NARROW-BORE LIQUID CHROMATOGRAPHY

- A PRACTICAL ASSESSMENT OF A COMMERCIAL INSTRUMENT

by

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ABSTRACT

This dissertation is concerned with the evaluation, in practice, of the technique of Narrow-Bore Liquid Chromatography using a commercial instrument. A relevant clinical problem is selected in order to enable a practical assessment to be made of the theoretical improvements in mass sensitivity attributed to this technique.

In Chapter 1 the theoretical basis of Narrow-Bore Liquid Chromatography is discussed. Particular emphasis is placed on the need for a minimal extra-column contribution to peak dispersion.

Chapter 2 describes the theoretical aspects of electrochemical detection and its suitability, in theory, for use in conjunction with Narrow-Bore columns. The performance characteristics of three commercial detectors are compared and contrasted.

Chapter 3 is divided into four parts.

Part I deals with the clinical significance of a series of important tryptophan-derived indolic compounds and also describes, in detail, the Narrow-Bore Liquid Chromatograph.

In Part II a separation by Reverse Phase Ion-Interaction Chromatography of five analytes (viz. tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindole-3-acetic acid and indoxyl-3-sulphate) and a possible internal standard (bufotenine) is developed. Evaluation of a separation on both 1mm and 2.1mm ID columns indicates gross discrepancies between the observed dispersion characteristics of the instrument and those necessary to operate columns of either geometry efficiently. The detector response time is highly significant in this respect.

Precision is examined in Part III and is found to be extremely poor. This is reflected in the limits of detection and quantitation, which are correspondingly high.

The exploratory experiments with blood samples, reported in Part IV, show sample clean-up to be highly problematical. Equipment limitations are recognised as the principal factor in determining the minimum sample volume which is manageable.

In Chapter 4 the multitude of practical difficulties experienced with the technique are detailed. It is concluded that the commercial Narrow-Bore Liquid Chromatograph under assessment is inadequate for efficient operation of these columns. Significant advances in instrument technology are necessary before this technique will achieve routinely its theoretical capabilities.

To Liz

for all her love, patience and understanding

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MEMORANDUM

The work described in this dissertation was conducted in the Department of Inorganic Chemistry of the University of Bristol, under the supervision of Dr. M. Cooke, between June, 1984 and November, 1986. The observations and recommendations herein are those of the author except where due reference has been made. No part of this work has been submitted for a degree to this or any other university.

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GLOSSARY OF SYMBOLS

1. General Terms

d = Diameter

L = Length

m = Mass of solute

P = Pressure

Q = Volumetric flow rate

r = Radius

(t) = (as a subscript) = Quantity in units of time

(v) = (as a subscript) = Quantity in units of volume

w = Width

η = Viscosity (of the mobile phase)

ρ = Density (of the mobile phase)

σ = Standard Deviation

σ^2 = Variance

2. Chromatography

A = van Deemter coefficient for eddy diffusion ($= 2\lambda d_p$)

B = van Deemter coefficient for longitudinal diffusion ($= 2\gamma D_m$)

C = van Deemter coefficient for resistance to mass transfer

$$\left[= \frac{a + bk' + ck'^2}{24\,(1 + k')}\ d_p^2/D_m + \frac{8k'}{\pi^2\,(1 + k')^2}\ d_f^2/D_s \right]$$

a,b,c = Quadratic coefficients in the van Deemter resistance to mass transfer in the mobile phase term

d_c = Column internal diameter

d_f = Effective film thickness of the stationary phase

d_p = Mean particle diameter

D_m = Diffusivity of a solute in the mobile phase

D_s = Diffusivity of a solute in the stationary phase

H = Height equivalent to a theoretical plate

$\hat{H}$ = Apparent height equivalent to a theoretical plate

H_c = Height equivalent to a theoretical plate in the column

k' = Capacity factor of a solute ($= (t_R - t_o)/t_o = (V_R - V_o)/V_o$)

L_c = Column length

L_δ = Detector flow cell length

L_t = Tubing length

m_i = Mass injected

N = Number of theoretical plates

$\hat{N}$ = Apparent number of theoretical plates

N_c = Number of theoretical plates in the column

r_c = Column radius

r_δ = Detector flow cell radius

r_t = Tubing radius

R_s = Resolution

t_o = Column dead time = retention time of an unretained solute

t_R = Retention time of a retained solute

u = Mobile phase linear velocity

V_c = Column volume

V_δ = Detector flow cell volume

V_i = Injection volume

V_o = Column dead volume = retention volume of an unretained solute

V_R = Retention volume of a retained solute

w_B = Peak width at baseline between tangents

$w_{0.5}$ = Peak width at half peak height

$w_{0.607}$ = Peak width at 60.7% of peak height = peak half-width

X_C = Concentration sensitivity of a HPLC system

X_δ = Concentration sensitivity of a detector

γ = Obstructive factor for molecular diffusion; a constant for a given column which is dependent upon the column packing efficiency

ε = Column porosity = fraction of the column volume occupied by mobile phase

ε^o = Snyder's solvent strength function

θ^2 = Factor representing the percentage contribution to the total variance of a system component

θ_i^2 = The percentage contribution of σ_i^2 to σ_{tot}^2

θ_δ^2 = The percentage contribution of σ_δ^2 to σ_{tot}^2

σ_ϕ^2 = The composite percentage contribution of σ_τ^2 and σ_Ω^2 to σ_{tot}^2

κ = A constant dependent upon the height from the baseline at which the peak width is measured

λ = A constant for a given column which is dependent upon the column packing efficiency

σ_{tot}^2 = Total or observed peak variance

σ_c^2 = Intra-column peak variance (= $\sigma_{ed}^2 + \sigma_{ld}^2 + \sigma_{rm}^2 + \sigma_{rs}^2$)

σ_{ed}^2 = Peak variance due to eddy diffusion

σ_{ld}^2 = Peak variance due to longitudinal diffusion

σ_{rm}^2 = Peak variance due to resistance to mass transfer in the mobile phase

σ_{rs}^2 = Peak variance due to resistance to mass transfer in the stationary phase

σ_{xc}^2 = Extra-column peak variance (= $\sigma_i^2 + \sigma_t^2 + \sigma_\delta^2 + \sigma_\tau^2 + \sigma_\Omega^2$)

σ_i^2 = Peak variance due to injection

σ_t^2 = Peak variance due to connecting tubes

σ_δ^2 = Peak variance due to the detector flow cell

σ_τ^2 = Peak variance due to detector response time

σ_Ω^2 = Peak variance due to recorder response time

3. Electrochemistry

a = Nozzle diameter

A_e = Electrode surface area

C = Bulk concentration of the electroactive species

D = Diffusivity (or diffusion coefficient) of the electroactive species

E = Potential

$E_{\frac{1}{2}}$ = Half-wave potential

E_{app} = Applied potential

E_{opt} = Optimum operating potential for a given series of electroactive species eluted under given chromatographic conditions

F = Faraday constant (96,487Cmol^{-1})

I = Current

I_a = Anodic (oxidation) current

I_c = Cathodic (reduction) current

I_ℓ = Limiting or diffusion current

k = Factor dependent upon cell geometry

l = Characteristic of electrode length

L_e = Electrode length

n = Number of electrons transferred in the electrode reaction

q = Function relating signal-to-noise ratio and electrode dimensions

r_e = Electrode radius

Re_x = Modified Reynolds Number (= $ul\nu^{-1}$)

Sc = Schmidt Number (= νD^{-1})

w_e = Electrode width

α = Factor dependent upon cell geometry

ν = Kinematic viscosity (= η/ρ)

ω = Characteristic of electrode width

4. Chemometrics

a = Intercept of the regression line with the ordinate (Y) axis

b = Gradient of the regression line

F = Snedecor's F statistic (Tabulated)

n = Number of observations

r = Correlation coefficient

s_{YX} = Sample standard error (Regression of Y on X)

s^2 = Sample variance

t = Student's t statistic (Tabulated)

X = "Controlled" variable (concentration or mass injected)

$\overline{X}$ = Mean value of X = $\Sigma X_i/n$

X_{LD} = Statistical detection limit of a substance based on its calibration data (99% confidence)

X_o = A selected value of X

x = $X-\overline{X}$

Y = Observed response (peak area or peak height)

$\overline{Y}$ = Mean value of Y = $\Sigma Y_i/n$

$\hat{Y}$ = Predicted value of the true response for a given value of X

Y_L = The lower limit on that predicted individual X value which exceeds the 99% upper prediction limit on the expected blank, Y_{UB}

Y_Q = The response calculated from the regression line which corresponds to X_{LD}

Y_{UB} = The 99% upper prediction limit on the expected blank response, a

y = $Y-\overline{Y}$

z_p = The normal deviate

α = Probability factor

ν = Number of degrees of freedom

Σ = Summation of

χ^2 = Chi-squared statistic (Tabulated)

ABBREVIATIONS

a.c.	=	Alternating current
Ac	=	Acetate
AE	=	Auxiliary electrode, counter electrode
AFID	=	Alkali Flame Ionisation Detector
AN	=	Aniline
ATP	=	Adenosine triphosphate
BF	=	Bufotenine, N,N-dimethyl-5-hydroxytryptamine
BMH	=	Bristol Maternity Hospital
b.p.	=	Boiling point
BRI	=	Bristol Royal Infirmary
CNS	=	Central nervous system
CPE	=	Carbon paste electrode
CS	=	Chromatographic Standard
csf	=	Cerebrospinal fluid
Ct	=	Citrate
DA	=	Dopamine
d.c.	=	Direct current
DHBA	=	3,4-dihydroxybenzoic acid
DME	=	Dropping mercury electrode
DOPAC	=	3,4-dihydroxyphenylacetic acid
DPV	=	Differential Pulse Voltammetry
DW	=	Single distilled water
EC,-EC	=	Electrochemistry, electrochemical (detection)
ECD	=	Electrochemical Detector
EDTA	=	Ethylenediaminetetraacetic acid
ELCD	=	Electron Capture Detector
EP	=	Epinephrine, adrenaline
FID	=	Flame Ionisation Detector
FL,-FL	=	Fluorescence Detector, fluorescence detection
f.s.d.	=	Full scale deflection
FTIR,-FTIR	=	Fourier-transform Infra-red Spectrometry, Fourier-transform infra-red detection
GC	=	Gas Chromatography
GCE	=	Glassy carbon electrode
5HI2A	=	5-hydroxyindole-2-carboxylic acid
5HIAA	=	5-hydroxyindole-3-acetic acid

HPLC	=	High Performance Liquid Chromatograph(y)
HSA	=	1-heptanesulphonic acid
5HT	=	5-hydroxytryptamine, serotonin
6HT	=	6-hydroxytryptamine
5HTP	=	5-hydroxytryptophan
HVA	=	Homovanillic acid, 3-methoxy-4-hydroxyphenylacetic acid
IAA	=	Indole-3-acetic acid
ID	=	Internal diameter
IIA	=	Ion-interaction agent
-IIC	=	Ion-Interaction Chromatography
IPA	=	Ion-pairing agent
-IPC	=	Ion-Pair Chromatography
IS	=	Internal standard
I3S	=	Indoxyl-3-sulphate, indican
IXC	=	Ion Exchange Chromatography
LC	=	Liquid Chromatography
LDV	=	Low dead volume
LOD	=	Limit of detection
LOQ	=	Limit of quantitation
MeCN	=	Acetonitrile
MeOH	=	Methanol
MS,-MS	=	Mass Spectrometry, mass spectrometric detection
MW	=	Molecular weight
$NAD^{\oplus}$,NADH	=	Nicotinamide adenine dinuclectide
$NADP^{\oplus}$,NADPH	=	Nicotinamide adenine dinucleotide phosphate
NB	=	Narrow-Bore
NE	=	Norepinephrine, noradrenaline
N-Me-5HT	=	N_{ω}-methyl-5-hydroxytryptamine
NPV	=	Normal Pulsed d.c. Voltammetry
OD	=	Outer (external) diameter
ODS	=	Octadecylsilane
PC	=	Paper Chromatography
-PC	=	Partition Chromatography
PKU	=	Phenylketonuria
PPP	=	Platelet-poor plasma
2-PrOH	=	Propan-2-ol
PRP	=	Platelet-rich plasma

p.s.i.	=	Pounds per square inch
PTFE	=	Polytetrafluoroethylene
RCF	=	Relative centrifugal force
RE	=	Reference electrode
REA	=	Radioenzymaticassay
RIA	=	Radioimmunoassay
RP	=	Reverse Phase
RPV	=	Reverse Pulsed d.c. Voltammetry
RSD	=	Relative standard deviation
SCOT	=	Surface coated open tubular
SD	=	Standard deviation
SE,SEM	=	Standard error (measurement)
sf	=	Solvent front
S/N	=	Signal-to-noise (ratio)
S/S	=	316 stainless steel
SW	=	Rapid-Scan Square Wave Voltammetry
T_4	=	Thyroxine
THF	=	Tetrahydrofuran
TLC	=	Thin Layer Chromatography
TP	=	Tryptophan
TSH	=	Thyrotropin, thyroid-stimulating hormone
T&SH	=	Taunton & Somerset Hospital
UV,-UV	=	Visible/ultra-violet spectrometry, visible/ultra-violet detection
WB	=	Wide-Bore
WCOT	=	Wall coated open tubular
WE	=	Working electrode
ZDV	=	Zero dead volume
α-Me TP	=	α-Methyltryptophan
μ(LC)	=	Micro-column

CHAPTER 1

General Introduction

Contents

1.1 Nomenclature of Liquid Chromatography Columns

Since 1976, when Scott and Kucera[1] used 1mm internal diameter (ID) liquid chromatography (LC) columns and introduced the term 'microbore' to refer to such columns, there has been growing confusion regarding LC column nomenclature. In the ten year period following this publication great interest has been aroused, and hence much research effort has been directed towards the development of small-diameter LC columns and associated hardware (see Section 1.2). The trend has been towards smaller and smaller diameter columns, resulting in the evolution and utilisation of both packed and open-tubular capillary columns with IDs of as little as 5μm.[2] This growth of small-diameter column research by a large number of unrelated groups has led to a plethora of undefined terms for such columns. Basey and Oliver[3] noted nine different descriptions of small-diameter LC columns together with the range of IDs to which each term referred. The descriptions were found to overlap to a large extent and so, recognising that a nomenclature problem existed, Basey and Oliver proposed that column descriptions such as 'microbore' be abandoned. They suggested that such terms simply be replaced by specified ID measurements in order to clarify texts and to aid electronic literature search processes by reducing the number of keywords required to extract relevant references from computer libraries. Unfortunately this proposal has gone largely unheeded and an abundance of ill-defined or undefined terms continues to abound in the literature.

The author finds it convenient to rigorously define terms for LC columns to be referred to in this dissertation. The selected descriptions are presented and defined in Table 1.1.

Table 1.1 Proposed Nomenclature of HPLC Columns

Column Description	Abbreviation For Column Type	ID range (mm)	Typical IDs Used (mm)
Micro-column	μ	ID < 0.5	-
Narrow-bore	NB	0.5 ≤ ID < 3.0	0.5, 1.0, 2.1
Conventional		3.0 ≤ ID ≤ 5.0	3.9, 4.6
Wide-bore or Preparative	WB	ID > 5.0	7.8

It should be noted that no such rigid boundaries in the behaviour of LC columns exist but, due to the typical IDs employed in general use it is convenient to compartmentalise columns in the above manner. The designation of 0.5mm as the lower limit of Narrow-Bore (NB) LC was made on instrumental grounds, i.e. down to 0.5mm ID conventional instrumentation may be utilised with some success under certain stringent conditions but at diameters smaller than this specialist instrumentation is essential. The term 'capillary' was rejected in favour of 'micro-column' because the only rigorous definition of capillary to be published[4] which refers to gas chromatography (GC) columns as: "... column(s) of capillary dimensions generally less than 1mm internal diameter" was inappropriate for this classification. The prefix 'micro-' to refer to columns of less than 0.5mm ID does appear to be coming into common usage. The original description 'microbore' coined by Scott and Kucera[1] was not considered suitable for reasons of confusion. The general term 'small-diameter' will be used to refer to all columns of smaller internal diameter than are used conventionally, i.e. < 3mm.

1.2 The Early Development of Small-Diameter Column Liquid Chromatography

Small-diameter columns are not new to the field of liquid chromatography. In the late 1960s Horváth and co-workers[5,6] employed columns of between 1 and 3m length and of 1mm ID dry-packed with their new pellicular materials. Not unexpectedly, due to the inefficient packing method employed and the primitive instrumentation these columns only produced 1000 theoretical plates, although because of the novel fabrication of the packing material this was a 300-500% improvement over typical efficiencies observed at that time. The potential of small-diameter columns was not recognised immediately, primarily because the importance of extra-column dispersion was neither understood nor appreciated then. Extra-column dispersion (or extra-column band broadening) results primarily from kinetic processes which manifest themselves in the finite volume of the instrument external to the column and between the point of injection and the point of detection (extra-column volume), processes which can seriously degrade the separation performance of the column. This phenomenon is discussed in detail in Section 1.4.3.

With the introduction of micro-particulate silica and the development of slurry-packing techniques in the early 1970s, columns of typically 25cm x *ca.* 5mm ID became adopted as standard. Columns of small diameter fell into general disuse, primarily due to packing difficulties and the absence of appropriate low volume HPLC hardware with which to utilise such columns effectively.

It was a paper published in 1976 by Scott and Kucera[1] that provoked the present interest in, and enthusiasm for, small-diameter columns. Novel column technology was of secondary importance in this particular publication; size exclusion chromatography on silica gel was the primary goal. The authors slurry-packed and coupled a series of ten 1m x 1mm ID columns with

20μm diameter particles and produced 250,000 theoretical plates (≡ 25,000 plates m^{-1}) which was by then the highest observed LC column efficiency ever reported. In order to achieve this, Scott and Kucera utilised a modified commercial UV flow cell to restrict extra-column band broadening.

The following year saw the publication of a paper by Machleidt et al.[7] which described the application to protein sequencing of 1m x 1-3mm ID columns slurry-packed with an ion exchanger. Also in 1977 a Japanese group under Ishii began publishing a series of papers[8-12] concerning the preparation of short (5-35cm), narrow (0.25-0.5mm ID) slurry-packed columns and the design, construction and application of equipment with which to utilise such columns. Specialist instrumentation was fabricated for this purpose including a syringe micro-feeder solvent delivery system and a novel home-built low volume UV flow cell.

In 1978, material appeared in the literature describing the preparation and use of columns of even smaller IDs. Tsuda and Novotný[13] reported the preparation of packed μLC columns of 50-200μm ID drawn from dry-packed 0.25mm ID glass capillaries. In order to overcome limitations imposed on the system with respect to band broadening Tsuda and Novotný introduced a split injector system and a post-column pre-detector solvent make-up flow, a technique commonly practiced in capillary GC. Even running under significant instrument limitations, plate counts of the order of 10,000m^{-1} were obtained.

Open-tubular μLC columns were developed concurrently with packed μLC columns and simultaneously by a number of research groups including Ishii and co-workers[14,15], Novotný and associates[16], Dewaele and Verzele[17], and Tijssen[18], following the earlier pioneering work of Horváth et al.[5] and Nota et al.[19] Columns of greatly varying lengths (2.5-100m) and internal

diameters (50-800μm) were experimented with, some were untreated or simply alkaline etched while others were coated to produce SCOT or WCOT columns similar in construction to those used in capillary GC work. Some workers elected to employ conventional LC hardware with few modifications[5,17,18], some used greatly adapted commercial equipment[16,19] while the remainder utilised home-made precision engineered specialist instrumentation.[14,15] Open tubes offer considerably less resistance to flow than packed tubes which enables longer lengths to be utilised at identical pressure drops which leads to higher efficiency potential. Furthermore, as theory predicts, workers found that the smaller the column diameter then the higher the column efficiency. However, gross practical difficulties with the fabrication of equipment with which to operate very long, extremely narrow columns at the very low flow rates required were encountered in these early studies.

In the meantime, Scott and Kucera had continued their work on 1mm ID NB columns and in 1979 published papers describing their general performance and characteristics[20], their use for extremely rapid analysis[21] and their application to the separation of substances of biological origin.[22] For all this work Scott and Kucera utilised a conventional LC instrument which they adapted in several ways in order to reduce extra-column volume and to provide accurate pulse-free solvent delivery at required flow rates. Scott and Kucera successfully demonstrated the use of NB columns to achieve high efficiency separations and high speed separations with solvent economy and high mass sensitivity and in so doing showed the potential of LC employing NB columns.

Two distinct schools of thought evolved around this time which led to the development of small-diameter column LC in two different directions.

These were: (1) moderate reduction in ID of columns and their utilisation with conventional or modified conventional LC hardware as advocated by Scott and Kucera (i.e. NBLC), and (2) gross miniaturisation of columns and the design and construction of specialist analytical instrumentation in which to accommodate such columns, the strategy adopted by Ishii's group, (i.e. μLC). Severe technological problems posed by the latter approach, particularly regarding the engineering and operation of ultra-micro high-specification LC equipment, have limited μLC to the research laboratory since its inception. The great promise of μLC, particularly in open-tubular configuration, has yet to be realised sufficiently to warrant commercial interest. It is the former approach advocated by Scott and Kucera that has been exploited commercially and is the subject of the research reported in this dissertation.

1.3 Advantages and Disadvantages Claimed for Narrow-Bore Liquid Chromatography Columns[23-26]

There are three basic advantages to NB columns, but one particular attribute, solvent economy, is probably the main cause of the present renaissance in the use of this column geometry. The efficiency realised from well-packed NB columns is exactly equivalent to their larger diameter counterparts, providing they are operated at the same mobile phase linear velocity. Consequently, as the mobile phase volumetric flow rate is proportional to the product of the linear velocity and the column cross sectional area, solvent consumption will be reduced proportionally to the square of the column radius. This situation yields certain economic and practical advantages. Operating costs may be drastically reduced[20] both in terms of the reduced solvent requirement and also the need for a substantially

smaller amount of expensive stationary phase in order to pack a column. As an alternative to a saving in the solvent costs, the use may be made of very high purity or exotic solvents which ordinarily would be discounted on ecomonic grounds.[27,28]

The second important benefit of NB columns is their high mass sensitivity which, like the concentration sensitivity, is a way of defining the overall detection limits of a chromatographic system. For a column of given length and efficiency, the smaller the ID of that column then the smaller the mass of solute that can be detected, and consequently the less sample is required for analysis. The advantage of high mass sensitivity becomes particularly apparent when dealing with trace quantities of materials in samples of restricted size, as is frequently encountered with the separation and determination of substances present in samples of biological origin.

The third attribute of NB columns is less obvious. Such columns have, as a result of their geometry, a very low heat capacity which allows them to be thermostatted to a precise and constant temperature fairly easily. This is because heat generated by viscous flow through a column can be dissipated through the column walls more efficiently with NB geometry.[29] Furthermore, less heat is actually generated in a NB column compared with a conventional column at a given linear flow rate.[29] In this way greater potential temperature stability is available with NB column technology, which is important for provision of improved accuracy and precision of retention times in the chromatogram. Thus, improved peak recognition is attainable when chromatographing authentic samples. Authentic samples may be defined as samples comprising analytes in a fairly complex matrix and are distinguished from synthetic samples which are simply comprised of pure standard compounds dissolved in an appropriate solvent.

In addition to the aforementioned advantages, a fourth attribute of NB columns has been recognised which arises essentially as a consequence of the low volumetric flow rates employed, and this is the ability to couple a mass spectrometer directly to a HPLC system. This is only beneficial if sample size is greatly restricted otherwise conventional interfacing with an effluent splitting device provides comparable performance.[25]

The disadvantages of NB columns also ironically arise from their geometry as, due to their small internal volume, they make certain stringent demands on the chromatographic equipment with which they are utilised. Similar mobile phase linear velocities are employed with NB columns as with conventional columns consequently the required volumetric flow rates are much smaller. As a result of this fact, pumps capable of delivering solvent at constant volumetric flow rates of the order of microlitres per minute are essential. Sample volumes will also be much smaller and thus specially designed sample injection valves are necessary. For identical reasons, detectors must possess low internal volume flow cells which generally, but not exclusively, require specialist design and construction. The application of a conventional 0.5µl volume electrochemical detector (ECD) to NB column technology is described in this dissertation (Chapter 3).

From this it follows that in order to realise the advantages of NBLC columns in routine analytical laboratories instrumentation of sufficiently high specification must be designed, manufactured and marketed by commercial companies. At the outset of this project at the beginning of 1984 such equipment had only very recently been introduced. The apparatus available for NBLC will be discussed in detail in Section 1.5.

A summary of the advantages and disadvantages of NB columns is presented in Table 1.2.

Table 1.2 Summary of the Major Advantages and Disadvantages Claimed for Narrow-Bore Columns

Advantages	Disadvantages
1. Solvent economy	1. The need for high specification instrumentation (pumps, injection valves, detectors)
2. High mass sensitivity	
3. High potential temperature stability	

1.4 Theoretical Aspects of Narrow-Bore Liquid Chromatography

1.4.1 Dispersion of a Solute Band - The Principle of the Summation of Variances

In order that the theory and properties of NB columns be appreciated, the fundamental importance of the contribution of physical processes to the dispersion of a solute band as it passes through a chromatographic system must be fully understood.

The width of the band of an eluted solute relative to its proximity to its nearest neighbour determines whether the two solutes are resolved or not and thus the band width directly controls the resolving power of the chromatographic system. This concept is illustrated diagrammatically in Figure 1.1. The ultimate band width of a solute is the result of a number of dispersion processes occurring in the chromatographic system, some of which take place in the column itself, and some in the sample injection valve, connecting tubing and detector. In order to determine the ultimate peak dispersion of the solute band as it is sensed by the detector, it is necessary to be able to calculate the final peak variance,

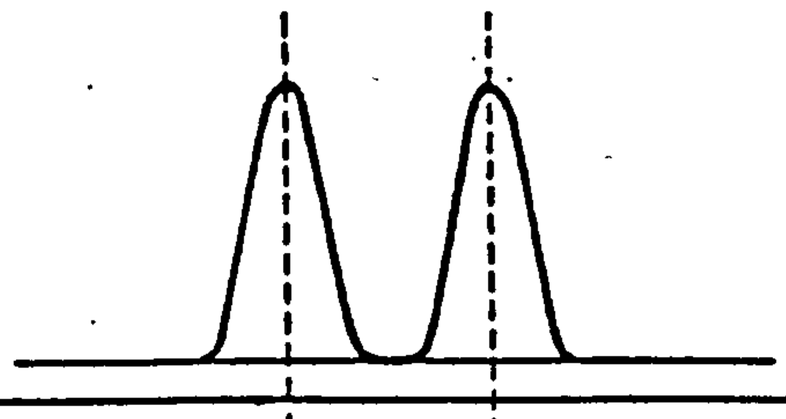
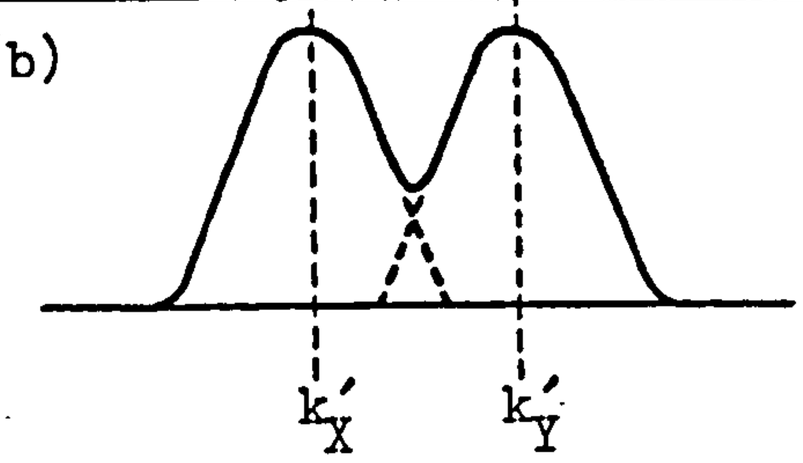
(a) 	Peaks selectively retained and narrow providing complete (baseline) resolution.
(b) 	Peaks equally selectively retained but poorly resolved due to broadness resulting from poorly controlled band spreading.

Figure 1.1 Illustration of the Dependence of Resolution on Bandwidth

which is the square of the standard deviation of the peak. This is achieved by taking into account the individual dispersion processes occurring in the total chromatographic system. It is not possible to sum the bandwidths resulting from the individual dispersion processes to determine the final bandwidth but it is possible to sum their variances. However, the summation of variances is only valid if all the dispersion processes are non-interacting and random in nature, i.e. the extent to which any one dispersion process progresses is independent of the development and progress of any other dispersive process.

Thus, assuming that there are z non-interacting, random dispersive processes occurring in the chromatographic system, then any process, p, acting alone will produce a Gaussian elution curve having a variance σ_p^2. Hence:

$$\sigma_1^2 + \sigma_2^2 + \sigma_3^2 \ldots + \sigma_z^2 = \sigma_{tot}^2 \qquad (1.1)$$

where σ^2_{tot} is the total observed variance of the solute band as displayed by the recording device.

Equation 1.1 is the algebraic enunciation of the principle of the summation of variances and is fundamentally important. It enables the critical factors controlling the chromatographic process in the column to be related to one another as was established by van Deemter et al.[30] (see Section 1.4.2). Furthermore, the summation of variances concept allows the contributions of the dispersive processes occurring in various parts of the chromatographic system to be combined to provide an assessment of the degree of the deleterious effect of the HPLC instrument on the chromatography.

1.4.2 Intra-Column Band Dispersion[24,31,32]

The column is the part of the chromatographic system in which the actual separation of solutes takes place. During the development of a chromatogram two mutually independent processes occur simultaneously in this region. First, the individual solutes are moved apart from one another and secondly, the width of each solute band is kept sufficiently narrow such that each solute is eluted discretely so preserving the quality of the initial separation.

The moving apart of the peaks is achieved by employing an appropriate stationary phase/mobile phase system that retains each solute within the column to different extents. This selective retention is achieved by exploiting the different molecular forces that can exist between the solute and the two phases. Those retention processes (and their operating mechanisms) that are relevant to this research project are discussed in detail in Section 3.1.4. For the purpose of this discussion it may simply be emphasised that the moving apart of the peaks in a column by selective

solute retention depends solely on the chemical nature of the phase system employed and is independent of the column geometry and the particle size of the packing material. Hence, NB columns and conventional columns are equivalent in this respect.

The other process that occurs in the column is the progressive dispersion of each solute band. It is important that this band broadening action is controlled and minimised, in order to ensure that once the solute bands have been moved apart they do not merge again by spreading. In contrast to solute selectivity, peak dispersion is strongly dependent on the particle size of the packing material and the physical properties of the solute and the mobile phase. The theory of band dispersion in a chromatographic column has been studied by many workers.[30,33-37] The equation that most accurately describes the relationship between peak dispersion, the mobile phase velocity and the physical properties of the phase system over the linear velocity range employed in HPLC is that of van Deemter et al.[30], which was first derived as long ago as 1956. The pertinence of the van Deemter equation has been well substantiated recently in a very extensive and thorough experimental investigation by Katz, Ogan and Scott.[38] Van Deemter postulated that three basic dispersion processes took place in the column and theoretically determined the variance contribution of each process. The individual variances so calculated were then integrated to determine the overall variance of the band using the principle of the summation of variances (Equation 1.1). The three basic dispersion processes that were postulated were eddy diffusion, longitudinal diffusion and resistance to mass transfer of the solute between the mobile and stationary phases. Eddy diffusion and resistance to mass transfer processes are illustrated in diagrammatic form in Figure 1.2 and are explained under their appropriate headings.

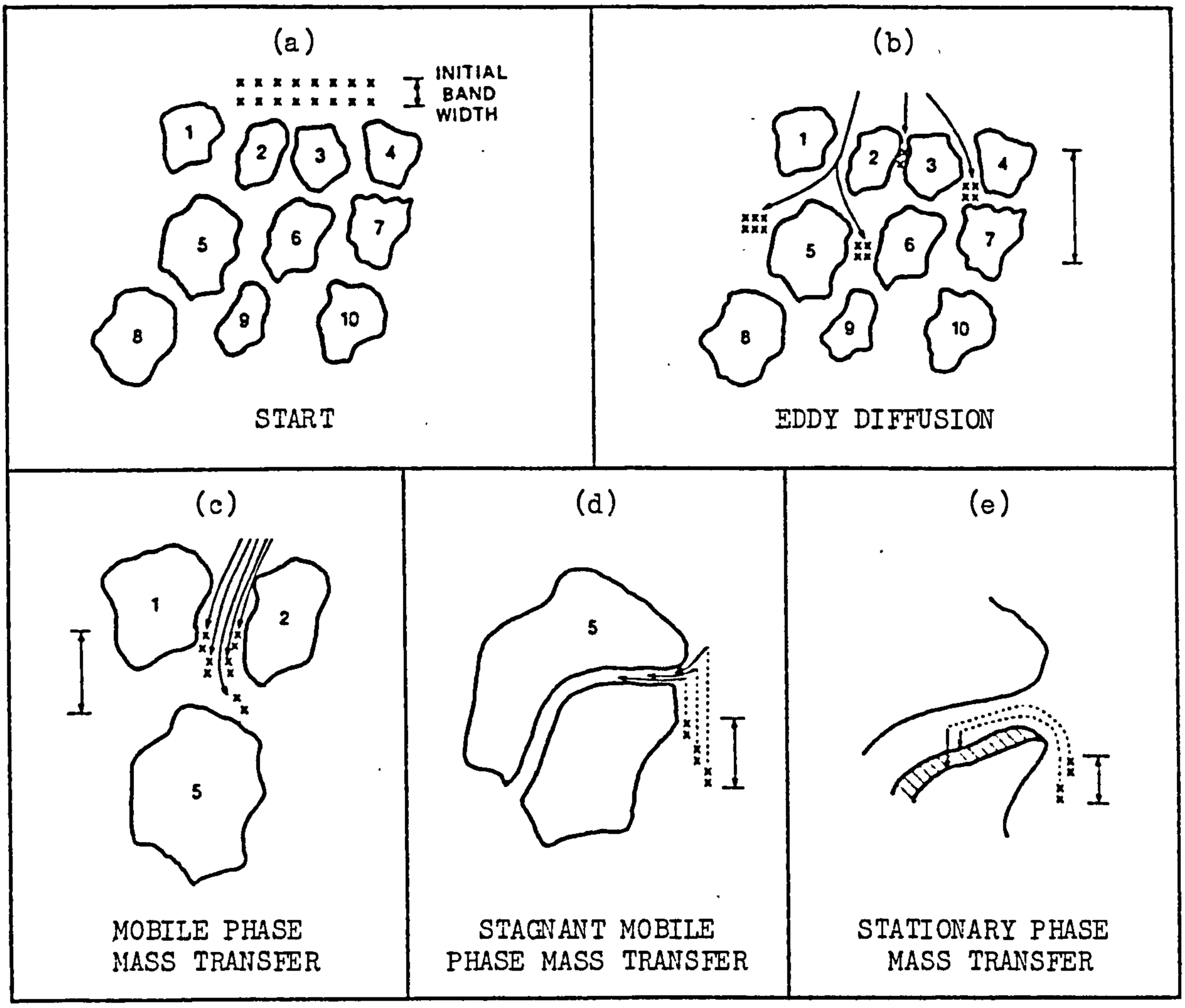


Figure 1.2 Diagrammatic Representation of some of the Dispersion Processes that occur within a Liquid Chromatography Column.

Figure 1.2(a) depicts a longitudinal section of the top of a column with the individual stationary phase particles numbered 1-10. Solute molecules are represented by x's and are shown immediately after injection where they form a narrow band at the top of the column as indicated.

1.4.2.1 Eddy Diffusion

Eddy diffusion, otherwise known as the multipath effect, arises from the different microscopic flowstreams that the solvent follows between different particles within the column. As a result, solute molecules take different paths through the interstices of the packed bed, depending on which flowstreams they migrate within. Liquid flows faster in wide channels and slower in narrow ones, hence in unit time some solute molecules will progress further down the column than others. This process is depicted in Figure 1.2(b). From this illustration it can be seen that molecules that follow the relatively wide flowpath between particles 1 and 2 (or 5 and 6) migrate further down the column in a given time than do molecules progressing through the relatively narrow flowpath between particles 2 and 3. Clearly such a process would contribute to band variance and van Deemter proposed that the band variance arising from eddy diffusion (σ^2_{ed}) could be expressed:

$$\sigma^2_{ed} = 2\lambda d_p \qquad (1.2)$$

where λ = a constant depending on the quality of the packing of the stationary phase,

and d_p = the mean particle diameter.

1.4.2.2 Resistance to Mass Transfer

Resistance to mass transfer of the solute between the two phases is the second effect that van Deemter considered to cause peak dispersion and, in most instances, especially at high mobile phase velocities, this effect is the major contributor to the overall peak variance. Essentially, three processes can be visualised giving rise to resistance to mass transfer, viz. mobile phase mass transfer, stagnant mobile phase mass transfer and

stationary phase mass transfer, which are illustrated in Figure 1.2, parts (c), (d) and (e) respectively.

Mobile phase mass transfer arises due to the existence of a flow profile across a single flowstream or path between surrounding particles. As is exemplified in Figure 1.2(c), where the flowstream between particles 1 and 2 is shown, the liquid adjacent to a particle moves slowly or is stationary, whereas liquid in the centre of the flowstream moves fastest. Hence, in unit time, solute molecules near to a particle move a shorter distance down the column than do solute molecules in the middle of the flowstream. This action necessarily results in a spreading of molecules along the column.

A second mobile phase mass transfer effect is what is termed stagnant mobile phase mass transfer, which is presented in Figure 1.2(d). With porous particulate stationary phases the mobile phase contained within the pores of a particle is stagnant or unmoving. Solute molecules diffuse into and out of these pores. Those molecules having a short residence time in a pore will spend correspondingly longer in the flowing external mobile phase and will migrate further down the column in a given time than molecules having a long residence time in a pore. Again an increase in band spreading results.

The effect of stationary phase mass transfer is demonstrated in Figure 1.2(e). After solute molecules diffuse into a pore they may penetrate the stationary phase (hatched region) or become attached to it in some fashion. In a similar way to stagnant mobile phase mass transfer, those molecules that penetrate deeper into the stationary phase have a greater residence time in the particle and so migrate down the column a shorter distance in unit time than do molecules which spend less time in the stationary phase. This too provides a band broadening effect.

Two functions have been proposed to describe the resistance to mass transfer between the two phases, (σ^2_{rm}, σ^2_{rs}). These are :

(1) Resistance to mass transfer in the mobile phase,

$$\sigma^2_{rm} = \frac{f_m(k')d_p^2\, u}{D_m} \qquad (1.3)$$

(2) Resistance to mass transfer in the stationary phase,

$$\sigma^2_{rs} = \frac{f_s(k')d_f^2\, u}{D_s} \qquad (1.4)$$

where k' = the capacity factor of the solute,

u = the mean mobile phase linear velocity,

D_m = the diffusivity of the solute in the mobile phase,

D_s = the diffusivity of the solute in the stationary phase,

and d_f = the effective film thickness of the stationary phase.

Van Deemter et al. in their original paper[30] showed that

$$f_s(k') = \frac{8k'}{\pi^2(1 + k')^2} \qquad (1.5)$$

In this work the authors dealt exclusively with the question of gas chromatography where resistance to mass transfer in the mobile phase is negligible, hence only resistance to mass transfer in the stationary phase was considered. When the van Deemter equation was generalised to include liquid chromatography it was expanded to include the then significant resistance to mass transfer in the mobile phase terms.

The generally accepted form of $f_m(k')$ for the resistance to mass transfer in the mobile phase is,

$$f_m(k') = \frac{a + bk' + ck'^2}{24(1 + k')} \qquad (1.6)$$

where the quadratic coefficients take the values a = 1, b = 6 and c = 11. However, Katz et al. recently conducted an extensive study[38], using an instrument of known and controlled extra-column dispersion, which generated accurate and precise experimental data from which they derived different values for the quadratic coefficients, i.e., a = 0.37, b = 4.69 and c = 4.04. These latter values are recommended and may be applied with confidence.

1.4.2.3 Longitudinal Diffusion

Finally, there is an additional contribution to molecular dispersion, known as longitudinal diffusion, which is not illustrated in Figure 1.2. Whether the mobile phase within the column is moving or at rest, normal diffusion processes occur and, apart from the aforementioned effects, this gives rise to a further spreading of solute molecules along the column. Obviously the longer the solute remains in the column, the more the solute will diffuse and thus the variance of the band due to this effect will be inversely proportional to the linear mobile phase velocity. Van Deemter proposed that the band variance due to longitudinal diffusion (σ^2_{ld}) could be expressed:

$$\sigma^2_{ld} = \frac{2\gamma D_m}{u} \qquad (1.7)$$

where γ = the obstructive factor for molecular diffusion, which is a constant and is dependent on how well the column is packed.

Longitudinal diffusion is often not an important effect, but is significant at low eluent flow rates for columns packed with small particles.

1.4.2.4 The van Deemter Equation

Van Deemter et al. considered that the total dispersion of a solute band eluting from a column was contributed to by all these individual processes. They expressed this dispersion as the variance per unit length of the column and assigned it the symbol H. The term H is known as the height equivalent to a theoretical plate in the column.

The van Deemter equation is compiled by utilising the principle of the summation of variances (Equation 1.1), i.e.,

$$\sigma_c^2 = \sigma_{ed}^2 + \sigma_{ld}^2 + \sigma_{rm}^2 + \sigma_{rs}^2 \qquad (1.8)$$

where σ_c^2 = the total peak variance generated in the column.

Therefore:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f_m(k')d_p^2 u}{D_m} + \frac{f_s(k')d_f^2 u}{D_s} \qquad (1.9)$$

Equation 1.9 is often expressed in the simplified form :

$$H = A + \frac{B}{u} + Cu \qquad (1.10)$$

where A, B, and C are all constants for a given solute in a given chromatographic column/eluent system.

Figure 1.3 shows a typical graph of column variance vs. mobile phase linear velocity. The points are experimentally determined values and the solid curve is that predicted by the van Deemter equation. The individual contributions of each dispersion process are also plotted.

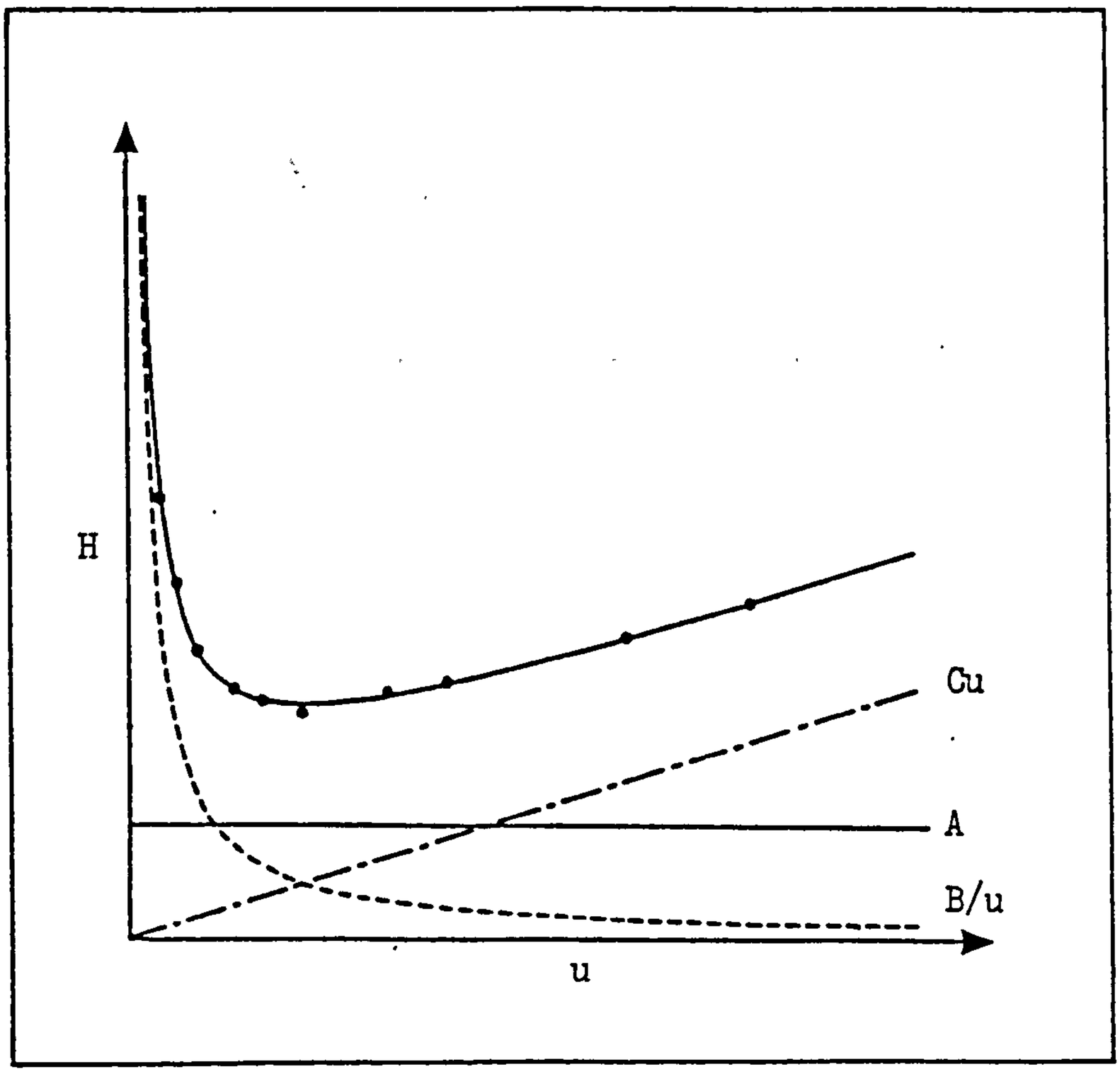


Figure 1.3 A Typical Plot of Height Equivalent to a Theoretical Plate (H) vs. Mobile Phase Linear Velocity (u) Demonstrating the Relative Contributions of the A, B and C Terms to the van Deemter Equation.

The linear velocity, u is obtained from,

$$u = L_c/t_o \tag{1.11}$$

where L_c = column length

and t_o = elution time of a fully excluded solute[38],

and the apparent plate height, $\hat{H}$, is given by,

$$\hat{H} = L_c/\hat{N} \tag{1.12}$$

where $\hat{N}$ = the apparent number of theoretical plates in the column.

$\hat{N}$ is calculated from one of several equivalent expressions having the general formula :

$$\hat{N} = \kappa\left(\frac{t_R}{w_{(t)}}\right)^2 \quad \text{or} \quad \kappa\left(\frac{V_R}{w_{(v)}}\right)^2 \tag{1.13}$$

where t_R = the retention time of the measured peak,

$w_{(t)}$ = the peak width (in time units),

V_R = the retention volume of the measured peak,

$w_{(v)}$ = the peak width (in volume units),

and κ = a constant dependent upon the height (from the baseline) at which the peak width is measured.

Equation 1.13 is derived from the statistics of a Gaussian distribution, the parameters of which are illustrated in Figure 1.4 and tabulated in Table 1.3.

Table 1.3 Alternatives for the Calculation of Apparent Column Efficiency

w	w in terms of SD*	κ	Method of calculation of $\hat{N}$
$w_{0.607}$	2σ	4	Half-width or inflection
$w_{0.5}$	2.35σ	5.54	Width at half-height
$w_{0.324}$	3σ	9	3σ
$w_{0.134}$	4σ	16	4σ
$w_{0.044}$	5σ	25	5σ
w_B	4σ	16	Tangential baseline width
$w_{B'}$	6σ	36	Total baseline width

* SD = standard deviation

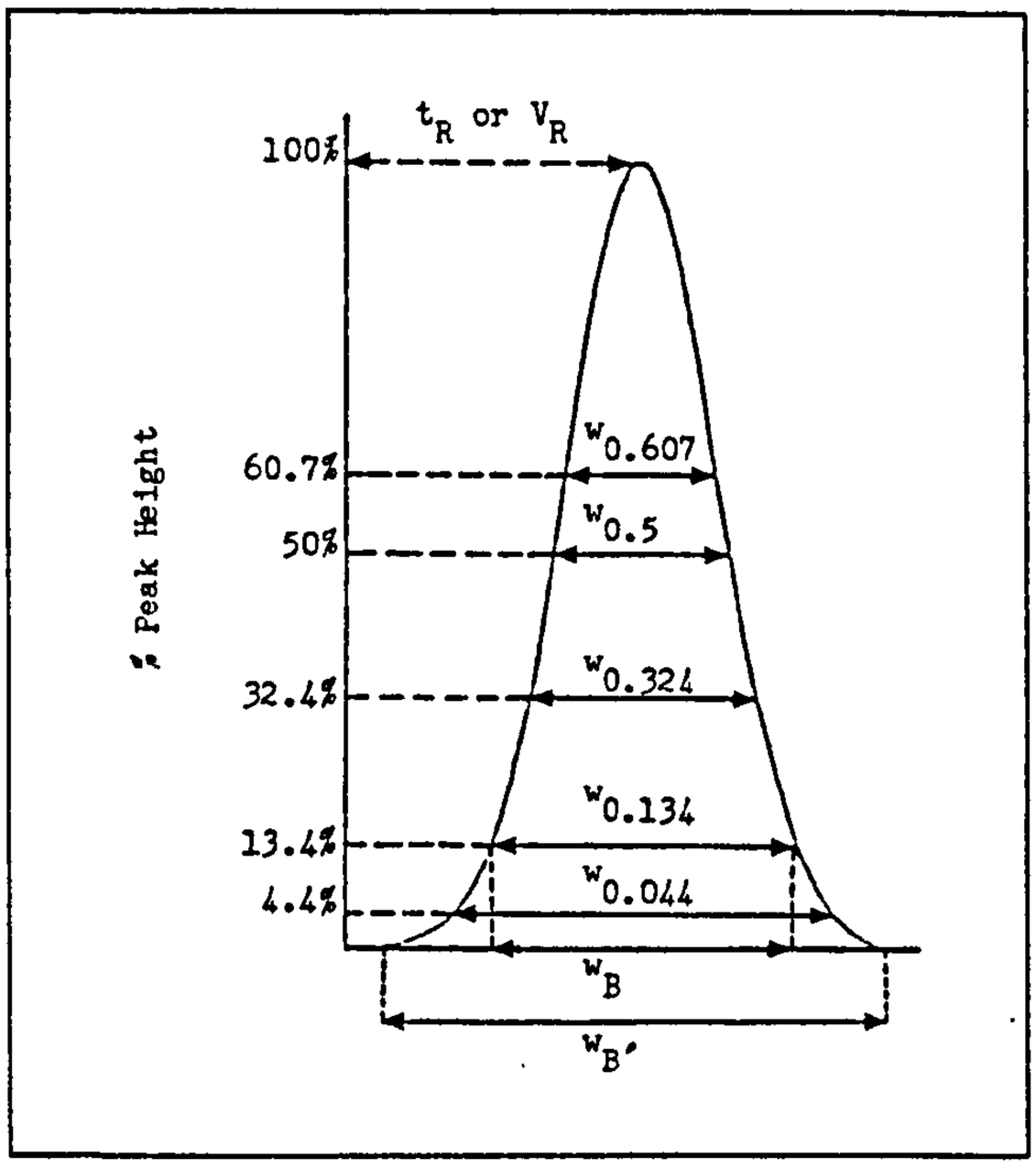


Figure 1.4 Illustration of the use of the Various Widths of a Gaussian Peak Profile for the Calculation of Apparent Column Efficiency

The 'half-width', 'width at half-height' and 'tangential baseline' methods are most commonly employed by chromatographers.

The characteristic of the van Deemter equation which is of great pertinence to NB columns is that peak dispersion is totally independent of column radius. This deduction has been confirmed experimentally.[39] Hence, on-column peak dispersion is identical for both NB and conventional columns provided that both columns are similarly packed and are employed with the same phase system. In fact, NB columns have frequently been found to be more difficult to pack in practice[40] so in many instances the plate count, and hence the resolution, obtained from these columns is often inferior to that obtained from conventional columns of equal length operated under identical conditions.

From the independence of peak dispersion on column radius it follows that any theoretical calculations of the chromatographic properties of a separation derived using the van Deemter equation (e.g., analysis time, resolving power) will all be independent of column radius. This is an extremely important point. Theoretically, any two columns of equivalent length packed equally well with the same stationary phase and operated with identical mobile phases delivered at identical linear velocities will yield exactly the same resolution and analysis time.[41]

1.4.3 Source and Control of Extra-Column Band Dispersion[25,26,42]

Extra-column band dispersion is that contribution to peak variance which takes place outside the column. Because this phenomenon may significantly degenerate the separation that has been previously obtained in the column itself, it is extremely important to understand the sources of extra-column dispersion and, consequently, how to control it.

1.4.3.1 Origins of Extra-Column Dispersion - Viscous Flow and the Parabolic Velocity Profile

Components of the apparatus such as sample injection loops, connecting tubes and detector flow cells can often be treated as open tubes with a circular cross-section through which the sample flows. In LC the flow nearly always can be considered to be laminar in nature. In a cylindrical tube laminar flow is accompanied by a parabolic velocity profile across the tube as illustrated schematically in Figure 1.5. Solute molecules introduced into a fluid stream where laminar flow exists will spread as a consequence of the flow profile, i.e. those molecules towards the centre of the tube will migrate down the tube faster than average whereas those

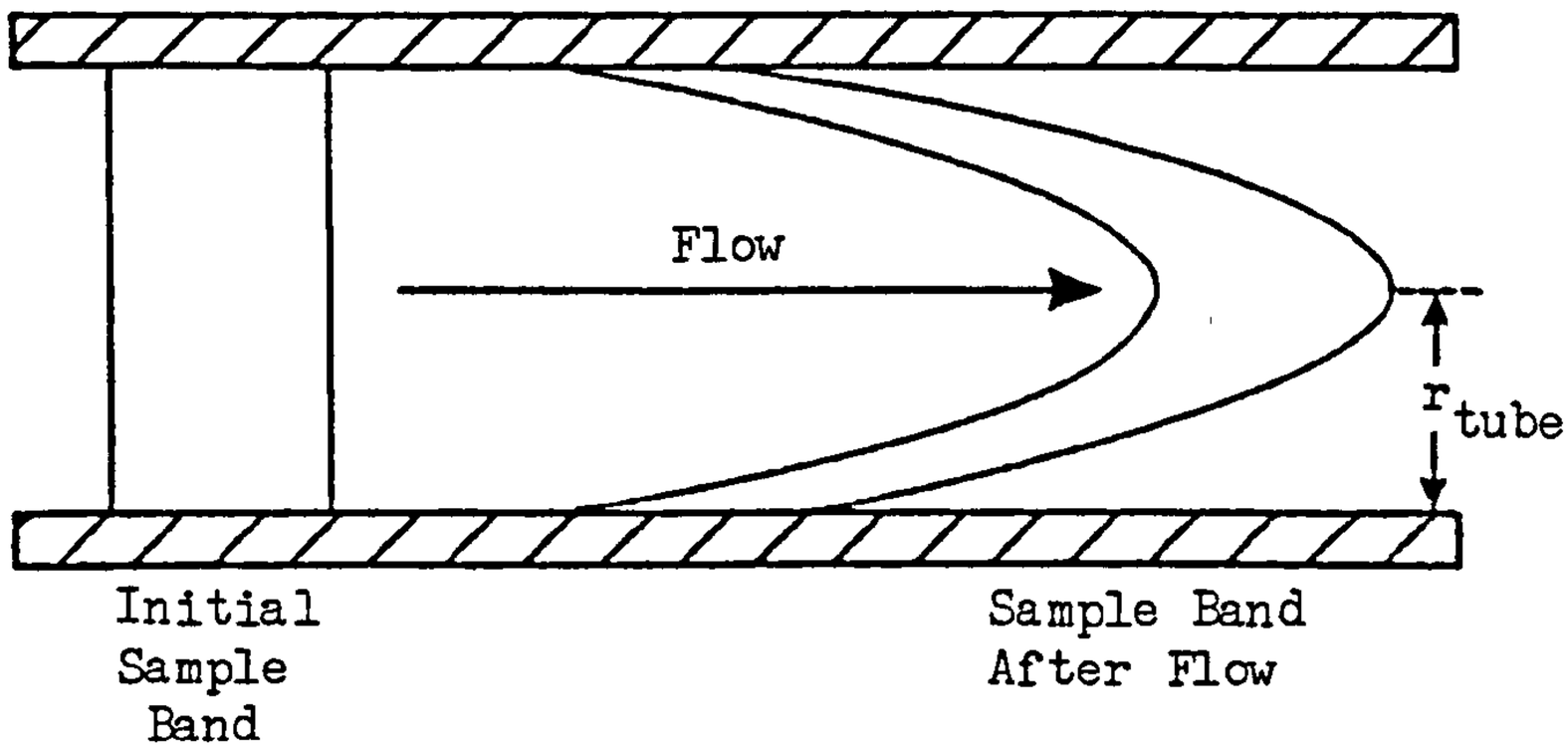


Figure 1.5 Schematic Representation of the Parabolic Velocity Profile Obtained from Laminar Flow in a Cylindrical Tube

molecules near the walls of the tube will travel down the tube slower than average. It follows that such relative velocities lead to a significant band dispersion which could probably be controlled if the dispersion can be quantitatively described and the inter-relationship between the parameters influencing the dispersion can be established.

The theory of the dispersion of a solute injected into fluid flowing through a straight open tube has been developed by Taylor[43,44], Aris[45] and Golay[46], and has been applied to chromatography by Sternberg[47] and Atwood and Golay.[48] By employing the Golay equation derived for solute dispersion in a straight tube with no retentive phase, the dispersion characteristics of a tube of any given length and radius can be predicted.

The Golay equation is given by :

$$H = 2D_m/u + r^2u/24D_m \qquad (1.14)$$

where r = the radius of the tube and all other symbols are as previously assigned.

If $u >> D_m/r$, as is the case for LC generally, then longitudinal diffusion is negligible and Equation 1.14 reduces to :

$$H = r^2u/24D_m \qquad (1.15)$$

From chromatographic theory the peak variance (in volume units) of a substance eluted from a tube with no retentive phase is defined as :

$$\sigma^2_{(v)} = (\pi r^2 L)^2/N \qquad (1.16)$$

where L = the length of the tube,

and N = the number of theoretical plates in the tube.

Hence, as N = L/H (Equation 1.12), Equations 1.15 and 1.16 may be combined to yield an expression for band dispersion in a straight open tube in terms of the tube dimensions, the solute diffusivity and the mobile phase linear velocity[26,42]

$$\sigma^2_{(v)} = \pi^2 r^6 Lu/24D_m \qquad (1.17a)$$

or alternatively in terms of the tube dimensions, the solute diffusivity and the mobile phase volumetric flow rate, $Q(=\pi r^2 u)$[26,49] :

$$\sigma^2_{(v)} = \pi r^4 LQ/24D_m \qquad (1.17b)$$

A further parameter pertinent to the successful operation of an LC instrument is the available inlet pressure which ultimately limits the performance of the total LC system. The pressure that develops in an LC system is determined primarily by the column permeability, but components of

the apparatus such as detector flow cells and connecting tubing produce additional flow impedence. Consequently, pressure gradients are set up within the apparatus and these must be controlled in order to achieve maximal performance. The pressure drop across a tube is expressed in terms of the physical properties of the mobile phase and the geometry of the tube by the Poiseuille equation :

$$\Delta P = 8\eta Lu/r^2 \qquad (1.18)$$

where η = the viscosity of the mobile phase.

Equations 1.17 and 1.18 are important in the assessment of chromatographic system design since the former expression enables the band dispersion arising in the connecting tubing and the detector flow cell to be calculated for a given solute in a given mobile phase, and the latter establishes the relationship between the physical properties of the mobile phase, the tube geometry and the pressure.

1.4.3.2 Extra-Column Dispersion - General Remarks

Dispersion in an LC instrument was discussed first by Scott and Kucera[49], who developed and experimentally validated equations that can be employed in the design of detector flow cells and connecting tubes. Aspects of equipment design were also considered with reference to extra-column dispersion by Martin et al.[50] The effect of extra-column dispersion on the separation performance of a column was determined by Kirkland et al.[51] Scott and Kucera[20] emphasised the necessity of appropriate low dispersion apparatus for NBLC while Knox and Gilbert[52] have discussed extra-column dispersion with regard to optimisation of open-tubular μLC. The overall

extra-column dispersion arising in commercially available chromatographs and its effect on column performance has been examined by Reese and Scott.[39] The extra-column dispersion phenomenon, because of its fundamental importance has been reviewed extensively in recent years, particularly with regard to small-diameter column technology, by several authors (e.g. 23-26, 42, 53-58).

The observed peak dispersion (σ^2_{tot}) of a solute chromatographed on an LC instrument may be represented by the equation :

$$\sigma^2_{tot} = \sigma^2_c + \sigma^2_{xc} \qquad (1.19)$$

where σ^2_c = the band variance resulting from within the column itself,

and σ^2_{xc} = the extra-column band variance, i.e. band variance generated in the various components of the LC instrument exterior to the column bed.

Depending on the particular LC system, σ^2_{xc} may be significant in which case the true performance of the column will be masked. Consequently, σ^2_{xc} must be minimised to enable realisation of as great a proportion of the potential of the column as possible. The amount of dispersion that might be tolerated before the separation is considered to be drastically affected ultimately depends upon the separation under investigation but most chromatographers adopt the recommendations of Klinkenberg[59] and arbitrarily take a 5% increase in peak standard deviation, or a 10% increase in peak variance, as the limit of acceptability. Applying this criterion, the extra-column variance may be written :

$$\sigma^2_{xc} \leq 0.1\sigma^2_c \qquad (1.20a)$$

or alternatively, in terms of band standard deviation,

$$\sigma_{xc} \leq 0.5\sigma_c \tag{1.20b}$$

From plate theory,

$$\sigma_c^2 = V_R^2/N = [V_o(1 + k')]^2/N \tag{1.21a}$$

where V_R = the retention volume of a retained solute,

and V_o = the column dead volume determined from the retention volume of an unretained solute.

The most stringent demands placed on the LC system apply with early eluting peaks, i.e. where $k' \to 0$. Hence, the following expression for σ_c^2 taken where $k' = 0$ represents the extreme case for early eluting peaks and will be used throughout the remainder of this discussion.

$$\sigma_c^2 = V_o^2/N \tag{1.21b}$$

Now, the dead volume of a column is given by :

$$V_o = \varepsilon\pi r_c^2 L_c \tag{1.22}$$

where ε = the column porosity (i.e., the fraction of the column volume occupied by the mobile phase = 0.7 for a well packed column),

r_c = the column radius,

and L_c = the column length.

Hence, by combining Equations 1.21b and 1.22 σ_c^2 may be expressed :

$$\sigma_c^2 = \varepsilon^2\pi^2 r_c^4 L_c^2/N \tag{1.23}$$

Since N = L/H (Equation 1.12) and for a well packed column H may be taken as equivalent to two particle diameters then :

$$\sigma_c^2 = 2\varepsilon^2\pi^2 r_c^4 L_c d_p \qquad (1.24)$$

From Equation 1.24 the on-column peak variance of any well packed column may be calculated and subsequently, by using Equation 1.20a, the maximum tolerable extra-column peak variance may be calculated. An acceptable value of σ_{xc}^2 for a conventional 25cm x 4.6mm ID column packed with 10μm particles can be as high as $64\mu l^2$ but for a 25cm x 1mm ID NB column also packed with 10μm particles σ_{xc}^2 must not exceed $0.15\mu l^2$.[42] Obviously there is little tolerance for extra-column dispersion in LC systems containing NB columns.

Extra-column dispersion arises from four main sources :

(i) the sample injection device (σ_i^2),

(ii) the injection valve/column and column/detector connecting tubes (σ_t^2),

(iii) the detector flow cell (σ_δ^2),

and (iv) the response times of the detector (σ_τ^2) and the recorder (σ_Ω^2).

Total extra-column dispersion can be expressed mathematically by utilising the principle of the summation of variances (Equation 1.1) thus :

$$\sigma_{xc}^2 = \sigma_i^2 + \sigma_t^2 + \sigma_\delta^2 + \sigma_\tau^2 + \sigma_\Omega^2 \qquad (1.25)$$

Dispersion in frits and unions can generally be assumed to be negligible if they are properly designed and fitted.[58] If guard columns or post-column reaction systems are employed then these components will also contribute significantly to the overall peak variance and must be considered in addition to the four major sources noted previously.

From Equation 1.25 it is clear that in order to fully assess the total extra-column variance of an LC system then the variance contributions of the individual LC components must be quantitatively determined. Scott and Simpson[60], and later Hupe et al.[61], addressed this problem and experimentally determined the dispersion arising from each component of an LC system. For the purposes of this discussion the aforementioned four principal sources of extra-column band dispersion will be considered further.

1.4.3.3 Dispersion in the Injection System (σ_i^2)

Dispersion occurring in the injector may take place due to the design of the injection device, due to the sample introduction technique, and due to the sample volume injected onto the column. Provided that the injector is of suitable, well-constructed design (i.e., with minimal internal volume) then transfer of the sample from the injection loop to the valve outlet is possible with negligible extra dispersion. Commonly, dispersion in the injection system is predominantly influenced by the limiting volume of the injection which can be made.[58] It is usually assumed that the contribution to the band variance originating from the injector is proportional to the injection volume[47,62], i.e.,

$$\sigma_i^2 = V_i^2/K^2 \qquad (1.26)$$

where V_i = the injection volume,

and K = a constant dependent upon the injection technique. For a rectangular plug injection (as encountered when utilising a loop), $K^2 = 12$.

If the percentage contribution of σ_i^2 to σ_{tot}^2 is symbolised θ_i^2 then :

$$\sigma_i^2 = \theta_i^2 \sigma_c^2 \qquad (1.27)$$

Combining Equations 1.21b and 1.26 with Equation 1.27 enables the derivation of an expression for the maximum tolerable injection volume, $V_{i(max)}$, i.e.,

$$V_{i(max)} = K\theta_i V_o / N^{\frac{1}{2}} \qquad (1.28)$$

From this equation it is clear that the lower is the column dead volume then the smaller is the injection volume that may be tolerated without serious loss of resolution. Consequently, NB columns, which possess low dead volumes, require sampling devices capable of delivering very small injection volumes.

If the extra-column contribution to band broadening were solely a function of the injection volume then, following the recommendations of Klinkenberg[59] that σ_{xc}^2 should contribute no more than 10% to σ_{tot}^2, i.e., $\theta_i^2 = 0.1$, and for a plug injection profile ($K^2 = 12$), $V_{i(max)}$ is given by :

$$V_{i(max)} = 1.1\ V_o / N^{\frac{1}{2}} \qquad (1.29a)$$

or

$$\frac{V_{i(max)}}{V_o} = 1.1\ N^{-\frac{1}{2}} \qquad (1.29b)$$

Hence, for a column of 5,000 plates the injection volume should not exceed 1.5% of the column void volume. Similarly, for columns of 10,000 and 20,000 plates the injection volume should not exceed 1.1% and 0.8% respectively of the column void volumes. Thus, for standard length (10-30cm) columns it may be surmised that the absolute maximum injection volume applied to any column should be no greater than about 1% of the void

volume of that column. However, it should be noted that as other factors apart from the injection volume contribute to the extra-column dispersion an even smaller volume is required in practice than that predicted by Equation 1.29.

Katz[42] assumed a 30% contribution to σ^2_{xc} from σ^2_i and followed the recommendations of Klinkenberg which together yielded the factor $\theta^2_i = 0.03$. For a plug injection profile ($K^2 = 12$), Katz obtained :

$$V_{i(max)} = 0.60\ V_o/N^{\frac{1}{2}} \qquad (1.30)$$

Guiochon and Colin[26] assumed a 50% contribution to σ^2_{xc} from σ^2_i, which together with Klinkenberg's criterion indicated $\theta^2_i = 0.05$. These authors utilised a practically determined value for K^2 of 4 and derived the following expression :

$$V_{i(max)} = 0.45\ V_o/N^{\frac{1}{2}} \qquad (1.31)$$

Guiochon and Colin extended their calculation further and arrived at an equivalent equation expressed in more fundamental terms, i.e.,

$$V_{i(max)} = 0.36\ d_c^2\ L_c^{\frac{1}{2}}\ d_p^{\frac{1}{2}} \qquad (1.32)$$

This equation enables the calculation of the maximum tolerable injection volume from the basic physical properties of the column, i.e. its dimensions and the mean particle size of the stationary phase.

1.4.3.4 Dispersion in the Connecting Tubing (σ^2_t)

In the initial work on NB columns by Scott and Kucera[20] the problem of dispersion in connecting tubing was eliminated by plumbing the column

directly into the injection valve and the detector flow cell. Unfortunately, this approach is rarely possible in practice because of the variations in column end fitting dimensions and the practical difficulties encountered with the architecture of most commercial equipment. The majority of marketed NBLC systems are supplied with connecting tubing and zero dead volume (ZDV) unions for column installation, hence dispersion in connecting tubes must be considered.

As has already been shown earlier in this section, dispersion in a straight open tube, σ_t^2, may be expressed in the following terms (Equation 1.17) :

$$\sigma_t^2 = \pi^2 r_t^6 L_t u/24D_m = \pi r_t^4 L_t Q/24D_m$$

where r_t = the radius of the tube,

and L_t = the length of the tube.

Hence, the extra-column dispersion arising from the connecting tubes can be calculated directly from the tube dimensions, the mobile phase linear velocity or volumetric flow rate and the diffusivity of the unretained solute in the mobile phase.

Several researchers[18,63-65] have described the geometrical deformation of connecting tubing in various ways (squeezing, twisting, coiling, bending, etc.) in order to introduce radial convection (or secondary flow) and so disrupt the parabolic flow profile. This phenomenon significantly improves radial mass transfer and consequently reduces the longitudinal dispersion characteristics of the tube. Katz and Scott[65] showed that serpentiform (or zigzag) tubes were the most effective configuration for reducing variance (they obtained values of only $0.05\mu l^2 cm^{-1}$) whilst generating relatively little back pressure (ca. 5 p.s.i. cm^{-1} at $Q = 3ml min^{-1}$).

Equation 1.17 therefore provides a maximum value for dispersion in the connecting tubing. Frequently the actual value is less than that predicted because of the incidence of increased radial convection resulting from the departure from linearity of such tubing used in practice.

1.4.3.5 Dispersion in the Detector Flow Cell (σ_δ^2)

Dispersion that occurs in detector flow cells often contributes greatly to the total extra-column dispersion because detection systems frequently require significant volumes in which to achieve satisfactory detection sensitivity. Indeed, this is the case for optical detectors such as visible/ultra-violet (UV) and fluorescence (FL) monitors which are commonly employed with LC.

Since a detector flow cell can often be considered as a straight open tube, the equation derived by Scott and Kucera[49] to describe dispersion under such circumstances (Equation 1.17) is applicable, i.e.,

$$\sigma_\delta^2 = \pi^2 r_\delta^6 L_\delta u/24D_m = \pi r_\delta^4 L_\delta Q/24D_m$$

where r_δ = the radius of the flow cell,

and L_δ = the length of the flow cell.

It should be noted that this is only a first approximation for a flow cell since the behaviour of a solute band eluted through a very short, narrow tube is far more complicated[48,66,67] than is discussed here. From the above expression, equations have been produced to permit geometrical parameters of the flow cell to be calculated in order to ascertain the maximum cell volume, $V_{\delta(max)}$, that may be tolerated without significant loss of column efficiency.

If the contribution of σ_δ^2 to σ_{tot}^2 (as a percentage) is symbolised θ_δ^2 then by Equation 1.27 :

$$\sigma_\delta^2 = \theta_\delta^2 \sigma_c^2$$

Combining Equations 1.17 and 1.27 yields an expression for the maximum cell radius, $r_{\delta(max)}$, that can be tolerated, i.e.,

$$r_{\delta(max)} = \left(\frac{24\theta_\delta^2 D_m V_o^2}{\pi^2 N L_\delta u}\right)^{1/6} = \left(\frac{24\theta_\delta^2 D_m V_o^2}{\pi N L_\delta Q}\right)^{1/4} \qquad (1.33)$$

Alternatively, this equation may be written in terms of maximum cell length, $L_{\delta(max)}$, i.e.,

$$L_{\delta(max)} = \frac{24\theta_\delta^2 D_m V_o^2}{\pi^2 r_\delta^6 N u} = \frac{24\theta_\delta^2 D_m V_o^2}{\pi r_\delta^4 N Q} \qquad (1.34)$$

The maximum tolerable cell volume may be elucidated from any of these equations by utilisation of the expression for the volume of a cylinder, *viz*. $V = \pi r^2 L$. Practical constraints exist on the range of values of the cell length and radius that may be employed, e.g. the smaller is the cell radius then the greater is the susceptibility of the cell to physical blockage. Furthermore, for optical detectors spectroscopic properties must also be considered in selecting suitable cell dimensions (see Section 1.5).

Katz[42] again assumed a 30% contribution to σ_{xc}^2 from σ_δ^2 and that σ_{xc}^2 should contribute no more than 10% to σ_{tot}^2[59], i.e. $\theta_\delta^2 = 0.03$, which yielded a value of 0.72 for $24\theta_\delta^2$. Scott[67] followed similar guidelines except that a 90% contribution to σ_{xc}^2 from σ_δ^2 was assumed, i.e. $\theta_\delta^2 = 0.09$, and obtained

a factor equivalent to $24\theta_\delta^2$ of 2.21. The difference in numerals in the basic equation reported by both researchers is accounted for solely by the difference in percentage contribution of σ_δ^2 to σ_{xc}^2 arbitrarily assigned by each author.

Following the assumptions of Katz and by utilising Equation 1.33 with the assignment of typical values for certain parameters (viz. $D_m = 3 \times 10^{-5} cm^2 s^{-1}$, $L_\delta = 1.0cm$ and $u = 0.1cms^{-1}$), the maximum cell radius (and consequently the maximum tolerable cell volume) may be calculated for any well packed column. Maximum cell volumes for some typical column geometries determined in this way are presented in Table 1.4.

Table 1.4 Maximum Tolerable Detector Flow Cell Volumes for some Typical Column Geometries Calculated Using Equation 1.33 and Following the Guidelines of Katz.

Column Type	L_c (cm)	d_c (mm)	d_p (μm)	V_o ($=\varepsilon\pi r_c^2 L_c$) (ml)	N ($=L/H=L/2d_p$)	L_δ (cm)	$r_{\delta(max)}$ (mm)	$V_{\delta(max)}$ (μl)
Conventional	25	4.6	10	2.9	12,500	1.0	0.50	7.7
NB (2mm)	10	2.1	5	0.24	10,000	1.0	0.22	1.6
NB (1mm)	25	1.0	10	0.14	12,500	1.0	0.18	1.0

The figures clearly show that an 8 fold reduction in cell volume (from 8μl to 1μl) is necessary when simply reducing the column ID from 4.6mm to 1.0mm in order to realise 90% of the potential column efficiency for early eluting peaks. Hence, NB columns require micro-flow cells of

$\leq$ 1µl volume for efficient operation. Guiochon and Colin[26] point out that the maximum tolerable detector cell volume is approximately half the maximum tolerable sample volume that can be put onto any particular column, i.e.,

$$V_{\delta(max)} \simeq 0.5\ V_{i(max)} \qquad (1.35)$$

All the equations and calculations regarding LC detectors have assumed a tubular cell geometry encapsulating a parabolic velocity profile. For all flow cells there is a certain degree of turbulence that occurs within the cell due to the manner in which the mobile phase enters the cell. This turbulence gives rise to increased radial diffusion which in turn produces favourable reduced dispersion. However, this effect is often accompanied by increased detector noise, especially in optical detectors, which can result in poorer detection sensitivity. There are detectors on the market in which the turbulence giving rise to reduced band dispersion also promotes improved detector sensitivity. Such an instrument is the wall-jet amperometric electrochemical detector (ECD) and this type of detector is discussed in great detail in Chapter 2.

1.4.3.6 Dispersion due to the Detector and Recorder Electronics (σ_τ^2, σ_Ω^2)

It is now well recognised that the response behaviour of the detector and recording system can seriously impair the apparent column efficiency in addition to volume-related contributions from the sample injection system, connecting tubes and detector flow cell. The contributions of the detector and recorder time-related variances to the extra-column variance arise due to the finite response time of the detector sensors (e.g.

photomultipliers, electrodes, etc.) and also due to the process of amplification and recording of the electrical signal generated in the detector.

Response behaviour of any electrical or electronic instrument is described by one of two terms, response time (RT) or time constant (TC). The response time of an instrument is defined as the time taken for the physical response of that instrument to come to rest at a new value after the quantity that the instrument measures is changed abruptly.[68] The time constant of an instrument is defined as the time taken for the instrument to register a given percentage (usually 63.2%) of its final reading in reaction to an abrupt change in input signal.[68] Response time and time constant are represented in graphical form in Figure 1.6.

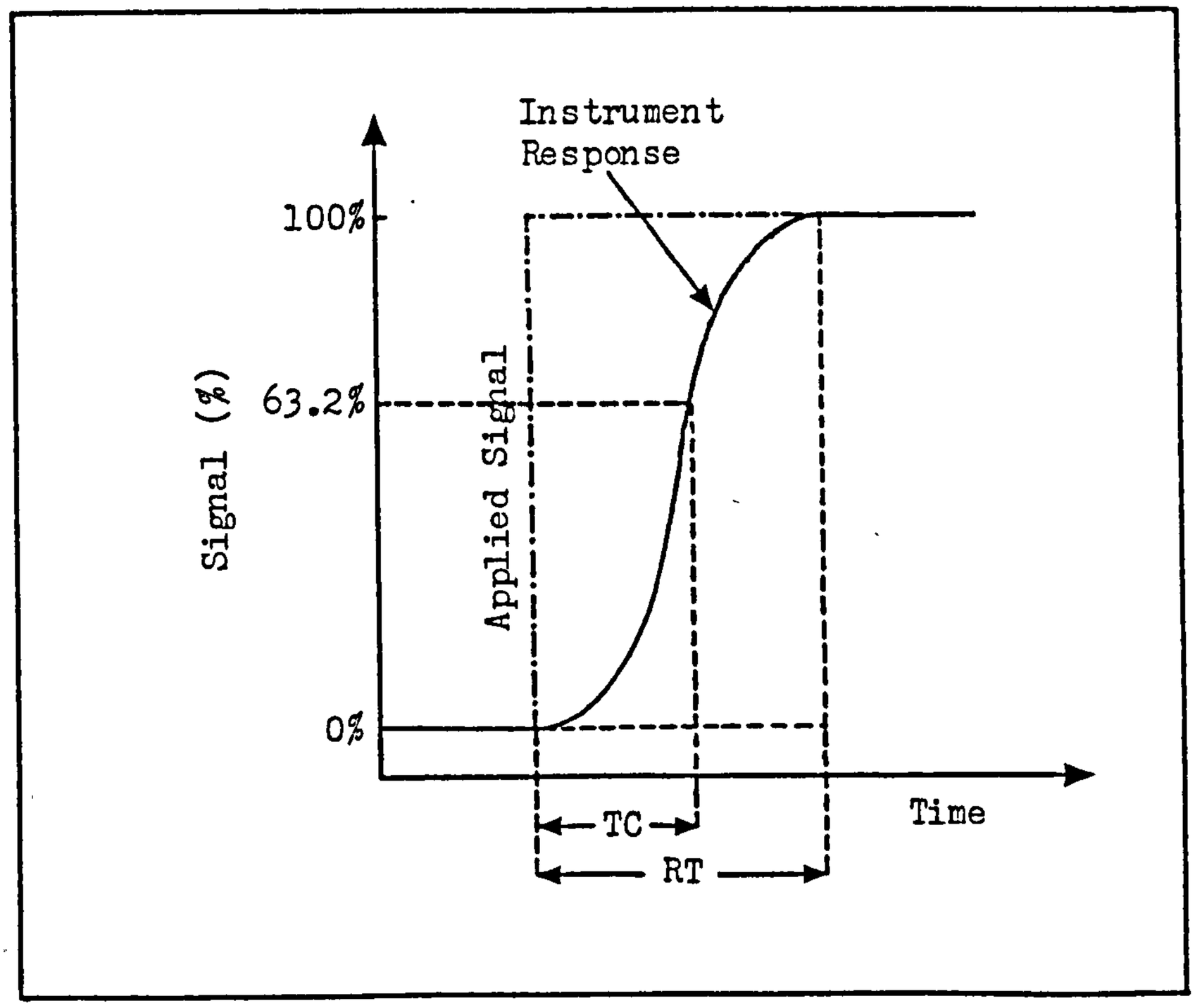


Figure 1.6 Illustration of the Origin of Response Time (RT) and Time Constant (TC) of an Electronic Instrument.

Detectors normally exhibit an exponential response (as depicted in Figure 1.6) resulting from a capacity resistance network inherent in the amplifier circuitry. It is the amplification of the signal, rather than its generation, that is the predominant factor which gives rise to detector-related band-broadening. This is particularly so when, as frequently occurs, an increase in the detector time constant is purposely introduced as a filter device in order to reduce short-term noise.

Potentiometric recorders do not have a response curve of the form presented in Figure 1.6. Recorder response is linear with respect to time because of the feedback circuitry incorporated in the instrument in order to provide output stability. Generally, for modern recorders employed with HPLC systems operated at mobile phase linear velocities near optimum, the recorder response time is insignificant compared with that of the detector. Usually it is only when the HPLC is operated at mobile phase linear velocities considerably in excess of optimum, i.e. when conducting fast LC, that the recorder contributes significantly to the extra-column band broadening. Since only the former case is to be discussed here, the contribution of the recorder can be considered to be negligible.[67]

The influence of detector response time on column efficiency has been examined by several researchers[50,67,69-71] and has been reviewed by Guiochon and Colin[26], Katz[42] and Kucera.[54] It is well recognised that because slow detector (and/or recorder) response times can significantly distort the shape of a peak (particularly for early eluting solutes) by decreasing peak height and increasing peak width, it is essential to be able to determine the maximum permissible detector and recorder response times that can be tolerated for any given LC column.

If the percentage contribution of the dispersion arising from the detector (σ_τ^2) and the recorder (σ_Ω^2) is designated θ_ϕ^2 then by Equation 1.27 :

$$\sigma_{\tau(t)}^2 + \sigma_{\Omega(t)}^2 = \theta_\phi^2 \sigma_{c(t)}^2 \qquad (1.36)$$

As was stated previously, when operating at mobile phase linear velocities approaching optimum the recorder contribution to band variance can normally be considered to be negligible, i.e. $\sigma_\tau^2 >> \sigma_\Omega^2$.[67] Hence, by substituting Equation 1.21b into Equation 1.36 and correcting for time variance, the maximum permissible detector response time approximates to :

$$\sigma_{\tau(t)}^2 = \theta_\phi^2 V_o^2 / NQ^2 \qquad (1.37)$$

or

$$\sigma_{\tau(t)} = \theta_\phi V_o / N^{\frac{1}{2}} Q = \theta_\phi t_o / N^{\frac{1}{2}} \qquad (1.38)$$

Scott[67] assumed the extreme condition of the entire extra-column dispersion arising from the detector response time. Hence, a 10% increase in peak variance (or 5% increase in peak width) was deemed tolerable[59] and values of $\theta_\phi^2 = 0.1$ and, consequently, $\theta_\phi = 0.32$ were calculated. By utilising Equation 1.38 with this value for θ_ϕ, the maximum permissible detector response time may be determined for columns of different dimensions, operated at various mobile phase volumetric flow rates. Typical detector response times are calculated and presented in Table 1.5.

The calculated maximum values of $\sigma_{\tau(t)}$ in Table 1.5 show that the maximum tolerable detector response time is independent of the column ID but is proportional to the column length and the particle size of the packing (to the first approximation of $H = 2d_p$ used here), and is inversely

Table 1.5 Maximum Permissible Detector Response Times for some Typical Column Geometries Calculated Using Equation 1.38

Column Type	L_c (cm)	d_c (mm)	d_p (μm)	V_o ($=\varepsilon\pi r_c^2 L_c$) (ml)	N ($=L/2d_p$)	u (cms^{-1})	Q ($mlmin^{-1}$)	$\sigma_{\tau(max)}(t)$ (s)
Conventional	25	4.6	10	2.9	12,500	0.1	1.0	0.5
NB (2mm)	25	2.1	10	0.61	12,500	0.1	0.21	0.5
NB (2mm)	25	2.1	5	0.61	25,000	0.1	0.21	0.35
NB (2mm)	10	2.1	5	0.24	10,000	0.1	0.21	0.2
NB (1mm)	25	1.0	10	0.14	12,500	0.1	0.05	0.5
NB (1mm)	25	1.0	10	0.14	12,500	0.85	0.4	0.06

proportional to the mobile phase linear velocity; in fact,

$$\sigma_\tau = f(L^{\frac{1}{2}}, d_p^{\frac{1}{2}}, u^{-1}) \qquad (1.39)$$

From this data it may also be surmised that the contribution of the detector response time to the column dispersion is minimal provided that the column in use is operated at the optimum linear velocity. It becomes more significant when shorter columns or smaller stationary phase particles are employed, and especially so if the column is operated at high linear velocities. High linear velocities are practical with NB columns because they may be generated at moderate volumetric flow rates with acceptable back pressure (see Equation 1.18, the Poiseuille Equation). High linear velocities are desirable in some instances where it is feasible to sacrifice some separation efficiency in favour of reduced analysis time and in this way an element of "fast NBLC" may be introduced.

In order to generate the figures in Table 1.4 it was assumed that the detector response time accounted for the entire permissible extra-column contribution to band dispersion. Detector response time ideally should be a factor of 10 or more better than calculated here to allow for contributions from other sources. In practice, most detectors have response times of greater than 0.4 sec which limits the speed of analysis and the resolution that can be attained. Current detector technology lags seriously behind column technology.[53]

In addition it should be noted that the contribution to extra-column dispersion arising from the recorder response time was considered to be negligible for the aforementioned calculations. In many instances this approximation is not valid in which case the calculated restrictions in response time presented in Table 1.5 should be applied to a combination of the detector and the chart recorder.

1.4.3.7 The Relationship Between Extra-Column Dispersion and Column Radius

The primary disadvantage of the use of NB columns that arises from their small dimensions is that specially designed LC equipment having low dispersion is required to fully realise all the potential advantages they might provide in practical applications. It follows that a function defining a relationship between extra-column dispersion and the column dead volume, and subsequently the column radius, would be valuable for careful column/LC system matching.

Throughout this theoretical treatment of extra-column dispersion it has been shown that the magnitude of σ^2_{xc} that can be tolerated is primarily determined by the column dead volume. Katz[42] developed an equation based

on this observation which allows rapid determination of permissible extra-column dispersion with a well packed column of any dimensions and particle size, i.e.,

$$\sigma_{xc} = 0.99\ r_c^2\ L_c^{\frac{1}{2}}\ d_p^{\frac{1}{2}} \qquad (1.40)$$

Conversely, this relationship allows the column parameters that may be used with a given chromatographic apparatus to be calculated.

1.4.4 Sample Sensitivity[24,25]

The principal theoretical advantage of NBLC over conventional LC of particular interest to this project is that of increased mass sensitivity.

There are two important characteristics of an LC system concerning sample sensitivity, viz. mass sensitivity and concentration sensitivity. The former is defined as the minimum mass of a solute that can be detected when the peak height is equal to twice the background noise level, and the latter is the smallest concentration of solute that can be detected whilst satisfying the same criterion. The mass sensitivity is crucial when the total mass of sample is limited, even when sample concentration methods are available.

1.4.4.1 Mass Sensitivity

Consider a peak of height equivalent to twice the noise level being sensed by a detector having a concentration sensitivity of X_δ. If m is the mass of solute giving rise to the peak, and the peak volume is $4\sigma_{(v)}$ (where $\sigma_{(v)}$ is the volume standard deviation of the peak), then,

$$2m/4\sigma_{(v)} = X_\delta \qquad (1.41)$$

Therefore,

$$m = 2\sigma_{(v)}X_{\delta} \tag{1.42}$$

Now, from plate theory,

$$\sigma_{(v)} = V_R/N^{\frac{1}{2}} \tag{1.43}$$

Hence,

$$m = 2V_R X_{\delta}/N^{\frac{1}{2}} \tag{1.44}$$

Now,

$$V_R = V_o(1 + k') \tag{1.45}$$

and $V_o = \varepsilon\pi r_c^2 L_c$ (Equation 1.22)

Consequently, Equation 1.43 may be written :

$$m = \frac{2\varepsilon\pi r_c^2 L_c (1 + k') X_{\delta}}{N^{\frac{1}{2}}} \tag{1.46}$$

Equation 1.46 clearly indicates that the minimum detectable mass is directly related to the square of the column radius. Hence, reducing the column ID from the conventional 4.6mm to 1.0mm results in a 21 times improvement in mass detectability, assuming that both columns are packed equally well. This constitutes a very significant improvement in performance.

1.4.4.2 Concentration Sensitivity

The concentration sensitivity of a chromatographic system, X_C, can be derived directly from the mass sensitivity. The minimum detectable solute

concentration is yielded by the ratio of the minimum detectable mass of solute and the maximum permissible sample volume. Assuming that a 10% increase in extra-column dispersion (or 5% increase in peak standard deviation) is the maximum that can be tolerated without serious loss of resolution[59], then the absolute maximum sample volume that may be placed on a column, $V_{i(max)}$, is given by Equation 1.29a :

$$V_{i(max)} = 1.1\ V_o/N^{\frac{1}{2}} = 1.1\ \sigma_{c(v)}$$

The system concentration sensitivity is obtained by combining Equations 1.42 and 1.29a, i.e.,

$$X_C = 2\sigma_{c(v)}X_\delta/1.1\ \sigma_{c(v)} = 1.8\ X_\delta \qquad (1.47)$$

Equation 1.47 shows that concentration sensitivity of a chromatographic system is independent of the column dimensions and is solely dependent on the detector sensitivity. Furthermore, Scott[24] demonstrated that the volume of sample that has to be employed to achieve this sensitivity is proportional to the square of the column radius. Thus, NB columns possess higher mass sensitivities than conventional columns and, in addition, require less sample volume to achieve the same concentration sensitivity. These properties are extremely desirable for solving the problem of the determination of trace components in a sample of severely restricted size such as is frequently encountered in the field of clinical analysis.

1.5 Instrumental Aspects of Narrow-Bore Liquid Chromatography

In Section 1.4 the need for small scale apparatus with very low extra-column dispersion, in order to achieve the advantages in performance

theoretically attainable from NB columns, was emphasised. The practical design characteristics of equipment suitable for the efficient operation of NB columns will now be discussed.

1.5.1 Pumps

Volume flow rates considerably lower than those applied to conventional columns are necessary for NBLC (viz. 5-500µlmin^{-1} cf. 0.1-10mlmin^{-1}). A pump employed for NBLC must be accurate, precise and pulse-free at these low flow rates, which is fairly difficult to achieve in practice. Scott and Kucera[20], in their pioneering work on NBLC, modified a commercial dual piston reciprocating pump to deliver flow rates as low as 2µlmin^{-1}. Other researchers since have adopted a similar approach but many conventional reciprocating pumps do not function properly at such low flow rates. This has primarily been attributed to the occurrence of considerable leakage past the check valves during the seating process over the lengthy cycle period of piston chamber empty and refill.

Manufacturers have avoided this problem in the new generation of LC pumps by significantly reducing the piston stroke volume and/or by radically modifying or redesigning the pump electronic drive circuits. A few pumps capable of delivering low flow rates with precision and accuracy began appearing on the market in 1982. By early 1984 several more instruments were available, a selection of which are listed in Table 1.6. Most common are dual piston reciprocating pumps or single piston rapid-refill pumps which generally incorporate a pulse dampener.

The comparatively large internal volume of many pulse dampeners, coupled with the low flow rates employed with NBLC, causes a significantly longer delay to the mobile phase change-

Table 1.6 Selected Commercial Low Flow Rate Pumps Available in January 1984

Manufacturer	Model	Flow Rate Range ($\mu l min^{-1}$)	Pump Type
Beckman	112	10-10,000	Single Piston
Gilson	302	5-5,000	Single Piston
Hewlett Packard	HP 1090	1-5,000	Piston Diaphragm
JASCO	Familic-100N	5-5,000	Syringe
LDC-Milton Roy	MicroMetric	1-559	Syringe
LKB-Bromma	2150	10-5,000	Dual Piston
Perkin Elmer	Series 4	10-9,900	Dual Piston ?
Pye Unicam	PU 4010	100-9,900	Dual Piston
Shimadzu	LC-5A	10-9,900	Single Piston
Varian	5560	10-15,000	Single Piston
Waters	590	10-15,000	Dual Piston

over than is common for conventional LC. However, this minor disadvantage is considered to be acceptable for the vast majority of problems where isocratic elution is to be employed though is unacceptable where gradient elution is required.

The greatly reduced retention volumes obtained from NB columns make syringe pumps more attractive than they are for conventional LC since many separations may be completed before the pump reservoir requires replenishing. Mobile phase compressibility still remains a problem with syringe pumps but, despite this drawback, commercial models are available specifically for NBLC.

The JASCO Familic-100N was developed from an original construction used by Ishii and associates (e.g. 8) for their many contributions to NB and μLC.

1.5.2 Injectors

As was noted in Section 1.4, sample injection is one of the major sources of extra-column dispersion. Because of this, design and construction of the injector to high specification is particularly crucial for NBLC. There are two different sources of dispersion in an injection system. First, there is a contribution directly proportional to the magnitude of the injection volume (since the greater the volume injected then the greater is the width of the band introduced onto the column). This contribution is given by $V_i^2/12$ for a cylindrical plug injection and, it may be noted, is totally independent of flow rate. Typical injection volumes employed in NBLC are between 0.05 and 5.0μl.

The second source of injector dispersion is a consequence of the design and construction of the injector itself. The transport of the sample plug out of the injection loop and through connecting channels results in distortion of the ideal cylindrical shape yielding leading and trailing edges to the band. The presence of unswept dead volumes can exacerbate this distortion. This second source of dispersion usually comprises an insignificant part of the total variance generated in injection valves in conventional LC systems. However, as the injection volume is reduced commensurate with reduction in column ID, these hardware-originating contributions become progressively more significant. Consequently, the correct design and construction of the micro-injection system for use with

NB columns, in addition to the actual sample volume introduced, is of prime importance.

Šlais and Kouřilová[72] identified these two separate dispersion contributions resulting from the injector and demonstrated that the short connecting channel in the valve is the dominant contributor to the injector-related variance in micro-injection valves. It was observed that the variance contribution of the capillary channel is strongly flow dependent as is predicted by Equation 1.17. This dispersion source is independent of the injection volume and hence represents a limit below which reducing the injection volume will not reduce the injection dispersion.

Certain injection valve manufacturers have addressed the problem of designing and producing low dispersion devices suitable for NBLC. By the beginning of 1984 a few small-volume valves had become available, a selection of which are presented in Table 1.7, together with the range of injection volumes that are obtainable with these models.

Table 1.7 Selected Commercial Small-Volume Injection Valves Available in January 1984

Manufacturer	Model	Injection Volumes Available (µl)
Rheodyne (Sunnyvale, CA., USA)	7410	0.5, 1.0, 2.0
Rheodyne (Sunnyvale, CA., USA)	7413	0.5, 1.0, 5.0
Valco Instruments (Houston, TX., USA)	C14W	0.06, 0.1, 0.2, 0.5, 1.0, 2.0

Other specialist valve manufacturers, e.g. Specac (Orpington, Kent, UK) and Negretti & Zambra (Southampton, Hants., UK) have since introduced comparable products onto the market.

All these commercial valves possess internal loops, where the loop comprises either a groove cut directly into the valve rotor or a short capillary tube connected directly to the rotor. Manufacturers have been careful to reduce the dimensions of the connecting channel between the loop and the valve outlet to a practical minimum and to eliminate unswept dead volume in order to reduce dispersion from this source as far as is practicable.

1.5.3 Connecting Tubing and Unions

Short lengths of tubing are usually used to couple the injection valve to the column and the column to the detector. This tubing contributes to extra-column dispersion, a contribution that becomes progressively more significant as the column ID is reduced. Very occasionally it is possible to eliminate connecting tubing altogether by plumbing the column directly into the injector and the detector flow cell[20] but such opportunities are rare because of practical difficulties (e.g. incompatibility of column end-fitting dimensions, injection valve mounting restrictions, detector flow cell geometry, etc.). Hence, the use of connecting tubing is still widespread. In general, connecting tube dimensions should be minimised while ensuring that the probability of tube blockage and the generation of back pressure are not unacceptably high. Tubing of 0.254mm (0.010") ID is commonly employed for conventional LC but tubing of 0.13-0.18mm (0.005-0.007") ID composed of 316 stainless steel (S/S) or PTFE is now readily available from many HPLC suppliers and is recommended to fulfill the aforementioned criteria for NBLC.

It has been shown that serpentiform geometry is the most favourable in reducing the dispersion contribution of the connecting tubing[65] but tubing is not generally available in this form. As a compromise straight tubing may be bent and/or twisted to achieve reduced dispersion and to facilitate coupling with minimum practicable tubing length.

Unions comprise a further source of extra-column dispersion and should be avoided wherever possible. Sometimes a union is required in order to link the column exit tubing to the detector flow cell inlet tubing. It has been shown that such a union can contribute significantly to the dispersion.[60] Where a union is necessary, use of a "drilled-out" coupling is advisable so that the two tubes make a butt-to-butt connection and dead volume is minimised. Optimum placement of ferrules is essential to achieve minimum dead volume at all couplings.

1.5.4 Column End-Fittings

Column end-fittings are a further potential source of band dispersion in the LC system. The end-fittings constitute a part of the hardware of the column but their contribution to band dispersion is external to that of the packed bed itself and therefore is considered to be extra-column dispersion. The function of the inlet end-fitting is to spread the incoming mobile phase stream (plus sample) evenly across the top of the packed bed. Conversely, the role of the outlet end-fitting is to collect uniformly the eluting mobile phase plus solute bands and funnel them into the narrow exit tube. Many types of end-fitting are available, a selection of which are illustrated in Figure 1.7.

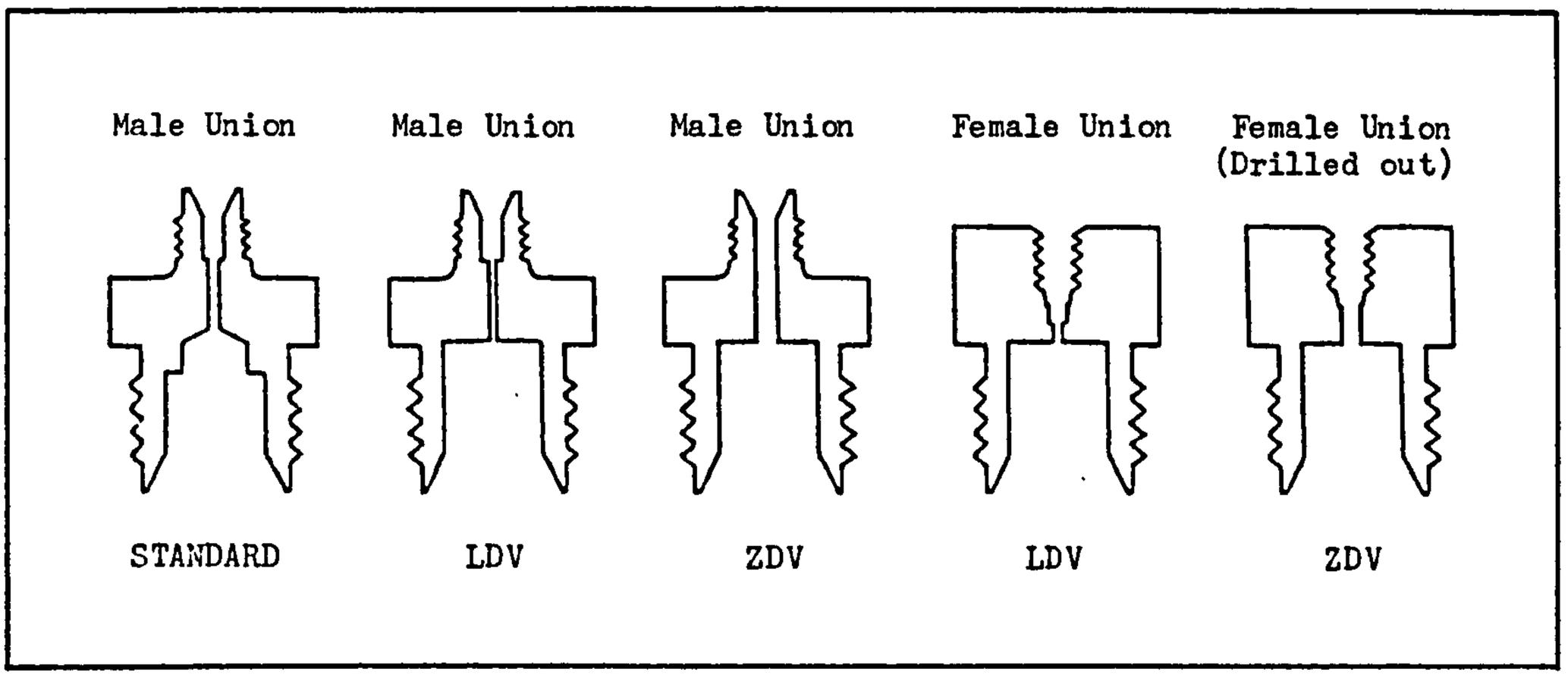


Figure 1.7 Selected Column End-Fitting Configurations

The very small peak volumes obtained from NB columns enhance the problem associated with dead volumes in these end-fittings and demand that the design and construction of column terminals be very precise. The contribution to dispersion by column end-fittings can be made very small by careful design. Satisfactory end-fittings for NB columns should have minimum dead volumes and wherever possible the connecting tubes should be directly in contact with the column end frits. In order to comply with this criterion, only zero dead volume (ZDV) fittings are acceptable; neither low dead volume (LDV) nor standard fittings permit the connecting tubing to butt up against the column end frit.

1.5.5 Detectors

The use of NB columns with their associated small volume peaks requires the use of detectors with comparably small dispersion characteristics in order to realise the full potential of such columns. This

reduction in dispersion must be accomplished without compromising the sensitivity of the detection system. The greatest problem facing manufacturers involves reducing the detector dispersion by a factor of 10 to 100 while maintaining high detector performance, i.e., high sensitivity and low noise. Some commercial conventional LC detectors could simply be modified to operate with cells of smaller volume and lower dispersion without reduction in detector sensitivity (e.g. 20). This advantageous situation arises where the signal to noise (S/N) ratio is maintained, i.e., where the reduction in detector signal is accompanied by an equivalent reduction in detector noise. However, for most commercial detectors a complete redesign of the detector optics (where appropriate), the flow cell and auxiliary connecting tubing was required before compatibility with NB columns was approached. It should be emphasised that cell volume is not the sole determinant of detector contribution to dispersion. Other contributing factors include cell design, inlet/outlet configurations and the existence of incoming-stream thermal equilibration volumes. Hence, the chromatographically important parameter to be used for comparison of detectors of different manufacture or design is the total contribution of the detector system to peak dispersion, and not merely the detector cell volume.

Significant reductions in detector cell volume and consequently in detector dispersion have been reported in the literature for the three major LC detectors, viz. visible/ultra-violet (UV), fluorescence (FL) and electrochemical (EC). The UV detector has received the most attention for NB column work as it has for conventional LC. Manufacturers have been obliged to give careful consideration to the means by which the UV detector cell volume is reduced because, by Beer's Law, the detector response or

signal is directly proportional to the path length of the cell. Hence, it is important to reduce the cell radius in preference to the cell length (assuming that the cell is illuminated longitudinally) in order to maintain signal intensity. However, there are practical limitations to miniaturisation. The radius of the cell must not be reduced so far that the probability of cell blockage or the generation of back pressure become unacceptably high. The severe restriction on cell volume imposed by NB column geometry has generally meant that some path length has had to be sacrificed in addition to cell radius and consequently some detector response is lost. Reduction of cell radius promotes a further practical difficulty, that of lamp/cell/photomultiplier alignment which becomes more and more critical with smaller and smaller cell radius because poorly aligned optics result in increased light scatter. The effect of miniaturisation on detector noise is largely unpredictable; much depends on the cell geometry together with temperature stability, flow stability, and characteristics of the electronics. However, in the majority of cases a reduction in cell volume gives rise to an increase in noise due to problems such as the aforementioned alignment difficulty.

Several small-volume UV detectors have been marketed for NBLC generally possessing cell volumes of between 0.5 and 2.5µl. Sagliano et al.[57] discussed the relative merits of different cell volumes for use with a 25cm x 1mm ID column packed with 10µm diameter particles. In their experience, the use of a 2.4µl cell offered a reasonable compromise between extra-column dispersion and sensitivity for practical separation problems when using a column of these dimensions.

Fluorescence detectors provide different challenges where miniaturisation is concerned. Fluorescence response is directly proportional to

both the incident (or excitation) light intensity and the cell path length, hence, reduction in cell volume may be compensated for in part by condensation of the incident light beam into the smaller volume by means of lenses. This approach has been adopted almost universally by manufacturers of NBLC FL detectors. The direct dependence of detector response on incident light intensity makes laser excitation very attractive for small volume systems. This idea has been exploited in research laboratories but at the outset of this project NB laser FL detectors were not commercially available.

Sources of noise in FL detectors include incident light scattered from the cell walls and from suspended particulate matter, stray light and dark current (which is strongly influenced by temperature). Noise arising from light scatter and dark current would be expected to increase with reduction in cell radius, but all these factors can be controlled to a substantial degree with careful detector design so providing a detector which goes some way towards satisfying the stringent criteria of high sensitivity and low dispersion required for NBLC. Commercial FL detectors with cell volumes of typically ca. 2µl were available for NB work from a few manufacturers at the commencement of this work.

Electrochemical detectors (ECDs) are becoming increasingly popular for conventional LC because of their intrinsically high sensitivity and selectivity. These properties are also desirable for application to many NBLC problems and consequently manufacturers have produced ECDs that are suitable for use with this column geometry. In EC detection the detector signal is related to the working electrode area, the mobile phase linear flow velocity and the cell geometry. The linear dependence of response on electrode area would suggest that large electrode systems would be

favoured but detector noise has also been found to increase with electrode area. In fact, the best *S/N* ratios (sensitivities) are obtained by reducing the electrode area, i.e., by miniaturising the electrode.

Miniaturisation of the flow cell in its entirity is also beneficial because response increases with linear velocity which itself increases with reduced cell thickness. Cell miniaturisation exactly matches the requirements of NB columns hence the ECD is ideally suited for use with NBLC.

Cell geometry intrinsically influences detector response. Geometries that promote turbulence within the cell serve to promote the detection process. That same turbulence also gives rise to reduced dispersion which, as has already been shown, is beneficial to NBLC,

There is one unfavourable consequence that arises from the signal dependence on mobile phase linear velocity, namely that the ECD is very flow sensitive. Hence, even more stringent specifications are imposed upon the pumping system to be pulse free otherwise an increase in noise and a consequent decrease in overall sensitivity can be expected.

Commercial ECDs have apparent flow cell volumes of as little as 0.5µl and a range of models are available. The ECD is discussed in great detail in Section 2.1.

1.5.6 System Extra-Column Dispersion - General Remarks

The total instrument dispersion governs the actual diameter of the column that can be used effectively with that chromatographic instrument. Smaller values of total extra-column dispersion permit the use of smaller bore columns. Hence, the extent to which the extra-column dispersion

needs to be reduced is motivated by the severity of the limitation of sample size or the degree of solvent economy desired.

All sources of extra-column dispersion are detrimental to the chromatography, causing additional broadening, and hence dilution, of the solute band. However, increases in the volume injected result in increased amounts of eluting solute, in addition to an increased variance contribution. Thus, the sensitivity of the method is not degraded although resolution may be impaired for closely eluting peaks. From this argument it may be concluded that the appropriate analytical strategy is to reduce the dispersion contribution from all sources other than the injector to levels significantly below that permitted for the total extra-column contribution. This allows the largest possible contribution from the injection step that is still within the 10% guideline. Hence, the maximum sensitivity is achieved by applying the largest possible injection volume.[73,74]

Extra-column variance maxima for efficient use of typical NB columns are calculated and presented in Table 1.8.

Table 1.8 Tolerable Instrument-Related Dispersion for Various Narrow-Bore Columns

L_c (cm)	d_c (mm)	d_p (μm)	V_o ($=\varepsilon\pi r_c^2 L_c$) (ml)	N ($=L/H=L_c/2d_p$)	σ_c ($=V_o/N^{\frac{1}{2}}$) (μl)	σ_c^2 (μl²)	σ_{xc} (μl)	σ_{xc}^2 ($=0.1\sigma_c^2$) (μl²)
25	2.1	10	0.61	12,500	5.46	29.8	1.73	2.98
10	2.1	5	0.24	10,000	2.40	5.76	0.76	0.58
25	1.0	10	0.14	12,500	1.25	1.57	0.39	0.16

In order that the major part of the extra-column variance may be accounted for by the injection volume, contributions due to the rest of the instrument must be significantly less than these values, say in the general region of 10% of σ^2_{xc} (i.e., ca. 0.015-0.3μl^2, depending upon the column dimensions). Measurements of extra-column dispersion have been made using both home-made and commercial instrumentation by several workers (e.g. 12,39,60,61,72,75-78). Extra-column variances of as little as 0.8μl^2 for early eluting peaks have been achieved on commercial equipment with extensive adaption[60] but many commercial liquid chromatographs yield much higher values than this.[39] Even a value of 0.8μl^2 for σ^2_{xc} is unsatisfactory for the efficient operation of many short NB columns (see Table 1.8), let alone enabling maximum sample volume to be introduced. Many small volume systems have been assembled and have achieved the benefits of solvent economy and high mass sensitivity from NB columns with moderate success. Not surprisingly however, no system has yet approached theoretical limits.

1.6 Commercial Columns for Narrow-Bore Liquid Chromatography Available in January 1984

Commercial NB column hardware is generally composed of 316 stainless steel (S/S) or glass-lined S/S although occasionally PTFE is the construction material of choice. Several types of column end-fitting are available from various manufacturers. For the majority of the work reported in this dissertation 'Swagelok' female ZDV fittings were employed (see Section 1.5.4, Figure 1.7). The other end-fitting design used was that of Shandon-Southern (Runcorn, Cheshire, UK). In addition to NB columns of conventional format, cartridge-type columns have recently been

marketed by manufacturers such as Brownlee Laboratories (Santa Clara, CA., USA) and Chrompack (London, UK). This novel column architecture was not generally available in NB format at the outset of this project and so will not be considered further here.

When NB columns began to appear in 1981 manufacturers were divided between 1mm and 2mm ID, some producing the former (e.g., Whatman, Maidstone, Kent, UK; the "Micro-B" range) and others producing the latter (e.g., Waters, Northwich, Cheshire, UK). 1mm and 2mm IDs were the two most popular column diameters, a fact directly attributable to the availability of suitable S/S tubing of these dimensions. The 1mm ID format provides a higher theoretical mass sensitivity (21x better than conventional 4.6mm ID columns) and better solvent economy but is comparatively difficult to pack efficiently and requires higher specification instrumentation with which to utilise the column effectively. Although the 2mm ID format provides less theoretical mass sensitivity (5x better than conventional 4.6mm ID columns) and less solvent economy than the 1mm columns, they are easier to pack efficiently and it is relatively easy to modify existing instrument design principles to meet the specifications required. By January 1984 some suppliers were offering a choice of column ID to the chromatographer.

Initially, only a limited range of stationary phases were available in commercial pre-packed NB columns but by the outset of this project the range was expanding rapidly. Table 1.9 provides a comprehensive overview of which pre-packed NB columns were on the market in January 1984. The major manufacturer of 1mm ID columns is seen to be Whatman whereas 2mm ID columns are produced by several companies including Beckman/Altex, Waters and HPLC Technology. For the work reported herein all pre-packed columns

Table 1.9 Pre-Packed Narrow-Bore Columns Commercially Available in the UK in January, 1984

Supplier	Manufacturer	ID (mm)	OD (in)	L_c (cm)	Construction Material	d_p (μm)	Stationary Phases
Alltech (Carnforth, Lancs.)	?	1	1/16	25, 50 < 25 to order	Flexible S/S[§]	5, 10	"μ bore series". Microsphere SiO_2, C_{18}, C_8 & C_2 as standard. Custom-made columns available with any packing material.
	?	2.1	?	10, 15, 20	S/S	3, 5	"Solvent miser series" SiO_2, C_{18}, C_8 & CN
Anachem (Luton, Beds.)	Whatman	1	1/8	25	Glass-lined S/S	3, 5, 10	Zorbax materials
	Beckman/Altex	2	1/8	25	S/S	?	Ultrasphere C_{18} only
Beckman (High Wycombe, Bucks.)	Beckman/Altex	2	1/8	25	S/S	?	Ultrasphere C_{18} only
Chrompack (London)	Chrompack	1	1/16	50 (C_{18}) 50, 100 (SiO_2) as standard. To order : 10 → 50 (C_{18}) 10 → 100 (SiO_2)	Flexible S/S	10	CP-Spher-Si (SiO_2) & CP-Spher-C_{18} (ODS)
	Chrompack	1.3	1/8	25	Rigid S/S	10	CP-Spher-Si (SiO_2) & CP-Spher-C_{18} (ODS)
Dyson (Houghton-Le-Spring, Tyne & Wear)	Shimadzu	1	?	25, 50 (C_{18}) 50, 100 (SiO_2)	S/S	10	MBC-SIL (SiO_2) & MBC-ODS (C_{18})
HPLC Technology (Macclesfield, Cheshire)	Whatman	1	1/8	25	Glass-lined S/S	10	Partisil SiO_2, ODS3, CCS/C_8, PAC, SAX & SCX
	HPLC Technology	2	1/8	10, 25 standard Any length to order	S/S	5*, 10†	Spherisorb SiO_2*†, Al_2O_3*†, C_{18}*†, CN*, NH_2*, Ph*, hexyl*. Partisil SiO_2*†, ODS1†, ODS2†, PAC†, SAX†, SCX†, C_{22}*. Other materials to order inc. Hypersil, Lichrosorb, Nucleosil, Polygosil, Techsil, Zorbax & μ-Bondapak
LDC/Milton Roy (Stone, Staffs.)	Whatman	1	1/8	25	Glass-lined S/S	10	Partisil ODS3 only.
	?	1	1/16	10	S/S	3	Spherisorb SiO_2, ODS2, C_8, CN & NH_2
LKB/Bromma (Croydon, Surrey)	Whatman	1	1/8	25	Glass-lined S/S	10	Ultrapac RP18 (C_{18}) & RP8 (C_8)
Perkin-Elmer (Beaconsfield, Bucks.)	?	?	?	?	S/S	10	C_{18} only. No more details available
Phase Sep (Queensferry, Clwyd)	?	1, 2	1/4	10, 15, 20, 25	Polished S/S	3, 5	Spherisorb SiO_2, ODS1, ODS2, C_8, C_6, C_1, CN, NH_2 & Ph
Waters (Northwich, Cheshire)	Waters	2	?	30	S/S	5*, 10†	Any Waters packing material inc. μ-Porasil†, μ-Bondapak C_{18}†, CN†, NH_2†, Ph†, Spherical SiO_2* & Spherical C_{18}*
Whatman (Maidstone, Kent)	Whatman	1	1/8	25	Glass-lined S/S	10	Partisil SiO_2, ODS3, CCS/C_8, PAC, SAX & SCX

§ S/S = Stainless steel

were supplied by HPLC Technology who deal in both Whatman "Micro-B" 1mm ID and their own laboratory-packed 2mm ID columns.

1.7 Applications of Narrow-Bore Liquid Chromatography Published to January 1984

Initially, many so-called applications of NBLC were devised primarily to illustrate some aspect of the NB technique rather than to solve practical analytical problems, consequently many of these "applications" did not utilise authentic samples. Relatively few genuine applications were published in the period to January 1984 and it is only these papers that are reviewed herein. The small number of genuine applications that have been reported generally encompass those cases where either the amount of sample available was extremely limited, where there was a need for the high resolution obtainable from long NB columns, or where the use of detection systems intolerant of conventional mobile phase flow rates (e.g., direct liquid introduction LC-MS) made the utilisation of NB columns essential. The potential for significant economy in solvent consumption was not originally a practical concern as the expense of modifying instrumentation usually outweighed any solvent savings that might have been achieved.

All the pioneering work of the application of NB columns was conducted on home-made or specially adapted commercially available conventional LC hardware. It was not until 1982 that a number of manufacturers began to introduce commercial instrumentation and columns designed to provide convenient NBLC systems for the routine analyst. The result has been an upsurge in published applications of NBLC and their more frequent use solely for solvent economy in otherwise routine applications. A summary of

important publications of NBLC applications reported in the review period is presented in Table 1.10. Included in the table is some indication of the properties of NB columns that were of greatest interest to the authors and which were directly responsible for the employment of the NB format. Applications are divided by field of research and are compiled chronologically within each such subject group. It may be noted that the majority of applications are from the fields of pharmaceutical and natural product research and primarily involve the analysis of samples of biological origin. Biological samples are, essentially, either quite complex mixtures of small and large molecules (e.g., drug metabolites in the presence of blood proteins) or they are composed of an abundance of low molecular weight species, most of which are present in trace amounts (e.g., urine). In a typical assay concentrates from such samples may contain only nanogram or sub-nanogram amounts of the analytes to be separated and quantified. The nature of biological samples makes them ideal candidates for analysis by NBLC because of the high plate numbers available and the enhanced mass sensitivity that can be obtained from this technique. The potential of NB column technology to solve analytical problems arising from biological samples was recognised early in the evolution of the technique (e.g., 22) and it is most probably this fact that has led to the large proportion of applications arising from this area of research.

From Table 1.10 it may be seen that little data is available regarding the LOD, sensitivity, repeatability and reproducibility of the reported methods. Furthermore, there is only one paper in which the question of column lifetime (which is an important factor in routine analysis) is addressed.[93] Hence, the overall ruggedness and reliability of these published analytical methods in which NB column technology has been utilised

Table 1.10 Summary of Applications of Narrow-Bore Liquid Chromatography Published to January, 1984

Field of Analysis	Sample(s) (Analytes)	Instrumentation	Column Dimensions (L_c x d_c)	Reasons for use of NB columns	LOD†/ Sensitivity	Repeatability/ Reproducibility	Ref.
Environmental Monitoring	Incinerator ash extract	Modified conventional LC/UV	25cm x 2.8mm	↑R_s* (↑N)**	n.d.***	n.d.	79
	Surface & tap waters (organics)	Modified conventional gradient LC/UV with pre-column sample concentration system	50cm x 1mm	↑sensitivity	n.d.	n.d.	22
	Diesel soot (nitro-PAHs)	Modified conventional LC/ECD	(50cm x 1mm) x 2 coupled	↑R_s (↑N)	n.d.	n.d.	80
	Surface waters (organics)	Commercial NBLC/MS (↑UV)	20cm x 0.5mm	Direct detector coupling	n.d.	n.d.	81
Forensic Science	Petrochemicals, wood, soot	Modified conventional LC/FL (cell home-made)	9cm x 1mm, 0.5-1.15m x 2.15mm	↑sensitivity	n.d.	n.d.	82
Petrochemicals	Petroleum	Modified conventional pumps for gradient work. Commercial NBLC/ UV otherwise	50cm x 1mm	↑R_s (↑N)	n.d.	n.d.	53
	Process solvent	Conventional LC/ modified RI and FTIR detectors	50cm x 1mm	Direct detector coupling	↑sensitivity observed. Not quantitated	n.d.	83
	Process solvent	Modified conventional LC/FTIR	(50cm x 1mm) x 2 coupled	Direct detector coupling, ↑R_s (↑N) & ↑sensitivity	LOD (S/N=3:1) ≈ 1µg. Sensitivity ↑8-10x with NBLC	n.d.	84
General Chemicals	Reagent solution	Assembled NBLC/ECD (cell home-made) No injection valve	15cm x 0.5mm	↑sensitivity	n.d.	n.d.	85
Pharmaceuticals and Natural Products	Human urine, serum whole blood (indoleamines)	Conventional gradient LC/UV and FL. No modifications	25↑50cm x 2.2mm single and coupled	↑R_s (↑N)	n.d.	n.d.	86
	Lapine and equine sera (steroids)	Commercial NBLC/UV with micro-pre-column extractor	13cm x 0.5mm	↑sensitivity	ng/200µl	n.d.	11
	Human urine supernatant	Conventional gradient LC/UV + FL. No modifications	100cm x 2mm	↑R_s (↑N)	n.d.	n.d.	87
	Fermentation broth, coal extract, bergamot and cinnamon oils	Modified conventional LC/UV	50cm, 1, 10, 14m x 1mm	↑R_s (↑N) vs. ↑speed of analysis @ reasonable P	n.d.	n.d.	20
	Citronella oil, human serum and whole blood extracts	Modified conventional gradient LC/UV with pre-column sample concentration system	25, 50, 100cm x 0.5-1mm	↑R_s (↑N) & ↑sensitivity	n.d.	n.d.	22
	Rat brain extract (5HT, an indoleamine)	Conventional LC/ECD No modifications	20cm x 2.1mm	↑sensitivity?	20pg/10mg tissue	SE§ < 3% @ 80% recovery	88
	Bacterial extract (plant hormones)	Conventional gradient LC/UV. No modifications	25cm x 2mm	↑sensitivity?	LOD < 5-20ng	n.d.	89

Continued .....

Table 1.10 continued

Field of Analysis	Sample(s) (Analytes)	Instrumentation	Column Dimensions (L_c x d_c)	Reasons for use of NB columns	LOD†/ Sensitivity	Repeatability/ Reproducibility	Ref.
Pharmaceuticals and Natural Products (continued)	Sandalwood oil	Modified conventional pumps for gradient work. Commercial NBLC/UV otherwise	50cm x 1mm	↑R_s (↑N) @ reasonable speed	n.d.	n.d.	53
	Hops extract	Conventional gradient LC/UV. No modifications	(25cm x 2mm) x 4 coupled	↑R_s (↑N), ↑sensitivity observed	n.d.	n.d.	90
	Equine body fluid extract (steroids)	Conventional LC/MS with modified interface	15cm x 1mm	Direct detector coupling	n.d.	n.d.	91
	Human urine (catecholamines)	Commercial NBLC/home-made dual ECD with on-line micro-pre-column	15cm x 0.5mm	↑sensitivity	n.d.	Repeatability: RSD$^{\Psi}$ (peak ht) = 0.5-5%	92
	Larval haemolymph extract (juvenile hormones)	Modified conventional pump and interface, commercial NBLC/MS otherwise	30-50cm x 1mm	Direct detector coupling & ↑sensitivity	n.d.	Short life-time (< 1 month)	93
	Pharmaceutical preparations	Modified conventional pump, commercial NBLC/UV otherwise	20, 25cm x 1mm	↑sensitivity ↑R_s (↑N) or ↑ speed of analysis	n.d.	Repeatability of fast LC: RSD ≈ 1%	94
	Lichen extract (depsides and depsidones)	Commercial NBLC with modified UV	25cm x 1mm	↑R_s (↑N) & ↑sensitivity	LOD = 23±1ng	n.d.	95
	Digitalis leaf extract (cardiac glycosides)	Commercial NBLC/UV	16cm x 0.5mm	↑sensitivity?	n.d.	Reproducibility RSD = 1-4%	96
	Equine urine and plasma extracts (corticosteroids)	Modified conventional pump and interface, commercial NBLC/MS otherwise	25cm x 1mm	Direct detector coupling & ↑sensitivity	n.d.	n.d.	97
	Human serum ultrafiltrate (catecholamines)	Commercial NBLC/home-made dual ECD with on-line micro-pre-column	15cm x 0.5mm	↑sensitivity	LOD = 3pg	Repeatability RSD 4-12% @ cathode RSD 8-27% @ anode	98
	Human urine filtrate (anti-cancer drugs)	Commercial NBLC pump, modified valve and FL with post-column extractor	20cm x 1mm	↑sensitivity (↑ speed of analysis)	LOD = 0.5-2μgml^{-1}	n.d.	99

† LOD = Limit of detection (S/N = 2:1 unless stated otherwise)

* R_s = Resolution ** N = Number of theoretical plates *** n.d. = No data

§ SE = Standard error Ψ RSD = Relative standard deviation

have not been rigorously examined. This practice is an essential prerequisite for analytical methods that are to be used routinely so that some assessment of confidence in the results produced by such procedures can be deduced.

1.8 Objectives

The primary objective of this investigation was to develop an analytical method for the determination of trace quantities of material in a complex matrix using a commercial isocratic NBLC instrument and column and, in so doing, to assess the practical advantages and limitations of the technique for a specific analytical problem. Particular regard was to be given to assessment of the suitability of the technique for routine employment in a hospital clinical laboratory.

The property of NB columns of special relevance to this research project is their ability to handle samples of severely restricted volume (and consequently limited analyte mass) because of their theoretically higher mass sensitivity compared to conventional columns (see Section 1.4.4). In addition, NB columns have been shown to exhibit comparable concentration sensitivity to conventional columns but a smaller volume of sample is required to achieve this. Furthermore, if there is sufficient sample available to enable some form of on-column or, more frequently, pre-column concentration technique to be employed then the intrinsic high mass sensitivity of a NB column can be used to provide a much higher concentration sensitivity. This is particularly important because all common LC detectors are concentration sensitive devices.

The application that was chosen with which to assess the NBLC system was the separation and quantitation of a series of important indolic

compounds which are present in blood at trace levels. The clinical basis for selecting this problem is discussed in detail in Section 3.1.2. In essence, it would be beneficial to be able to determine this class of endogenous compounds routinely in the blood of neonates* and young children. A general screening procedure is envisaged, whereby detection of biochemical abnormalities is possible leading to diagnosis and therapy. Sample availability in such cases is extremely restricted, particularly with regard to neonates where specimens of no greater than 1ml and usually far less are obtainable (normally via a heel prick). From the theoretical standpoint it would appear that this problem may well be solved best by the introduction of NB column technology to LC.

The development of the method was to be carried out initially using standards and the parameters to be evaluated were limit of detection (LOD), limit of quantitation (LOQ), repeatability, reproducibility, and column lifetime. Analyte isolation and concentration procedures were then to be developed and assessed with respect to recovery efficiency. Penultimately, the optimised method was to be applied to a series of samples from healthy individuals in order to establish a normal range. Finally, the efficacy of the method would be demonstrated by the analysis of blood taken from children of appropriate diagnosed disease states.

The performance of the instrument components and their ease of use in practice were to be scrutinised. The overall ruggedness of the NBLC instrument was to be considered and a decision with respect to the practicability of employing this technique for routine clinical analysis was to be made based on the findings of this study.

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* Neonate = "new born", an infant of up to 4 weeks of age.

Prior to the evaluation of the NBLC system available to the author, a study was proposed in which three commercial electrochemical detectors (ECDs) nominally suitable for NBLC were to be evaluated, compared and contrasted under a range of experimental conditions. Assessment with respect to warm-up characteristics, noise levels, operating characteristics, and typical LODs and LOQs for standard compounds was to be made. The detector that was deemed to perform the best was then to be utilised for the analysis of authentic samples, i.e. for the classical determination of catecholamines in brain tissue, in order to demonstrate and evaluate its capabilities with respect to "dirty" biological samples. The selected detector was then to be incorporated into a commercial NBLC system and this instrument was to be evaluated as previously outlined.

CHAPTER 2

The Evaluation and Comparison of Amperometric Electrochemical Detectors for High Performance Liquid Chromatography

Contents

2.1 Introduction

2.1.1 The Evolution of Electrochemical Detectors

The first on-line application of EC detection to LC was reported by Drake in 1950.[100] Drake utilised a crude polarographic detector employing a dropping mercury electrode (DME) as a means of detecting proteins eluting from a classical LC column. It was Kemula[101] who extensively developed the technique of polarographic detection in LC and christened the process 'chromatopolarography'. Over the next decade several designs and a wide range of applications of DME flow-through cells with fixed applied potential were reported (e.g., 102-105) but the technique was not then adopted commercially. The general lack of availability, the practical difficulties associated with the design, operation and maintenance of such a system and the overall inefficiencies of classical LC all contributed to the decline of EC detection.

It was the advent of high performance liquid chromatography (HPLC), which occurred primarily as a result of considerable advances in column technology, that brought about a renaissance in EC detection. After considerable initial effort was directed (unsuccessfully) towards developing a sensitive universal detector for HPLC equivalent to the flame ionisation detector for gas chromatography (GC), research became focused on the development of more selective detectors. During the early 1970s the potential of ECDs as selective LC detectors was recognised and this promoted a resurgence of interest in the technique. In recent years enormous advances have been made in flow cell design, microelectronics and electrode material technology enabling a wide range of detector types (e.g., amperometric, coulometric) and operational modes (e.g., d.c., a.c.,

pulsed, square waveform) to be made available to the analyst. In 1974 the first commercial ECD was marketed by Bioanalytical Systems (West Lafayette, IN., USA) which was quickly followed by ECDs from other companies. This gave rise to a rapid increase in activity in the field of EC detection. Many significant advances are still being made in research laboratories primarily in the areas of novel electrode materials, new applications of classical EC techniques, in flow cell and electronic circuitry design (especially for dual-working electrode detectors), and in miniaturisation for small-diameter column LC, particularly μLC.

2.1.2 Advantages and Disadvantages of Electrochemical Detection

Certain characteristics of an ideal detector for HPLC have been identified.[106] These characteristics include high sensitivity, low limit of detection (LOD), wide linear dynamic range, continuous operation, small internal volume, and independence of column parameters such as flow rate. Nowadays, robustness, economy and ease of automation must also be considered, particularly with regard to routine operation. Under the proper conditions ECDs can satisfy all these criteria to a large extent. To date the three types of HPLC detector that have achieved the most widespread popularity are ultra-violet/visible light absorption (UV), fluorescence (FL), and electrochemical (EC). EC detection has exhibited a number of advantages, primarily sensitivity, selectivity, and economy.[107] The advantages of EC detection may be summarised as follows :

(1) Sensitivity. The detection method is generally sensitive down to the nanogram level, and in some cases even to the picogram level, depending on the compound characteristics, chromatographic retention time,

and the applied potential. In applicable systems the LOD is typically lower (by as much as a factor of 100) than that of commercially available UV detectors. State-of-the-art EC detectors can achieve even lower LODs in the region of 100 femtograms.[108] Table 2.1 summarises the typical performances of EC, UV and FL detectors with respect to LOD.

Table 2.1* Typical Performances of HPLC Detectors

LC Detector	LOD (Commercial detectors)†	LOD (State-of-the-art)§
Electrochemical	10^{-9}-10^{-11}g	10^{-13}g
UV Absorbance	10^{-9}-10^{-10}g	10^{-12}g
Fluorescence	10^{-11}-10^{-12}g	10^{-14}g

* Table adapted from Yeung and Synovec.[109]

† LOD is calculated as the injected mass that yields a signal-to-noise ratio of 5:1 using a molecular weight of 200gmol^{-1}, 10µl injected for conventional or 1µl injected for NBLC.

§ Same definition as † above, but the injected volume is generally smaller.

(2) Selectivity. EC detection is selective to species containing an electrochemically oxidisable or reducible functional group. While a vast number of organic compounds yield absorption spectra in the UV or visible region, most organic compounds are not electroactive in an easily accessible potential range. Fortunately, a large number of important biochemicals, pharmaceuticals, food additives, pesticides and other compounds of bioanalytical and commercial interest are electroactive and amenable to EC

detection.[107] In addition to selectivity arising from the electroactivity requirement, the potential applied to the detector can be adjusted to discriminate between two or more incompletely separated electroactive compounds with different oxidation or reduction potentials.[110] The same degree of selectivity is rarely possible with a UV detector since the absorption bands are broad and usually less sensitive to change in substituent than electrochemical response is.

(3) Wide linear range. Typical linear response ranges cover 4-5 orders of magnitude of concentration.

(4) Facility of automation. EC detection is easily adapted to automatic operation and data acquisition.

(5) Small dead volumes. Modern commercial ECD flow cells have internal volumes of a few microlitres or less, giving negligible hydrodynamic broadening of chromatographic bands. State-of-the-art ECD flow cell internal volumes in the sub-nanolitre range have been reported.[108]

(6) No derivatisation required. In the vast majority of cases derivatisation is unnecessary for EC detection, unlike for FL detection where reaction is frequently required, either pre-column or post-column, to enable detection of analytes.

(7) Low cost. Direct conversion of a chemical phenomenon into an electrical signal, using no (expensive) optical components, results in inexpensive, simple and reliable electronic instrumentation.

EC detection in flowing systems is not without its problems however. The dependence of current flow per unit concentration on mobile phase flow-

rate, solution pH and ionic strength, cell geometry, condition of the working electrode (WE) surface, and injection volume requires careful control of experimental parameters. Purity of electrolytes and solvents is critical because electroactive impurities increase the observed background current and hence reduce sensitivity. For similar reasons it is advisable to minimise contact of the flow-stream with metal surfaces from which metal contaminants may be leached. A major drawback of EC detection is the need for an electrically conductive mobile phase, so limiting its applications largely to aqueous systems containing inorganic salts or acids or to mixtures of water with miscible organic solvents. Non-aqueous systems have been utilised (in conjunction with normal phase LC), for example by Gunasingham and co-workers[111,112] and Schieffer[113], but instances are rare. A further limitation resulting from the dependence of current flow on overall mobile phase composition is the general inapplicability of gradient elution to EC detection.[114] Occasional incidences of gradient elution with EC detection have been reported[115] but large baseline perturbations are inherent in the method.

2.1.3 Classification of Electrochemical Detectors

Electrochemical detectors, by the widest definition, are detection systems that utilise the measurement of an electrical property to monitor a solute in an effluent from an LC column. To date capacitance, resistance, potential and current have all been used to form the basis of a variety of EC detection techniques and these are listed in Table 2.2

Permittivity detectors have been marketed but are rarely used because of their poor sensitivity and poor selectivity (both are comparable with refractive index detection), their pronounced temperature dependence

Table 2.2 Types of Electrochemical Detection Systems Currently Used in HPLC[116]

Electrical Property Monitored	Type of Detector
Capacitance	Permittivity (Dielectric Constant)
Resistance	Conductimetric
Potential	Potentiometric
Current	Voltammetric (e.g., Polarographic, Amperometric & Coulometric)

(stability to 10^{-3} °C is required) and their limited applicability (primarily to the detection of hydrocarbons and to monitor gel permeation chromatography effluents).

Conductivity detectors are also commercially available and have been applied very successfully to modern ion chromatography since 1975.[117]

Potentiometric detectors have also been utilised for the detection of ions in HPLC eluates. Ion selective electrodes are generally employed as WEs, so providing excellent specificity but sensitivity is highly dependent on temperature and the quality of the instrumentation. Because of their very high specificity this type of potentiometric detector has found only limited use. Differential membrane-based[118,120] and solid-state metal electrode-based[121,122] potentiometric detectors have also been constructed and studied in research laboratories but detectors of these types are not currently available commercially.

The electrical property most widely utilised for LC-EC is current, i.e. the technique of voltammetry. All voltammetric methods are founded

on the same basic principles viz. if the WE is maintained at any fixed potential (relative to a reference electrode; RE) at or near the limiting-current plateau for the electroactive analyte, then the background current will remain constant so long as the solution velocity and the composition of the supporting electrolyte remain constant; as the electroactive analyte flows past the WE, electrolysis occurs and the resulting current generated (additional to the background current) is proportional to the concentration of the electroactive species. The theoretical basis of EC detection is presented in detail in Section 2.1.4.

Two different approaches to voltammetry can be applied to the detection of electroactive species; viz.

(1) complete (100%) electrolysis of a solute which is called coulometry, and
(2) partial electrolysis of a solute which is termed amperometry. If amperometry is performed with Hg as the WE material this is known as polarography.

Coulometric detectors operate using a solid WE (composed of glassy carbon, carbon gauze or a metal) of large surface area in order to achieve complete electrolysis of an eluting electroactive species. In order to maximise efficiency, coulometric cells are normally based on a thin-layer design, although tubular configurations have also been produced.[123] Coulometric detectors have been marketed but have not experienced much popularity because their sensitivity is often below that of amperometric detection[107,123], in spite of the latter rarely achieving greater than 20%, and frequently only between 1 and 10%, electrolysis efficiency.[116] The reasons for lack of sensitivity are primarily concerned with the difficulty in obtaining 100% conversion, the increase in background currents with

increased WE surface area, the comparatively large cell volume necessary to house a large WE, and increased electrode surface contamination. Coulometric detectors are also disadvantaged by complicated cell design and by decreased detector selectivity for closely eluting peaks arising from the difficulty in maintaining strict potential control over the entire electrode surface. However, coulometric detection does offer certain advantages should 100% efficiency be achieved and maintained. These advantages include the lack of effect of flow rate, temperature or electrode area on detector response and the superfluousness of calibration standards (except to check detector efficiency) due to the absolute nature of the detection process.[116] In practice these advantages are rarely attained.

Detectors that operate in the amperometric mode are far more widely available than any other type of ECD. Modern polarographic detection systems are commercially available from manufacturers such as EG & G Princeton Applied Research (Princeton, NJ., USA).[124] This is in spite of the many practical difficulties associated with the technique and its limitation primarily to electro-reduction. Not unexpectedly, polarographic detectors only account for a very small part of world-wide ECD sales. By far the greatest share of the ECD market is cornered by the much more versatile solid-state amperometric detector. These instruments will be discussed in detail in the following sections (2.1.5-2.1.7).

So far only relatively simple detector configurations operated at discrete and constant d.c. potentials have been discussed. There has been considerable interest in recent years in techniques employing more than one voltammetric detector in order to try to improve the overall selectivity and/or sensitivity of the detector systems. Various approaches have been investigated.

The operation of two ECDs in series has been studied by a number of research groups (e.g., 125-128), a coulometric ECD usually being employed prior to one of amperometric operation. Other workers (e.g., 128-130) have experimented with the concept of differential amperometric detection where two identical amperometric ECDs are run in parallel. One ECD receives the column effluent (Sample) while the other ECD, which is operated at the same potential, receives the unadulterated mobile phase (Reference). The signals obtained from the 'Reference' ECD are subtracted electronically from those obtained from the 'Sample' ECD by means of a differential amplifier yielding a consequent decrease in magnitude of the background current.

Further advances in dual-electrode detection systems have been accomplished based on the design and use of a single cell fitted with two WEs (e.g., 131-141). Great versatility is possible by variation of the configuration and operating potentials of the WEs and the extent of electrolytic conversion at each WE. This type of approach is still under experimentation from many research groups.

Increased selectivity, and in some cases sensitivity too, has been achieved by many of these dual-detection systems (e.g., 134,136) but their routine use has been prohibited by the high complexity and cost of the instrumentation.

Another area that has received extensive research has been the application of dynamic potential to detector systems in an effort to improve sensitivity and/or selectivity. A variety of pulsed waveforms adopted from classical electrochemistry have been utilised to this end and these are summarised in Table 2.3.

Table 2.3 Some Pulsed Voltage Waveforms used in HPLC Voltammetric Detection

Pulsed Waveform	Selected Reference(s)
Normal Pulsed d.c. (NPV)	142,143
Reverse Pulsed d.c. (RPV)	144
Differential Pulse (DPV)	135,143,145-155
A.c.	156,157
Rapid-Scan Square-Wave (SW)	158,159

All pulsed techniques rely on achieving increased discrimination against background effects afforded by the difference in decay rates of the charging current (due to the capacitive nature of the WE surface) and the Faradaic current (due to electron transfer at the WE surface which is proportional to the concentration of the electroactive species). The improvement of polarographic sensitivity resulting from the application of NPV waveforms, as well as the enhanced selectivity from SW and DPV waveforms, are well documented for Hg electrodes.[150-152,158,160] However, similar improvements with constant d.c. operation have not been observed for potentiodynamic voltammetry at solid electrodes. As expected from theory, analytical sensitivity (current per unit mass of analyte) for NPV- or DPV-EC detection is greater than for constant d.c. detection.[150,151] However, except for one report[142], applications of NPV or DPV have not yielded improved detectability.[135,143,145-149] This is because solid-state WEs experience considerably greater background currents and noise under pulsed waveform operation than do Hg electrodes.[146-149] The increase in back-

ground current originates primarily from Faradaic current generated by WE surface reactions which concern the formation/reduction of oxygen-containing functional groups at carbon, or of oxides at noble metals.[107,161,162] These currents decay very slowly, in comparison to charging current at Hg electrodes, and the pulsed techniques demonstrate significantly lower success in discriminating against these processes. In addition, double-layer charging at glassy carbon electrodes (GCEs) decays slowly[146], probably because of the existence of micropores in this material .[163]

The differential pulse voltammetric (DPV) technique, by virtue of its differential nature, does offer enhanced selectivity over d.c. potentiostatic operation with solid-state WEs.[107,135,143,145-147,149,153,161] The rapid-scan square-wave technique can also demonstrate improved selectivity by generation of 3-dimensional outputs of current *vs.* time *vs.* potential[158,160] but this technique is prohibitively expensive due to the complexity of the associated instrumentation.

Another current area of ECD research is that of the development of miniature flow cells and electrodes for application to small-diameter column LC, especially μLC. Theoretically, in some instances conventional ECD flow cells are of small enough effective volume (0.5-1μl) to be adequate for use in the NBLC format. It is the necessity for extremely small internal volumes in order to realise the full potential of very narrow packed and open-tubular capillary columns that has prompted this current trend in ECD development. Specialist ECD flow cells, including a few of dual WE design, have been constructed and evaluated by a number of research groups for application to μLC[85,92,108,164,165] but as μLC itself is currently only a research technique no such flow cells are yet available commercially. In

addition to the flow cell itself major advances are also being made in electrode design. Microelectrodes composed of carbon fibre have been developed to fit within the end of an open-tubular μLC column to enable on-column detection.[166,168]

However, to reiterate, it is the simple sensitive, selective and inexpensive constant d.c. amperometric ECD that is most widespread and it is this type of detector that was available for use by the author. Specific details of the amperometric detector are discussed in Sections 2.1.5 and 2.1.6.

