The Column

7 December 2015 Volume 11 Issue 22

the interactive e-publication for the global separation science industry

Cover Story

Features

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Instrumental Innovations

Selected highlights of innovative chromatography products

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Instrumental Innovations 2015

Photo Credit: Mina De La O/Getty Images

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Gas Generators

GC Gas Purifier

In 2015 GasTrap introduced a self-regenerating nitrogen and air purifier to extend the life of disposable gas chromatography (GC) gas filters. According to the company, the purifier reduces trace levels of oxygen, water, hydrocarbons, and CO_2 by a factor of 10. The company reports that the disposable filter life is extended 10 times using this system.

Back in 1982 CEO Gary Hosmer patented the method of air separation by pressure swing adsorption to produce high purity nitrogen. This spawned the production of laboratory gas generators.

His latest innovation allows the scavenging of trace levels of contaminant gases with full regeneration every 6 min. According to the company, the GasTrap purifier reduces ordering, storing, changing, and disposal of filters but, more importantly, it saves money while improving the quality of gas supply to GC systems and detectors.

According to the company, this is a new invention and is the only self-regenerating gas purifier designed specifically for gas chromatography.

GasTrap have launched purifiers for nitrogen and air with versions for oxygen and hydrogen due in Spring 2016.

www.GasTrap.com

Nitrogen Generator

Peak Scientific introduced i-FlowLab, the scalable high-flow high-purity nitrogen solution, to their product range at the start of 2015. According to the company, the i-FlowLab promises to deliver a total solution for on-site generation of nitrogen gas, delivering a continuous and consistent supply of high-purity nitrogen at the pressure and flow rates required to meet the full and varied demands of each laboratory or research facility.

Engineered around PSA technology, i-FlowLab is available in various pre-configured specifications to suit specific flow and purity demands. A single i-FlowLab generator installation can provide nitrogen at flow rates from 40–3402 LPM. Purities are specified at the time of system design to meet the needs of the application up to 99.999%*. Additional CMS column banks can be added to each i-FlowLab generator after installation to increase maximum flow rate.

The streamlined and compact footprint design of the i-FlowLab system allows far more efficient use of available space, in comparison to bulk tanks, large quantities of dewars, or pressurized cylinders. According to the company, it promises to remove the uncertainties, administrative hassle, and volatile on-going costs associated with delivered gas supply, as well as being safe and practical — decreasing potential health and safety concerns.

*depending on model

www.peakscientific.com

GPC/SEC

Polyolefin Characterization

Polymer Char, a leading company in the development of instruments for polyolefin characterization, has gone a step further in 2015 with the addition of more instruments to its portfolio of equipment for process and quality control in polyolefin production plants.

The GPC-QC is a simplified and compact high-temperature GPC system that has been developed to fulfil the need in industry for robust and precise molar mass distribution (MMD) data for process control in polyolefin manufacturing plant laboratories.

According to the company, the GPC-QC delivers complete MMD, short chain branching, and intrinsic viscosity information for one sample in approximately 30 min, including dissolution, allowing the polyolefin manufacturer to perform a more accurate production control thanks to a precise and complete characterization of the resins. It also helps to save costs by reducing off-grade material production.

The entire process is fully automated in GPC-QC, including delivery of solvent, dissolution, and injections. All with a simplified and reliable hardware and software, especially designed with the needs of a quality control environment in mind.

http://www.polymerchar.com

High Resolution GPC/SEC

The SECcurity µRI from PSS is the detector of choice to make full use of the enhanced separation power of smaller particles, according to the company. The cell volume of 1.7 µL reduces band broadening to a minimum and allows operation with all types of gel permeation chromatography/size-exclusion chromatography (GPC/SEC) columns, including micro-columns with smaller dimensions. The detector can be used with any LC hardware and fits into PSS SECcurity stacks as well as Agilent 1260/1290 stacks.

The µRI is optimized for flow-rates between 0.1 mL/min and 1.2 mL/min. The integrated solvent recycle valve saves mobile phase and reduces waste. Electronic temperature regulation maintains a steady optical unit temperature up to 55° C resulting in a stable baseline. Software control is available from PSS WinGPC UniChrom and from all software packages that can control Agilent 1260 modules.

An upgrade package is also available to modify existing Agilent 1200/1260 RI detectors or PSS SECcurity 1260 RI detectors to high resolution µRI detectors.

The company reports that together with the PSS DVD1260M µ-viscometer and the PSS SLD1000M µ-right angle light scattering detector the µRI allows any GPC/SEC instrument to be turned into a high resolution triple detection system.

www.pss-polymer.com

Instrument Services

Analytical Services

In 2015, Phenomenex announced the creation of a new, stand-alone facility for its PhenoLogix analytical services group. The 9000-square-foot, fully functional laboratory is equipped and staffed to develop methods for customers and address unique user challenges in ultrahigh-pressure liquid chromatography (UHPLC), LC, LC coupled to mass spectrometry (MS), gas chromatography (GC), and sample preparation. PhenoLogix provides support for the company's broad product line to customers in drug discovery and development, environmental testing, food safety and quality, forensics and clinical research.

The company provides services by phone or on-line communication and at the customer site if necessary.

PhenoLogix grew out of Phenomenex's research and development department into a self-contained organization staffed with a team of applications scientists dedicated to developing solutions for customers. The new building houses LC, GC, MS, and liquid handling instruments from multiple manufacturers to cover a complete range of applications. The organization's analysts take on projects involving high performance liquid chromatography (HPLC), UHPLC, GC, GC–MS, LC–MS–MS, solid-phase extraction (SPE), and method development and optimization, as well as sample preparation, impurity isolations, chiral screening, and small- to medium-scale purifications.

www.phenomenex.com/Home/Phenologix

LC Instrumentation

Liquid Chromatography Autosampler

The Cecil Instruments' AutoQuest autosampler can be used with virtually any high performance liquid chromatography (HPLC) and ion chromatography (IC) system, according to the company. The company also reports that this autosampler is useful if users want more from an existing HPLC/IC system; have an increased workload, or are building a modular HPLC/IC system. The autosampler is reportedly a low maintenance addition to most chromatography laboratories. The 100-sample position autosampler provides low carryover, very high injection precision, priority sampling, replicate injections, and sample volumes from 5 µL to 2 mL. According to the company, the low carryover provides effective sampling for food trace residue analysis and contaminant levels.

With simple and reliable use via PC control or the stand-alone keys, and with easy third party connection and injection synchronization, the autosampler will work together with an existing system.

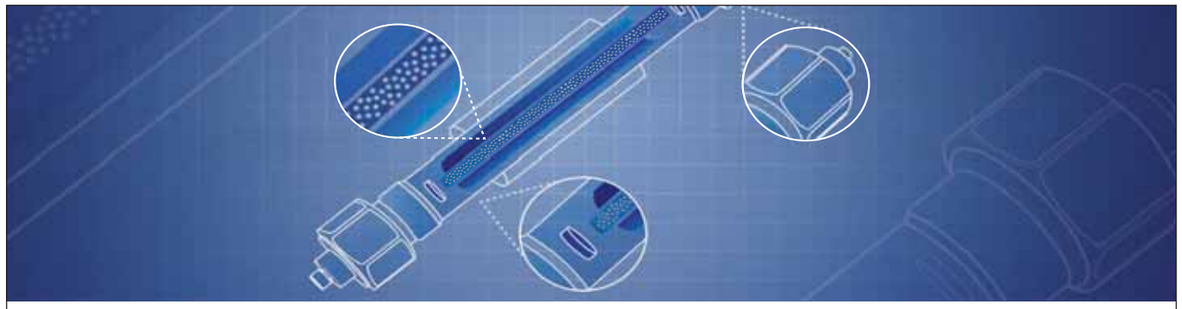
The AutoQuest autosampler fits within the Cecil Adept HPLC, Q-Adept HPLC, Merit HPLC, and IonQuest Ion Chromatography modular systems. According to the company, each chromatography system is designed to achieve long-term, fast, and reliable measurements with ease.

www.cecilinstruments.com/adept-autosamplers.html

IC Cation Suppressor

Trace analysis of cations, amines, and transition metals is possible with or without suppression. However, some applications require a particularly high analysis sensitivity. This can be achieved by suppression because suppression considerably lowers the detection limits. Such analyses are common, for instance, in pharmaceutical or power plant analyses. There are also a number of norms and standards that request cation analysis with suppression. Cation suppression reduces background conductivity to a minimum and decreases baseline noise. Both effects together improve the signal-to-noise ratio, which increases the sensitivity of the measuring system. Therefore, whenever very low concentrations of cations are required, analysis with suppression is the method of choice. Typical applications include ultratrace analysis of sodium in the presence of excess monoethanolamine in the water-steam circuits of power plants or the determination of alkali and alkaline earth metals and ammonium in ultrapure water. Traces of transition metals such as cobalt, nickel, zinc, manganese, and cadmium can be analyzed in various types of water samples as well as aliphatic and aromatic amines in pharmaceuticals, such as piperazine in cetirizine·HCl, tetrabutylammonium in atorvastatin, dimethylamine in meropenem, dimethylamine in imatinib mesylate, or meglumine in meglumine salts.

www.metrohm.com



SEPARATION OF FREE AMINO ACIDS AND PRIMARY AMINES USING DAICEL CROWN ETHER COLUMNS: CROWNPAK CR-I(+) AND CROWNPAK CR-I(-)

APPLICATION NOTE

INTRODUCTION

Daicel Corporation recently introduced a new generation of CROWNPAK® chiral selectors that can be used for the separation of free amino acids and primary amines: CROWNPAK CR-I(+) and CR-I(-). These crown ether selectors are immobilized on 5-µm silica support. Immobilization allows use of organic solvents in a wider range for both reversed-phase and normal-phase chromatography modes, thus, enhancing enantioselective resolution of chiral compounds in a shorter analysis time.

The CROWNPAK CR-I(+) and CR-I(-) chiral selectors are complementary to our CHIRALPAK® ZWIX chiral selectors. The complementarity provides a total solution for enantiorecognition of a wide variety of amino acids. For example, CR-I selectors afford challenging separations of asparagine, glutamine and serine.

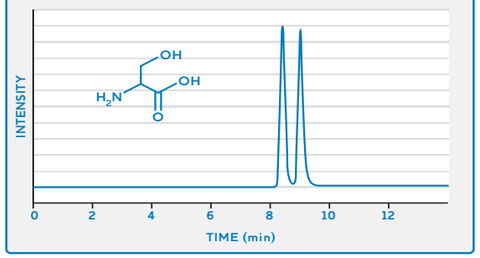
One important feature of both CHIRALPAK ZWIX and CROWNPAK CR-I chiral selectors is the ability to control the elution order. Typically, use of ZWIX(+) and CR-I(+) columns would lead to the reversal of the elution order for free amino acids eluted from ZWIX(-) and CR-I(-) columns.

EXPERIMENTAL AND DISCUSSION

A CROWNPAK CR-I(+) column, 3.0 mm i.d. x 150 mm, packed with 5-µm particles was used to develop the separation of DL-serine. The mobile phase was a mixture of perchloric acid and acetonitrile.

The CR-I(+) and CR-I(-) selectors are packed in columns of 3.0 mm i.d. and 150 mm long.

SEPARATION OF DL-SERINE ON CROWNPAK CR-I(+)



CHROMATOGRAPHIC CONDITIONS

Column Size:	Daicel CROWNPAK CR-I(+) 3.0 mm i.d. x 150 mm long, 5-µm
Mobile Phase:	$HClO_4$ a.q.(pH1.0) / ACN=85/15(v/v)
Flow Rate:	0.1 ml/min
UV Detection:	200 nm
Column Temperature:	25 °C

Note: *Recent scientific studies have demonstrated that the brain of Alzheimer's disease patients contain unusually high levels of D-serine. The potential association of the D-serine level with cognitive decline in the patients may lead to the development of a novel and effective biomarker for early detection of the disease.*

DAICEL

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CHIRAL TECHNOLOGIES DAICEL GROUP

Unified Chromatography System

Shimadzu's Nexera UC unified chromatography system is reportedly the world's first-ever unified and fully automated instrument combining supercritical fluid extraction (SFE) with supercritical fluid chromatography (SFC). According to the company, the SFE–SFC–MS platform merges quick and easy on-line sample preparation with advanced chromatographic analysis and high sensitivity detection.

The system serves a wide range of applications, for example, food control, research in biopharmaceuticals, and environmental analysis. The company reports that it enables highly reproducible extraction and stable analysis of unstable samples prone to oxidation or dissociation if exposed to light or air.

The highlights include:

1. Unified and fully automated system combining SFE with SFC or LC analysis.
2. Sample throughput for SFE, enabling up to 48 samples to be continuously and automatically processed.
3. Excellent levels of sensitivity as a result of splitless injection of the entire volume of eluent into the mass spectrometer.

