LC GC
europe
solutions for separation scientists

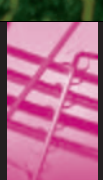
September 2018
Volume 31 Number 9
www.chromatographyonline.com

Profiling Plant Metabolites

A review of LC methods for analyzing phenolic compounds in plants and plant-derived food

UBM

LC TROUBLESHOOTING
Separating proteins using size-exclusion chromatography



GC CONNECTIONS
How to safely deliver gases to your system

QUESTIONS OF QUALITY
Data and *USP* <1058>

Feel good in your method development?

Feel better with Merck

Trust our analytical products in every stage of your drug product development. Whether you're working on bioanalytical LC-MS, formulation, or final quality control and release, we have the expertise you need.

Feel better with full workflow solutions at
SigmaAldrich.com/feel-better

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

Merck

GILSON®

CENTRIFUGAL PARTITION CHROMATOGRAPHY

Your cost-effective purification alternative

- Envrionmentally friendly, silica-free technique
- One-step purification process
- High recovery and purity

www.gilson.com/cpc

September | 2018

Volume 31 Number 9

Editorial Policy:
All articles submitted to *LC•GC Europe* are subject to a peer-review process in association with the magazine's Editorial Advisory Board.

Cover:
Original materials courtesy: Skyler Ewing/ Shutterstock.com

COVER STORY

480 **Fundamental and Practical Aspects of Liquid Chromatography and Capillary Electromigration Techniques for the Analysis of Phenolic Compounds in Plants and Plant-Derived Food, Part 1: Liquid Chromatography**
Danilo Corradini, Francesca Orsini, Laura De Gara, and Isabella Nicoletti
This paper is the first of a two-part review article discussing fundamental and practical aspects of both liquid chromatography (LC) and capillary electromigration techniques used for the analysis of phenolic compounds occurring in plant-derived food and in edible and medicinal plants. Part 1 focuses on LC.

Columns

492 **LC TROUBLESHOOTING**
Tips, Tricks, and Troubleshooting for Separations of Biomolecules, Part 2: Contemporary Separations of Proteins by Size-Exclusion Chromatography
Szabolcs Fekete, Davy Guillarme, and Dwight R. Stoll
Several new materials and columns have been introduced in recent years for size-exclusion separations of proteins. How do I know which one to choose, and which separation conditions will be the best for my protein separation?

500 **GC CONNECTIONS**
Safely Delivering the Best Possible Carrier and Detector Gases to Your GC System
John V. Hinshaw
The quality of a gas chromatogram depends heavily on the quality of the separation and detection gases, among many other factors. In this month's instalment, "GC Connections" discusses ways in which chromatographers can ensure a safe working environment while delivering gases that are up to the requirements of the separations at hand, in the context of moving a laboratory to a new location.

504 **QUESTIONS OF QUALITY**
Data Integrity and *USP* <1058>, Part 2: OQ Supervision and Execution
Paul Smith and R.D. McDowall
This is the second of three articles looking at the impact of the new *USP* <1058> on AIQ on data integrity in a regulated chromatography laboratory and focuses on how the laboratory should supervise the execution of OQ protocols by a third-party service provider.

512 **COLUMN WATCH**
LC–MS Sensitivity: Practical Strategies to Boost Your Signal and Lower Your Noise
Sharon Lupo
Several strategies are discussed to improve method sensitivity through the reduction of contaminants, the careful selection of LC method conditions, and the optimization of MS interface settings.

Departments

522 **Products**
524 **Events**
525 **The Applications Book**

SHIMADZU
Excellence in Science

50th ANNIVERSARY Shimadzu Europa

imake the difference

The i-volution in HPLC analysis continues

The popular i-Series of compact (U)HPLC systems has now evolved to the Plus family of Prominence-i and Nexera-i systems. The new line of products combines high-speed analysis with simplified method transfer, automated sample pre-treatment, minimized environmental impact and easy maintenance. It targets pharmaceutical, chemical and food industries.

Significantly improved analytical productivity
through automated pre-treatment functionality resulting in increased efficiency and reduced risk of human error

Wide range of application fields
such as R&D activities, specification tests and quality control

Flexible software control
by Shimadzu LabSolutions LC/GC, LC-MS or DB/CS for full FDA 21 CFR Part 11 compliance. Software packages from other vendors are also supported

Plus family Prominence and Nexera-i

www.shimadzu.eu/i-series

LC GC europe
solutions for separation scientists

Published by
UBM Americas

Vice President/ Group Publisher
Mike Tessalone
michael.tessalone@ubm.com

Editorial Director
Laura Bush
laura.bush@ubm.com

Editor-in-Chief
Alasdair Matheson
alasdair.matheson@ubm.com

Managing Editor
Kate Mosford
kate.mosford@ubm.com

Associate Editor
Lewis Botcherby
lewis.botcherby@ubm.com

Associate Publisher
Oliver Waters
oliver.waters@ubm.com

Sales Executive
Liz Mclean
liz.mclean@ubm.com

Senior Director, Digital Media
Michael Kushner
michael.kushner@ubm.com

Webcast Operations Manager
Kristen Moore
kristen.moore@ubm.com

Project Manager
Vania Oliveira
vania.oliveira@ubm.com

Subscribe on-line at www.chromatographyonline.com

Digital Production Manager
Sabina Advani
sabina.advani@ubm.com

Managing Editor Special Projects
Kaylynn Chiarello-Ebner
kaylynn.chiarello.ebner@ubm.com

Art Director
Dan Ward
dward@hcl.com

Subscriber Customer Service
Visit (chromatographyonline.com) to request or change a subscription or call our customer service department on +001 218 740 6877

Hinderton Point,
Lloyd Drive,
Cheshire Oaks,
Cheshire,
CH65 9HQ, UK
Tel. +44 (0)151 353 3500
Fax +44 (0)151 353 3601

SUBSCRIPTIONS: LC•GC Europe is free to qualified readers in Europe.
To apply for a free subscription, or to change your name or address, go to www.chromatographyonline.com, click on Subscribe, and follow the prompts.
To cancel your subscription or to order back issues, please email your request to fulfill@hcl.com, putting LCE in the subject line.
Please quote your subscription number if you have it.
MANUSCRIPTS: For manuscript preparation guidelines, visit www.chromatographyonline.com or call the Editor, +44 (0)151 353 3500. All submissions will be handled with reasonable care, but the publisher assumes no responsibility for safety of artwork, photographs or manuscripts. Every precaution is taken to ensure accuracy, but the publisher cannot accept responsibility for the accuracy of information supplied herein or for any opinion expressed.
DIRECT MAIL LIST: Telephone: +44 (0)151 353 3500.
Reprints: Reprints of all articles in this issue and past issues of this publication are available (250 minimum). Licensing and Reuse of Content: Contact our official partner, Wright's Media, about available usages, license fees, and award seal artwork at Advanstar@wrightsmedia.com for more information. Please note that Wright's Media is the only authorized company that we've partnered with for Advanstar UBM materials.

LC GC europe
solutions for separation scientists

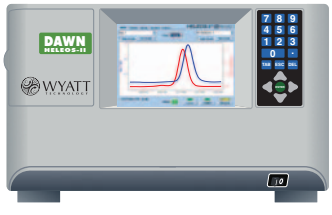
Editorial Advisory Board

Follow us @ LC_GC *'Like' our page LCGC* *Join the LCGC LinkedIn group*

The Publishers of LC•GC Europe would like to thank the members of the Editorial Advisory Board for their continuing support and expert advice. The high standards and editorial quality associated with LC•GC Europe are maintained largely through the tireless efforts of these individuals.

LCGC Europe provides troubleshooting information and application solutions on all aspects of separation science so that laboratory-based analytical chemists can enhance their practical knowledge to gain competitive advantage. Our scientific quality and commercial objectivity provide readers with the tools necessary to deal with real-world analysis issues, thereby increasing their efficiency, productivity and value to their employer.

If your standards and your samples are the same size, they must be the same mass, right?

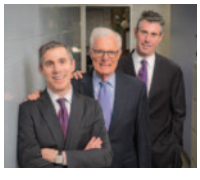


With DAWN you can determine absolute molecular weights and sizes without using reference standards made in someone else's lab.

That's the problem with calibration-based measuring techniques. You don't know—you can only assume. Which is why every major pharmaceutical and biotechnology company, as well as most federal regulatory agencies are switching from relative methods to Wyatt Technology's absolute measurements. Our DAWN® multi-angle light scattering (MALS) instruments allow you to determine absolute molecular weights and sizes without relying on so-called standards, or measurements made in someone else's lab. Wyatt instruments measure all of the quantities required for determining absolute molar masses directly.

Learn how to end you dependence on reference standards forever at wyatt.com/NoRef

WYATT TECHNOLOGY
wyatt.com



Fundamental and Practical Aspects of Liquid Chromatography and Capillary Electromigration Techniques for the Analysis of Phenolic Compounds in Plants and Plant-Derived Food, Part 1: Liquid Chromatography

Danilo Corradini[1], Francesca Orsini[1,2], Laura De Gara[2], and Isabella Nicoletti[1], [1]CNR, Institute of Chemical Methodologies, Area della Ricerca di Roma 1, Monterotondo Stazione (Rome), Italy, [2]Unit of Food Science and Nutrition, University Campus Bio-Medico of Rome, Rome, Italy

Column-based liquid phase separation techniques, such as liquid chromatography (LC) in reversed phase separation mode and capillary electromigration techniques, using continuous electrolyte systems, are widely used for the identification and quantification of phenolic compounds in plants and food matrices of plant origin. This paper is the first of a two-part review article discussing fundamental and practical aspects of both LC and capillary electromigration techniques used for the analysis of phenolic compounds occurring in plant-derived food and in edible and medicinal plants. The chemical structure and distribution of the major phenolic compounds occurring in the plant kingdom, as well as the main methods used for their extraction and sample preparation, are also discussed. Part 1 will focus on liquid chromatography.

Plant secondary metabolites, also known as specialized metabolites, are organic compounds produced along with the primary biosynthetic and metabolic routes that are believed to be mainly produced in response to the interactions of the plant with the environment.

These compounds protect the plant against biotic and abiotic stresses, including pathogens, predators, ultraviolet light, and drought (1). In addition, secondary metabolites may confer specific sensory characteristics to food products and play important roles in disease prevention and health-promoting effects of edible plants and plant-derived food products (2–5). Based on their biosynthetic origins, plant secondary metabolites are usually classified into three major classes: terpenoids, alkaloids, and phenolic compounds, the last of which are one of the most important and widespread class of secondary metabolites in the plant kingdom (6).

Phenolic compounds form an integral part of the human diet, contributing to the sensorial properties of food products and to the beneficial effects of the Mediterranean diet on human health, mainly as a result of their antioxidant properties (7). These compounds, as well as many other plant secondary metabolites, are also important as bioactive components in medicinal plants and have numerous biological activities and a variety of health benefits for chronic and degenerative human diseases (8).

KEY POINTS

- This article discusses the main separation modes of LC used to identify and quantify phenolic compounds in plants and food matrices of plant origin.
- Part 1 of the article also briefly describes the chemical structure and distribution of the major phenolic compounds occurring in the plant kingdom and the main methods used for their extraction and sample preparation.
- Reversed phase chromatography, either with HPLC or UHPLC columns, is the technique of choice for the analysis of phenolic compounds.
- HILIC is both a valuable alternative to reversed phase chromatography and the main separation modes used in multidimensional chromatography of phenolic compounds.

Photo Credit: Skyler Ewing/Shutterstock.com

Chromatography

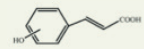
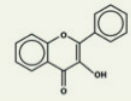
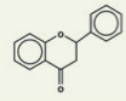
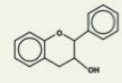
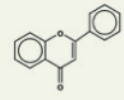
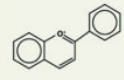
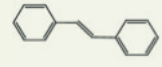
MACHEREY-NAGEL

Manufacturer of premium chromatography media

Find what you are looking for!

- CHROMABOND® columns with classical and innovative SPE phases
- Original NUCLEOSIL®, professional NUCLEODUR® and highly efficient NUCLEOSHELL® HPLC columns
- Robust TLC glass plates, economical aluminum and polyester sheets

Table 1: Main classes of plant phenolic compounds

Phenolic acids (C_6-C_1) (C_6-C_3)	Hydroxybenzoic acid derivatives	
	Hydroxycinnamic acid derivatives	
Flavonoids (C_6-C_3-C_6)	Flavonols	
	Flavanones	
	Flavanols (or flavan-3-ol)	
	Flavones	
	Anthocyanidins	
Stilbenes (C_6-C_2-C_6)	*trans*-Resveratrol derivatives	

Important steps for the assay of phenolic compounds in plants and plant-derived food are sample preparation and extraction, followed by identification and quantification using various instrumental analytical methods, most of which use column-based high performance liquid phase separation techniques coupled to a suitable detection method. Traditionally, total phenolic compounds are determined using spectrophotometric methods based on the Folin-Ciocalteau reaction (9), which generate a coloured product as the result of the oxidative titration of phenolate anions by phosphotungstate and phosphomolybdate. More sophisticated methods, based on instrumental analytical techniques, are needed to identify and quantify each of the main phenolic compounds present in a given sample. Among them, liquid chromatography (LC), generally in the reversed phase separation mode, is the technique of choice, while capillary electrophoresis (CE) is gaining increasing acceptance because of its high separation efficiency, short analysis time, and extremely small sample and reagent volume requirements.

This is the first of a two-part review article aimed at discussing fundamental and practical aspects of both LC and capillary electromigration techniques used for the analysis of phenolic compounds occurring in edible and medicinal plants and in plant-derived food and dietary supplements. Part 1 will focus on liquid chromatography of these compounds and the chemical structure and distribution of the major phenolic compounds, as well as the main methods used for sample preparation and extraction.

Major Phenolic Compounds

Phenolic compounds, also referred to as phenolics, comprise a large number of heterogeneous structures that range from simple molecules to highly polymerized compounds, which are commonly bound to other molecules, frequently to sugars, although phenolics in free form also occur. Among glycosylated phenolic compounds, both *C*- and *O*-glycosylations are found. A common structural characteristic of phenolics is the presence of at least one aromatic ring hydroxyl-substituted. They are commonly divided into different subclasses according to the number of aromatic rings, the structural elements that bind these rings to each other, and the substituents linked to the rings (see Table 1).

The simplest form of phenolics are the phenolic acids, which can be divided into benzoic acid and cinnamic acid derivatives, with basic carbon skeletons C_6-C_1 and C_6-C_3, respectively. Other main phenolic compounds comprise flavonoids, with carbon skeletons C_6-C_3-C_6, and a variety of nonflavonoid phenolic compounds with basic skeletons C_6-C_2-C_6 (stilbenes, anthraquinones), $(C_6$-C_3-$C_6)_n$ (condensed tannins), $(C_6$-$C_3)_2$ (lignans), $(C_6$-$C_3)_n$ (lignins), C_6-C_4 (naphtochinones), and C_6-C_1-C_6 (xanthones), just to mention the main subclasses.

Flavonoids are the largest group of phenolic compounds and are distributed in several subclasses of structurally diverse composition. Their classification is determined by the arrangements of the three-carbon atoms group occurring in the C_6-C_3-C_6 structure. Several studies have reported that flavonoids possess a variety of beneficial effects on human health, including the properties of acting as chemopreventive agents interfering with several cancer mechanisms (10).

One of the most abundant and widely distributed flavonoid subclasses are flavonols, which comprise quercetin, myricetin, kaempferol, and fisentin, and are found in wine, onion, apples, and a variety of leafy vegetables. Flavanones, commonly found in plants and plant-derived foods and beverages, include naringenin, naringin, nariruri, hesperidin, and hesperitin. A high intake of flavanones in the diet has been associated with a reduced risk of degenerative and cardiovascular diseases (11).

Flavanols (or flavan-3ol) include catechin and epicatechin, which can be hydroxylated to form gallocatechins, and are the building blocks of oligomeric and polymeric proanthocyanidins, which are called procyanidins when they consist exclusively of epicatechin units. Flavanols are found in wine, broccoli, and other food products, such as cocoa, tea, beans, and a variety of fruits, such as apples, pomegranate, blackberries, and red grapes.

Flavones are widely found in many medicinal plants, spices, fruits, and leafy vegetables. Common flavones include apigenin, luteolin, and their glycosylated forms, apigenin-O-glucuronide, apigenin-O-glucoside, and luteolin-C-glucoside, which have proven to have potential antioxidant and anti-inflammatory activity (12).

Anthocyanins are glycosylated derivatives of a flavylium cation carrying a positive charge on the heterocyclic oxygen, which, owing to its conjugated double bonds, absorbs visible light and is therefore responsible for the intensive red-orange to blue-violet colour of many fruits and flowers. The aglycon of anthocyanins, also known as anthocyanidins, occurring more frequently in nature and of dietary importance are cyanidin, delphinidin,

Introducing UCT's All-New Lipifiltr® Push-Thru Cartridge

LipiFiltr®

MAXIMIZE FAT AND LIPID REMOVAL FROM BOTH PLANT AND ANIMAL-BASED MATRICES

UCT's All-new Lipifiltr® traps unwanted lipids and fats using a streamlined push-thru cartridge format, leaving you with a purified extract that is suitable for LC-MS/MS analysis. A simple, one step procedure following a QuEChERS extraction can easily be incorporated into your current sample preparation work flow. No need for additional finishing steps!

Ideal for nuts, oils, fish, poultry and livestock samples, Lipifiltr® will not compromise the recoveries of your analytes of interest and will provide clean sample profiles.

LIPIFILTR® CARTRIDGES EASILY FIT ON THE END OF ALL STANDARD DISPOSAL SYRINGES

Sample Type	Fat Removal %
	Lipifiltr®
Beef	79.1
Swordfish	80.9
Olives	84.3
Nuts	84.3

The performance of the Lipifiltr® push-thru cartridge to remove fatty matrix was evaluated gravimetrically. The gravimetric analysis was done by collecting 2mL of sample before and after clean-up in pre-weighed test tubes and heating them to dryness at 110°C.

LIPIFILTR®

petunidin, peonidin, pelargonidin, and malvidin (13). Other subclasses of flavonoids include chalcones, aurones, dihydrochalcones, isoflavonoids, neoflavanoids, and biflavonoids (7,10).

Sample Preparation and Extraction

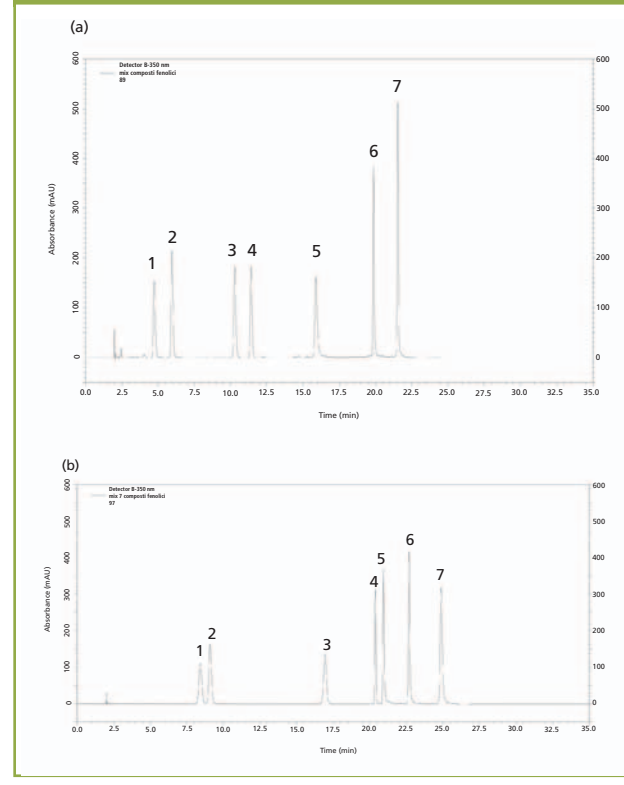
Edible plants and a variety of processed food and beverages of plant origin are major sources of bioactive secondary metabolites in the human diet. Very often, such matrices need to be processed by selected sample preparation methods before the extraction of the targeted secondary metabolites and, in some cases, also after their extraction (sample clean up and sample enrichment). The preparation of the samples and the extraction of the compounds of interest are critical steps in obtaining accurate analytical data and reliable interpretation of their values. The proper selection of the above processes depends on the nature of the sample matrix and the chemical properties of the targeted secondary metabolites, including their molecular structure and polarity. Other factors to be considered are the chemical stability of the compounds of interest during sample preparation, storage, and extraction, in addition to their concentration in the matrix, which is frequently washed, milled, dried, and homogenized before sample preparation and extraction.

The extraction of phenolics from the huge number of plant species and food matrices is performed by a variety of techniques, involving the use of solvents, steam, or supercritical fluids (14). These techniques include the conventional solid-liquid extraction (SLE) methods, such as maceration, infusion, percolation, hydrodistillation, decoction, and boiling under reflux (Soxhlet extraction). Most of these techniques, which are based on the application of heat or mixing, are cumbersome, time-consuming, and require the use of relatively large volumes of expensive hazardous organic solvents. In addition, their extraction yield is very often limited.

An alternative extraction technique, particularly suitable for thermolabile compounds, is supercritical fluid extraction (SFE) (15), which can be operated at room temperature and uses as the extracting media a supercritical fluid such as carbon dioxide. This compound, as well as all supercritical fluids, possesses liquid-like density and extraction power, as well as gas-like properties of viscosity, diffusion, and surface tension that facilitate its penetration to the sample matrix, with the result of improved extraction efficiency. However, carbon dioxide is nonpolar and therefore, because most phenolic compounds are polar, is generally used in combination with a polar cosolvent, such as ethanol, ethyl acetate, or acetone. The extraction rate can be further enhanced by using ultrasound during SFE (ultrasound-assisted SFE).

Other recent and efficient extraction techniques include ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE),

Figure 1: Reversed-phase HPLC separation of standard phenolic compounds. Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a linear gradient of increasing concentration of either (a) acetonitrile or (b) methanol in water containing 0.1% (*v/v*) formic acid; flow rate: 0.2 mL/min. UV detection at 280 nm. Standard phenolic compounds: 1. chlorogenic acid, 2. caffeic acid, 3. ferulic acid, 4. rutin, 5. myricetin, 6. quercetin, 7. kaempferol (personal data).



pulsed-electric field extraction (PEF), and pressurized liquid extraction (PLE). A few of these modern extraction techniques have been further advanced, such as MAE, with the recent development of high-pressure MAE (HPMAE), nitrogen-protected MAE (NPMAE), vacuum MAE (VMAE) ultrasonic MAE (UMAE), solvent-free MAE (SFMAE), and dynamic MAE (DMAE) (16).

The removal of potential interferents (sample clean-up), and the enrichment of the target analytes in the extracted samples are traditionally performed by liquid–liquid extraction (LLE) and solid-phase extraction (SPE). With the advent of miniaturization, these methods have been evolved in a variety of microscale extraction techniques, referred to as liquid–liquid microextraction (LLME) and solid-phase microextraction (SPME). High-molecular-weight polymeric phenolic compounds or individual low-molecular-weight phenolics associated to macromolecules, the so-called nonextractable phenolics (NEPs), are usually extracted after acid, alkaline, or enzymatic hydrolysis, which is performed to release NEPs from the matrix. Other advanced clean-up methods use liquid membrane extraction (LME), pipette-tip SPE (PT-SPE), molecular imprinted SPME (MI-SPME), and microfluid extraction systems (17).

HPLC and UHPLC

Conventional high performance liquid chromatography in reversed phase separation mode is the technique of choice for the analysis of phenolic compounds, which is generally performed using analytical size columns with an internal diameter (i.d.) in the range of 4.0–4.6 mm. Narrow-bore columns, with an i.d. of 2.0 mm or 2.1 mm, have recently gained increasing acceptance as a result of their positive impact on the environment and the analysis costs, which is a result of the reduced consumption of hazardous and expensive organic solvents. Additional advantages of using narrow-bore columns include the flow rate compatibility with mass spectrometry (MS) detection, in addition to the expected higher sensitivity of UV–vis absorbance detection, owing to the minor dilution of samples during separation, in comparison to using a conventional analytical size column.

Columns packed with microparticulate (2.5–5.0 µm) spherical porous octadecyl (C18) bonded silica are very popular, but other bonded stationary phases are also used including octyl (C8), phenyl-hexyl, pentafluorophenyl, and diphenyl bonded silica. Efficient and rapid separations are also obtained using superficially porous particles, also known as fused-core or core–shell particles, consisting of an impenetrable inner core surrounded by a layer of fully porous silica, which provide higher efficiency and more homogeneous packing density for the same particle diameter than conventional fully porous silica particles.

As well as packed silica-based columns, polymeric microparticulate packing materials and monolithic columns (either polymeric or silica-based) are also used in reversed-phase HPLC of phenolic compounds. Monolithic columns consist of a continuous rod of the chromatographic

Figure 2: Reversed-phase HPLC separation and identification of phenolic compounds extracted from grape berries, variety "Uva di Troia". Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a multisegment gradient of increasing concentration of acetonitrile in water containing 0.5% (*v/v*) formic acid; flow rate: 0.2 mL/min; photodiode array detection at 280 nm, 320 nm, 370 nm, and 520 nm. Peak identity (confirmed by ESI-MS detection): 1. gallic acid, 2. protocatechuic acid, 3. caftaric acid, 4. p-hydroxybenzoic acid, 5. catechin, 6. m-salicylic acid, 7. caffeic acid, 8. epicatechin, 9. delphinidin 3-O-glucoside, 10. p-coumaric acid, 11. cyaniding 3-O-glucoside, 12. pelargonidin 3-O-glucoside, 13. sinapic acid, 14. peonidin 3-O-glucoside, 15. *trans*-piceid, 16. malvidin 3-O-glucoside, 17. naringenine-7-glucoside, 18. rutin, 19. quercetin 3-O-glucoside, 20. myricetin, 21. kaempferol 3-O-glucoside, 22. *trans*-resveratrol, 23. quercetin, 24. naringenin, 25. kaempferol (personal data).

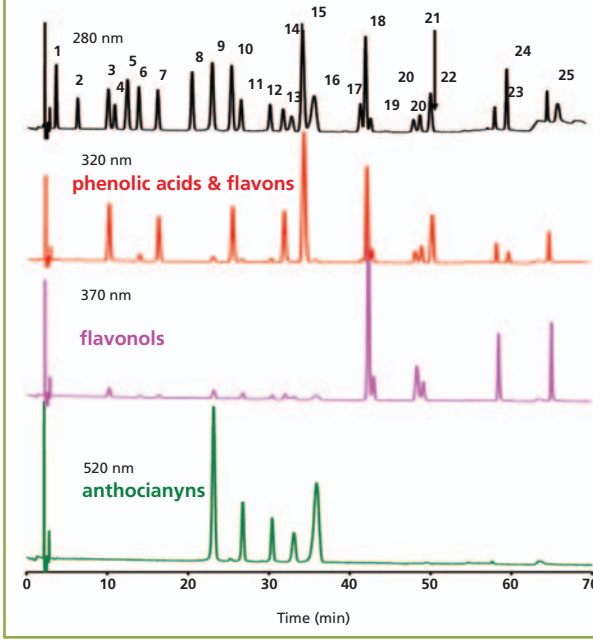
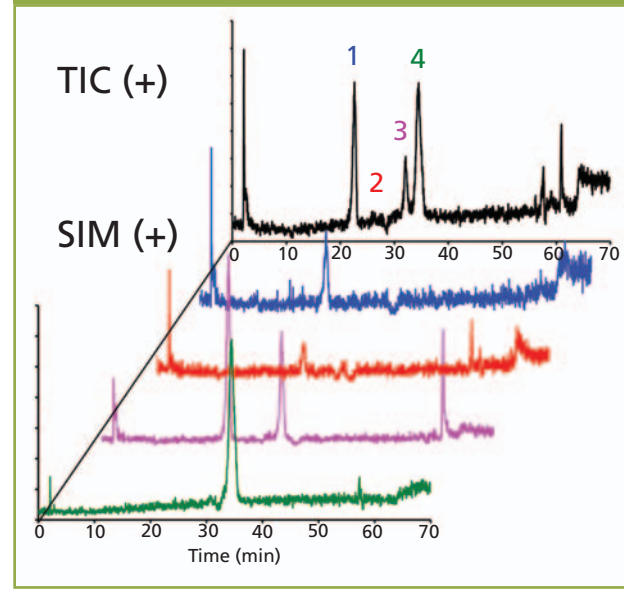


Figure 3: Separation and identification of antocyanins extracted from grape berries, variety "Merlot". Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a multisegment gradient of increasing concentration of acetonitrile in water containing 0.5% (v/v) formic acid; flow rate: 0.2 mL/min; ESI-MS detection in positive ionization mode. Peak identity: 1. delphinidin-3-glucoside (*m/z* 466.4), 2. cyanidin-3-glucoside (*m/z* 450.4), 3. peonidin-3-glucoside (*m/z* 464.4), 4. malvidin-3-glucoside (*m/z* 494.4) (personal data).



support with bimodal porosity. They are synthesized using either organic or inorganic precursors and exhibit enhanced mass transfer characteristics in comparison to conventional columns (18). Both core–shell packed and monolithic columns can be operated at higher mobile phase flow rates, with lower back pressures, than conventional columns, while providing high efficiency and resolving power for a variety of analytes, including phenolic compounds of natural origin (19–20).