2.1.4 Origins and General Theory of Voltammetric Electrochemical Detection

Voltammetric EC detection for HPLC is derived from classical voltammetry which essentially is based on the recording of potential-current (E-I) curves called voltammograms which are produced by the electrolysis of an electroactive substance at the surface of a WE. A pictorial representation of three such voltammograms is presented in Figure 2.1. This Figure illustrates the voltammograms obtained from the oxidation of substances X and Y and the reduction of substance Z. Qualitative data can be obtained because the potential at which the oxidation or reduction wave has reached half the total wave height (half-wave potential, $E_{\frac{1}{2}}$, shown only for compound X) is characteristic of each solute in a given electrolyte solution with a given electrode system. Quantitation is based on the measurement of the height of the wave, called the limiting or diffusion current (I_l), which is proportional to the bulk concentration of the electroactive solute giving rise to the wave.

At potentials at which electrolysis of the sample substance occurs, the concentration of the substance at the WE surface approaches zero.

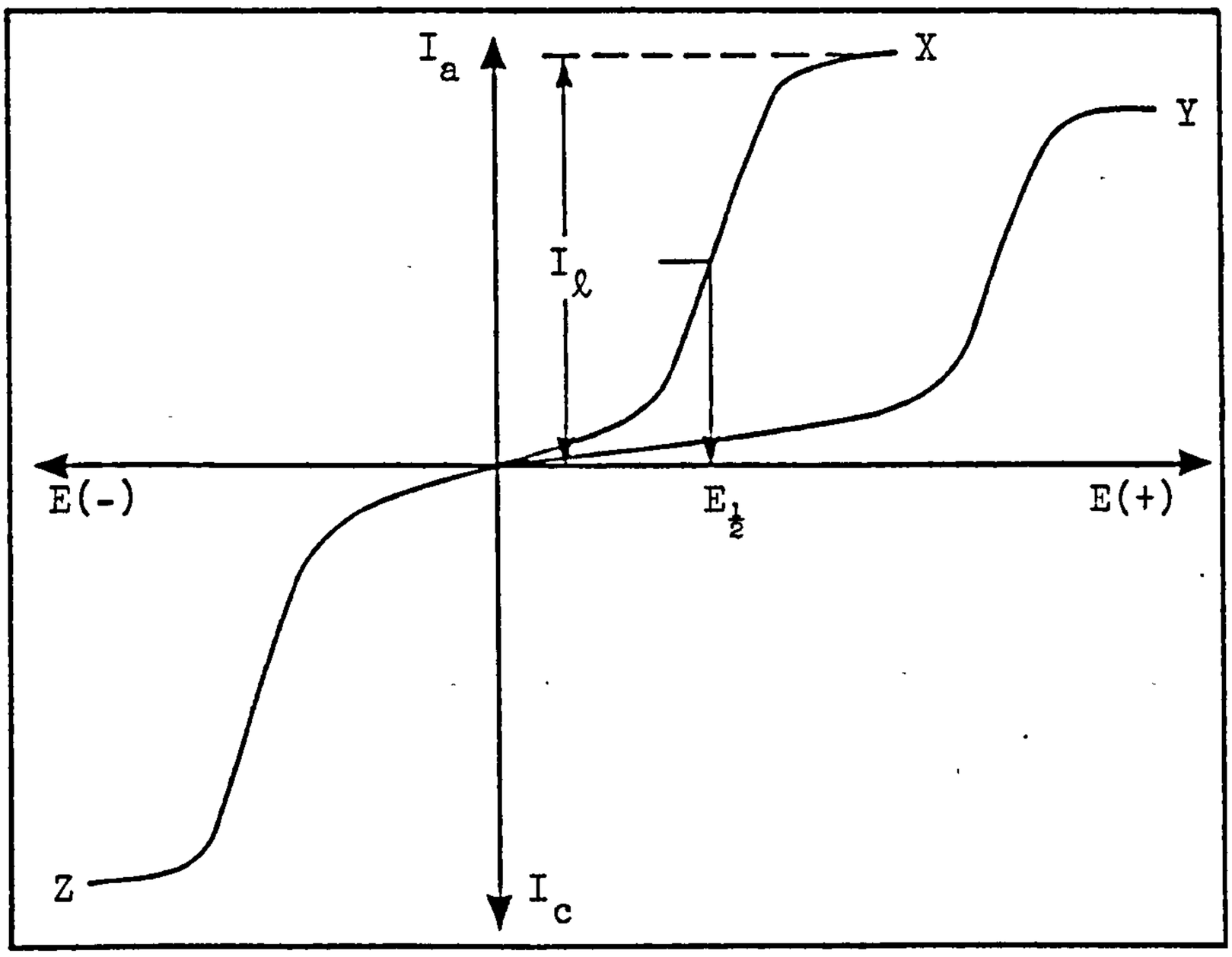


Figure 2.1 Potential-Current Curves (Voltammograms) for Three Compounds, X, Y and Z.

E(+) and E(-) = applied potential; $E_{\frac{1}{2}}$ = half-wave potential; I_a = anodic (oxidation) current; I_c = cathodic (reduction) current; and I_ℓ = limiting or diffusion current.

Current flow is sustained and increased by fresh sample substance being transported from the bulk of the solution by some mass-transfer process to the WE surface. As the applied potential is increased beyond the half-wave potential, the rate of mass-transfer becomes current-limiting. The mass-transfer processes include diffusion (movement in response to the concentration gradient generated between the WE surface and the bulk of the solution when electrolysis is initiated), migration (caused by the influence of electrical charge at the WE on charged electroactive species), and

convection (caused by solution agitation such as stirring or flowing). In a static system containing an excess of charged, non-electroactive (in the region of interest) supporting electrolyte, convection and migration are negligible, and diffusion alone controls mass transfer. Theory is well established to describe this process in the classical quiescent system and can be found in many standard texts (e.g., 169).

However, EC detection for HPLC by its very nature, takes place in a dynamic system. D.c. voltammetric detection is achieved as follows: a fixed potential, at or near the potential which yields the limiting current in a static system of similar composition, is applied. After a constant background current (resulting from electrolysis of other species in the electrolyte solution and other factors relating to the electrode system and the instrument) is established, the current generated by the oxidation or reduction of the analyte as it flows past the WE is recorded as a function of time, i.e. as an I vs. t plot.

Now, under the forced convection conditions experienced in flowing systems, both diffusion and convection contribute to the limiting current. This introduces further complications to the theoretical treatment required to fully describe the limiting current generated on electrolysis of an electroactive species.

Levich[170], by applying solution hydrodynamics utilising the concept of a thin hydrodynamic diffusion boundary layer close to the WE surface[171], derived equations for convective-diffusive voltammetry. Subsequently, other workers[129,172-176] have derived equations for their particular systems in a similar manner. Three basic assumptions are made in order to calculate the convectional-diffusional flow towards an electrode :

(1) The flow pattern is laminar,

(2) The reaction rate is infinitely large compared to the rate of mass transfer,

and (3) the electrode length and width are significantly larger than the thickness of the hydrodynamic boundary layer.

Hanekamp and van Nieuwkerk[177] have summarised all the equations for convective-diffusive voltammetry at a solid-state electrode derived by this method in the general form :

$$I_l = knFCD(Sc)^{1/3}\omega(Re_x)^{\alpha} \qquad (2.1)$$

where k = dimensionless factor dependent on the cell geometry,

n = the number of electrons involved in the electrode reaction,

F = Faraday constant (=96487 $Cmol^{-1}$),

C = bulk concentration of the electroactive species (mol l^{-1}),

D = diffusivity (or diffusion coefficient) of the electroactive species (cm^2s^{-1}),

Sc = Schmidt number = νD^{-1} [where ν = kinematic viscosity = absolute viscosity (η)/density (ρ), (cm^2s^{-1})],

ω = characteristic of the electrode width (cm),

Re_x = modified Reynolds number = u l ν^{-1} [where u = average linear fluid velocity (cms^{-1}) and l = characteristic of electrode length (cm)],

and α = factor dependent on cell geometry.

This equation applies to flow cells of all geometries. There are four basic cell geometries that have been utilised for EC detection and these are shown diagrammatically in Figure 2.2.

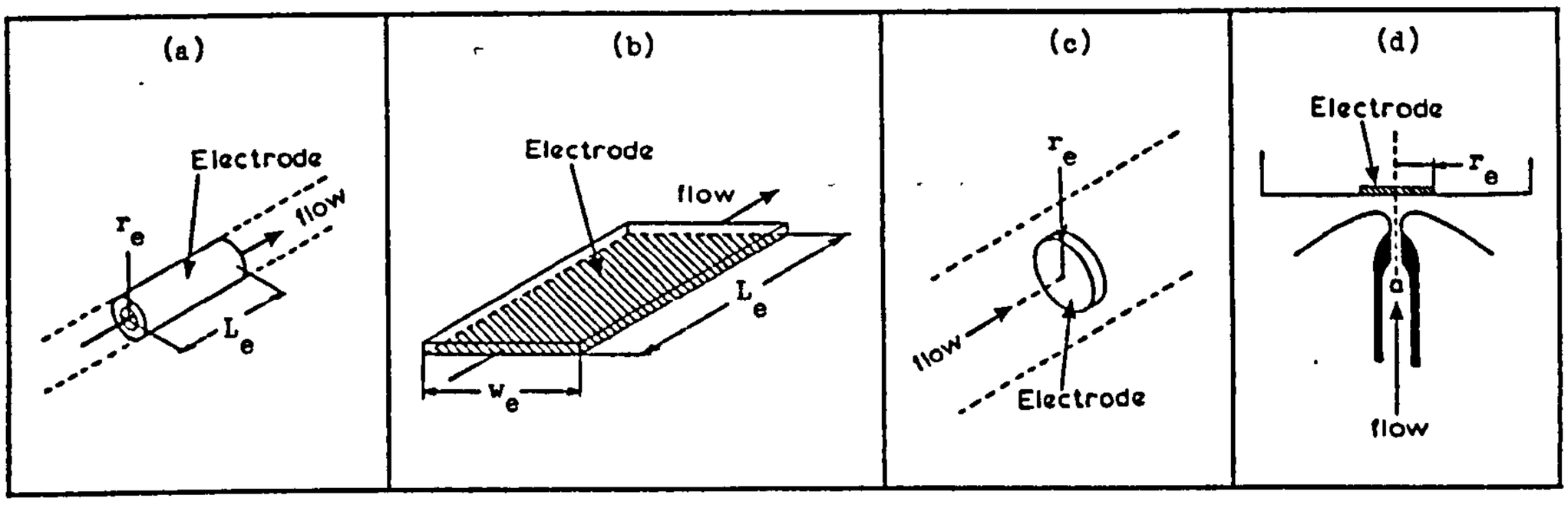


Figure 2.2 Solid-State Electrode Voltammetric Detector Configurations : (a) Tubular Electrode; (b) Thin-Layer Cell; (c) Disc Electrode; (d) Wall-Jet Cell.

The values of the geometry-related parameters for Equation 2.1 are presented in Table 2.4 for each of these configurations.

In all ECDs the signal (i.e. the output current) increases with the Reynolds number. This means that it is advantageous to build a detector with a small cross sectional area in order to increase the mean linear fluid velocity and to choose a geometry such that the highest value of α is obtained.

Another way to improve the signal would be to increase the electrode length but this can cause problems with peak dispersion due to the greater cell volume required in which to house a larger electrode.

A second important parameter is the noise as this is directly related to the LOD, which is defined empirically as the amount of compound which gives a chosen signal-to-noise (S/N) ratio. The noise in EC detection is generally accepted to be proportional to the WE surface area[178,179],

Table 2.4 Geometry-Related Parameters for Equation 2.1

Detector Configuration	k	ω*	l†	α
Tubular	8.0	$L_e^{1/3} r_e^{2/3}$	L_e	$^1/_3$
Thin-Layer	0.8	w_e	L_e	$^1/_2$
Disc	3.0/3.3	r_e	r_e	$^1/_2$
Wall-Jet	1.2	a	r_e	$^3/_4$

* ω = Characteristic of electrode width

(L_e = length; w_e = width; r_e = radius; a = nozzle diameter)

† l = Characteristic of electrode length from $Re_x = u\ l\ \nu^{-1}$

(L_e = length; r_e = radius)

i.e. noise $\simeq A_e$. Combination of this approximation with Equation 2.1 allows the *S/N* ratio to be given as a function of the electrode dimensions, q. Table 2.5 indicates the relevant terms.

It is advantageous to keep q as small as possible. The limit for decreasing q is given by the amplification system. For a tubular electrode, this means a cell with a small radius and length, for a thin-layer cell geometry a short but wide electrode, and for disc and wall-jet configuration cells an electrode of small radius.[180,181]

In practice, for amperometric EC detection the thin-layer and wall-jet configurations are strongly favoured commercially, although examples of tubular geometry do exist, e.g., the LDC/Milton Roy e.c.Monitor.[182]

Table 2.5 *S/N* Ratio as a Function of the WE Dimensions

Detector Configuration	A_e	$S \simeq$	$(S/N)_q \simeq$
Tubular	$2\pi r_e L_e$	$r_e^{2/3} L_e^{2/3}$	$(r_e L_e)^{-1/3}$
Thin-Layer	$w_e L_e$	$w_e L_e^{1/2}$	$L_e^{-1/2}$
Disc	πr_e^2	$r_e^{3/2}$	$r_e^{-1/2}$
Wall-Jet	πr_e^2	$r_e^{3/4}$	$r_e^{-5/4}$

2.1.5 Design, Construction and Range of Commercial Amperometric Electrochemical Detectors

Many cell designs for solid WE amperometric detectors have been devised, and with very few exceptions they are all based on a generally accepted three-electrode configuration. Usually the reference electrode (RE) and auxiliary electrode (AE) are placed on the downstream side of the WE so that either leakage from the RE or the formation of any electrochemical products at the AE do not interfere with the WE. The RE is normally placed in close proximity to the WE to ensure that the electrical resistance of the cell is kept to a minimum. These electrodes are fitted into the body of a flow cell, which is constructed from a non-conducting material (commonly Kel-F, a fluorocarbon). The WE surface can be formed from part of a tube, wire, sheet or disc of the desired material. Developments with planar surfaces have been the most popular as very small (sub-microlitre) cell volumes can be achieved.

There are basically two designs that have been adopted commercially, namely the thin-layer and wall-jet configurations (see Figure 2.2). Three commercial flow cells are illustrated in Figures2.3-2.5. The modern thin-layer cell was introduced by Kissinger and co-workers in 1973[183] and is now marketed worldwide by Bioanalytical Systems (West Lafayette, IN., USA). The BAS thin-layer cell (Figure 2.3) is very simple in construction and is supplied with a choice of planar WEs. The volume of the cell is controlled by the use of an appropriate thickness of PTFE spacer between the two halves of the cell. Volumes typically less than 5µl are obtained by this method. In the BAS cell the outlet tubing is composed of stainless steel which is wired as the AE thereby simplifying the cell design. No flow cell of thin-layer format was available for assessment and comparison in this study.

The wall-jet design was devised by Yamada and Matsuda in 1973.[184] Fleet and Little[185] were the first of many groups of workers to use this popular method for monitoring HPLC eluates. The so-called 'wall-jet' system comprises a nozzle of small diameter (typically 100µm) through which the mobile phase flowstream enters the cell and impinges perpendicularly onto the WE surface, which is commonly constructed of glassy carbon. The cell illustrated in Figure 2.4 is the EDT Research LCA 13 (London, UK) in which a stainless steel sleeve surrounding the inlet jet acts as the AE. An alternative version is illustrated in Figure 2.5 (the Metrohm 656; Metrohm, Herisau, Switzerland), in which a second discrete GCE is mounted in the cell to function as the AE.

Wall-jet amperometric ECDs possess certain practically useful characteristics which their thin-layer counterparts do not. Rapid convective mass transfer resulting from the perpendicular impact of the

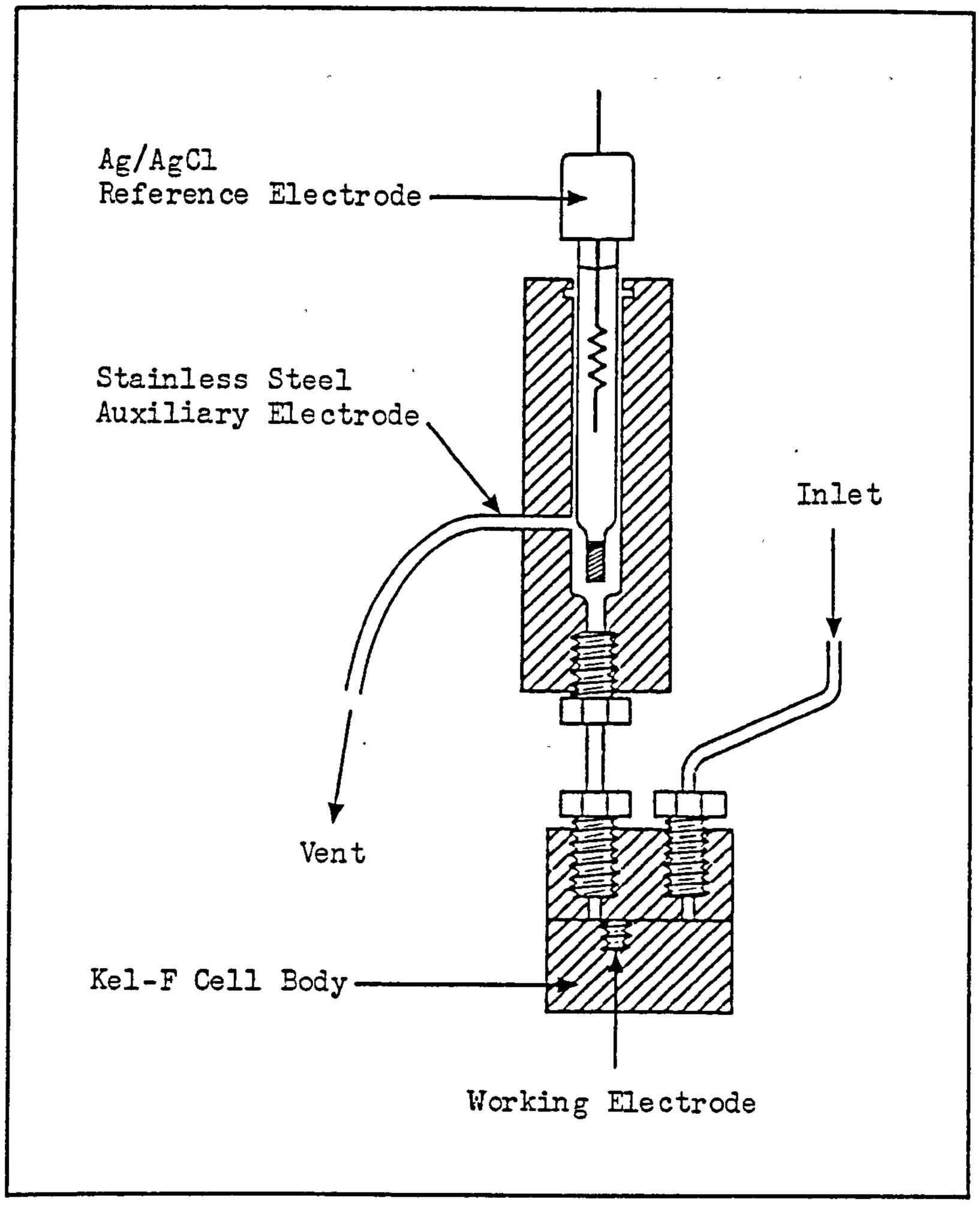


Figure 2.3 The Bioanalytical Systems LC4A Amperometric Thin-Layer Flow Cell

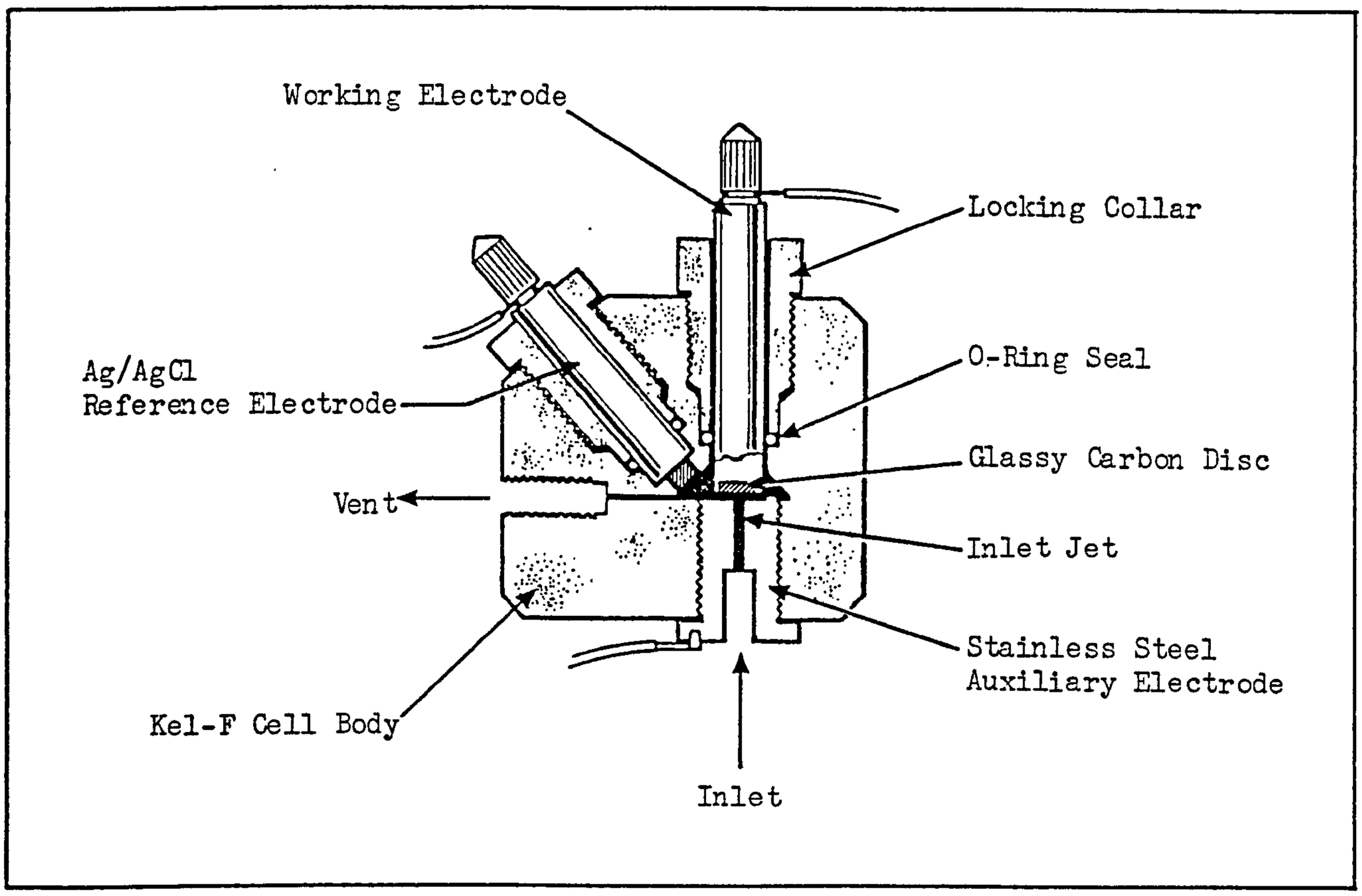


Figure 2.4 The EDT LCA 13 Amperometric Wall-Jet Flow Cell

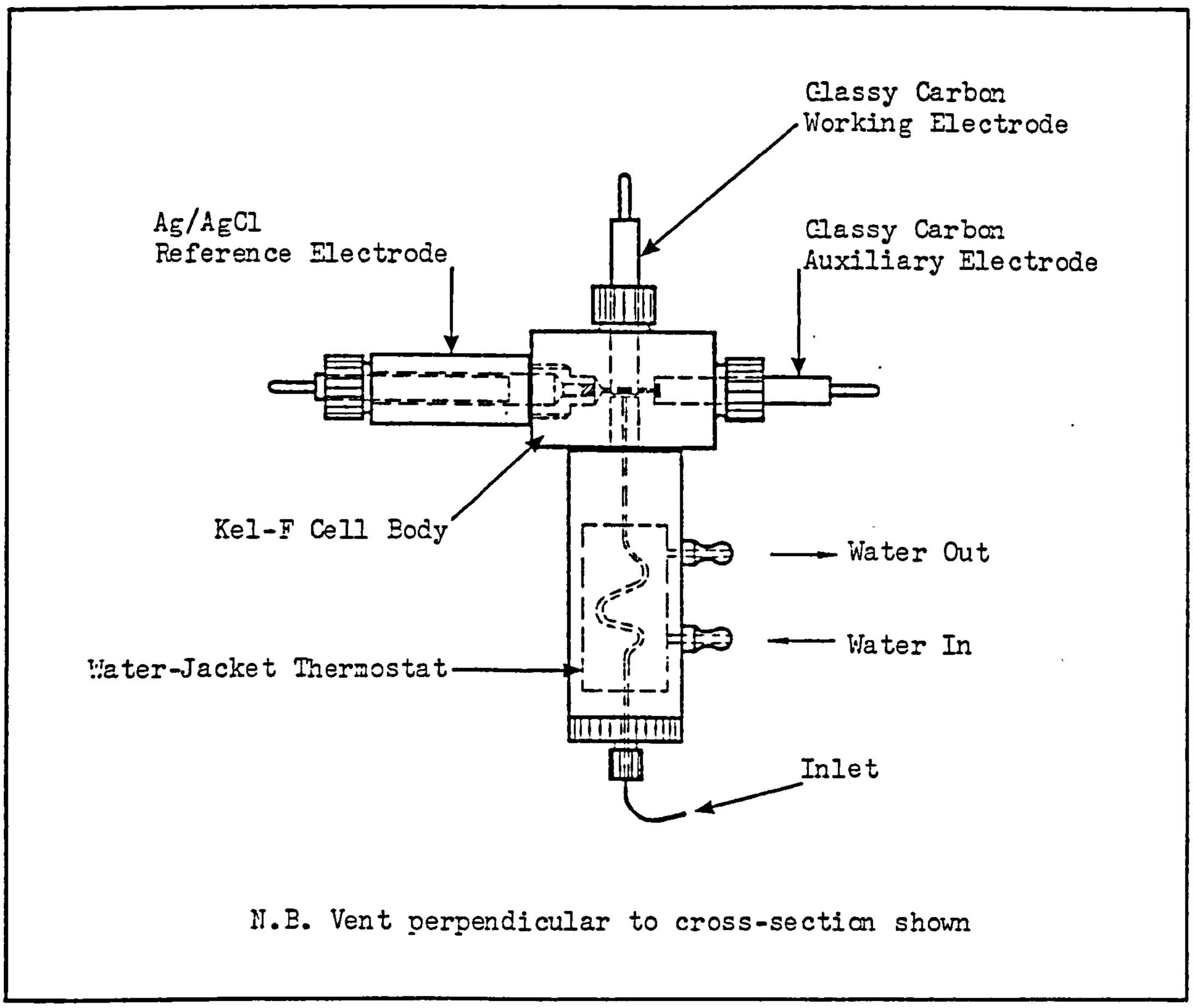


Figure 2.5 The Metrohm 656 Amperometric Wall-Jet Flow Cell

mobile phase on the WE yields very high sensitivity (picogram LODs are not uncommon (e.g., 185)). Also, the problems of surface adsorption are greatly reduced by the washing effect of the rapidly incoming solution.

At the outset of this project in 1984 there was only a small range of amperometric ECDs on the market in the UK. Since then many more amperometric ECDs have been launched. A list of currently available models (December 1986) is presented in Table 2.6. The increasing number of ECDs being produced is a reflection of the increasing demand for such instruments by liquid chromatographers.

Three amperometric ECDs, all of wall-jet format, were available to the author. These were the Metrohm 641-VA/656 and the EDT LCA 15 (the flow cells of which are illustrated in Figures 2.5 and 2.4 respectively), along with a recently marketed ECD from Pye Unicam (Cambridge, UK) viz. the PU4022. The PU4022 was identical in construction to the EDT model except in the power circuitry employed. Wall-jet configuration ECDs are considered in more detail in Section 2.1.6.

2.1.6 Theory and Principles of Operation of Wall-Jet Amperometric Electrochemical Detectors

In all modern flow-through amperometric detector cells three electrodes are required, namely the working (WE), reference (RE) and auxiliary (AE) (as previously described). It is possible to produce a detector with only two electrodes (i.e. WE and AE) and monitor the current that flows when a potential is applied across them but this arrangement produces an undesirable intrinsic non-linear response because a voltage drop will occur in the eluate as the current flow changes. The incorporation of a RE enables the potential of the WE to be monitored. Current still flows between the WE

Table 2.6 Amperometric Electrochemical Detectors Available in the UK, December 1986

UK Supplier	Model(s)	Year of Launch (if known)
Anachem (Luton, Beds.)	BAS LC4A, BAS LC4B	1974
Applied Chromatography Systems (Macclesfield, Cheshire)	ACS 350/06	1986
Bruker-Spectrospin (Coventry, W. Mids.)	Bruker LC314	-
Cecil Instruments (Cambridge)	Cecil CE1500	-
Dionex (Farnborough, Hants.)	ECD Pulsed Amperometric Detector	1983
Dyson Instruments (Houghton-Le-Spring, Tyne & Wear)	Shimadzu L-ECD-6A	1986
EG & G Instruments (Bracknell, Berks.)	BAS LC4A, BAS LC4B	1974
EDT Research (London)	EDT LCA 15 EDT LCA 16	ca. 1978 1986
LDC-Milton Roy (Stone, Staffs.)	LDC e.c.Monitor	1983/84
LKB-Bromma (Croydon, Surrey)	LKB 2143	1983/84
Perkin Elmer (Beaconsfield, Bucks.)	PE LC4B (≡BAS LC4B)	pre 1982
Pye Unicam (Cambridge)	PU4022 (≡EDT LCA 15)	1982
Roth Scientific (Farnborough, Hants.)	Metrohm 641-VA/656	pre 1982
Thames Chromatography (Maidenhead, Berks.)	Thames TC 100	-
Waters/Millipore (Harrow, Middx.)	Waters 460 ECD	1985/86

and AE but, if there is any deviation in the potential of the WE from the pre-set applied potential then current feedback *via* the AE can be employed to restore the balance. To a large extent it is this potentiostatic electronic feedback loop which enables amperometric ECDs to exhibit such a wide linear dynamic range (typically 10^4-10^5 concentration units). The current flowing off the WE is converted to a voltage by appropriate electronic circuitry and, after signal amplification and filtering, this voltage is channelled to a potentiometric recording device (chart recorder, integrator, computer) for visual presentation and/or data analysis.

Wall-jet flow cells, as stated previously, were developed by Yamada and Matsuda.[184] These researchers derived an equation for the limiting current,

$$I_\ell = 1.38nFCD^{2/3}\nu^{-5/12}Q^{3/4}a^{-1/2}r_e^{3/4} \qquad (2.2)$$

where Q = volume flow rate of the solution issuing from the circular nozzle,

and all other symbols are as previously defined (Section 2.1.4). The volume flow rate is equal to the mean linear velocity of the fluid in the nozzle multiplied by the area of the nozzle outlet :

$$Q = \frac{1}{4}\pi a^2 u \qquad (2.3)$$

When substituted into Equation 2.2 this yields an expression for the limiting current in terms of the linear flow rate :

$$I_\ell = 1.15nFCD^{2/3}\nu^{-5/12}au^{3/4}r_e^{3/4} \qquad (2.4)*$$

* N.B. With suitable substitution Equation 2.4 may be presented in the general form reported by Hanekamp and van Nieuwkerk[177], i.e. Equation 2.1.

With so many variables involved, the deduction of the current-concentration relationship from the fundamental parameters is difficult. However, Equation 2.4 may be expressed in a more general format, i.e.

$$I_\ell = f(n,C,D,\nu,a,u,r_e) \qquad (2.5)$$

In practical terms, for a given electrode reaction in a given flow cell n, a and r_e are constant. Hence, the expression for the limiting current may be simplified under these conditions to :

$$I_\ell = f(C,D,\nu,u) \qquad (2.6)$$

Now, at constant flow rate of a uniform mobile phase at a constant temperature, u and the two temperature dependent terms D and ν are all constant so yielding the direct proportionality between the limiting current and the bulk concentration of the electroactive compound being determined, i.e. :

$$I_\ell \propto C \qquad (2.7)$$

It is on this basis that quantitation is possible with an ECD.

The practical effects on detector signal of certain parameters have been investigated. Detector output increases with temperature; a typical relationship is illustrated in Figure 2.6.[186]

As would be expected, elevation of temperature increases the energy of the system and this in turn improves the rate of diffusion to the electrode surface so yielding a signal of increased intensity. Over the range 10-30 ^{o}C signal increases by about a factor of 1.5% per ^{o}C.[186] This degree of variation, although not marked over one or two ^{o}C, does serve to stress the advisability of thermostatting the detection system in order to

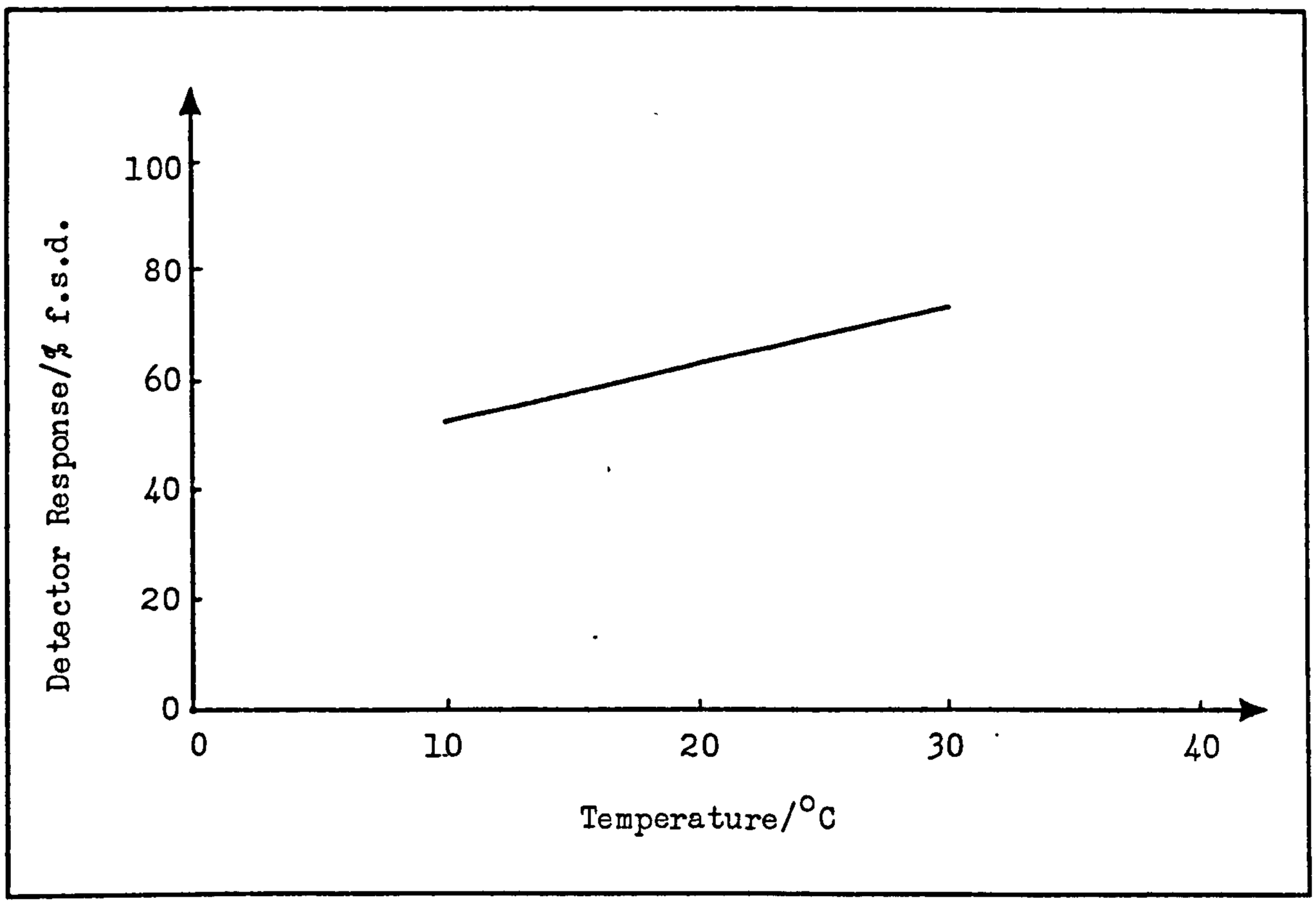


Figure 2.6 Dependence of the Signal from an Amperometric Electrochemical Detector on Temperature

reduce the error introduced in this way. The Metrohm 656 flow cell inlet tubing is thermostatted with a water jacket whereas the EDT LCA 15 and Pye Unicam PU4022 cells are operated at ambient temperature. There is one inherent disadvantage of thermostatting the inlet tubing, however. That is that a lengthy inlet tube is required to enable temperature uniformity to be attained, tubing in which increased chromatographic peak broadening will occur so reducing chromatographic efficiency. This situation is particularly detrimental where NBLC is concerned.

Detector output is also influenced by the mobile phase volumetric flow rate. A typical relationship is illustrated in Figure 2.7.[186]

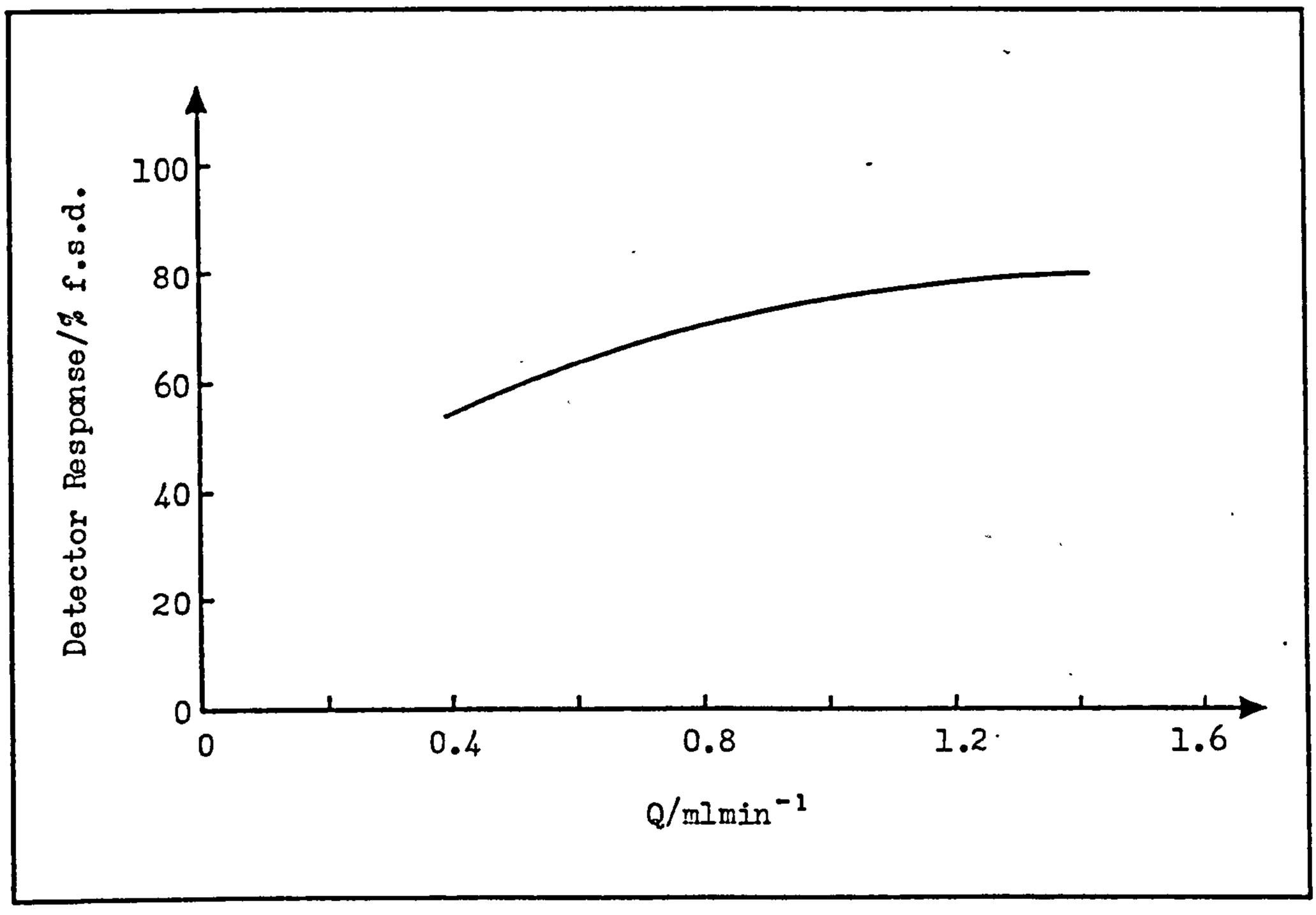


Figure 2.7 Dependence of the Signal from an Amperometric Electrochemical Detector on Mobile Phase Volumetric Flow Rate, Q

This curve represents the resultant effect of two antagonistically acting processes. As flow rate increases, greater convection is generated within the cell. As a consequence of this, the rate of mass transport towards the WE surface is increased leading to an increase in signal. However, the magnitude of the convection effect lessens with increasing flow rate because of the influence of flow rate on sample turnover at the WE surface (i.e. coulometric yield), which is illustrated in Figure 2.8.

As flow rate increases, the residence time of the electroactive species in the cell diminishes resulting in ever increasing amounts of analyte being swept out of the cell before it can be transported to the

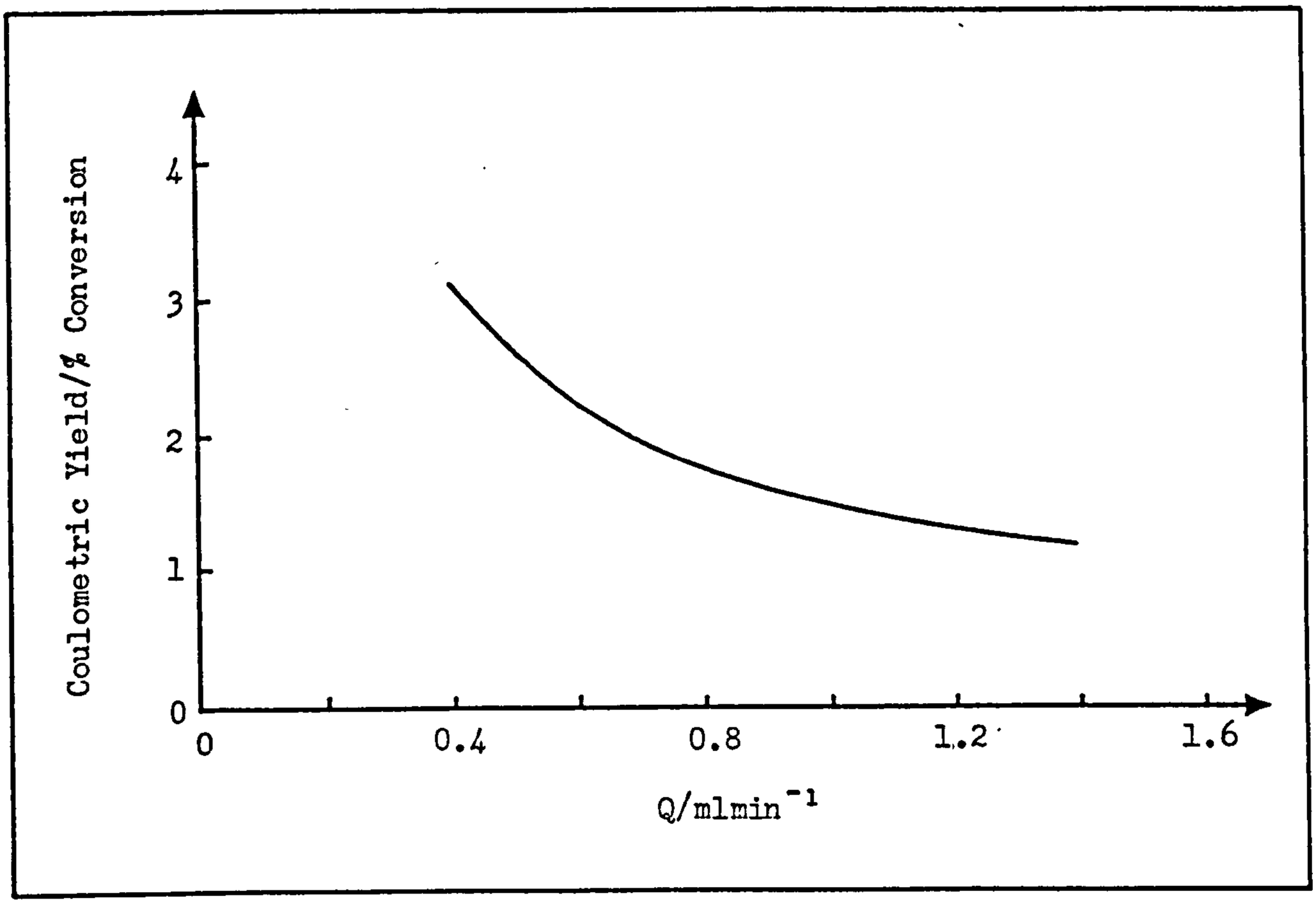


Figure 2.8 Dependence of Coulometric Yield on Mobile Phase Volumetric Flow Rate, Q

WE surface for reaction. Thus, the coulometric yield falls off with increasing flow rate as is depicted in Figure 2.8. The curve shown in Figure 2.7 is typical, increasing convection being the dominant factor at low to medium flow rates with the coulometric yield factor exerting greater influence at high flow rates.

2.1.7 Electrodes for Electrochemical Detectors[124,163]

While the choice of RE (usually saturated calomel or Ag/AgCl) and, where applicable, AE (usually a metal such as Pt or stainless steel) is generally not critical to detector performance, the choice of WE material is.

Although most of the early work in this technique involved the DME, solid-state electrodes (including various forms of carbon, noble metals, and metal amalgans) have been extensively studied and successfully applied in recent years. Each type of electrode material has both advantages and disadvantages.

The DME has an extensive cathodic range of polarisation which leads to its application for electro-reductions. However, the usable anodic potential range is severely limited by the oxidation of Hg metal itself (at +0.2V vs. Ag/AgCl) which precludes its use for most electro-oxidisable compounds. The DME provides a constantly renewed electrode surface, effectively eliminating the problem of surface contamination. Considerable disadvantages of the DME include the need for electronic damping, the need to remove oxygen from the supporting electrolyte solution, the problems of cell design by virtue of the nature of the WE material, and the effects of mobile phase motion on drop-time, limiting current and charging-current background.

Solid-state electrodes do not exhibit the restrictions in applied potential that are found with DMEs. Solid electrodes can be used to analyse easily reducible compounds but they are best suited to the study of oxidation processes because of their wide anodic (positive) polarisation range and low residual current within this range. Generally, greater sensitivity, simpler cell design and lower noise levels are realised with solid electrodes compared with DMEs. The major problem with solid electrodes is non-reproducibility of the electrode surface, primarily caused by adsorption and surface oxide formation. Thus, cleaning of the solid electrode, or surface renewal, becomes a major consideration.

Metal WEs are the least used. Pt[106,108,142,187,188], Au[142], Ag[187], Cd[189], Ni (as oxide)[190] and Cu[191] have all been utilised in ECD flow cells

to a limited extent but suffer greatly from oxide film formation during operation (especially in aqueous media), which yields a large residual current and affects electrode reactions. Metallic electrodes are available for commercial ECDs but are rarely used in practice.

The most prevalent electrode material is carbon which has been utilised in many forms including carbon paste, glassy carbon, graphite impregnated silicone rubber, pyrolytic carbon, reticulated vitreous carbon and, more recently, carbon fibre.

Carbon paste electrodes (CPEs) were the most popular WEs for EC detection until the early 1980s and are still widely employed. Made from high purity graphite dispersed in a water-immiscible organic solvent, CPEs can be prepared quickly. Other advantages of CPEs include very low residual current, high stability, rare exhibition of memory effects (unlike noble metal electrodes), and ease of surface renewal. The major disadvantage of CPEs in flowing systems is the restriction on mobile phase composition which must be largely aqueous. Organic solvents tend to cause rapid electrode deterioration by dissolution of the graphite dispersion medium. Furthermore, CPEs are unsuitable for the detection of most reducible compounds due to high cathodic residual currents, they require exceptionally long equilibration times to achieve constant background current (several hours from start-up at high sensitivity settings[114]), and they are poorly reproducible on replacement.

Over the last 5 years CPEs have largely been superseded by glassy carbon electrodes (GCEs)[192], as was predicted by Fleet and Little[185] as long ago as 1974. GCEs are now widely available and fitted as standard to most commercial ECDs. Glassy carbon is an impermeable, electrically conductive material, resistant to chemical effects and can be used directly as an

electrode.[193] The material is prepared by the heating of phenol-formaldehyde resins in an inert atmosphere. GCEs are more durable than CPEs and can be used with non-aqueous solvents. The wider useful potential range of glassy carbon compared with carbon paste (from -1.3V to +1.5V _vs_. Ag/AgCl has been reported[185]) enables the electroysis of both oxidisable and reducible species. The residual current at GCEs is higher than at CPEs.[194] GCEs are more susceptible to surface contamination than CPEs, and so require more frequent cleaning but cleaning, in many instances, may be accomplished by electrochemical treatments without the need for cell disassembly.[195] It is the great versatility and durability of GCEs that makes them particularly attractive for LC-EC work.

Of the other forms of carbon employed, graphite impregnated silicone rubber[193] and pyrolytic carbon electrodes[196,197] are similar to GCEs in ruggedness, applicability to non-aqueous systems, low residual current, large anodic as well as cathodic potential ranges and moderate susceptibility to surface adsorption. Reticulated vitreous carbon[198,199] and carbon fibres[200,201] have only recently been applied to LC-EC but both of these materials have also been found to perform similarly to glassy carbon, although carbon fibres have exhibited improved detection limits relative to GCEs at high positive potentials.[201] To the knowledge of the author, none of these other forms of carbon are currently marketed as WEs for ECD cells in the UK.

For the work reported herein glassy carbon was the material of choice for WEs and was used exclusively.

2.1.8 Objectives

The major objective of the comparison between the three amperometric ECDs, the EDT LCA 15, the Pye Unicam PU4022 and the Metrohm 641-VA/656 was to select that one which performed best, primarily with regard to signal-to-noise (*S/N*) characteristics, so that it may be applied in future work with NBLC. It was proposed that warm-up procedure, noise levels at various instrument sensitivities, 'user-friendliness' of the units, and limits of detection and quantitation of the three ECDs towards standard compounds would be evaluated and compared under a range of experimental conditions.

Secondary objectives included the direct comparison of the EDT and Pye Unicam ECDs which differed solely in their power circuits. The power packs employed are reported to differ in stability by a factor of three which would be expected to be reflected in their comparative LODs.[202] Any difference in performance, primarily with regard to *S/N*, that is directly attributable to the different power packs would be of value to the manufacturers in improving instrument design.

A further objective was the comparison of the Metrohm ECD with the EDT and Pye Unicam models primarily to determine to what degree structural differences affect overall performance. There are considerable differences between the designs of the Metrohm and the EDT and Pye Unicam ECDs, and these are summarised in Table 2.7. The Metrohm flow cell, although of wall-jet configuration like the other two, possesses a water-jacket thermostat around the inlet pipe, the cell itself is of different construction containing a discrete GCE as the AE and the entire cell assembly is housed in an enamelled metal cage in order to minimise the influence of external electrical fields. The cell design of the EDT and Pye Unicam models is much simpler; there is no thermostat or shield and the stainless steel inlet

Table 2.7 Structural Differences Between the Amperometric Electrochemical Detectors Available for Comparison

Feature	ECD Model		
	EDT LCA 15[203,204]	Pye Unicam PU4022[202,205]	Metrohm 641-VA/656[206,207]
FLOWCELL			
WE	Glassy Carbon(5mm diameter)	Glassy Carbon(5mm diameter)	Glassy Carbon(3mm diameter)
AE	Stainless Steel	Stainless Steel	Glassy Carbon(3mm diameter)
RE system	Ag/AgCl/KClgel, ceramic gel junction	Ag/AgCl/KClgel, ceramic gel junction	Ag/AgCl/KCl(3moll^{-1})
Cell volume	0.5µl	0.5µl	<1µl
Thermostatted inlet?	No	No	Yes
Screened?	No	No	Yes
CONTROLLER UNIT			
Instrument Sensitivity Range (f.s.d.)	1,3,10,30,100,300nA 1,3µA	1,3,10,30,100,300nA 1,3µA	0.1,0.5,1,5,10,50nA 0.1,0.5,1,5,10^2 and 10^{4}µA
Time Constants/Damping	1,3 and 10sec	1,3 and 10sec	@ <0.1nA = 5sec @ <0.5nA = 0.6sec @ <1nA = 0.3sec @ <5nA = 0.1sec Damping ≃ +1.7sec
Auto zero?	Yes	Yes	No
Event Marker?	Yes	Yes	No
Power Supply Requirements	210-240V only	110 or 220-240V by interchanging power cable and engaging/disengaging transformer	100,117,220 or 240V with internal transformer

nozzle is utilised as the AE. All three flow cells have a GCE as the WE and a Ag/AgCl RE. The electronic controller units also differ, primarily in the output range settings available. The EDT and Pye Unicam models offer a less extensive range of instrument sensitivities but a greater degree of electronic baseline noise-smoothing control.

The final objective of these experiments was to enable the operator to familiarise himself with the general practices of LC-EC and to gain 'hands-on' experience of the technique.

2.2 Experimental

2.2.1 Solvents and Reagents

Methanol (MeOH; GPR grade) was supplied by Wilcott Industrial, Bristol, Avon and Charles Tennants, London and was single distilled into glass before use. Water was single distilled into polythene before use. Acetone (GPR grade) was supplied by Charles Tennants, London and was used as received in the routine cleaning of glassware. All other solvents, viz. propan-2-ol (2-PrOH; GPR grade) and cyclohexanol (GPR grade) which were used in column packing, were obtained from BDH, Poole, Dorset.

Buffer components citric acid (HCt; Laboratory reagent grade) and sodium hydroxide (NaOH, AnalaR grade) were both supplied by BDH, Poole, Dorset, and sodium acetate (NaAc, anhydrous, GPR grade) was supplied by Hopkin and Williams, Chadwell Heath, Essex. Other mobile phase components ethylenediaminetetraacetic acid (EDTA, disodium salt dihydrate, AnalaR grade) and 1-heptanesulphonic acid (HSA), sodium salt monohydrate, laboratory reagent grade) were supplied by BDH, Poole, Dorset.

The catecholamines epinephrine (EP, as free base), norepinephrine (NE, as hydrochloride salt) and dopamine (DA, as free base) were kindly donated by Dr. P.J. Naish of Pye Unicam, Cambridge. The internal standard for the rat brain study viz. 3,4-dihydroxybenzoic acid (DHBA) was a gift from Dr. I. Kilpatrick, Department of Anatomy, University of Bristol.

Helium gas for the degassing of mobile phases was supplied in cylinders by BOC (Special Gases), Bristol, Avon.

2.2.2 Mobile Phase Preparation (1 litre)

2.2.2.1 For all Experiments Except Chromatography of Rat Brain Homogenates

Measured volumes of MeOH and single distilled water (DW) were combined in a 1 litre volumetric flask to provide the desired solvent ratio. The solvent mixture was transferred to a 2 litre-capacity beaker and HCt (6.3g), NaAc (2.27g) and NaOH (2.0g) were dissolved in the solvent mixture to provide a mobile phase of pH 4.8.

2.2.2.2 For Chromatography of Rat Brain Homogenates

MeOH (70ml) was placed in a 1 litre volumetric flask and to this was added an aq. 0.1M solution of NaAc up to the mark. The solvent mixture was transferred to a 2 litre-capacity beaker and to the solution were added NaHSA.H_2O (123mg ≡ 100mgl^{-1} HSA) and Na_2EDTA.$2H_2O$ (64.6mg ≡ 50mgl^{-1} EDTA). The pH of the mixture was then adjusted to 4.6 by the addition of HCt.

2.2.2.3 General Procedure

Each prepared batch of mobile phase was transferred to a 1 litre reagent bottle and was then degassed under a gentle stream of He for at least 5 minutes prior to delivery. Degassing was continued throughout the course of the experiments in order to maintain the exclusion of oxygen from the mobile phase. Preferential evaporation of solvent was restricted by plugging the reagent bottle neck around the tubing with lint-free paper towelling.

2.2.3 Instrumentation

Pumps	:	Pye Unicam PU4010, Pye Unicam/Altex LC-XPD Model 100.
Injection Valves	:	Rheodyne Model 7010 fitted with a Model 7012 loop filler port.
Loop Capacity	:	20µl except for the rat brain homogenate analysis where a 50µl loop was employed.
Columns	:	Spherisorb S5 ODS1 (25cm x 5mm ID, d_p = 5µm).
Detectors	:	Pye Unicam PU4022 ECD (comprising control unit and flow cell), EDT LCA 15 (comprising control unit and flow cell), and Metrohm 641-VA ECD with a Metrohm 656 flow cell. All flow cells were equipped with a glassy carbon WE, a Ag/AgCl/KCl RE and an AE. The AE was composed of stainless steel in the Pye Unicam and EDT instruments and glassy carbon in the Metrohm system.
Chart Recorders	:	Servoscribe Model 1s potentiometric recorders (1V f.s.d.).

2.2.4 Columns

The columns used throughout the course of this study were ones comprised of Spherisorb S5 ODS1 (25cm x 5mm ID, d_p = 5µm) which were packed in this laboratory using a modification of the high pressure balanced-density technique.[208] A slurry of the packing material (Spherisorb S5 ODS1, supplied by Phase Sep, Queensferry, Clwyd) was prepared by homogenisation and degassing of the packing material in supporting balanced-density solvent

(ca. 1g of packing to 10ml of solvent) by means of ultrasonic vibration for 10 minutes. This slurry was placed in a reservoir over 2-PrOH and was pumped into the column using a N_2-driven constant pressure hydraulic pump (Haskel MCP-71) set at 6500p.s.i. The flow rate during the packing was maintained within the range 15-20mlmin^{-1}. MeOH containing cyclohexanol (3 drops per 10mls MeOH) was employed as the supporting liquid.

2.2.5 Standard Solution Preparation

For general purposes standard solutions of the catecholamines EP, NE.HCl and DA were prepared from methanolic stock solutions (100μgml^{-1} active ingredient) by serial dilution using mobile phase.

For optimisation of the cell polarisation potential for catecholamine analysis a working standard containing 0.4μgml^{-1} of each catecholamine was prepared by serial dilution.

For determination of the limits of quantitation and detection of different makes of ECD towards EP, NE and DA a working standard containing 0.01μgml^{-1} of each catecholamine was prepared by serial dilution.

For the analysis of rat brain homogenates experiment an aqueous standard containing NE (0.2μgml^{-1}), DA (0.2μgml^{-1}), 3,4-dihydroxyphenyl-acetic acid (DOPAC, 0.2μgml^{-1}) and DHBA (0.65μgml^{-1}) was provided by Dr. I. Kilpatrick (Department of Anatomy, University of Bristol).

All standards were stored in a refrigerator maintained at ca. 4 ^{o}C until required. Vessels containing stock solutions were wrapped an Al foil in order to exclude light.

2.2.6 HPLC Operating Conditions

2.2.6.1 Conditions Employed for Warm-Up Procedures and Baseline Noise Comparison Studies[209]

Column : Spherisorb S5 ODS1 (L = 25cm, ID = 5mm, d_p = 5µm, C Loading = 7% w/w, Surface Area = $220 m^2 g^{-1}$).

Mobile Phase : 10% MeOH : 90% aq. buffer containing HCt ($6.3 g l^{-1}$), NaAc ($2.27 g l^{-1}$) and NaOH ($2.0 g l^{-1}$), pH 4.8.

Flow Rate : $1.0 ml min^{-1}$.

Injection Volume : 20µl (*via* loop).

Detection : ECD (Potential : +0.65V *vs.* Ag/AgCl reference; Mode : Oxidation; Instrument Sensitivity : 30nA f.s.d.; Time Constant : 1sec).

Chart Speed : $5mm\ min^{-1}$.

2.2.6.2 Conditions Employed for the Catecholamine Limit of Detection and Limit of Quantitation Study

As in 2.2.6.1 except ECD potential = +0.70V.

2.2.6.3 Conditions Employed for the Analysis of Rat Brain Homogenates for Neurochemicals[210]

As in 2.2.6.1 except ECD potential = +0.70V *vs.* Ag/AgCl and,

Mobile Phase : 7% MeOH : 93% aq. 0.1M NaAc/HCt buffer containing HSA ($100 mg l^{-1}$) and EDTA ($50 mg l^{-1}$), pH 4.6.

2.2.7 Detector Warm-Up Procedure

An HPLC system including the PU4010 pump, and with the first ECD to be evaluated on-line, was set up. The standard mobile phase comprising 10% MeOH : 90% aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$) was pumped through the apparatus at a flow rate of $1.0mlmin^{-1}$. Any air bubbles that became trapped in the flow cell were removed by holding the cell in a suitable orientation and tapping it vigorously. A free flow of solvent through the cell was ensured prior to start up.

The chart recorder was zeroed and set in motion at a velocity of $5mm\ min^{-1}$. The ECD controller unit was programmed as follows :

Power	:	Off
Applied Potential	:	+1.00V
Mode	:	Oxidation
Cell	:	Off
Sensitivity	:	1μA f.s.d.
Time Constant/Damping	:	1sec (PU and EDT models), Off (Metrohm model)

The power was engaged then the operating potential was applied to the cell. The behaviour of the baseline was observed and recorded. The time taken to settle to +10% f.s.d. was noted. A working potential of +0.65V was then selected. The instrument sensitivity was increased from 1μA to 30nA (EDT and PU ECDs) or 50nA (Metrohm ECD) in a stepwise manner, allowing the baseline to stabilise between increments. The time taken to achieve a steady baseline of $\leq$ 5% drift was noted. The instrument sensitivity was then further increased to 10nA f.s.d. and again the time taken to achieve a stable baseline was noted. Further increases in instrument sensitivity

were investigated as was deemed appropriate based on previous findings.

Subsequently, identical warm-up procedures were applied to the remaining two detectors so that the instruments could be directly compared.

2.2.8 Recording of Baselines

Detector outputs were recorded from the PU4022, EDT LCA 15 and Metrohm 641-VA/656 ECDs under a variety of experimental conditions. The variables were pump model (PU4010 or Altex LC-XPD), mobile phase organic modifier content (10% or 90%), mobile phase flow rate (0.5, 1.0, 2.0 and 3.0mlmin^{-1}) and detector sensitivity (30nA (or 50nA for the Metrohm model) or 10nA f.s.d., and 3nA or 1nA f.s.d. where appropriate).

Each required combination of variables was applied in turn and the baseline was recorded. Sufficient time was allowed for equilibration of the system, i.e. until no discernible change in the recorded baseline over a 20 minute period was noted, before a representative section of the trace was taken and a new set of conditions was applied.

2.2.9 Procedure for the Optimisation of Electrochemical Detector Applied Potential

An HPLC apparatus comprising the Altex LC-XPD pump, a Rheodyne injection valve fitted with a 20µl loop, a Spherisorb S5 ODS1 column (25cm x 5mm ID, d_p = 5µm) and the EDT LCA 15 ECD was constructed. Through this apparatus was pumped the standard mobile phase (10% MeOH : 90% aq. buffer containing HCt (6.3gl^{-1}), NaAc (2.27gl^{-1}) and NaOH (2.0gl^{-1}), pH 4.8) at a flow rate of 1.0mlmin^{-1}. An initial cell polarising voltage of +0.50V was programmed and the baseline at 30nA f.s.d. sensitivity with a

time constant of 1sec was allowed to settle. A standard containing EP, NE and DA (all components at concentrations of 0.4μgml^{-1}) was then chromatographed in duplicate. The applied potential was increased in 0.10V increments (except for +0.65V) to +0.90V and the procedure was repeated for each selected potential. Peak heights and baseline noise levels of all the chromatograms were measured manually.

2.2.10 Procedure for the Determination of Limits of Detection and Quantitation of EP, NE and DA

A second HPLC apparatus was assembled of identical format to the one used for the optimisation of ECD applied potential (see Section 2.2.9) except that the PU4010 pump and the PU4022 ECD were incorporated. An identical Spherisorb S5 ODS1 column (25cm x 5mm ID, d_p = 5μm) was connected into this second system. Through both instruments was pumped the same standard mobile phase (see Section 2.2.6) at 1.0mlmin^{-1}. Both ECDs were set to an instrument sensitivity of 1nA f.s.d. with a time constant of 10sec. The baseline was allowed to stabilise in each case. A standard containing EP, NE and DA (all at a concentration of 0.01μgml^{-1}) was chromatographed in duplicate on each system. Peak heights and baseline noise levels of all the chromatograms were measured manually.

2.2.11 Preparation of Rat Brain Samples

A laboratory rat was sacrificed by cervical dislocation and its brain was removed quickly on to an iced surfaced. The brain was carefully dissected and samples of cerebral cortex (2 x ca. 15mg), striatum (2 x ca. 15mg) and olfactory tubercles (2 x ca. 15mg) were taken and placed in PTFE

centrifuge vials. To one sample from each area of the brain was added aq. DHBA ($9\mu g ml^{-1}$, $25\mu l$) and aq. $HClO_4$ (0.4M, $325\mu l$) containing EDTA ($50\mu g ml^{-1}$). To the other sample from each area of the brain was added aq. $HClO_4$ (0.4M, $350\mu l$) containing EDTA ($50\mu g ml^{-1}$) only. Each tissue sample was homogenised then centrifuged to obtain the supernatant. The prepared samples were kept on ice until they were chromatographed.

2.3 Results and Discussion

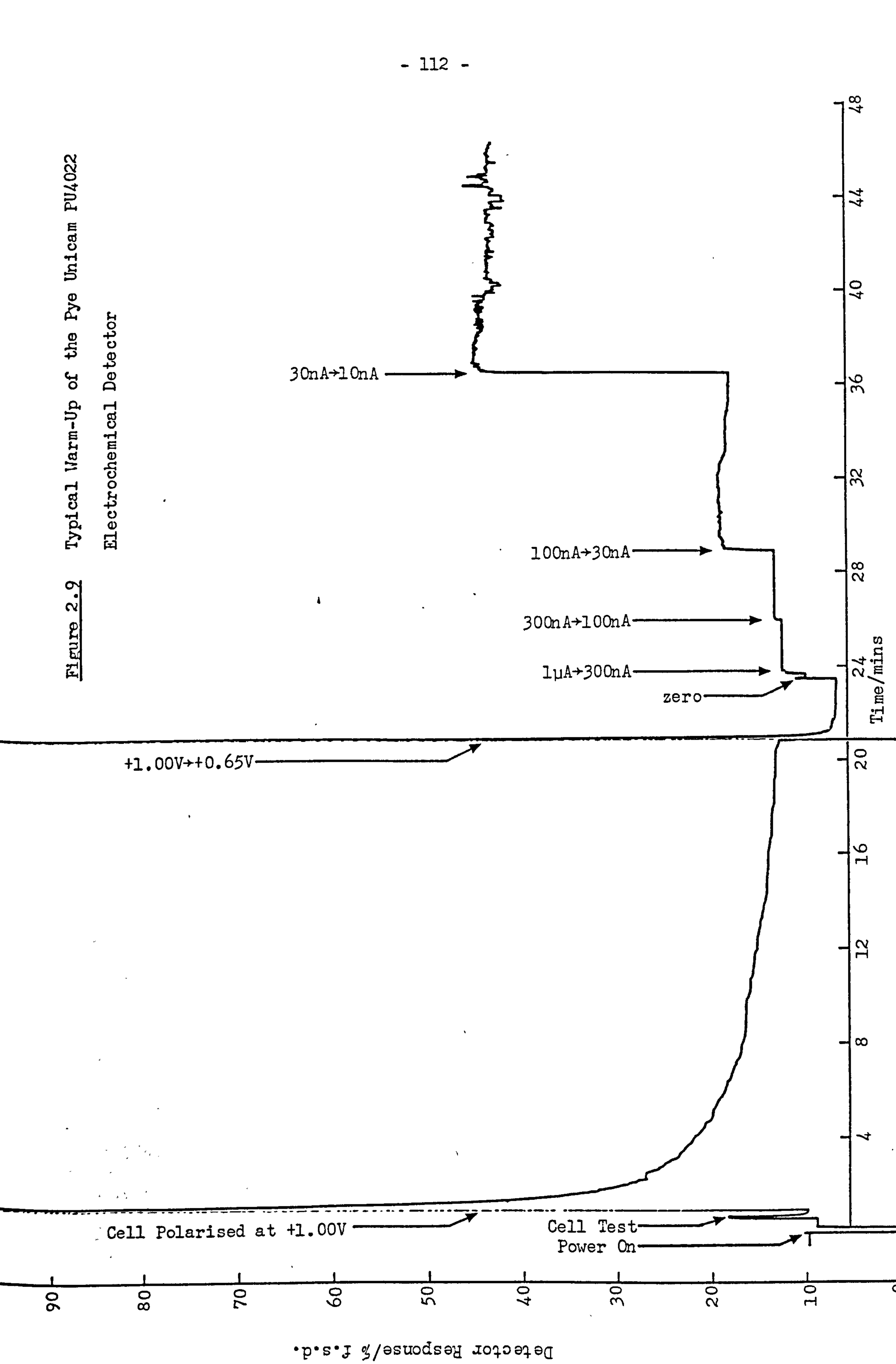
2.3.1 Evaluation and Comparison of Electrochemical Detector Warm-Up Characteristics

Each ECD to be investigated was incorporated in turn into a standard HPLC system and each instrument was then subjected to an identical warm-up procedure as outlined in Section 2.2.7.

Pye Unicam PU4022

From 'power on' the Pye Unicam PU4022 ECD took 10-20 minutes to return to the nominal +10% f.s.d. signal level whereupon the working potential of +0.65V was set. Under the influence of this applied potential the arbitrary criteria selected to describe a practicable baseline were that the baseline obtained should be steady, i.e. not cycling or generally subject to long-term (low frequency) noise, and should be subject to a drift of not greater than 5%. A practicable working baseline was achieved after a further 10 minutes at a stepwise selected instrument sensitivity of 30nA f.s.d. Following an increase in this parameter to 10nA f.s.d., another 5-10 minutes was required to obtain a reasonable baseline, although this suffered short-term (high frequency) noise of approximately 200pA (2% f.s.d.) and exhibited a tendency to drift. After overnight flushing of the flow cell, a relatively noise-free, drift-free baseline was attained at this sensitivity setting, Figure 2.9 shows a typical warm-up for this detector. At even higher instrument sensitivities (3nA and 1nA f.s.d.) the use of a time constant of >1sec was essential and the baseline at these levels was only utilisable after a considerable settling time of 12 hours (overnight) at the very least.

Figure 2.9 Typical Warm-Up of the Pye Unicam PU4022 Electrochemical Detector



EDT LCA 15

Upon subjection to the same warm-up procedure the EDT LCA 15 behaved identically to the PU4022, a practicable baseline at the 10nA f.s.d. sensitivity setting being achieved 25-40 minutes after switching on the mains supply. A typical warm-up for this detector is illustrated in Figure 2.10. At the higher instrument sensitivities of 3nA and 1nA f.s.d. very similar behaviour to that exhibited by the PU4022 was observed with the exception that the LCA 15 appeared to settle a little quicker than did the Pye model. This observation was relatively insignificant as equilibration time was still very long (>10 hours) and any small differences in performance could be attributed to minor differences in the state of the electrodes in the two flow cells.

The experimentally determined warm-up times for the Pye and EDT ECDs of 25-40 minutes from the engagement of the power supply to a steady base-line at 10nA f.s.d. are better than those claimed by the manufacturers.[203-205] It is expected to take between 40 and 60 minutes to achieve a steady base-line at 30nA f.s.d. according to the relevant operator manuals. However, these figures quoted by the manufacturers encompass a range of applied potentials and chromatographic conditions and criteria for acceptability are not stated;therefore direct comparison with literature claims is difficult.

Metrohm 641-VA/656

Application of the standard warm-up procedure to the Metrohm 641-VA/656 yielded many problems. From 'power on' exceptionally great noise and drift were observed at moderate instrument sensitivities (100nA and 50nA f.s.d.) for extremely long periods such that use was impossible. The baseline took

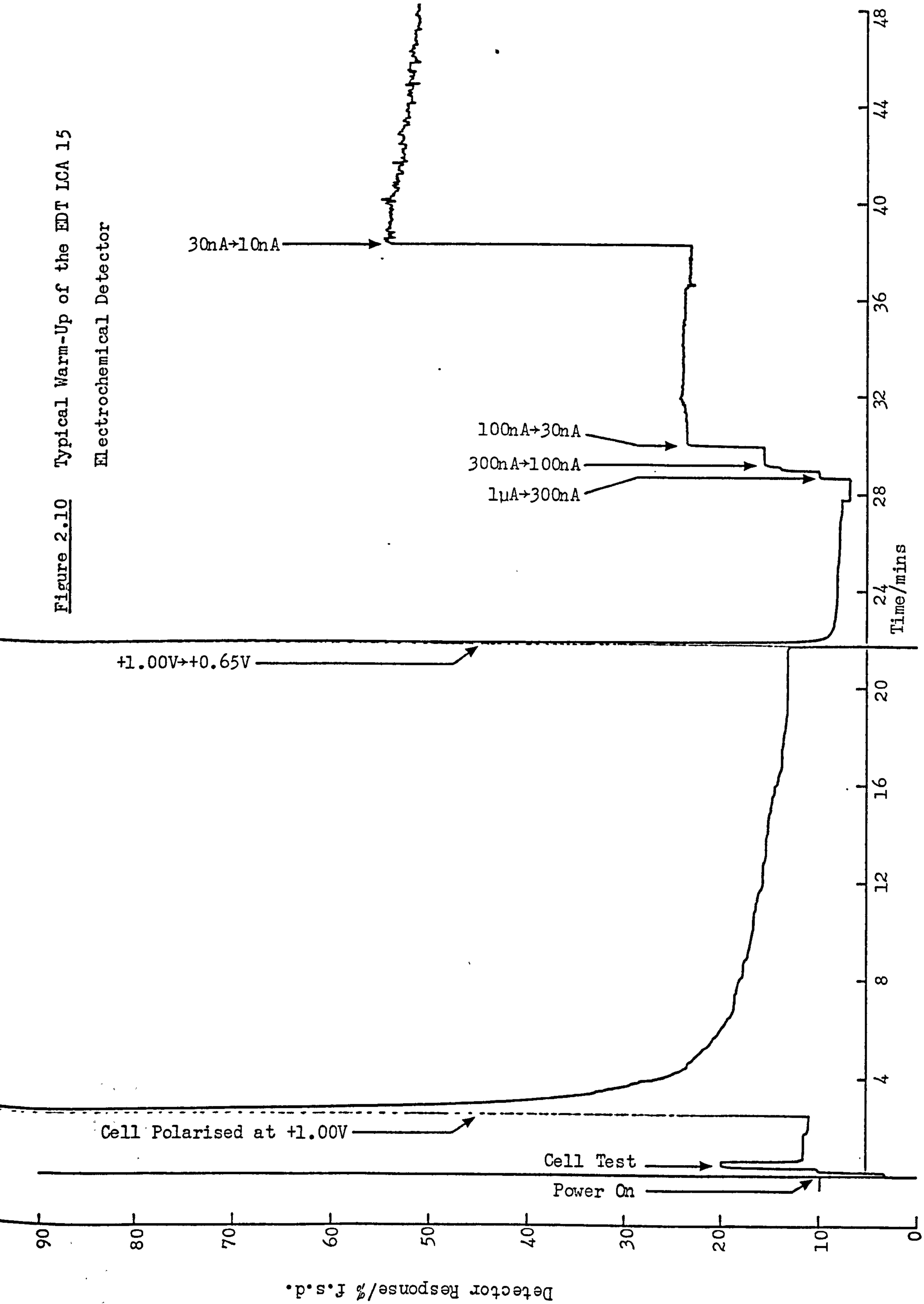


Figure 2.10 Typical Warm-Up of the EDT LCA 15 Electrochemical Detector

between 48 and 72 hours to settle to a reasonable state (although still not within the arbitrarily defined criteria), the signal continuing to be fairly erratic in nature. Prolonged application of a highly positive operating potential (+1.2V) served to stabilise the detection system to a small degree, probably by electrolytic cleaning of the WE. Regular servicing of the 656 flow cell was carried out. The GCEs were removed, polished by mechanical abrasion with a methanolic slurry of fine alumina powder (d_p = 0.3μm) on a soft, lint-free tissue and were replaced; the RE was removed, cleaned, refilled and reinstalled, and any trapped air bubbles were carefully excluded from the reassembled flow cell. Unfortunately this treatment yielded no significant improvement in performance. Hence, due to the instability of the system no relevant data was obtained regarding the warm-up characteristics of the Metrohm ECD.

The performance of the 641-VA/656 was considerably worse than had been observed on previous occasions when operating this instrument.[211] Furthermore, equilibration time was not within the one hour specified by the manufacturers.[186] Initially it was thought that the 6 month period of inactivity of the 641-VA/656 prior to this study (which was in contrast to the other two ECDs which had been in regular operation) may have contributed to the poor performance of this detector, although this explanation was not considered to be entirely satisfactory.

2.3.2 Baseline Noise Comparison Studies

In the following sections, baselines are discussed with respect to noise, which is the term given to any perturbation of the detector output that is not related to an eluted solute. The origins of noise in ECDs

are not completely understood.[212] Van Rooijen and Poppe[213] speculated that the predominant source of noise in amperometric ECDs is the RE. More recently, Morgan and Weber[214] examined, quantified and compared noise originating from current-, potential- and impedance-based sources. They concluded that noise in the frequency range below 1Hz is dominated by impedance noise while for frequencies between 1 and 20Hz the voltage noise in the current-to-voltage converter is predominant. Unfortunately, the most ubiquitous noises are those at lower frequencies. Baseline noise has been arbitrarily divided into three types, known as short-term (high frequency) noise, long-term (low frequency) noise and drift.

Short-Term Noise

Short-term noise consists of baseline fluctuations that have a frequency that is significantly higher than that of an eluted peak, and which often appears as "grass" on the output trace. Short-term noise is not usually a great problem because it does not seriously obscure the presence of a solute peak and further, it can often be eliminated totally. If the short-term noise persists at low detector sensitivities the source is often in the recorder and can be eliminated by either slightly reducing instrument sensitivity or increasing the degree of damping or, failing these measures, by repairing or replacing the recording apparatus. If the short-term noise is only prevalent at high sensitivity, then this arises from the detector amplifier and can be eliminated by interposing an electronic filter device between the amplifer output and recorder. Another source of short-term noise originates from the pulsations generated by the solvent delivery system and can be identified by the fact that the frequency of the noise matches the piston stroke frequency. Pump noise is often reduced by the incorporation of a pulse dampener in the system.

Long-Term Noise

Long-term noise consists of baseline perturbations having a frequency of the same order as eluted peaks. Consequently, this type of noise is the most serious as it cannot be differentiated from an eluted peak of the same amplitude. Furthermore, any filter that would eliminate long-term noise would virtually remove such a solute peak too and thus is inappropriate. It is this type of noise which ultimately limits the sensitivity of the detector.

Long-term noise arises primarily from the sensing system of the detector itself and not from the detector electronics and frequently results from component instability or small changes in ambient conditions. Noise of this type can increase progressively during the lifetime of a column due to the irregular elution of column contaminants. Noise originating from this source can only be reduced or eliminated by thorough column cleansing or replacement.

Drift

Perturbation of the detector output having a frequency significantly less than the frequency of the eluted peaks is called drift. Drift does not obscure eluted peaks but detectors operating with significant drift require frequent adjustment of the baseline level. Drift can result from slowly changing output from the power supply to the detector but more often originates from two other sources, both due to non-equilibrium conditions in the column and detector. If the detector, column and mobile phase are not in thermal equilibrium then serious drift can result. Thermostatting of the apparatus will eliminate this problem. The second major source of drift arises because of incomplete mobile phase equilibrium with the

stationary phase or inhomogeneity of the mobile phase. Such drift always occurs on changing the composition of the mobile phase and will generally decrease until a stable baseline is obtained with continued elution of the new mobile phase through the HPLC system. The presence of trace impurities in the mobile phase can exacerbate this problem thus again the need for high purity solvents and reagents is emphasised. When detectors are operated at or near to maximum instrument sensitivity, all three of the aforementioned types of noise are usually present.

Measurement of Detector Noise

The noise associated with a particular detector is defined as the maximum amplitude of the combined short- and long-term noise measured over a period of about 10 minutes[67], which is illustrated in Figure 2.11.

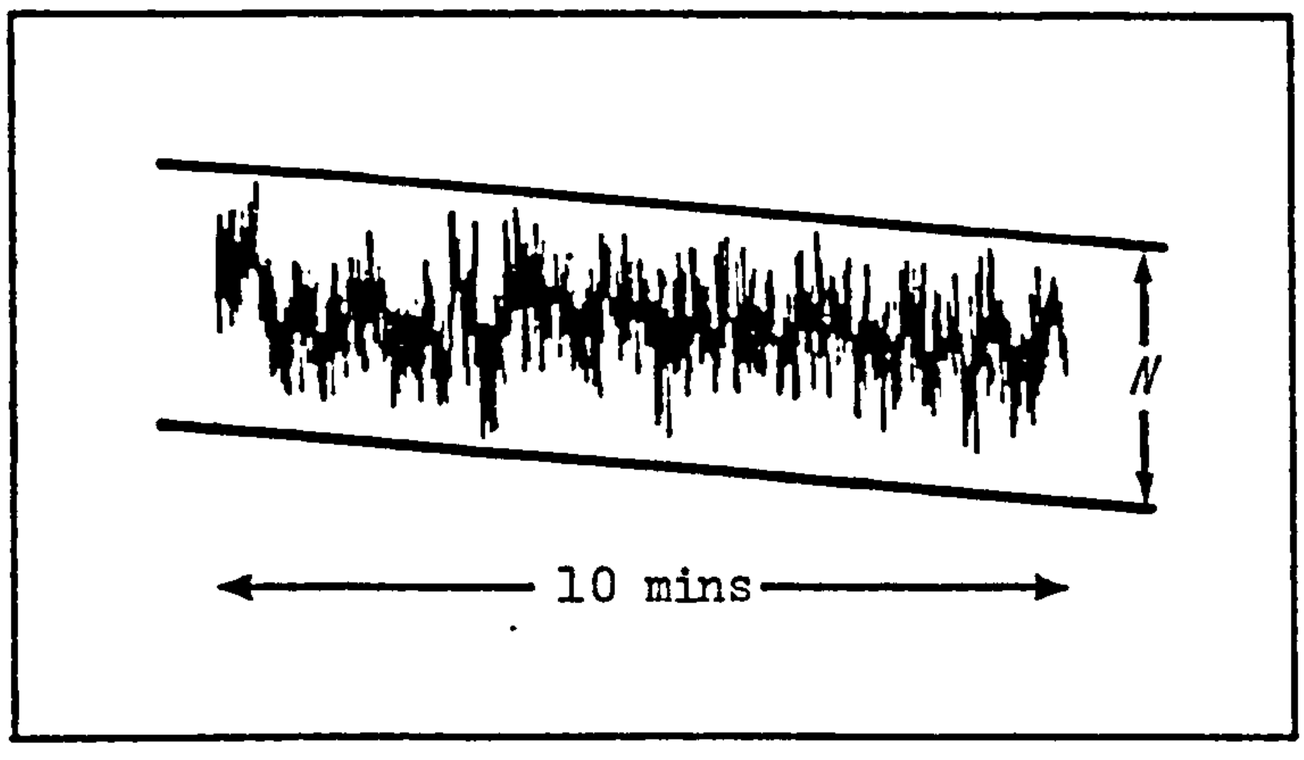


Figure 2.11 Measurement of Detector Noise Level

The HPLC system must be fully assembled and mobile phase must be pumped through it over the entire monitoring period. It is recommended[67] that the maximum amplitude of the combined short- and long-term noise be

measured in milliVolts (mV) and corrected to an amplification (or attenuation) of unity. In addition, the noise level should be determined at the highest amplification (or lowest attenuation) which does not include noise-filtering devices.

Total detector noise levels in this study were measured as in Figure 2.11 and, in addition, estimates of the magnitude of the individual contributions of short-term and long-term noise to the total noise were made. However, the recommendations of Scott noted above were not strictly followed. First, all noise levels were quantified in terms of the current that such voltage perturbations represented and not in units of potential as was suggested. Presentation of noise in this form was deemed to be more meaningful regarding amperometric ECDs. Secondly, a full assessment of the available electronic noise-filtration circuitry incorporated in each instrument under examination was sought. To this end certain noise measurements were conducted with noise smoothing functions engaged.

Baseline signals were recorded from each ECD under scrutiny over a range of experimental conditions. The variables that were exploited were pump model (PU4010 or LC-XPD), mobile phase flow rate (0.5,1.0,2.0 and 3.0mlmin^{-1}), mobile phase MeOH : aq. buffer ratio (10:90 or 90:10), and instrument sensitivity (30nA or 50nA, 10nA and, where appropriate, 3nA and 1nA f.s.d. with suitable time constant/damping settings). A standard mobile phase comprising 10% MeOH : 90% aq. buffer containing HCl, NaAc and NaOH was pumped through the system for all measurements except for the experiment where high organic modifier content was required when a 90% MeOH : 10% aq. buffer mixture was eluted. All experiments were carried out at two instrument sensitivity settings, viz. 30nA (or 50nA for the Metrohm ECD)

and 10nA f.s.d. These settings are commonly applied to trace analysis and were selected in order to demonstrate fully the noise levels to be expected during actual analyses.

Following initiation of each new set of experimental conditions employed, sufficient equilibration time was allowed (i.e. until, by inspection, no significant improvement in the baseline was evident over a 20 minute period) before a representative section of baseline was sampled and evaluated for noise. Ten minute duration lengths of baseline, as recommended by Scott[67], are assembled in Tables 2.8-2.15 for comparison purposes. Signals obtained from the Metrohm ECD are included in these tables but these are considered to be unrepresentative of the instrument in a properly functioning state.

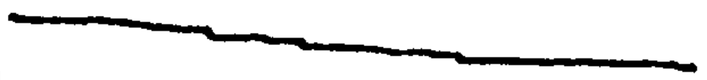
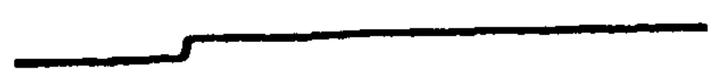
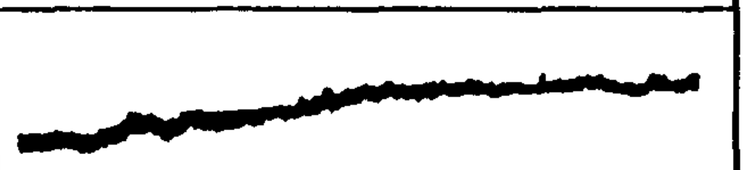
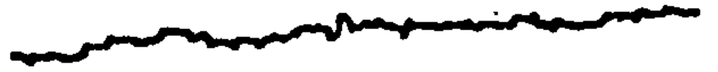
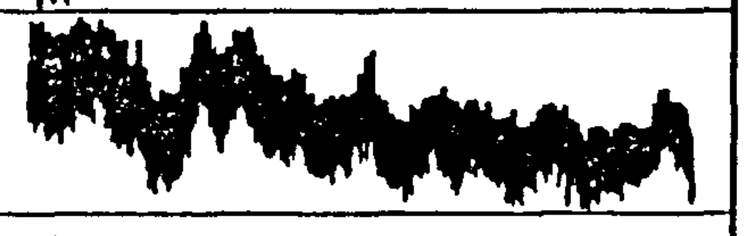
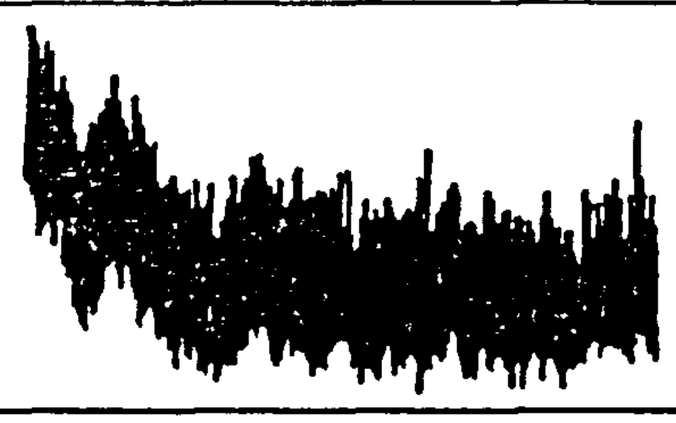
2.3.2.1 Variation of Mobile Phase Flow Rate

Tables 2.8 and 2.9 illustrate the effect of flow rate on the baseline noise generated within each LC-EC system. Table 2.8 depicts baselines obtained by delivering standard mobile phase at a range of flow rates using the PU4010 pump and recorded at ECD sensitivities of 30nA (or 50nA) and 10nA f.s.d. In a similar fashion Table 2.9 comprises baselines obtained using the Altex-manufactured LC-XPD pump.

By inspection, it is evident that under identical experimental conditions the signals from the PU4022 and the EDT LCA 15 are virtually identical with respect to short-term noise. No change in short-term noise is distinguishable with increase in flow rate from these two ECDs, short-term noise being essentially less than 150pA for all traces. Long-term noise from the PU4022 is generally a little greater than that from the

Table 2.8 Baselines Illustrating the Effect of Flow Rate on Noise Level (PU4010 Pump).

Parameters:- Pump: PU4010; Mobile Phase: 10% MeOH/90% aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$); Flow Rate: as stated; Instrument Sensitivity: as stated.

Instrument Sensitivity (nA f.s.d.)	Flow Rate ($mlmin^{-1}$)	Electrochemical Detector Model		
		PU4022	EDT LCA 15	Metrohm 640-VA/656*
30 (or 50*)	0.5			
	1.0			
	2.0			
	3.0			
10	0.5			
	1.0			
	2.0			
	3.0			

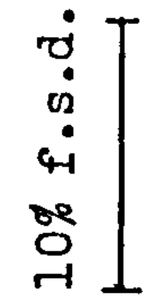
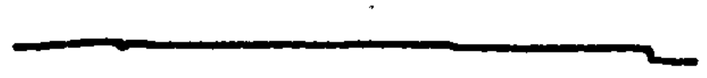
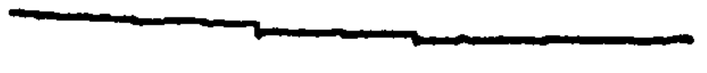
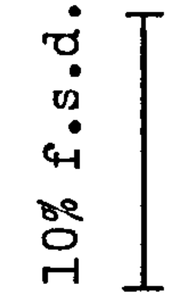


Table 2.9 Baselines Illustrating the Effect of Flow Rate on Noise Level (LC-XPD Pump).

Parameters:- as for Table 2.8 except Pump: LC-XPD.

Instrument Sensitivity (nA f.s.d.)	Flow Rate ($ml min^{-1}$)	Electrochemical Detector Model		
		PU4022	EDT LCA 15	Metrohm 641-VA/656*
30 (or 50*)	0.5			
	1.0			
	2.0			
	3.0			
10	0.5			
	1.0			
	2.0			
	3.0			



EDT LCA 15 (ca. 100-175pA cf. ca. 75-125pA) indicating slightly poorer stability.

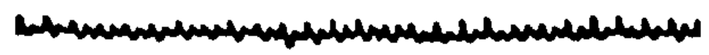
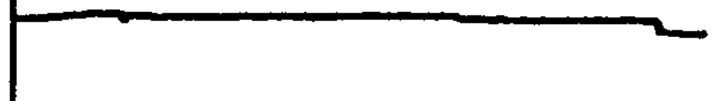
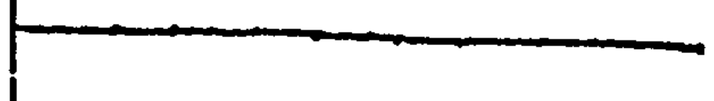
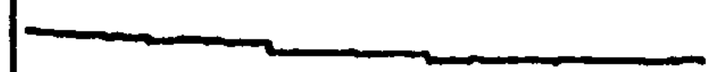
The Metrohm 641-VA/656 noise output is much greater than the outputs of the other two ECDs. Both short-term and long-term noise are increased by about a factor of four; the former ranges between ca. 75 and 500pA, the latter between 200 and 1100pA. Total noise is observed to increase in both magnitude and frequency with increasing flow rate. The nature of the short-term noise suggests that it originates primarily from pressure pulsations which increase in frequency with increase in piston stroke speed. Superimposed on top of this is a small amount of high frequency electronic noise.

2.3.2.2 Variation of Pump Model

A considerable difference between the Metrohm ECD baselines obtained when using the Pye Unicam PU4010 pump and those obtained when using the Altex LC-XPD pump was noted. In Tables 2.10 and 2.11 are presented compilations of baselines recorded from all three ECDs enabling direct visual comparison of outputs with respect to pump model over a range of experimental conditions. The effect of pump characteristics on noise is demonstrated most prominently by the Metrohm ECD traces, particularly those obtained at 10nA f.s.d. At this instrument sensitivity, and over the range of flow rates investigated, the long-term baseline noise is comparable between pumps. However, as Table 2.11 clearly shows, short-term noise originating from the PU4010 is markedly greater than that generated by the LC-XPD. Both pumps are of dual piston reciprocating-stroke design, and possess piston chambers of similar dimensions and internal pulse dampeners. However, the Altex LC-XPD exhibits superior performance with respect to

Table 2.10 Baselines Illustrating the Effect of Pump Model on Noise Level.

Parameters:- Pump: as stated; Mobile Phase: 10% MeOH/90% aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$); Flow Rate: as stated; Instrument Sensitivity: 30nA or 50nA(*) f.s.d.

Flow Rate ($mlmin^{-1}$)	Pump Model	Electrochemical Detector Model		
		PU4022	EDT LCA 15	Metrohm 641-VA/656*
0.5	PU4010			
	LC-XPD			
1.0	PU4010			
	LC-XPD			
2.0	PU4010			
	LC-XPD			
3.0	PU4010			
	LC-XPD			

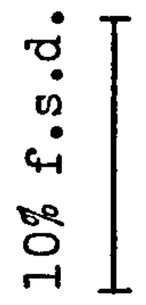
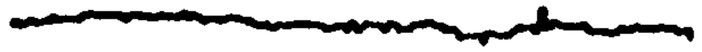
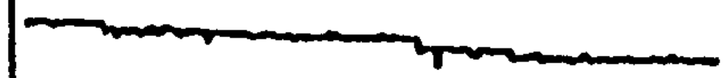
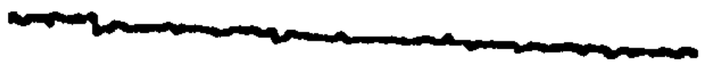
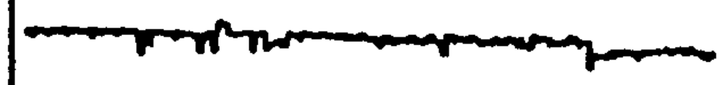
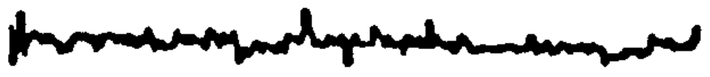
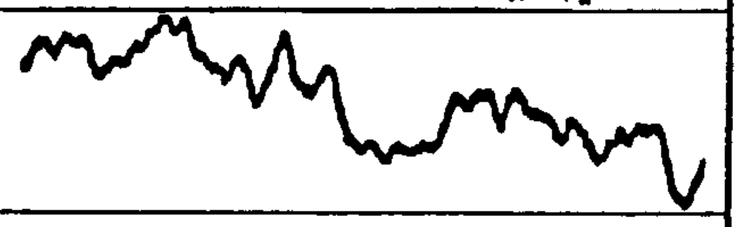
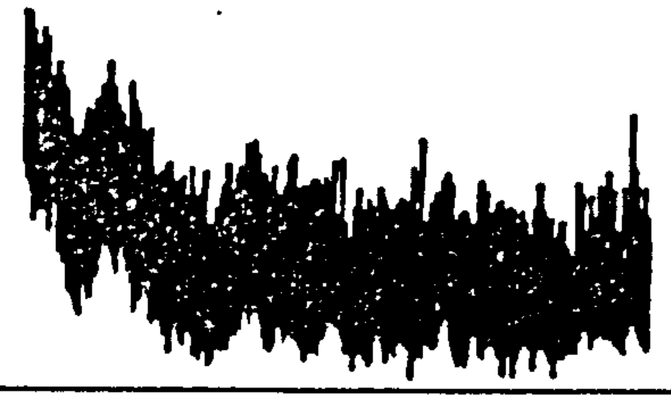
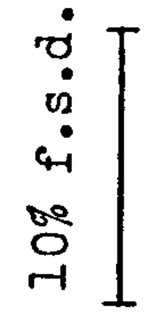


Table 2.11 Baselines Illustrating the Effect of Pump Model on Noise Level.

Parameters:- as for Table 2.10 except Instrument Sensitivity: 10nA f.s.d.

Flow Rate ($ml min^{-1}$)	Pump Model	Electrochemical Detector Model		
		PU4022	EDT LCA 15	Metrohm 641-VA/656*
0.5	PU4010			
	LC-XPD			
1.0	PU4010			
	LC-XPD			
2.0	PU4010			
	LC-XPD			
3.0	PU4010			
	LC-XPD			



pulsing, as exemplified by the Metrohm 641-VA/656 output, but this superiority is inconsequential with regard to the outputs of the PU4022 and the EDT LCA 15.

2.3.2.3 Variations of MeOH Content of the Mobile Phase

An experiment was carried out in order to determine to what extent the organic modifier content of the mobile phase contributed to baseline noise. Two mobile phases comprising MeOH and water containing fixed concentrations of HCt, NaAc and NaOH were compared, the first incorporating 10% MeOH and the second 90% MeOH. A standard flow rate of 1.0mlmin^{-1} was employed for all recordings. Baselines obtained from all three ECDs are compiled and presented in Tables 2.12 and 2.13.

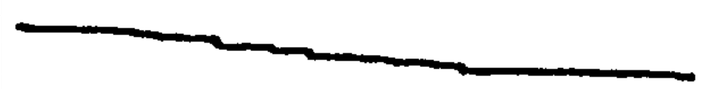
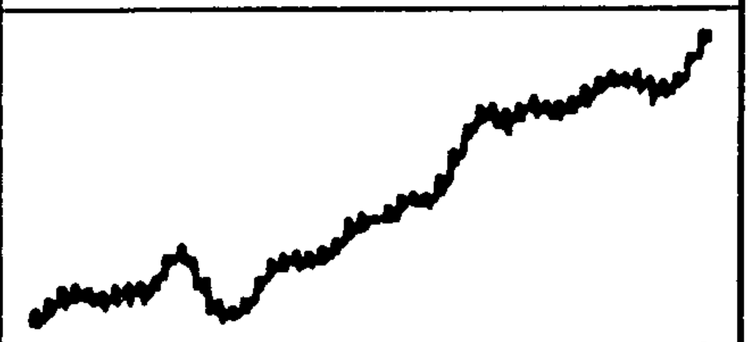
From these traces it can be deduced that both short-term and long-term noise are generally greater at high MeOH content. This is to be expected because MeOH is less able to support a current than is water. However, the signals recorded from the Metrohm 641-VA/656 do not entirely follow this pattern with respect to long-term noise, which is greater at low MeOH content. This situation may have arisen merely as a result of the unreliable performance of the Metrohm ECD and consequently is not considered further.

2.3.2.4 Baselines at Higher Sensitivity Settings

Utilising the Pye Unicam system comprising the PU4010 pump (with which standard mobile phase was delivered at a flow rate of 1.0mlmin^{-1}) and the PU4022 ECD, baselines were recorded at higher instrument sensitivities than had previously been examined, viz. 3nA and 1nA f.s.d. Time constants of 1,3 and 10secs were employed at both these instrument sensitivities and the

Table 2.12 Baselines Illustrating the Effect of Percent MeOH in the Mobile Phase on Noise Level (PU4010 Pump).

Parameters:- Pump: PU4010; Mobile Phase: MeOH/aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$), % composition as stated; Flow Rate: $1.0mlmin^{-1}$; Instrument Sensitivity: as stated.

Instrument Sensitivity (nA f.s.d.)	%MeOH	Electrochemical Detector Model		
		PU4022	EDT LCA 15	Metrohm 641-VA/656*
30 (or 50*)	10			
	90			
10	10			
	90			

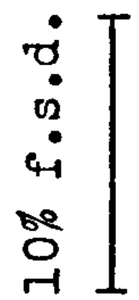
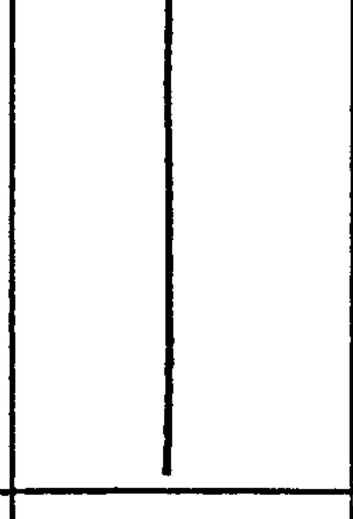
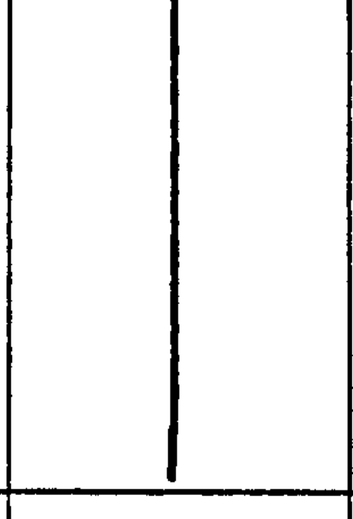
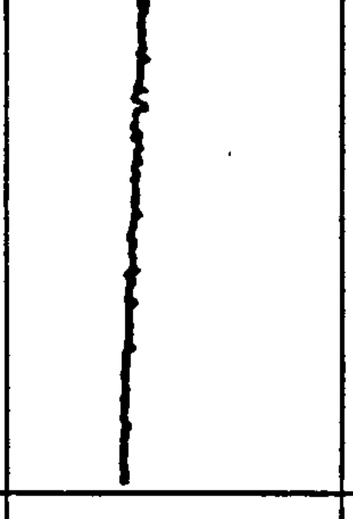
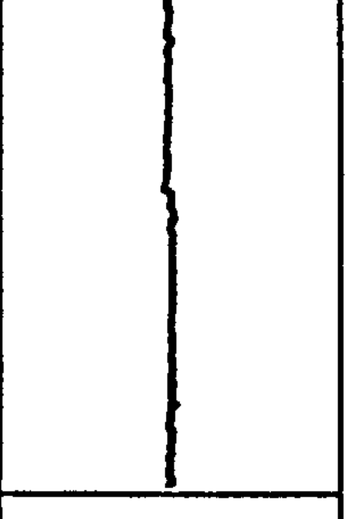
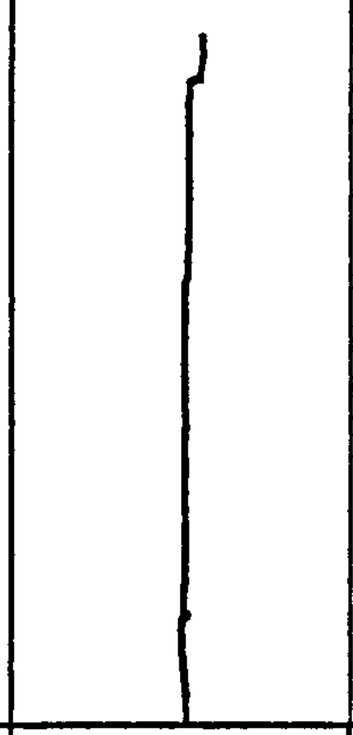
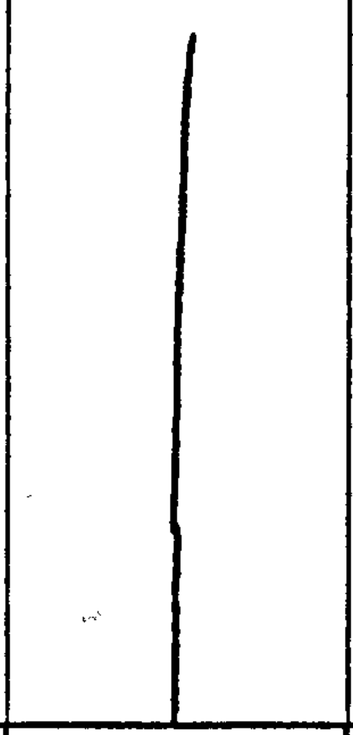
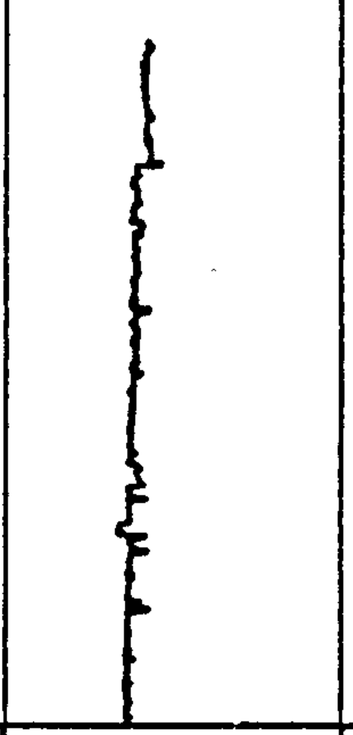
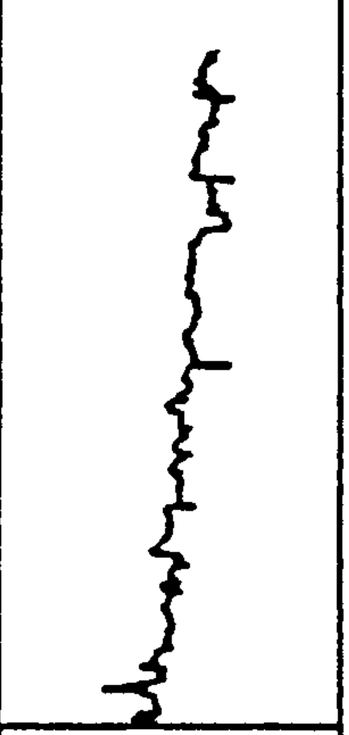
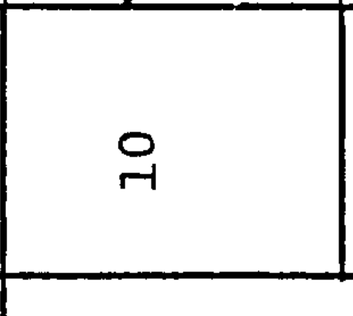
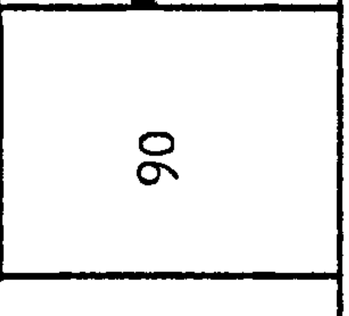
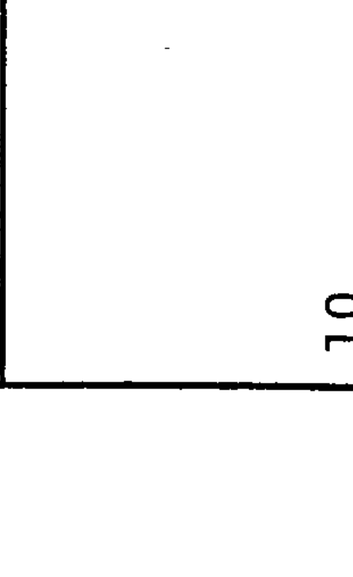
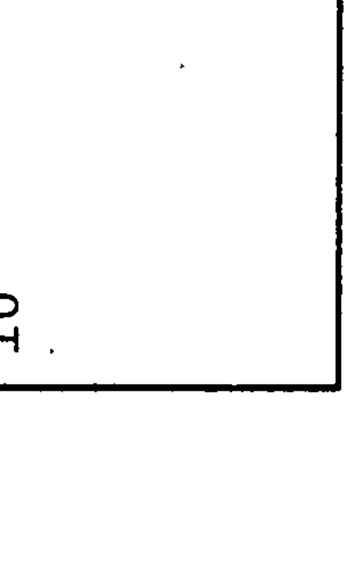


Table 2.13 Baselines Illustrating the Effect of Percent MeOH in the Mobile Phase on Noise Level (LC-XPD Pump).

Parameters:- as for Table 2.12 except Pump: LC-XPD

Instrument Sensitivity (nA f.s.d.)	%MeOH	Electrochemical Detector Model			
		PU4022	EDT LCA 15	Metrohm 641-VA/656*	
30 (or 50*)	10				
	90				
10	10				
	90				

resulting traces are displayed in Table 2.14. This procedure was repeated for the Altex LC-XPD pump/EDT LCA 15 system and the resulting traces are presented in Table 2.15. A comparison of these baselines shows that the output from the EDT LCA 15 was marginally noisier at the 3nA f.s.d. setting but there was no discernible difference at the 1nA f.s.d. setting.

The action of the electronic smoothing function is most clearly demonstrated at the 3nA f.s.d. sensitivity setting where noise is reduced from ca. 200pA to ca. 40pA (7% to 1.5% f.s.d.) with an increase in time constant from 1sec to 10sec. The baselines obtained at 1nA f.s.d. sensitivity plainly demonstrate that this setting is wholly unsuitable for analytical work with noise extending between ca. 150-250pA (15-25% f.s.d.). Thus, the highest practicable instrument sensitivity available on the PU4022 and EDT LCA 15 ECDs under these experimental conditions is 3nA f.s.d., ideally with greater than nominal signal smoothing introduced.

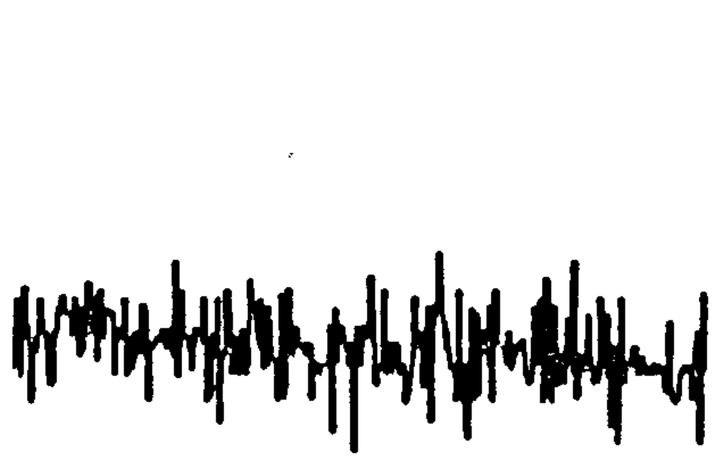
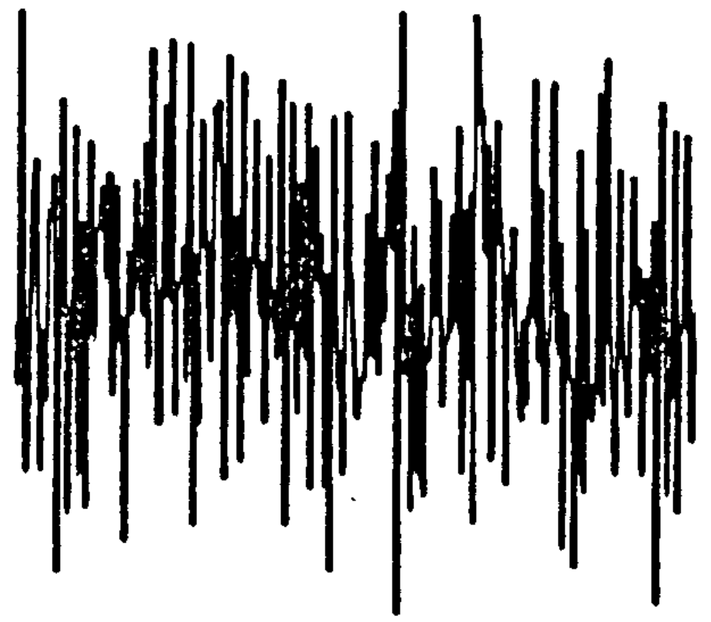
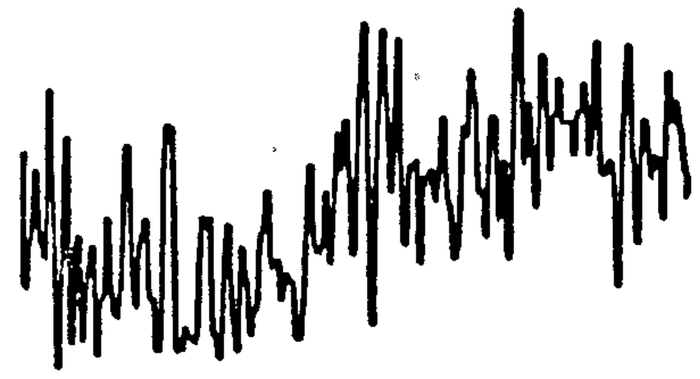
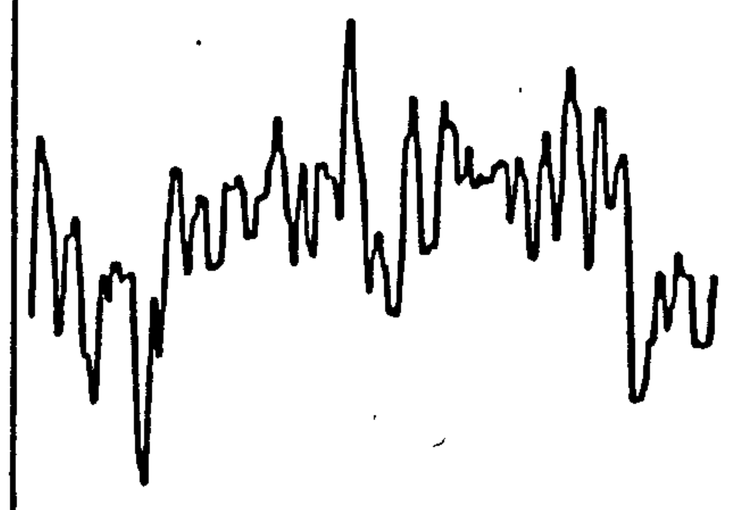
2.3.2.5 Summary

Over the range of experimental conditions employed the EDT LCA 15 fared best with respect to baseline noise. The performance of the PU4022 was generally very slightly inferior to that of the EDT model, primarily regarding long-term noise. The Metrohm 641-VA/656 was found to be far noisier in all respects than the other two detectors. Drawing conclusions regarding the Metrohm ECD proved to be difficult due to the failure to achieve a 'normal' standard of performance from the instrument.

A few months after the completion of this investigation, the Metrohm ECD was dispatched back to the manufacturers for servicing. The controller unit was found to contain faulty electronic components which undoubtedly were the major contributors to the erratic noisy signal output. For this

Table 2.14 Baselines at High Instrument Sensitivities, Illustrating the Effect of an Electronic Filter (PU4022 ECD).

Parameters:- Pump: PU4010; Mobile Phase: 10% MeOH/90% aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$); Flow Rate: $1.0mlmin^{-1}$; Instrument Sensitivity: as stated; Detector Time Constant: as stated.

Detector Time Constant (sec)	Instrument Sensitivity	
	3nA f.s.d.	1nA f.s.d.
1		
3		
10		

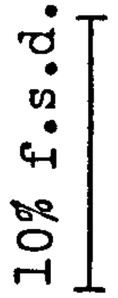
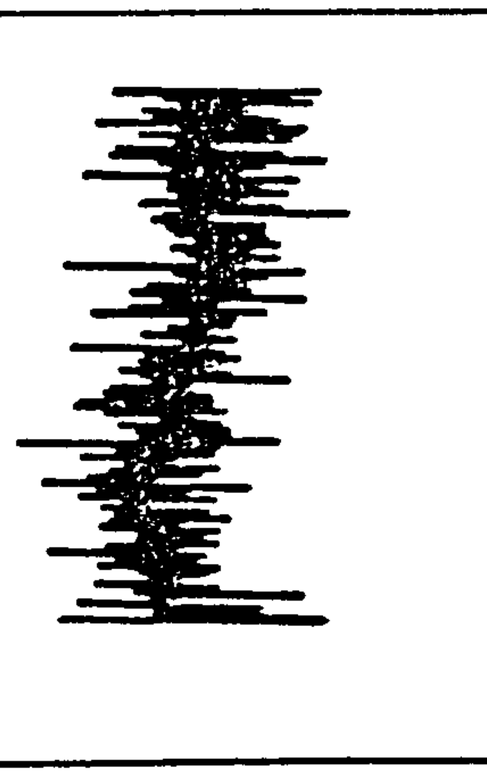
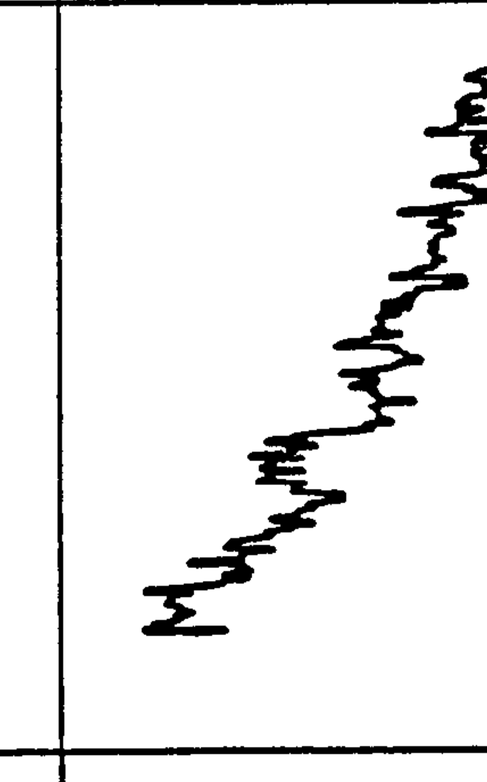
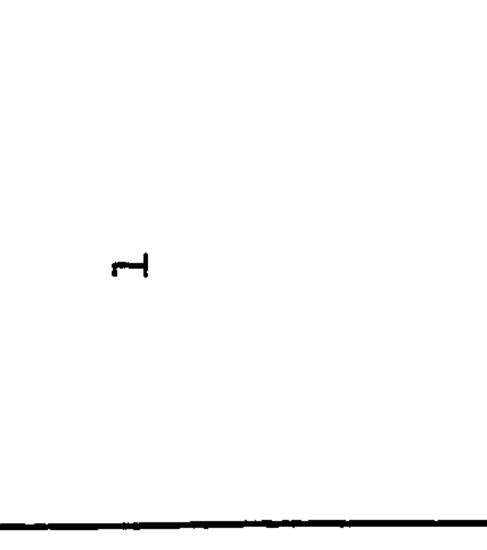
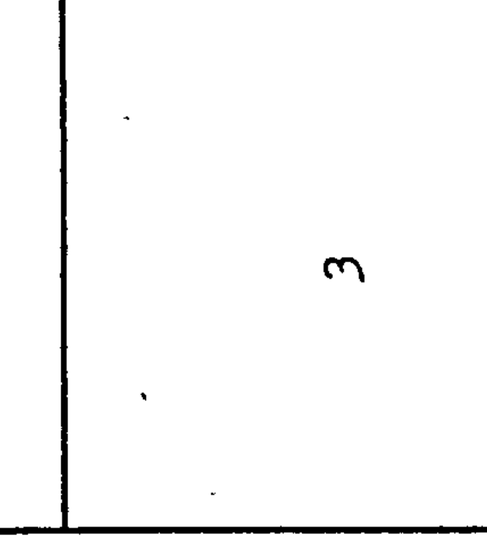


Table 2.15 Baselines at High Instrument Sensitivities, Illustrating the Effect of an Electronic Filter (EDT LCA 15).

Parameters:- as for Table 2.14 except Pump: LC-XPD.

Detector Time Constant (sec)	Instrument Sensitivity	
	3nA f.s.d.	1nA f.s.d.
1		
3		
10		

10% f.s.d.

reason any comparison made between the EDT/Pye ECDs and the Metrohm model in this study would be unreliable hence the comparison of ECD performance with respect to major design differences was not justifiable in this instance.

A comparison of the PU4022 and the EDT LCA 15 with respect to back-ground noise was justifiable however. The factor of three difference in noise levels resulting from the different power packs incorporated in these two ECDs[202] was not fully realised within the confines of these experiments. The overall noise difference between the EDT and Pye Unicam models was only of the order of 20-60%, not 300% as might have been expected. From this it must be concluded that other factors must contribute to the observed noise to a greater extent than does the instrument power system. The findings of the baseline noise study lead to the conclusion that the PU4022 and the EDT LCA 15 are of similar specification and are virtually interchangeable over the range of instrument sensitivities compared.

Because of the major problems encountered with the performance of the Metrohm ECD the instrument was abandoned at this point. Only the PU4022 and the EDT LCA 15 were assessed further.

2.3.3 Operational Evaluation of the ECDs

The three ECDs were assessed regarding their ease of operation and the utility of the features available to the operator.

The PU4022 and the EDT LCA 15 were considered to be superior to the Metrohm 641-VA/656 with regard to the facilities offered. The former two instruments both contain autozero and event marker features which were very useful for output presentation purposes. However, the push buttons provided for operation of these features were found to be a little crowded

and awkward to operate and were prone to manipulative error. An earlier design of the EDT controller[204] isolated both the autozero and the event marker buttons from the other controls on the unit facia. A return to this original arrangement of buttons and switches on the EDT controller, and adoption of the same by Pye Unicam, would provide an instrument that is far more accessible and easy to use.

The Metrohm ECD offers a more extensive range of operating sensitivities (100pA→10mA f.s.d.) than does either the Pye Unicam or the EDT ECD (1nA→3μA). Unfortunately, because of the uncharacteristically poor performance of the Metrohm instrument this greater sensitivity range could not be evaluated satisfactorily. However, it is evident from the literature that this wide range of instrument sensitivity is unnecessary for the vast majority of ECD applications reported to date, the most commonly employed sensitivity settings being between 10 and 100nA f.s.d. Hence, the Metrohm 641-VA/656 offers little advantage in this respect over the Pye Unicam and EDT detectors.

The flow cell supplied with the Pye Unicam and EDT detectors is both simple and readily accessible for cleaning and servicing. In contrast, the 656 flow cell provided with the Metrohm ECD is cumbersome. The screening cage and the water jacket thermostat themselves, as well as the multitude of wires and pipes passing through the rear of the screening cage, restrict access to the cell for routine maintenance. Thus, in terms of facility of operation the PU4022 and the EDT LCA 15 flow cells are preferred to the Metrohm 656 flow cell.

2.3.4 Evaluation of Electrochemical Detectors with Reference to the Determination of Catecholamines

A classical application of the use of the ECD is for the detection of the catecholamines epinephrine (EP), norepinephrine (NE) and dopamine (DA). The structure of these compounds are given in Figure 2.12.

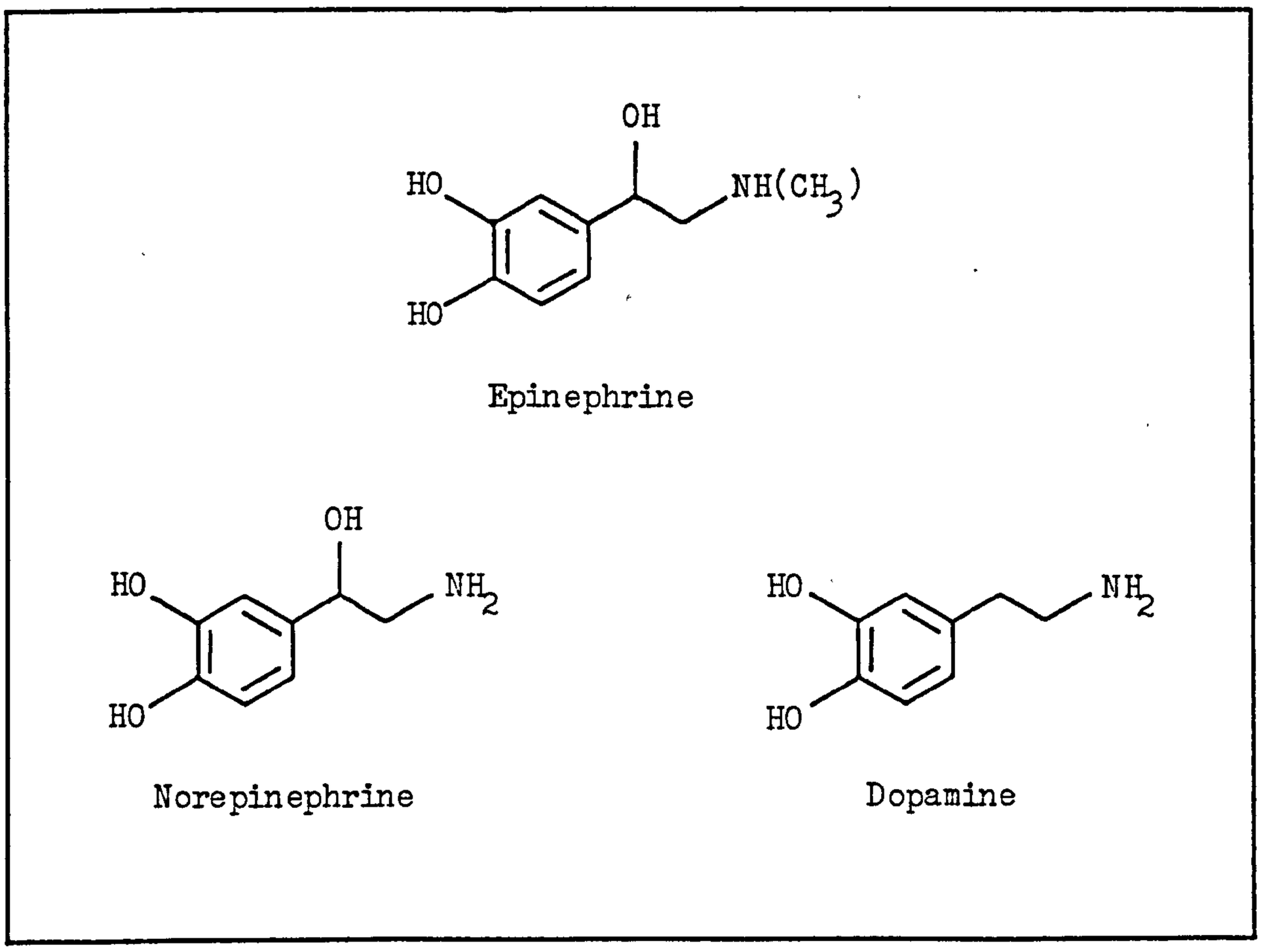


Figure 2.12 Structures of some Common Catecholamines

EP, NE and DA are the final products of a biosynthetic pathway which commences with the amino-acid, phenylalanine. These three catecholamines are widely occurring and perform extremely important bioregulatory functions. EP and NE are hormones secreted from the chromaffin cells in the adrenal medulla, and are fundamental in the control of heart rate, blood pressure and lipid and carbohydrate metabolisms. All three catecholamines also act

as neurotransmitters in both the sympathetic and central nervous systems. The great importance of these compounds is admirably demonstrated by the abundance of scientific literature pertaining to them.

The three catecholamines EP, NE and DA all possess a 1,2-diol(catechol) structure which readily oxidises at moderate positive potentials generating the corresponding 1,2-dione(orthoquinone), two protons and two electrons as illustrated in Figure 2.13.

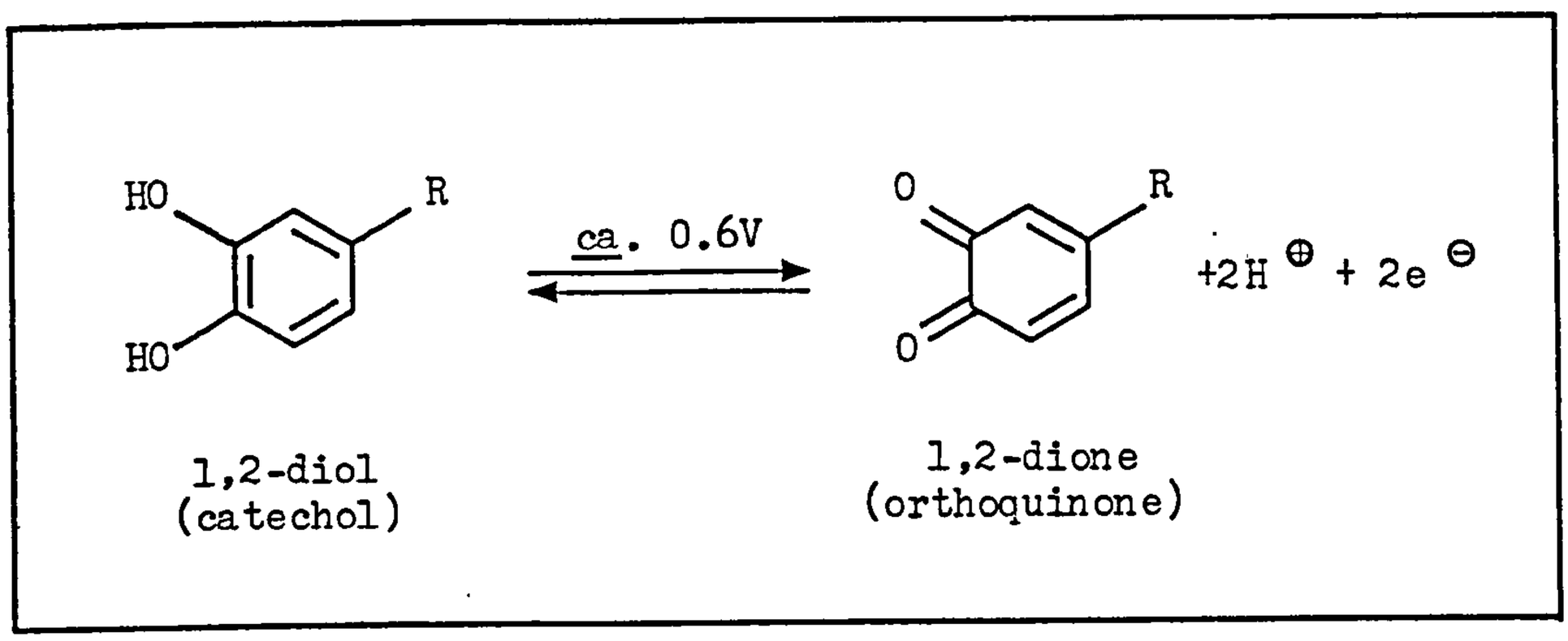


Figure 2.13 Electro-oxidation of a 4-substituted 1,2-diol(catechol)

It is this highly electroactive site which permits the EC detection process to take place with such ease. As a direct result of this property, and coupled with the great interest in measuring EP, NE and DA expressed by clinicians, these materials are commonly used for demonstration of ECD performance by manufacturers and researchers alike. The catecholamines are utilised herein for this purpose.

The initial objective of this study was to determine the optimum applied potential (E_{opt}) for the simultaneous detection of EP, NE and DA under the predetermined experimental conditions.[209] The optimum applied potential is defined herein as that potential at which *S/N* ratio is maximised for all three analytes. Secondly, using this optimum applied

potential the two ECDs were to be compared with respect to limit of detection (LOD) and limit of quantitation (LOQ) by means of their application to the determination of the three catecholamines in standard solutions. There is considerable ambiguity surrounding the term LOD in the field of chromatography. Foley and Dorsey[215] recently defined the LOD specifically as the smallest mass of analyte that can be detected with reasonable certainty for a given analytical procedure, and they expressed this quantity as :

$$\text{LOD} = \frac{\text{arbitrary detector signal level}}{\text{analytical sensitivity}}$$

where analytical sensitivity is defined as the signal output per unit mass of analyte introduced in a given analytical procedure. Chromatographers often define a second empirical parameter, namely the LOQ (sometimes known as the 'method sensitivity') which is arbitrarily taken as ten times the LOD and it is generally accepted that quantitation below this level is "unreliable". There is no consensus regarding which *S/N* ratio should be employed for the determination of the LOD[215], and subsequently the LOQ. Values of 2:1 (e.g., 216), 3:1 (e.g., 217) and 5:1 (e.g., 62) for LOD are commonplace in the chromatography literature. For the purpose of the investigations reported in this dissertation *S/N* ratios of 2:1 for LOD, and consequently 20:1 for LOQ, were selected so that the LOD was identical to the mass sensitivity (see Section 1.4). Although this empirical approach is generally accepted, it should be noted that neither of the aforementioned quantities is in fact statistically valid. However, this does not affect the ability to compare detectors using the empirical approach.

The final objective of these experiments was to demonstrate the applicability of a selected ECD to the determination of EP, NE, DA and other endogenous electroactive substances in selected rat brain regions.

2.3.4.1 Optimisation of Applied Potential

In the literature there is some confusion regarding the optimum cell polarising voltage (E_{opt}) for the detection of EP, NE and DA. This variation is attributable to the individual differences between detectors (architecture, electrode materials, etc.), to the supporting electrolyte composition and to the complete range of analytes under consideration. Typically, potentials between +0.5V and +1.0V vs. Ag/AgCl reference are reported. For this investigation E_{opt} was determined experimentally under the predetermined operating parameters.

An HPLC system was assembled using the Altex LC-XPD pump and the EDT LCA 15 detector and incorporating a Spherisorb S5 ODS1 column (25cm x 5mm ID, d_p = 5µm). The standard mobile phase (Section 2.2.7) was pumped through the apparatus at a flow rate of 1.0mlmin^{-1}. The operating potential of the ECD was set to +0.5V and the system was allowed to equilibrate. A standard containing EP, NE and DA was then chromatographed in duplicate. The operating potential was increased stepwise and at each selected potential the procedure was repeated. The series of chromatograms so obtained are presented in Figure 2.14. Baseline noise and peak heights of all three peaks in each chromatogram were measured and *S/N* ratios were calculated. These data are tabulated in Table 2.16. Graphs of *S/N* ratio vs. applied potential for all three analytes are compiled in Figure 2.15.

The data presented in Figure 2.15 reveals that, within the limits of the experiment, an applied potential of +0.70V provides the optimum *S/N*

Figure 2.14 Chromatograms Obtained at Various Applied Potentials for a Standard Solution Containing Catecholamines

Parameters :- Column : Spherisorb S5 ODS1 (25cm x 5mm, d_p = 5µm); Mobile Phase : 10% MeOH : 90% aq. buffer containing HCt ($6.3gl^{-1}$), NaAc ($2.27gl^{-1}$) and NaOH ($2.0gl^{-1}$), pH 4.8; Flow Rate: $1.0mlmin^{-1}$; Pump : PU4010; Detector : EDT LCA 15 (Eapp vs. Ag/AgCl as stated, TC : 1sec); Sample : 20µl via loop of EP, NE.HCl and DA in mobile phase (All constituents @ $0.4\mu gml^{-1}$ ≡ 8ng injected)

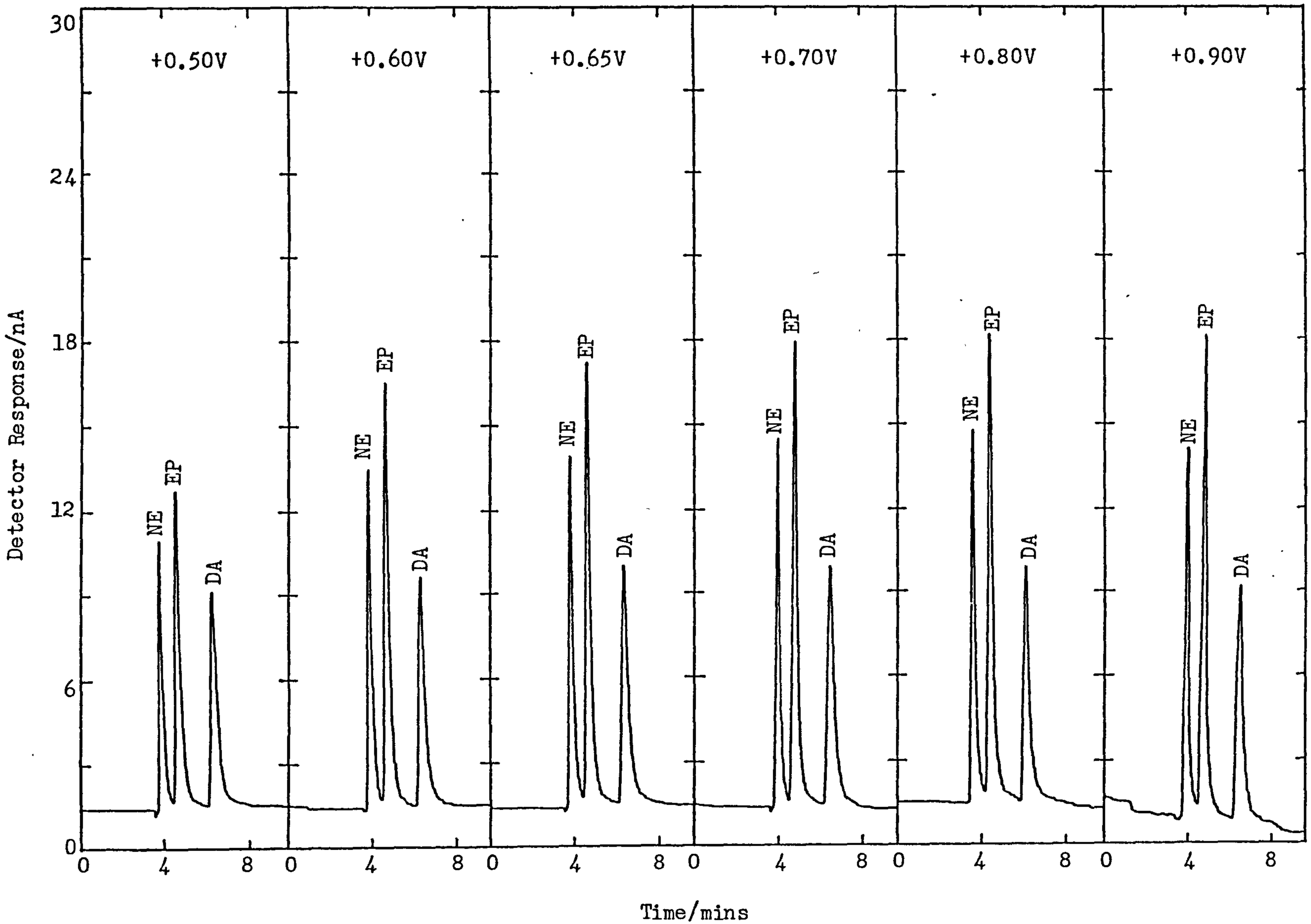


Table 2.16 Signal and Baseline Noise Data for EP, NE and DA Obtained at Various Applied Potentials

Applied Potential (V)	Baseline Noise		EP			NE			DA		
			Peak ht.		*S/N*	Peak ht.		*S/N*	Peak ht.		*S/N*
	(mm)	(pA)	(mm)	(nA)		(mm)	(nA)		(mm)	(nA)	
0.50	0.5	75	75	11.3	150	65	9.8	130	51	7.7	102
0.60	0.5	75	100	15.0	200	81	12.2	162	55	8.3	110
0.65	0.5	75	106	15.9	212	85	12.8	170	57	8.6	114
0.70	0.5	75	111	16.7	222	87	13.1	174	57	8.6	114
0.80	1.0	150	116	17.4	116	89	13.4	89	57	8.6	57
0.90	2.5	375	118	17.7	47	91	13.7	36	57	8.6	23

ratio for EP, NE and DA. The deterioration in baseline stability experienced at applied potentials of > +0.70V, without equivalent improvement in signal for the analytes, can be identified as the limiting factor.

Commonly, detector response alone rather than *S/N* ratio is considered in determining E_{opt}. Indeed, this method is recommended in the ECD operator manuals.[203,205] Strictly, this approach is unsatisfactory because ultimately it is *S/N* ratio that is the important factor in determining LOD and LOQ. The fallibility of using ECD response alone for the elucidation of E_{opt} is ably demonstrated by a comparison of the plot of Mean *S/N* ratio vs. applied potential (Figure 2.15) with a plot of mean detector response (measured as peak height) vs. applied potential, which is given in Figure 2.16. From the latter graph it may be deduced that, at least for E and NE, E_{opt} defined by ECD response alone lies at a value $\geq$ +0.90V which is a much more positive potential than the value of +0.70V selected by the *S/N* ratio criterion. This increased potential will in fact yield poorer LODs and LOQs than will the +0.70V value because of the ultimate dependence of these parameters on background noise level.

An applied potential of +0.70V, based on the more rigorous *S/N* ratio criterion, was programmed into the ECDs for the remainder of this study.

