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Mono and disaccharides are of increasing importance in the growing industrialized food industry. With the rise of diabetes and other metabolic diseases, nutritional content has found itself at the forefront of consumer concern. Investigation of the constituents found on the packaging is now commonplace. Consumer concern, coupled with the increase in FDA regulation on consumer products, has brought about a change in individual ingredients reported on labels. Identification of the five most common mono and disaccharides found in packaged foods, lactose, maltose, sucrose, fructose, and glucose, is easily resolved on any of the RCX-30 family of columns offered by Hamilton Company.

Ultraviolet absorption, normally the detection method of choice for most chromatography separations, is unsuitable for carbohydrates. This means other analyte detection methods must be utilized, such as Refractive Index (RI), Conductivity, and Pulsed Amperometric Detection (PAD). Both RI and Conductivity are excellent choices for detection if only isocratic methods are needed. PAD, however, offers the advantage of detection from either gradient or isocratic elution. An additional consideration when choosing a detector is sensitivity. RI and Conductivity provide nanomolar detection levels while the detection limit for PAD is an order of magnitude lower.

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Column Information

<table>
<thead>
<tr>
<th>Packing Material</th>
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Chromatographic Conditions

<table>
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<tr>
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</tbody>
</table>

### Compounds:
1: Glucose
2: Fructose
3: Lactose
4: Sucrose
5: Maltose

### PAD Sequence:
- $E_1$: 0.1 V
- $t_1$: 440 ms
- $t_2$: 220 ms
- $E_2$: 0.65 V
- $t_3$: 300 ms
- $E_3$: -1.5 V
- $t_4$: 250 ms

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Mike Hennessy, Jr.
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The presence of per- and polyfluoroalkyl substances (PFAS) compounds in food consumed by infants, such as milk and infant formula, points to a particular need for highly sensitive methods that can measure these compounds at parts-per-billion levels in these matrices. In this month’s cover story, Lukas Vaclavík and colleagues at Eurofins present a sensitive, robust, and high-throughput liquid chromatography–mass spectrometry (LC–MS) method for the determination of 16 priority PFAS analytes.

Other articles in this issue address fundamental concerns in chromatography. In “LC Troubleshooting,” Dwight Stoll and Yehia Baghdady discuss system suitability tests for two-dimensional liquid chromatography systems, which are critical as 2D-LC becomes more widely used, particularly in regulated laboratories. In “GC Connections,” Nick Snow explains that failing to properly address uncertainty can lead to presenting data as more accurate and robust than they really are. In “Column Watch,” guest authors Fabrice Gritti and coworkers present a research prototype ultrahigh-pressure liquid chromatography–mass spectrometry (UHPLC–MS) instrument built to improve the resolution power and the usability of LC–MS for routine analyses in pharmaceutical applications. In a contributed feature, Jun Zhang of Bristol Myers Squibb and coworkers assess a research tool for automated data processing that can match the speed of acoustic ejection mass spectrometry.

In “The Next Generation,” Katarína Maráková of Comenius University in Bratislava, Slovakia, addresses the need for sample preparation methods that can target proteins at very low concentration levels (pg/mL to ng/mL) in complex biological matrices. And in this month’s “Viewpoints” column, André Striegel of NIST asks the question, “Whence the next generation of macromolecular separation scientists?”

In closing, I’d like to take a moment to recognize Michael Tessalone. This issue marks the final time Mike’s name will be on the masthead of LCGC, as he is retiring after an impressive 29-year career. Mike started as a sales representative in 1993. He quickly moved up to become the publisher of both LCGC and our sister brand Spectroscopy, and ultimately came to lead the entire Industry Sciences division as the Senior Vice President. In all his roles, Mike was passionate, hardworking, and innovative. He always found new ways to grow the business, and drove to make it happen, while leading by example and defending his teams. It’s hard to imagine our brands without him! We will all be proud to carry on his legacy.

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Development of a System Suitability Test for Two-Dimensional Liquid Chromatography

Verifying system performance is important when using chromatographic instrumentation for analyzing both known and unknown samples. Typically, system suitability tests (SSTs) are used to verify performance, which involves running an established method and comparing the results to pre-established acceptance criteria. As two-dimensional liquid chromatography (2D-LC) becomes used more widely and in regulated laboratory environments, development and implementation of SSTs will be critical for successful routine use of the 2D-LC technique.

Yehia Z. Baghdady and Dwight R. Stoll

Although I have not written in the past about system suitability tests (SSTs) for the “LC Troubleshooting” column, they were mentioned several times in the past by John Dolan, the previous “LC Troubleshooting” columnist. The keyword “system suitability” was used in 11 prior “LC Troubleshooting” articles, which speaks to the importance of SSTs in the routine implementation of conventional one-dimensional liquid chromatography (1D-LC). As discussed by John many times, one benefit of regularly running SSTs is that the test results can provide early indications of a problem that is developing, which can be acted on by troubleshooting and solving the problem before it develops into a full-blown failure and results in instrument down time. However, I am not aware of any prior description of the development of a generic SST for two-dimensional LC (2D-LC) (although some are definitely expressing interest in this [1,2]), which we continue to see move in the direction of regulated laboratories, especially in the pharmaceutical industry. For this installment of LC Troubleshooting, I asked Dr. Yehia Baghdady, a Senior Scientist in Chemical Process Development at Bristol Myers Squibb, to share his experiences so far related to the development and implementation of a SST for routine use of 2D-LC for peak purity checks of small molecule separations in his laboratory. I’m hopeful that sharing these ideas and experiences will kick off a broader conversation around this topic in the community.

Dwight Stoll

One of the most attractive features of 2D-LC in the context of pharmaceutical analysis is the utilization of orthogonal (that is, complementary) selectivities in the first (1D) and second dimension (2D) separations. This feature is particularly valuable for the separation of structurally similar impurities that tend to be coeluted with the main compounds of interest in the pharmaceutical industry, such as active pharmaceutical ingredients (APIs), synthetic intermediates, and starting materials (SMs) (3–6). In these challenging separation scenarios, the peak capacity and resolving power of conventional 1D-LC is often insufficient—even for small molecules where the molecular complexity is lower compared to the challenges encountered in the analysis of large biomolecules.

Despite the recent increased availability of commercial 2D-LC instruments, 2D-LC technology is still not considered to be ready for routine use in quality control (QC) laboratories for small molecule in the same way that 1D-LC is (1). Transferability and robustness across different laboratories have not yet been demonstrated on the global scale, and 2D-LC also requires special hands-on experience, equipment, and software that are not widely available like they are for 1D-LC. Additionally, from a regulatory perspective, one or two independent 1D-LC methods are usually sufficient to provide the data needed to meet the analytical target profile of a small molecule pharmaceutical product. As a result, to the best of our knowledge there are no published articles in the literature providing guidelines about how to develop a periodic 2D-LC system suitability test (SST) capable of ensuring the quality of generated results and triggering early alarms for the need to replace a column or fix the instrument components before running real samples. Having a generic SST in place is important to enable routine use as well as to ensure consistent and desirable performance of 2D-LC as a whole integrated system.

Role of System SSTs for Chromatography Methods

The United States Pharmacopeia (USP) Chromatography General Chapter <621> provides guidance for the implementation of chromatographic methods in the pharmaceutical industry (7). It states: *System Suitability Tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.*
The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.”

Usually, analyte mixtures used in SSTs for conventional 1D-LC are designed to contain analytes that can be resolved with good peak shapes and acceptable symmetry or tailing factors (8,9). The situation with 2D-LC is different in the sense that some additional requirements need to be met. At a minimum, the mixture must contain two analytes that are well separated in the first dimension, but it is also helpful to have additional compounds that are both coeluted in the first dimension and separate in the second dimension. The behavior of these particular compounds can be used to evaluate and monitor the performance of both separation dimensions at the same time. In addition to the implementation of this 2D-LC SST mixture to monitor system performance, the separation of peaks in the second dimension can help to initially adjust system parameters to improve the performance of the 1D separation before utilizing the 2D-LC system in routine pharmaceutical analysis. That is to say, selecting and examining the effect of various 1D chromatographic conditions, such as injection volume (volume of sampling loops), column dimensions, gradient, and flow rate on the peak shape, efficiency, and resolution, when collected 1D fractions of coeluted analytes are transferred to the second dimension.

Development of a SST Method for 2D-LC
Selection of Test Analytes
Selecting proper SST test analytes is essential to ensure the reliability and accuracy of the data generated by the 2D-LC system. Because the system is comprised of two dimensions integrated together through an online interface (single or multiple valves), the probes should be sensitive to the performance of both dimensions. We selected test analytes to fulfill the following criteria:

- Good UV absorbance (because the scope here is for 2D-LC systems with UV detectors only)
- Good solubility in common solvents;
- Commercially available at reasonable cost;
- Span a range of hydrophilicity and hydrophobicity that reflects the range of properties that might be encountered in future, real samples;
- Most importantly, respond differently to the selectivities used in the two-dimensional reversed-phase separation (for example, a pair of analytes were coeluted in the first dimension, but is separated in the second dimension).

With these criteria in mind, a four-component mixture was designed such that two peaks are observed in the 1D separation, and each of the two peaks contains a pair of coeluted compounds. The structures of these molecules are shown in Figure 1, and representative chromatograms are shown in Figure 2. The first early eluting peak contains a co-

![Figure 1: Structures of the SST test analytes (paraxanthine, theophylline, ketoprofen, and β-estradiol). Properties were calculated using PerkinElmer ChemDraw Professional software (v.16.0.1.4(77)).](image)

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TABLE I: Key 2D-LC performance metrics monitored over the period of one year

<table>
<thead>
<tr>
<th>Time of SST (months)</th>
<th>Paraxanthine $t_R$ (min)</th>
<th>Theophylline $t_R$ (min)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.35</td>
<td>2.40</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>2.35</td>
<td>2.40</td>
<td>1.8</td>
</tr>
<tr>
<td>8</td>
<td>2.34</td>
<td>2.40</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>2.34</td>
<td>2.40</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The two hydrophilic compounds are structurally similar analogues that are difficult to separate, whereas the two hydrophobic compounds were chosen to be coeluted under the conditions of the $^1D$ gradient. Generic chromatographic conditions (that is, flow rates, gradient slopes, and solvents) are used for both dimensions, and the two stationary phases used have complementary chemistries. The first is a traditional C18 column and the phase is used as a starting point in development of reversed-phase (RP) methods in our laboratory (that is, our platform method). The second is a polar-embedded RP column that is known to have selectivity highly complementary to most C18 columns (10,11).

Establishment of Acceptance Criteria

The SST criteria are established to represent the minimum acceptable 2D-LC system performance rather than typical performance levels. This ensures that the predefined acceptance criteria are neither too wide—which would prevent detection of unexpected system problems—nor too narrow, which could trigger unnecessary alarms. The acceptance criteria we have settled on for this SST are as follows:

1. The number of peaks within the selected integration time window—two $^1D$ peaks in the 0.3–0.9-min window, and four $^2D$ peaks in the 2.0–3.0-min window for peaks 1a and 1b, and 4.0–5.0 min for peaks 2a and 2b;
2. The relative standard deviation (RSD) of retention time ($t_R$) and peak area <2% for triplicate injections;
3. A USP tailing factor <2; and
4. A USP resolution >1.5 for peaks in the second dimension.

Performance of the SST So Far (One Year)

We monitored the performance of our developed SST with respect to $t_R$ and resolution of the more challenging hydrophilic pair over the period of one year as shown in Figure 3 and Table I. These data show consistent performance of the 2D-LC system for its intended purpose as a whole integrated system, and they show the convenience of this developed SST over an extended period of use.

