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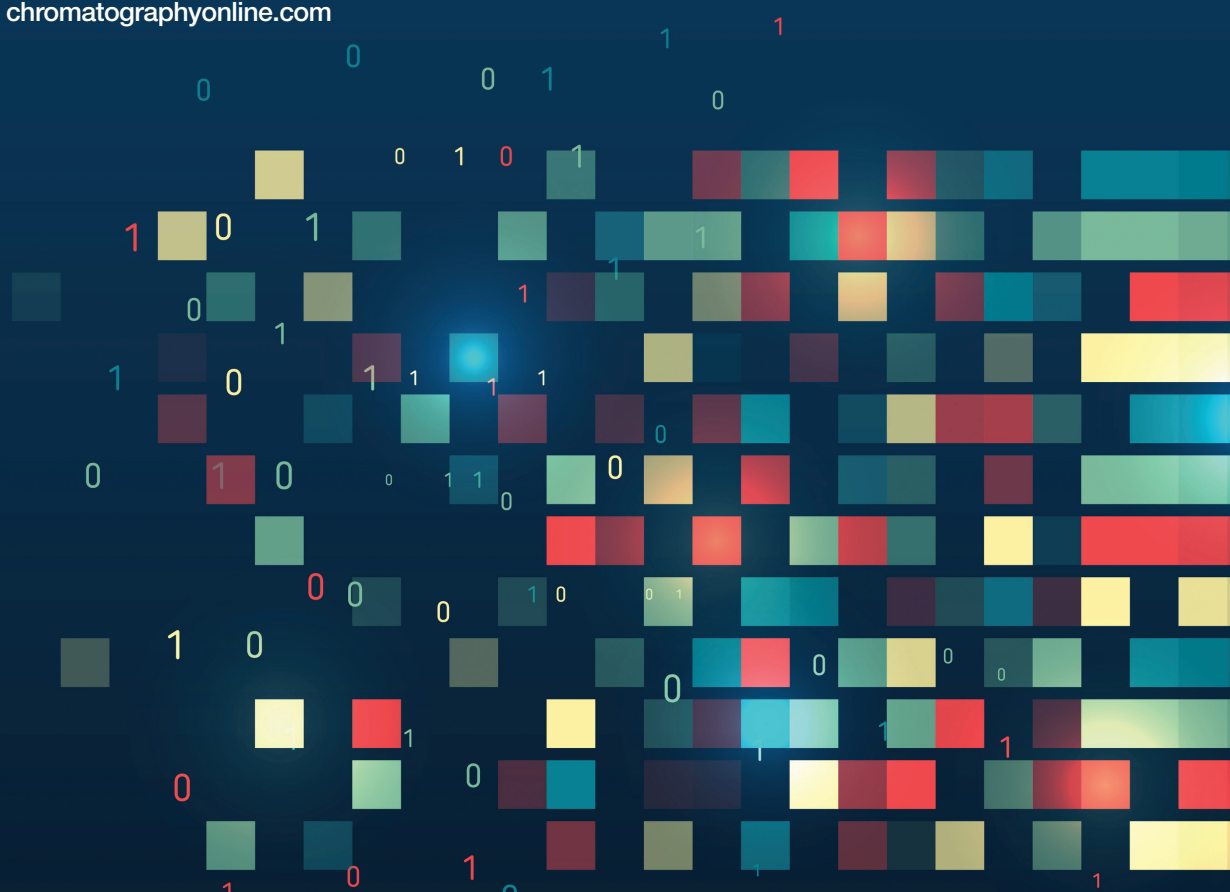

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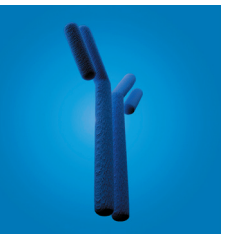
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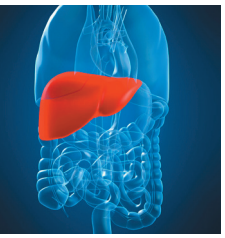
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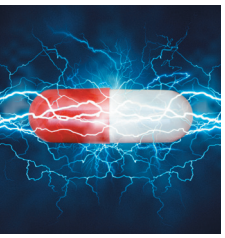
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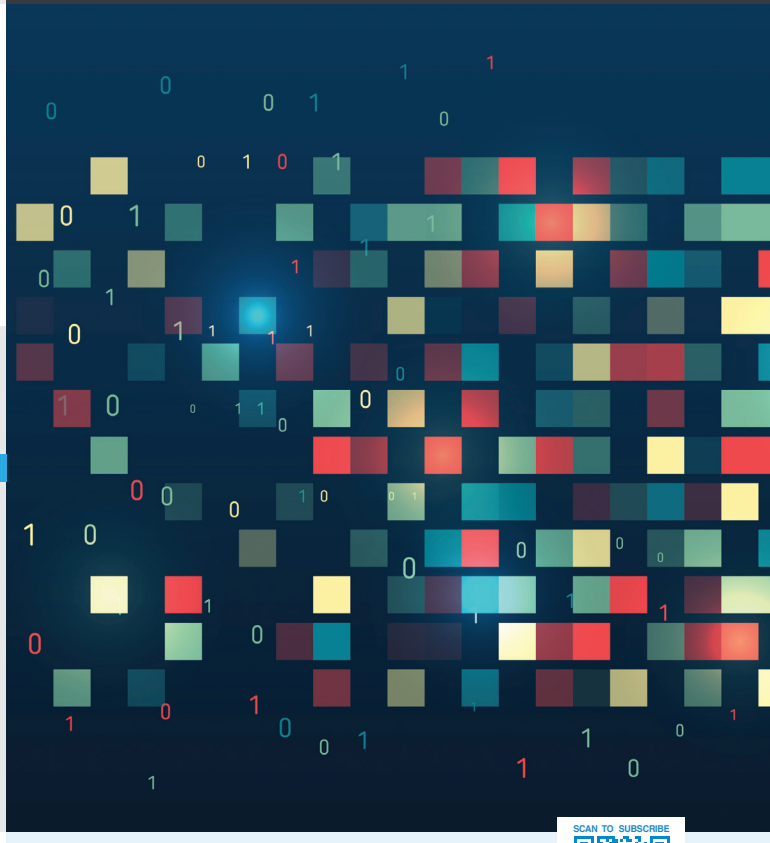

COVER STORY

COLUMNS

DEPARTMENTS

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AN MJH life sciences BRAND

Executive Vice President, Healthcare and Industry Sciences
Brian Haug
bhaug@mjhlifesciences.com

Vice President, Industry Sciences
Todd Baker
tbaker@mjhlifesciences.com

Group Publisher
Oliver Waters
owaters@mjhlifesciences.com

Associate Editorial Director
Caroline Hroncich
chroncich@mjhlifesciences.com

Editor-in-Chief
Alasdair Matheson
amatheson@mjhlifesciences.com

Managing Editor
Kate Jones
kjones@mjhlifesciences.com

Sales Manager
Liz Mclean
lmclean@mjhlifesciences.com

Sales Operations Executive
Sarah Darcy
sdarcy@mjhlifesciences.com

Senior Virtual Program Manager
Lindsay Gilardi
LGilardi@mjhevents.com

Digital Production Manager
Sabina Advani
sadvani@mjhlifesciences.com

Creative Director, Publishing
Melissa Feinen
mfeinen@mjhlifesciences.com

Senior Art Director
Marie Maresco
mmaresco@mjhlifesciences.com

Associate Art Director
Courtney Soden
csoden@mjhlifesciences.com

Custom Projects

Vice President, Content, Custom Projects
Colleen Hall
chall@mjhlifesciences.com

Director
Robert Alaburda
ralaburda@mjhlifesciences.com

Managing Editor
Jeanne Linke Northrop
jlinke@clinicalcomm.com

Senior Editor
Megan Manzano
mmanzano@mjhlifesciences.com

Senior Editor
Shannon Stolz
sstolz@mjhlifesciences.com

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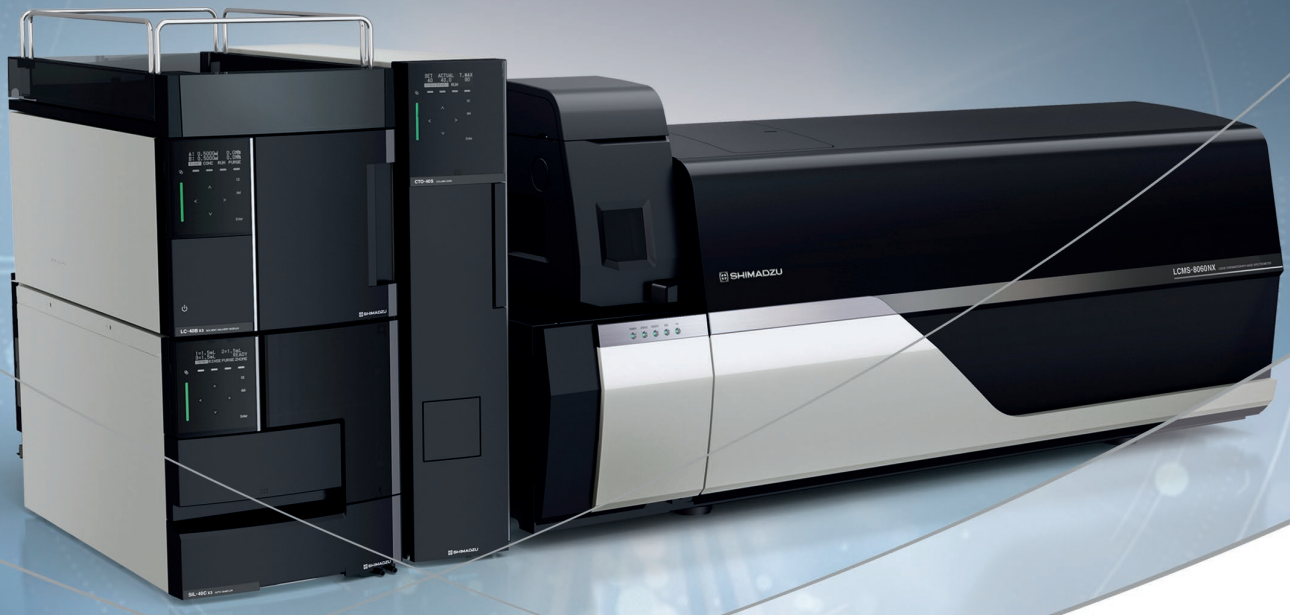

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September Update

Welcome to your September issue of *LCGC Europe*! Our cover story for this issue discusses inconsistent retention prediction in liquid chromatography (LC). The myriad benefits of screening column and mobile phase combinations that generate differing chromatographic selectivities in reversed-phase gradient ultrahigh-pressure liquid chromatographic (UHPLC) method development strategies are presented.

Continuing with the theme of method development, Dwight Stoll resumes his examination of how to improve your separation by looking more specifically at increasing speed for both complex and more simple samples.

Are you ready to make the switch to comprehensive two-dimensional gas chromatography (GC×GC)? Nick Snow looks at how GC×GC has become more amenable to routine use and can be applied across a diverse range of fields.

The 51st International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2023), chaired by Michael Lämmerhofer and Oliver Schmitz, was recently held in Düsseldorf, Germany. If you missed out on attending, Dave Bell was on the ground to offer his take on the topics and trends observed at the symposium.

"Questions of Quality" is celebrating a milestone this month—30 years young! Bob McDowall gives an overview of the generation and management of data in a chromatography laboratory from 1993 up to now. What's changed for the better? And what stubbornly remains the same?

Happy reading!

Mike Hennessy Jr
President and CEO, MJH Life Sciences®

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RISING STAR

Rising Stars of Separation Science: Selina Tisler

We spoke to Selina Tisler from the University of Copenhagen, about her work using SFC to detect very polar compounds and compounds of unknown toxicity in wastewater effluents.

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Sustainability in Pharmaceutical Separation Science

Sustainability is a driving force across the globe, but what steps are separation scientists taking to reduce the environmental impact of their activities?

Anomalous Retention Prediction Using Modelling Software in Gradient Reversed-Phase Liquid Chromatography: Why it Can Occur and How to Prevent It

Jennifer K. Field[1,2], Stuart N. Berry[3], James Hogbin[3], Earl McKoy[1], Veronica Paget[3], Patrik Petersson[4], Raymond Wong[1], and Melvin R. Euerby[1,2,5], [1]Shimadzu UK, Milton Keynes, UK, [2]The Open University, Faculty of Science, Milton Keynes, UK, [3]Advanced Chemistry Development, UK Ltd. (ACD/Labs), Bracknell, UK, [4]Ferring Pharmaceuticals A/S, Kastrup, Denmark, [5]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

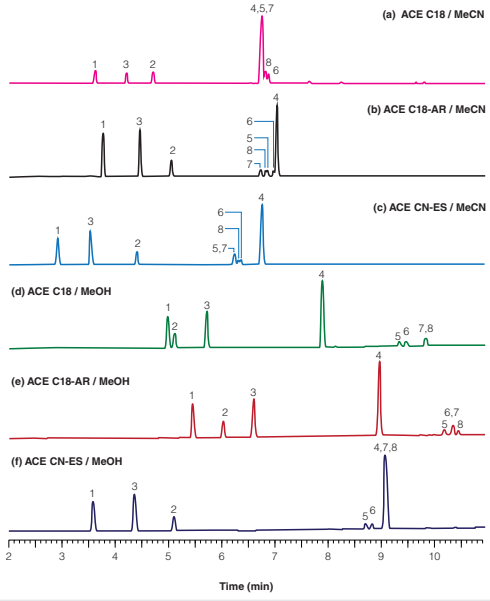
This article presents the benefits of screening column and mobile phase combinations that generate differing chromatographic selectivities in reversed-phase gradient ultrahigh-pressure liquid chromatographic (UHPLC) method development strategies. Photodiode array (PDA) and mass spectrophotometric (MS) detection was necessary to facilitate peak tracking or identification of components in the sample mixture to build retention models. Retention time prediction accuracies of < 0.3% were obtained from a two-dimensional gradient time vs. temperature model when the initial gradient conditions were maintained. However, anomalous retention predictions were observed when higher %B initial gradient conditions were employed. Polar analytes in the sample mixture that started to migrate down the column in the dwell volume of the UHPLC system produced inaccurate retention time predictions if an inappropriate dwell volume was used in the retention model. An iterative dwell volume estimation was demonstrated to generate more accurate retention time predictions than when a practically determined dwell volume was used. However, to obtain good predictions the analyst should endeavour to use initial chromatographic conditions that promote focusing of all analytes on top of the column (that is, retention factor > 10).

KEY POINTS

- **The success of screening column and mobile phase combinations that generate dissimilar selectivity is highlighted in a typical method development strategy.**
- **Excellent accuracy of retention predictions in RPLC was demonstrated.**
- **Anomalous retention predictions can be observed when polar analytes migrate down the reversed-phase column in the dwell volume.**

A popular strategy in reversed-phase liquid chromatographic (RPLC) method development for small-molecular-weight active pharmaceutical ingredients (APIs) is to screen various stationary and mobile phase combinations using a fixed %B/min gradient and temperature (classed as Wave 1, see Figure 1) using automated column and mobile phase screening technologies (1). The most promising stationary and mobile phase combination based on the number of peaks detected, peak shape, and resolution is then taken through to Wave 2 (see Figure 2), in which a two-dimensional gradient time vs. temperature resolution model is constructed. In the case of ionizable compounds, it may be beneficial to screen mobile phases of low, intermediate, and high pH in Wave 1 to completely deprotonate or protonate the analyte(s). To obtain a robust method, mobile phases with aqueous pH values > ±2 pH units away from the pK_a of the analyte(s) should ideally be used. In so doing, differing retentivity and selectivity may be acquired. In most instances, the authors do not recommend modelling pH in Wave 2 where differing selectivity would only occur over a narrow pH range close to the pK_a of

FIGURE 1: Chromatographic selectivity differences observed for Wave 1. Chromatographic conditions and peak assignments are as described in the experimental section for Wave 1, unless otherwise stated. (a) ACE C18/MeCN; (b) ACE C18-AR/MeCN; (c) ACE CN-ES/MeCN; (d) ACE C18/MeOH; (e) ACE C18-AR/MeOH; (f) ACE CN-ES/MeOH.



the analyte(s), which typically would result in poor method robustness. In addition, the modelling of pH, even over a narrow pH range, requires a relatively large number of input experiments.

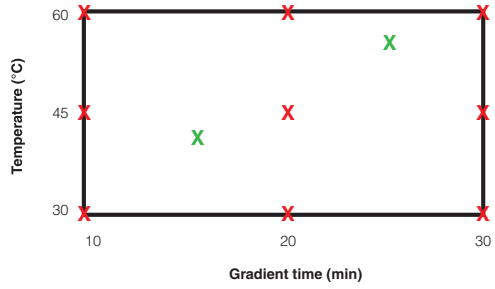
From the resultant resolution model, the optimum separation conditions can be predicted. A recent project was undertaken to provide educational material for the promotion of such a method development strategy using pre-selected analytes that would highlight the following prerequisites: 1) peak tracking using a combination of photodiode array (PDA) and mass spectrophotometric (MS) detection; 2) the ability to switch the elution order of analytes based on using differing organic modifier/stationary phase combinations; and 3) the ability to optimize the separation in terms of fine-tuning the percentage organic of the initial, final mobile phase compositions and gradient time.

During the optimization step there is always a tendency for the chromatographer to speed up the analysis by increasing the initial %B (that is, the mobile phase containing the high organic content). While this is a valid approach, particularly if the %B/min is kept constant to maintain the same desired chromatographic selectivity, if too high a %B is chosen, then the polar analytes in a RPLC separation may start to migrate in the dwell volume and may not be focused on top of the column. If this is the case, this can result in discrepancies between the predicted and experimental retention times unless a more accurate dwell volume value is used in the retention model.

TABLE 1: Predicted and actual retention times for the UHPLC separation (gradient time of 15 min; initial and final %B of 35–85%B; 30 °C) of six phenone derivatives based on a two-dimensional mode as described in the experimental section. Practically estimated V_D = 353 µL, iteratively estimated V_D = 391 µL with a t_M = 1.27 min.

Analyte	Actual t_R (min)	Predicted t_R (min)		Predicted - Actual t_R (min)		%Δ t_R	
		Practically Determined	Iteratively Determined	Practically Determined	Iteratively Determined	Practically Determined	Iteratively Determined
		V_D = 353 µL	V_D = 391 µL	V_D = 353 µL	V_D = 391 µL	V_D = 353 µL	V_D = 391 µL
Acetophenone	3.90	4.21	4.11	0.31	0.21	7.9	5.4
Butyrophenone	6.91	7.11	7.06	0.20	0.15	3.0	2.2
Benzophenone	7.76	7.97	7.93	0.21	0.17	2.7	2.1
Hexanophenone	9.60	9.77	9.74	0.17	0.14	1.7	1.4
Heptanophenone	10.85	11.01	10.99	0.16	0.14	1.5	1.3
Octanophenone	12.02	12.17	12.16	0.15	0.14	1.2	1.2

FIGURE 2: Wave 2: Two-dimensional gradient time vs. temperature model design. Chromatographic conditions are described in the experimental section for Wave 2, unless otherwise stated. X – input experiment used for the modelling; X – validation experiment (t_G = 15 min / 40 °C and t_G = 25 min / 55 °C, t_M = 1.272 min).