Nexera UC was developed in collaboration between Shimadzu Corporation, Osaka University, Kobe University, and Miyazaki Agricultural Research Institute, which is funded by the Japan Science and Technology Agency (JST).

Please click **www.shimadzu.eu** for further information.

Light Scattering Detectors

Multi-Angle Light Scattering Detector

Accurate, rapid, and reliable molecular weight determinations are high on the wish list of many chromatographers. While ultrahigh-pressure liquid chromatography (UHPLC) overcomes many speed and resolution issues, the reliability of traditional gel permeation/size-exclusion chromatography (GPC/SEC) analysis based on column calibration can be limited by the reliance on reference standards. Multi-angle light scattering (MALS) coupled to SEC eliminates the need for calibration because MALS determines the molecular weights and sizes of polymers and biopolymers independently of retention time.

However, standard MALS detectors are not capable of handling the very narrow peaks produced in UHPLC. The µDAWN MALS detector coupled with an Optilab UT-rEX refractive index (RI) detector (both by Wyatt Technology) create a µSEC–MALS instrument solution. When used with the latest chromatographic systems, such as Waters ACQUITY UPLC and Waters' Advanced Polymer Chromatography (APC), µSEC–MALS brings absolute molecular weight and size determinations to the micro scale. According to Wyatt, µSEC–MALS maintains the narrow, well-resolved peaks seen in UHPLC and with the APC, for samples spanning molecular weights from just 200 g/mol to millions of g/mol.

µSEC–MALS provides additional benefits beyond typical molar mass and size analyses. It can also characterize aggregates, conjugated proteins, and protein conformation as part of an essential quality control program. In the polymer realm, µSEC–MALS is useful in analyzing samples for which reference molecules do not exist, such as co-polymers and branched polymers, determining co-polymer composition and branching ratios. Accompanied by ASTRA software, a wide range of analytical methods are available for the µDAWN.

http://www.wyatt.com/uDAWN

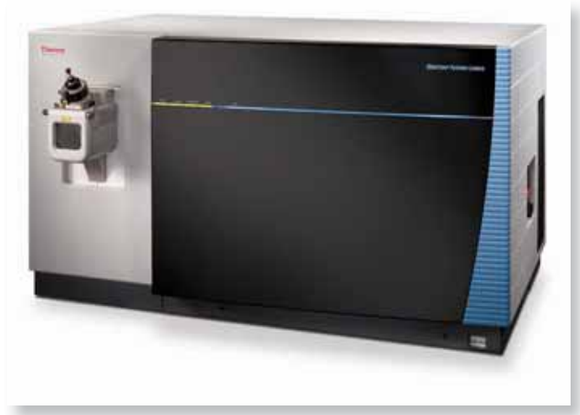
Mass Spectrometry Instrumentation

Routine Testing

Sciex has recently launched the new X-series mass spectrometry platform. According to the company, the X500R is the first robust and easy-to-use high-resolution MS system for routine food, environmental, and forensic testing. The platform was developed with users to ensure maximum balance of reliability and speed, as well as multiple workflow capacity and high productivity through the Sciex OS software. The software includes full data review and data analytics, improved algorithms, and new user interface designed for enhanced productivity. The breadth of data acquisition capabilities has been extended to include full scan high resolution TOF-MS, information dependent acquisition for complete MS–MS spectra of all detected MS peaks, SWATH Acquisition, and MRM^{HR} acquisition. Sciex reports that the X500R is the result of extensive routine user input and is unmatched in many ways, from its compact benchtop-sized footprint and modern design for easy serviceability, to its Turbo V ionization source for performance. Sciex plans to announce other new X-Series models for application-specific needs.

http://ter.li/68nvls

Protein Characterization



In 2015, Thermo Fisher Scientific released the Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer (MS) for protein and small molecule quantitation and characterization for research in proteomics, biopharma, and metabolomics. The newest addition to the Orbitrap family offers the two fundamental aspects of system performance critical to proteomics researchers: intact protein analysis, which allows scientists to perform more comprehensive protein characterization; and sensitivity, now 3 to 5 times greater, resulting in more accurate qualitative and quantitative sample analyses faster.

According to the company, the mass spectrometer combines the versatility of a Tribrid system with the selectivity of high-resolution accurate mass Orbitrap technology to achieve proteome-wide coverage. The instrument uses a very bright ion source to improve quantitation limits. The segmented quadrupole mass filter, powered by Advanced Quadrupole Technology, reportedly improves selectivity, and the advanced vacuum technology increases the transmission of ions, expanding the detection limits of the system. The company reports that the high-definition electron-transfer dissociation fragmentation capabilities increase the peptide precursor ion populations available. The ADAPT technology improves ease-of-use by adjusting key parameters without prior knowledge of sample amount. Researchers can reportedly quantify up to 10,000 proteins simultaneously across 10 samples in a single day.

http://www.thermoscientific.com/lumos

QTOF Mass Spectrometer

The Waters Vion IMS QTof Mass Spectrometer combines the benefits of both high-resolution mass spectrometry (HR-MS) and ion mobility separation (IMS) to bring clarity and confidence to routine analyses, according to the company. Ion mobility can separate spectral interferences from molecular ions and generates collision cross-section (CCS) values for every ion, giving scientists more information than they can get from traditional liquid chromatography–mass spectrometry (LC–MS) experiments, according to the company.

By separating ions from one another based on their shape, size, mass, and charge, ion mobility provides an additional, or orthogonal, dimension of separation. The selectivity of ion mobility refines spectra and allows the analyst to discriminate analyte ions from chromatographically co-eluting compounds and background interferences so compounds may be characterized better.

With Vion IMS QTof, CCS measurements are automatically available for every ion in an LC–MS experiment. Scientists can now identify sample analytes and overcome typical day-to-day challenges such as shifting retention times caused by matrix complexity and the use of different chromatography conditions.

According to Waters, for scientists involved in sample screening, metabolite identification, or classical -omics experiments, their ability to detect and identify all sample analytes in single analysis at the bench has reached a new level with the Vion IMS QTof.

www.waters.com/vion

Sample Preparation Instrumentation

Dynamic Headspace Analysis

The Dynamic Headspace System for large samples (DHS L) from Gerstel extracts and concentrates volatile organic compounds (VOCs) from liquid or solid samples placed in sample containers of up to 1 L volume. The system analyzes large or inhomogeneous bulk samples without cumbersome representative sample cutting; for example, the determination of volatiles in consumer products, foods, or packaging from which small representative samples cannot easily be taken. The sample is thermostated while the headspace is purged with a controlled flow of inert gas, providing controlled and efficient extraction conditions. Purged analytes are trapped in a replaceable sorbent-filled tube. Sample holders for flooring material eliminate interfering emissions from the edges of the cut sample. Non-sampled purge periods can be introduced to simulate air exchange conditions in environmental chambers and a series of analyses can be performed on a sample to determine emission behaviour over time. According to the company, the system has very low blank values and greatly diminished sink effects compared with environmental chambers. DHS screening can eliminate time consuming and expensive chamber work. The analysis method and sequence table are set up with just a few mouse-clicks. A single sample model DHS L and the DHS LS Autosampler with 11 positions are available.

www.gerstel.com

Sorbent Tube Conditioning

This year, Markes International launched TC-20 TAG, a stand-alone sorbent sampling tube conditioning unit for thoroughly cleaning thermal desorption (TD) tubes in preparation for sampling. The unit conditions up to 20 sorbent tubes simultaneously to free up the analytical TD instrument, which saves a huge amount of time, according to the company. TD tubes would otherwise have to be conditioned sequentially using the analytical TD instrument. The TC-20 TAG's added advantage is that it can accommodate tubes fitted with Markes' TubeTAG — a radio frequency identification (RFID)-based system for associating data with a tag fitted to the tube itself. According to the company, this releases analysts from the burden of manually logging data, and the associated risk of error. A key application of the new system is the newly-released US EPA Method 325 for refinery fenceline monitoring of benzene and other volatile organic compounds (VOCs). This demands regular passive sampling of air around petrochemical plants, requiring refineries to put in place efficient sampling and analysis protocols. Markes reports that the release of TC-20 TAG is extremely timely, because it allows analysts to free up their TD–GC (gas chromatography) systems to run the large number of samples specified in the method, while also ensuring a rigorous chain of custody for their tubes.

www.markes.com/Products/Instrumentation/TC-20.aspx

News

LGC Acquires Maine Standards Company

LGC (London, UK) has announced its acquisition of Maine Standards Company (Cumberland Foreside, Maine, USA). A leading provider of calibration verification materials in the US, Maine Standards Company bolsters LGC's portfolio of reference materials and proficiency testing schemes available for the clinical market.

Euan O'Sullivan, Managing Director of LGC's Standards Divison, expressed his delight in welcoming the Maine based company to LGC, and commented "Our two organizations represent a highly complementary commercial and cultural fit, with a common commitment to developing products of the very highest quality for the benefit of our customers. We look forward to working with Maine Standards Company to complete its leading portfolio of calibration verification materials for the US market, whilst using our global network to expand sales of these products internationally."

Maine Standards Company will continue to operate from its recently opened facility near Portland, Maine. Tom Happe, Founder and President of Maine Standards Company, hoped the union of the two companies capabilities would ease the transition of Maine Standards Company into the global market and address growing interest in their services from overseas.

Both companies hope the combination of their respective analytical capabilities will lead to the development of new products for their mutual customers.

For more information please visit **www.lgcgroup.com**

Quantifying Ocular Besifloxacin with LC–MS–MS

A new liquid chromatography tandem mass spectrometry (LC–MS–MS) method has been developed to study besifloxacin, which is used to treat bacterial conjunctivitis.[1] Besifloxacin, along with other fluoroquinolones, is used to treat a wide range of ocular infections. However, the overuse of the fluoroquinolone class of antibiotics puts them at high risk of bacteria developing resistance to them.

Besifloxacin, developed specifically for ophthalmic use, displays potent efficiency against a wide range of Gram-positive and Gram-negative ocular pathogens, including multi-resistant strains. This complex mode of action and rapid distribution within ocular tissues theoretically reduces the risk of resistance making besifloxacin important pharmaceutically.

Two chromatographic methods were previously developed to study besifloxacin; however, both the chiral high performance liquid chromatography (HPLC) and HPLC with UV detection methods were insufficient according to the Chinese authors and did not meet the desired sensitivity, speed, and throughput in bio-sample analysis.

The new method developed for quantification of besifloxacin in ocular and plasma tissues used LC–MS–MS to reach the desired analytical requirements.

Tissues were obtained from rabbits and analyzed using this new method. The lower limit of quantification for besioflaxin was 0.103 ng/mL for plasma and 2.06 ng/mL for other ocular tissues with good accuracy, precision, and a short run time of 3.0 min. This was across single and multiple topical applications.

According to the authors, this method was rapid and reproducible and therefore suitable for the pharmacokinetic study of besifloxacin. — L.B.

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1. X.F Gu *et al.*, *J. Pharm. Biom. Anal.* **117**, 37–46 (2016).

Photo Credit: Lew Robertson/Getty Images

Professor Michel Dreux

It is with great sadness we announce the death of Professor Michel Dreux. Professor Dreux was a professor at the University of Orléans, France. Head of a Chromatography Laboratory, he merged his team with a team of organic chemists to create the Bioorganic and Analytical Chemistry Laboratory (LCBA), which became the Institute of Organic and Analytical Chemistry (ICOA) in 1995, by merging with other carbohydrate-synthesis-focused laboratories.

Head of the Analytical Department in this Institute, he continued his research on both liquid chromatography and capillary electrophoresis. He taught hundreds of students at the University of Orléans, and supported several Ph.D. studies in his laboratories. He was highly and unanimously appreciated by both his colleagues and students for his readiness to help, the relevance of his advice, and his kindness.

Focused on carbohydrate and non-chromophoric chemical and biochemical species (amino acids, ions) analysis in the 1980s, Professor Dreux developed on his own a first prototype of an evaporative light-scattering detector (ELSD). This first device was the pioneer of several generations of low-temperature evaporative light-scattering detectors (LT-ELSD).

He produced numerous scientific publications and several patents to improve the LT-ELSD technology and to emphasize the relevance of this detection mode in liquid chromatography and supercritical fluid chromatography. His works on the detector originated several Ph.D. and scientists research programmes within the University. He also received the "Palmes Académiques" award, which is a specific decoration for services to Education in France.

In 2002, he entirely joined Sedere as the Scientific Director in order to continue the development of new ELSDs. He was a friend and a mentor for most people at his University and at Sedere, and is terribly missed today. — **Rodolphe Pennanec, Sedere**

Pharmaceuticals Found in Grocery Fish Fillets

Anti-histamines and compounds used to medicate anxiety and seizures were among those identified in grocery market fish fillets using a novel gas chromatography–mass spectrometry (GC–MS) technique.[1]

Consumption of pharmaceuticals has risen dramatically with tons of chemicals being produced annually worldwide. However, after consumption these compounds are excreted as parent compounds or their metabolites into ecosystems from urine, faeces, or residues. Currently the main routes of contamination are wastewater treatment plants that are not commonly designed to eliminate the drugs because they are non-regulated water contaminants.

