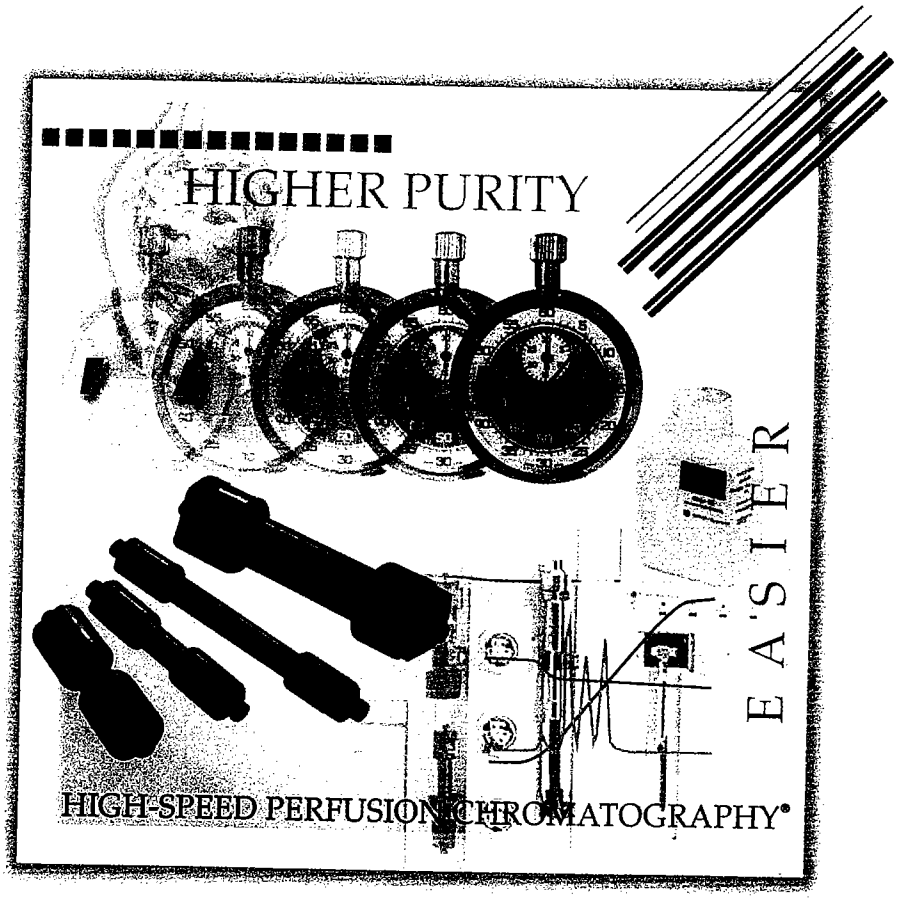
$29.95

THE BUSY RESEARCHER'S GUIDE TO
BIOMOLECULE CHROMATOGRAPHY



Applied Biosystems

THE BUSY RESEARCHER'S GUIDE TO
BIOMOLECULE CHROMATOGRAPHY

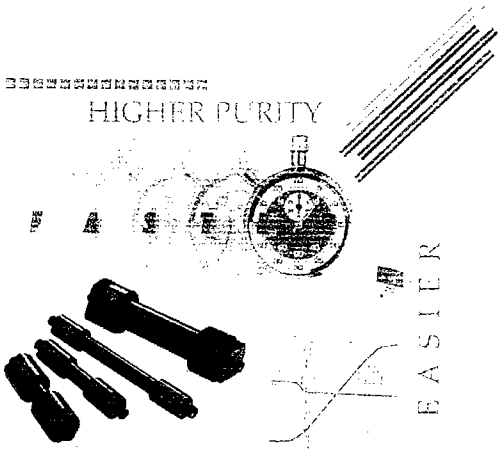


TABLE OF CONTENTS

How to Use this Book

This handbook is a practical guide to developing methods for the chromatography of biomolecules using a new technique known as Perfusion Chromatography® technology. It is intended to be useful to both beginners and experienced chromatographers. It is organized into 5 major sections with a Glossary and a Reference List:

Section 1 — *Introduction* describes Perfusion Chromatography technology and how it compares with conventional chromatographic methods you may be currently using.

Section 2 — *Principles of Systematic Method Development* outlines a systematic approach to chromatography method development that is enabled by Perfusion Chromatography technology. The section includes how to define the separation problem, set up a program of experiments, evaluate the results, implement the method and troubleshoot any problems that arise. This section should be reviewed even by experienced chromatographers, since many of the ideas discussed may be different from conventional practice.

Section 3 — *Developing Your Application* covers the source materials, molecular characteristics and suggested chromatographic approaches for each major class of biomolecules (proteins, antibodies, peptides and nucleic acids).

Section 4 — *Modes of Chromatography* details the basic mechanism of each mode (ion exchange, hydrophobic interaction, reversed-phase, affinity and gel filtration) and gives a starting point method protocol, a set of minimal method development experiments, and a comprehensive list of key variables for a full method development in each mode.

Section 5 — *Basics of Chromatography* provides a concise background on the various elements of chromatographic technology for those who are getting involved in it for the first time.

Throughout, words appearing in *italics* are defined in the glossary.

The reader who requires further information on the chromatography products referred to in the book should consult the *POROS® Columns and Media Selection Guide* also available from PerSeptive Biosystems.

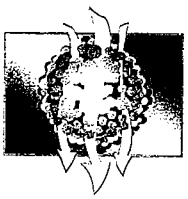
For those already familiar with chromatography, once the basic principles of Perfusion Chromatography and Systematic Method Development are understood, the handbook can be used as a practical reference for a particular application. If you are converting an existing protocol for use with POROS media, refer to the beginning of Section 3 — *Developing Your Application.*

If you are developing a new method, Section 3 — *Developing Your Application* should be used as a starting point, based on the class of biomolecule you are separating and your general approach to method development. This section should give you a good general idea of which chromatographic modes will be most useful for your application, and some specific suggestions for your kind of molecule. Section 4 — *Modes of Chromatography* will then provide the detailed method development protocols for each mode.

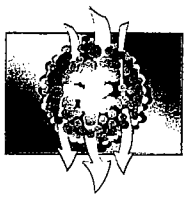
If you are new to using chromatography, after reading Section 1 — *Introduction* you should begin with Section 5 — *Basics of Chromatography,* followed by the introductory parts for each mode in Section 4 — *Modes of Chromatography.* You should then be well prepared to study Section 2 — *Principles of Systematic Method Development.* The text also has common *RULES OF THUMB* identified throughout, which should prove useful for the beginner to learn.

Users of any type of chromatographic instrumentation can benefit from systematic method development and Perfusion Chromatography media, and the experimental protocols and approaches described in this handbook have been carefully designed for use on any system. However, some specific tips are provided in Section 4 — *Modes of Chromatography* for users of the PerSeptive Biosystems BioCAD™ Workstation and BioCAD/*SPRINT*™ Systems (which were designed as workstations for systematic development).

It is our hope that, by using the techniques and products covered in this practical guide, you will discover a new power and ease of use in one of the fundamental tools of life science research. Perhaps you may even find that chromatography can be fun!



SECTION 1
INTRODUCTION



SECTION 1
INTRODUCTION

CHROMATOGRAPHY IN LIFE SCIENCE RESEARCH

Liquid chromatography is a vitally important technology in life science research. The technique provides a unique combination of capabilities. It provides the separation power needed to purify even subtle molecular variants from complex mixtures, combined with gentle chemical and physical conditions which enable recovery of biological activity for complex, biological macromolecules. Chromatography is highly scaleable and can be used for applications ranging from analysis of nanogram quantities in tiny samples on columns with sub-millimeter diameters to multi-kilogram scale production of bulk drugs in columns a meter or more in diameter.

However, for the vast majority of researchers, chromatography itself does *not* represent the focal point of their work. Instead it represents a means to an end, an important *tool* to help them isolate or identify a particular molecule of interest along the way toward answering some larger research questions (form/function etc.). Although the product of a chromatographic separation may be the most important starting point for a research project (e.g. a pure protein for X-ray crystallography studies), actually performing the chromatography itself is often viewed as the most tedious part of the project and can be frought with frustration.

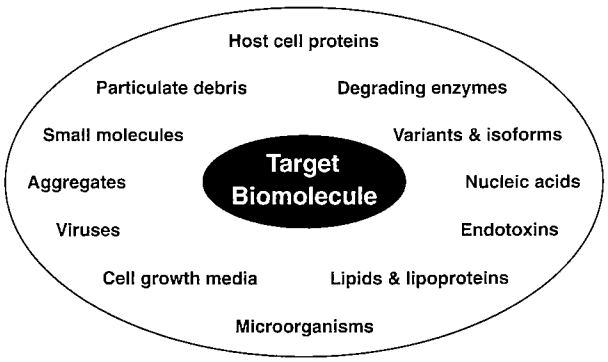


Figure 1-1. Biological systems present an unusually complex sample matrix and a difficult separations challenge.

One of the most challenging aspects for many researchers is actually developing a chromatographic separation protocol or method. The enormous flexibility of the technique, one of its most powerful features, as well as the complexity of both the target biomolecules and the biological sample matrix make it hard for the casual user to identify, much less optimize all the parameters that affect a separation. To develop a method ideally calls for a systematic experimental approach where each of the variables that could impact the separation is comprehensively tested in turn.

Unfortunately, the speed limitations of conventional chromatography media usually make it impractical to take such a systematic approach. Individual chromatographic run times on conventional media typically range from half an hour to a day or more and are the result of inherent limitations of the media (see opposite).

In an attempt to circumvent time-consuming development, researchers often rely on past experience, tips from colleagues, or previously published papers. Unfortunately, what worked in the past for one application is often not appropriate for a new research problem, leaving the researcher with a less-than-satisfactory separation and few ideas for what to do

next. When faced with a totally new separation problem, there seems to be no alternative to a lengthy, "trial and error" development process.

This handbook describes a novel systematic approach which enables effective chromatographic separations to be developed simply and quickly, allowing the researcher to spend more productive time toward their true research goals by spending less time on chromatography or by being more efficient in its use. This whole approach is made possible by the introduction of Perfusion Chromatography technology — a fundamental advance in the design and manufacture of chromatographic media developed by PerSeptive Biosystems to overcome the limitations of conventional chromatography media.

THE LIMITS OF CONVENTIONAL CHROMATOGRAPHY

It is commonly understood that with most currently available chromatography materials, one must make tradeoffs between speed, resolution and capacity. This relationship is often depicted as a triangle with each apex labelled with one of these parameters. The practical ramification is that there is an interdependence among these parameters, and that an increase in resolution or capacity must be at the expense of speed, or an increase in speed must be at the expense of resolution or capacity.

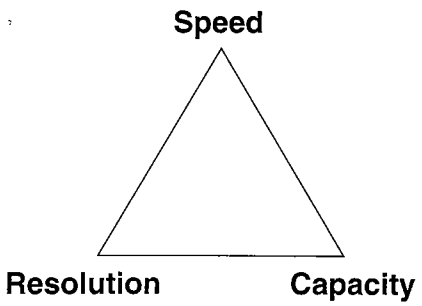


Figure 1-2. The tradeoff with conventional media between speed, resolution and capacity is depicted by many media manufacturers as a triangle.

The reason for this tradeoff lies in the nature of the particles that make up conventional chromatography media. In order for a chromatographic separation to occur, solute molecules must interact with the surface of the media particles. A typical chromatography particle is highly porous (with pores in the 100-1000 Å range) in order to maximize the internal surface area for binding. Solute molecules are carried to the perimeter of the particles by the liquid stream as it flows through the packed bed of the column. Transport of the solute molecules to the *inside surfaces of the particles occurs by diffusion within the pores (intraparticle diffusion).*

Diffusion is a slow process, especially for large macromolecules, and becomes the limiting factor in a conventional chromatography separation. As flow rate across the column increases, there is less time available for this intraparticle diffusion to occur, therefore less ability for solute molecules to interact with the surface area inside the particles. Band-spreading increases, and resolution and capacity are lost if the flow rate is too high. For first-generation biological chromatography packings (so-called soft gels) with 90-200 µm particle diameters, the typical time for a single separation run is on the order of hours or even days.

The introduction of modern *high performance liquid chromatography (HPLC)* materials brought a 10-fold advance in speed over the first-generation packings. HPLC enables higher speed separations by a reduction in the particle diameter to the range 3-30 µm. This reduces the distance for solute diffusion within the particles, and thus allows operation at higher flow rates. However, the relative speed improvement with HPLC is not the result of any fundamental advance in the pore structure of the particles themselves. Diffusion is still limiting in conventional HPLC columns. A typical "lab scale" size HPLC column usually cannot be run much faster than 1 ml/min (30 minute to 1 hour total run time) without an unacceptable loss of resolution and capacity.

PERFUSION CHROMATOGRAPHY — A NEW APPROACH

Perfusion Chromatography technology was introduced by PerSeptive Biosystems in 1989 and represents a fundamental advance in the design of chromatography particles. POROS Perfusion Chromatography media are designed to radically speed access to the interior of the chromatography particles by overcoming the diffusional mass transfer limitations of conventional chromatographic media.

Unlike conventional chromatography particles, POROS particles have two distinct types of pores — large *throughpores* that transect the particle and short *diffusive pores* that branch off from the throughpores, providing a large internal surface area for solute/particle interactions to occur. Flow through the packed column produces a pressure differential across each particle that induces flow through the throughpores. Sample molecules are carried by this throughpore flow into the interior of the particle and into contact with the network of diffusive pores. Since the length of the diffusive pores is small in comparison to the total particle diameter (typically less than 1 μm), the time required for sample molecules to diffuse to and from internal binding sites is very short.

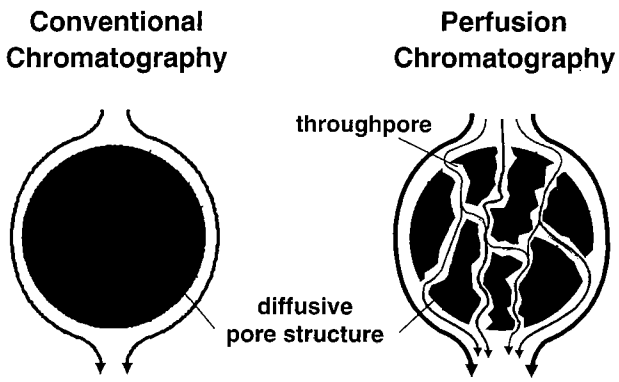


Figure 1-3. Pore structure and mass transport in conventional and perfusive particles

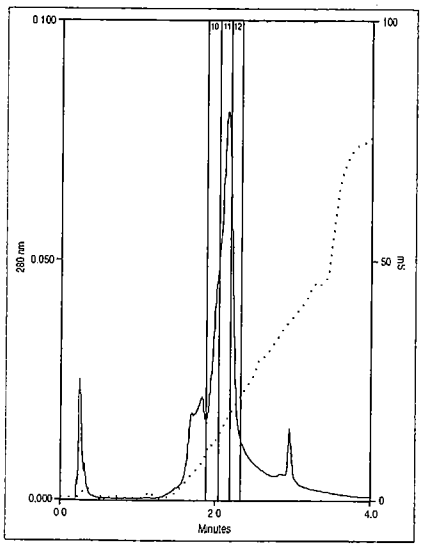
The combination of intra-particle flow and short diffusive pores effectively serves to access the entire surface area inside the particles much more rapidly than would be possible with conventional media, which rely solely on diffusion to achieve the same effect. Separations may therefore be carried out much faster on Perfusion Chromatography media with little or no loss in resolution or capacity. A more complete theoretical treatment of Perfusion Chromatography can be found in the companion booklet, *An Introduction to Perfusion Chromatography,* also available from PerSeptive Biosystems.

APPLICATIONS OF PERFUSION CHROMATOGRAPHY

The speed advantage of Perfusion Chromatography is on the order of 10 times over that of conventional HPLC columns, bringing typical separation times for a lab scale size column down to 3 - 5 minutes. In some ways, Perfusion Chromatography with POROS media redefines the way that researchers use and view chromatography in life science research. Many of the old trade-offs no longer come into play, and new application possibilities are opened.

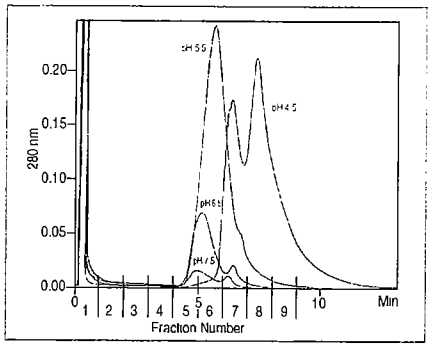
Improve and Simplify Method Development

Perhaps the most important advancement enabled by Perfusion Chromatography is a practical, systematic approach to the development of a separation method, which overcomes many of the problems discussed above. The short run times make it possible to complete a whole series of experiments in the same time it would take to make just a single run on conventional chromatography media. With this capability, the researcher can examine the critical separation variables *one at a time,* building a comprehensive picture of the behavior of the system, which makes it straightforward to design and test an optimal protocol. This systematic method development approach is the primary subject of this handbook and can be used on any chromatography system, although



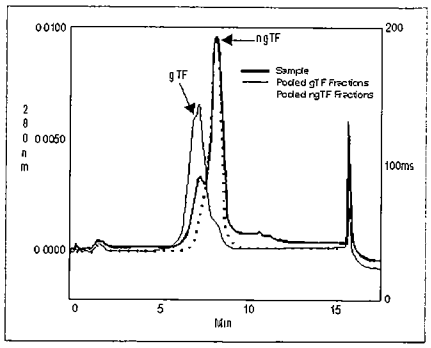
Sample:	Inclusion bodies solubilized in 10 M urea, 50 mM Tris, 5 mM EDTA, pH 8.0. Filtered and diluted 1:1 prior to injection (500 µl)
Column:	POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis-tris propane, pH 9.0
Eluent (B):	20 mM Tris/bis-tris propane, pH 9.0 + 3M NaCl
Flow Rate::	10 ml/min (3600 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 25% B in 15 CV

Lab scale purifications are routinely performed in 3-5 minutes, as in the first step of *Giardia lamblia* recombinant vacuolar ATPase A subunit purification from *E. coli* inclusion bodies. SDS PAGE revealed fractions 11 and 12 contain protein of interest with only two minor contaminants still remaining. From work conducted by Elena Hilario and Dr. Peter Gogarten at Univ. of Connecticut.



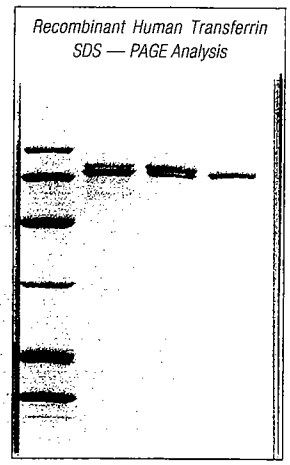
Sample:	50 µl *E. coli* cell culture supernatant (2.5 mg total protein)
Column:	POROS HS/M 4.6 mmD/100 mmL
Starting Buffer (A):	33 mM HEPES/MES/acetate, pH's as shown
Eluent (B):	33 mM HEPES/MES/acetate + 1.5 M NaCl
Flow Rate:	5 ml/min (1800 cm/hr)
System:	BioCAD Workstation
Detection:	280 nm
Gradient:	0 - 100% B in 15 CV

pH is one of the variables that can be systematically and conveniently explored with the short run times of Perfusion Chromatography, as shown in these overlaid traces of recombinant human heat shock protein (hsp 60) run on a POROS HS cation exchange column. pH 6.5 yielded fractions containing the least amount of contaminating proteins, and the highest concentration of the target molecule. After a final immunoaffinity purification to ensure no contamination with its bacterial counterpart, the hsp 60 was greater than 95% pure. PerSeptive Biosystems Application Note PA 419.

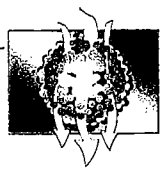


Sample:	BHK cell culture supernatant, 250 µl
Column:	POROS QE/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis/propane, pH 8.5
Eluent (B):	20 mM Tris/bis/propane, pH 8.5 + 1 M NaCl
Flow Rate::	5 ml/min (1800 cm/hrr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0-25% B in 45 CV

The result of taking a systematic approach to methods development. In this example, attempts with conventional chromatography media to develop an effective purification of recombinant non-glycosylated human transferrin (nghTF) from natural glycosylated form (gTF) present in tissue culture medium were met with frustration for two years. Using Perfusion Chromatography, this method was developed in a single day. Lanes 2-4 of electrophoresis gel show increasing purity of nghTF (lower band) through purification process. From work conducted by Dr. Anne B. Mason, U. Vermont College of Medicine. PerSeptive Biosystems Application Note PA 413.



Lane 1: Molecular weight markers
Lane 2-4: Progression through purification process showing increased purity of nonglycosylated hTF (lower band)



PerSeptive Biosystems has developed its own line of instrumentation (BioCAD family of systems) that further facilitates the process.

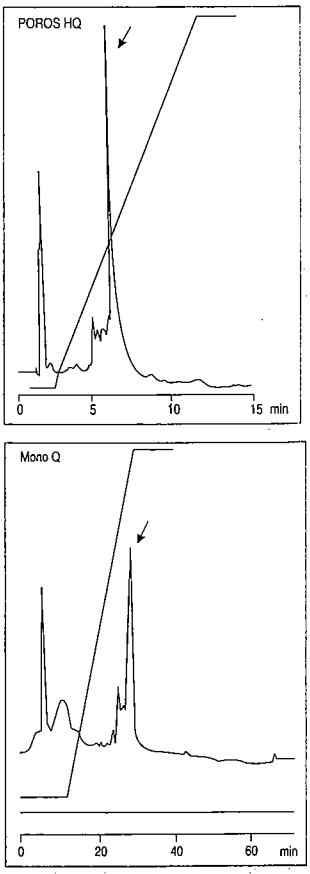
Taking the systematic Perfusion Chromatography approach can mean the difference between quickly zeroing in on the separation conditions that yield the molecule of interest in highly pure form, or experiencing frustration over yet another setback.

Even in cases where time and sample are limited, the high throughput of Perfusion Chromatography columns can make effective method development possible with fewer experiments than conventional techniques. For example, relatively shallow gradients can be used in ion exchange scouting runs, eliminating the need for gradient slope optimization once proper pH conditions are found. (See Section 4 — *Modes of Chromatography* for more details.)

Methods developed for use in an industrial quality control or production environment will need to be validated to confirm and document that the results meet the intended objectives. The systematic development approach is a good foundation for later validation, since the comprehensive information developed is essential to determining operating limits. The speed of Perfusion Chromatography allows final validation experiments to be performed quickly and efficiently.

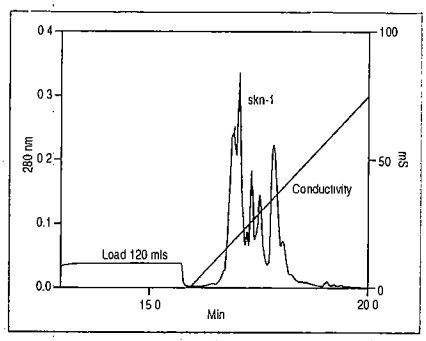
Enhance Recovery of Biological Activity

Compared to conventional chromatography, the fast run times of Perfusion Chromatography mean that the target molecules spend less time out of their natural state while being purified, under conditions that may promote denaturation. This also means less time in contact with potentially degrading enzymes that may be present in the sample. All of these factors can have a significant impact on the yield of biologically active product.



Sample:	2.5 ml partially purified and dialyzed into start buffer
Column 1:	POROS HQ/F 4.6 mmD/50 mmL
Column 2:	MonoQ® HR 5/5
Starting Buffer (A):	20 mM Tris HCl, 1 mM DTT, pH 7.5
Eluent (B):	Buffer A + 1 M KCl
Flow Rate 1:	4 ml/min
Flow Rate 2:	1 ml/min
System:	FPLC® System
Detection:	280 nm
Elution:	5 - 100% B in 40 CV

The purification of recombinant *E. coli* Mrr restriction enzyme on POROS anion exchange column was completed almost 5 times faster than the previously developed method on the conventional column. The enzyme was recovered from the POROS column in higher quantity and retained higher biological activity. From work conducted by Dr. J. Benner, New England Biolabs.



Sample:	120 ml pooled fractions from initial crude purification (0.5 - 1 mg/ml total protein)
Column:	POROS S/M 10 mmD/100 mmL
Starting Buffer (A):	20 mM HEPES, pH 6.5
Eluent (B):	20 mM HEPES, pH 6.5 + 1.5 M NaCl
Flow Rate: 1:	Sample load: 7.5 ml/min (575 cm/hr)
Flow Rate: 2:	Elution: 20 ml/min (1525 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 100% B in 15 CV

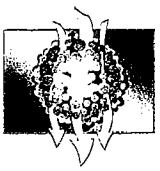
In the final step of the purification of recombinant skn-1 DNA binding protein, 120 ml of sample was processed in less than 20 minutes, eliminating the need for a separate concentration step that might have led to product loss. The yield was 25 mg at greater than 99% purity, sufficient for subsequent NMR studies. From work conducted by Dr. Gerhard Wagner and Dr. Dara Gilbert, Harvard Medical School. PerSeptive Biosystems Application Brief PA 421.

Simplify or Eliminate Sample Preparation Steps

The recovery of product (activity *and* mass) can be affected by sample preparation steps that take place before, between, or after the chromatography steps in a purification protocol. Concentration by ultrafiltration is often used at the outset of a purification to reduce the initial volume of a dilute sample, often simply to reduce the time required to load the chromatography column at the low flow rates necessitated by conventional media. In between purification steps, fractions coming off one chromatography column frequently need to be exchanged (often by dialysis) into a new buffer for the next column. When the first chromatography column is relatively large, fraction volumes for dialysis can also be large.

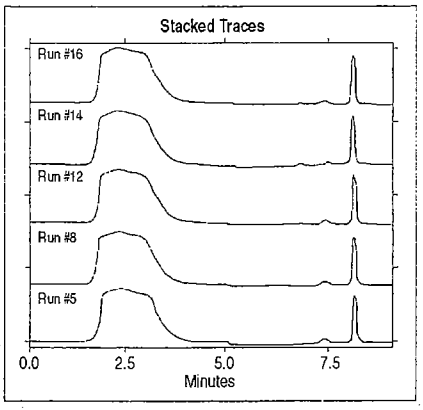
In both cases, the high capture efficiency at high flow rates of Perfusion Chromatography media can be employed to simplify or eliminate these steps, as well as the additional sample manipulation steps that can lead to product loss. In the first case, the large sample volume can be applied directly onto the chromatography column since the time to do so will no longer be a rate-limiting step. When the buffer conditions must be changed, it may be faster and simpler to *dilute* the original fraction in order to establish conditions for binding to a second column, after which you can use a high flow rate to apply and concentrate the sample on the column.

A second way in which POROS media can be used to simplify sample preparation stems from their pH stability. In the case of reversed-phase purification of synthetic oligonucleotides, detritylation with 2% TFA (which is normally done in solution) can be performed on-column in minutes, saving considerable time and possibly product loss through extra handling. This also holds true for Oligo R3™ medium, a PerSeptive Biosystems' reversed-phase packing specifically designed for preparative purification of synthetic oligonucleotides. On-column detritylation should not be performed with conventional silica C18 packings because of their limited ability to withstand the low pH of the detritylation conditions.



Reduce Column Size

In applications where large amounts of sample need to be purified, high speed Perfusion Chromatography enables the use of small columns. Since a perfusive column can be operated many times faster than a conventional column, several runs can be made on a smaller column to purify as much product in the same time as a scaled-up conventional column. This approach reduces the cost and uncertainty in scaling up, especially in the research environment.



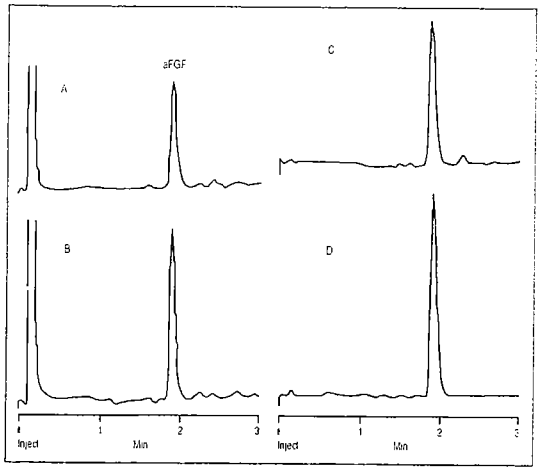
Sample:	Each 250 µl crude goat antiserum
Column:	Human transferrin immobilized on POROS EP/M 4.6 mmD/100 mmL
Starting Buffer (A):	PBS, pH 7.2
Eluent (B):	5 mM HCl
Flowrate:	2 ml/min (725 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

Use of POROS activated affinity to selectively purify antibody of desired specificity (anti-transferrin) from polyclonal population. Five of 16 separate injections are shown demonstrating how repeat cycling can be conveniently used to process larger samples. PerSeptive Biosystems Application Note PA 412.

Eliminate Analysis as a Bottleneck

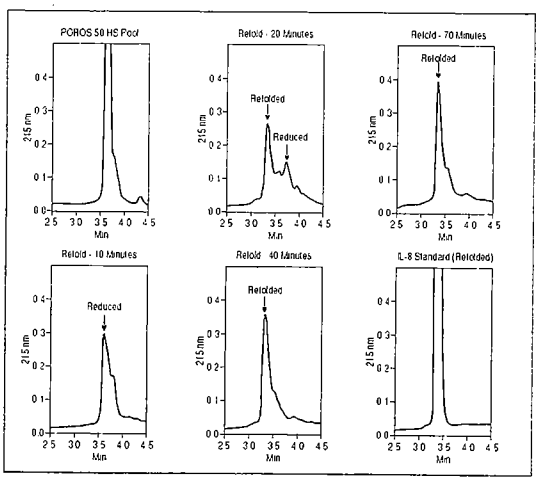
In addition to purification, chromatography is also often used for analysis (e.g. to analyze fractions of a purification step, to detect the presence of the target molecule in biological samples, etc.). When there are a large number of fractions or samples to assay, the analysis portion of the research project can represent a significant bottleneck. If a chromatographic assay can be adapted to POROS, the productivity of the lab can be increased significantly.

Not only can the impact be felt from a strict time-saving point of view, but in some cases from an efficiency point of view as well. For example, if the assay can be reduced to a few seconds or minutes, the output of a purification column can be monitored for the product in real time, and fractions only collected when product is present. This approach [which is embodied in the PerSeptive Biosystems' Real-Time Process Monitor (RPM®) System] can significantly reduce the number of fractions which must be collected and analyzed, and actually increase the yield of pure product.



Sample A:	Prepared cell paste suspension
Sample B:	In-process cell lysate
Sample C:	Anion exchange capture column product
Sample D:	Final affinity purification column product
Column:	POROS R1/H 4.6 mmD/50 mmL
Starting Buffer (A):	0.1% TFA in water
Eluent (B):	0.1% TFA, 80:20 acetonitrile-water
Flow Rate::	4 ml/min (1450 cm/hr)
System:	Conventional HPLC
Detection:	280 nm
Elution:	10 - 35% B in 20 s
	35 - 51% B in 2:25 min

A reversed-phase chromatographic assay was adapted to POROS to facilitate the quantitation of recombinant acidic Fibroblast Growth Factor (aFGF) during all stages of fermentation and purification. Three minute run times meant that *E. coli* expression levels could be closely monitored, and a full process recovery analysis (60 to 80 samples) could be completed in 4 hours. All samples 20 µl. Reprinted from *J. Chromatogr. A,* 663, DePhillips *et. al.,* Reversed-phase high performance liquid chromatography assay for recombinant acidic fibroblast growth factor in *E. coli* cell suspensions and lysate samples, 43-51, 1994 with kind permission of Elsevier Science, The Netherlands.



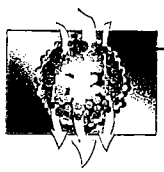
Sample:	50 µl aliquots from refolding reaction
Column:	POROS R2/M 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA in water
Eluent (B):	0.1% TFA in 95% acetonitrile
Flow Rate::	8.5 ml/min (3000 cm/hr)
System:	BioCAD workstation
Detection:	215 nm
Elution:	1 - 75% acetonitrile

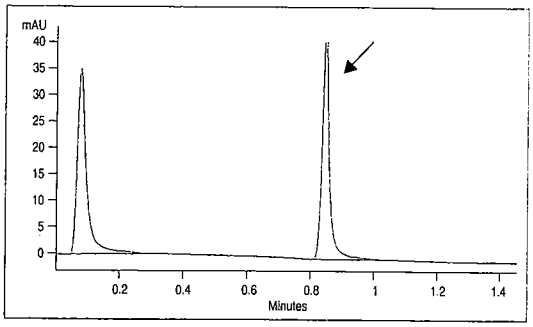
The first step in the purification of recombinant Interleukin-8 (IL-8) required solubilization of the cytokine from *E. coli* inclusion bodies with 8 M urea. The denatured product recovered from cation exchange column had to be refolded before loading onto the second (hydrophobic interaction) column. The existing method called for an overnight reaction to ensure refolding was complete. A rapid POROS reversed-phase separation was developed that allowed the refolding reaction to be monitored in "real time". It revealed that refolding was >90% complete within 70 minutes, as indicated by peak retention time shift in the series of chromatograms shown. The extra waiting time built into the original procedure was eliminated, creating time efficiencies in addition to those already realized in converting the purification method itself to POROS. PerSeptive Biosystems Application Note PA 422.

Develop Novel Assay Techniques

The high speed of Perfusion Chromatography enables a number of highly novel assay techniques. For example, it is possible to immobilize antibodies on POROS media and carry out on-column immunoassays using ImmunoDetection™ cartridges. The very short diffusion paths, high surface area, and rapid washing can reduce the many hours required for conventional microplate-based immunoassays to a few minutes, and allow complete automation with conventional HPLC instrumentation. (See the booklet *Introduction to ImmunoDetection,* available from PerSeptive Biosystems, for more details on this technique.) Enzymes immobilized on POROS perfusive supports (available as Poroszyme™ products) allow protein digestion and other analytical processes to proceed far more quickly and efficiently, without enzyme autodigestion.

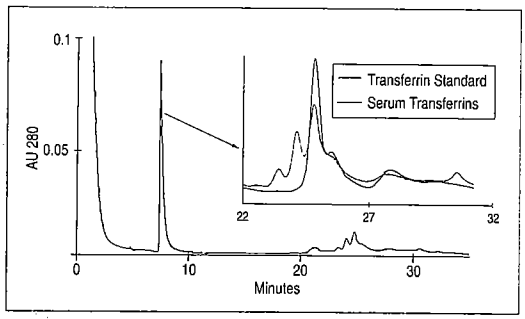
The short run times of perfusive media also make complex, multi-column and "hyphenated" analyses much more practical. For example, an ImmunoDetection cartridge immobilized with antibody can be used to quickly separate a particular type of biomolecule from a complex biological sample, and the bound target can then be eluted onto a reversed-phase column to separate the various isoforms. Perfusion Chromatography media packed into capillary columns allow for more efficient utilization and detection of sample when coupled directly to mass spectrometry instrumentation. The combination of high speed media and flexible, automated instrumentation (such as the PerSeptive Biosystems' INTEGRAL™ Micro-Analytical Workstation) enable the development of multi-dimensional chromatographic assays to solve many challenging analytical problems that could not be addressed before.





Sample:	25 µl reference standard (approx. 5 µg protein)
Column:	POROS 20 EP ID cartridge (2.1 mmD/30 mmL) with immobilized Lewis Y antigen
Starting Buffer (A):	PBS
Eluent (B):	0.1 phosphoric acid, pH 2.3 + 0.15 M NaCl
Flow Rate::	2 ml/min (3465 cm/hr)
System:	HP 1090
Detection:	280 nm
Elution:	Step to 100% B

Adaptation of an immunoassay to a POROS column. Lewis Y antigen is covalently attached to an activated affinity cartridge and specifically binds the target analyte, a chimeric IgG, which is eluted with acid and quantitated by OD 280 nm. The assay takes less than 2 minutes and is highly reproducible (<5% CV), eliminating limitations of the conventional assay techniques and lending itself to automation with readily-available HPLC equipment. Schenerman and Collins, *Anal. Biochem.* 217: 241-247. Reprinted with permission.



Sample:	1/10 human serum (50 µl)
Column 1:	POROS ID cartridge immobilized with anti-human transferrin antibody
Column 2:	POROS HQ/H 2.1 mmD/100 mmL
Starting Buffer (A):	50 mM Na Borate, pH 9
Eluent (B):	Buffer A + 1 M NaCl
Flow Rate:	2 mL/min
System:	INTEGRAL workstation
Detection:	280 nm
Elution:	6.5 - 11.5% B in 30 CV then to 50% B in 10 CV

Coupling of POROS immunoaffinity and anion exchange columns for simultaneous determination of target molecule concentration and isoform analysis. In this example, transferrin is selectively purified from the serum sample by the first immunoaffinity column. Concentration can be determined by peak area. After detection, the eluent from the ID cartridge is passed directly onto a POROS HQ column. The elution profile, when compared to that of transferrin standard (enlarged area, colored trace), indicates the presence of two additional isoforms in the serum sample.

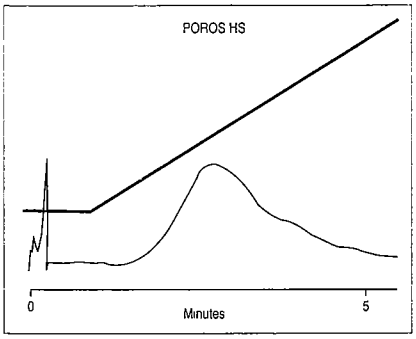
PERFUSION CHROMATOGRAPHY IN A RANGE OF TECHNIQUES

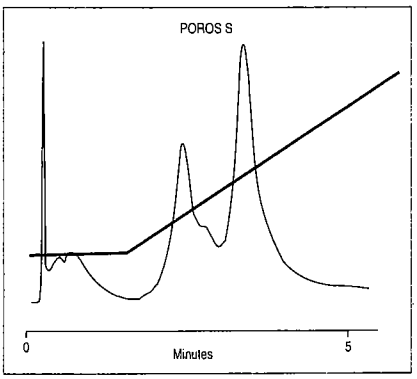
The fundamental performance characteristics (resolution and capacity at high speed) of Perfusion Chromatography technology are directly related to the unique pore structure of the poly(styrene-divinylbenzene) (PSDVB) particles that form the basis for all POROS media. The base particles themselves, being hydrophobic in nature, are available for *reversed-phase* applications.

To create Perfusion Chromatography media suitable for other modes, the base PSDVB particles are subsequently coated with a hydrophilic polymer and then functionalized for *ion exhange, hydrophobic interaction, affinity, activated affinity,* and *metal chelate* chromatography. The coating process does not interfere with the unique mass transport properties of the POROS particles, thus ensuring that the speed benefits of Perfusion Chromatography are available in each case.

POROS CHEMISTRIES

Ion Exchange	Reversed-Phase
HQ strong anion	R1
QE strong anion	R2
PI weak anion	
DEAE weak anion	**Affinity**
	A protein A
HS strong cation	G protein G
SP strong cation	HE heparin
S strong cation	MC metal chelate
CM weak cation	
	Activated Affinity
Hydrophobic Interaction	EP epoxide
HP2 high density phenyl	AL aldehyde
PE phenyl ether	HY hydrazide
ET ether	NH amine
	OH hydroxyl





Sample:	20 µl crude peptide (10 mg/ml) in start buffer
Column 1:	POROS HS/M 4.6 mmD/100 mmL
Column 2:	POROS S/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Trls/HCl, pH 8.5
Eluent (B):	20 mM Trls/HCl, pH 8.5 + 0.5 M NaCl
Flow Rate:	5 ml/min (1800 cm/hrr)
System:	HP 1050
Detection:	214 nm
Elution:	0 - 40% B in 5 min

Separation of a 28 residue synthetic vasoactive peptide (VIP) on different POROS cation exchange chemistries. A less hydrophobic functional group coupled with a lower ligand density on POROS S provided much better selectivity for this very basic, very hydrophobic peptide. From work conducted for LSU medical school.

Within certain chromatographic modes, there is a further range of *selectivities* available in POROS media. For example, there are four different POROS media available for cation exchange chromatography, each with a different functional group and/or ligand density. Depending on the nature of the sample, the separation profile on each packing in a series can be markedly different — a factor that can be exploited when trying to develop an effective separation method.

The range of selectivities on conventional high performance chromatography media is typically not as extensive as that found on POROS. Whether this relates to a manufacturing issue or not, even if a broader range of chemistries were available, the speed limitations of conventional media would make it impractical to thoroughly test them. This is not the case with Perfusion Chromatography media where the speed of the runs makes it convenient to bring all the power of differing selectivity to bear on a separation problem as required.

To reduce the cost associated with trying several different column chemistries in order to take advantage of selectivity, POROS media are available in a unique Self Pack® format (in addition to the prepacked column format).

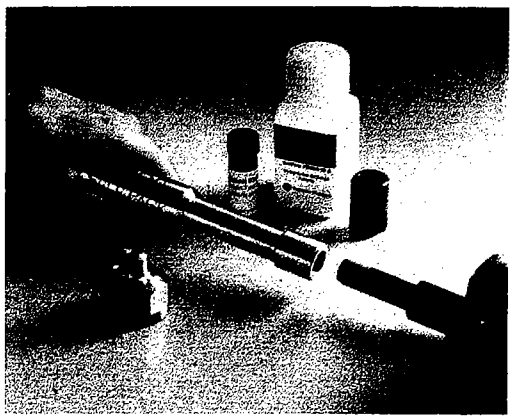
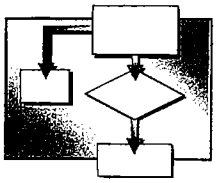


Figure 1-4. The Self Pack system gives researchers the ability to pack their high performance POROS columns, using their existing chromatography system.

One common technique for which there is not an analagous POROS product is *gel filtration* (or *size exclusion*) chromatography. Gel filtration relies on diffusion within the particles to create the "sieving" effect that fractionates based on molecular size. The effective pore structure of Perfusion Chromatography particles minimizes this diffusion so POROS media is not well suited for gel filtration. However, this does not mean that POROS cannot be used to advantage for a separation that currently uses gel filtration. In some instances, a Perfusion Chromatography alternative can be found. If possible this option should be explored, especially if the acute speed and capacity limitations of gel filtration pose a problem.

Refer to the companion piece, *POROS Columns and Media Selection Guide*, also available from PerSeptive Biosystems for a complete description of the POROS chemistries, column geometries and product formats available.



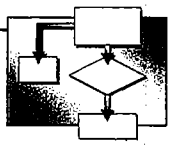
SECTION 2
PRINCIPLES OF SYSTEMATIC METHOD DEVELOPMENT

DEFINE

The Target Molecule & Sample
Class of Molecule
Molecular Characteristics
Sample Source
Analytical Methods
Screening Analysis vs. Final Analysis
Key Analytical Techniques
Tracking of Peaks
Separation Goals
Analytical
Preparative
Overall Separation Strategy
Resources
Sample
Time
Equipment

EXPERIMENT

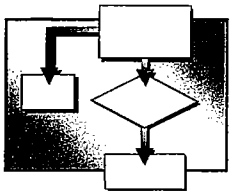
General Experimental Framework
Column
pH Map
Gradient/Elution Optimization
Loading Study



EVALUATE
- *Resolution*
- *Recovery*
- *Purification Table*
- *Capacity*
- *Practicality*

IMPLEMENT
- *Analytical Method Development*
- *Multi-step Separations*
- *Scale up*

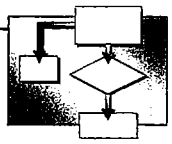
TROUBLESHOOT
- *Bandspreading*
- *Peak Shape*
- *Selectivity*
- *Recovery*
- *Pressure*
- *Reproducibility*
- *Column Cleaning and Reuse*
- *Cycle Time*



SECTION 2
PRINCIPLES OF SYSTEMATIC METHOD DEVELOPMENT

This section discusses the fundamental principles that you should follow in carrying out any chromatographic method development. It assumes that you have a good understanding of how chromatography works, which is covered in Section 5 — *Basics of Chromatography*. Specific approaches for different classes of biomolecules are discussed in Section 3 — *Developing Your Application*. Detailed method protocol recommendations are given in Section 4 — *Modes of Chromatography*.

Many users of chromatography, when developing a method using conventional media, have had to face very long experimental times, not only to set up and perform individual chromatographic runs, but also to analyze the resulting fractions. The length of the experiments inevitably limits the number of parameters that can be examined during the development process. This has forced many to resort to a kind of "hit and miss" approach, in which the chromatographic runs are viewed as trial solutions which either succeed or fail, rather than as useful data points about the behavior of the system. Often many parameters are changed at one time in moving from one trial solution to another, making it difficult to develop the kind of understanding that would allow an optimal method to be effectively designed and maintained.



With the advent of high speed Perfusion Chromatography media, individual run times (for both the chromatography itself and much of the analysis) have been sharply reduced. This makes it practical to take a more systematic approach, in which the critical parameters of the separation system are empirically examined, one at a time. The resulting information about the behavior of the system enables the user to actually design an optimal solution based on real data, as well as implement and maintain the method with a great deal of assurance.

Systematic method development may be viewed as a process with five stages, as follows:

Define	This is the critical stage in which you carefully delineate the problem to be solved, including the nature of the target molecule itself and the sample from which you are separating it, the analytical methods you will use, the overall goals of the separation, and the resources available.
Experiment	Once the problem is defined, you gather empirical information about the behavior of the system with respect to each of the key variables. Experimentation is carried out in a cycle of selecting systems of columns and mobile phases, then testing the effects of key parameters such as pH, gradient slope and, perhaps, sample load on each system.
Evaluate	Throughout the experimentation, you must continually evaluate the resolution, recovery, capacity and practicality of the method. The results of the evaluation are fed back into the design of the experimentation.
Implement	Based on the results of the experimental program and evaluation, you can then design your final method, optimize it and put it into practice. Details of the implementation vary, depending upon whether you are developing an analytical method, a multi-step separation or need to scale up.
Troubleshoot	Inevitably, you will be faced at some point with the need to solve problems with or fine tune the performance of your method. Chromatographic efficiency, selectivity, recovery, reproducibility, column equilibration, regeneration and reuse and method cycle time are all factors that might need optimization.

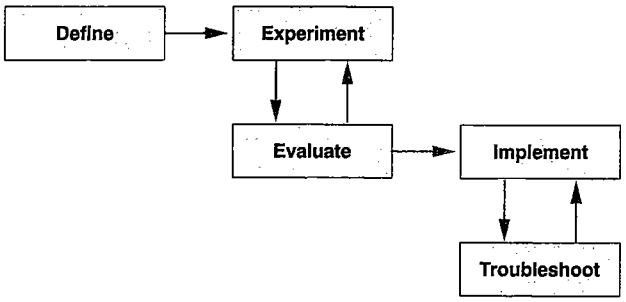


Figure 2-1. Stages of systematic chromatography method development

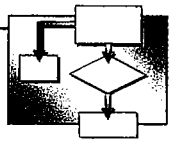
The following sections discuss in detail the considerations for each of these systematic method development stages.

DEFINE

In any field as complex as biomolecule chromatography, you must carefully define the problem you are trying to solve in order to be successful. You will not regret time spent at the beginning (*before* you go into the lab) examining the nature of the target molecule and sample source, choosing the analytical methods you will be using during the development, and evaluating the resources (time, equipment and sample) you will have available. Perhaps most importantly, you will need to clearly understand the goals or objectives of the separation (particularly whether it is analytical or preparative) and consider the specific method you are developing in the context of the overall separation strategy.

The Target Molecule & Sample

The starting point for defining a separation problem is obviously the target molecule and the sample from which it is to be separated. The class of molecule and molecular characteristics of the target relative to the other molecules in the sample



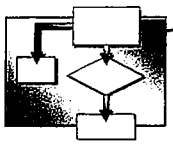
determine which mode(s) of chromatography you should use or try. You must also consider the important impurities from the sample or samples that must be separated for you to be successful.

Class of Molecule

You can classify biomolecules in many different ways, either by source, form or function. One basic classification is by size and structure. So-called "small organic molecules" generally consist of a single unit structure and are less than ~1000 MW. These include many hormones, cofactors, simple carbohydrates, etc. Although chromatographic separations of these molecules are important, the techniques used are very different from the larger, polymeric biomolecules which are the subject of this handbook.

There are four general classes of these larger biomolecules you will find most useful for determining a chromatographic separation strategy:

Proteins	Proteins have a great diversity of molecular and chromatographic characteristics. Some useful generalizations about separation strategies can be made.
Antibodies	Although all antibodies are proteins, all possess sets of common characteristics, including common sample sources. Because of their importance and widespread usage, it is worth considering them independently for the development of selective separation methods.
Peptides	Peptides are also related to proteins, but their lower molecular weight and limited 3D conformation make for a very different separation problem. Often peptides are produced synthetically, which creates contaminants very similar to the target, making separation difficult.
Nucleic acids	Nucleic acids have distinctly different characteristics from proteins and peptides, and demand a different separation strategy. As with peptides, the challenge is often to make fine separations between molecules which are structurally very similar.



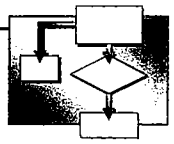
Each of these classes is considered in detail in Section 3 — *Developing Your Application*. Some useful distinctions within these classes may be made (such as membrane vs. soluble proteins or oligonucleotides vs. plasmid DNA), which are considered there as well. There are other significant classes of biomolecules (such as complex carbohydrates) which are not as common and are not covered in this handbook. However, the basic concepts of chromatography also apply to these classes, and separation strategies may be easily developed by considering the key molecular characteristics discussed below.

