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printing methods has fallen short of the requirements to make an effective analytical or preparative-scale column. In this article, we describe a new 3D-printing method that can create large-volume columns with a feature resolution of 10 μm.
The Publishers of LCGC Europe would like to thank the members of the Editorial Advisory Board for their continued support and expert advice. The high standards and editorial quality associated with LCGC Europe are maintained largely through the tireless efforts of these individuals. LCGC Europe provides troubleshooting information and application solutions on all aspects of separation science so that laboratory-based analytical chemists can enhance their practical knowledge to gain competitive advantage. Our scientific quality and commercial objectivity provide readers with the tools necessary to deal with real-world analysis issues, thereby increasing their efficiency, productivity and value to their employer.
September Update

In this issue of *LCGC Europe*, we pay tribute to Professor Harold Monroe McNair, who passed away peacefully at his home in Blacksburg, Virginia, USA, on Sunday 27 June 2021. He was 88 years old. In a remarkable career spanning over 60 years, he was a powerhouse in the chromatography world, from his gas chromatography (GC) doctoral thesis on stationary phase chemistry—the first in the USA—to the publication of his classic book, *Basic Gas Chromatography*—the “go-to” first book for all GC analysts starting out—his achievements were vast.

This month’s peer-review article looks at the benefits of using multiple ionization techniques (namely, atmospheric pressure photoionization [APPI] and electrospray ionization [ESI]) in environmental analysis. The authors discuss how APPI is an excellent complement to ESI in the detection of pharmaceuticals in the environment.

*LC Troubleshooting* continues last month’s examination of peak asymmetry with a focus on the physical causes, such as heterogeneity of the particle density inside the column, rearrangement of the particles over time, and accumulation of debris at the column inlet frit.

The promise of three-dimensional (3D) columns has fallen short in reality. In this month’s *Column Watch*, the authors present a novel 3D-printing method for liquid chromatography (LC) columns. Could this soon be the future of chromatography?

*Sample Preparation Perspectives* discusses why having knowledge of the fundamental principles underlying sample preparation methods is so important, and how these methods fit into the analytical “toolbox”.

*LCGC* is a multimedia platform that helps chromatographers keep up to date with the latest trends and developments in separation science and supports them to perform more effectively in the workplace. Keep updated with our print and digital content by visiting the global website (www.chromatographyonline.com), subscribing to our newsletters, and attending our wide range of educational virtual symposiums and webinars.

**Mike Hennessy Senior**, Chairman and Founder, MJH Life Sciences™
Ionization Efficiency for Environmentally Relevant Compounds Using Atmospheric Pressure Photoionization Versus Electrospray Ionization

Prakriya Shrestha, Katherine A. Maloof, Alayna Stephens, Clayton P. Donald, and Kevin R. Tucker, Department of Chemistry, Southern Illinois University Edwardsville, Edwardsville, Illinois, USA

For solution-phase samples, the world of mass spectrometry defaults to electrospray ionization (ESI). ESI is used for the analysis of a broad variety of compounds, ranging from polar to moderately nonpolar. However, ESI possesses limitations that prevent the ionization of certain analytes—particularly nonpolar compounds. This study aims to compare the ionization efficiency of complementary ionization techniques, and to demonstrate that multiple methods can improve the analytical results with respect to limits of detection and matrix tolerance. Atmospheric pressure photoionization (APPI) is an ionization method that complements ESI, excelling in the analysis of nonpolar and moderately polar analytes. In this study, we optimized methods using APPI and ESI for the detection and quantitation of pharmaceuticals frequently detected in the environment, including antibiotics, beta blockers, and selective-serotonin reuptake inhibitors, and tested their matrix tolerance relative to artificial wastewater. While most of these compounds ionized preferentially by ESI, some performed significantly better using APPI.

Every day, in the world of analytical chemistry, we strive to discover better ways to analyze, to achieve goals such as lower limits of detection, broader dynamic ranges, and techniques that tolerate greater interferences. Along with the need for improved methods of quantitation, there is pressure on the field of mass spectrometry (MS) to be able to successfully detect multitudes of analytes of interest from single injections. The question becomes, “How do we improve upon the detection of analytes?” However, sometimes using a single injection leads to a reduction in performance because it limits us to using a single ionization method. When you can optimize not only your separation but also your ionization method, your method may truly improve. The basic principle of MS is to ionize molecules under study into gaseous ions, separate these ions in accordance with their mass-to-charge (m/z) ratio, and detect them (1). Today’s commercial instruments are capable of transferring 97–99% of ions successfully from the source to the detector, so the greatest improvements in detection and methods today are focused on the source. The ultimate question is: “When ionizing a sample, will the use of complementary ionization techniques improve the figures of merit of the individual analyses, or not?” Note: All tables

KEY POINTS
• APPI and ESI are compared for the MS quantitation of pharmaceuticals in environmental waters.
• APPI is an excellent complement to ESI as it is highly efficient in the ionization of analytes that ESI is unable to ionize.
• Multiple ionization techniques for analyte detection can help to improve the outcome of the analysis.
ESI and APPI: Complementary Ionization Techniques
By choosing the appropriate ionization source, liquid chromatography with tandem mass spectrometry (LC–MS/MS) can be used for the detection of trace levels of contaminants, such as antibiotics and endocrine disrupting compounds (EDCs) from environmental samples. The electrospray ionization (ESI) source has been used as a powerful soft ionization technique for the analysis by MS of a wide array of sample types, ranging from polar to nonpolar, and has also been used for the analysis of thermolabile molecules of high molecular weight (MW) (2). Although ESI is popularly used for the analysis of environmental pollutants, it may not be able to ionize all contaminants efficiently. Certain contaminants are either poorly ionized, or not ionized at all. ESI is limited to analytes that are of low to high polarity and moderate to high MW (3).

Atmospheric pressure photoionization (APPI), introduced in 2000, is also a soft ionization technique. APPI was found to have success with the analysis of compounds with low to no polarity, and compounds of low to moderate MW, but cannot be used on thermolabile compounds. These parameters are what make APPI and ESI complementary to each other (4). This complementarity opens new doors for studies already utilizing the ESI method, because APPI can be used for complementary analysis of compounds that may not be detected by ESI. Additionally, APPI has shown tolerance to matrix components beyond what ESI has, due to its ionization pathway (5). APPI can be used for the analysis of a wide range of compounds, including drugs, human endogenous compounds, lipids, natural compounds, pesticides, synthetic organics, and petroleum derivatives (6). There are very few research papers reporting comparative studies of ionization efficiencies in MS for the detection of antibiotics and EDCs (7–9).

A triple-quadrupole mass analyzer was used in this study, utilizing both the full-scan mode optimization and multiple-reaction monitoring (MRM) quantitation. Full-scan mode can give qualitative analysis of a sample’s composition under study, and MRM mode is a highly selective mass monitoring mode with a wider linearity dynamic range, improved limit of quantitation (LOQ), increased sensitivity, and superior accuracy. One of the advantages of the MRM scan mode is an increase in signal-to-noise ratio because of removal of nonanalyte ions and isobaric precursors by monitoring fragments.

There are different MS acquisition parameters that affect the signal intensity of ions. The MassHunter Data Acquisition software sets a default value for all acquisition parameters for each ionization source (S1). There is a sheath gas flow chamber in the electrospray ionization source that is absent in the atmospheric pressure photoionization source. As a result, the sheath gas temperature and sheath gas flow rate parameters are present only for ESI while APPI has an additional vaporizer parameter that is not present in ESI. Fragmentor voltage, collision energy, cell accelerator voltage, gas temperature, vaporizer, gas flow (L/min), nebulizer (psi), sheath gas temperature, and sheath gas flow rate were all optimized for each analyte prior to data acquisition in this study.

Analytes of Interest
The current global population is growing at the annual rate of 1.09%, and pharmaceuticals are continuing to be prescribed and consumed at an alarming rate. In 76 countries across the globe, antibiotic consumption as described in defined daily doses (DDD) increased by 65%—from 21.1 billion doses in 2000 to 34.8 billion doses in 2015—and the overall antibiotic consumption rate has increased by 39% (10). In addition to antibiotics, beta blockers and antidepressants are two classes of pharmaceuticals gaining popularity. Beta blockers are a class of drugs frequently used to treat hypertension, heart disease, and other cardiovascular events. Although the true nature of their efficacy remains to be questioned in certain studies, beta blockers are still highly prescribed due to the diverse range of clinical symptoms they can successfully treat (11,12). In addition, according to 2017 data from the National Center for Health Statistics (NCHS), the rate of antidepressant use in America has increased by 65% since 1999 (13). Unfortunately, this increase in pharmaceutical use means more pharmaceutical waste is likely to end up in the environment. While there is an urgency to know the exact harm this excess will cause, the priority is to increase our ability to detect as many pharmaceuticals.
in environmental samples as possible. This will then allow for proper removal techniques to be employed before the harmful substances have a chance to contaminate the environment (14).

Low Concentrations, Large Impact
Numerous studies have shown that some pharmaceuticals are not completely removed during wastewater treatment, and ultimately enter the environment in low concentrations. Important effects of pharmaceuticals entering the environment in low concentrations include antibiotic resistance, genotoxicity, acute or chronic toxicity, and endocrine disruption (15). Antibiotics and EDCs are emerging pollutants detected throughout the world, yet they remain unregulated by the U.S. Environmental Protection Agency (EPA) (16).

Sir Alexander Fleming, the British bacteriologist, discovered penicillin in 1928 from the fungus *Penicillium notatum*, spurring a novel era of antibiotics derived from microorganisms and antibiotic synthesis (17). The ability antibiotics have to eradicate a wide range of bacterial infections led to their increased use over time. Unfortunately, these bacteria have developed mechanisms to combat the actions of antibiotics. Thus, the over-prescribing of antibiotics, along with a lack of patient knowledge regarding the importance of correct antibiotic administration, has become an insidious issue that is known as *antibiotic resistance*. Antibiotic resistance arises as microorganisms develop the ability to survive against the action of antibiotics, meaning that when antibiotic-resistant bacteria infect animals and humans, the antibiotic regimen that would normally eradicate the bacteria becomes useless. This is the reason being able to successfully detect antibiotics from wastewater samples is so important. In this study, five different classes of antibiotics were used: beta-lactams, macrolides, nitroimidazoles, sulfonamides, and tetracyclines.

Antibiotics can either be bacteriostatic, meaning they prevent the growth of bacteria, or they can be bactericidal, meaning that they destroy the bacteria entirely.

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Humanity has had a tumultuous history with nicotine, with use of nicotine-rich tobacco for ceremonial and social functions starting as early as 1400 BC, before being introduced to Europe as a medicinal item in the 16th century. In the last 100 years, nicotine reached peak popularity through cigarettes, and then fell from favor, as their addictive nature and danger to health became apparent.

But has nicotine been given an unfairly bad rap? More recent research has shown that, outside its association with smoking, nicotine is actually a promising treatment for psychiatric and cognitive disorders, including Alzheimer’s disease, Parkinson’s disease, and dementia.

THE HISTORY
Nicotine is an organic compound present in the leaves of plants of the genus Nicotiana, which numbers more than 60 species, generally characterized by tubular flowers and large leaves. Nicotiana species are native to North and South America, Australia, Africa, and the South Pacific, and are widely grown in China, India, and elsewhere.

While all are commonly called tobacco plants, it is Nicotiana tabacum that is cultivated worldwide for tobacco and is the principal source of nicotine, with many other species ornamental. Other plants in the genus containing nicotine at a significant level include:

- N. glauca (Brazilian tree tobacco)
- N. alata (Jasmine tobacco)
- N. rustica

Nicotiana is part of the nightshade family (Solanaceae), which may also contain nicotine. Duboisia hopwoodii (pituri) from Australia is one example, from which the leaves have been chewed as a stimulant by indigenous communities for centuries. Even potatoes, tomatoes, aubergines, and peppers contain very small amounts of nicotine!

As an addictive stimulant, nicotine is the ingredient in tobacco that makes its use pleasurable, but it wasn’t until 1828 that it was isolated from the tobacco plant and recognized as a chemical. Use of nicotine in tobacco, however, began long before that, potentially as early as 1400 BC, with evidence of tobacco cultivation by Mexicans and Native Americans. Tobacco was smoked socially, medicinally, and ceremonially, but also used for trade and currency: in colonial Virginia...
promissory notes payable in tobacco were accepted, and the cost of commodities from goods to wives was given in pounds of tobacco.