A more advanced form of HPLC, namely ultrahigh-pressure liquid chromatography (UHPLC), uses narrow-bore columns (1.0–2.1 mm [i.d.]), packed with sub-2-µm particles, which are eluted at high flow rates and require the use of a chromatographic system that withstands pressures up to 600 and even 1000 bar (60–100 MPa). According to theory (van Deemter equation), the use of columns packed with sub-2-µm particles implies a significant gain in efficiency, even at high values of the mobile phase linear velocity, which is proportional to the mobile phase flow rate. Because of this higher efficiency, the chromatographic peaks are narrower and the maximum number of resolvable peaks (peak capacity) is larger and the detection limits are lower, which means that both resolving power and sensitivity are expected to be higher in UHPLC than in conventional HPLC.

A further advantage of performing the chromatographic separation at high flow rates is the significant decrease in the analysis time, while the more evident disadvantage is the high column back pressure that can easily reach the upper pressure limits of conventional HPLC systems. The column back pressure can be lowered by running the chromatographic separation at higher than ambient temperature, with the advantage of the possible use of a conventional HPLC instrument. However, to take full advantage of UHPLC, the separation should be perfomed using dedicated instrumentation with extended pressure capability, a sampling valve with a fast injection cycle and low injection volume, tubing ensuring minimum extra-column volume, and a detector with fast time constant and acquisition rate.

Most of the reversed-phase columns used in either HPLC or UHPLC analysis of phenolic compounds are operated under gradient elution mode with the starting eluent and the gradient former consisting of a water-rich and an organic solvent-rich solution, respectively. A suitable acid is generally incorporated into the starting eluent and, less frequently, into the gradient former solution to control the protonic equilibrium at acidic pH values. Acidic conditions are requested to improve the hydrophobic interactions of the phenolic compounds with the stationary phase by ensuring that both carboxyl and hydroxyl groups of the analytes are

in their protonated form. Acetonitrile and methanol are the organic solvents generally used as the gradient former. The two solvents exhibit different elution strength and separation selectivity (Figure 1). However, whenever possible, acetonitrile is preferred to methanol because of its lower UV cut-off and viscosity.

The primary detection method used in LC of phenolic compounds is based on the absorbance of UV or, as in the case of anthocyanins, visible light. These detectors comprise fixed-wavelength, variable-wavelength, scanning, and photodiode array (PDA) detectors. Low-pressure discharge lamps are used as the source of intensive line UV radiations, such as mercury (254 nm) or cadmium (229, 326 nm), whereas deuterium lamps, covering the range 190–700 nm, are used in variable-wavelength and photodiode array detectors, where a tungsten-halogen lamp may also be used to improve the performance in the visible region.

Each class of phenolic compounds has unique spectral characteristics. Therefore, general information on the different classes of phenolic compounds occurring in a complex sample mixture can be obtained by performing UV–vis detection at the wavelength corresponding to the absorption maxima of the phenolic compounds expected to occur in the sample. An example of this approach is depicted in Figure 2, which displays the reversed-phase HPLC separation of phenolic compounds extracted from grape berries and detected by PDA at wavelength values corresponding to the absorption maxima of flavonols (370 nm), anthocyanins (520 nm), and phenolic acids and flavons (320 nm), respectively.

Fluorescence detection is used to detect the limited number of phenolic compounds that naturally fluoresce or that are chemically modified to produce molecules containing a fluorescent tag, usually using on-line post-column derivatization methods. On the other hand, indirect detection, performed by incorporating a fluorescent probe into the mobile phase, as well as chemiluminescence detection with post-column addition of suitable reagents has found limited application.

The hyphenation of either HPLC or UHPLC with MS or high resolution mass spectrometry (HRMS), mainly using time-of-flight (TOF) or orbital trap mass analyzers, allows the elucidation of the chemical structure of the investigated phenolic compounds. Prominent among the different ionization sources used in LC–MS is electrospray ionization (ESI) in negative ionization mode, although ESI in positive ionization mode is also used. For example, anthocyanins are glycosylated derivatives of a flavylium cation that are detected by ESI-MS in positive ionization mode (21) (Figure 3). Other, less common, atmospheric pressure ionization (API) interfaces used in LC–MS of phenolics include atmospheric-pressure chemical ionization (APCI) (22) and, to a minor extent, atmospheric-pressure photochemical ionization (APPI) (23).

The hyphenation of liquid chromatography with nuclear magnetic resonance spectroscopy (NMR), which is widely used in analytical chemistry for the unambiguous

identification of known and novel organic compounds, is also promising. Direct on-line hyphenation can be realized using flow NMR probes, either of the double saddle Helmholtz coil design or of solenoidal microcoil design, also in combination with methods designed to improve the otherwise low-detection sensitivity of NMR, which is negatively affected by the background absorption of the liquid phase used in the separation step (24). Noticeable among these methods is the hyphenation of LC with NMR using SPE. According to this approach, each phenolic compound eluting the chromatographic column as a separated peak is trapped on an SPE cartridge, which is subsequently dried with nitrogen and then eluted with a deuterated solvent into proper tubes for off-line NMR analysis. An application of this approach has been reported by Goulas *et al.* for the identification of the methoxylated flavones hispidulin, salvigenin, and cirsimaritin in extracts of *Salvia fruticosa*, exhibiting antifungal activity (25).

Hydrophilic interaction chromatography (HILIC) is receiving increasing attention, either as a valuable alternative to reversed-phase chromatography or as one of the separation modes used in multidimensional chromatography. The chromatographic retention in HILIC is governed by the hydrophilic partitioning of the analyte between an organic-rich mobile phase and a water layer formed at the surface of a polar stationary phase, with the possible contribution of hydrogen bonding, dipole-dipole interactions, and ion-exchange mechanism. Therefore, HILIC can be considered orthogonal to reversed phase chromatography, whose retention mechanism is based on hydrophobic interactions. On the other hand, similar to reversed-phase chromatography, HILIC uses hydro-organic mobile phases that are fully compatible with MS detection. Further advantages of HILIC include its suitability for the analysis of polar phenolic compounds that are not sufficiently retained in reversed phase chromatography, and the availability of an alternative separation mechanism, which implies that compounds not easily separated by reversed phase chromatography may be resolved in HILIC. Examples of the applicability of HILIC to the analysis of phenolics include the resolution of individual oligomeric and polymeric procyanidins in apples and apple extracts (26), and the separation and identification of anthocyanins in blueberries, red grape skins, black beans, red cabbage, and red radish (27).

Comprehensive Two-Dimensional Liquid Chromatography

HILIC is very promising for the development of two-dimensional liquid chromatographic methods for the analysis of phenolic compounds in food and beverages (28). This technique is performed by passing the sample through two columns, each containing a different stationary phase that separate the analytes either according to a diverse separation mechanism or with different selectivity. The technique is performed according to various approaches, which comprise “heart-cutting” and “comprehensive” two-dimensional LC, also referred to as

LC-LC and LC×LC, respectively. In LC-LC systems, only the fractions of the effluent from the first column containing the analytes of interest are further separated in the second column, whereas in comprehensive two-dimensional LC the entire effluent from the first column is transferred to the second one.

Comprehensive two-dimensional LC systems, created by coupling HILIC and reversed-phase chromatography, combine the different selectivity and resolving power of the two independent separation mechanisms operating in the first (HILIC) and second (reversed-phase chromatography) columns, which are based on hydrophilicity and hydrophobicity, respectively. HILIC×reversed-phase chromatography techniques are generally operated according to the on-line approach, using a multi-port switching valve with one or two sampling loops, whereas the off-line mode is less popular. On-line, off-line, and stop-flow modalities of HILIC×reversed-phase chromatography have been deeply investigated and successfully applied for the analysis of cocoa procyanidins (29) and for the separation and identification of anthocyanins and their derived pigments in aged red wine (30). Recently, 265 compounds, comprising 196 potentially new phenolic acids, were separated and tentatively characterized in *Salvia miltiorrhiza* by an off-line two-dimensional HILIC×reversed phase chromatography system, hyphenated to an ion trap time-of-flight mass spectrometer (31). However, because mobile phases with a high content of acetonitrile, such as those used in HILIC, are strong eluents in reversed phase chromatography, system compatibility problems may arise in comprehensive two-dimensional HILIC×reversed-phase chromatography systems.

A less problematic approach of comprehensive two-dimensional liquid chromatography to the analyses of phenolic compounds is based on the use of two reversed-phase columns of different selectivity. According to this method, phenolic acids and flavonoid antioxidants have been successfully separated in beer and wine using combinations of single or serially coupled polyethylene glycol (PEG)-, phenyl-, and C18- reversed-phase columns in the first dimension and single or two alternating C18 or Zr-carbon columns in the second dimension (32–33). The use of a microbore phenyl column in the first dimension coupled to a monolithic or superficially porous C18 column in the second dimension for comprehensive reversed-phase chromatography×reversed-phase chromatography analysis of phenolic compounds in red wine has also been reported (34). The same group has described a comprehensive two-dimensional reversed-phase chromatography×reversed-phase chromatography system for the analysis of phenolics in sugarcane leaf extract, consisting of a micro-cyano column and a partially porous C18 column as the first and the second dimension, respectively (35). A total of 34 phenolic compounds, comprising phenolic acids, ellagitannins, flavan-3-ols, flavonols, and ellagic acid conjugates, have been identified in the shoots of *Rubus idaeus* "Glen Ample" by a two-dimensional reversed-phase chromatography×reversed-phase chromatography system consisting of a C18 silica column in the first dimension and a pentafluorophenyl column in the second dimension (36).

Besides reversed-phase chromatography and HILIC, other chromatographic separation modes, such as size-exclusion (SEC) or ion exchange chromatography (IEC), have found limited applications in both LC-LC and LC×LC of phenolic compounds. Also limited is the use of dual-retention mechanism columns (37). The combination of SEC with reversed-phase chromatography has been reported for the analysis of green and black teas (*Camellia sinensis*) (38). The developed comprehensive two-dimensional SEC×reversed-phase chromatography method has allowed the separation and identification of a variety of phenolic compounds, including catechins, theaflavins and their gallate derivatives, kaempferol, quercetin and myricetin mono-, di-, tri-, and tetraglycosides, esters of quinic acid, and gallic or hydroxycinnamic acids. More recently, a zwitterionic polymethacrylate monolithic column, which provides a dual-retention mechanism, HILIC at high concentrations of acetonitrile, and reversed-phase in water-rich mobile phases, has been used in the first dimension of either HILIC×reversed-phase chromatography or reversed phase chromatography×reversed-phase chromatography comprehensive two-dimensional separations of flavones and related phenolic compounds (39).

Conclusions

To summarize, the expanding interest in phenolic compounds and their positive effects on human health has promoted the development of a variety of techniques

for the analysis of these compounds in edible and medicinal plants and in plant-derived food products and dietary supplements. The complexity of such matrices and the heterogeneous chemical structures of the variety of phenolic compounds occurring in the plant kingdom require, besides reliable analytical methods, well-designed sample preparation methods and extraction processes.

Specialized LC separation techniques, combined with state-of-art mass spectrometric detection, are currently used for the separation, identification, and quantification of phenolic compounds in plants and in plant-derived food products. Such techniques comprise HPLC, performed with either analytical size or narrow-bore columns, and UHPLC, with dedicated columns and instrumentation. The majority of HPLC and UHPLC methods currently used for the analysis of phenolic compounds are performed in reversed-phase separation mode, although HILIC is also used, either as a valuable alternative to reversed-phase chromatography or as one of the chromatographic modes used in in 2D LC, in combination with reversed-phase chromatography or with other chromatographic techniques.

References

(1) B. Wielkopolan and A. Obrępalska-Stęplowska, *Planta* **244**, 313–332 (2016).
(2) A.Scalbert, C. Andres-Lacueva, M. Arita, P. Kroon, C. Manach, M. Urpi-Sarda, and D. Wishart, *J. Agric. Food Chem.* **59**, 4331–4348 (2011).
(3) A. Soto-Vaca, A. Gutierrez, J.N. Losso, Z. Xu, and J.W. Finley, *J. Agric. Food Chem.* **60**, 6658–6677 (2012).
(4) U .Lewandowska, K. Szewczyk, E. Hrabec, A. Janecka, and S. Gorlach, *J. Agric. Food Chem.* **61**, 12183–12199 (2013).
(5) M. Iriti and S. Vitalini, Pol. *J. Food Nutr. Sci.* **62**, 71–76 (2012).
(6) J.N. Kabera, E. Semana, A.R. Mussa, and X. He, *J. Pharm. Pharmacol.* **2**, 377–392 (2014).
(7) F. Shahidi and P. Ambigaipalan, *J. Funct. Foods* **18**, 820–897 (2015).
(8) D. Granato, J.S. Santos, L.G. Maciel, and D.S. Nunes, *TrAC - Trends Anal. Chem.* **80**, 266–279 (2016).
(9) E. de Oliveira and R. Battista, *Compr. Rev. Food Sci. Food Saf.* **16**, 580–616 (2017).
(10) V.C. George, G. Dellaire, and H.P.V. Rupasinghe, *J. Nutr. Biochem.* **45**, 1–14 (2017).
(11) M.E. Goetz, S.E. Judd, T.J. Hartman, W. McClellan, A. Anderson, and V. Vaccarino, *J. Nutr.* **146**, 2233–2243 (2016).
(12) A.R. Carvalho, G. Costa, A. Figueirinha, J. Liberal, J.A.V. Prior, M.C. Lopes, M.T. Cruz, and M.T. Batista, *Food Res. Int.* **99**, 485–494 (2017).
(13) E. Skates, J. Overall, K. De Zego, M. Wilson, D. Esposito, M.A. Lila, and S. Komarnytsky, *Food Chem. Toxicol.* **111**, 445–453 (2018).
(14) L.C. Klein-Júnior, Y. Vander Heyden, and A.T. Henriques, *Trends Anal. Chem.* **80**, 66–82 (2016).
(15) K.-Y. Khaw, M.-O. Parat, P.N. Shaw, and J.R. Falconer, *Molecules* **22**, 1286–1208 (2017).
(16) K. Ameer, H.M. Shahbaz, and J-H. Kwon, *Compr. Rev. Food Sci. F.* **16**, 295–315 (2017).
(17) S. Tang, H. Zhang, and H.K. Lee, *Anal. Chem.* **88**, 228–249 (2016).
(18) L. Trojer, A. Greiderer, C. Bisjak, W. Wieder, C.W. Huck, and G.K. Bonn, in *Handbook of HPLC*, D. Corradini, Ed. (CRC Press, Boca Raton, USA, 2nd ed., 2010) pp. 3–45.
(19) A.A. Rahim, S. Nofrizal, and B. Saad, *Food Chem.* **147**, 262–268 (2014).
(20) P. Chocholouš, J. Vacková, I. Šrámková, D. Šatínský, and P. Solich, *Talanta* **103**, 221–227 (2013).
(21) P. Garcia-Herrera, M.L. Pérez-Rodríguez, T. Aguilera-Delgado, M.J. Labari-Reyes, B. Olmedilla-Alonso, M. Camara, and S. de Pascual-Teresa, *Int. J. Food Sci. Technol.* **51**, 2290–2300 (2016).
(22) A.P. Singh, T. Wilson, D. Luthria, M.R. Freeman, R.M. Scott, D. Bilenker, S. Shah, S. Somasundaram, and N. Vorsa, *Food Chem.* **127**, 80–85 (2011).
(23) L. Riffault, C. Colas, E. Destandau, L. Pasquier, P. André, and C. Elfakir, *Phytochem. Anal.* **26**, 189–201 (2015).
(24) R. Brkliača and S. Urban, *J. Liq. Chrom. Rel. Technol.* **34**, 1063–1076 (2011).
(25) V. Exarchou, L. Kanetis, Z. Charalambous, S. Apers, L. Pieters, V. Gekas, and V. Goulas, *J. Agric. Food Chem.* **63**, 457–463 (2015).
(26) W.J. Hollands, S. Voorspoels, G. Jacobs, K. Aaby, A. Meisland, R. Garcia-Villalba, F. Tomas-Barberan, M.K. Piskula, D. Mawson, I. Vovk, P.W. Needs, and P.A. Kroon, *J. Chromatogr. A* **1495**, 46–56 (2017).
(27) C.M. Willemse, M.A. Stander, and A. Villiers, *J. Chromatogr. A* **1319**, 127–140 (2013).
(28) F. Cacciola, S. Farnetti, P. Dugo, P.J. Marriott, and L. Mondello, *J. Sep. Sci.* **40**, 7–24 (2017).
(29) K.M. Kalili and A. de Villiers, *J. Chrmatogr. A* **1289**, 69-79 (2013).
(30) M. Willemse, M. Stander, J. Vestner, A.G.J. Tredoux, and A. de Villiers, *Anal. Chem.* **87**, 12006–12015 (2015).
(31) W. Sun, L. Tong, J. Miao J. Huang, D. Li, Y. Li, H. Xiao, H. Sun, and K. Bi, *J. Chromatogr. A* **1431**, 79–88 (2016).
(32) F. Cacciola, P. Jandera, E. Blahová, and L. Mondello, *J. Sep. Sci.* **29**, 2500–2513 (2006).
(33) E. Blahovà, P. Jandera, F. Cacciola, and L. Mondello, *J. Sep. Sci.* **29**, 555–566 (2006).
(34) P. Dugo, F. Cacciola, M. Herrero, P. Donato, and L. Mondello, *J. Sep. Sci.* **31**, 3297–3308 (2008).
(35) G. Mazzi Leme, F. Cacciola, P. Donato, A.J. Cavalheiro, P. Dugo, and L. Mondello, *Anal Bioanal Chem* **406**, 4315–4324 (2014).
(36) M. Kula D. Głód, and M. Krauze-Baranowska, *J. Pharm. Biomed. Anal.* **121**, 99–106 (2016).
(37) P. Jandera, M. Staňková, and T. Hájek, *J. Sep. Sci.* **36**, 2430–2440 (2013).
(38) C.T. Scoparo, L.M. de Souza, N. Dartora, G.L. Sassaki, P.A.J. Gorin, and M. Iacomini, *J. Chromatogr. A* **1222**, 29–37 (2012).
(39) T. Hájek, P. Jandera, M. Staňková, and P. Česla, *J. Chromatogr. A* **1446**, 91–102 (2016).

Danilo Corradini earned his Ph.D. in chemistry at Sapienza University of Rome (Italy) and received his postdoctoral education in separation science at the Department of Chemical Engineering, Yale University (USA). His research interests include the study of fundamental and practical aspects of HPLC and electromigration techniques for analytical separations of biomolecules, mainly of plant origin. In recognition of his scientific contributions, he has received the Csaba Horváth Memorial Award in 2009, the Central European Group for Separation Science Award in 2011 and the Arnaldo Liberti Medal in 2014.

Francesca Orsini is a registered nutritionist in the register of the Italian Biologists. She received her bachelor degree in food science and human nutrition in 2012 and master's degree in food technology and science nutrition in 2014. She completed her education with a Ph.D. in bioengineering and biosciences in 2018 at the University Campus Bio-Medico of Rome. Her research domains include development of sample preparation procedures and chromatographic analytical methods for bioactive compounds in plant material.

Laura De Gara is Full Professor of plant physiology and head of the master's degree in food science and human nutrition at the Campus Bio-Medico University of Rome. She has almost 30 years of experience in studying plant metabolism with particular attention to redox homeostasis and signalling in response to environmental conditions. She is also interested in plant metabolites with nutritional and healthy effects, in particular polyphenols, fructans, and other fibres, as well as antioxidant vitamins.

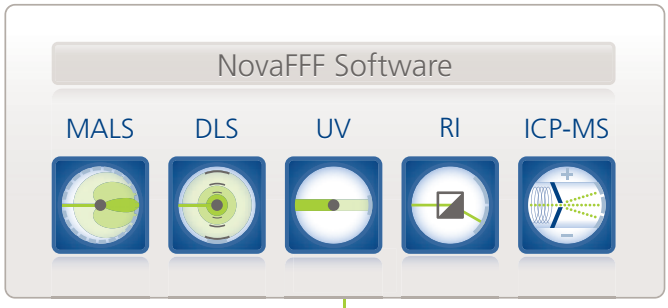
Isabella Nicoletti was educated as a biologist at Sapienza University of Rome, where she was also a postdoctoral fellow at the Department of Chemistry from 1982 to 1985. Since 1985 she has been a research scientist at the Institute of Chemical Methodologies of the Italian Research Council in Montelibretti (Rome). She is an expert in the field of separation science, and is interested in the development of chromatographic techniques for innovative analytical methods for foods with determined healthy prerogatives.

The FFF - MALS Platform

Next Level Nano, Bio and Polymer Analysis

POSTNOVA

NEW
With SEC Option!



Contact us for more information: **www.postnova.com**

Tips, Tricks, and Troubleshooting for Separations of Biomolecules, Part 2: Contemporary Separations of Proteins by Size-Exclusion Chromatography

Szabolcs Fekete[1], Davy Guillarme[1], and Dwight R. Stoll[2], [1]University of Geneva, Geneva, Switzerland, [2]LC Troubleshooting Editor

Several new materials and columns have been introduced in recent years for size-exclusion separations of proteins. How do I know which one to choose, and which separation conditions will be the best for my protein separation?

In Part 1 of this series (1), we focused on reversed-phase separations of proteins. In recent years, many new materials and columns have been introduced that provide potential for substantially better separations compared with those from one or two decades ago. Although some things have stayed the same, much of the old conventional wisdom has been overturned with the development of better stationary-phase chemistries and new research that has provided deeper insights into why we observe some phenomena (for example, low recovery of proteins from reversed-phase materials under some conditions). This research has also led to new guidance for operating conditions that improve the likelihood of obtaining acceptable chromatographic results.

Over the past few years, we have seen tremendous expansion in commercially available offerings for size-based separations of proteins as well. These separations are most commonly referred to as size-exclusion chromatography (SEC), and we will use that term here. As with reversed-phase separations of proteins, the upside to having more commercially available columns to choose from is that we can more precisely tailor our column choices to the needs of our applications. However, the downside to more options is that we have to choose which one is the most suitable, and in some cases, this can be a challenging task in itself. On the other hand, recent research studies have added considerable insights to the existing knowledge base to support this decision-making process. Even if we don't fully understand why SEC materials behave the way they do in every situation (for example, see reference 2), we are in a much better position today to make good choices about columns and operating conditions than we were five years ago.

For this instalment of "LC Troubleshooting", I have asked two of my collaborators in the biomolecule application space, and genuine experts in SEC separations of proteins, to join me in sharing some of the details that we have found to be particularly important to successful SEC separations.

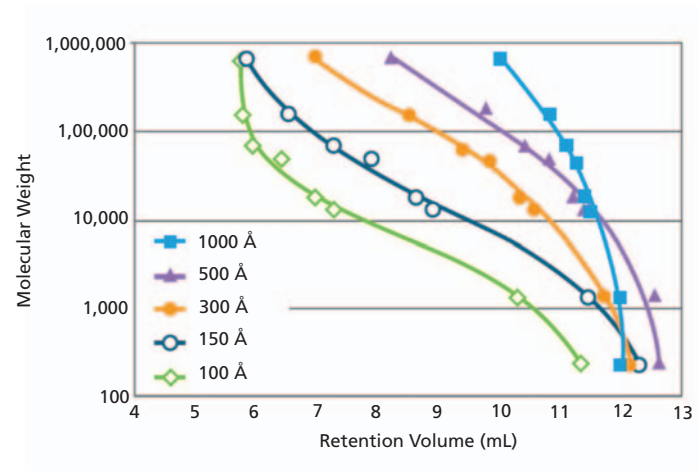
Dwight Stoll

Basics of SEC Separations

From a theoretical point of view, SEC is arguably the simplest of all chromatographic separation modes. In reversed-phase mode and other separation modes, we spend a lot of time thinking and talking about retention (that is, retention factors greater than zero are very important!), which is a function of differences between the strength of intermolecular interactions between analytes, mobile phase, and stationary phase. It is differences between the way one analyte interacts with the mobile and stationary phases compared to another analyte that give rise to differences in retention (that is, selectivity) and ultimately resolution of two analytes. In this way, resolution in reversed-phase and similar separation modes (sorptive modes) is inherently chemically driven. SEC, on the other hand, is completely different, at least in the ideal case. Here, resolution has a physical basis, rather than a chemical one, and in the ideal case, there is no retention of the analyte by the stationary phase (that is, retention factors are zero or apparently negative). Instead, separation arises

from differences in the physical limitations that analytes of certain sizes experience preventing them from exploring the entire pore network of porous particles used in SEC columns. Very small analytes in a sample will be able to explore most of the pore network. On the other hand, larger analytes that are too big to explore all of the pores will travel through the column with a higher velocity, and be observed flowing from the column earlier than the small analytes. From the point of view of the large analytes, the mobile phase volume inside the column is effectively smaller. Under ideal circumstances (that is, no retention as a result of intermolecular interactions), very small analytes will be eluted at what we would normally refer to as the dead time (t_m) in reversed-phase separations. The mobile-phase volume associated with this time (that is, $t_m \times F$) is referred to as the inclusion volume (corresponding to the total porosity of the column). Larger analytes will elute at earlier times, before the inclusion volume.

Figure 1: Molecular weight vs. retention volume plots for SEC columns having particles with different average pore sizes. The smallest two molecules in the dataset are uracil (112 Da) and vitamin B12 (1350 Da), and the largest two molecules are gamma-globulin (158 kDa) and thyroglobulin (670 kDa). Adapted with permission from reference 6.



Decision 1—Choosing the Column

Before we dive into the details here, we want to be clear about our intent for this instalment. A tremendous amount of very good information on the following topics has been published in recent years. Our discussion here is limited to a survey of highlights of that work. Readers interested in the details behind our discussion are strongly encouraged to engage the literature cited here to learn more.

Figure 2: Impact of mobile phase salt concentration on detected concentration of aggregates in a sample of the therapeutic protein adalimumab. Conditions: Column: 150 mm × 4.6 mm, 2.7-µm Agilent AdvanceBioSEC; mobile phase: 100 mM phosphate buffer, pH 6.8, + indicated concentrations of sodium chloride; flow rate: 350 µL/min. Peaks in order of elution: Dimer, monomer, and fragment (3). Unpublished data from the laboratory of D. Guillarme.