Molecular Characteristics

From the point of view of chromatography, all molecules share certain common characteristics, whether they are simple organic compounds or complex, multi-subunit proteins. These characteristics may be summarized with the acronym CHASM:

- **C** harge
- **H** ydrophobicity
- **A** ffinity
- **S** olubility & stability
- **M** olecular weight

Charge is a measure of the number of ionic charges (positive and/or negative) which are accessible on the surface of the molecule. *Hydrophobicity* (the inverse of which is called polarity) is a measure of the "oiliness" or "water-hating" character of the molecule or of functional groups within the molecule. *Affinity* refers to the presence of sites on the molecule which can interact with other molecules in a biospecific or "lock and key" binding interaction. *Solubility* and *stability* are the ranges of chemical conditions and concentrations in which the molecule can stay in solution and maintain its biological activity. *Molecular weight* is a measure of the size of the molecule. The shape of the molecule may also be important in some cases.



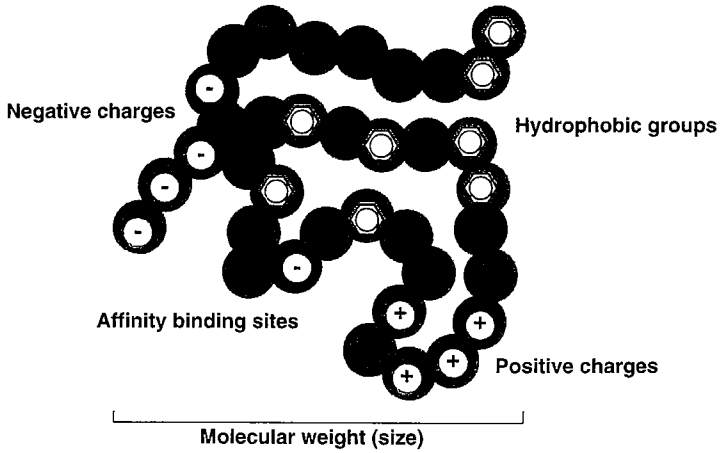
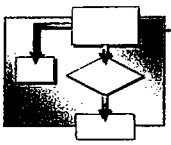


Figure 2-2. Molecular characteristics that control surface binding interactions in chromatography

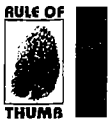
By analyzing the CHASM characteristics of the target molecule relative to other molecules in a mixture, you can begin to look for properties which differentiate the target and will allow its separation. You should try to utilize any unique characteristics as a "handle" for chromatographic binding, since this will give the maximum degree of separation. Once these properties are identified, you can then select the appropriate modes of chromatography, each of which is designed to interact selectively with a particular CHASM characteristic.

Note that you should not only consider the characteristics of the target molecule itself, but also the key impurities that must be removed. Sometimes, (such as when you are in the polishing stage of a separation process) it is the impurities rather than the target that become the focus of your efforts.

Another aspect of CHASM analysis is to understand the limitations on the separation imposed by the biochemistry of the target molecule and sample. Solubility and stability, in



particular, restrict the range of chemical conditions that you may use during chromatography and even the mode of chromatography you may select. You should gather any information about the acceptable pH range, ionic strength or organic solvent concentration your target will withstand with good solubility and stability before you design your experiments.



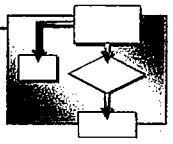
Don't rely solely on literature statements on these subjects. Conflicting information can exist. Verify key values yourself.

You should also consider any cofactors or other buffer additives that the target molecule may require for either solubility or stability. Some of these additives may be essential for the biomolecule, but may interfere with one or more modes of chromatography, restricting your development possibilities.

Sample Source

Although you may have detailed information about the target and its molecular characteristics, you will most often not have such information about every other molecule in the sample. In some cases, the sample may be extremely complex. However, you must remember that each and every molecule in the mixture will be participating in the separation and may be competing with the target for binding sites of the column.

One approach you may use in dealing with this problem is to consider the most important classes of molecules in the sample and to identify the key impurities that are the focus of your separation efforts. Understanding your goal is important here. For example, if you are cleaning up an antibody for use in an assay, simply obtaining a reasonable general purity may be sufficient. If, however, you are attempting to do an X-ray crystallography structural study of that same antibody binding to its antigen, it may be critical to remove traces of all contaminating proteins, including all other, non-specific antibodies.



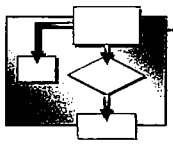
Natural sources offer the most complex array of potential impurities. Single purification steps are rarely sufficient for samples this complex, and you will almost always need two or more different chromatographic steps in sequence. Some natural sources (such as serum) are complex but are relatively well-defined. Understanding the overall behavior of these samples on a column of interest can be very useful for developing the separation of a particular molecule.

Biomolecules made by recombinant DNA and other "biosynthetic" methods offer some control over the source composition, and your choices can have a major impact on the chromatography. For example, it makes a significant difference if the product is secreted or if the cells must be lysed. Even in cell culture, where the product is often secreted, the culture medium is important (e.g. serum-free, defined media are much less difficult than media with a high concentration of fetal bovine serum), as is the culture method (e.g. high density methods such as hollow fibers often have a higher product concentration but more contaminants from accidental cell lysis).

Synthetic sources of biomolecules such as peptides and oligonucleotides present a normally more defined array of key impurities than natural or biosynthetic sources. However, many of the impurities are very similar to the target molecule in structure, since they represent minor modifications in the synthesis process. These "variant forms" can be extremely difficult to separate. For this reason high resolution methods such as HPLC are often required for synthetic biomolecule separations.

Analytical Methods

The analytical methods used during development are an absolutely critical part of defining your separation problem. In many cases, the target molecule is actually only identified by its activity in a particular analysis, and little else about it may be known.



Define a way of quantitating or at least identifying the presence of your target molecule, and of assessing its purity, before you undertake a chromatographic method development.

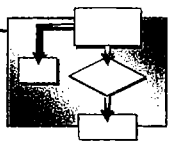
Screening Analysis vs. Final Analysis

During a Perfusion Chromatography method development, you will generate a potentially very large number of samples from separate chromatographic runs as well as individual fractions within those runs. The more samples you can analyze, the more variables you can examine experimentally and the more likely you are to find an optimal solution. Analysis is likely to be the most significant bottleneck you will encounter in the development process. A key strategy in breaking this bottleneck is to differentiate between *screening analysis* and *final analysis*.

You use screening analysis simply to identify which of a series of chromatographic conditions gives the best separation, or is the best direction to take for further development. Screening analytical methods are fast and must be suitable for a large number of samples, either in parallel or sequentially through automation. Semi-quantitative or even qualitative information is often sufficient. The key is that you can use the screening analysis in a practical way to differentiate between a large number of different chromatographic results.

You use final analysis to fully characterize the results of your optimized or semi-optimized separation. Final analysis must be precise, accurate and quantitative, and need not be suitable for large numbers of samples. The key is that you can use the final analysis to reliably determine if your optimized method is fully viable.

You should spend some time to select or develop good screening analysis methods. Often you will need two methods — one to identify and possibly quantitate the target molecule itself or its biological activity (such as an enzyme activity assay), and



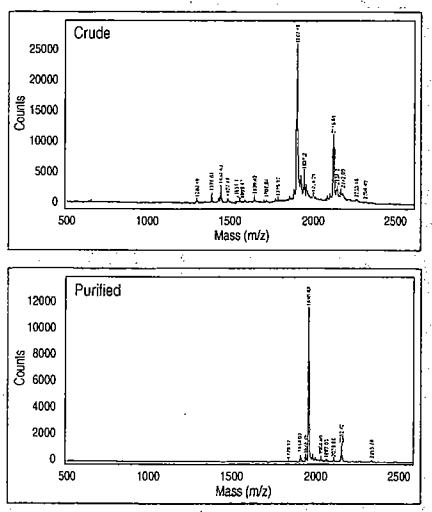
the other to give at least a rough assessment of purity (such as a total protein assay). Sometimes you can use a single method (such as SDS-PAGE) for both identification and purity assessment. In other cases, you may need an additional method for identifying or quantitating one or more critical impurities that must be removed from the target. In all cases, however, you should limit the screening assays to only the information needed to identify "good" results and where to move forward with the chromatographic development.

Key Analytical Techniques

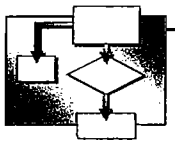
An incredible range of different analytical techniques are available for biomolecules. Each class of biomolecules has its own appropriate methods, each of which may be essential for a particular application. A thorough discussion of each technique and its relative merits is well beyond the scope of this handbook. However, a number of useful general comments may be made with respect to their use in screening analysis during method development.

Analytical techniques fall into a number of broad categories:

Bioassays	Assays in which a sample is introduced into a living biological system or complex extract and some response is measured. Bioassays are often expensive, time-consuming and qualitative in nature, but may be the only way to positively identify the activity of a particular molecule.
Binding Assays	Assays in which the specific binding to a particular entity (antibody, antigen, cell surface receptor, etc.) is measured. Immunoassays are a common example. Binding assays can be extremely sensitive and precise, and are frequently done in a microplate format.
Chemical Assays	Assays involving specific chemical reactions, which are quantitated by physical measurements such as spectrophotometry. Total protein (Lowry or Bradford dye-binding) and enzyme activity assays are examples.
Physical Assays	Direct assays using physical instrumentation, such as light scattering, UV absorbance, mass spectrometry, electrochemical properties, etc.
Separation Assays	Assays involving separation techniques such as electrophoresis or chromatography.



With the introduction of Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry instruments such as the Voyager™ Biospectrometry™ Workstation from PerSeptive Biosystems, mass spectrometry can now be conveniently used to help characterize biological samples. In this example, mass spectrometry was used to assess the quality of a difficult peptide synthesis as well as the quality of the subsequent HPLC purification. Analytical HPLC of the purified fractions gave an indication of the relative abundance of sample components, but information contained in the mass spectrum quickly identified whether contaminants resulted from incomplete deprotection or from failure or deletion sequences. PerSeptive Biosystems Application Note PA 437.

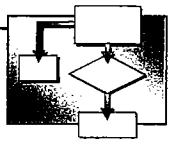


Binding, chemical and physical assays are the most widely useful for screening analysis. These techniques are relatively fast (although conventional binding assays can be time consuming) and can be easily run in large numbers. Both highly specific assays (for target quantitation) and more general assays (for purity assessment) are available. These kinds of assays also have an advantage in that they typically produce a single, simple result (a quantitative number or a +/- identification), which allows you to easily correlate the results with the chromatography.

Bioassays, while sometimes essential (especially at the early stages of research), can be very problematic. They are very often time- and labor-intensive and are frequently not suitable for running large numbers of samples. Whenever possible, you should seek alternatives or "surrogate assays", which can be at least correlated with the results of the bioassays and are more suitable for screening.

Separation-based assays such as electrophoresis can also be extremely valuable in screening analysis. These assays frequently allow you to obtain information about the specific product concentration and the total impurity concentration in the same assay. The tradeoff is that these assays often resist practical quantitation and instead rely on your visual interpretation. While this may be easy to do for individual samples, it can be difficult to correlate all the data from a complete set of chromatographic runs (in which complex parameter changes are made) with complex, qualitative assay results for each fraction.

An increasing trend in analysis is to combine different analytical techniques together in a single, automated method. An example of this is automated peptide mapping techniques in which a protein sample can be digested on-line with subsequent chromatographic separation of the peptide fragments. Recent instrument designs (such as the PerSeptive Biosystems' INTEGRAL workstation) enable multi-dimensional chromatographic assays in combination with on-line immunoassays,



enzymatic digestions, and other techniques to allow a number of quantitative analyses to be made at once from a single, highly complex sample. This kind of approach should make the analytical bottleneck in chromatographic development less of a problem in the future.

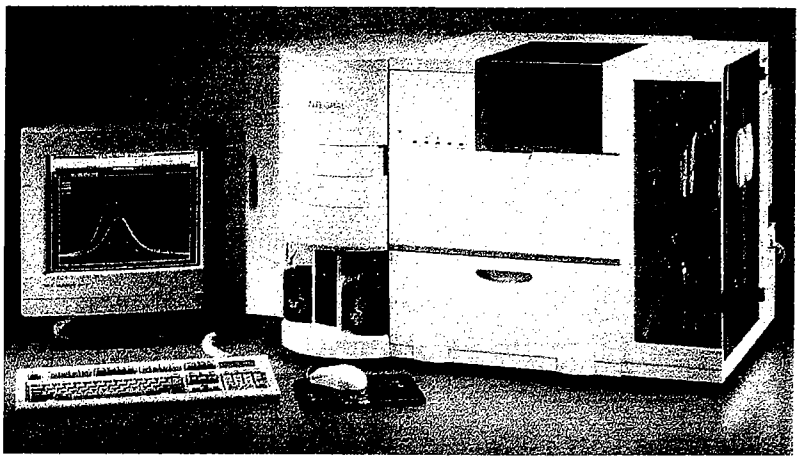


Figure 2-3. The INTEGRAL Micro-Analytical workstation enables automated multi-step assays combining immunoassay, enzymatic analysis and chromatography.

Tracking of Peaks

Even if you have a relatively fast and straightforward screening assay, it still may be difficult or cumbersome to collect and analyze every fraction from every chromatographic run. One technique you can use to alleviate the analytical burden is tracking of peaks.

The starting point is to identify the peak of interest in an initial chromatogram by one of two basic methods — either collect fractions and assay via screening analysis or run a purified sample of the target molecule (if this is available) under identical chromatographic conditions and compare the results with the chromatogram of the actual sample. Once the peak is

identified, you can often make an incremental change in the chromatographic conditions (shifting the pH, for example), and still be able to identify the peak of interest from the pattern of the chromatogram. If this is the case, you may not need to do anything else to analyze the later chromatograms.

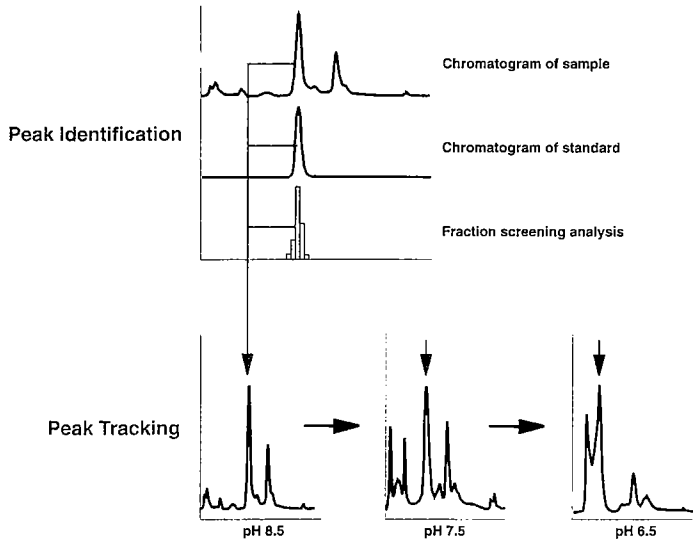
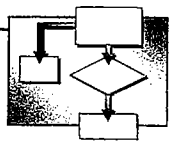


Figure 2-4. You can use either chromatography of a purified standard or screening analysis of fractions to initially identify peaks. Once identified, you can often visually track peaks in the chromatograms, as long as incremental changes in the chromatographic conditions are made.

This process of peak tracking may be continued as long as you are reasonably confident that you can still identify the peak of interest. Often the final chromatogram may be very different looking from the first, but because the changes have been incremental, you have been able to track your target peak throughout the process. Naturally, you should confirm the identity of the peak once you have achieved a reasonably optimal result.



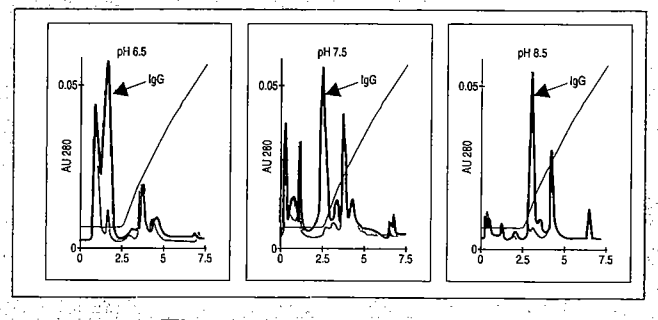
Sometimes (such as when the peak of interest is very small or even invisible) peak tracking is not appropriate. However, when it does work, tracking of peaks can save a great deal of analysis time, and enable practical fine tuning of a chromatographic method.

Separation Goals

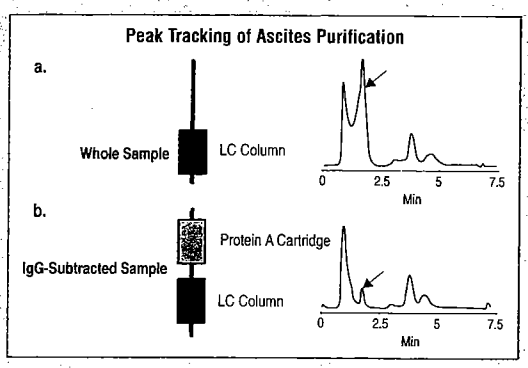
Understanding your goals and objectives in detail is vital in successfully developing a chromatographic method. There is a fundamental differentiation in goals between analytical and preparative chromatography.

Analytical chromatography is defined as a procedure carried out for the sole purpose of obtaining a measurement of the chemical composition of a sample. The "product" is simply a quantitative set of numbers and/or a qualitative chromatogram. The separated sample components are not usually collected but rather sent to waste. Analytical chromatography is generally done on a very small scale.

Preparative chromatography is defined as a process carried out for the purpose of purifying and collecting one or more components of a mixture. The "product" is the component(s) of interest in the sample which have been separated and collected, and may be used for further analysis, research testing, assay reagents, clinical trials, or commercial sale. Preparative chromatography may be done on a wide range of scales, from nanograms to 10's or 100's of kilograms, depending upon the application.



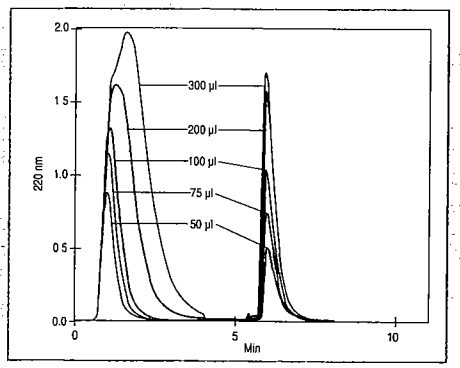
Sample:	50 µl crude ascites diluted 1:3 with 10 mM Tris, pH 8.0
Column:	POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis/propane, pH as indicated
Eluent (B):	Buffer A + 1 M NaCl
Flowrate:	8 ml/min (3000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 50% B in 20 CV

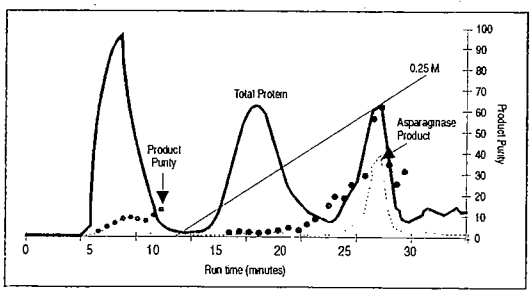


Peak tracking with POROS media can be facilitated on BioCAD workstation by using automated valving to selectively plumb a second column specific to the target molecule ahead of the main column. In this case the detection column is a POROS A (protein A) cartridge, which has specificity for IgG_2b (the target molecule).

By comparing chromatograms of two separate injections — the first with the POROS A cartridge in line (colored line), the second with it out-of-line (solid line) — the target peak on the anion exchange chromatogram can be identified. When in line, the POROS A cartridge effectively "subtracts" the peak of interest out of the POROS HQ run by binding the IgG out of the sample (see schematic). A co-eluting contaminant peak (mouse transferrin) is revealed during the subtraction.

Tracking the peak of interest in this manner during a mapping study, pH 8.5 is determined to be the optimum pH for additional scale up studies. At pH 6.5, the IgG_2b is not bound. At pH 7.5 it is barely retained, limiting the amount of protein that could be loaded before breakthrough occurs. PerSeptive Biosystems Application Note PA 415.

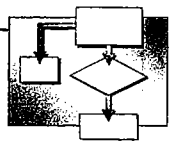




Sample:	30 ml crude *E. coli* supernatant diluted in start buffer
Column:	POROS 50 HQ 22 mmD/100 mmL
Starting Buffer (A):	20 mM Tris HCl, pH 8.0
Eluent (B):	20 mM Tris HCl, pH 8.0 + 1 M NaCl
Flowrate:	10 ml/min (160 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 25% B in 10 CV

The concept of peak tracking can be expanded to allow real time monitoring of process chromatography separations using the BioCAD/RPM system. In this example, the purification of asparaginase is monitored with a cartridge derivatized with anti-asparaginase antibodies. The cartridge is first calibrated with successive asparaginase injections of known concentrations (top figure). The BioCAD/RPM then uses it to automatically conduct rapid subtractive assays and calculate product concentration (dotted line) as well as purity (dots) as the separation develops.

Being able to monitor downstream processes on a real-time basis leads to more informed decision making and ultimately superior process performance. PerSeptive Biosystems Application Note PA 416.



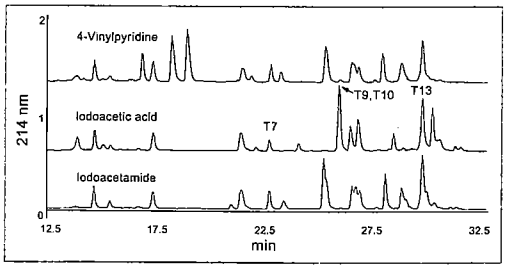
Analytical

Analytical chromatography can be used for a wide range of different types of applications, which fall into three general categories:

Single target	Quantitation of a single target molecule in the sample. This is the most common type of assay, and includes both product and contaminant quantitation.
Multiple targets	Quantitation of more than one (or sometimes all) of the molecules in the sample. An example is tryptic digest mapping, in which all the peptides should be separated and quantifiable.
Molecular variants	Quantitation or profiling of variant forms of the same molecule.

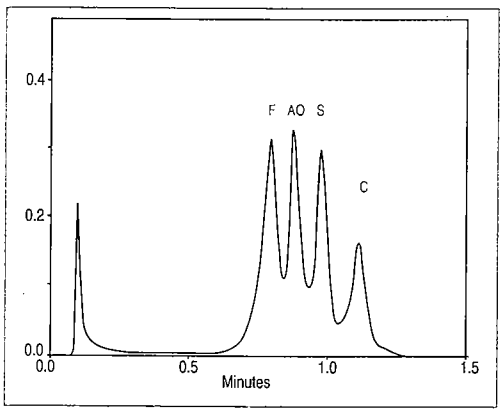
The class of assay can make a major difference in the method development. For example, you can optimize a single target assay so that the peak of interest is the only one that is well separated, with all the other components poorly resolved or even non-resolved. This approach can often save significant amounts of assay time. This option is usually not available when multiple targets must be separated.

When setting goals you should also consider the requirements for linearity, precision, accuracy and sensitivity, based on the proposed application of the assay. Assay reproducibility and robustness may also be important, especially if the assay is to be used for quality control or clinical diagnostics. The required assay throughput (number of assays that can be run per unit time) is also an important practical consideration.



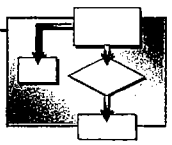
Sample:	50 µg Poroszyme tryptic digest of lysozyme, modified by various alkylation reagents as indicated
Column:	PepMap C18™ 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA in 1% acetonitrile
Eluent (B):	0.1% TFA in 95% acetonitrile
Flowrate:	1 ml/min (360 cm/hr)
System:	INTEGRAL workstation
Detection:	214 nm
Elution:	1 - 56% acetonitrile in 30 min

Example of a multiple target assay. In this application, a Poroszyme Immobilized trypsin column was used to digest lysozyme after treatment with different alkylation reagents. A PepMap C18 column was connected online after the Poroszyme column to separate the resulting peptide fragments. This entire analytical method (including alkylation) was fully automated on the INTEGRAL workstation.



Sample:	50 µl hemolyzed human blood diluted 1:10 in start buffer
Column:	POROS S/H 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM MES, pH 6.2
Eluent (B):	20 mM MES, pH 6.2 + 1 M NaCl
Flowrate:	10 ml/min (3600 cm/hr)
System:	BioCAD workstation
Detection:	415 nm
Elution:	0 - 10% B in 1 minute

High speed analysis of hemoglobin variants using a POROS cation exchange column.



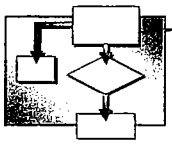
Preparative

As with analytical chromatography, preparative methods fall into a number of general categories:

Sample preparation	Some preparative methods may be viewed as preparing a sample for some other operation. Desalting, concentration and buffer exchange are typical applications. Sometimes these operations can be carried out non-chromatographically, but with high speed perfusive media, they may be faster or more practical on a column.
Capture	The goal of a capture step is to extract the target molecule from a crude solution, concentrate it and remove some of the bulk impurities. Capture steps are often the first stage in a longer purification process.
Purification	The goal of a purification step is to move the target molecule from low or intermediate purity to high purity, where it is typically 90 - 99% of the final product.
Polishing	In polishing, the goal is to remove trace quantities of contaminants from the target product, which has already been purified.

The approaches used for different preparative separation categories may be very different. For example, while capture steps often employ large particle, high capacity media to handle large quantities of crude solutions, final purification and polishing steps are often run with small particle HPLC media at lower loading to maximize resolution. *Negative chromatography* steps (in which the target does not bind to the column while contaminants do bind) are very useful for polishing, but rarely used for capture or sample prep.

Appropriate goals to consider for preparative chromatography include recovery, final product purity, and capacity. The ability to scale up may be important, as well as economic factors such as media costs and cycle life. Where large quantities of material must be processed, process throughput (amount of material produced per given time on a given column) can also be critical. The speed of Perfusion Chromatography can have tremendous impact here.



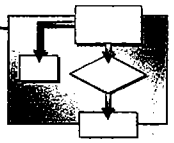
Overall Separation Strategy

When establishing your goals for a particular separation method under development, you should consider the entire separation process from start to finish. This is most important with preparative applications, which typically involve a number of chromatographic steps, usually interspersed with sample preparation and processing steps. Even analytical methods may require multiple steps before a final answer is obtained.

You should always examine how the method you are developing will fit into the overall separation strategy. Sometimes, you can be clever and eliminate steps. For example, by placing a reversed-phase step after an ion exchange step, you can take advantage of the buffer exchange or desalting capability of reversed-phase chromatography, and eliminate a sample preparation step. Elimination of steps not only saves time, but also can result in dramatic improvements in yield of final activity.

Resources

After you have considered the characteristics of the target molecule and sample, the analytical methods you will use and your separation goals, you should think about the resources you have available for developing the method. The resources are the most important determinant in the overall method development approach you will use. Sample, time and equipment are generally the most limiting resources for most researchers.

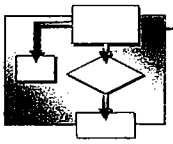


Sample

Sample availability is a limiting resource that is often not considered, but may be the most critical. You may have plenty of time and equipment available for an extensive method optimization, but if you only have a small amount of precious or expensive sample to use, you must limit your experiments carefully. To maximize your chances of obtaining optimal results, determine first how many runs you might be able to perform with the sample you have, and plan your experiments so that the most important variables are covered first.

Time

Time is obviously a precious quantity for everyone, and you will almost always want to limit the time spent in method development as much as possible — even with the capabilities of Perfusion Chromatography media. Deadlines and other factors will determine the time available, but you should also consider how much time and effort *should* be put into a new method. For instance, if you are purifying a protein only once for a limited study, it will certainly not be worthwhile developing a robust preparative method in which you have examined every variable (although some minimal development will probably be necessary). On the other hand, if you are developing a critical analytical method that will be used for thousands of different samples in different labs over a period of years, time spent in fully characterizing and optimizing all the key parameters will almost certainly be well spent.

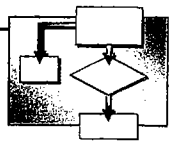


Equipment

The system and equipment you have for chromatography is also an important limiting resource. The most important constraints are the flow rate range and pressure limit of your column and chromatographic system, particularly in light of the flow rates you'll be operating with Perfusion Chromatography. These determine the column size (and thus sample size) that you can comfortably run, as well as the particle size of the packing, which is an important determinant of resolution.

With POROS prepacked columns, system pressure constraints can by and large be overcome by the availability of high and low pressure packed versions (column series) of each column chemistry. By choosing the column series with the appropriate pressure flow characteristics, flow rates can be maximized for Perfusion Chromatography within the pressure limits of your particular system. Refer to the *POROS Columns and Media Selection Guide* for more information.

By selecting the right column series, you can use POROS columns to advantage on most chromatography systems. The BioCAD workstation and BioCAD/*SPRINT* system from PerSeptive Biosystems were designed specifically for Perfusion Chromatography and so contain features not normally found on conventional systems that further facilitate the systematic experimental approach described on the following pages. Some of these features and tips on how to use them can be found in the method protocols outlined in Section 4 — *Modes of Chromatography.*

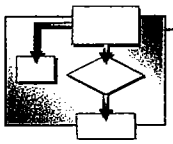


EXPERIMENT

Once your separation problem has been fully defined, you next conduct a program of experiments to find a solution. An empirical approach is necessary because of the complexity of chromatography, the biomolecules themselves and the sample matrices. Even though mathematical models or software simulations may provide useful insights into a development direction, they have limited usefullness for biomolecules. There is no substitute for reasonably comprehensive experimental data.

Unfortunately, the run times associated with conventional chromatography materials often make it impractical for a researcher to take a true systematic approach to generating this data. Instead, if a given run or trial solution does not work, it is highly tempting (in view of the limited number of runs that can be practically performed with conventional media) to change several variables at once in a "best guess" at the answer based on the data obtained so far.

The problem with this "hit or miss" approach is that it can be almost impossible to understand *why* a given method works, since the amount of actual information gained about the separation system is limited. It is easy to miss solutions that may be far more advantageous (for example, that may eliminate additional steps on the way to the final goal). In addition, if something changes (e.g. in the sample or the requirements of the separation itself), there is little information to use in making the necessary adjustments, and the "hit or miss" approach must be started over again.

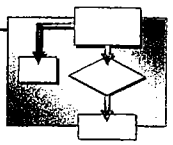


With systematic method development, in contrast, you utilize the short run times enabled by Perfusion Chromatography media to execute a program of experiments to examine each critical variable, one at a time — often in an automated way as with the BioCAD workstation. The idea is to build a coherent picture of how the separation system operates. This picture, in turn, enables you to then rationally design and implement the best available solution, and can provide a range of options for modifying the system to meet changing demands.

Note that this systematic approach does not preclude the use of statistical design techniques, in which more than one variable is changed in a controlled way. Statistical experimental design can be a very powerful way to limit the number of runs, conserving both sample and time. Although several parameters may be varied at once, the objective is actually to gain more information about the system behavior with respect to those parameters. A detailed discussion of statistical experimental design is beyond the scope of this handbook, but these techniques are clearly recommended as an adjunct to the methods covered here.

General Experimental Framework

One major issue in designing an experimental program is that many of the variables are interrelated, so that changing one will affect the results of changing the others. The surface chemistry of the column packing is the most critical variable in this regard, since it has the most complex effect on both the ultimate separation and on the effects of all the other variables. The pH has a similar effect in the case of ion exchange. It is important to take these dependencies into account so that you will not miss any important effects, but in such a way as not to waste time and sample with unnecessary runs.



An example of this is the method commonly used for "screening" different column packings. In this method, each packing is tested under a single, standard set of operating conditions. Sometimes this works, but very often the best performance of each packing will not be observed because the single set of conditions selected for screening is not even close to optimal for all the packings. It is easy to miss a unique characteristic of a particular packing because it was not tested under suitable conditions.

Taking these dependencies into account, it is possible to utilize a general framework for designing experiments in the systematic approach, as illustrated in figure 2-5. Of course, you should always consider your own resources and goals, and limit the work as much as is necessary or appropriate. However, you will frequently find it worthwhile to at least consider the entire framework, even when your work must be more restricted.

Experimentation begins with selecting one or more column packings to be tested, together with a starting mobile phase chemistry. You should then at least roughly optimize the elution gradient for each packing. In the case of ion exchange, this should be performed at a number of different pH values. Once you have completed this "mini-optimization" for each packing, you then choose the best one for further development.

With the selected column, you can then "fine tune" by modifying other variables in the mobile phase one at a time (such as organic solvents, eluent composition, buffer salt, etc.). If you discover a modification that has a large and significant impact on the selectivity or recovery characteristics, you should ideally return to the beginning and check the effect of the new mobile phase on each packing in your test panel. This ensures that your "discovery" will be thoroughly evaluated.

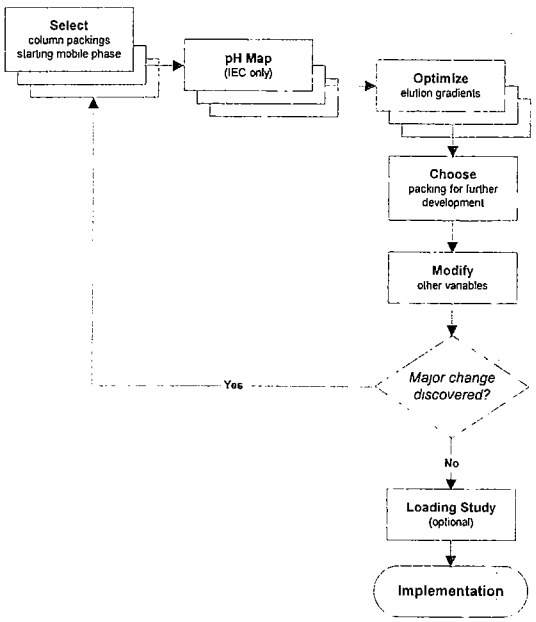
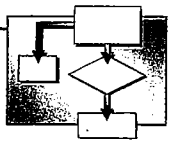


Figure 2-5. Basic experimental framework for complete systematic development

You can often streamline the experiments, as suggested in the *Minimal Method Development* schemes detailed in Section 4 — *Modes of Chromatography*. For example, in the case of ion exchange, you can use a shallow (e.g. 100 CV) gradient which covers a broad ionic strength range (0-1 M) when testing each pH on each column to eliminate the need for gradient optimization. Although this gradient is always longer and possibly more shallow than you will need in the final separation, it ensures that you will obtain close to optimal resolution for each set of conditions, and does not take long to run with Perfusion Chromatography media.

It may seem extreme to retest each packing material with any major mobile phase modification, but often this is important. For example, a high ligand density ion exchange packing (such as POROS HS) may give poor results with a very hydrophobic biomolecule due to secondary binding interactions. With a standard starting mobile phase, a low ligand density support



(such as POROS S) may give better peak shape, selectivity and recovery. However, if an organic solvent is added to the mobile phase, similar performance may be achieved on the POROS HS, but with a much higher loading capacity.

The number, nature and range of the variables you should examine in a systematic development approach depend upon a number of factors:

- The class of biomolecule you are separating and its characteristics
- The mode of chromatography you are using
- The resources (time, system and sample) you have available
- Your objectives for the separation

The variables and approaches you should consider based on the biomolecule, resources and objectives are covered in Section 3 — *Developing Your Application*. Detailed experimental plans for each mode of chromatography are covered in Section 4 — *Modes of Chromatography*. Some additional considerations in designing systematic development experiments are given below.

Column

The most important aspect of the column is the surface chemistry of the packing in a given chromatographic mode. Some differences in surface chemistry are obvious (such as between strong and weak ion exchange packings) while others are more subtle (such as between the different ligand density packings POROS S and POROS HS). In all cases, however, the effects on the separation of changing the surface chemistry are complex and usually unpredictable, so a systematic testing approach is necessary. Refer to the *POROS Columns and Media Selection Guide* for a complete description of different chemistries available from PerSeptive Biosystems.

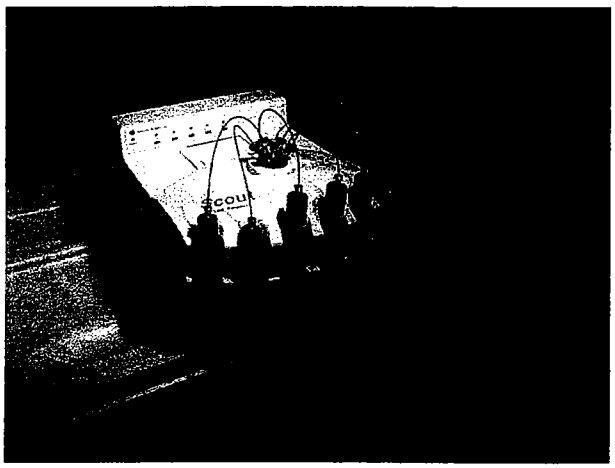
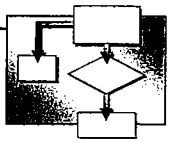


Figure 2-6. The optional SCOUT™ column selection valve system automates column chemistry experiments on the BioCAD system.

Other aspects of the column are not so complex, and may be optimized for a particular application as part of the *Implementation* stage of method development (see page 63). The packing particle size has only a very weak effect on the selectivity, and can normally be increased or decreased once the separation chemistry is optimized to balance the requirements for low bandspreading and low back pressure. The column dimensions are generally selected based on the amount of sample being run, and the column hardware and format are chosen based on cost and convenience.

Column length affects a number of parameters and should be changed with some care. The number of *theoretical plates* the column can generate is proportional to length. Total capacity is proportional to column volume, which is a function of column length (and diameter). Pressure drop is proportional to length, which directly affects the maximum possible flow rate. The run cycle time tends to increase with increasing column length but this is not a major consideration with Perfusion Chromatography. The column length usually does not have a critical effect on the selectivity of the separation itself.

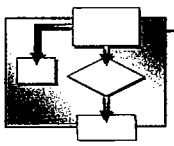


pH Map

The pH of the mobile phase is often the most important single variable in a separation. This is almost always true for ion exchange, but is frequently the case with other modes of chromatography such as hydrophobic interaction, reversed-phase or even affinity. The pH affects not only the overall charge and charge distribution of the biomolecules in solution, but also can cause significant changes in their conformation in solution, which can change the functional groups which are accessible to the binding surface.

One convenient means for changing the pH is a four-solvent blending system. Two of the solvent channels are used for a concentrated buffer, adjusted to the high and low pH's of the buffering range, respectively. The ratio of these two channels sets the pH. The other channels are used for water and concentrated eluent, respectively. By blending these channels appropriately with the ratioed buffer channels, you can vary both the buffer and eluent concentrations at any given pH.

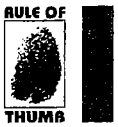
The BioCAD system provides both hardware and software for performing this blending automatically and even includes a template for designing and executing a complete pH mapping experiment. However, any quaternary blending system can be used. In this case, you will need to perform an experiment to determine the relationship between the high pH/low pH buffer blend ratio and the final pH. Once this is determined, a spreadsheet can be set up to determine the % composition values for given buffer concentrations and eluent concentration gradients.



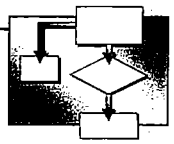
Gradient/Elution Optimization

Gradient optimization is, in principle, a rather simple matter of adjusting the starting and ending eluent concentrations and gradient duration in column volumes (which sets the slope) for optimal resolution of all the molecules of interest in the mixture. One would think that results from a quick run with a steep gradient covering a wide range in eluent concentration could be used directly by setting the starting concentration at the elution point of the first peak of interest, the ending concentration at the elution point of the last peak of interest, and the duration by the number of peaks in between. Unfortunately, due to the behavior of molecules on a column, such a simplistic approach rarely, if ever, actually works.

The problem stems from the fact that even when eluting in a gradient, molecules are *always* moving down the column. The rate at which a molecule moves at any time is determined by its isocratic retention at the instantaneous eluent concentration. At very low concentration, it may move very slowly, often imperceptibly. As the eluent concentration increases during the gradient, the molecule begins to move down the column at an ever-increasing rate until it shows no retention and moves at the eluent flow rate, at which point it certainly will elute from the column. However, elution may occur well before that point, if the gradient slope is low enough that the molecule reaches the bottom of the column during the "slow movement" stage. This same effect also serves to increase the peak width with decreasing gradient slope.



As the gradient slope decreases, peaks will tend to become lower and wider, and elute at lower eluent concentrations.



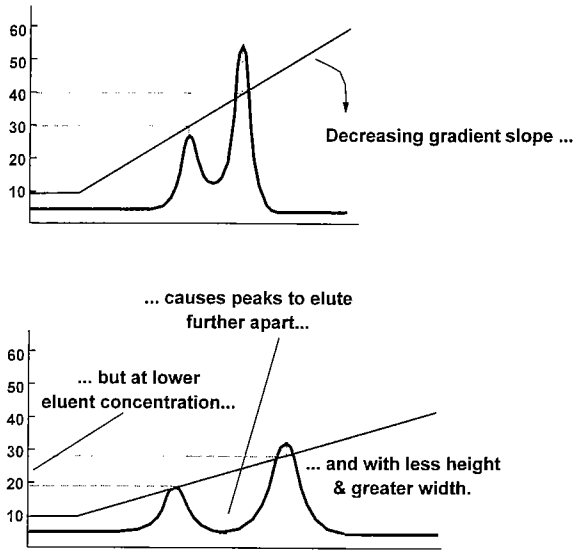


Figure 2-7. Effects of gradient slope on elution

The degree to which the elution point shifts with changing gradient slope depends upon the number of sites of interaction between the bound molecule and the surface, as well as the mode of chromatography. For smaller molecules, such as peptides, the isocratic retention often changes rather slowly with changes in eluent concentration, whereas for large proteins the change may be extremely sharp. For this reason, changing the gradient slope can actually change the selectivity and even cause a reversal in elution order in extreme cases. Thus the conclusions you may draw from a quick screening experiment with a steep gradient may not be valid as the gradient is optimized. The high throughput of Perfusion Chromatography media allows you to perform even initial optimization experiments at low gradient slopes, which can eliminate some of the need to perform detailed optimization experiments.

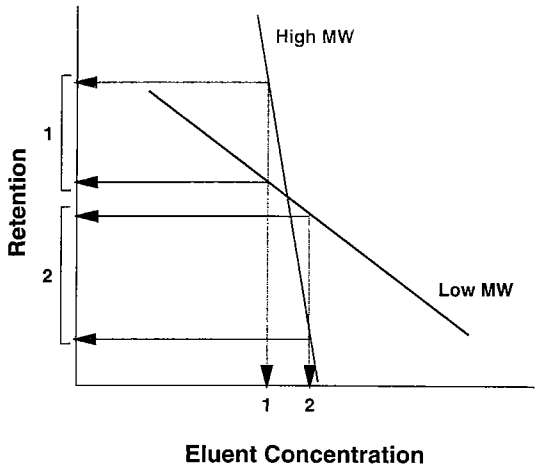
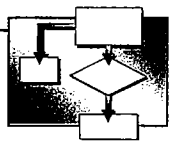


Figure 2-8. The effect of eluent concentration on isocratic retention varies with molecular weight. In some cases, a subtle change can not only shift retention dramatically, but even reverse the elution order.

This effect also can make it difficult to design step elution protocols based on the results of gradient runs. At best, the gradient results provide a useful starting point, but the optimal elution steps may well be at a significantly different eluent concentration than the gradient results suggest.

Caution should be observed when using steps to isocratically separate closely retained molecules, because even very small changes in the eluent concentration can dramatically affect the retention, especially for larger proteins. Often a gradient, even a shallow one, may provide a more reproducible separation. Step elution should be used when the selectivity is high, and the molecule of interest can be eluted under non-binding rather than weakly-binding conditions.

When working with gradient elution, always be sure to take into account pre-column delay volume (which delays the formation of the actual gradient) before assigning an eluent concentration based on the theoretical, programmed gradient. The BioCAD system can display both the theoretical and actual gradient to aid in the interpretation of gradient runs.

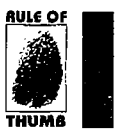


Loading Study

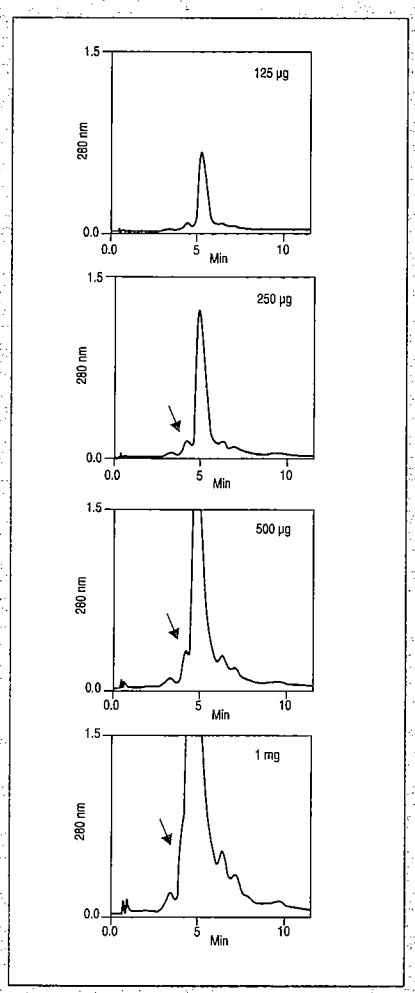
Method development is often performed at very low loading, primarily to reduce the amount of sample consumed. In some applications, especially for preparative work, you will then need to perform a loading study in order to determine how much sample can practically be run upon scale up. (*NOTE*: If sample is not at all limiting, you should perform method development experiments at higher loads. If loads are too low, you may not be able to detect some contaminants that will show up later when loading is higher.)

At relatively low loads (typical of analytical applications) the behavior of a column as the load is increased is straightforward — the retention time and the peak width stay relatively constant, peaks usually have an ideal "Gaussian" shape, and the peak height increases linearly with the load. The range of loading in which this condition holds is called the *linear range*, which corresponds to the range on the adsorption isotherm in which bound concentration increases roughly linearly with solution concentration (see Section 5 — *Basics of Chromatography)*.

At some point, however, the load exceeds the linear range and the column enters the *overload range*. In overload, the peak width increases, the peak shape becomes more triangular, and the retention time generally decreases with increasing load. This behavior is more typical of preparative chromatography. Eventually, the surface binding capacity of the column is exceeded, and some of the injected sample that would ordinarily bind breaks through during injection.

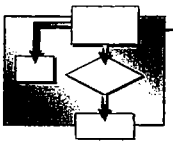


In preparative chromatography, expect the peak width to increase and the retention to decrease with increasing sample load.



Sample:	Synthetic 16-mer peptide, loading as shown
Column:	Oligo R3 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA
Eluent (B):	80% acetonitrile, 0.08% TFA
Flowrate:	2.77 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	215 nm
Elution:	15 - 50% B in 20 CV

Purification loading study of synthetic 16 mer peptide on Oligo R3 reversed-phase medium. Note reduced retention time, increased peak width and reduced resolution on the leading edge (arrow) as loading is increased — acceptable as long as original purity/recovery objectives are met.



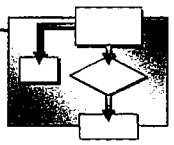
Unfortunately, the precise behavior of the separation with respect to sample load is not easy to predict, particularly in the overload range. The retention and bandspreading change in a non-linear way with loading. Different molecules can behave very differently, and the mode of chromatography and even mobile phase chemistry can have a major effect. Solubility can also be an important limiting factor, since molecules can become extremely concentrated within a peak on a column, even exceeding their solubility in the mobile phase.

The only way to reasonably determine the effect of loading is by experiment — simply running the separation with increasing sample loads, as can be done automatically with BioCAD Workstation Loading Study Templates. The maximum load at which the separation still meets the original objectives for resolution and recovery is referred to as the *loadability* of the column.

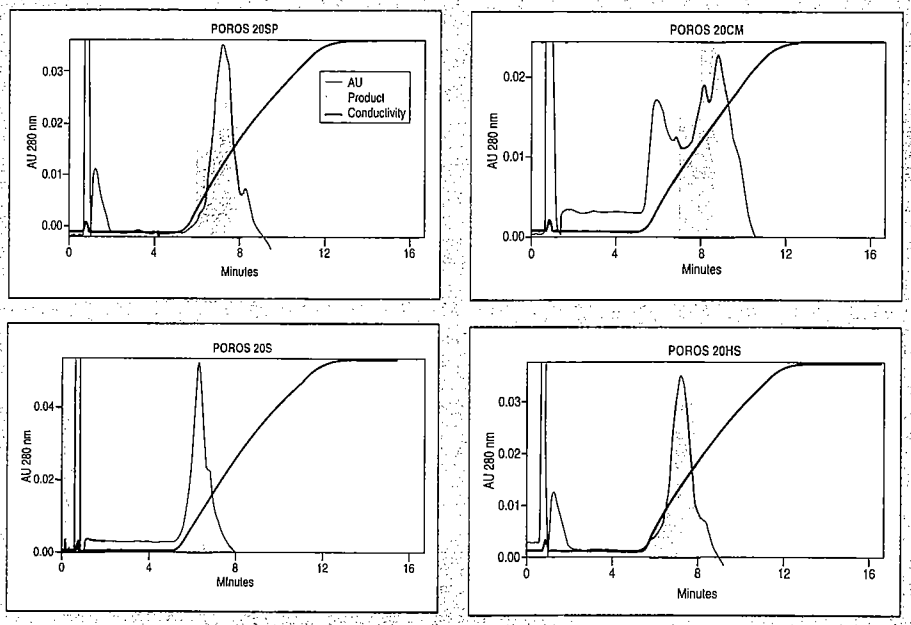
Loadability is a function of all the other chromatographic conditions, and should thus only be determined after a separation is optimized.

Sometimes a full loading study is not practical because of sample limitations. In this case, you can estimate the loadability based on the total binding capacity of the sample (or of protein in general) on the column. If a substantial fraction of the sample does not bind to the column under the loading conditions, take this into account. The rule is as follows:

For high resolution preparative separations, load 10-20% of the total binding capacity on to the column. For simple capture, load up to 30-50% of the total binding capacity.