When tobacco was introduced to Europe in 1559, it was promoted as a medicinal treatment; the French ambassador to Portugal, Jean Nicot de Villemain—from whose name we get Nicotiana and nicotine—helped popularize its use in society, sending tobacco and its seeds to the French king in 1560.

Smoking was believed to help prevent people from contracting diseases, in particular the plague—this is why those involved with burying the dead during outbreaks smoked clay pipes of tobacco.

This belief persisted for centuries, until concerns about smoking began to grow in the early 20th century, leading to increasing government campaigns encouraging people to quit, and eventually bans on smoking in enclosed places in many countries in the 2000s.

As tobacco’s star waned, nicotine replacement treatments such as patches and gum started to be used as an aid to help people overcome nicotine withdrawal, and e-cigarettes (vapes) were developed, which replace tobacco leaves with nicotine and water vapor. A 2019 clinical trial found that e-cigarettes were almost twice as effective as traditional nicotine replacement treatments in helping smokers to quit, though the Royal College of Physicians has cautioned that “there is a need for regulation to reduce direct and indirect adverse effects” and ensure quality standards for e-cigarettes are in place.

**CHEMISTRY AND PHARMACOLOGY**

Nicotine is a chiral alkaloid that makes up over 90% of tobacco alkaloid content, constituting 2-8% of the dry mass of tobacco leaves. The nicotine used in medication and e-cigarettes is also sourced from tobacco plants, and the extraction process can produce many potential impurities.

Most alkaloids contain oxygen in their molecular structure, and exist as colorless crystals. Nicotine contains no oxygen atoms, and is one of the few alkaloids that exist in liquid form when pure. It turns brown after exposure to light or air.

Other nicotine-related alkaloids include cotinine (a metabolite of nicotine in humans), nicotine N-oxides, myosmine, beta-nicotyrine, and beta-nornicotyrine.

Nicotine is a powerful psychoactive neurotoxin that acts both as a stimulant and a sedative and is absorbed and metabolized by the liver. It binds to receptors in the adrenal glands, stimulating an adrenaline release that causes an increase in heart rate, blood pressure and respiration. At the same time, it also activates nicotinic acetylcholine receptors (nAChR) in the brain and appears to cause a release of both dopamine and endogenous opioids, which activate opioid pathways in the neural reward system, producing feelings of pleasure and contributing to its reinforcing effects.

Its pharmacologic and psychodynamic effects make stopping nicotine consumption very difficult, with potential withdrawal symptoms including cravings, anxiety, difficulty focusing, irritability and depression. A 2011 National Institute on Drug Abuse study found evidence that nicotine consumption made cocaine more addictive in mice, and the American Heart Association has said that nicotine consumed through tobacco is one of the hardest substances to quit—on a par with heroin.

The next segment of this series will cover the most recent research behind the potential health benefits of nicotine.
However, an antibiotic’s mechanism of action is more important when considering treatment options. Beta-lactams inhibit the biosynthesis of bacterial cell walls by making penicillin-binding proteins unavailable for new peptidoglycan synthesis, which causes the lysing of bacteria. The beta-lactams used in this study are ampicillin, ceftriaxone, cephalexin, and penicillin G. Macrolides inhibit protein synthesis during translocation in bacteria by dissociating peptidyl-tRNA from the middle of the 23S rRNA of the ribosome’s 50S subunit, causing early detachment of unfinished peptide chains. The macrolides used in this study are erythromycin and tylosin, an antibiotic popularly used in farm animals. Tetracyclines prevent the attachment of aminoacyl t-RNA to the A site in bacterial ribosomes by acting on the 16S rRNA of the 30S subunit inhibiting protein synthesis. Oxytetracycline and tetracycline were used in this study. Sulfonamides prevent the multiplication and growth of bacteria by inhibiting certain steps in the metabolism of folic acid. Sulfamethoxazole and trimethoprim were the sulfonamides used in this study. Nitroimidazole antibiotics inhibit nucleic acid synthesis that occurs in bacterial cells by disruption of the DNA in microorganisms. Metronidazole and 1,2 dimethyl-5-nitroimidazole were the nitroimidazoles used in this study (18).

EDCs are natural compounds or synthetic chemicals that mimic natural hormones in the body and interfere with the action of the natural hormones (19). These compounds most profoundly cause adverse effects on reproduction, developmental, neural, and immune systems of human beings and animals. Research suggests that EDCs reduce fertility and increase the risk of cancer, diabetes, obesity, and endometriosis (20). Among various EDCs, beta blockers (acebutolol, atenolol, metoprolol, and propranolol) and SSRI antidepressants (citalopram, paroxetine, and venlafaxine) were used in this study.

Make or Break for Successful Analysis: Matrix Effects and Wastewater

Properly dealing with impurities is a necessary complication in every field of research. In MS, matrix effects variability in ionization efficiency of analytes of interest as coeluted species serve to either enhance or inhibit the ionization process for an analyte. This issue becomes increasingly problematic when trying to discern analytes of interest from wastewater. Water that is obtained as a byproduct of agricultural, industrial, domestic, and commercial activity is termed wastewater. Wastewater contains nutrients such as calcium, iron, nitrogen, phosphorus, and potassium, and components such as fats, sugars, and proteins. In this study, synthetic wastewater was made to mimic the wastewater from the influent of a “typical” wastewater treatment plant, with its composition designed to imitate the dissolved inorganic solids and dissolved organic solids of real wastewater. The synthetic wastewater prepared in this study followed the protocol described by H.E. Gray, 2012 (21).

APPI has been found to be less susceptible to matrix effects compared to ESI. This is likely due to the fact that APPI is more selective in ionization. The photon emitted from the krypton lamp, at 10.6 eV, can ionize analytes, but potentially not the matrix components; the difference in how a sample is ionized can be the difference between more or less matrix interference (22). Using APPI involves the ejection of an electron from the analyte molecule to produce the gaseous radical cation (23). It is also possible, however, that the matrix component can act as a dopant and ionize sample components with high ionization energy through electron transfer, leading to signal enhancement. Although matrix effects cannot be removed completely, they can be minimized by optimizing the sample preparation procedure and LC–MS parameters. Solid-phase extraction (SPE) with an appropriate sorbent can reduce matrix effects by eliminating interfering matrices. The formula for calculation of the matrix effect is:

$$\text{Matrix effect} = \left( \frac{\text{analyte in matrix}}{\text{same conc. of analyte in neat solvent}} - 1 \right) \times 100$$

Physicochemical characteristics of antibiotics and EDCs help to determine the environmental fate of these compounds. Table 1 contains important physicochemical characteristics of antibiotics and EDCs under study, including solubility, $pK_a$, and $K_{ow}$. A compound with $K_{ow}$ less than 2.5 means the compound is hydrophilic and readily found in the aqueous phase.

Methods

Specific Analytes Used: A total of 12 antibiotics and 7 EDCs were
analyzed in this study. Ceftriaxone sodium salt hemi(heptahydrate) and erythromycin with a purity of >98% were purchased from Acros Organics. Propranolol hydrochloride (99%), metronidazole (99%), acebutolol hydrochloride, metoprolol tartrate (98%), tetracycline hydrochloride (96%), and oxytetracycline hydrochloride were purchased from Alfa Aesar. TCI Co. was the main supplier of chemicals: cephalexin monohydrate (>98%), sulfamethoxazole (>98%), penicillin G potassium salt (>98%), atenolol (98%), venlafaxine hydrochloride (>98%), trimethoprim (>98%), citalopram hydrobromide (>98%), and 1,2 dimethyl-5-nitroimidazole. Ampicillin sodium salt was procured from Affymetrix Inc. Tylosin tartrate (95+%) and paroxetine hydrochloride (98+%) were bought from Ark Pharm Inc. All antibiotics and EDCs were used without further purification. All the solvents used in the analysis, such as acetonitrile, methanol, and toluene, are of HPLC-grade and purchased from Fisher Chemical. Potassium phosphate monobasic (99.8%) was purchased from EK Industries Inc. Sodium acetate trihydrate (100.7%), magnesium sulfate heptahydrate (99.9%), ammonium chloride (99.7%), and calcium chloride dihydrate (99.9%) were bought from Fisher Scientific. All solvents and chemicals were used without further purification. Optimized Parameters: The MS parameters were optimized as follows: 1.00 ppm sample of each analyte was analyzed for the selection of the precursor ion, optimization of fragmentor voltage, optimization of cell accelerator voltage, optimization of gas temperature, optimization of gas flow rate, and optimization of collision energy. All the ions formed were analyzed for intensity. Ions with m/z equal to and greater than the MW of each analyte were considered to determine the precursor ion. See Figure 1 for the flowchart of optimization strategy. A calibration curve for each analyte was obtained by the internal calibration method using the optimized MS parameters. Calibration was performed in the range of 1.00 ppt to 10.0 ppm for each analyte under study. Each of the standard solutions for antibiotics was spiked with the mixture of
internal standards of antibiotics (azithromycin d₃, cephalixin d₅, ciprofloxacin d₆, penicillin G d₇, sulfamethoxazole d₄, and trimethoprim d₈) to produce a final concentration of 100 ppb of each internal standard. Each of the standard solutions for EDCs was spiked with the mixture of internal standards of EDCs (metoprolol d₃, and paroxetine d₈) to produce a final concentration of 10.0 ppb of each internal standard.

A synthetic wastewater matrix solution was prepared by dissolving potassium phosphate monobasic, sodium acetate trihydrate, magnesium sulfate heptahydrate, ammonium chloride, and calcium chloride dihydrate in MilliQ water. The concentration and quantity of reagents used for synthetic wastewater matrix preparation is given in S2.

Analysis of the antibiotics and EDCs was performed in triplicate for each parameter using an Agilent Technologies 1290-6460 Triple Quadrupole LC–MS/MS instrument using two ionization modes.

### TABLE 1: Physicochemical characteristics of antibiotics and EDCs (24)

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g/mol)</th>
<th>Water Solubility (mg/mL)</th>
<th>pKₐ</th>
<th>Log Kₒw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactam antibiotics</td>
<td>Ceftriazone</td>
<td>C₁₈H₁₇N₅O₅S</td>
<td>554.6</td>
<td>0.105</td>
<td>2.5</td>
<td>0.68*</td>
</tr>
<tr>
<td></td>
<td>Cephalixin</td>
<td>C₁₈H₁₇N₅O₅S</td>
<td>347.4</td>
<td>0.297</td>
<td>5.2</td>
<td>0.65*</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>C₁₀H₁₇N₄O₅S</td>
<td>349.4</td>
<td>10.1</td>
<td>2.5</td>
<td>1.35*</td>
</tr>
<tr>
<td></td>
<td>Penicillin G</td>
<td>C₁₀H₁₇N₄O₅S</td>
<td>334.4</td>
<td>0.285</td>
<td>2.74</td>
<td>1.83*</td>
</tr>
<tr>
<td>Macrolide antibiotics</td>
<td>Erythromycin</td>
<td>C₂₁H₂₃NO₁₃</td>
<td>733.9</td>
<td>2</td>
<td>8.9</td>
<td>3.06</td>
</tr>
<tr>
<td></td>
<td>Tylosin</td>
<td>C₄₆H₇₅NO₁₇</td>
<td>916.1</td>
<td>5</td>
<td>7.73</td>
<td>3.5</td>
</tr>
<tr>
<td>Sulfonamide antibiotics</td>
<td>Sulfamethoxazole</td>
<td>C₅H₇N₃O₅S</td>
<td>253.3</td>
<td>0.61</td>
<td>1.6</td>
<td>0.89*</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>C₃H₇N₃O₃</td>
<td>290.3</td>
<td>0.4</td>
<td>7.12</td>
<td>0.91*</td>
</tr>
<tr>
<td>Tetracycline antibiotics</td>
<td>Oxytetracycline</td>
<td>C₂₀H₂₁N₄O₉</td>
<td>460.4</td>
<td>0.313</td>
<td>9.5</td>
<td>-0.92*</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>C₂₀H₂₁N₄O₉</td>
<td>444.4</td>
<td>0.231</td>
<td>3.3</td>
<td>-1.37*</td>
</tr>
<tr>
<td>Nitroimidazole antibiotics</td>
<td>Metronidazole</td>
<td>C₅H₃N₃O₃</td>
<td>171.16</td>
<td>9.5</td>
<td>14.58</td>
<td>-0.02*</td>
</tr>
<tr>
<td></td>
<td>1,2 dimethyl-5-nitroimidazole</td>
<td>C₄H₅N₃O₃</td>
<td>141.13</td>
<td>18.3</td>
<td>2.81</td>
<td>0.31*</td>
</tr>
<tr>
<td>β₁-selective beta-blocker</td>
<td>Acebutolol</td>
<td>C₈H₁₅N₄O₄</td>
<td>336.43</td>
<td>0.259</td>
<td>9.57</td>
<td>1.77*</td>
</tr>
<tr>
<td>β₁-selective beta-blocker</td>
<td>Metoprolol</td>
<td>C₁₀H₁₉NO₃</td>
<td>267.36</td>
<td>0.402</td>
<td>9.68</td>
<td>1.95*</td>
</tr>
<tr>
<td>β₁-adrenergic blocker</td>
<td>Propranolol</td>
<td>C₁₀H₁₉NO₂</td>
<td>259.34</td>
<td>0.0794</td>
<td>9.45</td>
<td>3.48</td>
</tr>
<tr>
<td>β₁-selective beta-blocker</td>
<td>Atenolol</td>
<td>C₄H₅NO₂</td>
<td>266.34</td>
<td>0.429</td>
<td>9.6</td>
<td>0.16*</td>
</tr>
<tr>
<td>SSNRI</td>
<td>Venlafaxine</td>
<td>C₁₀H₁₀NO₂</td>
<td>277.40</td>
<td>0.230</td>
<td>10.09</td>
<td>3.20</td>
</tr>
<tr>
<td>SSRI</td>
<td>Citalopram</td>
<td>C₁₀H₁₅F₈N₄O</td>
<td>324.39</td>
<td>0.00588</td>
<td>9.78</td>
<td>3.74</td>
</tr>
<tr>
<td>SSRI</td>
<td>Paroxetine</td>
<td>C₁₀H₁₅F₈NO₆</td>
<td>329.37</td>
<td>0.00853</td>
<td>9.6</td>
<td>3.87*</td>
</tr>
</tbody>
</table>

