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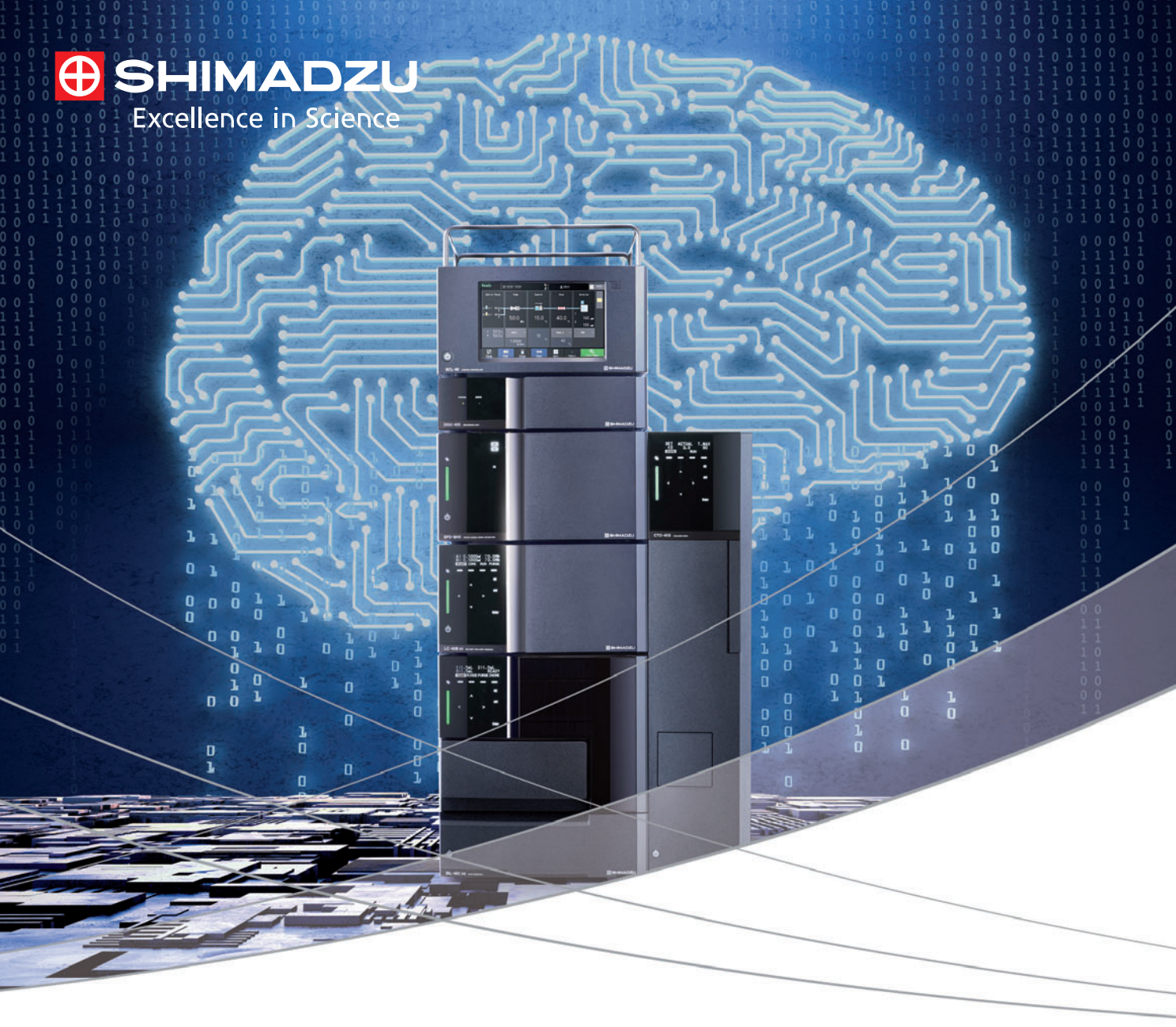

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INTERVIEW

The Challenges of Climate Change for Wine Production

The Column spoke to Julia C. Gouot from the National Wine and Grape Industry Centre in Australia about the importance of chromatography to understand the effects of temperature change on grape physiology and composition.

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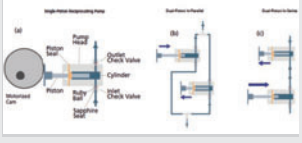
PERSPECTIVES IN MODERN HPLC

Modern HPLC Pumps: Perspectives, Principles, and Practices



This instalment of "Perspectives in Modern HPLC" provides an overview for analytical-scale HPLC pumps, including their requirements, modern designs, operating principles, trends, and best practices for trouble-free operation.

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LCGC BLOG

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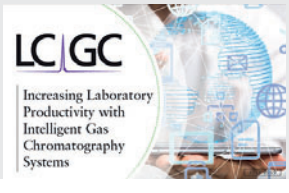

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PEER REVIEW

Design of Experiment Strategy for an Electrospray Ionization Optimization in Supercritical Fluid Chromatography–Mass Spectrometry Coupling

Stefan Bieber[1], Stefan Moser[2], Hans-Werner Bilke[3] and Thomas Letzel[1,4], [1]AFIN-TS GmbH, Augsburg, Germany, [2]Stefan Moser Process Optimization, Nußdorf am Inn, Germany, [3]LC-Pharm HPLC Expert Service, Brannenburg, Germany, [4]Technical University of Munich, Chair of Urban Water Systems Engineering, Garching, Germany

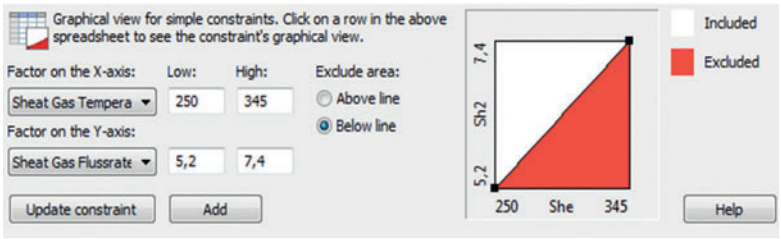
The optimization of adjustable factors in electrospray ionization (ESI) by design of experiments (DoE) is presented in this article. This approach allows a comprehensive and systematic optimization of all relevant factors for the ionization of compounds and increases understanding about factors that influence ionization efficiency. In total, 32 different compounds were separated by supercritical fluid chromatography (SFC), ionized, and then detected by time-of-flight mass spectrometry (TOF-MS). A three-stage optimization process was used to find a robust setting point for eight factors influencing the ionization for SFC-separated compounds. The results obtained for all eight factors were evaluated by multivariate statistics. At first, the influence of all eight factors on the ionization of compounds and applicable factor ranges was investigated in a geometric experimental design. Subsequently, a more comprehensive design was used to identify optimal factor settings to reduce factor ranges. Finally, the robustness of the derived setting point was assessed. The robust setting point obtained provided a sufficient ionization of all investigated compounds. The presented optimization approach allows a systematic optimization of several ionization-influencing factors, resulting in robust and statistically-assessed ionization for all the analytes investigated. In addition, design of experiments provides further information about factors that influence the ionization of compounds by electrospray.

KEY POINTS

- **Design-of-experiment approaches enable professional analytical method optimization.**
- **Rechtschaffner, D-optimal designs, and similar data evaluation can easily be handled by every analyst.**
- **Efficient mass spectrometric ionization provides foresight for multicomponent analysis (such as nontargeted screening).**
- **Chemometrics is alive and still needed.**

Electrospray ionization (ESI) is a well-established technique and is commonly applied in various disciplines such as metabolomics (1–3), proteomics (4,5), food analysis (6,7), and environmental analysis (8,9). ESI interfaces are used for transferring and ionizing analytes from the liquid phase into the gas phase to connect chromatographic techniques with liquid mobile phases and mass spectrometric (MS) detection. Ionization in ESI occurs under atmospheric pressure, by spraying the effluent of a chromatographic technique into an electrical field (10). Drying and sheath gas flows are used to dry the droplets and to enhance the transition of analytes to ions (11). The formation of ions can be explained by the ion evaporation (12,13) and the charged residue model (11). The efficiency of ionization directly depends the sensitivity of the subsequent MS detection and is related to physicochemical properties of the analytes as well as the mobile phase (3,14,15). An optimization of ionization parameters in ESI is therefore a necessary and critical step for method development to achieve optimal detection of analytes.

FIGURE 1: Constraint of sheath gas flow rate (*y*-axis) and sheath gas temperature (*x*-axis). Picture taken from Modde Pro (Sartorius Stedim Biotech, Malmö, Sweden, Version 12.1).

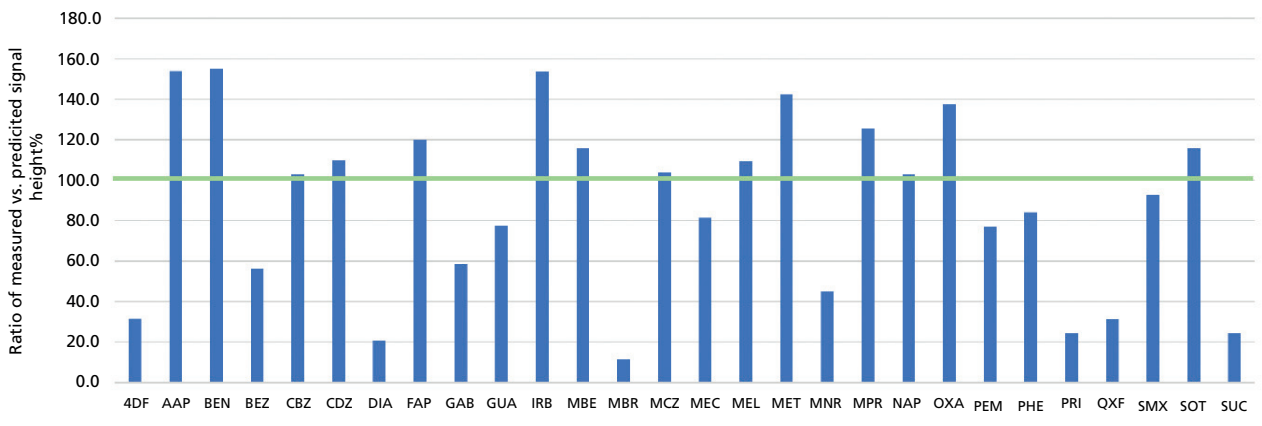


Optimization strategies can strongly differ from one-parameter optimization (16) to advanced statistics-based approaches (17,18). When coupling chromatography with ESI-MS, mobile phase composition, compound coelution, and ion suppression as well as enhancement caused by matrix compounds need to be considered as important influences on ionization efficiency and MS detection limits (19–21). The optimization of ESI parameters should be performed with the same analytes and chromatographic method as in the analyses. Optimizing ESI parameters to allow for the simultaneous (most sensitive) detection of several compounds can be a complex challenge. One-factor-at-a-time optimization (OFAT) approaches are time-consuming as all adjustable ESI parameters should be considered and the number of analytes or sample complexity increases. In addition, not all factors might contribute equally to the efficiency of the ESI ionization and interactions between factors cannot be determined this way. Chemometrics or design of experiments (DoE) can provide alternative but powerful approaches to investigate and optimize such complex systems as ESI (22). In DoE, all adjustable factors create a design space in which the number of dimensions is equal to the number of factors. For each factor, certain levels of variation are defined and the performed, balanced, orthogonal, and symmetrical positioned DoE experiments mark the corners of the design space. The significance of the effects resulting from changing factors can be determined by the analysis of variances (ANOVA) (23). The number of DoE experiments that have to be performed depends on the used experimental design and its resolution. A high-design resolution allows the investigation of two factor interactions, but requires more experiments than low-resolution designs (23).

In this study, an analytical supercritical fluid chromatography system (SFC) was applied in coupling time-of-flight mass spectrometry (TOF-MS) via electrospray ionization. The mobile phase in SFC separations is mainly comprised of carbon dioxide (CO_2) and organic solvents, such as methanol, which are added to modify the elution strength of the mobile phase (24). The system was aimed to be used for the analysis of environmental water samples, which can easily contain several hundreds of compounds. As well as mass accuracy, high sensitivity in MS detection is required here. This can also be improved by the optimization of ESI parameters, resulting in more effective ionization.

Since exclusively ESI-specific parameters were optimized, this strategy can easily be transferred to the

FIGURE 2: Ratio in percent of measured vs. predicted signal height from ESI-TOF detection of the investigated compounds.



optimization of MS ionization for other chromatographic techniques, and can be applied without detailed knowledge about ionization mechanisms.

Experimental

Reagents: Carbon dioxide (99.995% purity) for SFC separations was obtained from Linde AG and Westfalen AG. Methanol (HiPerSolv Chromasolv liquid chromatography [LC]–MS-grade) was bought from VWR, water (LC–MS Chromasolv-grade) was purchased from Fluka and ammonium acetate from Sigma Aldrich. Standard substances were obtained from Sigma Aldrich, Fluka, Dr. Ehrenstorfer, and Cimachem. Details are listed in Table 1. Standard substances were dissolved in 50:50 methanol–water (*v/v*) and united to a working mixture, containing 500 µg/L of each compound.

Chromatography and Mass Spectrometric Detection: The chromatographic separations of the working mixture were performed with an analytical SFC system (Agilent Technologies) using a 150 × 2.0 mm, 5-µm zwitterionic HILIC column (Knauer). The mobile phase consisted of carbon dioxide and methanol, containing 20-mM ammonium acetate. Compounds were eluted from the column using a gradient from 5% to 40% 20-mM ammonium acetate in methanol. The flow rate was 1.5 mL/min, with a constant back pressure of 150 bar. Column temperature was set to 40 °C. The sample injection volume was 5 µL. The outlet of the SFC system was directly connected to a JetStream ESI source, which was used as the ion source of a 6230 time-of-flight mass spectrometer (Agilent Technologies). The SFC separation method allowed the generic separation of all investigated compounds in the log D (pH 5) range -3.66 to +5.44 (see Table 1) and required no further optimization. Mass spectrometric data were evaluated using Agilent MassHunter Workstation Software Profinder (B.06.00).

Optimizable ESI Parameters: In order to perform a comprehensive optimization of ESI ionization, eight ESI factors were included in the experiments. These were drying gas temperature and flow rate, sheath gas temperature and flow rate, nebulizer pressure, nozzle voltage, capillary voltage, and fragmentor voltage. The adjustable factor ranges and the setting accuracies are summarized in Table 2. Factor settings of individual experiments were rounded according to the setting accuracies of the factors. All factors could be chosen freely, except for sheath gas temperature and flow rate; here the flow rate had to be increased when increasing sheath gas temperature. This constraint was considered in all experimental designs.

