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As I write this, I’m also grading the final exam from my Chromatography and Separations course. Here’s your opportunity to test yourself; answers are provided.

This article presents a new hydrophilic SAX hybrid monolithic column prepared by in-capillary coating 5 μm bare silica particles with functional organic polymers. After preparation conditions were investigated and selected, the resulting column was characterized in detail.

Key insights on how to get the most out of gas chromatography for the most difficult problem solving, the best quantitative analysis, and the highest data integrity

This instalment of “Column Watch” presents many of the highlighted topics and trends observed at the symposium.
The Publishers of LCGC Europe would like to thank the members of the Editorial Advisory Board for their continuing support and expert advice. The high standards and editorial objectivity provide readers with the tools necessary to deal with real-world problems and commercial objectivity provides readers with the tools necessary to deal with real-world problems and commercial objectivity.

The Editors of LCGC Europe would like to thank the members of the Editorial Advisory Board for their continuing support and expert advice. The high standards and editorial quality associated with LCGC Europe are maintained mainly 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.
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Welcome to the September issue of *LCGC Europe*! Our cover story this month focuses on column technology and presents a hydrophilic strong anion-exchange monolithic column, combining the typical merits of silica particle-packed and polymer-based monolithic columns. It has real potential applications for rapid analysis of some acidic drugs, inorganic anions, organic weak acids, phenols, and nucleotides.

In *LC Troubleshooting*, Dwight Stoll examines a longstanding problem in liquid chromatography (LC)—bad peaks—and explains how to fix some of the most common causes.

*GC Connections* takes us "under the hood" of gas chromatography (GC), and offers advice on maintaining, optimizing, and troubleshooting across a wide range of GC tools.

Highlights from the recent HPLC 2022 symposium are presented in the latest instalment of *Column Watch*, and Dave Bell and Cory Muraco discuss the welcome return of face-to-face meetings.

How confident are you in your sample prep fundamentals? In *Sample Preparation Perspectives*, we take a dive into the world of separation preparation concepts.

In this month’s Analysis Focus, the power of two-dimensional (2D-) LC to help unravel the complexity of biopharmaceutical and pharmaceutical samples is presented. Given the ever-increasing structural complexity of the new therapeutic modalities that are being developed, the risks involved in missing sample information continue to grow, and 2D-LC offers distinct advantages over one-dimensional (1D-) LC in this arena.

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### Multimedia Highlights

**Note from the CEO**

Mike Hennessy Jr, President and CEO, MJH Life Sciences®

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### LCGC Online

Selected highlights of digital content from *LCGC Europe* and *The Column*.

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The first part of a review of the recent ChromSoc spring symposium meeting focusing on small molecule analysis.

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Capillary liquid chromatography (cLC) offers high column efficiency, low consumption of sample and solvent, and good compatibility to mass spectrometry (MS) (1−4), which has led to widespread applications. Strong anion-exchange (SAX) columns are widely used to separate inorganic anions, organic weak acids, drugs, nucleotides, and proteins (5−9). For proteome analysis, SAX is often coupled with a reverse-phase column (5,10) and a two-dimensional separation system to substantially enlarge peak capacity. For the applications described previously, high hydrophilicity is desired. This allows a low content of organic modifier in the mobile phase to be used, and improves the orthogonality between SAX and reversed-phase mechanisms (5).

SAX columns can be prepared in the laboratory by packing capillaries with silica particles modified chemically with compounds containing quaternary ammonium groups (QAGs). QAGs are usually selected as the functional groups in SAX columns due to their ability to remain positive, even at high pH values, and their strong retention for anions. Packed columns possess good mechanical stability and almost no swelling-shrinking effects, but they do require retaining frits at both ends to trap the particles within the columns. The sintering process for the frits leads to band broadening, capillary fragility, chromatographic functionality degradation, and bubble formation in capillary electrochromatography (CEC) and cLC (4,11).

SAX columns can also be prepared by in-capillary synthesis of porous monoliths. Monolithic columns not only possess high permeability and column capacity but they also have none of the problems associated with the retaining frits. Depending on the matrix, reported monoliths are commonly classified into inorganically silica-based, organically polymer-based, or organically-inorganically hybrid monoliths (4). Jaafar et al. (12) have reported a silica-based SAX monolith. This column was prepared by

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**KEY POINTS**

- A hydrophilic strong anion-exchange monolithic column can be prepared by in-capillary coating 5 µm bare silica particles with the copolymers of methacryloxyethyltrimethyl ammonium chloride and pentaerythritol triacrylate.
- The column combines the typical merits of silica particle-packed and polymer-based monolithic columns.
- The column has potential applications for rapid analysis of some acidic drugs, inorganic anions, organic weak acids, phenols, and nucleotides.

A hydrophilic strong anion-exchange monolithic hybrid column was prepared by in-capillary coating 5-µm bare silica particles with the copolymers of methacryloxyethyltrimethyl ammonium chloride and pentaerythritol triacrylate in the presence of a porogen consisting of water, methanol, and cyclohexanol. The composition of the porogen and the concentration of the monomers were investigated and selected. The resulting column was characterized. The column had an uniform pore structure and could withstand a back pressure up to 3500 psi. Its permeability was comparable to that of packed columns and the swelling-shrinking behaviour negligible. Its hydrophobicity could be suppressed at acetonitrile concentrations above 40% (v/v) and the minimum theoretical plate height was about 10 µm for Br−. The column-to-column relative standard deviations (RSDs) were 2.2% and 3.5% (n = 9) and the batch-to-batch RSDs were 2.4% and 5.5% (n = 3) for k and H values, respectively. The column exhibited a remarkable performance for the separation of inorganic anions, organic weak acids, phenols, and nucleotides.

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chemically modifying a porous silica-based monolithic matrix with a quaternary salt to introduce QAGs. Such silica-based monoliths have high permeability, little swelling-shrinking effect, and high mechanical stability, but they suffer from poor preparation reproducibility and tolerability to extreme pH. In addition, post-modification is time-consuming (4,12,13). As a result, silica-based SAX monoliths have not been researched much. Several organic polymer-based SAX monoliths have been prepared through in-capillary copolymerizing QAGs-containing monomers and cross-linkers in the presence of proper porogens (14–18). Such monoliths possess the merits of easy preparation, good tolerability to extreme pH, high permeability, and wide commercial availability of functional monomers (4,19), but they suffer from the swelling-shrinking effect (14,15). The swelling-shrinking effect is serious, particularly for organic polymer-based strong ion-exchange monoliths, and is very harmful to mechanical stability, permeability, analytical performance, and lifetime. In addition, hydrocarbon moieties of the monomers and the C–C backbone of the monoliths contribute to undesired hydrophobicity (20). Such hydrophobicity can be lowered by using polar cross-linkers, such as pentaerythritol triacrylate (PETA) (16) or poly (ethylene glycol) diacrylate (21). Organically-inorganically hybrid monoliths combine the merits of organic polymer-based monoliths with inorganic silica-based monoliths, such as good mechanical stability, good tolerability to extreme pH, and high permeability. Several approaches have been reported for the preparation of hybrid monoliths (4,20,22–24). Among them, the most widely used is the “one-pot” method. Wang et al. (22) reported a hybrid monolith with mixed-mode of hydrophilic interaction and SAX using the “one-pot” method. This column was prepared through polycondensation of pre-hydrolyzed precursors of tetramethoxysilane and vinyltrimethoxysilane and then copolymerization of precondensed vinyltrimethoxysilane and 2-(acryloyloxy) ethyltrimethyl ammonium methyl sulfate, and was used in CEC.

Our team reported a zwitterionic monolith (25) and a hydrophilic strong cation-exchange monolith (26). They were prepared by in-capillary coating 5-µm bare silica particles with functional organic polymers. By adjusting the polarity of porogen and the total concentration of monomers, a thin layer of the polymers was formed only on the particle surface, leaving the interstitial space around the particles free for eluent flow. Such columns can be considered to be a hybrid of...
silica particles-packed and organic polymer-based monolithic columns, and thus combine the merits of both of them. This article presents a new hydrophilic SAX hybrid monolithic column (shortened to H-column below), prepared in a similar way. After preparation conditions were investigated and selected, the resulting H-column was characterized in detail.

**Experimental**

**Chemicals and Materials:**
Methacryloxyethyltrimethyl ammonium chloride (MATE) was produced by ACRS. Other chemicals and materials are described elsewhere (26).

**Instruments:**
Chromatographic separations were conducted on a setup composed of a Model 500 liquid delivery pump from Lab Alliance, an in-house made T-splitter, and a Micro 21 UV-01 detector from Jasco (Japan). Morphology of monoliths was observed using a SU-8010 scanning electron microscope (SEM) from Hitachi. Data acquisition was implemented using a Lenovo computer equipped with a 24 bit analog-to-digital converter and a N3000 software package from Zhejiang University. Chromatograms were reproduced using Origin software (OriginLab) based on the original data.

**Preparation of the H-Column:** The capillary was pretreated and pre-vinylized as described elsewhere (25). A retaining frit was fabricated at one end of the capillary by packing a 1–2 mm section of the capillary with 5-µm bare silica particles and then sintering this section in a hydrogen flame. The capillary with the retaining frit was packed with 5-µm bare silica particles using a slurry packing technique. A polymerization mixture, composed of the monomers MATE from Jasco (Japan), was used.

**Figure 4:** (a) Separation of three inorganic anions, (b) three organic weak acids, (c) five phenols, and (d) four nucleotides on H-columns. Conditions: (a) mobile phase, 50 mM NaCl in 10 mM NaH₂PO₄ buffer at pH 3.0; flow rate, 1.2 µL/min; effective column length, 2.5 cm; UV detection wavelength, 210 nm; solutes, 1. bromate, 2. bromide, 3. nitrate. (b) Mobile phase, 10% v/v acetonitrile in 50 mM NaH₂PO₄ buffer at pH 4.2; flow rate, 1.0 µL/min; effective column length, 2.5 cm; UV detection wavelength, 235 nm; solutes, 1. P-hydroxybenzoic acid, 2. M-hydroxybenzoic acid, 3. O-hydroxybenzoic acid. (c) Mobile phase, 10% v/v acetonitrile in 10 mM NaH₂PO₄ buffer at pH 9.3; flow rate, 1.0 µL/min; effective column length, 4.0 cm; UV detection wavelength, 277 nm; solutes, 1. phenol, 2. hydroquinone, 3. resorcinol, 4. catechol, 5. phloroglucinol. (d) Mobile phase, 5% v/v acetonitrile in 10 mM NaH₂PO₄ buffer at pH 6.3; flow rate, 1.0 µL/min; effective column length, 4.0 cm; UV detection wavelength, 254 nm; solutes, 1. UMP, 2. AMP, 3. CMP, 4. GMP.

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and PETA (2:1 MATE-PETA [v/v]; total monomer concentration, 25%, v/v), the initiator 2, 2’-azobisobutyronitrile (2%, w/v, with respect to monomers), and the porogen (1:1:6 water–methanol–cyclohexanol [v/v/v]), was prepared. After being degassed and homogenized by purging with nitrogen gas and ultrasonicating for 15 min, the mixture was pumped into the capillary packed with silica particles. After reaction in a water bath at 60 °C for 7 h, the retaining frit was cut off. Finally, plenty of methanol and water was sequentially pumped through the capillary to remove the porogen and unreacted monomers, and a H-column was obtained.

Results and Discussion

Conditions for Preparation: In order to reduce the swelling-shrinking effect and increase permeability, the polymers should be allowed to form on the silica particles rather than in the interstitial space around the particles, and the polymer layer on the particles should be controlled to be as thin as possible.

According to the principle of “similarity-intermiscibility”, it can be anticipated that the polar polymers of MATE and PETA trend to associate with the silica particles after forming nuclei if the polarity of porogen is lower than that of the particles. Therefore, a porogen of low polarity should be favourable for the H-column preparation. In this work, a ternary porogen consisting of water, methanol, and cyclohexanol was used, and its polarity was adjusted by changing the volumetric ratios. Figure 1 shows the SEM images of H-columns prepared with the porogens of different ratios (water–methanol–cyclohexanol: (a) 1:1:2, (b) 1:1:4, (c) 1:1:6, and (d) 1:1:8). As anticipated, the polymers formed on both on the particles and in the interstitial space because of the high polarity of the porogens (Figure 1[a] and 1[b]), and formed only on the particles when the polarity of porogens was properly lowered (Figure 1[c] and 1[d]). By close comparison of Figure 1(c) with 1(d), it can be found that the polymer layer becomes thick with the decrease in the polarity of the porogens. The thick polymer layers, however, resulted in the decrease in permeability, which was proven by the curves of back pressure to flow rate (not shown). The ratio of 1:1:6 was selected in this work.

Total concentration of monomers affects morphology, mechanical stability, and permeability (22). Undoubtedly, high monomer concentrations lead to many polymers being formed. Four monoliths were prepared at the concentrations of 20%, 25%, 30%, and 35% (v/v), and the curves of back pressure to flow rate were plotted for them (not shown). Mechanical stability of the H-column prepared at the concentration of 20% was so poor that the monolith might be extruded out from the capillary at the pressures used for routine flushing. This was probably because the amount of forming polymers was too little to link the particles to the capillary inner wall strongly. At concentrations higher than 20%, the obtained H-columns could withstand a pressure up to 3500 psi, but permeability of the monoliths decreased gradually with the increase of the monomer concentration. The concentration of 25% was used for the preparation of the H-column.

Morphology and Mechanical Stability: In order to observe axial and radial uniformity, a 10-cm H-column was cut into three sections; six SEM images were taken and are shown in Figure 2. Five observations can be made. First, polymers formed on the particles rather than in the interstitial space around particles (Figure 2[d], 2[e], and 2[f]). Second, thickness of the polymer layer on particles was estimated to be no more than 1 µm (Figure 2[d], 2[e], and 2[f]). Third, the particles interconnected through the polymers, forming a monolith. Fourth, both axial and radial distributions of the particles were uniform. Fifth, there were no cracks between the monolith and the inner wall of the capillary (Figure 2[a], 2[b], and 2[c]). The mechanical stability of the H-column prepared under the selected conditions was evaluated based on the curve of flow rate to back pressure (not shown). With the increase in back pressure from 350 to 3600 psi, flow rate remained increasing linearly from 1.6 to 10.7 µL/min. The back pressure above 3500 psi was not tested because of the failure in connection between column and solvent tubing. The flow rate of 10.7 µL/min at 3500 psi should be sufficient for practical use in cLC.

Permeability and Swelling-Shrinking Behaviour: The permeability was evaluated with Darcy’s Law (26). The K value was measured to be 2.0 × 10⁻¹⁴ m². This value was comparable to that of 1.3 × 10⁻¹⁴ m² obtained with the packed column (27), demonstrating that there was no remarkable loss in permeability for the H-column. This was because the layer of the polymer was very thin and there were no polymers in the interstitial space.