* A compound with Log Kₒw less than 2.5 means the compound is hydrophilic and readily found in the aqueous phase.  
SSNRI: selective serotonin & norepinephrine reuptake inhibitor  
SSRI: selective serotonin reuptake inhibitor
sources: a Jet Spray ESI source and an APPI source operated in positive mode. Full-scan mode was used for the optimization of MS parameters and MRM mode was used for calibration and analysis of wastewater. Data interpretation was performed using the instrument’s MassHunter Workstation Software. HPLC parameters for analysis are given in S3.

Calibration was performed in the range of 1.00 ppt to 10.0 ppm in matrix for all the analytes under study. Internal standards were added as described previously. Limits of quantitation were calculated based on the quadratic regression lines and were calculated in the instrument software. LOQs lower than 1.00 ppt were reported as <1.00 ppt as they were below the lowest calibrator.

A setup of Waters Oasis Prime HLB cartridges and a SPE vacuum manifold was used for offline SPE to extract antibiotics and EDCs from the synthetic wastewater matrix calibration sample. Waters Oasis PRIME HLB cartridges, 1 mL barrel syringe with 30 mg universal polymeric reversed-phase sorbent, were employed. SPE pretreatment was performed by washing the column with 2 mL of HPLC-grade methanol, 2 mL of Millipore deionized water, and 2 mL of Millipore deionized water at pH 2 under gravity. Then the samples were loaded on the column under vacuum at 10 to 20 mL/min rate.

Washing and Elution Step for Antibiotics Sample: After loading the sample on the column, the cartridge was washed with 2 mL Millipore deionized water for the antibiotic sample. The column was then eluted first with 2 mL of methanol, and then with 1 mL of 1:1 methanol–acetone under gravity and collected and combined in test tubes.

Washing and Elution Step for EDCs Sample: EDCs sample cartridges were washed with 1 mL of 5:95 methanol–water solvent. After the first washing step, the column was dried for 15 to 30 min under a vacuum. The column was eluted first with 1 mL of ethyl 9:1 acetate–methanol under gravity (this eluate was collected in a test tube labelled as fraction 1), then washed with 1 mL of 5% methanol/2% acetic acid in water, then with 1 mL of 5% methanol/2% NH₄OH in water under a vacuum and dried for 10 to 15 min under a vacuum. After drying, the column was eluted with 1 mL of 2% NH₄OH in methanol and combined with eluate present in the test tubes labelled as fraction 1.

Wastewater Sample: Wastewater samples were collected from the influent of the aeration treatment at the Environmental Resources Training Center (ERTC), a training center for drinking water and wastewater treatment at Southern Illinois University–Edwardsville, USA. The samples were analyzed for the presence of antibiotics and EDCs to demonstrate the effectiveness of the method development on real wastewater samples.

The wastewater samples were sequentially filtered through VWR 417 (40 µm) filter paper, then through VWR 696 (1.2 µm) glass microfibre filter paper, and then through Ahlstrom 193 (0.7 µm) microfibre glass filter. The filtrate was separated into six bottles each with 250 mL of filtrate, three samples for analysis of antibiotics and three for EDCs. All samples were spiked with appropriate internal standards as described previously.

Samples were adjusted to pH 3.0 using 6.0 M sulfuric acid before performing the SPE. Waters Oasis Prime HLB cartridges (6 mL, 200 mg universal polymeric reverse-phase sorbent) were used for wastewater sample analyte extraction. The SPE method for wastewater was identical to the synthetic matrix sample preparation, except the quantity of reagent
solvents used was five times greater, due to the increased volume and cartridge bed mass.

**Results and Discussion**

The optimized MS parameters for the precursor ion of antibiotics for ESI as an ionization source can be found in S4 and S5, while parameters for APPI are shown in S6 and S7. Ions with the highest intensity peak with a m/z equal to or greater than the MW of the analyte were selected as potential precursor ions and MS parameters were optimized using these ions. The optimized MS parameters for the product ion of antibiotics for ESI as an ionization source can be found in S8 and S9, while parameters for APPI are shown in S10 and S11. At most, three ions with m/z less than the MW of the analyte were selected to determine the optimized collision energy of the product ions.

The regression equations for antibiotics and EDCs without matrix were selected such that they were equivalent for the calibration curve performed with and without matrix.

The optimized MS parameters were established as higher than 0.99 for all the antibiotics and EDCs without matrix using ESI, shown in S12 and S13. Ampicillin, ceftriaxone, cephalixin, sulfamethoxazole, oxytetracycline, tetracycline, and metronidazole have limits of detection lower than 1 ppt using ESI and sulfamethoxazole has the highest LOQ among the antibiotics analyzed (226.1 ppb). All standard calibration curves are shown in the Supplemental Information (See S13). Paroxetine, propranolol, and venlafaxine have a limit of detection lower than 1 ppt using ESI, and acebutolol has the highest LOQ (14.81 ppb). Ampicillin, cephalixin, penicillin G, trimethoprim, and tetracycline have limits of detection lower than 1 ppt using APPI as the ionization source, and tylosin has the highest LOQ among the antibiotics analyzed (3401 ppb), as shown in S14. Citalopram, paroxetine, and propranolol have limits of detection lower than 1 ppt using APPI.

### TABLE 2: Limits of detection by ionization source for antibiotics

<table>
<thead>
<tr>
<th>Class</th>
<th>Antibiotics</th>
<th>ESI LOQ (ppb)</th>
<th>APPI LOQ (ppb)</th>
<th>Efficient Ionization Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactams</td>
<td>Ampicillin</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ceftriazone</td>
<td>&lt;0.001</td>
<td>-</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>Cephalixin</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Penicillin G</td>
<td>139.1</td>
<td>&lt;0.001</td>
<td>APPI</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>1.158</td>
<td>162.5</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>Tylosin</td>
<td>5.337</td>
<td>3401</td>
<td>ESI</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Sulfamethoxazole</td>
<td>226.1</td>
<td>70.87</td>
<td>APPI</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>81.1063</td>
<td>&lt;0.001</td>
<td>APPI</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Oxytetracycline</td>
<td>&lt;0.001</td>
<td>-</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>Nitroimidazoles</td>
<td>Metronidazole</td>
<td>&lt;0.001</td>
<td>2.945</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>1,2 dimethyl-5-nitroimidazole</td>
<td>169.2</td>
<td>19.91</td>
<td>APPI</td>
</tr>
</tbody>
</table>

### TABLE 3: Limits of detection by ionization source for EDCs

<table>
<thead>
<tr>
<th>Class</th>
<th>Antibiotics</th>
<th>ESI LOQ (ppb)</th>
<th>APPI LOQ (ppb)</th>
<th>Efficient Ionization Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta blockers</td>
<td>Acebutolol</td>
<td>14.81</td>
<td>38.37</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>Atenolol</td>
<td>9.68</td>
<td>22.44</td>
<td>ESI</td>
</tr>
<tr>
<td></td>
<td>Propranolol</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Metoprolol</td>
<td>8.690</td>
<td>21.700</td>
<td>ESI</td>
</tr>
<tr>
<td>Selective serotonin reuptake inhibitors</td>
<td>Paroxetine</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Citalopram</td>
<td>166.2</td>
<td>&lt;0.001</td>
<td>APPI</td>
</tr>
<tr>
<td></td>
<td>Venlafaxine</td>
<td>&lt;0.001</td>
<td>3788</td>
<td>ESI</td>
</tr>
</tbody>
</table>

The calibration curve correlation coefficient ($R^2$) criteria were established as higher than 0.99 for all the antibiotics and EDCs without matrix using ESI, shown in S12 and S13. Ampicillin, ceftriaxone, cephalixin, sulfamethoxazole, oxytetracycline, tetracycline, and metronidazole have limits of detection lower than 1 ppt using ESI and sulfamethoxazole has the highest LOQ among the antibiotics analyzed (226.1 ppb). All standard calibration curves are shown in the Supplemental Information (See S13). Paroxetine, propranolol, and venlafaxine have a limit of detection lower than 1 ppt using ESI, and acebutolol has the highest LOQ (14.81 ppb).

Ampicillin, cephalixin, penicillin G, trimethoprim, and tetracycline have limits of detection lower than 1 ppt using APPI as the ionization source, and tylosin has the highest LOQ among the antibiotics analyzed (3401 ppb), as shown in S14. Citalopram, paroxetine, and propranolol have limits of detection lower than 1 ppt using APPI.
have limits of detection lower than 1 ppt using APPI as the ionization source and venlafaxine has the highest LOQ (3,788 ppb) as shown in S15.

The efficiency of both ionization sources was determined by comparing the LOQ for each of the pharmaceuticals obtained using ESI and APPI. LOQ less than 1 ppt in both the ionization sources means the most efficient ionization source could not be determined. Many compounds that are thermolabile will degrade using APPI, meaning the compound will not be detected by APPI and ESI was the ionization source that was preferred here. Erythromycin, tylosin and metronidazole ionized efficiently by ESI based on the comparative LOQ result. This is hypothesized to be due to the pKₐ of each compound (Table 1) being greater than the pH of the mobile phase (3.80) used in the analysis of antibiotics, allowing it to protonate easily. Acebutolol, atenolol, metoprolol, and venlafaxine were ionized efficiently by ESI based on the comparative LOQ results obtained from the calibration curve performed with and without matrix. A complete breakdown of this analysis can be found in Table 2 and Table 3.

Penicillin G, sulfamethoxazole, and 1,2 dimethyl-5-nitroimidazole have lower LOQs when ionized by APPI; APPI is the preferred ionization source for these analytes. The pKₐ values of these compounds (Table 1) are less than the pH of the mobile phase (3.80), leading them not to be protonated in solution. In addition, sulfamethoxazole and 1,2 dimethyl-5-nitroimidazole each have a high degree of conjugation in their structures, facilitating the absorption of photons and molecular radical ion formation (M⁺•) (24). This specific trend was not observed in the case of trimethoprim, indicating that some other preferred ion formation pathway must be present. Citalopram also has a higher degree of conjugation in its structure, facilitating the absorption of photons and M⁺•, making the APPI source highly efficient for the analysis of these compounds.

Paired t-tests were conducted at a significance level (α) of 0.05 on the data of the calibration curve performed with an artificial matrix and without a matrix. Paired t-tests were conducted at a significance level (α) of 0.05 to determine if there was a significant difference between the concentration of antibiotics and EDCs in the real wastewater samples collected from the ERTC wastewater treatment plant using the calibration equations obtained with artificial matrix and without matrix. Only three of the p-values, shown in S20 and S21, demonstrated a statistical
difference between using the matrix calibration curve versus the water curve shown in S22, illustrating that while important for some analytes, overall it had insignificant effect in this specific study.

