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GC CONNECTIONS

Control or Chaos: How Can We Run a Gas Chromatograph from Anywhere?
Nicholas H. Snow,
This installment explores how the data system controls the functions of the GC instrument. Drawing on classical electronics and instrument designs, the article describes the evolution of instrument controls from knobs and gauges on the front panel of the instrument to computer control and current web-based systems.

QUESTIONS OF QUALITY

What’s Good About the WHO Good Chromatography Practices Guidance? Part 1
R.D. McDowall,
In September the World Health Organisation (WHO) issued a new guidance document on Good Chromatography Practices. What guidance does it contain and is it useful? Has the document failed its SST acceptance criteria?

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LC TROUBLESHOOTING

Troubleshooting for Two-Dimensional Liquid Chromatography: Breakthrough in the Second Dimension
Dwight Stoll
A common problem encountered in the development of 2D-LC methods is that the first dimension mobile phase properties can negatively affect the quality of subsequent second dimension separations. This installment reviews the origin of this problem and discusses potential solutions.

SAMPLE PREPARATION PERSPECTIVES

Exploring the Efficiency of Various Extraction Approaches for Determination of Crude (4-methylcyclohexyl)methanol (MCHM) Constituents in Environmental Samples
Ronald V. Emmons, Amila M. Devasurendra, Nipunika H. Godage, and Emanuela Gionfriddo
It is important to develop analytical methods to detect crude MCHM components in environmental water samples. This article describes two microextractive methods based on solid-phase microextraction (SPME) in fibre format and thin film microextraction (TFME) that were developed and validated for 4-MCHM and other constituents of crude MCHM.

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**INTERVIEW**

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**COLUMN WATCH**

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**EBOOK**

**Optimizing the Analysis of Viral Vectors with SEC-MALS**

This free e-book discusses how light scattering determines multiple CQAs of viral vectors, where multi-angle light scattering fits into a CMO analytical platform, and the benefits of SEC-MALS for characterizing oligonucleotides.  
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Mass spectrometry (MS) enables the structural characterization of molecular components of samples, allowing for the determination of their mass and elemental formula. Both intact mass and fragmentation pattern (such as using tandem MS/MS experiments, for example) lead to unique molecular information, which is crucial for compound identification in a wide variety of targeted and untargeted applications, from forensic research to synthetic polymer characterization.

Direct MS experiments (such as flow injection MS and ambient ionization techniques such as paper spray MS) are high-throughput approaches where the analytes are directly ionized and detected without prior chromatographic steps. Yet, direct MS is typically not suited for the analysis of complex samples due to possible matrix effects and relatively narrow dynamic range, which can significantly decrease the amount of information that is gathered per sample.

The marriage between liquid chromatography (LC) and MS has been made possible by the introduction of atmospheric ionization interfaces, notably electrospray ionization (ESI) (1,2). With LC–MS, the matrix effects can be reduced, the dynamic range significantly extended, and the sample components concentrated (3,4). Remarkable results in terms of sensitivity have been reported. For instance, identification of sub-zeptomolar (zM) peptides using low-flow separations (pL/min) allowed for the determination

**KEY POINTS**

- An overview of current trends in LC–MS, focusing on the advantages of alternative chromatographic selectivity versus RPLC–MS
- Recent advances in HILIC–MS for small and large molecules are described.
- An update on current SFC–MS approaches and prospective application areas.
- Applications of SEC–MS for synthetic and biopharmaceutical analysis are illustrated.
- The benefits of pH gradient-based IEC-MS to characterize biopharmaceuticals and perspectives on HIC-MS are described.
of the protein content in a small number of cells (5–8).

The research publication output of LC–MS and LC–MS-based studies between 1970 and 2019 is illustrated in Figure 1a. The steady growth of both LC and MS is accompanied by a steep increase in the percentage of publications using LC–MS (plotted as a function of LC publications). This trend is especially visible starting from the late 1990s, when LC–MS platforms became commercially available and, with time, developed to more and more robust instruments.

Although the results shown in Figure 1a are mostly reflecting academic research trends, a similar development has also been observed in industries and clinical laboratories, where an increasing number of LC–MS methods have been introduced in R&D departments, for end-product characterization as well as (although less frequently) for quality control purposes (9,10).

Since the introduction of the first commercial LC–MS instruments in the 1990s, numerous technological developments have been carried out in this field, further expanding the separation resolution. The advent of ultrahigh-pressure liquid chromatography (UHPLC) and high-resolution mass spectrometry (HRMS) has unquestionably revolutionized the field of LC–MS. Besides the commercialization of columns equipped with sub-2-µm particles, the development of columns equipped with core–shell particles and, more recently, micropillar array columns have brought new exciting perspectives in terms of separation efficiency, significantly decreasing

![Figure 1: (a) Number of publications per year in LC (dark blue), MS (red) and %LC–MS (black) literature between 1970 and 2019. Results have been extracted from Scopus using the following search: “liquid chromatography”, “mass spectrometry” and “LC–MS” as keywords. The result on the right y-axis “%LC–MS (vs. LC publications)” are obtained by calculating the percentage of LC–MS publications with respect to the LC publications in the same year. (b) Overview of the chromatographic modes used in LC–MS analysis. Results for reversed-phase LC–MS have been retrieved from Scopus using the following search: RPLC–MS (TITLE-ABS-KEY (liquid AND chromatography AND mass AND spectrometry) AND TITLE-ABS-KEY (reversed-phase AND chromatography) AND (LIMIT-TO [DOCTYPE, "ar"] AND (LIMIT-TO (SUBJAREA, "CHEM"))). Similarly, the same filters have been applied for the other chromatographic modes with the exception of the descriptor “TITLE-ABS-KEY” which has been replaced by “hydrophilic-interaction” for HILIC–MS, “ion-exchange” for IEC–MS, “size-exclusion” for SEC–MS, “normal-phase” for NPLC–MS, “supercritical-fluid” for SFC–MS, and “hydrophobic-interaction” for HIC–MS.](image-url)
Improvement in peak shape observed with addition of trace phosphate in the HILIC mobile phase: (a) epinephrine, (b) isocitrate. Upper red trace, without phosphate; lower green trace, with 5 µM phosphate added to the mobile phase. Separation performed using a SeQuant ZIC-pHILIC column (50 x 2.1 mm, 5-µm); mobile phase composition: (1) 20 mM ammonium acetate in water:acetonitrile (95:5, v/v) containing 5 µM ammonium phosphate, and (2) 100% acetonitrile. Adapted from reference (21) with permission.

FIGURE 3: HILIC–MS analysis of a lipase enzyme. (a) Total ion current (TIC) with annotated glycoform elution windows (xN-ym), where (x) indicates the number of N-glycosylation sites occupied (0–3) and (y) the number of mannose units present next to a single N-acetylglucosamine present in each glycan. (b) Deconvoluted mass spectra obtained for the corresponding elution windows (7.5–7.7 min, 8.7–8.8 min, 8.8–9.0 min and 9.0–9.3 min), showing signals for the mature form of the protein (intact sequence, Ma), a mature form without a C-terminal tryptophan (Ma-W) and a mature form without a C-terminal serine and tryptophan (Ma-SW). (c) SDS-PAGE of the lipase showing the derived densitogram (top trace) aligned with the TIC trace obtained with HILIC-MS. Reproduced from reference (32) with permission.

Hydrophilic Interaction Chromatography (HILIC)

HILIC-MS is considered the second most common chromatographic mode used in LC–MS analysis (Figure 1b). The reasons for its widespread diffusion include an orthogonal selectivity to reversed-phase LC, allowing for the retention of polar-ionizable compounds often poorly retained in reversed-phase LC (11), a lower mobile phase viscosity (enabling the use of longer columns leading to higher efficiencies), and a high sensitivity when using ESI-MS due to the use of high organic percentages in the mobile phase (12,13).
A large number of papers in the literature have investigated HILIC retention mechanisms, describing the multimodal nature of this chromatographic mode (14). The retention is driven by the partition of analytes between a polar stationary phase and a relatively hydrophobic mobile phase (for example, an aqueous–organic mixture containing a high proportion of acetonitrile). Under appropriate conditions, namely, a concentration of 5–40% water in the eluent, a water-enriched layer is formed at the surface of the stationary phase, allowing for the hydrophilic partitioning. HILIC retention also involves ionic interactions, dipole–dipole interaction, and hydrogen bonding (15,16). The stationary phases can be classified on the basis of their chemistry as neutral, such as diol and amide, and charged stationary phases, such as bare silica, zwitterionic, and amine (17).

The analyte retention strongly depends on the selection of the stationary phase chemistry, as well as the buffer composition used in the mobile phase. Moreover, in order to ensure reproducible experiments, careful attention has to be paid to essential operating parameters, such as the composition of the injection solvent and the use of a reproducible mobile phase composition, as well as adequate equilibration time between runs. We refer to published literature for more insights on method development in HILIC–MS (18,19).

