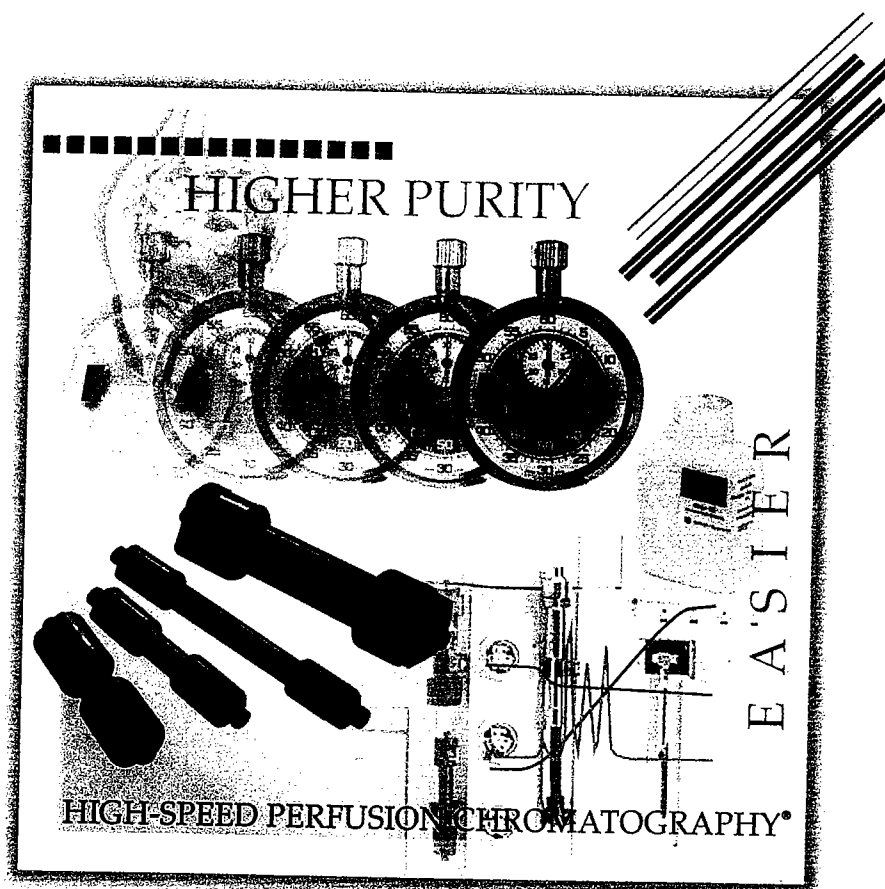


\$29.95

THE BUSY RESEARCHER'S GUIDE TO BIOMOLECULE CHROMATOGRAPHY



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THE BUSY RESEARCHER'S GUIDE TO BIOMOLECULE CHROMATOGRAPHY

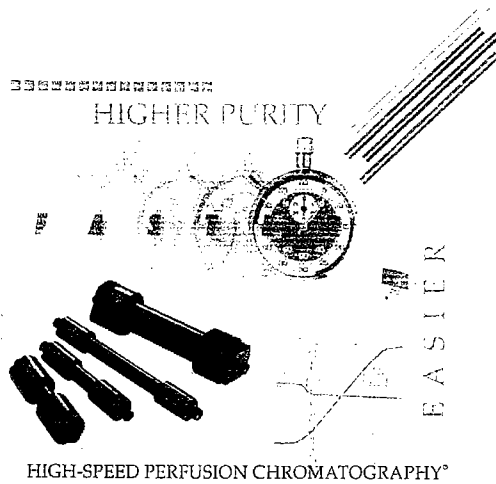












TABLE OF CONTENTS

HOW TO USE THIS BOOK	i	
SECTION 1 INTRODUCTION	1	
Chromatography in Life Science Research	3	
The Limits of Conventional Chromatography	5	
Perfusion Chromatography — A New Approach	7	
Applications of Perfusion Chromatography	8	
Perfusion Chromatography in a Range of Techniques	16	
SECTION 2 PRINCIPLES OF SYSTEMATIC METHOD DEVELOPMENT	19	
Define	23	
Experiment	42	
Evaluate	56	
Implement	63	
Troubleshoot	68	
SECTION 3 DEVELOPING YOUR APPLICATION	81	
Method Development Approaches	83	
Transferring an Existing Method	85	
Proteins	90	
Antibodies	102	
Peptides	113	
Nucleic Acids	122	
SECTION 4 MODES OF CHROMATOGRAPHY	133	
Ion Exchange Chromatography	135	
Hydrophobic Interaction Chromatography	149	
Reversed-Phase Chromatography	157	
Affinity Chromatography	165	
Gel Filtration Chromatography	175	
SECTION 5 BASICS OF CHROMATOGRAPHY	177	
Principle of Chromatography	179	
Column Hardware	189	
Chromatography Systems	195	
Evaluation of Chromatographic Performance	200	
GLOSSARY	207	
REFERENCES	219	
ACKNOWLEDGEMENTS	227	
INDEX	229	