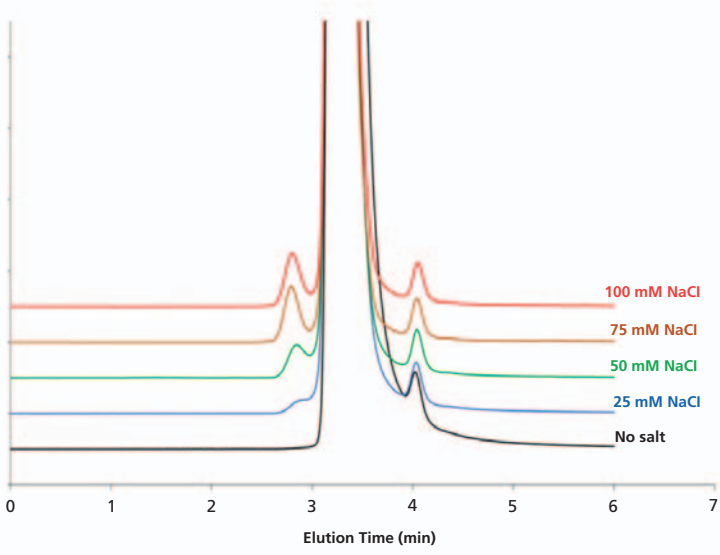
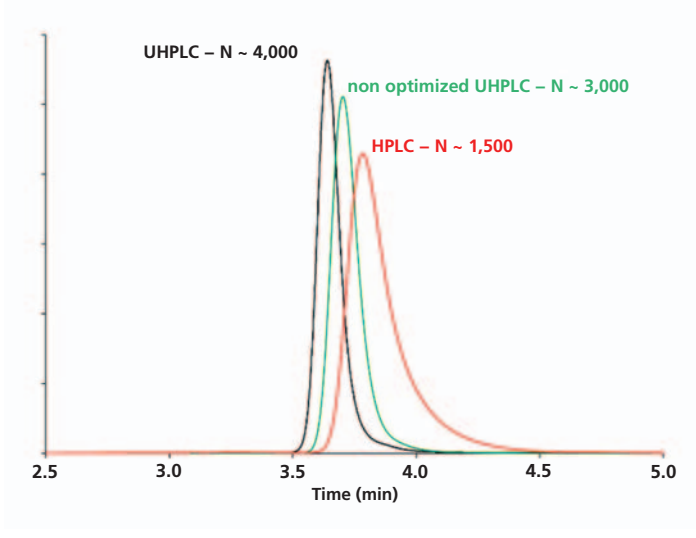


Figure 3: Observed chromatograms for a mAb monomer on three different LC systems. Column: 150 mm × 4.6 mm, 1.8-µm; flow rate: 0.3 mL/min; temperature: ambient. Adapted with permission from reference 11.



Particle Size and Column Length

Before the advances in column technology for SEC in recent years, most SEC columns in use were relatively large—typically 7.8 mm in diameter, and 150 to 300 mm in length. The long column lengths were required because of the large particles that were used, most of which did not have high mechanical strength and had to be used at relatively low pressures. The recent trend in column technology for SEC has been focused on the development of columns with smaller particles (<3 µm), in shorter columns (the standard now is 15 cm), and in smaller diameters (typically 4.6 mm). This trend has been supported by the development of particle chemistries that are both sufficiently mechanically stable to be used at the higher pressures that accompany the smaller particle sizes, and sufficiently inert toward biomolecules, to produce separations based mostly on molecular size. The move to smaller particle diameters also provides opportunities to improve separation speed by using higher flow rates through these columns. Whereas with larger particles, using high flow rates tends to result in decreases in efficiency (that is, plate number) and resolution, the price paid for doing so with smaller particles is not as severe.

Although we must be careful with generalizations, it is useful to think a bit about what the trend towards the use of small particles can do for us, in a practical sense. In rough terms the plate height scales with the particle diameter. So, upon moving from a 5-µm particle to a 2-µm particle, the plate height should decrease by about a factor of two (3). There are two main ways we can capitalize on this improvement in plate height—we can either improve resolution while using a column of the same length, or we can decrease analysis time while maintaining resolution. In the first case, if we use two columns of the same length—one with 5-µm particles and one with 2-µm particles—the plate number for the 2-µm particle should be approximately double that of the column with 5-µm particles. Since resolution scales with the square root of plate number, we should expect the resolution to improve by about 40%. In the second case, the plate number is directly proportional to column length, and inversely proportional to plate height. If the plate height decreases by a factor of two with the smaller particles, then we can decrease the column length by a factor of two, while maintaining the same plate number and resolution. If the same flow rate is used in both cases, we should expect this to immediately result in a 50% decrease in analysis time. This is a simple but useful view of these scenarios. There are a number of other factors to think about when considering the move to smaller particles, including the pressure limitations of the column and particles, and specifications of the instrument. More detailed discussions of the theory relevant to these considerations can be found elsewhere (4,5).

Average Pore Size and Distribution

As described above, the velocity of a particular molecule through a SEC column depends on the extent to which it can explore the pores of the particles. For particles with a well-defined pore size distribution, there is a range of molecular sizes for which a particular particle will be effective for size-based separations. The calibration curve shown in Figure 1 shows the selectivity (that is, difference in elution volume for a given change in molecular weight) for particles with different average pore diameters. We see that with small-pore columns there is good selectivity for small molecules, but the largest molecules will effectively be coeluted. On the other hand, the very large pore materials effectively separate the largest molecules, but the smallest molecules are coeluted. This type of plot can be used to decide which pore size will be most effective for the application at hand. For protein characterization, typical pore sizes between 150 and 500 Å are used. For common therapeutic proteins (MW ≈15–80 kDa), a pore size of 150–200 Å works well, while a 200–300 Å pore size is usually used for monoclonal antibodies (mAbs, MW ≈ 150 kDa). For very large proteins (MW > 200 kDa, for example, pegloticase or PEGylated proteins), typically the 500–1000 Å materials offer the most appropriate selectivity.

The pore size distribution has an impact on the slope of the calibration curves. The wider the pore size distribution, the steeper the curve is. Therefore, with a wide pore size distribution, the selectivity will be lower but the range of the analytes that can be separated will be broader. A narrow pore size distribution provides higher selectivity between species with slight differences in size, but only a limited size range of analytes can be separated.

The challenge in practice is that the only data that is readily available from column manufacturers is the nominal pore size. Unfortunately there is not broad agreement about how exactly to report pore size, and most of these measurements are based on gas adsorption/desorption measurements and may not be very meaningful for protein analyes. Thus, from the point of view of users of these columns, it is practically useful to experimentally determine the calibration curve by injecting a mixture of standard proteins in order to have a good sense for the selectivity that can be expected for a given protein sample.

Decision 2—Choosing the Mobile Phase

After choosing the column, the next most important decision involves choosing exactly what will go into the mobile phase. As described above, one of the basic tenets of SEC separations is that conditions should be chosen so that retention (in a chemical sense) is minimized. If achieved, this approach ensures that the elution volume is an indicator of molecular size (as in a calibration curve of the type shown in Figure 1) and nothing else. At first glance, this seems like it should be straightforward—we should just choose a stationary phase that does not interact strongly through specific types of interactions with the analyte, and choose a mobile phase in which the analyte has a high solubility

and that is able to minimize analyte–stationary phase interactions. But, if we've learned anything from 50 years of liquid chromatography, one of the big lessons has been that apparently tiny changes in the chemistry or structure of stationary phase or analyte can lead to big changes in retention. Indeed, we often exploit these interactions to great effect in reversed-phase separations when developing a new method. However, implementing this approach also means that achieving the "no retention" condition in SEC separations of proteins can be quite difficult in practice. There is a rich literature describing studies that have explored the use of different mobile phase modifiers and conditions to minimize stationary phase–analyte interactions.

It has been our experience that many of the specific effects of different mobile-phase conditions are protein or stationary-phase specific (or both), and thus some amount of exploration of variables is a necessary part of method development when starting work with a new molecule. However, based on our experience and the literature available to date, we can provide some suggestions for starting conditions:

- **pH:** When the isoelectric point (pI) of the protein is known, the mobile-phase pH should be adjusted to approximately match the pI of the protein. If the pI is not known, pH 6.5 is a good starting point. One should ensure, either based on existing literature or by experiment, that the protein is both highly soluble and chemically stable at the pH that is planned for.
- **Salts:** Various additives have been tested as a means to reduce nonspecific interactions and retention of proteins under SEC conditions. For example, high concentrations (~0.2 M) of arginine have been used in the past (7). Arginine and other amino acids can interact with the protein and therefore decrease the accessible charges and possible electrostatic (ion-exchange) interactions. More commonly though, significant concentrations of sodium and potassium salts are used to suppress electrostatic interactions between the stationary phase and protein (8,9). An example of the effect of adding increasing levels of sodium chloride to a phosphate buffered mobile phase at pH 6.8 is shown in Figure 2 for the therapeutic protein adalimumab. Here, we see two major effects, both of which evidently result from decreased interactions between the protein and the stationary phase. First, the detected concentration of the mAb dimer (peak eluted before the monomer) increases dramatically (higher recovery) from barely detectable with no salt added, to easily detected at 100 mM sodium chloride added. Second, the elution volume of the dimer also decreases, again because interactions with the stationary phase are decreased, such that the resolution of the dimer and monomer increases.
- **Organic solvents:** Although most proteins are sufficiently hydrophilic that completely aqueous mobile phases will yield acceptable SEC results, hydrophobic proteins may require small additions of solvent to improve recovery and peak shape. In particular, antibody–drug conjugates (ADCs) are a class of molecules of current interest that may benefit from addition of organic solvent (10). In these cases, addition of 10–15% of isopropanol to the mobile phase is a good starting point.

And What About the Instrument?

There are at least two major issues we could discuss here—the impact of system dispersion on the performance of high-quality SEC separations, and the impact of instrument construction and the use of bioinert, biocompatible materials. The latter topic is complex and we will reserve that discussion for a later date. On the topic of system dispersion, we have to recognize that SEC separations are particularly prone to the negative effects of peak dispersion outside of the column (that is, extracolumn dispersion) because, again, the peaks are eluted with no retention or even before the inclusion volume. In separation modes where retention is desirable, the effects of extracolumn dispersion are less severe for peaks that are more retained, and in the case of gradient elution in many cases nearly all precolumn dispersion can be eliminated. Not so in SEC, because no peaks are retained, and all separations are isocratic.

As discussed above, until relatively recently most SEC columns in use were large in diameter (~7.8 mm) and long (300 mm). This resulted in separations where the peak volumes (that is, the peak width in time units, times the flow rate) were large enough in comparison to the injector-to-detector volumes of LC systems they were connected to. However, with the improved plate heights and smaller volumes of state-of-the-art columns, the peak volumes are small enough that extracolumn dispersion has become a very important issue again (11). Figure 3 shows a comparison of the detected peak for a monoclonal antibody monomer obtained on three different LC systems with different levels of extracolumn peak dispersion. Given that resolution is often very valuable in SEC separations, this comparison makes it clear that one should seriously consider the effect of extracolumn dispersion on the observed chromatography, particularly when using modern SEC columns with small volumes and small particles.

When working with a state-of-the-art 150 mm × 4.6 mm SEC column, for a small analyte that is eluted near the inclusion volume, only 25–60% of the intrinsic column efficiency can be attained on conventional high performance liquid chromatography (HPLC) systems. The situation is even worse with a partially excluded analyte. Optimized ultrahigh-pressure liquid chromatography (UHPLC) systems having very low extracolumn volumes (typically V_{ec} < 10 µL) have to be used to properly operate these columns. Therefore connector tubing volume and detector cell volume must be as low as possible. As most SEC separations are performed at ambient temperature, the mobile-phase preheater unit can also be removed to further gain in apparent efficiency. Another interesting finding is that conventional HPLC systems also have a big impact on the apparent elution time of proteins—and therefore on mass-calibration curve—when working with 150 mm × 4.6 mm columns. Under these conditions the resulting calibration data will not be reliable, except if corrected for extracolumn residence time.

Summary

Developing effective and high performing SEC separations for proteins requires attention to all facets of the method, including choices around stationary phase, particle size, and column dimensions, mobile-phase

conditions, and instrument effects on chromatographic efficiency and resolution. Several research groups are continually contributing to our understanding of the effects of all of these decisions on separation performance. Although we certainly are very far from a complete understanding, we are in a better position than ever before to leverage the information we do have to develop the best methods possible today.

With this instalment of "LC Troubleshooting", I am approaching my first full year of writing monthly columns that address some of the pain points we experience as practitioners of liquid chromatography. As I have said many times already here, some new problems emerge as technology changes and we adapt to the new behaviours of instruments and columns, but there are also many problems that nominally remain the same over time. I will continue working to bring a mix of discussions of old and new topics to the column, but I am also particularly interested to hear what you, as a regular consumer of the column, have to say about topics you would like to see addressed here. Are there topics that are emerging challenges that you have not seen addressed in the past? Are there "old" topics that you would like to see addressed in more depth? I'd love to hear your topic suggestions! Please send them along to LCGCedit@ubm.com

References

(1) S. Fekete, D. Guillarme, and D.R. Stoll, *LCGC Europe* **31**(8), 427–431 (2018).
(2) A. Goyon, S. Fekete, A. Beck, J.-L. Veuthey, and D. Guillarme, *J. Chromatog. B* **1092**, 368–378 (2018). doi:10.1016/j.jchromb.2018.06.029.
(3) A. Goyon, A. Beck, O. Colas, K. Sandra, D. Guillarme, and S. Fekete, *J. Chromatog. A* **1498**, 80–89 (2017). doi:10.1016/j.chroma.2016.11.056.
(4) K. Broeckhoven and G. Desmet, *TrAC, Trends Anal. Chem.* **63**, 65–75 (2014). doi:10.1016/j.trac.2014.06.022.
(5) P.W. Carr, X. Wang, and D.R. Stoll, *Anal. Chem.* **81**, 5342–5353 (2009). doi:10.1021/ac9001244.
(6) *How to Guide for Size Exclusion Chromatography for Biomolecule Analysis,* (n.d.). https://www.agilent.com/cs/library/primers/public/5991-3651EN_LR.pdf (accessed 30 June 2018).
(7) D. Ejima, R. Yumioka, T. Arakawa, and K. Tsumoto, *J. Chromatog. A* **1094**, 49–55 (2005). doi:10.1016/j.chroma.2005.07.086.
(8) A. Goyon, A. Beck, J.-L. Veuthey, D. Guillarme, and S. Fekete, *J. Pharm. Biomed. Anal.* **144**, 242–251 (2017). doi:10.1016/j.jpba.2016.09.031.
(9) F. Bickel, E.M. Herold, A. Signes, S. Romeijn, W. Jiskoot, and H. Kiefer, *Eur. J. Pharm. Biopharm.* **107**, 310–320 (2016). doi:10.1016/j.ejpb.2016.07.020.
(10) A. Goyon, L. Sciascera, A. Clarke, D. Guillarme, and R. Pell, *J. Chromatog. A* **1539**, 19–29 (2018). doi:10.1016/j.chroma.2018.01.039.
(11) A. Goyon, D. Guillarme, and S. Fekete, *J. Pharm. Biomed. Anal.* **135**, 50–60 (2017). doi:10.1016/j.jpba.2016.12.004.

Szabolcs Fekete holds a Ph.D. degree in analytical chemistry from the Technical University of Budapest, Hungary. He worked at the Chemical Works of Gedeon Richter Plc at the analytical R&D department for 10 years. Since 2011, he has been working at the University of Geneva in Switzerland. He has contributed to more than 100 journal articles and authored book chapters and edited handbooks. His main interests include liquid chromatography (reversed-phase, IEX, SEC, HIC, SFC, HILIC), column technology, method development, pharmaceutical, and protein analysis.

Davy Guillarme holds a Ph.D. degree in analytical chemistry from the University of Lyon, France. He is now senior lecturer at the University of Geneva in Switzerland. He has authored more than 200 journal articles related to pharmaceutical analysis. His expertise includes HPLC, UHPLC, HILIC, LC–MS, SFC, and analysis of proteins and mAbs. He is an associate editor of *Journal of Chromatography B* and editorial advisory board member of several journals including *LCGC North America*, *Journal of Chromatography A*, *Journal of Separation Science*, and others.

Dwight R. Stoll is the editor of "LC Troubleshooting". Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota, USA. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 50 peer-reviewed publications and three book chapters in separation science and more than 100 conference presentations. He is also a member of *LCGC*'s editorial advisory board. Direct correspondence to: LCGCedit@ubm.com

A Q&A

GPC/SEC Adventures in (Bio)Polymer Analysis: *"Perfect Separation Solutions"* from Our Contract Analysis Lab

Claudia Lohmann, PhD
Independent Polymer Consultant
PSS-Polymer Standards Service – USA Inc.

More sophisticated chromatographic techniques are needed to characterize increasingly complex macromolecular materials.

Modern macromolecular materials can be tailored to fit virtually any application, but their characterization has become more challenging as a result. More sophisticated chromatographic techniques are required to reveal important architectural details in the molecular structures. The separation techniques applied can be as diverse as the macromolecules themselves. *LCGC* recently sat down with Claudia Lohmann, PhD, independent polymer consultant to PSS-Polymer Standards Service – USA Inc., to discuss how PSS can help solve these analytical problems, who its customers are, and samples that are particularly challenging to analyze.

LCGC: Why is it an adventure when you work with macromolecules?

Lohmann: Macromolecules, especially modern purpose-design materials, have so many interesting features and applications. Macromolecules can be tailored to fit virtually any requirement and are used in all industries. For instance, without plastic, there would be no civilization. At PSS, we are constantly surprised and amazed to learn about the different applications of macromolecules.

Because of the complex and sophisticated nature of these macromolecular materials, conventional calibration techniques based on narrow standards no longer provide a complete answer with respect to molar mass and chemical composition distribution. It is very challenging. The analysis of modern polymers can be compared with trying to find the best way out of a maze or a jungle.

At PSS, we appreciate and understand the complexities of these materials. Therefore, we can apply our expertise to the characterization of large molecules with respect to correct sample preparation and choice of separation technique/s to produce accurate, reliable, and useful data.

For example, PSS was able to identify the components in a polymer protection film made the late 1950s that nearly destroyed a historic document from the 12th century. In other examples, we discovered why paint wouldn't stick on the heels of shoes and how milling degrades different types of starches. In a more modern instance, PSS quantified the copolymer in a tablet coating, and we developed and implemented a method for European gelatin manufacturers.

LCGC: How can PSS Labs help with solving problems?

Lohmann: The scope is broad, ranging from simple batch-to-batch comparisons to complex detective work. PSS can establish and perform quality-control procedures to run samples under standardized conditions with dedicated instrumentation or column sets.

SPONSORED BY

PSS POLYMER STANDARDS SERVICE

LC GC europe

PSS can also deformulate products to investigate possible patent or intellectual property infringement and provide a project plan for how to identify specific sample components, such as polymers and additives etc. PSS can help develop gel permeation chromatography (GPC) and interaction polymer chromatography (IPC) methods that can be easily transferred to a customer's lab.

Importantly, PSS tailors conditions to accommodate the customer's sample requirements instead of trying to make things fit that just do not fit. The bottom line is, PSS does not just give numbers to its customers. We supply answers that are both needed and useful.

LCGC: How is PSS of service and whom does PSS serve?

Lohmann: PSS works with customers in all aspects of academia and industry. Our industrial clients dealing with polymers are in the automotive, food, wood, biotech, and pharmaceutical industries and also work with medical devices, and specialty chemicals. Our customers in academia, at universities, and in renowned research facilities all value PSS's scientific input.

Customers contact PSS with either a specific application problem or a request for routine analysis. We then have an interactive discussion with the customer to confirm the initial analysis request. In some cases, we can provide extra information that the customer was not aware would be possible.

For example, in addition to molar mass PSS can supply composition information. If the request is straightforward (e.g., determination of a mass distribution by a conventional calibration or light-scattering detection or a simple batch-to-batch comparison), PSS provides the customer with a quote and a sample information sheet. The turnaround time is rapid.

LCGC: In your experience, what has been the most challenging sample to analyze, and why?

Lohmann: A very challenging (and smelly!) project was on sewage sludge. The objective was to find out if the flocculants would degrade over time, when the sludge is spread on fields. The degradation was monitored over time under close-to-natural conditions after extracting the polymer from the soil. The biggest challenge was the sample matrix because it contained humic acids, which made detection nearly impossible. With a lot of effort, PSS determined that the polymers in the soil do degrade over time, thus allowing the sewage sludge to be spread on fields that are cultivated.

Another interesting example, from a chromatographic point of view, was a comparison of two polyvinylchloride samples. The molar masses of the polymers were almost identical. There seemed to be only one small peak, coming from an additive, on the low molecular end of the chromatogram.

However, these materials behaved differently. With the help of FT-IR and ESI-MS hyphenation, PSS was able to finally identify that the small additive peak of one sample consisted of three co-eluting species, whereas the one of the other sample contained only one specie. In this case, there was much more than met the eye.

LCGC: Are there any samples that cannot be analyzed directly by GPC/SEC?

Lohmann: The first things that come to mind are usually gels, cross-linked samples, or ultra-high molar mass samples. Generally speaking, if the sample is not soluble, then gel permeation chromatography (GPC) or interaction polymer chromatography (IPC) cannot be used. However, other options and alternative techniques are available.

For example, inverse GPC allows PSS to measure the pore size distribution. Or, PSS could determine the soluble portion of a sample or its gel content, if the sample contains a gel. High molar mass samples are definitely a challenge. However, PSS has experience with those fragile samples. PSS scientists know how to prepare them with care and how to make the chromatography work.

Another example is reactive samples. These must be analyzed in close cooperation with the customer to prevent any damage to the equipment.

LCGC: To summarize, why is PSS the perfect partner for liquid chromatography (LC) of polymers and biopolymers?

Lohmann: PSS, which has been in business for more than 30 years, has a creative team of scientists who have extensive experience with macromolecules. We have state-of-the-art equipment for ambient-temperature liquid chromatography and high-temperature GPC. Multiple LC systems are set up, featuring specialty detectors necessary for sophisticated characterization.

Solvent-wise, we can cover the entire polarity chart. Analyses are performed in common (e.g., tetrahydrofuran) or uncommon (e.g., hexafluoroisopropanol or N-methyl-2-pyrrolidone) GPC solvents. PSS produces GPC columns, has its own software, and makes polymeric reference materials.

PSS's close interdepartmental cooperation allows the development of long-term stable methods, grants unparalleled in-house support, and provides access to advanced products and the latest developments. In addition, PSS is certified according to ISO 9001:2015.

Safely Delivering the Best Possible Carrier and Detector Gases to Your GC System

John V. Hinshaw, GC Connections Editor

The quality of a gas chromatogram depends heavily on the quality of the separation and detection gases, among many other factors. In this month's instalment, "GC Connections" discusses ways in which chromatographers can ensure a safe working environment while delivering gases that are up to the requirements of the separations at hand, in the context of moving a laboratory to a new location.

Earlier this year, the laboratory I use was moved to a new location several miles away. In the course of setting up the new laboratory, the gas chromatography (GC) carrier and detector gas supplies had to be torn down and rebuilt. This laboratory is similar to many industrial GC laboratories—it contains a number of GC systems, plus a variety of specialized test equipment. The laboratory has two double-wide gas cylinder corrals that hold helium, air, nitrogen, hydrogen, and an assortment of gas standards. Cylinders not in use are stored in gas safety storage cages in another room. Occasionally a cylinder is secured next to an instrument, but lengths of manifolded tubing anchored to the walls deliver the gases for permanent use from the corrals to the instruments.

The new laboratory is somewhat larger and requires longer tubing runs between the cylinder corrals and the instruments. The challenge was to reuse as much of the existing hardware—regulators, tubing, fittings, and valves—as possible, to save costs, while maintaining the integrity of the connections and purity of the delivered gases. The leak-tight aspect is absolutely crucial for laboratory safety, because some of the gas standards contain high levels of toxic substances, and, of course, hydrogen is quite flammable.

How did it go, and what lessons were learned? Let's take a look.

Safety First

Gas cylinder safety has been addressed many times in this column as well as in multiple safety guides and government regulations. The topic was covered recently in two "GC Connections" instalments from 2016 (1,2). Good safety practice centres around proper training and equipment. Gas safety training should include both general procedures and practices as well as topics specific to the gases in use, emergency procedures, and appropriate training on how to make and break the various gas-tight fittings found in the work environment. Beyond cylinder restraints and carts, safety equipment also includes goggles, gloves, and safety shoes, plus correctly sized and rated regulators, tubing, valves, gas filters, and fittings. Please see the two instalments (1,2), as well as the references inside, for additional gas safety information.

In this laboratory, tanks of air, hydrogen, nitrogen, and helium are secured alongside gas standard cylinders. Liquid nitrogen tanks are used occasionally. Beyond dangers directly attributable to the gas cylinders, such as high internal pressures and risks from handling heavy objects improperly, the cylinder contents create hazards of toxicity, flammability, oxygen displacement, and cryohazards. See table 1 in reference 1 for more details about the various commonly used gases and associated hazards.

The primary lines of defense against these gas hazards are proper cylinder handling and regular verification of the leak-free state of all gas feeds. These hazards are mitigated further by providing the laboratory with high-volume heating, ventilation, and air conditioning (HVAC) air flow and suitable ventilation of gas streams, along with toxic, flammable, and oxygen-depletion gas sensors wired to the building alarm system.

Note that flushing a hydrogen line with helium for the initial leak check is a good idea, because it avoids potentially venting a lot of hydrogen into the air uncontrollably in the event of a large unintentional leak.

When the new laboratory was configured, the gas sensors were brought over intact, and a larger HVAC system was installed to create an improved gas-safety work environment. During the interim period between moving the gas sensors to the new building and leaving the old laboratory, sets of similar portable gas sensors were leased and placed in key spots in the old location, where some work continued right up to the move date.

Ultra smooth bores, low dead volume, clear graduations, and precision calibration.

VICI® Precision Sampling Pressure Lok® Syringes

Syringe options
HPLC syringes, liquid syringes, gas/liquid syringes and autosampler syringes.

PTFE plunger tips
Stress-formed to ensure leak-tight seals.

Self-lubricating plunger tips
Designed to remain smooth, without the seizing or residue of conventional metal plungers.

Needles sealed by PTFE sleeves
Isolates samples and prevents adhesive dissolution and contamination.

Easily replaceable parts
Depending on the specific model, some or all of the wear parts can be easily replaced.

VICI® PRECISION SAMPLING

Visit www.vici.com for more information.

Immediately after the move, some of the built-in sensors were found to be near their rated service period and were replaced. It is difficult to perform regular checks for this type of safety failure. Fire extinguishers, for example, are checked periodically for expiration as specified in fire safety regulations. Equipment like the gas sensors sits quietly for extended periods of time without alarming and so can fade into the background and not receive sufficient attention. Another example like this that I have encountered is eye wash equipment in a very expired condition, the type that has a fluid reservoir instead of a plumbed water connection. Thus gas safety sensors, as well as all of the other safety equipment related to gases or for other purposes, should be checked regularly for function and expiration date where appropriate.

I can say, from the experience of unintentionally testing a combustible gas detector, that it takes about two seconds for hydrogen to make its way up to a combustible gas detector near a 10-ft ceiling and halfway across the lab!

Making the Move

The move was a multistep process. One of the first steps was to disconnect the GC gas filters. The incoming gas pressures on the carrier and detector lines were reduced, the gas lines to the instrument were disconnected, and the filter fittings were quickly capped under gas flow. After disconnecting and capping the bulkhead fittings at the back of the instruments, the gas line pressures were shut off at the regulator outlet valves, the tubing was disconnected from the filters one by one, and the filter inlet fittings were capped. This approach allowed as much pure gas as possible to be retained inside the filters. The intent was to reuse each filter at the new location.

The tubing, fittings, and valves were disconnected from the regulators, and the longer tubing runs with intermediate unions were disconnected as well. The first tubing sections, starting at the tank regulators, are six-foot lengths of flexible hose, which makes connecting the cylinders much easier and adaptable. The same is true for the gas connections at the outlet ends of the carrier gas lines. Where possible, fittings were left intact, because they were likely to remain leak-tight through the move. The 0.125-in. diameter tubing was coiled as smoothly as possible, while the 0.25-in. pieces had to be moved with straight sections and bends left intact. It was not practical to cover all of the exposed fitting ends, so they were taped over with low-residue blue painter's tape. Of course, the new laboratory configuration is not the same, and so a significant number of pieces of bent tubing would not fit anywhere at the destination.

The tank regulators were vented and then packed a few to a box with bubble wrap or foam surrounding them. Although cylinder regulators don't look very fragile, their gauges and valve stems are prone to impact damage during transport. It's a good idea to cover the regulator gas inlets with low-residue blue painter's tape as well, to prevent ingress of particles. Regulator outlets are best sealed with matching caps.