HILIC–MS has significantly matured over the last years, along with the commercialization of columns ensuring an improved batch-to-batch reproducibility and the introduction of stationary phases equipped with sub-2-µm particles. HILIC–MS is currently used in a wide range of fields, including pharmaceutical analysis, metabolomics, lipidomics, and glycomics. Moreover, recent investigations have demonstrated the suitability of HILIC–MS to study large molecules, such as intact proteins and their subunits, making it an attractive orthogonal tool for the analysis of glycosylated biotechnological products.

From Metabolomics to Lipidomics
One of the major challenges in metabolomics is the wide diversity in physico-chemical properties observed between metabolites, showing the need for complementary approaches enabling a larger coverage of the metabolome. The human metabolome encompasses
a large number of (highly) polar metabolite classes such as amino acids, small organic acids, nucleosides, nucleotides, and phosphate derivatives, as well as saccharides, which are playing key roles in multiple (patho)physiological processes. Such metabolites show poor retention, and suffer from severe matrix effects using reversed-phase LC, leading to poor quantitative accuracy and low sensitivity. However, they are typically well-retained using HILIC. HILIC–MS also typically leads to a broader metabolome coverage in untargeted metabolomics compared with reversed-phase LC–MS (14,20). For all these reasons, HILIC–MS is now considered by the metabolomics community as essential as reversed-phase LC–MS, being integrated in the state-of-the-art analytical toolbox.

One of the major challenges in HILIC-based metabolomics is to develop methods offering a good compromise between metabolome coverage and acceptable peak shapes for different metabolite classes. The presence of broad peaks results in additional challenges during data pre-processing that may lead to poor quantitative accuracy. Better peak shapes for all compounds can be obtained by using multiple HILIC methods in successive experiments. However, this strategy is often not possible, due to time constraints and sample or resource availability. Most reported applications have therefore been carried out using one single HILIC method; for example, using a zwitterionic or diol column in untargeted metabolomics approaches, sacrificing part of the coverage for a better analytical efficiency.

Peak broadening has been significantly reduced by adding micromolar concentrations of phosphate to the mobile phase buffer (5 μM phosphate, corresponding to an estimation of ca. 40 nmol introduced to the column during each run). In the presence of trace amounts of phosphate, a significantly better peak shape, signal intensity, and improved coverage have been observed for a set of 65 polar compounds, including neurotransmitters, small organic acids, nucleosides, nucleotides, biogenic amines, and sugars (Figure 2) (21). Similar improvements have been observed when a comparable amount of phosphate was added to the sample injection solvent. Moreover, in addition to the chromatographic effects, a slight increase of ionization efficiency has been observed in presence of phosphate (5 μM in the mobile phase), that is, 6% for negatively ionizing compounds and 16% for positively ionizing compounds, respectively. The significant improvement in peak shapes led to a more accurate automatic peak detection, representing a key advantage in the data preprocessing pipeline for

FIGURE 4: Hyphenation of SFC with MS. (a) The two commercially available interfaces used to hyphenate SFC with MS (pre-BPR splitter with sheath pump interface and BPR and sheath pump with no splitter interface). (b) UHPSFC–MS interface design developed to improve the spray stability. Note: A is commercially available interface and B is the modified prototype interface. Adapted from references (42,45) with permission.

FIGURE 5: SFC–MS for the simultaneous analysis of hydrophobic and hydrophilic metabolites. (a) SFC–MS chromatogram showing the simultaneous injection of tricosanoic acid and raffinose (see reference [47] for experimental conditions); (b) Scatter plot showing the molecular weights of the detected metabolites as a function of their retention times. Blue dots, metabolites detected from the library; red dots, metabolites from the selected experimental set of metabolites. Adapted from reference (47,48) with permission.
untargeted applications. Interestingly, no sign of source contamination or instrument failure was observed after a year of experiments.

With additional experiments, the authors showed that the presence of trace phosphate improved the chromatographic performance by shielding electrostatic interactions between the analytes and the polymeric-based zwitterionic stationary phase, similar to what is observed when increasing the concentration of salts in the mobile phase. Indeed, a similar behaviour in performance enhancement was observed when increasing the concentration of ammonium acetate in the mobile phase (that is, from 5 to 200 mM) compared with the addition of trace phosphate (21). The analytes being the most positively affected by the presence of phosphate were also the ones requiring a higher concentration of ammonium acetate to achieve optimal peak shape. Moreover, phosphate—which has a high charge density—showed to be highly beneficial to improve the peak shape of compounds whose elution profiles were negatively affected by strong electrostatic interactions.

The exact mechanisms underlying this shielding effect remain to be fully understood, as different effects were observed depending on the column batch used. Indeed, irregularities in column manufacturing—as well as column conditioning—may modify the accessibility of phosphate to the electrostatic sites of the stationary phase. Moreover, phosphate blocks trace metals within the stationary phase matrix, influencing the peak shape. Interestingly, this study not only highlighted the benefits of adding trace phosphate to the mobile phase, but also revealed that electrostatic interactions may be the predominant cause of poor chromatographic performance when using HILIC, notably in metabolomics.

Lipidomics is a subdiscipline of metabolomics focusing on the large-scale study of the structure and functions of lipids. Lipids are involved in a plethora of physiological functions, including energy storage, signalling, and regulation of protein function. More than 60% of all human metabolites have been annotated as lipids (22). Reversed-phase LC–MS is widely used in lipidomics, typically using a C\textsubscript{18}– or C\textsubscript{8}–based column combined with a highly organic gradient, allowing for the separation of lipids based on their fatty acyl chain length and unsaturation.

**FIGURE 6:** Comparison of normal LC and SFC for the analysis of a protein mixture. (a) UV chromatograms obtained with a PolyPentyl A column at 25 °C using a normal LC gradient (upper panel) and a CO\textsubscript{2} dual gradient (lower panel). (b) Mass spectra observed for ribonuclease A from bovine pancreas (RNaseA) and lysozyme from chicken egg white (Lys) using a normal LC gradient (upper panel) and a CO\textsubscript{2} dual gradient (lower panel). Adapted from reference (51) with permission.

**FIGURE 7:** Analysis of an industrial branched polyester resin sample using SEC–MS. Left panel: total ion current chromatogram (TIC), extracted ion chromatogram (EIC) for polymers of different masses and UV trace. Right panel: average mass spectra collected at different time intervals, as indicated in the colored boxes of the TIC. Annotation highlights the increase of charge states for higher mass species detected at earlier retention times. Supercharging conditions are described in reference (55,56).
**FIGURE 8:** Online SEC-native MS analysis of a temperature-stressed NIST mAb sample. (a) Overlaid UV chromatogram of stressed (red trace) and unstressed (blue trace) NIST mAb. (b) Native mass spectra of peaks (1) and (2) observed for the SEC analysis of the temperature-stressed NIST mAb sample. Figure adapted from reference 58 with permission.

**FIGURE 9:** Analysis of trastuzumab using IEC-MS. (a) Simulated spectrum for the deamidated (†) and relatively unmodified (*) isomers of trastuzumab G0F/G0F 27+ charge state. The centroid peak apices of the two isoforms are distinguished by a calculated mass difference of 6.75 ppm. (b) Experimentally measured m/z values for deamidated and unmodified G0F/G0F 27+ ions, shown as profile and centroid spectra. (c) Base peak chromatogram (BPC, upper panel) with labelled peaks corresponding to deamidated and relatively unmodified isoforms, extracted ion chromatogram (XIC, lower panels) traces for deamidated and unmodified isoforms rounded to nearest hundredth m/z value and plotted using a 3.3-ppm extraction width. (d) Major isoforms (corresponding to the top three glycan combinations ± deamidation) are detected using Sliding Window deconvolution and plotted as individual extracted deconvoluted mass traces (3.3 ppm mass/merge tolerances). Reproduced from reference (64) with permission.

HILIC–MS, on the other hand, offers an orthogonal chromatographic separation where lipids are separated based on the lipid headgroup polarity, however typically leading to the co-elution of all lipids of a specific lipid class (23). The latter is considered advantageous in quantitative analysis, as the target lipids of different classes are co-eluting with their labelled internal standard (ISTD), chosen for each class. In reversed-phase LC–MS, ISTDs are usually eluted at a different retention time, possibly showing different matrix effects than the target lipids. Lange and associates evaluated the quantitative performance of HILIC–MS and reversed-phase LC–MS for the analysis of five lipid classes in human plasma (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysosphatidylcholine, and lysosphatidylethanolamine) using the so-called “one ISTD-per-lipid class” approach (23). They demonstrated that, despite the obvious difference in matrix effects, both workflows can be equally used for quantitative analysis, as similar concentrations were measured for most of the lipid classes assessed, consistent with reported NIST consensus values (except for highly unsaturated phosphatidylcholines).