Example of a Failed 2D-LC SST

Several 2D-LC SST failures could be attributed to various causes such as problems with the autosampler, interface valves, pumps, column aging, and mobile phase preparation errors. The aforementioned acceptance
criteria are sensitive to these errors and may trigger early alarms that prevent the acquisition of unreliable data that lead to inaccurate results and the need to retest samples. Figure 4 shows an example of SST failure in our laboratory where the 1D performance was out of specification. Figure 4 shows 1D chromatograms from two SST injections that illustrate a sudden significant difference in performance. With respect to the acceptance criteria, it is clear that the peak number check fails to meet the required number within the specified integration time window because of the large shift in $t_r$ of both peaks (blue trace in the chromatogram, where each peak represents a pair of coeluted peaks).

Investigating the cause of this problem resulted in the identification of a small leak in the pump B head, which delivers the organic solvent component of the mobile phase. The leakage was too small to trigger the built-in leak sensor of the pump. Therefore, if the SST did not reveal this issue, it would have passed undetected with negative consequences for the reliability of the 2D-LC result. In this context, a difference in the peak shape (broader peak) and the integration time window (i.e., the time at which the peak is considered to be fully integrated) may be observed. A notable point to mention here is that the peak shape was also another indicator of poor performance, which could be captured by either adopting peak efficiency ($N$) as an additional SST acceptance criterion or by the visual examination of the chromatogram. The peak shape (broader peak) and $N$ for the coeluted hydrophilic pair was more affected than the later one because a small difference of the delivered gradient has a significant effect on the initial low fraction of organic solvent in the mobile phase (1%) that elutes polar analytes.

Summary
In this installment of “LC Troubleshooting,” we described the development and implementation of a SST for multiple heart-cut 2D-LC. Such SSTs are routinely used for conventional LC systems to verify that the performance of the LC system is suitable for use in analyzing pharmaceutical samples. However, there has been little discussion of SSTs for 2D-LC in the literature to date. The 2D-LC SST described here is intended to serve as a starting point and will likely become more sophisticated over time as we learn more about the criteria needed for the most effective use of the SST. Initial results from the regular use of the SST over a period of one year both demonstrated consistent performance of the 2D-LC method and identified one instance where the system performance was not suitable because of a small leak in the 1D pump that was subsequently repaired to restore system performance.

Acknowledgment
The authors would like to thank Qinggang Wang for his feedback and suggestions while preparing this article.

References
(1) A. Clarke and A. Ehkirch, LCGC Europe 33, 136–150 (2020).
(5) C.J. Venkatramani, LCGC North Am. 31(s10), 22–29 (2018).

Figure 4: Comparison of 1D chromatograms from cases (a) where the system was operating properly, or (b) where a small leak in the 1D flow path caused a significant shift of the 1D peaks out of their respective sampling windows. The failure of the SST triggered a repair of the 2D-LC to fix the leak.
Improved Performance of UHPLC–MS Hyphenated Systems

An ultrahigh-pressure liquid chromatography–mass spectrometry (UHPLC–MS) research prototype instrument was built to improve the resolution power and the usability of conventional LC–MS hyphenated instruments for routine analyses in pharmaceutical applications. The improved characteristics of this UHPLC–MS system include: 1) the dramatic reduction of post-column sample dispersion; 2) the adoption of vacuum jacketed columns (VJC) for the reduction of undesirable radial temperature gradients across the column diameter; and 3) the presence of a column outlet end nut heater to refocus the distorted peaks prior to analyte ionization. The benefits of each of these added features are analyzed with a rigorous approach from a peak broadening perspective. A 2x improvement in peak capacities recorded with this prototype UHPLC–MS system compared to a standard system (Acquity UHPLC I-class/Xevo TQ-S) is illustrated for the gradient separation of seven small pharmaceutical compounds using a 2.1 mm x 100 mm column packed with sub-2-μm core-shell particles (1.6 μm Acquity UHPLC Cortecs C18 column).

Fabrice Gritti, Sornanathan Meyyappan, Wade Leveille, and Jason Hill

Hyphenated liquid chromatography–mass spectrometry (LC–MS) systems have become the most widespread and routine separation detection techniques in metabolomics, proteomics, and lipidomics, as well as in the pharmaceutical and agricultural industries (1–10). The move from LC–MS to ultrahigh-pressure liquid chromatography (UHPLC)–MS during the 2000s after adopting small volume columns (2.1 mm i.d., 5–10 cm long) packed with very fine particles (sub-2-μm) has revealed the resolution limit of standard LC–UV and LC–MS hyphenated instruments (11–17). In gradient elution, the observed loss of peak resolution relative to the expected performance of the column alone is essentially because of the large post-column sample dispersion (18). The transport of the analyte from the LC column outlet to the electrospray ionization (ESI) source usually occurs via several lengthy connecting tubes and a divert (infusion) valve, which together generates a significant amount of sample dispersion relative to that caused by the column itself. Peak capacity is then severely reduced unless a solution is found to eliminate or at least minimize post-column dispersion (13,16,17).

The practical problem encountered in conventional UHPLC–MS instruments is that the position of the column oven in the LC instrument stack is remote from the ESI source of the mass spectrometer. Therefore, the UHPLC-to-MS connecting tube dimensions are unsuitable for UHPLC columns to perform at their best. For instance, the Acquity UHPLC I-class/Xevo TQ-S hyphenated system is equipped with a 60 cm x 100 μm tube connecting the column outlet to a divert/infusion valve and a 75 cm x 125 μm tube connecting this valve to the ESI source: overall, they account for a total post-column dispersion variance close to 13 μL² (small molecules, 1 mL/min). In contrast, the dispersion variance of a 2.1 mm x 100 mm column packed with 1.6 μm core-shell particles is only of the order of 3 μL² in gradient elution for a retention factor approximately 1 at elution. In practice, it amounts to observing a peak capacity that is approximately half that theoretically expected from new generations of UHPLC columns.

This work proposes a solution by redesigning the interface between the UHPLC instrument and the mass spectrometer. In the first part of this article, a research prototype UHPLC–MS instrument is built. Its specific features are described to illustrate the main problem of excessive post-column dispersion while maintaining the integrity of the column performance. In the second part, each modification of the conventional UHPLC–MS instrument is justified from a sound and quantitative approach. Finally, it is illustrated from a concrete pharmaceutical separation problem how much gain in peak resolution and sensitivity the users can benefit from the prototype system relative to conventional UHPLC–MS instruments.
Materials and Methods

LC–MS Gradient Experiments

Seven pharmaceutical compounds (acetaminophen 10 µg/L, valine-tyrosine-valine 2.5 µg/L, leucine-enkephalin 2.5 µg/L, verapamil 0.6 µg/L, reserpine 1.9 µg/L, and terfenadine 0.6 µg/L) were prepared in a mixture of acetonitrile and water (75/25, v/v) and injected under a gradient condition in a 2.1 mm x 100 mm Acquity UHPLC Cortecs C18 1.6 µm column (Waters). The LC system is the Acquity UHPLC I-class equipped with a binary solvent manager and a fixed loop sample manager (Waters). The vacuum jacketed columns were outfitted in house with custom vacuum sleeves. The injection volume, the flow rate, and the inlet eluent temperature were set at 0.3 µL, 0.6 mL/min, and 50 °C, respectively. Two solvent bottles (A and B) were prepared: solvent bottle A contained 0.1% (v/v) formic acid in water, and solvent bottle B contained 0.1% (v/v) formic acid in acetonitrile. The volume fraction of B was programmed to increase linearly from 2% to 98% over 3 min. The analyte detection was performed under the multireaction monitoring (MRM) mode of the Xevo TQ-S mass spectrometer (Waters). The dwell time, the capillary voltage, the source temperature, the desolvation temperature, the desolvation flow rate, and the cone flow rate were fixed at 3 ms, 3.0 kV, 120 °C, 600 °C, 1000 L/hr, and 150 L/hr, respectively, for all compounds. All the other MS detector details specific to the analyte (cone voltage, masses of precursor, and product ions) are given in Table I.

LC–UV Isocratic Experiments

In this series of isocratic experiments, the same column and UHPLC system as those described above were used, except the column outlet was connected to the optical TUV detector (250 nL volume, 254 nm wavelength, 40 Hz sampling rate) using a 15 cm x 75 µm connecting tube. The sample mixture is a solution of acenaphthene (0.2 g/L) and octanophenone (0.1 g/L) prepared in a 25:75 (v/v) mixture of water:acetonitrile, which was also the solvent composition used as the mobile phase. The injection volume, the flow rate, and the inlet eluent temperature were set at 0.5 µL, 0.6 mL/min, and 50 °C, respectively.

Simulation of the Temperature Profiles

In this work, the radial temperature profiles (from r = 0 to r = 1.05 mm) expected across the packed bed diameter at the column outlet (z = 10 cm) were simulated under a steady-state temperature regime by considering the most realistic design and structure of the vacuum jacketed column (VJC) as shown in Figure 2. All the modeling and post-analysis were done using Ansys Fluent 2020 R2 software.

Briefly, a minimum element size of 75 µm was selected for the packed bed zone and features under 5 µm were not featured. A total of 6,940,547 nodes and 14,984,108 elements were
A laminar viscous model with viscous heating was used for the eluent mixture (acetonitrile:water 50/50, v/v). The thermal profile inside the packed bed zone was modeled assuming an energy source term for the eluent mixture without modeling the actual details of the macroporous zone. The viscous heat that was generated was calculated from the total pressure drop, the volumetric flow rate, the fluid inlet temperature, and the thermal expansion coefficient of the eluent. The surrounding air was modeled as an incompressible ideal gas. The inlet boundary condition is set by the inlet superficial linear velocity of the eluent at a fixed temperature of 50 °C. The post-column heater was set at a uniform and constant temperature boundary condition, which was increased from 60 °C to 75 °C in subsequent simulations. Table II lists all the necessary physicochemical parameters assumed in the calculation.

Results and Discussion

Overall Description of the Novel Research Prototype UHPLC–MS System

The key motivation underlying the proposed design of the UHPLC–MS research prototype system is to eliminate most of the post-column sample dispersion occurring from the column outlet to the divert (infusion) valve and the ESI source. Figure 1 shows schematics of the standard and modified systems (left) and photography of the research prototype system (right).

The chromatographic column (2.1 mm x 100 mm, 1.6 μm Cortecs C18 superficially porous particles) was placed on a prototype column holder fixed directly onto the atmospheric pressure ionization (API) source enclosure. The post-column tubing assembly of the modified UHPLC–MS system was optimized to minimize the post-column dispersion. Overall, the post-column dispersion variance is reduced from approximately 13 μL² (for a 60 cm x 100 μm tube + a divert
The relatively large precolumn dispersion of the APH (~6 μL²) is not a severe issue in gradient elution because of the sample focusing at the column head. The eluent temperature at the column inlet can still be controlled over a wide range from room temperature to 90 °C. The anticipated problem of placing the column outside the standard air oven compartment (the oven temperature is usually equal to the inlet eluent temperature) is the cooling of the column wall because of heat exchange with the surrounding air at room temperature. Consequently, the lack of control of the column wall temperature and the loss of column performance because of the column-to-laboratory temperature mismatch requires a solution to avoid the formation of undesirable radial temperature gradients (19–22). Therefore, the column was wrapped in a vacuum jacket (see Figure 2), described in great detail in two previous special issues of LCGC North America (23,24). The benefit of the VJC is that the heat delivered at the column inlet (25 °C < Tinlet < 90 °C) can no longer be dissipated by heat exchange between the column wall (hot, T ≥ 25 °C) and the surrounding air (cool, room temperature), so the overall column temperature can be controlled.