EXAMPLE OF SYSTEMATIC METHOD DEVELOPMENT



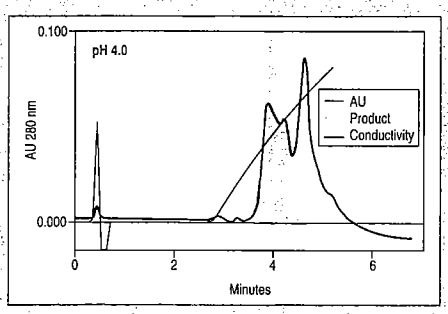
Sample:	0.5 ml concentrated cell culture supernatant (1.33 mg/ml)
Column:	Self Pack POROS 4.6 mmD/100 mmL, chemistry as indicated
Starting Buffer (A):	50 mM acetate, pH 4.5
Eluent (B):	50 mM acetate, pH 4.5 + 1 M NaCl
Flow Rate:	5 ml/min (1805 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 100% B in 20 CV

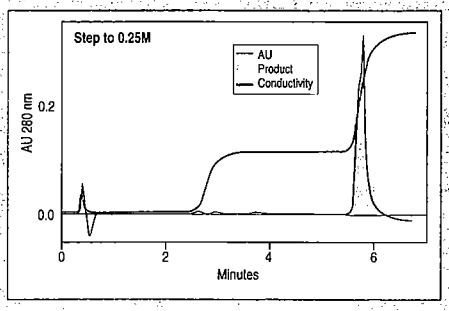
Example of a full separation method development on a recombinant human enzyme intended for therapeutic use. As shown on this page, the first step was to evaluate the range of POROS cation exchange chemistries to determine which had the best selectivity for the product of interest. Self Pack technology was employed to facilitate chemistry screening. POROS SP was chosen for further studies.

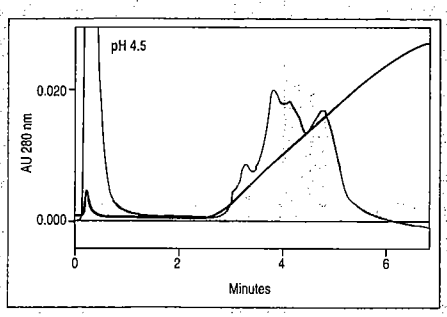
A pH mapping experiment was next conducted (next page, left hand series). pH 4.0 was determined to be optimal due to maximal product recovery. At other pH's the product was only partially bound; activity was spread out over the entire chromatogram.

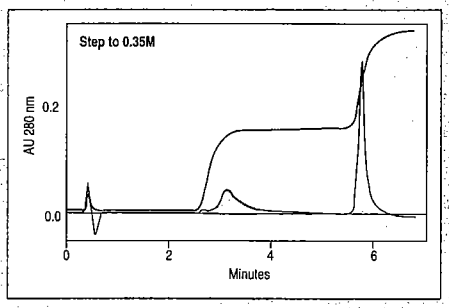
Because the separation was being developed for future scale-up, gradient optimization was conducted in the form of step elution (next page, right hand series). The optimum step was chosen at 0.45M NaCl because the product eluted in a small volume and was well separated from contaminating proteins.

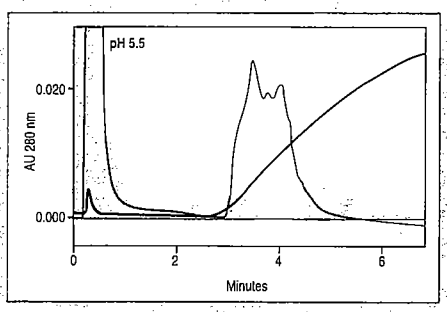
The entire method development was completed in two days. Using conventional resins and equipment, a similar series of experiments, if conducted at all, would have taken weeks. Work conducted by Anastasia Moisidis, Michael McNamara, Isabel Roberts, and Peter Schoofs, CSL Limited, Australia. *Analysis*, April 1995. Reprinted with permission.

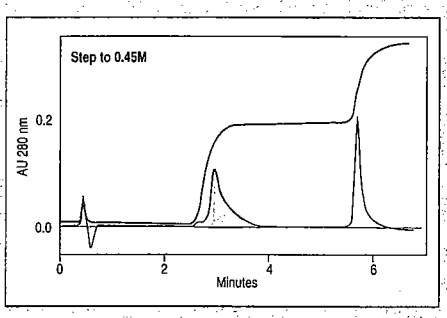






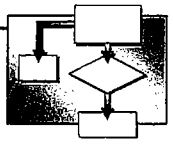






Sample:	0.5 ml partially purified cell harvest (5 mg/ml)
Column:	Self Pack POROS SP 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM acetate (Chromatogram 1); 20 mM HEPES/MES/acetate (Chromatograms 2&3), pH as indicated
Eluent (B):	Buffer A + 1 M NaCl
Flow Rate:	8.5 ml/min (3000 cm/hr)
Elution:	0 - 100% B in 20 CV

Sample:	0.5 ml of partially purified cell harvest (5 mg/ml) used in previous experiments
Column:	Self Pack POROS SP 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM acetate, pH 4.0
Eluent (B):	50 mM acetate, pH 4.0 + 1 M NaCl
Flow Rate:	8.5 ml/min (3000 cm/hr)
Elution:	Step elution as indicated



EVALUATE

During the experimental process, you will constantly need to evaluate the performance of the chromatographic runs against your objectives. The primary criteria are the resolution of the separation and the recovery of the product. In addition, for many applications, the capacity of the column is also important. Formal measurements of these parameters are discussed at the end of Section 5 — *Basics of Chromatography*. Finally, there are a number of practical aspects that should be considered in evaluating a separation.

Resolution

Resolution is the actual measurement of the degree of separation between the target molecule and other molecules in the mixture. Although resolution is a convenient single parameter for characterization, you must always remember that it is the result of two completely independent effects — *selectivity* (the relative difference in retention between two peaks) and *efficiency* (the width of each peak).

Selectivity (α) is perhaps the most critical parameter in achieving a satisfactory result, whether the objective is an analysis or a kilogram-scale preparative separation. The retention (k') of each molecule in a mixture is affected by almost every aspect of the chromatographic method, including the selection of packing material, the mobile phase chemistry and the elution profile. Because it is critically affected by so many parameters, selectivity can be challenging to optimize.

Bandspreading (which serves to reduce efficiency) becomes important when the selectivity has been optimized and the peaks are still not completely separated. Peak width is determined primarily by the particle size of the packing material, but is also affected by sample load, column length, elution profile and even the details of the surface and mobile phase chemistries. Minimizing bandspreading is especially critical for analytical chromatography.

RULE OF THUMB

Use a smaller particle size, longer column and/or smaller sample size to reduce the effects of bandspreading.

Poor resolution can be corrected by dealing with either one of these factors (as illustrated in the following figure), but the techniques employed to do so and the costs involved in time and money may be quite different.

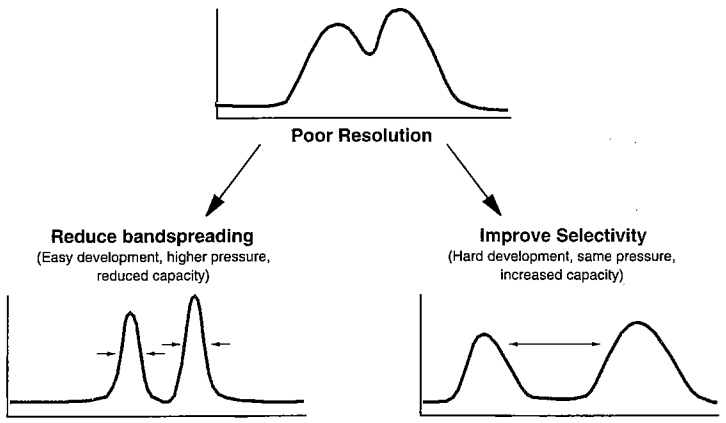
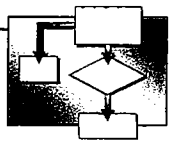


Figure 2-9. Effects of improving selectivity vs. efficiency

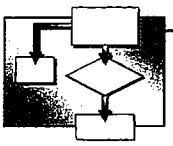
Particularly in large scale preparative chromatography, it is often a better strategy to focus on improving the selectivity through method development (even though this is more challenging) since improvements in efficiency almost always come at the expense of higher pressure drop, lower capacity, and higher media costs. However, in cases where a purification is being run a few times on a small scale, using the "brute force" approach of higher efficiency may take less time and be quite effective. High efficiency is more important in analytical chromatography, where complete separation between peaks is critical and sensitivity is a direct function of peak height.



Formal measurements of resolution based on peak separation are not the only measurements of the separation. *Purity* or *specific activity* refers to the amount of the specific target molecule (or target molecule activity) divided by the amount of total material (such as protein) present in the sample. The *purification factor* refers to the specific activity of the final eluted product divided by the purity of the starting sample. In the case of preparative chromatography, the purification factor may be a better, more meaningful measurement of the performance of the chromatographic separation than more abstract parameters such as resolution. In the case of a polishing step, where the target product is already at high purity, performance may be better measured simply by determining the concentration of the contaminants being removed.

Recovery

It is not sufficient for a chromatographic column to simply bind molecules from a sample and separate them. Those molecules must also be eluted completely from the column. *Recovery* of product or sample can be measured in two different ways. *Mass recovery* simply refers to the amount of material eluted from the column divided by the amount injected. This is usually the most important measurement for analytical chromatography. *Activity recovery* refers to the amount of biological or functional activity eluted from the column divided by the amount injected. This may be very different than the mass recovery for complex protein molecules, since the conditions used for elution of all the mass may, in some cases, give rise to denaturation of the proteins and loss of their activity. Often the term *yield* is used instead of recovery.



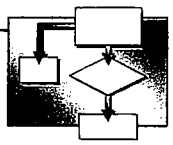
Purification Table

Because purity and recovery are so critical for preparative chromatography performance, it is important to keep track of both parameters for each step in a complex separation. A convenient method for summarizing this information is the *purification table*. This table lists the total concentration (often of protein), target molecule concentration (or activity) and volume after each significant step in the purification process. By calculation and comparison with the previous step you can then determine the total mass, total target molecule mass (or activity), specific activity (target divided by total), recovery and purification factor for each step.

TYPICAL PURIFICATION TABLE

	[Protein] *(mg/ml)*	[Target] *(units/ml)*	Volume *(ml)*	Total protein *(mg)*	Total target *(units)*	Specific activity *(units/mg)*	Step recovery (%)	Step purification *(fold)*	Total recovery (%)	Total purification *(fold)*
Starting Material	25	0.5	100	2500	50	0.02	100%	—	100%	0
Step 1	15	3.5	10	150	35	0.23	70%	11.5 X	70%	11.5 X
Step 2	6	2.0	8	48	16	0.33	46%	1.5 X	32%	16.5 X
Step 3	0.2	7.0	2	0.4	14	35	88%	105 X	28%	1750 X

The purification table can be a very useful tool for trouble-shooting a purification process. In the example shown, Step 1, the capture step, provides both reasonable recovery and a good purification. The final step also provides excellent recovery and a very large purification. Step 2, however, has a significant yield loss and provides little in the way of purification factor. This step is a possible candidate for elimination or reoptimization. In some cases, you should include non-chromatographic steps in a purification table, in order to identify possible improvements. A blank purification table is provided for your convenience. Copy it as needed to help you in your work.

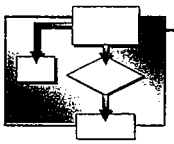


PURIFICATION TABLE

	[Protein] *(mg/ml)*	[Target] *(units/ml)*	Volume *(ml)*	Total protein *(mg)*	Total target *(units)*	Specific activity *(units/mg)*	Step recovery (%)	Step purification *(fold)*	Total recovery (%)	Total purification *(fold)*
Starting Material										
Step										
Step										
Step										
Step										
Step										
Step										
Step										
Step										
Step										
Step										
Step										

NOTES:

PerSeptive Biosystems



It is important to realize that a purification table alone sometimes does not show the complete picture. Sometimes a step provides little in the way of a purification factor, but may remove a critical contaminant, and thus be worthwhile, even with a significant recovery loss. Step 2 in the example might be such a case. As always, you must remember your goals when evaluating a separation under development.

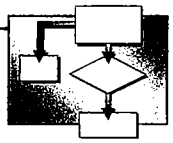
Capacity

Capacity (the amount of sample that can be loaded on the column) can be important in both preparative and analytical chromatography. The formal measurements of saturation and dynamic capacities are an important starting point (see Section 5 — *Basics of Chromatography*). However, the full capacity of the column can almost never be utilized completely. This is because bandspreading always increases and resolution thus decreases as the load of sample on the column is increased (as discussed above). The rate of decrease is a function of the particle diameter, with smaller (more efficient) particles showing a much more rapid decrease in resolution with load. This is one reason that larger particle packings are acceptable for large scale preparative applications.

A more relevant parameter is the *loadability*, defined as maximum effective sample load at which the required resolution can be obtained. Loadability must be determined empirically in a loading study, which should be performed after the rest of the separation is fully optimized.

Practicality

A wide range of different factors determine the practicality of a separation method for a given application. Issues such as the pressure drop, scaleability, reproducibility or cost can all play an important role. The relative importance of these factors depends upon the specific objectives and situation.

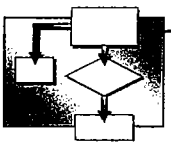


Robustness is one practical factor that can be important in both preparative and analytical separations. Robustness is defined as the ability of a separation to withstand small changes in operating conditions (such as sample composition or mobile phase pH) and still meet the objectives. While not critical for methods that will only be run a few times, robustness is a highly desirable characteristic for protocols that must be used for long periods or by different laboratories. Systematic development is key in producing robust separation methods.

One interesting practical issue is that of *throughput*. While *capacity* is a measurement of the amount of material a chromatographic column can process in a single run, *throughput* adds the time element to give a measurement of the actual productivity of the system. The measure used for throughput depends upon whether you are performing a preparative or an analytical separation.

Preparative throughput is normally defined as the capacity (or, better, the *loadability*) divided by the cycle time (the minimum time from one injection on a column to the next) and so is an indicator of how much material can be processed per unit time by the system. The distinction between capacity and throughput is especially important when designing a process-scale separation. If, as with Perfusion Chromatography, a column can be operated at very high speed (short cycle time), then the throughput can be much higher than a column of equivalent (or even higher) capacity that only operates at a very slow speed (long cycle time). As discussed on pages 66 to 68, higher throughput opens up a number of practical options for reducing development time and purification cost.

Throughput in an analytical system is defined simply as the number of samples that can be processed in a given unit of time. Analytical throughput is thus completely unrelated to the capacity or loadability of the column, but is a function of the cycle time for an assay.



IMPLEMENT

Once the basic parameters of the separation system have been experimentally explored and evaluated, you then can actually design and test a separation protocol to meet your objectives. How you approach this implementation stage depends very much upon your original goals. The following are some implementation considerations for analytical method development, multi-step separations and scale up.

Analytical Method Development

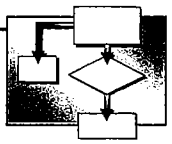
Analytical methods may be classified as either *qualitative, semi-quantitative* or *quantitative,* depending upon the type of information obtained. Chromatographic assays may be of any one of these types. In particular, chromatography works very well for more qualitative assays, because of the visual nature of the raw data. With care, highly quantitative assays can also be developed. These generally require baseline resolution of the sample components being assayed.

The key parameters that must be considered and examined for any assay are as follows:

- Accuracy
- Precision
- Linearity
- Detection limit (sensitivity)
- System suitability
- Specificity

The definitions of these parameters are given in the Glossary.

In general, analytical chromatography is performed with small particle (3 - 10 μm diameter), high efficiency media packed in small columns. The standard column diameter for analytical work has been 4.6 mmD. However, in recent years smaller diameter columns have come into use (2.1 and 1.0 mmD),



called *microbore* or *narrow bore* columns. More recently, technology has been developed to use even smaller diameter *capillary* columns (50 - 800 µm diameter).

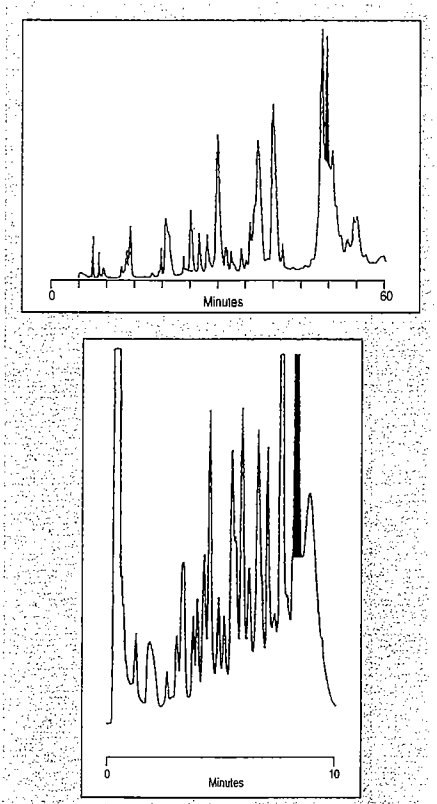
There are several motivations for reducing the column diameter. Smaller columns require less sample to achieve the same sensitivity. In addition, all other things being equal, the detection limit or sensitivity is an inverse function of the column diameter, so smaller columns provide more sensitivity. Finally, the solvent consumption is less, which is a growing concern in all laboratories.

Smaller bore columns demand extreme care in the choice and setup of the plumbing system components to prevent excessive amounts of bandspreading.

Multi-step Separations

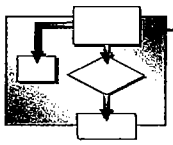
Purification from complex samples almost always requires multiple steps to achieve the degree of purity and level of removal of critical contaminants required. These stages can be divided into several categories:

Capture	The product is in a crude, often highly dilute form. The objective of this stage is to greatly reduce the volume of the product, and remove as much as possible of the bulk contaminants. It is particularly desirable to remove proteases and other degrading enzymes as early as possible in the process in order to maximize product yield.
Purification	The product moves from being a fraction of the feed stream to a high purity level (normally well over 95% pure and often over 99% pure). Purification usually involves several chromatographic steps with different modes or selectivities, although sometimes a highly selective affinity column can purify in one step.
Polishing	The product itself constitutes the vast majority of the sample and the objective is to remove trace contaminants down to undetectable levels. This is a very different problem from capture or purification, and often media with high resolution are employed.



Sample:	5 µl endoprotease Lys-C digestion of phosphodiesterase PDE-IV protein
Column:	POROS 10 R2 0.32 mmD/150 mmL
Starting Buffer (A):	0.05% TFA in water
Eluent (B):	0.05% TFA in 90/10 acetonitrile/water
Flowrate:	45 µl/min (3600 cm/hr)
System:	ABI 140A microbore system
Detection:	210 nm
Elution:	0 - 60% B in 10 min

Comparison of POROS reversed-phase separation in packed capillary column (lower chromatogram) vs. conventional microbore C18 column (upper chromatogram). The POROS separation was completed in one-fifth the time while maintaining similar chromatographic separation efficiencies. Reprinted with permisson from Kassel *et al. Anal. Chem. 1994. 66:*236-243. Copyright 1994 American Chemical Society.



When integrating multiple chromatographic steps, one important consideration is the sample preparation required for each step. For example, samples for ion exchange must be introduced to the column in low salt and are eluted in medium-to-high salt concentrations. Samples for HIC must be introduced in high salt and are eluted in medium-to-low salt concentrations. If an HIC step is placed before an ion exchange step, the salt would have to be removed, which can involve an extra dialysis or desalting step. If ion exchange is run first, however, salt only must be added prior to the HIC step, which is a much simpler process.

Whenever possible, the elution conditions of a preceding step should be compatible with the binding conditions for the following step.

Simple dilution is a sample preparation strategy with Perfusion Chromatography media that is not often practical with conventional approaches. Because the column can be loaded at very high speed, it may well be faster to simply dilute a sample until the conditions are suitable, and directly load the diluted sample onto the column at high flow rate. This approach can often eliminate or at least simplify sample preparation, even with "non-compatible" steps.

Example:
Interrelationship between flow rate, loading and gradient duration if the rules of scale up are followed.

Column	1	2	3
Dimensions (mm)	5 x 100	10 x 100	10 x 200
Bed Volume	2 ml	8 ml	16 ml
Flow rate (ml/min)	x	4x	4x
Loading (ml)	y	4y	8y
Gradient Duration (min)	z	z	2z

Bed volume can be increased by increasing diameter (Column 1 to Column 2), length (Column 2 to Column 3) or both (Column 1 to Column 3).

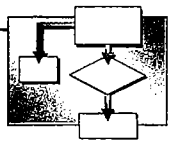
Doubling column diameter requires a four-fold increase in flow rate and load. Provided gradient duration is held constant in terms of column volumes, the gradient time will be the same.

Doubling column length necessitates keeping the flow rate the same and a doubling of load, but gradient time will double in order to keep it the same in terms of column volumes.

Scale up

Chromatography generally scales linearly without too much difficulty, provided a few key rules are followed:

- First develop the method at a small scale so that impurities are well separated from the peak of interest (i.e. optimize selectivity)
- Determine the loadability by increasing the amount loaded on the column until resolution is compromised and purity specification is no longer met
- Increase the bed volume in proportion to the required increase in sample volume

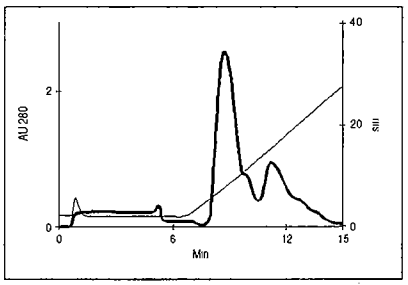


- Keep the linear velocity constant
- Keep the sample and mobile phase compositions constant
- Keep the elution durations the same in terms of column volumes

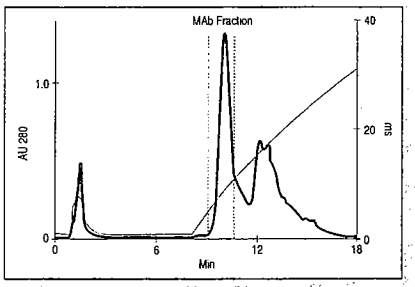
Some practical problems may interfere with scale up. It is critical that the column design maintain a uniform flow distribution as the diameter is increased. The solvent blending and pumping systems may require different technologies for operation at the different scales. Sometimes the pressure drop may be limiting at large scale. Cost factors may prevent a direct linear scale up.

One key concept when designing and developing a large scale process is to think about the constraints of operation at large scale and run the small scale development system within those constraints for testing and optimization. For example, at large scale, adding to column diameter is generally more expensive than adding column length, so a long, narrow column may be more practical than a short, fat one. On the other hand, increasing the column length increases the back pressure and decreases the maximum flow rate, increasing the separation time. Since the effect of column length is so critical, the effect of column length should be tested at small scale.

The advent of high speed, high throughput Perfusion Chromatography media has opened up a number of different design options for purification processes. With conventional media, capture of dilute feed streams is often a two-step process, in which a preconcentration stage using something like ultrafiltration is used to remove the water and a slow chromatography column is used to do the crude purification. With high speed media, these two can be combined in a single chromatographic dilute feed capture step.

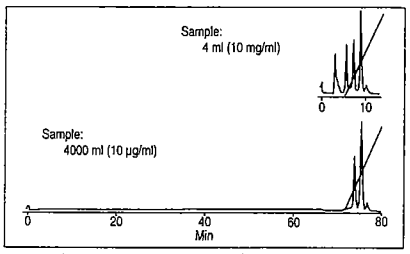


Sample:	3 ml crude ascites diluted 1:3 with start buffer (total 14 mg Mab)
Column:	POROS 50 HQ 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis-tris propane, pH 8.5
Eluent (B):	20 mM Tris/bis-tris propane, pH 8.5 + 1 M NaCl
Flowrate:	2.7 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 50% B in 18 CV



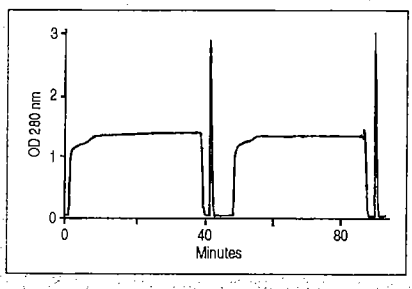
Sample:	10 ml crude ascites diluted 1:2 with start buffer (total 70 mg Mab)
Column:	POROS 50 HQ 10 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis-tris propane, pH 8.5
Eluent (B):	20 mM Tris/bis-tris propane, pH 8.5 + 1 M NaCl
Flowrate:	13 ml/min (1000 cm/hr)
Elution:	0 - 50% B in 20 CV

The principles of scale up are demonstrated in this ascites purification. Column length is constant (100 mm). The 5-fold sample load increase (70 mg vs. 14 mg) is proportional to the bed volume increase (8 ml vs. 1.7 ml). Volumetric flow rate is increased proportionally to ensure a constant linear velocity of 1000 cm/hr. By following these simple rules, the separations are very similar and take place over the same time. PerSeptive Biosystems Application Note PA 415.



Sample:	Horse muscle extract, concentration & loading as indicated
Column:	POROS 50 HQ 22 mmD/100 mmL glass column
Starting Buffer (A):	50 mM Tris/HCl, pH 7.2
Eluent (B):	Starting Buffer + 0.5 M NaCl
Flowrate:	60 ml/min (950 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 100% B in 10 CV

Example of dilute feed capture on POROS anion exchange column. After a 1000X dilution, the very weakly binding first elution no longer binds to the column. Otherwise, the elution profile is very similar. 100 column volumes were loaded in just over 1 hour.



Sample:	320 L (160 L/cycle) cell culture supernatant (39.7 mg IgG/L; 12.7 g total IgG)
Column:	180 mmD/40 mmL (1.0 L bed)
Starting Buffer (A):	PBS
Eluent (B):	PBS + 0.25 M NaCl
Flowrate:	4 L/min (850 cm/hr)
System:	AutoPilot® System
Detection:	280 nm
Elution:	Step to 100% B

Process scale separation of monoclonal IgG showing how the high flow rates on POROS media for dilute feed capture can be used in conjunction with cycling to allow the use of a smaller column in a short period of time. The process resulted in a 45X concentration of the product with >95% recovery.

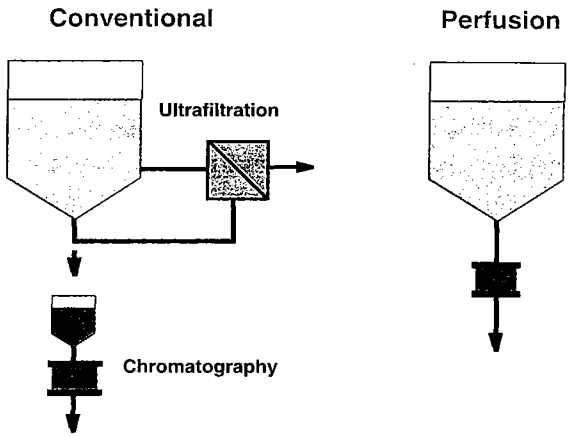


Figure 2-10. Dilute feed capture using perfusive media can eliminate preconcentration steps.

A second interesting option is to use the high throughput to reduce the size of the columns needed through rapid cycling. If a high speed column can be operated with a cycle time, say, 1/4 that of a conventional column, then a column 1/4 the volume could be run 4 times in a row to get the same throughput.

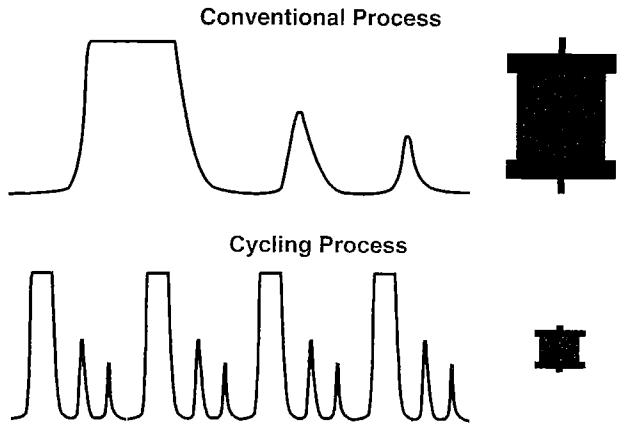
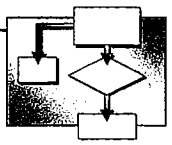


Figure 2-11. Cycling purification with high throughput column



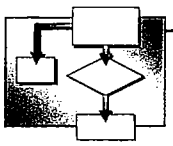
Cycling operations are not always desirable, for reasons of validation, quality control, etc. However, in many cases there are significant benefits of reduced space requirements, reduced operating costs and greater flexibility of operation. Cycling can confer a particular advantage when the column packing is quite expensive, as is the case for affinity chromatography media. In research, cycling can completely eliminate the need to scale up the column itself, but allow large amounts of material to be produced with the same column used for method development. This not only eliminates the cost of a larger column, but also reduces the uncertainty involved in scaling up.

TROUBLESHOOT

Even when following a systematic approach, at some point you may need to simply solve problems that arise with the method. Problems can arise in a number of different areas — bandspreading, peak shape, selectivity, recovery, pressure, reproducibility, column life, etc. Sometimes a method that has worked perfectly well for some time can suddenly fail. Solutions can range from the very obvious or simple to the very subtle or complex. Sometimes intuition honed by years of experience is the only good guide, but even the beginner can solve many of the common problems that may arise.

One cardinal rule in troubleshooting is to *only change one variable at a time*. This is the key to systematic development as well, and, in fact, the data you obtain by using a systematic approach will prove to be invaluable when any problems do arise. In troubleshooting, however, the one-at-a-time approach is absolutely critical, because you not only need to solve a problem, but you also need to know why it happened so that it will not recur.

The focus in this section is on fixing problems during method optimization, rather than those that arise after an optimized method is put into practice. However, the basic principles of troubleshooting chromatography are the same in both cases. A

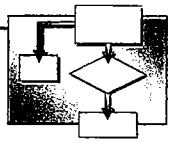


complete guide to troubleshooting chromatographic methods is beyond the scope of this book, but the following points will provide a starting point for the most common problems. Please contact PerSeptive Biosystems' Technical Support for assistance with any issues you cannot solve on your own.

Bandspreading

Excessive bandspreading (or insufficient chromatographic efficiency) is one cause of poor resolution. If the peak shape is good (see next page), try the following to reduce bandspreading:

- *Use a smaller particle size.* Efficiency is roughly proportional to particle diameter. However, pressure drop is inversely proportional to the particle diameter squared, so that you may have to reduce the flow rate to stay within the pressure limits of your system.
- *Use a longer column.* Efficiency is proportional to column length. Note that the elution gradient may need to be adjusted to stay constant in column volumes, and that the pressure drop at a given linear velocity is proportional to column length.
- *Repack the column.* Sometimes efficiency is lost due to deterioration of the column bed or voiding at the top of the bed.
- *Check your system.* If you are trying to achieve very high efficiency, use the smallest practical tubing diameter, and keep the tubing runs as short as possible. Also check fittings, valves, flow cells, etc. for dead volumes which can reduce efficiency. These considerations are especially important when working with 2.1 mm diameter POROS columns.
- *Reduce the sample load.* Loading may effect both bandspreading and retention time, especially in the overload range.
- *Reduce the flow rate.* This is a reasonable strategy for conventional (non-perfusive) supports. However, with Perfusion Chromatography media, the flow rate effect is much less.



Peak Shape

The overall peak width part of bandspreading is controlled by the chromatographic efficiency. Many chromatographic peaks, especially under analytical conditions, have the rough "bell curve" shape of a Gaussian probability distribution. Several factors can cause a deviation from this shape. Some are normal, but some can be good indications of specific problems in either the column itself or the method.

For a normal "Gaussian" peak, the peak shape can be represented by a number called the *asymmetry factor (As)*, which is equal to the ratio between the back half of the peak and the front half measured in time (or volume). This ratio is typically measured at 10% of the peak height, although 5% is sometimes used. The "normal" range for asymmetry is around 0.7 - 1.5, although a wider range is often tolerated, especially in preparative work. Normal preparative peaks often exhibit a roughly triangular shape, with the front half very steep.

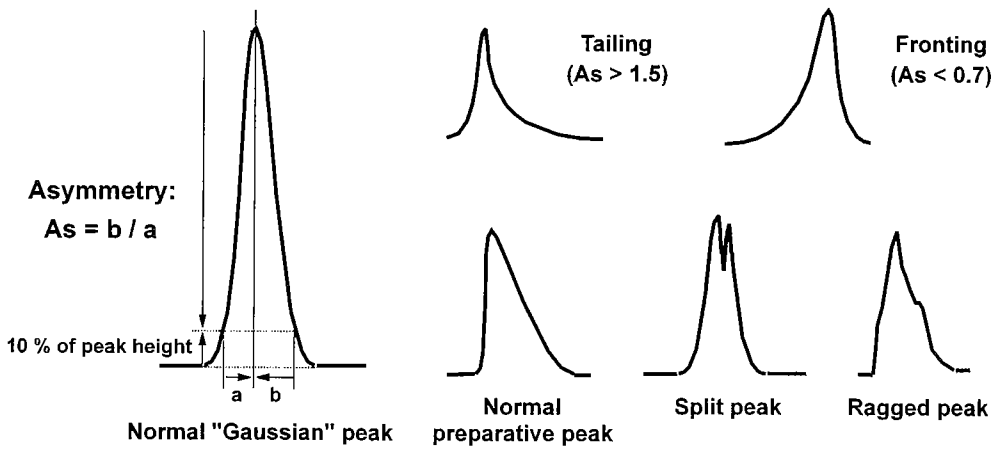
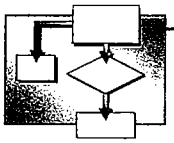


Figure 2-12. Asymmetry is the ratio between the back and front halves of the peak. Some typical normal and abnormal peak shapes.



The following are several types of problems with peak shapes, with suggested solutions:

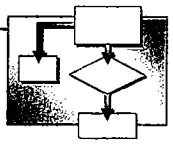
Tailing (As >1.5) is a long extension on the back of the peak, and is the most common peak shape problem. It can arise from either problems with the packing of the column or secondary, non-specific interactions between the sample molecule and the stationary phase.

- *Check for dead volumes in the column or system.* Repair or replace components if needed. Plugged column frits or screens are a common cause.
- *Repack the column.* If the column is simply voided, it can sometimes be repaired by moving down the adjuster assembly. Air trapped in a low pressure column is a common cause of tailing, and usually requires repacking.
- *Modify the mobile phase.* Try adding organic solvent (in ion exchange), or salt (for affinity). Try changing solvents, ion pairing agents (for reversed-phase), or pH.
- *Reduce the sample load.*

Fronting (As <0.7) is a long extension at the front of the peak Fronting is somewhat rarer than tailing and is usually a more serious problem. The most common cause is a channel through the column bed, although unusual interaction thermodynamics between the sample molecule and the column can cause the problem.

- *Repack the column.*
- *Modify the mobile phase.* Anything that modifies the solubility or selectivity could help.

Split peaks have a normal peak shape, except for a splitting near the top of the peak. Since split peaks can be caused by coeluting sample components, you should run an efficiency test to see if the problem is with the column. If peak splitting



is found upon isocratic elution of a small volume injection of pure tracer, consider the following solutions:

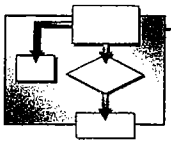
- *Clean the column.* Backflushing to remove trapped particles is particularly useful.
- *Replace the frit.* Sometimes a plugged or blinded area in the frit is a source of peak splitting.
- *Check the system plumbing.* Look for any poor connections, crimped tubing, etc., especially between the injector and the detector flow cell. Many instances of peak splitting can be traced back to the autosampler.
- *Repack the column.*

Ragged peaks are unusual, irregular, frequently irreproducible peak shapes. Sometimes these are caused by several co-eluting sample components (in which case the results are usually quite reproducible). A common problem is also poor solubility of the sample in the mobile phase, causing elution to occur in irregular bursts instead of a single band.

- *Check the system.* Any problem with the pump or blending system can cause ragged peaks.
- *Modify the mobile phase.* Anything that modifies the solubility or selectivity could help.
- *Reduce the sample load.*
- *Clean and/or regenerate the column.* Non-eluted components can cause ragged peak shape. In ion exchange, improper regeneration can leave the column with mixed counter ions, which can cause the problem.

Selectivity

Troubleshooting problems with selectivity is certainly among the most complex problems faced by the chromatographer. Finding the proper selectivity in the first place is the major



purpose of method development, and the techniques used to modify the selectivity to solve a particular problem are exactly the same.

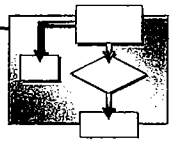
The systematic Perfusion Chromatography approach is extremely useful when trying to modify the selectivity of a separation. If you have followed this approach in initially developing the method, you will already have a good sense of the most fruitful avenues to take (or not take) in order to make the required changes. If nothing else, you should know how the variables you have already checked will affect the separation and can begin your search elsewhere if needed.

A good place to look if you are adopting an existing method is the *Minimal Method Development* for the particular mode you are using in Section 4 — *Modes of Chromatography*. If you have already performed the minimum development experiments, check the *Full Development Variables* for ideas to try.

Recovery

Poor recovery can be a problem in both analytical and preparative chromatography. In preparative work, not only must you recover the material itself, but often you must also recover biological activity. Recovery problems are quite specific to the target molecule characteristics, and you should always carefully examine those characteristics (especially solubility and stability), as well as the rest of the sample when approaching a recovery problem.

- *Modify the mobile phase.* Consider adding any reagents which will stabilize or solubilize the target molecule, although be careful to check their effects on the chromatography. Also consider the pH, which can have a significant effect on recovery. Additives which reduce secondary non-specific interactions (such as salt or organic solvents) may also help.



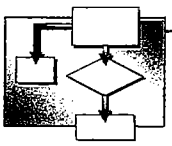
- *Reduce exposure to harsh conditions.* If the elution conditions are detrimental to recovery, consider rapid sample processing to remove those conditions as quickly as possible after the product is eluted. For example, collecting fractions eluted by acid from an affinity column into tubes containing concentrated neutral buffer can improve the yield of activity.
- *Change the packing surface chemistry.* Even in the same mode of chromatography, a lower ligand density packing material can greatly improve recovery. Sometimes you must trade capacity for recovery.
- *Speed up the run.* A limited option with conventional packings. However, using Perfusion Chromatography to significantly decrease the run time may improve recovery if there are enzymes degrading the target (e.g. proteases, nucleases or glycosidases) or harsh elution conditions.

Pressure

Excessive pressure is usually a rather simple mechanical problem. Plugging of the column from either particulate material or precipitation on the column is the most common cause. Column and system hardware problems are another source.

- *Filter the sample.* For most columns, the sample should be able to pass through a 0.45 µm microporous filter before being applied to the column. If this is not possible or practical, consider centrifugation or other kinds of filtration.
- *Filter the mobile phase.* For small particle analytical columns, it is also important to filter the mobile phase itself through a 0.45 µm microporous filter.

For most applications, both the sample and the mobile phase should pass through a 0.45 µm filter before being applied to the column.

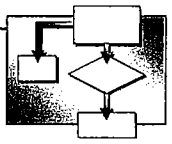


- *Backflush the column.* Sometimes material builds up on the top of the column bed, and can be removed by reversing the flow direction for a short period (backflushing). In extreme cases this should be done as part of every run.
- *Check the column and system.* Plugged column screens or frits, crimped tubing, overtightened fittings and too small a tubing size are all common problems.
- *Modify the mobile phase.* Anything which will help solubilize the sample or prevent precipitation should be considered. Also check the mobile phases for compatibility. Organic solvents can cause precipitation of salts on the column. The metal salts used in IMAC can also easily precipitate under some conditions.

Reproducibility

Reproducibility problems can be the trickiest to solve because the potential causes are quite numerous and do not necessarily occur one at a time. To begin troubleshooting a reproducibility problem, list *all* of the items which could *possibly* have changed, even things that may seem ridiculous. These should include the following:

Sample	New preparation methods, different source, different composition, different volume
Reagents	New bottles, new supplier, expiration dates, improper storage
Water	Different source, new cartridges in filter system, broken cartridge, poor maintenance of filter system
Mobile phases	pH, reagent concentration, temperature, mixing, labeling of bottles
Environmental	Seasonal changes in temperature or water quality, power surges or fluctuations



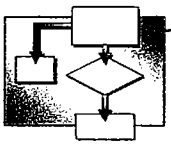
Once the obvious sources have been eliminated, the following are some suggested starting points:

- *Check the column equilibration.* Incomplete reequilibration of the column before each run is one of the most common causes of poor reproducibility, especially with weak ion exchange packings. If the column is fully equilibrated the chemical composition of the mobile phase coming out should be the same as that entering the column. Consider increasing the volume used. At the flow rates of Perfusion Chromatography, this won't add significant time to your experiments. Another possibility is to use a higher strength buffer for a small volume (to adjust the pH) followed by the more dilute starting buffer (to adjust the ionic strength).

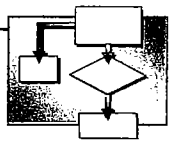
For most modes of chromatography, 10 column volumes of buffer is sufficient for equilibration.

- *Check the system.* Problems with the pump or gradient system are another common source of reproducibility problems. Erratic pressure readings are an indicator of pump problems.
- *Check delay volumes.* With very small volume columns, the system delay volume (including the volume in the sample loop) may be quite large relative to the column size. Make sure you have taken this into account.
- *Degas or sparge the mobile phase.* Sometimes, when mobile phases are blended, the gas solubility in the blend is lower than in the starting liquids, causing outgassing and bubble formation in the pump, which can lead to erratic performance. Try vacuum degassing or helium sparging the mobile phases, especially if organic solvents are involved.

If organic solvent is in one or more of the mobile phases, you should degas or sparge to prevent outgassing.



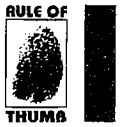
- *Preblend mobile phases.* With some instruments, asking the gradient system to make very fine changes in mobile phase composition may lead to reproducibility problems. In these cases, consider mixing the mobile phases off-line to the proper composition.
- *Control the temperature.* Retention behavior is always temperature sensitive, although to varying degrees, depending upon the sample and mode. If you need high precision in retention, control the temperature with a column heater or chromatography refrigerator.
- *Check column regeneration.* With ion exchange columns, it is important to control the counter ions for the functional groups on the surface at the beginning of the run. You should follow the regeneration procedure recommended in the column Operating Instructions.
- *Check column cleaning.* Some reproducibility problems are caused by sample that is not eluted fully from the column, which builds up and changes the packing surface characteristics. One diagnostic for this problem is to do a blank run, in which the full elution protocol is followed, but no sample is injected. Any "ghost" peaks which show up are a sign of uneluted sample or impurities in the buffer. If uneluted peaks are the problem, you should use a more rigorous column cleaning protocol (see next page).
- *Check mobile phase chemicals.* Sometimes a reproducibility problem is in the mobile phase. Occasionally the chemicals themselves degrade over time (triethylamine or TEA used in reversed-phase, which oxidizes easily, is a common example) or are variable in quality (non-biochemical grade ammonium sulfate is a common example). Even water can be a problem in critical applications.



Column Cleaning & Reuse

After a chromatographic run is complete, the column should be returned to the state in which it started, with no residual sample remaining behind to interfere with subsequent runs. Unfortunately, since the column is a solid phase, it is difficult or impossible to directly measure what is bound to the surface. Indicators of a problem include loss in capacity, changes in peak shape or retention, "ghost" peaks showing up on blank runs, and increasing back pressure. In the end, however, only actual reuse itself will allow you to determine if there is a problem.

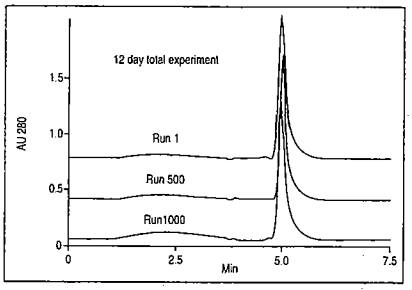
When developing a cleaning method, always remember the following rule:



To clean a column, all bound molecules must be* simultaneously *solubilized and released from the binding surface.

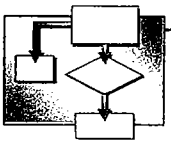
Sometimes this rule creates problems, especially in reversed-phase, where the high concentrations of organic solvent needed to release a very hydrophobic molecule from the surface may not be compatible with solubility. Mixed agents and steep gradients from a strong solubilizer to a strong eluent are good approaches in these cases. High temperature can also help. Consult the Operating Instructions of the packing material for specific suggestions for each chemistry.

POROS Self Pack technology gives you a cost-effective way to replace a column if there is ever any doubt as to its condition after subjecting it to cleaning protocols designed with the best intent.



Sample:	0.8 ml of 1 mg/ml Cohn Fraction II
Column:	POROS 50A 2.1 mmD/30 mmL
Starting Buffer (A):	PBS
Eluent (B):	12 mM HCl
Flow Rate::	0.6 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

The study of column lifetime under process conditions is important for the process development scientist, and convenient to undertake with POROS media. In this example, the effect of cleaning the media every second cycle with 2 M acetic acid was examined: 1000 runs were completed in just 12 days. The column packing showed no ill effect from such a rigorous cleaning protocol.

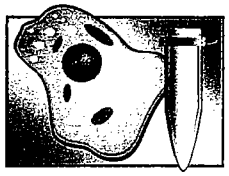


Cycle Time

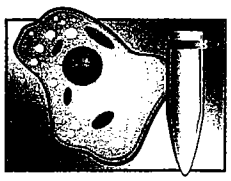
Often a method is not really practical unless the cycle time can be reduced. This is an issue in the analysis of large numbers of samples, or for monitoring applications. It can also be an issue in preparative applications where high throughput is needed, as for direct capture of dilute feeds or for cycling applications. The use of Perfusion Chromatography media is a major step toward reducing cycle time. Even with perfusive media, however, it may be important to reduce cycle time even further in some cases.

The basic tradeoff in shortening the cycle time is to reduce everything in the separation until it is *just* good enough to meet the objectives. The following are some suggestions:

- *Use a steeper gradient.* Sometimes the gradient is considerably shallower (i.e. longer in duration) than necessary for the separation. Reducing the gradient time or volume will both shorten the run and increase the peak height, increasing sensitivity.
- *Use a shorter column.* Shorter columns allow a shorter gradient time at the same column volume duration, as well as reduce the pressure drop to allow an increased flow rate.
- *Increase the flow rate.* Often the column can simply be run faster, especially with perfusive packings. Shorter columns or even larger packing particles will help. Even if it is not suitable to run faster during the separation, the equilibration steps can often be done at faster flow rates.
- *Reduce the separation scope.* If there is only one target, you should look at reducing the separation until just that one peak is well-separated, and sacrifice the separation of everything else. Time is often wasted separating peaks of no consequence for the application.



SECTION 3
DEVELOPING YOUR APPLICATION



SECTION 3
DEVELOPING YOUR APPLICATION

The power of chromatography allows you to precisely control separation selectivity for a particular application. While this power enables you to perform very challenging separations, it demands that you consider a potentially bewildering range of different variables in developing and optimizing a chromatographic method. The advent of Perfusion Chromatography facilitates the process of exploring each of these variables in turn. Nevertheless, the potential complexity of chromatography can pose a formidable obstacle to beginners. Even an expert can overlook important options and possibilities. This section of the *Guide* constitutes the first part of a "roadmap" for working through variables to perform an effective chromatographic separation.

METHOD DEVELOPMENT APPROACHES

The approach that you intend to use to develop your method is based on your needs, expertise, the availability of existing methods and the time and resources available to you. In general, you may be in a situation to

- Transfer an existing method to a POROS column
- Adapt a method that exists for a similar biomolecule (e.g. same enzyme family)
- Develop an entirely new method.

The simplest situation is when a method already exists on a conventional packing (e.g. from your own protocols, from the literature) for the specific molecule of interest. You can frequently transfer an existing method by keeping constant a number of key parameters and following a few simple rules for modifying the elution gradient and flow rate. This procedure is detailed on the following page (*Transferring an Existing Method*).

Any time that a method is transferred there is a good chance that the new packing surface chemistry differs enough that some modifications to the method will be needed. More commonly, an existing method is not available. Either way, you will have to develop a new method.

For modification or development of new methods, the best starting place is the class of biomolecule you are trying to separate — protein, antibody, peptide or nucleic acid. While every individual molecule is unique, useful generalizations can be made about the significant molecular characteristics of each class, the impurities of concern in different samples, and the chromatographic modes that are most useful. This section summarizes these generalizations, along with specific protocol suggestions. Based on this information, you should be able to design an overall separation scheme.

Once you have chosen your scheme, Section 4 — *Modes of Chromatography* contains specific information about how each mode works (for those who need it) and gives specific recommendations on how to develop and optimize a method in that mode by taking advantage of the benefits of Perfusion Chromatography.

When developing a new method, there are three possibilities:

- You may be extremely limited by either the time or sample available. You will need to make a "best guess" about the method for a single experiment. In this case, you should use the most common chromatographic mode(s) for the separation as outlined in this section, and use the *Starting Point*

Method given for each mode in Section 4. You should modify the base method as appropriate whenever you have specific knowledge about the chromatographic behavior or molecular characteristics of your own sample.

- You may be able to do a few optimization runs, but still want to keep things as short and simple as possible. In this case, you should perform the *Minimal Development Experiments* detailed for each mode in Section 4 — *Modes of Chromatography*. These experiments are designed to produce a workable method in the shortest possible time, and should produce reasonable results in a majority of applications. These *Minimum Development Experiments* take optimal advantage of the speed and high flow rate possible with POROS media.
- You may need to consider or test all of the possible variables affecting a separation, especially when you will be running a method repeatedly (as for an analysis or an industrial process), or for an especially difficult separation. Section 4 includes lists of all the variables that should be considered for each mode. Again, the short run times possible with POROS media makes comprehensive method testing practical.