**Analysis of Intact or Subunits of (Glyco)Proteins**

HILIC–MS has recently emerged as an attractive analytical tool for proteins analysis, in particular for the characterization of glycoproteins. Glycosylation is a common post translational modification (PTM), where oligosaccharides (referred to as glycans) are covalently bonded to an amino acid residue of the protein. Glycoproteins have an heterogeneous glycan composition; differences in their distribution may have a major impact on the biological functions of the protein and its stability, solubility, antigenicity, folding, and half-life (24,25). The glycosylation pattern is therefore considered a Critical Quality Attribute (CQA) of recombinant pharmaceutical proteins, and should be carefully monitored in drug development pipelines to ensure quality, safety, and efficacy of the biopharmaceutical product.

Similar to what is observed for small molecules, HILIC allows for the discrimination between protein sample components based on a mechanism orthogonal to reversed-phase LC (26). In particular, optimized HILIC–MS methods have led to the separation of glycoforms of glycoproteins, such as monoclonal antibodies (27,28) and their fragment crystallizable portion (29), biopharmaceuticals (30), and neo-glycoproteins (31).
In most cases, protein samples are solubilized in water–acetonitrile mixtures, typically high in water content and containing an acidic ion-pair (such as trifluoroacetic acid). Small volumes (<10% of column volume) of this solution are injected and analyzed using acetonitrile–water gradients on amide stationary phases. The mobile phase conditions allow for ion-pair formation, with the basic protein residues at low pH reducing ion-exchange interactions with the stationary phase. As a result, the (neutral) sugars of protein glycans substantially contribute to the retention, resulting in glycoform resolution based on the overall glycan size and composition.

Figure 3 shows an example of the resolving power that can be obtained with HILIC–MS for the characterization of a lipase enzyme used in the food industry (results obtained in our laboratory) (32). Whereas the reversed-phase LC–MS analysis resulted in a single peak, the HILIC–MS method revealed the different glycoforms of the glycoprotein, resolved into distinct peaks according to the number of glycose units and the glycosylation site occupied (Figure 3). The HILIC–MS method resulted in the detection of over 100 glyco-proteoforms, allowing for the detection of glycoforms with two and three glycosylation sites occupied that were not observed with reversed-phase LC–MS. Interestingly, the total ion current profile obtained was similar to the SDS-PAGE densitogram. Off-line fractionation of the HILIC-UV separation confirmed this observation.

An important aspect in HILIC–MS being currently actively investigated is the replacement of ion-pair additives, such as trifluoroacetic acid, with more MS-friendly acidic ion-pair, such as formic acid, to increase the sensitivity of the method while maintaining its selectivity. In this context, an interesting development has been presented by the group of Wirth and co-workers, who used stationary phases with a special polymer coating to reduce the ionic interactions between the stationary phase and the proteins, preserving the same HILIC selectivity while significantly reducing the concentration of trifluoroacetic acid needed (33–35).
**Supercritical Fluid Chromatography (SFC)**

First introduced in the last century with minor success, supercritical fluid chromatography (SFC) has regained a substantial popularity in recent years due to the remarkable technology advances carried out in modern SFC instrumentation, which have greatly improved its reliability, reproducibility, and robustness (36). SFC is based on the use of supercritical fluids as mobile phase, typically carbon dioxide ($CO_2$), which is considered the solvent of choice, due to its low critical temperature (31 °C) and critical pressure (73.8 bar). Due to the low polarity of $CO_2$, a small proportion of a miscible organic modifier, usually methanol, is added to the mobile phase, extending the application of SFC to the analysis of more polar compounds. Compared with HPLC, the high proportion of supercritical fluids in the mobile phase offers a faster mass transfer thanks to an enhanced diffusivity. Moreover, the low viscosity of the SFC mobile phase leads to a lower pressure drop, which allows for the use of significantly higher flow rates compared with HPLC. Besides the organic modifiers, acids, bases, or salts at low concentrations, as well as water, can be added to the mobile to increase selectivity and separation efficiency, improve peak shape, and enable the elution of polar compounds (37).

However, the presence of a modifier and additives in the mobile phase influences the critical parameters of the fluid, which is then closer to the so-called subcritical conditions (38).

Most of the reversed-phase, normal-phase, and HILIC stationary phase chemistries can be used in SFC, overall offering a wide selectivity range (39). Moreover, multiple SFC-specific stationary phases have been developed and commercialized over the last years. A large number of the commercially available columns are also available with fully porous sub-2-$μm$ and superficially porous sub-3-$μm$ particles, enabling ultrahigh-performance SFC (UHPSFC) analysis which leads to excellent kinetic performance with a low pressure drop compared with UHPLC (40,41).

Hyphenating SFC with MS remains more challenging than the rather straightforward LC–MS configuration, due to the depressurization of the mobile phase which may lead to compound precipitation and cooling effects (36). Many approaches have been recently proposed to interface SFC with MS, with two designs currently available on commercial instruments, namely, the pre-backpressure regulator (pre-BPR) splitter with sheath pump, and the backpressure regulator (BPR) and sheath pump with no splitter interfaces, both illustrated in Figure 4a (42). In the pre-BPR splitter with sheath pump interface, the effluent is mixed with an added make-up solvent flow, and split into two parts, with the smaller proportion directed to the MS, and the remaining larger proportion directed to BPR to maintain the adequate pressure in the system. In the BPR and sheath pump with no splitter interface, the effluent is mixed with the make-up solvent flow, and directed to the MS inlet placed after the BPR. Adding a make-up flow to the SFC–MS interface has two purposes: enhancing the ionization efficiency, and avoiding a possible precipitation along the tubing. With such interfaces, the conventional ESI and atmospheric-pressure chemical ionization (APCI) sources can be used. Compared to LC–MS, SFC–MS typically shows higher sensitivities, due to an improved evaporation process, as a rapid evaporation of $CO_2$ occurs at the MS inlet, which leaves the analytes in a small amount of easily-evaporated organic modifier (42). Finally, most of the common mass analyzers used in LC–MS, such as single quadrupole, triple quadrupole, and time-of-flight, as well as hybrid instruments are also suited for SFC–MS analysis (41,43,44).

Due to the nonpolar nature of $CO_2$, SFC–MS has been mostly used for the analysis of relatively non-polar lipids, steroids, tocopherols, and vitamins. As an example, a validated workflow for the quantitative analysis of 14 fat-soluble vitamins and carotenoids in human plasma was developed using UHPSFC–MS (45). Using a $C_{18}$ stationary phase and adding a small proportion of water to the mobile phase, multiple isomers and tocopherols were successfully separated in a single 8-min run. The commercial SFC–MS interface hardware was improved to minimize the post-decompression volume and allow for a better control of the chromatographic effluent density before the ESI process (Figure 4b). Combined with specific make-up solvent conditions, this new prototype interface led to a more stable spray, reducing the occurrences of spiky peaks, and resulting in an improved repeatability and sensitivity.

SFC–MS is not only well-suited for the analysis of hydrophobic compounds, but also fully applicable to the analysis of (highly) hydrophilic compounds using optimal experimental conditions. Moreover, with one of the major advantages of SFC–MS being its great versatility, the simultaneous analysis of both hydrophobic and hydrophilic compounds has become
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achievable, as demonstrated in recent studies (46–50). As an example, a comprehensive study has demonstrated the applicability of UHPSFC–MS in metabolomics for the simultaneous analysis of both nonpolar and highly polar metabolites within one single run, which remains very challenging using UHPLC-based methods (47). The complete separation of the compounds set composed of 57 metabolites of different polarities (-6 < logP <11) was achieved in a single UHPSFC–MS run, using optimized conditions (a mobile phase gradient ranging from 2% to 100% of modifier, a column packed with sub-3-μm superficially porous particles and a mix of water:acetonitrile [50:50, v/v] as sample diluent) (47). Figure 5a shows the separation of two compounds with a large polarity difference (tricosanoic acid [logP = 9.3] and raffinose [logP = -6.3]), thus demonstrating the suitability of UHPSFC–MS for metabolomics.