Swelling-shrinking behaviour of a monolith affects its separation performance and lifetime (28). When swelling-shrinking behaviour of a monolith occurs, the size of its through pores will alter, and correspondingly the flow rate of mobile phase will change at a constant back pressure. Based on this principle, an experiment was designed and conducted to investigate the swelling-shrinking behaviour of the H-column. Acetonitrile was pumped through a H-column until swelling-shrinking equilibrium (for 12 h at least), and then water for 10 h at a fixed pressure of 600 psi. During the 10 h for water, the water flow rate was measured once per hour, and the curve of flow rate to time was plotted (not shown). The curve showed that the flow rate had nearly no change, indicating that no remarkable swelling-shrinking behaviour had arisen during the 10 h. This was principally owing to the silica particles, which were less swellable or shrinkable and accounted for a larger proportion in the column (above 65%) than the organic polymers in the H-column.
Hydrophilicity: The curves of retention factors ($k$) on acetonitrile content were plotted for toluene, acrylamide, and thiourea (Figure 3). With the increase in acetonitrile content, the $k$ values of nonpolar toluene and polar acrylamide and thiourea decreased and increased, respectively, showing the hydrophilic characteristics of the H-column. Lin et al. (9,28) suggest that hydrophilicity of a column can be measured using the acetonitrile concentration at the intercross point of the curves obtained with toluene and acrylamide as a critical value. The smaller the critical value, the higher the hydrophilicity of the column. The critical value of the H-column was about 40%, much lower than those (50–70%) reported for other hydrophilic columns (9,22,29). The high hydrophilicity of the H-column should be mostly ascribed to QAGs of the MATE monomers and hydroxyl groups of the PETA cross-linkers.

Column Efficiency: A van Deemter plot of the H-column was obtained by using Br$^-$ (not shown). The minimum of theoretical plate height ($H_{min}$) and the value of the $C$ term were about 10 µm and 9 ms, respectively. For the columns packed with 5 µm particles and silica-based monoliths, the reported values of $H_{min}$ were typically in the range of 5 to 10 µm (11,30). For some commercial silica-based monolithic columns and polymer-based monolithic columns, the reported values of the $C$ term are 10 and 70 ms on average, respectively (31). Therefore, the column efficiency of the H-column was comparable to those of the columns packed with 5 µm particles and silica-based monoliths (11,30), and much higher than those of polymer-based monolithic columns (31).

Preparation Reproducibility: Retention factor ($k$) and column efficiency ($H$) were measured to evaluate preparation reproducibility of the H-column using Br$^-$. Nine H-columns (5.0-cm long) were prepared in three batches (three columns per batch). The column-to-column relative standard deviations (RSDs) were 2.2% and 3.5% ($n = 9$) and the batch-to-batch RSDs were 2.4% and 5.5% ($n = 3$) for $k$ and $H$ values, respectively. The preparation reproducibility of the H-column was comparable to that reported by Wang et al. (13) for a SAX column.

Applications: Figure 4(a) shows three monovalent inorganic anions, including bromate, bromide, and nitrate, that were baseline separated on a 2.5-cm H-column within 2.5 min and with good peak symmetry. Evenhuis et al. (7) also separated the three anions using a 5.0-cm commercial polymethacrylate-based monolithic SAX column within 8 min.

It is difficult to separate P, M, O-hydroxybenzoic acids due to the similarity in structure. As illustrated in Figure 4(b), the three isomers were baseline separated on a 2.5-cm H-column in less than 4 min based on a mixed mechanism of ion exchange and hydrophilic interaction. Ayaz et al. (8) also separated the three isomers using a reversed-phase column based on hydrophobic interaction, but retention times were more than 30 min.
A new hydrophilic SAX monolithic column was exhibited a remarkable performance in fast exchange and hydrophilic interaction. Overcame the serious swelling-shrinking problem with the polymer-based monolithic column. The column is a significant complement to SAX monolithic columns, and has potential applications in the analysis of some acidic drugs and two-dimensional separations of a proteome.

**Conclusion**

A new hydrophilic SAX monolithic column was prepared by in-capillary coating 5 μm bare silica particles with poly (MATE-co-PETA). This column can be considered to be a hybrid of silica particles-packed and polymer-based monolithic columns, and thus combines the typical merits of them both. The column overcame the serious swelling-shrinking problem with the polymer-based monolithic SAX columns reported previously, and exhibited a remarkable performance in fast separations of inorganic anions, organic weak acids, phenols, and nucleotides. This column is a significant complement to SAX monolithic columns, and has potential applications in the analysis of some acidic drugs and two-dimensional separations of a proteome.

**References**

Some liquid chromatography (LC) troubleshooting topics never get old, because there are some problems that persist in the practice of LC, even as instrument technology improves over time. There are many ways for things to go wrong in an LC system that ultimately manifest as peak shapes that are not good. Developing a short list of the likely causes of these results can help streamline our troubleshooting experience when peak shape-related problems occur.

Writing this “LC Troubleshooting” column and thinking about topics each month is interesting in the sense that there are some topics that just never get old. Whereas in the chromatography research world certain topics or ideas become obsolete as they are displaced by newer and better ideas, in the troubleshooting world there are certain topics that have remained relevant since the very first troubleshooting article appeared in this magazine (LC Magazine at that time) in 1983 (1). Over the last few years, I’ve focused several “LC Troubleshooting” instalments on contemporary trends (for example, the relatively recent advances in our understanding of the effects of pressure on retention [2]) in liquid chromatography (LC) that are affecting the way we approach our interpretation of LC results, and approach troubleshooting with modern LC instruments. With this month’s instalment, I am continuing a series I started in January 2022 (3) that is focused on some of the “bread and butter” topics of LC troubleshooting—those elements that are essential for any troubleshooter, no matter the vintage of the system we are working with.

The topics at the heart of this series are highly related to LCGC's well-known “Guide to LC Troubleshooting” wall chart (4) that hangs in many laboratories. For the third instalment in this series, I’ve chosen to focus on problems related to peak shape, or perhaps peak characteristics. Incredibly, the wall chart lists 44 different potential causes of bad peak shapes! We won’t be able to consider all of these in any detail in a single article, so in this first instalment on this topic, I focus on some of the problems that I see most frequently. I hope LC users young and old will find some useful tips and reminders related to this important topic.

Everything is Possible

More and more, I find myself responding to troubleshooting questions with “Everything is possible”. This response might seem like an easy out when considering observations that are hard to explain, but I find that this response is appropriate more often than not. With many possible causes of poor peak shape, it is important to keep an open mind when considering what might be the problem, and it is important to be able to prioritize the potential causes to start our troubleshooting effort, focusing on those possibilities that are most likely.

What Is to Be Expected?

A critical step in any troubleshooting exercise—but one that I think is underappreciated—is recognizing that there is a problem to be solved. Recognizing that there is a problem usually amounts to recognizing that what is happening with the instrument is different from our expectations, which are formed from theories, empirical knowledge, and experience (5). By “peak shape”, we are really referring here to not only the shape of the peak (symmetric, asymmetric, smooth, shaggy, fronting, tailing, and so on) but also to the width. Our expectation regarding the actual peak shape is straightforward. The textbook expectation, which is well supported by theory (6), is that, under most conditions, chromatographic peaks should be symmetric and consistent with the shape of a Gaussian distribution, as shown in Figure 1(a). Our expectations for the peak width is a more complicated matter, and we’ll deal with that topic in a future instalment. The other peak shapes
in Figure 1 show some of the other possibilities that can be observed—in other words, some of the ways that things can go wrong. In the rest of this instalment, we take the time to discuss some specific examples of situations that can lead to these types of shapes.

**No Peak**

Sometimes peaks are simply not observed where they are expected to be eluted in the chromatogram. The aforementioned wall chart suggests that the absence of peaks (assuming the sample actually contains the analyte of interest at a concentration that should give a detector response sufficient to see it above the noise) is usually related to some instrumental problem, or mobile phase conditions that are incorrect (typically too “weak”, if peaks are observed at all). A short list of potential problems and solutions in this category can be found in Table 1.

**Too Broad**

As stated above, the question of how much peak broadening one should tolerate before getting concerned and trying to troubleshoot the problem is a complex topic that I will address in a future instalment. My experience has been that significant peak broadening is usually accompanied by a significant change in the peak shape, and more often peak tailing than fronting or splitting. Nevertheless, broadening of the peak that is nominally symmetrical can also occur, and could be caused by a handful of different reasons:

- “Volume overload”, where a large volume of injected sample can cause the peak to broaden (7);
- Data acquisition rate that is too slow (8);
- Connecting components of the system (for example, column and detector) with tubing that is too long or too large in diameter (9).

**TABLE 1: Potential problems leading to no peaks observed**

<table>
<thead>
<tr>
<th>Incorrect Mobile Phase</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty “B solvent” bottle (for example, organic solvent in reversed-phase, or high salt in ion-exchange)</td>
<td>Refill solvent bottle and purge pump</td>
</tr>
<tr>
<td>Malfunctioning solvent proportioning valve (that is, low-pressure mixing in ternary pump designs)</td>
<td>Run diagnostic test using instrument software to determine if the valve is failing; if it is, replace the valve</td>
</tr>
<tr>
<td>Malfunctioning check valve in pump (that is, high-pressure mixing in binary pump designs)</td>
<td>Run diagnostic test using instrument software to measure the mobile phase composition accuracy; if it fails the test, troubleshoot to determine which pump head is producing the problem</td>
</tr>
<tr>
<td>Mobile phase prepared incorrectly by the operator</td>
<td>Prepare and try a new batch of mobile phase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Instrumental Problems</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partially blocked autosampler needle, such that sample is not withdrawn by the syringe from the sample vial</td>
<td>Check pressure drop across needle; replace needle</td>
</tr>
<tr>
<td>Liquid level too low in the vial, such that the needle cannot reach it</td>
<td>Add more sample to vial; if sample is limited, use low volume vial inserts, or vial “bottom-sensing” capabilities of sampler</td>
</tr>
</tbody>
</table>
Each of these problems has been discussed in detail in prior instalments of “LC Troubleshooting”, and readers interested in those topics are referred to those articles for more detail on the root causes and potential solutions to these problems.

**Tailing**

Peak tailing, fronting, and splitting can each be caused by chemical or physical phenomena, and the list of potential solutions for each of these problems differs greatly, depending on whether we are dealing with a chemical or physical problem. Often, an important clue about which one is the culprit can be found by comparing different peaks in a chromatogram. If all peaks in a chromatogram exhibit similar shapes, then the cause is more likely than not to have a physical origin. If only one or a couple of peaks are affected, but the rest look good, then it is more likely than not that the cause is chemical in nature.

The chemical causes of peak tailing are too complex to discuss in a short section here. Interested readers are referred to a recent instalment of “LC Troubleshooting” for a more thorough discussion (10). However, one easy thing to try is to decrease the mass of analyte injected and see if the peak shape improves. If it does, then this is a good clue that the problem is “mass overload”. In this situation, the method has to be limited to injecting less analyte mass, or the chromatographic conditions must be changed so that good peak shapes can be obtained even when injecting larger masses.

The potential physical causes of peak tailing are also numerous; readers interested in a detailed discussion of the possibilities are referred to another recent instalment of “LC Troubleshooting” (11). One of the more common physical causes of peak tailing is a bad connection at some point between the sample injector and the detector (12). An extreme example of this is shown in Figure 1(c), which was acquired in my laboratory a couple of weeks ago. In this case, we set up a system with a new injection valve we had not used previously, and installed a small volume injection loop that had ferrules already swaged onto the stainless steel capillary. After a few initial troubleshooting experiments, we realized that the port depth in the stator of the injection valve was much deeper than we are accustomed to, resulting in a large dead volume at the bottom of the port. This issue was easily...
resolved by replacing the injection loop with a different piece of tubing where we could adjust the ferrule to the proper position to eliminate the dead volume at the bottom of the port.

**Fronting**

Peak fronting like that shown in Figure 1(e) can also result from physical or chemical problems. A common physical cause of fronting is channelling in the particle bed of a column that is not well packed, or where the particles have reorganized over time. As with peak tailing, which is caused by this kind of physical phenomenon, the best solution for this problem is to replace the column and move on.

Fundamentally, a fronting peak shape that has a chemical origin usually results from what we refer to as “nonlinear” retention conditions. Under ideal (linear) conditions, the amount of analyte retained by the stationary phase (and thus, the retention factor) is linearly related to the concentration of the analyte in the column. Chromatographically, this means that when the mass of analyte injected into the column is increased, the peak gets taller, but not wider. When retention behaviour is nonlinear, this relationship is broken, and when more mass is injected, the peak not only gets taller but also wider. Furthermore, the shape of the nonlinearity determines the shape of the resulting chromatographic peaks, leading to either fronting or tailing. As with mass overload that leads to peak tailing (10), peak fronting caused by nonlinear retention can also be diagnosed by reducing the mass of analyte injected. If the peak shape improves, then either the method has to be modified to not exceed the injected mass that leads to fronting, or the chromatographic conditions have to be changed to minimize this behaviour.

**Splitting and Shouldering**

Sometimes we observe what appears to be a “split” peak, like that in Figure 1(f). The first step to solving this problem is to determine whether this peak shape is due to partial coelution (that is, the presence of two different but closely eluted compounds) or not. If there are actually two different analytes eluting close together, then this is a problem of increasing their resolution (for example, by increasing selectivity, retention, or plate number), and the apparently “split” peak has nothing to do with the physical performance of the column per se. Usually, the most important clue for making this determination is whether or not all peaks in the chromatogram exhibit the splitting shapes, or just one or two. If it is just one or two, then it is probably a coelution problem; if all peaks are split, then it is probably a physical problem, most likely with the column itself.

Split peaks that are related to the physical performance of the column itself are most often because of a partially occluded inlet or outlet frit, or a reorganization of particles in the column such that channels form where mobile phase flows faster in some regions of the column than in other regions (11). A partially occluded frit can sometimes be cleared by reversing the flow through the column; however, in my experience, this is usually a short-term rather than long-term solution. If the particles have reorganized within the column, this is usually lethal with modern columns. At this point, it is best to replace the column and move on.

**“Fuzzy”, “Shaggy”, “Spiky”, or “Flat-Topped” Peaks**

The peak in Figure 1(g), again from a recent instance in my own laboratory, most often indicates that the signal is so high that the high end of the response range has been reached. In the case of an optical absorbance detector (UV–vis, in this case), when the analyte concentration is very high, the analyte absorbs most of the light going through the detector flow cell, leaving very little light to be detected. Under these conditions, the electrical signal from the light detector is heavily influenced by a variety of noise sources (for example, stray light and “dark currents”), such that the signal becomes very “fuzzy” in appearance, and independent of analyte concentration. When this occurs, the problem can usually be easily fixed by injecting less analyte—either by reducing the injection volume, diluting the sample, or both.