However, small residual heat leaks are detected at the column ends and negatively affect the column performance (24). Therefore, an outlet end nut heater is placed at the outlet of the column to reverse the heat flow direction from outside to inside the column. The anticipated effect is to refocus the tailing peaks and recover the full column performance. The benefit of the outlet end nut heater maximizing column performance will be demonstrated and explained in detail in the next section (using an infrared [IR] camera and simulation of the temperature profiles across the packed bed).

**The Rationale Behind the UHPLC–MS System Modifications**

In this section, it is justified from scientific arguments (analysis of the peak volume variance, IR surface temperature profiles, simulation of the column temperature profiles, and explanation of the observed change in peak asymmetry) why the configuration of standard LC–MS systems had to be modified. It is explained how the gradient performance of current LC–MS systems is improved every step of the way by 1) reducing the post-column sample dispersion; 2) wrapping the column in a vacuum jacket; and 3) delivering heat locally to the column outlet end fitting using an end nut heater.

**Post-Column Dispersion**

Figure 3 justifies why the post-column modifications are critical in LC–MS when operating a narrow-bore 2.1 mm i.d. x 100 mm long column packed with 1.6 μm Cortecs-C18 core-shell particles. Each solid-colored curve represents all the possible combinations of post-column dispersion (y-axis) and retention factor, k_{elution}, at elution (x-axis), leading to the same apparent gradient peak capacity equal to a fraction, p (0.32 < p < 0.98), of the maximum theoretical gradient peak capacity expected when the post-column dispersion is strictly equal to zero. Assuming that the gradient compression factor is close to 1 (that is, small molecules) and the retention factor at elution is 1, then a standard UHPLC–MS system with a post-column sample dispersion equal to 13 μL² would lead to a frac-
The laboratory air. Therefore, radial temperature gradients are formed across the column diameter and negatively affect its performance. To cope with that problem, the column was wrapped in a vacuum jacket to prevent the heated eluent from cooling off as it flows further down along the column length. The principle of this ideal vacuum jacket column has been presented and explained in several previous communications (25,26). Not only is the eluent temperature maintained constant (at low flow rates, no viscous heating) along the column length, but it also preserves the integrity of the column performance when viscous heating becomes significant (high flow rates and pressure drops). The amplitude of the radial temperature gradients across the column inner diameter is minimized, and the column efficiency remains close to its theoretical maximum.

Figure 4 shows that the peak capacity increases from 187 (“no sleeve” configuration) up to 209 (“with sleeve” configuration) after the column is wrapped with the vacuum jacket. The relative gain of peak capacity increases to 79%, which is closer to 116% (as expected theoretically) than the 60% reported in the absence of a vacuum jacket. The remaining gap to be covered is explained when looking at the surface of the VJC using an IR camera.

**Outlet End Nut Heater**

The IR image in Figure 5 is a 2.1 mm x 100 mm stainless steel (SS) column packed with 1.6 μm Cortecs-C18 particles installed in the “with sleeve” UHPLC–MS system configuration. The inlet temperature is kept free at room temperature (~23 °C), the flow rate of pure water was fixed at 0.65 mL/min, and the pressure drop was recorded close to ΔP = 12,000 psi. The heat capacity of water at room temperature is \( C_p = 4.2 \times 10^6 \text{ J/m}^3\cdot\text{K} \), and its coefficient of thermal expansion is \( \alpha = 3.1 \times 10^{-4} \text{ K}^{-1} \).

Accordingly, if the column is strictly operated under adiabatic conditions,
the expected increase, $\Delta T$, of the water temperature from the column inlet to the column outlet is expected to be $\Delta T = (1-\Delta P/C_p) = +18 \, ^\circ C$. The red color at the column outlet end fitting revealed by the IR camera is an indication of heat leaks. It is noteworthy that the heat somewhat diffuses back towards the column inlet along a short section of the vacuum jacket. This IR camera image confirms that the whole column is not entirely thermally insulated and is still exchanging some heat with the surrounding laboratory air environment. The same phenomenon occurs at the column inlet when the imposed inlet temperature is above laboratory temperature. The overall result is a measurable loss of column performance caused by the presence of undesirable radial temperature gradients at both the inlet and the outlet regions of the column. The mechanism underlying this performance loss was previously proposed in a short communication (24).

A physical solution to this problem is proposed and consists of placing an end nut heater at the column outlet in direct contact with the exposed SS surface of the outlet end fitting. The anticipated effect is to refocus the distorted peak shape as much as possible. The graph in Figure 5 considers the case where the temperature of the inlet mobile phase is fixed at 50 °C. The experimental section gives all the experimental details regarding the gradient conditions and the measurement of the reported peak capacity. The temperature, $T_{outlet}$ imposed by the outlet end nut heater was then increased stepwise from room temperature (“off” or passive) to 40, 50, 60, 70, and 80 °C. The most remarkable result is that the observed peak capacity passes through a maximum (249) for $T_{outlet} = T_{outlet, opt} = 60–70$ °C. The gain of peak capacity relative to that observed for the standard UHPLC–MS system (117) is now equal to +113%, close to the maximum of +116% expected theoretically. Overall, this is because of 1) the drastic reduction of the post-column sample dispersion from approximately 13 $\mu$L$^2$ down to 0.3 $\mu$L$^2$ and 2) the elimination of the negative effect of radial temperature gradients on column performance. The proposed explanation is as follows: when $T_{outlet}$ is not sufficiently high (<60 °C), the temperature of the bed in the inlet and outlet regions of the column is more prominent in the center than in the wall region, and the loss of column performance is maximum. When $T_{outlet}$ is optimum (~70 °C), the wall temperature is higher than the bed temperature at the column outlet (that is, the direction of the heat flow is reversed from outside to inside). The initial
Peak distortion occurring at the column inlet is somewhat compensated at the column outlet according to a refocusing phenomenon (27–29), leading to nearly symmetric peaks. Finally, if $T_{\text{outlet}}$ is too high (>75 °C), the heat flow imposed in the outlet region of the column is too large and overcompensates for the initial peak tailing (the peaks are then fronting). This mechanistic interpretation is well supported by 1) the calculation of the radial temperature profiles at the column outlet (Figure 6), according to the simulation approach described in the method section, which reveals that the amplitude of the radial temperature gradient is minimized for $T_{\text{outlet}}$ ~70 °C and 2) from the evolution of the peak asymmetry (from tailing to symmetrical, and to fronting) observed under isocratic elution when increasing $T_{\text{outlet}}$ from 50 °C to 80 °C (Figure 7).

**Application and User Benefits**

In this section, we illustrate the practical advantage of performing gradient elution with the modified UHPLC–MS system. The experimental section details the experimental conditions (column, gradient, flow rate, nature and concentration of the samples injected, and temperatures). In particular, Figure 8 compares the observed peak shapes (MS detection, MRM mode, and the total ion count) of three of the seven analyte products (the precursor ions are verapamil, reserpine, and terfenadine), which are recorded under the same four UHPLC–MS configurations as those discussed in the previous section (see Figure 4). After complete system modifications (from “standard” to “with sleeve 70 °C outlet”), it is remarkable that the peak widths are virtually reduced by a factor of two for all the compounds injected irrespective of their elution time. The total ion chromatogram in Figure 8 also confirms that the insulation of the column using a VJ and the application of heat at the column outlet participate both to further increase the resolution power and the sensitivity of the very same LC gradient method.
Conclusion
In this work, a research prototype UHPLC–MS system was built to significantly improve the resolution performance compared to conventional UHPLC–MS systems. The improved system integration between the column outlet and the API source was made possible by replacing the LC oven with a column support placed directly against the API enclosure. In the absence of the column oven, maintaining the full integrity of the column efficiency consisted of wrapping the column with a vacuum jacket and delivering a well-controlled heat flow at the exposed outlet of the column. At pressure drops of about 12,000 psi, the temperature of the outlet end nut heater should be approximately 20 °C higher than the inlet temperature imposed by the active eluent preheater for maximum column performance. Additionally, the risk of eluent leaks at the column inlet and outlet and poor data reproducibility caused by the improper manual column installation and the aging of column-to-tube connections at very high pressures were avoided by adopting an easy-to-use cam-actuated mechanism for the column installation.

In conclusion, the overall relative increase of the peak capacity observed was quantitatively interpreted from the combination of the drastic reduction of the post-column sample dispersion (13 μL² → 0.3 μL²) along with the elimination of most of the radial temperature heterogeneities (ΔT < 0.3K) across the packed bed diameter by adjusting the temperature of the column outlet (T_{outlet} − T_{inlet} < 20 °C at ΔP ~12,000 psi and F_r = 0.6 mL/min).

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References
(17) G. Desmet and K. Broeckhoven, TrAC 119, 115619 (2019).
(23) F. Gritti, LCGC North Am. 36(6), 18–23 (2018).

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Is the Solution Dilution? Hidden Uncertainty in Gas Chromatography Methods

Used by chemists and nonchemists alike, gas chromatography (GC) is considered mature and among the most widely used instrumental techniques for chemical analysis. Because instruments have become both more sensitive and easier to use, columns have achieved higher resolution and stationary phases have greater selectivity. As a result, gas chromatographs have taken on a “black box” view. With greater sensitivity, resolution, and advanced data handling capabilities, a new set of experimental uncertainties emerge that may not be apparent to most users, especially those who were not formally trained as analytical chemists. In this installment of “GC Connections,” we examine these uncertainties in typical GC methods, especially as they relate to quantitative analysis. We look at hidden experimental uncertainty, especially in the glassware used for sample preparation. We also comment on injection and detection with an eye toward understanding the sources of the errors. It is important to understand the experimental error and uncertainty are inherent in all analytical techniques; they can be reduced but cannot be eliminated.

Nicholas H. Snow

A n increasing portion of the chromatographic literature of today describes applications and quantitative analysis rather than fundamental advances in chromatographic techniques and principles. I am not offering an opinion about whether this is good or bad, but it is apparent. Articles describing chromatographic methods that show quantitation at parts per billion (ppb) and lower levels are now commonplace. However, much of this literature shows common mistakes and problems with experimental uncertainty in quantitative analysis. Most commonly, uncertainties, usually in the form of standard deviations, are presented with too many significant digits. Uncertainty should be expressed in the least significant digit. The uncertainty then determines the number of significant digits in the result regardless of the number of digits provided by the data system. Often, I see both uncertainties and quantitative results presented with too many significant digits.

In addition to this first challenge, the basic reporting of results often confuses the presentation of both the experimental result and the uncertainty. Classically, results larger than 10 and smaller than 0.1 should be reported using scientific notation. This guideline is often stretched to 0.01–100, but it should not be stretched further. Strict adherence to this rule by itself reduces confusion in result reporting and significant figures.

An additional challenge relates to the common use of the standard deviation and relative standard deviation (RSD) as a figure of merit for the precision of chromatographic results. As we know from basic population statistics, one standard deviation from the mean indicates that approximately 68% of results should be expected to be in that range. The true experimental uncertainty is larger, typically around two standard deviations, which would include approximately 95% of the results, or three standard deviations, which would include about 99.7% of expected results. However, most quantitative analytical chemistry experiments do not generate enough results for population statistics, so these generalizations do not hold. Classically, as taught in quantitative and instrumental analysis texts for decades, a 95% confidence interval about the data is best used as the experimental uncertainty, which for small populations of data would be significantly wider than one standard deviation. In much of the chromatography literature, this additional step is not performed. In short, much of the literature underestimates experimental uncertainty and overestimates significant figures.

