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An overview of microcolumn HPLC

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AN OVERVIEW OF MICROCOLUMN HPLC

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

By

Wei Zhang

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Abstract

An Overview of Microcolumn HPLC

By Wei Zhang

Over the last twenty years, many laboratories increasingly are using microcolumns with inner diameters smaller than 4.6 mm. Microbore column HPLC has become a very important separation technique nowadays. This paper reviews the microcolumn HPLC techniques; its advantages over conventional HPLC and its applications in various fields with particular attention to their applications in the analysis of pharmaceutical and chiral compound separation. The underlying theory of miniaturization of HPLC, column preparation and instrumentation are discussed. Techniques of LC/LC and LC/MS coupling are also presented.

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1. General view of liquid chromatography

1.1 Introduction

The analytical importance of modern liquid chromatography (LC) has been widely recognized. Indeed, high-performance liquid chromatography (HPLC) is in some respects more versatile than gas chromatography since it is not limited to volatile and thermally stable samples and the choice of mobile and stationary phases is wider. Therefore, HPLC is frequently referred to as the single most successful method in modern analytical chemistry.

In liquid chromatography, analytes are separated based on their different distribution between in a liquid mobile phase and a stationary phase. While the basic principles remained essentially the same, high-performance liquid chromatography uses small particles of uniform size and shape with a narrow pore size distribution. The use of high-efficiency packing materials of small particles requires high-pressure solvent pumping systems that can deliver the mobile phase at uniform and reproducible flow rates. A basic configuration of an LC instrument is given in Figure 1 [1].

1.2 Fundamentals

The separation principle is based upon the distribution of solute molecules between two immiscible liquid phases according to their relative solubilities. The separating medium consists of a finely divided inert support (e.g. silica gel, kieselguhr, etc.) holding a fixed (stationary) liquid phase, and separation is achieved by passing a

mobile phase over the stationary phase. The stationary phase is usually in the form of a packed column.

Based on the relative polarities of the stationary and mobile phases, LC can be divided into two categories. When the stationary phase is polar and the mobile phase is non-polar, it is called Normal Phase Chromatography (NPC). In this case, the solute elution order is based on the principle that non-polar solutes prefer the mobile phase and elute first, while polar solutes prefer the stationary phase and elute later. In Reverse Phase Chromatography (RPC), the stationary phase is non-polar and the mobile phase is polar. The elution order is commonly the reverse of that observed in Normal Phase Chromatography, i.e. with polar compounds eluting first and non-polar ones later.

Because nearly all organic molecules have hydrophobic regions in their structures, they are therefore capable of interacting with the non-polar stationary phase. Since the mobile phase in RPC is polar, and commonly contains water, the method is particularly suited to the separation of polar substances which are insoluble in organic solvents. Therefore, most separations performed today use RPC. Table 1 shows typical stationary and mobile phases that are used in normal and reverse phase chromatography [2].

Ion-exchange chromatography is another useful separation mode, especially for the analysis of ionized or ionizable compounds. In this process the species of interest associates with a counter ion of opposite charge, the latter being selected to confer solubility in an organic solvent on the resulting ion pair. The stationary phase consists of an aqueous medium containing a high concentration of a counter ion and at an

appropriate pH. The mobile phase is generally an organic medium having low to moderate solvating power. Ion-exchange chromatography is widely used in the separation of mixtures of inorganic salts and biomolecules such as amino acids, nucleic acids, and proteins [3].

The high pressure and speeds involved in HPLC sometimes tend to mechanically remove the stationary phase from the support material. Column packings with chemically bonded stationary phase have been developed to overcome this disadvantage. This form of LC, in which both monomeric and polymeric phases have been bonded to a wide range of support materials, is called bonded-phase chromatography. The silylation reaction has been widely used to prepare bonded phases.

1.3 Instrumentation

High Performance Liquid Chromatography apparatus consists of five essential components as illustrated in Figure 1 [1].

1. Mobile phase delivery system
2. Sample injection system
3. The column
4. The detector
5. The recorder (data acquisition)

1.3.1 Mobile phase delivery system

The mobile phase delivery system is required to force the mobile phase through the injector, column and detectors and from there to the waste. The system includes a pump to provide the high pressures required and usually contains some means of providing gradient elution. The solvent reservoirs can be filled with a range of solvents of different polarities and are mixed with the buffer. The pump should be able to deliver the mobile phase at flow rates of 1-5 mL/min and at pressures up to 5000 psi. Probably 80-90% of HPLC separations are performed with a pressure of less than 1200 psi. However, the supply of mobile phase to the column should be constant, reproducible and pulse-free. The accuracy of the flow rate is important when comparing a method worked out on one instrument to another instrument. If the flows are different, the retention time will be different. The effect could be crucial in gradient elution during which the solvent composition could be affected. Most pumps for HPLC now set the flow rate electronically, with an accuracy of 1% or better [4].

Gradient elution vs. Isocratic elution

Gradient elution is a technique of programming the strength of the mobile phase as a function of time. The initial composition of the mobile phase is chosen to be a poor eluent under the mode selected; therefore, most of the components of the sample are retained on the column. The eluting power of the mobile phase is increased by mixing with a miscible stronger solvent, which elutes the more strongly retained components from the column. Gradient elution is used in many separations when the number of the

sample components is large or covers a wide range in retention. The eluting strength of the mobile phase is directly related to the composition of the mobile phase. The simplest way is to control the composition by changing the speeds of the pumps, each metering one solvent. First start with 100% of the flow from the pump with the weaker solvent and then increase the flow from the pump with the stronger solvent. The schematic of gradient elution HPLC is shown in Figure 2 [5].

1.3.2 Sample injection system

A typical injection system is shown in Figure 3 [6]. The sample is loaded at atmospheric pressure into an external loop that connects port 1 and port 4. Then the sample is introduced into the high-pressure mobile phase by an appropriate rotation of the valve from load to inject position so that the sample loop is connected to the column from port 3. In this way, the sample can be introduced reproducibly into pressurised columns without significant interruption of the mobile phase flow. Samples of a few microliters can be injected at pressures up to 6000 psi. Therefore, this type of stop-flow valve injection is currently the most widely utilized for its high precision.

1.3.3 The column

1.3.3.1 Column material

As Table 2 shows [7], silica gel is the mainstay base material for most HPLC packings. Scanning electron micrographs of silica gel are shown in Figure 4. The structure of the silica gel is shown in Figure 5. Silica gel is rigid, provides excellent

efficiency, is reasonably priced, and can be converted to bonded phase materials by virtue of its reactive surface silanol (SiOH) groups and its relatively high surface area. A single gram of a typical silica gel - just enough to fit on the end of the thumb - has the surface area of a football field. When its surface is fully hydroxylated, silica gel has a maximum silanol density of $8.0 \mu\text{mol}/\text{m}^2$. It is available with irregular and spherical shaped particles and in a wide variety of pore diameters and pore volumes. It can be used in nonaqueous and aqueous environments. However, since its silanols are acidic, they can cause tailing or adsorption of basic compounds. Also, silica gel can be dissolved in water rapidly at a pH greater than 9 [7].

Since the majority of phases for HPLC consist of silica as the support material, the bonding of various organic moieties to the substrate is based on the chemistry of the silanol at the surface [8]. There are several approaches developed. A summary of these reactions is given in Table 3 [9].

Esterification is the first and the simplest method used for making chemically bonded stationary phases for HPLC [10]. It involves bonding of an alcohol to the silanol to form a Si-O-C linkage. However, this linkage is hydrolytically unstable and not suitable for aqueous mobile phases.

For the organosilanization approach, there are two forms of the reaction. The monomeric reaction involves the use of an organosilane with only one leaving group (X), such as chlorine or methoxy. Therefore, only one tie-down point to the silica surface is possible. This reaction provides well-defined single layer coverage and is easily reproducible. A polymeric reaction involves the use of an organosilane with either two

or three leaving groups. In this case, the surface chemistry is more complicated with extensive cross-linking between adjacent organosilane moieties resulting in a polymeric network extending away from the surface of the silica. This yields more bonded mass but less well-defined surface coverage, because different silanes may anchor to the surface or to other silanes at some distance from the surface. Reproducibility is more difficult to control in this reaction.

The third approach involves chlorination of the silica surface with thionyl chloride followed by attachment of the organic moiety through a Grignard reagent or organolithium compound [9]. The linkage formed from this method is a direct Si-C bond which is more stable than the linkage formed during organosilanization.

The silanization/hydrosilation process has achieved great success lately [9]. In the first step, bare silica is reacted with the hydrolysis product of the triethoxysilane (TES) to form a monolayer of hydride on the surface.

The appropriate organic moiety is then attached to silica via a hydrosilation reaction between the hydride on the surface and a terminal olefin which is catalyzed by a transition metal complex like hexachloroplatinic acid.

Polymeric materials are used in approximately 20% of liquid chromatography applications [7]. Similar to silica gel, polymeric packings also are available with a variety of particle diameters, porosities, and mechanical strengths. The most popular matrix is polystyrene cross-linked with divinylbenzene (PS-DVB). Generally, polymeric packings are stable over a wider pH range than silica gel, and some can operate throughout the full pH 0-14 range. Therefore, polymeric materials are favored for ion-exchange chromatography. Figure 6 shows the separation of basic drugs on a polymeric reversed-phase column at 50°C. The mobile phase consisted of an aqueous phosphate buffer of pH 12 and acetonitrile. The column is filled with a PS-DVB packing with octadecylsilane groups extending from its surface [7].

Other packing materials are niche packings frequently used for specialized applications. Because of their specialized nature, columns packed with these materials are more expensive.

1.3.3.2 Column phase design

Column developers have used several types of chemical bonds to bond stationary phases to silica gel, but the most utilized is the siloxane bond (Si-O-Si-C). It results from the reaction of the surface silanol group on silica gel with an organochloro- or organoalkoxysilane. Organomonochlorosilanes are used most frequently, and they provide a monolayer of the organo phase. This reaction is shown below.

In column surveys conducted by LC-GC, since 1984, reversed-phase chromatography has dominated HPLC applications. More than 50% of the HPLC applications are in reversed phased mode while normal phase mode makes up about 24% of the LC analysis. Most reversed phase packings consist of a hydrophobic phase bonded to silica or polymeric support. C18 is the most popular phase by far followed by C8, phenyl-diphenyl, and C4 phases [11].

Normal phase chromatography encompasses adsorption chromatography on silica gels and partitioning-adsorption on polar bonded phases such as amino, cyano, and diol. With the development of LC-MS, NPC is getting more attention because its solvents can accommodate MS inlet systems much better.

The use of chiral phases has grown very fast recently especially in the pharmaceutical and agrochemical industries. More than half of the drugs on the market are organic compounds with asymmetric centers. One enantiomeric form may be toxic and the other form has therapeutic value. Some types of chiral stationary phases that display enantiomeric selectivity are based on proteins, cyclodextrins, π -complexes and chiral polymers.

1.3.3.3 Column and particle dimensions

To achieve faster analytical separations, chromatographers recently have begun using smaller particles packed into shorter and sometimes narrower columns. Today, 5- μm d_p is the standard, while particles as small as 1.5 μm are also commercially available. Although 3- μm d_p particles have been available for many years, they have been used sparingly for routine separations because of their tendency to plug, higher column back pressures, and lower-than-predicted efficiency. The advent of columns with 3.5- μm d_p particles has solved the practical problems previously associated with traditional 3- μm d_p particles. Columns packed with 3.5- μm d_p particles are narrowly sized and contain no fines, so 2- μm frits can be used instead of the easily plugged 0.5- μm frits normally required for columns of 3- μm d_p particles. The approach results in columns that are as rugged as traditional 5- μm d_p columns but allow separations to be performed in one half the time using one half the solvent and providing equivalent resolution. Figure 7 shows an example of this effect for the separation of aspartame (the synthetic sweetener) and its metabolites [12].

1.3.4 The detector

Over 95% of all LC analysis utilizes one of four types of detectors: the UV detector, the electrical conductivity detector, the fluorescence detector, or the refractive index detector. Among these four, the UV detector is, by far, the most commonly used detector. The fluorescence detector is the most sensitive general detector. However, it is limited to those materials that will fluoresce or by appropriate derivatization can be made to fluoresce. The electrical conductivity is the detector of choice for the separation and analysis of ions of all types and can also be relatively inexpensive. Even though the refractive index detector is not in common use, it can often be successful for the separation of carbohydrates that have no UV chromophores, do not fluoresce and contain no ions [13].

1.3.5 The recorder (data acquisition)

Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. In addition, most systems nowadays can store data in a retrievable form for highly sophisticated computer analysis at a later time.

In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. In recent years, with the application of computer and software development, complex data acquisition and analysis becomes more feasible. Additional

automation options become much easier to implement such as computer controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors [13].

2. Introduction of microcolumn HPLC

2.1 General aspects

In conventional HPLC, 4-6 mm I.D. columns packed with 3-10 μm materials are commonly employed. Research on miniaturization of the column in liquid chromatography began at the same time as HPLC was introduced. Many laboratories increasingly are using microcolumns with inner diameters smaller than 4.6 mm. Microcolumn HPLC has become a very important separation technique nowadays.