Experimental

Water, methanol (MeOH), and acetonitrile (MeCN) used were of LC–MS grade and were supplied by Romil. All compounds used in this study and the mobile phase additives (ammonium formate and formic acid) were supplied by Sigma Aldrich. Ultrahigh-pressure liquid chromatographic (UHPLC)-PDA analysis was performed on a Shimadzu Nexera XS UHPLC (Shimadzu UK Ltd) equipped with two binary pumps (LC-40D XS) and proportionating valves, degassers (DGU-405), flow-through needle autosampler (SIL-40C XS), column oven (CTO-40C), diode array detector (SPD-M30A) with a 1 µL/10 mm pathlength flow cell, 40 µL mixer (practically determined dwell volume = 353 µL and system volume = 14 µL [2]), single quadrupole mass spectrometer (LCMS 2020) with a Shimadzu electrospray ion source working in the positive ionization mode, communication bus module (CBM-40lite), and a six-position column switching valve. Selected ion monitoring of the $M+H^+$ peaks was used to track the components. The system was controlled and data collected using LabSolutions software (Shimadzu UK Ltd, version 5.114). Retention modelling and log D value determinations were performed with ACD/LC Simulator and ACD/Percepta software (versions 2021.2.2), respectively. At least 20 column volumes of the appropriate mobile phase were flushed through the columns prior to commencing the testing, or on changing the mobile phase conditions.

FIGURE 3: Wave 2: Two-dimensional gradient time vs. temperature input chromatograms. Chromatographic conditions are described in the experimental section for Wave 2, unless otherwise stated. (a) 10 min/30 °C; (b) 20 min/30 °C; (c) 30 min/30 °C; (d) 10 min/45 °C; (e) 20 min/45 °C; (f) 30 min/45 °C; (g) 10 min/60 °C; (h) 20 min/60 °C; (i) 30 min/60 °C.

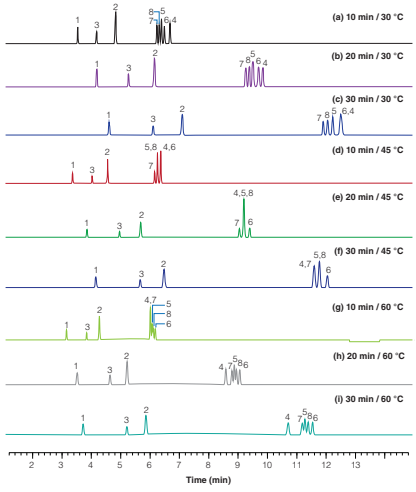
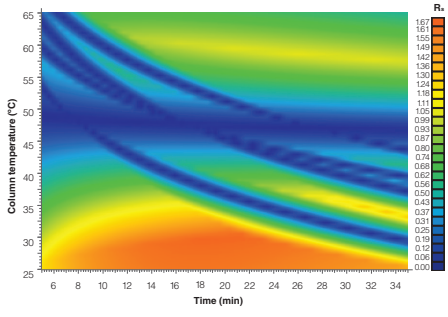


FIGURE 4: Two-dimensional resolution surface model. The colour scale on the right indicates the resolution for the least resolved pair of analytes at that temperature and gradient time condition.



Wave 1 Chromatographic Conditions (see Figure 1): Totally porous Avantor ACE Excel C18, ACE Excel C18-AR, and ACE Excel CN-ES columns (all 100 × 3 mm, 3-µm, 100 Å) were supplied by VWR Avantor. Stock 100 mM aqueous ammonium formate was prepared by dissolving 6.306 g of ammonium formate in 900 mL of water, adjusting the pH to 3.0 using formic acid, and then made up to 1000 mL with water. The degassed mobile phases A and B corresponded to 10 mM aqueous ammonium formate (pH 3.0) in water and 10 mM aqueous ammonium formate in 9:1 (v/v) MeCN–water or 9:1 (v/v) MeOH–water, respectively. Unless otherwise stated, the following UHPLC conditions were used flow rate 0.43 mL/min, temperature 40 °C, 1 µL injection volume, linear gradient 5–95% B over 10 min, hold at 95% B for 1 min, linear gradient 95–5% B over 0.1 min, and hold at 5% B for 9.9 min to equilibrate the column. The first baseline disturbance for a water injection was used as the dead time (t_M) marker. The PDA detector was set to monitor a wavelength of 254 nm (bandwidth 8 nm), with a reference at 360 nm (bandwidth 100 nm). The data sampling rate was set at 40 Hz. The mass spectrometer utilized positive polarity mode electrospray ionization, and used 1.5 L/min nebulizing gas flow and 15 L/min drying gas flow. The interface temperature was set to 350 °C, the desolvation line (DL) temperature 250 °C, and heat block temperature 200 °C.

Wave 2 Chromatographic Conditions (see Figure 2): Conditions as described in Wave 1 were used unless otherwise specified. The Avantor ACE Excel C18-AR column and MeCN were used to construct a two-dimensional 3 × 3 (that is, nine input experiments) gradient time vs. temperature (t_G vs. T) retention model (3). Temperatures of 30, 45, and 60 °C were examined at gradient times of 10, 20, and 30 min. The integrity of the input data was established by repeating the initial conditions at the beginning and end of the batch.

Results and Discussion

Method Development Activity: Wave 1: The aim of this work was to demonstrate the large selectivity differences that can be exploited when differing stationary phases (C18, C18 with aromatic selectivity, and a cyano with high hydrophobic character) are combined with differing organic modifiers (MeOH and MeCN) in gradient RPLC at a fixed gradient slope of 9%B/min and a column temperature of 40 °C using column dimensions of 100 × 3 mm, 3-µm. This dimension is popular because it is not significantly affected by the system volume of the liquid chromatograph and can still provide a relatively low solvent consumption. A 10 mm ammonium formate buffer (pH 3) was maintained in both mobile phases A and B and a flow rate of 0.43 mL/min was employed because

this was ideal for mass spectrometry with an electrospray ionization source.

The test mixture was composed of eight analytes (1, theophylline; 2, sulfamerazine; 3, caffeine; 4, sulfaquinoxaline; 5, prednisone; 6, cortisone; 7, prednisolone; and 8, hydrocortisone), all of which possessed varying hydrophobicity (that is, log D at pH 3 ranged between 0.09–1.66). Sulfaquinoxaline was selected to represent the API, as ideally this should elute after the other components. MS could not discriminate between cortisone and prednisolone, as they both possess the same *m/z* charge ratio; however, their PDA spectra differed significantly enough to allow discrimination of these components. In contrast, the steroidal pairs prednisolone/hydrocortisone and prednisone/cortisone had similar PDA spectra but could be discriminated via their different *m/z* charge ratios. Therefore, by using a combination of PDA and MS all the components of the mixture could be peak tracked successfully.

From Wave 1, it could be concluded that the column possessing a C18 with aromatic functionality and MeCN was the most promising combination, as all eight components could be observed with good peak shape, with sulfaquinoxaline eluting last (see Figure 1). Large selectivity differences in the elution profile between MeOH and MeCN could be observed across both C18 columns; sulfamerazine eluted before caffeine with MeOH, while the reverse was true for MeCN. This observation was not seen with the cyano column with enhanced hydrophobicity. In addition, with MeOH sulfaquinoxaline (4) eluted before the four steroidal components, whereas with MeCN it eluted much later.

Method Development Activity:

Wave 2: A 3 × 3 (nine input experiments) gradient time vs. temperature (t_G vs. T) retention model (3) was constructed using the C18 column with aromatic functionality and MeCN as the organic modifier (see Figure 2). The same initial and final starting %B and flow rate was employed as in Wave 1.

As can be observed in Figure 3, there is considerable peak movement as a function of temperature and gradient time. Peak tracking was performed using positive ion MS using an electrospray ionization source and PDA spectroscopy.

The input data were transferred to the retention modelling software

FIGURE 5: Actual and predicted optimized UHPLC chromatograms. Chromatographic conditions are described in the experimental section, unless otherwise stated; gradient time, 10 min; initial and final %B, 35 to 50%B, t_M = 1.272 min. (a) Predicted chromatogram using the practically determined V_D = 353 µL; (b) actual chromatogram; (c) predicted chromatograms using the iteratively determined V_D = 420 µL.

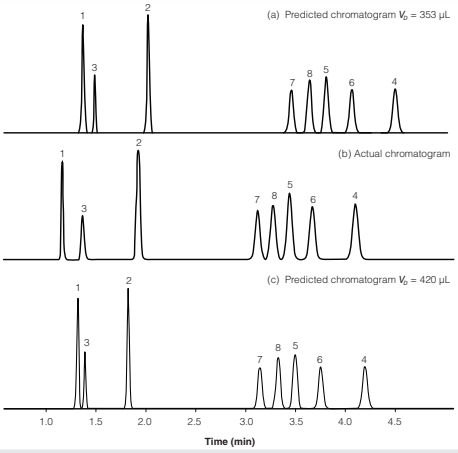
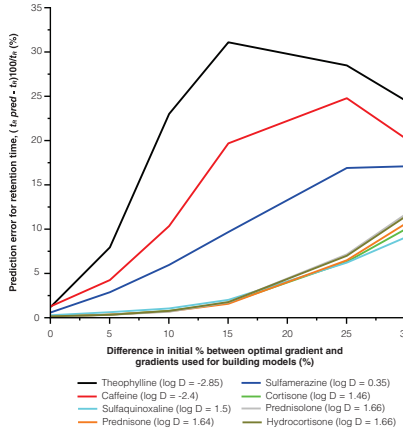


FIGURE 6: Effect of the difference in the initial %B to that used to construct the model on the retention time accuracy of the final optimized condition.



and a resolution response surface was constructed (see Figure 4, V_D = 353 µL, t_M = 1.27 min). The percentage difference between the actual and predicted retention time (%Δt_R) was found to be in excellent agreement (%Δt_R < 0.3%) for the validation conditions, confirming the accuracy of the retention model. Improved separation was achieved at temperatures below 35 °C. The presence of dark blue regions corresponding to poor resolution sandwiched between orange and green colours (higher resolution) indicated that there are switches of peak elution order.

The Problem: Discrepancies Between Actual Retention Times and Predictions: From the two-dimensional model of the dependence of retention on gradient time and temperature (see Figure 4), the elution conditions (that is, temperature, initial and final %B in the gradient, and the gradient slope) can be adjusted to produce an optimized separation. By investigating the retention behaviour of the analytes in the test mixture using the retention modelling software, it could be predicted that a t_G of 10 min with an initial to final %B of 35 to 50%B would yield an acceptable separation (see Figure 5[a]). However, surprisingly, the retention prediction did not match with the actual retention times (that is, %Δt_R values between 5–18% were obtained, see Figures 5[a] and [b]), even though validation runs were in excellent agreement (%Δt_R values < 0.3%). Similar observations were also encountered using different UHPLC instrumentation, differing types of stationary phase, and with a different type of test mixture.

The dwell volume of a UHPLC instrument is an input variable that the retention model uses to predict analyte retention. It is defined as the volume from the point at which the mobile phases first mix in the pump to the head of the column. It can be determined in different ways; in many earlier publications it is suggested that a wide linear gradient range should be employed accompanied with a high flow rate (4,5). In comparison, several instrument manufacturers utilize a step gradient with a low flow rate and a narrow organic range. It has previously been observed that the gradient type (step or linear), flow rate, and gradient range are all critical for the accurate estimation of dwell volumes (differences of up to 80% have been observed [2]). It is the authors' opinion that the dwell volume should be determined using gradient conditions that are appropriate for the type of analyte and LC instrumentation that will be used

(reference 2 describes a procedure for the estimation of dwell volumes suitable for modelling purposes). Since the dwell volume determination is based on a linear gradient, it can also be used to ensure that linear gradients can be generated by the UHPLC instrumentation. The dwell time volume was estimated at a flow rate of 0.43 mL/min to be 353 µL, as described in reference 2, and was used in the retention model. It has been previously demonstrated (2,6,7) that even relatively large errors (up to ±20%) in the estimation of the dwell volume used in the retention model do not have a significant impact on the accuracy of the predicted retention times (that is, <<1%).

Further investigations highlighted that excellent retention time predictions could be achieved for this and other test mixtures when the initial %B was kept the same as that used to generate the retention model input data; however, when higher %B conditions were used, inaccuracies in retention time prediction became significant. The degree of error in the retention time predictions was observed to be related to the difference in initial %B of the optimized method relative to that used to construct the model (that is, when Δ%B initial [%B initial optimized – %B initial used in the model] was large, poorer retention predictions were observed, see Figure 6). The more polar analytes exhibited the phenomenon to a greater extent compared to the more nonpolar analytes. This was also shown to be the case with an additional test mixture of phenone standards.

The Solution: An alternative approach for estimating the dwell volume—as well as to compensate for errors in other parameters—is to iteratively try a few different dwell volumes while fitting the retention model, compare the residuals obtained, and, based on this, select the dwell volume that gives the lowest residual (8). This has been implemented recently in the software that we have used (9). When the retention times for the test analytes for the 30 °C input data were used in this iterative approach, the dwell volume was estimated to be 420 µL instead of the experimentally estimated values of 353 µL. When this dwell volume was used in the retention model, a much better retention prediction accuracy was achieved (< 2.5% for the steroids, however, the more polar analytes still gave larger errors), see Figures 5(b) and 5(c).

While it is true that relatively large inaccuracies in the dwell volume do not impact on the retention time prediction accuracy when initial %B remains the same as (or lower than) that used to construct the retention model, this is not the case when higher initial %B conditions are employed (that is, the analytes are not focused on top of the column and start to migrate isocratically down the column).

If the analyte is focused on top of the column (that is, it possesses a large isocratic retention factor [*k*]) at the higher %B initial conditions, this phenomenon is not observed, and dwell volume accuracy is not that critical. However, if analytes are of a low to medium retentivity (low hydrophobicity when considering a reversed-phase mechanism, low isocratic *k* values) and therefore are not focused on top of the column and start to migrate down into the dwell volume, then this phenomenon is profound, particularly if an inaccurate dwell volume is used.

It appears that the established methods for determining dwell volumes are not appropriate for accurate retention time predictions and that the iterative methodology generates a more meaningful estimation for retention modelling purposes.

To investigate this further, a test mixture of six phenones of varying hydrophobicity was evaluated. Table 1 demonstrates that the inaccuracy of the retention prediction of the six phenones becomes less pronounced as a function of the analytes' retentivity on the column when the practically determined dwell volume (353 µL) was used to construct the model. However, when the iteratively estimated dwell volume was used (391 µL), acceptable retention time predictions were obtained for all the phenones (%Δt_R values < 2.2%), except for the more polar acetophenone, which was least retained.

From the log *k* vs. %B relationship it is possible to predict the retention factor of the analytes at differing %B conditions. For the eight-component test mixture all components had *k* values <3 when run isocratically at 35%B, and generated poor retention predictions if an inappropriate dwell volume was used. For this relatively polar mixture of components, Figure 6 suggests that using an initial Δ%B > 10%B (that is, 15%B initial concentration) should not be considered if acceptable retention predictions are required. In comparison, for the phenone mixture the more hydrophobic components such as benzophenone, hexanophenone, heptanophenone, and octanophenone possessed *k* values > 10 at 35%B and generated acceptable retention prediction accuracy of ≤2%.

Conclusions

This article has highlighted the usefulness of method development strategies that 1) utilize the complementary chromatographic selectivity of differing stationary and mobile phase combinations to screen initial conditions (Wave 1), 2) the necessity of combining MS and PDA detection for peak tracking purposes, 3) the power of two-dimensional gradient time vs. temperature retention modelling (Wave 2), 4) the high retention time prediction accuracy of gradient RPLC within the resolution model, and 5) the ability to optimize the separation further by refining the % initial, final, and gradient time. It has also been surprisingly demonstrated that small errors in the dwell volume used for the retention model may give rise to large retention prediction inaccuracies depending on the retentivity of the analyte. It is therefore recommended that an iterative methodology for estimating dwell volume should be used, as this is a more accurate reflection of the true dwell volume than the well-established approaches previously recommended (2,4,5).

If the analyte is retained on top of the column (a process often referred to as *peak focusing* or *compression*), then this anomalous retention prediction inaccuracy is not observed, and the accuracy of the dwell volume estimation is not critical. It is recommended that the isocratic retention factor must be >10 at the predicted initial %B composition. It is anticipated that a warning message in newer versions of retention modelling programmes will be incorporated that will highlight if a too high initial %B condition is selected, which may result in unacceptable retention prediction errors.

Analytes of high to medium polarity may not be focused on top of reversed-phase columns and will start to migrate down the column in the dwell volume; therefore, if an incorrect dwell volume is used, the software will not model the retention properly, which may result in large retention prediction errors along with possible selectivity differences, as can be seen in Figure 5. As an approximation for accurate retention time predictions (Δt_R < 2%), it is recommended that the optimized initial gradient conditions should correspond to the %B initial used to construct the model, plus no more than 10% MeCN (or that analytes should ideally possess isocratic *k* values >10).

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Jennifer K. Field is a research scientist at Shimadzu Centre of Excellence.
Melvin R. Euerby is principal scientist at Shimadzu Centre of Excellence and visiting professor at the Open University and the University of Strathclyde.
Patrik Petersson is a principal scientist at Ferring Pharmaceuticals.
Stuart N. Berry is an application scientist at Advanced Chemistry Development, UK Ltd. (ACD/Labs).
James Hogbin is an application scientist at Advanced Chemistry Development, UK Ltd. (ACD/Labs).
Veronica Paget is an account manager, UK, and Ireland at Advanced Chemistry Development (ACD/Labs).
Earl McKoy is a senior product specialist in LC at Shimadzu UK.
Raymond Wong is national sales manager at Shimadzu UK.

What Are Options to Improve My Separation? Part 4: Solutions to Consider for Improving Separation Speed

Dwight R. Stoll, LC Troubleshooting Editor

Many high performance liquid chromatography (HPLC) users are confronted with questions about how to improve upon the performance of an existing or recently developed method. These days, we have many technological options to consider, but how do we choose one (or a few) to try? The variables that are most important for improving the speed of a separation are the maximum pressure available to drive the separation, and column temperature. Understanding the relationship between these variables and analysis time, and their effects on other choices made during method development, is helpful for developing methods that are both effective and time-efficient.