The presence of these compounds in concentrations ranging from ng/L to µg/L is of great concern with a number of studies finding similar results across many environments, indicating that many pharmaceuticals and metabolites are environmentally persistent, bioactive, and have potential to bioaccumulate.[2,3,4,5,6]

By investigating pharmaceuticals in fish fillets available across multiple stores, researchers developed and validated a GC–MS method using selected ion monitoring mode (GC–SIM–MS). While liquid chromatography coupled to tandem MS (LC–MS–MS) analysis is favoured for monitoring polar pharmaceuticals and their metabolites from environmental matrices, the method requires about 50–150 times more volume of samples or pure standards for each injection than GC–MS, as well as being considerably more expensive.

The GC–SIM–MS method identified a total of nine pharmaceuticals and their metabolites at various concentrations including diphenhydramine (DPH), diazepam (DZP), and carbamazepine (CZP); DZP was reported for the first time in edible fish fillets collected from grocery stores.

Pharmaceuticals DPH and DZP were further investigated using GC–SIM–MS. Silica gel cleaned extracts of 14 different species of edible fish were found to contain concentrations ranging between 0.61–6.21 ng/g for DPH and 1.99–16.57 ng/g for DZP.

Previous studies have explored the effect pharmaceuticals have upon organisms in the wild[7,8,9] finding a wide range of responses from reductions in alertness to stress. This study highlights the need for further investigation into emerging contaminants including non-regulated pharmaceuticals and personal care products. — L.B.

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Photo Credit: Eugene Mymrin/Getty Images

LCGC TV Highlights

***LCGC* TV: Rudolf Krska on Mycotoxin Analysis Using LC–MS–MS**
Mycotoxins are an important group of secondary metabolites produced by fungi that can cause disease in humans and animals. Rudolf Krska from BOKU, IFA-Tulln, in Vienna, Austria, explains why mycotoxin analysis is important and describes the analytical challenges faced.
Watch Here>>

***LCGC* TV: Advancing Chromatographic Methods**
Kate Rimmer of the National Institute of Standards and Technology (NIST) discusses separation science research carried out at NIST — on using 2D LC to quantitate polycyclic aromatic hydrocarbons, the challenges of identifying the best peak integration approach, and where 2D LC research is heading next.
Watch Here>>

Peaks of the Week

The Best of Column Watch and Sample Prep Perspectives: A Farewell to Ron Majors
Throughout his career, Ron Majors always had his finger on the pulse of the latest chromatography developments, and he kept readers in the know too. This special supplement from *LCGC North America* celebrates his career. **Read Here>>**

The Evolution of UHPLC in Pharmaceutical Analysis
Michael Dong offers advice on using ultrahigh-pressure liquid chromatography (UHPLC) for method development in pharmaceutical analysis and the important distinction between method conversion and method transfer. **Read Here>>**

GC Troubleshooting in 20 Pictures (Part 1)
The ability to recognize baseline and separation problems and identify their causes from the everyday pictures we see on our data systems, is a fundamental skill that every chromatographer should learn. The first 10 of these is presented, with a brief explanation of the potential cause and suggested fixes.
Read Here>>

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News In Brief

A review focusing on new developments in microextraction techniques within bioanalysis has been published by *Analytica Chemica Acta*. The authors detail new microextraction methods and discuss potential applications. Trends towards miniaturization and on-chip extraction are highlighted.
doi:10.1016/j.aca.2015.10.041

Researchers from Chongqing Medical University, China, have developed a simple and rapid HPLC method for the determination of bioamines. Deploying a boron-doped diamond electrode (BDD), researchers simultaneously determined 11 compounds related to the metabolism of bioamines in rat cortices and hippocampi.
doi:10.1016/j.jpba.2015.10.020

A new study has developed a rapid chiral separation technique for racemic cetirizine in human plasma. Using subcritical fluid chromatography–tandem mass spectrometry researchers successfully separated cetirizine and quantified levocetirzine. The method was compared to current normal phase-HPLC or reversed phase-HPLC methods finding a reduction in analysis time and cost.
doi:10.1016/j.jpba.2015.09.012

The Power of Planar Chromatography

High performance thin-layer chromatography (HPTLC) offers many advantages over conventional separation techniques when applied to complex samples. *The Column* interviewed Gertrud Morlock from Justus Liebig University Giessen in Germany, on her current research, which involves hyphenating HPTLC with multiple analytical techniques.

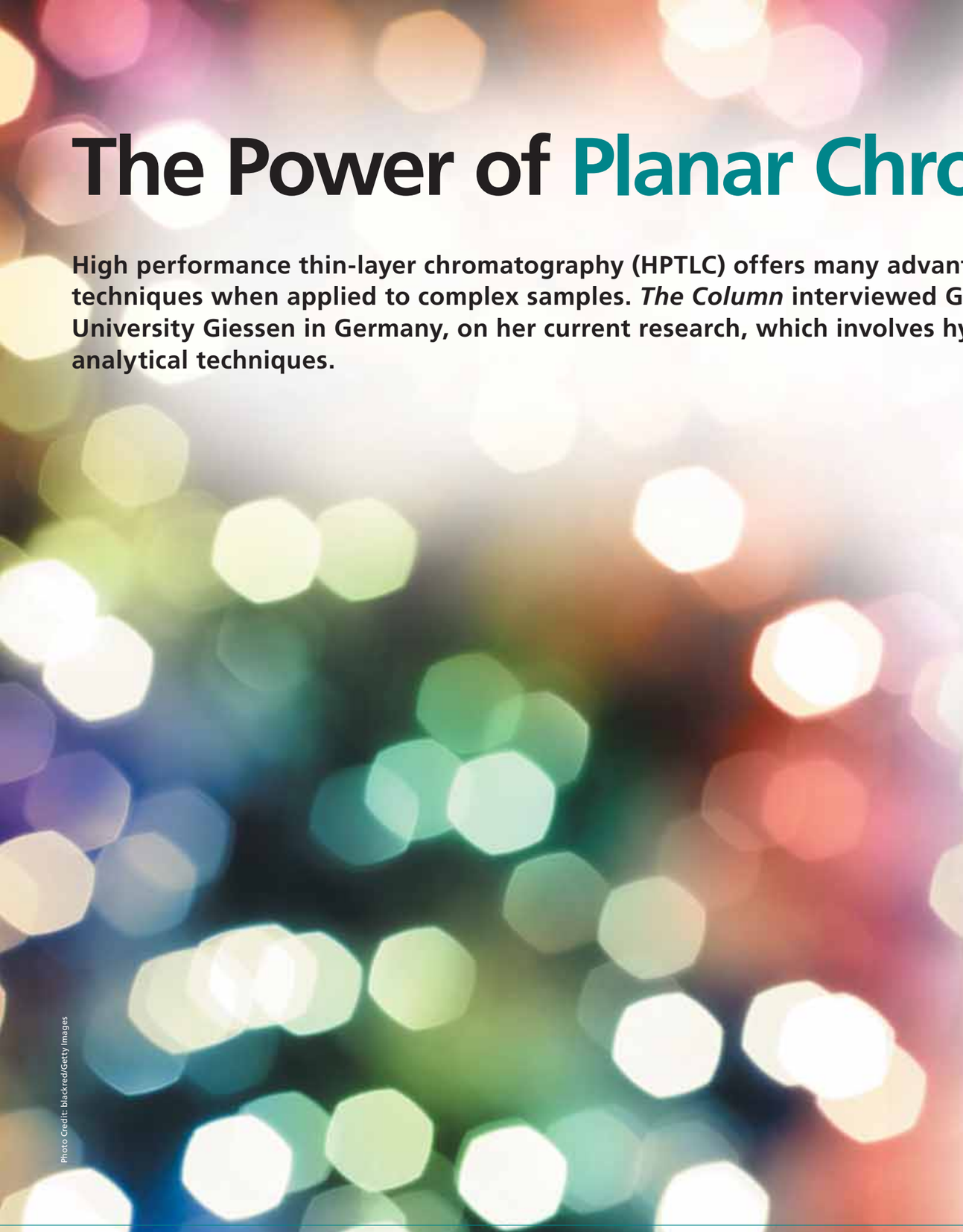

Photo Credit: blackred/Getty Images

Q. Your research focuses on the development of planar chromatography. For those who are not familiar with the field, can you describe the principles behind it?

A: Planar chromatography comprises all chromatographic techniques that have an open planar stationary phase present as — or on — a plane. Thin-layer chromatography (TLC) is the most commonly used and simple planar chromatographic technique, whereas high performance TLC (HPTLC) is the most efficient and powerful one and refers to an optimized coating material combined with advanced instrumentation to utilize its full potential.

Hyphenations of HPTLC, particularly with mass spectrometry (HPTLC–MS), were mainly developed in the last decade. A key feature of elution head-based HPTLC–MS is the cost-effective, sensitive, and targeted recording of mass spectra, whereas for fast scan along a separated sample track desorption-based HPTLC–MS is useful, and also more discriminative for the transfer of compounds. Optimized solutions are also commercially available. Hyphenations of HPTLC with attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy are also possible with an elution head-based interface. Recent progress was also made in the hyphenation with bioassays.

HPTLC is a technique that can be used for many kinds of hyphenation because of its open system and stepwise operation as well as its image-giving feature. Thus, HPTLC–UV–vis–fluorescence detection (FLD)–bioassay–heartcut high performance liquid chromatography (HPLC)–HRMS could become an interesting robust workflow for analysts searching for effective compounds in complex samples.

Q. In a study published in 2014, you developed a method using quantitative direct bioautography coupled with MS to discover endocrine disrupting compounds (EDCs).[1] Can you explain what bioautography refers to?

A: Bioautography has been known since

1946 and it is the combination of planar chromatography with bioassays. There are different subcategories, but direct bioautography is the most effective. The chromatogram is immersed into the cell suspension and this seeded plate is incubated for a few hours, depending on the bioassay selected. After a visualization step, only the effective compound zones are visible in a complex sample that might consist of, for example, up to 4000 compounds. Direct bioautography may answer analytical questions rapidly, which may not be solved with other techniques that fast.

Q. Can you explain how you developed this novel approach using HPTLC?

A: The diffusion of zones during the incubation period has been a fundamental challenge since 1946 and past approaches to solve it have failed. Water has a high elution power on normal-phases like silica gel, resulting in diffusion of polar to middle polar compounds, and so we chose to study the application of water-wettable reversed phases. Water-wettable reversed layers have been available since 1970, and so it was unsurprising that we initially obtained no response — other researchers who might have tried this must have got the same result! But because the approach made sense theoretically, we did not give up. We studied several parameters of influence, as well as the pH value of the plate. This pH measurement revealed the crucial shortcoming: the plate's pH needed adjustment. Buffering of the layer before the bioassay application was studied next and led to a successful response and sharply bounded bioactive zones! After optimization of further bioassay parameters, a streamlined workflow was obtained that detected important estrogens down to the fg/band level and allowed those estrogens without enrichment to be detected down to the ng/L range.[3]

Q. In a recent study, you developed a HPTLC–MS method for the detection of endocrine disrupting compounds (EDCs) in food.[2] Why did you focus on the detection of EDCs in food samples?

A: Endocrine-disrupting compounds (EDCs) may affect the metabolism at very low concentrations. In this bioassay example, xeno-oestrogens are detectable, as well as phyto-oestrogens, oestrogen-effective contaminants, degradation products, or by-products of food processing. We were looking for all compounds in a complex sample that can generate this endocrine effect. A holistic food profiling for bioactivity is urgent on the food intake side, as exemplarily shown in this paper. The demonstrated potential of HPTLC in combination with cell assays in general can contribute to the discovery of not only ECDs present but also, for example, of new antibiotics against antibiotic-resistant bacteria and especially to the understanding of potential developments of a resistance against antibiotics or of anabolic effects caused by food intake. Commonly used target analyses cannot provide comprehensive answers, and such limited results that focus only on known bioactive compounds may mislead decision makers. Hence, optimized bioautographic workflows are important tools in the analytical toolbox of experts.

Q. Can you describe the principles of the method and how it was developed?

A: You only see and get what you extract and what you detect — not every compound is ionizable using the standard settings of MS, and so on… analysts have to be very cautious in the interpretation of results. The principle behind this method is that the sample is used as naturally as possible; it is only extracted with different solvents and then filtered. As the layer is used only once, sample preparation on the plate and chromatography can be performed simultaneously. Matrix can remain at the start zone or be shifted to the front. However, it is still accessible for the non-target bioassay detection and not out-of-focus. At this point bioactive compounds are discovered — the next question is "Which compounds are these?"