Besides metabolome coverage, another important aspect in metabolomics is the presence of matrix effects, which may significantly affect the quality of the quantitative data and represent a crucial parameter during method optimization. In their study, Guillarme and co-workers also investigated the importance of matrix effects in plasma and urine for a set of representative metabolites (48). Overall, limited matrix effects were observed for the analysis of both biological fluids, with 30% of the metabolites suffering from matrix effects in plasma and 25% in urine, respectively. Moreover, the repeatability of the UHPSFC–MS coupling was remarkable, with average relative standard deviation (RSD) values for retention time repeatability between 0.3 and 0.5% over a period of three weeks. The authors also evaluated the performance of UHPSFC–MS in metabolomics using a commercial library containing 597 metabolites. With UHPSFC–MS, 66% of the compounds present in the library were detected, notably highly polar compounds such as amino acids, nucleosides, and carbohydrates, as well as hydrophobic analytes such as steroids and lipids (Figure 5b). Phosphorylated metabolites, however, led to poor performance. SFC–MS does not only show interesting advantages for the analysis of low-molecular weight compounds, but has also demonstrated a great potential for the analysis of proteins. As an example, SFC (here referred to as enhanced fluidity liquid chromatography by the authors) was used to separate intact proteins using hydrophobic interaction chromatography stationary phase combined with a dual gradient elution that consisted of an LC solvent gradient with simultaneous addition of an increasing amount of CO2 (51). Compared to conventional LC analysis, improved efficiency was observed using SFC, due to the faster mass transfer caused by the presence of CO2 (Figure 6a). As the desolvation of the mobile phase in the MS was also enhanced by the addition of CO2, a “supercharging” effect was also observed, resulting in higher ionization efficiencies and shifts of charge state (Figure 6b).

Overall, the major advantage of SFC–MS compared with LC–MS is its suitability for the simultaneous analysis of a wide range of compounds showing very different chemical properties. SFC–MS offers excellent flexibility, due to the large diversity of commercially available stationary phases and the multiple options possible for the composition of the mobile phase. Together with the ongoing instrumentation development and improvements, SFC–MS is expected to play a major role and become increasingly used in multiple analytical applications.

**Size-Exclusion Chromatography (SEC–MS for the Analysis of Synthetic Polymers and Non-Covalent Protein Complexes**

Size-exclusion chromatography (SEC) separates molecules according to their hydrodynamic volume (or size) based on the difference in exclusion from the pores of the stationary phase. Under ideal SEC conditions (where there is no interaction of the solutes with the stationary phase), the retention time can be translated to molecular weight using appropriate calibration standards. SEC–MS is considered an attractive tool for the analysis of mixtures of molecules showing a wide molecular weight distribution, such as synthetic polymers and protein complexes. Although chemically different, proteins and synthetic polymers share the characteristics of being heterogeneous, distributing in an ensemble of polymer-forms, differing in properties such as chemical composition, molecular weight, and higher order structure organization.

Synthetic polymers are chemical products used in a large diversity of everyday life applications, such as plastics and coatings. Polymers are produced through polymerization of chemical units (referred to as monomers), resulting in a heterogeneous distribution of molecules. The development
of high performance polymers (used in applications such as drug delivery systems) relies on tailoring the distribution of chemical features to achieve the desired product properties. Such chemical features include molecular weight distribution, end-group functionality, topology (branching vs. cyclic), and polymer sequence distribution.

SEC-UV is typically used to determine the molecular-weight distribution of soluble polymeric products, but it does not provide any information on the molecular composition of the sample. On the other hand, MS can provide information on multiple chemical distributions (52). Therefore, the combination of SEC with MS is highly relevant in the field of polymer analysis. However, the common mobile phase systems used in SEC-UV (apolar solvents like THF) are not compatible with ESI-MS. An interesting approach is to couple SEC–MS using a post-column make-up flow (53). In this setup, the LC eluent is mixed post-column with an organic solvent (such as methanol containing an ionization agent such as NaI) (54). Furthermore, supercharging agents can be used as additives to improve the ionization and detect higher molecular weight (MW) polymers showing a wide variety of chemistries (such as polystyrene, acrylates, and polyesters), also enabling the ionization of hydrophobic polymers (55).

The combination of the resolving power of SEC, partly discriminating the sample polydispersity, with ESI-MS with supercharging agents significantly extends the MW range that can be measured. As a result, SEC–MS with supercharging agents allow for the analysis of complex synthetic polymers up to 10 kDa range. Figure 7 shows an example of SEC–MS for the analysis of a branched polyester resin sample (unpublished data from Groeneveld and associates [56]). Species up to [M + 6Na]+ were observed, allowing for the detection of species up to 6 kDa distributed in a highly complex MS spectrum.

Beside polymer characterization, SEC–MS is often used for the characterization of biotechnological products, notably native SEC–MS, which is gaining more attention. Size and conformation based separation are of interest as often the active form of a protein is determined by its higher order structure distribution (dimerization). The information on the protein structural organization is lost when using denaturing methods such as reversed-phase LC and HILIC for the analysis of biopharmaceuticals. Native (or non-denaturing) separation approaches are indeed only accessible using mild conditions, such as water-based solutions at neutral pH and separation at room temperature. Coupling native SEC to MS allows for the analysis of protein complexes and aggregates. Under native separation conditions, water-based mobile phases with relatively high concentrations (100 mM) of volatile salts are used. These conditions result in a mitigation of ionic interactions with the stationary phase and maintain non-covalent interactions during measurements, enabling to obtain protein structural information (57).

An example is illustrated in Figure 8 with the analysis of monoclonal antibodies (mAbs), where dimer, monomers and mAbs fragments of are separated using SEC–MS (58). Similar workflows can also be used to desalt and buffer-exchange protein products, allowing for the analysis of very large molecular complexes (59).

**The (Not Necessarily) Salty: Ion-Exchange Chromatography (IEC) and Hydrophobic Interaction Chromatography (HIC)–MS**

The growing interest in characterizing protein-based biotechnological products in their intact forms under nondenaturing conditions has fostered further developments in alternative chromatographic approaches such as ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and capillary electrophoresis, focusing on the development of MS-compatible liquid-phase conditions.

IEC is commonly used to characterize charge variants of mAbs, such as those from amino acid variants, or PTMs such as deamidation. Typically, IEC methods are based on the use of cation-exchange stationary phases and mobile-phase gradients where either the concentration of salts (NaCl) is increased during analysis, or the pH is changed during analysis. Reference methods however make use of nonvolatile buffers or salt components; they are therefore not suitable for direct MS coupling and can only be used combined with desalting procedures.

Recent results, such as reported by Füssl and associates (60, 61), have demonstrated the feasibility of performing pH gradient-based IEC separations using low concentrations of volatile salts such as ammonium acetate. These results underline the importance of a separation step to...
obtain a detailed characterization of molecules having similar masses (62,63). Figure 9 illustrates a striking example of the analytical power of such a method with the separation of the deamidated forms of the mAb trastuzumab, differing in only 1 Da on a molecule with a MW of ca. 150 kDa (64). This detailed characterization is not possible with direct MS (65) or using denaturing reversed-phase LC–MS methods, as they lack the sufficient selectivity to differentiate the charge variants.

Conclusions

Reversed-phase LC–MS has long remained the gold standard chromatographic technique for the analysis of a large diversity of compounds, from metabolites to biopharmaceuticals via lipids, glycans, and peptides. Over the last decade, remarkable technological developments have been carried out in the fields of HILIC, SFC, SEC, IEC, and HIC, fostering their use in many different applications. Those developments include the commercialization of novel stationary phase chemistries and new instruments, as well as experimental conditions enabling the direct coupling to MS. Overall, these innovations have raised the interest of the analytical science community, seeking alternative chromatographic options that would help in solving the challenges encountered with reversed-phase LC–MS. Moreover, alternative selectivities may be a valuable tool to address analytical questions when not having access to the latest MS technology platforms.

Most of the alternative chromatographic techniques are now reaching levels of maturity that allow them to be used outside academic research. HILIC, SFC, SEC, IEC, and HIC, together with important liquid-based separation approaches that were not covered here, such as capillary electrophoresis, multi-dimensional liquid chromatography, and chiral chromatography, are not expected to replace reversed-phase LC–MS, but will become increasingly important in analytical sciences, enabling the scientific community to tackle analytical challenges that cannot be entirely solved with reversed-phase LC alone.

We, therefore, find it essential that the new generation of young scientists become familiar with those techniques as early as possible, together with reversed-phase LC–MS.

References

Adeno-associated viruses (AAVs) are increasingly used for gene therapy due to their versatility and safety. One of the biggest concerns for manufacturing a uniform AAV suspension is the presence of viral aggregates, which can create problems with transduction efficiency, biodistribution, and immunogenicity. These large AAV aggregates are challenging to separate and characterize by traditional column-based chromatography techniques such as size exclusion chromatography (SEC).

Asymmetrical Flow Field-Flow Fractionation with Multi Angle Light Scattering (AF4-MALS) can separate and size large AAV aggregates, and discern a difference in aggregate concentration due to the stressing protocol. Some or all of the large aggregates would be filtered out by SEC, resulting in incorrect determination of the aggregate content or the false conclusion that no aggregates are present.

For more details visit www.postnova.com and search for ‘virus’.
Two-dimensional liquid chromatography (2D-LC) separations can pose troubleshooting challenges not normally encountered in conventional high-performance liquid chromatography (HPLC) separations. A common problem encountered in the development of 2D-LC methods is that the first dimension mobile phase properties can negatively affect the quality of subsequent second dimension separations. In this instalment the origin of this problem and potential solutions are discussed.

