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Welcome to the July/August issue of *LCGC Europe*! The development of robust analytical assays for separation and analysis of complex multicomponent mixtures can often be challenging, reflecting the increased complexity of new medicine and vaccine processes. In our cover story this month, *Pharmaceutical Perspectives* addresses in silico LC method development strategies for large molecules using modelling software.

The peer review article this month demonstrates how pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) is a powerful tool for conducting qualitative and quantitative analysis of thermolytic decomposition products from cembratriene in tobacco products.

Verifying system performance is so important when using chromatographic instrumentation for analyzing both known and unknown samples. As 2D-LC is becoming more widely used, *LC Troubleshooting* discusses how the development of a system suitability test (SST) for 2D-LC is critical for successful use of the technique.

*Questions of Quality* takes a look at the draft publications ICH Q2(R2) and Q14 for analytical procedure validation and development and what they mean for a regulated good manufacturing practice (GMP) laboratory. Are they consistent with the approach taken by the *United States Pharmacopeia* (USP) <1220> on Analytical Procedure Life Cycle?

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.

This issue also features a compilation of product profiles highlighting what leading chromatography vendors are showcasing at live and virtual events this year.

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The epimeric macrocycles, α- and β-2,7,11-cembratriene-4,6-diol (α- and β-CBT), are the two most abundant cembranoids found in tobacco (1–3) and are the main components of the glandular secretions formed on the surface of tobacco leaves. They play a number of important roles during plant growth and development. The absolute configuration of α-CBT is (1S, 2E, 4S, 6R, 7E, 11E)-2,7,11-cembratriene-4,6-diol and that of β-CBT is (1S, 2E, 4R, 6R, 7E, 11E)-2,7,11-cembratriene-4,6-diol (Figure 1).

As two latent fragrant substances, α- and β-CBT are of interest because they are the precursors of key flavour constituents in Nicotiana species (4). They can be biochemically degraded and transformed into neutral flavouring substances such as solanone, benzaldehyde, and 4-methylbenzaldehyde. More pyrolysis products were obtained at higher temperatures, and almost all of the harmful aromatic ingredients were produced at 900 °C. Importantly, solanone, a significant flavour component, was only obtained from the pyrolysis of α-CBT under 10% O₂ in N₂ at both 600 and 900 °C. The number of the pyrolysates changed with the change in pyrolysis temperature and the presence of oxygen. The study of the thermal behaviour and pyrolysis products of these terpenoids could possibly suggest flavour precursors that could be used to provide specific flavours.

**KEY POINTS**

- Py-GC–MS is a powerful tool for conducting qualitative and quantitative analysis of thermolytic decomposition products from cembratriene in tobacco products.
- The results were able to suggest flavour precursors that could be used to provide specific flavours to tobacco.

Py-GC–MS Investigation of Pyrolysis Behaviours and Decomposition Products of α- and β-2,7,11-Cembratriene-4,6-Diols

Shen Huang, Chen Yang, Ning Ma, Lifeng Zhou, Chunxiao Jia, Tao Wei, and Duobin Mao, College of Food and Biological Engineering, Zhengzhou University of Light Industry, Henan, China

To study the thermal stability of cembratriene, α- and β-2, 7, 11-cembratriene-4, 6-diols (α- and β-CBT) were isolated from tobacco leaves. Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses were used to evaluate the cleavage differences between the two compounds. The TGA results showed the peak temperatures (T_p) were 263.3 °C and 254.1 °C with the largest weight loss rate; the significant weight losses were 90.96% and 99.45%.

Pyrolysis gas chromatography–mass spectrometry (Py-GC–MS) was employed for the pyrolysis products at different temperatures (300, 600, and 900 °C) under either N₂ or an O₂–N₂ (10%:90%) mixture. The results showed that the major pyrolysates from α- and β-CBT were simple hydrocarbons, such as toluene, 1, 4-pentadiene, and p-xylene, as well as several important flavour compounds, such as 2-methylfuran, benzaldehyde, and 4-methylbenzaldehyde. More pyrolysis products were obtained at higher temperatures, and almost all of the harmful aromatic ingredients were produced at 900 °C. Importantly, solanone, a significant flavour component, was only obtained from the pyrolysis of α-CBT under 10% O₂ in N₂ at both 600 and 900 °C. The number of the pyrolysates changed with the change in pyrolysis temperature and the presence of oxygen. The study of the thermal behaviour and pyrolysis products of these terpenoids could possibly suggest flavour precursors that could be used to provide specific flavours.
of α-CBT was based on chemical degradation and later confirmed using multiple methods (13,14). The two compounds, which constitute 0.01% and 0.005% of the dry Burley leaf, have also been found in flue-cured, Turkish, and unaged Burley tobaccos.

To improve the initial complicated process for the isolation and purification of α- and β-CBT, optimized methods were reported by Severson et al. (2) and Guanghui and Zong (15). Mature fresh tobacco leaves were extracted using dichloromethane and subjected to liquid–liquid extraction (LLE) to obtain a crude extract. This extract was separated by high performance liquid chromatography (HPLC), crystallization, and recrystallization, to successively produce α- and β-CBT. Ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and high-resolution mass spectrometry (HRMS) techniques, as well as other modern organic chemical analyses, were used to characterize their structures and absolute stereochemistries. The structural variation between α-CBT and its epimer β-CBT was ultimately distinguished by two-dimensional (2D)-NMR.
Previous studies on \( \alpha \)- and \( \beta \)-CBT have typically concentrated on their separation, identification, qualitative and quantitative analysis (15, 16), biosynthesis (17, 18), biodegradation (19), and chemical synthesis. In this study, we applied pyrolysis–gas chromatography–mass spectroscopy (Py-GC–MS), which served as a powerful tool for conducting qualitative and quantitative analysis of thermolytic decomposition products from \( \alpha \)- and \( \beta \)-CBT (20). Although studies on the thermal behaviour and pyrolysis of glycosides have been reported (21–24), their application of cembrane for the purpose of producing aroma and flavour compounds, such as solanone, has not been reported. By studying the product distributions obtained under different pyrolysis conditions, important basic information can be obtained that suggests appropriate precursors that could be used to provide specific flavours.

**Materials and Methods**

**Plant Material:** Mature tobacco leaves of *Nicotiana tabacum* L were collected in an experimental tobacco field in Xuchang City, Henan Province, China. A voucher specimen (TL20150804) was deposited in our laboratory.

**General Characterization Methods:**

UV spectra were obtained using a Puxi TU-1901 spectrophotometer. The IR spectra were recorded using a Bruker Vertex 70 Fourier transform IR (FT-IR) ESP spectrometer. The samples were analyzed as KBr micropellets. First dimension (\(^1\)D) and second dimension (\(^2\)D) NMR spectra were recorded on Bruker Avance AMX-400 spectrometers, with TMS as the internal standard. HRMS spectra were recorded with a Thermo Fisher Exacta Orbitrap spectrometer. Py-GC–MS analysis was performed on a combined system consisting of an Agilent 7890A-5975C GC–MS instrument and a CDS Analytical Pyroprobe 5200.

Semipreparative HPLC was performed on a Waters Delta 600 preparative liquid chromatograph with a Waters 2489a UV detector and a 4.6 mm \( \times \) 250 mm, 5-\( \mu \)m C18 column, with the detection wavelength being 210 nm.

**Sample Preparation:** Whole fresh tobacco leaves (100 kg) were extracted three times with \( \text{CH}_2\text{Cl}_2 \) (3 \( \times \) 5.0 L). Each of the 3–5 leaves were swirled for 3–5 s, and filtered to yield a filtrate. A crude extract was concentrated by rotary evaporation at 45 °C. The dried extract (2.0 g) was reconstituted in \( \text{CH}_2\text{Cl}_2 \) was washed twice with 5% phosphoric acid (20 mL) by a separatory funnel, and the separation then left the organic phase. The organic phase was rinsed 2–3 times with deionized water (20 mL) until a neutral pH was obtained, and then it was separated and dried over anhydrous sodium sulphate. The filtered organic solution was concentrated by rotary evaporation at 45 °C to obtain the extract of cembratriene (1.8672 g), which was subjected to silica gel (100–200 mesh) chromatography using a petroleum ether/ethyl acetate gradient system (6:1), with final elution using 100% ethyl acetate. The collected ethyl acetate fractions were further purified by HPLC (50% acetonitrile–\( \text{H}_2\text{O} \); flow rate, 4.00 mL/min) to yield solid \( \alpha \)- and \( \beta \)-CBT samples.

**Thermal Analysis:**

**Thermogravimetric Analysis (TG) and differential thermogravimetric (DTG) analysis was performed using a Diamond TG analyzer from PerkinElmer. A sample (\(-4.0\) mg) was heated at a rate of 10 °C/min from ambient temperature to 800 °C under \( \text{N}_2 \).

**Py-GC–MS Method:** In the pyrolysis experiments, the mass of the solid sample was 0.40 mg, and the atmosphere was either \( \text{N}_2 \) or a mixture of \( \text{O}_2 \) (10%) and \( \text{N}_2 \) (90%). The initial pyrolysis temperature of 50 °C was increased to 300, 600, and 900 °C at 20 °C/min over 15 s. The pyrolysis products were directly transferred to the GC–MS instrument for analysis.

Qualitative GC–MS analysis was conducted with a 30 m \( \times \) 0.25 mm, 0.25–\( \mu \)g Agilent DB-5MX wax fused silica capillary column. The column temperature was held at 50 °C for 2 min, subsequently raised to 280 °C at a rate of 5 °C/min, and then maintained at 280 °C for 20 min. The injector and detector temperatures were set at 280 °C. The sampling volume was 1 \( \mu \)L, and the shunt ratio was 25:1. The temperature of the ion source was 230 °C, and the temperature of the transmission line was 280 °C. The EI ionization energy was 70 eV, and the scan range was 30–650 amu. The spectra of the pyrolysis products were simulated and matched against the PerkinElmer National Institute of Standards and Technology (NIST) library and other published data.

**Results and Discussion**

**Structural Characterization:**

**Compound 1:** The HRMS spectrum gave the molecular formula \( \text{C}_{20}\text{H}_{16}\text{O}_6 \) by HRMS ([\( \text{M}+\text{Na} \)]\(^+\); \( \text{m}/\text{z} \): 329.24493), suggesting four degrees of unsaturation. Secondary and tertiary mass-spectrogram showed that the structure of \( \alpha \)- and \( \beta \)-CBT lost two -OH and one -C=H was consistent. The IR spectrum showed absorption bands at 3351, 2919, 1653, 1430, 1170, and 976 cm\(^{-1}\), indicating the presence of the -OH, the C-H, C=C, C-H, C-O, and trans-C=C. The \(^{13}\text{C}\) NMR spectrum of compound 1 displayed 20 carbon signals, respectively, corresponding to a cembranoids nucleus, \( \delta \): 137.4, 136.8, 133.3, 130.5, 127.7, 124.3, 72.4, 66.2, 52.1, 46.3, 38.8, 36.7, 32.9, 30.0, 27.9, 23.2, 20.6, 19.3, 16.0, and 14.9 ppm. Its \(^1\)H NMR spectrum exhibited 34 signals: \( \delta \): 5.33–5.32 (m, H-2,3,7), 5.04–5.02 (t, HO-6), 4.50–4.45 (m, J = 8.9, 1.9Hz, H-6), 2.16–2.13 (m, H-9, 10), 2.02–1.96 (m, 4H, H-13, 14), 1.82 (s, HO-4), 1.67 (d, H-19),
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δ_1, 1.57–1.58 (m, H-5), δ_1, 1.52–1.49 (m, H-1, 11, 20), δ_1, 1.34 (S, H-18), δ_1, 1.28 (m, H-15), δ_1, 0.83–0.81 (d, H-16), and δ_1, 0.80–0.78 (d, H-17) ppm. These data indicated compound 1 was α-CBT.