Before the move, the gas cylinders were inventoried, and any that were no longer needed were returned to the supplier. When the time came to break down and pack the laboratory gas systems, rather than attempting to put the cylinders on a truck and run afoul of state or federal Department of Transportation, Occupational Safety and Health Administration (OSHA), and who knows what other regulations while creating a true public safety hazard, the commercial gas suppliers were engaged instead to move their cylinders themselves. The cylinders were disconnected from their regulators, capped, and then packed by the gas suppliers onto suitable pallets for the short journey to the new location. Once on site they were unloaded from the pallets and placed back into the in-lab corrals or into one of the gas storage cages.

Reassembly

At the destination, the lengths of tubing were assembled as best they could be positioned to bring the carrier and other gases to the instrumentation. Nearly all the tubing is stainless steel, so there was little concern that the tubing would fail as a result of stress fracturing across multiple bends. A few of the lines are the heat-treated copper 0.125-in. outer diameter type provided by some instrument manufacturers. These lines were left intact and were disconnected only at the instrument bulkhead and the corresponding gas filter, then reassembled to exactly the same fittings with as little rebending as possible. No fractures occurred.

New tubing was used in some places where nothing fit the required lengths. When all was complete, about 75% of the tubing in the new laboratory was recovered from the old lab. Much of the new tubing used was needed to extend the tubing runs for the increased distance between the tanks and the instruments.

Special attention was paid to any reused swaged fittings. Each fitting was inspected first for over-tightening symptoms of a bulging tubing end, or distorted ferrules. Another problem can arise from mis-matched fittings from two different manufacturers. A recent instalment of "GC Connections" (3) has some good photos of what to look for in this regard as well as an informative discussion on how to make the connections.

A trial attempt at making each connection was performed. If the fitting nut did not engage the threaded union or valve thread smoothly and without requiring the force of a wrench, then the nut and ferrule portion on the tubing were discarded. If the union or valve was not new, it was also discarded since it was likely that the damage extended to both sides of the connection.

After making a new clean cut on the tubing, and using a new union or valve, a new connection was made following the fitting manufacturer's procedures. Overall a good recovery of used fittings was achieved, around 80%. This good recovery was due to having paid attention to the quality of the original installation of the fittings in the old laboratory, which paid off handsomely for the move to the new lab.

After a tubing run was complete, the exit was sealed temporarily with a plug and the line was pressurized with helium. The first leak check consisted of turning off the tank valve after pressurization, leaving the regulator's outlet valve open, and observing the high-pressure

tank gauge for up to 30 min. If any observable pressure drop was seen, a quick check of the fittings with a helium leak detector usually revealed one or more leaking fittings, which were duly repaired or replaced. Sometimes all that was needed was the audible hiss of an untightened fitting! If no pressure drop was observed, then the fittings were checked more carefully to be sure there were no microleaks. Liquid leak checking solutions were not used.

Another observation: Treat your fittings well and they will repay you with multiple make–break cycles. You will avoid having to replace them often to keep the gas system leak tight, and reduce correspondingly the expense of new fittings.

Finally the intended gas, if other than helium, was connected and another pressure drop check was made for air or nitrogen, or a leak tester check for hydrogen, after which the line was deemed ready for service. Note that flushing a hydrogen line with helium for the initial leak check is a good idea, because it avoids potentially venting a lot of hydrogen into the air uncontrollably in the event of a large unintentional leak.

Leak checking was performed before connecting tubing to the inlets of any gas-scrubbing filters, to avoid forcing any more air than necessary into them. After a line was leak tight, it was purged with the appropriate gas before connecting to the filter, and then the filter was purged before connecting to the instrument. Fortuitously, none of the water- or oxygen-indicating filters exhibited significant degradation from before to after the move after following the above procedures.

Caution is advised when venting hydrogen lines. Hydrogen diffuses away into the room air quite rapidly, because of its buoyancy and high diffusivity. Using a low pressure in the line during purging helps limit the amount of hydrogen that is released. I can say, from the experience of unintentionally testing a combustible gas detector, that it takes about two seconds for hydrogen to make its way up to a combustible gas detector near a 10-ft ceiling and halfway across the lab!

After the gas lines were set, one of the GC systems was powered on and a series of baseline runs were made. They followed a normal sequence with some ghost peaks and baseline instability in the first couple of runs, and then settled down nicely. The move was deemed successful, and we resumed our normal work.

Conclusions

One of the lessons learned in the move was that it is absolutely necessary to maintain accurate records of the age of consumable components in the laboratory. In this case, expired gas detectors were discovered and replaced. The GC gas filters, at least the indicating ones, appeared to survive the move well, but the nonindicating filters may or may not be in good shape today. It is difficult to track how well they perform, unless a small indicating filter is inserted downline. These will be replaced as necessary and feasible.

Another observation: Treat your fittings well and they will repay you with multiple make–break cycles. You will avoid having to replace them often to keep the gas system leak tight, and reduce correspondingly the expense of new fittings.

Although I would not choose to move a workplace very often, with proper planning, organization, and attention to the technical requirements for both safety and gas handling, a move can be made without major unplanned interruption or equipment losses.

References

(1) J.V. Hinshaw, *LCGC Europe* **29**(10), 576–580 (2016).

(2) J.V. Hinshaw, *LCGC Europe* **29**(11), 629–633 (2016).

(3) N.H. Snow, *LCGC Europe* **31**(1), 28–34 (2018).

"GC Connections" editor **John V. Hinshaw** is a Senior Scientist at Serveron Corporation in Beaverton, Oregon, USA, and a member of *LCGC Europe*'s editorial advisory board. Direct correspondence about this column to the author via e-mail: LCGCedit@ubm.com

Data Integrity and *USP* <1058>, Part 2: OQ Supervision and Execution

Paul Smith[1] and R.D. McDowall[2], [1]Agilent Technologies, Inc., Stockport, UK, [2]R.D. McDowall Ltd, Ltd, Bromley, Kent, UK

This is the second of three articles looking at the impact of the new *United States Pharmacopeia (USP)* <1058> on analytical instrument qualification (AIQ) on data integrity in a regulated chromatography laboratory. This part focuses on how the laboratory should supervise the execution of operational qualification (OQ) protocols by a third-party service provider. The principles described also apply to in-house metrology departments.

The first part of this three-part series focused on the impact of the new version of the *United States Pharmacopeia (USP)* <1058> (1) and looked at instrument specifications and the role of suppliers (2). As demonstrated in part 1, analytical instrument qualification (AIQ) is level 1 of the data integrity model (2–4) and is therefore an essential component for ensuring data integrity.

The Qualification Continuum

Before going into detail about operational qualification (OQ), it is important to understand that AIQ is a dynamic continuum that spans all instrument stages associated with:

- Specification, purchase, and implementation
- Ongoing qualification
- Routine use.

A key component of this continuum is the relationship between the user requirement specification (URS), OQ, and performance qualification (PQ) as shown in Figure 1. The new *USP* <1058> (1) states that users must write a URS for their instrument (yes, even for a pH meter) and this must be tested in the OQ. What is equally important, and will be covered in Part 3, is that the PQ must also demonstrate that the instrument meets intended use requirements during the operational phase. Therefore, as tests for the OQ are developed, consideration should also be given to the plans and tests that need to be performed during the PQ phase to demonstrate conformance with URS requirements.

- Will any OQ tests be repeated in the PQ?
- Are new tests required for the PQ?
- What is different and why?

For chromatography instruments, many laboratories have historically applied a life cycle process based on a minimum sub-set of the life cycle in the 2008 version of *USP* <1058> (5):

- **Supplier:** Responsible for IQ/OQ, preventative maintenance, and an annual OQ
- **Laboratory:** Responsible for system suitability tests (SST) and in some mindsets SST = PQ

Limitations of this segmented approach include the fact that the AIQ is disconnected from routine use or applications and components of the full life cycle are missing. There is no laboratory URS and requirements are informally based on pharmacopoeial general chapters. Diagnostic tests performed by the instrument are another important component of assuring instrument functionality, for example, when a UV–vis high performance liquid chromatography (HPLC) detector is powered up a wavelength test based on emission lines from the lamp is performed.

A Quick Recap

Historically, when process validation principles were first applied to laboratory instruments, there was an over reliance on documentation (6) and many of the life cycle steps, such as URS and design qualification (DQ), were considered clerical steps that were the responsibility of the supplier and potentially independent of how the instrument would be used in an individual laboratory. This is wrong (7). In Part 1 (2), we discussed why users must write their own specifications for the analytical instruments that they purchase based on their intended use, and not blindly use those from a supplier.

This means that the URS has evolved from what was a tick box activity to becoming the most important document in the AIQ life cycle.

USP <1058> requires that suppliers must publish meaningful specifications relating to instrument parameters that users can test practically in their own laboratories, rather than in a controlled environment. In addition, the URS for a chromatograph must be the basis for intended use testing in the OQ phase (1). In this part, we will address the writing, execution, supervision, and execution of OQ tests as shown in Figure 2.

CONNECT WITH LCGC ON SOCIAL MEDIA

Join your colleagues in conversation, respond to hot topic questions, and stay up-to-date on breaking news. "Like" and follow us on Twitter, LinkedIn, Facebook, and YouTube today!

LCGC

www.chromatographyonline.com/LCGCSocialMedia

Figure 1: The qualification continuum: relationships between URS - OQ - PQ.

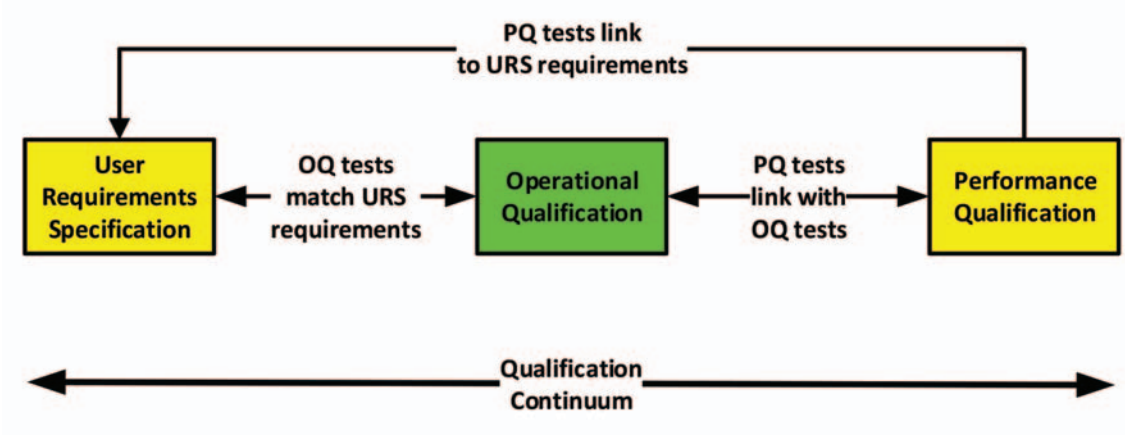


Table 1: Qualification instructions for HPLC pump flow rate (9)

Instruction	Acceptance Criterion
1. Prime all the solvent lines with HPLC-grade water. 2. Set the flow rate to 0.500 mL/min. 3. Wait for about 15 min to stabilize the system and ensure that the pressure is stable. 4. Insert the outlet tubing into a 10 mL volumetric flask and start the stopwatch simultaneously. 5. Stop the stopwatch when the lower meniscus reaches the 10 mL mark on the flask. 6. Record the elapsed time. 7. Similarly check the flow for 1.0 mL/min and 2.0 mL/min.	The time taken to collect the water should be within ± 2.0% of the actual value

Merging IQ and OQ?

There is harmonization between the new *USP* <1058> and EU GMP Annex 15 on Qualification and Validation when it comes to merging qualification documents because both allow this (8). The main documents that can be merged are typically the IQ and OQ protocols. This has advantages for both the laboratory and the supplier because there is a single document to prepare, review, and execute. It also permits a service engineer to move from the IQ to the OQ without having to wait for a laboratory approval, thereby allowing for more efficient execution.

Quality by Design or Compliance by Stupidity?

When designing OQ tests it is important to understand the following factors:

- How an instrument and any associated software works;
- Know the applicable pharmacopoeial and regulatory requirements;
- The application of sound science and risk-based qualification

Let us explain what we mean. How would you measure flow rate accuracy and precision of an HPLC pump? Table 1 shows an example of how to measure pump flow rate (9).

Let us analyze this approach. Is it scientifically sound? No!

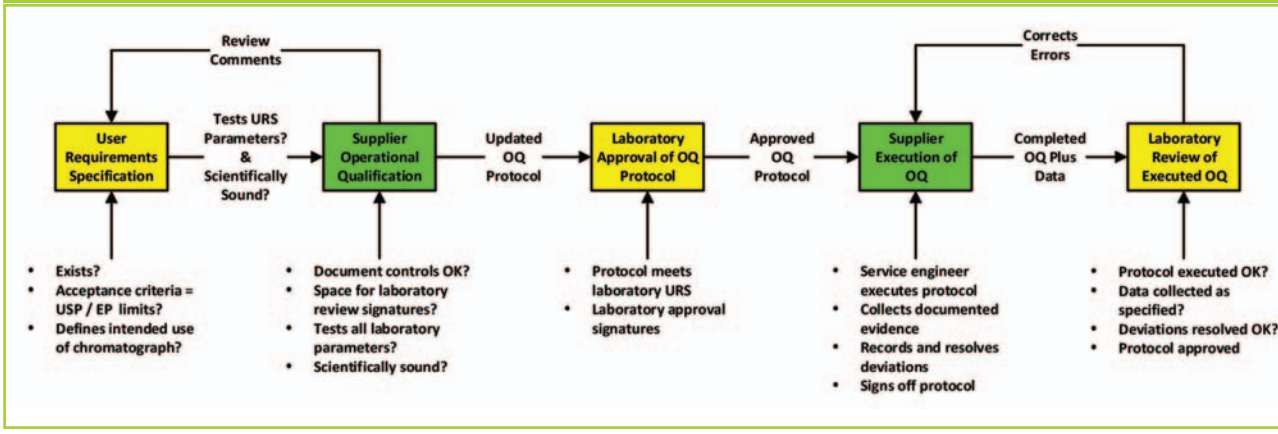
- Where is the user requirement for flow rate and acceptance criteria? The paper does not mention the instrument specification
- Flow rate accuracy is measured but there is only one measurement, therefore flow rate precision cannot be determined
- Is the stopwatch calibrated? Probably not, as it does not say.
- Is there a column attached to the pump? The paper does not say, but if not then the measurement does not cover intended use.

Compliance by stupidity? Yes!

- The normal qualification approach would be to measure the lower and upper limit of flow rate. Interpolation is acceptable, extrapolation is not.
- In the example, an additional middle flow rate is added "just in case".
- There is no objective evidence of the measurement, only a single observation by a person. Yet, the laboratory has agreed to this approach.

Instead, simplify the process by purchasing a calibrated digital flow meter and conduct six measurements at the top and bottom flow rates of the user specification. This is faster and more accurate than shown above and if the flow meter is linked to a printer or laboratory informatics application

Figure 2: Tasks involved with defining and executing an operational qualification.



there is objective evidence of the test as well as better data integrity.

Our second example is a UV diode array HPLC detector. Apart from the initial wavelength check during the first OQ, why would you want to check the wavelength again? This last sentence may raise some eyebrows. Why would you not check wavelength accuracy? Remember, know your instrument: the diode array is superglued to the optical bench. It does not move. Therefore, why check the wavelength annually? Most chromatographers will take the path of least resistance and perform an unnecessary qualification because it is easier to defend than take a justified risk-based approach.

Tests performed on a chromatography instrument during qualification typically fall into the following categories:

- Metrology performance (for example, flow, temperature, pressure)
- Checks versus reference materials (wavelength accuracy)
- Detector noise evaluation
- Injection-based tests (for example, injector precision, accuracy, linearity, and carryover)
- Usage-based tests (typically requires an injection to "trigger" another measurement such as gradient formation but are not specific to the injector)

The URS becoming the most important life cycle document will challenge many laboratories current interpretation of AIQ and *USP* <1058> (based on how the 2008 version was implemented where instrument specification was the responsibility of the supplier). In the absence of a strong industry or regulatory driver to harmonize AIQ, many laboratories have implemented a diverse interpretation of *USP* <1058> requirements. Although there is variation from laboratory to laboratory, most quality control (QC) laboratories have a clear understanding of <1058> requirements and what should be included in an OQ and will typically be comfortable with testing the instrument range of use based on current applications.

In contrast, R&D laboratories are more likely to base requirements on the instrument specification, some of which cannot be measured effectively outside of controlled conditions. Thermal drift of the instrument environment contributes significantly to detector drift measurements for an HPLC system. This is why "factory testing" of the instrument occurs in a controlled environment and is a scientific requirement to test the instrument noise and drift specification. However, this causes a challenge when performing detector OQ tests if the laboratory tries to test the instrument performance to the instrument specification, because the laboratory temperature control is not as good as the controlled environment used at the factory. This is an example of where trying to test an instrument to its specification may be less than scientifically valid.

Do I Need a Contract?

EU GMP Chapter 7 is focused on outsourcing. Using a third party such as the supplier or a service agent is outsourcing and as required by Chapter 7 (10):

> 7.1. There should be a written Contract covering the outsourced activities, the products or operations to which they are related, and any technical arrangements made in connection with it.

A contract or agreement outlining the roles and responsibilities between the laboratory and the supplier is essential for any qualification work. However, what if you use an internal supplier such as an in-house metrology department? We suggest that the approach offered by EU GMP Annex 11 clause 3.1 (11) is appropriate here: there needs to be an agreement with third-party suppliers and the same applies to IT departments. An agreement should also apply to in-house service groups performing qualification services. Do you have a quality or technical agreement with your metrology department?

OQ Tests Must Reflect the Laboratory URS

The new version of *USP* <1058> makes the following statements related to OQ:

> OQ demonstrates fitness for the selected use, and should reflect URS.
>
> Holistic tests, which involve the entire system, demonstrate that the whole system complies with URS (1).

Therefore, the principle for any qualification is that experiments should be designed to reflect the

way that you work and that are documented in an instrument URS.

However, the big problem is that most laboratories do not have a user requirements specification for their instruments. Many chromatographers don't see the need for an instrument specification, but definition of intended use is a requirement of all GXP regulations (12–15). Why can't you use the supplier's specification? Our response is read Part 1 of this series (2) and then go and write one.

The URS is an essential requirement for input into the OQ protocol; without one you cannot qualify an analytical instrument. Many laboratories just use a supplier's OQ protocol and ignore the first two stages shown on the left of Figure 2.

Review and Approval of OQ Protocols

The new *USP* <1058> states:

> For OQ test packages purchased from a service provider or supplier, the user must review the material to assure themselves of the scientific soundness of the tests and compliance with applicable regulations (1).

There are several points to consider that arise from this sentence (Figure 2):

- Are the document controls of the OQ adequate?
- Do the tests match your URS requirements?
- If not, can the supplier configure the OQ to match your requirements (such as adding additional tests and set points) or will the laboratory have to conduct additional qualification experiments?
- How will any "gaps" be managed?
- Are the acceptance criteria set to limits defined in a pharmacopoeial general chapter?
- Is each test in the OQ scientifically sound, for example, is it conducted with calibrated instruments, traceable reference standards, etc?
- How is documented evidence of each test collected and managed?
- Does the laboratory have the electronic records of the testing available for later review?
- Is there space for the laboratory to sign off the final OQ protocol prior to execution?
- Does the laboratory's organization require a quality assurance (QA) signature prior to execution?

Why? See our discussion later in this column.

Most qualification protocols are paper-based documents and, as such, will typically include instructions for performing the test, spaces to write in the results, tables for documenting deviations (where deviations from the protocol are documented), and a list of attachments. A paper OQ protocol is almost a "stand-alone" quality system. In contrast, as laboratories consider implementation of electronic protocols, the content and format of the protocol does not need to be the same as the paper-based approach (for example, the instructions will be in the application used to execute the qualification and generate the report).

Laboratory Training and Understanding

The laboratory is always responsible for the quality, data integrity, and scientific validity of any AIQ work performed, irrespective of who performs the work (1,14). Historically, where the instrument manufacturer performs an OQ, many laboratories may have approved OQ protocols without a thorough understanding of the test design and how the work will be performed. This "manufacturer knows best" interpretation of <1058> requirements can result in problems during a regulatory audit or inspection—the highest risk a laboratory can take during an audit is to try and explain something they don't understand! The change in FDA reporting practices means it is not unusual for software vendors and instrument manufacturers to be named in an FDA warning letter or 483 report. This means that laboratories who approve documents without understanding the content represent the greatest risk to the perceived reputation of the supplier. Historical laboratory audits and defence processes (pre-2010) tended to concentrate on the scientific validity of the information. In data integrity focused audits, providing evidence that the results of the OQ are not fraudulent represents a very different kind of audit.

This means that companies performing OQ testing should provide training to laboratories on the OQ protocol, including how to review the electronic information and associated metadata from the OQ. Some OQ tests are performed manually, typically those based on metrology measurements, and currently this can be interpreted as a data integrity risk. Until secure, fully electronic measurement tools are developed (for example, for an HPLC flow meter), there are no other ways the work can be performed. The options available to the laboratory include second person observation of the test being performed. When performing laboratory analysis, sample preparation is one of the most critical steps. Should all sample preparation be observed by a second person?

Where there is an OQ test failure, this can represent a high focus area for an auditor. Because of this, it is not unusual for some laboratories to request that the OQ qualification report does not contain any deviations (by implication, if there is a test failure, don't include it in the report!). How often an OQ test fails will depend on how tight the OQ specification is for the test. If a laboratory has implemented very tight limits for a test, the test is more likely to fail, prompting additional work for the laboratory and an investigation into the potential impact of the instrument failure on analytical results generated since the previous OQ. Qualification work must include all results, which is easier to enforce with electronic protocols and supporting software, rather than relying on procedural control (instructions) of paper protocols.

One of the areas that needs to be clearly understood relates to limits defined in pharmacopoeia general chapters and drug testing monographs. Instruments must be capable of meeting the requirements of the pharmacopoeia, but this does not have to be performed during the OQ. This is where the performance qualification (PQ) and the valid role of meaningful SSTs come in as part of the continuum of qualification. For example, injection precision is a requirement that is almost always included in SSTs when performing chromatographic testing. The requirements specific in *USP* <621>

(16) must be satisfied. Injection precision tests are included in OQ protocols, but they are based on the test design of the OQ protocol and associated methodology, not on pharmacopoeia requirements. As well as being scientifically valid and developed through an appropriate quality life cycle process, OQ tests are typically developed by suppliers to test instruments in a time efficient way. A supplier or service provider could test every function or combination of parameters of an instrument during an OQ protocol, but this would significantly increase the duration of the OQ protocol to several days as well as the cost. The qualification continuum extends qualification thinking beyond the OQ and across multiple stages of the life cycle. It is this extended continuum that provides robust assurance of the performance of the instrument.

Review and Approval Signatures

USP <1058> is very explicit in this respect:

> The user should review the documents before execution and approve the tests after execution to ensure completeness and accuracy of the completed document and the test data generated (1).

Who in the laboratory is going to review and approve qualification protocols and are they trained?

Technical personnel such as those in the laboratory should review and approve the OQ protocol not QA, who may not have the necessary understanding of the underlying analytical technique. This raises a complication: under European GMP, the qualified person (QP) is legally responsible for the quality of the medicines—should they approve the OQ protocol?

Just as many laboratories have approved OQ protocols without fully understanding the OQ test design and rationale, many have also approved the OQ report without a detailed understanding of the information or electronic review of the data. Many laboratories may have a policy where an external service provider cannot access or use the laboratory's chromatography data system (CDS). This means that the service provider has to control the instrument using software run from a laptop, which the laboratory does not have control over. Additionally, unplugging the instrument from the network to plug into a laptop possibly running different CDS software to that used in the laboratory is not testing the instrument under actual conditions of use. How can the laboratory review audit trail entries if the computer where the records reside is taken off site at the end of the qualification? It is better to work with service providers and define appropriate CDS access for the service personnel to perform the work.

As can be seen in Figure 2, there is typically iteration to agree the OQ protocol before execution and then post-execution approval.

- Pre-execution review should be simple and efficient, not convoluted.
- If there are changes to be made to the OQ protocol, does the supplier,

laboratory, or both change control procedures apply?
- Should a supplier refuse to perform work if the protocol has not been approved?
- Should a risk-based approach be applied to review of qualification e-records or should all records be reviewed?

Not reviewing and approving qualification protocols can seriously damage your compliance reputation as seen in 2000 when Spolana, a Czech active pharmaceutical ingredient (API) supplier, received a warning letter with the following citation (17):

> 1. Written procedures had not been established for the calibration of analytical instruments and equipment in the Quality Control laboratories used for raw material, finished API and stability testing. Furthermore, calibration data and results provided by an outside contractor were not checked, reviewed and approved by a responsible Q.C. or Q.A. official
>
> 2. The <redacted> systems calibrated by an outside contractor did not include verification of the precision (% RSD) of the autoinjector at more than one injection volume, the flow rate below 1 ml/min, or the wavelength accuracy for the wavelength regions used for testing of <redacted>

Although this warning letter is nearly 20 years old, the warning is clear. The laboratory is responsible for the technical content and compliance with regulations of the OQ protocols.

Roles and Responsibilities

The role of the laboratory is defined clearly in *USP* <1058> (1):

> For OQ test packages purchased from a service provider or supplier, the user must review the material to assure themselves of the scientific soundness of the tests and compliance with applicable regulations. The user should review the documents before execution and approve the tests after execution to ensure completeness and accuracy of the completed document and the test data generated.

The supplier of the OQ protocol has the following responsibilities:
- They must provide base OQ protocols.
- They must clarify roles and responsibilities between the two parties for update, review, execution, and approval of the work.
- Is the protocol static where no changes can be made or dynamic where the testing can be adapted to a laboratory's specification?
- The engineer should be trained not just in executing the protocol but also in data integrity (ALCOA principles) with certification.
- The engineer must not falsify data and must record all work including deviations and their resolution.
- All AIQ activities should be predefined and contemporaneously documented (1).
- Electronic data and the means to read it must be left with the laboratory if the user's CDS is not used.

Don't Forget the Software!

Although this discussion is focused on the qualification of the chromatograph system, it is important that the CDS software is not forgotten. The new *USP* <1058> (1) integrates qualification of the instrument with validation of the software to reflect what happens in practice with Group C instruments: the instrument needs the software and the software needs the instrument to operate. For the most part, the new *USP* <1058> is now aligned with GAMP 5 principles (18).
- For the installation of a new system there will be both software and instrument qualification protocols (3,19).
- If the laboratory's CDS is going to be used to execute the instrument OQ, then create a supplier account where the identity of the engineer executing the test can be identified directly or indirectly. Never use the default account in a CDS for performing qualification.
- A periodic review of the overall system is the equivalent of an instrument preventative maintenance and regular OQ.

Dude, Where's My Data?

In an ideal world, the instrument should be controlled through the laboratory CDS during OQ protocol execution, with all the chromatographic data generated in a single location and under laboratory control. The data generated during the OQ execution are essential to support a claim that any instrument is qualified. If an organization does not allow a supplier to access the CDS, the OQ protocol will be performed using the engineer's laptop and the data will reside there—not an ideal solution. A situation could arise that the engineer leaves the laboratory and there are only paper records available—a worse situation still. Where data are provided on a CD at the end of the qualification work, this is still a higher risk than having the data stored and backed up automatically within the laboratory's IT domain (CDs have to be managed through procedural control and can go "missing"!). Regardless of how an OQ is executed, does the engineer have access to the system clock, the data in the operating system directories, and the recycle bin?

Again, in an ideal world, the protocol should be executed and signed electronically, rather than having a hybrid situation of electronic records in a CDS and signed paper printouts. This is the worst situation.

Summary

In part 2, we have discussed the qualification continuum from URS to OQ and PQ. The OQ protocol must be based on the requirements in the laboratory URS for the chromatograph system. Users must review the protocol prior to execution to check for scientific soundness and coverage vs. their URS. The supplier executes the reviewed protocol and the trained engineer collects the data, completes the protocol, and resolves any deviations. The executed protocol is then collected by an engineer trained both in execution of protocol and ALCOA principles. The user reviews after execution both protocol execution and data collected.

The instrument is then released for operational use. In Part 3 we will look at the new requirements for performance qualification.

References

(1) General Chapter <1058> "Analytical Instrument Qualification" in *United States Pharmacopeia* 41 (United States Pharmacopeial Rockville, Maryland, USA, 2018).