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

CHROMATOGRAPHY IN LIFE SCIENCE RESEARCH

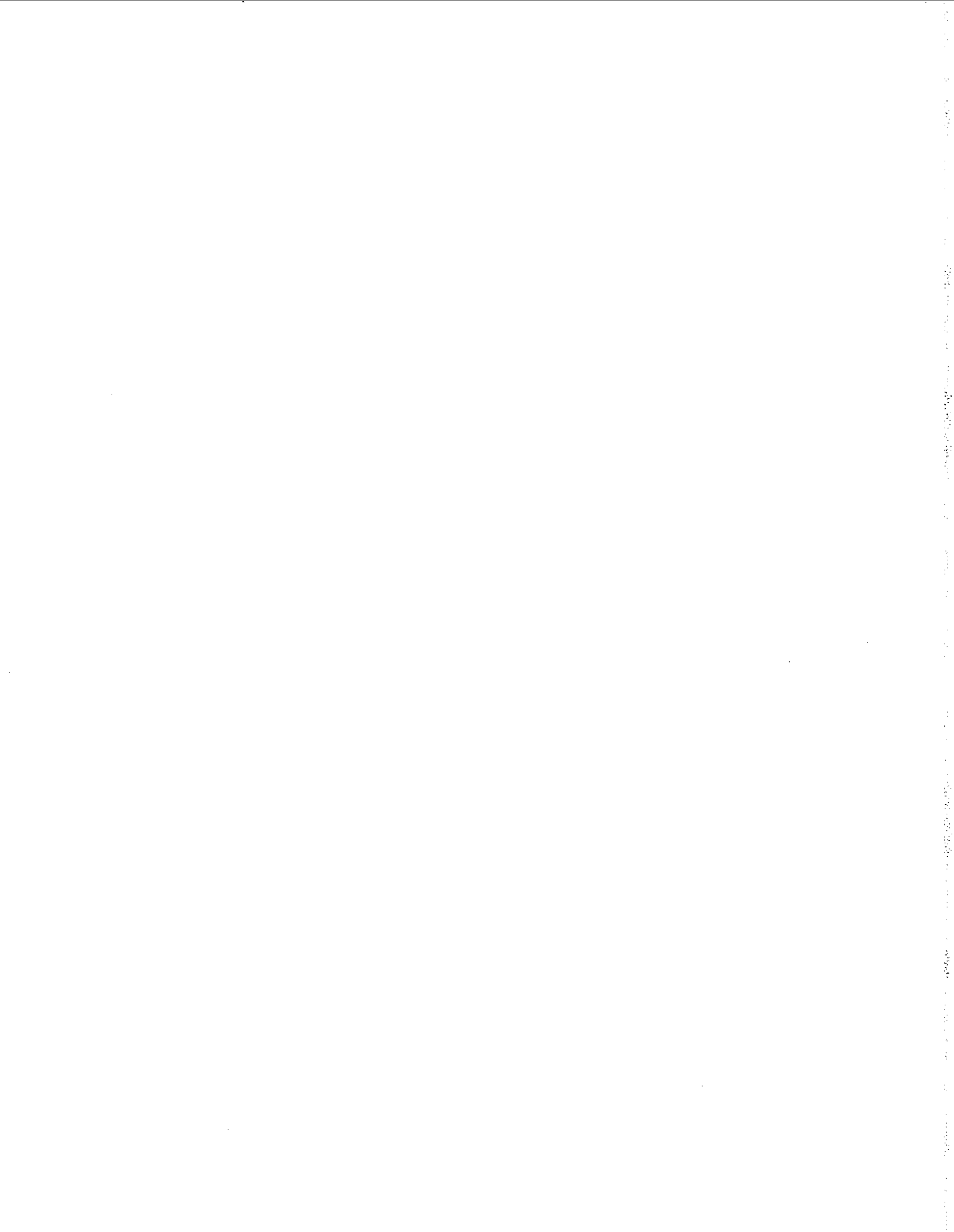
THE LIMITS OF CONVENTIONAL CHROMATOGRAPHY

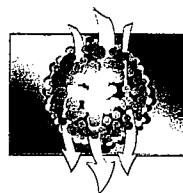
PERFUSION CHROMATOGRAPHY —
A NEW APPROACH

APPLICATIONS OF PERFUSION CHROMATOGRAPHY

- Improve and Simplify Method Development*
- Enhance Recovery of Biological Activity*
- Simplify or Eliminate Sample Preparation Steps*
- Reduce Column Size*
- Eliminate Analysis as a Bottleneck*
- Develop Novel Assay Techniques*

PERFUSION CHROMATOGRAPHY
IN A RANGE OF TECHNIQUES





