Improvement of the selectivity in column liquid chromatography : design of post-column reaction systems

IMPROVEMENT OF THE SELECTIVITY IN COLUMN LIQUID CHROMATOGRAPHY

DESIGN OF POST-COLUMN REACTION SYSTEMS

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE TECHNISCHE WETENSCHAPPEN AAN DE TECHNISCHE HOGESCHOOL EINDHOVEN, OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF.DR.P.VAN DER LEEDEN, VOOR EEN COMMISSIE AANGEWEZEN DOOR HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 17 FEBRUARI 1978 TE 16.00 UUR

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Dr.Ir. C.A.M.G. Cramers en Prof.Dr. J.F.K. Huber.

aan Truus
aan mijn ouders

CONTENTS

SUMMARY

Improvement of separation in column liquid chromatography can be accomplished by increasing either the selectivity or the efficiency. The achievement of large selectivity factors is fundamentally of greater importance in liquid chromatography. However, the optimation of selectivity in practice is of limited value when the samples to be analyzed are getting more and more complex.

In Part II the use of selective phase systems is demonstrated for the analysis of extracts from biological material: cortisol and tricyclic antidepressants in human plasma.

A quantitative assay of cortisol in human plasma by modern liquid chromatography using a selective *p*-nitroaniline modified silicagel is described. The extraction and subsequent chromatographic analysis is optimized, resulting in a recovery for cortisol of 96% and a minimum detectable amount of 1 ng cortisol respectively. The specificity of the method was tested by field desorption mass spectrometry. Quantitative analysis of cortisol in human plasma by liquid chromatography is compared with a number of very specific and sensitive techniques: two fluorometric assays, competitive protein binding and three radio immuno techniques. The radio immuno assay methods offer the best prospects on routine basis for laboratories equipped for radioactive tracers, while liquid chromatography gives a good alternative.

A liquid chromatographic system with high separation efficiency for the analysis of tricyclic antidepressants is obtained with eluents consisting of mixtures of ethyl acetate, n-hexane and methylamine on a silicagel column. The retention behaviour of the components is regulated by varying the concentration of n-hexane, the modifier methylamine and the water content of ethyl acetate.

In complex samples often the combination of optimal selectivity and maximum efficiency is still insufficient

to enable complete separation by high performance liquid chromatography. The use of selective detection methods is imperative to perform analysis. Part III deals with this subject. The sensitivity and selectivity of detection for many classes of compounds by standard liquid chromatographic detection devices such as photometers, fluorometers and coulometric cells can be improved substantially by coupling the exit of the chromatographic column to a chemical reaction system. This can be carried out by continuously adding a suitable reagent to the column effluent and continuously monitoring the reaction mixture.

It is shown in the present study that additional band-broadening and the consequent loss of resolution can be reduced to an acceptable level by careful design of the reaction system.

Two types of reactors are studied: open tubular reactors and packed bed reactors (packed with non-porous spherical glass beads). Performance and characteristics of these reactors are evaluated and rules are given for their optimum design.

The main mechanisms of dispersion in a packed bed are molecular diffusion and mixing arising from streamsplitting, generally termed eddy diffusion. This eddy diffusion is mainly determined by geometry parameters; the estimation of these geometry parameters is of the utmost importance for the prediction of band-broadening and consequently in the design of packed bed reactors. A suitable choice of reactor length and particle size of the glass beads should be made for a given combination of reaction time and allowable additional band-broadening in the reactor. Another restriction in practice is the maximal allowable pressure drop over the reactor. Favourable conditions are found near the minimum of the pressure drop *vs* particle size curve (for constant values of the residence time and the additional band-broadening in the reactor), giving acceptable reactor lengths.

Axial dispersion in a helically coiled tube is smaller than in a straight tube, because of a secondary flow,

perpendicular to the main direction of flow. The ratio of the dispersion coefficient in a helix and the dispersion coefficient in a straight tube is related to the dimensionless group $DnSc^{\frac{1}{2}}$ by a single curve. This relation enables the prediction of band-broadening in coiled tubular reactors. Similar as in packed bed reactors a suitable choice of the internal diameter, length and coiling ratio should be made to obtain the fixed reaction time and not to exceed the allowable extra band-broadening and pressure drop. It appears that little additional band-broadening will be obtained for narrow bore tubes. The coiling ratio, defined as the ratio of the diameter of the coil and the internal diameter of the tube, is to be kept small too.

Selection criteria for a reactor type depend on the speed and the complexity of the reactions involved. It appears that for fast reactions (residence time < 5 min) packed bed reactors give the lowest additional band-broadening, whereas the axial dispersion in helically coiled open tubes is relatively large. Therefore open tubular reactors should only be selected when packed reactors can not be used because of the reaction conditions. For slow reactions (residence time > 5 min) neither glass bead columns nor helically coiled tubes can be used, without accepting large values for the extra-column peak-broadening; then gas-segmented liquid flow reactors should be considered.

The use of a packed bed reactor in combination with a colorimetric detection system is described for the analysis of hydroperoxides. Another application described of this reactor type is the derivatization of amino-acids to fluorescent products by reaction with *o*-phthalaldehyde. A coiled tubular reactor is described which enables the electro chemical detection of reducing sugars. Data on band-broadening and minimum detectable amounts are listed for the reaction systems described above.

SAMENVATTING

Verbetering van scheidingen in de vloeistofchromatografie kan verkregen worden door verhoging van de selectiviteit of van de efficiency. Het streven naar grotere selectiviteitsfactoren is echter fundamenteel van groter belang. De optimalisering van selectiviteit is echter in de practijk beperkt wanneer de te analyseren monsters complexer van aard zijn.

In deel II worden selectieve fasen systemen beschreven voor de analyse van stoffen uit extracten van biologisch materiaal; cortisol en tricyclische antidepressiva in plasma.

Een kwantitatieve bepalings methode van cortisol in menselijk plasma wordt beschreven met moderne vloeistofchromatografie met behulp van een selective chemisch gebonden stationaire fase, *p*-nitroaniline op silicagel. De extractie en chromatografische analyse is geoptimaliseerd en resulteert respectievelijk in een recovery voor cortisol van 96% en een minimaal aantoonbare hoeveelheid van 1 ng cortisol. De specificiteit van de methode is getest met veld desorptie massaspectrometrie. De kwantitatieve analyse van cortisol met vloeistofchromatografie is vergeleken met een aantal zeer specifieke en gevoelige bepalingsmethoden; twee fluorometrische technieken, drie radio immuno assays en competitive protein binding. De radio immuno assays bieden goede perspectieven voor routine analyses in laboratoria, uitgerust voor bepalingen met radioactief materiaal, terwijl vloeistofchromatografie een goed alternatief is.

Een vloeistofchromatografisch systeem met een hoog scheidend vermogen voor de analyse van tricyclische antidepressiva wordt verkregen met eluenten bestaande uit ethylacetaat, n-hexaan en methylamine op een silicagel kolom. Het retentie gedrag van de komponenten wordt geregeld door de concentratie van n-hexaan, methylamine en het water gehalte van ethylacetaat.

Voor complexe monsters is de combinatie van optimale

selectiviteit en maximale efficiency vaak nog onvoldoende om volledige scheiding te bewerkstelligen. Het gebruik van selectieve detectie methoden kan dan noodzakelijk zijn. In deel III wordt dit beschreven. De gevoeligheid en selectiviteit van de detectie van vele stofklassen door standaard vloeistofchromatografische detectoren, zoals spectrofotometers, fluorometers en coulometrische cellen, kan aanzienlijk verbeterd worden door de uitgang van de chromatografische kolom te koppelen aan een chemisch reactie systeem. Dit wordt practisch uitgevoerd door continu een reagens aan het kolom effluent toe te voegen en voortdurend het reactiemengsel door de detector te leiden.

In dit proefschrift wordt aangetoond dat additionele piekverbreding in de reactor en het resulterende verlies in scheidend vermogen tot een acceptabel niveau beperkt kan blijven door een geschikt ontwerp van het reactie systeem.

Twee typen reactoren worden beschreven: buis reactoren en gepakte reactoren (gepakt met niet-poreuze inerte glasbollen). Karakteristieke eigenschappen van deze reactoren worden geëvalueerd en vuistregels voor een optimaal ontwerp worden gepresenteerd.

De dispersie mechanismen in een gepakt bed zijn moleculaire diffusie en convectieve menging, meestal eddy diffusie genaamd. Deze eddy diffusie wordt hoofdzakelijk bepaald door geometrie parameters; de afschatting van deze geometrie parameters is van groot belang voor de beschrijving van piekverbreding en dus voor het ontwerp van gepakte reactoren.

Een geschikte keuze van reactor lengte en deeltjesgrootte van de glasbollen moet worden gemaakt voor een bepaalde combinatie van reactietijd en toegestane extra piekverbreding in de reactor. Een extra restrictie in de practijk is de maximaal toelaatbare drukval over de reactor. Geschikte condities worden gevonden in het minimum van de drukval-deeltjesgrootte curve (bij constante waarde van de verblijftijd en de additionele piekverbreding in de reactor), waarbij acceptabele reactorlengtes verkregen worden.

Vanwege een secundaire stroom is de axiale dispersie in een schroefvormig gekromde buis kleiner dan in een rechte buis. Het quotient van de dispersiecoefficient in een helix en de dispersiecoefficient in een rechte buis is gerelateerd aan de dimensieloze groep $DnSc^{\frac{1}{2}}$ door één enkele curve. Deze relatie maakt het mogelijk de piekverbreding in een gekromde buis reactor te voorspellen. Zoals bij gepakte reactoren dient een geschikte keuze voor de inwendige diameter, lengte en krommingsgraad gemaakt te worden, om een bepaalde reactietijd te verkrijgen en de maximaal toegestane piekverbreding en drukval niet te overschrijden. Weinig piekverbreding wordt verkregen voor buizen met een kleine inwendige diameter en een hoge krommingsgraad.

Criteria voor de keuze voor een reactortype hangen samen met de reactie snelheid en de complexheid van de betreffende reactie. Het blijkt dat voor snelle reacties (verblijftijden kleiner dan 5 min) gepakte bedden de kleinste additionele piekverbreding geven, terwijl de axiale dispersie in schroefvormig gekromde buizen relatief groot is. Daarom dienen buisreactoren alleen dan gekozen te worden, wanneer gebruik van gepakte reactoren door de reactie condities onmogelijk is. Voor langzame reacties (verblijftijden groter dan 5 min) zijn noch gepakte reactoren noch buisreactoren geschikt, tenzij hoge waarden voor de extra piekverbreding acceptabel zijn; in die gevallen kunnen gas-gesegmenteerde vloeistof stromen in overweging genomen worden.

Het gebruik van een gepakte reactor in combinatie met een colorimetrisch detectiesysteem wordt beschreven voor de analyse van hydroperoxiden. Tevens wordt de derivatisering van aminozuren tot fluorescerende producten in een gepakte reactor door een reactie met *o*-ftaalaldehyde beschreven. Een buisreactor wordt gebruikt om de electochemische detectie van reducerende suikers mogelijk te maken. Gegevens over piekverbreding en minimaal aantoonbare hoeveelheden voor de genoemde reactie systemen worden vermeld.

PART I

INTRODUCTION

It is well established that by any standard the analytical technique of chromatography is one of the most powerful that has ever been developed. The isolation of species from complex mixtures requires such efficient separation methods. During the last two decades column liquid chromatography[1-8] has developed into a position where it can offer highly efficient and fast separations comparable to those of gas chromatography, although the capability of capillary gas chromatography has not yet been reached.

The most recent developments in column liquid chromatography have been made in the direction of faster separations, optimization of column design and operating parameters, improvement of the quantitative aspects of the technique and more sensitive and specific detectors.

The ability of any chromatographic technique to perform separations is expressed in the resolution R. The resolution is a measure of separation of two adjacent peaks and is defined as:

$$R_{1,2} = \frac{\Delta t_R}{2(\sigma_{t,1} + \sigma_{t,2})} = \frac{\Delta t_R}{2\sigma_{t,2}(n + 1)} \qquad (I.1)$$

where $\Delta t_R = t_{R,2} - t_{R,1}$ is difference of retention time of peak 1 and peak 2 and $\sigma_{t,1}$ and $\sigma_{t,2}$ are the standard deviations of the elution functions in time units and $n = \frac{\sigma_{t,1}}{\sigma_{t,2}}$.

Assuming equal band widths (n=1) a relationship can be derived between resolution and three fundamental chromatographic parameters:

$$R_{1,2} = \frac{1}{4} \frac{k_2}{k_2 + 1} \frac{r_{2,1} - 1}{r_{2,1}} \sqrt{N_2} \qquad (I.2)$$

As can be seen from Equation (I.2) the resolution depends upon both the selectivity r and the efficiency N of the chromatographic system and on the capacity factor k. The former is expressed in terms of the selectivity factor, r, defined as:

$$r_{2,1} = \frac{k_2}{k_1} = \frac{K_2}{K_1} \qquad (I.3)$$

where k_1 and k_2 are respectively the capacity factors of the components 1 and 2. The capacity factor, k, is the ratio of the times spent by a compound in respectively the stationary and the mobile phase. The ratio of the concentrations of a component in mobile and stationary phase is defined as the distribution coefficient K. More fundamentally, r is equal to the ratio of distribution coefficients. Column efficiency is generally expressed as the theoretical plate number N, defined as:

$$N = \left(\frac{t_R}{\sigma_t}\right)^2 \qquad (I.4)$$

or the related quantity height equivalent to a theoretical plate, H, defined as:

$$H = \frac{L}{N} \qquad (I.5)$$

where L is the column length.

While both selectivity and efficiency are important, achievement of large selectivity factors is fundamentally more critical in liquid chromatography. The equation for the resolution shows that the degree of separation of two peaks increases with $N^{\frac{1}{2}}$. Broadly, the number of plates depends on operating conditions such as eluent flow-rate, packing particle size, stationary phase loading and so forth, in short on the dynamics of the system. In liquid chromatography the number of theoretical plates that can be obtained is limited to about 30,000 (for a 50 cm column) whereas in gas chromatography up to 500,000 plates (for a 150 m open tubular column) are reported.

The second parameter in Equation (I.2) which determines resolution is the capacity factor for a component. The

resolution improves as k increases until, at larger values of k, the factor $\frac{k}{k+1}$ goes to unity and therefore no longer plays a role in resolution. A consequence of large k values is a large analysis time. In high speed analysis k is small and the factor $\frac{k}{k+1}$ must be considered. A compromise between analysis time and resolution must obviously be made, that is, there is an optimal k value ($k = 2$)[9].

Relative retention, or the selectivity factor, $r_{2,1}$, on the other hand, is an equilibrium property of a specific system, independent of all its extensive properties. Basically, to increase r, it is necessary to change one of the chromatographic phases at least, so as to improve the selectivity of the system. In contrast to gas chromatography, where interactions in the gas phase are negligible, in liquid chromatography r is determined by the composition of both the stationary as well as the mobile phase. A main goal of research in liquid chromatography is the selection of appropriate phase systems for a given separation problem.

In liquid liquid chromatography (LLC) selectivity is determined by the relative solubility of the solutes in the two immiscible phases, which are usually prepared from binary or ternary liquid-liquid systems. Secondary effects on retention are caused by the support. Two variations exist in LLC. In straight phase LLC the most polar phase is used as stationary liquid and in reversed phase LLC the most apolar phase is the stationary one.

In liquid solid chromatography (LSC) the same phase variation is possible. Using a polar support and an unpolar solvent, selectivity is governed by the relative strength of interactions between the solute molecules and the surface of the support. In reversed phase LSC, however, using an unpolar support and a polar solvent, these interactions are very weak and r is mainly determined by the solubility of the solutes in the mobile phase. This last mode of operation is commonly performed on chemically bonded stationary phases. In LSC the mobile phase is in selective competition with solute molecules for adsorption sites on the adsorbent.

In ion exchange chromatography (IEC) r depends on a set of parameters such as the type of ion-exchange matrix, its poor structure and its degree of crosslinking, respectively the type, surface concentration and distribution of functional groups, the type of the eluent ion, its concentration, the ionic strength and pH-value of the eluent, the temperature.

Selectivity in ion pair chromatography (IPC) is controlled not only by the volume ratio of the two phases and the temperature, but also by the type and concentration of the counter ion, the type and composition of the organic mobile phase, the ion strength of the aqueous stationary phase in straight phase chromatography and the pH of this aqueous phase. Ion pair chromatography can also be performed in a reversed phase and in an adsorption mode.

Improvement of selectivity in general is limited when the samples to be analysed are getting more and more complex. Some additional selectivity is obtained by extraction procedures of the sample. Finally the situation is reached where improvement of chromatographic selectivity of one solute pair results in a decrease of selectivity of an other pair of components. Then increase of the efficiency of the separation column is the only way to improve separation. The major reason why classical liquid chromatography has been so successful was the sufficiently high values of r, but it was dealing with relatively simple separation problems.

Within the last decades, there has been a revival of interest in liquid chromatography, resulting primarily from the theoretical understanding of gas chromatography, which have greatly speeded up separations and improved the efficiency of the columns. If the combination of the advantages of high r values with high efficiencies is still insufficient, the only possibility for analysis left, is the use of selective detectors.

An extremely selective detection method is based on reaction detection. The reaction is performed on line in a flow reactor in front of the detection device. Such a

reaction detector generally also has the advantage of decreasing the detection limits.

In Part II the improvement of chromatographic selectivity is demonstrated for extracts from biological material; cortisol in plasma and tricyclic antidepressant drugs in plasma. Also a comparison is made with very selective and sensitive determination methods (radio immuno assay (RIA), competitive protein binding (CPB and fluorometry), which determine directly in the bulk without prior profiling the mixture by means of a chromatographic separation procedure. This comparison is made with respect to quantitative analysis.

In Part III the performance and characteristics of packed bed reactors and tubular reactors are discussed. Rules for optimum design are given and some chromatographic applications of flow reactors are shown.

PART II

IMPROVEMENT OF CHROMATOGRAPHIC SELECTIVITY — APPLICATION TO THE ANALYSIS OF SAMPLES OF BIOLOGICAL ORIGIN

Parts have already been published elsewhere:

J.H.M. van den Berg, Ch.R. Mol, R.S. Deelder, J.H.H. Thijssen, "A quantitative assay of cortisol in human plasma by high performance liquid chromatography using a selective chemically bonded stationary phase", Clin. Chim. Acta, 78 (1977) 165.

J.H.H. Thijssen, J.H.M. van den Berg, H. Adlercreutz, "The determination of cortisol in human plasma. Evaluation and comparison of seven assays". Submitted for publication in Clin. Chim. Acta.

J.H.M. van den Berg, J. Milley, N. Vonk, R.S. Deelder, "Mechanisms of separation using the ternary mixture dichloromethane-ethanol-water in high performance liquid chromatography", J. Chromatogr., 132 (1977) 421.

J.H.M. van den Berg, H.J.J.M. de Ruwe, R.S. Deelder, Th.A. Plomp, "Column liquid chromatography of tricyclic antidepressants", J. Chromatogr., 138 (1977) 431.

1. A selective chemically bonded stationary phase for the quantitative assay of cortisol in human plasma.

1.1 Introduction.

Adequate information concerning the function of steroid-producing endocrine organs and the interaction of the hypothalamic-hypophyseal system with these organs can be derived from estimations in urine and in plasma. Steroid analyses are applied regularly during diagnosis and control of therapy in cases of hypo-, hyper-, and dysfunctions of the adrenal cortex and the gonads.

A determination of corticosteroids in human plasma, demands that specificity is combined with high sensitivity. For single components these conditions can be fulfilled by very specific, analytical methods like radio immuno assay (RIA), competitive protein binding (CPB) and to some extent by fluorometry. A comparative study will be given in Section II.1.5. When different steroids have to be determined in one run, separation of the complex mixture prior to detection is a necessity. Chromatography is the separation method of choice. In this study high performance liquid chromatography is selected for the analysis of cortisol in plasma. The presence of synthetic steroids like dexamethasone, used in diagnosis and therapy, should not interfere during the analysis. Chromatographic parameters and extraction methods should be optimized to determine low concentrations (about 0.28 µmole cortisol is present in 1000 ml plasma) with reasonable reliability. In addition it is desirable not to overburden the patient by requiring a large amount of blood.

1.2 Separations of corticosteroids by column liquid chromatography.

For test mixtures of corticosteroids Fitzpatrick *et al.* [10] described pre-column benzoylation of the non-UV-absorbing hydroxy steroids. This aim was the improvement of

detection-selectivity and detection-sensitivity for these compounds. Henry *et al.*[11] proposed the use of 2,4 dinitrophenylhydrazones as derivatives. This derivative may be used as an alternative to benzoate esters.

With respect to chromatographic selectivity several investigations were performed on test mixtures. Liquid-liquid partition mechanisms were studied in order to find a method for the prediction of partition coefficients[12,13]. Tymes[14] reported the reversed phase chromatography of steroids and gave retention data on 28 corticoids and related steroid analogs on a chemically bonded phase.