(2) P. Smith and R.D. McDowall, *LCGC Europe* **31**(7), 385–389 (2018).

(3) R.D. McDowall, *Validation of Chromatography Data Systems: Ensuring Data Integrity, Meeting Business and Regulatory Requirements* (Second Edition, Royal Society of Chemistry, Cambridge, UK, 2017).

(4) R.D. McDowall, *Data Integrity and Data Governance: Practical Implementation in Regulated Laboratories* (Royal Society of Chemistry, Cambridge, UK, 2018).

(5) General Chapter <1058> "Analytical Instrument Qualification" in *United States Pharmacopeia* 31 (United States Pharmacopeial Rockville, Maryland, USA, 2008).

(6) AAPS White Paper on Analytical Instrument Qualification (American Association of Pharmaceutical Scientists, Arlington, Virginia, USA, 2004).

(7) R.D. McDowall, *Spectroscopy* **25**(11), 24–31 (2010).

(8) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) Guidelines, Annex 15 Qualification and Validation*

(9) D. Gowrisankar *et al., Journal of Biomedical Science and Research* **2**(2), 89–99 (2010).

(10) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) Guidelines, Chapter 7 Outsourced Activities* (European Commission, Brussels, Belgium, 2013).

(11) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) Guidelines, Annex 11 Computerised Systems* (European Commission, Brussels, Belgium, 2011).

(12) 21 CFR 58, *Good Laboratory Practice for Non-Clinical Laboratory Studies* (Food and Drug Administration, Washington, DC, USA, 1978).

(13) OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 1, OECD Principles on Good Laboratory Practice (Organisation for Economic Co-operation and Development, Paris, France, 1998).

(14) 21 CFR 211, *Current Good Manufacturing Practice for Finished Pharmaceutical Products* (Food and Drug Administration, Silver Spring, Maryland, USA, 2008).

(15) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) Guidelines, Chapter 3 Premise and Equipment* (European Commission, Brussels, Belgium, 2014).

(16) United States Pharmacopeia General Chapter <621> "Chromatography" (United States Pharmacopoeia Convention, Rockville, Maryland, USA).

(17) FDA, Warning Letter Spolana (Food and Drug Administration, Rockville, Maryland, USA, 2000).

(18) L. Vuolo-Schuessler *et al., Pharmaceutical Engineering* **34**(1), 46–56 (2014).

(19) GAMP Good Practice Guide A Risk Based Approach to GXP Compliant Laboratory Computerised Systems, Second Edition (International Society for Pharmaceutical Engineering, Tampa, Florida, USA, 2012).

Paul Smith is a Global Strategic Compliance Specialist at Agilent Technologies. After initially specializing in spectroscopy and application of chemometrics to spectroscopic data, Paul developed his compliance expertise in a variety of quality and management roles within the 17 years he spent in the pharmaceutical industry. Paul worked as an independent consultant and university lecturer before moving into laboratory compliance consultancy roles.

"Questions of Quality" editor **Bob McDowall** is Director at R.D. McDowall Ltd., Bromley, Kent, UK. He is also a member of *LCGC Europe's* editorial advisory board. Direct correspondence about this column to the editor-in-chief, Alasdair Matheson, at alasdair.matheson@ubm.com

LC–MS Sensitivity: **Practical Strategies to Boost Your Signal and Lower Your Noise**

Sharon Lupo, Restek, Bellefonte, Pennsylvania, USA

Liquid chromatography–mass spectrometry (LC–MS) has become the preferred analytical technique for many challenging assays based on its selectivity, sensitivity, and broad applicability to compounds of varying polarity. Despite its advantages, the complexity of LC–MS systems often leaves analysts struggling to meet method detection limits. In this instalment of "Column Watch", several strategies will be discussed to improve method sensitivity through the reduction of contaminants, the careful selection of LC method conditions, and the optimization of MS interface settings. By understanding the relationship between these parameters and ionization efficiency, analysts can enhance their signal-to-noise ratio and realize the hidden potential of their LC–MS.

The term "sensitivity" can have several meanings in mass spectrometry (MS) that are often used interchangeably. Sensitivity may be defined as the change in signal for unit change in concentration of an analyte (such as the slope of the calibration curve) (1). More commonly, it is used to reference the magnitude of the signal produced by the analyte in the MS detector. In this latter usage, MS sensitivity is often used to compare detectors.

Fundamentally, the ability of a detector to provide quantitative data is a function of the signal-to-noise ratio (*S/N*) for an analyte. The limit of detection (LOD) is determined from the analyte *S/N* and is the lowest concentration of a substance where its signal can be distinguished from system noise (2). As shown in Figure 1, the higher the sensitivity of the MS system, the greater the value of *S/N* for a given method LOD if background noise remains constant. Therefore, improvements in sensitivity can occur through manipulation of *S/N*. MS optimization, sample pretreatment strategies, mobile-phase composition, and LC column characteristics are all integral to ionization efficiency and will improve analyte signal when optimized. Likewise, limiting contaminants that contribute to signal suppression or adduct formation may also enhance response.

MS Optimization

In liquid chromatography–mass spectrometry (LC–MS), sensitivity directly relates to the effectiveness of producing gas-phase ions from analytes in solution (ionization efficiency) and the ability to transfer them from atmospheric pressure to the low pressure zone of the MS system (transmission efficiency) (3). The optimization of ionization and transmission efficiency is dependent on the LC method parameters and the target analyte or analytes. To make the appropriate adjustments, it is necessary to have a basic understanding of the mechanisms taking place within the MS source.

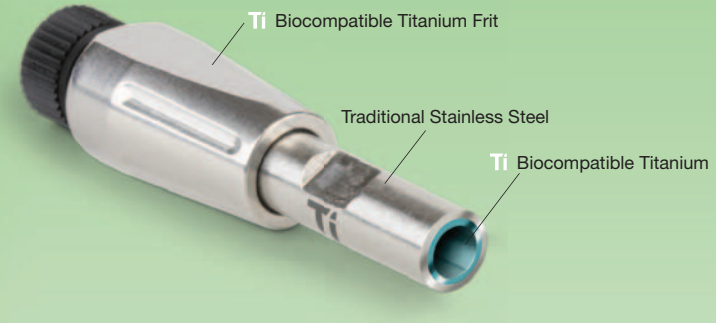
Choosing the appropriate polarity is the first step in developing a sensitive LC–MS method. The capillary polarity is selected to match the charge of the analytes of interest.

Electrospray ionization (ESI) is one of the most popular ionization techniques; therefore, it will be the focus of this column instalment. It is important to note, however, that optimization of the source parameters is necessary regardless of the ionization mode selected. As the LC mobile phase flows into the sample capillary, positive and negative ions are separated based on the polarity chosen. In positive ESI, the negative ions are neutralized on the capillary wall, and the positive ions continue with the mobile phase to the capillary tip where the charged analytes accumulate into a droplet. Under the influence of an applied voltage, a Taylor cone is formed (4). Electrostatic repulsion causes the cone to break up into small, electrically charged droplets, which then travel towards the sampling orifice under the guidance of the applied potential difference between the capillary tip and the sampling plate. As the tiny droplets progress towards the orifice, the solvent evaporates with the aid of drying gas and heat, causing the droplet surface area to decrease and an increase in charge density. Ultimately, repulsive forces overcome the droplet surface tension and it explodes into even smaller droplets. The process repeats itself until the droplets are so small that gas-phase ions are emitted (5). The cloud of ions formed is known as *the ion plume*.

Choosing the appropriate polarity is the first step in developing a sensitive LC–MS method. The capillary polarity

Not Zen

Mucho Zen

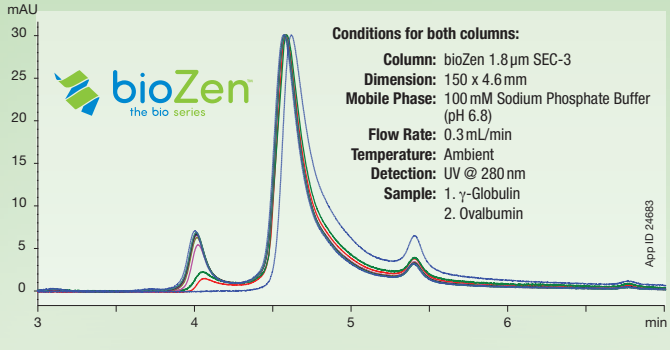


bioZen Biocompatible LC Columns for:

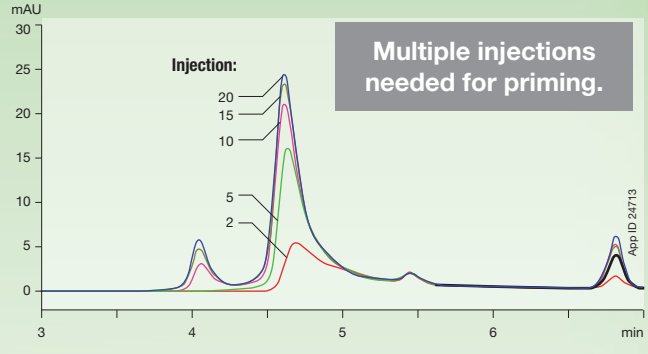
- Peptide Mapping
- Aggregate Analysis
- Glycan Analysis
- Peptide Quantitation
- Drug Antibody Ratio
- Intact Mass
- Intact and Fragment Analysis

Overlaid Successive Injections – Protein Priming Comparison

bioZen Titanium BioTi™ Hardware



Traditional Stainless Steel



PAS9530318_W_1

phenomenex®
...breaking with tradition℠

www.phenomenex.com/muchozen

Figure 1: A hypothetical demonstration of the effect of increased sensitivity on limits of detection (LOD) assuming linear calibration and fixed background noise.

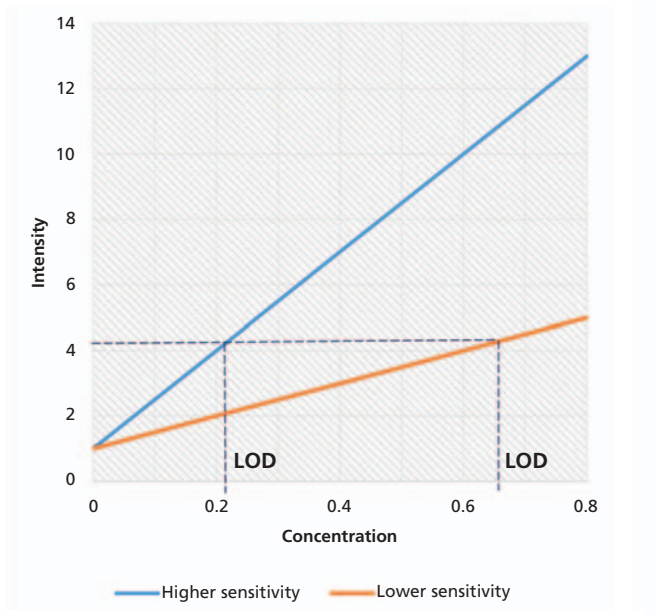
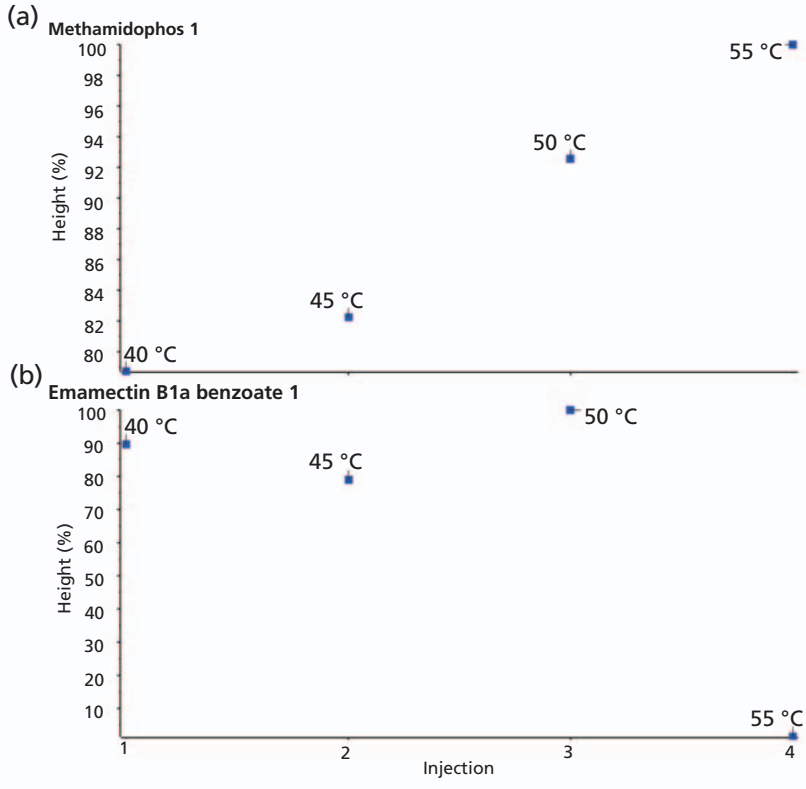


Figure 2: LC–MS/MS pptimization of desolvation temperature for (a) methamidophos and (b) emamectin B1a benzoate over four successive injections. Column: 100 mm × 2.1 mm, 3-µm fully porous C18; mobile-phase A: water + 2 mM ammonium acetate + 0.1% formic acid; mobile-phase B: methanol + 2 mM ammonium acetate + 0.1% formic acid; gradient %B (time): 5% (0 min), 5% (1.5 min), 70% (6 min), 70% (9 min), 100% (10 min), 100% (12 min), equilibrate; flow rate: 0.5 mL/min; polarity: ESI+; curtain gas: 30 psi; nebulizer gas: 45 psi; drying gas: 55 psi; capillary voltage: 5.5 kV; collision gas: 10 psi.



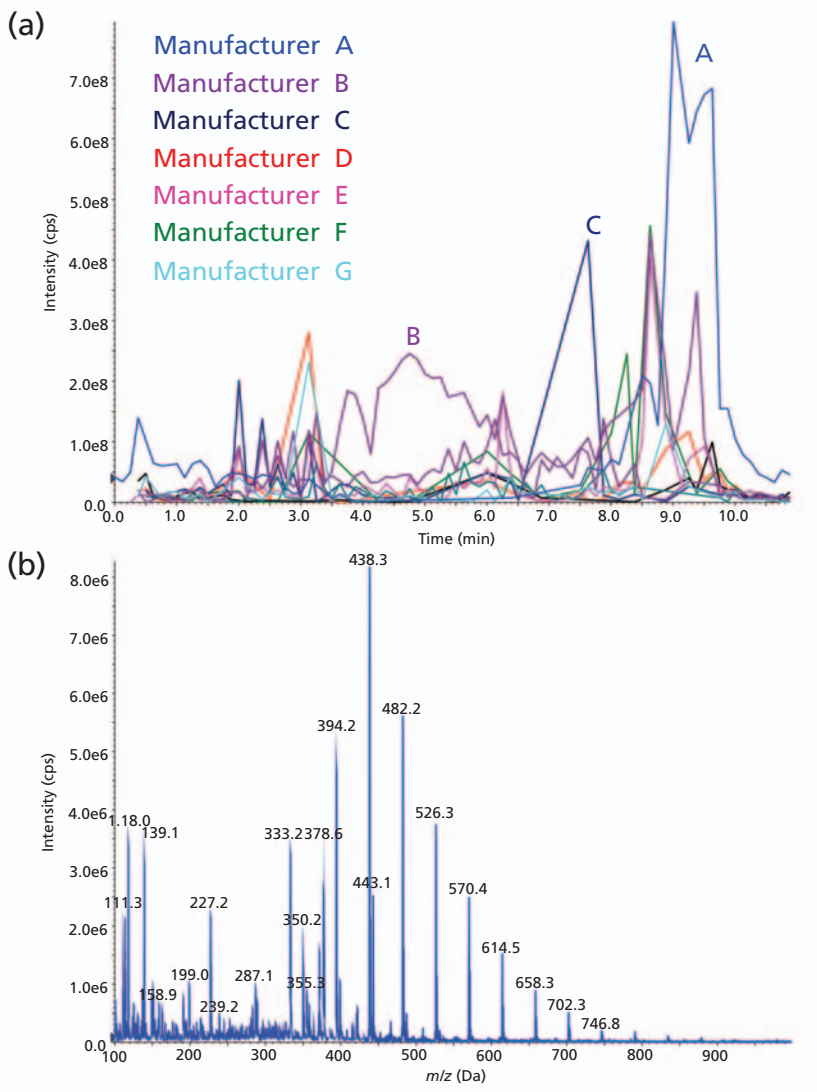
is selected to match the charge of the analytes of interest. Typically, basic analytes will ionize most efficiently in positive ion mode by accepting a proton $(M+H)^+$, while acidic analytes will produce the strongest signal in negative ion mode by donating a proton $(M-H)^-$. However, it can be difficult to predict the best polarity mode for more-complex molecules. In addition, analyte behaviour and response varies by instrument platform. Therefore, it is beneficial to screen analytes using both polarity modes during initial method development or when transferring an existing method to a new instrument (6).

At faster flow rates, the capillary tip should be placed further from the sampling orifice to allow for adequate desolvation and an increased number of fission events.

Ionization efficiency is strongly influenced by flow rate, mobile-phase composition, and the physicochemical properties of the target analytes. The capillary voltage setting is dependent on the analytes, eluent, and flow rate and can have a significant impact on method reproducibility. The applied potential difference between the capillary tip and sampling plate is responsible for maintaining a stable and reproducible spray (7). Problems with variable ionization and precision can arise if the capillary voltage is set incorrectly. Optimal nebulizing gas flow and temperature are also eluent dependent. The nebulizing gas constrains the growth of the droplet while charge accumulates and also affects the size of the droplets emitted from the capillary. The nebulizing gas flow and temperature should be increased for faster LC flow rates or when using highly aqueous mobile phases. Similarly, drying gas flow and temperature can be critical for effective desolvation of the LC eluent and the successful production of gas-phase ions. As a caution, when analyzing thermally labile analytes, care must be exercised to prevent their degradation in the source.

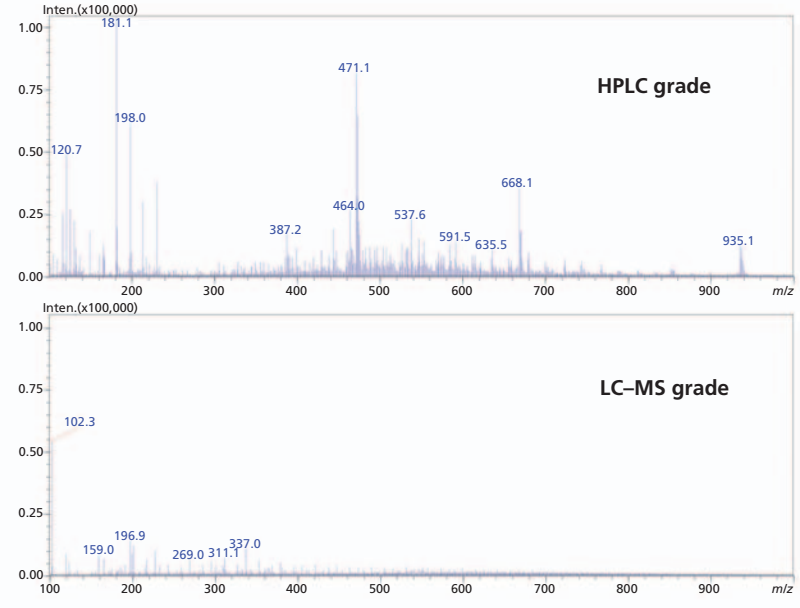
The location at which gas-phase ions are produced within the ionization source is important for optimal

Figure 3: Contaminants extracted with acetonitrile from polymeric solid-phase extraction reversed-phase 96-well plates and analyzed by LC–MS/MS: (a) Overlay of background subtracted TIC from seven manufacturers. (b) Averaged spectra collected from peak C located at 6.5–8 min. Column: 100 mm × 2.1 mm, 2.7-µm superficially porous C18; mobile-phase A: water + 1 mM ammonium acetate + 1% acetic acid; mobile-phase B: methanol; gradient %B (time): 5% (0 min), 100% (8 min), 100% (9 min), equilibrate; flow rate: 0.5 mL/min. (Methodology developed by Hua and Jenke, reference 10.).



transmission into the MS system. The size of the ion plume is dependent on the number of fission events required to emit gas-phase ions and its distance from the sampling orifice. Sampling of the ion plume can be optimized by adjusting the position of the capillary tip in relation to the orifice based on the LC flow rate. At faster flow rates, the capillary tip should be placed further from the sampling orifice to allow for adequate desolvation and an increased number of fission events. Although extending the distance will allow for an increased number of gas-phase ions to be produced, repulsive forces will also increase proportionally, causing the size of the ion plume to expand and the density of gas-phase ions to be reduced. As a result, the number of ions entering the sampling orifice could decrease, causing a drop in signal intensity (3). At slower flow rates, smaller droplets are formed, allowing the capillary tip to be placed closer to the sampling orifice. Smaller droplets desolvate more easily and require fewer fission events, reducing the impact

Figure 4: Comparison of averaged spectra for HPLC-grade methanol and LC–MS-grade methanol. Mobile phase: unmodified methanol as indicated; flow rate: 0.5 mL/min; system: LC–MS with ESI+ ionization; scan range: 100–2000 *m/z*.



of repulsive forces and inhibiting the size of the ion plume. The decreased distance between the capillary tip and sampling orifice increases ion plume density and improves analyte ionization efficiency and transmission (3).

Optimization of the ionization source parameters described above could potentially bring sensitivity gains of two- to threefold as demonstrated by Szerkus and colleagues for the analysis of 7-methylguanine and glucuronic acid in urine (8). When optimizing the source conditions, it is important to use the intended LC mobile phase and flow rate. One method of optimization is to inject a standard solution several times and alter a specific source parameter stepwise with each injection. Figure 2 demonstrates this process for the evaluation of optimal desolvation temperature for two pesticides: methamidophos and emamectin B1a benzoate. A 20% increase in response for methamidophos was achieved by increasing the desolvation temperature from 400 °C to 550 °C. In contrast, emamectin benzoate B1a experiences complete signal loss if the desolvation temperature is increased beyond 500 °C because of the thermal lability of that compound. Alternatively, source conditions may be optimized by teeing a constant flow of analyte into the LC eluent and monitoring the analyte TIC. This technique allows for adjustments to be made on the fly. Methods using gradient elution of multiple compounds should be optimized by estimating the organic concentration at the time of elution. Although this step can be overwhelming, the process can be simplified by concentrating efforts on only critical or low intensity analytes.

Sample Pretreatment

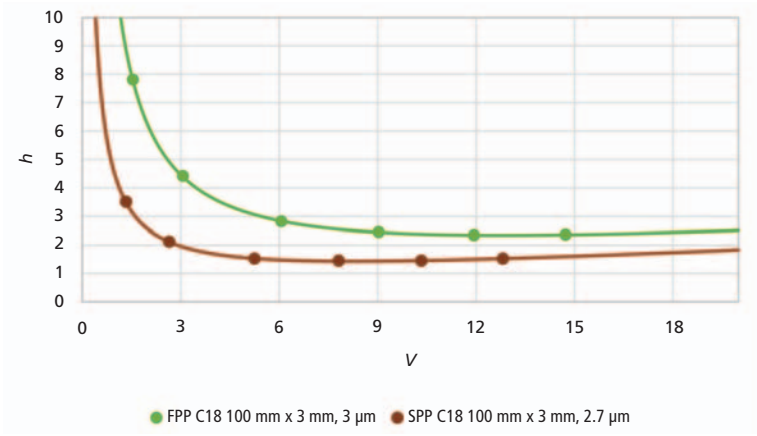
Sample pretreatment is an essential part of the LC–MS analytical workflow, particularly when analyzing complex samples containing target analytes at low concentrations. Removal of nontarget sample components can minimize matrix interferences and improve the *S/N* ratio for the analytes of interest. Matrix compounds coeluted with a target analyte may cause suppression or enhancement of the analyte signal; these interferences are known as *matrix effects*. Matrix effects often manifest as a loss in MS sensitivity or specificity and are prevalent in ESI because of the potential for charge competition on the droplet surface prior to emitting gas-phase ions. As an alternative, atmospheric pressure chemical ionization (APCI) may be employed if the analytes of interest are thermally stabile and of moderate polarity (1). In APCI, the LC eluent is completely evaporated into a gas before ionization by the applied voltage of the corona needle. The ionized mobile-phase vapour then reacts with the analyte molecules to produce charged ions. Matrix effects tend to be less extensive in APCI since ions are produced through gas-phase reactions instead of liquid-phase reactions (9).

Removal of nontarget sample components can minimize matrix interferences and improve the S/N ratio for the analytes of interest.

Various sample preparation strategies are available to extract target analytes from potential interfering matrix components. The appropriate technique is dependent on the sample matrix, sample volume, target analyte concentration, and analyte physicochemical properties. If the sample is clean and known to contain high concentrations of the target analyte, simple filtration and dilution is a quick and convenient way to reduce the concentration of potential interferences. On the other hand, complex samples known to contain low target analyte concentrations will require a more rigorous extraction procedure to improve signal intensity. Although more stringent sample preparation procedures may not be desirable because of the cost and time investment required, injecting cleaner samples will decrease the likelihood of matrix effects from endogenous interferences while simultaneously increasing analyte response and instrument reproducibility.

Regardless of the sample preparation technique chosen, it is important to consider that matrix effects can result from the presence of endogenous or exogenous substances. Whereas endogenous constituents are already present in the sample (proteins, lipids, pigments, and so forth), exogenous compounds are introduced into the sample during the sample pretreatment process. These compounds can leach from plastics used in centrifuge tubes, well plates, and pipette tips and may include by-products and residues

Figure 5: Van Deemter plot comparing efficiency between a 3-µm fully porous C18 column and a 2.7-µm superficially porous C18 column. Mobile-phase A: 45% water; mobile-phase B: 55% acetonitrile; detection: photodiode array, 254 nm; injection volume: 1 µL; sample: 0.03 mg/mL biphenyl prepared in 25:75 acetonitrile–water.



from the manufacturing processes (for example, molding agents, plasticizers, stabilizers, and releasing agents). The amount and type of contaminants varies from manufacturer to manufacturer, as shown in Figure 3(a). In this experiment, contaminants extracted from polymeric solid-phase extraction (SPE) reversed-phase 96-well plates were compared for seven manufacturers. The extracts were analyzed by LC–MS, and the resulting data were background subtracted to remove contributions from the solvent and the analytical column. An overlay of the resulting chromatograms shows the presence of multiple chemical contaminants between the various manufacturers. The spectra for polyethylene glycol (PEG) was clearly identified in manufacturer C based on the series of repeating ions separated by 44 Da (Figure 3[b]).

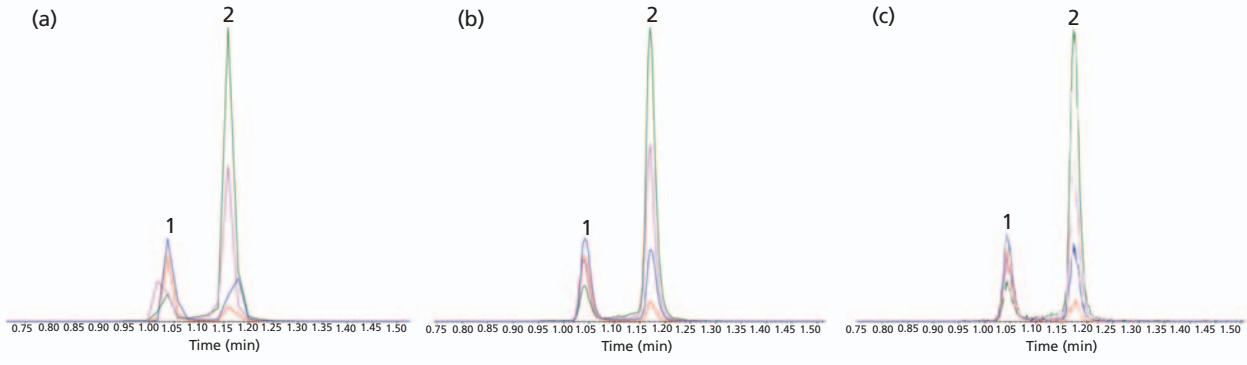
Other sources of exogenous chemicals include glassware (especially when cleaned with detergents), the use of non-MS-grade solvents and additives, or careless work practices that can introduce chemicals from the skin or surrounding environment. Without appropriate laboratory procedures and careful screening of sample pretreatment products, it is possible to inadvertently introduce contaminants into a sample. If samples are subjected to a concentration step, a decrease in *S/N* ratio may be observed, because both analytes and contaminants will be concentrated (9).