TRANSFERRING AN EXISTING METHOD

To transfer an existing method to POROS packings, you must keep constant (or nearly so) some parameters of the existing method, while adjusting others appropriately in order to adapt the method to the Perfusion Chromatography column. By doing so, transfer to POROS media can be straightforward and the desired advantages immediately achieved. Keep in mind that any time you switch from one chromatographic media to another, simple direct methods transfer may not always give the exact results, due to the unique nature of each packing's surface chemistry. Some reoptimization may be needed (see *Limitations on Method Transfer*). However, such optimization can be quickly carried out on POROS media.

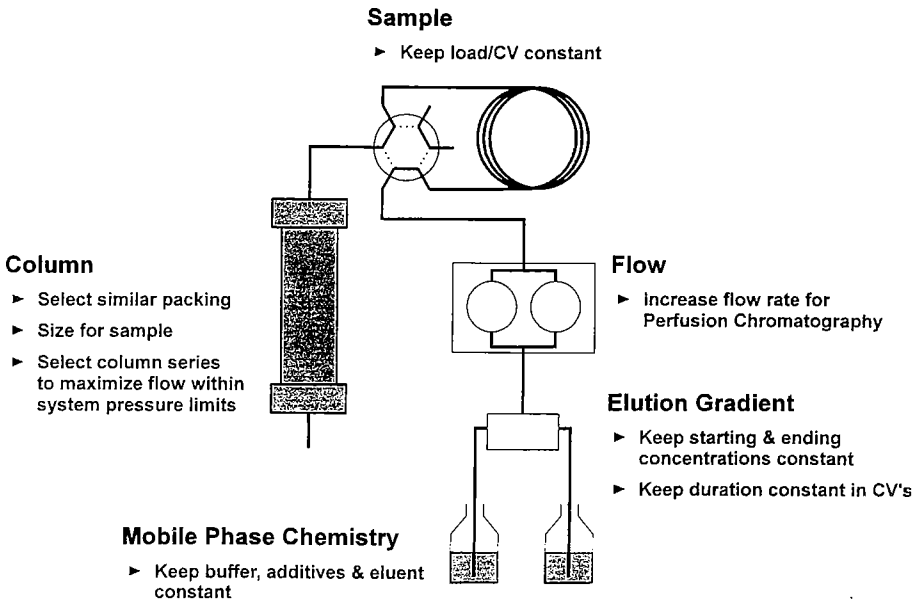
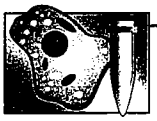


Figure 3-1. Rules for transferring an existing method to POROS

Flow Rate

- When using conventional media it is recommended to keep the linear velocity constant when transferring a method to a new column (linear velocity equals the volumetric flow rate divided by the cross sectional area of the column. See Section 5 — *Basics of Chromatography*). The same is not true for POROS media, since flow rate does not substantially affect chromatographic performance.

You should set the flow rate as high as desired within the pressure and pumping constraints of your system and column to take full advantage of Perfusion Chromatography.



Packing & Column

- Choose a POROS column packing with chemistry resembling as closely as possible the packing used in the existing method. Refer to the *POROS Columns and Media Selection Guide*, or consult with PerSeptive Biosystems' Technical Support group.
- If possible, choose a column of the same diameter and length as in the existing method. If a smaller or larger column is required to accommodate varying sample sizes, determine the appropriate column size using the calculation opposite.
- If using POROS prepacked columns, select the column series with the appropriate pressure/flow characteristics to maximize flow rate within the pressure constraints of your chromatography system. Refer to the *POROS Columns and Media Selection Guide*, or consult with PerSeptive Biosystems' Technical Support group.

Example:
The existing method protocol calls for loading 400 mg of total sample protein on to a 16 mm (1.6 cm) diameter (D) by 200 mm (20 cm) length (L) column. The column volume in this case is

$$CV = \pi (D/2)^2 L$$
$$= \pi (1.6\ cm/2)^2 (20\ cm)$$
$$= 40\ ml$$

The sample load per unit column volume (loadability) is

$$Loadability = 400\ mg/40\ ml$$
$$= 10\ mg\ sample/ml\ CV$$

For a 10 mm diameter by 100 mm length column the column volume is

$$CV = \pi (1.0\ cm/2)^2 (10\ cm)$$
$$= 8\ ml$$

The total amount of sample appropriate for this column is

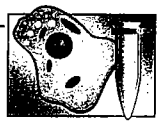
$$Total\ Sample\ Load = (CV)(Loadability)$$
$$= (8\ ml)(10\ mg/ml\ CV)$$
$$= 80\ mg$$

Sample

- Use the same sample preparation as that in the original protocol.
- Keep the mass or volume of sample per unit column volume very similar to the original protocol.

Mobile Phase Composition

- Keep the mobile phase the same as the existing method. Critical elements include buffer salt and concentration, pH, stabilizing and solubilizing agents, and the eluent(s).



Elution Gradient

- Use the same starting and ending eluent concentrations for all gradient segments.
- Keep the duration of each gradient segment constant in terms of **column volumes**. NOTE: You will need to change the gradient time (which is how most instruments are programmed) if either the column volume or flow rate is changed.

This latter point is particularly important when converting to Perfusion Chromatography. As you increase the flow rate, be sure to decrease the gradient time accordingly. Otherwise, you will throw off the gradient profile and consume excessive amount of buffer. This is often easy to overlook the first time you transfer a method to POROS media because of habits developed with conventional media, i.e., gradient **times** are kept constant when converting between columns of identical sizes because flow rates remain unchanged.

The BioCAD workstation allows programming in column volumes, which simplifies method transfer by eliminating the need for calculation on your part.

Example:
A simple linear gradient was originally developed for the 16 mm diameter by 200 mm length column mentioned in the previous example. The original method specified a gradient of 0-30% B over 5 hours (300 min) at a flow rate of 2 ml/min.

Gradient Volume = (300 min)(2 ml/min)
= 600 ml

As shown, the original column volume (CV) is 40 ml. Therefore:

Gradient Duration = (600 ml)/(40 ml/CV)
= 15 CV

To run an equivalent gradient on the 10 mm diameter by 100 mm length column (CV = 8 ml) at a flow rate of 10 ml/min, keep the starting and end points of the gradient the same at 0 and 30% B, respectively. Also keep the gradient duration the same at 15 CV. The gradient volume in this case would be

Gradient Volume = (15 CV)(8 ml/CV)
= 120 ml

The gradient time is thus

Gradient Time = (120 ml)/(10 ml/min)
= 12 min

Method Transfer Worksheet

The following worksheet (easily incorporated into an electronic spreadsheet format, if desired) gives the calculations required to transfer an existing method. To utilize the worksheet, first determine all the values shown for the current method. [*NOTE:* If the gradient consists of more than one segment, determine the gradient start, end, time and duration (in column volumes) for each segment.] Once the values have been determined for the current method, the column diameter, column length, and flow rate are inserted for the POROS-based method. These values are then used with those calculated for the current method to determine the final values for the POROS method.

METHOD TRANSFER WORKSHEET

		Current Method		POROS Method	
Column Diameter *(cm)*	d_C			d_P	
Column Cross Sectional Area *(cm^2)*	$A_C = \pi(d_C/2)^2$			$A_P = \pi(d_P/2)^2$	
Column Length *(cm)*	L_C			L_P	
Column Volume *(ml)*	$CV_C = A_C L_C$			$CV_P = A_P L_P$	
Flow Rate *(ml/min)*	Q_C			Q_P	
Linear Velocity *(cm/hr)*	$U_c = 60(Q_C/A_C)$			$U_p = 60(Q_P/A_P)$	
Sample Volume *(ml)*	V_S			$= V_S (CV_P/CV_C)$	
Gradient Start *(%B)*	$[E]_1$			$= [E]_1$	
Gradient End *(%B)*	$[E]_2$			$= [E]_2$	
Gradient Time *(min)*	T_G			$= CV_P D_G / Q_P$	
Gradient Duration *(in column volumes)*	$D_G = Q_C T_G / CV_C$			$= D_G$	

Limitations on Method Transfer

Transferring a method from one packing material to another rarely gives identical results. Sometimes this is simply a matter of all the peaks eluting earlier or later, in which case you can compensate by adjusting the elution gradient. In other cases, however, the overall separation is quite different, and simple gradient adjustments do not suffice.

When this occurs, the temptation is to make small adjustments to method parameters (such as the pH) in order to try to get equivalent results. Before doing this, first determine if it is really necessary. It may not matter if the chromatographic profile is exactly the same, as long as the peak(s) of interest can be identified and the objectives of the separation are met.

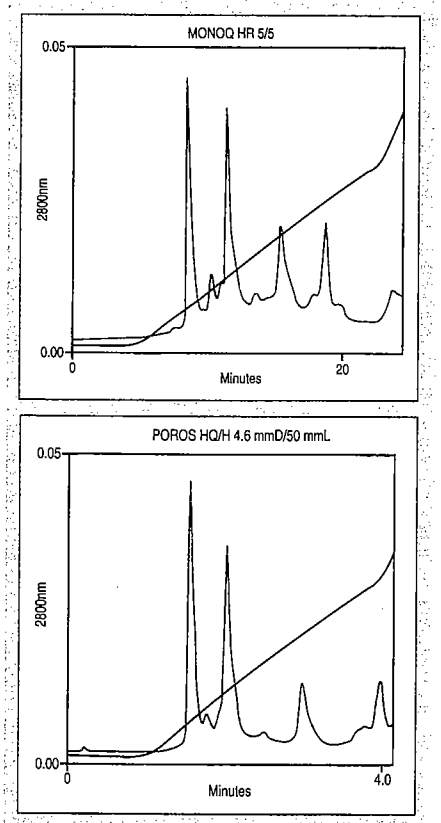
If additional methods development is deemed necessary, the speed of Perfusion Chromatography allows this to be done conveniently. However, instead of beginning with small adjustments to the existing method, you will almost always find it more efficient and effective to redevelop the method from the beginning. Try the *Minimal Development Experiment* as detailed in Section 4 — *Modes of Chromatography* to develop a new protocol.

PROTEINS

Proteins are probably the most widely purified biomolecules, as they constitute most of the key structural and functional elements in all biological systems. Much of modern biochemistry is concerned with understanding the structure and function of individual proteins and protein complexes. The biopharmaceutical industry was founded on the ability of genetic engineering and other tools of biotechnology to reliably produce any protein in large quantities at very high purity. Chromatography is an absolutely vital tool for both purification and analysis of proteins, both in research laboratories and at production scale.

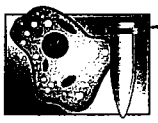
Sources & Key Impurities

The sources of proteins and the impurities present are incredibly varied. Some proteins are extracted from natural sources, including virtually any organism, organ, cell, virus or bodily fluid. Often the proteins of interest are present in these sources in very low concentrations in the presence of many other (sometimes highly concentrated) proteins and other biomolecules.



Sample:	10 µl injection of standard protein mix (5 mg/ml each of conalbumin, apotransferrin, ovalbumin, soybean trypsin inhibitor)
Starting Buffer (A):	20 mM Tris/Bis-Tris (1:1), pH 7.0
Eluent (B):	Buffer (A) + 0.5 M NaCl
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 70%2 B in 8 CV
Column 1:	MonoQ HR 5/5
Flowrate:	1 ml/min (306 cm/hr)
Column 2:	POROS HQ/H 4.6 mmD/50 mmL
Flowrate:	5 ml/min (1800 cm/hr)

Example of transferring a method to POROS from a conventional high performance column. Flow rate is increased on POROS to take advantage of Perfusion Chromatography. Profiles are similar. The last 2 major peaks are more retained on the POROS column.



Extraction of an active and intact protein from a natural source can be quite challenging. Homogenization, lysis, extraction, precipitation, centrifugation and filtration are all used to prepare the solubilized, particulate-free protein mixture required for chromatographic separation. Some proteins, such as membrane proteins, require special treatment with detergents or other agents to get them into solution.

Once a protein has been identified from a natural source, it is often produced by recombinant DNA methods in bacteria or cultured cells for further research or other uses. The most common systems are

- Bacteria (*E. coli, B. subtillus*)
- Yeast
- Insect cells (using the *Baculovirus* system)
- Chinese hamster ovary (CHO) cells
- Transgenic animals and plants

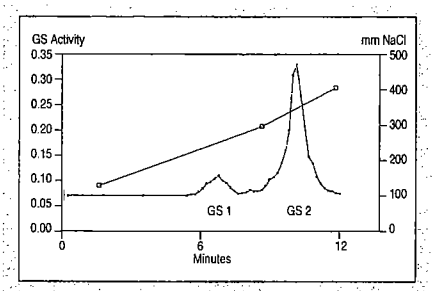
In some cases, the protein is produced intracellularly in soluble form or as an inclusion body (an insoluble mass of denatured protein, often disulfide crosslinked protein). With these systems the cells must be lysed, the cell debris removed and the protein purified away from all the host cell proteins, nucleic acids and other biomolecules. Inclusion bodies may be easily separated from the cell lysate and washed by centrifugation, yielding fairly pure protein, which must be solubilized, properly refolded and then further purified.

In other cases, the cells secrete the protein. This makes the purification task much easier, as there are fewer potential contaminants. However, the proteins and other components of the cell culture medium are present. If fetal bovine serum is used, its removal presents a formidable purification challenge. The use of serum-free, defined media can ease the purification problem considerably.

The following are the key impurity classes with some considerations for chromatography:

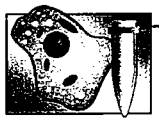
KEY IMPURITY CLASSES

Particulate debris	Critical to remove (by centrifugation or filtration) prior to chromatography to prevent column plugging.
General source proteins	Also called host cell proteins. Generally present in large quantities with very diverse characteristics. Difficult to assay at low levels due to extreme diversity.
Specific proteins	Some proteins (such as enzymes with activities in competition with the target) must be removed to very low levels, even if overall purity requirements are not stringent.
Variant target proteins	Target protein modified (by denaturation, incorrect sequence, cleavage, modified side chains, etc.) either *in vivo* or during purification. Also *isoforms*, which are different natural forms (particularly in glycosylation). May be very similar to the target in chromatographic behavior.
Aggregates	Most proteins aggregate at least to some extent with almost any change in chemical conditions. Removal must use gentle conditions to prevent more aggregation.
Small molecules	Various salts, nucleotides, carbohydrates and other small species (<1000 MW). Can also include chemicals (salts, solvents, etc.) used in previous purification steps (reagent carryover).
Proteases	Proteases (and other protein-modifying enzymes) may be active during purification. Recovery is often greatly improved if these enzymes can be removed quickly, early in the purification process.
Nucleic acids	High concentrations of nucleic acids can cause problems due to high charge and viscosity. Trace quantities must be removed from proteins purified for injection.
Virus	Viruses and related organisms are trace contaminants in any protein sample produced in cell culture. Removal is difficult to quantify.
Lipids & lipoproteins	Critical to remove (especially prior to anion exchange chromatography) to prevent column fouling.
Pyrogens	Trace quantities cause a fever response when injected. Lipopolysaccharides from the cell walls of gram negative bacteria such as *E. coli* (called *endotoxins*) are the most common.



Sample:	2 ml plant extract supernatant after removal of phenols (3.1 mg total protein)
Column:	POROS HQ/P 4.6 mmD/50 mmL
Starting Buffer (A):	50 mM Imidazole/HCl, pH 7.3 + 1 mM $MgSO_4$
Eluent (B):	Buffer A + 1 M NaCl
Flowrate:	3.5 ml/min (1260 cm/hr)
System:	FPLC
Detection:	280 nm
Elution:	0 - 50% B

Purification of glutamine synthetase isoforms (GS1 & GS2) from sugar beet leaves on POROS anion exchange. Conventional methods reported in the literature require separation times of 1.7 to 10 hours. The 13 minute separation time with Perfusion Chromatography allowed operation at room temperature without concern for enzyme activity. From work conducted by Dr. Gisela Mäck, University of Göttingen.



Molecular Characteristics

All proteins are composed of linear chains of amino acids linked together by peptide bonds. Each individual protein molecule may consist of one or more polypeptide chains, bound together by covalent (disulfide), ionic, hydrophobic, and hydrogen bonds. The side chains of the 20 common amino acids (together with a few uncommon amino acids) produce an enormous range of chemical and functional characteristics, including charge and hydrophobicity. Additionally, some of the amino acid side chains may be chemically modified after the protein is formed (post-translational modification), or chemically bound to other prosthetic groups (such as the oxygen-carrying heme group) to provide additional functionality.

One extremely important molecular characteristic of proteins is their three-dimensional (3-D) structure or conformation. Once the *primary structure* (the basic polypeptide chain sequence) is formed, the protein first folds into a localized *secondary structure* and finally a *tertiary structure* encompassing the entire molecule. In some cases, binding between different polypeptide subunits (called *quaternary structure*) is required to form an entire molecule. The 3-D conformation is stabilized by both non-covalent and covalent (disulfide bonds between cysteines) interactions. For many proteins, conformational stability largely results from the sequestration of the hydrophobic amino acids inside the molecule, away from water.

Usually a protein has proper biological activity in only one 3-D conformation. When the protein assumes a different, inactive conformation (which may also be thermodynamically stable) it is said to be *denatured*. While some proteins spontaneously refold (assume the proper conformation) after denaturation, most require a careful, critical process for refolding, if they can be refolded at all. Some proteins are stable against denaturation under a wide range of chemical conditions, while others are stable under very limited conditions. Understanding the conditions for stability is critical when developing a chromatographic method.

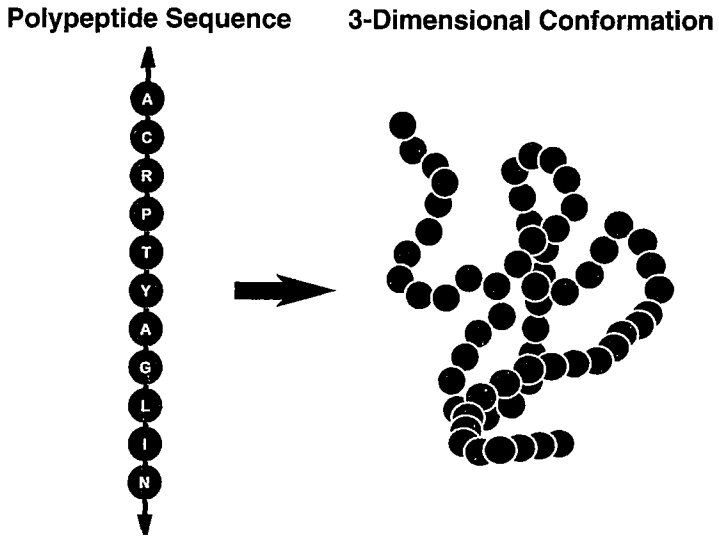


Figure 3-2. Three-dimensional conformation of proteins

The 3-D conformation of proteins critically affects chromatography in another way. If the protein did not have a stable conformation, all of the amino acid side chains would potentially influence its interaction with a chromatographic surface. However, when the protein assumes a 3-D conformation, only the amino acids on the "outside" of the molecule are available for interactions with a surface. The amino acids "buried on the inside" of the molecule cannot interact with a surface, and therefore do not affect the chromatography. However, any change in the chromatographic conditions (especially mobile phase parameters such as pH) can potentially modify the 3-D conformation, exposing new amino acids to the surface, and alter (sometimes dramatically) the binding and elution characteristics of the molecule.

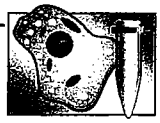
The 3-D conformation also creates binding sites for affinity interactions. Affinity results from the same basic physical-chemical interactions (ionic, hydrophobic, hydrogen bonding), but only when certain functional groups are arranged in a particular stereochemical orientation. The arrayed functional groups in the binding site of one protein fit like a "lock and

key" into complementary groups in the binding site of the other protein. Obviously, correct 3-D conformation is necessary for affinity interactions to occur.

Aside from these considerations, it is almost impossible to generalize about the molecular characteristics of proteins. Each molecule can have quite unique charge, hydrophobicity, affinity, solubility/stability, and molecular weight (CHASM — See Section 2 — *Principles of Systematic Method Development*). The following table summarizes techniques that can be used to get information about these characteristics.

CHASM ANALYSIS — *PROTEINS*

Charge	The most easily determined characteristic is the isoelectric point or pI (measured by isoelectric focusing electrophoresis). However, the *surface charge distribution* determines binding in chromatography, which may be very different from that predicted by the pI. This effect is best determined empirically by chromatographic pH mapping techniques.
Hydrophobicity	A good general measure is the ammonium sulfate concentration required for precipitation. Higher concentrations indicate lower hydrophobicity and *vice versa*. Some proteins (such as membrane proteins) are so hydrophobic that they are not soluble without solvents or detergents.
Affinity	Various binding interaction assay techniques can be used to find affinity binding ligands. Often a biological activity is based on an affinity binding interaction. In addition, antibodies can be made to almost any protein and immobilized on chromatographic supports for assay or purification.
Solubility & stability	Solubility and stability must be empirically evaluated. The effects of pH, salt ions and ionic strength, organic solvents, detergents and chaotropes and temperature are important in chromatography. Stability to proteases and other degrading enzymes is important. Exposure to various agents for long periods of time may result in greater loss than if the separation is carried out quickly, as it can be with Perfusion Chromatography.
Molecular weight	Proteins in general are in the range of 5000-1,000,000 MW. SDS PAGE or mass spectrometry are common measurement techniques. The effective molecular weight in chromatography may be effected by subunit interactions.

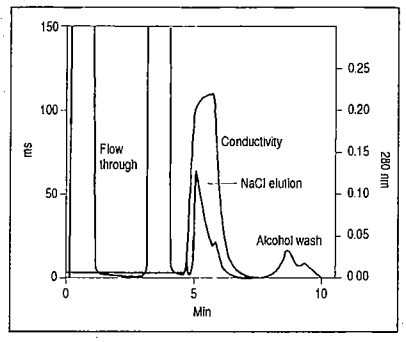


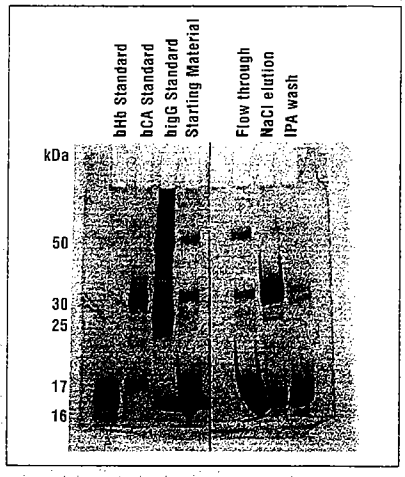
Chromatography of Proteins

As might be expected, chromatographic separations of proteins can be complex and diverse. Both target molecules and impurities show considerable variation and complexity. The size and complexity of proteins means that changes in chromatographic conditions can have a profound effect, making even seemingly impossible separations possible with fine tuning. However, the chromatographer must also balance separation performance with the preservation of biological activity and protein stability.

All the modes of chromatography can potentially be used for protein purification:

- *Ion exchange chromatography (IEC)* is used in virtually all protein purification methods and many analyses. All proteins have some ionic character, and the conditions used for ion exchange (aqueous, buffered salt solutions) are highly compatible with most proteins.
- *Hydrophobic interaction chromatography (HIC)* is less widely used, but is becoming more popular. Most proteins can be made to bind and elute in HIC (and those that do not are often quite useful in negative chromatography) with good stability and recovery, since the high salt elution conditions help to maintain the 3-D conformation. HIC is highly complementary to ion exchange.
- *Reversed-phase chromatography (RPC)*. Caution must be exercised when using RPC, because the strongly hydrophobic binding surface and organic solvent elution conditions often lead to protein denaturation, which may not be readily reversible. For this reason, RPC is more often used for protein analysis than for purification. However, it is sometimes used for final purification of proteins of <30k MW. Because of this denaturation, the selectivity of RPC is usually very different from HIC, even though both techniques involve hydrophobic binding.





Sample:	Delipidated bovine erythrocyte lysate (200 mg)
Column:	Self Pack POROS 20 EP 4.6 mmD/ 100 mmL coupled with 4-amino-methylbenzene sulfonamide
Starting Buffer (A):	20 mM Na_2HPO_4, pH 7.5
Eluent (B):	20 mM Na_2HPO_4 + 2 M NaCl
Flowrate:	8 ml/min (3000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

Affinity chromatography removal of carbonic anhydrase (a metalloenzyme present in high concentrations in mammalian erythrocytes) from hemoglobin. The column was made by coupling a sulfonamide enzyme inhibitor to an epoxy-activated POROS column. Spot densitometry performed on the bands in lane 4 (starting material) and lane 5 (flow through) indicated that ~90% of carbonic anhydrase present in the starting material was removed by the affinity column. The method could also be used for purification — note the enrichment of carbonic anhydrase in lane 6 (NaCl elution) compared to starting material. PerSeptive Biosystems Application Note PA 423.

- *Affinity chromatography* can provide the most spectacular purification of proteins in any one step, often with very high recovery. The limitation is the complexity of implementation — an appropriate ligand or antibody must be found, purified and covalently immobilized while maintaining its specific binding characteristics. The exceptions are pre-immobilized general affinity techniques such as protein A or G for IgG separations, heparin for coagulation factors, or immobilized metal affinity chromatography (IMAC) for binding to surface histidines.
- *Gel filtration chromatography* (or *size elution chromatography*) is also very widely used, especially for desalting and as a final polishing step. This mode is unique in that fractionation is achieved without a change in mobile phase conditions and is a good method for removal of protein aggregates.

Sample preparation can be as important as the chromatography itself. Techniques vary widely, depending upon the source. However, the ultimate objective is the same — to produce a particulate-free, fully solubilized protein mixture in an appropriate solvent medium for binding to the first column.

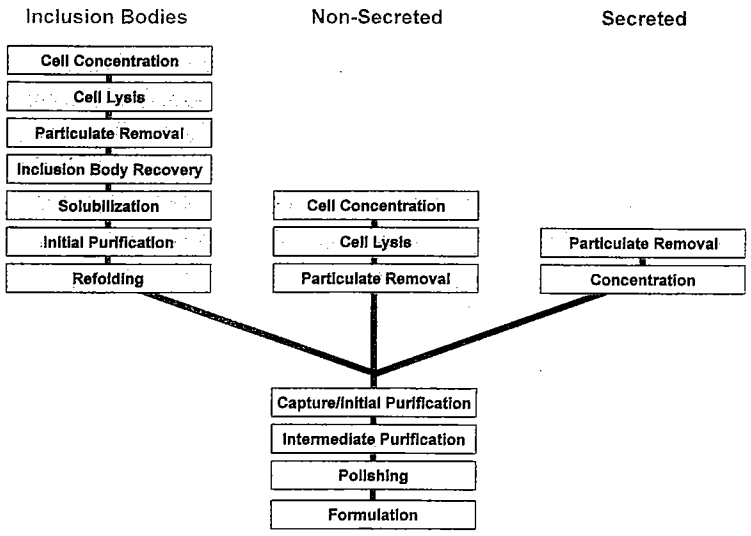
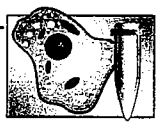


Figure 3-3. General protein purification schemes



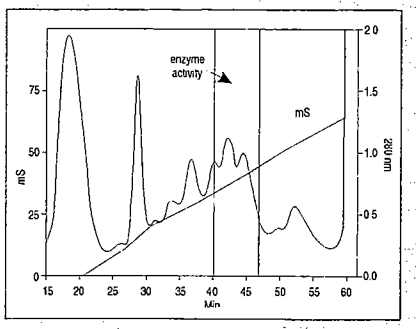
Because of the complexity of typical samples, most protein purifications involve more than one chromatographic step.

- *Capture* steps are to bind most of the protein from the crude sample while removing a large fraction of the impurities. Removal of proteases and other degrading enzymes is an important consideration at this stage. Note that rapid separations (such as are enabled by Perfusion Chromatography media) reduce the time for these degrading enzymes to operate.
- *Intermediate purification* steps remove most impurities to make the target molecule the majority of the product — in some cases >99%. Most protein purification protocols involve more than one intermediate purification step.
- *Polishing* steps remove trace contaminants, particularly specific proteins that must be at a very low level or variant forms of the target protein. Polishing is not needed for all research applications, but is vital for making injectable proteins or for critical applications such as X-ray crystallography.

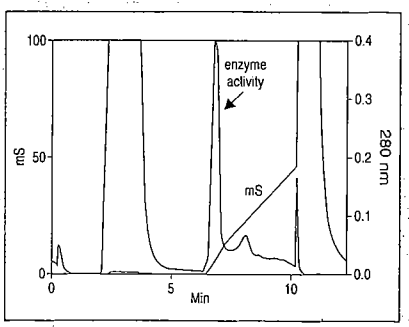
Following the chromatographic purification, it is often necessary to "formulate" the product, to remove purification reagents, concentrate the protein, and/or add components to the mixture to stabilize the protein or make it suitable for the final application.

While it is very hard to generalize about methods for protein separation, a number of specific suggestions can be made:

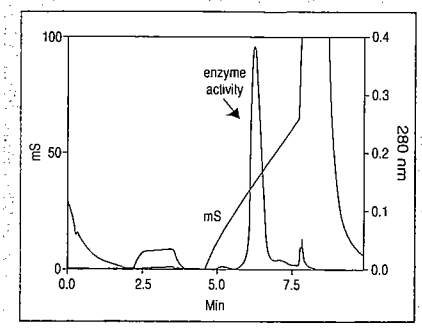
- Generally avoid using the same column surface chemistry for more than one step in a purification, unless the mobile phase conditions are significantly changed. You will usually get better results in fewer steps by using a range of different modes with complementary selectivity.



Sample:	25 ml crude cell lysate
Column:	POROS 50 HQ 20 mmD/120 mmL glass column
Starting Buffer (A):	50 mM Tris/bis-tris propane, pH 7.0
Eluent (B):	50 mM Tris/bis-tris propane, pH 7.0 + KCl
Flowrate:	10 ml/min (190 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 600 mM KCl in 10 CV

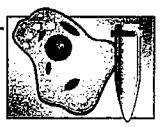


Sample:	50 ml dialyzed pool from previous purification step
Column:	Self Pack POROS 20 HE (heparin) 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM Tris/bis-tris propane, pH 7.0
Eluent (B):	50 mM Tris/bis-tris propane, pH 7.0 + KCl
Flowrate:	10 ml/min (3600 cm/hr)
Elution:	0 - 300 mM KCl in 20 CV

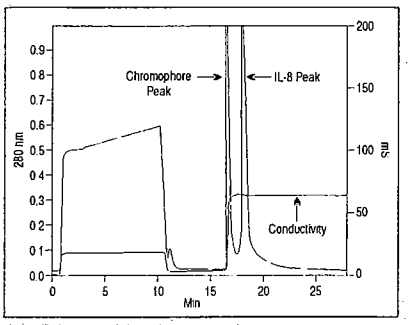


Sample:	5 ml fraction from previous purification step
Column:	POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM Tris/bis-tris propane, pH 7.0
Eluent (B):	50 mM Tris/bis-tris propane, pH 7.0 + KCl
Flowrate:	10 ml/min (3600 cm/hr)
Elution:	0 - 600 mM KCl in 20 CV

A three-step procedure for purification of recombinant THF-dependent enzyme from *E. coli* cell culture supernatant for X-ray crystallography studies. The entire protocol was developed in one week, eliminating dependence on outside collaborators for supply of the protein. From work conducted by Dave Dyer and Dr. Barry Stoddard, Fred Hutchinson Cancer Research Institute. PerSeptive Biosystems Application Note PA 429.



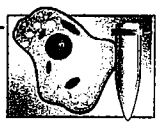
- Use of high resolution columns can help eliminate purification steps. For research purposes, it can be sufficient to use a single, small particle size HPLC column, with a shallow, well-optimized gradient, instead of a series of low resolution columns.
- Anion exchange is commonly used as a first step although cation exchange columns can be equally effective depending on the objective of the separation. For example, cation exchange can be used to eliminate nucleic acids because DNA does not bind to cation exchange media, whereas it does bind to anion exchange media. You may find it useful to employ both anion and cation exchange in the same purification.
- Use hydrophobic interaction later in a purification, after ion exchange. There is less likelihood of contaminating proteins precipitating, and the volume of the sample (to which salt must be added) is usually greatly reduced. An exception to this is when ammonium sulfate precipitation is used as a preliminary purification step. In this case, HIC should be used immediately after.
- Use reversed-phase, when feasible, as a late polishing step. Many contaminant proteins can be difficult to remove from reversed-phase columns due to low solubility in organic solvents. Reversed-phase is often most useful for separation of variant protein forms. Care should be taken in removing the organic solvent (by evaporation, lyophilization, desalting or dialysis) to refold the protein correctly and preserve activity.
- Consider using affinity columns after a preliminary ion exchange or other capture step. These columns are often expensive, susceptible to fouling and to degrading enzymes present in the sample. An exception to this is IMAC, which can be as robust as ion exchange but more selective.



Sample:	140 ml solubilized inclusion body extract in 8 M urea (approx. 1 mg/ml target protein)
Column:	POROS 50 HS 10 mmD/100 mmL
Starting Buffer (A):	25 mM acetate, pH 4.5
Eluent (B):	50 mM Tris, pH 8.0 + 1 M NaCl
Flowrate:	13 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

First step (cation exchange) in the purification of interleukin 8 (IL-8) from *E. coli* inclusion bodies. Converting this step (previously done on S Sepharose® Fast Flow) to POROS allowed a 20-fold increase in flow rate. The entire purification protocol was reduced from 3 days to about 4 hours. PerSeptive Biosystems Application Note PA 422.

- Membrane proteins are very difficult to purify chromatographically because they require detergent to be soluble. However, you can often use ion exchange for these proteins with non-ionic detergents present in the mobile phase. Normal salt elution and method development techniques such as pH mapping may be used.
- Purify difficult-to-solubilize proteins, such as non-refolded inclusion body proteins, by ion exchange in the presence of up to 8 M urea in the mobile phase. Normal salt elution and method development techniques such as pH mapping may be used.
- Some proteins require special buffer additives for stability. Be sure these are compatible with the buffers and eluents required for the chromatography. If they are not, you can sometimes take advantage of the speed of Perfusion Chromatography by performing the separation very quickly without the additive and capturing the product in a tube containing the additive.
- When performing repeated analyses of complex samples, run periodic blanks (full elution runs with no sample injected). Some peaks appearing in an analysis may be "ghosts" carrying over from previous runs and not be actually present in the sample. When this occurs, you should consider a new cleaning or regeneration protocol for the column.
- Effective reduction of endotoxin can be accomplished by using ion exchange or reversed-phase steps in a purification process. A commercially available endotoxin assay can be used to measure the level of endotoxin removal after each step in the purification process.



ANTIBODIES

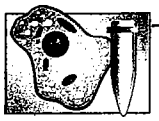
Antibodies represent a very important class of biomolecules. The highly specific binding properties of these molecules toward their *antigens*, together with the relative ease with which individual antibodies can be generated makes them extremely useful in a broad range of applications in quantitative and qualitative analysis, purification, clinical diagnostics and even therapeutics. Because of their central role in the immune system, antibodies themselves are the subject of intensive research.

All antibodies are proteins but share a number of important structural characteristics. As a result the approaches used to specifically separate antibodies are sufficiently distinct from proteins in general to warrant separate consideration.

Sources & Key Impurities

Virtually all antibodies are made by injecting an *immunogen* into an animal and letting the natural immune response occur. Individual B-lymphocytes in the blood are stimulated by contact with the immunogen to differentiate into plasma cells and to generate plasma cell clones secreting the unique antibody. Each clone stimulated by the immunogen produces a slightly different antibody molecule with binding specificity for the immunogen. The antibodies from different clones may recognize different parts of the immunogen, referred to as the *epitopes*.

One important source of antibodies is the blood serum from the injected animal, called the *antiserum*. Antibodies from serum are referred to as *polyclonal* antibodies, because all of the individual antibody species with binding activity toward the antigen are present (along with all of the other antibodies produced by the animal). Polyclonal antisera are easy to produce, and the heterogeneity of antibody binding activity can be very useful for some applications.



However, for most applications, it is desirable to produce a pure antibody with a unique molecular structure, produced by an individual plasma cell clone line, referred to as a *monoclonal antibody*. Although individual cells can be extracted and multiplied or cloned through cell culture techniques, the antibody-producing plasma cells found in normal blood will die after only a few cell divisions. On the other hand, plasma cells from an animal with the form of cancer called myeloma will multiply in culture indefinitely.

In the mid-1970's, a technique was developed for fusing normal, antibody-producing plasma cells with a line of myeloma cells which do not produce any of their own antibodies. The resulting hybrid cell line, called a *hybridoma*, both multiplies in culture and produces the desired monoclonal antibody. By carefully selecting and culturing a line of hybridomas produced from the plasma cells of an immunized animal, it is possible to create a hybridoma line that will produce an almost unlimited supply of monoclonal antibody in culture.

Hybridoma cells are cultured by two general techniques. In the first, the cells are injected into the abdomen of a mouse, where they grow to produce a liquid tumor called an *ascites* that contains a high concentration of the monoclonal antibody. While this method is relatively simple and can produce up to 100 mg or so of antibody per mouse, it is difficult to produce larger quantities, and each ascites sample (even from a single mouse) can contain a different range of contaminants. The technique has also been banned in some countries.

Hybridomas can also be cultured *in vitro* using a very wide range of methods, from petri dishes and roller bottles, to suspension culture in deep tank fermentors or perfusion systems such as hollow fibers. The product of these methods can be very reproducible and, with the use of modern serum-free media, relatively free of impurities. In addition, cell culture methods can be scaled almost indefinitely.

SOURCES OF ANTIBODIES

	Protein (mg/ml)	Antibody (mg/ml)	Key Impurities
Antiserum	50 - 70	10 - 20 total 0.1 - 2 specific	Serum proteins Non-specific antibody Lipids/lipoproteins
Ascites	15 - 25	5 - 20 total 5 - 15 specific	Serum proteins Cell lysis products (protein, DNA) Lipids/lipoproteins Non-specific mouse antibody
Cell Culture	1.5 - 2.5 (5% FBS) 0.05 - 0.1 (serum-free) 5 - 7 (perfusion)	0.3 - 0.5 (5% FBS) 0.005 - 0.1 (serum-free) 4 - 6 (perfusion)	Defined media proteins Fetal bovine serum Cell lysis products (protein, DNA) Non-specific bovine antibody

Antiserum is relatively defined in terms of protein composition (see table). Lipids and lipoproteins can also be present in high concentrations (causing the serum to be cloudy), depending upon the diet of the animal prior to bleeding. These can irreversibly foul columns, and so should be removed (usually by solvent extraction) prior to chromatography.

Ascites is variable in composition, but is normally similar to serum. Ascites may also contain proteins and DNA from lysed cells. The specific monoclonal antibody may represent anywhere from 10 - 40% of the total protein. In addition, there may also be small amounts of polyclonal mouse antibody present. Ascites very often contain a high particulate load and lipids, which must both be removed prior to chromatography.

Cell culture supernatants are the most varied in composition, depending upon the production system and cell culture media used. Purity of the monoclonal antibody can be very high, but the concentration is often very low. The most critical variable is the amount of fetal bovine serum (FBS) used in the culture medium. FBS not only contributes contaminating serum proteins (such as bovine serum albumin), but also adds bovine

polyclonal antibodies. Many modern systems use low serum or totally serum-free (in some cases protein-free) media, which greatly ease the purification problem.

Perfusion cell culture systems produce supernatants with much higher antibody concentrations than suspension cell culture. However, because of the much higher cell density in these systems, cell lysis products are also much more concentrated. In some systems, the DNA concentration in the supernatant may be so high as to present a significant purification challenge.

Molecular Characteristics

Almost all antibodies are relatively high molecular weight glycoproteins, sharing a common basic unit structure (see figure), composed of two identical heavy and two identical light polypeptide chains, linked together with disulfide bonds. There are one or more of these basic subunits per antibody molecule, depending upon the antibody class. The antigen binding sites in the Fab domains of the molecule are highly variable in structure, which gives the antibodies different binding specificities. For a given source species and subclass, the rest of the antibody molecule is relatively constant in structure.

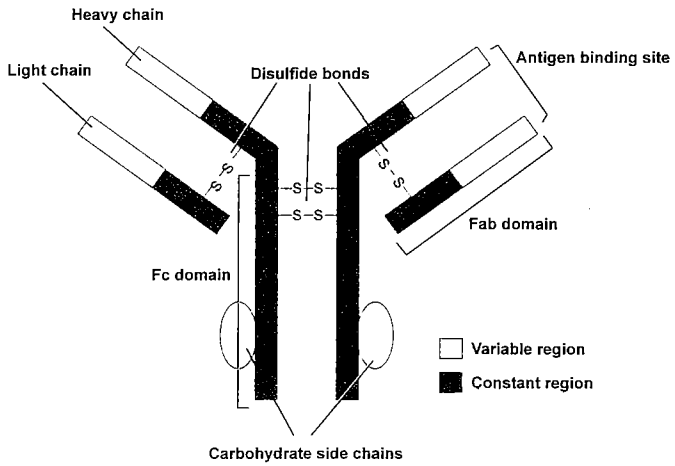


Figure 3-4. Schematic of the basic unit structure of an antibody molecule

There are 5 major classes of antibodies in mammals, each with different molecular characteristics. The most widely used and purified are the IgG and IgM classes. The other classes are becoming of increasing research interest. Within some of these classes are subclasses (four for IgG in humans), differing primarily from one another in the amino acid sequence of the heavy chain constant region.

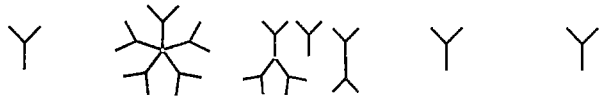
	IgG	IgM	IgA	IgE	IgD
Structure					
Size (MW)	160 K	1000 K	160 - 500 K	160 K	160 K
Serum mg/ml	10 - 20	0.5 - 2	1 - 4	0.0001	0 - 0.4
Function	Secondary response	Primary response	Mucosal membranes	Allergic response	Cell surface markers

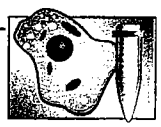
Figure 3-5. Major classes of antibodies

From the separation standpoint, antibodies present an interesting paradox. On the one hand, their shared characteristics should make them amenable to the use of common purification schemes. To a large extent this is true for many applications. On the other hand, the variable regions comprise a significant portion of the molecular structure which is, by "design", highly critical in determining the binding characteristics. Thus each antibody is a unique molecule with its own properties and purification challenges. In addition, the applications (as well as the sources) of antibodies are quite diverse, and each presents different problems.

The key molecular characteristics of antibodies are summarized in the CHASM analysis (see Section 2 — *Principles of Systematic Method Development*) shown in the following table. Always note that any individual antibody (especially a monoclonal) may be very different in behavior.

CHASM ANALYSIS — *ANTIBODIES*

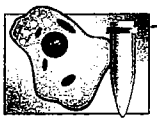
Charge	Polyclonals cover a range of pI's, but the majority tend to be basic (pI > 6), usually among the most basic proteins in the mixture. Monoclonals follow this trend, but an individual antibody can have any pI, usually between 4 and 10.
Hydrophobicity	The apparent hydrophobicity is quite high, due to both actual surface hydrophobicity and the high molecular weight. Antibodies are usually among the first proteins to precipitate with ammonium sulfate (30 - 50 % saturation).
Affinity	The antibody's antigen is highly specific in binding. Most antibodies can be bound selectively by protein A or G (although these often cannot distinguish species or subclasses). Antibodies to antibodies of particular species or subclasses can also be used.
Solubility & stability	Antibodies are usually soluble in aqueous buffers, except near the isoelectric point. Some antibodies are more soluble in the presence of salt. Some monoclonals and IgM may present solubility problems. Antibodies differ greatly in stability. Most antibodies are relatively stable to low pH, but lose activity at pH 9 and above. Organic solvents often *reduce* both solubility and stability.
Molecular weight	See table of antibody classes on previous page.



Chromatography of Antibodies

All of the modes of chromatography (with the exception of reversed-phase) can be used effectively for the separation of antibodies. Each has uniquely useful characteristics:

- *Ion exchange* can separate different polyclonal antibodies from each other as well as different subclasses and species. However, you must optimize the separation carefully in each case. As a group-selective method to separate antibodies as a class, ion exchange is less effective. Cation exchange is widely used since antibodies are among the most basic proteins in a mixture.
- *Hydrophobic interaction* can be a very useful group-selective method, since antibodies as a class usually have high apparent surface hydrophobicity and are often similar to each other in this respect. HIC is less useful for separating antibodies from each other.
- *Affinity* techniques include protein A or G, immobilized anti-antibodies and immobilized antigens. The use of proteins A and G is very widespread, and has largely superseded the use of anti-antibodies. Immobilized antigens are most often used for isolating mono-specific antibodies from polyclonal sera.
- *Gel filtration* is very useful as a final polishing technique, to remove aggregates and other contaminants based on size. Desalting is useful for removing small molecule free ligands when making conjugates. Gel filtration can also be used for IgM separations.



Although there are many variations, most chromatographic separations of antibodies fall into four major schemes:

Classical

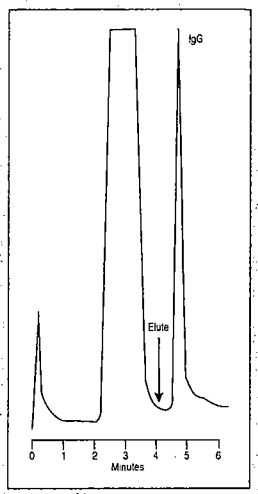
Although precipitation is not widely used as a general protein purification technique, antibodies tend to precipitate in salt well before other proteins in the sample, so you can use ammonium sulfate or other agents (such as caprylic acid) very selectively. Further purification is done using ion exchange chromatography.

Orthogonal

This is a high resolution version of the *Classical* scheme. Use ion exchange first, followed by hydrophobic interaction (HIC). With this order you can apply the ion exchange fractions to the HIC column with just the addition of salt.

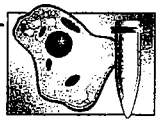
Affinity

The most popular and simplest scheme is to simply use protein A or G affinity to selectively purify the antibody fraction. You can also use immobilized antigen affinity columns for separating specific antibodies from polyclonals antisera.



Sample:	2 ml rabbit antisera diluted to 20 ml with PBS
Column:	POROS 20A 16 mmD/20 mmL glass column
Starting Buffer (A):	200 mM Tris, pH 9.0
Eluent (B):	0.1 M citrate, pH 3.0
Flowrate:	10 ml/min
System:	Peristaltic Pump
Detection:	280 nm
Elution:	Step to 100% B

Low pressure purification of polyclonal IgG from antiserum on POROS A using a peristaltic pump.



Ultra High Purity

The highest purity antibodies are usually made by combining affinity (normally protein A or G or immobilized antigen) with physical-chemical separations based on ion exchange and/or HIC. Multi-step techniques such as this are required for making therapeutics.

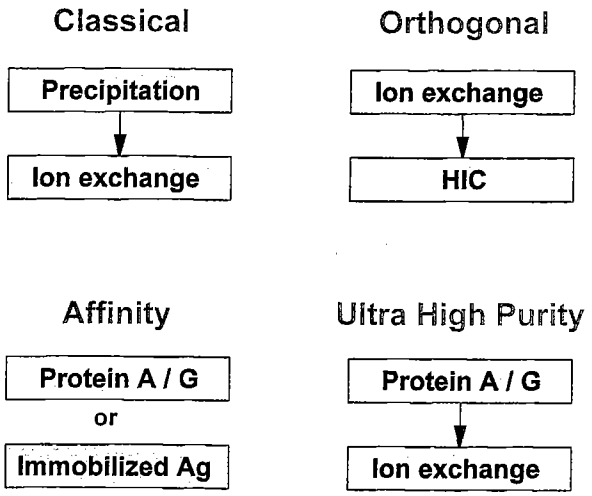
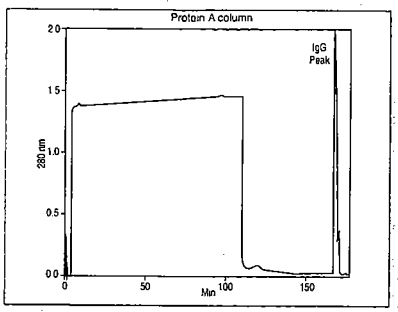
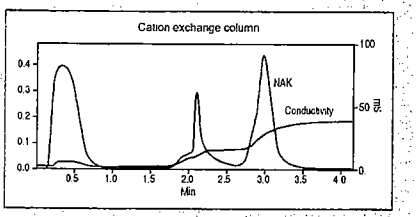


Figure 3-6. Chromatographic separation schemes for antibodies

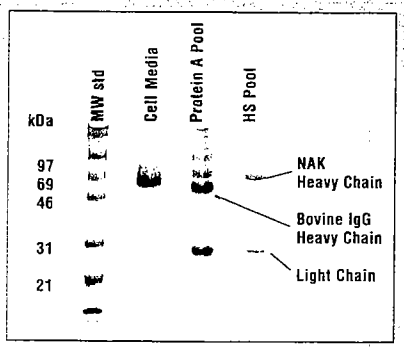
Many researchers use the *classical* scheme, often limited to just the precipitation, especially with polyclonal antisera. However, the *affinity* approach, using protein A or G, is much more convenient, and usually produces a significantly purer product with minimal or no method development. The *affinity* scheme works well with various types of monoclonal antibodies, as well as antisera. Where even greater purity is needed, the *ultra high purity* scheme is recommended. The *orthogonal* scheme, because of the significant method development required, has typically been recommended only when the use of the *affinity* scheme is not possible or is uneconomical, but becomes a viable option when using Perfusion Chromatography media.