At the moment we are working on coupling the method to MS directly from the bioactive zone. Using an orthogonal separation principle, coeluting compounds can be separated. The elution of a zone via an integrated HPLC column and valve into the MS increases the overall separation number at almost no or few extra-time for the second dimension. This way most of the very polar salt load of the cell culture medium can be directed to the waste. This streamlined hyphenated workflow, HPTLC–UV–vis–FLD–bioassay–HPLC–HRMS, is fascinating because of its stepwise, image-giving feature. Despite its super-hyphenation, it offers the potential to work robustly in routine analysis if the analyst has suitable expertise. More education and training in HPTLC is necessary to achieve this.

Q. What are the advantages of using HPTLC–MS over existing methods, for example, HPLC–MS?

A: For non-target analysis by HPTLC–UV–vis–FLD–MS, matrix or background is not directed into the MS because visual evaluation is performed prior to MS recording as a result of the stepwise operation. I consider this and the parallel screening of many samples as a real benefit. This multi-detection preselection via the UV–vis–FLD images for MS recordings of natural complex samples may reduce the cleaning circles and improve robustness of the MS system. Knowing that the focus on UV–vis–FLD-detectable zones only may mislead a decision, a universal derivatization reaction follows on the same plate after the MS recordings. Any further compound can thus be detected and attract attention. Of course, there is a point where HPTLC analysts have to stop, despite all technical flexibility.

Q. What are you working on next?

A: "Next" is a scientific secret… At the moment: On instrumental developments such as office chromatography, hyphenations with NMR, HPLC–HPTLC, further bioassays, and MS such as direct analysis in real time MS (DART–MS), matrix-assisted laser desorption/ionization–time-of-flight MS (MALDI–TOF-MS), etc. On the application side, we are working on different projects such as profiling and pattern recognition of a hundred packaging ink formulations, bio profiling of coffee powders prepared differently, wine samples and traditional German medicines, steviol glycosides in leaves cultivated differently, various projects with fermentation broths, and amino acids in dates. There are so many because we have so many dedicated young students who want to learn this at JLU Giessen. Cost-effective bioprofilings are definitely part of the analytical future. We are working on these — and more separation scientists should too.

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Related Links:
Course day on HPTLC-MS - 335/15 of the German Chemical Society

Course day on HPTLC-bioassay-HRMS - 338/15 of the German Chemical Society

[Both courses in English on request.]

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Tips & Tricks GPC/SEC: Finding the Right Standards

Daniela Held, Jasmin Preis, and Friedhelm Gores, PSS Polymer Standards Service GmbH, Mainz, Germany.

Polystyrenes (PS) are the most commonly used reference standards in gel permeation chromatography/size-exclusion chromatography (GPC/SEC) for nearly all organic GPC/SEC separations including high temperature GPC and pullulan or dextran for aqueous GPC/SEC. The majority of users rely on these standards for reproducible results. However, there is potential for improvements and this instalment of Tips & Tricks will discuss some general points that should be considered when determining calibration standards.

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Gel permeation chromatography/size-exclusion chromatography (GPC/SEC) reference materials and standards are used for many different purposes. In addition to using them to validate a system, a detector, or to verify your own operational procedures, one of their main uses is to create a GPC/SEC calibration curve. Calibration curves provide extensive information about the chromatographic system and they allow us to:

- determine molar mass distributions and averages for unknown samples;
- measure pore sizes using inverse GPC/SEC for porous materials;
- quantify the resolution of GPC/SEC columns;
- identify the optimum separation range of GPC/SEC columns.

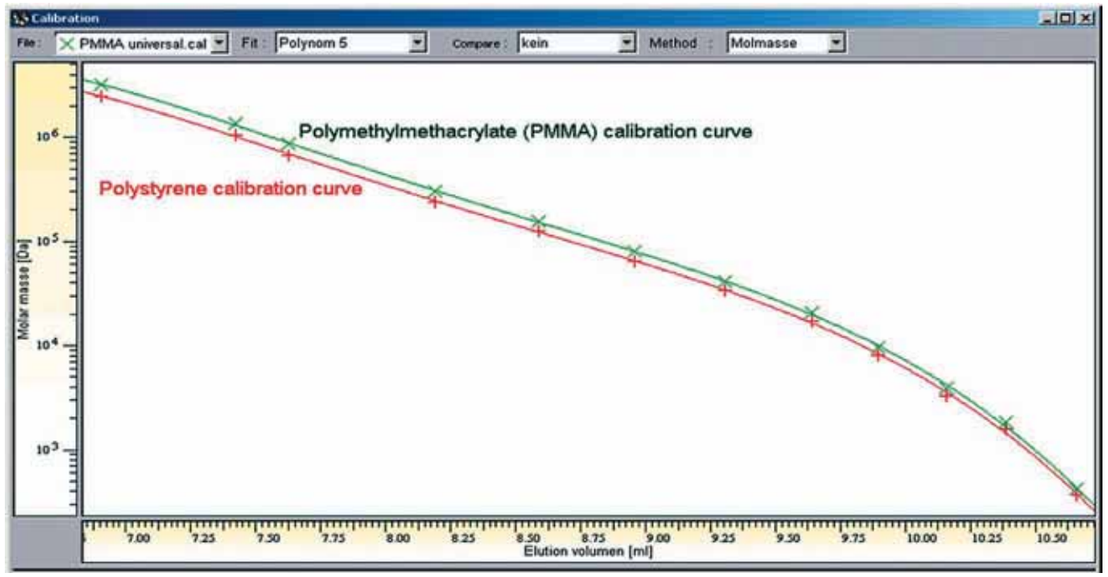
GPC/SEC reference materials are available for many different polymer types. Just as for GPC/SEC stationary phase materials, standards are very often classified either as aqueous standards for water-based GPC/SEC or as organic standards for GPC/SEC in organic solvents. There are a few standards that can be used for both applications.

Before deciding on a reference materials the following points should be considered.

What Reference Material is the Best Match for my Macromolecules?

GPC/SEC is a relative method and the separation is based on the size (not the molar mass) of the molecule in solution. For example, a 100,000 Da polystyrene and a 100,000 Da polymethyl methacrylate (PMMA) differ in tetrahydrofuran (THF) by approximately 20% in size. Their calibration curves will not be the same, and therefore different results will be obtained for the molar mass distribution. Figure 1 shows an overlay of a PS and a PMMA calibration

Figure 1: Overlay of PS and PMMA calibration curve in THF.



curve where it is clearly visible that the curves do not match.

Please note that the shift between the curves is not just a linear shift with a constant factor. It is possible to use a mathematical procedure to transfer calibration curves into each other, but this process requires four constants in total, with the Mark-Houwink constants K and α for the two types of macromolecules involved.[1]

To obtain accurate molar masses it is best to use standards that are chemically and structurally alike to your samples. Therefore, a system that is used to characterize 1,4 polyisoprene, is best calibrated with narrowly distributed 1,4 polyisoprene standards. There are two advantages to this method. Firstly, all chromatographic problems can be easily identified when reference materials with known elution profiles are measured, and secondly accurate molar masses are obtained.

If no perfectly matching narrowly distributed reference materials are available several strategies can be applied to achieve reproducible or even accurate results:

- Choose the standards that have the most

similarity with your samples. Refer to the molar masses as "based on calibration with" as they will not be accurate but precise and reproducible.

- If accurate molar masses are needed check for the availability of broad standards to perform a broad standard calibration.[2] Producing your own (broad) standards is also possible. Another option is advanced detection. On-line light scattering[3] is perfect for accurate molar masses of homo-polymers and proteins; on-line viscometry[4] offers the chance to measure a universal calibration curve independent of the chemistry, structure, and composition of the calibrants.

Can the Standard be Used with my Mobile Phase/Stationary Phase?

An absolute prerequisite for using GPC/SEC is that the sample is completely soluble. This also holds true for the reference materials. Therefore, the reference materials are classified into materials for organic and for aqueous GPC/SEC. There are only a few examples where materials can be used in both solvent systems.

Polyethylene glycol (PEG) can be used in organic solvents such as THF for molar masses < 20,000 Da, but is also applied as a calibrant in aqueous GPC/SEC over a wider molar mass range. To overcome the high crystallinity, it is often required to heat the calibrant solution for a short time.

Also soluble in both solvent systems is poly(2-vinylpyridine). This material is often used for the calibration of GPC/SEC systems for polycation characterizations, but can also be used in other systems such as THF.

Polymethyl methacrylate (PMMA) is also widely used as a calibrant for organic GPC/SEC. Historically it has often been used in medium polar solvents such as dimethylacetamide (DMAc), dimethylformamide (DMF), or dimethyl sulphoxide (DMSO). The main reason for this was that previously only styrene-divinylbenzene polymeric phases were available; polystyrene could not be used because of interaction and retarded elution. Since the mid-1990s medium polar stationary phases have become commercially available allowing calibration with PS. However, in order to compare with older data, many results are still reported using PMMA as calibrants.

Similarly, PMMA is also used as a calibrant in hexafluoroisopropanol (HFIP) and is recommended for use in other solvents, such as THF or chloroform, especially if, for example, methacrylates are being investigated.

Table 1: Overview of a selection of reference materials. Please note that this table does not automatically list the best reference substance for your samples. Its purpose is to provide quick information to decide if this material is suitable for your detection system and in general for your mobile phase.

Reference material	Typically recommended for	UV detection	Comments
Pullulan	Aqueous GPC/SEC, Organic GPC/SEC: DMSO	-	Advantage: linear polysaccharide.
Dextran	Aqueous GPC/SEC	-	Less expensive, but branched (especially at higher molar masses).
PEG/PEO	Aqueous GPC/SEC	-	Organic GPC/SEC < 20 KDa in THF.
Poly(styrene sulphonate) sodium salt	Aqueous GPC/ SEC of neutral macromolecules and polyanions	254 nm	UV detection depends on the salt added: possible with NaCl, problematic with phosphate buffer, not possible with $NaNO_3$.
Poly(2-vinylpyridine)	Aqueous GPC/SEC of polycations	254 nm	Can also be used in THF.
Proteins	Aqueous GPC/SEC	215 nm / 280 nm	
Polystyrene	Organic GPC/SEC, HT-GPC	254 nm / (280 nm)	No UV detection in HT-GPC/SEC with HT solvents. 280 nm can be used for DMAc, DMF, DMSO.
Polymethyl methacrylate	Organic GPC/SEC	230 nm (THF)	Widely used for medium polar solvents such as DMAc, DMF, DMSO (UV-detection not recommended). Used for calibrations in HFIP.

Pullulan is a reference standard used for aqueous GPC/SEC or for GPC/SEC in DMSO, for example when starches are characterized. It is a linear polysaccharide and therefore has some advantages over dextran. Dextran is normally branched and the branching increases with molar mass, that is, the structure changes. As the size of the higher molar mass dextrans only increase slightly with changing molar mass, it makes them

less suitable for construction a calibration curve that makes full use of the complete separation range of the GPC/SEC columns.

Is Detection Possible with my GPC/SEC?

The standard detector for GPC/SEC is the refractive index detector (RI). Fortunately this detector is very universal and there are only a few isorefractive systems where this detector cannot be used in the applied solvent/sample combination. Therefore, nearly all reference materials can be used with RI detection. As an alternative to RI detection, evaporative light scattering detector (ELSD) can be used if there is sufficient difference in volatility between the standards and the eluent being used and if the eluent can be completely evaporated. It should also be noted that the response characteristics of an ELSD at low concentrations are slightly different from those of an RI.

If only an ultraviolet (UV) (diode array detector [DAD], photodiode array [PDA]) detector is available the choice of reference materials, especially for aqueous systems, is limited. Both dextran and pullulan, the two most common calibrants in aqueous GPC/SEC, are not UV-detectable in the vast majority of solvents applied. A UV-detectable alternative for neutral polymers and polyanions is poly(styrene sulphonate) sodium salt and poly(2-vinylpyridine) for polycations. Proteins can also be used.

Table 1 shows a list of the most commonly applied reference materials with application recommendations. If UV detection is possible, an applicable UV wavelength is provided. Please note that this table does not automatically list the best reference substance for your samples, there might be better choices available. Its purpose is to provide quick information to decide if this material is suitable for your detection system and in general for your mobile phase.

Packaging of Calibration Standards

Normally polymeric reference materials are available as single standards with different molar masses. Kits are available to enable the fast and precise creation of calibration curves, which cover a dedicated molar mass range. The single standards selected for the kits ensure that the molar masses cover the complete range without gaps so that the data fitting process is more precise. The most convenient calibration kits consist of a mixture of different molar masses in the right concentration, which is premixed in autosampler vials. It is then only necessary to add the GPC/SEC mobile phase, wait a short time for full dissolution, and then inject directly from the vial.

