This year marks the 15th anniversary of the Waters Sep-Pak® cartridge. Sep-Pak cartridges have been used successfully in thousands of unique applications, and have been integrated into many official analytical methods. Although these miniature columns are simple to use in practice, the science behind them is more sophisticated. The object of this article is to review parameters that will affect the performance of these Sep-Pak cartridges.

Two common problems in analytical chemistry are: (1) the removal of contaminants from a sample that interfere with an analysis, and (2) the enrichment of an analyte in a sample to enhance sensitivity. These two problems have been solved classically by liquid-liquid extraction (LLE). In this technique, the desired analyte or analytes of interest are selectively extracted into one of two immiscible liquids. Typically one liquid is water, and the other is an organic solvent, such as hexane or methylene chloride. By judicious selection of the aqueous phase pH and the proper organic phase, the extraction may be optimized to obtain a favorably high partition coefficient of the analyte into one of the phases, while the interferences selectively partition into the other phase. If the partition coefficient is too low, a second or a third extraction may be required. The sample usually must then be concentrated by evaporation prior to analysis. Besides requiring large amounts of organic solvents (which will need disposal), the time required for the sample preparation is often quite long, even with automation. Also, if more than one analyte requires extraction, it may be impossible to isolate all desired components with one LLE procedure.

Consider how these problems could be solved by using a solid phase (such as a chromatographic sorbent) instead of the second liquid phase. There are two distinct phases that can be readily segregated. Also, by proper selection of pH and type of solid phase sorbent, one could optimize the conditions to selectively partition the component(s) of interest on the solid phase. After removal of the liquid phase, a second liquid phase could be employed to desorb these components. As in the case of LLE, a second liquid-solid extraction may be required.

Now consider the case in which the solid phase is put into a column or similar flow-through device; nearly 100% loading or elution may be achieved, assuming no breakthrough or irreversible adsorption. This approach of column-liquid-solid extraction (CLSE) may reduce the final liquid volume significantly compared with LLE, thus requiring no evaporation step. The key to the success of this solid phase extraction (SPE) technique depends on the reproducibility of the solid phase sorbent, as well as developing a rugged procedure that can withstand possible variations in the sample matrix or the experimental protocol. With regard to the solid phase sorbent, the SPE vendor must provide consistent media that does not change from lot-to-lot. Waters takes significant steps to ensure that the solid phase chemistry does not change between batches. Each batch of Sep-Pak cartridge stationary phase undergoes a variety of stringent analytical and functional chromatographic testing to ensure that your application will perform identically on every batch of sorbent.
The Science Behind SPE

The reason SPE works is the same reason why HPLC works, because SPE is liquid chromatography. In fact, the Waters Sep-Pak cartridges employ the same raw silica and bonding chemistry as are found in the Porasil™ and Bondapak™ family of analytical and preparative HPLC columns. The only difference between the packing found in an analytical µBondapak™ column and a Sep-Pak cartridge is the particle size. Sep-Pak cartridges contain a nominal 40 or 80 µm particle, while analytical µBondapak and Bondapak sorbents are 10 or 20 µm in size. Can a Sep-Pak cartridge be used as an inexpensive analytical column? The answer can be found by examining the fundamental HPLC equation for resolution between two components:

$$R_s = \frac{1}{4} \left(\frac{\alpha - 1}{\alpha}\right) \sqrt{\frac{k_2}{k_2 + 1}} N$$

where $\alpha$ is selectivity, $k_2$ is the retention factor for the more retained component, and $N$ is the number of plates. The selectivity term is a measure of how the two different components behave on the chromatographic system. The higher the $\alpha$, the greater the difference in chromatographic behavior. The retention factor is a measure of how strongly retained the component is, and $N$ is a measure of the efficiency of the chromatographic system. If one compares a Sep-Pak cartridge to a µBondapak analytical column, one would find a similar $\alpha$ and $k$ for both. The difference between the two systems resides in $N$, the number of plates ($N$). Typically a µBondapak analytical column (3.9 x 150 mm) will have approximately 7500 theoretical plates, while a Sep-Pak short body cartridge will have less than 50 plates. Using the resolution equation, a minimum $\alpha$ can be determined for baseline resolution. Assuming a resolution of 1.2 (typical value for baseline resolution), one can solve the above equation for $\alpha$ and find that for $k_2=10$, a minimum $\alpha$ of 1.06 is required for baseline resolution on the HPLC system, while an $\alpha$ of 3.9 is necessary with a Sep-Pak cartridge. This means that a Sep-Pak cartridge is limited in scope. The HPLC column will be able to separate components within a single class, while the Sep-Pak cartridge can separate one class of components from another. This explains why Sep-Pak cartridges are used most often in a step-gradient mode, as one can elute components with high $\alpha$’s in relatively small volumes.

Effect of $k$

Most SPE applications consist of five basic steps (1) cartridge conditioning, (2) cartridge equilibration, (3) sample loading, (4) intermediate elutions or rinses, and (5) product elution. The first step is of greatest importance with reversed-phase sorbents, as they are not easily wetted by water. Typically, acetonitrile or methanol is used to completely wet the solid phase support as well as to solvate the hydrocarbonaceous ligand. The equilibration step simply removes this organic solvent from the cartridge and prepares it for the sample. Care must be taken to prevent the support from drying at this point in order to keep it completely wetted. For this equilibration and the sample load step, typically one wants to use conditions that will make the analytes of interest adsorb strongly to the sorbent, i.e., to drive $k\rightarrow\infty$. (Note that in some SPE applications, loading solvents are chosen to let the analytes of interest pass through the column unretained, while the interferences are adsorbed. In this case, one wants to drive $k\rightarrow0$.) For the case of strong adsorption, this typically means equilibrating the cartridge with neat water or aqueous buffer for a reversed-phase Sep-Pak cartridge, or using a non-polar solvent such as hexane for a silica cartridge. In practice, $k$ values are finite, say 100-500. This means that for trace enrichment applications in which large sample volumes are passed through a Sep-Pak cartridge, one needs to be concerned about sample breakthrough, especially of the least retained analyte of interest. Consider the case in which $k=500$. The retention volume of an injected peak can be calculated from the relation:

$$k = \frac{V_R - V_0}{V_0}$$

If $V_0=1$ mL, then $V_R=501$ mL. However, because of peak dispersion, the component will start to elute well before this volume. This is shown graphically in Figure 1. Note that the more efficient the Sep-Pak cartridge, the greater the volume one can load before breakthrough occurs. This effect will be discussed again shortly when we examine the effect of flow rate on cartridge performance.

Figure 1. Plot of breakthrough profile for frontal loading of sample onto Sep-Pak with $k'=500$ at different cartridge efficiencies ($N$)
If interferences are present, they can be minimized or eliminated by implementing a cartridge rinse procedure and/or choosing an appropriate elution solvent. The cartridge rinse step is important to remove more weakly retained interferences from the components of interest by using a rinse solvent that is of sufficient polarity or solvent strength to elute undesired contaminants without coelution of the desired analytes. Typically, this means using a mixture of water (or buffer) and miscible organic solvent (such as methanol or acetonitrile) for a reversed-phase Sep-Pak cartridge, or moderately polar solvent with a silica sorbent, such as a mixture of hexane and ethyl acetate. In choosing appropriate rinse and elution conditions, knowledge about the behavior of analytes and contaminants on an HPLC system is extremely useful. Injection of the components isocratically on an HPLC at a few different mobile-phase compositions will provide valuable information when developing an optimized SPE protocol.

For the elution step, the objective is to elute the analytes of interest in as small a volume as possible without also coeluting more strongly adsorbed contaminants. If possible, use a solvent where all analytes of interest are unretracted. When interferences are present, this may not be possible. However, keep in mind that the more retained the analyte is, the greater is the elution volume required for complete recovery. Thus as a result, the analyte will be more dilute, and this will result in less sensitivity for the analytical method. Figure 2 shows the effect that k has on the recovery for different k values with N=20. Figure 3 shows the effect that flow rate has on cartridge performance for two different Sep-Pak Plus cartridges, one a C18 Sep-Pak cartridge, the other a tC18 Sep-Pak cartridge. The cartridge efficiency was determined by connecting the Sep-Pak cartridge to an HPLC system, and measuring the number of plates for a neutral marker with a k of 5 using a mobile phase of 50/50 water/acetonitrile. One difference between the two sorbents is the particle size. The C18 material contains 55-105 µm bonded silica, while the tC18 material contains 37-55 µm particles. In both cases, the Sep-Pak cartridge dimensions are identical.

**Figure 2. Plot of Recovery as a Function of Elution Volume for Different k’s with N=20**

**Figure 3. Plot of Number of Plates vs. Flow Rate for Sep-Pak Cartridges**

* trifunctional C18
Effect of Cartridge Size

The standard Sep-Pak Plus Cartridge has been used for the work reviewed in this article. For other cartridge sizes, the linear velocity will change at a given flow rate due to differences in the inner diameters. The linear velocity is proportional to the cross-sectional area of the cartridge, or proportional to the square of the cartridge inner diameter. One must take this into account and scale flow rate appropriately. For example, the dimensions of the Sep-Pak Light Cartridge are 5.44 x 12 mm. The ID is ~1.9 x less than that of the Sep-Pak Plus Cartridge, so flow rates must be reduced by a factor of 3.6 to achieve comparable linear velocities.