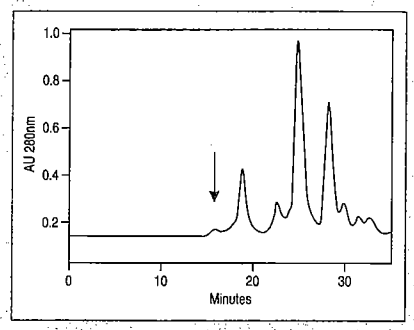
Sample:	1 L cell culture supernatant
Column:	POROS 50A 10mmD/100mmL
Starting Buffer (A):	50 mM Tris, pH 8.5 + 0.5 M NaCl
Eluent (B):	100 mM glycine, pH 3.0
Flowrate:	6.5 ml/min (500 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B



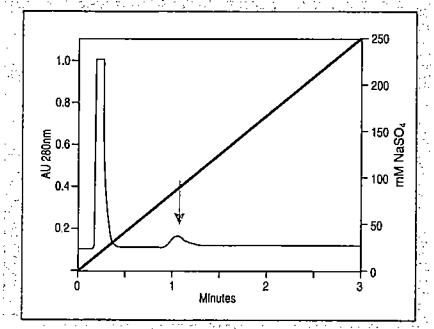
Sample:	3 ml pooled fractions from above
Column:	POROS HS/M 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM Tris, pH 7.5 + 150 mM NaCl
Eluent (B):	50 mM Tris, pH 7.5 + 500 mM NaCl
Flowrate:	8 ml/min (3000 cm/hr)
Elution:	Step to 100% B



Example of ultra high purity method for purification of a human nerve growth factor/monoclonal antibody fusion protein. Reprinted from *J. Chromatogr. A*, 708, Hunt *et. al.*, 61-70, 1995 with kind permission of Elsevier Science, The Netherlands.



Sample:	60 µl clarified cell culture (200 µg/ml)
Column:	TosoHaas TSK G5000PWxl, 7.8 mmD/300 mmL
Starting Buffer (A):	PBS, pH 7.4
Flowrate:	0.5 ml/min (65 cm/hr)
System:	System Gold™
Detection:	280 nm



Sample:	60 µl clarified cell culture (200 µg/ml)
Column:	POROS S/H 4.6 mmD/50 mmL
Starting Buffer (A):	20 mM $NaPO_4$, pH 7.4
Eluent (B):	20 mM $NaPO_4$, pH 7.4 + 250 mM Na_2SO_4
Flowrate:	3.5 ml/min (1260 cm/hr)
System:	System Gold
Detection:	280 nm
Elution:	0 - 100% B in 3 min

An analytical method for IgM (marked with arrow) based on gel filtration was converted to POROS cation exchange, with a 10 X reduction in separation time. Data provided courtesy of David Wood, Zhen-hong Li, Teresa Genthe of MedImmune, Inc.

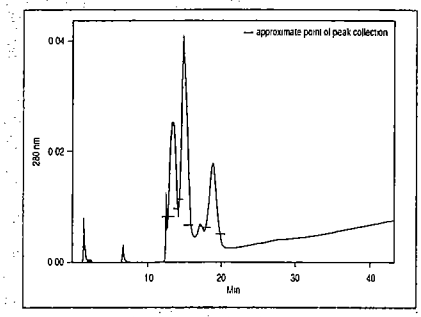
Some special cases with antibodies require particular techniques:

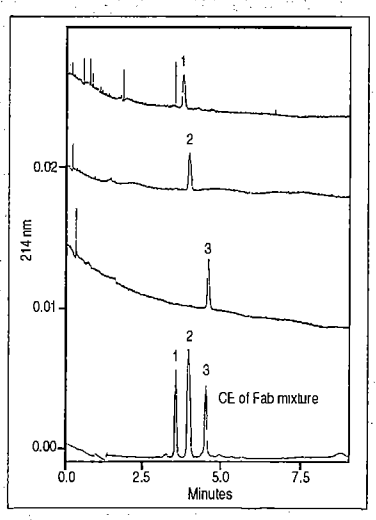
IgM To make use of the very high molecular weight of this class, gel filtration is frequently used, although the low throughput of this mode can be a problem. Ion exchange with a low ligand density support (such as POROS S) can also be very effective. Protein G has a very high specificity for IgG over IgM (unlike protein A) and can therefore be used to separate the two.

IgG subclasses You can sometimes effectively fractionate subclasses using protein A with elution in steps of descending pH over a range of 6-3. Immobilized antibodies to particular subclasses are also effective, although sometimes impractical.

Fab fragments Antibody molecules may be cleaved with the proteases papain or pepsin to produce Fab or $(Fab')_2$ fragments respectively. While the Fc fragment is constant in behavior, the Fab fragments can be quite variable. You can sometimes use protein A (not G) for this separation, with care taken in the selection of buffers. Ion exchange is also very useful.

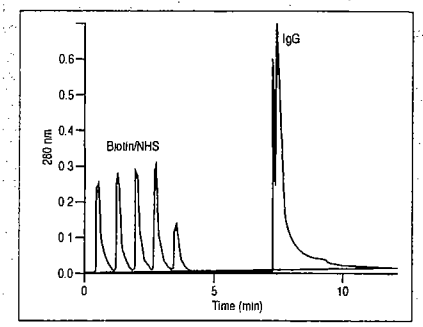
Antibody conjugates Antibodies or Fab fragments are sometimes chemically coupled to other molecules (such as enzymes or dyes) or ligands to form conjugates for use in assays or other applications. You may easily remove low molecular weight free ligands using desalting chromatography or ultrafiltration. High molecular weight free ligands and free antibody can be more difficult to remove. Often these separations must be treated like any typical protein purification problem, with optimized affinity or physical-chemical separation methods such as ion exchange and HIC being the most effective approaches.





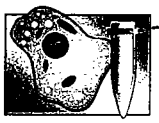
Sample:	Papain digestion of anti-cortisol IgG_2b
Column:	POROS S/M 4.6 mmD/100 mmL
Starting Buffer (A):	10 mM MES, pH 4.5
Eluent (B):	10 mM MES, pH 7.5
Flowrate:	1 ml/min (360 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 40% B in 20 CV

Purification of three Fab isoforms using a pH gradient on POROS cation exchange column. Capillary electrophoresis (bottom trace) of the collected fractions indicated pure isoforms were isolated. Recovery was about 90%. The pI values of two of the isoforms differed by only 0.1 pH unit. Reprinted with permission from *J. Chromatogr. A*, 707 Mhatre *et. al.*, Purification of antibody Fab fragments by cation-exchange chromatography and pH gradient elution, 225-231, 1995, with kind permission of Elsevier Science, The Netherlands.



Sample:	5 x 200 µl biotinylation mixture diluted 1:1 in start buffer
Column:	POROS PE/M 4.6 mmD/100 mmL
Starting Buffer (A):	50 mM $NaHCO_3$ + 1.2 M Na_2SO_4, pH 8.5
Eluent (B):	10mM phosphate, pH 7.4 + 150 mM NaCl
Flowrate:	10 ml/min (3600 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

Use of POROS HIC column to facilitate the preparation and purification of biotinylated antibody conjugates for Perfusion Chromatography based immunoassays. The biotinylation reaction mixture is passed over the column in multiple injections. With each injection, unreacted biotin and hydrolyzed NHS flow through (first five peaks). Biotinylated IgG accumulates on the column and is later eluted. The same column was also used for buffer exchange prior to biotinylation, eliminating the need for time-consuming ammonium sulfate precipitations. PerSeptive Biosystems Application Note PA 432.



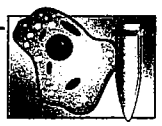
PEPTIDES

Peptides play a number of important roles in research. These short polymers of amino acids can function as the basic recognition unit for receptors and other biological binding proteins, and are thus used for studying and affecting a very wide range of biochemical interactions. Some peptides themselves are biologically important, serving as hormones, neurotransmitters, growth factors, etc. Peptides are also important structural and functional elements of proteins, and the analysis of peptide from enzymatic digests of proteins is a critical tool in understanding protein structure.

Sources & Key Impurities

Peptides are separated from several quite distinct sources. Some peptides are obtained from natural biological extracts such as serum, cell lysates, organ extracts, etc. In these cases, the peptides are often present at extremely low concentrations, in the presence of large amounts of various proteins, nucleic acids and small molecules. Purification of these natural peptides can be quite challenging, and is very much analogous to protein purification.

Another source of peptides is enzymatic cleavage of proteins. In this case, a protein is obtained in highly pure form, and is treated with a specific proteolytic enzyme (often trypsin) to produce a complex mixture of 5 - 100 or more defined peptide fragments. Separation of these fragments (most often using reversed-phase chromatography) and analysis (by looking at the chromatograms or by analysis of individual peaks using techniques such as mass spectroscopy) can reveal a great deal of information about the protein structure. This enzymatic digestion technique has become a primary means of protein identification and characterization, and is now an important quality control method in the biopharmaceutical industry.



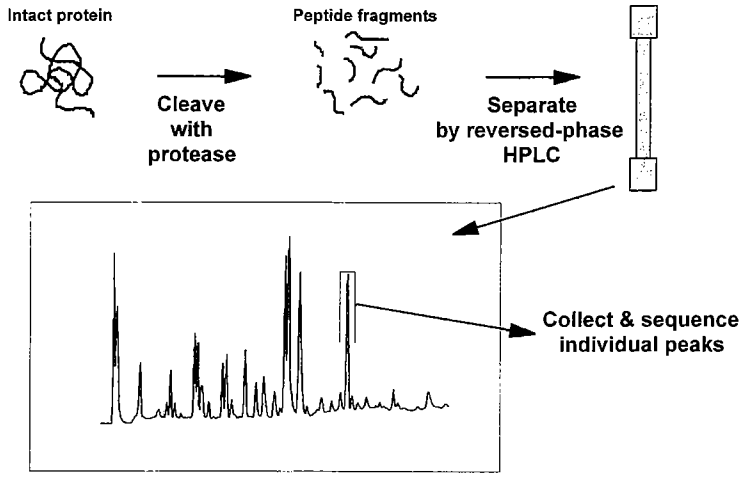
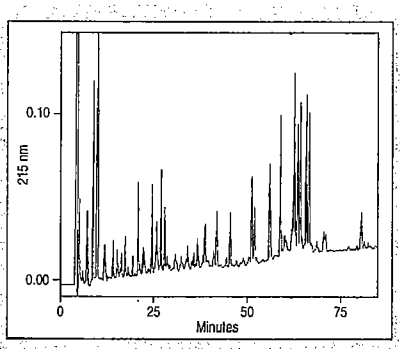


Figure 3-7. Enzymatic digestion of proteins to produce peptide fragments

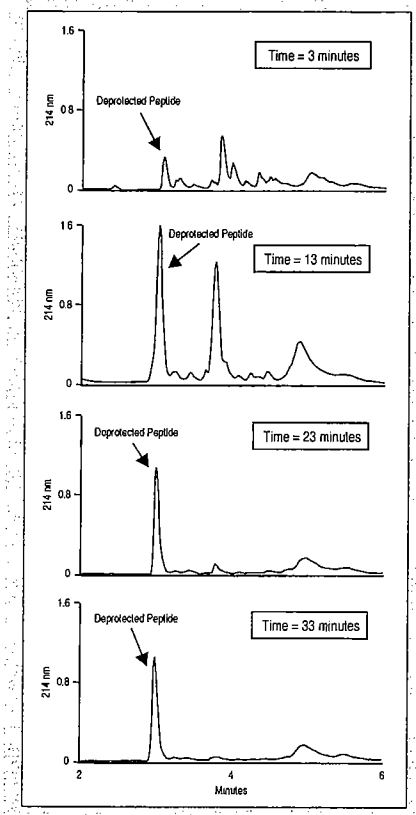


Sample:	50 µl recombinant protein A (1 mg/ml) trypsinized with Poroszyme Immobilized Trypsin column.
Column:	PepMap C18 4.6 mmD/250 mmL
Starting Buffer (A):	0.1% TFA in water
Eluent (B):	0.1% TFA in acetonitrile
Flowrate:	0.8 ml/min (290 cm/h)
System:	INTEGRAL Micro-Analytical workstation
Detection:	215 nm
Gradient:	0 - 60% B in 16 CV

Typical high resolution reversed-phase separation, most commonly used for peptide mapping, where resolution of each of the individual components in the sample is critical. Poroszyme Immobilized enzyme columns utilize the enhanced mass transfer of POROS media to perform rapid enzyme digestions on column, overcoming many of the limitations of conventional solution-based digestions.

The most common source of peptides in the research laboratory is chemical synthesis. Using either manual methods or automated peptide synthesis instruments, it is possible to manufacture even relatively large peptides in up to gram quantities. These synthesized peptides are purified and utilized in research applications.

The chemistry used in peptide synthesis involves a cyclical process. Both reactive side chains and the N-terminal amine groups of the amino acids are chemically blocked to prevent unwanted side reactions. Most lab scale synthesis is carried out on a solid phase, with the first amino acid immobilized by the carboxyl group. The N-terminal amine blocking group is unblocked to begin the reaction cycle and is reacted with a blocked amino acid (or small peptide) in which the C-terminal carboxyl is activated with a facile leaving group, allowing a nucleophilic substitution to take place and the formation of an amide bond. This cycle is repeated with different blocked amino acids until the peptide chain is complete.



Sample:	Model peptide in cleavage cocktail (90% TFA/ 5% triisopropylsilane/ 5% water)
Column:	POROS R2/H 4.6 mmD/100 mmL
Starting Buffer (A):	0.1 TFA
Eluent (B):	0.1 TFA in acetonitrile
Flowrate:	5 ml/min (1800 cm/hr)
System:	BioCAD workstation
Detection:	214 nm

Short run times make it possible to follow the deprotection of a synthetic peptide in real time by making injections every ten minutes directly onto a POROS reversed-phase column from the cleavage cocktail. Optimizing deprotection time can help maximize lab throughput, at the same time ensuring maximal recovery of product.

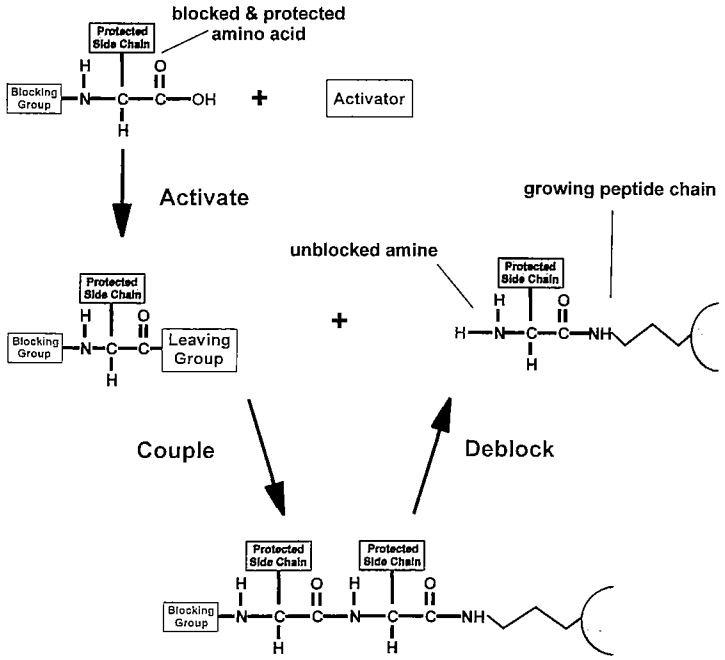


Figure 3-8. Schematic of peptide synthesis reaction cycle

Many different impurities can arise during peptide synthesis. No reaction cycle is 100% efficient, so there will be some "failure sequences", missing one or more amino acids, the percentage of which increases with increasing length of the peptide. Some amino acids do not react well (due to the bulk of their side chains or protecting groups) and give rise to still more failure sequences. In addition, some amino acids are much more difficult to effectively block, giving rise to branched peptide impurities. Leftover synthesis reagents are often present. Finally, some of the harsh reaction conditions used can chemically modify the peptides, producing other impurities.

Molecular Characteristics

Peptides are generally linear polymers of the 20 common *amino acids* (as well as some less common ones) held together by peptide bonds formed by coupling the primary amine on one amino acid to the carboxyl group on the next. Peptides larger than ~5000 MW (~30 amino acids) are generally referred to as proteins. A few peptides are branched, and some have the N- and C-terminal ends coupled to form a cyclic ring polymer. Most peptides are flexible, showing little or no higher order structure such as is the case with proteins.

Peptides possess a bewildering array of molecular characteristics. In some ways, peptides are more diverse in their behavior than proteins, because the effects of individual amino acids are more important. However, a few useful generalizations can be made.

The binding of peptides to chromatographic media is based largely (although sometimes not entirely) upon the sum of the interactions of the various amino acid side chain functional groups, as well as the peptide bond backbone. A useful way to characterize peptides for separation purposes is to sum the numbers of basic (+ charge), acidic (- charge) and hydrophobic amino acids (including the N- and C-terminal ends) to get a rough "functional profile".

COMMON AMINO ACID GROUPS

Basic (+ charge)	Acidic (- charge)	Hydrophobic	Neutral
Lysine (K, Lys)	Aspartic Acid (D, Asp)	Alanine (A, Ala)	Glycine (G, Gly)
Arginine (R, Arg)	Glutamic Acid (E, Glu)	Valine (V, Val)	Serine (S, Ser)
Histidine (H, His)		Leucine (L, Leu)	Threonine (T, Thr)
		Isoleucine (I, Ile)	Cysteine (C, Cys)
		Proline (P, Pro)	Tyrosine (Y, Tyr)
		Methionine (M, Met)	Asparagine (N, Asn)
		Phenylalanine (F, Phe)	Glutamine (Q, Gln)
		Tryptophan (W, Trp)	

For example, the peptide LIPSKNV has one basic (K or lysine), no acidic, four hydrophobic (L or leucine, I or isoleucine, P or proline and V or valine) and two neutral (S or serine and N or asparagine) amino acids. The functional profile is thus +2 / -1 / H4 (two positive, one negative and 4 hydrophobic groups), indicating a rather hydrophobic peptide.

The key molecular characteristics of peptides are summarized in the CHASM analysis (see Section 2 — *Principles of Systematic Method Development*) shown in the following table. Always note that each individual peptide is quite unique.

CHASM ANALYSIS — *PEPTIDES*

Charge	Determined by the sum of the amino acid side chains, N-terminal amine and C-terminal carboxyl (both of which may be modified). Use a "functional profile" (see above) to characterize. The actual charge varies with pH. At pH 2, most acidic groups are neutralized. Unlike proteins, all peptide charge groups are usually accessible for binding interactions.
Hydrophobicity	Very broad range in hydrophobicity; use the "functional profile" (see above) to characterize. Hydrophobicity may vary with pH, since some charged amino acids (such as lysine) are very hydrophobic in their uncharged state.
Affinity	Peptides sometimes have a binding affinity for receptors and other ligands. Antibodies can be made to many peptides. For separation purposes, the binding capacity of an affinity column for a peptide is often very low because of the stoichiometry of these interactions. IMAC can be used for peptides containing histidines or tryptophans.
Solubility & stability	Very wide range in solubility. Some only soluble in the presence of organic solvents or chaotropes; others insoluble except in fully aqueous solutions. Likewise, stability is highly variable. Some amino acids are sensitive to extremes of pH, others are not. Susceptibility to proteolysis depends upon sequence.
Molecular weight	Up to ~5000 MW. Amino acids average 150 MW per monomer unit.

Chromatography of Peptides

Reversed-phase HPLC is the most commonly-used mode of chromatography to either purify or analyze peptides. In part this is simply due to historical reasons, since reversed-phase was the only high performance separation technique available when peptide synthesis was developed. However, it is also true that reversed-phase HPLC can be highly effective. The high resolution offered by the technique is essential for separating the many very closely related species present in a typical peptide mixture. Through the use of proper ion pairing agents, reversed-phase can be made to resolve peptides based on charge as well as hydrophobic characteristics. Both the eluent and ion pairing agents can be volatile, simplifying sample cleanup after purification.

As powerful and popular as reversed-phase may be, it does not solve all peptide separation problems. Some impurities simply do not separate by reversed-phase, especially when they are due to a single charge group difference. The capacity of reversed-phase columns for preparative separations is also typically very low relative to other separation modes. The use of organic solvents and expensive columns also can make the technique expensive and impractical when gram-scale purifications are required.

Ion exchange chromatography can be equally effective in peptide separations, and does not suffer from some of the disadvantages of reversed-phase. Modern ion exchange HPLC columns can provide resolution as sharp as many reversed-phase media, with a completely different and complementary selectivity.

A very powerful approach combines ion exchange and reversed-phase. These two techniques are complementary, in that the solvents used for reversed-phase do not affect binding to ion exchange columns, and the buffer and eluent salts and pH's used with ion exchange do not affect binding to reversed-

phase. If the ion exchange column is used first in the sequence, the reversed-phase column will simultaneously desalt and purify the peptide.

Other modes of chromatography, such as hydrophobic interaction and affinity, have somewhat more limited usefulness. HIC can be effective for hydrophobic peptides, but is not widely used. Similarly, for peptides with significant histidine or tryptophan content (particularly with two or more histidines in a row), immobilized metal affinity chromatography can be useful. Gel filtration is less widely used with synthetic peptides because most of the contaminants are of very similar molecular weight.

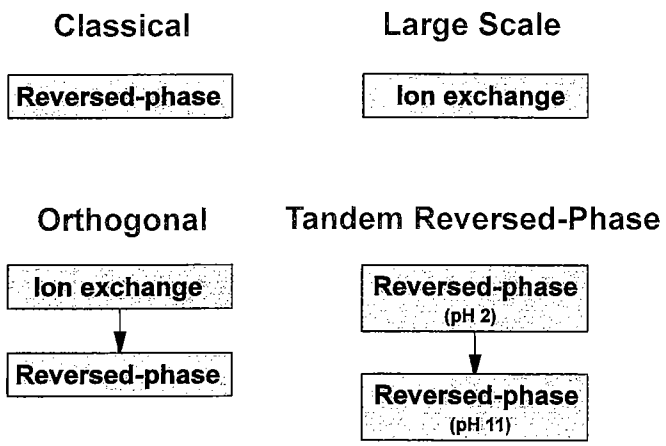
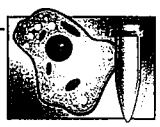
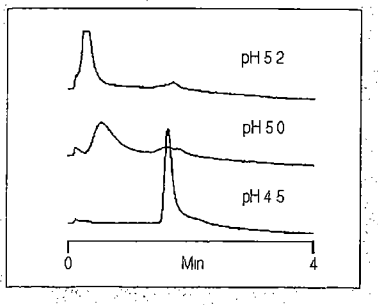
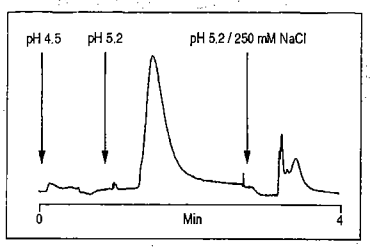


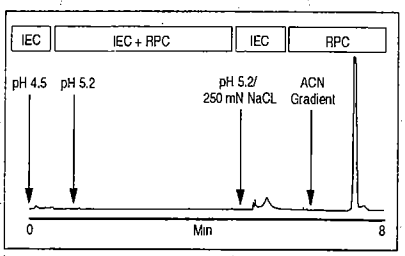
Figure 3-9. Chromatographic separation schemes for peptides

The *classical* (reversed-phase) scheme is generally recommended as a starting point for most applications. Where a large number of different peptides must all be resolved, as in the case of protein digest analysis, a shallow gradient over a wide range in organic concentration should be used together with a very high resolution, small particle (5 µm) column (such as PepMap C18). In general, low pH, with TFA as the ion-pairing agent is a good starting point. However, different ion-pairing agents and pH can dramatically alter the selectivity.





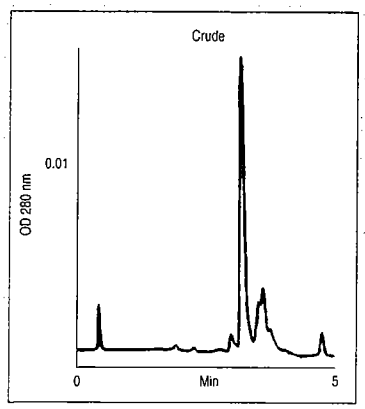


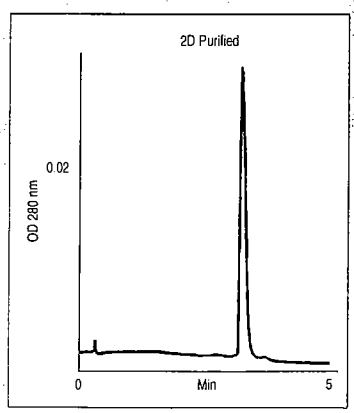


Sample:	14 mer synthetic peptide
System:	BioCAD workstation
Detection:	214 nm
First dimension	
Column:	POROS HS/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM MES, pH as shown
Eluent (B):	as indicated
Flowrate:	10 ml/min (3600 cm/hr)
Second dimension	
Column:	POROS R2/M 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA
Eluent (B):	0.1% TFA in acetonitrile
Flowrate:	10 ml/min (3600 cm/hr)
Elution:	0 - 45% B in 15 CV
Analysis	
Column:	POROS R2/H 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA
Eluent (B):	0.1% TFA in acetonitrile
Flowrate:	5 ml/min (1800 cm/hr)
Elution	0 - 45% B in 20 CV

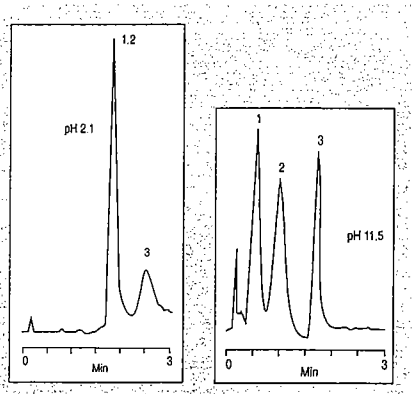
To develop a high purity separation of a synthetic peptide, a pH map was first conducted on POROS cation exchange column (chromatogram #1). The peptide was found to bind to the column at pH 4.5 but flowed through in the wash at pH 5.2. Taking advantage of this fact, peptide was loaded at pH 4.5, allowing some contaminants to flow through. Stepping to pH 5.2 caused elution of the target molecule. A final step to 250 mM NaCl removed remaining contaminants on the column (chromatogram #2).

Automated valving on the BioCAD workstation linked the ion exchange method with a reversed-phase method developed separately (chromatogram #3). The first dimension separation constitutes the ion exchange method. No peak appears at pH 5.2 because peptide eluting from the first dimension is immediately bound to the reversed-phase column, which is switched into line during ion exchange elution. After contaminants are washed off the ion exchange column, the peptide is eluted from the POROS R2 column.





The 2-D method provided higher purity than either method alone, eliminating a key impurity not removable by reversed-phase. The reversed-phase step provided added benefit of desalting and sample concentration. The total time for the automated 2D separation was 8 minutes, far less than the time for a single run on a conventional column. The last two chromatograms show purity analysis by reversed-phase.



Sample:	Anglotensin peptide mixture
Column:	POROS R1/M 4.6 mmD/100 mmL
Starting Buffer (A):	1) 0.1% TFA, pH 2.1 2) 10 mM tribasic sodium phosphate, pH 11.5
Eluent (B):	acetonitrile
Flowrate:	5 ml/min (1800 cm/hr)
System:	HP 1090
Detection:	220 nm
Elution:	0 - 30% B in each case

High pH elution on the stable POROS reversed-phase base matrix provides additional selectivity to resolve two forms of anglotensin that coelute at low pH conditions. Shifts in selectivity at low and high pH can be used to advantage in the tandem reversed-phase scheme described in the text. Peaks 1, 2, and 3 represent Anglotensin II, III, and I respectively.

In the case of *large scale* peptide purifications, ion exchange is often the better starting point, for the practical reasons stated above. When ion exchange is used for peptides, either anion or cation exchange may be used, depending upon the charge characteristics of the particular peptide.

Often, these single column approaches provide sufficient purity or resolution. However, in some cases particular impurities may be very difficult to remove with just one mode of chromatography. For "problem peptides" or where very high purity is needed, the *orthogonal* approach combining ion exchange and reversed-phase is recommended. Ion exchange is used as the first step, so that the reversed-phase column both desalts and purifies the peptide.

Another interesting approach is the *tandem reversed-phase* scheme, which uses a single reversed-phase column. The peptide is first separated under typical conditions at low pH. The partially purified peptide is then applied to the same column and run at very high pH, which completely changes the charge distribution of the peptide and often shifts the selectivity enough to remove the remaining impurities. [*NOTE:* The *tandem reversed-phase* scheme cannot be used with conventional silica-based reversed-phase columns. Silica cannot withstand pH greater than 7. POROS and Oligo R3, being polymeric in nature, have high pH stability, enabling the tandem reversed-phase scheme to be used to advantage. The technique should also be limited to peptides that can withstand exposure to very high pH.]

SECTION 3

NUCLEIC ACIDS

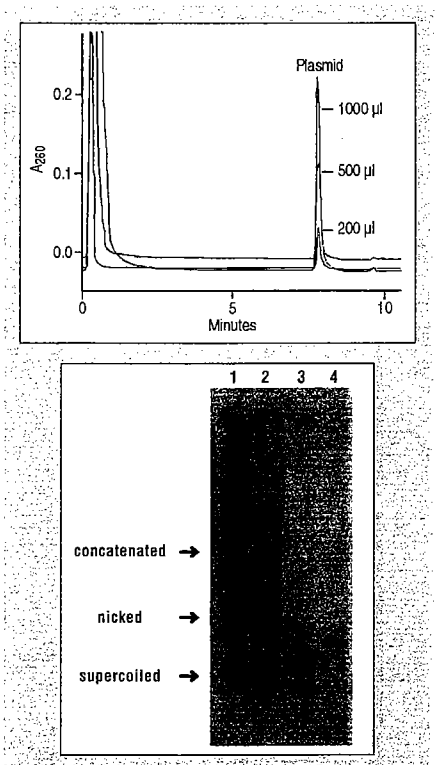
While proteins and peptides are the primary structural elements and effectors in biological systems, nucleic acids carry information. The biotechnology revolution is based largely upon techniques for synthesizing nucleic acids and using them to manipulate the genetic material in living cells. Thus nucleic acids of all kinds constitute important tools for researchers.

Sources & Key Impurities

Nucleic acids may be either natural or synthetic. Natural nucleic acids, including genomic DNA and the various forms of RNA are extracted generally by cell lysis, followed by a series of precipitation and centrifugation steps. Virtually any organism, organ, cell type or virus may be a source. Typical impurities to be removed include cell debris, small molecules, proteins, and unwanted types of nucleic acids.

An important type of "natural" DNA is the plasmid. Plasmids are circular double-stranded DNA (usually supercoiled) produced by bacteria separate from their genomic DNA. Gene-sized pieces of DNA (up to several thousand base pairs) can be easily added to plasmids by artificial means, and the plasmids then inserted into bacteria which are then cultured, producing the plasmid (and gene) on a large scale. This is a critical manufacturing method for gene therapy.

The problem of extracting and purifying plasmid DNA is very similar to that of other natural nucleic acids, except that it is often done on a much larger scale. The same impurities are present, as well as bacterial endotoxins and variant forms of the plasmids themselves, including non-supercoiled and nicked (plasmids with breaks in one or more places on one of the nucleic acid strands) forms.



Sample:	Crude plasmid extract, diluted 1:10 in water, sample load as indicated
Column:	POROS 50 HQ 4.6 mmD/100 mmL
Starting Buffer (A):	25 mM Tris/HCl, pH 8.1, 1 mM EDTA
Eluent (B):	Buffer A + 2 M NaCl
Flowrate:	2.8 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	260 nm
Elution:	10 - 100% B in 10 CV

Loading study for preparative purification of plasmid DNA. A recovery pattern (supercoiled, nicked, concatenated) similar to that obtained with commercially available cartridge type purification systems was demonstrated by an agarose gel. PerSeptive Biosystems Application Note PA 428.

The other major source of nucleic acids, especially single-stranded oligonucleotides, is chemical synthesis. Although the chemistry is very different, the general process for oligonucleotide synthesis is a cyclical one similar in principle to peptide synthesis. The process may be carried out either manually or (more commonly) on an automated system.

In the synthesis cycle, nucleotides are used with the 5' hydroxyls blocked with dimethyl trityl groups and the phosphate blocked and activated to form a phosphoramidite. This activated nucleotide is coupled to the 5' end of the growing oligonucleotide chain, which has been unblocked to expose the primary hydroxyl group. Once the phosphoester bond is formed, the phosphate group is oxidized (either with oxygen to form a natural phosphodiester bond, or with sulfur, to form a phosphorothioate bond, which is more stable to enzymatic attack). The trityl group is then removed by treatment with acid to start the cycle again.

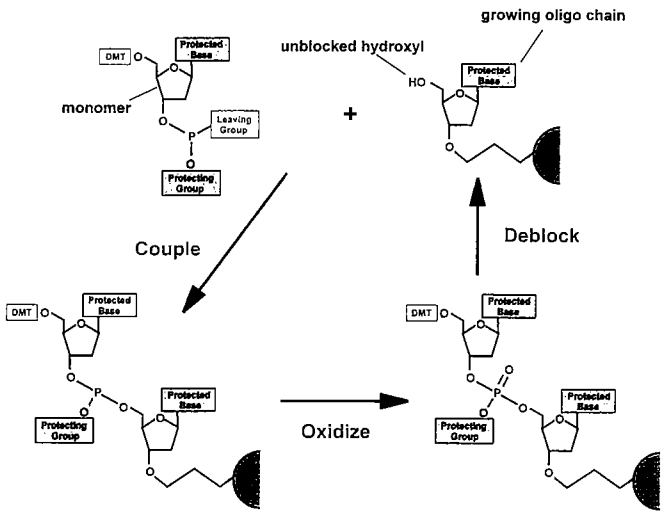
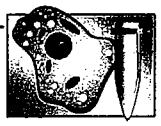


Figure 3-10. Schematic of oligonucleotide synthesis cycle



Although the efficiency of the reaction cycle often exceeds 98%, it is never absolutely complete, so failure sequences (missing one or more nucleotides) are always formed. Some failure sequences do not participate in any further reaction cycles, and thus end up with no trityl group present (trityl-off failures). Some failure sequences involve a missed early reaction cycle, but participate in later cycles, and so end up trityl-on. In addition to failure sequences, incomplete blocking of the bases can result in branched oligonucleotides. It is also possible for other chemical modifications of the bases to occur. Finally, when synthesizing phosphorothioates, some mixed thioate/diester species can form.

Molecular Characteristics

Nucleic acids are polymers of a basic nucleotide unit structure. Each nucleotide unit is based on a pentose sugar — ribose for RNA and deoxyribose for DNA. The pentose sugars are joined together by phosphate groups through phosphoester bonds between the 3' carbon of one sugar and the 5' carbon of the next sugar in the chain. Small nucleic acids (<50 monomer units) are generally called oligonucleotides.

Attached to the 1' carbon of each sugar is one of five different bases — either one of the purines, adenine or guanine, or one of the pyrimidines cytosine, thymine (on DNA) or uracil (on RNA). The sequence of the bases carries the information content of the molecule. The bases are able to form relatively strong hydrogen bonds with each other in specific complementary pairs (C-G, A-T or A-U). This base pairing characteristic allows single strands of nucleic acids with complementary sequences to specifically bind together to form so-called "double stranded" nucleic acids. These double stranded molecules are normally in the form of a double helix.

The size of nucleic acids is quite variable. Synthetic oligonucleotides are often in the range of 15-30 bases, since this is the minimum size required to define an "unambiguous" binding sequence. On the other extreme, plasmids and genomic DNA can be many thousands of base pairs long.

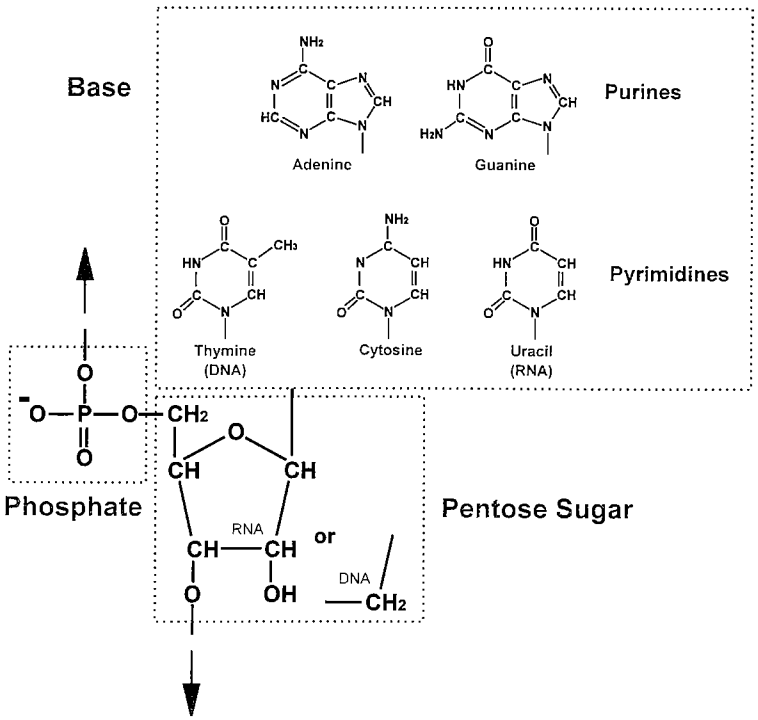


Figure 3-11. Basic structure of nucleic acids

Nucleic acids are linear molecules with no branching. In some cases (particularly plasmids) the 5' carbon on one end of the molecule is connected to the 3' carbon on the other end of the molecule to form a circular loop. The base-pairing and other hydrogen bonding allows the nucleic acids to form considerable higher order structure in solution, especially for larger nucleic acids. One very important type of structure is "super-

coiling", in which the nucleic acid double helix is itself twisted into a helical structure, much like a twisted telephone handset cord. Supercoiling allows very long nucleic acid molecules to occupy a relatively small space. RNA can adopt a number of other complex structures, similar to proteins, which are necessary for its function.

In addition to these conventional nucleic acid structures, researchers are increasingly synthesizing more complex structures, including RNA/DNA hybrids and conjugates between nucleic acids and other molecules such as biotin or fluorescent dyes. There is also considerable research on alternative backbone structures, including uncharged backbones such as PNA (peptide nucleic acids), in which a modified peptide backbone substitutes for the conventional sugar-phosphate. PNA's have a number of very interesting applications because of their ability to hybridize much more tightly than conventional nucleic acids.

From a chromatographic separation perspective nucleic acids have a relatively uniform structure. The phosphate groups in the backbone provide a strong negative charge, which dominates ion exchange interactions. The molecules are relatively hydrophilic, with little difference between the base units. Although these features allow nucleic acids to be relatively easily separated from other biomolecules, fractionation of mixtures of different, closely related nucleic acids can be difficult. Most separations are based on differences in charge and other characteristics as a function of chain length.

The key molecular characteristics of nucleic acids are summarized in the CHASM analysis (see Section 2 — *Principles of Systematic Method Development*) shown in the following table.

CHASM ANALYSIS — *NUCLEIC ACIDS*

Charge	Backbone has very strong negative charge over entire pH range due to phosphate groups, with total charge proportional to chain length. Some bases are positively charged at pH < 5.
Hydrophobicity	Backbone is very hydrophilic. Bases weakly hydrophobic, especially guanine. Trityl blocking group of synthetic oligonucleotides is extremely hydrophobic, allowing easy separation of trityl-on from trityl-off by reversed-phase.
Affinity	Under proper conditions, nucleic acids can specifically hybridize to complementary sequences. This characteristic is used in chromatography to purify nucleic acids with poly dT or poly A tails.
Solubility & stability	Oligonucleotides are soluble under a broad range of conditions, except at low pH. C-rich oligos may precipitate at pH < 8. Larger nucleic acids may require salt for solubility and will precipitate in high concentrations of organic solvent. G-rich oligos are a special problem, due to the strong non-specific intermolecular interactions due to base-stacking. These can only be overcome by operation at very high pH (10-12). Nucleic acids are generally stable to most conditions, including high pH, but prolonged exposure to strong acids may cause decomposition of purine bases (depurination). Trityl-on oligos begin to detritylate at pH < 8. RNA is highly susceptible to degradation by ribonuclease, which is ubiquitous.
Molecular weight	Highly variable. Typical synthetic oligonucleotides are 5000 - 10000 MW. Genomic and plasmid DNA may be very large (>10^6 to 10^8 MW). Monomer units average 300 MW.

Chromatography of Nucleic Acids

Unlike the case with proteins, antibodies, and peptides, chromatography is *not* the predominant separation technique used for nucleic acid applications. The most common techniques for purification from natural sources include extraction & precipitation (with cold ethanol, phenol and other methods) and

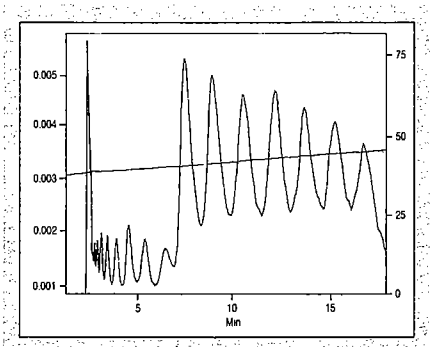
density gradient ultracentrifugation. Fine separations and analysis of nucleic acids are most frequently performed with various types of gel electrophoresis. These techniques work quite well in most cases, and are highly appropriate and convenient for the very small scale at which many nucleic acid separations take place.

There are several nucleic acid applications in which chromatography plays a very important role. One is the large-scale purification of plasmid DNA, for which the traditional extraction/precipitation and ultracentrifugation methods are not practical. Another is the purification of synthetic oligonucleotides.

Anion exchange chromatography is the predominant method used for nucleic acid purification, due to the high negative charge density from the backbone phosphates. Under the proper conditions, anion exchange can resolve single failures (so-called "N-1") from full-length strands with oligonucleotides of up to 30 mer in length. Anion exchange can also separate various types of nucleic acids from each other (such as RNA from DNA). Very small particle anion exchange media (2.5 µm) can separate variant forms of the same molecule (such as supercoiled, intact plasmid from the uncoiled or nicked forms).

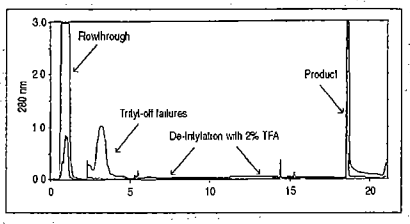
Reversed-phase chromatography has somewhat limited utility for separating mixtures of nucleic acids. However, reversed-phase is excellent for separating trityl-on from trityl-off synthetic oligonucleotides. This enables many of the failure sequences from a synthesis to be easily separated from full length product.

Other modes of chromatography, such as hydrophobic interaction or affinity, are rarely, if ever, used with nucleic acids. Gel filtration is sometimes used for desalting but rarely used for fractionation of synthetic oligonucleotides because most nucleic acid contaminants are of approximately the same molecular weight as the product.



Sample:	40 µl poly dT diluted in start buffer
Column:	2 x POROS HQ/H 4.6 mmD/ 100 mmL connected in series
Starting Buffer (A):	20 mM triethyl ammonium bicarbonate, pH 8.5
Eluent (B):	Buffer A + 1 M NaCl
Flowrate:	4 ml/min
System:	BioCAD workstation
Detection:	260 nm
Elution:	48 - 60% B in 30 CV

Separation of synthetic polyT 12-18 mer ladder on POROS anion exchange column. To get the resolution shown, two columns were plumbed in series (giving an effective column length of 200 mm) and a very shallow gradient was run. However, the fast flow rate still enabled the separation to be completed in under 20 minutes.



Sample:	75 mg (9.6 mg/ml CV) of 18 mer phosphorothioate DNA in start buffer
Column:	Oligo R3 10 mmD/100 mmL
Starting Buffer (A):	50 mM ammonium acetate, pH 10
Eluent (B):	100% acetonitrile
Flowrate:	13 ml/min (1000 cm/hr)
System:	BioCAD workstation
Detection:	260 nm
Elute trityl-off failures:	Step to 8% B
Detritylation:	2% TFA at 180 cm/hr
Elute product:	50% B

Example of simultaneous purification and on-column detritylation made possible by the pH stability of Oligo R3 polymeric reversed-phase media. Flow rate was reduced during detritylation to increase contact time while conserving solvent. Detritylation was quenched with a brief wash of starting buffer prior to final elution of product.

Several different schemes may be used to process and purify synthetic oligonucleotides using chromatography. After the synthesis is completed, the oligo is generally cleaved from the support and the blocking agents removed with ammonium hydroxide (which must be at least partially evaporated or lyophilized to allow binding to the reversed-phase column). Because many of the failure sequence impurities are trityl-off, it is advantageous to purify the oligo by reversed-phase prior to detritylation. Oligo R3 is the packing of choice for this application. The trityl-off impurities elute at a significantly lower solvent concentration than the trityl-on product. This easy separation will usually yield a purity around 90% full length product (assuming a reasonably efficient synthesis) with good recovery.

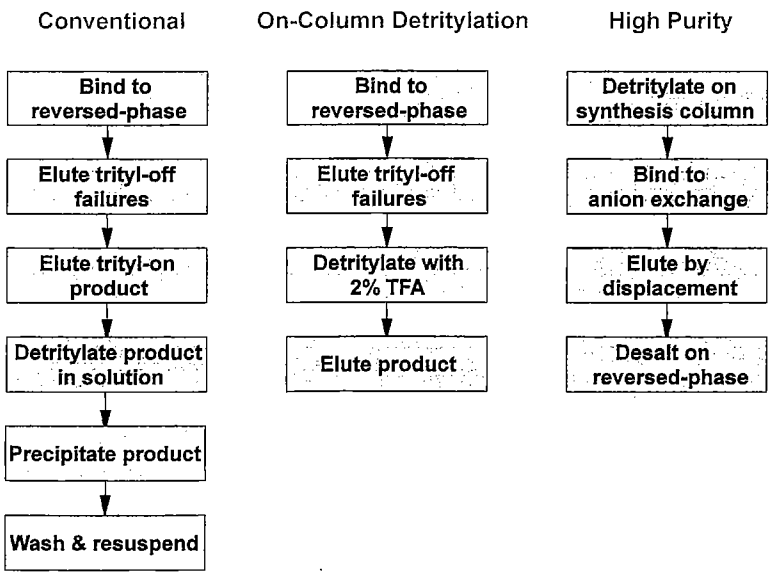
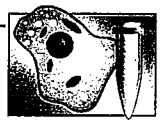


Figure 3-12. Processing and separation schemes for synthetic oligonucleotides

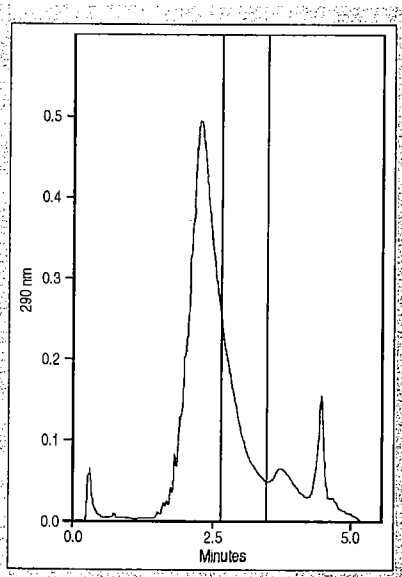


Once this separation is complete, the trityl blocking groups are removed by treatment with acid. Conventionally, this is done in solution after the product is eluted from the reversed-phase column. After the reaction, the product can be precipitated with ethanol, then washed by filtration and resuspended. Done this way, detritylation typically takes a day, is sometimes not complete, and product losses can be high due to the handling involved.

A faster, more convenient and higher yield approach is to take advantage of the pH stability of POROS or Oligo R3 media to carry out the detritylation while the oligonucleotide is still bound to the reversed-phase column. After eluting the trityl-off failures, wash for several column volumes with 2% trifluoroacetic acid (TFA), then pump at very low flow rate for 5-10 minutes to detritylate the product. Elute the now detritylated product with the same solvent concentration used for the trityl-off failures. Elute any remaining trityl-on material and the released trityl with a steep gradient to 95% organic. This approach should not be used on silica-based columns.

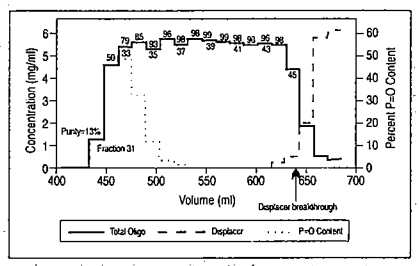
When very high purity (>95% full length product) oligos (<30-mer) are required, as for therapeutic work or crystallographic studies, anion exchange must be used. Conventional salt gradient elution will work, but because the impurities are so similar in structure to the product, there is often a tradeoff between yield and purity. The fraction cutting required for very high purity will often sacrifice a significant quantity of product.

An interesting alternative to conventional gradient elution is displacement chromatography. In this technique, the sample is loaded on the column, followed by a solution of a molecule that binds to the column more tightly than any of the samples components. This *displacer* "pushes" the bound sample molecules down the column, during which time they form a "displacement train" of adjacent bands in order of increasing binding strength. Once the sample molecules have all been displaced off, the displacer is eluted with salt or another eluent and the column is reequilibrated.



Sample:	485 µl of 1 mol synthesis (0.6 µmol full length product), deprotected, dried, and resuspended in water
Column:	Self Pack POROS 10 HQ 4.6 mmD/ 100 mmL
Starting Buffer (A):	0.1 M NaCl, 0.01 M NaOH
Eluent (B):	0.8 M NaCl, 0.01 M NaOH
Flowrate:	8 ml/min (3000 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	290 nm
Elution:	Two part gradient: 10-62% B, 62-72% B

Preparative purification of 26-mer synthetic oligonucleotide on POROS anion exchange column. Fractions in the area marked by vertical bars were pooled as determined by radio-labelled electrophoretic analysis. The speed of the Perfusion Chromatography runs facilitated the screening of mg quantities of oligonucleotides varying in length and location of binding site for crystallographic studies of DNA/DNA binding protein complexes. From work conducted by Drs. Tom Ellenberger and Sylvie Doublie, Harvard Medical School. PerSeptive Biosystems Application Note PA 443.