2.2 Early stage studies

The introduction of microcolumn LC is attributed to Horvath and Lipsky in 1967, who used 0.5-1.0 mm I.D. stainless steel columns packed with pellicular particles for the separation of ribonucleotides [14]. Since then several types of columns have been developed and proposed, based on the superiority of their performance as compared to conventional LC columns.

Horvath's study was to develop a highly sensitive and rapid technique for quantitative analysis of nucleic acid constituents. High sensitivity is often required for the minute quantities of nucleic acids or nucleic acid fragments that may be available – e.g., by zonal centrifugation or single cell isolation. In his investigation, small-bore columns (0.5 and 1.0 mm I.D.) packed with pellicular cation-exchange resin were used to separate four major bases (uracil, guanine, adenine and cytosine) and nucleosides of RNA (uridine, guanosine, adenosine and cytidine). Small-bore columns are particularly suitable for the analysis of minute samples because the high column efficiency and low

flow rates make possible the elution of the sample components in a small volume of the effluent. The sensitivity of a LC system is determined by the maximum solute concentration in the column effluent and the sensitivity of the detector for the solute in question. Peaks can be eluted from small-bore columns in much smaller volumes than from conventional columns; therefore, the concentration of the effluent peaks is much larger for the same amount injected. Most commercial UV detectors, however, are not suitable for working with small-bore columns because of their relatively large cell volume, which causes peak distortion and reduction of the concentration maximum. Horvath used micro UV detector which has a very small volume-to-light-path ratio (about 0.8 mm^2) and low noise level (about 2×10^{-5} absorbancy unit with stagnant liquid), which makes it possible to detect peaks having small volumes and low concentration. The columns were made of 1-mm I.D. #316 stainless steel tubing. Approximately 150-cm lengths were packed dry under vibration, then deaerated eluent was pumped through the bead at 200 atm while the column was kept in a perpendicular position. A thin porous Teflon plug was used at the column outlet. The separation of the four nucleosides within 4 minutes is shown in Figure 8. The chromatogram in Figure 9 shows the separation of the four bases within 6 minutes [15].

The analysis of nucleic acid hydrolyzates was most commonly carried out by means of a conventional column on an anion exchange polystyrene resin. By using a small bore column system with gradient elution, Horvath separated the nucleoside mono-, di-, and triphosphates in the nanomole range within 90 minutes (Figure 10), which compared well with a conventional column that usually requires at least 20 hours. In

conclusion, it is possible that the analysis of subpicomole quantities could be achieved by slight modification of the LC system, such as a decrease of the diameter of the column and volume-to-path-length ratio of the detector cell [14].

In the early 1970's, Ishii's group started research on miniaturization in HPLC [16]. Also in 1984, Kucera published his work using a 1 mm I.D. column [17].

2.3 Classification of microcolumns

Although the initial motives to develop such columns in different research groups varied, the resulting column technologies and emerging instrumentation appear to have both overlapping and complementary features. The nomenclature of these small diameter columns varies. Microcolumn liquid chromatography is used frequently as the overall term to describe small I.D. LC columns in most scientific journals.

Novotny categorized the small I.D. columns into three basic types: open tubular column, packed microcapillary column and small-bore packed column [18]. The features of the three columns are illustrated in Figure 11. Yang suggested four types of microcolumns (narrow-bore, micro-bore, capillary and nano) with a comparison of the characteristics shown in Table 4 [19].

The inner diameter of open tubular column is ideally very small to compensate for slow diffusion processes in liquids, and the retentive medium is uniformly distributed over the column wall. The packed microcapillary column has a larger inner diameter, and the adsorbent or particles of solid support are uniformly distributed throughout the column, with a substantial number of them actually embedded in the column wall. Such

columns are characteristically semi-permeable. The small-bore packed column has the same geometrical characteristics as conventional HPLC columns with the exception of a smaller internal diameter. The characteristics and operational parameters for those micro-bore columns are summarized in Table 5 [18].

2.4 Advantages of microcolumn HPLC

The application of 1-2 mm I.D. microbore columns and 50-530 μm I.D. packed capillary columns in microcolumn HPLC has grown significantly in recent years. The driving forces for the rapid development and growth are (1) lower cost of solvents and waste disposal; (2) improvement of performance when coupling with mass spectrometers; and (3) increased sensitivity and resolution when only a small volume of sample is available.

2.4.1 Solvents and waste

From Tables 4 and 5, compared to conventional column, the flow rate in a microcolumn is reduced significantly. While a typical value for an ordinary HPLC packed column is a few mL/min, microbore columns utilize only 40-50 $\mu\text{L}/\text{min}$. A typical value for a packed microcapillary is only 1 $\mu\text{L}/\text{min}$. The reduction in the flow rate drastically reduces the consumption of expensive mobile phases and toxic solvent waste disposal. A total saving of up to 99.9% can be achieved when narrow-bore microparticle packed columns or open tubular micro-capillary columns are used. For example, in a micro HPLC application 1 gal of acetonitrile would last for more than 60

days of continuous pumping with a 50 $\mu\text{L}/\text{min}$ flow rate. A 4.6 mm I.D. conventional column would use more than 1 gal in less than three days at 1 mL/min flow rate. The flow rate can be reduced to 1 $\mu\text{L}/\text{min}$ for a typical packed capillary column. At this rate, it will last more than 8 years. Reduction in column diameter also permits the use of exotic solvents, such as CDCl_3 , $\text{CF}_3\text{CF}_2\text{OH}$, or one of the pure chiral isomers of a conventional solvent, like 2-butanol or an additive. Therefore, the cost saving that comes with reduced solvent consumption and toxic solvent waste disposal is enormous [19].

2.4.2 Convenient for direct coupling to detectors

The low eluent flow rates used in micro-columns allow not only solvent saving, but also provide the potential for direct interfacing to mass spectrometer and flame based detectors. As most compounds analyzed by LC/MS are of high molecular weight, the solvent velocity must be reduced. The diffusion coefficient is usually lower than $5 \times 10^{-6} \text{ cm}^2/\text{sec}$ and the same value of the reduced velocity corresponds a lower flow rate, typically between 10 and 30 $\mu\text{L}/\text{min}$. A 1 mm I.D. packed column is easily accommodated by all interfaces. In this case, the use of a microcolumn often facilitates the operation of the mass spectrometer. The ability to connect a microcolumn directly to a low-cost, bench-top mass spectrometer has been facilitated by the development of an electrospray interface. Microcolumns commonly are used for several ionization techniques, including fast-atom bombardment [20-25], particle beam [26-28], electron and chemical ionization [29-32] and electrospray-nanospray ionization [33-39]. Among those techniques, the first successfully applied microcolumn LC-MS interface was

continuous-flow fast atom bombardment (CF-FAB), which has been applied mainly for the analysis and identification of biochemical compounds. CF-FAB has contributed to a very large extent to the current success of microcolumn LC, and has been one of the major driving forces behind the development of microcolumn LC [40].

2.4.3 Efficiency

Another major advantage of micro HPLC is its high efficiency, fairly close to theoretically predicted values. Conventional columns can also produce similar efficiency per unit length; however, in the case of microcolumns, one can connect a number of microcolumns in series or prepare a long column, much longer than conventional columns. Therefore, a much higher total theoretical plate numbers can be obtained.

The decrease in the peak volume allows direct coupling with a secondary separation system. A micro-HPLC system facilitates direct combination with a conventional HPLC system, because the peak volumes of micro-HPLC are so small that a whole primary band can be quantitatively transferred to the secondary system [41].

Figure 12 shows the application of LC-LC to the analysis of aromatic hydrocarbons in a light kerosene. The primary separation was carried out on an NH₂-bonded micro-HPLC column (150 x 0.35 mm I.D.) in the normal-phase mode. The analytes were separated on the basis of differences in the number and size of their aromatic rings. Fractions containing each peak were then transferred to the secondary separation system and subjected to reversed-phase separation using a conventional column (250 x 4.6 mm I.D.).

Several components were identified on the basis of their retention time and the use of a multi-channel UV detector gave additional qualitative information [42].

Hartwick and Meyer's study found out that high speed analysis using 1 mm I.D. columns are quite practical using commercially available instrumentation. Time reduction on the order of 100 fold can often be achieved with no loss in accuracy. The use of micro LC techniques becomes almost mandatory in these types of applications, since reduced solvent consumption and uniquely optimized column lengths would be difficult to achieve using conventional systems [43]. Also, the low heat capacity of the microcolumn allows the easy use of temperature programming in HPLC and this can partly replace solvent gradient elution [44,45].

2.4.4 Sensitivity

Use of narrower columns could also result in better sensitivity when the sample size is limited because it will be diluted in a smaller volume of mobile phase. The increase in the mass sensitivity is especially important when dealing with the analysis of samples of biological origin since such samples often contain very limited quantities of the individual analytes. For microcolumns, the peak volume containing the mass of the sample is proportional to the ratio of column cross sectional areas [46]. Therefore, theoretically a 20-fold enhancement can be obtained if the column I.D. is reduced from 4.6 mm (conventional LC) to 1 mm (microcolumn). If the column I.D. is further reduced to 0.1 mm packed capillary column, it would result in a 2000-fold enhancement of the peak concentration.

2.5 Disadvantages of microcolumn HPLC

Because of the miniaturization of the columns, the flow-rate, sample volume, detection volume must be decreased in proportion to the column volume that generates stringent instrumental requirements. Also, the enhancement of the mass sensitivity can be offset by the difficulties encountered in the miniaturization of detectors, requiring compromises that result in a higher detection limit [41].

3. Theoretical aspects

3.1 Chromatographic dilution

During the chromatographic separation process, the sample is subjected to dilution. The chromatographic dilution factor can be expressed in terms of the maximum peak concentration $C_{\max}$.

$$C_{\max} = \frac{2^{3/2} m_s N^{1/2}}{L\varepsilon(1+k)\pi^{3/2}d^2} \quad (1)$$

Where $C_{\max}$ = final compound concentration at the peak maximum

m_s = injected sample mass

N = number of theoretical plate

L = column length

ε = column porosity

k = capacity factor

d = column diameter.

For the same sample mass injected into a column, the maximum peak concentration is inversely proportional to the length of the column and the square of the column diameter. Therefore, by decreasing the column diameter, the maximum peak concentration can be increased [40]. For example, using a 100 μm I.D. packed capillary column would result in a 2116-fold increase in peak height compared to a conventional 4.6 mm I.D. column. The response of a UV detector is compared for the same aromatic hydrocarbon test

sample eluted under near identical conditions from packed columns of 4.6, 2.1, and 1 mm I.D. and is shown in Figure 13. A substantial gain in sample delectability using microbore columns is demonstrated [47]. However, this advantage of small I.D. columns can only be fully exploited when the same sample size can be loaded and the operating characteristics of such columns are identical compared to their conventional counterparts.

3.2 Separation impedance

In assessing the separation potential of various microcolumns, one must consider not only the plate height H-value, but also the column length, L, and the analysis time, t. A large number of theoretical plates, $N = L/H$, is often achieved in LC at the expense of long analysis time. Knox and Bristow introduced the concept of separation impedance, E, to evaluate columns under dissimilar conditions of chromatographic analysis [48].

$$E = h^2 \phi = H^2 / K^0 \quad (2)$$

Where E is the separation impedance or performance factor. A lower value of E ultimately translates into either faster or more efficient separations for a given set of instrumental conditions. The equation shows that the separation impedance is proportional to the square of the plate height H divided by the column permeability K^0 , or alternatively, is equal to the square of the reduced plate height ($h = H/d$) multiplied by a column resistance factor, $\phi = d^2/K^0$, where d is the particle diameter for packed columns or the column I.D. of an open tubular column. Table 6 gives estimates of such values for different types of microcolumns [49].

Conventional LC has a E value around 2000 while microcapillary packed columns have a separation impedance factor between 100 and 300. Narrow-bore fused-silica packed columns of I.D. < 0.4 mm packed with 3-10 μm particles normally have a separation impedance factor below 500.

4. Instrumentation

4.1 Column development

Considering the small amount silica gel needed for micro-HPLC, the possibility of using very small particles is most important for column development. The curves of H versus u (flow rate) in Figure 14 demonstrate that smaller particles have the desirable feature of not showing an early upswing compare to larger particles [50]. Even though it is difficult to prepare very small particles, it is not a drawback for microcolumn HPLC since only a very small amount of packing material is needed.

The particle size distribution range is also very important. Numerically the ratio $d_p 90 / d_p 10$ can describe this property ($d_p 90$ and $d_p 10$ are the particle sizes limiting 90 and 10% of the total weight of the particles). For micro column HPLC silica gel packing materials, the $d_p 90 / d_p 10$ ratio should not be greater than 1.5 [50].

Different filling techniques have been reported for the preparation of packed capillary LC columns, applying gases, supercritical fluids or liquids to transport the stationary phase particles into the column. For microcolumns, liquids are used exclusively to pack columns. Small, compressible particles can be packed efficiently only by a slurry technique. Basically, the packing process can be regarded as filtration. The slurry is pressed in the column and the solids (packing material) are filtered off in the column under high pressure. The procedures for packing microbore and packed capillary column are similar. Yang reported the general steps are as follows [51]:

- Connect a column end restrictor to the end of the column while the column inlet is attached to a slurry reservoir.