Effect of Mass Loaded

As has been shown, maximum sample load depends on the volume one can load. It can also depend on the mass, both of the component of interest as well as interferences. For trace enrichment, mass overload is not usually a problem. But for concentrated samples, product can break through earlier than expected due to mass overload. This condition is similar to mass overload in HPLC. A simplified explanation of this phenomena is that the cartridge has a finite loading capacity. Injection of a large mass causes saturation of the binding sites, and as a result product elutes with a sharper front earlier than expected. (For a more detailed discussion of mass overload and volume overload, see Fundamental Effects in Preparative Chromatography on page 7.) Because of the relatively low number of plates in a Sep-Pak Cartridge, it may be possible to load up to 100 mg of sample onto the cartridge. This value will depend on the retention factor of the analytes as well as the amount of impurities present in the sample. One may need to look at using a cartridge with greater sorbent mass to prevent mass overload. Conversely, a smaller cartridge, such as the Sep-Pak Light Cartridge may be advantageous when doing trace analyses. This will allow elution in a smaller volume, resulting in greater sensitivity without the need for concentration.

One brief note on the Environmental tC18 Sep-Pak cartridge. This material was formulated specifically for environmental trace enrichment applications, such as the preconcentration of organic compounds in drinking water (e.g., EPA Method 525.1). The trifunctional-C18 material was made by bonding with octadeyltrichlorosilane, which provides low extractables as observed by GC or GC/MS in comparison to monofunctional bonded phases found in the standard C18 Sep-Pak cartridge. Also the smaller particle size was chosen to maximize sample load before breakthrough occurs, which provides the greatest possible recoveries.

Figure 4a. Plot of Recovery as a Function of Volume Loaded for an Analyte with k=500

Figure 4b. Plot of Recovery as a Function of Elution Volume
Conclusions

Sep-Pak cartridges are simple devices to use for trace enrichment and removal of interferences from liquid matrices. The devices are small liquid chromatography columns. Because SPE is fundamentally liquid chromatography, one can examine parameters that affect recovery. Knowledge about these parameters can help in developing a more rugged SPE procedure. The flow rate, in particular, is important to control because of its effect on chromatographic efficiency, which in turn will affect the breakthrough volumes for both SPE loading as well as the elution. HPLC can be used to assist in the development and optimization of an SPE procedure; an understanding of the chromatographic behavior of analytes and interferences will help in rapidly developing and optimizing appropriate conditions for a given application.

Table I. Comparison of analytical HPLC column and Sep-Pak cartridge.

<table>
<thead>
<tr>
<th></th>
<th>Analytical Column</th>
<th>Sep-Pak Short Body</th>
<th>Sep-Pak Long Body</th>
<th>Sep-Pak Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>150 mm</td>
<td>10 mm</td>
<td>20 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>ID</td>
<td>3.9 mm</td>
<td>10 mm</td>
<td>10 mm</td>
<td>5.44 mm</td>
</tr>
<tr>
<td>Particle size</td>
<td>10 µm</td>
<td>40 or 80 µm</td>
<td>40 or 80 µm</td>
<td>40 or 80 µm</td>
</tr>
<tr>
<td>Plates (N)</td>
<td>7500</td>
<td>50</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Plates/cm</td>
<td>500</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Minimum α required</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for k=5, Rs=1.2</td>
<td>1.07</td>
<td>5.39</td>
<td>2.36</td>
<td>5.39</td>
</tr>
<tr>
<td>k=10, Rs=1.2</td>
<td>1.06</td>
<td>3.95</td>
<td>2.12</td>
<td>3.95</td>
</tr>
<tr>
<td>k=10, Rs=1.5</td>
<td>1.08</td>
<td>1.5</td>
<td>2.94</td>
<td>1.5</td>
</tr>
<tr>
<td>k=100, Rs=1.5</td>
<td>1.08</td>
<td>6.99</td>
<td>2.54</td>
<td>6.99</td>
</tr>
<tr>
<td>Cross-sectional area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12 cm²</td>
<td>0.79 cm²</td>
<td>0.79 cm²</td>
<td>0.23 cm²</td>
</tr>
<tr>
<td>Flow rate for linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>velocity</td>
<td>1 mL/min</td>
<td>6.6 mL/min</td>
<td>6.6 mL/min</td>
<td>1.9 mL/min</td>
</tr>
</tbody>
</table>

An analytical column provides about fifteen fold more plates than a Sep-Pak cartridge. This is due to the longer length of the HPLC column, the smaller particle size, and the more highly efficient packed bed. Note that when optimizing the flow rate with different size Sep-Pak cartridges, the flow rate should be optimized to give an equivalent linear velocity.

---

For more information on Waters Sep-Pak cartridges, check box number 3 on the reply card and return today. You will receive an eight-page brochure entitled “How many steps does it take to do sample preparation”, ordering information and a valuable limited-time free offer.

---

Does Your HPLC Method Stand the Test of Time?
Find Out Using the Waters Method Validation Kit.

When validating your method, you need a column to produce the same separation time after time. The Waters Method Validation Kit is an essential tool for checking the ruggedness and reproducibility of your new method.

From column traceability to batch information for packing material, you are guaranteed complete reproducibility when validating your method time and time again.

The Waters Method Validation Kit contains three NovaPak™ C₁₈ columns (3.9 mm x 150 mm) from two different batches. Two columns packed with the same batch of packing allow you to validate column to column method reproducibility. A third column, packed with a different batch of packing lets you validate batch-to-batch method reproducibility. The kit also contains a Certificate of Analysis and a comprehensive care and use manual.

For more information on the Waters New Method Validation Kit, check box number 4 on the reply card and return today.
New Waters Sentry™ Guard Column—A Lifesaver for Your Analytical Column

The new Waters Sentry Guard Columns (3.9 x 20mm) are designed with two special features: high efficiency and high capacity. Packed with the same high efficiency Nova-Pak and uBondapak packings used in our analytical HPLC columns, Sentry Guard columns actually improve the plate count of your analytical column, and have sufficient capacity to protect your columns from even the dirtiest samples. The reusable Sentry column holder allows you to insert and remove guard columns without tools, and is sealed with fingertight fittings. The column holder fits into Waters column heater box (is this the right name?) and most commercially available column ovens.

High Efficiency

Other guard columns act to reduce the efficiency of analytical HPLC columns. When a Sentry guard column is used in-line with an HPLC column, efficiency for the total column system is actually increased. For example, a Nova-Pak C_{18} columns (3.9 x 150mm) alone delivered an efficiency of 13,200 plates. With the 20mm Nova-Pak C_{18} Sentry guard column in line, plate count increased to 13,900.

High Capacity

HPLC samples often contain soluble debris and other contaminants that readily foul or irreversible bind to high performance columns and shorten their lifetime.

The 0.24 mL packed bed of the Sentry guard column has the capacity required to significantly extend analytical column lifetime. The figures to the right demonstrate the dramatic results that are possible—a full 25mg of protein from direct injection of mouse serum were adsorbed on the Nova-Pak C_{18} Sentry guard column before a change in efficiency and resolution of the separation were noted.

Two Ways to Get the Benefits

Waters Sentry Guard Column is available as a stand-alone holder/column for use with any analytical HPLC column. If you use Waters new cartridge columns with removable enfittings, the Sentry guard column fits directly into the existing column enfitting.

For more information on the new Waters Sentry Guard Column check box number 5 on the reply card and return today.

For ordering information, please see page 28 and 29.
Summary
The differences between analytical and preparative separations lie in the desire to process large amounts of sample in preparative chromatography. Consequently, the chromatographic column is used frequently in “overload” mode, i.e., either large sample volumes or high concentrations or a combination of these are applied to the column. This leads to broadening of peaks and shifts in retention compared to analytical chromatography. This article will demonstrate and explain these effects with a simple model.

Introduction
In analytical chromatography, the position of the peak is determined by the distribution coefficient of the sample between mobile phase and stationary phase, and its width is determined by band-spreading effects originating in the packed bed. In preparative chromatography additional phenomena occur, which ultimately completely overshadow the effects encountered in analytical chromatography.

A rigorous treatment of these influences is mathematically complex and is the subject of the research of many scientists (e.g., Refs. 1–3). Nevertheless, some simplified systems can demonstrate the underlying phenomena without undue complexity and help in the understanding of the complete phenomenon (Ref. 4). In the following, we will look separately at the effect of volume overload, mass overload, and the effect of using columns of different lengths and/or different plate-counts.

Experimental
System
The entire study was conducted using a Waters™ 845 Chromatography Data and Control System, a Waters™ 717 Autosampler, and a Waters™ 590 programmable HPLC pump. Depending on the sample concentrations, either a Waters™ 410 Differential Refractometer or a Waters™ 490E Programmable Multiwavelength Detector were used. The data were processed and analyzed on the Waters™ 845 Data Station using the System Suitability Software Package with ExpertEase™ V.3.0. Efficiency calculations were performed using the 5-Sigma Method.

Columns and Mobile Phase
The columns used were 6 µm Nova-Pak® C₁₈ 8 x 100 mm Radial-Pak™ cartridges in the RCM™ 8 x 10 Cartridge Holder. With this system it is possible to stack cartridges in series and thus manipulate column efficiency and column volume. The mobile phase consisted of 37% acetonitrile and 63% water.

Samples
The samples for the mass overload study were prepared as follows. A stock solution consisting of propylparaben dissolved in 37% acetonitrile and 63% water solution was made. This saturated solution contained 23.9 mg/mL of propylparaben. From this stock, serial dilutions by a factor of two were made to produce solutions with the concentrations of 12.0 mg/mL, 5.98 mg/mL, 2.99 mg/mL, 1.49 mg/mL, 747 µg/mL, 373 µg/mL, 187 µg/mL, 93.4 µg/mL, and 46.7 µg/mL.