Mobile-Phase Composition

The mobile phase plays a key role in LC–MS sensitivity by influencing the retention and ionization of target analytes. The use of high-purity solvents and additives is of utmost importance to prevent unwanted adduct formation and increased MS background. Similarly, only ultrapure water from a water purification system or bottled water suitable for LC–MS should be used for mobile-phase preparation. LC–MS spectra collected for MS-grade and HPLC-grade methanol showed significantly increased impurities in the HPLC-grade methanol, particularly in the low-molecular-weight ranges common for small-molecule analysis (Figure 4). It is apparent from this data how the use of lower grade solvents could contribute to reduced sensitivity and convoluted spectra, making accurate quantitation or spectra interpretation difficult. Mobile phases should be stored in borosilicate glass containers and "topping off" solvents should be avoided to prevent the accumulation of contaminants.

The incorporation of volatile buffers and acids into the mobile phase enables control over the ionization state of the target analytes so that retention can be manipulated. Analyte retention affords the LC–MS analyst several advantages. First, increased retention of analytes means a higher organic solvent concentration is required to elute the analyte from the column during gradient LC. It has been shown that droplets with a higher

Figure 6: Comparison of data collected with dwell times of (a) 300 ms, (b) 50 ms, and (c) 5 ms, and their contribution to time-related band broadening. System: LC–MS/MS; polarity: ESI+. Peaks: 1 = morphine, 2 = hydromorphone.



organic concentration are desolvated more efficiently in the MS source, leading to improved MS sensitivity (11). Second, greater chromatographic selectivity makes it possible to avoid coeluted matrix effects that can be detrimental to analyte response. Areas of retention and matrix suppression can be monitored chromatographically by simultaneously infusing the analyte post column while performing an LC injection of an extracted blank matrix sample through the analytical column (12). Areas of matrix suppression are characterized by a decrease in analyte signal. In this way, analyte retention can be adjusted to avoid zones of significant suppression in the chromatogram.

Mobile-phase buffers and acids also affect ionization efficiency. This statement is especially true for ESI because it is susceptible to the reduction of detector response because of competition for ionization. To reduce the likelihood of buffer-induced suppression, concentrations should generally be kept to a minimum. Alternatively, mobile phases containing formic acid can minimize unwanted metal adducts. The excess in protons provided by the acid drives the majority of ion formation to the protonated molecule $[M+H]^+$, resulting in an overall improvement in response since it would no longer be distributed across multiple charged species (13).

Enhancements in ionization efficiency have been observed by donating protons in the case of an acid modifier in positive-ion mode or by accepting protons in the case of a basic modifier in negative-ion mode. The latter was demonstrated for the negative ionization of two neutral estrogens, estrone and estriol, where their response triples when they are prepared in diluent containing 0.2% ammonium hydroxide compared to one that contains 0.2% acetic acid (14). Buffer salts containing ammonia (for example, ammonium formate or ammonium acetate) can increase the ionization efficiency of polar neutral compounds that cannot be ionized on their own by forming ammonium adducts. Ammonium salts can be used to prevent the formation of unwanted adducts by providing a constant supply of ammonium. For example, the LC–MS analysis of two cardiac glycosides, digoxin and digitoxin, is performed almost exclusively with ammonium formate modified mobile phases. Without ammonium formate, these compounds tend to form sodium adducts, which are difficult to fragment when analyzed by tandem MS (15).

LC Column Characteristics

The desire for increased LC–MS sensitivity has trended towards the implementation of highly efficient LC columns using smaller particles (sub-2 µm) in combination with reduced column diameters (≤ 2.1 mm). The introduction of superficially porous particles (SPPs) has allowed for increased efficiency while reducing system pressure when compared to fully porous particles (FPPs). High efficiency columns theoretically translate to improved sensitivity; however, LC–MS system extracolumn volume, ionization efficiency, and data sampling rates must be considered to fully realize the benefits.

The ability of a column to provide narrow chromatographic peaks is characterized as its efficiency (*N*), and is defined by its plate height (*H*). The efficiency of a peak is a function of its width and retention time. There are several processes that contribute to peak broadening inside and outside of the column. The injector, connecting tubing, and detector are all sources of extracolumn peak broadening.

Inside the column, eddy diffusion (*A*), longitudinal mass transfer (*B*), and mobile-phase and stationary-phase mass transfer (*C*) all contribute to peak dispersion. Collectively, these terms make up the van Deemter equation:

$$h = A + B/v + Cu \qquad [1]$$

where h is reduce plate height and v is the mobile-phase linear velocity (2). The van Deemter equation serves as a basis from which column performance is compared.

One way to increase column efficiency is by decreasing the particle size. Decreasing the overall peak width will cause an overall increase in peak height. Assuming that detector noise remains constant, taller peaks result in improvements in *S/N* and a boost in sensitivity. Additionally, highly efficient peaks are likely to be more resolved, reducing the likelihood that matrix interferences will impact ionization efficiency.

Smaller-particle columns also allow for the use of faster optimal linear velocities, and by extension, faster flow rates—without experiencing significant losses in efficiency. Unfortunately, because of the mechanisms that govern ESI, faster flow rates are

generally a detriment to sensitivity since all eluent must be removed for successful formation of gas-phase ions. Although some manufacturers claim instrument compatibility with eluent flow rates up to 1 mL/min, the best performance for standard flow LC–ESI–MS systems has been reported to occur in the range of 10–300 µL/min (16). To accommodate small particles and their associated high linear velocities, 2.1-mm i.d. columns have become the preferred size for standard-flow LC–ESI–MS systems with optimal flow rates of 200–300 µL/min.

Changing the particle morphology is yet another way to improve column efficiency. Superficially porous particles are different from fully porous particles in that they have a thin porous shell surrounding a solid core. They are able to provide a significant increase in efficiency because of decreases in longitudinal diffusion (*B*) and eddy diffusion (*A*) that result from their narrow particle size distribution, reduced permeability, and rough surface exterior (17). Figure 5 compares the kinetic performance of a fully porous 3-µm C18 column to a superficially porous 2.7-µm C18 column of the same dimension using a van Deemter plot. The superficially porous column displays up to a 60% increase in efficiency over the fully porous particle.

Utilizing columns with narrow inner diameters minimizes analyte dilution, which takes place during the chromatographic separation. Because of on-column dilution, analyte sensitivity is inversely proportional to the square of the column inner diameter for concentration dependent detectors (18). Therefore, switching from a 2.1-mm i.d. column to a 0.3-mm i.d. column would theoretically increase sensitivity by a factor of 50, assuming the same volume of sample can be injected in both cases (19). Likewise, smaller inner diameter columns maintain the same linear velocity with reduced flow rates, which is beneficial in terms of ionization efficiency. The use of very slow flow rates (nanolitres per minute) has gained popularity for applications that require high sensitivity with limited sample amounts. At these flow rates, desolvation becomes so efficient that matrix effects actually cease to be a concern (1).

There are several implications when coupling high-efficiency, narrow-bore columns with mass spectrometry. The decrease in column inner diameter in conjunction with increased efficiency leads to substantial decreases in peak volume. Without minimizing extracolumn volume in the instrument, column performance will be compromised, making it difficult to realize any significant gains in sensitivity. Most extracolumn-volume contributions can be attributed to the LC system with MS contributions being negligible. However, the tubing used to interface the LC column to the MS system was found to be critical since this tubing is located post-column, where the focusing effects that compensate for band broadening do not occur (20). As a rule of thumb, the extracolumn volume should not exceed one-third of the peak volume of the narrowest peak in the chromatogram (21). For example, a 1.8-µm, 100 mm × 2.1 mm

column produces a peak volume of approximately 8 µL (16). Therefore, the maximum extracolumn volume should be <3 µL to negate system related losses to efficiency.

The easiest and most effective way to improve sensitivity is through optimization of the ionization source conditions to ensure maximum production and transfer of gas-phase ions into the MS system.

Smaller peak volumes also imply a fast acquisition rate is required to collect the minimum 15–20 data points across a peak needed for quantitative data. Time-related band broadening effects can result from insufficient dwell times and excessive data smoothing. In Figure 6, morphine and hydromorphone were analyzed using three scan rates (300 ms, 50 ms, and 5 ms). Artificial broadening is apparent for the 300-ms data whereas the 5-ms data show excessive noise that is characteristic of over sampling. Improper dwell time settings can have a profound impact on data quality and *S/N* ratios. When analyzing a large number of compounds, increased cycle times can be achieved by collecting data in selected-ion monitoring (SIM) or multiple reaction monitoring (MRM) mode to reduce the occurrence of time-related band broadening effects. In addition, most software allows for timed data collection, enabling data to be collected for a particular compound over a predefined window of time, extending the cycle time of a system.

Conclusion

Developing a sensitive and robust LC–MS method is a difficult task. Equipped with an understanding of the physicochemical properties of their target analytes, as well as the mechanisms and limitations of MS ionization and transmission efficiency, analysts can begin to make educated decisions to optimize overall response. The easiest and most effective way to improve sensitivity is through optimization of the ionization source conditions to ensure maximum production and transfer of gas-phase ions into the MS system. Careful selection of sample pretreatment procedures can reduce limits of detection by improving response and reducing interferences that could contribute to matrix effects and baseline noise. The use of efficient, narrow-bore LC columns, slower LC flow rates, and logical mobile phases can facilitate gains in signal intensity assuming extracolumn volumes are minimized and the data acquisition rates are appropriately set.

References

(1) R.K. Boyd, C. Basic, and R.A. Bethem, *Trace Quantitative Analysis by Mass Spectrometry, 1st Edition* (John Wiley & Sons Ltd, West Sussex, UK, 2008), pp. 242, 249.

(2) L.R. Snyder, J.J. Kirkland, and J.W. Dolan, *Introduction to Modern Liquid Chromatography, 3rd Edition* (John Wiley & Sons, Hoboken, New Jersey, USA, 2010), pp. 39–45, 157.

(3) J.S. Page, R.T. Kelly, K. Tang, and R.D. Smith, *J. Am. Soc. Mass Spectrom.* **18**, 1582–1590 (2007).

(4) G.I. Taylor, *Proc. R. Soc. Lond. A.* **280**, 383 (1964).

(5) M. Wilm, *Mol Cell Proteomics* **10**, 1–8 (2011).

(6) A. Kiontke, A. Oliveira-Birkmeier, A. Opitz, and C. Birkemeyer, *PLoS One* **11**, 1–16 (2016).

(7) T. Taylor, *The LCGC Blog* (7 November 2017).

(8) O. Szerkus, A.Y. Mpanga, M.J. Markuszewski, R. Kaliszan, and D. Siluk, *Spectroscopy* **14**, 8–16 (2016).

(9) R. Dams, M.A. Huestis, W.E. Lambert, and C.M. Murphy, *J. Am. Soc. Mass Spectrom.* **14**, 190–1294 (2003).

(10) Y. Hua and D. Jenke, *J. of Chromatogr. Sci.* **50**, 213–227 (2012).

(11) S.R. Needham, P.R. Brown, K. Duff, and D. Bell, *J. Chromatogr. A* **869**, 159–170 (2000).

(12) R. Bonfiglio, R.C. King, T.V. Olah, and K. Merkle, *Rapid Commun. Mass Spectrom.* **13**, 1175–1185 (1999).

(13) F. Klink, MS Solutions #3, Sepscience.com/Information/Archive/MS-Solutions.

(14) S. Lupo and T. Kahler, *LCGC North America* **35**, 424–433 (2017).

(15) J. Boertz, X. Lu, H. Brandes, S. Squillario, D. Bell, and W. Way, Poster Session presented at the Annual Meeting of the German Society for Mass Spectrometry (DGMS), Wuppertal, Germany (2015).

(16) S. Buckenmaier, C.A. Miller, T. van de Goor, and M.M. Dittman, *J. Chromatogr. A* **1377**, 64–74 (2015).

(17) G. Guiochon and F. Gritti, *J. Chromatogr. A* **1218**, 1915–1938 (2011).

(18) J.P.C. Vissers, H.A. Classens, and C.A. Cramers, *J. Chromatogr. A* **779**, 1–28 (1997).

(19) J. Abian, A.J. Oosterkamp and E. Gelpi, *J. Mass. Spectrom.* **34**, 244–254 (1999).

(20) D. Spaggiari, S. Fekete, P.J. Eugster, J. Veuthey, L. Geiser, S. Rudaz, and D. Guillarme, *J. Chromatogr. A* **1310**, 45–55 (2013).

(21) A.J. Alexander, T.J. Waeghe, K.W. Himes, F.P. Tomasella, and T.F. Hooker, *J. Chromatogr. A* **1218**, 5456–5469 (2011).

Sharon Lupo joined Restek in 2010 as an LC Applications Chemist. While in this position, she focused on developing LC–MS/MS applications and providing LC technical support for the environmental, food safety, and clinical markets. Currently, Sharon is a Senior Scientist for the LC Product Development group and uses her market knowledge and analytical skills to develop new and innovative LC products. Sharon has nearly 20 years of experience in the field of liquid chromatography. Prior to joining Restek, she was a principal investigator, study director, and analytical chemist for good laboratory practice (GLP) and good manufacturing practice (GMP) regulated environmental, bioanalytical, and pharmaceutical studies.

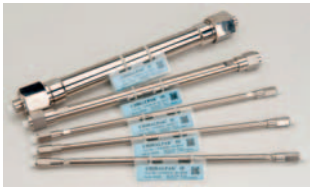
David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for *LCGC* and is the Editor for "Column Watch". Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His undergraduate studies in chemistry were completed at the State University of New York at Plattsburgh (SUNY Plattsburgh). He received his Ph.D. in analytical chemistry from The Pennsylvania State University and spent the first decade of his career in the pharmaceutical industry performing analytical method development and validation using various forms of chromatography and electrophoresis. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: LCGCedit@ubm.com

Pressure sensors

Blockages can be immediately detected and risks mitigated, before important samples are compromised using Idex QuickStart Pressure Sensors. Engineered for precision sensing, these sensors continually monitor system parameters to provide the user with the information that they need to keep the instrument operating reliably. The sensors connect in-line to a system easily with minimal solution carryover and bubble trapping, according to the company.

www.biotech.se
Biotech AB, Onsala, Sweden.

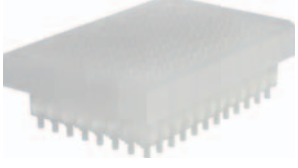
Immobilized Chiral Columns



Daicel launches the newest addition to its family of immobilised chiral stationary phases, ChiralPak IH. This phase is the immobilized version of the coated ChiralPak AS and offers significant solvent versatility and robustness. The ChiralPak IH is available in 5- and 3-µm particle size and can be used in both HPLC and SFC modes to develop methods for the most challenging separations.

http://chiraltech.com/polysaccharide-selectors
Chiral Technologies Europe SAS, Illkirch Cedex, France.

SPE adsorbent



The hydrophilic-lipophilic balanced polymeric SPE adsorbent, Chromabond HLB is specially designed for the enrichment of hydrophilic analytes, such as pharmaceuticals and pesticides, from polar matrices, including blood, water, and food. As well as the typical polypropylene columns, Macherey-Nagel offers Chromabond Multi 96 HLB plates for high-throughput SPE in 96-well format.

www.mn-net.com
Macherey-Nagel GmbH & Co. KG, Düren, Germany.

FID gas station

The VICI FID gas station combines the reliability of the VICI DBS hydrogen and zero-air generators into one compact and convenient package. Available in High and Ultra-High purity for all GC detector and carrier gas applications. The generator is available in two styles: flat for placement under a GC, or the Tower. Available in H_2 flow ranges up to 1 L/min and 10.5 bar.

www.vicidbs.com
VICI AG International, Schenkon, Switzerland.

Evaporation system

A new six-position evaporation station (mVAP) is available for fully automated sample concentration at user-defined temperature and vacuum. The mVAP can evaporate solvents after SPE or liquid/liquid extraction including changing to a compatible solvent for the best possible chromatographic analysis and MS ionization. According to the compnay, significantly improved limits of detection are achieved. Injection to the GC–MS or LC–MS is automated.

www.gerstel.com
Gerstel GmbH & Co. KG, Mülheim an de Ruhr, Germany.

EAF4

Postnova's new simultaneous Electrical and Asymmetrical Flow Field-Flow Fractionation (EAF4) system is designed to enhance separation and characterization of biopharmaceutical, environmental, and nanomaterials. In an EAF2000 system Electrical and Cross Flow Fields are applied simultaneously enabling separations by particle size and particle charge based on electrophoretic mobility for characterizing complex proteins, antibodies, and viruses as well as environmental and charged nanoparticles or polymers.

www.postnova.com
Postnova Analytics GmbH, Landberg, Germany.

SEC columns

YMC has developed a new high-resolution column phase designed specifically for the HPLC analysis of antibodies, their fragments, or aggregates by size exclusion chromatography. The YMC-SEC MAB column contains a diol-bonded silica-based phase that is suitable for the high-resolution analysis of both the high-molecular-weight- and low-molecular-weight- species, and reportedly offers excellent lot-to-lot reproducibility.
www.ymc.de
YMC Europe GmbH, Dinslaken, Germany.

Flow field-flow fractionation

The Eclipse DualTec system offers both Hollow-Fiber Flow-FFF (HF5) and Asymmetric Flow-FFF (AF4) techniques. Both may be integrated into one instrument and coupled seamlessly to the most advanced light scattering detector for determination of molar mass and nanoparticle size, Wyatt's Dawn Heleos II.
https://www.wyatt.com/eclipse
Wyatt Technology, Santa Barbara, California, USA.

Mass spectrometer

Knauer have launched a single quadrupole mass spectrometer, the Knauer 4000 MiD, which covers a mass range of 50–800 m/z. As a result of the integrated vacuum system, the 4000 MiD has a very small footprint and can be operated in laboratories with limited space. Together with the MiDas automated sampling unit, the 4000 MiD enables high-throughput preparative HPLC. The Azura prep LC for mass-directed fractionation is controlled with ClarityChrom, an easy-to-use chromatography data system for workstations.
www.knauer.net
Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany.

(U)HPLC columns

Kromasil EternityXT is the well-known chromatographic material based on patented technology that offers chromatographers opportunities to run separations between pH 1 and 12; from analytical with UHPLC to HPLC semi-prep prepacked columns and bulk for production.
www.kromasil.com/lcgc_1807a
AkzoNobel PPC AB, Bohus, Sweden.

Microchip column

µPAC is PharmaFluidics' chip-based chromatography column for nano-liquid chromatography. Perfect order in the separation bed is achieved by etching a regular pattern of pillars into a silicon wafer using micromachining technology, according to the company. The column allows high-resolution separation of tiny, complex biological samples, with an unprecedented robustness. µPAC is suitable for lipidomic, metabolomic, and peptide profiling.
www.pharmafluidics.com
PharmaFluidics, Ghent, Belgium.

TD sorbent sampling tubes

Markes thermal desorption sorbent sampling tubes are for VOC and SVOC analysis from air and solids. According to the company, they are manufactured to the highest quality, fully quality-controlled, and provide optimum sampling and analytical performance. Available in metal, inert-coated, or glass with a wide range of packing materials for any application. Compatible with all major thermal desorption instrument manufacturers.
http://chem.markes.com/TDCat
Markes International, Llantrisant, UK.

The 38th International Symposium on the Purification of Proteins, Peptides, and Polynucleotides (ISPPP 2018)

Photo Credit: Eo naya/Shutterstock.com

The 38th International Symposium on the Purification of Proteins, Peptides and Polynucleotides (ISPPP 2018) will be held on **November 4–7 2018** in **Berlin, Germany.**

ISPPP 2018 is a leading event to meet scientists from around the world in the exciting and innovative field of biomolecule separation and purification. With new milestones in processing, materials, methods, and instrumentation being reached and an endless list of interesting and challenging target molecules, there is never a better time to attend **ISPPP**, according to the organisers. The event is expected to attract around 120 participants from universities, research institutions, and industry working in the field of biopolymers purification, **ISPPP 2018** offers a unique chance to learn about the wide array of novel research in one place.

Key Note Lectures have been confirmed by Rainer Bischoff (University of Groningen, Germany), My Hedhammar (Royal Institute of Technology, Stockholm, Sweden) and Herbert Lindner (Medical University of Innsbruck, Austria). The symposium is preceded by three highly interesting pre-conference workshops held by members of the scientific committee on Mechanistic Understanding of Biomolecules Adsorption, Surface Plasmon Resonance Technology, and Magnetic Separation of Proteins.

The conference programme includes scientific sessions on Biopharmaceuticals, Bioanalytics, Simulation/Automation/Modelling, Advances in Stationary Phases, Continuous Processing, and Scale-up and -down of Chromatography. Furthermore, several young scientists will present their work in short talks referring to their respective posters. **ISPPP 2018** also hosts an exhibition of companies offering innovative solutions for separation and purification tasks of biomolecules.

The participation and inclusion of young researchers is an important issue for the scientific committee of **ISPPP 2018**. A travel grant has been awarded to several doctoral students and the social programme aims to intergrate young scientists. Beyond the welcome reception and poster party, a casual conference dinner will be held that will enable and facilitate interaction between all participants.

ISPPP 2018 will take place in Berlin, the booming capital of Germany, which is an international innovation hub on the way to become Europe's leading start-up metropolis and the home of several renowned universities and research facilities. Berlin, is renowned for its exceptional variety of attractions. The city's flourishing cultural scene and a way of life that is both fast-paced and relaxed. Contrasts between historical buildings and modern architecture, between the traditional and the modern are what set the city apart from the rest. The sights of Berlin, from the Brandenburg Gate to the Federal Chancellery, tell the story of an entire nation.

The **ISPPP** conference series is a well-established scientific event taking place alternately in Europe and the US for more than 30 years. Some of the scientific committee members have been part of **ISPPP** since its beginning in the early 1980s. The symposium is multi-disciplinary in nature to provide attendees with information about the latest advances in their own fields of interest as well as insights into related fields.

The scientific committee headed by Prof. Dr. Sonja Berensmeier (Technical University of Munich, Germany) will be pleased to welcome you in Berlin as an active participant to this scientific event and is looking forward to discussing and solving your difficult and challenging analytical or preparative problems. For more information, please visit: **www.isppp2018.net.**

23–27 September 2018
32nd International Symposium on Chromatography (ISC 2018)
Cannes-Mandelieu, France
E-mail: info@isc2018.fr
Website: www.isc2018.fr

5–8 October 2018
Grass Roots 2018
The Waterhead Hotel, Ambleside, Cumbria, UK
E-mail: paul.ferguson@chromsoc.com
Website: www.chromsoc.com/event/grass-roots-2018

21–24 October 2018
7th International Conference on Polyolefin Characterization
Houston, Texas, USA
E-mail: Raquel.ubeda@icpc-conference.org
Website: www.icpc-conference.org

27 November 2018
Advances in Clinical and Forensic Analysis 2018
RSC Burlington House, London, UK
E-mail: adrian.clarke@novartis.com
Website: www.chromsoc.com/events/

21–23 January 2019
10th Multidimentional Chromatography Workshop
Liège University, Liège, Belgium
Website: www.multidimensionalchromatography.com

31 January–1 February 2019
SCM–9
Rhone Congress Centre, Amsterdam
E-mail: info@scm-9.nl
Website: www.scm-9.nl

10–13 March 2019
DGMS 2019
University of Rostock, Germany
E-mail: ralf.zimmermann@uni-rostock.de
Website: www.dgms.eu

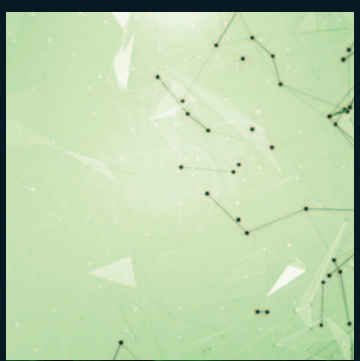
17–21 March 2019
Pittcon 2019
Pennsylvania Convention Center, Philadelphia, Pennsylvania, USA
E-mail: info@pittcon.org
Website: https://pittcon.org/pittcon-2019/

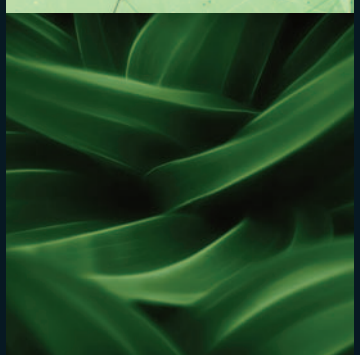
Please send any upcoming event information to Lewis Botcherby at lewis.botcherby@ubm.com

LC GC europe

solutions for separation scientists

THE APPLICATIONS BOOK





September 2018

www.chromatographyonline.com

UBM

THE APPLICATIONS BOOK

Photo Credit: Yarygin/Shutterstock.com

Photo Credit: Cesare Sent/Shutterstock.com

Photo Credit: 1163998627/Shutterstock.com

Photo Credit: Elesey/Shutterstock.com

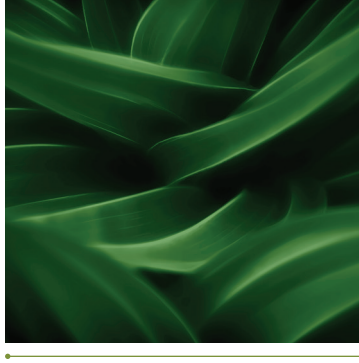


Photo Credit: Dee Browning/Shutterstock.com

Food and Beverage

527 **Analysis of 213 Pesticide Residues in Grapes by LC–MS/MS with Time-Managed MRM**
PerkinElmer

528 **Streamlined Method for Pesticide Residues in High-Lipid Food Samples Using QuEChERS Extraction, LipiFiltr® Cleanup, and UHPLC–MS/MS Analysis**
Bikash Bhattarai and Brian Kinsella, UCT, LLC

Medical/Biological

529 **Analysis of Phospholipid Classes by iHILIC®-Fusion**
Christian Vosse[1], Carina Wienken[1], Wen Jiang[2], and Heiko Hayen[1], [1]Institute of Inorganic and Analytical Chemistry, University of Münster, Münster, Germany, [2]HILICON AB

531 **Analysis of Fentanyl and Its Analogues in Human Urine by LC–MS/MS**
Shun-Hsin Liang and Frances Carroll, Restek Corporation

Pharmaceutical/Drug Discovery

533 **Determination of Pharmaceuticals from Serum**
Hans Rainer Wollseifen, Johannes Brand, and Detlef Lambrecht, MACHEREY-NAGEL GmbH & Co. KG

535 **UHP-SEC Analysis of Biosimilars**
Tosoh Bioscience GmbH

General

536 **Analysis of Polyethylene by Pyrolysis-GC×GC–MS**
Daniela Peroni, CDS Analytical

537 **Analysis of Cellulose Molecular Weight Distributions in DMAC**
Wyatt Technology

538 **Absolute Molar Mass Analysis of Chitosans**
Wyatt Technology

Cover Photography: Shutterstock.com

Analysis of 213 Pesticide Residues in Grapes by LC–MS/MS with Time-Managed MRM

PerkinElmer

The grape crop is one of the most important fruit crops consumed in the world. Grapes are consumed both as fresh and as processed products, such as wine, jam, juice, jelly, grape seed extract, raisins, vinegar, and grape seed oil. A large variety of pesticides are used in grape production throughout its growing season to control pests and diseases in vineyards and to increase crop yield. Pesticide residue is a major concern for the stakeholders of the grape industry, due to more and more stringent regulations and safety standards in most countries. It is also a concern for the general consumers, due to increased demand for safer products. Therefore, to prevent health risks, it is important to monitor the presence of pesticides and regulate their levels in grapes.