In the April 2023 instalment of "LC Troubleshooting", I kicked off a series of articles aimed at addressing the general question, "How do I improve my separation?" Given the diversity of analytical challenges that are addressed using high performance liquid chromatography (HPLC), there are several variants of the general question. In the first part of the series (1), I reviewed some basic and foundational concepts that are relevant to these discussions. In Parts 2 (2) and 3 (3), I discussed the ideas of sample complexity, the likelihood of fully resolving the components of relatively simple samples, and options for dealing with more complex samples. In this fourth part in the series, I address the issue of separation speed, and discuss aspects ranging from the kinetics of the separation itself to other nonchromatographic factors such as autosampler throughput. As with the other parts of this series, building up knowledge of how different factors affect separation speed from a theoretical point of view, as well as what changes are possible within practical constraints, is powerful when we are confronted with a separation that needs to be improved, or when an existing separation is not performing as expected.

How Fast is Fast Enough?

What constitutes a "fast" separation can be very different in the diverse application areas where LC is used. In some cases, we expect analysis times on the order of a few seconds, and in other cases, we tolerate analysis times on the order of a few hours. Usually, "faster" does not necessarily mean "better", and very often we end up having to make a compromise between analysis speed and performance as measured by resolution and robustness, for example. Sometimes both speed and resolution are important, but speed is more important because we have hundreds or thousands of samples to analyze. In other cases, resolution is more important, but we would like to achieve a certain resolution as fast as possible—for example, in the quantitation of a low-concentration impurity that is closely related to a main product. Here, resolution is critically important, as if the resolution is insufficient or decreases over time, we lose the ability to quantify the impurity accurately. While it is flashy and attractive to think about pushing for faster separations, we need to be careful that we don't sacrifice data quality in doing so.

Improving Speed by Adjusting the Dynamics of the Separation

The dynamics of analytical-scale chromatographic separations is arguably one of the most thoroughly studied areas of separation science. Indeed, one of the most well-known early texts

FIGURE 1: Comparison of chromatograms for a simulated mixture of small molecules using: (a) an "old method" involving a 150 mm × 4.6 mm i.d. column packed with 5-µm particles that yields a plate number of 15,000; (b) a hypothetical method using conditions corresponding to the KSL with a maximum pressure of 800 bar, where the optimal particle size and column are 1.1 µm and 33 mm, respectively, and (c) a method using conditions near the KSL, but with a commercially available 100 mm × 2.1 mm i.d. column with 1.8-µm particles. Other conditions: D_m, 1 × 10^{-5} cm^2/s; Φ, 500 (interstitial porosity based); λ, 0.75; temperature, 30 °C; η, 0.8 cP; mobile phase, 38% acetonitrile in water; van Deemter parameters, A = 1, B = 5, C = 0.05. All chromatograms were simulated and exported from www.multidlc.org/hplcsim.

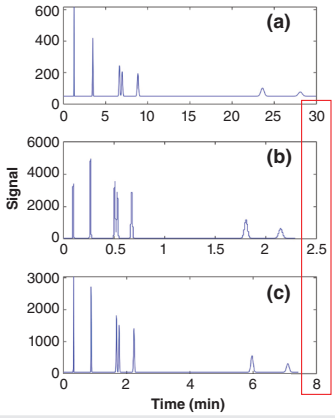
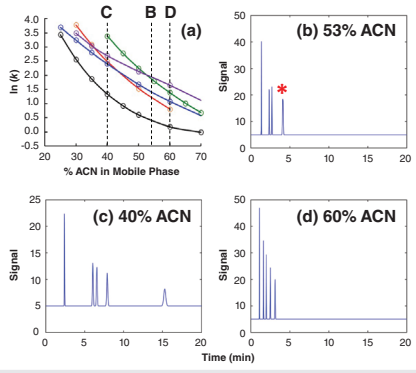


FIGURE 2: (a–d) Effect of mobile phase concentration of organic solvent on elution pattern for a simple mixture. The mixture is full resolved (five peaks) when mobile phases with either 40 or 60% acetonitrile (ACN) are used; however, the separation with 60% acetonitrile is clearly more desirable because the analysis is complete in a much shorter time. The red star in (b) indicates two compounds are coeluted.



in the field, authored by the late Cal Giddings, carries the title "Dynamics of Chromatography" (4). Although many of the fundamental principles governing the optimization of separations for speed were described in the 1960s and 1970s, the dynamics of separation has continued to be a popular area of study, motivated largely by the continuing evolution of the field, and involving developments including working with smaller particles, superficially porous particles, monolithic columns, and microfabricated columns (5,6).

From a distance, the number of variables impacting the speed and performance of a separation can seem overwhelming. For example, flow rate, van Deemter parameters, particle size, column dimensions, temperature, and mobile phase composition can all affect how long it takes to achieve a separation of a certain mixture of compounds with a desired target resolution. Readers interested in developing a thorough understanding of these details are referred to other publications of the shorter (7) or longer (8) variety. However, we can simplify the thought process quite a bit by following two ideas:

1) **Stay focused on the major factors influencing speed.** All the variables listed above affect speed, but some are much more influential than others. For this part of the discussion, we should focus on particle size, column length, operating pressure, and column temperature.
2) **Assume that we are working at the Knox-Saleem Limit (KSL).** At the KSL, the particle size, flow rate, and column length are all chosen such that the pressure drop across the column is exactly equal to a target pressure drop, and the flow rate is exactly that needed to work at the van Deemter optimum velocity.

Under these conditions, we have the following relatively simple relationships that are highly instructive for thinking about how to improve speed. First, equation 1 shows the relationship between analysis time, pressure drop across the column (*P*), and temperature (implicitly through its effect on the mobile phase viscosity (η) (7). The other parameters in this equation are nominally fixed in the case where we know what plate number (*N*) is needed to achieve a desired resolution: h_{min} is the minimum reduced plate height in the van Deemter curve relevant to the technology being used, and Φ and λ are constants related to the way fluid moves through the column.

$$t_{anal} \propto (N \cdot h_{min})^2 \cdot \left(\frac{\Phi}{\lambda}\right) \cdot \left(\frac{\eta}{P}\right) \quad [1]$$

We see here that the analysis time is inversely proportional to the first power of the pressure, and directly proportional to the first power of the viscosity. This means that, assuming everything else is held constant, if we increase the pressure by a factor of two (for example, from 400 to 800 bar), we can cut the analysis time in half. On the other hand, if we increase the column temperature such that the mobile phase viscosity decreases by a factor of two (as is the case with water in the mobile phase when the temperature is increased from 40 to 80 °C), this will also cut the analysis time in half.

That's it. It's beautifully simple. However, it is critical to emphasize again here that the preceding discussion assumes that we are working at the KSL. Readers interested in understanding the implications of this in more detail are referred to a recent series of "LC Troubleshooting" articles focused on this topic (9).

At this point, we can bring the discussion back to focus on how these ideas can be applied in a practical context. Thinking back to my recent experience visiting a local laboratory that eventually led to this series of articles, I find very often that people have existing methods that are nowhere near the KSL conditions mentioned above. This means that there are opportunities for improvement of analysis speed that are much greater than the factors of two or three that would be expected by moving to a newer pump that could provide two or three times more pressure than what was available with an older instrument. For example, a lot of older methods for reversed-phase separations of small molecules call for 150 mm × 4.6 mm internal diameter (i.d.) columns packed with 5-µm particles, operated around room temperature, with a flow rate of 1 mL/min. Under these conditions, the plate number is about 15,000, and the pressure drop across the column is about 60 bar, which is nowhere near the KSL considering that these methods are typically run on an instrument with a pressure limit of 400 bar. A representative chromatogram for a separation of small molecules under these conditions is shown in Figure 1(a), where we see that the last peak is eluted around 30 min. At this point, we can ask two questions:

1) How much could the analysis time be improved if we moved to a pressure limit of 800 bar and worked at the KSL?
2) How does the situation change if we consider practical constraints on particle size and column length?

At the KSL we have not only equation 1 to guide our thinking but also

equations for the optimal particle (d_p^*) size and optimal column length (L^*) that correspond to the KSL condition; these are shown in equations 2 and 3, where B and C are van Deemter parameters, t_m is the column dead time, and D_m is the diffusion coefficient of an analyte in the mobile phase.

$$d_p^* = \left[\frac{\Phi \cdot \eta \cdot B/C}{P}\right]^{1/4} (\lambda \cdot t_m)^{1/4} D_m^{1/2} \quad [2]$$

$$L^* = \left[\frac{P \cdot B/C}{\Phi \cdot \eta}\right]^{1/4} (\lambda \cdot t_m)^{3/4} D_m^{1/2} \quad [3]$$

Once a target is set for the plate number (15,000 in this case) and the constants in equations 2 and 3 are known (see Figure 1 caption for details), then d_p^* and L^* can be calculated easily. In this case, we find that they are 1.1 µm and 33 mm, respectively, and we would have to use a flow rate of 0.6 mL/min, with a 2.1 mm i.d. column to work at the KSL. If we could do this, we would get the separation shown in Figure 1(b), where we see that the analysis time has improved dramatically to just 2.5 min without sacrificing resolution (a factor of 12!). The problem is that there are no commercially available columns sold with 1.1 µm particles, so this improvement is completely theoretical.

At this point we move to the second question, where we recalculate the improvement in analysis time, but assume that we will use a column with a length and particle size that is commercially available. If we move to a 1.8-µm particle, then the shortest column that would give us at least 15,000 plates under these conditions would be 64 mm—also an unconventional length. The next best option would be a 100 mm × 2.1 mm i.d. column, which we could operate at 0.55 mL/min at our pressure limit of 800 bar. Such a column would yield the chromatogram shown in Figure 1(c), where we see that the analysis time is about 7.5 min—not fast as the separation at the true KPL, but still about fourfold faster than our old method with the 30 min analysis time.

Through this example, we see that the simple relationships in equations 1–3 can provide tremendous insight into the potential for gains in analysis time and the columns needed to provide these gains, but also that, in some cases, we aren't able to realize the entire gain in practice because of the constraints of commercial column offerings.

Improving Speed by Adjusting Selectivity

The preceding discussion was focused on kinetic factors that affect analysis time. These most certainly are very important, but we should not lose sight of the value of adjusting separation selectivity because this can be an incredibly powerful tool for improving analysis time, even after kinetic parameters have been optimized. A fuller discussion of this topic can be found in Part 2 of this "LC Troubleshooting" series (2). Please note Figure 2, where I have repeated figure 3 from that article, which emphasizes the point that, when developing separations for relatively simple mixtures, there often are multiple mobile phase compositions that will provide the selectivity needed to fully resolve the mixture, but with one requiring significantly less analysis time than the others.

When the Chromatography is Not the Slow Step

While the effects of chromatographic variables on separation speed discussed above are obviously important to any method optimization process, we should also not lose sight of the fact that other, nonchromatographic factors can strongly affect method throughput. Two examples are between-analysis data processing time, and the time associated with loading a sample into the injection apparatus prior to the actual introduction of the sample into the mobile phase stream (that is, the actual sample injection). In modern chromatography data systems, the time intensive steps associated with data processing (for example, peak integration and report generation) can be turned off entirely, deferred and completed at a later time, or completed using a computer different from the one used to acquire the data. In recent years, the recognition that manipulation of samples prior to injection into the mobile phase can be too time-consuming for some applications has motivated instrument manufacturers to improve the efficiency of this step using hardware- (for example, using multiple flow paths in parallel) or software-oriented (for example, enabling the start of the next analysis before the prior analysis is completed) solutions. A detailed discussion of these solutions is beyond the scope of this article, but at this point it is useful to be aware that a sampling handling cycle can be more than 30 s before the injection even occurs. If the actual chromatographic separation step is only 15 s in the case of a very fast method, for example, then it would be worthwhile to consider implementing a hardware- or software-oriented solution to decrease the time overhead associated with the sampling step so that it occupies much less than 50% of each analysis cycle.

Summary

Many HPLC users are confronted with questions about how to improve upon the performance of an existing method. In this instalment, I have discussed the factors that most strongly influence separation speed, including the pressure available to drive the separation, column temperature, particle size, and column length. Although the relationships between these factors and other

chromatographic variables such as flow rate and column diameter can seem overwhelming, they can be reduced to relatively simple expressions that form the foundation of a way of thinking about how to improve separation speed. In addition to this theoretical guidance, it is also important to keep in mind that other nonchromatographic factors, such as the time needed for the instrument to handle a sample prior to injection, can contribute to the total analysis cycle time and limit throughput. In these situations, it is worthwhile to consider investing in software- or hardware-oriented solutions that decrease the sample handling time.

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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. He has authored or coauthored more than 75 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of *LCGC*'s editorial advisory board. Direct correspondence to: amatheson@mjhlifesciences.com

Are You Ready to Switch to Comprehensive Two-Dimensional Gas Chromatography?

Nicholas H. Snow, GC Connections Editor

In the past two decades, comprehensive two-dimensional gas chromatography (GC×GC) has progressed from an interesting concept to the forefront of thinking and research in gas chromatography. When combined with mass spectrometry (MS) detection, GC×GC provides high resolution, high sensitivity, and massive chromatographic data sets, which are useful in diverse fields such as petroleum analysis, metabolomics, food, flavour, fragrance, and environmental analysis. In this instalment, recent developments in GC×GC that make it more amenable for routine use are discussed. These include advances in instrumentation, particularly modulation, column sets, data analysis, and the range and types of samples amenable to GC×GC. We will ponder the question—are you ready to make the switch to GC×GC?

Comprehensive two-dimensional gas chromatography (GC×GC) was developed in the 1990s in the laboratory of the late Professor John Phillips at Southern Illinois University (1). A book chapter by Dimandja provides an excellent overview of the development of GC×GC in the Phillips laboratory (2). Dimandja traces the development of coupled columns in GC, in which columns of differing stationary phase chemistries are connected in tandem, from simple coupling of columns to heart-cutting to comprehensive GC×GC. The goal of any chromatographic technique involving multiple stationary phases in the same separation is to achieve a desired selectivity.

When two capillary columns of differing stationary phases are connected in tandem, there are three possibilities, as summarized in Table 1. If the columns are simply connected end-to-end, the final selectivity will be a combination of the selectivity of the two initial columns. If the two columns are of equal dimensions, they will roughly contribute equally to the selectivity of the final separation. A second possibility is to add a simple switching valve at the outlet of the first column that directs a portion of the effluent, such as one peak, or a timed portion, into the second column. This is called *heart-cutting* and allows the second stationary phase to separate a small set of analytes not separated on the first. If heart-cutting is extended to its limit, sampling the first column effluent continuously every few seconds, and then assembling each short cut or slice into a three-dimensional plot, we obtain GC×GC.

Figure 1 shows a simplified instrumental diagram of a tandem column system. The device connecting the two columns is called a *modulator*. In GC×GC, the two columns are usually housed in separately controlled column ovens, although this is not always required. Tandem columns provide an advantage of allowing a multidimensional separation using a single detector. With the timing of each slide being carefully controlled, a single chromatogram is generated. The data system then slices the single plot into each short second-dimension slice and aligns the slices into a single plot, with the two separation dimensions as the *x*- and *y*-axes and the signal intensity as the *z*-axis.

The Modulator

If the column is considered the heart of a one-dimensional separation rather than the modulator, a device

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FIGURE 1: Diagram of a tandem-column multidimensional gas chromatograph. The modulator can be any of the configurations described in Table 1.

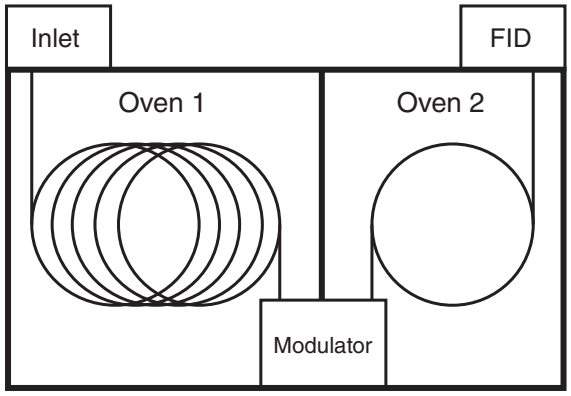
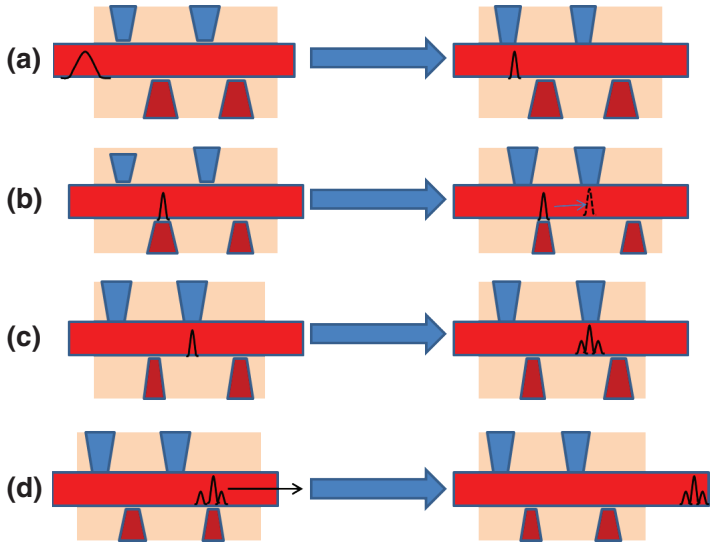


FIGURE 2: Schematic of the cryotrap modulator and peak modulation process. (a) First cold jet focuses peak of single component; (b) first hot jet moves peak to second cold jet; (c) second cold jet splits peak of component; and (d) second hot jet moves peaks for component into secondary oven.



that both connects the columns and transfers the first-dimension column effluent into the second-dimension column effluent will act as the heart of multidimensional chromatography. Like the inlet on a traditional gas chromatograph, the modulator must collect a chosen aliquot of the first-dimension effluent, focus it into a very narrow band at the head of the second column, and then inject it rapidly into the second column. Keep in mind that in GC×GC the second column is usually very short, 1–2 metres long, with retention times measured in a few seconds.

There are three types of modulators used for GC×GC—thermal, valve, and flow—with thermal and flow being most used in commercial systems. Each has advantages and disadvantages that relate to both analytical performance and ease of use. Figure 2 shows a diagram of the steps involved with a thermal modulator; it is easy to understand and illustrates the steps needed to ensure that the effluent is efficiently transferred between the two columns, while preventing carryover from one second-dimension injection into the next.

This thermal modulator uses two cold jets and two hot jets that operate in sequence to transfer the sample. Typically, the cold jets employ liquid nitrogen, and the hot jets employ nitrogen gas. One obvious disadvantage of thermal modulation is the need for cryogens to provide the best peak focusing. The first cold jet activates and traps the analytes in a narrow band. This is followed by the first hot jet activating and rapidly transferring the analytes to the second stage, where the second cold jet activates and traps them in a narrow band. The first cold jet then re-activates to trap the next analyte band, while the second hot jet injects the first band into the second column.