2.3.4.2 Limits of Detection and Quantitation for Catecholamines

The PU4022 and EDT LCA 15 ECDs were compared and contrasted with respect to the LODs and LOQs attainable for the catecholamines EP, NE and DA. In order to do this two parallel HPLC systems were assembled. The first one was that used for the optimisation of the applied potential which included the Altex LC-XPD pump and the EDT LCA 15 ECD. The second set-up was of similar composition except that the PU4010 pump and the PU4022 ECD were

Figure 2.16 Graphs of Measured Electrochemical Detector Response (Peak Height) vs. Applied Potential for the Catecholamines EP (□), NE (○) and DA (Δ).

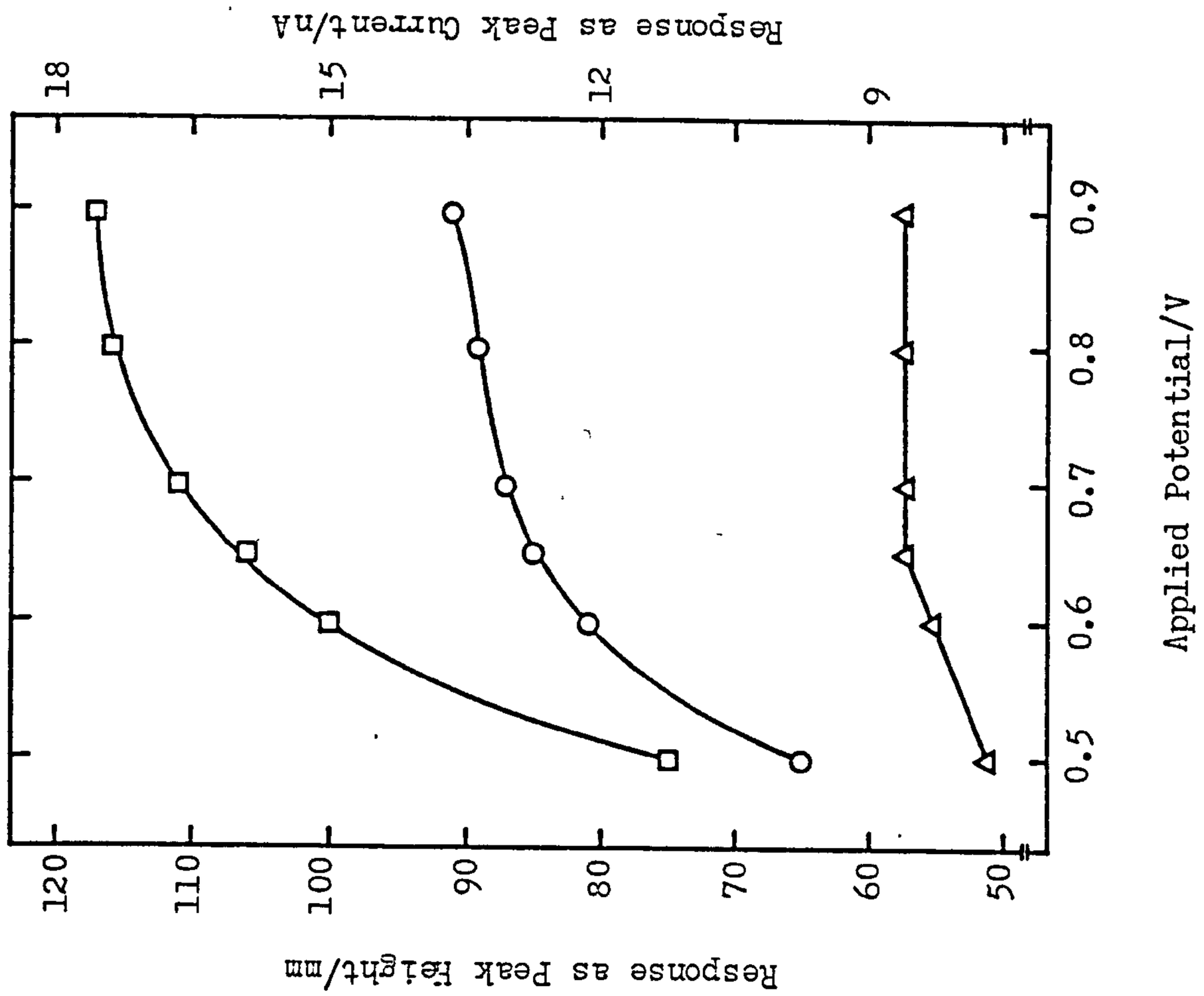
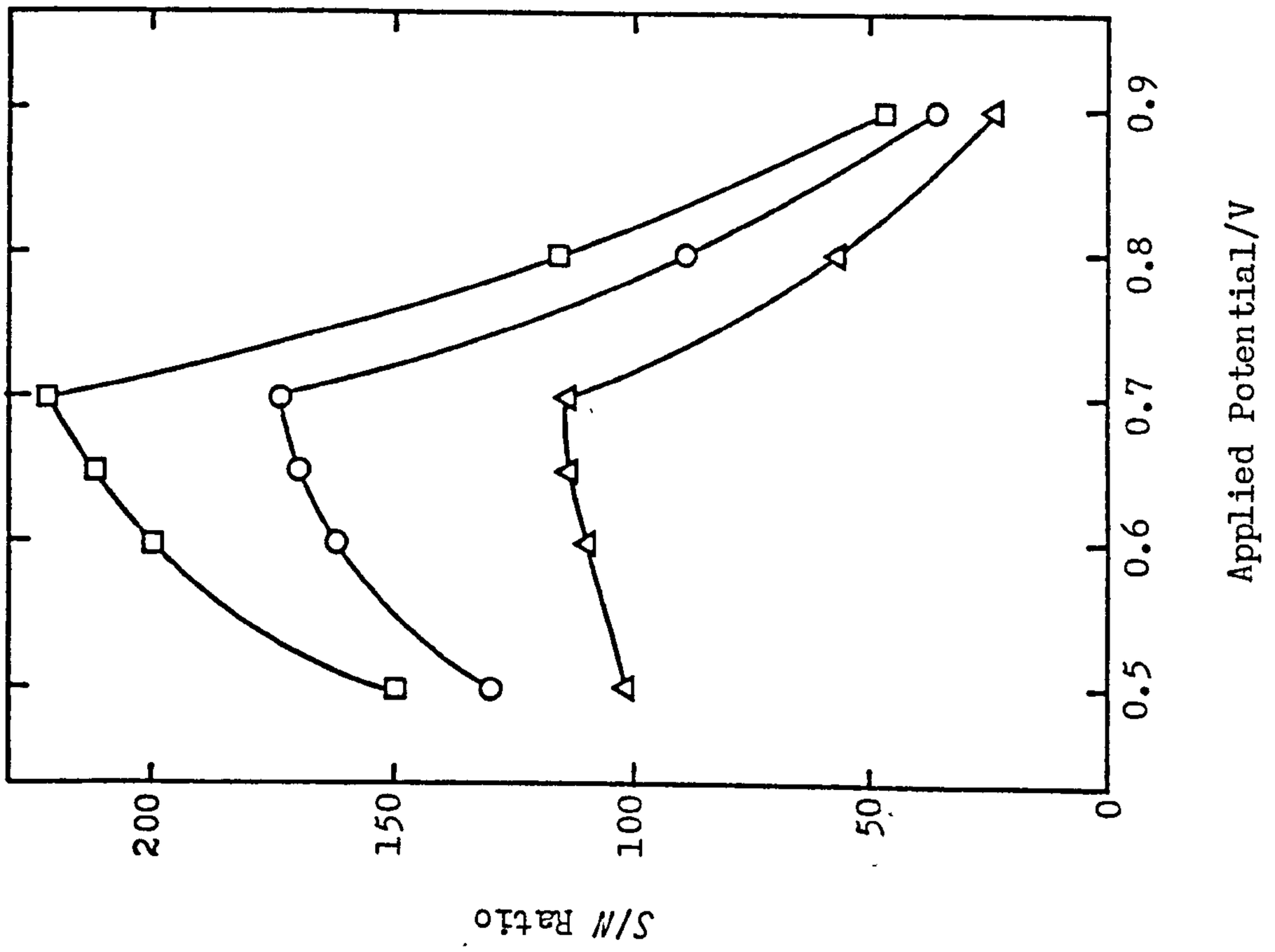


Figure 2.15 Graphs of Experimentally Determined Signal-to-Noise Ratios vs. Applied Potential for the Catecholamines EP (□), NE (○) and DA (Δ).



employed. Identical columns of Spherisorb S5 ODS1 were interposed into both systems.

Each ECD was programmed with an instrument sensitivity of lnA f.s.d. (the maximum setting available on each control unit) and a time constant of 10sec. Through each system was pumped the standard mobile phase used to date for catecholamine chromatography and the baselines obtained were allowed to settle as far as was possible. A standard mixture of EP, NE and DA ($0.01\mu g ml^{-1} \equiv 200pg$ injected of each compound) was chromatographed in duplicate on each system. Baseline noise and peak heights were measured for all chromatograms.

As stated previously, LOD is defined herein by a *S/N* ratio of 2:1 and LOQ by a *S/N* ratio of 20:1, therefore masses of each catecholamine required to provide these *S/N* ratios were calculated and are presented in Tables 2.17 and 2.18.

A comparison of the mean LODs and mean LOQs for each catecholamine with respect to detector model points to the conclusion that there is little difference between the two ECDs on this basis. The rough calculations yield slightly lower values from the PU4022 than from the EDT LCA 15 but within the limits of the original measurement error no clear distinction may be drawn.

A more reliable study might be achieved perhaps by applying a more rigorous approach and by increasing the number of injections on each system. However, within the bounds of this experiment, which was restricted greatly by the short time that the EDT LCA 15 was available for assessment, the two ECDs were found to be equivalent.

As it was known at this point that the EDT LCA 15 would not be retainable for future work beyond this comparison study, the PU4022 alone

Table 2.17 Recorded Responses from the Pye Unicam PU4022 Electrochemical Detector for the Catecholamines EP, NE and DA

Compound	Signal		Noise		*S/N*	Wt. required for *S/N* = 2:1 (pg)	Mean LOD (±SD) (pg)	Wt. required for *S/N* = 20:1 (pg)	Mean LOQ (±SD) (pg)
	(mm)	(pA)	(mm)	(pA)					
EP	162	810	15	75	10.8	37	37	370	370
	162	810	15	75	10.8	37		370	
NE	105	525	15	75	7.0	57	57 ± 1	571	567 ± 1
	106	530	15	75	7.1	56		563	
DA	90	450	15	75	6.0	67	66 ± 1	667	662 ± 8
	92	460	15	75	6.1	66		656	

Table 2.18 Recorded Responses from the EDT LCA 15 Electrochemical Detector for the Catecholamines EP, NE and DA

Compound	Signal		Noise		*S/N*	Wt. required for *S/N* = 2:1 (pg)	Mean LOD (±SD) (pg)	Wt. required for *S/N* = 20:1 (pg)	Mean LOQ (±SD) (pg)
	(mm)	(pA)	(mm)	(pA)					
EP	135	675	15	75	9.0	44	43 ± 2	444	428 ± 23
	155	775	16	80	9.7	41		412	
NE	102	510	15	75	6.8	59	59 ± 1	588	593 ± 6
	107	535	16	80	6.7	60		597	
DA	81	405	15	75	5.4	74	70 ± 6	741	699 ± 60
	97	485	16	80	6.1	66		656	

was evaluated regarding its performance in the analysis of samples of biological origin.

2.3.5 Demonstration of the Application of the PU4022 Electrochemical Detector to the Determination of Neurochemicals in Specific Rat Brain Regions

In order to demonstrate the effectiveness of the Pye Unicam system (PU4010 pump and PU4022 ECD), the instrument was used to detect, and in some cases identify, a number of electroactive neurochemicals present in different regions of the brain of a rat.

The Pye Unicam apparatus from the previous study, which included the Spherisorb S5 ODS1 column, (see Section 2.3.4) was used again except that the 20µl injection loop was replaced by one of 50µl capacity. A new solvent system comprising 7% MeOH : 93% aq. NaAc/HCt buffer containing HSA as an ion interaction agent and EDTA as a metal ion complexing agent (pH 4.6) was recommended by Dr. I. Kilpatrick (Department of Anatomy, University of Bristol)[210] who routinely determines the aforementioned substances using a similar HPLC system to that being examined. The mobile phase was pumped through the apparatus and a standard provided by Dr. Kilpatrick was chromatographed. The resulting chromatogram is presented in Figure 2.17.

A series of 6 supernatants was obtained by homogenisation and centrifugation of small parts of a rat brain. Three brain regions were studied, viz. the cerebral cortex, the striatum and the olfactory tubercles. Two samples of each region were taken, to only one of which was added 3,4-dihydroxybenzoic acid (DHBA), the commonly employed internal standard for this work. The six supernatants were chromatographed and the resulting chromatograms are presented in Figures 2.18 to 2.23.

Figure 2.17 Chromatogram of Catecholamine Standards Obtained Using the Pye Unicam Instrumentation

Parameters :- Column : Spherisorb S5 ODS1 (25cm x 5mm ID, d_p = 5µm); Mobile phase : 7% MeOH : 93% aq. 0.1M NaAc/HCt buffer containing HSA ($100mgl^{-1}$) and EDTA ($50mgl^{-1}$), pH 4.6; Flow Rate : $1.0ml min^{-1}$; Pump : PU4010; Detector : PU4022 ECD (E_{app} = +0.7V *vs.* Ag/AgCl, TC = 1sec); Sample : 50µl *via* loop of an aq. standard containing NE, DHBA, DOPAC and DA

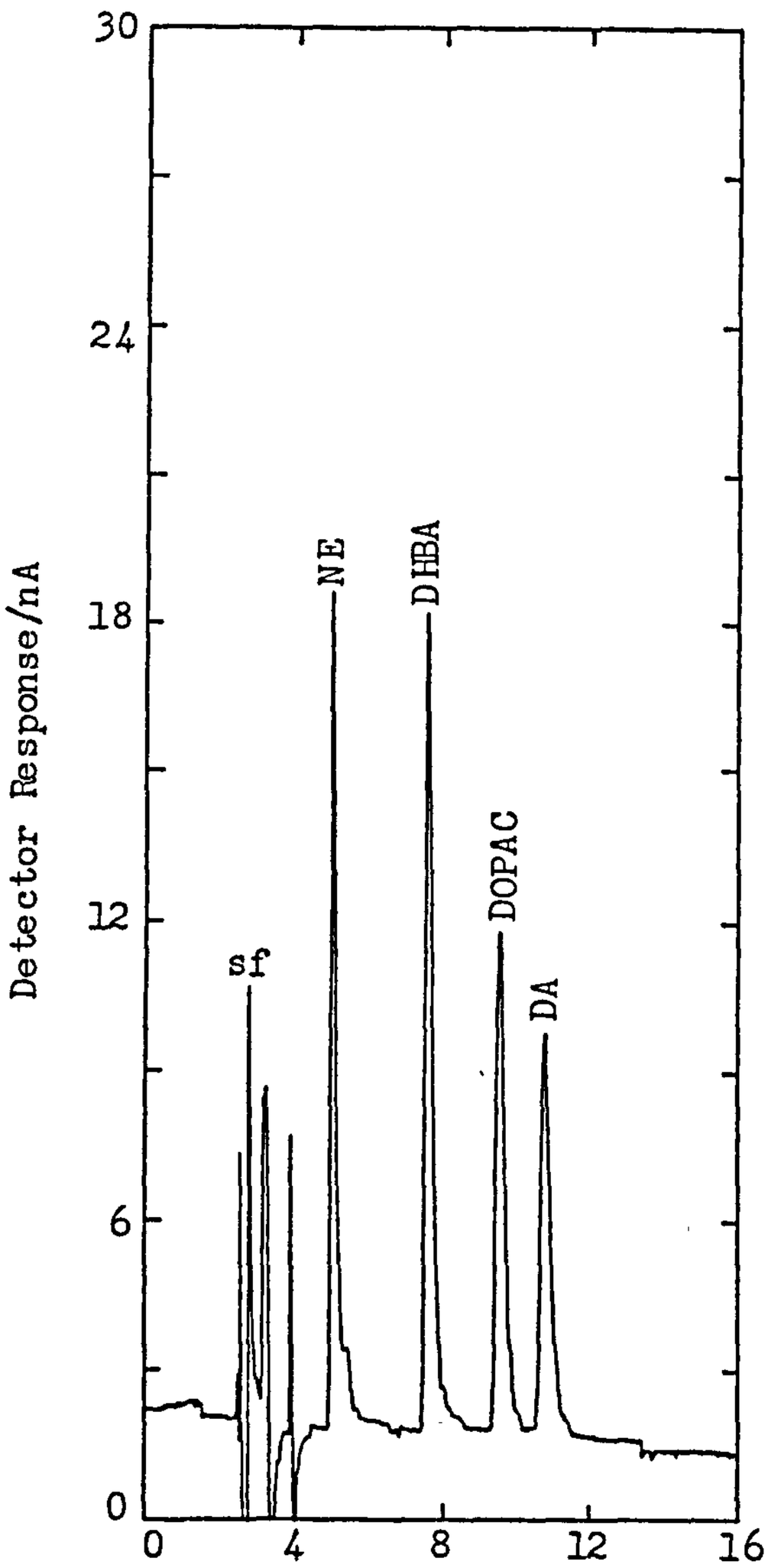


Figure 2.18 Chromatogram of Rat Cerebral Cortex Homogenate

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl via loop of a $HClO_4$/EDTA homogenate (350µl) of rat cerebral cortex (ca. 15mg)

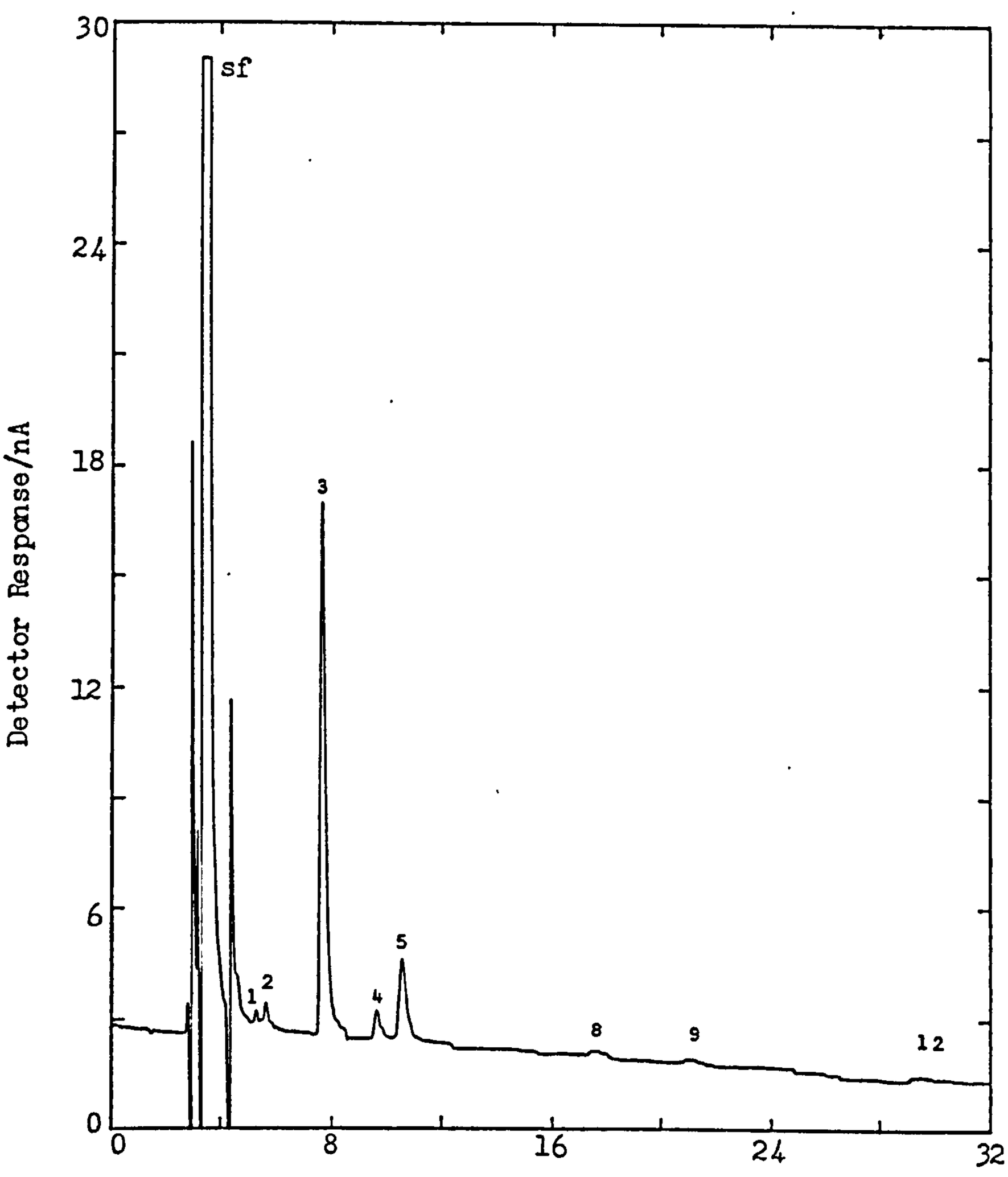


Figure 2.19 Chromatogram of Rat Cerebral Cortex Homogenate with Added Internal Standard

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl via loop of a $HClO_4$/EDTA homogenate (350µl) of rat cerebral cortex (ca. 15mg) incorporating DHBA as an IS

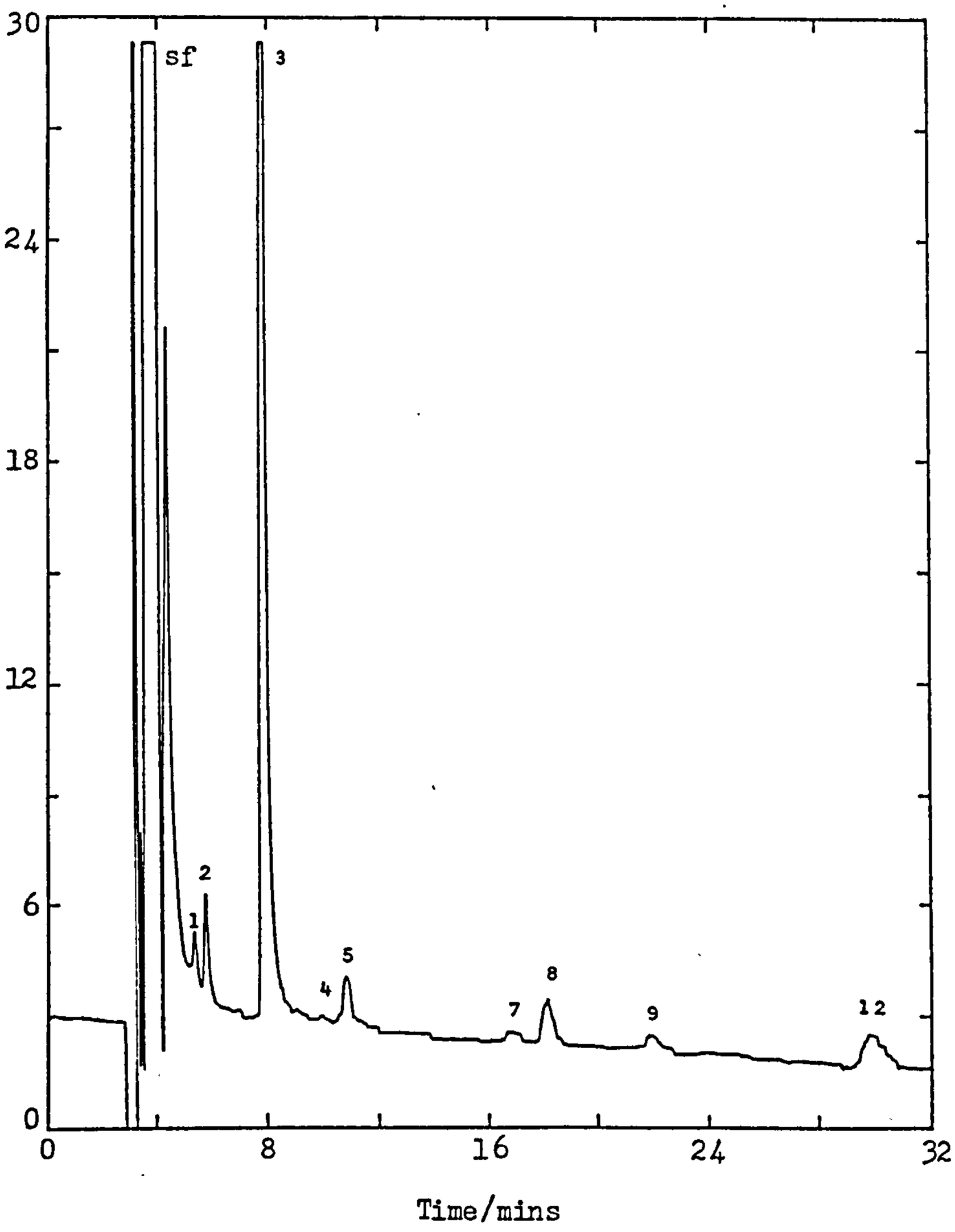


Figure 2.20 Chromatogram of Rat Striatum Homogenate

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl *via* loop of a $HClO_4$/EDTA homogenate (350µl) of rat striatum (*ca*. 15mg)

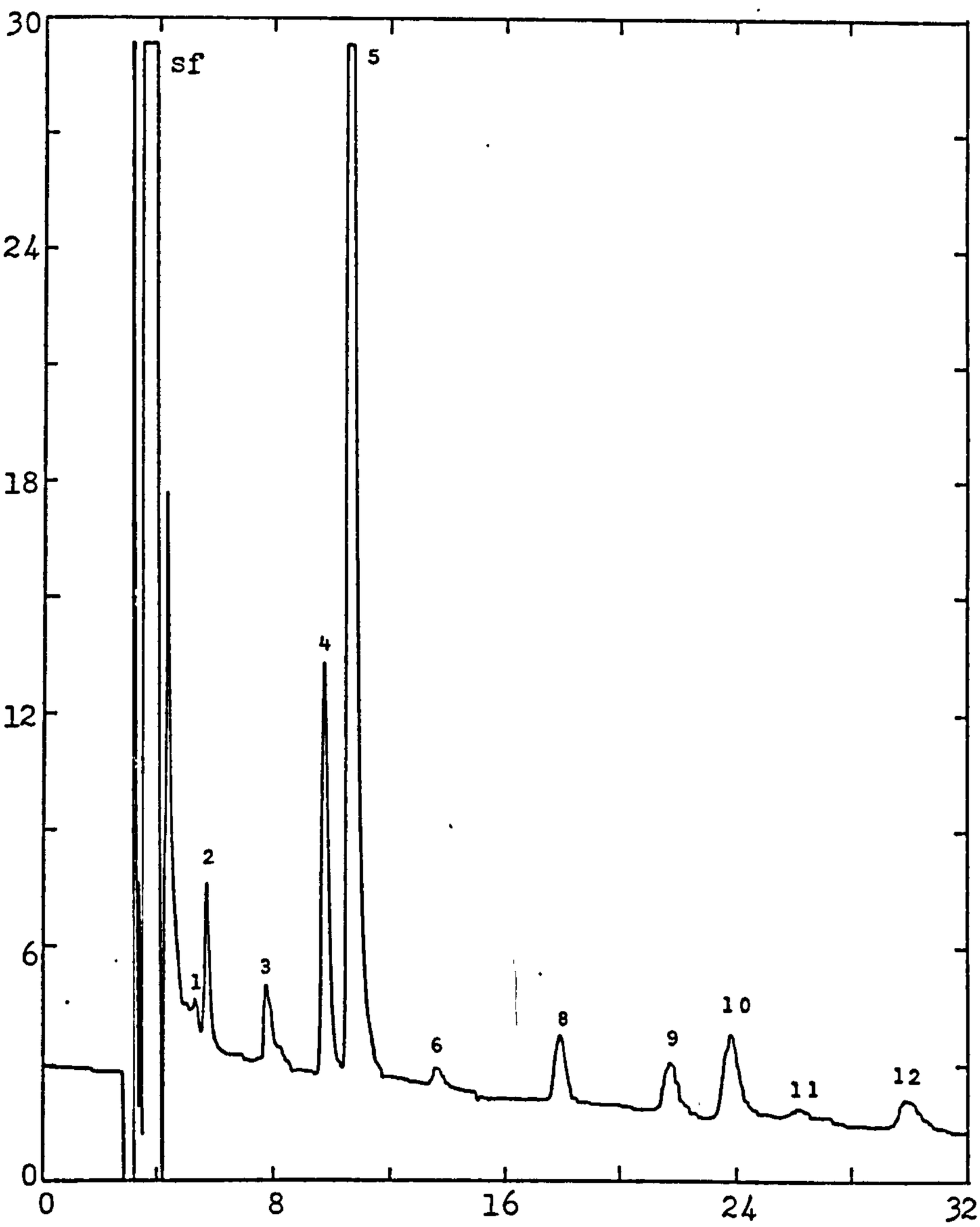


Figure 2.21 Chromatogram of Rat Striatum Homogenate with Added Internal Standard

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl via loop of a $HClO_4$/EDTA homogenate (350µl) of rat striatum (ca. 15mg) incorporating DHBA as IS

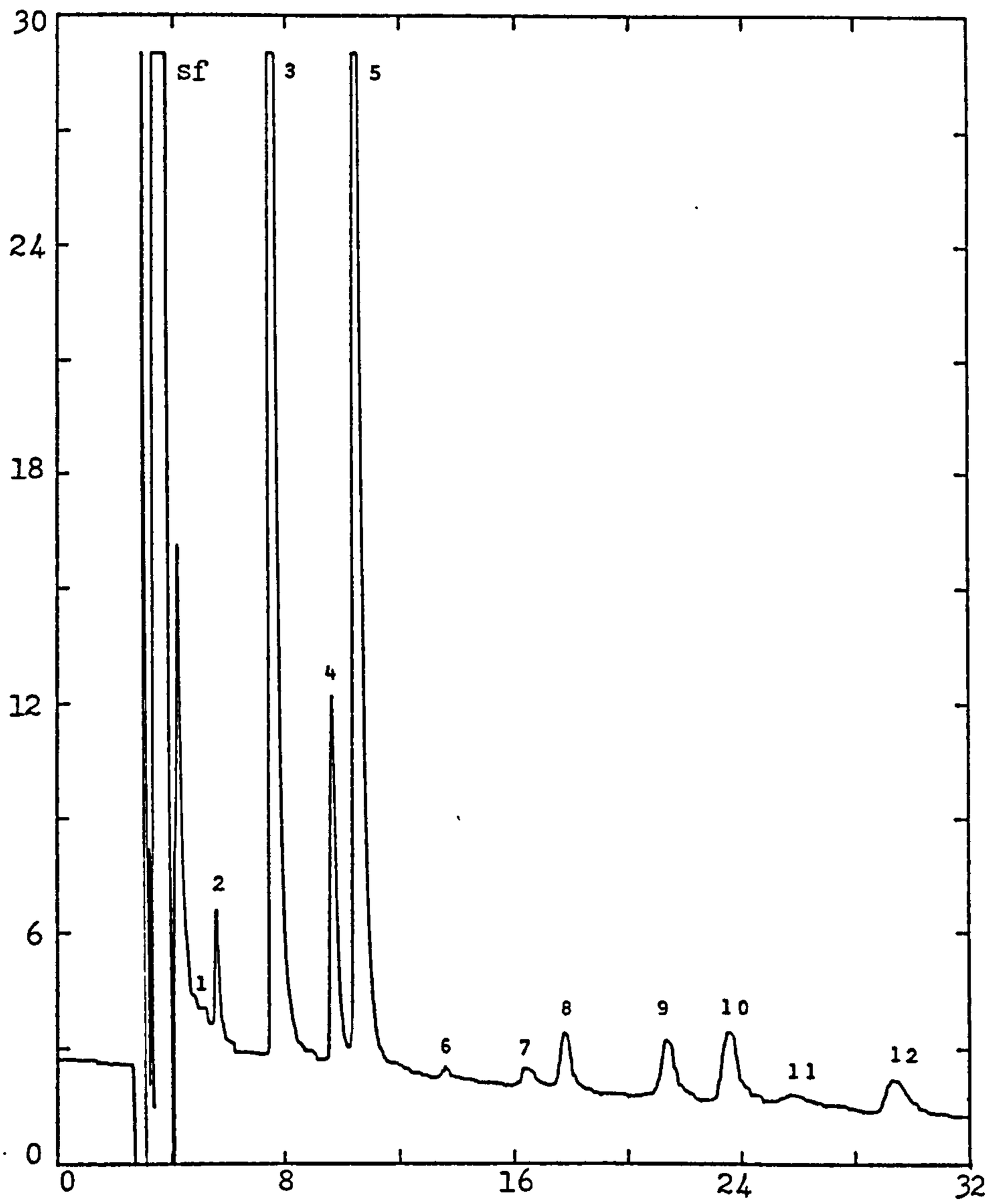


Figure 2.22 Chromatogram of Rat Olfactory Tubercles Homogenate

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl via loop of a $HClO_4$/EDTA homogenate (350µl) of rat olfactory tubercles

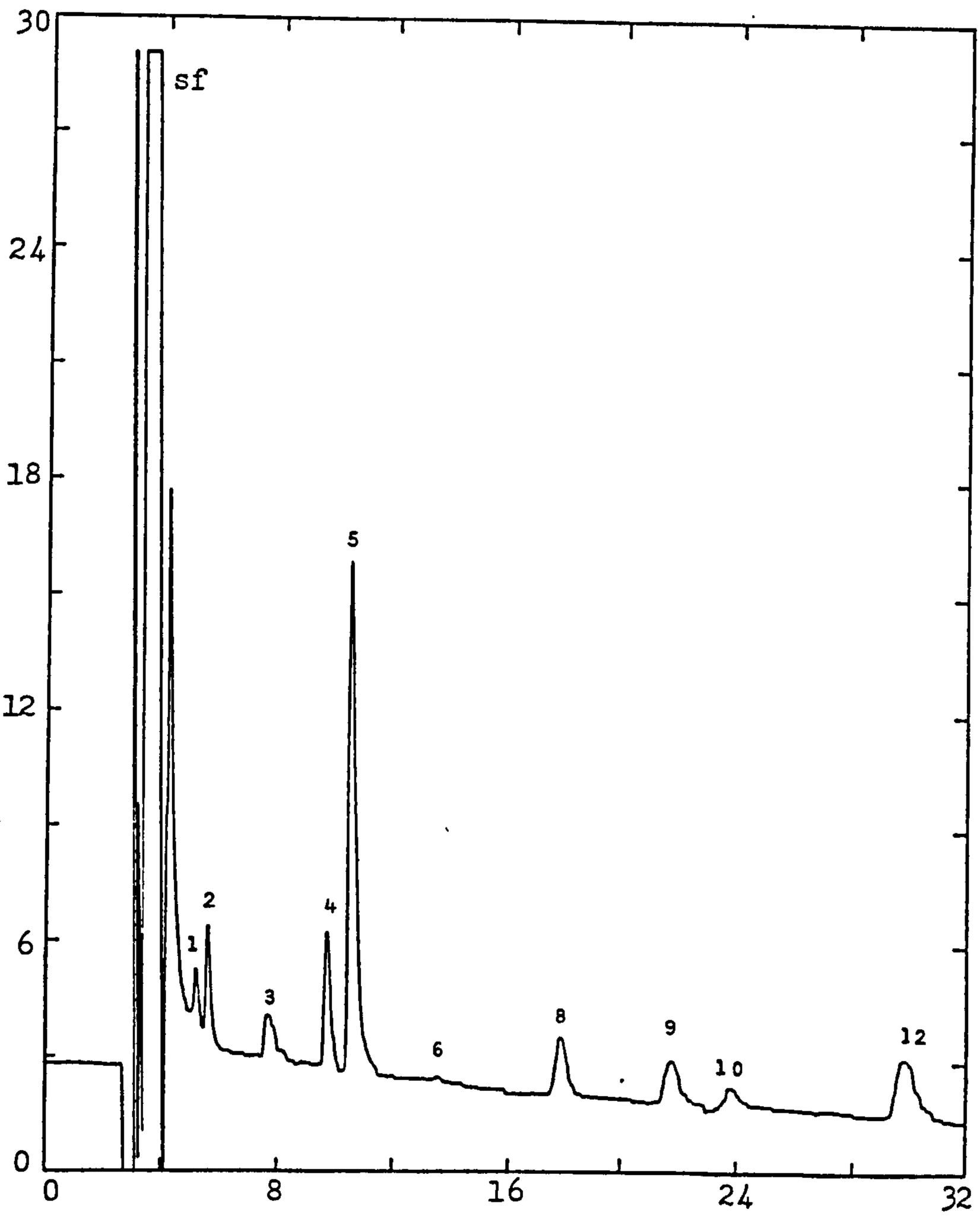
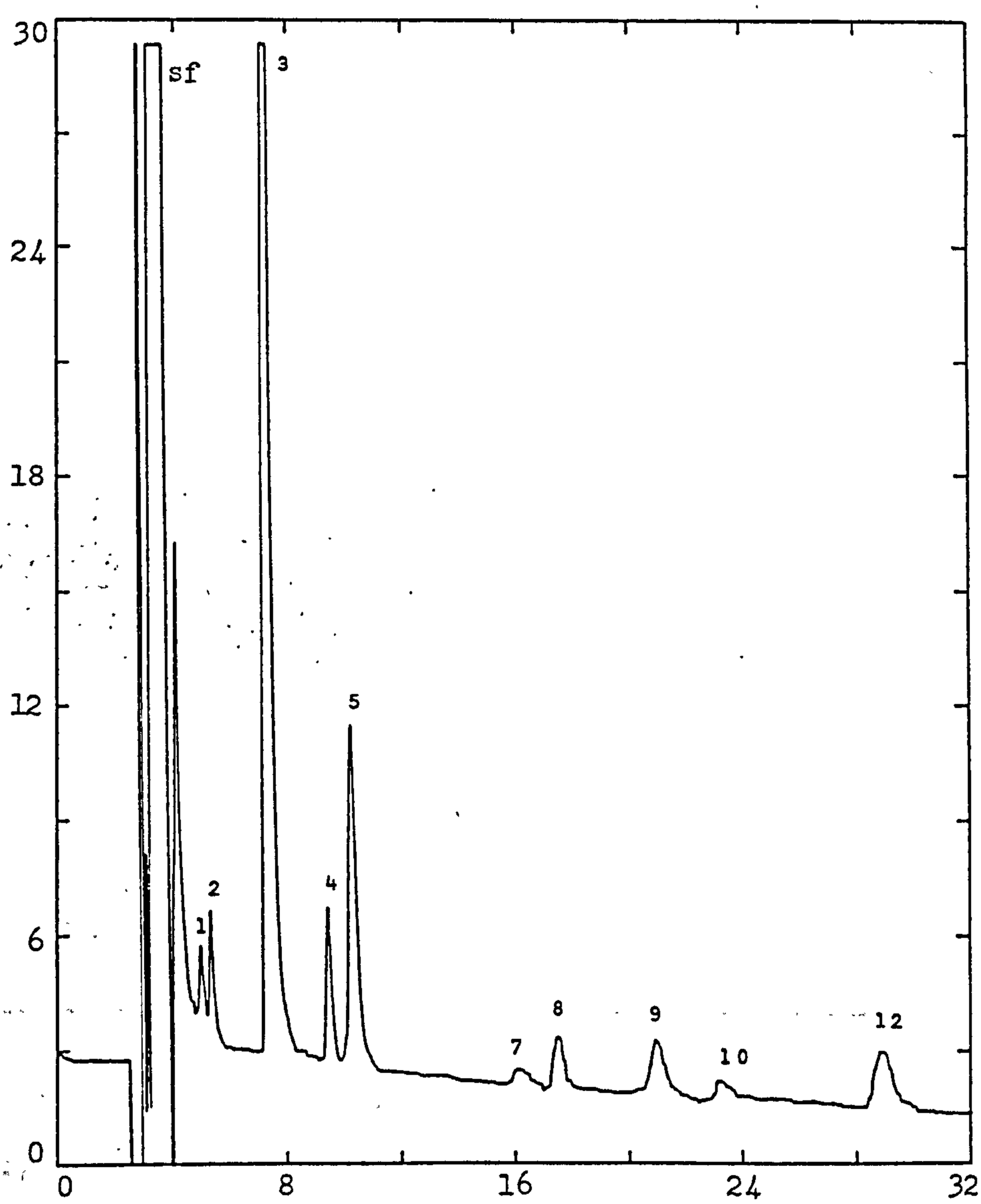


Figure 2.23 Chromatogram of Rat Olfactory Tubercles Homogenate with Added Internal Standard

Parameters :- For conditions see Figure 2.17 except for Sample : 50µl via loop of a $HClO_4$/EDTA homogenate (350µl) of rat olfactory tubercles (ca. 15mg) incorporating DHBA as IS



In conjunction with Dr. Kilpatrick, the peaks obtained were tentatively assigned and these assignments are tabulated in Table 2.19. More rigorous identification may be made by chromatographing individual standards of known neurochemicals but this was not deemed necessary for the purposes of this demonstration.

Table 2.19 Tentative Assignments of Peaks Obtained by Chromatographing Rat Brain Region Homogenates

Peak Number	t_R (min)	k′	Assignment
1	5.3	0.77	Unknown
2	5.7	0.90	Norepinephrine (NE)
3	7.7	1.57	5-hydroxyindole-3-acetic acid (5HIAA) & 3,4-dihydroxybenzoic acid (DHBA)
4	9.7	2.23	3,4-dihydroxyphenylacetic acid (DOPAC)
5	10.6	2.53	Dopamine (DA)
6	13.6	3.53	Unknown
7	16.6	4.53	Artefact associated with DHBA addition
8	18.0	5.00	3-methoxy-4-hydroxyphenylacetic acid (Homovanillic acid, HVA)
9	21.6	6.20	Unknown
10	23.8	6.93	Unknown
11	25.6	7.53	Unknown
12	30.0	9.00	5-hydroxytryptamine (Serotonin, 5HT)

These chromatograms illustrate the variation in the general neurochemical composition between different regions of the rat brain. For

example, the striatum contains detectable quantities (at 30nA f.s.d.) of compounds 6 and 11 which are not observed in the chromatograms of the cerebral cortex and olfactory tubercles homogenates (with the possible exception of compound 6 in the olfactory tubercles, Figure 2.22). Similarly, compound 10 is absent in the chromatograms of cerebral cortex extract but is detectable in the other two brain regions examined.

From the chromatograms, information regarding the relative abundances of those compounds found in more than one brain region may also be deduced. This is best exemplified by peak 5, which is assigned to DA. The amount of DA present increases markedly from the cerebral cortex (ca. 2-3ng injected) to the olfactory tubercles (ca. 10-20ng injected) and again to the striatum region (>> 30ng injected). All DA quantities quoted assume linearity of response of the ECD over the range examined, i.e. constant analytical sensitivity.

The PU4022 is observed to be able to detect a range of neurochemicals in samples of biological origin in low ng quantities. The detectability of these compounds would be expected to be enhanced at more sensitive instrument settings, and possibly at a higher operating potential whilst using this alternative mobile phase composition. The PU4022 has been demonstrated to perform effectively for the analysis of rat brain extracts at 30nA f.s.d., with increased capacity for detectability remaining within the instrument.

2.4 Conclusions

A comparison of the performances of three wall-jet amperometric ECDs has been carried out.

The Metrohm 641-VA/656 was found to exhibit very poor performance with respect to baseline noise. This was uncharacteristic of the instrument based on previous experience with this detector.[211] The cause of this problem was almost certainly faulty electronic components in the controller unit. This was only discovered when the unit was serviced by the manufacturer several months after the completion of this study. No reliable evaluation of the Metrohm ECD could be made therefore a comparison of detector performance related to structural differences in the flow cell was not possible.

The EDT LCA 15 and the PU4022 were compared over a range of experimental conditions in order to establish what effect the power circuitry of each model (the only difference between the two ECDs) had on overall performance. The two ECDs were almost identical regarding warm-up, the EDT model perhaps settling a little quicker at high instrument sensitivity settings (3nA and 1nA f.s.d.). Over the range of conditions applied to the systems the PU4022 was generally slightly noisier in the long term than was the EDT LCA 15 but they were practically identical regarding short-term interference.

With respect to *S/N* ratio (as demonstrated by the detection of chromatographically separated catecholamines), the EDT and Pye Unicam detectors were shown to be equivalent within experimental error.

Overall, the slightly poorer performance from the PU4022 was of little consequence in practical terms. The use of a noise smoothing function at high instrument sensitivity is recommended. This serves to reduce the

baseline noise level but also lessens the difference between the outputs of the two ECDs. For small-diameter column LC work operation at high sensitivity is envisaged so the two detectors may be considered to be practically equivalent.

The selection of ECD to be used in conjunction with NBLC was in fact forced upon the author. The EDT LCA 15 was required to be returned to the manufacturers immediately following this comparison study but the PU4022 was made available for use for the duration of the investigations reported in this dissertation.

EC detection was selected for application to NBLC because of the small cell volumes (as little as 0.5µl) readily available. The constraints imposed on the HPLC system regarding minimisation of extra-column volumes in order to achieve maximum practicable mass sensitivity of the detector require cell volumes of this order and smaller. At the outset of this project UV flow cell volumes were typically of the order of 5-10µl and those of FL cells were even greater (commonly 10-30µl). Both detection techniques were inferior to EC detection on the grounds of cell volume, although in recent years both UV and FL flow cells have been miniaturised for LC with small-diameter columns. However, because UV and FL detection are solution phenomena the reduction in cell volume leads to a reduction in intrinsic sensitivity with respect to the detection process, although a moderate overall gain may be achieved in some instances by the lower dispersion of the chromatographic peak. EC detection is a surface phenomenon not a solution phenomenon, in contrast to other detection methods, and so it does not suffer the aforementioned restriction. Indeed, because EC detection is a surface phenomenon it is uniquely well suited to be scaled down for µLC.[109]

NBLC coupled with EC detection was to be assessed, primarily with regard to mass sensitivity, for the determination of indolic compounds. Special emphasis was to be placed on the problems of limited sample size experienced with neonatal and paediatric patients, and to the restrictions of routine analysis in a clinical laboratory.

CHAPTER 3

Part I

Clinical and Analytical Aspects of a Selected Series of Indolic Substances Derived from Tryptophan

Contents

3.1 Introduction

3.1.1 Origin and Metabolism of Tryptophan in the Human Body[218,222]

In 1901, Hopkins and Cole[223] first isolated and identified the naturally occurring indole-based amino-acid tryptophan (TP), the structure of which is given in Figure 3.1.

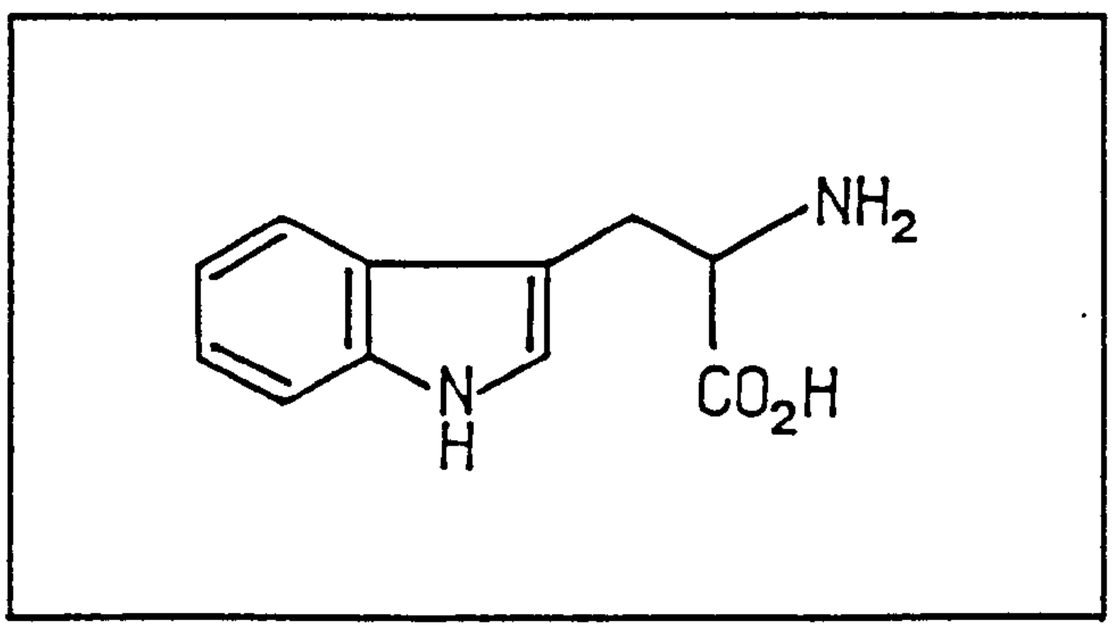


Figure 3.1 The Structure of Tryptophan

Willcock and Hopkins[224] examined the biochemical implications of TP in mammalian species and demonstrated the amino-acid to be nutritionally indispensable. Today it is known that mammals are unable to synthesise TP internally, hence requirements must be supplied entirely from external sources, i.e. in the diet primarily in the form of TP-containing proteins. TP is one of ten such substances which are known as essential amino-acids.

The metabolism of TP has been studied extensively, primarily because of the vital role played by several of its metabolites in a wide range of bodily processes. The major pathways are presented in Figures 3.2 and 3.3.

The kynurenine pathway is the primary route by which TP is utilised in healthy individuals. In excess of 95% of available TP is metabolised

Figure 3.2 The Primary Metabolism of Tryptophan in Humans (Abridged)[218-221]

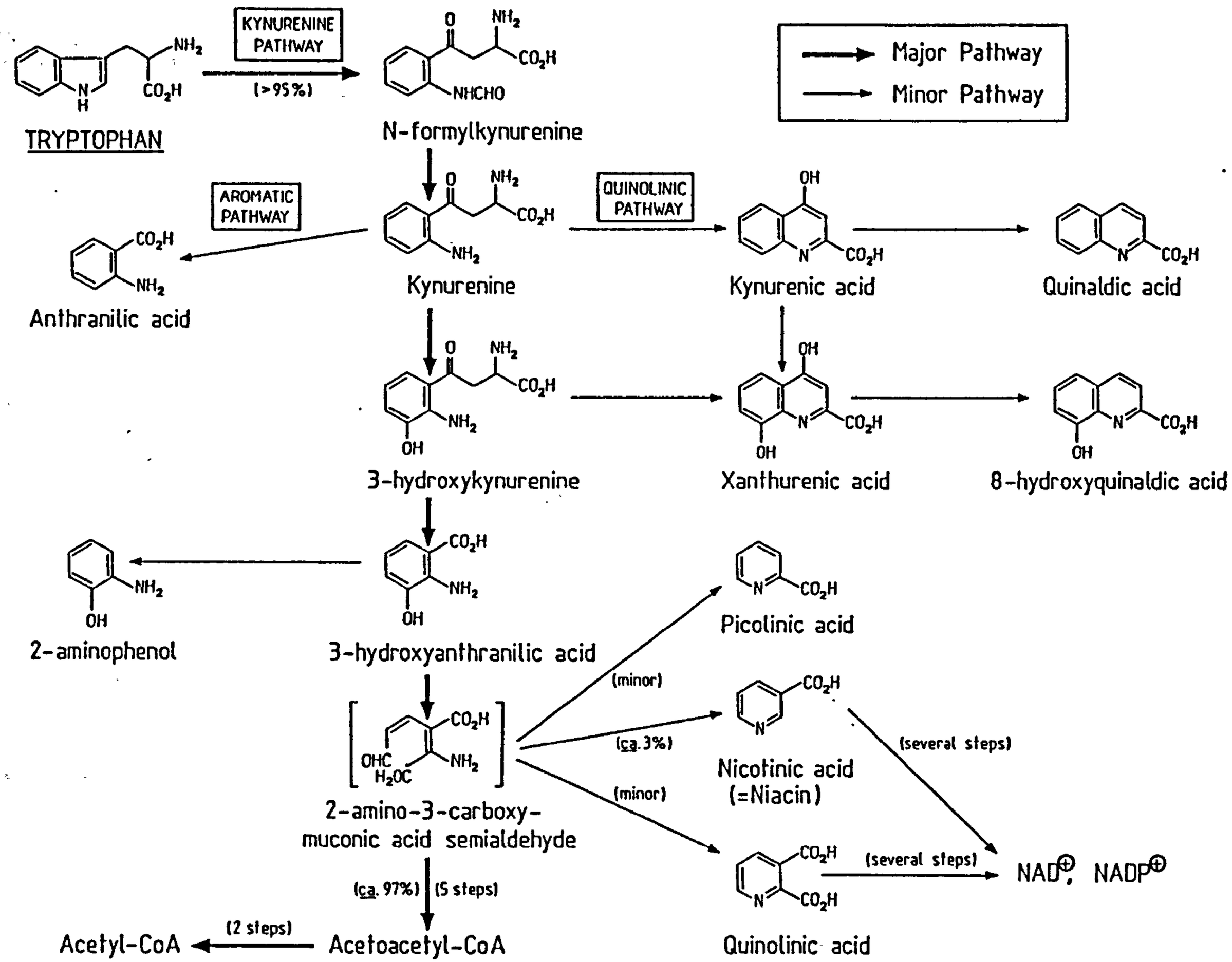
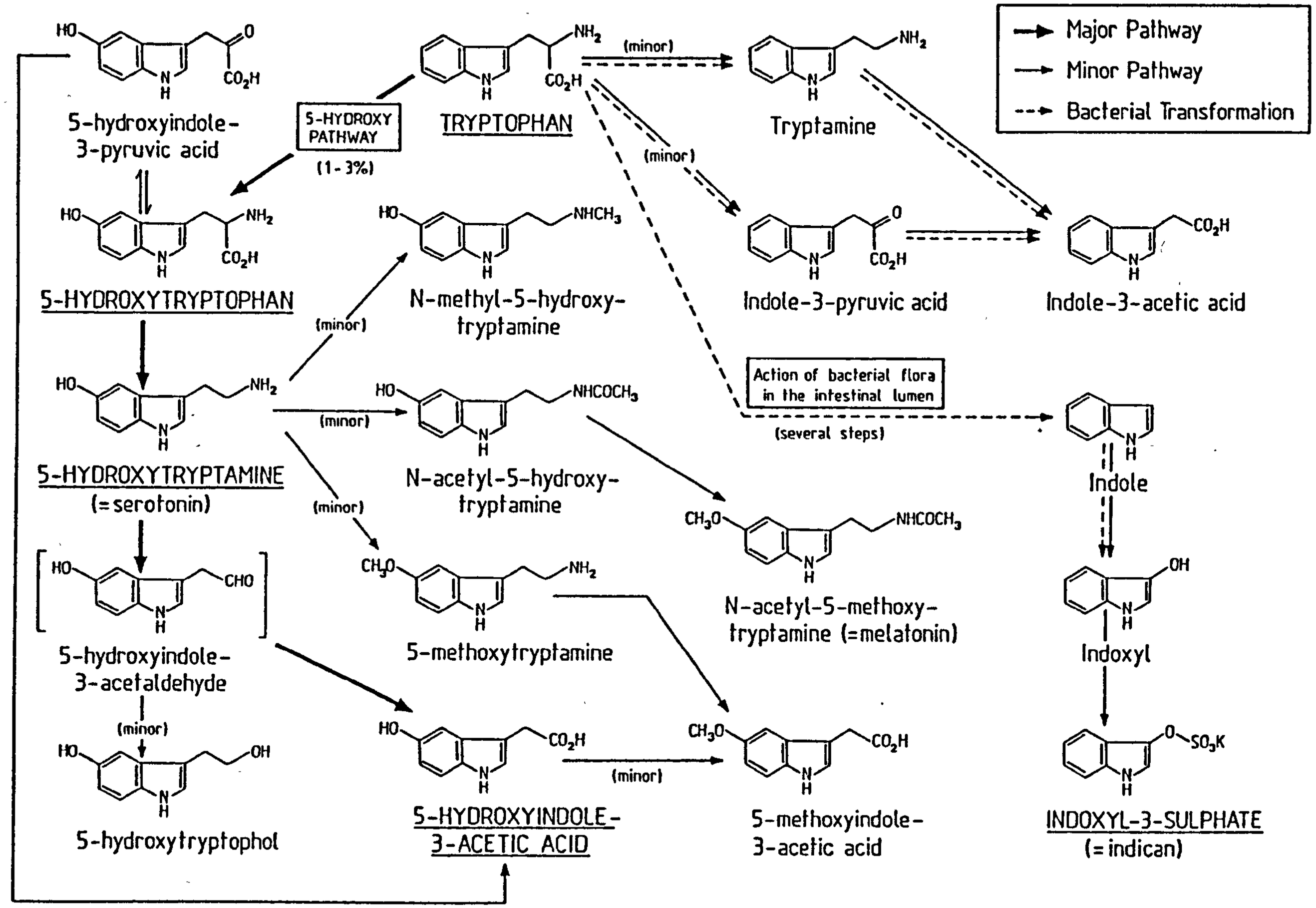


Figure 3.3 The Metabolism of Tryptophan in Humans by Secondary and Minor Pathways (Abridged)[218-221]



via this pathway under normal circumstances. Amongst the products generated are nicotinic acid (which is also known as the B vitamin, niacin), nicotinamide adenine dinucleotide ($NAD^{\oplus}$ or NADH, corresponding to the oxidised and reduced forms respectively) and the phosphorylated analogue of NAD, nicotinamide adenine dinucleotide phosphate ($NADP^{\oplus}$ or NADPH). All three of these substances are extremely important coenzymes.

Ordinarily only 1-3% of dietary TP is metabolised *via* the secondary 5-hydroxy pathway. Nevertheless one vitally important compound is generated by this route, *viz*. 5-hydroxytryptamine (serotonin, 5HT). 5HT is synthesised by the following series of reactions. TP liberated from dietary proteins by digestive action is absorbed, either actively or passively[225], through the wall of the small intestine and into the circulatory system whereby it is transported around the body. At certain specific tryptophan hydroxylase-containing sites within the body the action of this enzyme on TP in the presence of a tetrahydrobiopterin cofactor yields 5-hydroxytryptophan (5HTP), the immediate precursor of 5HT. Transformation of 5HTP to 5HT proceeds rapidly catalysed by the enzyme, aromatic-L-amino acid decarboxylase.

5HT is synthesised primarily in enterochromaffin (argentaffin) cells[226] which are widely distributed throughout the intestinal mucosa, biliary tract and gall bladder, pancreatic ducts and bronchial tree and are also found in the spleen, thymus, salivary glands, ovaries and uterus.[222] Additional synthesis of 5HT occurs in serotonergic neurons in the central nervous system (CNS).[227] Following synthesis, 5HT is thought to be stored as a 5HT-adenosine triphosphate(ATP)-$Mg^{2\oplus}$ micelle[228,229] primarily (i.e., *ca*. 90%[230]) in the enterochromaffin cells themselves and additionally in thrombocytes (platelets) within the blood, in basicytes (specifically mast

cells) in connective tissue and within serotonergic neurons. Whilst bound to platelets or tissues 5HT is pharmacologically inactive.[222] Following release into the circulation, the bulk of the free 5HT is eliminated in a single passage through the liver or lungs, hence plasma levels of free 5HT are low.[231,232] Metabolic destruction occurs predominantly by a two stage conversion process proceeding *via* the intermediate 5-hydroxyindole-3-acetaldehyde to 5-hydroxyindole-3-acetic acid (5HIAA). These two reactions are catalysed by the enzymes monoamine oxidase and aldehyde dehydrogenase respectively. 5HIAA is the principal excreted species from the 5-hydroxy pathway although TP, 5HTP, 5HT and several other minor metabolites are also excreted in healthy individuals.

In the unbound state, 5HT is the most pharmacologically active amine, serving a multitude of functions.[233] 5HT is an important neurotransmitter[234,235], a powerful target-dependent vasoconstrictor or vasodilator of the respiratory tract and vascular system[219], and a potent smooth muscle stimulator.[219] Furthermore, 5HT has been associated with the regulation of sleep[236,237], body temperature[238], several neuroendocrine functions[233] and peristalsis[239,240], and it is implicated in the perception of pain.[233,237] 5HT is also known to play a minor role in blood clotting where it functions as a weak platelet aggregating agent.[241] Because 5HT performs such a diverse range of functions there are a correspondingly large number of clinical conditions which directly or indirectly affect the concentrations of 5HT, its precursors and metabolites in body fluids and tissues (see Section 3.1.2). Hence, the ability to quantify the levels of the major (and minor) indole compounds formed by means of the 5-hydroxy pathway would be of great value clinically.

In addition to the kynurenine and 5-hydroxy pathways, other minor routes exist for TP metabolism. One of particular interest is that leading to the formation of indoxyl-3-sulphate (indican, I3S). Any TP-containing protein which is present in the lumen and which reaches the distal section of the gastrointestinal tract is frequently subject to putrefaction by the anaerobic bacterial population resident in this region. These micro-organisms absorb TP and metabolise it, excreting indole as the primary waste product. This compound is readily absorbed through the colon wall into the circulatory system. On reaching the liver, detoxification occurs by hydroxylation at the 3-position to produce indoxyl followed by sulphonation of this hydroxyl group to yield I3S which is excreted in the urine. The ability to quantify I3S in blood and urine is of particular importance in a number of gastrointestinally-related problems, especially when assessed in conjunction with the determination of 5-hydroxy pathway metabolites (see Section 3.1.2).

Because of the intense interest expressed by clinicians in the products of TP metabolism synthesised by the secondary and minor pathways, the significance of these particular substances with respect to disease in humans is discussed further.

3.1.2 Clinical Significance of the Tryptophan 5-Hydroxy Pathway Metabolites and Closely Related Indolic Substances

Abnormalities in the concentrations of TP and its 5-hydroxy pathway metabolites in body fluids and tissues may arise due to the influence of hormonal or dietary factors on the metabolic pathways. Nevertheless, measurement of the levels of these substances in whole blood or blood

fractions, urine, cerebrospinal fluid (csf) or tissue, as appropriate, affords valid biochemical indices for a variety of diseases.

3.1.2.1 Inborn Errors of Tryptophan Metabolism[242]

In spite of the highly complex nature of TP metabolism (see Figures 3.2 & 3.3), very few inborn errors of this metabolism have been characterised to date. Hypertryptophanaemia and hypertryptophanuria[243] are recognised by elevated concentrations of TP in the blood or urine respectively, often accompanied by elevated levels of the 5-hydroxy metabolites but with no corresponding increases in the levels of I3S or indole-3-acetic acid (IAA). The condition arises because of a deficiency of tryptophan pyrrolase, the enzyme which catalyses the initial step of the principal kynurenine pathway. Because the kynurenine pathway is inhibited a higher than normal proportion of absorbed TP is metabolised *via* alternative routes, e.g., the 5-hydroxy pathway, whilst much remains unchanged. Hypertryptophanaemia/hypertryptophanuria invariably exhibits symptoms of pellagra (a disease corresponding to niacin deficiency) as a secondary condition because the synthetic pathway which yields this vitamin is inhibited so preventing normal metabolic supplementation of dietary intake. Approximately 50% of daily niacin requirement is synthesised from TP in healthy individuals.[244]

A second inborn error of metabolism gives rise to 3-hydroxykynureninuria[245] and xanthurenicaciduria.[241] A deficiency in the enzyme kynurininase, which is essential to catalyse the cleavage of alanine from kynurenine and 3-hydroxykynurenine, leads to a breakdown in the kynurenine pathway. Because of this inhibitory effect, elevated quantities of 3-hydroxykynurenine and its alternative metabolite, xanthurenic acid,

are excreted in the urine. Again, because the pathway to niacin is inhibited, pellagra commonly results as one of the consequences of this metabolic disorder. No diversion to the 5-hydroxy pathway occurs therefore levels of 5HTP, 5HT and 5HIAA are normal.

Quantitation of TP and its 5-hydroxy metabolites in blood and urine provides supportive diagnostic evidence of the former metabolic defect.

3.1.2.2 Neurological and Psychological Disorders[229,233]

Because 5HT functions as a neurotransmitter in the CNS, a large number of neurological and psychiatric diseases have been linked, to greater or lesser extents, to this substance, its precursors and metabolites. A selection of the most important examples of clinical conditions which fit into the above category, together with observed abnormalities in 5-hydroxy pathway metabolite concentrations, is presented in Table 3.1. Neurological and psychiatric abnormalities also present as secondary effects in many other conditions which give rise to disturbances in TP metabolism.

Stahl, in an excellent review article[229], advocated the use of the blood platelet as a suitable model of serotonergic neuron function with respect to transport, metabolism and release of 5HT. Although a direct pharmacological link between blood levels of TP and its 5-hydroxy metabolites and CNS function (or malfunction) has not been established for many of the ailments listed in Table 3.1, experimental evidence does tend to indicate that a close connection exists in many instances. This is in spite of the blood-brain barrier which is impervious to 5HT[233,246] although TP, 5HTP and 5HIAA may traverse.[247]

Table 3.1 Selected Central Nervous System-Related Disorders Presenting Abnormalities in Tryptophan 5-Hydroxy Metabolite Levels (229 and references therein)

Clinical Condition	Important Pathological Symptons	Abnormalities in Indole Levels			Remarks
		Sample*	Indole	Status†	
Down syndrome	Mental retardation	wb	TP	↓	Diminished platelet 5HT due to defect in active uptake mechanism
		wb	5HT	↓	
		plt	5HT	↓	
Other mental retardation syndromes	Mental retardation: Phenylketonuria (PKU) Histidinaemia Lange's Syndrome	wb	5HT	↓	Mechanism unknown; probably transport-related in PKU and histidinaemia
		plt	5HT	↓	
	Infantile hypothyroidism Maternal rubella Kernicterus Various retardation syndromes with and without motor impairment	wb	5HT	↑	
		plt	5HT	↑	
Infantile autism	Inaccessibility, aloneness, inability to relate, rage reactions and language disturbances	wb	5HT	↑	Elevated levels in ca. 30% of cases only
		plt	5HT	↑	
		wb	5HIAA	↑	
		plt	5HIAA	↑	
		csf	5HIAA	↓	
Minimal brain dysfunction	Hyperactivity, short attention span, aggressiveness	wb	5HT	↓	
Schizophrenia	Delusions, hallucinations, thought disturbances, impaired daily functions	wb	5HT	↑ ?	Elevated in some types. Possibly due to low monoamine oxidase activity
Affective disorders (inc. despression and manic-depression)	Depression, or alternating periods of elation and depression	plt	5HT	↓ ?	No consistent change in levels. They may be reduced
		csf	5HIAA	↓ ?	
		ur	5HIAA	↑ or ↓	
Duchenne muscular dystrophy	Progressive disease. Pseudohypertrophy of shoulder and pelvic muscles, weakness	pl	5HT	normal	
		plt	5HT	↓	
Parkinsonism	Movement disorder	br	5HT	↓	
		br	5HIAA	↓	
		csf	5HIAA	↓	
		plt	5HT	abnormal	
Huntington chorea	Hereditary brain infection. Progressive mental deterioration and movement disorder	plt	5HT	↑	Enhanced platelet 5HT uptake
Migraine headaches	Head pains, distorted vision, gastrointestinal disturbances	plt	5HT	↓	
		pl	5HT	↓	
		ur	5HIAA	↑	

* wb = whole blood, plt = platelet, pl = plasma, ur = urine, csf = cerebrospinal fluid, br = brain

† ↑ elevated, ↓ diminished

3.1.2.3 Carcinoidosis and Carcinoid Syndrome[248-250]

Lembeck[251], in 1953, first identified 5HT as a pharmacologically active constituent of a carcinoid tumour and in the following year Thorsen et al.[252] recognised the carcinoid syndrome. Since this time concentrations, particularly urinary, of TP 5-hydroxy metabolites have been of value in the diagnosis and therapeutic monitoring of metastatic carcinoids and carcinoid syndrome.

Carcinoid tumours are the second most common endocrine tumours (after thyroid tumours) but less than one in ten is functional. Metastasising carcinoid tumours usually arise in the small intestine (duodenum, jejunum and ileum; 10% incidence), appendix (45% incidence) and rectum (15% incidence)[250], and in very rare cases they are found in the colon, stomach, gall bladder, pancreas, bronchi and ovaries. These tumours develop from enterochromaffin cells and, not surprisingly, they normally secrete large amounts of TP 5-hydroxy metabolites.[253] As much as 60% of dietary TP may be metabolised along this pathway within carcinoids (cf. 1-3% normally utilised[249]). The principal indole secreted by a carcinoid tumour depends upon the tissue from which the growth originates. Fore-gut tumours (bronchial and gastric) secrete 5HTP[254], some of which is excreted unchanged. Increased quantities of 5HT and 5HIAA are also excreted, presumably because further transformation of 5HTP occurs in other tissues, especially the kidney. Mid-gut tumours (primarily ileal) secrete 5HT predominantly, most of which is normally metabolised to 5HIAA by the widespread enzyme, monoamine oxidase. Carcinoids derived from the hind-gut (colon and rectum) rarely produce excess 5HT or 5HIAA.

In about 10% of cases this excretion is associated with the carcinoid syndrome[255] which includes such features as diarrhoea, valvular damage in

the heart and flushing attacks of various types.[256] Carcinoid syndrome usually occurs only with extensive metastases of a carcinoid tumour.

Because grossly elevated amounts of 5-hydroxy indoles are produced by carcinoids, the determination of these substances and their metabolites in blood and/or urine is an invaluable diagnostic aid.

3.1.2.4 Gastroenterologically-Related Conditions[249,255]

There are a series of gastroenterologically-related disorders which promote pathological changes affecting TP absorption and/or metabolism.

The small intestine is affected by a host of diseases which prevent normal aboral propagation of intestinal contents. There are two major categories of problem. Firstly, there are disorders producing an actual physical barrier to the passage of intestinal contents which may be classified as 'intestinal obstruction' and secondly, there are disorders which induce an erratic or paralysed intestinal peristalsis which does not support the normal propagation of intestinal contents. This latter group of motility disorders give rise to a clinical picture mimicking intestinal obstruction and include paralytic ileus and intestinal pseudo-obstruction.

3.1.2.4.1 Intestinal Obstruction

Obstruction of the small intestine can be caused by one of three types of physical barrier. Blockage can be due to (1) intraluminal objects (e.g., gallstones or foreign bodies) which may lodge in a narrow portion of the small intestine, especially the ileocaecal valve, or (2) an intramural process (e.g., a primary small bowel carcinoma or ileitis) which may encroach on the lumen of the small intestine sufficiently to produce

an obstruction, or (3) a process extrinsic to the small intestine itself (e.g., adhesive fibrous bands, incarcerated hernias, volvulus or compression by an adjacent neoplasm) which can cause the small intestine to obstruct. This last aetiology is by far the most common accounting for between 70% and 90% of all cases in adults. Greater than 50% of all paediatric cases also originate from processes extrinsic to the small intestine, particularly incarcerated inguinal hernia and intussusception. The latter is the single most common cause of obstruction in children aged between 2 months and 5 years. Furthermore, intussusception occurs predominantly in individuals under 2 years of age.

3.1.2.4.2 Paralytic Ileus and Intestinal Pseudo-Obstruction[257,258]

This group of motility disorders are characterised by a loss of propulsive peristalsis for which the underlying cause may be systemic illness (e.g., sepsis, uraemia, electrolyte imbalance) but is usually idiopathic, i.e. the disturbance arises spontaneously and is of unknown or uncertain origin. The most common motility disorder is paralytic ileus. This condition is of unknown induction, occurs frequently post-operatively and is usually transient. A far more chronic form of disordered bowel motility is that known as intestinal pseudo-obstruction. Primary or idiopathic pseudo-obstruction may sometimes be characterised by histological abnormalities of the ganglion cells in the myenteric plexus (the autonomic nervous system concerned with bowel motility control) or of the smooth muscle cells of the bowel wall. Secondary pseudo-obstruction has also been recognised and its relationship to a number of systemic problems, e.g., endocrine or neurological disorders, has been established.

3.1.2.4.3 Consequences of Intestinal Obstruction or Pseudo-Obstruction on Tryptophan Absorption and Metabolism

Physical blockage or motility disorders of the small intestine often cause a stagnation of the contents of the lumen. Frequently bacterial overgrowth occurs in the affected region and this abnormally high population of anaerobes metabolises a significantly higher proportion of dietary TP than usual. This event leads to elevated blood and urinary levels of I3S and, occasionally, of IAA and other indolic species too. As a consequence, general malabsorption of TP ensues resulting in diminished quantities of the 5-hydroxy (and kynurenine) metabolites in the body fluids. One exception to this is where an indole-secreting carcinoma is the causal factor of the physical obstruction or where the pseudo-obstruction relates to a TP-based endocrinological or neurological disorder. Under these circumstances 5-hydroxy metabolite concentrations may be abnormally high at the expense of the kynurenine pathway metabolites, with all the attendant consequences (e.g., pellagra).

Any situation like the above, where a combination of bacterial overgrowth and a demonstrable metabolic disturbance reversible by administration of oral antibiotics occurs, is known by the general term 'blind loop syndrome'.

3.1.2.4.4 Mucosal Abnormalities which Impair Absorption

Coeliac disease (synonyms : non-tropical sprue, coeliac sprue)[249,259] is a common ailment with an incidence of 1:3000 in the UK.[249] It is a wasting condition which often presents at an early age, frequently within the first 6-9 months of life. The exact mechanism is unknown but coeliac

disease is induced by a sensitivity to gluten, a protein present in many cereal grains, especially wheat, but not in corn or rice. The condition is associated with structural abnormalities of the mucosal cells which promote villous atrophy which reduces the internal surface area of the small intestine considerably. In addition, enzyme deficiency often appears secondary to these architectural abnormalities.

The primary consequence of the disease is general intestinal malabsorption, particularly inhibition of fat uptake which presents as steatorrhoea. Protein assimilation is also grossly affected by coeliac disease. The absorption rate is reduced, less than the normal amount is absorbed, and a significant proportion is lost from the lumen to metabolism by colonic bacteria. This last problem is sometimes exacerbated by the occurrence of pseudo-obstruction which accompanies coeliac disease in many cases.

Thus, TP absorption, and consequently its metabolism, is adversely affected. Coeliac disease has been associated with slightly elevated excretion of 5HIAA[240,260-270] together with increased urinary levels of I3S[264-266] and in some cases IAA and other indolic compounds produced by bacterial degradation of TP.[240,264-266] Whole blood[262,272] and platelet[240,262] 5HT concentrations are also elevated as a result of this disease. The reasons for the high blood 5HT levels, and consequently the raised 5HIAA excretion, have not been clearly established. Abnormally high tissue 5HT concentrations have been found in the duodenal mucosa of coeliac patients.[272] Abnormally high enterochromaffin cell counts in similar samples have also been demonstrated by the same group.[273] Hence, hyperplasia of the enterochromaffin cells in the small intestine most probably accounts for this phenomenon although hyperactivity cannot be ruled out as an additional causal factor.

In addition to structural abnormalities of the mucosa giving rise to protein-derived amino-acid (specifically TP) malabsorption, two biochemical abnormalities have also been discovered, viz. Hartnup disease[244,274] and 'blue diaper' syndrome.[244] Both these diseases are congenital and occur due to derangements of absorptive transport mechanisms.

Hartnup disease was first described in 1956 by Baron and co-workers[275] and was named after the family in which it was first recognised. By 1978 a total of 53 cases of Hartnup disease had been reported[274], and today an incidence of 1:18,000 is estimated.[222] The illness is characterised by a photosensitive pellagra-like skin rash, temporary loss of control of bodily functions (cerebellar ataxia) and occasionally by psychiatric changes ranging from emotional instability to delerium, but not mental retardation or deterioration. Acute renal aminoaciduria which is selective for free monoamino-monocarboxylic amino-acids with neutral or aromatic side chains is the only constant feature. A characteristic amino-acid pattern in urine and faeces from patients suffering from Hartnup disease has been demonstrated.[244,276,277] Nemeth and Nachmias[278] suggested that the disease might originate from a deficiency of TP pyrrolase while Milne et al.[279] proposed that a selective intestinal and renal transport defect in one biochemical system peculiar to the aforementioned group of amino-acids, is responsible for the disease. This latter view has since been substantiated[280] and is now widely accepted. The source of the flaw is still obscure but the synthesis of a protein intrinsic to the defective carrier mechanism is suspect.[241]

Although the basic biochemical breakdown involves the intestinal and renal transport of several amino-acids, the symptoms appear to be secondary to the malabsorption of only one, viz. TP. Aminoaciduria occurs because

of impaired reabsorption, following ultra-filtration, of neutral amino-acids in the kidney tubules. Only ca. 50-80% reabsorption efficiency is attained in patients with Hartnup disease which may be compared with ca. 98% for normal subjects.[274] This finding is indicative of the biochemical defect blocking only one or possibly a few of several transport mechanisms applicable to each neutral amino-acid.[281] Confirmation of this view is provided by the discovery that the absorption of TP-containing oligopeptides proceeds normally in Hartnup patients.[282,283] The remaining symptoms of this complaint pertain to diminished TP absorption in the small intestine which is supported by diminished plasma TP levels[284] while faecal TP levels are significantly raised.[277] Impaired intestinal absorption consequently results in correspondingly high plasma and urinary levels of the bacterial breakdown products of TP, i.e. I3S and sometimes IAA.[285,286] It has been suggested that the ataxia attacks may be induced by a toxic agent derived from bacterial action. Pellagra most probably arises as a consequence of restricted TP availability as reflected by diminished levels of kynurenine pathway metabolites in the urine of Hartnup sufferers.[285,286] The reported mental disturbances may be manifestations of the abnormally low TP 5-hydroxy metabolite concentrations in plasma and urine which are often associated with this disorder.[285,286]

A second rare biochemical abnormality has been postulated as the cause of 'blue diaper' syndrome, a condition first reported in 1964 by Drummond et al.[287] Symptoms of this illness include fever, growth retardation, irritability and constipation, and of particular note is the blue discolouration observed on nappies. This colour is due to indigotonin (indigo blue), an oxidation product of I3S. Urinary indole excretion is gross in 'blue diaper' syndrome. Besides I3S, tyramine, IAA and indole-3-lactic acid are

also present in large amounts in the urine. TP is only found in minimal quantities in plasma and urine but in high quantities in faeces. All the evidence suggests that a selective absorptive defect for TP in the intestine is responsible.

'Blue diaper' syndrome, although of similar aetiology to Hartnup Disease, is remarkably different. Although they share indicanuria because of colonic bacterial action on unabsorbed TP, there the pathophysiological similarity ends. 'Blue diaper' syndrome does not have associated with it the acute aminoaciduria (presumably because renal function is normal), the pellagra-like skin rash or the neurological defects of Hartnup disease. Conversely, Hartnup disease does not present with nappy staining, presumably because I3S excretion, although elevated, is not as great as in patients with selective TP malabsorption.

The prominent manifestations of disturbed TP metabolism in coeliac disease, Hartnup disease and 'blue diaper' syndrome imply that an analytical method capable of quantitating TP, 5HTP, 5HT, 5HIAA and I3S simultaneously in blood and in urine would provide a useful diagnostic tool in the clinical laboratory.

3.1.2.5 Miscellaneous Diseases

A diverse range of other diseases have also been observed to present disturbances in TP metabolism as a secondary effect of the complaint. Such illnesses include a variety of dermatitic problems[242], scurvy[242], phenylketonuria (PKU)[242,288], rheumatoid arthritis[242,289], osteoarthritis[242], diabetes[242], alcoholism[242,290] and typhoid fever.[242]

3.1.2.6 Clinical Problems of Particular Relevance to Neonates, Infants and Young Children†

Those diseases giving rise to disturbances in TP metabolism which apply to the title subject group are listed in Table 3.2.

Table 3.2 Disorders giving rise to Deranged Tryptophan Metabolism Suffered by Individuals in Early Life

Disease Category	Specific Illness
Inborn Errors of Metabolism	Tryptophanaemia/Tryptophanuria
Neurological and Psychological Disorders	Down Syndrome Various Mental Retardation Syndromes Infantile Autism Minimal Brain Dysfunction
Cancer	Carcinoid Tumours
Gastrointestinal Problems and Malabsorption Syndromes	Intestinal Obstruction Pseudo-intestinal Obstruction Coeliac Disease Hartnup Disease Blue Diaper Syndrome

Those substances deemed to be of greatest interest diagnostically are those compounds which constitute the major route of the TP 5-hydroxy pathway, viz. TP itself, 5HTP, 5HT and 5HIAA. In addition, for gastrointestinal conditions where TP malabsorption occurs the measurement

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† Neonate = individual up to 4 weeks of age.

Infant = individual up to 1 year of age.

Child = individual of between 1 year of age and puberty, i.e. ca. 14 years of age.

of I3S would also prove to be of value. Screening biological fluids taken from neonates, infants and young children for these five indoles would provide supportive diagnostic information for all of the ailments listed in Table 3.2. Of special interest to one local hospital (the Taunton and Somerset Hospital, T&SH) are the gastrointestinal complaints, particularly coeliac disease and other conditions resulting from food allergies. Emphasis will be given to this group of disorders in this investigation although it should be noted that a general analytical procedure capable of yielding diagnostic information for all diseases exhibiting clinical changes in levels of the selected analytes in biological fluids is sought.

Some of the disorders listed in Table 3.2 have the effect of elevating TP metabolite levels in biological fluids whereas others give rise to diminution of the same as has been intimated previously. It is those conditions in which the latter situation prevails which pose the greatest challenge to the analyst. Analytical sensitivity must be sufficiently high to facilitate the detection and quantitation of the target compounds at both normal and abnormal levels and to differentiate between such states with confidence. The problems involved in achieving this goal are exacerbated by the limitation of sample size available from subjects who are very young. This constraint is discussed in more detail in Section 3.1.7 of this Chapter.

3.1.3 Analytical Techniques that have been Applied to the Determination of Indoles in Biological Samples

Several techniques have been applied, with varying degrees of success, to the determination of indoles in samples of biological origin. These techniques include colorimetry, fluorimetry, radioenzymaticassay (REA),

radioimmunoassay (RIA), and the chromatographic procedures, viz. classical column LC, paper chromatography (PC), TLC, GC and HPLC. Most of the developed methods comprise a number of extraction stages followed by some identification and quantitation technique.

The chemical methods, i.e. colorimetry and fluorimetry, are laborious, often exhibit poor sensitivity and may be subject to gross errors by losses in extractions, by simultaneous extraction of interfering compounds and/or by low specificity of colorimetric or fluorimetric reagents. In addition to the above drawbacks, these chemical methods, by their very nature, do not enable simultaneous multi-analyte analysis to be performed, a distinct disadvantage where the problem currently under consideration is concerned.

REA methods (e.g., 291, 292) and RIA methods (e.g., 293-295) have found occasional application to indole analysis. REA and RIA both combine the high degree of sensitivity of radiochemistry with the high specificity of enzymatic reactions or immunological reactions, respectively. However, these procedures are very time consuming and furthermore each REA or RIA method is only capable of the determination of a single species thus precluding simultaneous multi-analyte determination.

It is the chromatographic techniques which provide the means whereby several species may be quantified concurrently with discrimination. Classical column LC is extremely slow and laborious, one chromatogram often taking many hours to complete (e.g., 296). Furthermore, this approach is frequently inadequate with respect to selectivity due to the low resolution attainable and with respect to sensitivity due to extensive peak dilution. With the advent of PC the separation of complex mixtures became more reliable and, in 1962, Sprince and co-workers[297] used this technique to separate the various indoles present in urine. More recently, TLC has

been utilised for the same purpose (e.g., 298-302). Unfortunately, the improvements in chromatographic resolution over classical column LC obtained from PC and TLC are still limiting. In addition, the sensitivities of the available detection methods, i.e. colorimetry or fluorimetry, are poor. Consequently neither of these techniques is suitable for quantitation.[302]

GC coupled with flame ionisation detection (FID), alkali flame ionisation detection (AFID), electron capture detection (ELCD) or mass spectrometric detection (MS) has been applied to the subject determination with reasonable success. Greatly improved column efficiency is attainable with GC compared with classical LC, PC or TLC, which provides greater chromatographic resolution. Further selectivity is possible through judicious choice of the detection method (N.B. MS is generally considered to be superior in this respect to the other alternatives listed). However, complicated extraction and derivatisation procedures are necessary[303] because of the incompatibility of the aqueous-based samples with GC stationary phases and the known instability of TP and 5-hydroxyindoles at elevated temperatures.[304,305] These procedures are time consuming and commonly give rise to significant analyte losses. The preferred GC-MS technique is further disadvantaged because the apparatus required is complex and prohibitively expensive for the majority of clinical laboratories.[303] Consequently, the reported GC-MS methods (e.g., 306-309) are rarely used routinely and are confined primarily to research establishments.

In recent years HPLC has become the method of choice for the routine determination of indoles in biological tissues and fluids.[310] With careful selection of the detection system, high sensitivity and selectivity are possible. HPLC can accommodate partially or wholly aqueous samples with

ease and in addition, analyte derivatisation is frequently unnecessary hence, sample work-up procedures prior to injection are generally faster and less complicated than for GC. Aspects of HPLC relevant to the separation and quantitation of the chosen analytes, viz. TP, 5HTP, 5HT, 5HIAA and I3S, are discussed in the following sections.

3.1.4 Mechanistic Aspects of Solute Retention in Liquid Chromatography Pertinent to the Separation of Indolic Tryptophan Metabolites

Four primary operational modes exist in HPLC, viz. liquid-solid adsorption, liquid-liquid partition, size exclusion (or gel permeation), and ion-exchange. This inherent versatility originates from the fact that the retention and subsequent separation of two or more solutes on an HPLC column occurs as a result of a combination of interactions between each sample component and the stationary and mobile phases. Several fundamental intermolecular forces may be exploited to achieve selectivity and which of these forces predominate depends solely upon the chemical nature of the solutes and the phase system employed. Now, the TP-derived analytes are all ionisable and are of moderate polarity, consequently the liquid-solid adsorption format in which a non-polar mobile phase is eluted over a polar stationary phase (commonly silica) is inappropriate. Furthermore, the analyte moieties are of low molecular weight (MW = 179-220) therefore size exclusion chromatography, which is only applicable to the separation of high molecular weight species (MW >2000), is similarly inappropriate to this problem. For analytes possessing the aforementioned properties only the liquid-liquid partition and ion-exchange modes are suitable hence only these two formats will be considered further.

3.1.4.1 Partition Chromatography

Retention in partition chromatography is achieved by means of the relative solubility of a sample component in the stationary and mobile phases. Separation occurs on the basis of differences between the relative solubilities of each solute. The reverse phase (RP) mode is applied predominantly to partition chromatography, i.e., the stationary phase is fairly non-polar (hydrophobic) while the mobile phase is of high polarity (hydrophilic). Commonly, the stationary phase is composed of an alkylsilyl-bonded silica, e.g., octadecylsilane (ODS), and the mobile phase is either partially or wholly aqueous in nature. This is a highly desirable property for the majority of clinical applications because the mobile phase and sample matrix are invariably compatible. Reverse phase partition chromatography (RP-PC) has been utilised extensively for the separation of both polar and non-polar materials. In addition, RP-PC has been extended to ionic and ionisable substances by the employment of secondary equilibrium phenomena.

3.1.4.2 Ion Suppression Chromatography

When ionogenic solutes are required to be separated, the mobile phase pH must be controlled by using a buffer system. Adjustment of the pH to greater acidity will serve to suppress the dissociation of weak acids, i.e.,

$$AH + H_2O \rightleftharpoons A^{\ominus} + H_3O^{\oplus}$$

hence enabling the acids to be separated by conventional reverse phase techniques. Similarly, but conversely, adjustment of the pH to greater

basicity will suppress ionisation of weak bases, i.e.,

$$B + H_2O \rightleftharpoons BH^{\oplus} + OH^{\ominus}$$

thus allowing separation of the bases by RP-PC. This general approach is termed ion suppression and is useful for the analysis of weak acids or bases in the pH 2-8 range. Strong acids and bases cannot be accommodated by ordinary HPLC techniques since the ion suppression method is limited by the instability of silica-based bonded stationary phases outside the above pH limits. However, by employing other, more versatile secondary equilibria these highly dissociated species can be separated in conjunction with weak acids and bases and non-ionisable solutes.

3.1.4.3 Ion-Pair (or Ion-Interaction) Chromatography

The development of ion-pair chromatography (IPC) is generally attributed to Schill and co-workers[311,312] who, in the mid-1970s, were first to apply well established ion-pair extraction techniques to modern HPLC. The basis of IPC lies in the addition of a suitable organic counter-ion, called an ion-pairing agent (IPA), to the chromatographic system which encourages the formation of coulombic association species between two ions of opposite charge, known as ion-pairs. In this way, ionic or ionisable substances can be converted (reversibly) into electrically neutral non-polar complexes which exhibit identical behaviour to electrically neutral non-polar compounds. Thus, in the RP mode the ion-pairs partition between the two phases as if they are electrically neutral non-polar compounds. The extent to which an ionised solute and counter-ion form an ion-pair complex affects the degree to which the retention of the sample species is increased.

This RP-IPC approach implies the need for pH control by addition of a buffer system to the mobile phase to promote dissociation of the ionisable analytes in order that ion-pair association may actually take place. There are many other factors which affect a RP-IPC separation and these are summarised in Table 3.3. Generally a separation is controlled by first selecting a column and an organic modifier. The aqueous:organic solvent ratio and the nature and pH of the buffer must then be optimised. Finally the type, size and concentration of IPA must be chosen, the last providing fine adjustment of the chromatography.[313]

Although the theory of batch extraction of ion-pairs is well understood, the exact mechanism of RP-IPC is still obscure. Three hypothetical models have been proposed to date, viz. an ion-pairing mechanism, an ion-exchange type mechanism, and an ion-interaction mechanism.

The first postulate stipulates that the formation of an ion-pair occurs in the aqueous mobile phase prior to partition into the hydrocarbonaceous bonded stationary phase. Retention is governed by the degree of non-polarity of the ion-pair complex which determines its affinity for the stationary phase. The longer the alkyl chain on the IPA the less polar is the formed ion-pair and the retention of the ion-pair increases due to its greater affinity for the stationary phase. This concept is illustrated in Figure 3.4.

The second postulate stipulates that the unpaired lipophilic counter-ions adsorb onto the non-polar surface so modifying the column to behave as an ion-exchanger (see Section 3.1.4.4). The longer the alkyl chain length of the IPA the greater will be the stationary phase surface coverage, hence providing more ionic sites for interaction to take place on and consequently the longer will be the retention of the ionic sample. This concept is depicted in Figure 3.5.

Table 3.3 Adjustable Variables in RP-IPC

Variable	Effect
Type of counter ion	The better the ability to ion pair then the greater is the retention
Size of counter ion	An increase in counter ion size will increase retention
Concentration of counter ion	An increase in counter ion concentration will increase retention to a limit beyond which retention will decrease
Type of organic modifier in mobile phase	Retention decreases with increasing lipophilicity
Concentration of organic modifier in mobile phase	Retention decreases with increasing modifier concentration
pH	Effect is dependent upon the nature of the IPA. Retention increases as pH changes cause the solutes to become more dissociated
Stationary phase	Retention increases with lipophilicity and degree of coverage
Temperature	Retention decreases with increasing temperature

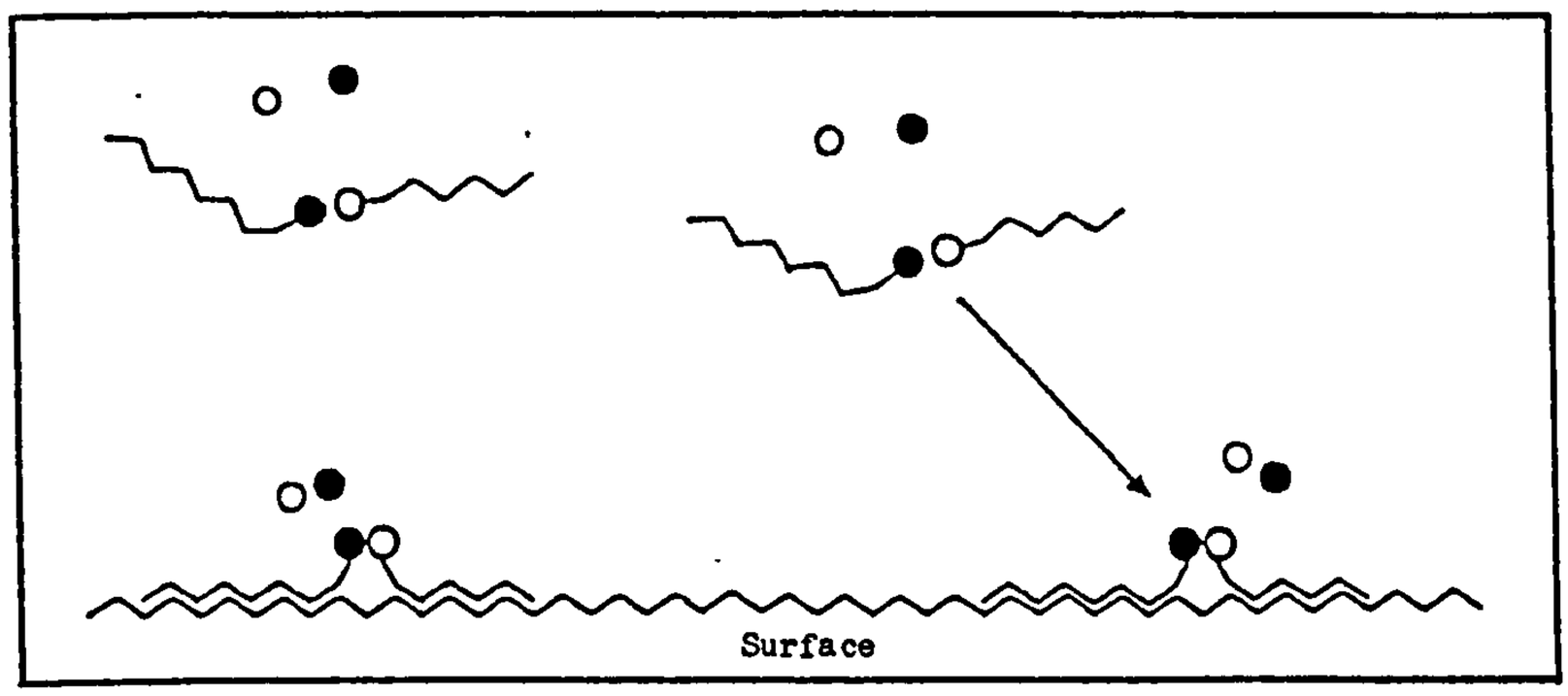


Figure 3.4 The Paired-Ion Model

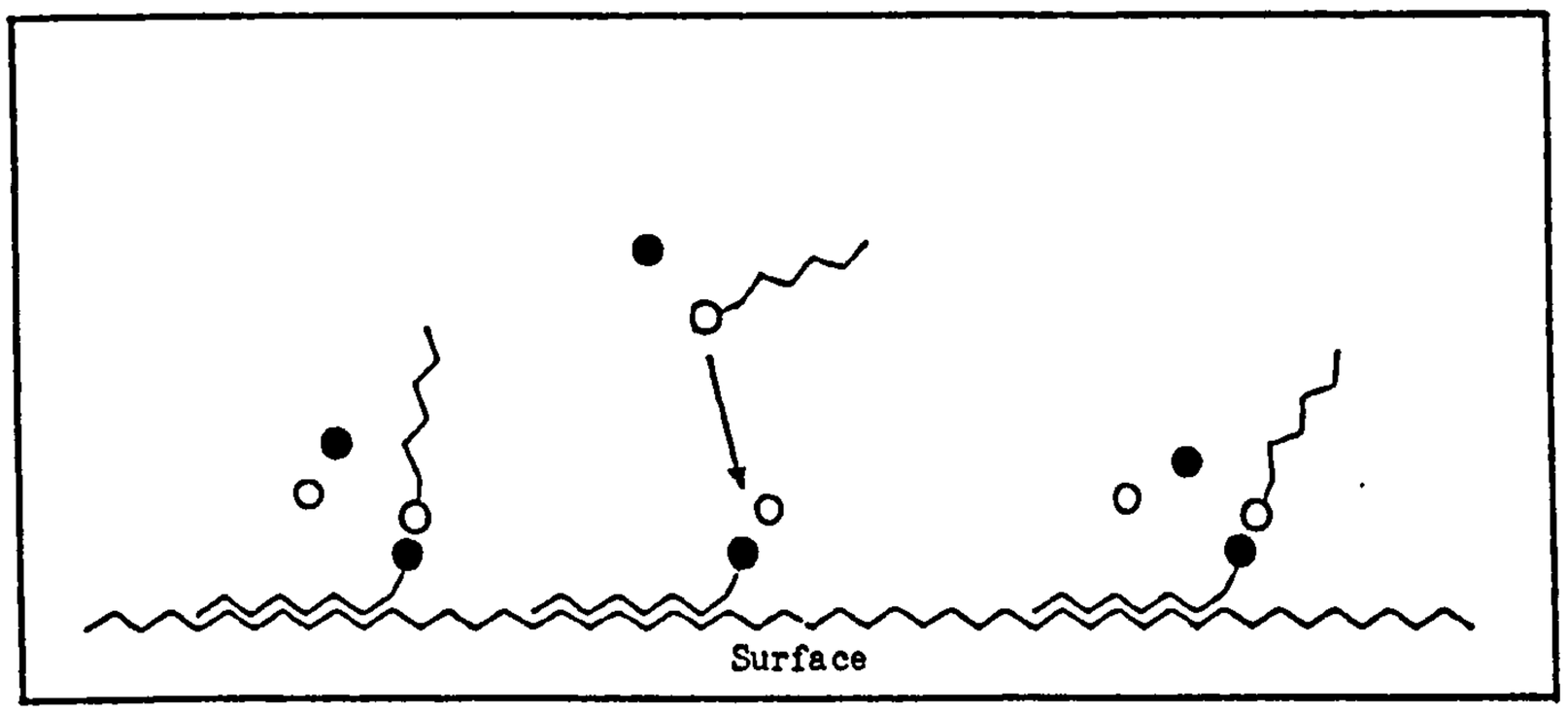


Figure 3.5 The Dynamic Ion-Exchange Model

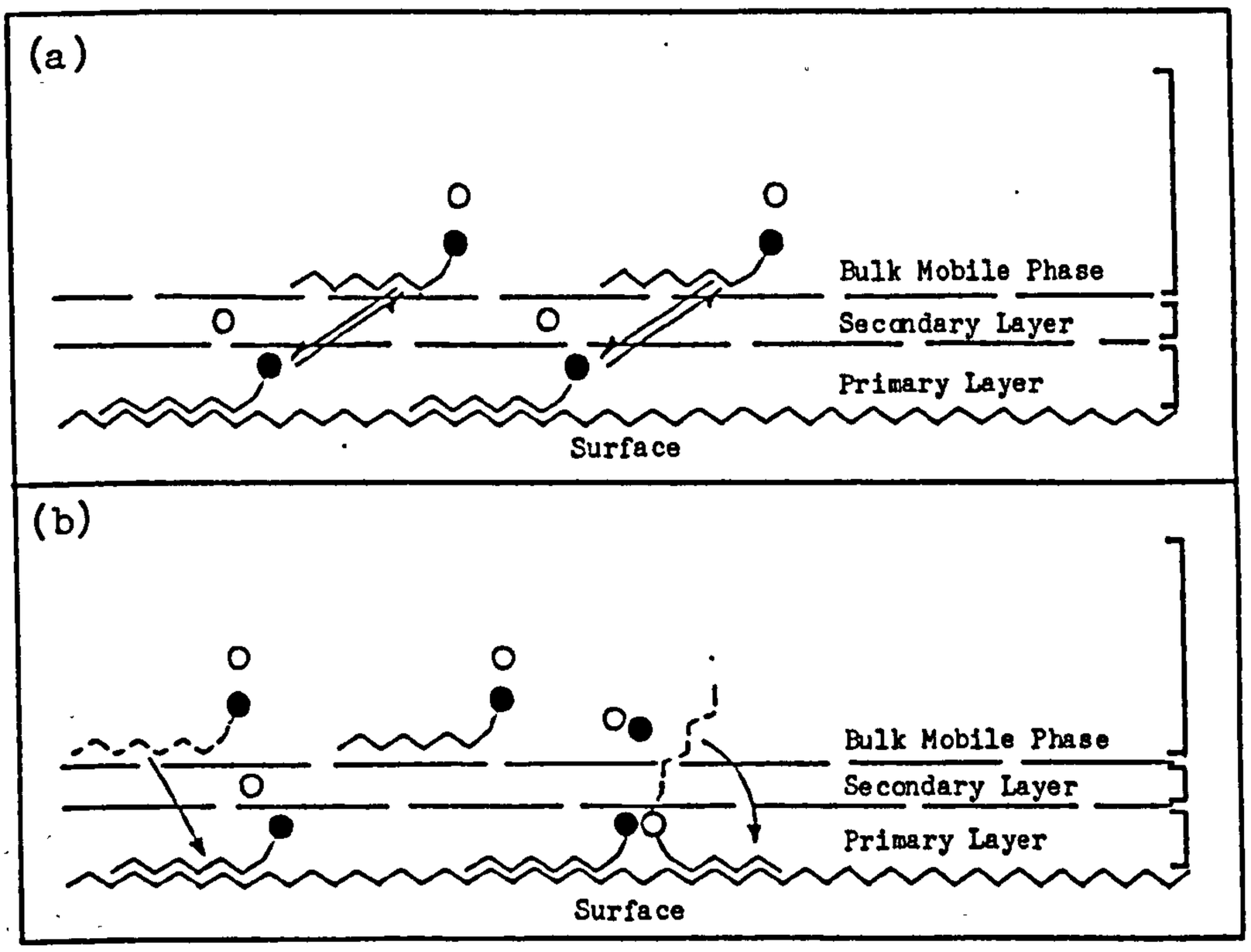
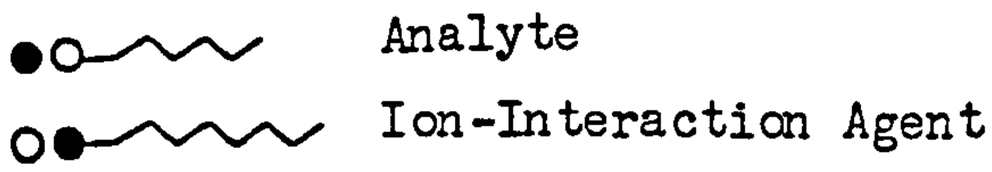


Figure 3.6 The Ion-Interaction Model :
(a) Double Layer Formation, (b) Retention



The third postulate, that of ion-interaction, assumes dynamic equilibrium of the lipophilic counter-ion resulting in the formation of an electrical double layer on the surface. The retention of an ionised solute results from an electrostatic force due to the surface charge density provided by the counter ion and from an additional "sorption" effect onto the non-polar surface. The two stages of this proposed mechanism are presented in Figure 3.6.

In actuality, the true mechanism most probably encompasses aspects of all three processes which undoubtedly will be further complicated by adsorption, micelle formation, and complexation of both the solute and the IPA. In view of the ambiguity associated with the mechanistic modelling of this chromatographic technique the terms 'ion-pairing agent' and 'reverse phase ion-pair chromatography' are misleading. It would be more appropriate to use more general terms such as ion-interaction agent (IIA) and reverse phase ion-interaction chromatography (RP-IIC). This latter nomenclature is adopted in this dissertation.

The literature is replete with examples of the application of RP-IIC to the separation of ionised and non-ionised solutes. The current status of the technique is similarly well documented.[31,313-315] The reader is referred to these sources for a more extensive review of the subject.

3.1.4.4 Ion-Exchange Chromatography

Ion-exchange chromatography (IXC) has been utilised traditionally for decades for the separation of ions and was the first of the various LC modes to be employed widely under HPLC conditions. However, for practical reasons, IXC was largely superseded by RP-IIC until 1975 when Small et al.[117]

developed a procedure called "Ion Chromatography" which overcame many of the early problems and enabled IXC to re-establish itself.

IXC is conducted on a stationary phase comprising a polymer matrix (an organic resin or silica) onto the surface of which are permanently bonded ionogenic functional groups. There are four types of ion-exchangers available and these are classified by the charge of the ions that may be exchanged on the ionised stationary phase surface and by the dissociation constant of the ionogenic functional group. The four categories of ion-exchanger are indicated in Table 3.4 below.

Table 3.4 Ionogenic Functional Groups on Ion-Exchange Stationary Phases

Class		Ionogenic Group
Strong cation exchanger	(SCX)	$-SO_3H$
Weak cation exchanger	(WCX)	$-CO_2H$
Strong anion exchanger	(SAX)	$-\overset{\oplus}{N}R_3$
Weak anion exchanger	(WAX)	$-\dot{N}H_2$

Strong ion exchangers are fully dissociated over the entire operating pH range (the limits of which depend on the stability of the polymer matrix) whereas for weak ion exchangers the degree of dissociation, and hence the retention properties of the stationary phase, is highly pH dependent. In fact, pH is the most influential variable that may be used to control the

separation in this mode. Ion exchangers are operated with a buffered, largely aqueous mobile phase in order to support ionisation. The ionised exchanger surface has a counter-ion associated with it which may be displaced by sample ions of the same charge. Retention is achieved by this coulombic association process and separation is obtained due to the differences in ability to cause displacement of the associated counter ion. The nature of the retention mechanism limits IXC solely to the separation of ions or ionisable compounds unlike RP-IIC which will also accommodate non-ionised materials.

3.1.5 High Performance Liquid Chromatographic Methods Reported for the Determination of Indoles in Biological Samples

Separations of indolic compounds of biological origin have been accomplished on both ion-exchange and hydrocarbonaceous bonded stationary phases. IXC (e.g., 88, 316-319) has rarely exhibited high efficiency. Furthermore, employment of conventional pellicular ion-exchange resins (d_p = 30-50µm) generally excludes the simultaneous determination of amine, amino-acid and acid metabolites[320] because of the widely differing polarities of these analytes.[305] Only when anion and cation exchangers are connected in series (e.g., 86) is it possible to determine these compound types concurrently by IXC, but restrictions are imposed upon the mobile phase composition and the flow rate which invariably results in significantly extended run times. Considerably greater success has been achieved by the utilisation of reverse-phase materials, especially ODS, to the extent that the versatile RPLC mode, with or without the inclusion of an IIA, is now widely accepted as the separation technique of choice for the determination of biogenic indolic substances.

In addition to the selectivity achievable chromatographically, further discrimination can be attained by judicious choice of the detection technique. UV, FL and EC detectors have all been applied to indole "visualisation" following RPLC. A few methods have employed two detectors in series (e.g., 303, 321-323) in order to enhance selectivity still further and to establish peak purity. The use of two detectors in itself adds increased complexity and expense to the analytical method, which is compounded by the need for extra recorder and/or integrator facilities. This level of instrumentation is considered impractical in many hospital clinical laboratories.

Of the discrete detection techniques, UV monitoring is not sufficiently selective at the wavelengths applicable to indoles (e.g., 324) and furthermore, it is fairly insensitive. Consequently, UV detection is deemed to be unsuitable for trace analysis of biological samples.[325] Detection of indoles by native FL has been widely exploited (e.g., 324, 326-331) and has been found to be both more sensitive and more selective than UV absorption.[324,326] Subsequently other workers have enhanced this fluorescence either by pre-column (e.g., 332) or post-column (e.g., 333) derivatisation techniques. However, derivatisation procedures require a chemical transformation stage that not only provides another source of error, but also increases analysis time.[334] Furthermore, FL procedures in general may be compromised by background fluorescence, fluorescence quenching and a variable relative response.[335] Hence, FL detection is not without its problems but this technique is still used fairly frequently for routine indole determinations.

However, it is amperometric EC detection which is regarded by many chromatographers as the technique of choice for the subject determination.[310]

Sensitivity is generally equal to or superior to that obtained from FL detection.[327,336-338] Detection limits for TP and the 5-hydroxyindoles in the low pg region have been reported.[320,335,339,340] Although some problems have been experienced with the operation of ECDs in practice (see Chapter 2, Section 2.1.2), these minor difficulties are usually of little consequence with modern instruments employed for routine analysis. A selection of recently published applications of RPLC-EC to the determination of biogenic indolic substances in biological specimens is presented in Table 3.5. This compilation is by no means complete, the few references cited here represent only a small fraction of the vast literature concerning this group of compounds.

RPLC-EC is capable of high selectivity in addition to high sensitivity. Overall method selectivity is influenced by three parameters: (1) the efficiency of the chromatographic separation, (2) the discrimination possible with the detection technique, and (3) a combination of the nature of the sample and the complexity of pre-injection clean-up procedures. A trade-off exists between these three parameters and the maximum total analysis time deemed to be acceptable. The high discriminating power of the ECD is of great advantage here, generally enabling the extent of sample manipulation necessary for trouble-free analysis to be curtailed to manageable proportions. Lengthy, multi-stage extraction procedures prior to chromatography are undesirable with respect to analysis time, analyte losses (sensitivity) and method precision. Some early methods for indole determination relied upon extensive sample work-up including pre-fractionation of the analytes such that each analyte-containing fraction was chromatographed separately.[338,341,343] This approach is clearly unsatisfactory for routine analytical work. More recent studies have utilised the inherent capabilities of the HPLC system

Table 3.5 Selected Applications of RPLC-EC to the Determination of Biogenic Indoles in Biological Tissues and Fluids Published to December 1984

Indoles Determined	Sample Type(s)*	LC Mode	LC Column(s)	Operating Potential (V)	Remarks	Ref.
TP, 5HT, 5HIAA	TH, csf, wb, pl, ur	RP-PC	ODS	+0.50, +1.00 (TP)	Indoles chromatographed separately after extractive pre-separation	341
TP, 5HIAA	csf	RP-PC	ODS	+0.80	FL in series	336
TP, 5HTP, 5HT, 5HIAA, I3S	ur	RP-PC	ODS	+1.20	Gradient elution	334
5HTP, 5HT, 5HIAA	TH	RP-IIC	ODS	+0.80	N -Me-5HT as IS	342
5HT	pl	RP-PC	ODS	+0.50	On-line trace enrichment	337
TP, 5HTP, 5HT, 5HIAA	TH, (ser, ur, sal)†	RP-PC	RP resin	+0.90		320
5HT, 5HIAA	TH, csf	RP-PC	ODS	+0.50	On-line trace enrichment, indoles chromatographed separately	338
5HT, 5HIAA	TH	RP-PC	ODS	+0.50	Indoles chromatographed separately after extractive pre-separation	343
(5HTP), 5HT, 5HIAA	TH, (csf)	RP-PC	ODS	+0.70		339
5HT, 5HIAA	TH	RP-IIC	Ph, ODS	+0.72, +0.78	Indoles chromatographed separately after extractive pre-separation	344
5HTP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.45		335
TP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.50	FL in series	321
5HTP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.50		340
TP, 5HTP, 5HT, 5HIAA	TH, csf, pl	RP-IIC	ODS	+0.60, +0.88 (TP)		345
TP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.70	FL in series	322
5HTP, 5HT, 5HIAA	TH	RP-IIC	ODS	+0.75	N -Me-5HT as IS	346
5HT, 5HIAA	TH	RP-PC	ODS	+0.50		347
5HIAA	TH	RP-PC	ODS	+0.70	FL in series	303
5HTP, 5HT, 5HIAA	csf, pl, ur	RP-PC	ODS	+0.50	6-F-5HT and 5HI2A as ISs	348
TP, 5HT, 5HIAA	TH	RP-IIC	ODS	+0.40-0.60, +0.80-1.20 (TP)		349
5HT, 5HIAA	TH, csf	RP-PC	ODS	+0.70		350
TP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.60, +0.70	Amines chromatographed separately from 5HIAA after extractive pre-separation	351
TP, 5HTP, 5HT 5HIAA	TH, csf, ser	RP-IIC	ODS, C_8	+0.90		352
5HTP, 5HT, 5HIAA	TH, csf, PRP, plt	RP-PC	ODS	+0.55, +0.65, +0.75	5HI as IS	353
5HT, 5HIAA	PPP	RP-PC	ODS	+0.55		354, 355
5HTP, 5HT, 5HIAA	TH	RP-IIC	ODS	+0.85 & -1.50	Dual ECD	139

Continued .....

Table 3.5 continued

Indoles Determined	Sample Type(s)*	LC Mode	LC Column(s)	Operating Potential (V)	Remarks	Refs.
5HT, 5HIAA	TH	RP-IIC	ODS	+0.72		356
TP, 5HTP, 5HT, 5HIAA	TH, ur	RP-IIC	Modified ODS	+0.80	ODS modified with n-Bu_3PO_4, FL also used	325
5HIAA	csf	RP-PC	ODS	+0.80		357
5HT, 5HIAA	TH	RP-IIC	ODS	+0.70		358
TP, 5HTP, 5HT, 5HIAA	csf	RP-IIC	Ph	+0.74	α-Me 5HT as IS	359
5HTP, 5HT, 5HIAA	ur	RP-IIC	ODS	+0.72	Column switching system, FL in series	323
5HTP, 5HT, 5HIAA	TH, csf	RP-PC	ODS (x 2), C_8	+0.70	Step gradient elution, column switching system	115
TP, 5HTP, 5HT, 5HIAA	ser	RP-PC	ODS	+1.00		360
5HT, 5HIAA	TH	RP-IIC	ODS	+0.80		361
TP, 5HTP, 5HT, 5HIAA	TH	RP-PC	ODS	+0.55-0.73	Pre-separation of analytes by complex extraction, chromatographed on 4 HPLC systems	362
5HIAA	pl	RP-PC	ODS	+0.60 & +0.75	Dual ECD, 5HI2A as IS	363
TP, 5HT, 5HIAA	TH, csf	RP-IIC	ODS (x 3)	+0.75	NBLC (2.1mm ID), FL in series	305
TP, 5HT, 5HIAA	TH	RP-IIC	ODS	+0.80		364
(TP, 5HTP, 5HT), 5HIAA	pl	RP-PC	ODS	+0.70	Dual ECD	365
5HTP, 5HT, 5HIAA	TH	RP-IIC	ODS (x 2)	+0.65-0.70, +0.90	Two HPLC systems. N -Me-5HT as IS	366
5HT	csf, PRP PPP, amf	RP-PC	ODS	+0.65	6HT as IS	367

* TH = tissue homogenate, csf = cerebrospinal fluid, wb = whole blood, ser = serum, pl = plasma, PRP = platelet-rich plasma, PPP = platelet-poor plasma, plt = platelets, ur = urine, sal = saliva, amf = amniotic fluid.

† Parentheses indicate author's claims which are not demonstrated in the cited publication.

to a much fuller extent and have performed acceptable chromatography with very little sample treatment and no pre-injection fractionation of the analytes (e.g., 345). A detailed discussion of the need for clean-up of biological samples prior to injection and the means by which this task may be performed is given in Part IV of this Chapter.

The majority of studies of TP, 5HTP, 5HT, 5HIAA, I3S and related compounds have been carried out on account of the neurochemical significance of these substances. Consequently, many applications concern the analysis of nervous tissue (especially brain); applications which have been developed for, and utilised extensively in, neurological and psychiatric investigations. Csf has also been studied in this regard. Methods also exist for the analysis of urine, principally for 5HIAA content as an indicator of the presence of carcinoid tumours. In addition, analyses of whole blood and various blood fractions (i.e., serum, platelet-rich plasma (PRP), platelet-poor plasma (PPP) and platelets themselves) have been developed and a few investigators have also examined saliva and amniotic fluid for endogenous indolic compounds.

It should be noted that the literature is replete with publications describing the separation and quantitation of one or more 5-hydroxyindoles, occasionally including TP too, and often in conjunction with endogenous catecholamine compounds. However, no application of RPLC-EC in which I3S was resolved and quantitated in addition to TP and its three major 5-hydroxy pathway metabolites was evident from a literature search conducted at the outset of this project. In fact, papers describing the HPLC determination of I3S in biological samples were very few in number. One RPLC-FL method which enables the simultaneous measurement of TP, 5HIAA and I3S in serum and haemodialysates from uraemic patients was reported

recently.[368] I3S appears to be determined almost exclusively by semi-quantitative TLC methods in many clinical laboratories, including those in local hospitals.

3.1.6 Current Practices in Local Hospitals

In spite of the proclaimed advantages of applying HPLC procedures to the determination of TP and its indole metabolites in biological samples this technique is not currently employed in local hospitals. The Bristol Royal Infirmary (BRI), the Bristol Maternity Hospital (BMH), and the Taunton & Somerset Hospital, Musgrove Park Branch (T&SH) use two methods to determine urinary constituents of this type. The first is specifically for the determination of 5HIAA and is a colorimetric procedure reported in 1973 by Goldenberg[369], which is itself an adaption of an earlier method published by Udenfriend and associates.[370] Secondly, a TLC procedure for the determination of TP, 5HTP, 5HT, 5HIAA, I3S and three other indole compounds adapted from a paper which appeared in 1968 by Berry et al.[371] is also applied. Both methods examine gross elevations of TP indolic metabolites in 24 hour urine specimens. The former approach, apart from lacking in sensitivity, is suspect regarding its specificity towards 5HIAA.[372,373] The second procedure resolves the analytes adequately but sensitivity is a major problem, especially with respect to 5HTP and 5HT which are not detectable at their normal levels in urine. In addition, the aforementioned problem with TLC regarding accurate quantitation of the spots on the plate gives cause for concern.

Currently only 24 hour urine specimens are analysed by these methods. Because of the difficulties in detection neither procedure is sufficiently

sensitive to enable detection of diminished levels of analyte species which occur in several clinical conditions of importance (see Section 3.1.2). Blood samples and fractions thereof (e.g., serum, PRP, PPP, platelets) contain considerably lower concentrations of many of these analytes than does urine ; consequently neither method is satisfactory for the analysis of these types of sample either. Where sample volume is highly restricted in addition clearly a new method is required.

There are several reasons why an HPLC procedure has not yet been adopted in the aforementioned institutions. First, the limited amount of necessary instrumentation is heavily committed to performing other tasks. Secondly, the cost of analysis by HPLC is greater than either colorimetry or TLC. Finally, few of the staff have sufficient expertise with HPLC equipment to be able to troubleshoot effectively. However, HPLC is now being introduced more into these laboratories. In recent years HPLC has largely supplanted colorimetry, fluorimetry and TLC for the determination of urinary phenolic acids and catecholamines, and has enabled plasma catecholamines to be quantitated.

Ideally, what is required for the determination of urinary or blood indole levels is a relatively simple HPLC procedure which is sufficiently sensitive, discriminatory, rapid, reliable, cost effective and of great enough worth to justify the acquisition of more instrumentation and an increase in staff training. This requirement is addressed herein.

3.1.7 Problems Specific to the Determination of Trace Level Constituents of Biological Fluids of Neonates, Infants and Young Children

The potential utility of a knowledge of indolic TP metabolite levels in biological fluids as an aid to the diagnosis of various debilitating and

sometimes life-threatening diseases suffered by the title subject group has already been proclaimed (see Section 3.1.2). It is desirable to screen individuals in an effective way in order to detect any abnormality at the earliest opportunity. This enables prompt action to be taken (i.e., chemotherapeutic, surgical, dietary control, etc.) in order to minimise the effects of the abnormality, e.g., mental retardation, or in certain cases to prevent infant fatality.

The principal decision to be taken from the clinical point of view is which biological fluids to analyse for TP and its indolic metabolites. Csf, urine and blood (or particular blood fractions) are all pertinent to the clinical conditions under investigation to various extents. Csf indole levels are useful where mental disturbances are involved but this sample medium is not necessarily a satisfactory indicator of other derangements. The acquisition of a csf sample requires epidural puncture which has a certain risk associated with it, hence csf is not very accessible and is deemed to be unsuitable for routine screening purposes.

Urine and blood are both more readily obtainable and are of greater applicability to the detection of TP metabolism abnormalities than is csf. However, in very young individuals such as those of particular interest here, urine collection is problematical. Generally, it is necessary to obtain the complete urine output over a lengthy time period (usually 24 hours) in order to take account of normal diurnal fluctuations of excreted analyte levels. Routine collection of the total urinary output over such a time span from neonates, infants and young children who are not yet toilet-trained is extremely difficult[268], primarily because of the incontinence of these subjects. Complete amassment of this urine output from nappies is not feasible and catheterisation (a process whereby a flexible plastic tube

is inserted into the urethra in order to drain the accumulating urine into an attached plastic collection bag) is undesirable since it may introduce infection and is not justifiable on a routine basis.

Unlike urine acquisition, blood sampling is simple and is already performed on all new-borns delivered in the BMH. Blood is obtained by making a heel prick with a lancet. This sample is utilised for metabolic screening for evidence of two harmful clinical conditions, viz. phenylketonuria which is assessed by fluorescence measurement of phenylalanine levels, and hypothyroidism which is investigated by RIA techniques for thyroxine (T_4) and thyrotropin (thyroid-stimulating hormone, TSH). Because blood is already sampled routinely from the subject group this medium is considered to be the most readily accessible for the determination of endogenous indolic compounds.

The decision to examine blood brings with it two fundamental obstacles that must be surmounted. First the question of whether to quantitate levels of TP, 5HTP, 5HT, 5HIAA and I3S in whole blood, serum, PRP, PPP or platelets alone has to be addressed. Whole blood is not conducive to HPLC analysis without treatment involving removal of blood cells yielding either serum or plasma. The estimation of 5HT in serum has been shown to be compromised by variability in the fraction of platelet-bound 5HT reaching the serum on clotting[374], therefore serum is an inappropriate medium for analysis. Controversy exists concerning the physiological significance of free 5HT in plasma (determined practically in PPP). Currently it is uncertain whether 5HT concentrations measured in PPP are a genuine reflection of freely circulating 5HT levels in the blood stream or merely an artefact of platelet lysis during sample collection and preparation.[375] In view of these comments, the decision was taken to analyse both PRP and PPP

for indolic TP metabolites in order to determine which sample type is of greatest worth diagnostically.

The second point to note is that normal concentrations of the analytes are generally very low in whole blood or blood fractions as is demonstrated in Table 3.6. It should be noted that available information is both scant and variable, most probably as a direct result of unsatisfactory methodology (which yields variable and uncompensated for analyte losses by decomposition and/or in sample manipulation, analytical interferences, etc.) and insufficient sample numbers for valid statistical evaluation. Furthermore, normal reference ranges specific to newborns, neonates, infants and children have not been established in many cases. The major changes in blood composition which are known to take place over the first few years of life, and particularly in the first few days, may have deterred experimenters from examining this difficult area in detail. However, the general agreement regarding the order of magnitude of concentration of the individual indoles in the blood fractions of interest (sub-$\mu g ml^{-1}$ in most cases) means that a highly sensitive analytical method (i.e. with extremely low LODs) is required to enable quantitation with differentiation between analyte contents which are in these general ranges and values which lie outside these ranges, both to higher and lower extremes. The problem of analytical sensitivity is further compounded by the sample size impediment associated with neonates, infants and young children. Since the total blood volume of a full-term new-born baby weighing 3kg is only of the order of 275ml *cf.* *ca.* 5.3 l for a 70kg adult, the amount of blood that may safely be taken for analysis is correspondingly small. In paediatric work the acquisition of sufficient blood (*ca.* 1.5ml) to yield 500µl of serum or plasma is considered to be a relatively large volume.[381] Hence, taking into account requirements for

Table 3.6 Normal Reference Ranges for Tryptophan and Selected Metabolites in Biological Fluids

(All figures are for adults unless stated otherwise. Ranges are given as either extreme values or mean ± SD except where indicated by an asterisk where mean ± SEM is quoted)

Biological Fluid	Indole				
	TP	5HTP	5HT	5HIAA	I3S
Whole Blood			186 ± 12* ngml^{-1} [375] 168 ± 13* ngml^{-1} [375] 337 ± 40ng/10^9 plts [375] 80-250ngml^{-1} [376] 115-440ngml^{-1} (Children) [377]	100-200ngml^{-1} [249]	
Plasma (PRP)	4-30µgml^{-1} [221] 0.43-1.71µgml^{-1} [378] 0.95 ± 0.03µgml^{-1} [378]		213-544ngml^{-1} [331] 127-501ng/10^9 plts [375] 341 ± 27* ng/10^9 plts [375]		
Plasma (PPP)			0-29ngml^{-1} [231] 0.9-2.5ngml^{-1} [295]		
Platelets			570 ± 180ng/10^9 plts [375]		
Serum	11.0 ± 1.3µgml^{-1} [378] 8.8 ± 0.4µgml^{-1} [378] 14.9 ± 2.9µgml^{-1} [378] 8.6-21.6µgml^{-1} [378] 6.9-9.5µgml^{-1} (Children) [378] 3.7 ± 2.0µgml^{-1} (Newborns) [378] 14.4 ± 1.9µgml^{-1} Total [379] 1.4 ± 0.4µgml^{-1} Free [379]		68-161ngml^{-1} [295] 46-166ngml^{-1} [331] 44-247ngml^{-1} [375] 70 ± 20ngml^{-1} [375] 210 ± 53ngml^{-1} [375] 144 ± 46ngml^{-1} [318]		770 ± 240ngml^{-1} Total [379] 100 ± 20ngml^{-1} Free [379]
Urine	10.9 ± 3.3mg/g creatinine [378]	0-50µg/24 hrs [376]	30-180µg/24 hrs [376] 46-103µg/g creatinine [331]	2-8mg/24 hrs [376] 2-9mg/24 hrs [249] 7-59mg/g creatinine (Neonates) [380]	4-20mg/24 hrs [241]
Csf	390 ± 73ng/ml^{-1} [378] 163 ± 286ng/ml^{-1} (Children) [378]	0.44-0.88ngml^{-1} [359]	0.36-0.72ngml^{-1} [359]	0.38-0.76ngml^{-1} [359]	

the established screening procedures, as little as 300-500µl of neonate blood is the maximum volume which can be allocated to indole determination. Now, the preference for HPLC-EC in order to determine TP indolic metabolites in biological fluids has been discussed in depth in this introduction. The severe restriction of sample size imposed by the nature of the subject group under study suggests that the potential for increased mass sensitivity available with NBLC-EC compared with that offered by conventional LC-EC would be of considerable value to this application. Hence, the determination of plasma indole levels in neonatal and paediatric patients provides a genuine and worthwhile clinical problem with which to assess the capabilities of the Pye Unicam NBLC-EC system under authentic conditions.

3.2 The Isocratic Narrow-Bore Liquid Chromatography Instrument

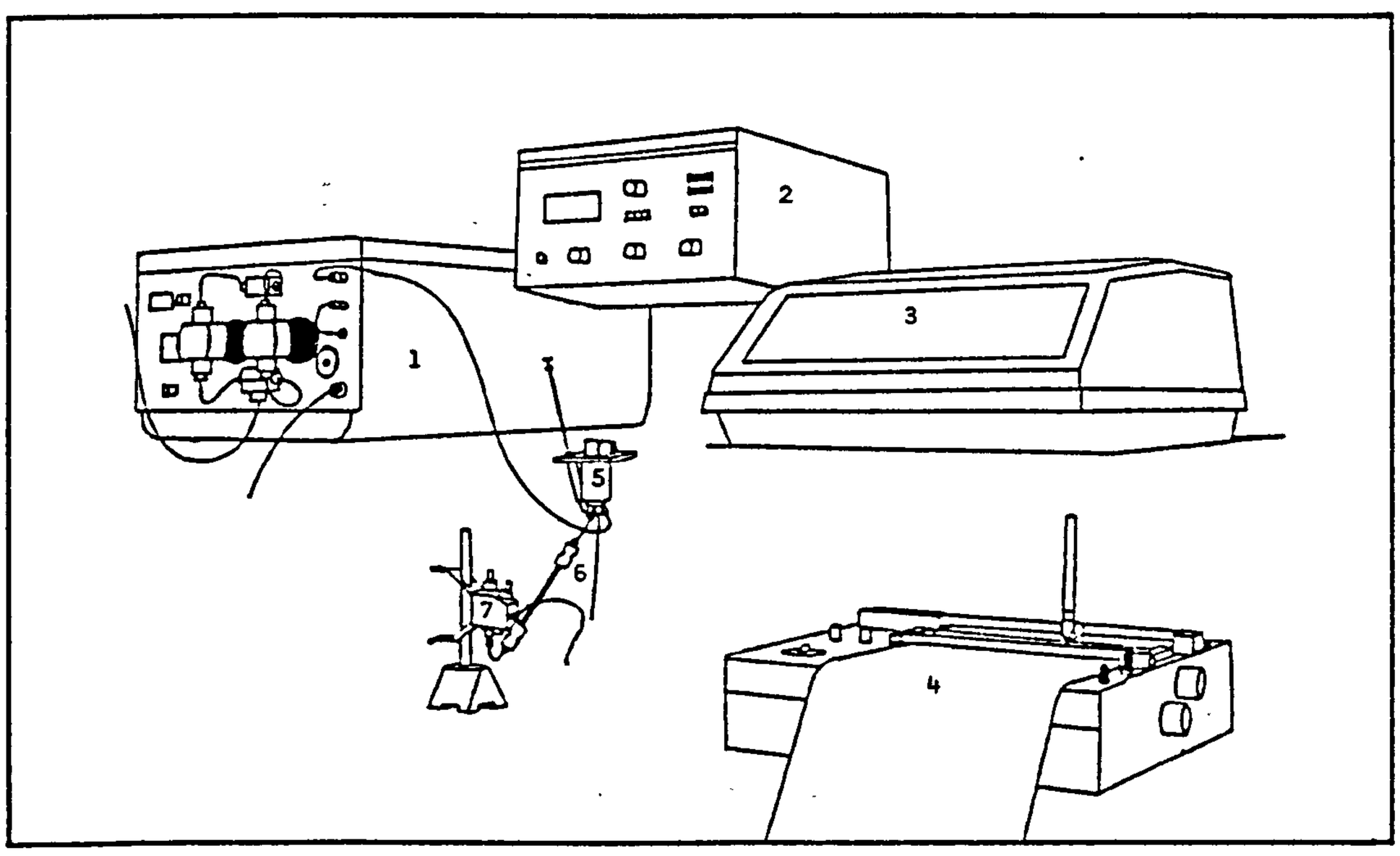
The instrument available for this evaluation study was supplied by Pye Unicam (Cambridge, UK). The apparatus consisted of a PU4010 dual-piston reciprocating pump, a Rheodyne model 7413 injection valve (containing an interchangeable internal loop disc of 0.5, 1.0 and 5.0µl capacity loops) with a Rheodyne model 7012 loop filler port, and a PU4022 wall-jet electrochemical detector comprising a 0.5µl internal volume flow cell and an electronic controller unit. A PU4810 electronic computing integrator was also supplied. In addition, a Servoscribe model 1s potentiometric recorder set at 1V f.s.d. was utilised for pictorial presentation of the detector output.

The entire apparatus incorporating a 10cm x 2.1mm ID, $^1/_8$" OD column is pictured in Plate 1. A close-up of the "business" part of the apparatus, i.e., the injector, column and detector flow cell assembly,is pictured in Plate 2.

3.2.1 Connections

Columns were installed by connection to the injection valve and the ECD flow cell with short lengths of 0.15mm (0.006") ID tubing. The connection tube linking the injector and the column inlet was composed of 316 stainless steel (S/S) whereas the connection between the column outlet and the ECD flow cell was fabricated from PTFE tubing which was supplied with the detector. All connecting tubes were carefully squared-off and deburred at both ends and all S/S ferrules were placed at optimum distances from the tubing ends for the appropriate fittings (as illustrated in Figure 3.7) in order to avoid the creation of unnecessary extra-column

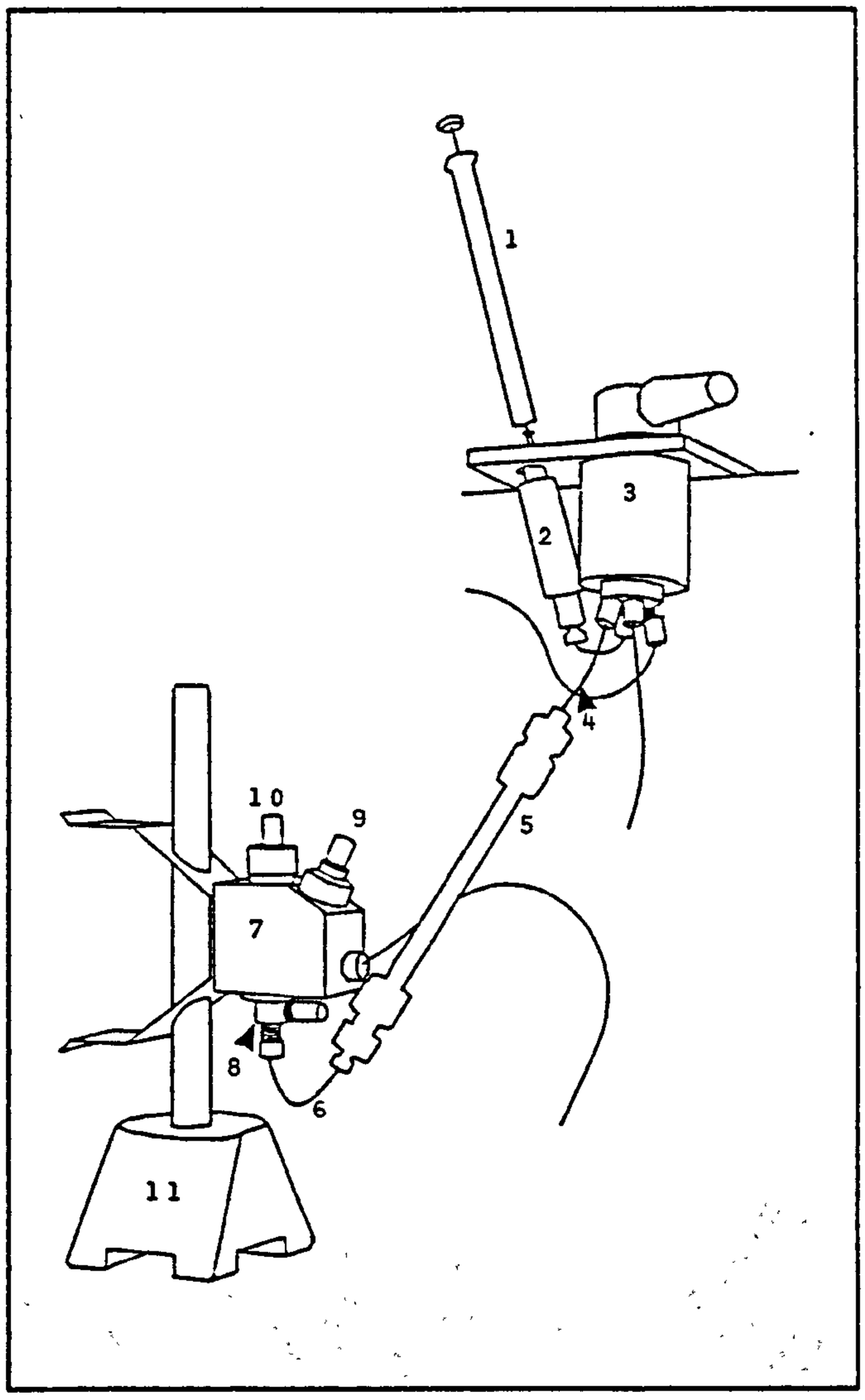
Plate 1 General View of the Pye Unicam Narrow-Bore Liquid Chromatography-Electrochemical Detection Instrument



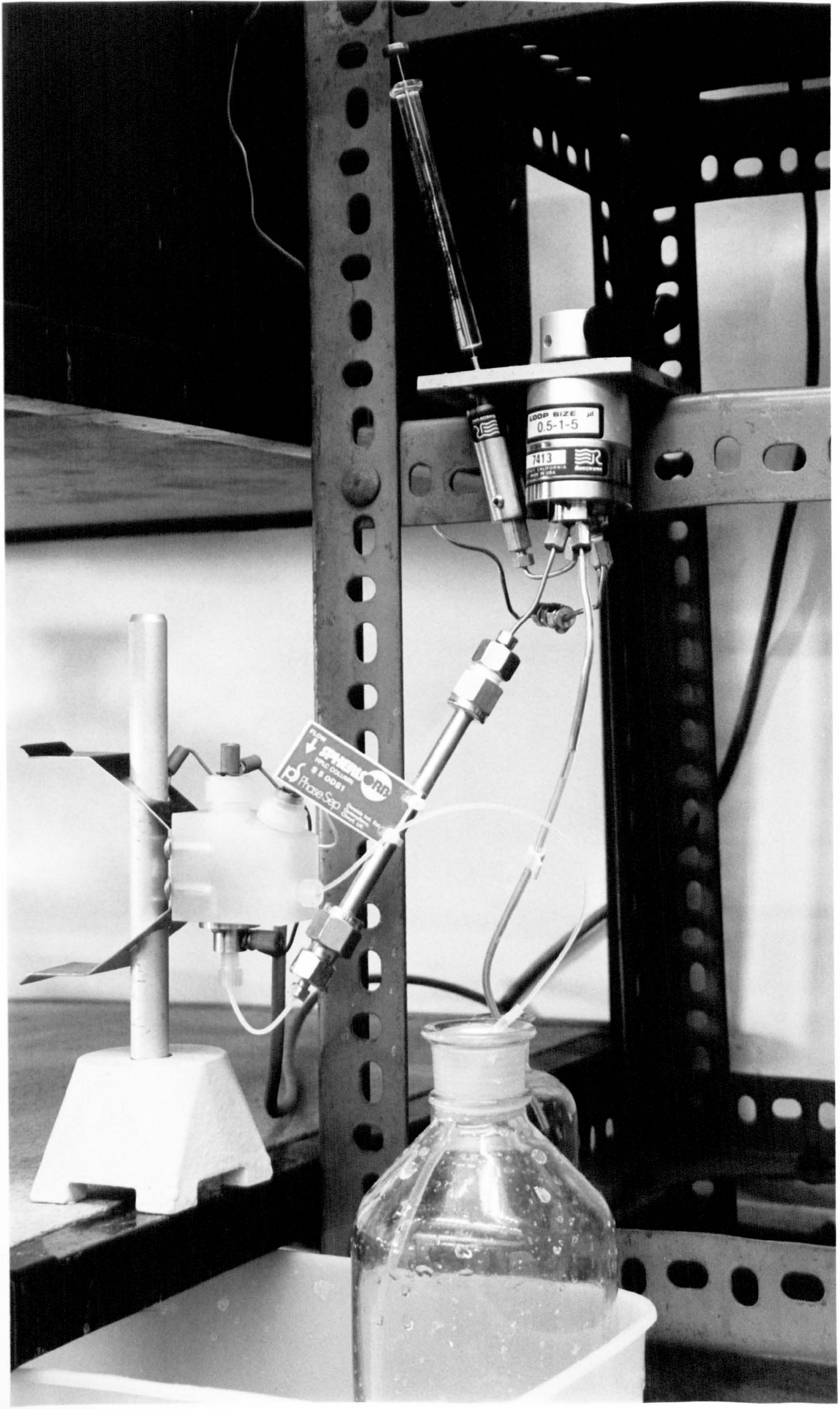
1. PU4010 pump, 2. PU4022 ECD controller, 3. PU4810 electronic computing integrator, 4. Servoscribe model 1s potentiometric recorder, 5. Injection system, 6. Column, 7. PU4022 ECD flow cell.


EXPERIMENT IN PROGRESS
PLEASE LEAVE
PYE UNICAM PU 4070 computing temperature
PHILLIPS
PYE UNICAM PU 4020 electrochemical detector
PHILLIPS
PYE UNICAM PU4070 pump
PHILLIPS

Plate 2 The Narrow-Bore Liquid Chromatograph Injector-Column-Detector Flow Cell Assembly



1. Syringe, 2. Rheodyne 7012 loop filler port, 3. Rheodyne 7413 injection valve, 4. Valve-column inlet connection tube, 5. Column, 6. Column outlet-detector connection tube, 7. PU4022 ECD flow cell, 8. Auxillary electrode (S/S), 9. Ag/AgCl/KCl reference electrode, 10. Glassy carbon working electrode, 11. Flow cell stand.


LOOP SIZE µl
0.5-1-5
7413
SPHERI-ORB
HPLC COLUMN
Phase Sep

dead volume which would consequently lead to undesirable extra-column band broadening.