DoE Approaches: The study included eight ESI factors that had to be optimized to achieve a sufficient ionization for 32 different compounds. For all parameters, the full adjustable ranges were considered for optimization. Modde Pro (Sartorius Stedim Biotech, Version 12.1) was used for the creation of experimental plans, based on Rechtschaffner or D-optimal designs and data evaluation. Randomization of experimental order was applied to minimize the impact of external procedure influences. To ensure that all temperatures were fully equilibrated in the ESI source, a blank SFC injection was placed prior to the next injection of reference standards. The ESI parameters of the blank injection were the same as those of the following reference injection.

Results and Discussion

Compound ionization is the key element for sensitive MS

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TABLE 1: List of standard compounds used for the optimization of ESI ionization

Compound	CAS	Acronym	Sum Formula	Monoisotopic Mass	Log D pH 5	Retention Time (min)	Distributor
4'-hydroxydiclofenac	64118-84-9	4DF	C14H11Cl2NO3	311.0116	2.75	11.93	Sigma
4-aminoantipyrine	83-07-8	AAP	C11H13N3O	203.1059	0.33	3.84	Sigma
1,2,3-Benzotriazole	95-14-7	BEN	C6H5N3	119.0483	1.3	3.82	Fluka
Bezafibrate	41859-67-0	BEZ	C19H20ClNO4	361.1081	2.79	8.96	Sigma
Carbetamide	16118-49-3	CBA	C12H16N2O3	236.1161	1.65	3.61	Fluka
Chlorbromuron	13360-45-7	CBR	C9H10BrClN2O2	291.9615	2.85	1.50	Dr. Ehrenstorfer
Carbamazepine	298-46-4	CBZ	C15H12N2O	236.095	2.77	3.23	Sigma
Chloridazon	1698-60-8	CDZ	C10H8ClN3O	221.0356	1.11	6.40	Sigma
Diclofenac	15307-86-5	DCF	C14H11Cl2NO2	295.0167	3.21	6.80	Fluka
Diazinon	333-41-5	DIA	C12H21N2O3PS	304.101	4.13	0.82	Dr. Ehrenstorfer
N-Formyl-4-aminoantipyrin	1672-58-8	FAP	C12H13N3O2	231.1008	0.11	4.48	Fluka
Fenofibrate	49562-28-9	FEN	C20H21ClO4	360.1128	5.28	1.00	Sigma
Gabapentin	60142-96-3	GAB	C9H17NO2	171.1259	-1.4	9.18	Sigma
Guanylurea	141-83-3	GUA	C2H6N4O	102.0542	-2.9	9.02	Sigma
Irbesartan	138402-11-6	IRB	C25H28N6O	428.2324	5.44	10.19	Sigma
5-Methyl-1H-benzotriazole	136-85-6	MBE	C7H7N3	133.064	1.81	3.73	Sigma
Metobromuron	3060-89-7	MBR	C9H11BrN2O2	258.0004	2.24	1.27	Sigma
Metconazole	125116-23-6	MCZ	C17H22ClN3O	319.1451	3.59	1.75	Sigma
Mecoprop	7085-19-0	MEC	C10H11ClO3	214.0397	1.44	6.51	Fluka
Melamin	108-78-1	MEL	C3H6N6	126.0654	-2.54	4.93	Sigma
Metformin	657-24-9	MET	C4H11N5	129.1014	-3.66	11.53	Sigma
Monuron	150-68-5	MNR	C9H11ClN2O	198.056	1.93	2.59	Sigma
Metoprolol	37350-58-6	MPR	C15H25NO3	267.1834	-1.47	6.10	Sigma
N-Acety-4-Aminoantipyrine	83-15-8	NAP	C13H15N3O2	245.1164	0.15	3.86	Sigma
Oxazepam	604-75-1	OXA	C15H11ClN2O2	286.0509	2.92	5.70	Cimachem
2-Phenyl-2-ethylmalonamid	80866-90-6	PEM	C11H14N2O2	206.1055	0.73	4.11	Sigma
Phenazone	60-80-0	PHE	C11H12N2O	188.095	1.22	2.60	Fluka
Primidone	125-33-7	PRI	C12H14N2O2	218.1055	1.12	5.07	Sigma
Quinoxyfen	124495-18-7	QXF	C15H8Cl2FNO	306.9967	4.95	1.01	Sigma
Sulfamethoxazole	723-46-6	SMX	C10H11N3O3S	253.0521	0.76	9.38	Sigma
Sotalol	3930-20-9	SOT	C12H20N2O3S	272.1195	-3.18	12.11	Sigma
Sucralose	56038-13-2	SUC	C12H19Cl3O8	396.0146	-0.47	12.91	Fluka

detection, regardless of which chromatographic system is connected. However, the connection of SFC to MS detectors requires special attention because of mobile phase composition and its depressurization (25). ESI is the most commonly used ionization source for SFC–MS applications and several connection modes involving make-up flows and/or utilizing a flow splitter have been investigated previously (26,27). In this study, the full mobile phase stream of the SFC system was introduced into the ESI-interface and additional make-up flow was added. The make-up flow was intended to support ionization independent from the composition of the mobile phase in SFC separations (28,29). Eight factors in total were used for the ESI optimization, including drying and sheath gas temperatures and flow rates, nozzle, capillary and fragmentor voltages, and nebulizer pressure. As a result of the number of optimizable factors and potential interactions of different factors, DoE was chosen as the optimization approach. The optimization was performed using 32 compounds (Table 1), all of which are likely to be detected in samples of the aqueous environment. The optimization procedure was specifically tailored to the technical requirements and contained three stages. In a first screening, the impact of all factors was investigated

TABLE 2: Optimizable ESI factors, adjustable ranges, and accuracy applied in ionization optimization experiments

Factor	Minimum	Maximum	Setting Accuracy
Drying gas temperature (°C)	250	350	± 0,5
Drying gas flow rate (L/min)	5	8	± 0,05
Sheath gas temperature (°C)	250	350	± 0,5
Sheath gas flow rate (L/min)	5	8	± 0,05
Nebulizer pressure (psi)	45	60	± 1
Capillary voltage (V)	500	4000	± 10
Nozzle voltage (V)	1000	2000	± 10
Fragmentor voltage (V)	150	300	± 5

TABLE 3: Factor robust setting points and contributions in ESI ionization, obtained from Rechtschaffner design

Factor	Most Robust Setting Point	Factor Contribution
Fragmentor voltage	166 V	78.6%
Drying gas flow rate	4.2 L/min	6.4%
Sheath gas flow rate	7.5 L/min	3.9%
Drying gas temperature	308 °C	3.6%
Nebulizer pressure	51 psi	2.6%
Sheath gas temperature	349 °C	2.5%
Nozzle voltage	1619 V	1.2%
Capillary voltage	3026 V	1.1%

TABLE 4: Factor robust setting points and contributions in ESI ionization, obtained from D-optimal design

Factor	Most Robust Setting Point	Factor Contribution
Nozzle voltage	1286 V	16.7%
Capillary voltage	1500 V	16.1%
Drying gas flow rate	6.7 L/min	15.2%
Nebulizer pressure	49 psi	14.9%
Sheath gas flow rate	6.7 L/min	14.8%
Drying gas temperature	321 °C	13.8%
Sheath gas temperature	314 °C	8.3%

in predefined adjustable ranges. The results were used to reduce the factor ranges to more relevant parameter and regions. These ranges were used in the second stage to find optimal conditions for all factors, resulting in a robust setting point within the design space. In stage three, the robustness of the obtained setting point was tested. As a minimum requirement, a signal height of 1000 counts was defined for all compounds.

Evaluation of Factor Influences and Parameter Ranges: The sensitivity of SFC–MS can be as good or even better than the sensitivity of LC–MS, but this requires a comprehensive optimization of the relevant factors in adequate ranges (30). To estimate the influences of the chosen factors and to evaluate their relevant ranges for the SFC–ESI-MS application, a Rechtschaffner experimental design was chosen. This geometric design

TABLE 5: Predicted signal intensities of compounds at the robust setting point in D-optimal design

Compound	Predicted Signal Intensity at Setting Point (counts)	Measured Signal Intensity at Setting Point (counts) (Average of Two Measurements)	Ratio of Measured vs. Predicted Signal Height (%)
4DF	78,813	24,864	31.5
AAP	403,083	620,699	154.0
BEN	393,902	611,365	155.2
BEZ	388,559	218.482	56.2
CBZ	1,924,820	1,984,296	103.1
CDZ	1,312,020	1,439,379	109.7
DIA	236,749	49,223	20.8
FAP	1,193,050	1,429,172	119.8
GAB	35,6541	208,815	58.6
GUA	36,262	28,170	77.7
IRB	778,034	1,197,112	153.9
MBE	605,490	700,428	115.7
MBR	153,369	17,443	11.4
MCZ	1,412,640	146,7767	103.9
MEC	73,753	60,100	81.5
MEL	978,358	1,068,568	109.2
MET	455,231	648,515	142.5
MNR	1,274,540	572,710	44.9
MPR	1,819,690	2,284,301	125.5
NAP	1,363,670	1,405,808	103.1
OXA	239,992	330,055	137.5
PEM	211,656	162,964	77.0
PHE	1,634,760	1,376,674	84.2
PRI	173,755	42,675	24.6
QXF	729,456	227,734	31.2
SMX	411,903	381,558	92.6
SOT	342,490	396,440	115.8
SUC	17,205	4216	24.5

employs response surface methodology and analyzes linear and nonlinear factor contributions. It is also very effective in terms of the low number of experiments needed in the test plan. All experiments were performed twice in randomized order, which resulted in 96 runs, including six experiments at the centre point of the experimental space. The ion chromatograms of all investigated compounds were extracted on the basis of the elemental formula, considering protonated ions, as well as sodium and ammonium adducts of each compound. Detected ions and adducts were combined by the software to features, of which the signal height was used for further evaluations.

The signal intensities of all compounds showed that the fragmentor voltage had the highest influence (78.6%) on the signal height of the detected compounds (Table 3). The fragmentor voltage can be adjusted to decluster ions in the mass spectrometer used, so a high impact on ionization efficiency would be expected. Effects on the ionization by other parameters were comparably small because overall effects were dominated by fragmentation voltage. As the fragmentor voltage is located at the transition from ionization to ion guiding, this parameter is possibly more related to ion optics than to ionization. Interactions of other factors, such as gas flow rates and temperatures, could be expected.

The sheath gas flow rate and temperature could exclusively be changed in certain ratios for the instument used. This additional term resulted in a limitation of the geometric investigation pattern, which had to be considered in the creation of the design space (Figure 1). Rechtschaffner designs are very suitable to investigate factors independently

between minimum and maximum levels. To consider limitations such as step-wise changes of parameter values, resulting in constraint investigation spaces, it is necessary to switch from a geometric design, such as Rechtschaffner, to a mathematical D-optimal design.

Optimization of Parameters: The D-optimal design calculates the optimal locations of the experiments within the given investigation space. For a given model, Y = Xb + e, the D-optimal approach maximizes the determinant of the matrix X'X (31). The new experiments, together with the former, span the largest volume possible in the experimental space. In summary, a D-optimal design can be tailored to support an irregular experimental region, or a very complex problem setup (31). For the D-optimal design, the fragmentor voltage—which showed the highest influence on the ionization in the previous experiments—was kept constant at 166 V, the most robust setting point for the investigations. The chosen candidate set of the D-optimal design with seven considered factors resulted in 47 runs. Twenty-nine of the 32 analyzed standard compounds were detectable within all investigated factor combinations. Chlorbromuron, fenofibrate, and sulfamethoxazole, not consistently detectable in this study, were excluded from the evaluation. The most robust setting point and individual factor contributions for the minimum signal height requirement of 1000 counts for all compounds are summarized in Table 4. Nearly all factors showed comparable contributions to the ionization efficiency, only the sheath gas temperature showed a minor impact. The most robust setting point values of the factors, however, were not comparable to those obtained from the Rechtschaffner design.

Grand-Guillaume Perrenoud and coworkers investigated the coupling of SFC to ESI-MS with integrated flow splitting with six optimizable factors and found the capillary voltage, desolvation temperature, and the drying gas flow rate as the three most impactful factors (26). Although the ESI and MS models and vendors were not the same as in this study, the results are comparable. The predicted signal heights at the robust set point were compared to measured signal heights (Table 5). Although the predicted and measured signal heights did not always match (Figure 2), this setting point guaranteed a sufficient detection of all considered compounds, with more than 1000 counts for each analyte.

Robustness of the Optimized Factors: To assess the robustness of the results provided by the D-optimal experimental design and to test the quality of the signal height prediction, the borders of the seven-dimensional design space were tested for compliance with the pre-set specifications. Therefore, results from the optimization were used and evaluated in a linear design by adjusting the minimum requirement for the signal height. The calculations, made using 1000, 5000, and 10,000 counts, resulted in different robust setting points for the optimized ionization method (Table 6). The acceptable minimum and maximum values for each factor were calculated using Monte Carlo simulations, leading to the determination of Manhattan distances (32). The altered minimum requirement for the signal height also led to a decrease of the available design space. While the requirement of 1000 counts could be reached in 7.8% of the tested design space volume, this space was decreased to 0.6% when increasing the requirement to 10,000 counts (Table 6). This is also

TABLE 6: Robust setting points, calculated for different minimum required signal heights. Low edge and high edge values for individual factors are added in ranges below factor values, where applicable. For contribution values the ranking of individual values in the experimental set is added in brackets

Factor	Minimum Signal Height					
	1000 Counts		5000 Counts		10000 Counts	
	Value	Contribution	Value	Contribution	Value	Contribution
Drying gas temperature (°C)	321 (284–346)	13.8% (6)	336 (286–348)	17.4% (3)	336 (311–348)	16.4% (3)
Drying gas flow rate (L/min)	6.7 (6.0–7.5)	15.2% (3)	6.7 (6.0–7.1)	9.2% (6)	6.7 (6.0–7.5)	12.7% (4)
Sheath gas temperature (°C)	314 (252–346)	8.4% (7)	332 (239–348)	5.4% (7)	279 (232–341)	5.0% (7)
Sheath gas flow rate (L/min)	6.7 (6.0–7.5)	14.8% (5)	5.4 (5.1–6.6)	20.6% (2)	5.4 (5.1–6.2)	20.9% (2)
Nebulizer pressure (psi)	49 (46–55)	14.9% (4)	47 (45–51)	26.1% (1)	47 (45–49)	25.1% (1)
Capillary voltage (V)	1500 (625–1938)	16.1% (2)	1000 (563–2313)	11.3% (4)	1000 (563–1875)	10.6% (5)
Nozzle voltage (V)	1286 (1036–1536)	16.8% (1)	1286 (1036–1536)	10.0% (5)	1429 (1179–1679)	9.3% (6)
Fragmentor voltage (V)	166 (constant)		166 (constant)		166 (constant)	
Relative volume of design space	7.8%		4.1%		0.6%	

true for the ranges in which factors could be changed without the risk of reaching signal heights below the pre-defined value. With the increasing signal height requirement, the ranges of most factors decreased. This can be observed at the drying gas temperature, which can be set between 284 and 346 °C (range of 62 °C) to reach 1000 counts as minimum signal height, and between 311 and 348 °C (37 °C difference) for a minimum of 10,000 counts. The contribution of the investigated factors changed with changing the minimum requirement. While nozzle voltage and capillary voltage showed the highest impact on the observed signal for the 1000 counts minimum requirement, the highest impact was observed for nebulizer pressure and sheath gas flow rate when increasing the minimum signal height to 10,000 counts. This can be explained by reduced factor ranges and shifting set point locations. Interestingly, all values of the robust set point for 1000 counts—except for the sheath gas flow rate—are in the ranges of the according values for the 10,000 counts requirement. This also reflects the increasing impact of the sheath gas flow rate with increasing signal height requirements.

