496	7.05	
9.7A	1	1.42	1.42	1.31	5.31	1856 7425	7.02	
9.7B	1.01	1.43	1.42	1.35	5.35	1874 7496	7.05	
9.7C	1.01	1.43	1.42	1.31	5.13	1721 6884	7.05	
10A	1.02	1.44	1.41	1.28	5.26	1892 7570	7.083	
10B	1.01	1.43	1.42	1.29	5.35	1874 7500	7.05	
10C	1	1.43	1.43	1.32	5.48	1874 7500	7.05	

Table 8.3-6 Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded at buffer pH on PLRP-1000 column.

Buffer pH data (continued)

Buffer pH	k'_{EX}	k'_{EA}	α_{EXA}	$R_{S\,EXA}$	$R^{N}_{S\,EXA}$	N(p/c) p/m	t_R (mins)	t_0 (mins)
10.5A	0.73	1.46	2	3.94	13.53	2090 8350	7.12	
10.5B	0.72	1.46	2.03	3.97	13.33	1910 7640	7.12	
10.5C	0.72	1.46	2.03	4	13.38	1920 7670	7.13	
11A	0.713	1.44	2.02	3.75	13.69	2070 8270	7.08	
11B	0.713	1.44	2.02	3.84	13.69	2070 8270	7.08	
11C	0.713	1.44	2.02	3.75	13.1	1892 7570	7.08	

Table 1.3.6 (cont.) Capacity factors, selectivity factors, resolutions, column efficiencies, retention times and dead times for the data recorded at buffer pH on PLRP-1000 column.

LEGEND:

k'_{EX}	the capacity factor of the impurity nearest to EA and eluted before EA
k'_{EA}	the capacity factor of EA
α_{EXA}	selectivity factor calculated from k'_{EX} and k'_{EA}
$R_{S\,EXA}$	the resolution factor calculated directly from the chromatogram
$R^{N}_{S\,EXA}$	the resolution factor calculated using the equation which contains selectivity factor, N and k'.
N(p/c)[p/m]	number of theoretical plates (plates/column or plates/metre)
t_R	retention time of EA
t_0	retention time of unretained peak

8.4 Appendix 4

8.4.1 Semipreparative separation of erythromycin on CG-300

150 x 4.6mm		
LOAD(µl)	N (p/m)	average values (p/m)
21a	1370	
21b	1370	1370
42a	1330	
42b	1370	1350
125a	1220	
125b	1070	1145
211a	870	
211b	900	884

Table 8.4-1 Column efficiency on 4.6mm ID column

150 x 7.8mm		
LOAD(µl)	N (p/m)	average values (p/m)
60.8a	1710	
60.8b	1290	1500
121a	1200	
121b	1380	1290
365a	1120	
365b	1120	1120
599a	860	
599b	890	875

Table 8.4-2 Column efficiency on 7.8mm ID column

150 x 10mm		
LOAD(µl)	N (p/m)	average values (p/m)
100a	1290	
100b	1290	1290
200a	1560	
200b	1290	1425
600	1140	1140
1000	772	772

Table 8.4-3 Column efficiency on 10mm ID column

Statistics on rows follow:

If all the data in the above table were subjected to statistical calculations then the standard mean would be 1180. The standard error (±) is 68 and the standard deviation (±) is 235 when the calculations are done on 12 species.

8.5 Appendix 5 - Prochrom

8.5.1 Reproducibility studies.

8 ml loop

Prochrom	loop: 8ml
injection number	N (p/m)
1	544
2	606
3	706
4	722
5	667

Table 8.5-1 Column efficiencies on the data recorded with 8ml loop on Prochrom

AVERAGE: 649 p/m
sd(±) = 72
coefficient of variation: 11.4%

15 ml loop

Prochrom	loop: 15ml
injection number	N (p/m)
1	683
2	745
3	683
4	700
5	840

Table 8.5-2 Column efficiencies, on the data recorded with 15ml loop on Prochrom

AVERAGE: 730p/m
sd(±) = 66
coefficient of variation: 9.1%

30 ml loop

Prochrom	loop: 30ml
injection number	N (p/m)
1	635
2	655
3	605
4	NA
5	NA

Table 8.5-3 Column efficiencies, on the data recorded with 15ml loop on Prochrom

AVERAGE: 632 p/m
sd(±) = 25
coefficient of variation: 4%

NA in the case of samples 4 and 5 is due to the peak acquiring a flat top. The calculations of standard deviation and error should therefore be taken with reserve. They do not reflect the true nature of the chromatographic profiles.

8.6 Appendix 6 - h/ν curves

8.6.1 ACETONE

Acetone (8ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	29.38	2.13	1059	0.0166	4.72	3.4
66	7	0.5	1086	0.0161	4.61	12.79
165	2.92	0.21667	1004	0.0174	4.98	31.97
210	2.35	0.18333	910	0.0192	5.49	40.69

Table 8.6-1 The derivation data for h and ν for acetone with 8ml loop calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation (see paragraph 8.6.4 below)

Acetone (15ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	29.73	2.2666	953	0.0184	5.24	3.4
66	6.87	0.5333	918	0.0191	5.44	12.79
165	3.07	0.2333	957	0.0183	5.22	31.97
210	2.4	0.2	798	0.0219	6.27	40.69

Table 8.6-2 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for acetone with 15ml loop(see paragraph 8.6.4 below)

Acetone (30ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	29.61	2.55	747	0.0234	6.69	3.4
66	7.17	0.625	728	0.024	6.86	12.79
165	3.01	0.28333	628	0.0278	7.96	31.97
210	2.43	0.21667	698	0.025	7.17	40.69

Table 8.6-3 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for acetone with 30ml loop(see paragraph 8.6.4 below)