Sample:	1.2 grams (6 mg/ml CV) of oligonucleotide, partially purified by reversed phase
Column:	POROS HQ/M 50 mmD/100 mmL
Mobile Phase:	100 mM NaCl, 10 mM NaOH, 5% Methanol
Displacer:	7 mg/ml 40 kD dextran sulfate
Flowrate:	50 ml/min (150 cm/h)
System:	BioCAD workstation
Detection:	300 nm

Preparative displacement chromatography of 24-mer phosphorothioate oligonucleotide. The displacement histogram is constructed by plotting N-length purity of collected fractions as determined by capillary electrophoresis. Fraction numbers are listed below the line, N-length purity above the line. The results of phosphodiester (P=O) analysis of fractions is also shown (dotted trace).

Failure sequences and phosphodiesters were concentrated in the initial displacement fractions. Fractions 38 to 44 contained essentially pure phosphorothioate full-length product. Using this method, 24.5 grams of purified oligonucleotide could be generated in a 24 hour period. PerSeptive Biosystems Application Note PA 424.

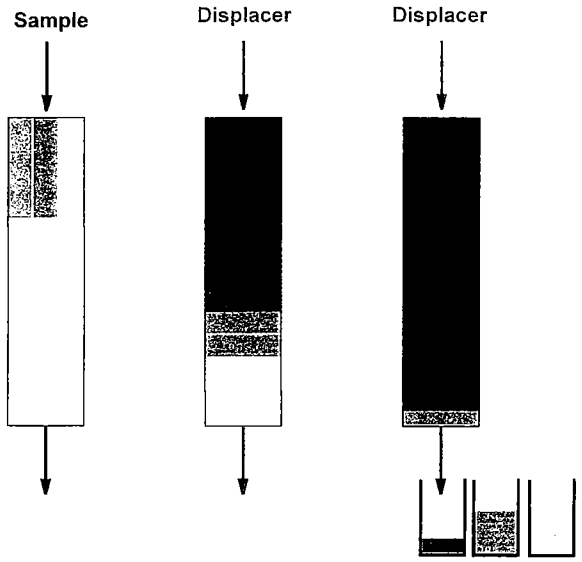
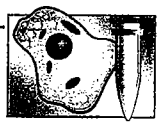


Figure 3-13. Displacement chromatography

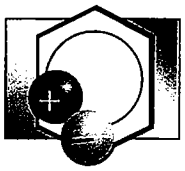
Displacement chromatography offers several advantages for oligonucleotide purification. The most important is that it significantly improves the yield vs. purity tradeoff. Purity can be very high (>95% full length) in a single step, even when crude synthesis product is used as the feed. In addition, the technique is very simple, and a single protocol can be used for most oligonucleotides without modification.

To utilize displacement anion exchange chromatography, first carry out the detritylation reaction while the oligo is still bound to the synthesis support. Then cleave and load the entire synthesis mixture (containing all the failure sequences together with the product) onto an anion exchange column (POROS HQ). Elute this column by displacement, using 40 kD dextran sulfate as the displacer. [*NOTE:* For a more detailed discussion of displacement chromatography as applied to oligonucleotides, see PerSeptive Biosystems' *Application Note PA424.*] You can then remove the salt and residual displacer by binding the product to a reversed-phase column (Oligo R3), washing with water and eluting with a low concentration of organic solvent.

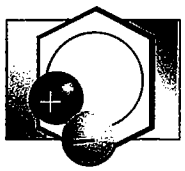


The following are some specific protocol suggestions for nucleic acid chromatography:

- You should use very high pH (10 - 12) for both anion exchange and reversed-phase chromatography of oligonucleotides. High pH reduces the intermolecular interactions between the bases, which otherwise can cause poor peak shape and recovery. This is especially a problem with G-rich oligos, where high pH is essential. Ammonium acetate is a useful buffer salt for these conditions due to its volatility, although NaOH is also often used. When operating under high pH conditions you must use strong anion exchange packings (POROS HQ and QE) instead of weak ones.
- In many cases, it is highly beneficial to include 10 - 50% organic solvent (alcohol or acetonitrile) in the mobile phase for anion exchange chromatography of nucleic acids. This applies both to oligonucleotides and large nucleic acids, especially plasmids.
- Elevated temperature can also be useful to reduce base stacking.
- You can separate phosphodiester from phosphorothioate oligonucleotides relatively easily using anion exchange, even at the same chain length. This is because the phosphorothioate groups have a more localized charge distribution, causing tighter binding to the ion exchanger.
- DNA/RNA separations can be enhanced by using borate as the buffer for anion exchange at pH 8.5 - 9.5. The borate ion forms a negatively charged complex with the cis-diol groups on the RNA ribose, but not on the deoxyribose of DNA.
- Modified oligonucleotides with uncharged backbones (such as PNA) can present major purification problems. One approach is to use cation exchange at relatively low pH (<4), to take advantage of the charges on the bases.



SECTION 4
MODES OF CHROMATOGRAPHY



SECTION 4
MODES OF CHROMATOGRAPHY

The key element in a chromatographic separation is the nature of the interaction between the bonded phase surface of the packing and the molecules in the sample solution. These interactions fall into several broad classes or *modes* of chromatography. Each mode represents a unique binding mechanism and utilizes a particular chemical characteristic of the mobile phase to effect elution of the bound sample molecules. The following sections describe each chromatographic mode in some detail. Each section also contains a set of specific experimental protocols for developing a method in the given chromatographic mode. Detailed suggestions for particular kinds of molecules will be found in Section 3 — *Developing Your Application*.

ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography separates molecules based on differences in their accessible surface charges. The technique can be used for virtually any charged molecule that is soluble in aqueous systems, and typically provides high resolving power and high binding capacity. Ion exchange is widely used in the separation of proteins because the relatively mild binding and elution conditions allow high protein recovery with intact biological activity. Most protein separation schemes involve one or more ion exchange steps. Ion exchange also plays a critical role in the purification of many antibodies and nucleic acids, and to a lesser extent peptides.

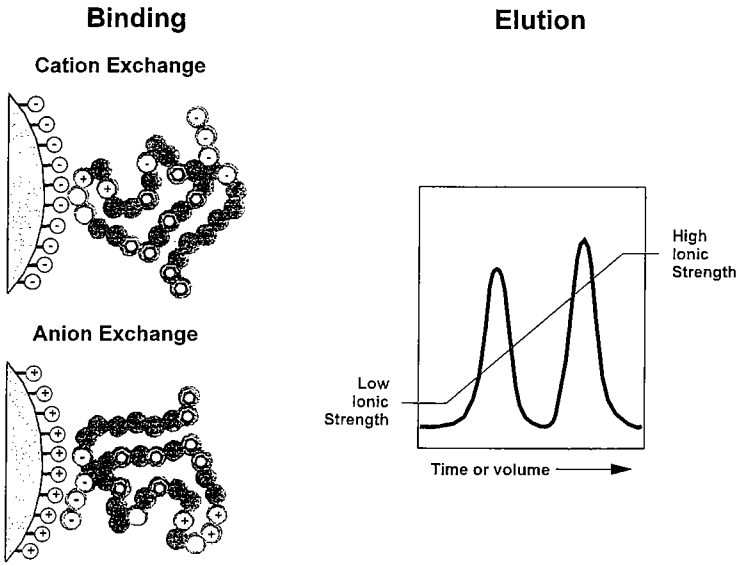


Figure 4-1. Binding and elution mechanisms of ion exchange chromatography

Binding Mechanism

Ion exchange chromatography is based on the ionic attraction between molecules of opposite electric charge. The bonded phase of an ion exchange packing consists of functional groups that have either a positive charge (*anion exchange*), used to separate negatively charged target molecules (anions), or a negative charge (*cation exchange*), used to separate positively charged target molecules (cations). The electrostatic interactions between the opposite charge groups on the surface of the chromatographic packing material and on the binding molecules take place over a relatively short distance. Because of this, in ion exchange chromatography, molecules are separated based on the number of positive or negative charges *accessible on their surfaces*.

Anion or cation exchange functional groups are classified as either "weak" or "strong". *Weak ion exchange* groups are titratable — i.e. they gain or lose electrical charge as the pH of the mobile phase changes. The most common weak anion exchange group is diethylaminoethyl or *DEAE*. The most common weak cation exchange group is carboxymethyl or *CM*. *Strong ion exchange* groups maintain their charge independent of the pH of the mobile phase. The most common strong anion exchange groups are quaternary amines. The most common strong cation exchange groups are sulfonates.

Note that the terms "strong" or "weak" do **not** refer to the strength of the binding but only to the effect of pH on the charge of the functional groups.

Elution Method

Because of the substantial energy involved in charge-charge interactions, the laws of physics dictate that the number of positive and negative charges in any given volume must be almost exactly equal. Thus each charged group in solution or on a surface has a corresponding *counter ion* nearby of the opposite charge. The most common counter ions are small salt or buffer molecules.

Ion exchange *binding* occurs when the salt concentration or *ionic strength* of the mobile phase is reduced to the point that the ionic groups on the sample molecules begin to serve as the counter ions for the charged groups on the stationary phase. This causes the sample molecules to bind to the surface. *Elution* takes place when the ionic strength of the mobile phase is increased. As this happens, salt molecules displace the bound sample molecules back into the mobile phase. At the same time, salt molecules with the same charge as the bonded phase (called *co-ions*) bind to the charge groups on the sample molecules.

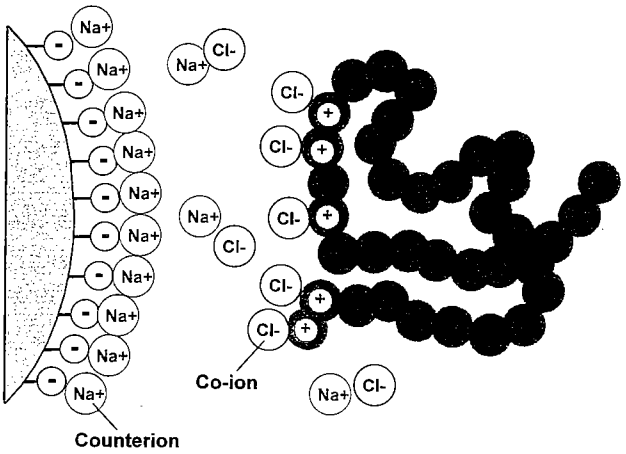


Figure 4-2. Counter ions and co-ions during cation exchange elution

It should be noted that all the charged species in the solution (including any salts present and even the buffer ions) interact in a complex way with either the bonded phase or the sample molecules during binding and elution. The precise concentrations and chemical nature of all these species have a significant impact on the selectivity of the separation.

Elution methods may also include changes in pH along with (or instead of) ionic strength increases. The pH can affect the charge of the sample molecules (see below) as well as (in the case of weak ion exchange media) the charge of the bonded phase. Changes in pH can thus be used to weaken or eliminate charge-charge interactions, thereby causing elution.

Charge Characteristics of Biomolecules

The behavior of molecules in an ion exchange separation obviously depends upon their charge characteristics. The total net charge (number of positive charges minus the number of negative charges) and, more importantly, the actual distribution of charges within the molecule critically affect both binding and elution. However, available information about the charge characteristics of complex biomolecules such as

proteins is usually very limited. In fact, the researcher may be purifying the protein specifically to obtain such detailed structural information.

The pH of the mobile phase strongly affects the charge characteristics of proteins and peptides. This is because virtually all of the charged amino acid side chains are weak ion exchange groups whose charge depends upon the pH. Thus both the charge distribution and the total net charge depend on pH. This dependency is usually highly non-linear. In all cases, however, the molecules will become more positively charged at lower pH values, and more negatively charged at higher pH values.

One important characteristic relating to the charge of a biomolecule is its *isoelectric point* (or pI), the pH at which the total number of positive charges equals the total number of negative charges, resulting in a *net* charge of zero. A molecule with a high isoelectric point (called a basic molecule) will thus tend to be positively charged at neutral pH, and a molecule with a low isoelectric point (called an acidic molecule) will tend to be negatively charged at neutral pH. The isoelectric point is usually measured by isoelectric focusing electrophoresis.

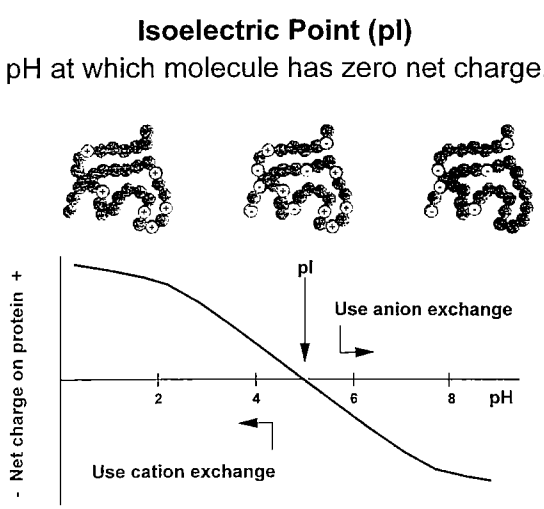


Figure 4-3. Isoelectric point

Although the isoelectric point provides a useful starting point, its value in predicting the behavior of a sample on an ion exchange column is limited. This is because binding and elution in ion exchange are determined by the *distribution of accessible charge groups on the surfaces* of the molecules, and not by the *total charge*. Although many molecules exhibit very weak or no binding when at their isoelectric point, this is not always the case. As Figure 4-4 illustrates, it is possible to have two molecules at their isoelectric point (with zero *net* charge), but which exhibit completely different binding behavior, because of differences in the actual distribution of the charge groups in the molecule.

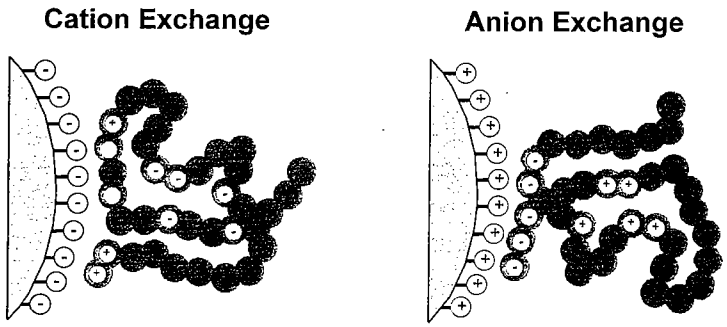


Figure 4-4. The effect of charge distribution on ion exchange binding. Both molecules are at their isoelectric point (zero net charge), but because of their different, highly asymmetric charge distributions, the one on the left will bind tightly to a cation exchange column, and the one on the right to an anion exchange column.

In addition to affecting the distribution of charge within the molecule, pH can also have a strong effect on the 3-D conformation of the molecule in solution. The conformation affects not only which charge groups are accessible to the bonded phase surface, but also secondary binding interactions (such as hydrogen bonding and hydrophobic interactions), which play a role in binding and elution behavior.

Because of these phenomena, the effect of pH on the chromatographic selectivity in an ion exchange separation can be quite profound and difficult to predict. For this reason, an empirical approach should be used to evaluate an ion exchange separation over a range of pH values when developing a new method. This can be done easily with Perfusion Chromatography.

Choosing an Ion Exchange Column

You must make several choices when selecting the column packing used to develop an ion exchange separation. The first is whether to use anion or cation exchange. The second is whether to use a weak or strong ion exchange functional group.

The choice between anion and cation exchange depends upon the charge characteristics and the effect of pH on stability and solubility of both the target molecule itself and the other molecules in the sample. To maximize binding strength, select an operating pH range that is either above or below the isoelectric point of the target, based on where the biomolecule is most stable and soluble. *(CAUTION: The solubility of most proteins is very limited at a pH close to the isoelectric point if the ionic strength is low.)* The ion exchanger is then chosen by the following rule:

At a pH above the pI, the molecule is negative — try anion exchange first. At a pH below the pI the molecule is positive — try cation exchange first.

Many proteins (such as enzymes and blood proteins) are most stable near neutral pH and have an acidic pI (negative charge at neutral pH), making anion exchange the technique of choice. However, many regulatory proteins (such as cytokines and growth factors) and peptides that are currently of great interest in research have a basic pI (strong positive

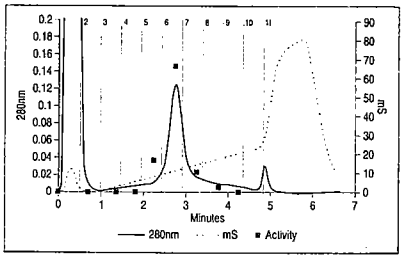
charge at neutral pH) and/or are only soluble at very low pH (2-4). For these applications you should choose strong cation exchange media.

Many biomolecules have a solubility and stability pH range that encompasses their isoelectric point, so that either anion or cation exchange can be used. The choice in this situation depends upon which column will give the best selectivity (e.g. which column will achieve the greatest separation between the target molecule and the contaminants). Unless you know enough about the charge characteristics of the target and contaminants to make a choice, you should try both anion and cation exchange in these cases. In many instances, a combination of the two methods in sequence will give the best separation.

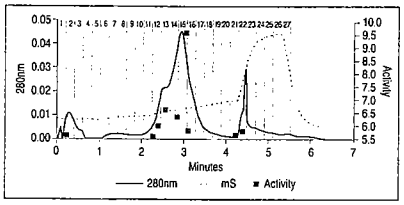
For most biomolecules and pH ranges, either strong or weak ion exchange media may be used. In extreme pH conditions (pH >10 for anion exchange and pH <3-5 for cation exchange), weak ion exchange media lose most of their charge, and thus bind molecules very weakly or not at all. In additio ak ion exchange media can take much longer to equilibrate, because the column itself has a significant buffering capacity.

As a starting point for method development, use strong ion exchange media, since they operate over a broader pH range and equilibrate more easily than weak ion exchange media.

Exceptions to this rule include separations of samples that contain molecules with large amounts of strong ion exchange functionality (such as large nucleic acids, phospholipids, or anionic detergents). These types of molecules bind to an ion exchange column so tightly they do not come off in high salt. In such cases, weak ion exchange media should be used, since a wash in either acid (for cation exchange) or base (for anion exchange) will eliminate the charge on the column and allow

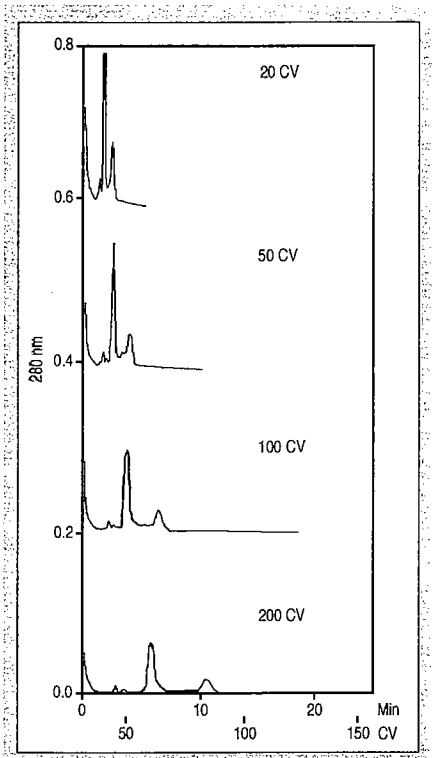


Sample:	2 ml crude ascites diluted 1:5 in PBS
Column:	Self Pack POROS 20 HS 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM NaH_2PO_4, pH 6.5
Eluent (B):	20 mM NaH_2PO_4, pH 6.5 + 3 M NaCl
Flow Rate:	8.5 ml/min (3000 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 350 mM NaCl in 20 CV



Sample:	2 ml pooled fractions 6 & 7 from previous run
Column:	Self Pack POROS 20 HQ 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis/propane, pH 8.0 + 150 mM NaCl
Eluent (B):	20 mM Tris/bis/propane, pH 8.0 + 3 M NaCl
Flow Rate:	10 ml/min (3600 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step from 150 to 200 mM NaCl for 2 CV 200 - 350 mM NaCl in 15 CV

Two-step purification of monoclonal IgM on POROS cation exchange followed by anion exchange. Final IgM product was >95% pure, as determined by SDS-PAGE silver stain. The high flow rates facilitated the use of the two-step method, minimized the risk of aggregation and helped preserve biological activity of the labile antibody, circumventing some of the problems typically experienced with conventional IgM purification techniques. Work done in conjunction with Dr. Y.P. Lim, Rhode Island Hospital. PerSeptive Biosystems Application Note PA 440.



Sample: 100 µl of test mixture
Column: POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A): 25 mM Tris/HCl, pH 8.5
Eluent (B): 25 mM Tris/HCl, pH 8.5 + 2 M NaCl
Flow Rate: 10 ml/min (3600 cm/hr)
System: BioCAD workstation
Detection: 280 nm
Elution: 0 - 50% B, CV as indicated

The effect of gradient slope on an ion exchange separation. As the gradient becomes shallower, peaks are spread out from each other, increasing resolving power. When the goal is to identify and recover the molecule of interest with a minimum of experiments, the extra dilution of peaks (bandspreading) that also results can often be accepted.

Programming a long, shallow gradient for such a minimal development experiment is practical with POROS media because of the high flow rates that are possible. The gradient can be truncated once all peaks have eluted, as was done in the 200 CV case. The run then provides a good starting point for additional gradient optimization, if required.

these tightly binding species to elute. In addition, strong and weak ion exchange functionalities will have subtle and sometimes useful differences in selectivity, which can be exploited when needed. However, most separations can be done acceptably on strong ion exchange media.

Developing an Ion Exchange Method

The most critical parameter in an ion exchange method is the buffer pH. As the starting point in developing a method, use a pH at an extreme end of the working range (pH 8.5 for anion exchange, pH 4.0 for cation exchange). At this pH, binding generally should be tightest, so that all molecules in the sample that can bind, will bind.

To insure good resolution and recovery with a minimum number of experiments, run a long, shallow elution gradient from 0 - 1.0 M NaCl. A gradient developed over 50 to 100, even 200 column volumes provides high resolving power, while the wide range in ionic strength ensures that all the molec hat can elute with good recovery will elute. Gradients of this length are typically not considered because of the time required to do the experiment at the flow rates of conventional media. The high flow rates of Perfusion Chromatography media, on the other hand, permit you to perform the shallow gradient experiment in a convenient time frame, as shown in the figure.

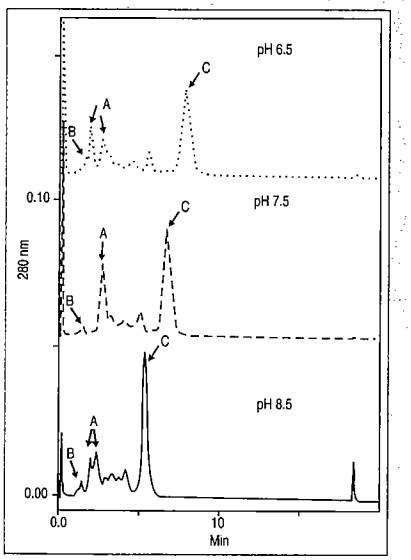
Note that as gradient slope gets shallower, bandspreading and dilution of the eluted peak generally increases. The initial choice of gradient slope will therefore depend on the amount of sample available, the sensitivity of detection, and the importance of conserving handling volumes for subsequent steps. A gradient duration of 100 CV is given as a general recommendation but adjust it according to your situation, keeping in mind that the shallower you can make the gradient without compromising elsewhere, the better chance of minimizing the number of subsequent experiments.

Although the extreme pH generally ensures maximal sample binding, take the time to try at least two other pH values across the range. This is because pH effects are non-linear and different for different molecules. Selectivity effects can be quite dramatic (see discussion on previous pages), as is illustrated quite well in the example of anion exchange chromatography of egg white protein shown here.

Some biomolecules are relatively hydrophobic and/or have low solubility in completely aqueous systems. If poor recovery or broad, tailing elution peaks are observed, try adding a water-miscible organic solvent (such as methanol, glycerol, ethylene glycol, isopropanol or acetonitrile) to the mobile phases. You want to add organic solvent in a concentration high enough to reduce hydrophobic interactions between the molecules and the surface (and in some cases enhance solubility), but not so high as to denature molecules or cause precipitation (of molecules *or* buffer salts).

A good general recommendation is to add 20% (v/v) organic solvent but you should really experiment with your own sample and buffer system to determine the optimum concentration. Adding organic solvent may also increase viscosity, which can have an impact on how fast you can run your column. If organic solvent is added, the effect of pH should be reinvestigated in the presence of the solvent.

Once you determine the optimal pH (and solvent concentration, if needed), the gradient may be shortened by jumping directly to the high salt wash after the target molecule is eluted. Following this, you may perform an appropriate loading study if needed to determine the maximum sample loading at which the target molecule still binds and is sufficiently resolved.



Sample:	100 µl egg white diluted 1:10 in start buffer
Column:	POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A):	25 mM HEPES, pH as indicated
Eluent (B):	Buffer A + 2 M NaCl
Flow Rate:	10 ml/min (3600 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 50% B in 100 CV

Typical Minimum Method Development experiment for anion exchange, illustrating the importance of pH mapping. Note that components labeled (A) are baseline separated from each other at pH 6.5, coelute at 7.5, and begin to separate again at pH 8.5 (reversed in elution order at the higher pH).

Peak labeled (C) does not behave as might be expected. Instead of being more retained as pH increases on anion exchange, it becomes less retained, probably due to changes in 3-D conformation with pH which alter the surface charge distribution.

Variables for Full Method Development

pH map	Examine a range of pH 6-9. Increments of 0.5 pH units are usually sufficient, although even finer differences may be significant.
Surface chemistry	POROS QE, PI and DEAE are available in addition to HQ. Use same experimental strategy for each surface chemistry. [NOTE: The POROS Self Pack Methods Investigation Kits represent an economical way to try different chemistries.]
pH gradient	The effects of pH gradient elution (from high to low) are very different from salt elution. You can also combine the two techniques. Keep in mind that pH gradients will be non-linear.
Gradient	If target elutes at end of the gradient, try including salt in the equilibration and/or wash to elute lower binding molecules before gradient. For some applications, you may wish to use step elution.
Buffer	Conventionally, buffer ions are either zwitterionic (as with Good's buffers) or positively charged. Negatively charged buffers (e.g. phosphate), act as a counter ion, which may create unusual selectivity effects and affect reproducibility. Sometimes this is useful, but examine with care.
Organics	Check organic solvents (methanol, ethanol, isopropanol, acetonitrile, ethylene glycol, etc.), non-ionic detergents or chaotropes (urea) to modify selectivity and improve peak shape and recovery, especially for very hydrophobic proteins.
Salt	Other salts may provide quite different selectivities as eluents. Both anion and cation have an effect.

BioCAD Tips

- The BioCAD pH Map template is an extremely convenient way to probe the effect of pH in detail. Up to 9 different pH values may be programmed at one time, with the computer-controlled buffer blending system setting the pH automatically.
- When using the pH Map template, use either the recommended BioCAD mapping buffer systems (which provide large linear buffering range) or any other appropriate buffer (after running a buffer calibration routine on the instrument). If BioCAD mapping buffers are used for developing a method, always switch to a more conventional single buffer at the same pH for the final method.
- BioCAD SCOUT Column Selector valve makes it extremely convenient to check different anion exchange chemistries on the same sample.
- Although most instruments program gradients only in time, the BioCAD allows programming in column volumes (CV). This feature is highly recommended for method development, since you can make changes in flow rate and even column size without changing the gradient or affecting the chromatography.
- BioCAD also allows programming in eluent concentration instead of percentages, which can make complex experiments simpler to interpret. When using this feature, define each solvent channel carefully in the Configuration screen. Consult BioCAD manual for details.
- The 6 solvent capability allows you to check different buffer salts or additives and concentrations automatically. Use water and concentrated salt in two solvent channels, leaving room for two different buffers (at high and low pH) for a pH mapping experiment. At constant pH, use the extra channel for additives at varying concentration, such as organic solvent, stabilizing agent, etc.
- Unless you pre-blend organic solvent with all the mobile phases (which is recommended once you have settled on a concentration), be careful to degas and/or helium sparge all solvent lines.

Variables for Full Method Development

pH map	Examine a range of pH 4.0-7.5. Increments of 0.5 pH units are usually sufficient, although even finer differences may be significant.
Surface chemistry	POROS S, SP and CM are available in addition to HS. Use same experimental strategy for each surface chemistry. [*NOTE:* The POROS Self Pack Methods Investigation Kits represent an economical way to try different chemistries.]
pH gradient	The effects of pH gradient elution (from low to high) are very different from salt elution. You can also combine the two techniques. Keep in mind that pH gradients will be non-linear.
Gradient	If target elutes at end of the gradient, try including salt in the equilibration and/or wash to elute lower binding molecules before gradient. For some applications, you may wish to use step elution.
Buffer	Conventionally, buffer ions are either zwitterionic (as with Good's buffers) or negatively charged. Positively charged buffers (e.g. Tris), act as a counter ion, which may create unusual selectivity effects and affect reproducibility. Sometimes this is useful, but examine with care.
Organics	Check organic solvents (methanol, ethanol, isopropanol, acetonitrile, ethylene glycol, etc.), non-ionic detergents or chaotropes (urea) to modify selectivity and improve peak shape and recovery, especially for very hydrophobic proteins.
Salt	Other salts may provide quite different selectivities as eluents. Both anion and cation have an effect.

BioCAD Tips

- The BioCAD *pH Map* template is an extremely convenient way to probe the effect of pH in detail. Up to 9 different pH values may be programmed at one time, with the computer-controlled buffer blending system setting the pH automatically.
- When using the *pH Map* template, use either the recommended BioCAD mapping buffer systems (which provide a large linear buffering range) or any other appropriate buffer (after running a buffer calibration routine on the instrument). If BioCAD mapping buffers are used for developing a method, always switch to a more conventional single buffer at the same pH for the final method.
- The BioCAD SCOUT Column Selector valve makes it extremely convenient to check different cation exchange chemistries on the same sample.
- Although most instruments program gradients only in time, the BioCAD allows programming in column volumes (CV). This feature is highly recommended for method development, since you can make changes in flow rate and even column size without changing the gradient or affecting the chromatography.
- BioCAD also allows programming in eluent concentration instead of percentages, which can make complex experiments simpler to interpret. When using this feature, define each solvent channel carefully in the Configuration screen. Consult BioCAD manual for details.
- The 6 solvent capability allows you to check different buffer salts or additives and concentrations automatically. Use water and concentrated salt in two solvent channels, leaving room for two different buffers (at high and low pH) for a pH mapping experiment. At constant pH, use the extra channel for additives at varying concentration, such as organic solvent, stabilizing agent, etc.
- Unless you pre-blend organic solvent with all the mobile phases (which is recommended once you have settled on a concentration), be careful to degas and/or helium sparge all solvent lines.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic Interaction Chromatography (*HIC*) separates biomolecules based on the hydrophobic groups on their surfaces. Binding of biomolecules to the mildly hydrophobic surface of an HIC column is induced by the addition of high salt concentrations to the sample and equilibration buffer. Elution is effected by decreasing the salt concentration. Although the mechanism is somewhat different, from a purely functional point of view, HIC can be viewed as a high resolution version of classical ammonium sulfate precipitation. Any type of protein (enzyme, antibody, blood protein, regulatory protein, etc.) or large peptide is a potential candidate for HIC.

HIC is a very powerful technique that can serve as a useful complement to ion exchange, providing separation based on hydrophobicity where ion exchange is based on charge. The conditions for binding and elution are mild, and recoveries and capacities are generally very good. Despite its potential usefulness, HIC is not nearly as widely practiced as IEC. In large part, the reason for this is that many researchers do not understand the technique.

Binding Mechanism

One common technique for concentrating and partially purifying proteins is *ammonium sulfate precipitation*. In this technique, the protein mixture is blended with a high concentration (1.0 - 5.0 M) of a "lyotropic salt", normally ammonium sulfate. The ammonium sulfate both damps out ionic interactions between the proteins and dramatically increases the strength of hydrophobic interactions. When the ammonium sulfate concentration is high enough, certain proteins in the mixture will aggregate and, when the aggregates become sufficiently large, precipitate out of solution.

Proteins differ significantly in the amount of ammonium sulfate required for precipitation, with the more hydrophobic precipitating at a lower concentration. This is the principle of

ammonium sulfate precipitation as a crude separation technique. If the protein solution is prepared in a buffer containing a high concentration of ammonium sulfate, and the solution is exposed to a chromatography bonded phase surface that is more hydrophobic than the solution itself, the more hydrophobic proteins will bind to the surface via an induced *hydrophobic interaction*. This is the principle of hydrophobic interaction chromatography or *HIC*.

The organizational structure of the solvent molecules (water) surrounding the solutes and the binding surface is the driving force for hydrophobic binding. When hydrophobic areas bind together (as when a protein binds to the surface of an HIC packing), water is effectively released from surrounding the hydrophobic areas, causing a thermodynamically favorable increase in entropy. Salts that induce hydrophobic interactions are those which increase the ordered structure (decrease the entropy) of the water. On the other hand, some salts or other agents that decrease the ordered structure of water (solvents or chaotropes) weaken hydrophobic interactions.

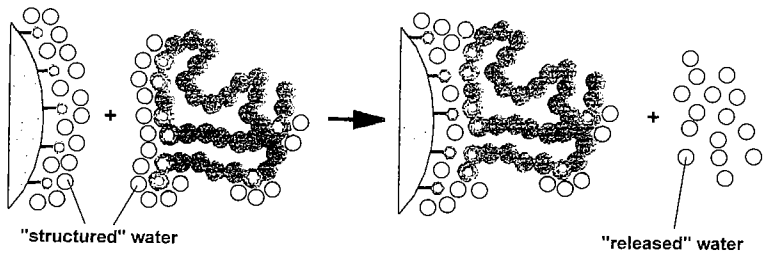


Figure 4-5. Hydrophobic binding interactions release "structured" water surrounding hydrophobic areas. This results in increased entropy, making the interactions thermodynamically favorable.

Because HIC is driven by entropy, temperature can have a strong effect. Generally, increasing the temperature increases the binding strength. However, temperature can also affect protein conformation, causing the resulting binding effects to be quite complex. Similarly, the effect of pH on hydrophobicity can be complex and hard to predict.

Ammonium sulfate, by virtue of its good "salting out" properties, high solubility in water, ready availability and low cost, is by far the most common salt used for HIC. The following table lists both anions and cations in order of increasing lyotropic ("salting out") effect and decreasing chaotropic effect.

Most Lyotropic ("Salting Out")	
PO_4^{3-}	NH_4^+
SO_4^{2-}	Rb^+
CH_3COO^-	K^+
Cl^-	Na^+
Br^-	Cs^+
NO_3^-	Li^+
ClO_4^-	Mg^{2+}
I^-	Ca^{2+}
SCN^-	Ba^{2+}
Most Chaotropic	

The salt concentration required for the induced hydrophobic interaction can be modulated by changing the hydrophobicity of the bonded phase. More hydrophobic surfaces require less salt for binding, causing less risk of protein precipitation. However, if the bonded phase is made too non-polar (which is the case with virtually all reversed-phase packings), the protein may not elute when the salt is removed and require organic solvent. In some cases, the protein may actually unfold on the hydrophobic surface, lose its 3-dimensional structure, and *denature*. Thus finding the proper hydrophobicity of the bonded phase surface for a particular application is quite important.

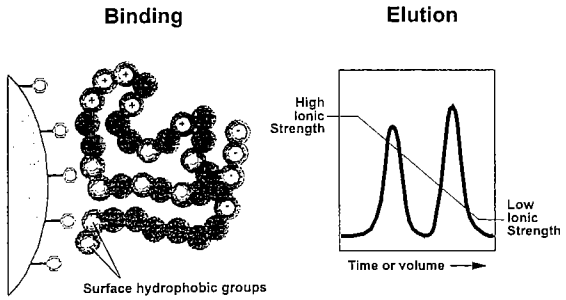
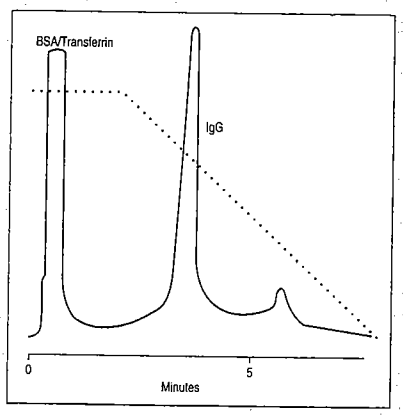


Figure 4-6. Binding and elution mechanisms of hydrophobic interaction chromatography

Elution Method

The binding interaction induced by salt between proteins and the HIC packing surface is quite reversible. Thus, reducing the salt concentration will elute proteins that are bound. As with ion exchange, using a continuous gradient (in this case a *decreasing* salt concentration) will separate different bound molecules from each other.

Some proteins (such as membrane proteins) are so hydrophobic that they will bind to the stationary phase in a low salt mobile phase (i.e. without any salt-induced hydrophobic effects). In these cases elution can be accomplished by adding an increasing gradient of a solvent, a *chaotropic agent* (such as ethylene glycol, urea, guanidine-HCl or thiocyanate salts) or a detergent. Sometimes these agents are used even in the presence of ammonium sulfate to aid in solubility of the protein.



Sample:	2 ml concentrated cell culture supernatant (1.2 mg IgG)
Column:	POROS PE/M 4.6 mmD/100 mmL
Starting Buffer (A):	10 mM phosphate, pH 7.0 + 1.5 M ammonium sulfate
Eluent (B):	10 mM phosphate, pH 7.0
Flow Rate:	5 ml/min (1800 cm/hr)
System:	HP 1050
Detection:	280 nm
Elution:	100 - 0% B in 15 CV

Purification of IgG by POROS hydrophobic interaction chromatography. The major contaminants (albumin and transferrin) do not bind to the column, making the technique quite selective for most antibodies.

The "Salting Out" Experiment

While HIC appears relatively simple, setting up a separation can be a bit tricky. The high salt conditions used to bind proteins to an HIC column can cause precipitation of either the target molecule or other molecules in a sample. While this is not always a negative, the possibility must at least be understood and taken into account.

A "salting out" experiment provides a useful preliminary step prior to developing an HIC method. This experiment determines what salt concentration (if any) causes the first significant precipitation of any proteins in the sample, and also what salt concentration (if any) causes the target molecule itself to precipitate. You can do this by mixing different amounts of saturated ammonium sulfate solution with a small amount of the sample, letting the mixture stand for at least 15 minutes and observing the precipitation behavior. This may be quantified by filtering or centrifuging off the precipitate and assaying

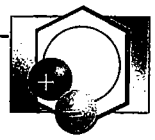
the supernatant formed for the desired protein. Usually concentrations of 1.0, 2.0 and 3.0 M ammonium sulfate provide a good "feel" for the sample behavior.

The most critical information to obtain from the salting out experiment is the highest salt concentration (up to 3.0 M ammonium sulfate) that will *not* precipitate the target molecule. This salt concentration becomes the starting point for HIC method development. The range of hydrophobicity between the target molecule and the most hydrophobic proteins in the sample (the ones that precipitate at the lowest salt concentration) is also important.

If the target molecule is the most hydrophobic (precipitates first), the target molecule should bind with good capacity to the right surface chemistry at a reasonable salt concentration, and HIC should provide excellent selectivity. You should choose the *least* hydrophobic chemistry that will easily bind the target and keep the starting salt concentration as low as possible to minimize binding and precipitation of other molecules in the sample.

If, on the other hand, there is a large difference in hydrophobicity between the target and the most hydrophobic proteins in the sample (i.e. a large number of other proteins precipitate before the target), it may be necessary for you to perform a low salt cut (i.e. precipitate the most hydrophobic proteins first) in order to get a salt concentration high enough to bind the target molecule. Care should be taken to clarify the sample after precipitation by centrifugation or filtration. You should use the *most* hydrophobic packing that still allows good recovery of the target.

If the target molecule is the *most* hydrophilic protein in the mixture, it may be possible to use conditions where the target does *not* bind and the other molecules do bind. This kind of *negative chromatography* can be very useful as a final polishing step.

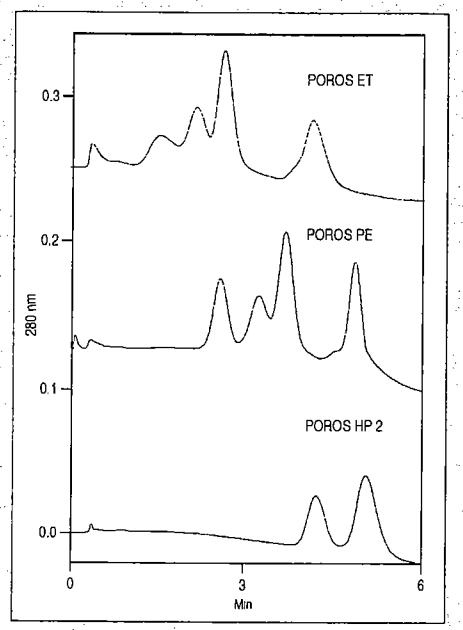


Developing a Hydrophobic Interaction Method

The strategy for developing an HIC method revolves around selecting the proper stationary phase. The POROS HIC media are (in order of increasing hydrophobicity) ET < PE < HP2. The *Starting Point Method* recommendation is to use POROS PE, since if it is too hydrophobic or too hydrophilic, you only need to try one other packing. Based on the results of the salting out experiment, use the highest salt concentration that does not precipitate the target molecule in the starting buffer as the starting point of the gradient, and elute by running a 15 CV gradient to zero salt concentration (more shallow gradients are not normally beneficial in HIC).

If the target fails to bind or elutes very early in the gradient, you should repeat the experiment on POROS HP2. If, on the other hand, the target binds but does not elute with good recovery, you should try POROS ET. [*NOTE:* The POROS Self Pack Methods Investigation Kits provide a simple and economical way to try different surface chemistries.] If either of these packings fails to bind or elute the product, then consider if HIC has usefulness as a negative chromatography step (i.e. bind the contaminants and let the target molecule flow through), otherwise it is probably not a good choice for this separation.

The retention behavior of proteins in HIC is extremely sensitive to both the sample load and the starting salt concentration. Once the proper chemistry is selected, you may perform an appropriate loading study if needed to determine the maximum sample loading at which the target molecule still binds and is resolved sufficiently. Once the maximum feasible loading is determined, if the target molecule is still eluting at the very end of the gradient, you can reduce the starting salt concentration.

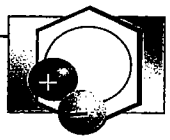


Sample:	20 µl of four protein test standard
Column:	4.6 mmD/100 mmL, chemistries as indicated
Starting Buffer (A):	20 mM phosphate + 2.5 M ammonium sulfate
Eluent (B):	20 mM phosphate
Flow Rate:	5 ml/min (1800 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	0 - 100% B in 15 CV

Selectivity differences of POROS HIC chemistries. POROS HP2 is too hydrophobic for this test mixture and is unable to resolve the four components as a result.

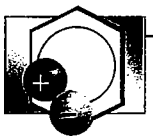
Variables for Full Method Development

pH map	pH can affect the binding strength and selectivity, although effects are highly unpredictable. Check a number of pH values over the stability range of the target.
Salt	Other salts may provide different selectivities, or have other utility. The ionic strength required may be different from ammonium sulfate. Sodium chloride requires 2-4 X ammonium sulfate concentration for similar binding, but may be useful for very hydrophobic proteins.
Organics	Use different organic solvents (ethylene glycol, isopropanol, methanol, ethanol, acetonitrile, etc.) if target does not elute at zero salt. Use up to 40% (v/v), although be careful not to exceed target molecule solubility limit. Detergents or chaotropes (urea) can be used instead of organics to elute tightly bound molecules, but are considerably less desireable (hard to remove and less reproducible).
Gradient	For some applications, optimize the gradient or develop a step elution method.
Temperature	Temperature can modify binding strength and selectivity, although this is rarely worth the trouble. However, perform development at final operating temperature (usually cold room vs room temperature).



BioCAD Tips

- The BioCAD SCOUT Column Selector valve makes it extremely convenient to check different HIC chemistries on the same sample.
- A *pH Map* template is a convenient way to check the effects of pH.
- Although most instruments program gradients only in time, the BioCAD allows programming in column volumes (CV). This feature is highly recommended for method development, since you can make changes in flow rate and even column size without changing the gradient or affecting the chromatography.
- BioCAD also allows programming in eluent concentration instead of percentages, which can make complex experiments simpler to interpret. When using this feature, define each solvent channel carefully in the Configuration screen. Consult BioCAD manual for details.
- The 6 solvent capability of BioCAD allows you to try the effects of different salts or additives and concentrations automatically. With one buffer, up to five different salts can be examined at once.
- The 4 buffer blending capability makes it easy to add an increasing organic concentration gradient at the end of the decreasing salt gradient in each screening run. This is useful to insure that no protein remains on the column.
- Unless you pre-blend organic solvent with all the mobile phases (which is recommended once you have settled on a concentration), be careful to degas and/or helium sparge all solvent lines.



REVERSED-PHASE CHROMATOGRAPHY

Reversed-phase chromatography is the most common chromatographic mode for analysis of any type of molecule, as well as for preparative separations of small molecules, peptides and oligonucleotides. Like hydrophobic interaction chromatography, reversed-phase chromatography separates molecules based on differences in hydrophobicity.

Reversed-phase is not widely used for preparative purification of proteins, except for small, robust proteins (generally below 30K MW). Because both the extremely nonpolar stationary phase and the organic solvents used for elution in reversed-phase can cause irreversible denaturation, protein separations based on hydrophobicity are usually carried out by hydrophobic interaction chromatography.

Binding Mechanism

The bonded phase in reversed-phase is an extremely hydrophobic or *nonpolar* surface. The mobile phase is *polar*, usually water or an aqueous solution. Nonpolar or hydrophobic molecules bind preferentially to the stationary phase and the polar molecules remain in the mobile phase. The separation is thus based on the principle that oil and water do not mix.

In the case of proteins, the hydrophobicity of the stationary phase is so extreme that the protein often loses its three-dimensional shape and denatures. This is because in aqueous solution, a major force holding the protein in its correct shape is the tendency for the hydrophobic amino acids to cluster at the core of the protein, where they can exclude water. When exposed to the reversed-phase surface, the protein may unfold allowing internal hydrophobic groups to bind.

This is not the case in hydrophobic interaction chromatography where the high salt concentration and weakly hydrophobic packing surface tend to keep the protein stable in its correct 3-D conformation. For this reason, the selectivity and binding characteristics of these two modes may be very different, even though they are both separating based on hydrophobicity.

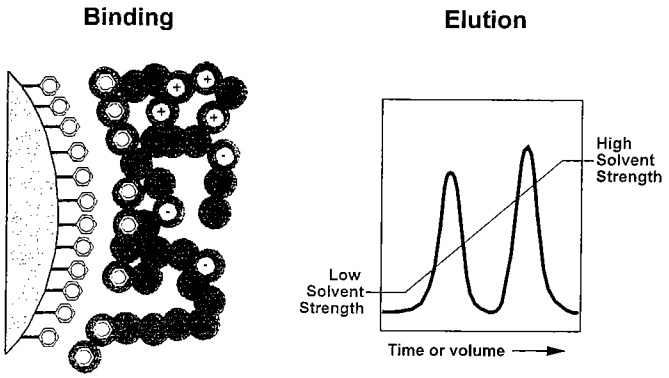


Figure 4-7. Binding and elution mechanisms of reversed-phase chromatography

Many common reversed-phase packings (including PepMap C18) are based on silica, with bonded hydrocarbon chains (usually C4, C8 or C18) referred to as *alkylsilane* groups. The base silica itself has a very high surface concentration of extremely polar, negatively charged *silanol* groups. Although it is desireable when manufacturing a reversed-phase packing to completely react the surface silanols with the silane alkyl groups, in practice complete coverage is impossible due to the bulk of the alkyl chains. This creates a potential for both non-specific binding of positively charged molecules (especially amines) and a *mixed mode* selectivity. One solution is to use an *endcapping* agent, which has a small, low molecular weight alkyl group (usually C1) that can react with unmodified silanols between the main bonded phase groups.

Another approach is to use a polymeric support matrix, such as poly(styrene-divinylbenzene), which does not have silanol groups at all. POROS and Oligo R3 packings are based on polystyrene. This gives them the additional advantage of being able to tolerate alkaline pH. Silica becomes increasingly soluble above pH 7, and is unusable above pH 8.

Elution Method

Reducing the polarity of the mobile phase allows the hydrophobically bound molecules to partition off the bonded phase surface, causing elution from a reversed-phase column. This is usually done by adding a water-miscible organic solvent such as acetonitrile or an alcohol (methanol or isopropanol are the most common).

Elution is most often performed in a continuous gradient. Reversed-phase separations (especially with peptides) often show the best resolution with a very shallow or "near isocratic" gradient. This contrasts with ion exchange or hydrophobic interaction, where the increased bandspreading overcomes any benefit from increased difference in retention in very shallow gradients. In reversed-phase there is usually a very narrow range in solvent concentration over which a given molecule goes from no elution at all to no binding at all. Very slight shifts in solvent concentration can greatly affect retention, so a shallow gradient is usually more reproducible than true isocratic elution.

Charged functional groups are generally hydrophilic, but when a charge is titrated out, the same functional group can exhibit significant hydrophobic character. The amino acid lysine is a good example of this effect. The pH of the mobile phase can thus have a significant effect on the selectivity of reversed-phase during elution.

Ion Pairing Agents

The effect of charge groups on sample molecules can be modified through the use of *ion pairing agents,* which are molecules with both charge and hydrophobic functionality. Ion pairing agents enhance the interaction of charge groups with the surface, by binding hydrophobically to the bonded phase surface and ionically to the charge groups. In the case of silica-based reversed-phase packings, ion pairing agents also serve a critical function of negating the effect of any residual silanol groups on the surface.