In addition to the literature cited above, numerous papers on the subject of separations of corticosteroids by high performance liquid chromatography appeared. Only a limited number dealt with the quantitative analysis of cortisol in plasma. Meijers *et al.*[15] used liquid liquid chromatography for the analysis of cortisol but at that time efficient columns were not available, so separation and detection limit were not optimal. A practical disadvantage of this method is the low solubility of corticosteroids in the mobile phase. Further, the high value of the partition coefficients required the use of packing materials of low specific area. Hesse and Hovermann[16] used an apolar layer of a ternary mixture of dichloromethane, ethanol and water as the eluent in a number of separation problems. In particular, they described a method for the determination of some corticosteroids in plasma, this eluent being a good solvent for corticosteroids[17,18]. It is assumed that a conjugated phase is deposited on the packing and that separations are based on partition between the apolar eluent and the polar conjugated phase in the pore system of the support (phase systems corresponding to points on the binodal curve of the phase diagram, see Figure II.1). For the chromatographic determination of cortisol in serum, it was decided to evaluate this method. However, columns were found to be unstable and a gradual decrease in the capacity ratios was observed by van den Berg *et al.*[19].

Similar negative results were obtained by Parris[20]. Good and stable separation conditions[19,181], however, could be obtained by working under adsorption chromatographic conditions. Therefore eluents were applied corresponding to points outside the miscibility gap of the three solvents (region 1 of Figure II.1). Efficient separations of test mixtures of steroids[19] were obtained as shown in Figure II.2.

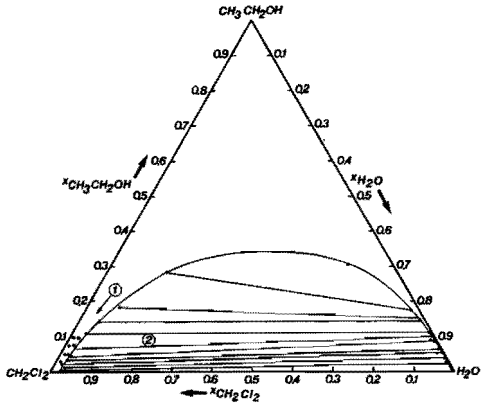


Figure II.1. *Phase diagram for the ternary system dichloromethane-ethanol-water at ambient temperature.*

In 1973 Touchstone and Wortmann[21] described the first liquid solid chromatographic assay of cortisol in plasma. Recently Loo *et al.*[22] and Schwedt *et al.*[23] reported on such systems.

Chemically bonded phases have not got the disadvantages as mentioned above. Wortmann *et al.*[24] used a reversed phase chromatographic procedure. Therefore it seemed useful to develop a liquid chromatograhic assay of cortisol in human plasma using a selective chemically bonded stationary phase on silica.

In our laboratory silicagel (LiChrosorb SI 60, Merck, Darmstadt, GFR) was chemically modified according to a method described by Brust *et al.*[25]. Irregular silica, with a mean particle diameter of 5 µm, was chlorinated by

thionylchloride. The product was dried and subjected to a reaction with *para*-nitroaniline. This slightly polar packing proved to be selective for steroid analysis and further investigations on plasma level were performed.

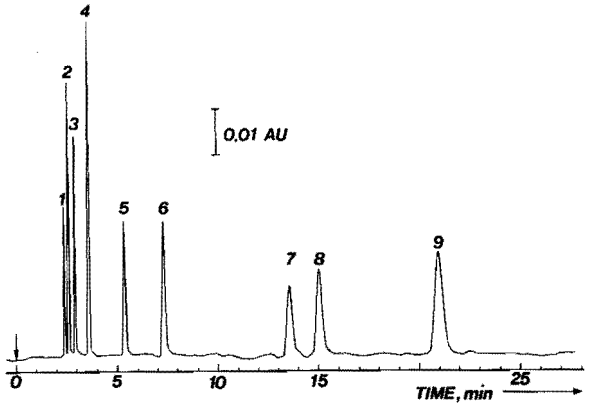


Figure II.2. *Chromatogram of a test mixture of steroids. Conditions: column, stainless-steel 316,300 x 4.6 mm I.D.; adsorbent, LiChrosorb SI 60, d_p = 5 µm; mobile phase, $x_{dichloromethane}$ = 0.940, $x_{ethanol}$ = 0.050, x_{water} = 0.010, (x = mole fraction); volume flow-rate, 1.5 cm^3/min; u = 0.2 cm/s; UV detection at 240 nm, 0.1 a.u.f.s. Peaks: 1 = monochlorobenzene; 2 = progesterone; 3 = 11-desoxycorticosterone; 4 = testosterone; 5 = corticosterone; 6 = cortisone; 7 = dexamethasone; 8 = cortisol; 9 = prednisolone.*

During these investigations a similar packing material became commercially available. Nucleosil NO_2 (Machery and Nagel, Dueren, GFR), a chemically modified spherical silica, was preferred in the later experiments.

The separation was optimized with a test mixture by varying the mobile phase composition in such a way that the capacity ratio of cortisol was about 5. Cortisol is separated from other substituents in the plasma extract without the loss of to much sensitivity. The selectivity of the chosen phase system, Nucleosil NO_2 and modified adsorbent and dichloromethane-2-propanol- water (97.5: 2.3: 0.2, v/v) as eluent is given in Table II.1.

It is practicable to perform analyses of cortisol without interference by dexamethasone. Prednisolone can be used as internal standard.

Recently it has been shown that spherical as well as irregular particles can be packed regularly to obtain efficient and reproducible columns with a balanced-density-slurry packing procedure. Short elution times and low heights equivalent to a theoretical plate have been achieved. Our columns gave plate heights of 30 µm for cortisol and prednisolone.

Table II.1. *Capacity ratios on Nucleosil NO_2 and p-nitroaniline modified LiChrosorb SI 60. Mobile phase: dichloromethane-2-propanol- water (97.5 : 2.3 : 0.2, v/v).*

Compound	Modified silica		Nucleosil NO_2	
	k	$r_{x,cortisol}$	k	$r_{x,cortisol}$
11-Desoxycorticosterone	0.37	0.07	0.2	0.04
Testosterone	0.86	0.15	0.3	0.06
Corticosterone	1.78	0.31	1.04	0.21
Cortisone	2.53	0.44	2.14	0.44
Cortisol	5.73	1	4.88	1
Dexamethasone	5.15	0.90	8.06	1.65
Prednisolone	8.95	1.56	8.84	1.81

1.3 Experimental.

Equipment.

A liquid chromatograph (model 771, Kipp & Zn., Delft, The Netherlands) was used in this study. A variable wavelength UV detector (type PM2D LC, Zeiss, Oberkochen, GFR) was operated at 240 nm. Column and detector cell were thermostatted at ambient temperature by a circulating liquid.

In addition a liquid chromatograph (type 8500, Varian, Palo Alto, USA.), equipped with an injection valve

(type HPSV 20, Spectra Physics, Berkeley, USA.), and an UV detector (type Variscan, Varian) was used. Columns were filled by the balanced-density-slurry method[121-123].

Chemicals.

Dichloromethane, 2-propanol and ethanol, all *p.a.* grade were purchased from Merck.

Sodium hydroxide solutions of 0.25 M were prepared.

Standard samples of cortisol and prednisolone were prepared in ethanol at a concentration of 10 µg/ml.

Dichloromethane for the extraction of the plasma samples was cleaned over an Al_2O_3 column (Woelm, Eschwege, GFR., basic, activity grade I).

Extraction procedure.

The extraction of corticosteroids from plasma was carried out with dichloromethane, which was washed over Al_2O_3.

To 1 ml plasma 7 ml dichloromethane was added and 20 µl internal standard (corresponding to 200 ng prednisolone).

At the same time 0.1 ml 0.25 M NaOH solution was added to exclude phenolic contaminations from the extract[26].

This mixture was stirred for 30 s in a vortex mixer. Then the water phase was separated by centrifugation (1000 *g*).

5 ml of the aliquot is transferred and evaporated to dryness under warm nitrogen gas (40°C.). The residue is dissolved in 100 µl eluent.

After 10 min in an ultrasonic bath the sample was homogeneous and could be injected into the liquid chromatograph (60 µl). The value of the injection volume is restricted because of the contribution to peak-broadening should not exceed certain limits. On the other hand, the injection volume should be large to ensure a minimal dilution, as it has been pointed out by several workers[27,28] (see also Section III.1.4).

1.4 Results and discussion.

Extraction recovery.

Extraction procedures were performed with ethyl acetate, diethyl ether and dichloromethane as solvents. The extraction was judged by means of measuring recovery of cortisol and prednisolone. Cortisol was quantitated by measuring peak heights and correcting against the internal standard. Calibration curves without the extraction procedure were determined with test mixtures; calibration curves including an extraction step with 1 ml pool-plasma samples to which increasing amounts of cortisol and prednisolone were added, were also determined. The ratio of the slopes of the calibration curves with and without extraction gives the recovery. Table II.2 presents the recovery of cortisol and prednisolone for the solvents mentioned. From these results it is apparent that the extraction with dichloromethane is to be preferred. Figure II.3 gives the calibration curves for cortisol and prednisolone in standard mixtures and plasma samples for the extraction with dichloromethane.

Washing with sodium hydroxide has no significant influence on recovery, but the purity of the sample is increased[19].

Table II.2. *Recovery of cortisol and prednisolone for some solvents.*

Solvent	Recovery cortisol %	Recovery prednisolone %
Ethyl acetate	89	82
Diethyl ether	78	72
Dichloromethane	96	93

Figures II.4 and II.5 show the chromatograms obtained with the Nucleosil NO_2 and nitroaniline-modified silicagel, respectively.

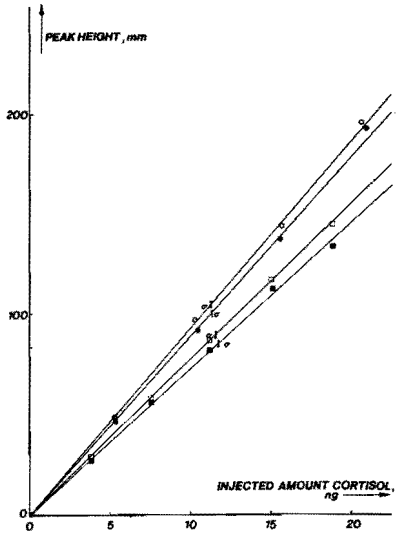


Figure II.3. *Calibration curves.* O, *cortisol test mixtures;* ●, *cortisol extracted from plasma;* □, *prednisolone test mixtures;* ■, *prednisolone added to plasma and extracted from plasma.*

Repeatability.

The precision of the method has been determined by the mean standard deviation per point of duplicate measurements. For each determination the whole procedure consisting of extraction and chromatographic analysis was carried out. 23 duplicate measurements gave a relative standard deviation of 3.3% (Table II.3).

Biological spreading of the cortisol content in plasma has been eliminated in this way.

Table II.3. *Plasma cortisol values in duplo of some randomly selected patients.*

Subject	Plasma-cortisol, µmole/l	
1	0.330	0.358
2	0.338	0.371
3	0.594	0.619
4	0.671	0.660
5	0.567	0.602
6	0.638	0.630
7	0.792	0.770
8	0.402	0.429
9	0.424	0.424
10	0.457	0.402
11	0.968	0.993
12	0.487	0.473
13	0.616	0.622
14	0.399	0.382
15	0.385	0.366
16	0.264	0.261
17	0.558	0.578
18	0.550	0.572
19	0.413	0.396
20	0.314	0.294
21	0.644	0.663
22	0.272	0.259
23	0.349	0.338

Mean 0.498 µmole/l; mean standard deviation per point 0.017 µmole/l; number of duplicates 23.

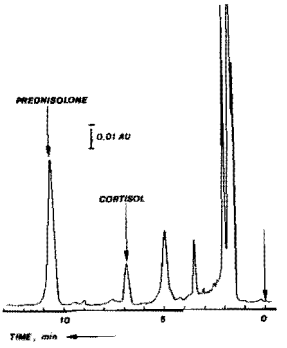


Figure II.4. *Chromatogram of plasma sample. Column: 200 x 4.6 mm I.D.; Nucleosil NO_2; d_p = 5 µm. Eluent: dichloromethane-2-propanol- water (97.5:2.3:0.2, v/v); flow-rate 1.15 ml/min. Detection: UV 240 nm.*

Confirmation of the identity.

To show the selectivity of the liquid chromatographic method a chromatogram of a plasma of a patient treated with dexamethasone was made. Figure II.6 shows the result; the cortisol content is decreased drastically by this treatment to 0.069 µmole cortisol/l plasma.

Figure II.7 demonstrates the results that can be obtained by the off-line coupling of liquid chromatography and field desorption-mass spectrometry (FD-MS).

The cortisol was collected as it eluted from the liquid chromatographic column and the wire emitter was submerged in the solution, containing 10^{-5} g cortisol/ml. The FD-MS of cortisol confirms the identification of the peak in the chromatogram of a plasma extract.

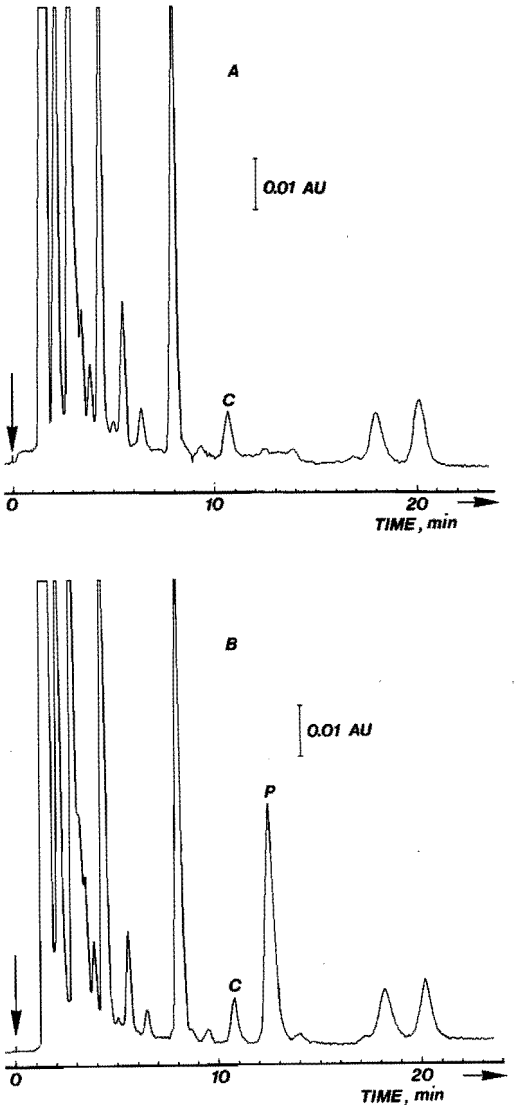


Figure II.5. *Chromatogram of plasma sample: A, without addition of internal standard; B, with addition of internal standard. Column: 220 x 2.1 mm I.D.; modified LiChrosorb SI 60; d_p = 5 µm. Eluent: dichloromethane-2-propanol-water (97.5:2.3:0.2, v/v); flow-rate: 25 ml/h. Detection: UV 240 nm. Sample: C = cortisol; P = prednisolone.*

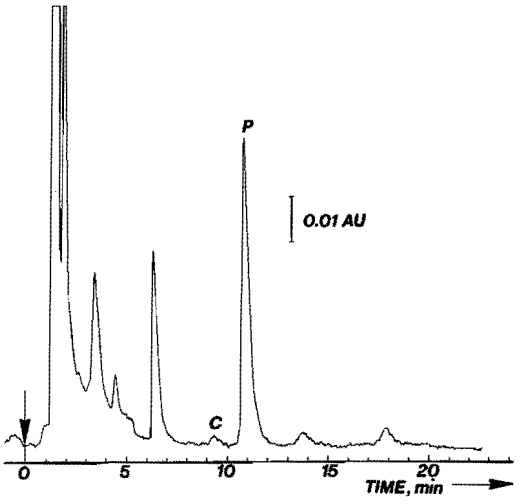


Figure II.6. *Chromatogram of a plasma sample of a patient treated with dexamethasone. Conditions see Figure II.5.*

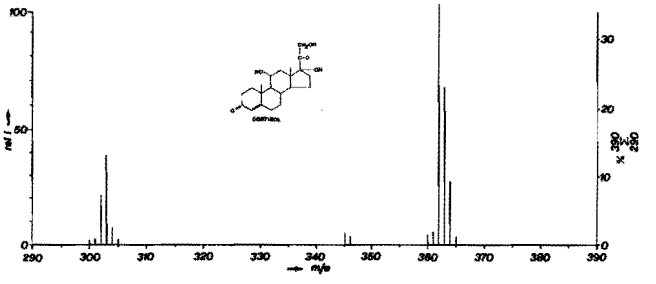


Figure II.7. *Field desorption-mass spectrum of cortisol. Equipment: Varian Mat 711; Emitter 10 µm heated by a current of 15 mA; source temperature 75°C.*
The FD-MS experiment is performed by Dr. J. van der Greef, University of Amsterdam.

1.5 The determination of cortisol in human plasma. Evaluation and comparison of seven assays.

1.5.1 Introduction.

For the estimation of cortisol concentrations in plasma many different methods have been published, in which fluorometry, colorimetry, double isotope derivative, competitive protein binding and radio immuno techniques were used. Since it is not easy to evaluate the practibility of all these techniques and only a limited number of comparative studies[29-34] are available a study was undertaken to compare a number of clinical useful assays. Here comparison on seven different assays will be described. Three of these do not rely on the use of radioactivity and four are using radioactive cortisol.

The methods which were compared consisted of:

Method 1: Fluorometric method. The technique as recommended by a Working Party of the British Medical Research Council was used as described previously[35,36].

Method 2: Modified, more specific fluorometric method, as described by Clark[37,38].

Method 3: High performance liquid chromatography, as recently reported[39]. (See Section II.2).

Method 4: Competitive protein binding technique, the modification as described by de Jong *et al.*[40].

Method 5: Solid phase radio immuno assay[41].

Method 6: Radio immuno assay using ^{125}I-cortisol[43].

Method 7: Direct radio immuno assay in diluted plasma, according to the method described by Foster *et al.*[42] and Thijssen *et al.*[43], using ^{3}H-cortisol as tracer.

Method 6 and method 7 have been described and evaluated by Thijssen *et al.*[43].

1.5.2 Comparison.

For the comparison of the different assays, plasma samples obtained during clinical investigations of

patients were used. No selection has been used during this procedure, random samples were taken for the tests. After collection and in the time between assays the samples were stored at -20°C. Samples were distributed frozen and were transported in this way.

In the comparative study using routinally obtained patient samples, no absolute method was available to measure the true cortisol content of each sample. Because the direct ^{3}H-radio immuno assay (method 7) has been compared in different series of plasma samples arbitrarily the values obtained with method 7 have been used in the statistical evaluation as the "independent variabel", as the reference method. In Table II.4 are shown the results of the comparative study. In this table the number of plasma samples assayed with the two compared techniques is given, the correlation coefficient, the intercept (± standard deviation) and the slope (± standard deviation) are given, compared with method 7, the direct ^{3}H-radio immuno assay. In addition, the calculated mean error per point is given.

From the individual points the regression lines were calculated by a least square method. The fitting function was composed of a constant and a first order term. Furthermore, the mean distance from each individual point to the fitted regression line was calculated (mean error per point), the correlation coefficient and the standard deviation of the fitting coefficients.
From the figures in Table II.4 given, there seems to be a fairly good correlation between the results obtained with the commonly used fluorometric method (method 1). The slope of the calculated regression line is not significantly different from unity. However, although we are dealing with a relative large number of samples, the estimated mean error per point is large. In addition, there is a highly significant intercept of 0.17 ± 0.02 µmole/l of the ordinate. Especially the intercept is an indication of the problems encountered with the fluorometric method because,except for the fluorescence given

Table II.4. *Correlation between the various methods that have been compared.*

method	number of estimations	slope (± s.d.) of the regression line	intercept ± s.d., µmole/l	correlation coefficient	mean error per point, µmole/l
1	288*	0.98 ± 0.032	0.171 ± 0.018	0.90	0.174
2	25	0.95 ± 0.033	0.013 ± 0.012	0.99	0.039
3	58	1.05 ± 0.055	0.102 ± 0.023	0.93	0.088
4	39	0.80 ± 0.034	0.065 ± 0.022	0.97	0.081
5	24	1.10 ± 0.057	0.060 ± 0.034	0.97	0.108
6	69	1.13 ± 0.032	0.012 ± 0.016	0.97	0.102

Arbitrarely the direct ^{3}H-radio immuno assay (method 7[42,43])has been taken as the independent variable.