The samples for the volume overload study were made in a similar fashion. However, this time the stock solution was first diluted to a concentration of 478 µg/mL and then serial dilutions of two were made from this solution. The concentrations of these solutions were 239 µg/mL, 120 µg/mL, 59.8 µg/mL, 29.9 µg/mL, 14.9 µg/mL, 7.47 µg/mL, 3.73 µg/mL, and 1.87 µg/mL.

Results and Discussion
In most practical studies of the effect of increased load on a chromatographic separation, the injection volume is increased at constant sample concentration. Under these circumstances, the effects of mass overload and volume overload are confounded. In our study, we purposely separated both effects to clearly demonstrate the phenomena that occur. Effects caused by increases in injection volume alone at constant and small mass load are termed volume overload, and effects caused by increasing the injected mass at constant and small injection volume are called mass overload.
1. Volume Overload

The increase in injection volume leads to both a change in the apparent retention time of the peak measured at the peak apex and to a change in peak width. The effect is identical to the effects caused by extra-column band broadening encountered in analytical mode. It is well understood and has been treated by many authors. We separated mass-overload from volume-overload effects by decreasing the concentration in proportion to the increase in injection volume, thus keeping the injected mass constant. One observes, that at low injection volumes the peak is unaffected, but at higher injection volumes the peak becomes broader, as the injection volume increases. This is shown in Figure 1.

In this graph, the square of the peak width is plotted against the square of the injection volume according to the following equation:

\[ w_t^2 = w_c^2 + f \cdot w_i^2 \]

\( w_t \) is the total width of the peak, \( w_c \) is the peak width of the column alone and \( w_i \) is the injection volume. The factor \( f \) is unequal to 1 due to the laminar flow profile in the injector.

Also, the retention time of the center of the peak is shifting with increasing injection volume. That is because the injection mark that starts the recording of the chromatogram is made at the beginning of the injection, while the injection volume still needs to be purged onto the column. The peak elution volume will therefore increase by about half the injection volume. This is shown in Figure 2. The actual delay is slightly larger than half the injection volume due to the laminar flow profile in the injector, which increases the injection volume.

---

**Figure 1. Peak Width as a Function of Injection Volume**

The square of the peak width is plotted against the square of the injection volume (rule of additivity of variances). The insert shows the results of the linear regression. The coefficient of 4.47 is due to the laminar flow profile in the injector tubing.

**Figure 2. Dependence of Retention Volume on Injection Volume**

The retention time of the center of the peak increases with increasing injection volume. This is because the recording of the chromatogram starts before the injected volume is purged out of the injector. The peak elution volume will therefore increase by about half the injection volume.
These phenomena can be observed in their pure form only, when the sample solvent and the mobile phase are identical. When the sample solvent differs from the mobile phase, both the increase in peak width and the shift in peak retention are a function of the elution strength of the solvent in which the sample is made up. If the elution strength of the sample solvent is higher than the elution strength of the mobile phase, the sample is initially washed through the column faster than under equilibrium conditions. This leads to additional peak distortion and an apparent overload of the column and is undesirable. On the other hand, if the sample is dissolved in a solvent that is a weaker eluent than the mobile phase, a concentration of the peak at the top of the column occurs, and larger volumes can be injected before volume overload occurs. Therefore it is preferred to dissolve the sample in either the mobile phase or in a weaker solvent than the mobile phase. Obviously, in practice this desire has to be balanced against the solubility of the sample in the various solvents.

2. Mass Overload

As the sample concentration in the mobile phase increases, the adsorption isotherm of the sample passes from the linear region into the non-linear region. The phenomena that occur under non-linear conditions are called mass overload.

For an ideal column of infinite plate-count, i.e., without band spreading, it can be shown with a little algebra that the tail of the peak is described by the adsorption isotherm of the sample (See Appendix) (Ref. 5). Figure 3 depicts the phenomenon.

As one can see, the tail of the peak follows a common line, while the front of the peak moves forward, i.e., elutes earlier. The actual description of this phenomenon in a real column with finite plate-count is fairly complex, and the subject of a large body of literature. Nevertheless, this simplified model is very useful for our understanding of the fundamental effects. It is customary to plot the plate-count against mass load in a double-logarithmic graph (Figure 4).

As the injected mass of sample increases, the apex of the peak moves to shorter retention times. The tail of the peak follows a common line, which is defined by the adsorption isotherm.

At low load, the plate-count is independent of sample load. When the load is increased, the plate-count is dominated by the adsorption isotherm and becomes independent of the inherent column efficiency.

At low load, the plate-count is independent of load. This is the linear region of the adsorption isotherm. The peak width is determined by column phenomena and injection volume. Different columns, either of different lengths or packed with particles of different sizes, exhibit a different plate-count under this condition. As the load increases, the peak width is determined by the derivative of the adsorption isotherm and increases steadily with increasing mass load. In this region, the intrinsic efficiency of the column decreases in importance until the curves for both columns merge into a single curve. In the next paragraph, we will demonstrate this by comparing column systems of different efficiencies.

Call for Papers

If you are using Waters columns or sample preparation products in your work and would like to submit an article for inclusion in a future issue of the Waters Column, please contact Uwe Neue at 508-478-2000, Ext. 2157.
3. Segmented Column Technology

The radial compression system used in this study makes it possible to stack columns in series. In this way, the amount of sample that can be processed in a single run can be increased. As a natural consequence, the intrinsic plate-count of the column system varies.

We studied the mass overload phenomenon by injecting a constant volume of ever-increasing sample concentrations. Peak width and retention times were recorded and the plate-count was calculated. This was first done on a single column. Then, two columns were coupled in series. The experiment was repeated first at the same flow rate as used for the single column and then at double the flow rate, which resulted in the same run-time as with the single column. The results are shown in Figure 5.

As expected, one can see that at low load the coupled columns resulted in a higher plate-count than a single column. The difference under these conditions became smaller as the flow rate for the coupled columns was doubled. At high mass load, expressed in mg injected per column, the difference in efficiency between all three experimental conditions became negligible. One can therefore increase the sample load in direct proportion to the number of columns and obtain identical resolution. If the flow rate is also increased, the run time on a bank of two columns is the same as on a single column. But since the load for equal resolution has doubled, an increase in throughput is observed. This is in perfect agreement with theoretical expectations.

![Figure 5. Plate-Count vs Mass Load for Segmented Columns](image)

The theoretical relationship shown in Figure 4 is demonstrated here with actual data. Segmented column technology allows you to tailor the column to the load requirements of the application.

**Conclusion**

A simple model system has been used to demonstrate fundamental phenomena in preparative chromatography. The understanding obtained from the model system helps in the design of the more complex systems encountered in practical applications. Segmented technology gives the user the versatility to tailor the performance of the preparative system to his or her needs.

---

**Develop a Method or Application in 1/3 Less Time . . . Introducing Two New Waters Columns Scouting Kits**

**Reversed Phase** for precise compound/chemistry match and **Method Development** for unknown and unique compound applications

Save time, money, and solvents by using fast, high-performance NovaPak® columns (available in 50-mm length C18, C8, and Phenyl and CN HP columns). These new columns feature reusable finger-tightened endfittings so changing columns is fast, easy and requires no tools.

Also, for batch-to-batch and lot-to-lot reproducibility and packing material traceability, Waters columns come with complete documentation so you can expect the same quality separation results time after time.

For more information on the Waters Reversed Phase and Method Development Scouting Kits, check box number 6 on the reply card and return today.

For ordering information, please see page 29.
Appendix

The following mathematical treatment explains the effect of mass overload in the case of an “ideal” column.

Let us consider a column, with a very high plate-count, i.e., which does not cause any dispersion. We also need to assume that the equilibrium between mobile phase and stationary phase is instantaneous. The mass balance for the sample in the column-length element dx is then:

\[ V_m \cdot \frac{dc_m}{dt} + V_s \cdot \frac{dc_s}{dt} + V_m \cdot u \cdot \frac{dc_m}{dx} = 0 \]

The subscripts \( m \) and \( s \) stand for mobile and stationary phase respectively. \( V \) is the volume of the phase and \( c \) is the sample concentration. \( u \) is the mobile phase velocity.

This equation can be rearranged:

\[ \frac{dc_m}{dt} \cdot \left( 1 + \frac{V_s}{V_m} \cdot \frac{dc_s}{dc_m} \right) + u \cdot \frac{dc_m}{dx} = 0 \]

The second factor in the last term in the parentheses is nothing but the derivative of the adsorption isotherm, and the factor preceding it is the phase-ratio

Further rearrangement yields the (fictitious) migration velocity of a sample band of the concentration \( c_m \):

\[ \frac{dx}{dt} = \frac{u}{\left( 1 + \frac{V_s}{V_m} \cdot \frac{dc_s}{dc_m} \right)} \]

Consequently, the \( k' \) of this band is:

\[ k' = \frac{V_s}{V_m} \cdot \frac{dc_s}{dc_m} \]

As mentioned above, the last term is the derivative of the adsorption isotherm.

References


ISO 9000

The Quality Is In How You Do It.

Early this year, Waters dedicated chromatography materials synthesis plant received ISO 9002 certification. But achieving registration alone simply isn’t good enough.

Putting processes in place to achieve ISO registration is very different from putting processes in place to achieve higher customer satisfaction and then subjecting them to the rigors of ISO standards.

At Waters, continuous efforts to improve customer satisfaction drive all our programs. Including the decision to achieve ISO 9000 certification. We knew if we designed the process with the objective of providing improvement to you, not just obtaining certification, then this considerable effort would be worthwhile.