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 fraught with frustration.

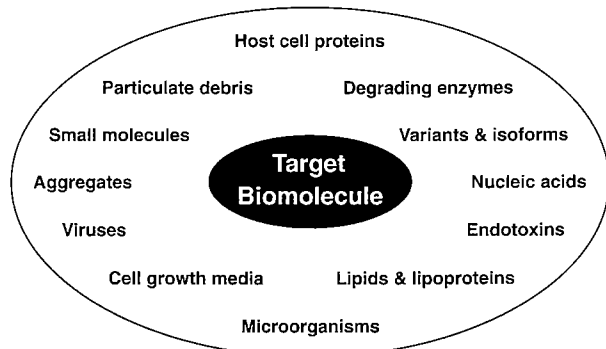


Figure 1-1. Biological systems present an unusually complex sample matrix and a difficult separations challenge.

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.

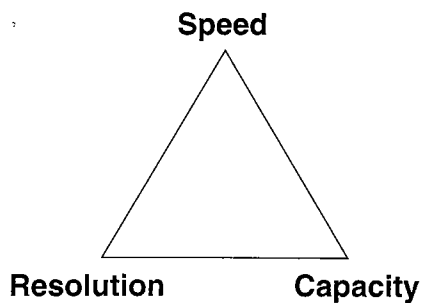


Figure 1-2. The tradeoff with conventional media between speed, resolution and capacity is depicted by many media manufacturers as a triangle.

INTRODUCTION



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. Bandspreading 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.