* Three values have not been included in the calculations (see Figure II.8.A).

method 1: fluorometric[35,36]

method 2: modified fluorometric[37,38]

method 3: high performance liquid chromatography[39]

method 4: competitive protein binding[40]

method 5: solid phase radio immuno assay[41]

method 6: ^{125}I-radio immuno assay[43]

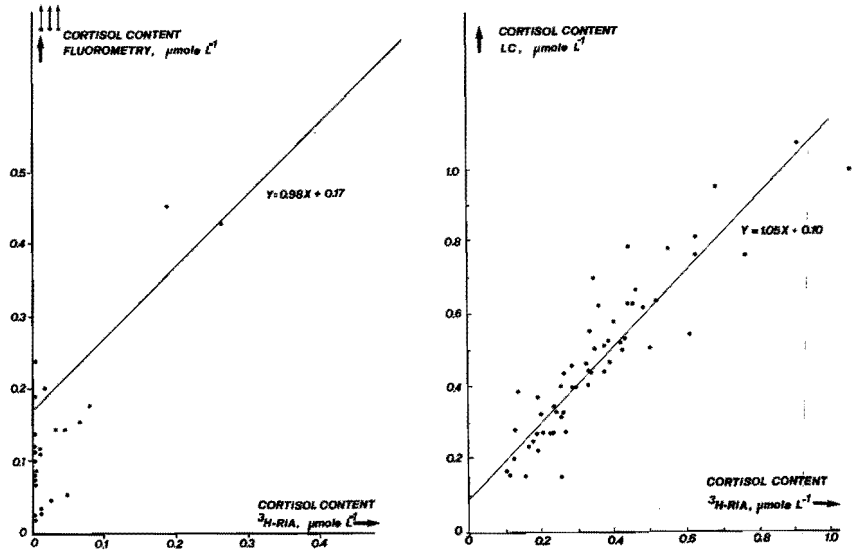


Figure II.8.A. *Cortisol content in plasma measured by fluorometry v.s. cortisol content in plasma measured by* ^{3}*H-radio immuno assay.*

Figure II.8.B. *Cortisol content in plasma measured by liquid chromatography v.s. cortisol content in plasma measured by* ^{3}*H-radio immuno assay.*

by steroids other than cortisol *i.e.* corticosterone, the interference of the assay by widely-used common pharmaceutics may be serious. To illustrate this problem, in Figure II.8.A are shown the cortisol values obtained after suppression by dexamethasone, as estimated by method 1 and 7. In 3 cases (indicated as ↕ in Figure II.8.A). The interference is evident from the highly increased levels measured by fluorometry, but in a number of cases, the interference is fairly large but disturbance can not easily by judged from the values obtained. The main troubles with the fluorometric estimation are caused by commonly used tranquilisers,derived from diazepam. The interference with the cortisol assay can be explained by

the properties of the substances, they show a clear fluorescence under the conditions used for cortisol[44]. The more specific and more time-consuming fluorometric method ad described by Clark (method 2) shows a more satisfactory correlation and a good regression with the ^{3}H-radio immuno assay. As well the slope as the intercept are not significantly different from 1 and 0 respectively.

The third method compared is a liquid chromatographic method (see Figure II.8.B). The slope of the regression line is not different from unity. The calculated intercept is puzzling because of the separation ability of the chromatographic step and the selective UV detection method employed. We have no explanation therefore for the intercept. The correlation coefficient with ^{3}H-radio immuno assay was satisfactory, it has values between the correlations seen with method 1 and method 2.

Method 4, 5 and 6 have the use of radioactive cortisol in common. Of these methods the competitive protein binding (method 4) shows the least satisfactory results. Although the correlation coefficient is almost equal to those found for method 5 and 6, both the slope and the intercept of the regression line are significantly different from 1 and 0 respectively. Again, these results are different from the ones expected while with the protein used for the estimation, more cross-reacting steroids may be present in plasma samples obtained from patients.

The two other radio immuno assays (method 5 and 6) which were investigated agree well with the ^{3}H-radio immuno assay. In both instances no significant intercept is seen, with the ^{125}I-cortisol estimation, a slope, slightly different from unity was observed.
Because differences in the standard used could be excluded, the questions remain unanswered whether a difference in specifity, due to the tracers used, might be important.

1.5.3 Discussion.

One of the purposes of this study was an evaluation of the practicability of various methods which can be used for the estimation of cortisol in human plasma. The first practical decision that must be made is whether radioactive labels may of may not be used. In case one can use radioactive tracers, the choice is obvious: performing a radio immuno assay offers several advantages *i.e.* small plasma sample required, simplicity and usually specificity, depending on the antiserum used. Although the competitive protein binding is a good alternative, larger variability of this method and less specificity are disadvantages. Making a choice between radio immuno assays based on the use of ^{3}H-cortisol or of ^{125}I-methyl-tyrosine-cortisol depends on:

a. availability of equipment for the measurement of radio activity.
b. the ^{125}I-cortisol can not be prepared easily. Therefore usually the commercially available tracer is used. At least in Europe, the quality of this tracer did show large variations. Moreover, the stability is less, partly due to the relative short half-life time of the ^{125}I.

For laboratories who are not planning to use radioactive tracers of cortisol for the estimation of the results of three different methods are offered.
The widely used relatively simple fluorometric method can hardly be recommended because of:

a. necessity of using agressive chemicals,
b. moderate specificity towards cortisol,
c. non-specific fluorescence due to plasma components,
d. interference of several drugs in the assay.

Especially the interference can lead to highly misleading results. The more specific fluorometric method of Clark does not have all the disadvantages mentioned above. The disadvantage left is the use of the agressive chemicals and more over more time is required to perform the analysis in a routine case.

The liquid chromatographic method offers a specific estimation only for these laboratories who have the technical facilities to perform this form of chromatography. Especially the separation power gives the method a special place as a reference method and in addition it offers the possibility of determining several components (*e.g.* prednisolone) during the same analysis.

2. Column liquid chromatography of tricyclic antidepressants. Optimation of phase systems.

2.1 Introduction.

Tricyclic antidepressants (TCA) are widely used for the treatment of anxiety and depression and it is not surprising that the incidence of overdosage with these drugs has considerably increased. In order to develop an adequate therapeutic treatment in cases of overdose or in chronic treatment of endogene depression a fast, selective and sensitive method for the analysis of TCA in plasma is required.

2.2 Separations of tricyclic antidepressants by column liquid chromatography.

The application of column liquid chromatography for the analysis of TCA has many advantages over gas-liquid chromatography[45-48], thin-layer chromatography[49,50], and spectrophotometric techniques[51,52]. Knox and Jurand[53] examined the application of amineperchlorate ion pairs on silica gel with dichloromethane and a higher aliphatic alcohol (n-butanol or isoamyl alcohol) as eluent, while Persson and Lagerström[54] preferred methanesulphonic acid as the stationary phase and dichloromethane,n-hexane and n-butanol as the mobile phase in ion pair partition chromatography. Mellström and Eksborg[55] chromatographed chlorimipramine, desmethylchlorimipramine and trimipramine on a diatomaceous earth coated with a mixture of hydrochloric acid and tetraethylammonium chloride as the stationary phase. The mobile phase was a mixture of n-hexane and isobutanol. Ion pair chromatography in an adsorption mode was performed on a chemically modified silicagel by Knox and Pryde[56] and by Westenberg[64]. Adsorption chromatography of TCA on alumina[53] and silicagel[57-64] was reported by several workers. The separation and determination of amitriptyline and some of its most

important metabolites in plasma, using a reversed phase system, was described by Kraak and Bijster[65]. The influence of the content of dichloromethane and methanol, type and content of base in the eluent was investigated. Similar procedures based on reversed phase systems were developped by Brodie *et al.*[66] and Salmon and Wood[67].

The aim of this study was to develop a complete separation of all TCA by liquid solid chromatography on silicagel using a versatile mobile phase system. The procedure should also permit the routine determination of TCA in plasma samples.

2.3 Experimental.

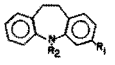
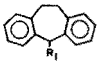
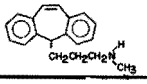
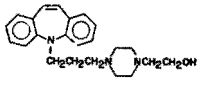
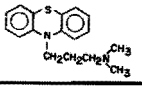
Apparatus, chemicals and materials.

The liquid chromatograph was constructed from custom-made and commercially available parts[68].

In all experiments, organic solvents of analytical grade (Merck, Darmstadt, GFR.) were used, including the 35% aqueous solution of methylamine. Solvents were degassed by ultrasonication immediately before use. LiChrosorb SI 60 and Lichrosorb Alox-T (Merck) with a mean particle diameter of 5 µm were used as packing materials. The names and formulae of the tranquillizers are listed in Table II.5.

The columns were packed by using the balanced-density-slurry-technique. Mixtures of ethyl acetate (or dichloromethane), n-hexane and methylamine were used as eluents. Capacity ratios (k) were calculated from the retention times of the components and an unretarded compound (monochlorobenzene). The samples were dissolved in the eluent and injected by means of a sampling valve device. The volume of the loop was varied during the experiments.

Table II.5. *Structures of TCA.*

Dihydrodibenzoazepines		
	R_1	R_2
Imipramine (IMI)	-H	$-CH_2CH_2CH_2N(CH_3)_2$
Desipramine (DESI)	-H	$-CH_2CH_2CH_2NH(CH_3)$
Trimipramine (TRIMI)	-H	$-CH_2CH(CH_3)CH_2N(CH_3)_2$
Clomipramine (CLOMI)	-Cl	$-CH_2CH_2CH_2N(CH_3)_2$
Dibenzocycloheptanes		
	R_1	
Amitriptyline (AMI)	$=CHCH_2CH_2N(CH_3)_2$	
Nortriptyline (NOR)	$=CHCH_2CH_2NH(CH_3)$	
Dibenzocycloheptene		
Protriptyline (PRO) 		
Others		
Opipramol (OPI) 		
Promazine (PROM) 		

Extraction procedure.

The procedure for the extraction of the TCA from plasma is outlined in Table II.6.

Table II.6. *Scheme for extraction of TCA from plasma.*

Stage	Operation
2 ml of plasma	(1) Add internal standard, add 40 µl of 1N NaOH solution. (2) Add 10 ml of diethyl ether. (3) Homogenize for 5 min. (4) Decant organic phase. (5) Repeat steps (2), (3) and (4) twice with aqueous phase. (6) Pour aqueous phase into glass tube, centrifuge for 1 min at 1000 *g*.
Diethyl ether phase	(1) Collect diethyl ether phase from steps (4) and (6). (2) Dry over anhydrous Na_2SO_4. (3) Evaporate solvent with a warm stream of nitrogen.
Residue	(1) Dissolve in 200 µl of eluent. (2) Ultrasonicate for 1 min. (3) Analyse aliquot by liquid chromatography (volume injected: 48 µl).

The extraction procedure resulted in recoveries of 91 ± 4% for amitriptyline and 95 ± 4% for imipramine.

2.4 Results and discussion.

2.4.1 Selection of the phase system.

The composition of the mobile phase was varied in order to find the optimal separation conditions. The

influence of the percentage of n-hexane and of the modifier methylamine on the capacity ratio was measured. The presence of methylamine is essential: when no methylamine is present in the eluent the basic TCA are irreversibly adsorbed on the column. Caude *et al.* [69] obtained similar results for the separation of phenothiazines on silica-gel with a mobile phase consisting of ethyl acetate, methanol and ethylamine.

In order to elucidate the influence of the modifier methylamine, the capacity ratios (k) of some compounds were plotted against the percentage of methylamine at a constant water content of the eluent (Figure II.9). It can be seen that the retention decreases rapidly at higher methylamine contents. Ammonia or other weak bases can be used instead of methylamine. Increasing the percentage of n-hexane produces a small increase in the capacity ratios, as can be seen in Figure II.10.

The order of elution of the components depends primarily on the acid-base properties of the substituents; primary amines are less basic than secondary, while secondary are more basic than tertiary amines, because of steric hindrance. Hence imipramine will be eluted before desipramine and amitriptyline before nortriptyline.

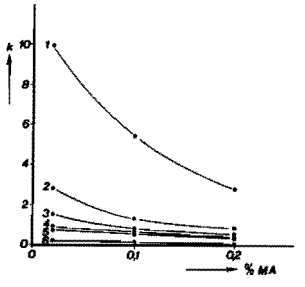


Figure II.9. *Dependence of k on volume percentage of methylamine (MA) in eluent consisting of ethyl acetate (20% saturated with water) and a 35% aqueous solution of methylamine. 1 = Desipramine; 2 = promazine; 3 = imipramine; 4 = amitriptyline; 5 = clomipramine; 6 = trimipramine.*

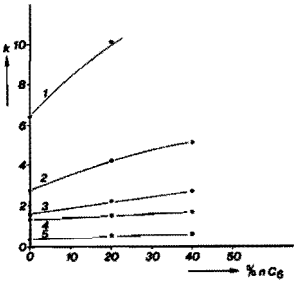


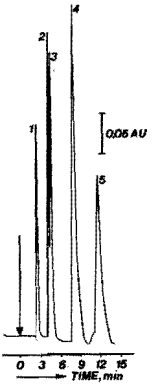
Figure II.10. *Effect of volume percentage of n-hexane (nC_6) on the capacity ratio. Mobile phase: ethyl acetate, (dry), n-hexane and 0.1% (v/v) methylamine. 1 = Nortriptyline; 2 = promazine; 3 = imipramine; 4 = amitriptyline; 5 = trimipramine.*

From Table II.7, it can be concluded that mobile phase compositions based on ethyl acetate give slightly less selective but faster separations than those based on dichloromethane.

Table II.7. *Capacity ratios of TCA on LiChrosorb SI 60 with different eluent compositions.*
Eluent 1: ethyl acetate (20% saturated with water) + 0.2% (v/v) of methylamine. Eluent 2: dichloromethane (20% saturated with water) + 0.2% (v/v) of methylamine. Eluent 3: dichloromethane (20% saturated with water) + 20% (v/v) of n-hexane + 0.2% (v/v) of methylamine.

Sample	Capacity ratio, k		
	Eluent 1	Eluent 2	Eluent 3
Trimipramine	0.08	0.17	0.30
Clomipramine	0.38	0.44	0.55
Amitriptyline	0.40	0.51	0.62
Imipramine	0.56	0.67	0.90
Nortriptyline	-	2.61	2.44
Desipramine	2.74	4.11	4.33
Opipramol	3.5	10.3	10.1

Some preliminary experiments had already shown a smaller selectivity on alumina with mobile phases consisting of dichloromethane, n-hexane and acetic acid. The presence of acetic acid in the eluent was also necessary for symmetrical elution of the components. Hence separations on silicagel were to be preferred, and this preference is clearly demonstrated in Figures II.12 and II.13.



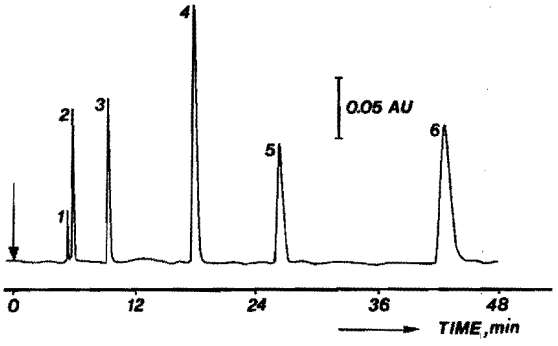


Figure II.11. *Chromatogram of TCA test mixture. Column: 170 x 4.6 mm I.D., LiChrosorb Alox-T, mean particle diameters 5 µm. Eluent: dichloromethane + 20% (v/v) of n-hexane + 0.06% (v/v) of acetic acid. Flow-rate: 0.9 ml/min. Detection: UV, 254 nm. Components: 1 = tetrachloromethane; 2 = clomipramine; 3 = amitriptyline + imipramine; 4 = desipramine; 5 = opipramol. Volume injected: 20 µl.*

Figure II.12. *Chromatogram of TCA test mixture. Column: 300 x 4.6 mm I.D. LiChrosorb SI 60, mean particle diameter 5 µm (HETP 20-30µm for all components). Eluent: ethylacetate (20% saturated with water) + 40% (v/v) of n-hexane + 0.02% (v/v) of methylamine. Flow-rate: 0.7 ml/min. Detection: UV, 254 nm. Components: 1 = solvent; 2 = monochlorobenzene; 3 = trimipramine; 4 = amitriptyline; 5 = imipramine; 6 = promazine. Volume injected: 48 µl.*

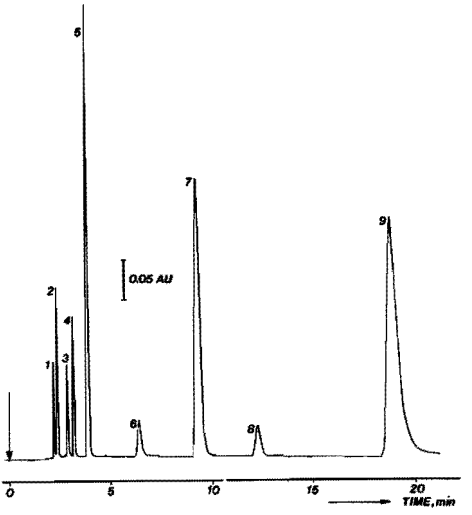


Figure II.13. *Chromatogram of TCA test mixture. Column: 300 x 4.6 mm I.D. LiChrosorb SI 60, mean particle diameter 5 µm (HETP 20-30 µm for all components). Eluent: ethyl acetate (20% saturated with water) + 0.2% (v/v) of methylamine. Flow-rate: 1.7 ml/min. Detection: UV, 254 nm. Components: 1 = monochlorobenzene; 2 = trimipramine; 3 = Clomipramine; 4 = amitriptyline; 5 = imipramine; 6 = promazine; 7 = nortriptyline; 8 = desipramine; 9 = protriptyline. Volume injected: 48 µl.*

2.4.2. Quantitative analysis of tricyclic antidepressants in plasma samples.

In order to determine TCA concentrations in plasma samples it is important to combine high sensitivity with high selectivity. The selectivity can be regulated by varying the content of n-hexane and methylamine and by the degree of water saturation of ethyl acetate. When working with a UV detector, the sensitivity is dependent mainly on the molar absorbance at the optimal wavelength (see Figure II.14) and noise of the detector. UV detection in this instance was limited by the choice of ethyl acetate (cut-off at 260 nm). Other workers[27,28] have

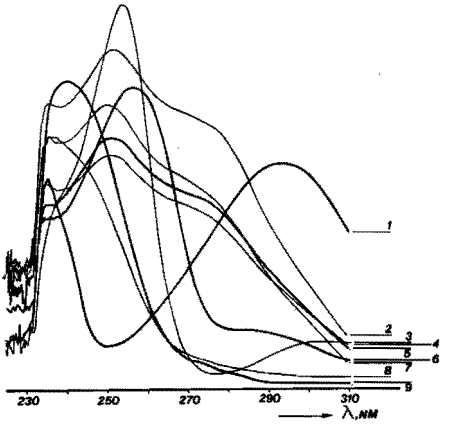


Figure II.14. *UV spectra of TCA. 1 = protriptyline; 2 = clomipramine; 3 = promazine; 4 = desipramine; 5 = imipramine 6 = opipramol; 7 = trimipramine; 8 = amitriptyline; 9 = nortriptyline.*

Table II.8. *Characteristic parameters for detection. Column: 300 x 4.6 mm LiChrosorb SI 60, mean particle diameter 5 µm. Eluent: ethyl acetate (dry) + 0.05% (v/v) of methylamine. Flow-rate: 1.3 ml/min. Detection: UV, 254 nm ; noise (peak to peak), 2.10^{-4} a.u.; time constant, 0.7 s.*

Parameter	Amitriptyline	Imipramine
Sensitivity, S (a.u. ml g^{-1})	$1.9\ 10^{13}$	$2.5\ 10^{13}$
Minimal detectable amount, M (g)	$13.5\ 10^{-9}$	$13\ 10^{-9}$
Capacity ratio, k	2.5	4.0
Molar absorbtivity at 254 nm, ε (a.u. 1 $mole^{-1}$ cm^{-1})	4900	6300

considered the determination of sensitivity (S), minimal detectable amount (M) and maximal allowable injection volume (V_o), and these properties are summarized in Table II.8.

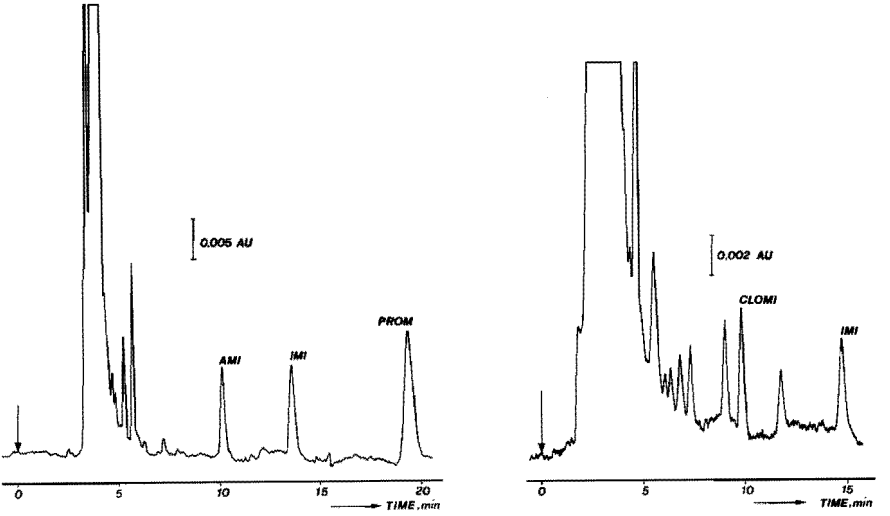


Figure II.15. *Chromatogram of an extract of plasma. Column: 300 x 4.6 mm I.D. LiChrosorb SI 60, mean particle diameter 5 µm. Eluent: ethyl acetate (dry) + 0.05% (v/v) of methylamine. Flow-rate: 1.0 ml/min. Pressure drop: 95 atm. Detection: UV, 254 nm. Components: AMI = amitriptyline (126 ng)(HETP = 25 µm); IMI = imipramine (132 ng) (HETP = 26 µm); PROM = promazine (186 ng)(HETP = 30 µm). Volume injected: 48 µl.*

Figure II.16. *Chromatogram of an extract from plasma. Column: 300 x 4.6 mm I.D. LiChrosorb SI 60, mean particle diameter 5 µm. Eluent: ethyl acetate (dry) + 0.04% (v/v) of methylamine. Flow-rate: 1.1 ml/min. Pressure drop: 110 atm. Detection: UV, 254 nm. Components: CLOMI = clomipramine (67 ng)(HETP = 24 µm); IMI = imipramine (70 ng) (HETP = 20 µm). Volume injected: 48 µl.*

The response of the UV detector was linear with amounts of sample injected of up to at least 300 ng, and injections of amounts up to 100 µl could be allowed without causing more than a 5% loss in resolution.