Conclusions

There are few studies comparing the efficiencies of ionization sources for the MS analysis of low MW pharmaceutical analytes. While many researchers are limited to using ESI as their only ionization source, the use of complementary ionization techniques produces better results for the quantitation of analytes and should thus be considered for future studies. When purchasing an instrument costing $250,000 and up, the addition of a $25,000 additional ion source to improve analyte coverage in the analysis should be viewed as a reasonable added cost, considering that it nearly doubles the analytical capabilities of the instrument in terms of analyte coverage.

It was found that ESI is preferable for the analysis of pharmaceuticals, such as antibiotics, beta blockers, and SSRI antidepressants. However, ESI is not suitable for the ionization of all the pharmaceuticals with high sensitivity. APPI is an excellent complement to ESI, as it is highly efficient in the ionization of analytes that ESI is unable to ionize.

There was no significant difference observed in the presence of matrix effects at very low analyte concentrations. With higher concentrations of analytes, however, matrix effects should be taken into consideration when using these methods due to the significant difference observed. Since the LOQ of most of the analytes was less than 1 ppt, further study is needed to determine the ionization efficiency of ESI and APPI for these compounds by calibrating at lower concentrations.

By determining the ionization energy of an analyte using the appropriate software, it can be predicted which compounds will ionize by APPI or ESI preferentially. This determination will aid in the analysis of other classes of environmental pollutants, including other groups of pharmaceuticals and pesticides.

Using ESI and APPI as complementary ionization techniques yields a more complete picture of what compounds are present when analyzing in full scan mode, as well as better quantitation of analytes when appropriately optimized. This information holds value because employing multiple ionization techniques is an easy fix that creates a cost-effective method for analyte detection that can improve the outcome of future research studies.

References

2) C.S. Ho, C.W.K. Lam, M.H.M. Chan,
But My Peaks Are Not Gaussian!
Part 2: Physical Causes of Peak Asymmetry

Dwight R. Stoll, LC Troubleshooting Editor

Although symmetric peaks with Gaussian shapes are predicted by models of the chromatographic process, “perfect peaks” are not observed very often outside of textbooks. Several physical phenomena can lead to asymmetric peak shapes, including heterogeneity of the particle density inside the column, rearrangement of the particles over time, and accumulation of debris at the column inlet frit. Understanding these phenomena can help identify whether the cause of asymmetry is most likely to have a physical or chemical origin, which, in turn, dictates which troubleshooting steps to start with when dealing with poor peak shapes.

Last month, in the first instalment of this series of “LC Troubleshooting” articles on peak asymmetry, I discussed some basic concepts in peak asymmetry, including commonly used models of chromatographic peak shapes, how to quantify peak tailing, and the impact of peak tailing on separation performance. I then went on to discuss in some detail how poorly-made connections between the column and the rest of the liquid chromatography (LC) system can lead to serious peak tailing. The good news is that this particular cause of peak tailing can usually be fixed rather easily by carefully considering the parts (such as capillaries and unions, to name two) used in the flow path between the injector and detector, and replacing those that are inappropriately sized (for example, a union with a very large through-hole) or somehow improperly connected (for example, a ferrule set too shallow). In addition to these problems, there are many other potential causes of peak asymmetry—too many to cover in a single one of these articles—some having primarily chemical origins, and some having primarily physical origins. In this month’s instalment, I will focus on several other potential physical causes, including problems with column packing, changes in the packed particle bed over time, and accumulation of debris in the column. A common symptom of all of the physical causes of peak asymmetry discussed here is that all peaks in a chromatogram will be affected similarly. This can be an important clue to help determine if the source of the asymmetry is more likely to have chemical or physical origins. If all of the peaks in a chromatogram are either fronting or tailing (or both [1]), the cause is most likely physical in origin. If only some of the peaks look good, then it is most likely that the cause of the poor peak shape is chemical in nature. This distinction is helpful when deciding which potential solutions to improve the peak shape to try first.

Brief Review of Flow Through a Packed Bed of Particles
To understand the different peak shapes that can be observed as a result of different physical problems within a LC column, it is helpful to first review some foundational concepts related to mobile phase flow through a column packed with small particles like those used in HPLC. Figure 1(a) shows a highly idealized illustration of the organization of particles (depicted as porous spheres) in a perfectly packed bed. Such perfect arrangements of particles are not achievable in practice, though some come close, in the case of very small (< 1 µm) particle columns [2]. The main point of this illustration is that a highly ordered, consistent arrangement of the particles in the column leads to highly consistent mobile phase velocities across the column radius. Since the rate of migration of analyte molecules (that is, analytic velocity) from the inlet to the outlet of the column is proportional to the mobile phase velocity, the consistency in mobile phase velocity directly translates into consistent analyte velocities, and thus a symmetric analyte peak observed at the detector. In the case of perfectly consistent mobile phase velocity across the column diameter, there would be no “A-term
broadening” of peaks, and their widths would be dictated primarily by diffusion of analytes along the long axis of the column, and into and out of pores in the particles. The consistency of the mobile phase velocity in this case is communicated in Figure 1(a) with yellow arrows of the same size. Analyte molecules are illustrated as green dots, and the resulting chromatographic peak observed at the detector is shown at the right of the column.

Variations in Particle Packing Tightness Throughout the Column Can Lead to Asymmetric Peaks

In contrast to the highly ordered bed structure illustrated in Figure 1(a), Figure 1(b) shows that, in real columns, the structure of the particle bed is rather disordered, leading to regions where the particle density (that is, the number of particles per volume of column, not the density of the particles themselves) is much higher than others. In recent years, several studies discussed in the literature have made clear that much of this heterogeneity in packing density throughout the column arises as a result of friction between the column wall and the particles as they are pushed into the tube. This results in large variations in density near the wall, moving from the wall towards the centre of the tube. This is sometimes referred to as a wall effect on the packing density. The net effect of this variation, though, is that the mobile phase velocity will be lower in regions of high density and higher in regions of low density, because there is less friction opposing the flow of mobile phase as the spaces between adjacent particles increase. This variation in mobile phase velocity then manifests as an “A-term type of peak broadening”, and results in peaks that are broader compared to the situation where there is no variation in mobile phase velocity across the radius of the column. In the particular situation shown in Figure 1(b), the packing density near the wall is significantly lower compared to the centre of the column. If the difference in densities is large enough, this can actually result in peak asymmetry like that shown in this case, which we refer to as peak fronting. We typically do not observe this behaviour much in commercial LC columns, because the manufacturers do a good job of mitigating this particular problem, but I’ve packed enough columns during my days working in the column business to know that this effect can be very serious, and must be solved through optimization of column packing procedures. During the development of new stationary phases and column technologies, column manufacturers study
Accumulation of insoluble debris from the mobile phase can lead to mobile phase mixing in the column inlet where there are no particles and lead to significant peak tailing like that shown in Figure 1(c). Sometimes reversing the flow direction can restore some of the column performance, but, in my experience, this performance is usually very short-lived, and when a void develops in a column it is best to just replace the column. Although many modern LC columns are highly resilient to various physical stressors, it is still helpful in the long run to avoid causes of such stress in order to increase column lifetime. This includes avoiding major pressure fluctuations (for example, due to air bubbles in pumps), and properly flushing columns according to manufacturer recommendations before storing the column for more than a few days.

Development of Particle-Free Void Spaces in the Column Leads to Peak Tailing

When packed particle LC columns are manufactured, the slurry containing the particles is pushed into the tube at very high pressures, typically exceeding 10,000 psi for modern materials. In principle, these high pressures force the particles into an arrangement that is unlikely to change significantly when used in an LC instrument at lower pressures. In the early days of HPLC it was not uncommon for column performance to deteriorate upon settling or rearrangement of the particle bed. Fortunately, modern manufacturing procedures have generally improved the robustness of LC columns significantly, and I have to say I am often impressed at just how resilient modern columns are, surviving instrument failures (for example, pressure spiking due to sticky check valves) or user error (for example, allowing columns to dry out without flushing first with an organic–water mixture to remove buffer salts). However, rearrangement of the particle bed can still happen—for example, in response to physical stress on the column (such as repeated pressure fluctuations [4]). Although there are many ways that the particle bed could conceivably rearrange, the classical observation is that the particle bed consolidates in the direction of the outlet (think of sand settling in a bucket when vibrated), leaving a significant “void” at the column inlet where there are no particles and only mobile phase. These void spaces can then act like small mixing chambers and lead to significant peak tailing like that shown in Figure 1(c). Sometimes reversing the flow direction can restore some of the column performance, but, in my experience, this performance is usually very short-lived, and when a void develops in a column it is best to just replace the column. Although many modern LC columns are highly resilient to various physical stressors, it is still helpful in the long run to avoid causes of such stress in order to increase column lifetime. This includes avoiding major pressure fluctuations (for example, due to air bubbles in pumps), and properly flushing columns according to manufacturer recommendations before storing the column for more than a few days.

Accumulation of Debris on Column Frits Can Lead to Asymmetric Peaks

As I’ve written about in the past, one of my favourite troubleshooting tips is to consistently use inline filters in LC systems, especially immediately upstream from the column [5]. There are many ways that insoluble debris can make its way to the column inlet, including particulate matter in the sample that is injected, particulate matter in the mobile phase that comes from unfiltered solvent feeding the pump, and polymeric material that is shed by valves (for example, from a rotor seal) like those found in autosamplers. If an inline filter is not used between the sample injector and the column, then much of this insoluble debris will accumulate on the inlet frit of the LC column. I’ve looked at many inline filters when replacing them after they’ve become blocked, and, without exception, I see that debris never accumulates across the frit in a uniform
way. Sometimes it is concentrated at the edges, sometimes in the middle, and other times there is no obvious pattern. However, the non-uniform distribution of the accumulated material again means that flow through the column inlet frit will also be non-uniform, leading to different mobile phase velocity streams at the column inlet, peak dispersion, and asymmetric peak shapes. In the illustration in Figure 1(d), I’ve attempted to show accumulation of debris at the centre of the inlet frit, and that in some cases this can lead to severely distorted split peaks as shown at the right. The good news is that this particular cause of peak asymmetry can largely be avoided through consistent use of inline mobile phase filters directly upstream from the analytical column. It is also possible in many cases to physically replace the inlet frit on the LC column, but this requires extraordinary care to avoid disrupting the particle bed, and I would not recommend this remedy as a routine practice.

**Summary**

In this instalment of “LC Troubleshooting”, I’ve discussed several of the common physical causes of peak asymmetry in LC, and remedies for some of them. In some cases there is not a whole lot the user can do to address the root cause of peak asymmetry, as some problems occur at the point of manufacture of the column, but in the spirit of increasing our troubleshooting knowledge, it is still helpful to know what can go wrong, and this knowledge can also help determine if the problem is likely to be solvable, or if the column must simply be replaced. In the next instalment in this series, I will discuss several chemical causes of peak asymmetry, where we as users typically have more opportunities to improve peak shape through changes in operating conditions.

**References**


**ABOUT THE COLUMN EDITOR**

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**A New Era in Universal Detection**

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Harold McNair had a remarkable 60-year career as a chromatographer, and his writings spanned the entire breadth of chromatography and separation science. By looking back at some of his key early writings, we have an opportunity to explore three key areas in gas chromatography (GC), which are still relevant today: stationary-phase chemistry, chromatography and computers, and liquid chromatography (LC) versus GC.

Understanding Stationary-Phase Chemistry
Harold McNair’s doctoral dissertation, presented to the faculty of Purdue University in 1959, entitled “Efficiency of Solvents in Gas Chromatography,” was the first doctoral thesis on GC in the United States (1). The original thesis is freely available by open access through the university and ProQuest. The thesis title alone offers interesting historical insight because the thesis was written prior to the definition of many of the currently used terms in chromatography (2). When we think of efficiency today, we mostly discuss peak widths and theoretical plates. In 1959, McNair was discussing retentiveness and selectivity. When we think of solvents today, we would likely think of sample preparation or dilution solvents; McNair was discussing the stationary phase. Translated to the terminology of today, this title might read something like: “Retentiveness of stationary phases in gas chromatography”. McNair performed the first systematic study of retention and polarity of stationary phases in GC, which is a topic that we are still debating and discussing today.

Figure 1 shows a schematic of McNair’s home-built GC instrument. A complete description of the construction can be found in the thesis. A few construction details about this instrument provide lessons about gas chromatographs and columns that are still relevant today. McNair describes making and packing the columns using 6–10 ft lengths of copper tubing, which can easily be coiled, handled, and fitted into the instrument. Although stainless steel and glass columns would be more inert, McNair was thinking practically about ease-of-use. Today’s fused-silica capillary columns are designed with ease-of-use in mind as well. Other materials might be more inert and more temperature stable, but fused silica columns are easy to use.