This observation is not to denigrate the work of the many scientists developing and optimizing gas chromatography (GC) methods. Because GC has become increasingly popular over the years, it has gone from a technique largely performed by formally trained analytical chemistry specialists to a much broader range of scientists, who may not have had formal training in quantitative analysis and analyzing experimental error and uncertainty. In the rest of this installment, we examine some common cases where experimental uncertainty arises in GC methods.

Dilute and Shoot

Even simple “dilute and shoot” methods have hidden experimental uncertainties. We start with this case because nearly all
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other sample preparation techniques and methods involve dilution in the preparation of samples and standards. Many analysts make the incorrect assumption that volumetric glassware is perfect. Figure 1 shows a close-up look at a typical “Class A” 100 mL volumetric flask, with the class indication circled. For any volumetric glassware, it is best to use Class A glassware and avoid any glassware that does not indicate class or has the markings worn off. If you look closely, you can note the uncertainty value of ±0.08 mL provided in the printing on the flask. Although the uncertainty seems small, the rules for propagation of errors tell us that each additional dilution step will add to the experimental uncertainty of the result.

Table I shows the uncertainties involved with some Class A volumetric flasks and transfer pipets. It illustrates one of the interesting problems in analytical method development. Because we are pressured to reduce the use of solvents, smaller volumes introduce higher relative experimental errors in each step. Table I shows that there is flask-to-flask and pipet-to-pipet experimental uncertainty involved with volumetric glassware that must be considered when developing methods. Drawing a sample to the mark in a pipet or filling to the mark in a flask may not deliver the exact volume stated on the device. To this end, experimental procedures for gravimetrically verifying the volume delivered by a pipet or contained in a flask have been published by the National Institute of Standards and Technology (NIST) (1).

Consider the experimental uncertainty involved in a 1:100 dilution of a pre-prepared stock standard solution by two different procedures. In the first procedure, a 1 mL class A transfer pipet is used to deliver 1 mL of the stock solution into a 100 mL class A volumetric flask and the flask is filled to the mark with the dilution solvent. In the second procedure, a 1 mL class A transfer pipet is used to deliver 1 mL of the stock solution into a 10 mL class A volumetric flask and is then filled to the mark with the dilution solvent. In pro-

---

**FIGURE 1:** Close-up photograph of a 100 mL Class A volumetric flask.

**TABLE I:** Class A volumetric glassware tolerances

<table>
<thead>
<tr>
<th>Device</th>
<th>Volume (mL)</th>
<th>Class A Tolerance (± mL)</th>
<th>How Volume is Expressed (mL ± mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volumetric flask</td>
<td>10</td>
<td>0.02</td>
<td>10.00 ± 0.02</td>
</tr>
<tr>
<td>Volumetric flask</td>
<td>25</td>
<td>0.03</td>
<td>25.00 ± 0.03</td>
</tr>
<tr>
<td>Volumetric flask</td>
<td>50</td>
<td>0.05</td>
<td>50.00 ± 0.05</td>
</tr>
<tr>
<td>Volumetric flask</td>
<td>100</td>
<td>0.08</td>
<td>100.00 ± 0.08</td>
</tr>
<tr>
<td>Volumetric flask</td>
<td>250</td>
<td>0.12</td>
<td>250.0 ± 0.1</td>
</tr>
<tr>
<td>Volumetric pipet</td>
<td>1</td>
<td>0.006</td>
<td>1.000 ± 0.006</td>
</tr>
<tr>
<td>Volumetric pipet</td>
<td>5</td>
<td>0.01</td>
<td>5.00 ± 0.01</td>
</tr>
<tr>
<td>Volumetric pipet</td>
<td>10</td>
<td>0.02</td>
<td>10.00 ± 0.02</td>
</tr>
<tr>
<td>Volumetric pipet</td>
<td>25</td>
<td>0.03</td>
<td>25.00 ± 0.03</td>
</tr>
<tr>
<td>Volumetric pipet</td>
<td>50</td>
<td>0.05</td>
<td>50.00 ± 0.05</td>
</tr>
</tbody>
</table>

**TABLE II:** Calculation of experimental uncertainty from the glassware for two 1:100 dilution schemes. Assumes ± 0.1% relative uncertainty in the concentration of the original stock solution, use of Class A glassware, and use of the classical dilution expression: \( M_1 V_1 = M_2 V_2 \).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Glassware Used</th>
<th>Glassware Percent Uncertainties (%)</th>
<th>Uncertainty in Final Dilution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 x 100 mL volumetric flask 1 x 1 mL volumetric pipet</td>
<td>0.08% (flask) 0.60% (pipet)</td>
<td>0.6%</td>
</tr>
<tr>
<td>2</td>
<td>2 x 10 mL volumetric lask 2 x 1 mL volumetric pipet</td>
<td>0.20% (flask) 0.60% (pipet)</td>
<td>0.9%</td>
</tr>
</tbody>
</table>
Procedure 1, a total of approximately 100 mL of dilution solvent is used; in procedure 2, the amount of dilution solvent is reduced by 80% to approximately 20 mL. Table II shows the propagation of errors comparison of procedures 1 and 2. Detailed equations and discussion of propagation of errors is not provided here but can be referenced in nearly any textbook on analytical chemistry or instrumental analysis (2). In that chapter, I share a specific example of a propagation of error analysis applied to a pharmaceutical analysis method. Table II shows that the serial dilution procedure increases the uncertainty from 0.6% to 0.9% over the single dilution, which is an increase of 50% in the uncertainty.

As seen in Table II, the serial dilution procedure, which most analysts would prefer today because it uses far less solvent, has significantly higher experimental uncertainty, and this experimental uncertainty would then be added to any uncertainty in the stock standard. Note that when making extremely low concentration standards, which is a common need with the extremely sensitive methods and instruments of today, the experimental uncertainty involved in using even the most precise available glassware to prepare standards and samples in multiple dilution steps can lead to relatively large uncertainties that may reduce the number of significant figures in the experimental results. As a result, it is likely that any determinations at sample concentrations lower than parts per million (ppm) level should be reported with no more than one or two significant figures.

### Uncertainty In Sample Injection

The inlet and injection process in GC is well-known to provide hidden uncertainty, as discussed in two recent “GC Connect” articles. Table III shows the propagation of uncertainty for simple operations with impact on relative uncertainty and significant digits of the result using two artificial data points: 110.0 ± 0.2 and 100.0 ± 0.2.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Raw Result</th>
<th>Uncertainty</th>
<th>Final Result</th>
<th>Final Uncertainty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original data</td>
<td>100.0</td>
<td>0.2</td>
<td>100.0 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Addition</td>
<td>210.0</td>
<td>0.3</td>
<td>210.0 ± 0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Multiplication</td>
<td>1.100 x 10^4</td>
<td>30</td>
<td>1.100 x 10^4 ± 3 x 10^1</td>
<td>0.3</td>
</tr>
<tr>
<td>Subtraction</td>
<td>10.0</td>
<td>0.3</td>
<td>10.0 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>Division</td>
<td>1.100</td>
<td>0.003</td>
<td>1.100 ± 0.003</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**TABLE III:** Propagation of uncertainty for simple operations with impact on relative uncertainty and significant digits of the result using two artificial data points: 110.0 ± 0.2 and 100.0 ± 0.2.
tions” installments and a classic book (3–5). As discussed above, as pipets and sample delivery system volumes get smaller, relative experimental uncertainty in the delivered volume increases. Most microliter volume syringes are accurate to approximately 1% of their nominal maximum volume. A typical 10 μL syringe has graduations representing 0.1 μL. Interestingly, it is common to inject 1 μL of liquid using a 10 μL syringe, providing a nominal error of up to 10% in the accuracy of the delivery. Furthermore, the graduations on the syringe barrel do not account for the internal volume of the syringe needle, which is approximately 0.6 μL. Therefore, our 1 μL injection using a 10 μL syringe may be an injection of 1.5–1.7 μL, depending on the accuracy of the syringe.

With the use of an auto-injector and proper maintenance, a syringe can remain highly reproducible for hundreds or possibly thousands of injections. Syringe manufacturers provide quick guides on how to take care of and perform maintenance on syringes (6) that include suggestions for extending syringe lifetime. With the high precision of auto-injectors, users can typically expect injections with less than 1% RSD in the resulting peak areas from injection to injection. Difficulty can arise when the syringe is changed; you can expect peak areas to vary by several percentage points up or down from the original syringe. With effective calibration, and either internal or external standard, this change may not be noticed unless working at or near the limit of detection (LOD) or limit of quantitation (LOQ), where a few percent of reduced injected sample volume might move results lower than the LOD or LOQ.

Uncertainty in Detection and Quantitation

In a recent installment, we discussed several hidden challenges in measuring the LOD for an instrument or as part of the method validation process (7). We saw that the classical International Union of Pure and Applied Chemistry (IUPAC) calculation for LOD includes terms only related to uncertainty in the measured signal, not in the calibration curve. We saw an alternate calculation, based on propagation of errors, for determining the LOD that includes terms for uncertainty in both the slope and y-intercept of the calibration curve. Earlier in this column, we discussed uncertainty in simple “dilute and shoot” procedures, which are similar to the procedures used to generate calibration curves.

In general, greater increases in experimental uncertainty from calculations occur when subtraction and division are used in equations and formulas. In both cases, the calculation result becomes smaller while the uncertainty becomes larger. Table III illustrates this principle with some simple calculations, in which the values 1.100 x 10^2 ± 0.2 and 1.000 x 10^2 ± 0.2 are combined by addition, subtraction, multiplication, and division. Note that both initial values would be seen as highly precise. As seen in Table III, the original data has a relative uncertainty of 0.2%. When the data points are multiplied or divided, the uncertainty is determined from the relative uncertainties, so the final uncertainty is the sum on a relative basis for both. Since experimental uncertainties are additive for addition and subtraction, adding the two data points results in a decreased relative uncertainty as the result increases faster than the uncertainty. However, subtracting them results in a much greater relative uncertainty, since the result is now smaller and the uncertainty larger. In this case, the four significant figure original data is reduced to three in the result. The relative experimental uncertainty increases from a fraction of a percent to nearly 3% in the subtraction case. When I examine GC methods with unsatisfactory reproducibility, the first places I look for the source of the problem are calculations and glassware choices, followed by injection and detection.

Summary

Error analysis and the consideration of experimental uncertainty may seem like a chore. However, it is one of the most important aspects of method development, optimization, and validation. GC instruments and data systems are highly precise, and they generate raw data that may have three, four, or more significant figures. In the past, we would look first at potential instrumental challenges when troubleshooting and optimizing problems with precision and accuracy. Today, I look first at the glassware and calculations, followed by the instrument. Chromatographers should be cautious and recognize that there are hidden uncertainties in almost all quantitative chromatographic methods that may increase experimental uncertainty, reduce the number of significant figures, and make the data coming from the data system look more precise and accurate than it really is.

References


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FOCUS ON FOOD ANALYSIS

Quantitative Analysis of PFAS in Milk, Infant Formula, and Related Ingredients Using Liquid Chromatography–Tandem Mass Spectrometry

Per- and polyfluoroalkyl substances (PFAS) are a large group of anthropogenic chemicals that have been applied in a wide range of industrial, commercial, and domestic products since the 1950s. Because of their toxicity, persistence, and bioaccumulation potential, PFAS have become global environmental pollutants. Besides the environment, the food chain represents another source of exposure, and the risk to consumers related to the presence of PFAS in foods has recently become of increased interest. In this respect, whole milk, infant formula, and ingredients used in infant formula production represent important foodstuffs that require sensitive methods with reporting limits at low parts per billion (ppb) levels or lower for multiple PFAS. This article summarizes optimization experiments and the validation of a complete workflow, including sample preparation and a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of 16 priority PFAS analytes listed by the U.S. Food and Drug Administration (FDA).