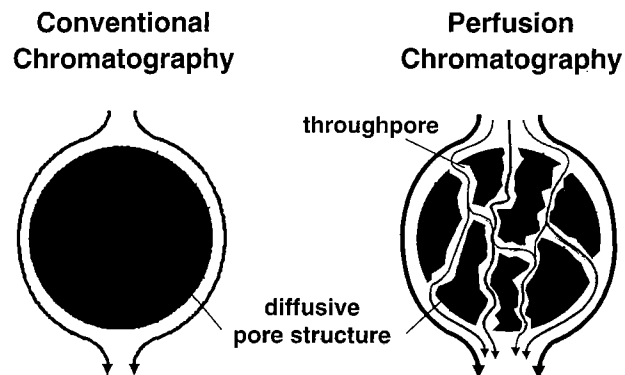


Figure 1-3. Pore structure and mass transport in conventional and perfusive particles

INTRODUCTION



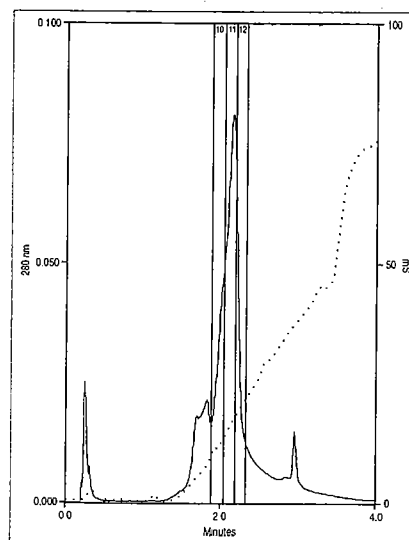
The combination of intra-particle flow and short diffusive pores effectively serves to access the entire surface area inside the particles much more rapidly than would be possible with conventional media, which rely solely on diffusion to achieve the same effect. Separations may therefore be carried 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 μ l)

Column: POROS HQ/M 4.6 mmD/100 mmL

Starting Buffer (A): 20 mM Tris/bis-tris propane, pH 8.0

Eluent (B): 20 mM Tris/bis-tris propane, pH 9.0 + 3M NaCl

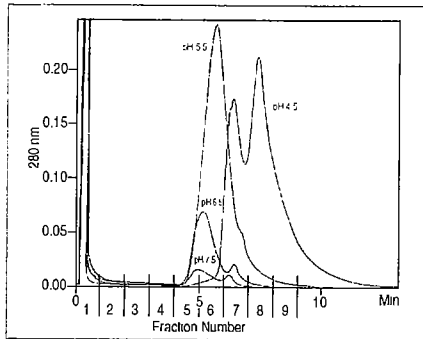
Flow Rate: 10 ml/min (3600 cm/hr)

System: BioCAD/*SPRINT* system

Detection: 280 nm

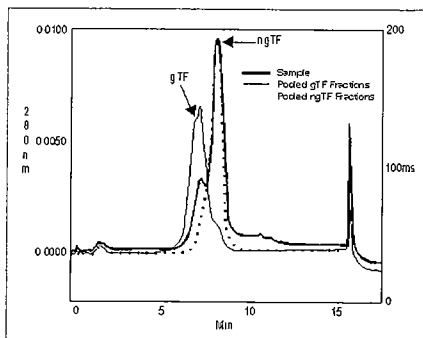
Elution: 0 - 25% B in 15 CV

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.



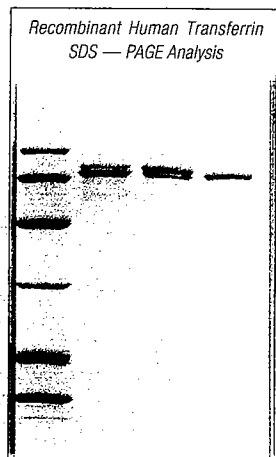
Sample: 50 μ l *E. coli* cell culture supernatant (2.5 mg total protein)
 Column: POROS HS/M 4.6 mmD/100 mL
 Starting Buffer (A): 33 mM HEPES/MES/acetate, pH's as shown
 Eluent (B): 33 mM HEPES/MES/acetate + 1.5 M NaCl
 Flow Rate: 5 ml/min (1800 cm/hr)
 System: BioCAD Workstation
 Detection: 280 nm
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