Now, everytime you select Waters columns for your world wide quality control methods you are assured that the column performance will be the same wherever your method is being used. The same ruggedness you built into your method will be available to your colleagues throughout the company and throughout the world.

When you choose Sep-Pak® cartridges to perform your initial sample preparation you not only benefit from the superior performance of the products but also the knowledge that you will not be redeveloping your methods when the batch changes.

Clearly the way in which companies approach ISO registration will make the difference as to what benefits (if any) their customers will see. At Millipore we believe our customer approach is the only way to better serve you. We hope you agree.

If you would like to tour our ISO certified, cGMP facility in Taunton, Massachusetts, please check box number 7 on the reply card and return today. If you would like more information, please check box number 7 on the reply card and return today.

For more information on Waters semi preparative and preparative products, check box 8 on the reply card and return today. You will receive a six-page brochure including ordering information and a valuable limited-time free offer.
Automated Electrophoretic Separation and Blotting System for the Analysis of Proteins

William Warren and Steven Cohen, Ph.D.

Introduction
Polyacrylamide gel electrophoresis (PAGE) performed in the slab gel format is a well established and frequently used technique for the separation and analysis of proteins. Following separation, these gels are commonly transferred to membranes in a process referred to as blotting. Once the separated proteins have been blotted, further analysis can be performed. This generally takes the form of probing with specific antibodies, or subjecting individual proteins to amino acid sequencing and amino acid analysis.

The use of capillary electrophoresis (CE) has become an alternative high-resolution method for the separation and analysis of proteins. CE offers the advantages over traditional PAGE of automation and more accurate quantitation. In addition, CE can be executed with considerably less time and labor than PAGE. However, to date it has been difficult to link this high-resolution protein separation to the standard biochemical analysis techniques of immunostaining, protein sequencing, and amino acid analysis. This is due to the difficulty in collecting and preserving very small amounts of sample fractions following the separation.

In this article we describe a new technique that easily links the CE separation to some of the standard analysis techniques used for proteins. Utilizing routine CE conditions, proteins are separated and immediately blotted onto PVDF membranes without loss of sample or of separation resolution. The blotted proteins are then subjected to antibody probes, or alternatively, subjected to amino acid sequencing and amino acid analysis.

Materials and Methods

CE Separations with Membrane Fraction Collector

All capillary zone electrophoretic (CZE) separations were performed on a 75 µm x 60 cm fused silica capillary at a constant voltage of +7 kV using a Waters Quanta™ 4000 System with a positive polarity power supply (Waters Chromatography, Milford, MA). The electrolyte was 100 mM sodium phosphate, pH 7.0, containing 1.0 M trimethylammonium propane sulfonate (Waters AccuPure™ Z1-methyl reagent). Egg white lysozyme, horse cytochrome c, horse heart myoglobin, human transferrin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in electrolyte and passed through Milllex™-HV 0.45 µm filter (Millipore, Bedford, MA). All samples, except for protein sequencing experiments, were loaded hydrostatically onto the capillary with volumes ranging from 18 to 100 nL. Horse heart myoglobin used for sequencing was loaded into the capillary at 8 mg/mL using electromigration at +6 kV for 10 seconds. Four replicate injections were collected on a single membrane prior to analysis.

Figure 1. Membrane Fraction Collector

The eluate of the capillary is continuously applied on a PVDF membrane (Immobilon-P™, Millipore, Bedford, MA) using a rotating platform consisting of a stepper motor-driven platen capped with a polyethylene disk as shown in Figure 1. Details of the fraction collector assembly and operation have been previously described (Refs. 1-3). The disk rotated at a rate of 0.033 rpm for this series of experiments.

Reprinted with permission from LC-GC, January 1994, Volume 12, Number 1.
Protein Staining Techniques

Twenty second injections of the five-protein standard mixture were loaded onto the capillary, separated by CZE and collected on the rotating Immobilon-P membrane. Protein component concentrations ranged from 250 ng/mL to 250 µg/mL. Total protein component detection was obtained by soaking the membranes for 15 minutes at room temperature with AuroDye™ Forte colloidal gold solution (Amersham International, Amersham, UK).

Immunodetection of proteins was carried out as follows. The membranes were first blocked for 1 hour at room temperature with 10 mM potassium phosphate, pH 7.5 containing 150 mM sodium chloride, 1% bovine albumin, and 0.1% sodium azide. The membranes were then incubated for 1 hour at room temperature with a 1:2000 dilution of goat anti-human transferrin IgG (Sigma T-2027) in 10 mM potassium phosphate, pH 7.5 containing 150 mM sodium chloride, 1% bovine albumin, 0.05% Tween-20 and 0.1% sodium azide (i.e., wash buffer) were performed, followed by a 1 hour incubation with rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma, 1:8000 dilution in conjugate diluent). Excess conjugate was removed by three washes with buffer followed by a Milli-Q® water rinse. The chromogenic signal was generated by incubation in BCIP/NBT alkaline phosphatase substrate per the manufacturer’s directions (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

Chemiluminescent signals were generated using the protocol above with the following modifications. Membranes were washed for 10 minutes in 10 mM Tris, pH 9.5 containing 10 mM sodium chloride and 1 mM magnesium chloride. The membranes were then covered with Lumigen-PPD substrate (Millipore) and allowed to incubate at room temperature for 10 minutes. Excess Lumigen-PPD reagent was removed and the membranes were sealed in a clear plastic bag. In a darkroom, a piece of X-ray film (Kodak XAR) was sandwiched against the sealed membranes and the film was exposed for 60 minutes prior to development.

Amino Acid Composition Determinations

One hundred nanoliters of cytochrome c and myoglobin (5 mg dry weight/mL each) were injected onto the capillary, separated and collected onto Immobilon-P membranes. The air-dried membranes were wet with methanol then rinsed copiously with Milli-Q water. Following redrying of the membranes, the proteins were visualized with 20% methanol and the bands excised with a clean razor. The strips were placed in a 6 x 50 mm glass test tube and hydrolyzed with 6N HCl with 1% phenol using a Waters Pico•Tag® Workstation and a vapor phase procedure previously described (Ref. 4). Following hydrolysis, the amino acids were extracted with 100 µL of 4% aqueous SDS: acetonitrile (1:1, v/v) following a 50 µL wash with the same solvent, and the combined extracts were vacuum dried. As controls, 2087 ng of cytochrome c and 1708 ng of myoglobin were individually hydrolyzed.

Amino acid derivatization was performed as follows. Standard amino acid mixtures (10 µL, 0.1 mM) were buffered with 70 µL of 0.2 M sodium borate, pH 8.8 containing 5 mM disodium ethylenediamine tetraacetic acid and derivatized with 20 µL of 10 mM (dimethylaminonitril)N-hydroxysuccinimidyl carbamate in acetonitrile (AQC, AccQ•Fluor™ Reagent, Waters Chromatography, Milford, MA) according to a previously published procedure (Ref. 5). Protein hydrolysates were reconstituted with 20 µL of 0.1 M HCl, buffered with 60 µL of sodium borate and derivatized in a similar fashion.

Separation of amino acid derivatives was performed as follows. Samples were separated on a Waters AccQ•Tag™ reversed-phase column (3.9 mm x 150 mm) using a ternary eluent system and a multistep gradient. The HPLC system was a Waters AccQ•Tag Amino Acid Analysis System (Waters Chromatography) consisting of a Waters™ 625 LC System equipped with a column heater, a 717 Plus Autosampler with heater/chiller accessory, a 470 Scanning Fluorescence Detector, and a Millennium 2010 Chromatography Manager™.

Compositional data were calculated according to previous publications (Ref. 6) where % error = 100 x [experimental – true residue value] / true residue value. The overall compositional accuracy was assessed as the Average % error where Average % error = Σ absolute % error / 16.

Amino Acid Sequencing

The protein bands were excised with a razor blade and subjected to automated Edman degradation in a Millipore Model 66000 ProSequencer. No precycling of solvent or reagents was performed and degradations were done using the ADS100 (adsorptive) protocol with automatic on-line high-performance liquid chromatographic identification of the PTH amino acids measured at 269 nm, with dehydro derivatives of serine and threonine residues confirmed by detection at 313 nm.
Results and Discussion

Reproducibility of CZE Separations

Previous work shows that proteins interact with the walls of untreated capillaries and generate irreproducible migration times (Ref. 2). This phenomenon was also observed in the separation of five protein standards under regular CZE separation conditions (Figure 2, bottom). The broad tailing peaks suggest protein interaction with the negatively charged capillary wall. Furthermore, not only was cytochrome c (pI=9.3) poorly recovered from the capillary but the overall migration time reproducibility was less than satisfactory. Under these conditions, the migration time relative standard deviation (M.T. RSD) was found to be 4.0% for six consecutive runs. Protein peak-shape and resolution were significantly improved by the addition of 1.0M zwitterion (i.e., trimethylammonium-1-methylpropane sulfonate, AccuPure Z1-Methyl, Waters) to the phosphate-buffer electrolyte (Figure 2, top). Furthermore, the M.T. RSD was improved from 4.0% to 0.4%.

Effect of Membrane Fraction Collector on CE Separations

This experiment was performed to determine whether the installation and utilization of the CE membrane fraction collector has a deleterious effect on the CZE separation of the five-protein standard mixture. Figure 3 shows the electropherograms for separations performed with (top) and without (bottom) the collective device. As indicated, the relative protein peak shapes, migration times, and resolution were nearly identical. These data indicate that methods developed for traditional CZE can be transferred directly to a system equipped with the described membrane fraction collector device.
Samples containing the five-protein component mixture spanning three orders of magnitude in concentration were prepared and separated by CZE. The amounts of each protein component injected were 4.5 ng, 450 pg, 45 pg, and 4.5 pg. The electropherograms from on-capillary, 185 nm absorbance detection are shown in Figure 4. At the 4.5 ng and 450 pg levels, the five protein components are easily detected. However, at the 45 pg level, the peaks are barely discernible and no proteins are detected at the 4.5 pg amount.