For more than three decades, this “LC Troubleshooting” column has focused on solving problems encountered in liquid chromatography methods involving a single column. Since the late 1970s, researchers have been working to develop two-dimensional liquid chromatography (2D-LC), which typically involves two different columns (1). For decades, 2D-LC has been used extensively and successfully in several specific application areas, including proteomics and polymer separations. Most recently, however, the number of commercially available options for off-the-shelf instrumentation for 2D-LC has increased. Because of this, users in a broad array of application areas have begun deploying 2D separations more widely (2). Given the steady increase of active users deploying 2D-LC, it is now time to dedicate some instalments of “LC Troubleshooting” to problems encountered in 2D-LC. Although it is true that many of the troubleshooting topics in the context of conventional HPLC separations also apply to 2D-LC (for example, best practices for mobile-phase preparation are nominally the same for 1D- and 2D-LC), it is also true that there are challenges that are unique to 2D-LC and deserve focused attention. Readers interested in learning much more about state-of-the-art 2D-LC separations are referred to several recent review articles (1–3). In the following paragraphs, I provide a brief review of the concept of 2D separation, and then discuss one of the common problems encountered by users developing 2D-LC methods: breakthrough peaks in the second dimension.

Two-Dimensional Separations: The Basic Idea
People are interested in 2D-LC separations because the 2D format provides a chance to resolve analytes that otherwise would not be resolved by a single column in conventional 1D-LC. This can be useful for separating very complex mixtures, such as tryptic digests of proteins where the analyst tries to extract as much information from the sample as possible. However, 2D separations are also useful for samples that are not so inherently complex, but contain several analytes that are difficult to separate. For example, samples of several different enantiomer pairs may be difficult to separate using a single chiral column, but may be relatively easy to separate in a 2D format using a combination of achiral and chiral columns (4).

There are several different modes or ways of executing 2D-LC separations. Among these, the single heartcut mode—denoted LC-LC—is the simplest in terms of the instrumentation, methods, and data analysis involved. The goal of such a separation is illustrated in Figure 1. A first dimension (1D) separation results in three clusters
of unresolved peaks. The first cluster contains a peak corresponding to the analyte of interest (blue peak). Current commercial instrumentation and software make it straightforward to target this peak and instruct the instrument to transfer a portion of effluent from the \( ^1 \text{D} \) column containing the peak (along with the overlapping pink and green peaks) to a second dimension (\( ^2 \text{D} \) column) for further separation. Then, provided a \( ^2 \text{D} \) column and separation conditions are chosen that are complementary to the \( ^1 \text{D} \) conditions, the analyte of interest can be separated from the compounds it overlapped with in the \( ^1 \text{D} \) separation, enabling accurate quantitation and a qualitative analysis free from interferents.

There are a number of ways that instrumentation can be configured to support such a separation, but one example of the interface between the \( ^1 \text{D} \) and \( ^2 \text{D} \) separations is shown in Figure 2. The interface valve has two positions, and the valve is toggled to either have the \( ^1 \text{D} \) column effluent flow to waste in regions of the separation that don’t require additional \( ^2 \text{D} \) separation (position B), or to collect the \( ^1 \text{D} \) effluent in the sample loop (position A). At the end of this sampling period, the contents of the loop are effectively injected into the \( ^2 \text{D} \) column for further separation by switching the valve from position A to B.

**Mobile-Phase Mismatch Can Lead to Breakthrough Peaks**

As indicated in the preceding section, an important determinant of successful \( ^2 \text{D} \)-LC separations is the degree of complementarity of separation conditions (that is, the separation selectivities should be different) used in the first and second dimensions. Sometimes, this can be realized without the conditions used in the \( ^1 \text{D} \) separation negatively impacting the quality of the \( ^2 \text{D} \) separation as measured by chromatographic efficiency or detection sensitivity. For example, many applications of \( ^2 \text{D} \)-LC in the field of proteomics involve a \( ^1 \text{D} \) separation based on a cation-exchange mechanism and a \( ^1 \text{D} \) separation that relies on a reversed-phase mechanism. In the case of \( ^2 \text{D} \)-LC, peptides are eluted from the \( ^1 \text{D} \) column in effluent composed mainly of a buffered aqueous solution. This is highly favourable for the \( ^2 \text{D} \) separation in the sense that relatively large volumes of this effluent can be injected into the \( ^2 \text{D} \) column without negatively impacting the widths or shapes of \( ^2 \text{D} \) peaks. However, a favourable relationship between the operating conditions used in the two dimensions is not inevitable. In other cases, the properties of the effluent from the \( ^1 \text{D} \) column can have a dramatic and negative effect on the quality of the \( ^2 \text{D} \) separation. We refer to problems of this type as originating from mismatch between the mobile phases used in the two dimensions. An example of this is where we use a hydrophilic-interaction liquid chromatography (HILIC) separation in the first dimension and a reversed-phase liquid chromatography (RPLC) separation in the second dimension. In general, HILIC
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separations tend to involve mobile phases containing much more than 50% acetonitrile, whereas RPLC separations of compounds that can be reasonably retained under HILIC conditions tend to involve mobile phases containing much less than 50% acetonitrile. If HILIC separation is used in the first dimension and RPLC separation is used in the second dimension, this then sets up a situation where a large volume of '1D effluent containing analytes of interest and more than 50% acetonitrile is injected into the RPLC column running with a mobile phase containing less than 50% acetonitrile. If HILIC separation is used in the first dimension and RPLC separation is used in the second dimension, this then sets up a situation where a large volume of '1D effluent containing analytes of interest and more than 50% acetonitrile is injected into the RPLC column running with a mobile phase containing less than 50% acetonitrile.

Figure 3 shows an illustration of the local environment inside the '1D column during the injection of '1D effluent into the '2D column. In principle, this injection step is not different from what happens in conventional 1D-LC. However, problems can arise in 2D-LC when the volume of effluent from the '1D column is not very small (less than 1%) relative to the volume of the '2D column itself (which is common in 2D-LC). I like to emphasize the impact of this difference by saying that in 2D-LC the '1D column effluent becomes the '2D mobile phase during the injection step. If analytes are weakly retained by the '2D column in the '1D effluent, then it hardly matters what the analyte retention is in the '1D column in the '2D reversed-phase separation. The severe breakthrough peaks in the second dimension of the separation in (b) are highlighted by the red rectangle. With the use of ASM in (c) these breakthrough peaks are completely eliminated. Adapted from reference (5).

A Variety of Solutions to Help
While the mobile-phase mismatch problem described above is certainly not new to the 2D-LC community, its

Figure 3: Illustration of the injection of a fraction of '1D effluent into the '2D column, emphasizing the point that sometimes the volume of the fraction is large enough relative to the volume of the '2D column that the '1D effluent effectively acts as the '2D mobile phase during the injection step.
significance in practical 2D-LC method development and the importance of developing solutions for the problem to the continued growth of the technique have garnered a lot of attention in recent years. A broad perspective on the significance of the problem in different application areas and for possible combinations of different separations modes has been discussed in detail by Pirok and co-workers (6). The good news is that currently there are commercially available technologies that can be used to address the problem, and other potential solutions are being explored by various research groups across the globe (7). Currently, two of the most effective commercially available solutions rely on interventions that disrupt the situation illustrated in Figure 3 that can lead to poor outcomes like that shown in Figure 4b, by adjusting the composition of the 1D effluent prior to injection of that material into the 2D column for further separation. In an approach known as at-column dilution (ACD), a pump is used to add diluent to the 1D column effluent as it exits the column, thereby adjusting the properties of the effluent prior to loading into the 2D column (8). In a different approach known as active solvent modulation (ASM), valve technology is used to enable temporary adjustment of the properties of the 1D effluent prior to injection into the 2D column (9). Figure 4 shows a representative result from our own work aimed at demonstrating the positive impact of an intervention like this on the quality of the 2D separation in a situation where the impact of the mobile-phase mismatch is severe if left unchecked. A 2D HILIC separation of the fragments of the monoclonal antibody atezolizumab after treatment with the IdeS enzyme and reduction with dithiothreitol is shown in panel (a). Adding a 2D RPLC separation either without or with ASM produces the 2D chromatograms shown in panels (b) and (c), respectively. Separating these protein fragments by HILIC requires a 1D mobile phase with about 70% acetonitrile, and a 2D mobile phase with about 30% acetonitrile for RPLC separation. When there is no mitigation of the effects of this mobile-phase mismatch, the 70% acetonitrile in the 1D effluent causes severe breakthrough of protein peaks in the dead volume of the 2D column due to very low RPLC retention in a high acetonitrile environment. These breakthrough peaks are highlighted by the red rectangle in Figure 4b. On the other hand, ASM enables a reduction in the acetonitrile level to below 30% in the sample that is injected into the 2D column, thereby completely eliminating the breakthrough, as shown in Figure 4c.