**“Negative” Peaks**

In chromatography school, we talk about the detector signal (that is, the y-axis in chromatograms) as an indicator of analyte concentration in the sample. Thus, it seems really weird to see a chromatogram where the signal goes below zero, as the simple interpretation would be that this indicates a negative analyte concentration—which, of course, is not physically possible. In my experience, negative peaks are most commonly observed when using optical absorbance detectors (for example, UV–vis).

In this context, a negative peak simply indicates that the molecules eluting from the column absorb less light than the mobile phase itself immediately before and after the peak. For example, this could happen when using a relatively low detection wavelength (<230 nm) and mobile phase additives that absorb a significant fraction of light at these wavelengths. Such additives could be mobile phase solvent components (such...
as methanol) or buffer constituents (such as acetate or formate). One can actually prepare calibration curves and obtain accurate quantitative information using negative peaks, so there is no fundamental reason to avoid them per se (this approach is sometimes referred to as indirect UV detection) (13). However, if we really want to avoid negative peaks altogether, then the best solutions in the case of absorbance detection are to either use a different detection wavelength where the analyte absorbs more than the mobile phase, or change the constituents of the mobile phase so they absorb less light than the analyte.

Negative peaks can also occur when using refractive index (RI) detection when the components of the sample other than the analyte (for example, the solvent matrix) have a refractive index different from the refractive index of the mobile phase. This can also occur when using UV–vis detection, but the effect tends to be muted relative to RI detection. In both cases, negative peaks can be minimized by more closely matching the composition of the sample matrix to the composition of the mobile phase.

Summary
In this third instalment on essential topics in LC troubleshooting, I have discussed situations where the observed peak shape is somehow different from what is expected or normal. Effective troubleshooting for this type of problem begins with a sense for what the expected peak shape is (based on theory, or prior experience with an existing method), so that a deviation from those expectations is noticeable.

There are many different potential causes of peak shape problems (too broad, tailing, fronting, and so forth). In this instalment, I discussed some of the causes I see most frequently in some detail. Understanding these details provides a good place to start troubleshooting, but does not capture all possibilities. Readers interested in learning about a deeper list of causes and solutions are referred to the LCGC “Guide to LC Troubleshooting” wallchart.

References
Getting “Under the Hood” of Gas Chromatography: Lessons from the 2022 GC ChromTalks

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The 2022 gas chromatography ChromTalks, sponsored by LCGC and ChromAcademy brought together six world-renowned gas chromatography experts who discussed some of the most important lessons in troubleshooting and “getting under the hood” of gas chromatography (GC). In this instalment, we re-visit the 2022 ChromTalks, discussing the most important troubleshooting lessons from the half-day symposium.

Speakers at the 2022 gas chromatography ChromTalks, sponsored by LCGC and ChromAcademy, brought practical advice for maintaining, optimizing, and troubleshooting sampling, inlets, detectors, data handling, and documentation. They discussed key insights on how to get the most out of gas chromatography (GC) for the most difficult problem solving, the best quantitative analysis, and the highest data integrity (1).

I led off the symposium with a discussion of some hidden errors in sampling and sample preparation, especially in glassware handling, that befall all of our methods, which was discussed in a previous instalment of this column (2). Our first two lessons are discussed in this article.

Lesson 1: Volumetric glassware is not perfect. It has finite uncertainty that must be considered in the development of even the simplest methods.

Lesson 2: Be careful of basic statistics and significant figures when reporting results. We often over-report significant figures and under-report experimental uncertainty, making our results appear better than they really are.

Now, we discuss highlights from the other five speakers’ talks, and provide information on how you can access them online. In my opinion, this was one of the strongest line-ups of gas chromatographers in one symposium in recent memory. There was far more takeaway information than we can discuss here, so please access the full session on-demand at ChromAcademy, LCGC’s online educational portal, or at chromatographyonline.com.

Jaap de Zeeuw of Creavision, an independent consulting firm, and a long-time column and instrumentation wizard with Varian and Restek, discussed “The 10 Most Important Maintenance Points You Need to Know About Your Split/Splitless Injection System”. Hans-Gerd Janssen of Unilever and Vlaardingen University then discussed “Sample Introduction Methods for Liquid Samples in GC: Classical Volumes and Large Volumes”. Matthew Klee of XO Associates, a long-time GC leader at Agilent Technologies, presented “Optimizing the Inlet-to-Column Transition in Gas Chromatography”. Mary Ellen McNally of FMC Corporation followed with “Detecting It All—Keeping Your Response Consistent with Common GC Detectors”. Finally, Gregory Slack of Boston Analytical discussed “Qualifying and Optimizing GC Instruments and Methods in High-Throughput Regulated Laboratories”. The titles alone provide some insight into the most common trouble points in GC: inlets, injection, detectors, data handling, and documentation.

A follow-up session at ChromTalks included talks from vendor scientists including Agilent Technologies, CEM/Lucidity, LECO, and Scion Instruments.

Lesson 3: The inlet is the most difficult part of the instrument to understand and optimize. Jaap de Zeeuw offered over 10 practical tips for optimizing and caring for split/splitless inlets, some of which are highlighted here.

Lesson 4: Take care with the septum and ferrules. Pre-seal ferrules onto the column prior to installation to ensure a secure leak-free seal. Ferrules should be tightened, but not too tight, using modern finger-tight fittings and...
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Lesson 7: “If the column is described as the heart of the chromatographic system, than sample introduction is the Achilles’ heel.”—Pretorius and Bertsch 1983 (4). Hans Gerd Janssen and co-author Sjaak de Koning discussed sample introduction for liquids using classical and large volumes. There is a wide range of injection techniques available; how do we select one? The main factors to consider include sample concentration, stability, boiling point of solvent and analytes, polarity of the solvent, and injection volume. A decision tree to assist in making the best choice of injection technique was provided in the presentation.

Lesson 8: Understand the impact of solvent and stationary phase focusing on splitless injections. Peak focusing is very important in sample introduction; we can only get narrow peaks at the end of the column if we introduce narrow peaks at the beginning of the column. Whatever injection technique is used, think about how the analytes are focused at the column head. In stationary phase focusing, bands are focused by interaction with the stationary phase, and is inherent in temperature programming. The cool temperature at the column head traps analyte bands near the head of the column. The second mechanism, solvent focusing, works for volatile analytes. After the injection, a band of liquid solvent is created at the column inlet. As the solvent band evaporates, analytes are concentrated until the solvent band fully evaporates. In a classical split injection, transfer through the inlet is very rapid, so peaks enter the column already focused. Classical splitless injection is a much slower process, requiring peak focusing, either by solvent or stationary phase focusing at the beginning of the run. Hans-Gerd presented several guidelines for splitless injections, including purge off time, initial oven temperature (keep it 20 degrees or more below the pressure corrected solvent boiling point), and avoiding sample discrimination.

Lesson 9: Try cold injection techniques. While split and splitless injections are most widely used, programmed temperature vaporization (PTV) injection combines the benefits of split, splitless, and on-column inlets. In short, the sample is injected cold as liquid into the liner, and analytes are transferred into the column by rapidly heating the inlet. There are three modes: cold split, cold splitless, and solvent elimination, with solvent...
elimination being the most useful. In solvent elimination, the syringe to liner and liner to column transfers are decoupled. Janssen provided an overview of how solvent elimination PTV injection can be used to inject both traditional volumes of liquid volumes and large volumes up to hundreds of microlitres. An overview of cold injection techniques with references was provided in a recent “GC Connections” column (5).

Lesson 10: Representative sampling is critical if the full sample is to be analyzed, as in untargeted analysis. It is less critical in targeted analysis. Matthew Klee discussed the inlet to column transition, including representative sampling, discrimination, droplet formation in the column, liner versus column decomposition, and maintenance principles. Regardless of the inlet or detector system, representative sampling is critical if the sample components must be fully characterized, as in untargeted analysis. Representative sampling is not always needed in targeted analysis. To ensure representative injection, install the insulation cup in the oven under the inlet in the Agilent GC system. The inlet temperature causes discrimination that results in nonrepresentative sampling, with sources including pressure surge or liner overload causing loss of volatile components and too low temperature, which causes loss of less volatile components. Splitless purge time also influences discrimination, with less volatile components requiring longer purge time.

Lesson 11: Use a tapered liner with glass wool for most liquid injections. While simple straight liners were recommended in the past, for simplicity, today, a liner with glass wool at the top and a taper at the bottom is recommended for most liquid injections. Wool wipes the needle and prevents septum pieces from falling further into the liner. This helps reduce inlet degradation, which can cause peak splitting or poor peak shapes. Related, column degradation will generally cause a broad increase in the baseline. If both are present, a “bridge” between two peaks might be seen. Split peaks also often result from sample condensation in the column; use of a retention gap or uncoated precolumn can ensure that a stable uniform solvent film is formed in a splitless injection. Split peaks commonly result from a mismatch in polarity between the solvent and the stationary phase. Finally, have a routine maintenance schedule that includes septum, liner, baseplate, and seal replacement, column trimming and installation, and solvent vent trap replacement.

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Lesson 12: Detectors are not just a “black box” at the end of the column. Mary Ellen McNally discussed detectors, with a focus on the fundamentals of how common and selective detectors operate, as well as principles common to detection. Some key practical considerations for all detectors include:

- Avoid temperature fluctuations when placing the instrument. Avoid air conditioning ducts, drafts, vents, and poorly insulated walls. Detectors are very sensitive. Also, allow time for the detector temperature to equilibrate when changed or cooled for maintenance.
- Watch for gas leaks. As in the inlet discussion, gas leaks can seriously degrade performance; for example, diffusion of air into a thermal conductivity detector (TCD) is particularly bad, since air has a different conductivity than helium or nitrogen. Use an electronic leak detector to ensure all connections are leak-free, and ensure that carrier gas is pure. Carrier gas impurities will cause both elevated baseline and noise.
- Disconnect the column from the detector during column conditioning. This prevents eluents from the conditioning from contaminating the detector.
- Bring the column oven to operating temperature before turning on the detector to prevent condensation build-up in the detector, since the oven heats more rapidly.
- Keep all side panels and covers in place on the instrument during operation to maintain overall thermal stability.
- Scrubbers and moisture traps are recommended to remove contaminants from detector gases, as well as carrier gases.
- Leave detector running at operating temperature overnight and on weekends, to ensure equilibration.

Lesson 13: Ensure that the detector is clean and equilibrated. Keep it running overnight. In short, for the best detector operation, think cleanliness and equilibration. The detector and system should be contaminant-free, and given time to equilibrate before use and between runs.

Lesson 14: Qualify your instrument at installation, for performance at installation, and ongoing, as according to standard operating procedure. Gregory Slack closed the session by providing a big-picture overview of challenges in optimizing and qualifying gas chromatographs in high-throughout labs. Instrument qualification requires four steps: design qualification, installation qualification, performance qualification, and ongoing calibration and maintenance. Greg emphasized the need for standard operating procedures, and documented processes for each of these steps. He recommends using real samples for ongoing qualification.

Lesson 15: Ensure that methods are fully documented, and that all analysts follow them. Greg also provided several examples of investigations and FDA inspection results. A common theme in the inspection results was that written methods and standard operating procedures were either incomplete or not followed. In regulated laboratories, once instruments are qualified, the methods that run on them must be validated, according to regulatory guidance and internal standard operating procedures. A broad audit finding might indicate a problem such as the assay method for the active pharmaceutical ingredient lacking a specificity determination. A more detailed finding might indicate a problem such as the use of a 10.0 μL syringe for a method requiring delivery of 0.2 μL of liquid. The finding might then ask for supportive data to prove that the 10.0 μL syringe can accurately and precisely deliver the 0.2 μL of liquid. This finding provides an appropriate close to this column. While it is possible that the 10 μL syringe can precisely deliver a given volume, it cannot accurately deliver 0.2 μL given the needle volume alone of approximately 0.5 μL. Our previous speakers discussed sampling and injection; in this case, an injection problem becomes evident in a regulatory inspection. Greg also provided several real-world troubleshooting investigation examples. Interestingly, and independently of the other speakers, he discussed a real-world example involving a poor choice of injection liner.

Conclusion
I was honoured to host and speak in the 2022 GC ChromTalks “Getting Under the Hood of Gas Chromatography”. These brief summaries only give us a quick look. I invite you to watch these presentations online, they are available on demand, at chromatographyonline.com or at LCGC’s ChromAcademy, to get a more detailed look “under the hood”.

References

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Highlights from the 50th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2022)

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After three “lost” years, the 50th International Symposium on High Performance Liquid Phase Separations and Related Techniques, or HPLC 2022, returned. The symposium was held at the Town and Country Hotel and Conference Center in sunny San Diego, California, USA, from 18–23 June 2022. The HPLC symposium continues to be the premier event bringing together leading scientists in the field of liquid chromatography (LC) and related techniques. The conference was chaired by Frantisek Svec of Charles University and, especially under the uncertain times leading up to the event, was very successful and welcomed by the attendees.

In this instalment of “Column Watch”, we report on the observed highlights and trends from the HPLC 2022 conference. In a similar fashion to the previous HPLC symposium review articles (1–3), many colleagues in attendance at the symposium were asked for their insights regarding the most interesting topics they observed at the event. Figure 1 attempts to capture the major topics of interest as determined by attendees in an infographic gleaned from these responses, and what follows is a synopsis of highlighted topics along with some personal views.

Column and System Technologies
The topic of column technologies immediately took centre stage with the opening plenary lecture by Gert Desmet of Vrije Universiteit Brussel called, “Technological (R)evolutions in Column Making: Quo Vadis Chromatography?” Desmet provided a historical review of column development and highlighted the concept of Moore’s Law and its pertinence to developments in column technology. The “law” predicts that there will be a certain steady rate of gain in a given area of development until something revolutionary happens to change that rate of gain. Desmet went on to demonstrate how measurements of performance, such as column efficiency, have followed this law pretty well since the 1980s (see Figure 2), and asked the question, “how do we break out of it?” The advantages, disadvantages, and potential for some emerging technologies intended to create highly ordered structures such as three-dimensional (3D)-printing, micro-machining, and micro-groove columns were discussed and contrasted. It was interesting to learn that the 3D-printing technology has developed enough to create the microstructures necessary for the creation of chromatographic devices, but the time to process is still extraordinarily long and materials amenable to printing are very limited.

In a plenary lecture entitled “3D-Printed Microfluidics for Solid-Phase Extraction and Biomolecule Separation,” Adam Woolley (Brigham Young University) discussed modern tools in 3D printing to create microfluidic devices for solid-phase extraction (SPE) and biomolecule separations. By utilizing such a device, Woolley’s group developed a reliable assay for monitoring protein biomarkers for pre-term birth. In the future, such a small device could be amenable to rapid detection in the clinic in real time; thus, it demonstrates an area of promise for 3D printing. In addition to the Woolley lecture, there was an excellent short course put on by Woolley and Greg Nordin (Brigham Young University) as well as a focus session within the programme featuring noted talks by Nordin and Rosanne Guijt (Deakin University). Although the 3D-printed
liquid chromatography (LC) column may be a long way away, 3D printing is certainly gaining ground in the area of microfluidics and integrated fluidic devices.