Compound 2: Compound 2 had a molecular formula C_{20}H_{34}O_2 that was established by the quasimolecular ion peak observed by HRMS measurement at m/z: 329.24316 (M+Na)^{+}, suggesting four degrees of unsaturation. The results of the IR spectrum were similar to those of compound 1. The 13C NMR spectrum of compound 2 exhibited 20 carbon signals. The NMR data, corresponding to a cembranoids nucleus δ_1: 137.4, 136.1, 133.0, 131.4, 130.3, 124.4, 71.4, 64.4, 52.4, 46.2, 38.8, 36.4, 32.9, 28.7, 27.6, 23.0, 20.5, 19.3, 15.9, and 14.9 ppm. The above also confirmed its 1H NMR spectrum at δ_1, 5.41–5.37 (d, H-3), δ_1, 5.27–5.18 (m, H-2, 7), δ_1, 5.00–4.99 (t, H-6), δ_1, 4.83–4.79 (t, H-6), δ_1, 2.22–1.83 (m, H-9, 10, 13, 14), δ_1, 1.70–1.64 (d, H-19), δ_1, 1.60–1.39 (m, H-1, 5, 11, 18, 20, OH-4), δ_1, 1.31–1.27 (m, H-15), and δ_1, 0.83–0.78 (m, H-16, 17) ppm. These data indicated that compound 2 was β-CBT.

Thermogravimetric Analysis: Figure 2 shows the TG and DTG curves recorded for α- and β-CBT from ambient temperature to 700 °C. Two stages are observed in the pyrolysis process for α-CBT, the first stage (1.39% weight loss) between 57–100 °C is attributed to the evaporation of water of crystallization (25). The second stage, representing the main weight loss with a sharp decrease of 90.96%, occurs between 123–329 °C. For β-CBT, the weight of the β-CBT sample decreases by 99.45% between 125–322 °C. No solid residue remains in the latter sample at 347 °C, whereas the α-CBT does not seem to be completely pyrolyzed at 700 °C, which shows that α- and β-CBT can complete pyrolysis at a lower temperature.

Study of Pyrolysis Products of α- and β-CBT: To investigate the main products obtained from the CBT pyrolysis processes, Py-GC–MS analyses were conducted (Figure 3). The structures of the pyrolysis products were identified by NIST 17, and their relative contents were determined by the peak area normalization method. To ensure the accuracy and repeatability of the experiments, we calculated the relative standard deviation (RSD) of the relative content of each component by averaging the results of three parallel measurements, and obtained RSD values between 0.48–5.02%. These results illustrate that the pyrolysis process had good repeatability. The results are shown in Tables 1 and 2 (which can be accessed through the QR code found at the end of this article).

FIGURE 3: Total ion current chromatograms of Py-GC–MS in (a) α- and (b) β-CBT in O_2 at different temperatures.

FIGURE 4: Classification of pyrolysis products of α- and β-CBT.
a significant effect on the pyrolysis product formation. Under N₂ atmosphere conditions, 0, 10, and 100 substances were detected from α-CBT at 300, 600, and 900 °C, respectively, compared to four, 53, and 104 substances from β-CBT. Under 10% O₂ atmosphere, four, 56, and 66 compounds were produced from α-CBT, respectively, whereas only a single compound was observed at any temperature from β-CBT. Generally, the higher the temperature, the greater the number of products observed for the epimeric diols, except in the case of the β-CBT in a 10% O₂ atmosphere, where only one pyrolysis product obtained at any temperature was methacrylaldehyde.

If the pyrolysis temperatures are divided into three zones, low (300 °C), medium (600 °C), and high (900 °C), one trend that emerged from the data was that the harmful aromatic components are mainly produced under high temperature conditions. A second interesting observation from the data was that the pyrolysis behaviours of the two substances were different under various conditions. For α-CBT, pyrolysis at high temperatures produced fewer oxygenated products, but oxygen incorporation was increased at low and moderate temperatures. In contrast, only one substance can be detected in 10% O₂ at any of the three β-CBT pyrolysis temperatures, which was far less than the number of pyrolysis products obtained under pure N₂. There were no obvious differences between the N₂ and 10% O₂ pyrolysis products from α-CBT at 900 °C, although the pyrolysate distributions from α-CBT at 300 and 600 °C resemble those from the 10% O₂ pyrolysis conditions. However, β-CBT displays the opposite behaviour: its pyrolysis products at 300, 600, and 900 °C are mainly under the N₂ atmosphere, which shows that α- and β-CBT were easier to pyrolyze in 10% O₂. When looking category-wise at levels of agreement for pyrolysis products for α- and β-CBT, aromatic hydrocarbons and enynes are the main volatiles detected, with alkanes, alcohols, aldehydes, ketones, and furans comprising a minor fraction (Figure 4). Among the pyrolysis products under various conditions of α- and β-CBT, aromatic hydrocarbons were the main components, accounting for 45.45% and 50%, respectively, followed by alkynes, which accounted for 31.10% and 37.08%, respectively, which is because of its unsaturated isoprene ring structure. Second, it can be found that the alcohols, aldehydes, and ketones in α-CBT were higher than those in β-CBT. At 300 °C, only a few pyrolysis products were obtained from α- and β-CBT were easier to pyrolyze in 10% O₂.
β-CBT, including high boiling components, butyaldehyde (α-CBT) and methacrylic aldehyde (α- and β-CBT), which indicates that bonds were broken in both α- and β-CBT, but complete pyrolysis did not occur. This result could be explained by their melting and boiling points. The melting point of α-CBT is 125–126 °C, and the melting point of β-CBT is 65–66 °C. Similarly, it can be seen that the boiling point of α-CBT is higher than that of β-CBT. The peak area percentage of β-CBT is 28.1%, whereas that of α-CBT is 0. At moderate and high temperatures, the results show that both α- and β-CBT are completely decomposed.

The most striking observation to emerge from the data comparison pertains to solanone, one of the most important pyrolysis products, which was produced only during the pyrolyses of α-CBT at 600 (0.4%) and 900 °C (0.6%) in 10% O₂. Two possible explanations for this result may apply: (1) both α- and β-CBT produce solanone, but in the case of β-CBT, this material is completely converted to other compounds, whereas that from α-CBT is not completely consumed; alternatively, (2) only α-CBT produces solanone. If the latter conclusion is correct, it can be determined that α-CBT contributes most to tobacco aroma substances. One definite conclusion is that α-CBT can be converted to solanone only in an atmosphere of 10% O₂.

Conclusions
Thermogravimetric analysis showed that epimeric α- and β-CBT underwent significant weight losses of 97.41% and 96.54% in the ranges of 170–700 °C and 180–660 °C, respectively. However, during pyrolysis, α-CBT alone was converted to solanone in low yields of 0.6% and 0.4% at 600 and 900 °C only under 10% O₂, respectively, whereas β-CBT produced no solanone at all.

Despite the low pyrolytic efficiency in the production of solanone, α- and β-CBT have great potential to be developed and utilized, especially because of their abundance. This work demonstrates that an approach such as the microbial degradation of α- and β-CBT might be more appropriate for conversion into aroma compounds such as solanone. Nevertheless, Py-GC–MS was shown to be a useful technique for assessing the pyrolytic potential of α- and β-CBT.

Acknowledgement
This work was supported by the National Natural Science Foundation of China under grant numbers 21406210 and 21546012; the Doctor Research Foundation under grant number 152106000058; and the Henan province foreign cooperation projects under grant number 152106000058.

References

Supplementary Materials
Tables 1 and 2 of this article can be accessed using the QR code to the left.

ABOUT THE AUTHORS
Shen Huang, Chen Yang, Ning Ma, Lifeng Zhou, Chunxiao Jia, Tao Wei, and Dukbin Mao are with the College of Food and Biological Engineering at the Zhengzhou University of Light Industry, in Henan, China.
Preventing Analyte Loss Due to Filter Membrane Adsorption

Vivek Joshi, Cat Lautenschlager, and Maricar Dube, MilliporeSigma, an affiliate of Merck KGaA, Darmstadt, Germany

Many analytical methods, including HPLC, UHPLC, and LC–MS, require sample filtration prior to analysis. When recovery or quantitation of analyte is critical, syringe filters used in sample preparation should be evaluated for analyte loss resulting from filter adsorption. Proper syringe filter validation can prevent analyte loss, improving recovery and yielding more accurate results.

The following factors should be considered when evaluating analyte binding of filter membranes:

1. **Filter Membrane Properties.** Analyte binding is dependent on the physicochemical properties of the membrane. Some common secondary interactions that lead to analyte binding include electrostatic interactions, hydrogen bonding, and hydrophobic interactions. Different membrane materials contain different functional groups, leading to distinct binding characteristics. Membranes made from hydrophilic PTFE, hydrophilic PVDF, and polypropylene tend to exhibit lower binding than those made from PES, glass fibre, or nylon. Nylon tends to exhibit the highest binding due to amino, carboxyl, and amide groups that promote electrostatic interactions and hydrogen bonding.

2. **Analyte Properties.** The physicochemical properties of the analyte are also important. The protonation state of the analyte changes the charge of the molecule, altering potential electrostatic interactions. Functional groups on the molecule also contribute to hydrophobic interactions and hydrogen bonding.

3. **Analyte Concentration.** Analyte concentration also impacts loss. Highest recovery is obtained after the filter membrane has become saturated with analyte. Once saturated, no further analyte binding is observed due to the limited number of functional sites that enable binding. For this reason, the first few millilitres of filtrate are often discarded prior to analysis. As analyte concentration decreases, the volume needed to saturate binding sites on the filter membrane increases.

To illustrate, Table 1 shows the binding behaviour of three different drugs to three different filter membranes. Tablets containing acetaminophen (phenolic – weakly acidic), acetylsalicylic acid (acidic), and caffeine (neutral) were dissolved and filtered through Millex syringe filters containing hydrophilic PTFE, nylon, or Durapore PVDF membranes. The first 1 mL of collected filtrate was analyzed by HPLC.

<table>
<thead>
<tr>
<th>Drug Component</th>
<th>Syringe Filter</th>
<th>Millex® LCR (Hydrophilic PTFE)</th>
<th>Millex® Nylon</th>
<th>Millex® Durapore® PVDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>110.4</td>
<td>58.4</td>
<td>109.0</td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>119.5</td>
<td>28.2</td>
<td>117.4</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>114.2</td>
<td>106.0</td>
<td>112.2</td>
<td></td>
</tr>
</tbody>
</table>

Syringe filters containing hydrophilic PTFE and Durapore PDVF membranes exhibited quantitative analyte recovery regardless of the physicochemical properties of the drug. Lower recovery was obtained with syringe filters containing nylon membrane, suggesting higher filter adsorption that varied based on the physicochemical properties of the analyte. This does not mean nylon should never be used for sample preparation. For these analytes, it is recommended that the first volume of filtrate be discarded to ensure complete saturation of the nylon membrane.

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

Yehia Z. Baghdady¹ and Dwight R. Stoll², ¹Bristol Myers Squibb, New Brunswick, New Jersey, USA, ²LC Troubleshooting Editor

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.

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 downtime. 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 instalment of LC Troubleshooting, I asked Yehia Baghdady, 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 behaviour 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 2D separation before utilizing the 2D-LC system in routine pharmaceutical analysis. That is to say, selecting and examining the effect of various 2D 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 coeluted pair of hydrophilic and highly polar compounds, whereas the late eluting peak contains a pair of coeluted hydrophobic compounds. 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 methods in our laboratory (that is, our platform method). The second is a polar-embedded reversed-phase 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

**TABLE 1:** Key 2D-LC performance metrics monitored over a 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>

**FIGURE 3:** 2D chromatograms obtained for peaks 1a and 1b using the SST method at several intervals over a period of one year. Conditions were the same as in Figure 2.

**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.
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 1. 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 ageing, and mobile phase preparation errors. The aforementioned acceptance criteria are sensitive to these errors and may trigger early alarms that may lead to inaccurate results and may trigger early alarms that otherwise separate, or vice versa. 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 impact on the initial low fraction of organic solvent in the mobile phase (1%) that elutes polar analytes.

**Summary**

In this instalment of “LC Troubleshooting”, we described the development and implementation of a SST for multiple heartcut 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 "D pump that was subsequently repaired to restore system performance.

**Acknowledgement**

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**References**


**ABOUT THE CO-AUTHOR**

**Yehia Z. Baghdady** is Senior Scientist in the Chemical Process Development group at Bristol Myers Squibb, in New Brunswick, New Jersey, USA.