Summary

- GPC/SEC calibration standards are available with different chemistries to enable the determination of accurate molar masses.
- Nearly all calibrants can be detected using RI detectors or ELSDs. Detection with only an UV (DAD/PDA) detector is limited, especially for the materials typically used in aqueous GPC/SEC.
- Calibrants can be ordered as single molar masses or already assembled in kits or autosampler vial kits.

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Protein Aggregates and Gel Filtration Chromatography: Improving Quantitation and Throughput of a UHPLC Method

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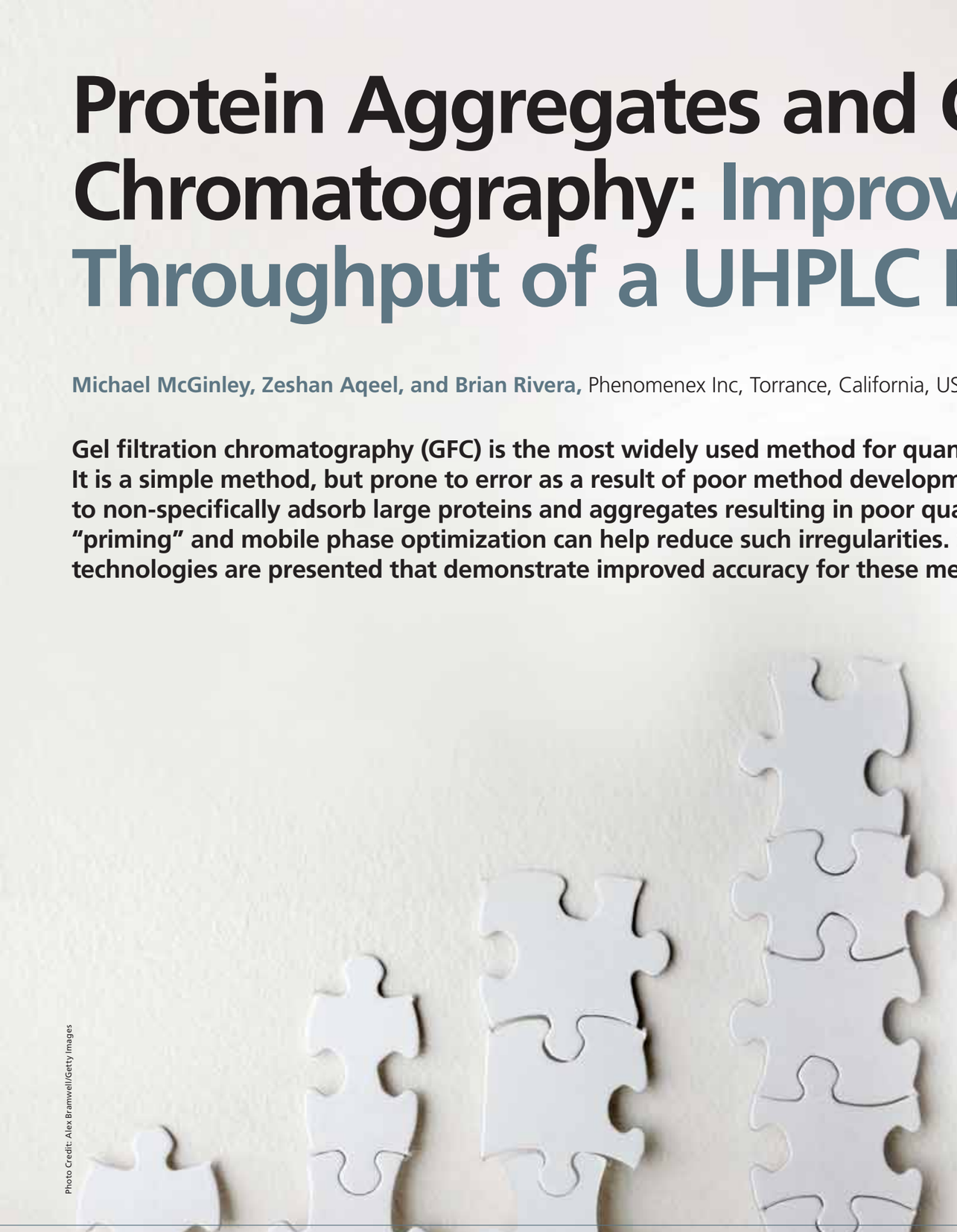
Gel filtration chromatography (GFC) is the most widely used method for quantitating protein aggregates in therapeutic drugs. It is a simple method, but prone to error as a result of poor method development and column selection. GFC columns tend to non-specifically adsorb large proteins and aggregates resulting in poor quantitation of "true" aggregate amount. Sample "priming" and mobile phase optimization can help reduce such irregularities. Simple method development rules using new column technologies are presented that demonstrate improved accuracy for these methods.



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In the world of protein therapeutic drugs there are several analytical assays that are routine for determining structure and function of proteins: peptide mapping, intact protein analysis by reversed phase, ion-exchange chromatography, and gel filtration chromatography (GFC). Although most of these analyses are used to look for structural changes in a protein, GFC is used to quantitate the aggregation state. This requires that the protein be in its native state, and therefore separation modes requiring denaturing conditions cannot be used. Aggregate analysis is necessary because of potential loss in specific activity of a protein as well as the strong possibility of immunogenicity for protein aggregates.

Unlike other chromatographic separation modes, GFC does not separate molecules by interactions with the stationary phase. Instead, molecules are primarily separated by differences in size and their ability to penetrate the pores of the stationary phase. Larger proteins can only partially permeate some of the pores of the fully porous media, which results in a reduced retention time; smaller proteins or small molecules can fully permeate all of the pore volume of a stationary phase, which results in longer retention times. Since protein aggregates are much larger (2× larger or more) than the protein therapeutic, they will elute earlier and relative peak heights or area can provide the analyst with an estimate of total aggregate present provided that all of the proteins remain soluble and do not bind to the GFC stationary phase. A key part

Figure 1: GFC analysis of a protein standard mixture (thyroglobulin, Ig-A, Ig-G, ovalbumin, myoglobin, uridine) using different salt concentrations in the mobile phase. A 300 × 7.8 mm Yarra SEC-3000 column (Phenomenex) was used on an LC system using 50 mM sodium phosphate pH 6.8 with varying amounts of sodium chloride up to 300 mM at a 1 mL/min flow rate. Note the change in the thyroglobulin and ovalbumin retention and peak shape with changes in salt concentration demonstrating optimization of mobile phase conditions maximizing recovery and resolution of peaks of interest.

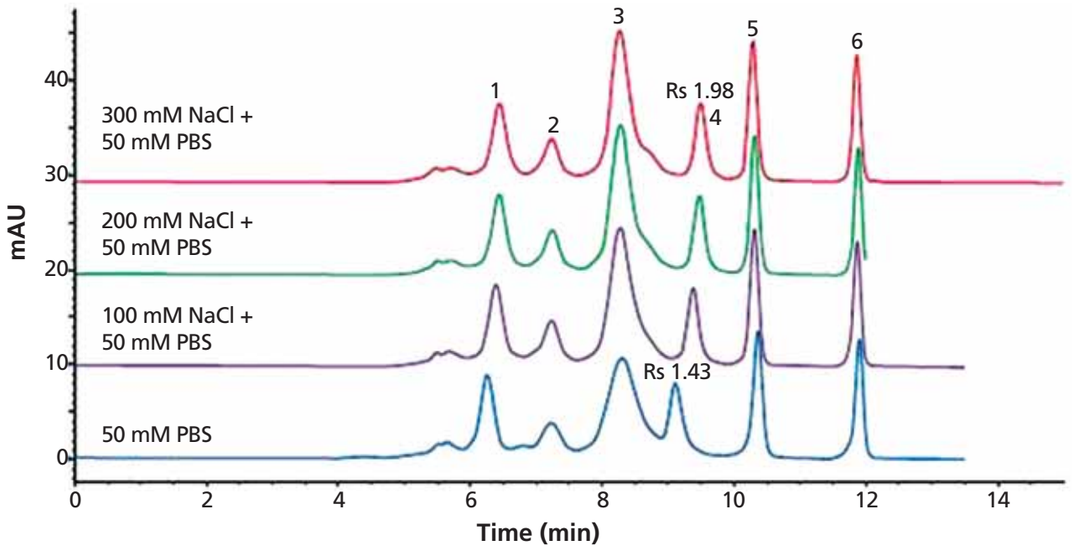
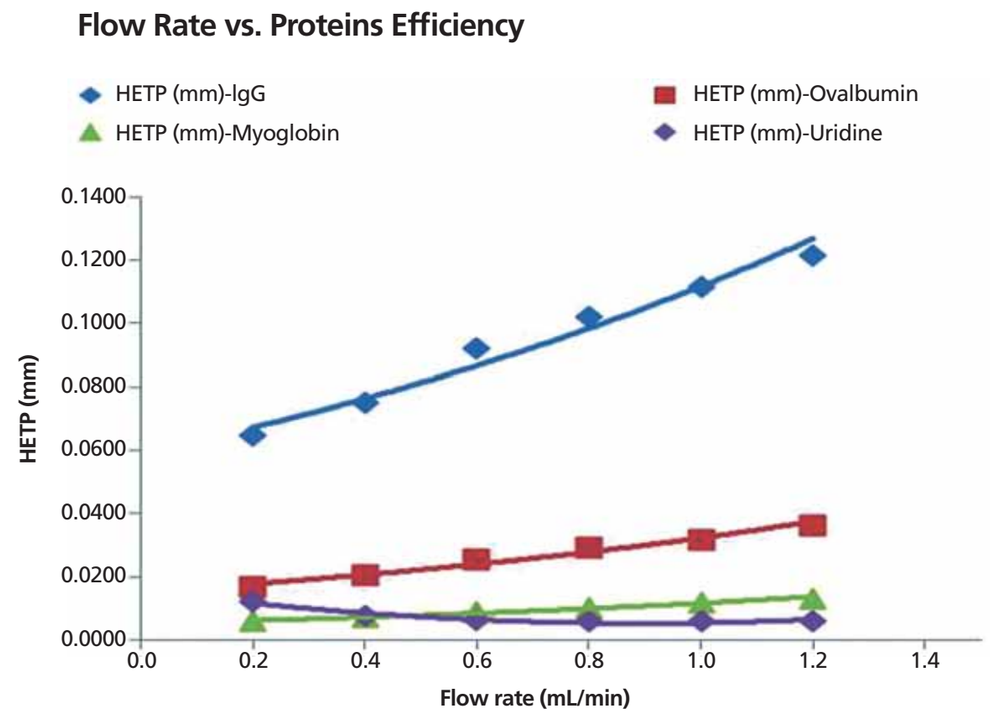


Figure 2: Plot of plate height of specific protein peaks at different flow rates. Protein standard (see Figure 1) was run on a 300 × 7.8 mm Yarra SEC-2000 column (Phenomenex) using 100 mM phosphate buffer pH 6.8 at varying flow rates. Efficiency and plate height was plotted on a Van deemter curve. Lower plate height indicates higher efficiency. Note that most proteins see reduced plate height with decreasing flow rate (uridine, a small molecule has an opposite effect). Slower flow rates improve resolution and efficiency but with a cost of increased run time and less throughput.