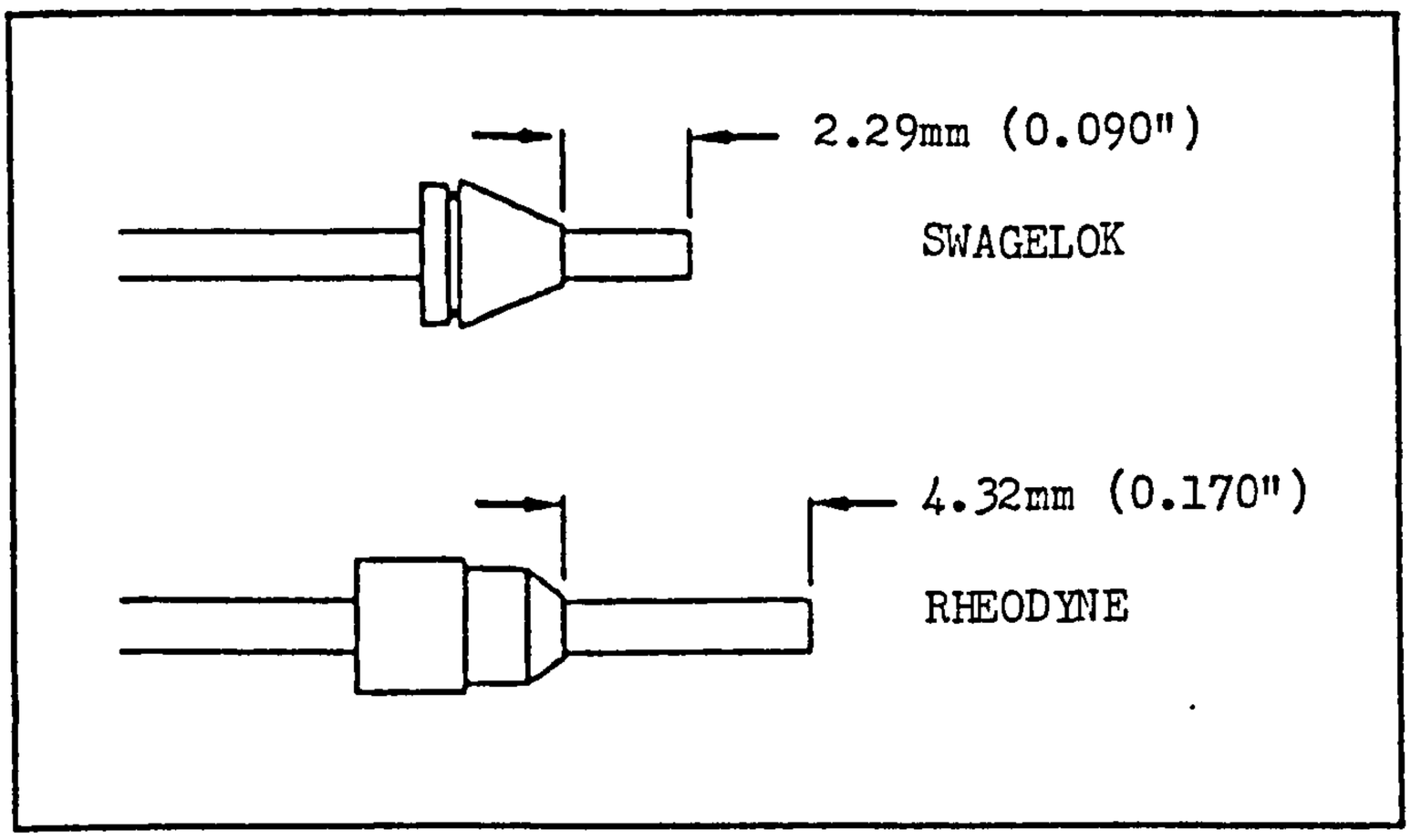


Figure 3.7 Optimum Placement of Ferrules for Connections to Swagelok and Rheodyne Fittings.

3.2.2 The PU4010 Pump[382]

The PU4010 pump operates by means of a dual-piston linear reciprocal pumping mechanism which is cam-driven from a single motor. The instrument is capable of delivering mobile phase at flow rates between $0.1 ml min^{-1}$ ($= 100 \mu l min^{-1}$) and $9.9 ml min^{-1}$ in $0.1 ml min^{-1}$ increments. Each piston chamber has an internal (displacement) volume of 100µl. Back flow is prevented by the presence of check valves stationed in the piston head at the inlet and outlet of each piston chamber. All check valves are protected from particulate matter by integrated porous S/S frits. Back pressure is measured by means of a Bourdon gauge and is displayed in units of bar on the pump facia. A pressure ceiling of ca. 6000p.s.i. (420 bar) is

indicated by the manufacturers. Baseline noise originating directly from pump pulsations is claimed to be minimal. The pump was tested by the manufacturers in their laboratory and was found to be within specification.

3.2.3 The Rheodyne Model 7413 Micro-Injection Valve (with Model 7012 Loop Filler Port)[383,384]

The Rheodyne model 7413 4-port injection valve is capable of measuring and injecting very small volumes in a highly reproducible manner. The valve incorporates three tiny sample loops on a removable disc which is held within the valve body. These internal sample loops have very narrow flow passages (the 0.5µl capacity loop has an ID of only 0.13mm (0.005")) in order to reduce injector-related extra-column band dispersion. The 3-loop disc supplied with the 7413, which is illustrated in Figure 3.8, carries capillaries of 0.5, 1 and 5µl capacities.

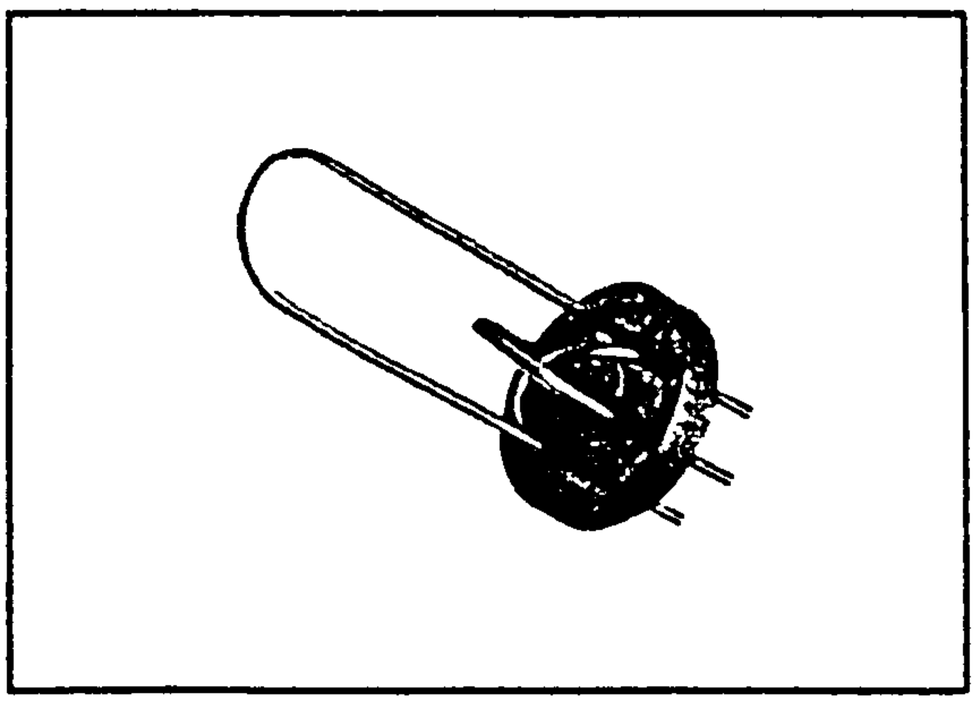


Figure 3.8 The 0.5-1-5µl 3-loop Disc for the Rheodyne Model 7413 Micro-Injection Valve

The loop disc is inserted directly into the rear of the valve rotor seal which is composed of Vespel, a fluoropolymer, which has been levelled and polished to a smooth finish. The rotor butts up against the valve

stator block into which are drilled four very short (<1mm), narrow (0.4mm, 0.016" ID) micro-flow passages. This design enables injector-related extra-column volume, and consequently, extra-column band dispersion to be cut to a minimum. Plate 3 depicts the important components of a partly disassembled Rheodyne 7413 valve. Also shown is a Rheodyne 7012 loop filler port.

The injection valve is claimed to be capable of high volumetric precision (ca. 0.05% RSD) because sample measurement occurs by the complete loop-filling method. An excess of sample is required to fill the loop. The loop is loaded by means of the 7012 loop filler port which, in conjunction with the 9cm x 0.3mm (0.012") ID connecting tube, has an internal volume of about 7µl. Total sample wastage is generally of the order of 10µl per injection taking into account both valve internal volume and exit tube volume. The manner by which sample is introduced into the HPLC flow stream is illustrated with flow diagrams (Figure 3.9). With the valve in

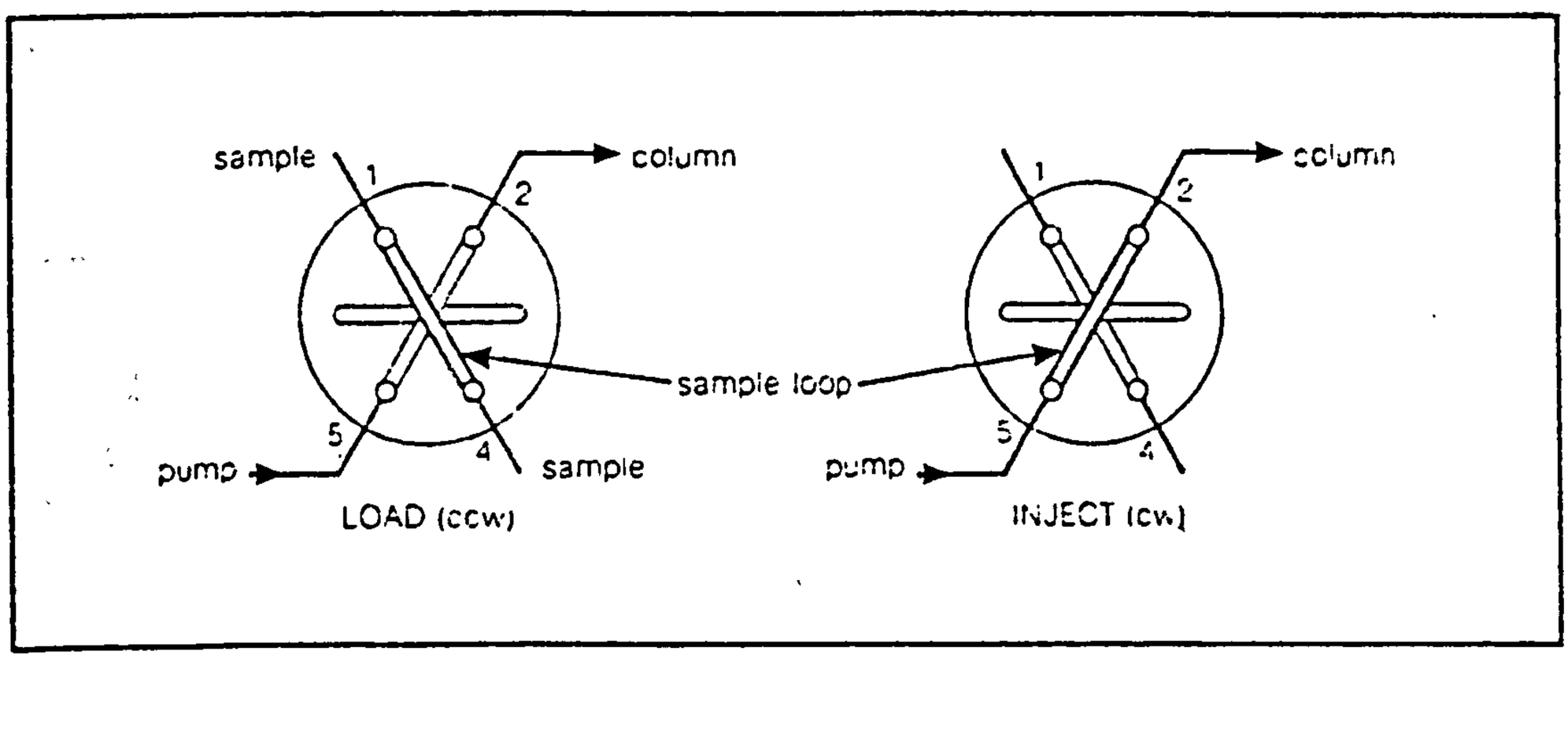
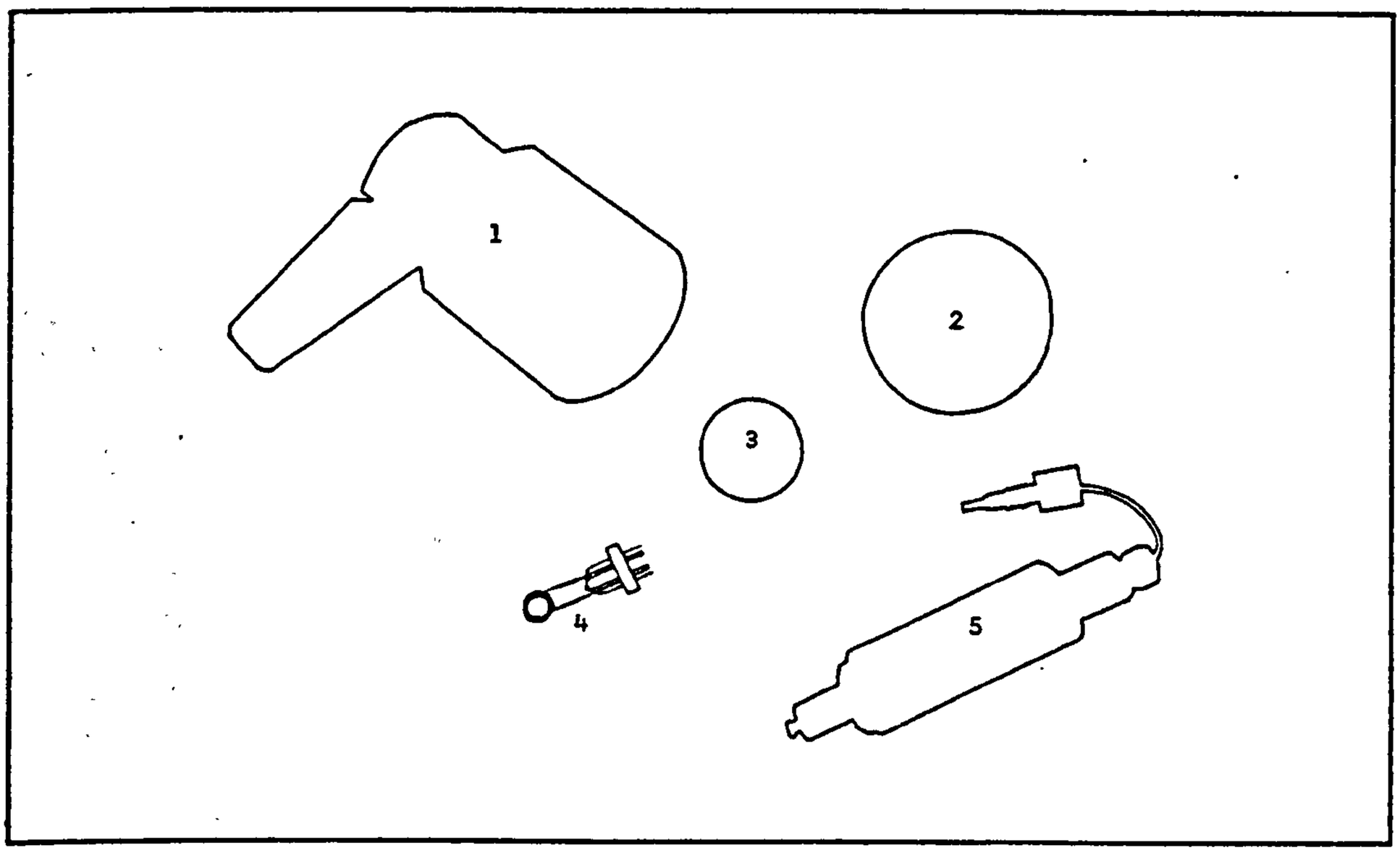


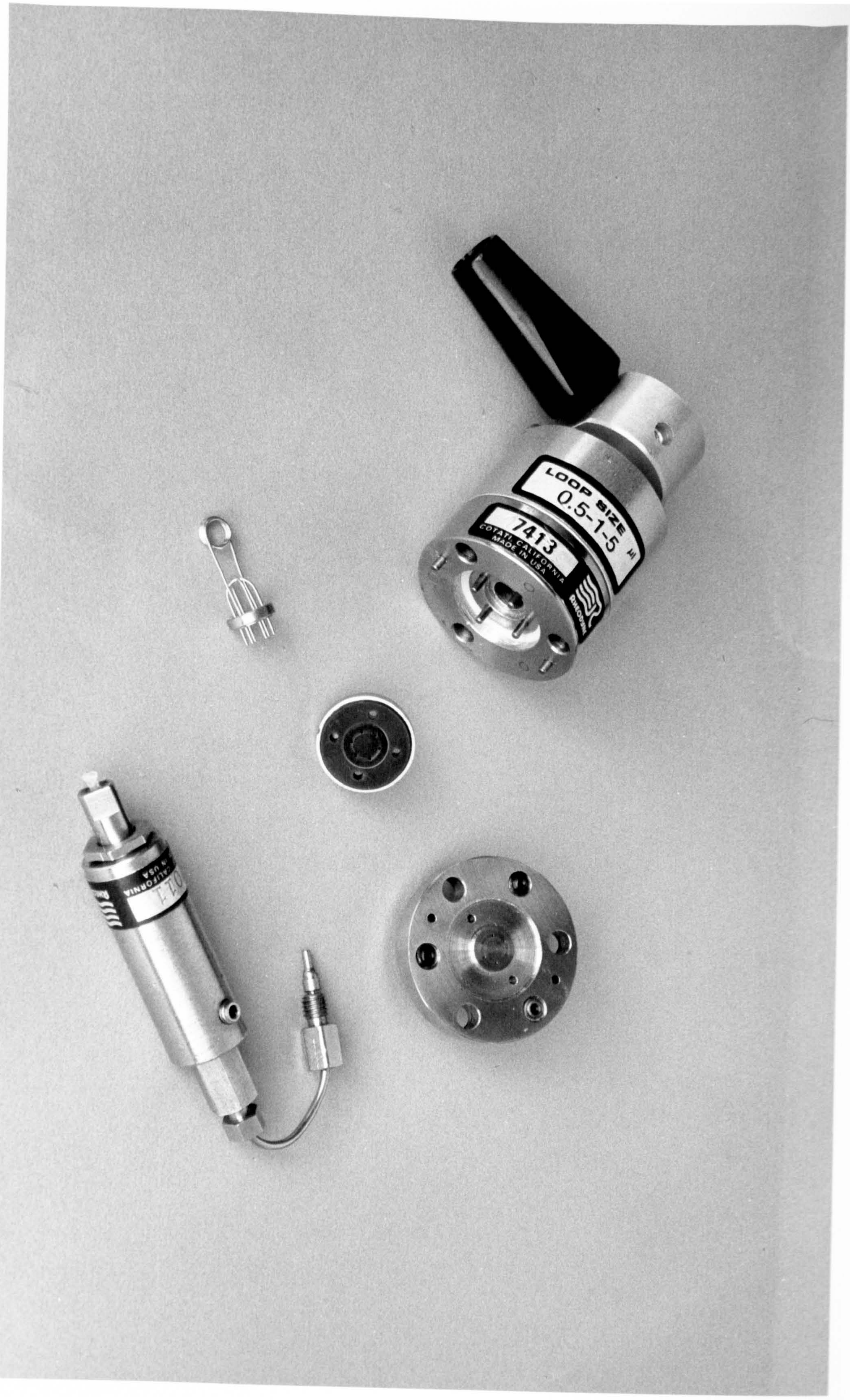
Figure 3.9 Flow Diagram for the Rheodyne Model 7413 Micro-Injection Valve

the LOAD position, sample solution is forced under pressure into the operational sample loop from a suitable syringe via the loop filler port.

Plate 3 Exploded View of the Rheodyne 7413/7012 Injection System



1. Valve body, 2. Stator block, 3. Valve rotor seal, 4. Loop disc, 5. Loop filler port.


LOOP SIZE
0.5-1-5
7413
COTATI, CALIFORNIA
MADE IN USA
CALIFORNIA
IN USA

The mobile phase flow from the pump to the column is carried by one of the other two capillary tubes not in use as the sample loop. A 60° clockwise rotation of the rotor shaft from the LOAD to the INJECT position translocates the sample loop into the pump-column mobile phase flow stream. While the valve is switched to the INJECT position free flow is possible from the sample loading port to waste via the third capillary tube. In order to avoid cross-contamination between consecutive samples it is essential to flush the sample lines with an appropriate solvent both while the valve is in the INJECT position and subsequently when the valve is returned to the LOAD position. The manufacturers state that this injector is capable of leak-free operation at pressures up to 7000 p.s.i. (500 bar).

For this work the injection valve was mounted in a vertical orientation with the four ports downmost in order to minimise the practicable length of the valve-to-column inlet connection tube.

3.2.4 Syringes[385]

The syringe used for sample introduction was a Hamilton # 702 'microliter' micro-syringe (Reno, NV, USA) of 25µl capacity. On this model the needle is fixed, 51mm (2") in length, 0.46mm (0.028") OD (# 22 gauge), and it possesses a square-ended tip. This syringe is recommended for use with the Rheodyne Model 7012 injection port.

In all manipulations of the syringe good practice was followed and care was taken not to handle the syringe barrel. However, because volume measurement was by injection loop and not by the syringe directly it was unlikely that significant errors would occur through temperature-derived sample density changes within the syringe.

The syringe was always cleaned prior to injection. Grasping the syringe by the flange and plunger button only, clean distilled water was drawn into the syringe and then expelled several times. The syringe was then pumped dry before use with sample.

The injection procedure was as follows. The needle was inserted into the sample solution and the plunger was pumped a few times to provide lubrication within the syringe. The syringe was filled to the required point with sample then was inverted and tapped gently to exclude any air bubbles. The needle was blotted dry using a tissue then the sample was drawn back into the syringe barrel leaving an air gap of about 5µl. The needle was inserted into the injection port and the syringe contents were discharged into the injection loop until the preceeding air bubble had emerged fully from the sample vent port of the valve. This can best be observed if a very short S/S tube extended with a transparent plastic tube is fitted to the valve sample vent port. The valve was switched to insert the sample loop into the flow stream.

Syringes require regular maintenance to ensure trouble-free operation. If the withdrawn plunger was handled or exposed to dust at any time it was carefully wiped with tissue prior to reinsertion into the barrel in order to avoid problems with the syringe seizing up. Periodically, the syringe was disassembled and was cleansed in warm water and detergent. On the rare occasions when the needle became plugged, the blockage was removed using compressed air or a cleaning wire or by ultrasonication in a suitable solvent or by any combination of these procedures.

3.2.5 The PU4022 Electrochemical Detector[204,205]

The PU4022 ECD has been discussed in some detail in Chapter 2 of this

dissertation. The inert Kel-F flow cell body contains three electrodes. Supplied with the instrument were a glassy carbon working electrode (WE) and a Ag/AgCl/KCl gel reference electrode (RE), along with a S/S auxiliary electrode (AE) which also serves as the solvent inlet. The various components of the PU4022 flow cell are depicted in Plate 4. A diagram showing how the cell parts are assembled is presented in Figure 3.10, while

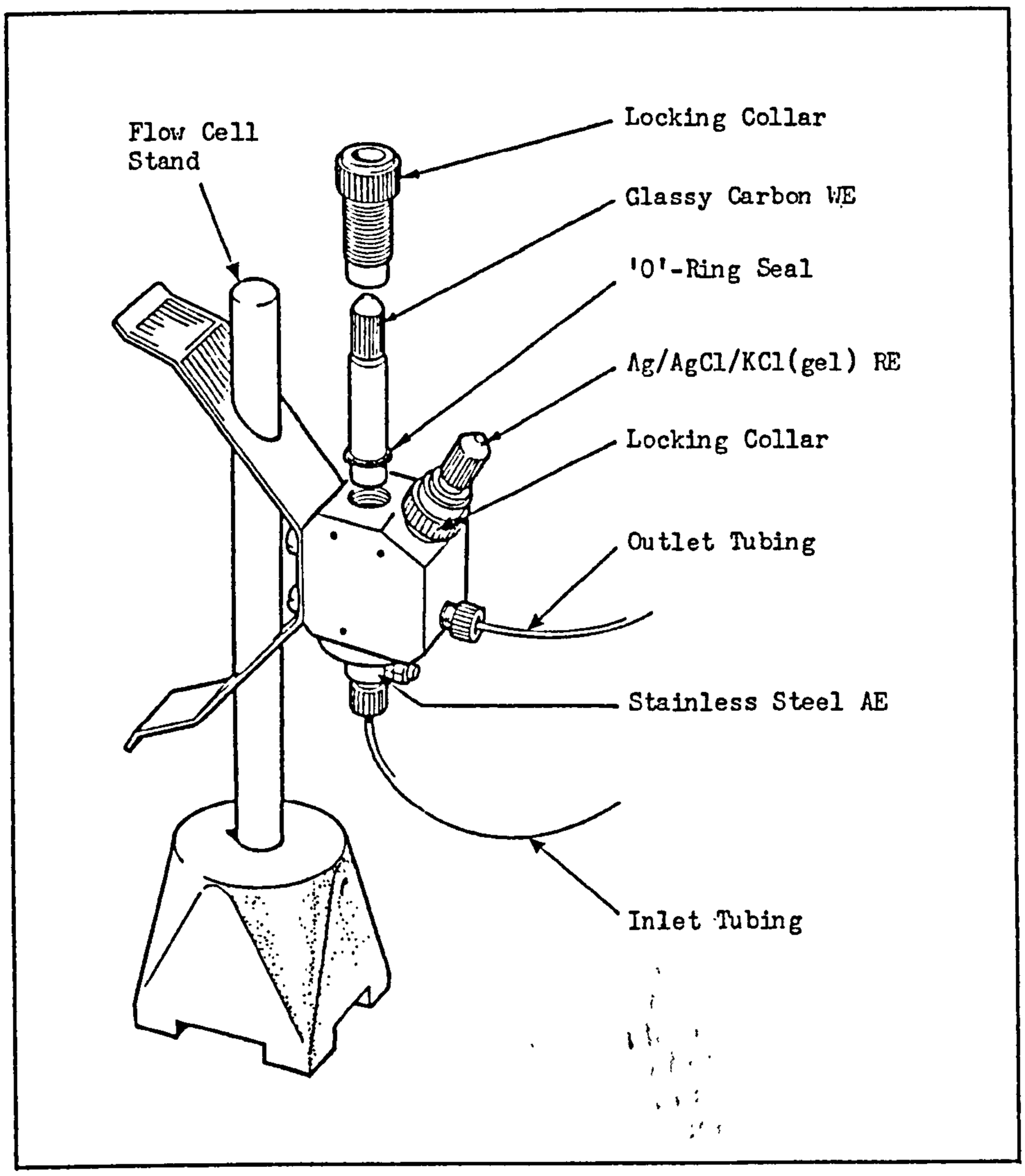
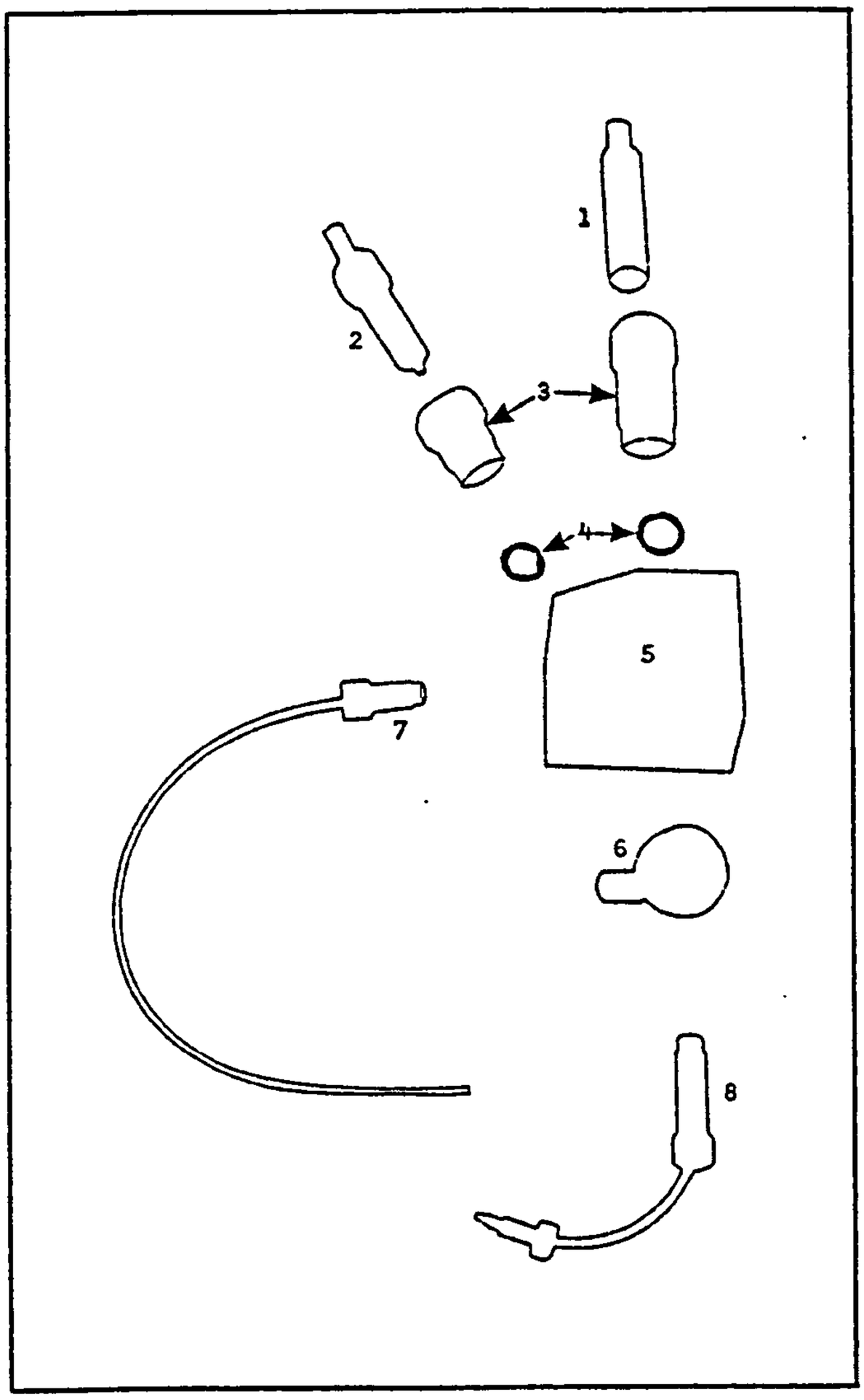
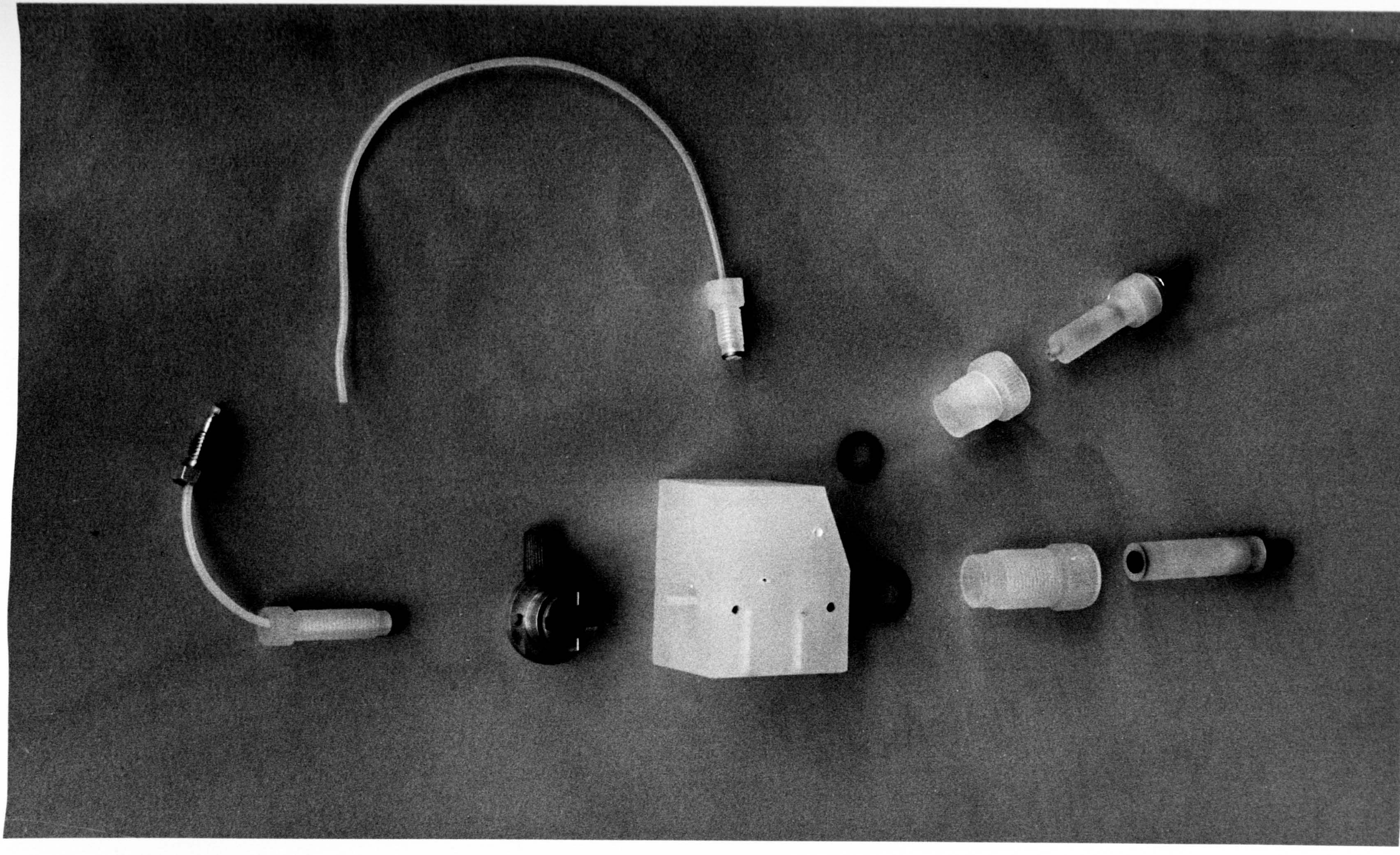


Figure 3.10 The PU4022 Electrochemical Detector Flow Cell (Partially Assembled) - External View Showing Component Parts

Plate 4 Exploded View of the PU4022 Electrochemical Detector Flow Cell Showing its Component Parts



1. Glassy carbon WE, 2. Ag/AgCl/KCl gel RE, 3. Locking collar,
4. O-Ring, 5. Kel-F cell body, 6. Stainless steel AE and inlet assembly,
7. Flow cell outlet tube, 8. Flow cell inlet tube.



a cross-sectional diagram of an identical flow cell (the EDT LCA 13) may be found in Chapter 2 (Figure 2.4).

Those specifications of the detection system that are of greatest relevance to NBLC are the flow cell internal volume, the baseline stability and the detector response time. As has been stated previously, the cell volume of the PU4022 is 0.5µl which is of the order required from a detector for operation with 1-2mm ID columns, (see Section 1.4.3.5). Baseline stability is a feature of the electronics, which the manufacturers state to be "stable and drift-free"[204], and various physical parameters of the system. A practical examination of baseline stability characteristics of this PU4022 detector unit is reported in Chapter 2. Detector response time varies with detector time constant which is programmed into the PU4022 by the operator. A choice of 1,3 or 10secs is available. The longer the time constant is then the greater is the degree of baseline noise smoothing but also the greater is the degree of peak broadening and consummate peak height attenuation. The manufacturers recommend that the time constant setting providing the best *S/N* ratio at the working instrument sensitivity is selected for use. The design of the electronic circuitry of the PU4022 ensures that data point sampling is in phase with the mains period so that mains-derived noise pulsations are rejected.

Further specifications of this instrument may be found in Chapter 2 (Table 2.7).

3.2.6 Ancillary Instrumentation

In addition to the aforementioned Pye Unicam NBLC apparatus other instruments were utilised for part of this research project. These included

a Pye Unicam/Altex LC-XPD model 100 dual-piston reciprocating pump, a Rheodyne model 7010 6-port injection valve (with model 7012 loop filler port), a Metrohm 641-VA electrochemical detector controller (with leads adapted to allow connection to the PU4022 flow cell), and a Hewlett Packard HP3390A electronic reporting integrator.

CHAPTER 3

Part II

Development and Optimisation of a Separation of Selected Indolic Compounds using Columns of Narrow-Bore Geometry

Contents

3.3 Experimental

3.3.1 Solvents and Reagents

The following solvents were all obtained in GPR grade and were single distilled into glass before employment as mobile phase modifiers: methanol (MeOH; from Wilcott Industrial, Bristol, Avon and Charles Tennants, London), propan-2-ol (2-PrOH; from BDH, Poole, Dorset), and tetrahydrofuran (THF, from May & Baker, Dagenham, Essex). Acetonitrile (MeCN, HPLC grade) was supplied by Fisons, Loughborough, Leics. and was used as received for the same purpose. Water was single distilled into polythene before use.

Solvents employed for column packing and repair, viz. MeOH (as above), 2-PrOH (as above), cyclohexanol (BDH, Poole, Dorset) and ethanol (EtOH; from James Burroughs, London) were all purchased in GPR grade and were utilised without further purification.

Acetone (Charles Tennants, London) and diluents of concentrated nitric acid (c.HNO_3; from BDH, Poole, Dorset) were used for the cleaning of glass-ware and other items of hardware.

Buffer components citric acid (HCt, GPR grade), acetic acid (HAc, glacial, GPR grade) sodium acetate (NaAc, anhydrous, AnalaR grade), orthophosphoric acid (H_3PO_4, GPR grade), potassium dihydrogen orthophosphate (KH_2PO_4, anhydrous, GPR grade), sodium hydroxide (NaOH, AnalaR grade) and potassium hydroxide (KOH, AnalaR grade) were all obtained from BDH, Poole, Dorest. Ion interaction agents, PIC-B7 reagent (containing 3.84% w/v 1-heptanesulphonic acid active ingredient in a pH 3.5 aqueous acetate buffer) which was purchased from Waters Associates, Northwich, Cheshire, or 1-heptanesulphonic acid (HSA, sodium salt monohydrate, laboratory reagent grade) which was supplied by BDH, Poole, Dorset, were incorporated as

required. Ethylenediaminetetra-acetic acid (EDTA, disodium salt dihydrate, AnalaR grade), which was also obtained from BDH, Poole, Dorset, was used as received for metal ion complexation.

The indole standard materials, D,L-tryptophan (TP), 5-hydroxy-D,L-tryptophan (5HTP), 5-hydroxytryptaminė (serotonin, 5HT, creatinine sulphate complex), 5-hydroxyindole-3-acetic acid (5HIAA), indoxyl-3-sulphate (urinary indican, I3S, potassium salt) and N,N-dimethyl-5-hydroxytryptamine (bufotenine, BF, mono-oxalate monohydrate complex) were all purchased from Sigma, Poole, Dorset and were of the highest purity available. Indole-3-acetic acid (IAA, GPR grade) and indole (GPR grade) were obtained from BDH.

All substances which were investigated as potential internal standards with the exception of BF were selected off the laboratory shelves. These materials were from various suppliers and were of laboratory reagent grade or better. All compounds were used as received except aniline (AN) which was freshly distilled into glass prior to use.

Helium gas for the deaeration of mobile phases was supplied in cylinders by BOC (Special Gases), Bristol, Avon.

3.3.2 Mobile Phase Preparation (1 litre)

3.3.2.1 General Method

Measured volumes of organic modifier and aqueous buffer salt solution of the selected concentration were combined in a 1 litre volumetric flask in the desired proportions. The solvent mixture was transferred to a 2 litre-capacity beaker. If an IIA was required then an appropriate amount of the chosen substance was weighed out and added at this point. The mixture was stirred to ensure complete dissolution of the IIA. Mobile phase

preparation was completed by adjustment of the solution pH to the required value. This was achieved by addition of a buffer acid, with stirring and with constant pH monitoring by means of a pre-calibrated hand-held portable pH meter (Gallenkamp pH Stick, Model PHK-120-B) which was capable of measuring to ± 0.01 pH units. When a mobile phase with a pH greater than the natural pH of the solvent mixture was required then buffer acid was added as usual until pH 4.00 was reached after which this combination was supplemented with aqueous NaOH or KOH as appropriate until the desired pH value was attained.

Each prepared batch of mobile phase was transferred to a 1 litre-capacity reagent bottle and was then degassed under a gentle stream of He for at least 5 minutes prior to delivery. Degassing was continued throughout the course of the experiments in order to maintain the exclusion of oxygen from the mobile phase. Preferential evaporation of solvent was restricted by plugging the reagent bottle neck around the tubing with lint-free paper towelling.

Mobile phases were stored at room temperature and were prepared freshly each week or more frequently as necessary.

3.3.2.2 Preparation of the Optimised Mobile Phase for RP-IIC of the Subject Indoles on Spherisorb S5 ODS1

MeOH (40ml) was placed in a 1 litre volumetric flask and the volume was made up to the mark with aqueous KH_2PO_4 (0.1M) to provide a 4:96 solvent ratio. The solvent mixture was transferred to a 2-litre capacity beaker and into this solution was dissolved $NaHSA.H_2O$ (246mg ≡ 200mgl^{-1} HSA) to act as an IIA. The natural pH of the resulting combination was ca. 4.63.

The pH was then adjusted to 4.00 by the dropwise addition of aqueous H_3PO_4 (ca. 30% v/v) to yield the optimised mobile phase.

3.3.3 Columns

3.3.3.1 1mm ID Column

A 25cm x 1mm ID, $^1/_8$" OD glass-lined 316 S/S column commercially packed with Partisil 10 ODS3 stationary phase ('micro-B', Whatman, Maidstone, Kent) was supplied by Pye Unicam, Cambridge. The column end-fittings were also composed of 316 S/S and were of the female ZDV Swagelok type illustrated in Figure 1.7 except that these end-fittings contained integral column end frits of 2µm porosity.

3.3.3.2 2mm ID Columns

A series of 2mm ID columns of two basic architectures were utilised. Those of conventional design comprised a 10cm x 2.1mm ID, $^1/_8$" OD internally-polished 316 S/S tube to which were attached similar end-fittings to those of the 1mm ID column except that the frits were not integral to the end-fitting but consisted of the more usual discrete 2µm porosity discs. The other 2mm ID column hardware design was that of Shandon-Southern which is illustrated in Figure 3.11. Columns consisted of either 15cm or 20cm lengths of 2mm ID, $^1/_8$" OD internally-polished 316 S/S tube with ZDV end-fittings individually tailored to each column. Leak-free seals were made by finger tightening each end-fitting against a teflon 'O'-ring. This system format necessitated the use of a ZDV union and an additional length of connecting tubing for attachment to the ECD so introducing a small additional contribution to extra-column band broadening.

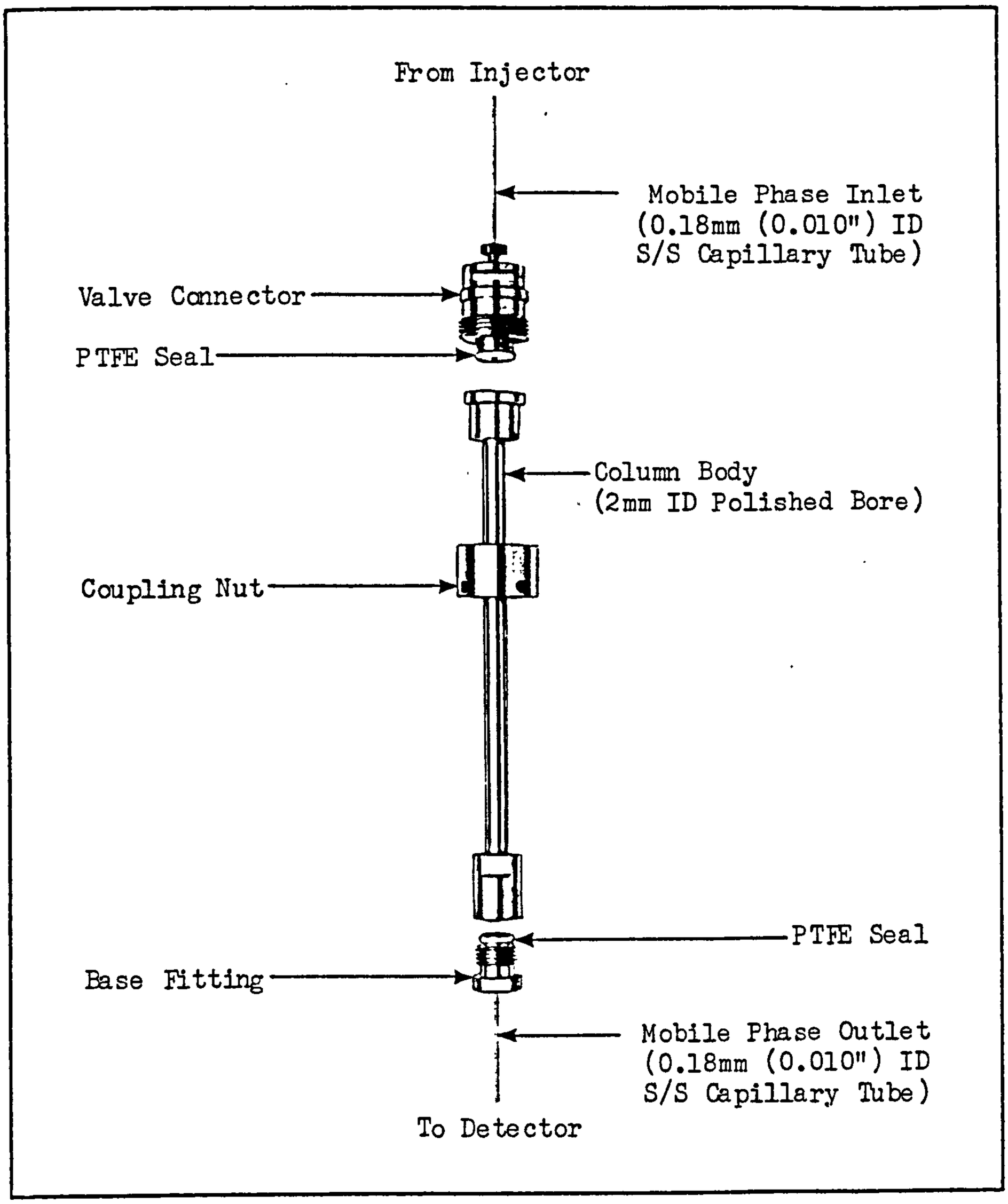


Figure 3.11 Shandon-Southern Column Architecture

For initial experiments only with the 2mm ID column geometry the three Shandon-Southern style columns were employed. These columns were slurry-packed in the laboratory with Hypersil ODS (15cm x 2mm ID), Spherisorb S5 ODS1 (15cm x 2mm ID) and μ-Bondapak ODS (20cm x 2mm ID) stationary phases. Also for initial experiments, a 10cm x 2.1mm ID column of conventional design commerically packed with Spherisorb S5 ODS2 (Phase Sep, Queensferry, Clwyd) was supplied by Pye Unicam.

All other columns utilised in this study were of the conventional design, 10cm long x 2.1mm ID, and were either commercially packed with Spherisorb S5 ODS1 (Phase Sep, Queensferry, Clwyd) and supplied by Pye Unicam or were slurry-packed in the laboratory with fresh stationary phase of identical type and using the same column hardware.

3.3.3.3 Column Packing Procedure

All columns packed in the laboratory were packed by the high pressure balanced-density slurry method outlined in Section 2.2.4. Certain hardware modifications were necessary to ensure successful packing of 2mm ID columns. First, an adaptor was fabricated to enable the interfacing of the Shandon-Southern column design to the N_2-driven constant pressure hydraulic pump. Secondly, for the packing of all 2mm ID columns a 2mm ID reservoir was incorporated immediately before the column in order to avoid problems with bed inconsistency resulting from an abrupt change in the ID of the tubing. In addition, the amounts of stationary phase utilised in packing were scaled down accordingly with the column length and ID.

3.3.3.4 Stationary Phases

A list of stationary phases employed in this study, together with their specifications is compiled in Table 3.7.

3.3.4 Standard Solution Preparation

For all experiments reported in Part II of Chapter 3 except selection

Table 3.7 Specifications of Stationary Phases Evaluated for the NBLC Separation of Indoles.

Stationary Phase	Manufacturer	d_p (μm)	Particle Shape*	Carbon Loading (%)	Mean Pore Diameter (nm)	Surface Area (m^2g^{-1})	Surface Coverage† (mgm^{-2})	Description	Ref(s)
Partisil 10 ODS3	Whatman	10	I	10.5	8.5	350+	0.30	End-capped	386,387
Hypersil ODS	Shandon-Southern Product	5	S	10	9-10	200	0.50	Monolayer, fully end-capped	386,388
Spherisorb S5 ODS1	Phase Separations Group	5	S	7	8	220	0.32	Partially end-capped	386,389
Spherisorb S5 ODS2	Phase Separations Group	5	S	12	8	220	0.55	Fully end-capped	389
μ-Bondapak ODS	Waters Associates	10	I	10	12.5	300-500	0.33	End-capped	386,390

* I = irregular; S = spherical

† Surface coverage (mgm^{-2}) = $\frac{\text{C loading (\%)} \times 1000}{\text{Surface area } (m^2g^{-1}) \times 100}$

of an internal standard (IS), stock solutions (500µgml^{-1} in standard material) of TP, 5HTP, 5HT creatinine sulphate complex, 5HIAA and I3S,$K^{\oplus}$ salt were prepared in a MeOH:water mixture and were acidified with HAc (5 drops) to aid dissolution.

RP-PC was conducted using a working standard mixture (5µgml^{-1} of each compound) and individual working standards of identical concentration which were prepared by serial dilution of the stock solutions with MeOH. For RP-IIC, similar standards were produced by serial dilution of the stock solutions but a mixture of MeOH:aq. 0.1M KH_2PO_4 (4:96) was used for these dilutions instead of MeOH.

To optimise the ECD cell polarisation potential for the detection of TP, 5HTP, 5HT, 5HIAA and I3S a working standard containing 1.0µgml^{-1} (active ingredient) of each analyte was prepared by serial dilution of the stock solutions with MeOH:aq. 0.1M KH_2PO_4 (4:96).

For investigation of the effect of injection volume on the chromatographic separation, a working standard containing 0.4µgml^{-1} (active ingredient) was prepared similarly.

In view of the known lability of TP and its 5-hydroxy metabolites in solution to light[391,393], heat (they degrade slowly at room temperature)[304,305], air[391] and in an alkaline environment[391] careful storage of stock and working standard solutions was essential. All stock solutions were acidified with HAc on preparation, and were stored in sealed flasks wrapped in Al foil in a refrigerator at 4 °C. Working standards were also stored in a refrigerator at 4 °C and were removed and brought to room temperature only immediately prior to use. Stock solutions were freshly prepared at intervals of no longer than 3 months. Working standard solutions were replaced every 4 weeks or sooner if deterioration was suspected.

3.3.5 HPLC Operating Conditions

3.3.5.1 Conditions Employed for all Development and Optimisation of Separation Experiments

Column	:	Variable
Mobile Phase	:	Variable
Flow Rate	:	0.1mlmin^{-1} (1mm ID columns) or 0.4mlmin^{-1} (2mm ID columns)
Injection Volume	:	1µl (*via* micro-loop)
Detection	:	ECD (Potential : +1.00V *vs.* Ag/AgCl reference; Mode : Oxidation; Instrument Sensitivity : 100nA f.s.d.; Time Constant : 1sec)
Chart Speed	:	5mm min^{-1}

3.3.5.2 Optimised Chromatographic Parameters for the Separation of TP, 5HTP, 5HT, 5HIAA and I3S by NBLC

Column	:	Spherisorb S5 ODS1 (L = 10cm, ID = 2.1mm, d_p = 5µm, C loading = 7% w/w, Surface Area = 220m^{2}g^{-1})
Mobile Phase	:	4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA (200mgl^{-1}), pH 4.00
Flow Rate	:	0.4mlmin^{-1} ($\equiv$ u = 0.19cms^{-1})
Injection Volume	:	1µl (*via* micro-loop)
Detection	:	ECD (Potential : +0.95V *vs.* Ag/AgCl reference; Mode : Oxidation; Instrument Sensitivity : 100nA f.s.d.; Time Constant : 1sec)
Chart Speed	:	5mm min^{-1}

The concentration of the IIA (i.e., HSA) was adjusted to a small extent in order to compensate for column deterioration with use.

3.3.6 Procedure for the Optimisation of Electrochemical Detector Applied Potential

The Pye Unicam NBLC system, incorporating a commercially-packed Spherisorb S5 ODS1 column (10cm x 2.1mm ID) was eluted with the optimised mobile phase (i.e., 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA ($200mgl^{-1}$) which was adjusted to pH 4.00) at the standard flow rate of $0.4mlmin^{-1}$. An initial cell polarising voltage of +0.30V was programmed into the ECD controller and the baseline (at 100nA f.s.d. sensitivity with a time constant of 1sec) was allowed to settle. Once a constant drift-free baseline was established then a working standard containing TP, 5HTP, 5HT, 5HIAA and I3S ($1.0\mu gml^{-1}$ active ingredient, each constituent) was chromatographed in duplicate. The operating potential was increased stepwise in increments of 0.05V to +1.00V and at each selected potential the procedure was repeated. Peak heights and baseline noise levels of all the chromatograms were measured manually.

3.3.7 Procedure for the Evaluation of the Effect of Injection Volume on the Chromatographic Separation of Indole Standards

The NBLC system incorporating the Rheodyne model 7413 injection valve was utilised for the first part of the title experiment. With the 0.5µl internal loop in the active position, a standard solution containing TP, 5HTP, 5HT, 5HIAA and I3S ($0.4\mu gml^{-1}$ active ingredient, each component) was chromatographed on a freshly-packed Spherisorb S5 ODS1 column (10cm x 2.1mm

ID) which was operated under optimised conditions. Chromatograms recorded at the standard chart speed of 5mm min^{-1} were obtained at two different ECD sensitivity settings, viz. 50nA f.s.d. and the highest sensitivity at which all indole peaks were still on-scale (which was 5nA f.s.d. for the 0.5µl volume ≡ 0.2ng each indole on-column). A further chromatogram was recorded at the latter instrument sensitivity using a chart speed of 30mm min^{-1} to enable manual measurement of peak parameters to be carried out with less error.

Subsequently, the above procedure was repeated employing the 1µl and 5µl injection loops in the active position.

For the second part of the experiment a Rheodyne model 7010 external loop injector was substituted for the model 7413 micro-valve. The conduit between the injector and the column inlet was retained. The above procedure was repeated with 10µl and 20µl capacity loops fitted.

For each elution band in the generated chromatograms, peak height, baseline noise, retention time and peak width at both 60.7% and 50% of peak height were measured.

3.3.8 Procedures Adopted for the Search for an Internal Standard

3.3.8.1 Non-Indolic Materials

A total of eighteen compounds of similar molecular weight to the analytes which were soluble in the mobile phase at low concentrations and which contained electrophores (i.e., principally phenol- or aniline-based substances) were selected from the laboratory shelf. A solution of each compound was prepared qualitatively by dissolving a small amount of each material in a pseudo-mobile phase mixture, viz. 4% MeOH : 96% aq. 0.1M

KH_2PO_4. Each solution was chromatographed in turn on a commercially-packed Spherisorb S5 ODS1 column (10cm x 2.1mm ID) under optimised conditions and was allowed to run for 1 hour or until a readily discernible peak eluted, whichever was the sooner. Injections of a standard containing TP, 5HTP, 5HT, 5HIAA and I3S (1.0$\mu g ml^{-1}$ active ingredient, each component) were interspersed between the other injections in order to ensure continuity of chromatographic behaviour. Repeat injections were performed of any of the substances under scrutiny which were observed to elute within an acceptable period. Dilution or concentration of the original solutions were made as deemed appropriate in order to obtain a peak which was both on-scale and of sufficient height with which to ascertain a retention time. Retention times of each substance were measured manually and capacity factors were calculated. Peak shapes were also noted.

Those compounds eluting within a chromatographic window or a short time after the longest retained analyte were then co-chromatographed with the standard indole mixture to establish whether full resolution from the analytes was realised in practice.

3.3.8.2 Indolic Materials

A little of the $BF(CO_2H)_2.H_2O$ complex was dissolved in MeOH and was chromatographed under optimised conditions. The concentration of the methanolic BF solution was then adjusted with aqueous KH_2PO_4 (0.1M) to produce a peak eluting on-scale. The retention time of this peak was then measured and its capacity factor calculated. Aliquots of this BF solution and the standard indole mixture were combined. Co-chromatography was undertaken to show the complete separation of BF from the indole analytes.

3.4 Development of a Separation of a Series of Indolic Standard Materials

3.4.1 Chromatography on a 1mm ID Reverse Phase Column

3.4.1.1 Separation Strategy - General Remarks

Reverse phase systems comprising the elution of a largely aqueous mobile phase (polar) across a C_{18} bonded stationary phase (non-polar) were chosen in preference to normal phase systems for application to the separation of selected indoles. This choice was based upon five major considerations. First, because indoles are soluble in polar solvents (e.g., water and lower alcohols) but are insoluble in relatively non-polar solvents (e.g., alkanes and halocarbons), this dictates that a polar mobile phase is required. Secondly, a polar mobile phase would be beneficial because the mobile phase and the sample matrix would then be compatible, so enabling sample preparation prior to injection to be kept to a minimum. A polar mobile phase is also necessary in order to carry an electrolyte which will support an electric current, hence enabling EC detection to be utilised. Furthermore, as most of the potential interfering species present in biological systems are ionic in nature, it would be advantageous to use a polar mobile phase and a non-polar stationary phase so that these ions exhibit little affinity for the column surface and consequently are eluted from the column with great rapidity. Finally, the reverse phase mode is highly versatile, especially with respect to the employment of secondary chemical equilibria, so providing the chromatographer with additional operational variables not available in the normal phase mode. For these reasons the reverse phase mode was a logical choice and was employed exclusively throughout the course of these investigations.

3.4.1.2 Selection of Operational Parameters

The first NB column obtained for incorporation into the Pye Unicam NBLC instrument was supplied by Pye Unicam. The 25cm x 1mm ID glass-lined 316 S/S column was of Whatman manufacture from their 'micro-B' range and was received pre-packed with Partisil 10 ODS3 stationary phase (d_p = 10μm). The column end fittings were of the female ZDV Swagelok type illustrated in Figure 1.7. All connections between the column and the rest of the LC instrument were custom-made from short lengths of 0.15mm (0.006") ID, $^1/_{16}$" OD tubing and all ferrules were positioned carefully according to the manufacturers' recommendations (see Figure 3.7) in order to avoid the introduction of unnecessary extra-column volume into the system.

The composition of the mobile phase employed in the first instance was based on that used for the separation of catecholamine standards reported in Chapter 2, i.e., a modified aqueous acetate/citrate "buffer". The buffer served a dual function, viz. control of pH (and consequently of solute speciation) and also provision of supporting electrolyte. The initial pH was 4.1 which was the natural value for the mobile phase containing citric acid ($6.3gl^{-1}$) and NaAc ($2.27gl^{-1}$) (NaOH was omitted). The organic modifier that was selected was MeOH. This choice was made on the grounds of its relatively high polarity, water miscibility, protic nature, ready availability and low cost. A volumetric flow rate of $0.1mlmin^{-1}$ ($100\mu lmin^{-1}$; $u = 0.21cms^{-1}$ for a 1mm ID column) was adopted, the lowest possible with the PU4010 pump.

The Rheodyne 7413 injection valve was received with the 1μl capacity loop in the active position. This injection volume was applied for all early experiments.

For this preliminary work the PU4022 ECD was programmed with an operating potential of +1.00V *vs*. Ag/AgCl, an instrument sensitivity of 100nA f.s.d. and a time constant of 1sec, the fastest available setting. The operating potential was selected following the general recommendations of the ECD manufacturers for the indole class of compounds.[204,206] Fine adjustment of this parameter to an optimum for the particular analytes under examination was to be carried out once a satisfactory separation had been achieved. The first approach to the separation of the selected indoles attempted was that of pure reverse phase partition chromatography (RP-PC).

3.4.1.3 Reverse Phase Partition Chromatography - Variation of Organic Modifier Content

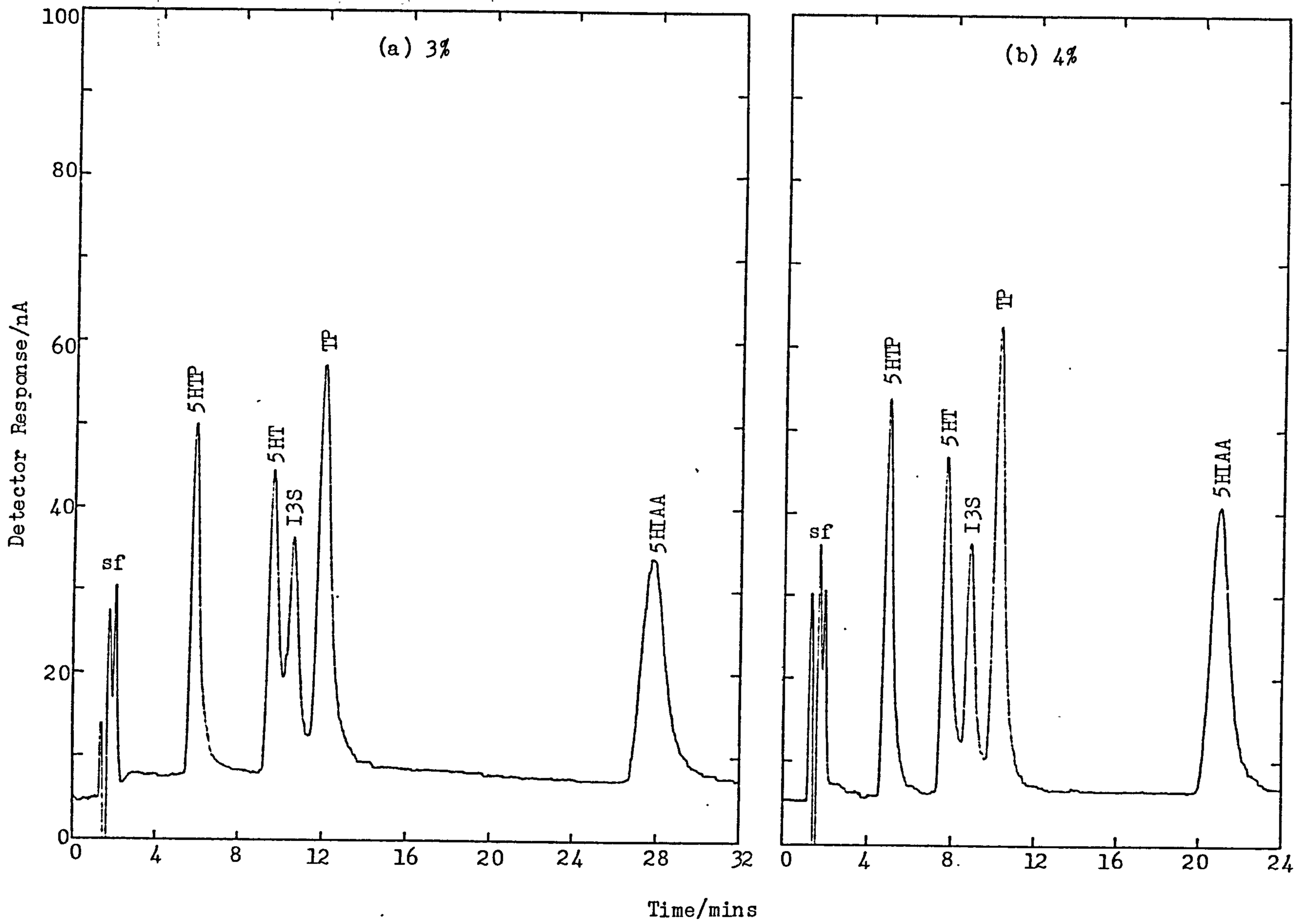
A methanolic five component standard containing the indolic compounds of interest was chromatographed in duplicate on the 25cm x 1mm ID Partisil 10 ODS3 column under the aforementioned conditions using mobile phases with MeOH contents ranging from 3-20%. The resulting chromatograms are compiled in Figure 3.12. Peaks were identified by matching capacity factors with those obtained by chromatographing standards containing single indoles. Mean capacity factors for each analyte were calculated and are presented in Table 3.8.

The relationship between indole capacity factor and mobile phase MeOH content is expressed graphically in Figure 3.13. From the chromatograms and the graph a MeOH content of 4% was considered to be optimal. This judgement was made on the basis of three parameters, *viz*. capacity factor, run time and resolution.

For this separation it was desirable to have the first peak of interest

Figure 3.12 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Partisil 10 ODS3 with Various Concentrations of MeOH in the Mobile Phase

Parameters :- Column : Partisil 10 ODS3 (25cm x 1mm, d_p = 10µm); Mobile Phase : MeOH : aq. 20mM NaAc/HCt buffer (modifier concentration as specified), pH 4.00; Flow Rate : 0.1mlmin^{-1}; Detection : ECD (Eapp = +1.00V vs. Ag/AgCl, TC = 1sec); Sample : 1µl via loop of TP, 5HTP, 5HT.creatinine sulphate.H_2O, 5HIAA and I3S (K^+ salt) in MeOH (All constituents @ 5µgml^{-1} ≡ 5ng injected)



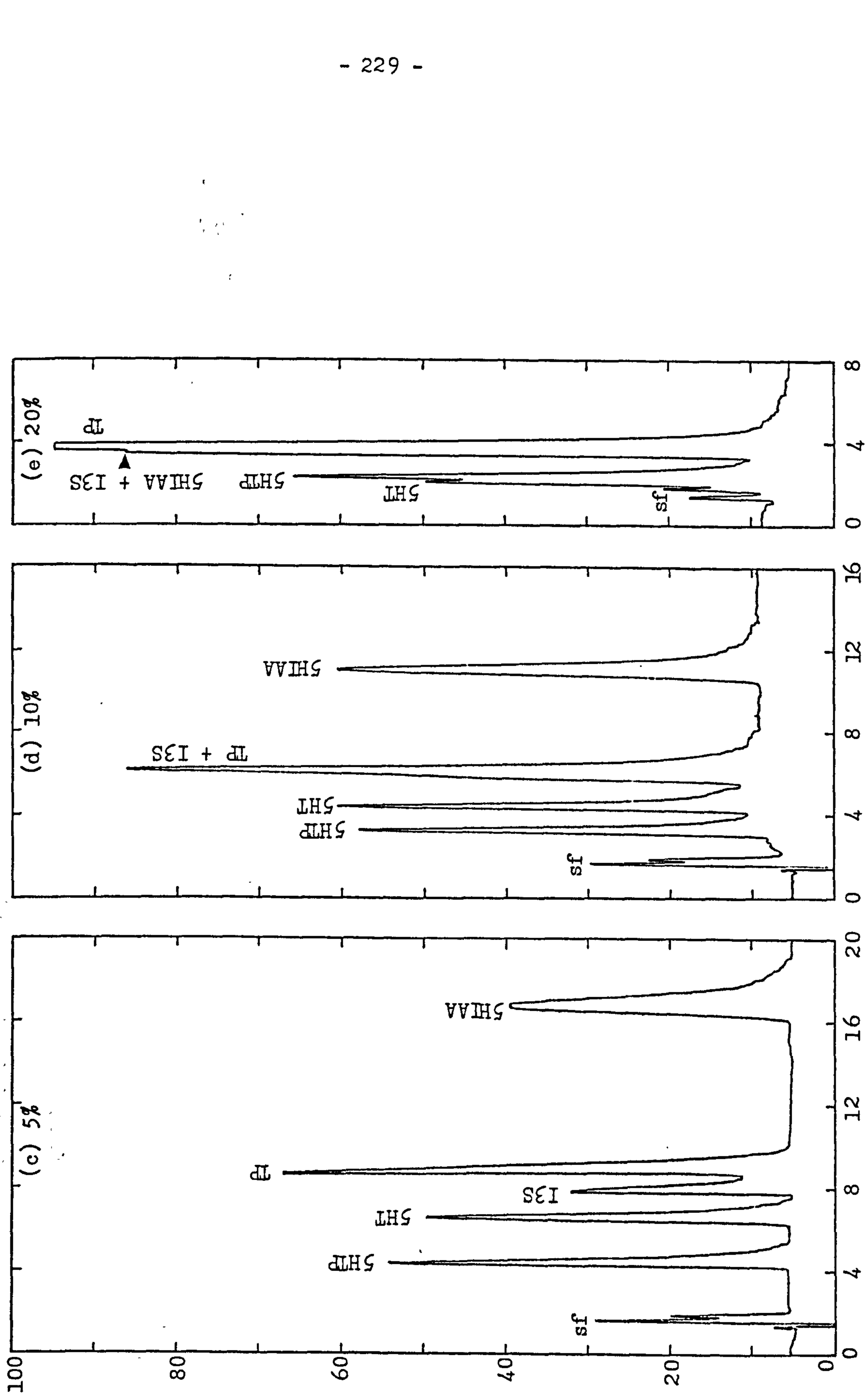


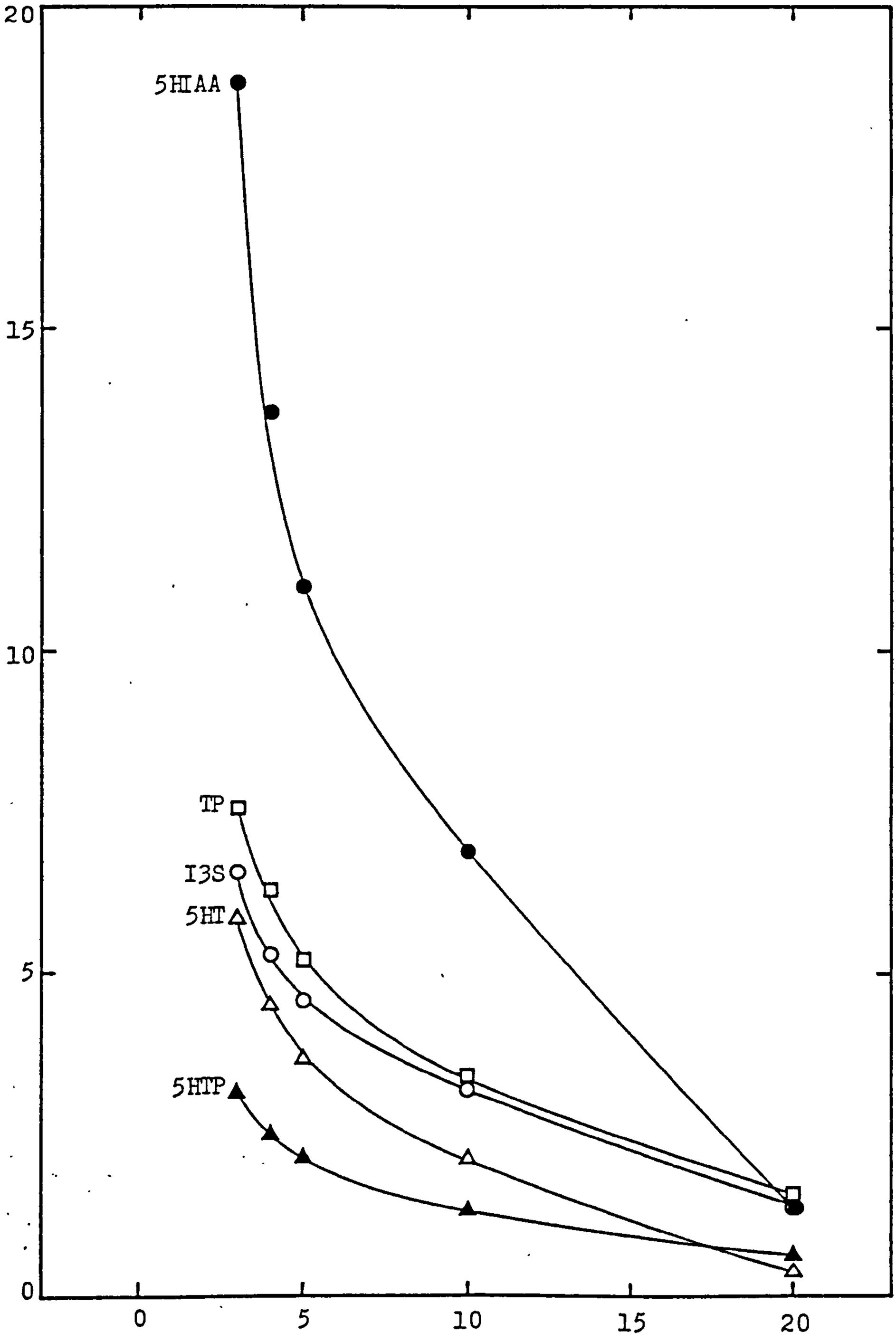
Figure 3.12 continued

Table 3.8 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on Partisil 10 ODS3 with Various Concentrations of MeOH Modifier in the Mobile Phase

% MeOH	sf*	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
3	1.4	12.0	7.57	5.8	3.14	9.6	5.86	27.8	18.86	10.6	6.57
4	1.4	10.2	6.29	4.9	2.50	7.7	4.50	20.6	13.71	8.8	5.29
5	1.4	8.7	5.21	4.4	2.14	6.6	3.71	16.8	11.00	7.8	4.57
10	1.4	6.2	3.43	3.3	1.36	4.4	2.14	11.1	6.93	5.9	3.21
20	1.4	3.6	1.57	2.3	0.64	2.0	0.43	3.4	1.43	3.4	1.43

* sf = Solvent front

Figure 3.13 The Relationship Between Capacity Factor and MeOH Content of the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Partition Conditions on Partisil 10 ODS3



elute at $k' \geq 2$ for two reasons, i.e., (1) to avoid potential interference from early eluting species expected to be present in samples of biological origin, and (2) in order to relax to some degree the severity of the demands for low extra-column dispersion placed upon the instrument by the operation of 1mm ID columns since the most stringent restrictions apply to peaks eluting close to the solvent front. In order to comply with this criterion a mobile phase MeOH content of $\leq$ 6% is required.

In addition, it was deemed necessary to keep the duration of the chromatographic analysis within reasonable limits (say $\leq$ 30 minutes if possible) in order to provide acceptable throughput for use in the clinical laboratory. From Figure 3.13 it is evident that the analysis time parameter increases rapidly with decreasing MeOH content, (as would be expected). Therefore a moderate organic modifier concentration would be desirable.

Finally, achievement of baseline resolution of all five analytes was sought. Within the range of mobile phase MeOH contents investigated, all three of these criteria could not be met in full, particularly that of resolution. Resolution (R_s) of an LC system depends on the relative retention times and tangential baseline peak widths of the two closest eluting components (X and Y) and is defined by the expression :

$$R_s = \frac{t_{R(Y)} - t_{R(X)}}{\frac{1}{2}(w_{B(Y)} + w_{B(X)})} \qquad (3.1)$$

where $t_{R(Y)} > t_{R(X)}$, i.e., solute X elutes before solute Y. Values of R_s were calculated using Equation 3.1 for the separation obtained with 3,4 and 5% MeOH in the mobile phase and these data are presented in Table 3.9.

Table 3.9 Resolution Obtained on a 25cm x 1mm ID Partisil 10 ODS3 Column for a Series of Indoles Chromatographed using Various Mobile Phase MeOH Contents

% MeOH	t_R(min)		$w_{0.607}$(min)		w_B(min)*		R_s
	X	Y	X	Y	X	Y	
3	9.6	10.4	0.46	0.51	0.92	1.02	1.03
4	7.7	8.8	0.39	0.43	0.78	0.86	1.34
5	7.8	8.7	0.35	0.41	0.71	0.82	1.18

* w_B is calculated from $w_{0.607}$ assuming Gaussian peak shape, i.e., $w_B = 2w_{0.607}$ (see Figure 1.4 for explanation).

Clearly the greatest resolution was achieved with a mobile phase MeOH content of 4%. The chromatographic run time using this mobile phase was ca. 24 minutes and the capacity factor of the earliest eluting analyte was $k' = 2.50$. Hence, a 4% MeOH-modified mobile phase best satisfied all the aforementioned criteria. Furthermore, this small percentage of MeOH did not appear to disrupt the electrical properties of the largely aqueous mobile phase to any significant extent or cause buffer precipitation problems. Thus a 4:96 MeOH/aq. buffer ratio was adopted for the next stage of the development of the separation.

3.4.1.4 Reverse Phase Partition Chromatography - Variation of Mobile Phase pH

Mobile phase pH is an important parameter where ionic or ionisable

solutes are to be separated because control of solute speciation is possible. The methanolic five component standard used in the preliminary experiments was again chromatographed (in duplicate) on the 25cm x 1mm ID Partisil 10 ODS3 column under the operational parameters outlined in Section 3.4.1.2 but this time using a series of mobile phases containing 4% MeOH that had been buffered to different pH values. Unfortunately, practical problems developed which restricted this investigation to only two mobile phases. The resulting chromatograms are displayed in Figure 3.14. Again peak identification was by means of the chromatography of individual indole standards. Mean capacity factors for each analyte were calculated and are tabulated in Table 3.10. These data are presented in graphical form in Figure 3.15.

Little of value could be deduced from this study except that pH has a profound effect on the chromatography of indoles. Of greater concern were the practical difficulties experienced with the NBLC column.

3.4.2 Practical Problems Encountered With the Operation of the 1mm ID Column

After only 80 injections of standards onto the 1mm ID column the column back pressure had risen to such a degree that the automatic pressure limiting circuitry of the pump came into operation and cut power to the motor so interrupting the solvent flow. The source of the increased pressure was traced to the column itself. On inspection, no discolouration or physical disruption of the top of the column bed was discernible. One problem with small-diameter columns that had been recognised by the column supplier in their own laboratory is blockage of the column exit frit by "fines" (very small diameter particles of stationary phase).[394] Hence, the

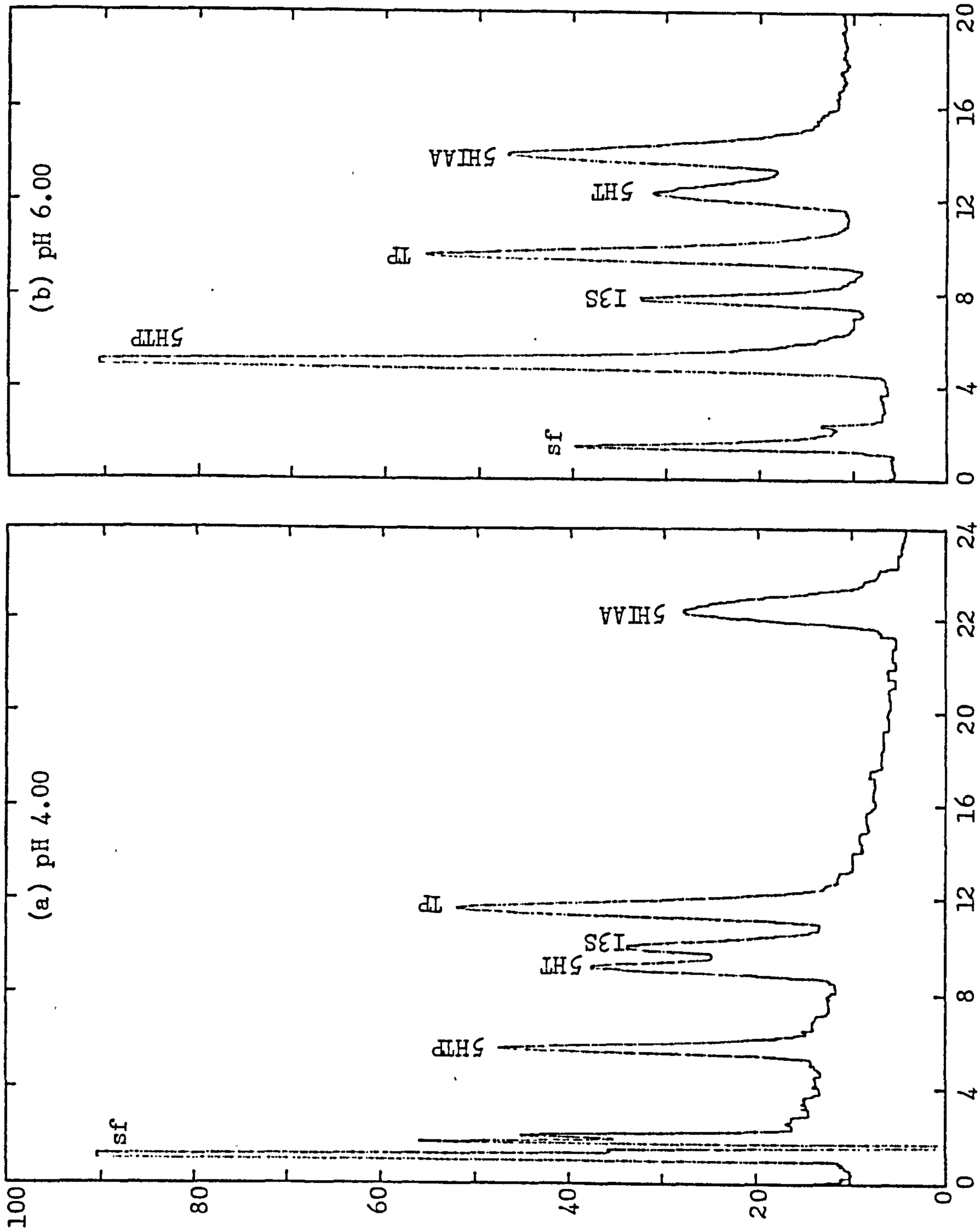


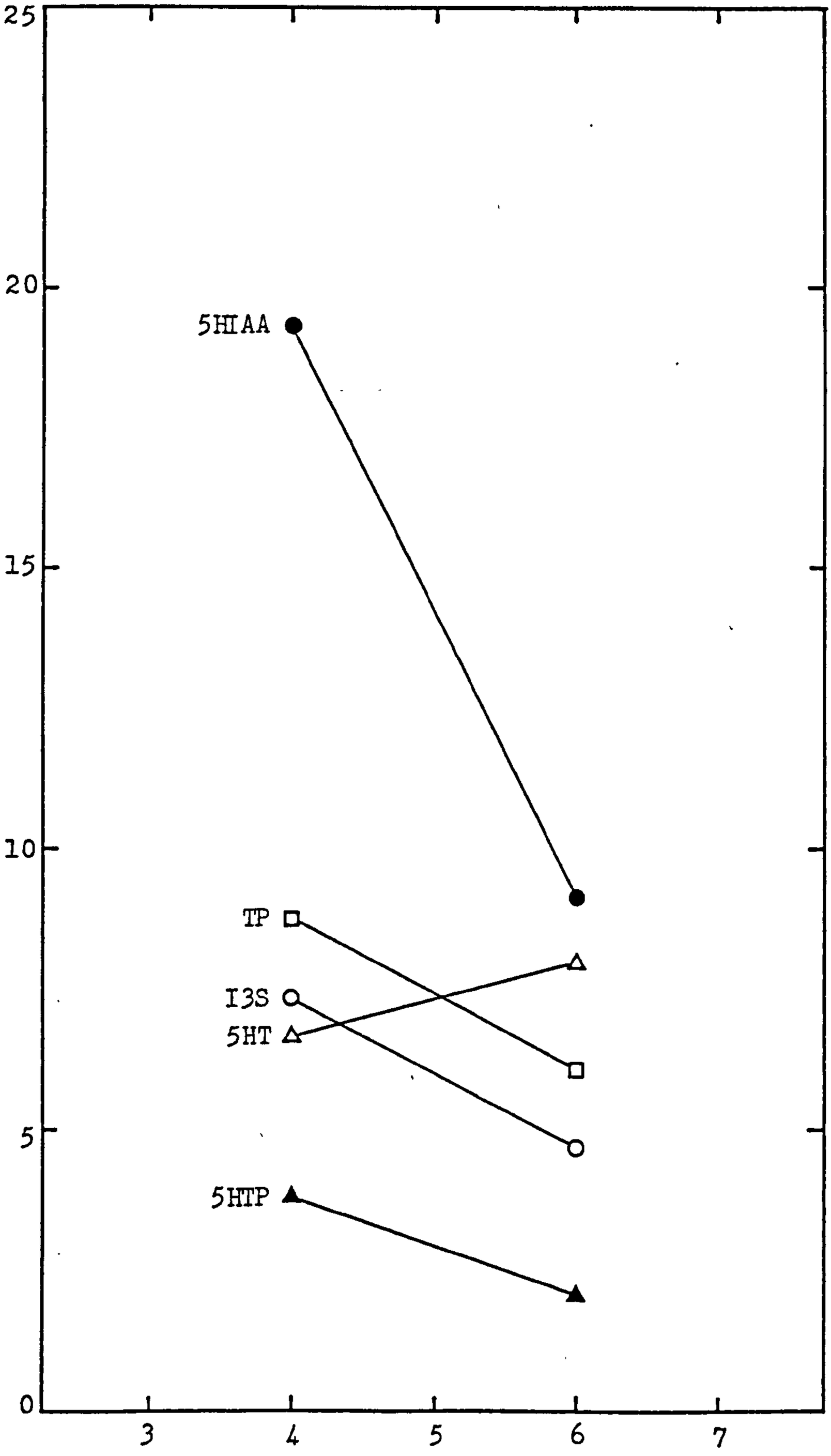
Figure 3.14 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Partisil 10 ODS3 at Various Mobile Phase pH Values

Parameters :- For conditions see Figure 3.12 except for Mobile Phase : 4% MeOH : 96% aq. 20mM NaAc/HCl buffer, pH as specified.

Table 3.10 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on Partisil 10 ODS3 at Various Mobile Phase pH Values

pH	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
4.0	1.2	11.7	8.75	5.8	3.83	9.2	6.67	24.4	19.33	10.0	7.33
6.0	1.4	9.9	6.07	4.3	2.07	12.6	8.00	14.2	9.14	8.0	4.71

Figure 3.15 The Relationship Between Capacity Factor and pH of the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Partition Conditions on Partisil 10 ODS3



column end fittings with integral frits were ultrasonicated in HNO_3 (20%, 30 mins) in order to dislodge any offending material.[395] Following a thorough water wash the column was reassembled and replaced in the NBLC instrument in its original orientation. On recommencement of mobile phase delivery, the system back pressure stabilised at a more reasonable and practicable level of 2250 p.s.i. (155 bar). However, the original problem rapidly re-established itself after only a further 8 injections of standards.

The excessive proneness to physical blockage of this 1mm ID column constituted a major problem. The probability of blockage occurring was expected to increase with decreasing column ID because of the correspondingly smaller cross-sectional area presented to the sample. A conventional column of 4.6mm ID possesses a cross-sectional area of 16.6mm^2 compared with only 0.8mm^2 for a 1mm ID NB column constituting a factor of 21 times difference. Consequently, the average lifetime of 1mm ID columns would be expected to be significantly shorter than that of conventional columns operated under identical conditions. The high blockage probability, encountered with 1mm ID columns is exacerbated by a general inability to apply many of the protective measures commonly used in conjunction with conventional columns[395-397] to systems incorporating NB columns. Devices such as guard columns would introduce considerable additional extra-column volume to the system which of course would give rise to additional extra-column dispersion resulting in a further degradation of system performance. The necessity of taking feasible preventative precautions when employing NB column technology is emphasised by Rabel.[58] One such precaution that may help prolong column lifetime whilst not increasing extra-column dispersion is the use of a scavenger pre-column.[395,396] A scavenger column is incorporated in the solvent line between the pump and the injection valve, the purpose

of which is to capture particulate material (e.g., piston seal debris, dust, precipitated material) and so to prevent analytical column contamination from this source. A 5cm x 4.6mm ID scavenger column was packed and interposed as indicated.

Unfortunately, no replacement column of 1mm ID was immediately available to enable completion of these studies and to allow a comparison of the lifetimes of such columns to be made. Since it was the column end-fittings that were suspected of being the source of the problem, a replacement pair were ordered with a view to continuing these experiments with the present column at a later date. When this investigation was recommenced the column performed adequately for only 89 injections of standards before the back pressure again exceeded tolerable limits. The problem was deduced to be a recurrence of the earlier failure. Such a reproducibly short column lifetime under moderate operating conditions was considered to be highly unsatisfactory. Should such behaviour be found to be typical from 1mm ID columns, then this column geometry would not be deemed to be acceptable for the rigours of routine application in a clinical laboratory.

3.4.3 Evaluation of Instrument Performance with Respect to 1mm ID Columns

The best separation of indoles obtained on the 1mm ID Partisil 10 ODS3 column to date was that displayed in Figure 3.12(b). Peak symmetry was generally considered to be satisfactory but all band profiles were actually non-Gaussian as a result of some peak tailing which most probably originated from adsorption-type interactions between the analytes and any accessible residual silanol (≡Si-OH) groups on the stationary phase surface. Baseline peak widths ranged from 1.2 mins for 5HTP to 3.2 mins for 5HIAA which are

equivalent to peak volumes of 120 and 320µl, respectively. The indole peaks are remarkably broad and this excessive broadness is almost certainly the primary factor responsible for the lack of baseline resolution between the peaks due to 5HT, I3S and TP. Such a conclusion immediately raises questions regarding the level of control exerted over the kinetic processes giving rise to extra-column dispersion within the Pye Unicam NBLC system.

In order to evaluate the NBLC system incorporating the 1mm ID column in a quantitative manner it is necessary to calculate values for a number of parameters. These parameters include the extra-column variance (σ^2_{xc}), the number of theoretical plates in the column (N_c) and the apparent number of theoretical plates observed in the chromatogram ($\hat{N}$). The respective plate heights (H_c &$\hat{H}$) associated with these plate counts may also be calculated if desired.

Several methods have been applied to the calculation of extra-column variance. Many workers have determined the value of σ^2_{xc} directly by measuring the band width of a solute probe peak eluted from a system in which the column is replaced by a ZDV union.[60,75,77,78,398,399] This approach is known as the "no-column" or "zero-length column" method and the principle of this technique is illustrated diagrammatically in Figure 3.16.

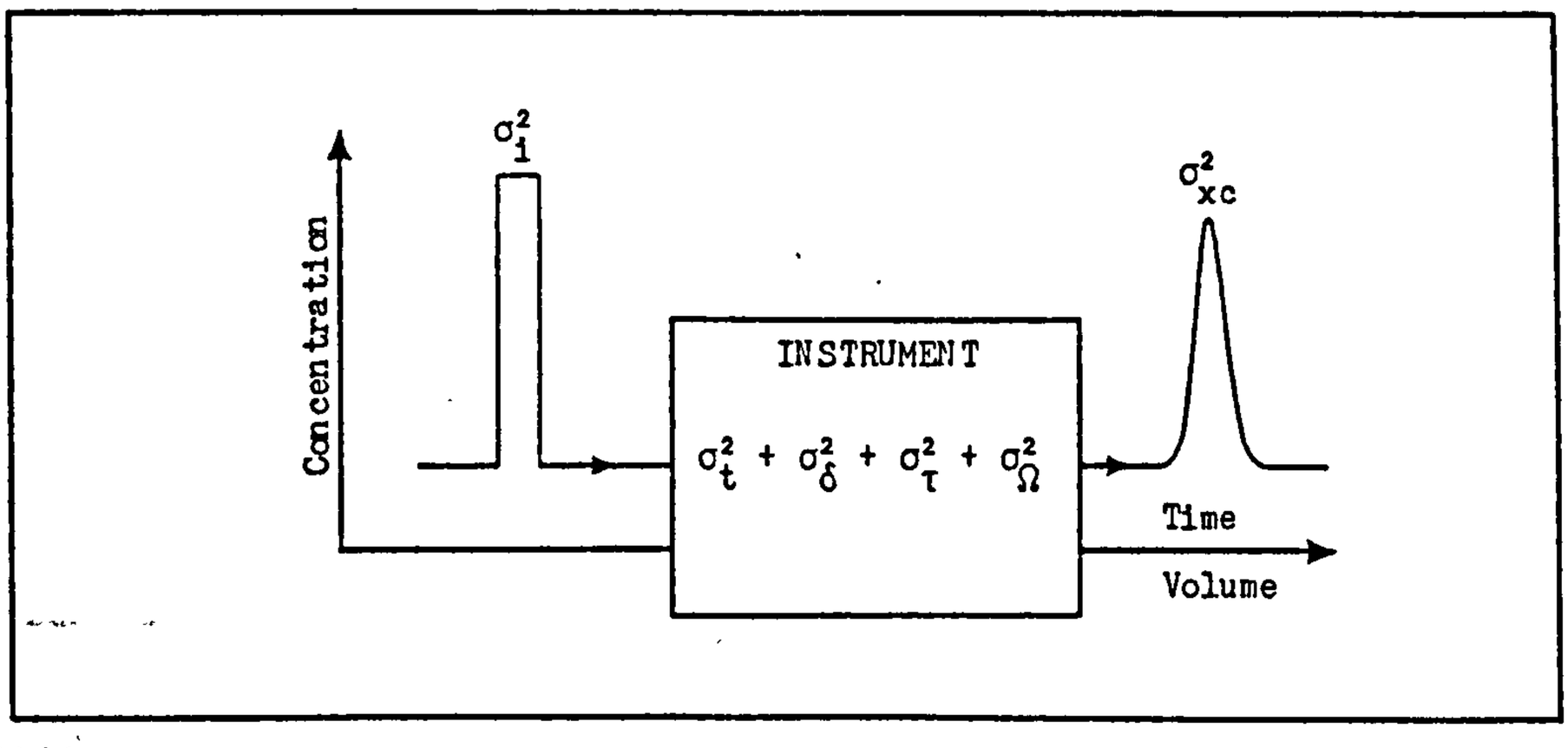


Figure 3.16 Schematic Representation of Band Broadening in the "No-Column" Mode

The "no-column" method suffers from the disadvantage that no account is taken of the contributions arising from the column hardware exterior to the packed bed such as the frits and end-fittings. Furthermore, as there is no column to retard the progress of the solute probe, it elutes very rapidly and so correspondingly fast-response electronics are required in order to allow effective measurement of the generated peak profile.

The extra-column variance has also been determined by a number of indirect methods. If a length of straight, open tubular capillary is substituted for the column then σ^2_{xc} can be obtained by measuring the band width of a solute probe peak and correcting for the contribution of the capillary[77] calculated according to Equation 1.17, which is derived from the work of Taylor, Aris and Golay (see Section 1.4.3.1). Alternatively, if the probe band is measured for a number of different lengths of otherwise identical capillary tubing, then by plotting the observed peak variance (σ^2_{tot}) against tube length and performing linear regression on these points σ^2_{xc} is yielded by extrapolation of the graph to zero tube length.[39,60] A diagrammatic representation of the principles behind these two techniques is presented in Figure 3.17. Of these two approaches the former depends

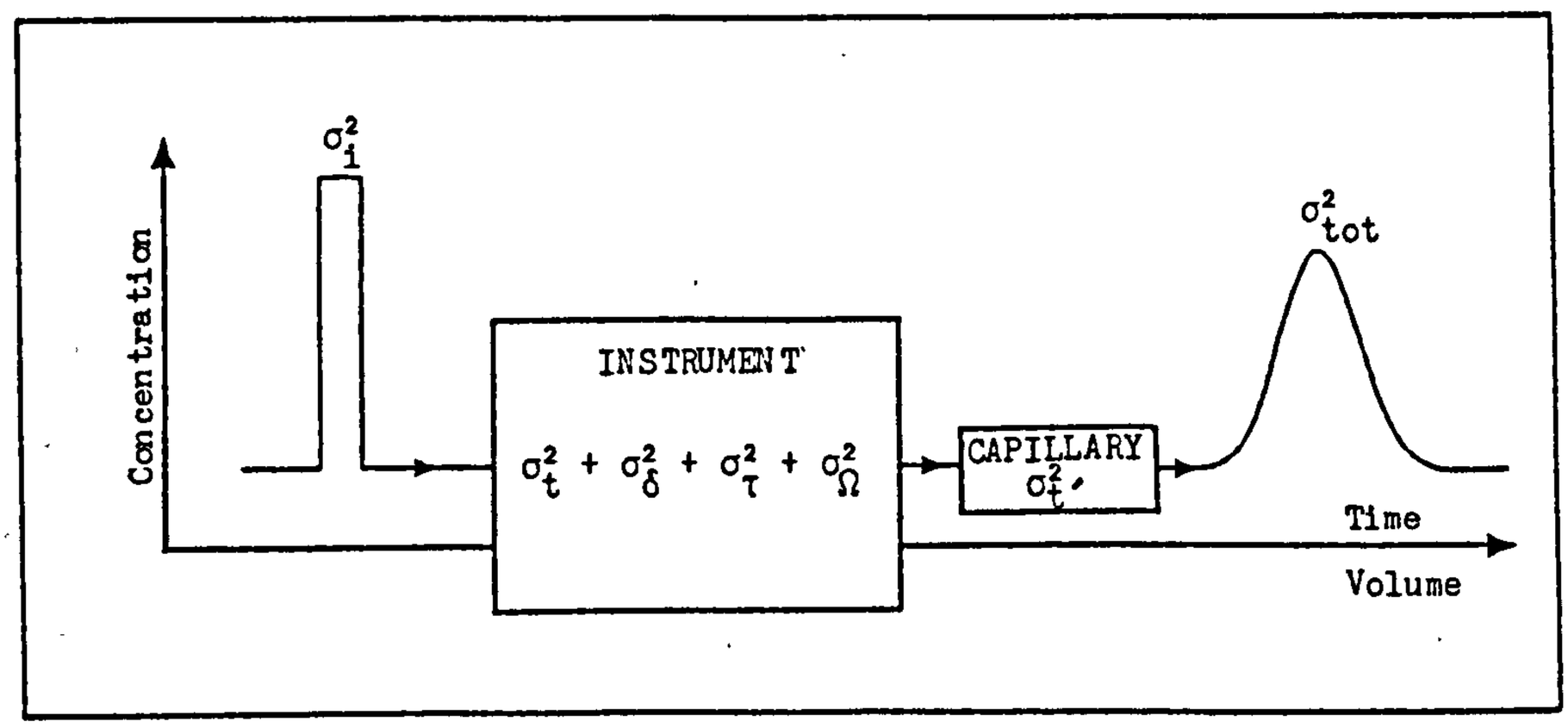


Figure 3.17 Schematic Representation of Band Broadening in the "Open Tubular Capillary" Mode

on the accuracy with which the values of the parameters in the governing equation are known (particularly the ID of the capillary), and the latter is time consuming. In addition, both techniques are also subject to the primary drawback inherent with the "no-column" method, i.e., that no account is taken of the effects of column hardware.

The most widely applied indirect method for the determination of the extra-column variance utilises a column and consequently is designated the "column" method.[75-78,400] Figure 3.18 demonstrates in schematic form the fundamental band broadening contributions inherent in the "column" method.

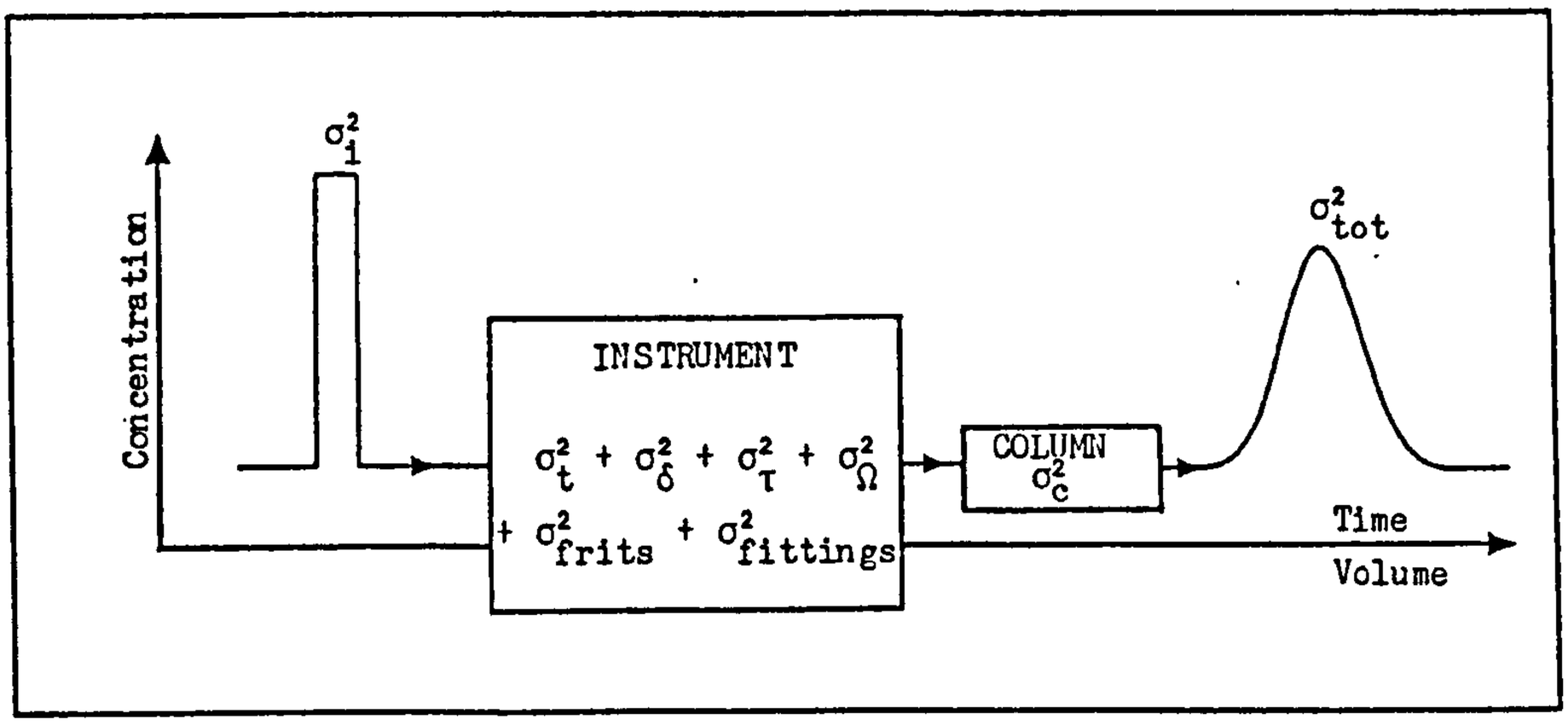


Figure 3.18 Schematic Representation of Band Broadening in the "Column" Mode

This technique relies upon the assumption that the true plate count of a column (N_c) is independent of k' (and consequently of t_R and V_R). This is, in fact, a conservative assumption since N_c will generally decrease with k' whereas extra-column dispersion will cause the apparent plate count ($\hat{N}$) to increase with k'[77], at least over small values of k'.[386] The "column" method is based on the relationship (Equation 1.19) :

$$\sigma^2_{tot} = \sigma^2_c + \sigma^2_{xc} \qquad (1.19)$$

where $$\sigma^2_{c(v)} = \frac{V_o(1 + k')^2}{N_c} = \frac{V_R^2}{N_c} \qquad (1.21)$$

or in time units :

$$\sigma^2_{c(t)} = \frac{t_o(1 + k')^2}{N_c} = \frac{t_R^2}{N_c} \qquad (1.21)$$

Hence :

$$\sigma^2_{tot(v)} = \frac{V_R^2}{\hat{N}} = \frac{V_R^2}{N_c} + \sigma^2_{xc(v)}$$

or :

$$\sigma^2_{tot(t)} = \frac{t_R^2}{\hat{N}} = \frac{t_R^2}{N_c} + \sigma^2_{xc(t)}$$

Thus, if the band widths of a series of consecutive peaks are measured and σ^2_{tot} is plotted versus the square of the peak retention times (or volumes), then after linear regression, σ^2_{xc} is given by the intercept at zero retention time (or volume). Furthermore, the true column efficiency is given by the reciprocal of the gradient. This procedure is preferred by the author because <u>all</u> the external sources of band broadening are incorporated in the assessment. The "column" method does suffer from two (minor) disadvantages. Firstly, deviations in behaviour from the assumed independence of N_c with respect to t_R, V_R and k' do occur as outlined previously, and secondly, this technique is substance dependent. The latter dependency results in part from the variability in diffusivities between

solutes and also, and probably more importantly, due to the influence of solute structure on peak shape. The occurrence of certain types of (essentially undesirable) physico-chemical interactions between the solute(s) and the stationary phase surface can give rise to phenomena such as peak fronting and/or tailing. Such departures from Gaussian peak shape will necessarily affect the peak width measurement and subsequently the value of σ^2_{tot} obtained. Consequently the values of σ^2_{xc} and N_c determined using σ^2_{tot} will ultimately be subject to error. In spite of these minor reservations, the "column" method of extra-column variance determination was applied to the current problem.

The preceding point regarding non-Gaussian peak profiles leads to another important consideration, namely that of the method of σ^2_{tot} determination. There are several alternatives available but all of these may be summarised into two categories, viz. graphical methods and statistical methods.

A number of graphical methods of peak variance estimation are available, and these have been described in Section 1.4.2.4. Peak width at half-height[400], peak half-width[51,61,75,76], and tangential baseline width[51,77,399] have all been applied by various workers in order to determine σ^2_{tot}. For all these graphical techniques Gaussian or near-Gaussian peak profiles are a prerequisite for accurate σ^2_{tot} estimation. Error in the determination of total peak variance by these methods becomes increasingly significant with increasing peak skew and under these circumstances serious under-estimation often results.[51,61,75,78]

The second central moments method is a statistical procedure whereby the total peak variance is yielded by the calculation of second central moments about each peak by point by point summation across each peak profile.

This technique requires computing facilities in order that the calculation may be performed within an acceptable time span. The second central moments method, although currently accepted, can over-estimate the peak variance due to excess weighting of the tails of peaks[51,78], and the heavy dependency on peak end-point determinations, leading to errors in the variance particularly for skewed peaks.[78] Comparisons of estimation by graphical techniques with estimation *via* second central moments have shown the latter technique to be considerably more accurate than the former[51,61,75]. However, for the experiments reported herein the necessary computing facilities were not available to enable second central moments to be calculated. Hence, the use of graphical techniques was mandatory and the strong possibility of under-estimation of σ^2_{tot} had to be accepted.

Tangents were drawn to all peaks in each of four chromatograms obtained under optimal conditions (e.g., Figure 3.12(b)). Peak widths were measured at three different points, *viz*. at 60.7% of peak height ($\equiv 2\sigma_{tot}$), at 50% of peak height ($\equiv 2.35\sigma_{tot}$), and at the baseline between the tangents ($\equiv 4\sigma_{tot}$). Retention times of every band were also quantified. Measurement error was estimated to be *ca*. ±3secs. Mean values of each parameter were determined and values of $\sigma^2_{tot(t)}$ and t^2_R were calculated for all three peak widths. All data are tabulated in Table 3.11. Graphs were plotted of mean total peak variance against square of mean retention time for each method of evaluation (Figure 3.19). Linear regression was performed on each series of points and values for $\sigma^2_{xc(t)}$ (and consequently $\sigma_{xc(v)}$) and N_c (and consequently H_c) were determined from the intercept and gradient respectively. This information is compiled in Table 3.12.

From Table 3.12 it may be seen that all three peak width parameters yield different values for the column plate height, H_c. These values range

Table 3.11 Mean Retention Time and Peak Width Data Together with Calculated Peak Standard Deviations and Peak Variances for a Separation of a Series of Indoles on the Pye Unicam NHLC System with a 25cm x 1mm ID Column (n = 4)

					Peak width measurement								
					Half-width			Width at half-height			Tangential baseline width		
Peak Number	Indole	t_R (min)	t_R (s)	t_R^2 (s^2)	$w_{0.607}$ (min)	σ_{tot} (s)	σ^2_{tot} (s^2)	$w_{0.5}$ (min)	σ_{tot} (s)	σ^2_{tot} (s^2)	w_B (min)	σ_{tot} (s)	σ^2_{tot} (s^2)
1	5HTP	5.03	301.5	90902	0.335	10.05	101.0	0.410	10.45	109.2	0.770	11.55	133.4
2	5HT	7.93	475.5	226100	0.435	13.05	170.3	0.535	13.64	186.0	0.975	14.63	213.9
3	I3S	9.03	541.5	293222	0.440	13.20	174.2	0.530	13.51	182.5	0.950	14.25	203.1
4	TP	10.40	624.0	389376	0.475	14.25	203.1	0.555	14.16	200.2	1.035	15.53	241.0
5	5HIAA	21.35	1281.0	1640961	0.815	24.45	597.8	1.000	25.49	649.8	1.830	27.45	753.5

Figure 3.19 The Relationship Between Mean Total Peak Variance and Square of Peak Retention Time for a Series of Indoles Chromatographed on the Pye Unicam NBLC System with a 25cm x 1mm ID Column Installed

(σ^2_{tot} derived from peak half-width, $w_{0.607}$[◆] , peak width at half-height, $w_{0.5}$[■], and tangential baseline width, w_B[▼])

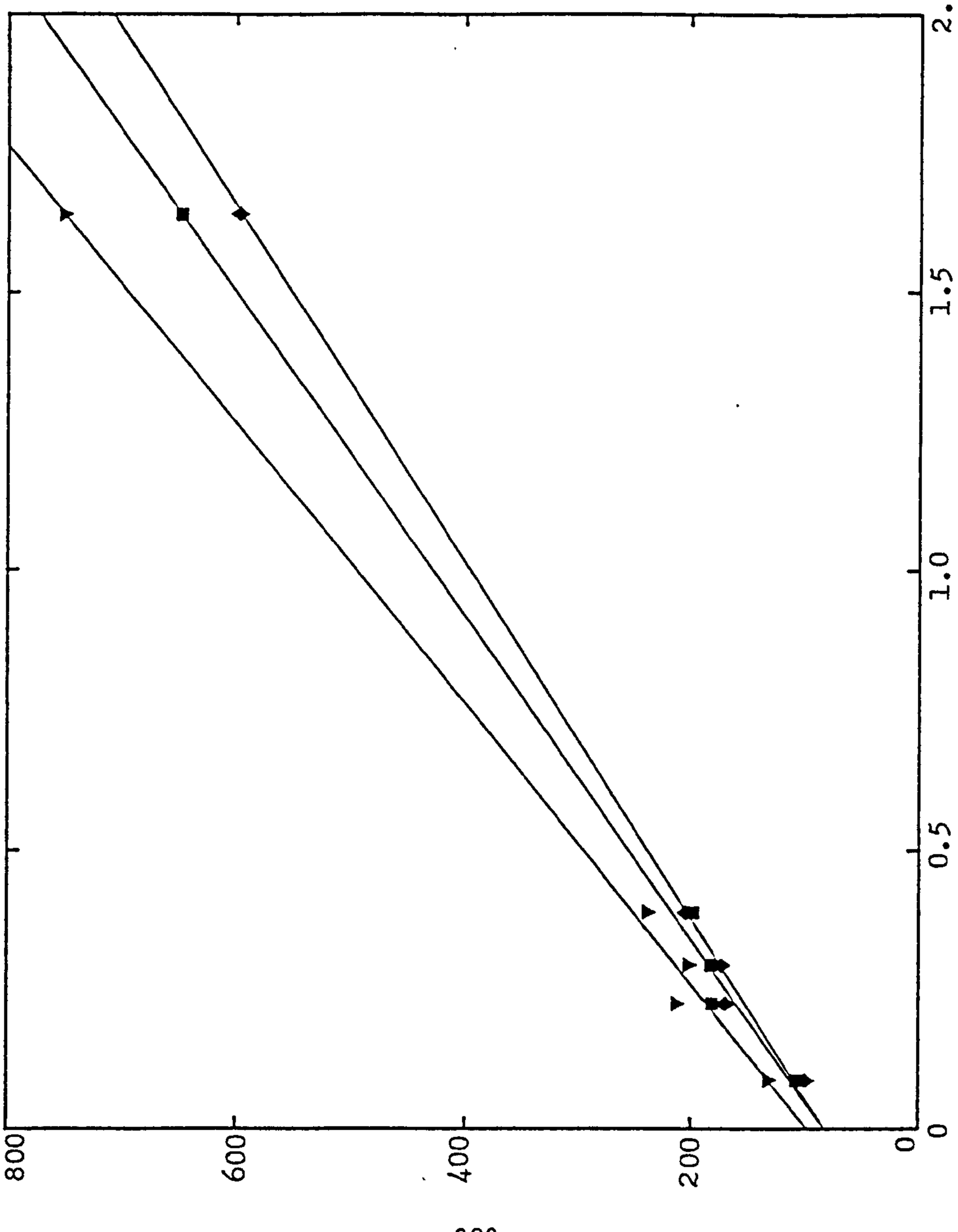


Table 3.12 Column Efficiency and Instrument Variance Calculated from Linear Regression Analysis of Peak Variance and Retention Data Presented in Table 3.11

Method of σ^2_{tot} estimation	Intercept ($=\sigma^2_{xc(t)}$) (s^2)	Gradient ($=1/N_c$)	Correlation Coefficient	N_c ($=1/$Gradient)	H_c ($=L_c/N_c$) (μm)	$\sigma^2_{xc(v)}$* (μl^2)	$\sigma_{xc(v)}$ (μl)
Half-width	83.6 ± 6.9	3.14×10^{-4} ± 0.09×10^{-4}	0.9988	3185 ± 94	78.5 ± 2.3	232.2 ± 19.2	15.2 ± 0.7
Width at half-height	83.9 ± 10.9	3.44×10^{-4} ± 0.14×10^{-4}	0.9975	2907 ± 123	86.2 ± 3.5	233.1 ± 30.3	15.3 ± 0.9
Tangential baseline width	98.9 ± 10.9	3.98×10^{-4} ± 0.14×10^{-4}	0.9981	2513 ± 91	99.5 ± 3.5	274.7 ± 30.3	16.6 ± 1.0

* $\sigma^2_{xc(v)} = \sigma^2_{xc(t)} \times Q^2$ (where $Q = 100 \mu l min^{-1}$)

from 79-100μm, the former being generated from peak half-width measurements whilst the latter is determined from tangential baseline width data. In addition, it may be noted that quantitation by the half-width and width at half-height methods yield equal values (within experimental error) for instrument variance of $232\mu l^2$ ($\equiv \sigma_{xc} = 15.2\mu l$). However, quantitation *via* the tangential baseline width method produces a significantly higher value for σ^2_{xc} of $274\mu l^2$ ($\equiv \sigma_{xc} = 16.6\mu l$).

Discrepancies in both the values obtained for H_c and those obtained for σ^2_{xc} determined *via* the three graphical techniques employed most probably arise due to the non-Gaussian nature of the peaks. Under these circumstances the peak SD equivalences used in the estimation of σ_{tot} and consequently σ^2_{tot}, are not strictly valid. A further source of error is inherent in the tangent technique, specifically that of tangent positioning. This may account for the significantly different value of σ^2_{xc} obtained by the tangent method compared with those obtained by the other two evaluation methods. On this basis, quantitation using the tangential baseline width was considered to be less reliable than the half-width and width at half-height techniques. Hence the tangential baseline width was rejected for further calculations.

Apparent plate heights were calculated by means of Equations 1.12 and 1.13 using both half-width and width at half-height measurements and these values were compared with the values for plate heights generated in the column which are indicated in Table 3.12. These data are compiled in Table 3.13. These data reveal that at a linear velocity of $0.21 cms^{-1}$, only about 28% of available column efficiency is realised for the first peak (5HTP, $k' = 2.66$) and even the last peak (5HIAA, $k' = 14.50$) falls short of realising the acceptable level according to Klinkenberg.[59] A graph of

Table 3.13 Proportions of Available Column Efficiency Realised for each Peak in a Standard Indole Chromatogram Run on the Pye Unicam NBLC System with a 25cm x 1mm ID Column Installed

			Peak Width Measurement						n = 4
			Half-width			Width at Half-height			
Peak Number	Indole	k′	H_c (μm)	$\hat{H}$ (μm)	$(H_c/\hat{H}) \times 100$ (%)	H_c (μm)	$\hat{H}$ (μm)	$(H_c/\hat{H}) \times 100$ (%)	Mean of two methods (%) ± SD
1	5HTP	2.66	78.5	278.5	28.2	86.2	300.5	28.7	28.5 ± 0.4
2	5HT	4.75	78.5	195.4	40.2	86.2	208.7	41.3	40.8 ± 0.8
3	I3S	5.53	78.5	146.5	53.6	86.2	156.7	55.0	54.3 ± 1.0
4	TP	6.55	78.5	130.5	60.2	86.2	131.0	65.8	63.0 ± 4.0
5	5HIAA	14.50	78.5	91.1	86.2	86.2	97.1	88.8	87.5 ± 1.8

percentage of column efficiency realised versus indole capacity factor (Figure 3.20) demonstrates the grossly detrimental effect of the apparatus under the operating conditions employed. The dotted line at 90% represents the level of acceptability.

It is notable that the peak due to 5HT suffers additional peak broadening compared with the other solutes; peak broadening which is not immediately obvious from the chromatogram (Figure 3.12(b)) due to the close proximity of the following I3S peak but is demonstrated in the peak width values obtained (Table 3.11). This additional broadening has the effect of increasing $\hat{H}$ and consequently leading to a relative decrease in the proportion of column efficiency realised which is observed in Figure 3.20. A similar deviation from linearity due to this phenomenon is exhibited by 5HT in Figure 3.19. The possible origins of additional band broadening will be discussed presently.

For early eluting peaks the maximum tolerable instrument dispersion without serious loss of resolution[59] may be elucidated for a well-packed column of any dimensions using Equation 1.40 (see Section 1.4.3.7), i.e.,

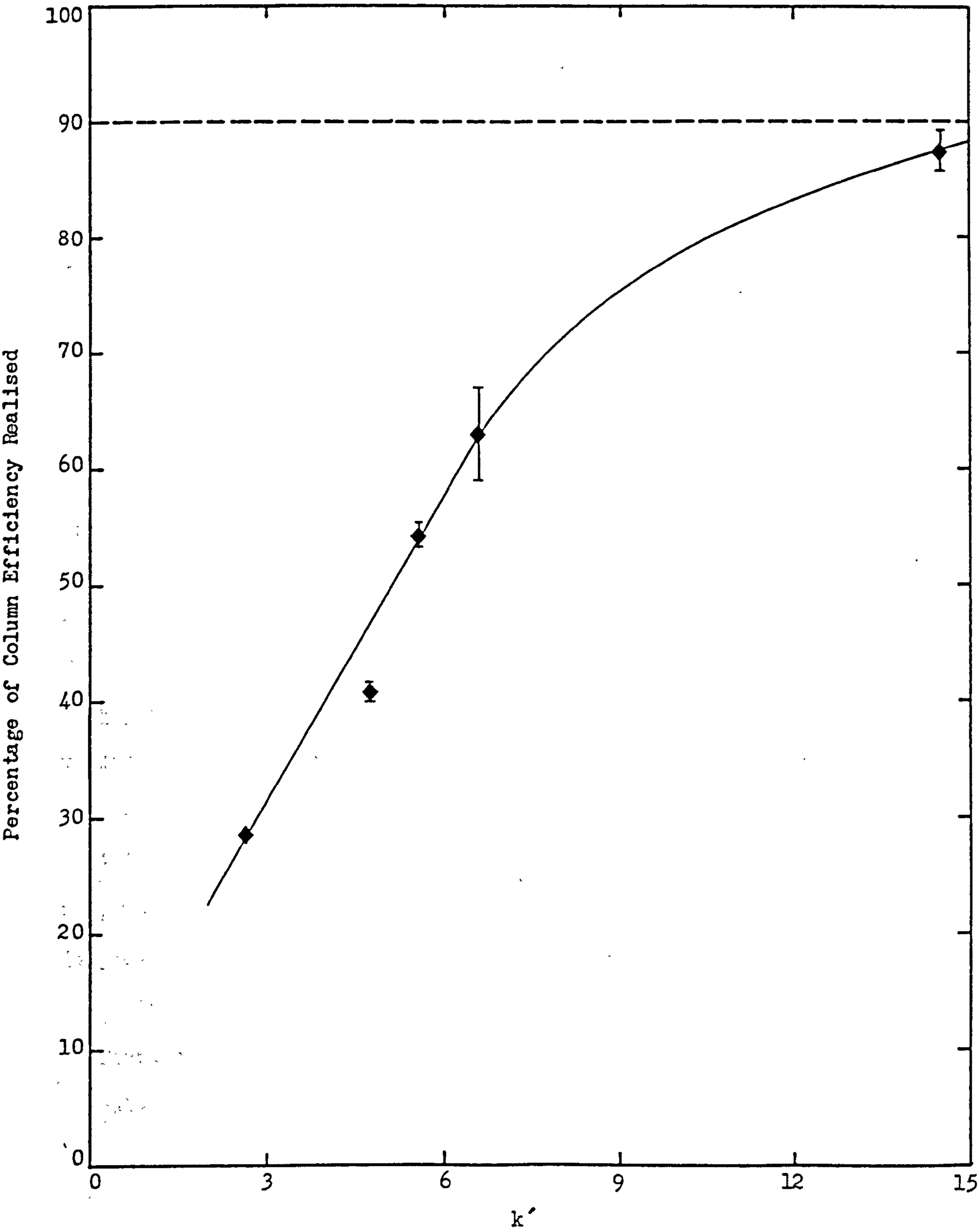
$$\sigma_{xc(v)} = 0.99\ r_c^2\ L_c^{\frac{1}{2}}\ d_p^{\frac{1}{2}} \qquad (1.40)$$

For the 25cm x 1mm ID Partisil 10 ODS3 column, $\sigma_{xc(v)}$ must be $\leq$ 0.39µl (see Table 1.8, Section 1.5.6). The experimentally determined value of $\sigma_{xc(v)}$ for the Pye Unicam NBLC system run under the conditions outlined in Figure 3.8(b) was 15.2µl which is forty fold greater than is permissible under the commonly accepted criterion for efficient operation! Equation 1.39 may also be employed in a converse sense to the above, i.e., to calculate column parameters that may be used effectively with a given chromatographic apparatus. Hence, for a 25cm long column packed with 10µm particles, a

Figure 3.20 Plot of Proportion of Available Column Efficiency Realised (Mean of Two Methods of Assessment) vs. Capacity Factor for a Series of Peaks Yielded by Chromatography of a Standard Indole Mixture on the Pye Unicam NBLC System with a 25cm x 1mm ID Column Installed

(Dashed line represents Klinkenberg's criterion of tolerability.

Standard deviation limits indicated)



column radius of 3.1mm (≡ column ID of 6.2mm) is required for efficient operation in the Pye Unicam NBLC system. Thus it may be concluded that this instrument is not even capable of running a column of conventional dimensions to the commonly accepted standard under these chromatographic conditions.

The instrument variance of 232μl^2 (≡ σ_{xc} = 15.2μl) determined for the Pye Unicam NBLC system used in this experiment was roughly comparable to variances reported in 1980 by Reese and Scott[39] for three unspecified commercial instruments operated at equivalent flow rates. However, by the outset of this project, equipment with an external band spreading contribution of not more than 3-5μl expressed as volume SD was on the market.[61] Indeed, using a similar instrument to that employed here and operating at an identical flow rate, Naish et al.[78] recently demonstrated an external band spreading contribution of this order. However, even the σ_{xc} of 4.7μl that Naish and co-workers actually obtained, is twelve times greater than permissible for effective operation of the 25cm x 1mm ID Partisil 10 ODS3 column.

The fundamental difference between the instrument evaluated by Naish et al. and that evaluated by the author was the detector. Naish et al. utilised a UV monitor with 1μl flow cell whilst an ECD with a 0.5μl flow cell was employed for the study reported herein. The implication of this fact is that, all other things being equal, the ECD is very probably largely responsible for the three fold difference in extra-column band broadening (expressed as volume SD) exhibited by the two systems.

There are a number of factors that may have contributed to the poor performance observed. It is unlikely that connections were at fault as all ferrules were carefully positioned and connecting tubing lengths were reduced

to a total of ca. 15cm, which was a little in excess of the practicable minimum for the instrumentation employed. Straight tubing of circular cross-section, 0.15mm (0.006") ID and a solute diffusivity of $3 \times 10^{-5} cm^2 s^{-1}$ will yield a maximum variance of $0.023 \mu l^2 cm^{-1}$, which is equivalent to a volume SD of $0.15 \mu l cm^{-1}$ (Equation 1.17). For the total length of tubing incorporated in the apparatus, a volume SD of less than 2.3µl would be expected, i.e., a contribution of approximately 15% to the total instrument-related band broadening. Shortening of the connecting tubing to an absolute practicable minimum length of ca. 12cm would reduce the connections contribution to band broadening by only 0.45µl. However, this would not affect the overall extra-column factor significantly.

An injection volume of 1µl was employed in this investigation. Such a volume would contribute a variance of $0.083 \mu l^2$, or a volume SD of 0.29µl (Equation 1.26). The valve internal connecting channel (< 1mm x 0.4mm ID) would contribute a maximum variance of $0.12 \mu l^2$ or a volume SD of 0.34µl (Equation 1.17). The total theoretical contribution (expressed as volume SD) of 0.63µl arising from the injection system comprises only about 4% of σ_{xc}. Hence, a reduction in injection volume would not be expected to reduce σ_{xc} significantly.

Assuming that the commercial column fittings are of satisfactory design, then by elimination, the major cause of poor performance most probably originates in the detector system as was first suspected. The wall-jet flow cell itself because of its design was unlikely to have generated much extra-column band broadening,unless the electrodes were not seated properly or a leak occurred in this part of the chromatographic system. Both these eventualities were minimised or actually eliminated as band broadening sources

by careful assembly and maintenance of the flow cell. The primary cause of the poor instrument performance is probably the electronic response characteristics of the detector which are represented by the time constant of the apparatus. A setting of 1sec, the fastest response available with the PU4022 ECD, was programmed into the unit for these experiments. This value, when compared with the maximum theoretical response time (corresponding to the entire permitted extra-column dispersion and calculated according to Equation 1.37) of 0.24sec, and a maximum practicable response time of 0.024sec, is seen to be well in excess of requirements for efficient operation of this column system at the flow rate employed.

A critical factor in the determination of maximum permissible response time is the mobile phase flow rate. A flow rate of $0.1ml min^{-1}$, which is equivalent to a linear velocity of $0.21cms^{-1}$ in a 1mm ID column, was employed for this work. Such a linear velocity, in addition to promoting the need for fast detector response characteristics, is also well above the typical optimum of ca. $0.1cms^{-1}$ for the operation of well-packed columns. Consequently a significant reduction in the inherent efficiency of the column will result, and this is reflected in the graphically-determined high values of H_c which are listed in Table 3.13. A plate height of the order of 80μm is about four times the theoretical minimum for a column well-packed with 10μm particles. Hence, it is inferred that the minimum solvent delivery rate of $0.1ml min^{-1}$ is a severely restrictive intrinsic limitation of the PU4010 pump, rendering this instrument barely satisfactory for operating 1mm ID columns except under "fast LC" conditions.

In addition to the kinetic factors considered above, thermodynamic factors can also influence peak symmetry and consequently give rise to reduced apparent column efficiency. Column overloading, a phenomenon which

occurs if a sufficiently large sample mass is introduced onto the column which prevents the attainment of equilibrium within each theoretical plate zone, is one thermodynamic phenomenon which causes skewed peaks. Effects of this nature are highly undesirable and so were avoided in these experiments by restricting the total sample mass applied to the NB column to within the loading capacity of that column. Conventionally this is taken to be 0.1% (by weight) of stationary phase in the column[401] (i.e., ca. 160ng injected).

A second factor of thermodynamic origin is the occurrence of mixed separation mechanisms within the column, e.g., solute adsorption on residual silanol groups in the presence of a dominating partition mechanism; a phenomenon which normally gives rise to peak tailing. Tailing is apparent in Figure 3.8(b). Such a situation may only be remedied in one of three ways. First, by addition of a surface modifier to the mobile phase which would be likely to influence the partition chromatography too. Secondly, by permanent modification of the stationary phase with a suitable reagent to "cap" the remaining silanol groups. This is inappropriate here because consistency and continuity of supply are necessary for routine operation purposes. Thirdly, by transferring the separation to an alternative commercial stationary phase. This possibility will be considered further in future experiments.

In summary, the Pye Unicam NBLC system under evaluation was found to be of insufficiently high specification to successfully operate 1mm ID columns by commonly accepted criteria. The major source of extra-column dispersion was almost certainly the PU4022 ECD time response characteristics. A critical limiting factor was the minimum flow rate obtainable with the PU4010 pump. In conjunction with the instrument inadequacies, the 1mm ID

column was subject to fairly rapid blockage, resulting in short column lifetime from the exclusive application of standard solutions. Injection of extracts from biological samples (e.g., isolates from blood and urine) would be expected to shorten the column lifetime even further by a process of contamination. Both the inability of the instrumentation to operate 1mm ID columns combined with the practical problems encountered with the latter, led to the decision to abandon columns of this geometry.

A compromise between potential advantages attainable with reduced ID columns and practicalities of operating such columns was settled upon. 2.1mm ID columns possess a cross-sectional area of $3.5mm^2$ which is only a factor of five times smaller than that of conventional 4.6mm ID columns. Consequently, compared with 1mm ID columns with cross-sectional areas of only $0.8mm^2$, 2.1mm ID columns would be expected to exhibit longer column lifetimes. Furthermore, as was shown in Chapter 1, the instrument specifications required to operate columns efficiently become less and less stringent with increasing column ID. Thus, columns of approximately 2mm ID were applied to the analytical problem under investigation.

3.4.4 Chromatography on 2mm ID Reverse Phase Columns

3.4.4.1 Stationary Phase Selection

Previous experiments with Partisil 10 ODS3 had shown this stationary phase, when operated in the reverse phase partition mode with a MeOH:water-based mobile phase, to exhibit reasonable selectivity towards the indoles of interest. However, Partisil 10 ODS3 was not generally available in pre-packed columns of ca. 2mm ID in 1984/5 (see Table 1.9). Hence, an alternative stationary phase that was currently marketed in the 2mm ID format

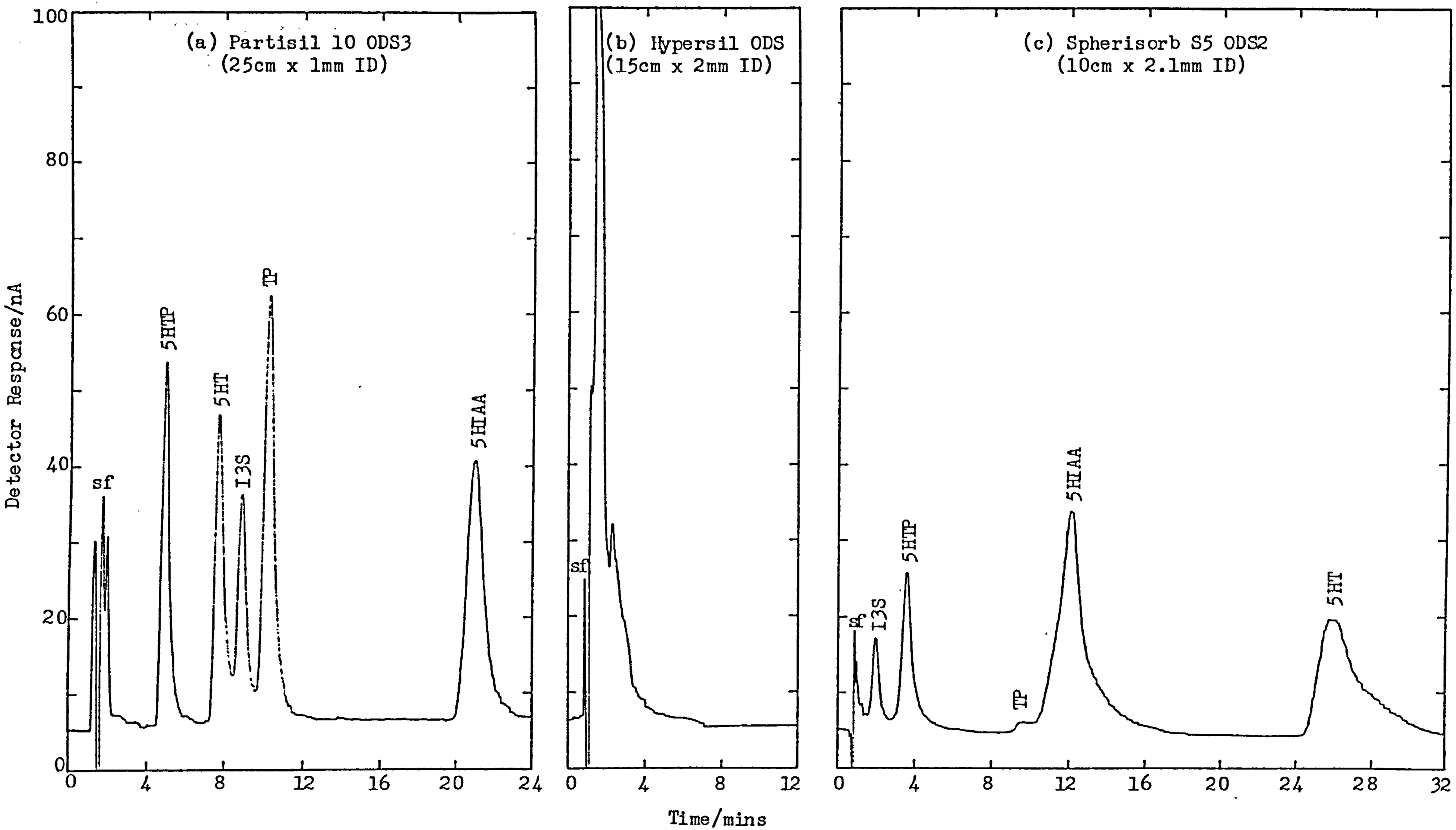
was sought. Four loose packing materials, *viz*. Hypersil ODS, Spherisorb S5 ODS1, Spherisorb S5 ODS2 and μ-Bondapak ODS were to hand in the laboratory; all of which were obtainable in pre-packed columns of the desired geometry. Specifications of these stationary phases, together with Partisil 10 ODS3 are presented in Table 3.7 (see Section 3.3.3.4). Available column hardware was of the Shandon-Southern format illustrated in Figure 3.11 (see Section 3.3.3.2). Three columns were slurry-packed in the laboratory, *viz*. Hypersil ODS (15cm x 2mm ID), Spherisorb S5 ODS1 (15cm x 2mm ID) and μ-Bondapak ODS (20cm x 2mm ID). In addition, two commercially packed columns were supplied by Pye Unicam for assessment, *viz*. Spherisorb S5 ODS1 (10cm x 2.1mm ID) and Spherisorb S5 ODS2 (10cm x 2.1mm ID); the former was supplied on the basis of preliminary results from this study.

All five columns were operated under identical conditions to those applied in conjunction with the 1mm ID Partisil 10 ODS3 column, i.e., with a mobile phase composition of 4% MeOH : 96% aq. 20mM NaAc/HCt "buffer" (pH 4.00) pumped at a flow rate of $0.4 ml min^{-1}$ ($\equiv u = 0.19\text{-}0.21 cms^{-1}$ depending upon the exact ID of the column), using an injection volume of 1μl and ECD settings of +1.00V *vs*. Ag/AgCl potential and a time constant of 1sec. A methanolic standard containing TP, 5HTP, 5HT, 5HIAA and I3S was chromatographed a minimum of twice on each stationary phase and individual indole standards were also chromatographed for peak identification and subsequent elucidation of elution order. Typical separations obtained on each stationary phase are presented in Figure 3.21. Mean capacity factors were calculated for each solute and these values are tabulated in Table 3.14, together with the values obtained previously with the Partisil 10 ODS3 phase.

From Figure 3.21(b) it is clear that the indoles exhibit very little affinity for the Hypersil ODS column. All five solutes elute coincidentally

Figure 3.21 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Various Stationary Phases

Parameters :- Column : as specified, Mobile Phase : 4% MeOH : 96% aq. 20mM NaAc/HCt buffer, pH 4.00; Flow Rate : 0.1mlmin^{-1} (1mm ID), 0.4mlmin^{-1} (2mm ID); Detection : ECD (Eapp = +1.00V vs. Ag/AgCl, TC = 1sec), Sample : 1µl via loop of TP, 5HTP, 5HT. creatinine sulphate.H_2O, 5HIAA and I3S ($K^{\oplus}$ salt) in MeOH (All constituents @ 5µgml^{-1} ≡ 5ng injected)



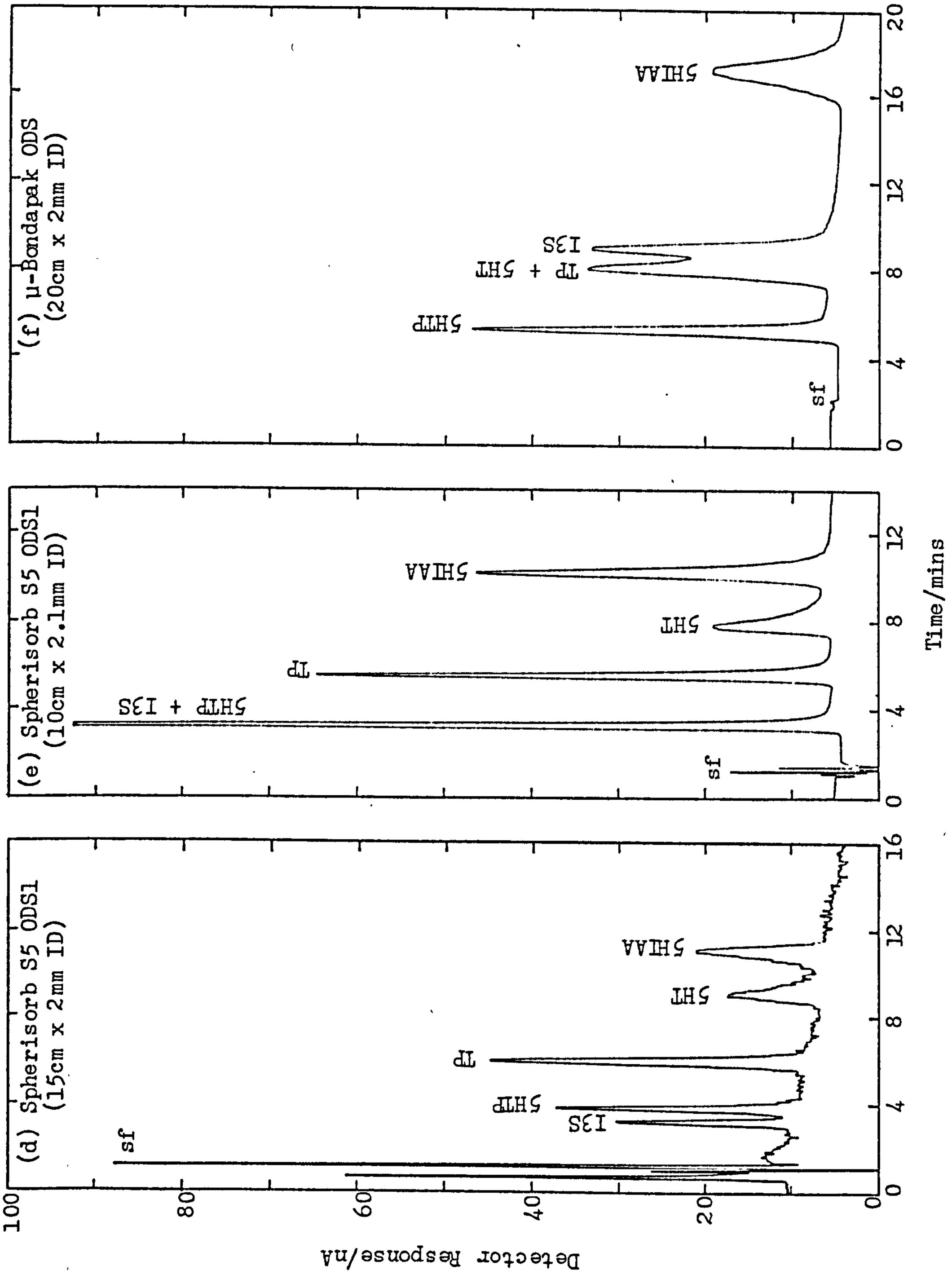


Figure 3.21 continued

Table 3.14 Retention Times and Capacity Factors of Indoles Chromatographed on Various Stationary Phases.

Stationary Phase	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
Partisil 10 ODS†	1.4	10.2	6.55	4.9	2.66	7.7	4.75	20.6	14.50	8.8	5.53
Hypersil ODS§	Not measured										
Spherisorb S5 ODS1§	0.7	6.0	7.57	3.8	4.43	9.0	11.86	11.0	14.71	3.2	3.57
Spherisorb S5 ODS1†	0.6	5.0	7.33	2.8	3.67	7.1	10.90	9.6	15.00	2.8	3.67
Spherisorb S5 ODS2†	0.7	9.6	12.71	3.7	4.23	25.9	36.00	12.2	16.43	2.0	1.86
μ-Bondapak ODS2§	2.2	8.2	2.73	5.5	1.48	8.1	2.68	17.9	7.14	9.2	3.18

† commercially packed column; § laboratory packed column

with the solvent front when employing the aforementioned mobile phase. Omission of the MeOH modifier from the mobile phase provided very little promotion of retention. The apparent plate count ($\hat{N}$) and subsequently the apparent plate height ($\hat{H}$) of the Hypersil column were calculated from a peak generated by indole-3-acetic acid (IAA, $k' = 18.67$), which was employed as a solute probe, in order to establish the quality of this laboratory-packed column. These quantities were calculated too for the remainder of the columns under assessment using the 5HIAA peak. Values were determined by employing both the width at half-height and the half-width and the results were averaged for each stationary phase. These data are compiled in Table 3.15. On comparison of the apparent plate counts, it is clear that the reason for the poor retention properties of the Hypersil ODS column, observed under the aforementioned operating conditions, is not the column efficiency (which is comparable with other stationary phases), but most probably results from the incompatible surface geometry and chemical nature of the packing material. For this reason the Hypersil ODS stationary phase was rejected.

Apart from Hypersil ODS all the other stationary phases exhibited some retention properties. The Spherisorb S5 ODS2 column provided reasonable selectivity and relatively long retention times (Figure 3.21(c)), the latter being expected of an ODS phase of high surface coverage. However, peak shape was highly unsatisfactory. Gross peak tailing was evident which reduced the apparent plate number for the column to only 550, which was extremely poor. Excess dead volume in the column system was eliminated as the source of this phenomenon. Therefore it was deduced that the tailing resulted from thermodynamic mechanisms, which was surprising because Spherisorb S5 ODS2 is claimed to be fully capped.[389] Tailing to this degree renders

Table 3.15 Apparent Plate Count and Apparent Plate Height Data for the Columns Under Evaluation

Figure Number	Stationary Phase	Column Origin*	L_c (cm)	$\hat{N}$			$\hat{H}$ (μm)		
				$w_{0.5}$	$w_{0.607}$	Mean ± SD	$w_{0.5}$	$w_{0.607}$	Mean ± SD
3.21(a)	Partisil 10 ODS3	C	25	2626	2730	2678 ± 74	95.2	91.5	93.4 ± 2.6
3.21(b)	Hypersil ODS	L	15	2347	2557	2452 ± 148	63.9	58.7	61.3 ± 3.7
3.21(c)	Spherisorb S5 ODS1	L	15	2138	2101	2120 ± 26	70.2	71.4	70.8 ± 0.8
3.21(d)	Spherisorb S5 ODS1	C	10	2413	2304	2359 ± 77	41.4	43.4	42.4 ± 1.4
3.21(e)	Spherisorb S5 ODS2	C	10	519	595	557 ± 54	192.7	168.1	180.4 ± 17.4
3.21(f)	μ-Bondapak ODS	L	20	1233	1334	1284 ± 71	162.2	149.9	156.1 ± 8.7

* C = commercially packed; L = laboratory packed

Spherisorb S5 ODS2 inappropriate for the separation of these indoles with MeOH : aqueous-type mobile phases under these operating conditions and so this phase too was rejected.