**ABOUT THE COLUMN EDITOR**

**Dwight R. Stoll** is the editor of “LC Troubleshooting.” Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota, USA. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. Direct correspondence to: amatheson@mjhlifesciences.com
Nonlinear Predictive Modelling Enables In Silico Optimization of Chromatographic Methods for Complex Stationary Phase-Analyte Interactions

Imad A. Haidar Ahmad¹, Gioacchino Luca Losacco¹, Adrian Clarke², and Erik L. Regalado¹, ¹Analytical Research and Development, MRL, Merck & Co., Inc., Rahway, New Jersey, USA, ²Chemical & Analytical Development, Novartis Pharma AG, Basel, Switzerland

The development of robust analytical assays for separation and analysis of complex multicomponent mixtures can often be challenging, reflecting the increased complexity of new medicine and vaccine processes. In silico liquid chromatography (LC) method development strategies for small molecules have reached a mature stage across the pharmaceutical industry. However, a straightforward approach for large molecules remains elusive because of conformational changes that can often influence chromatographic retention. Nonetheless, an excellent correlation between experimental and predicted retention time is possible by deploying the correct regression retention models in terms of ln \( k \) vs. %B and ln \( k \) vs. 1/T (\( \Delta t_R < 0.1\% \)). Excellent outcomes generated through in silico chromatographic method development of large molecules using different chaotropic and denaturing mobile phases are illustrated. Linear and nonlinear (polynomial regression) retention models using readily available software were deployed as a function of several chromatographic parameters (gradient slope and column temperature) for a variety of proteins (12–670 kDa) and peptides.

Modern pharmaceutical R&D of increasingly challenging therapeutics and vaccines requires a commensurate level of innovation to deploy critical analytical methods across functional areas (1). Several strategies are commonly applied to the separation of biomolecules, such as ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and reversed-phase liquid chromatography (LC). While reversed-phase LC is known to cause conformational changes of biomolecules under chromatographic conditions, it remains one of the most utilized techniques for purity assays, with a convenient deployment across manufacturing settings. In addition, reversed-phase LC allows for a straightforward hyphenation to mass spectrometry (MS), which is critical in enabling characterization efforts. The use of acids, ion-pairing reagents, and chaotropic reagents in reversed-phase LC mobile phases disrupts noncovalent interactions and results in protein unfolding (2,3). In contrast to small molecule pharmaceuticals, these protein-based targets are highly sensitive to small changes in mobile phase composition and temperature, requiring more challenging method development and optimization endeavours (4). Reversed-phase LC method development of a protein-based sample can benefit from one-dimensional (1D)- and two-dimensional (2D)-LC multicolumn screenings, especially when dealing with complex mixtures (5,6). After the selection of suitable separation conditions, in silico
method development software can be leveraged to facilitate an effective optimization output. During this process, it is crucial to deploy the correct retention models of \( \ln k \) as a function of \( %B \) and \( 1/T \), which are more sophisticated than those deployed for small molecules. Previous studies have demonstrated that it is vital to use second-degree polynomial fits of \( \ln k \) vs. \( 1/T \) when the protein is not completely denatured (4,7,8). However, in the presence of strong chaotropic or denaturing agents, first-degree polynomial regression can deliver good retention time prediction.

**Experimental**

Optima-grade acetonitrile (CH\(_3\)CN) was purchased from Fisher Scientific. Trifluoroacetic acid (TFA) and perchloric acid (HClO\(_4\)) were purchased from Sigma-Aldrich, Inc. Water (H\(_2\)O) was filtered through a Milli-Q UHPLC-grade filter (Waters). All above reagents were used to prepare different mobile phases, solvents, and diluents. The protein standards were obtained from Sigma-Aldrich, Inc. The cyclic peptide (MW ~ 10–21 kDa) was synthesized by an in-house procedure. The protein mixture consisted of Cytochrome C (MW ~ 12.4 kDa), Ribonuclease A (MW ~ 16.9 kDa), Apomyoglobin (MW ~ 44.7 kDa), y-globulins from bovine blood (MW ~ 150 kDa), and Thyroglobulin bovine (MW ~ 670 kDa). A 2.1 mm × 75 mm, 2.7-µm particles, 1000 Å Halo C4 column (Advanced Material Technology, Inc.) was selected. The following chromatographic conditions were selected for reversed-phase method chromatographic modelling and simulation of the protein separation: three eluent gradients (10–70% B in 10 min, 10–70% B in 20 min, and 10–70% B in 30 min) with a flow rate = 0.5 mL/min. All three gradients were executed at three different temperatures (20, 40, and 60 °C) to build a 3D resolution map. For the cyclic peptide sample, the separation was modelled on a 2.1 mm × 100 mm, 2.7-µm particles, 300 Å Halo C4 column (Advanced Material Technology, Inc.) using three gradients (40–70% B in 5 min, 40–70% B in 10 min, and 40–70% B in 15 min). All three gradients were executed at three different temperatures (20, 40, and 60 °C) to build a 3D resolution map. The resultant experimental data were processed using ACD/LC Simulator 2015 Release (Version L10R41) (Advanced Chemistry Development, Inc. [ACD]). Linear and polynomial regression retention models using
ACD/Labs software were built as a function of gradient slope, column temperature, and mobile phase buffer.

**Results and Discussion**

The importance of deploying a second-degree polynomial fit of ln $k$ vs. $1/T$ when modelling the separation of proteins in the absence of strong chaotropic or denaturing reagents is illustrated in Figure 1. In this example, a mobile phase composed of 0.1% trifluoracetic acid in water and acetonitrile was used to separate a mixture of six proteins (Cytochrome C, Ribonuclease A, Apomyoglobin, Albumin chicken egg, y-globulin, and Thyroglobulin bovine).

The resolution map obtained using this nonlinear fit indicated the following optimum conditions: 10–70 %B in 30 min at 50 ºC, as shown in Figure 1(a). Under these conditions, the experimental chromatogram was compared to the predicted chromatograms when first- and second-degree polynomial fits of ln $k$ vs. $1/T$ were used to build the chromatographic model. Figure 1(b) highlights an excellent accuracy of the retention time of the proteins predicted using the second-degree polynomial fit (%,$\Delta t_R < 0.1$). A significant difference between predicted and experimental retention time can be observed when a first-degree polynomial fit was used.

To further understand this difference, the predicted and experimental retention time of Cytochrome C are compared in Figure 2 under 21 conditions (three gradients, seven temperatures). When a first-degree polynomial fit was used for establishing ln $k$ vs. $1/T$ model (left graph), the retentions times at each of the three gradients show a defined curvature as a function of temperature. These results are indicative of an improper fit of modelled retention time vs. temperature (Figure 2[a]). However, a linear trend appeared when a second-degree fit was deployed (Figure 2[b]). This suggests that such deviation of the experimental retention times from the predicted ones was due to an incorrect polynomial fit in the equation relating ln $k$ vs. $1/T$, which could be easily addressed during software simulation.

In the presence of a chaotropic reagent (perchloric acid), the accuracy of the retention time modelled using a first-degree fit was significantly enhanced. Figure 3(a) shows the resolution map for the protein mixture modelled using 0.1 M perchloric acid, a stronger chaotrope than TFA. The following optimum conditions (10–70%B in 30 min at 50 ºC) were extracted from the resolution map to investigate the accuracy
of the modelling when different polynomial fits were used to model retention coefficient and temperature. It should be noted that a very good accuracy (\(\Delta t_R < 0.5\)) between predicted and experimental retention times was observed regardless of which regression model was employed during modelling. Nonetheless, the second-degree fit (\(\ln k \text{ vs. } 1/T\)) resulted in a better prediction of retention time when compared to the first-degree one. This conclusion agrees with previous studies reporting the effect of chaotropic and denaturing conditions on the retention modelling of such complex molecules.

Using the correct equations (\(\ln k \text{ vs. } \%B\) and \(\ln k \text{ vs. } 1/T\)) to model the retention of proteins was essential to establish an accurate and precise computer-assisted simulation for method development purposes. The separation of a pipeline sample composed of a cyclic peptide (MW ~ 20 kDa) and related components (10–21 kDa) was modelled on a 2.1 mm \(\times\) 100 mm, 2.7-μm column using 0.1% TFA in water as mobile phase A and CH\(_2\)CN as mobile phase B. The resolution map obtained highlighted how sensitive the quality of the separation was, specifically in relation to minor changes in percentage composition and temperature (see Figure 4[a]). The optimum conditions obtained from the resolution map, which were established using a second-degree fit between \(\ln k\) and \(1/T\), were localized in a very defined spot. This served to illustrate the importance of computer-assisted modelling to map the entire separation landscape of a given mixture. The experimental chromatogram under the conditions obtained from the resolution map (51%B, 35 °C) is shown in Figure 4(b). To further assess the effect of using a first-degree polynomial fit rather than a second-degree one, the separation was simulated using a first-degree fit, as shown in Figure 4(c). The risk of using the incorrect model to build retention time must be highlighted as this can lead to incorrect optimum conditions, as demonstrated in Figure 4(a) and 4(c) when comparing the two resolution maps.

**FIGURE 2:** Calculated versus experimental retention time of Cytochrome C for 21 conditions (three gradient times [10, 20, and 30 min] each at seven temperatures [20, 30, 40, 50, 60, and 80 °C]) for a set of protein standards under different mobile phase A: 0.1% TFA in water, mobile phase B: acetonitrile, F = 0.5 mL/min, wavelength: 210 nm. In each cluster, the data points correspond to retention times across a temperature range (20–80 °C). (a) A first-degree polynomial is used to fit retention time (\(\Delta t_R\)) as a function of \(1/T\). (b) A second-degree polynomial is used to fit retention time (\(\Delta t_R\)) as a function of \(1/T\).

**FIGURE 3:** (a) Resolution map of the separation of a mixture of proteins obtained using three gradient times (10, 20, and 30 min) at three temperatures (20, 40, and 60 °C) for a set of protein standards (Cytochrome C, Ribonuclease A, Apomyoglobin, Albumin chicken egg, y-globulins, and Thyroglobulin bovine). Mobile phase A: 1M HClO\(_4\). Mobile phase B: CH\(_2\)CN. Column: 2.1 mm \(\times\) 75 mm, 2.7-μm, 1000 Å Halo C4, gradient: 10–70%B, flow rate = 0.5 mL/min, \(\lambda = 220\) nm, F = 0.5 mL/min. (b) Experimental and predicted chromatograms at the optimum conditions obtained from the resolution map. Gradient: 10–70%B in 20 min. Column temperature: 50 °C.
Conclusions
Although recent instrumentation and column technology advances have facilitated chromatographic method development for biomolecules, in silico modelling of retention behaviour has been less straightforward because of conformational changes of biomolecules under chromatographic conditions. To reach satisfactory results in terms of accurate retention time prediction, new in silico optimization approaches must be considered, as illustrated in this article. The choice of the correct polynomial fit is highly important in order to better model the retention time of protein-based targets. In the absence of chaotrophic and denaturing reagents, second-degree polynomial fits of In k vs. 1/T demonstrate better correlation of experimental and predicted retention time.

References

ABOUT THE AUTHORS
Imad A. Haidar Ahmad received his Ph.D. from Florida State University under the mentorship of André Striegel. He completed his postdoctoral research with Peter Carr at the University of Minnesota. He is currently Associate Principal Scientist and Scientific Supervisor in the Analytical Enabling Capabilities Group within MRL’s Analytical Research & Development Department.

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**FIGURE 4:** Separation of a mixture of the components of a cyclic peptide and related products. (a) Resolution map obtained by employing a second-degree fit between ln k and 1/T using three gradient times (5, 10, and 15 min) at three temperatures (20, 40, and 60 °C). Mobile phase A: 0.1% TFA in water. Mobile phase B: CH₃CN. Column: 2.1 mm × 100 mm, 2.7-μm, 300 Å, Halo C4, gradient: 40–70% B, flow rate = 0.5 mL/min, λ = 280 nm, F = 0.5 mL/min. (b) Experimental chromatograms at the optimum conditions obtained from the resolution map (51%B at 35 °C). (c) Resolution map and incorrect optimum conditions obtained by employing a first-degree (linear) fit between ln k and 1/T.
Quo Vadis Analytical Procedure Development and Validation?