In addition to 3D printing, there were several other prominent column and system technologies that stood out. These include an increase in the presence and number of portable LC systems, continued advances in microscale separations, and the current trend towards bio-inert or metal-passivated columns and systems.

**Portable Instrumentation:** Prominent at the symposium was the concept of miniaturized and portable LC instrumentation. Several talks were given, including “A Modular Approach to a Portable Analytical Separation System for Pharmaceutical Analysis” by Llewellyn Coates; “Taking the Chromatograph Out of the Lab: High and Low Pressure Field Deployable Instruments for Environmental Applications” by Brett Paull; “Evolving Capabilities of Compact, Portable Capillary LC” by Milton Lee; “Analysis of Small Molecules in Complex Mixtures using Compact LC–UV-MS Instrumentation” by Samuel Foster; and “Implementation of Compact Capillary LC into Analytical Workflows” by James Grinias.

The latter presenter noted in his talk that portability affords “laboratory-to-sample” rather than “sample-to-laboratory”, which could be utilized in improving point-of-care and many environmental analyses. The reduced solvent and power consumption also leads to a lower overall cost as well as moving towards a more “green analytical chemistry” approach. Grinias demonstrated several applications where the portable LC systems were fit for purpose.

The upward trend in the development of portable instruments was also apparent on the exhibition floor with Axcend, Trajan, and CEM (Lucidity) all promoting their latest high performance LC (HPLC) systems.

**Microscale Separations:** Robert Kennedy of the University of Michigan presented “Capillary LC at 35,000 psi and Beyond for...
Complex Mixture Analysis”, a talk centred on using elevated pressure, combined with capillary LC–mass spectrometry (MS), to analyze complex mixtures. Kennedy’s group employed longer columns (20–100 cm) in length running at pressures of 35,000 psi to separate lipid and metabolite mixtures. With 1-m long columns, he demonstrated a peak capacity of ~1000 in 3-h analysis times, suitable for complex mixtures. When employing a novel 1.1 µm particle in this format, analysis times were reduced to 1/3 of those achieved by a more conventional 1.7 µm particle in a 15-cm long column at 15,000 psi. Taking this aspect one step further, Kennedy used two-dimensional (2D-) LC (hydrophilic-interaction chromatography [HILIC] and reversed-phase liquid chromatography) to analyze the lipid complement of plasma to yield a peak capacity of 1900 in a MS-compatible format (see Figure 3). He also reported packing HILIC particles into 75 cm columns at 35,000 psi to yield 213,000 plate separations.

In his talk entitled “Nanoscale Separations to Enable Bottom-up, Top-down and Native Proteomic Profiling of ng- and Sub-ng-level Samples”, Alexander Ivanov (Northeastern University) employed nano-flow methodologies with narrow-bore monolith columns to achieve sensitive results for analyzing sample-limited protein mixtures in a proteomics workflow. The use of low flow solvent delivery system (12 nL/min) was key to achieving the sensitivity needed in very low abundant protein biomarkers in the clinical specimen.

The need for small sample analyses (for example, in single cells) is proving the need for very narrow columns. Examples
were also shown by Ryan Kelly of Brigham Young University in his talk, “Miniaturized LC Separations for Single-Cell Proteomics”. The need for reduced dilution and high resolution is, and will be, key for small samples. The technique also appears to be moving closer to clinical applications as exemplified later in this article under the “Highlighted Areas of Application” section.

Bio-Inert Systems and Columns:
The topic of bio-inert or metal-passivated systems and columns was prevalent at HPLC 2022. As noted in the LCGC 2022 review of new LC columns and accessories (4), column and system hardware with various metal passivation strategies have been on the rise recently. David McCalley (University of the West of England, Bristol) kicked off the discussion with a highly cited, Monday morning keynote lecture entitled, “Managing Deleterious Solute Interactions with Metals in Hydrophilic Interaction Chromatography”. McCalley noted that interactions of analytes such as nucleotides and catecholamines with metal surfaces can occur, and there are several ways one may mitigate these undesirable interactions: (1) use columns/systems constructed from metals other than stainless steel; (2) use PEEK- or PTFE-coated systems; (3) use systems and columns deactivated by vapour-deposition of some “inert” material; or (4) use complexing agents in the mobile phase. Although the focus of the work was geared towards the favourable impact of vapour-deposition deactivated columns and systems, the additional use of high pH as well as low-level complexing agents in the mobile phase provided the best overall separation of the adenosine phosphate probes employed.

In addition to several posters, many vendors were demonstrating their various approaches to bio-inert systems and columns. ThermoFisher displayed their Vanquish systems that replace stainless steel with titanium or MP35N components. Shimadzu showcased Nexera XS Inert, their bio-inert system that utilizes PEEK-lined stainless steel to offer a metal-free flow path. Waters delivered numerous talks and presented quite a few posters on their Premier systems that utilize chemical vapour deposition of hybrid silica to block metal interactions. Agilent also had their suite of biosystems, which were either metal-free (PEEK) or utilize alternative metal alloys labelled as “iron-free”. Silcotek was also present and showcased their chemical vapour deposition solutions and how their product performance is on par with PEEK when it comes to keeping LC flow paths metal-free.

Multidimensional Chromatography:
Multidimensional chromatography was a large part of the show, with two dedicated sessions as well as many lectures utilizing various multidimensional techniques.
throughout the conference. Peilin Yang (Dow Chemical) presented an excellent lecture entitled, “Multidimensional Separations with Simultaneous Evaporative Light Scattering Detection (ELSD) and MS Detections for Complex Industrial Polymers”, where she showed examples of how comprehensive 2D-LC can be a powerful tool for polymer analysis. Yang focused her examples on industrial applications such as terpolymer composition heterogeneity, tri-block polymeric impurities, and nonionic copolymer surfactants analysis. Combinations of size-exclusion chromatography (SEC) and reversed-phase LC, as well as HILIC and reversed-phase LC, were highlighted along with the intelligent use of various detectors based on information needs. Unfortunately, little detail on any additional multidimensional talks and topics were available at the time of writing. However, it is interesting to note that attendee thoughts on the adoption of multidimensional chromatography in the industry varies widely—some indicate a clear adoption whereas others feel this is still solidly in the hands of academics.

**Separation Modes**

Several separation modes were considered dominant in their presence this year by conference attendees. Notably supercritical fluid chromatography (SFC), HILIC, and multidimensional separations seemed to take centre stage.

**SFC:** A number of colleagues expressed surprise at the number of presentations focusing on SFC, and many were perplexed by the lack of adoption of SFC in general practice. Some also expressed the sentiment that perhaps SFC is finally breaking through towards acceptance. As for the conference, there were presentations on SFC fundamentals, practical tips and tricks, and, as noted in the forthcoming “Highlighted Areas of Application” section, important real-world applications in cancer diagnosis.

In his talk entitled, “Measurement and Modelling of Longitudinal Diffusion in Supercritical Fluid Chromatography”, Ken Broechkoven of Vrije Universiteit Brussel measured both molecular diffusion and longitudinal diffusion (that is, the $B$-term) in SFC, which allowed the authors to model particle diffusion and surface diffusion, possibly for the first time, in SFC. In another notable talk, Chun-Kai Chang of the University of Minnesota used molecular simulations of the mobile phase composition stationary phase pores to better understand retention mechanisms in SFC in the talk entitled, “Exploration of the Retention Mechanism in Supercritical Fluid Chromatography by Molecular Simulations”. Although they only showed results for C18 surfaces, this work might shed some light on the rather poorly understood retention mechanisms in SFC.

Attila Felinger of the University of Pecs discussed the adoption of methanol on SFC stationary phases in his talk entitled, “Solvent Interactions in Supercritical Fluid Chromatography”. Lucie Novakova of Charles University (“Problems of Stationary Phases in SFC”) followed this up by providing highly practical evidence on how the history of using a column in SFC can strongly affect separation. Columns exposed to additives and organic modifiers may be chemically changed so as to perform differently than the original or a new column. Novakova suggested dedicating columns to certain methods because of this, but also noted that the change is not necessarily permanent. The group is running a large amount of experiments to test a wide range of columns and conditions to find adequate procedures to better understand these findings.

SFC was also noted for its potential as a more “green” technique compared to HPLC. Stefan Lamotte (from BASF) provided a keynote lecture entitled, “The Good, the Bad, and the Ugly - Towards the Separation of Polar Analytes in Supercritical Fluid Chromatography”, where he provided comparisons of SFC with HPLC and HILIC as well as examples of important SFC separations. Lamotte noted that SFC has several real advantages over HPLC including speed of analysis, but noted solubility issues in SFC may need to be overcome. In a post-conference communication, Lamotte added that the chemical industry is clearly being asked to minimize waste and develop sustainable methodologies, and his efforts are clearly moving in the direction of SFC.

**HILIC:** HILIC was a hot topic of discussion. The topic garnered its own session, and there were several additional HILIC-based talks throughout the conference. As mentioned previously, David McCalley’s talk on the impact of metal passivation in HILIC was a noted highlight. Brady Anderson (University of Michigan) presented a well-received lecture on the power of HILIC at pressures up to 50,000 psi. Thomas Walter and Jinchuan Yang from Waters Corporation gave excellent talks on characterizing and using a zwitterionic HILIC phase, respectively. Joe Pesek from San Jose State University discussed method development for hydrophilic compounds using aqueous normal phase (ANP) chromatography and contrasted this to HILIC. Although the modes employ similar mobile phase compositions, ANP uses silica hydrate-based stationary phases as opposed to more common silica-based chemistries (see Figure 4). Pesek noted that this silica hydrate surface exhibits less than one-half a monolayer of water, whereas silica-based columns can have between 4–10 monolayers under similar mobile phase conditions. It is this difference that Pesek surmises results in differences in retention mechanisms (dominant adsorption in ANP compared to dominant.
partition in HILIC) and other attributes such as fast equilibration. Separation examples are then provided for several important classes of polar analytes.

**Emerging Topics and Trends**

**Pressure-Enhanced-Liquid Chromatography (PE–LC):** Many colleagues noted Szabolcs Fekete’s talk on how pressure can be used as an additional and unique parameter to tune the selectivity of large molecules in LC as an interesting highlight of the conference. Fekete introduced pressure as a potentially important parameter for selectivity tuning, especially for large molecules, as pressure may impact analyte tertiary structure. Equilibria associated with separation processes such as ionization, adsorption, partition, and complexation are also pressure-dependent. Fekete also noted that pressure may also impact the stationary phase structure and thus the interactions available for retention and selectivity. A system utilizing a pressure-regulating fluid interface was described and utilized to test the retention and selectivity dependence of a set of large molecules. PE–LC was shown to provide unique separations and be applicable to large molecules. PE–LC also showed efficacy in several modes of chromatography including reversed-phase LC, ion-exchange, hydrophobic interaction chromatography (HIC), and ion-pair reversed-phase LC.

**Machine Learning and Molecular Dynamics Simulations:** Many attendees noted the increased interest and prevalence of machine learning and molecular dynamics as tools for improving our understanding of chromatographic principles. Two talks on molecular dynamic simulations and model reconstruction of porous media to predict chromatographic separations were given by Ulrich Tallarek from Philipps-Universität Marburg and Fabrice Gritti from Waters Corporation. Gritti contrasted the molecular dynamics approach to more traditional modelling methods such as empirical modelling and rate theory measurements (both with limitations). Although the molecular modelling requires a deep understanding of physical chemistry method and techniques, it may lead to a vast expansion of our knowledge of retention and mass transfer mechanisms as well as improve the robustness of LC modelling.

Machine learning approaches were also noted as an emerging trend. Torgny Fornstedt from Karlstad University gave a talk on ion-pair chromatography of oligonucleotides, discussing the importance of machine learning to interpret and act on the abundance of data collected for these complex analytes. In the following lecture, Imad Haider Ahmad from Merck & Co. discussed the multifactorial modelling of data for multidimensional chromatography in an attempt to facilitate method development in both one- and two-dimensional separations.

**Green Analytical Chemistry:** A major theme throughout the conference centered on green analytical chemistry and some of the major topics discussed previously, such as SFC, microscale separations, and even modelling, can be related to this theme. As with other areas of chemistry and life in general, analytical chemists will certainly be called upon to get creative and generate less waste, use less power, and establish overall sustainable methods of analyses moving forwards.

**Highlighted Areas of Application**

The application of modern separation tools in life sciences was once again prominent at the symposium. Whereas recent HPLC meetings have focused largely on proteomics and related therapeutics (that is, monoclonal antibodies [mAbs] and antibody–drug conjugates [ADCs]), HPLC 2022 also had a strong focus on oligonucleotides, lipids, and the emerging general topic of personalized medicine.

Oligonucleotides, RNA, DNA, and Nucleic Acids: The most recognized applied topic for the conference was centered on the separation of oligonucleotides, including various ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) classes. A simple search of the conference technical programme using the term “oligonucleotide” revealed the topic was highlighted not only in many lectures and posters but also in several vendor seminars and short courses. The focus on oligonucleotides likely stems from the location of the conference (San Diego is a recognized hotbed for oligonucleotide research), recent success of oligonucleotide-based therapeutics, and, of course, the elevated general community recognition of oligonucleotides because of Covid-19.

In a session dedicated to nucleic acids and oligonucleotides, Kelly Zhang, Claus Rentel, and Alexandre Goyon from Genentech impressed many attendees with their recent efforts in analyzing oligonucleotides using clustered regularly interspaced short palindromic repeats (CRISPR). In her lecture entitled, “Characterization of Guide RNA for CRISPR Gene Editing”, Zhang described methods for characterizing guide RNA (gRNA) for CRISPR gene editing systems and therapeutics. Zhang described ion-pair reversed-phase LC (IP-reversed-phase LC) strategies and noted the best results were achieved by using large pore stationary phases, triethylamine (TEA)- and hexafluoroisopropanol (HFIP)-supplemented mobile phases, and temperatures less than 40 °C to resolve variants. For fine discrimination of gRNA that only differ by one nucleotide, Zhang employed the use of HILIC–MS. Overall, Zhang’s platform of methods includes IP-reversed-phase LC, SEC, and HILIC–MS in analyzing these biotherapeutics.

Rentel discussed the analysis challenges and methods for synthetic
oligonucleotides by LC–MS in the paper “Analytical Techniques for Oligonucleotide Therapeutics”. Three different modes of chromatography were employed in characterizing oligonucleotides: ion-pairing, reversed-phase chromatography (IP-RPC) using various IP reagents, weak anion exchange (WAX) chromatography, and metal ion complexation chromatography. The IP-RPC was used to resolve positional isomers of nucleic acids to detect truncated or modified oligos. The WAX chromatography was employed to resolve diastereoisomers and deamidation by-products during formulation. Alternatively, the metal ion complexation chromatography methods were also used to examine diastereoisomers.