Lukas Vaclavik, John Schmitz, Matthew Eckert, Katerina Mastovska, and Tarun Anumol

There are several ways humans can be exposed to per- and polyfluoroalkyl substances (PFAS). These include exposure during normal use of a wide range of commercial consumer products that have been treated with fluoropolymers or PFAS—for example, to make them non-stick, water- or stain-repellent. Drinking water can also be a source of exposure in locations where these chemicals have contaminated water supplies. Finally, humans can be exposed to these chemicals through contaminated food. When it comes to contamination of food with PFAS, there are two main processes to consider. Bioaccumulation of PFAS in both aquatic and terrestrial food chains is a result of using contaminated water and soil for growing food. Additionally, contamination of finished products and ingredients may occur because of the use of PFAS containing materials for food packaging and food processing. The presence of PFAS in food and related health risks to consumers have become of increased concern to many regulatory agencies worldwide that have initiated monitoring programs. Besides various foods of animal and plant origin, infant formula and baby food represent another important food commodity of concern regarding the vulnerability of infants and toddlers (1,2).

Rapid, sensitive, and reliable analytical techniques are required for the monitoring of PFAS in the above matrices. Liquid chromatography coupled to tandem quadrupole mass spectrometry (LC–MS/MS) with electrospray ionization (ESI) operated in negative ionization mode has been the instrumental technique most frequently used for the analysis of PFAS in foods as well as in other biotic matrices (3).

In this study, an LC–MS/MS assay based on the U.S. FDA method C-010.01 was optimized and validated for the analysis of 16 PFAS compounds in whole milk, ready-to-feed (RTF) and powdered infant formula, milk powder, soybean oil, and maltodextrin matrices (4).

Experimental Standards, Reagents and Samples
The target PFAS and the stable isotope-labeled internal standards employed in this study are listed in Table I. The PFAS standards, including isotopically labeled analogs, were purchased from Wellington Laboratories. The formic acid (99%, for analysis) was from Thermo Scientific. LC–MS grade ammonium formate, acetonitrile, and methanol and were from MilliporeSigma. LC–MS grade isopropanol and HPLC grade hexane were from Thermo Fisher Scientific. Ultrapure water (UPW) was obtained from Elga Purelab Ultra purification system. QuEChERS salts (6 g
TABLE I: PFAS compounds analyzed, with software optimized MRM parameters and stable isotope-labeled internal standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acronym</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion (m/z)</th>
<th>RT (min)</th>
<th>CE (V)</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfluoro-n-butanoic acid</td>
<td>PFBA</td>
<td>213</td>
<td>169</td>
<td>1.83</td>
<td>6</td>
<td>M3PFBA</td>
</tr>
<tr>
<td>Perfluoro-n-pentanoic acid</td>
<td>PFPeA</td>
<td>263</td>
<td>219</td>
<td>3.04</td>
<td>4</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-hexanoic acid</td>
<td>PFHxA</td>
<td>313</td>
<td>269 (119)</td>
<td>3.78</td>
<td>6,18</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-heptanoic acid</td>
<td>PFHpA</td>
<td>363</td>
<td>319 (169,119)</td>
<td>4.54</td>
<td>6,16,20</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-octanoic acid</td>
<td>PFOA</td>
<td>413</td>
<td>369 (219,169)</td>
<td>5.42</td>
<td>8,12,16</td>
<td>MPFOOA</td>
</tr>
<tr>
<td>Perfluoro-n-nonanoic acid</td>
<td>PFNA</td>
<td>463</td>
<td>419 (269,219,169)</td>
<td>6.32</td>
<td>8,12,16,17</td>
<td>MPFOOA</td>
</tr>
<tr>
<td>Perfluoro-n-decanoic acid</td>
<td>PFDA</td>
<td>513</td>
<td>469 (269,219,169)</td>
<td>7.21</td>
<td>8,16,16,20</td>
<td>MPFOOA</td>
</tr>
<tr>
<td>Perfluorobutane-1-sulfonic acid</td>
<td>PFBS</td>
<td>299</td>
<td>99 (80)</td>
<td>3.53</td>
<td>33,40</td>
<td>M3PFBS</td>
</tr>
<tr>
<td>Perfluoropentane-1-sulfonic acid</td>
<td>PFPeS</td>
<td>349</td>
<td>80 (119,99)</td>
<td>4.25</td>
<td>48,40,36</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluorohexane-1-sulfonic acid</td>
<td>PFHxS</td>
<td>399</td>
<td>80 (169,119,99)</td>
<td>5.10</td>
<td>45,35,40,40</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoroheptane-1-sulfonic acid</td>
<td>PFHpS</td>
<td>449</td>
<td>80 (169,99)</td>
<td>6.01</td>
<td>60,40,44</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluorooctane-1-sulfonic acid</td>
<td>PFOS</td>
<td>499</td>
<td>80 (99)</td>
<td>6.90</td>
<td>50,50</td>
<td>M8PFOS</td>
</tr>
<tr>
<td>4,8-Dioxa-3H-perfluorononanoic acid</td>
<td>ADONA</td>
<td>377</td>
<td>251 (85)</td>
<td>4.77</td>
<td>8,32</td>
<td>M8PFOA</td>
</tr>
</tbody>
</table>
| 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-
  heptafluoropropoxy)-13C3-propanoic acid    | HFPO-DA (Gen X) | 285                | 169 (185,119)    | 4.04     | 4,16,30| M3HFPO-DA         |
| 9-Chlorohexadecafluoro-3-oxa-    
  anonane-1-sulfonic acid                    | 9Cl-PF3ONS      | 531                | 351 (99,83)      | 7.52     | 28,40,32| M8PFHxS           |
| 11-Chloreicosfluoro-3-oxaun-    
  decane-1-sulfonic acid                    | 11Cl-PF3OuDS    | 631                | 451 (199,83)     | 9.06     | 32,30,32| M8PFHxS           |
| Perfluoro-n-[2,3,4-13C3] butanoic acid     | M3PFBA          | 216                | 172              | 1.82     | 6      | NA                |
| Perfluoro-n-[1,2-13C2]hexanoic acid        | MPFHxA          | 315                | 270              | 3.78     | 4      | NA                |
| Perfluoro-n-[13C8]octanoic acid            | M8PFHOA         | 421                | 376 (172)        | 5.39     | 8,18   | NA                |
| Perfluoro-1-[2,3,4-13C3]butanesulfonic acid| M3PFBS          | 302                | 80 (99)          | 3.53     | 41,35  | NA                |
| Perfluoro-1-hexane-[18O2]sulfonic acid     | MPFHxS          | 403                | 84 (169,103)     | 5.10     | 50,35,45| NA                |
| Perfluoro-1-[13C8]octane sulfonic acid     | M8PFOS          | 507                | 80 (99)          | 6.87     | 58,50  | NA                |
| 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-
  heptafluoropropoxy)-13C3-propanoic acid   | M3HFPO-DA       | 287                | 185 (169)        | 4.04     | 18,4   | NA                |

Sample Preparation
Sample preparation followed a modified QuEChERS method based on the U.S. FDA method C-010.01. Homogenous samples (2 g for solids and 5 g for liquids) were weighed into a 50-mL polypropylene (PP) centrifuge tube (900 mg MgSO₄, 300 mg PSA, 150 mg graphitized carbon black [GCB]) supplied by UCT. PP centrifuge tubes were from Sarstedt. Disposable PP syringes were obtained from Becton Dickinson. Nylon syringe filters (15 mm, 0.2 μm), and PP vials (1 mL) with caps were from Agilent Technologies.

Method validation was performed with the use of whole milk, RTF and powdered infant formula, milk powder, soybean oil, and maltodextrin samples obtained from local retail market and previously demonstrated to be free of target PFAS.

anhydrous MgSO₄ and 1.5 g NaCl) and dispersive-solid phase extraction (SPE) sorbents in 15-mL polypropylene (PP) centrifuge tubes (900 mg MgSO₄, 300 mg PSA, 150 mg graphitized carbon black [GCB]) were supplied by UCT. PP centrifuge tubes were from Sarstedt. Disposable PP syringes were obtained from Becton Dickinson. Nylon syringe filters (15 mm, 0.2 μm), and PP vials (1 mL) with caps were from Agilent Technologies.

Method validation was performed with the use of whole milk, RTF and powdered infant formula, milk powder, soybean oil, and maltodextrin samples obtained from local retail market and previously demonstrated to be free of target PFAS.

Sample Preparation
Sample preparation followed a modified QuEChERS method based on the U.S. FDA method C-010.01. Homogenous samples (2 g for solids and 5 g for liquids) were weighed into a 50-mL polypropylene (PP) tube followed by the addition of UPW (15 mL for solids and oils and 5 mL for liquids). Hexane (10 mL) was added to oil samples prior to aliquotting UPW. Each sample was spiked with 10 ng of isotope labeled PFAS, added with 10 mL acetonitrile and 150 μL formic acid. The tube was vortexed for 1 min. Preweighed QuEChERS salt mixture was added to the sample extract, and the mixture was immediately shaken to ensure crystalline agglomerates were broken up sufficiently. Samples were further vortexed on a multi-tube vortexer set to 1500 rpm for 5 min. Following centrifugation at >2,000 x g, the upper acetonitrile layer was transferred into a 15-mL tube while avoiding lipid layer or hexane. In the next step, pre-weighed dispersive-SPE sorbent mixture (900 mg MgSO₄, 300 mg PSA and 150 mg GCB) was added to the sample extract, and the tube was vortexed for 2 min and centrifuged (>2,000 x g, 5 min). A 5 mL aliquot of the supernatant was transferred into a fresh 15-mL centrifuge tube, and evaporated at 60 °C to near dryness using a gentle stream of nitrogen. The residues were reconstituted in 500 μL of methanol and briefly vor-
texed to mix. The sample was filtered through a 0.2 µm nylon filter into a PP autosampler vial, and capped.

**LC–MS/MS Instrumentation**

LC–MS/MS analysis of the PFAS was carried out using an Agilent 1290 Infinity II liquid chromatograph (LC), coupled with an Agilent 6495 triple quadrupole LC–MS system.

The LC parameters are listed in Table II. The 1290 Infinity II LC was equipped with an Agilent Zorbax Eclipse Plus rapid resolution high definition (RRHD) C18 column (2.1 x 50 mm, 1.8 µm) with an Agilent Zorbax Eclipse Plus guard column. Gradient elution was performed with 1 mM ammonium formate in UPW (A) and a mixture of acetonitrile and methanol (1:1, v/v) (B) at a flow of 600 µL/min. The eluent was diverted to waste during the first 1.0 min and after 10 min of the run. The total run time from injection to injection was approximately 12.5 min.

To eliminate PFAS background, an Agilent perfluorinated compound (PFC)-free high-performance liquid chromatography (HPLC) conversion kit was installed on the Agilent 1290 Infinity II LC system (5). Additionally, an Agilent Poroshell 120, 3.0 x 30 mm, 1.9 µm delay column was connected between the solvent mixer and injector module to further control potential background contamination from the mobile phases. To reduce contamination because of sorption after injection, the needle wash procedure consisted of 10-sec washes with acetonitrile:isopropanol (1:1, v/v), followed by methanol and UPW:methanol (9:1, v/v).

The LC–MS parameters are listed in Table III. The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in negative ion and multiple reaction monitoring (MRM) modes. The MRM parameters (listed in Table I) were optimized for best response using the Agilent MassHunter Optimizer software.