This process illustrates the basic principles for all modulators, transferring the analytes from the first column to the second in repeating, reproducible narrow bands, with no bleed or carryover between slices. The need for no carryover is why modulators generally have two stages. Analytes exiting from the first column are stopped while injection into the second column is happening.

Advances in Data Analysis

Not surprisingly, data sets in GC×GC and especially GC×GC–

mass spectrometry (MS) are quite large and include many levels of information, including first- and second-dimension retention times, peak widths, peak height, peak area, peak shape in both dimensions, and, if using MS, a full mass spectrum of each peak. In many untargeted situations, this can mean hundreds of peaks and mass spectra in a single analysis. With such large data sets, automated chemometric techniques are needed. If a chromatogram is seen as a fingerprint of a sample, think about the complexities involved if two or more are to be compared, now in multiple dimensions and with a spectrometric detector, such as a mass spectrometer, providing yet another data dimension.

FIGURE 3: Two-dimensional chromatogram of 58 pharmaceutical solvents, showing multiple selectivities possible in GC×GC. Reprinted from reference 6 with permission from the author.

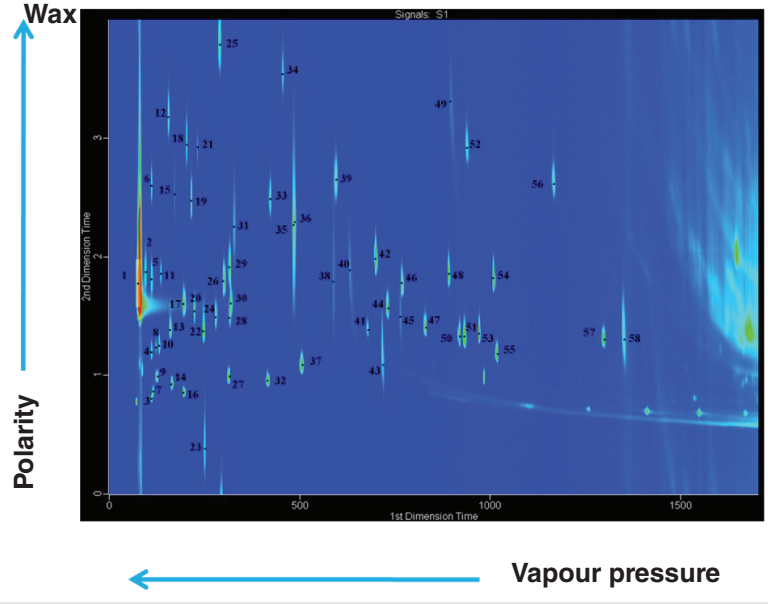


TABLE 1: Summary of tandem column configurations

Tandem Column Configuration	What it Does	Selectivity	When to Use It
Simple Connection	Connects two columns in sequence	Combines selectivity of the two stationary phases as if it were one phase	A specific selectivity not obtainable with a single stationary phase is needed
Switching Valve "Heart-Cutting"	Diverts a portion of effluent from the first column into the second	Second stationary phase selective for components not separated on first	Further separation of a few unresolved components on first stationary phase is needed
Modulator "GC×GC"	Collects and injects effluent from first column into second at regular, short intervals	Full selectivity of both stationary phases throughout the chromatogram	Separation of mixtures too complex for a single stationary phase

There are several statistical techniques for both qualitative and quantitative comparison of these complex multidimensional chromatograms that perform basic functions: i) deconvolution, in which the individual signals that generate unseparated peaks can be separated; ii) qualitative and quantitative pattern recognition, in which compounds of interest are identified and quantified, likely among large numbers of peaks that are not of interest. Berrier, Prebihalo, and Synovec introduce the basics of these techniques, many of which are available as components of commercial data systems for GC×GC (3).

Most recently, chromatogram tiling has become available, in which pattern recognition is performed by separating the larger two-dimensional chromatogram into smaller tiles, accounting for small variations in retention times in both dimensions and aligning the chromatograms for component identification and pattern recognition (4). The best way for a new user to distinguish between and choose the necessary techniques is to discuss their needs and possibilities in detail with instrument vendors' technical support teams.

When Should I Use GC×GC?

There are several considerations in making a transition from GC to GC×GC, with situations in both targeted and untargeted analyses lending themselves to GC×GC, and some specific cases in petroleum and drug analysis having been discussed in a previous column (5). The utility of GC×GC in untargeted analysis is straightforward. We want to see as many components in the mixture as possible and may be concerned with the identity of some or all of them. This started with petroleum and related analyses and has moved to metabolomic studies in clinical, pharmaceutical, food, environmental, and other situations where there are complex mixtures and all the peaks may be of interest. In many of these cases, there may be many samples with large numbers of analytes in each one that may not all be the same. GC×GC, especially GC×GC–MS, lends itself well to this situation.

In targeted analysis, we either have many specific analytes that need to be identified or quantified, or a small number of analytes mixed with a complex sample matrix. GC×GC and GC×GC–MS also lend themselves well to these situations. If there are many possible analytes to quantify in a single method, such as in drug testing or pesticide analysis, GC×GC allows for more separation space, and more potentially fully separated peaks can fit into the space of one chromatogram. In pharmaceutical residual solvents analysis, there are more than 50 possible analytes, but only a few of them may be present in any single analysis. However, the method must still account for fully separating all of them, with required selectivity stated by regulatory bodies (6). Figure 3 shows a contour plot chromatogram of pharmaceutical residual solvents, illustrating the power of GC×GC for targeted analysis. Each analyte is represented as a bright spot against the blue background of the baseline. The specific analytes are listed in the reference (6).

Figure 3 also illustrates the power of GC×GC to separate analytes from matrix components. In this example, the analytes were dissolved in methanol, which shows up as a large peak to the left in the chromatogram.

Note that the methanol peak shows tails in both dimensions, seen as stripes moving up and to the right from the main peak. With the second-dimension separation, the analyte peaks are separated from the methanol peak tails. In a traditional one-dimensional separation, the analyte peaks would lie on top of the methanol peak tail, potentially impacting the baseline and integration of the analyte peaks.

Finally, note that this chromatogram is presented roughly as a square contour plot, yet the *x*-axis time is in minutes and the *y*-axis in seconds. While this makes it easier to visualize the peaks, be aware that the actual chromatogram, if drawn to scale, would look like a narrow strip, not a square.

Conclusion

GC×GC and GC×GC–MS have come a long way in the last several years. With increasingly robust modulators and advanced data handling capabilities, application has expanded well beyond its roots in petroleum-related analysis to nearly all fields in which complex mixtures are separated or where individual analytes may be separated from complex matrices. For new users, the best way to consider transitioning into GC×GC is the same as with any new instrument: carefully outline the problem and scientific questions you are working to solve, then explore possible solutions with the scientific literature and instrument vendors. In the GC×GC space, which remains somewhat specialized, vendors are an excellent place to start.

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ABOUT THE COLUMN EDITOR

Nicholas H. Snow is the founding endowed professor in the Department of Chemistry and Biochemistry at Seton Hall University, USA, and an adjunct professor of medical science. During his 30 years as a chromatographer, he has published more than 70 refereed articles and book chapters and has given more than 200 presentations and short courses. He is interested in the fundamentals and applications of separation science, especially gas chromatography, sampling, and sample preparation for chemical analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to amatheson@mjhlifesciences.com

Highlights from the 51st International Symposium on High Performance Liquid Phase Separations and Related Techniques

David S. Bell, Column Watch Editor

The 51st International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2023), chaired by Michael Lämmerhofer and Oliver J. Schmitz, was held from 18–22 June in Düsseldorf, Germany. This instalment of "Column Watch" presents many of the highlighted topics and trends observed at this exceptionally well-run symposium.

The 51st International Symposium on High Performance Liquid Phase Separations and Related Techniques, or HPLC 2023, was held in Düsseldorf, Germany, and was chaired by Michael Lämmerhofer (University of Tübingen) and Oliver J. Schmitz (University of Duisburg-Essen). The HPLC symposium continues to be the premier event bringing together leading scientists in liquid chromatography (LC) and related techniques. The conference was well organized, packed with an abundance of solid science, and provided for high-quality interactions with colleagues and friends.

In this instalment of "Column Watch", observed highlights and trends from the conference are reported. In a similar fashion to the previous HPLC symposium review articles (1–5), many colleagues in attendance at the symposium were asked for their insights regarding the most interesting topics they observed at the event. Figure 1 captures the major topics of interest as an infographic gleaned from these responses. What follows is a synopsis of these highlighted topics, along with some personal views and observations.

Biopharmaceutical and Oligonucleotide Drivers

The topic of biopharmaceuticals took a large share of the attention at HPLC 2023. Many talks discussed the importance of the high performance liquid chromatography (HPLC) technique for biotherapeutics. Oligonucleotide separations, as with HPLC 2022, were again a dominant topic. In addition, many "omics" topics, such as proteomics, lipidomics, metabolomics, "multi-omics", and even "valvomics" (Schoenmaker's talk, "New LC Characterization Methods: What a (Few) Valve(s) Can Do") were discussed. A few of the many fine talks on the topic of biopharmaceuticals and related omics fields at the conference are discussed here.

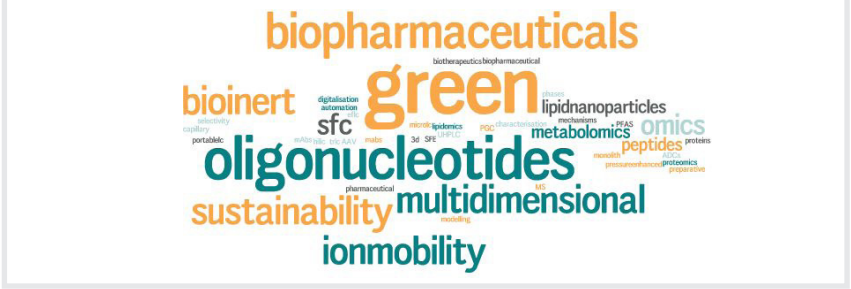
The subject of biopharmaceuticals was immediately evident in an opening plenary lecture by John McLean (Vanderbilt University), where he introduced a new area of "omics" research, phenomics, or the systematic study of traits that make up a phenotype. One of the main challenges in this area of research is that many key markers of diseases are isomeric pairs, and thus require separation prior to mass spectrometric detection. McLean also noted another dominant topic of the conference when he discussed the use of ion mobility in conjunction with liquid chromatography–mass spectrometry (MS). (See additional information on ion mobility in subsequent sections). The technique was used by the researchers to examine lipids and other drug metabolites in organ-on-a-chip models. In one striking demonstration of the technique, the researchers detected biomarker changes to the system almost immediately after a liver model was dosed with acetaminophen.

In a highly touted lecture, Robert Kennedy (University of Michigan) discussed his group's quest to push HPLC efficiency and throughput to new levels. Kennedy demonstrated excellent peak capacity for a metabolomics sample with a 50-cm-long column packed with 1.7 µm particles; however, this generated a back pressure of 35k psi, which is beyond the limits of commercial ultrahigh-pressure liquid chromatography (UHPLC) systems. Working with Waters Corporation, Kennedy

employed novel 1.1 µm particles, which led to comparable efficiency but only requiring a 20 cm column. This novel particle technology was then demonstrated with a two-dimensional (2D)-LC–MS (hydrophilic interaction chromatography [HILIC] × reversed-phase liquid chromatography [RPLC]) lipidomics assay where a peak capacity of 1900 was achieved in 60 min.

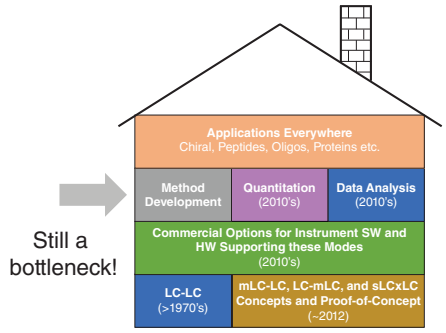
Govert Somsen (Vrije Universiteit Amsterdam) presented strategies to improve sensitivity and MS-compatibility for native, size-based separations of proteins. Utilizing a 1.0 mm internal diameter (i.d.) size-exclusion chromatography (SEC) column at low flow rates resulted in a 12–16-fold increase in sensitivity for various proteins over conventional 2.1 mm i.d. columns. By utilizing this low flow rate, ion suppression at the source was minimized, which also allowed the researchers to use higher concentrations of volatile salts (600 mM ammonium acetate) to further prevent protein–stationary phase interactions. This strategy was used to analyze the asparaginase enzyme without the loss of the protein's quaternary structure.

FIGURE 1: Highlighted topics of interest at HPLC 2023 as generated with Pro Word Cloud. (Image courtesy of the author).



Christian Hubert (University of Salzburg) presented his group's work developing a platform of chromatographic methods fully characterizing protein glycoforms.

FIGURE 2: Depiction of the current status of non-comprehensive 2D-LC: Where does the 2D-LC field stand today? (Image courtesy of Dwight Stoll [Gustavus Adolphus College]).



Utilizing a combination of a nano porous graphitic carbon (PGC) column, a nano C18 column, and native MS combined with anion-exchange chromatography, the researchers were able to discover and resolve more than 42k glycoforms of myozyme, as in one application example. Comparison of innovator vs. biosimilar therapeutics using this platform approach also led to significant differences in the glyco profile of the two drugs, which could not be elucidated using conventional approaches.

Davy Guillarme (University of Geneva) presented a compelling case for using ultrashort columns for analyzing protein biotherapeutics. Proteins are known for their on/off adsorption mechanism in RPLC. Theory predicts that proteins are infinitely retained at the column inlet (on or near the frit), and are only released after a certain concentration of organic is entered into the flow path. To test this theory, Guillarme and collaborators from Waters developed columns with lengths from 2–20 mm packed with RPLC, HILIC, and ion exchange (IEC) particles. Indeed, comparable efficiencies were achieved with these ultrashort columns as could be achieved with conventional 5–15 cm length columns, but with analysis times in the order of seconds. Select applications included peptide mapping of National Institute of Standards and Technology (NIST) monoclonal antibodies (mAb) in less than 30 s and the calculation of the drug-to-antibody ratio of an antibody–drug conjugate (ADC) in 24 s. Guillarme concluded the talk saying that as good as these columns are, their performance could be even better if instruments are redesigned to significantly reduce postcolumn path length to the detector as band broadening does occur.

As has been the case for more than a decade, biopharmaceutical needs are a major driver for HPLC innovations. The few talks mentioned above demonstrate that this industry is poised to continue to provide challenges that promote further developments in all aspects of analytical chemistry, including separation science.

Green and Sustainable Analytical Chemistry

At HPLC 2022, it was noted that green and sustainable analytical chemistry was fast becoming a dominant topic of interest (5). That trend continued at HPLC 2023, with an even greater presence as demonstrated by the number of talks focused on developments in this area. Elia Psillakis (Technical University of Crete) provided an excellent recount on the recent history of developments in understanding the goals and systems to measure "greenness" and sustainability of analytical methods. A crucial point made in this presentation was that an additional condition of a green method on top of the more obvious attributes is to reduce or eliminate waste. Psillakis noted that the complete definition of what "green" or "sustainable" means is still actively under development.

Tadeusz Gorecki (University of Waterloo) also discussed different measurements used to assess how green an analytical procedure is by taking a step back from the methodology itself and looking at the entire process. Alternative renewable and less toxic solvents usage was also discussed.

A couple of talks, including one from Martina Catani (University of Ferrara) and another from myself (Restek Corporation, *LCGC*), discussed research probing for suitable "greener" alternative solvents for use in chromatography. While Catani focused on linear and preparative RPLC systems, my focus was on attempting to replace acetonitrile in HILIC systems. Catani reported promising results substituting dimethyl carbamate for common, less green solvents, such as acetonitrile and methanol. While acetonitrile still reigns supreme for HILIC, as this author's work suggests, more environmentally suitable and sustainable solvents such as ethanol and ethyl lactate may be substituted in certain cases. It is clear from both talks that much more research is required to achieve green and sustainable methods while maintaining the necessary qualities of the overall separation achieved.

The direct substitution of green solvents for traditionally used "less green" solvents is only one means of lowering the impact of current analytical methods. Frederic Lynen (Ghent University) presented on a unique mode of chromatography: temperature-responsive liquid chromatography (TRLC), which can eliminate the use of organic solvents in the mobile phase. Using thermal-responsive polymers, Lynen developed stationary phases that could change their degree of hydrophobicity based on the temperature of the column. No organic solvent is required to elute analytes from the

column, making this approach a green alternative to typical RPLC. For more detailed information on TRLC, please see two recent articles on the subject (6,7).

Employing alternative techniques such as supercritical fluid chromatography (SFC), reducing consumption through microsampling or HPLC miniaturization, and the use of *in silico* modelling to reduce experimental waste are all additional areas of active exploration towards greener methods. The quest for green and sustainable analytical methods is, and will continue to be, a significant driver for HPLC innovation for the near future.

Multidimensional LC and Ion Mobility

Multidimensional liquid chromatography (MDLC) has been increasingly prominent at recent HPLC meetings. This year there were three full sessions dedicated to the topic, indicating the trend is continuing. As noted in a talk by Dwight Stoll (Gustavus Adolphus College and the ChromSoc Silver Medal Jubilee 2021 awardee), scientists have been conducting heart-cutting 2D-LC for about 45 years. More sophisticated concepts, such as multiple heart-cutting 2D-LC (mLC-LC) and selective comprehensive 2D-LC (sLC-LC), are more recent (circa 2010). These latter techniques have become popular modes, and have thus prompted the introduction of commercial hardware and software to support them. In addition, quantitation strategies and data analysis tools have been defined and refined to meet the needs of non-comprehensive 2D-LC. Stoll stressed that what is required now are tools and strategies for method development in this arena, which is where his group is now focusing. Figure 2 from Stoll's presentation nicely depicts the past, current state, and future of this exciting field.

André de Villiers (Stellenbosch University) provided a similar message, noting that automated method development for 2D-LC is needed due to the complex, often counterintuitive nature of the process, and that the task is complex because of the need to accommodate the interplay between many variables. He went on to note that, although the algorithms are complex, development is possible, and the results are useful. Limitations such as the shortcoming of theoretical models to model injection band broadening in the second dimension, and the need for experimental measurement of analyte/separation/system parameters were noted; however, experimental verification of the predicted separations obtained using the software is extremely promising in terms of accuracy. de Villiers demonstrated this using HILIC×RPLC and RPLC×HILIC separation of phenolic compounds.

Koen Sandra (RIC group and Ghent University) presented an excellent talk on further understanding the structure/function relationship of therapeutic antibodies. In this study, Sandra utilized novel affinity columns in 2D-LC–MS assays, relying on specific antibody binding interactions to interrogate the antibody-dependent cellular cytotoxicity (ADCC) of the biotherapeutics. Using this technique, Sandra resolved oxidized variants from main antibody peaks, which provided circumstantial evidence for lower therapeutic efficacy. In an alternative approach, Sandra used capillary electrophoresis coupled to MS and middle and bottom-up digestion to further gauge primary structure of the therapeutics. Sandra and colleagues discovered key point mutations (cysteine to tyrosine substitution) in the mAb.