From the stationary phase specifications (Table 3.7), surface coverage calculations alone suggest that Spherisorb S5 ODS1 ($0.32 mgm^{-2}$) and µ-Bondapak ODS ($0.33 mgm^{-2}$) most closely resemble Partisil 10 ODS3 ($0.30 mgm^{-2}$). Indeed, this is observed to be the case from the chromatograms (Figures 3.21a, d,e and f) and is similarly evident when indole capacity factors are plotted against stationary phase (Figure 3.22) or, more revealingly, versus stationary phase surface coverage (Figure 3.23). It may be noted that elution orders vary between stationary phases. This differing behaviour is well recognised and results from a congregation of various factors such as differences in the base silica (particle shape, pore diameter, etc.), C_{18} loading, surface geometry, and the degree of residual silanol group capping. The method of manufacture is instrumental in controlling the majority of these factors and this may be responsible for the break in the general trend of increasing k′ with increasing surface coverage exhibited by the µ-Bondapak ODS phase (see Figure 3.23). It may be noted too that resolution is better on the laboratory-packed Spherisorb S5 ODS1 column rather than on the commercially-packed equivalent, in spite of the former demonstrating a smaller apparent plate count (see Table 3.15). This minor but significant difference in behaviour is probably attributable to batch-to-batch variation in stationary phase manufacture.

From these initial studies the laboratory-packed µ-Bondapak and the commercially-packed Spherisorb S5 ODS1 columns were selected for further investigation.

Figure 3.22 Relative Capacity Factors for a Series of Indoles Chromatographed under Identical Conditions on Various Stationary Phases

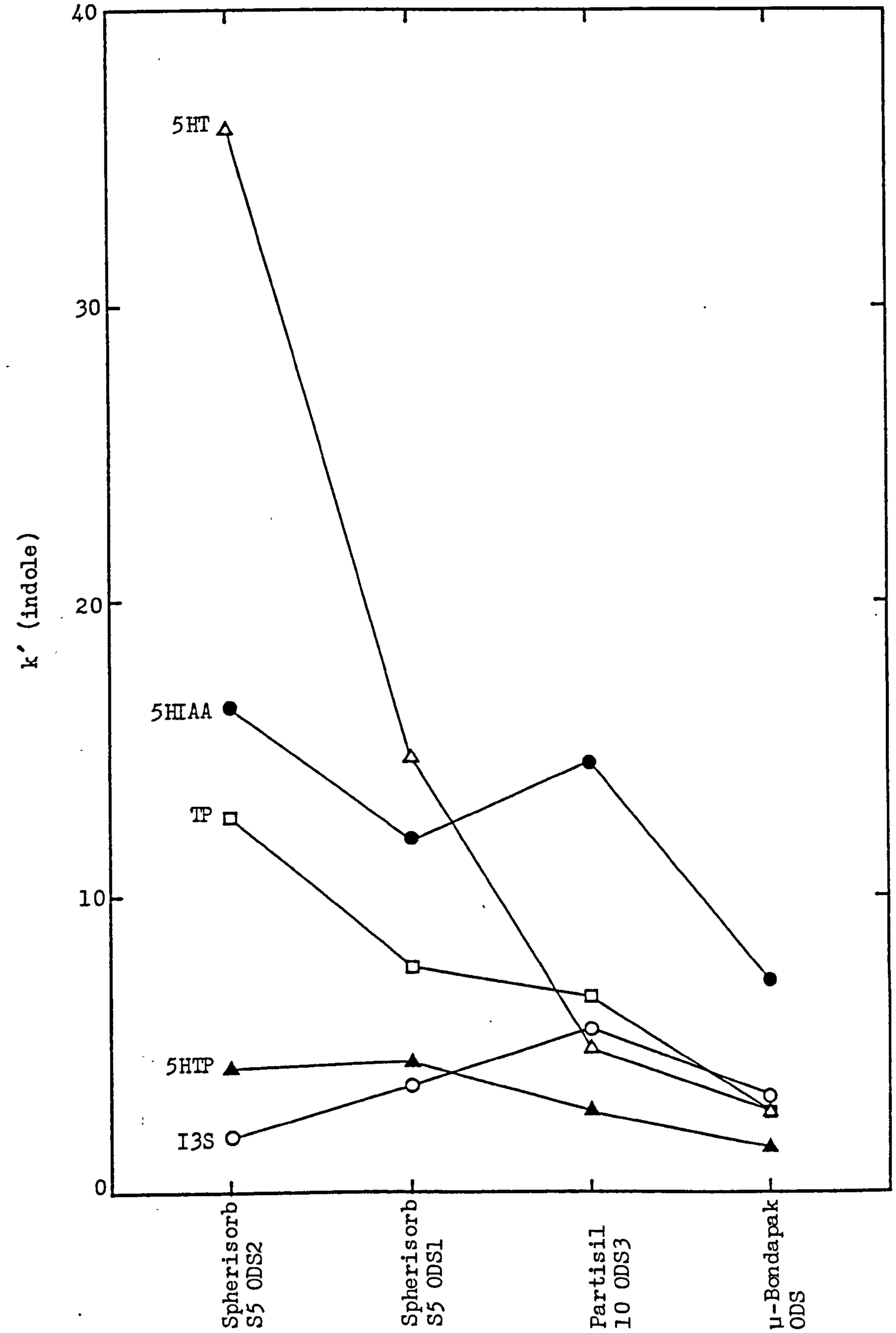
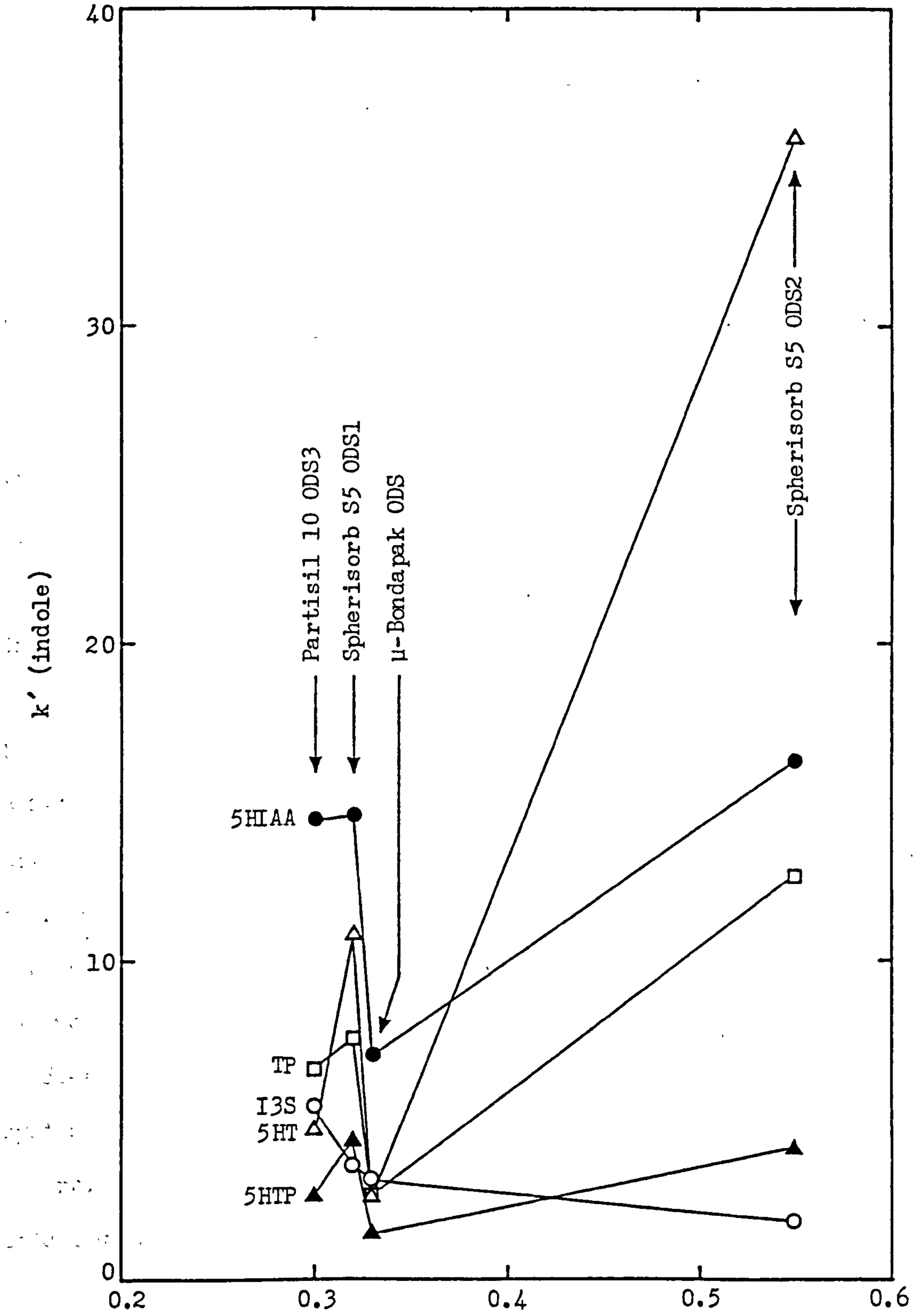


Figure 3.23 The Relationship Between Capacity Factor and Stationary Phase Surface Coverage for a Series of Indoles Chromatographed under Identical Conditions on Various Stationary Phases



3.4.4.2 Optimisation of the Reverse Phase Partition Chromatography of Indoles on μ-Bondapak ODS

3.4.4.2.1 Variation of Buffer Type

A pH adjustment buffer is included in the mobile phase for solute speciation control. The composition selected originally comprised a NaAc/HCt combination which is not a true buffer. A true buffer consists of a solution of an acid and its conjugate base (utilised in the form of a soluble salt of the acid), in approximately equal concentrations. The effect of using a true buffer system, in this case NaAc/HAc, on the chromatography of indoles is illustrated in Figure 3.24. The same indole standard mixture was chromatographed in duplicate, with identical mobile phases except for buffer type and individual indoles were also run for peak identification. Mean capacity factors were calculated for each solute and are presented in Table 3.16. The relative retentions under the influence of each buffer system are illustrated in Figure 3.25.

The observed difference in separation performance attributable to buffer type is remarkable. When an acetate buffer is employed (Figure 3.24(b)) resolution is much improved over that achieved with the acetate/citrate combination (Figure 3.24(a)), but concomitantly, solute retention is significantly reduced. This effect implies that the citrate moiety in some way, directly or indirectly, promotes retention of the indoles in the μ-Bondapak ODS surface, which the acetate moiety either does not do at all or does to a lesser extent. One possibility is that the acetate/citrate system, because it is not a true buffer, is not veryefficient at maintaining control of pH. Perhaps it is changes in the pH of the enviroment inside the column which promote changes in indole speciation with consequent changes

Figure 3.24 Reverse Phase Partition Chromatography of a Standard Indole Mixture on μ-Bondapak ODS Employing Two Different Buffer Systems at Identical pHs (4.00)

Parameters :- Column : μ-Bondapak ODS (20cm x 2mm, d_p = 10μm), Mobile Phase : 4% MeOH : 96% aq. 20mM buffer (constituents as specified), pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : ECD (Eapp = +1.00V vs. Ag/AgCl, TC = 1sec); Sample : 1μl via loop of TP, 5HTP, 5HT.creatinine sulphate.H_2O, 5HIAA and I3S ($K^{\oplus}$ salt) in MeOH (All constituents @ 5μgml^{-1} ≡ 5ng injected)

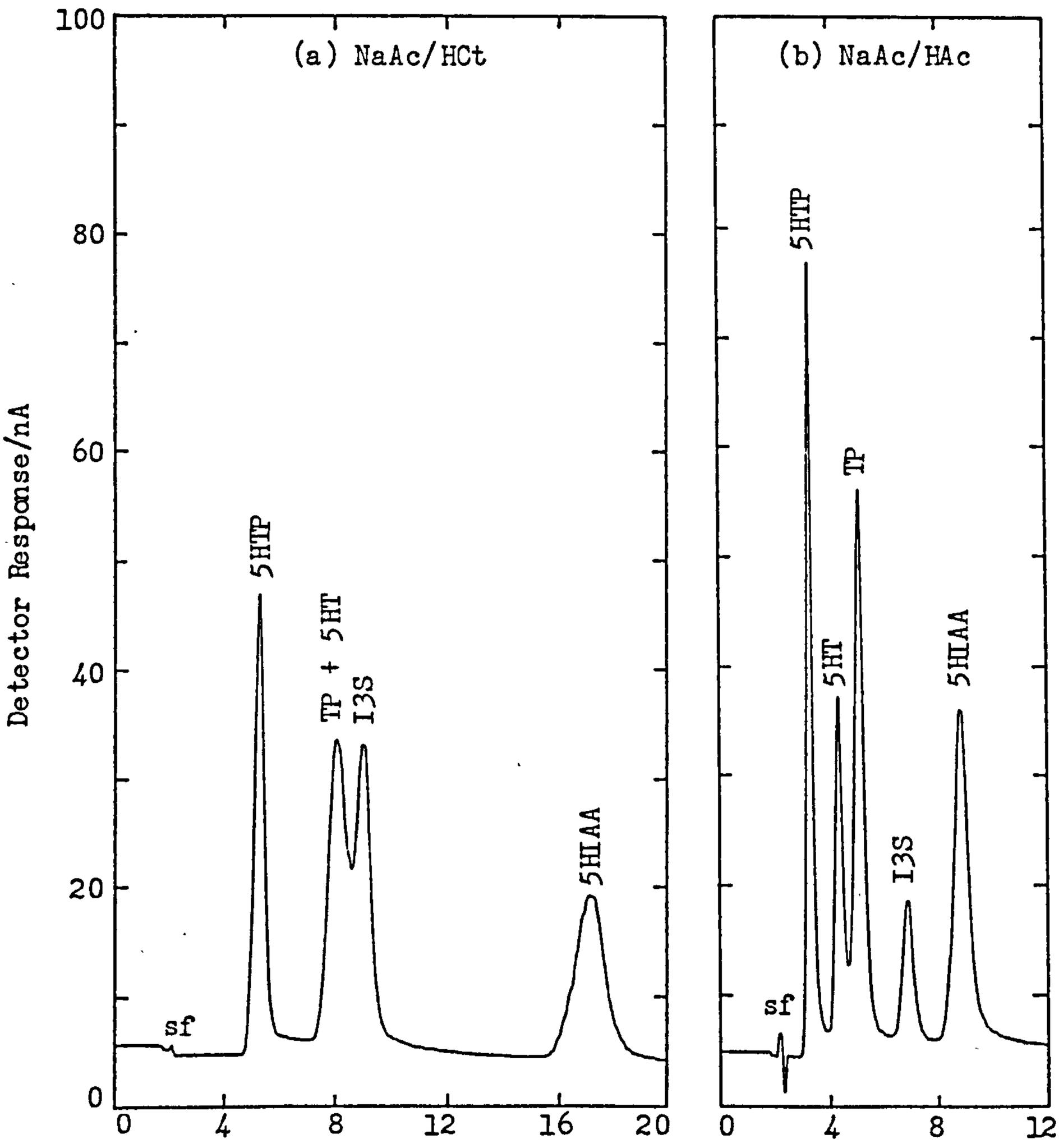
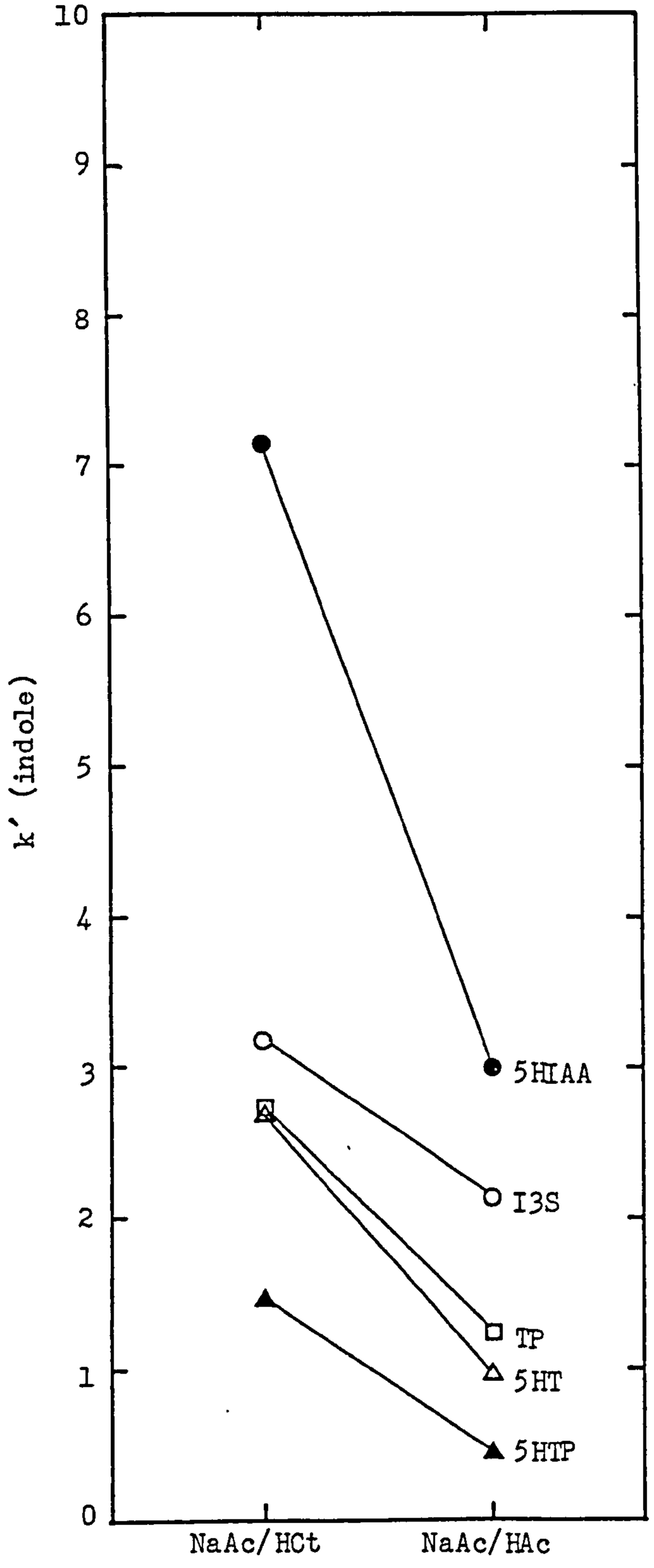


Table 3.16 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on μ-Bondapak ODS using Various Buffer Compositions

Buffer System	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
NaAc/HCt	2.2	8.2	2.73	5.5	1.48	8.1	2.68	17.9	7.14	9.2	3.18
NaAc/HAc	2.2	5.0	1.27	3.2	0.45	4.3	0.95	8.8	3.00	6.9	2.14

Figure 3.25 Relative Capacity Factors for a Series of Indoles Chromatographed on μ-Bondapak ODS Employing Two Different Buffer Systems at Identical pHs (4.00)



in affinity for the stationary phase. A second explanation of this behaviour may be that the citrate moiety acts as an ion-interaction agent in some fashion and promotes indole retention by ion-pair association. This phenomenon has been reported for the acetate moiety[402] but the very weak nature of the interaction was also noted. The citrate anion would be expected to give rise to a stronger retentive interaction than acetate in view of their respective structures. This postulation is consistent with the experimental observations. The true reason for the marked variation in capacity factor with buffer type was not investigated further.

The acetate buffer system, although favourable because it provides improved and acceptable resolution, does suffer one disadvantage, namely that the indoles elute over the k′ range 0.45-3.00. For reasons of minimisation of the effects of extra-column dispersion, it was deemed necessary for the first peak of interest to elute at $k' \geq 2$. Hence, while employing an acetate buffer for selectivity, promotion of solute retention by reduction of the mobile phase MeOH content was attempted.

3.4.4.2.2 Variation of MeOH Content

The indole standard mixture was chromatographed (in duplicate at least) on the μ-Bondapak ODS column under the aforementioned conditions, using mobile phases incorporating an acetate buffer with MeOH contents ranging from 1-4%. The resulting chromatograms are compiled in Figure 3.26. Peaks were identified in the usual manner. Mean capacity factors for each analyte were calculated and are displayed in Table 3.17.

Indole peak shapes were excellent with these phase systems, near-Gaussian profiles being observed for all solutes. From the chromatograms

Figure 3.26 Reverse Phase Partition Chromatography of a Standard Indole Mixture on μ-Bondapak ODS with Various Concentrations of MeOH in the Mobile Phase

Parameters :- For conditions see Figure 3.24 except for Mobile Phase : MeOH : aq. 20mM NaAc/HAc buffer (modifier concentration as specified), pH 4.00

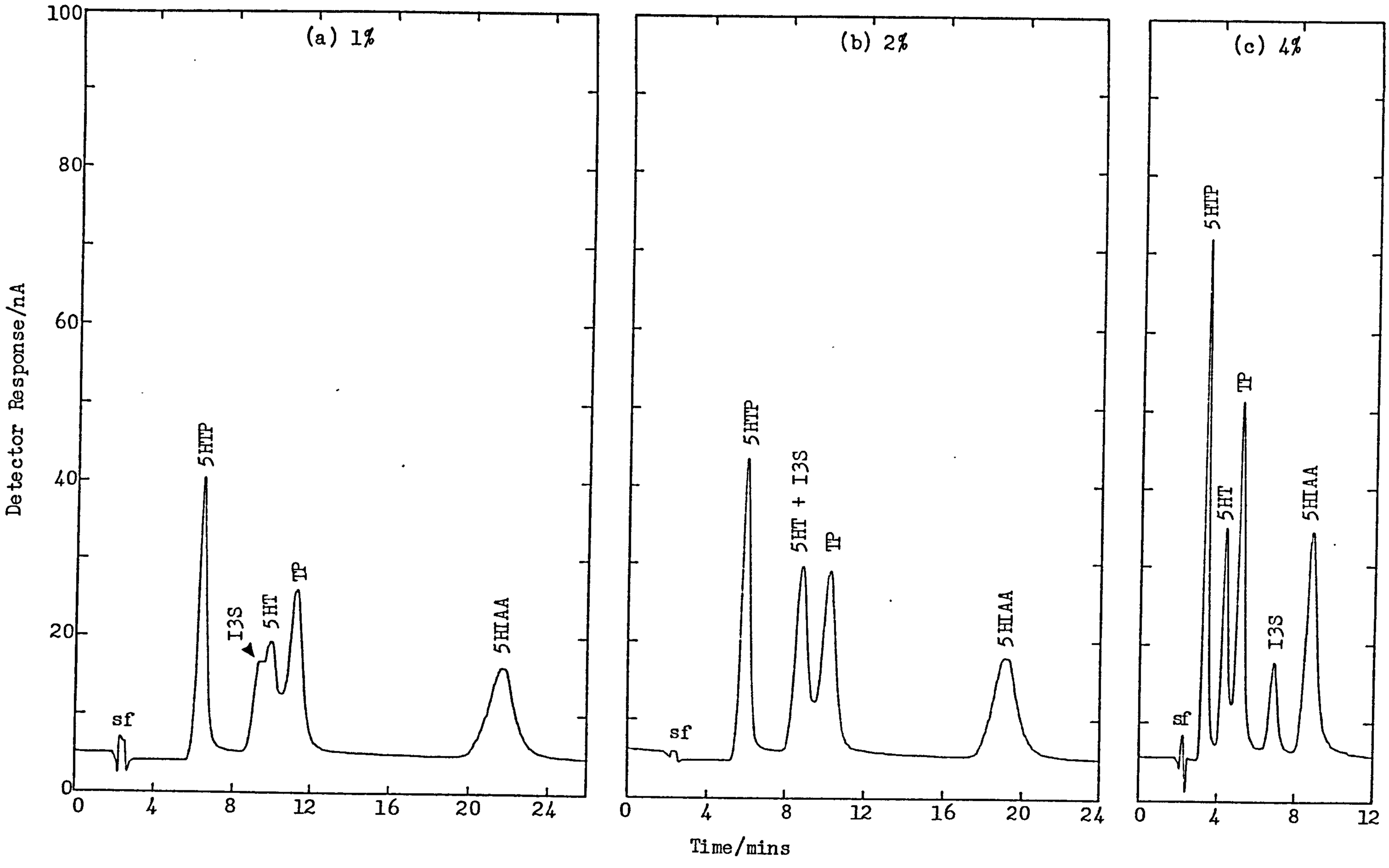


Table 3.17 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on μ-Bondapak ODS with Various Concentrations of MeOH Modifier in the Mobile Phase.

% MeOH	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
1	2.2	11.1	4.05	6.3	1.86	9.8	3.45	21.5	8.77	9.4	3.27
2	2.2	10.0	3.55	4.7	1.14	8.6	2.91	19.00	7.64	8.6	2.91
4	2.2	5.0	1.27	3.2	0.45	4.3	0.95	8.8	3.00	6.9	2.14

(Figure 3.26) and the extracted data (Table 3.17), it was evident that decreasing the MeOH content of the mobile phase (i.e., decreasing mobile phase polarity) had the desired effect of increasing solute capacity factors. However, the degree of increased retention observed was substance dependent and led to an unacceptable loss of resolution. Figure 3.27 comprises a plot of indole k′ values versus percent MeOH modifier which demonstrates the resolution problem graphically. It may also be noted that the MeOH content of the mobile phase must be reduced to < 1%, in order to satisfy the imposed condition of the first peak of interest eluting at k′ $\geq$ 2. Such a level would be difficult to reproduce accurately in practice. It would also leave no possibility for reductions in MeOH content, which are frequently required to compensate for the gradual but inevitable deterioration of the column with extended use. In an effort to increase indole capacity factors without loss of resolution, but with sufficient organic modifier in the mobile phase to enable minor adjustments to be made, the use of other less polar modifiers was investigated.

3.4.4.2.3 Variation of Organic Modifier

Propan-2-ol (2-PrOH; ε^{o} = 0.82*) and acetonitrile (MeCN; ε^{o} = 0.65) were readily available as less polar alternatives to MeOH (ε^{o} = 0.95). Mobile phases were prepared comprising 4% v/v modifier in aqueous acetate buffer adjusted to pH 4.00. Duplicate chromatograms were obtained of the five component indole standard on the μ-Bondapak ODS column using each mobile phase and these chromatograms are presented in Figure 3.28. Again, peak

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* ε^{o} = Snyder's solvent strength function.[32]

Figure 3.27 The Relationship Between Capacity Factor and MeOH Content of the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Partition Conditions on μ-Bondapak ODS

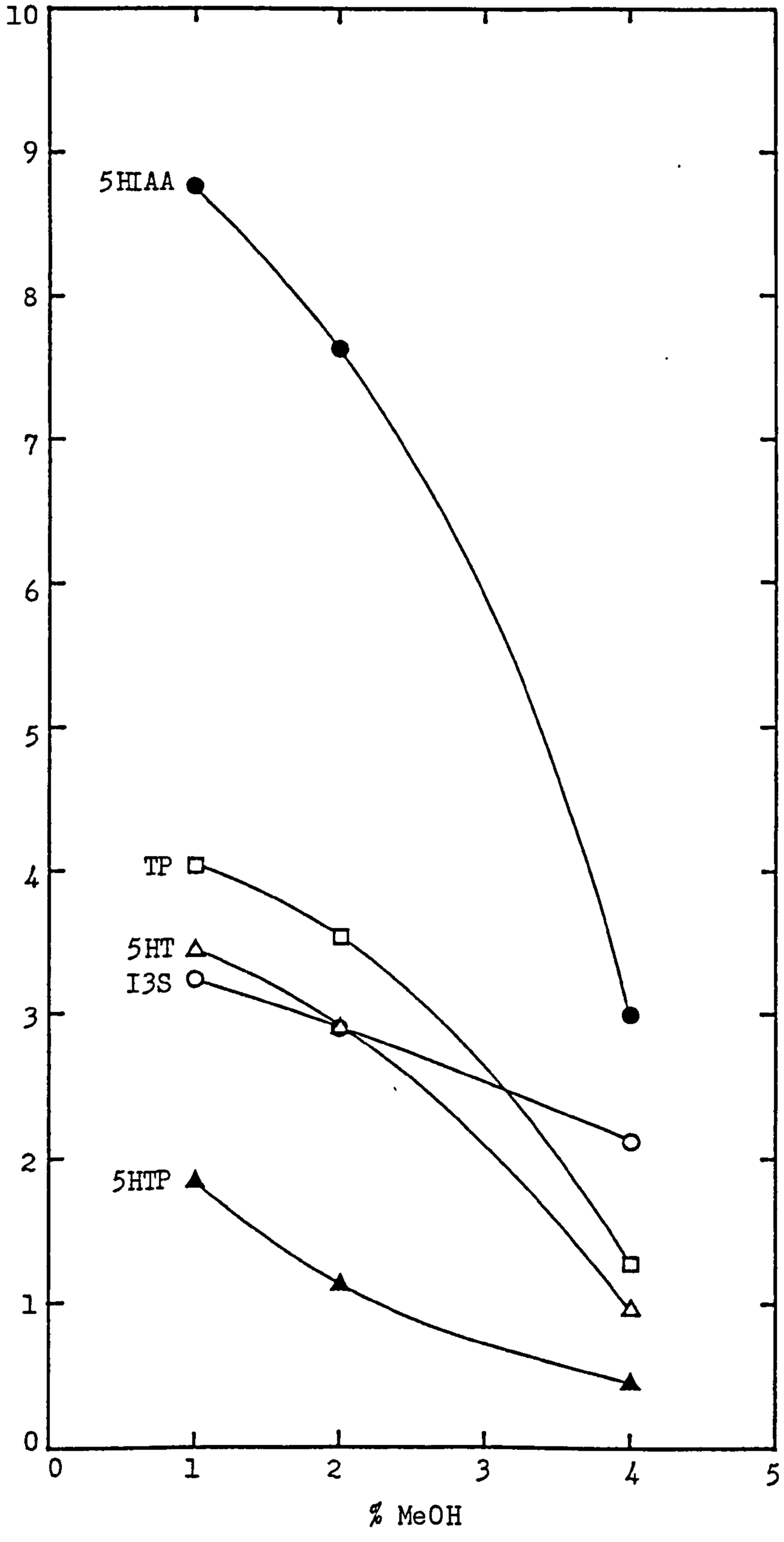
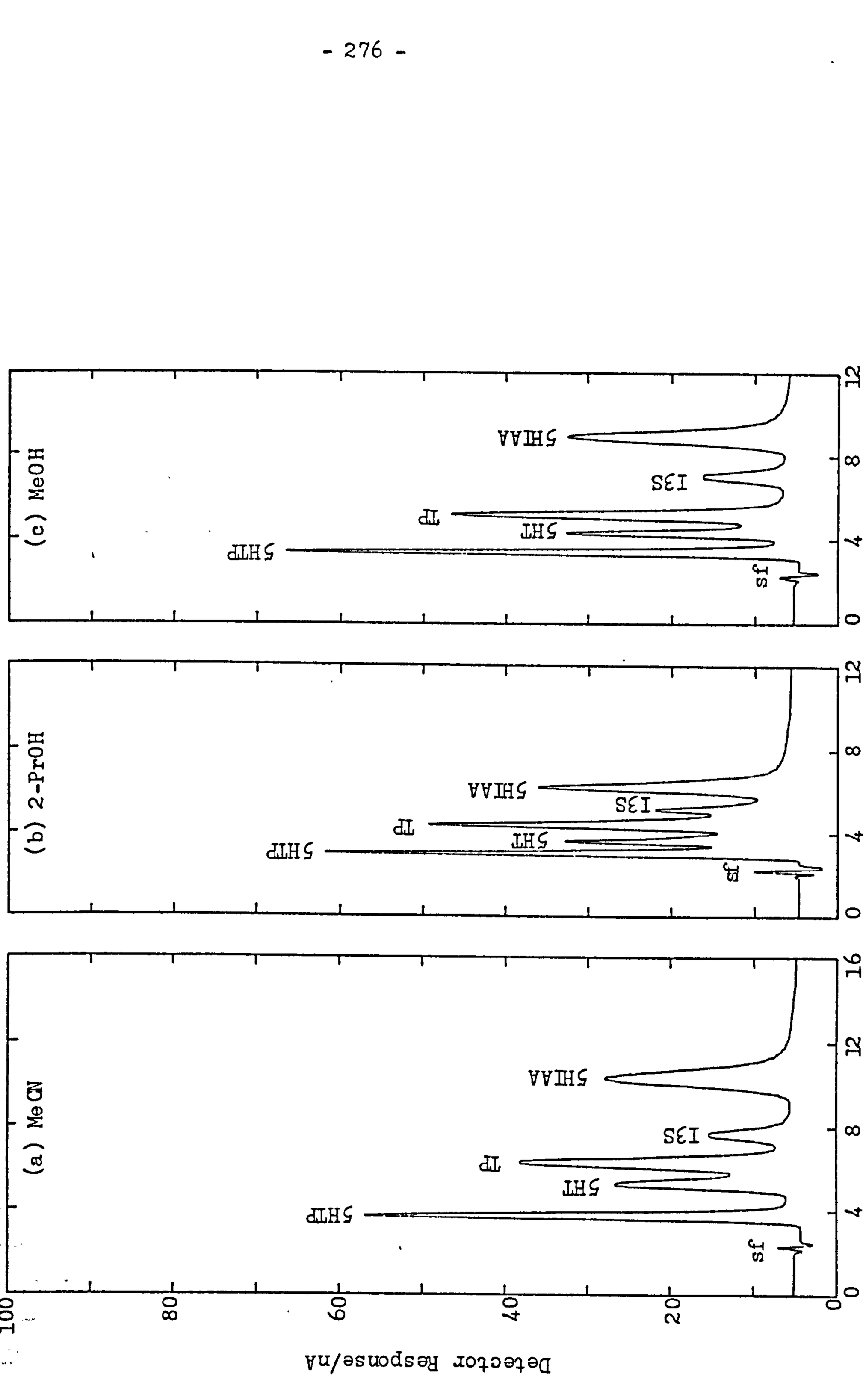


Figure 3.28 Reverse Phase Partition Chromatography of a Standard Indole Mixture on μ-Bondapak ODS Employing Various Organic Modifiers at Identical Concentrations (4% v/v)

Parameters :- For conditions see Figure 3.24 except for Mobile Phase : 4% modifier : 96% aq. 20mM NaAc/HAc buffer (organic modifier as specified), pH 4.00



identification was achieved by chromatography of individual indole standards. Mean capacity factors for each solute calculated from these traces are given in Table 3.18. The influence of organic modifier polarity on solute capacity factor is illustrated in Figure 3.29.

Great care was taken to isolate the effects of each modifier by thorough and prolonged washing of the column between elution of the different mobile phases. For similar reasons, a minimum of 1 hour equilibration time was allowed for each mobile phase before commencement of chromatography. This period was equivalent to greater than 50 column volumes of solvent at the flow rate employed. The results obtained indicate no perceptible change in selectivity with change in organic modifier at the 4% level and excellent near-Gaussian peak shapes were observed in each chromatogram. Substitution of MeCN for MeOH resulted in the desired increase in k′ values, but bandwidths also increased significantly yielding an unfavourable decrease in resolution. Resolution was quantitated using Equation 3.1 and assuming Gaussian peak profiles such that $w_B = 2w_{0.607}$. Calculated values of resolution are presented in Table 3.19.

Table 3.19 Resolution Obtained on a μ-Bondapak ODS Column for a Series of Indoles Chromatographed using Various Organic Modifiers (4% v/v) in the Mobile Phase

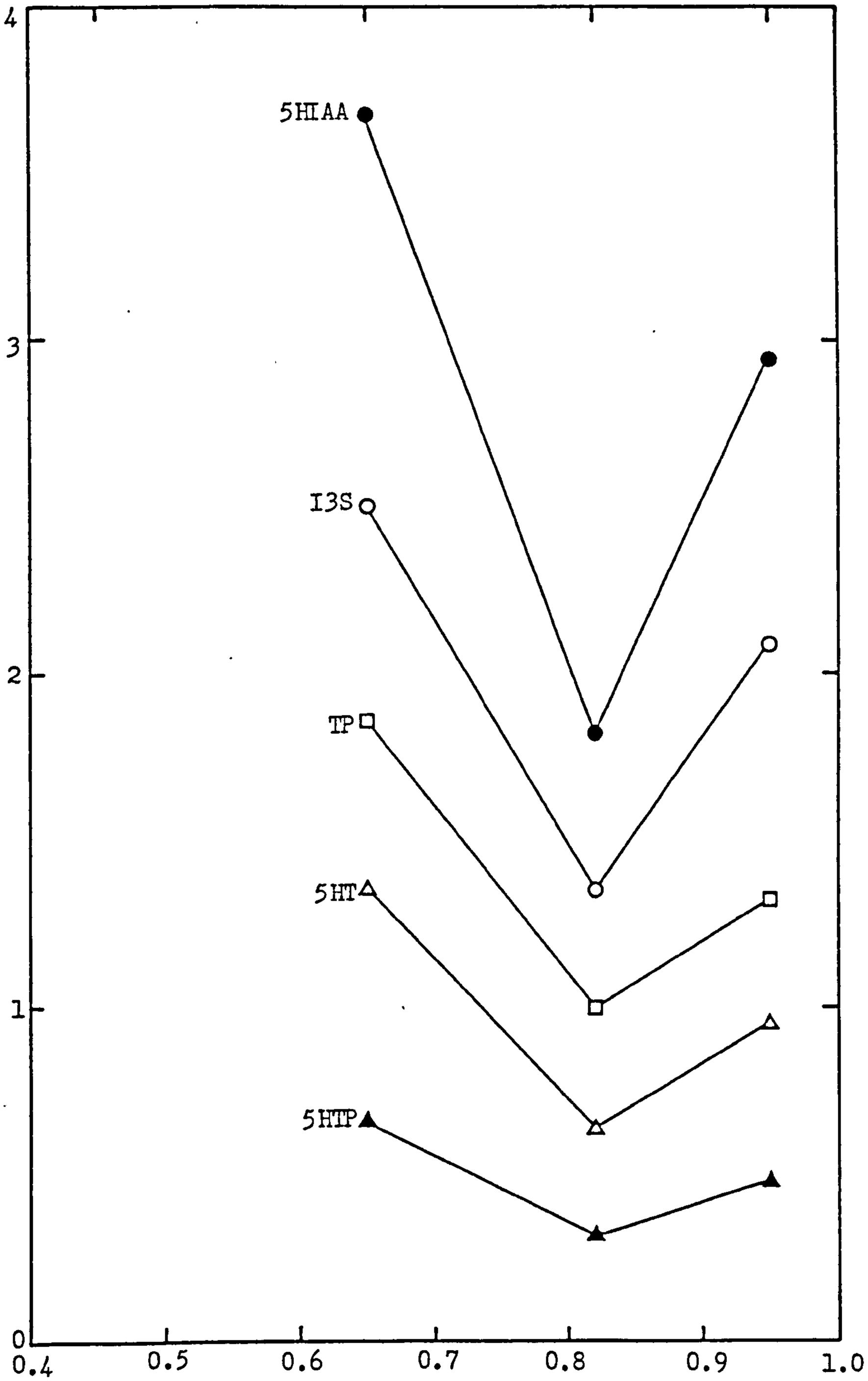
Organic Modifier	t_R(min)		$w_{0.607}$(min)		w_B(min)*		R_s
	X	Y	X	Y	X	Y	
MeCN	5.2	6.3	0.46	0.50	0.92	1.00	1.15
2-PrOH	4.4	5.16	0.30	0.34	0.60	0.68	1.19
MeOH	4.3	5.1	0.28	0.30	0.56	0.60	1.38

* $w_B = 2w_{0.607}$ for a Gaussian peak profile

Table 3.18 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on μ-Bondapak ODS with Various Organic Modifiers (4% v/v) in the Mobile Phase.

Organic Modifier (4% v/v)	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
MeCN	2.2	6.3	1.86	3.7	0.66	5.2	1.36	10.3	3.68	7.7	2.50
2-PrOH	2.2	4.4	1.00	2.9	0.32	3.6	0.64	6.2	1.82	5.2	1.35
MeOH	2.2	5.0	1.27	3.2	0.45	4.3	0.95	8.8	3.00	6.9	2.14

Figure 3.29 The Relationship Between Capacity Factor and Organic Modifier Polarity (Represented by Snyder's Solvent Strength Parameter, ε^o) for a Series of Indoles Chromatographed under Reverse Phase Partition Conditions on μ-Bondapak ODS



The (undesirable) decrease in resolution which ensued when MeCN was used in place of MeOH is clearly demonstrated. This incidence of poorer resolution is entirely due to the disproportionately larger bandwidths observed in the presence of MeCN. This difference in chromatographic behaviour most probably results from the difference in the solvation properties of MeOH and MeCN. MeOH is a protic solvent which interacts largely by hydrogen bonding, whereas MeCN is an aprotic solvent which solvates predominantly via dipole interactions. This fundamental difference in physical properties would be expected to influence the type and extent of any secondary processes occurring in the system and thus could conceivably give rise to the discrepancy indicated.

The utilisation of 2-PrOH as modifier also gave rise to reduced resolution compared with MeOH. Moreover, all peaks eluted more rapidly with the use of the former alcohol than the latter. This observation was surprising because 2-PrOH, the less polar solvent, was expected to promote retention on the column. A further hour for equilibration (≡ 50+ column volumes of solvent) was allowed, but an identical chromatogram was obtained following this period. Hence, the influence of 2-PrOH on the partition chromatography of indoles on μ-Bondapak ODS was confirmed. Such behaviour may possibly be explained by competition for sites on the column surface between 2-PrOH and the analytes but this postulation is entirely speculative and there is no confirmatory evidence to support this proposal.

3.4.4.2.4 Summary of the Reverse Phase Partition Chromatography of Indoles on μ-Bondapak ODS

The greatest resolution achieved to date for the five indoles TP, 5HTP, 5HT, 5HIAA and I3S, on μ-Bondapak ODS was obtained employing a mobile

phase of 4% MeOH : 96% aqueous acetate buffer adjusted to pH 4.00 (e.g., Figure 3.24(b)). Capacity factors of the first three eluted solutes were considered to be too small, in view of the fact that extra-column dispersion effects become increasingly dominant as k′ tends towards zero. Furthermore, interference from poorly retained components frequently present in authentic sample extracts could be problematical. The use of lower concentrations of MeOH in the mobile phase gave rise to unacceptably decreased resolution, as did the employment of MeCN and 2-PrOH as alternative organic modifiers. Because of the difficulties encountered with the promotion of retention on μ-Bondapak ODS, without unacceptable loss of resolution resulting, Spherisorb S5 ODS1, the promising alternative stationary phase, was investigated.

3.4.4.3 Optimisation of the Reverse Phase Partition Chromatography of Indoles on Spherisorb S5 ODS1

3.4.4.3.1 Variation of Buffer Type

The major problem with the separation on the commercial Spherisorb S5 ODS1 column was not retention but rather selectivity. In the initial investigation on this stationary phase, 5HTP and I3S coeluted when a 4% MeOH : 96% aqueous NaAc/HCt mobile phase adjusted to pH 4.00 was employed (see Figure 3.21(e)). On the μ-Bondapak ODS material, it was found that the use of a NaAc/HAc buffer improved selectivity. Hence this approach to selectivity modification was examined for Spherisorb S5 ODS1.

Chromatograms of the five component indole standard were obtained in duplicate using the above mobile phase, incorporating the NaAc/HCt "buffer" system and one of identical composition except that a NcAc/HAc buffer was substituted. Typical examples of these chromatograms are given in

Figure 3.30. Peaks were identified by single indole standard injection. Mean capacity factors for each solute were calculated and are compiled in Table 3.20 and the relative retentions under the influence of each buffer system are illustrated in Figure 3.31.

As was the case on the μ-Bondapak ODS column, all indole capacity factors were reduced when an acetate buffer was employed. As Table 3.20 shows, the k′ value of the first peak was still greater than 2 and thus was still acceptable. However, in contrast to the behaviour observed on μ-Bondapak ODS, no improvement in selectivity was achieved by the utilisation of an acetate buffer (5HTP and I3S still coeluted). In fact, a serious loss in resolution between the later eluting peaks due to 5HT and 5HIAA resulted. A discussion of possible reasons for the variations in retention brought about by changes in buffer composition may be found in Section 3.4.4.2.1.

Hence, from this experiment it is obvious that neither mobile phase system investigated herein was satisfactory for the separation of the five analytes. Because the capacity factor of the first peak was relatively close to the minimum acceptable value, there was little scope for increase in mobile phase MeOH content in order to improve selectivity. Reduction in MeOH content was very limited also, and based on the experiments with the μ-Bondapak ODS material, was deemed unlikely to produce the major changes in selectivity required. The effect of organic modifier nature was investigated however.

3.4.4.3.2 Variation of Organic Modifier

In addition to the modifiers employed with the μ-Bondapak ODS column (i.e., MeOH, 2-PrOH and MeCN) a further solvent was available for trial,

Figure 3.30 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Spherisorb S5 ODS1 Employing Two Different Buffer Systems at Identical pHs (4.00)

Parameters :- Column : Spherisorb S5 ODS1 (10cm x 2,1mm, dp = 5µm); Mobile Phase : 4% MeOH : 96% aq. 20mM buffer (constituents as specified), pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : ECD (Eapp = +1.00V *vs.* Ag/AgCl, TC = 1sec); Sample : 1µl *via* loop of TP, 5HTP, 5HT.creatinine sulphate.H_2O, 5HIAA and I3S ($K^{\oplus}$ salt) in MeOH (All constituents @ 5µgml^{-1} ≡ 5ng injected)

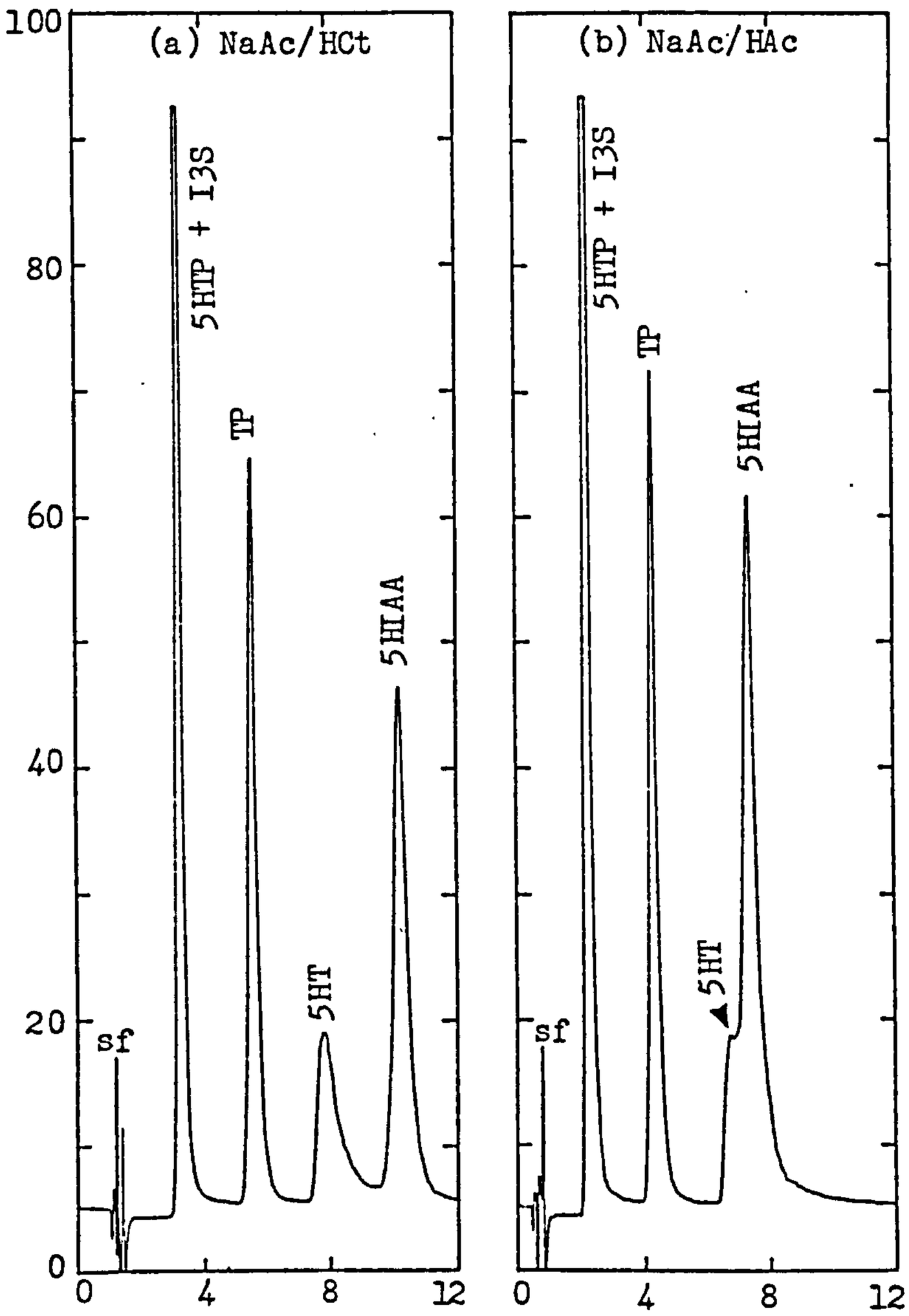
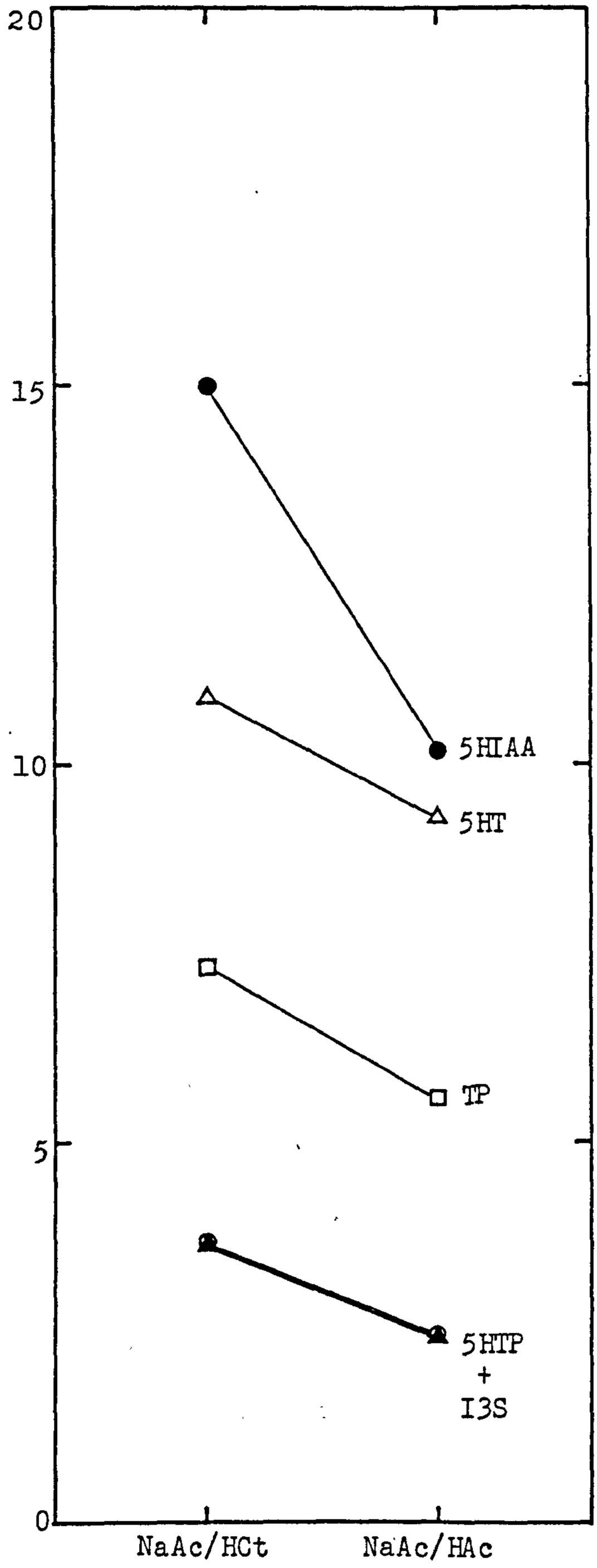


Table 3.20 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on Spherisorb S5 ODS1 using Various Buffer Compositions.

Buffer System	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
NaAc/HCt	0.6	5.0	7.33	2.8	3.67	7.1	10.90	9.6	15.00	2.8	3.67
NaAc/HAc	0.66	4.3	5.58	2.3	2.48	6.8	9.30	7.4	10.21	2.3	2.48

Figure 3.31 Relative Capacity Factors for a Series of Indoles Chromatographed on Spherisorb S5 ODS1 Employing Two Different Buffer Systems



<u>viz</u>. tetrahydrofuran (THF; ε^{o} = 0.45). Mobile phases were prepared comprising 4% v/v modifier in aqueous acetate buffer adjusted to pH 4.00. The acetate buffer system was selected in preference to the acetate/citrate "buffer" (which, with MeOH modifier, was slightly superior) in order to enable direct comparison of behaviour on the Spherisorb S5 ODS1 and µ-Bondapak ODS stationary phases to be made.

The standard indole mixture was chromatographed in duplicate on the Spherisorb S5 ODS1 column with each mobile phase system in turn. As was the case when carrying out this experiment with the µ-Bondapak ODS phase, sufficient time was allowed for column washing and re-equilibration between mobile phases in order to isolate the effects of each organic modifier. Typical chromatograms are compiled in Figure 3.32. As usual, peaks were identified by chromatography of single indole standards. In Table 3.21 are displayed the mean capacity factors calculated for each analyte in each phase system. Figure 3.33 illustrates the effect of organic modifier polarity on k′.

These results show that organic modifier type exerts a profound influence on both selectivity and retention. With the use of THF as modifier, 5HTP and I3S were separated but I3S was observed to coelute with 5HT resulting in poor and unsatisfactory resolution. Furthermore, all solutes eluted rapidly such that only 5HT and 5HIAA satisfied the $k' \geq 2$ criterion.

With MeCN in the mobile phase all five components were well separated initially (R_s = 2.22). Peak shapes were generally near-Gaussian except for that of 5HT which exhibited considerable tailing. In addition, only 5HTP failed to satisfy the minimum capacity factor criterion, but it was considered that a slight reduction in MeCN content may serve to promote retention times sufficiently to comply with this requirement. Unfortunately, this separation

Figure 3.32 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Spherisorb S5 ODS1 Employing Various Organic Modifiers at Identical Concentrations (4% v/v)

Parameters :- For conditions see Figure 3.30 except for Mobile Phase : 4% modifier : 96% aq. 20mM NaAc/HAc buffer (organic modifiers as specified), pH 4.00

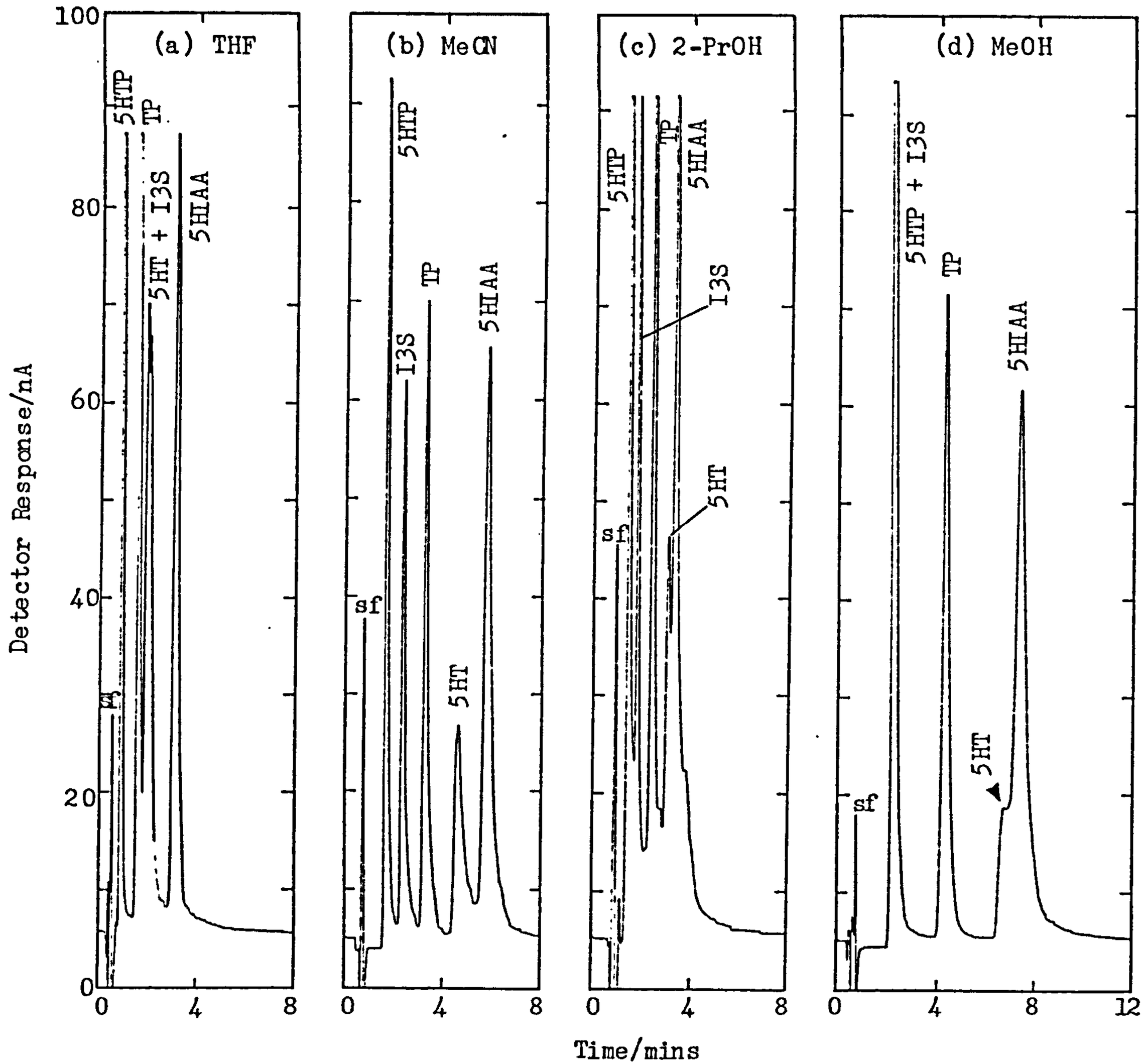
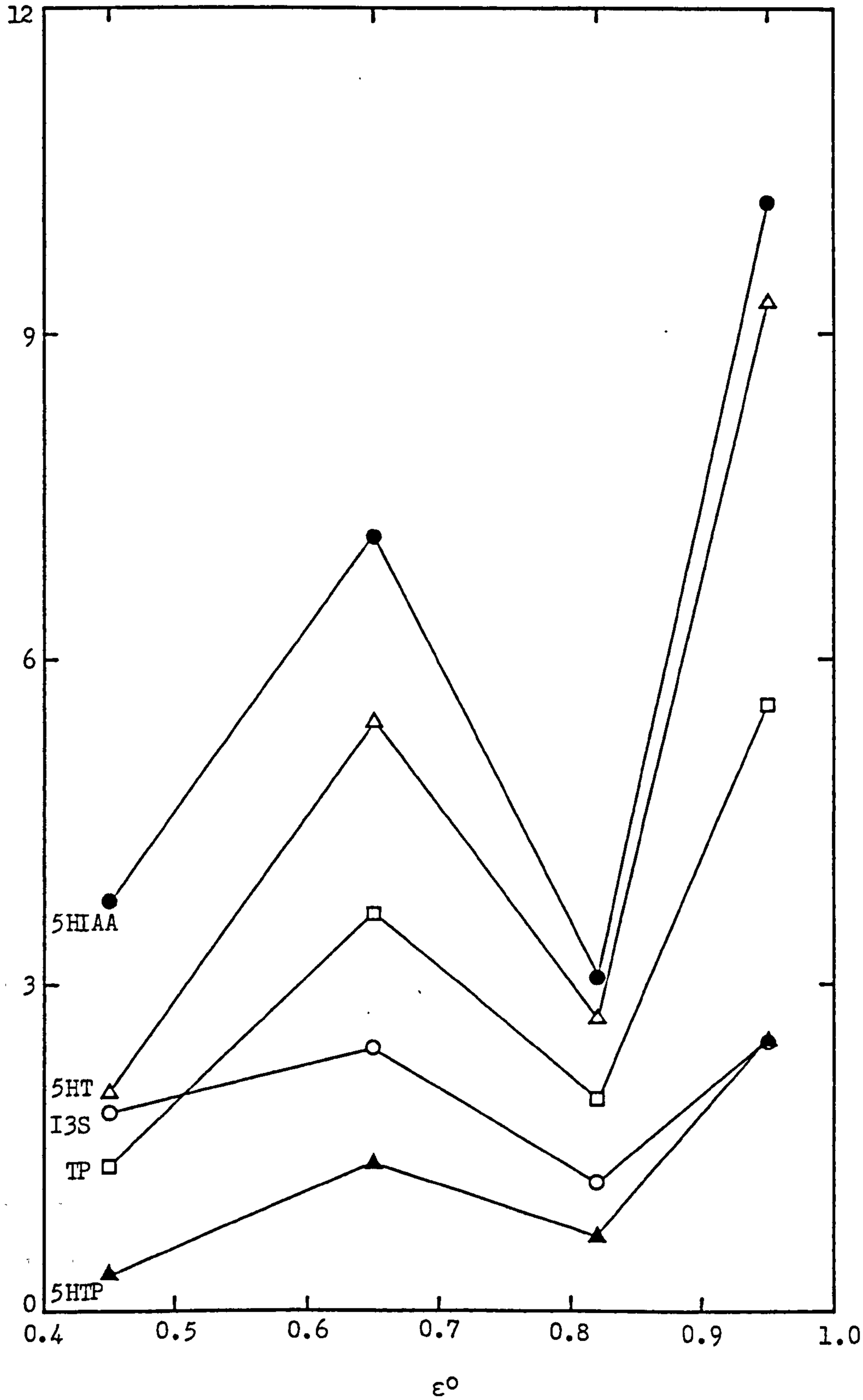


Table 3.21 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on Spherisorb S5 ODS1 with Various Organic Modifiers (4% v/v) in the Mobile Phase.

Organic Modifier (4% v/v)	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
THF	0.6	1.4	1.33	0.8	0.33	1.8	2.00	2.86	3.77	1.7	1.83
MeCN	0.7	3.26	3.66	1.66	1.37	4.5	5.43	5.7	7.14	2.4	2.43
2-PrOH	0.8	2.36	1.95	1.36	0.70	2.94	2.68	3.26	3.08	1.74	1.18
MeOH	0.66	4.3	5.58	2.3	2.48	6.8	9.30	7.4	10.21	2.3	2.48

Figure 3.33 The Relationship Between Capacity Factor and Organic Modifier Polarity (Represented by Snyder's Solvent Strength Parameter, ε^o) for a Series of Indoles Chromatographed under Reverse Phase Partition Conditions on Spherisorb S5 ODS1



deteriorated over only a few injections (R_s = 1.29) as illustrated in Figure 3.34.

The utilisation of 2-PrOH as modifier, like THF and MeCN, also achieved the separation of 5HTP and I3S. In addition, 5HT and 5HIAA were a little better resolved than was the case with the MeOH modifier. However, all solutes eluted very rapidly resulting in only the two most retarded solutes eluting with sufficiently large k′ values. A similar situation was encountered when 2-PrOH was employed in conjunction with μ-Bondapak ODS.

From the plot of capacity factor versus organic modifier polarity (Figure 3.33), it may be noted that there is a general trend to shorter retention with reduction in modifier polarity. This trend is opposite to expectations; a reduction in mobile phase polarity being predicted to bring about an increase of retention. Clearly modifier polarity does not appear to be the controlling factor here and, more probably, differences in mechanism of solvation are instrumental in influencing the state of the chromatography. A similar conclusion was reached to explain the chromatographic behaviour on the μ-Bondapak ODS column.

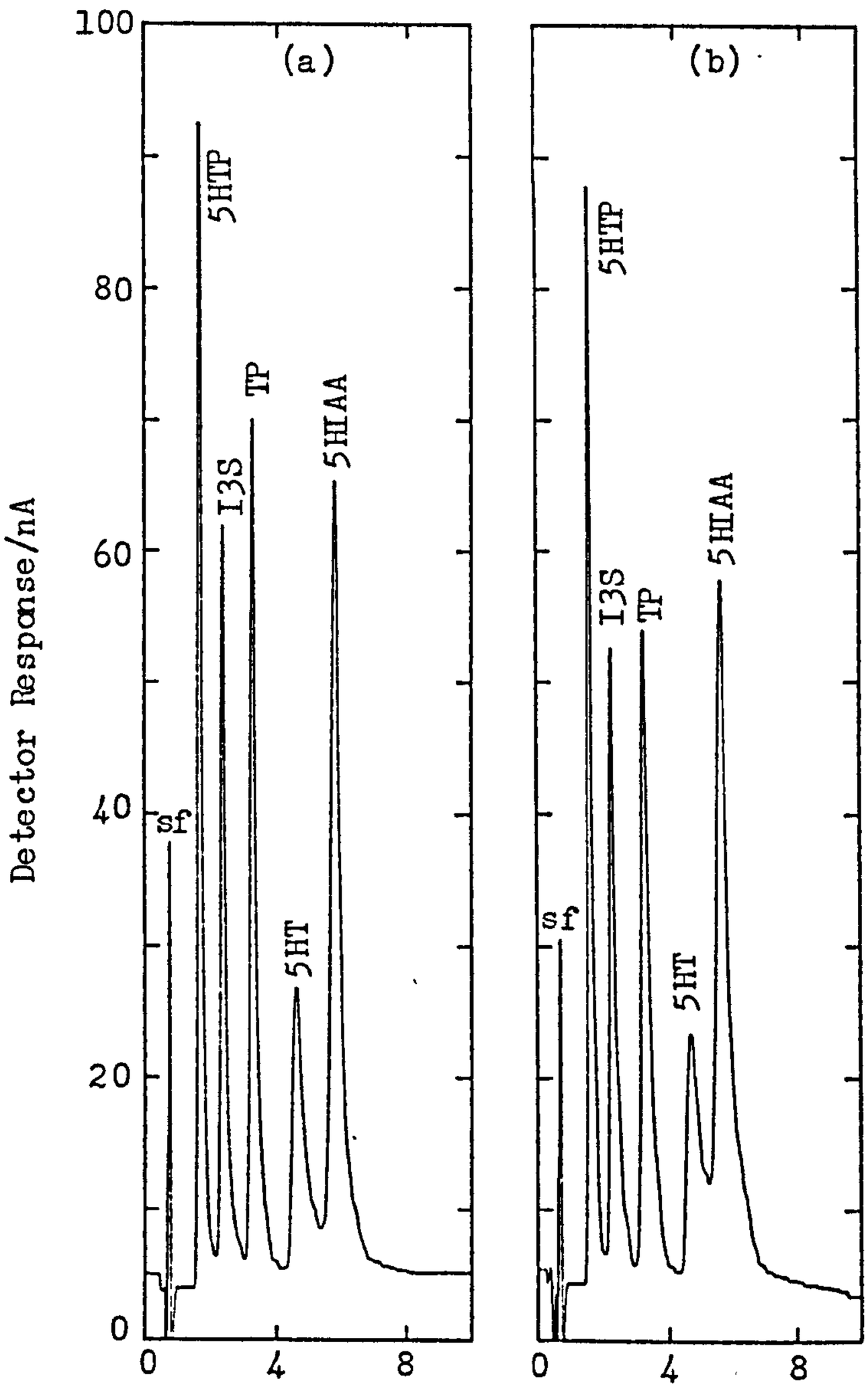
3.4.4.3.3 Summary of the Reverse Phase Partition Chromatography of Indoles on Spherisorb S5 ODS1

The use of MeOH as organic modifier provided sufficient retention of all five indoles on Spherisorb S5 ODS1 ($k'_{(min)}$ = 2.48) but selectivity was unsatisfactory (see Figures 3.30(b) and 3.32(d)).

The best separation of the five indoles TP, 5HTP, 5HT, 5HIAA and I3S achieved to date on Spherisorb S5 ODS1 is illustrated in Figures 3.32(b) and 3.34(a) for which a mobile phase comprising 4% MeCN : 96% aqueous acetate buffer adjusted to pH 4.00 was employed. This degree of

Figure 3.34 Chromatograms Illustrating the Deterioration with Time of the Reverse Phase Partition Separation of Indoles on Spherisorb S5 ODS1 Observed when MeCN is used as Organic Modifier

Parameters :- For conditions see Figure 3.30 except for Mobile Phase : 4% MeCN : 96% aq. 20mM NaAc/HAc buffer, pH 4.00



performance was not sustained however and the chromatogram presented in Figure 3.34(b), is more representative. The first peak in this chromatogram elutes at $k' < 2$. Hence promotion of retention would be advantageous in order to relax to some degree the severity of instrument specifications required to operate 2mm ID columns efficiently, and to avoid potential interferences from early eluting impurities frequently present in authentic samples. Slight reduction of mobile phase MeCN content may achieve this goal. However, this experiment was not performed due to the manifestation of a major practical problem.

3.4.4.4 Practical Problems Encountered with the Operation of 2mm ID Columns

Acute instrument problems were experienced during these RP-PC studies which resulted in long periods of system downtime. The PU4010 pump developed two major faults over a period of only a few weeks. Serious pulsing originating from the pump gave rise to intolerable baseline noise. The primary cause of this phenomenon was the uneven wearing of a pump cam follower, which functions as a part of the mechanism which converts the circular rotation produced by the motor into a dual-piston reciprocal action at the pump heads. The worn cam follower was replaced. Pulsations emanating from the pump were not generally an important factor at the instrument sensitivities employed thus far (100nA f.s.d.), but could present great problems when operating at the high sensitivity settings that will be required for clinical monitoring purposes. A second difficulty arose with the PU4010 pump, namely that the pump rate control mechanism malfunctioned leading to the pump 'racing away'. This situation was again solved by component replacement.

In addition to pump breakdowns, the PU4022 ECD suffered many practical problems leading to considerable system downtime. The notable symptoms of

detector malfunction were gross high frequency noise, considerably reduced detector response and, to a certain extent, pulsations on the baseline.

High frequency noise is generally of electrical or electronic origin. Two causes of this type of noise were recognised during the course of these experiments. Firstly, poisoning or fouling of the porous frit in the RE was noted, even following chromatography solely of standard solutions. Such an occurrence impresses the importance of injecting only "clean" samples, and of utilising mobile phase components of the highest purity affordable, with the consequent necessity of employing efficient solvent clean-up techniques. The RE was replaced with a new one, following unsuccessful attempts to clean the frit and all standard solutions and mobile phases were discarded. Solvent stills were cleaned out thoroughly to eliminate contamination from this source. New standard solutions and mobile phases were prepared from freshly distilled solvents and newly acquired reagents as far as was possible.

The second major source of high frequency noise originated as a result of corrosion of the S/S inlet locking nipple and washer. The action of the moderately aggressive mobile phase employed caused erosion of the chromium oxide protective layer on the S/S surface. Once activated, subsequent erosion and reaction of the iron constituent of the construction material could take place, as described by Mowery.[403]

Conditions in the flow cell inlet were ideal for these processes because of the aid to erosion provided by the narrow restricted flow path, and to corrosion by the application of a potential difference and the presence of dissolved oxygen (because the degassing is not 100% efficient). Metal ions formed by these processes could then be swept into the flow cell by the flow stream and electro-oxidised at the WE surface, so generating high frequency noise.

Prior to this discovery of the source of the noise, the system was flushed with aqueous Na_2EDTA, a commonly employed metal ion complexing agent. Background high frequency noise was reduced but rapidly returned to its previous intolerable level when the system was eluted with an unadulterated aqueous/organic modifier mobile phase. Addition of Na_2EDTA to the mobile phase itself gave rise to reduced solute retention with an unacceptable resolution loss. Hence this practice was discontinued.

On discovery of the corrosion problem, a replacement flow cell for the PU4022 ECD was ordered from the suppliers. On receipt of the new flow cell, it was noted that the new inlet locking nipple was composed of PTFE (unlike the original S/S component) which eliminated the possibility of corrosion problems at this point. However, the washer provided with the new flow cell was still composed of S/S. Hence, in order to protect it from potential solvent attack, a small ring washer was fabricated from PTFE tape and was placed between the S/S washer and the PTFE tube flange. Furthermore, the ability of phosphate buffer to inhibit corrosion of S/S parts has been noted.[403,404] Thus, the application of this buffer system to the chromatography of indoles was investigated and is described in the following section.

A further problem experienced with the PU4022 ECD was the gradual decline of detector response, due primarily to adsorption onto the glassy carbon WE surface. This event is well known and has been discussed in Section 2.1.7. Temporary restoration of response may be achieved by electrochemical cleaning of the WE (i.e., by increasing the cell operating potential for a period), but for longer lasting effect the WE must be removed from the flow cell and polished mechanically. It was found that frequent delays ensued because of the need for flow cell servicing in this way. The rapidity of electrode fouling may have been a consequence of the

aforementioned buffer precipitation and corrosive action of the mobile phases in use. Replacement of the WE provided a brief respite from these problems. Yet it was only when the new flow cell was procured and steps were taken to avoid buffer precipitation and inhibit corrosion that the need for physical cleaning of the WE was reduced to an acceptable frequency (i.e., every 4-6 weeks of operation with standards).

One other performance-restricting factor originating from the PU4022 ECD was recognised during the course of this study. It was observed that pulsations on the baseline were accentuated when the PU4022 flow cell was connected. This problem was thought to be caused by the movement of the electrodes in the cell body with the pulsating flow produced by the PU4010 pump. The construction of the flow cell is such that both the WE and the RE are held in place by screw-in locking collars bearing down on 'O'-ring seals (see Figure 3.10 and Plate 4). This system does not anchor the electrodes very firmly. A small design modification of the locking collars would probably alleviate this problem. If the locking collars were built such that they capped the electrodes and in so doing exerted a downward force on the electrodes whilst in position, then there would be very little possibility of movement within the cell body. In practice, little could be done at the ECD regarding the accentuated pulsations. The best course of action was to tackle these pulsations at source, i.e., the PU4010 pump.

The general lack of robustness displayed by the NBLC-EC instrument was very disconcerting. Such major operational difficulties could well be considered unacceptable in many clinical laboratories, where instrument ruggedness and reliability are of great importance.

Over the course of these RP-PC experiments, system back pressure increased steadily until the level became so great that routine operation

was impossible. The primary underlying cause of this problem was discovered to be the precipitation out of solution of white crystalline material (very probably organic buffer constituents) in the pump, connecting tubing, injection valve and column. This resulted in the gradual blockage of flow passages within the instrument and of the column itself. This situation was remedied by disassembling the liquid heads and filters of the pump and thoroughly cleaning with distilled water all the parts which come into contact with the mobile phase. Similarly, the injection valve was dismantled and serviced. Following reassembly of the apparatus, the column was thoroughly back-flushed with distilled water in order to wash off the offending material. The system was then returned to the mobile phase of choice for further experiments to be conducted.

On the second occasion of this problem arising, it was noted that system breakdown related to suspected buffer precipitation correlated with the use of MeCN as organic modifier. This observation brings into question the solubility of the organic buffer systems under the conditions employed. Because of the existence of this buffer precipitation problem and suspicion of the role of MeCN in this process, MeCN was rejected for use as an organic modifier with this type of buffer system. Consequently, it was decided at this point to return to the use of MeOH as modifier and to investigate the practicalities of a third alternative buffer system, _viz_. KH_2PO_4/H_3PO_4.

A mobile phase of the original format, i.e., 4% MeOH : 96% aqueous buffer adjusted to pH 4.00 was prepared using the KH_2PO_4/H_3PO_4 buffer system. This was eluted through the Spherisorb S5 ODS1 column and the indole standard mixture was chromatographed in duplicate along with single indole standards for peak recognition. The separation so obtained

is illustrated in Figure 3.35 and mean capacity factors for each indole are tabulated in Table 3.22. The chromatogram in Figure 3.35 may be compared with those obtained with acetate/citrate and pure acetate buffers under otherwise identical conditions (see Figure 3.30). A plot of indole capacity factor versus buffer composition for all three buffer systems is presented for this purpose (Figure 3.36).

The adoption of a phosphate buffer in a MeOH-modified mobile phase resulted in no discernible precipitation and consequent clogging of the instrument. Moreover, considerably higher resolution (concomitant with a significant change in elution order) than that obtained with either of the organic buffer systems under the same conditions was observed. The effective removal of ion interactions, by employment of a buffer moiety with little or no affinity for the stationary phase, provides for uncomplicated 'pure' RP-PC on the non-polar surface, which would be expected to give rise to elution in order of decreasing solute polarity. Essentially, such behaviour is observed in practice.

The partial separation of indoles achieved with non-interacting buffer components is very promising for future manipulation. However, the limitations of pure RP-PC for the separation under investigation are amply demonstrated by the experiments conducted thus far. Insufficient breadth of retention and selectivity control exists in this mode for this particular separation problem. Hence, a decision was made to introduce secondary equilibria to the system to provide more parameters for variation, so enlarging the scope available for management of solute retention.

In this instance, the ion suppression technique was considered to be of little value. The reasons for this lie in the great variation of acid/

Figure 3.35 Reverse Phase Partition Chromatography of a Standard Indole Mixture on Spherisorb S5 ODS1 Employing a Phosphate Buffer in the Mobile Phase (pH 4.00)

Parameters :- For conditions see Figure 3.30 except for Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer, pH 4.00

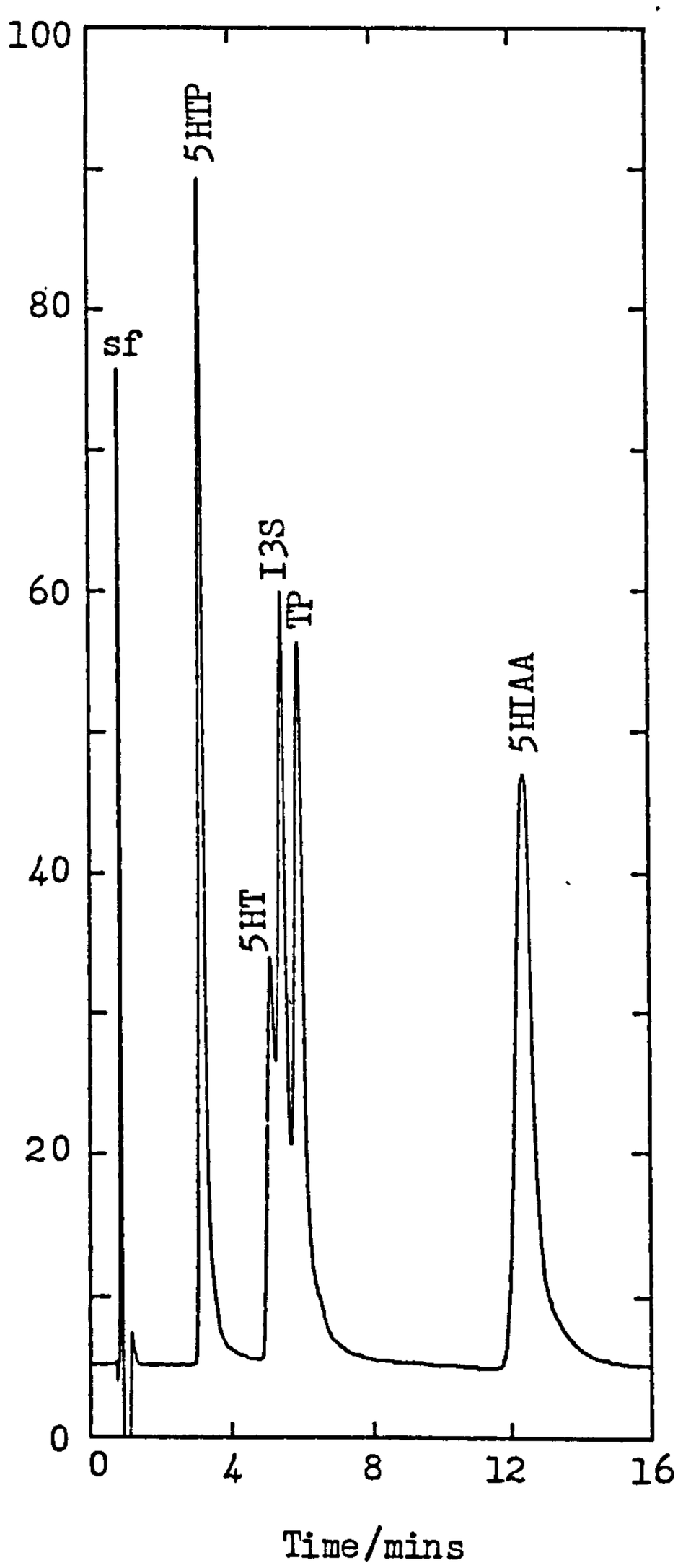
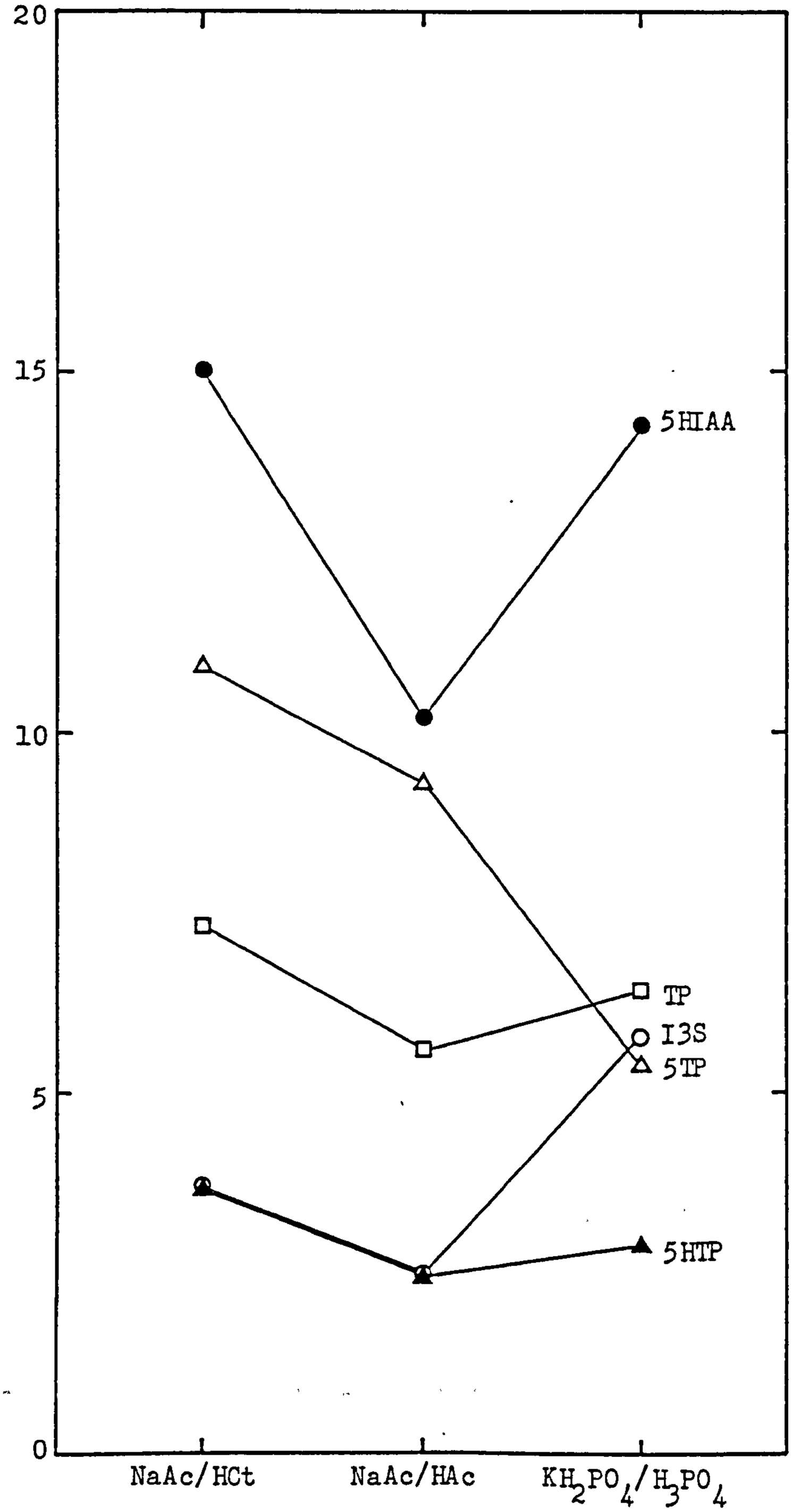


Table 3.22 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Partition Chromatography on Spherisorb S5 ODS1 using a Further Alternative Buffer System in the Mobile Phase.

Buffer System	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
KH_2PO_4/H_3PO_4	0.8	5.9	6.38	3.1	2.88	5.1	5.38	12.2	14.25	5.4	5.75

Figure 3.36 Relative Capacity Factors for a Series of Indoles Chromatographed on Spherisorb S5 ODS1 Employing Three Different Buffer Systems at Identical pHs (4.00)



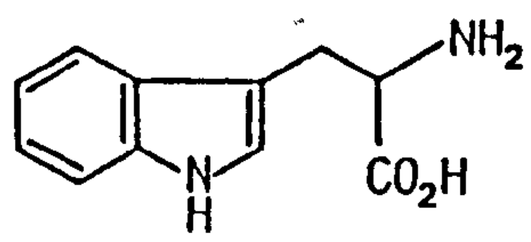
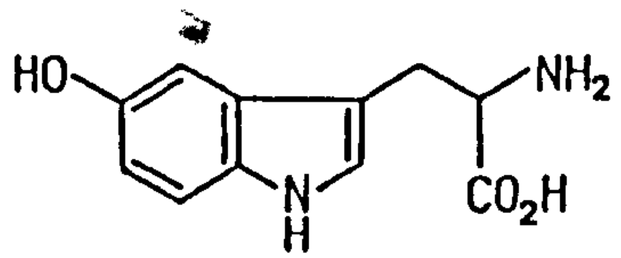
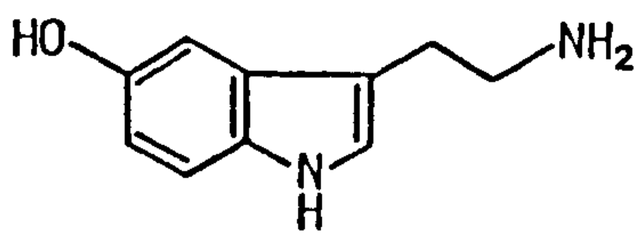
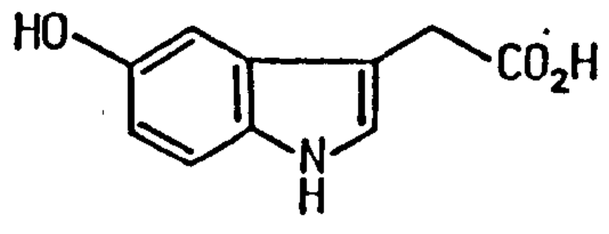
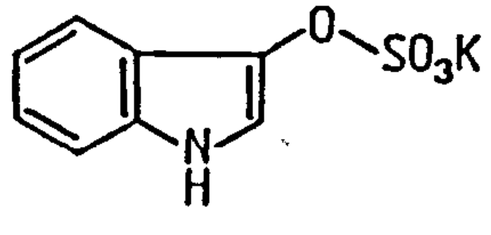
base properties exhibited by the five analytes. In Table 3.23, pK_a values for the dissociation of TP, 5HTP, 5HT, 5HIAA and I3S are compiled and the dissociation processes to which these pk_a values refer are presented in Figure 3.37. It may be noted that the indole >NH group is never protonated. This is because the lone pair associated with the N atom is not discrete and available for donation, but is delocalised within the π-electron system, so conferring aromaticity on the molecule. From this data it is clear that over the accessible pH range of 2-8 for silica-based columns, only the ionisation of 5HIAA may be suppressed. All the other analytes possess dissociation constants at the extremes of or outside of these limits where the limitations of the column material prevent the application of ion suppression. Hence, the introduction of another variable, namely an ion-interaction agent (IIA), was considered. This approach to the separation of the indole class of compounds and related substances has been attempted by many workers with some success (see Section 3.1.5).

3.4.4.5 Experiments with Reverse Phase Ion Interaction Chromatography for the Separation of Indoles

3.4.4.5.1 Preliminary Experiments

Reverse phase ion-interaction chromatography was applied to the separation of the indoles of interest, in order to exert greater chromatographic flexibility and control *via* interactions with charged sites on the analyte molecules. The choice of IIA was arbitrary since both the amino-acid TP and its 5-hydroxy derivative 5HTP are zwitterionic in nature over almost the entire accessible pH range. Furthermore, 5HT harbours a positive charge whilst I3S carries a negative charge in this region. An

Table 3.23 Dissociation Constants of Selected Indoles[405,406]

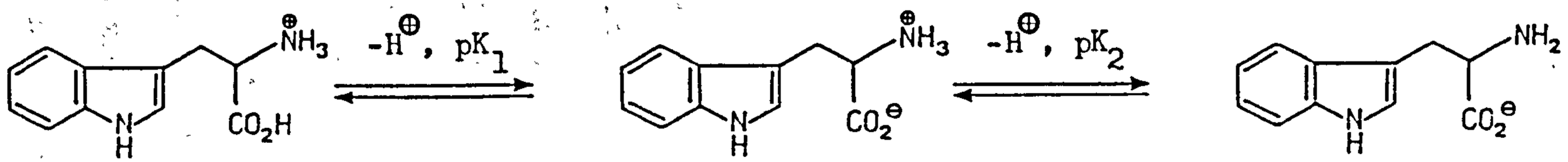
Indole	Structure	pK_1	pK_2	pK_3	Remarks	Refs.
TP		2.46	9.63	-	pK_1 (CO_2H), pK_2 (NH_2)	407
5HTP		2.65	9.63	10.68	pK_1 (CO_2H), pK_2 (NH_2), pK_3 (Ar-OH)	408
5HT		9.98	11.26	-	pK_1 (NH_2), pK_2 (Ar-OH)	408,409
5HIAA		4.6*	10.5-11.5†	-	pK_1 (CO_2H), pK_2 (Ar-OH)	-
I3S		<1	-	-	pK_1 (SO_3K)	-

* Estimated value based on that for indole-3-acetic acid (IAA)[410]

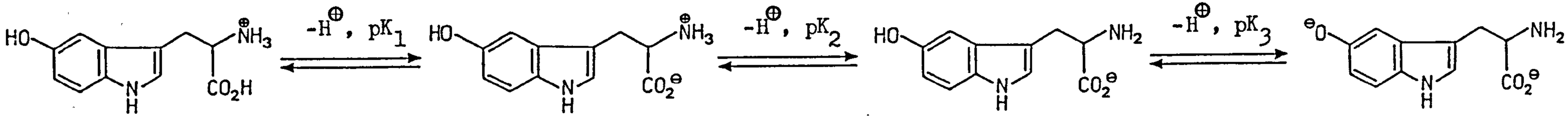
† Estimated pK_a range for dissociation of a phenolic proton

Figure 3.37 The Dissociation of Selected Indoles

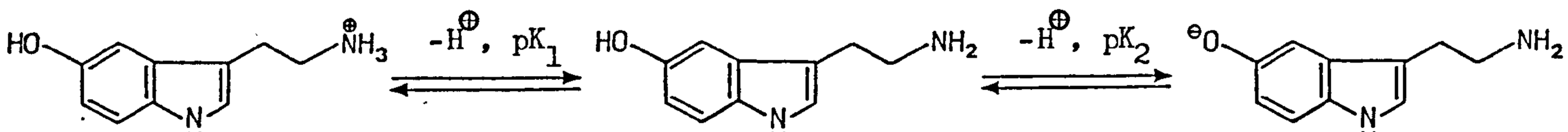
TP



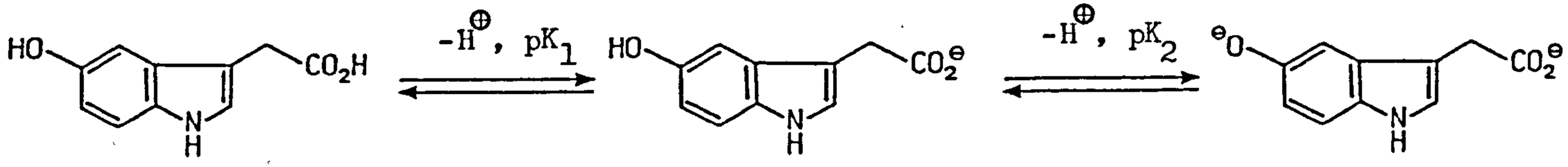
5HTP



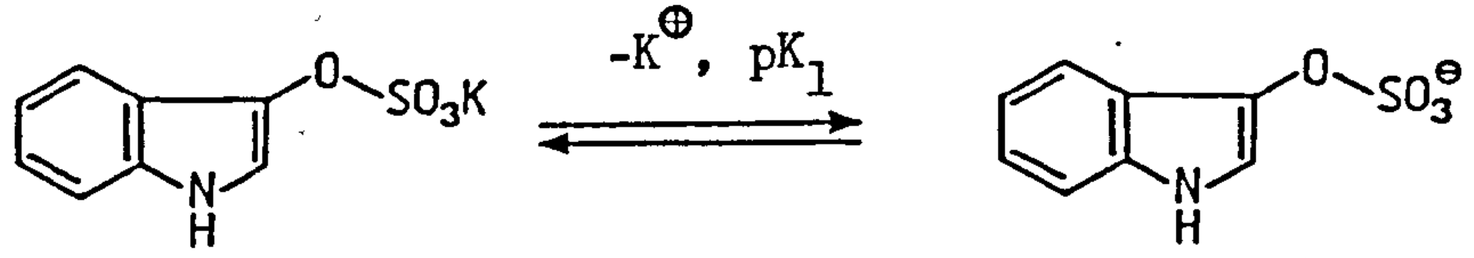
5HT



5HIAA



I3S



IIA possessing a negatively charged lipophilic moiety was selected initially, with a view to promoting the retention of those species presenting a positive site to the IIA (i.e., the protonated amine function of TP, 5HTP and 5HT), in an attempt to achieve considerably greater resolution of the three closely eluting peaks corresponding to 5HT, I3S and TP in Figure 3.35.

A preliminary experiment was conducted by chromatographing a five indole standard on the Spherisorb S5 ODS1 column. An identical mobile phase to that employed for the production of Figure 3.35 was used, except that an aliquot of Waters PIC-B7 reagent (3.84% w/v 1-heptanesulphonic acid (HSA) active ingredient in an aqueous acetate buffer of pH 3.5) was added to the solvent mixture prior to pH adjustment, to provide an HSA concentration of approximately $100mgl^{-1}$. The chromatogram so obtained is presented in Figure 3.38(a) along with a chromatogram obtained without IIA addition (Figure 3.38(b)) for comparison. Peak identification was carried out in the usual way. Capacity factors for each solute were calculated and are compiled in Table 3.24.

The addition of PIC-B7 reagent to the mobile phase produced an excellent near-baseline separation of all five indoles (R_s = 2.11). The smallest analyte capacity factor was 2.63 (I3S) and the total run time was 15 mins. Both of these parameters were of reasonable magnitude, the smallest capacity factor just exceeding the proposed lower limit of 2, whilst the run time allowed a throughput of four samples per hour (standards) which was well within acceptability.

However, difficulties were experienced in aliquoting a suitable volume of the PIC-B7 solution, both accurately and reproducibly, for incorporation into the mobile phase. This problem, compounded by the high cost of PIC-B7 reagent, prompted substitution of a more economical and easier to manage

Figure 3.38 Chromatograms Illustrating the Separation on Spherisorb S5 ODS1 of a Series of Indoles With and Without the Addition of PIC-B7 Reagent to the Mobile Phase

Parameters :- Column : Spherisorb S5 ODS1 (10cm x 2.1mm, d_p = 5µm); Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer (a) containing PIC-B7 reagent (≡ HSA @ 100mgl^{-1}), (b) unmodified, pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : 1µl via loop of TP, 5HTP, 5HT, 5HIAA and I3S in 4% MeOH : 96% aq. 0.1M KH_2PO_4 (All constituents @ 5µgml^{-1} ≡ 5ng injected)

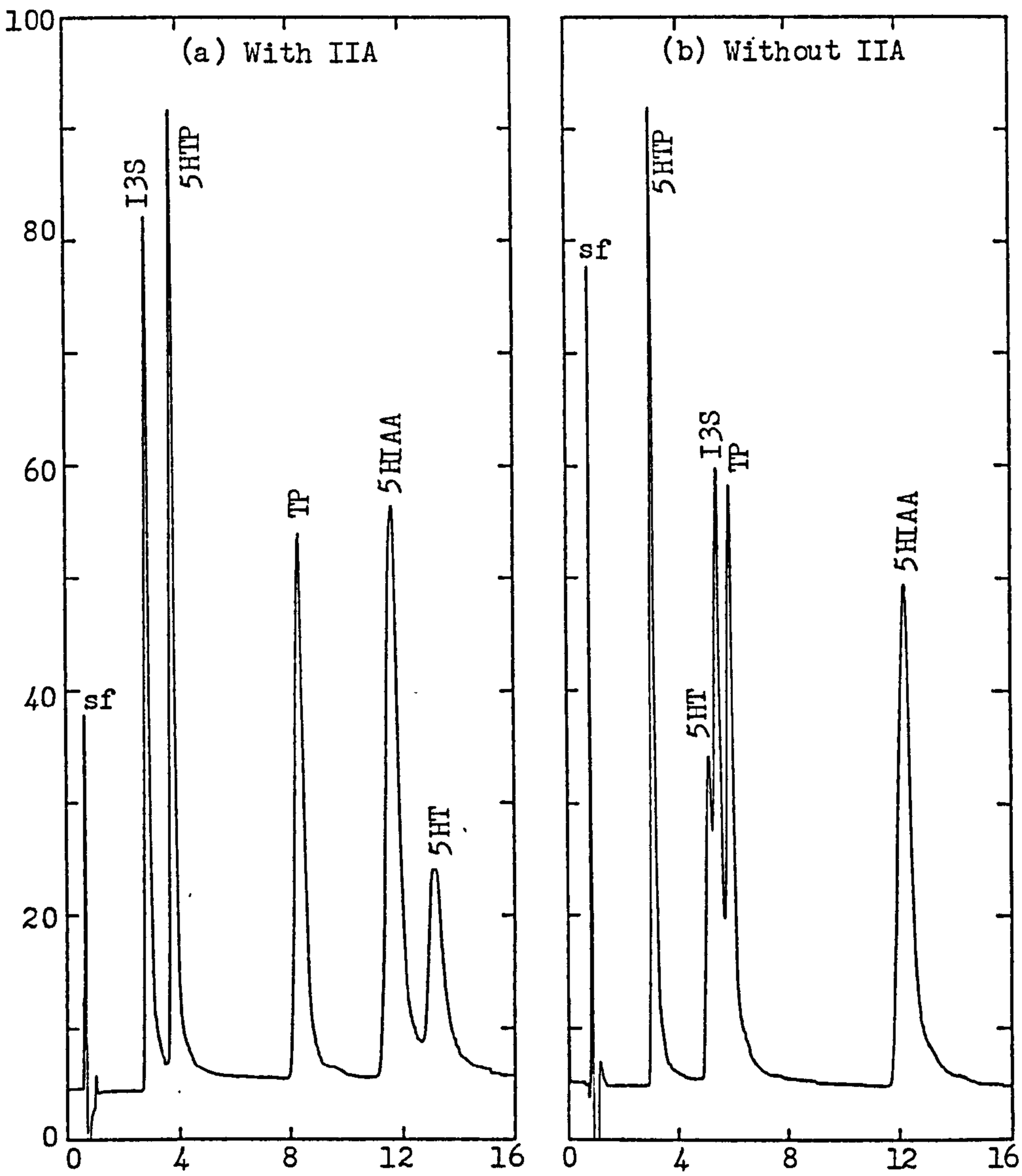


Table 3.24 Retention Times and Capacity Factors of Indoles Chromatographed on Spherisorb S5 ODS1 With and Without the Addition of PIC-B7 Reagent to the Mobile Phase.

IIA Added?	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
Yes	0.8	8.4	9.50	3.8	3.75	13.1	15.38	11.6	13.50	2.9	2.63
No	0.8	5.9	6.38	3.1	2.88	5.1	5.38	12.2	14.25	5.4	5.75

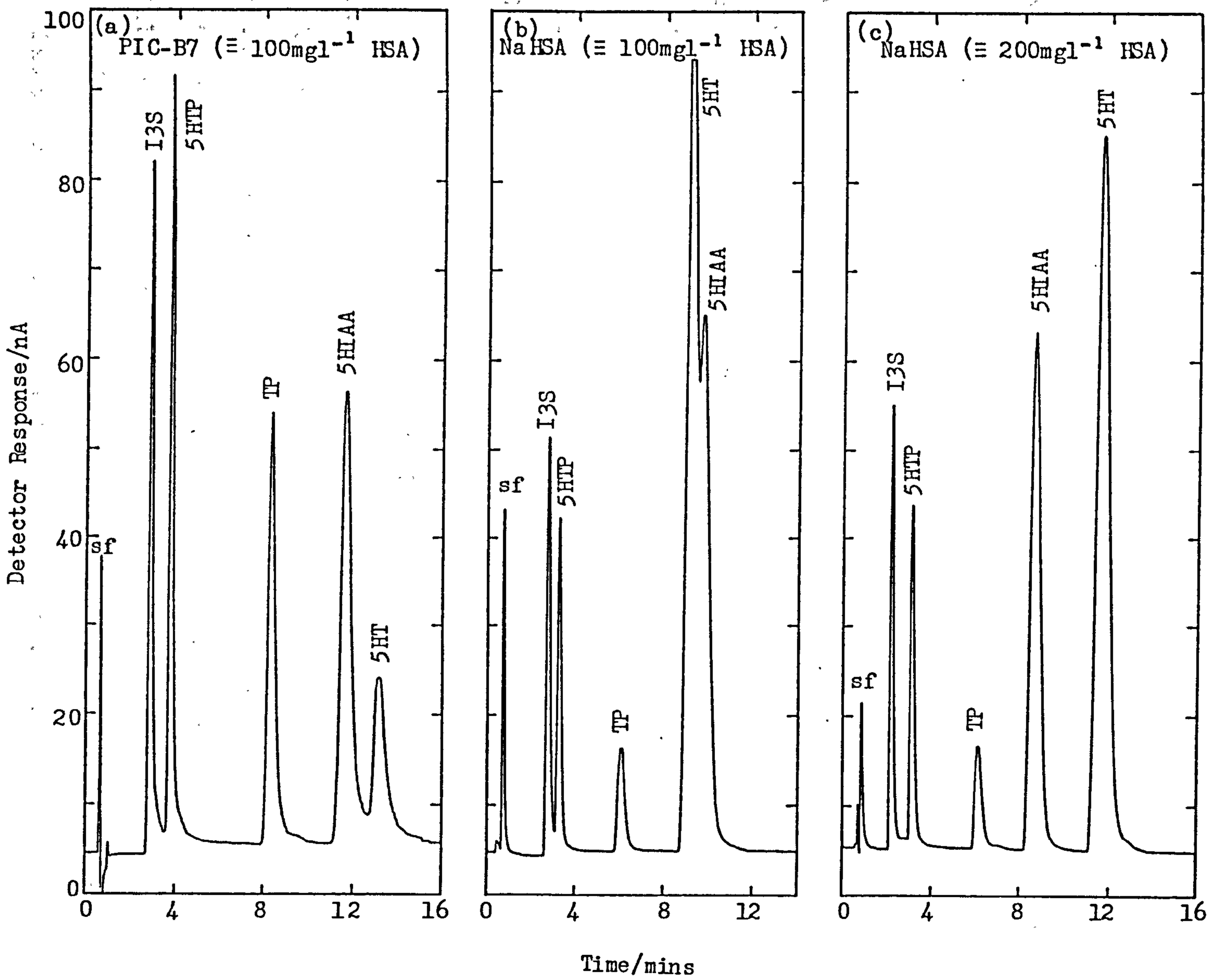
alternative. The obvious choice was the sodium salt of the PIC-B7 active ingredient, HSA. Chromatography of the indole standard mixture using a mobile phase prepared with NaHSA, nominally containing the same concentration of HSA ($100\mu gl^{-1}$) as was employed when PIC-B7 was utilised, did not yield an identical separation. Only when the HSA concentration was increased to $200mgl^{-1}$ was a similar separation obtained. This situation is depicted in Figure 3.39. The observed discrepancy in behaviour may in part be due to the inaccuracy in measurement of the HSA concentration of the PIC-B7-prepared mobile phase. Another factor may have been column deterioration incurred between the acquisition of the representative traces. Furthermore, the small amount of acetate moiety present in PIC-B7, which was absent from the NaHSA-based mobile phase, probably exerted an influence on the chromatography.

Since a satisfactory separation of the analytes had been accomplished by RP-IIC on Spherisorb S5 ODS1, the fate of the μ-Bondapak ODS column was considered. Preference for employment of the Spherisorb phase was expressed by our suppliers (Pye Unicam), because they routinely stock and market Phase Sep packing materials (e.g., Spherisorb), but they do not carry stationary phases manufactured by Waters (e.g., μ-Bondapak). Hence, for this reason utilisation of the μ-Bondapak ODS column was discontinued in favour of the Spherisorb S5 ODS1 column.

A detailed examination of the effects of concentration of HSA, MeOH content, and pH of the mobile phase on the elution properties of the indoles on Spherisorb S5 ODS1 was undertaken, in order to enhance understanding of the nature and magnitude of any such effects. These experiments are reported in the following sections.

Figure 3.39 Chromatograms Illustrating the Effect of Ion-Interation Agent Form on the Separation of a Series of Indoles on Spherisorb S5 ODS1

Parameters :- For conditions see Figure 3.38 except for Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer modified with ion-interaction agent as specified, pH 4.00



3.4.4.5.2 Variation of Mobile Phase MeOH Content

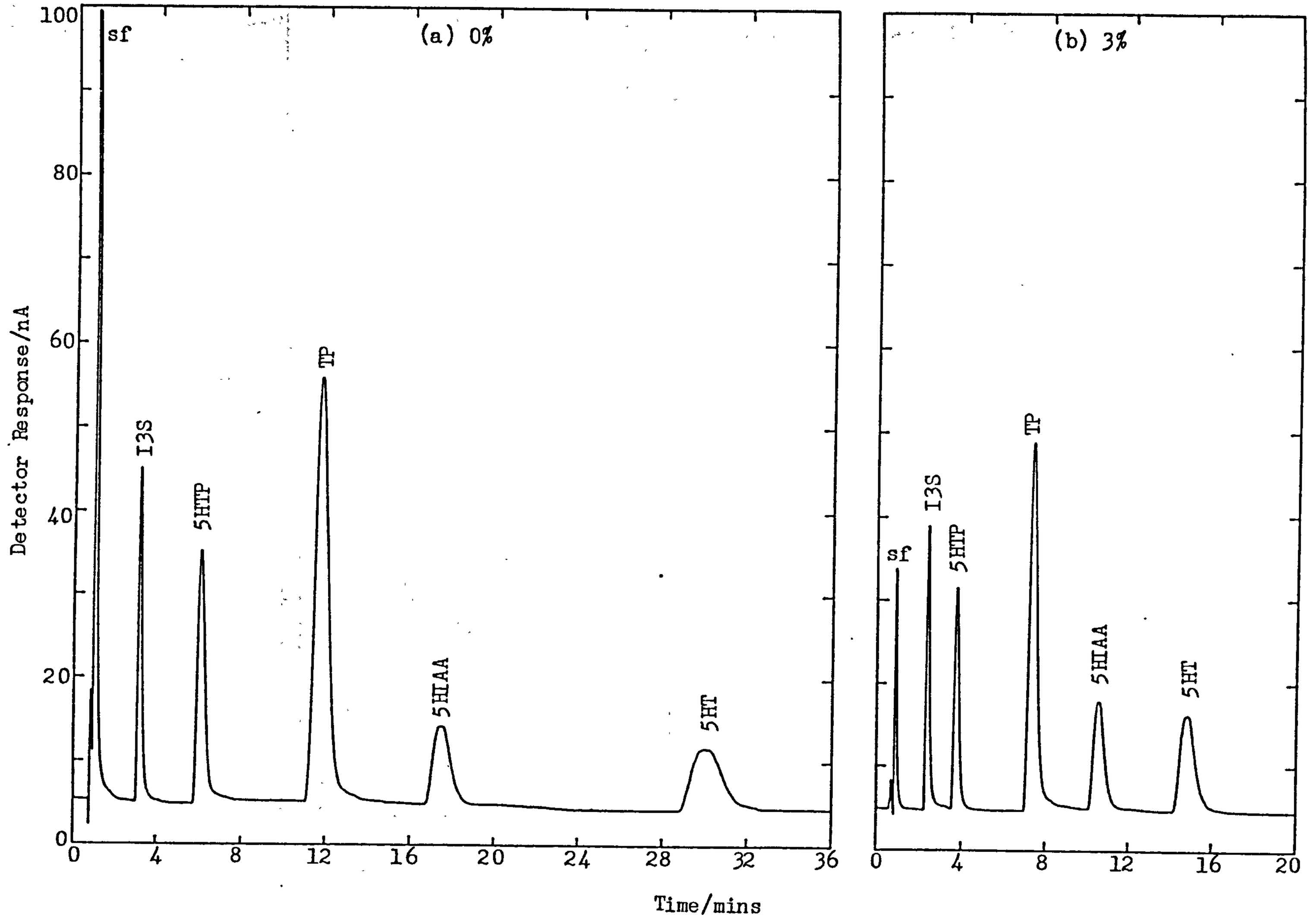
Chromatography of a standard solution containing TP, 5HTP, 5HT, 5HIAA and I3S was carried out on the Spherisorb S5 ODS1 column, using mobile phases of the general composition MeOH/aqueous phosphate buffer containing NaHSA (200mgl^{-1} active ingredient) adjusted to pH 4.00. MeOH contents in the range 0-20% were employed. Chromatography was conducted in ascending order of MeOH percentage. Great care was exercised in ensuring that equilibrium had been attained in the HPLC system with each mobile phase before representative traces were taken. The establishment of equilibrium was recognised by settling of the baseline to drift-free status and confirmation was derived from the observation of chromatographic reproducibility. Single indole standards were injected at each MeOH percentage for peak identification.

Typical traces are compiled in Figure 3.40 and mean capacity factors calculated for each solute are given in Table 3.25. Figure 3.41 depicts the relationship between indole capacity factor and percent MeOH in the mobile phase plotted from these data.

Capacity factors for all analytes are observed to decrease with increasing proportion of MeOH in the mobile phase as was to be expected. Moderation of the mobile phase polarity with MeOH yields a mobile phase for which the indoles have a greater affinity and faster elution results as a consequence. A change in elution order occurs at a MeOH content of approximately 14-15%, which presumably is due to changes in the relative solubilities of the five analytes in the increasingly more methanolic mobile phase. From the above data (Table 3.25) and the graphs (Figure 3.41) it is clear that under the operating conditions employed and to satisfy the criterion $k'_{min} \geq 2$, a MeOH content of $\leq 4\%$ must be utilised.

Figure 3.40 Reverse Phase Ion Interaction Chromatography of a Standard Indole Mixture on Spherisorb S5 ODS1 with Various Concentrations of MeOH in the Mobile Phase

Parameters :- For conditions see Figure 3.38 except for Mobile Phase : MeOH : aq. 0.1M KH_2PO_4/H_3PO_4 buffer (modifier concentrations as specified) containing HSA ($200mgl^{-1}$), pH 4.00



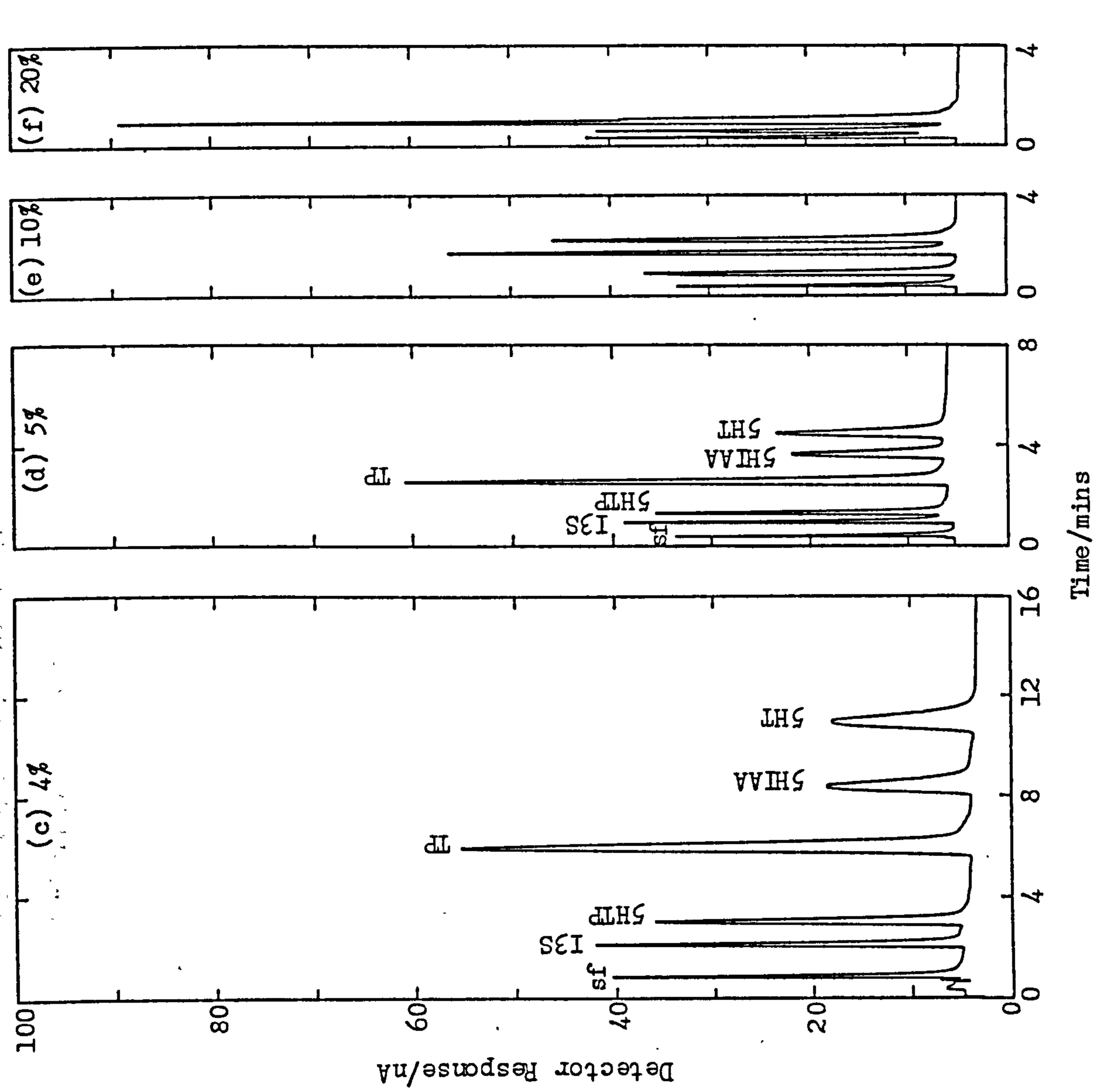
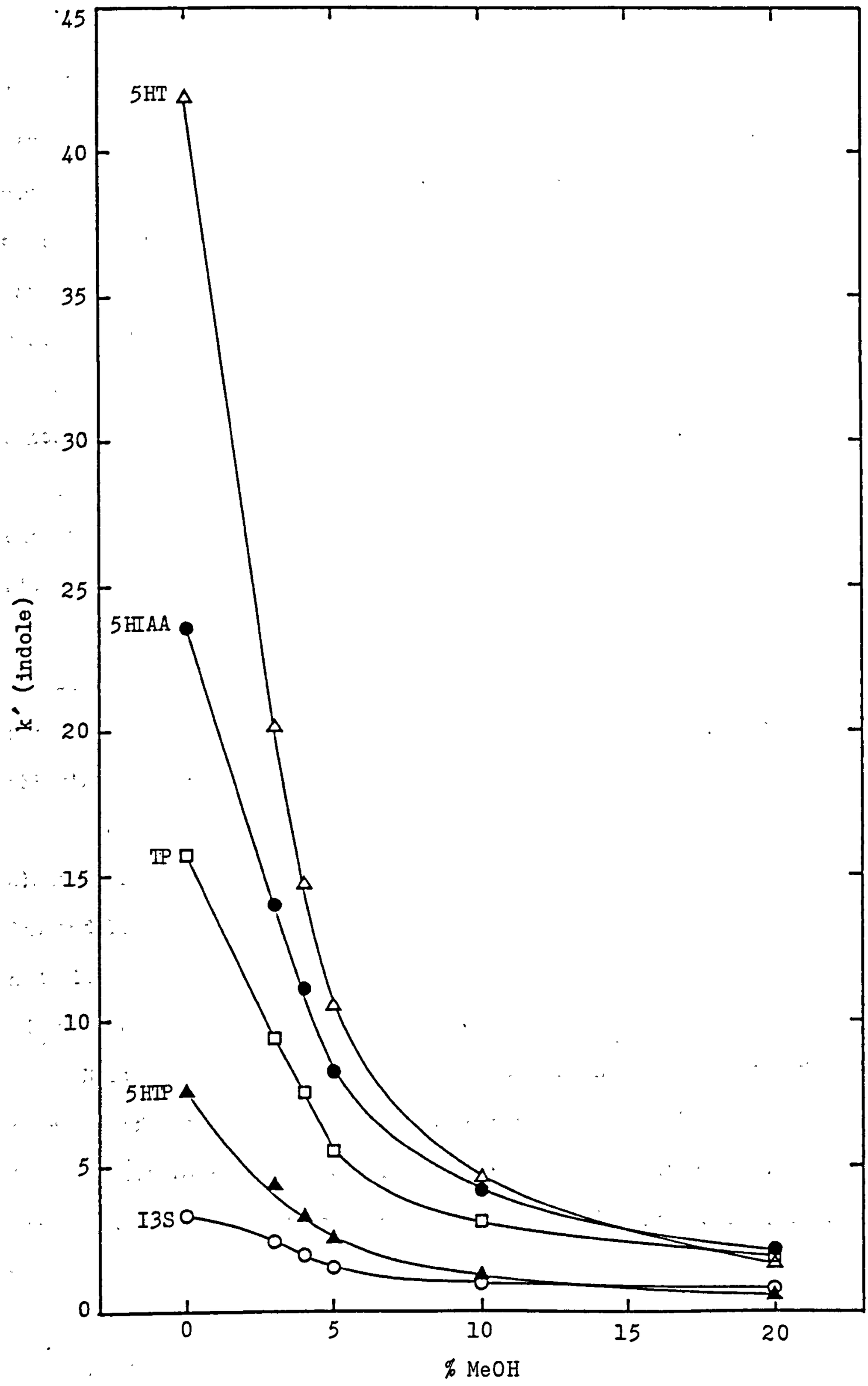


Figure 3.40 continued

Table 3.25 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Ion Interaction Chromatography on Spherisorb S5 ODS1 at Various MeOH:Aqueous Phosphate Buffer Ratios.

% MeOH	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
0	0.7	11.7	15.71	6.0	7.57	30.0	41.86	17.2	23.57	3.0	3.29
3	0.7	7.3	9.43	3.8	4.43	14.8	20.14	10.5	14.00	2.4	2.43
4	0.7	6.0	7.57	3.0	3.29	11.0	14.71	8.5	11.14	2.1	2.00
5	0.4	2.6	5.50	1.4	2.50	4.6	10.50	3.7	8.25	1.0	1.50
10	0.4	1.7	3.13	0.9	1.25	2.3	4.63	2.1	4.25	0.8	1.00
20	0.33	0.93	1.80	0.53	0.60	0.88	1.65	1.05	2.15	0.60	0.80

Figure 3.41 The Relationship Between Capacity Factor and MeOH Content of the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Ion-Interaction Conditions on Spherisorb S5 ODS1



3.4.4.5.3 Variation of Mobile Phase pH

The five component indole standard was chromatographed on the Spherisorb S5 ODS1 column using a series of mobile phases adjusted to different pHs between 3.00 and 7.00. The foundation of these mobile phases consisted of 4% MeOH : 96% aq. 0.1M KH_2PO_4 containing NaHSA (200mgl^{-1} as HSA), a mixture which exhibited a natural pH of 4.62. Adjustments of pH to greater acidity were made by addition of H_3PO_4 while adjustments to greater basicity were achieved by addition of H_3PO_4 to ca. pH 4 (to provide buffering capacity) followed by appropriate introduction of KOH. Chromatography was conducted in ascending order of pH (i.e., acid → base). The establishment of equilibrium was ascertained prior to acquisition of representative chromatograms. Elution order at each pH was elucidated by individual indole standard injection.

Typical chromatograms obtained at each mobile phase pH are presented in Figure 3.42. Table 3.26 indicates the mean capacity factors calculated for each analyte run at the various mobile phase pHs while Figure 3.43 illustrates graphically the relationship between these two parameters.

The results show that mobile phase pH has a profound effect on the elution of TP and 5HIAA, a lesser effect on 5HTP and little or no effect on 5HT and I3S over the range studied. The chromatographic behaviour of these materials at different environmental pHs may be explained in terms of their pK_a values.

Neither 5HT nor I3S have a pK_a value within two pH units of the extremes of the region investigated herein, therefore little or no variation in retention behaviour is observed. In aqueous solution, 5HT exists in the form of a protonated amine at acid pHs whereas I3S is a strong acid and

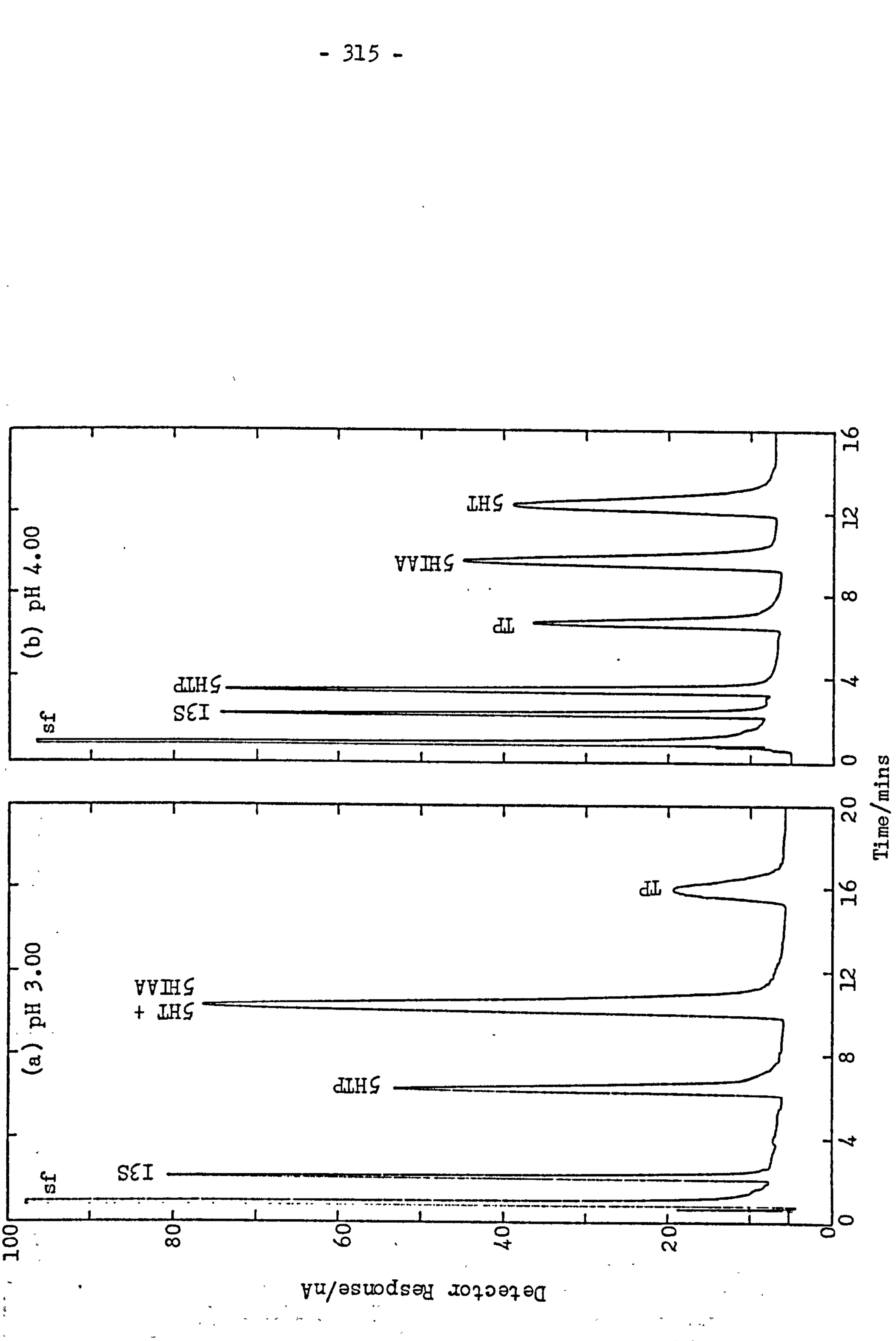


Figure 3.42 Reverse Phase Ion-Interaction Chromatography of a Standard Indole Mixture on Sphorisorb S5 ODS1 at Various Mobile Phase pH Values

Parameters :- For conditions see Figure 3.38 except for Mobile Phase : 4% MeOH : 96% aq. 0.1M $KH_2PO_4/H_3PO_4/KOH$ buffer containing HSA (200mgl^{-1}), pH as specified

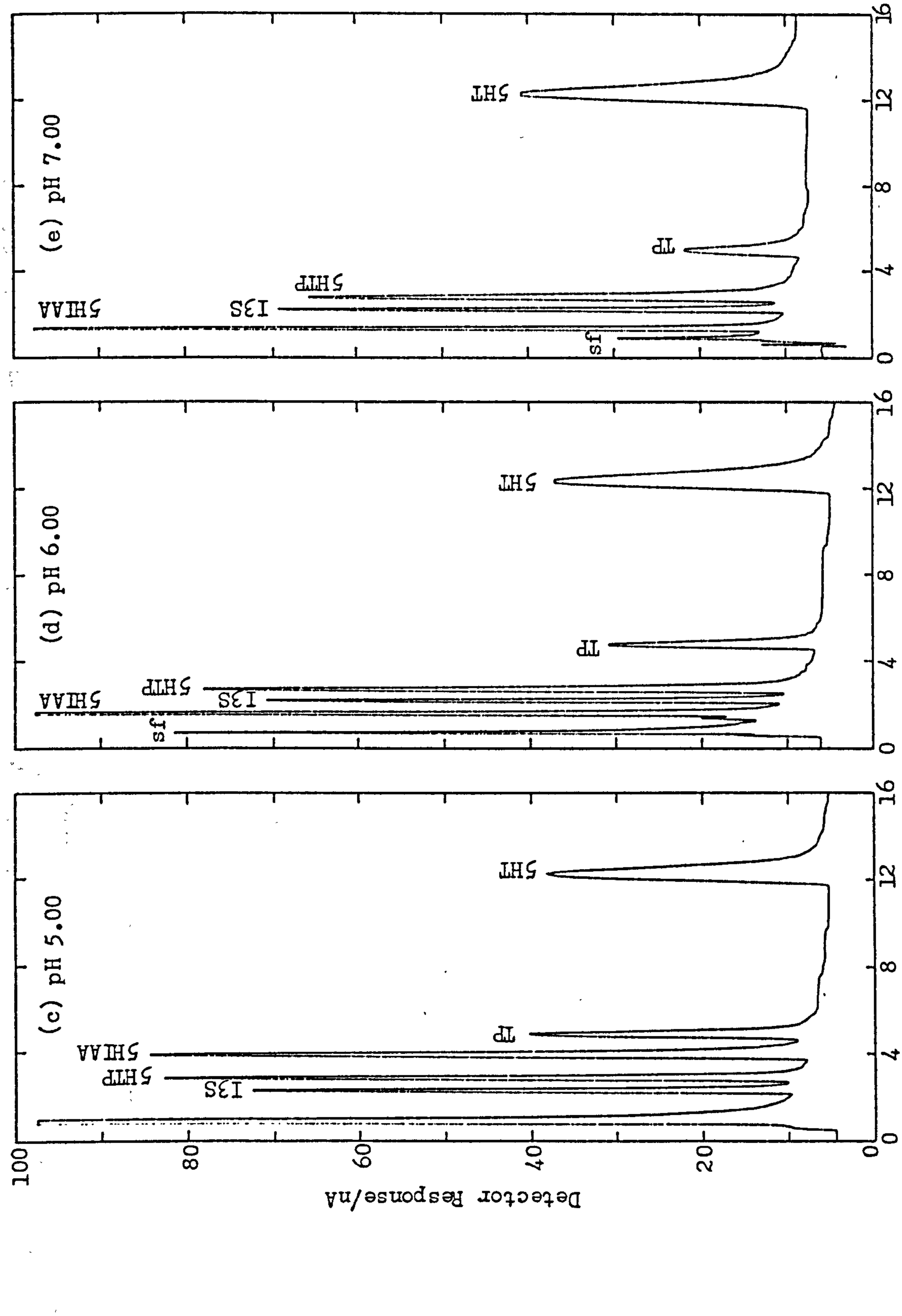
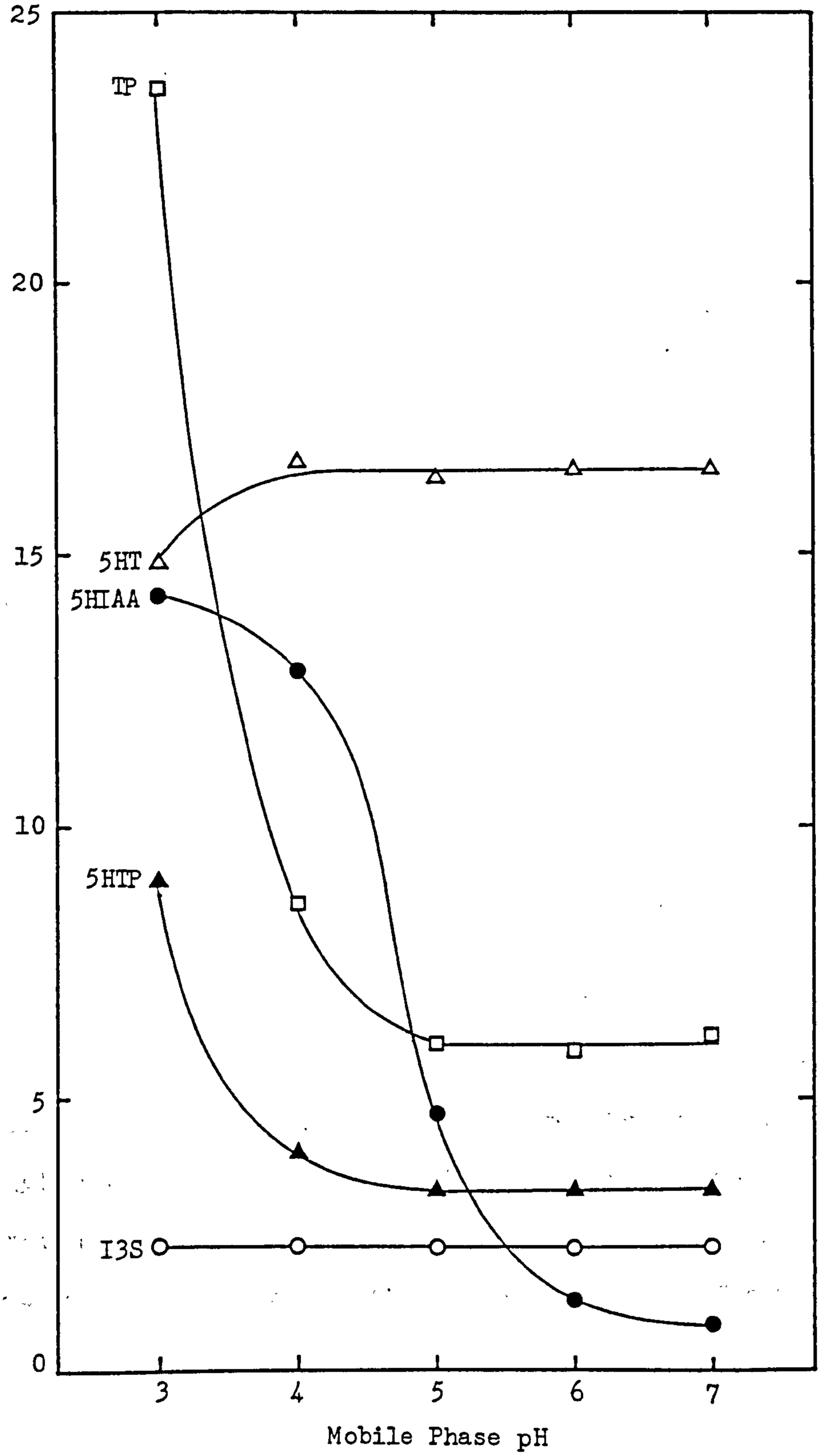


Figure 3.42 continued

Table 3.26 Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Ion Interaction Chromatography on Spherisorb S5 ODS1 at Various Mobile Phase pH Values.

Mobile Phase pH	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
3.00	0.7	17.2	23.57	7.0	9.00	11.1	14.86	10.7	14.29	2.3	2.29
4.00	0.7	6.7	8.57	3.5	4.00	12.4	16.71	9.7	12.86	2.3	2.29
5.00	0.7	4.9	6.00	2.8	3.29	12.2	16.43	4.0	4.71	2.3	2.29
6.00	0.7	4.8	5.86	2.8	3.29	12.3	16.57	1.6	1.29	2.3	2.29
7.00	0.7	5.0	6.14	2.8	3.29	12.3	16.57	1.3	0.86	2.3	2.29

Figure 3.43 The Relationship Between Capacity Factor and pH of the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Ion-Interaction Conditions on Spherisorb S5 ODS1



consequently is fully dissociated over the entire pH range 1-14 (see Table 3.23 and Figure 3.37, Section 3.4.4.4).

TP and 5HTP are amphoteric possessing both weakly acidic and weakly basic functional groups. Dissociation of the carboxyl group in these two substances occurs at a pK_a of ca. 2.5 (see Table 3.23). Horváth and co-workers[411,412] have demonstrated that weakly acidic (and weakly basic) materials show a sigmoidal relationship between pH and capacity factor over a range of approximately four pH units with the inflection point occurring at the pK_a value of the acidic (or basic) functional group. The rapid shortening of k′ between pHs 3 and 4 followed by a levelling off of the curve to higher pH for TP and 5HTP is representative of the tail of a sigmoidal curve. This almost certainly corresponds to the aforementioned equilibrium.

5HIAA also possesses a carboxyl group which dissociates over the examined pH range. The graph of k′ against mobile phase pH for this substance depicts a sigmoidal curve with an inflection point at ca. pH 4.6 which, according to Horváth et al.[411,412], is equivalent to the dissociation of a weak acid with a pK_a of ca. 4.6. No information regarding the dissociation constant(s) of 5HIAA was available but the pK_a of the non-hydroxylated compound, indole-3-acetic acid (IAA) has been measured[410] and was found to be 4.6. Now, as is exemplified by TP and 5HTP, substitution of a hydroxyl group at the 5-position on the indole ring has negligible effect on the pK_as of functional groups at the 3-position (see Table 3.23). Consequently, very similar pK_as would be expected for the dissociation of the carboxyl group in both IAA and 5HIAA. The chromatographic evidence confirms this hypothesis; the literature pK_a value for IAA being in very close agreement with the experimentally derived pK_a value for 5HIAA.

The observed changes in chromatographic behaviour to smaller k′ with increasing pH for TP, 5HTP and 5HIAA may be rationalised in fundamental terms. As the elution medium becomes less acidic over the region in which carboxyl group dissociation occurs, more of the anionic carboxylate species is generated. This negatively charged moiety is repelled by the negatively charged IIA which may be envisaged as being in dynamic equilibrium with the stationary phase surface. As a result of the increased influence of the IIA with increased ionic nature of the solutes, TP, 5HTP and 5HIAA all elute progressively more rapidly until complete ionisation is attained.

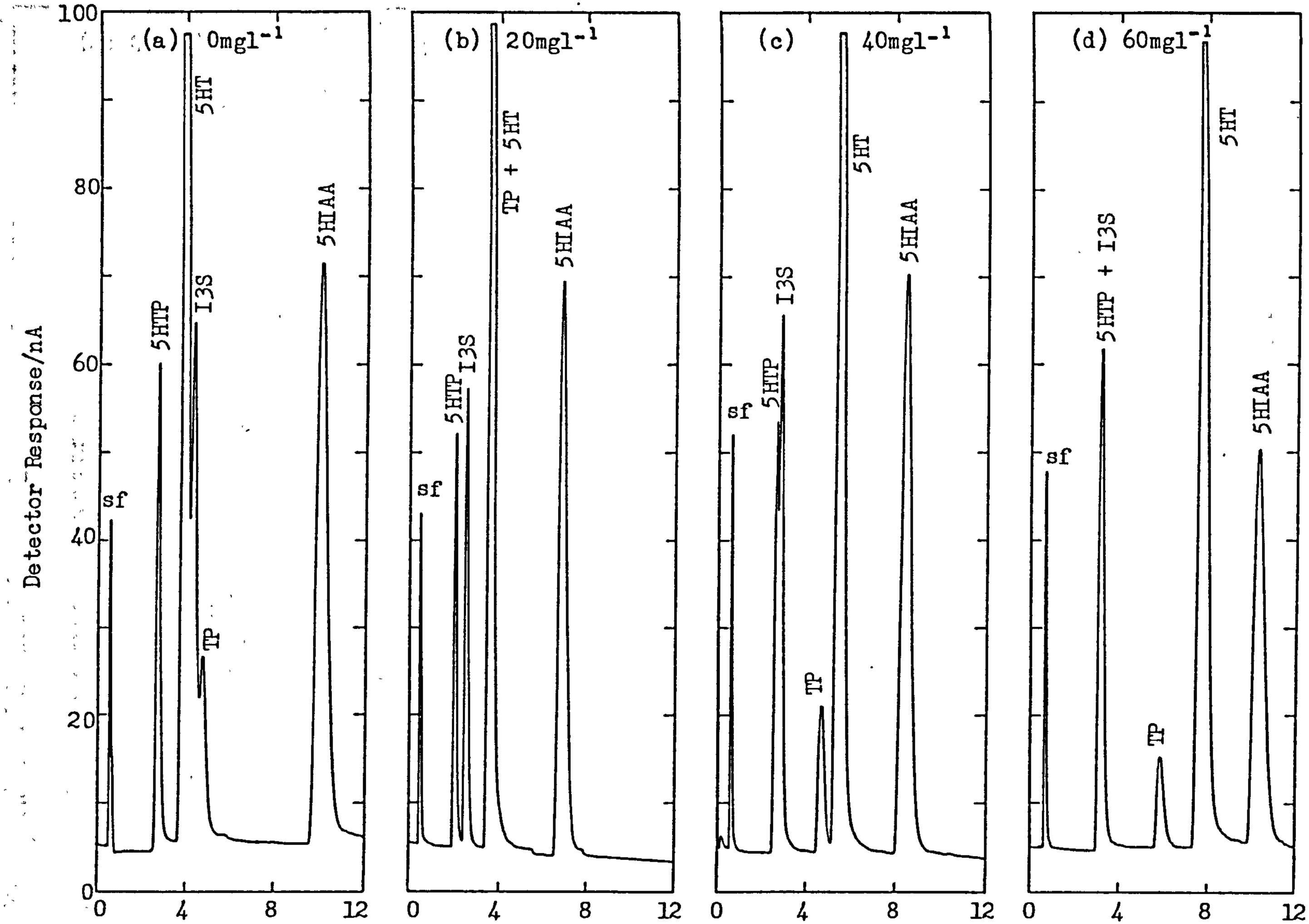
3.4.4.5.4 Variation of 1-Heptanesulphonic Acid Concentration

An indole standard mixture was chromatographed at least twice with each mobile phase on a Spherisorb S5 ODS1 column. Mobile phases comprising 4% MeOH : 96% aq. phosphate buffer containing HSA and adjusted to pH 4.00 were utilised. The concentration of HSA was increased stepwise from 0-200mgl^{-1} active ingredient. Establishment of equilibrium at each IIA concentration was ensured prior to recording the chromatograms. These chromatograms are compiled in Figure 3.44. Elution order in each instance was ascertained by single indole standard injection. Calculated mean capacity factors are tabulated in Table 3.27 and a graph of these values plotted versus HSA concentration is presented in Figure 3.45.

The observed changes in elution behaviour with HSA concentration are most easily explained by way of the dynamic ion exchange mechanism outlined in Section 3.1.4.3. The indoles largely fit into two groups, one which includes TP, 5HTP and 5HT in which promotion of retention with increase in HSA concentration is predominant, and a second which includes 5HIAA and I3S in which increasing HSA concentration gives rise to an overall reduction in retention.