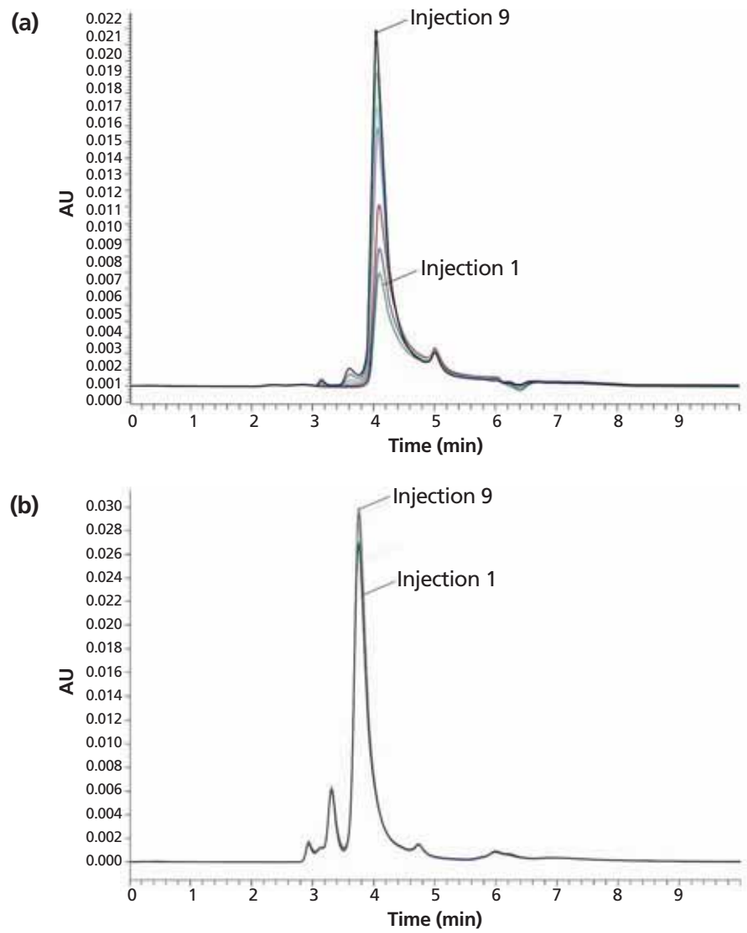


of method development for GFC is based on maintaining solubility of protein and aggregate, as well as minimizing unwanted interactions between the stationary phase and analytes of interest.[1]

Secondary Interactions

The highest resolution gel filtration media on the market are typically fully porous silica media bonded with a highly polar ligand, usually a glycol- or diol-based chemistry. This "water-like" ligand minimizes any secondary interaction between the stationary phase and the protein analytes, thus leading to separation based primarily on the ability of the proteins to permeate the porous silica particle. Unfortunately, some secondary interactions can lead to anomalous retention characteristics for some proteins (especially very basic or hydrophobic proteins). Basic proteins can have electrostatic interactions with negatively charged silanol groups beneath the bonded layer of the silica particles. These interactions can lead to tailing peak shape, poor recovery, and increased retention of some basic proteins unless method development efforts are undertaken to reduce such interactions. Conversely, depending on the mobile phase, the glycol-/diol-based surface ligand can exhibit some hydrophobic behaviour. In the presence of a highly polar liquid phase

Figure 3: Overlay of the first 10 injections of a GFC column demonstrating the priming effect. A 150 × 4.6 mm, sub-2-µm column using metal hardware is shown in Figure 3(a), in Figure 3(b) a 150 × 4.6 mm, sub-2-µm PEEK encapsulated Yarra X-150 column (Phenomenex) was used. Both columns used a mixture of dimer Ig-G, Ig-G, and albumin run at 0.3 mL/min with a 100 mM sodium phosphate buffer with 100 mM sodium sulphate pH 6.8 mobile phase. Note that the sub-2-µm stainless steel column demonstrated significant adsorption of the Ig-G and dimer peak that required 10+ injections to provide a stable recovery of Ig-G dimer. The PEEK encapsulated column reached steady state within 1–2 injections. Priming is critical in GFC for most materials to get accurate quantitation of aggregate peaks.



(for example, a high salt buffer like 2X PBS [phosphate buffered saline]) these polar bonded phases can exhibit hydrophobic interactions between hydrophobic proteins and the stationary phase, similar to what is seen with hydrophobic interaction chromatography (HIC).

Therefore, every GFC method would benefit from optimization to properly minimize ionic and hydrophobic interactions. An excellent example of this behaviour is shown in Figure 1 where a mixture of standard proteins are run using different mobile phases of varying ionic strength. Depending on the recovery and resolution of proteins desired, one can optimize a separation while maintaining proteins in a native state. Some variant of a phosphate buffer either with or without supplemental salt is used to maximize the recovery and resolution of protein. An increasing concentration of phosphate buffer (50–150 millimolar, pH ~7) is preferred over using MS-friendly buffer systems, which tend to demonstrate low recovery and resolution of hydrophobic or ionic proteins; aggregates are especially negatively impacted. For very hydrophobic proteins (for example, antibody-drug conjugates [ADCs]), the addition of up to 15% isopropanol or acetonitrile to a phosphate buffer system has been reported by some to assist with the recovery of very hydrophobic proteins and is therefore an additional method development option to consider. Finally, some groups have shown that using arginine in the mobile phase can reduce secondary interactions. While such mobile phases minimize secondary interactions, the use of arginine in the mobile phase interferes with UV detection at lower wavelengths, thereby limiting detection to 280 nm wavelength.[2]

Stationary phase chemistry can also have some impact on the selection of the mobile phase. Some of the more classical GFC chemistries bonded to high silanol silica tend to benefit from the addition of excess salt (300 mM sodium chloride +) while newer column chemistries with low silanol activity tend to perform better with phosphate-only buffer systems. Of course, the pore size of the media (150Å vs. 250Å vs. 500Å) will have a major impact on the size range of the proteins being separated. It typically benefits a researcher to examine multiple pore sizes to get the optimal separation for a critical pair.

Flow Rate

Flow rate is another overlooked optimization parameter to consider for

GFC. Unlike other separation methods, GFC is not typically run at optimal flow rates. Large proteins diffuse much slower than small molecules and resolution of higher molecular weight species tend to improve as flow rate is decreased but at a cost of dramatically increased run time. Therefore, researchers trying to balance throughput in their laboratories are usually resigned to running their GFC separations at less than optimal conditions. A great example of the impact of flow rate on a separation is shown on Figure 2 where a standard is run at different flow rates using the same standard and mobile phase conditions. In the last few years new column chemistries have been introduced that use smaller particles (≤3 µm) to maintain higher resolution at accelerated flow rates. These columns offer an additional alternative when throughput is a consideration.

Prime Time

Minimizing secondary effects through good method development is crucial for GFC, but even with optimal conditions and an inert stationary phase some secondary interactions do occur. Residual silanol activity, interactions with the stainless steel of the high performance liquid chromatography (HPLC) column, and interactions with the frit material can all influence a GFC method. Often protein aggregate recovery is most impacted by non-specific adsorption, which can result in the aggregate present in a sample being underestimated. A common method to overcome such column limitations is the use of several "priming" injections of sample to "saturate" non-specific sites to get maximum recovery of any aggregate peak.[3] An overlay of several injections in Figure 3(a) demonstrates the increasing recovery of protein with repetitive injections. While column chemistry has some impact on this phenomena, the recent introduction of a ultrahigh-pressure liquid chromatography (UHPLC) compatible PEEK hardware suggests that metal hardware of traditional columns also plays a role in non-specific adsorption and that the use of PEEK hardware (Figure 3[b]) might be a future solution for reducing the frequency of priming a GFC column before analysis.

Summary

Gel filtration is an easy separation mode to optimize to achieve good resolution and recovery of critical analytes of interest; however, many scientists just run PBS buffer and struggle through poor peak shape and reproducibility with questionable accuracy for quantitating aggregate protein. After screening columns that give the best separation with standard mobile phase (100 mM sodium phosphate pH 6.8), one can adjust salt concentration to optimize recovery and resolution of critical components. Alternate mobile phases or additives (arginine, 5–15% isopropanol, or even alternate salts) can also be considered if standard optimization is not efficient enough. Flow rate can also be adjusted based on resolution or throughput requirements. Recent introduction of 3-µm and sub-2-µm GFC columns allow for higher resolution in shorter analysis times compared to older GFC columns/chemistries. Finally, considering column priming and coming up with planned sequences of runs to assure a properly primed column are important for achieving accurate quantitation of aggregate in the sample of interest. Recent introductions of new materials and PEEK-encapsulated hardware can minimize or potentially eliminate the need for column priming. Using such method development strategies with a logical workflow can in most cases deliver an assay that gives accurate quantitation of the aggregation state of a therapeutic protein.

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Meeting the Challenges of Bioanalysis with Time-of-Flight High Resolution Mass Spectrometry (TOF-HRMS)

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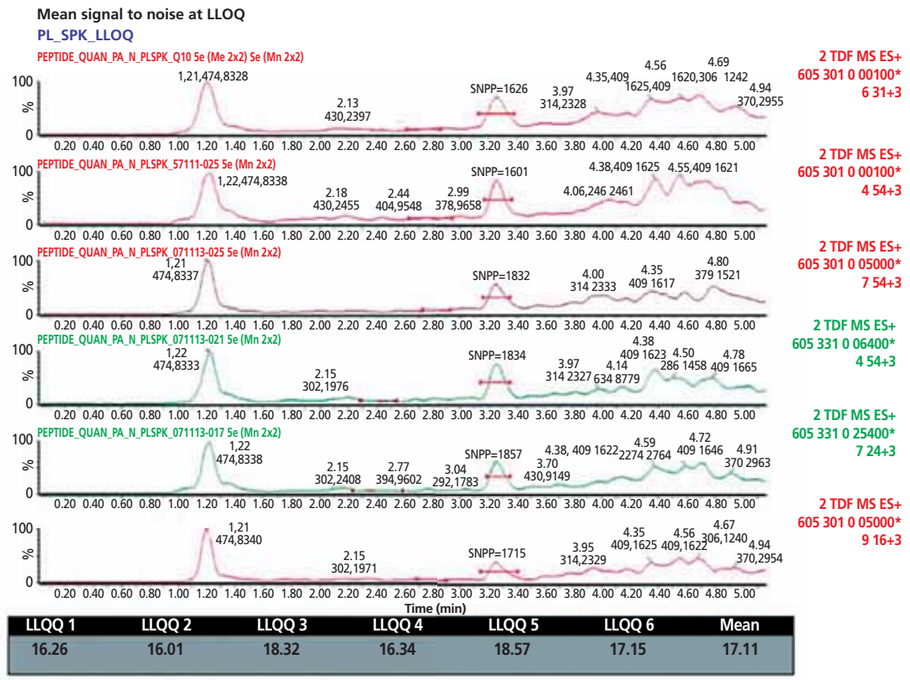
In an era where "more data in less time" is expected, bioanalytical scientists have had to become more resourceful. Consequently, bioanalytical laboratories are adopting and extending the use of high-resolution mass spectrometry (HRMS) and, in particular, time-of-flight high-resolution mass spectrometry (TOF-HRMS). One of the key benefits of TOF-HRMS is consistent resolution, sensitivity, and mass accuracy — even at high scan speeds with large molecules being analyzed.

Bioanalytical scientists have had to become more agile, resourceful, and flexible to obtain "more data and insight in less time". As a result, many bioanalytical laboratories are adopting high-resolution mass spectrometry (HRMS) into their quantitative workflows.

HRMS technology comes in different forms, including electrostatic ion resonance mass spectrometry (ion trap MS); time of flight mass spectrometry (TOF-MS); hybrid quadrupole time-of-flight mass spectrometry (QTOF-MS), which involves tandem quadrupole isolation and/or fragmentation before transfer to TOF detection; and Fourier-transform ion cyclotron resonance (FTICR-) MS. The versatility of HRMS has been demonstrated in both quantitative and qualitative analyses and for small molecules, large molecules, and hybrid biotherapeutics.[1,2] The recent evolution of more complex, targeted delivery methods such as antibody drug conjugates (ADCs) has resulted in HRMS becoming popular as the MS platform to complement traditional ligand binding assays.

Figure 1: The mean signal-to-noise (S/N) ratio for six replicate injections of leuprolide extracted from plasma and analyzed via full scan TOF at 2 pg/mL. The consistency in S/N means it's feasible for bioanalysts to quantify analytes at extremely low concentration levels with TOF-MS.



LLQQ 1	LLQQ 2	LLQQ 3	LLQQ 4	LLQQ 5	LLQQ 6	Mean
16.26	16.01	18.32	16.34	18.57	17.15	17.11

Despite the different capabilities of the respective HRMS platforms available, the unique advantages of TOF-HRMS will be the focus of this article. One of the key benefits of TOF-HRMS is consistent resolution, sensitivity, and mass accuracy — regardless of scan speed and molecule size. Recent modifications in both hardware and software have advanced analytical improvements in selectivity, sensitivity, and linear range. As a result, validated and transferrable methods using HRMS with both ultrahigh-pressure liquid chromatography (UHPLC) and microflow LC are being reported and one example employing leuprolide is shown in Figure 1.[3,4,5,6] With this evolution in technology, the dossier of evidence for the acceptance and adoption of HRMS quantitation into regulatory environments is increasing.