Ion mobility–mass spectrometry (IM–MS) was also a prominent technique at the symposium, with two sessions dedicated specifically to it, as well as utilization in research discussed outside these sessions. Valerie Gabelica (University of Bordeaux) gave an interesting plenary talk on using IM–MS to elucidate biopolymer folding and interactions. Gabelica used IM–MS, along with super-charging agents (salts that shift spectra to higher charge states) to get more accurate mass spectra of proteins. The researchers could elucidate folded, unfolded, and partially folded proteins by using this technique. IM–MS was first noted as a highlight at the HPLC 2016 meeting (2); however, there seemed to be a significant increase in the attention given to the technique this year. While the reason for this is unclear, improvements in IM–MS instrumentation and improved understanding of the structural information the technique offers may be contributing to the trend.

Bioinert

The topic of bioinert or metal-passivated systems and columns was highly prevalent at HPLC 2023. As noted in the *LCGC* 2022 review of new LC columns and accessories (8), system hardware with various metal passivation strategies have been on the rise recently. This trend continued into HPLC 2023 with several talks and posters highlighting bioinert systems and columns. Biopharmaceutical and specifically oligonucleotide applications dominated, but other areas such as environmental, food, and metabolomics are also utilizing modern technology. In terms of new offerings from column vendors and instrument manufacturers, new stationary phases and systems that offer some form of bioinert sample pathway were the most prevalent at the meeting.

Other Observations and Conference Highlights

Poster Flash Presentations: Posters sessions contribute significantly to the HPLC programme year after year. Unfortunately, due to other commitments and the fast-paced schedule this year, I was personally unable to spend much time within poster sessions. Fortunately, there were two poster flash sessions where authors of committee-selected posters

were provided the opportunity to present five-minute lectures to the audience. The quality of the work and the presentations was one of my personal highlights, and one of the most noted events by colleagues at the conference. One colleague said the quality of the talks in this session was at the level of or surpassing those in other organized sessions.

Separation Science Slam and HPLC Tube: The Separation Science Slam and HPLC Tube events, originating at the 2019 HPLC meeting in Milan (3), were again a big hit at HPLC 2023. Both events provide a fun and engaging means of delivering scientific information, as well as showing off "other talents" our scientific community members possess. Based on the number of positive mentions, the events have established good traction, and will hopefully be continued in some fashion at future meetings.

In Memory of Andrew Alpert: Andy Alpert, often regarded as the "Father of HILIC", sadly passed prior to the HPLC meeting this year. His contributions and enthusiasm were, however, very present. As coined by Alpert, HILIC was again a major topic of discussion during the meeting, and his impact on science, as well as on the professional and personal lives of others was noted by several speakers. He will be missed.

Conclusions

The HPLC 2023 symposium, in its first return to Europe since 2019, was well organized, packed with great science, and highly engaging. Biopharmaceutical and especially oligonucleotide analysis needs are continuing to drive innovation in separation science and expanding the application of liquid separations to solve crucial challenges. Green and sustainable concerns are emerging as, perhaps, the next major innovation producer. Multidimensional liquid chromatography continues to make strides towards more routine analysis, and the attention paid to ion mobility coupled to liquid chromatography reminds us of the importance hyphenated techniques such as mass spectrometry play in the development of separation tools. Product development from both column and system manufacturers has largely focused on providing inert sample pathways. It was noted in last year's HPLC meeting review that our scientific community is awaiting that next revolutionary discovery. Although this revolution was not readily apparent from HPLC 2023, the inspiration for it may just have been seeded as a result of it.

The next HPLC meeting will be in Denver, Colorado, USA, from 20–25 July 2024.

Acknowledgements

Coverage of such a large symposium is impossible without a great amount of assistance. The author would like to acknowledge the invaluable input and discussion from Professor Steven Ray Haakon Wilson, Professor Martina Catani, Professor Alla Chernobrovkina, Dr. Adrian Clarke, Professor James Grinias, Dr. Xiaoli Wang, Dr. Diego Lopez, Dr. Jesse Bischof, Dr. Jason Anspach, Dr. Tom Walter, Dr. Paul Jacobs, Dr. Mark Woodruff, Professor Dwight Stoll, Professor André de Villiers and Professor Gert Desmet for providing notes, text, insights, figures and enlightening discussions regarding the content of various sessions. The author would like to especially thank Mr. Cory Muraco for his incredibly valuable and highly detailed notes

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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 *LCGC* 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 articles. Direct correspondence to: amatheson@mjhlifesciences.com

What Goes Around Comes Around?

R.D. McDowall, R.D. McDowall Ltd, Bromley, Kent, UK

"Questions of Quality" is 30 years old! What, if anything, has changed in chromatography laboratories over that time? What, if anything, remains the same?

Back to the future! The origins of this column lie at the 1993 Pittsburgh Conference, when I was asked by Paul Moss, then editor of *LCGC International,* to write a six-part series on—wait for it—data integrity. The first column was published in September (1), and the original theme of data integrity has been the subject of many columns since then. Let's start with a summary of what I wrote 30 years ago and see what, if anything, has changed.

Ground Zero

The aim of the first column was to consider data integrity within an automated chromatography laboratory (1). My key points were:

- A manual paper system can demonstrate integrity, but it is very laborious to maintain, slow, inefficient, and error-prone.
- Point solution or integrated laboratory? There is not enough thought put into ensuring the integrity of data generated by a process. Unless systems are planned, with data integrity issues discussed and resolved, then problems can arise when operational.
- Sampling and sample management are the basis of data integrity. Therefore, it is critical that sample labels should be barcoded for automated sample management, rather than using manual logbooks that are slow and laborious to maintain.
- Instruments should be interfaced to laboratory information management systems (LIMS) and automated processes must have integrated data integrity (DI) checks, therefore avoiding paper records where data are manually entered into a computerized system.
- Raw data: move from paper to electronic data management for efficient searching.

Readers of this column will see that these are recurrent themes that I have written about in later instalments. Recently, I discussed how laboratories are still printing paper instead of working electronically (2)—some chromatographers never learn.

References to outdated technology such as optical disks and binary-coded decimal (BCD) communication of vial numbers to the chromatography data system (CDS) have not stood the test of time. This only goes to show that you can't win them all.

Sample Labels and Other Disasters

Two of my past columns have discussed sample labelling using barcodes, with the suggestion of looking at your local supermarket to see how they use barcodes to manage stock and then taking these principles and applying them in your own laboratory (3,4). There was practical advice to ensure that you had the right tack label adhesive for the type of container and storage conditions. This was an example of Horner's Five Thumbed Postulate in action (the amount of experience is directly proportional to the number of systems ruined), as I had bought a quarter of a million sample labels with the wrong tack adhesive.

There were columns on how to evaluate and purchase a chromatograph and a CDS. In the former there was mention of a cupboard that all laboratories have, typically furthest from the head of the lab's office, where they stash all instrument disasters (5). If your laboratory suffers from an end of fiscal year slush fund spend where you have a month to use it or lose it, you'll find plenty of candidates for the cupboard. At least you have a fighting chance of hiding your mistakes, unlike a CDS where the wrong system is visible with no hiding place (6). Writing a specification is critical and varies from minimal for a chromatograph to more extensive for a CDS (7).

The Alchemist's Laboratory

In an early column I discussed why kitchen designers have a better understanding of layout and the need to integrate the sink, cooker, and food preparation areas than the average chromatographer has in laboratory design (8). My evidence was based on the fact that laboratory benches have been unchanged since 1609 (the year not the time) as shown in an antique print. Alternative ways of designing laboratories were presented, including putting chromatographs on moveable trolleys with overhead services or having

a space behind a single bench for easy access to all parts of an instrument.

Chromatography Data Systems

CDS applications are much improved since 1993. An early column for evaluation of a CDS mentioned the lack of calibration models and the inability to perform all post-run calculations (6), which are now much improved. CDS architecture has changed from files stored in operating system directories or folders to databases, which has resulted in much improved data integrity functions and access to data across all chromatographs. There are functions to control different supplier's chromatographs, electronic signature capability, and interfacing with LIMS to transfer sample identities, weights, and individual and reportable results between the two applications.

However, chromatographers are still buying standalone rather than networked systems because the former suffer from a number of issues such as keyboard contention, inability to share data easily—leaving chromatographers responsible for backup and recovery—and suffer a lack of resilience with single points of failure. Networked systems are much better. Standalone systems tend to be used in hybrid mode, with lax technical controls and potential conflicts of interest. In many cases, raw data or complete data (9,10) are defined as paper printouts, which is most certainly not the case, as it is the electronic record that is the foundation of raw data or complete data (11,12). Networked systems that are designed to work electronically provide both regulatory compliance and business efficiencies.

Process, Process, Process

The CDS is only half the story. As stated in the very first column, the process must be considered when automating. In 2005, Jens Donath and I published a case study about the validation of a site-wide CDS (13). The key to success was the mapping of the current (As-Is) process and the identification of bottlenecks and improvement ideas, which were used to develop the future (To-Be) process. This was achieved by two two-day workshops, with a report describing each process and highlighting the process improvements. The aim of the redesign was to have an electronic process with signatures that met the requirements of both good manufacturing practice (GMP) (14,15) and 21 CFR 11 (13). The CDS was then implemented and validated based on the To-Be process. Management is critical to supporting such an initiative, but the payback on the investment is considerable. Twenty years on, the same unchanged process is implemented across all six business units and all sites within the organization (16), which shows the power of process design and getting it right first time. Incidentally, screenshots to support the validation were kept to a minimum.

Since the Able Laboratories fraud case in 2005 involving a CDS (17), regulators have found more CDS data integrity problems, such as test injections, deletion of data, and integration into compliance. This culminated in a column instalment on the role of a CDS in falsification and fraud, with 10 suggestions made to avoid data integrity problems (18). Peak integration is a major way of ensuring compliance with specifications, but is also a subtle way to falsify results; tips to manage the process featured in two columns in 2015 and 2019 (19,20). Historically, 25% of data integrity warning letters were integration related (21)—so control peak integration.

Always remember that the role of a CDS is not to correct your crummy chromatography, good peak shapes result in good integration.

The War on Spreadsheets

Allied to process redesign is my war on spreadsheets. As a chromatographer and a consultant, get rid of them! As an auditor, this is a gift that keeps on giving, so please keep using them.

Why use a spreadsheet? A CDS can perform calculations without the need to print the peak areas and manually enter the values into a spreadsheet. The number of instances where a CDS is only used as an electronic ruler is amazing, even today. It appears that the lure of the spreadsheet is greater than the common-sense approach of using a CDS to perform calculations, such as system suitability test (SST) parameters and sample results.

To illustrate the silliness of this approach, a 2020 "Questions of Quality" column (22) grew from an FDA warning letter (23) that cited an unvalidated spreadsheet where the inspector had found a calculation error. This was compounded during remediation by the company finding another error!

I presented a current process where the CDS was an electronic ruler and two spreadsheets, one for SST calculations and one for the results calculations, were used. The process featured a stellar cast of characters, including Dopey, the head of quality control (QC), and Stupid from quality assurance (QA) (22), who between them allowed such an abysmal process to be established. This is not a fairy tale and names were changed to protect the guilty.

Analytical Instrument Qualification

Having the right instrument for the job is a key requirement for data integrity, and requires qualification of each chromatograph. Since the publication of *United States Pharmacopeia* (*USP*) <1058> on Analytical Instrument Qualification (AIQ) in 2008 (24), discussion and interpretation of the approach has featured in this column with co-authors Chris Burgess or Paul Smith (25–28). The current version of the general chapter integrates instrument qualification with computerized system validation (CSV) (29). An update to *USP* <1058> is

scheduled soon, with a name change to Analytical Instrument and System Qualification (AISQ). Articles outlining some possible changes to the general chapter have been published (30–33).

A Question of Balance

Analytical balances are at the heart of any chromatographic analysis, and the principles of operation must be understood and controlled to ensure the foundation of reliable and trustworthy results. Chris Burgess and I wrote two columns looking at the principles of an analytical balance and how to apply those principles in practice (34,35).

Blank Forms and Master Templates

For 30 years regulators have tried to persuade or cajole laboratories to control blank forms for recording analytical work (36–39). If results don't meet expectations, the original record can be replaced by a new form straight from the photocopier and the analysis repeated. This is not compliant and is actually falsification. Chris Burgess and I wrote an article on control of the master templates and blank forms (40). There is a high administrative overhead to manage this; it is much better to work electronically and eliminate paper.

LIMS Projects and Data Integrity

LIMS have featured in "Questions of Quality" over the years. The first two articles explained what a LIMS is and the 10 steps to heaven, sorry, to effective LIMS implementation (41,42). As a result of data integrity problems, I also wrote a column on how a LIMS can help ensure data integrity (43).

Review of Regulatory Guidance Documents

Regulatory documents from the World Health Organization (WHO), Indian Pharmacopoeia, and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) have been on the receiving end of extremely critical comments by both myself and in collaboration with Chris Burgess. I described the WHO guidance for good chromatography practices (44) as having sections organized at random with no logical order, and document numbering not following a numerical sequence (45,46). It should be fast-tracked to the round filing cabinet under your desk or the recycle bin.

Chris Burgess and I reviewed the Indian Pharmacopoeia's general chapter for qualification of high performance liquid chromatography (HPLC) instruments as unscientific dross—enough said (47). We also reviewed the draft ICH Q2(R2) and ICH Q14 for validation of analytical procedures (48,49). The original aim of the working party was to write a single document but they failed; it is not an integrated approach to development, validation,

and operation of analytical procedures (50). Use *USP* <1220> instead for a better integrated approach (51).

Summary

My philosophy when writing "Questions of Quality" has been to present a topic in simple terms that is intended to stimulate you to think. I'm not interested in whether you agree or disagree with me, I want you to think and come to your own conclusions. Quality, integrity, and compliance may be the world's second most boring subject, so I try to inject a degree of humour into my writing, although scepticism and sarcasm also appear.

The trigger for starting this column was data integrity, and throughout the 30 years of this column the quality and integrity of data have been my focus. With the major problems of data integrity in the pharmaceutical industry since 2005, what goes around comes around.

Let me answer the questions posed at the start of this column:

What has changed?

- Improvements in CDS functions: databases over directories, better compliance features, and electronic signatures for business efficiency, if you design the processes well. However, there is still further to go and Chris Burgess and I wrote a four-part article on the ideal CDS in *LCGC North America* (52–55).
- Focus on process improvement and elimination of paper make full use of the investment in a CDS. Good process design is about understanding workflow, identification of data and records created, and using risk-based technical controls to protect them.
- Once electronic, stay electronic, never print paper.

What remains the same?

- A failure to grasp common sense risk management throughout the laboratory, resulting in death by compliance.
- Unchanged working practices based on paper records coupled with a failure to automate processes effectively.
- Using spreadsheets for calculations instead of the CDS (just read the user manual!).
- Screenshotting all validation testing to death.

Acknowledgements

I would like to thank all my co-authors and reviewers of all "Questions of Quality" columns over the past 30 years, it has been very satisfying and enjoyable collaborating with you all. Thanks to Chris Burgess, Paul Smith, and Mahboubeh Lotfinia for constructive review of this column.

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ABOUT THE COLUMN EDITOR

Bob McDowall is Director of R.D. McDowall Ltd., Bromley, UK. He is also a member of *LCGC Europe*'s editorial advisory board. Direct correspondence to: amatheson@mjhlifesciences.com

Performance Maintenance Kits

Sciencix HPLC and mass spec performance maintenance kits are designed for comparability to the corresponding top brand OEM kits. The CTS-21939 PM Kit for Waters Acquity Arc SM-FTN contains all the essential components needed for optimal performance of the UHPLC system.

www.sciencix.com
Sciencix, Inc., Cary, North Carolina, USA.

LC/GC–MS Data Workflow

Mnova Gears is a vendor-neutral software solution that supports multiple LC/GC–MS applications, such as purification, affinity screening, small molecules QC, and puts automated analysis and reporting into the analyst's hands. According to the company, the setup using a no-code GUI, and the integration with existing systems makes Mnova Gears the go-to solution for scientists and IT teams.

https://mestrelab.com/automation-of-data-workflows-with-mnova-gears-software-suite-for-lc-gc-ms-analysis/
Mestrelab Research, S.L., Santiago de Compostela, Spain

Polymeric HILIC Columns

iHILIC-Fusion(P) and iHILIC-(P) Classic are two lines of polymeric HILIC columns with different surface chemistries. They provide complementary selectivity, ultra-low column bleeding, and excellent durability at basic conditions. According to the company, the columns are particularly suitable for LC–MS-based analysis of polar and hydrophilic compounds.

www.hilicon.com
Hilicon AB,
Umeå, Sweden.

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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.

Achiral Column

Daicel's DCpak PMPC column is the 4th achiral column in Daicel's DCpak series. According to the company, this polymeric-based achiral selector is highly effective for SFC and HILIC modes. PMPC offers new selectivity for peptide and lipid separations. Available in 3 and 5 micrometres.

www.chiraltech.com
Daicel Chiral Technologies, Illkirch, France.

Bioinert (U)HPLC Columns

YMC-Triart Bio C18 columns offer a pore size of 300 Å, specifically designed for analysis of peptides, proteins, and oligonucleotides. The robust hybrid silica-based columns can be used within a wide temperature (up to 90°C) and pH range (1–12). A robust bioinert surface column hardware coating is available for permanent inertness against challenging substances.

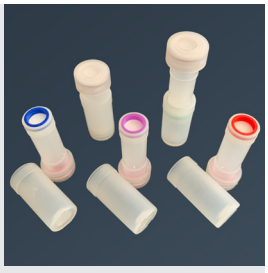
https://ymc.eu/d/brDfN
YMC Europe GmbH, Dinslaken, Germany.

Dual Detector

Tosoh Bioscience has launched LenS$_3$ MALS-V, an integrated light scattering and viscometry dual detector. Combined with the EcoSEC Elite GPC system and TSKgel columns, the LenS$_3$ MALS-V offers a complete, cost-effective, and high-performance approach for triple detection, according to the company. It features accessible and advanced multi-detection GPC/SEC software.

www.tosohbioscience.de
Tosoh Bioscience GmbH, Darmstadt, Germany.

Filter Vials



Elevate sample preparation in chemical analysis with OlimPeak filter vials. According to the company, this solution streamlines filtration, ensuring convenience, top-tier quality results, and significant time savings.

www.teknokroma.es/es/
Teknokroma Analítica S.A., Sant Cugat del Vallés, Barcelona, Spain.