Samples separated by CZE and collected on Immobilon-P membranes were subjected to total protein staining as well as antigen specific, anti-human transferrin immunodetection procedures. Figure 5 shows data from the 4.5 ng analysis, at which level total protein band staining with the colloidal gold reagent was clearly visible. Bands were faintly seen at the 450 pg level and not detected at or below the 45 pg level (data not shown).

Immunodetection was performed using antibodies specific to human transferrin, and as such, only the transferrin protein bands were detected on the membranes. Non-specific immunodetection was not seen at any protein concentration tested. Visualization of the antibody-bound transferrin with the chromogenic and chemiluminescent substrates was also clearly evident at the 4.5 ng level with minimum level of transferrin detection being 45 pg (data not shown). This level is comparable to that of detection at 185 nm.
Amino Acid Analysis of Proteins Recovered from PVDF Membranes

The CE/membrane fraction collector separation of 100 mL of horse cytochrome c and myoglobin is shown in Figure 6. These results demonstrate that successful component resolution can be obtained even with relatively large loading volumes.

The amino acid compositional analysis data from these samples are presented in Table 1. Protein recovery from the hydrolyzed and extracted membranes were 88% and 73%. The compositional accuracy for cytochrome c and myoglobin were 9.5% and 11.6% respectively. These values were only slightly higher than those obtained from the analysis of the cytochrome c and myoglobin controls (i.e., 58% and 7.4% respectively) and are significantly better than previous reports of hydrolysate analysis of membrane adsorbed samples with microgram or lower amounts (Refs. 7-9).

As expected, increased CE component resolution was observed using decreased sample load volumes. However, this compromises amino acid compositional accuracy (data not shown). Techniques such as capillary isoelectric focusing or collection of repetitive low-volume injections are currently being explored which may help to overcome this apparent tradeoff.

![Figure 6. Electropherogram of a Mixture of Cytochrome c and Myoglobin Using the CE Membrane Fraction Collector](image)

Table 1. Representative Amino Acid Analysis Results for Samples Purified by CE and Collected on PVDF Membranes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lit. Value</th>
<th>CE Sample</th>
<th>Average Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>8</td>
<td>9.6</td>
<td>9.5%</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>10</td>
<td>12.9</td>
<td>11.6%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>ng Injected on CE</th>
<th>ng Hydrolyzed</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>415</td>
<td>341</td>
<td>88%</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>365</td>
<td>249</td>
<td>73%</td>
</tr>
</tbody>
</table>
Amino Acid Sequencing of Proteins Recovered from PVDF Membranes

Protein bands from the four replicate injections were excised and subjected to sequence analysis. A representative sequence of the amino acid terminal fragment is shown in Figure 7. The repetitive yield for myoglobin was 97.8% with an initial yield of 1.9 pmol. Initial yield is defined as the amount of protein loaded onto the sequencer that gives a PTH signal in the first sequencing cycle.

Conclusions

Post-run analysis of samples separated by capillary electrophoresis has been problematic due to limited sample masses as well as to difficulties encountered collecting discrete fractions from the capillary. The data presented in this study provide evidence of the utility of a membrane fraction collector interface for performing post-run analysis of CZE-separated proteins. This interface, since it is based on the continuous transfer of molecules emerging from the end of the capillary onto the moving membrane surface, preserves the spatial resolution of the separation and permits CE to be successfully coupled with other analytical methods including immunodetection and amino acid compositional analysis.

Significant findings of these studies include:

1. Proteins collected with the membrane fraction collector following CZE can be visualized at the low picogram level using antigen-specific immunodetection procedures. This level of sensitivity is comparable to that obtained using non-selective, on-capillary detection at 185 nm.

2. Membrane-bound samples are readily amenable to reversed-phase HPLC amino acid analysis following vapor-phase hydrolysis and derivatization with AQC, as well as standard protocols for protein sequencing.

For more information on capillary electrophoresis, check box number 9 on the reply card and return today.

For ordering information, please see pages 29-30.

References

HPLC-Purification of Sticky Peptides Obtained from Membrane Proteins

Keith A. Lerro, Ron Orlando, Hongzhi Zhang, Peter N.R. Usherwood, and Koji Nakanishi

Department of Chemistry, Columbia University, New York, New York, USA, and Suntory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka, Japan, and Department of Life Science, University of Nottingham, Nottingham, United Kingdom

Introduction
The amphiphilic peptides obtained upon cleavage of membrane proteins, including numerous receptors, are recalcitrant to most separation techniques because of their limited solubility and tendency to aggregate and adsorb to surfaces. This article describes HPLC techniques that can be used to separate these “sticky” peptides on silica and aminopropyl-modified silica columns. Mobile phases consist of mixtures of chloroform/methanol/isopropylamine. The protocols developed have been applied to synthetic M1 and M2 peptides, which constitute part of the transmembrane domain of glutamate-gated ion-channel proteins. Four of these M1 and M2 peptides were separated from minor synthetic impurities, and a 23-mer was baseline separated from a 28-mer. The HPLC procedures have also led to purification of the 10 peptides resulting from cyanogen bromide cleavage of bacteriorhodopsin, peptides which have so far eluded HPLC separation despite numerous attempts.

Experimental
All separations were performed at room temperature. Solvent mixtures were degassed under an aspirator before use. The columns, µPorasil™ (silica) and µBondapak™ NH2 (aminopropyl-modified silica), were obtained from the Waters Division of Millipore Corporation. Peptides were dissolved in trifluoroethanol or 7:1:1 chloroform/methanol/isopropylamine (solvent A), sonicated briefly, and centrifuged at 14,000 x g in a microcentrifuge. Alternatively, samples were dissolved in 1:7:1 chloroform/methanol/isopropylamine (solvent B) and passed through a Waters Sep-Pak® Light NH2 cartridge, then dried on a rotary evaporator and suspended in solvent A for injection onto the HPLC columns. The analytical scale columns had been preequilibrated to solvent A for at least 10 minutes at 1 mL/min (semiprep columns: 4 mL/min). Then the peptides were injected and the columns washed for 10 minutes with the same solvent. For separations employing the µBondapak NH2 column, a linear gradient was developed over 45 minutes from 100% solvent A to 100% solvent B. Separations on the µPorasil silica column used a 30-minute linear gradient from 100% solvent A to 50% solvent B. After the gradients, the columns were washed for 10 minutes with the final solvent. Peptides were detected at 272 nm at a sensitivity of 0.5 absorbance unit full scale. Peaks were collected into glass round-bottom flasks or polypropylene tubes and dried on a rotary evaporator or a Speed-Vac concentrator (Savant). The baseline change was caused by the changing refractive index of the solvent, and varied with the batch of solvent and the optics of the UV detector.

Results
Synthetic peptides. The transmembrane domain of many ion-channel proteins consists of four peptides, M1/M2/M3/M4, which span the lipid bilayer in the α-helical conformation (1, 2). Table 1 shows the four synthetic M1 and M2 peptides of the quisqualate (Quis) and N-methylD-aspartate (NMDA) subtypes of glutamate receptors (3, 4) which were employed in the present studies. The M2 peptides could not be purified by conventional reversed-phase HPLC procedures owing to their low solubility, aggregation, etc. In Table 1 the hydrophobic amino acids of these peptides are underscored to illustrate the amphiphilic nature of the M2 helices, which surround the ion-channel pores with their hydrophilic faces directed inward (5). In contrast to the M2 peptides, the M1 is more hydrophobic because it is positioned away from the pore opening, further back into the lipid bilayer (5).

<table>
<thead>
<tr>
<th>Table 1.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1 peptide</strong></td>
</tr>
<tr>
<td>Quis</td>
</tr>
<tr>
<td><strong>M2 peptides</strong></td>
</tr>
<tr>
<td>Quis</td>
</tr>
<tr>
<td>NMDA-1</td>
</tr>
<tr>
<td>NMDA-2</td>
</tr>
</tbody>
</table>

1. Abstracted with permission from Analytical Biochemistry, Volume 215, Number 1, pages 38-44, November 1993.
Figure 1A shows the HPLC separation of crude synthetic quisqualate M2 peptide on the µBondapak NH₂ column. Figure 1B is the HPLC trace after two semi-prep-scale HPLC separations of the crude M2 peptide; it shows that the impurities appearing as shoulders on the main peak in Figure 1A were removed by the two successive HPLC steps. All four synthetic M1 and M2 peptides were purified on the semi-prep-scale aminopropyl column, the purity and identity being confirmed by electrospray MS and amino acid composition analysis. Approximately 5 mg of pure peptides were obtained upon combining the eluates of 3-4 HPLC runs, the recovery yield being over 95% as judged by UV spectrophotometry.

Figure 2A shows the HPLC separation of the two NMDA peptides on the µBondapak NH₂ column, whereas Figure 2B shows the separation on the µPorasil silica column. The NMDA-1 peptide is the five-residue amino-terminal extension of NMDA-2 (Table 1); the two peptides were baseline separated on both columns, although the aminopropyl column Figure 2A resulted in better removal of the minor synthetic impurities appearing as shoulders on the main peptide peaks. The aminopropyl column generally provided sharper peaks and better resolution; however, the silica column is useful for certain separations in which a different profile is obtained.