Figures II.15 and II.16 demonstrate the analysis of amitriptyline, imipramine and clomipramine in human plasma (with promazine and imipramine, respectively, as internal standards).

PART III

DESIGN OF POST-COLUMN REACTORS FOR SELECTIVE DETECTION

Parts have already been published elsewhere:

R.S. Deelder, M.G.F. Kroll, J.H.M. van den Berg, "Determination of trace amounts of hydroperoxides by column liquid chromatography and colorimetric detection", presented at the "Second International Symposium on Column Liquid Chromatography", Wilmington, U.S.A., May 1976. J. Chromatogr., 125 (1976) 307.

R.S. Deelder, M.G.F. Kroll, A.J.B. Beeren, J.H.M. van den Berg, "Post column reactor systems in liquid chromatography", presented at the "Third International Symposium on Column Liquid Chromatography", Salzburg, Austria, September 1977. Submitted for publication in J. Chromatogr.

1. Reaction detection in column liquid chromatography.

1.1 Introduction.

Photometric flow detectors are probably the most widely used detection devices in modern liquid chromatography. They are mainly operated in the UV-region of the spectrum, where numerous compounds of chemical or biological interest show absorption.

Sometimes, non-absorbing molecules are converted into absorbing by appropriate derivatization reactions prior to separation[70-72]. Although the sensitivity of the UV detector is extended in this way to a wide range of substances, it adversily affects the chromatographic selectivity. Also formation of artifacts or of several derivatives of one compound can occasionally occur.

A number of methods of chemical analysis are based on specific colour reactions followed by photometric measurements. However, the specificity of the reactions is frequently unsatisfactory and pre-separations by extraction or chromatography may be required to eliminate interfering substances.

Direct coupling of a chromatographic column to a chemical reaction system (Figure III.1) not only enables the elimination of interfering substances. It also permits the selective determination of a group of compounds, if reagents are used specific to this group of compounds, in this way substantially increasing the selectivity of liquid chromatographic photometric detection. Formation of artifacts in post-column derivatization reactions is not as likely as in pre-column derivatization. However, the solubility of the reagents and the effluent may limit this mode of operation. It is obvious that the derivatization reagents are not to be detected and the rate of post-column reactions must be relatively high as will be demonstrated in Section III.4. Automatic on-stream detection by measuring the developed absorbance or fluorescence after a fixed reaction time, is currently

applied in the analysis of biologically important mixtures by ion-exchange chromatography[73,74].

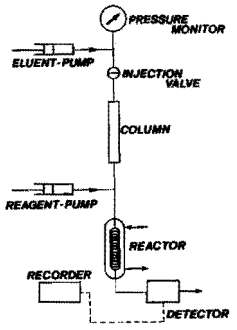


Figure III.1. *Schematic diagram of the chromatographic system with reaction detection.*

A number of papers were introducing continuous reaction procedures[75-85]. The number of publications dealing with the technological aspects of reactors in column liquid chromatography is rather low, this in contrast to the importance of reactor design and optimation. It is shown in the present study that additional band-broadening and the consequent loss of resolution can be reduced to an acceptable level by careful design of the reaction system (see Sections III.2 and III.3), even if high performance columns are used for separation.

1.2 Reactor types.

Currently three post-column reactor designs are applied:

1. Tubular reactors.
   In the simplest design flow reactors consist of narrow stainless steel coiled tubes.
2. Gas-segmented reactors.
   In detection systems based upon this principle, the column effluent is fed into a continuous series of short slugs of reagent separated by gas bubbles.
3. Packed reactors.
   Columns packed with inert particles, such as glass

beads, serve as a reactor.

Packed and tubular reactors are investigated in this thesis in Sections III.2 and III.3, respectively. The axial dispersion in these reactors is investigated as well as some general characteristics and rules for the optimal design are given.

Some general properties of reaction systems will be treated in this chapter.

Axial dispersion in open tubular reactors can be reduced by using a gas-segmented liquid flow. Even in carefully designed gas-segmented reaction systems[86] the axial dispersion is such, that it exceeds the limits imposed by modern liquid chromatography. Therefore reactors of type 2 will only be treated summarily here. The drawbacks of gas-segmented flows are well-known:

a. because of the compressibility of gas, the stream tends to pulsate rather than to flow regularly;
b. streams have to be debubbled before they enter the flow cell;
c. the size of the gas-bubbles has to be controlled and volume-increase because of temperature-increase has to be avoided;
d. the pressure drop and flow velocities vary in the presence of gas for different tubing materials.

Nevertheless this type of reactors is widely used in automated colorimetric analyzers such as the Technicon Auto Analyzer systems. In these reactors the column effluent is fed into a continuous series of short slugs of reagent separated by gas bubbles. A leakage between the segments of the reaction mixture is brought about by fluid from a segment wetting the wall of the reactor tube and mixing with the next segment[73]. Mixing in the acceptor slug is mainly due to the circulating flow within each segment[87-89]. Prediction of the axial dispersion resulting from this mechanism is only possible if the leakage flow and mixing within the slugs can be described accurately.

To our knowledge no rigorous theoretical treatment of this problem has been reported. Recently Snyder[90] gave the following semi-empirical expression for the dispersion in segmented flow reactors:

$$\Delta\sigma^2_{t,r} = \left[\frac{0.14\ d_t^{2/3}\ (\Phi_r + nV_G)^{5/3}\eta^{2/3}}{\gamma^{2/3}\ ID_r\Phi_r} + \frac{1}{n} \right] \cdot \left[\frac{2.35\ (\Phi_r + nV_G)^{5/3}\eta^{2/3}\ t_v}{\gamma^{2/3}\ \Phi_r d_t^{4/3}} \right] \qquad \text{(III.1)}$$

where d_t is the internal diameter of the reactor tube, Φ_r the liquid flow through the reactor, V_G the volume of a gas bubble, η the viscosity of the reaction mixture and γ its surface tension. Further, ID_r is a dispersion coefficient for the tracer substance in water and n the segmentation frequency. Mixing the fluid slugs is represented by a flow with a flat velocity profile equal to the actual mean velocity of the slug and with an effective radial dispersion coefficient, ID_r, which accounts for the enhanced mass transfer[91,92]. Moreover, the thickness of the liquid slug left behind by the fluid slug is calculated by using a well-known equation from fluid mechanics [93,94]. In fact, the radial dispersion coefficient, ID_r, depends on the actual flow pattern in the slug, and

$$ID_r = ID_r\ (Re,\ Sc,\ \beta,\ \lambda,\ \ldots) \qquad \text{(III.2)}$$

where $\beta = l/d_t$, the dimensionless slug length, l is the actual slug length and $\lambda = d_c/d_t$, the curvature ratio. However, in Equation (III.1) ID_r is assumed to have a constant value and Snyder[95,96] has calculated a mean value for ID_r from an extensive series of experimental $\Delta\sigma^2_{t,r}$ values using this equation. The measurements were carried out for a single tracer substance and for Sc ~ 2500, $10 < Re < 100$ and $10 < \beta < 200$. In these ranges variations in the radial mass transfer rate are likely

limited[24].

In practice the values of Φ_r, η, γ, t_v and ID_r are fixed by the chromatographic separation and the reaction involved. Now, $\Delta\sigma_{t,r}$ can be minimized by a proper choice of the tube diameter, d_t, and the slug frequency, n. From Equation (III.1) it can be shown that $\Delta\sigma_{t,r}$ values of approximately 1 s are within reach. However, Equation (III.1) only expresses band-broadening from the reaction coil itself. Practical experience[86] with gas-segmented flow reactors in liquid chromatography shows that mixing tees, connectors and the debubbler overrule the contribution of the reactor coil itself[97,98]. Particulary the debubbler seems to have an adverse effect at this point, as liquid holdup is needed to prevent gas bubbles from being sucked into the flow cell. To make the most of the theoretical performance of segmented flow reactors, the conventional debubblers should be eliminated. Systems which permit photometric measurements on gas-segmented liquid flows by means of so-called electronic debubbling[99] are used in highly automated clinical analyzers; these systems are not yet commercially available for use in liquid chromatography.

1.3 Effect of the reactor on the overall resolution.

The reaction system can be considered as to consist of a reactor and a measuring cell (e.g. a flow cell of a spectrophotometer). A chromatographic peak leaving the column will undergo an additional broadening in the reactor. This has an adverse effect on the analytical performance, especially for narrow peaks.

Let the variance, expressed in time units, of a solute band passing from the column into the reaction system be $\Delta\sigma^2_{t,c}$, the variance of the residence time distribution function in the reactor system $\Delta\sigma^2_{t,r}$ and the variance of the peak at the end of the reactor-detector system σ^2_t. These peak broadening processes are independent and therefore:

$$\sigma_t^2 = \Delta\sigma_{t,c}^2 + \Delta\sigma_{t,r}^2 \qquad \text{(III.3)}$$

It is assumed that the variance $\Delta\sigma_{t,r}^2$ caused by the reactor system can be approximated by the variance of the residence time distribution function of an inert non-reacting component.

The increase in band-width, due to the dispersion phenomena in the reaction system, reduces the separation between the components A and B. The separation between two peaks that differ by $\Delta t_R = t_{R,B} - t_{R,A}$ in their retention time is characterized by the resolution $R_{A,B}$ (see Part I):

$$R_{A,B} = \frac{t_{R,B} - t_{R,A}}{2(\sigma_{t,A} + \sigma_{t,B})} = \frac{\Delta t_R}{2\sigma_{t,B}\ (n + 1)} \qquad \text{(III.4)}$$

where $\sigma_{t,A}$ and $\sigma_{t,B}$ are the standard deviations of the elution functions and $n = \sigma_{t,A}/\sigma_{t,B}$.
Assuming $n = 1$, the following equation can then be derived for reactors:

$$\frac{R}{R_{max}} = \left[1 + \left(\frac{\Delta\sigma_{t,r}}{\Delta\sigma_{t,c}}\right)^2\right]^{\frac{-1}{2}} \qquad \text{(III.5)}$$

The maximum value of the resolution, R_{max}, will be obtained with $\Delta\sigma_{t,r} = 0$ and $\sigma_t = \Delta\sigma_{t,c}$.
This relationship is graphically represented in Figure III.2. If a 5% decrease in resolution is considered acceptable, then:

$$\Delta\sigma_{t,r} = 0.33\ \Delta\sigma_{t,c} \qquad \text{(III.6)}$$

Now, $\Delta\sigma_{t,c}$ can be calculated from the plate number, N, of the separation column used and the retention time, t_R, of the solute.
The number of theoretical plates is defined as:

$$N = \left(\frac{t_R}{\sigma_t}\right)^2 \qquad \text{(III.7)}$$

Where t_R is the retention time of the solute and σ_t is the standard deviation of the elution profile.

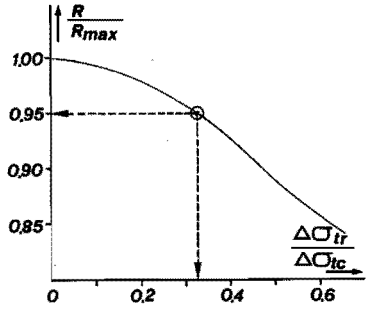


Figure III.2. *Relationship between* R/R_{max} *and* $\Delta\sigma_{tr}/\Delta\sigma_{tc}$.

The height equivalent to a theoretical plate is:

$$H = \frac{L}{N} \qquad \text{(III.8)}$$

where L is the column length.
The column length, L, giving a resolution $R_{A,B}$ at the column outlet can be calculated from the equation (neglecting extra column effects):

$$L = 4(n+1)^2 R_{A,B}^2 \left(\frac{r_{B,A}}{r_{B,A}-1}\right)^2 \left(\frac{k_B+1}{k_B}\right)^2 H_B \qquad \text{(III.9)}$$

where $r_{B,A} = K_B/K_A$ (the selectivity factor, which is greater than unity), K_B and K_A are the distribution coefficients of the components B and A, respectively, k_B the capacity ratio of component B and H_B represents the plate height for the slower migrating component B. If the standard deviation σ_t increases according to Equation (III.3) due to extra column dispersion R becomes smaller (Equation (III.4)). Consequently a longer column is

required to obtain the original resolution, R_{max}, by applying Equation (III.9).

1.4 Effect of the reactor on the minimum detectable amount of sample.

Most detectors used in liquid chromatography are dependent on concentration, in contrast to gas chromatography where mass flow devices are more common (e.g. flame ionisation detector). The chromatographic process always involves dilution of the sample components, which increases the minimal detection concentration limit of any chromatographic method as compared with a direct measurement of concentration.

This dilution-effect can be described as the ratio of the maximum of the concentration peak leaving the column, C_{max}, to the concentration of the component in the sample, C_o[27,28,97]:

$$\frac{C_{max}}{C_o} = \frac{V_o}{A\ (1 + k)(2\pi L\ H)^{\frac{1}{2}}} \qquad \text{(III.10)}$$

where V_o is the sample volume, L the length of the column and H the plate height of the column. A represents the free cross sectional area of the separation column and equals $A = \varepsilon_T \pi r^2$, with ε_T the total porosity of the column and r the column radius. The product $C_o V_o$ corresponds to the amount of the component injected.

If a reaction system is used as a detector, further dilution occurs owing to the addition of the reagent and the broadening in the reaction system:

$$\frac{C_{max}}{C_o} = \frac{\Phi_c}{\Phi_c + \Phi_r} \frac{\Delta\sigma_{t,c}}{\sigma_t} \frac{V_o}{A(1+k)(2\pi LH)^{\frac{1}{2}}} \qquad \text{(III.11)}$$

where Φ_c and Φ_r represent, respectively, the volumetric flow-rate of the eluent and the reagent.

From this it can be concluded that if small concentrations of component have to be detected, Φ_c and $\Delta\sigma_{t,r}$ should be kept as small as possible.

If the additional peak-broadening due to the reactor, $\Delta\sigma_{t,r}$, is not allowed to be larger than 0.33 $\Delta\sigma_{t,c}$ the factor $\frac{\Delta\sigma_{t,c}}{\sigma_t}$ in Equation (III.11) is about 0.95.

The increase of the volumetric flow-rate has a larger influence on the dilution, because the factor $\frac{\Phi_c}{\Phi_c+\Phi_r}$ can vary from 1 to 0.

This loss in dilution, which increases the minimal detectable concentration, should be compensated by a large increase in molar absorbtivity to make the derivatization in the post-column reactor worth while.

2. Design and application of packed bed reactors.

2.1 Introduction.

It has been demonstrated that columns with non-porous spherical glass beads of less than 100 µm in mean particle diameter are suitable for use as packed reactors.
Jolley and co-workers[100-103] used a packed tubular reactor in a colorimetric detection system for a carbohydrate analyser, with glass beads serving as the packing material. In this study the reactor design was not critical because of the long retention times and thus rather broad elution peaks involved. Since then several workers attempted to employ packed bed reactors in their liquid chromatographic equipment in order to improve the sensitivity and selectivity of their analysis.
Regnier *et al.*[104,105] developed a post-column system for enzymatic reactions consisting of a substrate pump, a post-column reactor and a photometric detector. Quantitation of enzyme activity in the effluent may be accomplished by incubating an enzyme with an appropriate substrate and continuously monitoring the formation of the product after a fixed time spent in the flow-through reactor.
Deelder and van den Berg *et al.*[86,106], and Little *et al.*[107] made a study of the design of chemical reactors for post-column derivatization. A theoretical treatment as well as experimental results were given for packed bed reactors.
Jonker[108,109] showed the application of this reactor-type in amino-acid analysis using the ninhydrin reaction.

2.2 Dispersion in packed beds.

To start with, mixing in its present meaning refers to random fluctuations of matter that diminish existing concentration gradients.

Axial and radial mixing in packed beds is important both in chemical reactors and in equipment devoted to separation processes. In separation columns extra dispersion processes caused by mass transfer occurs, as is the case in many chemical reactors. Axial dispersion is usually an undesirable feature since it causes residence time distribution in chemical reactors as well as in separation columns. Radial mixing, on the other hand, is desirable.

An attempt to increase the degree of radial mixing may also increase the degree of axial mixing. Therefore a good knowledge of the inter-relationship is necessary.

As the dispersion in the axial direction is likely to differ from that radial to it, one defines two effective diffusion or mixing coefficients ID_a and ID_r. It is well understood that in the case of physical similarity both mixing coefficients are proportional to the mean velocity of flow u, as well as, to the mean particle diameter d_p. Therefore the Peclet numbers

$$\left(Pe_a = \frac{ud_p}{ID_a} \text{ and } Pe_r = \frac{ud_p}{ID_r}\right)$$

appear to be the dimensionless measure of effective diffusion in a packing.

2.2.1 Axial dispersion.

In Figure III.3 a composite of diffusional data is given with the Peclet group as ordinate and Reynolds number based on particle diameter ($Re = \frac{udp}{\nu}$) as abscissa for liquids. In these curves three major regions can be distinguished. In the zone with Re > 250 the behaviour of the Peclet group is independent of the Reynolds number. The constancy of the Pe-group gives information that in this regime the dispersion ID_a increases linearly with the fluid velocity and the particle diameter. Such a linear variation with fluid velocity is characteristic of a fully developped turbulence. In the performance of rapid reactions a reactor operation corresponding to a point in zone 3, will be optimal if a high pressure drop would be acceptable. But this is not easily realizable in the present state of art of liquid chromatography.

Below the Reynolds number 3.10^{-4} the curve tends to approach directly the curve for molecular diffusion (the first zone). This region has not yet been explored for liquids.

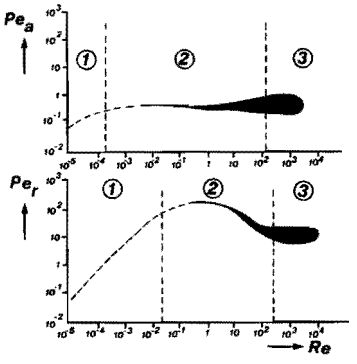


Figure III.3. *The experimental relationship between the axial and radial Peclet number and the Reynolds number.*

The two main mechanisms of dispersion in a packed bed are first molecular diffusion and secondly mixing arising from streamsplitting, generally termed eddy diffusion. The former will be of predominant importance at low Reynolds numbers (zone 1) and the latter at high Reynolds

numbers (zone 3), with both mechanisms making a contribution in the intermediate region (zone 2: 3.10^{-4} < Re < 250). This intermediate region is of importance in modern liquid chromatography. If molecular diffusion is the only mechanism operating, the axial dispersion coefficient ID_a is related to the molecular diffusivity D_m by the equation:

$$ID_a = \gamma D_m \qquad \text{(III.12)}$$

where γ is known as the tortuosity factor; $\gamma = 0.67$ for $\varepsilon_u = 0.4$, with ε_u the interparticle porosity for non-porous particles. According to theoretical analysis by Aris *et al.*[110] at high Reynolds numbers the Peclet number should then approximate a value of about 2 for liquids for axial dispersion. So:

$$ID_a = \frac{1}{2} ud_p \qquad \text{(III.13)}$$

For the intermediate region (zone 2) liquids will obey the relation according to Hiby[111]:

$$ID_a = \gamma D_m + \frac{\frac{1}{2}\lambda_1 ud_p}{1 + \lambda_2 \sqrt{\frac{D_m}{ud_p}}} \qquad \text{(III.14)}$$

with λ_1 and λ_2 constants and d_p the mean particle diameter of the packing.

Recently Horvath and Lin[112] treated the influence of interstitial stagnant mobile phase on the interplay of convective and diffusional mixing. An equation has been derived for unsorbed solutes, being:

$$ID_a = \gamma D_m + \frac{\frac{1}{2}\lambda_1 ud_p}{1 + \lambda_2 (\frac{D_m}{ud_p})^{\frac{1}{3}}} \qquad \text{(III.15)}$$

Equation (III.14), however, is preferred, because of the lack of experimental data to prove the validity of

Equation (III.15).

In Equation (III.14) a reduction of axial dispersion is taken into account. Because of the wide distribution of pore sizes in the bed, a tracer will flow at greater velocities through some pores than through the others. Therefore, at the scale of particle size, an axial concentration gradient will set up. The issuing streams from neighbouring pores come into contact again, radial dispersion will take place, thus diminishing the magnitude of the effects of axial dispersion. An equation often used for the comparison of gas and liquid chromatography in dimensionless form reads:

$$Pe_a^{-1} = \gamma(ReSc)^{-1} + \frac{\frac{1}{2}\lambda_1}{1 + \lambda_2(ReSc)^{-\frac{1}{2}}} \qquad \text{(III.16)}$$

where $Re = \frac{ud_p}{\nu}$, with ν the kinematic viscosity and Sc the Schmidt number equal to $\frac{\nu}{D_m}$.

Excellent reviews on the subject of axial and radial dispersion are published by Gunn *et al.*[113-115] and others[116-117].