Push-Thru Cartridge

UCT's LipiFiltr is a push-thru cartridge that removes lipids from acetonitrile extracts. Following initial QuEChERS, the supernatant is pushed through the cartridge using a disposable syringe and the extract is collected in an autosampler vial. Cleaner extracts, convenience, and cost savings make LipiFiltr a compelling choice for pesticide residue analysis in complex fatty samples, according to the company.

www.unitedchem.com
UCT, LLC, Bristol, Pennsylvania, USA.

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
VICI AG International, Schenkon, Switzerland.

GPC/SEC Systems

The Knauer Azura SEC provides analytical and preparative solutions for macromolecule studies. These systems analyze, separate, and fractionate sample components depending on size and molecular weight, and can be used in the polymer, food, biochemistry, and pharmaceutical sectors, as well as research. They are available in different configurations.

www.knauer.net/GPC
Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany.

HPAE Columns

SweetSep is a new line of high-performance anion exchange columns from Antec Scientific for fast and superior separation of a wide variety of carbohydrates using PAD or MS detection. They are reportedly ideally suited for the separation of monosaccharides present in food, plants, and glycoproteins up to oligosaccharides such as FOS and N-linked glycans.

www.AntecScientific.com
Antec Scientific, Alphen a/d Rijn, Netherlands.

Thermal Desorption

An automated thermal desorption system, TD100-xr is used for the unattended analysis of VOCs/SVOCs in up to 100 samples with GC–MS. The system offers sample re-collection to ensure sample security, cryogen-free cooling to save on operating costs, and it can be run with a choice of carrier gases (helium, nitrogen, or hydrogen) for future-proofing.

https://markes.com/thermal-desorption-instrumentation/sorbent-tube/td100-xr
Markes International Ltd., Bridgend, UK.

GC Column

Designed specifically for semivolatiles (SVOC) analysis, Restek's Rxi-SVOCms columns reportedly ensure consistent performance that will keep calibrations passing longer so more samples can be run before needing to recalibrate the instrument or replace the column.

www.restek.com/Rxi-SVOCms
Restek, Bellefonte, Pennsylvania, USA.

LC System

The LCMS-2050 combines high-performance liquid chromatography with the qualitative possibilities of mass spectrometry. With an extensive range of features such as a dual ion source (HESI/APCI), high sensitivity, and the MASS-IT function, the system provides reliable analysis with high-end results, according to the company.

www.shimadzu.eu/countless-benefits
Shimadzu Europa GmbH, Duisburg, Germany.

Preparative System

Prepbox is a compact alternative to Ecom 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, an optional secondary pump for repetitive sampling or with injection valve, a switching valve for CCC applications, and a PDA four-channel detector up to 800 nm.

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

Nitrogen Solution

Genius XE Nitrogen is a cutting-edge evolution combining advanced technology with refined, robust engineering, according to the company. With two models—XE 35 (up to 35 L/min) and XE 70 (up to 70 L/min)—it provides a standalone nitrogen solution for high performance LC–MS/MS and other mission-critical laboratory applications.

www.peakscientific.com
Peak Scientific, Inchinnan, Scotland.

Microplates

Unlike traditional loose-packed SPE methods, Microlute CP uses a hybrid structure, a solid interconnected network of evenly distributed pores combined with the retentive media. According to the company, the 96-well plates are designed to enhance the flow-through of samples to maximize interactions between analytes and the solid phase to deliver a reproducible SPE method that excels in performance, cleanliness, and sensitivity.

www.microplates.com/
Porvair Sciences LTD, Wrexham, UK.

LC GC europe
solutions for separation scientists

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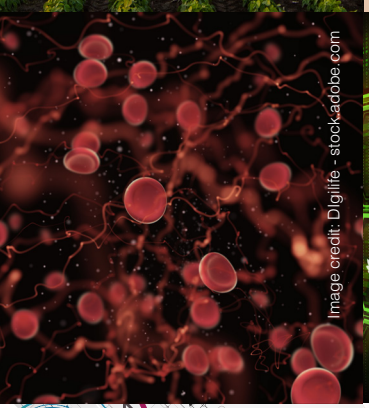
The Applications Book



CONTENTS

Analysis of Pesticide and Mycotoxin Residues in Cannabis Using QuEChERS Extraction and LC–MS/MS

UCT

This application note outlines a QuEChERS method for the simultaneous analysis of cannabis for 67 pesticides and 5 mycotoxins residues in cannabis flower.

UCT Part Numbers

ECMSSC-MP: Mylar pouch containing 4 g $MgSO_4$ and 1 g NaCl
ECQUSF154CT: SpinFiltr® dSPE cleanup tube 50 mg $MgSO_4$, 150 mg endcapped C18, 150 mg ChloroFiltr® and 150 mg PSA

Instrumentation

HPLC Column: UCT Selectra® PFPP, 100 × 2.1 mm, 3 µm
(p/n: SLPFPP100ID21-3UM)
Guard Column: UCT Selectra PFPP, 10 × 2.0 mm, 3 µm
(p/n: SLPFPPGDC20-3UM)
Guard Column Holder: UCT Selectra Guard Cartridge Holder
(p/n: SLGRDHLDR)
Column Temperature: 40 °C
Flow Rate: 0.4 mL/min
Injection Volume: 2 µL
Mobile Phase A: 10 mM ammonium formate with 0.1% formic acid in DI water
Mobile Phase B: Acetonitrile
Gradient Program: Conc. B. 2% (0 min) — 100% (12–13 min) — 2% (13.1–16.5 min)

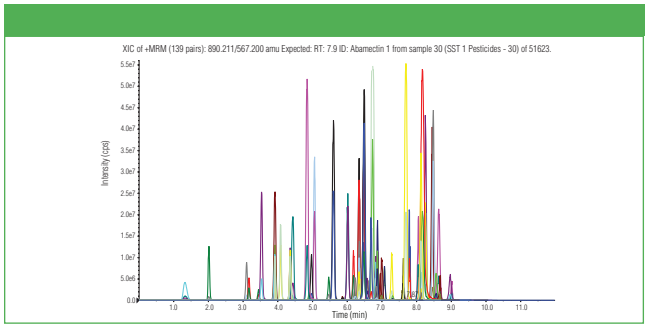


Figure 1: Pesticides panel TIC.

Cannabis Flower Extraction Procedure

Sample Preparation

a) Grind and homogenize a 10 g measure of cannabis flower using 2 mL DI water.
b) Mix the sample in a Spex 2010 Geno/Grinder® for 10 min.
c) Thoroughly mix and vortex the sample to achieve homogeneity.

UCT, LLC
Email: methods@unitedchem.com
Website: www.unitedchem.com

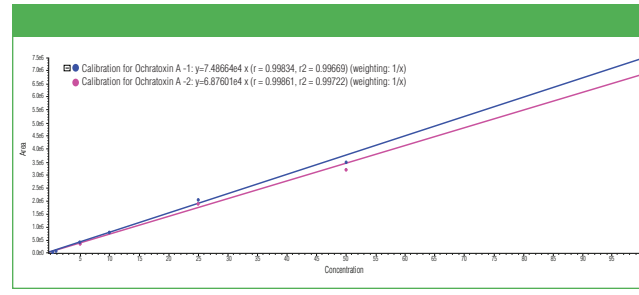


Figure 2: Calibration curve example: Ochratoxin A. A 7-point calibration curve prepared at 0.5, 1, 5, 10, 25, 50, and 100 ng/mL.

d) Weigh 10 different samples at 1 g each.
e) Spike five samples at low (5 ng) and five samples at high (25 ng) fortification levels

Extraction Procedure

a) Place each prepared sample in a 50 mL centrifuge tube.
b) Add 10 µL of internal standard(s).
c) Add 5 mL of DI water to each sample and vortex mix well to ensure the analyte concentration is distributed as equally as possible throughout the sample.
d) Add 10 mL of acetonitrile containing 2% formic acid.
e) Add the contents of the ECMSSC-MP Mylar pouch (4 g $MgSO_4$ and 1 g NaCl) and shake for 10 min using the Spex 2010 Geno/Grinder.
f) The sample is centrifuged at ≥ 3000 × g for 5 min.

Cleanup Procedure

a) Transfer 1 mL aliquot of supernatant into ECQUSF154CT dSPE cleanup tube containing 50 mg $MgSO_4$, 150 mg endcapped C18, 150 mg ChloroFiltr, and 150 mg PSA.
b) Vortex the sample for 30 s.
c) Centrifuge the sample at ≥ 3000 × g for 5 min.
d) Transfer the purified and filtered sample extract into an autosampler vial for analysis on ABSciex 6500+ Triple Quad LC–MS/MS.

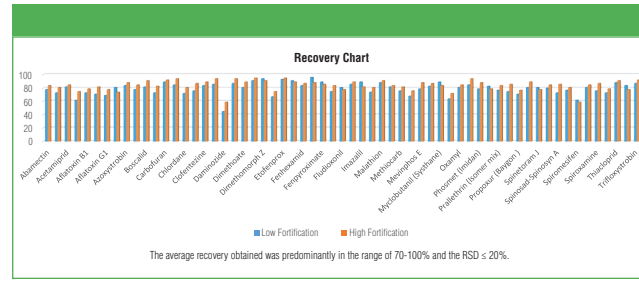


Figure 3: Recovery chart. The average recovery obtained was predominantly in the range of 70–100% and the RSD ≤ 20%.

Targeted Analysis of Phosphorylated Metabolites in Biological Samples by HILIC–LC–MS

Ondrej Hodek[1], Wen Jiang[2], and Thomas Moritz[2,3], [1]Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå, Sweden, [2]HILICON AB,[3]Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Nucleotides, deoxynucleotides, and coenzymes comprise a wide range of phosphorylated metabolites with zwitterionic nature and high polarity. They constitute a family of compounds that participate in key metabolic pathways, such as glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, and synthesis of nucleic acids. Due to their physicochemical properties, separation and sensitive quantification by LC–MS/MS without using ion-pairing reagents is challenging.

Historically, hydrophilic interaction liquid chromatography (HILIC) has demanded high concentrations of buffers in mobile phase to achieve better retention and separation efficiency for the analysis of phosphorylated metabolites, especially for di- or triphosphates (1). However, a high concentration of salts in the mobile phase suppresses transfer of metabolites to the gaseous phase in electrospray ionization-mass spectrometry (ESI-MS), which results in poor sensitivity and contamination in the ion source. Previous studies have shown that medronic acid binds to the active sites of trace metal in stainless steel tubing of LC, which prevents undesired interactions of phosphorylated compounds with the active sites (2). As a result, the addition of medronic acid in the mobile phase significantly improves peak shapes (less tailing), thus boosting the separation efficiency.

In this application, we describe the methodology applied to targeted analysis of various biological samples such as human *in vitro* differentiated adipocytes (3), plant tissue (*Arabidopsis thaliana* leaves) (4), or mouse skeletal muscle using polymer-based iHILIC-(P) Classic HILIC columns.

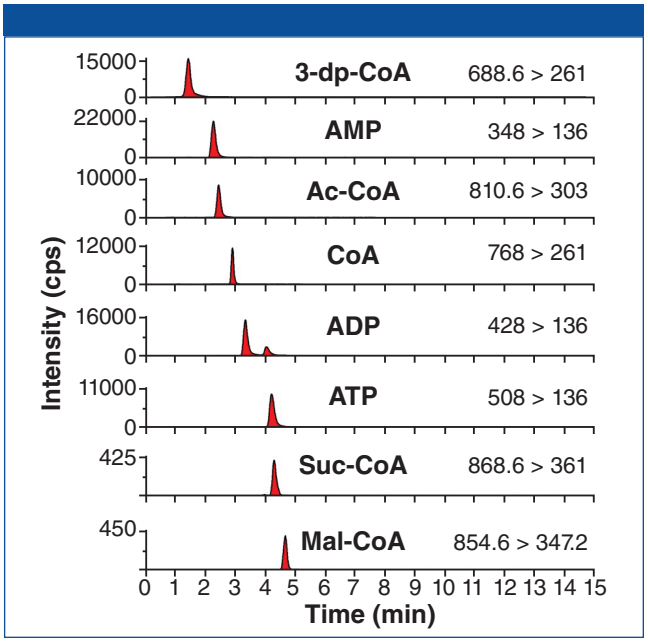


Figure 1: MRM chromatograms from 1 µM mixture of standards in 50% methanol. Column: 50 × 2.1 mm iHILIC-(P) Classic. Elution: Gradient 2.

Experimental

Sample Preparation:

1) Human *in vitro* differentiated adipocytes were lysed at day 14 post-induction of differentiation in 1 mL of 90:10 (v/v) methanol–H_2O solution containing 0.5 µmol/L creatine-D_3 as an internal standard, shaken with metal beads at 30 kHz for 3 min, and centrifuged at 14,000g for 10 min. The supernatant was evaporated and reconstituted in 50 µL of 50:50 (v/v) methanol–H_2O. Targeted metabolites: phosphocreatine, creatine.

2) Rosette leaves (10 mg) of *Arabidopsis thaliana* were extracted with 250 µL of ice-cold extraction medium (chloroform–methanol, 3:7) and incubated at -20 °C for 2 h. Thereafter, 10 µL 50 µM UDP-Glc-$^{13}C_6$ was added to each sample as an internal standard. Samples were then extracted twice with 200 µL of ice-cold water and the aqueous layers combined and dried in a freeze-dryer. The dried samples were dissolved in 50 µL of 50:50 (v/v) methanol–H_2O and diluted 10-fold with the same solvent before the analysis by LC–MS/MS. Targeted metabolites: UDP-Glc.

3) Mouse skeletal muscle (20 mg) was extracted with 500 µL of 90:10 (v/v) methanol–H_2O solution containing 1 µM labelled standards (creatine-D_3, ADP-$^{15}N_5$, ATP-D_4) as in Method 1. Targeted metabolites: 3-dp-CoA, AMP, ADP, ATP, Ac-CoA, CoA, Suc-CoA, Mal-CoA, cyclocreatine, creatine, β-GPA, phosphocreatine.

LC–MS/MS System: An Agilent 1290 UHPLC system with an Agilent 6490 triple quadrupole. Analytes were ionized in an electrospray source operated in both positive and negative mode. The source and gas parameters were set as follows: ion spray voltage at -3.5 kV (+4.0 kV in positive), gas temperature at 150°C, drying gas flow at 11 L/min, nebulizer pressure at 20 psi, sheath gas temperature at 350 °C, sheath gas flow at 12 L/min, and fragmentor at 380 V.

HILIC Separation:

Columns:

1) 150 × 2.1 mm, 5-µm, iHILIC®-(P) Classic (P/N 160.152.0520, HILICON); Flow rate: 0.2 mL/min

2) 50 × 2.1 mm, 5-µm, iHILIC-(P) Classic (P/N 160.052.0520, HILICON); Flow rate: 0.35 mL/min

Eluents:

A) 10 mM ammonium acetate in water (pH 6.8) with 5 µM medronic acid

B) 10 mM ammonium acetate (pH 6.8) in 90:10 (v/v) acetonitrile–water

Column Temperature:

40 °C

Table 1: Gradient programs for separation of phosphorylated metabolites with iHILIC-(P) Classic

15-cm Column		5-cm Column			
Gradient 1		Gradient 2		Gradient 3	
Time (min)	% B	Time (min)	% B	Time (min)	% B
0	90	0	85	0	95
15	30	5	60	3	90
18	30	7	30	8	30
19	90	8	30	9	30
27	90	9	85	10	95
		15	85	15	95

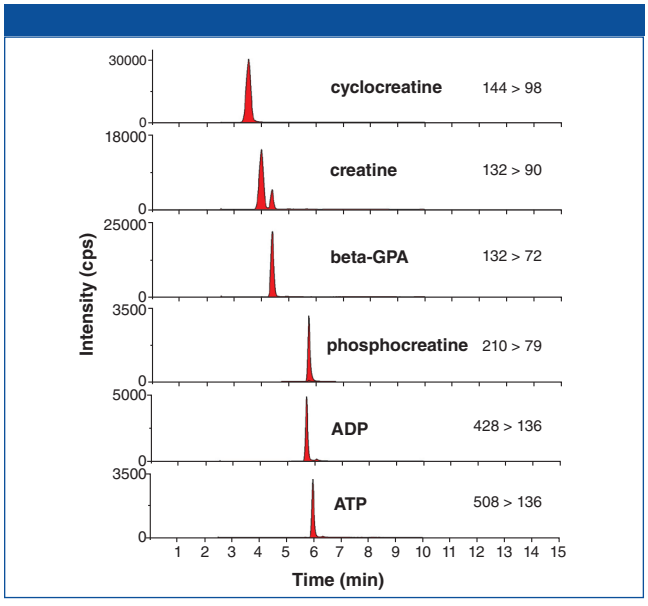


Figure 2: MRM chromatograms from 1 µM mixture of standards dissolved in 50% methanol. Column: 50 × 2.1 mm iHILIC-(P) Classic. Gradient program 3. Phosphocreatine detected in negative ESI.

Results and Discussion

The developed gradient elution methods with iHILIC-(P) Classic columns were optimized to fit the needs of the specific sample matrix and metabolites of interests. Initially, we have been using a 150-mm column and Gradient 1 program (Table 1) for quantification of creatine and phosphocreatine (3) and UDP-Glc (4). However, we discovered that a 50-mm iHILIC-(P) Classic column allows the separation of many phosphorylated metabolites within a 15-min run (Figure 1) whilst retaining a similar separation efficiency as the 15-cm columns. These findings significantly helped us to increase the analysis throughput.

Moreover, by fine-tuning the gradient profile, we managed to separate creatine analogues and their isomeric compounds-β-guanidinopropionic acid (β-GPA) and creatine that share the MRM transition of 132>90. Thus, baseline separation is needed, as shown in Figures 2 and 3.

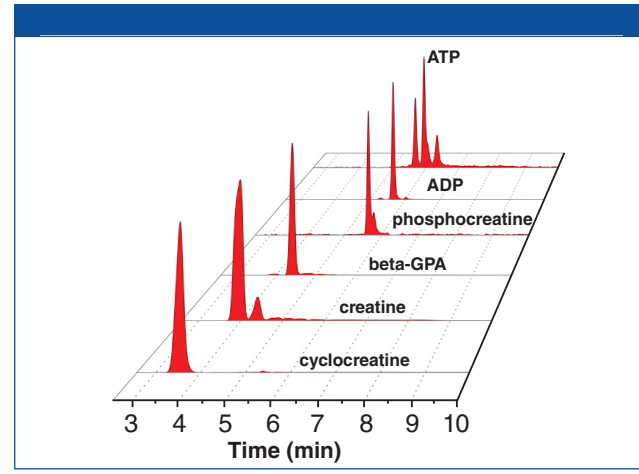


Figure 3: MRM chromatograms from a skeletal muscle extract (10× diluted with 50% methanol). Column: 50 × 2.1 mm iHILIC-(P) Classic. Elution: Gradient 3.