- Prepare a 20-30% (w/v) packing material in slurry solvent such as acetone, methanol, tetrahydrofuran, or a mixture of solvents.
- Apply an ultrasonic or vacuum degasser to degas the packing slurry.
- Quickly fill the slurry reservoir with the packing slurry. Then rapidly increase the reservoir pressure to approximately 200-300 atm, depending on column length and internal diameter.
- Increase the packing pressure gradually or stepwise to more than 500 atm after the packing has filled one-half the length of the column.
- Maintain the final packing pressure for at least 30 minutes under ultrasonic agitation, then stop the ultrasonic agitation, and slowly release the column pressure to prevent back-flow disruptions in the column bed.
- Install the column end unions and condition the column with the elution solvent.

The packing methods described above are generally applicable. When the size of the particles becomes very small, spherical particles are needed for better results. Also higher pressure and using low-viscosity solvents will be very helpful during the packing. Table 7 compares the packing parameters for conventional, microbore, and fused silica packed capillary columns [50].

The separation efficiency of a microcolumn is influenced by a large number of parameters – not only so-called packing parameters (such as solvent selection, packing pressure, slurry concentration, the use of surfactants to stabilize the particle suspension, slurry vessel dimensions, etc.), but also by column parameters (such as column blank material, frit selection, etc.).

A well packed microcolumn has a stable packed bed as measured by a characteristic pressure drop against a given solvent at a specific flow rate. The visual identification of an acceptable packed column includes the absence of air pockets along the packed column after drying. The column should maintain packed-bed stability after repeated pressurization and depressurization [19].

4.2 Pumping system

Micro HPLC requires a pump providing flow rates between 1 to 10 $\mu\text{l}/\text{min}$, while flow-rates between 10 to 100 $\mu\text{l}/\text{min}$ are common in semi-micro-HPLC. High-pressure micro-pumps, both the syringe-type and reciprocating pumps, which provide these low flow-rates, are commercially available. For isocratic elution, these pumps work with good precision at flow-rates down to 1 $\mu\text{l}/\text{min}$. The reciprocating pumping systems are, however, favorable over syringe pumps for their large column back pressure compensation abilities, rapid flow equilibrium and stability, and good possibilities for micro-gradient delivery. Delivery of flows in the nl range for columns with ids of approximately $<500\text{ }\mu\text{m}$ cannot be readily performed by direct pumping. For such flows, stream-splitting pumping systems can be applied. It can be set up easily by using a constant back-pressure regulator to maintain pressure at the split point (Figure 15A) or by using a needle valve or long fused-silica tubing as a flow restrictor for fixing a constant split ratio (Figure 15B) [52].

4.3 Injection system

The injection volume should be carefully selected with due consideration given to the column dimensions. Roughly speaking, the injection volume should be less than one-hundredth of the column volume. Manual injections from the μL range down to approximately 20 nL can be routinely performed with (micro)-injection valves equipped with a replaceable internal loop (Figure 16) [53]. Below 20 nL, manual valve injections can be performed by positioning a split vent between the injector and the column (shown in Figure 17)[54]. Alternatively, for these very small volumes, the moving injection technique, the static split or the pressure-pulse-driven stopped-flow injection technique can be used too. These techniques have in common that, by controlling the injection time and flow through the injector, only a small part of the injection plug is injected on the top of the column. Figure 18 shows a schematic diagram of such a system [55]. The purge valve is initially open so that no flow enters the column. The sample is then injected into the injection valve and flows all the way through the connecting tube until reaching the column inlet. The purge valve is closed for a specific time period (Δt). Only during that time, the sample is allowed to enter the column. After the appropriate injection time has elapsed, the purge valve is re-opened so that the remainder of the initial sample plug is not introduced into the column. Therefore, the sample volumes can be conveniently controlled by varying the injection time (Δt) and the flow rate from the pump. As an injection variable, time is much easier to control accurately with the aid of a computer. The timing sequence can be readily automated using either pneumatically or electrically

actuated valves. Automated injection in the μl range can easily be performed by many of the autosamplers currently available [56, 57].

4.4 Detector

Various types of detectors are employed in HPLC, with UV-visible, fluorescence, electrochemical and refractometer detectors being the most common. However, the flow cell volumes must be reduced so that the band broadening in the detection part should not degrade the overall efficiency. A general rule for detector selection is to choose one with a flow cell volume less than or equal to 10% of the elution peak volume. Therefore, for microcolumns, existing commercially available refractive index detectors must be modified to reduce the extracolumn band-broadening effects. On the other hand, the most universally applied detector in microcolumn LC is the UV absorbance detector because of its ease of use and broad application area. To prevent extracolumn band broadening, on-column detection is often a first approach. On-column detection was originally proposed for micro-HPLC in order to eliminate the dispersion of analytes caused by the connections between the microcolumn and the detector flow cell. On-column detection is defined as the case where the analytes are detected in the presence of a stationary phase, while the term post-column detection is used for the case where the analytes are detected in the absence of the stationary phase. Since the stationary phase focuses the analytes into a narrow zone, on-column detection has a potential to increase sensitivity by a factor of $(1+k')$ in comparison with post-column detection, where k' is the capacity factor of the analyte. Therefore, on-column detection has a potential to

improve particularly the mass detection limits of late-eluted analytes with large k' values. On-column UV detectors, in which a part of the capillary column is used as the optical window, often yield subnanogram sensitivity in spite of an extremely small path length [58]. Fluorescence and electrochemical detection are the other two most important and commonly used LC detectors. Both are concentration sensitive, like the UV detector, so that a decrease in their volume for small-diameter columns is beneficial in terms of substantially improved mass sensitivity.

5. Hyphenation

The most attractive application of microcolumn HPLC is in the hyphenated techniques. Chromatography can only give us retention information and this is not sufficient enough for the identification of a separated component. On the other hand, the combination of spectroscopy with chromatography is a versatile technique. Such combinations are called “hyphenated techniques”. Another commonly used hyphenated system is called multidimensional chromatography in which two or more chromatographic systems are connected in series to increase the separation efficiency.

5.1 Multidimensional chromatography

In multidimensional chromatography, selected components separated in the first separation system are transferred in some manner to a second separation system, where further resolution can be obtained. Thus column-coupling attempts to achieve in a chromatographic separation what one column cannot satisfactorily do alone. Column coupling can be either direct (on-line) or indirect (off-line). In the off-line (indirect) coupling application, fractions eluted from the first separation system are collected. They can be treated (e.g. removal of incompatible solvent) prior to introduction into the next chromatographic system. Indirect coupling is very tedious and time consuming. Therefore, it is not routinely used. In the on-line (direct) coupling technique, two or more separation systems are connected in series. Figure 19 shows the butt-to-butt coupling of two microbore columns connected within a drilled-through union [59]. A block diagram of a LC/GC coupling system is shown in Figure 20 [60]. In those on-line systems, the

selected fractions eluted from the first system are introduced into the second system through appropriate valves or pressure control. This technique has the advantages of simplicity, time efficiency and reproducibility.

The coupling of LC with GC offers variety of opportunities in multidimensional chromatographic separations. Because each technique has its unique advantages (selectivity, sensitivity, mobile phase and stationary phase range, etc.), one can get a significant improvement in efficiency, selectivity, sensitivity, and resolution by combining these two different separation systems. In a LC-GC coupled system, the reduced column I.D. of microcolumn LC offers many advantages over conventional LC columns. The volume of eluent used is significantly reduced, which means the solutes of interest are diluted in much less eluent. Much larger sections of the LC chromatogram can be introduced into the GC, allowing quantitative transfer of the components of the sample, resulting in greater reproducibility. Because microcolumns can be effectively prepared at lengths greater than the conventional 25 cm, greater total column efficiencies can be obtained. One of the challenges for microcolumn LC/GC coupling is to convert the liquid mobile phase into a GC compatible (gaseous) sample. An on-column injector interface was developed to facilitate this transition. An uncoated inlet capillary is placed in front of the GC column. The large solvent volume is vaporized and the solute is concentrated at the beginning of the separation column. This technique was used to analyze a sample of fuel oil for the presence of various chlorinated benzenes shown in Figure 21. In this experiment, a normal phase eluent was used in a capillary fused silica column. Heart-cutting was applied to introduce a part of the first separation system onto

the secondary separation system. With heart-cutting techniques, only a fraction of the original sample (section x in Figure 21-a) is introduced into the GC and separated (Figure 21-b) [61].

The chromatogram of a test mixture of alkylbenzenes on nine coupled microcolumns (50 cm x 1 mm I.D.) is shown in Figure 22. An extraordinarily high number of theoretical plates (~ 200,000) has been achieved, which demonstrated a super efficiency. Meanwhile, the total solvent consumption was only 24 ml for almost a 20 hour run time [59].

5.2 LC-MS coupling

The most favorable aspect of micro LC is that it may provide most of the advantages of conventional HPLC (speed, efficiency, and gradient elution), but with a 20 to 50-fold decrease in eluent flow rate. In general, as low a flow-rate as possible to the mass spectroscopy is most favorable for ionization and ion extraction efficiency. Prior to the introduction of the analyte into the MS ion source, the mobile phase solvent from LC needs to be removed quickly to avoid its interferences in spectroscopic measurements. Therefore, the lower flow rate of microcolumn HPLC greatly facilitates the direct coupling with MS.

In 1987, Jorgenson et al. coupled open-tubular capillary columns (1 m x 10 μ m I.D.) with both chemical ionization (CI) and electron-impact (EI) modes of MS measurement. The mobile phase (methanol/water = 75/25) flow rate was 30 nl/min. The interface worked well in both CI and EI (Figure 23 and Figure 24). Using a mobile phase

flow rate in the order of nl/min made it possible for direct introduction of the entire column effluent into the mass spectrometer [62]. Figure 25 is the schematic drawing of a typical micro LC/MS system with a direct liquid introduction interface in CI measurement mode [63]. A conventional chemical ionization (CI) MS vacuum system equipped with a cryopump can handle from 10-60 $\mu\text{L}/\text{min}$ of reversed phase eluents and this flow rate readily accommodates the optimum performance requirements of today's packed metal microbore LC columns.

The continuous-flow fast atom bombardment (CF-FAB) and electrospray ionization (ESI) interface also takes advantage of the low flow rate supplied by microcolumn HPLC. In a CF-FAB interface, a liquid flow, typically 5-10 $\mu\text{L}/\text{min}$ with standard pumping systems, enters a fast atom bombardment ion source through a narrow fused silica capillary (50-75 μm I.D.). This capillary is generally contained within a probe that can be brought up to or removed from the ion source by means of a standard vacuum lock. In LC-MS applications, CF-FAB cannot be coupled directly with conventional HPLC columns because of the incompatibility in flow rate. Various devices must be used to split the column effluent from 1.5 ml/min to 5 $\mu\text{L}/\text{min}$. However, split ratios up to 0.004 are not favorable with respect to the detection limits. Therefore, microcolumn HPLC is the ideal candidate. Packed fused-silica columns can be directly compatible with CF-FAB while some minor splitting is necessary when microbore columns are used [64]. The schematic diagram of the first prototype CF-FAB interface is shown in Figure 26 [65].

In the electrospray ionization process, a flow of sample solution is pumped through a hypodermic needle held at a potential of a few kilovolts relative to an opposing metal plate. Charging of the liquid occurs and it emerges from the needle as a mist of very fine charged droplets. This spraying process takes place in the atmosphere. A typical ESP interface for LC-MS was developed by Whitehouse et al, as shown in Figure 27. Sample solutions enter into a spray chamber by pumping through a stainless-steel hypodermic needle at a flow-rate of 5-20 $\mu\text{L}/\text{min}$. The needle is kept at ground potential. The electrode is set at -3.5 kV for positive ion detection. The metallized inlet and outlet ends of the glass capillary are set at -4.5 kV and $+40\text{ V}$. For negative ion detection, the polarities of the various are reversed accordingly. The liquid is electrosprayed from the tip of the hypodermic needle and the droplets formed are further dispersed by means of a countercurrent stream of heated nitrogen gas. The solvent vapor from the rapidly evaporating droplets is swept away by the bath gas while the ions formed that are coming near the inlet of the glass capillary are entrained in a dry bath gas and transported into the first vacuum chamber to form a supersonic beam. Ions at supersonic speeds can easily overcome the potential barrier in the glass capillary. The core of the supersonic jet is sampled by a 2 mm I.D. skimmer, kept at -20V , and transported into the quadrupole analyzer region for mass analysis [66]. Various studies have found the electrospray LC-MS interface is very promising. However the major drawback of the ESP interface in LC-MS applications lies in the fact that the maximum allowable flow-rate is limited to 10 $\mu\text{L}/\text{min}$ with the lower the flow-rate the better the performance. Therefore, the low flow

rate accomplished by microcolumn HPLC offers great advantages over conventional HPLC to interface with ESP.

6. Applications

Microcolumn HPLC has evolved from a research laboratory curiosity to a routine, eminently practical technique suited for the real separation problems in many different fields. In this chapter, a number of relevant applications utilizing microcolumn HPLC separation methods are presented.