2.2.2 Radial dispersion.

Figure III.3 shows the log-log plot for liquid phase radial dispersion in packed beds. At high Reynolds numbers the radial Peclet number approaches a value of about eleven. The dominating convective dispersion is determined by deviations in the flow path caused by particles. The reduction effect as pointed out in the second term of Equation (III.14) for axial dispersion is not observed for radial mixing. Therefore in the intermediate region the Peclet number (Pe_r) is even larger than in zone 3. Since liquid chromatographic conditions are situated in the intermediate zone (zone 2), it is evident that the radial mixing of a reagent and the column effluent, containing the component molecules, is not accomplished to high extent in a packed reactor.

In practice this problem is solved by mixing these two

flows in front of the packed bed reactor in a tee-piece with 0.15 mm I.D. channels. The reagent was added to the column effluent through a 0.15 mm I.D. stainless steel capillary tube (see Figure III.4). A similar approach was described by Stahl *et al.*[78] and Nachtmann[118]. This contruction assures a maximum impulse exchange which favours rapid mixing. The completeness of mixing was experimentally verified by using indicator techniques[119-120].

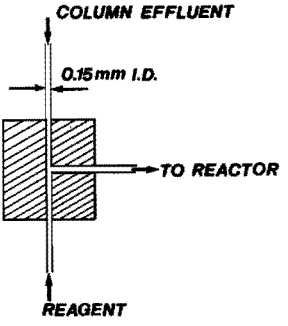


Figure III.4. *Tee-piece between column and reactor.*

2.3 Determination of geometry factors from the band-broadening of unsorbed solutes.

The chromatograph used for the measurement of band-spreading in packed beds was assembled from the following moduli: reciprocating pump (type DMP 1515, Orlita, Giessen, G.F.R.); sample valve (type HPSV-20, Spectra Physiscs Berkeley, U.S.A.), with a 20 µl loop; UV detector (type PM2D, Zeiss, Oberkochen, G.F.R.). The dead volume between the sampling valve and the column inlet as well as between the column outlet and the flow cell was minimized by using the shortest possible length of 0.25 mm I.D. connector tubing. All measurements were corrected for the contribution to band-broadening by injection and detection. Narrow sieve fractions of the glass beads were prepared by an air classifier (Zichzack sichter, type MZR, Alpine, Augsburg, G.F.R.). The columns were prepared from stainless steel tubing. In Figure III.5 the dimensions as well as the

mean particle size of the glass beads (Sovitec, Charleroi, Belgium) are given.

The filling of the stainless steel tubing with glass beads was achieved by a balanced-density-slurry-procedure as proposed by several workers[121-123]. The slurry liquid consisted of dioxane, tetrachloromethane and 1,1,2,2-tetrabromoethane (23:23:54 v/v %) and sodiumlaurylsulphate (0.05 w/v %). Afterwards the glass beads are added; 2.3 g glass beads are required per cm^3 column volume. The slurry is pushed through the column by means of hexane using pressures up to 600 atm..

For the measurements of band-spreading 2,2,4-trimethyl pentane was used as eluent and diluted solutions of nitrobenzene , the tracer, in 2,2,4-trimethylpentane were injected. The flow-rate was varied. The peaks obtained were symmetrical. Plate heights were calculated from half-height widths (Equations (III.7) and (III.8)). In each run, the flow-rate was measured. The linear flow velocity, u, for non-porous particles can be calculated from the elution time of the peak top and the column length. The reduced velocities were obtained by using the solute diffusivity of nitrobenzene in 2,2,4-trimethylpentane of 2.5×10^{-5} cm^2s^{-1}, measured separately (see Section III. 3.4).

The resulting plots of the reduced plate height $h = \frac{H}{d_p}$ versus the reduced linear velocity $\nu = \frac{ud_p}{D_m}$ is represented in Figure III.5. It should be noted that in the practice of chromatography the term reduced linear velocity, ν, is used for the quantity ReSc. The results of Done *et al.*[124] for a Zipax (a porous-layer glass-bead) packing and Knox and Parcher[125] for glass beads are also shown. The agreement with the results of Done *et al.*[124] is striking. Sternberg and Poulson[126] made a systematic study of the effect of column to particle ratio, d_r/d_p, on the spreading of unretained component peaks in gas chromatography. They noticed a decreasing value of h with increasing particle size in a fixed diameter column (thus with increasing d_p/d_r ratio). This phenomenon could explain

that the performance of the Zipax columns and our glass-bead columns is slightly worse (higher h values) over the entire range of ν compared with the glass-bead columns of Knox. The particle sizes are approximately the same. The use of larger diameter columns in our case, however, is a necessity to get larger volumetric flow-rates at the same linear velocity of the liquid.

The estimation of the geometry parameters λ_1 and λ_2 (Hiby constants) seemed to be of the utmost importance in the design of packed bed reactors (see Section III.2.4.).

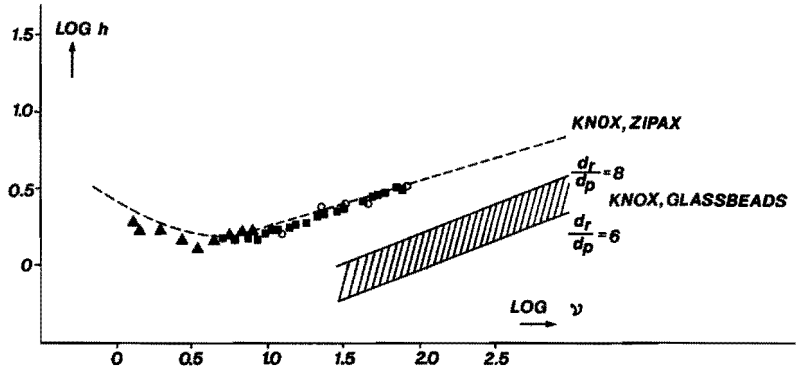


Figure III.5. *Reduced plate height-reduced velocity plot for unretained solutes in glass bead columns.*

○ 28 x 0.4 cm column, d_p = 17 μm; ■ 48 x 0.6 cm column, d_p = 35 μm; ▲ 29 x 0.9 cm column, d_p = 15 μm; --- Done, Kennedy, Knox[124]*; ▨ Knox, Parcher*[125]

A curve fitting procedure to the data of Figure III.5. by means of the least squares method was used to estimate λ_1 and λ_2. The fitting function was in the case of a packed bed without mass transfer, derived from Equation (III.14):

$$H = \frac{2ID_a}{u} = \frac{2\gamma D_m}{u} + \frac{\lambda_1 d_p}{1 + \lambda_2 (\frac{D_m}{ud_p})^{\frac{1}{2}}} \qquad \text{(III.17)}$$

In Table III.1 our results are summarized with the estimations, reduced from published experimental results. It can be seen that our experimental results agree reasonably well, especially with the extensive investigations of band-spreading in glass beads and Zipax columns by

Knox and coworkers[124,125,127].

Table III.1. *Comparison of experimental results of* λ_1 *and* λ_2.

Investigator	λ_1	λ_2	Remarks
Hiby[111]	0.6	0.7	gases and liquids, glass beads
Edwards, Richardson[116]	0.5	9.7	gases, glass beads
Knox, Parcher[125]	7.2	33.9	liquids, glass beads $d_r/d_p = 8$
	4.2	31.8	liquids, glass beads $d_r/d_p = 6$
Horvath, Lin[112]	6.9	11.1	liquids, glass beads
Done, Kennedy, Knox[124]	10.3	18.2	liquids, Zipax $d_r/d_p = 20\text{-}60$
Our results			
o (Figure III.5)	10.7	18.6	liquids, glass beads $d_r/d_p = 235$
■	9.0	15.7	liquids, glass beads $d_r/d_p = 171$
▲	10.0	16.8	liquids, glass beads $d_r/d_p = 600$

Generally speaking a lower value of λ_1 and a higher λ_2 represent a "better" column packing. Together with the tortuosity factor γ they represent the parameters of the column packing. The value of γ, however, does not vary significantly from column to column and is assumed to be 0.7[117,127]. Consequently the quality of a given column packing can be quantified by the overall values of λ_1 and λ_2. As evident from our data in the following treatment values of $\lambda_1 = 10$ and $\lambda_2 = 18$ will be used.

2.4 Design of packed bed reactors.

As in chromatographic columns, peak-broadening, $\Delta\sigma_{t,r}$, in packed tubular reactors is proportional to the mean residence time, t_v, in the reactor. The proportional factor being $N^{-\frac{1}{2}}$, where N is defined as the plate number, or:

$$\Delta\sigma_{t,r} = \frac{t_v}{\sqrt{N}} = t_v \sqrt{\frac{H}{L}} \qquad \text{(III.18)}$$

If it is assumed that there is no mass transfer, then band-broadening is due to the combination of axial molecular diffusion and convective mixing (as pointed out in Section III.2.2) and can be expressed through the empirical relation (III.17). The contribution of convective mixing, expressed in the second term on the right-hand side of Equation (III.17), can be reduced considerably by using appropriate column packing techniques. Plate heights corresponding to 1 or 2 times d_p (this implies h = 1-2, see Figure III.5) are obtainable[128]. These findings suggest that columns packed with small particle size glass beads can be put in good use as post-column reaction systems, because of the small values of H (see Equation (III.18)).

The problems of reactor design are similar to those encountered in choosing the optimal conditions for liquid chromatographic separations[129-134]. In fact, a suitable choice of reactor length, L, particle size of the glass beads, d_p, should be made for a given combination of reaction time, t_v, and allowable additional band-broadening, $\Delta\sigma_{t,r}$. In practice limits are set to the pressure drop, Δp, over the reactor. The variance, $\Delta\sigma^2_{t,r}$, of the residence time distribution in the reactor should meet the requirements of the chromatographic column concerned (see Section III.1.3). The residence time, t_v, is fixed by the reaction kinetics. A procedure for the design of a packed reactor is based on Equation (III.17) and the following set of equations:

$$u = \frac{L}{t_v} \qquad \text{(III.19)}$$

and

$$\Delta p = \frac{u\eta L}{k_o d_p^{\,2}} \qquad \text{(III.20)}$$

Equation (III.20) is the Darcy equation giving the pressure drop, Δp, over a packed column, where k_o is the permeability constant of the reactor and η the dynamic viscosity of the reaction mixture. In fact we have three independent Equations (III.17), (III.19) and (III.20). This implies that of the five parameters L, d_p, Δp, t_v and $\Delta\sigma_{t,r}$ three can be chosen at will. As t_v and $\Delta\sigma_{t,r}$ are fixed, only one of the three remaining parameters need be chosen. Substitution of Equation (III.19) for u and of Equation (III.21) for H and of Equation (III.20) for d_p, in Equation (III.17) gives Equation (III.22.a):

$$H = \frac{L}{N} \qquad \text{(III.21)}$$

$$\xi^{3/2} - \psi\xi^{1/2} - \psi \frac{\lambda_2}{L} (D_m t_v)^{1/2} = 0 \qquad \text{(III.22.a)}$$

where

$$\xi = \left(\frac{\eta}{k_o t_v \Delta p}\right)^{1/2} \qquad \text{(III.22.b)}$$

and

$$\psi = \frac{1}{\lambda_1} \left(\frac{\Delta\sigma_{t,r}^2}{t_v^{\,2}} - \frac{2\gamma D_m t_v}{L^2}\right) \qquad \text{(III.22.c)}$$

For fixed t_v and $\Delta\sigma_{t,r}$ and chosen value of L, Equation (III.22.a) gives the corresponding value of Δp. From Equation (III.20) d_p is found. These calculations were

carried out for $\gamma = 0.7$, $D_m = 2 \cdot 10^{-5}$ cm^2 s^{-1}, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 2 \cdot 10^{-3}$ and $\eta = 0.5$ cP.
These calculations were carried out for $\sigma_{t,r} = 0.5, 1, 2, 4$ s; in Figures III.6 - III.9 Δp and L are plotted against d_p for the various $\Delta\sigma_{t,r}$. In these figures also the independent variable t_v is varied between reasonable limits (30 - 480 s).

Favourable conditions are found near the minimum of the Δp versus d_p curves, giving short reactors and low pressure drops. The smallest contribution to peak-broadening obtainable in practice, is 0.5 s, because of the unavailability of glass beads smaller than 10 μm and because the use of very small glass beads leads to excessively high pressure drops (see Figure III.6). If $\Delta\sigma_{t,r}$ is allowed to have a larger value, from the practice of liquid chromatography 5 s is already an extreme value, a larger range of particle sizes are reasonable, because the slopes of the Δp versus d_p curves decrease, as can be seen in the right parts of Figures III.6 - III.9.

Larger values of the residence time, t_v, can only be applied when $\Delta\sigma_{t,r}$ is also increasing (Equation (III.18)). This is due to a practical limit set to the number of plates that can be realized in a reactor column.
In order to facilitate comparison of reactor types (see Section III.4) in Figures III.10 and III.11 d_p is plotted against $\Delta\sigma_{t,r}$ for various reaction times, t_v, and for reactor lengths of 25 and 50 cm respectively. In practice limits are set to the pressure drop, Δp, over the reactor and the broken lines interconnect points of equal pressure.

The values for the parameters γ, η, D_m, λ_1, λ_2, k_o were chosen such as to cover the conditions met in specific reaction-detection systems. The influence of these physical constants D_m, η and γ and the packing geometry factors λ_1 and λ_2 on the graphs will be discussed here. The influence of γ is very small (see Figure III.12). The slope of the Δp versus d_p curves increase with lower D_m values while the minima of these curves move to lower d_p values (see Figure III.13). When varying the viscosity of the reaction mixture, consequently the molecular diffusion

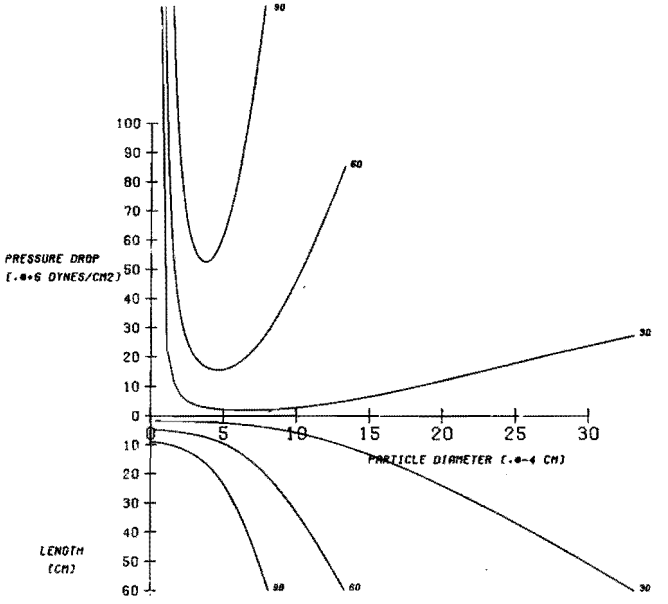


Figure III.6. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various reaction times, t_v, at a constant residence time distribution in the reactor, $\Delta\sigma_{t,r}$, of 0.5 s. Numbers plotted near the curves: t_v in s. Conditions: $\gamma = 0.7$, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

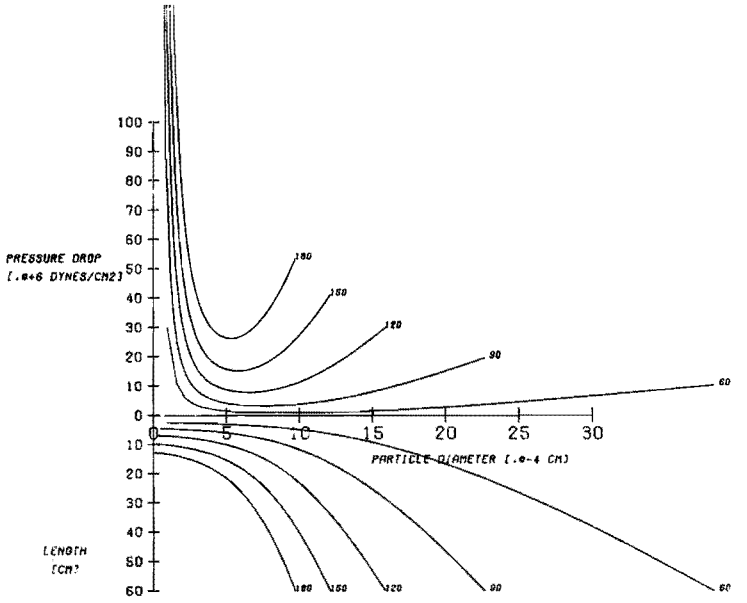


Figure III.7. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various reaction times, t_v, at a constant residence time distribution in the reactor, $\Delta\sigma_{t,r}$, of 1.0 s. Numbers plotted near the curves: t_v in s. Conditions: $\gamma = 0.7$, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

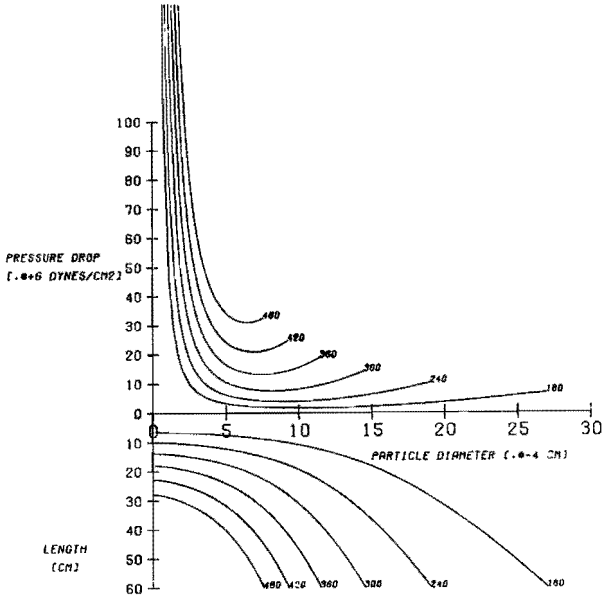


Figure III.8. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various reaction times, t_v, at a constant residence time distribution in the reactor, $\Delta\sigma_{t,r}$, of 2.0 s. Numbers plotted near the curves: t_v in s. Conditions: $\gamma = 0.7$, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

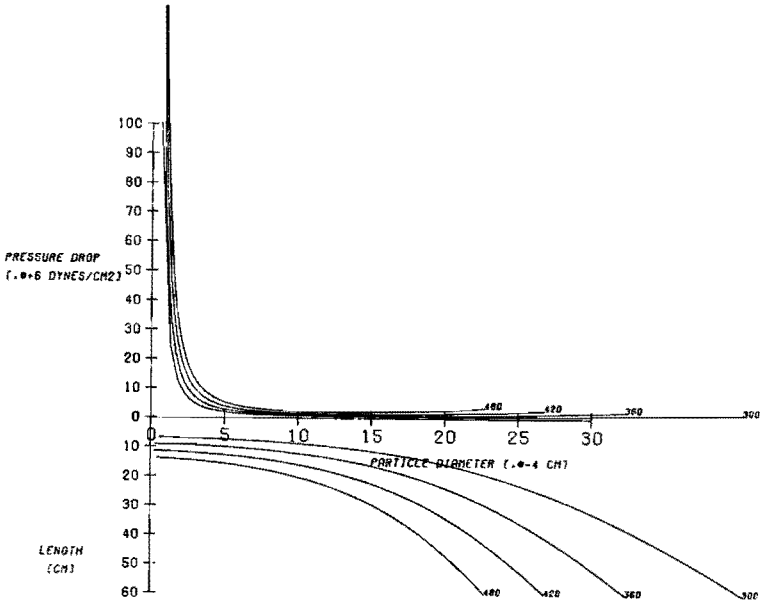


Figure III.9. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various reaction times, t_v, at a constant residence rime distribution in the reactor, $\Delta\sigma_{t,r}$, of 4.0 s. Numbers plotted near the curves: t_v in s. Conditions: $\gamma = 0.7$, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

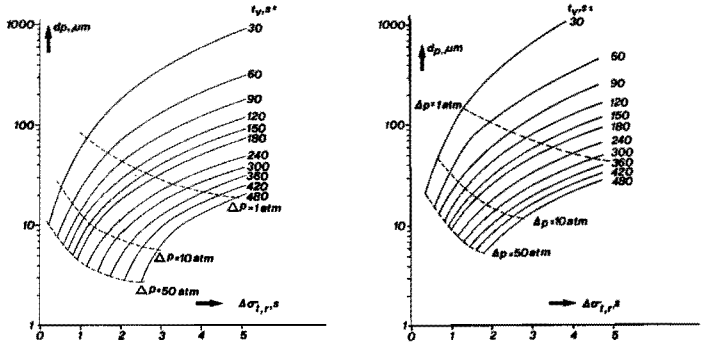


Figure III.10. *The particle diameter, d_p, as a function of the band broadening, $\Delta\sigma_{t,r}$, for various reaction times, t_v, assuming a constant reactor length L = 25 cm. The broken lines interconnect points of equal pressure drop.*

Figure III.11. *The particle diameter, d_p, as a function of the band broadening, $\Delta\sigma_{t,r}$, for various reaction times, t_v, assuming a constant reactor length L = 50 cm. The broken lines interconnect points of equal pressure drop.*

coefficient will alter, since D_m is proportional to $1/\eta$. In Figure III.14 the influence of D_m is superposed on the pressure drop increase because of the increase of η according to Darcy in the $\Delta p - d_p$ plot. But the influence of all these parameters is small in comparison with the effect of the Hiby constants (Figures III.15 - III.17). The estimation of the values of these parameters seems to be very important (see Section III.2.4). To be sure of having constant and reliable values of these geometry factors, the packing procedure should be reproducible. The values of the Hiby constants λ_1 and λ_2 were determined in separate experiments with glass bead columns (see Section III.2.4).