C. Burgess1 and R.D. McDowall2, 1Burgess Analytical Consultancy Ltd, Barnard Castle, Co Durham, UK, 2RD McDowall Ltd, Bromley, Kent, UK

What do the draft publications ICH Q2(R2) and Q14 for analytical procedure validation and development mean for a regulated good manufacturing practice (GMP) laboratory? Are they consistent with the approach taken by the United States Pharmacopeia (USP)<1220> on Analytical Procedure Life Cycle? Why does it take the ICH two documents to describe what the USP can do in one?

Good analytical science, as well as ensuring data integrity, requires that analytical procedures should be validated for their intended use (1,2). The applicable Food and Drug Administration (FDA) good manufacturing practice (GMP) clause is 21 CFR 211.194(a)(2), which has been in the regulation since 1978 (3):

The suitability of all testing methods used shall be verified under actual conditions of use.

Similarly, EU GMP Chapter 6.15 on Quality Control requires validation of test methods (4):

Testing methods should be validated. A laboratory that is using a testing method and which did not perform the original validation, should verify the appropriateness of the testing method. All testing operations described in the marketing authorization or technical dossier should be carried out according to the approved methods.

Does Your Analytical Procedure Generate OOS Results?
One of the continuing sources of schadenfreude with FDA warning letters and 483 observations is the invalidation of out of specification (OOS) results for a variety of unscientific reasons, such as the ever-popular “human error”—often without a scientific investigation. This results in invalidation of the result. This has resulted in the FDA generating a quality metric for any quality control (QC) laboratory of a percentage of invalidated OOS results (5), as well as a revision of the guidance on investigating OOS results in May 2022 (6).

Why are there many OOS results in some laboratories? After all, pharmacopoeial procedures always work as written? Right? Wrong! Could a laboratory have rushed to validate a hastily developed procedure with the result that a poor procedure is the root cause of OOS results? We need a robust method development phase for any analytical procedure to have a sound foundation for validation and to ensure that OOS results are rare.

ICH and USP Approaches to Procedure Validation
We will focus mainly on publications from two organizations: United States Pharmacopeia (USP) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) for analytical procedure validation. For those who are unaware, ICH was initially
called the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use from its inception in 1990. It was a consortium of the regulatory authorities and pharmaceutical industry bodies from the US, Japan, and Europe. The aim was to develop unified approaches to key regulatory topics so that the pharmaceutical industry only had to perform one activity and it was acceptable in the three regions. Since then, additional regulatory authorities have joined (for example, Brazil and South Korea) and the name of the organization has changed. Conference has been replaced by Council, but it is still known as ICH.

As you can see in Figure 1, the FDA have also published two draft and one final guidance document on validation for analytical procedures and methods. Which brings us to an interesting question: procedure or method?

**Analytical Method or Analytical Procedure?**

It is important to understand what is meant by analytical procedure and if there is a difference with analytical method:

- Analytical procedure refers to the whole process, from sampling through to reporting the result.
- Analytical method usually only refers to the instrument portion or analytical principle/separation technique of the analytical procedure.

Therefore, analytical procedure is the preferred term, as there may be problems when sampling (light sensitive analyte) during preparation of the sample dilution/extraction before analysis or during transport or storage (unstable analyte), which will not be considered if you are focused on the analytical method. Unless you are the FDA, who have bet on both terms.

**In The Beginning—1: Initial Validation Guidance**

Although 21 CFR 211 was published in 1978, there was no official guidance available on how to validate an analytical procedure; eventually the USP issued an informational general chapter <1225> entitled Validation of Compendial Methods in 1989 (7).

In addition to general chapter <1225>, USP has also issued two more general chapters:

- USP <1226> Verification of Compendial Procedures (8)
- USP <1224> Transfer of Analytical Procedures (9).

**In the Beginning—2: ICH Q2**

USP <1225> served as the foundation for the development of ICH Q2 Guidance on Validation of Analytical Procedures, which was published in two parts:

- ICH Q2A Validation of Analytical Methods: Definitions and Terminology in 1994 and is the parent guideline;

In 2005, ICH Q2A was renamed ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology and incorporated into ICH Q2B, with no changes to the content of the combined document (10) (see Figure 1). However, there are some problems with Q2:

- Procedure development is ignored; this is vitally important as cobbling a procedure together can result in variability, contributing to OOS results.
- Q2(R1) can be followed slavishly; for example, an analytical procedure for measuring assay between 90 and 110% of label claim often have limits of quantification (LOQ) and limits of detection (LOD) determined in the validation. The reason? It is in ICH Q2(R1)! This is stupid; sound science has not been applied, as required by 21 CFR 211.160(b) (3). We bet you didn’t know that a regulation required you to use sound science.
- The focus is mainly on chromatographic analysis.
In The Beginning—3: USP Life Cycle of Analytical Procedures

However, our interest lies in the latest USP general chapter <1220> on Analytical Procedure Life Cycle (11), which became effective on 1 May 2022. Bet you didn’t know that either! USP <1220> has had a long gestation; starting in 2011 it released the first stimulus paper to the Revision Process in 2012 (12), with further stimuli articles (13–15) and two draft versions <1220> (16,17) to support the development of the general chapter.

The life cycle approach is now a regulatory expectation and features in ICH Q8 Pharmaceutical Development (18) and ICH Q12 Technical and Regulatory Considerations for Pharmaceutical Product Life Cycle Management (19).

The aim of ICH Q8 is to move from quality by testing to quality by design (QbD), using knowledge and science as well as statistical design to achieve more robust pharmaceutical products. QbD is defined by ICH Q8 (18) as:

A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.

The key points are:
- Predefined objectives
- Product and process understanding
- Sound science
- Risk management.

These four points are incorporated in the three-stage life cycle of <1220> (11), which is shown in Figure 2 and comprises:

1. Procedure Design and Development (PPD): PPD or method development is based on the analytical target profile (ATP) for the procedure. Within this phase of the life cycle, the critical parameters are identified and risk-based ways to control them and an analytical control strategy defined. A design space is defined and tested using experimental design software.

2. Procedure Performance Qualification: Otherwise known as method validation of the final design space against the parameters for intended use of the analytical procedure as defined in the ATP and elucidated during the PPD phase of the life cycle.

3. Procedure Performance Verification: After formal release, ongoing assessment is necessary to monitor the procedure’s performance during operational use, for example, trending data and results over time. Any changes made within the design space are considered validated and can be made without formal change control. Changes outside the design space return to either phase 2 for qualification or, if major, revise the ATP with further development. This is the longest portion of the life cycle and it is imperative that it is controlled and monitored to ensure verification against intended purpose.

The key to a life cycle approach for any analytical procedure is to define the intended purpose as required by the GMP regulations. This is achieved by writing an ATP to provide the foundation for all other work in the life cycle. Get the ATP wrong and the procedure is not fit for intended purpose.

ICH Catchup: Revision of Q2(R1) and Writing Q14

In 2018, the ICH established an Expert Working Group (EWG) to revise Q2(R1) and write Q14 (20). The aims of the EWG were:
- Q2 required revision, as it lacked guidance for near-infrared spectroscopy (NIR) and Raman spectroscopy, particularly using multivariate models, resulting in inadequate validation reports in regulatory submissions and multiple questions being asked by regulators.
- Development of Q14 as a new guideline outlining the principles and scientific approaches for analytical procedure development
- Specifically, the feasibility of combining both documents into one for simplification and clarity should be examined.

ICH guidances go through a five-step process:
- Step 1: Define the scope and drafting by the Expert Working Group (EWG)
- Step 2: Issue of the draft for public comment (the stage that Q2[R2] and Q14 are at now)
• Step 3: Review of public comments and update of the document
• Step 4: Final approved version released
• Step 5: Incorporation into a member’s national regulatory framework. This can vary from updating regulations to issuing a guidance for industry.

Let us now look at the contents of these two draft guidance documents. Avid readers should also read a review of these two draft documents by Teasdale et al. (21), who have been involved in analytical procedure life cycle development for a number of years.

Two Into One? No Chance!
The first thing to realize is that the EWG have failed to combine both documents to define, simplify, and clarify a unified approach to life cycle management of analytical procedures. This leaves analysts in regulated laboratories juggling two regulatory documents with differing approaches on the same subject. A great start it is not.

This leaves ICH Q2(R2) (22) and its continuing failure to mention the most critical parts of the life cycle: method development and on-going performance verification. Given the pharmaceutical industry’s inability to change, this means perpetuating poor science. Furthermore, the failure to consider a life cycle approach breaks the principles outlined in ICH’s own publications of ICH Q8 and Q12.

In summary, the revision of ICH Q2(R2) is a tweak not a substantive advance; the issues mentioned about lack of validation in regulatory submissions are discussed in ICH Q14. To be fair to the EWG, it is argued that they wanted to maintain the status quo, but this brings with it its own problems. Will analysts read ICH Q14 and Q2(R2) and integrate them together? Probably not. It has not stopped USP <1220>, which has managed to incorporate all phases in a single general chapter (11).

ICH Q2(R2): The Bad Bits
Let’s start with the major flaws in the draft (22):
• Does Q2(R2) define an analytical procedure life cycle? No.
• Does it define an ATP to document intended use of the procedure? No. Q2(R2) does mention “performance characteristics” as the nearest to ATP.
• Method development is still conspicuous by its absence. This is a critical component of the life cycle that has been missing throughout the various versions of Q2.
• There is no mention of validating the analytical procedures against the intended purpose as defined by an ATP. Although Figure 1 shows the linkage between ICH Q2(R2) and Q14, the real linkage is the ATP. But if these documents are to remain separate, they should be linked via procedure of the ATP.
• The only mention of ATP in the whole of the Q2(R2) is in the glossary, which is copied from ICH Q14.

The draft guidance jumps straight into a validation study for the procedure, assuming that development is not done, undocumented, or cobbled together. Given the fact that ICH Q8 and Q12 both focus on life cycle and the proposed ICH Q2(R2) does not, this leaves us with the assumption that the authors are happy to accept a suboptimal and unscientific approach.

ICH Q2(R2): The Good Bits
On the plus side is the expansion of the scope of ICH Q2(R2) to include:
• Spectroscopic methods such as nuclear magnetic resonance (NMR), NIR, and Raman assays for both identification and quantitation.
• Given the large number of biological products and biosimilars that are available and in development, biological assays (polymerase chain reaction [PCR], cell-based, and binding assays) are included in an expanded ICH Q2(R2) draft. This has resulted in an expansion of calibration methods to accommodate them.
• Section (3.1) covers validation during the life cycle, which was not mentioned in R1. However, it only mentions validation due to changes or transfer, but not as continuous performance verification.
• Good results require a good calibration model and the draft guidance includes calibration models that are linear, nonlinear, and multivariate. Use any calibration method but keep it appropriate—and use the simplest model for your data.
• Linearity as a validation parameter is only applicable now for linear calibration models.

In Annex 1, there is a good process flow diagram leading from the objective of an analytical procedure that then defines which parameters need to be considered during a validation (not forgetting development as well): specificity, range, accuracy, and precision. For example, a procedure for identification would focus on specificity in contrast to an assay where all four would be determined. This is better than the table in the current version of ICH Q2(R1) (10).

ICH Q14: A Life Cycle Approach (Mostly)
In contrast, ICH Q14 (23) aligns with ICH Q8 in terms of a life cycle approach to analytical procedures but with a big gap—validation of the
ICH Q14 Enhanced Approach

ICH Q14

USP

Stage 1: Procedure Design

ICH Q2(R2)

Stage 2: Procedure

ICH Q14 Minimal Approach

Stage 3: Procedure

ICH Q14 Enhanced Approach

ICH Q14 (23)

USP <1220> (11) versus draft ICH Q2(R2) (22) and ICH Q14 (23)

TABLE 1: Comparison of minimal and enhanced life cycle approaches for analytical procedures

<table>
<thead>
<tr>
<th>ICH Q14 Minimal Approach</th>
<th>ICH Q14 Enhanced Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Identify attributes of the procedure to be tested</td>
<td>• Evaluate sample properties</td>
</tr>
<tr>
<td>• Select the instrumental technique</td>
<td>• Evaluate prior knowledge of the analyte and sample matrix</td>
</tr>
<tr>
<td>• Conduct development studies</td>
<td>• Define the ATP</td>
</tr>
<tr>
<td>• Define and document the analytical procedure</td>
<td>• Conduct risk assessment</td>
</tr>
</tbody>
</table>

The ATP defines what is wanted from the procedure but not how it is achieved. However, from the ATP comes the outline of the analytical procedure with knowledge of the analyte and matrix:

- Any sampling and sample storage requirements
- Any sample preparation required
- Instrumental technique used to analyze the sample
- Any data interpretation or calculation required
- Nature of the reportable result.