McNair’s GC instrument did not have a column oven as we have come to know them. Traditional ovens of the day at a
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reasonable cost would not likely have provided precise enough temperature control for systematic and reproducible studies of retention. In McNair’s gas chromatograph, the column and detector were placed in a vapour jacket that contained the vapour of a refluxing solvent at constant temperature. Using acetone, water, and anisole provided stable constant temperatures at 55 °C, 100 °C, and 153 °C, respectively. Although this arrangement provided stable temperatures, it was obviously difficult to change temperature. Temperature programming, a new concept at the time, was out of the question.

McNair recognized the need for strong analyte retention in studies of stationary phase properties, so he prepared packed columns using 30% by weight of each stationary phase, with the other 70% being the solid support particles. He commented that this was a higher than usual loading of the stationary liquid phase onto the solid particles to ensure greater retention of the analytes through having a higher mass of stationary phase in the column. Today, the equivalent thinking is to use a thicker film capillary column to increase retention.

Finally, McNair provides a summary of classical early works on chromatography, some from before the beginnings we think of today. He notes an original work describing gas-solid chromatography but not described as chromatography, as it predates Tswett’s coining of the term chromatography by several years (3,4). In 2010, McNair provided an excellent summary of the history of GC for LCGC North America (5).

McNair’s classic book, Basic Gas Chromatography, was first published in 1964 and became the standard by which all other books intended for new users to learn analytical instruments is measured (6). Figure 2 is a photograph of my own well-worn copy that I purchased upon joining his research group in the 1980s. Of course, this book was required reading in his courses and at the time it still contained much timely information. Today, the original versions with the instantly recognized classical green cover seem dated but the concise descriptions of stationary phases, detectors, and troubleshooting are still relevant. Over the decades, the original Basic Gas Chromatography sold more than 130,000 copies and was published in eight languages. The original paperback is out of print, but second-hand copies are often available through online booksellers. The most recent edition, published by John Wiley and Sons, with Professor James Miller and I as co-authors, was published in 2019.

The more modern discussion of stationary phase chemistry and polarity presented in the later editions of Basic Gas Chromatography demonstrate the continuing timeliness and relevance of this discussion, which began with McNair’s thesis and was re-energized with the development of ionic liquid-based stationary phases in the 2000s (7). When these highly polar stationary phases were being developed, it was quickly discovered that the chemical names of the compounds used to make them were far too complex for marketing purposes, or even for most chemists to remember. In 2011, to simplify the discussion of the polarity of these new stationary phases, Luigi Mondello seized on an idea presented in Basic Gas Chromatography that an overall stationary phase polarity value could be generated using the sum of the McReynolds constants for that phase (8). Mondello’s polarity numbers, calculated from the sum of McReynolds constants, are now widely used to express the overall polarity of stationary phases (9).
Since its publication in 1964 and written in a style that does not require the reader to be a highly experienced chemist, *Basic Gas Chromatography* has been the “go-to” first book on GC for analysts all over the world.

McNair’s publications also provide insight into major developments in chromatography over the years. Although he is best known as a pioneer in GC, McNair’s publication list includes many important works in high performance liquid chromatography (HPLC), supercritical fluid extraction (SFE), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), mass spectrometry (MS), and many more techniques. Perhaps the most important lesson from McNair’s publication list is to not just focus on a single technique but think about solving problems. Thinking about or learning one technique is too limiting for the complex scientific problems of today.

**Chromatography and Computers**

In 1972, McNair hosted the first International Symposium on Computer Chromatography and Associated Techniques in Mainz, Germany. His editorial introducing the symposium, which was captured in a special issue of *Chromatographia*, included several comments about data systems and chromatographic data analysis that remain relevant today (10). McNair asked the following two questions: “What are we doing with computers?” and “What should we be doing with computers?” The symposium volume provides an excellent set of answers to the first question, which included developing interfaces for single and multiple instruments, acquiring the data, and processing the data. McNair commented that the introduction of computers to chromatography caused chemists to complain about the large amounts of data generated each morning. This complaint rings true today in the fast GC and comprehensive two-dimensional gas chromatography (GC×GC) communities, which often deal with large data sets. A few of the article titles from this 1972 symposium, including Kaiser’s “PPB-Analysis Computerized?” and Battista’s “Computerized Blood Alcohol Analysis” demonstrate the intense interest in quantitative analysis. Several other articles on data analysis and evaluation include diagrams showing integration schemes for overlapping and asymmetrical peaks that look very similar to those used to describe these processes in today’s modern data systems.

McNair emphasized the need to use computers for qualitative analysis, mentioning the early work of Schomburg and Ziegler on the use of Kovats retention indices to identify analytes. These early ideas led to the development of retention index libraries that are still in common use today. I was fortunate to work with McNair and Sadttler Research Laboratories in the 1980s on the development of their retention index library into a “poor man’s gas chromatography–mass spectrometry (GC–MS)” concept, whereby retention indices measured on two significantly different stationary phases could be compared to libraries to generate an accurate qualitative analysis. With the rise of benchtop GC–MS and GC×GC–MS instruments, tools for qualitative analysis have been included in many of today’s data systems. McNair’s comments about qualitative analysis and large data sets still resonate in the GC×GC community, where both are the norm, and the chromatograms themselves are increasingly complex.

**LC Versus GC**

In 1974, McNair posed a question that is still commonly asked today in some form: “How soon will liquid chromatography replace gas chromatography?” (11). He
then comments that asking the question at all may demonstrate a general lack of understanding about the two techniques. Difficulties in fundamental understanding exist today. In a comprehensive edited volume on GC published this year, Poole devotes a short section and commentary to this challenge (12). Interestingly, having published exclusively on GC until then, McNair built an extensive publication record on HPLC and other techniques, including GC. When I was in his group, there were two active laboratories in the group: the “LC group” and the “GC group”. The LC and GC laboratories were even in separate buildings for a time. McNair kept us all working together: LC group members worked with and challenged GC group members and vice versa. He made sure we were all cross-trained and that we all understood the fundamentals of separation science, not just individual techniques, and problem solving. The answer to the question of LC replacing GC was never fully decided, and McNair’s response is as true today as it was back in 1974. McNair’s concise two-page comparison of LC and GC, including thoughts on the separation principle, sample types, minimum detectable quantities, analysis time, theoretical plates, preparative capability, and price range, is as useful today as it was back then. Viewing LC and GC as complementary highlights an important principle of analytical chemistry—that the problem should be considered before the technique, and the technique should be designed to solve the problem, not the other way around. Viewing LC, GC, and other analytical methods as competitive to one another only limits the work and experience to a single technique, which is an elementary mistake new analytical scientists can make.

In 1984, Bowermaster and McNair demonstrated the fundamental complementary nature of LC and GC, describing temperature programming in HPLC (13). In both GC and LC, partitioning between the mobile and stationary phases is an equilibrium-controlled process so both techniques are subject to similar phase thermodynamic principles that govern retention. As a simple analogy, mobile-phase strength in LC is like temperature in GC. However, in LC, temperature can also be used as an additional variable to control retention. Temperature-programmed versus isothermal LC runs showed very similar behaviour to temperature-programmed runs in GC, with the temperature-programmed runs showing roughly equal spacing of the peaks versus the exponential spacing seen in isothermal runs. Although temperature-programmed HPLC has not caught on very much, precise temperature control, obviously necessary in GC, which Bowermaster and McNair also discussed in detail, has been incorporated into nearly all of today’s commercial HPLC systems.

These brief vignettes provide a small taste of Professor Harold McNair’s massive contributions to separation science. His publication record alone does not capture the whole story of a career and life devoted not only to the science but also to people all over the world. There are few separation scientists of my own and previous generations that did not meet or interact with McNair at a conference, a short course, or in the laboratory. He was quick to invite visitors from all over the world to visit his laboratory in Blacksburg, Virginia, USA, to share knowledge, experience, and good times. He was a world traveller, who routinely visited the laboratories of colleagues all over the globe. We had a globe of the earth in our laboratory with a small arrow attached, pointing to wherever in the world “Doc” was, often not Blacksburg. Whether through publication, working in the laboratory together with other scientists, or from conversations, Harold McNair freely shared his knowledge and experience. His work and spirit influenced separation science far beyond GC and he is easily considered among the most influential analytical scientists of the past 60 years.

Thank you, Harold.

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A Novel 3D-Printing Method to Create Liquid Chromatography Columns

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For approximately a decade, three-dimensional (3D)-printed columns have been hailed as the future of liquid chromatography (LC). However, the resolution of typical printing methods has fallen short of the requirements to make an effective analytical- or preparative-scale column. In this article, we describe a new 3D-printing method that can create large-volume columns with a feature resolution of 10 µm.

During the ongoing quest for fundamental advancements in high performance liquid chromatography (HPLC) column performance in the past two decades, several methods of producing ordered stationary phases have been explored. To quote John Knox, “The overall effect of a very homogenous bed is to greatly enhance chromatographic efficiency” (1). Over the past decade, the emergence of additive manufacturing, or three-dimensional (3D)-printing, led to many in the chromatography field to consider it as the eventual method to create ordered stationary phases with tailored geometries (2).

Studies have been performed on orientations (3), element shapes (4), and novel porous geometries that are best suited for 3D-printed columns. The efficiency of such structures were demonstrated in both computational simulations and experimental tests, with reduced chromatographic plate heights as low as 1.12. Furthermore, a bifunctional resin has been developed that allows for direct printing of anion exchange columns (5,6).

However, a limitation in such studies has been the feature size of the porous beds that were created. This limitation is because of the limited resolution of most 3D-printing methods. Common printing methods, such as fused deposition modelling, stereolithography, and selective laser sintering, typically operate with nominal resolutions in the order of 20–100 µm. By operating with nominal resolutions, common printing methods generate feature sizes of porous beds typically greater than 300 µm, which is two orders of magnitude short of the feature sizes that are necessary for use in HPLC columns.

In contrast to conventional methods, the high-resolution 3D-printing method called two-photon polymerization is capable of creating pore sizes of under 1 µm with high fidelity. However, as detailed in a previous article by De Malsche and coworkers (7), the printing method requires impractically long production times for analytical-scale columns typically used in HPLC. Therefore, the current capabilities of 3D-printing technologies are inadequate to create analytical- or preparative-scale columns because of limitations in both resolution and print speeds.

To address these limitations, we have developed a 3D-printing technique called hybrid stereolithography (HSLA) (8), which combines traditional stereolithography and photolithography, and is capable of high-resolution and fast printing of high-volume structures.

Figure 1(a) shows a prototype HSLA setup during the printing process. A lower resolution pattern is illuminated, using an ultraviolet (UV) light source (shown in blue in Figure 1[a]) and a digital mirror device. To achieve higher resolution...
than traditional stereolithographic methods, a high-resolution photomask with predefined patterns is used between the resin tray and the digital pattern. After a layer is cured, the build platform lifts to peel the printed piece from the resin tray.

The primary advantage of such a system is that it prints high-resolution repetitive features such as ordered LC column microstructures. The photomask patterns can be used to print grids with feature sizes as low as 10 µm without compromising on printing speeds. Because all regions of a pattern are cured simultaneously, the column internal diameter (i.d.) is entirely independent of the print speed. For example, the 40-mm i.d. column seen in Figure 1(a) was printed at a speed of 5 mm of column length per hour, translating to 6.5 mL/hr. The 40-mm i.d. column represents an improvement of greater than three orders of magnitude compared to the fastest two photon polymerization systems for a similar resolution (9). The high printing speed can be used to create large preparative-scale columns, or several analytical-scale columns in parallel (up to 32 4.6-mm i.d. columns in the setup shown in Figure 1(a)).

Figures 1(b) and 1(c) show axial and radial cutaways of two different lattices printed using HSLA. Figure 1(b) shows a 50 µm simple cubic grid printed in the setup shown above, with a design porosity of 50% and a layer thickness of 25 µm. Figure 1(c) shows a lattice with a feature size of 20 µm with a layer thickness of 10 µm. In both cases, the ordered nature of the printed structures is evident, with the photomask
features being imprinted on the structure with good fidelity.

To create a 3D structure, several patterns were defined on the photomask, with a linear stage moving the photomask after a layer is cured. A photomask-switching system with two patterns, shown in Figure 2, was used to create the lattice seen in Figure 1(b). More complex 3D structures can also be created using more photomask patterns. Because LC column microstructures typically consist of simple, repeatable geometries, a photomask-switching system is well-suited for column production.