Conclusions

The optimization of the electrospray ionization procedure was conducted in three stages. The first rather simple experimental design offered an overview on relevant factors and identified reasonable factor ranges. The results gained were used in a more complex design, which resulted in a robust set point at which a pre-set signal height could be achieved. As a last step, the robustness of the final optimized ionization method was assessed. This resulted in factor ranges in which a sufficient ionization for all considered analytes should be achievable. This approach allows a systematic optimization of all ionization-influencing factors and offers a robust and statistically assessed ionization for all considered analytes. In contrast to other "trial and error" optimization approaches, the quality by design (QbD) approach allows a comprehensive, systematic, and a less laborious optimization.

In practice, this approach is very useful to several analysts because, subsequent to a detailed and consequent optimization, the results may remain robust and sensitive for a long time (not only in ionization optimization). This approach can be used for a variety of ESI systems and several types of mass spectrometer (coupled with different chromatographic techniques).

To conclude, a smart DoE approach seems to be time-consuming, however, in the long term it saves a lot of time compared to an OFAT approach.

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Minimizing Fluctuating Peptide Retention in 2D-LC: How to Address a Moving Target

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Retention of peptides is strongly dependent on solvent composition in reversed-phase separations with gradient elution. In this instalment we provide tips, tricks, and suggestions for best practices to help minimize retention time variations over time.

At a conference I attended in the summer of 2019, I listened to Dr. Patrik Petersson share results from his work on the development of two-dimensional liquid chromatography (2D-LC) methods for the characterization of therapeutic peptides and related impurities. A significant part of his talk was focused on the need to take special precautions in mobile-phase preparation and operation of the LC system to reduce retention-time variation, particularly in the first dimension of the 2D-LC system. Some of his suggestions are routinely recommended by instrument manufacturers, and will be familiar to many users. However, some of the other suggestions will not be so familiar, so I've asked Patrik to summarize his deep experience in this area in a way that captures all of these tips and tricks in one place. Minimizing retention variation is particularly important in some types of 2D-LC separation, but the strategies presented will also be useful to anyone looking to obtain more consistent separations using conventional one-dimensional (1D)-LC systems as well.

Dwight Stoll

Heart-cutting 2D-LC is an off-the-shelf technique offering important advantages over conventional 1D-LC separations. For example, unknown molecules eluted from separations involving salty mobile phases can be characterized—nearly in real time—by transferring them to a second dimension separation running with a mass spectrometry (MS)-compatible mobile phase and directly into a mass spectrometer (1,2). In other situations, combining different selectivities in the two dimensions (^{1}D and ^{2}D) provides a possibility to significantly increase resolution compared to what can be achieved with a single column. This selectivity-based approach to increase resolution is already available today, and can serve as an alternative to increasing resolution by increasing chromatographic efficiency of a single separation. The latter would require smaller particles in capillary columns, and an entirely new type of LC system capable of handling extremely high pressure and dissipation of frictional heating (3). Another approach to increase efficiency is to use very long columns packed with large particles and a very low flow; however, that approach is not very practical, nor popular because it requires very long analysis times (4,5).

Heart-cutting 2D-LC works very well for small molecules. It also works for larger molecules, such as peptides and proteins. However, for larger molecules, it can be more challenging due to ^{1}D retention fluctuations resulting in a moving target—that is, trapping peak(s) of interest can be a challenge, as illustrated in Figure 1(a) to 1(c) (II), where a 40 µL cut defined based on a 1D analysis (a) would miss its target in subsequent analysis (b) and (c). These fluctuations are related to a very strong response of these large molecules to small changes in mobile-phase composition. Snyder and co-workers have shown that the following expression is valid for reversed-phase chromatography of large molecules such as peptides and proteins (6):

$$\log k \approx \log k_0 + 0.25\,(M)^{0.5}\,\Phi \qquad [1]$$

where k is the isocratic retention factor, M is the molecular weight of the analyte, and Φ the fraction of organic modifier in the mobile phase; k_0 is the retention factor with no organic modifier in the mobile phase. As shown in Figure 2, this means that the retention of large molecules can change dramatically in response to even small changes in mobile-phase composition.

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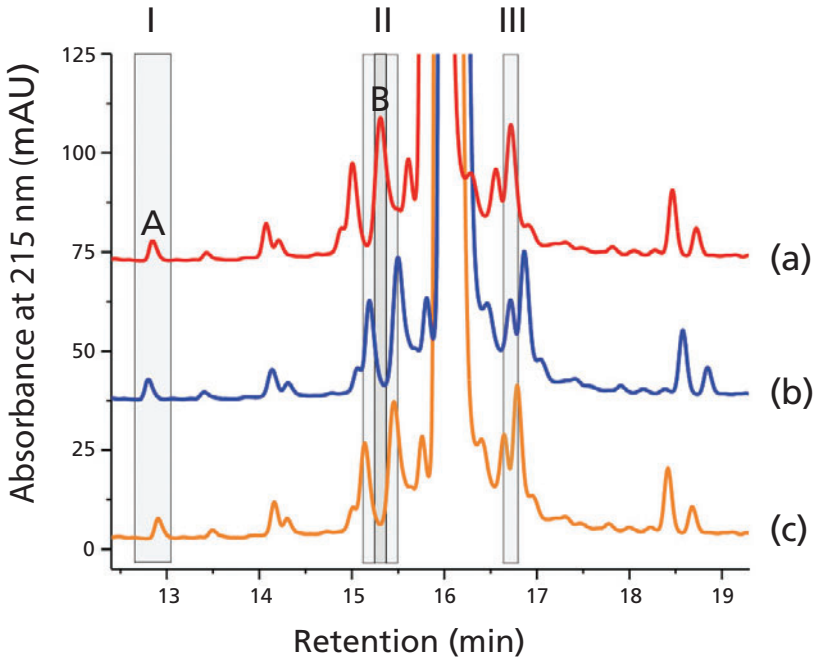
FIGURE 1: Representative 1D chromatograms from a series of injections of a ~10 kDa peptide and related impurities separated using a UHPLC instrument in good condition. (a) The trace with shortest main peak retention, (b) the trace with longest retention, and (c) the trace with median retention. Shaded area (I) illustrates a 120 µL cut to capture peak A based on trace (a). Shaded area (II) illustrates three adjacent 40 µL cuts to capture peak (B) based on trace (a). It also illustrates the problem of trying to capture a peak B in one 40 µL cut based on run (a) when there is retention variation; that is, the peak of interest is not captured in subsequent runs (b) and (c). (III) Depicts a typical 95% confidence interval for retention variation in this case corresponding to ±24 µL at 0.3 mL/min.



Thus, small variations in the composition of mobile phase delivered by the pump to the column can result in practically significant variations in retention time for large molecules that would not otherwise be noticed for small molecules.

Since a modification of one functional group in a large molecule can result in a relatively small change in physical and chemical properties of the molecule compared to the same modification of a small molecule, it is also more challenging to separate related impurities for large molecules. Consequently, large molecules require very shallow solvent gradients in order to separate related impurities by reversed-phase LC. In our work, we often have LC purity methods for peptides with a slope of about 0.2%/min at a flow of about 0.3 mL/min, and still chromatograms typically show a cluster of poorly separated impurities around the main peak, such as that shown in Figure 1.

One solution to the problem could be to use peak-based capture of cuts (that is, cuts defined by a change in UV signal slope, or rise above a threshold absorbance). This works nicely for well isolated peaks, but for peptide impurity analysis this approach is less viable due to the sample complexity and poor separation. Therefore, peptides and proteins are typically analyzed using time-based cuts.

In this "LC Troubleshooting" column, I present what I believe to be current best practice to address the problem with ^{1}D retention variation. The recommendations are based on four years of experience working with 2D-LC within the biopharmaceutical industry. Some of the recommendations are based on hard data, and some are less well founded.

> **It is important to use a high-pressure mixing pump (typically a binary pump) in the first dimension of a 2D-LC system to obtain the best possible retention stability.**

Strategies to Minimize Pump Related Contributions

In our experience, it is important to use a high-pressure mixing pump (typically a binary pump) in the first dimension of a 2D-LC system to obtain the best possible retention stability. In a recent head-to-head comparison of state-of-the-art ultrahigh-pressure liquid chromatography (UHPLC) instruments conducted in our laboratories, we found that a low pressure mixing pump (typically a quaternary pump) gave 2.4-times higher retention variation than a high pressure mixing pump from the same vendor (four systems of each type were tested on two occasions over a period of six months). Another conclusion from this work was that high pressure mixing pumps from three different vendors produced results with very similar retention variation. For a 6 kDa peptide and an LC system in good condition, the retention variation was approximately ±0.2% relative standard deviation (RSD) for a 0.15%/min reversed-phase LC

gradient at 0.3 mL/min (four systems of each type tested on two occasions over a period of six months).

It is also important to have sufficient re-equilibration time between gradients. In the previously mentioned head-to-head comparison of UHPLC instruments, we found that the retention variation could be reduced by a factor of three by simply increasing the re-equilibration time to match the flush-out volume of the system; that is, the volume it takes for the mobile phase composition delivered to the column inlet to reach steady state after a programmed step change in composition. To determine the mixing characteristics of the ^{1}D system and thereby define a sufficient re-equilibration time, we use the approach illustrated in Figure 3 and the following equation:

FIGURE 2: Isocratic retention factor versus amount of organic modifier for molecules of different sizes. All molecules are assumed to have strong retention in totally aqueous conditions—that is, logk_0 assumed to be 5, as in equation 1.

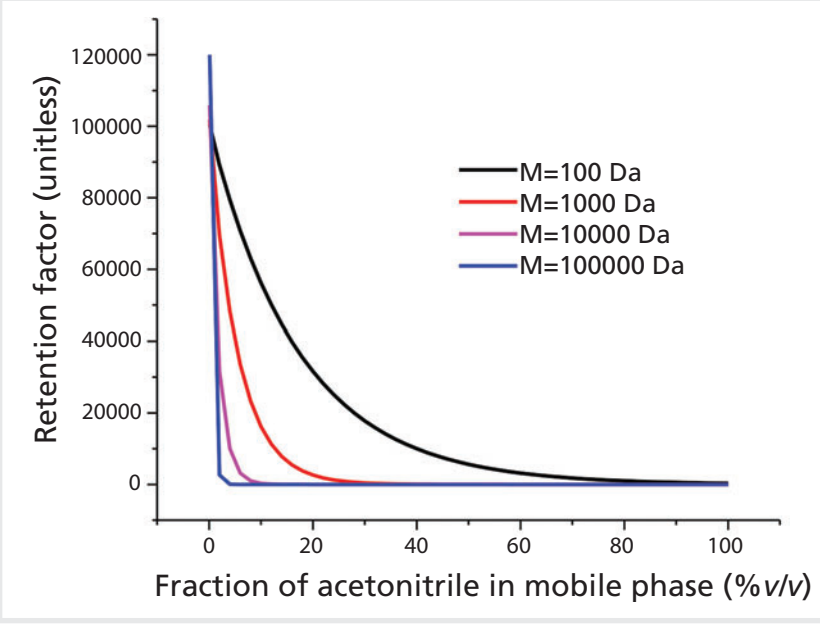
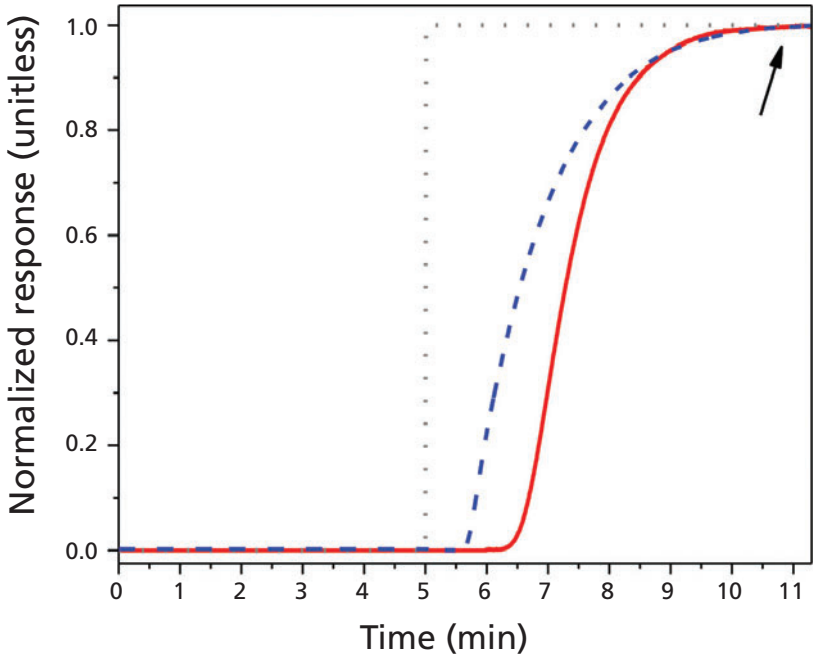


FIGURE 3: Determination of flush-out volume and subsequently a suitable re-equilibration time based on a step gradient with a UV-absorbing compound in channel B after replacing the column with a restrictor capillary. Chromatographic conditions: column replaced with 100 cm of 0.13 mm tubing; injection of 1 µL of water; flow rate, 0.3 mL/min; A-solvent is water, and B-solvent is 10 mg/L of uracil in water; mobile-phase composition program - 10% B from 0 to 5 min, then 15% B from 5.01 to 20 min (dotted line); detection at 253 nm and 20 Hz. Flush-out times for the two different systems characterized in this example were both determined to 10.8 – 5.0 = 5.8 min corresponding to a flush-out volume of 1.7 mL (solid line = LC system X and dashed line = LC system Y).



$$t_{eq} = (5\ V_m + V_f)/F \quad [2]$$

where t_{eq} is re-equilibration time, V_m is column dead volume, V_f is flush-out volume, and F is flow rate. For the high-pressure mixing UHPLC systems equipped with trifluoroacetic acid (TFA) mixers from three different manufacturers that we have characterized, we find that $V_f \leq 1.7$ mL. Knowing this and that V_m is approximately 0.3 mL for a 150 × 2.1-mm reversed-phase LC column, it is possible to estimate a re-equilibration time suitable for current UHPLC systems.