6.1 Chiral separation

Much research has been focused on chiral separations, especially in the pharmaceutical industry, and interest is growing with the demands on documentation of drug safety. Optical isomers differ drastically in bioavailability, biological activity and metabolism. A racemic mixture is therefore to be considered as a mixture of two different substances. Consequently, it is of great importance to determine the relative abundance of each isomer, possibly through stereoselective LC. Chiral separations can be achieved by the use of chiral stationary phases, a chiral selector that is attached to the packing material, or by using chiral mobile phase additives. Micro HPLC is a very attractive technique for chiral separations since it is possible to apply new types of stationary phases that are normally too expensive. In addition, the consumption of expensive stereoselective mobile phase additives is lower, and chromatographic efficiency and selectivity is higher.

There are three chiral selectors that have been extensively studied for chiral separations. Those three methods utilize either β -cyclodextrin (β -CD) as a mobile phase additive, bovine serum albumin (BSA) as a stationary phase attached to underivatized

silica or a commercially available Pirkle phase, covalently bound 1-dinitrobenzoyl-phenylglycine as chiral selectors. Wannman et al. studied those three methods and transferred them to micro-LC columns with a 0.32 mm I.D. They compared the results with those previously reported using conventional sized columns and found out that every originally developed method for chiral separations on conventional HPLC columns can be transferred to capillary LC systems with little effort [67].

The separation of ($\pm$)-terbutalin with β -CD as a chiral mobile phase additive is shown in Figure 28 along with the chromatographic data listed in Table 8. It shows that the retention time (t_R) and the separation factor (α) are dependent on the concentration of β -CD. The values of R_s and α are in good agreement with those generated from conventional HPLC [68]. The flow rate in the separation is only 1.8 μ L/min that will greatly reduce the consumption of the expensive mobile phase additive.

In the case of separation using BSA attached to silica as a chiral stationary phase, similar α and R_s values were obtained from Wannman's experiments as compared to the results reported using 1.6 and 4.6 mm I.D. stainless-steel columns [69, 70]. Figure 29-a shows the separation of D- and L-tryptophan. It seems the separation is highly dependent on the pH of the mobile phase. Hu et al. later explained that it was the result because of cyclodextrin's low solubility in aqueous/organic solution [71]. At a pH of 5, one can not separate the mixture. When the pH is increased to 7, a reasonable selectivity can be obtained. For an even better separation, the pH can be further increased up to the limit of the breakdown of silica. However, for the separation of ($\pm$)-benzoin, the pH of the mobile phase does not have much impact. (Figure 29-b). In order to get a better baseline

resolution, higher flow-rate is needed for the separation of ($\pm$)-oxazepam as shown in Figure 29-c. It is because the enantiomers of oxazepam rapidly racemize in aqueous solutions [72]. This will cause the formation of a diffusion zone between the two separated peaks containing a mixture of the separated species called coalescence. Therefore, when the flow rate is increased from 2.0 $\mu\text{L}/\text{min}$ to 6.0 $\mu\text{L}/\text{min}$, a better separation is obtained despite lower column efficiency. The faster flow rate helped to reduce the intra-column racemization.

The Pirkle phase was first developed by Pirkle et al. in 1980. The Pirkle phase columns (100 mm x 4 mm I.D.) are packed with Daltosil 100 L-DNB-phenylglycine (4 μm , 90 $\text{\AA}$, 300 m^2/g) and run with a hexane-isopropanol mixture as the mobile phase [73]. Separations of ($\pm$)-2,2,2-trifluoro-1-(9-anthryl)ethanol and ($\pm$)-propranolol using the Pirkle phase in a microcolumn are shown in Figure 30 and Table 9. Both results are similar to data obtained by using a conventional column.

Although the solubility of the CD can be improved by increasing the pH of the mobile phase, the conditions adopted in turn restrict the applicability. Hu et al. discovered that methylation of β -CD can improve this problem without applying stringent mobile phase conditions [71]. The two commercially available methylated β -CDs are heptakis(2,6-di-*O*-methyl)- β -CD (DM- β -CD) and heptakis(2,3,6-tri-*O*-methyl)- β -CD (TM- β -CD). Figure 31 illustrates the reciprocal of the capacity factor ($1/k'$) and the separation factor (α) of the BNDHP enantiomers as a function of TM- β -CD concentration in the mobile phase. The results show that the relationship between the separation factor and TM- β -CD concentration is not linear. By adjusting the TM- β -CD

concentration, one can get an optimal α of 1.7 at a concentration of about 25 mM. This is a considerable improvement compared to that observed for native β -CD ($\alpha = 1.1$) and native γ -CD ($\alpha = 1.12$). It is presumed that the methylation of β -CD may stabilize the inclusion complex formed by BNDHP and TM- β -CD. This leads to the abnormally large separation factors observed for TM- β -CD.

The separation of FERET enantiomers was also studied, (Figure 32 and Figure 33). In Figure 32, it is shown that the concentration of TM- β -CD has little effect on the separation factor. The separation factor remains at 1.2 when the concentration of TM- β -CD increases from 25 to 50 mM. The separation factor achieved with native β -CD was 1.1. [74]. However, no resolution of FERET enantiomers was achieved when γ -CD was used.

Kientz and Langenberg studied microcolumn LC separation of some chiral organophosphorus pesticides and found that capillary LC has a 5 fold improved separation impedance, an approximately 2-fold increase in column permeability and greater inertness compared with conventional LC [75]. The structures of the organophosphorus compounds studied are shown in Figure 34. The columns were fused silica micro-LC columns (0.32 mm I.D.; 250, 300, or 720 mm length) packed with Chiralcel OD (cellulose) material that was used in conventional columns. A comparison of the results is summarized in Table 10. The separation factor (α) is the same for both columns. For the same linear velocity, the back pressure in micro-LC was lower and the efficiency and peak symmetry were generally better. The column permeability (K^0) is defined as:

$$K^0 = UL\eta/\Delta P$$

Where U = eluent linear velocity

L = column length

η = eluent viscosity

ΔP = pressure drop across the column

Because the column length and eluent viscosity are the same in both columns, a 1.8-fold improvement in column permeability can be obtained based on the above equation.

Based on this improvement, the actual column performance, the separation impedance (E) can be calculated from the equation given in section 3.2

$$E = h^2 \cdot \phi = h^2 \cdot d_p^2 / K^0$$

The comparison indicates that microcolumn LC has a 6-fold improvement for (-) enantiomers and a 3.5 fold improvement for (+) enantiomers in terms of separation impedance.

Acephate enantiomers are the most difficult pesticides to separate with conventional LC. The best resolution obtained from conventional LC ($R_s = 0.55$) still has largely unresolved peaks. With micro-LC, near baseline separation ($R_s = 1.0$) can be easily achieved using identical conditions (Figure 35).

Another advantage of microcolumn LC that Kientz discovered is the better chemical inertness of microcolumn LC. During the conventional LC separation, decomposition of ethyl 4-nitrophenyl phosphoramidate was found. When the eluent flow rate was decreased, the UV response decreased substantially. At the same time, an extra large peak showed up at 16 min which represented p-nitrophenol (Figure 36-a). This

result is an indication of decomposition. However, when the micro-LC system was used, no breakdown was observed, (Figure 36-b). In a separate experiment, a 720 mm long microcolumn was used with a 3 $\mu\text{L}/\text{min}$ flow rate to increase the contact time 7 fold longer. The result also confirmed that no p-nitrophenyl was present. This demonstrated that the degradation observed in conventional LC is a result of interaction of the phosphoramidate with the stainless steel wall of the column. In microcolumn-LC, the inertness of the fused silica capillary greatly improved the stability of phosphoramidate [75]. Verzele et al. also confirmed the improved chemical inertness of micro-LC for the analysis of iso-alpha acids [76].

Cortes and Nicholson did a feasibility study on the potential of chiral separations using packed capillary LC columns. They claimed that the resolution, efficiency, and peak asymmetry values obtained using microcolumns are significantly better than conventional size columns [77]. The chiral analytes used in this study were 1,3,5-tri-*t*-butylbenzene, 1-phenylethanol, 2-phenylcyclohexanone, 2,2,2-trifluoro-1-(9-anthryl)ethanol, and 2-hydroxy-2-phenylacetophenone. The structures of the analytes are shown in Figure 37. The columns used are 25 cm x 250 μm I.D. and 80 cm x 250 μm I.D. Chiracel OD microcolumns packed with acetonitrile, hexane, or isopropanol as the slurry solvent. The results are compared with those obtained on the conventional size 25 cm x 4.6 mm I.D. columns (Figure 38). The chromatograms obtained from microcolumns prepared using acetonitrile, hexane, and isopropanol as packing and slurry solvents are shown in Figures 39, 40 and 41 respectively. Of the three slurry solvents, isopropanol has the best performance probably because its higher viscosity makes it more

difficult for the packing material to settle in the reservoir during the packing process. Therefore, columns packed using isopropanol were used for further investigations. An 80 cm long microcolumn packed with isopropanol as the slurry solvent operated at the optimum linear velocity (1.5 $\mu\text{L}/\text{min}$) will increase the separation time from 12 minutes to 90 minutes (Figure 42). In order to yield an equivalent retention time, the long microcolumn needs to operate at a higher linear velocity (9 $\mu\text{L}/\text{min}$) as shown in Figure 43. Separation data obtained for 2-hydroxy-2-phenylacetophenone is summarized in Table 11-A. The asymmetry values (B/A) for peaks generated from microcolumns were much better than those from conventional columns. The resolution factors for the enantiomer pairs are presented in Table 11-B. Better performance was also obtained for all pairs using the microcolumns packed with isopropanol. Therefore, Cortes concluded that operation of the long (80 cm) microcolumns at high linear velocities resulted in increased enantiomer resolution (by up to 200-300%) over conventional columns without a decrease in analysis time. In addition to improved performance, the slow operating flow rate (5 $\mu\text{L}/\text{min}$) offers great savings in expensive solvents and solvent disposal cost compared to the 1-2 mL/min flow rate for conventional columns. Meanwhile, the cost of column preparation is also reduced because a smaller amount of packing material is needed for microcolumns.

Racemic dansyl amino acids are considered difficult-to-separate enantiomers. However, Han and Armstrong reported great success by using microcolumn LC with a β -cyclodextrin bonded chiral stationary phase [78]. The column was made from fused silica tubing (250 μm I.D.) and packed with 5 μm spherical silica gel to which β -

cyclodextrin was bonded through an eight-atom spacer. Figure 44 shows the influence of acetonitrile concentration in the mobile phase on the capacity factor (k') of the dansyl amino acids. Both retention and enantiomeric resolution increased with increasing acetonitrile concentration. The chromatogram of enantiomeric separation of dansyl butyric acid (Figure 45) shows the solute was not retained when the mobile phase was acetonitrile-buffer (85:15). A sharp peak representing the void volume showed up at 46 minutes. When the acetonitrile concentration was raised to 90%, the retention time increased to ca. 56-60 minutes. Then two sharp peaks were obtained with a separation factor α of 1.12. Baseline resolution can be accomplished when the acetonitrile concentration was increased to 95%. A separation factor of 1.23 was obtained with an increased retention time longer than 80 minutes. It shows that chiral recognition increases at high acetonitrile concentration which is an indication of increasing interaction with the β -cyclodextrin chiral stationary phase. The separation data for various enantiomers of dansyl amino acids are presented in Table 12. All of the separations yield a resolution greater than 1.2. In one case, a resolution as high as 2.83 was achieved for dansyl DL-valine. Three of the low resolution enantiomers, dansyl tryptophan, aspartic acid and glutamic acid, could not be separated on conventional 25 cm LC columns of the same packing [79]. All of three can be resolved on the microcolumn with satisfactory resolution. Figure 46 shows the chromatogram of dansyl DL-tryptophan and aspartic acid.

6.2 Protein and peptide

HPLC is an established separation technique for characterization of proteins and peptides. However, the often-low concentrations or small amount of available sample always limit the wide usage of its applications. Microcolumn HPLC, especially capillary LC with its higher mass sensitivity provides enhanced detection sensitivities, excellent reproducibility and good separation. Therefore it is an ideal technique to deal with such samples.

Hsieh et al. reported the successful separation and identification of peptides present in single neurons by microcolumn HPLC coupled with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Structural information about the peptides from the LC fractions can be partially elucidated by MALDI-PSD analysis [80]. The microcolumn LC system used was a capillary column (30 cm x 50 μ m I.D.) packed with 5- μ m silica particles having a 120-Å pore size. The syringe pump was operated at a 0.1 mL/min flow rate. A splitter capillary was used to split the 0.1 mL/min pump flow to 85 nL/min flow through the microcolumn. The chromatographic eluent was collected at the end of the column every 60 seconds. A schematic diagram for the collection of the microcolumn effluent is shown in Figure 47. The argon gas will supply 1 bar of pressure to deliver the 2,5-dihydroxybenzoic acid (DHB) matrix through a capillary at 60 nL/min flow rate. Both capillary column and matrix were threaded into a 1-mm-I.D. glass capillary. Therefore, the eluent from the LC column and the matrix fused into a single droplet. The droplet was deposited onto the steel target and then collected every 60 seconds as one fraction. At the end of the chromatographic separation, each fraction was

transferred for MALDI-MS analysis. Figure 48 shows the three-dimensional plot of the peptide signals by MALDI-MS using a DHB matrix after microcolumn separation. Figure 49 shows the direct single cell MALDI-MS analysis. The same set of neuropeptides detected in the direct analysis was found in the microcolumn HPLC fractions. However, the reliability of the assignment of the peptide identities, based on their measured masses along with their elution position, is higher than that obtained from the direct MS measurement alone. Thus, the combination of microcolumn HPLC and MALDI-MS made it possible for rapid screening of the entire peptide profile of a single cell and the separation of the peptides into nearly pure fractions for subsequent analysis. In order to detect novel peptides and further confirmation of a peptide species, postsource decay analysis was performed after the microcolumn LC fractionation. Based on the PSD results, Hsieh concluded that a microcolumn LC fraction step brings about “cleaner” mass spectra and eliminates the stringency for very high precursor ion selection capabilities of the PSD analysis system to a great extent.