Peptides derived from BR, a membrane protein.

Figure 3 is a diagram of the BR secondary structure showing the seven transmembrane α-helices (A-G) and the Met residues where CNBr cleavage produces fragments 1-10. Separation of the CNBr fragments on the µBondapak NH₂ column gave the profile shown in Figure 4. Peaks are labeled according to the fragment number, which was determined by electrospray MS. The profile varied slightly in different trials, some overlapping peaks (i.e., fragments 7, 9, and 10) being somewhat better resolved in other experiments (Figure 4, insert).
Peak 2' (Thr24-Met32) is a previously characterized truncation of peptide 2, which loses its three N-terminal amino acids as a result of hydrolysis by the 70% formic acid used in the CNBr cleavage (Orlando et al., in preparation); the formic acid also led to formylation of some of the BR-peptides, which were obtained as mono- to hexaformate esters.

We demonstrated with mass spectrometry that the single HPLC run (Figure 4) resulted in high-purity BR-peptide 5. However, the large overlapping peaks in Figure 4 eluting at around 23 minutes and 32 minutes respectively, were shown by MS and electrophoresis to be mixtures of fragments 1/3 and 7/9/10, respectively. It was found that a second HPLC step, this time on µPorasil silica, was capable of separating the two overlapping peaks into their respective component peptides. Purity of the peptides was verified by SDS-polyacrylamide gel electrophoresis. Thus, usage of the two consecutive HPLC procedures described has led to a clear separation of the peptides resulting from CNBr cleavage of BR for the first time.

It is interesting to note that there is a linear relationship (correlation coefficient = 0.984) between the retention of the BR peptides on the µBondapak NH2 column and their chain length. The larger peptides are eluted later in the gradient. This linear relationship held for peptides of up to 27 amino acids.

The HPLC procedures described above are best suited for the separation of intermediate-sized polypeptides comprising 5-50 amino acids, although smaller peptides can also be separated provided they are sufficiently polar to be retained on the columns. The small hydrophobic peptides N-Cbz-Val-Phe-methyl ester and N-Cbz-Val-Gly-Gly-benzyl ester were eluted with the solvent front from both the silica and amino-propyl columns. Likewise the BR fragment 4, a tetrapeptide, was eluted with the solvent front (Figure 4); however, Phe and the dipeptides Gly-Tyr and Tyr-Arg were retained on both column types, being eluted as sharp peaks during the respective solvent gradients.

To continue to receive information on this type of work please check box number 10 on the reply card and return today. For ordering information, please see page 30.
Resolution in Gel Permeation Chromatography
Uwe D. Neue, Ph.D.

It is not uncommon to find differences among molecular weight distributions when the same sample is analyzed in different laboratories. Although this can be caused by many factors, an important but often overlooked origin of the different results may be the column bank used for the analysis. In this article, we will discuss the resolution of the column bank for the molecular weight range of the sample as the underlying reason for these differences.

If all or part of the molecular weight range of a polymer is excluded from the pores of GPC columns, no separation of the different molecular sizes can occur. In this case it is obvious that a correct molecular weight cannot be obtained and the analysis is invalid. This extreme example illustrates clearly the importance of resolution for the quality of the analysis: the accuracy of the analysis is a function of the resolution of the column bank for the sample (Ref. 1). Poor resolution results in low accuracy.

Resolution is determined by both the efficiency and the selectivity of the column bank for the sample. The relative influence of these parameters can easily be examined by inspecting the resolution equation.

Generally, resolution in chromatography is defined as:

\[ R_s = \frac{\Delta V}{w} \]

where \( \Delta V \) is the difference in elution volume between the two species of interest and \( w \) is the average peak width of the species. In GPC, usually another parameter is used, the specific resolution:

\[ R_s = \frac{\Delta V}{w \cdot (\log M_2 - \log M_1)} \]

where the resolution is normalized for a 10-fold difference in molecular weight.

\[ W = \frac{4 \cdot V_r}{\sqrt{N}} \]

\( V_r \) is the retention volume of a species, \( N \) is the column plate-count. The factor 4 implies that the peak-width is defined as the distance between the intersection points of the tangents to the peak with the baseline. It is important to understand that plate-count is not a constant for a particular column. It is a complicated function of the molecular diffusion coefficient of the sample molecule, the particle size, the quality of the packed bed, and the partition coefficient of the sample molecule into the pores (Ref. 2). Since it is usually not possible to measure the plate-count of the column bank for the polymer of interest, the plate-count of a small molecule is usually used as a substitute. It is however a very poor substitute, since there are orders of magnitude differences in the diffusion coefficients of polymers and small molecules.

As can be seen from the last equation, the peak width \( w \) improves only with the square root of the plate-count of a column, and little improvement in resolution results from optimizing the plate-count of a column bank by, for example, changing the flow rate.

For a bank of columns packed with the same packing material and having equal performance, the elution volume and the plate-count increase in proportion to the number of columns in the bank. On one hand, this means that the difference in the elution volume between two species grows with the number of columns. On the other hand, the peak width also grows, but with the square root of the number of columns. Therefore, the gain in resolution with each column added to the bank is only proportional to the square root of the number of columns. This is shown in Figure 1.

Figure 1. Resolution as a Function of the Number of Columns in a Bank

Resolution increases with the square root of the number of columns in the bank. This relationship holds only for columns of equal plate count and volume.

Resolution as a Function of the Efficiency of a Column Bank

Plate-count or efficiency of a column is described as follows:

\[ W = \frac{4 \cdot V_r}{\sqrt{N}} \]

Plate count or efficiency of a column is an important parameter in chromatography.
At the same time, the analysis time increases with the number of columns added to the bank. The analyst therefore has to trade off the quality of information obtained by the GPC analysis against the time needed to perform the analysis.

It should be stressed that this relationship holds only for columns that are packed with the same particles and are of equal performance. It does not hold for banks of columns of different pore sizes, as is common practice in GPC. Columns that do not show good selectivity in the molecular weight range of interest will still contribute to the plate-count, but not to the separation. As a matter of fact, a column in the bank that does not contribute to the selectivity effectively destroys the separation obtained in the remainder of the bank. Consequently, the plate-count of a bank is a very poor measure of the quality of the separation. Therefore the optimization of selectivity, which is outlined in the next paragraph, is of higher importance.

Resolution as a Function of Column Selectivity

The selectivity of GPC is defined as the ratio of the difference in elution volume to the difference in the logarithm of the molecular weight:

\[ S = \frac{\Delta V}{\log M_2 - \log M_1} \]

column bank. The flatter the calibration curve, the larger is the difference in the elution volume between two species, and the selectivity increases. At constant dispersion, the accuracy of the GPC analysis is directly proportional to the selectivity.

In Figure 2, the calibration curves of a typical column with a Single pore size is compared to the calibration curve of a column prepared from a mixture of particles with different pore sizes.

As can be seen, the calibration curve for the column prepared from the single pore-size material is flatter than the calibration curve of the mixed-bed. The consequence of this is that the selectivity, and, therefore the resolution obtained with a carefully chosen single pore-size column, is superior to the resolution obtained with a mixed-bed. This is shown in Figure 3:

At the maximum, the single pore-size column has over two times the resolution than the mixed-bed, while the mixed-bed covers a broader range of molecular weight. The example column shown in Figure 3 would be ideal for the molecular weight determination of a polymer with an average molecular weight of 10,000 Dalton. It still shows superior resolution to the mixed-bed column in the range from less than 1,000 Dalton to above 100,000 Dalton. On the other hand, a different column or column bank should be chosen if the average molecular weight of the sample were 1,000 or 100,000 Dalton. The availability of columns with individual pore sizes allows the analyst to optimize resolution for the analytical problem at hand.
Figure 4 demonstrates this with actual data obtained on a single pore-size column and a mixed-bed column. The selectivity (difference in elution volume in mL per MW decade) is plotted against molecular weight. Both columns are packed with the same particle size (10 µm) and have consequently about the same efficiency. The Waters Styragel® HT 4 column has a better selectivity and therefore a superior resolution for a molecular weight range from about two thousand to about two million. At the maximum, its resolution is 2.5 times higher than the mixed-bed column. This results in a higher accuracy of the analysis for this molecular weight range. If accuracy of the analysis is important, it is always better to tailor the column bank to the sample to be analyzed than using mixed-bed columns or column banks that contain columns that do not contribute to selectivity. However, mixed-bed columns have their place for screening purposes, or if samples with very different MW distributions need to be analyzed. But it is important to remember that under these circumstances the accuracy of the analysis is compromised.

Polymer Characterization Made Economical and Environmentally Friendly

Waters is pleased to announce the introduction of our new narrow-bore GPC columns in January 1994. Waters solvent efficient Styragel® columns in a 4.6 mm x 300 mm format are designed to reduce your solvent consumption and solvent delivery costs by two thirds, while still delivering the high-performance characteristics of our 7.8 mm x 300 mm Waters Styragel columns.

The new Waters solvent efficient Styragel column series consists of:

- 5 µm HR (High Resolution) columns for low molecular weight applications, where high resolution is critical. These columns offer the highest resolution in the market today.
- 10 µm HT (High Temperature) columns for mid to high molecular weight applications, where durability and high temperature capabilities are often required. The narrow particle size distribution results in a more stable packed-bed structure making these columns very durable and long lasting.
- 20 µm HMW (High Molecular Weight) columns for high to ultrahigh molecular weight applications, where polymer shearing is a concern. These columns use a specifically designed 10 µm frit which minimizes shear effects.