Conclusion

The HILIC–MS methods described in this application are generic for the analysis of polar metabolites in targeted and nontargeted metabolomics. It is straightforward and fast in both sample preparation and separation, which empowers high-throughput analysis.

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HILICON AB
Tvistevägen 48 A, SE-90736 Umeå, Sweden
Tel.: +46 (90) 193469
Email: info@hilicon.com
Website: www.hilicon.com

Analysis of Phosphorylated Peptides Using a Bioinert YMC-Accura Triart C18 Column

Kirstin Arend and Daniel Eßer, YMC Europe GmbH

The YMC-Accura column hardware is specifically designed for biomolecules, which contain phosphate group(s). This includes phosphopeptides, oligonucleotides, nucleotides, or phospholipids. The hardware eliminates any interaction between sample and stainless steel because of a strict bioinert coating on the column body and frit. It allows sharp peaks, stable recoveries, and eliminates consequential carryover effects. This makes it a great choice for working at trace levels.

This application note presents results obtained with standard column hardware and compares them with the bioinert coated YMC-Accura Triart C18 column. To demonstrate the beneficial effects, four phosphorylated peptides (Figure 1) were selected. To eliminate the influence of any potential interaction between these critical analytes and any metal parts in the liquid chromatography (LC) system, all measurements were performed on a fully bioinert LC system.

The use of the bioinert YMC-Accura Triart C18 column led to higher intensities and peak areas for all peaks (Figure 2). In addition, the high recovery rate from the YMC-Accura Triart C18 column also enabled the detection of the challenging phosphopeptide T43pp, which contains two phosphate residues. In contrast, the analysis with the standard column hardware showed no signal, even after thorough equilibration after several sample injections.

The bioinert YMC-Accura Triart C18 column provided very stable peak areas. The deviation within the first 10 injections was about 6–9% for T18p, T19p, and T43p. Only T43pp saturated after 10 injections with about a 32% increase in peak area. This proves that little or no column conditioning is necessary with the YMC-Accura Triart C18 column. Further, the column showed very reproducible peak performance. Almost no peak tailing could be observed (Figure 3).

These results demonstrate that the bioinert hardware is an essential tool for the analysis of biomolecules. All peptides were detected with much higher recovery rates than with the standard column hardware. Even challenging peptides can be analyzed reproducibly. The use of bioinert YMC-Accura Triart columns has several benefits: higher recovery, better peak shapes, greater reproducibility, and little or no requirement for conditioning. This makes YMC-Accura Triart columns an excellent choice for the analysis of critical biomolecules such as phosphopeptides.

Chromatographic Conditions

Columns: 100 × 2.1 mm, 1.9-µm, 12 nm YMC-Accura Triart C18 (bioinert hardware); 100 × 2.1 mm, 1.9-µm, 12 nm YMC-Triart C18 (standard hardware)

Part Nos.: TA12SP9-10Q1PTC; TA12SP9-10Q1PT

Eluent: A) water + 0.1% formic acid; B) acetonitrile + 0.1% formic acid

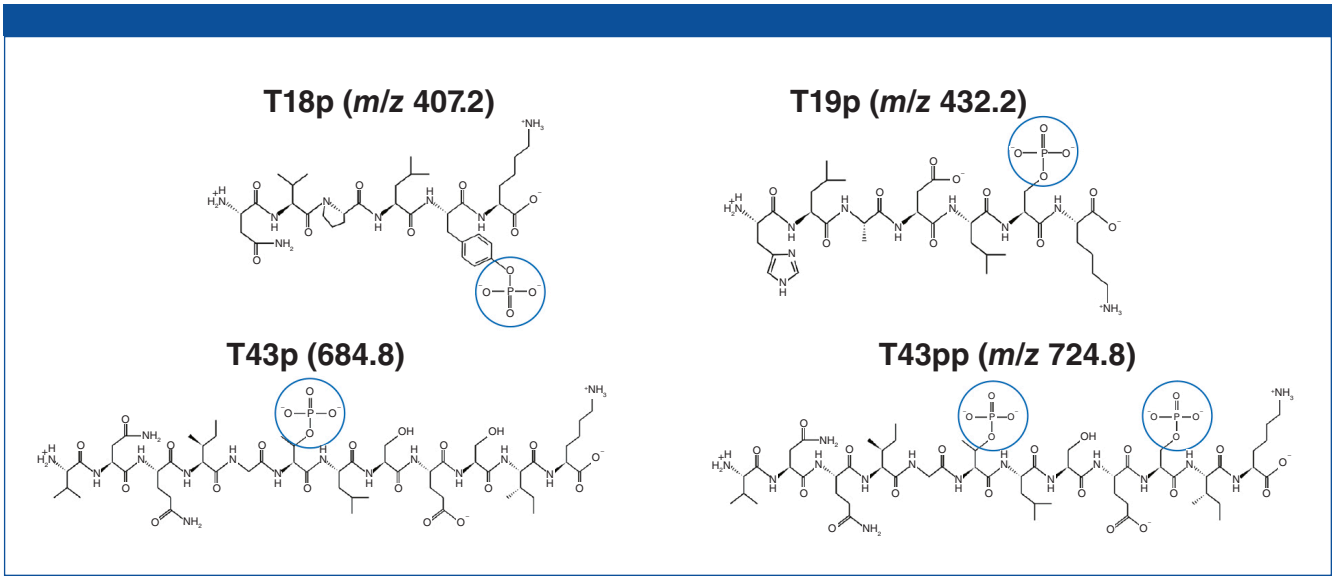

Figure 1: Synthetic phosphorylated peptides used in this application.

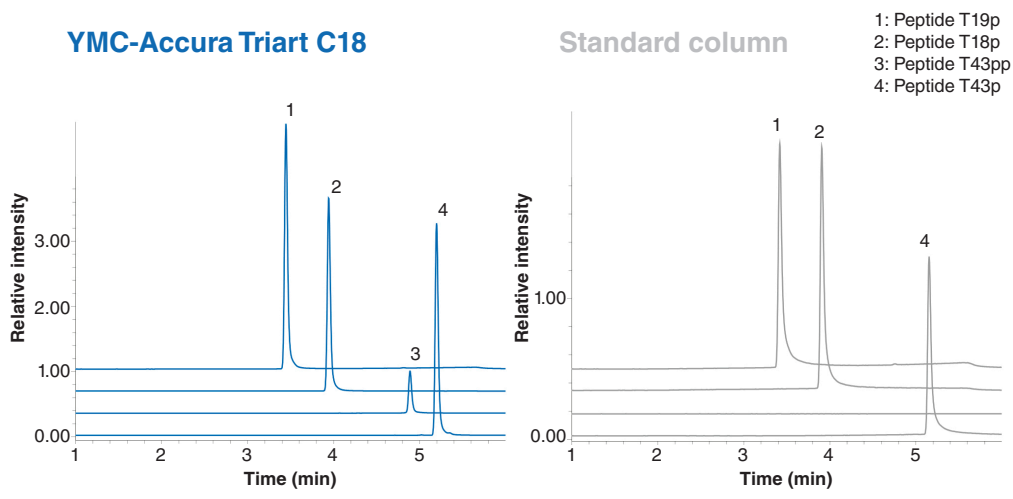

Figure 2: Extracted ion chromatograms (EICs) of phosphopeptide mixture separated with the bioinert YMC-Accura Triart C18 column and the stainless-steel column hardware equilibrated with 10 injections.

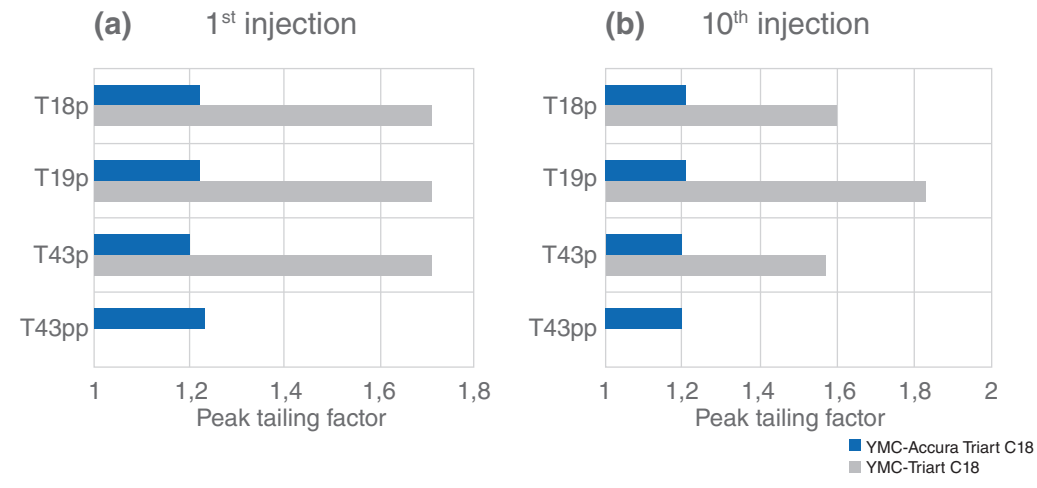

Figure 3: Peak tailing factor of the four phosphopeptides analyzed by the bioinert YMC-Accura Triart C18 column (blue) and the stainless-steel standard column (grey) after (a) the first injection and (b) the tenth injection.

Gradient: 0.7–25%B (0–5 min), 25%B (5–6.6 min), 0.7%B (6.6–8 min)
Flow rate: 0.6 mL/min
Temperature: 60 °C
Detection: ESI-MS
Injection: 2 µL (10 pmol/µL)
Sample: Massprep phosphopeptide enolase standard (Waters)
System: Shimadzu Nexera XS inert, Shimadzu LCMS-2020

YMC Europe GmbH
Schöttmannshof 19, 46539 Dinslaken, Germany
Email: info@ymc.eu
Website: www.ymc.eu

Size Distribution and Surface Charge of Liposomal Doxorubicin Assessed by Electrical Asymmetrical Flow Field-Flow Fractionation Coupled With Multi-Angle Light Scattering

Postnova Analytics GmbH

Doxorubicin is a well-established chemotherapeutic agent frequently used in cancer treatment. In 1995, Doxil®, the brand name of the first liposomal doxorubicin formulation, became the very first FDA-approved nano-drug. Encapsulation of doxorubicin is a useful way to mitigate its cardiotoxicity and to ensure a high, stable dose, as well as a prolonged circulation time of the drug in the human body (1). Today, several liposomal doxorubicin formulations (Figure 1) are available for clinical application, and the accurate characterization of their physicochemical properties—including size distribution, shape, and physicochemical stability—is a prerequisite for market approval.

Asymmetrical flow field-flow fractionation (AF4) coupled with multiple detectors, providing particle size separation and characterization in one system, has proven to be a powerful analytical setup to assess these critical quality attributes, both from a regulatory (2) and a standardization (3) perspective.

Besides the size distribution, precise knowledge of the surface charge (Zeta potential) is a key parameter towards tailor-made liposomal drug formulations with enhanced physicochemical stability, particularly under physiological conditions. Here we present the application of electrical asymmetrical flow field-flow fractionation coupled with multi-angle light scattering to derive both essential physicochemical parameters, size distribution, and surface charge of a commercial liposomal doxorubicin formulation.

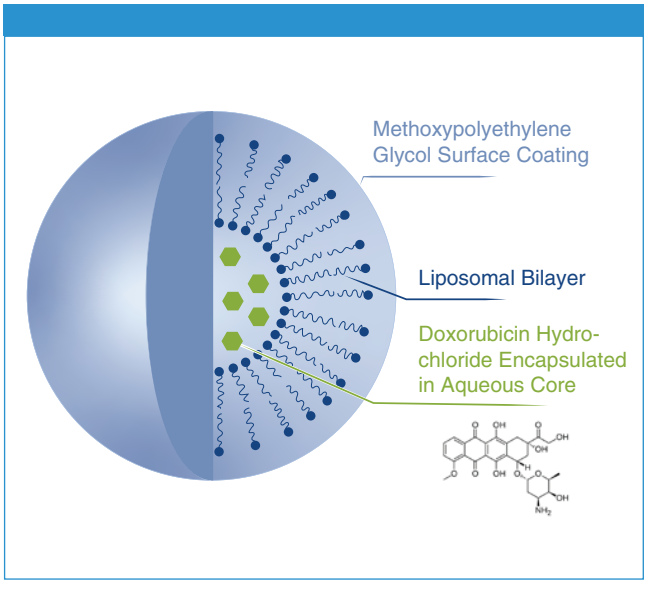

Figure 1: Schematic of liposomal doxorubicin.

Table 1: Overview of the derived size distribution, electrophoretic mobility, and surface Zeta potential of the investigated liposomal doxorubicin sample

Radius of Gyration R_g (nm)	Electrophoretic Mobility ($1*10^{-8}$ m^2 V^{-1} s^{-1})	Zeta Potential, Smoluchowski (mV)
27–45	-2.6 ± 0.3	-34.3 ± 2.8

Electrical Asymmetrical Flow Field-Flow Fractionation

Electrical asymmetrical flow field-flow fractionation (EAF4) combines the principle of electrical and asymmetrical flow field-flow fractionation in one single channel, with an electrical and a cross flow field applied simultaneously across the channel (Figure 2). By this means, EAF4 enables access to both size distribution and surface charge of an individual sample.

To obtain the surface charge from an EAF4 experiment, measurements with and without application of an electrical field have to be performed. The instrument software can then automatically calculate the Zeta potential via the following steps: First, the ratio of the obtained retention times, with and without electrical field, allows the calculation of the drift velocity induced by field. Then, the effective electrical field is determined by taking into account the applied electrical current and the simultaneously measured conductivity of the eluent. A simple linear fit of drift velocity vs. applied field yields the electrophoretic mobility, from which the surface Zeta potential can be derived.

Size Distribution and Surface Zeta Potential of Liposomal Doxorubicin

EAF4 measurements were performed using 0.5 mM NaCl as eluent at three different applied electrical currents (0.0 mA, 0.1 mA, 0.2 mA). In the experiments where a charge was applied, the top plate of the EAF4 channel was negatively charged. In addition, an online multi-angle light scattering (PN3621 MALS) detector was used to derive the size distribution of the liposomal doxorubicin sample. Figure 3 displays the obtained EAF4-MALS fractogram and indicates a broad size distribution of the investigated sample (radius of gyration R_g = 27–45 nm, Berry model fit) with no detrimental influence of the applied electrical field on the size distribution.

Plotting the calculated drift velocity against the applied electrical field strength (Figure 4) enables the determination of the electrophoretic mobility from which the surface Zeta potential can be derived using the Smoluchowski approximation. Obtained results indicate a negative surface Zeta potential of the liposomal doxorubicin sample in 0.5 mM NaCl (-34.3 mV ± 2.8 mV).

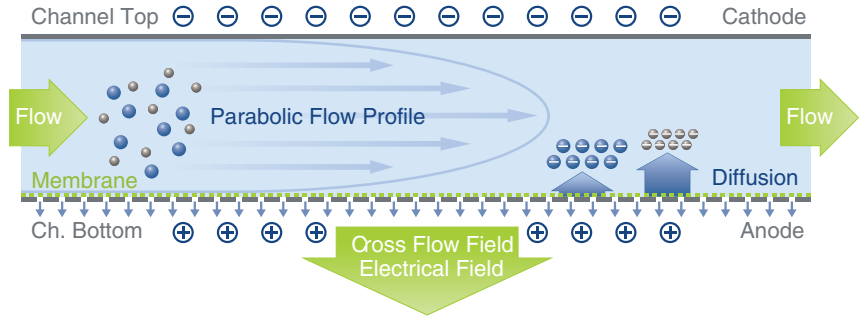

Figure 2: Schematic of the fractionation in an EAF4 channel both by size and surface charge.

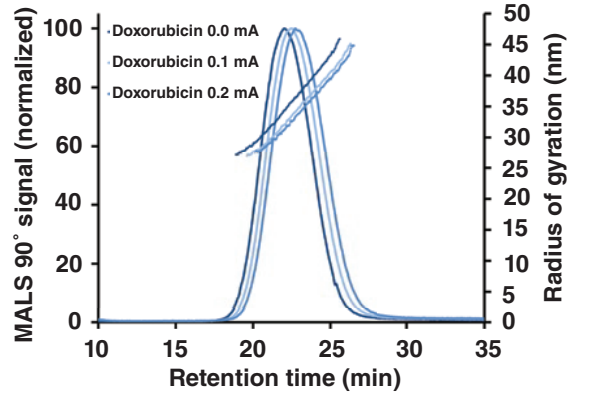

Figure 3: EAF4-MALS-fractograms of the investigated liposomal doxorubicin sample obtained at three different applied electrical fields (left *y*-axis: normalized MALS signal intensity at 90°; right *y*-axis: radius of gyration).

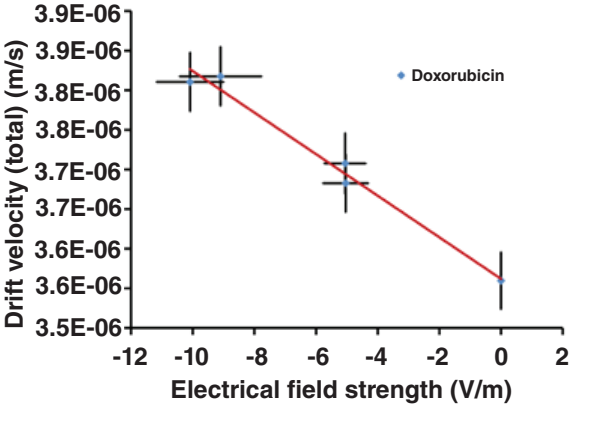

Figure 4: Drift velocity vs. electrical field strength plot to determine the electrophoretic mobility of the liposomal doxorubicin sample.

The results from the EAF4-MALS experiment are summarized in Table 1.

Conclusion

EAF4-MALS is a powerful analytical technique for the characterization of nano-enabled pharmaceuticals such as liposomal drug formulations. Besides access to the size distribution, it also provides information about the surface charge even under physiological conditions. By enabling access to these crucial physicochemical parameters, EAF4-MALS is an indispensable tool not only for quality control purposes but also when it comes to a more rational development of novel nano-enabled products in this extremely innovative field.

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Postnova Analytics GmbH
86899 Landsberg, Germany
Email: info@postnova.com
Website: www.postnova.com

Optimization of Ion Analytical Conditions in Pharmaceuticals Using LabSolutions MD

Hiromasa Iboshi, Shimadzu

Using software, ion analytics in pharmaceuticals can be enhanced. Each parameter can be varied comprehensively and easily, enabling efficient analysis method development. Thus, resolution and analysis parameter relationships can be visually assessed and the valid parameter areas can be confirmed.