A good idea is to define the ATP for any analytical procedure, including the minimal approach. However, without an ATP is the minimal approach scientifically sound (3)? No. Outlines of the minimal and enhanced approaches are shown in Table 1 (23). Apart from defining the ATP, the critical component of the enhanced approach is the definition of an analytical control strategy. Here time is taken to identify the parameters that are critical to ensuring a robust analytical procedure and how they must be controlled. Knowing how a component can influence the outcome is essential, such as, for example, what happens to the separation if you have more or less organic modifier or aqueous buffer in the mobile phase.

What is not shown in Table 1 is the iterative nature of the development process between the analytical control strategy and the results from experiments. As more information and knowledge about the procedure are generated, the procedure and control strategy can be refined. Risk management is used to identify critical parameters that may need to be carefully controlled and others that have a lesser or zero impact on the outcome of the procedure. Experimental design software can be used to devise experiments that will define the procedure design space. Applications can be either standalone or can control instruments via a chromatography data system. Having this information before the validation of the procedure as well as when it is used operationally is essential, as changes within the design space can be made without the need for revalidation.

ICH Q10 Section 10 deals with submission of analytical procedure. One question that arises is why is this not in ICH Q2(R2)? This is where the bulk of the data are generated, and is a good argument for merging the two documents.

ICH Grand Canyon of Emptiness

It is often said that a camel is a horse designed by committee and with the two ICH documents we have two camels. Let us explain.

What do we mean by the word life cycle? A reasonable definition would be a series of changes from birth to death. Thus, an analytical procedure life cycle should encompass the beginning of the method through its development, validation, and operation. What is missing from the two ICH documents? The operational phase and monitoring of the validated procedure! This is the critical Grand Canyon of omission by
the ICH EWG and the longest part of the life cycle. Table 2 maps three phases of a procedure life cycle for the two ICH documents and USP <1220>; it is clear that ICH has no consideration for operational monitoring of a procedure.

In addition, we suggest that by this omission the ICH approach fails to comply with the EU GMP Chapter 6 requirements (4):

6.9 Some kinds of data (e.g. tests results, yields, environmental controls) should be recorded in a manner permitting trend evaluation...

6.16 The results obtained should be recorded. Results of parameters identified as quality attribute or as critical should be trended and checked to make sure that they are consistent with each other...

In conclusion, the ICH EWG must revise these documents to include a full life cycle; alternatively, use the USP <1220> approach for a complete life cycle in a single document.

Summary
Whilst Q2(R2) and Q14 represent significant progress, the lack of consistency between them is disappointing. Had the EWG seen fit to produce a combined document then this would have been avoided. However, the largest issue remains from a life cycle perspective.

Validation is a journey not an event. The USP <1220> has clearly got this journey process right. ICH has only managed to cover stage 1 with Q14 and stage 2 with a revised Q2(R2). Why has ICH ignored the longest and most important life cycle component of performance verification during actual use (stage 3)? ICH should now consider a new guideline to cover this deficiency and complete the validation life cycle process.

Perhaps history will repeat itself and, just as USP<1225> was a precursor to ICH Q2, USP<1220> could provide the stimulus and input to a new ICH guideline for stage 3. We hope so.

Acknowledgements
We would like to thank Jane Weitzel and Margarita Sabater for their review comments.

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ABOUT THE AUTHOR
Chris Burgess is Managing Director of Burgess Analytical Consultancy Limited, in Barnard Castle, UK. He is also a member of Pharmaceutical Technology Europe’s editorial advisory board.

ABOUT THE COLUMN EDITOR
Bob McDowall is Director of R.D. McDowall Limited, Bromley, UK. He is also a member of LCGC Europe’s editorial advisory board. Direct correspondence to: amatheson@mjhlifesciences.com
Improved Performance of UHPLC–MS Hyphenated Systems

Fabrice Gritti, Sornanathan Meyyappan, Wade Leveille, and Jason Hill, Waters Corporation, Milford, Massachusetts, USA

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 postcolumn 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 2× 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 × 100 mm column packed with sub-2-μm core–shell particles (1.6 μm Acquity UHPLC Cortecs C18 column).
to illustrate the main problem of excessive postcolumn 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, sulfadimethoxine 1 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 × 100 mm, 1.6-µm Acquity UHPLC Cortecs C18 column (Waters). The LC system was 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

### TABLE 1: MRM settings of the mass spectrometer

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass Precursor Ion</th>
<th>Mass Product Ion</th>
<th>Cone Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>152.06</td>
<td>110</td>
<td>35</td>
</tr>
<tr>
<td>Valine-Tyrosine-Valine</td>
<td>380.2</td>
<td>136.1</td>
<td>25</td>
</tr>
<tr>
<td>Leucine-Enkephalin</td>
<td>556.2</td>
<td>397.2</td>
<td>35</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>311</td>
<td>156</td>
<td>25</td>
</tr>
<tr>
<td>Verapamil</td>
<td>455.4</td>
<td>165.2</td>
<td>35</td>
</tr>
<tr>
<td>Reserpine</td>
<td>609.3</td>
<td>195</td>
<td>35</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>472.2</td>
<td>436.2</td>
<td>35</td>
</tr>
</tbody>
</table>
98% over 3 min. The analyte detection was performed under the multiple reaction 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 1.

**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 × 75 μm connecting tube. The sample mixture was a solution of acenaphthene (0.2 g/L) and octanophenone (0.1 g/L) prepared in a 25:75 (v/v) water–acetonitrile mixture, 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 1. All the modelling 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 generated. The model is based on a pressure-based steady-state solver with double precision and considers gravity to resolve natural convection. A coupled solver was used for solving pressure-velocity coupling of the Navier-Stokes momentum, Navier-Stokes energy, and the continuity equation. Based on its bulk superficial velocity and Reynold’s number, a laminar viscous model with viscous heating was used for the eluent mixture (50:50 [v/v] acetonitrile–water). The thermal profile inside the packed bed zone was modelled assuming an energy source term for the eluent mixture without modelling 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 modelled as an incompressible ideal gas. The inlet boundary condition was set by the inlet superficial linear velocity of the eluent at a fixed temperature of 50 °C. The postcolumn heater was set at a uniform and constant temperature boundary condition, which was increased from 60 °C to 75 °C in subsequent simulations. Table 2 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 postcolumn sample dispersion occurring from the column outlet to the divert (infusion) valve and the ESI source. Figure 2 shows schematics of the standard and modified systems (left) and photography of the research prototype system (right). The chromatographic column (2.1 mm × 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 postcolumn tubing assembly of the modified UHPLC–MS system was optimized to minimize the postcolumn dispersion. Overall, the postcolumn dispersion variance was reduced from approximately 13 μL² (for a 60 cm × 100 μm tube + a divert [infusion] valve + a 75 cm × 125 μm tube) to approximately 0.3 μL² only. The gain in performance is discussed quantitatively in the next section.

The injection valve of the I-class Acquity UHPLC system is now connected to the column inlet via a 60 cm × 120 μm i.d. Peek tube in addition to an active eluent preheater (APH). 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 (19–21). Therefore, the column was wrapped in a vacuum jacket (see Figure 1), described in great detail in two previous special issues of *LCGC North America* (22,23). The benefit of the VJC is that the heat delivered at the column inlet (25 °C < T_{inlet} < 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 (23). 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 postcolumn 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.

Postcolumn Dispersion: Figure 3 justifies why the postcolumn modifications are critical in LC–MS when operating a narrow-bore 2.1 mm × 100 mm long column packed with 1.6-μm Cortecs-C18 core–shell particles. Each solid-coloured curve represents all the possible combinations of postcolumn dispersion (y-axis) and retention factor, $k_{\text{elution}}$, 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 postcolumn 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 postcolumn sample dispersion equal to 13 μL² would lead to a fraction $p \approx 0.44$. Therefore, the gradient peak capacity observed is no more than 44% of the maximum peak capacity. Reducing the postcolumn sample dispersion from 13 μL² down to only 0.3 μL² (see the vertical blue arrow) increases $p$ from 0.44 to 0.95: the observed peak capacity is now equal to 95% of the maximum peak capacity.

![Figure 5: Evidence of heat leaks because of viscous heating ($F_v = 0.65$ mL/min, $\Delta P = 12,000$ psi, 100% water) at the column outlet revealed by an IR camera image. (b) End nut heater T$_{\text{outlet}}$ optimization ($T_{\text{inlet}} = 50 \degree C$). Experimental gradient peak capacity is measured as a function of the outlet end nut heater temperature (from “off” or passive to 40, 50, 60, 70, and 80 °C). Note the optimum peak capacity for T$_{\text{outlet}} \approx 70 \degree C$ when T$_{\text{inlet}} = 50 \degree C$.](image1)

![Figure 6: Calculation of the radial temperature profiles across the diameter (2.1 mm) of the packed bed at the column outlet (z = 10 cm) for three different outlet end nut temperatures ($T_{\text{outlet}} = 60$, 70, and 75 °C) and fixed $T_{\text{inlet}} = 50 \degree C$. Note the quasi-flat temperature profile when the optimum outlet end nut temperature is set at 70 °C.](image2)

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**OUTLET RADIAL TEMPERATURE PROFILE**

- Large and negative wall-to-centre temperature differences → Not optimum peak capacity
- Wall-to-centre temperature differences minimized → Optimum peak capacity
- Large and positive wall-to-centre temperature differences → Not optimum peak capacity

<table>
<thead>
<tr>
<th>Radial position from column centre (mm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.5</td>
<td>60.1</td>
</tr>
<tr>
<td>-1</td>
<td>61.4</td>
</tr>
<tr>
<td>-0.5</td>
<td>62.8</td>
</tr>
<tr>
<td>0</td>
<td>64.2</td>
</tr>
<tr>
<td>0.5</td>
<td>66.1</td>
</tr>
<tr>
<td>1</td>
<td>68.0</td>
</tr>
<tr>
<td>1.5</td>
<td>70.0</td>
</tr>
</tbody>
</table>

**OUTLET Radial Temperature Profile**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Radial position from column centre (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>-1.5</td>
</tr>
<tr>
<td>72</td>
<td>-1</td>
</tr>
<tr>
<td>70</td>
<td>-0.5</td>
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<td>64</td>
<td>1</td>
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<tr>
<td>62</td>
<td>1.5</td>
</tr>
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</table>

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**FIGURE 5:**
(a) Evidence of heat leaks because of viscous heating ($F_v = 0.65$ mL/min, $\Delta P = 12,000$ psi, 100% water) at the column outlet revealed by an IR camera image. (b) End nut heater T$_{\text{outlet}}$ optimization ($T_{\text{inlet}} = 50 \degree C$). Experimental gradient peak capacity is measured as a function of the outlet end nut heater temperature (from “off” or passive to 40, 50, 60, 70, and 80 °C). Note the optimum peak capacity for T$_{\text{outlet}} \approx 70 \degree C$ when T$_{\text{inlet}} = 50 \degree C$.

**FIGURE 6:** Calculation of the radial temperature profiles across the diameter (2.1 mm) of the packed bed at the column outlet (z = 10 cm) for three different outlet end nut temperatures ($T_{\text{outlet}} = 60$, 70, and 75 °C) and fixed $T_{\text{inlet}} = 50 \degree C$. Note the quasi-flat temperature profile when the optimum outlet end nut temperature is set at 70 °C.
capacity or a relative increase of 116% compared to that observed for the standard UHPLC–MS system. Nevertheless, in contrast, Figure 4 reveals experimentally that the peak capacity increases from 117 (reference LC–MS system configuration: “Standard”) to only 187 (60%, modified “No sleeve” configuration) with reduced postcolumn sample dispersion. This less-than-expected performance gain is explained by the fact that the eluent temperature cannot be maintained at \( T_{\text{inlet}} = 50 \, ^\circ \text{C} \) along the entire column length because of heat losses towards the external environment. This problem calls for additional modification of the instrument.