At a given length of the reactor, the internal diameter, d_r, of the reactor should be chosen such as to fit in with

the residence time and the volumetric flow rate, $\Phi_r + \Phi_c$, through the reactor. The internal diameter is found from:

$$d_r = \left[\frac{4t_v (\Phi_c + \Phi_r)}{\pi \varepsilon_T L} \right]^{\frac{1}{2}} \qquad \text{(III.23)}$$

where Φ_c is the volumetric flowrate through the column and ε_T the void fraction of the reactor ($\varepsilon_T = 0.4$ for non-porous particles).

The use of packed reactors in high performance liquid chromatography is possible with relatively fast reactions as will be shown in Sections III.2.5 and III.2.6.

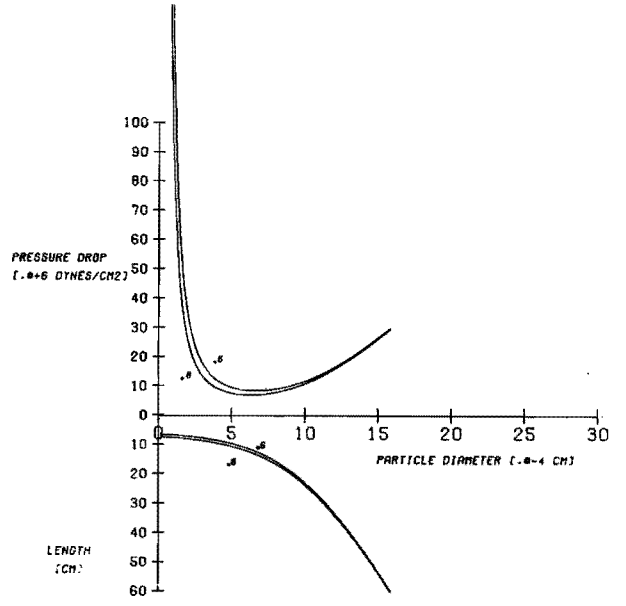


Figure III.12. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various tortuosity factors, γ. Numbers plotted near the curves: γ. Conditions: $t_v = 120$ s, $\Delta\sigma_{t,r} = 1.0$ s, $\lambda_1 = 10$, $\lambda_2 = 18$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5}$ cm^{2}s^{-1}.*

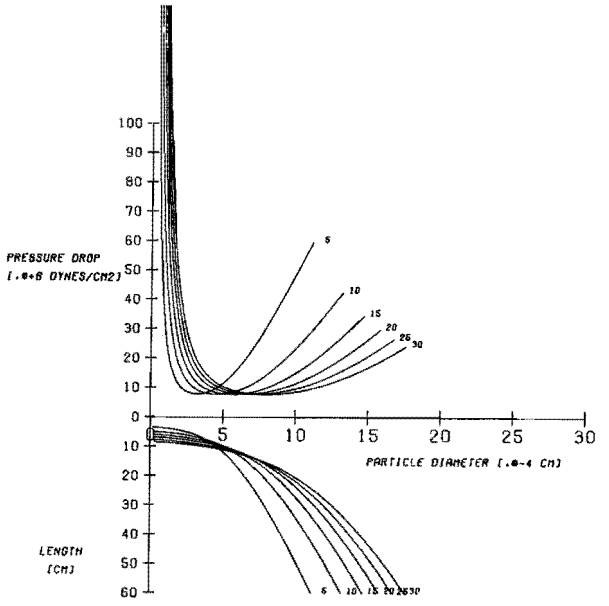


Figure III.13. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, varying the value of the molecular diffusion coefficient, D_m, of a component in the reaction mixture. Numbers plotted near the curves: D_m in 10^{-6} $cm^2 s^{-1}$. Conditions: t_v = 120 s, $\Delta\sigma_{t,r}$ = 1.0 s, γ = 0.7, λ_1 = 10, λ_2 = 18, k_o = 0.002, η = 0.5 cP.*

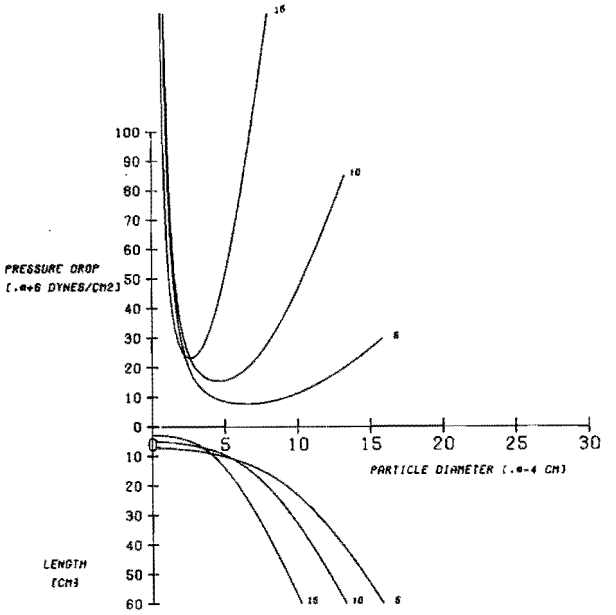


Figure III.14. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various values of the viscosity, η, of the reaction mixture. Numbers plotted near the curves: η in 10^{-3} P. Conditions: t_v = 120 s, $\Delta\sigma_{t,r}$ = 1.0 s, γ = 0.7, λ_1 = 10, λ_2 = 18, k_o = 0.002.*

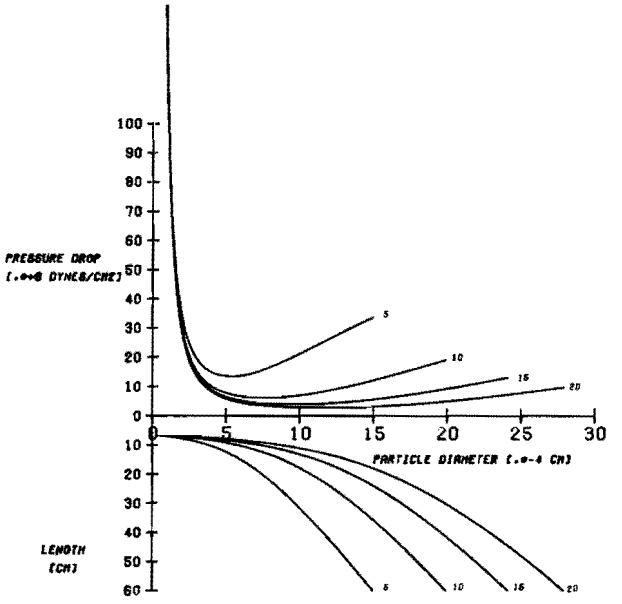


Figure III.15. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various values of the geometry factor λ_2 and at constant $\lambda_1 = 5$. Numbers plotted near the curves: λ_2. Conditions: $t_v = 120$ s, $\Delta\sigma_{t,r} = 1.0$ s, $\gamma = 0.7$, $\lambda_1 = 5$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

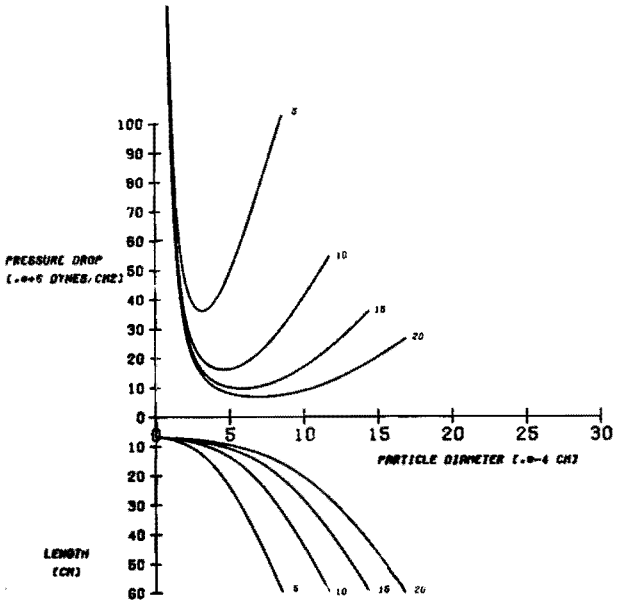


Figure III.16. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various values of the geometry factor λ_2 and at constant $\lambda_1 = 10$. Numbers plotted near the curves: λ_2. Conditions: $t_v = 120$ s, $\Delta\sigma_{t,r} = 1.0$ s, $\gamma = 0.7$, $\lambda_1 = 10$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5} cm^2 s^{-1}$.*

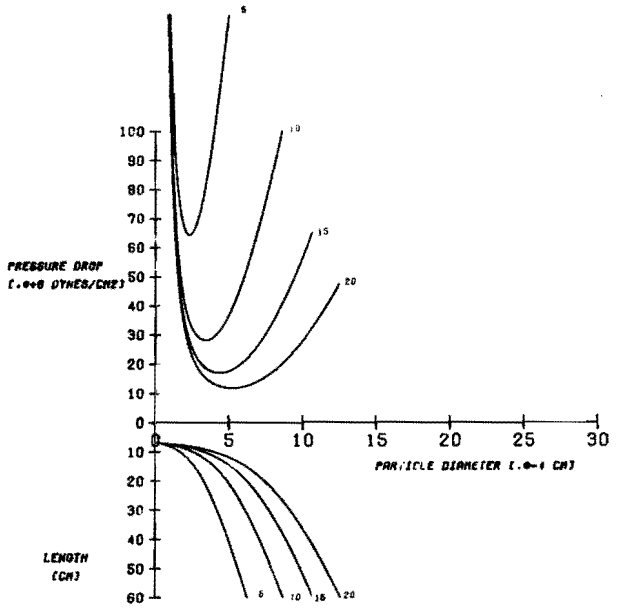


Figure III.17. *Reactor length, L, and reactor pressure drop, Δp, as a function of the particle diameter, d_p, for various values of the geometry factor λ_2 and at constant $\lambda_1 = 15$. Numbers plotted near the curves: λ_2. Conditions: $t_v = 120$ s, $\Delta\sigma_{t,r} = 1.0$ s, $\gamma = 0.7$, $\lambda_1 = 15$, $k_o = 0.002$, $\eta = 0.5$ cP, $D_m = 2.10^{-5}$ cm^{2}s^{-1}.*

2.5 Application of the packed bed reactor for the determination of trace amounts of hydroperoxides.

2.5.1 Introduction.

Hydroperoxides are important intermediates in a number of (industrial) oxidation processes and the study and control of these processes require sensitive methods for determining the individual hydroperoxides in the reaction mixtures. Because of their thermal instability, the compounds should preferably be separated by column liquid chromatography.

Direct UV-absorption detection has been used in the liquid chromatography of hydroperoxides. However, this

approach proved to be unsuitable when using complex reaction mixtures containing high concentrations of other reaction products. A well-known sensitive and selective method for the colorimetric determination of hydroperoxides is based upon the reaction with sodium iodide in a mixture of 2-propanol, acetic acid and water; the absorbance of the reaction mixture due to I_3^- is usually measured at 362 nm. The direct objective of this work was the construction of an on-stream system for this reaction which should be used in combination with the chromatographic separation of hydroperoxides.

2.5.2 Experimental.

The liquid chromatograph (Figure III.18) was constructed in our laboratory and has been described elsewhere[97]. Chromatographic columns (30 cm x 4.6 mm) were made from stainless-steel tubing. The columns were filled with 5 µm silicagel (LiChrosorb SI 60, Merck, Darmstadt, G.F.R.) by a slurry packing procedure.

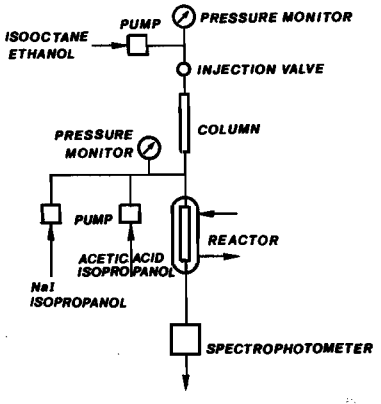


Figure III.18. *Schematic diagram of the chromatographic system.*

The plate numbers achieved in these columns under normal operating conditions appeared to range from 12,000 to 15,000. A 50% water-saturated mixture of 2,2,4-trimethyl-

pentane and ethanol (95 : 5 v/v %) was used as the eluent. Normally, the columns were operated at a liquid flow rate (Φ_c) of about 1.1 $cm^3\ min^{-1}$, which corresponds to a linear eluent velocity of ca. 0.2 cm s^{-1}.

The reagent used for colorimetric detection of hydroperoxides was formed *in situ* by mixing two streams of equal flow-rates (0.55 $cm^3\ min^{-1}$), one consisting of a solution of sodiumiodide in 2-propanol (12.5 g l^{-1}), the other being a mixture of acetic acid and 2-propanol (10 : 90 v/v %). *In situ* formation of the reagent is necessary, because of its instability. The reagent streams were supplied by reciprocating high-pressure pumps (Type DMP-AE-10.4, Orlita, Giessen, G.F.R.). An effective pulse damping system is essential, because of pulsating reagent flow will disturb the homogeneity of the reaction mixture and, consequently, produce a high detector noise. A pulse damping circuit consisting of a large volume bourdon tube and capillary tubing proved to be sufficient. The reagent was added to the column effluent through a 0.15 mm I.D. stainless steel capillary tube. The connection between the column and reactor consisted of a short piece of 0.15 mm I.D. capillary tubing (see Section III. 2.2.2). The stainless-steel reactor (50 cm x 4.6 mm I.D.) was filled with glass beads (Sovitec, Charleroi, Belgium) of 15 µm mean particle size. Narrow sieve fraction of these glass beads were prepared by an air classifier (Zichzacksichter MZR, Alpine, Augsburg, G.F.R). A balanced-density-slurry method (see Section III.2.3) was used for packing the columns. The reactor column was thermostated at 70°C. The absorbance of the reaction mixture due to the presence of I_3^- was continuously measured at 362 nm with a PM2D spectrophotometer (Zeiss, Oberkochen, G.F.R.) equipped with a thermostated low-dead-volume flow cell. The contribution to peak-broadening from injection and detection device is 0.4 s.

2.5.3 Results and discussion.

The reaction conditions were derived from a standard colorimetric method for the assay of low concentrations of hydroperoxides in hydrocarbons. It was found that the reaction time for hydroperoxides could be considerably reduced from that in the original procedure by heating the reaction mixture.

In view of the boiling points of the components of this mixture, a temperature of 70°C was chosen. At this temperature, the influence of the reaction time on the formation of I_3^- from NaI by cyclohexyl hydroperoxide was investigated; the results are shown in Figure III.19. The measurements were carried out on mixtures containing equal volumes of the reagent mixture sodium iodide, 2-propanol and acetic acid and the eluent mixture 2,2,4-trimethylpentane-ethanol. The curve shows this at the selected residence time of 90 s almost 80% of the maximum absorbance is attained.

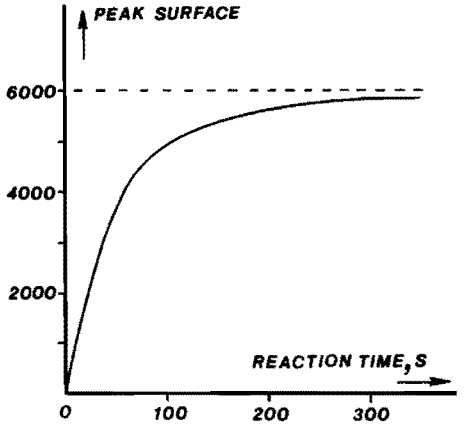


Figure III.19. *Influence of reaction time on the formation of I_3^- from sodium iodide by cyclohexyl hydroperoxide, in reaction mixture at 70°C.*

The linear eluent velocity in the chromatographic column corresponds to a volume flow-rate of about 1,1 cm^3 min^{-1}. The total volume flow-rate for the reagent was set

arbitrarily at the same value. This choice is a compromise; on the one hand, high flow-rates cause undesirable dilution of the sample compounds (see Section III.1.4), while on the other hand difficulties, such as irreproducible pump setting and irregular flow, will arise from too low flow-rates.

In order to estimate $\Delta\sigma_{t,r}$ from practical liquid chromatography, we consider a 30 cm column. The application of a 5 μm packing enables plate numbers of about 15,000 to be attained in such a column. Suppose that the column is operated slightly above its optimal linear velocity, for instance at 0.2 cm s^{-1}. In practical separations, the capacity ratio, k, will be at least 1, which corresponds to a retention time of 300 s. Using these values for t_R and N, we find $\Delta\sigma_{t,c}$ = 2.45 s (k = 1) and hence $\Delta\sigma_{t,r}$ = 0.81 s, when a loss of 5 % is accepted (see Section III.13, Equation (III.6)).The condition $\Delta\sigma_{t,r}$ = 0.8 s can be met by using short columns (L = 10 cm) packed with glass beads of 6 μm, at a pressure drop of 8 atm. It can be calculated from Equation (III.23) that with $\Phi_c + \Phi_r$ = 2.2 cm^3 min^{-1}, the internal diameter of the reactor column should be 0.72 cm.

For practical reasons, it was decided to construct the reactor of standard 4.6 mm I.D. stainless-steel tubing. Consequently, standard low-dead-volume end-fittings could be used. From Equation (III.23) it is found that L = 50 cm. It can be shown that the column should be packed with 15 μm glass beads in order to meet the condition $\Delta\sigma_{t,r}$ = 0.8 s (graph not included). The viscosity of the reaction mixture is almost equal to the value assumed (0.54 compared with 0.5 cP). Therefore, the pressure-drop over the reactor was expected to be 29 atm.; the experimental result was 20 atm.

The additional band-broadening in the reaction system, $\Delta\sigma_{t,r}$, was measured experimentally. For this purpose, a UV-absorbing compound, *m*-nitrophenol (k = 1.1) was injected into the chromatographic column, which had been connected directly to the spectrophotometer. The elution peak was

observed at 280 nm.

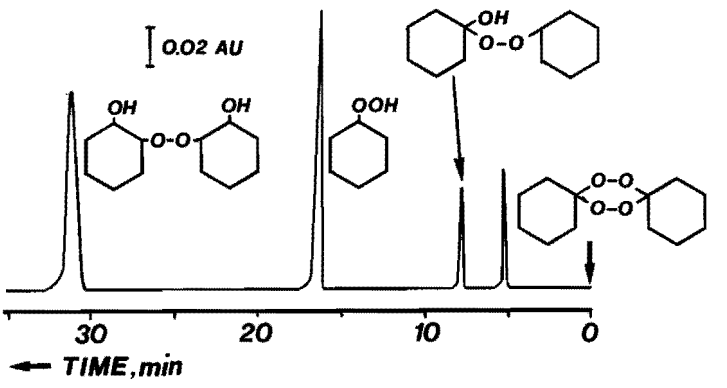


Figure III.20. *Chromatogram of a test mixture of peroxides. Column: 300 x 4.6 mm, packed with 5 µm LiChrosorb SI 60. Eluent: 2,2,4-trimethylpentane-ethanol (95:5 v/v %), 50% water saturated. Flow-rate: 1.1 cm^3 min^{-1}. Flow-rate reagent* Φ_r *= 1.1 cm^3 min^{-1}. Detection by reation system, consisting of a 50 x 0.46 cm packed bed reactor, with 15 µm glass beads, and an UV detector, operated at 360 nm, temperature: 70°C.*

The reactor was then placed between the chromatographic column and the spectrophotometer; the reagent flow was replaced by a flow of pure 2-propanol. The same compound was injected again and $\Delta\sigma_{t,r}$ could be calculated by comparing the band widths of the peaks. The value of 0.9 ± 0.2 s which was found, agrees with the theoretical value fairly well.

Figure III.20 shows the chromatogram obtained for a model mixture of peroxides derived from cyclohexane; the peak of cyclohexyl hydroperoxide (k ~ 5) corresponds to about 1 µg. The minimum detectable amount of this peroxide was 5 ng; injection of this amount produces a peak five times the standard deviation of the detector noise signal

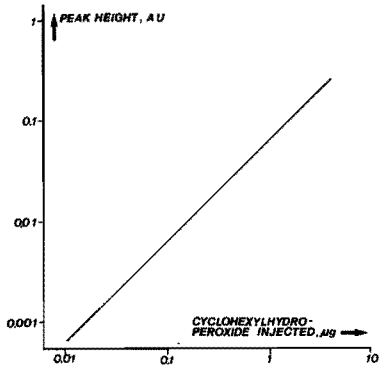


Figure III.21. *Calibration curve for cyclohexylhydroperoxide.*

(noise equals 2.10^{-4} A.U.).
In Figure III.21 a calibration curve is given for cyclohexylhydroperoxide.