The physicochemical and pharmacokinetic properties of active pharmaceutical ingredients change depending on the counterion. In the drug development stage, various counterions are tested and selected as appropriate salts. Residual inorganic impurities such as catalysts and ions used in the synthesis stage can affect product safety, solubility, and stability, so it is very important to analyze ions as impurities. In this article, an analysis example using ion exclusion chromatography is described. Formic acid, acetic acid, fumaric acid, and maleic acid, which are organic acids frequently used for drug counterions, were analyzed in the example. Response was visualized by drawing design spaces while comprehensively changing each parameter, and analytical conditions were optimized by using LabSolutions™ MD and LC-2050C 3D.

Analytical Conditions

In ion exclusion chromatography, retention strength mainly depends on column temperature and acid concentration. In addition, there are components that greatly change the retention time, so it is necessary to consider the analytical conditions. We examined the analytical conditions that can achieve good separation of the four components of formic acid, acetic acid, fumaric acid, and maleic acid by using LabSolutions MD. Table 1 shows the analytical conditions used for the separation study of each component.

Table 1: Analytical conditions

Mobile Phase A:	Water
Mobile Phase B:	10 mmol/L perchloric acid
Column:	Shim-pack™ Fast-OA (100 mm × 7.8 mm, 5-µm) × 2
	Shim-pack Fast-OA (G) (10 mm × 4.0 mm, 5-µm)
B Conc.:	10, 20, 30, 40, 50% (5 patterns)
Column Temp.:	30, 35, 40, 45, 50 °C (5 patterns)
Flow Rate:	0.8 mL/min
Vial:	SHIMADZU LabTotal™ for LC 1.5 mL, Glass
Injection Vol.:	10 µL
Detection:	PDA at 210 nm

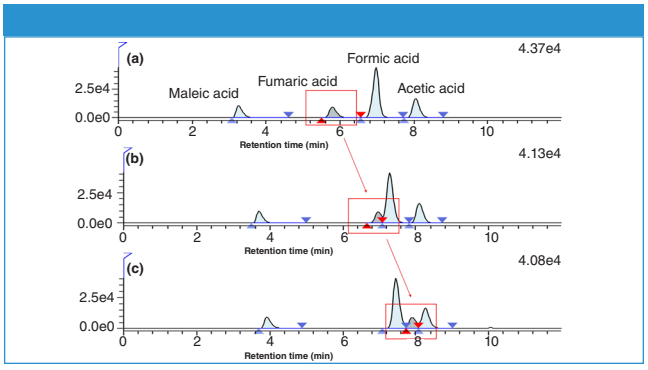

Figure 1: Chromatogram for each analytical condition. (a). Column temp.: 50 °C, B conc.: 10%. (b). Column temp. 50 °C, B conc.: 30%. (c). Column temp.: 35 °C, B conc.: 40%.

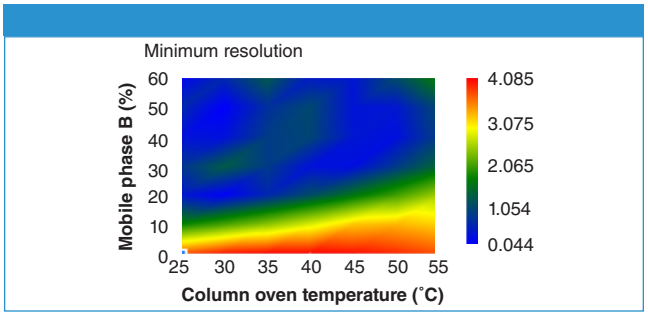

Figure 2: Design space for parameters and responses.

The resolution of four organic acids was comprehensively examined by changing the column temperature and acid concentrations in the mobile phase. Acid concentration was changed from 1 to 5 mmol/L in 1 mmol/L increments, and column temperature from 30 °C to 50 °C in 5 °C increments.

Peak Tracking

LabSolutions MD has a function to identify peaks using multiple parameters. Each peak was identified and peak tracking was performed by combining the two parameters of height percentage and peak elution number for each component (Figure 1).

It was found that the retention time of fumaric acid changed significantly compared to other peaks. In this case, each peak could be identified automatically by filtering by peak number and peak height percentage (maleic acid), or by peak height percentage only (other components). It was also possible to automatically identify each peak for fumaric acid, for which the peak number changed.

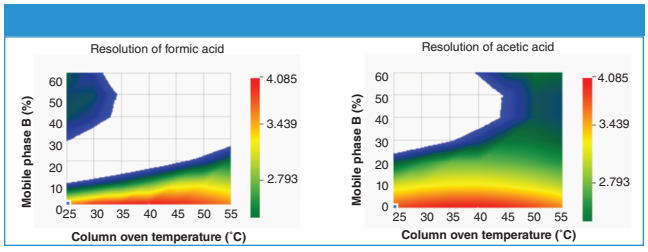

Figure 3: Design space for the resolution of each component.

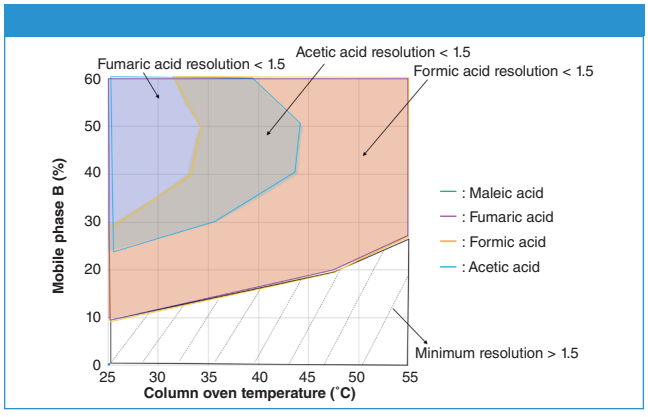

Figure 4: Overlay 2D contour lines.

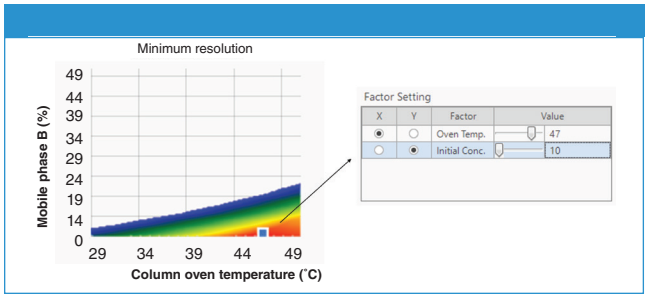

Figure 5: Optimal analysis parameters proposed.

Visualizing Separation by Design Space

LabSolutions MD can visually evaluate the relationship between analytical conditions and separation by drawing design spaces. Based on the identified retention time, a design space was produced that shows the minimum separation of each peak in the height direction, with mobile phase B concentration in the vertical axis and the column oven temperature on the horizontal axis (Figure 2). The warm areas indicate a high response and minimum resolution, allowing the effective analytical conditions to be visually determined.

LabSolutions MD can also describe design spaces that focus on specific compounds. Figure 3 shows the design space when the lower limit of resolution for formic acid and acetic acid is set to 1.5. For formic acid, the region with a resolution of 1.5 or higher is confirmed in the upper left, but with acetic acid, it can be seen that the resolution from other components is not good in the corresponding region. It is also possible to evaluate the resolution and analytical conditions for each component. In addition, by overwriting the resolution with 2D contour lines, it is possible to evaluate

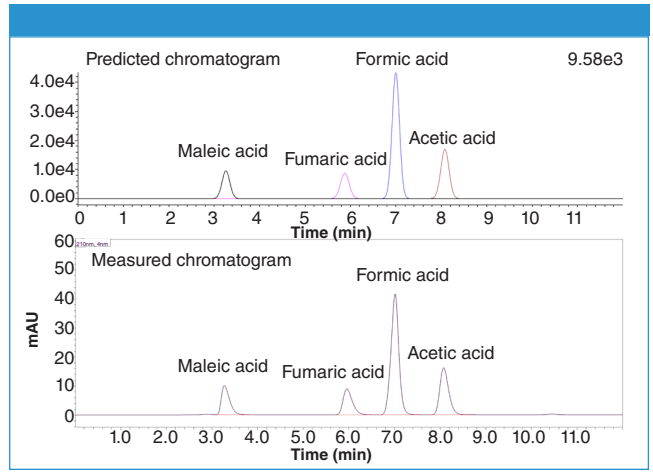

Figure 6: Predicted and measured chromatogram.

the effective area from multiple perspectives. It was possible to visually confirm the relationship between the effective region showing a resolution of 1.5 or more and the relationship for each component (Figure 4).

Proposal of Optimal Analytical Conditions

LabSolutions MD has a function to search for optimal conditions based on model analysis results. By using this function, it is possible to propose analytical conditions with good separation and high robustness in the entire variation region of various analysis parameters. A search was performed for the optimal point for the minimum resolution, and the corresponding parameters were confirmed (Figure 5). The predicted and measured chromatograms for the presented analysis parameters are shown in Figure 6. It was confirmed that there were no large discrepancies in the separation and retention time of each component.

Conclusion

The analytical conditions of the four organic acid components were examined using LabSolutions MD. By using a complex of parameters, it was possible to automatically identify peaks for components whose peak elution order changed. In addition, by drawing the design space, it was possible to visually determine the effect made on the resolution of a number of parameters. It was also possible to confirm the optimal analytical conditions. Using LabSolutions MD makes it possible to optimize analytical conditions based on scientific evidence without depending on the analyst's experience or intuition.

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Shimadzu Europa GmbH
Albert-Hahn-Str. 6-10, 47269 Duisburg, Germany
Tel.: + 49 203 76 87 0
Email: shimadzu@shimadzu.eu
Website: www.shimadzu.eu

Analysis of PEGylated Antibody Fragments by SEC-MALS

Tosoh Bioscience

The covalent modification of proteins with polyethylene glycol (PEG) is a way of improving the pharmacokinetic behaviour of small biotherapeutic drugs (Fabs, scFvs, or peptides). PEGylation is mainly used to improve drug solubility, increase serum half-life, reduce sensitivity to proteolysis, and reduce renal uptake. One major challenge is the characterization of PEGylated protein samples. Since the PEGylation reaction is unspecific, mono-PEGylated as well as poly-PEGylated proteins may be present. Furthermore, by evaluating the amount of free PEG, PEGylated and free protein, PEGylation efficiency can be determined and the PEGylation reaction optimized. Since PEGylation changes the hydrodynamic volume of the molecule, proteins with various degrees of PEGylation can be separated by size-exclusion chromatography (SEC). In this application note, SEC was combined with multi-angle light scattering (MALS), refractive index (RI), and UV detection to calculate the molecular weight (MW) of the individual peaks in a PEGylated scFv sample. This allows the determination of the degree of conjugation (DOC) as well as the analysis of reaction byproducts.

Experimental Conditions

Instrumentation: Vanquish™ UHPLC System with RI detector and LenS3™ MALS detector
Data Acquisition and Processing: SECview™ software
Column: 4.6 mm × 30 cm, 2-µm TSKgel® UP-SW2000
Mobile Phase: 100 mmol/L sodium phosphate (pH 6.2) + 300 mmol/L arginine + 10% isopropyl alcohol
Flow Rate: 0.175 mL/min
Temperature: 25 °C
Detection: UV @ 280 nm; RI and MALS
Sample: 10 kDa PEGylated single chain variable fragment (10 kDa PEG-scFv) (10 µL injected)

Results

To determine the molecular weight of the protein conjugate mixture by light scattering, the concentration and refractive index increment (dn/dc) of the individual components are required (equation 1). In the case of PEG-scFv, the PEG part is not UV-active whereas the scFv adsorbs UV. In contrast, both components generate different RI responses because of their different dn/dc values (Figure 1[a]). By comparing the RI and UV signals for the different peaks, it is thus possible to determine each peak's exact composition (weight fractions of the two components) and thereby the dn/dc for each corresponding species (equations 2 and 3).

$$MW \sim \frac{LS\ signal}{Concentration * (\frac{dn}{dc})^2} \quad [1]$$

$$RI\ signal \sim dn/dc_{PEG} * Conc_{PEG} + dn/dc_{Protein} * Conc_{Protein} \quad [2]$$

$$UV\ signal \sim dA/dc_{PEG} * Conc_{PEG} + dA/dc_{Protein} * Conc_{Protein} \quad [3]$$

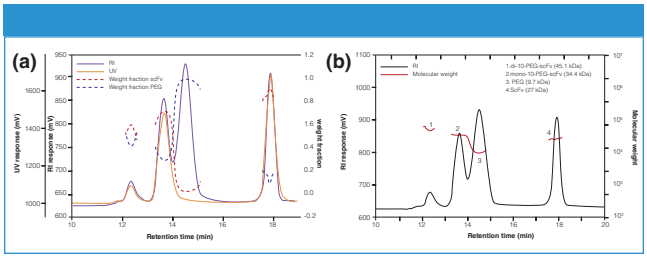


Figure 1: SEC separation of PEGylated scFv. (a) UV and RI detector signals and calculated weight fractions of protein and PEG. (b) Molecular weight profile of a 10-PEG-scFv sample.

Table 1: Literature values for dn/dc, dA/dc, and expected MW of analyzed molecules

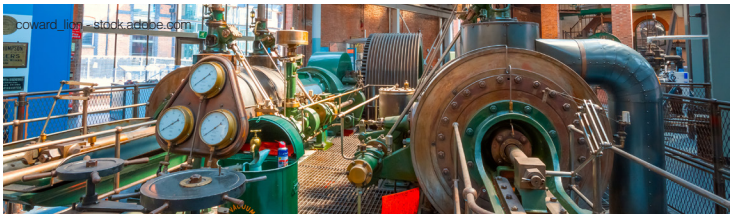
Molecule	dn/dc	dA/dc	Expected MW (kDa)
scFv	0.185	1.927	26.8
10 kDa PEG	0.134	0	10

The dn/dc distribution obtained over the full chromatogram was used to calculate the molecular weight of the multiple species in the 10-PEG-scFv sample. The obtained molecular weight trace is illustrated in Figure 1(b). The RI signal shows a small shoulder on the high-molecular-weight region of the chromatograms (~11.8 min), indicating a bimodal pattern that represents the poly-PEGylated conjugates. The MW of peak 4 and peak 3 were determined at 27 kDa and 9.7 kDa, respectively, which is in agreement with the expected MW of free unreacted scFv and PEG. Peak 1 was calculated at 45.1 kDa, which corresponds to the di-10-PEG-scFv, whereas peak 2 shows a MW of 34.4 kDa corresponding to the mono-10-PEG-scFv.

Conclusion

The complex mixture obtained from an undirected PEGylation of scFv was successfully characterized by SEC-MALS. A versatile characterization method was established by separating the sample components on a TSKgel UP-SW2000 UHPLC column, combined with the LenS3 MALS detector as well as UV and RI detectors. By determining the MW of the individual peaks, the degree of conjugation could be identified. The SECview software streamlines the conjugation analysis so that it can be done in a matter of minutes.

Tosoh Bioscience GmbH
Im Leuschnerpark 4 64347 Griesheim, Darmstadt, Germany
Tel: +49 6155 7043700 Fax: +49 6155 8357900
Email: info.tbg@tosoh.com
Website: www.tosohbioscience.de



Recent Advances in Gas Chromatography

The Chromatographic Society (ChromSoc) has announced that the **Recent Advances in Gas Chromatography** meeting will be held **Thursday 28 September 2023** at the **Science and Industry Museum** in **Manchester, UK**.

As we move to increasingly difficult sample matrices and challenging separations, more is demanded of gas chromatography (GC) in terms of separation and limits of detection. Co-organized by Tony Taylor and Dan Carrier, the meeting will address the latest GC solutions alongside emerging new approaches to analytical problems.

The following talks and speakers are confirmed:

- *Aroma and Fragrance Analysis: In Which Cases is GC×GC/TOF-MS Overshadowing GC–TOF-MS for Firm and Hard Conclusions?* Tatiana Cucu, RIC, Kortrijk, Belgium
- *2D-GC–TOF-(MS) in the Analysis of Extractables in Pharmaceutical Packaging Material*, Nicholas Morley, Element Materials Technology, Cambridge, UK
- *Helium to Hydrogen—The Analytical Scientists Perspective*, Paul O'Nion RSSL, Reading, UK
- *From Data to Decisions: Automated Workflows for Comparing 1D- and 2D-GC–MS Chromatograms*, Laura McGregor, SepSolve, Peterborough, UK
- *A Fully Automated System for Simultaneous High Sensitivity Detection of PAHs, PCBs, and Multi-Residue Pesticides in Water Using SPME Arrow and APGC–MS/MS*, Janitha de-Alwis, JSB, Birmingham, UK
- *Improved Confidence in Unknowns Analysis Using Accurate Mass Screening in Wastewater*, Richard Davis, Agilent, Cheadle, UK
- *Approaches to Significantly Reduce Helium Consumption*, Ian Parry, Thermo Fisher Scientific, Hemel Hempstead, UK
- *The Use of Vacuum-Assisted Headspace Solid-Phase Microextraction for Analysis of Volatiles in Food*, Kathy Ridgway, Element Materials Technology, Cambridge, UK
- *Fundamentals of Low Pressure Gas Chromatography (LPGC or Vacuum GC) for Realizing Shortest Possible Analysis Time in Existing GC–MS Systems*, Jaap de Zeeuw, CreaVisions
- *Chromatographic Performance Comparison Between Single and Multidimensional Chromatograph, Hydrogen and Helium, and the Relevance of Sub Nominal and High Resolution Mass Spectrometry*, Alan Griffiths, Leco, Stockport, UK
- *Breath Biopsy Using Accurate Mass GC*, Jenny Mizen, Owlstone Medical, Cambridge, UK.

Email: **registration@sasevents.co.uk** | Website: **https://na.eventscloud.com/ereg/index.php?eventid=743617**

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17–22 SEPTEMBER 2023
6th International Mass Spectrometry School
Cagliaria, Sardinia (Italy)
✉ gianluca.giorgi@unisi.it

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24–27 SEPTEMBER 2023
27th International Syposium on Separation Sciences (ISSS 2023)
Cluj-Napoca, Romania
✉ isss2023.conference@ubbcluj.ro

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5–8 NOVEMBER 2023
42nd International Symposium on the Separation of Proteins, Peptides, and Polynucleotides (ISPPP)
Parkhotel Schönbrunn, Vienna, Austria
✉ nico.lingg@boku.ac.at

SCAN FOR WEBSITE

13–15 NOVEMBER 2023
Eastern Analytical Symposium (EAS) and Exposition 2023
Crowne Plaza Princeton Conference Center, Plainsboro, New Jersey, USA
✉ askEAS@eas.org

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5–6 FEBRUARY 2024
1st Green Analytical Chemistry Workshop
Novotel Paris Charenton-le-Pont, France
✉ david.benanou@veolia.com

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24–28 FEBRUARY 2024
Pittcon 2024
San Diego Convention Center, San Diego, California, USA
✉ info@pittcon.org

SCAN FOR WEBSITE

28–31 MAY 2024
18th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-18)
Leuven, Belgium
✉ info@htc-18.com

Send upcoming event information to Kate Jones at kjones@mjhlifesciences.com

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