Summary

Currently, it is most important that 2D-LC users recognize that breakthrough in the second dimension of 2D-LC separations can be a severe problem, particularly in cases where the degree of mismatch between the mobile phases used in the two dimensions is large. Currently, ACD and ASM are two popular and commercially available solutions that address many, but not all, of these challenges. There are many other potential “homemade” solutions, and several research groups are exploring other potential solutions involving membrane and sorbent-based approaches, to name a few (7,10). Given the importance of this problem it seems likely that we will see other commercial solutions emerge in the not-too-distant future.

References


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Exploring the Efficiency of Various Extraction Approaches for Determination of Crude (4-methylcyclohexyl)methanol (MCHM) Constituents in Environmental Samples

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Crude (4-methylcyclohexyl)methanol (MCHM) is a chemical blend, mainly used in the coal industry for the separation of usable coal from rocks, debris, and coal dust by froth flotation. Following a 2014 MCHM spill in the Elk River in West Virginia, USA, studies demonstrated that MCHM sorbed into water pipes and linings readily desorbed from polyethylene into water at levels above the odour threshold, confirming the risk of its long-term exposure from contaminated tap water pipelines. In light of this, it is imperative to develop analytical methods able to detect crude MCHM components in environmental water samples. In this work, two microextractive methods based on solid-phase microextraction (SPME) in fibre format and thin film microextraction (TFME) were developed and validated. Their performance was compared with a modified solid-phase extraction (SPE) method based on U.S. Environmental Protection Agency (EPA) Method 522 for analysis of volatiles in water. SPME and TFME methods both showed enhanced performance in terms of achievable limit of quantitation (LOQ) compared to the SPE protocol. Moreover, the sensitivity of the TFME method, coupled with its higher analytical throughput, established TFME as the optimal extraction approach for 4-MCHM and other constituents of crude MCHM, with limits of quantitation below the odour threshold for aqueous crude MCHM in 19–21 °C deionized water (0.55 µg/L).

On January 9, 2014, an estimated 37,800 litres (9,986 gallons) of a chemical mixture was spilled into the Elk River upriver from Charleston, West Virginia, USA. This mixture, used in a purification process for coal, contained crude (4-methylcyclohexyl)methanol (MCHM; 88.5%), a proprietary blend of stripped polyglycol ethers (PPh; 7.3%) and water (4.2%). Crude MCHM is composed of (4-methylcyclohexyl)methanol (4-MCHM; 68–89%), 4-(methoxymethyl)cyclohexanemethanol (4MMCH; 4–22%), methyl 4-methylcyclohexanecarboxylate (MMCHC; 5%), dimethyl-1,4-cyclohexanedicarboxylate (DM-1-4-CHC; 1%), 1,4-cyclohexanediethanol (1,4CHDM; 1-2%), water (4–10%) and methanol (1%) (1). The drinking water treatment plant located in Charleston, operated by West Virginia America Water (WVAW), was contaminated along with the water supply of approximately 300,000 residences. This may be an ongoing problem, given that it has been demonstrated that 4-MCHM readily adsorbs and desorbs from pipes made from polyethylene.
materials (2), creating a risk of chronic exposure for contaminated households. Analysis was not performed on the day of the spill, but the next day contamination levels as high as 2400 µg/L 4-MCHM were detected exiting the WVAW treatment plant (3). Concentrations of 4-MCHM in the range of 2–5 µg/L were found persisting from January 20 to February 2, 2014 (4). Analysis of tap water from households indicated that contamination was not limited to the WVAW facility, but also affected the entire water distribution system, evidenced by concentrations as high as 420 µg/L being found in drinking water two weeks after the spill (5). Tap water from residences with different water suppliers were demonstrated to be contaminated by crude MCHM, with 4-MCHM being identified over 600 km from the spill site in Louisville, Kentucky, USA (6). Of the approximate 300,000 households directly affected by the spill, an estimated 25,623 families exhibited health problems, such as rash, skin irritation, respiratory problems, nausea, diarrhoea, and other symptoms linked to crude MCHM, according to the Centers for Disease Control and Prevention (7). It has been established that 4-MCHM damages DNA, with its metabolites being more toxic as a result of their potential to cause greater oxidative stress (8). It has also been shown that low levels of 4-MCHM can be cytotoxic when paired with PPh, with concentrations as low as 1.28 µg/L 4-MCHM and 1.52 µg/L PPh (9). It is thus important to develop analytical methods able to quantitate the components of crude MCHM, as well as the associated metabolites, so as to monitor long term accumulation and release of these contaminants in environmental waters. Previous studies employed liquid–liquid extraction (LLE), coupled with gas chromatography with flame ionization detection (GC–FID) (10), or mass spectrometry (GC–MS) (11). Also, headspace (HS) sampling coupled with GC–FID (2,12), purge and trap (P&T) with GC–MS (6), and headspace solid-phase microextraction (HS-SPME) with GC–MS (13) have also been used to detect 4-MCHM only, given that other components of crude MCHM contribute less in the leaked mixture. In light of new toxicological data (8,9), accompanied by a lack of adequate research on the toxicology and sorption behaviour of the minor constituents of crude MCHM, we herein propose quantitative methods for analysis of 4-MCHM and all associated compounds, including a primary metabolite of 4-MCHM, trans-4-methyl-1-cyclohexanecarboxylic acid (MCHCA) (14). The goal of this study is to quantitate, for the first time, all known components of crude MCHM, comparing the efficiency of various extraction approaches across different methods. The methods developed in this study utilized solid-phase extraction (SPE), direct immersion solid-phase microextraction (DI-SPME), and direct immersion thin film microextraction (DI-TFME). Each method employed GC–MS for separation and detection. SPE is a sample preparation technique commonly used in the analysis of environmental matrices, utilizing a solid-phase sorbent to exhaustively extract and preconcentrate target analytes in the same procedure (15). SPME is a non-exhaustive sample
extraction method accomplished by a supported solid-phase coating, sharing the same advantages as SPE, but with a geometry that allows ease of automation and direct desorption of the SPME device into analytical instrumentation (16). TFME differs in geometry compared to SPME, with the extraction phase being coated on a carbon-mesh film in contrast to the fibre geometry of SPME. The increased surface area of a TFME device compared to a SPME fibre allows it to achieve greater sensitivity, thus making it more suitable for multiresidue trace analysis in environmental matrices (17). Among the methods developed, TFME provided the best results in terms of throughput and limits of quantitation (LOQs) achievable. The TFME based method was thus used for analysis of tap, lake, and river water.

**Experimental Method and Apparatus**

Reference standards of 4-MCHM, MMCHC, DM-1-4CHC, and MCHCA were purchased from Sigma Aldrich (St. Louis, Missouri, USA), along with the internal standards toluene-D₈, and methyl benzoate-D₈. A standard solution of 4MMCH was obtained from Toronto Research Chemicals Inc (Toronto, Ontario, Canada) and 1,4-CHDM was purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). Buffer solutions with pH 4, 6, 8, and 10 were purchased from Honeywell Specialty Chemicals (Seelze, Germany). Materials for SPE included Sep-Pak AC2 Plus short Cartridge (400 mg) from Waters (Milford, Massachusetts, USA), and Cole-Parmer 2 mL wide neck volumetric vial with PTFE/silicone lined cap (Vernon Hills, Illinois, USA). Disposable Falcon tubes (3, 10, and 50 mL) were obtained from Becton Dickinson and Company (Franklin Lakes, New Jersey, USA). HPLC grade water, methanol, and dichloromethane were bought from ThermoFisher Scientific (Fair Lawn, New Jersey, USA). SPME fibres were purchased from Supelco (Bellefonte, Pennsylvania, USA). Car/PDMS thin films were obtained from Gerstel (Linthicum, Maryland, USA). River and tap water samples were collected in glass containers, being completely filled to minimize headspace. The river sample was collected ~0.5 m below the water surface along the bank of the Ohio River located at Portsmouth, Ohio. The lake water was obtained from the west fork of Keuka Lake near Pulteney, New York, in Steuben County, USA, approximately 10 m off shore. The lake water was collected in a plastic container ~1.5 m below the surface. Tap (drinking) water was obtained at a residence in Louisville, Kentucky, USA. Samples were stored in their original containers at 4 °C until analysis. An Agilent 7890 B GC instrument hyphenated to a 5977 B single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA) and equipped with a DB-5ms column (30 m × 250 μm × 0.25 μm, Agilent Technologies), MultiPurpose Sampler, MPS, (Gerstel, Inc., Linthicum, Maryland, USA), a cooled injection system, CIS 4, (Gerstel, Inc., Linthicum, Maryland, USA) and a thermal desorption unit, TDU, (Gerstel, Inc., Linthicum, Maryland, USA) was used for analysis of the crude MCHM constituents. Ultrapure helium (99.999%) was used as the carrier gas at a flow of 1.5 mL/min for each method. For SPE and SPME analysis, the GC oven temperature was held at 50 °C for 2 min, then raised to 220 °C at a rate of 20 °C/min, then held at 220 °C for 2 min. During TFME analysis, the GC oven was programmed at an initial temperature of 50 °C (held for 2 min), then raised to 290 °C at a rate of 20 °C/min, then held at 290 °C for 2 min. The mass