A decade ago, tandem quadrupoles were the instrument of choice for LC–MS quantitation.[3] Improvements in scan speed, sensitivity and selectivity, reduction in data file size, and instrument robustness have made TOF-HRMS a viable and complementary option for both qualitative and quantitative applications. For small molecule analysis, TOF-HRMS maintains its analytical performance, even with the narrow peaks obtained with fast UHPLC methods. Consequently, both full scan and multiple reaction monitoring (MRM) scan data can be collected without losing peak definition. In one example, quantitation of the parent molecule with complementary characterization of putative metabolites in a biotransformation workflow has been reported.[2] This approach has also been implemented for residual analysis of pesticides and analysis of veterinary drugs in animal-based foods.[5] In a recent review article, Rainville and colleagues described the robust use of a chip-based microfluidic separation device coupled with TOF-HRMS for characterization and bioanalysis of various biofluids with excellent inter-assay reproducibility. In the same report the beta-blocker, propranolol, showed clear improvement in the signal intensity and overall assay sensitivity when analyzed with the chip-based microfluidic platform compared with UHPLC.[7]

As the industry continues to shift from small molecule development and analysis to larger, more complex biomimetics and protein-based therapeutics, TOF-HRMS has proven to be a trusted companion along this journey. Although ligand binding assays still maintain a stronghold for quantitation of intact biotherapeutics like monoclonal antibodies (mAbs) and ADCs, the challenges associated with this approach (significant lead time for antibody generation, limited linear range of the assay, and the potential for cross-reactivity) are creating a space for ultrahigh-pressure liquid chromatography (UHPLC) or microflow LC–TOF-HRMS.[1] These LC–MS platforms equip the analyst with the specificity, sensitivity, and flexibility to quantitate biomolecules. Although TOF-HRMS is well suited for the characterization of large, intact molecules, and desalted, deglycosylated proteins can be ionized and detected,[8] quantitation is typically achieved through enzymatic digestion and analysis of a signature peptide with concomitant analysis of a stable isotope-labelled peptide analogue. For instance, Plumb *et al.* demonstrated the quantitation of a 70 kDa protein from plasma using a hybrid QTOF after enzymatic digestion. Although these authors observed good analytical merits (good linear regression and specificity), the ultimate desired outcome is reliable LC–MS quantification of intact

proteins. However, as sample preparation, MS ionization, ion transfer, and detection techniques improve, it is anticipated that TOF-HRMS will lead the way to robust and routine quantitation of large intact biomolecules as a routine complement to ligand binding assays.

Although the regulatory agencies have widely accepted qualitative data from HRMS platforms for applications such as protein characterization, metabolite profiling, proteomics, and glycan analysis, evidence for the validation of quantitative data generated by TOF-HRMS is still growing, and the agencies have been slow to embrace this quantitative platform. Ramanathan notes that in regulated bioanalysis, the need for validation demands that the scope of the assay be carefully defined and that assay parameters stay validated for use over several years and even decades spanning the development life of the new chemical entity (NCE) from early clinical trials to late stage efforts and sometimes post-new drug application approval.[3] As a result, it is more challenging in the regulated environment to establish methods on instrumentation that has been used historically for non-specific, non-targeted analysis because no precedent exists. This limitation is eroding. As investigators continue to show the utility of TOF-HRMS and transfer the methods to contract research organizations (CROs), it is anticipated that adoption and acceptance by the overseer of regulatory compliance in pharma and by extension, the regulatory agencies, will continue to increase.

In the last five years, a number of studies supporting the narrowing of differences in analytical sensitivity between tandem quadrupole and TOF-HRMS have been reported. In a study measuring prednisone and prednisolone in human plasma using full scan HRMS, the authors reported good accuracy (96–106%), good linear range (5–2500 ng/mL), and a lower limit of quantitation (LLOQ) of 5 ng/mL that matched the gold standard tandem quadrupole approach compared in the study.[5] More recently, Evans and colleagues at GlaxoSmithKline (GSK) described the quantitation of a small molecule NCE using TOF-HRMS for improved selectivity, sensitivity, and successful transfer to a CRO. Dr. Evans's team also developed a sensitive and selective TOF-HRMS method using chip-based microflow LC analysis for a large intact biomolecule with successful CRO transfer.[9]

The analytical challenges encountered by the bioanalytical scientist are growing and changing in complexity. In response to these obstacles, analysts have reached into their analytical tool box by using a new and improved HRMS resource. In the last 10 years, the scientific community has shown numerous examples where HRMS can match tandem quadrupoles for sensitivity. There is no compromise in speed of acquisition because the performance of TOF-HRMS is consistent at low and high data acquisition rates. TOF-HRMS maintains its resolution and is not affected by space charge effects associated with multiple charged biomolecules. As a result of these enhanced capabilities, HRMS in conjunction with UHPLC and microflow LC is knocking on the regulatory agencies' doors and windows, serenading the investigators to pay attention and acknowledge its presence. In particular, as the applications and demand for characterization and analysis of biomolecules and drug conjugates continue to rise, TOF-HRMS will take wing and serve as a complementary analytical tool for ligand binding assays and enable the successful passage of these complicated biotherapies to the ultimate goal — new drug therapies for improved patient outcomes.

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9. C. Evans, "The ever expanding role of HRMS in regulated bioanalysis applications to both small molecule and biopharmaceutical study support http://www.bioanalysis-zone.com/2015/07/28/the-ever-expanding-role-of-hrms-in-regulated-bioanalysis-application-to-both-small-molecule-and-biopharmaceutical-study-support/

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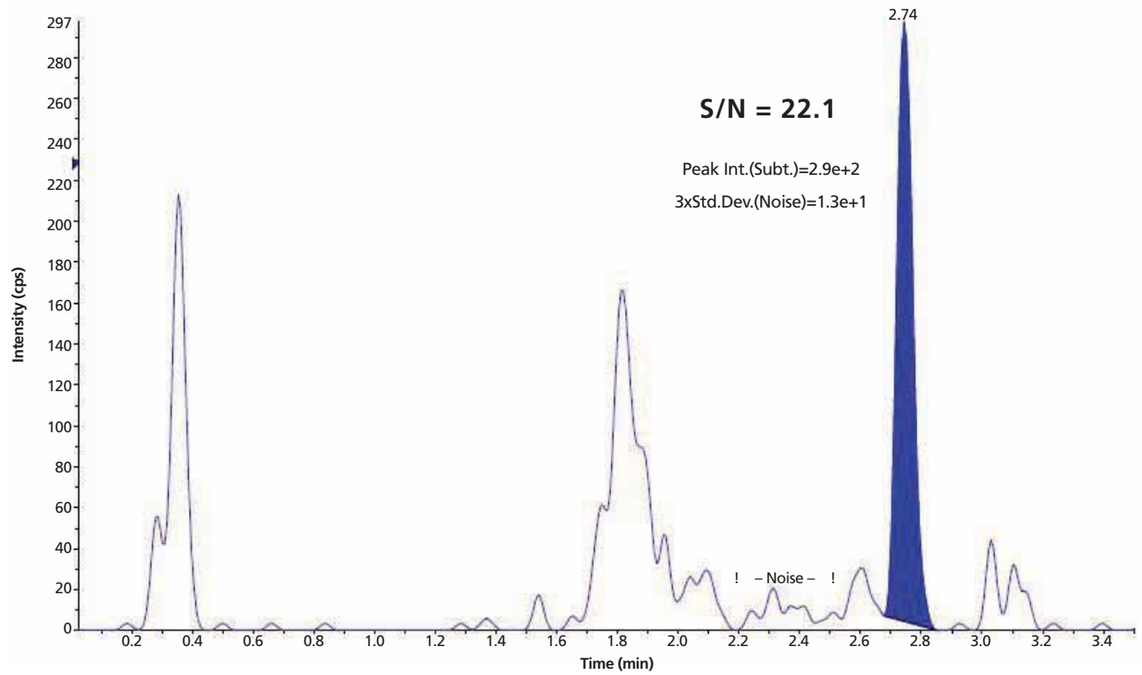
Trends in Bioanalysis
Using LC–MS–MS

Suma Ramagiri, Sciex, Toronto, Canada.

Bioanalysis of biologics presents a number of technical challenges. Ligand binding assays (LBA) are the gold standard bioanalytical technique for quantification of biologics in complex matrices such as serum and plasma but selectivity issues and the need for specific capture reagents limit their applicability in the drug discovery and development phase. Liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) is widely used for highly selective and sensitive bioanalysis of small molecules. However, large molecule bioanalysis presents challenges including the need for extensive and complex sample preparation for LC–MS–MS. This article explores the limitations of LC–MS–MS for bioanalysis of biologics and some of the latest trends for overcoming these in bioanalysis laboratories.

The biologics market has grown rapidly in recent years, with several new therapeutics being approved annually.[1] Traditional approaches for quantification of biologic drugs typically involve either ligand-binding assays (LBAs) such as enzyme-linked immunosorbent assays (ELISA), or UV detection of individual peptides using high performance liquid chromatography (HPLC) separation. LBAs rely on immunoaffinity detection of a unique epitope on the protein or peptide of interest. The high specificity of the antibody-based interactions can track an analyte at high sensitivity, although the dynamic range is narrowed to just one or two orders of magnitude.[2] LBAs can detect both physiologically active and "free" forms of circulating large molecule drugs in samples. They are popular for their ease of implementation, but are typically expensive and time-consuming to develop,[3] and are subject to limited selectivity and antibody cross-reactivity. This results in lack of specificity from interference, and high background levels that are not suitable for meeting the biopharmaceutical industry's requirements to detect diverse proteins and peptides with ever-increasing sensitivity and reproducibility.

Figure 1: Example chromatograms of glucagon in plasma at 10 pg/mL (697.6/813.4), using conventional flow LC.



Since the 1980s, liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) has been used extensively in pharmaceutical laboratories for small molecule bioanalysis, and is favoured for its high throughput, sensitivity, accuracy, and selectivity. LC–MS–MS has similar advantages for biologics; importantly, it is not subject to antibody cross-reactivity because LC–MS–MS involves direct evaluation of the chemical properties of the analyte.[2] LC–MS–MS also offers excellent selectivity, being able to distinguish and quantify highly homologous isoforms — even at low levels — with accuracy and precision over a wide linear dynamic range.[4]

However, performing LC–MS–MS-based bioanalysis for large molecule drugs raises a number of new challenges

that can be especially difficult for laboratories switching from small to large molecule workflows. In particular, sample preparation and extraction steps for quantification of large molecules can be complicated and very laborious to optimize because bioanalysis samples are highly complex biological matrices that contain numerous background peptides and proteins. Furthermore, the analysis of such samples is more challenging, because the background peptides and proteins compete with the biotherapeutic molecule of interest, creating interference problems and impacting on accuracy. LC–MS–MS approaches are also unable to capture free drugs that may be circulating in serum, thereby missing important data during quantification.

As a result, bioanalysis laboratories face several common issues when studying biologics, including insufficient selectivity amid background interference; insufficient sensitivity at low levels of detection (picogram quantities); and sub-standard data quality and reproducibility, associated with the challenges of quantifying peptides and proteins. These issues have driven several new technology and method developments, including improvements in sample preparation, detector technologies and sensitivity, and selection methods.

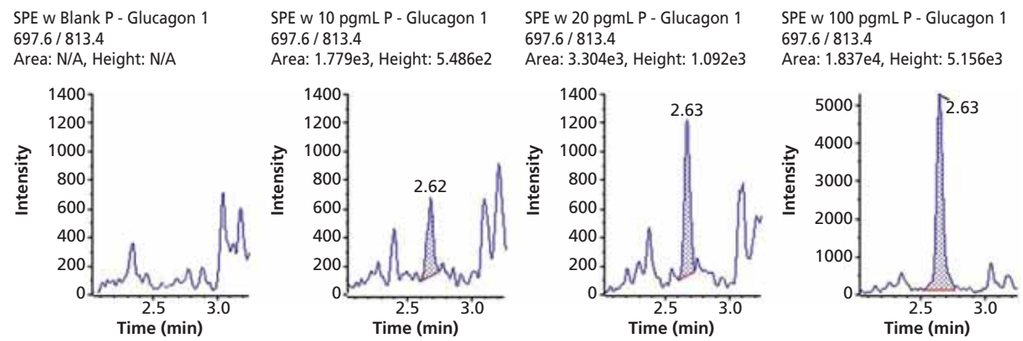
Improving Sample Preparation and Selectivity

Quantification of large molecules requires sample extraction, which typically involves one of several different immunocapture approaches. These can be drug target-specific, using an antibody developed for the specific drug of interest; target specific, using receptor-specific antibodies to the drug molecule's complementarity determining region (CDR); or semi-targeted capture, using antibodies to the Fc (fragment-crystallizable) region of the molecule. While drug target-specific strategies deliver better specificity, developing specific antibodies for each biologic of interest takes considerable time and investment. More general approaches have recently been developed using sample enrichment steps such as immunoaffinity enrichment using magnetic beads, combined with semi-targeted capture for successful sample extraction.[5] These enrichment steps concentrate the target drug molecule and help to lower the complexity of the sample background.

Sample preparation for LC–MS–MS-based quantification of large molecules can involve extensive workflows, especially when dealing with proteins or peptides above 10 kDa in molecular weight. These are not always suitable for direct MS–MS analysis, so quantification must be performed on just a small portion of the protein, using a "signature" peptide that has a unique *m/z* ratio to provide a representative concentration for the intact protein.[2] The drug target therefore has to undergo proteolytic digestion and quantification of multiple unique signature peptides; this process is widely accepted but is not always straightforward to incorporate into GLP laboratory workflows.