A commercial nonporous high-resolution photopolymer resin called Nanoclear (FTD, Alkmaar) was used for all tests. For this study, 1-, 2.1-, 4.6-, 10-, and 20-mm i.d. columns were produced and thoroughly flushed using isopropanol to clear the porous structure of uncured resin. An external casing with fittings and flow distributors was used to connect the column to an HPLC system.

To perform residence time distribution (RTD) tests on the printed 4.6-mm i.d. × 5-cm L structures, 1 µL injections of 1 mg/mL of uracil were performed on a Waters Acquity H-Class system across a range of flow rates (0.01–4 mL/min). For the RTD tests, two flow orientations were tested—with and against the print direction of the column, which is shown in Figure 3(a). In addition to the RTD tests, pycnometric measurements were performed to determine the total void volumes of the printed columns.

Uracil injections were performed in triplicate on the 4.6-mm i.d. columns from two directions. The resulting plate heights show a classical van Deemter curve, with a clear column efficiency minimum (Qmin) of 0.25 mL/min. As seen in Figure 3(b), aligning the flow direction with the print direction (that is, top to bottom as seen in Figure 3[a]) is clearly advantageous compared to orienting the column against the direction of the print. A minimum absolute plate height of \( H = 112.4 \mu m \) (a reduced plate height of \( h = 2.25 \)) was demonstrated with the column oriented in the direction of the print. To the best of our knowledge, these represent the lowest absolute plate

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heights for 3D-printed columns, and a fivefold improvement compared to previous tests using conventional printing methods (3). In the region of the optimum flow rate, the column also exhibited low relative standard deviations of 4% and 7% for the first moment and plate height, respectively.

Switching the flow direction of the column resulted in a near-doubling of plate heights. This difference can be explained by the minor printing artifacts that occur because of a phenomenon called back-curing, where a small portion at the bottom of a pore in a lattice layer cures and solidifies (10).

In addition to determining the plate heights, the scalability of the printing method was tested by measuring the void volumes of columns of different internal diameters. Columns with internal diameters of 1–20 mm × 2.5-cm L were printed in triplicate and measured. As seen in Figure 4, the volume of the printed columns were consistently between 90% and 100% of the design volume, indicating good fidelity between the designed photomasks and the printed structures. However, larger columns (10 and 20 mm i.d.) showed a greater variation in measured void volumes. A possible cause for larger columns showing a greater variation in void volumes is the post-processing method that was used to remove the uncured resin from the printed lattices. Isopropanol was flushed through the columns using an analytical-scale HPLC system for a fixed column volume. However, to achieve constant flushing velocity, the optimum flow rates for the larger internal diameter columns were significantly higher than the maximum flow rate limits HPLC systems will allow. As a result, the larger printed columns delivered incomplete removal of the uncured resin. Tests with preparative-scale systems are necessary, not just to perform separations on such larger internal diameter columns, but to achieve the desired porosities and void volumes.

**Conclusion**

This article outlines the development and operation of a novel 3D-printed method, HSLA, geared towards the production of liquid chromatography columns. The method is capable of operating on length scales of micrometres to >40 cm to produce analytical- and preparative-scale column structures in reasonable timeframes, representing a significant improvement compared to existing 3D-printing methods. The characterization of the printed structures show good agreement between the designed and measured void volumes and reduced plate heights as $h = 2.25$. The results here show that full-scale HPLC and even industrial preparative-scale 3D-printed columns with an organized internal structure that is consciously designed to fit the application are soon to be a widespread reality.

**References**

FIGURE 4: Pycnometric measurements of column void volume compared to the designed volumes of printed lattices at different internal diameters, with a constant length of 2.5 cm.
The Origins of Sample Preparation Technologies

Douglas E. Raynie, Sample Preparation Perspectives Editor

In this column, and elsewhere throughout the research community devoted to chromatographic sample preparation, the importance of the knowledge of the fundamental principles underlying sample preparation is emphasized. What is the role of solubility, diffusion, surface tension, and other parameters on the efficacy of extraction, selectivity, and so on? Of course, the answer is partly based on insight into the physicochemical principles upon which they are based, and partly based on serendipity. This month, we take a look at some of the more popular sample preparation methods, and present a discussion of their initial conception and development, to shed light on their current roles in our analytical “toolbox.”

Two years ago, we presented a retrospective look at the classical Soxhlet extraction, including the origins, operation, standard methods, and derivations (1). One can learn about the principles of modern techniques for extracting solid samples, such as via supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and microwave-assisted extraction (MAE), from a working knowledge of Soxhlet extraction, according to our premise. Now, let’s take it to the next step and investigate the origins of more modern treatments of extraction. To do this, we look to the chemical literature, but more importantly, some of the folklore is presented based on my conversations or subsequent communications with key players in the field of sample preparation. Last year, my colleague Brian Logue described his thought process in combining freeze concentration with stir-bar sorptive extraction (SBSE) to form a novel approach called ice concentration linked with extractive stirrer (ICECLES) (2). This is a great example of combining natural curiosity and an understanding of fundamental principles to develop modern approaches to analytical problem solving. Stories about the development of modern analytical extractions follow.

Solid-Phase Extraction
Perhaps the place to start is the beginning of chromatography. Of course, A.J.P. Martin and R.L.M. Synge are foremost among the pioneers of analytical separations. In 1941, they separated amino acids via partition between aqueous and organic phases by designing a “mixer-settler” extractor (3). They abandoned this approach when they encountered mechanical difficulties with their apparatus, and turned their attention towards the development of liquid–liquid (partition) chromatography (LC). However, L.C. Craig picked up on this work in refining his countercurrent extractor.

As a side note, when I was in graduate school, Milton Lee always showed a Craig countercurrent apparatus during his separations course. Each time, one or more of the glass tubes broke off. I feel sorry for the more recent students that probably never observed a somewhat intact device. We start this discussion with the advent of liquid chromatography (LC), because in 1977, James Waters, founder of the LC company that bears his name, challenged company scientist Patrick McDonald to “find new, faster, more convenient ways to do traditional sample preparation operations” (4). McDonald proposed to “solve a real analytical problem using, whenever possible, our LC technology”. The team at Waters used their silica-based adsorbent technology, along with triaxial bed compression and individual cartridge packaging, to create the SEP-PAK product. Within a few years, countless competitors began appearing and the J.T. Baker Company rearranged the SEP (which stood for “Sample Enrichment and Purification”) acronym into SPE, or the more generic solid-phase extraction. This application of LC technology to analytical sample preparation was forward thinking to the extent that many of us are still asking the questions posed by Waters in their original marketing literature; see Figure 1 (4).
QuEChERS
One of the most recent, and more intriguing, techniques in the sample preparation arsenal is that known as QuEChERS or quick, easy, cheap, effective, rugged, and safe extraction, developed in the 2000s. This approach has been adapted for use with the extraction of almost any analyte from almost any matrix, or so it seems. Steve Lehotay in the U.S. Department of Agriculture’s Agricultural Research Service Laboratory in suburban Philadelphia and his colleague Michelangelo Asastassiates at the EU Reference Laboratory for Pesticides set out to develop what Lehotay described in an email message as, “the most efficient method for multiclass, multiresidue analysis of pesticides in foods” (5).

Previously, both researchers had explored SFE and taxed it to its limits. Steve describes their collaboration as something like, “the Holy Grail of regulatory and industry laboratories for decades.” He explained further (5):

Increasing concerns about excessive glassware, use of chlorinated solvents, high labour needs, and low sample throughput were putting more pressure...
on laboratories to adopt more efficient practices. More sensitive, smaller, and less expensive gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) instruments were also being introduced at about that time, which permitted even broader scope of analysis while still maintaining high selectivity in detection. This meant that sample preparation needed to recover a wide range of polar and nonpolar pesticides, but cleanup was still needed to avoid instrument contamination.

Ultimately, it became a brute force effort. They were aware of magnesium sulfate as both a drying agent and an extraction aid, so that was a starting point. They isolated and studied solvent type, ratio of sample size to solvent volume, sample and buffer pH, salting-out salts and amounts, and extraction time and temperature. They determined recoveries depending on analyte polarity relative to the amounts of water and solvent used, along with measuring co-extractives. After tailoring the extraction for a broad range of pesticides and food samples, their attention turned to the dispersive-SPE step, addressing the perceived disadvantages of matrix-solid phase dispersion, the cost of cartridges, and related factors. Since that time, several vendors have modified the salts or parameters to expand the scope of the technique, and Lehotay has refined the approach to a new QuEChERSER which will be the subject of an upcoming “Sample Preparation Perspectives” column.

In the food industry, it is customary to name extraction techniques after those who developed them, such as the Luke or Randall methods. However, the inventors apparently felt that the “Lehotay and Asastassiasides Method” didn’t roll off the tongue with the same impact as listing the key attributes of the technique!

Solid-Phase Microextraction

Continuing along the theme of extraction techniques based on chromatographic sorbents is solid-phase microextraction (SPME). Despite its name, SPME is not a miniaturized version of SPE. It is described as an open bed, diffusion rate-controlled technique (6). While numerous versions of the approach continue to appear, our discussion will focus on the original, traditional variant of SPME, and this is perhaps a case where sometimes a researcher can be too advanced.

The story of SPME begins in 1987, when Pawliszyn and Liu described sample introduction in capillary gas chromatography (GC) using laser desorption of the sample from the end of an optical fibre, as seen in Figure 2 (7). This provided inspiration to Pawliszyn, who realized that the fibre, clad with a stationary phase like polydimethylsiloxane (PDMS), could also extract organic compounds. The fibre optic replaced the wire used in microsyringes.

Since then, SPME continues to develop, with myriad configurations providing unique advantages. For example, thin-film...
SPME, the SPE Arrow, and SBSE all provide enhanced sample loading and sensitivity compared with the original device. One distinctive format is the coated-blade spray, also developed in Pawliszyn’s laboratory. Coated-blade spray builds from the substrate-spray ionization approaches to MS developed by John Fenn at Yale University, and later by Virginia Commonwealth University, Jentaie Shiea in Taiwan, and Graham Cooks at Purdue University. Coated-blade spray marries SPME and substrate-spray ionization for direct-to-MS analysis. A flattened, sword-shaped stainless steel blade is coated with stationary phase, much like SPME but with a broader array of phases. Then, after the sample is applied, an electrospray solution is added, and the device receives a high potential difference across the uncoated portion of the blade, resulting in electrospray ionization in the mass spectrometer.

Stir-Bar Sorptive Extraction
SBSE represents an interesting example of applying your expertise to a seemingly unrelated situation, at least partially according to the folklore. We remember overhearing a conversation involving Pat Sandra from Belgium’s Research Institute of Chromatography, where he claimed that his inspiration was reading one of his colleague’s papers where, in performing a mass balance for an extraction, the author looked at sample losses due to volatilization, adsorption to the walls of the glassware and stir bar, and other reasons. Sandra thought he could apply a stationary phase to the stir bar and intentionally adsorb the analyte onto the device for subsequent desorption. There is more to this story, again demonstrating knowledge of fundamental principles reapplied in a novel manner.

David, Ochiai, and Sandra note that, in the early days of SPME, a controversy existed regarding the nature of the sorption phenomenon observed (8). Apolar solutes could potentially adsorb onto the Teflon-coated stir bar used for sample agitation, as well as onto the PDMS SPME coating. This led to the development, and subsequent commercialization, of PDMS-coated stir bars for extraction, the advent of SBSE. In the two decades since Sandra’s keen insight, approximately 1000 reports of SBSE are in the literature.

Pressurized Liquid Extraction
The 1990s are considered the heyday of the development of modern instrumental approaches to sample preparation, including SFE and MAE. In the early part of that decade, Dionex/Lee Scientific
introduced the Model 703 supercritical fluid extractor. Within a very short period, their competitors responded with their SFE offerings and with the more advanced developments, shortcomings in the earlier equipment rose to the forefront. In response, the Dionex Salt Lake City Technical Center, home to the SFE development, convened a customer focus group to “fix the SFE”. Led by Bruce Richter and Brian Jones, the conversation led to questions like “If nearly all SFE requires the use of an organic co-solvent, what is the role of the carbon dioxide?” From here, the concept evolved into the concept of an “analytical pressure cooker”—that is, the application of pressure to an extracting solvent with the intent of increasing its boiling point to create enhanced extracting conditions. Both kinetic and thermodynamic factors become favoured at temperatures of 100–150 °C. The resulting technique became accelerated solvent extraction (ASE), a trade name more generally referred to as PLE. During the development of SFE instrumentation in the 1990s, the U.S. Environmental Protection Agency’s Office of Solid Waste issued a memo to equipment manufacturers delineating the requirements to gain approval for an official EPA method utilizing SFE. Dionex followed this guidance during their development of the ASE technique and they received early market acceptance. As a result of the introduction of ASE, not only did other vendors follow suit in creating competitive instrumentation but the overtype of SFE also declined. Applications of SFE became less risky and increasingly oriented towards those applications, like foods, flavours, or polymers, where supercritical fluids truly had advantages.