Figure 3.44 Reverse Phase Ion-Interaction Chromatography of a Standard Indole Mixture on Spherisorb S5 ODS1 with Various Concentrations of 1-Heptanesulphonic Acid in the Mobile Phase

Parameters :- For conditions see Figure 3.38 except for Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA (concentration as specified), pH 4.00



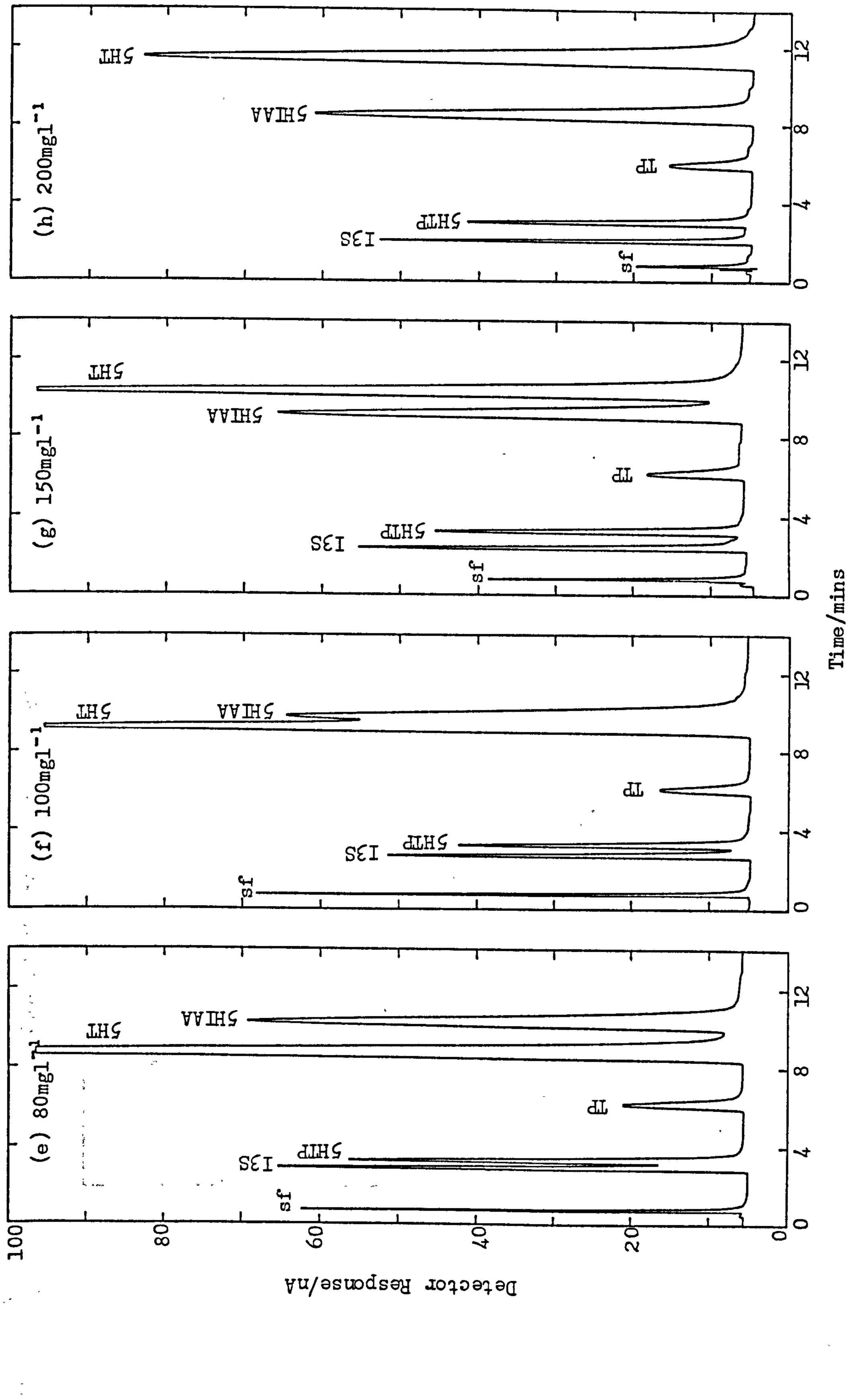
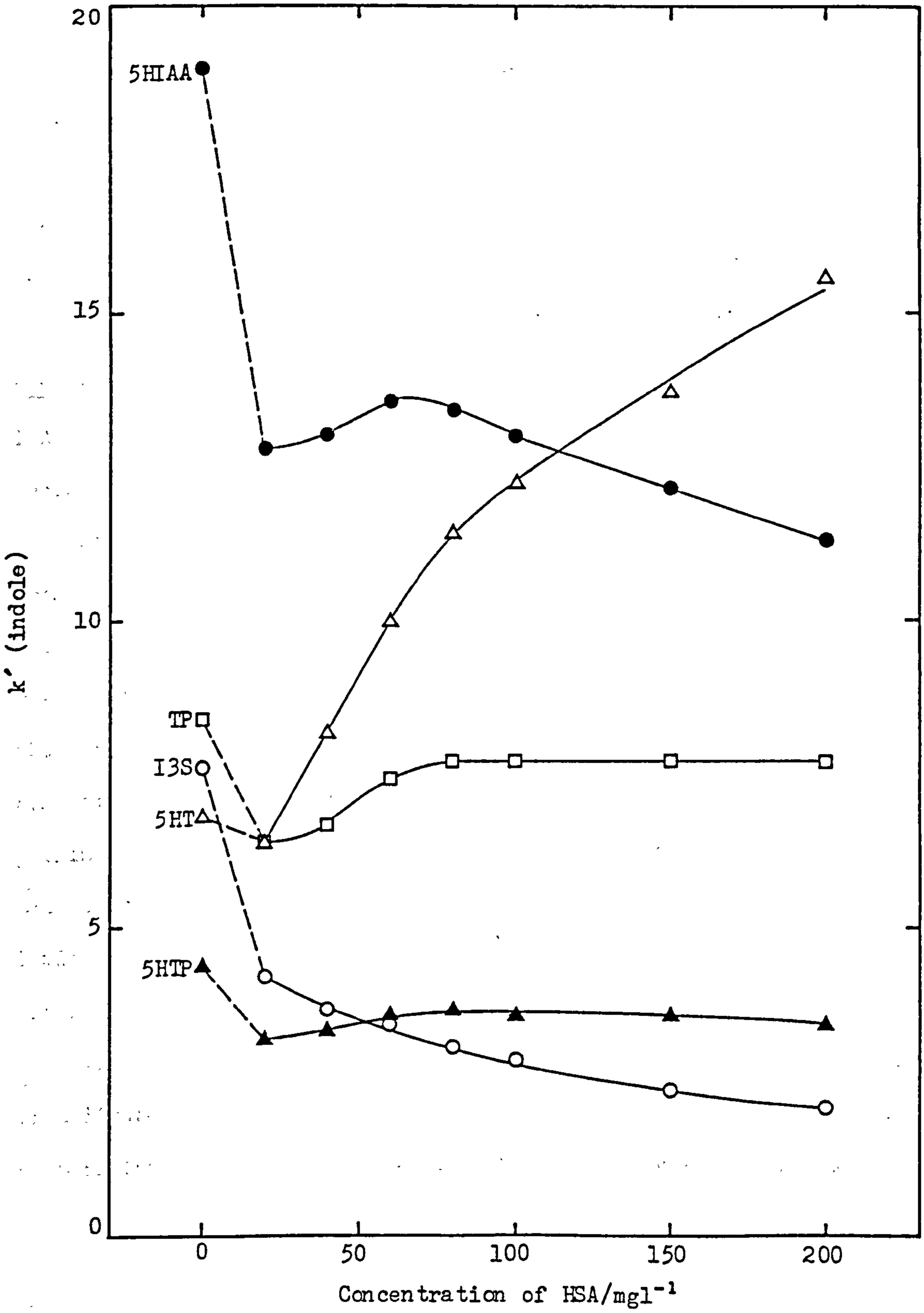


Figure 3.44 continued

Table 3.27 · Retention Times and Capacity Factors of Indoles Obtained by Reverse Phase Ion Interaction Chromatography on Spherisorb S5 ODS1 with Various Concentrations of 1-Heptanesulphonic Acid in the Mobile Phase.

Concentration of HSA (mgl^{-1})	sf	TP		5HTP		5HT		5HIAA		I3S	
	t_o (min)	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′	t_R (min)	k′
0	0.5	4.7	8.40	2.7	4.40	3.9	6.80	10.0	19.00	4.3	7.60
20	0.5	3.7	6.40	2.1	3.20	3.7	6.40	4.9	12.80	2.6	4.20
40	0.6	4.6	6.67	2.6	3.33	5.5	8.17	8.4	13.00	2.8	3.67
60	0.7	5.9	7.43	3.2	3.57	7.7	10.00	10.3	13.57	3.1	3.43
80	0.7	6.1	7.71	3.3	3.66	8.7	11.43	10.1	13.43	2.9	3.09
100	0.7	6.1	7.71	3.2	3.57	9.3	12.23	9.8	13.00	2.7	2.86
150	0.7	6.1	7.71	3.2	3.57	10.3	13.71	9.2	12.14	2.4	2.37
200	0.7	6.1	7.71	3.1	3.43	11.6	15.57	8.6	11.29	2.2	2.09

Figure 3.45 The Relationship Between Capacity Factor and Concentration of 1-Heptanesulphonic Acid in the Mobile Phase for a Series of Indoles Chromatographed under Reverse Phase Ion-Interaction Conditions on Spherisorb S5 ODS1



At the operating pH of 4.00 the speciation of all five indoles has already been established. TP and 5HTP carry both ammonium ($-\overset{\oplus}{N}H_3$) and carboxylate ($-CO_2^{\ominus}$) functions, the former which is very nearly completely associated and the latter which is largely dissociated. 5HT possesses only the protonated amine group, the proton of which is associated to a similar extent to that of TP and 5HTP. Conversely, the carboxyl group pertaining to 5HIAA only exhibits a little ionic character (estimated at about 10%). I3S, however, incorporates the strongly acidic sulphate structure which completely dissociates in an aqueous environment to generate the $-OSO_3^{\ominus}$ anion.

As was to be expected, the three indoles presenting positive sites, via which coulombic associative interactions with the negatively charged HSA moiety could take place, displayed increased retention. However, the degree to which this promotion was effected differed markedly between species thought to be similarly associated. Clearly, 5HT exhibits the most pronounced change and this is attributable to the free and unhindered interactions possible between the $-\overset{\oplus}{N}H_3$ site on the molecule and the anionic HSA moiety. TP and 5HTP are far less affected by increasing HSA concentration than is 5HT, with only slight promotion of retention over the decade of concentration investigated. Presumably this is because of the close proximity of a $-CO_2^{\ominus}$ site to the $-\overset{\oplus}{N}H_3$ site in TP and 5HTP. Repulsion between the carboxylate group and the isocoulombic IIA would be expected to limit the directions from which the HSA moiety may approach the ammonium group so moderating any retardation process.

The mechanism of retention of 5HIAA and I3S may also be postulated from Figure 3.45. Both analytes are most probably retained predominantly by parition into the ODS surface. The general decrease in capacity factor

with increasing HSA concentration is almost certainly due to competition with other species, particularly HSA, for the ODS surface and will be accentuated by repulsions between the largely negatively charged dynamically modified stationary phase surface and the $-CO_2^{\ominus}$ and $-OSO_3^{\ominus}$ sites on 5HIAA and I3S respectively. This type of "ion exclusion" interaction obtained by introduction of an isocoulombic species to the mobile phase is frequently utilised for modification of the chromatography of bases in order to improve peak shapes.[390]

3.4.4.5.5 Choice of Mobile Phase for Reverse Phase Ion Interaction Chromatography of Indoles on Spherisorb S5 ODS1

A mobile phase comprising 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA ($200mgl^{-1} \equiv 1.11mM$) adjusted to pH 4.00 was selected as being most suitable for the separation of the five indoles TP, 5HTP, 5HT, 5HIAA and I3S on Spherisorb S5 ODS1. The primary criteria applied in reaching this decision were as stated previously, i.e., that the minimum capacity factor should be equal to, or should exceed 2, that the total run time should be acceptable - preferably within 30 mins, and that all five components should be resolved satisfactorily from one another.

A MeOH content of $\leq$ 4% was dictated by minimum capacity factor considerations. Noting that the total run time increased markedly with decreasing MeOH percentage in this region, a value of 4% was preferred although a completely aqueous mobile phase yielded a run of only 30 mins duration. Low MeOH content also favoured the detection technique because current stability is greater in a largely aqueous environment.

The employment of a pH of 4.00 was arrived at as a compromise between

all three criteria together with the added requirement that small variations in mobile phase pH should not give rise to large variations in capacity factor for the analytes. This additional proviso was entertained so that sufficient control over analyte elution could be exerted. At pH 3 5HT and 5HIAA were unresolved. Between pHs 3 and 4 TP and 5HTP elution was drastically affected by small changes in pH. Between pHs 4 and 6 the chromatography of 5HIAA was similarly afflicted. At pH values in excess of ca. 5.6 the peak due to 5HIAA eluted too rapidly presenting an unacceptably low capacity factor. The pH value of 4.00 provided the best separation of all five analytes with all capacity factors in excess of 2 while exhibiting acceptable tolerance of pH change.

The choice of IIA concentration to be employed was governed primarily by resolution requirements. Figure 3.45 shows that coelution of analytes occurs at HSA concentrations of approximately 20, 50 and 115mgl^{-1}. The maximum concentration tolerable on minimum k′ grounds was ca. 220mgl^{-1} (determined by extrapolation of the curve relating to I3S). The selected level of 200mgl^{-1} provided baseline resolution of all five indoles (see Figure 3.44(h) for example) and allowed for minor modification to compensate for column performance deterioration with time.

3.4.5 Evaluation of Instrument Performance with Respect to 2.1mm ID Columns

The Pye Unicam NBLC system incorporating the 10cm x 2.1mm ID Spherisorb S5 ODS1 column was assessed in an identical manner to that described for the 25cm x 1mm ID Partisil 10 ODS3 column in Section 3.4.3. For seven chromatograms of the five indoles obtained under identical experimental conditions, peak widths were measured at 60.7% of peak height (the half-width

method) and at 50% of peak height (the width at half-height method). Tangential baseline widths were not measured because of the greater error involved in this process. All retention times were also quantified. Mean values of each quantity were determined and values of $\sigma^2_{tot(t)}$ and t^2_R were calculated for both peak width measurements This information is compiled in tabular form in Table 3.28. Plots of mean total peak variance versus square of mean retention time for each method of evaluation are presented in Figure 3.46. From these graphs, values for $\sigma^2_{xc(t)}$ (and consequently $\sigma_{xc(v)}$) and N_c (and consequently H_c) were obtained from the intercept and gradient respectively by linear regression analysis. All these data are compiled in Table 3.29.

Column plate heights (H_c) determined <u>via</u> both peak width measurements were in very close agreement, the mean value being 51.8μm. Instrument variances too were equivalent within experimental error, the mean value of σ^2_{xc} being 236μl^2 ($\equiv \sigma_{xc}$ = 15.3μl). This was almost identical to the value of 232μl^2 ($\equiv \sigma_{xc}$ = 15.2μl) obtained for this HPLC system operating with the 1mm ID Partisil 10 ODS3 column. The insignificant difference in instrument variance determined for the system incorporating the 1mm ID column and that with the 2.1mm ID column is indicative of the minor contribution of the connecting tubing which was reduced in length from 15cm to 12cm, i.e., by 20%, between the former and the latter determinations.

Apparent plate heights were calculated using both half-width and width at half-height measurements by means of Equations 1.12 and 1.13. The values so obtained were compared with plate heights generated in the column (Table 3.29), and these data are given in Table 3.30. These figures show that only 55% of available column efficiency is realised for the first eluted peak (I3S, $k' = 1.64$) rising to approximately 99% for the last peak (5HT,

Table 3.28 Mean Retention Time and Peak Width Data Together with Calculated Peak Standard Deviations and Peak Variances for a Separation of a Series of Indoles on the Pye Unicam NBLC System with a 10cm x 2.1mm ID Column (n = 7)

					Peak Width Measurement					
					Half-Width			Width at half-height		
Peak Number	Indole	t_R (min)	t_R (s)	t_R^2 (s^2)	$w_{0.607}$ (min)	σ_{tot} (s)	σ^2_{tot} (s^2)	$w_{0.5}$ (min)	σ_{tot} (s)	σ^2_{tot} (s^2)
1	5HTP	2.19	131.4	17266	0.129	3.87	15.0	0.163	4.16	17.3
2	5HT	3.19	191.4	36634	0.174	5.22	27.2	0.211	5.39	29.0
3	I3S	6.20	372.0	138384	0.303	9.09	82.6	0.366	9.34	87.3
4	TP	8.84	530.4	281324	0.411	12.33	152.0	0.489	12.49	155.9
5	5HIAA	11.66	699.6	489440	0.540	16.20	262.4	0.638	16.19	262.0

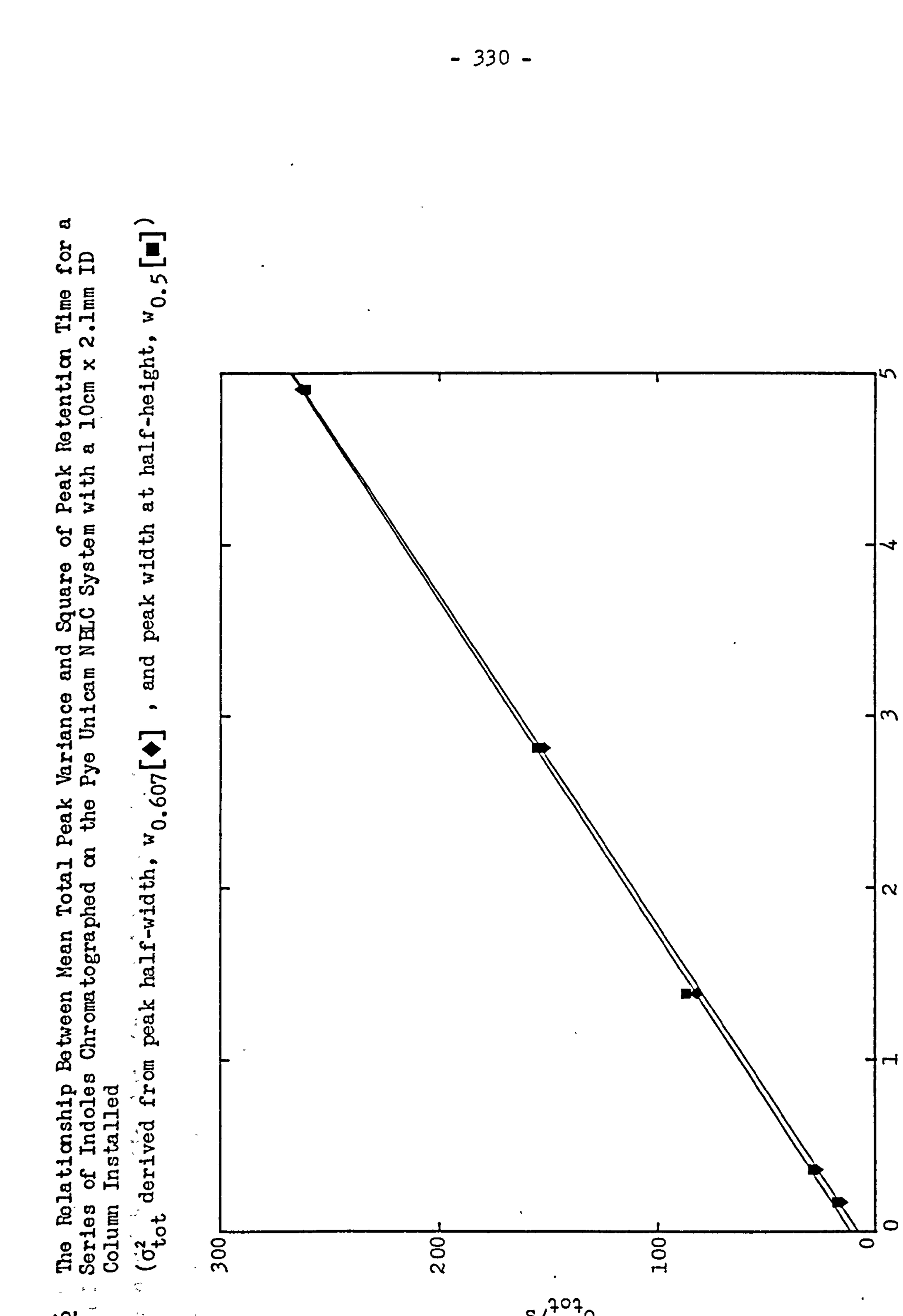

The Relationship Between Mean Total Peak Variance and Square of Peak Retention Time for a Series of Indoles Chromatographed on the Pye Unicam NBLC System with a 10cm x 2.1mm ID Column Installed
(σ^2_{tot} derived from peak half-width, $w_{0.607}$[◆] , and peak width at half-height, $w_{0.5}$[■])
σ^2_{tot}/s^2
300
200
100
0
0
1
2
3
4
5
$t_R^2/x\ 10^{-5}s^2$

Figure 3.46

Table 3.29 Column Efficiency and Instrument Variance Calculated from Linear Regression Analysis of Peak Variance and Retention Data Presented in Table 3.28

Method of σ^2_{tot} estimation	Intercept ($=\sigma^2_{xc(t)}$) (s^2)	Gradient ($=^1/N_c$)	Correlation Coefficient	N_c ($=^1$/Gradient)	H_c ($=L_c/N_c$) (μm)	$\sigma^2_{xc(v)}$* (μl^2)	$\sigma_{xc(v)}$ (μl)
Half-width	7.75 ± 1.52	$5.20 \times 10^{-4} \pm 0.06 \times 10^{-4}$	0.9998	1923 ± 23	52.0 ± 0.6	193.8 ± 38.0	13.9 ± 1.4
Width at half-height	11.10 ± 2.23	$5.15 \times 10^{-4} \pm 0.09 \times 10^{-4}$	0.9996	1942 ± 34	51.5 ± 0.9	277.5 ± 55.8	16.6 ± 1.7

* $\sigma^2_{xc(v)} = \sigma^2_{xc(t)} \times Q^2$ (where $Q = 300 \mu l min^{-1}$)

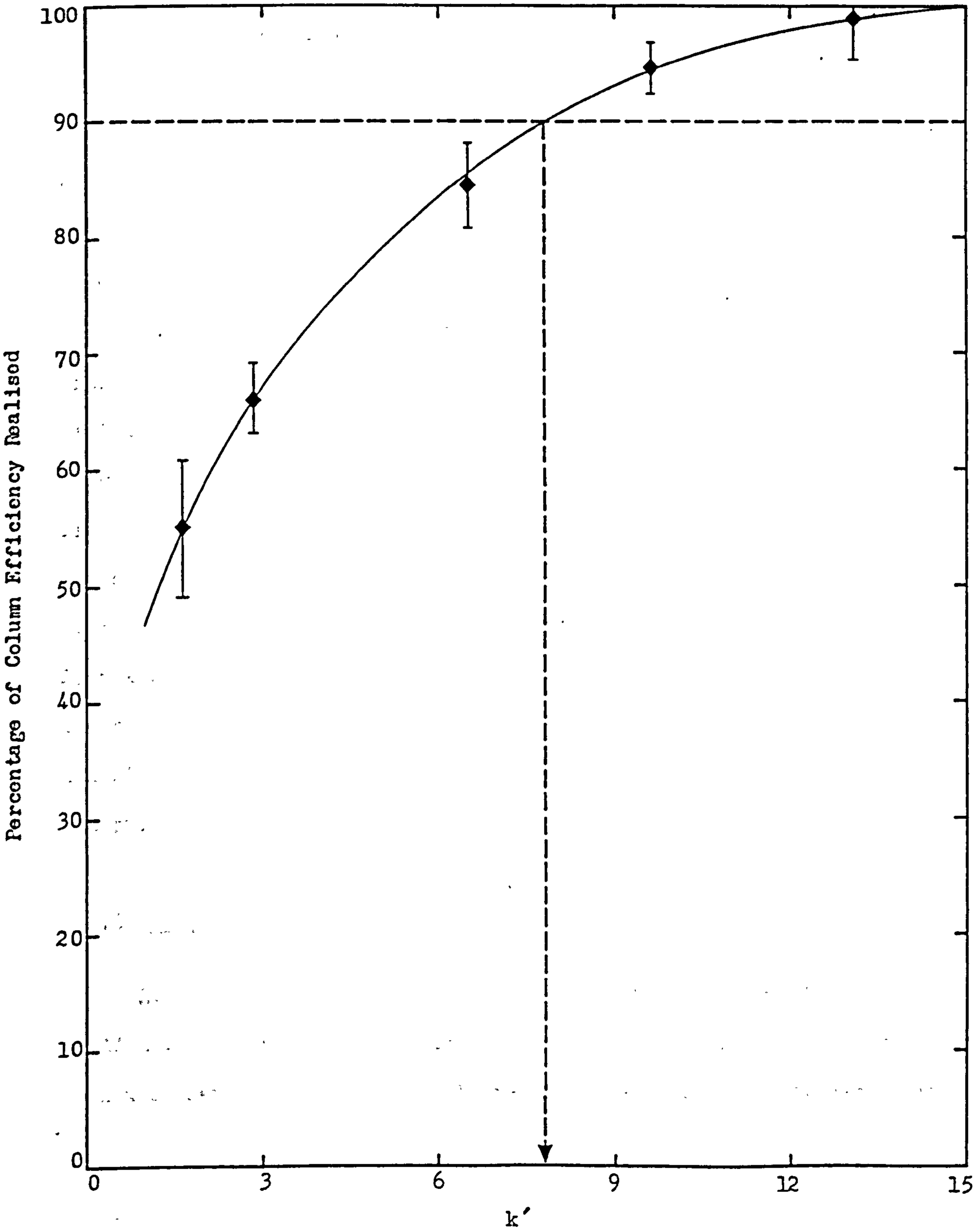
Table 3.30 Proportions of Available Column Efficiency Realised for each Peak in a Standard Indole Chromatogram Run on the Pye Unicam NBLC System with a 10cm x 2.1mm ID Column Installed

			Peak Width Measurement						n = 7
			Half-width			Width at Half-height			
Peak Number	Indole	k′	H_c (µm)	$\hat{H}$ (µm)	$(H_c/\hat{H}) \times 100$ (%)	H_c (µm)	$\hat{H}$ (µm)	$(H_c/\hat{H}) \times 100$ (%)	Mean of two methods (%) ± SD
1	I3S	1.64	52.0	88.2	59.0	51.5	101.5	50.7	54.9 ± 5.9
2	5HTP	2.85	52.0	76.3	68.2	51.5	80.6	63.9	66.1 ± 3.0
3	TP	6.48	52.0	59.8	87.0	51.5	63.0	81.7	84.4 ± 3.7
4	5HIAA	9.67	52.0	54.1	96.1	51.5	55.4	93.0	94.6 ± 2.2
5	5HT	13.08	52.0	51.3	101.4	51.5	53.5	96.3	98.9 ± 3.6

Figure 3.47 Plot of Proportion of Available Column Efficiency Realised (Mean of Two Methods of Assessment) vs. Capacity Factor for a Series of Peaks Yielded by Chromatography of a Standard Indole Mixture on the Pye Unicam NBLC System with a 10cm x 2.1mm ID Column Installed

(Dashed line represents Klinkenberg's criterion of tolerability.

Standard deviation limits indicated)



k′ = 13.08). Percentage of column efficiency realised was plotted against indole capacity factor and this graph is presented in Figure 3.47. The dotted line at 90% represents the tolerance level according to Klinkenberg[59] as before (cf. Figure 3.20). The detrimental effect of the instrument variance on chromatography with the 10cm x 2.1mm ID column is clearly illustrated. Figure 3.47 shows that all peaks eluting prior to a k′ of 7.8 are adversely affected by extra-column band broadening processes beyond the acceptable level. This curve may be compared with the curve representing the deterimental effect of the instrument variance on chromatography when a 25cm x 1mm ID column is employed (i.e., Figure 3.20). Here, all peaks in the chromatogram suffered unacceptable extra-column band broadening. By extrapolation, the minimum capacity factor at which the system operated efficiently with the 1mm ID column was estimated to be approximately 16.2. A comparison of the two values reveals that changing the column dimensions from 25cm x 1mm ID to 10cm x 2.1mm ID served to reduce the minimum k′ at which the system could be operated efficiently by about 50%. Thus, the 10cm x 2.1mm ID column packed with 5µm particles was preferred to the 25cm x 1mm ID column packed with 10µm particles for this reason. However, it should be emphasised that the Pye Unicam NBLC instrument was not of satisfactory specification to operate columns of either geometry to commonly accepted standards.

3.4.6 Optimisation of Applied Potential

Having optimised the mobile phase for the separation of TP, 5HTP, 5HT, 5HIAA and I3S, the potential applied across the electrodes in the PU4022 flow cell was then refined to yield the maximum *S/N* ratio. A standard containing

the five indoles of interest was chromatographed in duplicate on the optimised chromatographic system at various applied potentials between +0.30V and +1.00V. The series of traces so generated are compiled in Figure 3.48. Baseline noise and peak heights of all peaks corresponding to indoles were measured and *S/N* ratios were calculated. These results are presented in Table 3.31. Graphs of *S/N* ratio versus applied potential were constructed for all five analytes and are compiled in Figure 3.49. Physical difficulty in measuring accurately the low noise levels prevalent even at high instrument sensitivity (3nA f.s.d.) determined the need for estimation of the error involved in this process. Error bars representing the effect of an estimated measurement error of ± 0.1mm (≡ 1.5pA) in the noise level are included in this Figure. Curves of detector response (signal) alone plotted against applied potential are presented in Figure 3.50, which is included for comparison purposes.

From Figure 3.50 it may be deduced that there are three distinct half-wave potentials associated with the indoles under investigation. The half-wave potential of between +0.35 and +0.40V observed for 5HTP, 5HT and 5HIAA is assigned to oxidation of the 5-hydroxyl group on the phenol ring.[334] I3S alone exhibits a half-wave potential of +0.55V which presumably is due to oxidation of the sulphate group on this molecule. All five indoles display a further half-wave potential in the region +0.80 to +0.85V which is attributable to oxidation of the ring nitrogen atom, possibly to $N^{\oplus}$.[334] This last assignment of oxidation site was confirmed by chromatographing a solution of indole, the unsubstituted parent molecule from which TP and its metabolites are derived, under standardised conditions at various applied potentials. By inspection, (Figure 3.49) the lowest utilisable applied potential for the examined separation problem was deemed to be ca. +0.75V.

Figure 3.48

(Overleaf)

Chromatograms Illustrating the Effect of Applied Potential on the Response of the Electrochemical Detector to the Indoles under Study

Parameters :- Column : Spherisorb S5 ODS1 (10cm x 2.1mm, d_p = 5µm); Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/ H_3PO_4 buffer containing HSA (200mgl^{-1}), pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : ECD (Eapp *vs.* Ag/AgCl as stated , TC = 1sec); Sample : 1µl *via* loop of TP, 5HTP, 5HT, 5HIAA and I3S in 4% MeOH : 96% aq. 0.1M KH_2PO_4 (All constituents at 1.0µgml^{-1} ≡ 1ng injected)

Figure 3.48

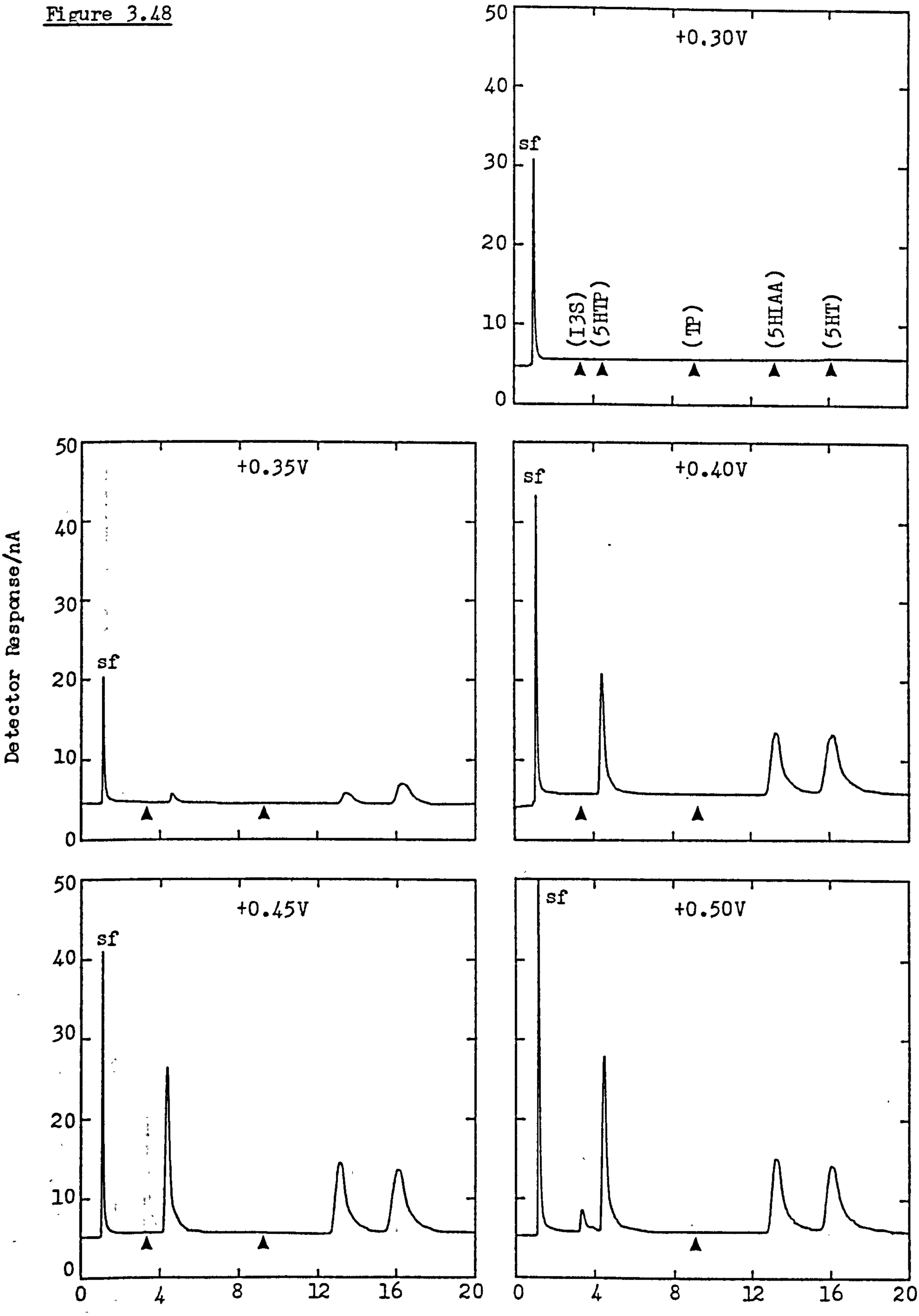

+0.30V
+0.35V
+0.40V
+0.45V
+0.50V
sf
(I3S)
(5HTP)
(TP)
(5HIAA)
(5HT)
Detector Response/nA
Time/mins
0
10
20
30
40
50
4
8
12
16
20

Figure 3.48 continued

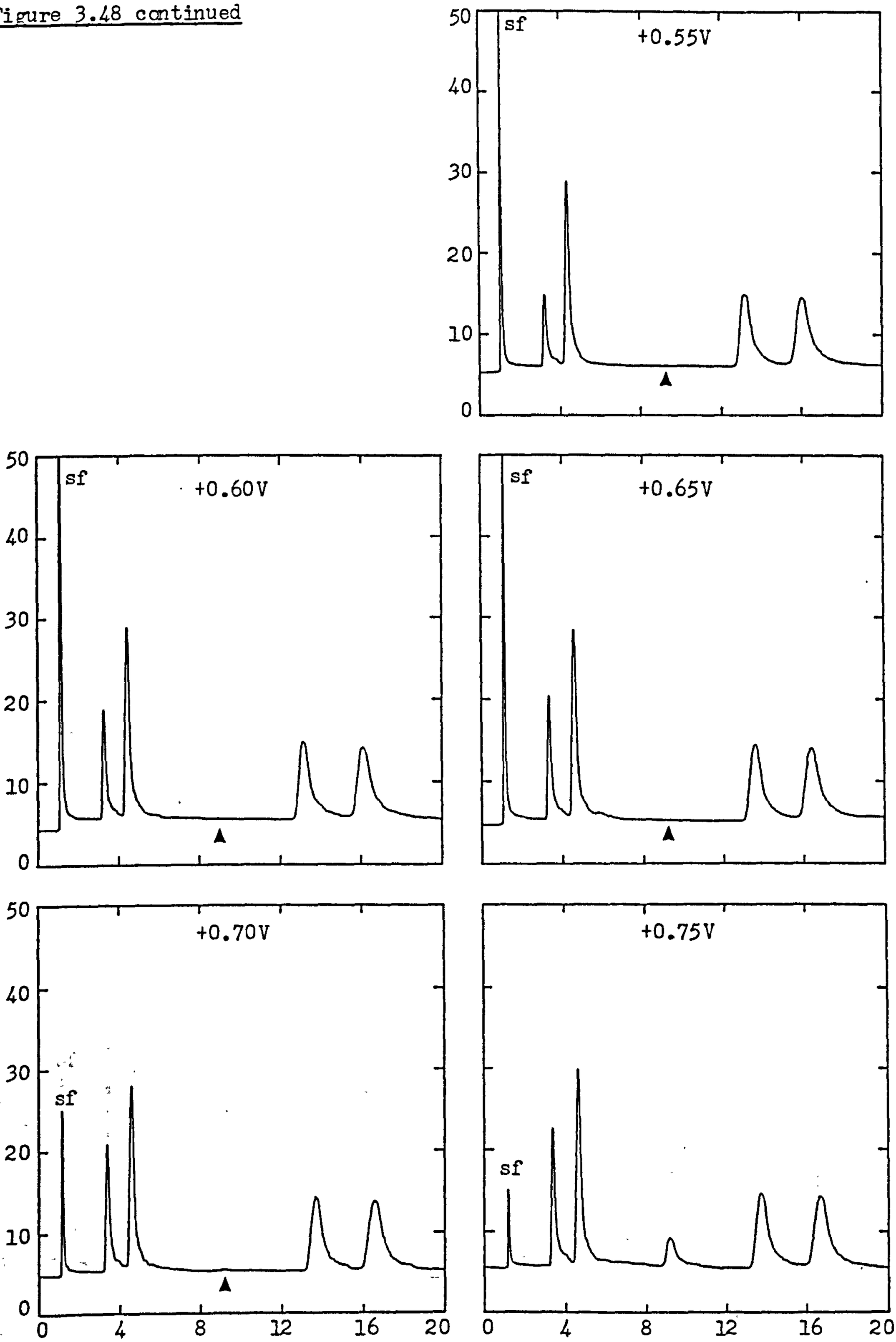


Figure 3.48 continued

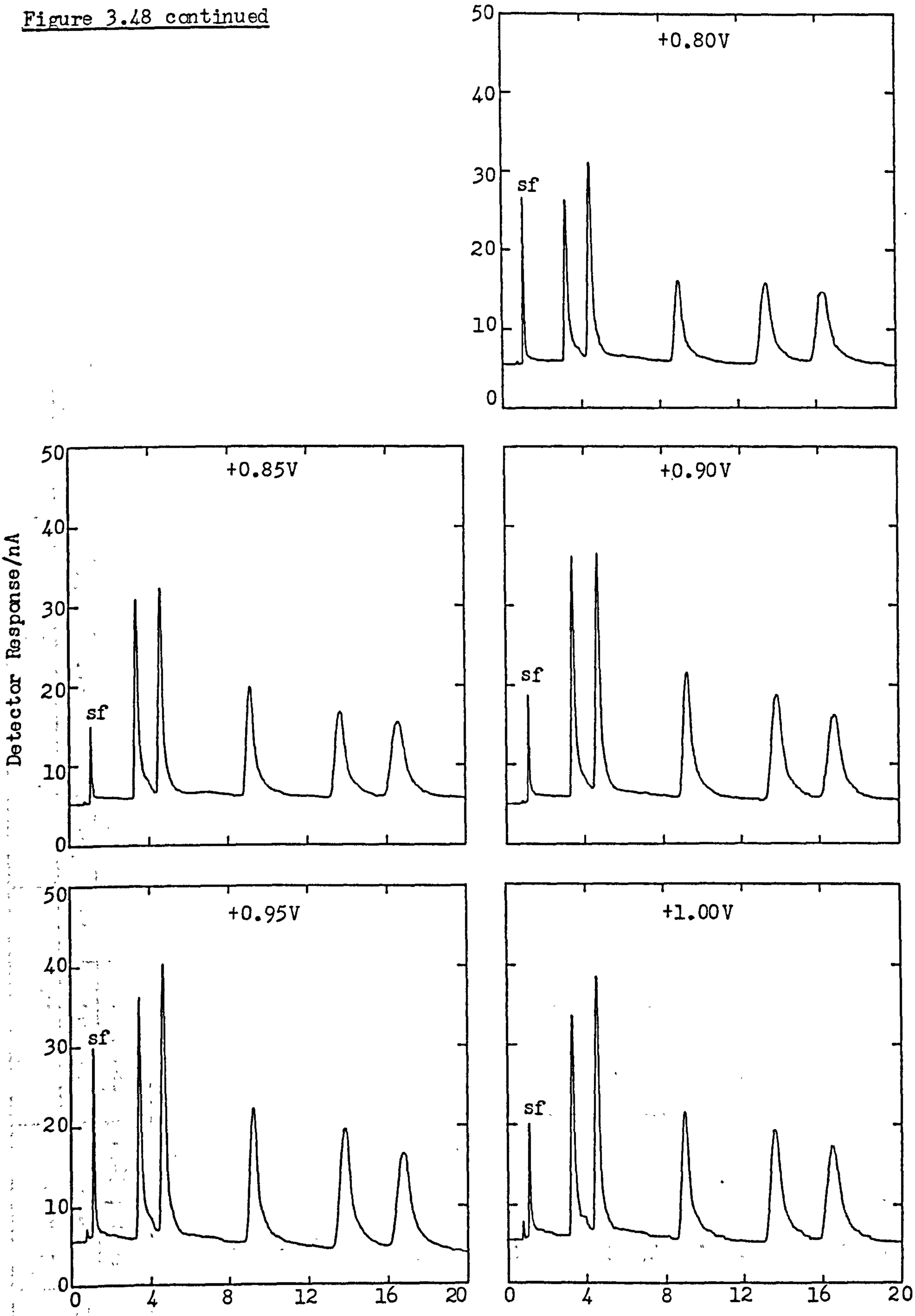


Table 3.31 Signal and Baseline Noise Data Obtained at Various Applied Potentials for Five Indoles

Applied Potential (V)	Baseline Noise		TP			5HTP			5HT			5HIAA			I3S		
			Peak ht.		S/N	Peak ht.		S/N	Peak ht.		S/N	Peak ht.		S/N	Peak ht.		S/N
	(mm)	(pA)	(mm)	(nA)		(mm)	(nA)		(mm)	(nA)		(mm)	(nA)		(mm)	(nA)	
+0.30	1.0	15	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-
+0.35	1.0	15	0	0	-	2	0.30	20	5	0.75	50	3	0.45	30	0	0	-
+0.40	1.2	18	0	0	-	30	4.50	250	15	2.25	125	16	2.40	133	0	0	-
+0.45	1.2	18	0	0	-	41	6.15	342	16	2.40	133	18	2.70	150	0	0	-
+0.50	1.2	18	0	0	-	44	6.60	367	16	2.40	133	18	2.70	150	5.5	0.83	46
+0.55	1.2	18	0	0	-	45	6.75	375	16	2.40	133	18	2.70	150	18	2.70	150
+0.60	1.2	18	0	0	-	46	6.90	383	16	2.40	133	18	2.70	150	26	3.90	217
+0.65	1.4	21	0	0	-	46	6.90	329	17	2.55	121	19	2.85	136	30	4.50	214
+0.70	1.4	21	1	0.15	7	46	6.90	329	17	2.55	121	18	2.70	129	31	4.65	221
+0.75	1.4	21	7	1.05	50	47	7.05	336	17	2.55	121	18	2.70	129	34	5.10	243
+0.80	1.4	21	20	3.00	143	49	7.35	350	18	2.70	129	20	3.00	143	41	6.15	293
+0.85	1.4	21	27.5	4.13	197	52	7.80	371	19	2.85	136	21.5	3.23	154	50	7.50	357
+0.90	1.4	21	31	4.65	221	62	9.30	443	21	3.15	150	26	3.90	186	61	9.15	436
+0.95	1.4	21	34	5.10	243	69	10.35	493	24	3.60	171	30	4.50	214	61	9.15	436
+1.00	1.6	24	32	4.80	200	64	9.60	400	23	3.45	144	28.5	4.28	178	55	8.25	344

Figure 3.49 Plots of Signal-to-Noise Ratio *vs.* Applied Potential for the Five Indoles under Study.

(Error bars represent an estimated measurement error of 0.1mm ≡ 1.5pA in the determination of the noise level)

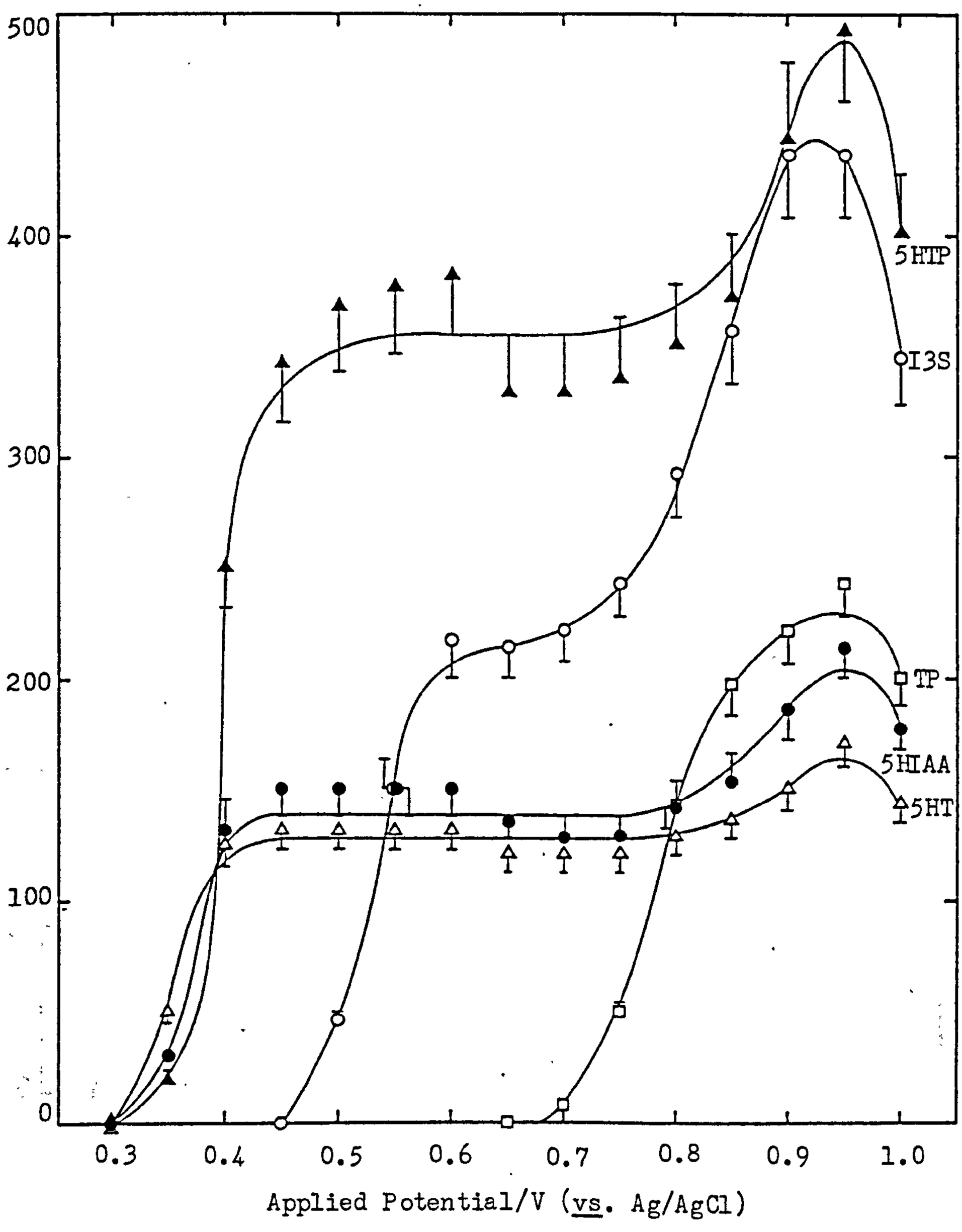
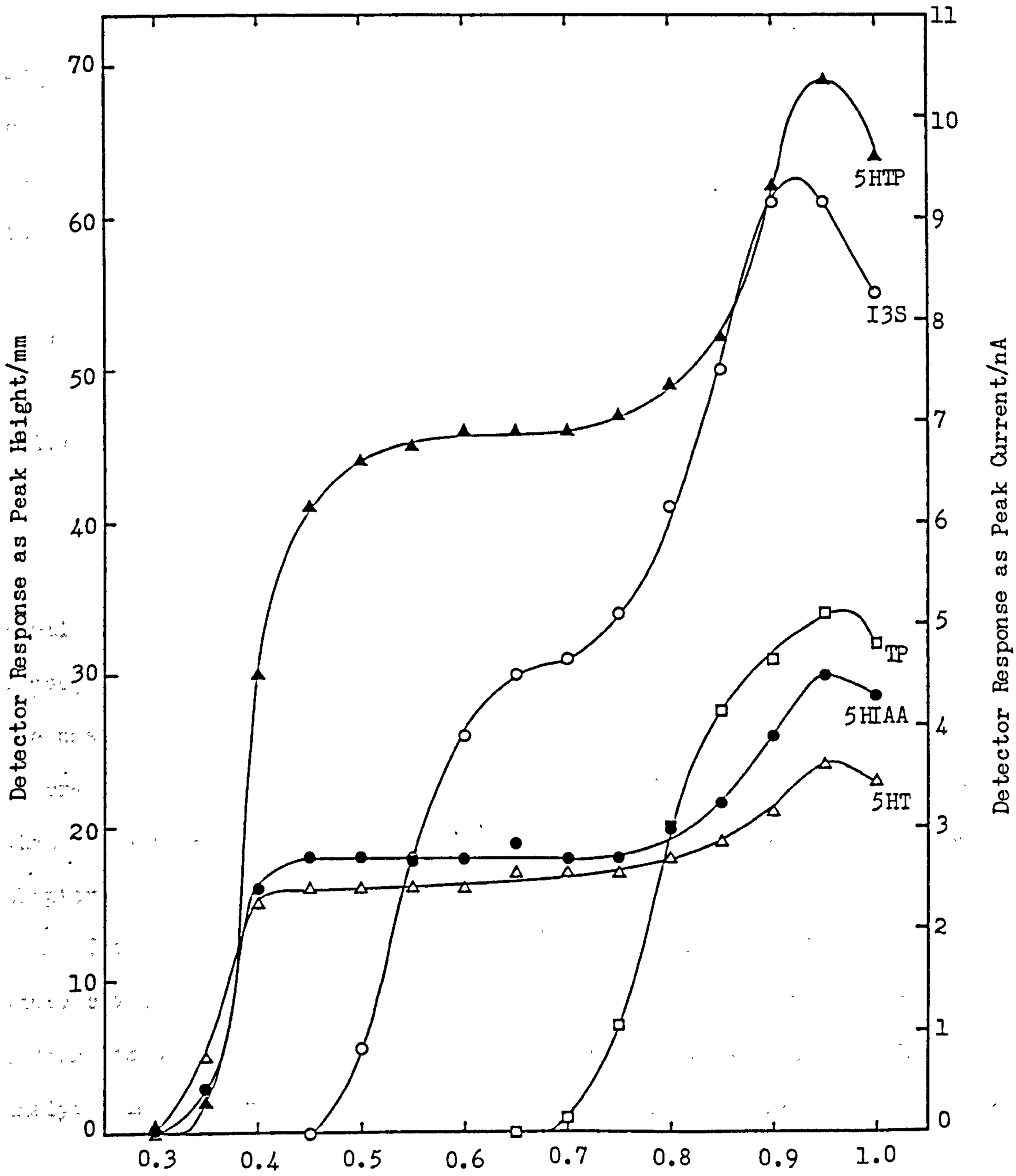


Figure 3.50 Plots of Electrochemical Detector Response (Expressed as Peak Height and Peak Current) vs. Applied Potential for the Five Indoles under Study



This limit is dictated by TP which possesses only one electrophore, viz. the indole ring nitrogen atom which oxidises between +0.70 and +1.00V. The other four analytes all possess one additional electrophore which oxidises at a lower potential. Based on signal-to-noise considerations (Figure 3.49) the optimum applied potential (E_{opt}) for this separation of standards was taken to be +0.95V. Should interference be prevalent when extracts of authentic samples are chromatographed, then greater selectivity is available by reduction of E_{opt} down as far as +0.75V. Should this strategy be unsatisfactory, then attention should be addressed to improvement of the isolation procedure and/or the chromatography to reduce this interference to a tolerable level.

For all subsequent chromatography an applied potential of +0.95V was selected unless stated otherwise.

3.4.7 Evaluation of the Effect of Injection Volume on the Chromatography of Indoles in a Sample-Limited Situation

Frequently in the field of biomedical analysis, sample volume is limited particularly where neonatal or paediatric patients are concerned. Consequently, for the determination of trace level components in the sample, the mass available is highly restricted. Under such circumstances, one theoretical advantage of NBLC over conventional HPLC would be of value, namely that of increased mass sensitivity, the origin of which has been discussed in Chapter 1 (Section 1.4.4).

The ideal approach to the analysis of a biological fluid specimen would consist of the extraction of the analyte substances from all the other matrix components (the isolation step) followed by dissolution of the analytes in a minimum volume of mobile phase (the concentration step) so that

the entire analyte mass could be introduced onto a NBLC column for separation and quantitation. Unfortunately, in practice this procedure is not entirely feasible and to some extent is extravagant. Complete isolation of the analytes from the remainder of the sample components prior to chromatography is very nearly impossible to accomplish where complex biological matrices are concerned and often is unnecessary anyway. Only those species which constitute an interference, either physical or chromatographic, need be removed.* Furthermore, there are practical limitations which govern the volume of mobile phase that may be employed to redissolve the analytes following isolation, which necessarily restrict the maximum concentration of each analyte that may be attained. First, sufficient volume must be utilised to ensure that complete dissolution of the analytes is possible. Secondly, this volume must be dispensable and transferable with acceptable accuracy and precision by means of available laboratory hardware (i.e., syringes, centrifuge tubes, sample tubes, etc.). Finally, the injection technique employed is subject to potentially significant sample loss, about 10µl in excess of the loop volume being required to enable sample introduction to be executed with the Rheodyne model 7413 micro-injection valve at the author's disposal.

Of the aforementioned three considerations, the controlling factor was found to be that of sample manipulability which imposed a practical

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* Physical interferences include strong (irreversible) adsorption of, for example, proteinaceous material onto the stationary phase surface which cause column deterioration. Chromatographic interference from a substance arises if that substance both coelutes with an analyte and produces a response from the detector in use.

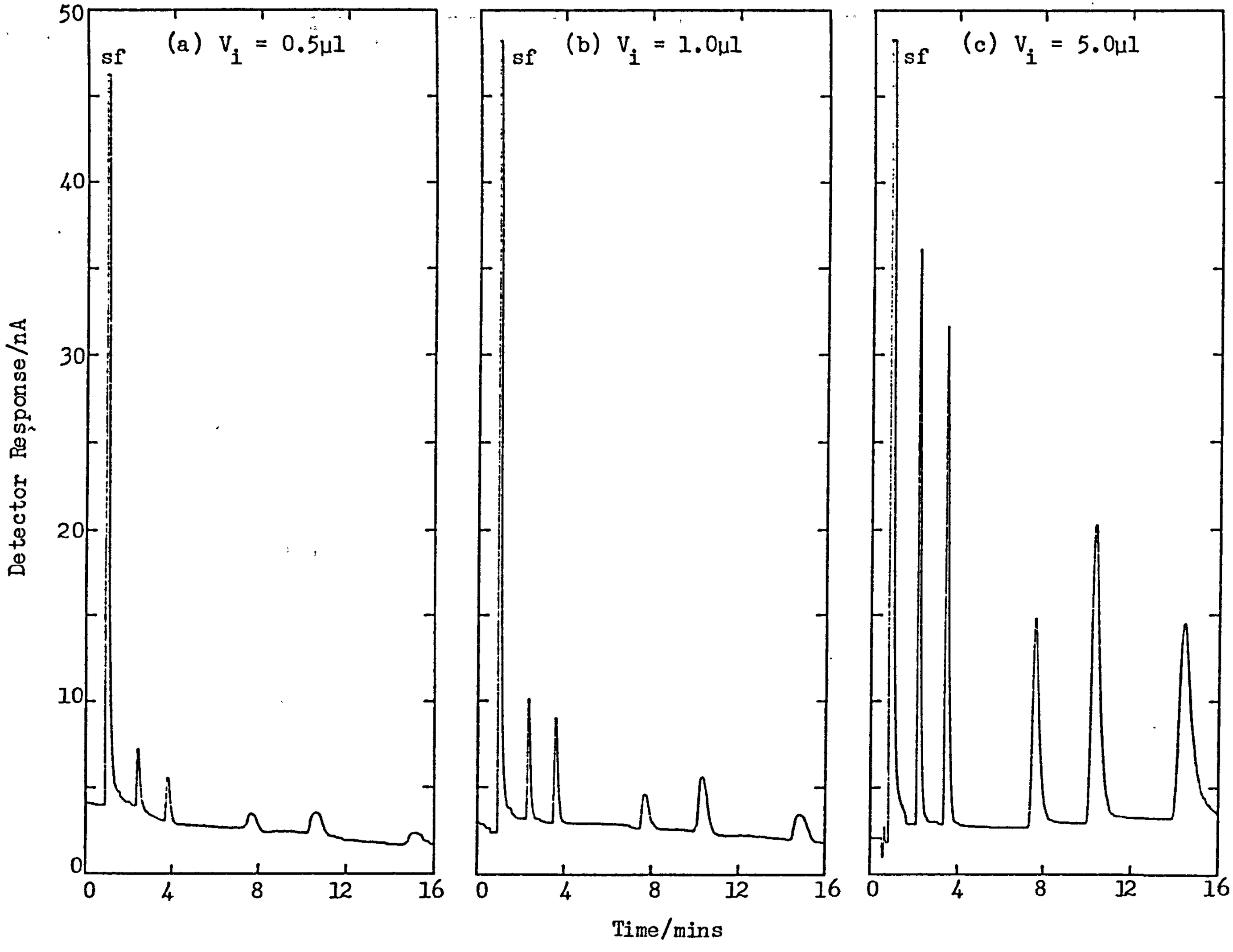
minimum volume limit of 50µl on the extract. Such a limit is of the order of 10-100 times greater than injection volumes commonly employed with NBLC which, in practice, represents a highly significant sample mass loss to the chromatographic process. Moreover, because of this constraint, the applicability of increased mass sensitivity with NBLC columns is brought into question since an increase in injection volume is also accompanied by an increase in the proportion of available analyte mass delivered onto the column. Clearly, it would be advantageous to discover what injection volume provides the greatest practicable detectability within the bounds of satisfactory chromatography. An experiment was conducted with the intention of determining this injection volume.

A standard solution containing a fixed concentration ($0.4\mu g ml^{-1}$) of each indole under investigation was utilised. Volumes of 0.5, 1, 5, 10 and 20µl (≡ 0.2, 0.4, 2, 4 and 8ng of each analyte on-column) were injected into the Pye Unicam NBLC system incorporating a freshly-packed Spherisorb S5 ODS1 column (10cm x 2.1mm ID, d_p = 5µm, N_c = 3280 plates, H_c = 30.5µm) which was operated under standardised conditions. The three smallest volumes were introduced via the Rheodyne model 7413 micro-injection valve whilst a conventional Rheodyne model 7010 external loop valve was employed for transference of the two largest volumes. All chromatograms were recorded at 50nA f.s.d. instrument sensitivity (Figure 3.51) and additionally at an appropriate sensitivity setting so that the peaks of interest generated at each injection volume were on-scale and of approximately equivalent height. Furthermore, the latter chromatograms were also re-run at elevated chart speed so that peak width measurements could be made with less error.

Peak heights were determined together with the baseline noise level and from these values LODs and LOQs for each indole at each injection volume,

Figure 3.51 Chromatograms Illustrating the Effect of Injection Volume of a Standard Indole Mixture of Fixed Concentration on Chromatographic Performance

Parameters :- Column : Spherisorb S5 ODS1 (10cm x 2.1mm, d_p = 5µm); Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA (200mgl^{-1}), pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : ECD (E_{app} = +0.95V vs. Ag/AgCl, Damp : On); Sample : TP, 5HTP, 5HT, 5HIAA and I3S in 4% MeOH : 96% aq. 0.1M KH_2PO_4 (All constituents @ 0.4µgml^{-1}), injection volume as specified



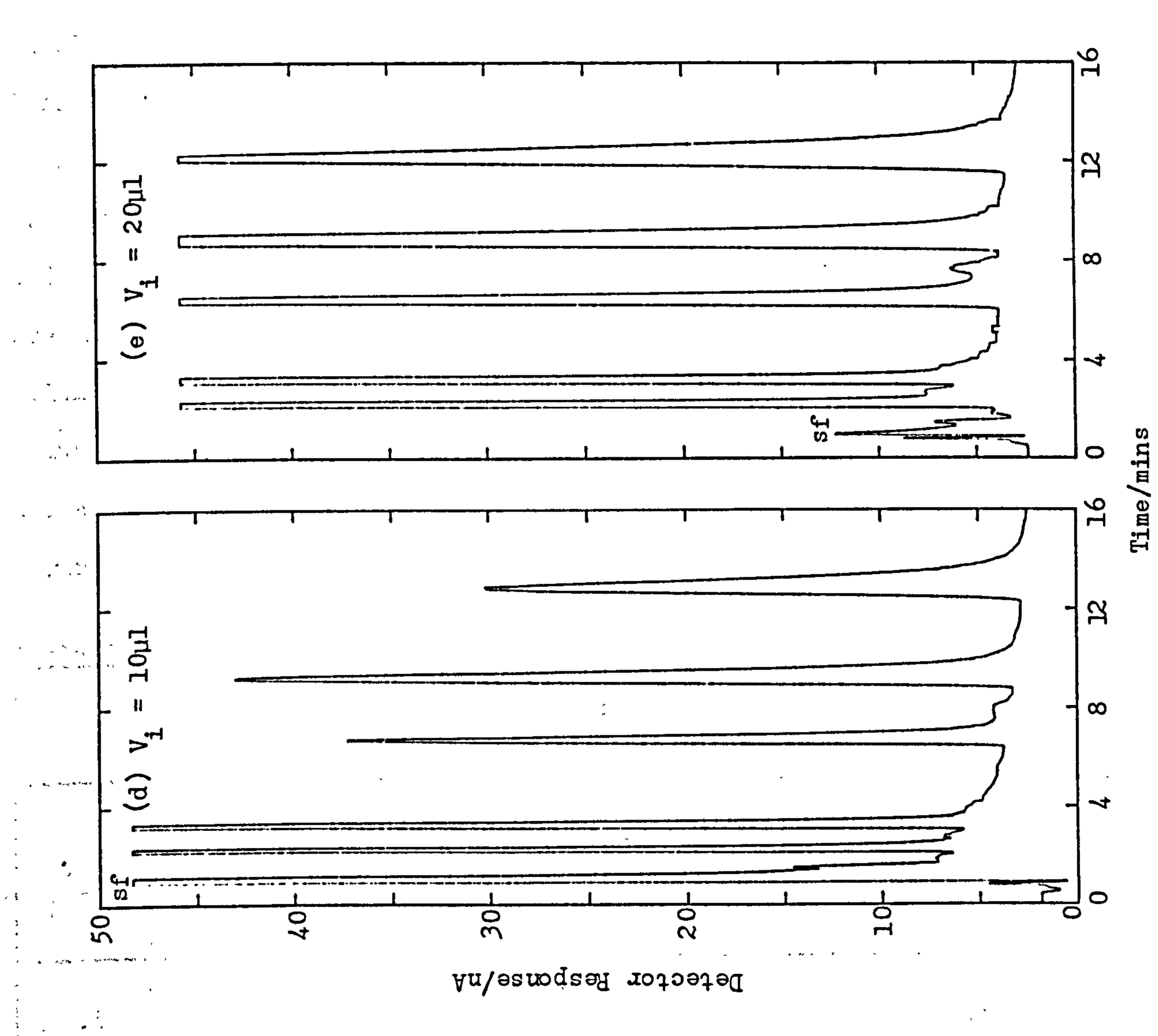


Figure 3.51 continued

V_i, were calculated. These data are tabulated in Table 3.32 and graphs of peak current (and *S/N*) versus V_i, and LOD (and LOQ) versus V_i are presented in Figures 3.52 and 3.53 respectively.

Retention times and peak widths at both 60.7% and 50% of peak height were measured and, from these parameters, values of $\hat{N}$ and $\hat{H}$ were elucidated. Results are reported for I3S and 5HT in Tables 3.33 and 3.34 respectively. Peaks corresponding to I3S, the fastest eluting analyte, and 5HT, the most highly retained analyte, were selected for presentation in order to show the extremes of behaviour exhibited. Graphs were constructed of mean $\hat{N}$ and mean $\hat{H}$ (for the two methods of calculations) against V_i. These plots are included as Figures 3.54 and 3.55. In addition, the percentage of column efficiency realised with respect to I3S and 5HT at each injection volume was determined and this was plotted versus V_i. The computed values are compiled in Table 3.35 while their relationship with V_i is depicted in Figure 3.56.

Table 3.35 Calculated Proportions of Available Column Efficiency Realised at Various Injection Volumes for I3S and 5HT

V_i (µl)	H_c (µm)	I3S		5HT	
		$\hat{H}$ (µm)	$(H_c/\hat{H}) \times 100$ (%)	$\hat{H}$ (µm)	$(H_c/\hat{H}) \times 100$ (%)
0.5	30.5	48.2	63.3	32.2	94.8
1.0	30.5	50.3	60.7	32.8	93.1
5.0	30.5	61.5	49.6	33.7	90.5
10.0	30.5	74.3	41.0	44.3	68.9
20.0	30.5	100.7	30.3	58.1	52.5

Table 3.32 Signal, Signal-to-Noise Ratio, Limit of Detection and Limit of Quantitation Data for the Indoles at Various Injection Volumes

Indole	V_i (µl)	m_i (ng)	Signal (nA)	*S/N**	LOD (pg)	LOQ (pg)
TP	0.5	0.2	2.1	21	18.8	188
	1.0	0.4	3.4	34	23.9	239
	5.0	2.0	13.0	130	30.8	308
	10.0	4.0	32.0	320	25.0	250
	20.0	8.0	65.0	650	24.6	246
5HTP	0.5	0.2	3.5	35	11.5	115
	1.0	0.4	7.4	74	10.9	109
	5.0	2.0	30.5	305	13.1	131
	10.0	4.0	60.0	600	13.3	133
	20.0	8.0	113.0	1130	14.2	142
5HT	0.5	0.2	1.8	18	22.2	222
	1.0	0.4	2.9	29	28.1	281
	5.0	2.0	11.5	115	34.8	348
	10.0	4.0	24.5	245	32.7	327
	20.0	8.0	47.0	470	34.0	340
5HIAA	0.5	0.2	2.7	27	14.8	148
	1.0	0.4	4.4	44	18.4	184
	5.0	2.0	18.5	185	21.6	216
	10.0	4.0	37.5	375	21.3	213
	20.0	8.0	74.0	740	21.6	216
I3S	0.5	0.2	4.0	40	10.0	100
	1.0	0.4	8.5	85	9.5	95
	5.0	2.0	35.0	350	11.4	114
	10.0	4.0	72.0	720	11.1	111
	20.0	8.0	135.0	1350	11.9	119

* *N* (measured at 5nA f.s.d.) = 0.10nA. *N* is unaffected by V_i

Figure 3.52 The Relationship Between Electrochemical Detector Response (Expressed as both Peak Current and Signal-to-Noise Ratio) and Injection Volume of a Standard Indole Mixture of Fixed Concentration

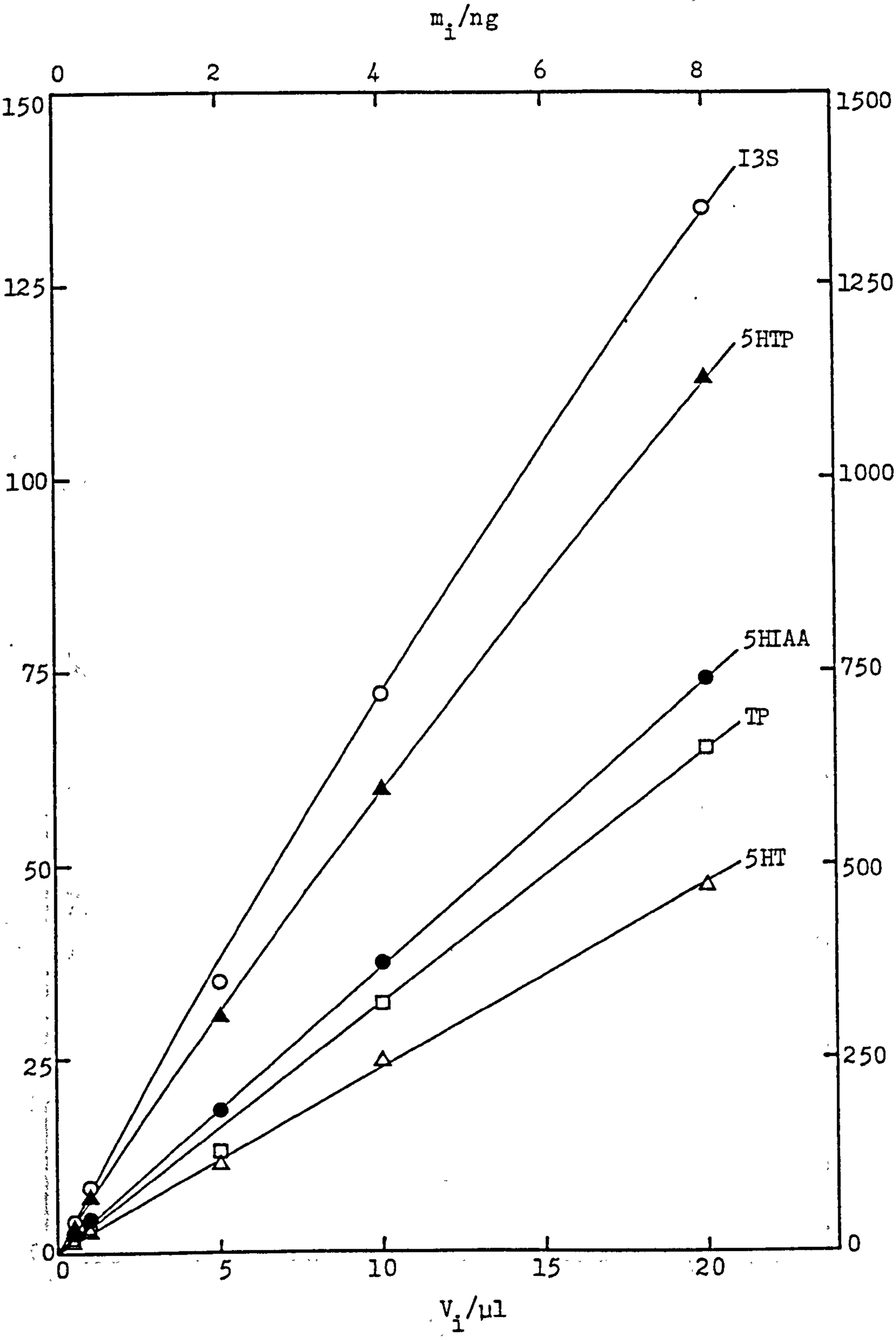


Figure 3.53 The Relationship Between Limit of Detection (*S/N* = 2:1) or Limit of Quantitation (*S/N* = 20:1) and Injection Volume of a Standard Indole Mixture of Fixed Concentration

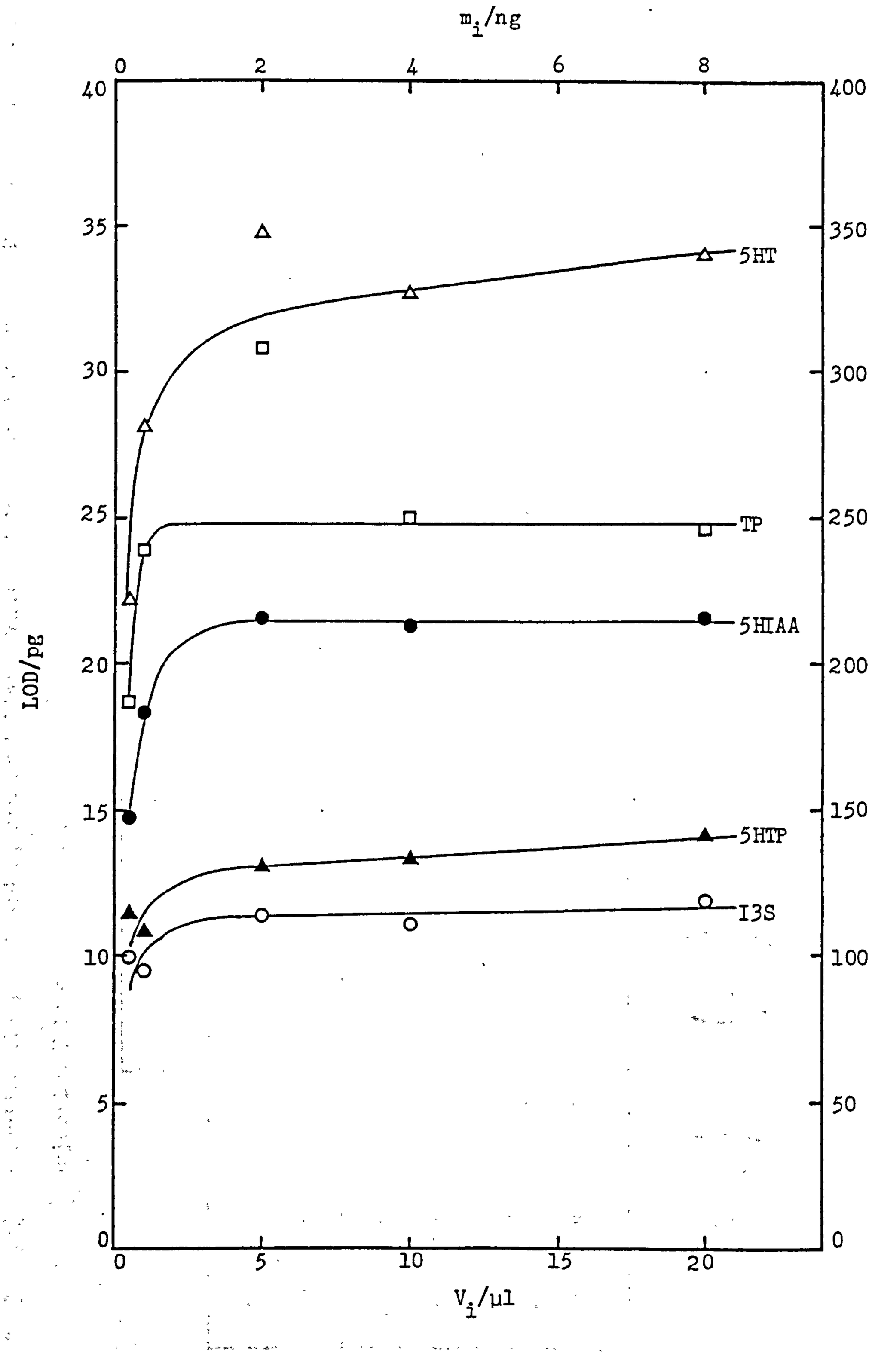


Table 3.33 Retention Time, Peak Width, Apparent Plate Count and Apparent Plate Height Data for I3S at Various Injection Volumes

			Peak Width Measurement									
			Half-width				Width at half-height					
V_i	t_R		$w_{0.607}$		$\hat{N}$*	$\hat{H}$†	$w_{0.5}$		$\hat{N}$*	$\hat{H}$†	Mean $\hat{N}$ for the two methods ± SD	Mean $\hat{H}$ for the two methods ± SD (μm)
(μl)	(mm)	(min)	(mm)	(min)		(μm)	(mm)	(min)		(μm)		
0.5	69	2.30	3.0	0.10	2116	47.3	3.6	0.12	2035	49.1	2076 ± 57	48.2 ± 1.3
1.0	65.5	2.18	2.9	0.10	2041	49.0	3.5	0.12	1940	51.5	1991 ± 71	50.3 ± 1.8
5.0	67.5	2.25	3.3	0.11	1674	59.7	4.0	0.13	1578	63.4	1626 ± 68	61.5 ± 2.6
10.0	68	2.27	3.6	0.12	1427	70.1	4.5	0.15	1265	79.1	1346 ± 115	74.3 ± 6.4
20.0	68	2.27	4.3	0.14	1000	100.1	5.1	0.17	985	101.5	993 ± 11	100.7 ± 1.1

* $\hat{N} = 4\left(\frac{t_R}{w_{0.607}}\right)^2 = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2$

† $\hat{H} = L_c/\hat{N}$ where L_c = 10cm

Table 3.34 Retention Time, Peak Width, Apparent Plate Count and Apparent Plate Height Data for 5HT at Various Injection Volumes

			Peak Width Measurement									
			Half-width				Width at half-height					
V_i	t_R		$w_{0.607}$		$\hat{N}$	$\hat{H}$	$w_{0.5}$		$\hat{N}$	$\hat{H}$	Mean $\hat{N}$ for the two methods ± SD	Mean $\hat{H}$ of the two methods ± SD (μm)
(μl)	(mm)	(min)	(mm)	(min)		(μm)	(mm)	(min)		(μm)		
0.5	420	14.00	15.0	0.50	3136	31.9	17.8	0.59	3084	32.4	3110 ± 37	32.2 ± 0.4
1.0	415	13.83	15.0	0.50	3062	32.7	17.7	0.59	3046	32.8	3054 ± 11	32.8 ± 0.1
5.0	435	14.50	15.8	0.53	3032	33.0	19.0	0.63	2904	34.4	2968 ± 91	33.7 ± 1.0
10.0	396	13.20	16.5	0.55	2304	43.4	19.8	0.66	2216	45.1	2260 ± 62	44.3 ± 1.2
20.0	377	12.57	18.0	0.60	1755	57.0	21.6	0.72	1688	59.2	1722 ± 47	58.1 ± 1.6

Figure 3.54 The Relationship Between Apparent Plate Count (Mean of Two Methods of Assessment) and Injection Volume of a Standard Indole Mixture of Fixed Concentration

(Standard deviation limits indicated)

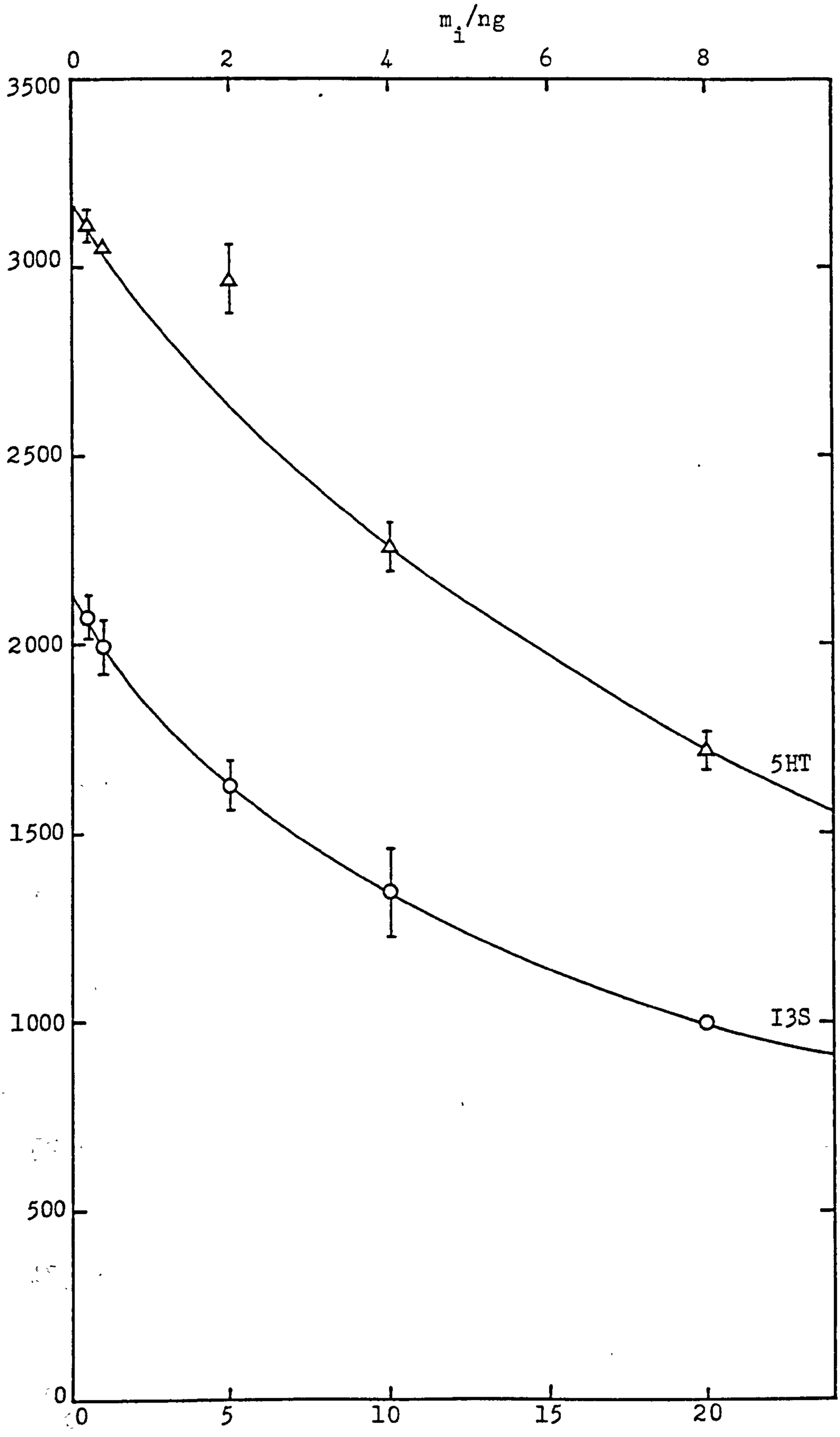
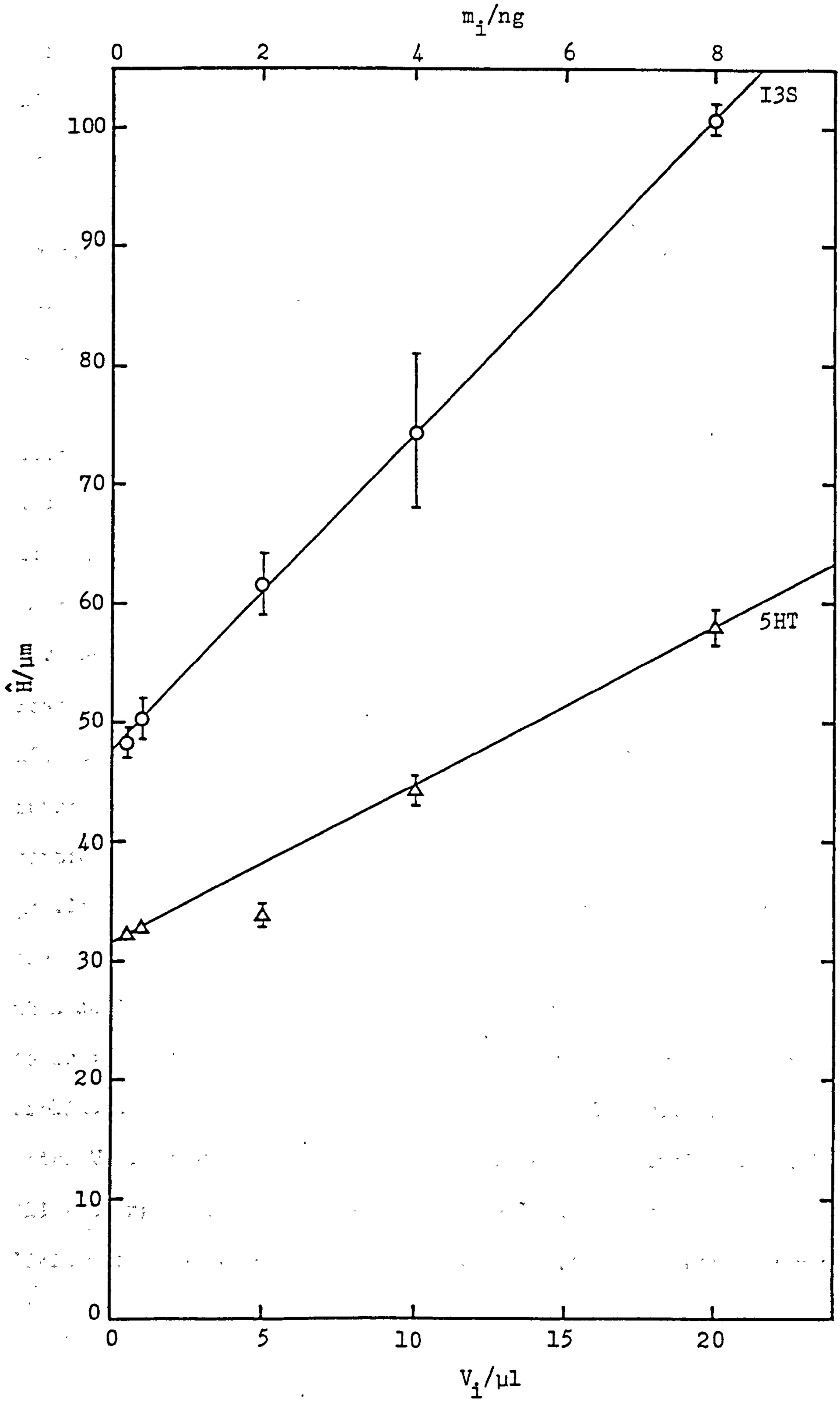


Figure 3.55 The Relationship Between Apparent Plate Height (Mean of Two Methods of Assessment) and Injection Volume of a Standard Indole Mixture of Fixed Concentration

(Standard deviation limits indicated)



It should be noted that all quantities measured and calculated from the chromatograms obtained by injection of a 5µl volume were suspect. These chromatograms were recorded first and exhibited significantly narrower peaks with lengthened capacity factors which yielded correspondingly higher apparent plate counts and smaller apparent plate heights. The freshly-packed column most probably had not been "worked-in" sufficiently prior to commencing this study. Attempts to repeat this part of the experiment were obstructed by instrument malfunction, hence, the values that were obtained initially are reported herein and are treated as outliers.

Now, it is well known that increase in injection volume gives rise to increased extra-column dispersion which manifests itself as a broadening and flattening of peaks. Consequently, the apparent plate count is reduced and the apparent plate height increased. This effect is amply demonstrated in Figures 3.54 and 3.55. Parameters which depend directly upon $\hat{N}$ such as mass sensitivity, (which is synonymous with LOD as defined herein) and resolution are correspondingly affected. The relationship between LOD (and LOQ) and V_i for each analyte is illustrated in Figure 3.53. The theoretical improvement in LOD with decreasing V_i is observed in practice with a particularly marked effect at low V_i, i.e. < 2-3µl. However, as was stated previously, improvement in LOD by reduction in V_i may not necessarily provide the best detectability because of the intrinsic increase in signal obtainable when an increase in V_i is accompanied by an increase in injected mass, m_i, as is the case here (see Figure 3.52). Hence, large V_i provides high signal amplitude while small V_i provides low LOD. The critical factor that defines which V_i is most appropriate is the degree of resolution which is acceptable. Klinkenberg[59] arbitrarily defined a 10% loss in resolution as the tolerable limit which today is widely accepted. Now, the theoretical maximum injection

volume that is acceptable is given by Equation 1.28, i.e.,

$$V_{i(max)} = K\theta_i V_o(1 + k')/N^{\frac{1}{2}} \qquad (1.28)$$

This expression may be written in terms of column parameters thus :

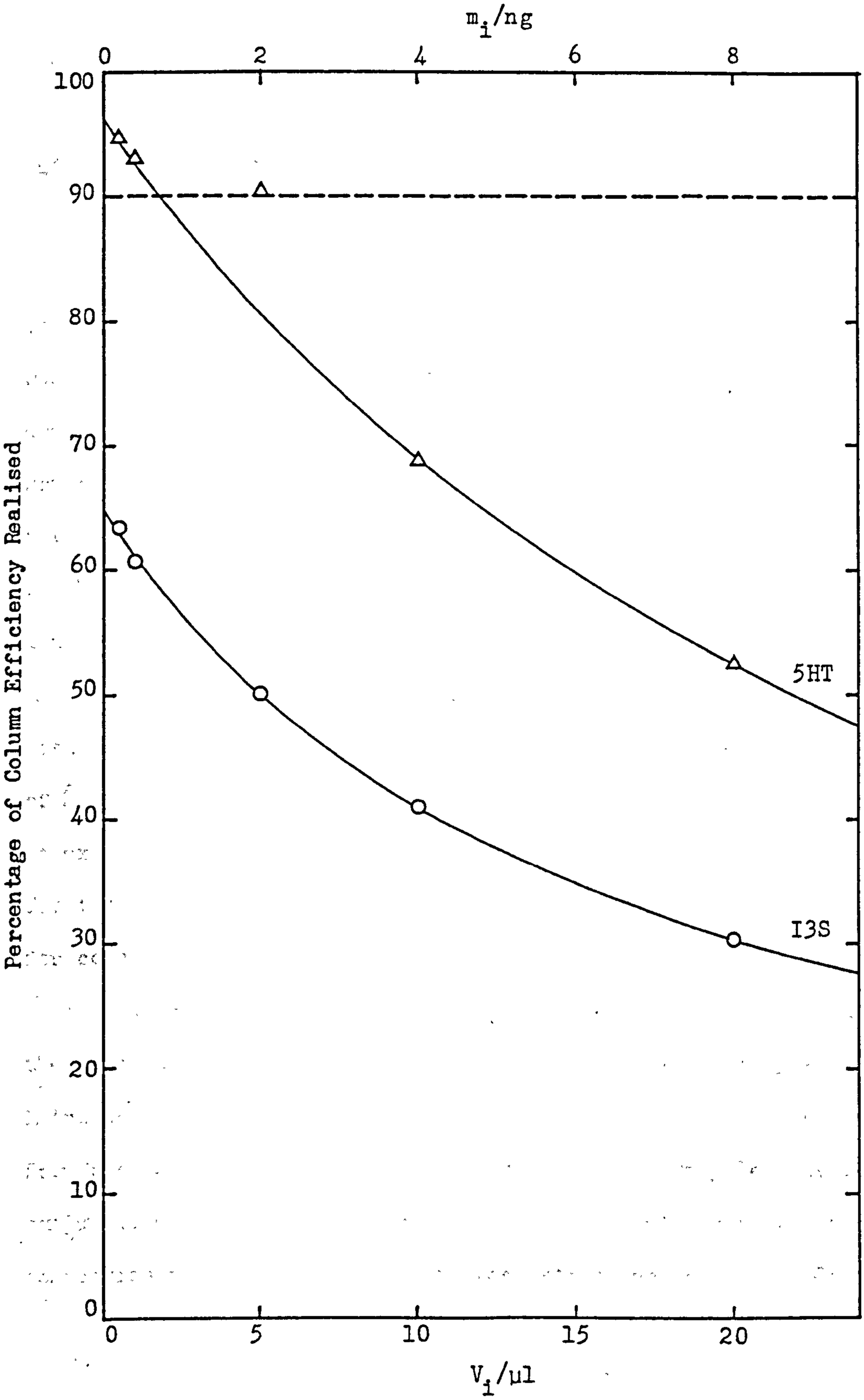
$$V_{i(max)} = K\theta_i \, 2\varepsilon\pi(1 + k')r_c^2 \, L_c^{\frac{1}{2}} \, d_p^{\frac{1}{2}}$$

Employing typical values for K and ε of 12 and 0.7 respectively and substituting in the column dimensions, the absolute theoretical maximum injection volume tolerable (i.e., where $\theta_i^2 = 0.1$) for a chromatogram in which $k'_{min} \simeq 2$ (as arranged here) is calculated to be 8.0µl. It should be noted that in a practical situation a volume substantially less than this would be considered acceptable due to the contributions to extra-column dispersion from other components of the system.

The imposition of Klinkenberg's criterion brings to the fore the problem that the NBLC system, when subjected to a 1µl injection volume, had previously been found to be operating well below the level of performance necessary to conform to this degree of tolerance (see Section 3.4.5). This difficulty is further exacerbated at higher V_i as is demonstrated by Figure 3.56 which depicts the proportion of available column efficiency realised for I3S and 5HT for values of V_i between 0.5 and 20µl. These graphs indicate that I3S ($k' = 2.3$) suffers gross extra-column band broadening over the entire volume range, only achieving 63% of possible column efficiency for an applied volume of 0.5µl, while 5HT ($k' \simeq 19$) is similarly subject to this phenomenon over the majority of the volume range examined, the theoretical criterion only being satisfied at injection volumes of $\leq$ 2µl. The other three solutes occupy the region between these two extremes. The gross

Figure 3.56 Plot of Proportion of Available Column Efficiency Realised (Mean of Two Methods of Assessment) vs. Injection Volume of a Standard Indole Mixture of Fixed Concentration

(Dashed line represents Klinkenberg's criterion of tolerability)



inadequacy of this system with respect to instrument dispersion, irrespective of injection volume, has been demonstrated conclusively. Clearly the arbitrary criterion of 10% resolution loss being acceptable is impractical where the Pye Unicam NBLC system under examination is concerned.

In practical terms, the important consideration is maintenance of sufficient resolution such that the peaks of interest in the chromatogram do not begin to coalesce but elute as discrete entities. For some separations 50% loss in resolution may be acceptable whereas for others 5% loss may not be. In terms of absolute resolution, a value of 1.5 affords baseline separation between two peaks of Gaussian or near-Gaussian shape even with a discrepancy in height of 100:1 (cf. 31). The actual chromatographic resolution experienced at each injection volume was calculated from retention time and peak width data for peaks cprresponding to I3S and 5HTP, which exhibited the lowest, and consequently, the controlling resolution in the chromatogram. These data are compiled in Table 3.36 and are presented in graphical form in Figure 3.57. As was expected, resolution decreases with increasing V_i because of the former's dependence on the square root of $\hat{N}$. Even with an injection volume as great as 20µl resolution was far in excess of the aforementioned value of 1.5 and consequently was satisfactory for the elution of standards with considerable leeway remaining to allow for column deterioration.

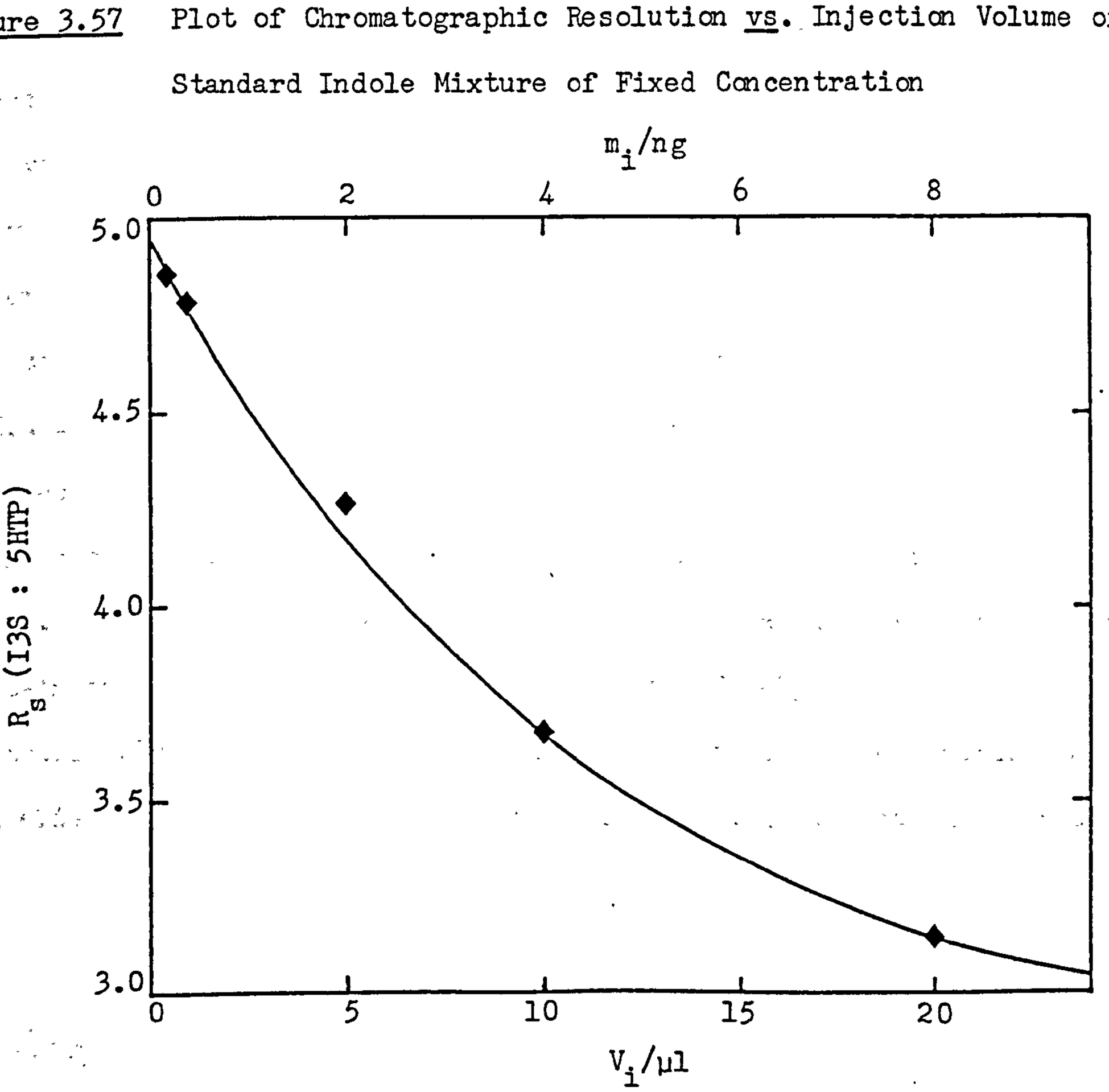
One fundamental point has not been addressed thus far, namely that the separation obtained for standards does not necessarily allow for the introduction and elution of endogenous electroactive coextracted materials from biological samples. Hence, resolution may only be assessed fully in conjunction with isolation procedures and authentic samples in order that a compromise may be settled upon between stringency of the isolation procedure

Table 3.36 Retention Time, Peak Width and Resulting Resolution Data for I3S and 5HTP Corresponding to Various Injection Volumes

V_i (µl)	I3S			5HTP			R_s (I3S:5HTP)
	t_R (min)	$w_{0.607}$ (min)	w_B* (min)	t_R (min)	$w_{0.607}$ (min)	w_B* (min)	
0.5	2.30	0.10	0.20	3.50	0.15	0.29	4.86
1.0	2.18	0.10	0.19	3.30	0.14	0.27	4.79
5.0	2.25	0.11	0.22	3.40	0.16	0.32	4.26
10.0	2.27	0.12	0.24	3.33	0.17	0.34	3.68
20.0	2.27	0.14	0.29	3.27	0.17	0.35	3.15

* w_B estimated as 2 x $w_{0.607}$

Figure 3.57 Plot of Chromatographic Resolution vs. Injection Volume of a Standard Indole Mixture of Fixed Concentration



and elution characteristics of any detectable coextracted substances in relation to those of the analytes.

An injection volume of 5µl was selected as a compromise between resolution, LOD and proportion of available sample mass introduced. A further consideration was the ability to conduct duplicate and possibly triplicate analyses of a single 50µl extract using the Rheodyne 7413 sample injection valve. This decision was open to review in view of resolution considerations where biological fluid extracts were concerned although the clear separation obtained (R_s > 4 between each analyte pair) provided considerable scope for avoidance of coelution problems.

The application of a 5µl volume does not allow realisation of the full improved mass sensitivity potential obtainable with small-diameter columns. However, this volume was selected on the basis of the limitations of the Pye Unicam NBLC system and the practical difficulties associated with accessible ancillary laboratory equipment. Improvements should be achievable in three ways. First, acquisition of higher specification laboratory equipment would enable manipulation of sample volumes approximately one order of magnitude smaller than is currently feasible. Should this be possible, then a less wasteful but equally precise sample introduction method would be advantageous. Within the last three years Rheodyne have marketed a new micro-injection valve, the model 7520, which is pictured in Figure 3.58. This injector represents a significant step forward in valve design. The sample is loaded directly into the sample "loop" *via* a central built-in injection port with a hold-up volume of only 0.3µl (*cf.* 7µl for the model 7413 valve) so reducing waste substantially. No sample loop as such exists; the injection volume (a choice of 0.2, 0.5 and 1µl is available)

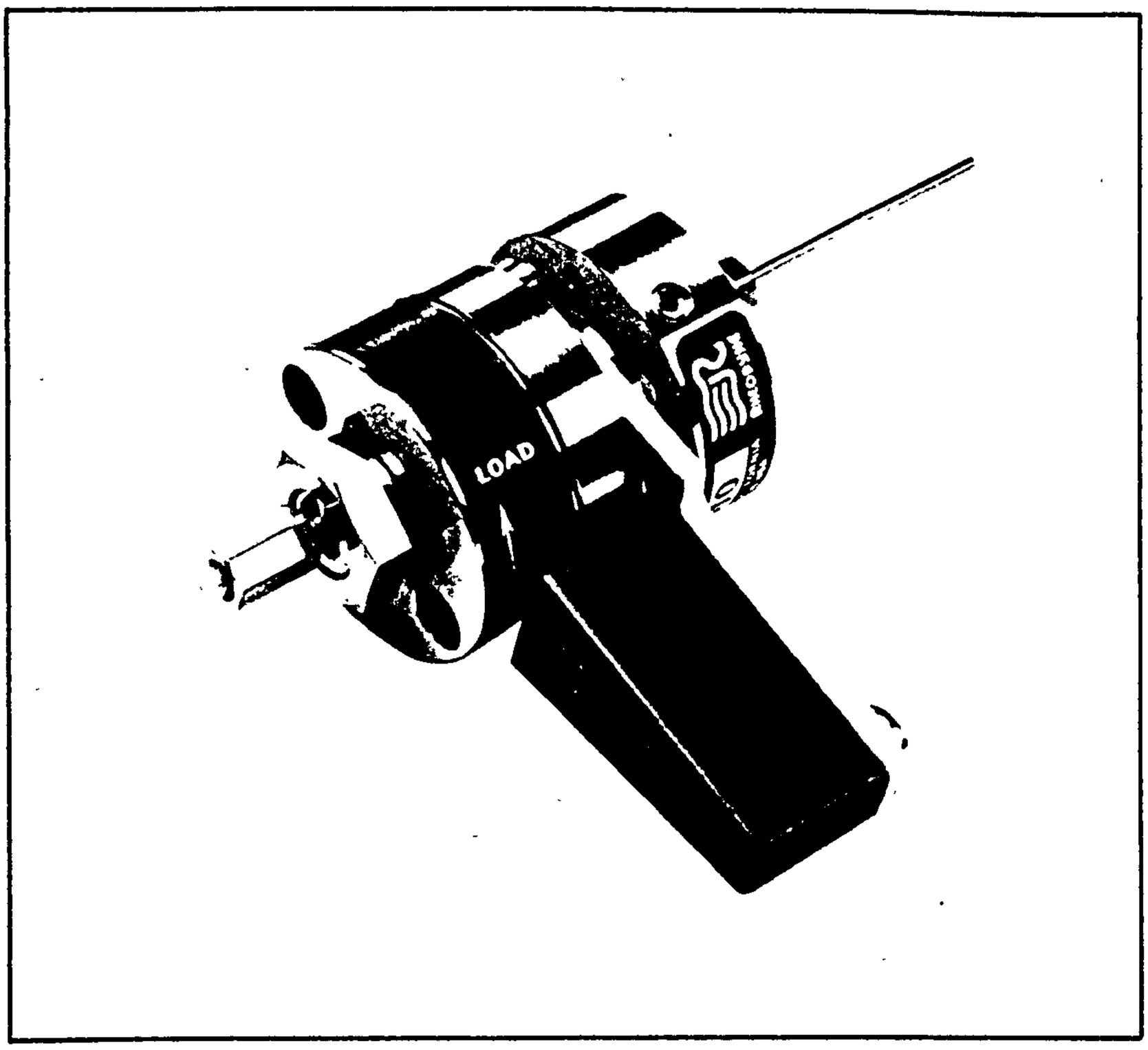


Figure 3.58 The Rheodyne Model 7520 Micro-Injection Valve

is housed in a chamber in the rotor block which is translocated into the flowstream in order to execute injection. This process is illustrated by means of flow diagrams in Figure 3.59.

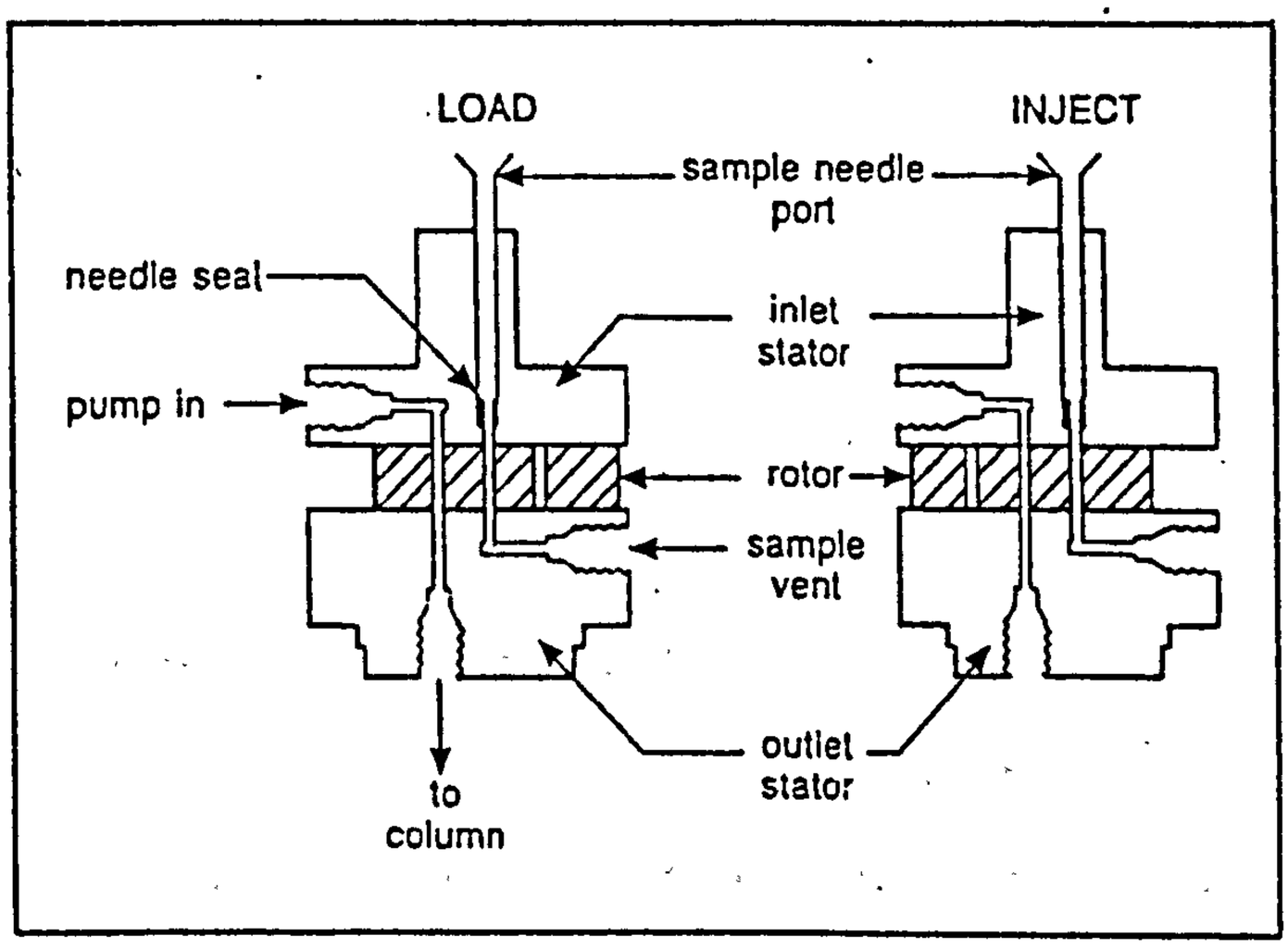


Figure 3.59 Flow Diagram for the Rheodyne Model 7520 Micro-Injection Valve

Hence, valve technology today is sufficiently advanced to accommodate the low sample wastage requirement. The third improvement of ultimate benefit would be gained by use of a NBLC system of considerably reduced dispersion characteristics. A pump of higher specification capable of delivering pulseless flow rates of one order of magnitude lower than the PU4010 can and an ECD with a considerably reduced time constant facility would be particularly beneficial.

3.4.8 The Search for an Internal Standard

The employment of an internal standard (IS) is desirable in trace analysis because some sources of random error inherent in the isolation and chromatographic procedures may be compensated for thus yielding improved precision. Generally the IS is added to the sample as early in the sample preparation procedure as possible and the final quantitative measurement is the ratio of either the peak heights or the peak areas of the species of interest and the IS.

Ideally an IS should be both chemically similar (i.e., possessing similar structure and functionality) and chromatographically similar (i.e., being of similar size and polarity) to the analyte species.[413] The importance of the IS being chemically similar to the species to be determined is twofold. First, the IS is required to behave similarly to the analyte species during the extraction procedure and secondly, the IS is required to generate a response from the detector in use. In this case the IS must undergo electro-oxidation at the selected potential (+0.95V vs. Ag/AgCl) and in order to do so it must possess suitable electrophores. Chromatographic similarity to the analyte species is necessary so that the IS elutes at a suitable point in the chromatogram as near to the analyte peaks as possible

whilst being completely resolved from all peaks in the chromatogram. Another essential criterion relating to an IS is that it should not be indigenous to the sample medium. Clearly this is important so that "interference" with the IS peak from (variable) media components does not occur and hence the potential for improved precision is preserved.

Other desirable features of an IS are that it should be chemically stable, non-hazardous, readily available and inexpensive.

In previous work with indoles a number of different substances have been applied as IS's for the analysis of whole blood, serum or plasma by LC-EC. Recent examples include 6-hydroxytryptamine (6HT)[367,414,415], N_{ω}-methyl-5-hydroxytryptamine (N-Me-5HT)[416], N,N-dimethyl-5-hydroxytryptamine (= bufotenine; BF)[417], 5-hydroxyindole-2-carboxylic acid (5HI2A)[363] and α-methyltryptophan (α-MeTP).[418] The choice of IS appears to have been made based on which TP derivative was of primary concern to the researchers. Regrettably, none of these materials was immediately to hand for trial as an IS in conjunction with the current separation although all the afore-mentioned compounds are commercially available, for example from Sigma Chemical Co. (Poole, Dorset, UK). In the absence of a non-biogenic indole, a series of chemicals was selected off the laboratory shelf. Three criteria were employed in making this selection i.e., that the molecules be of similar molecular weight to the analytes, that small quantities of them be soluble in an aqueous MeOH environment, and that they possess at least one suitable electrophore. These conditions were most closely satisfied by aromatic hydroxy- and amino-compounds, primarily phenol, aniline and derivatives thereof. A total of eighteen compounds were assessed as potential IS's for the determination of indoles. These compounds were :

(i) phenol

(ii) 1,2-dihydroxybenzene (catechol)

(iii) 1,2,3-trihydroxybenzene (pyrogallol)

(iv) 1,3,5-trihydroxybenzene (phloroglucinol)

(v) 3-methylphenol (m-cresol)

(vi) 3-chlorophenol

(vii) 4-chlorophenol

(viii) pentachlorophenol

(ix) 2-nitrophenol

(x) 4-nitrophenol

(xi) 4-hydroxybenzoic acid

(xii) 4-methoxybenzyl alcohol (anisyl alcohol)

(xiii) aniline

(xiv) 4-bromoaniline

(xv) 4-nitroaniline

(xvi) 2-aminophenol (2-hydroxyaniline)

(xvii) 2-methoxyaniline (o-anisidine)

(xviii) 4-methoxyaniline (p-anisidine)

Solutions were prepared in a pseudo-mobile phase comprising 4% MeOH : 96% aq. 0.1M KH_2PO_4 and were chromatographed sequentially under conditions optimised for the separations of TP, 5HTP, 5HT, 5HIAA and I3S. These injections were interspersed with injections of a five component indole standard which were made in order to ensure that the chromatography was stable with time and to enable direct comparison of the retention and peak shape of each potential IS with that of the indoles. Retention times were measured and capacity factors were calculated for each substance. These values are listed in Table 3.37 and may be compared with the typical retention times and capacity factors for the five analyte species listed in Table 3.38.

Table 3.37 Retention Times and Capacity Factors for Potential Internal Standards Chromatographed under Optimised Conditions.

Compound	t_R (min)	k′
phenol	6.9	7.63
1,2-dihydroxybenzene	3.6	3.50
1,2,3-trihydroxybenzene	1.8	1.25
1,3,5-trihydroxybenzene	2.0	1.50
3-methylphenol	14.4	17.00
3-chlorophenol	34.6	42.25
4-chlorophenol	32.4	39.50
pentachlorophenol	51.0	62.75
2-nitrophenol	20.8	25.00
4-nitrophenol	19.6	23.50
4-hydroxybenzoic acid	7.3	8.13
4-methoxybenzyl alcohol	10.8	12.50
aniline	5.0	5.25
4-bromoaniline	>60.0	>74.00
4-nitroaniline	15.0	17.75
2-aminophenol	3.8	3.75
2-methoxyaniline	11.0	12.75
4-methoxyaniline	9.2	10.50

Table 3.38 Retention Times and Capacity Factors of Indoles Chromatographed Under Optimised Conditions

Indole	t_R (min)	k′
I3S	2.8	2.50
5HTP	3.6	3.50
TP	7.4	8.25
5HIAA	10.3	11.88
5HT	14.1	16.63

"Windows" appear in the chromatogram of the five component indole standard between peaks corresponding to 5HTP and TP (k′ = 5.3-7.3), TP and 5HIAA (k′ = 9.8-10.8), and 5HIAA and 5HT (k′ = 13.3-15.3). In addition, the region following the final peak in the chromatogram (i.e., k′ > 18.5) is available for IS elution although placement of an IS to greater retention than the most highly retained analyte necessarily increases analysis time.

Of the 18 substances evaluated as IS's, the two trihydroxybenzenes exhibited very little retention (k′ < 2) therefore they were rejected. The four halogenated compounds, 3- and 4-chlorophenol, pentachlorophenol and 4-bromoaniline, all eluted at unacceptably long retention times and consequently, they too were rejected. 1,2-dihydroxybenzene and 2-aminophenol were eliminated because peaks corresponding to these substances interfered with the 5HTP peak. For similar reasons, phenol and 4-hydroxybenzoic acid (coelution with TP), 2-methoxyaniline (coelution with 5HIAA), and 3-methylphenol, 4-methoxybenzyl alcohol and 4-nitroaniline (coelution with 5HT) were

also unacceptable. The two nitrophenols eluted with capacity factors of 23.5 and 25.0 which, if utilised as IS's, would represent an increase in analysis time of about 30% which was not ideal. Of these two compounds the 4-nitrophenol was rejected on peak shape grounds, the band being unacceptably broad. Both substances yielded poor response characteristics, most probably as a result of the powerful electron-withdrawing nature of the $-NO_2$ group on the aromatic ring.

Only two substances were favoured chromatographically in terms of k′, viz. aniline and 4-methoxyaniline. The peak shape for aniline was satisfactory but that for the 4-methoxy derivative was broad and tailing, the tail of which encroached into the region in which 5HIAA elutes and consequently may cause minor interference if used. Hence, aniline (AN) was selected as the substance that was best suited on chromatographic grounds to be employed as an IS for indole chromatography. This assumes that the region of the chromatogram in which AN elutes is free for any electro-oxidisable substances coextracted from samples of biological origin.

The well known instability of AN to aeriel oxidation was considered not to represent a great problem because the analytes are also known to be labile (e.g., 304,305,391-393) thus frequent standard preparation is necessitated for quantitative work regardless of the stability of the chosen IS.

Experiments investigating the extraction of indoles and AN from spiked blood samples (reported in Part IV of this Chapter) revealed that AN was not well suited chemically to the role of IS. Because of the difficult isolation problem, a more closely similar compound to the analytes in structural terms was deemed necessary to act as an IS. AN, however, is suitable as a chromatographic standard (CS), i.e., a substance which is added

to the sample extract following isolation and prior to chromatography in order to compensate for random errors in the chromatographic step alone. However, when using a modern high precision injection valve, a CS is of very limited value.[413]

A sample of N,N-dimethyl-5-hydroxytryptamine (= bufotenine; BF) was obtained from Sigma Chemical Co. (Poole, Dorset, UK) for investigation as an IS on the recommendation of Dr.E. Gelpí of the Instituto de Química Bio-Orgánica, Barcelona, Spain.[417,419] The structure of the BF molecule is shown in Figure 3.60. BF closely resembles 5HT, differing only in the

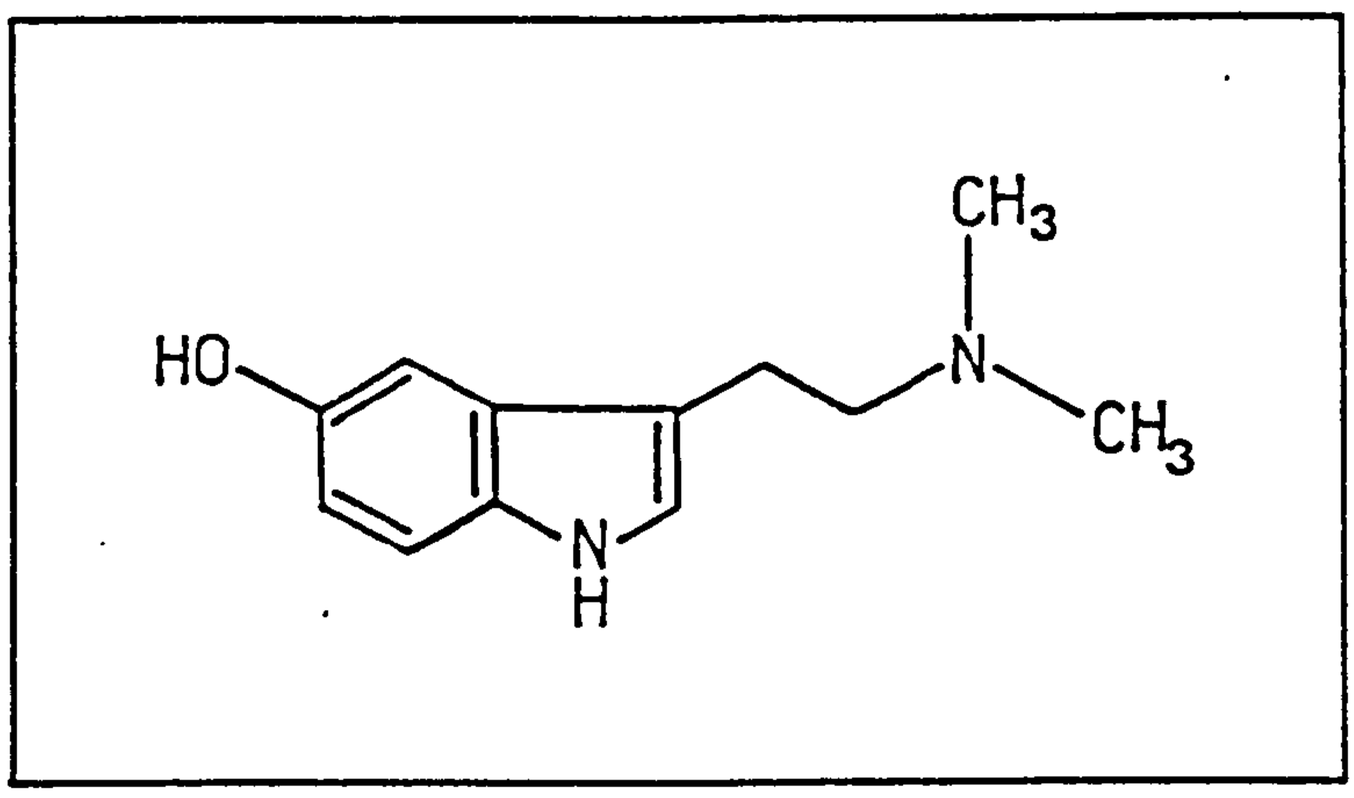


Figure 3.60 The Structure of Bufotenine

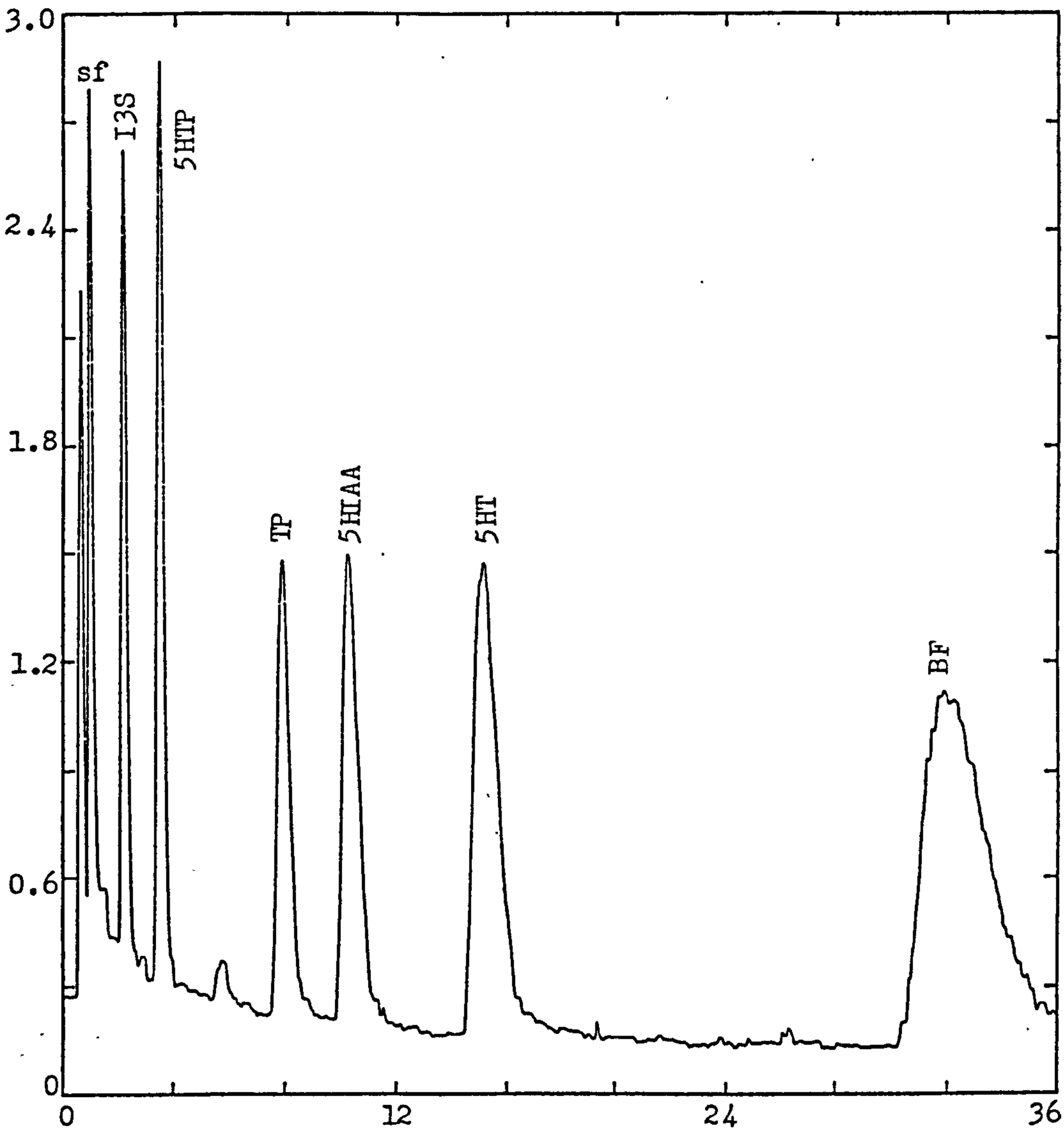
fact that the amine group is dimethylated in the former compound. As the two molecules are so similar, two half-wave (oxidation) potentials would be predicted for BF analogous to 5HT, i.e., at ca. +0.4V for the 5-hydroxyl group and ca. +0.8V for the indole ring nitrogen atom. Since the amine group is isolated from these electrophores, it would not be expected to exert any discernible influence on these oxidations. Thus, an adequate response to EC detection at an applied potential of +0.95V for the BF molecule would be foreseen.

Following initial investigative experiments with a methanolic BF solution, a standard containing TP, 5HTP, 5HT, 5HIAA, I3S and BF was freshly prepared in pseudo-mobile phase. A typical chromatogram obtained under optimised conditions from injection of this six component standard is presented in Figure 3.61. BF is observed to be well resolved from the five analyte species, eluting with a capacity factor of 44.71 (t_R = 32.0 mins). This represents an undesirable twofold increase in chromatographic run time from ca. 18 mins to ca. 38 mins. In addition, the peak corresponding to BF was observed to suffer from tailing to a not insubstantial degree which caused concern regarding precision of peak area integration in quantitative analysis. Regrettably, gradient elution, which would normally be contemplated to reduce the overall run time and improve peak shape under such circumstances, has been stated to be extremely difficult to apply with EC detection because of problems with maintenance of detector stability.[114] Furthermore, a gradient elution facility was not available with the Pye Unicam NBLC instrument under assessment. As it was considered to be prohibitively expensive to acquire and test other closely related substances (e.g., 6HT, N-Me-5HT, 5HI2A) as potential IS's, the less-than-ideal chromatographic behaviour demonstrated by BF, under conditions optimised for the separation of TP and its 5-hydroxylated metabolites, had to be persevered with, especially in view of the expected suitability of BF with respect to its chemical properties. The evaluation of BF for use with blood samples is reported in Part IV of this chapter.

The chromatographic behaviour of BF can be rationalised by considering its speciation at the working pH of 4.00. BF, like 5HT, possesses two pK_as in an aqueous environment. These pK_a values are 9.8 and 11.2[409] (cf. values for 5HT of 9.98 and 11.26, Table 3.23). The equilibria represented by these constants are indicated in Figure 3.62.

Figure 3.61 Typical Chromatogram of a Five Indole Standard Incorporating Bufotenine as an Internal Standard

Parameters :- Spherisorb S5 ODS1 (10cm x 2.1mm, d_p = 5µm); Mobile Phase : 4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA (200mgl^{-1}) pH 4.00; Flow Rate : 0.4mlmin^{-1}; Detection : ECD (E_{app} = +0.95V *vs.* Ag/AgCl, TC = 10sec); Sample : 5µl *via* loop of a standard containing TP, 5HTP, 5HT, 5HIAA, I3S and BF in 4% MeOH : 96% aq. 0.1M KH_2PO_4



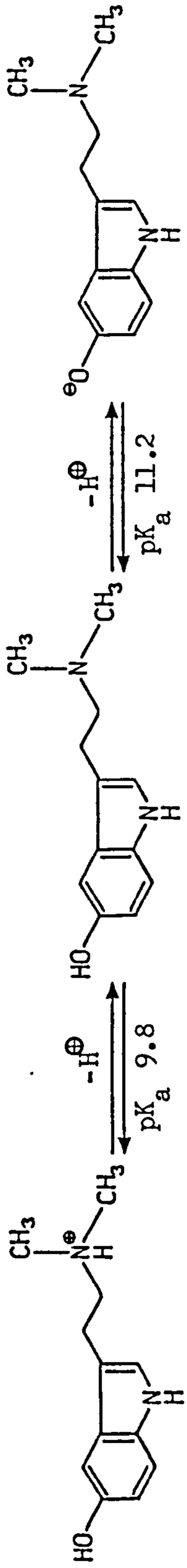


Figure 3.62 The Dissociation of Bufotenine

From this scheme, it is clear that in a pH 4.00 environment, BF exists almost exclusively as the protonated quaternary ammonion ion. This is analogous to the behaviour of 5HT. Consequently, BF would be expected to be retained on the reverse phase column in an identical manner to 5HT, i.e., by ion-pair association with the lipophilic HSA moiety. BF is retained considerably longer than is 5HT under identical conditions ($k' = 44.71$ *cf.* $k' = 20.43$ for 5HT). This is probably a direct result of the increased stability of the positively charged site in BF compared with 5HT which is afforded by the inductive effects of the two methyl groups. The increased stability of the cationic site in BF will necessarily lead to stronger coulombic association with the anionic HSA species. This in turn would result in an increase in the retention of BF compared with 5HT.

CHAPTER 3

Part III

Quantitative Evaluation of the Narrow-Bore Liquid Chromatography System Applied to the Determination of Indoles in Standard Solutions

Contents

3.5 Experimental

3.5.1 Solvents and Reagents

All chemicals were obtained from the sources noted in Section 3.3.1 and were used as received with the exception of solvents which were treated prior to use as stipulated previously.

3.5.2 Mobile Phase Preparation

The optimised mobile phase was prepared as described in Section 3.3.2.2.

3.5.3 Columns

A 10cm x 2.1mm ID column was packed in the laboratory with Spherisorb S5 ODS1. The packing procedure employed was that described in Section 2.2.4 with the equipment modifications noted in Section 3.3.3.3.

3.5.4 Standard Solution Preparation

For all experiments reported in Part III of Chapter 3 stock solutions (400$\mu g ml^{-1}$ active ingredient) of TP, 5HTP, 5HT.creatinine sulphate complex, 5HIAA and I3S.$K^{\oplus}$ salt were prepared in a MeOH:water mixture and were acidified with HAc (5 drops) to aid dissolution. A stock solution of BF.oxalate.H_2O (1.700 ± 0.001mg complex/100mls ≡ 11.1$\mu g ml^{-1}$ as active ingredient) was prepared in MeOH.

Working standards were prepared by combination and serial dilution with "pseudo"-mobile phase (4% MeOH : 96% aq. 0.1M KH_2PO_4). A series of solutions containing 0.02, 0.04, 0.06, 0.08 and 0.10$\mu g ml^{-1}$ of each of TP, 5HTP, 5HT, 5HIAA and I3S (as active ingredient) together with BF (0.22$\mu g ml^{-1}$ as active ingredient) were obtained.

For determination of within-batch precision the 0.06μgml^{-1} standard was employed. For determination of between-batch precision and LOD for each analyte, all five standards were utilised.

3.5.5 HPLC Operating Conditions

Column	:	Spherisorb S5 ODS1 (L = 10cm, ID = 2.1mm, d_p = 5μm, C Loading = 7% w/w, Surface Area = 220m^{2}g^{-1})
Mobile Phase	:	4% MeOH : 96% aq. 0.1M KH_2PO_4/H_3PO_4 buffer containing HSA (200mgl^{-1}), pH 4.00
Flow Rate	:	0.4mlmin^{-1} (≡ u = 0.19cms^{-1})
Injection Volume	:	5μl (*via* micro-loop)
Detection	:	ECD (Potential : +0.95V *vs.* Ag/AgCl Reference; Mode : Oxidation; Instrument Sensitivity : 3nA f.s.d.; Time Constant : 10sec)
Chart Speed	:	5mm min^{-1}
Integrator Parameters (HP3390A)	:	ATT : 2^{10}, PK WD : 0.16, THRSH : 11, AR REJ : 1 x 10^7

3.6 System Evaluation with Respect to Precision, Limit of Detection and Limit of Quantitation

3.6.1 General Remarks

In order to investigate the capabilities of the NBLC-EC system with respect to within-batch precision (repeatability), between-batch precision (reproducibility), LOD and LOQ, the PU4022 detector was set at the highest sensitivity available which yielded an acceptable baseline. This condition was satisfied at 3nA f.s.d. with a time constant of 10sec (see Section 2.3.2.4). A time constant of this order would be expected to be detrimental to performance causing broadening and attenuation of the detector output to some degree. However, the above settings, provided the greatest *S/N* ratio with baseline noise at a tolerable level which is the recommended criterion for optimum usage.[205]

An injection volume of 5µl was employed and the magnitude of concentration of indole standards to be utilised was determined by preliminary experiments. Solutions containing the five analytes each at concentrations of 0.02, 0.04, 0.06, 0.08 and 0.10µgml^{-1} were applied. All standards incorporated BF at 0.22µgml^{-1} as an IS. This represented on-column masses of 100, 200, 300, 400 or 500pg for each analyte together with 1.1ng of BF per injection.

3.6.2 Within-Batch Precision of Peak Measurements

An indole standard containing TP, 5HTP, 5HT, 5HIAA and I3S (all constituents at a concentration of 0.06µgml^{-1} as active ingredient), together with BF, the IS (0.22µgml^{-1} as active ingredient), was chromatographed

(V_i = 5µl) ten times consecutively. The Pye Unicam NBLC system equipped with a freshly-packed Spherisorb S5 ODS1 column (10cm x 2.1mm ID) was utilised. Chromatography was performed under the optimised operating conditions (see Section 3.5.5). A typical trace is presented in Figure 3.63. The detector signal was fed to both a chart recorder and a Hewlett Packard HP3390A electronic integrator which were connected in parallel. Integration parameters were determined from preliminary investigations and were programmed into the HP3390A prior to commencement of this experiment.

For each standard run peak areas were obtained from the integrator and peak heights were measured manually. Peak area ratios and peak height ratios (indole/IS) were calculated. All these data are presented in Table 3.39. The within-batch precision of each quantitation methodology for each analyte was determined and is reported both in absolute terms (as mean ± SD) and in relative terms as a percentage (the relative standard deviation, RSD) in Table 3.40.

These results demonstrate that the peak height ratio method of quantitation yields the highest within-batch precision (mean RSD = 4.7%). The two external calibration methods, peak area and peak height were found to be equivalent in relation to within-batch precision (mean RSDs of 6.6% and 6.5%, respectively). This observation is in agreement with the findings of Janik[420], who demonstrated that manually measured peak heights and electronically integrated peak areas obtained from a GC output were equivalent with respect to precision. This statement is only true under carefully controlled conditions because peak height is sensitive to instrumental and operational variations whereas peak area suffers less from this disadvantage.[421] More recently, two extensive cooperative studies comparing the precision of peak height and peak area measurements in LC have

Figure 3.63 Typical Chromatogram Obtained During Within-Batch Precision Determination

Parameters :- For conditions see Figure 3.61 except for Sample @ 0.06µgml-1 ≡ 300pg injected) with BF (0.22µgml-1 ≡ 1.1ng injected) in 4% MeOH : 96% aq. 0.1M KH_2PO_4 and I3S (All constituents

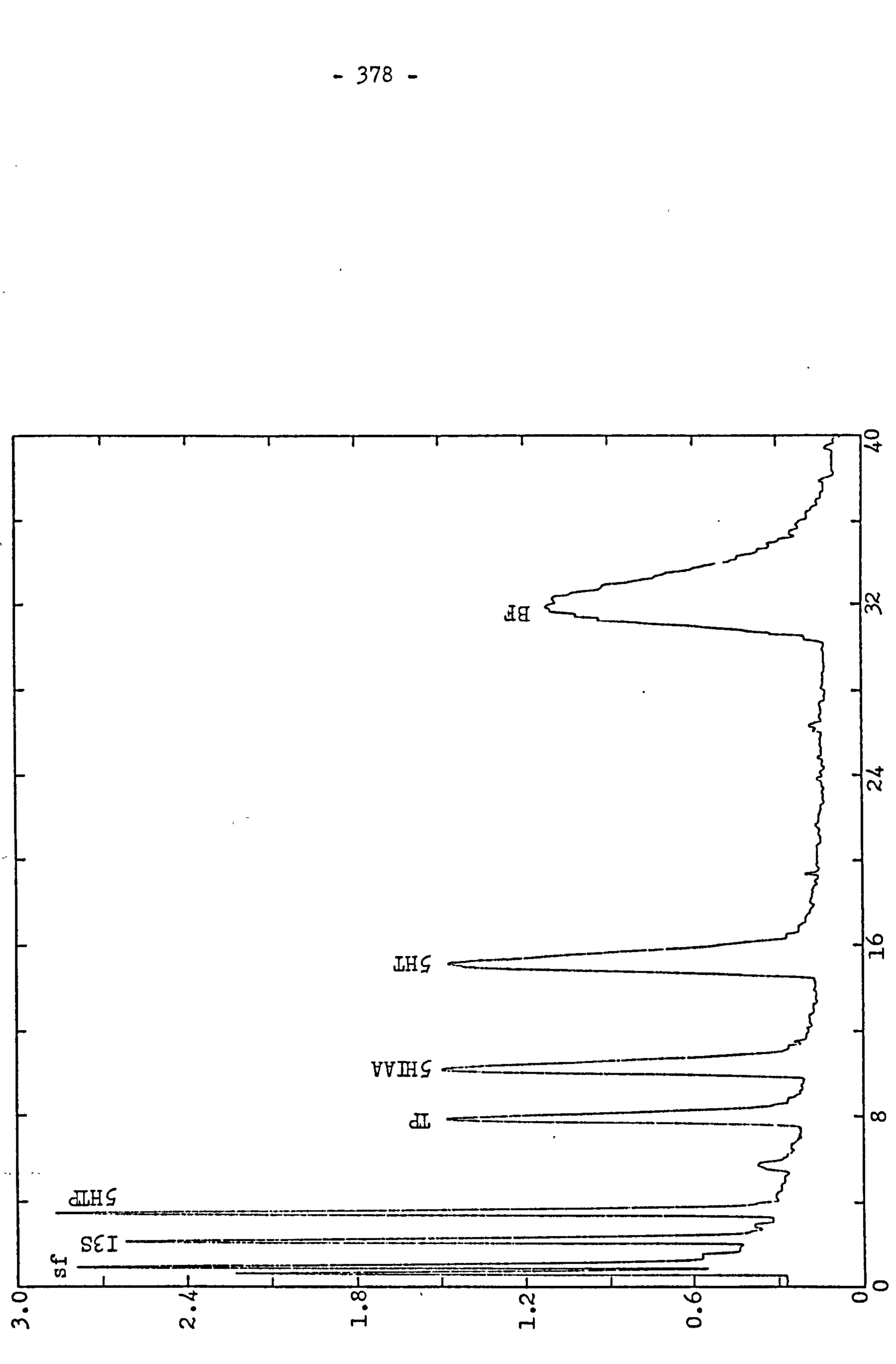


Table 3.39 Peak Measurement Data for Replicate Injections of a Six Component Indole Standard (n = 10)

Measurement Parameter	Run Number	TP	5HTP	5HT	5HIAA	I3S	BF (IS)
Area	1	11.463	10.408	19.093	12.237	6.2801	27.459
(counts*, x 10^{-7})	2	9.561	10.568	18.687	12.212	7.1601	32.404
	3	10.876	11.023	20.968	15.406	7.8887	38.194
	4	10.379	10.331	19.973	14.047	7.1312	35.892
	5	11.592	11.519	21.197	14.544	8.0877	33.681
	6	10.998	11.340	20.491	14.594	7.8110	44.162
	7	10.109	11.526	19.212	13.123	7.7727	42.427
	8	9.918	11.771	22.405	14.101	7.7600	46.277
	9	10.217	11.440	21.921	13.498	7.6767	42.521
	10	11.137	11.231	22.135	15.315	7.4815	47.455
Area Ratio	1	0.4175	0.3790	0.6953	0.4456	0.2287	-
	2	0.2950	0.3261	0.5767	0.3769	0.2310	-
	3	0.2848	0.2886	0.5490	0.4034	0.2065	-
	4	0.2892	0.2878	0.5565	0.3914	0.1987	-
	5	0.3442	0.3420	0.6293	0.4318	0.2401	-
	6	0.2490	0.2568	0.4640	0.3305	0.1769	-
	7	0.2383	0.2717	0.4528	0.3093	0.1832	-
	8	0.2143	0.2544	0.4841	0.3047	0.1677	-
	9	0.2403	0.2690	0.5155	0.3174	0.1805	-
	10	0.2347	0.2367	0.4664	0.3227	0.1577	-
Height (mm)	1	73	143	74	78	124	58.5
	2	74	146	78	76.5	132	56
	3	77	162	80	80.5	144.5	59.5
	4	75	149	76.5	77	130.5	56.5
	5	81.5	164	83.5	87.5	144	59.5
	6	83.5	165	89	88	143	67
	7	84	170	86.5	85.5	146.5	65.5
	8	83	165	90.5	89	144	69
	9	84.5	171	88	95	147	68
	10	82.5	165	88.5	90	145	69
Height Ratio	1	1.2479	2.4444	1.2650	1.3333	2.1197	-
	2	1.3214	2.6071	1.3929	1.3661	2.3571	-
	3	1.2941	2.7227	1.3445	1.3529	2.4286	-
	4	1.3274	2.6372	1.3540	1.3628	2.3097	-
	5	1.3697	2.7563	1.4034	1.4706	2.4202	-
	6	1.2463	2.4627	1.3284	1.3134	2.1343	-
	7	1.2537	2.5373	1.2910	1.2761	2.1866	-
	8	1.2029	2.3913	1.3116	1.2899	2.0870	-
	9	1.2426	2.5147	1.2941	1.3971	2.1618	-
	10	1.1957	2.3913	1.2826	1.3043	2.1014	-

* 1 area count = 0.125µVs (± 10%)

Table 3.40 Statistical Data for Replicate Injections of a Six Component Indole Standard (n = 10)

Measurement Parameter	Statistic	TP	5HTP	5HT	5HIAA	I3S	BF (IS)	Mean RSD for all Analyte Peaks
Area	Mean (counts*, x 10^{-7})	10.625	11.116	20.608	13.908	7.505	39.047	
	± SD (counts*, x 10^{-7})	0.685	0.511	1.338	1.136	0.528	6.587	
	RSD (%)	6.5	4.6	6.5	8.2	7.0	16.9	6.6
Area Ratio	Mean	0.2807	0.2912	0.5390	0.3634	0.1971	-	
	± SD	0.0615	0.0446	0.0791	0.0529	0.0286	-	
	RSD (%)	21.9	15.3	14.7	14.6	14.5	-	16.2
Height	Mean (mm)	79.8	160.0	83.5	84.7	140.1	62.9	
	± SD (mm)	4.5	10.1	5.9	6.3	8.1	5.3	
	RSD (%)	5.7	6.3	7.1	7.5	5.8	8.5	6.5
Height Ratio	Mean	1.2702	2.5465	1.3268	1.3467	2.2306	-	
	± SD	0.0563	0.1307	0.0467	0.0576	0.1346	-	
	RSD (%)	4.4	5.1	3.5	4.3	6.0	-	4.7

* 1 area count = 0.125µVs (± 10%)

been conducted.[422,423] The authors concluded that peak area derived by electronic integration was equivalent or superior in terms of precision to peak height determined either manually or by electronic means for "well-behaved" peaks (i.e., bands which are not subject to chromatographic interference). For "poorly-behaved" peaks (i.e., bands which are subject to chromatographic interference) peak height was shown to yield higher precision than peak area. In the case of the "well-behaved" peaks under assessment here the former statement applies.