Grimm et al. also utilized the advantages of the capillary LC based on an upgraded conventional HPLC system coupled with electrospray interface and MALDI MS detection for protein-peptide mapping at subpicomole levels [81]. Figure 50 is a schematic diagram of the capillary LC system. The microcolumn is a 25 cm x 300 μ m I.D. capillary column connected to the UV detector with a capillary flow cell. The outlet capillary of the UV flow cell is then connected to electrospray needle's inlet port. The flow rate for the system is 4 μ L/min. With such a low flow rate, one can introduce much higher analyte concentrations into the capillary and a larger portion of the electrospray

plume into the mass spectrometer. Therefore, such a capillary LC system coupled with an electrospray MS system is the most feasible route for the analysis of minute and dilute samples. The on-line capillary LC-UV-electrospray MS traces of a tryptic digest of myoglobin are shown in Figure 51. The UV-absorbance signal in Figure 51-a has an excellent agreement with the total ion current trace shown in Figure 51-b. In addition, Grimm was able to detect all fragments of the identified tryptic peptide residues and some of the chymotryptic fragments (Figure 52).

Similar to Hsieh's approach, Grimm performed off-line mass determination with the MALDI-TOF system. The small volume of the column eluent enables the microfraction collection of the separated compounds directly onto MALDI targets. The same capillary LC equipment described in Figure 50 was used but the outlet capillary of the UV flow cell was threaded with the matrix capillary to connect with an automated microfraction collection system. Each microfraction can be collected at 30-second intervals with a flow rate of 2 $\mu\text{L}/\text{min}$. Therefore, each target has a total of 1 μL in volume for MALDI-MS analysis. The chromatogram of a 700-fmol myoglobin digest with automated collection onto MALDI targets is shown in Figure 53. The insert figures show the MALDI spectra of two peptide fragments.

Elicone et al. also utilized microbore reversed-phase HPLC for the purification of low picomole amount (<5 pmol) of the peptide SKI PepMix6 [82]. PepMix6 is a mixture of 14 synthetic peptides with the size ranging from 9 to 26 amino acids. The purpose of the experiment was to provide peptide, sufficiently homogeneous to yield interpretable chemical sequencing data. A Vydac C18 (15 cm x 1.0 mm I.D) column was used for

micro-preparative peptide purification and re-purification at a flow rate of 30 $\mu\text{L}/\text{min}$. Sensitivity of peptide detection was evaluated with various quantities of PepMix6 (from 625 fmol to 5 pmol). The chromatogram shown in Figure 54 indicates that the mixture can be satisfactorily separated and collected at the 1-2 pmol level. Even with 0.6 pmol of peptide mix, most of the peaks are still distinct. During the first chromatography run, the peak fraction was collected for rerun (re-purification). Elicone claimed that the low flow rate allows efficient manual collection of closely eluting peaks at the end of the outlet tubing without a complicated automated fraction collector. The peak fraction collected (ca. 20 μL) is diluted and then re-injected onto the same column for re-purification. Recoveries after such a rerun were about 40-60% (corresponding to 2-3 pmol of peptides). The re-purified peptides can later be chemical sequenced and found to be homogeneous. Figure 55 shows the re-purification of peptide peak fractions. The inserts are T17, T26, and T37 peaks rerun on the same column. Therefore, Elicone concluded that with adequate instrumentation and handling procedures in place, low-picomole amounts of peptide could be easily prepared for analysis with the help of micro HPLC.

6.3 Pharmaceutical and biological compounds

The need for efficiency, sensitivity, and economy in the pharmaceutical and biotech industries make micro LC more and more attractive in terms of evaluation of the purity and identification of a wide range of pharmaceutical and biological compounds. Battersby et al. at Genentech developed a procedure for the isolation and purification of trace amounts of recombinant DNA-derived human growth hormone (rhGH) from an in

vivo mode [83]. The rat serum sample was taken at 5, 15, 45 and 60 min after being dosed with rhGH. The blood sample was first partially purified through an affinity chromatography. Then the eluate from the affinity column was further purified, concentrated, and quantitated by a conventional reverse phase HPLC. Approximately 18 μg of the rhGH recovered from the secondary RP-HPLC was digested with trypsin and later injected onto a C18 capillary column (15 cm x 320 μm I.D.) at a flow rate of 3.5 $\mu\text{L}/\text{min}$. Figure 56 is the chromatogram of rhGH recovered from rat serum taken 5 minutes after an intravenous dose of rhGH (upper profile) compared to the peptide map of native rhGH (lower profile). The two chromatograms are almost identical except for an extra tiny peak at 66 min for the 5 min sample. This extra peak was later identified as oxidized T2 by on-line electrospray mass spectrometry. This oxidized T2 fragment represents ~9% of the total T2 content. Figure 57 shows the partial peptide maps for the 15 and 45 min samples. It clearly indicates the trend of decreasing T2 signals at 76 minutes and increasing amounts of the corresponding oxidized T2 (66 min). This result also confirmed that the extra peak in the in vivo trace is attributed to the metabolism process (oxidation of T2). By using the recent advances in microcolumn separation techniques, one can get a closer look at a metabolic event in terms of chemical information about the metabolic fate of unlabeled proteins. Such an approach offers huge advantages over a conventional radioactive labeling method which requires tedious sample handling.

Moore et al. developed analytical procedures to determine the cephalosporin antibiotics that is entirely compatible with direct liquid interfacing (DLI) for MS analysis

[84]. Although conventional HPLC have been used for this purpose, most of these methods have various physical or chemical constraints on the instrument or cannot reach sufficient detection limits. Moore found out that with the low flow-rate, microbore columns make it ideal for interfacing with a mass spectrometer by direct liquid introduction. The column used is 0.3 mm I.D. with 5- μ m C18 packing at a flow rate of 4 μ L/min. The mobile phase contained no buffers or salts to prevent column blocking problems. The four cephalosporins, cefazolin, ceftizoxime, cefaloridine and cefaclor can be separated satisfactory as shown in Figure 58. Compared with the results using conventional HPLC [85], this method obtained a 10-fold increase in sensitivity. The detection limits were 1 ng for cefazolin and cefaloridine and 5 ng for cefaclor and ceftizoxime.

Tsuji and Binns demonstrated the application of a micro-bore HPLC system for the analysis of various pharmaceutical compounds, such as antibiotics, ibuprofen and steroids [86]. A RP-8 micro-bore column (50 cm x 1 mm I.D.) was used for the analysis of ibuprofen. A plate number of 36,800 can be obtained when operated at the optimal flow rate of 20 μ L/min . The chromatogram of ibuprofen with an internal standard, valerophenone is shown in Figure 59. For the analysis of steroids, the results from conventional and micro-bore HPLC are compared side by side. Both used identical compounds for the mobile phase and a similar composition. The chromatograms are shown in Figures 60 and 61. The micro-bore column gave satisfactory separation while the conventional column could not resolve the methylprednisolone, prednisolone and fluorometholone peaks.

The feasibility of high speed analysis by micro-bore HPLC was also presented. It took 20 minutes for a conventional HPLC system to complete the separation of methylprednisolone acetate when prednisone was used as an internal standard. With a flow rate of 300 $\mu\text{L}/\text{min}$, a commercially available micro-bore HPLC can obtain a satisfactory result in 3.5 minutes. (Figure 62.)

7 Conclusion

Microcolumn HPLC is growing rapidly and widely accepted in many fields. It has already been demonstrated that microcolumn separation methods hold the key to improvements in mass detection sensitivity, column efficiency and peak resolution. The low flow rate makes it ideal for direct coupling with a mass spectrometer and secondary chromatographic systems. In addition, the low consumption of both mobile and stationary phases offered by microcolumn HPLC facilitate the use of expensive materials and a reduction of cost in waste disposal. It is likely that 'the microcolumns will become the analytical columns of the future' [18].

Bibliography

1. http://hplc.chem.shu.edu/NEW/HPLC_Book/index.html
2. A. Weston, P.R. Brown, "HPLC and CE: Principles and Practice", Chapter 2, Academic Press, San Diego, 1997.
3. E. Katz, R. Eksteen, P. Schoenmakers, "Handbook of HPLC", Chapter 10, Marcel Dekker, New York, 1998.
4. L.R. Snyder, J.J. Kirkland, "Introduction to Modern Liquid Chromatography" 2nd edition, Chapter 3, John Wiley & Sons, New York, 1979.
5. M. C. McMaster, "HPLC: A Practical User's Guide", p105, Wiley-VCH, New York, 1994.
6. S. Lindsay, "High Performance Liquid Chromatography", 2nd ed., Chapter 3, John Wiley & Sons, Chichester, 1992.
7. R.E. Majors, "Current issues in HPLC Technology", LC-GC, (1997) S8.
8. E.F. Vansant, P. Van Der Voort, K.C. Vrancken, "Characterization and Chemical Modification of the Silica Surface", Elsevier, Amsterdam, 1995.
9. J.J. Pesek, S.A. Swedberg, J. Chromatogr., 361(1986) 83.
10. J.F.K. Huber, F.F.M. Kolder, J.M. Miller, Anal. Chem., 44 (1972) 105.
11. LC-GC HPLC Columns and Packing, Advanstar Communications, Edison, New Jersey, 1997.
12. K.M Kirkland, D.A. McCombs, J.J. Kirkland, J. Chromatogr. 660 (1994) 327.
13. H. Colin, G. Guiochon, M. Martin in H. Engelhardt, ed. "Practice of High Performance Liquid Chromatography: Applications, Equipment and Quantitative Analysis", Springer-Verlag, Berlin, 1986.
14. C.G. Horvath, B.A. Preiss, S.R. Lipsky, Anal. Chem., 39 (1967) 1422.
15. C.G. Horvath, S.R. Lipsky, Anal. Chem., 41 (1969) 1227.
16. D. Ishii, JASCO Report, 11 (6) 1974.

17. P. Kucera, "Small Bore Liquid Chromatography Columns: Their Properties and Uses", Wiley, New York, 1984.
18. M. Novotny, in M.V. Novotny, D Ishii (Editor), "Microcolumn Separations", p20, Elsevier, Amsterdam 1985.
19. F.J. Yang, W. Lai, D.C. Park, C.T. Yang, Current Issues in HPLC Technology. LC-GC (1997) S34.
20. M.A. Mosely, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, Anal. Chem. 63 (1991) 1467.
21. J.E. Coutant, T.M. Chen, B.L. Ackerman, J. Chromatogr. 529 (1990) 265.
22. S. Pleasance, P. Thibault, M.A. Mosely, L.J. Deterding, K.B. Tomer, and J.W. Jorgenson, J. Am. Soc. Mass Spectrom. 1 (1990) 312.
23. J.P. Gagne, A. Carrier, L. Varfalvy, M.J. Bertrand, J. Chromatogr. 647 (1993) 13.
24. J.P. Gagne, A. Carrier, L. Varfalvy, M.J. Bertrand, J. Chromatogr. 647 (1993) 21.
25. K.B. Tomer, J.R. Perkins, C.E. Parker, L.J. Deterding, Biol. Mass Spectrom. 20 (1991) 783.
26. A. Cappiello, F. Bruner, Anal. Chem. 65 (1993) 1281.
27. A. Cappiello, G. Famiglini, F. Bruner, Anal. Chem. 66 (1994) 1416.
28. A. Cappiello, G. Famiglini, Anal. Chem. 66 (1994) 3970.
29. H. Alborn, G. Stenhagen, J. Chromatogr. 394 (1987) 35.
30. G. Stenhagen, H. Alborn, J. Chromatogr. 474 (1988) 285.
31. A.C. Barefoot, R.W. Reiser, J. Chromatogr. 398 (1987) 217.
32. A.C. Barefoot, R.W. Reiser, Biomed. Environ. Mass Spectrom. 18 (1989) 77.
33. M. Hail, S. Lewis, I. Jardine, J. Liu, M. Novotny, J. Microcol. Sep. 2 (1990) 285.
34. L.J. Deterding, C.E. Parker, J.R. Perkins, M.A. Mosely, J.W. Jorgenson, and K.B. Tomer, J. Chromatogr. 554 (1991) 329.
35. E.C. Huang, J.D. Henion, Anal. Chem. 63 (1991) 732.