For your convenience, Waters solvent efficient Styragel columns are available in toluene, THF, and DMF.

For more information on Waters solvent efficient Styragel columns, check box number 11 on the reply card and return today.
To make the selection of the column bank for an analysis possible, it is important to know the selectivity of the columns. Figure 5 shows the selectivity for Waters Styragel HR single pore-size columns. The Styragel HR 0.5 column is ideal for the separation of low molecular weight additives and oligomers. Its selectivity drops off sharply above MW 1000. The columns Styragel HR 1 and HR 2 show increasingly higher selectivity in the molecular weight range of 1000 to 10000. The Styragel HR 3 column exhibits good selectivity from a molecular weight of 100 to above 50000. The selectivity of the Styragel HR 4 column is identical to the selectivity of the Styragel HT 4 column, which has already been discussed above.

Figure 6 shows the GPC selectivities for the Waters Styragel HT series of individual pore-size columns. The selectivities for the Styragel HT 3 and HT 4 columns are identical to their HR equivalents. The Styragel HT 5 column extends into the molecular weight range of a few millions, while the Styragel HT 6 column excels above 1 million MW.

**Conclusion**

To maximize the accuracy of the GPC analysis, it is desirable to optimize the column bank for the polymer to be analyzed. Single pore-size columns give superior selectivity to mixed-bed columns and are ideal for tailoring the column bank to the needs of the analysis.

**References**


For more information on our new series of Styragel columns, check box 12 on the reply card and return today.

For ordering information, please see page 28.
Waters™ High-Performance Carbohydrate Column is an applications-specific column for fast analysis of monosaccharides and disaccharides. Simple sugars (the mono- and disaccharides) are required on food labels by the new Food and Drug Administration mandate. If a food contains more than 1% simple sugars, the content must be included on the food label.

The chemistry of choice for meeting this analysis is a propylamine bonded phase, such as the Waters High-Performance Carbohydrate Column. The introduction of the propylamine chemistry on a 4 µm spherical particle provides the ability to analyze the required sugars in 10 to 12 minutes. The Waters High-Performance Carbohydrate Column is tested in Waters cGMP Quality Control laboratory to ensure the separation of fructose from glucose and maltose from lactose in less than 12 minutes (chromatography on the right).

Currently HPLC with a propylamine column is an official AOAC (Association of Official Analytical Chemists) method for only a few matrices: presweetened cereals (shown in Figure 2), milk chocolate, licorice, and honey. These methods require ≥ 2 mL/min flow rates and ≥ 80% acetonitrile. The short analysis time at low flow rates and lower acetonitrile concentration required for the Waters High-Performance Carbohydrate Column drives these methods to be expanded across a broader matrix range. A major advantage of the AOAC methods developed on the new Waters column is solvent reduction; lower flow rates and acetonitrile concentrations as well as shorter analysis times will result in the use of less solvent.

For more information on the Waters High-Performance Carbohydrate Column, check box number 13 on the reply card and return today.

For ordering information, please see page 30.
After more than 20 years, silica remains the foundation of reversed-phase chromatography. Why does silica continue to play this central role? Silica as an inorganic material has excellent properties for chromatography; high pore volumes give good mass transfer and larger surface areas per column volume. These parameters translate into high efficiency, fast separations, and good dynamic range in terms of sample load. Silica’s basic material chemistry guarantees its continued importance for HPLC.

Since silica has had such a long tenure as a chromatographic material, a common view (not just of silica but of octadecylsilane-functionalized silicas) is that this is mature technology. In fact, today’s silicas are as different from their predecessors as the computer processors of today are from the transistors of the 1960s.

Waters has been active in this advancement of silica technology since the introduction of µBondapak™ in 1973. Ten years later we began to manufacture a spherical high-performance silica, Nova-Pak®, providing not only high-performance analytical materials but also state-of-the-art 6 micron preparative products. Five years ago, Waters purchased a solid-state NMR to perform 29Si analyses of silica and bonded phases. Of course this capability is augmented by FTIR and a host of more conventional analyses. These investments produced highly ordered trifunctional bonded phases — for example our tC₁₈ products.

Our ongoing investment in silica technology and processes will result in the introduction of both a new silica particle and new bonded phase in 1994. This new silica was produced after years of investigation of two issues: the relationship of pore size and pore volume to mass transfer (column efficiency) and the relationship of surface heterogeneity to chromatographic performance. The new bonded phase was developed after investigations of the silanol/bonded phase interplay in the chromatography of polar compounds.

How will these new products benefit chromatographers? Improved mass transfer will produce separations that will be more tolerant of flow rate (flatter Van Deemter profiles) and enable the use of shorter columns. Faster analyses will result. The focus on surface homogeneity will produce better peak shapes and a reduced dependence on buffers. And, the extremely high tolerances built into the manufacturing process and specifications for these products will give chromatographers a level of confidence in the long-term integrity of their HPLC methods that has never before been possible. The new bonded phase will provide another tool in the selectivity arsenal of chromatographic columns while raising silica-based reversed phase to a new performance level.

Of course these new products, like our existing ones, will be produced in our cGMP, ISO 9002 certified manufacturing facility in Taunton, Massachusetts. And it is Waters’ focus on the need for still more reproducible chromatographic materials that sets the stage for our future research. We’ve made tremendous progress since the introduction of our first column based on 100 grams of µBondapak C₁₈ 20 years ago. Today, we manufacture tens of kilograms per batch representing thousands of columns. Although our columns are the most reproducible on the market, this is not our endpoint. Our goal is still the production of columns that give the same retention, selectivity, and efficiency on their first and last injections—columns that are indistinguishable from each other, not just on some simple test mixture but for all types of chromatography. I look forward to reporting our progress over the next few years.

Dr. John S. Petersen
Director of Research and Development
1994 WATERS TRAINING PROGRAMS — INCLUDING 2-DAY DEVELOPING HPLC SEPARATIONS SHORT COURSES

DEVELOPING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS

January 11-14
February 15-18
March 15-18
April 12-15
May 10-13
June 7-10
July 12-15
August 9-12
September 13-16
October 11-14
November 8-11

MILLENNIUM 2010 CHROMATOGRAPHY MANAGER

January 18-21
March 22-25
May 17-20
July 19-22
September 20-23
November 15-18

LC TROUBLESHOOTING AND MAINTENANCE

January 25-28
March 22-25
May 24-27
July 26-29
September 27-30
November 15-18

MILLENNIUM 996 PDA

January 25-28
May 24-27
September 27-30
November 29-December 2

THEORY AND PRACTICE—GEL PERMEATION CHROMATOGRAPHY

February 8-11
May 3-6
August 16-19
November 1-4

150C OPERATIONS AND THEORY

April 4-8
August 1-5
December 5-9

MILLENNIUM 2010 GEL PERMEATION CHROMATOGRAPHY SOFTWARE

April 26-29
August 23-26
October 18-21

ExpertEase SOFTWARE CONCEPTS

February 1-4
October 4-7

CAPILLARY ION ANALYSIS

April 20-21
October 19-20

DEVELOPING HPLC SEPARATIONS SHORT COURSES

Houston, TX
March 7-8
Research Triangle Park, NC
March 10-11
Chicago, IL
June 14-15
Saddle Brook, NJ
June 21-22
Chicago, IL
October 25-26

For more information on these and many other regional courses, please call the Customer Education Coordinator at 508-624-8502 or check box 14 on the reply card and return today.
## Ordering Information

### Waters Styragel HR, HT, and HMW Columns (4.6 mm x 300 mm)

<table>
<thead>
<tr>
<th>Column</th>
<th>Effective Molecular Weight Range</th>
<th>Part No.</th>
<th>Part No.</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styragel HR 0.5</td>
<td>0-1,000</td>
<td>WAT045830</td>
<td>WAT045835</td>
<td>WAT045840</td>
<td>$900</td>
</tr>
<tr>
<td>Styragel HR 1</td>
<td>100-5,000</td>
<td>WAT045845</td>
<td>WAT045850</td>
<td>WAT045855</td>
<td>900</td>
</tr>
<tr>
<td>Styragel HR 2</td>
<td>500-20,000</td>
<td>WAT045860</td>
<td>WAT045865</td>
<td>WAT045870</td>
<td>900</td>
</tr>
<tr>
<td>Styragel HR 3</td>
<td>500-30,000</td>
<td>WAT045875</td>
<td>WAT045880</td>
<td>WAT045885</td>
<td>900</td>
</tr>
<tr>
<td>Styragel HR 4</td>
<td>5,000-600,000</td>
<td>WAT045890</td>
<td>WAT045895</td>
<td>WAT045900</td>
<td>900</td>
</tr>
<tr>
<td>Styragel HR 4E</td>
<td>50-100,000</td>
<td>WAT045800</td>
<td>WAT045805</td>
<td>WAT045810</td>
<td>950</td>
</tr>
<tr>
<td>Styragel HR 5E</td>
<td>2,000-4 x 10^6</td>
<td>WAT045815</td>
<td>WAT045820</td>
<td>WAT045825</td>
<td>950</td>
</tr>
<tr>
<td>Styragel HT 3</td>
<td>500-30,000</td>
<td>WAT045915</td>
<td>WAT045920</td>
<td>WAT045925</td>
<td>790</td>
</tr>
<tr>
<td>Styragel HT 4</td>
<td>5,000-600,000</td>
<td>WAT045930</td>
<td>WAT045935</td>
<td>WAT045940</td>
<td>790</td>
</tr>
<tr>
<td>Styragel HT 5</td>
<td>50,000-4 x 10^6</td>
<td>WAT045945</td>
<td>WAT045950</td>
<td>WAT045955</td>
<td>790</td>
</tr>
<tr>
<td>Styragel HT 6</td>
<td>200,000-1 x 10^7</td>
<td>WAT045960</td>
<td>WAT045965</td>
<td>WAT045970</td>
<td>790</td>
</tr>
<tr>
<td>Styragel HT 6E</td>
<td>5,000-1 x 10^7</td>
<td>WAT045975</td>
<td>WAT045980</td>
<td>WAT045985</td>
<td>840</td>
</tr>
<tr>
<td>Styragel HMW 7</td>
<td>500,000-1 x 10^8</td>
<td>WAT046800</td>
<td>WAT046805</td>
<td>WAT046810</td>
<td>730</td>
</tr>
<tr>
<td>Styragel HMW 6E</td>
<td>5,000-1 x 10^7</td>
<td>WAT046815</td>
<td>WAT046820</td>
<td>WAT046825</td>
<td>790</td>
</tr>
</tbody>
</table>