By far the most imprecise quantitation technique considered in this investigation was that of peak area ratio, with a mean RSD of 16.2%. The primary reason for such poor precision was the variability in the integration of the IS peak, which occurred due to problems encountered with reproducibility in recognition of the start and end points of the IS peak. Variation of integration parameters failed to provide improved precision. In previous studies difficulties of this nature have been recognised at low *S/N* levels.[424] Furthermore, the problem was certainly exacerbated by the considerable deviation from Gaussian peak shape exhibited by the BF band. Haefelfinger[413] remarked upon the limitations of the IS technique and using the law of propagation of error derived the formula :

$$RSD_{IS} < 2r.\ RSD_{analyte} \qquad (3.2)$$

where, RSD_{IS} = the relative standard deviation of the area (or height) of the IS.

$RSD_{analyte}$ = the relative standard deviation of the area (or height) of the analyte.

and r = the correlation coefficient which is in the range $0 < r \leq 1$ since a positive correlation must exist between the area (or height) of the IS and that of the analyte.

Only if the relationship stated in Equation 3.2 holds true will inclusion of an IS improve the precision of a particular method. By following the above procedure for the peak areas it was found that the expression was not satisfied for any analyte, therefore no improvement in precision was possible by the peak area ratio method using BF as an IS. In fact, an impairment of precision was observed in practice between the use of analyte peak areas alone (i.e., external calibration) and peak area ratios (see Table 3.40). This impairment would be expected to be intensified when extraction techniques are considered in addition.[413] In contrast to the circumstances encountered with peak areas, employment of peak heights with Equation 3.2 yielded valid statements for TP, 5HTP, 5HT and 5HIAA, but I3S just failed to achieve acceptability. Consequently, improved precision is expected by the use of peak height ratios as compared with peak heights alone for all the analytes except I3S where slight impairment is predicted. This situation occurs in practice (see Table 3.40).

Of the four methods considered, quantitation <u>via</u> peak height ratios provides the best within-batch precision for standard solutions. The limitation of peak area ratios in this application is the irreproducibility of the IS peak intregration. In addition, it should be noted that even the best RSDs are well in excess of the claimed precision for the injector of 0.05%.[383,384] The reasons for this occurrence are unclear. There are obviously factors present which give rise to short-term (intra-day) variability in detector response. ECDs are flow sensitive so perhaps insufficient control of flow rate <u>via</u> the PU4010 pump leads to short-term instability. Progressive electrode contamination over the analysis period albeit only a few hours, would also be expected to contribute to the imprecision.

3.6.3 Between-Batch Precision of Peak Measurements

In order to assess between-batch precision with standard solutions, a series of indole standards (0.02, 0.04, 0.06, 0.08 and 0.10μgml^{-1} each analyte + 0.22μgml^{-1} BF) was chromatographed (V_i = 5µl) a total of 5 times over a period of 8 months. A typical series of chromatograms so obtained is presented in Figure 3.64. The detector signal was processed as described for the determination of within-batch precision. Peak area and peak height measurements were obtained from each run and peak area and peak height ratios (indole/IS) were calculated. All values are reported in Tables 3.41-3.45.

At the instrument sensitivity setting employed for this work (3nA f.s.d.) early eluting peaks (I3S and 5HTP) went off-scale on the chart recorder during the first three experiments on chromatographing the 0.08 and 0.10μgml^{-1} standards. Consequently, peak height measurements could not be made for these two analytes, so rendering impossible subsequent calculation of peak height ratios.

Between-batch precision was determined separately with respect to each analyte, concentration and method of quantitation investigated. Results were expressed in terms of mean ± SD at each concentration and, in order to be able to compare values obtained at different concentrations, the SD in each case was expressed as a percentage of the mean, i.e., as a RSD. The calculated RSDs were averaged over the entire concentration range for each analyte and are reported (± SD thereof) in Table 3.46. In addition, the collective means averaged over all concentrations and substances for each particular method of quantitation were determined and these values are also included in Table 3.46.

RSD values ranged from 18% for I3S quantified by either peak area ratio or peak height ratio to 46% for TP measured directly by peak area.

Figure 3.64 A Typical Series of Chromatograms Obtained by Injection of Indole Calibration Standards

Parameters :- For conditions see Figure 3.61 except for Sample : 5µl via loop of TP, 5HTP, 5HT, 5HIAA and I3S (All constituents at the specified concentration) with BF ($0.22\mu g ml^{-1}$ ≡ 1.1ng injected) in 4% MeOH : 96% aq. 0.1M KH_2PO_4

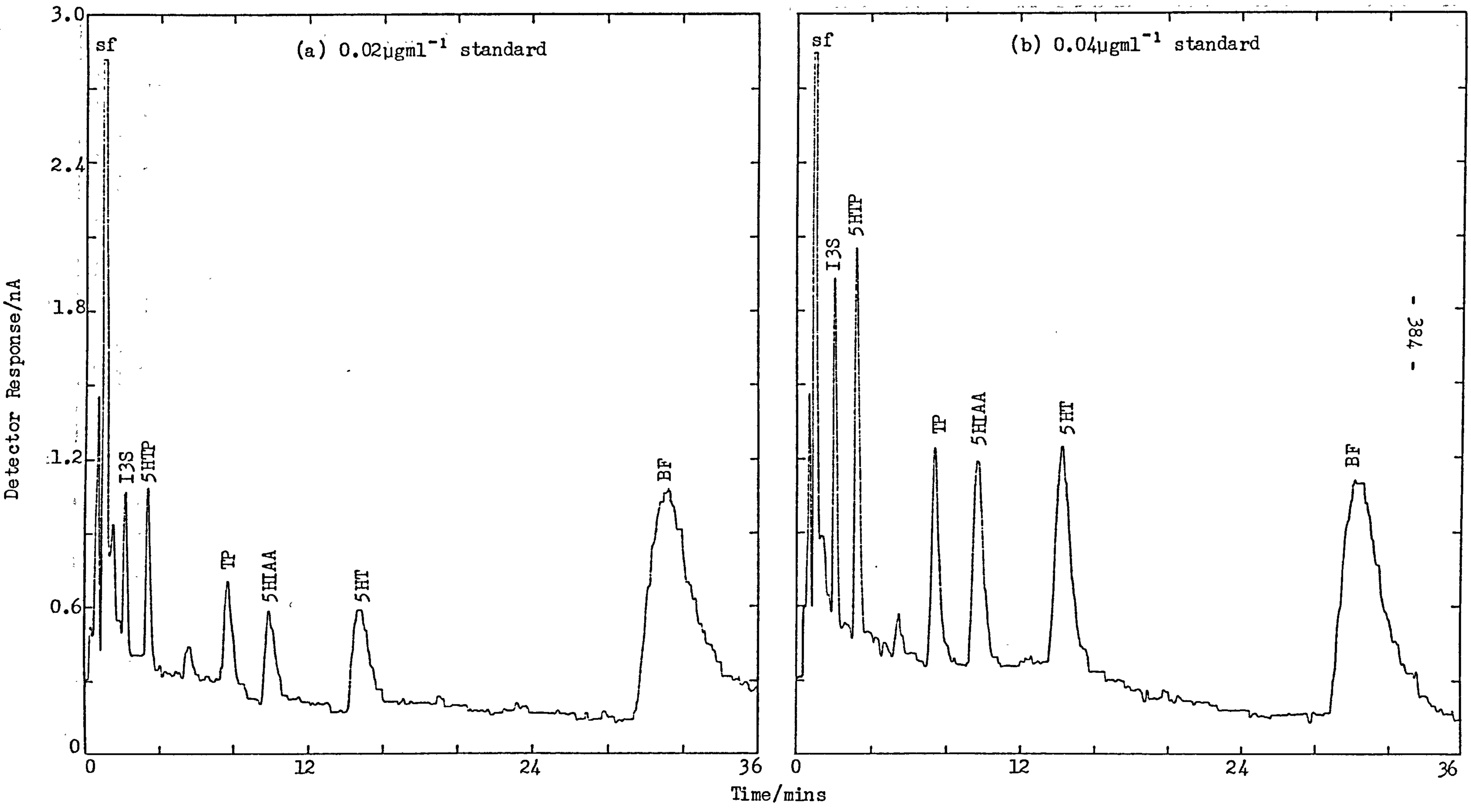
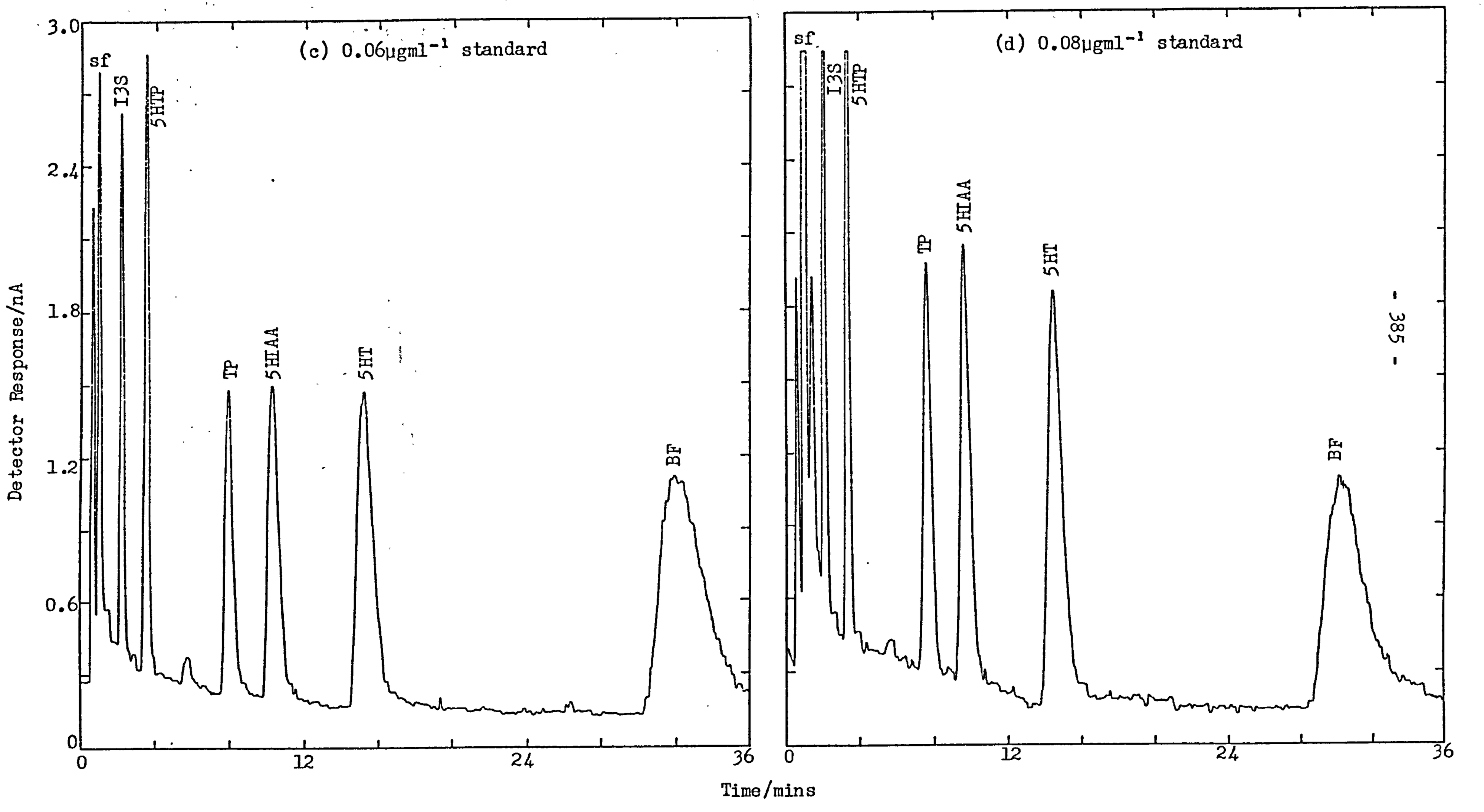


Figure 3.64 continued



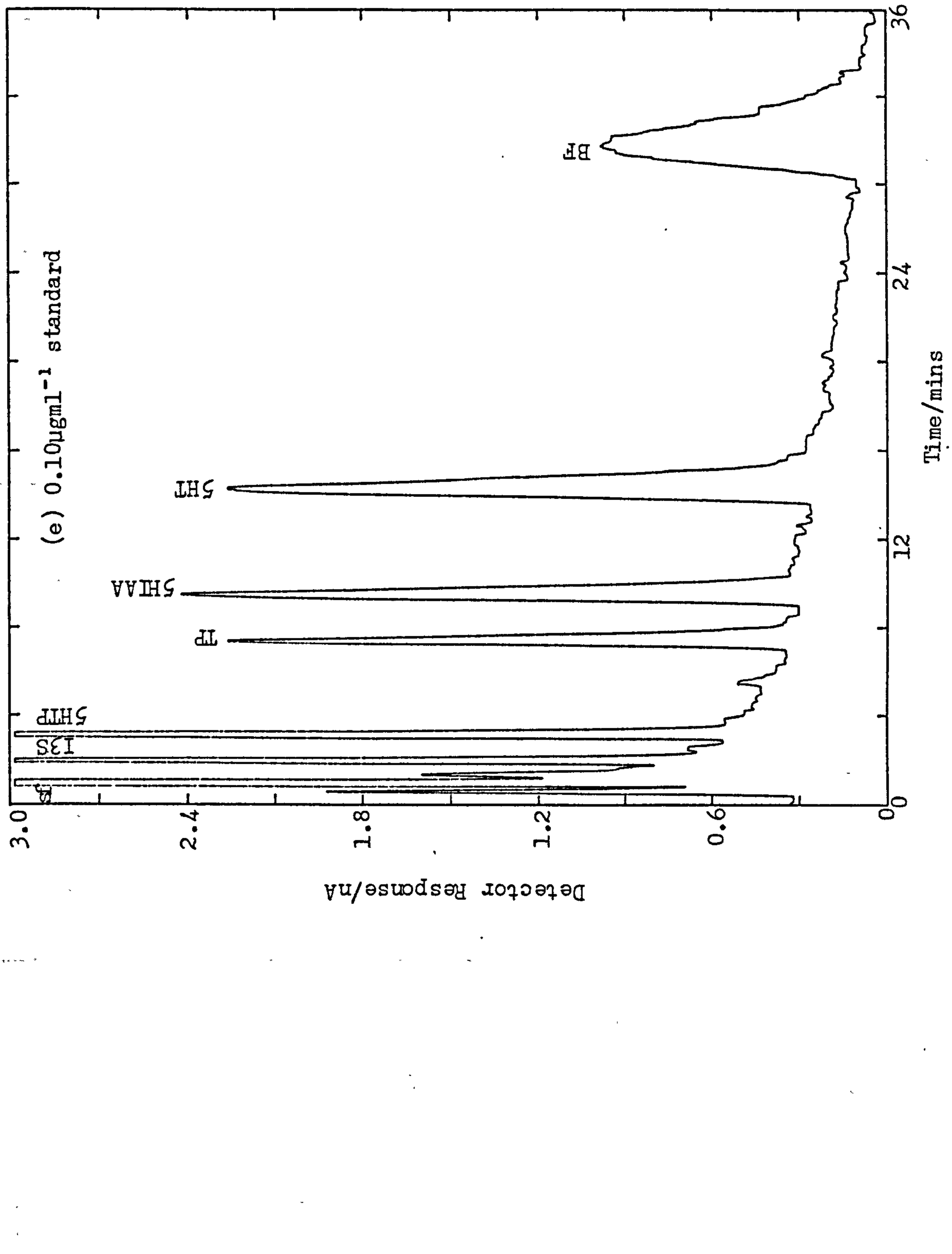


Figure 3.64 continued

Table 3.41 Experimental Data for Tryptophan

X	Y				IS (= BF)	
Concentration ($\mu g ml^{-1}$)	Peak Area (counts x 10^{-7})	Peak Area Ratio	Peak Height (mm)	Peak Height Ratio	Peak Area (counts x 10^{-7})	Peak Height (mm)
I						
0.02	3.8555	0.1141	28	0.4516	33.802	62
0.04	7.0800	0.2162	49.5	0.7500	32.746	66
0.06	9.3328	0.2794	75	1.1905	33.408	63
0.08	12.395	0.3882	111	1.8049	31.928	61.5
0.10	15.262	0.4943	123	2.0847	30.876	59
II						
0.02	4.0538	0.1073	23.5	0.4123	37.785	57
0.04	6.7251	0.1906	45	0.8182	35.292	55
0.06	11.463	0.4175	73	1.2479	27.459	58.5
0.08	14.545	0.4747	107	1.7833	30.640	60
0.10	17.427	0.5900	123	2.0500	29.537	60
III						
0.02	3.5438	0.0868	27	0.4426	40.823	61
0.04	7.2645	0.1667	59	0.9219	43.568	64
0.06	11.137	0.2347	82	1.1884	47.455	69
0.08	12.448	0.3008	106	1.7377	41.378	61
0.10	16.935	0.4517	130	2.0968	37.491	62
IV						
0.02	1.1799	0.0572	11	0.2340	20.642	47
0.04	2.5028	0.1184	24	0.4800	21.132	50
0.06	3.5853	0.1442	40.5	0.7864	24.862	51.5
0.08	5.3221	0.2115	52.5	1.0096	25.167	52
0.10	5.9705	0.2498	62	1.2277	23.897	50.5
V						
0.02	1.5220	0.0758	14.5	0.2788	20.088	52
0.04	2.4673	0.1060	26	0.4727	23.287	55
0.06	3.8734	0.1574	45	0.7563	24.601	59.5
0.08	5.5402	0.2313	60	1.0000	23.955	60
0.10	7.1952	0.2861	74.5	1.2957	25.152	57.5

Table 3.42 Experimental Data for 5-Hydroxytryptophan

X	Y				IS (= BF)	
Concentration ($\mu g ml^{-1}$)	Peak Area (counts x 10^{-7})	Peak Area Ratio	Peak Height (mm)	Peak Height Ratio	Peak Area (counts x 10^{-7})	Peak Height (mm)
I						
0.02	3.9025	0.1155	54	0.8710	33.802	62
0.04	6.7375	0.2058	104	1.5758	32.746	66
0.06	10.183	0.3048	151	2.3968	33.408	63
0.08	12.400	0.3884	o/s	nc	31.928	61.5
0.10	13.827	0.4478	o/s	nc	30.876	59
II						
0.02	4.6366	0.1227	50	0.8772	37.785	57
0.04	7.0666	0.2002	93	1.6909	35.292	55
0.06	10.408	0.3790	143	2.4444	27.459	58.5
0.08	14.799	0.4830	o/s	nc	30.640	60
0.10	15.230	0.5156	o/s	nc	29.537	60
III						
0.02	3.6981	0.0906	46	0.7541	40.823	61
0.04	6.6770	0.1533	105	1.6406	43.568	64
0.06	11.231	0.2367	163	2.3623	47.455	69
0.08	13.430	0.3246	o/s	nc	41.378	61
0.10	14.779	0.3942	o/s	nc	37.491	62
IV						
0.02	1.1666	0.0565	21	0.4468	20.642	47
0.04	2.8556	0.1351	47	0.9400	21.132	50
0.06	4.1009	0.1649	72	1.3981	24.862	51.5
0.08	5.2695	0.2094	97	1.8654	25.167	52
0.10	6.8134	0.2851	123	2.4356	23.897	50.5
V						
0.02	1.2989	0.0647	21	0.4038	20.088	52
0.04	2.5927	0.1113	49.5	0.9000	23.287	55
0.06	4.5162	0.1836	78	1.3109	24.601	59.5
0.08	5.9405	0.2480	101	1.6833	23.955	60
0.10	7.1439	0.2840	127	2.2087	25.152	57.5

o/s = off scale, nc = not calculable

Table 3.43 Experimental Data for 5-Hydroxytryptamine

X	Y				IS (= BF)	
Concentration ($\mu g ml^{-1}$)	Peak Area (counts x 10^{-7})	Peak Area Ratio	Peak Height (mm)	Peak Height Ratio	Peak Area (counts x 10^{-7})	Peak Height (mm)
I						
0.02	6.9678	0.2061	28	0.4516	33.802	62
0.04	12.681	0.3873	54	0.8182	32.746	66
0.06	19.517	0.5842	83	1.3175	33.408	63
0.08	25.599	0.8018	113	1.8374	31.928	61.5
0.10	31.929	1.0341	134	2.2712	30.876	59
II						
0.02	7.3850	0.1954	22	0.3860	37.785	57
0.04	10.678	0.3026	49	0.8909	35.292	55
0.06	19.093	0.6953	74	1.2650	27.459	58.5
0.08	27.595	0.9006	109	1.8167	30.640	60
0.10	33.438	1.1321	135	2.2500	29.537	60
III						
0.02	7.3301	0.1796	27	0.4426	40.823	61
0.04	13.276	0.3047	59	0.9219	43.568	64
0.06	22.135	0.4664	88	1.2754	47.455	69
0.08	24.533	0.5929	114	1.8689	41.378	61
0.10	32.508	0.8671	141.5	2.2823	37.491	62
IV						
0.02	2.3530	0.1140	12	0.2553	20.642	47
0.04	4.7079	0.2228	23.5	0.4700	21.132	50
0.06	7.2889	0.2932	45.5	0.8835	24.862	51.5
0.08	9.1243	0.3626	62	1.1923	25.167	52
0.10	12.817	0.5363	78.5	1.5545	23.897	50.5
V						
0.02	3.7349	0.1859	12.5	0.2404	20.088	52
0.04	4.4836	0.1925	28	0.5091	23.287	55
0.06	8.8983	0.3617	48	0.8067	24.601	59.5
0.08	10.456	0.4365	65	1.0833	23.955	60
0.10	13.025	0.5179	79.5	1.3826	25.152	57.5

Table 3.44 Experimental Data for 5-Hydroxyindole-3-acetic Acid

X	Y				IS (= BF)	
Concentration ($\mu g ml^{-1}$)	Peak Area (counts x 10^{-7})	Peak Area Ratio	Peak Height (mm)	Peak Height Ratio	Peak Area (counts x 10^{-7})	Peak Height (mm)
I						
0.02	4.1379	0.1224	29	0.4677	33.802	62
0.04	9.4181	0.2876	57	0.8636	32.746	66
0.06	13.160	0.3939	85	1.3492	33.408	63
0.08	17.390	0.5447	118	1.9187	31.928	61.5
0.10	20.115	0.6515	141	2.3898	30.876	59
II						
0.02	6.1103	0.1617	24.5	0.4298	37.785	57
0.04	9.8988	0.2805	54	0.9818	35.292	55
0.06	12.237	0.4456	78	1.3333	27.459	58.5
0.08	20.306	0.6627	117	1.9500	30.640	60
0.10	22.957	0.7772	134	2.2333	29.537	60
III						
0.02	4.0620	0.0995	25	0.4098	40.823	61
0.04	7.9531	0.1825	55	0.8594	43.568	64
0.06	15.315	0.3227	89.5	1.2971	47.455	69
0.08	18.809	0.4546	121	1.9836	41.378	61
0.10	22.810	0.6084	145	2.3387	37.491	62
IV						
0.02	2.2834	0.1106	15	0.3191	20.642	47
0.04	4.3790	0.2072	32.5	0.6500	21.132	50
0.06	6.6091	0.2658	53	1.0291	24.862	51.5
0.08	8.7523	0.3478	69.5	1.3365	25.167	52
0.10	10.726	0.4488	88.5	1.7525	23.897	50.5
V						
0.02	2.2267	0.1108	16	0.3077	20.088	52
0.04	3.6013	0.1546	30	0.5455	23.287	55
0.06	7.3232	0.2977	52.5	0.8824	24.601	59.5
0.08	8.4134	0.3512	69.5	1.1583	23.955	60
0.10	10.316	0.4101	91	1.5826	25.152	57.5

Table 3.45 Experimental Data for Indoxyl-3-sulphate

X	Y				IS (= BF)	
Concentration ($\mu g ml^{-1}$)	Peak Area (counts x 10^{-7})	Peak Area Ratio	Peak Height (mm)	Peak Height Ratio	Peak Area (counts x 10^{-7})	Peak Height (mm)
I						
0.02	2.2696	0.0671	48	0.7442	33.802	62
0.04	4.7519	0.1451	99	1.5000	32.746	66
0.06	6.7889	0.2032	135	2.1429	33.408	63
0.08	8.7879	0.2752	o/s	nc	31.928	61.5
0.10	9.9319	0.3217	o/s	nc	30.876	59
II						
0.02	2.0119	0.0532	45	0.7895	37.785	57
0.04	4.9073	0.1390	89	1.6182	35.292	55
0.06	6.2801	0.2287	124	2.1197	27.459	58.5
0.08	9.6515	0.3150	o/s	nc	30.640	60
0.10	10.464	0.3543	o/s	nc	29.537	60
III						
0.02	2.1789	0.0534	40	0.6557	40.823	61
0.04	4.7781	0.1097	91	1.4219	43.568	64
0.06	7.4815	0.1577	145	2.1014	47.455	69
0.08	8.9779	0.2170	o/s	nc	41.378	61
0.10	10.399	0.2774	o/s	nc	37.491	62
IV						
0.02	1.2141	0.0588	25	0.5319	20.642	47
0.04	2.4286	0.1149	54.5	1.0900	21.132	50
0.06	3.4306	0.1380	87	1.6893	24.862	51.5
0.08	4.8280	0.1918	118	2.2692	25.167	52
0.10	6.0200	0.2519	148	2.9307	23.897	50.5
V						
0.02	1.1153	0.0555	27.5	0.5288	20.088	52
0.04	2.0638	0.0886	53.5	0.9727	23.287	55
0.06	3.3663	0.1368	90.5	1.5210	24.601	59.5
0.08	4.6825	0.1955	119	1.9833	23.955	60
0.10	6.3616	0.2529	151	2.6261	25.152	57.5

o/s = off scale, nc = not calculable

Table 3.46 Reproducibility Data (Expressed as Relative Standard Deviations) for Indole Quantitation by Various Measurement Parameters over a Range of Concentrations Reported for each Analyte, Individually and also Collectively, by Measurement Parameter

Measurement Parameter	Statistic (%)	TP	5HTP	5HT	5HIAA	I3S	Mean RSD for all Analyte Peaks
Area	Range	42.8-49.2	36.4-54.4	42.3-46.7	34.7-42.6	26.0-37.3	
	Mean ± SD	46 ± 3	44 ± 7	45 ± 2	39 ± 3	33 ± 4	41
Area Ratio	Range	26.3-44.8	25.5-34.9	20.5-37.2	20.0-28.4	10.1-23.8	
	Mean ± SD	34 ± 7	31 ± 4	30 ± 7	25 ± 4	18 ± 6	28
Height	Range	30.0-37.3	35.4-41.9	28.0-37.9	23.2-29.1	22.6-28.0	
	Mean ± SD	34 ± 3	38 ± 4*	32 ± 5	26 ± 2	26 ± 3*	29
Height Ratio	Range	23.3-29.5	29.0-34.3	22.0-29.9	17.8-22.7	15.1-20.9	
	Mean ± SD	27 ± 3	31 ± 3*	26 ± 4	20 ± 3	18 ± 6*	24

All means are of 5 RSDs except those marked with an asterisk which are means of 3 RSDs

Between-batch variation was generally greatest for peak area quantitation (average 41% RSD) and least for the peak height ratio method (average 24% RSD).

All the RSD values obtained from this experiment were very high, which is indicative of poor reproducibility by every quantitation method. This general imprecision does not include the contribution from variability in analyte losses, which invariably occur during clean-up of authentic samples, and consequently is considered to be a conservative estimate with respect to the analysis of biological fluids.

The possible origins of such low between-batch precision require comment. Variable detector sensitivity, most probably attributable to variable degrees of electrode contamination over the evaluation period, is highly suspect. Support for this supposition is provided by the observation that the reproducibility of the ratioing methods of quantitation was considerably superior to that of their respective direct measurement alternatives (i.e., 28% *cf.* 41% with respect to area and 24% *cf.* 29% with respect to height). The ratio methods compensate to some degree for systematic variations in peak response. These systematic variations in area and height are evident from inspection of the experimental data (Tables 3.41-3.45). General response in Experiments IV and V is considerably diminished compared with the typical level of response in Experiments I-III for equivalent concentrations.

In addition to fouling of the WE, other long-term causes of variability are almost certainly present. These will include the errors in preparation of standards and the mobile phase, level of standard deterioration, column condition (i.e., packing uniformity, age, level of contamination, etc.) and general factors relating to NBLC system performance (e.g., constancy of flow-

rate, cleanliness of injection valve, in-line filters and frits, occurrence of leakage). Ambient temperature can also be expected to cause wider variations over the course of many weeks than it does within one day.

It should be noted that major technical difficulties were experienced with the Pye Unicam NBLC system over the period in which this investigation was conducted. These problems will be discussed in detail in Chapter 4. Since chronic instrument failure was prevalent during this period it is highly likely that the influence of instrument-related factors on the reproducibilities would predominate over other factors.

The mean RSDs obtained for all analytes over the entire range of concentration examined follow the trend :

$$\text{peak area} > \underset{\text{ratio}}{\text{peak height}} \simeq \underset{\text{ratio}}{\text{peak area}} > \text{peak height}$$

Since the greater is the RSD, the lesser is the precision then in terms of between-batch precision the reverse trend is evident, i.e.,

$$\underset{\text{ratio}}{\text{peak height}} > \text{peak area} \simeq \underset{\text{ratio}}{\text{peak height}} > \text{peak area}$$

Consequently the peak height ratio approach is preferred because it exhibits the best reproducibility. This is in agreement with the result of the within-batch precision determination where peak height ratio provided the best repeatability.

There are problems associated with the use of the peak height ratio however. The concentration range that may be retained on-scale in a single run is highly constrained compared with the range that may be integrated electronically. Where concentrations are within this range, peak height ratio quantitation is feasible. For samples in which higher concentrations

are present three options are possible; (1) Instrument sensitivity may be altered with regard to the initial chromatogram and a repeat analysis be performed. In view of the extreme limitation of sample size this approach is not satisfactory. (2) The applied potential of the detector may be reduced prior to chromatography but this would have the effect of raising the LODs of all the analytes and would require re-calibration. (3) Instrument sensitivity may be changed in situ with respect to a chromatogram. This third approach requires prior knowledge of the sample, which is unavailable in routine screening, and is prone to operator error. Furthermore, it is impractical at high sensitivity because of the baseline disruption inherent with the process (see Section 2.3.1). Consequently, no totally satisfactory peak height-based quantitation method is available. The applicability of peak height ratio to the determination of indole compounds in plasma samples can only be investigated experimentally.

Of the area-based methods available, peak area ratio suffers poor repeatability whereas analyte peak area is not very reproducible.

Clearly it is essential to take all possible steps to control and reduce the excessive variability exhibited by the NBLC system before acceptable performance can be achieved.

3.6.4 Generation of Calibration Curves

In order to quantitate measurements made on an authentic sample a calibration curve is constructed from standards by plotting true values of a precise measurement (concentration or mass injected) on the abscissa against observed values of an economical, less precise measurement (peak area, peak height, peak area ratio or peak height ratio) on the ordinate

axis. The line of best fit is yielded by applying least squares regression to obtain the fitted model

$$Y = a + bX$$

where a = the intercept on the ordinate axis,

and b = the gradient of the regression line.

The appropriate procedure required to generate this regression line is given in most standard texts (e.g., 425).

In order to estimate the errors inherent in the determination of this line it is possible to construct a confidence region in which the true line will be contained, say, 95% of the time. The 95% confidence region for the entire line is given by Working-Hotelling statistics which employ Snedecor's F statistic, i.e.,

$$Y = a + bX \pm \left[2F_{2,\nu,\alpha} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}} \qquad (3.3)$$

where F = Snedecor's F statistic (Tables),

ν = degrees of freedom

α = probability setting for required confidence level (= 0.05 for 95%)

n = number of observations

$\bar{X}$ = mean of observed X values from standards

$\Sigma x^2 = \Sigma(X-\bar{X})^2$

and s^2 = variance

A full discussion of the application of Working-Hotelling statistics to the calibration function is presented in a fundamental paper by Hunter.[426]

Calibration curves serve two main functions, i.e., (1) the analytical function, and (2) determination of the LOD.

3.6.4.1 Calibration Curves Employed as Analytical Aids[426]

Once a fitted line is obtained then it is often required to be used over and over again for all future values of the abscissa variable X (concentration or mass injected) to estimate all associated future observations Y* (peak area, peak height, etc.). When the fitted line is to be employed repeatedly in this way, then account is taken of the error involved by employing the Working-Hotelling confidence region for the true line combined with tolerance limits for the observations. These tolerance limits provide bounds within which a certain proportion (say, 90%) of the future observations will be found. Commonly a 95% confidence region for 90% of future observations is employed. These tolerance bounds are given by :

$$Y = a + bX \pm (A + z_p B) \qquad (3.4)$$

where $$A = \left[{}^{2}F_{2,\nu,\alpha/2} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}},$$

$$B = \left[\nu s^2 / \chi^2_{\nu,1-\alpha/2} \right]^{\frac{1}{2}},$$

and z_p = the normal deviate.

For 100 (1-α)% overall confidence, the critical values of both F and χ^2 are set at α/2, i.e., for 95% overall confidence tabulated values corresponding to $F_{2,\nu,0.025}$ and $\chi^2_{\nu,0.975}$ are appropriate.

Regression lines, Working-Hotelling 95% confidence regions and 95% confidence bounds for 90% of future observations were calculated for each indole quantified via each of the four different peak parameters. Exceptions were 5HTP and I3S by peak height and peak height ratio because since the higher concentration standards employed herein produced peaks

which went off-scale at the instrument sensitivity setting used then the total data was insufficient for computation in these cases. The calculations performed on data from the quantitation of TP by peak area are given by way of example. All calculated calibration data is summarised in Tables 3.48-3.52. Plots of the regression lines, with attendant Working-Hotelling 95% confidence limits and 95% confidence bounds for 90% of future observations, are compiled in Figures 3.65-3.69.

The calibration curves presented here were constructed principally for quantitation experiments utilising blood samples.

The broadness of the 95% confidence bounds for 90% of future observations reflects the uncertainty with which the estimation of the true regression line is held. Thus, a comparative examination of the confidence bounds reflects the precision of the technique used to derive the original data and the method of measuring that data. In effect, the confidence bounds reflect the between-batch precision obtained. The arguments put forward regarding the possible origins of such variation and the steps required to be taken to reduce it therefore apply (see Section 3.6.3).

Construction of the Calibration Curve of Tryptophan from Peak Area Measurements with Fitted Regression Line, Working-Hotelling 95% Confidence Region and 95% Confidence Bounds for 90% of Future Observations

Table 3.47 Calibration Data

(X = concentration in $\mu g ml^{-1}$, Y = peak area in counts x 10^{-7})

X	Y	x (= X-$\bar{X}$)	y (= Y-$\bar{Y}$)	x^2	y^2	xy
0.02	2.83100	-0.04	-4.874048	0.0016	23.75634391	0.19496192
0.04	5.20794	-0.02	-2.497108	0.0004	6.235548364	0.04994216
0.06	7.87830	0.00	0.173252	0.0000	0.030016255	0.00000000
0.08	10.05006	0.02	2.345012	0.0004	5.49908128	0.04690024
0.10	12.55794	0.04	4.852892	0.0016	23.55056076	0.19411568
$\Sigma X = 0.30$	$\Sigma Y = 38.52524$			$\Sigma x =$ 0.0040	$\Sigma y =$ 59.07155057	$\Sigma xy =$ 0.48592000
$\bar{X} = 0.06$	$\bar{Y} = 7.705048$					

$Y = a + bX$

Gradient, $b = \frac{\Sigma xy}{\Sigma x^2} = \frac{0.48592}{0.004} = \underline{121.48}$

Intercept, $a = \bar{Y} - b\bar{X} = 7.705048 - (121.48 \times 0.06) = \underline{0.416248}$

$\therefore$ Regression Line is given by $\underline{\hat{Y} = 0.416248 + 121.48X}$

Variance, $s^2 = \frac{\Sigma y^2 - b\Sigma xy}{n-2} = \frac{59.07155057 - (121.48 \times 0.48592)}{3}$

$\underline{s^2 = 0.04198897}$

$\therefore \underline{s_{YX} = 0.204912103}$

Working-Hotelling 95% Confidence Region

$$Y = a + bX \pm \left[2F_{2,\nu,\alpha}\left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}} \qquad (3.3)$$

Since $F_{2,3,0.05} = 9.552$ (Tables)

Then

$$Y' = 0.416248 + 121.48X' \pm \left[2 \times 9.552 \left(0.2 + \frac{(X'-0.06)^2}{0.004} \right) 0.04198897 \right]^{\frac{1}{2}}$$

$$= 0.416248 + 121.48X' \pm \left[0.802157282 \left(0.2 + \frac{(X'-0.06)^2}{0.004} \right) \right]^{\frac{1}{2}}$$

$\therefore$ For $X_1 = 0.02$, $Y_1 = 2.85 \pm 0.69$

$= 3.54$ or 2.15

For $X_2 = 0.04$, $Y_2 = 5.28 \pm 0.49$

$= 5.77$ or 4.78

For $X_3 = 0.06$, $Y_3 = 7.71 \pm 0.40$

$= 8.11$ or 7.30

For $X_4 = 0.08$, $Y_4 = 10.13 \pm 0.49$

$= 10.63$ or 9.64

For $X_5 = 0.10$, $Y_5 = 12.56 \pm 0.69$

$= 13.26$ or 11.87

95% Confidence Bounds for 90% of Future Observations

$$Y = a + bX \pm (A + z_p B) \tag{3.4}$$

where $A = \left[{}^{2}F_{2,\nu,\alpha/2} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2} \right) s^2 \right]^{\frac{1}{2}}$

and $B = \left[\nu s^2 / \chi^2_{\nu,1-\alpha/2} \right]^{\frac{1}{2}}$

Since $z_p = 1.29$, $F_{2,3,0.025} = 16.04$, and $\chi^2_{3,0.975} = 0.216$ (Tables)

Then

$$Y' = 0.416248 + 121.48X' \pm \left\{ \left[2 \times 16.04 \left(0.2 + \frac{(X'-0.06)^2}{0.004} \right) 0.04198897 \right]^{\frac{1}{2}} + \left[1.29 \left(\frac{3 \times 0.04198897}{0.216} \right)^{\frac{1}{2}} \right] \right\}$$

$$= 0.416248 + 121.48X' \pm \left\{ \left[1.347006158 \left(0.2 + \frac{(X'-0.06)^2}{0.004} \right) \right]^{\frac{1}{2}} + 0.985124392 \right\}$$

$\therefore$ For $X_1 = 0.02$, $Y_1 = 2.85 \pm 1.88$

$= 4.73$ or 0.96

For $X_2 = 0.04$, $Y_2 = 5.28 \pm 1.62$

$= 6.90$ or 3.65

For $X_3 = 0.06$, $Y_3 = 7.71 \pm 1.50$

$= 9.21$ or 6.20

For $X_4 = 0.08$, $Y_4 = 10.13 \pm 1.62$

$= 11.76$ or 8.51

For $X_5 = 0.10$, $Y_5 = 12.56 \pm 1.88$

$= 14.45$ or 10.68

Table 3.48 Summary of Calibration Data for Tryptophan

(X = concentration in $\mu g ml^{-1}$, Y = (a) peak area in counts x 10^{-7}, (b) peak area ratio, (c) peak height in mm, and (d) peak height ratio)

(a) $\hat{Y} = 0.4162 + 121.5X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	2.8310	2.8458	3.54 or 2.15	4.73 or 0.96
0.04	5.2079	5.2754	5.77 or 4.78	6.90 or 3.65
0.06	7.8783	7.7050	8.11 or 7.30	9.21 or 6.20
0.08	10.0501	10.1346	10.63 or 9.64	11.76 or 8.51
0.10	12.5579	12.5642	13.26 or 11.87	14.45 or 10.68

(b) $\hat{Y} = 0.0019 + 4.069X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.0882	0.0832	0.12 or 0.05	0.18 or -0.02
0.04	0.1596	0.1646	0.19 or 0.14	0.25 or 0.08
0.06	0.2466	0.2460	0.28 or 0.22	0.33 or 0.17
0.08	0.3213	0.3274	0.35 or 0.30	0.41 or 0.24
0.10	0.4142	0.4069	0.44 or 0.37	0.51 or 0.31

(c) $\hat{Y} = -0.12 + 1050X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	20.8	20.9	29.4 or 12.4	43.9 or -2.1
0.04	40.7	41.9	47.9 or 35.9	61.7 or 22.1
0.06	63.1	62.9	67.8 or 58.0	81.3 or 44.5
0.08	87.3	83.9	89.9 or 77.9	103.7 or 64.1
0.10	102.5	104.9	113.4 or 96.4	127.9 or 81.9

(d) $\hat{Y} = -0.0049 + 17.76X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.3639	0.3504	0.48 or 0.22	0.70 or 0.01
0.04	0.6886	0.7056	0.80 or 0.62	1.00 or 0.41
0.06	1.0339	1.0609	1.13 or 0.99	1.34 or 0.79
0.08	1.4671	1.4162	1.51 or 1.33	1.71 or 1.12
0.10	1.7510	1.7714	1.90 or 1.64	2.12 or 1.43

Table 3.49 Summary of Calibration Data for 5-Hydroxytryptophan

(X = concentration in $\mu g ml^{-1}$, Y = (a) peak area in counts x 10^{-7}, and (b) peak area ratio)

(a) $\hat{Y} = 0.9205 + 111.2X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	2.9405	3.1445	4.77 or 1.52	7.56 or -1.27
0.04	5.1859	5.3685	6.52 or 4.22	9.17 or 1.57
0.06	8.0878	7.5925	8.53 or 6.65	11.12 or 4.07
0.08	10.1898	9.8166	10.97 or 8.67	13.61 or 6.02
0.10	11.5587	12.0406	13.67 or 10.41	16.46 or 7.63

(b) $\hat{Y} = 0.1612 + 3.801X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.0900	0.0921	0.16 or 0.03	0.27 or -0.09
0.04	0.1611	0.1682	0.21 or 0.12	0.32 or 0.01
0.06	0.2538	0.2442	0.28 or 0.21	0.39 or 0.10
0.08	0.3307	0.3202	0.37 or 0.27	0.47 or 0.17
0.10	0.3853	0.3962	0.46 or 0.33	0.57 or 0.22

Table 3.50 Summary of Calibration Data for 5-Hydroxytryptamine

(X = concentration in $\mu g ml^{-1}$, Y = (a) peak area in counts x 10^{-7}, (b) peak area ratio, (c) peak height in mm, and (d) peak height ratio)

(a) $\hat{Y} = 0.2598 + 243.4X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	5.5542	5.1272	7.30 or 2.95	11.03 or -0.78
0.04	9.1653	9.9947	11.53 or 8.46	15.08 or 4.91
0.06	15.3864	14.8622	16.12 or 13.61	19.58 or 10.15
0.08	19.4615	19.7296	21.27 or 18.19	24.81 or 14.65
0.10	24.7434	24.5971	26.77 or 22.42	30.50 or 18.69

(b) $\hat{Y} = -0.0109 + 8.098X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.1762	0.1511	0.24 or 0.06	0.41 or -0.10
0.04	0.2820	0.3130	0.38 or 0.25	0.53 or 0.09
0.06	0.4802	0.4750	0.53 or 0.42	0.68 or 0.27
0.08	0.6189	0.6369	0.70 or 0.57	0.86 or 0.42
0.10	0.8175	0.7989	0.89 or 0.71	1.05 or 0.54

(c) $\hat{Y} = -3.61 + 1184X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	20.3	20.1	24.3 or 15.9	31.5 or 8.6
0.04	42.7	43.7	46.7 or 40.4	53.6 or 33.9
0.06	67.7	67.4	69.8 or 65.0	76.5 or 58.3
0.08	92.6	91.1	94.0 or 88.1	100.9 or 81.2
0.10	113.7	114.7	119.0 or 110.5	126.2 or 103.3

(d) $\hat{Y} = -0.0681 + 20.12X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.3552	0.3342	0.42 or 0.25	0.56 or 0.10
0.04	0.7220	0.7366	0.80 or 0.68	0.93 or 0.54
0.06	1.1096	1.1389	1.19 or 1.09	1.32 or 0.96
0.08	1.5597	1.5413	1.60 or 1.48	1.74 or 1.34
0.10	1.9481	1.9436	2.03 or 1.86	2.17 or 1.71

Table 3.51 Summary of Calibration Data for 5-Hydroxyindole-3-acetic Acid

(X = concentration in $\mu g ml^{-1}$, Y = (a) peak area in counts x 10^{-7}, (b) peak area ratio, (c) peak height in mm, and (d) peak height ratio)

(a) $\hat{Y} = 0.2947 + 174.6X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	3.7641	3.7873	5.08 or 2.50	7.30 or 0.28
0.04	7.0501	7.2798	8.19 or 6.37	10.30 or 4.26
0.06	10.9289	10.7724	11.52 or 10.03	13.57 or 7.97
0.08	14.7341	14.2649	15.18 or 13.35	17.28 or 11.25
0.10	17.3848	17.7575	19.05 or 16.47	21.27 or 14.25

(b) $\hat{Y} = -0.0018 + 5.831X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.1210	0.1148	0.14 or 0.09	0.19 or 0.04
0.04	0.2225	0.2314	0.25 or 0.21	0.29 or 0.17
0.06	0.3451	0.3480	0.36 or 0.33	0.41 or 0.29
0.08	0.4722	0.4646	0.48 or 0.45	0.53 or 0.40
0.10	0.5792	0.5812	0.61 or 0.55	0.65 or 0.51

(c) $\hat{Y} = -3.17 + 1247X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	21.9	21.8	27.8 or 15.7	38.1 or 5.4
0.04	45.7	46.7	51.0 or 42.4	60.7 or 32.6
0.06	71.6	71.6	75.1 or 68.1	84.7 or 58.6
0.08	99.0	96.6	100.8 or 92.3	110.6 or 82.5
0.10	119.9	121.5	127.5 or 115.5	137.8 or 105.1

(d) $\hat{Y} = -0.0556 + 21.17X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.3868	0.3679	0.55 or 0.19	0.85 or -0.12
0.04	0.7801	0.7913	0.92 or 0.66	1.21 or 0.37
0.06	1.1782	1.2148	1.32 or 1.11	1.60 or 0.83
0.08	1.6694	1.6382	1.76 or 1.51	2.06 or 1.22
0.10	2.0594	2.0617	2.24 or 1.88	2.55 or 1.58

Table 3.52 Summary of Calibration Data for Indoxyl-3-sulphate

(X = concentration in μgml^{-1}, Y = (a) peak area in counts x 10^{-7}, and (b) peak area ratio)

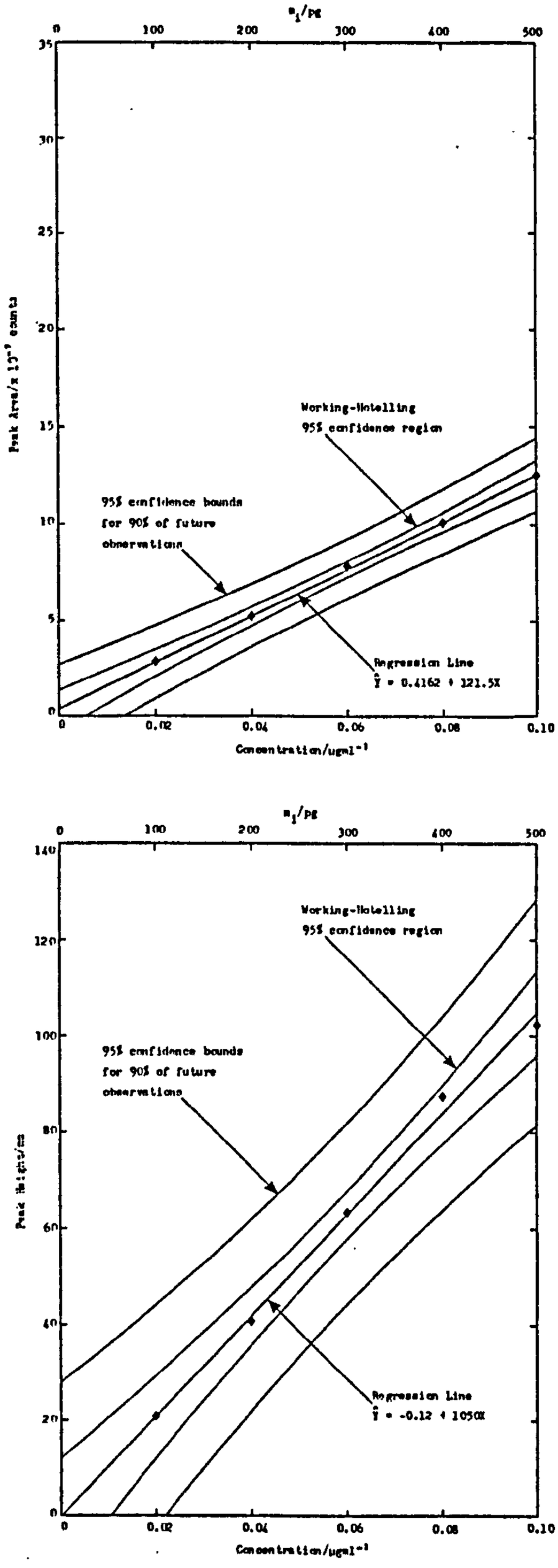
(a) $\hat{Y} = 0.2005 + 86.77X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	1.7580	1.9360	2.73 or 1.14	4.10 or -0.23
0.04	3.7859	3.6714	4.24 or 3.11	5.54 or 1.81
0.06	5.4694	5.4068	5.87 or 4.95	7.14 or 3.68
0.08	7.3856	7.1423	7.71 or 6.58	9.01 or 5.28
0.10	8.6353	8.8777	9.68 or 8.08	11.04 or 6.71

(b) $\hat{Y} = -0.0001 + 2.937X$

X	Y	$\hat{Y}$	Working-Hotelling	Confidence Bounds
0.02	0.0576	0.0586	0.07 or 0.06	0.09 or 0.03
0.04	0.1195	0.1174	0.13 or 0.11	0.14 or 0.09
0.06	0.1729	0.1761	0.18 or 0.17	0.20 or 0.15
0.08	0.2389	0.2348	0.24 or 0.23	0.26 or 0.21
0.10	0.2916	0.2936	0.31 or 0.28	0.33 or 0.26

Figure 3.65 Calibration Curves for Tryptophan by Peak Area, Peak Area Ratio, Peak Height and Peak Height Ratio



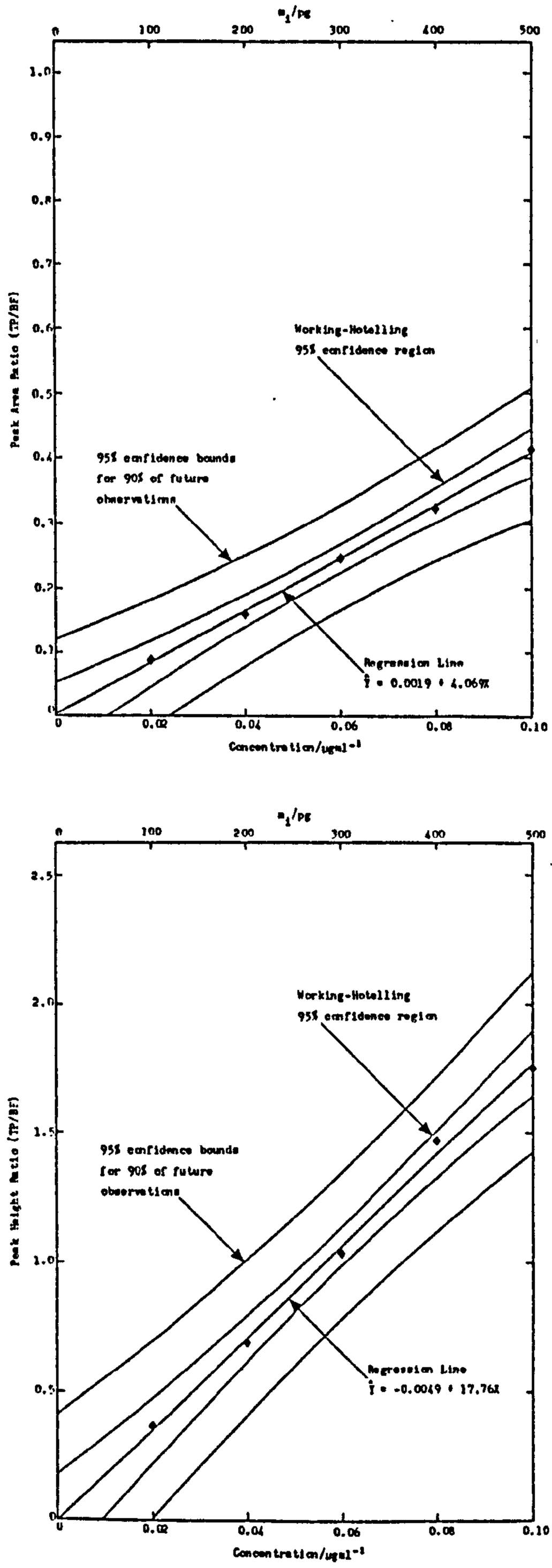
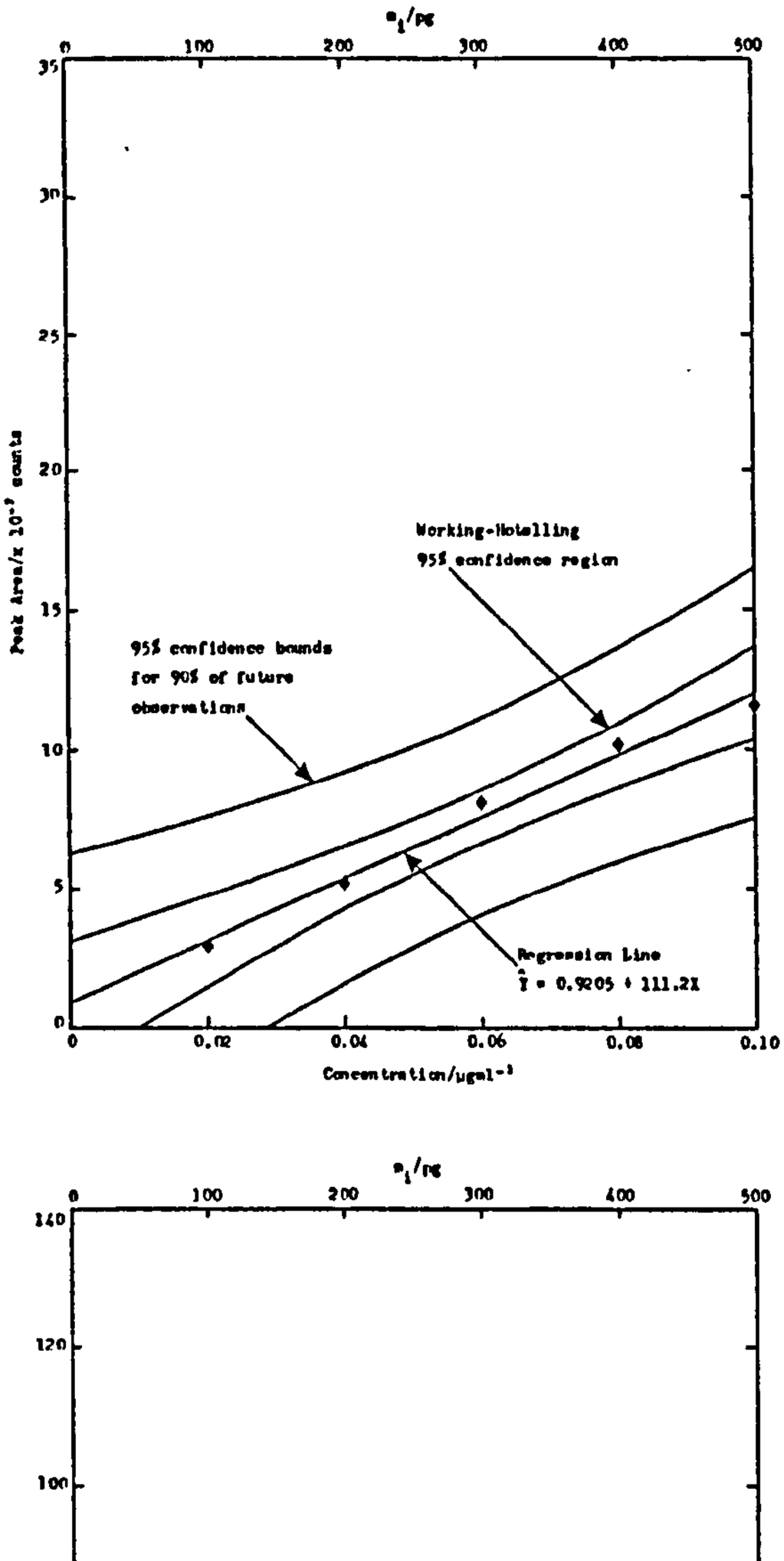
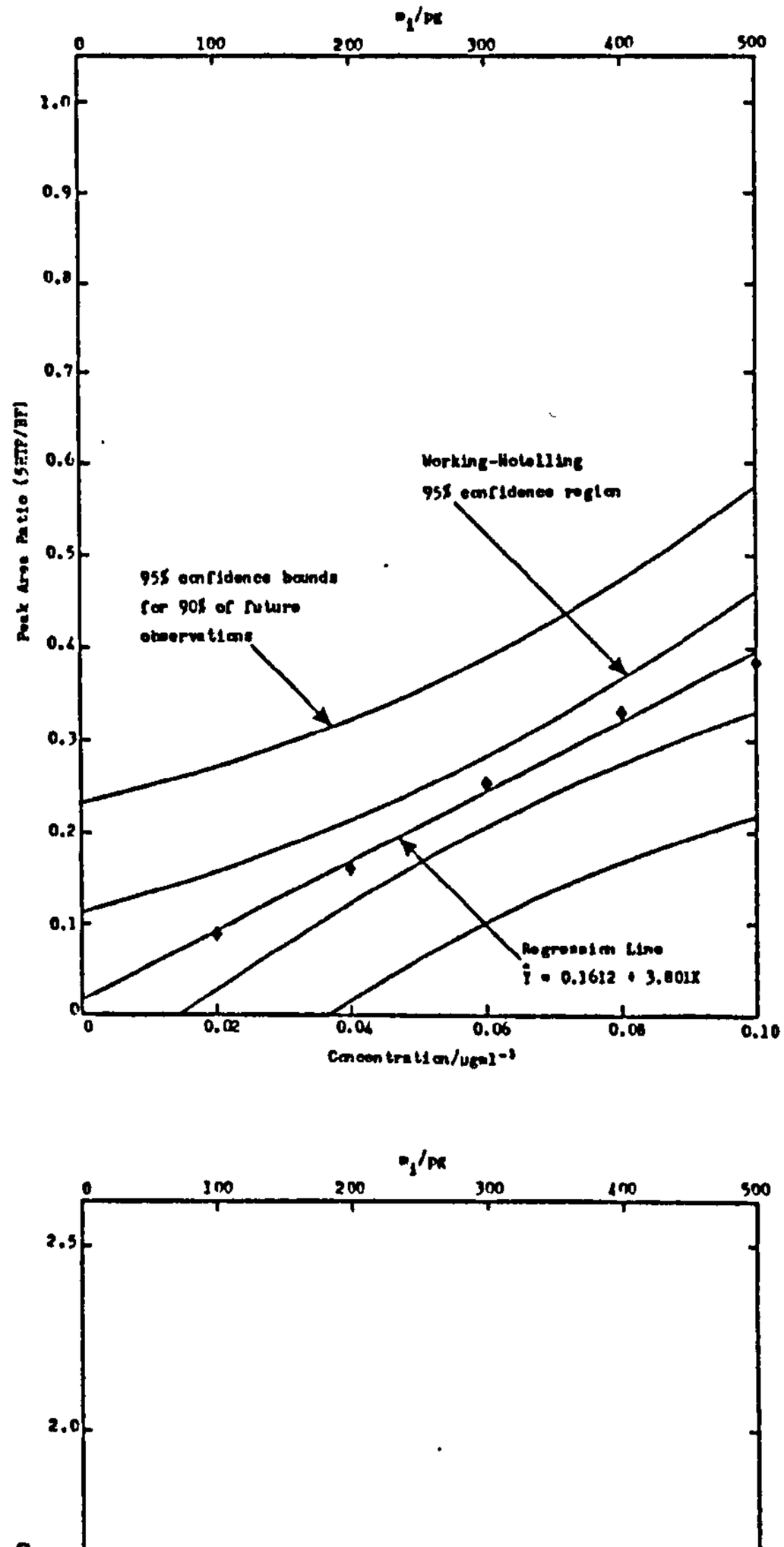
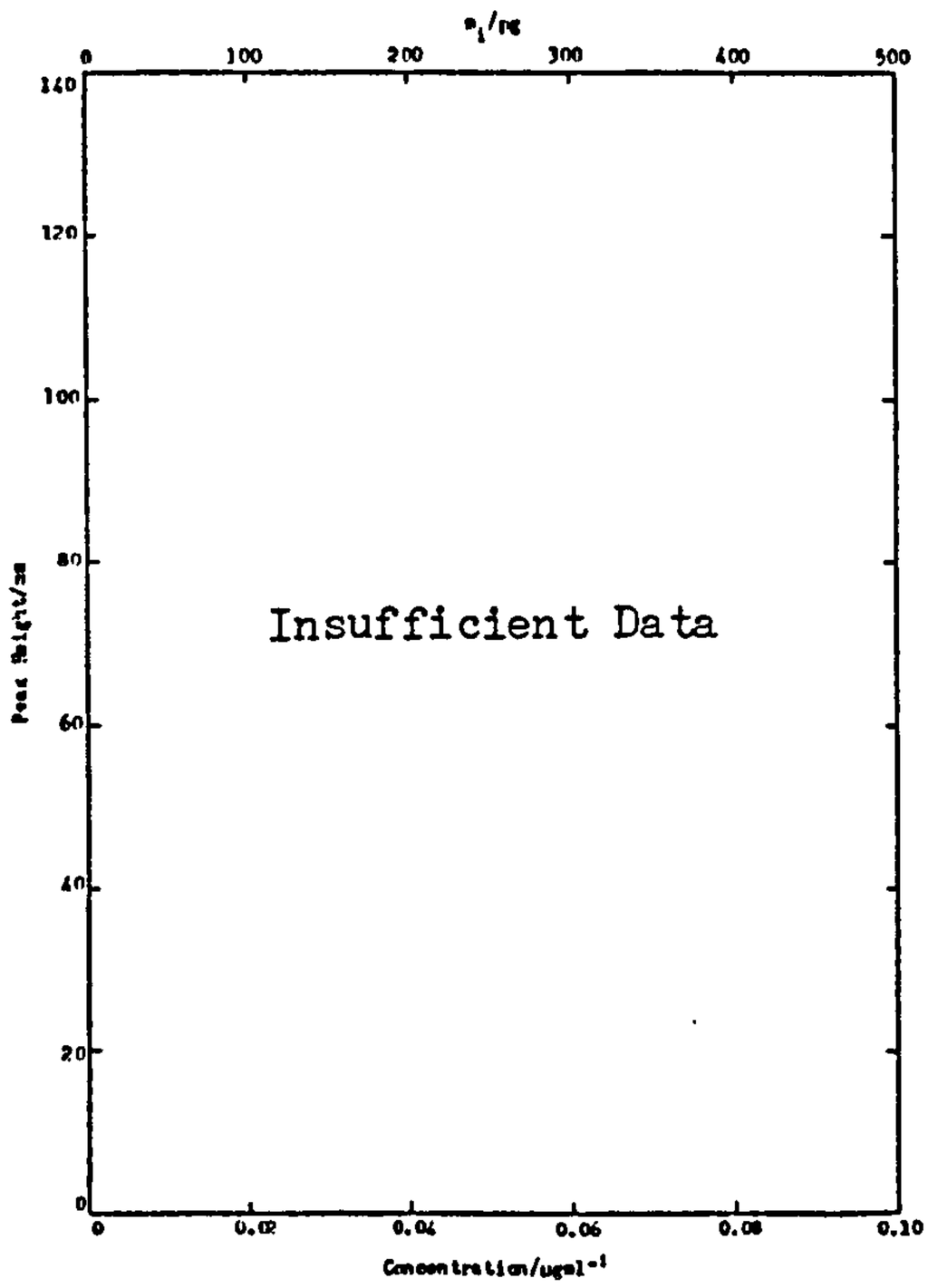


Figure 3.66 Calibration Curves for 5-Hydroxytryptophan by Peak Area and Peak Area Ratio







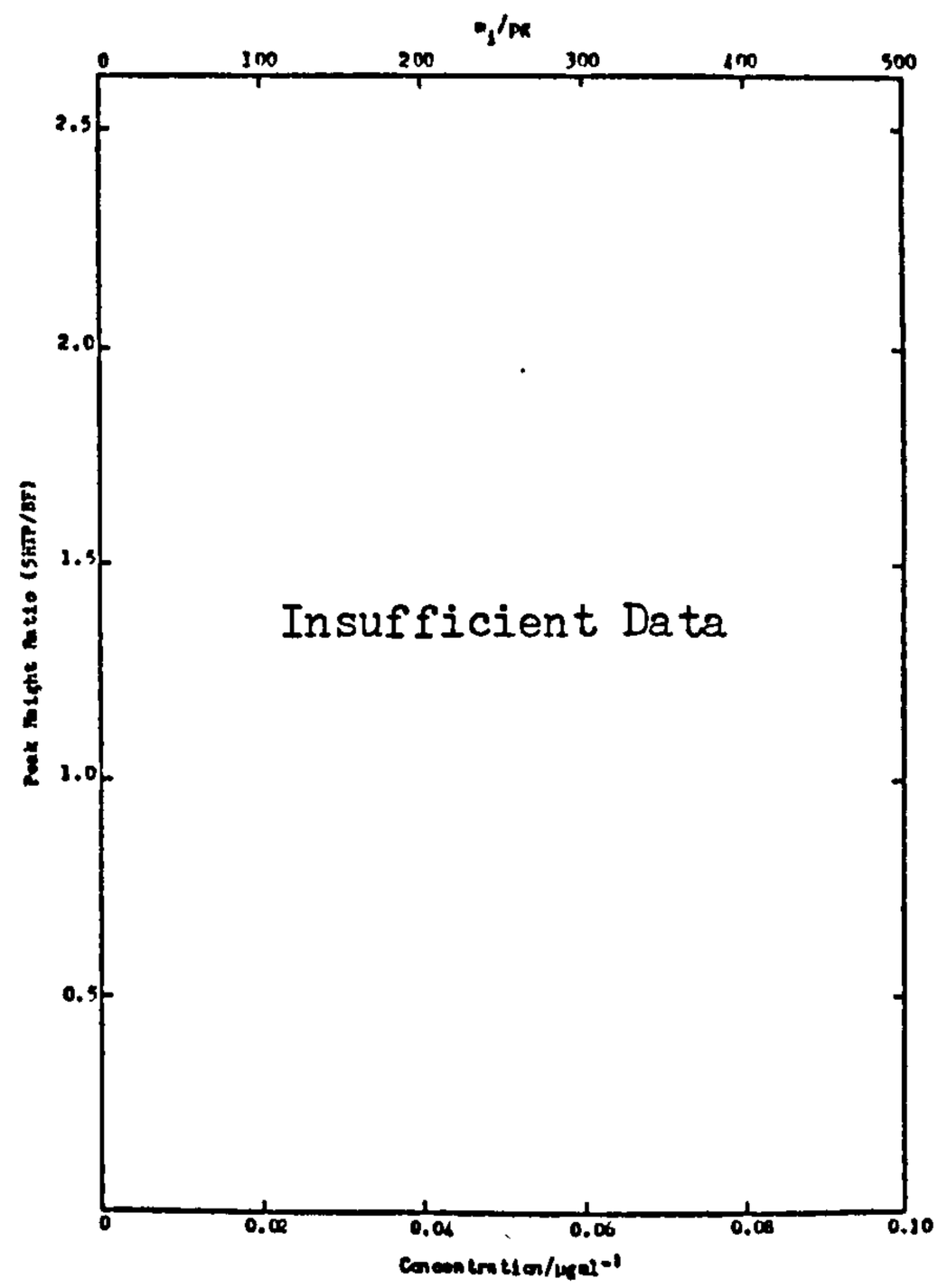
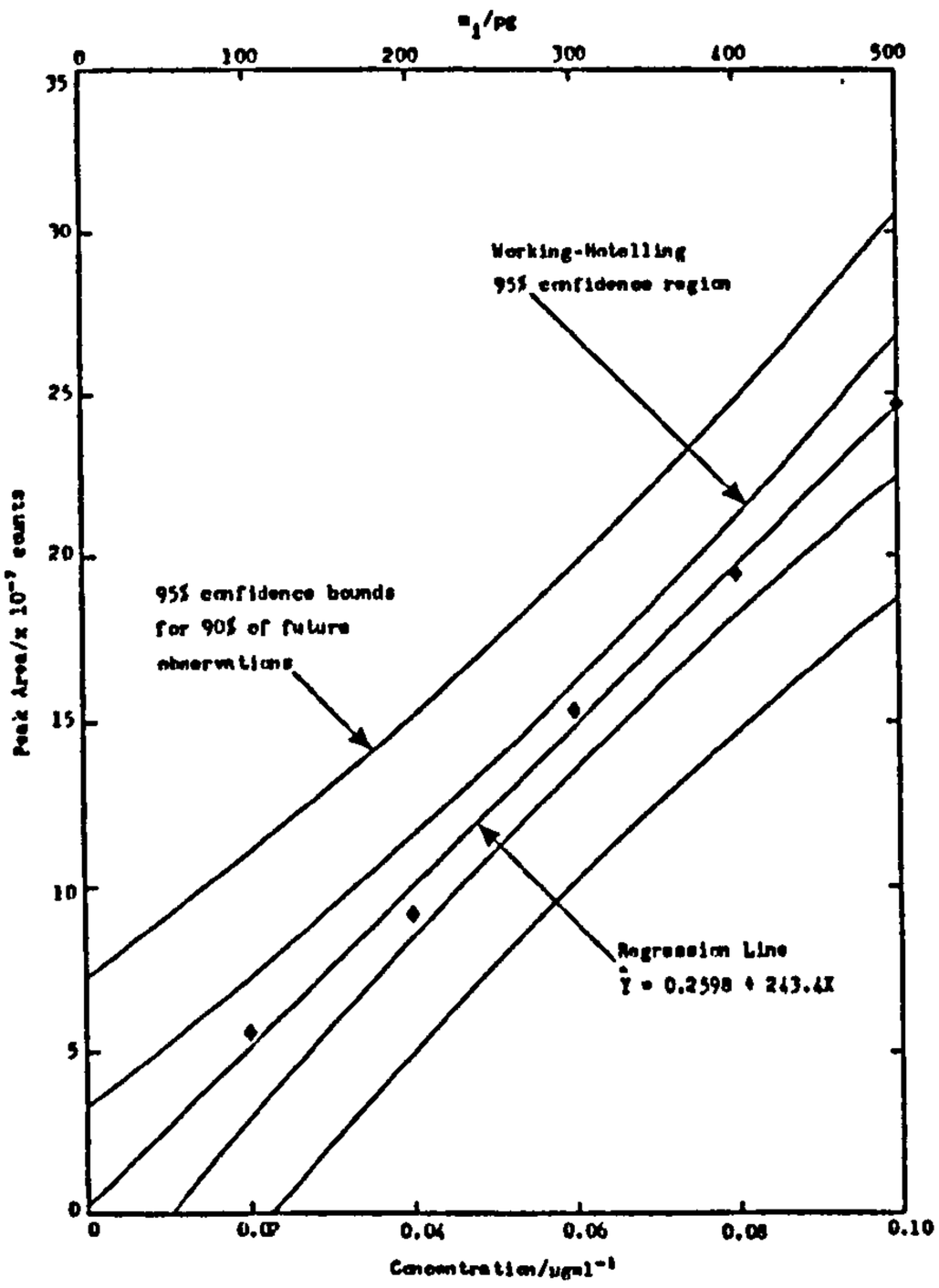
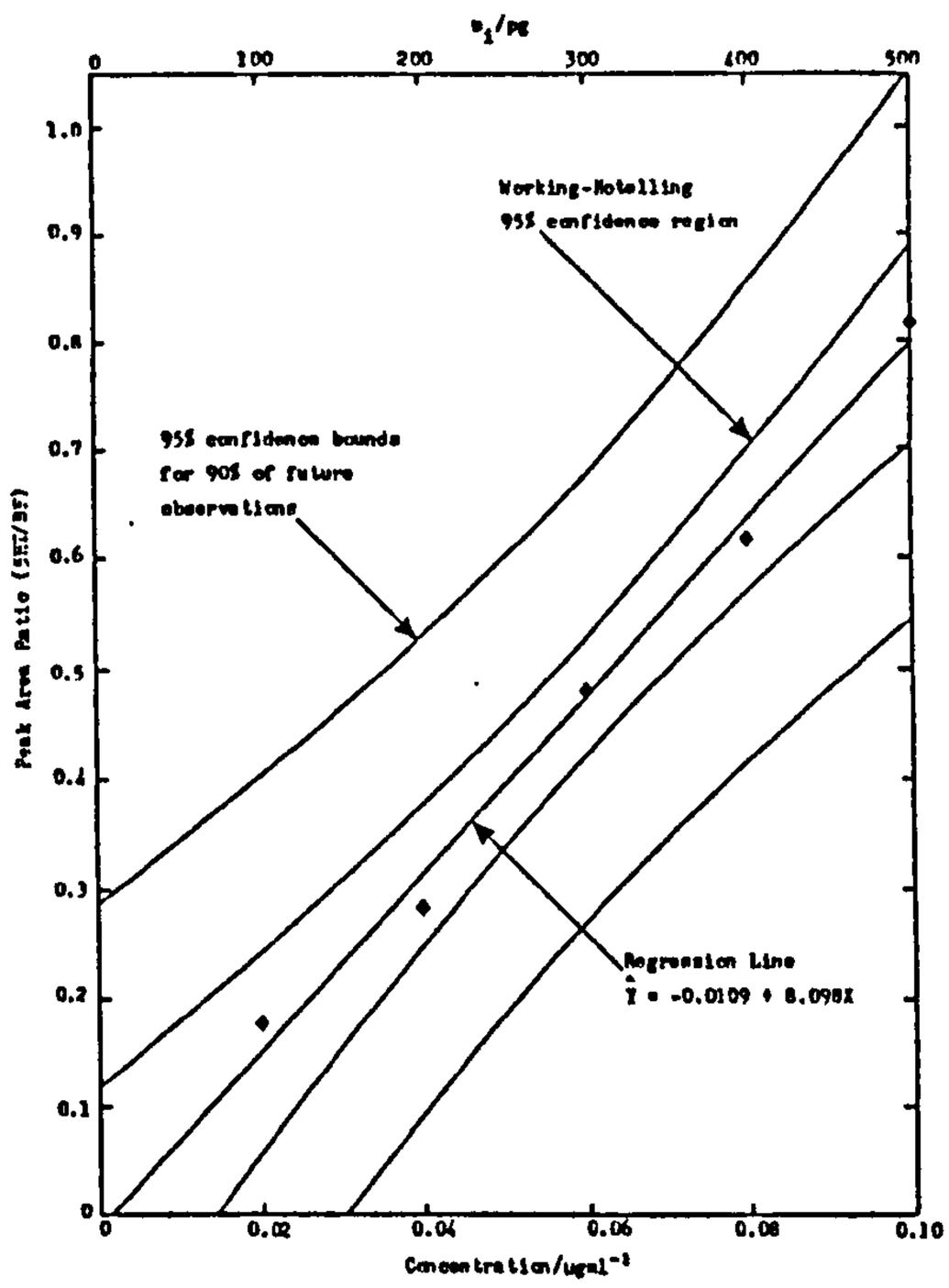
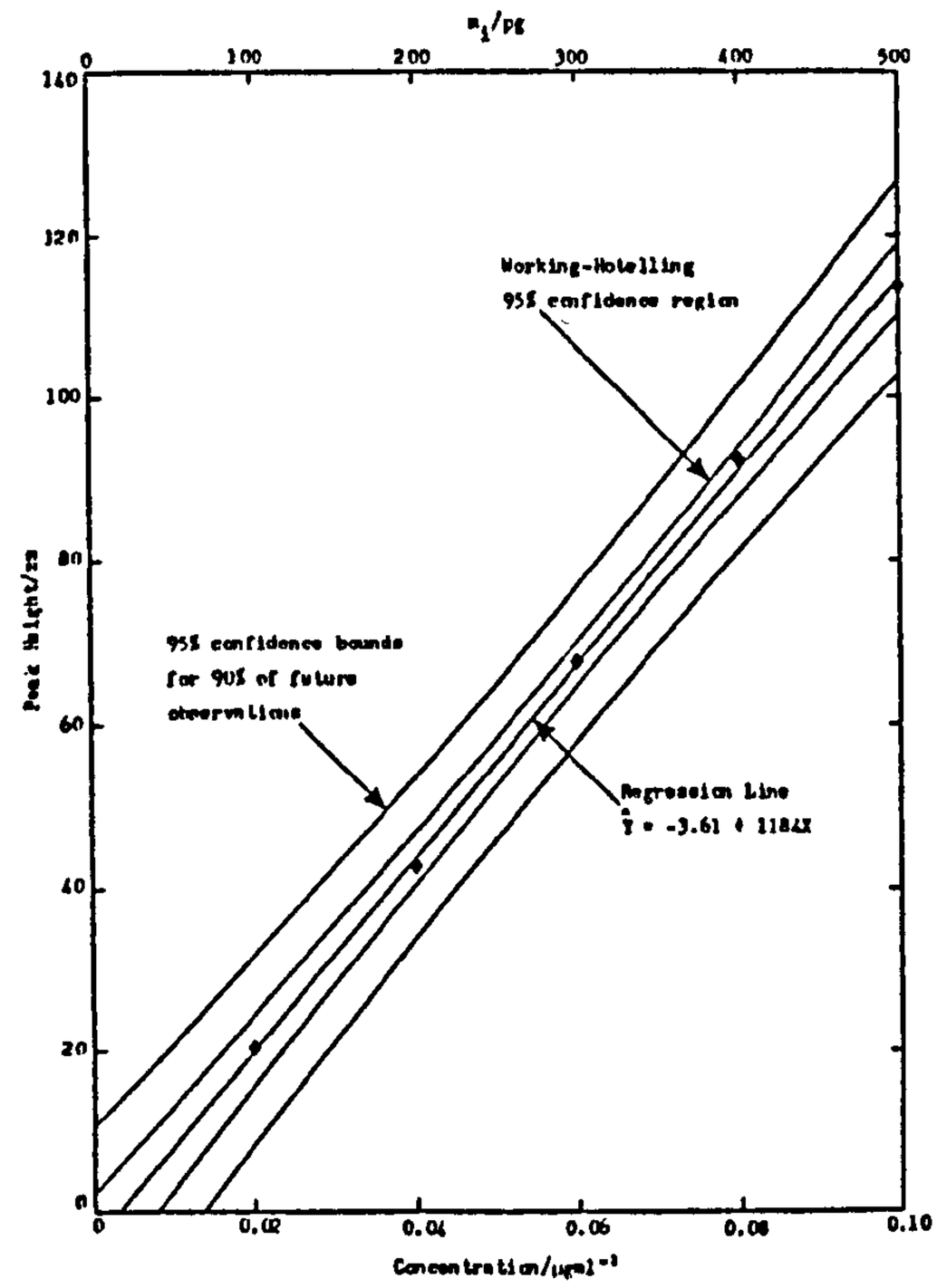


Figure 3.67 Calibration Curves for 5-Hydroxytryptamine by Peak Area, Peak Area Ratio, Peak Height and Peak Height Ratio







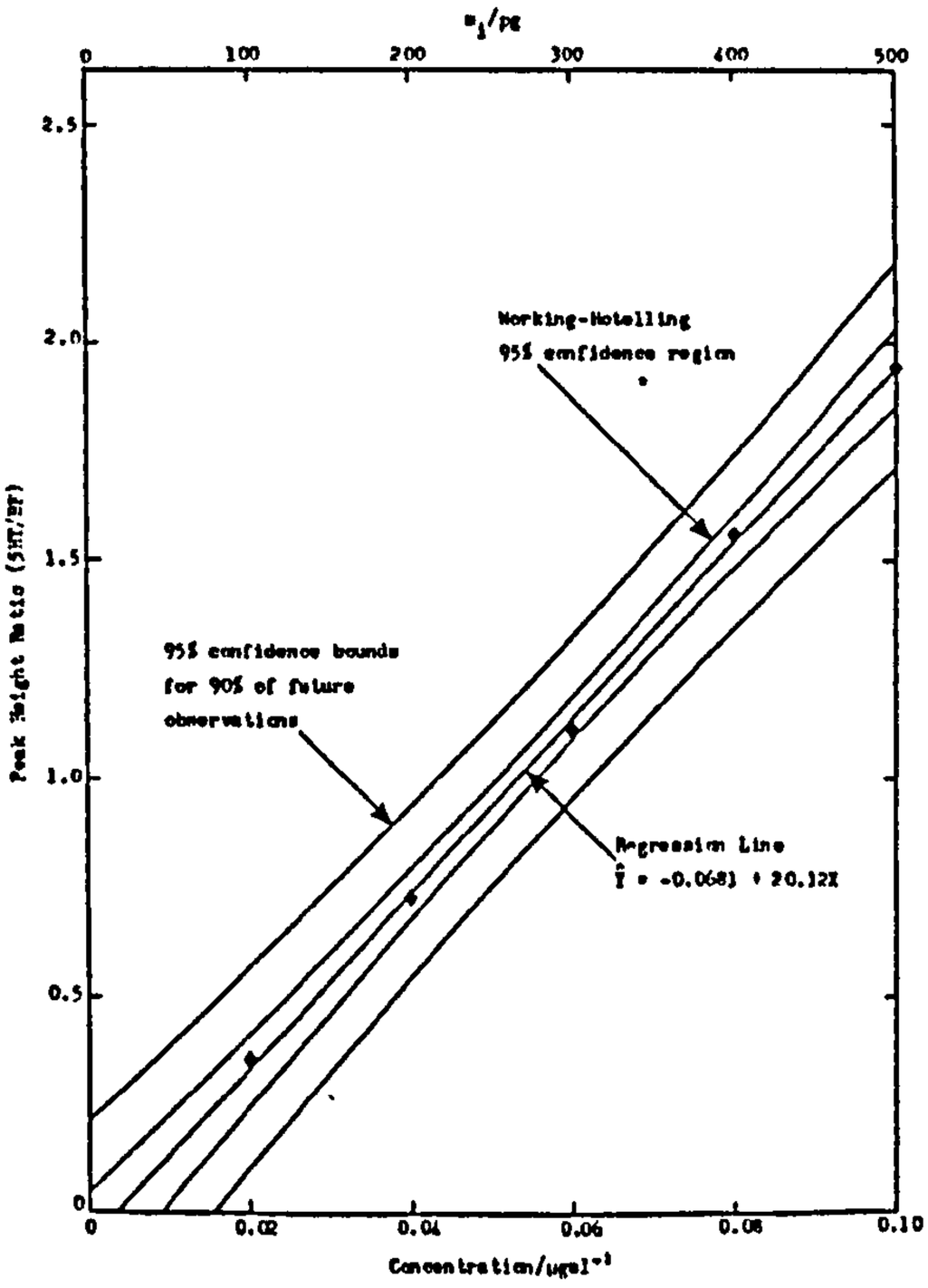
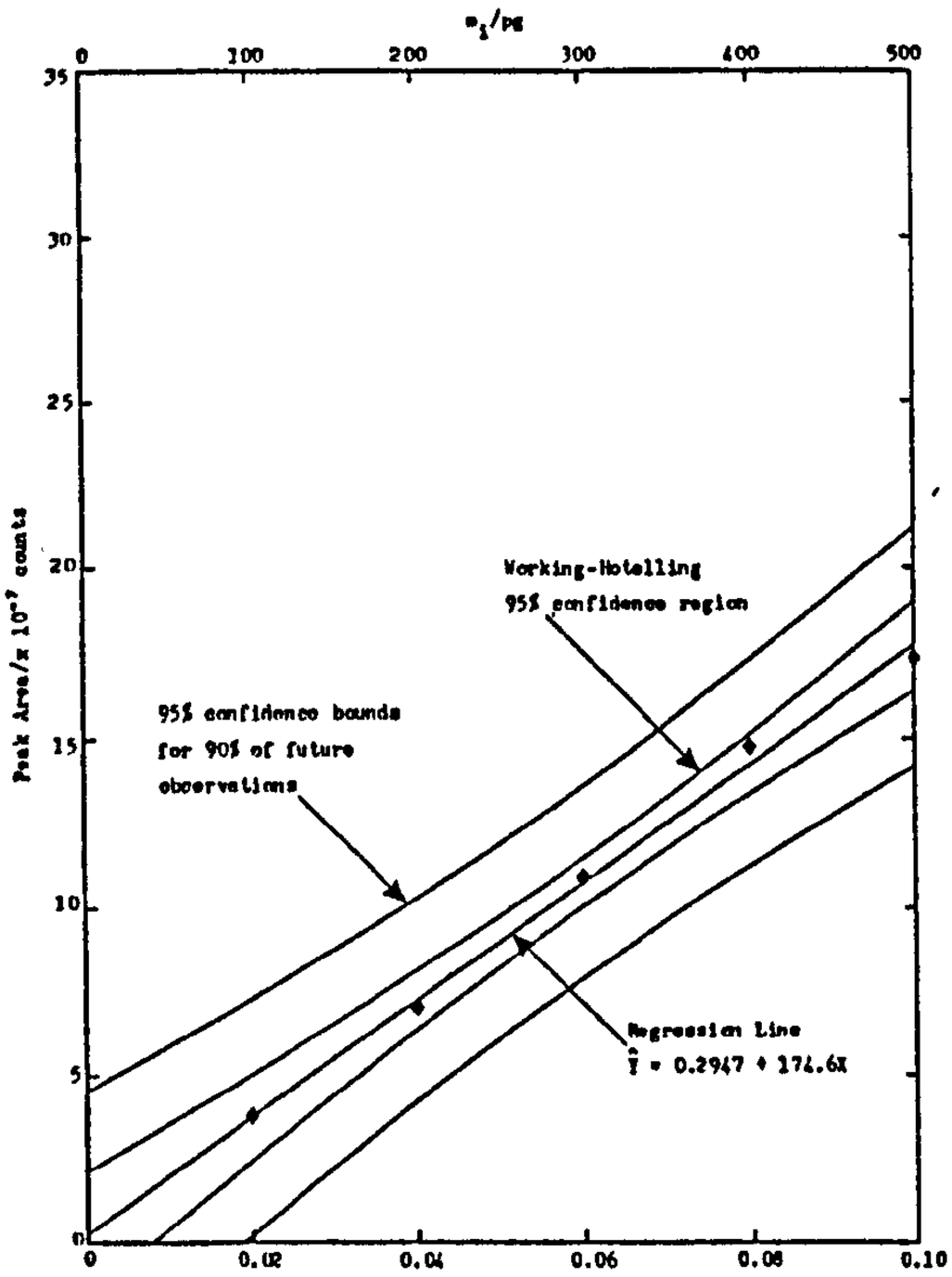
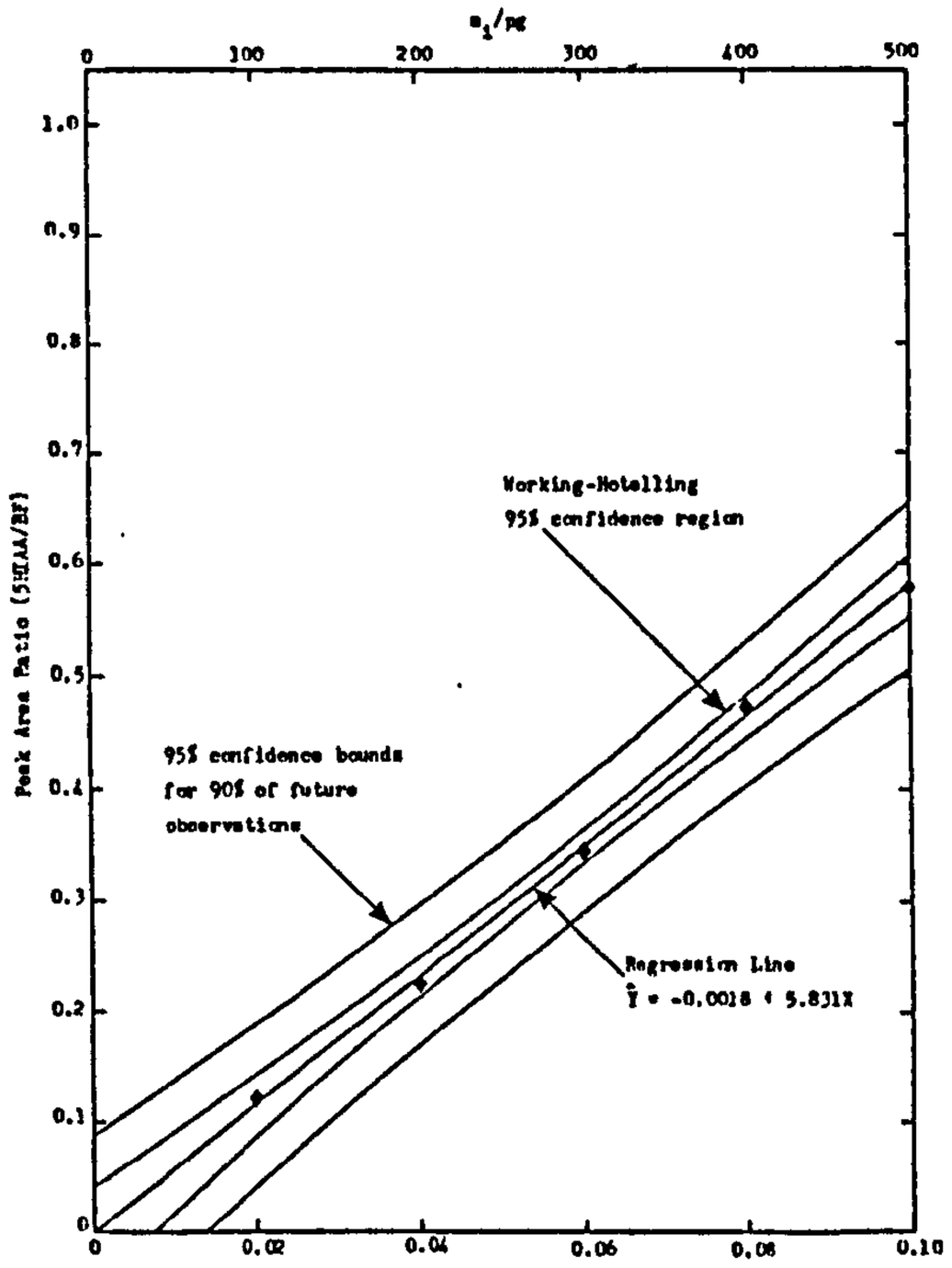
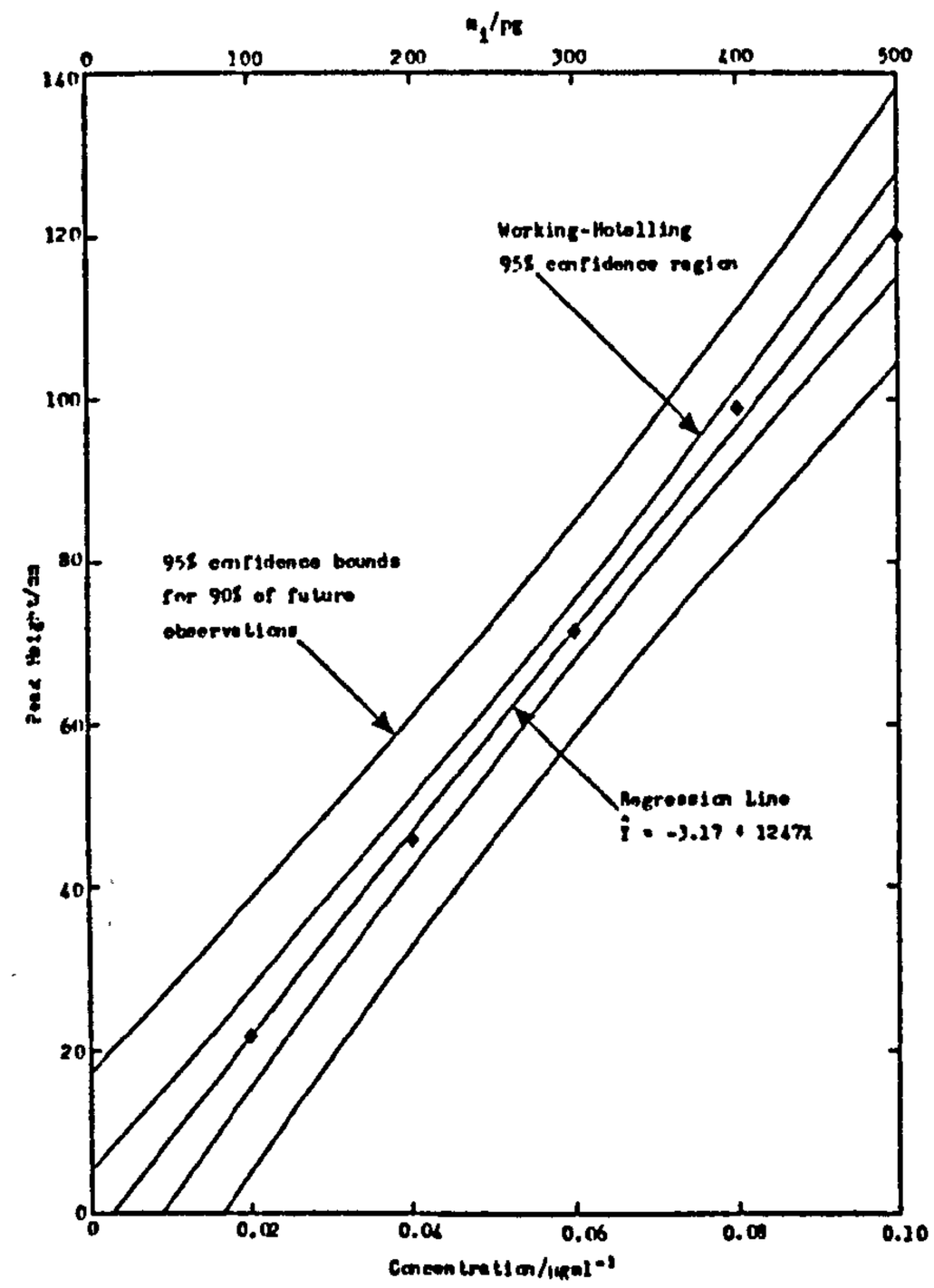


Figure 3.68 Calibration Curves for 5-Hydroxyindole-3-acetic Acid by Peak Area, Peak Area Ratio, Peak Height and Peak Height Ratio







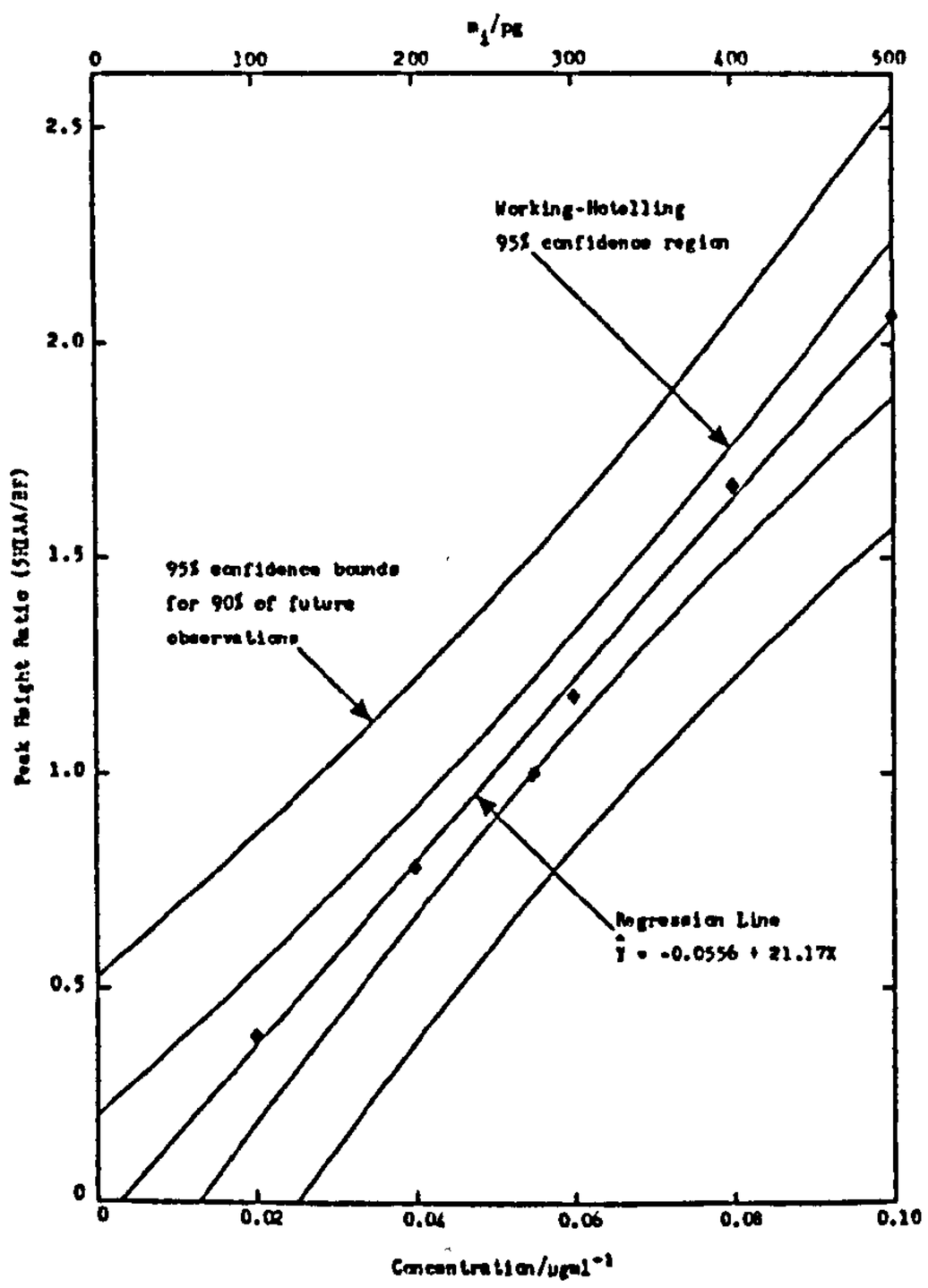
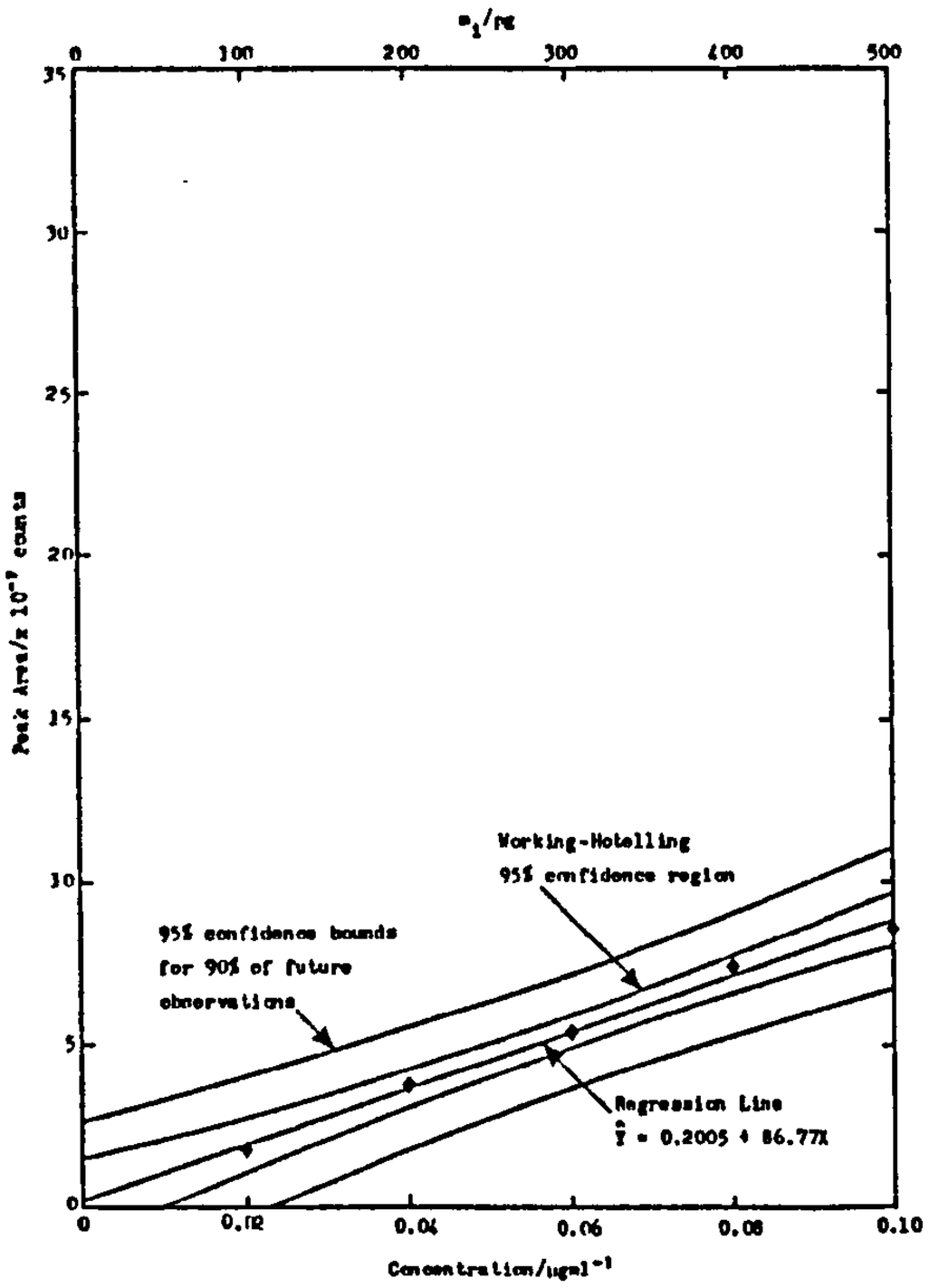
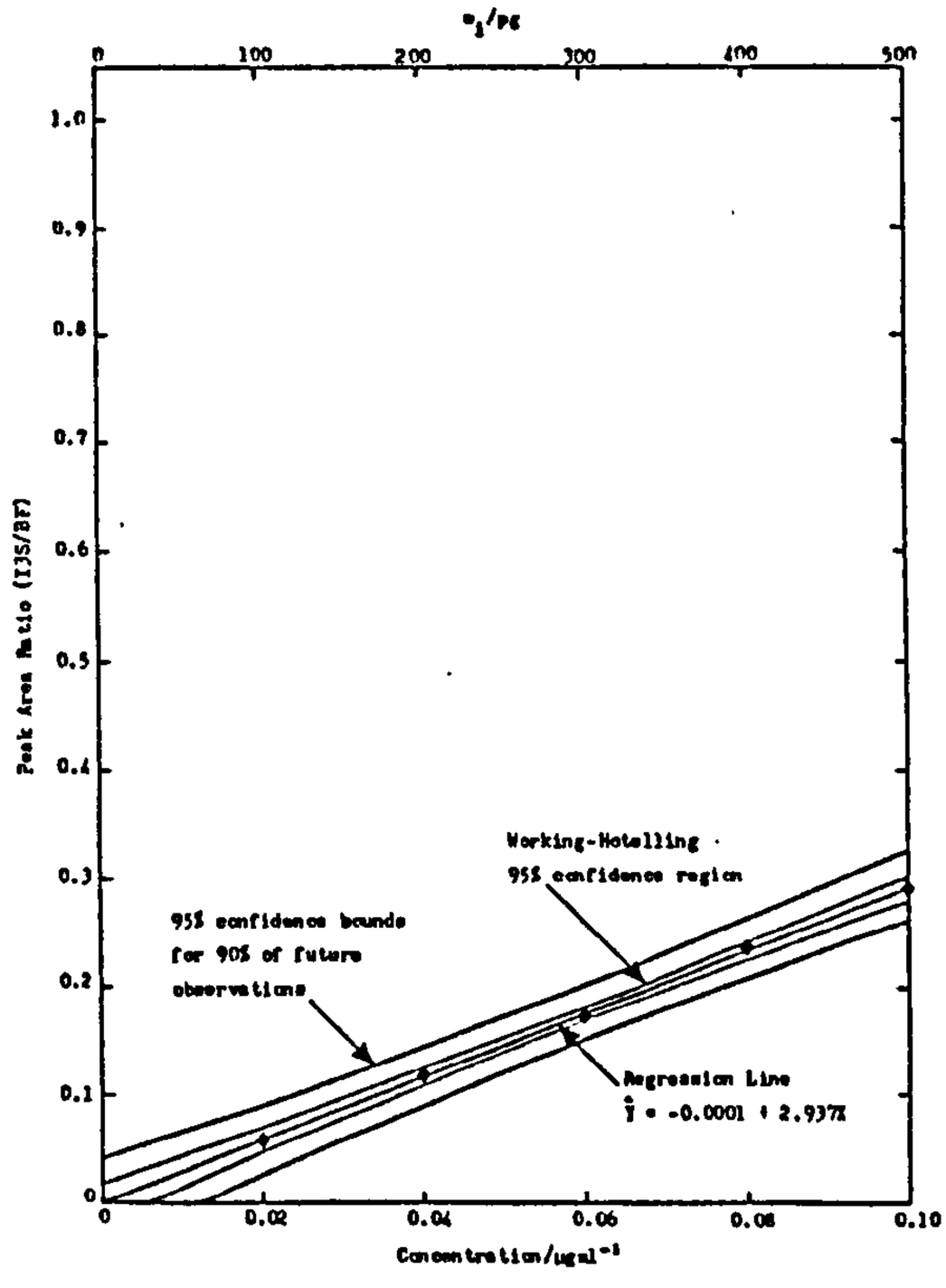
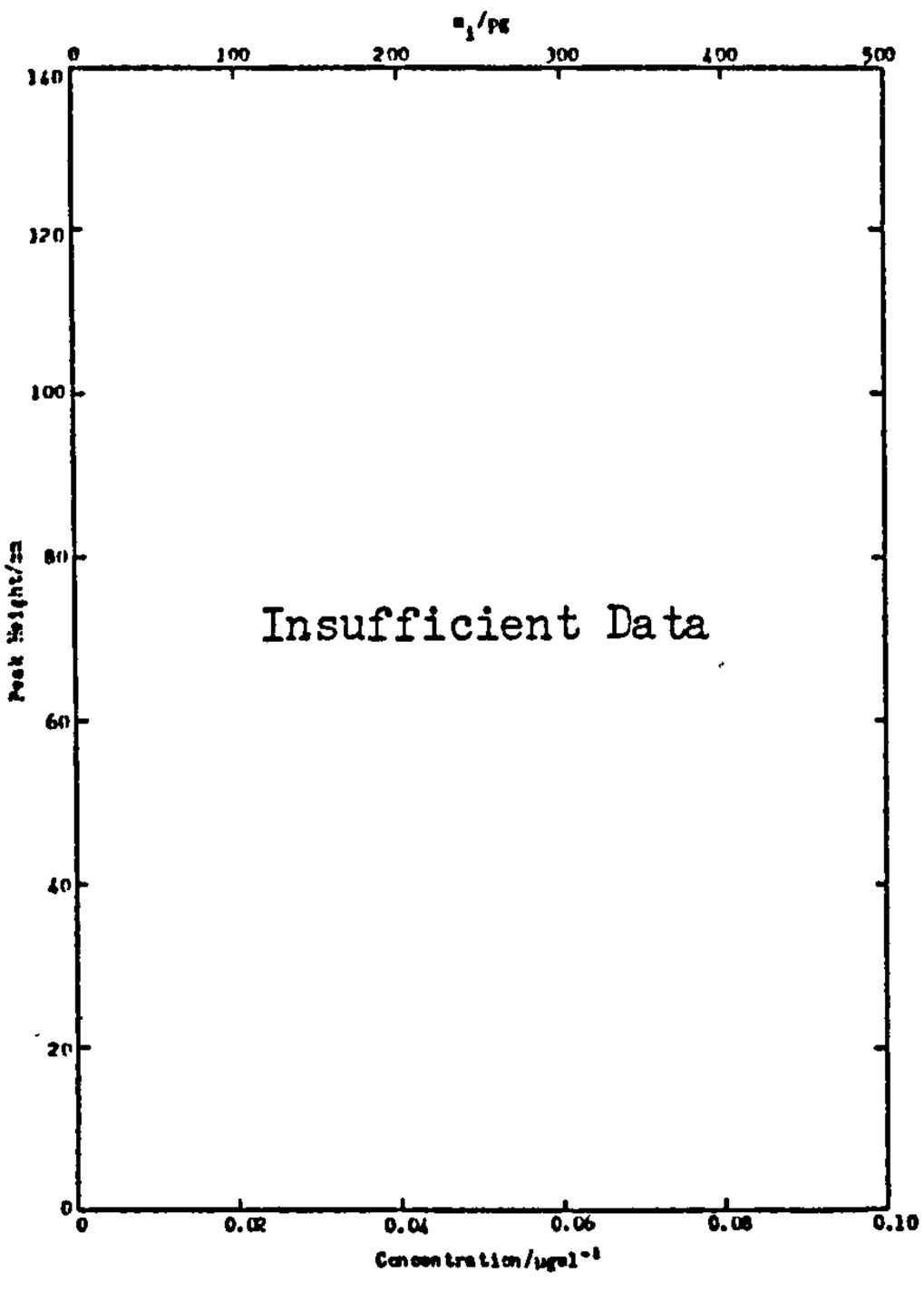
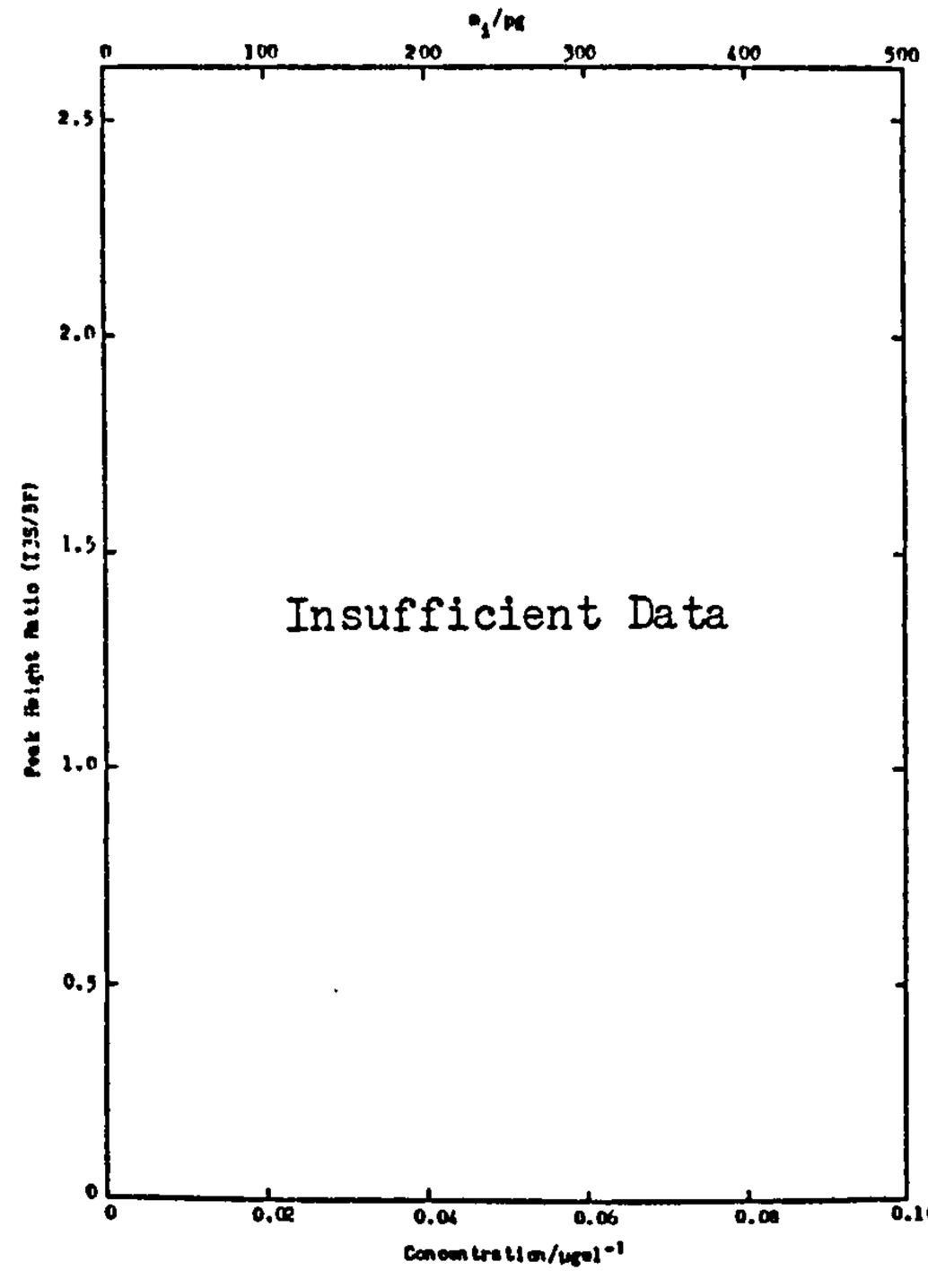


Figure 3.69 Calibration Curves for Indoxyl-3-sulphate by Peak Area and Peak Area Ratio









3.6.4.2 Calibration Curves Employed for Statistical Determination of the Limit of Detection

The second function of a calibration curve is that of elucidation of the LOD. It is generally agreed that the LOD is statistical in nature[427] although an arbitrary non-statistical method of determination is commonly employed! (see Section 2.3.4 for example). Because the electrochemical response observed on a blank is virtually a horizontal straight line, this precludes a measurement of the background in units of area, - the units in which quantitation is often performed. Hubaux and Vos[428] and subsequently Bailey et al.[427] have both advocated calculation of what they term a "detection limit" from other available information, i.e., the calibration data.

The regression line equation is solved for a concentration (or mass injected) of zero to yield a peak response which is the expected blank value (a in Figures 3.70(a) & (b)).

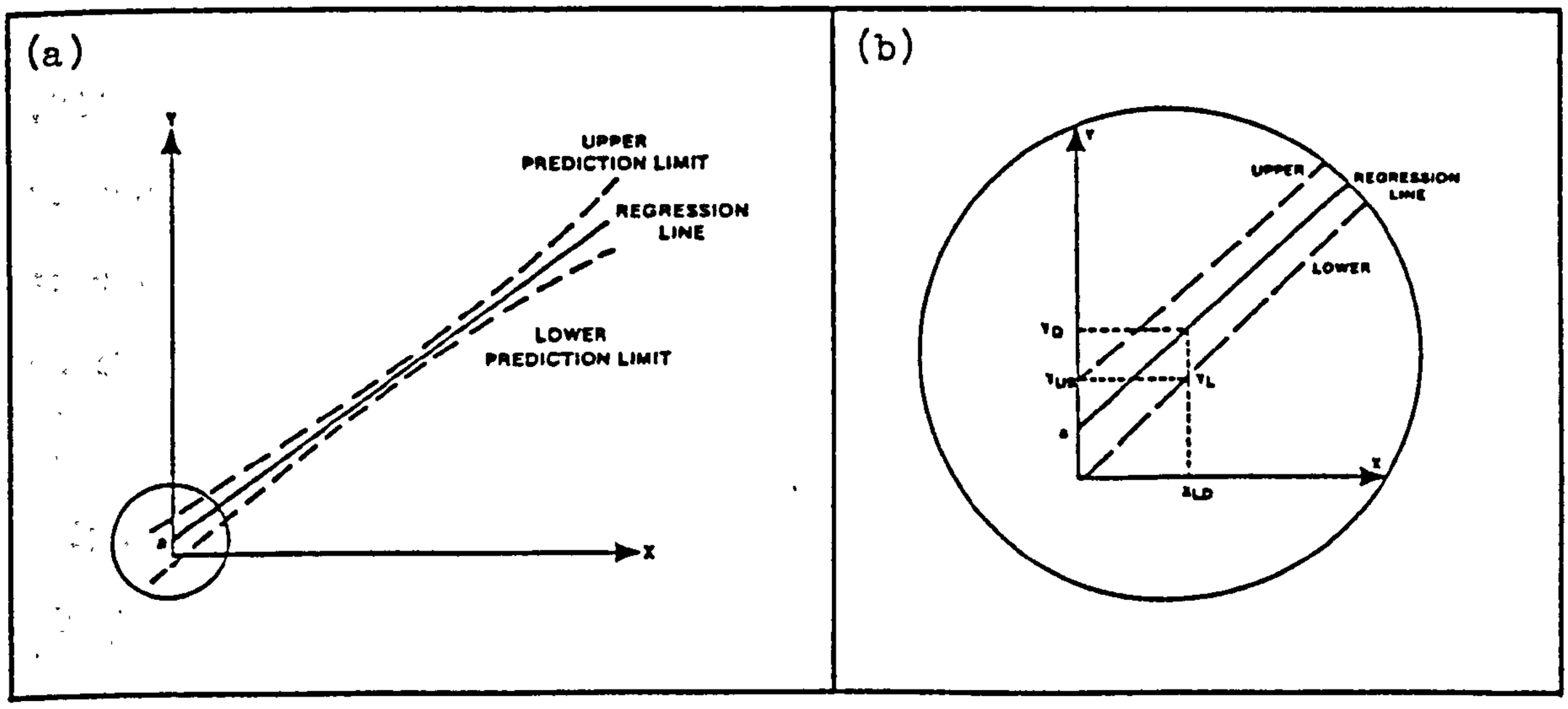


Figure 3.70 (a) Regression Line with Prediction Limits, and Expected Blank Value, a. (b) Enlargement of part of (a) showing the Additional Parameters Y_{UB}, Y_L, Y_Q and X_{LD} (see text for details)

The LOD is calculated by comparing two prediction limits. Y_{UB}, the 99% upper prediction limit on a, the expected blank value, is given by the expression :

$$Y_{UB} = a + bX + \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(\bar{X})^2}{\Sigma x^2} \right)^{\frac{1}{2}} \right] \qquad (3.5)$$

where t = Student's t statistic (Tables),

s_{YX} = standard error of the estimate = $\left[\frac{\Sigma y^2 - b\Sigma xy}{n-2} \right]^{\frac{1}{2}}$,

and all other symbols are as previously defined.

Y_L, the 99% lower prediction limit on the expected peak response at a given concentration (or mass injected), X_o, is calculated from :

$$Y_L = a + bX_o - \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(X_o - \bar{X})^2}{\Sigma x^2} \right)^{\frac{1}{2}} \right] \qquad (3.6)$$

Values for X_o are substituted into Equation 3.6 and the equation is solved for Y_L. The lowest value of X_o which yields a value for Y_L equalling or just exceeding Y_{UB} is the LOD for a single analysis (denoted X_{LD} in Figure 3.70(b)). The peak response corresponding to X_{LD} may be determined using the regression line. This peak response (denoted Y_Q in Figure 3.70(b)) represents the peak response above which quantitation may be performed (at the 95% confidence level).

This statistical procedure was employed to determine the LOD for each analyte by each method of quantitation (except for 5HTP and I3S by peak height and peak height ratio for the reasons noted earlier). The calculations performed on data from the quantitation of TP by peak area are presented as an example. A summary of the statistically derived LOD expressed both in terms of concentration and the more relevant quantity,

mass injected on-column, is compiled in Table 3.53. LODs (and LOQs) were also determined from signal and noise (height) measurements of the chromatograms by the more commonly employed empirical method (i.e., LOD = *S/N* ratio of 2:1, LOQ = *S/N* ratio of 20:1). Ranges and means of these values for each indole are also reported in Table 3.53 for comparison.

Calculation of the Detection Limit for Tryptophan Measured by Peak Area (Statistical Method)

(i) Y_{UB}, the 99% upper prediction limit on the blank :

$$= a + bX + \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(\bar{X})^2}{\Sigma x^2} \right)^{\frac{1}{2}} \right] \qquad (3.5)$$

Since $n = 5$, $\nu = n-2 = 3$, and $\alpha = 0.01$, $t_{3,0.01} = 5.84$ (Tables)

Then at $X = 0$

$$Y_{UB} = 0.416248 + 121.48 \times 0 + \left[5.84 \times 0.204912103 \left(1 + 0.2 + \frac{(0.06)^2}{0.004} \right)^{\frac{1}{2}} \right]$$

$$Y_{UB} = \underline{2.150412}$$

(ii) Y_L, the 99% lower prediction limit of the expected response at a given concentration, X_o :

$$= a + bX_o - \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(X_o - \bar{X})^2}{\Sigma x^2} \right)^{\frac{1}{2}} \right] \qquad (3.6)$$

$$= 0.416248 + 121.48X_o - \left[5.84 \times 0.204912103 \left(1 + 0.2 + \frac{(X_o - 0.06)^2}{0.004} \right)^{\frac{1}{2}} \right]$$

For $X_o = 0.02$,

$Y_L = 2.845848 - 1.513702 = 1.332146$ i.e., $< Y_{UB}$

For $X_o = 0.025$,

$Y_L = 3.453248 - 1.468686 = 1.985462$ i.e., $< Y_{UB}$

For $X_o = 0.026$,

$Y_L = 3.574728 - 1.460252 = 2.114476$ i.e., $< Y_{UB}$

For $X_o = 0.027$,

$Y_L = 3.696208 - 1.452015 = 2.244193$ i.e., $> Y_{UB}$

$\therefore$ $\underline{X_{LD} = 0.027 \mu g ml^{-1}}$

Since $V_i = 5\mu l$ then $\underline{X_{LD} = 135pg \text{ injected on-column}}$

Table 3.53 Limits of Detection Determined Statistically and Empirically for Indole Standards Chromatographed Using the Pye Unicam NBLC System

(Statistically derived values reported with respect to method of quantitation. Empirically derived values are means of 5 observations obtained from signal and noise height data)

Indole	X_{LD} (pg (≡ $\mu g ml^{-1}$))				LOD by *S/N* = 2:1 (pg (≡ $\mu g ml^{-1}$))	LOQ by *S/N* = 20:1 (pg (≡ $\mu g ml^{-1}$))
	Peak Area	Peak Area Ratio	Peak Height	Peak Height Ratio		
TP	135 (0.027)	195 (0.039)	185 (0.037)	165 (0.033)	39 (0.0078)	390 (0.078)
5HTP	325 (0.065)	400 (0.080)	nc*	nc	21 (0.0042)	210 (0.042)
5HT	200 (0.040)	255 (0.051)	85 (0.017)	100 (0.020)	36 (0.0072)	360 (0.072)
5HIAA	170 (0.034)	110 (0.022)	115 (0.023)	190 (0.038)	34 (0.0068)	340 (0.068)
I3S	210 (0.042)	95 (0.019)	nc	nc	21 (0.0042)	210 (0.042)

* nc = not calculable

Not surprisingly, the statistically derived LODs fall into no readily discernible pattern with respect to elution order (i.e., I3S < 5HTP < TP < 5HIAA < 5HT). In contrast, the empirically calculated quantities do follow this general trend, with the notable exception of TP. The lack of obvious trend within the statistically derived LODs is almost certainly a reflection of the great uncertainty in the calibration lines, a direct result of the extensive variability of the raw data.

No single quantitation method can be said to be significantly better with regard to LOD than any other because of the large error estimate. Only in the case of 5HT do the peak height-based methods appear to yield lower LODs than their area-based counterparts. This may be explained because of the poorer reproducibility of peak start and end point recognition in area determination to longer k′. Regrettably, complete appraisal is not possible due to the insufficiency of height data relating to 5HTP and I3S to enable calibration and subsequent determination of their LODs by statistical calculation.

The high values obtained for the statistically derived parameter, X_{LD} also reflect the uncertainty in the calibration lines. These LODs are considerably greater than their empirically-derived supposed-equivalents. This observation highlights a fundamental problem which is the source of much confusion. Currie[429] noted 8 different definitions of LOD appearing under various titles and then proceeded to define the parameter another way! Currie also remarked upon the widely differing values for "LOD" that these various definitions yielded from a single set of data. The empirical definition which employs a *S/N* ratio of 2:1 was one of these 8 alternatives. This quantity represents the smallest amount of a substance which is deemed to be detectable. Thus it is essentially a qualitative limit by which it

may be deduced whether a peak is actually present or not rather than a quantitative limit above which "reliable" quantitation of said peak may be preformed. This latter limit is arbitrarily taken herein to be the amount of substance which gives rise to a peak of a height of 20 times the noise level and is denoted LOQ. No estimate of reliability is incorporated in these definitions. In contrast, the statistically-derived limit, X_{LD}, represents the minimum amount of substance that produces a signal which can be detected and quantified with 95% confidence. Thus, this definition of "detection limit" is actually a "quantitation limit" and consequently is more akin to the empirical LOQ than the empirical LOD. This is reflected in the values obtained for X_{LD} and LOQ which are of the same order of magnitude, unlike the values of LOD which are one order of magnitude lower (see Table 3.53).

For quantitative work, it is the values of X_{LD} and/or LOQ which are important. These limits are generally of the order of 100-400pg. No reliable data is available concerning normal levels of indolic TP metabolites in plasma but the information compiled in Table 3.6 suggests that concentrations of the order of 50ngml^{-1} or less may be expected for certain analytes. With sample size limited to only ca. 100µl plasma, this corresponds to 5ng of material for determination. Now, a maximum of 10% of this (i.e., 500pg) will be introduced on-column assuming no losses during clean-up (see Section 3.9). Hence, by this argument it may still be possible to quantify normal and perhaps diminished levels of indoles in plasma in spite of the poor specifications of the Pye Unicam NBLC-EC system under appraisal. However, this postulation may only be substantiated in practice.

CHAPTER 3

Part IV

Preservation, Fractionation and Clean-up of Blood Samples for Narrow-Bore Liquid Chromatographic Analysis

Contents

3.7 Introduction

In order to obtain a plasma sample in a form conducive to analysis by HPLC, four specific considerations must be addressed. These are : (1) prevention of blood clotting, (2) preservation of the character of the sample (i.e., maintenance of the analyte composition), (3) fractionation of the sample to yield the part(s) of interest, in this case PRP and PPP, and (4) extraction or isolation of the analyte compounds from other components in the matrix which may cause either physical or chromatographic interference in the analytical process.

3.7.1 Anticoagulation[222,430]

Plasma constitutes the extra-cellular liquid fraction of the blood inclusive of endogenous coagulating agents as distinct from the cellular matter (e.g., erythrocytes, leucocytes, granulocytes) suspended within it. Consequently, in order to obtain the plasma fraction for analysis, clotting of the blood must be prevented *in vitro*. Because the mechanism responsible for coagulation is highly complex and requires many components (or factors) to be present in order to function, there are several possible ways of achieving disruption of this process. Most commonly employed anticoagulants rely on the fact that free $Ca^{2\oplus}$ ions are essential for coagulation. $Ca^{2\oplus}$ ions may be rendered unavailable very effectively either by complexation with EDTA or by precipitation as insoluble salts with oxalate ($C_2O_4^{2\ominus}$) or, to a lesser extent, with $F^{\ominus}$. Thus, Na_2EDTA, $Li_2C_2O_4$, $Na_2C_2O_4$, $K_2C_2O_4.H_2O$ and NaF (at levels of 1-2mg per ml of blood) have all found application as anticoagulants.

The most widely used anticoagulant, probably because it is known

generally to cause the least interference, is heparin. Heparin (MW = 6,000-25,000) is a glycosaminoglucuronan (acid mucopolysaccharide) that occurs in most tissues. This substance is physiologically active in relation to haemostasis. It interferes with the coagulation mechanism, enhances fibrinolysis (the dissolution of fibrin which forms the basis of a blood clot) and it can also inhibit platelet aggregation. A concentration of 0.2mg per ml of blood is sufficient to suppress coagulation.

Other anticoagulants utilised, especially for donated blood, include citrate, citrate-dextrose, citrate-phosphate-dextrose and citrate-phosphate-dextrose-adenine combinations.

For routine acquisition of blood specimens of ca. 10ml, a series of pre-treated, evacuated blood collection tubes ('Vacutainers') is available. The marketed product range is indicated in Table 3.54.

Table 3.54 The Range of Evacuated Blood Collection Tubes Currently Available[222]

Colour Code	Use	Additive
Lavender	Plasma or whole blood	EDTA (Na_2 or K_2)
Grey	Plasma or whole blood with glycolysis inhibition	Oxalate (Na or K), $F^{\ominus}$ (Na) or iodoacetate (Na)
Green	Plasma or whole blood	Heparin (Na, Li or NH_4)
Blue	Plasma or whole blood	Citrate (Na)
Red	Serum	None
Yellow	Serum	None, sterile interior

These 'Vacutainers' permit rapid blood collection with appropriate treatment and with reduced risk of infection for the staff. For these reasons they are employed routinely in many hospitals.

3.7.2 Preservation

Since the measurement of indole levels cannot be performed *in vivo*, a sample is taken and analysis is carried out *in vitro*. The integrity of this sample must be maintained in order to enable the attainment of an accurate estimation of the status of the chosen analytes as they were at the time of sampling. It is important therefore to prevent further transformations of the analytes by inducing cessation of enzyme activity within the sample as quickly as possible following collection. In the case of TP and its indolic metabolites this is achieved principally by cooling the specimen. The use of ice-cold reagents in future manipulations is also recommended. In addition, the rapid precipitation of enzymatic material (predominantly protein) is desirable (see also Section 3.7.4).

Furthermore, if the compounds of interest are themselves labile with respect to external factors such as temperature, aerial oxidation and/or exposure to light (as are the indoles) then steps must be taken to prevent, or at least minimise, the effects of these factors. As noted above temperature is often reduced as a matter of course. Some experimenters have advocated the addition of chemical antioxidants to blood samples in order to preserve their readily oxidisable constituents. The two most widely used additives applicable to indolic substances are ascorbic acid[318,354,377,417] and sodium metabisulphite ($Na_2S_2O_5$).[363,416] It may also be advantageous to exclude bright light in order to prevent photo-decomposition in situations where particularly photosensitive compounds are under consideration.

Blood samples (or fractions thereof) which are not to be analysed immediately are generally frozen and stored at low temperature ($\leq$ -20 °C).

3.7.3 Fractionation

Once the blood sample has been collected the plasma part must be separated from the unwanted cellular material. This is usually accomplished by centrifugation. Platelet-rich plasma (PRP) is yielded by low speed centrifugation of whole blood. Preparation conditions ranging from 100 x g for 10 min at 4 °C[416] to 500 x g for 30 min at 4 °C[331] and 750 x g for 10 min at ambient temperature[348] have been utilised to obtain PRP for indole determination. Platelet-poor plasma (PPP) is produced by centrifugation at higher speed of either previously separated PRP or whole blood. Again various conditions have been employed for preparation. Martínez and co-workers[354] used 1,000 x g for 10 min at 4 °C, Petruccelli et al.[348] utilised 1,000 x g for 20 min at ambient temperature, whereas Morita and associates[431] employed parameters of 1,500 x g for 10 min at 25 °C. Recently, Picard et al.[416] examined various spin speeds for the production of PPP for 5HT determination. They demonstrated that centrifugation conditions of 6,000 x g for 15 min at 4 °C were optimum for deposition of the platelets without causing them to be damaged. Spin rates producing relative centrifugal forces (RCFs) of less than 6,000 x g resulted in incomplete platelet separation, hence erroneously high "free" 5HT values were obtained on analysis. At spin rates producing RCFs in excess of 6,000 x g platelet disruption occurred liberating intra-platelet 5HT into the plasma which again produced false "free" 5HT levels on quantitation.

The employment of refrigeration during centrifugation is recommended for the reasons of analyte preservation given previously.

3.7.4 Extraction

Direct injection of plasma into the HPLC system is favoured by some experimenters because this substantially reduces sample preparation time and eliminates errors due to variability in extraction efficiency.[432] However, most workers consider the attendant problems involved to be intolerable. These problems include:(1) physical blockage of the column by strongly adsorbed endogenous substances leading to greatly reduced column lifetime[432,433] and (2) because normal levels of TP and its indolic metabolites are considerably lower in plasma than in nervous tissue, csf and urine[363], combined with the fact that there are more potential interferents in plasma than in these other samples[363], this gives rise to complex chromatograms which render peak identification difficult and a consequent increase in LOQ (and LOD) inevitable.[362,432] The first of these two problems is particularly great for NBLC columns where the cross-sectional surface area is substantially less than that of conventional columns. Consequently, even shorter column lifetimes would be anticipated especially since the use of protective devices is excluded by extra-column dispersion considerations.

Since direct injection is so detrimental to column performance, some form of further sample manipulation is normally performed. There are four main reasons for further sample work-up, viz. (1) the removal of column degrading substances (e.g., proteinaceous materials and lipids), (2) the isolation of the subject compounds from other compounds in the matrix which are electroactive at the selected operating potential and which would otherwise cause interference or elongation of run time in the chromatographic step, (3) the replacement of the sample matrix by one better suited to the chromatographic conditions, i.e., matched to the mobile phase, and (4) the

concentration of the analytes (without corresponding concentration of endogenous interferents) in order to improve their detectability.

To some degree a compromise may be considered between the applied potential and the extent of clean-up required. The more selective is the detection process then the less pre-chromatographic extraction is necessary.

Generally a protein elimination step is deemed to be essential for the viability of the method. There are several means by which this aim may be achieved.[434,435] Precipitation and subsequent centrifugation is the usual method employed. Various precipitating agents have been applied including hydrochloric, perchloric, trichloroacetic and 5-sulphosalicylic (2-hydroxy-5-sulphobenzoic) acids, alkaline $ZnSO_4$ and the organic solvents, MeOH and MeCN. Deproteinisation with any of the strongly acidic precipitants is ill-advised where "free" indole levels are required because they will necessarily bring about a change in sample pH. Now, both protein and platelet binding of indolic substances is pH dependent and such a dramatic pH change would be expected to cause imbalances in the "bound" to "free" equilibria of the analytes. In addition, highly acidic (and highly alkaline) environments have been observed to promote indole decomposition.[338,379] Precipitation by means of MeOH or MeCN is considered preferable in this instance because the pH shift would be minimal.

Alternatively to precipitation, proteins may be removed by ultra-filtration, active filtration, acid hydrolysis or by enzymatic digestion. Ultra-filtration is a very efficient method but problems have been encountered with coincident loss of analytes[360], presumably by adsorption onto the filtration membrane. Problems have also arisen regarding active filtration on commercial solid-phase extraction cartridges, where analyte losses have been found to be high.[360] Acid hydrolysis is rarely employed

today because the process is quite severe and can cause decomposition of the analytes.[433] The use of proteolytic enzymes has also been discontinued.

For relatively simple samples such as brain tissue homogenates and csf, deproteinisation is often sufficient preparation prior to indole determination by HPLC-EC (see Section 2.3.5 for example). However, for a sample of the general complexity of plasma further purification is frequently necessary in spite of the high discrimination power of the ECD. Matrix exchange and/or concentration of the analytes may also be incorporated into this clean-up procedure. The two techniques which are particularly applicable are liquid-liquid extraction and liquid-solid extraction. Both processes are not without their difficulties with respect to the isolation of TP and its indolic metabolites.

One general problem experienced is associated with the wide polarity range of the analyte species.[305] Under analytical conditions the various indoles exist as cationic, anionic and zwitterionic moieties (see Figure 3.37) so rendering differentiation of these substances from potential interferents by chemical means a formidable task.

Liquid-liquid separation techniques suffer many disadvantages. First, the analytes are all highly soluble in water hence extraction with water-immiscible organic solvents is usually poor.[436] In addition, this procedure involves attempting to replace one RP-LC-compatible matrix with one which is incompatible, thus dictating the need for back-extraction which adds another step in which analyte losses can occur. Liquid-liquid extraction is invariably a multi-stage process and generally the more steps that are incorporated then the greater are the analyte losses incurred. Significant loss of analyte material during clean-up may well be intolerable

when trace components in a sample of highly restricted size are to be quantified. Furthermore, considerable dilution of the analyte-containing solution is inherent with this technique, hence requiring removal of comparatively large volumes of solvent before chromatographic analysis, which is both time consuming and subject to degradation losses. Moreover, liquid-liquid extraction is laborious and is not readily automated.

Liquid-solid separation techniques, although not problem-free, do offer several advantages over liquid-liquid separation methods. Liquid-solid extraction is more versatile, adsorption-desorption, partition, ion-interaction and ion-exchange modes of separation all being accessible and allowing improved discrimination. Generally, smaller volumes of solvent are required to achieve comparable extractions, hence, little or no reduction in volume is necessary. Frequently, extraction efficiencies are greater than those realised by liquid-liquid-based methods. Furthermore, liquid-solid techniques are more rapid and easier to automate. Consequently, the use of small commercial solid-phase extraction cartridges has found favour with many experimenters in recent years.

At various stages during the progress of the investigations reported in this dissertation, blood samples were examined. Taking due note of the comments contained herein, several procedures for blood collection, preservation, fractionation and extraction of the resulting plasma were devised and evaluated for effectiveness.

3.8 Experimental

3.8.1 Solvents and Reagents

All solvents and reagents utilised for production of the mobile phase, and as standard materials were obtained from the sources indicated previously (Section 3.3.1) and were treated as specified therein. Additional chemicals employed in sample preparation procedures included ascorbic acid (vitamin C, AnalaR grade) and perchloric acid ($HClO_4$, 70% w/v solution, AnalaR grade). Both substances were purchased from BDH, Poole, Dorset. Oxygen-free N_2 for solvent evaporation was supplied by BOC, Bristol, Avon.

3.8.2 Standard Solution Preparation

Stock solutions ($500\mu gml^{-1}$ in standard material) of TP, 5HTP, 5HT. creatinine sulphate complex, 5HIAA and I3S.K^+ salt were prepared in a MeOH:water mixture and were acidified with HAc (5 drops) to aid dissolution. An indole solution ($0.5\mu gml^{-1}$ each analyte) for spiking plasma was acquired by combination and serial dilution with DW of these stock solutions.

Stock solutions of selected internal standards, AN ($333\mu gml^{-1}$ and $100\mu gml^{-1}$) and $BF.(CO_2H)_2.H_2O$ ($11.1\mu gml^{-1}$ as BF) were prepared in MeOH. Stock solutions were diluted with MeOH to provide working solutions (3.33 and $0.5\mu gml^{-1}$ AN and $0.11\mu gml^{-1}$ BF) for addition of IS to the plasma samples.

$HClO_4$ (7% w/v ≡ 0.7M) for protein precipitation was prepared by aqueous dilution of the concentrated acid. For neutralisation, KOH (3.92g) was dissolved in water (100ml) to yield a solution ($39.2mgml^{-1}$, 0.7M).

For all procedures in which an extract was reduced to a residue, a mixture of 4% MeOH : 96% aq. 0.1M KH_2PO_4 ("pseudo"-mobile phase) was made up from existing mobile phase precursors for reconstitution purposes.

An aqueous solution of ascorbic acid ($5mgml^{-1}$) was prepared for addition to some blood samples to act as an antioxidant.