**Results and Discussion**

**Method Optimization**

The main focus of the optimization efforts was on high throughput of the analysis. To meet this requirement, a 50 mm ultrahigh-pressure liquid chromatography (UHPLC) C18 column with sub-2-µm particle size and a corresponding 5 mm guard column maintained at 50 °C was used. Under the optimized conditions, baseline separation of all analytes was achieved in approximately 9 min, a significant improvement over the U.S. FDA method C-010.0 (4). The analyte peak widths at the baseline were below 0.2 min. A representative chromatogram of RTF infant formula extract spiked with target analytes at a concentration of 1 µg/kg is presented in Figure 1.

Target precursor ions represented deprotonated molecules for all analytes except for hexafluoropropyl-
ene oxide-dimer acid (HFPO-DA, or Gen X) that fragmented during the ionization to form a decarboxylation product. Considerable time was spent evaluating the type and concentration of modifier in the aqueous mobile phase component A. Various concentrations of ammonium acetate, ammonium fluoride, and ammonium formate were tested. There was only a minimal impact on separation, but the type and concentration of the modifier impacted the response of certain analytes, especially HFPO-DA. When 1 mM ammonium formate was used as mobile phase A, it allowed for the best response for HFPO-DA, while not sacrificing response for the other PFAS.

**Method Validation**
The method validation evaluated selectivity, linearity, accuracy and precision, limit of quantification (LOQ), and method detection limit (MDL).

Selectivity of the method was demonstrated based on analyses of unspiked samples and a method (procedural) blank in each validation batch. The responses of target PFAS in matrix and method blank injections were <30% of the response in samples spiked at the LOQ. Analytes present in validation samples at or above the LOQ were successfully identified. The analyte retention times in samples were well within 0.1 min of the retention time in standards. Ion ratio in samples were within ±30% of the average ratio established in calibration standards for at least one quantifier/qualifier transition pair. Ion ratio-based identification could not be performed for perfluorobutyrate (PFBA) and perfluoro-n-
pentanoic acid (PFPeA) because only a single MRM transition was available for each of these analytes.

Linearity was evaluated with each validation batch at eight concentration levels ranging from 0.1 to 50.0 ng/mL injected at minimum at the beginning and at the end of the measurement sequence. This concentration range corresponded to 0.050–25 µg/kg and 0.020–10 µg/kg in 2-gram and 5-gram samples, respectively. Linear regression calibration curves with 1/x weighting were constructed by plotting the analyte-to-internal standard peak area ratio against the analyte concentrations. The coefficients of determination (R²) were consistently above 0.995 for all analytes with residuals within ± 20%.

Accuracy (marginal recovery) and precision (repeatability as relative standard deviation (RSD)) and intermediate precision (RSD_{INT}) were determined based on replicate analyses of unspiked samples and samples spiked with target PFAS at multiple concentrations ranging from 0.05 to 5.0 µg/kg. The spiking experiments were performed at minimum in six replicates over two different days. The mean recoveries calculated at individual spiking levels above method LOQ were within 70–120% with RSDs and RSD_{INT} values ≤20%, indicating robust recoveries in a variety of matrices with high reproducibility. The mean recoveries obtained at LOQ for each analyte are shown in Figure 2.

Method detection limit (MDL) was determined for each analyte/matrix combination based on accuracy and precision data after samples were taken through the entire workflow including sample extraction and LC–MS/MS analysis. Standard deviation was calculated based on precision data at low level spiking concentration and the obtained value was multiplied by t-value corresponding to the appropriate degrees of freedom (6). LOQ was determined for each PFAS as the lowest spiking level for which the recoveries within 70–120 and RSDs ≤20% were achieved in all matrices while successfully identifying the analyte. MDLs and LOQs are summarized in Table IV. MDLs were typically lower than 25 ng/kg for most PFAS analytes in all matrices except for PFBA which was significantly higher in matrices other than infant powder.

Conclusions

This paper described optimization and validation of a sensitive, robust and high-throughput LC–MS method based on a modified QuEChERS procedure for the determination of 16 priority U.S. FDA PFAS analytes including perfluoroalkyl carboxylic acids, perfluoroalkyl sulfonic acids and major replacement chemicals in whole milk, infant formula and related ingredients. The validation experiments demonstrated adequate performance of the method in the studied matrices that proves to be robust, reliable, and reproducible with scope to expand the list of PFAS in the future.

References


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FIGURE 2: Mean marginal recoveries determined in validation matrices at LOQ.
A Full Scan Data Review Tool to Match the Speed of Acoustic Ejection Mass Spectrometry

Acoustic ejection mass spectrometry (AEMS) has recently emerged as the premier ultrahigh-throughput mass spectrometric methodology for drug discovery and related fields. The ultrahigh analytical speed (~ 1 s/sample) of AEMS has significantly enhanced the efficiency of many high throughput applications. As a result, a data processing and reviewing tool with a matching speed is in high demand for the large amount of data generated, especially for applications such as quality control (QC) of compound collections and high throughput chemistry, where full-scan MS data required convoluted subsequent peak extraction and evaluation. In this study, we demonstrated the feasibility of a tool developed specifically for this purpose. The process using the tool involved automated splitting of the full scan data to correlate well positions with each signal peak, extraction of expected mass traces, and subsequent peak integration. Data evaluation based on verification rules, such as detected mass accuracy, isotopic pattern, and signal-to-noise ratio (S/N), enabled a comprehensive assessment of sample quality that was complemented by visualization in the form of a plate heat map generated from the selected rules. The tool demonstrated fast and straightforward data review and reporting and, more importantly, at a matching speed of sample analysis by acoustic ejection mass spectrometry. The choice of data processing and storage over the cloud further facilitated results sharing among data users.

Jun Zhang, Chang Liu, Cristiano Veiga, Tom Covey, Wilson Shou, and Harold Weller

The recent emergence and utilization of acoustic ejection mass spectrometry (AEMS) in various miniaturized drug discovery campaigns has significantly increased the throughput and turnaround of these applications (1–3). The enhanced efficiency is driven by the ultrafast analytical speed of AEMS of ~1 s/sample, the direct sampling of a dimethyl sulfoxide (DMSO) solution, and the negligible sample consumption of a few nanoliters. As a result, the high-speed, chromatography-free analysis generates an unprecedented amount of data from a range of high throughput assays arrayed in high-density microtiter plates, shifting the bottleneck of the high throughput workflow from conventionally sample analysis to data review. For quantitative AEMS assays, such as bioanalysis of cytochrome P450 or transporter inhibition, where a single probe substrate is detected across plates by multiple reaction monitoring (MRM)–mass spectrometry (MS), the data review is usually straightforward, involving mainly MRM peak integration and subsequent intensity comparison across wells or plates if needed (4). However, for qualitative or semiquantitative analysis using full scan MS detection, the data review is much more involved. Examples of such analysis include reaction monitoring in high throughput chemistry (HTC) (5), compound integrity check in high throughput screening (HTS) (6,7), and quality control (QC) in compound management and storage (8). The full scan data review on these occasions requires generating an extracted ion chromatogram (XIC) from the complex total ion chromatogram (TIC), for each well of the microtiter plate typically containing discrete targeted analytes to help identify the presence or absence of the expected compounds of interest. Subsequently, a verification needs to be established for each sample based on both target identity (qualitative) and amount (quantitative). Manual or even semiautomatic execution of this process is tedious and time-consuming, more so for full scan data acquired on quadrupole MS when the unit resolution alone is inadequate for definitive identity confirmation, compared to full scan data acquired on high resolution MS (5). The requirement for speedy full scan data processing propelled by the ultrafast AEMS has made the automated data processing tool in high demand, especially when better understanding of AEMS mechanism and exploration of novel operation modes continue to push for even faster data acquisition speed (9,10). In addition, the availability of an automated full scan data processing tool can facilitate the utilization of full scan AEMS for nonsubstrate-based or compound-based quantitative assays, such as in vitro metabolic stability, to eliminate the MRM optimization required for these assays (11). Without any commercially available tools in place, significant effort and resources have been spent on the development and maintenance of custom-built analytical tools for full scan data processing (12), or combinations of commercial software supported by home-built scripts (13). These user-built tools, mainly developed for specific applications, lack the comprehensive considerations required for various full scan functions from expansive user groups.

We have previously reported a fully vendor-supported, semiautomated full scan data review platform, using a research version data splitting tool and commercially available software (8). Although the platform has provided the basic functions required for the full scan AEMS data review, the lack of needed speed still made data processing the bottleneck of the entire analytical process. In this platform, the correlation
between AEMS signal peaks in the data file with their respective well positions in the sample plate had to be made manually by assigning the retention time for the first ejection, which sometimes could be confusing and unreliable. Because the software was initially designed for processing high resolution MS (HRMS) data, it was not entirely applicable to quadrupole full scan data for which more stringent data survey and verification rules were needed to compensate for the unit resolution by quadrupole MS. In addition, the lack of any plate-based visualization capability in the tool, such as plate heat maps, compelled users to perform plotting and graphing in excel using exported results.

In this study, we evaluated a commercially available, automated full scan AEMS data review tool developed and supported by the AEMS instrument vendor Sciex. The tool specifically incorporated verification rules to support processing of full scan AEMS data acquired.
The balanced solvent and gas flows

The process using the tool included data splitting using an add-on app as an integral part of the system operating software for aligning signal peaks in the data file with the corresponding well positions in the sample plate. It also included cloud-based data processing with unique features such as isotopic pattern comparison of target compounds between the observed and theoretical spectra (as opposed to confirmation of just protonated molecules previously) and background subtraction for eliminating matrix interference. The tool allowed for results visualization in the format of a heat map for a plate-based survey of the sample characteristics. The effectiveness of the tool, including rates for false positive or false negative, data processing speed, user friendliness and versatility for AEMS full scan data, were evaluated.

Materials and Methods

A Sciex Echo MS system was used for all of the sample analysis and data acquisition. The system comprised of an acoustic droplet ejection module coupled to a triple quadrupole mass spectrometer 6500+. An open-port interface (OPI), designed to capture and transport the ejected samples, was mounted between the acoustic dispenser and an OptiFlow ion source equipped on the MS instrument. The OPI carrier solvent used was 0.1% (v/v) formic acid (J.T. Baker) in methanol (MilliporeSigma), delivered by a gear pump. The system was operated by the Sciex OS operating software with both the MS instrument and acoustic dispenser built into the system hardware configuration. The ion source gas 1 (nebulizing gas) was set at a fixed value of 90 psi, and the carrier solvent flow was fine-tuned (typically around 0.43 mL/min) to achieve the proper formation of a “stable vortex” at the inlet of OPI, commanded by a balanced flow of the carrier solvent and nebulizing gas. The balanced solvent and gas flows allowed for the most efficient transportation of ejected samples through the OPI sample transfer tube to the MS for detection. The ion source gas 2 was set at 50 psi, curtain gas at 20 psi, and the source temperature at 300 °C. The MS was operated under a positive electrospray ionization (ESI) mode at a spray voltage of 5500V, and full-scan data (m/z 150–800) were acquired at a scan speed of 2000 Da/s. All samples from an entire plate (or user assigned wells from an AEMS run) were acquired into a single MS data file. An ejection volume of 5 nL was employed, with a pause time of 1 s between each sample ejection. The sample plates used were the Echo Qualified 384-Well flat-bottom plate or the LDV COC MS 1536-well plate (Beckman Coulter).