If the pumping system allows definition of solvent composition and compressibility for the mobile-phase components, it is recommended to set these according to the solvents in use to obtain best possible performance.

Depletion of the volatile organic modifier from pre-mixed mobile phases results in constantly increasing retention time over a sequence of injections. To minimize this, we avoid using organic modifier in the A-solvent during 2D-LC analysis. Removing water from the B-solvent would be beneficial; however, this is often impractical, due to solubility problems associated with mixing salt-based mobile phases and organic solvents.

Twenty years ago, Dolan and co-workers (7) addressed the problem with varying peptide retention in shallow high performance liquid chromatography (HPLC) gradients by increasing the amount of organic modifier in the A-solvent and decreasing it in the B-solvent while maintaining the slope of the gradient expressed as %acetonitrile/min. This results in an increase in %B/min, and should be less demanding because a larger volume has to be pumped to achieve a certain increase in %acetonitrile. The approach seems very logical, but when we evaluated it on four of our UHPLC systems, it did not significantly reduce the retention variation. It should be stressed, however, that we so far have only evaluated the approach for one peptide.

Another approach that we have evaluated is to scale the method for a wider column (8). The idea was that using a method with a higher flow rate would be less demanding to generate an accurate gradient. As with the previous approach, this would require a larger volume to be pumped to maintain the same %acetonitrile/min. The approach was evaluated using both 3- and 4.6-mm internal diameter (i.d.) columns, but no improvement in retention variation was observed for the four UHPLC systems evaluated.

Other considerations that promote high pump performance include the following:

- In order to obtain the best possible homogeneity of the mobile phase delivered to the ^{1}D column as well as reduce UV baseline noise we use large volume TFA mixers of approximately 400 µL.
- Ensure that the piston seal wash solution has been primed to wash and lubricate pistons.
- It is also helpful to monitor the pump pressure ripple and run a

leak test before analyzing real samples to spot pump related problems, such as a slightly leaking piston seal, air bubbles, or a malfunctioning check valve.

Strategies to Minimize Temperature-Related Contributions

Variations in ambient temperature may slightly influence the system operation. This becomes prominent when analyzing large molecules, whose retention is highly sensitive to changes in temperature as well as mobile-phase composition. It is therefore important to feed the pump with solvents at a consistent temperature. The density of the solvents going into the pump changes with temperature and therefore also the composition of the mobile phase coming out of the pump. The temperature consistency of the solvent at the pump inlet is affected both by the laboratory temperature and the temperature of the liquid in the bottle. For this reason, it is an advantage to have a stable ambient temperature. The temperature control in our laboratory is tight at ±0.6 °C (maximal deviation over 24 h). To maintain this temperature stability, it is helpful to avoid direct exposure of sunlight into the laboratory. Finally, we also place the mobile-phase bottles on the system the day before usage to allow thermal equilibration after preparation (considering the length of the tubing from the flask to the pump this is probably not necessary).

Heat of friction related to compression or decompression of the mobile phase affects the temperature in both the pump heads and in the column. This change of temperature depends on flow rate and pressure. Since the pressure changes during the gradient, there will always be a fluctuation in temperature over the gradient, but eventually some kind of repeating pattern from injection to injection will be established (9). In order to achieve this, we program a sequence of gradients. Once we observe a stable retention (typically after 2–3 gradients), we define where cuts should be taken and replace the method in subsequent sequence lines without interrupting the sequence. The time must be the same for all gradients in the sequence. In 2D-LC separations, the re-equilibration time for the ^{1}D separation often needs to be extended to account for the fact that the total analysis time for different separations will vary depending on the number of cuts made in each method. Thus, the total analysis should be adjusted to be consistent across different methods, independent of how many cuts are made. This approach with conditioning of the system also addresses other factors that influence retention, such as column

priming effects (that is, saturation of slowly equilibrating active sites on the column). Note that the conditioning needs to be repeated even if leaving the system pumping isocratically between sequences for a while.

To obtain a stable retention, it is of course also important to have an efficient column thermostat with pre-column heat exchanger configured for the flow rate to be used.

Strategies to Minimize Mobile Phase Related Contributions

Thorough mixing of the mobile phase during preparation should be applied to ensure the solution is homogeneous before connecting the bottle to the pump inlet. In practice, we continue to stir our solvents with a significant vortex for several minutes, even after any salts have been dissolved.

After changing solvents on the pump, the system is extensively primed. As a rule of thumb, we use five times the volume to be replaced in the sinker, tubing, and degasser (in total, ~30 mL depending on system). In order to refresh the system (for example, when it has been pumping at low flow overnight), we flush with five times the volume in the degasser (~10 mL) in order to compensate for changes in composition that may take place in the degasser (that is, loss of volatile additives like TFA or organic modifier if pre-mixed solvents are used).

To minimize problems related to evaporation of volatile solvent components from the mobile phase bottles, we also use caps with one-way valves that allow air to enter the flask, but do not allow vapour to exit.

Other Ways to Reduce the Problem with Varying Retention Specific to 2D-LC

For well-separated peaks, it is possible to use peak-based cuts—that is, a cut is triggered by a certain threshold or slope in the detector signal. In this mode of operation, small fluctuations in retention are not critical. However, as mentioned above, related impurities are usually poorly separated around the main component and therefore peak-based cuts are usually not helpful for peptides. For well-separated peaks, another alternative is to use a very wide time-based cut (Figure 1, cut I A). This does, however, usually require an on-line dilution with a weak solvent to focus the analyte at the head of the ^{2}D column (2).

For poorly separated peaks, another approach is to place two adjacent narrow cuts bracketing the peak of interest as shown in Figure 1, cut II. This increases the probability that the peak of interest (Figure 1[a], cut II B) is captured in one or other of the two cuts in a subsequent analysis (Figure 1[b] or 1[c], cut II).

Sometimes, it is also possible to reduce retention instability by increasing the slope of the gradient slightly. Determination of what constitutes an acceptable increase in slope is done by an iterative approach where the gradient range for the critical step (that is, change in %B) is increased during a few injections while monitoring resolution to ensure that selectivity and resolution do not change too much.

Summary

For large (bio)molecules, successful operation of current instrumentation for heart-cutting 2D-LC requires some precautions to minimize ^{1}D retention variation. However, this is a reasonable price to pay for the tremendous benefits of 2D-LC for some applications, such as the ability to obtain nearly real time LC–MS identifications of unknowns, even for ^{1}D separations involving salt-containing mobile phases. In this instalment of "LC Troubleshooting", I have presented some tips and tricks that I believe reduce or circumvent the problem. Some might also be applicable for conventional 1D-LC, if stable retention times are important to a particular application.

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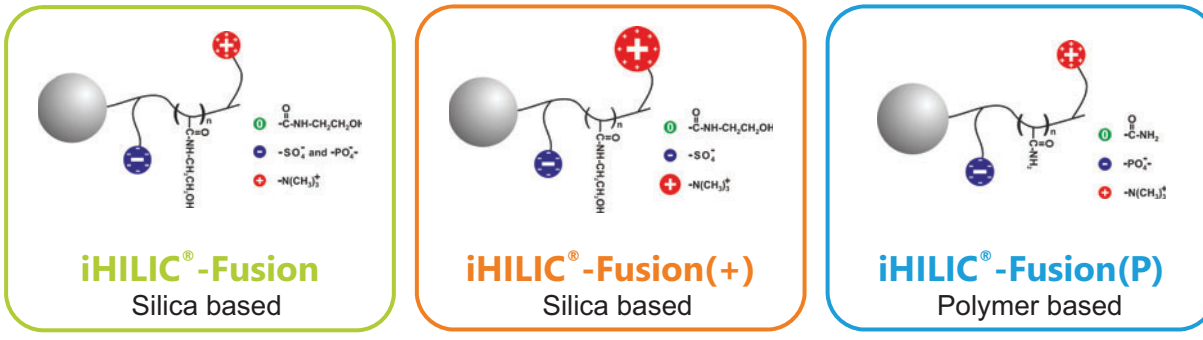

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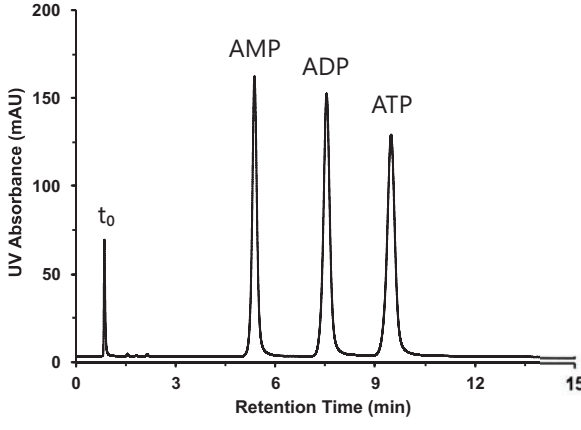

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Highlights from the HPLC 2019 Symposium

David S. Bell, Column Watch Editor

The 48th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2019), chaired by Alberto Cavazzini and Massimo Morbidelli, was held from 16 to 20 June in Milan, Italy. This instalment of "Column Watch" covers many of the highlights observed at the symposium. In addition, trends and perspectives on future developments in HPLC, and related techniques gleaned from the conference, are presented.

The 48th International Symposium on High Performance Liquid Phase Separations and Related Techniques, or HPLC 2019, was held in the beautiful city of Milan, Italy. This was the first time this important conference has convened in Italy. The conference primarily took place at the University of Milano-Bicocca and spanned 16–20 June 2019. The HPLC symposium, which continues to be the premier event bringing together leading scientists in the field of liquid chromatography (LC) and related techniques, attracted approximately 1200 delegates from numerous countries. The conference was co-chaired by Professor Alberto Cavazzini of the University of Ferrara and Professor Massimo Morbidelli of the Polytechnic Institute of Milan. The programme had a strong focus on encouraging contributions from young scientists, as well as promoting the more traditional fundamentals of separations science.

The conference included 308 oral presentations and 508 posters. Of special note were a couple of specific events geared towards young scientists, namely "Separation Science Slam" and "HPLC Tube" (see further discussions below), both of which were highly entertaining and well attended. The social programmes included a beautiful opening ceremony at the Milan Conservatorio and Gala Dinner at the Central Courtyard of the Università degli Studi di Milano. In this instalment of "Column Watch", observed highlights and trends from the conference are reported.

Highlights and Trends

In a similar fashion to previous HPLC review articles (1,2), several colleagues in attendance at the symposium were asked for their insights regarding the most interesting topics they observed at the event. Much of what follows is a synopsis of their responses along with some personal views.

In reviewing highlights from the past several symposia, multiple areas of interest stood out, including three-dimensional (3D) printing, advances in large-molecule separations, multidimensional separations, and hydrophilic interaction liquid chromatography (HILIC). The main 2019 symposium topics were largely similar to recent years. One notable difference, however, was the inclusion of dedicated sessions to thin-layer chromatography (TLC).

3D Printing and Microfabricated Structures

Three-dimensional printing and microfabricated structures continue to increase in interest. The 2019 programme was the first year that there was a session dedicated solely to 3D printing and microfabricated structures. As an illustration of the level of interest this topic generated, many attendees were willing to wait approximately 40 minutes in humid air and hot temperatures, due to an air conditioning issue, for the session to start. Simone Dimartino led off the session, speaking about the design of 3D-printed stationary phases (3). There were also a couple of presentations demonstrating the advantages of 3D printing by Peter Schoenmaker's group, who continue to actively investigate multidimensional separations (4,5). Along with new developments in column design through 3D printing, devices can be readily designed and tested, using the technique as shown by Jackie Sosnowski in the talk entitled, "3D Printing as a Flexible Tool to Customize Liquid Introduction with Electrospray Mass Spectrometry (6)." Dimartino also conducted a well-attended short course on 3D printing, which provided an overview of how 3D printing is influencing separation science. As noted in a recent review of 3D-printed stationary phases, the manufacture of highly efficient chromatographic columns is becoming a reality as 3D printers

become more affordable and accessible and their resolution, speed, and material flexibility continue to grow (7).

Large-Molecule Separations, Proteomics and Lipidomics

Large-molecule separations were again at centre stage during the 2019 conference. The importance of the topic was indicated by the high number of sessions dedicated to advances in the separation of proteins and related molecules. As noted in previous symposia reviews (1,2), many different modes of chromatography are necessary to fully characterize such complex molecules. Chen, for example, presented on the development of suitable columns based on sub-2-µm particles for size-exclusion chromatography (SEC), an invaluable technique used to assess molecular size variants of protein therapeutics (8). Chen described the importance of blending adequate pore volume, mechanical strength of particles, and bonding coverage when designing SEC phases in small-particle formats. The reader is referred to a recent *LCGC* article authored by Chen and colleagues for more details (9).

It was noted by several colleagues that alternative particle types are being used for large-molecule separations. Several talks were presented involving large-molecule separations using porous graphitic carbon (PGC) and polymeric supports. It was observed that these alternative supports are finding utility for "omics" studies that rely on resolving highly complex samples prior to analysis by mass spectrometry (MS). This trend has been apparent for a number of years where alternative supports that once found uses for small-molecule separations are now being applied to large molecule challenges. One observer noted that there seems to be a shift of emphasis from monoclonal antibody (mAb) and antibody–drug conjugate (ADC) analyte separations, and more towards bispecific antibodies (bsAbs) and fusion proteins, which are being proposed as new formats for biotherapeutics.

One "omics" talk that was identified as being particularly interesting was presented by Michael Lämmerhofer, future co-chair of the HPLC conference, to be held in Düsseldorf, Germany, in 2021. The talk entitled, "Lipidomics: A Window of Opportunity for Clinical Analysis", compared and contrasted a number of different approaches towards performing lipidomic studies (10). A focus of the talk centred on the benefits of both HILIC–MS/MS and reversed-phase MS/MS methods coupled to quadrupole time of flight (QTOF)-MS.