In the lecture entitled “Development of Innovative Analytical Approaches for the Separation of Large RNA Oligonucleotide Impurities and Their Sequencing”, Goyon discussed a 2D-LC–MS platform to analyze these impurities. The first-dimension column was packed with various nuclease enzymes to digest the large oligonucleotides into smaller fragments that would be more amenable to ionization and interpretation in the MS instrument. Goyon shared with the audience that this technique is successful in allowing for the detection of very minute modifications in the oligonucleotide product, including even single oxidation events on one nucleotide. The second-dimension column was a HILIC phase because it provided better resolution than several IP-RPC columns and methods.

In a second session dedicated to nucleic acids and oligonucleotides, Gregory Jones of Alnylam Pharmaceuticals discussed ways to automate impurity characterization of oligos utilizing multidimensional chromatography in his talk, “RNAi Therapeutic Impurity Characterization: Workflow Automation using Multidimensional Chromatography”. Also noted as a highlight by several attendees was Ken Cook’s presentation, “Optimization of mRNA Sequence Analysis by UHPLC/HRMS”.

Separation of oligonucleotides is complicated and, as expressed by many of the presenters, requires a number of separation tools. IP-RPC and HILIC appear to be the main modes of separation, sometimes in combination (multidimensional approaches). It was also evident that column and system metal-passivation (bio-inert) is highly desirable for this class of analytes. Lipids: A highly touted lecture from Michal Holčapek, University of Pardubice, highlighted the use of ultrahigh-performance supercritical fluid chromatography coupled with MS (UHSFC–MS) for high-throughput quantitation of lipids in biological samples. Holčapek described the SFC approach and...
compared it to HILIC and reversed-phase LC separation modes (also coupled with MS). Although each mode has its advantages, the UHSFC–MS system offered excellent selectivity and sensitivity (among other attributes) at comparatively short analysis times (see Figure 5). Holčapek went on to describe how the methodology was impressively applied to the early diagnosis of pancreatic and other cancer types. In a post-conference communication, Holčapek noted that “We have already established the spin-off company LipiDiCa (abbreviation of Lipidomic Diagnosis of Cancer), hired [our] first employees, are installing instruments in the laboratories now, and we are working on the preparation of clinical validation, which is a crucial step in our ambitious effort.” The reader is referred to two recent key papers that the presentation was derived from in the literature (5,6).

Lipid research was also well represented by both Robert Kennedy (“Capillary LC at 35,000 psi and Beyond for Complex Mixture Analysis”) and Erin Baker (“Combining Multidimensional Measurements with Standards and Isotopologue Workflows to Detect, Identify, and Validate Molecules in Omic Studies”). Analysis of lipids continues to be a big opportunity for separation science, as they are challenging analytes to identify with MS alone. Isomers, underdeveloped databases, and a huge range of sizes and polarities make them a gold mine for separations research.

Personalized Medicine: The emerging topic of personalized medicine was very apparent at the meeting. In her plenary lecture in the opening session, titled “Next Phase of Precision Medicine: Application of LC–MS”, Jennifer Van Eyk (Cedars-Sinai Medical Center) discussed the utilization of LC–MS in personalized medicine.

Organ on a Chip/Human on a Chip: Perhaps the most personally memorable lecture of the conference was given by Steven Ray Wilson (University of Oslo) entitled “Organoids, Organ-on-a-chip, and HPLC”. After describing the advantages and disadvantages of current biomedical research models for disease modelling, Wilson introduced the ideas of using organoids, or laboratory-grown organ models derived from human pluripotent stem cells (Figure 6). The research effort aims to couple organoids with LC as a means for improving, among others, precision medicine and drug development. After describing initial success studying the role of oxysterols in non-alcoholic fatty liver disease using a basic “offline” setup, Wilson went on to describe a more elaborate liver “organ-on-a-chip” for drug metabolism studies that utilized electromembrane extraction (EME) to sample solutions in contact with the operative organoid. Lastly, Wilson described packing “columns” with organoids, representing a new area of research where devices could be developed for research purposes (that is, drug metabolism studies) or as bioreactors. For the latter, the potential of a “pancreas-on-a-chip” was assessed by the online coupling of a disulfide bond cleavage reactor prior to MS analysis for measuring excreted insulin. The disulfide bond cleavage was used to fully characterize the insulin released from the organoids when using a nano-LC-based setup.

Conclusions

The HPLC 2022 symposium was a welcome return to face-to-face communication that was well-organized and lively. The meeting once again engaged researchers interested in analytical science from around the globe. The conference was full of new and expanding column and system technologies, offered new insights into many different separation modes, and exhibited important application to our craft in real-world situations. The meeting also revealed some clear emerging topics such as a focus on oligonucleotide separations, the need for metal passivation of instruments and columns, and the increased focus on green analytical technologies. That said, it was mentioned by many attendees that there were no monumental or revolutionary developments noted at the conference, so it appears the science is still trending in accordance with Moore’s Law. Be it from 3D-printing advancements, developments in microscale separations, or perhaps revelations from molecular dynamics studies, the scientific community lies in anticipation for that next revolutionary discovery.

Acknowledgements

Coverage of such a large symposium is impossible without a great amount of assistance. The authors would like to acknowledge the invaluable input and discussion from Robert Kennedy, Joseph Pesek, David McCalley, Steven Ray Haakon Wilson, Ken Broeckhoven, Mariosimone Zoccali, Michal Holčapek, Alan McKeown, Michael Lämmerhofer, Kevin Schug, Stephen Groekreutz, Andrea Gargano, Martina Catani, Alberto Cavazzini, Fabrice Gritti, Szabolcs Fekete, James Grinias, Michael Dong, Nathaly Reyes, Diego Lopez, Jesse Bischof, Jason Anspach, Mark Schure, and Gert Desmet for providing notes, text, insights, figures, and enlightening discussions.

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Testing the Fundamentals: An Examination of Your Extraction Knowledge

Douglas E. Raynie, Sample Preparation Perspectives Editor

As I write this, I’m also grading the final exam from my “Chromatography and Separations” course. We spent approximately the first 75% of the course covering column chromatography, capillary electrophoresis (CE), and field-flow fractionation (FFF). The remainder of the course focused on chromatographic sample preparation. The final exam was based on this last section of the course. Some questions make a good review of the fundamentals of various extraction techniques. The basis for these questions may have been discussed in previous iterations of this column, in analytical chemistry textbooks, or in books on chemical separations. I generally give the final exam in my graduate courses as a take-home exam, so you are free to use whatever resources you have available to you. Here’s your opportunity to test yourself, as the answers are provided.

Question 1: Consider a system comprised of water and a neutral solute. An aliquot of this mixture is used to partially fill a septum-capped vial and solid-phase microextraction (SPME) is used to extract the solute from the headspace vapour. Using Gibbs’ Phase Rule, how many degrees of freedom describe this extraction system? Considering these degrees of freedom, what are the important parameters in developing this extraction?

The phase rule, \( F = C - P + 2 \), describes systems at thermodynamic equilibrium, where \( F \) is the number of degrees of freedom describing the system; \( C \) is the number of components in the system; and \( P \) is the number of phases. Because the phase rule applies to “pressure/volume/temperature [pvT]” systems, the constant 2 accounts for the pressure and temperature of the system. For example, if we have a single solute that may partition between an aqueous and an organic phase, there are three components (the aqueous and organic phases and the solute) and two phases (the aqueous and organic), so \( F = 3 - 2 + 2 = 3 \). Thus, if the temperature and pressure are constant, the concentration of solute in one phase dictates the concentration in the other phase because the system is at equilibrium. In other words, concentration is the third degree of freedom, which provides the basis for the concept of distribution ratios. In a slightly more complex, illustrative example, consider that the solute in the previous example is an amine. Now we have four components (the aqueous and organic phases, the deprotonated amine and its protonated counterpart) with the two phases, or \( F = 4 - 2 + 2 = 4 \). As in the previous example, temperature, pressure, and concentration are degrees of freedom that describe the system. The fourth degree of freedom is the pH (relative to the pKb of the amine), which determines whether the amine is protonated or deprotonated.

Getting back to our question, in this case, we have four components (water, solute, headspace vapour, and SPME sorbent) and three phases (water, headspace vapour, and solid-phase microextraction [SPME] sorbent), or \( F = 4 - 3 + 2 = 3 \). In addition to the temperature and pressure, because all three phases are in equilibrium with each other, \([\text{solute}]_{aq} = [\text{solute}]_{g} = [\text{solute}]_{SPME}\), the concentration of solute in one phase dictates the solute concentration in all phases. Thus, headspace SPME can be considered a quantitative technique provided the SPME fibre is exposed to the headspace for a sufficient period to allow equilibria to be attained.

Question 2: Refer to the graph in Figure 1 for a given pair of extractions. For each of the two data sets depicted, state what property limits the extraction from producing quantitative yields? How might you adjust the extraction conditions to produce a more quantitative yield in each case?

The kinetics of most extractions generally follows the curve shown in Figure 1. In these plots, the cumulative amount extracted is shown as a function of extraction progress, which may be, for
example, solvent volume or time in a flow-based extraction like pressurized liquid extraction (PLE) or supercritical fluid extraction (SFE), individual separative funnel extractions, or cycles in a Soxhlet apparatus. The kinetics show a rapid extraction period because of analyte solubility in the extraction solvent under the prescribed conditions, followed by a slowing of the curve, where the extraction kinetics become dependent on the diffusion of the solvent (with or without dissolved analyte) through the sample phase, asymptotic to the maximum amount extracted. Figure 1 demonstrates two different extraction examples.

In the upper blue curve, the initial slope is quite large, demonstrating high solubility of the analyte in the extracting solvent. Approximately 55% of the analyte is extracted in one cycle and a combined two-thirds following the second cycle. However, the total extraction never exceeds 70%. We can conclude that the rate-limiting step for this extraction is the diffusion of the extracting solvent into the pores of the sample particles (these diffusion-limited situations are much less prevalent when extracting liquid or gaseous samples) to solvate the analyte trapped inside the sample. There are two primary means of overcoming the diffusion limitation. The first is to shorten the diffusion pathlength (that is, increase the sample surface area) by grinding the sample. Extractions of solids are generally faster as sample particle size decreases. For example, consider making a pot of coffee with whole beans as opposed to grinding the beans! The one thing to watch out for in this situation is to minimize grinding the sample too fine (say the consistency of flour or talcum powder) to avoid the particles packing together and impeding solvent flow. The second means to address this situation is to enhance the diffusivity of the solvent. One may choose a less viscous solvent, but solubility is not the issue, so we would likely stay with the original solvent used. Supercritical fluids are one type of potential extracting solvent exhibiting fast diffusion rates. The easiest way to improve solvent diffusivity is to heat the system. As the temperature increases, the diffusion is faster, which is the principle behind techniques such as PLE or microwave-assisted extraction (MAE). PLE and MAE allow for higher temperatures than the atmospheric boiling point to be used. One caveat is that we must avoid thermal decomposition of the sample or analyte. Another less often used and more difficult way to increase solvent diffusivity is to add a secondary chemical, such as a surfactant or a gas like carbon dioxide (perhaps in the supercritical region), which lowers the surface tension and enhances diffusion.

The example shown in the lower, red curve in Figure 1 is limited by the analyte.

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<th>TABLE 1: Comparison of the attributes of modern extraction techniques</th>
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<td>Extraction time</td>
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solubility in the extracting solvent. Only approximately 20% of the analyte is extracting during the first cycle before the curve flattens, though it still rises at a visible rate. This extraction is solubility limited. Again, increasing temperature generally results in increased analyte solubility. A different solvent, or a solvent additive, is most likely needed.

Plotting the extraction kinetics during method development, especially when extracting solid samples, often helps guide the extraction process: Should I switch solvents, heat, or grind the sample? However, the analyst should keep in mind two common situations where the shape of the kinetic plot may differ from that shown in Figure 1. The first common situation is where the analyte may decompose (because of light, heat, or oxidation) during the course of the extraction. In the second common situation, the analyte may be tightly adsorbed onto the sample matrix or may even be encapsulated by the matrix. These situations may require more complex method development.

**Question 3:** Over the past 20–25 years, there have been serious concerns over the environmental consequences (for example, flammability, toxicity, and waste generation) of the use of organic solvents in chemical extractions. Discuss how modern extraction methods reduce or eliminate the use of organic solvents from the perspectives of (a) extracting liquid samples and (b) extracting solid samples. Since the 1990s, the chemistry community has seen the rise of green chemistry, a thought process on the practice of our discipline considering the health and environmental consequences of our products and processes. At approximately the same time, there has been the concurrent development of the next generation of extraction techniques with improved performance. Techniques like solid-phase extraction (SPE), SPME, hollow-fibre membranes, SFE, PLE, and MAE are among the techniques that possess significant performance advantages compared to their traditional counterparts and happen to have green advantages as well. These approaches are preferred because methods cannot possess green advantages only; they must also be cheaper, faster, and more quantitative (equal performance will not cut it) than the methods they are replacing.

Extracting liquid samples has been greatly influenced by the use of adsorbents, such as chromatographic stationary phases. This led to the development of SPE, followed by SPME, stir-bar sorptive extraction (SBSE), immobilized liquid extraction (ILE), and others. The key feature of each of these

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techniques is that the dissolved (typically aqueous) sample is added to the sorbent phase, with a variety of configurations that define the techniques, and small amounts of organic solvent are used in the washing steps, the desorption steps, or both. The same organic solvents may be used in conventional extractions or different solvents may be used. As these methods—particularly SPME and SBSE—were developed, thermal desorption of the analyte from the sorbent led to the complete elimination of organic solvent use. The various formats of these sorbent-based extractions became the basis for other techniques such as hollow-fibre membranes and support-assisted liquid–liquid extraction (SALLE), which also dramatically reduced organic solvent use. Single-drop microextraction (SDME) can be envisioned as a derivative of SPME where microlitres of immiscible organic solvent may be considered as the sorbent phase.

Meanwhile, the extraction of solid samples was advanced by the development of instrumental approaches that utilize energy to impact the extraction. Generally, the energy is manifested in the form of heat to improve solubility, diffusion, and desorption energy, as discussed in Question 2. These techniques include PLE, MAE, and ultrasound-assisted extraction (UAE). The advantage of these techniques is a reduction of solvent use from hundreds of millilitres in Soxhlet extraction, for example, to tens of millilitres. The techniques also happen to be faster (from up to a day to 15–30 min per extraction) and more reproducible.

In addition to the newer techniques described above, another approach applied to both liquid and solid samples is the use of alternative solvents with green advantages. Beginning in the mid-1980s, SFE saw increasing use. Supercritical carbon dioxide has solubility and selectivity advantages for certain compounds and favourable diffusion. The instrumental configuration of SFE also inspired the subsequent development of PLE and MAE. Natural products like D-limonene, α-pinene, ethyl lactate, deep eutectic solvents, and ionic liquids are all finding use in analytical extractions. Coacervative extractions, such as cloud-point extraction (CPE), use surfactants to encapsulate solutes in the form of micelles to isolate and separate the solutes from the liquid solution.