In this study, a fast, sensitive, and selective multiresidue method has been developed by coupling QuEChERS sample preparation with LC–MS/MS. Using time-managed-MRM™ in the QSight® triple quadrupole mass spectrometer, the optimum dwell time can be generated automatically for MRM transitions based on the number of coeluting transitions, expected cycle time, retention time, and tolerance time window of the targeted analytes. Such method automation results in improved data quality and better sensitivity, accuracy, and reproducibility, as demonstrated in this study by the results of 213 pesticide residues analyzed in grape samples.

Table 1: Example matrix effects for the pesticides identified from the grape samples in this work

Pesticides	Peak Area (In Neat Solution)	Peak Area (In Matrix Spiked Solution)	Matrix Effect
Acetamiprid	1024279	1068162	104.3
Boscalid	696490	734080	105.4
Chlorantraniliprole	197766	190834	96.5
Cyprodinil	453221	464004	102.4
Diafenthiuron	957108	692523	72.4
Difenoconazole	1260903	1344640	106.6
Dimethomorph	435381	366000	84.1
Fenhexamid	373104	411145	110.2
Fludioxonil	293046	322095	109.9
Fluopyram	2281108	2379392	104.3
Imidacloprid	305674	304391	99.6
Pyrimethanil	366881	381018	103.9
Pyraclostrobin	1682524	1764409	104.9
Spinosad	666859	698195	104.7
Spirotetramat	517179	485990	94.0
Spiroxamine	1273651	1354261	106.3
Spinetoram	641735	665530	103.7
Trifloxystrobin	2132708	2219020	104.0
Tebuconazole	653128	620261	95.0

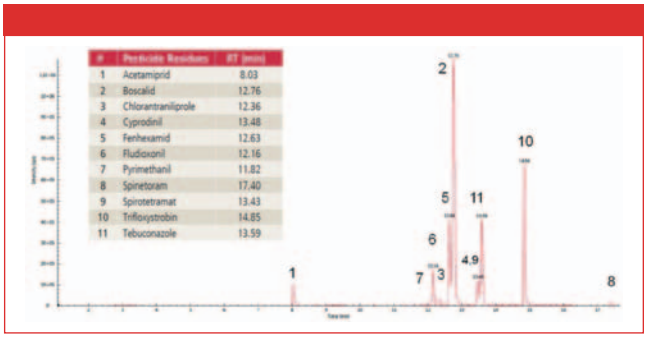


Figure 1. Example of the chromatogram that lists pesticides positively identified in one of seven brand samples.

Experimental

Hardware and Software: The chromatographic separation was performed by a PerkinElmer® UHPLC system and detection was achieved using a PerkinElmer QSight 220 triple quadrupole mass spectrometer, equipped with both ESI and APCI ionization sources. All instrument control, data acquisition, and data processing were performed using the Simplicity™ 3Q software.

Organic and non-organic grape samples were obtained from local grocery stores in Ontario, Canada. The mixed pesticide standards were obtained from ULTRA Scientific®. The samples were prepared using Supra-d QuEChERS kits (AOAC 2007.01 method).

Conclusion

An LC–MS/MS method for multiresidue pesticides analysis in grapes was developed by coupling a UHPLC system to a QSight 220 triple quadrupole mass spectrometer. This method can be applied for the determination of pesticide residues in grapes, with LOQs well below the limits set by regulatory boards.

The same LC–MS/MS method has also been applied successfully to other fruit analyses, such as berries, orange, and grapefruit, all with good performance. These results demonstrated the method's applicability and effectiveness in detecting and quantifying pesticide residues in fruit samples.

PerkinElmer
710 Bridgeport Ave, Shelton, Connecticut 06484, USA
Website: www.perkinelmer.com

Streamlined Method for Pesticide Residues in High-Lipid Food Samples Using QuEChERS Extraction, LipiFiltr® Cleanup, and UHPLC–MS/MS Analysis

Bikash Bhattarai and Brian Kinsella, UCT, LLC

The easy to use, new LipiFiltr® push-through purification cartridge was designed to remove lipids from acetonitrile extracts. This application will outline the performance benefits achieved using the new LipiFiltr® cartridges in applications involving multiclass, multiresidue analysis for pesticides in complex, high fat samples. Samples are extracted using a standard QuEChERS procedure and an aliquot of the supernatant is simply pushed through the LipiFiltr® purification cartridge using a disposable syringe. The purified extract is collected in an autosampler vial and analyzed by LC–MS/MS. The ability to obtain significantly cleaner extracts, the ease of use, and the time and cost savings make the new LipiFiltr® push-through cartridges an attractive cleanup option for laboratories conducting pesticide residue analysis in complex fatty samples.

Table 1: Extraction and analytical materials

ECQUEU7-MP	Mylar pouch containing 4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3Cit•2H_2O$, and 0.5 g $Na_2Cit•1.5H_2O$
LPFLTR01	LipiFiltr® Push-Through Cartridge
SLAQ100ID21-18UM	100 × 2.1 mm, 1.8-µm Selectra® Aqueous C18 UHPLC column
SLAQGDC20-18UM	10 × 2.1 mm, 1.8-µm Selectra® Aqueous C18 guard column
SLGRDHLDR-HP	High pressure guard cartridge holder

Procedure

Sample Extraction

(a) Weigh 5–10g of homogenized sample into a 50-mL centrifuge tube. Add 5 mL reagent water if necessary.
(b) Add 10 mL acetonitrile and internal standards.
(c) Shake or vortex samples for 5 min at 1000 strokes/min.
(d) Add ECQUEU7-MP packet to each sample and shake for 1 additional min at 1000 strokes/min.
(e) Centrifuge at ≥3000 rcf for 15 min.
(f) Attach LipiFiltr® push-through cartridge to disposable syringe.
(g) Take 1.5 mL of supernatant into syringe barrel. Attach the plunger and gently push the sample through the LipiFiltr® cartridge into an autosampler vial.

Instrumental

LC–MS/MS: Shimadzu Nexera X2 coupled with Shimadzu LCMS-8050
UHPLC column: 100 × 2.1 mm, 1.8-µm Selectra® Aqueous C18
Guard column: 10 × 2.1 mm, 1.8-µm Selectra® Aqueous C18
Injection volume: 2 µL
Mobile phase A: H_2O containing 0.1% formic acid + 5 mM ammonium formate
Mobile phase B: Methanol containing 0.1% formic acid + 5 mM ammonium formate
Column flow rate: 0.45 mL/min

Results

The performance of the LipiFiltr® push-thru cartridges to remove fatty matrix was evaluated gravimetrically. The gravimetric analysis was done by collecting 2 mL of sample before and after clean-up in pre-weighed test tubes and heating them to dryness at 110 °C.

Table 2: Gravimetric analysis

Matrix	Matrix Removal (%)
Beef	79.1
Black Olives	84.3
Avocado	54.7
Salmon	80.9
Chicken fat	71.7
Olive oil	61.5
Nuts	84.3
Swordfish	80.9

Conclusion

This application note demonstrates the performance benefits achieved using the new LipiFiltr® cartridge in applications involving multiclass, multiresidue analysis for a wide range of pesticides (n = 189) in complex, high fat samples. Individualized recoveries obtained for each pesticide in a variety of matrices, such as avocado, olives, beef, and swordfish, can be found at unitedchem.com

UCT, LLC
2731 Bartram Rd, Bristol, Pennsylvania 19007, USA
Tel: (800) 385 3153
Email: methods@unitedchem.com
Website: www.unitedchem.com

Analysis of Phospholipid Classes by iHILIC®-Fusion

Christian Vosse[1], Carina Wienken[1], Wen Jiang[2], and Heiko Hayen[1], [1]Institute of Inorganic and Analytical Chemistry, University of Münster, Münster, Germany, [2]HILICON AB

Phospholipids (PLs) are the major components of cellular membranes. They are important for the functionality of membrane proteins or serve as precursors for second messengers. Several studies reveal the role of PL alterations in various diseases such as cancer (1). Therefore, it is crucial to identify and quantify PLs in complex biological samples for lipidomic studies and clinical research.

The PL classification is based on their different polar head groups. The diversity within each PL class is generated by combination of different fatty acids (FAs) with variation of chain length and the number of double bonds. Nowadays, tandem mass spectrometry (MS/MS) is the most important technique for the identification and quantification of PL species. By hyphenation with liquid chromatography (LC), LC–MS/MS provides prodigious power for the separation of isobaric or isomeric lipids. The amphiphilic properties of PLs mean that both reversed phase LC and hydrophilic interaction liquid chromatography (HILIC) are applicable for the separations. However, HILIC enables the separation of PL classes according to their polar head groups and minimizes ion suppression effects between PLs. The HILIC–MS/MS approach makes it possible to quantify lipid species based on similar retention times with internal standards of each lipid class (2) and also their characteristic fragments. Therefore, the assignment of lipid species to a specific lipid class is straightforward.

In this application, 10 PL classes are separated by HILIC using an iHILIC®-Fusion column. Detection was carried out by electrospray-MS/MS. The applicability of this method for potential clinical application is demonstrated by the analysis of PL species in a lipid extract of a MCF-7 breast cancer cell culture (3).

Experimental

LC–MS system: Thermo Scientific Ultimate 3000 HPLC system coupled to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer, which is equipped with a HESI II source and operated in negative ionization mode (ESI-).

Column: 150 × 2.1 mm, 3.5 µm 100 Å iHILIC®-Fusion (P/N 110.152.0310, HILICON).

Eluent: A) ammonium acetate solution (35 mM, pH 5.75) and acetonitrile (95:5, *v/v*); B) acetonitrile.

Gradient elution: 0–0.5 min, 97% B; 0.5–26.5 min, from 97% to 75% B; 27–33 min, 60% B; 35–45 min 97% B.

Flow rate: 0.3 mL/min

Column temperature: 40 °C

Injection volume: 10 µL

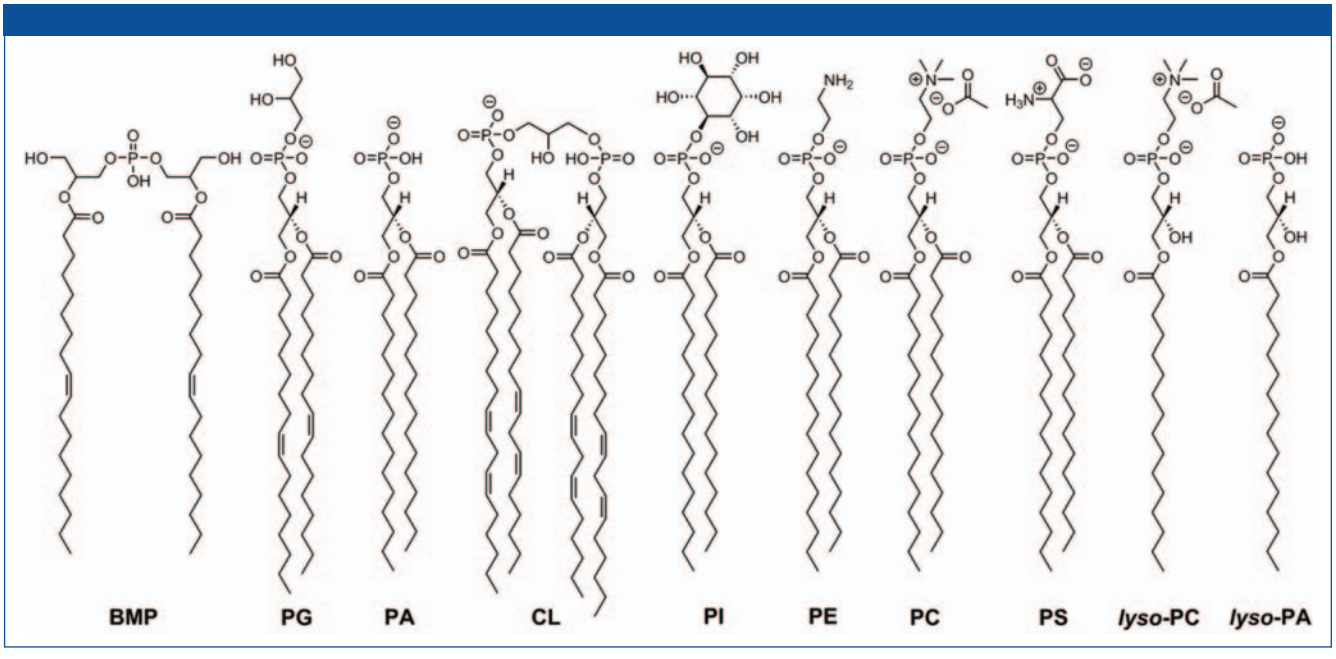


Figure 1: Chemical structures of phospholipids used in the study.

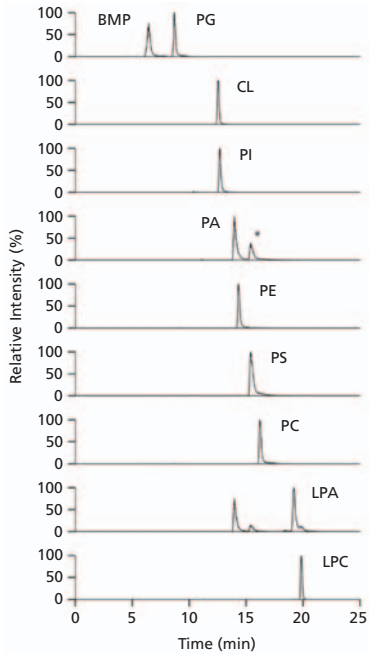


Figure 2: Separation of PL standards in ascending retention order with iHILIC®-Fusion. *Double peak of PA is caused by in-source fragmentation of the PS head group resulting in corresponding PA species.

Phospholipid standards: Bis(monoacylglycero)phosphate (BMP 36:2), phosphatidylglycerol (PG 36:2), phosphatidylcholine (PC 32:0), phosphatidylethanolamine (PE 32:0), phosphatidylserine (PS 32:0), phosphatidylinositol (PI 32:0), lyso-phosphatidylcholine (LPC 16:0), phosphatidic acid (PA 32:0), lyso-phosphatidic acid (LPA 16:0), and cardiolipin (CL 72:8). The chemical structures are shown in Figure 1.

MCF-7 breast cancer cells: The extract of MCF-7 cell sample was provided by the working group of Dr. Cristina Cadenas (Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany).

Results and Conclusion

Figure 2 shows the separation of 10 PL standards according to their polar head groups and different chain lengths using an iHILIC®-Fusion column. Importantly, the constitutional isomers BMP and PG were baseline separated. It was found that the other PL classes were also well separated. This is very important for the separation between PA and PS due to the potential in-source fragmentation of the PS head group leading to a mass-to-charge ratio (*m/z*) of the corresponding PA species. In short, the iHILIC®-Fusion provides good peak shapes and narrow peaks

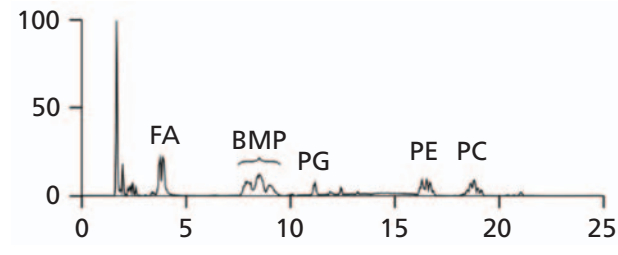


Figure 3: Chromatogram of PL class separation of MCF-7 cell extract with iHILIC®-Fusion.

for all PL classes. The buffer concentration has turned out to be the most crucial factor for method development. It affected both separation efficiency and elution order. Furthermore, the acetate buffer also facilitated the detection of positively charged PC and lyso-PC species in the negative ionization mode as acetate adducts. All other investigated PL classes were detected as deprotonated molecules $[M-H]^-$. Figure 3 shows the base peak chromatogram of a separation of MCF-7 cell extract. Besides our focused BMP and PG class, further PL classes such as PE or PC could also be well detected.

The combination of HILIC and tandem MS is a powerful tool for analysis of PL classes and PL species. The iHILIC®-Fusion provides an efficient separation of PL classes, which is mandatory for isomers such as PG and BMP (3). This facilitates a quantification approach based on single class-specific internal standards.

References

(1) C. Cadenas *et al., Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1821**, 1256–1268 (2012).

(2) E. Cífková *et al., Anal. Chem.* **84**, 10064–10070 (2012).

(3) C. Vosse, C. Wienken, C. Cadenas, and H. Hayen, *J. Chromatogr. A* **1565**, 105–113 (2018).

HILICON AB
Tvistevägen 48, SE-90736 Umeå, Sweden
Tel.: +46 (90) 193469
E-mail: info@hilicon.com
Website: www.hilicon.com

Analysis of Fentanyl and Its Analogues in Human Urine by LC–MS/MS

Shun-Hsin Liang and Frances Carroll, Restek Corporation

Abuse of synthetic opioid prescription painkillers such as fentanyl, along with a rapidly growing list of illicit analogues, is a significant public health problem. In this study, we developed a simple dilute-and-shoot method that provides a fast 3.5-min analysis of fentanyl and related compounds (norfentanyl, acetyl fentanyl, alfentanil, butyryl fentanyl, carfentanil, remifentanil, and sufentanil) in human urine by LC–MS/MS using a Raptor Biphenyl column.

In recent years, the illicit use of synthetic opioids has skyrocketed, and communities worldwide are now dealing with an ongoing epidemic. Of the thousands of synthetic opioid overdose deaths per year, most are related to fentanyl and its analogues. With their very high analgesic properties, synthetic opioid drugs such as fentanyl, alfentanil, remifentanil, and sufentanil are potent painkillers that have valid medical applications; however, they are also extremely addictive and are targets for abuse. In addition to abuse of these prescription drugs, the current opioid crisis is fueled by a growing number of illicit analogues, such as acetyl fentanyl and butyryl fentanyl, which have been designed specifically to evade prosecution by drug enforcement agencies.

As the number of opioid drugs and deaths increases, so does the need for a fast, accurate method for the simultaneous analysis of fentanyl and its analogues. Therefore, we developed this LC–MS/MS method for measuring fentanyl, six analogues, and one metabolite (norfentanyl) in human urine. A simple dilute-and-shoot sample preparation procedure was coupled with a fast (3.5 min) chromatographic analysis using a Raptor Biphenyl column. This method provides accurate, precise identification and quantitation of fentanyl and related compounds, making it suitable for a variety of testing applications, including clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

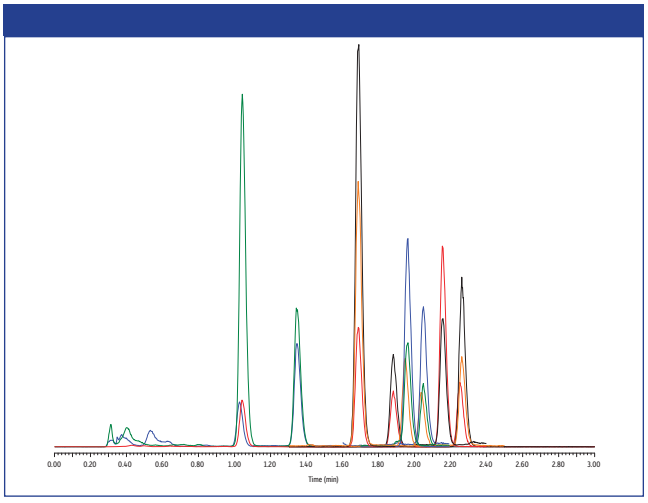


Figure 1: The Raptor Biphenyl column effectively separated all target compounds in urine with no observed matrix interferences. Peak elution order: norfentanyl-D_5, norfentanyl, remifentanil, acetyl fentanyl-$^{13}C_6$, acetyl fentanyl, alfentanil, fentanyl-D_5, fentanyl, carfentanil-D_5, carfentanil, butyryl fentanyl, sufentanil-D_5, sufentanil.

Table 1: Analyte transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier	Internal Standard
Norfentanyl	233.27	84.15	56.06	Norfentanyl-D_5
Acetyl fentanyl	323.37	188.25	105.15	Acetyl fentanyl-$^{13}C_6$
Fentanyl	337.37	188.26	105.08	Fentanyl-D_5
Butyryl fentanyl	351.43	188.20	105.15	Carfentanil-D_5
Remifentanil	377.37	113.15	317.30	Norfentanyl-D_5
Sufentanil	387.40	238.19	111.06	Sufentanil-D_5
Carfentanil	395.40	113.14	335.35	Carfentanil-D_5
Alfentanil	417.47	268.31	197.23	Acetyl fentanyl-$^{13}C_6$
Norfentanyl-D_5	238.30	84.15	—	—
Acetyl fentanyl-$^{13}C_6$	329.37	188.25	—	—
Fentanyl-D_5	342.47	188.27	—	—
Sufentanil-D_5	392.40	238.25	—	—
Carfentanil-D_5	400.40	340.41	—	—

Experimental Conditions

Sample Preparation: The analytes were fortified into pooled human urine. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water–methanol solution (fivefold dilution) and 10 µL of internal standard (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat. #25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected into the LC–MS/MS.

Calibration Standards and Quality Control Samples: The calibration standards were prepared in pooled human urine at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL. Three levels of QC samples (0.75, 4.0, and 20 ng/mL) were prepared in urine for testing accuracy and precision with established calibration standard curves. Recovery analyses were performed on three different days. All standards and QC samples were subjected to the sample preparation procedure described.

LC–MS/MS analysis of fentanyl and its analogues was performed on an ACQUITY UPLC instrument coupled with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows, and analyte transitions are provided in Table 1.

Analytical column:	Raptor Biphenyl (5 µm, 50 mm × 2.1 mm; cat. #9309552)	
Guard column:	Raptor Biphenyl EXP guard column cartridge, (5 µm, 5 mm × 2.1 mm; cat. #930950252)	
Mobile phase A:	0.1% Formic acid in water	
Mobile phase B:	0.1% Formic acid in methanol	
Gradient	Time (min)	%B
	0.00	30
	2.50	70
	2.51	30
	3.50	30
Flow rate:	0.4 mL/min	
Injection volume:	5 µL	
Column temp.:	40 °C	
Ion mode:	Positive ESI	

Results

Chromatographic Performance: All eight analytes were well separated within a 2.5-min gradient elution (3.5-min total analysis time) on a Raptor Biphenyl column (Figure 1). No significant matrix interference was observed to negatively affect quantification of the fivefold diluted urine samples. The 5-µm particle Raptor Biphenyl column used here is a superficially porous particle (SPP) column. It was selected for this method in part because it provides similar performance to a smaller particle size fully porous particle (FPP) column, but it generates less system back pressure.

Linearity: Linear responses were obtained for all compounds and the calibration ranges encompassed typical concentration levels monitored for both research and abuse. Using 1/x weighted linear regression ($1/x^2$ for butyryl fentanyl), calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, alfentanil, acetyl fentanyl, butyryl fentanyl, and sufentanil; from 0.10 to 50 ng/mL for remifentanil; and from 0.25 to 50 ng/mL for norfentanyl and carfentanil. All analytes showed acceptable linearity with r^2 values of 0.996 or greater and deviations of <12% (<20% for the lowest concentrated standard).

Accuracy and Precision: Based on three independent experiments conducted on multiple days, method accuracy for the analysis of fentanyl and its analogues was demonstrated by the %recovery values, which were within 10% of the nominal concentration for all compounds at all QC levels. The %RSD range was 0.5–8.3% and 3.4–8.4% for intraday and interday comparisons, respectively, indicating acceptable method precision (Table 2).

Conclusions

A simple dilute-and-shoot method was developed for the quantitative analysis of fentanyl and its analogues in human urine. The analytical method was demonstrated to be fast, rugged, and sensitive with acceptable accuracy and precision for urine sample analysis. The Raptor Biphenyl column is well suited for the analysis of these synthetic opioid compounds and this method can be applied to clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

Restek Corporation
110 Benner Circle, Bellefonte, Pennsylvania 16823, USA
Tel. 1 (814) 353 1300
Website: www.restek.com

Table 2: Accuracy and precision results for fentanyl and related compounds in urine QC samples

Analyte	QC Level 1 (0.750 ng/mL)			QC Level 2 (4.00 ng/mL)			QC Level 3 (20.0 ng/mL)		
	Average Conc. (ng/mL)	Average % Accuracy	%RSD	Average Conc. (ng/mL)	Average % Accuracy	%RSD	Average Conc. (ng/mL)	Average % Accuracy	%RSD
Acetyl fentanyl	0.761	102	1.54	3.99	99.7	2.08	19.9	99.3	0.856
Alfentanil	0.733	97.6	3.34	3.96	98.9	8.38	20.9	104	6.73
Butyryl fentanyl	0.741	98.9	6.29	3.77	94.3	6.01	20.8	104	4.95
Carfentanil	0.757	101	7.34	3.76	94.0	4.64	20.6	103	4.24
Fentanyl	0.761	102	1.98	3.96	99.1	2.31	19.9	99.6	1.04
Norfentanyl	0.768	103	6.50	4.04	101	1.84	20.1	101	2.55
Remifentanil	0.765	102	3.42	3.97	99.2	3.68	20.8	104	4.14
Sufentanil	0.752	100	1.67	3.93	98.3	1.28	20.1	100	0.943

Determination of Pharmaceuticals from Serum

Hans Rainer Wollseifen, Johannes Brand, and Detlef Lambrecht, MACHEREY-NAGEL GmbH & Co. KG

This application note describes the determination of pharmaceuticals from serum using solid-phase extraction (SPE) with the hydrophilic-lipophilic balanced SPE phase CHROMABOND® HLB for analyte enrichment and for sample cleanup. The eluates from SPE are finally analyzed by HPLC–MS/MS on a NUCLEOSHELL® PFP core–shell phase.

Nowadays, people suffer from various diseases, and are prescribed many types of pharmaceuticals as part of their treatment, for example, anesthetics, antibiotics, anticholinergics, anticonvulsants. In order for the treatment to be successful, it is necessary to keep controlling the levels of the pharmaceuticals to provide an accurate dosage. This has led to an increasing demand for the development of accurate and sensitive analytical methods to analyze the pharmaceuticals from serum to protect human health.

Table 1: SRM transitions for the investigated pharmaceuticals

Analyte	Retention Time (min)	$[M-H]^-$	Q_1 (Quantifier)	Q_2 (Qualifier)
Atenolol	1.12	267.2	145.2	74.1
Sulfapyridine	1.72	242.9	130.9	96.9
Atropine	1.81	290.2	124.2	93.0
Sulfamerazine	1.82	265.1	156.0	91.9
Ketamine	1.87	238.2	125.1	179.1
Chlorpheniramine	2.27	275.1	230.0	167.0
Sulfachloropyridazine	2.47	285.1	156.0	91.9
Sulfadoxine	2.63	311.1	156.0	92.1
Sulfamethoxazole	2.70	254.1	155.8	91.8
Propanolol	2.74	260.2	116.2	182.9
Diphenhydramine	2.94	256.1	166.9	152.1
Amitriptyline	3.04	278.2	223.0	91.0
Sulfaquinozaline	3.14	301.1	156.1	92.1
Nortriptyline	3.32	264.2	232.9	91.1
Verapamil	3.36	455.2	165.0	150.1
Trimipramine	3.41	295.2	100.1	58.0
Carbamazepine	3.50	237.1	194.1	193.0
Clomipramine	3.67	315.1	86.1	58.0
Indapamide	3.77	366.1	132.1	91.1
Ketoprofen	4.28	255.1	77.0	105.0

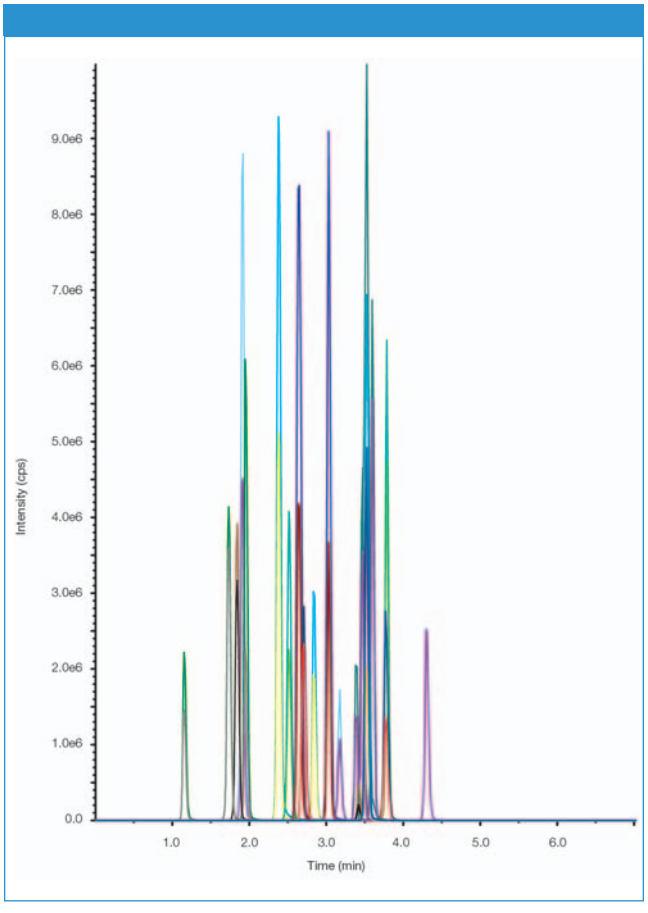


Figure 1: Chromatogram of serum sample spiked with 10 ng/mL for each pharmaceutical.

