Agilent InfinityLab

PRINCIPLES AND PRACTICAL ASPECTS OF PREPARATIVE LIQUID CHROMATOGRAPHY

A Primer

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Trusted Answers

Principles and practical aspects of preparative liquid chromatography

A primer

Content

1 Foreword

As a synthetic chemist, biologist or engineer, it's your job to study the impact of chemical compounds. To achieve this goal, you need to stay at the leading edge of your field of research. Consequently, you often find yourself with less time for tasks that are not necessarily your core competences but nevertheless important for your workflows. Isolation and purification of chemical compounds are typical of these tasks.

In situations where the compound of interest is not available in pure form, you are challenged to purify it yourself. Possible scenarios include synthesis of the compound in multiple stages, using purification as an interim step, or isolation of the compound from a natural source when synthesis is too complex and tedious. Other scenarios that require you to turn to purification techniques include, for example, when flash chromatography did not yield the desired purity, or when crystallization did not work the way you expected.

Isolation of pure compounds was, in fact, the original purpose of liquid chromatography and as such drove the development of separation science during the last century – with close linkages to the discovery of natural sources and new synthetic pathways. The increasing need for high-value compounds deployed as pharmaceuticals, agrochemicals or nutraceuticals, has in turn justified the extra effort required for optimization of purification processes.

Today, preparative chromatography is no longer based on guesswork but is founded solidly on a set of well-documented rules to be followed for optimum results. Scouting for appropriate starting conditions, optimizing for speed, yield, and purity are fundamental considerations. The desired sample throughput determines priorities: high yields for a few different samples justify optimization of yield, whereas dealing with large numbers of different samples at the milligram scale demands proper automation.

Now, it is all about getting started with preparative liquid chromatography without having to spend time delving deeply into the literature. Although a primer will never replace textbooks on preparative liquid chromatography to gain a full understanding of the theoretical background, this publication nevertheless bridges the gap between textbook literature and a typical system's user documentation that provides specific guidance on how to achieve optimum results.

Analytical liquid and gas chromatography are the techniques of choice for purity determination and indispensable tools for confirming the progress of purification processes. Preferably, you should have made yourself familiar with these techniques prior to reading this primer. This also includes the concepts of choosing the appropriate column chemistries as part of LC method development.

At this point, we would like to give a word of caution to those who have already gained a high level of expertise in analytical liquid chromatography. In preparative chromatography, there are additional rules and priorities as you try to optimize for speed, purity, and yield. Hence, we hope this primer proves to be worthwhile reading for everyone starting to care about efficient purification of compounds.

2 Introduction

In this primer, we give an introduction into the basic principles of preparative liquid chromatography, describe the components of a purification system, discuss strategies for collection of fractions, and offer some practical solutions for common purification tasks.

We begin by redefining the difference between analytical and preparative liquid chromatography – not classically in terms of column dimensions or flow rates – but from the modern-day perspective of solutions for specific applications.

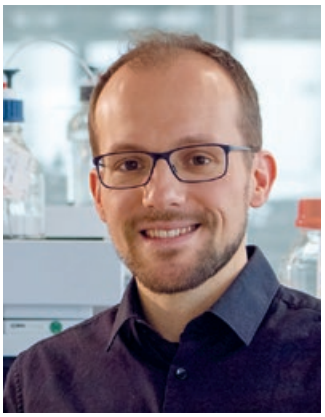
3 About the Authors

Dr. Helmut Schulenberg-Schell

Helmut Schulenberg-Schell has a master's degree in chemistry and a Ph.D. in biochemistry from Münster University, Germany. Isolation of naturally occurring cyclopentenyl fatty acids and bacterial hopanoids, and purification of bovine lipid binding proteins introduced him to preparative chromatography early in his career. Later, his interest focused on combining membrane separation techniques with biotechnology. Over the past 25 years he has worked in various positions in product and market development for Hewlett-Packard and Agilent Technologies to educate chemists, biologists, and engineers about new technologies and products. Helmut currently holds the position of director of business development for liquid phase separations at Agilent Technologies.