**Vacuum Jacket:** Bringing the column outlet near the API source enclosure leaves the heated column \( (T_{\text{inlet}} = 50 \, ^\circ \text{C}) \) in a still-air environment at room temperature. The column temperature cannot be maintained uniform at the temperature set by the active eluent preheater at the column inlet. The temperature difference between the column walls and the surrounding air drives a heat flux from the column body towards 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 (24,25). 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 7:** Experimental variation of the peak asymmetry (at 5% peak height) of (a) acenaphthene and (b) octanophenone eluted under isocratic conditions (see details in the experimental section) as a function of the outlet end nut temperature \( (T_{\text{outlet}} = 50, 70, \) and \( 80 \, ^\circ \text{C}) \) for \( T_{\text{inlet}} = 50 \, ^\circ \text{C} \). Note the progressive evolution of the peak shape from tailing to fronting when increasing the heat flux from the column outlet wall to the packed bed. Quasi-symmetric peaks are observed at the optimum temperature of 70 °C.

**FIGURE 8:** Application: LC–MS gradient chromatograms of a pharmaceutical sample mixture (See experimental section and Table 1 for the gradient conditions) recorded for the same four system configurations as those described in Figure 3. Note the constant reduction of the peak widths of verapamil, reserpine, and terfenadine compounds from the “Standard” to the “No sleeve,” “With the sleeve,” and to the “With sleeve and opt outlet T” system configurations.
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 × 100 mm stainless steel (SS) column packed with 1.6 μm Cortecs-C<sub>18</sub> particles installed in the “with sleeve” UHPLC–MS system configuration. The inlet temperature was 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<sub>p</sub> = 4.2 × 10<sup>6</sup> J/m<sup>3</sup>-K, and its coefficient of thermal expansion is α = 3.1 × 10<sup>-4</sup> K<sup>-1</sup> (9).

Accordingly, if the column is strictly operated under adiabatic conditions, the expected increase, ΔT, of the water temperature from the column inlet to the column outlet is expected to be ΔT = (1-ΔT) ΔP/(C<sub>p</sub>). The red colour 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.

Table 2: Physicochemical parameters of all the materials involved in the VJC-based LC–MS system considered in calculating the radial temperature profiles across the column diameter (2.1 mm × 100 mm) and reported in Figure 7

<table>
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<tr>
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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 (23).

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, \( T_{\text{outlet}} \), 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 \( T_{\text{outlet}} = T_{\text{outlet, opt}} \approx 60–70 ^\circ \text{C} \). 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 postcolumn sample dispersion from approximately 13 µL² down to 0.3 µL², and 2) the elimination of the negative effect of radial temperature gradients on column performance. The proposed explanation is as follows: when \( T_{\text{outlet}} \) 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 centre than in the wall region, and the loss of column performance is maximum. When \( T_{\text{outlet}} \) 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 (26–28), 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 \( T_{\text{outlet}} \approx 70 ^\circ \text{C} \), 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 ageing 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 postcolumn sample dispersion (13 μL² → 0.3 μL²) along with the elimination of most of the radial temperature heterogeneities (∆T < 0.3K) across the packed bed diameter by adjusting the temperature of the column outlet (T_{outlet}−T_{inlet}~20 °C at ∆P~12,000 psi and Fv = 0.6 mL/min).

Acknowledgements
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References
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22) F. Gritti, Supplement to LC GC North America 36(s6), 18–23 (2018).
23) F. Gritti, Supplement to LC GC Europe 32(s5), 8–13 (2019).

ABOUT THE CO-AUTHORS
Fabrice G. Gritti is Principal Consulting Scientist in the Instrument/Core Research/ Fundamentals Department at Waters Corporation in Milford, Massachusetts, USA. Sornanathan Meyyappan is Research Mechanical Engineer II at Waters Corporation in Milford, Massachusetts.

Wade P. Leveille is Principal Mechanical Engineer working for Waters Corporation in Milford, Massachusetts.

Jason Hill is Principal Research Scientist at Waters Corporation in Milford, Massachusetts.

ABOUT THE COLUMN EDITOR
David S. Bell is a Research Fellow in Research and Development at Restek. He also serves on the Editorial Advisory Board for LC GC and is the Editor for “Column Watch” Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: amatheson@mjlifesciences.com.
Advanced Detection for Size-Exclusion Analysis of Proteins: What it Offers and What it Demands

Biotherapeutics, such as molecules derived from a monoclonal antibody (mAb) and their impurity profiles, are becoming more complex. These complexities challenge the routinely applied analysis of high and low molecular weight impurities by size-exclusion chromatography (SEC) and UV absorbance. Possible limitations of SEC-UV can be overcome by adding advanced detection technologies such as multiangle-light scattering (MALS) and mass spectrometry (MS).

LCGC: SEC-UV is the gold standard for analyzing protein biotherapeutics and their high- and low-molecular-weight impurities—why have there been limitations recently?
KRUMM: Not all molecules experience limitations with SEC-UV analysis. However, biotherapeutics are developing and getting more complex. So what used to be a classic mAb with two identical heavy chains and two identical light chains is now a bi-specific, that in its simplest form, comprises a half mAb that targets one antigen and a half mAb that targets another antigen. In some cases, we encounter tri- and multi-specifics. These contain additional parts of antibodies that convey specific binding to a target molecule to get tri- or multi-specificity. This may be, for example, a variable domain of a heavy chain.

These modifications result in a more complex impurity profile—more difficult to separate using SEC. Think of a nanobody that is attached to a full mAb structure—it makes a difference of 15 kilodaltons whether it is attached or not, which is 10% of the total weight of our molecule. And this gets challenging for SEC. Another example is light-chain mispairing, where the molecular weight (MW) difference is minimal. However, it does make a difference if on a bispecific there are two times the same light chains with identical specificity or if there are two different light chains and specificities—this affects efficacy and needs to be analyzed.

LCGC: For more complex impurity profiles, SEC might not be sufficient. How can we still characterize those samples?
KRUMM: The good news is we don’t have to start at square one; it’s the opposite, in fact. We can still use SEC separations and extend them with more sensitive detection methods, meaning more sensitive than UV: MALS and MS.

LCGC: How does MALS characterize complex samples?
KRUMM: MALS exploits the fact that molecules scatter light, and the intensity of the scattered light changes with their concentration and MW. This means if we detect the light that a molecule scatters, and if we know the concentration from concentration detectors, e.g., UV, we can directly determine the MW of the sample component. This now allows us to draw additional information from a size-exclusion run. We can directly determine the MW of the molecule separated, which, yes,
you can do with SEC, but there are drawbacks and inaccuracies to SEC and using a standard calibration curve: It may determine the wrong MW, as the separation is size-dependent and not MW-dependent. So, if a molecule has a different size and shape than what was used for the calibration, then the result will not be accurate. Furthermore, if the separation not only works 100% on a size-exclusion mechanism, but we come across secondary interactions, this leads to undesired retention of our molecules and can also lead to incorrect MW results. These uncertainties do not exist with MALS, as the MW is directly determined independently of the retention time. Furthermore, it allows us to assign peaks more accurately to a specific structure. We can also identify species that are hidden in UV. If we don’t know if something is a shoulder or if it might be a tailing, then we use MALS to tell us if we have two different molecules or if we have a tailing due to any secondary interactions.

Last, as large molecules scatter more light, MALS is particularly sensitive for aggregates that we want to analyze in our mAb or protein samples. MALS can characterize high-MW impurities down to the nanogram range if the most sensitive device for MALS currently on the market is used: the LenS3® detector.

**LCGC:** Which additional information can be derived from MS?
**KRUMM:** High-resolution mass spectrometers can be used in combination with SEC to analyze the intact proteins that are eluting from it. When we use electrospray ionization (ESI) in combination with a mass analyzer, we receive the exact masses for the sample components at a higher resolution than MALS, for example. This way, it is possible to get back to minor structural differences in our samples, e.g., post-translational modifications such as glycosylations. And it’s the most accurate way to analyze our samples and their impurities.

**LCGC:** What about possible disadvantages?
**KRUMM:** The additional information we get from these two methods does come at a price. MALS and MS need a standalone detector, which costs money. A MALS detector is more affordable than a high-resolution mass spectrometer. Due to their sensitivity, both detectors make impurities of the system visible that are not seen in UV. For MALS, larger particles are problematic, such as salt crystals or microbes, as it is sensitive to big molecules. These particles may stem from the buffer, which can be avoided by multiple filtrations, or from the high-performance liquid chromatography (HPLC) or ultra HPLC (UHPLC) that is connected to the detector, which can be avoided by thoroughly cleaning and flushing the instrument upfront.
**Bioinert System**
The Nexera XS inert, a bioinert UHPLC system, from Shimadzu, offers reliable, robust performance. According to the company, the system represents a new peak in the analysis of biopolymers. In combination with a new range of consumables, Shimadzu extends its offering by a complete solution for bioanalysis.

www.shimadzu.eu/higher-peaks
Shimadzu Europa GmbH, Duisburg, Germany.

**UV–vis Detector**
Luma from VUV Analytics is a new UV–vis detector for gas chromatography that is designed to be universal, sensitive, selective, and simple. Providing up to 12 channels of data from 120–500 nm, Luma is the ideal detector for trace-level analysis, according to the company.

www.vuvanalytics.com
VUV Analytics, Inc., Cedar Park, Texas, USA.

**Prep System**
Prepbox is a compact alternative to Ecom’s modular preparative systems. It is used for high-capacity systems or with centrifugal chromatography systems, such as in pharmaceutical purification of cannabis extracts. One basic gradient pump, optionally a secondary pump for repetitive sampling or with injection valve, switching valve for CCC applications, PDA 4-channel detector up to 800 nm.

www.ecomsro.com/systems/compact-preparative
ECOM spol. s r.o., Chrastany u Prahy, Czech Republic.

**Thermal Desorption System**
According to the company, the Unity-xr is versatile—upgrade the single-tube unit to include canisters and online automation; secure—re-collect a portion of every sample for storage/re-analysis; and flexible—choice of carrier gas.

https://bit.ly/3PsNsOF
Markes International Ltd, Bridgend, UK.

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

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

**UHPLC Columns**
The newly released YMC-Accura Triart (U)HPLC columns are characterized by a bioinert surface coating for challenging substances such as oligonucleotides or peptides/proteins. According to the company, they provide excellent peak shapes, prevent carryover without any preconditioning, secure recovery, and are ideal for use in highly sensitive LC–MS analyses. They are available with all eight YMC-Triart stationary phases.

https://ymc.eu/d/brDnU
YMC Europe GmbH, Dinslaken, Germany.
**Process Analytical Technology**

The ultraDawn reportedly represents a breakthrough in process analytical technology for production of nanoparticles, biopharmaceuticals, and polymers. It measures multi-angle light scattering and reports the results—molecular weight, size, and particle concentration—in real time. With RT-MALS, critical quality attributes can be monitored directly, for rapid feedback on product and process quality.

[www.wyatt.com/ultraDAWN](http://www.wyatt.com/ultraDAWN)
Wyatt Technologies, California, USA.

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**Nitrogen Generator**

The VICI DBS HP Tower Nitrogen Generator produces a 24/7 on-demand supply of high-purity nitrogen with flow rates from 500 to 4000 mL/min, purity up to 99.999% and less than 0.1 ppm, and THC pressure up to 5 bar. The generator can be placed close to the instrument, which eliminates the need for long gas lines from external cylinder supplies.

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

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**Protein Kit**

This kit contains 10 different proteins preweighed into autosampler vials for the convenient molecular weight and radius calibration of aqueous GPC/SEC systems. The concentrations are optimized for UV detection @ 280 nm.

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

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**LC Purification Software**

PurityChrom 6 is the next generation of Knauer’s control software for LC purification systems. The GAMP 5 and 21 CFR part 11 compliant software addresses separation tasks in biopurification and preparative HPLC. An animated flow path visualization facilitates operation and method creation. The software features intelligent fraction collection and is multi-system capable.