36. F.J. Yang, J. Chromatogr. 20 (1982) 241.
37. D.F. Hunt, J.E. Alexander, A.L. McCormack, P.A. Martino, H. Michel, J. Shabanowitz, N. Sherman, M.A. Mosely, J.W. Jorgenson, and K.B. Tomer, in Techniques in Protein Chemistry II, J.J. Villafranca, Ed. (Academic Press, San Diego, 1991), p441.
38. P.R. Griffin, J.A. Coffman, L.E. Hood, J.R. Yates III, Int. J. Mass Spectrom. Ion Proc. 111 (1991) 131.
39. G.A. Schutz, J.N. Alexander, J.B. Poli, The 44th ASMS Conference Proceedings (American Society for Mass Spectrometry, Santa Fe, New Mexico, 1996), p909.
40. J.P.C. Vissers, H.A. Claessens, C.A. Cramers, J. Chromatogr. A, 779 (1997) 1.
41. T. Takeuchi, M. Asai, H. Haraguchi, D. Ishii, J. Chromatogr., 499 (1990) 549.
42. D. Ishii, T. Takeuchi, Trends Anal. Chem. 9,5 (1990) 152.
43. R.A. Hartwick, R.F. Meyer in M.V. Novotny, D. Ishii (Editor), "Microcolumn Separations", Elsevier, Amsterdam (1985) 87.
44. T. Takeuchi, Y. Watanabe, D. Ishii, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 300.
45. Y. Hirata, K. Jinno, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 196.
46. M. Novotny, in P. Kucera (Editor), "Microcolumn High Performance Liquid Chromatography", Elsevier, Amsterdam (1984) p194.
47. F.J. Yang, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 348.
48. P.A. Bristow, J.H. Knox, Chromatographia, 10 (1977) 279.
49. J.H. Knox, J. Chromatogr. Sci. 18 (1980) 453.
50. M. Verzele, C. Dewaele in "Microbore Column Chromatography", Marcel Dekker, New York (1989) 51.
51. F. J. Yang, J. Chromatogr. 236 (1982) 284.
52. S.J. Van der Wal, F.Y. Yang, HRC & CC 6 (1983) 216.

53. P. Kucera in P. Kucera (Editor), "Microcolumn High Performance Liquid Chromatography", Elsevier, Amsterdam (1984) p39.
54. F. J. Yang, J. Chromatogr. 236 (1982) 265.
55. V.L. McGuffin, M. Novotny, Anal. Chem. 55 (1981) 580.
56. R.C. Simpson, J. Chromatogr. A 691 (1995) 163.
57. H.J. Cones, J.R. Larson, G.M. McGowan, J. Chromatogr. 607 (1992) 131.
58. M. Novotny, Anal. Chem. 60 (1988) 500.
59. P. Kucera, G. Manius in P. Kucera (Editor), "Microcolumn High Performance Liquid Chromatography", Elsevier, Amsterdam 1984.
60. H.J. Cortes, C.D. Pfeiffer, B.E. Richter, J. High resolute. Chromatogr. Chromatogr. Commun. 8 (1985) 469.
61. H.J. Cortes, C.D. Pfeiffer, D.E. Jenssen, J. Chromatogr. 349 (1985) 55.
62. K.Jinno, Chromatographia, 25 (1988) 1009.
63. J.D. Henion, J. Chromatogr. Sci. 19 (1981) 57.
64. W.M.A. Niessen, J. van der Greef "Liquid Chromatography-Mass Spectrometry", Marcel Dekker, New York (1989) 207.
65. R.M. Caprioli, T. Fan, J.S. Cottrell, Anal. Chem. 58 (1986) 2949.
66. C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem., 57 (1985) 675.
67. H. Wannman, A. Walhagen, P. Erlandsson, J. Chromatogr. 603 (1992) 121.
68. A. Walhagen, L.E. Edholm, Chromatographia, 32 (1991) 215.
69. P. Erlandsson, L. Hansson, R. Isaksson, J. Chromatogr., 370 (1986) 475.
70. P. Erlandsson, S. Nilsson, J. Chromatogr., 482 (1989) 35.
71. R. Hu, T. Takeuchi, J. Jin, T. Miwa, Anal. Chin. Acta., 295 (1994) 173.

72. Y. Aso, S. Yoshioka, T. Shibazaki, M. Uchiyama, *Chem. Pharm. Bull.*, 36 (1988) 1834.
73. W.H. Pirkle, D.W. House, J.M. Finn, *J. Chromatogr.*, 192 (1980) 143.
74. T. Takeuchi, T. Miwa, *J. Chromatogr.*, 666 (1994) 439.
75. C.E. Kientz, J.P. Langenberg, *J. High Resolut. Chromatogr.* 14 (1991) 460.
76. M. Verzele, C. Dewaele, M. De Weerd, *LC-GC International* 2 (1988) 12.
77. H.J. Cortes, L.W. Nicholson, *J. Microcol. Sep.* 6 (1994) 257.
78. S.M. Han, D.W. Armstrong, *J. Chromatogr.* 389 (1987) 256.
79. W.L. Hinze, T.E. Riehl, D.W. Armstrong, W. DeMond, A. Alak, J.J. Ward, *Anal. Chem.* 57 (1985) 237.
80. S. Hsieh, K. Dreisewerd, R.C. Van der Schors, C.R. Jimenez, J.S. Zheng, F. Hillenkamp, J.W. Jorgenson, W.P.M. Geraerts, K.W. Li, *Anal. Chem.* 170 (1998) 1847.
81. R. Grimm, M. Serwe, J.P. Chervet, *LC-GC* 15 (1997) 960.
82. C. Elicone, M. Lui, S. Geromanos, H.E. Bromage, P. Tempst, *J. Chromatogr. A* 676 (1994) 121.
83. J.E. Battersby, V.R. Mukku, R.G. Clerk, W.S. Hancock, *Anal. Chem.* 67 (1991) 215.
84. C.M. Moore, K. Sato, Y. Katsumata, *J. Chromatogr.* 539 (1991) 215.
85. C.M. More, K. Sato, H. Hattori, Y. Katsumata, *Clin. Chim. Acta*, 190 (1990) 121.
86. K. Tsuji, R.B. Binns, *J. Chromatogr.* 253 (1982) 227.

HPLC Mode	Stationary Phase	Mobile Phase
Normal Phase	Silica, Alumina, Cyano, Amino, Diol	Hexane, Heptane, Octane, Methylene Chloride, Ethyl Acetate, THF, Acetonitrile
Reversed Phase	C18, C8, Phenyl, C4, C1-C2	Water, Dimethyl Sulfoxide, Ethylene Glycol, Acetonitrile, Methanol, Acetone, Dioxane

Table 1. Typical stationary and mobile phases used in normal and reverse phase chromatography. [2]

Base Material	Examples	Estimated Usage(%)
Silica gel	High surface area, with pore	75
Polymer	Polymethacrylates, PS-DVB, Polyethylene glycol	20
Alumina	Neutral, acidic	1
Other	Graphitized carbon, Zirconia, hydroxyapatite	4

Table 2. Base materials used for HPLC column packings. [7]

REACTION TYPE	REACTION	SURFACE LINKAGES
ESTERIFICATION	$\text{Si} - \text{OH} + \text{R} - \text{OH} \rightarrow \text{Si} - \text{OR} + \text{H}_2\text{O}$	$\text{Si} - \text{O} - \text{C}$
ORGANO-SILANIZATION	a). $\text{Si} - \text{OH} + \text{X} - \text{SiR}'_2\text{R} \rightarrow \text{Si} - \text{O} - \text{SiR}'_2\text{R} + \text{HX}$ OY b). $\text{Si} - \text{OH} + \text{X}_2 - \text{Si} - \text{R} \rightarrow \text{Si} - \text{O} - \text{Si} - \text{R} + 3\text{HX}$ OY $\text{Y} = \text{Si} \text{ or } \text{H}$	$\text{Si} - \text{O} - \text{Si} - \text{C}$
CHLORINATION FOLLOWED BY REACTION OF GRIGNARD REAGENTS AND ORGANOLITHIUM COMPOUNDS	$\text{Si} - \text{OH} + \text{SOCl}_2 \xrightarrow{\text{Toluene}} \text{Si} - \text{Cl} + \text{SO}_2 + \text{HCl}$ a). $\text{Si} - \text{Cl} + \text{BrMgR} \rightarrow \text{Si} - \text{R} + \text{MgClBr}$ or b). $\text{Si} - \text{Cl} + \text{Li} - \text{R} \rightarrow \text{Si} - \text{R} + \text{LiCl}$	$\text{Si} - \text{C}$
a). TES SILANIZATION -	$ \begin{array}{ccc} \begin{array}{c} \\ \text{O} \\ \\ -\text{Si} - \text{OH} \\ \\ \text{O} \\ \\ -\text{Si} - \text{OH} \\ \\ \text{O} \\ \\ -\text{Si} - \text{OH} \\ \\ \text{O} \\ \end{array} & \rightarrow & \begin{array}{ccc} \begin{array}{c} \\ \text{O} \\ \\ -\text{Si} - \text{O} - \text{Si} - \text{H} \\ \\ \text{O} \\ \\ -\text{Si} - \text{O} - \text{Si} - \text{H} \\ \\ \text{O} \\ \\ -\text{Si} - \text{O} - \text{Si} - \text{H} \\ \\ \text{O} \\ \end{array} & & \begin{array}{c} \\ \text{O} \\ \\ -\text{Si} - \text{H} \\ \\ \text{O} \\ \\ -\text{Si} - \text{H} \\ \\ \text{O} \\ \\ -\text{Si} - \text{H} \\ \\ \text{O} \\ \end{array} \end{array} $	a). $\text{Si} - \text{H}$ monolayer
b). HYDROSILATION	$ \text{b). } \text{Si} - \text{H} + \text{CH}_2 = \text{CH} - \text{R} \xrightarrow{\text{Catalyst}} \text{Si} - \text{CH}_2 - \text{CH}_2 - \text{R} $	b). $\text{Si} - \text{C}$

Table 3. Chemical reactions used to modify silica surface. [9]

Column Type	Column Inner Diameter	Flow Rate	Loading	Detection Enhance
Conventional	4.6 mm	0.5-2 mL/min	2-10 mg	1
Narrow bore	2.1 mm	200-400 μ L/min	0.5-2 mg	5
Microbore	0.8-1.0 mm	25-60 μ L/min	50-500 μ g	20-25
Capillary	100-500 μ m	1-15 μ L/min	1-50 μ g	80-2000
Nano	< 100 μ m	<1 μ L/min	<1 μ g	2000-10,000

Table 4. Comparison of column types by Yang et al. [19]

Column Type	Column I.D.	Column Length	Flow Rate	Loading
Conventional	4.6 mm	10-25 cm	1 mL/min	10-100 µg
Small-Bore Packed	0.2-1 mm	1-10 m	1-20 µL/min	1-10 µg
Packed Capillary	40-80 µm	1-100 m	0.5-2 µL/min	100 ng-1µg
Open-Tubular Capillary	15-50 µm	1-100 m	<1 µL/min	< 100 ng

Table 5. Characteristics of conventional and miniaturized columns for LC by Novotny. [18]

Column type	$h_{\min}$	ϕ	E
Conventional or small bore packed capillary	2	500-1000	2000
Semipermeable packed capillary column	2	150	600
Open tubular capillary	0.8	32	20

Table 6. Comparison of the theoretical performance of different column types.

$h_{\min}$ = reduced plate height, ϕ = column resistance factor, E = separation impedance [49].

Parameter	Conventional, i.d.: 4-5 mm	Microbore, i.d.: 0.5-2 mm	Fused silica, i.d.: 0.1-0.5 mm
1. Slurry solvent	See text	Viscous solvents (MeOH/glycol)	Nonviscous acetonitrile
2. Slurry concentration	5-30%	1-5%	20%
3. Packing pressure	300-1000 atm	600-1800 atm	300-500 atm
4. Down or up	Both	Down	Down
5. Column	As such or polished	Polished or glass-lined	As such or inner wall coated

Table 7. Column packing parameters for conventional, microbore and fused silica packed capillary columns. [50]

[β -CD] (mM)	k' ₁	k' ₂	α	R _s	N ₁
8	4.14	4.70	1.14	1.17	2300
12	3.00	3.53	1.18	1.39	2100
16	2.31	2.78	1.2	1.38	2000

Table 8. Chromatographic data from Figure 28. Chiral separation of ($\pm$)-terbutalin with β -cyclodextrin (β -CD) as a chiral mobile phase additive. [67]

Substance	Mobile phase ^a	k' ₁	k' ₂	α	R _s	N ₁
Tri-F ^b	95:5:-	2.22	2.87	1.29	2.54	3200
Propranolol	90:10:2	12.0 ^c	13.3	1.11	1.12	2500

Table 9. Chromatographic data from Figure 30. Chiral separation on a Pirkle column. a Mobile phase : n-hexane-isopropanol-acetonitrile. b 2,2,2-trifluoro-1-(9-anthryl)ethanol. c (+)-propranolol [67]

Comparison of micro-LC and conventional LC columns^{a)}.