Solid-Phase Extraction (1)

SPE column: CHROMABOND® HLB, 1 mL, 30 mg, MACHEREY-NAGEL REF 730921
Column conditioning: 1 mL methanol, then 1 mL water
Sample application: 1 mL spiked serum sample is passed through the column by vacuum.
Washing: 1 mL water
Drying: 10 min with vacuum
Elution: 2 mL methanol
Eluent exchange: Eluate is evaporated to dryness at 40 °C under a stream of nitrogen and reconstituted in 1 mL 95:5 (*v/v*) water–acetonitrile

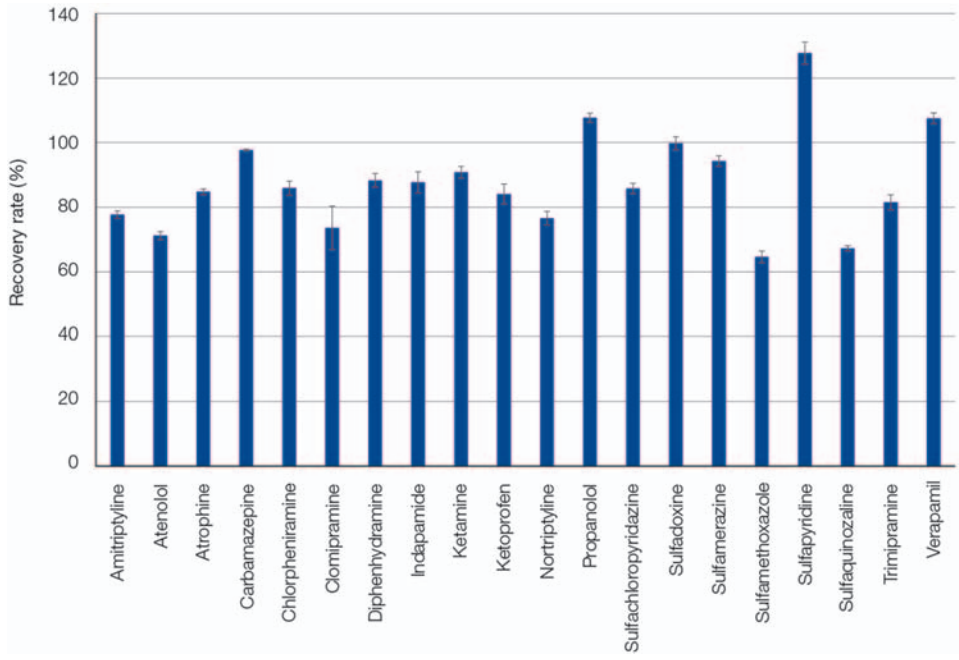


Figure 2: Recovery rates for solid-phase extraction method of pharmaceuticals from serum.

Subsequent Analysis: HPLC–MS/MS (2)

HPLC column: EC 50/2 NUCLEOSHELL® PFP, 2.7 µm, MACHEREY-NAGEL REF 763532.20
Eluent A: 0.1% formic acid in water
Eluent B: 0.1% formic acid in acetonitrile
Gradient: 5–95% B in 7.5 min, 95% B for 1 min, 95–5% B in 0.5 min, 5% B for 5 min
Flow rate: 0.3 mL/min
Temperature: 30 °C
Injection volume: 5 µL
MS/MS detection: API 5500 (AB Sciex GmbH); ion source: ESI; positive ionization mode; scan type: selected reaction monitoring (SRM, for transitions see Table 1); detection window: 90 s; curtain gas: 40 psig; ion spray voltage: 5500 V; temperature: 500 °C; nebulizer gas: 45 psig; turbo gas: 45 psig; CAD: medium.

Results

The recovery rates show that the determination of pharmaceuticals from serum could be carried out successfully (Figure 2). By using SPE with CHROMABOND® HLB, it was possible to recover nearly all pharmaceuticals from serum, with good reproducibility on average. Regarding the different types of pharmaceuticals, the average recovery rates were: for anesthetics 90.8%, antibiotics 94.4%, anticholinergics 84.8%, anticonvulsants 97.7%, antidepressants 77.4%, antihistamines 87.1%, anti-inflammatory drugs 84.1%, beta blockers 89.5%, calcium channel blockers 107.5%, and for diuretics 87.7%.

The identification and quantification of pharmaceuticals in the solid-phase extracts were performed using ESI-MS on an EC 50/2 NUCLEOSHELL® PFP column. The chromatogram in Figure 1 shows the results of solid-phase eluate spiked with 10 ng/mL serum for each pharmaceutical.

Conclusion

The presented application describes a quick and convenient method for the determination of pharmaceuticals from serum by SPE with a hydrophilic-lipophilic balanced phase, followed by HPLC–MS/MS analysis.

References

1. Application No. 306510, MACHEREY-NAGEL, available from www.mn-net.com/apps
2. Application No. 128200, MACHEREY-NAGEL, available from www.mn-net.com/apps

MACHEREY-NAGEL GmbH & Co. KG
Neumann-Neander-Str. 6 – 8, 52355 Düren, Germany
Tel.: +49 24 21 969 0 Fax: +49 24 21 969 199
E-mail: info@mn-net.com Website: www.mn-net.com

UHP-SEC Analysis of Biosimilars

Tosoh Bioscience GmbH

The amounts of high- and low-molecular-weight impurities are critical quality attributes for a therapeutic protein. Size-exclusion chromatography (SEC), the standard technology for analyzing aggregation and fragmentation, was applied to elucidate the molecular similarity between an Adalimumab biosimilar antibody and the corresponding innovator product Humira®.

A biosimilar is a biological medicine highly similar to an approved biological medicine (originator) in terms of structure, biological activity and efficacy, safety, and immunogenicity profile. In order to demonstrate biosimilarity, the biosimilar manufacturer must generate an array of data comparing the proposed product to the approved reference product; a detailed analytical characterization and comparison of the products marks the first step in this process.

A series of analytical chromatographic techniques are used to characterize similarity. When it comes to the monitoring of aggregation and fragmentation of a therapeutic protein, SEC is the standard technology for the characterization of a biosimilar molecule versus the reference product. In this application, a TSKgel® UP-SW3000 UHP-SEC column was used to elucidate the molecular similarity between an Adalimumab biosimilar and the reference product Humira®.

UHPLC Conditions

Column: 30 cm × 4.6 mm, 2 µm TSKgel UP-SW3000
Mobile phase: 100 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4, 0.05% NaN_3
Gradient: Isocratic
Flow rate: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol.: 5 µL
Samples: Humira Innovator Reference Product (5 mg/mL); Humira Biosimilar (4 mg/mL)

Results

Humira and its biosimilar were then analyzed on a UHPLC instrument with UV detection using a TSKgel UP-SW3000, 2 µm SEC column in order to elucidate and compare the SEC profiles of the two molecules. Figure 1 shows that Humira and its biosimilar show slight variability in retention times. The zoomed-in profile provides a better look at the aggregates (HMW impurities and fragments) present in each sample.

Table 1: Comparison of retention time and percent peak area for Humira and biosimilar

Retention Time (min)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	6.387	7.37	7.883, 9.097
Humira Biosimilar	5.843	6.283	7.46	7.960, 9.217
% Peak Area (mAU*min)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	0.27	99.59	0.14
Humira Biosimilar	0.15	1.73	97.66	0.46

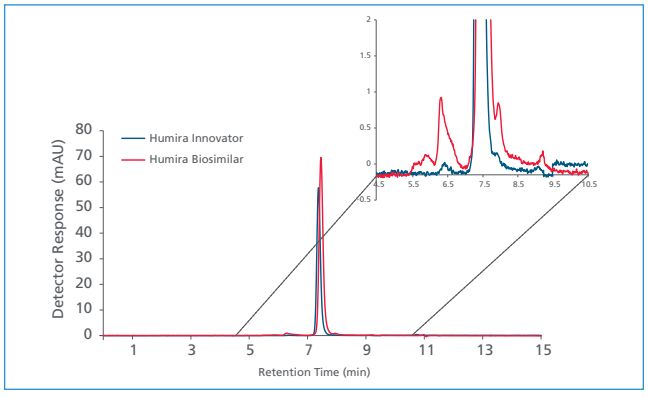


Figure 1: UHPLC analysis of Humira (blue) and biosimilar (red) with TSKgel UP-SW3000.

Table 1 outlines the retention time and relative percent peak area values that are shown in the chromatogram. The relative peak area calculation suggests a larger percentage of impurities, predominantly high-molecular-weight species (antibody aggregates), are present in the biosimilar compared to the innovator drug.

Analysis of six consecutive injections for Humira and its biosimilar. showed that results were highly reproducible.

Conclusion

UHP-SEC using a TSKgel UP-SW3000 column is a powerful tool to show the similarity between the innovator drug and the biosimilar with regard to the HMW and LMW impurities, as a function of hydrodynamic radii of the molecules. Reproducibility of consecutive injections yielded very low percent RSD when analyzing peak parameters such as retention time, peak area, peak asymmetry, and number of theoretical plates. Robustness and reproducibility are indispensable prerequisites for using SEC columns in quantitative analysis.

Tosoh Bioscience GmbH
Im Leuschnerpark 4 64347 Griesheim, Darmstadt, Germany
Tel: +49 6155 7043700 Fax: +49 6155 8357900
E-mail: info.tbg@tosoh.com
Website: www.tosohbioscience.de

Analysis of Polyethylene by Pyrolysis-GC×GC–MS

Daniela Peroni, CDS Analytical

This application note shows the pyrolysis-GC×GC–HRMS profiles of crude oils for more detailed separations and more complete characterization of complex matrices, especially on the speciation of heteroatoms such as sulphur-containing compounds.

Pyrolysis, coupled to comprehensive two-dimensional gas chromatography-high resolution mass spectrometry (pyGC×GC–HRMS), is a very powerful technique for the characterization of complex, heavy matrices such as crude oils. The two-dimensional resolution provides enhanced separation of the pyrolysis products, leading to improved classification for groups and individual analytes. Additionally, the 2D pyrograms make sample comparison easier and more informative. Here we show the use of py-GC×GC for improved characterization and comparison of crude oils and the advantages arising from HRMS detection for speciation of heteroatoms such as sulphur-containing compounds.

Experimental Details

Three different crude oil samples were pyrolyzed at 750 °C for 15 s using a CDS Pyroprobe coupled to an Agilent 7890B GC equipped with a Zoex ZX2 cryogen-free thermal modulator and an Agilent 7200B QTOF detector.

Results and Discussions

Figure 1 shows the 2D separation obtained for the crude oils with corresponding percent response for paraffins, naphthenes and olefins, and aromatics (green). The two-dimensional chromatographic separation allows for the efficient separation of different chemical groups fully coeluting on the nonpolar primary column and are hereby effectively separated based on polarity on the second dimension. The 2D plots include a template designed to outline the distribution of the main aliphatic and aromatics hydrocarbons groups. The amount of each group is clearly different in the three samples: sample B is mostly aromatic, with significant paraffinic content and very low in naphthenes; samples A and C are mostly naphthenic, but sample C has more paraffins while being clearly poorer in aromatics.

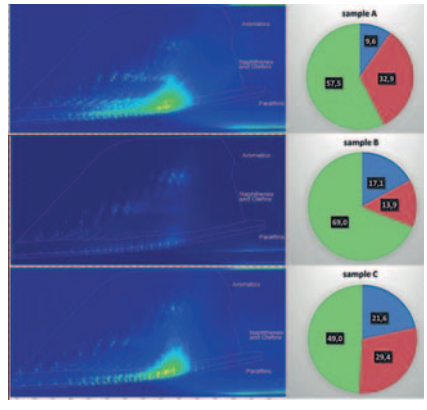


Figure 1: TIC 2D plots for crude oil A (top), B (middle), and C (bottom) and corresponding calculated percent response for paraffins (blue), naphthenes and olefins (red), and aromatics (green) for each sample.

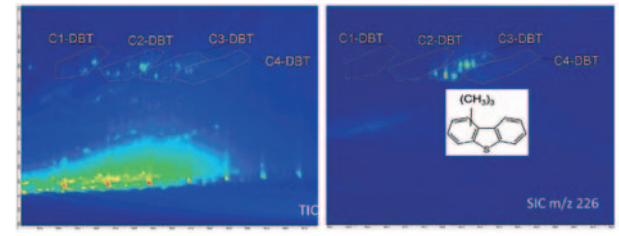


Figure 2: TIC 2D plot (left) and selected ion chromatogram (right) with template showing the location of dibenzothiophene (DBT) groups with different degree of alkyl-substitution for sample A.

Given their high relevance for the petrochemical industry, we focused on evaluating the presence of sulphur-containing compounds of potential interest. Figure 2 shows part of a template that indicates the location of the dibenzothiophenes with different degrees of alkyl-substitution, constructed by looking for the masses corresponding to the specific molecular formulas. This figure shows the example for dibenzothiophenes with three methyl groups. These are fully separated by the large aliphatic signal on the secondary column, making detection and identification (and potentially quantification) much easier and more accurate. Numerous isomers are present, differing only on the position of the alkyl-substitutions. Some blobs are fully coeluting on the primary column and, given the fact that the MS spectra will be close to identical, can be resolved and distinguished as different compounds only thanks to the enhanced two-dimensional resolving power.

CDS Analytical
465 Limestone Road
Oxford, Pennsylvania 19363, USA
Website: www.cdsanalytical.com

Analysis of Cellulose Molecular Weight Distributions in DMAC

Wyatt Technology

SEC-MALS analysis of cellulose provides absolute molar mass distributions to understand the impact of different extraction processes. The biopolymer is solubilized in DMAC, enabling liquid chromatography without degradation.

Cellulose, a biopolymer of great importance to the fibre and paper industries, is difficult to characterize because of its high molar mass. Its intractable nature means it cannot be dissolved in conventional solvents without chemical modification. With tedious effort, it can be modified so that it can be dissolved in an easy-to-use solvent like THF, but when the cellulose is so modified it is degraded and the analysis does not represent the source material.

Unmodified cellulose can be dissolved in dimethyl acetamide (DMAC) with LiCl added. The problem remains: how to characterize it without reference to column calibration standards that typically do not have the same conformation as cellulose. Absolute characterization is performed by combining multi-angle light scattering with size-exclusion chromatography (SEC-MALS) to determine molar mass, independently of elution standards.

Experimental Conditions

Separations were performed on a set of SDV-GPC columns in DMAC and LiCl. The separation columns were followed by the HPLC's UV detector, a DAWN® MALS detector (Wyatt Technology), and an Optilab® differential refractive index (dRI) detector (Wyatt Technology).

Data collection and analysis were performed in the ASTRA® software (Wyatt Technology) using empirically determined differential refractive index increments (*dn/dc*). Polymer molar mass, *M*, was calculated at each elution volume using signals from the two detectors.

Results

Molar masses determined by MALS in Figure 1 follow the usual logarithmic variation with elution volume. For the sake of comparison, a run of two mixed polystyrene standards was overlaid in a plot of molar mass versus elution volume. As can be clearly seen, a calibration based on polystyrene standards would overestimate the molar mass by more than a factor of five. This discrepancy is usually a result of branching, typical for cellulose in the MW range of 10^5–10^6 and above.

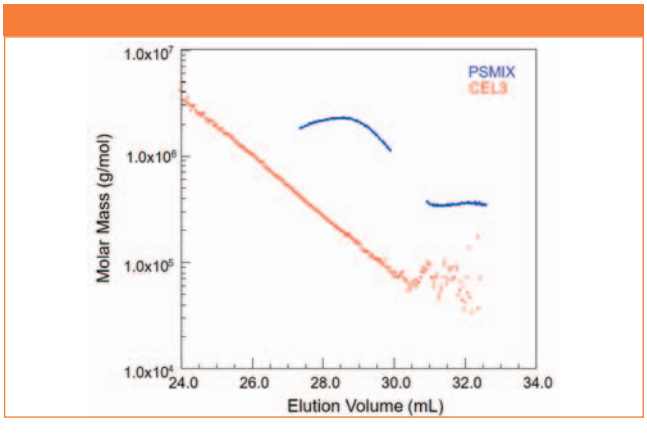


Figure 1: Two narrow polystyrene standards and a cellulose. Note that at the same elution volume, the "standard" gives a molar mass 10 times larger than the cellulose value.

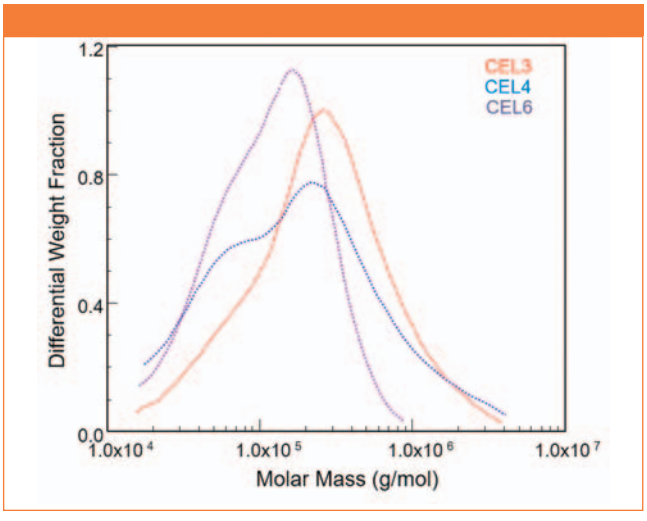


Figure 2: ASTRA's Differential Weight Distribution plot shows how different extraction processes create large variations in cellulose molar mass distributions.

The technical process of extracting the cellulose from the wood pulp can have a profound effect on the molar mass distributions. Figure 2 shows the differences in molar mass distributions arising from different extraction processes. Only a MALS detector can reveal and quantify those differences and thereby MALS has become an important tool in optimizing the production processes for cellulose.

Conclusions

The SEC-MALS results prove that the lengthy process of solubilizing the cellulose has been mastered, enabling the manufacturer to optimize the cellulose extraction process.

Wyatt Technology
6330 Hollister Avenue, Santa Barbara, California 93117, USA
Tel.: +1 (805) 681 9009
Website: www.wyatt.com

Absolute Molar Mass Analysis of Chitosans

Wyatt Technology

Chitosans are analyzed by SEC-MALS to determine molar mass moments and distributions.

Chitin is one of the most abundant biopolymers on earth (the other is cellulose). Chitin, or poly-N-acetylglucosamine, is the major polymer in the exoskeleton of marine arthropods and can also be found in fungi and yeasts.

Chitosan is deacetylated chitin. It can be obtained from shrimp or crab shells. Its applications vary from the therapeutic, such as wound healing, to cosmetics and dietary supplements.

For many of these applications, it is useful to fully characterize the molar mass moments and distributions of the chitosan products. Size-exclusion chromatography in combination with multi-angle light scattering detection (SEC-MALS) provides an easy method to obtain these properties in an absolute manner, free of molecular references. In this note, we describe the results for two chitosan samples analyzed by SEC-MALS.

Experimental Conditions

A DAWN® MALS detector (Wyatt Technology) and an Optilab® differential refractive index (dRI) detector (Wyatt Technology) were plumbed downstream of the GPC column. Data collection and analysis were performed in the ASTRA® software (Wyatt Technology) using empirically determined differential refractive index increments (*dn/dc*). Polymer molar mass, *M*, was calculated at each elution volume using signals from the two detectors.

The differential refractive index is a property of the polymer and solvent system. It is measured by injecting a series of known concentrations into the Optilab, using solutions that are often prepared by the dry weight method for accuracy, and the GPC mobile phase as solvent. ASTRA collects and analyzes the results to determine *dn/dc*.

Results

The molar masses of two chitosan samples were plotted as a function of elution volume in Figure 1. The molar mass decreases logarithmically, indicating that chromatographic conditions were optimal. The offset in elution volume for a given molar mass may be a result of conformation (short-chain branching, SCB) or non-ideal analyte-column interaction. SCB may be further investigated by making use of simultaneous size (radius of gyration, Rg) and molar mass analysis by MALS.

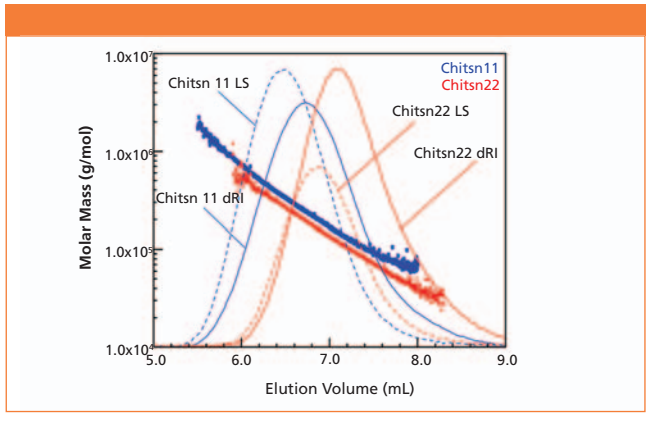


Figure 1: Molar mass vs. elution volume plots superimposed over chromatograms.

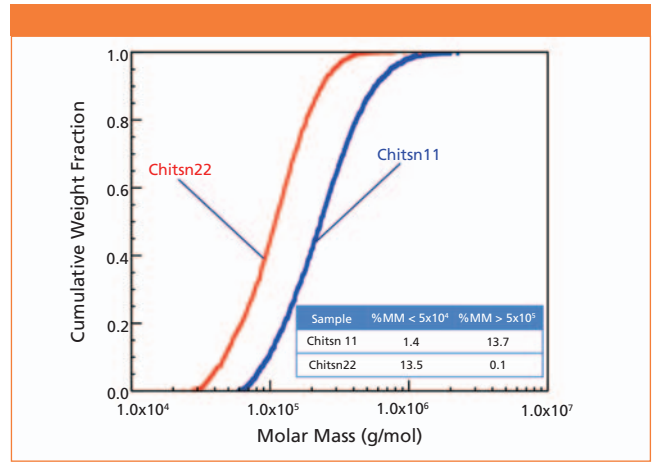


Figure 2: Cumulative molar mass distribution plot of two chitosan samples with quite different spans.

A cumulative molar mass distribution plot, depicted in Figure 2, clearly differentiates the two chitosan samples. ASTRA software can also report weight fractions above, below, or between the molar masses of interest. As an example, the weight fraction of molar masses below 50 kDa and above 500 kDa for these samples are given in the table in Figure 2. These calculations and the cumulative molar mass distribution plot are ideal for quality control applications.

Conclusions

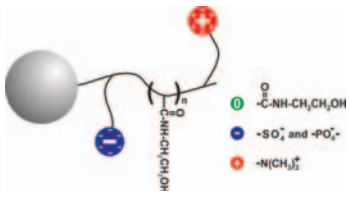
The results described herein show that MALS detection combined with SEC provides an indispensable tool for biopolymer characterization. Absolute molar mass and molar mass distribution can be readily obtained without the use of any standards or empirical relations, simplifying QC and routine analyses while enhancing in-depth analysis with absolute measurements of size as well as molar mass.

Wyatt Technology
6330 Hollister Avenue, Santa Barbara, California 93117, USA
Tel.: +1 (805) 681 9009
Website: www.wyatt.com

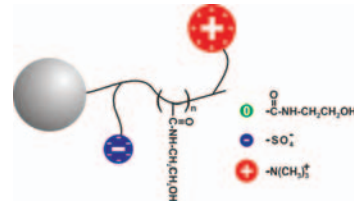
HILICON

iHILIC®-Fusion

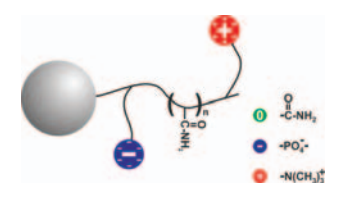
advances HILIC separations in UHPLC and HPLC



iHILIC®-Fusion
Silica based

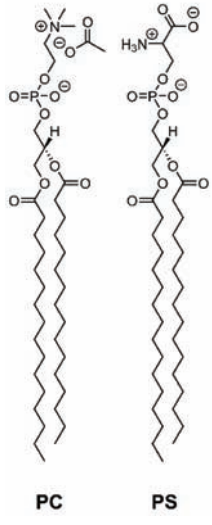


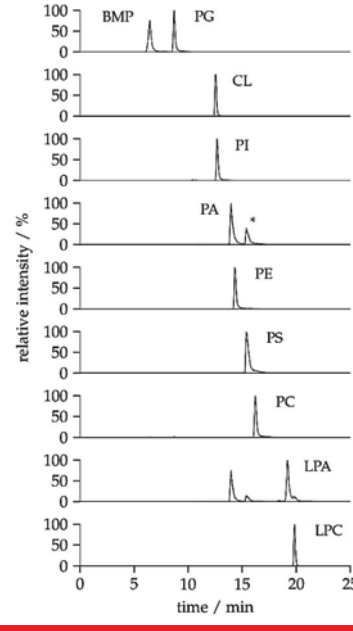
iHILIC®-Fusion(+)
Silica based



iHILIC®-Fusion(P)
Polymer based

- Charge modulated hydroxyethyl amide/amide HILIC columns
- Three complementary selectivities for separation of polar analytes
- Excellent durability and ultra-low bleeding for LC-MS
- Versatile columns for "Omics" studies and other applications
- iHILIC®-Fusion and iHILIC®-Fusion(+): pH 2-8; 1.8, 3.5, and 5 µm
- iHILIC®-Fusion(P): pH 1-10; 5 µm





For more information:
Email: info@hilicon.com | Website: www.hilicon.com

GERSTEL

AppNotes

Food Safety • Flavor and Fragrance • Quality Control

GC-MS and LC-MS

Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC–MS Based on the Commonly Used Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10)

Automated determination of 3-MCPD and glycidol in edible oils by GC–MS. An evaporation step helps reach the required LODs using a standard MSD, while removing excess derivatization reagent for improved uptime and stability.

Automated determination of Acrylamide in Brewed Coffee samples by Solid Phase Extraction (SPE)–LC–MS/MS

A manual SPE method used for the determination of acrylamide in brewed coffee was automated. Calibration standards prepared in freshly brewed green (unroasted) coffee produced good linearity and precision.

A Combination of Standard (SBSE) and Solvent-Assisted (SA-SBSE) Stir Bar Sorptive Extraction for Comprehensive Analysis of Flavor Compounds in Beverages

Smoothie News

Comprehensive GC/MS profiling of flavors in beverages such as smoothies with a significant amount of matrix. Standard SBSE and Solvent-Assisted-SBSE using liquid desorption and LVI-GC/MS enable good recovery of polar compounds.

Analysis of Aroma Compounds in Edible Oils by Direct Thermal Desorption GC–MS Using Slitted Micro-Vials

Hexanal, 2-(E)-nonenal and 2,4-(E,E)-decadienal, edible oil off-flavors derived from unsaturated fatty acid degradation were determined by direct thermal desorption in disposable micro-vials.

Qualitative Analysis of Coconut Water Products Using Stir Bar Sorptive Extraction (SBSE) combined with Thermal Desorption-GC–MS

Flavor compounds, off-flavors, pesticides, antioxidants, and compounds migrating from packaging materials were successfully determined in coconut water products by stir bar sorptive extraction (SBSE)-TD-GC–MS.

For more information about these and other GERSTEL applications, please go to www.gerstel.com/en/apps-food-beverages.htm

What can we do for you?

Agilent Technologies
Premier Solution Partner

GERSTEL

www.gerstel.com