2.6 Application of packed bed reactors for the analysis of amino-acids.

Recently the detection of primary amines is significantly enhanced by using a selective fluorescence reaction [135-136]. *o*-phthalaldehyde reacts in alkaline medium in the presence of a reducing agent such as 2-mercapto-ethanol by giving rise to strongly fluorescing compounds. Benson and Hare[137] modified the reagent employed by Roth[135] and demonstrated that, by using *o*-phthalaldehyde reaction-detection in combination with an efficient separation, a highly sensitive analysis of protein amino-acids and peptides could be achieved.
However, packed beds should be preferred as reactors, because they give the lowest possible additional band-broadening. Figure III.22 gives the scheme of a reaction system for the fluorometric detection of amino-acids separated by "soap chromatography"[138]. The separation conditions are summarized in Figure III.23. The reagent

consisted of a solution of *o*-phthalaldehyde (0.8 g/l), ethanol (10 ml/l) and 2-mercapto-ethanol (2 ml/l) in 0.1 M sodium tetraborate buffer (pH = 10.4). The linear eluent velocity in the chromatographic column corresponds to a volume flow-rate of 1 cm^3 min^{-1}. The flow-rate for the reagent was arbitrarely set at the same value. Mixing of the column effluent and reagent was achieved by using a tee in which both streams were fed through 0.15 mm I.D. channels (see Section III.2.2.2). At 20°C the reaction was almost complete for t_v = 60 s. The reaction was carried out in a 30 cm x 4.6 mm I.D. stainless steel reactor packed with 15 µm glass beads. It can be calculated from Equation (III.23) that for $\Phi_c + \Phi_r = 2$ cm^3 min^{-1} the mean residence time in the reactor will be about 60 s. Further, as can be seen from Figure III.10, this reaction can be carried out for $\Delta\sigma_{t,r} < 1$ s at a moderate pressure drop. The fluorescence of the reaction products was continuously measured at 455 nm with a fluorometer (type 135800/50, Farrand, New York, U.S.A.) equipped with a home made flow-cell; the excitation wavelength was 340 nm. Figure III.23 shows a chromatogram for a mixture of amino-acids.

The minimum detectable amount of the amino-acids is about 100 pg, even for strongly retained components. It should be emphasized that these detection limits are only attained when fluctuations in column and reagent flow are virtually eliminated. Reciprocating high-pressure pumps (type DMPSK 15, Orlita, Giessen, G.F.R.) equipped with an effective pulse-damping circuit were used. The additional band-broadening in the reaction system, $\Delta\sigma_{t,r}$, was measured experimentally. To this purpose, an UV-absorbing amino-acid, tyrosine, was injected into the chromatographic column, which had been connected directly to an UV detector (PM2D, Zeiss, Oberkochen, G.F.R.). The eluent had been modified by adding methanol in order to reduce the capacity factor for tyrosine to about 1. The reactor was placed between the column and the detector; the reagent flow was replaced by water. The same compound

was injected again and $\Delta\sigma_{t,r}$ was calculated by comparing the widths of the peaks. A value of about 1 s was found.

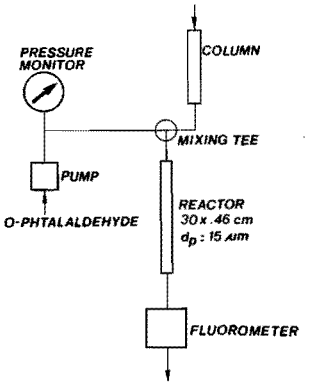


Figure III.22. *Schematic diagram of the system for separation and fluorometric detection of amino-acids.*

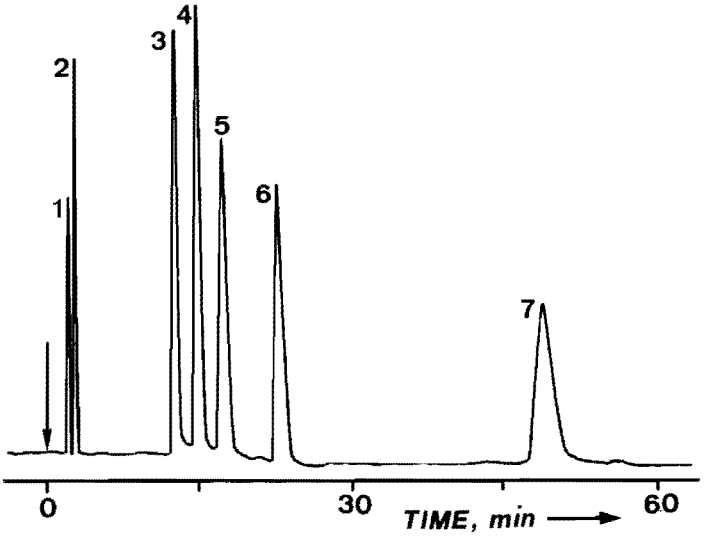


Figure III.23. *Chromatogram for a mixture of amino-acids. Column: 20 cm x 0.46 cm I.D. packed with 5 µm LiChrosorb RP-8. Eluent: 0.01 M sodium phosphate buffer, pH = 2.5 in water + 1 v/v % 2-propanol + 0.06 w/v % sodium laurylsulphate. Flow-rate eluent: 1 $cm^3 min^{-1}$, reagent 1$cm^3 min^{-1}$. Components: 1 = cystine.H_2O, 2 = taurine, 3 = aspartic acid, 4 = glutamic acid, 5 = serine, 6 = threonine, 7 = alanine.*

3. Design and application of tubular reactors.

3.1 Introduction.

In analytical chemistry, process industry and medical technique, mass and heat transfer is frequently accomplished with tubes and laminar flow. This laminar flow is expected especially in the case of viscous liquids and flow in capillary tubes. Generally one strives for:

- a good radial concentration mixing,
- a minimal axial dispersion.

Turbulent flow is favourable for this aim. In many practical application no turbulent flow can be realized.

Laminar flow in straight tubes is less favourable. In comparison with laminar flow, helical coiled tubes give better results, because of a secondary flow perpendicular on the axial flow direction. The transfer in radial direction is realized by diffusion and by convection. By the secondary flow a smoothing effect on the distribution of the axial velocities is noticed, which reduces the residence time distribution. A small increase of the axial pressure gradient is the result.

To derivatize eluted components after the separation columns, numerous workers[75-85] added a suitable reagent to the column effluent using a tee piece and short lengths of highly coiled tubing. At the flow-rates employed, these coils provide time for mixing reagent with effluent and the time is ample for the reaction to go to completion and occasionally for destruction of excess reagent[80].

The basis of the design of such tubular reactors will be given in this section.

3.2 Axial dispersion in straight tubes.

The problem of dispersion of soluble matter in a solvent flowing through a straight tube of circular cross section was first treated by Taylor[139,140], and was subsequently extended significantly by Aris[141]. Aris

showed the form of the effective dispersion coefficient to be equal to the sum of the molecular diffusion coefficient and Taylor's dispersion coefficient:

$$ID_{a,s} = D_m + \frac{d_t^2 u^2}{192 D_m} = D_m \left(1 + \frac{Pe_a^2}{192}\right) \qquad \text{(III.24)}$$

where d_t is the diameter of the tube.
At high Peclet numbers the effective dispersion coefficient is just $\frac{d_t^2 u^2}{192 D_m}$ which was found originally by Taylor. However, at low Peclet numbers, the relative effect of axial diffusion increases, and in the limiting case, when Pe_a goes to zero, the effective coefficient $ID_{a,s}$ is simply the molecular diffusion coefficient D_m.

Figure III.24, which summarizes the region of application of the various solutions, can be used to compare the applicability and limitations of the various equations. This graphical summary of the regions of application was introduced by Ananthakrishnan *et al.*[142-144]. A very detailed analysis of the problem for straight tubes has been successfully completed by these investigators. According to Ananthakrishnan *et al.*[142] and as indicated in Figure III.24 the Taylor-Aris equation is applicable if:

$$\tau = \frac{4 D_m t_v}{d_t^2} > 0.8 \qquad \text{(III.25)}$$

where τ = dimensionless time, defined as the ratio of characteristic time for convective transport to that for radial diffusion.
t_v = residence time.
D_m = molecular diffusion coefficient
d_t = internal diameter of the tube.

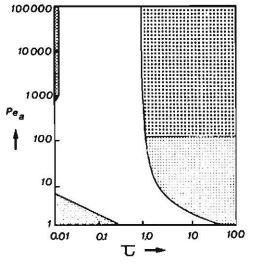


Figure III.24. *Summary of all operating conditions, τ vs Pe.*

- *Pure diffusion*
- *Pure convection*
- *Taylor's solution*
- *Taylor solution with Aris' modification*
- *Numerical solution only* [142].

A second restriction given for the application of Equation (III.24) is:

$$Pe_a > 100 \qquad \text{(III.26)}$$

The first limitation is in fact similar to the condition according to Taylor:

$$\frac{L}{d_t} >> \frac{Pe_a}{30} \qquad \text{(III.27)}$$

A third criterium for the validity of the Taylor-Aris equation is that the capillary tube has sufficient theoretical plates or:

$$\Delta\sigma_{t,r} << t_v \qquad \text{(III.28)}$$

where t_v is the residence time and $\Delta\sigma_{t,r}$ the standard deviation of the residence distribution. Equation (III.28) also implies a symmetrical elution pattern.

From the effective dispersion coefficient in axial direction the variance of the residence time distribution $\Delta\sigma_{t,r}^2$ can be derived because according to Einstein[145]:

$$\sigma_{L,a}^2 = 2ID_a t_v \qquad \text{(III.29)}$$

where t_v is the mean residence time equal to $\frac{L}{u}$ and $\sigma_{L,a}^2$ is the variance of the band in distance units. Furtheron:

$$\frac{\Delta\sigma_{t,r}^2}{t_v^2} = \frac{\sigma_{L,a}^2}{L^2} = \frac{1}{N} = \frac{H}{L} \qquad \text{(III.30)}$$

so:

$$\Delta\sigma_{t,r}^2 = \frac{2D_m t_v^2}{uL} + \frac{d_t^2 u^2 t_v^3}{96\, D_m L^2} \qquad \text{(III.31)}$$

with $u = \frac{L}{t_v}$ Equation (III.31) becomes:

$$\Delta\sigma_{t,r}^2 = \frac{2D_m t_v^3}{L^2} + \frac{d_t^2 t_v}{96D_m} \qquad \text{(III.32)}$$

Introducing the plate height Equation (III.32) becomes:

$$H = \frac{2D_m}{u} + \frac{d_t^2 u}{96D_m} \qquad \text{(III.33)}$$

Except for extremely low flow velocities the first term on the right hand side of Equation (III.32) can be neglected. At velocities as used in practice the variance per second of residence time in the reactor, can be expressed as:

$$\frac{\Delta\sigma_{t,r}^2}{t_v} = \frac{d_t^2}{96D_m} \qquad \text{(III.34)}$$

For a non-parabolic flow profile this equation should be modified as:

$$\frac{\Delta\sigma_{t,r}^2}{t_v} = \frac{\kappa d_t^2}{96D_m} \qquad \text{(III.35)}$$

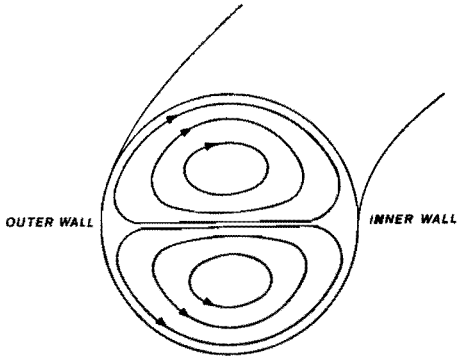
where κ depends on the particular flow profile.

3.3 Axial dispersion in coiled tubes.

Curved configurations of circular tubes have some definite advantages over straight tubes. The nature of curved-tube fluid motion as compared with straight tube parabolic flow, causes a higher axial pressure gradient, a higher critical Reynolds number for transition to turbulent flow and last but not least a narrower residence time distribution. Besides the advantage of the compactness of coils, higher heat and mass transfer coefficients are measured. The reason is that when a fluid flows through a helically coiled tube, a secondary flow, that is, flow perpendicular to the main direction of flow, sets in. This secondary flow (Figure III.25) is due to the centrifugal forces acting on the flowing fluid. Near the tube center the axial velocity is greatest and here centrifugal forces will act most strongly. The fluid near the center is thrown outward and replaced by recirculating fluid moving inward along the tube wall.

Dean[146,147] was the first to analyse mathematically the phenomenon of secondary flow in tubes of circular cross-section coiled in the form of a circle, and obtained approximate expressions for the fluid velocity profile. The theoretical analysis of laminar dispersion in helical coils or the simpler case of a curved tube consist in solving the convective diffusion equation along with the equations of motion.
Dean proposed the use of a new dimensionless parameter, now known as the Dean-number, $Dn = Re \sqrt{\frac{d_t}{d_c}}$ for characterizing helical flow. Here Re is the Reynolds number, d_t the diameter of the tube, and d_c the coil diameter. However, Deans analytical solutions are only valid for a very limited range of flow conditions.

More recently several investigators[148-154] have obtained analytical and numerical methods for solving the equations of motion over a wide range of Reynolds numbers and curvature ratios ($\lambda = \frac{d_c}{d_t}$) (See Figure III.26).



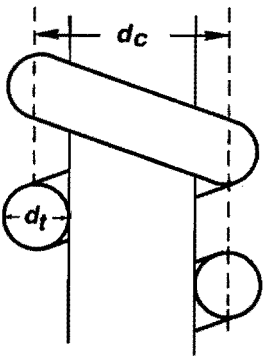


Figure III.25. *Secondary flow pattern in the cross section of a coiled tube.*

Figure III.26. *A coiled tube.*

The calculated axial flow velocity profiles were found to deviate strongly from their parabolic straight-tube counterparts; the difference between mean axial velocity and the velocity on the various axial streamlines are greatly reduced. These results are confirmed by experimental data[149]. The secondary flow in coiled tubes causes an increase in radial mass transfer and, therefore, reduces axial dispersion in comparison with a flow through straight pipes. When defining

$$\kappa = \frac{(\Delta\sigma_{t,r}^{2})_{coiled}}{(\Delta\sigma_{t,r}^{2})_{straight}}$$

Equation (III.34) can be written as Equation (III.35) as stated before for non-parabolic flow patterns ($\kappa \lesssim 1$). Horvath *et al.* [155] tried to exploit this phenomenon in liquid chromatography and investigated solute band-spreading in coiled uncoated capillary columns. Experimentally a considerable decrease in band-broadening was observed due to the "coiling effect", but the residual dispersion was still unacceptable for liquid chromatography.

An exact treatment of dispersion in laminar flow of liquid in coiled tubes essentially concludes the solution of three-dimensional mass-transfer equation accounting for molecular as well as convective diffusion at a given flow field. No satisfactory solution for this problem has been found up to now, due to the complexity of the equations involved.

Recently Trivedi and Vasuveda[156,157] have presented experimental results for dispersion in coiled tubes over a wide range of Dean numbers; these authors found that the experimental κ-values when plotted against Dn were represented by a series of curves according to the Schmidt-number Sc. However, as the κ-values were derived at least in part from asymmetrical step response curves, the accuracy of the results leaves to be desired (condition of Equation (III.28)).

A recent theoretical study[158-160, 178,179] shows that κ can be given as a function of Dn^2Sc in the case of large molecular diffusion and at least for $Dn < 20$ and $\lambda > 20$. κ is the ratio of the dispersion coefficient of the helix ID_c and the dispersion coefficient of the straight tube ID_s. The Schmidt number, Sc, is defined as $Sc = \frac{\eta}{\rho D_m}$ with η the viscosity and ρ the density of the fluid. As already stated in Section III.3.2 for coiled capillaries the variance per second of residence time is:

$$\frac{\Delta\sigma_{t,r}^2}{t_v} = \kappa \frac{d_t^2}{96D_m} \quad \text{with } \kappa \leqslant 1 \qquad \text{(III.36)}$$

Analogous to the derivation of Equation (III.34) from Equation (III.24), Equation (III.36) resulted from:

$$ID_{a,c} = D_m + \kappa \frac{d_t^2 u^2}{192D_m} \qquad \text{(III.37)}$$

again by neglecting the molecular diffusion term.

Here the plate height equation is:

$$H = \frac{2D_m}{u} + \kappa \frac{d_t^2 u}{96 D_m} \qquad \text{(III.38)}$$

To asses the significance of secondary flow in capillary columns, solute band-spreading in coiled tubes was investigated experimentally. The results form the basis of the design of open tubular reactors.

3.4 Determination of the effect of coiling on the residence time distribution.

In order to measure κ-values, helical coils of various internal diameter and coiling ratio were prepared. The geometries of the helical tubes, made of thin walled stainless-steel tubing are summarized in Table III.2. All coils were tightly wound.

The viscosities and densities of the solvents and the molecular diffusion coefficients of the tracers in the solvents are mentioned in Table III.3. The molecular diffusion coefficients are measured by the Taylor method [139,140] in a straight tube.

Table III.2. *Dimensions of the coiled tubes used.*

Coil no.	d_t cm	L cm	d_c cm	$\lambda = \frac{d_c}{d_t}$
1.	0.0508	1150	15.5	305
2.	0.0508	1150	52.0	1024
3.	0.11	610	6.0	54.5
4.	0.11	610	17.0	154.5
5.	0.11	610	2.0	18.2

Table III.3. *Physical constants of the systems measured.*

Solvent	Tracer	$\rho_{solvent}$ g cm^{-3}	$\eta_{solvent}$ 10^2P	$D_{m,tracer}$ 10^5cm^{2}s^{-1}	Sc
2,2,4-tri-methylpentane	nitro-benzene	0.69	0.52	2.5	300
chloroform	benzene	1.48	0.54	2.05	180
hexane	benzene	0.66	0.29	4.1	110

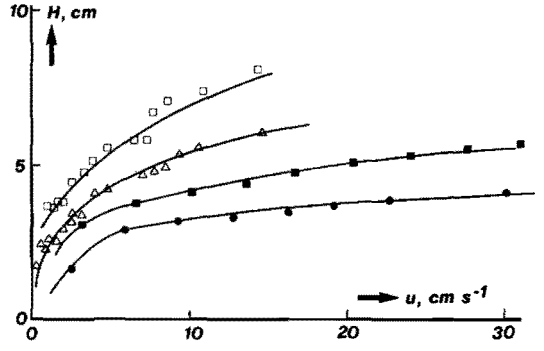


Figure III.27. *Plate height versus linear velocity of different coiled tubes.*

- ■ *benzene-chloroform* (λ = 305)
- ● *benzene-hexane* (λ = 1024)
- □ *nitrobenzene-iso-octane* (λ = 155)
- △ *nitrobenzene-iso-octane* (λ = 55)

The procedure for determining the residence time distribution essentially consisted in measuring the standard deviation of a peak of the tracer. This was done as a function of the flow-rate. Injection was made by means of a sample valve and detection by UV-absorbance. All measurements were corrected for injection and detection contributions, being < 3%. Besides the limitations given in Equations (III.25), (III.26), and (III.28) it should be noted that the time constant of the registration system should not exceed 0.2 $\Delta\sigma_{t,r}$ in order to avoid distortion of the peak shape[161].

The symmetry of the peaks obtained was better than 98%

measured at 0.6 of the peak height. The symmetry is defined as the ratio of the peak width of the front and the rear end of the peak.

The resulting H-u curves are given in Figure III.27. Flattening of the H *versus* u curves in coiled tubes is observed as expected as a consequence of secondary flow. It is also clearly demonstrated that there is a strong effect of diffusivity. Higher plate heights are obtained with slow diffusing components, indicating molecular diffusion plays a significant role in the dispersion mechanism under these conditions (second term on the right hand of Equation (III.33)). The effect of coiling is once again clearly demonstrated in Figure III.28 where $\Delta\sigma_{t,r}$ is plotted *versus* $1/d_c$.