FIGURE 1: The Milan Cathedral (Duomo di Milano), the landmark for which the city of Milan is best known (Photo courtesy of the author).

FIGURE 2: Central Courtyard of the Università degli Studi di Milano (Photo courtesy of the author).

Multidimensional Chromatography

Multidimensional chromatography (MDLC) continues to be a hot topic. Sessions on two-dimensional and multidimensional separations were held each day, and on several days there were more than one. MDLC is finding its way in to a number of areas of application, including pharmaceutical analysis, indicating this is more than an academic exercise. Koen Sandra presented within a pharmaceutical session where he emphasized the utility of MDLC for biopharmaceutical analysis (11). Sandra noted many different approaches to MDLC, HILIC × reversed-phase, and SEC × reversed-phase, among others, as being important for characterization of large biopharmaceutical analytes.

Frederic Lynen presented an interesting talk on yet another combination of techniques: temperature responsive chromatography combined with reversed-phase LC (12). Lynen described recent efforts to prepare and utilize temperature responsive polymer stationary phases, such as poly(N-isopropylacrylamide) coupled to reversed-phase separations. One possible advantage of temperature responsive chromatography versus other forms is that the aqueous mobile phase employed in the temperature responsive dimension is generally a weak solvent for the second dimension, thus allowing for refocusing of the bands. Further information can be found in a recent article from the Lynen group (13).

Andre de Villiers presented on the topic of incorporating ion-mobility mass spectrometry (IM-MS) into one- and two-dimensional workflows for the analysis of complex sets of phenolic compounds in natural products (14). For the one-dimensional analysis, de Villiers combined ultrahigh-performance liquid chromatography (UHPLC) with electrospray ionization (ESI), IM-MS, and TOF-MS for the analysis of the ornamental flower *Protea*. The author noted that 41 phenolic acid esters, 25 flavonoid derivatives, and five anthocyanins were observed. During the presentation, de Villiers went on to add a second dimension of HILIC chromatography for the analysis of a variety of phenolic classes in chestnuts. The author noted that the incorporation of IM-MS can provide improved mass spectra quality, complementary separation, and

automation. Ion mobility continues to improve and many mobility techniques are being adopted for the successful separation of complex samples.

HILIC

HILIC was discussed in a number of different sections, including one session solely dedicated to the technique. Marti Roses of the University of Barcelona presented an interesting talk attempting to utilize the Abraham model, or linear solvation energy relationships (LSER), to predict retention and help elucidate HILIC retention mechanisms (15). The model was applied to a ZIC-pHiLIC column with acetonitrile–water and methanol–water mobile phases in the range 80–95% *v/v* of organic solvent. The study cast some light on the most important retention contributions to HILIC, but has not as yet been applied to ionizable solutes.

FIGURE 3: Conference best poster awardees (Photo courtesy of the author).

David McCalley presented on the often-discussed topic of re-equilibration in HILIC (16). Although full equilibration is considerably longer in HILIC versus reversed-phase chromatography, McCalley's work demonstrates that reproducible chromatography is obtained as long as re-equilibration times are controlled, even if full equilibration is not achieved. McCalley also discussed the impact of flow rate and temperature on equilibration rates in HILIC. The results indicate steps can be taken to optimize the process even when full equilibration is required. For more details, the

reader is referred to recent papers by the McCalley group (17,18).

As noted in last year's review of the HPLC meeting (2), HILIC is being applied to large-molecule analyses. Work in this area continues as demonstrated by Andrea Gargano of the University of Amsterdam in his work characterizing an enzyme used in bread production (19). Compared to reversed-phase chromatography, HILIC has found particular utility for the separation of intact protein glycoforms. The reader is referred to recent publications from Gargano and colleagues for further details (20,21).

Thin-Layer Chromatography

For the first time in memory, thin-layer chromatography (TLC) was the focus of not one but two full sessions. Both sessions focused on advanced TLC techniques utilizing modern, high performance TLC plates (often referred to as *HPTLC*). Modern plates are characterized as being relatively fast, providing excellent resolution due to the sharp bands produced, and of higher throughput due to lower dispersion (compared to, for instance, traditional 20 cm × 20 cm silica gel plates). The presentations demonstrated continued interest in utilizing HPTLC for studying a wide variety of natural products, several of which were focused on studying bioactivity of components. Another area of interest lies in the coupling of HPTLC with MS to aid in identifying active molecules in complex samples.

Focus on Young Scientists

Most notable in all the impressions received from colleagues regarding the 2019 programme was that the symposium was highly geared towards young scientists. The 20-minute oral format allowed for the inclusion of many talks during the allotted time, providing many opportunities for young investigators to present. Mariosimone Zoccali, a young scientist from the University of Messina, presented on carbon dioxide extraction and subsequent separation techniques for the analysis of a complex set of carotenoids and derivatives (22). Zoccali explained the steps of supercritical fluid extraction (SFE) in detail, and then went on to demonstrate that SFE coupled to supercritical fluid chromatography (SFC) and triple-quadrupole MS could reduce extraction time, provide faster run times, reduce the potential for sample contamination, and improve precision as compared to published methods. Zoccali went on to note that the developed method allowed the observance of previously never reported apocarotenoids in human blood and colostrum. Omar Ismail of the University of Ferrara gave another notable young investigator presentation on the topic of zwitterionic teicoplanin-based chiral selectors (23). The study centred on kinetic and thermodynamic comparisons of the chiral selector bonded to core–shell silica supports of varying particle sizes (2.0-, 2.7-, and 3.4-µm) and pore sizes (90, 160, and 400 Å). Ismail concluded that the 2.7-µm, 160 Å support provided the greatest overall performance.

In addition to the excellent talks from the young scientists, two innovative evening programmes, the "Separation Science Slam" and the "HPLC Tube" contests, provided young scientists with an alternative means of communicating their research in a fun and creative manner. The "Separation Science Slam" asked young scientists to engage in a competition to creatively inform the audience about their research projects in a manner of their choice. From inspiring videos to, let us say, "interesting" raps, the audience was indeed entertained and inspired. The "HPLC Tube" contest provided young participants the opportunity to create a 3-minute video to communicate their research. Many of the videos were highly enjoyable, and exhibited ingenuity in delivering their message. Both of the sessions were very well attended. Many young scientists were also included in the organizing committee and as session co-chairs for the conference. The consensus from discussions with colleagues on the topic is that the engagement of young scientists in the programme is important, and that the co-chairs of HPLC 2019 set the bar very high in this regard.

Poster Sessions

Posters represent a significant part of the HPLC symposia. In 2019, there were 508 submitted posters, separated into 14 scientific themes and six poster sessions. Agilent, once again, sponsored the "Best Poster Awards" that are graded by a group of expert reviewers for novelty, quality, presentation, and impact of the work.

Conclusions

HPLC 2019 was a lively symposium that engaged researchers interested in analytical science from around the globe. Most of the "hot topics" noted in recent symposium reviews remained the same at this year's conference. Three-dimensional printing, multidimensional chromatography, large-molecule separations, and specific techniques such as HILIC continue to dominate discussions. The 2019 programme also included two sessions focused on TLC.

The most significant impact from HPLC 2019 may be the focus of engaging our young scientists. From innovative programmes such as the HPLC tube to the inclusion of many young investigators in the organizational committee, the conference set a new and hopefully continuing precedence for encouraging, inspiring, and engaging our talented young scientists. Based on the success of HPLC 2019, I suspect this is not the last time we will see Italy host this important conference.

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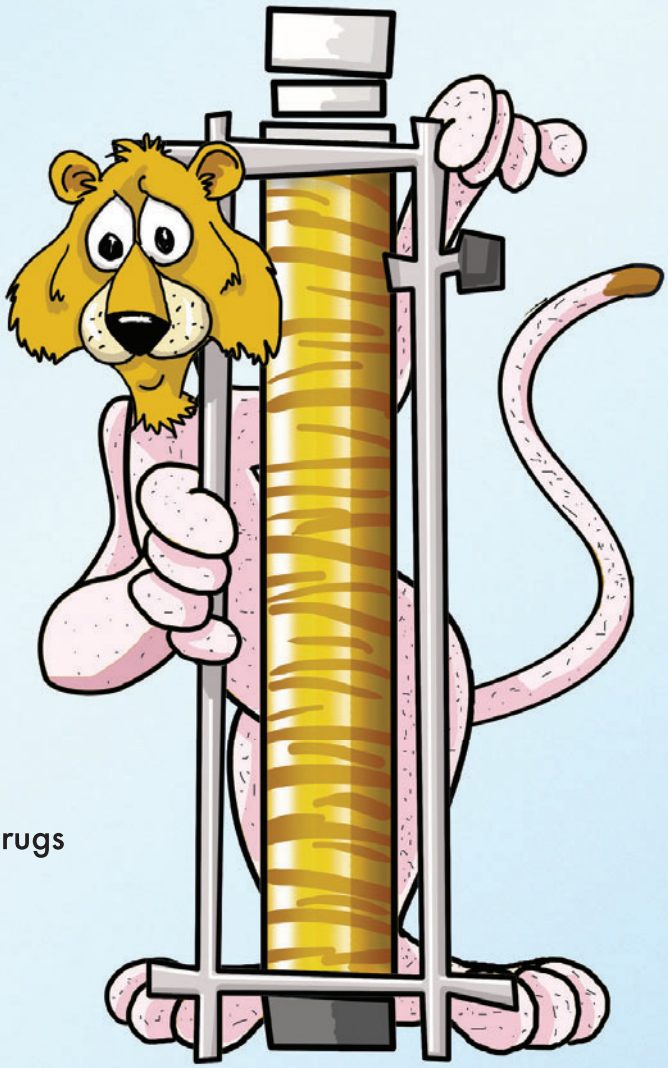

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Host Cell Protein Monitoring During Downstream Processing Using Micro-Pillar Array Columns Combined with Mass Spectrometry

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Protein biopharmaceuticals have substantially reshaped the pharmaceutical market and today over 350 products have been approved for human use in the United States and the European Union. Protein biopharmaceuticals are commonly produced recombinantly in mammalian, yeast, or bacterial expression systems. Next to the therapeutic protein, these cells produce endogenous host cell proteins (HCPs) that can contaminate the biopharmaceutical product despite multiple purification steps in a process. Since these process-related impurities can affect product safety and efficacy, they need to be closely monitored. Enzyme-linked immunosorbent assays (ELISA) are recognized as the gold standard for measuring HCPs because of their high sensitivity and high throughput. Mass spectrometry (MS), however, is gaining acceptance as an alternative and complementary technology for HCP characterization. This article reports on the use of micro-pillar array columns combined with MS for the characterization and in-depth monitoring of HCPs during downstream processing.

In contrast to small molecule drugs, which are commonly synthesized by chemical means, protein biopharmaceuticals result from recombinant expression in bacterial, yeast, and mammalian cells. As a consequence, the biotherapeutic is co-expressed with hundreds of host cell proteins (HCPs) with different physicochemical properties present in a wide dynamic concentration range. During downstream processing, the levels of HCPs are substantially reduced to a point considered acceptable to regulatory authorities (typically < 100 ppm – ng HCP/mg product). These process-related impurities are considered as critical quality attributes because they might induce an immune response, cause adjuvant activity, exert a direct biological activity (for example, cytokines) or act on the therapeutic itself (for example, proteases) or excipients (for example, phospholipase on co-formulated Tween) (1,2,3).

Multicomponent enzyme-linked immunosorbent assays (ELISA) is currently the workhorse method for HCP testing because of its high throughput, sensitivity, and selectivity (1). Polyclonal antibodies used in the test are typically generated by the immunization of animals with an appropriate preparation derived from the production cell minus the product-coding gene. However, ELISA does not comprehensively recognize all HCP species, that is, it cannot detect HCPs to which no antibody was raised, it only provides information on the total amount of HCPs without providing insight into individual HCPs and, in a multicomponent setup, it has poor quantitation power. In that respect, mass spectrometry (MS) nicely complements ELISA because it can provide both qualitative and quantitative information on individual HCPs. In recent years, various papers have appeared dealing with the mass spectrometric analysis of HCPs (4–23). These studies typically rely on bottom-up proteomic approaches in which peptides derived from the protein following proteolytic digestion are handled. Evidently, one is confronted with an enormous complexity and dynamic range and the separation space is dominated by peptides derived from the therapeutic protein. For successful HCP analysis, there is clearly a need for highly efficient up-front MS separations. In that

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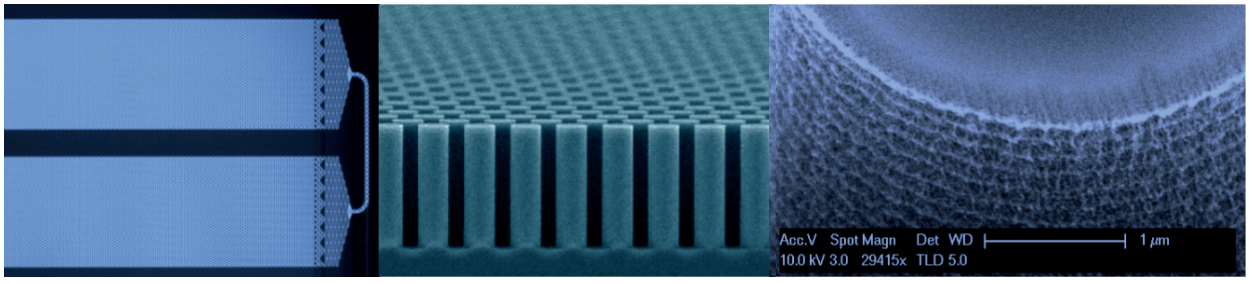
FIGURE 1: Micro-pillar array column. From left to right: (left) Top view of two parallel 315 µm wide separation channels that have been interconnected with proprietary flow distributor structures, (middle) scanning electron microscope (SEM) image showing a transverse section of a separation channel containing 5 µm diameter cylindrical pillars, (right) high resolution (HR)-SEM image of the 300 nm porous-shell layer incorporated into a 5 µm diameter pillar.



respect, micro-pillar array columns are highly promising. The origin of this technology dates back to the late 1990s when Regnier *et al.* addressed the problem of miniaturizing capillary electrochromatography (CEC) columns and introduced microfabricated supports as an alternative for the conventional packed beds (24,25). The theoretical benefit (reduction of the van Deemter A-term) of such supports was elucidated only a few years later by Knox (26). In the years to follow, Desmet *et al.* conducted several quantitative studies on Knox's argument, taking a column filled with an array of pillars as a representative example (27). Finally, in 2007, the first proof-of-concept of micromachined liquid chromatography (LC) columns operated by pressure-driven liquid flow, later termed *micro-pillar array columns* was reported (28). The inherent high permeability and low "on-column" dispersion obtained by the "perfect order" of the separation bed makes micro-pillar array column-based chromatography unique and offers several advantages compared to conventional column technologies (packed beds and monoliths). The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated (sharp peaks) during separation. The freestanding nature of the pillars also leads to much lower back pressure, which permits the use of very long columns. These properties result in excellent chromatographic performance with high resolution and high sensitivity. This article reports on the use of micro-pillar array columns combined with MS for the characterization of HCPs and their monitoring during downstream processing.