<table>
<thead>
<tr>
<th></th>
<th>SPE</th>
<th>SPME</th>
<th>TFME</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDR (mg/L)</td>
<td>LDR (µg/L)</td>
<td>LDR (µg/L)</td>
<td>LDR (µg/L)</td>
</tr>
<tr>
<td>LOQ (mg/L)</td>
<td>R²</td>
<td>LOQ (µg/L)</td>
<td>R²</td>
</tr>
<tr>
<td>MCHM</td>
<td>0.5–25</td>
<td>0.983</td>
<td>0.5</td>
</tr>
<tr>
<td>MMCHC</td>
<td>0.25–25</td>
<td>0.987</td>
<td>0.25</td>
</tr>
<tr>
<td>4MMCH</td>
<td>0.5–25</td>
<td>0.984</td>
<td>0.5</td>
</tr>
<tr>
<td>1-4CHDM</td>
<td>5–25</td>
<td>0.987</td>
<td>5</td>
</tr>
<tr>
<td>DM-1-4CHC</td>
<td>0.5–25</td>
<td>0.987</td>
<td>0.5</td>
</tr>
<tr>
<td>MCHCA</td>
<td>25–50</td>
<td>0.983</td>
<td>25</td>
</tr>
</tbody>
</table>
### TABLE 2: Accuracy values of the validated SPE, SPME, and TFME protocols

<table>
<thead>
<tr>
<th></th>
<th>SPE</th>
<th>SPME</th>
<th>TFME</th>
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</thead>
<tbody>
<tr>
<td><strong>Accuracy (%)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Relative Standard Deviation (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5 (mg/L)</td>
<td>95.2 (7.5)</td>
<td>95.3* (28.1)</td>
<td>92.0 (5.2)</td>
</tr>
<tr>
<td>30 (mg/L)</td>
<td>100.0 (9.2)</td>
<td>93.3* (20.5)</td>
<td>95.6 (11.3)</td>
</tr>
<tr>
<td>3.5 (µg/L)</td>
<td>97.1 (6.3)</td>
<td>102.6* (24.7)</td>
<td>99.7 (7.9)</td>
</tr>
<tr>
<td>35 (µg/L)</td>
<td>95.3 (6.3)</td>
<td>98.3* (18.7)</td>
<td>73.2 (22.2)</td>
</tr>
<tr>
<td>75 (µg/L)</td>
<td>106.8 (7.6)</td>
<td>90.8* (28.7)</td>
<td>95.4 (15.6)</td>
</tr>
<tr>
<td>3.5 (µg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 (µg/L)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>50 (µg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Concentration value above upper limit of quantitation
† Accuracy values for MCHCA were calculated for TFME at
  a: 7 (µg/L)
  b: 70 (µg/L)
  c: 150 (µg/L)
spectrometer was used in electronic ionization (EI) mode at 70 eV with the MS source being set at 230 °C and the quadrupole at 150 °C, collecting full scan mass spectra. All data analysis was performed with Agilent Masshunter Workstation Quantitative Analysis software (Agilent Technologies, Santa Clara, California, USA) for GC–MS. The response for each analyte was obtained as the sum of individual peak areas for trans and cis isomers, with the exception of DM1-4-CHC and MCHCA.

SPE Protocol
The SPE extractions were conducted according to a modified-protocol based on EPA method 522 (18) before GC–MS analysis. SPE cartridges were first conditioned with 2 mL of dichloromethane. The cartridges were then equilibrated by passing 2 mL of HPLC grade methanol, followed by 10 mL of HPLC grade water through the cartridge. Throughout the equilibration step and onward, care was taken not to allow the cartridges to come to dryness during solvent exchange and sample loading. For extraction, 18 mL of each aqueous solution spiked with the targeted analytes was loaded onto the SPE cartridge. Any water residues was removed from the cartridge by purging it with high purity argon gas for 3 min until dryness. A 2 mL volumetric vial was used for collection of the eluate. Elution of the analytes was performed with the addition of dichloromethane to the cartridge. During this step, dichloromethane was initially used to soak the SPE cartridge for 1 min. Elution then proceeded in a dropwise fashion into a volumetric vial. Additional dichloromethane was added to the cartridge until the eluted volume in the vial was 1.5 mL. To eliminate residual water in the eluate, 0.4 g of anhydrous sodium sulfate was added to each vial. The samples were sealed, and then stored in the refrigerator at 4 °C until GC–MS analysis.

SPME Protocol
The extraction efficiency of five commercially available SPME fibres, namely Carboxen/polydimethylsiloxane (Car/PDMS), divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Car/PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), polyacrylate (PA), and polydimethylsiloxane 100 µm (PDMS), was assessed using a 100 µg/L aqueous solution containing the targeted analytes. Optimization of pH was performed using both Car/PDMS and DVB/Car/PDMS by adjusting the pH of the 100 µg/L aqueous solution containing the targeted analytes to pH 4, 6, 8, and 10 using disodium hydrogen citrate plus sodium dihydrogen citrate plus sodium chloride buffer solution at pH 4, trisodium citrate-2-hydrate plus disodium hydrogen citrate buffer solution at pH 6, sodium chloride plus disodium tetraborate buffer solution at pH 8, and disodium tetraborate plus sodium hydroxide buffer solution at pH 10. The optimized SPME protocol consisted of 1 min incubation, followed by 30 min extraction, both performed at 65 °C and 300 rpm agitation speed. Desorption was performed for 10 min in splitless mode at 300 °C for Car/PDMS, 270 °C for DVB/Car/PDMS, 300 °C for PA, 270 °C for DVB/PDMS and 280 °C for PDMS. The conditions used for desorption prevented the occurrence of carryover of the analytes on the SPME coatings. Carryover was tested by re-desorbing the fibres.

| TABLE 3: Comparison of extraction efficiency between the methods developed in this work and other methods found in the literature |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Total 4-MCHM    | trans-4-MCHM    | cis-4-MCHM      | Reference       |
| SPME-GC–MS      | 1               | –               | –               | This work       |
| TFME-GC–MS      | 0.1             | –               | –               | This work       |
| LLE-GC–FID      | –               | [100]           | [100]           | (10)            |
| HS-GC–FID       | 5380            | –               | –               | (12)            |
| H–P&T-GC–MS     | [0.4]           | [0.16]          | [0.28]          | (6)             |
| HS–SPME-GC–MS   | –               | [23]            | [10]            | (13)            |

[ ] Indicates the value to be reported as a limit of detection
immediately after the first extraction and desorption cycle and verifying the absence of peaks attributable to the targeted analytes in the obtained chromatograms.

**TFME Protocol**

Samples for TFME method optimization were prepared following the same procedures described for SPE and SPME to ensure direct comparison between extraction methods. During extraction, TFME devices were held in place by stainless steel pins penetrating the vial septum, with the thin films fully submerged in the sample solution. Samples were placed in a water bath at 65 °C for 5 min without agitation, and subsequently stirred at 900 rpm for 15 min by a magnetic stir bar placed in the vial. After agitation, the TFME device was quickly wiped, and then placed into a baffled glass desorption liner prior to insertion into the TDU. TDU parameters were first set at 30 °C, held at 30 °C for 0.5 min, raised to 270 °C at a rate of 700 °C/min, then held at 270 °C for 8 min, all performed in splitless mode at a 280 °C transfer temperature. Coupled to the TDU was a CIS 4, with an initial temperature of −50 °C, raised to a final temperature of 280 °C at 10 °C/s, then held at 280 °C for 3 min. Under the desorption conditions used for TFME devices, minimum carry over (<1%) was achieved for the targeted analytes.