Question 4: Choose one of the more “advanced” methods (that is, automated Soxlet, UAE, PLE, SFE, or MAE) for extracting solid samples. Compare this method with the traditional Soxhlet procedure in terms of method operation, time, solvent use, cost, safety, and other performance variables. Table 1 summarizes the attributes of each of the subject extraction techniques. Each of the techniques is much quicker than Soxhlet extraction, which takes up to 6–24 h. SFE, PLE, and MAE use the least amount of solvent, even though automated Soxhlet and UAE are also reduced-solvent use techniques. The automation characteristics of SFE, PLE, and MAE result in less labour. The instrumental methods suffer from a high acquisition cost, but that is amortized to a lower operating cost per sample. Although each technique is safe if operated properly (Soxhlet extraction should be performed in a fume hood), the instrumental methods have built-in features to avoid exposure to solvent vapours.

Question 5: Caffeine has a $K_{ow}$ of 0.616. A cup (237 mL) of coffee contains 95 mg of caffeine. If you want to extract caffeine from a cup of coffee using LLE with a separatory funnel and ethyl acetate as the solvent, how many extractions and how much solvent will be used to extract 92 mg of caffeine, if 237 mL each of coffee and ethyl acetate are used in each extraction? The octanol–water partition coefficient, or $K_{ow}$, can be used to estimate the amount of solvent used in a LLE. This value is tabulated for many common compounds of biochemical or environmental interest in several references. The $K_{ow}$ is the equilibrium constant showing the distribution of an analyte (A) between a nonpolar solvent (n-octanol) and water: $K_{ow} = [A]_{org}/[A]_{wat}$ assuming n-octanol approximates all nonpolar organics. One thing I try teaching students is to estimate an answer using what is known. With a $K_{ow}$ of approximately 0.5, one-third of the solute should partition into the organic solvent with each extraction, or seven steps should extract approximately 95% of the caffeine in this case.

The equation:

$$[A] = ([V_{org}K + V_{wat}][A]_i)$$  \[1\]

where $[A]_i$ is the concentration of analyte remaining in the sample following $i$ number of extractions, $[A]_i$ is the initial concentration of analyte in the sample, and $V_{org}$ and $V_{wat}$ are the volumes of aqueous sample and organic extracting solvent, tells of the efficiency of an extraction. Using our ballpark estimate of seven extractions: $[A]_i = (237\text{ mL coffee})/((237\text{ mL ethyl acetate} \times 0.616) + 237 \text{ mL coffee})^7 \times 95 \text{ mg caffeine} = 3.3 \text{ mg caffeine}$ remaining. This will result in a whopping 1659 mL of ethyl acetate to extract the cup (237 mL) of coffee. Therefore, seven extractions will result in a cup of coffee with 91.7 mg caffeine remaining. From an analytical perspective, selection of a solvent with a greater $K_{ow}$ for caffeine will provide a more efficient extraction.
From a coffee drinker perspective, start with decaffeinated beans!

**Question 6:** Both benzoic acid and ethyl benzoate (structures shown below) are soluble in organic solvents. How can you use extraction to separate them from a solution?

Knowledge of the acid-base properties of benzoic acid is key to solving this problem, similar to the amine example in Question 1. The $pK_a$ of benzoic acid is 4.20, meaning that it will be in the deprotonated, or anionic, form at neutral pH or at any pH greater than 1.5 pH units higher than the $pK_a$, or pH 5.60. Hence it should be water soluble. The ethyl ester is not impacted by pH and is essentially insoluble in water. Thus, a LLE using neutral water and a water-immiscible organic solvent should achieve the desired separation.

As seen in Question 5, selection of the organic solvent should be based on the $K_{ow}$ of ethyl benzoate in that solvent.

**Benzoic acid**

**Ethyl benzoate**

**Conclusion**

I hope that this month’s column provides an understanding of miscellaneous extraction and sample preparation concepts. You are challenged to recall your knowledge, or learn it for the first time, using the format of exam questions. Because our course is taught in alternate years, I’m likely to teach the course no more than one more time before retirement. Therefore, other professors are welcome to reuse or modify my questions, but it is also likely that these questions will be available to your students on various internet sites. Future instalments of this column may present other exam questions I’ve developed over the years.

**ABOUT THE COLUMN EDITOR**

Douglas E. Raynie is a Department Head and Associate Professor at South Dakota State University, USA. His research interests include green chemistry, alternative solvents, sample preparation, high-resolution chromatography, and bioprocessing in supercritical fluids. He earned his Ph.D. in 1990 at Brigham Young University under the direction of Milton L. Lee. Raynie is a member of *LCGC*’s editorial advisory board. Direct correspondence about this column via e-mail to amatheson@mjhlifesciences.com
Chromatographic instrumentation and column technology are continuously evolving and the state-of-the-art equipment used regularly today is quite impressive. Ultrahigh-pressure liquid chromatography (UHPLC) has enabled laboratories to boost the chromatographic resolution, and hence the number of compounds that can be separated in an analytical run. Even with high-end systems and well-developed one-dimensional LC (1D-LC) methods, the probability of missing critical information on the sample composition, for example, because of chromatographically overlapping peaks, is very real. Figuring out ways to minimize this risk must be continuously explored. The hyphenation of chromatography with mass spectrometry (MS) generally improves analytical sensitivity and selectivity significantly. Using MS can help the user to detect compounds that otherwise would be overlooked as a result of a lack in signal intensity or interference from other compounds present in the sample. LC–MS techniques are a big leap forwards, but even these instruments are not always able to provide the answers, and LC–MS methods have more restrictions in terms of the chemicals (such as the mobile-phase buffers and additives) that can be used to execute the analyses compared to other more traditional detectors. Furthermore, high-end MS instrumentation is expensive to acquire and operate, requiring substantial user expertise. All of the above leads to the conclusion that even with the high performance of UHPLC and MS equipment, the probability of missing critical information about the sample persists. Missing relevant compounds in the sample and not meeting the given analytical or regulatory requirements can have serious economic consequences (delays), damage the reputation of the department or company, and above all, affect safety and efficacy. The latter is obviously the reason for the considerable regulatory burden associated with drug development.

An emerging tool to substantially reduce the above risk is two-dimensional LC (2D-LC). In 2D-LC, two different chromatographic separation mechanisms are combined and peaks that are eluted from the first column are further separated on a second column that ideally has an orthogonal separation behaviour. As a result, separation power can to a great extent be boosted, complementary information can readily be obtained (such as information related to molecular properties like size, charge, hydrophobicity, and affinity), and separations can be

Minimizing the Risk of Missing Critical Sample Information by Using Two-Dimensional Liquid Chromatography

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Analytical requirements in the biopharmaceutical, pharmaceutical, and food industries, among several others, are more demanding than ever. Chromatographic techniques are excellent tools to acquire detailed information on a vast number of molecules and sample types. The present challenge in research and development (R&D) as well as in quality control (QC) laboratories is to collect as much sample information as possible. However, even with the current one-dimensional (1D) analytical portfolio, it is not possible to fully ensure that all the relevant information from a sample is captured. This article illustrates the power of an online two-dimensional liquid chromatographic (2D-LC) setup to unravel the complexity of biopharmaceutical and pharmaceutical samples. This technology tremendously increases the resolving power in all areas where LC is applied and drastically reduces the risk of missing an unknown in the samples.

Chromatographic instrumentation and column technology are continuously evolving and the state-of-the-art equipment used regularly today is quite impressive. Ultrahigh-pressure liquid chromatography (UHPLC) has enabled laboratories to boost the chromatographic resolution, and hence the number of compounds that can be separated in an analytical run. Even with high-end systems and well-developed one-dimensional LC (1D-LC) methods, the probability of missing critical information on the sample composition, for example, because of chromatographically overlapping peaks, is very real. Figuring out ways to minimize this risk must be continuously explored.

The hyphenation of chromatography with mass spectrometry (MS) generally improves analytical sensitivity and selectivity significantly. Using MS can help the user to detect compounds that otherwise would be overlooked as a result of a lack in signal intensity or interference from other compounds present in the sample. LC–MS techniques are a big leap forwards, but even these instruments are not always able to provide the answers, and LC–MS methods have more restrictions in terms of the chemicals (such as the mobile-phase buffers and additives) that can be used to execute the analyses compared to other more traditional detectors. Furthermore, high-end MS instrumentation is expensive to acquire and operate, requiring substantial user expertise. All of the above leads to the conclusion that even with the high performance of UHPLC and MS equipment, the probability of missing critical information about the sample persists. Missing relevant compounds in the sample and not meeting the given analytical or regulatory requirements can have serious economic consequences (delays), damage the reputation of the department or company, and above all, affect safety and efficacy. The latter is obviously the reason for the considerable regulatory burden associated with drug development.

An emerging tool to substantially reduce the above risk is two-dimensional LC (2D-LC). In 2D-LC, two different chromatographic separation mechanisms are combined and peaks that are eluted from the first column are further separated on a second column that ideally has an orthogonal separation behaviour. As a result, separation power can to a great extent be boosted, complementary information can readily be obtained (such as information related to molecular properties like size, charge, hydrophobicity, and affinity), and separations can be
rendered MS-compatible, allowing the analyst to obtain a detailed understanding of the sample under investigation. Peaks can be transferred from one dimension to the other, either in an online or an offline manner. Historically, offline transfer has been the method of choice, but with robust, state-of-the-art, online 2D-LC instrumentation and the elegant software solutions made commercially available in recent years, this technology has found its way to mainstream laboratories. Such advanced systems offer the further benefit of automation and reduced sample manipulation, thus preventing solute loss and degradation. In addition, automation inherently adds more reliable tracking of the entire workflow, in contrast to manual or external tracking applied in offline approaches, thereby improving traceability in regulated environments.

This short contribution discusses the potential pitfalls associated with 1D-LC, and the 2D-LC approaches that can address those shortcomings. This discussion is briefly illustrated with some relevant applications related to pharmaceutical and biopharmaceutical analysis (1–17). However, the reader should take note that the same principles apply in other disciplines such as food, environmental, toxicological, chemical, and petrochemical analysis, to name a few.

### Coelution Resulting from Lack of Selectivity

One of the most recurring analytical concerns in the pharmaceutical industry relates to the lack of selectivity of methods, causing peaks to be coeluted, or a failure to prove that the results demonstrate adequate selectivity. Hence, it is crucial to incorporate as much selectivity as possible into any chromatographic method. This goal of ensuring sufficient selectivity is often supported by using forced degradation or even process samples during method development. However, it is impossible to ensure full selectivity for all potential and real impurities and degradants in the drug substance or drug product using a single LC method. Therefore, evaluating other methods with alternative selectivity is a common and good practice to assess peak coelution and prove peak purity.

One obvious example is the investigation of chirality for a given active pharmaceutical ingredient (API) and its impurities separated in a purity LC method, typically in reversed-phase LC mode. Because the purity method is achiral, it would not supply information about the presence of enantiomeric impurities in a 1D-LC setup. The sample or collected fractions would need to be analyzed with another method and alternative (chiral) stationary phases. By using an online 2D-LC instrument that combines a reversed-phase LC and a chiral LC column, data on both the achiral and chiral impurities can be obtained from a single injection in a fully automated manner.

Another typical challenge is related to being able to demonstrate the absence or presence of impurities due to degradation or the synthesis reactions that might be coeluted with the main peak. The main
peak is often overloaded and too broad to reach the 0.05% reporting limit and can mask the presence of low-abundance compounds. Selective detectors, such as MS, can sometimes reveal these impurities based on differential m/z; however, the high API and matrix content loaded onto the LC–MS system can suppress the ionization and detection of coeluted impurities. In addition, coeluted isomers would often not be differentiated. Therefore, it is advised to investigate such samples with chromatographic methods with alternative selectivity. An example of reversed-phase LC analysis of a small-molecule API (metoclopramide) is shown in Figure 1. The large peak of the API observed in the UV chromatogram hides any impurities that may be eluted in this region. MS data on this run only show the presence of the API. On the other hand, data from comprehensive 2D-LC (LC×LC), in which the entire first dimension is sent in discrete fractions to the second dimension with alternative selectivity, reveal the presence of an unknown impurity hidden underneath the main peak.

It is crucial to detect these impurities and degradation products early in the method development process to streamline final method development. It is because of this that multiple 1D-LC methods are frequently developed and applied—to cover as many impurities as possible. However, such an approach is time and resource consuming, and peak tracking is not an easy task. Bundling alternative selectivities in a single 2D-LC method may be the better and more productive approach.

A multitude of examples can be found in the literature that apply to small molecules, oligonucleotides, peptides, proteins, monoclonal antibodies (mAbs), antibody–drug conjugates (ADCs), and more, illustrating the lack of selectivity of 1D-LC and how 2D-LC sheds light on peak and drug purity and drug quality.

**Insufficient Resolving Power for a Given Sample Complexity**

The above discussion focuses on potential chromatographic issues encountered in relatively simple yet challenging and difficult-to-resolve mixtures. The biopharmaceutical industry is evidently also confronted with many samples of high complexity such as natural products, fermentation samples, reaction mixtures, digests, antibodies, mRNA, and so on. It is obvious that when sample complexity increases, the risk of losing or not detecting valuable information rises.

Peptide mapping is an appreciated methodology for the in-depth characterization of therapeutic proteins, such as mAbs and ADCs. The analysis is part of an extensive portfolio of chromatographic methods that, on their own, already demonstrate the need for complementary selectivities. Interestingly, there is a tendency to combine several of these methods in multidimensional and parallel setups, a principle known as multi-attribute analysis (12). Peptide mapping demands the best in terms of chromatographic separation because the complexity of the sample is substantially increased following the generation of peptides. In digests of larger therapeutic proteins such as mAbs, it is not uncommon to encounter hundreds of peptides with different physicochemical properties that are present in a wide concentration range. Despite the feasibility of generating peak capacities up to 1000 in UHPLC, the random distribution of peaks requires peak capacities in excess of 10,000 to resolve 98% of a tryptic digest containing 100 peptides. As a result, peptide mapping using 1D-LC does not provide the complete picture. In contrast, 2D-LC offers a substantially higher peak capacity as long as the two dimensions are orthogonal and separation obtained in the first dimension is maintained upon transfer to the second dimension (13).

Regulations do not only require full characterization of the API; guidelines concerning the evaluation of excipients are also becoming more demanding. Some excipients, such as surfactants, are highly heterogeneous and their analysis as a raw material or in a formulation represents an additional challenge.
In addition, composition can change over time and stability needs to be evaluated with the best chromatographic performance. Polysorbates (Tween), for example, are commonly used surfactants in pharmaceutical and biopharmaceutical formulations and exhibit high complexity resulting from the synthesis process and the inherent distribution already present in the raw materials used. If the distribution within each group were relatively narrow (degree of ethoxylation, fatty acid), a well-developed reversed-phase LC method could potentially suffice to profile the polysorbate sample. Generally, however, natural mixtures of fatty acids are used in synthesis and the ethoxylation degree is relatively broad, making it very difficult to clearly separate all groups from each other. MS can be used to increase selectivity to a certain extent, but care should be taken for components with the same elemental composition and molecular weight. Consequently, 1D-LC will not be able to resolve all compounds within those samples. Nevertheless, impressive profiles and complexities have been revealed using 2D-LC (Figure 2) (14).