Materials and Methods

Materials: Water and acetonitrile were purchased from Biosolve. Formic acid (FA), trifluoroacetic acid (TFA), dithiothreitol (DTT), and 2-iodoacetamide (IAA) were from Sigma-Aldrich. Tris-HCl pH 7.5 was purchased as a 1 M solution from Thermo Fisher Scientific. Porcine sequencing-grade modified trypsin was acquired from Promega. Rapigest, MassPREP enolase (ENL), bovine serum albumin (BSA), alcohol dehydrogenase (ADH), and phosphorylase b (PHO) digestion standards were purchased from Waters. Downstream process samples were obtained from a local biotechnology company.

Sample Preparation: To a volume corresponding to 100 µg of protein, 105 µL of 0.1% Rapigest in 100 mM Tris-HCl pH 7.5 was added followed by the addition of 100 mM Tris-HCl pH 7.5 to a final volume of 192.5 µL. The sample was subsequently reduced at 60 °C for 30 min by the addition of 5 mM DTT (2.5 µL of 400 mM DTT in 100 mM Tris-HCl) and alkylated at 37 °C for 1 h by adding 10 mM IAA (5 µL of 400 mM IAA in 100 mM Tris-HCl). Digestion proceeded for 16 h at 37 °C using trypsin as protease added at an enzyme to substrate ratio of 1:25 (*w/w*). Lyophilized trypsin (20 µg) dissolved in 100 mM Tris-HCl (50 µL) was added in a volume of 10 µL giving rise to a final sample volume of 210 µL. Rapigest removal was achieved by adding TFA to a final concentration of 0.5%. Following incubation for 30 min at 37 °C, the sample was centrifuged for 10 min at 13,000 rpm and the supernatant was carefully transferred to an autosampler vial. MassPREP ADH, PHO, BSA, and ENL digestion standards were added at a concentration of 1000 fmol, 100 fmol, 50 fmol, and 10 fmol / 100 µg therapeutic, respectively.

LC–MS: An Ultimate 3000 RSLCnano system (Thermo Scientific) was used for LC–MS measurements. Tryptic

TABLE 1: Metrics obtained in the different downstream process samples

Sample	mAb Sequence Coverage (%)	Protein Groups (≥1PSM)	Protein Groups (≥2PSM)	Total Peptide Groups	Total PSMs	MS/MS Spectra
Harvest	97	2444	**1821**	12,731	16,560	68,695
Purification Step 1	100	39	**10**	133	680	45,723
Purification Step 2	99	24	**5**	115	660	44,299
Purification Step 3	100	11	**2**	76	581	44,793

digests were analyzed on a 200 cm C18 µPAC column (PharmaFluidics) at 50 °C. Samples were loaded on a 1 cm C18 µPAC trapping column (PharmaFluidics) using 0.1% TFA in 1:99 acetonitrile–H_2O (*v/v*) at a flow rate of 10 µL/min. Elution was carried out with a gradient of (A) 0.1% FA in H_2O and (B) 0.1% FA in 80:10 acetonitrile–H_2O (*v/v*) from 1% B to 30% B in the first 115 min and from 30% B to 45%B in the following 11 min. The flow rate was 750 nL/min from 0 to 10 min and 500 nL/min from 10 min to 150 min. An injection program allowed the introduction of 4 µL of sample in between two plugs of loading buffer. Loop size was 20 µL and samples were kept at 10 °C in the autosampler tray while waiting for injection.

High-resolution accurate mass measurements were performed on a Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) equipped with a Nanospray Flex source (Thermo Scientific). The micro-pillar array column was connected via a 20 µm internal diameter (i.d.)/360 µm outside diameter (o.d.) fused-silica capillary to a PicoTip emitter (10 µm tip i.d. – New Objective) via a 50 µm bore stainless steel union (C360UFS2 360 µm union from VICI AG International) and true no twist one-piece PEEK fittings (C360NFPK 360 µm fittings for fused-silica tubing from VICI AG International). Spray voltage was set at +2.0 kV, the capillary temperature was 275 °C, and a S-lens RF level of 40 was used. The Q Exactive HF-X was operated in DDA (data dependent acquisition) mode, where one cycle consisted of a MS1 survey scan followed by TopN MS/MS scans. MS1 spectra were collected in centroid mode in a scan range from 380 *m/z* to 1500 *m/z* at a resolution of 120,000 at *m/z* 200. Automatic gain control (AGC) parameters included 3e6 target value and maximum injection time of 100 ms. The ten most abundant multiply-charged precursors were selected for fragmentation by higher-energy collisional dissociation (HCD) (28% NCE). MS/MS spectra were acquired at 15,000 resolution at *m/z* 200 and MS2 AGC parameters included target of 5e4 and maximum injection time of 200 ms. The precursor isolation window was set to 1.2 Th and dynamic exclusion was set to 90 s.

Protein Identification and Quantification: Data files were processed using Proteome Discoverer (v2.3) (Thermo Scientific). Spectra were searched against protein sequences consisting of CHO-K1 protein database (08/24/2014) (downloaded from www.chogenome.org), therapeutic mAb fragment, and ADH, PHO, BSA, and ENL using the Sequest HT search engine. Search parameters

FIGURE 2: Bar plots showing the number of HCPs identified in function of the number of uniquely identified peptides in the different downstream process samples.

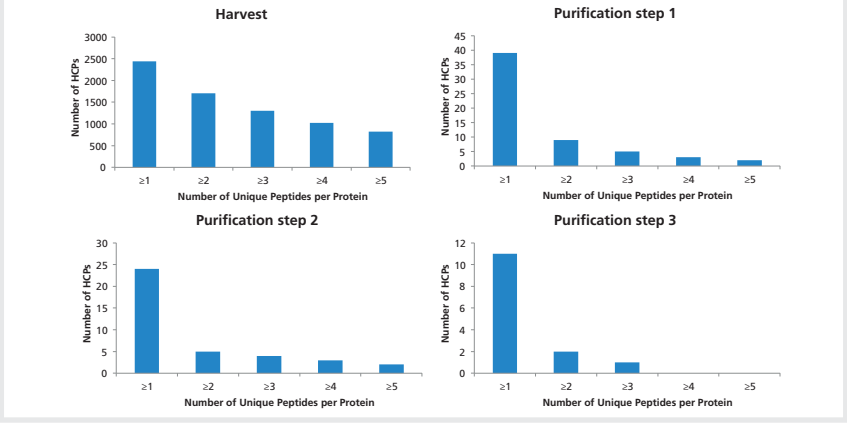
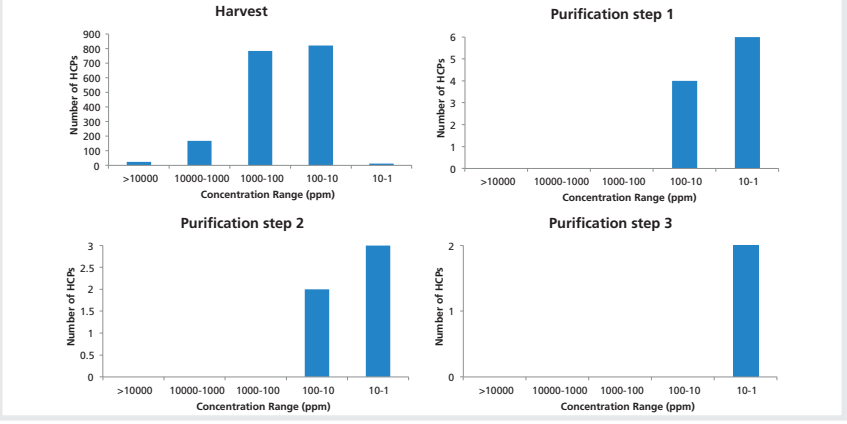


FIGURE 3: Bar plots showing the number of HCPs detected at a given concentration in the different samples.



were precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.02 Da, carbamidomethylation of cysteine was selected as a fixed modification, and a maximum of two trypsin missed cleavages were allowed. False discovery rate (FDR) targets were set to 0.01 (99%) and 0.05 (95%) as strict and relaxed, respectively, calculated by the target decoy peptide spectral match (PSM) validator node. Keratin and trypsin peptides and proteins were excluded from the final list.

Protein abundances, calculated from the average of the three most abundant unique peptides, were obtained via the Precursor Ions Quantifier node in the Proteome Discoverer software.

Results and Discussion

The separation beds of micro-pillar array columns are fabricated by etching the interstitial volumes out of a silicon substrate following lithographic definition of an array of pillars. This creates a stationary phase support structure that is organized in a reproducible and ordered pattern. Concatenation of several of these channels allows long column lengths to be fabricated on a small footprint (29). The most important characteristics of the micro-pillar array separation bed design are: pillar diameter, 5 µm; inter pillar distance, 2.5 µm; pillar height or bed depth, 18 µm; external porosity ($V_{interstitial}/V_{total}$), 59%; bed channel width, 315 µm; and bed length, 200 cm. To increase the retentive surface, the pillars are rendered superficially porous with a typical porous shell thickness of 300 nm and pore sizes in the nanometre range. The porous surface has been uniformly modified with octadecyl chains to create a hydrophobic stationary phase suited for reversed-phase LC separations. Figure 1 shows some relevant characteristics of the micro-pillar array column.

Because of the high permeability, the 200 cm column used in this study can be operated at moderate LC pump pressures (50 to 300 bar) over a wide range of flow rates (100–1000 nL/min). Van Deemter measurements with heptyl-phenyl ketone demonstrated that a total of 400,000 theoretical plates could be generated at the optimal linear solvent velocity, corresponding to a flow rate of 200–250 nL/min and generating a column back pressure of only 70 bar.

In this study, micro-pillar array columns were used in combination with a hybrid quadrupole-orbital trap MS system for the characterization of HCPs throughout the downstream processing of a therapeutic mAb fragment recombinantly expressed in Chinese Hamster Ovary (CHO) cells. Proteinaceous samples collected at different purification steps were reduced using DTT and alkylated using IAA prior to overnight trypsin digestion. Two µg of digested sample, spiked with four pre-digested protein calibrants (ADH at 10 fmol/µg, PHO at 1 fmol/µg, BSA at 0.5 fmol/µg, and ENL at 0.1 fmol/µg), was subsequently loaded (via a pillar-based pre-column) onto a 200 cm long micro-pillar array column. Peptides were separated

using a 116 min gradient and the MS system was operated in DDA mode. Data were subsequently searched against the CHO database extended with the sequence of the mAb fragment and the four protein calibrants (ADH, PHO, BSA, and ENL).

Table 1 shows some important metrics obtained on the downstream process samples. In the harvest sample, close to 70,000 MS/MS spectra were obtained, and, following database searching, 12,731 unique peptides could be identified corresponding to 2444 protein groups with at least one PSM and 1812 proteins with more than one PSM. A nice clearance of these proteins throughout the purification process was observed and in the last purification step the number of HCPs could be reduced to two. Note that in the table, the mAb fragment, ADH, PHO, BSA, ENL, as well as keratin and trypsin, are excluded from the protein groups columns. Figure 2 plots the number of HCP protein identifications by function of the number of uniquely identified peptides. In the harvest sample, a substantial number of HCPs are identified with more than one peptide. In the downstream purification samples, one-hit wonders start to dominate as a result of the much lower abundance of HCPs.

Semiquantitation of the HCPs identified with more than one PSM was achieved by spiking pre-digested proteins (ADH, PHO, BSA, and ENL) at known concentrations in every sample. The average intensity of the three most intense peptides for each protein calibrant was plotted in function of column load (expressed in fmole), and the slope of the resulting calibration curve (counts/fmole) was applied for semiquantitation of the HCPs in the corresponding sample. This is an adaptation of the procedure described by Silva *et al.* (30) and commonly used in HCP quantitation. The average slope measured in the different downstream samples was 15,918,593 counts/fmole with a relative standard deviation (RSD) of 14.1%. This precision allows for a good comparison in HCP quantity throughout the process. Upon back-calculating the concentration of the spiked protein digests (ADH, PHO, BSA, ENL) in every sample using the corresponding slope, an accuracy well between 50% and 200% was obtained, which is more than acceptable for semiquantitation. Figure 3 plots the number of HCPs detected at a given concentration in the different samples.

FIGURE 4: Evolution of CHO HCPs clusterin and phospholipid transfer protein isoform X2 throughout the process.

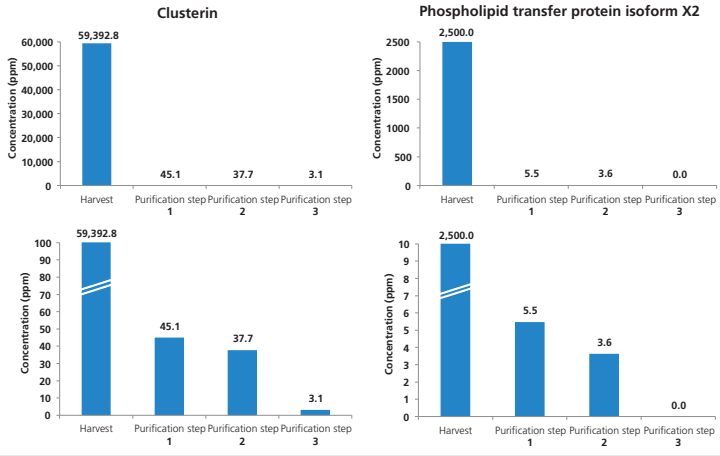
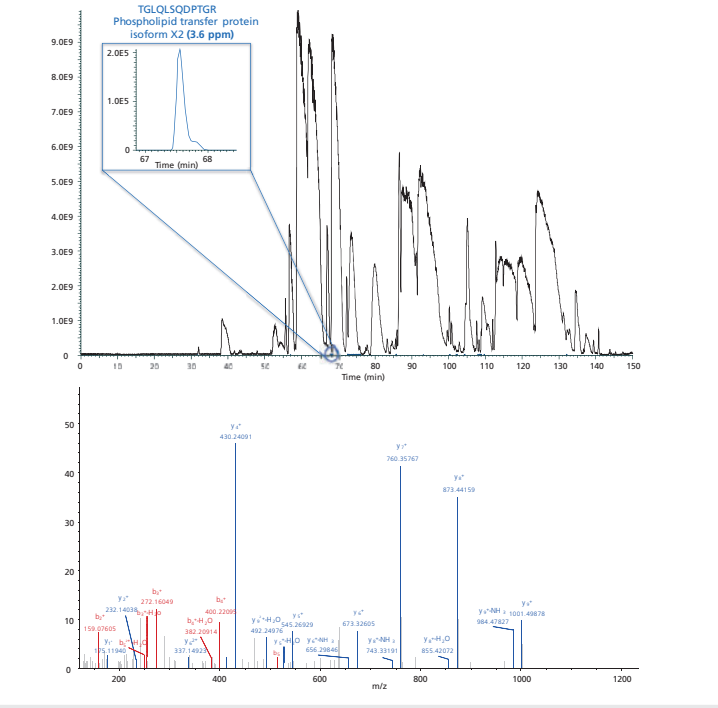


FIGURE 5: Base peak chromatogram, extracted ion chromatogram, and MS/MS spectrum of peptide TGLQLSQDPTGR corresponding to the phospholipid transfer protein isoform X2 peptide detected at 3.6 ppm in purification step 2.