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

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**UHPLC Columns**

TSKgel UP-SW3000-LS UHPLC columns analyze monoclonal antibodies and biotherapeutics by SEC with high resolution. According to the company, the key feature of this column is its noise suppression in downstream detection methods such as light scattering and MS.

Tosoh Bioscience GmbH, Griesheim, Germany.

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**Chiral Phase**

Daicel’s immobilized chiral selector, ChiralPak IK, builds on the ChiralPak IG, an effective chiral chromatography phase used in life science laboratories. ChiralPak IK uses the same chiral derivative as ChiralPak IG but with a cellulose polymer, offering the opportunity to re-evaluate and discover new chiral separations not previously separated on other polysaccharide-based phases. Available in 3 and 5 µm.

[www.chiraltech.com](http://www.chiraltech.com)
Daicel Chiral Technologies Europe, France.
SEC-MALS of Antibody Therapeutics—A Robust Method for In-Depth Sample Characterization

Tosoh Bioscience GmbH

Modern biotherapeutics such as bi- or multispecific antibody-derived molecules can have complex impurity profiles. To characterize these, conventional size-exclusion chromatography (SEC) is increasingly combined with multi-angle light scattering (MALS) to determine the true molecular weight (MW) of sample components. We determined the MW of a monoclonal antibody and its impurities with the LenS\textsuperscript{3} MALS detector, and analyzed the robustness and sensitivity of the method.

Experimental Conditions
A reference mAb was used to demonstrate the benefits of multi-angle light scattering detection combined with size-exclusion analysis of a monoclonal antibody. Adaptations were required to optimize MALS data quality, as light scattering is more sensitive for particles such as salt crystals, microbes, or column shedding than UV detection: the mobile phase was filtered twice and a light-scattering-dedicated UHPLC column (TSKgel UP-SW3000-LS) was employed. It had previously proven to have the lowest noise levels when compared to two other columns. The LenS\textsuperscript{3} MALS detector was chosen due to its high sensitivity.

- Column: 4.6 mm × 30 cm, 2-μm TSKgel\textsuperscript{®} UP-SW3000-LS (P/N 0023546)
- Mobile phase: 100 mmol/L NaH\textsubscript{2}PO\textsubscript{4}Na\textsubscript{2}HPO\textsubscript{4}, pH 6.7, 100 mmol/L, Na\textsubscript{2}SO\textsubscript{4} (filtered twice using a 0.1 μm pore size PES vacuum filter)
- Flow rate: 0.35 mL/min
- Detection: UV-absorbance @280 nm, multi-angle light scattering (RALS) for MW calculation
- Injection volume: 10 μL
- Sample: SILu™Lite SigmaMAb (1 mg/ml), IgG1 monoclonal antibody standard (Sigma #MSQC4)
- Instrument: Thermo Scientific Vanquish UHPLC

Results
The MW of the monoclonal antibody and its impurities was analyzed using UV 280 as a concentration detector and the right-angle light scattering signal (RALS) for MW calculation. Molecules with 290.3 kDa (dimer), 148.8 kDa (monomer), 122.4 kDa (mAb missing one light chain), and 48.7 kDa (Fab fragment or two aggregated light chains) were found (Figure 1).

For sensitivity and robustness determination, triplicate injections of a dilution series of the antibody were analyzed. Accurate and robust results were achieved down to 50 ng injected protein, while at lower concentrations, MW determinations were still possible but with lower accuracy and higher variation (Table 1).

Conclusion
Analyzing monoclonal antibody samples with SEC-MALS directly determines the MW of sample components, facilitating the assignment of peaks to the structures of impurities. By employing the sensitive LenS\textsuperscript{3} MALS detector in combination with the light-scattering dedicated UP-SW3000-LS column, the method was sensitive down to less than 50 ng of dimerized antibody. However, MW determination of monomer and impurities was possible at concentrations down to 15 ng but with less accuracy and higher variation.

Table 1: Robustness and sensitivity of triplicate mAb MW determinations with SEC-MALS

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</table>

Figure 1: Molecular weight determination of high- and low-molecular-weight impurities of mAbs.

- Sample: SILu™Lite SigmaMAb (1 mg/ml), IgG1 monoclonal antibody standard (Sigma #MSQC4)
- Instrument: Thermo Scientific Vanquish UHPLC

Tosoh Bioscience GmbH
Im Leuschnerpark 4 64347 Griesheim, Darmstadt, Germany
Tel: +49 6155 7043700 Fax: +49 6155 8357900
E-mail: info.tb@tosoh.com
Website: www.tosohbioscience.de
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Advanced Materials Technology’s new generation HALO® 1.5-mm-i.d. column technology represents a breakthrough in traditional column hardware options. The new internal diameter column offers greater sensitivity in UHPLC and LC–MS systems, without sacrificing the convenience of an analytical system solution.

While microflow and nanoflow systems deliver increased sensitivity, they are generally more expensive and complicated systems to operate and maintain, and therefore not as convenient to adopt broadly.

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**Chiral Chromatography Solutions**

Expanding its range of innovative chiral chromatography solutions, Daicel Chiral Technologies has announced the introduction of CHIRALPAK® IK-3, adding new separation capabilities to the Daicel portfolio.

This new immobilized selector builds on the success of CHIRALPAK IG, one of the most effective chiral chromatography phases in use in life science laboratories worldwide. CHIRALPAK IK uses the same chiral derivative as CHIRALPAK IG, but with a cellulose polymer, adding new separations and enhancing some separations already achieved using IG and other selectors.

CHIRALPAK IK is now available in 3- and 5-μm particle size and will be extended into other particle sizes over the coming weeks. Daicel 3-μm chiral columns are extensively used by life science companies for faster separations and enhanced resolution.

Daicel will continue to add new immobilized columns throughout 2022, offering more ways to achieve successful enantiomeric separations. The extended life and new selectivities of Daicel’s robust immobilized columns ensure their increasing success.

By continuously extending its range of robust immobilized columns, Daicel now offers more than 2000 chiral products, giving analysts a broad range of enantiomeric separation answers. Each new product builds upon Daicel’s 40 years of experience specializing in chiral chromatography.
**Liquid Handler**

Laboratory automation and complex tasks are becoming increasingly important. With the Liquid Handler LH 8.1 for analytical applications, KNAUER offers a solution to make these tasks easier for laboratory professionals. Designed for high-throughput applications, the LH 8.1 can be used in both (U)HPLC and GC. The combination of lossless sample introduction with lowest carryover makes the LH 8.1 the ideal autosampler for LC–MS/MS or GC–MS/MS applications. The expandable platform not only allows the integration of multiple switching valves for complex applications—depending on the configuration—but it also allows the use of up to 12 well plates or 576 standard HPLC vials (including cooling). The LH 8.1 platform will be extended in the future with modules for sample preparation. Communication protocols are available for OEM customers, allowing easy integration into their own software.

**Particle Size Analysis**

The newly developed PSS Nano Particle Distribution Analyzer, PSS NaPDA, based on the principles of hydrodynamic chromatography, provides a cost-effective entry into the fast analysis of particle sizes and their distributions. It consists of a chromatographic system and dedicated software for data acquisition and analysis. Specially developed algorithms in the software are used to process the high speed chromatograms obtained from the system, providing better resolution—especially for multicomponent samples. This allows for a more precise determination of the particle size distributions and improved quantification of incompletely separated peaks. Typical analysis time per sample is 10 min.

Typical applications are:
- Polymer latices
- Polymer emulsions and dispersions
- Liposomes
- Colloids
- Fillers
- Nanoparticles
**LC PRODUCTS**

**Multi-Angle Light Scattering**

Over the past four decades, multiple light scattering instruments have been commercially available, each design having its own benefits and drawbacks. Tosoh Bioscience’s latest technology addresses the shortcomings of these traditional approaches, to expand the boundaries of light scattering measurements for polymers and biomolecules.

The LenS\textsuperscript{3} MALS detector features a novel flow path geometry that maximizes scattered light collection, to increase the detector’s response, while the nonrefractive material of the flow chamber eliminates noise from undesired stray light. The advanced optical design also provides exceptional signal-to-noise at the crucial extreme angles (10° and 170°) for molecular weight (MW) and size measurements.

The resulting enhanced capabilities and unprecedented sensitivity of the LenS\textsuperscript{3} MALS detector provide a direct and accurate measurement of molecular weight, even for low MW or low concentration samples, or trace amounts of aggregates and fragments. By allowing the angular dependence to be reliably detected to a considerably lower level, this novel design extends the range of radius of gyration (Rg) determination by light scattering down to a few nanometres for the very first time. In addition, the instrument is fully compatible with standard HPLC as well as UHPLC systems and columns.

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**YMC EUROPE**

**(U)HPLC Columns**

Charged compounds including oligonucleotides and nucleotides are able to interact with inert column hardware materials such as stainless steel or titanium. In addition, several peptides, proteins, and coordinating small molecules are sensitive to these materials. The adsorption on metallic surfaces typically leads to peak tailing, loss of recovery, and sample carryover. This behaviour can especially be seen when new columns are used without conditioning before the actual analysis.

For these challenging substances, YMC offers different bioinert hardware options for the YMC-Triart columns. The newly released YMC-Accura Triart (U)HPLC columns are characterized by a bioinert surface coating that is less hydrophobic compared to the PEEK lining used in the YMC-Triart metal-free columns. These two options allow for optimum hardware for analyses of any sensitive compounds. Further, YMC-Accura columns are ready to use without any preconditioning, which is usually required for the analysis of sensitive substances using standard columns.

The bioinert YMC-Accura Triart columns show excellent peak shapes for sensitive compounds, prevent carryover, secure recovery, and are ideal for use in highly sensitive LC–MS analyses. They are available with all eight YMC-Triart stationary phases and particle sizes of 1.9-, 3-, and 5-\(\mu\)m.

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**TOSOH BIOSCIENCE**

**Tosoh Bioscience**

Im Leuschnerpark 4
64347 Darmstadt-Griesheim, Germany
E-mail: info.tbg@tosoh.com
Website: www.tosohbioscience.de

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**YMC EUROPE**

**YMC Europe**

Schöttmannshof 19
43549 Dinslaken, Germany
E-mail: support-lca@ymc.de
Website: www.ymc.eu
Fully Automated High-Performance Thin-Layer Chromatography

Designed for high-throughput analysis in routine quality control, the CAMAG® HPTLC PRO SYSTEM revolutionizes the HPTLC handling process while maintaining the method’s flexibility. With the launch of the Module DERIVATIZATION, the HPTLC PRO SYSTEM autonomously handles the entire chromatographic processing of multiple plates—thanks to time-controlled operations—even overnight.

Once the user has prepared the system, the fully automated processing is started with a single mouse-click in the visionCATS software. Responsible for plate handling within the system, the Module PLATE STORAGE feeds the system with clean plates, which are transported to the subsequent modules APPLICATION, DEVELOPMENT, and DERIVATIZATION. Processed plates are transported back to the module PLATE STORAGE. Manual user intervention between the individual process steps is no longer required.

With maximized standardization and full automation, the cGMP compliant HPTLC PRO SYSTEM is capable of delivering reproducible and reliable results, while low solvent consumption keeps operating costs low.

Low-Pressure GC–MS

Low-pressure GC–MS (LPGC–MS) provides significant speed gains using standard instrumentation. Multiresidue pesticides, for example, can be analyzed in a third of the time required by conventional methods. While LPGC–MS is an effective way to increase sample throughput, it can be difficult to implement because manual connections between different tubing diameters are prone to leaks. Restek's new factory-coupled, low-pressure GC column kit makes getting set up for LPGC–MS as simple as a column change. In addition, the robust, preassembled connection and integrated transfer line ensure consistent, leak-free performance. Ease of use and consistent results make this column kit an effective way to implement LPGC–MS, making the benefits of this advanced technique widely attainable for routine use in high-throughput labs.
Thermal Desorption for GC–MS
Markes’ range of thermal desorption (TD) instruments that work with any GC–(MS) system to detect semivolatile and volatile organic compounds have been designed to make life easier for analysts. At the heart of every instrument is Markes’ proprietary valve and focusing trap, working together to provide capabilities only Markes’ TD systems can offer.