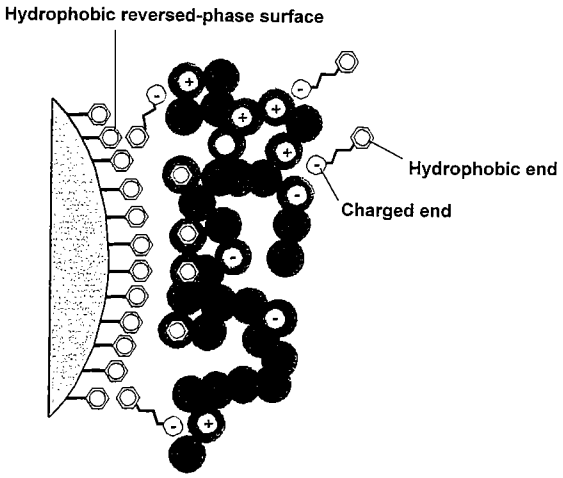
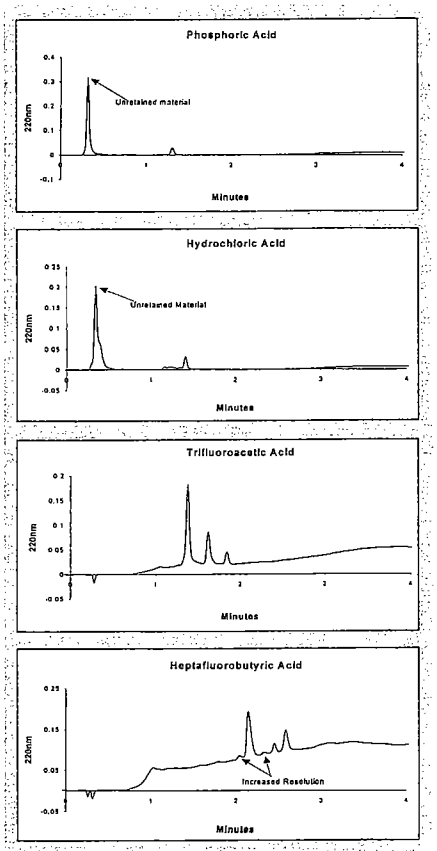


Figure 4-8. Ion pairing agents

Trifluoroacetic acid *(TFA)* is the most common ion pairing agent used with silica reversed-phase packing for chromatography of biomolecules. It is a good ion pairing agent with amine functionalities and is such a strong acid that it suppresses the ionization of silanols and helps to solubilize difficult peptides and proteins.



Sample:	15 mer crude synthetic peptide
Column:	Self Pack POROS 10 R2
	4.6 mmD/100 mmL
Starting Buffer, (A):	0.9% acetonitrile,
	0.1% ion pairing agent as indicated
Eluent (B):	95% acetonitrile,
	0.1% ion pairing agent
Flow Rate:	6 ml/min (2,200 cm/hr)
System:	BioCAD workstation
Detection:	214 nm
Elution:	0 - 50% B in 10 CV

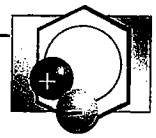
The effect of ion pairing agent on selectivity in reversed-phase chromatography of a synthetic peptide. The relatively hydrophilic peptide elutes before the gradient starts with the weakly hydrophobic pairing agents H_3PO_4 and HCl. With TFA and HFBA, the sample is retained and eluted in the gradient. Maximum retention time and resolution is achieved with the very hydrophobic HFBA. PerSeptive Biosystems Application Note PA 445.

POROS or Oligo R3 offer considerably more flexibility in the selection of mobile phase additives, since there are no residual silanols which must be blocked by ion pairing agents. Alternatives to TFA such as formic, phosphoric, acetic or hydrochloric acid can be successfully used, each with different effects on selectivity. The stability of POROS media to high pH also allows reversed-phase to be performed under conditions where the charge distribution of the sample molecules is completely different, further increasing the selectivity options.

Developing a Reversed-Phase Method

The starting point in developing a reversed-phase method is to select the packing material. POROS R2 packing has the most generally useful selectivity and is recommended for most applications. If in the initial run the target molecule(s) do not elute with good recovery, it is possible that the less hydrophobic POROS R1 packing, which allows molecules to elute at lower solvent concentration, may allow better recovery. Oligo R3 is recommended for preparative separations of oligonucleotides (and can also be used for peptides) especially if maximizing loadability is a key objective.

The central task in developing a reversed-phase method is to determine the starting and ending solvent concentrations of the gradient which give the most optimal separation. The required elution concentration depends upon the stationary phase, sample molecules, sample load, and mobile phase pH and modifiers. The problem is that the actual solvent concentration at which a given molecule elutes depends critically upon the slope of the gradient, because molecules begin to move slowly down the column at a lower eluent concentration than when they actually emerge from the column. As the gradient slope is decreased, molecules tend to elute at a lower solvent strength. This effect is most pronounced with smaller molecules such as peptides, but is also significant with larger molecules.



Although this effect can be mathematically modeled, it is not easy to predict the behavior of a complex mixture of molecules as the gradient slope is reduced. The simplest experimental approach is to start with a relatively steep, broad range gradient. The solvent concentration of the earliest and latest eluting molecules of interest is then determined. These are then used to set the starting and ending points for the next experimental gradient using the "adjusting by halves" technique shown in the figure.

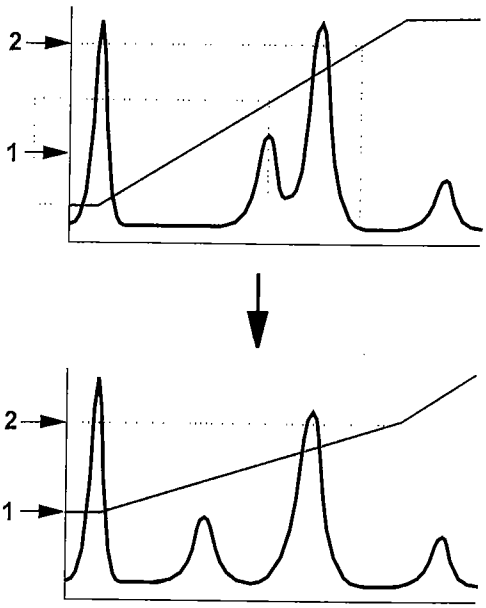
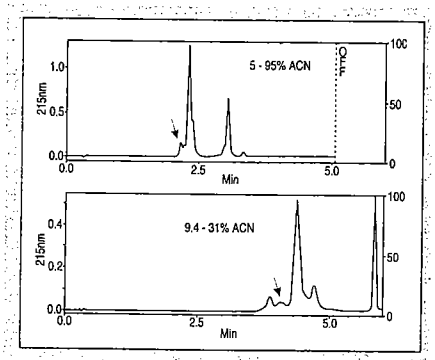


Figure 4-9. Optimizing a reversed-phase gradient by "adjusting by halves". Using the results of the previous gradient run, the new gradient starting point (1) is set halfway between the previous gradient starting point and the elution point of the earliest eluting molecule of interest. The new gradient end point (2) is set just beyond the elution point of the latest eluting molecule of interest. This procedure may be continued until the resolution is optimized.



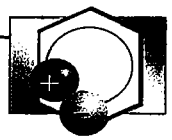
Sample:	20 µl commercially available soybean trypsin inhibitor (5 mg/ml)
Column:	POROS R2/H 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA
Eluent (B):	0.1% TFA, 95% acetonitrile
Flow Rate:	5 ml/min (1800 cm/hr)
System:	BioCAD workstation
Detection:	215 nm
Elution:	as indicated

An example of the "adjusting by halves" technique for optimizing a reversed-phase separation. Note the improved resolution in the area indicated.

For preparative applications, it is desirable to perform a loading study. The low solubility in the organic solvent concentration required for elution often limits the loading of biomolecules in reversed-phase. Loading capacity is, of course, not a major concern in analytical applications.

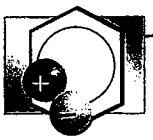
Variables for Full Method Development

Ion pairing agents	Check a variety of different ion pairing agents in addition to TFA. HCl is UV transparent and provides very different selectivity and higher sensitivity. Formic, phosphoric and acetic acid are also often used. Heptafluorobutyric acid (HFBA) is more hydrophobic than TFA, and can often simulate the selectivity of silica-based media on polymeric media such as POROS or Oligo R3. At neutral and basic pH, triethanolamine (TEA) is a common ion pairing agent. Take care to insure the TEA is relatively fresh, since it easily oxidizes to form UV-absorbing products. Ammonium acetate and even NaOH are also used at higher pH ranges.
pH	pH changes the charge distribution in the sample molecules, which can dramatically affect the selectivity. A very useful experiment is pH 2, 7 and 12, to check at the three major charge states of a protein or peptide. [NOTE: Do not use pH >7 on silica-based media such as PepMap C18.]
Organic solvent	On POROS media, acetonitrile is almost always the solvent of choice. Alcohols (such as methanol, 1- or 2-propanol or butanol) or other water miscible solvents such as DMSO can be added up to 20% (v/v) to improve selectivity or peak shape.
Gradient	To insure reproducibility and robustness of the method, do not generally go below a span of 5% ACN between the start and end of the gradient for proteins and peptides. Increase the duration to increase resolution. A more complex, multi-step gradient may be useful to resolve a number of species with markedly different retention ranges.
Packing material	For preparative applications with peptides and oligonucleotides Oligo R3 will provide higher capacity and retentivity than POROS R2. If POROS R1 still does not give good recovery, try the hydrophobic interaction supports (POROS HP2 or PE) as "weak reversed-phase" media.
Temperature	Temperature affects the retention time and resolution. Control the temperature of the column for high precision analysis.



BioCAD Tips

- The BioCAD SCOUT selector valve makes it extremely convenient to check a range of different reversed-phase chemistries on the same sample.
- Although most instruments program gradients only in time, the BioCAD allows programming in column volumes (CV). This feature is highly recommended for method development, since you can make changes in flow rate and even column size without changing the gradient or affecting the chromatography.
- The BioCAD also allows programming in eluent concentration units instead of percentages, making complex experiments much simpler to interpret. This enables programming in actual % ACN when using blended solvents as the A and B mobile phases. When using this feature, define each of the solvent channels carefully in the Configuration screen. Consult the BioCAD manual for more details.
- The 6 solvent capability of BioCAD allows you to try the effects of different ion pairing agents, solvent additives and concentrations automatically. With set of A and B mobile phases (water/ACN mixtures), up to four different additives can be examined at once. (See example on page 161.)
- Be careful to degas and helium sparge all solvent lines to prevent outgassing. This should always be done when running in reversed-phase mode.



AFFINITY CHROMATOGRAPHY

Affinity chromatography is based on biospecific binding interactions between a ligand chemically bound to the chromatographic packing and a target molecule in the sample. The technique is used almost exclusively to purify proteins and antibodies, although there are a few applications with peptides and nucleic acids. Immobilized ligands may include either proteins or small molecules that interact specifically with the target of interest. A common application is the use of antibodies as immobilized ligands.

Binding Mechanism

Virtually all biological molecules interact in some selective way with some other molecules through binding at a specific site. Common examples include the binding of an antigen to an antibody, of a substrate, inhibitor or cofactor to an enzyme, or a regulatory protein to a cell surface receptor. The forces involved in the binding include the same ionic and hydrophobic interactions that cause more non-specific ion exchange or HIC binding. However, in the case of *biospecific* or *affinity binding*, the charged and hydrophobic groups are arranged on the two binding molecules or *ligands* in a unique orientation. Weaker forces such as hydrogen bonding also play an important role. The two ligands thus fit together very much like a lock and key, with a high degree of specificity.

In affinity chromatography, one member of the ligand pair is *immobilized* (i.e. covalently coupled) as a bonded phase. Sometimes a *spacer arm* or *linker* is used to place some distance between the bound ligand and the support matrix to improve accessibility. The immobilized ligand/support matrix combination forms a highly selective stationary phase that, in theory,

will only bind to the other molecule (usually a protein) of the ligand pair. In affinity chromatography, the molecules are thus separated based on their biospecific binding to the immobilized ligand.

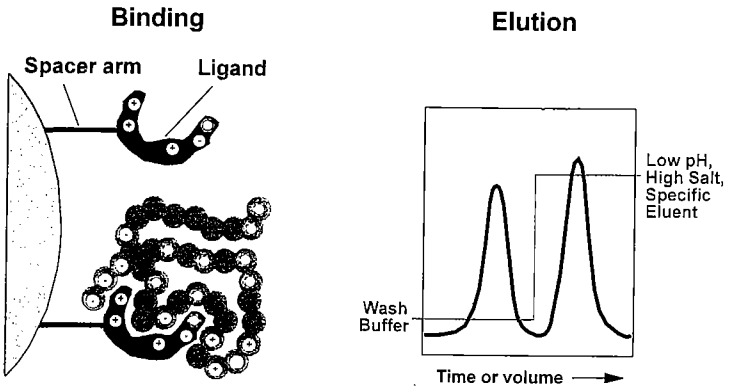


Figure 4-10. Binding and elution mechanisms of affinity chromatography

In order for completely specific binding to occur, it is important that the support matrix and spacer arm themselves have minimal binding interaction (*nonspecific adsorption*) with any of the molecules in solution. The surface of the support matrix should be very hydrophilic and have no ionic charge. *Ligand leakage,* which can contaminate the final product, is also very much a function of the support matrix and linking chemistry.

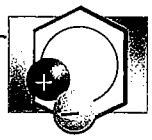
Because of the tremendous diversity of affinity ligands, it is not possible for any manufacturer to supply them in all pre-immobilized form. Thus most vendors offer *activated affinity media,* which are sold coated with covalently reactive groups ready for end-users to couple their own ligands. Several different POROS coupling chemistries are available to target

different functional groups and to allow coupling under different conditions. The most common target functionality for coupling chemistries is the primary amine group, found primarily on the side chain of lysines in a protein. A detailed discussion of the selection of coupling chemistry and optimization of coupling is beyond the scope of this book. (See the *Appendix* for suggested references, or contact PerSeptive Biosytems' Technical Support.)

Three major affinity systems in which pre-immobilized ligands are available on Perfusion Chromatography media are protein A/G affinity chromatography, heparin and immobilized metal affinity chromatography (IMAC). In protein A or G affinity chromatography, a specific protein (originally extracted from the surface of certain gram negative bacteria, but now usually made recombinantly) is immobilized. These proteins selectively bind to a broad range of antibody molecules, thus forming an affinity column for antibodies. Proteins A and G differ in both their species and subclass specificity for antibody binding.

In heparin affinity chromatography, the heparin ligand selectively binds coagulation proteins, lipoproteins, restriction endonucleases and nucleic acid polymerases.

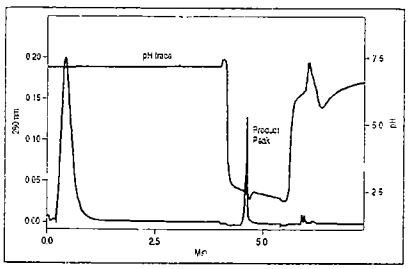
In IMAC, a metal chelating group (typically imidodiacetate) is immobilized and a multivalent transition metal ion (typically Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} or Fe^{2+}) is bound in such a way that one or more coordination sites are available for interaction with proteins. Certain surface amino acids (primarily histidines) bind specifically to these free coordination sites, allowing separation based on the surface concentration of these amino acids. In some cases a "tail" of usually 5 or 6 histidines can be added to the end of a protein by genetic engineering to provide a very effective "handle" for purification by IMAC.



Elution Method

Elution from affinity media can be done with any agent that disrupts the ligand-ligand interaction. The most common technique is to employ a shift to acidic pH (usually to pH 2 - 4), which is used extensively for protein A/G and for antibody ligand affinity methods. Other elution techniques include increases in ionic strength or the use of specific eluents such as the immobilized ligand or an analog in free solution. Affinity elution is usually in the form of a step gradient.

In the case of IMAC, the binding is not usually as selective as other affinity methods, so a gradient elution technique is used to separate the bound molecules from each other. While a variety of eluents can be used, the most effective and reproducible is to use gradients in imidazole concentration. Imidazole is the active functionality in histidine which binds to the metal coordination site, so the elution in this case is by mass action competitive binding.



Sample:	50 µl concentrated pooled fractions from cation exchange column
Column:	POROS G 2.1 mmD/30 mmL derivatized with anti Hsp-60 antibodies
Starting Buffer (A):	PBS, pH 7.2
Eluent (B):	12 mM HCl
Flow Rate:	2 ml/min (3465 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

Micro-scale immunoaffinity purification of recombinant human heat shock protein (hsp 60) from *E. coli*. Anti human hsp-60 monoclonal antibody was immobilized to Protein G surface, then crosslinked in place. The high specificity of immunoaffinity was utilized as a last step in the purification protocol to ensure the specific recovery of human hsp and not its bacterial counterpart. PerSeptive Biosystems Application Note PA 419.

Developing an Affinity Chromatography Method

For Perfusion Chromatography based on *activated affinity media,* the primary task of method development is to select a coupling chemistry and couple the ligand to the support. As discussed before, the selection and development of coupling chemistry is beyond the scope of this handbook. Please refer to the *POROS Columns and Media Selection Guide* or contact PerSeptive Biosystems' Technical Support for more information.

For protein A/G affinity chromatography, the first task is to select which protein ligand to use. The primary criterion is binding affinity, although cost and ease of elution are also

important. Once the ligand is selected, a satisfactory separation can usually be performed with little or no development using the starting point method, since the selectivity is in the ligand and the "chromatography" consists of little more than binding and step elution.

Choosing between Protein A and G

Proteins A and G primarily differ in species and subclass binding specificity, as shown in the following table. Protein G is clearly more universally useful, showing superior binding to all major antibody classes and species except IgM.

Where both proteins will work, protein A is always recommended. This protein is available at lower cost than protein G. In addition, protein A withstands harsher conditions used in cleaning and regeneration. Finally, and perhaps most importantly, while protein G will bind more antibody types than protein A, the binding is often stronger, making elution and complete recovery more difficult.

The most important type of antibody bound by protein G and not normally by protein A is mouse IgG_1. This is the most common subclass of monoclonal antibodies. Under normal conditions, mouse IgG_1 does not generally bind well to protein A. However, the addition of high salt (2-3 M NaCl) with high pH (8 - 9) to the binding/wash buffer will cause these antibodies to bind.

Binding Specificity of Proteins A & G

Antibody Type	Protein A	Protein G
Human		
IgG	++	++
IgG_1	++	++
IgG_2	++	++
IgG_3	-	++
IgG_4	++	++
IgM	++	-
IgA	++	-
IgE	++	-
IgD	-	-
Mouse		
IgG_1	-*	++
Other	++	++
Rabbit	++	++
Sheep	-	++
Goat	+/-	++
Horse	-	++
Sheep	-	++
Pig	++	++
Cat	++	-
Dog	++	+/-
Rat	-	+/-
Chicken	-	-

*May bind at high salt & pH

Starting Point Method — PROTEIN A / G

Column:	POROS A or G 4.6 mmD/50 mmL (0.8 ml)
Starting buffer (A):	50 mM phosphate pH 7.0, 0.15 M NaCl
Eluent (B):	50 mM glycine pH 3.0, 0.15 M NaCl
Sample:	0.5 - 10 mg total antibody (plus other proteins)
Flowrate:	10 ml/min (3600 cm/hr)
Elution sequence:	10 CV equilibration in 100% A Inject sample 10 CV wash in 100% A 10 CV elution in 100% B

Notes on Starting Point Method

- Carefully filter or centrifuge the sample to remove particulates and any precipitated protein. Ascites should ideally be delipidated to extend column life.
- Sample pH and salt concentration is usually not critical, except that the pH should be >6.0. Protein A or G columns can serve as a good salt removal step. The salt concentration must be high enough to prevent non-specific binding (>0.1 M).
- Column size is for method development on HPLC or FPLC system. Use other sizes if desired, and adjust flow rate and sample size accordingly.
- Increase or decrease the flow rate to meet your system requirements. For POROS 50 media, the maximum recommended linear velocity is 1000 cm/hr (2.5 ml/min in a 4.6 mmD column).

Variables for Full Method Development

pH	The elution pH is the most critical variable. For protein G, usually use more extreme pH conditions [up to 12 mM HCl (0.1% v/v)] to obtain full mass recovery. For protein A, elution by pH steps (starting at pH 6) may fractionate different species (weaker binding bovine IgG from target antibodies) or subclasses. High pH (8 - 9) in conjunction with high salt may promote binding of mouse IgG_1 to protein A. Otherwise, binding pH is not critical, as long as it is above pH 6.
Salt	Salt is not generally an effective eluent. Use a 0.1 - 0.5 M to reduce non-specific adsorption. On protein A, use high salt (2 - 3 M NaCl) with high pH to promote the binding of mouse IgG_1.
Divalent cations	Sometimes salts containing divalent cations (particularly Mg^{2+}) function as eluents in place of low pH if activity loss is a concern. Use concentrations up to 1 M.

IMAC method development is somewhat more complex, since gradient elution is generally used. However, the starting point conditions will often provide satisfactory results with little or no further development in many cases. Optimization steps include selection of the metal used and optimizing the gradient.

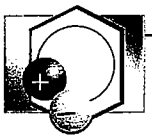
Choosing the Metal Ion for IMAC

The ligand most commonly used for immobilized metal affinity chromatography is imidodiacetate with bound metal. Imidodiacetate binds the metal ion at three of its coordination sites, leaving the remaining coordination sites free to bind to the target biomolecules. Although the general mechanism of binding is the same for all metals, the precise geometry of the coordination sites and the binding strength differs for different metals. Thus the choice of metal ion has an effect on the selectivity of the separation.

The most commonly used metal ions are (in order of binding strength):

$$Cu^{2+} > Ni^{2+} > Zn^{2+} \sim Co^{2+}$$

The best development strategy is usually to use Cu^{2+} for the initial screening studies, since it binds the most tightly. If the selectivity or recovery need to be improved, the other metals should be tried. Ni^{2+} is often used for proteins which contain a "polyhistidine tail" of 4 - 8 histidine residues added by genetic engineering to allow easy recovery by IMAC. The very high binding strength of the tag allows greater selectivity through the use of the less tightly binding metal.



Starting Point Method — IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Column:	POROS MC 4.6 mmD/100 mmL (1.7 ml) Charge column with Cu^{2+} ions (see column Operating Instructions)
Starting buffer (A):	50 mM phosphate pH 7.0, 0.5 M NaCl, 0.5 mM imidazole
Eluent (B):	100mM imidazole in starting buffer
Sample:	100 - 500 µg total protein
Flowrate:	10 ml/min (3600 cm/hr)
Elution sequence:	10 CV wash with 100% B 5 CV equilibration in 100% A Inject sample 5 CV wash in 100% A 15 CV gradient 0-100 mM imidazole (0-100% B) 5 CV hold at 100% B Strip and recharge column (see column Operating Instructions)

Notes on Starting Point Method

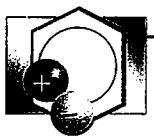
- Carefully filter or centrifuge the sample to remove particulates and any precipitated protein.
- The pH and salt concentration in the sample is usually not critical. Use pH 4.5 - 8.5, but best results usually pH 7 - 8. Use 0.1 - 1.0 M salt to suppress non-specific ionic interactions.
- You will generally obtain the best binding in phosphate and acetate buffers. You should avoid amine-containing buffers such as Tris.
- Chaotropic agents such as guanidine or urea may be used if needed for solubility.
- Column size is for method development on HPLC or FPLC system. Use other sizes if desired, and adjust flow rate and sample size accordingly.
- Increase or decrease the flow rate to meet your system requirements. For POROS 50 media, the maximum recommended linear velocity is 1000 cm/hr (2.5 ml/min in a 4.6 mmD column).
- Charge the column with metal before each run and strip with EDTA after each run to remove tightly bound protein and insure reproducibility (especially with weaker binding metals such as Zn^{2+} or Co^{2+}). Follow the sequence of washes (including washes with water) recommended in the Operating Instructions to prevent precipitation of the metal salts.
- 0.5 mM imidazole in the starting buffer keeps the metal ion in the "imidazolo" form rather than the "aquo" form, which allows easier exchange with proteins, and thus more reproducible results.

Variables for Full Method Development

Metal ion	See discussion on page 172.
Gradient	Improve selectivity and shorten run time by optimizing the gradient. The simplest approach is a 15 CV gradient from the starting buffer to just above the point where the latest eluting target molecule elutes.
pH	Elution can sometimes be effected by reducing the pH (3 - 6) in steps or gradients using phosphate or acetate buffers. This sometimes gives unique selectivity, but obtaining full recovery or good reproducibility can be a problem.
Elution agent	Imidazole competes with histidine and other binding functional groups on target molecules to cause elution. Other competing agents (glycine and various ammonium salts in concentrations up to 1.0 M) have been used, but generally do not give reproducible results. If the binding is very weak, use 2-methyl pyridine.

BioCAD Tips

- Although most instruments require that gradients be programmed in time, the BioCAD allows programming in column volume units (CV). This feature is highly recommended for method development, since it allows you to make changes in flow rate and even column size without changing the gradient programming or affecting the chromatography.
- The 6 solvent capability of BioCAD is particularly useful in IMAC due to the need for stripping and recharging of the column with metal. One line can be used for the starting/wash buffer, one for the eluent (e.g. imidazole), one for water, one for EDTA for stripping, leaving two lines for different metal salts (e.g. copper and nickel).
- After loading metal, be sure to wash the system and column thoroughly with water to prevent hydroxide precipitation of metal salts.



GEL FILTRATION CHROMATOGRAPHY

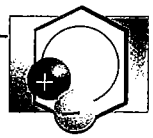
Gel filtration chromatography (GFC) [also known as *size exclusion chromatography (SEC)* or *gel permeation chromatography (GPC)*] separates molecules on the basis of their size. Although POROS Perfusion Chromatography media are not available for gel filtration it can be an important separation technique. It is most often used as a final polishing method, since it is the only separation method available to remove aggregated protein species without any chemical or physical change that may cause more aggregates to form.

Gel filtration has limited usefulness as a high throughput technique. The separation mechanism requires a slow flow rate, and in most cases, sample load should be only 1-5% of the column bed volume to ensure good results.

Therefore, depending on the separation objectives, it may be worth exploring alternatives to gel filtration, particularly if by doing so you can take advantage of the speed of Perfusion Chromatography. A good example of this can be found on page 111 where a gel filtration assay of IgM was replaced with a Perfusion Chromatography assay, speeding analysis 10 fold.

Separation Mechanism

Gel filtration is different from other modes of chromatography in that one goes to great lengths to *prevent* any binding interaction at all between the sample molecules and the bonded phase. Gel filtration depends on the fact that within each particle of the stationary phase there is a distribution of pore sizes. For small enough molecules, the pores are so large that the molecules can penetrate all of the internal volume of the particle. If the molecules are large enough, the pores are so small that the molecule is completely excluded from the internal volume. Molecules in between will have access via diffusion to a portion of the internal volume but will be excluded by the smaller pores from the rest. In gel filtration, molecules are thus separated based on size or molecular weight.



A special application of gel filtration is *desalting*, or *buffer exchange*. In this application, also known as *group separation*, a packing is used which completely excludes the molecule of interest (usually a protein) and has no exclusion for small salt, solvent or buffer molecules. The column is equilibrated with the mobile phase or buffer in which the target molecule is to be placed. Then a sample containing the target molecule and undesirable salts or solvents is applied to the column. During elution, the protein target elutes well ahead of the sample salts or solvents which serves to rapidly exchange it into the new mobile phase. In small volumes, this technique is often much faster and more effective than diafiltration or dialysis.

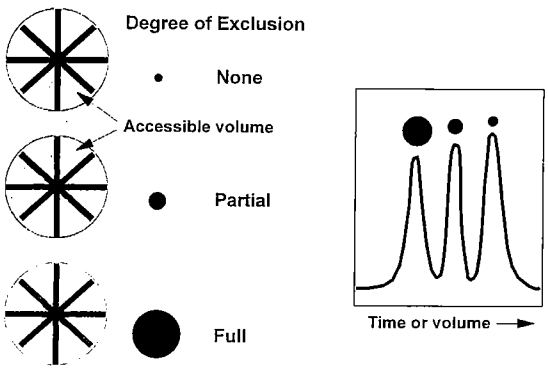
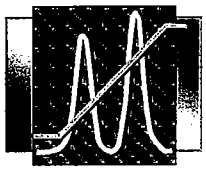
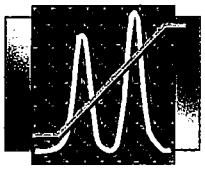


Figure 4-11. Mechanism of gel filtration chromatography

In gel filtration a band of sample is applied to the inlet of the column and the column is eluted isocratically with virtually any mobile phase (the only requirement is that the sample molecules must stay in solution and not bind to the stationary phase). As the band moves down the column, the fully excluded molecules will simply move with the mobile phase and will elute with a retention volume equal to the volume between the packed particles. Small molecules with no exclusion (see diagram) will elute in a volume equal to the pore volume plus the volume between packed particles. Intermediate-sized molecules will elute in between. The range of molecular weights that can be separated is determined by the pore size distribution of the packing material.



SECTION 5
BASICS OF CHROMATOGRAPHY



SECTION 5
BASICS OF CHROMATOGRAPHY

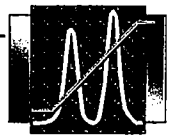
This section describes the basic principles of liquid chromatography for those who are getting involved in the technique for the first time. Where appropriate, practical aspects of Perfusion Chromatography technology that differ from conventional chromatography are described. However, for a more in-depth theoretical discussion of Perfusion Chromatography, the reader is asked to refer to the companion piece *Introduction to Perfusion Chromatography*, also available from PerSeptive Biosystems. Use the handy fax-back order form at the back of this book.

PRINCIPLE OF CHROMATOGRAPHY

Chromatography is a molecular separation method based on dynamic binding interactions or partitioning between chemical compounds, a fluid *mobile phase* and the surface of a solid *stationary phase*. By manipulating the chemistry or composition of the mobile and stationary phases along with other operating conditions, one can effect very subtle separations due to minor differences in molecular structure or configuration (e.g. substitution or oxidation of a single amino acid in a large protein).

Chromatographic separations require five basic elements:

- A stationary phase with controlled structure and surface chemistry



- A column packed with the stationary phase
- One or more mobile phases of controlled chemical composition
- A protocol for running the mobile phases through the column to effect the separation
- An instrument system for accurately delivering the sample, controlling the blend and flow of mobile phases to the column and detecting the separated products

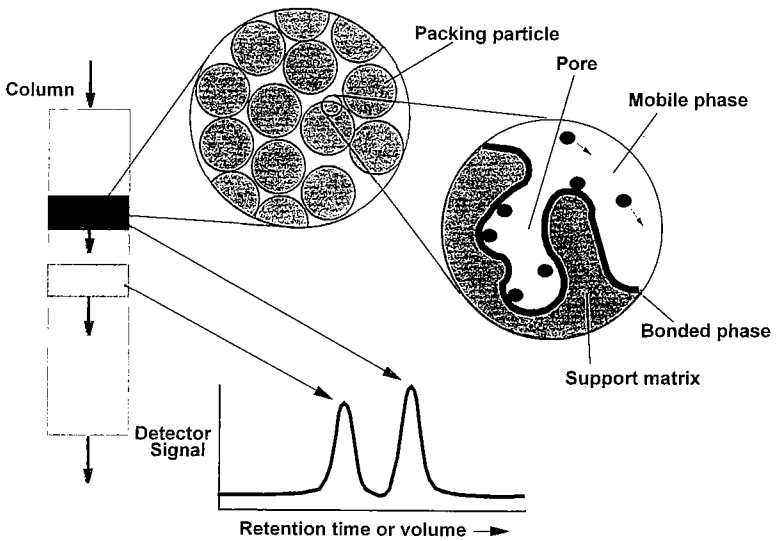


Figure 5-1. Basic elements of chromatography

Stationary & Mobile Phases

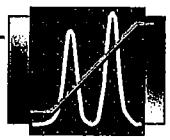
The heart of the chromatographic system is the *stationary phase* (also called the *column packing* or *media*). The packing must provide a large amount of surface area which is accessible to

the mobile phase and sample molecules. Normally, this is done by using small particles of a porous material, called the *support matrix,* in which all or most of the surface area within the pores is accessible to the mobile phase. These particles are usually coated with a *bonded phase* designed to provide functional groups for the desired specific binding interaction. The support matrix and bonded phase are also chosen to reduce *non-specific binding* interactions as much as possible. The stationary phase is discussed in more detail below.

The stationary phase is packed into a *column* that contains the packing in a bed and distributes the liquid being pumped into the bed in a uniform *plug flow*. Normally, columns are cylindrical in design with the flow in the axial direction. See Figure 5-8 for a more complete description of column geometry.

The column is pumped with one or more *mobile phases* (or *eluents*), which carry the sample into the column and effect controlled adsorption and desorption (*binding* and *elution*) of different sample components at different times.

During binding and elution, molecules from the sample move through the spaces between the packing particles at the same rate as the mobile phase. However, the molecules also freely diffuse in and out of the packing particle pores, thus gaining access to the bonded phase surface. Some of the molecules will bind to the surface with greater or lesser strength. While inside the particles or bound to the surface, the molecules do not move down the column. Thus the molecules that bind most tightly will move more slowly down the column (or not move at all unless the mobile phase conditions are changed), and the ones that bind weakly or not at all will move more quickly. Molecules which do not bind at all to the column elute in the solvent front in a volume which is equal to the *void volume* of the column.



Elution Approaches

The next element required to perform a chromatographic separation is the method or protocol. One of the most powerful aspects of chromatography as a technique is that by varying the precise nature of the different elements (such as stationary and mobile phase chemistries, column geometry, elution sequence, sample loading, etc.) one can tailor the separation result over a very broad range and to a very fine degree to solve nearly any problem. However, with so many critical parameters to be adjusted, development of a protocol can be quite challenging. Therefore most of this handbook has been dedicated to the topic of method development.

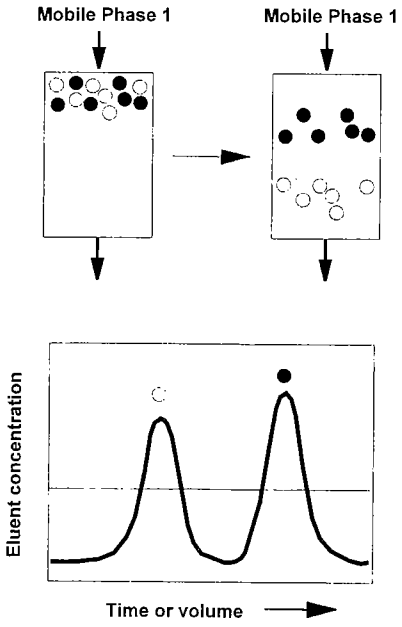


Figure 5-2. Isocratic elution

In isocratic elution, some of the molecules in the sample may bind so tightly under loading conditions that they essentially do not move at all from the top of the column. In this case it is necessary to change the mobile phase chemistry in such a way to weaken the binding interaction enough for the very tightly binding molecules to be eluted. This is done by modifying the pH, salt concentration, solvent concentration and/or other aspects of the mobile phase chemistry in a method called *gradient elution*. Gradient elution can either be *step*, in which the change occurs in one or more discreet stages, or *continuous*, in which the change is effected gradually. Step gradients are usually used for simpler mixtures, continuous for more complex.

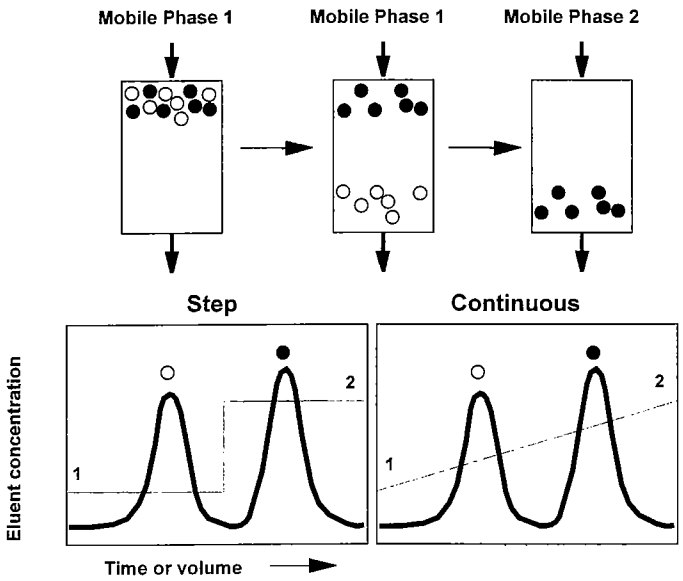
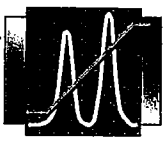


Figure 5-3. Step and continuous gradient elution



The Chromatography Column

The chromatography column packed with stationary phase or packing material constitutes the most critical component of the chromatography system. The key characteristics of the stationary phase include the support matrix (material, particle structure and pore structure) and the surface chemistry. These characteristics determine the function and separation performance, as well as the kind of column hardware and packing techniques required, and the expected pressure-flow behavior.

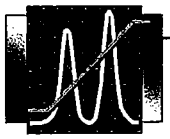
Support Matrix

The *support matrix* (generally highly porous particles packed into a bed) forms the "skeleton" of the stationary phase. The support matrix provides the surface upon which functional groups may be attached which provide the chemical binding interactions enabling a separation to occur. The support matrix must provide a high surface area in a form easily accessible to the sample and mobile phase molecules.

A large number of different materials have been used as chromatographic support matrices. These have included natural polymers (cellulose, dextran and agarose), synthetic polymers [poly(styrene-divinylbenzene), polyacrylics, polyvinyl alcohols, etc.] and inorganic materials (silica, alumina and zirconia).

The following are the basic requirements for the support matrix:

- Suitable mechanical strength and chemical resistance
- An economical process for manufacturing particles of the material
- Controlled particle structure and pore structure
- A means of adding a range of different bonded phases to the surface



In many particle designs, the surface of the support matrix is exposed, thus forming part of the bonded phase. In these cases, the material would also have low non-specific binding characteristics (usually hydrophilic and charge-neutral for protein applications).

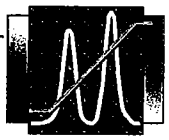
Particle Structure

The particle structure is a critical determinant of the chromatographic *efficiency*, and thus the *resolution* attainable in a given separation. Chromatographic media may be either irregular or spherical in shape, with spherical materials being greatly preferred. The two key characteristics of the particle structure are the average particle diameter and the particle size distribution. The distribution should be quite tight, since the smallest particles in the mix determine the pressure drop across the bed at a given flow rate, while the largest particles determine the obtainable efficiency. In addition, a very broad particle distribution packing is difficult to pack into a stable and uniform bed, also required for maximum performance.

For reference, HPLC media used for analytical applications generally range from 3 - 10 μm in diameter. Particles less than around 1 - 3 μm have proven impractical due to difficulty with packing and the need for very high operating pressures. Particles in the 10 - 40 μm diameter range are used for high performance preparative separations. Particles in the 40 - 150 μm diameter range are used for low pressure and large scale applications. Larger particles (up to around 300 μm diameter) are used in very large scale operations.

Pore Structure

The pore structure of the stationary phase critically affects capacity. The pore diameter must be large enough (typically >5 times the size of the molecules being purified) to allow free access to all the internal surface area by molecular diffusion.



However, since the surface area per unit volume is inversely related to the pore diameter, use of materials with overly large pores will result in loss of capacity. For these reasons, both the average pore diameter and the pore size distribution are critical parameters.

In conventional chromatographic packings, pores in the range of 60 - 150 *ångströms (Å)* are optimal for small molecules (<500 MW). For peptides and oligonucleotides, pores in the range of 150 - 500 Å are best. Larger proteins require still larger pores (500 - 2000 Å). POROS Perfusion Chromatography packings have a more complex bimodal pore distribution, with diffusive pores typically in the 500 - 2000 Å range and throughpores in the 4000 - 8000 Å range. As discussed in Section 1 — *Introduction,* this pore structure enables flow through the particles allowing rapid access of molecules to and from the binding surface and thus much faster separations. Refer to the companion piece, *Introduction to Perfusion Chromatography,* for more detail.

Figure 5-4. SEM of POROS 10 particle, showing both particle and pore structure

Bonded Phase

While the support matrix provides the stationary phase structural "skeleton", the *bonded phase* or surface chemistry actually enables selective binding and thus the separation itself. The bonded phase consists of the functional groups, such as charge or hydrophobic groups, that engage in binding interactions with the sample molecules. In addition, the underlying surface itself (aside from the functional groups) must be "neutral" and have as little binding interaction as possible with the sample.

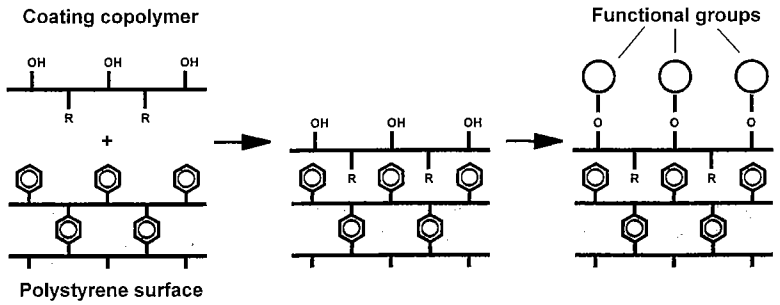
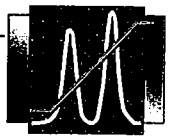


Figure 5-5. Bonded phase chemistry system used with POROS media. A copolymer with both hydrophobic and hydroxyl groups is adsorbed and crosslinked onto the surface of the polystyrene support matrix. The hydroxyl groups are then functionalized to form the final bonded phase.

Several key characteristics of the bonded phase critically influence chromatographic selectivity and capacity. These include the precise chemical structure of the functional groups, how they are chemically bonded to the support matrix surface and the density of functional groups (on both a per unit volume and per unit surface area basis). The physical and chemical stability of the bonded phase, both under normal operating conditions and conditions used to clean and regenerate the column, can also be important considerations.



HPLC vs. LC

High chromatographic efficiency is only possible through the use of small particle size packing materials. As liquid chromatography has become a high precision analytical technique, many developments have been made in order to utilize ever-smaller particle diameters. These developments included techniques for making precisely sized, high strength porous particles, appropriate surface chemistries, high pressure/high precision pumps, etc. Collectively, this technology is referred to as *HPLC*, for High Performance Liquid Chromatography or, alternatively, High Pressure Liquid Chromatography.

There is a rather sharp distinction between the user environments of HPLC and conventional low pressure chromatography, often called simply LC. HPLC is always an instrument-intensive technique, while LC can be performed with no more equipment than a gravity-fed column attached to a ring stand. HPLC has been primarily an analytical and milligram-scale preparative method (although some large scale HPLC systems have been developed over the years), while LC is generally used for preparative applications at a wide range of scales. HPLC is most often used with small molecules, with application to proteins being a more recent development, while LC as a technique has been highly associated with biomolecule applications.

Aside from these critical differences, the actual separation mechanisms of HPLC and LC are identical, and the approaches used to develop protocols or methods are essentially the same as well. This handbook makes little distinction between the two, and the user should select the POROS media suitable for the application at hand and the equipment available.

COLUMN HARDWARE

Chromatography column hardware is relatively simple, although the details of the design are critical for optimum performance. The basic component is a cylindrical tube, usually made out of either stainless steel, glass or plastic. Fitted on the ends of the column tube are end cells, consisting of a bed support (a screen or fritted material with fine enough mesh to retain the packing particles but allow liquid to pass through), a flow distributor (sometimes consisting of baffles or channels to insure even flow across the entire bed) and a tube to connect to the system plumbing.

The end cell design is critical for good capacity and efficiency in the column. On some columns, one or both of the end cells are adjustable along the length of the column, with some type of sliding seal and a mechanism for fixing the end cell in place once the bed support is in contact with the column bed itself. These adjustable end cells allow variation in the column bed length (and therefore volume) without introducing a void space at the top of the bed. If there are no void spaces, the flow in the column usually can be either up or down, although in some cases it is desirable to flow the column in the direction in which it was packed.

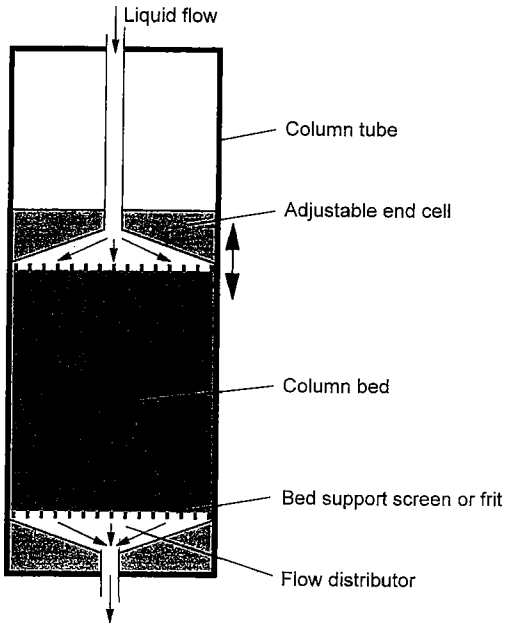


Figure 5-6. Elements of column hardware

The wetted parts of the column must be able to withstand exposure to all of the desired mobile phases and must not contaminate the sample or product through leaching of plasticizers or other materials. The column must be designed to operate at the desired pressure and flow rate. The column design should also include some features to allow the column to be easily packed and unpacked.

Packing a Column

Most columns are packed using a so-called flow packing method. In this technique a slurry of the stationary phase is made in a suitable packing solvent. The column (with only one end cell) is connected to a packing tube, which is usually just an extension of the column tube. The slurry is poured

all at once into the assembly. Liquid is then pumped in the top of the packing tube (normally at a flow rate significantly above the desired operating flow rate), forcing the slurry to flow downward into the end cell (which retains the stationary phase) forming a packed bed. The top of the packed bed moves upward until all of the slurry has been packed. The packing tube is then removed from the column, and the other end cell is attached.

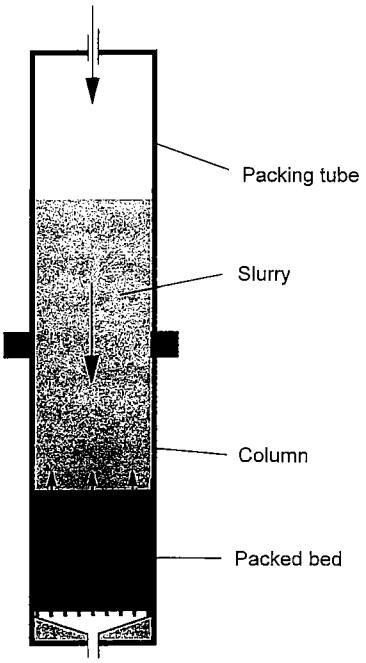
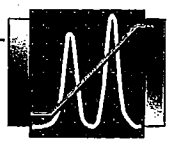


Figure 5-7. Slurry packing a column

There are many variations in this technique, and the exact details of the procedure can be quite critical for obtaining good results. The POROS Self Pack format contains preweighed quantities of media and detailed step-by-step instructions so that even a user with no prior chromatography experience can immediately begin producing highly reproducible, high performance Perfusion Chromatography columns.



Column Geometry & Flow

The chromatographic bed is almost always cylindrical with flow parallel to the axis. The geometry of the column is specified by the diameter and length of the bed itself (inside the wall of the column). The *cross-sectional area* is the area of a slice through the column bed perpendicular to the axis, and is equal to pi (π) times the radius of the bed squared. The *column (or bed) volume* (*CV*) is thus equal to the length times the cross-sectional area.

Flow rate can be measured in two ways. The most obvious is just the *volumetric flow rate*, measured in ml/min or liters/min. However, it is preferable to express the flow rate as the *linear velocity*, which is equal to the volumetric flow rate divided by the cross-sectional area of the column. It is usually measured in ml/hr/cm^2 or just cm/hr. The critical properties of the system, such as the efficiency and the pressure drop, are a function of the linear velocity, which is independent of the size of the column and thus much easier to use when designing a scale up. Linear flow rate used with conventional chromatography media are typically in the range of 50 to 360 cm/hr. Perfusion Chromatography media are typically operated in the range of 1000 to 3600 cm/hr.

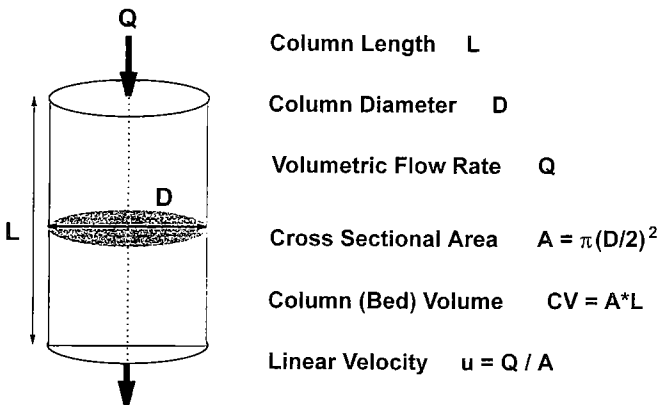


Figure 5-8. Column geometry and flow rate measurements

Pressure/Flow Characteristics

Pressure is required in order to drive flow through the packed column bed. If the support matrix is perfectly rigid, the amount of pressure should be a linear function of the flow rate and the length of the column bed. However, for many kinds of packings, especially soft polymeric gels, the pressure/flow profile is non-linear. This means that there is a maximum flow rate that can be achieved before the bed crushes and the pressure increases beyond the limits of the system. For these kinds of materials, the pressure/flow profile may depend on the bed diameter, since friction between the wall of the column and the bed helps to support the packing. This "wall effect" diminishes as the bed diameter increases, causing increased pressure at a given flow.

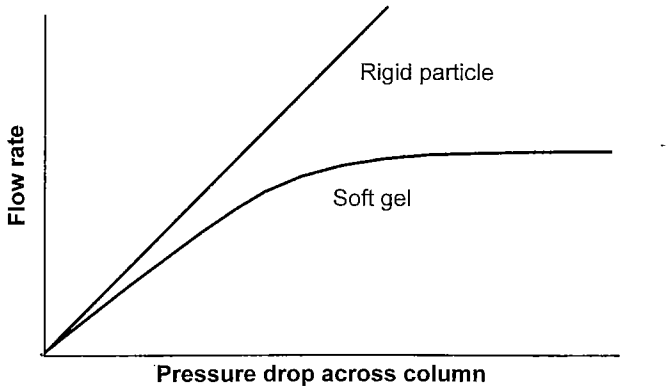
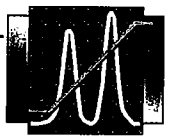


Figure 5-9. Typical pressure/flow characteristics for rigid and soft gel media



One critical point to understand is the difference between *absolute pressure* and *pressure drop*. Absolute pressure is the actual measured pressure at any point in the column or system. The pressure drop is the *difference* in absolute pressure *between* two points in the system, usually caused by the flow of liquid between those points. The pressure drop across the column is usually the most important for maintaining the integrity of the packing.