The bar plot presented in Figure 4 follows two specific HCPs throughout the process. Clusterin, for example, is present at 59,393 ppm in the harvest but is reduced to 3.1 ppm in the final purification step. Clusterin can easily associate in a nonspecific manner with both the Fc and Fab fragments of antibodies (2,31). Phospholipid transfer protein isoform X2 is present at 2500 ppm in the harvest and reaches 0 ppm in the final purification step. Figure 5 shows the extracted ion chromatogram of one of the phospholipid transfer protein isoform X2 peptides detected at 3.6 ppm overlaid with the base peak chromatogram. This figure illustrates the large protein dynamic range within the sample, one of the major challenges in HCP characterization. This particular HCP was present at low ppm levels, however, high-quality MS/MS data could be obtained allowing its confident identification. When surveying the base peak chromatogram, it becomes clear that the column is overloaded at 2 µg. The broad peaks observed can all be traced back to the therapeutic antibody. Peptides originating from the HCPs on the other hand give nice Gaussian and sharp peaks.

Conclusion

The method described is useful for the characterization of HCPs and their monitoring throughout downstream processing. Downstream process samples were directly digested following reduction and alkylation and the resulting peptides separated using one-dimensional (1D) LC. The MS system was operated in data dependent mode. HCP content and sensitivity can potentially be further improved by implementing a second chromatographic dimension (two-dimensional [2D]-LC) (5,12), by optimizing sample preparation by depletion or HCP-directed digestion (14,17), by MS-based exclusion of precursors originating from the therapeutic itself, or by operating the MS system in data independent mode (17).

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Petroleum Pollution Analysis with Ramped Pyrolysis GC–MS

***LCGC Europe* spoke to Zhanfei Liu from the Marine Science Institute at the University of Texas at Austin, Texas USA, about the advantages of ramped pyrolysis GC–MS to analyze petroleum pollution from the Deepwater Horizon oil spill.**

Interview by Alasdair Matheson, Editor-in-Chief, *LCGC Europe*

Q. You recently developed a method using ramped pyrolysis-gas chromatography–mass (Py-GC–MS) spectrometry to analyze petroleum pollution related to the Deepwater Horizon oil spill (1). How did this project arise?

A: Understanding the chemical composition of oil residues is essential for the oil-spill community, response team, and decision makers because the chemistry is related to evaluating toxicity of oil in environments and developing the appropriate response and remediation strategies. The traditional analytical tools for hydrocarbon analysis, mainly GC–flame ionization detection (FID) and gas chromatography mass spectrometry (GC–MS), are rugged in the quantitative perspective, and have long been applied in oil spill research. Unfortunately, GC–FID and GC–MS can only quantify a small fraction of hydrocarbons in crude oil, not to mention oil residues on a molecular level. Take the Deepwater Horizon oil as an example, only less than 25% of the crude is GC amenable (2). This fraction dramatically decreases with weathering in environments by processes such as biodegradation and photooxidation, forming unresolved complex mixture (UCM) as frequently observed in GC–FID spectra. Advanced mass spectrometry, such as Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), offers superb resolving power and greatly expands the analytical window to include a variety of polar compounds formed during the oil weathering, such as oxygenated hydrocarbons (2,3). However, this technique is not quantitative, and samples need to be in a solution before the analysis, which may not always be possible for highly weathered oil, such as those hardened tars often found along beaches. To further oil spill science, therefore, there is a need to develop new analytical techniques to expand the analytical window for crude oil and residues. My laboratory has been developing and applying the Py-GC–MS technique after the Deepwater Horizon oil spill, funded by the Gulf of Mexico Research Initiative through the DROPPS (Dispersion Research on Oil: Physics & Plankton Studies) consortium.

Q. Why did you decide to use ramped pyrolysis-gas chromatography–mass spectrometry? What advantages does it offer compared to other techniques?

A: Ramped Py-GC–MS offers a unique angle to interrogate the chemical composition of oil and oil residues, providing many advantages that other techniques cannot offer, from sample pretreatment to expanded analytical window. For example, the

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only pretreatment needed for an oil residue sample is freeze-drying. The sample can then be directly pyrolyzed. In comparison, the steps for regular hydrocarbon analysis in an oil residue are tedious and time-consuming, and include freeze-drying, extraction using organic solvent, oil fractionation using chromatographic columns, and condensation using a rotary evaporator before GC–FID or GC–MS analysis. If relatively low temperatures, less than 300 °C, are used in the pyrolyzer the same quantitative data on hydrocarbons, including steranes and hopanes, would be obtained with the GC–MS component as those from routine GC–MS analysis, but without the pretreatment. More importantly, Py-GC–MS can offer insights into chemical composition of highly weathered oil, such as the resin and asphaltene components, through pyrolyzing the samples at high temperatures, say 350–650 °C. The fragments produced under high temperature in the pyrolyzer can be analyzed through GC–MS analysis, and thus the chemical structures of highly weathered oil, analogous to putting the puzzle pieces together, can be pinpointed. For example, the high-temperature pyrolysis generated a large quantify of long-chain n-alkanes/1-alkenes in the Deepwater Horizon tar samples (1). This clearly confirms that the asphaltenes contain a high fraction of aliphatic components, and that the alkyl tails connected to aromatic centres are long. This type of molecular information in highly weathered oil cannot be obtained through any other techniques. Certainly, you can get a quantitative idea about carbon functionalities using nuclear magnetic resonance (NMR) or Fourier-transform infrared spectrometry (FT-IR), but not a molecular level of information. Another advantage is that the CO_2 peak in the GC–MS spectrum for a weathered oil sample can be seen, because during the cracking process the oxygen atom in weather oil will be released mainly in the form of CO_2 (CO is another component, but minor). Therefore, based on the peak area of CO_2, the amount of oxygen in a highly weathered oil residue can be estimated.

Q. You used both bulk pyrolysis and thermal splicing pyrolysis in this project. Can you explain these two approaches and what they were used to investigate?

A: In this technique there are basically two modes you can use: bulk pyrolysis and thermal slicing pyrolysis. In the bulk pyrolysis, a very short GC column,

2.5 m, and a ramped temperature programme for pyrolysis are used, so individual compounds cannot be separated. As such, a bulk pyrogram is obtained with temperature against ion intensity. The bulk pyrogram offers a general idea of the distribution of pyrolysates with temperature. For all the oil samples we have analyzed, including crude and residues, there are basically two "humps" in the pyrogram, one at the low temperature range from 100–300 °C, and the other at 300–600 °C. This is expected as the low temperature hump represents the volatile fraction of oil and the high temperature the weathered oil components such as asphaltenes. However, it turned out that the ratio of these two humps is proportional to the weathering degree of oil, based on the wet-chemical analysis. In other words, the hump ratio can be used to examine the extent of weathering to oil samples in an oil spill, as an easy and quick method. The bulk pyrogram can also be used to inform the thermal slicing pyrolysis, or the temperature ranges to be selected for pyrolysis on a molecular level.

Understanding the chemical composition of oil residues is essential for the oil-spill community, response team, and decision makers because the chemistry is related to evaluating toxicity of oil in environments and developing the appropriate response and remediation strategies.

In the thermal slicing mode, the pyrolysates are condensed in a cryotrap between the pyrolyzer and GC–MS system during the pyrolysis at a certain temperature range or slice. After the sample is lifted out of the furnace, the pyrolysates are heated, eluted through the GC column (30 m length), and on to the MS system. When this GC–MS run is done, the sample is re-dropped to the furnace and the process is repeated for a second slice, and on and on until all pre-selected slices are finished. At the end, you will get consecutive GC–MS spectra at different thermal slices. For the oil and oil residues, there are two distinct temperature zones in terms of the pyrolysis: thermal desorption and cracking zones. In the thermal desorption zone (50–370 °C), not surprisingly, there are essentially no cracking products detected, so all compounds in this zone belong to intact hydrocarbons. Individual hydrocarbons in this zone can be used for quantitative analysis, similar to those from traditional GC–FID and GC–MS analysis, but without the need for pretreatment. In addition, the thermal slicing data (which compounds occur in which thermal slice [or in what percentages]) can be used to describe the matrix effect of oil weathering in a semiquantitative way. It has been well documented that some intact hydrocarbons are sealed or encapsulated in oil matrix or asphaltenes to prevent further degradation (4), but this is difficult to quantify. Thermal slicing offers a way of doing just this, as one can convert the temperature data to activation energy following a recent computing method (5). We are still working on this. The cracking zone (370–650 °C) offers data on the small fragments from the cracking of large or polar hydrocarbons, and this data can be used to pinpoint the original structure of those components, which are otherwise difficult to analyze. For example, we found the pyrolysates in this zone are dominated by long-chained n-alkanes/1-alkenes as compared to small aromatics, confirming the highly aliphatic nature of the weathered Deepwater Horizon oil.

Ramped Py-GC–MS offers a unique angle to interrogate the chemical composition of oil and oil residues, providing many advantages that other techniques cannot offer.

Q. What were the main analytical challenges you had to overcome?

A: To quantify the hydrocarbons in the thermal desorption zone for oil residues, we needed to establish an external standard calibration curve. However, we found that the small hydrocarbon standards, such as n-alkanes C_8-C_{15}, could easily get lost when they are sitting in the pyrolysis cup before the pyrolysis as a result of evaporation (the cup is in a constant helium flow). We were struggling with this issue, but finally we were able to stabilize the small hydrocarbons using polystyrene as the matrix. Another aspect was how to deal with the data. Even though pyrolysis GC–MS has long been applied in many different fields including kerogens, it had not been applied to analyze crude and weathered oil. Thus, how to analyze the samples appropriately and how to interpret the data were challenging to us at the time. To conquer this, there was a lot of trial and error during sample analysis and a lot of background reading, particularly about kerogens.

Q. What is novel about this approach?

A: First of all, solid samples such as tars can be directly analyzed without the need for pretreatment. However,

I think the key novel aspect of this approach is that a single sample can be pyrolyzed in multiple slices in a consecutive way, and essentially we will obtain two-dimensional data, not only the specific pyrolysates but also at what temperature they are being released. The data are more enriched than those from other pyrolysis techniques such as the Curie point ones. For example, in terms of oil residue, this approach will allow us to differentiate what compounds are intact or bound in the matrix, and what are cracking products.

Q. What were your main findings?
A: There were several findings in this work. The bulk pyrolysis offers an easy and quick way of estimating the weathering degree of oil residues. From the thermal slicing pyrolysis, we found that certain free/intact hydrocarbons, particularly n-alkanes, were bound more tightly in oil residues as weathering proceeded. This is the first data to show the matrix effect in preserving hydrocarbons in weathered oil residues in a semiquantitative way. Using the cracking products or pyrolysates in the cracking zone, we found that the highly weathered Deepwater Horizon oil was dominated by aliphatic components, mainly long-chained alkyl tails, and that aromatic moieties, mostly small ones, represented a minor fraction. These data offer key insights into the structural composition of weathered oil after the Deepwater Horizon oil spill.

Q. Are you planning to develop this research further?
A: Yes, we are. We have been developing this approach further and have optimized the thermal slicing that is needed to calculate the binding energy for a given compound or compound class in the organic matrix. In addition to the oil work, we are working on other types of samples using this approach, such as dissolved and particulate organic matter in aquatic systems. We also upgraded our system from single to double GC, which would offer a new dimension of information, so stay tuned!

> **I think the key novel aspect of this approach is that a single sample can be pyrolyzed in multiple slices in a consecutive way.**

Q. Anything else you would like to add?
A: As an environmental chemist, I think it is very helpful and rewarding to have the mentality of developing or applying new approaches in our research, which can often provide unique ways of looking at the samples and produce breakthroughs to the fundamental questions we would like to solve.

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5) J.D. Hemingway, D.H. Rothman, S.Z. Rosengard, and V.V. Galy, *Biogeosciences* **14**, 5099–5114 (2017).

Flow Modulator

LECO has introduced a new Flux flow modulator option for routine GC×GC analysis. While LECO's traditional thermal modulation alternative is still available and provides high sensitivity, the Flux flow modulator is a cost-effective option that makes GC×GC more accessible and easy to use, according to the company. Another advantage of the Flux is that it does not require cryogens to perform GC×GC,which can save the user time and resources in the laboratory.

https://info.leco.com/flux-lcgc
LECO Corporation, Saint Joseph, Michigan, USA.

UHPLC system

The new Nexera UHPLC series LC-40 of Shimadzu was awarded a Red Dot Design Award 2019 in the product design category. By incorporating the internet of things (IoT) and artificial intelligence (AI), and various sensor technologies, the "Nexera Series" applies groundbreaking technology in terms of intelligence, efficiency, and design, according to the company.

www.shimadzu.eu
Shimadzu Europa GmbH, Duisburg, Germany.

Sampling Tubes

Markes' industry standard-sized thermal desorption tubes are manufactured to the highest quality, delivering optimum results time after time, according to the company. The complete range of tube materials and sorbent packings offers flexibility, making them suitable for VOC and SVOC analysis for all TD applications, including environmental air monitoring, fragrance analysis, and breath monitoring.

http://chem.markes.com/sampling-tubes
Markes International Ltd., Llantrisant, UK.

IEX Columns

YMC's porous and non-porous BioPro IEX columns are designed for reproducible separation of antibodies, proteins, peptides, nucleic acids, and oligonucleotides, showing high binding capacity and recovery, according to the company.

https://ymc.de/ymc-biopro-iex-143.html
YMC Europe GmbH, Dinslaken, Germany.