In setting up sample analysis, a “marker well,” typically containing a compound of a known identity, was ejected at the beginning of the sample sequence. Unlike other samples or wells, the marker well underwent a unique ejection pattern containing three ejection events, a single-droplet ejection, a continuous droplet ejection at a low frequency of 10 Hz for 1.5 s, followed by another single-droplet ejection. The recognition of this ejection pattern of the marker well by the data processing algorithm enabled a determination of the delay time from the acoustic ejection event to the MS signal, which was then used to correlate all other MS signal peaks with their appropriate sample well positions. The user may also choose to incorporate a blank matrix well in the plate to generate its mass spectrum that can be used for background subtraction in later data processing. The blank matrix spectrum may also be generated on a separate analysis occasion and saved in a separate data file.

The tool used for full scan AEMS data processing consisted of a Splitter, a Sciex OS add-on app, and OneOmics, a web-based data review suite. The workflow using the tool included four major steps: TIC data file splitting, XIC extraction, sample verification, and plate visualization (Figure 1). The post-acquisition TIC data file contained all of the ejections from a run and was first split into individual sub-files, with each containing a TIC peak from one ejection. The split subfiles were then uploaded to the cloud via the WIFFDB Manager app in the OneOmics Suit, followed by data processing and review by the Echo MS app. For peak extraction, a Microsoft Excel file was first browsed into the app tabulated with the well position, expected target compound formula and charge agent (H+, NH4+, Na+ and others) for up to five targets per well. The expected m/z value was then searched in the full scan data based on the extracting window set by the user, typically ±0.5 amu of the targeted mass calculated from the formula and charge agent provided for quadrupole data, to produce XIC for each ejection. From the XIC generated, the full scan mass spectrum can be visualized for any selected wells of interest. With a user-defined window (for instance, for ±10 amu of the targeted mass), the observed mass spectrum may be overlaid with the theoretical isotopic distribution calculated from the formula of the target compound. A score of correlation was then deduced from the isotopic pattern comparison for each compound well. Combined with the detected mass accuracy, as well as the signal-to-noise ratio (S/N) of the XIC peak for respective wells, the sample quality was evaluated for verification purposes. The results can then be visualized in the format of a plate heat map, and color-coded for each well based on their verification status before being exported in the form of an Excel file.

Results and Discussion

AEMS analysis achieved an ultrafast analytical speed of seconds or subseconds per sample, using acoustic technology for direct liquid sampling coupled with MS detection. Unlike the conventional LC–MS analysis where each injection is typically acquired to a single data file, AEMS analysis has a single MS file remained open for the acquisition of all ejections from an applicable microtitre plate, either 384-well or 1536-well. This way, the overhead associated with opening and closing the individual acquisition files for each ejection is eliminated to achieve an overall maximized analytical speed. Therefore, the resulting data file may contain hundreds or more signal peaks, which presents challenges in the downstream data processing in terms of appropriately correlating...
the microtiter plate well positions with their corresponding signal peaks in TIC. This matching or correlating process could be perplexing if the correlation is solely based on finding the expected mass in the respective wells, especially when the wells under survey may be absent of the expected mass or present with the same expected mass. In AEMS, the scanning of the marker well was designed to eliminate that confusion via the unique signal pattern of a continuous infusion bracketed by two single-droplet ejections. The confirmation of the marker well at both the beginning and end of the sample sequence provided unequivocal association of its retention time in the TIC to the marker well position, which enabled a definitive correlation of all other well positions with their respective signal peaks based on the delay time set across each ejection during acquisition (Figure 2a).

The provision of the marker well retention time in the Splitter then ensured an appropriate assignment of post-split sub-files to their respective well positions in the sample plate (Figure 2b).

From the post-split subfiles, XIC was generated based on the imported compound information and user-defined search criteria (Figure 3a). Simultaneous search and extraction of multiple targets in each well offered the much-needed versatility for applications like HTC to help identify not only the expected products, but also the remaining starting materials and potential side products (Figure 3b). The choice of different charge agents, such as proton adduct and sodium adduct for the target compounds, enabled a comprehensive survey of the targets in the full scan data and minimized potential false negatives in cases where unexpected adducts were formed. Although generating XICs multiple times with one charge agent in each could be implemented as previously reported (8), it would significantly slow down the data extraction process.

**FIGURE 2:** Pre-splitting TIC and post-splitting subfiles: (a) TIC with marker well scanned at the beginning and end of the sample sequence and (b) post-splitting subfiles.

**FIGURE 3:** XIC and corresponding mass spectrum (a) XIC and (b) full scan mass spectrum of the corresponding XIC.
In the data review tool, based on the XIC, the observed mass spectrum was then displayed and overlaid with the theoretical isotopic distribution of the target compound, enabling a direct visualization of the correlation between the two (Figure 4). A correlation score, or “average ratio offset,” was also calculated for a more quantitative comparison. For each target compound, the observed intensity ratios of the found isotopic mass $M+2$ and $M+1$ over $M$ were compared respectively with the corresponding theoretical ratios calculated from the formula, and the average ratio difference from the comparison was defined as the average ratio offset. The lower the offset was, the better the overlay was between the observed and theoretical isotopic distribution; hence, the higher the confidence was in the match to the expected target compound. The average ratio offset offered additional assurance in identity confirmation compared to the simple matching of the protonated molecule, which was especially helpful for quadrupole full scan data where unit resolution was inadequate in differentiating isobaric interferences, which could shift the observed peak mass centroid and affect the apparent isotope ratios. In this case, a poor average ratio offset from the targeted mass would raise a red flag for potentially false positive findings.

In addition to its utility for small molecule identification in HTC or compound QC, the isotopic distribution overlay was also an effective approach for identifying trace metal chelated organic complexes, which typically displayed characteristic isotopic fingerprints unique to the chelating metal involved (14). We have demonstrated the utility of this approach in the investigation evaluating chelator effectiveness and chelating ratios between thiourea (TU, $C_{23}H_{26}N_4OS$) and palladium (Figure 5). The metal-chelated organic complexes have been demonstrated to produce false positives in HTS campaigns (15), whereas an ultrafast AEMS scanning of the HTS plate would effectively detect the complexes to avoid misleading HTS results.

Direct MS analysis of small molecules in biological assays or chemical reactions by AEMS can be subject to interferences by the matrix that is otherwise removed by chromatography in LC–MS analysis. The presence of interfering background ions in the matrix, especially when their isotopic distribution fully or partially overlapped with that of the target compound, may result in a false positive target identification or an overestimation of target quantity. Subtracting common background ions from those of the analytes was an effective approach to minimize the interference, which was especially helpful for full scan data acquired on quadrupole
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The tool we evaluated has demonstrated fast and straightforward data processing and review, with well-designed features for reliable data extraction, compound identification, sample verification, and results visualization. The unique scan feature of the marker well in the sample plate ensured proper extraction of appropriate mass from the corresponding well positions. The identification and verification features were especially beneficial for full scan data acquired on unit-resolution quadrupole MS, which could more likely generate false positives or false negatives compared to data obtained on HRMS. Enabled by the user selectable verification rules, the comprehensive assessment of the sample quality in both qualitative and quantitative terms was complemented by results visualization in the form of a plate heat map and an exportable results table. The tool demonstrated fast and straightforward data processing and review. More importantly, this process was done at a matching speed to that of sample analysis by AEMS, effectively eliminating the bottleneck of the analytical process. The choice of data processing and storage over the cloud further facilitated results sharing among data users.

Acknowledgment
We would like to thank Dr. Anthony Cauley and Dr. Yong Zhang of Bristol-Myers Squibb, Small Molecule Drug Discovery, for generating the HTC samples for this study.

References

Jun Zhang, Wilson Shou, and Harold Weller are with Bristol Myers Squibb, in their Research and Early Development department, in Princeton, New Jersey. Chang Liu, Cristiano Veiga, and Tom Covey are with Sciex, in Concord, Ontario. Direct correspondence to: jun.zhang1@bms.com.
The Crucial Step in Every Analytical Workflow: Sample Preparation—Are We Ready For a Growing Area of Intact Protein Analysis?

Proteins are biomolecules with a lot of essential functions in a human body. Their varied expression during complex disorders, including cancer, predicts their potential use as biomarkers. Therefore, there is a need for reliable analytical workflows for their analysis in complex biological matrices. Despite the very sensitive and advanced instrumentation we have available for protein analysis today, sample preparation still remains one of the biggest challenges.

Katarína Maráková

Targeted top-down proteomics based on mass spectrometry (MS) platforms connected with liquid chromatography (LC) or capillary electrophoresis (CE) as separation techniques is a fast-growing research area (1–3). Whether it is the pharmaceutical industry moving in the direction of biopharmaceuticals, or clinical researchers looking for novel protein biomarkers, there is immense demand for reliable bioanalytical workflows to target these biomolecules in complex biological matrices (4,5). There have been more than 15,000 different proteins identified in major human body fluids. Those circulating proteins can play an important role as biomarkers and potential drug targets in various disorders, including cancer, neurodegenerative, and inflammatory-mediated diseases (1).

Biological fluids also contain vast amounts of other chemical species including salts, metabolites, and lipids. Therefore, reliable quantitative analysis of intact proteins in complex biological samples using MS still comes with significant challenges. The drawbacks of existing techniques include low overall sensitivity because of the poor ionization and fragmentation efficiencies of intact proteins, the nonspecific binding of proteins to various surfaces of analytical instruments, and the often time-consuming, expensive, and laborious sample preparation steps needed before analysis. All these concerns make the development of a successful analytical workflow for intact proteins even more difficult because an efficient sample preparation protocol is essential before a powerful separation technique with sensitive and selective detection is used. On the other end, current advanced analytical workflows bring several benefits to the proteomics field, including high accuracy, precision, selectivity, and a multiplexing ability that could be beneficial for determining a whole panel of multiple biomarkers in one run (2,6–8).

Sample Preparation for Intact Proteins in Biological Matrices

The go-to sample preparation methods for proteomics analysis are highly selective and quite expensive immunoaffinity-based methods. Other sample pretreatment techniques frequently used for intact protein analysis involve mainly complex and laborious size-exclusion chromatography (SEC), two-dimensional (2D) gel electrophoresis, or their combinations (1,2,9). During our recent work (6) studying the development of an LC–triple quadrupole mass spectrometry (LC-MS/MS) method for direct quantitation of multiple intact growth factors and cytokines in human body fluids, we realized that there was limited commercial availability of simpler, non-immunoaffinity sample preparation options for intact proteins. These could be different extraction techniques or coacervation systems (10–12), but few options could be found. Therefore, a strong emphasis on the development of such new potential sample preparation methods for isolation and enrichment of multiple intact proteins present in complex samples should be of great interest.
Micro-EIution
Solid-Phase Extraction (µSPE)

A common and relatively simple solution for removing interferences caused by the presence of cellular and extracellular matrix components in biological samples is protein precipitation (13,14). However, for proteomics analysis, precipitation can often cause deleterious protein loss. Another potential simple non-immunoaffinity sample preparation technique, already frequently used for analysis of small peptides after protein digest, is solid-phase extraction (SPE) (15,16). SPE generally shows limited selectivity, which can be advantageous if one wishes to purify a wider range of proteins from the sample. Developments in the area of new SPE sorbents has started to focus on new materials and formats to target intact proteins as analytes, although commercially available SPE products of this type are still lacking in the market. SPE in a microplate format (µSPE) is designed for effective isolation and preconcentration of various substances. Microplate SPE has multiple advantages, including the use of small sample volumes, elution into volumes as low as 25 μL, and no need for drying or reconstitution steps. The latter is especially important for intact proteins, which can suffer serious recovery losses during such processes.