Furthermore, digestion of the target protein may cause variable peptide release, which can have a detrimental impact on the accuracy of signature peptide quantification data. These sample preparation steps therefore need to be highly optimized with a robust and proven protocol to achieve reproducible data with lower limits of quantitation (LLOQ) in the low ng/mL range.[6] Such optimization typically involves enrichment and semi-purification of the analyte, and may also require controlling digestion of the target protein to avoid irregular signature peptide release.[4] These procedures can be lengthy and complex, and knowing which sample preparation and clean-up procedures to use for different peptides requires substantial expert knowledge. Recently, there has been increasing interest in combining LBA immunoaffinity enrichment with LC–MS–MS quantification to combine the greater specificity and broader immunocapture capabilities of LBAs with the sensitivity and selectivity of LC–MS–MS technologies.[5] This should result in the wider availability of pre-optimized published methods that will assist biopharmaceutical laboratories

Figure 2: Example chromatograms of glucagon (697.6/813.4) in plasma at 0, 10, 20, and 100 pg/mL, using microflow LC.



in developing sample preparation more efficiently for robust and stringent data.

There has been increasing demand for automated sample preparation technologies to increase the precision and accuracy of assays, as well as save time and expense. Automation of many laboratory procedures has become commonplace over the past 20 years, and the particularly laborious analytics needs of biologics is driving further demand for automation within LC–MS–MS workflows.

A number of front-end autosamplers and platforms have become available that automate sample preparation. More recently, instrumentation providers have begun developing complete workflow-orientated solutions that provide the hardware and software required for LC–MS–MS with biologics.

Improving Sensitivity of Detection

LC–MS–MS-based bioanalysis of biologics requires excellent MS–MS sensitivity and selectivity to quantify proteins and peptides accurately in the low ng/mL range. Quantification issues that may arise often relate to the polarity or adsorptive properties of peptides, as well as interference from background proteins, which can reduce both sensitivity and selectivity.[2] Non-specific binding, poorly soluble, and poorly fragmenting peptides can also affect MS–MS quantitation and multiple reaction monitoring (MRM), all of which affect data accuracy.

Several LC–MS–MS technology advances have recently been made that can help to overcome many of these problems. In particular, the enhancement of ionization efficiency and ion transmission in the latest triple quadrupole instruments have significantly improved sensitivity, making it possible to detect biologics at picogram levels (Figure 1) or even sub-femtogram levels.[7] Technology advances within these instruments include better collisional focusing of ions, which brings more ions to the detector, as well as improvements to the detector's dynamic range, to enhance sensitivity and performance for bioanalysis.

They can also be combined with microflow LC systems, allowing reduced injection volumes to be used when samples are limited, while still obtaining robust sensitivity at low picogram levels[7] (Figure 2). Another advantage can come from using mass spectrometers with dual (switchable) mass ranges, which allows ions of different mass to pass through the quadrupole so that both small and large molecules can be analyzed without compromising sensitivity.

Improving Selectivity During Analysis

Selectivity can still be problematic when detecting peptides that are expressed at low levels, making it difficult to separate these out from background proteins. Methods have been developed to improve selectivity by providing an additional degree of separation, either after ions enter the mass spectrometer or after MS–MS selection. One approach is to use MRM^3 scanning techniques to improve MRM peak shapes and signal-to-noise ratios. MRM^3 can be performed using hybrid triple quadrupole mass spectrometers that have a linear ion trap for additional fragmentation of the primary product ions. Similarly to standard MRM, precursor ions are filtered then fragmented and filtered again in the third quadrupole. In MRM^3, however, these second filtered precursor ions are fragmented and scanned again for improved selectivity.[8–10] This approach means that overlapping or competing ions are filtered out, resulting in improved quantitation of the secondary product ions and lower limits of quantitation, which is advantageous for bioanalysis of biologics.

Alternatively, high resolution triple quadrupole time-of-flight (TOF) mass spectrometers enable the use of so-called MRM^{HR} workflows,[11,12] where all precursor ion fragments are detected by the TOF analyzer at high resolution and high mass accuracy. During MRM^{HR}, ions are extracted post-acquisition at narrower extraction widths than are typically used with triple quadrupole-based experiments. This allows more accurate and specific analyte detection, even in complex biological matrices.[2]

Another approach is to use differential ion mobility separation techniques, where the ions are separated on mobility differences (that is, in trajectory rather than in time) as they move between a set of planar plates with high and low energy fields applied.[13] This differential mobility spectrometry removes background components by providing orthogonal separation between the LC and MS stages, and results in a highly robust and fast technique with short MRM cycle times.[13]

Conclusions

LBAs are widely used for bioanalysis of small and large molecules, but these assays can be lengthy to develop and have limited selectivity and sensitivity. LC–MS–MS-based bioanalysis can offer important advantages over immunoassay-based techniques, but LC–MS–MS with large molecules introduces several challenges

affecting sensitivity, selectivity, and data analysis. By combining traditional LBA immunoaffinity assays with the latest LC–MS–MS technologies, bioanalysts can achieve efficient immunocapture and sample extraction of large molecules, followed by highly sensitive and selective data analysis. Significantly improved selectivity can also be achieved in biological samples that have numerous highly abundant proteins by using MRM^3 and differential ion mobility separation technology. Further advances in software and automation are being made to increase throughput, and enhance method development and optimization.

Although LC–MS–MS technologies have certainly advanced to be more suitable for biologics bioanalysis, the array of mass spectrometry technologies and techniques, sample preparation methods and reagents could be daunting for non-experts needing to develop a new biologics bioanalysis workflow. Accordingly, some instrumentation vendors offer integrated packages for biologics analysis that include sample preparation kits, separation and mass spectrometry technologies, and standardized software.

These developments are making LC–MS–MS more accessible for biologics bioanalysis, helping scientists to accelerate their large molecule drug development. The latest instrumentation and software developments will deliver significant improvements in the quality and reliability of bioanalysis studies, delivering more robust and compliant data that have important implications for patient safety.

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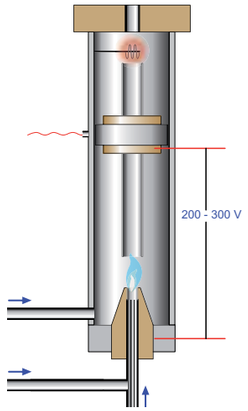

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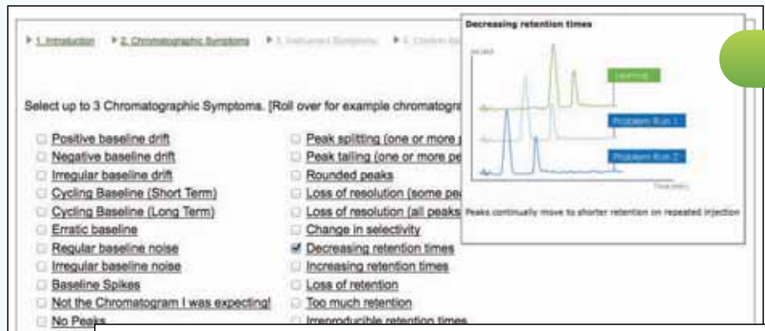

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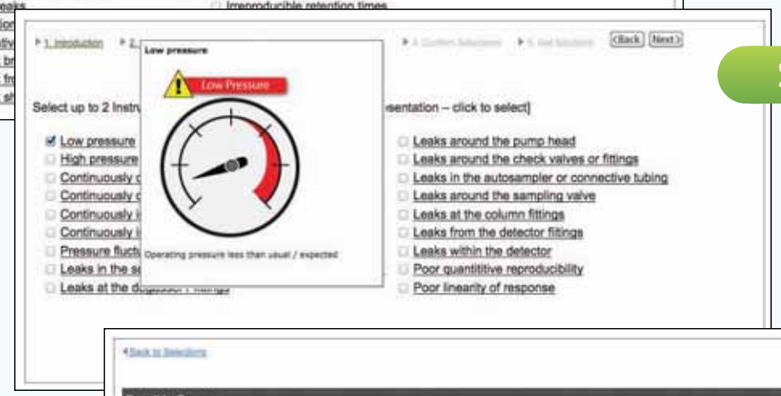

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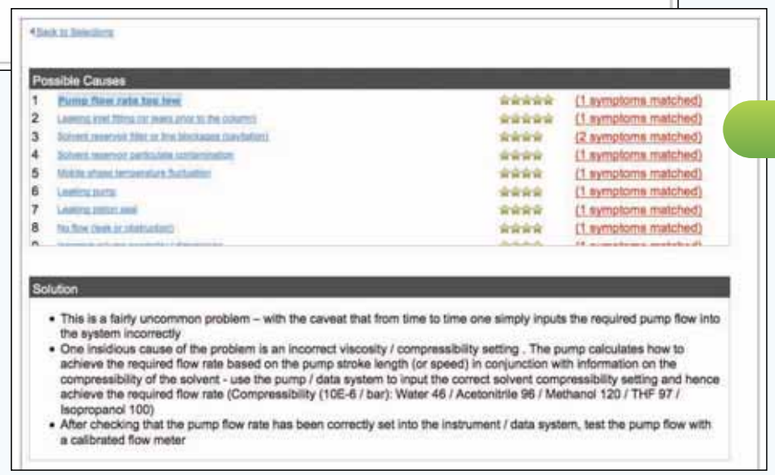
Select your instrument symptoms.



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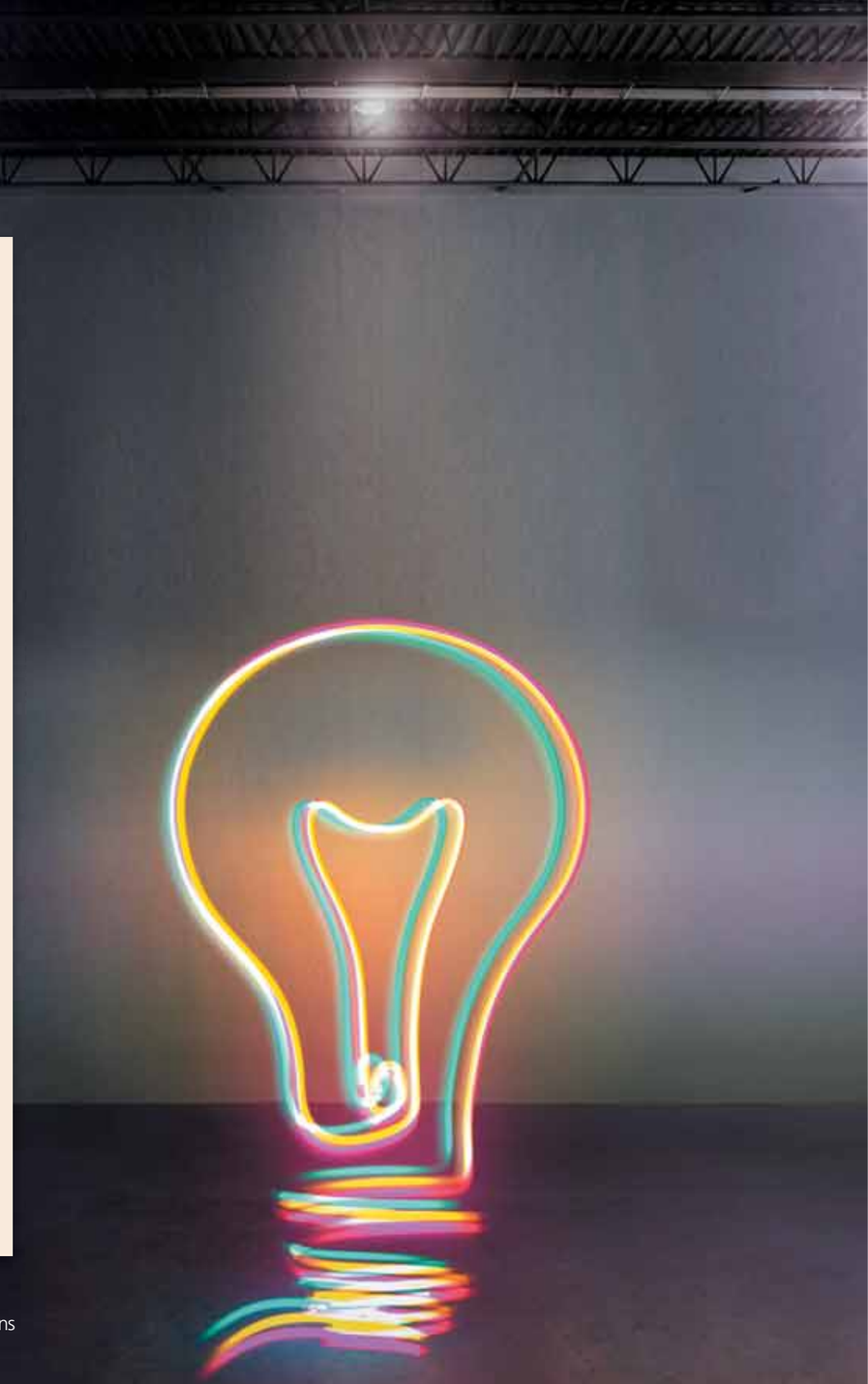

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