**MS Compatibility**

MS is nowadays an indispensable tool in analytical laboratories, both in research and development (R&D) and quality control (QC) environments. However, the direct hyphenation of LC methods to MS is not always an option because of mobile-phase incompatibility. For legacy and compendial LC methods, the main reason is the use of involatile mobile-phase additives such as phosphate and ion-pairing reagents that are incompatible with MS. In addition, chromatographic modes, such as ion exchange (IEC), hydrophobic interaction (HIC), and size-exclusion chromatography (SEC), are used to highlight charge, hydrophobic, and size variants of protein biopharmaceuticals, and are ideally run with inorganic or nonvolatile mobile phases, which are also incompatible with MS.

In the absence of MS data, the probability of missing crucial sample information is real. A solution could be to perform an LC–MS experiment using similar yet MS-compatible conditions, by, for example, replacing phosphoric acid with formic acid. In certain cases, success will lurk around the corner. However, more frequently, selectivity would have changed too drastically, and, in a worst-case scenario, separation would be completely destroyed. Another approach to retrieve an MS readout from an MS-incompatible run is to collect the...
peaks of interest offline using a fraction collector (or manually in tubes) and desalt or concentrate them before MS analysis. Desalting or concentration is ideally carried out during the re-injection of the collected fraction onto an MS-compatible LC method, typically reversed-phase LC-based, in which salts are eluted before the compounds of interest.

The above process can be fully automated using (multiple) heart-cutting 2D-LC in which one or a couple of first-dimension peaks or fractions are collected in loops installed on a valve followed by sequential transfer to a second chromatographic dimension that takes care of the desalting, concentration, or both. The second dimension optionally adds further separating power or selectivity, which is further beneficial to discriminate in-source fragments from real signals. The existing method, and therefore the previously acquired chromatographic profile, remains unchanged and peaks or regions of interest observed in the first-dimension profile can be identified. Additional advantages of online 2D-LC–MS are full traceability and the limited sample manipulation, which prevents sample loss and degradation. It is not uncommon for low-abundance peaks to remain undetectable when they are collected in an offline manner and yet to be fully retrieved using the online 2D-LC approach.

Some of the common applications of this approach are MS characterization of protein variants observed in the SEC, IEC, or HIC chromatograms of mAbs or the identification of unknown or new impurities detected with legacy and validated methods that are not compatible with MS. Figure 3 shows the analysis of the peptide glucagon using an MS-incompatible mobile phase in accordance with the monograph in USP 39 and the automated desalting of peaks of interest using a short reversed-phase LC cartridge and MS-compatible mobile phases in the second dimension. Clean and adduct-free MS spectra are obtained for major and minor peaks (15). As a more advanced example, a fully automated four-dimensional (4D)-LC–MS protein analyzer was recently described for characterizing mAbs. Charge variants resolved by IEC were collected in loops installed on a multiple heart-cutting valve and consequently subjected to online desalting, denaturation, reduction, and trypsin digestion prior to LC–MS-based peptide mapping (16). This innovation substantially reduces turnaround time, sample manipulation, loss, and artifacts, and increases information gathering. Unstable protein modifications, such as succinimide intermediates, which were not maintained when performing classical in-solution overnight digestion of offline collected IEC peaks, were revealed.

Sample Matrix Effects
In addition to the impact of mobile-phase additives, the sample matrix can drastically influence MS behaviour. Electrospray ionization (ESI) may be suppressed as a result of the presence of high levels of salt, polymer, surfactant, lipid, protein, and so on in the sample (drug product, biological matrix). In addition to causing signal suppression and the concomitant limited sensitivity, a heavy matrix can lead to contamination and loss of performance of the MS system. Consequently, it is necessary to carry out extensive sample cleanup to remove as much matrix as possible prior to LC–MS analysis. A challenge is to minimize the loss of target analytes and to reduce the workload to a minimum in case large numbers of samples are to be analyzed (such as in pharmacokinetic studies). In contrast, 2D-LC, in which the relevant section in the first-dimension chromatogram is transferred to a second dimension with altered selectivity, is an elegant approach to substantially reduce matrix effects and detect the undetectable or improve sensitivity by at least an order of magnitude.

Conclusions
Despite the fact that the state-of-the-art in 1D-LC is impressive, there is an ongoing risk of missing relevant sample information and not complying with regulatory requirements, particularly given increasing demands. In fact, these risks are growing, given the ever-increasing structural complexity of all the new therapeutic modalities that are being developed and introduced today. To continue to bring safe and effective products to the patient, today and in the future, pharmaceutical and biopharmaceutical scientists need to continuously explore the analytical landscape and guarantee that the latest and greatest tools are utilized.

In recent decades, the chromatographic community has devoted a lot of attention to the development of 2D-LC. Recently, 2D-LC hardware and software became commercially available and the technology has quickly been adopted by, and shown to be of enormous value to, the pharmaceutical and biopharmaceutical industry. In comparison to 1D-LC, the main features of 2D-LC in pharmaceutical and biopharmaceutical analysis are 1) the increased selectivity, limiting the risk of coeluted peaks and making it possible to obtain complementary information about the sample (such as size, charge, hydrophobicity, and affinity); 2) a substantially increased resolving power for complex samples; 3) MS compatibility and the option to hyphenate all LC modes and methods to MS; and 4) the reduction or even elimination of matrix interferences, thus boosting detectability. Furthermore,
all these benefits are available in 2D separations that can be run in a fully automated manner with minimal sample manipulation, thereby reducing the risk of sample loss and degradation while offering full traceability.

Moreover, given the ruggedness of current instrumentation, developing 2D-LC methods is not particularly any more challenging than developing 1D-LC methods, and method validation in 2D-LC has been demonstrated (17).

With the implementation of 2D-LC in the pharmaceutical and biopharmaceutical industry, the risk of missing relevant sample information is substantially reduced to the benefit of all, but not in the least the patient! Furthermore, although the examples shown here relate to pharmaceutical analysis, the application domain of 2D-LC stretches way beyond drugs to food, environmental, toxicological, chemical, and petrochemical analysis, and beyond.

References
2) K. Sandra and P. Sandra, Bioanalysis 7(22), 2843–2847 (2015).

Gerd Vanhoenacker is a senior scientist for HPLC in the RIC group in Kortrijk, Belgium. Pat Sandra is the Founder and Advisor of the RIC group and Emeritus Professor of Ghent University, in Ghent, Belgium. Koen Sandra is the CEO of the RIC group and a Visiting Professor at Ghent University.
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FOOD AND BEVERAGE

356 Food Metabolomics of Alcoholic Beverages Using Single-Quadrupole Mass Spectrometer
Shimadzu Europa GmbH

MEDICAL/BIOLOGICAL

359 Analysis of 26 Natural and Synthetic Opioids in Blood and Urine Using Clean Screen® DAU SPE and Selectra® DA UHPLC Column
UCT

PHARMACEUTICAL/DRUG DISCOVERY

360 Exosome Characterization in Biological Matrices Using Asymmetrical Flow Field-Flow Fractionation and Nanoparticle Tracking Analysis
Postnova Analytics GmbH

362 Monitoring Structural Changes in Polysaccharides Using SEC-MALS
Tosoh Bioscience GmbH
Food Metabolomics of Alcoholic Beverages Using Single-Quadrupole Mass Spectrometer

Shimadzu Europa GmbH

In recent years, metabolomics has gained more attention. Food metabolomics focuses on targeted analysis of low-molecular-weight metabolites, such as amino acids and organic acids, generated by the activities of cells, to clarify differences among multiple sample groups. This is used for various purposes, such as food quality assessment, quality prediction, improvement of manufacturing and storage processes, and evaluation of functional properties. Food contains a great many metabolites and previous research has revealed many of the metabolites involved in flavour, quality, and functional properties. Therefore, targeted analysis is common in food metabolomics. By focusing on important components and analyzing them comprehensively, metabolomics can efficiently provide useful results.

This article introduces an example of food metabolomics using a single-quadrupole (SQ) liquid chromatography–mass spectrometry (LC–MS) system. Compared to triple-quadrupole LC–MS systems, SQ LC–MS systems are cheaper and involve simpler analytical conditions. So even those with minimal mass spectrometry experience can easily perform metabolomics.

Samples and Pretreatment
For samples, we prepared six types of alcoholic and non-alcoholic beers. Table 1 shows the details of the samples. At dilution, 1 μmol/L 2-morpholinoethanesulfonic acid (MES) was added as an internal standard.

Analytical Conditions
LC–MS analysis was performed using a Nexera™ XR HPLC system coupled with an LCMS-2050 single-quadrupole mass spectrometer (Figure 1). The LCMS-2050 is compact, easy to use, and is equipped with a heated dual ESI and APCI ion source offering a wide mass range of m/z 2 to 2000. This is especially useful in metabolomics, where metabolites with a wide range of physical characteristics are analyzed. Table 2 shows the analytical conditions for high performance liquid chromatography (HPLC) and MS analysis. Analytical conditions for simultaneous analysis by single-quadrupole LC–MS were developed by referring to the analytical conditions in the ion pair-free LC–MS/MS method included in the LC–MS/MS Method Package for Primary Metabolites Ver. 3. It enables simultaneous analysis of 143 hydrophilic metabolites, such as amino acids, organic acids, nucleosides, and nucleotides, which are important in food analysis.

Multivariate Analysis
Using simultaneous analysis of hydrophilic metabolites, 82 compounds were detected. The main metabolites were amino acids, organic acids, and nucleoside metabolites. Table 3 shows the number of metabolites detected in each sample. More than 70 compounds were detected in beer 1, beer 2, and non-alcoholic beer 2, but 22 compounds were detected in the low-malt beer, showing a different tendency. The Multi-omics Analysis Package (Figure 2) is a metabolic engineering software that can automatically generate metabolic maps and perform a variety of data analyses based on the vast amounts of mass spectrometry data generated. The intuitive visualization of data provides powerful support to increase the efficiency of metabolomic data analysis work.

The package includes connected gadgets (software tools) for data analysis and data processing, making it easy to perform various multivariate analysis tasks, as though using a single software program. Figure 3 shows the results of principal component analysis (PCA). From the score plot, the low-malt beer and non-alcoholic beer 1 were plotted close together and had similar trends in the amount of hydrophilic compounds. Other beers and non-alcoholic beers were successfully classified and had different features. From the first principal component (PC1), two groups (Group A: low-malt beer, non-alcoholic

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**Table 1: Details of samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beer 1</td>
<td>Lager beer (bottom fermentation)</td>
</tr>
<tr>
<td>Beer 2</td>
<td>Ale beer (top fermentation)</td>
</tr>
<tr>
<td>Low-malt beer</td>
<td>Purine free</td>
</tr>
<tr>
<td>Beer 3</td>
<td>Soy protein as ingredients</td>
</tr>
<tr>
<td>Non-alcoholic beer 1</td>
<td>Made in Japan</td>
</tr>
<tr>
<td>Non-alcoholic beer 2</td>
<td>Made in Germany</td>
</tr>
</tbody>
</table>

**Table 2: Analytical conditions**

<table>
<thead>
<tr>
<th>HPLC Conditions (Nexera XR)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column: Shim-pack™ GIST PFPP*1</td>
<td>(2.1 mm × 150 mmL, 3.0-µm)</td>
</tr>
<tr>
<td>Mobile phases: A) 0.1% formic acid in water</td>
<td>B) 0.1% formic acid in acetonitrile</td>
</tr>
<tr>
<td>Mode: Gradient elution</td>
<td></td>
</tr>
<tr>
<td>Flow rate: 0.25 mL/min (17 to 19 min, 0.5 mL/min)</td>
<td></td>
</tr>
<tr>
<td>Injection volume: 3 µL</td>
<td></td>
</tr>
</tbody>
</table>

**MS Conditions (LCMS-2050)**

<table>
<thead>
<tr>
<th>Ionization: ESI/APCI (DUIS), Positive and negative modes</th>
<th>SIM (143 events)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizing gas flow: 3.0 L/min</td>
<td></td>
</tr>
<tr>
<td>Drying gas flow: 5.0 L/min</td>
<td></td>
</tr>
<tr>
<td>Heating gas flow: 7.0 L/min</td>
<td></td>
</tr>
<tr>
<td>Desolvation temp.: 500 °C</td>
<td></td>
</tr>
<tr>
<td>DL temp.: 250 °C</td>
<td></td>
</tr>
</tbody>
</table>

*1 P/N: 227-30858-07
beer 1, and beer 3; Group B: non-alcoholic beer 2, beer 1, and beer 2) were differentiated. This suggests that PC1 shows the difference in ingredients because ingredients that are not used for beer are used for Group A and ingredients that are used for beer are used for Group B.

From the loading plot, the characteristic compounds contained in each sample were identified. Beer 2 contained a lot of amino acids and nucleoside metabolites, as shown in blue. PCA makes it easy to classify each sample by feature and find the compounds that cause a difference.

Figure 4 shows the result of hierarchical cluster analysis (HCA). Similar to the results of PCA, HCA classified samples into two groups (Group A: low-malt beer, non-alcoholic beer 1, and beer 3; Group B: non-alcoholic beer 2, beer 1, and beer 2). Non-alcoholic beer 1 and non-alcoholic beer 2 are both non-alcoholic beers, but they are classified in different groups. Non-alcoholic beer 1 is made in Japan and is made by seasoning wort without fermentation. Non-alcoholic beer 2 is made in Germany and is made from the same ingredients as beer and fermented in a way that suppresses the production of alcohol. This suggests that the differences in the ingredients and the manufacturing processes affect the tendencies of hydrophilic compounds in the non-alcoholic beers. Beer 1 and non-alcoholic beer 2 were classified in similar groups. This may be because the ingredients of beer 1 and non-alcoholic beer 2 are beer-based and they are made by bottom fermentation. HCA provides a visual understanding of the degree of similarity between the compounds in each sample.

Compounds Related to Purine

The results of PCA and HCA showed significant differences in nucleoside metabolites, so compounds related to purine in each sample were compared. Adenine, adenosine, cyclic adenosine monophosphate, adenosine monophosphate, inosine, hypoxanthine, xanthine, guanine, guanosine, and guanosine monophosphate were detected. Figure 5 shows the sum of the peak area ratios of each compound. It was found that beer 2 contains the most compounds related to purine, and the low-malt beer, beer 3, and non-alcoholic beer 1 contain the fewest of them. In particular, these compounds were hardly detected in the low-malt beer (purine free).