Parameter	Micro-LC		Conventional LC [11]	
<i>U</i> [mm/s]	0.77		0.74	
<i>F</i> [μl/min]	3		500	
<i>P</i> [MPa]	0.85		1.5	
<i>α</i>	2.13		2.12	
	(-)	(+)	(-)	(+)
<i>t_R</i> [min]	10.2	14.9	10.7	16.1
<i>h</i>	4.2	6.4	7.2	8.8
<i>B/A</i>	1.4	2.0	1.8	2.5

^{a)} Test compound, (-) and (+) *trans-stilbene oxide*; column, 250 mm × 0.32 mm and 4.6 mm i.d., respectively; eluent, hexane – isopropanol (9 : 1).

Table 10. Comparison of micro-LC and conventional LC columns [75].

Column ^b	h_1	h_2	B/A	B/A
Conventional	6.2	6.5	1.72	1.62
Hexane packed	11	13	1.17	1.20
Acetonitrile packed	15	15	1.13	1.16
Isopropanol packed	4.6	5.2	1.14	1.20
Isopropanol packed ^c	3.5	3.6	1.20	1.11
Isopropanol packed ^d	17	20	1.28	1.29

^aMeasured for 2-hydroxy-2-phenylacetophenone (pair 5).

^b25 cm length unless otherwise noted.

Table 11-A

Reduced plate height (h) and asymmetry (B/A) value comparison for micro and conventional columns in chiral separation^a[77].

Column ^a	$R_{S2,2}$	$R_{S3,3}$	$R_{S4,4}$	$R_{S5,5}$
Conventional	1.02	1.18	6.52	4.33
Hexane packed	0.59	0.50	5.83	3.34
Acetonitrile packed	0.52	0.53	5.44	3.18
Isopropanol packed	1.13	1.07	7.38	5.03
Isopropanol packed ^b	2.76	2.10	18.5	10.4
Isopropanol packed ^c	1.31	0.98	8.16	4.92

^a25 cm length unless otherwise noted.

^b80 cm x 250 μ m i.d. operated at 0.07 cm s⁻¹.

^c80 cm x 250 μ m i.d. operated at 0.67 cm s⁻¹.

Table 11-B

Resolution factors for solute pairs 2-5 obtained using microcolumns and conventional columns in chiral separation [77].

TABLE I

OPTICAL RESOLUTION OF THE ENANTIOMERS OF DANSYL AMINO ACIDS .

Amino acid	k'		α	R_f	Mobile phase
	L	D			
Tryptophan*	3.22	3.65	1.14	1.68	97:3**
Aspartic acid*	0.98	1.20	1.22	1.20	95:5***
Glutamic acid*	0.81	0.96	1.19	1.20	95:5***
Valine	2.35	3.27	1.39	2.83	97:3**
Butyric acid	0.90	1.11	1.23	1.67	95:5**
Serine	0.90	1.06	1.18	1.50	95:5**
Norleucine	0.80	0.99	1.23	1.75	95:5**
Threonine	0.61	0.80	1.31	1.86	95:5**
Phenylalanine	1.49	1.86	1.25	2.33	97:3**
Methionine	0.57	0.79	1.39	1.78	95:5**
Leucine	1.14	1.15	1.28	2.20	95:5**
Norvaline	0.81	1.01	1.25	2.00	95:5**

* Could not be separated on ordinary 25-cm LC columns of the same packing.

** Numbers represent the volume percent of acetonitrile to buffer (pH 7.1). Data obtained using a 190-cm β -cyclodextrin bonded microcolumn.

*** Numbers represent the volume percent of acetonitrile to buffer (pH 4.1). Data obtained using a 120-cm β -cyclodextrin bonded micro column.

Table 12. Optical resolution of the enantiomers of dansyl amino acids [78].

Figure 1. Basic configuration of a high-performance liquid chromatography [1].

Figure 2. High-pressure mixing gradient system [5].

Figure 3. Flow diagram for Rheodyne 7125 valve [6].

Figure 4. Scanning electron micrographs of (a) sil-gel and (b) xerogel spherical silica gelused in HPLC. (a) Structure: uniform subparticles; porosity: 50%; surface area: 150 m²/g (10 Å pore diameter); strength: high; high-pH stability: good; purity: high; pore-diameter distribution: narrow. (b) Structure: sponge-like polymeric network; porosity: 70%; surface area: 300 m²/g (100-Å pore diameter); strength: low; high-pH stability: poor; purity: low-high; pore-diameter distribution: broad. [7]

Figure 5. Structure of silica gel used in HPLC [7].

Figure 6: Separation of basic drugs at pH 12 using polymeric packing. Column: 125 mm X 4.0 mm Ashipak ODP-50; mobile phase A: phosphate buffer (pH 12); mobile phase B: acetonitrile; gradient: 10-70% B in 8min; temperature: 50 °C. Peaks: 1 = tocalnarnide, 2 = procainamide, 3 = quinine, 4 = diisopyramide, 5 = dihydroquinine, 6 = diphenhydramine.[7]

Figure 7: Separation of aspartame and its metabolites using (a) 5- and (b) 3.5- μ m dp supports. Columns: (a) 15 cm x 0.46 cm Zorbax SB-C18, (b) 7.5 cm x 0.46 cm Zorbax SB-C18; mobile phase: 15:85 acetonitrile-0.1% trifluoroacetic acid; flow rate: 1.0 mL/min; temperature: ambient. Peaks: 1 = phenylalanine, 2 = 5-benzyl-3,6-dioxo-2-piperazineacetic acid, 3 = aspartic acid-phenylalanine, 4 = aspartame [12].

Figure 8. Rapid separation of ribonucleosides Column: 151.7 cm long; 1-mm i.d.; packed with pellicular cation exchange resin, sieve fraction #270/325 Eluent: 0.02M $\text{NH}_4\text{H}_2\text{PO}_4$, pH 5.6. Temperature 39 °C Flow rate 25.5 mL/h. Inlet pressure 131 atm Sample size: 300 picomoles of each component Full scale 0.04A.U [15].

Figure 9. Rapid separation of bases Column: 300.7 cm long; 1-mm i.d.; packed with pellicular cation exchange resin, sieve fraction #270/325 Eluent: 0.02M $\text{NH}_4\text{H}_2\text{PO}_4$ pH 5.5. Temperature 68°C, flow rate 33.4 mL/h, inlet pressure 162 atm, sample size: 800 picomoles of each component 4, fullscale 0.02A.U [15].

Figure 10. Separation of ribonucleosides mono-, di-, and tri-phosphoric acids
Column : 1.0-mm i.d., 193-cm length; packed with pellicular anion
 exchanger (strong basic), sieve fraction No.270-325; 8 micro-equivalent per
 gram. **Eluent:** linear gradient of ammonium formate buffer, pH 4.35 at 25°C,
 from 0.04 M to 1.5 M. **Temperature:** 71° C. **Flow rate:** 12 ml per hour. **Flow**
velocity: 1 cm/second **Pressure:** 51 atm. **Sample:** 1.5-3.5 nanomoles (0.6-1.5
 μg) per component [14].

TYPES OF MICROCOLUMNS

Figure 11. Types of LC Microcolumns [18].

Figure 12. Coupled-column chromatography of aromatic hydrocarbons in a light kerosine. Operating conditions of micro-HPLC system: column, TSKgel NH2-60, 150 x 0.35 mm I.D., mobile phase; hexane, flow-rate: 4.2 μ l/min; sample: 0.02 μ l of 10% light kerosine. Wavelength of UV detection: 220 nm. Operating conditions of conventional HPLC system: Column, Develosil ODS, 250 X 4.6 mm I.D., mobile phase, acetonitrile-water (70:30), flow-rate, 1.0 ml/min; wavelengths of UV detection, 210 nm for monocyclic aromatic hydrocarbons fraction and 220 nm for bicyclic aromatic hydrocarbons fraction. Peak identification, 1 a = benzene; 1b = toluene, 1c = o-xylene; 1d = m- and p-xylene; 1e = ethylbenzene; 1f = cumene; 1g = mesitylene; 1h = n-propylbenzene; 2m = naphthalene; 2n = biphenyl, 2o = 1-methylnaphthalene; 2p = 2-methylnaphthalene [42].

**INJ: 1 μ l, 4 μ g BENZ., TOL.
0.5 μ g NAPH.
0.2 μ g FLUORENE
0.03 μ g ANTHR.**

MOBILE PHASE: 55% ACN/H₂O

**DETECTOR: VARIAN MODEL
UV-200 WITH
SUBMICROLITER
FLOW CELL**

**COLUMN A:
15 cm x 1 mm
SP-C₁₈-5
FLOW: 50 μ l/MIN**

**0.02
A₂₅₄**

**COLUMN B
15 cm x 2.1 mm
SP-C₁₈-5
FLOW: 200 μ l/MIN**

**COLUMN C
15 cm x 4.6 mm
SP-C₁₈-5
FLOW: 1000 μ l/MIN**

Figure 13. Detectability differences of microbore and conventional HPLC columns with a UV detector [47].

Figure 14. Curves of H versus u (flow rate) for small particles. Column: 25 x 0.46 cm for 8- and 5- μ m ROSil-C18-D, 15 x 0.46 cm for 3- μ m ROSil-C 18- D , and 4 x 0.46 cm for 2 μ m; mobile phase: 75: 25 acetonitrile/water; sample: pyrene ($k' \sim 6$) . [50]

Figure 15. Diagram of stream splitting set up using (a) constant pressure regulator and (b) a constant flow restrictor. [19]

Figure 16. Connection between a Valco valve and a microbore column. [53]

Figure 17. The modified Valco 0.5 µl internal loop sampling valve for split injection in narrow-bore packed column HPLC. [54]

Figure 18. Schematic diagram of the “heart-cut” injection system [55].

Figure. 19 . Detail of microbore column coupling utilizing a modified Swagelok union [59].

Figure 20 Block diagram of on-line packed capillary LC-capillary GC system. (1) Pump; (2) injection valve; (3) packed capillary LC column; (4) detector; (5) recorder; (6) switching valve; (7) effluent waste; (8) retention gap; (9) butt connector; (10) capillary GC column; (11) detector; (12) gas chromatograph oven; (13) recorder. [60]

Figure 21 (A) Micro-LC chromatogram of fuel oil sample. Column 105 cm x 250 μ m i. d .packed with Zorbax silica, $dp = 7 \mu$ m; mobile phase, heptane; flow, 10.6 μ l/min; detector, Jasco Uvidex II at 214 nm; pressure, 3800 psig. Sample prepared to give concentration of 1.00 g/10 ml in heptane. X = section introduced into the gas chromatograph. (B) Capillary gas chromatogram of fuel oil from LC Column, 30 m x 0.25 mm i.d. Supelcowax 10 ($df = 0.25 \mu$ m); retention gap, 15 m x 0.25 mm fused silica; oven temperature 105°C for 9 min, program to 245°C at 5°C /min ; carrier, helium at 70 cm/s; makeup gas, nitrogen at 30 ml/min; detector, Fill at 275°C; injected volume, 21 μ l. Retention times of chlorobenzenes of interest are indicated. (1) Chlorobenzene, (2) 1,2-dichloro-benzene, (3) 1, 2, 4,5-tetrachlorobenzene, (4) 1,2,3,4-tetrachloro-benzene, (5) pentachlorobenzene, (6) hexachlorobenzene [61].

Fig. 22. High-resolution chromatogram of a test mixture of alkylbenzenes on nine 4.5 m x 1 mm I.D. C-18 reversed-phase microbore column. Eluent: methanol :water (85:15); flow rate: 20 μ l/min; detection: 254 nm. [59]

Figure 23 . Total-ion-current chromatogram of five compounds, metribuzir methyl parathion, ethyl parathion, merphos and trifluralin, obtaine by using negative methane chemical ionization [62].

Figure 24. Separation of the two isomeric pesticides propazine (scan 176) and trietazine (scan 183-184) (molecular weight: 229); ion current traces for molecular ions (D) and several characteristic fragment ions (A, B, C) [62].

Fig. 25. Schematic drawing of the micro LC/MS system 1: pump; 2: 250 μ l gas-tight syringe; 3: PTFE tubing, 0.5 mm i.d.; 4: sample inlet; 5: micro column; 6: UV variable wavelength detector; 7: micro UV cell, 0.3 μ l volume; 8: stainless steel capillary; 9: PTFE tubing; 10: glass-to-Teflon connector; 11: glass capillary micro LC/MS probe; 12: direct insertion probe inlet of MS; 13: CI mass spectrometer ion source. Note the close proximity of the UV exit and the micro LC/MS glass capillary probe. [63]

Figure 26 Schematic diagram of the first prototype CF-FAB interface probe, built by Caprioli et al. [65].