8.6.2 URACIL

uracil (8ml)					
F (ml/min)	t_r	$t_{w1/2}$	N	H	h
17.5	21.13	1.95	650	0.0269	7.7
66	5.2	0.46667	690	0.0254	7.3
165	2.27	0.2	711	0.0246	7.03
210	1.82	0.16667	658	0.0265	7.6

Table 8.6-4 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for uracil with 8ml loop(see paragraph 8.6.5 below)

uracil (15ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	22.53	2.16667	599	0.0292	8.344	
66	5.05	0.45	698	0.025	7.143	
165	2.27	0.2	711	0.0245	7.03	
210	1.83	0.16667	670	0.026	7.43	

Table 8.6-5 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for uracil with 15ml loop(see paragraph 8.6.5 below)

uracil (30ml)					
F (ml/min)	t_r	$t_{w1/2}$	N	H	h
17.5	22.63	1.98334	721	0.0242	6.93
66	5.33	0.56667	490	0.0355	1018
165	2.32	0.25	476	0.0368	105
210	1.85	0.2	474	0.0369	10.55

Table 8.6-6 The derivation data for h calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for uracil with 30ml loop(see paragraph 8.6.5 below)

8.6.3 ERYTHROMYCIN

8.6.3.1 with normal retention time

erythromycin (8ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	94.25	9.67	526	0.0166	4.756	12
66	20.1	2.55	34	0.05084	14.526	46
165	8.63	1.4	211	0.083	23.74	115
210	6.83	1.3	153	0.1146	32.74	147

Table 8.6-7 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 8ml loop(see paragraph 8.6.6 below)

erythromycin (15ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	91.13	7.8	756	0.0231	6.6	12
66	21.4	2.4	440	0.0397	11.4	46
165	8.5	1.25	256	0.0683	19.5	115
210	6.75	1.25	162	0.1083	31	147

Table 8.6-8 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 15ml loop(see paragraph 8.6.6 below)

erythromycin (30ml)						
F (ml/min)	t_r	$t_{w1/2}$	N	H	h	ν
17.5	85.43	8.433	569	0.0308	8.8	12
66	18.88	2.29	376	0.0465	13.3	46
165	8.57	1.2833	244	0.07172	20.5	115
210	6.7	1.1	206	0.085	24.3	147

Table 8.6-9 The derivation data for h and ν calculated from t_r , $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 30ml loop(see paragraph 8.6.6 below)

8.6.3.2 with a reduced retention time

erythromycin (8ml)							
F (ml/min)	t_r	t_0	$t_{w1/2}$	N	H	h	ν
17.5	94.25	20.47	9.67	322	0.0543	15.52	12
66	20.1	4.8	2.55	199	0.0877	25	46
165	8.63	1.815	1.4	129	0.1355	38.73	115
210	6.83	1.402	1.3	96	0.1815	1.86	147

Table 8.6-10 The derivation data for h and ν calculated from t_r, $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 8ml loop(see paragraph 8.6.6 below)

erythromycin (15ml)							
F (ml/min)	t_r	t_0	$t_{w1/2}$	N	H	h	ν
17.5	91.13	20.473	7.8	455	0.0385	11	12
66	21.4	4.8	2.4	265	0.066	18.9	46
165	8.5	1.815	1.25	158	0.11	31.55	115
210	6.75	1.402	1.25	101	0.173	49.3	147

Table 8.6-11 The derivation data for h and ν calculated from t_r, $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 15ml loop(see paragraph 8.6.6 below)

erythromycin (30ml)							
F (ml/min)	t_r	t_0	$t_{w1/2}$	N	H	h	ν
17.5	85.43	20.473	8.433	329	0.0532	15.21	12
66	18.88	4.8	2.29	209	0.08364	23.89	46
165	8.517	1.815	1.2833	151	0.116	33.1	115
210	6.7	1.402	1.1	129	0.136	38.91	147

Table 8.6-12 The derivation data for h and ν calculated from t_r, $t_{w1/2}$ and Wilke-Chang equation for erythromycin with 30ml loop(see paragraph 8.6.6 below)

8.6.4 Molar volume of acetone

Molar volume of acetone, used in Wilke-Chang equation, was calculated:

$$V = \frac{M_{acetone}}{\rho_{acetone}} = \frac{58g/mol}{0.79g/cm^3} = 73.4cm^3/mol$$

where M is a molecular weight of the solute

ρ is density of the solute

8.6.5 Molar volume of uracil

Molar volume of uracil, used in Wilke-Chang equation, was calculated:(Parry 1954)

$$V = \frac{M_{uracil}}{\rho_{uracil}} = \frac{112.1g/mol}{1.617g/cm^3} = 69.3cm^3/mol$$

Molar volume of erythromycin, used in Wilke-Chang equation, was calculated using density of erythromycin crystal of EA dihydrate ρ=1.226 g/ml (Stephenson et.al.1997)

8.6.6 Molar volume of erythromycin

Molar volume of erythromycin, used in Wilke-Chang equation, was calculated:

$$V = \frac{M_{erythromycin}}{\rho_{erythromycin}} = \frac{769.97g/mol}{1.226g/cm^3} = 628cm^3/mol$$

8.6.7 Reynolds numbers calculated for different temperatures

F (ml/min)	25	50	75	100	150	200	250	300	350	viscosity (cPoise)
$Re_{@25°C)}$	353	706	1059	1413	2120	2826	3532	4240	4946	0.86
$Re_{@30°C)}$	393	786	1177	1571	2357	3142	3930	4713	5500	0.77
$Re_{@35°C)}$	431	863	1294	1726	2590	3452	4315	5178	6041	0.70

Table 8.6-13 Reynolds number calculated using viscosity at different temperatures.