Dr. Andreas Tei

Andreas Tei attained his master's degree in organic chemistry and his Ph.D. in natural sciences from Heidelberg University in Germany. He started his industrial career with one of the market-leading scientific instrument manufacturers and held various positions in technical support, application support, sales and marketing for LC/MS and GC/MS systems. In his years as an application scientist he was involved with the automation of mass-based purification for medicinal chemistry laboratories. In 2011, Andreas joined Agilent as product manager for preparative chromatography systems. In this role he was responsible for the design of new modules and automation software. He went on to become a marketing segment manager for pharma small molecules and now manages the customer success portfolio at Agilent.



Dr. Florian Rieck

Florian Rieck holds a master's degree in food chemistry and a Ph.D. in analytical chemistry from Karlsruhe Institute of Technology (KIT) in Germany. Detecting and characterizing new potentially bioactive plant metabolites during his Ph.D. studies, he gained first experience with preparative liquid chromatography. Florian joined Agilent Technologies in 2015, where he started as an applications chemist for liquid chromatography in Waldbronn, Germany. Working initially on general LC applications projects, Florian soon focused on preparative liquid chromatography and mass-based purification applications.

Ronald Guilliet

Ronald Guilliet received his education in analytical chemistry from the University of Applied Sciences in Vlissingen, The Netherlands. He started his career with a scientific instrument and consumables manufacturer and held positions in production and marketing for HPLC columns. Later, his interest focused on large-scale (process) purification. He gained a global view on pharmaceutical large-scale workflows as business development manager and sales specialist with a strong focus on India and Asia where he successfully offered total solutions. Ronald joined Agilent in 2010 as product manager for preparative chromatography and supercritical fluid chromatography. At this stage of his career, he is currently responsible for the design of the Agilent InfinityLab LC Purification portfolio.

4 Symbols and Abbreviations

Symbols

A	absorption [AU]
c	concentration [mol/L]
d	path length [cm]
$\mathbf{d_A}$	diameter of analytical column [mm]
$\mathbf{d_P}$	diameter of preparative column [mm]
$\boldsymbol{\varepsilon_\lambda}$	molar extinction coefficient
$\mathbf{f_{a,A}}$	actual flow in analytical system [mL/min]
$\mathbf{f_{p,P}}$	proposed flow in preparative system [mL/min]
k	retention factor
$\mathbf{k_e}$	retention factor efficiency
$\mathbf{L_A}$	length of analytical column [mm]
$\mathbf{L_P}$	length of preparative column [mm]
N	number of theoretical plates
$\mathbf{p_A}$	column particle size in analytical system [µm]
$\mathbf{p_P}$	column particle size in preparative system [µm]
$\mathbf{t_R}$	retention time [s]
$\mathbf{t_{D,A}}$	dwell time of analytical system [s]
$\mathbf{t_{I,A}}$	initial hold of analytical system generic gradient [s]
$\mathbf{t_{c,A}}$	column pass time in analytical system [s]
$\mathbf{t_{D,P}}$	dwell time of preparative system [s]
$\mathbf{t_{I,P}}$	initial hold of preparative system gradient [s]
$\mathbf{t_{c,P}}$	column pass time in preparative system [s]
$\mathbf{v_{D,A}}$	dwell volume of analytical system [mL]
$\mathbf{v_{c,A}}$	column void volume of analytical system [mL]
$\mathbf{v_{D,P}}$	dwell volume of preparative system [mL]
$\mathbf{v_{inj,A}}$	injection volume for analytical system [µL]
$\mathbf{v_{inj,P}}$	injection volume for preparative system [µL]
$\mathbf{w_h}$	peak width at half-height (in time units) [s]

Abbreviations

DAC	dynamic axial compression
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EIC	extracted ion chromatogram
id	inside diameter
IPA	isopropyl alcohol
SAC	static axial compression
TIC	total ion chromatogram

Principles and Practical Aspects of Preparative Liquid Chromatography

5 Introducing Preparative Liquid Chromatography

In this chapter, we introduce preparative liquid chromatography (LC) by first making a clear distinction between preparative and analytical LC, and then discussing the diverse priorities that laboratories face when required to enrich or purify target compounds from mixtures.