Fig. 27. Schematic representation of an electrospray interface [66]

Fig. 28. Chromatograms showing the resolution of (±)-terbutalin with different concentrations of β-cyclodextrin in the mobile phase. Column, 190 mm x 0.32 mm I.D. packed with Nucleosil 100-5 C18; mobile phase, 0.05 M ammonium acetate (pH 6.0) with 2% methanol as modifier; flow-rate, 1.8 μl/min; volume injected, 60 nl of a 0.75 mM solution in water; UV detection at 215 nm. [67]

Figure 29. Resolution of (a) D,L-tryptophan. (b) (±)-benzoin and (C) (±)-oxazepam on a 250 mm x 0.32 mm I.D. column packed with 5- μm BSA-silica. (a) Mobile phase. 0.05 M phosphate buffer (pH 5.0 and 7.0) with 2% of n-propanol as modifier; flow-rate 2.5 $\mu\text{L}/\text{min}$; volume injected, 200 nl of a 0.02 mg/ml solution in the mobile phase; UV detection at 279 nm. (b) Mobile phase. 0.05 M phosphate buffer (pH 5.0) with 2% of n-propanol as modifier; flow-rate. 2.4 $\mu\text{L}/\text{min}$; volume injected, 200 nl of a 0.02 mg/ml solution in water-methanol (98:2, v/v); UV detection at 279 nm. (c) Mobile phase. 0.05 M phosphate buffer (pH 7.0) with 2% of n-propanol as modifier; flow-rate, 2.0 and 6.0 $\mu\text{L}/\text{min}$; volume injected. 200 nl of a 0.02 mg/ml solution in water-methanol (75:25, v/v); UV detection at 249 nm. [67]

Fig. 30. Resolution of (a) (±)-2,2,2-trifluoro-1-(9-anthryl)ethanol and (b) (±)-propranolol as its naphthyl isocyanate derivative on a 260 mm x 0.32 mm I.D. column packed with 4- μ m L-DNB-phenylglycine. (a) Mobile phase, n-hexane-2-propanol (95:5, v/v); flow-rate, 1.9 μ l/min; volume injected, 60 nl of a 0.08 mg/ml solution in 2-propanol; UV detection at 253 nm. (b) Mobile phase, n-hexane-2-propanol-acetonitrile (90:10:2, v/v/v); flow-rate, 1.9 μ l/min; volume injected, 60 nl of a 2-propanol-diluted reaction mixture (less than 1.5 mM); UV detection at 214 nm. [67]

Figure 31. Retention behaviour of BNDHP enantiomers as a function of TM-β-CD concentration in the mobile phase. Column, Develosil ODS-5 (150 x 0.35 mm i.d); mobile phase, aqueous acetonitrile solution (15%, v/v) containing TM-β-CD; flow-rate, 2.8 μl/min; sample volume, 20 nl; wavelength of UV detection, 220 nm, O = 1/k' of (S)-(+)-BNDHP; Δ = 1/k' of (R)-(-)-BNDHP; ■ = α of both analytes. [71]

Figure 32 Retention behaviour of FERET enantiomers as a function of TM-β-CD concentration in the mobile phase. Mobile phase, aqueous acetonitrile solution (20%, v/v) containing TM-β-CD; other operating conditions as in Fig. 31. O= $1/k'$ of (+)-FERET; Δ = $1/k'$ of (-)-FERET; $\blacksquare$ = α of both analytes. [71]

Figure 33. Separation of FERET enantiomers with TM- β -CD as the mobile phase additive. Mobile phase, aqueous acetonitrile solution (20%, v/v) containing 25 mM TM- β -CD; analytes, 0.05% (w/v) of each isomer; other operating conditions as in Fig.31. [71]

Figure 34 Structures of organophosphorus pesticides: A) methamidophos; B) ethyl p-nitrophenyl phosphoramidate; C) acephate. [75]

Figure 35 Micro-LC chromatogram of acephate: column, 720 x 0.32 mm i.d. packed with Chiralcel OD; eluent, hexane-isopropanol (8:2); flow rate, 10 μ l/min; amount injected, 360 ng of the racemic mixture; detection, UV at 215 nm; peak assignment is unknown because only racemic mixtures were available.[75]

Figure 36 LC of ethyl *p*-nitrophenyl phosphoramidate on Chiralcel OD using hexane -isopropanol (8:2) as eluent: detection, UV at 254 nm; A) 250 x 4.6 mm i.d. column; flow rate, 2 mU/min; B) 300 mm x 0.32 mm i.d. column; flow rate, 10 μ l/min; the peak assignment is unknown because only racemic mixtures were available. [75]

Figure 37. Chiral analytes used in this study. (1) 1,3,5-tri-*t*-butylbenzene , (2) 1-phenylethanol, (3) 2-phenylcyclohexanone, (4) 2,2,2-trifluoro-1-(9-anthryl)ethanol, and (5) 2-hydroxy-2-phenylacetophenone [77].

Figure 38. Chromatogram of the test mixture on a conventional size column. Conditions: 25 cm x 4.6 mm i.d. ChiracelOD column; hexane/isopropanol (80:20) mobile phase, 1.6 mL/min ; 10 μ L injection size,. UV detection at 254 nm. For peak identifications, see Figure 37. [77]

Figure 39. Chromatogram of the test mixture obtained using a microcolumn packed with acetonitrile as slurry solvent. Conditions: 25 cm x 250 $\mu\text{m.i.d.}$ ChiracelOD column; 5 $\mu\text{L}/\text{min}$ flow; 60 nL injection size. Other conditions as in Figure 38. [77]

Figure 40. Chromatogram of the test mixture obtained using a microcolumn packed with hexane as slurry solvent. Conditions as in Figure 39. [77]

Figure 41. Chromatogram of the test mixture obtained using a microcolumn packed with isopropanol as slurry solvent. Conditions as in Figure 39. [77]

Figure 42. Chromatogram of the test mixture, obtained using a long microcolumn packed with isopropanol as slurry solvent. Conditions: 80 cm x 250 μ m i.d. column; 1.5 μ L/min flow. Other conditions as in Figure 39. [77]

Figure 43. Chromatogram of the mixture obtained using a long micro-column packed with isopropanol as slurry solvent, and operated at linear velocity. Conditions: 9 μ L/min flow. Other conditions as in Figure 39. [77]

Figure 44. Influence of percentage acetonitrile on the capacity factors of some dansyl amino acid, A = Dansyl D-tryptophan; B = dansyl D-leucine; C = dansyl D-phenylalanine. [78]

Figure 45. Enantiomeric separation of dansyl butyric acid as a function of the percentage of acetonitrile in the mobile phase. The α values were 0.0, 1.12, and 1.23 for mobile phase compositions of 85:15, 90:10 and 95:5. respectively [78] .

Figure 46. Enantiomeric separation of dansyl tryptophan and dansyl aspartic acid. See Table 13 for experimental conditions. A = Dansyl DL-tryptophan; B = dansyl DL-aspartic acid [78].

Figure 47 Schematic diagram of the system for coupling of the microcolumn LC system with MALDI-MS. Splitter capillaries were used to split the 0.1 mL/min pump flow to 85 nL/min flow through the microcolumn. The matrix solution at 60 nL/min was mixed with effluent at the end of the column. Every 60 s, the droplet is deposited onto the target by adjusting the manipulator. [80]

Figure 48. 3-D plot of neuropeptides in a single VD1 neuron fractionated by microcolumn LC and analyzed by MALDI-MS. The microcolumn effluent mixed with DHB matrix solution was obtained every 60 s, producing fraction volume sizes of approximately 145 nL. After drying, the samples with spot sizes of approximately 1 mm in diameter were analyzed by MALDI-MS. Only spectra with clearly detectable analyte signals are shown. The synthetic peptide eluting in fractions 26 and 27 was added to the cell supernatant before injection into the microcolumn. The numbers at the intensity bars reflect the (different) absolute signal intensities in the mass spectra for different sets of fractions. Peptides derived for the VD1 precursors, i.e., α_1 , α_2 , and β , are indicated, as well as the SCPs derived from the SCP precursor. The peptides C, D, and E are the putative modified forms of α_2 peptide. The novel putative peptides are labeled F, G, and I, respectively. For larger peptides, doubly charged molecular species $(M + 2H)^{2+}$ in addition to the singly charged species are detected and are also indicated. Matrix: 2,5-DHB. [80]

Figure 49 Direct MALDI-MS spectrum of a VD1 neuron. After dissection from the snail brain, the cell was directly placed on target in a 0.5-μL drop of DHB matrix. See Figure 48 and text for explanation of the peak labels. [80]

Figure 50. Schematic of a capillary LC system coupling with MS. [81]

Figure 51: On-line capillary LC-UV-electrospray MS analysis of a tryptic digest of myoglobin. Shown are (a) UV-absorbance and (b) total ion current chromatograms. Flow rate: 4 μ L/min. [81]

Figure 52: Total ion current trace with identified tryptic peptide residues (T1-T22) and chymotryptic and fragments (x). The inset figure is the electrospray MS spectrum of fragment T3. Other conditions were the same as in Figure 51. [81]

Figure 53: Capillary LC analysis of a 700-fmol myoglobin digest with automated microfraction collection onto MALDI targets. The inset figures show MALDI spectra of two arbitrarily chosen peptide fragments. Flow rate: 2 μ L/min; collected fraction volume: 1 μ L/spot. [81]

Figure 54. Sensitivity of peptide detection in microbore RP-HPLC. RP-HPLC profiles of various quantities of PepMix6 (composition listed in Table 1) are shown. HPLC configuration was as described under Experimental; a 1.0 mm I.D. Vydac 218 TP5115 C18 column was used. Amounts of PepMix6 are indicated on the panels; samples were injected in 50 μ l of 2 or 20% TFA(indicated). Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5-35%/22.5min, 35-70%/12.5 min at a flow-rate of 30 μ l/min and at ambient temperature. Time scale is from 15 to 32 min; AUFS values vary between panels, as indicated. Symbols: O, $\square$, and Δ indicate the peaks corresponding to peptides 7, 4 and 13, respectively. [82]

Figure 55. Repurification of peptide peaks obtained from micro-bore RP-HPLC. RP-HPLC profile of a peptide mixture, obtained after in situ tryptic digestion of protein CM-CRKA_{p110}; precise amount of protein was not known but estimated, from amido black staining of the nitrocellulose blot, to be about 10 to 15 pmol. Peaks T17, T26, and T37 (indicated) were rechromatographed on the same column after addition of 5 μ l neat TFA and two-fold dilution with water; results are shown in the insets. HPLC configuration was as described under Experimental; a 1.0 mm I.D. SGE Inertsil 100 GL-1-ODS-I10/5 C18 column and an LC packings U-Z view flow cell were used. Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5-35%/45 min, 35-70%/22.5 min at a flow-rate of 30 μ l/min and at ambient temperature, for the primary separation; gradient during each rechromatography was 3.5-35%/90 min. Full scale corresponds to 0.004 AU at 214 nm; the same absolute scale also applies to the sections of the secondary chromatograms (inserts). [82]

Figure 56. Capillary HPLC peptide map of rhGH recovered from rat serum taken 5 min after an intravenous dose of rhGH (upper profile). The sample was recovered using the purification scheme described in the text. The lower profile is the peptide map of native rhGH. The peptides were separated on a Nucleosil C18 capillary column (320 μm i.d. x 15 cm) using an acetonitrile gradient (0-40% in 2 h) with a flow rate of 3.5 $\mu\text{l}/\text{min}$. The separation details are described in the Experimental Section. [83]

Figure 57. Capillary HPLC peptide maps of rhGH recovered from rat serum taken 15 (middle profile) and 45 min (upper profile) after an intravenous dose of rhGH. The lower profile is the tryptic map of native rhGH. Arrows link methionine-containing peptides with their corresponding oxidized forms. The separation conditions are described in Figure 56. [83]

Figure. 58. Cephalosporin standards (0.1 $\mu\text{g/ml}$; 100 μl). Mobile phase, deionized water-methanol-acetic acid (60:40:0.5); flow-rate, 4 $\mu\text{l/min}$; detection wavelength, 262 nm; C18 column, 15 cm x 0.3 mm I.D. Samples: (a) ceftizoxime; (b) cefaclor; (c) cefaloridine; (d) cefazolin. [84]

Figure 59. A chromatogram of ibuprofen (1) with an internal standard, valerophenone (2), using an RP-8 packed micro-bore column. Mobile phase: acetonitrile-water (with 1% chloroacetic acid) (65:35) at pH 3.0. Flow-rate: 80 μ l/min. [86]

Figure 60. A normal-phase micro-bore chromatogram indicating high column efficiency separation of cortisone acetate (1), fluorometholone (2), hydrocortisone (3), hydrocortisone hemisuccinate (4), methylprednisolone (5), prednisolone (6) and prednisone (7). Mobile phase: water-saturated butyl chloride-butyl chloride-THF-methanol-glacial acetic acid (450:450:105:53:44). [86]

Figure 61. Conventional 5- μ m particle size packed normal-phase HPLC (25 cm x 4.6 mm) chromatogram of cortisone acetate (1), prednisone (2), hydrocortisone (3), methylprednisolone (4), prednisolone (5) and fluorometholone (6). Mobile phase: water-saturated butyl chloride-butyl chloride- THF-methanol-glacial acetic acid (450:450:70:35:30). Flow-rate: 1 ml/min. [86]

Fig. 62. Normal-phase chromatograms for the analysis of methylprednisolone acetate (1) with prednisone (2) used as an internal standard. A. Conventional HPLC: mobile phase, water-saturated butyl chloride-butyl chloride-THF-methanol-glacial acetic acid (475:475:70:35:30); flow-rate, 1 ml/min. B. High-speed micro-bore packed LC: mobile phase, as in A but 450:450:105:53:44; flow-rate, 300 μ l/min. [86]