3.8.3 Chromatography

Standard solutions, column, mobile phase, and chromatographic conditions which had all been optimised (see Section 3.3) were employed throughout.

3.8.4 Auxiliary Apparatus

3.8.4.1 Blood Collection Vessels

A standard blood collection bag (500ml capacity) containing $NaCt.2H_2O$ (13.2g), $HCt.H_2O$ (1.6g), $NaH_2PO_4.2H_2O$ (1.3g) anhydrous dextrose (11.6g) and adenine (0.14g), and green-top (heparinised) 'Vacutainers' were provided by the Bristol Royal Infirmary.

3.8.4.2 Centrifuges

1) Gallenkamp AM3G fixed speed centrifuge (r_{rotor} = 10.5cm; spin speed = 3000rpm; RCF = 1,050 x g).
2) BTL bench centrifuge (r_{rotor} = 15.0cm; spin speed = 0-6,000rpm; RCF = 0-6,040 x g).
3) MSE Mistral 6L refrigerated centrifuge (r_{rotor} = 26.0cm; spin speed : variable, low; head capacity : 12 x 20 tubes; head type : pivoted).
4) MSE HS18 refrigerated ultra-centrifuge (r_{rotor} = 30.0cm; spin speed : variable, high; head capacity : 8 x 50 tubes; head type : fixed angle).

3.8.4.3 Platelet Counter

Ortho Diagnostic Systems ELT 800 cell counter.

3.8.4.4 Specialised Apparatus for Sample Clean-Up

All attempts at sample filtration were made using Swinnex filtration units (Millipore, Harrow, Middlesex) fitted with filter membranes (Type HA, $d_{membrane}$ = 13mm, d_{pore} = 0.1μm).

Solid-phase extraction cartridges ('Sep-Pak', C_{18} type, Waters Associates, Northwich, Cheshire) were employed in several procedures. All C_{18} Sep-Paks were activated prior to use by sequential elution with MeOH (2ml) and DW (2ml) at flow rates of ca. 3-5mlmin^{-1}.

3.8.5 Blood Collection, Preservation and Fractionation

3.8.5.1 For Preliminary Qualitative Studies and Initial Experiments Incorporating Aniline as an Internal Standard

Blood was sampled from an outpatient at the Bristol Royal Infirmary who suffered from polycythaemia (erythrocytic hyperplasia). These patients routinely undergo blood letting and are not known to have any abnormality of the plasma constituents. Blood (500ml) was drawn by venepuncture into a blood collection bag containing citrate-phosphate-dextrose-adenine. The blood was immediately cooled in an ice-water bath. After thorough mixing, ca. 100ml of the blood was transferred to centrifuge tubes wherein it was subjected to centrifugation (BTL bench centrifuge, 2,700 x g, 15 min, ambient temperature) in order to spin down the cells. Aliquots of the supernatant (plasma) were transferred by pipette to plastic vials which were then stoppered and stored deep-frozen (-20 °C) until required.

3.8.5.2 <u>For Further Experiments Incorporating Aniline as an Internal Standard</u>

Blood was sampled from three outpatients at the Bristol Royal Infirmary. All three individuals were attending for routine blood testing and were not known to exhibit any pertinent abnormalities of the blood plasma. Venous blood (<u>ca</u>. 10ml) was drawn into heparinised evacuated tubes (Vacutainers) which were placed in ice immediately following collection. The three samples were pooled and six aliquots (4ml) were placed in centrifuge tubes which were stoppered immediately. Centrifugation (BTL bench centrifuge, 3,400 x g, 5 min, ambient temperature) was performed within 30 min of collection. The resulting plasma samples were pipetted into a series of plastic vials which were then stoppered and stored deep-frozen (-20 ^{o}C) until required.

3.8.5.3 <u>For Methodological Studies Incorporating Bufotenine as an Internal Standard</u>

Blood (2 x <u>ca</u>. 10ml) was collected from two similar volunteers and in an identical manner to that outlined above (see Section 3.8.5.2). Immediately after pooling of the samples, aqueous ascorbic acid (5mgml^{-1}, 250µl per 10ml blood) was added as a preservative. Four aliquots (5ml) were centrifuged (MSE Mistral 6L, 350 x g, 10 min, 1-2 ^{o}C) to obtain ostensibly platelet-rich plasma (PRP). One sub-sample was transferred to a plastic vial, stoppered and retained. The other three sub-samples were pipetted into centrifuge tubes and were further centrifuged (MSE HS18, second sub-sample : 7,300 x g, third sub-sample : 16,400 x g, fourth sub-sample : 29,100 x g, each for 15 min at 3 ^{o}C). The supernatants were

removed and placed in plastic vials which were then stoppered. All plasma samples were kept on ice until a platelet count had been performed (Ortho Diagnostic Systems ELT 800 cell counter) after which they were stored deep-frozen (-20 °C) until required.

3.8.6 Investigated Extraction Procedures

Experiment 1

Plasma (1ml) derived from citrate-phosphate-dextrose-adenine-treated blood was transferred onto a pre-activated C_{18} Sep-Pak cartridge at a flow rate of *ca*. 0.5mlmin^{-1} by means of a syringe. The effluent was collected in a sample tube and stored in a refrigerator (4 °C). The cartridge was eluted with MeOH (1ml, Q ≃ 0.5mlmin^{-1}) applied *via* a syringe, and the washings were collected in a second sample tube which was subsequently stored in a refrigerator (4 °C). Chromatograms of both fractions were recorded (1µl injected). This procedure is summarised in Figure 3.71.

Experiment 2

Two aliquots (200µl) of plasma obtained from the citrate-phosphate-dextrose-adenine-treated blood were placed into plastic centrifuge tubes (3ml capacity). The first portion was spiked with methanolic AN (3.33µgml^{-1}, 300µl ≡ 1µg), whereas to the second was added MeOH (300µl). Each sample was vortex mixed (15-20sec) then centrifuged (Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature) to remove the white gelatinous precipitate which is formed. The resulting supernatants were transferred to clean sample tubes and were stored in a refrigerator (4 °C) prior to being chromatographed (1µl injected). Figure 3.72 depicts this procedure in schematic form.

Figure 3.71 Extraction Scheme 1

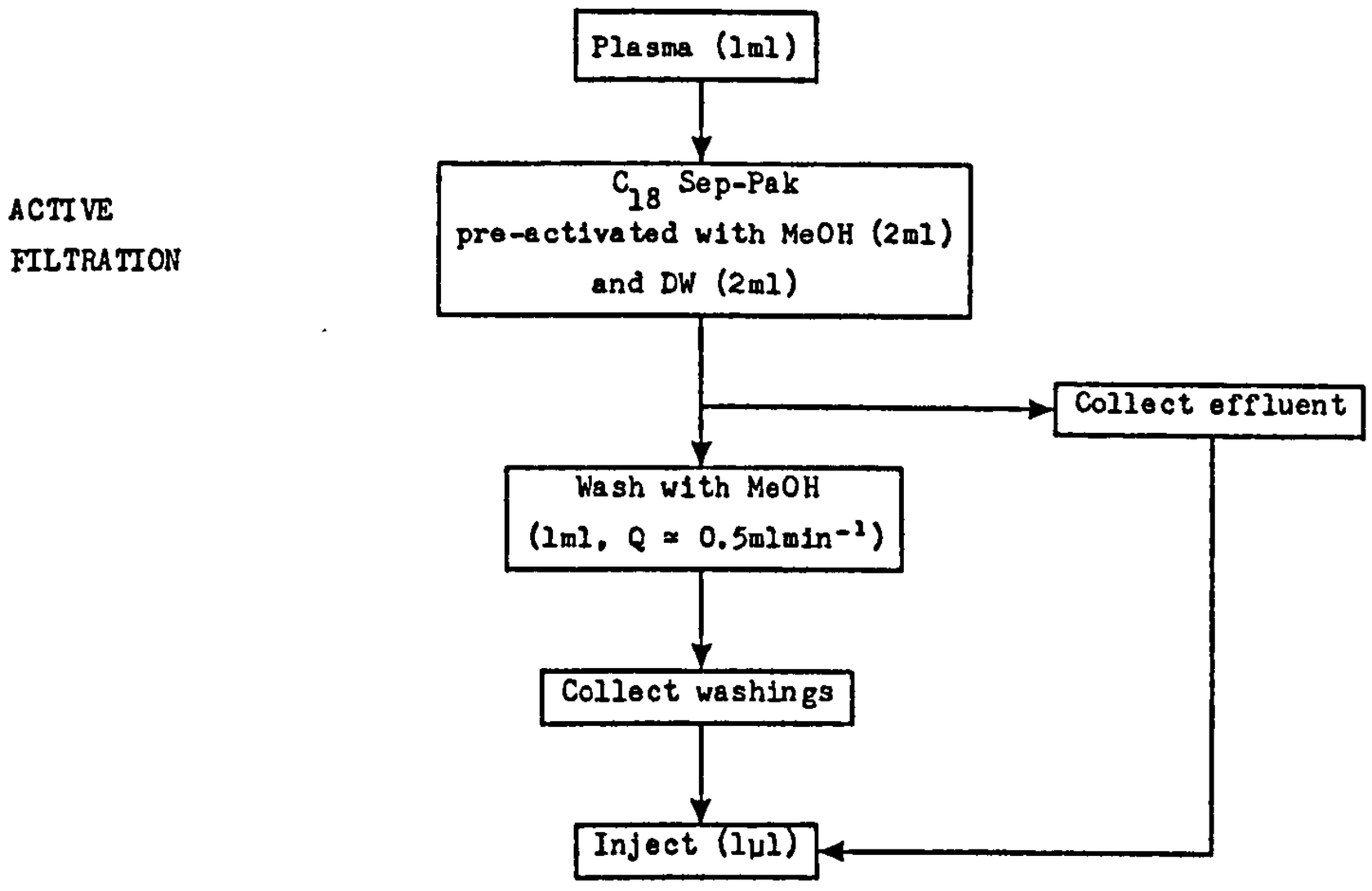
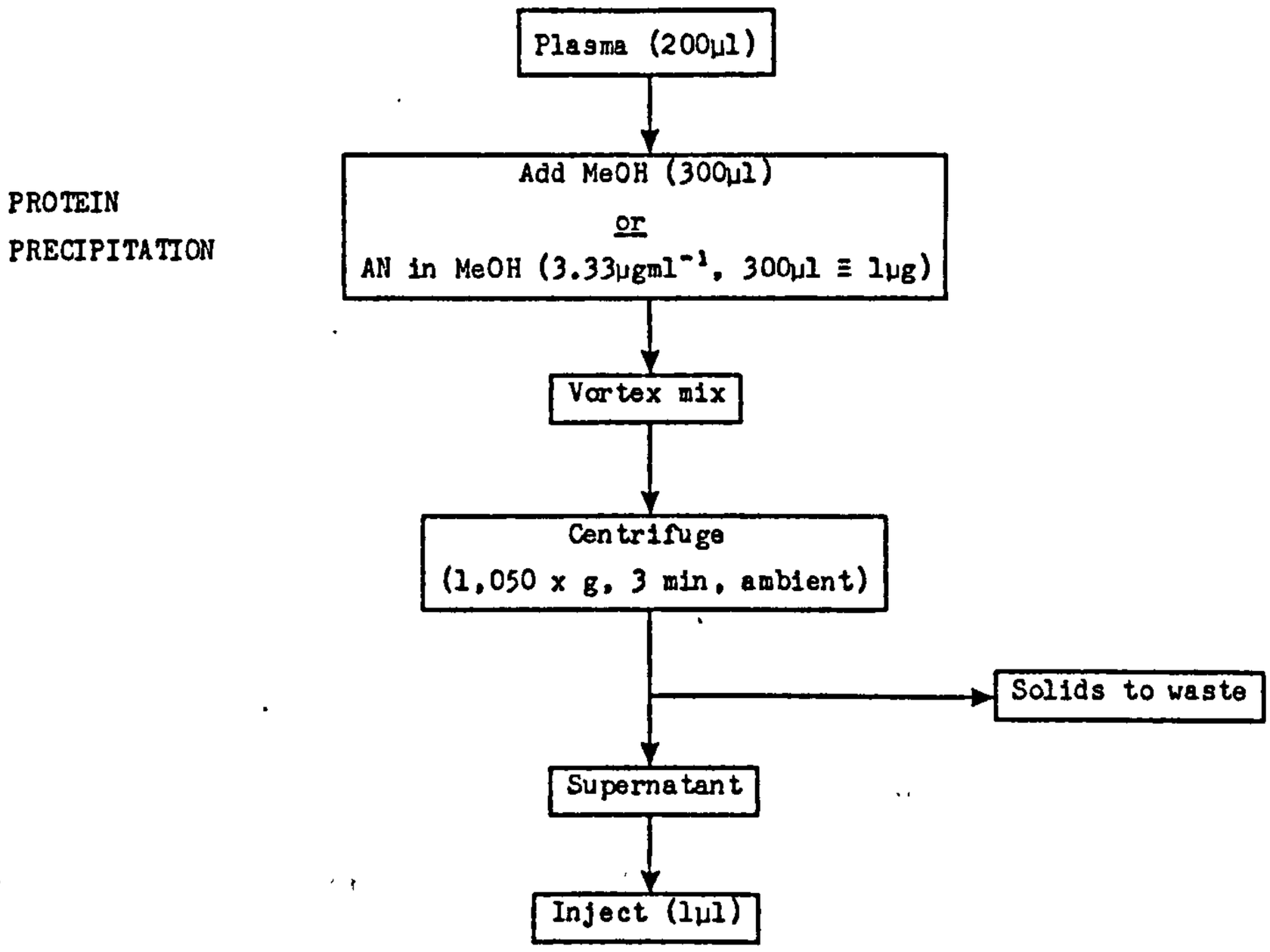


Figure 3.72 Extraction Scheme 2



Experiment 3

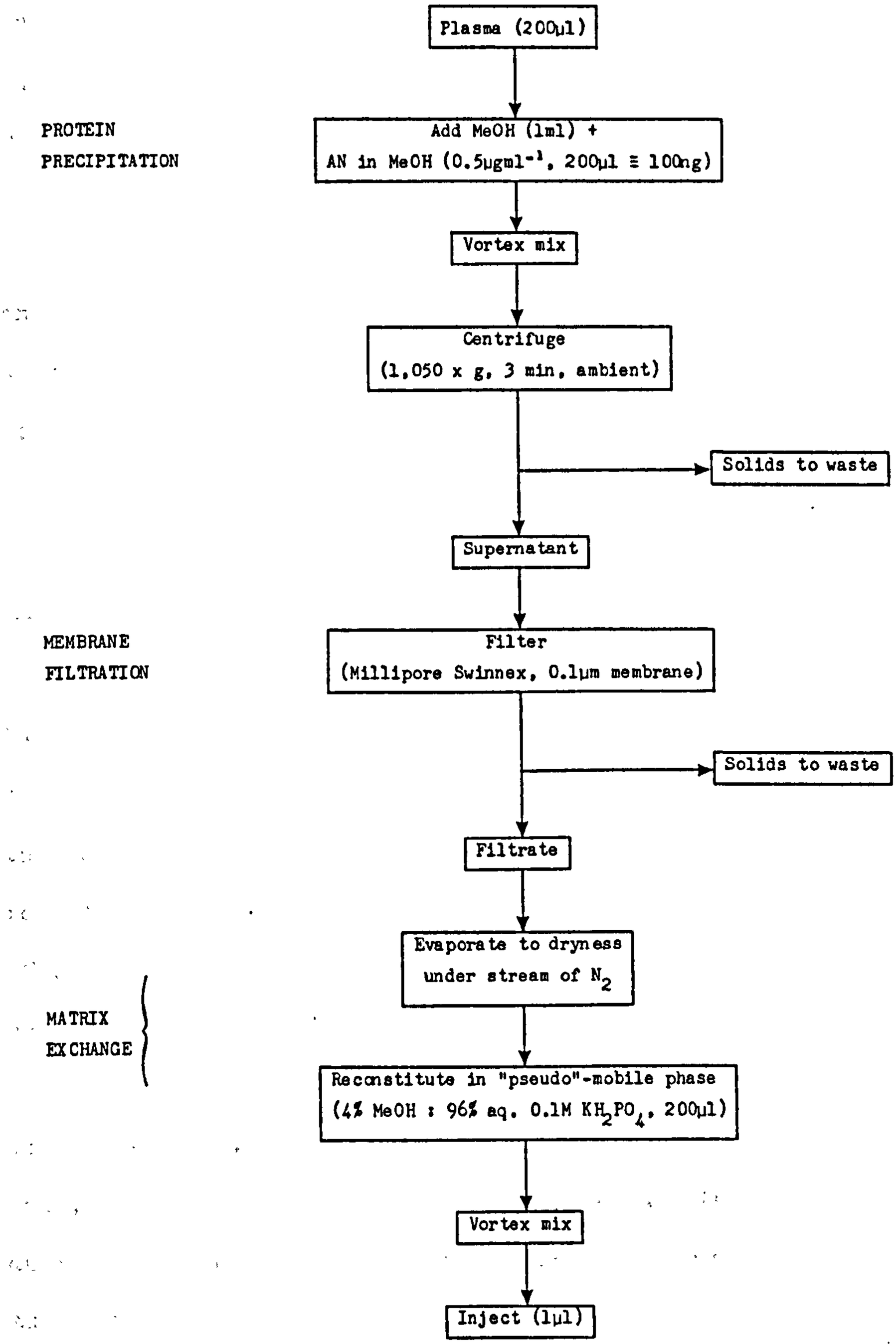
Two further aliquots (200µl) of plasma taken from the same source as that used in the first two experiments were transferred into plastic centrifuge tubes (3ml capacity). MeOH (1ml) and methanolic AN (0.5µgml^{-1}, 200µl ≡ 100ng) were placed in each tube. The mixtures were homogenised by vortex mixing (10-15sec) and centrifuged (Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature) to separate the precipitated material. The supernatants were taken by syringe, the needles were removed and membrane filtration units (Millipore Swinnex) were substituted. Membranes (Type HA, d_{pore} = 0.1µm) were pre-wetted with water before sample application. Filtration was achieved by applying positive back pressure behind the sample by means of the syringe plunger. The filtrate was collected in each case and these solutions were evaporated to dryness under a gentle stream of N_2. The residues were reconstituted in "pseudo"-mobile phase (200µl) and were vortex mixed (20-30sec). The resulting extract solutions were chromatographed (1µl injected).

This procedure is presented as a flow diagram in Figure 3.73.

Experiment 4

Pooled fresh plasma separated from blood taken into heparin was utilised. Two aliquots (200µl) were dispensed into plastic centrifuge tubes (3ml capacity) and one sample was spiked with an aqueous solution containing the indoles TP, 5HTP, 5HT, 5HIAA and I3S (0.5µgml^{-1} as active ingredient, 200µl ≡ 100ng each compound). Henceforth, both the spiked and the unspiked plasmas were subjected to idential manipulations. MeOH (1ml) and methanolic AN (0.5µgml^{-1}, 50µl ≡ 25ng) were added, the mixture was vortex mixed (10-15sec) and the resulting precipitate was spun down

Figure 3.73 Extraction Scheme 3



(Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature). The supernatant was removed and transferred onto a pre-activated C_{18} Sep-Pak cartridge ($Q \simeq 0.5 ml min^{-1}$) under positive syringe pressure. The effluent was collected. The cartridge was washed with MeOH (1µl, $Q \simeq 0.5 ml min^{-1}$) and the eluant was collected in combination with the original effluent. The corporate solution was evaporated to dryness under a gentle stream of N_2 and was subsequently reconstituted in "pseudo"-mobile phase (50µl) and was vortex mixed (15-20sec). NBLC-EC analysis (1µl injected) was performed of the reconstituted sample. These operations are illustrated schematically in Figure 3.74.

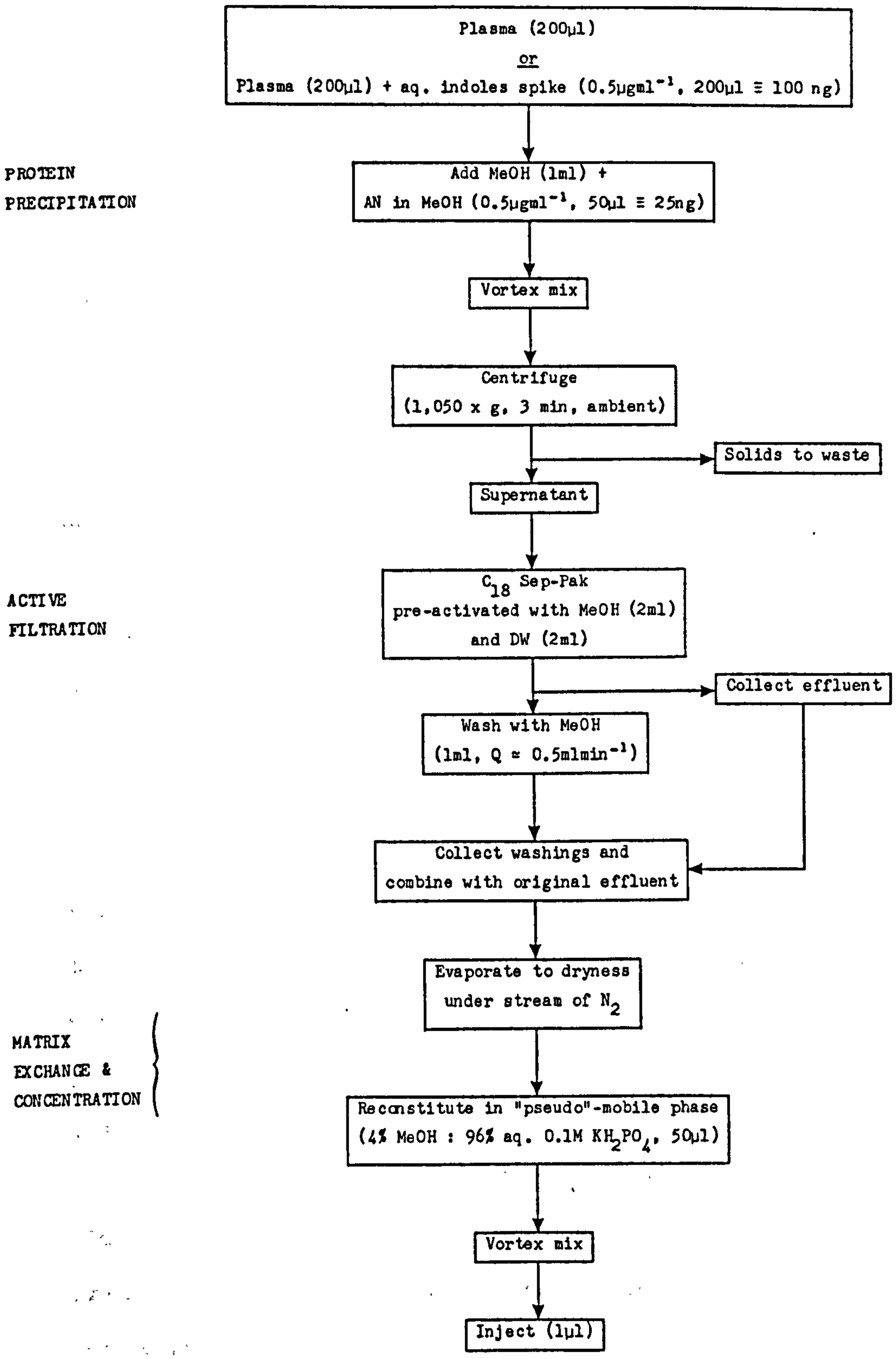
Experiment 5

Four aliquots (200µl) of the same plasma pool as was utilised for Experiment 4 were placed in plastic centrifuge tubes (3ml capacity). Two of these aliquots were spiked as detailed in the previous experiment. To one pair of samples (spiked and unspiked), MeOH (1ml) was added and the resulting combinations were vortex mixed (10-15sec) then centrifuged (Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature). The supernatants were transferred into clean centrifuge tubes.

The remaining pair of plasma samples (spiked and unspiked) were treated with aq. $HClO_4$ (7% w/v, 0.7M, 200µl). After vortex mixing (10-15 sec), the samples were centrifuged (Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature). The supernatants were conveyed to clean centrifuge tubes and aq. KOH (0.7M, 200µl) was added. The samples were vortex mixed (10-15sec) and then allowed to stand for 5 min in a refrigerator (4 °C).

At this stage all four extracts were spiked with methanolic AN

Figure 3.74 Extraction Scheme 4



($0.5\mu g ml^{-1}$, 200µl ≡ 100ng) and were subjected to centrifugation (Gallenkamp AM3G, 1,050 x g, 3 min, ambient temperature). Supernatants were removed and processed through an identical concluding work-up procedure to that described for the previous experiment, i.e., C_{18} Sep-Pak extraction, evaporation and reconstitution. Each extract was chromatographed (1µl injected).

A schematic representation of this experimental approach is given in Figure 3.75.

Experiment 6

Four plasma fractions obtained from a freshly taken blood sample were extracted by the following procedure. All actions prior to the Sep-Pak cartridge procedure were conducted in a cold room (ca. 4 °C). MeOH (1.5ml) and methanolic BF ($0.11\mu g ml^{-1}$, 100µl ≡ 11ng) were added to an aliquot of plasma (500µl) housed in a centrifuge tube (5ml capacity) which was kept on ice. The contents of the tube were vortex mixed (15-20sec) and subsequently centrifuged (MSE Mistral 6L, 1,700 x g, 15 min, 4 °C) in order to spin down the precipitate. The supernatant was removed and applied to a pre-activated C_{18} Sep-Pak cartridge. The effluent was collected and placed on ice. The extraction cartridge was washed first with DW (1ml, $Q \simeq 0.5 ml min^{-1}$) then with MeOH (1ml, $Q \simeq 0.5 ml min^{-1}$). The eluates from each wash were received in two further identical glass tubes and were also placed on ice. All extracts were freeze-dried. On completion of the drying process, the residue from each extract was redissolved in "pseudo"-mobile phase (50µl) and was vortex mixed (20-30sec). Injections (5µl) were made into the NBLC system at various instrument sensitivity settings.

This entire procedure is depicted in flow diagram format in Figure 3.76.

Figure 3.75 Extraction Scheme 5

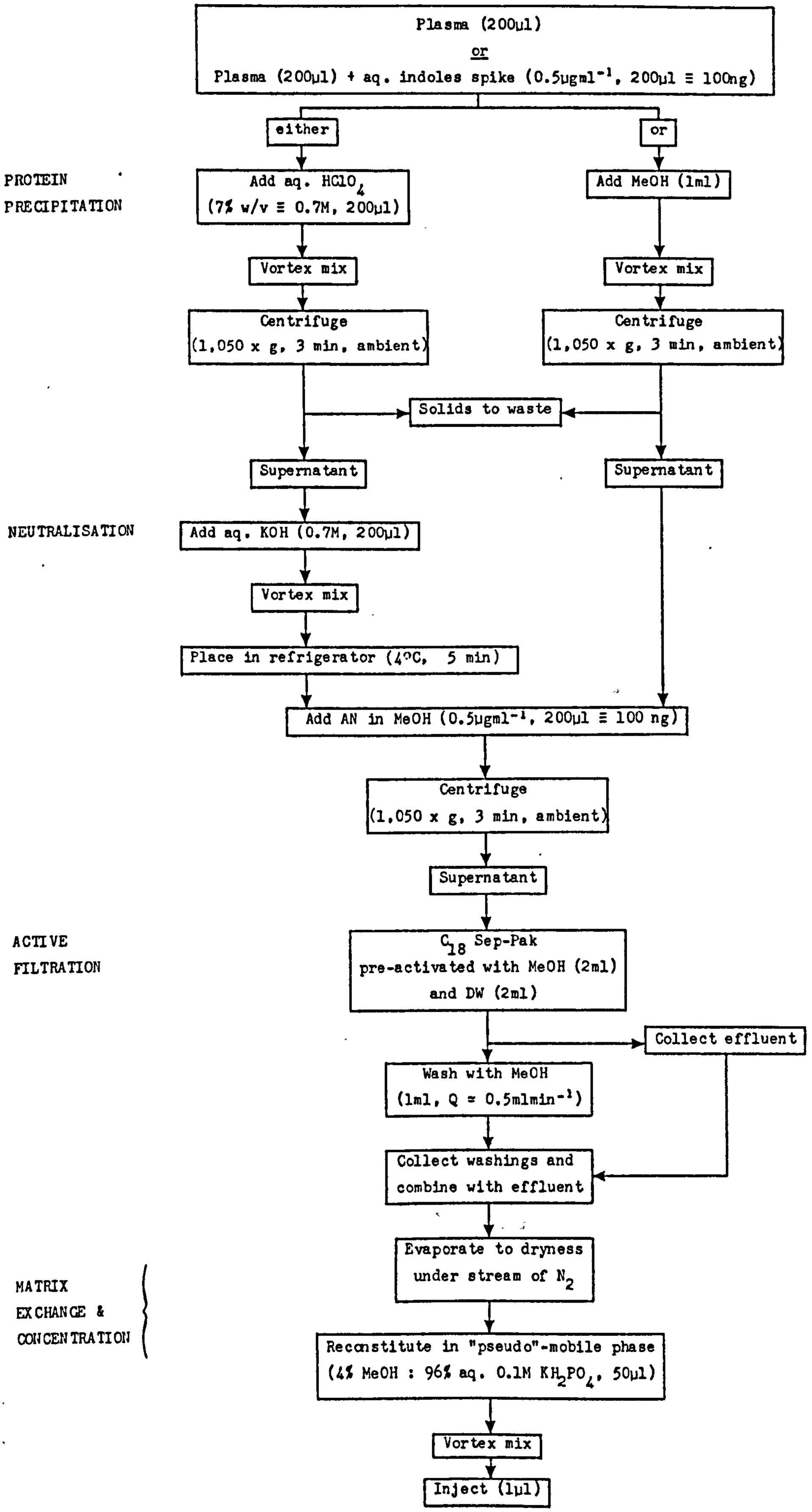
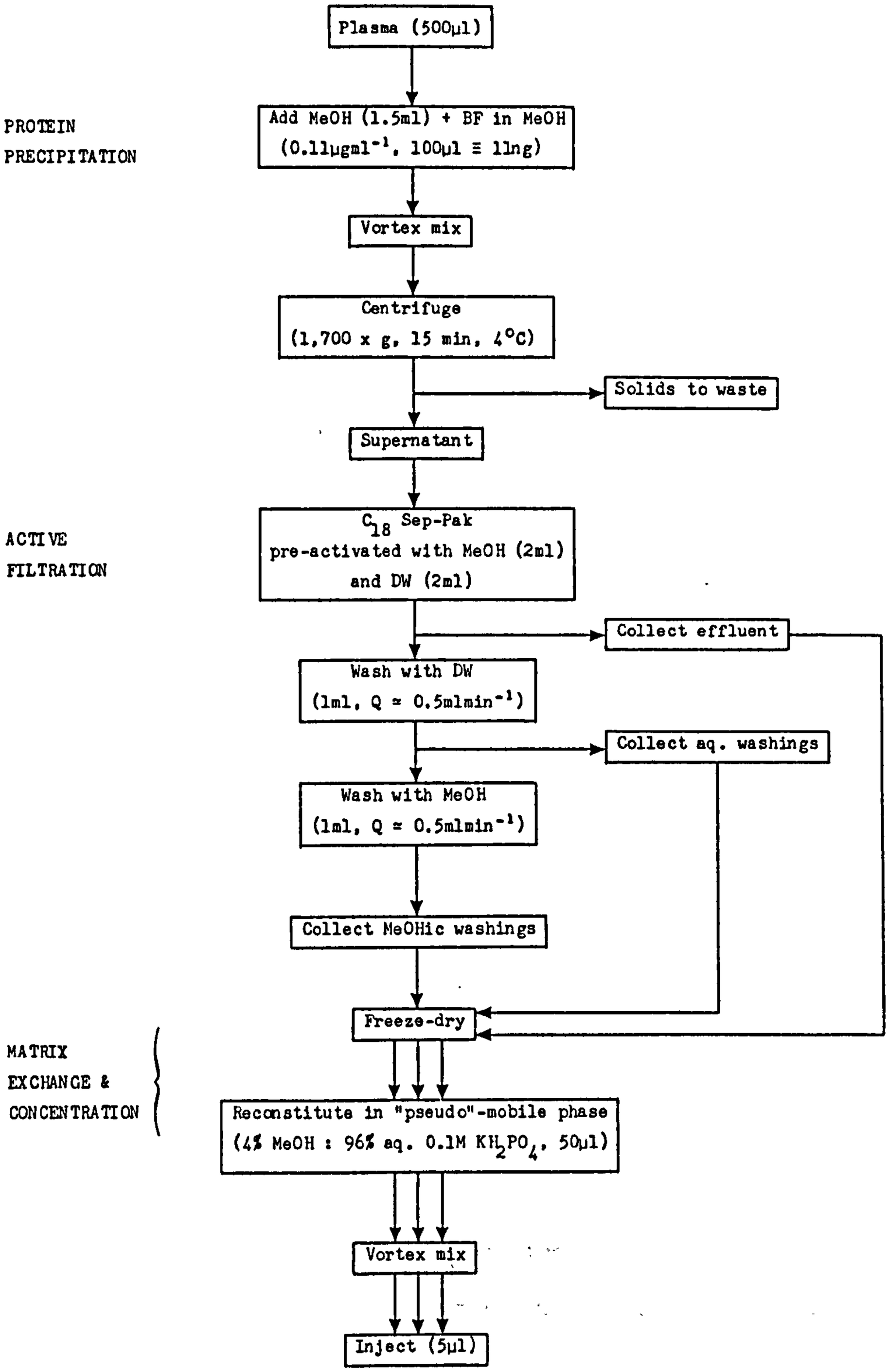


Figure 3.76 Extraction Scheme 6



3.9 Development of a Clean-Up Procedure

3.9.1 Early Qualitative Experiments

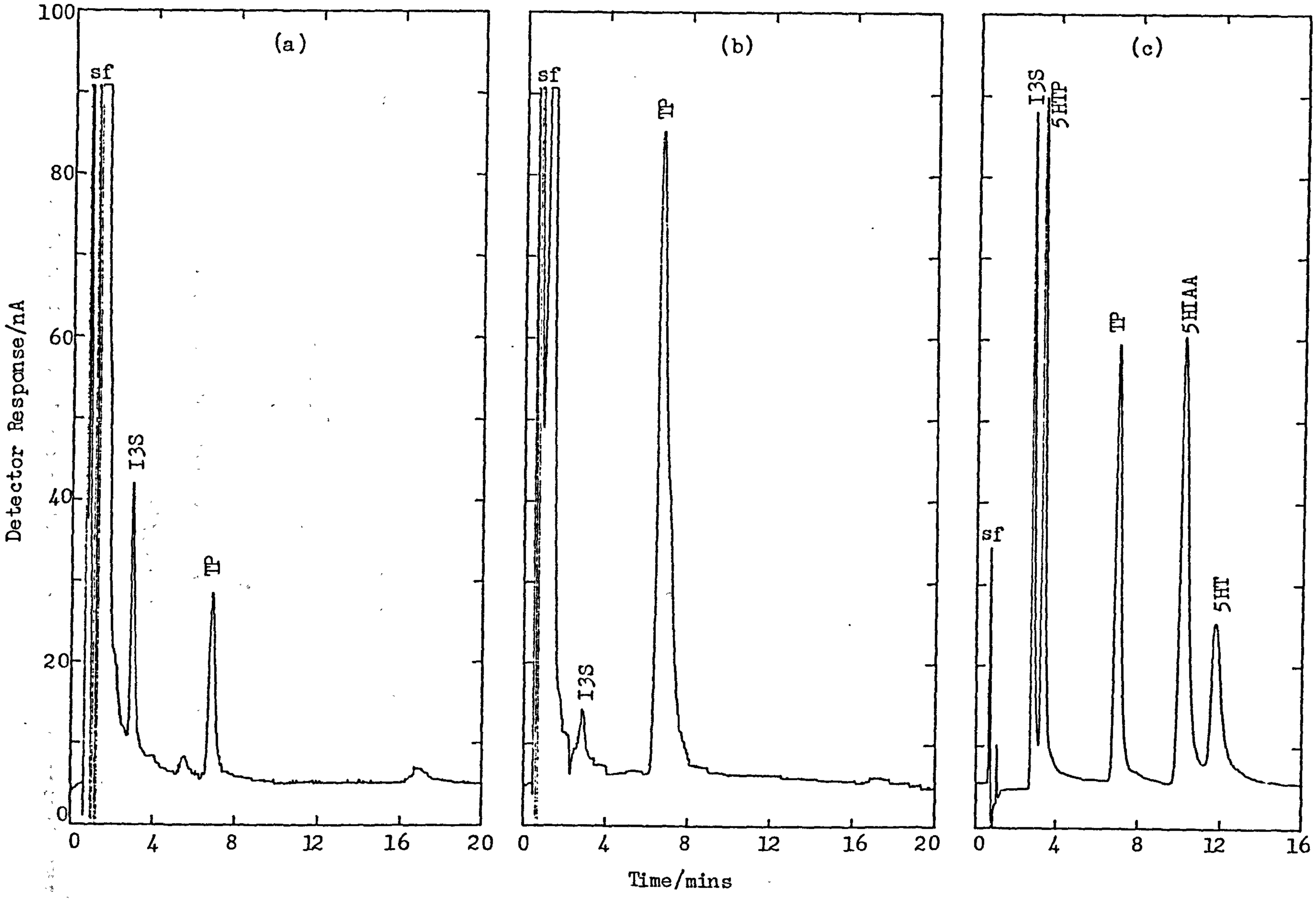
Using plasma separated from a blood sample taken into citrate-phosphate-dextrose-adenine the efficacy of a simple active filtration preparation procedure was investigated. The principle applied to the elimination of protein was that if the plasma sample was eluted through a pre-column, the protein would be irreversibly adsorbed onto the stationary phase surface so yielding a protein-free sample which poses no threat to the analytical column. There are several brands of small disposable solid-phase extraction cartridge currently on the market which may perform this function; examples include Sep-Pak (Waters Associates, Northwich, Cheshire) and Bond-Elut (Analytichem International, supplied in the UK by Jones Chromatography, Llanbradach, Mid-Glamorgan). These cartridges consist of a coarse grade (d_p = 20-30μm) silica or bonded-silica phase retained between two sieves and within a moulded plastic case. A wide variety of stationary phase chemistries are available in this form. C_{18} Sep-Paks (ODS-filled) were immediately accessible therefore these cartridges were applied to the current problem.

Plasma was treated according to Extraction Scheme 1 (Figure 3.71). Chromatograms were recorded of the plasma effluent (Figure 3.77(a)) and the methanolic eluate (Figure 3.77(b)). These traces were compared with a chromatogram of a standard indole mixture run under identical conditions (Figure 3.77(c)).

At the moderate instrument sensitivity settings employed for this work two peaks corresponding to indoles were observed in both the plasma effluent and the methanolic washings. These peaks were tentatively

Figure 3.77 Chromatograms Resulting from Plasma Extraction According to Scheme 1 (Figure 3.71)

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (E_{app} = +1.00V *vs.* Ag/AgCl, TC = 1sec) and Sample : 1µl *via* loop of (a) effluent from plasma introduction on to a Sep-Pak, (b) MeOH eluate from a Sep-Pak, and (c) a standard solution containing TP, 5HTP, 5HT, 5HIAA and I3S



assigned to TP and I3S. Their occurrence in both media could have been the result of insufficient capacity of the extraction cartridge for such a large sample or employment of too rapid an elution rate during plasma introduction on to the Sep-Pak or even low partition ratios under the experimental conditions.

There were no discernible peaks corresponding to the 5-hydroxy indoles. This may have been because these substances occur at concentrations too low to produce a noticable peak at these instrument sensitivities. Alternatively, the 5-hydroxy indoles may have reacted or decomposed within the sample prior to analysis or they may simply have been retained on the extraction cartridge. Considering the noted elution behaviour of these compounds from ODS stationary phases employed for HPLC this last possibility is highly unlikely.

Experimental difficulties were encountered with the attempted extraction. On addition of MeOH to the cartridge a white precipitate formed, presumably due to precipitation of protein and, perhaps, inorganic salts. This precipitate tended to clog the extraction cartridge so rendering further elution extremely difficult. Consequently, active filtration alone was considered to be ineffective for protein elimination.

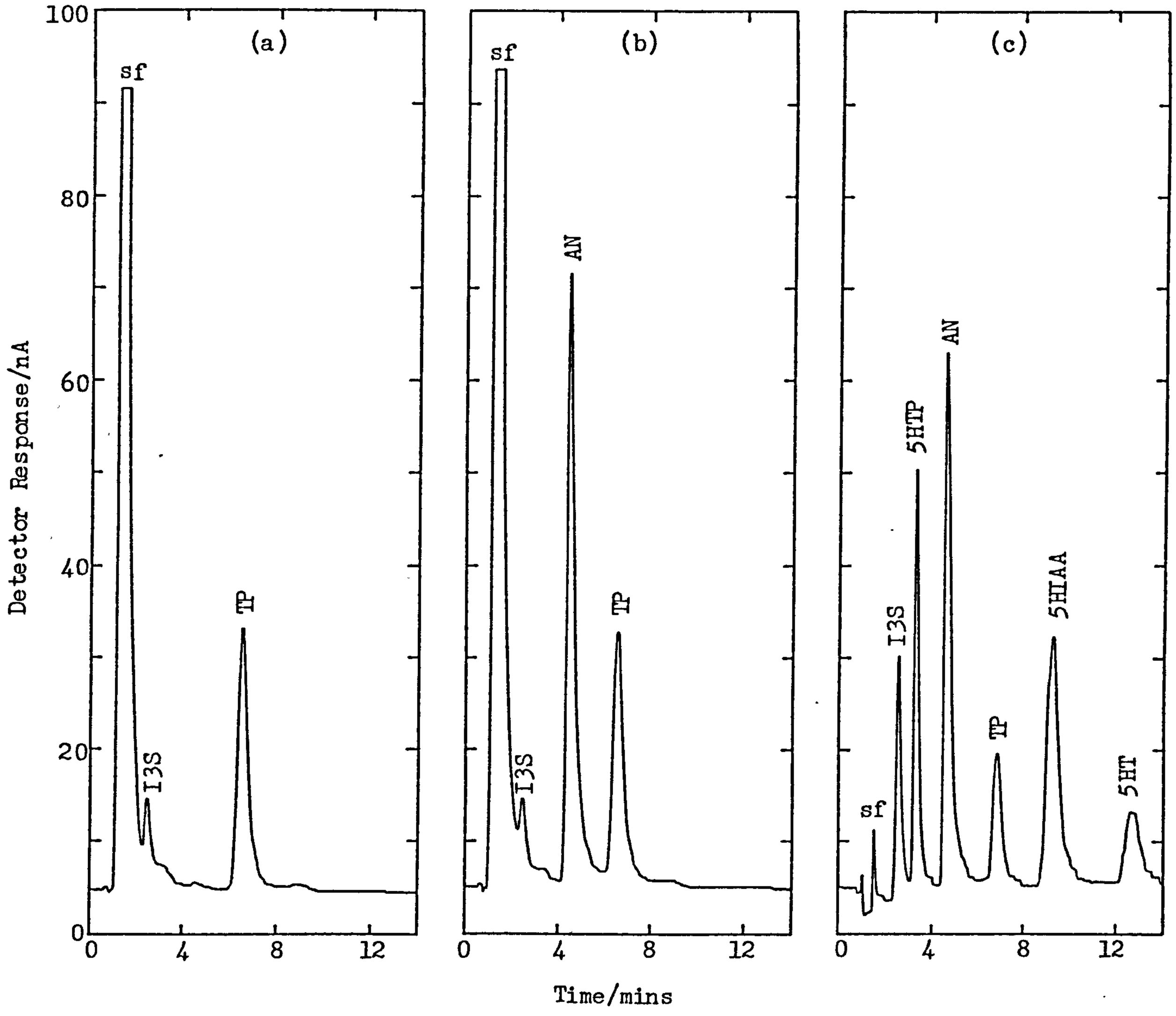
3.9.2 Experiments Incorporating Aniline as an Internal Standard

3.9.2.1 Protein Precipitation with MeOH

Figures 3.78(a) and (b) show chromatograms of supernatants obtained from plasma samples which were deproteinised with MeOH and subsequently centrifuged, according to Extraction Scheme 2 (Figure 3.72). Figure 3.78(a) corresponds to unadulterated plasma while Figure 3.78(b) is representative of plasma with the addition of AN at the precipitation stage. A chromatogram

Figure 3.78 Chromatograms Resulting from Plasma Extraction According to Scheme 2 (Figure 3.72)

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (E_{app} = +1.00V vs. Ag/AgCl, TC = 1sec) and Sample : 1µl via loop of (a) a plasma extract, (b) a plasma extract containing AN and (c) a standard solution containing TP, 5HTP, 5HT, 5HIAA I3S and AN



of a standard indole mixture run under identical conditions is included for comparison (Figure 3.78(c)).

Chromatograms of similar format to Figures 3.77(a) and (b) were afforded, peaks tentatively assigned to TP and I3S being observed but no peaks corresponding to the 5-hydroxy indoles were detectable at the currently selected instrument sensitivity. AN eluted in an otherwise unoccupied area of the chromatogram. Instrument problems yielding high background noise prevented the utilisation of higher sensitivity settings at this time.

The supernatant obtained from the centrifugation was observed to be slightly turbid. The importance of effecting complete removal of suspended solids from the sample, especially prior to NBLC analysis, cannot be emphasised highly enough. Hence, the need to improve centrifugation or introduce a second method for the exclusion of particulate matter was recognised. The severe limitations of the only centrifuge for use with small samples that was available to the author at this time (i.e., fixed rotation speed, manually operated spring-loaded switch) prompted inclusion of a filtration step in the procedure. Further refinements were also introduced at this juncture.

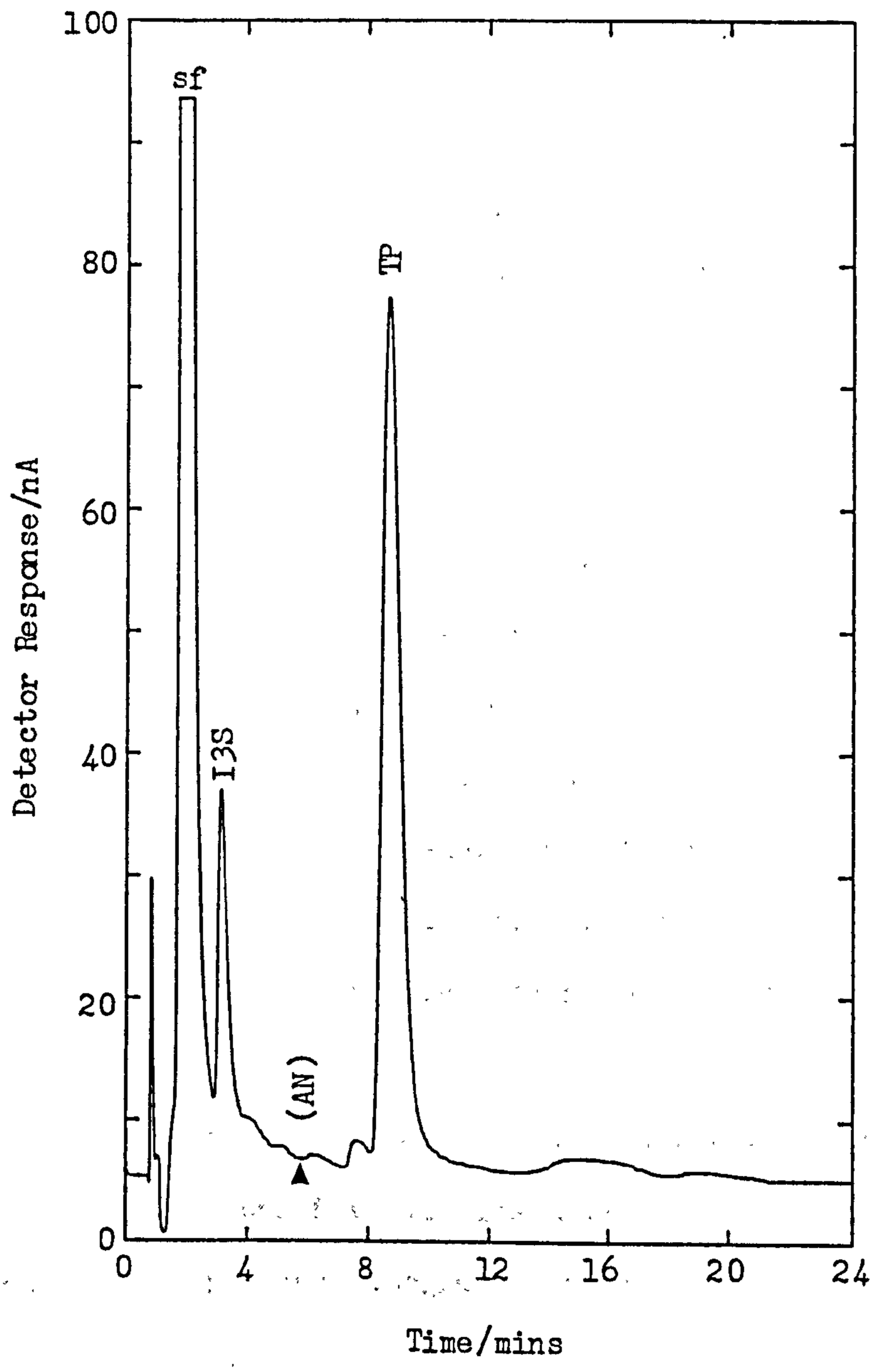
3.9.2.2 Experiment Combining Protein Precipitation and Membrane Filtration

Identical plasma samples to those employed to generate Figures 3.78 (a) and (b) were processed according to Extraction Scheme 3 (Figure 3.73). Deproteinisation with 5 volumes of MeOH (see Blanchard[435]) was effected.

This procedure resulted in a chromatogram (Figure 3.79) which is very similar to that depicted in Figure 3.78(a), i.e., peaks corresponding to TP and I3S were observed but the expected AN peak was absent. Thus, the IS

Figure 3.79 Chromatogram Resulting from Plasma Extraction According to Scheme 3 (Figure 3.73)

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (E_{app} = +1.00V vs. Ag/AgCl, TC = 1sec) and Sample : 1µl via loop of a plasma extract containing AN



was ajudged to have been "lost" in the filtration step, probably by adsorption on to the membrane since AN is not particularly volatile (b.p. = 184 °C). Another problem also existed, namely that great difficulty was experienced in applying sufficient back pressure to the syringe to force the methanolic plasma supernatant through the filtration membrane. Additional experiments employing this method of clean-up were similarly afflicted to such a degree that on occasions filtration could not be performed successfully. Hence, attempts at filtration using this system were discontinued.

3.9.2.3 Experiment Combining Protein Precipitation and Active Filtration

The clean-up procedure investigated previously (Scheme 3, Figure 3.73) was adapted because of the physical difficulties experienced in filtering through a membrane. An active filtration step similar to that first examined was substituted in place of membrane filtration. The adopted approach is illustrated in Figure 3.74 (Scheme 4).

Two important changes were made to previously employed schemes. First, the plasma effluent and washings from the extraction cartridge were combined prior to evaporation. Secondly, the final volume of solvent utilised to redissolve the resultant residues was restricted to 50µl. This represented a four fold concentration step hence the amount of AN incorporated was reduced accordingly. A final volume of 50µl was the smallest volume considered to be feasible within the limitations of available laboratory equipment.

Two aliquots of a recently prepared plasma pool were extracted, one of which was first spiked with the five indoles under study (100ng each compound). Their respective chromatograms (1µl injected) are presented in

Figures 3.80(a) and (b), along with a typical chromatogram of an indole standard mixture (Figure 3.80(c)) for comparison.

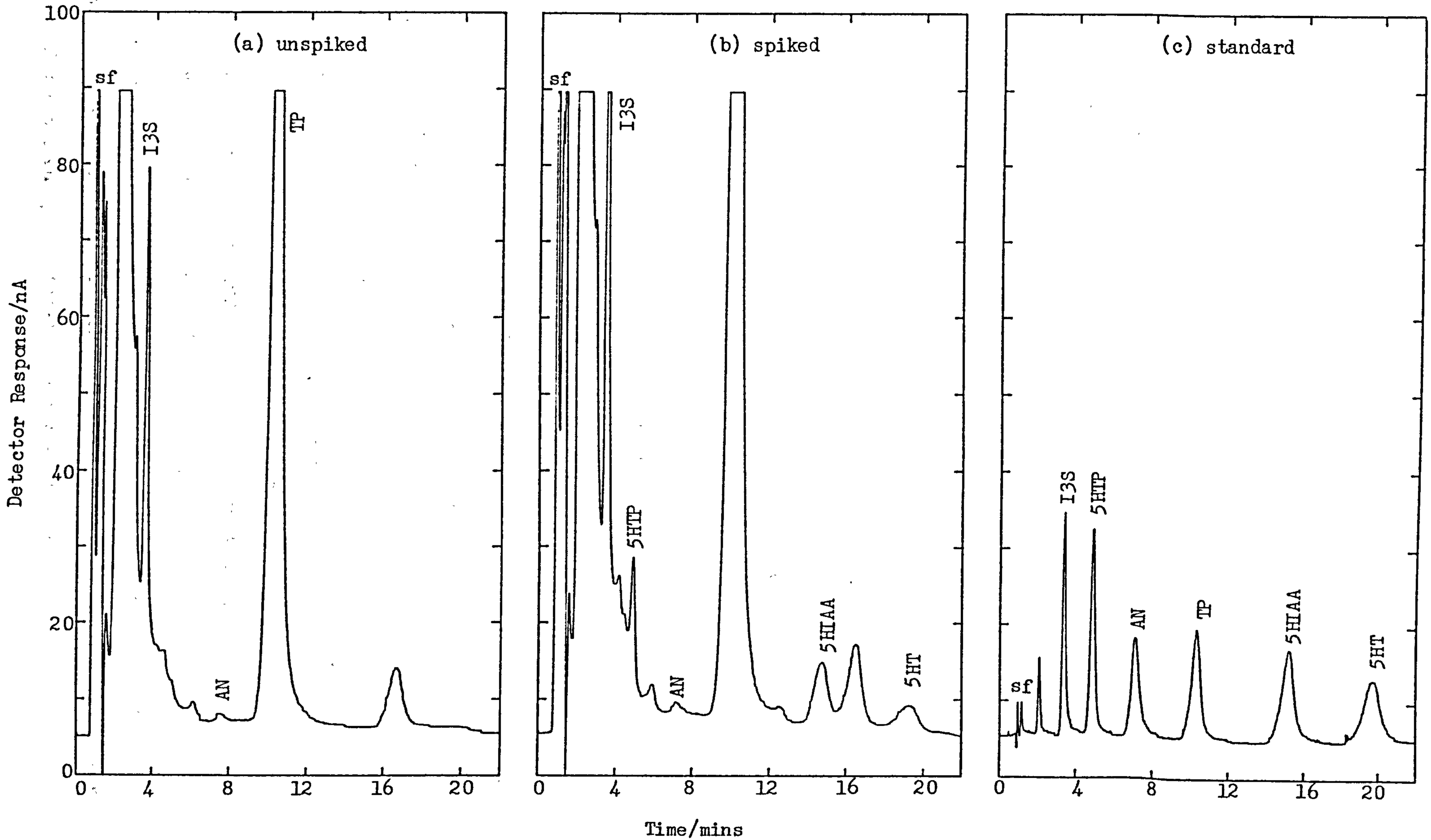
The trace obtained from unspiked plasma contains a very large peak corresponding to TP ($k' \simeq 11.8$) and also one corresponding to I3S ($k' = 3.4$). No peak due to a 5-hydroxy indole was observed. In the chromatogram of the spiked plasma sample small peaks due to 5HTP, 5HT and 5HIAA (with capacity factors of 5.1, 23.0 and 17.3, respectively) were identified and, in addition, the peaks corresponding to TP and I3S increased in size. Evidently insufficient sensitivity was demonstrated for detection of the 5-hydroxy indoles at endogenous concentrations under the conditions and procedures utilised. Increase of instrument sensitivity is necessary (e.g., 3nA f.s.d.) but noise levels become unacceptable.

It is evident that AN is poorly extracted by this clean-up method, with only a very small peak eluting at the relevant point in the chromatograms. Such behaviour necessarily determines that AN is unsatisfactory as an IS where an extraction procedure of this general ilk is employed. However, in view of its chromatographic properties it may have limited use as a CS.

During the course of this experiment, instrument troubles continued to surface, particularly with regard to background noise level which, of course, affected overall sensitivity. A full discussion of these problems is presented in Chapter 4. In addition to the NBLC apparatus, the column exhibited poor stability. The necessity of frequent column repacking was great because of intolerable pressure increases and the creation of voids in the stationary phase bed. These effects are characteristic of progressive column blockage which, in this instance, was suspected to have been caused by residual protein in the injected sample.

Figure 3.80 Chromatograms Resulting from Plasma Extraction According to Scheme 4 (Figure 3.74)

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (E_{app} = +1.00V vs. Ag/AgCl, TC = 1sec) and Sample : 1µl via loop of (a) an extract of unspiked plasma containing AN, (b) an extract of spiked plasma (TP, 5HTP, 5HT, 5HIAA and I3S added, 100ng of each) containing AN and (c) a standard solution containing TP, 5HTP, 5HT, 5HIAA, I3S and AN



3.9.2.4 Comparison of Two Commonly Employed Protein Precipitants

In view of the suspicion aroused regarding incomplete deposition of plasma proteins by treatment with MeOH, an experiment was conducted in order to ascertain whether the use of $HClO_4$ would be preferable, in spite of the reservations expressed previously regarding its possible detrimental effects on analyte levels (see Section 3.7.4). Using identical plasma samples to those employed for the previous extractions, two experiments were conducted in parallel. Four plasma aliquots were utilised, two of which were spiked with TP, 5HTP, 5HT, 5HIAA and I3S. One spiked sample and one unspiked sample were taken through the procedure in Scheme 4, Figure 3.74 with the exception that the IS was added to the supernatant prior to active filtration. The second pair of plasma samples were treated similarly except instead of MeOH addition, vortex mixing and subsequent centrifugation, protein was eliminated by $HClO_4$ treatment, vortex mixing and centrifugation. Where $HClO_4$ had been utilised, an additional series of steps were included in order to neutralise the highly acid environment with KOH. Figure 3.75 (Scheme 5) depicts these extractions.

Chromatograms (1µl injected) were recorded of the four extracts and are presented in Figures 3.81 and 3.82. The former Figure contains traces obtained after protein precipitation with MeOH while the latter shows the results of $HClO_4$/KOH treatment.

Figures 3.81(a) and (b) are very similar to Figures 3.80(a) and (b), as was to be expected since identical samples were prepared for chromatography by procedures which differed only in one very minor detail. Peaks tentatively assigned to TP and I3S were present and a very small peak possibly due to AN was noted in the unspiked extract. In the spiked extract the 5-hydroxy indoles were observed in addition, but any signal due to the IS was masked by electro-oxidised co-extractives.

Figure 3.81 *Chromatograms Resulting from Plasma Extraction According to Scheme 5 (Figure 3.75) Employing NaOH as Protein Precipitant*

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (Eapp = +1.00V vs. Ag/AgCl, TC = 1sec) and Sample : 1µl via loop of (a) an extract of unspiked plasma containing AN and (b) an extract of spiked plasma (TP, 5HTP, 5HT, 5HIAA and I3S added, 100ng of each) containing AN

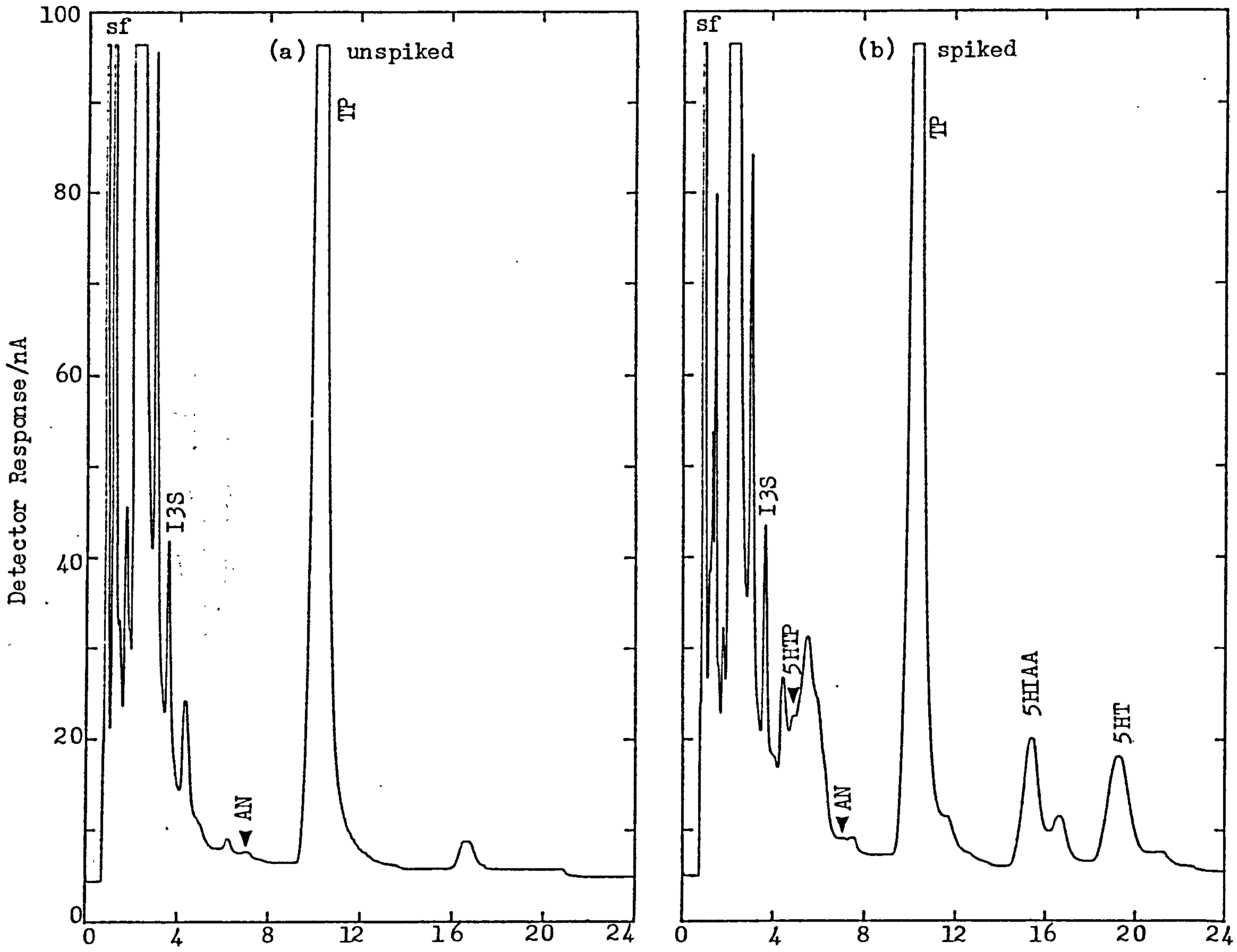
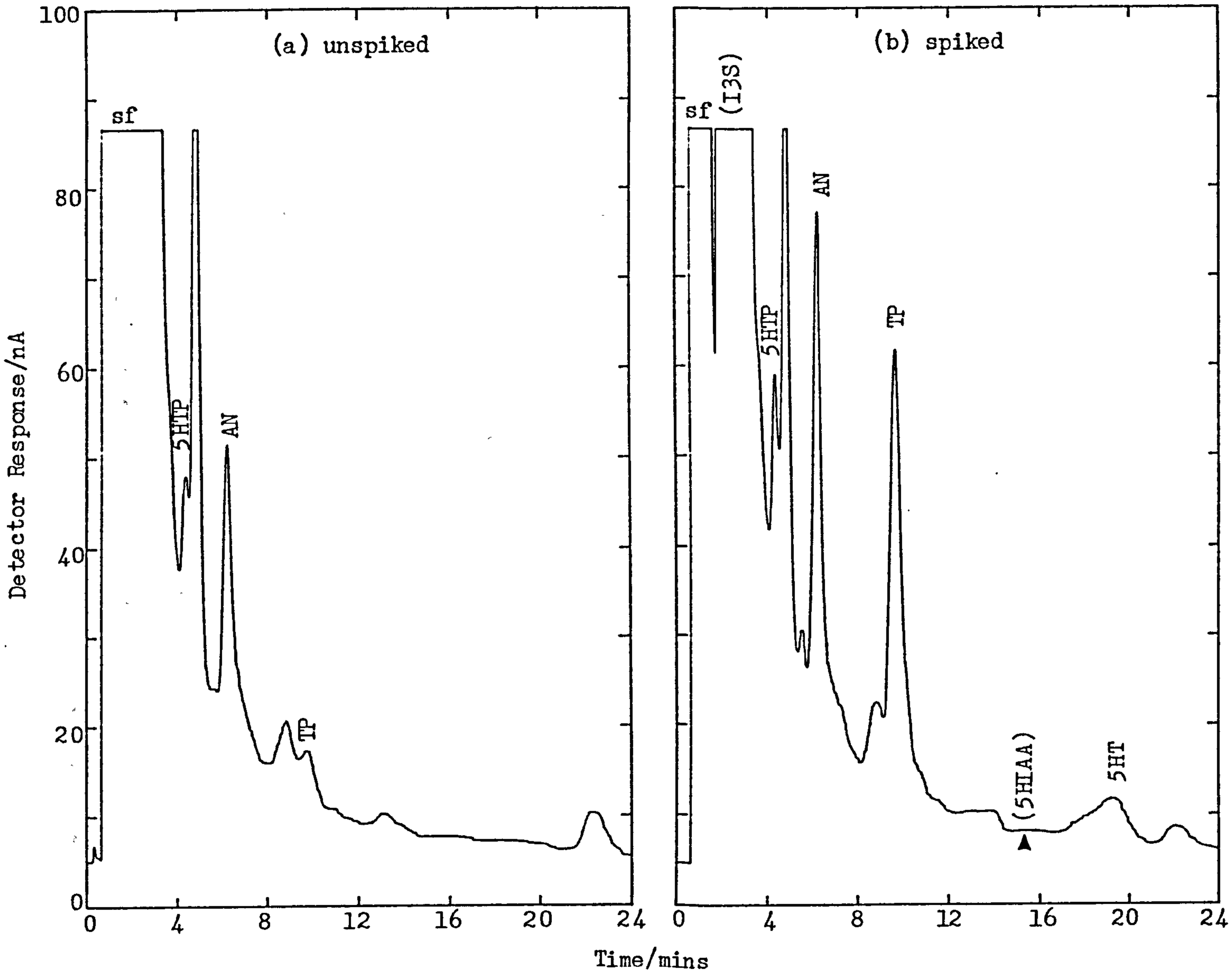


Figure 3.82 *Chromatograms Resulting from Plasma Extraction According to Scheme 5 (Figure 3.75) Employing $HClO_4$ as Protein Precipitant*

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (Eapp = +1.00V vs. Ag/AgCl, TC = 1sec) and Sample : 1µl via loop of (a) an extract of unspiked plasma containing AN and (b) an extract of spiked plasma (TP, 5HTP, 5HT, 5HIAA and I3S added, 100ng of each) containing AN



In contrast to the above findings, the $HClO_4$/KOH based procedure produced very different chromatograms. Unspiked plasma gave rise to peaks corresponding to the amino-acids TP and 5HTP and a substantial peak due to AN (assigned on the basis of retention compared with standards chromatographed under identical conditions). No peaks were identified as originating from the acids (5HIAA and I3S) or the amine (5HT). In the spiked plasma chromatograms (Figure 3.81(b)) the TP and 5HTP signal intensities increased and, in addition, a peak of very poor shape (broad and fronting) was obtained for 5HT. Neither acid metabolite was distinguishable in this trace at the instrument sensitivity employed.

The use of $HClO_4$, with KOH neutralisation, as a protein precipitant appears to be very detrimental to the extraction of the indoles generally. Although extraction efficiencies of 5HTP and AN were enhanced (assuming peak purity), that of TP was greatly inhibited (cf. Figure 3.81(a) and 3.82(a)). Furthermore, the acidic metabolites were not extracted to any discernible degree, even when the plasma sample was spiked. The reasons for this behaviour probably lie in part with the lability of some of the analytes in the strongly acid conditions and also possibly with the variability of speciation of the indoles under conditions of largely unknown and uncontrolled pH. Since "neutralisation" with aqueous KOH was only carried out on a very crude equimolar basis without pH monitoring (because of the difficulties entailed in being able to measure the pH of a small sample without suffering loss of sample), the likelihood of the latter possibility was quite high. Comparing the chromatograms obtained via treatment with the two precipitants, the use of MeOH is preferred on current evidence although further studies of the $HClO_4$/KOH combination with pH control introduced were called for to ascertain the latter's full potential.

During the course of this experiment practical problems with both the column and the instrument continued to occur. In addition, one difficulty arose regarding the inability to evaporate to dryness under a gentle stream of N_2 the substantially aqueous solutions yielded from active filtration. The use of a freeze-drying technique could perhaps alleviate this problem and also, as a consequence, provide improved sample stability through maintenance of low temperature.

3.9.3 Experiments Incorporating Bufotenine as an Internal Standard

The difficulty experienced in extracting AN in conjunction with TP and its indolic metabolites prompted the investigation of another substance more chemically similar to the analytes to function as an IS. BF was selected in this regard (see Section 3.4.8). The fractionation of blood samples was studied concurrently with regard to the preparation of platelet-rich and platelet-poor plasma for indole analysis.

Blood which had been taken into heparin was immediately treated with ascorbic acid preservative (125ng per ml of blood). This was in accordance with the recommendations of Artigas et al.[355] who advocated addition of ascorbic acid in the region of 100-250ng per ml of plasma in order to fulfil the antioxidant requirement while not producing too gross a signal at the solvent front.

Fractionation of the blood to produce PRP was performed using a MSE Mistral 6L refrigerated centrifuge (BRI). This instrument was not ideal for two reasons. First, the smallest capacity tube it could accommodate was 5ml, which was far too large for application to neonate blood samples of as little as 300µl. This disadvantage also applied to the MSE HS18 centrifuge (see below). Secondly, the spin rate was not easy to control at low speed settings hence precluding the attainment of a RCF of 100 x g

recommended by Picard et al.[416] for preparation of PRP. A spin speed producing a RCF of 350 x g was used. This yielded ostensibly PRP however the platelet count was only 16 x $10^9 l^{-1}$ compared with normal counts in whole blood of 150-400 x $10^9 l^{-1}$. Consequently, over 90% of the platelets had been lost from the plasma under these centrifugation conditions.

Three aliquots of this "PRP" were subjected to further centrifugation using a MSE HS18 refrigerated centrifuge (BRI), while a fourth was retained for study. Spin rates generating RCFs of 7,300 x g (PPP1), 16,400 x g (PPP2) and 29,100 x g (PPP3) were employed. The resulting specimens exhibited platelet counts of 6 x 10^9, 5 x 10^9 and 4 x $10^9 l^{-1}$ respectively.

The four plasma samples (PRP, PPP1, PPP2 and PPP3) were all extracted following the scheme depicted in Figure 3.76 consisting of protein elimination with MeOH, with subsequent active filtration using a C_{18} Sep-Pak cartridge. The plasma effluent, water washings and MeOH washings were all collected separately and were freeze-dried on a vacuum frame. Following reconstitution, chromatograms (5µl injected) were recorded of all samples at two instrument sensitivities, i.e., 3nA and 30nA f.s.d.

It was evident from the chromatograms that all four samples, viz. PRP, PPP1, PPP2 and PPP3, yielded profiles with no significant differences. Typical illustrations are presented in Figures 3.83 and 3.84 (chromatograms recorded at 3nA and 30nA f.s.d., respectively). In addition, profiles of the deproteinised plasma effluents, the aqueous washings and the methanolic washings were all identical except in respect of peak amplitude where a progressive and sequential reduction in intensity was observed, as would reasonably be expected. Examples of this occurrence are provided in Figures 3.83 and 3.84.

Figure 3.83(a) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) - Effluent from Plasma Introduction on to a Sep-Pak Cartridge via loop of effluent from the introduction on to a C_{18} Sep-Pak Cartridge

Parameters : - For conditions see Figure 3.61 except for Sample : 5µl volume of PPP2 (500µl) containing BF (11ng)

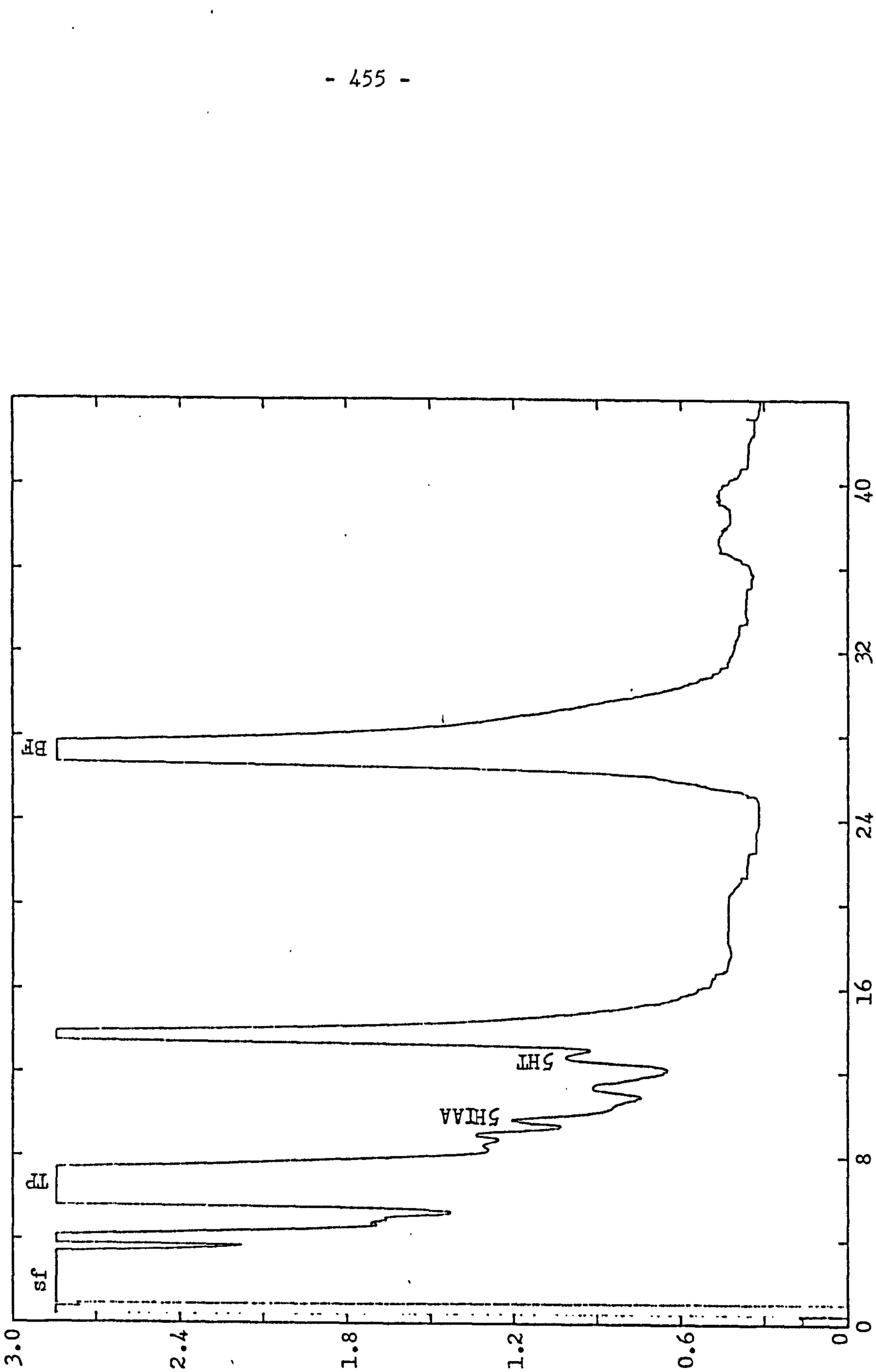


Figure 3.83(b) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) from a C_{18} Sep-Pak Cartridge

Parameters :- For conditions see Figure 3.61 except for Sample PPP2 (500µl) containing BF (6) - Aqueous Eluate : 5µl via loop of the aqueous eluate from a Sep-Pak treated with

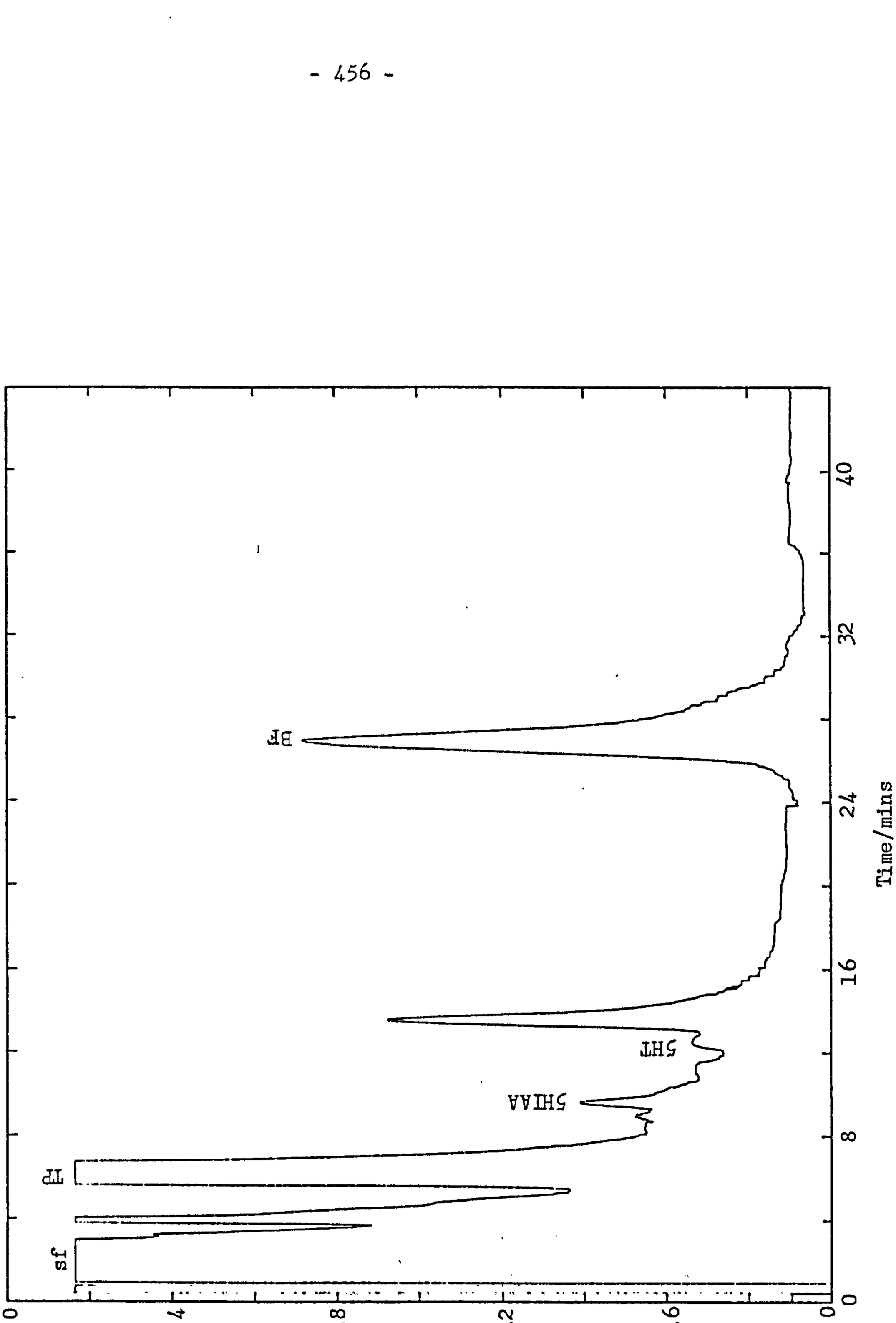


Figure 3.83(c) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) from a C_{18} Sep-Pak Cartridge - Methanolic Eluate

Parameters :- For conditions see Figure 3.61 except for Sample : 5µl via a loop of the methanolic eluate from a Sep-Pak treated with PPP2 (500µl) containing BF (11ng)

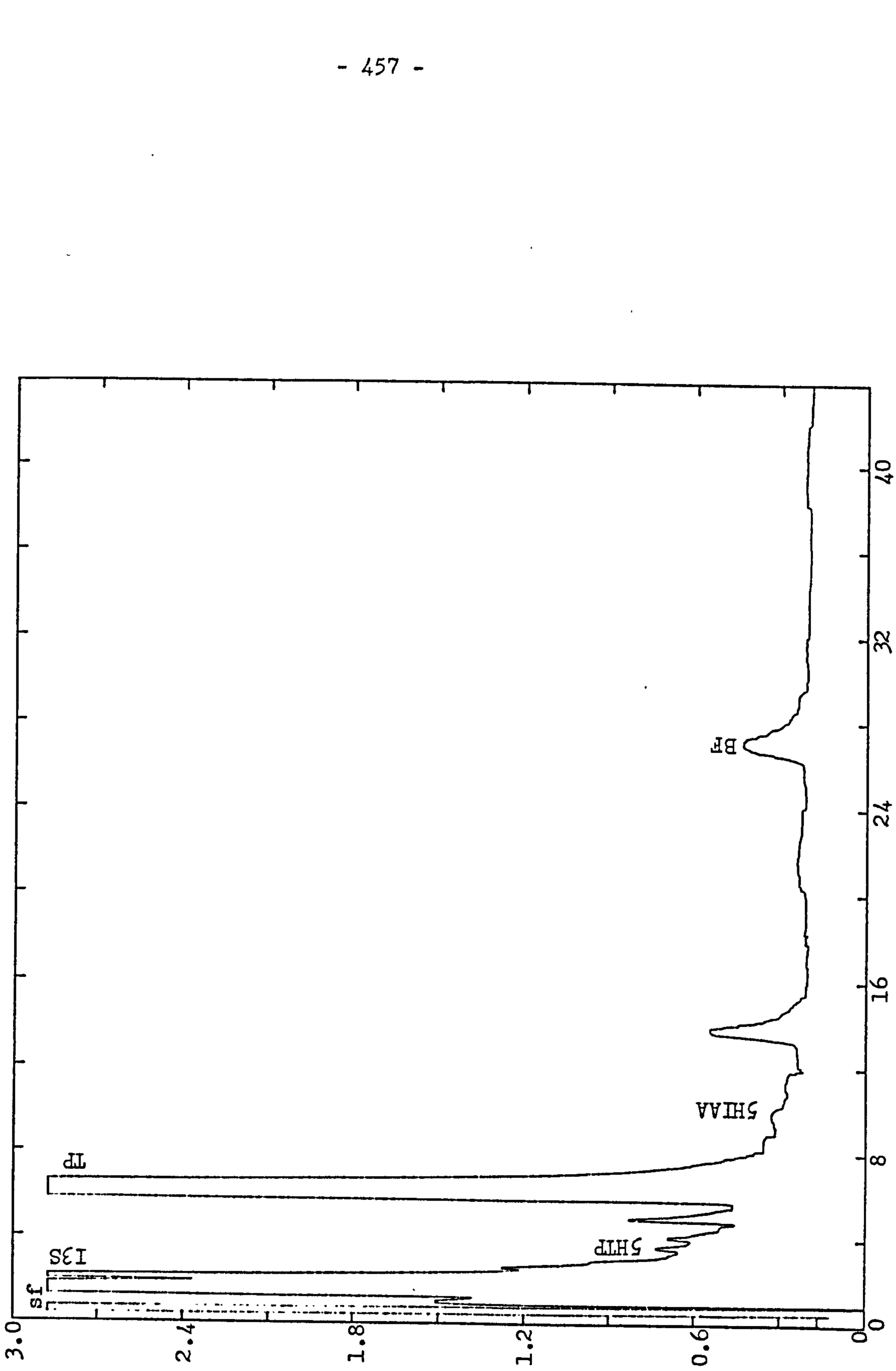


Figure 3.84(a) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) - Effluent from Plasma Introduction on to a C_{18} Sep-Pak Cartridge

Parameters :- For conditions see Figure 3.61 except for Sample : 5µl via loop of effluent from the introduction on to a Sep-Pak of PPP3 (500µl) containing BF (11ng)

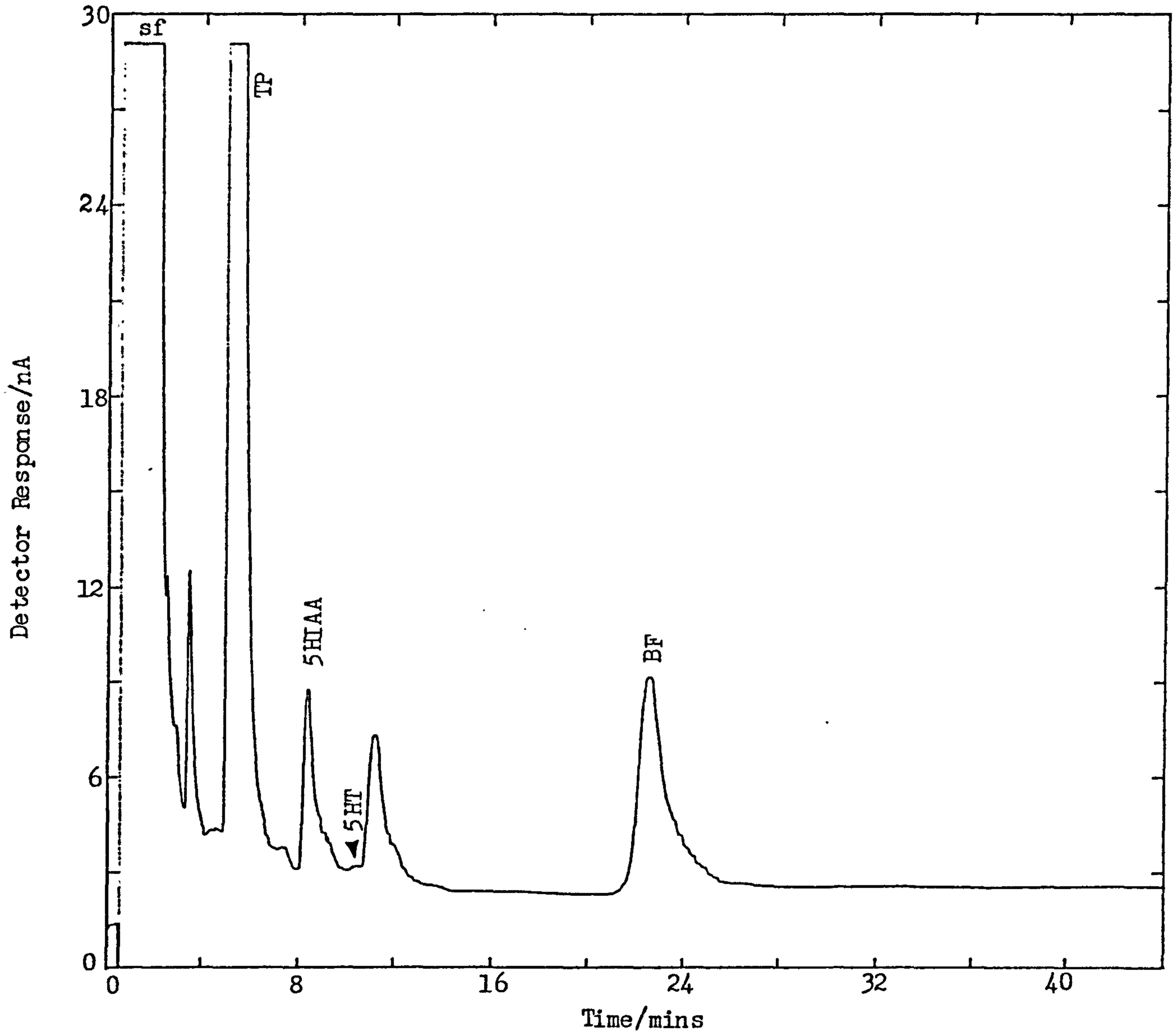


Figure 3.84(b) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) - Aqueous Eluate from a C_{18} Sep-Pak Cartridge

Parameters :- For conditions see Figure 3.61 except for Sample : 5µl via loop of the aqueous eluate from a Sep-Pak treated with PPP3 (500µl) containing BF (11ng)

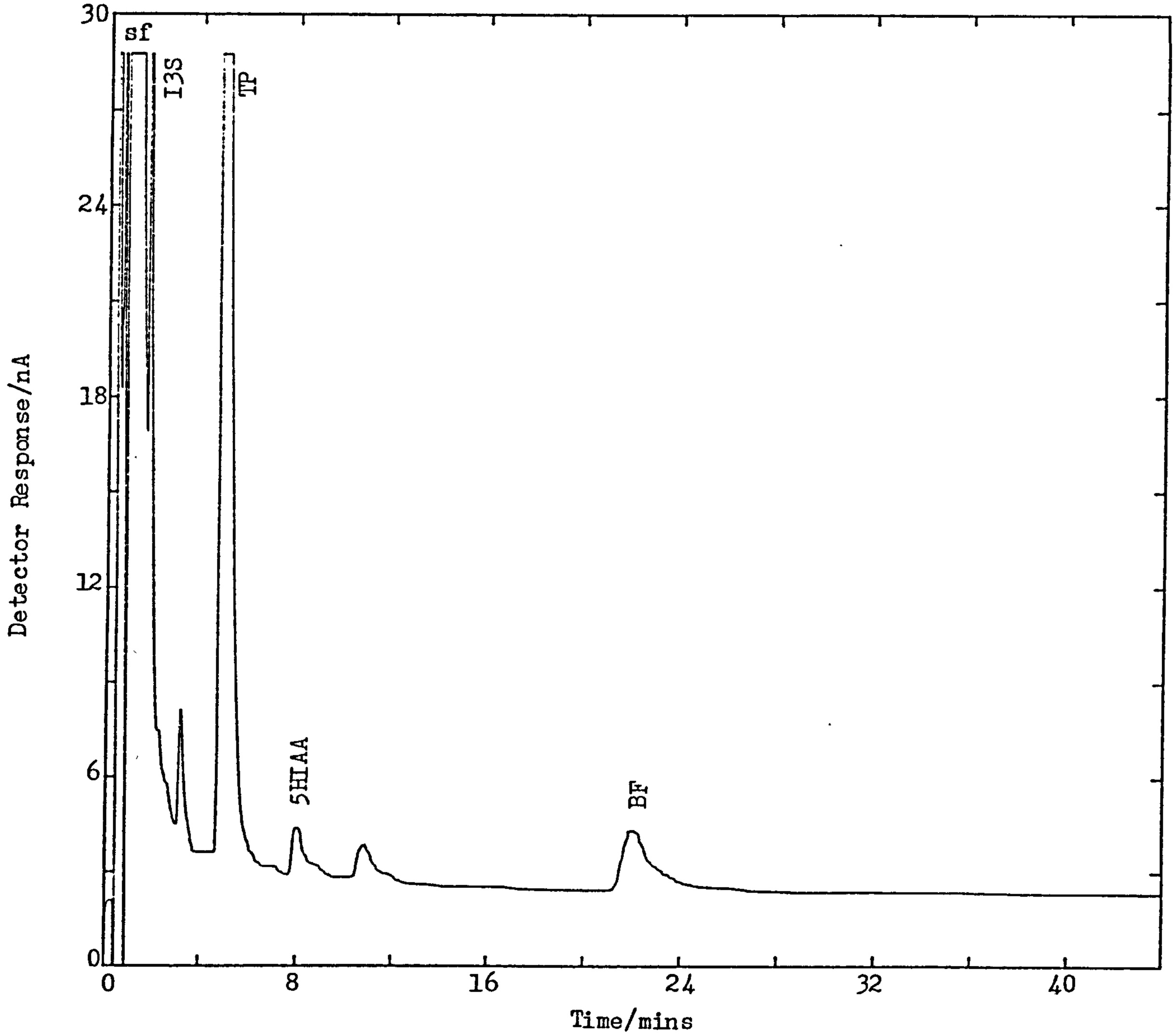
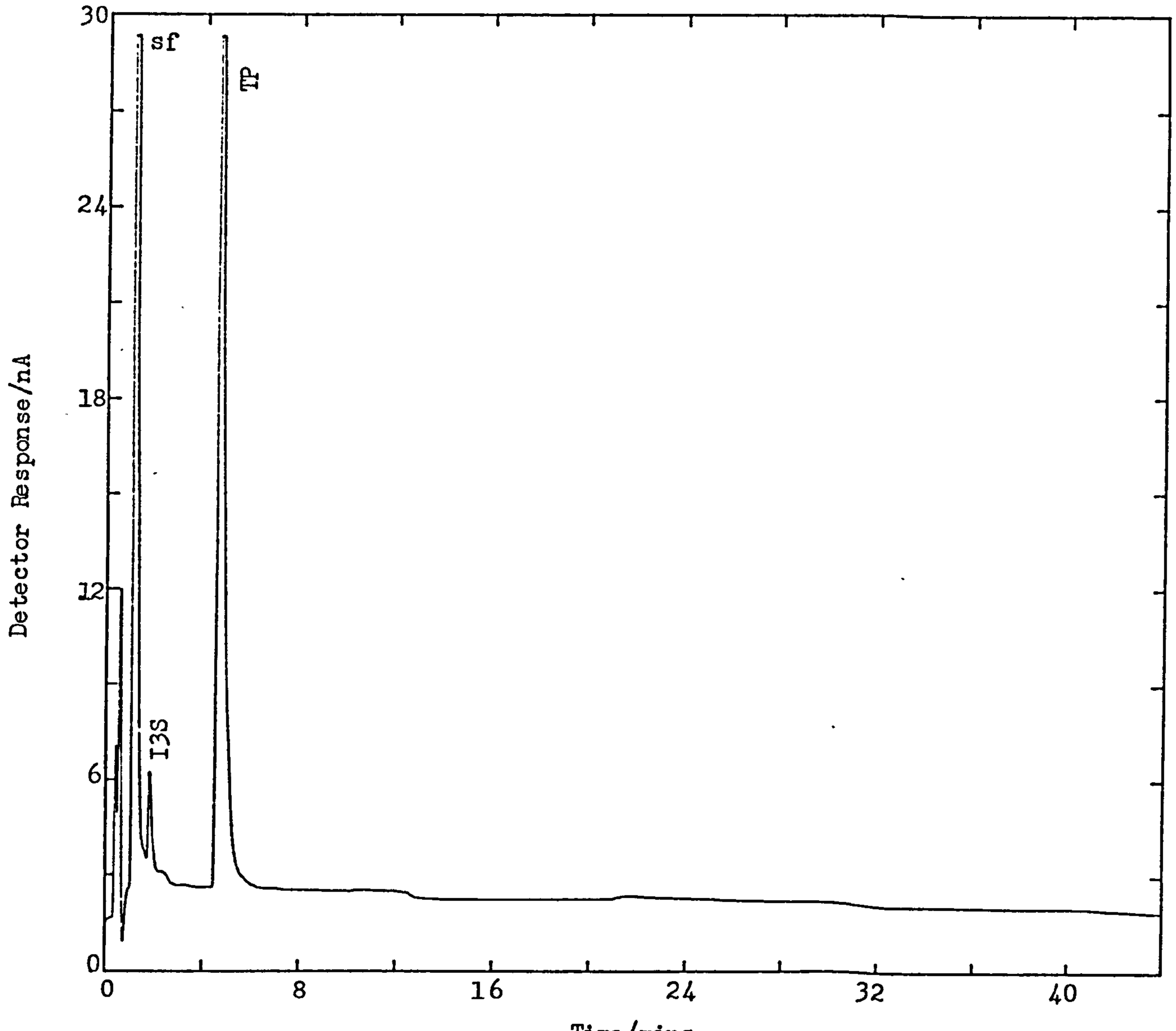


Figure 3.84(c) Typical Chromatogram Resulting from Plasma Extraction According to Scheme 6 (Figure 3.76) - Methanolic Eluate from a C_{18} Sep-Pak Cartridge

Parameters :- For conditions see Figure 3.61 except for Sample : 5µl via loop of the methanolic eluate from a Sep-Pak treated with PPP3 (500µl) containing BF (11ng)



A substantial peak corresponding to BF was observed in all chromatograms recorded at 3nA f.s.d. instrument sensitivity (Figure 3.83). Hence, BF exhibited similar extraction behaviour to the analyte indoles (as was predicted) and, although not ideal chromatographically, these observations provide encouragement regarding the potential of BF to act as an IS for the subject determination.

In all the chromatograms recorded at both instrument sensitivities, the signal attributed to I3S was almost completely swamped by a gross solvent front. In order that this substance may be quantitated the amount of interference must be reduced. Greater discrimination is necessary either in the extraction procedure or in the detection potential. The applied potential was reduced from +0.95V to +0.80V and a further chromatogram of the extract derived from the Sep-Pak effluent of PRP was recorded at 30nA f.s.d. (Figure 3.85(a)). A comparison with the equivalent chromatogram obtained at +0.95V (Figure 3.85(b)) indicates that no improvement in I3S detectability is forthcoming through reduction of the applied potential because of the concomitant reduction in signal amplitude which results. Furthermore, detectabilities of the other analytes are correspondingly affected. In view of this finding,it is deemed essential that discrimination in extraction be markedly improved.

In addition to increased discrimination, the maximisation of extraction efficiency is also vitally important. The overall sensitivity of this method is currently insufficient for the determination of the majority of the substances of interest in plasma samples of restricted volume. Plasma aliquots of 500µl were applied herein. In a genuine case of neonatal or paediatric illness, the volume available may be as little as 100µl which represents a reduction of 80% in available analyte mass compared with that at the analyst's disposal in this experiment.

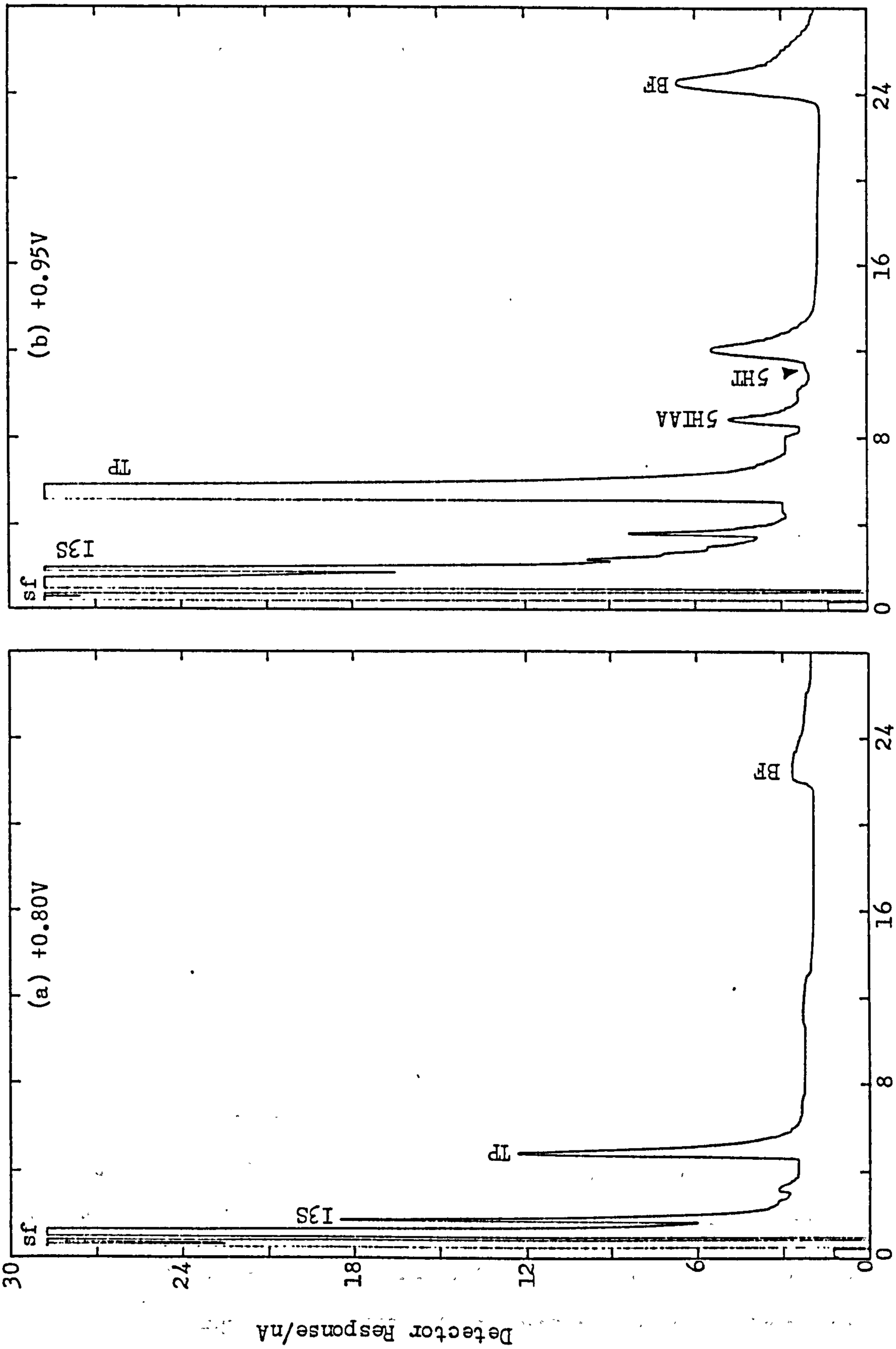
A substantial peak corresponding to BF was observed in all chromatograms recorded at 3nA f.s.d. instrument sensitivity (Figure 3.83). Hence, BF exhibited similar extraction behaviour to the analyte indoles (as was predicted) and, although not ideal chromatographically, these observations provide encouragement regarding the potential of BF to act as an IS for the subject determination.

In all the chromatograms recorded at both instrument sensitivities, the signal attributed to I3S was almost completely swamped by a gross solvent front. In order that this substance may be quantitated the amount of interference must be reduced. Greater discrimination is necessary either in the extraction procedure or in the detection potential. The applied potential was reduced from +0.95V to +0.80V and a further chromatogram of the extract derived from the Sep-Pak effluent of PRP was recorded at 30nA f.s.d. (Figure 3.85(a)). A comparison with the equivalent chromatogram obtained at +0.95V (Figure 3.85(b)) indicates that no improvement in I3S detectability is forthcoming through reduction of the applied potential because of the concomitant reduction in signal amplitude which results. Furthermore, detectabilities of the other analytes are correspondingly affected. In view of this finding, it is deemed essential that discrimination in extraction be markedly improved.

In addition to increased discrimination, the maximisation of extraction efficiency is also vitally important. The overall sensitivity of this method is currently insufficient for the determination of the majority of the substances of interest in plasma samples of restricted volume. Plasma aliquots of 500µl were applied herein. In a genuine case of neonatal or paediatric illness, the volume available may be as little as 100µl which represents a reduction of 80% in available analyte mass compared with that at the analyst's disposal in this experiment.

Figure 3.85 Chromatograms of the same Plasma Extract Recorded at Two Different Applied Potentials

Parameters :- For conditions see Figure 3.61 except for Detection : ECD (E_{app} vs. Ag/AgCl as specified, 5µl via loop of the effluent from the introduction on to a Sep-Pak of PRP (500µl) containing BF (11ng)



3.10 Plasma Clean-Up : General Remarks

All peak assignments herein have been made on the basis of the comparison of retention parameters (t_R, k') with those of standard materials. It is preferable to confirm peak identity by co-chromatography with a known standard where possible. The identification of peaks by retention parameter alone is far from ideal. No account is taken of the possibility of co-incident elution of another extracted substance. In addition, the problem of identification was complicated during these experiments by the observation of a progressive decrease in retention times of all peaks with successive injections of plasma extracts. It may be assumed that contamination, almost certainly arising from these injected samples, was the causal factor. The presence of a concomitant increase in back pressure with time supports this opinion. This irreproducibility of chromatographic behaviour must be considered prohibitive for application to routine analytical work.

Column stability was generally poor over the course of this study, both in terms of contamination and physical disturbance of the stationary phase bed. The latter effect was most probably a direct consequence of the increase in back pressure generated by the former problem. It may be inferred from the experimental observations made that even a small amount of residual protein in extractives is intolerable for the successful operation of NB columns.

In addition to column instability, poor instrument performance influenced the detectability of the analytes. High background noise emanating from both the PU4010 pump and the PU4022 ECD caused a narrowing of the range of instrument sensitivities that were utilisable. General unreliability stemming from various and frequent equipment malfunctions was also unacceptable (see Chapter 4).

The upshot of all the attempted methods employed was that insufficient sensitivity was obtained for indole determination in volumes of plasma of the order of 100µl. This was in part due to lack of discrimination in extraction, part probably due to poor extraction efficiencies and part due to instrument failings or limitations. Improved discrimination and efficiency of extraction may be attainable through optimisation of solvent compositions and flow rates and perhaps by the use of other Sep-Pak cartridge chemistries either in place of or in conjunction with the currently employed ODS type. Furthermore, the control of pH at all stages may prove to be helpful, although ion suppression, IIC and IXC separation techniques as such are all considered to be untenable in this instance because of the extent of variation in analyte polarity which exists.

One factor which appears to be crucial to the objective sought is improvement of equipment. Fractionation of small blood samples (<500µl) requires a micro-centrifuge which is both highly controllable and capable of a wide range of spin rates suitable for the purpose intended. It should also have a refrigeration facility. The best centrifuges available to the author were of too large a scale and were not utilisable at sufficiently low speed to yield plasma without very substantial platelet loss. Furthermore, general laboratory apparatus of a design and specification to allow performance of micro-scale manipulations with high precision is important. The apparatus at the author's disposal was severely limited in this respect; the minimum tenable sample volume was ajudged to be 50µl. Ideally a volume in the region of 5µl would be better suited to yield a sensitivity increase. Ultimately, the performance characteristics of the NBLC system itself may also require considerable improvement before success is achieved with regard to the analytical problem faced.

In summary, from these preliminary experiments the need for extensive clean-up is apparent. The difficulty of the extraction, because of the wide polarity range of the five analytes, is exacerbated by the heightened demands for sample purity invoked by the use of the NB column format. These demands are not readily satisfied thus the development of a rapid and simple extraction procedure appears to be very improbable under these circumstances. Considerable extra study is necessary before the extraction difficulties encountered may be overcome.

CHAPTER 4

Overview and General Conclusions

Contents

A commercial isocratic NELC-EC instrument comprising a PU4010 pump, a Rheodyne model 7413 micro-injection valve and a PU4022 electrochemical detector has been evaluated, in conjunction with a variety of column geometries, with respect to both performance and practicability.

4.1 Appraisal of the Pye Unicam PU4022 Electrochemical Detector

The detector characteristics were assessed in comparison with an EDT LCA 15 and a Metrohm 641-VA/656. Regrettably the Metrohm 641-VA/656 could not be compared satisfactorily due to the occurrence of a major malfunction which was later discovered to have arisen principally from faulty circuitry in the electronic control unit. Warm-up properties, baseline noise (generated under various conditions), ease of operation and utility of incorporated features were considered. The PU4022 was practically equivalent to the EDT LCA 15 in all these respects. The former instrument exhibited slightly greater long-term baseline instability than did the latter. This difference most probably originated from the transformer unit incorporated in the PU4022 to provide dual mains-voltage operation capability, a feature which is not included in the EDT model. All other structural aspects of these two ECDs were identical, which was to be expected since both instruments were manufactured by EDT Research. Instrument sensitivity settings of between 1nA and 3μA f.s.d. were available. The highest practicable settings for analytical purposes, within the confines of the systems studied, were 10nA f.s.d. with minimum noise filtration (time constant = 1sec) or 3nA f.s.d. with increased noise filtration. The PU4022 and the EDT LCA 15 yielded very similar limits of detection (S/N = 2:1) and limits of quantitation (S/N = 20:1) for a series of catecholamine compounds chromatographed on a conventional column

(25cm x 5mm ID). The PU4022 was demonstrated to be applicable to the post-chromatographic detection of several neurochemicals present in discrete regions of a rat brain. The PU4022 was considered to be reasonably easy to use although slight design changes to the controller facia, notably separation and isolation of the auto-zero, event marker and x10 instrument sensitivity switches, would greatly decrease the incidence of manipulative error on the part of the operator. Access to the flow cell for maintenance purposes was relatively unimpeded and, consequently, electrode inspection was undemanding.

Possessing a flow cell volume of only 0.5µl, the PU4022 ECD offered the possibility of deployment in a NBLC system without modification. The property of NBLC of particular interest to the author is the ability, in theory, to achieve much improved mass sensitivity (i.e., lower LODs) in instances where sample size is highly restricted. In clinical chemistry this need frequently arises such as in the case of the determination , for diagnostic purposes, of trace components in the blood of neonates, infants and young children. The Pye Unicam NBLC-EC has been evaluated with respect to the determination of a series of indolic metabolites of TP, initially in the form of standard solutions and later in blood samples. Separations of the five analytes viz. TP, 5HTP, 5HT, 5HIAA and I3S have been developed and optimised on columns of 1mm and 2.1mm ID.

4.2 Appraisal of the Narrow-Bore Instrument with Respect to Extra-Column Dispersion

Extra-column dispersion within the Pye Unicam instrument amounted to $232\mu l^2$ (≡ an effective extra-column volume of 15.2µl) when a 25cm x 1mm ID column with Swagelok end-fittings containing integral frits was coupled. When a 10cm x 2.1mm ID column possessing similar Swagelok end-fittings (except that discrete disc frits were included) was connected a value of $236\mu l^2$ ($\equiv \sigma_{xc}$ = 15.3µl) was obtained. The systems were run at similar but not identical linear velocities and differed only in the above noted respects and in the fact that the total length of connecting tube was reduced in the latter case by 20% from 15cm to 12cm, the absolute minimum possible within the constraints of the system architecture.

The above values for extra-column dispersion are in accordance with the findings of Reese and Scott[39] for three unspecified commercial NBLC systems. Extra-column dispersions in this region are considerably greater than the theoretical maxima (applicable for early eluting peaks) to enable efficient operation with the columns under evaluation. For a column of 25cm length and 1mm ID packed with 10µm diameter particles the effective extra-column volume must not exceed 0.39µl, which the Pye Unicam NBLC-EC system does 40 fold! In the case of the 10cm long by 2.1mm ID column packed with 5µm diameter particles, the maximum tolerable value is 2.4µl which is exceeded over 6 fold. The Pye Unicam instrument was determined to be barely capable of operating short conventional-bore columns to accepted standards.

The efficiency of the system was evaluated in relation to capacity factor for both column geometries. The 1mm ID column was only able to achieve 28% of available column efficiency for the first analyte peak ($k' = 2.66$)

increasing to just short of 90% (representing the 10% loss due to the detrimental effect of the instrument which is generally accepted to be tolerable) for the fifth analyte peak ($k' = 14.50$). Clearly, even at $k' = 15$ the Pye Unicam NBLC-EC instrument was woefully inadequate for the effective operation of short 1mm ID columns. The system was better able to handle the 2.1mm ID column, as was to be expected from theory. In this case 55% of available column efficiency was realised by the first eluted analyte peak ($k' = 1.64$) up to as much as 99% by the fifth analyte peak ($k' = 13.08$). Acceptable losses in efficiency were only attained at $k' \geq 7.8$ which represented a significant improvement when compared with the 1mm ID column. From these findings it must be concluded that in theoretical terms the Pye Unicam NBLC-EC system under scrutiny fell considerably short of the specifications required to operate columns of either geometry to commonly accepted standards.

The major source of this gross extra-column dispersion was deduced to be the PU4022 detector. The volume and kinetic properties of the flow cell were considered to be adequate for application to NB column geometries. However, the electronic response time characteristics of the controller unit were extremely poor. Time constant settings (which are faster than their corresponding response times) of 1, 3 and 10sec were available with the instrument. These values are well in excess of the theoretical maximum tolerable response times of 0.24sec for operation of the 25cm x 1mm ID column packed with 10µm diameter particles, and 0.12sec for operation of the 10cm x 2.1mm ID column packed with 5µm diameter particles. These calculated limits do not allow for extra-column dispersion arising from other sources and consequently for practical applications, limits of an order of magnitude faster, i.e., 0.024 and 0.012sec respectively, are more appropriate.

Evidently, even the fastest time constant available with the PU4022 detector is two orders of magnitude too slow for efficient operation of NBLC columns.

In addition to the contribution from the response characteristics of the detector, calculations from theory predict that only ca. 4% of the observed extra-column dispersion originated in the injection valve. A further 15% was estimated to have arisen in the connecting tubing of the system employing the 1mm ID column while the corresponding figure was 12% for the 2.1mm ID column-containing system. The only effective way to reduce the extra-column dispersion created in the connecting tubing is to eliminate these conduits entirely and redesign the apparatus so that the column may be plumbed directly into the injection valve and the flow cell. This approach has been adopted by LDC/Milton Roy in the design of their NBLC system[182] but not by Pye Unicam.

A major practical limitation of the evaluated instrument was the capability of the PU4010 pump with respect to flow rate. The minimum available setting was $0.1 ml min^{-1}$ ($\equiv 100 \mu l min^{-1}$) which, for NB columns, corresponds to linear velocities far greater than optimum. Consequently, since efficiency is directly dependent upon linear velocity, the maximum achievable efficiency for any given NB column is reduced considerably. However, it was deemed to be necessary to sacrifice some of the attainable efficiency when 2.1mm ID columns were employed in order to operate within an acceptable analysis time.

4.3 Narrow-Bore Liquid Chromatography in Practice

A vast range of problems were experienced with NBLC in practice, resulting in extremely long and frequent periods of system downtime. On average, the Pye Unicam system is estimated to have functioned satisfactorily, in practical terms, for only one day in every four or five which is clearly unacceptable for research activities let alone routine daily operation. All components of the instrumentation were affected in some way.

The PU4010 pump suffered many breakdowns for various reasons and often required attention. The most prominent signs of trouble were :(1) unacceptable baseline noise in the form of pulsations following a cycle corresponding to the dual-piston reciprocating pump action, (2) a gradual increase or large fluctuations in back pressure under constant operating conditions, and (3) leaking joints. Progressive blockage of flow passages with solid material was indicated by pressure problems and/or pulsing. On separate occasions the cause of the trouble was deduced to be an accumulation of either buffer precipitate, débris from scored or shredded piston seals or micro-flora within the pump. Buffer crystallisation was remedied by flushing the pump with water until the deposited material had been removed. Piston seal damage, which was also recognisable by the appearance of white crystalline matter (i.e., buffer constituents) around the piston rods, required seal replacement and thorough cleaning of the piston chambers and pistons. In addition, check valves were ultrasonicated in a series of appropriate solvents to dislodge all débris from the filters and valve chambers. The third blockage problem resulted from the growth of micro-floral colonies in the mobile phase which subsequently entered the pump. The intrusion of this proteinaceous material into the pump necessitated

drastic action which included flushing with 30% HNO_3 followed by copious quantities of water. All plastic tubing was then replaced and the inlet filter and check valves were thoroughly ultrasonicated as described above. To avoid future occurrences of this problem the addition of a small amount of NaN_3 to the mobile phase to act as a pesticide was contemplated but was rejected in favour of more frequent renewal of the mobile phase in the interests of simplicity. In order to protect the NB column from solid material emanating from the pump, a scavenger column was interposed between the pump and the injection valve.

Two critical malfunctions of the PU4010 pump occurred over the course of this study. The cam followers became worn and seized up thus requiring renewal. Secondly, the flow rate control mechanism became defective which also necessitated replacement. In view of the seriousness of, and recurrence of, these problems, the entire pump was exchanged for another identical model to enable completion of the experiments reported in this dissertation.

Other practical problems of note concerning the pump were predominantly due to structural fatigue. On numerous occasions screw threads had to be sealed with PTFE tape, plastic tubing required reflanging and eventual substitution, and plastic nuts broke also necessitating replacement.

The high incidence of mechanical failure of the PU4010 pump almost certainly resulted from the strain put upon the unit in being required to operate continually in order to maintain a flow over the glassy carbon WE in the detector flow cell. The general frailty of the PU4010 gave great cause for concern in addition to its failings regarding specifications.

Connecting tubing did on occasion become partially blocked and required flushing, back flushing or ultrasonication before becoming serviceable. Total replacement of a conduit was carried out if necessary.

The Rheodyne model 7413 micro-injection valve required attention relatively infrequently compared with the pump and detector. Typical problems included leakage and blockage. Leaks occurred around nuts which were remedied by the application of PTFE tape and eventually by installation of new parts. In addition, leaks were discovered internally, both at the rear of the loop disc whichwere repaired by the application of a little silicone adhesive ('Silicoset'), and at the rotor itself(resulting from regular wear and tear) whichwere remedied initially by tightening the stator screws and eventually by insertion of a new rotor. The incidence of blockage was largely prevented by regular and frequent flushing of the valve with water between injections. On the rare occasions when blockage which was not removable by flushing occurred, the offending parts were ultrasonicated in appropriate solvents to dislodge the obstruction.

The PU4022 ECD posed numerous difficulties in its operation throughout the duration of this project. The majority of system downtime can be attributed to the PU4010 pump and this instrument.

The flow cell required frequent attention in several ways. The glassy carbon WE needed polishing at least every four weeks with introduction of standard solutions only, and even more often when extractives were chromatographed. The lifetime of a pair of electrodes (WE and RE) was approximately one year. Some difficulties were experienced with seating of the aforementioned electrodes giving rise to accentuated baseline pulsation with the flow of the mobile phase. Redesign of the locking collars to enclose the electrode assembly and so provide a firmer anchorage may prove beneficial in this respect. The construction of the cell to enable the rapid passage of any incident air bubbles through the detection volume was particularly efficacious hence this occurrence was not problematical. With age, leaks

were observed to develop around the inlet and outlet conduits. Treatment of the screw threads with PTFE tape and/or reflanging tubing ends solved the problem on most occasions. Complete replacement of tubing and nuts was carried out when leaks developed with regularity.

One serious problem was encountered with the original flow cell. Excessive short-term noise was evident and persistent. The source of this noise was found to be corrosion and subsequent erosion of the S/S inlet sleeve which liberated metallic species randomly into the mobile phase. These ions reacted at the WE surface causing the problem. Clean up was unsuccessful, especially since the RE had become poisoned. Consequently, a replacement flow cell was obtained with which to continue these investigations.

The PU4022 electronic controller unit did not provide trouble-free operation either. The general short-term (electronic) noise level was fairly substantial throughout these experiments. In addition, difficulties developed regarding the auto-zero facility. On servicing the instrument to find and repair the cause of the latter fault (a worn and malfunctioning switch), a general pitting of the circuit boards was discovered. This presumably originated from the action of atmospheric acid pollutants and may have constituted the source of the general noise problem.

Little difficulty was encountered with the peripheral recording devices. Regular maintenance (cleaning of electrical contacts, and guide wire and bar in the case of the potentiometric recorder) ensured efficient operation.

In addition to instrument malfunctions, difficulties were experienced with the columns employed. These troubles could be categorised broadly into two types; contamination of the column packing and the creation of

voids in the same. Certain types of contamination were reversible, e.g., buffer precipitation. In these cases column performance could be reinstated by back-flushing with water. Other contamination sources were irreversible, e.g., the adsorption of protein or blockage with insoluble particulates. In order to overcome these problems, columns required either removal of the contaminated material and manual repacking of the resulting void or complete renewal. These strategies were also appropriate when packing homogeneity became unsound particularly under high back pressure.

The lifetime of a column was considered to be an important parameter in this assessment. The 1mm ID column failed after only 80 injections of standard solutions. The primary reason for this was thought to be obstruction of the exit frit by micro-particulate "fines". This problem recurred rapidly rendering the column unserviceable. Such a short lifetime, if typical of 1mm ID columns, is considered to be unacceptable in routine work, especially as the useful duration would be expected to be even shorter with the application of biological fluid extracts. The physical problems experienced with the operation of 1mm ID columns together with the extremely poor performance exhibited in respect of their employment in the Pye Unicam NBLC-EC instrument prompted their abandonment for further study. Consequently, on the basis of this evidence the use of the Pye Unicam NBLC-EC system with 1mm ID columns cannot be recommended. Furthermore, in view of the very short lifetimes, columns of this geometry are considered to be unacceptable for general use.

2.1mm ID columns generally exhibited lifetimes of longer duration than did the 1mm ID column, although these fell short of expectations when compared on a pro rata basis with typical conventional columns. This state of affairs was predictable since few of the normally employed protective

measures could be utilised because of the inevitable creation of more detrimental extra-column dispersion. The situation was exacerbated by the smaller cross-sectional area of 2.1mm ID columns presented to the flow stream, providing greater opportunity for obstruction to take place.

2.1mm ID columns were assessed in greater depth with respect to the chosen separation.

4.4 Selection of Injection Volume

Some choice was available regarding injection volume introduced on-column as a result of practical restrictions, viz. a minimum volume limit of 50µl for the extract and an inherent loss of 10µl with each injection made using the Rheodyne model 7413 micro-injection valve. It has been shown that the greater the volume injected then the greater is the signal amplitude because of the increase in mass injected. However, increased injection volume gives rise to a concomitant increase in extra-column dispersion which is manifested as a reduction in apparent column efficiency which, in turn, yields decreases in resolution and mass sensitivity.

In practice, resolution is the critical factor which governs acceptability. The arbitrary theoretical limit of tolerability of 10% resolution loss was shown to be impractical with regard to the Pye Unicam NBLC-EC system due to the poor inherent extra-column dispersion characteristics of this instrument. A limit whereby sufficient resolution remains to enable discrete elution of every analyte peak from all other peaks in the chromatogram is most appropriate in practical terms. From the data obtained, an injection volume of 5µl appeared to satisfy this criterion best. Such a volume also demonstrated the advantage of allowing duplicate (or possibly triplicate) chromatographic analyses to be performed on a single sample. The next smallest volume (1µl) provided much poorer detectability while the next largest volume (10µl) was considered to leave insufficient room within the chromatogram for the elution of co-extractives. The chosen value of 5µl is open to review depending upon the severity of interference encountered with actual plasma extracts.

4.5 Quantitative Aspects of Narrow-Bore Liquid Chromatography

Gross levels of imprecision were apparent from this study, both by calculation and by inspection of the broadness of 95% confidence bounds constructed around plotted calibration lines. Mean within-batch precision ranged from 4.7% to 16.2% while mean between-batch precision ranged from 24% to 41%, depending upon the method of quantitation employed. These values may be treated as conservative estimates of precision to be expected from the chromatography of plasma extracts because no account is taken of the inevitable variability in analyte loss during extraction.

Peak height ratio provided the greatest precision (smallest RSD), both within-batch and between-batch, and consequently was considered to represent the quantitation method of choice on these grounds. However, peak height (and consequently peak height ratio) is highly restricted in practical range at high instrument sensitivity. The area-based alternatives both suffer considerably greater imprecision. Peak area ratio is not very repeatable, largely because of variability in peak start and end point recognition by the integrator. Peak area measurements alone are subject to extremely poor reproducibility.

A major source of this great imprecision was thought to be variability in the degree of WE contamination incurred. In addition, other instrument-based factors are most probably fundamental to this phenomenon because of the chronic equipment malfunctions which occurred during the assessment period. The high incidence of breakdown was the sole reason why only five calibrations were successfully completed in 8 months. The obvious implication of these findings is that all possible steps must be taken to improve the reliability of the instrumentation before an acceptable standard of performance can possibly be achieved.

The poor precision of quantitation is reflected in values for the LOD and LOQ determined both in terms of empirical definitions which are not accompanied by any estimate of reliability, and in terms of a statistical definition which embodies an overall confidence limit of 95%. The statistical definition of what is termed a "detection limit" has been argued to in effect represent a "quantitation limit". Indeed, the values obtained from the statistical calculations for this quantity are in reasonable agreement with the empirically-derived LOQs. Values of between 100 and 400pg on-column for the five analytes were obtained. Expected levels of TP metabolites in small volumes of plasma were deduced to perhaps be measurable using the Pye Unicam NBLC system, in spite of its inadequencies, so long as losses of analyte material during extraction are insignificant.

4.6 Practical Aspects of Narrow-Bore Liquid Chromatography Arising from the Analysis of Blood Samples

Sample preparation for the determination of indoles in plasma fractions has been examined. Available equipment was found to be severely restrictive. High specification apparatus, providing the capability of handling very small volumes with high precision, is required for both blood fractionation and general measurement, dispensation and manipulation of samples and reagents. In the absence of such items, the ability to achieve high detectability is seriously inhibited. In addition, selectivity of extraction proved to be extremely difficult to accomplish because of the wide ranging polarities of TP and its indolic metabolites. Furthermore, the need for extreme cleanliness of the injected sample is inferred to be crucial to the reliable operation of NB columns since even small amounts of residual degradative substances lead to rapid contamination, blockage and physical disturbance of the packed bed. As a consequence of the progressive action of these phenomena, reproducibility of retention becomes very poor thus peak identification and subsequent recognition in further chromatograms is seriously hampered.

Problems with the operation of the NBLC instrument, combined with poor extra-column dispersion characteristics of said instrument, restricted greatly the practically attainable detectabilities of the various analytes. No satisfactory extraction procedure was developed to enable reliable quantitation of endogenous levels of TP, 5HTP, 5HT, 5HIAA and I3S in plasma samples of the order of 100µl in volume.

At high instrument sensitivities the observation and quantitation of early eluting peaks, especially I3S, is prevented by the presence of a gross solvent front. This emphasises the need for far greater discrimination

which must be accomplished through the extraction procedure since reduction of detection potential failed to provide improved detectability.

The extraction behaviour of BF was encouraging regarding the possible use of this substance as an IS, although its chromatographic behaviour is not ideal. Investigation of alternative materials as potential ISs (e.g., N-Me5HT, 6HT) may be rewarding.

4.7 General Conclusions

From the evidence amassed and reported in this dissertation it is surmised that the Pye Unicam NBLC-EC system is unsuitable for application to routine clinical analysis both on the grounds of its extra-column dispersion characteristics and its impracticality. The system components are neither sufficiently rugged nor of high enough specification to operate columns of NB geometry to anywhere near their optimum potential. If the Pye Unicam instrument is typical of commerical NBLC equipment then these comments will apply to the technique generally. The fact that there are still few reports in the literature concerning the routine application of NBLC, in spite of the wealth of interest expressed in the technique, may be interpreted as implying that other experimenters have perhaps encountered similar difficulties to those described herein. Generally, only highly customised or entirely custom-built research instruments have yielded some of the theoretical benefits pertaining to NBLC.

Professor John Knox (Edinburgh University), in a presentation at a recent conference[437], remarked that so-called specialist commercial instruments were not attaining sufficiently low dispersion. Effective extra-column volumes of 25µl were prevalent rather than figures of 0.25µl or even 2.5µl which are required. Furthermore, he noted that commercial NBLC equipment from 75% of manufacturers was of insufficiently high quality to run columns of this geometry to a standard approaching acceptability. The best instruments on the market were claimed to be just suitable for the operation of conventional length 3mm ID columns packed with 3µm diameter particles and it was concluded that there is no real value in reducing the column ID below 3mm at present. From the findings of this investigation of the Pye Unicam system the author must concur with these remarks.

The attitude of the manufacturers towards NBLC has changed markedly over the period in which this study was conducted. In 1983 and 1984 "micro-bore LC", as NBLC was then called, was heavily promoted with respect to claims of attainable high mass sensitivity, improved interfacing capabilities and low solvent consumption. 1mm ID columns were marketed but by the end of 1985 most companies had tended towards producing columns of around 2mm ID. The problems entailed with the efficient operation of the narrower format columns almost certainly became apparent and, rather than completely redesign their equipment range to produce dedicated, high quality, high specification (and consequently expensive) apparatus, many manufacturers elected for a compromise by adapting their current instruments to accept 2mm ID columns. This policy was adopted by Pye Unicam in the production of the instrument under appraisal in this study. In 1987, NBLC has generally been relegated in stature within the manufacturers' literature and is currently marketed purely on the grounds of solvent economy. This retrogression in publicity for the technique was probably initiated by technical difficulties, insurmountable within the restrictions of current company policy in many cases, associated with the efficient use of even 2mm ID columns.

The preceding remarks lend weight to the argument that compromise is not good enough in NBLC. Although a general recognition of the problems inherent with NBLC has led to greater awareness and understanding and, subsequently, has stimulated the improvement of conventional HPLC system design, instrument technology is not yet sufficiently advanced to enable the theoretical advantages of NBLC to be realised fully in practice. Consequently, when combined with the severe practical difficulties encountered, the technique of narrow-bore liquid chromatography cannot presently be recommended for routine use in the clinical laboratory.

CHAPTER 5

General Experimental Procedures

Contents

5.1 Routine Maintenance of HPLC Hardware

5.1.1 Pumps

Periodically, and routinely after changing over mobile phases, the pump was primed to remove any accumulated air bubbles from the system. Routine maintenance of the instrument included regular cleaning of the reservoir solvent inlet filter and the pump check valves. This was achieved by sequentially submerging the aforementioned parts in an appropriate series of solvents (usually HNO_3 (30%), DW then MeOH) and subjecting the parts to ultrasonic vibration for 10-30 minutes in each.

When using buffer solutions continually it was found to be necessary to flush the pump with DW occasionally in order to wash out any precipitated buffer material and so prevent residues accumulating on internal mechanical parts of the pump.

On a few occasions the pump was isolated as the source of high detector background current and baseline noise. This was thought to arise from $Fe^{2\oplus}$ ions being stripped from the internal surfaces of the pump, being carried to the ECD in the mobile phase and there oxidising to $Fe^{3\oplus}$ so producing the observed effects. This problem was overcome by flushing the pump first with HNO_3 (30%) to clean it out then with aq. EDTA (ca. 10^{-3}M) to complex any stripped metal ions and so passivate the S/S surfaces. The pump was returned to mobile phase via DW to ensure that precipitation of salts did not occur within the instrument.

Over the course of this work certain mechanical parts were replaced in the pumps. These parts included plastic piping, piston seals, check valves and, on one PU4010 pump, a pair of cam followers.

5.1.2 Injection Valves

As general practice, injection valves were flushed thoroughly with copious amounts of DW between sample injections to prevent accumulation of residues within the valve. Periodically a more aggressive solvent (e.g., HNO_3 (30%) or MeOH) was used for this purpose, especially when valve blockage was suspected. When this procedure did not manage to free the trapped particulate matter, the valve was dismantled and the various attached lengths of tubing, injection loop(s) and rotor seal were ultrasonicated sequentially in a series of solvents (usually HNO_3 (30%), DW then MeOH) as were pump parts (see Section 5.1.1).

Leaks that occurred around the long-bush nuts used to connect the various tubes into the rear of the injection valve were repaired temporarily with PTFE tape. Eventually, the nuts and ferrules were replaced.

With general wear and tear rotor seals become scored and leak. At such time, or before this point was reached, the rotor seal was renewed.

A problem was encountered with the Rheodyne 7413 internal-loop micro-injection valve, namely that a leak occurred at the rear of the loop disc in which the three injection loops were mounted. This was remedied by applying a little 'Silicoset' silicone adhesive to the base of the loops at the rear of the loop disc which sealed the gap.

5.1.3 Analytical Columns

With general use, a gradual decline in chromatographic efficiency, usually coupled with an increase in system back pressure, is observed. This situation is caused partly by contamination and partly by physical disturb-ance of the packed bed leading to a depression in the column top so creating

a void in which band broadening occurs. ODS columns were regenerated routinely by washing with ca. 20 column volumes each of DW, MeOH, DW, HNO_3 (0.1M) and DW again before returning to the mobile phase.

This procedure rarely re-established maximum column performance, particularly on those occasions when disruption of the packed bed had taken place. In these instances the inlet fittings were removed from the column and the top of the column bed was examined. If the packing was discoloured or depressed or cracked then column repair was attempted. The top 1-2mm of material was removed with a small spatula and the new top was examined again for faults. The procedure was repeated until clean, undisturbed packing was seen. The resulting void was then filled manually under gravity with an ethanolic slurry of new packing material, preferably from the same batch. The column was tapped lightly to allow even setting of the packing material. This process was repeated until the column void was full. The top of the column was compressed and made flush to the S/S using the spatula then the column inlet fittings were replaced. Inlet fittings were ultrasonicated to clean them prior to reuse. This procedure enabled column lifetime to be prolonged but an efficiency loss of 10-20% was commonplace when columns were repaired in this manner.

When efficiency loss was unacceptable, the whole column was removed and replaced with a new one. The old column was emptied, cleaned out and repacked with fresh material using a N_2-driven constant pressure hydraulic packing pump and following an appropriate procedure (see Sections 2.2.4 and 3.3.3.3). Column end-frits and fittings were examined for damage at all stages and were replaced as necessary.

Analytical columns were flushed thoroughly with DW prior to storage or if they were to be dormant for any lengthy period of time in order to displace potentially harmful buffers.

5.1.4 Scavenger Columns

Unacceptable increases in system back pressure commonly resulted from particulate contamination of the scavenger column which was incorporated between the pump and the injection valve to protect the analytical column from such problems. On these occasions the scavenger column was replaced with a new one. The contaminated scavenger column was then repaired as was the analytical column (see Section 5.1.3 above) or was emptied and repacked with fresh material as outlined in Section 5.2.1. If the top surface was badly polluted with piston seal debris then the piston seals were examined with view to replacement.

5.1.5 Electrochemical Detector Flow Cells

Flow cells were assembled as follows. Solvent was pumped into the cell while the outlet tubing was raised above the level of the cell so that the cell body gradually filled with solvent. The RE was installed first using an 'O'-ring seal (composed of rubber in the EDT and PU detectors and PTFE in the Metrohm one) and was secured in place with a locking collar. Care was taken to avoid trapping air bubbles in the cell. The WE was then fitted in a similar fashion, again taking care not to trap air bubbles. For the Metrohm ECD only, a discrete AE was also fitted as above. 'O'-ring seals were inspected for damage and were replaced as necessary prior to installation. Finally, appropriate electrical connections were made to the controller unit and the system was ready for start up.

The occurrence of air bubble formation in the flow cells themselves was discouraged by submerging the effluent pipe in the waste fluid so creating a slight back pressure on the cell. This procedure also offered

the added benefit of reducing flow-associated noise normally arising from drop formation and release at the tube exit.[214] The flow cells were designed to encourage the free flow of air bubbles through to waste should they form. However, in the event of air becoming trapped in the cell, then the cell body was held in an appropriate orientation and was tapped gently to dislodge the bubbles.

Glassy carbon WEs became contaminated with use. In order to decontaminate the GCE surface, the cell was depolarised and the electrical cable to the WE was disconnected. The GCE was removed and was cleaned by mechanical abrasion with a methanolic slurry of fine alumina powder (d_p = 3μm) on a lint-free tissue. Gentle circular motions were applied to avoid scratching the surface as much as possible. The GCE was thoroughly rinsed with DW and the flow cell was reassembled as outlined above.

Periodically the PU4022 S/S WE was removed and cleaned by ultra-sonication and gentle brushing in HNO_3 (30%) followed by DW.

All electrical contacts were cleaned routinely to ensure least resistance.

The first PU4022 flow cell used in this study eventually had to be replaced due to a gross contamination problem generated by the corrosion of the S/S inlet tube locking collar. An identical flow cell was obtained except that a PTFE collar was incorporated in place of a S/S one.

Electrodes were replaced occasionally in the PU4022 flow cell. GCEs had a lifetime of ca. 1 year whereas Ag/AgCl REs lasted ca. 2 years in the course of this project.

5.1.6 Electrochemical Detector Control Units

Switch contacts on the PU4022 Controller were cleaned occasionally by technicians.

Great problems were encountered eventually with the operation of the PU4022. Faults developed initially in the auto-zero circuitry and later in other parts of the instrument. After various unsuccessful attempts to repair the unit it was abandoned and was replaced by the Metrohm 641-VA ECD which had been repaired. The PU4022 flow cell was retained so that comparisons with previous work could be made. Suitable cables to connect the 641-VA to the PU4022 cell were prepared and utilised.

5.1.7 Potentiometric Recorders

The guide wire, bar and pen holder were cleaned routinely with MeOH or 2-PrOH to ensure smooth, unrestricted movement. Periodically a high grade metal polish was also used on S/S parts. Electrical connections were polished frequently to reduce resistance and provide good contact.

Potentiometric recorders were serviced as required to obtain optimum performance.

5.2 General Operating Procedures

5.2.1 Preparation of a Scavenger Column

The purpose of this scavenger column was to protect the analytical column from particulate matter (e.g., pump piston seal débris, micro-flora) and irreversibly adsorbed chemical contaminants carried in the mobile phase.[397]

An empty column (45mm x 4.6mm ID) possessing $^{1}/_{16}$" male 'Swagelok' end-fittings was cleaned thoroughly with MeOH. A slurry in MeOH of Resolve ODS (d_p = 10μm, Waters Associates, Northwich, Cheshire) recovered from Radial-Pak cartridges was prepared (ca. 0.9g in 10mls) by ultrasonication for 10 minutes. The resulting paste was then packed manually under gravity into the column. The column top-fitting was connected and the assembly was installed in the solvent line immediately following the pump. The scavenger column was flushed, first with DW and then with the mobile phase. The column was removed and topped up as deemed necessary. Finally, the column was connected to the injection valve via the remaining length of solvent line. The column was repacked and replaced when system back pressure increased to an unacceptable level.

5.2.2 Determination of Accuracy of Pump Rate

All pumps used in this investigation were assessed regarding the accuracy of their solvent delivery. A suitable mobile phase was degassed under a gentle stream of He and was eluted through the system at the desired flow rate setting. For a predetermined time a clean, dry measuring cylinder of appropriate capacity was introduced into the flow stream to

collect the effluent going to waste. (It was not possible to use a burette due to the high surface tensions of the predominently aqueous mobile phase). The volume collected in the time was noted and from this the actual flow rate was calculated. The procedure was then repeated and an average flow rate was determined. Any deviation from the set flow rate was noted.

Both of the Pye Unicam PU4010 pumps and the Altex LC-XPD used throughout the course of this work were found to pump fast consistently by about 2-3% at a nominal flow rate of $0.4 ml min^{-1}$ (NBLC systems) using a 4% MeOH : 96% aq. buffer mobile phase. At the $1.0 ml min^{-1}$ flow rate setting (Conventional LC systems) using a 10% MeOH : 90% aq. buffer mobile phase the first PU4010 and the Altex LC-XPD both pumped slightly fast by about 1%. None of these deviations from the nominal settings were considered to be sufficiently great as to be significant and are within specification for the individual pumps concerned.[438]

5.2.3 Injection Technique

Grasping only the syringe flange and the plunger button, a quantity of standard or sample solution was drawn into a clean, dry glass syringe of 1ml capacity for conventional LC work or 25µl capacity (# 702 'Microliter', Hamilton, NV, USA) for NBLC work. Air bubbles were excluded from the syringe and the needle was wiped gently with a clean tissue to remove excess liquid. Care was taken not to draw solution out of the syringe needle by capillary action. The needle was then placed in the injection valve loop filler port and, with the injection valve in the 'load' position, the plunger was depressed steadily in order to transfer the sample into the loop. As good practice, handling of the micro-syringe barrel

(used for NBLC injections) was avoided so preventing any volume change taking place which would affect absolute concentrations injected. This was not important when chromatographic or internal standards were incorporated in the sample for quantitation purposes. Injection onto the column was carried out by switching the valve.

After the injection, the syringe was removed and was flushed several times with clean DW and finally with acetone. The syringe was then dried by pumping air in and out of the barrel using the plunger or, alternatively, by directing a stream of compressed air over the disassembled syringe parts.

After the completion of the chromatographic run a 1ml capacity syringe filled with clean DW was placed in the injection port and the valve was flushed liberally, first in the 'inject' position then in the 'load' position. This procedure served to prevent cross-contamination between successive injections should traces of the previous injection have remained within the valve after the termination of the previous chromatographic run.

5.2.4 Overnight Status

It is recommended that the flow stream passing through an ECD flow cell should never be interrupted whilst the cell is subjected to an applied voltage because rapid deterioration of the WE due to surface contamination can be expected to result.[439] The excessive length of time required to start up each day from reintroduction of the working mobile phase and polarisation of the cell to achieving a practicable baseline, together with the time required at the end of each day to wash the system prior to shut down was considered to be prohibitive. Hence, the alternative strategy of continual elution was adopted.

Initially, mobile phase cycling[205,440] was carried out overnight; a practice whereby the effluent emerging from the flow cell vent is directed back into the solvent reservoir, the contents of which are sparged continually with He. This practice was ceased due to problems encountered with contamination and, to a lesser extent, column deterioration.

Generally, mobile phase was pumped through the system without cycling when left overnight. Flow rate was reduced to 0.1-0.2mlmin^{-1} in order to economise on solvent. Re-equilibration of the baseline on returning to the working flow rate was fairly rapid so this action was not very restrictive.

Periodically the applied voltage was increased to a high positive value (ca. +1.2-1.5V) overnight in order to promote electrochemical cleaning of the WE surface.

5.2.5 Thermostatting of the Apparatus

The Metrohm 656 ECD flow cell inlet tube was thermostatted with a water jacket. All other apparatus was operated at ambient conditions, although the action of direct sunlight on the instrument was avoided.

No column thermostat was available for use so, in order to reduce short-term temperature fluctuations, the column was wrapped securely in a strip of polyurethane foam which was surrounded by a length of PVC pipe (120mm x 37mm ID, 45mm OD) which was utilised to house the assembly and to provide extra insulation.

Ideally the entire solvent, injector, column and flow cell should be thermostatted in order to gain greater stability and improved precision. The use of a Pye Unicam PU104 GC oven system was considered for thermostatting

purposes but was rejected because (1) temperature control was poor at around 20 °C, (2) installation would have proved difficult - connecting tubing would have required elongation hence increasing extra-column volume and, consequently, extra-column dispersion, and (3) clinical laboratories would not generally have such facilities available to them.

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