The valve enables sample re-collection, in which the sample can be split during desorption so that the unanalyzed portion is sent to a clean sorbent tube for storage or later re-analysis, giving peace of mind and enabling easier method development. Analysts will find it easy to switch between sampling methods—industry-standard-sized tubes, canisters, bags, and online sampling—all on a single TD–GC instrument, a versatile option for those wishing to quickly analyze different types of samples without the hassle of switching between TD instruments. In Markes’ systems, the trap is cooled with a Peltier cooler, avoiding the inconvenience of using a liquid cryogen and build-up of ice in the trap box, and high-quality data and precision are guaranteed with internal standard addition and inherent leak testing, reassuring analysts that they can be confident with every analysis.

Helium shortage problems?
Switch to Hydrogen
Multi-Gas thermal desorbers from Markes International

A powerful leap in analytical instrument technology, Markes’ Multi-Gas enabled thermal desorbers have been independently certified to work with three carrier gases: helium, nitrogen and now hydrogen.

Taking your lab to the next level, using hydrogen as a carrier gas with Markes’ Multi-Gas -xr series TD–GC–MS:

- Increases productivity,
- Lowers running costs,
- Gives superior analytical performance...and much more.

Explore the Multi-Gas -xr series:
chem.markes.com/MG
Data Analysis Software for GC and GC×GC–MS

ChromSpace is a powerful and versatile software platform for GC and multidimensional GC that reduces the workload for analysts in busy laboratories. Analysts previously put off by the complex data processing associated with two-dimensional gas chromatography (GC×GC) will benefit from ChromSpace’s quick-to-learn and easy-to-use interface. Following feedback from experts in industry and academia, ChromSpace was designed to meet the needs of those working in high-throughput laboratories. ChromSpace enables analysts to enjoy full instrument control and the flexibility to explore multiple GC×GC data files in a single window. Data navigation can even begin while a sample is still running, saving precious time and streamlining the process. An instant display of library matches also helps analysts to make a quick identification. Multiple file formats are supported in ChromSpace, enabling files from MS and single-channel detectors from most major manufacturers to be read, taking the hassle out of the process and avoiding the expense of purchasing multiple software packages. ChromSpace uses unique algorithms to ensure that trace or masked peaks are not overlooked or incorrectly merged, a deconvolution tool to identify coeluting peaks, classification “stencils” for simple group-type reporting, and a scripting tool to filter complex data and identify target compounds or chemical classes. The tools are easy to use and give analysts confidence in their data while ensuring a fast and high-quality data output.

SepSolve Analytical Ltd
14 Swan Court, Cygnet Park, Peterborough, PE7 8GX, UK
E-mail: hello@sepsolve.com
Website: www.sepsolve.com
**UV–vis Detector**

LUMA™ from VUV Analytics, is an innovative new UV–vis detector for gas chromatography that is designed to be sensitive, selective, and simple. Taking advantage of the vacuum ultraviolet (VUV) range of the electromagnetic spectrum, LUMA is a universal GC detector that provides up to 12 independent channels or bands of data between 120 and 500 nm.

LUMA is the ideal detector for routine analysis applications that have high sensitivity requirements, such as nitrosamines. Because of LUMA’s unique operating characteristics and innovative design, it is ideal for trace-level analysis. Observed IDL’s for compounds like benzene are 4 ppb.

Because LUMA provides up to 12 channels of data, it has a high degree of selectivity. This selectivity is useful for impurity analysis, simple deconvolution, and application consolidation. For example, because different compounds absorb differently at different wavelengths LUMA can be used to combine different analysis into a single injection—saving time and money.

LUMA was designed to be user installable in less than one hour and works with existing laboratory chromatography data system software; reducing laboratory training requirements while increasing productivity.

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**TEKNOKROMA**

**Headspace Sampling**

The Teknokroma 2t Head Space Sampler for Head Space technique within your reach with a low cost and high precision level. The 2t sampler is the first manual system for Static Head Space that allows the application of this technique in a quantitative manner. Until now it was only possible to use the technique of Static Head Space with automatic equipment. This “equipment” has a high cost, low versatility and complex operations. For this reason the Static Head Space technique has not been fully used in most laboratories. The new 2t sampler solves these problems making the technique available to all gas chromatography users in a economical and simple way. It complies with all requeriments of the European CE. Suitable for applications such as volatiles in pharmaceuticals, flavours analysis in food and cosmetic products, alcohol and other toxic compounds in blood, screening of volatiles in all type of environmental samples (soils, waters, plastics, polymers, etc.) and others. The clear advantages of the 2t HS Sampler are agility and analysis optimization on headspace technique.
Cannabis Sample Analysis

The Chromatrap Spin Column Homogenizer from Porvair Sciences is for laboratories preparing cannabis samples for analysis of tetrahydrocannabinol (THC) and cannabidiol (CBD). Accurate THC/CBD determination relies on effective sample preparation from samples of cannabis leaf, bud, and flower. Offering a gentle, quick, and clean alternative to mechanical syringe and needle homogenization, the Chromatrap Spin Column Homogenizer quickly and efficiently homogenizes cells, tissue lysates, and plant extracts in a single step. Porvair’s unique proprietary bio shredding Vyon® polymer advantageously reduces lysate viscosity and captures insoluble debris by centrifugation. Used in conjunction with a uniquely robust Porvair specialist deep well microplate, the Chromatrap Spin Column Homogenizer provides efficient sample preparation clean-up and cannabis THC/CBD analysis set-up.

Microplate for Cannabis Sample Preparation

Start the first step in sample preparation the right way. The 96-well deep well processing microplate from Porvair Sciences is designed and built to robustly handle and process solid plant material for cannabis sample preparation.

- Reinforced structure to withstand applied forces up to 6,000 x g
- Made from high-grade polypropylene for low extractables and leachables
- High working volume of 2 ml for sample clean and extraction methods

Website: www.microplates.com
Enquiries: hello@porvairsciences.com
**Solid-Phase Extraction**

HydraFlow® is the latest addition to the UCT family of SPE manifolds geared towards efficient sample processing. Its unique design provides enhanced precision and streamlined sample extractions for large-volume water analysis. While the assembly is simple, the compact footprint is perfect for fume hood set-up and the overall weight of only 12 kgs makes it easy to relocate the unit in the lab. HydraFlow is equipped with four independent channels that allow the user to process the samples separately or simultaneously without the risk of cross-contamination during extraction. PEEK, PTFE, and stainless steel in the sample flow pathways ensure minimal to no corrosion from exposure to organic solvents such as dichloromethane and acids.

The distinctive feature of diverting the organic solvents and the aqueous solvents in separate collection bottles contributes to an effective waste management system and long-term cost savings. The waste and eluent collection channel switching valve adds convenience to the overall user experience.

Compared to traditional manifolds, HydraFlow eliminates the need to turn off the vacuum pump, drain the waste liquid out, and place the collection vials in the chamber. The precision control valves are another excellent feature used to achieve desired flow rates of waste and elution solvents.

**ACD/LABS (ADVANCED CHEMISTRY DEVELOPMENT, INC.)**

**Software Instrument Control**

New functionality and improvements in ACD/Labs’ upcoming software release will help you make better use of your time. AutoChrom® delivers expanded instrument control. In addition to ChemStation, you will be able to automate screening and optimization trials using OpenLab CDS. Alternatively, import processed or raw data. Support for Waters’ Empower CDS sees expansion with the capability to control the ACQ-ISM and SFC-MS modules. This expansion of hardware support means you can set up your experiments and walk away. In various software applications you will be able to define and quantitate multiple compounds on calibration curves; carry out targeted processing for easier identification and tracking of isobaric compounds across data sets; use a new set of filters for auto-peak picking; better understand data by visualizing xC/UV/MS data as a series; determine best unique hits in xC/MS data with improvements to the deconvolution and spectral search tool; and benefit from increased flexibility in reporting across all chromatography and MS software. As with every version of Spectrus software, there will be improved data import for most major instrument vendor formats. For those that manage open access instruments, version 2022 of Spectrus JS introduces browser-based processing of xC/UV/MS data with importing, processing, annotation, and reporting capabilities.
**POSTNOVA ANALYTICS GMBH**

**Electrical/Asymmetrical Flow Field-Flow Fractionation**

Traditional separation technologies for biopharmaceutical and nanoparticle applications provide particle size or molar mass distributions as the final result. However, it is clear that particle and molecular charge play a primary role in many applications such as protein aggregation, polymer flocculation, particle agglomeration, and in pharmaceutical formulations in general. The Postnova EAF2000 instrument using electrical asymmetrical flow field-flow fractionation (EAF4) technology allows the particle size or molar mass distributions to be further differentiated and transformed into charge distributions. This identifies charge heterogeneities present within the different size and molar mass fractions and will help to aid research or establish more efficient product development processes.

The instrument works by combining the principle of electrical and AF4 in one system. This instrument is a key tool, particularly for protein research, because existing techniques for zeta-potential are limited by concentration and are batch techniques giving an average value for all components in the solution. The EAF2000 can determine the zeta-potential of each individually separated component, such as protein monomer and dimer (or higher aggregates) or antibody monomer and fragments–aggregates.

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**ECOM spol s.r.o.**

**TRI-CLAMP Flow Cells**

As part of its increasing emphasis on the development of new products and innovations to cover customers’ requirements, ECOM presents a new TC flow cell series for high flow rate and high-pressure installation on pipelines. These TC cells allow measuring using optical cables connected to ECOM UV detectors.

The ECOM series of flow cells with TRI-CLAMP pipeline connection is used in preparative process chromatography. The entire product range of the TC flow cells can be supplied in various materials—stainless steel, Hastelloy, or PEEK material design. The optional optical path length ranges from 0.1 to 10 mm. The maximum operating pressure is 30 MPa (300 bar, 4351 psi) for DN8–DN20, and for DN25–DN100 it is 2 MPa (20 bar, 2950 psi). The advantage of ECOM’s TRI-CLAMP flow cells technical design is the elimination of dead volume inside the cell. The TC cells are used out of the detector, and are connected by special UV optical cables with SMA 905 connectors. The products have been approved by FDA certification for use in food and pharmacy.
N-Rich®—Impurity Isolation Technology

YMC ChromaCon’s proprietary N-Rich technology is an automated purification technique run by the Contichrom CUBE, a specialized twin-column chromatography system. The purification of low abundance target compounds from a complex mixture is often a difficult challenge, typically done by pooling 10 s or 100 s of analytical HPLC runs to accumulate enough target material for further testing. By contrast, N-Rich employs a more efficient two-step enrichment and separation strategy yielding high purity material in far less time. In step one, N-Rich uses cyclical enrichment where the desired molecule is selectively accumulated while interfering compounds are depleted. In step two, the enriched fraction is then automatically separated using a shallow, high-resolution gradient. Crucially, N-Rich can be done at semipreparative scale, while achieving analytical purity. Due to the much higher loading afforded by semipreparative resins, N-Rich completes task orders of magnitude faster than accumulating fractions from standard analytical HPLC runs. N-Rich is an invaluable tool for:

- Isolation of product-related impurities for active pharmaceutical ingredients
- Biosimilar CMC characterization
- Characterization of antibody–drug conjugate isoforms
- Identification of biomarkers and compounds in proteomics and metabolomics research
- Purification of ultra-pure compounds as reference materials
- Isolation of impurities after stability and formulation studies

YMC ChromaCon AG
Technoparkstrasse 1
8005 Zürich, Switzerland
E-mail: sales@chromacon.com
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The Gold Standard in Field-Flow Fractionation
FROM THE COMPANY THAT INVENTED FFF

The Postnova FFF-MALS-DLS analytical characterization platform is the premier solution for the advanced analysis of nanoparticles, vesicles, proteins and macromolecules.

Direct access to molar mass, size, charge, structure, conjugation and elemental speciation are provided by hyphenation of our unique Field-Flow Fractionation platform technologies with:

- Multi-Angle Light Scattering
- Dynamic Light Scattering
- Mass Spectroscopy
- Size Exclusion Chromatography
- Intrinsic Viscometry

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