Even though success in the adoption of commercially available products aimed for small molecules for preparation of intact proteins may be limited, and these methods still suffer from poor recoveries and detrimental matrix effects, in our latest work we explored the practical opportunities and challenges of applying µSPE to the preparation of lower molecular weight intact proteins (<30 kDa). Under optimum conditions, final recoveries of >65% in urine for all targeted proteins and >50% in serum and plasma for most of the proteins were achieved, as shown in Figure 1. Limits of quantitation (LOQ) were at ng/mL concentration levels (corresponding to a range between approximately 0.35 and 97.6 nM). The preliminary results of this work were presented at 69th ASMS Conference on Mass Spectrometry and Allied Topics (17) and are currently being prepared for publication.

Conclusion

Every year, we see the development of cutting-edge analytical instrumentation and the improvement of the performance parameters, particularly sensitivity, of these instruments. However, even with the most sensitive instrumentation, we are not able to reliably analyze multiple intact proteins in complex biological samples without improvements in the front-end to create a highly efficient, robust, and reproducible sample purification and fractionation strategy. There are no simple universal strategies for sample preparation workflows for intact proteins and there is plenty of room to invent. We must continue to develop workflows capable of reaching proteins at very low concentration levels (pg/mL to ng/mL) in complex matrices, providing the appropriate selectivity for proteins of interest and removing potential interferences. The systematic study of the application potential of non-immunoaffinity-based sample preparation methods for intact protein analysis in biological fluids will continue to be a focus of our future work. We hope that others will also see this need and opportunity to contribute to an important field of research.

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References


Transforming growth factor-α (TGF, 5.5 kDa)
Epidermal growth factor (EGF, 6.3 kDa)
Insulin-like growth factor 1 (IGF, 7.5 kDa)
Cytochrome c from bovine/equine heart (CYT CB, 12.3 kDa/CYT CE, 12.4 kDa)
Ribonuclease A (RNA, 13.7 kDa)
Lysozyme (LYZ, 14.3 kDa)
α-lactalbumin (LAC, 14.1 kDa)
Myoglobin (MYO, 17.1 kDa)
β-lactoglobulin A (BLA, 18.4 kDa)
Carbonic anhydrase (CA, 29.0 kDa)
VENDOR TIPS & TRICKS

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Stephanie Schuster, Senior Technical Support Scientist, Advanced Materials Technologies

What makes a HALO 1.5 mm column the best choice of column dimension for UHPLC and UHPLC–MS?

1. Obtain increased sensitivity
When the same volume of the same concentration sample is injected on a 1.5 mm column compared to a 2.1 mm ID column, the response on the 1.5 mm ID column will be about double what is observed on the 2.1 mm ID column. Why does this happen? With a 1.5 mm ID column, the operating flow rate is half that of a 2.1 mm ID column. This means that the sample spends twice as much time in the flow cell, thus giving a higher response.

2. Obtain improved ionization efficiencies
Using a lower flow rate means that there is less solvent present that needs to be ionized when using ESI. To get the best performance using HALO 1.5 mm columns, the source parameters will require optimization along with minimization of any extra column volume, especially the connection from the column outlet to the MS source.

3. Reduce solvent consumption
The optimum flow rate for a HALO 1.5 mm column is half that of a 2.1 mm ID column. This means that the solvent used and subsequent waste generated is cut in half. The solvent will last twice as long and there will be half as much waste for disposal.

4. Easy-to-use microflow solution
HALO 1.5 mm ID columns offer ease of use without having to make the investment into a capillary or nanoflow system. HALO 1.5 mm ID columns are loaded into stainless steel hardware unlike capillary columns which are typically loaded into PEEKsil™ tubes. This difference in hardware enables the 1.5 mm ID columns to be more robust compared to capillary columns.

These benefits are perfectly timed for the days when supply chains are broken, lead times are long, and inventories are depleted.

ABOUT THE AUTHOR

Katarína Maráková is an Assistant Professor at the Department of Pharmaceutical Analysis and Nuclear Pharmacy, Faculty of Pharmacy, Comenius University in Bratislava. Her research is focused at the development of high performance separation methods (capillary electrophoresis, liquid chromatography, and mass spectrometry), including multidimensional approaches for advanced pharmaceutical and biomedical applications. She has spent two post-doctoral research stays at the University of Texas at Arlington (prof. K.A. Schug), supported by the National Scholarship program of the Slovak Republic and the Fulbright Commission.
Verify sample application pH. Analytes that are not in their proper form (such as neutral or charged), will not effectively bind to the sorbent and may result in low or erratic recoveries. The pH of deionized water cannot be correctly determined using pH paper. Use of a calibrated pH meter is necessary.

Always pre-rinse the cartridge with the strongest solution the cartridge will see to ensure the cleanest extraction of your eluate. Do not allow the sorbent to completely dry out between conditioning steps or before sample application. To ensure properly solvated cartridges, apply each solvent immediately after the previous solvent. Improperly conditioned cartridges may lead to inconsistent recoveries.

Prior to elution, fully dried cartridges, especially when the last wash solution is aqueous, will ensure optimal analyte recovery. To confirm cartridge dryness, touch the sides of the cartridge at the sorbent level at full vacuum and pressure. Cartridges should feel about room temperature but not cool. If the cartridge feels cool, water is probably present and still evaporating. Continue drying the cartridge unless otherwise specified in the application note.

Elution rates and soak times specified in the applications are critical for acceptable and consistent recoveries. Hint: When in doubt, slower is always better. Using 1 mL to 2 mL per min is a good general guideline for the sample addition and analyte extraction steps. This recommendation is required for suitable ion exchange extractions.

Always use fresh ammonium hydroxide (NH₄OH) for elutions. NH₄OH rapidly loses its effectiveness when exposed to air. A good time to prepare elution solvent mix with NH₄OH is while the columns are drying after the wash procedure.

NH₄OH is more soluble in IPA than CH₂Cl₂. To ensure complete mixing of eluate solvents, add NH₄OH to IPA, then add CH₂Cl₂.

Addition of 1% HCl in MeOH assists in reducing volatility of certain analytes, especially sympathomimetic amines. Add 2–3 drops after approximately 4 min of evaporation.

Certain compounds are slightly volatile. Closely monitor eluate concentration to prevent loss of analyte. Hint: Higher water bath temperatures and lower nitrogen flow rates usually provide optimal results. However, do not exceed 40 °C. Optimal evaporation temp ranges from 35–40 °C. Optimal Nitrogen flow rates are from 5–15 psi.

Solvent quantities for methods are suggested and might be further reduced to meet particular laboratory sample size needs.

### Table I: Non-chlorinated elution solvent mixes for commonly encountered drug panels

<table>
<thead>
<tr>
<th>Assay</th>
<th>Chlorinated</th>
<th>Non-chlorinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opiates</td>
<td>CH₂Cl₂ / IPA/NH₄OH (78:20:2)</td>
<td>EtAc / IPA/NH₄OH (90:6:4)</td>
</tr>
<tr>
<td>Cocaine/BE</td>
<td>CH₂Cl₂ / IPA/NH₄OH (78:20:2)</td>
<td>EtAc / CH₃OH/ NH₄OH (68:28:4)</td>
</tr>
<tr>
<td>Amphetamines</td>
<td>CH₂Cl₂ / IPA/NH₄OH (78:20:2)</td>
<td>EtAc / IPA/NH₄OH (90:6:4)</td>
</tr>
</tbody>
</table>

### Use of non-chlorinated elution solvents

In response to environmental concerns over the use of chlorinated compounds in the laboratory, UCT offers these suggested non-chlorinated elution solvents. The recommended parameters have been used successfully on UCT columns by our customers throughout the world and may be routinely used as an alternative to chlorinated elution solvents. You may however see subtle differences on certain compounds due to solubility effects.

UCT would like to thank Dr. Leon Glass for his efforts in developing these non-chlorinated mixtures.
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**VIEWPOINTS**

**Whence the Next Generation of Macromolecular Separations Scientists?**

André M. Striegel

During my time in industry, I developed an interactive gradient polymer elution chromatography (i-GPEC) method to determine the vinyl alcohol (VOH) distribution in the vinyl butyral terpolymer that comprises the middle, polymeric layer of most automotive and architectural safety glass. Applying this method during one of the “routine crises” that arise in the industry, I was able to demonstrate that the undesirable behavior of a particular batch of polymer was because of its chemical composition distribution (CCD)—specifically, to the distribution in the percentage of VOH among terpolymer chains. This type of i-GPEC method is not one that is developed “on the spot” and without a good fundamental knowledge of polymer chromatography. Fortunately, building upon expertise in this area I had already taken the time to painstakingly develop and perfect this and related methods so that, when needed, fine-tuning one of them to a particular application could be done relatively quickly. However, waiting for a problem to manifest itself to then try and find an expert who can develop a non-trivial macromolecular separation is not the way to solve time-sensitive behavior in products. As a result, it is of paramount importance that companies develop their in-house macromolecular separations expertise.

Industrial managers and supervisors will recognize two immediate and related problems with my recommendation. First, they may not have any internal personnel in place with this type of knowledge who can further develop along these lines. Second, even if hiring is approved in this area, there is an almost complete lack of suitable candidates, even at the junior, entry-level stage. This leaves companies with three options: leaving an important characterization gap, hiring an unsuitable candidate with only a cursory knowledge of the subject matter, or getting involved into a bidding war for the few senior-level experts still working in industry. Worse, there is currently virtually no refilling of the supply pipeline of chemists trained in this area.

Training in macromolecular separations involves much more than “just” being trained in chromatography. One must also be an expert, or at least have knowledge of several layers beyond the superficial, in polymer and colloid science (and biochemistry or carbohydrate chemistry, or both) as well as in certain aspects of physics, fluid mechanics, and so on. There are a number of reasons for this. For example, as part of a macromolecular separations experiment, one will likely be dealing with light-scattering and viscometric measurements (among others) and interpretation of the results within the context of polymer science. As an example, one cares less about how much light is scattered by a polymer in solution upon irradiation by a light beam than about what this can tell us about the polymer’s molar mass and size, about the thermodynamic state of the solution, and more. In a general way, this is also true of any chromatographer in that they should have a decent knowledge of the intended analytes and of the detectors connected to the separation system. The additional challenge with macromolecular separations is the types of analytes studied, and the types of detectors most commonly employed, are not topics commonly taught in most chemistry departments. Polymers are usually given an at-best cursory look in most traditional organic and physical chemistry courses and detectors, such as light-scattering photometers and differential viscometers, are rarely mentioned in an instrumental analysis course. More fundamentally, the types of separation methods employed in polymer characterization are not usually taught even in traditional separations courses, save for some brief mention of size-exclusion chromatography (SEC) with, perhaps, a nod to field-flow fractionation. How many people who took a separations course learned anything about, for example, gradient polymer elution chromatography, temperature gradient interaction chromatography, or liquid chromatography (LC) at the critical condition, to name just a few macromolecular separations techniques? I bet the answer is “not many.”

Developing this type of program in an academic setting requires support. It is industry that will reap the most benefit from having a workforce trained in macromolecular separations. It is industry that continually expresses to me the need for trained polymer chromatographers. Thus, it is industry that should make its needs strongly felt to funding bodies while simultaneously providing funding for professors, especially assistant professors just starting their academic careers, who are willing to perform research and train students in macromolecular separation science. Without this type of financial support, young faculty are sure to either leave academia or to take their programs in a different direction, one in which funding is being provided; either case will result in faculty no longer training students in macromolecular separation science. Will the lack of trained students be noticed? Probably not—at least not until the next “routine crisis” occurs at a company.

**André M. Striegel** is with the National Institute of Standards and Technology (NIST), where he is currently Scientific Advisor in the Chemical Sciences Division.
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