Vaping as Sample Preparation

Finally, we introduce a novel sample preparation approach based on knowledge of extraction fundamentals and creative reapplication of casual observations. While I know little about the phenomenon of vaping, I realized that this approach is fundamentally sample preparation. The vaping device, via application of elevated temperature, vaporized sample components for delivery by inhalation. We obtained a dry herb vaporizer and, as the final part of his dissertation (9), one of our graduate students, Ahsan Ahmed, evaluated its use in analytical extractions. The particular device we used allowed controllable temperatures up to 240 °C. What was intriguing was that, not only could we employ temperatures similar to conventional headspace techniques and thermal desorption but also at often unexplored temperatures intermediate between these techniques and pyrolysis. Ahsan used the vaping pen with little modification and SPME to collect the emitted vapours. Due to the volume of the vaping chamber, improved sensitivity resulted. Figure 3 shows the GC–MS results for the extraction, or vaping, of horseradish root as a function of temperatures from 150 °C to 240 °C. While further investigation is warranted and improvements to the device may be necessary, it shows promise for use in analytical laboratories, perhaps even for applications done “in the field”. Our initial paper describing this approach, our application to food and environmental samples, and comparison to headspace sampling is in preparation, with the intent of journal submission this autumn.

Conclusions

We’ve presented the told and untold stories surrounding the development of several modern extraction techniques. These examples confirm that the invention process requires understanding of the fundamental principles in a given field, often reapplied in a unique setting. While some of these reapplications may seem intuitive in retrospect, what is obvious to one investigator may not be so to others. Of course, serendipity also plays a role in the creative process. Perhaps, sometime down the road, readers of this column may be inspired to develop new approaches by employing their knowledge and experience.

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Sedere offers a complete range of evaporative light scattering detectors for HPLC, UHPLC, microscale LC, and SFC, as well as purification techniques. The new range of LT-ELSD reportedly offers outstanding performances such as sub-ppm sensitivity, more than five decades of direct dynamic range (SAGA feature), in addition to a choice of several drivers to integrate the detector in most configurations.

www.sedere.com
Sedere, Olivet, France.

**EAF4 System**
Postnova’s simultaneous electrical and asymmetrical flow field-flow fractionation (EAF4) system is designed to enhance separation and characterization of biopharmaceutical, environmental, and nanomaterials. In an EAF2000 system, electrical and cross-flow fields are applied simultaneously, enabling separations by particle size and particle charge based on electrophoretic mobility to characterize complex proteins, antibodies, and viruses, as well as environmental and charged nanoparticles or polymers.

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

**Hydrogen Generator**
Designed for GC-FID, Precision SL is a small and easy to use laboratory-grade hydrogen generator, according to the company, producing hydrogen gas at the push of a button. Available in both 100 cc and 200 cc, the hydrogen generator is reportedly simple to use and maintain, with advanced fail-safe technology, providing a safer solution for flame detectors.

www.peakscientific.com/precisionSL
Peak Scientific, Scotland, UK.

**Sample Filtration**
Teknokroma’s range of Olimpeak filter vials provide efficient, safe, fast, and sustainable sample filtration prior to HPLC analysis, according to the company. Manufactured from highly inert, high purity materials and designed for easy use, they fit directly into most autosamplers and are reportedly the ideal solution for laboratories of all sizes. They are available with a wide variety of membranes.

Teknokroma Analítica S.A., Barcelona, Spain.

**HPLC Column**
Antibodies regulate their immune response by binding Fc receptors on immune cells. The TSKgel FcR-IIIa column analyzes this interaction: in screenings, quality control, and by providing fractions with different FcR affinities.

https://www.separations.eu.tosohbioscience.com
Tosoh Bioscience GmbH, Darmstadt, Germany.
**GC Detector**
VICI’s Model D-3-1-8890 is a plug-and-play pulsed discharge detector for easy installation and configuration on the Agilent 8890 GC. It is optimized for trace-level work in helium photoionization mode, and is a non-radioactive, low maintenance universal detector with a wide linear range, according to the company. It also utilizes the electronics and power supply of the host GC.

- [www.vici.com](http://www.vici.com)
- VICI AG International, Schenkon, Switzerland.

**Lab System**
The Verity CPC Lab MS System is an automated, cost-effective, liquid-liquid purification system that streamlines your workflow and allows you to isolate added-value molecules with high yields and purity quickly. According to the company, this system is accurate, efficient, easy-to-use, and requires minimal maintenance.

- [www.gilson.com](http://www.gilson.com)
- Gilson Inc, Middleton, Wisconsin, USA.

**High Performance Pulse Dampers**
Baseline fluctuation is a thing of the past. Knauer’s Pulse Dampers provide excellent damping performance whilst remaining simple to integrate into HPLC systems due to their membrane-free assembly, according to the company.

- [https://www.knauer.net/](https://www.knauer.net/)
  Knauer Wissenschaftliche Geräte, Berlin, Germany.

**Electrochemical Detector for HPLC**
Antec Scientific’s Decade Elite is designed as an easy-to-use electrochemical detector that can integrate with any LC system. When used with the SenCell, it becomes the most sensitive electrochemical detector by today’s standard, according to the company. With software drivers for Chromeleon, OpenLab, ChemStation, Clarity, and many more.

- [https://antecscientific.com/decade-elite](https://antecscientific.com/decade-elite)

**Bioinert (U)HPLC Columns**
The bioinert YMC-Triart (U)HPLC columns are suitable for critical substances such as selected proteins/peptides, nucleotides, oligonucleotides, and metal-coordinating small molecules. According to the company, this provides excellent peak shapes, recoveries, and no carryover effects. The columns are fully inert due to their pressure-stable PEEK-lined stainless steel column body and the use of PEEK frits.

- [www.ymc.de](http://www.ymc.de)
  YMC Co., LTD., Kyoto, Japan.

**Capillary Columns**
Fortis Technologies has a full range of capillary and narrow-bore columns for those requiring increased sensitivity over traditional LC columns—75 µm and 200 µm capillary columns, 0.5 mm and 1 mm narrow-bore—all packed with Bio 300 Å range or traditional 100 Å phases. Stationary phase choice is not limited and particle size can be 1.7 µm to 5 µm.

- [www.fortis-technologies.com](http://www.fortis-technologies.com)
  Fortis Technologies Ltd, Cheshire, UK.
**Nutrient Pad Sets**
NPS are dehydrated media pads in petri dishes, packed in a double-layer aluminium protective bag, and come with suitable membrane filters for economical, time-saving, microbiological quality control of water and brewery samples. They have a two-year shelf life and there’s no need for refrigeration. 3.0–3.5 mL of sterile water is required to reactivate the media on the NPS.

[www.sartorius.com](http://www.sartorius.com)
Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany.

**Phospholipid Removal**
The Microlute PLR from Porvair Sciences is a 96-well microplate that provides effective removal of phospholipids and proteins with high levels of reproducibility from plasma and serum samples while maintaining maximum recovery of target analytes. According to the company, the microlutes enable you to increase the sensitivity and integrity of your UHPLC and HPLC methods.

[www.microplates.com/microlute-plr](http://www.microplates.com/microlute-plr)
Porvair Sciences Ltd, Wrexham, UK.

**Method Translator**
Pro EZLC method translation software makes it possible to scale down an existing LC method to a smaller column format so that users can speed up run time, increase sample throughput, and reduce solvent use, according to the company. The user can input current column dimensions and method conditions, then specify the dimensions of the new column that they want to try.

[www.restek.com/Pages/Pro-EZLC-Method-Translator](http://www.restek.com/Pages/Pro-EZLC-Method-Translator)
Restek Corporation, Bellefonte, Pennsylvania, USA.

**Environmental Analysis**
Halo Enviroclass is the newest family of HPLC columns for environmental separations. From performance-designed application-specific phases to separate per-and polyfluorinated alkyl substances (PFAS) and polycyclic aromatic hydrocarbons (PAH), Halo Enviroclass represents more than method assured products, according to the company.

[www.halocolumns.com](http://www.halocolumns.com)
Advanced Materials Technology, Delaware, USA.

**SEC Columns**
PSS MAB, for size-exclusion chromatography (SEC) of monoclonal antibodies, is the latest addition to the PSS column family. Analytical and semi-micro columns, which cover a wide molar mass range and are pre-equilibrated for light scattering detection, are available. Bio-inert coated hardware is also optional for separations that are required to be metal-free.

[www.pss-polymer.com](http://www.pss-polymer.com)
PSS GmbH, Mainz, Germany.

**GC Autosampler**
The HT2800T is HTA’s multi-functional autosampler that combines the functions of an autosampler for liquid, static headspace, and SPME in a single unit. Compatible with GC and GC–MS systems of any brand or model. According to the company, the autosampler features cutting-edge technologies that allow usability optimization and excellent analytical performance.

[www.hta-it.com](http://www.hta-it.com)
HTA s.r.l., Brescia, Italy.
The Applications Book
MEDICAL/BIOLOGICAL

397 Analysis of Redox and Bioenergetics Metabolites with Polymeric iHILIC®-(P) Classic HILIC Column and Mass Spectrometry
Susan Kim¹, Wen Jiang¹, and Joshua D. Chandler¹,³
¹Department of Pediatrics, Division of Pulmonology, Allergy & Immunology, Cystic Fibrosis and Sleep Medicine, Emory University, Atlanta, Georgia, USA, ²HILICON AB, ³Children’s Healthcare of Atlanta, Atlanta, Georgia, USA

PHARMACEUTICAL/DRUG DISCOVERY

401 Molecular Weight Determination of VLPs Using LenS³ Multi-Angle Light Scattering Detector
Tosoh Bioscience GmbH

402 Liposome Size, Concentration, and Structural Characterization by FFF-MALS-DLS
Wyatt Technology
Aerobic organisms use oxygen as the terminal electron acceptor in cellular respiration. Molecular oxygen is capable of accepting up to four electrons to become water through the reaction catalyzed by mitochondrial complex IV (1). However, intermediate reactive oxygen species (ROS) are formed by partial reduction of oxygen. Aerobes have evolved both physiological and biochemical strategies to mitigate molecular damage caused by ROS while maintaining oxygen availability for cellular respiration (2). Core metabolism and redox control depend on a number of metabolites, including nucleotides (NADP⁺/NADPH and NAD⁺/NADH) and redox-active amino acids and peptides (particularly glutathione, cysteine, and methionine). For example, reduced glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is an important regulator of local hydrogen peroxide signalling by serving as a cofactor of glutathione peroxidases (3). In turn, oxidized glutathione (GSSG) is reduced back to GSH in an NADPH-dependent fashion by GSSG reductase (4). Both cysteine and methionine in proteins may act as redox switches linked to NADPH-dependent repair mechanisms (5,6). NADPH is generated by the pentose phosphate pathway that links to glycolysis, which generates ATP and relies on NAD⁺ as a cofactor. Multiple steps of the citric acid cycle generate NADH that supports chemiosmotic ATP synthesis.

Liquid chromatography–mass spectrometry (LC–MS)‑based metabolomics presents an opportunity to monitor many molecules simultaneously, relying on a combination of chromatographic and mass-based resolution to quantify hundreds to thousands of molecules in a typical experiment (7). However, many of the metabolites noted above could be challenging to profile simultaneously because of poor chromatographic separation or peak shape (8). We sought to develop a simple method using hydrophilic interaction liquid chromatography (HILIC) that could simultaneously profile the metabolites that are important in redox regulation and bioenergetics. By incorporating these metabolites into a nontargeted-compatible and derivatization-free approach, information about redox and bioenergetics can be gained as part of routine profiling.

**Experimental**

**LC–MS System:** A Vanquish Horizon binary pump was hyphenated to a Q Exactive HF (Thermo Fisher Scientific), set at 120,000 FWHM, 1e6 AGC target, and 200 ms max IT. Connecting tubings between the column, autosampler, and MS system were 100 µm i.d. PEEK‑lined stainless steel MarvelXACT tubing (IDEX Health & Science) instead of original Viper MP35N. The HESI‑II probe was held at 320 °C and +3.5 kV with 40 sheath gas, eight auxiliary gas, and one sweep gas flow (arbitrary units). **Column:** 150 × 2.1 mm, 5-µm, 200 Å, iHILIC®-(P) Classic (P/N 160.152.0520, HILICON AB), coupled to a 20 mm guard column via a PEEK coupler. **Eluent:** A) 15 mM ammonium acetate pH 9.4, B) acetonitrile.