Most chromatography packings will withstand a high *absolute pressure,* but are limited in the *pressure drop* across the column which is allowable before the support matrix crushes. The total absolute pressure which must be supplied by the pump (and is usually measured by the pressure monitor) is the sum of the column pressure drop plus the pressure drop contribution of the plumbing and other system components (called the *system pressure*).

You must therefore pay attention to two different pressure limits for the system — the total pressure limit (determined by the pump and column hardware capabilities) and the column pressure drop limit (determined by the packing material). At flow rates used with conventional chromatography, the system pressure and pressure drop across the column are essentially equal. When you begin using Perfusion Chromatography media, however, the high flow rates may cause a significant increase in the system pressure (due to a plugged fitting, too small a tubing ID, or flow cell restrictions, for example).

When using Perfusion Chromatography media for the first time, perform a blank run with a union in place of a column to check the system pressure at high flow and correct any problems that may occur.

CHROMATOGRAPHY SYSTEMS

Chromatography is usually carried out using an instrument system containing the elements illustrated below. The system may be simple or complex, homemade or commercial, modular or integral. The simplest system uses gravity to "pump" a mobile phase through the column, with fractions collected manually. Such a system can be effective, but has clear limitations. Most researchers use a more complex system to both improve flexibility and automate the process.

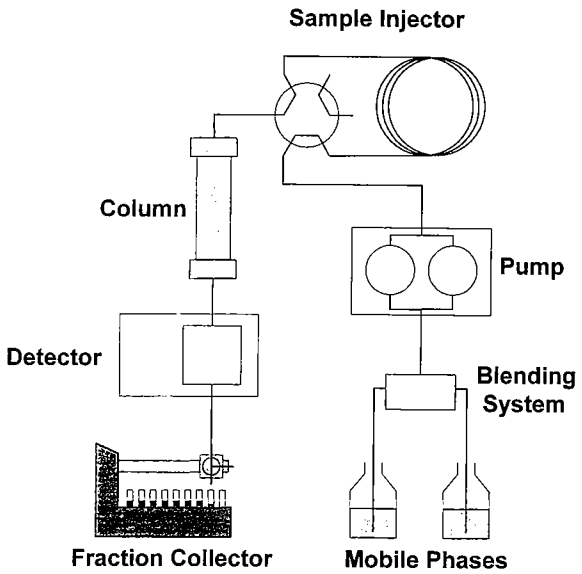
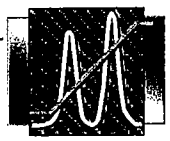


Figure 5-10. Elements of a chromatography instrument system

The selection of the specific components of the instrument system will affect the kind of chromatography that can be performed. These are each discussed briefly on the following pages.



Blending System

The *blending system* creates a controlled mixture of different mobile phases, used for producing gradients. The simplest approach is to manually change the reservoir used as the source for the mobile phase, which limits the user to step gradients. Simple linear gradients can be accurately and reproducibly produced by connecting two reservoirs together with a tube and withdrawing from one which is stirred. As the level in the stirred reservoir goes down, liquid from the other will flow in to maintain an equal level, causing the formation of a linear gradient. This type of blending system is inexpensive and quite useful with simple peristaltic pump-based systems.

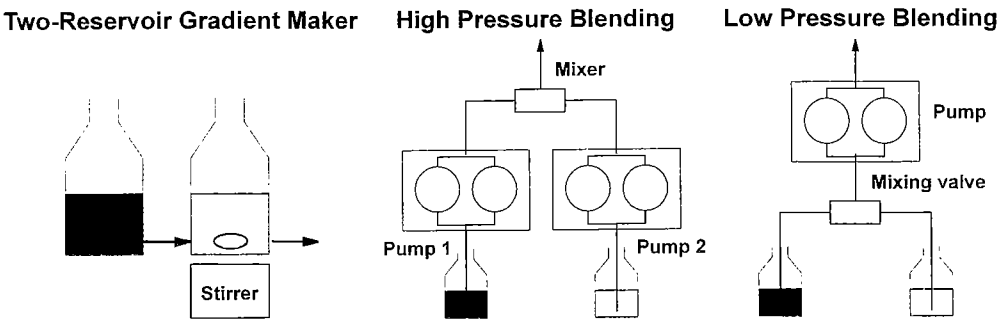


Figure 5-11. Types of blending systems

In order to easily vary the gradient produced or to make more than single linear or step gradient segments, a computer-controlled blending system is required. Two basic types are available. In a *high pressure blending system,* the output from two (or more) pumps are blended together, and the pump speeds are controlled to set the blend. In a *low pressure blending system,* a single pump is used, and blending is done by using a fast switching valve to proportion blend in time.

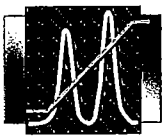
Modern versions of both of these system types are generally quite functional, although there are a few engineering tradeoffs. High pressure systems are often slightly more precise and reproducible, but are more expensive, especially if more than two mobile phases are to be blended. Low pressure systems give a broader flow rate range and are often more economical (especially for 3 or more mobile phases), but can have problems achieving high precision.

Pump

The *pump* delivers mobile phase at a controlled flow rate to the column. The type of pump determines the maximum pressure available to force liquid through the column, which in turn determines particle size of chromatography resins that can be used. Most chromatography systems employ pumps which generate a constant flow rate over a fairly wide pressure range (positive displacement pumps). Several types of pumps are typically used.

A peristaltic pump uses flexible tubing which is pinched at 2 or more locations with rollers, which are moved in a circle to force a bolus of liquid through the tube. Peristaltic pumps are widely employed for low pressure (< 3 bar) chromatography with fairly large particle size resins (50 - 150 μm). This type of pump is relatively trouble-free, if the tubing is replaced fairly frequently.

A reciprocating piston pump uses one or more small pistons with inlet and outlet check valves to deliver very precise flow against back pressures up to several hundred bar. These pumps are the most common in HPLC systems. The seals and check valves can fail, and require care and maintenance.



Some laboratory systems also use syringe pumps, in which one or two syringes are pushed by linear drives. These systems can be quite accurate, but generate only moderate pressures. Pumps used for large-scale process chromatography include diaphragm and rotary lobe pumps.

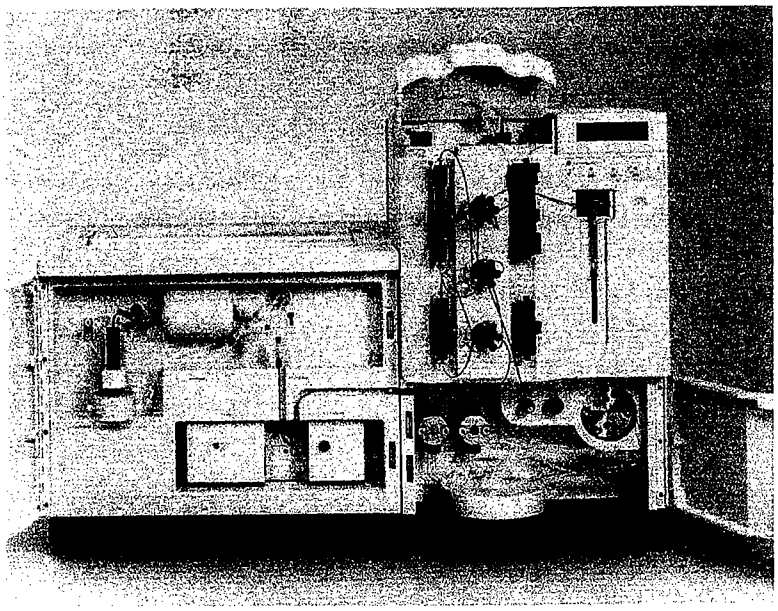


Figure 5-12. Photograph of BioCAD system, showing key system components

Valves & Plumbing

A variety of valves, tubing, fittings and other plumbing components are used with chromatography systems. The plumbing components must have a sufficient pressure rating to work with the pump and column requirements. They must also have very low unswept or "dead" volume, to prevent undue band-spreading and loss of chromatographic resolution. It is also best to use the smallest tubing diameter possible (given the flow rate required) to minimize these bandspreading effects.

One critical use for valves is for injection of the sample onto the column. If the sample volume is very large, the sample can be simply pumped onto the column using the system pump itself, just like the mobile phase. However, for high resolution chromatography with smaller samples, a *sample injector* composed of a switching valve coupled with a *sample loop* is often employed. The sample loop (just a coil of tubing with enough volume to hold the sample) is filled completely or partially with sample, and the switching valve is used to place the loop in the stream between the pump and the column to load the sample onto the column.

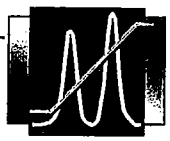
Detectors

The *detector* is used to instantaneously measure the presence and concentration of sample components in the eluate stream. UV absorption through a small flow cell at a controlled wavelength (spectrophotometry) is the most common detection method, although fluorescence, electrochemical and other detectors are also used for special applications. A record of the concentration vs. time or volume of eluate (the *chromatogram*) will show *peaks* corresponding to the various bands of sample components as they elute from the column.

In some systems other types of sensors are also used to monitor performance. High pressure systems almost always have a pressure monitor, usually built into the pump, which is critical to prevent overpressurization of the column or plumbing. Some systems also have flow-through pH and/or conductivity sensors, which are extremely useful for monitoring the state of the mobile phase coming out of the column.

Fraction Collector

The *fraction collector* is an apparatus used to collect the separated sample components into individual test tubes or other vessels in preparative applications. Fraction collectors are usually controlled to change fractions based on either time, volume, or detection of peaks coming out of the column.

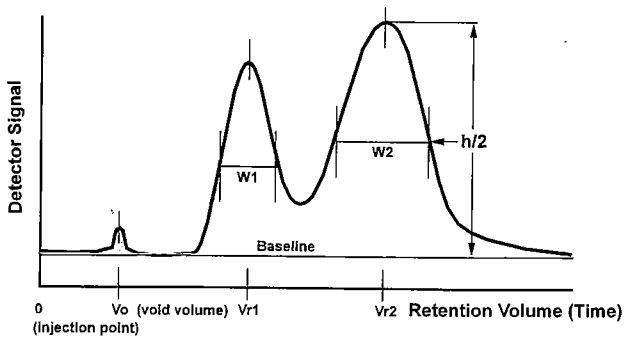


System Controller

In most modern chromatography systems a computer controls the separation and analyzes the resulting data. The computer may either be a microprocessor embedded in the system, or a stand-alone PC connected to the system through an interface board.

EVALUATION OF CHROMATOGRAPHIC PERFORMANCE

During the experimental phase of method development, you need to evaluate the performance of your separation. Chromatographic performance is not simple and must be characterized by a variety of different parameters. A number of formal techniques and measurements have been developed to evaluate the key parameters of *resolution* (*selectivity* and *bandspreading*) and *capacity*. Section 2 — *Principles of Systematic Method Development* describes the use of these parameters during the method development process.



Retention	$k' = (Vr - Vo) / Vo$
Selectivity	$\alpha = k'2 / k'1$
Efficiency	$N = 5.54 (Vr / W)^2$
Resolution	$Rs = (Vr2 - Vr1) / 0.85 (W2 + W1)$

Figure 5-13. Quantitative performance analysis of chromatography

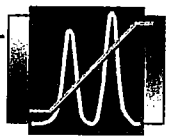
Resolution

Resolution is a measurement of the actual amount of separation between two peaks. It is effectively the "bottom line" result, since without resolution you do not have a separation. In quantitative terms, the resolution (Rs) between two peaks is equal to the difference in retention (in either time or volume) divided by the average of the peak widths at the base of the peak. In practice, it is much easier to measure the peak width at half the peak height, so a factor is included to account for the difference between the base width and half-height width. A resolution of 1.0 is a nearly baseline separation, although a higher resolution is normally required for high precision analysis.

It is critical to realize that resolution is actually a composite function of two completely independent parameters — *selectivity* and *bandspreading* (or *efficiency*).

Selectivity

Selectivity is a measurement of the difference in *retention* between the molecule of interest and the other molecules in the sample. The retention is simply the time or volume after injection of a sample at which a particular peak elutes. In isocratic elution, the retention is often normalized by subtracting the void volume or time (elution point of an unretained peak, equal to the liquid volume in the column) from the retention and dividing by the void volume or time. This normalized retention is called the k' (or k). Selectivity is sometimes expressed as the ratio of the k' (or k) of two molecules being separated, also called the *alpha*. In gradient elution, k' is not a valid measurement, and the simple retention time or volume is used.



Bandspreading or Efficiency

As peaks move down a column or through a chromatography system, they often increase in volume. This so-called *bandspreading* is due to a wide range of different factors, including simple molecular diffusion, flow profiles, mixing within the column hardware and plumbing, and the diffusion into and out of the packing particles. Obviously, the narrower and sharper the peaks, the better the ability to separate sample components, so keeping bandspreading to a minimum is a critical performance objective.

The chromatographic *efficiency* is a conventional measure of the bandspreading that occurs during elution. Efficiency is a function of both the width of the peak (most easily measured at the half-height) and the retention volume. Efficiency is measured by injecting a small volume of a small molecule tracer, which is eluted isocratically from the column.

The unit of measure is the *theoretical plate*, an unusual term which arises from the analogy between chromatography and distillation, for which a mathematical plate theory was originally developed. The higher the number of theoretical plates, the sharper the peaks (i.e. the lower the bandspreading). Since the number of plates is proportional to the length of the column, column efficiency (which is a function of the particle size of the packing and how well packed the column) is often expressed in plates per meter or *plate height*.

Efficiency (or the number of plates) is primarily controlled by the size of the packing particles and the length of the column. In some cases, chemistry effects, such as non-specific adsorption, can cause unusually high bandspreading. In addition, with conventional media, the efficiency is also a function of the flow rate. The typical relationship, normally shown as a plot of plate height vs. flow rate, is called a Van Deemter curve. The Van Deemter curves for conventional and Perfusion Chromatography media are quite different. Refer to the *Introduction to Perfusion Chromatography* companion piece for more detail.

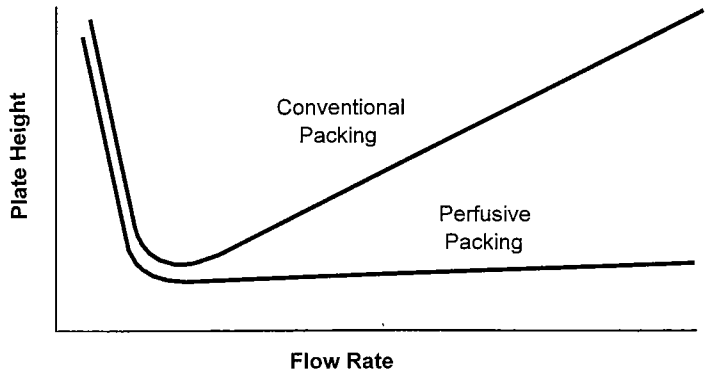
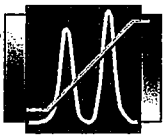


Figure 5-14. Van Deemter curves for conventional diffusion-based and perfusive media of the same particle size. Note that with the perfusive media, the bandspreading increases much more slowly with increasing flow rate.

Capacity

Capacity is defined as the amount of sample that a chromatography system can separate. Capacity is clearly an important parameter for preparative chromatography, since it determines the size of column and system required to process a given sample load. Capacity can also be important in analytical chromatography, since it is one determinant of the dynamic range of an assay.

Capacity can be expressed in a number of distinct ways. The first is the *saturation* or *equilibrium capacity,* which is the amount of sample the packing will bind at complete saturation using the given mobile phase. This is the maximum amount of material the column can possibly bind, and is the number usually reported by media vendors in their specifications. Saturation capacity is normally measured by mixing a predetermined amount of packing material with an excess of the binding molecule in solution in the mobile phase, allowing the mixture to come to binding equilibrium (usually overnight) and measuring the bound vs. free binding molecule.



A second measurement is to determine the capacity in a packed column under flowing conditions, using a technique called *frontal adsorption analysis*. In this method, the sample is pumped onto the column continuously until the entire column with all its binding sites is completely saturated and the concentration measured in the eluate is equal to the level in the feed (see figure). The volume of sample required to reach one half saturation times the feed concentration will be just equal to the saturation capacity.

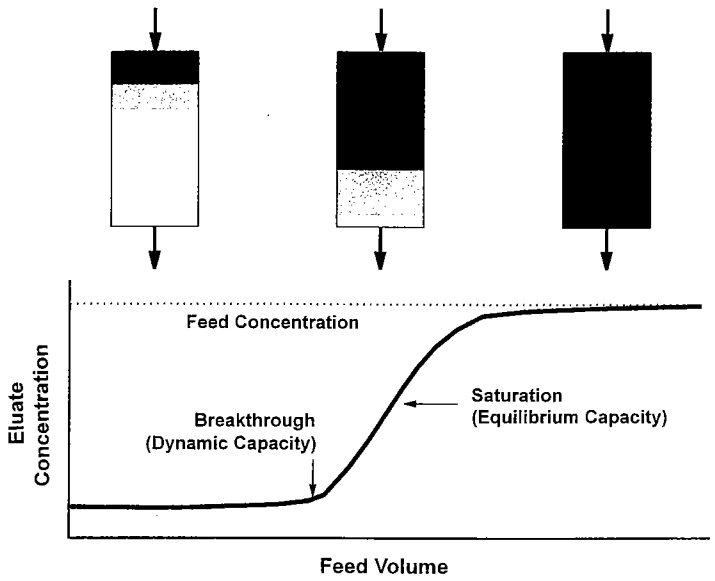


Figure 5-15. Frontal adsorption analysis for dynamic capacity measurement

Frontal adsorption analysis is more than just a convenient way to measure the saturation capacity, however. A more interesting number derived from a frontal curve is the volume of sample required to reach the first measurable breakthrough of

sample (often measured at 1, 5 or 10% of the feed concentration). This value is called the *dynamic capacity*. In conventional, non-perfusive media, the dynamic capacity decreases with increasing flow rate. This is because the slow rate of diffusion into the pores of the packing allows some molecules to flow all the way to the bottom of the column before they are able to bind to the internal surface of the stationary phase. Because of this effect, the dynamic capacity is less than the saturation capacity at any reasonable operating flow rate for conventional media. A major advantage of perfusive media is the elimination of this effect, allowing full capacity at a wide range of flow rates.

For analytical chromatography, it is important for the amount of the sample bound to the column to be linearly dependent upon the amount of sample injected, so that the eluted peak area can be easily used to quantitate the concentration of the sample. At lower concentrations of binding molecule, the amount bound *does* depend linearly upon the concentration of the binding molecule itself, allowing linear analysis. At higher concentrations, however, the column enters the so-called "overload" range, in which the bound concentration asymptotically approaches the saturation capacity. In the overload range, it is more difficult to use peak area as a measurement of sample concentration. Preparative chromatography, however, is almost always carried out in the overload range.

The relationship between bound and free concentrations is referred to as the *adsorption isotherm*. The precise shape of the isotherm is a critical determinant of chromatographic behavior, and depends upon the nature of the binding molecule and packing material, as well as the mobile phase conditions. Normally, detailed knowledge of the isotherm is not required to practice chromatography.

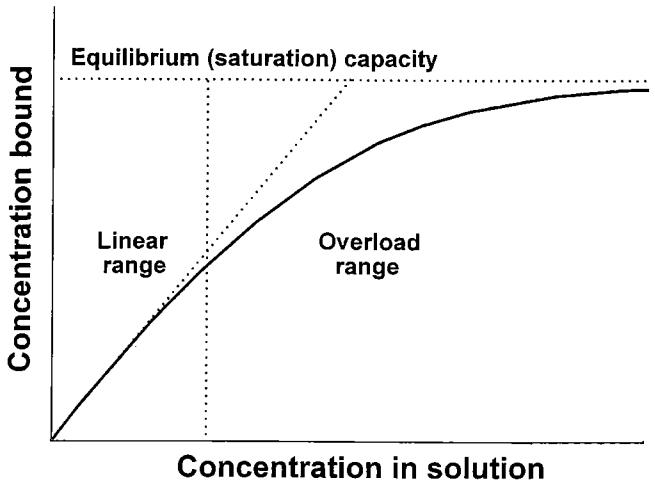


Figure 5-16. Typical adsorption isotherm, showing the saturation capacity and linear and overload regions

In the overload range, both the retention and peak width for any given molecule can be strongly dependent upon the sample load. For this reason, the strict capacity of a column is not always relevant. A more important parameter may be the *loadability*, which is defined as the maximum amount of sample load under a given set of conditions that yields a separation which meets whatever goals are required. Loadability must always be measured empirically by doing a loading study once the separation conditions have been developed.

GLOSSARY

accuracy — the degree of agreement between a value determined by an assay for a given sample and the accepted or true value for that sample.

activated affinity media — packing materials for use in affinity chromatography supplied with covalently reactive surface functionality to allow the end user to couple their own affinity ligands.

activity recovery — the fraction of the biological activity (such as antibody binding activity, enzyme activity, etc.) applied to a chromatographic step that is recovered in the eluted product fraction. Also referred to as *activity yield*.

affinity chromatography — chromatographic separation based on a specific binding interaction between an immobilized ligand and its binding partner in the sample. Examples include antibody/antigen and enzyme/substrate or enzyme/inhibitor interactions. The degree of purification in affinity chromatography can be very high, depending upon the specificity of the interaction used.

alkylsilane — a chemical functional group used to form a reversed-phase bonded phase on silica support matrices, consisting of a chemically reactive silane group coupled to a long alkyl carbon chain (usually C1, C4, C8 or C18).

alpha (α) — a measure of chromatographic selectivity, equal to the capacity factor (k') of the later eluting molecule, divided by the k' of the earlier eluting molecule. An alpha of 1.0 indicates no resolution.

ammonium sulfate precipitation — a widely used, non-chromatographic technique for protein concentration and enrichment, consisting of adding salt (ammonium sulfate) to a protein solution until the protein of interest precipitates. Because the precipitation is related to the surface hydrophobicity of the protein, more hydrophobic proteins will drop out of solution at lower salt concentrations. Hydrophobic interaction chromatography is in some ways a higher resolution, chromatographic version of this technique.

analyte — the substance to be detected by an assay; the target molecule.

analytical chromatography — any chromatographic method in which the desired outcome is a quantitative or qualitative determination of the chemical composition of the sample, rather than recovery of actual purified material (which is preparative chromatography).

ångström (Å) — a unit of length commonly used for measurement of pore sizes in chromatographic packings, equal to 10^{-4} µm (10^{-10} meters).

GLOSSARY

anion exchange chromatography — a form of ion exchange chromatography in which anionic (negatively charged) molecules are separated by binding to a positively charged stationary phase.

antibody (Ab) — an *immunoglobulin* (see below) produced by the immune system of vertebrates in response to exposure to a foreign substance (the immunogen, see below). Characterized by a highly specific binding interaction with the corresponding *antigen*.

antigen (Ag) — a molecule which can bind specifically to an antibody. Note that the antigen may not necessarily be the same molecule that induced the formation of the antibody (which is the *immunogen*).

antiserum — the serum fraction (whole blood with cells and clotting proteins removed) from an animal that has been immunized or exposed to an immunogen and contains antibodies to a particular antigen.

ascites — a liquid tumor formed by injection of a hybridoma cell line into the peritoneal cavity (generally of a mouse). A common method for producing small quantities of monoclonal antibodies.

bandspreading — the increase in volume in an injected sample band caused by its movement through the column and chromatographic system.

bed volume — the total volume occupied by the chromatographic packed bed. For a conventional cylindrical column, the bed volume is equal to the cross sectional area (π times the radius of the bed squared) times the bed length. Also called the column volume or CV.

biospecific binding — a specific binding interaction between an immobilized ligand and its binding partner in the sample. Examples include antibody/antigen and enzyme/substrate or enzyme/inhibitor interactions. Used for affinity chromatography.

blending system — the part of the chromatographic instrument system which continuously blends between two or more solvents or buffers at a controllable, time-variable ratio to form specific mixes and gradients. Blending systems fall into two general categories. In low pressure systems, the blending is done using a high speed switching valve to proportion small volume slugs from two or more streams feeding into the low pressure inlet of a single system pump. In high pressure systems, the output of two or more separate controlled system pumps are mixed together on the high pressure outlet side.

bonded phase — the chemical functional groups bonded to the surface of a support matrix of a stationary phase. The bonded phase is responsible for providing the selectivity of the binding interaction.

buffer exchange — an application of gel filtration chromatography in which a packing is used that completely excludes from the pores any proteins and allows buffer salts and other small molecules to penetrate the pores. On passage through the column, the protein molecules are exchanged from the buffer and salt conditions of the sample to the conditions in which the column was originally equilibrated. Also called desalting.

capacity — any measure of the amount of sample material (mass, volume or activity) which can either bind to or be separated by a chromatographic column and system. Several important capacity measurements include the static or equilibrium binding capacity, dynamic binding capacity and loadability.

capacity factor (k') — a normalized measure of retention, equal to the retention time (or volume) of a given molecule minus the void time (or volume, equal to the time or volume of a completely unretained peak) divided by the void time (or volume).

capillary chromatography — chromatography in columns less than 1 mm in internal diameter.

cation exchange chromatography — a form of ion exchange chromatography in which cationic (positively charged) molecules are separated by binding to a negatively charged stationary phase.

cell culture supernatant — the fluid made during cell culture (either roller bottle, suspension or perfusion), containing culture media components and the product of interest. A large scale manufacturing source of monoclonal antibodies.

chaotropic agent — any type of molecule which interferes with hydrophobic interactions by disrupting the ordered structure of water molecules. Common examples include urea, guanidine and thiocyanate salts. Chaotropic agents can be powerful solubilizing agents and can be used to reduce strong hydrophobic adsorption.

chromatogram — a trace of the detector signal vs. time (or volume) produced during a chromatographic run. Peaks above the baseline indicate the presence of molecules coming out of the column in the eluate stream.

column volume (CV) — the total volume occupied by the chromatographic packed bed. For a conventional cylindrical column, the column volume is equal to the cross sectional area (π times the radius of the bed squared) times the bed length. Also called the bed volume.

conjugate — a covalently-linked complex between two or more molecules. Conjugates are typically made between an antibody or an antigen and a label such as a dye or an enzyme.

GLOSSARY

continuous gradient elution — a form of gradient elution in which the mobile phase composition or blend is changed gradually over time or volume. Continuous gradients can range in shape from simple linear profiles to complex functions, sometimes combined with step gradients.

counter ion — any molecule of opposite charge associated with the bonded phase charged functional groups on the surface of an ion exchange stationary phase. There are always an exactly equal number of counterions to balance the charges on the stationary phase. Counterions can be salt molecules from the solution or sample molecules.

cross-sectional area — the area of a circular slice taken through a column perpendicular to the cylindrical axis, equal to π times the radius of the column squared.

CV (coefficient of variation) — the statistical standard deviation of replicate runs of an assay divided by the average value of the assay for the sample, expressed as a percent. Used as a measure of *precision*.

denature — cause a protein molecule to lose its correct three-dimensional folded structure, and thus, usually, its biological activity. Some proteins can be correctly refolded and renatured under the right conditions, but for other proteins the process of denaturation is irreversible.

desalting — an application of gel filtration chromatography in which a packing is used that completely excludes from the pores any proteins and allows buffer salts and other small molecules to penetrate the pores. On passage through the column, the protein molecules are exchanged from the buffer and salt conditions of the sample to the conditions in which the column was originally equilibrated. Also called buffer exchange.

detection limit — the minimum amount of the analyte that can be detected (although not necessarily accurately quantitated) above the background noise under a given set of analytical conditions in a given sample matrix. The standard formal definition is the signal of a blank sample (sample with no analyte) plus two standard deviations, where at least 20 replicates of the blank are run.

diffusive pore — a small pore in a chromatographic packing material which is only accessible to a molecule by molecular diffusion. See *throughpore*.

dynamic capacity — the binding capacity of a chromatographic stationary phase in a packed bed with a flowing sample stream. Usually measured by frontal adsorption analysis, and defined based on the volume of a known concentration sample that can be applied at a given flow rate before any measurable amount of the binding molecule breaks through into the eluate. For conventional media, dynamic capacity is usually a function of the flow rate through the bed.

efficiency — a standardized measure of the bandspreading of a column. Efficiency can be expressed as the number of theoretical plates ($N = 5.54 (Vr / w1/2)^2$, where Vr is the retention volume [time can also be used] and w1/2 is the peak width at half the maximum peak height) or the plates/m (equal to N / L, where L is the column length in meters) or the HETP.

eluent — a mobile phase used to elute molecules from a column.

eluate — the stream of mobile phase emerging from the outlet of the column.

elution — the process of removing injected sample molecules from a column by pumping through one or more different mobile phases.

endcapping — a chemical treatment for reversed-phase silicas designed to reduce the effects of residual silanol activity on chromatographic selectivity and recovery, consisting of bonding very short chain alkylsilanes (such as C1) after the longer chain bonded phase is applied.

epitope — the portion of the antigen molecule which is recognized by and is directly involved in the binding to the antibody.

equilibrium capacity — the maximum possible binding capacity of a chromatographic stationary phase, usually measured by an equilibrium adsorption experiment, in which a known amount of stationary phase is mixed with an excess of a binding molecule and the mixture allowed to come to binding equilibrium. The amount of binding molecule remaining in solution is measured and used to determine the amount bound. Also called the saturation capacity.

frontal adsorption analysis — a technique usually used for determining dynamic binding characteristics, in which a sample with a fixed concentration of a binding target molecule is continuously pumped into a column and the amount of target molecule in the eluate is continuously measured. The experiment is generally complete when the eluate concentration of the target molecule equals the inlet concentration, indicating the column is completely saturated.

gel filtration chromatography — a chromatographic separation based on molecular size, in which a stationary phase with a controlled pore size range and very low or zero adsorption characteristics for the sample is used. Molecules elute in decreasing order or size due to restricted access into the pore volume of the packing. Also called size exclusion or gel permeation chromatography.

gel permeation chromatography — see *gel filtration chromatography.*

gradient elution — any elution method in which the composition of the mobile phase is changed, either abruptly (step gradient) or gradually (continuous gradient).

HETP — Height Equivalent of a Theoretical Plate, a measure of chromatographic efficiency, equal to the length of a column divided by the plate count (see *efficiency*). The lower the HETP, the more efficient the column. Also called the plate height.

HIC — Hydrophobic Interaction Chromatography

HPLC — High Performance Liquid Chromatography or High Pressure Liquid Chromatography, generally defined as chromatography run with high efficiency, small particle stationary phases, normally in a highly instrumented system.

hybridoma — a hybrid cell line produced by fusing an antibody-producing cells with a myeloma (tumor) cells to produce an immortal, reproducing cells that manufactures the specific antibodies. The hybridoma cells are then selected to find a cell line producing a single, desired *monoclonal antibody* indefinitely in cell culture.

hydrophilic — the opposite of hydrophobic; a tendency to bind or include water. Also called polar.

hydrophobic — a molecular property of a functional group which causes water to be repelled or excluded. In aqueous mobile phases, hydrophobic groups tend to bind together. The hydrophobic effect is what causes oil (hydrophobic) and water to separate. Also called nonpolar.

hydrophobic interaction chromatography — chromatographic separation based on surface hydrophobic functionality of proteins and peptides. Usually performed using packings with weakly hydrophobic bonded phases, using a starting mobile phase of very high ionic strength (to promote hydrophobic binding) and eluting using decreasing salt concentration.

immobilized — bound to a surface, usually through covalent chemical bonds.

immunoglobulin (Ig) — one of a family of globular proteins capable of acting as antibodies. These include IgG (the most common), IgM, IgA, IgD and IgE.

ion exchange chromatography — chromatographic separation based on binding of opposite ionic charge groups on the stationary phase and the molecules in the sample.

ionic strength — a measure of the concentration of salt in a solution, which includes a factor for the chemical activity of the salt ions.

isocratic elution — elution with a constant mobile phase composition.

isoelectric point — the pH at which the net or total charge of a molecule is zero (i.e. the total number of positive charges equals the total number of negative charges).

isotherm — the relationship between the concentration of a test molecule bound to a stationary phase (under given experimental conditions) and the concentration left in free solution at equilibrium. The shape of the isotherm contains a great deal of information about the nature of the binding interaction and the chromatographic behavior of the test molecule.

k or k' — see *capacity factor.*

LC — liquid chromatography. Sometimes used to refer to a lower resolution form than HPLC, with lower pressure system and larger stationary phase particles.

ligand — a term for a molecule non-covalently but selectively bound to a protein. It is often used for an antigen or hapten.

ligand leakage — leakage of ligand immobilized on a support into the mobile phase.

linear velocity — a normalized measure of the flow in a column, equal to the volumetric flow rate divided by the cross-sectional area.

linearity — the measure of the correlation between the assay response and the concentration of an analyte. The correlation may be obtained through a well-defined mathematical transformation (e.g. semi-log or log-log). Note that over a broad enough range, *all* immunoassays require a transformation for linearity, due to the equilibrium nature of the binding.

linker — a molecular chain used to connect an affinity ligand to a stationary phase surface in immobilization. The purpose of the linker is to increase the accessibility of the ligand in a large target molecule. Also called a spacer or spacer arm.

loadability — the maximum amount of sample that can be run in a given separation and still maintain the required chromatographic resolution.

mass recovery — the mass of sample material recovered in the eluate divided by the mass injected on the column.

media — another name for chromatographic stationary phases or packing materials. Technically, the word media is plural, but it is often used as a singular.

microbore chromatography — use of columns with very small internal diameters, usually around 1 mmD.

mixed mode — any chromatographic separation in which there is more than one mode of binding interaction at work at the same time. Note that virtually all chromatographic packings have some mixed mode effects.

GLOSSARY

ruggedness — the ability of an assay to withstand small changes in particular operating parameters without affecting the performance. A critical component of *precision*.

sample matrix — all of the molecular components in a sample aside from the analyte itself. Changes in the sample matrix may affect the outcome of an assay in a range of different ways.

saturation capacity — see *equilibrium capacity*.

selectivity — the actual degree of separation or difference in retention between different molecules in a sample on a chromatographic column. Highly sensitive to a wide range of parameters in the chromatographic method.

semi-quantitative analysis — giving a result that indicates there is more or less than particular amount(s) of an analyte in a sample, without indicating the actual concentration. See *quantitative analysis* and *qualitative analysis*.

sensitivity — the ability of an assay to detect very small amounts of the target analyte. Often expressed as the *detection limit*.

silanol — a highly polar, sometimes negatively charged surface group present in very high concentrations on native silica gel that has strong non-specific adsorption properties when exposed on reversed-phase silica stationary phases.

size exclusion chromatography — see *gel filtration chromatography*.

spacer arm — see *linker*.

specificity — the ability of an assay to measure only the target analyte without interference from other *sample matrix* (see above) components or undesired molecules closely related to the target. In the case of immunoassays, the cross-reactivity of the antibody is a major element of the specificity.

stationary phase — the solid material packed into the column on which the chromatographic separation takes place. Consists of a support matrix, usually in the form of porous particles, with a controlled bonded phase surface.

step gradient elution — a form of gradient elution in which the mobile phase is changed abruptly from one composition to another.

strong ion exchange chromatography — a form of ion exchange bonded phase functionality in which the charge density does not change with mobile phase pH.

suitability — the ability of an assay to meet the required performance criteria and produce data with acceptable *accuracy* and *precision* for a given type of sample.

support matrix — the solid "skeleton" or substructure of a stationary phase to which the bonded phase is applied as a surface coating.

system pressure — the pressure drop caused by all of the plumbing and other system components aside from the column.

TFA — trifluoroacetic acid, often used as a mobile phase additive in reversed-phase chromatography of peptides and proteins.

theoretical plate — see *efficiency*.

throughpore — a large pore in a Perfusion Chromatography packing which, as part of an interconnected network, allows flow to pass through the particle under normal operating conditions to enhance mass transport within the particle.

throughput — in preparative chromatography the amount of feed material that can be processed per unit time. In analytical chromatography, the number of samples that can be processed per unit time.

validation — the establishment of documented evidence that an analytical test or process system will consistently produce results of sufficient quality to meet its intended purpose.

void volume — the retention volume of a completely non-retained peak, usually nearly equal to the actual liquid volume in the column and plumbing system.

weak ion exchange chromatography — a form of ion exchange bonded phase functionality in which the charge density changes with mobile phase pH.

yield — the amount of material or activity recovered from a purification step divided by the amount applied to the step, often expressed as a percent.

REFERENCES

The following reference list is divided into three sections. This first section is a listing of applications literature available from PerSeptive Biosystems on chromatographic purification and analysis. Use the fax-back form at the back of this book to request copies. The second section is a listing of standard textbooks on various aspects of chromatography, for readers who desire more detailed background information. The third section is a listing of published journal articles with purification or analysis applications of Perfusion Chromatography media or on the general principles, chemistry or theory of the method.

PERSEPTIVE BIOSYSTEMS' APPLICATION LITERATURE

PA401. Preparative method development with Perfusion Chromatography: A one day case study

PA402. Perfusion Chromatography for dilute feed capture: An introduction

PA409. Preparative peptide purification by cation exchange and reversed-phase Perfusion Chromatography

PA410. pH mapping template for proteins and peptides

PA411. Contaminant IgA monitoring: Automated, online product monitoring with the Real-Time Process Monitor

PA412. Purification of anti-transferrin antibodies using affinity Perfusion Chromatography

PA413. Resolution of human recombinant nonglycosylated transferrin from natural human glycosylated transferrin

PA415. Optimization of monoclonal antibody purification from ascites: Rapid method development

PA416. Development and use of a subtractive ImmunoDetection assay for the real-time process monitoring of the purification of recombinant asparaginase

PA417. Protein aggregate detection using the INTEGRAL Micro-Analytical workstation and ImmunoDetection technology

PA419. Use of cation exchange and affinity Perfusion Chromatography for the purification of recombinant human heat shock protein 60

PA420. Use of the Real-Time Process Monitor system during scale up of the purification of a human nerve growth factor/antibody fusion protein

PA421. Purification of recombinant DNA binding protein skn-1

PA422. Conversion of a recombinant IL-8 purification procedure to POROS perfusive media: Increased capacity and decreased process time with Perfusion Chromatography

PA423. Rapid development and optimization of an affinity Perfusion Chromatography method for removal of carbonic anhydrase from hemoglobin preparations

PA424. A displacement chromatography approach to gram-scale purification of phosphoramidite oligonucleotides for therapeutic applications

PA425. Preliminary development of a Perfusion Immunoassay for the detection of neural acetylcholinesterase in amniotic fluid

PA426. High speed resolution of snake venom components

PA427. Use of a Poroszyme immobilized trypsin cartridge and a Voyager-Elite Biospectrometry Research Station for analysis of myoglobin tryptic peptides

PA428. Rapid, preparative purification of plasmid DNA by anion exchange Perfusion Chromatography

PA429. Purification of a bifunctional folate-dependent enzyme for x-ray crystallography studies

PA431. Rapid analysis of hemoglobin variants: Direct coupling of protein digestion, reversed-phase chromatography and mass spectrometry via the INTEGRAL workstation

PA432. Automated, online biotinylation of IgG using the BioCAD workstation for Perfusion Chromatography

PA433. Real-time monitoring of monoclonal antibody production from mammalian cell culture fermentation using the BioCAD/RPM system

PA435. Peptide ladder sequencing using the Voyager™-RP Biospectrometry workstation

PA437. Use of the Voyager Biospectrometry workstation for rapid characterization of synthetic peptides prior to and following HPLC purification

PA440. Rapid purification of Mab 46.3, an IgM monoclonal antibody, from crude mouse ascites fluid

PA443. Use of Perfusion Chromatography technology for the rapid purification of synthetic oligonucleotides used in DNA/DNA-binding protein structural studies

PA444. Rapid, automated peptide mapping using the INTEGRAL Micro-Analytical workstation

PA445. Optimization of reversed-phase peptide separations using automated pairing agent mapping on the BioCAD Workstation

PA446. Real-time process monitoring of the purification of an anti-interleukin 8 monoclonal antibody from mouse ascites using the BioCAD/RPM Workstation

PT901. Advantages of POROS 50 HQ over DEAE-Sephacel® for the purification of human transferrin

PT902. Compatibility of ConSep LC100 liquid chromatography system with POROS 50 HQ medium

PT906. Compatibility of ConSep LC100 liquid chromatography system with POROS A protein A medium

PT907. POROS 50A medium compatibility with common sanitization and clean in place reagents

PT916. Direct control of chromatographic separations through on-line process analytics

PT918. Developed ImmunoDetection assays

TEXTBOOKS ON CHROMATOGRAPHY

Dean, Johnson & Middle *Affinity Chromatography — A Practical Approach,* IRL Press, Ltd. 1985.

Deutscher *Methods in Enzymology Vol. 182, Guide to Protein Purification,* Academic Press 1990.

Gooding & Regnier *HPLC of Biological Macromolecules, Methods and Applications,* Marcel Dekker, Inc. 1990.

Hancock *High Performance Liquid Chromatography In Biotechnology,* J. Wiley & Sons 1990.

Harris & Angal *Protein Purification Applications — A Practical Approach,* IRL Press, Ltd. 1990.

Hermanson, Mallia & Smith *Immobilized Affinity Ligand Techniques,* Academic Press, Inc. 1992.

Janson & Ryden *Protein Purification; Principles, High Resolution Methods and Applications,* VCH Publishers, Inc. 1989.

Karger, Snyder & Horvath *An Introduction to Separation Science,* J. Wiley & Sons 1973.

Mant & Hodges *High Performance Liquid Chromatography of Peptides and Proteins; Separation, Analysis and Conformation,* CRC Press 1991.

Oliver *HPLC of Macromolecules — A Practical Approach,* IRL Press, Ltd. 1989.

Scopes *Protein Purification Principles and Practice, 3rd Edition,* Springer Verlag 1992.

Snyder & Kirkland *Introduction to Modern Liquid Chromatography,* J. Wiley & Sons, Inc. 1979.

Snyder, Glajch & Kirkland *Practical HPLC Method Development,* J. Wiley & Sons 1988.

JOURNAL REFERENCES

Purification

Ball, H. & Mascagni, P. Solid phase synthesis of proteins using large (15-25 residues) protected fragments: methodology, in *Innovation and Perspectives in Solid Phase Synthesis,* Epton, R. ed. 61-68 (1995).

Garza, K. & Tung, K. Frequency of molecular mimicry among T-cell peptides as the basis for autoimmune disease and autoantibody induction, *J. Immunology 5444* (1995).

Hunt, A. et al. Development and monitoring of purification process for nerve growth factor fusion antibody, *J. Chromatogr. A 708*:61 (1995).

Edmonds, B. et al. pH regulation of the F-actin binding properties of *Dictyostelium* elongation factor 1-alpha, *J. Biol. Chem. 270*:15222 (1995).

Fulton, S. Large scale processing of macromolecules, *Current Opinions in Biotechnology 5*:201 (1994).

Roberts, E. et al. Identification of active site peptides from 3H-labelled 2-ethynylnapthalene P450 2B1 and 2B4 using amino acid sequencing and mass spectrometry, *Biochemistry 33*:3766 (1994).

Roberts, E. et al. Mechanism-based inactivation of cytochrome P450 2B1 by 2-ethynylnapthalene: identification of a new active site peptide, *Chemical Research in Toxicology 6*:470 (1994).

Hackett, M. et al. Internal lysine palmitoylation in adenylate cyclase toxin from B. pertussis, *Science 266*:433 (1994).

Burton, J. et al. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell line, stimulates T-cell proliferation and the induction of LAK cells, *PNAS 91*:4935 (1994).

Rose, D. & Opiteck, G. Two dimensional gel electrophoresis/LC for the micropreparative isolation of peptides, *Anal. Chem. 66*:2529 (1994).

Zeidel, M. et al. Ultrastructure, pharmacologic inhibition and transport selectivity of aquaporin channel-forming integral membrane protein in proteoliposomes, *Biochemistry 33*:1606 (1994).

Landro, J. & Schimmel, P. Zinc-dependent cell growth conferred by mutant tRNA synthetase, *J. Biol. Chem. 269*:20217 (1994).

Wilkins, J. et al. A study of intermediates involved in the folding pathway for recombinant human macrophage colony stimulating factor: evidence for two distinct folding pathways, *Protein Science 2*:224 (1993).

Ellis, B. et al. A rapid purification procedure for human synovial phospholipase-A2 using Perfusion Chromatography, *J. Cell Biol. Supplement 17A* p. 49 (1993).

Lehman, E. et al. A novel process for the large scale purification of recombinant tick anticoagulant peptide using Perfusion Chromatography, *Bio/Technology 11*:207 (1993).

Fulton, S. et al. Large-scale processing and high throughput Perfusion Chromatography, *Bio/Technology 12*:742 (1992).

Fulton, S. et al. Preparative peptide purification by cation exchange and reversed-phase Perfusion Chromatography, *BioTechniques 12*:742 (1992).

Baker, B. et al. Deacetolycephalosporin C hydroxylase of *Streptimyces clavuligerus*: purification, characterization, bifunctionality and evolutionary implications, *J. Biol. Chem. 266*:5087 (1991).

Analysis

Evans, D. et al. A tandem column chromatographic method for studying the interaction between ligands and their targets: lipopolysaccharide (LPS) as a model, *Anal. Biochem. 229*:42 (1995).

DePhillips, P. et al. Reversed-phase HPLC assay for recombinant acidic fibroblast growth factor in *E. coli* suspensions and cell lysate samples, *J. Chromatogr. A 663*:43 (1994).

Schenerman, M. & Colins, M. Determination of a monoclonal antibody using ImmunoDetection, *Anal. Biochem. 217*:241 (1994).

Paliwal, S. et al. Process monitoring of the production of gamma interferon in recombinant Chinese hamster ovary cells, *J. Chromatogr. A 659*:317 (1994).

Palmer, D. et al. Rapid fluoresence flow injection immunoassay using a novel Perfusion Chromatography material, *Analyst 119*:943 (1994).

Chen, Y. et al. Mapping the retinoid binding domain of cellular retinaldehyde-binding protein, *Techniques in Protein Chemistry*, Academic Press (1994).

Paliwal, S. et al. Rapid process monitoring in biotechnology, *Trends in Biotechnology 11*:95 (1993).

Kassel, D. et al. Rapid purification, separation and identification of proteins and enzyme digests using packed capillary perfusion column LC and LC/MS, in *Techniques in Protein Chemistry IV*, Angeletti, R. ed. Academic Press (1993) p. 55.

Paliwal, S. et al. Automated process monitoring of monoclonal antibody production, *Anal. Chemistry 65*:3363 (1993).

Afeyan, N. et al. Automated real-time immunoassay of biomolecules, *Nature 358*:603 (1992).

Fulton, S. et al. Very high speed separation of proteins by Perfusion Chromatography, *J. Chromatogr. A 547*:452 (1991).

Fulton, S. et al. Antibody quantitation in seconds using affinity Perfusion Chromatography, *BioTechniques 11*:226 (1991).

General Principles, Chemistry & Theory

McCoy,M. et al. Application of mathematical modelling to the simulation of binary Perfusion Chromatography, *J. Chromatogr. A 644*:1 (1993).

Rodrigues, A. An extended van Deemter equation (Rodrigues equation) for performing chromatographic processes using large-pore, permeable packings, *LC-GC Intl. 6*:20 (1993).

Carta, G. & Rodrigues, A. Diffusion and convection in chromatographic processes using permeable supports with a bidisperse pore structure, *Chem. Eng. Sci. 48*:3927 (1993).

Davis, R. & Stone, H. Flow-through beds of porous particles, *Chem. Eng. Sci. 48*:3993 (1993).

Varady, L. et al. Fimbriated stationary phases for proteins, *J. Chromatogr. A 631*:107 (1993).

Afeyan, N. et al. Perfusion Chromatography: a novel tool for protein purification and analyis, in *Techniques in Protein Chemistry III*, Angeletti, R. ed. Academic Press (1992) p. 135.

Frey, D. et al. Effect of intraparticle convection on the chromatography of biomacromolecules, *Biotechnol. Prog. 9*:273 (1993).

Liapis, A. & McCoy, M. Theory of Perfusion Chromatography, *J. Chromatogr. A 599*:87 (1992).

Regnier, F. Perfusion Chromatography, *Nature 350*:634 (1991).

Glad, M. & Larsson, P. New methods for separation and recovery of biomolecules, *Current Opinions in Biotechnology 2*:413 (1991).

Afeyan, N. et al. Perfusion Chromatography packing materials for proteins and peptides, *J. Chromatogr. A 544*:267 (1991).

REFERENCES

Afeyan, N. et al. Flow-through particles for the high performance liquid chromatographic separation of biomolecules: Perfusion Chromatography, *J. Chromatogr. A. 519*:1 (1990).

Afeyan, N. et al. Perfusion Chromatography: an approach to purifying biomolecules, *Bio/Technology 8*:203 (1989).

ACKNOWLEDGEMENTS

This book was authored and illustrated by Scott Fulton and Daryl Vanderburgh. Editorial assistance was provided by John Curling, Patricia Dimond, Mary Duggan, Joseph Gerstner, Tony Hunt, Krishna Kalghatgi, Paul Lynch, Michael Meys, Rohin Mhatre, Tim Nadler, Larry Schwartz, Gordon Siek, Vern Tisdale, George Vella, and Fred Regnier. Karen Christie provided much-needed administrative support. Some applications were developed by outside researchers, who are individually credited in the text.

Design and production was done by Buck, Harrison/Louis of Waltham, MA.

PerSeptive Biosystems thanks all of these individuals for their many contributions.

INDEX

Q

R

S

T

V

W

X

Y