### Waters Sentry Guard Holders and Columns (20 mm length)

<table>
<thead>
<tr>
<th>Description</th>
<th>Quantity</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrated Guard Holder</td>
<td></td>
<td>WAT046905</td>
<td>$75</td>
</tr>
<tr>
<td>Universal Guard Holder</td>
<td></td>
<td>WAT046910</td>
<td>95</td>
</tr>
<tr>
<td>NovaPak C_{18}</td>
<td>2/pkg</td>
<td>WAT044380</td>
<td>99</td>
</tr>
<tr>
<td>NovaPak C_{8}</td>
<td>2/pkg</td>
<td>WAT046830</td>
<td>99</td>
</tr>
<tr>
<td>NovaPak Phenyl</td>
<td>2/pkg</td>
<td>WAT046835</td>
<td>99</td>
</tr>
<tr>
<td>NovaPak CN HP</td>
<td>2/pkg</td>
<td>WAT046840</td>
<td>99</td>
</tr>
<tr>
<td>NovaPak Silica</td>
<td>2/pkg</td>
<td>WAT046845</td>
<td>99</td>
</tr>
<tr>
<td>µBondapak C_{18}</td>
<td>2/pkg</td>
<td>WAT044480</td>
<td>99</td>
</tr>
<tr>
<td>µBondapak CN</td>
<td>2/pkg</td>
<td>WAT046855</td>
<td>99</td>
</tr>
<tr>
<td>µBondapak NH_{2}</td>
<td>2/pkg</td>
<td>WAT046865</td>
<td>99</td>
</tr>
<tr>
<td>µBondapak Phenyl</td>
<td>2/pkg</td>
<td>WAT046850</td>
<td>99</td>
</tr>
</tbody>
</table>
### Waters Sentry Guard Holders and Columns (20 mm length) continued

<table>
<thead>
<tr>
<th>Description</th>
<th>Quantity</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>µPorasil</td>
<td>2/pkg</td>
<td>WAT046860</td>
<td>$99</td>
</tr>
<tr>
<td>Puresil C₁₈</td>
<td>2/pkg</td>
<td>WAT046870</td>
<td>105</td>
</tr>
<tr>
<td>Delta-Pak C₁₈ 100Å</td>
<td>2/pkg</td>
<td>WAT046880</td>
<td>105</td>
</tr>
<tr>
<td>Delta-Pak C₁₈ 300Å</td>
<td>2/pkg</td>
<td>WAT046890</td>
<td>105</td>
</tr>
<tr>
<td>Delta-Pak C₄ 100Å</td>
<td>2/pkg</td>
<td>WAT046915</td>
<td>105</td>
</tr>
<tr>
<td>Delta-Pak C₄ 300Å</td>
<td>2/pkg</td>
<td>WAT046920</td>
<td>105</td>
</tr>
<tr>
<td>Resolve C₁₈</td>
<td>2/pkg</td>
<td>WAT046925</td>
<td>105</td>
</tr>
<tr>
<td>Resolve C₈</td>
<td>2/pkg</td>
<td>WAT046925</td>
<td>105</td>
</tr>
<tr>
<td>Resolve CN</td>
<td>2/pkg</td>
<td>WAT046925</td>
<td>105</td>
</tr>
</tbody>
</table>

### Analytical Kits

<table>
<thead>
<tr>
<th>Description</th>
<th>Dimensions</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waters Reversed-Phase Scouting Kit</td>
<td>3.9 mm x 50 mm</td>
<td>WAT044360</td>
<td>$360</td>
</tr>
<tr>
<td>(includes 1 each Nova-Pak C₁₈, C₈, and Phenyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steel cartridge column)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waters Method Development Scouting Kit</td>
<td>3.9 mm x 50 mm</td>
<td>WAT044255</td>
<td>360</td>
</tr>
<tr>
<td>(includes 1 each Nova-Pak C₈, Phenyl, and CN HP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steel cartridge column)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waters Method Validation Kit</td>
<td>3.9 mm x 150 mm</td>
<td>WAT052770</td>
<td>895</td>
</tr>
<tr>
<td>(includes 3 Nova-Pak C₁₈ steel columns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>representing 2 different bulk bonded silica</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>batches)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### AccuSep CE/CIA Capillary Assemblies (2/pkg)

<table>
<thead>
<tr>
<th>Capillary Assembly</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µm I.D. x 35 cm length</td>
<td>WAT250-01</td>
<td>$125.00</td>
</tr>
<tr>
<td>50 µm I.D. x 60 cm length</td>
<td>WAT250-02</td>
<td>125.00</td>
</tr>
<tr>
<td>50 µm I.D. x 100 cm length</td>
<td>WAT250-03</td>
<td>125.00</td>
</tr>
<tr>
<td>75 µm I.D. x 35 cm length</td>
<td>WAT250-04</td>
<td>125.00</td>
</tr>
<tr>
<td>75 µm I.D. x 60 cm length</td>
<td>WAT250-05</td>
<td>125.00</td>
</tr>
<tr>
<td>75 µm I.D. x 100 cm length</td>
<td>WAT250-06</td>
<td>125.00</td>
</tr>
<tr>
<td>100 µm I.D. x 60 cm length</td>
<td>WAT250-08</td>
<td>125.00</td>
</tr>
<tr>
<td>100 µm I.D. x 100 cm length</td>
<td>WAT250-09</td>
<td>125.00</td>
</tr>
</tbody>
</table>
### AccuPure Z1-Methyl Reagent

<table>
<thead>
<tr>
<th>Description</th>
<th>Quantity</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccuPure Z1-Methyl Reagent</td>
<td>50 g</td>
<td>WAT037825</td>
<td>$350.00</td>
</tr>
<tr>
<td></td>
<td>250 g</td>
<td>WAT054825</td>
<td>$900.00</td>
</tr>
</tbody>
</table>

### AccQ•Tag Amino Acid Analysis of Protein Hydrolysates

#### Column and Accessories

<table>
<thead>
<tr>
<th>Description</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccQ•Tag Chemistry Package</td>
<td>WAT052875</td>
<td>$1,450.00</td>
</tr>
</tbody>
</table>

for up to 250 analyses.

Includes:

- AccQ•Fluor™ Reagent 1  
  5 x 6 mL vials
- AccQ•Fluor Reagent 2A  
  5 x 3 mg vials
- AccQ•Fluor Reagent 2B  
  5 x 4 mL vials
- AccQ•Tag column  
  3.9 mm x 150 mm
- AccQ•Tag Eluent A, concentrate  
  2 x 1 liter
- Sample tubes  
  4 x 72/pk
- Amino Acid standard, hydrolysate  
  10 x 1 mL ampoules
- AccQ•Tag User Guide

**AccQ•Fluor Reagent Kit***

<table>
<thead>
<tr>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAT052880</td>
<td>$315.00</td>
</tr>
</tbody>
</table>

Includes:

- AccQ•Fluor Reagent 1  
  5 x 6 mL vials
- AccQ•Fluor Reagent 2A  
  5 x 3 mg vials
- AccQ•Fluor Reagent 2B  
  5 x 4 mL vials

*The components of this kit are not available separately.

#### AccQ•Tag Components

<table>
<thead>
<tr>
<th>Description</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccQ•Tag column</td>
<td>WAT052885</td>
<td>$460.00</td>
</tr>
<tr>
<td>AccQ•Tag Eluent A, concentrate</td>
<td>WAT052890</td>
<td>$450.00</td>
</tr>
<tr>
<td>AccQ•Tag Eluent B **</td>
<td>WAT052895</td>
<td>$52.00</td>
</tr>
</tbody>
</table>

**For use with multi-pump gradient systems.

### Waters High-Performance Carbohydrate Column*

<table>
<thead>
<tr>
<th>Description</th>
<th>Dimensions</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-Performance Carbohydrate Column</td>
<td>4.6 mm x 250 mm</td>
<td>WAT044355</td>
<td>$450</td>
</tr>
<tr>
<td>Carbohydrate Guard-Pak™ inserts 10/pk</td>
<td></td>
<td>WAT044356</td>
<td>165</td>
</tr>
</tbody>
</table>

*Requires reusable endfittings,

**Requires Guard Pak Holder.

### Analytical Columns

<table>
<thead>
<tr>
<th>Description</th>
<th>Dimensions</th>
<th>Part No.</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>µBondapak NH₂</td>
<td>3.9 mm x 300 mm</td>
<td>WAT084040</td>
<td>$365</td>
</tr>
<tr>
<td>µPorasil</td>
<td>3.9 mm x 150 mm</td>
<td>WAT086692</td>
<td>$260</td>
</tr>
</tbody>
</table>