HILIC Columns

Hilicon offers a broad range of HILIC products to separate polar compounds. Three column chemistries in UHPLC and HPLC, iHILIC-Fusion, iHILIC-Fusion(+), and iHILIC-Fusion(P), provide customized and complementary selectivity, excellent durability, and very low column bleeding, according to the company. The columns are suitable for the analysis of polar compounds in "omics" research, food and beverage analysis, pharma discovery, and clinical diagnostics.

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

Chromatography Accessories

Action Europe offers bottles, closures, caps, seals, and syringe filters. Crimping and screwing bottles from 2 mL to 1 L. Closures and seals for all types of bottles and caps. Caps, aluminium standard caps, flip-off caps, flip tear-off caps, flip tear-up caps, and magnetic caps are available. Electric crimping machines are also available. Samples and catalogue are available from the company.

www.sertir.fr
Action Europe, Sausheim, France.

FID Gas Station

The VICI FID gas station combines the reliability of the VICI DBS hydrogen and zero-air generators into one compact and convenient package, according to the company. Available in high- and ultrahigh purity for all GC detector and carrier gas applications. The generator is available in two styles: flat for placement under a GC, or the Tower. Available in H_2 flow ranges up to 1 L/min and 10.5 bar.

www.vicidbs.com
VICI AG International, Schenkon, Switzerland.

Asymmetric Flow Field-Flow Fractionation

Asymmetric flow field-flow fractionation (AF4) is an advanced technique for achieving analytical and semi-preparative separations. Wyatt Technology's Eclipse instruments cover range of sizes from 1 nm to 10 µm. Eclipse AF4 systems allow separation to occur without shear or adverse, non-ideal column interactions.

www.wyatt.com/separation
Wyatt Technology, Santa Barbara, California, USA.

Olfactory Detection Port for GC

The ODP 4 from Gerstel has a heated mixing chamber and is highly inert, resulting in good recovery and sensitive olfactory detection even for high-boiling and polar compounds, according to the company. The detection port is easily positioned for optimized ergonomics. The Olfactory Data Interpreter (ODI) software enables time-aligned sensory evaluation of compounds eluting from the GC–MS system. Fractions can be collected for further analysis.

www.gerstel.com
Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany.

Leak Detector

Restek's new electronic leak detector can pinpoint small gas leaks quickly and accurately before they cause bigger problems, according to the company. The unit can be operated during charging or used up to 12 h between charges. In addition, it now comes with a flexible charging kit that includes both a universal AC power adaptor and a USB charging cable.

www.restek.com/leakdetector
Restek Corporation, Bellefonte, USA.

Hydrogen Generator

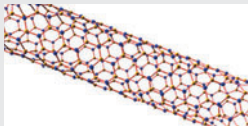
Designed for GC–FID, Precision SL is the smallest and easiest to use laboratory-grade hydrogen generator of its kind, according to the company, producing hydrogen gas at a push of a button. Available in both 100 cc and 200 cc, the hydrogen generator is reportedly simple to use and maintain, with advanced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

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

Boron Nitride Nanotubes

Goodfellow has a new addition to its range of boron nitride nanotubes (BNNTs) products. According to the company, there are many advantages of using BNNTs over carbon nanotubes (CNTs) such as: BNNTs are electrical insulators with bandgap of ~5.5 eV; BNNTs demonstrate superior thermal and chemical stability compared to CNTs and have 200,000 times higher thermal neutron absorption capacity than that of CNTs; applications include electrical insulation and reinforced ceramic materials for aerospace applications, among others.

www.goodfellow.com
Goodfellow Cambridge Limited, Huntingdon, UK.



Biphenyl Phases

Biphenyl-modified silica gels are an interesting alternative to octadecyl and octyl-modified HPLC sorbents. Macherey-Nagel offers columns packed with its biphenylpropyl silica gel, Nucleodur π2, and the core–shell phase, Nucleoshell Biphenyl, characterized by excellent performance under highly aqueous conditions, according to the company.

www.mn-net.com
Macherey-Nagel GmbH & Co. KG, Düren, Germany.

Chip-Based Chromatography

The 200-cm µPAC column is suitable for comprehensive proteomics, while the 50-cm µPAC column is suitable for performing higher throughput analyses with shorter gradient solvent times, according to the company. The µPAC Trapping columns were reportedly developed to ensure optimal chromatographic performance for peptide sample enrichment.

www.pharmafluidics.com
PharmaFluidics, Ghent, Belgium.

HPLC Phases

Kromasil by Nouryon presents a new wettable C18 phase specifically engineered for bioseparations and API manufacturing. Kromasil 100 Å, C18(w) can be loaded and run under 100% aqueous conditions, enabling separation and removal of impurities from small molecule samples and biosubstances mixtures including peptides.

www.kromasil.com.
Nouryon Pulp and Performance Chemicals AB, Bohus, Sweden.

SEC Mobile Phases

Arg-SEC, the universal mobile phase for SEC, enhances protein separations by reducing nonspecific interaction while maintaining protein structure, according to the company. The company reports correct determination of protein aggregates, which tend to stick to columns, is possible and that column lifetime may also be improved.

www.nacalai.com
Nacalai Tesque, Inc., Kyoto, Japan.

GPC/SEC Validation Kit

PSS EasyValid is a system suitability test that reportedly evaluates the entire GPC/SEC/GFC system, equipment, electronics, and analytical operations, to ensure that "true" molar mass results are obtained. According to the company, the system is ideal for various aspects of quality assurance qualification, whether mandated by stringent requirements or good management practices.

www.pss-polymer.com
PSS GmbH, Mainz, Germany.

Ultra-Pure Gas Delivery

By starting with ultra-pure gas and delivering it through award-winning BIP technology cylinders, gases are up to 300 times purer than normal gas cylinders, according to the company. Low levels of impurities are reported guaranteed. BIP gases are suitable for gas chromatography where impurities in the carrier gas can cause baseline noise and damage in the column.

www.airproducts.co.uk/BIP
Air Products PLC, Hersham, UK.

The 13th International Scientific Conference on Ion Chromatography and Related Techniques 2020

The 13th International Scientific Conference on Ion Chromatography and Related Techniques will be held **21–22 April 2020 in Zabrze, Poland.**

Ion chromatography, which was established in 1975, has evolved from a simple method for separating the main inorganic anions and cations in water into a sophisticated separation technique that may be coupled with modern detectors to detect trace substances in a variety of gaseous, liquid, and solid samples. Many changes introduced in the stationary phases of the ion-exchange columns have helped to extend the range of the applied eluents and detection methods. At present, the greatest challenges in ion chromatography are related to:

- Introducing new ion-exchange stationary phases
- Improving the suppressor operation efficiency
- Lowering the limits of detection and quantification for analyte ions
- Elaborating new sample preparation methods
- Extending the analysis range with new organic and inorganic substances
- Increasing the use of different ion chromatography types in the molecular biology and genetics research (genomics, proteomics, metabolomics, transcriptomics)
- Establishing new standard and detection methods
- Apparatus miniaturization.

These challenges will all be discussed at the conference. Although ion chromatography is in its early forties, the technique is still evolving and has many applications beyond the determination of inorganic ions. The organizers of the ion chromatography and related techniques conferences have sought to further this development by bringing together researchers from across the globe to present and discuss their research on ion chromatography.

The organizers welcome participants to the 13th International Conference, which will be held in Zabrze, Poland. Participation in the conference is free of charge, and is an excellent opportunity to discuss new developments in the field of ion chromatography and related techniques, share experiences, and present results to your peer group.

Conference proceedings with cutting-edge peer-reviewed papers in the field of ion chromatography and related techniques will be published prior to the conference.

As the host city for the conference, Zabrze has a lot of to offer with a wide variety of bars and restaurants featuring traditional Polish cuisine. For those looking for a museum with a difference, the "Guido Mine" allows visitors to travel as far as 355 metres underground to get first-hand experience of coal mining life.

The city also features a Municipal Botanical Garden created in 1928, which is spread across six hectares of land. The garden is home to thousands of specimens of plants, trees, and shrubs, as well as two greenhouses with 5000 plants from climate zones across the planet.

Information from previous conferences can be found at:
http://ipis.pan.pl/pl/pm-konferencje/konferencje-planowane

For additional information please e-mail Rajmund Michalski at **rajmund.michalski@ipis.zabrze.pl**

Image credit: Maksym/stock.adobe.com

14–16 OCTOBER 2019

The 11th Conference of The World Mycotoxin Forum and the XVth IUPAC International Symposium on Mycotoxins (WMFmeetsIUPAC)
Belfast, Northern Ireland
E: WMF@bastiaanse-communication.com
W: www.worldmycotoxinforum.org

21–23 OCTOBER 2019

Solutions and Workflows in (Environmental) Molecular Screening and Analysis (SWEMSA 2019)
Erding, Germany
E: info@swemsa.eu
W: www.swemsa.eu

5–8 NOVEMBER 2019

The 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019)
Prague, Czech Republic
E: RAFA2019@vscht.cz
W: www.rafa2019.eu

28–29 NOVEMBER 2019

The 7th Workshop on Field-Flow Fractionation – Mass Spectrometry (FFF-MS)
Leipzig, Germany
E: nanoanalytics@univie.ac.at
W: https://www.ufz.de/index.php?en=46025

29–31 JANUARY 2020

The 16th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology
Ghent, Belgium
E: htc16@kuleuven.be
W: https://kuleuvencongres.be/htc16

24–29 MAY 2020

44th International Symposium on Capillary Chromatography and 17th GC×GC Symposium
Congress Centre, Riva del Garda, Italy
E: meeting@rivafc.it
W: http://iscc44.chromaleont.it

Please send any upcoming event information to Lewis Botcherby at lbotcherby@mmhgroup.com

What a GC–MS Tune Report Can Tell You

An excerpt from *LCGC*'s professional development platform, CHROMacademy.com

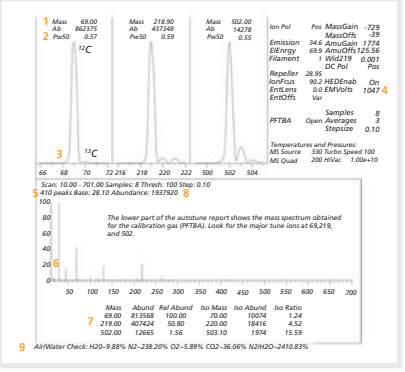
Tuning in gas chromatography–mass spectrometry (GC–MS) involves adjusting several mass spectrometer parameters through the infusion of a tune compound, commonly perfluorotributylamine (PFTBA). The tune report generated is invaluable because it indicates how well the instrument is operating, and is an essential tool when troubleshooting is required. A typical autotune report is shown in Figure 1.

There are several parameters that need to be examined in this autotune report:

- Correct mass assignments. Check that the tune has correctly assigned the masses of peaks 69 (base peak), 219, and 502. The tolerance for unit mass resolution are: 69 (68.8–69.2), 219 (218.8–219.2), 502 (501.8–502.2).
- The mass peak widths (PW50) should be 0.55±0.1.
- Peaks should be smooth and Gaussian. Do not be concerned with peak shoulders unless they become a major component of the spectral peak. The shoulders should increase in area to reflect the isotopic abundance of C13.
- Note the electron multiplier (EM) voltage. A clean source with a relatively new EM horn should report a gain voltage of 1400 to 1600. As the lifetime of the dynode increases and the source becomes contaminated, this value may approach values of 3000 V. If the voltage consistently needs to be ramped to 2800–3000 V, it may be time for a source clean or a new dynode.
- Low background. The presence of a large number of peaks across the spectrum is known as a *high background*. This can arise from several sources of contamination, such as column bleed, septum bleed, oil contamination, and various other sources.
- Low water and air. Presence of large peaks at 18, 28, or 32 indicate an air or water leak.
- The tables below give the relative abundances of each of the ions, as well as isotopic masses, abundances, and ratios. The isotopic mass should always be an M+1 ion, for example, 70, 220, and 503 amu. As PFTBA contains only C13 and N15, the relative isotopic ratios can be easily calculated and should be approximately 69 (1.0), 219 (4.0), and 502 (10.0). Any drift in these ratios can indicate that the spectrometer mass axis has not been correctly calibrated, or that the system resolution is grossly degraded. The following conditions are typical if everything is functioning correctly. The absolute abundance of mass 69 should be ≥200,000 but ≤400,000. For a maximum sensitivity autotune, the relative ratios of each of the peaks should be within the following ranges: 219/69 (20–35%) and 502/69 (0.51%).
- Proper absolute abundance.
- The data system will allow the operator to perform an air and water check to quantify the amounts within the spectrometer at any given point in time. This can be a useful tool to check the vacuum system and spectrometer operating temperatures are equilibrated, or to check if a suspected leak has been properly fixed.

All other parameters within the autotune will differ from instrument to instrument, but general trends from subsequent autotune reports should be noted. Any consistent drift in either the positive or negative direction should be noted and then re-autotune, manual tune, or maintenance should be carried out accordingly.

FIGURE 1: Example autotune report.



Target relative abundances	
Mass	**Target Relative**
50	1.0
69	100.00
131	55.0
219	45.0
414	3.5
502	2.5

Relative ratios for prominent masses	
***m/z* 69**	**Base Peak**
70/69	≥0.5 but ≤1.6%
219/69	≥40% but ≤85%
220/219	≥3.2 but ≤5.4%
502/69	≥2.0% but ≤5%
503/502	≥7.9 but ≤12.3%

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Direct Thermal Extraction-GC/MS Analysis of Food Packaging Material for crème-filled cookies, cheese-filled crackers and soft and chewy candy.

Recent Addition

Thermal Extraction requires almost no sample preparation and is well suited for trace analysis of migrating compounds in food packaging material. The packaging of three products was analyzed and benzaldehyde was quantified in one case.

Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC–MS Based on the Commonly Used Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10)

Automated determination of 3-MCPD and glycidol in edible oils by GC–MS. An evaporation step helps reach the required LODs using a standard MSD, while removing excess derivatization reagent for improved uptime and stability.

Automated determination of Acrylamide in Brewed Coffee samples by Solid Phase Extraction (SPE)–LC–MS/MS

A manual SPE method used for the determination of acrylamide in brewed coffee was automated. Calibration standards prepared in freshly brewed green (unroasted) coffee produced good linearity and precision.

Qualitative Analysis of Coconut Water Products Using Stir Bar Sorptive Extraction (SBSE) combined with Thermal Desorption-GC–MS

Flavor compounds, off-flavors, pesticides, antioxidants, and compounds migrating from packaging materials were successfully determined in coconut water products by stir bar sorptive extraction (SBSE)-TD-GC–MS.

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