5.1 Distinguishing between analytical and preparative liquid chromatography

Analytical liquid chromatography is a standard technique that needs to be embraced by any scientist or engineer interested in investigating mixtures of chemical compounds or biologically derived molecules. A thorough qualitative or quantitative analysis of such mixtures can be achieved through chromatographic separation and selective detection of the mixtures' components.

In contrast, the need to enrich or purify target compounds from mixtures for further investigation or for commercial purposes is the key motivation to adopt and deploy preparative LC. For centuries, multiple absorptive procedures have been developed to extract and enrich valuable substances. Towards the end of the 20th century, the demand for compounds of highest purity in the food and pharmaceutical industries increased the pressure to advance preparative LC methodologies.

If we were to write a single statement that describes the distinction between liquid chromatography for analytical and preparative purposes, it would read like this:

"In preparative LC, the separated compounds are collected in individual containers for further processing, whereas in analytical LC, the laboriously separated compounds are simply diverted to waste or destroyed by a destructive detection technique"

The classical approach of distinguishing between analytical and preparative chromatography in terms of column dimensions or flows rates is no longer appropriate.

Analyzing our generic description gives us an impression of just how common the use of preparative LC is. Completely independent of flow rates, preparative LC is deployed for collecting tiny protein fractions at flows of nanoliters or microliters per minute as well as at high flow rates in industrial-scale purification of proteins.

In this primer, we focus on preparative LC as a simple yet sophisticated technique to separate and extract one or more target compounds from a mixture. A sample of the mixture is driven batch-wise through a tube containing absorptive layers of stationary phase. This process separates the mixture into its constituent components. Subsequently, the target compounds are collected from the eluent stream.

5.2 Setting priorities for compound purification

When only limited amounts of raw material are available such as in the fractionation of complex natural product mixtures, preparative LC at lowest flow rates in the nanoliter or microliter range is deployed – possibly enabling novel discoveries in life sciences.

In contrast, high flow rates of multiple liters per minute are common in manufacturing processes for highly valuable compounds. Exact scale-up procedures and tightly controlled, manual collection of fractions by experienced process engineers with a sound understanding of chromatography yield several kilograms of pure product – with a potential market value of millions of dollars.

Synthetic chemists working in pharmaceutical drug discovery or agrochemical research laboratories are focused constantly on the compromise between sample throughput, yield, and purity. The injected amounts of crude sample are typically in the range of 100 to 500 mg. Key pharmaceutical laboratories are often purifying between 50 and 100 different samples on each system every day. High levels of system automation allow even nonexpert chromatographers to purify their precious samples in self-service purification labs.

To ensure that every chemist can purify samples quickly and securely, and to be able to continue with synthetic work, the systems must be highly robust. With large numbers of different samples, it is impossible to individually optimize the purification parameters unless the processes can be automated [1–4].

In process development, chemists and engineers are focused mainly on pilot-scale purification in the range of multiple grams to kilograms of intermediates, fine chemicals or biological compounds. When it is required to purify large quantities of the same compound repetitively, it is worthwhile to tune the process thoroughly. Consequently, experienced chromatographers carefully elaborate scale-up processes for each compound. Optimized gradients and often manually controlled fraction collection are common practice. The purified compounds are usually precious and although the number of purified samples per system per day is low, the value of the product could be huge. Hence, an efficient purification process is mandatory to sustain a profitable business model.

When separating complex samples such as metabolites in a biological matrix, the chromatographic resolution has the highest priority. For these challenges, column sizes of 4.6 × 150 mm with 3 to 5-micron particles or even sub-2-micron particles are required, together with chromatographic conditions close or even identical to those used for typical analytical separations. Slow gradients combined with low-carryover autosamplers and fraction collectors are used to guarantee highest purity and recovery of the separated compounds. Typically, the concentrations in the crude sample are low. Hence, the compounds must be enriched from large volumes of dilute sample (for example, urine) or recovered with maximum yield from small amounts of biological tissue.

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