![Figure 1](image-url)

**Figure 1:** Base intensity-normalized chromatograms of (a) GSH/GSSG; (b) Met/MetO/MetO₂; (c) NAD⁺/NADH/NADP⁺/NADPH; (d) AMP/ADP/ATP.
Table 1: Peak areas of reduced and oxidized compounds from reduced standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>REDUCED</th>
<th>OXIDIZED</th>
<th>OXIDIZED %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>6.82×10⁷</td>
<td>2.16×10⁴</td>
<td>0.03</td>
</tr>
<tr>
<td>METHIONINE</td>
<td>2.87×10⁸</td>
<td>5.89×10⁵</td>
<td>0.21</td>
</tr>
<tr>
<td>NADH</td>
<td>2.76×10⁷</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>NADPH</td>
<td>8.32×10⁸</td>
<td>8.32×10⁵</td>
<td>ND</td>
</tr>
</tbody>
</table>

* *Metabolites were quantified in positive mode as their [M+H]+ ions with 5 ppm mass accuracy. The result is based on a semi-quantitative comparison of peak areas.

Gradient Elution: 0–15 min, gradient from 10–90% A; 15–17 min, 10% A (column re-equilibration)

Flow Rate: 200 µL/min

Column Temperature: 40 °C

Injection Volume: 2.5 µL

Metabolite Samples: Individual metabolites were prepared fresh from reference standards in water (50 µmol/L). We focused on the following 14 metabolites: oxidized/reduced nicotinamide adenine dinucleotide (NAD+/NADH), oxidized/reduced nicotinamide adenine dinucleotide phosphate (NADP+/NADPH), adenosine mono-di-triphosphate (AMP/ADP/ATP), glutathione/glutathione disulfide (GSH/GSSG), methionine/methionine sulfoxide/methionine sulfone (Met/MetO/MetO₂), and cysteine/cystine (Cys/CySS). We also analyzed human plasma Standard Reference Material 1950 (MilliporeSigma) and metabolite extract of *E. coli* (Cambridge Isotope Labs), which were either reconstituted with a solution of 1:1:1 acetonitrile–methanol–water + 8.33 µM D5-hippuric acid or extracted with addition of 2 vol 1:1 acetonitrile–methanol + 12.5 µM D5-hippuric acid followed by vortexing and incubation on ice for 30 min. Samples were then centrifuged at 20,000 g and 4 °C for 10 min. The supernatant was then applied for HILIC–MS analysis.

Results and Conclusion

The newly developed method provides excellent peak separation and overall good peak quality for all of the metabolites as shown in Figure 1. We analyzed all of the reduced metabolite standards to determine if autooxidation was rapidly occurring (Table 1). Peak areas of most oxidized species from injections of reduced standards were <1% that of the reduced species at pH 9.4. However, in the case of the Cys standard, only oxidized CySS was detectible. When using neutral pH instead of 9.4, Cys was preserved and detected (though considerable autooxidation was still noted). Cys may be unstable at alkaline pH as a result of enhanced deprotonation to the nucleophilic thiolate form (pK = 8.3) (3).

We then determined whether the 12 metabolites shown in Figure 1 were well represented in standard reference materials for untargeted metabolomics experiments. Human plasma SRM 1950 and the unlabelled *E. coli* metabolite extracts were used as probes. Across the two matrices, signals for all metabolites except NADP⁺ were observed (Table 2).

This work demonstrates the feasibility of profiling core metabolites related to redox metabolism and bioenergetics, which might be incorporated into a nontargeted workflow for more extensive profiling.

We note that thiol metabolites are labile and nontargeted profiling of these should be experimentally validated with derivatization-based methods (9). A HILIC method with an acidic mobile phase may offer better stability for thiol compounds as well.

Acknowledgements

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References

Antisense oligonucleotides (ASOs) have an outstanding therapeutic potential and are used for the therapy of genetic diseases. Due to the specific binding of an ASO to the complementary sequence of the RNA of a target protein, protein expression can be suppressed.

Recent Approvals of Commercial ASOs
Recently, gapmer ASOs with increased in vivo stability have been introduced. They are chimeric RNA/DNA hybrids containing modified RNA nucleotides at the flanks and DNA nucleotides at the central block. Several gapmer ASOs have been clinically approved in 2020, including inotersen (Tegsedi®), volanesorsen (Waylivra®), and mipomersen (Kynamro®). These gapmer ASOs typically comprise phosphorothioate (PS) linkers and 2’-O-methoxyethyl (2’-MOE) modifications.

Mipomersen, which is serving as the model compound here, was developed to treat homozygous familial hypercholesterolemia. It inhibits the production of the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) and therefore reduces their concentration in blood.

Bioanalytical Methods Required
Despite their promising therapeutic potential, bioanalytical methods for ASOs have not been well developed and require further optimization. In addition, fully validated LC–HRMS methods have not been established yet. Y. Sun et al. have recently reported a successful development of a sensitive LC–HRMS method for mipomersen in rat plasma as a model compound for 2’-MOE gapmers (1).

LC–HRMS Method Using Bioinert YMC Column
In this application note based on the study by Y. Sun et al., a bioinert YMC-Triart C8 metal-free UHPLC column was used. In addition, the versatile YMC-Triart C18 and the very hydrophobic YMC-Triart C18 ExRS (both also in metal-free column hardware) were tested as well, but YMC-Triart C8 demonstrated the best separation of mipomersen and LNA-mipomersen as internal standard. However, the use of a bioinert column turned out to be crucial as the use of a standard C18 column in stainless steel hardware showed severe carryover.

Optimized Conditions
TEA/HFIP (triethylamine/1,1,1,3,3,3-hexafluoro-2-propanol) were used as the ion pairing reagents. Their concentrations were optimized to improve the separation, the peak shapes, avoid carryover, and ensure high reproducibility. The final concentrations are 28.0 mM TEA and 135.8 mM HFIP. In addition, it was essential that the column was prewashed prior to the first use with 0.1% phosphoric acid in water–

**Figure 1:** Metal-free YMC-Triart columns are fully bioinert thanks to their PEEK lining and the use of PEEK frits.
methanol (30:70) for 1 h to prevent nonspecific binding of ASOs to the column. The resulting method not only showed potential for measuring preclinical samples of very low ASO concentrations but also for the future analyses of ASOs of the gapmer type.

Reference

Viruses and virus-like particles (VLPs) are multimeric protein structures that mimic native viruses but are non-infectious. VLPs are potential candidates in new vaccines and gene therapy products. For example, commercially available VLP-based vaccines are available for hepatitis B and human papillomavirus. Robust analytical techniques are needed to not only ensure quality of final products but also provide data for informed decision-making during the development process.

Size-exclusion chromatography (SEC) is an analytical technique that provides results on the size and purity of macromolecules. When coupled with multi-angle light scattering (MALS), it offers both molecular weight (MW) and radius of gyration ($R_g$) or size. Importantly, AU$_{280}$ detection is only concentration-dependent, whereas MALS corresponds to both concentration and molecular weight. Thus, the large molecular weight characteristic of VLPs inherently provides MALS with a strong scattered light response and enables VLP detection even in a dilute solution that is well below AU$_{280}$ detection limit.

**Material and Methods**

**Instrument**: Thermo Scientific UltiMate® 3000 with multiple wavelength UV detector and Shodex RI-504 semi-micro RI detector

**MALS**: LenS3 MALS detector

**Columns**: TSKgel® GMPWxl, 13 μm, 7.8 mm ID × 30 cm

**Mobile Phase**: 0.145 mol/L NaCl, 0.01 mol/L HEPES, 0.05% sodium azide, pH 7.4

**Flow Rate**: 0.3 mL/min

**Sample**: Parvovirus VLP (MVM-MVP) (Cygnus Technologies), stock 1 × 10$^{12}$ particles/mL (15 μL injection), (dn/dc = 0.19)

**MALS Calibrant**: BSA, 5 mg/mL (dn/dc = 0.185)

In this application, parvovirus VLP was analyzed on a TSKgel GMPWxl SEC column coupled with the LenS3 MALS detector. Either RI or UV can function as the concentration detector. RI was used with the right-angle light scattering signal (RALS) to measure MW. Extreme low angle (LALS), right angle, and extreme high angle (HALS) signals were used to plot angular dissymmetry and to determine $R_g$. The MALS detector was calibrated with BSA prior to sample analysis and all data were processed and analyzed using SECview® software.

Analysis of parvovirus VLP by SEC-MALS using the TSKgel GMPWxl column revealed a MW of ~4 megadaltons and $R_g$ of 12.8 nm (Figure 1). These results closely align with reported values for this VLP (1).

Parvovirus VLP was diluted up to 64-fold and injected onto a TSKgel G5000PWxl column. Approximately 3 × 10$^{10}$ particles per mL can still be detected using the RALS signal from the LenS3 MALS detector, which allows for analysis of materials with low concentration or when working with limited sample.

**Conclusion**

Mass spectrometry is the most common method previously used for VLP size determination, but this technique is costly and impractical for frequent analysis. Inclusion of SEC-MALS as an analytical technique to determine the MW and $R_g$ is a preferred alternative and allows for both routine analysis and process monitoring. The wide range in pore sizes and separation ranges of TSKgel PWxl SEC columns overcomes challenges in analytical SEC where separations of large macromolecules require a larger pore sized stationary phase. When these SEC columns are then combined with the greatly enhanced sensitivity of Tosoh Bioscience’s LenS3 MALS detector, fast and easy analysis of MW and $R_g$ with an improved level of detection (LOD) is provided.

**Reference**

Liposomes and lipid nanoparticles are often used as nanocarriers to encapsulate fragile nucleic acids, hydrophobic or highly toxic drugs, and to deliver these drugs safely to target tissue. During drug nanocarrier product and process development, as well as quality control, it is of great importance to monitor liposome size distributions accurately while also verifying drug encapsulation. FFF-MALS-DLS, consisting of field-flow fractionation (FFF) combined with multi-angle light scattering (MALS) and dynamic light scattering (DLS), is a powerful tool for characterizing the size, concentration, and structure of large nanoparticle ensembles.

Method
Encapsulation might cause changes in liposomal dimensions, but that is not always the case and other effects could cause such changes. Therefore, a more sophisticated analysis is warranted than mere size. Here, we report the analyses of two liposome samples, one empty and one filled with drug, by means of an Eclipse™ FFF system followed by a DAWN® MALS detector with embedded WyattQELS™ DLS module. The FFF separation method was optimized with the aid of Wyatt’s proprietary FFF simulation software. ASTRA software was used to collect and analyze the light scattering data to determine size and concentration (number density).

Results and Discussion
FFF separates particles according to hydrodynamic radius. Thanks to upstream separation, quantitative size distributions by FFF-MALS-DLS provide far more resolution and quantification than batch (unfractionated) DLS. Online DLS directly measures the hydrodynamic radius, $R_h$, sequentially for each eluting size fraction, while MALS simultaneously measures the root-mean square radius, $R_g$. The shape factor, $\rho$, which is defined as the ratio $R_g/R_h$, provides important structural information: it can discriminate between empty and filled shells or quantify the axial ratio of a uniform ellipsoid.

Both $R_h$ and $R_g$ are plotted against elution time in Figure 1. The results from duplicate runs demonstrate clean separation and excellent reproducibility of the FFF-MALS-DLS method. Figure 1(a) shows that the $R_h$ values for both empty and filled liposomes are well-overlaid, which is expected since FFF separates according to hydrodynamic size. However, as shown in Figure 1(b), $R_g$ values for these two liposomes do not overlay, which indicates different internal structures.

Figure 2(a) plots $R_g$ against $R_h$; $\rho$ is the slope of the linear fit. Interpretation of $\rho$ requires an assumption about the shape of the particles; here we make use of a priori knowledge that they are spherical. The values of $\rho$ for these two populations then correlate precisely to empty and filled liposomal structures.

In addition to size and structure, FFF-MALS can determine quantitative size distributions (number density vs. size) of size-fractionated nanoparticles if the refractive index of the constituent material is known. For lipids this is quite straightforward and Figure 2(b) provides the quantitative nanoparticle concentration analysis.

Conclusion
For liposomes or other nanoparticles, FFF-MALS-QELS provides an easily adaptable yet powerful characterization tool to obtain information on particle size, size distribution, particle count, as well as structure—all without making assumptions about the particles or their composition. FFF-MALS-DLS instrumentation is essential for robust drug nanocarrier development and quality control.
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