Xanthine differences between samples was quantified. Figure 6 shows the calibration curve obtained using the standard solution of xanthine. Good linearity with a coefficient of determination ($R^2$) of 0.999 was obtained for the calibration curve range of 0.1 to 50 µmol/L. Table 4 shows concentrations of xanthine in each sample. Beer 2 contained the highest amount (102 µmol/L) of xanthine.
In addition to amino acids, organic acids, and nucleoside metabolites, components with functional properties were also detected in each sample. For example, GABA ($\gamma$-aminobutyric acid), which is known to improve blood pressure, relieve stress, and reduce fatigue, and ferulic acid, vanillic acid, sinapic acid, and caffeic acid, which have antioxidant effects, were detected. The peak area ratios of these functional components were compared. As shown in Figure 7, beer 1, beer 2, and non-alcoholic beer 2 were rich in these functional components. Ferulic acid and vanillic acid are the main antioxidants in beer and are known to be contained in malt. More of these functional components were detected presumably because the proportion of malt is high in the ingredients of beer 1, beer 2, and non-alcoholic beer 2.

**Conclusion**

This article introduces an example of food metabolomics using single-quadrupole LC–MS. Although triple-quadrupole LC–MS/MS is normally used for targeted metabolomics, it was found that single-quadrupole LC–MS also has sufficient potential for targeted metabolomics. Compared to triple-quadrupole LC–MS, single-quadrupole LC–MS systems are cheaper and easier to operate, making analysis easier for many people, including those who have no experience with mass spectrometry. The spread of food metabolomics using single-quadrupole LC–MS is expected to lead to further development of technologies and products in the food industry.
Analysis of 26 Natural and Synthetic Opioids in Blood and Urine Using Clean Screen® DAU SPE and Selectra® DA UHPLC Column

UCT

In this application, UCT offers a simple procedure to extract opioids from biological matrices with excellent recoveries and overall precision. Additionally, UCT’s Selectra DA UHPLC column provides excellent retention and peak shape for all the opioids in the method, including baseline separation of the critical isobaric compounds. Furthermore, all compounds were eluted in less than 8 min from the Selectra DA UHPLC column.

Table 1: Instrument parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC System</td>
<td>Thermo Scientific Dionex Ultimate 3000</td>
</tr>
<tr>
<td>MS System</td>
<td>Thermo Scientific TSQ Vantage</td>
</tr>
<tr>
<td>UHPLC Column</td>
<td>Selectra DA Column 50 × 2.1 mm, 1.8 μm PN: SLDAGDC20-18UMOPT</td>
</tr>
<tr>
<td>Guard Column</td>
<td>Selectra DA 5 × 2.1 mm, 1.8 μm PN: SLDAGDC20-18UMOPT</td>
</tr>
<tr>
<td>Column Temp.</td>
<td>40 °C</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.4 mL/min</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>5 μL</td>
</tr>
<tr>
<td>Mobile Phase A</td>
<td>0.1% formic acid in water</td>
</tr>
<tr>
<td>Mobile Phase B</td>
<td>0.1% formic acid in MeOH</td>
</tr>
<tr>
<td>Gradient</td>
<td>B Conc: 0% (0 min) – 15% (0.5 min) – 30% (3.5 min) – 100% (7.5 to 8.5 min) – 0% (8.6 to 11 min)</td>
</tr>
</tbody>
</table>

Table 2: SPE procedure

<table>
<thead>
<tr>
<th>Sample Pre-treatment</th>
<th>Blood</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>1 mL sample + 4 mL pH 6.0 phosphate buffer (0.1 M) + ISTDs. Vortex briefly and centrifuge</td>
<td>1 mL sample + 1 mL pH 6.0 phosphate buffer (0.1 M) + ISTDs. Vortex briefly and centrifuge</td>
</tr>
<tr>
<td>Load</td>
<td>- Load the samples at 1–2 mL/min</td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>- 3 mL 1% formic acid in DI H₂O</td>
<td>- 3 mL MeOH</td>
</tr>
<tr>
<td>Dry</td>
<td>- Dry column for 1–2 min at full vacuum or pressure</td>
<td></td>
</tr>
<tr>
<td>Elute</td>
<td>- 3 mL 5% NH₄OH in MeOH (95:5 v/v MeOH:NH₄OH)</td>
<td>- Evaporate and reconstitute in mobile phase or 1 mL MeOH</td>
</tr>
<tr>
<td>Post Elution</td>
<td>- Evaporate and reconstitute in mobile phase or 1 mL MeOH</td>
<td>Note: Smaller reconstitution volumes can be used to achieve higher concentrations</td>
</tr>
</tbody>
</table>

Results

Extraction of urine and blood quality control samples resulted in excellent recoveries and reproducibility. Urine samples spiked at 5 and 25 ng/mL concentrations had recoveries >80%, with the corresponding RSD values <3%. Blood samples spiked at 5 and 25 ng/mL concentrations showed recoveries >85%, with the corresponding RSD values <4%. The simple protocol outlined in this application note can be readily implemented in pain management, clinical diagnostics, and forensic analysis.

Figure 1: A representative chromatogram showing complete separation of isobaric compounds; (1) morphine (2) hydromorphone, (3) norcodeine, and (4) norhydrocodone.

Figure 2: Analyte recovery from urine samples prepared at 5 ng/mL and 25 ng/mL using an optimized extraction method on CSDAU SPE column.

Figure 3: Analyte recovery from blood samples prepared at 5 ng/mL and 25 ng/mL using an optimized extraction method on CSDAU SPE column.
Exosome Characterization in Biological Matrices Using Asymmetrical Flow Field-Flow Fractionation and Nanoparticle Tracking Analysis

Postnova Analytics GmbH

For therapeutics development, a comprehensive investigation of formulations during the optimization process is essential for later clinical trials. Often the in vivo behaviour differs significantly from the previous in vitro activity. Therefore a characterization under conditions mimicking the in vivo environment is gaining in interest (1).

Both field-flow fractionation (FFF) and nanoparticle tracking analysis (NTA) have gained in increasing popularity for the analysis of complex biological samples, including extracellular vesicles such as exosomes, over recent years (2–4).

In this application note, we present the online hyphenation of asymmetrical flow field-flow fractionation (AF4), multi-angle light scattering (MALS), and NTA to analyze exosomes spiked into rabbit serum.

**Experimental**

The exosome pellet (exosome standard, which was extracted from human urine) was recovered according to the manufacturer’s guidelines. Prior to injection, the exosomes were diluted to the final concentration of $1.64 \times 10^{10}$ particles mL$^{-1}$ in phosphate-buffered saline in a 1:10 dilution of rabbit serum in ultrapure water (UPW).

The experimental setup is illustrated in Figure 1. The samples were fractionated in the AF4 channel according to their hydrodynamic size. To reduce the flow rate for NTA measurement and to increase the sample concentration after the dilution in the channel, the Slot Outlet technique (also known as Smart Stream Splitting) was used: the upper sample-free solvent stream was split away at the end of the fractionation channel. The AF4 system was also coupled with a MALS detector to derive the size (radius of gyration, $R_g$) of the fractionated sample constituents. To deliver the sample at an appropriate flow rate (here approximately 12 μL min$^{-1}$) for the NTA measurement, an additional flow splitter was used for coupling both systems. The NTA determined the hydrodynamic diameter, $D_h$, and the concentration of the separated sample constituents.

**Results**

The rabbit serum represents a highly complex mixture containing a variety of proteins and electrolytes. To exclude matrix-induced interferences, a comprehensive fractionation step is advised prior to the analysis. Otherwise, various interactions between the matrix components and the incident laser light, such as fluorescence, would overlap with the scattered light and may render an accurate size determination by MALS and NTA virtually impossible. In addition, in NTA, the significantly lower intensity of scattered light from small particles may also result in a size distribution that is skewed towards the bigger particles.

Figure 2 shows the obtained AF4-MALS fractograms overlaying the exosome standard, the serum blank, and the exosomes in serum, including the obtained $R_g$ distributions. The data confirm the successful separation of rabbit serum constituents from the exosomes. However, the serum blank also shows particles in the same size range as the exosomes, indicating that there may also be exosomes already present in the serum itself.
The $R_g$ distribution of the exosomes ranged from 23 nm to 100 nm and was not affected by the spiking into rabbit serum. In Figure 3, AF4-NTA fractograms with an overlay of $D_h$ and the particle number concentration are displayed for (a) the exosome standard and (b) the exosomes in serum. The $D_h$ distribution of the exosomes ranged from around 43 nm up to a maximum of 150 nm. The $D_h$ distribution of the exosome-serum sample ranged from roughly 35 nm up to 90 nm, with increasing variation to the exosome standard. The particle concentration and the relationship of $R_g$ and $D_h$ in a blank medium and in serum provide information on the morphology of the exosomes and indications of a corona formation. The hyphenation of AF4-MALS-NTA delivers this information within one single measurement, which reduces time, costs, and sample material.

**Conclusions**

Asymmetrical flow field-flow fractionation hyphenated to multi-angle light scattering detection and nanoparticle tracking analysis represents a powerful analytical platform to study the behaviour of promising drug delivery vehicles under in vivo-like conditions. Both techniques complement each other perfectly. The online matrix removal and sample purification capability of AF4 support the NTA to overcome its limitations, while NTA acts as a true particle-counting detector for AF4. Together with MALS, AF4-NTA may also enable particle shape analysis.

**References**


As the most abundant natural biopolymer, polysaccharides’ unique chemical and physical properties, as well as excellent biocompatibility, make them the materials of choice in many industries. Due to their wide application range and the complexity of their structure, such polymers need to be examined very thoroughly to fully understand their molecular characteristics. For example, to study their diffusion properties, the size of the molecules is an important parameter. Also, the size of dextran is by far the most important determinant of red blood cell aggregation, where low size molecules inhibit aggregation while larger molecules promote aggregation. In addition, a great variety of conformations and branching behaviours make specific polysaccharides either suitable or problematic for certain applications. This application note explains how size-exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS) can be used for the determination of structural changes in polysaccharides, with pullulans and dextrans as examples.

**Experimental Conditions**

**System:** EcoSEC Elite® (HLC-8420) GPC system

**Columns:** 2 × TSKgel® GMPWXL

**Mobile phase:** Water + 0.01 mol/L NaNO₃ and 0.02% NaN₃

**Flow rate:** 0.7 mL/min

**Detectors:** Refractive index (RI) and LenS³ MALS detector

**Results and Discussion**

A set of linear pullulan standards with molecular weight (MW) ranging from 21 kDa to 915 kDa was analyzed using the same experimental conditions. Using the SECview software, the radius of gyration (Rg) and the MW of the pullulan standards were determined; the conformation plot is depicted in Figure 1. It is noticeable that Rg values were obtained for all the standards, even the smaller ones. Technically, traditional MALS detectors cannot detect the angular dependence of scattered light to measure Rg for sizes below 12 nm, at best. However, the novel design of the LenS³ MALS detector extends its Rg measurement range to much smaller polymers. Here, an Rg of 5.1 nm for the lowest MW pullulan standard (21 kDa) was obtained.

In addition to the linear pullulan standards, dextrans with different MW distributions were also analyzed and the results are presented in Figure 1. The Rg of the low MW dextran (dextran 2) across its entire distribution overlays perfectly with the pullulan’s conformation plot. This relationship is an indicator of molecular density, suggesting that the low MW dextran has the same linear structure as the pullulans. Conversely, the Rg to MW relationships of dextran 1 and dextran 3 have a slope of 0.22 and 0.35, respectively. These are much smaller slope values than the linear pullulans, suggesting a comparatively denser structure.

The linear pullulans form a random coil structure in the eluent. In the case of dextrans, there is a possible long chain branching formation by an α-1,3 (and infrequently α-1,2) linkages, especially in the high MW dextrans. As a result, a more compact random coil structure is formed while dissolving in the eluent. Due to this compact structure, the size of the molecule is smaller and therefore elutes at a higher elution time from the column.

**Conclusion**

This study demonstrates that structural differences in polymers can be investigated in depth by SEC-MALS analyses. In the given example, higher MW dextrans tend to exhibit increased branching on their backbone, leading to the formation of a more compact structure in solution. Ultimately, this results in higher retention volumes and lower Rg values compared to linear pullulans of similar MW. In practice, elucidating structural changes in the low MW and low Rg region requires a light scattering instrument with high sensitivity, capable of detecting very slightly anisotropic scattering, such as the LenS³ MALS detector.

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**Figure 1:** Conformation plot of pullulan and dextran.
The 33rd International Symposium on Chromatography (ISC 2022) will be held from 18–22 September 2022 in Budapest, Hungary. The International Symposium on Chromatography (ISC) represents the oldest conference series on separation science. ISC symposia have been organized since 1956 in each even year. ISC is one of the premier meetings series of chromatography and separation science and covers a broad range of techniques and applications.

ISC 2022 is going to be the first significant live event in Europe since 2019 and will cover all fields of chromatography and separation science.

The major focus of the symposium will be on the impact of chromatography and separation science to meet the needs of the pharmaceutical, environmental, food, and health industry, as well as science and medicine. The symposium programme will reflect these themes and highlight new challenges and emerging opportunities for the science and marketing of separation and detection systems and methods.

As an integral part of the scientific programme of lectures and poster sessions, an international exhibition and vendor seminars on instrumentation and services for chromatography, separation science, and mass spectrometry will be organized. ISC 2022 will provide the perfect forum for attendees from academia, industry, and government research institutions to meet for scientific exchange and networking.

The symposium programme reflects all aspects of separation science and aims to highlight new challenges and emerging opportunities in the field. The scientific programme is set to be wide ranging and diverse with topics including: new technologies, instrumentation, and separation media for gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and electrodrevon separations; mass spectrometry hyphenation and applications; pharmaceuticals; biopharmaceutical and biologics; foods, natural products, health, security; chemometrics, quality by design, data processing; all modes of chromatography and electrodrevon separation techniques; miniaturized and lab-on-chip systems; clinical, biomedical, and toxicological analysis and diagnosis; process chromatography and process analytical technology; multidimensional and hyphenated techniques; sample handling and trace analysis; ’omics; complementary and emerging techniques; 3D-printing of separation systems.

During the symposium, prominent separation scientists will be honoured. The Halász Medal Award, the Csaba Horváth Memorial Award, the Ervin sz. Kováts Award for Young Scientists of the Hungarian Society for Separation Sciences, and the Martin and Jubilee Medals of the Chromatographic Society will be presented during the ISC 2022 Symposium. The Joseph Huber Name Lecture Award of the Austrian Society of Analytical Chemistry will also be presented at the award ceremony.

Budapest, the capital of Hungary, is an economic, financial, and cultural centre with two million inhabitants. The city, which is beautifully situated on both sides of the Danube River, has a history dating back over 2000 years. The venue—Budapest Congress Centre—is equipped with a complete range of conference facilities and is easily reached by public transport. It is situated in a pleasant and peaceful chestnut park and directly connected to Novotel Budapest City.

For more information, please visit the symposium website at www.isc2022.hu. For further details contact Attila Varga, Symposium Manager, email: diamond@diamond-congress.hu
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