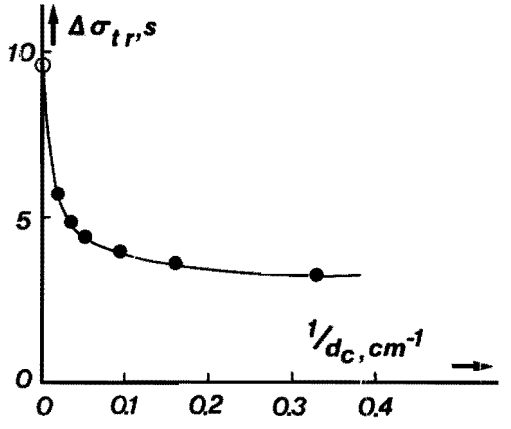


Figure III.28. *Influence of tube coiling on* $\Delta\sigma_{t,r}$. *Tube: 25 m x 0.0508 cm. Benzene in iso-octane, flow-rate 2.056 cm^3 min^{-1}.* O *calculated from Equation III.32.*

In Figure III.29 K-values from Equation (III.36) are plotted against $DnSc^{\frac{1}{2}}$. The data points are represented by a single curve as predicted by Janssen[160]. In this figure three regions can be distinguished. The differen-

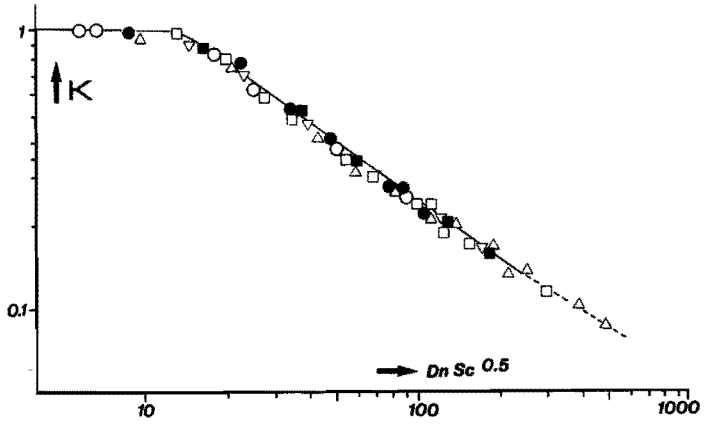


Figure III.29. *Reduction of axial dispersion due to secondary flow in coiled tubes, κ-values were calculated from experimental data by using Equation (III.36). Nitrobenzene-iso-octane:* ○ λ = *1024,* ▽ λ = *305,* □ λ = *155,* △ λ = *55. Benzene-chloroform:* ■ λ = *305. Benzene-hexane:* ● λ = *1024.*

tiation is related to the ratio of the inertia forces and friction forces in the flow. For values of the group $DnSc^{\frac{1}{2}} < 10$, there is no significant difference between the axial dispersion in helical coils and in straight tubes (zone 1). For increasing values of $DnSc^{\frac{1}{2}}$ ($DnSc^{\frac{1}{2}} > 10$) the axial dispersion in the helical coils decreases as compared with axial dispersion in straight capillaries (zone 2). Janssen *et al.*[160] observed a very small decrease of κ for values of $DnSc^{\frac{1}{2}}$ larger than 100 (zone 3). In our measurements this flattening is not observed experimentally and κ had not yet reached a constant value at $DnSc^{\frac{1}{2}}$ of even 500. Zone 2 is relevant for practical use of helical coils as tubular reactors. Zone 3 is only reached for high values of Sc and/or Re and small values of λ. Values of λ smaller than 25 are practically inconvenient for d_t smaller than 0.1 cm.

The measurements in region 2 were represented by a single curve which could approximately be fitted by the following equation:

$$\kappa = 5.6\ (DnSc^{\frac{1}{2}})^{-0.68} \qquad \text{(III.39.a)}$$

for

$$200 > DnSc^{\frac{1}{2}} > 10$$

whereas $\kappa = 1$ (III.39.b)

for

$$DnSc^{\frac{1}{2}} < 10$$

By using the κ-values from Equations (III.39.a) and (III.39.b) it is possible to predict band-broadening in coiled tubular reaction systems. This opens possibilities to optimize the design of capillary reactors in high performance liquid chromatography.

3.5 The pressure drop in a coiled tube.

In the case of a non-compressible fluid the pressure drop Δp in a laminar flow in a straight pipe is expressed by the Hagen-Poisseuille equation:

$$\Delta p = \frac{512\eta\ \Phi^2 t_v}{\pi^2\ d_t^6} \qquad \text{(III.40)}$$

with η the viscosity and Φ the flow-rate of the fluid, d_t the internal diameter of the tube and t_v the residence time.

The circulation in the cross-section of a coiled tube causes an increase of the axial pressure gradient. Considerable data are available on pressure drop in coiled tubes and a good agreement exists between theoretical and experimental values[162-164] (Figure III.30).
For coiled tubes the following equation is stated:

$$\Delta p = \psi \frac{512\eta\ \Phi^2 t_v}{\pi^2\ d_t^6} \qquad \text{(III.41)}$$

with $\psi > 1$

ψ is defined as the ratio of the pressure drop for a coiled tube and for an equivalent straight pipe at the same flow-rate. ψ is a function of the Dean number and the coiling ratio λ. It turns out that for $Dn \leqslant 25$ the increase in pressure drop in comparison with flow through a straight pipe is less than 10% ($\psi < 1.1$). As in practice this condition will always be fulfilled for post-column reactors, Equation (III.40) can be used as a good approximation and the pressure drop increase was neglected.

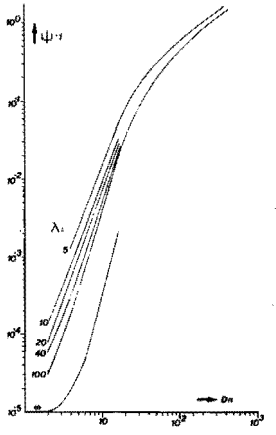


Figure III.30. *Pressure drop in coiled tubes* [162].

3.6 Design of tubular reactors.

Similar as in packed bed reactors, peak-broadening $\Delta\sigma_{t,r}$ in capillary reactors can be expressed by the mean residence time t_v and the plate number N. Here, a suitable choice of the internal diameter, d_t, length L, and coiling ratio λ, should be made to fulfill the fixed reaction time t_v, allowable $\Delta\sigma_{t,r}$ and without exceeding a maximal allowable pressure drop Δp.

The three criteria are expressed in the following equations:

$$t_v = \frac{\pi d_t^2 L}{4\Phi_r} \qquad \text{(III.42.a)}$$

$$\Delta\sigma^2_{t,r} = \kappa \frac{d_t^2 t_v}{96\, D_m} \qquad \text{(III.42.b)}$$

$$\Delta p = \psi \frac{512\eta\Phi_r^2 t_v}{\pi^2 d_t^6} \qquad \text{(III.42.c)}$$

By using κ values from Equation (III.42.a) it is possible to predict band-broadening in coiled tubular reactor systems. To simplify the procedure for each value of Φ_r the relation of $\Delta\sigma^2_{r,t}/t_v$ and d_t can be plotted for various values of λ. This is done in Figure III.32 for Φ_r = 1 cm^3 min^{-1}. With the aid of Figure III.31, in Figure III.32 $\Delta\sigma_{t,r}$ is plotted against the internal diameter of the reactor tube, d_t, for reaction times varying from 30 to 420 s. The curves were calculated from Equation (III.42.b) or with the aid of Figure III.31 with Φ_r = 1 cm^3 min^{-1}, ($D_m = 2.10^{-5} cm^2 s^{-1}$, η = 0.5 cP and ρ = 0.7 g cm^{-3}, λ = 200).

It can be observed from Figure III.32 that little additional band-broadening will be obtained for narrow bore tubings and small residence times, however, with the consequence of long reactors, small internal diameters and high pressure drops.

At a given residence time in the reactor, t_v, the minimum internal diameter of the reactor, d_t, will be limited for practical reasons by the pressure drop and tube length. At a constant flow-rate a decrease of the internal diameter, d_t, generates higher linear velocities, u, consequently it requires longer capillaries at a fixed delay time, t_v. A decrease of the internal diameter also increases the pressure drop over the reactor drastically. Also the chance of clogging is higher. Arbitrarily a pressure limit of 50 atm (ψ = 1) and a maximum length of 50 m were chosen.

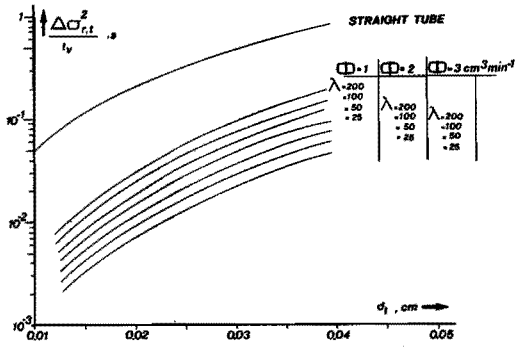


Figure III.31. *Band-broadening per time unit in coiled tubular reactors as a function of the tube diameter, d_t, for various volumetric flow-rates and curvature ratios* λ.

For the various residence times indicated in Figure III.32 the minimum value for d_t was calculated with Equation (III.42.b) for Δp = 50 atm. On the other hand the internal tube diameters for a reactor length of 50 m were calculated from Equation (III.42.a). The practical limits for d_t are indicated in Figures III.32 - III.35 by broken lines. Similar figures are given for Φ = 2, and 3 cm^3 min^{-1} both for λ = 200 (Figures III.33 and III.34) and also for Φ = 3 cm^3 min^{-1} with λ = 25 (Figure III.35). As can be concluded from Figures III.34 and III.35 where Φ is kept constant and λ is varied from 200 to 25 it is worth while to select a smaller coiling ratio λ. This results in a larger internal reactor diameter and thus in a smaller length. The effect is an appreciable decrease in pressure drop.

3.7 Application of a coiled tubular reactor for the determination of sugars.

The use of high performance liquid chromatography for the analysis of trace amounts of sugars has previously been almost impossible owing to the low sensitivity of detection. Hitherto, liquid chromatographic separations

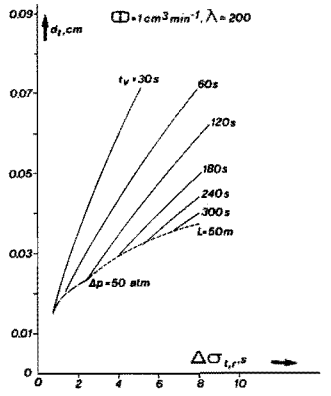


Figure III.32. *Band-broadening, $\Delta\sigma_{t,r}$, versus tube diameter, d_t, for various reaction times, t_v, assuming a constant total volumetric flow-rate (Φ_r = 1 cm^3 min^{-1}) and a constant coiling ratio (λ = 200); D_m = 2 x 10^{-5} cm^2 s^{-1}, η = 0.5 cP, ρ = 0.7 g cm^{-3}.*

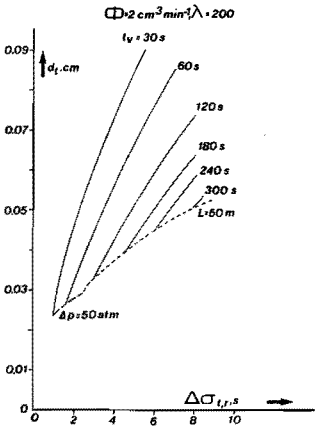


Figure III.33. *Band-broadening, $\Delta\sigma_{t,r}$, versus tube diameter, d_t, for various reaction times, t_v, assuming a constant total volumetric flow-rate (Φ_r = 2 cm^3 min^{-1}) and a constant coiling ratio (λ = 200); D_m = 2 x 10^{-5} cm^2 s^{-1}. η = 0.5 cP, ρ = 0.7 g cm^{-3}.*

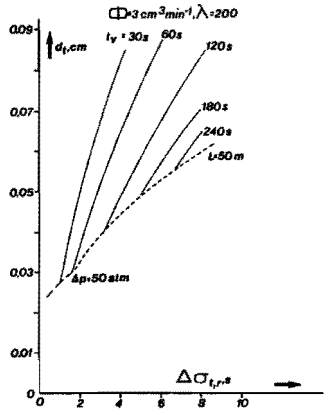


Figure III.34. *Band-broadening, $\Delta\sigma_{t,r}$, versus tube diameter, d_t, for various reaction times, t_v, assuming a constant total volumetric flow-rate (Φ_r = 3 cm^3 min^{-1}) and a constant coiling ratio (λ = 200); D_m = 2 x 10^{-5} cm^2 s^{-1}, η = 0.5 cP, ρ = 0.7 g cm^{-3}.*

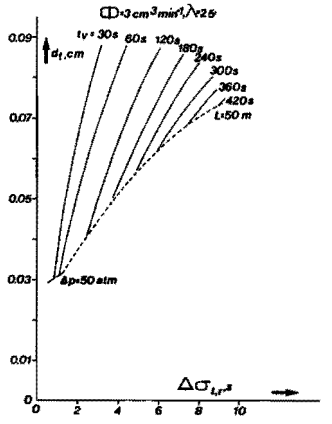


Figure III.35. *Band-broadening, $\Delta\sigma_{t,r}$, versus tube daimeter, d_t, for various reaction times, t_v, assuming a constant total volumetric flow-rate (Φ_r = 3 cm^3 min^{-1}) and a constant coiling ratio (λ = 25); D_m = 2 x 10^{-5} cm^2 s^{-1}, η = 0.5 cP, ρ = 0.7 g cm^{-3}.*

of carbohydrates with various stationary phases have been described[165-169]. The absence of suitable chromophores demands the use of refractive index detectors, and the detection limit obtained by these detectors is about 20 µg of sugar[169]. Classical liquid chromatographic methods involve the use of cation exchangers or sugar-borate complexes on anion exchangers with gradient elution for separation, which is the principle of commercial sugar analyzers[170-173]. The detection is effected by colour reactions after elution[170-172] and detection limits in the microgram region have been achieved. Better results can be obtained by using an oxidative reaction that relies on the reduction of cerium (IV) to fluorescent cerium (III) by the eluted carbohydrates[174].

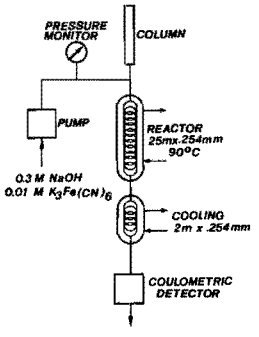


Figure III.36. *Schematic diagram of the system for the separation and detection of reducing sugars.*

Recently Takata *et al.*[175] described a highly selective method for detecting reducing sugars by combination of a reaction system and electrochemical detection:

$$\text{reducing sugar} + Fe(CN)_6^{3-} \longrightarrow Fe(CN)_6^{4-} \qquad \text{(III.43)}$$

$$Fe(CN)_6^{4-} \longrightarrow Fe(CN)_6^{3-} + e \qquad \text{(III.44)}$$

The reaction occurs in alkaline solution at high temperature (t_v = 2 min at 90°C). Glass-bead columns can not be

used for this reaction since alkaline solutions attack the packing material.
For this reaction a metal tubular reactor was used. The scheme of the reaction system is given in Figure III.36. Sugars are separated on a strongly acidic cation exchange resin (Aminex A-9, Biorad, Richmond, U.S.A.) in the K^+-form [176]. Deionized water was used as the eluent at a flow-rate of 0.35 cm^3 min^{-1}. The column effluent is mixed with the reagent consisting of a solution of 0.01 M $K_3Fe(CN)_6$ in 0.3 M NaOH, flow-rate 0.3 cm^3 min^{-1}. A coiled 316 stainless-steel capillary tube 25 m x 0.254 mm I.D. was used as the reactor; the coil diameter was 2.5 cm. A coulometric detector as described by Lankelma and Poppe[177] is used for measuring the concentration of the reduced ion $Fe(CN)_6^{4-}$. The particular reaction mixture made it necessary to modify the detector slightly. The glassy carbon electrodes were replaced by platinum sheets. Moreover, an external calomel electrode was used instead of the internal reference electrode of the original design. The working electrode had a constant potential of +0.5 V.

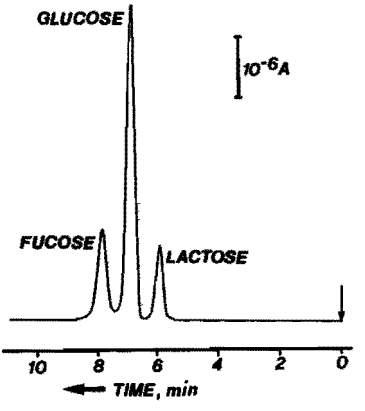


Figure III.37. *Chromatogram of a mixture of sugars. Column: 25 cm x 4.6 mm I.D. Aminex A-9 cation exchanger (K^+-form). Eluent: water. Flow-rate ,eluent: 0.35 cm^3 min^{-1}, reagent: 0.3 cm^3 min^{-1}.*

Figure III.37 shows a chromatogram for a mixture of sugars. The additional band-broadening in the reaction system was 4.5 s and the pressure drop 18 atm. The band-broadening was measured by injecting fructose directly into the reactor.

This value of $\Delta\sigma_{t,r}$ = 4.5 s agrees with the theoretical value of 3.9 s fairly well. The contribution of the separation column is $\Delta\sigma_{t,c}$ = 6.2 s for glucose. The band-broadening caused by the coiled capillary reactor tube is 22% of the band-broadening of a similar straight capillary tube. The minimum detectable amount reached with this type of detection is about 100 ng.

4. Selection criteria for post-column reactors.

In applying reactor systems the "chemistry" of the reaction involved is of the utmost importance for the choice of the reactor type. For simple, one-step fast reactions and, consequently, low values of t_v, *i.e.* 2-4 minutes at most, this choice is obvious. Packed reactors give the lowest additional band-broadening and should be preferred therefore.

Helically coiled tubes are attractive as reactors because of the simple construction and the easily predictable dispersion. However, in spite of the "coiling-effect" the dispersion in these reactors is relatively important: even for fast reactions, *i.e.* t_v = 30 s, the additional band-broadening, $\Delta\sigma_{t,r}$, will be about 1 s and packed reactors should be preferred. In some applications the properties of the reagent are such as to preclude the use of reactors packed with glass beads. Glass-bead columns can not be used for reactions in alkaline solutions since the packing material will be attacked.

For these reactions a metal tubular reactor can be applied. By comparison of Figure III.10 for packed reactors and Figure III.32 for coiled tubular reactors it may be concluded that in general packed reactors should be preferred over tubular reactors.

For slow reactions (reaction times larger than 5 minutes) neither glass-bead columns nor helically coiled reactors can be used without accepting large values for the additional band-broadening, $\Delta\sigma_{t,r}$. It should be noted, however, that many reputedly slow reactions can considerably be speeded up, because a closed pressurized reactor allows the reaction mixture to be heated up to temperatures exceeding the boiling point at atmospheric pressure of the solvent[109]. For particular applications, however, this approach is not appropriate, e.g. when aggressive reagents such as concentrated acids are used. Then a segmented flow reactor is suitable.

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BIOGRAPHY

The author was born on April 14, 1950 in Weert, The Netherlands. After following advanced grammar school (hbs-b) at the "Bisschoppelijk College St. Jozef" in Weert, he started his studies in the chemical engineering at the Eindhoven University of Technology in 1968.
The title of "Scheikundig ingenieur" was obtained in May 1973 after graduate work on gas chromatography performed under the guidance of the late Professor Dr.Ir. A.I.M. Keulemans.
Since 1973, the author has been employed as a research-assistant in the laboratory of Instrumental Analysis.
Since January 1978 he has been appointed as a research chemist with DSM Research at Geleen, The Netherlands.

STELLINGEN

1. Hesse en Hövermann[1] beschrijven het gebruik van geconjugeerde fasenparen van het ternaire stelsel dichloormethaan-ethanol-water als fasensysteem in de vloeistofchromatografie. In tegenstelling tot hun bevindingen kunnen hiermee geen stabiele retentiekarakteristieken verkregen worden[2].

   1. Chr. Hesse, W. Hövermann, *Chromatographia*, 6 (1973) 345.
   2. J.H.M. van den Berg, J. Milley, N. Vonk, R.S. Deelder, *J. Chromatogr.*, 132 (1977) 421.

2. Bij de door Quano[1] geponeerde bepalingsmethode van moleculaire diffusie coëfficienten in de vloeistoffase wordt ten onrechte geen rekening gehouden met de invloed van de secundaire stroming op de axiale dispersie in helices.

   1. A.C. Quano, *Ind. Eng. Chem. Fundam.*, 11 (1972) 268.

3. De beschrijving van chromatografische kolomprocessen met gebruikmaking van vijfdimensionale nomogrammen[1] leidt eerder tot verwarring dan tot vergroting van inzicht in de optimalisering van de vloeistofchromatografie.

   1. I. Halász, *Twelfth International Symposium on Advances in Chromatography*, Amsterdam, 1977.

4. De bewijsvoering van de door Horváth *et al.*[1,2] gelegde relatie tussen de ionsterkte van het eluent en de capaciteitsverhouding voor niet-geïoniseerde verbindingen is onjuist.

1. C. Horváth, W. Melander, I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
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5. Voor een optimale analyse met behulp van vloeistofchromatografie verdient kolomkoppeling de voorkeur boven gradientsystemen.

H. Engelhardt, *Z. Anal. Chem.*, 277 (1975) 267.

6. De on-line koppeling van vloeistofchromatografie en massaspectrometrie wordt alleen dan zinvol, als methoden beschikbaar komen om de componenten te ioniseren zonder deze vooraf in de gasfase te brengen.

7. Bij de interpretatie van de ^{13}C NMR chemical shifts gemeten aan lagere koolwaterstoffen in oplossing zijn de mogelijke invloeden van het solvent ernstig onderschat[1,2].

1. J.B. Stothers, *Carbon-13 NMR Spectroscopy*, Academic Press, New York, 1972, 55.
2. L.J.M. van de Ven, J.W. de Haan, *J. Chem. Soc., Chem. Commun.*, in druk.

8. Titels voor wetenschappelijke artikelen, zoals "Chairman Mao's brilliant philosophic thought guides me in winning triple cropping with high yield"[1] zijn in hoge mate dubieus.

1. Li-Kuang-ching, *Scientia Sinia*, 10 (1977) 391.

9. Ten onrechte stelt Crambach[1], dat het baanbrekend werk op het gebied van de analytische scheidingsmethoden[2] door Dr. A.J.P. Martin*, C.B.E., F.R.S., tot diens sanctificatie[3] heeft geleid.

1. A. Crambach, *Trends Biochem. Sci.*, 2 (1977) 260.
2. *The International Who's Who 1977-78*, Europa Publications Ltd., London, 1977, 1111.
3. *Osservatore Romano*, 1910-heden.

* Bijzonder hoogleraar van de Technische Hogeschool Eindhoven, 1964-1974.

Eindhoven, 17 februari 1978. J.H.M. van den Berg.