**Method Validation**

The methods developed were validated with respect to linearity, precision, accuracy, and limits of quantitation (LOQs). Calibration curves were obtained for each target by plotting the signal ratio of the analyte and the isotopically labelled internal standards (A/Is) for various concentration levels (8 for the SPE protocol and 10 for the SPME and TFME protocols) in three independent replicates. Toluene-D8 was used as internal standard for the SPE method, while methylbenzoate-D8 was used for the SPME and TFME methods. Furthermore, three validation points selected within the linear range of each method were analyzed to assess precision and accuracy. LOQs were calculated as the lowest calibration point, with precision values lower than 20% and accuracy within 70–120%.
**Results and Discussion**

**SPME Optimization**

Various parameters affecting SPME were optimized to achieve the most suitable conditions for extraction of the major components of crude MCHM and a metabolite of 4-MCHM, namely MCHCA. At first, we tested the extraction performance of 5 commercially available SPME coatings, PDMS 100 µm, PA, PDMS/DVB, Car/PDMS, and DVB/Car/PDMS in order to select the most suitable extraction phase. Results shown in Figure 1(a) reveal that the best performance was achieved with Car/PDMS and DVB/Car/PDMS. Considering that SPME coating optimization was performed before optimization of other parameters such as pH and ionic strength of the solution, it was not possible to achieve extraction of MCHCA (a carboxylic acid with pKa = 4.89). Consequently, Car/PDMS and DVB/Car/PDMS were both used to perform the optimization of matrix pH with the purpose of determining which of the coatings could best extract MCHCA. Figures 1(b) and 1(c) compare results obtained adjusting the pH of the aqueous solution at 4, 6, 8, and 10 pH units. The results demonstrate that MCHCA is most efficiently extracted by the Car/PDMS coating, which was selected for further optimization and method validation. Moreover, it was also noticed that adjusting the pH of the aqueous solution to 4 guaranteed simultaneous and best extraction of all the analytes targeted in this study. The next step was the optimization of the sample ionic strength. The adjustment of ionic strength was performed by adding opportune concentrations of NaCl to aqueous samples adjusted to pH 4. However, changes in ionic strength also resulted in variations of the final pH of the sample originally adjusted at 4. A similar effect was noticed by changing the type of salt (potassium nitrate and magnesium nitrate) used for ionic strengths adjustments. Therefore, we decided to perform two separate sample preparations to extract MCHCA. The sample was adjusted to pH 4 with no adjustment of ionic strength; for the remaining analytes, the sample was kept at pH 7, adjusting the ionic strength to 20% with NaCl, per the results obtained in Figure 1(d).

Extraction temperature was also evaluated from 35 to 85 ºC (Figure 2(a), representative results for 4-MCHM extraction time profile at 75 ºC and (c) 65 ºC. (d) Extraction time profile at 65 ºC of four crude MCHM constituents.)
MCHM). From the trend obtained, the best extraction performance was achieved at 85 °C. However, due to pressure built up in the vial with consequent partial deformation of the vial cap septum, 75 °C was selected as the optimum, since no pressure buildup occurred in the vial. While performing the extraction time profile for MCHM at optimized extraction conditions (pH 7, 20% NaCl content, 75 °C extraction), high variability of the measurements and a decline in the MCHM response over time were observed (Figure 2(b)). This indicates that a probable degradation of the analyte occurred under the used experimental conditions. To investigate if the extraction temperature were the main contributing factor to the trend observed, the extraction time profile was repeated at 65 °C, while keeping all the other parameters constant. As can be seen in Figures 2(c) and 2(d), good reproducibility was obtained with a reasonable trend for the equilibration process. Therefore, 65 °C was considered the optimum extraction temperature for further testing and validation. Moreover, 30 min extraction time was selected as the best compromise between analyte response and analysis throughput. In summary, the extraction conditions used for 4-MCHM, 4MMCH, MMCHC, 1-4CHDM, and DM-1-4-CHC were Car/PDMS SPME fibre, 30 min extraction time, 65 °C extraction temperature with the sample adjusted at pH 7 with 20% (w:w) of NaCl. For analysis of the MCHM metabolite MCHCA, the optimized extraction conditions were: Car/PDMS SPME fibre, 30 min extraction time, 65 °C extraction temperature, with the sample adjusted at pH 4 with no adjustment of the ionic strength of the solution.

**TFME Optimization**

During SPME optimization, it was determined that the extraction phase Car/PDMS was best suited for analysis of crude MCHM components. Consequently, we used a Car/PDMS TFME device for further method optimization and validation. Some of the optimized operating conditions, such as sample ionic strength, pH, and extraction temperature, are unaffected by the geometry of the microextraction device, and thus further optimization of these parameters for the TFME method was unnecessary. However, parameters such as extraction speed and extraction time required further optimization as extraction by TFME devices was not automated (Figure 3[a]). Moreover, the microextraction device geometry, as well the agitation method used, directly influence the kinetics of the extraction process. Figure 3(b) compares the extraction performance achieved at agitation speeds of 600, 900, and 1200 rpm during extraction, each experiment being performed at optimized conditions. Results show that at 1200 rpm extraction efficiency is the greatest, however, with greater variability compared to the comparably efficient 900 rpm; thus, 900 rpm was chosen as the optimal agitation speed. Using this new optimal agitation speed, figures 3(c) and 3(d) show the extraction time profile for the analysis of crude MCHM constituents by TFME using all optimized parameters. Obtained results clearly demonstrate that all analytes, with the exception of MMCHC, equilibrate at the optimal 15 min extraction time. MMCHC equilibrated soon after 15 min, however, considering its high affinity for the extraction phase, extraction was performed conveniently at 15 min for all the target analytes.

**Method Validation and Analysis of Real Samples**

A summary of the results obtained from the method validation of the SPE, DI-SPME, and DI-TFME methods are presented in Table 1. LOQs obtained with the SPE protocol were several orders of magnitude higher than the results of the other two extraction techniques tested, indicating that this technique is not suitable for extraction of crude MCHM constituents at trace level.

### TABLE 4: Analysis of crude MCHM constituents spiked at 45 µg/L in different environmental matrices

<table>
<thead>
<tr>
<th></th>
<th>Tap Water</th>
<th>Lake Water</th>
<th>River Water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Accuracy (%)</strong></td>
<td>RSD (%)</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>4-MCHM</td>
<td>127.2</td>
<td>13.1</td>
<td>123.6</td>
</tr>
<tr>
<td>MMCHC</td>
<td>69.6</td>
<td>5.9</td>
<td>87.4</td>
</tr>
<tr>
<td>4MMCH</td>
<td>63.3</td>
<td>6.2</td>
<td>63.9</td>
</tr>
<tr>
<td>1-4CHDM</td>
<td>117.5</td>
<td>12.6</td>
<td>115.0</td>
</tr>
<tr>
<td>DM-1-4CHC</td>
<td>77.1</td>
<td>13.9</td>
<td>94.6</td>
</tr>
<tr>
<td>MCHCA</td>
<td>61.6</td>
<td>20.6</td>
<td>65.8</td>
</tr>
</tbody>
</table>
Linear ranges of most compounds were very narrow, notably for MCHCA; some ranges spanned only one order of magnitude or less. SPME and TFME both circumvent the caveats of using SPE, with the two microextraction protocols exhibiting similar linear ranges and coefficients of determination. TFME recorded lower limits of quantitation than both SPE and SPME for most compounds (Table 1) with comparable accuracy, except for the accuracy level at 3.5 ppb (Table 2). Further comparison with previously published work demonstrates that the microextraction methods developed in this study, in addition to being the first to simultaneously detect and quantitate all known components of crude MCHM, also quantitate 4-MCHM at concentrations at levels lower than any previous published method. These results are shown in Table 3. Compared to other extraction techniques evaluated in this study, TFME recorded lower limits of quantitation and better throughput; extraction times are shorter than SPME (15 min for TFME vs. 30 min for SPME). Consequently, TFME was chosen as the best extraction protocol to follow for analyzing real matrices. Tap, river, and lake water samples were chosen to compare different possible matrices where 4-MCHM and its constituents might be detected. No detectable amount of the target analytes was discovered in the samples. Subsequently, each sample was fortified with the targeted analytes and tested for accuracy using the TFME method developed and validated as mentioned above. Results shown in Table 4 reasonably validate the TFME protocol for analysis of real matrices for the contributing compounds of crude MCHM and one 4-MCHM metabolite, MCHCA.

Conclusions
For the first time, methods utilizing SPME and TFME were developed and optimized for simultaneous analysis of 4-MCHM and all other known components of crude MCHM. MCHCA, a primary metabolite of 4-MCHM, was also determined independently with only minor modifications to the sample preparation protocol. The performance of these microextraction-based analytical methodologies was compared to an SPE method that was also tested in this study. Our results show that lower limits of quantitation can be achieved with microextraction methods. Relating these results to previously developed methods, both SPME and TFME are competitive in detecting and quantitating 4-MCHM. The TFME-GC–MS method recorded a limit of quantitation for 4-MCHM lower than any currently known method, with only 15 min of extraction time needed. To validate this, water samples from various sources were analyzed using TFME and the accuracy of the method was determined. The sensitivity of the TFME method, coupled with its increase in analytical throughput, made it clear that TFME is the optimal extraction approach for 4-MCHM and its constituents found in crude MCHM.

Acknowledgements
This work was supported by funds provided by The University of Toledo. The authors are grateful to Gerstel USA, particularly to Robert Collins for enabling the use of Gerstel TDU unit in our laboratory, to John Stuff for the useful scientific discussions and for providing the TFME devices used in this work.
and to Daniel Gatch for the expert technical support with the MPS autosampler and the TDU unit. The authors also thank Jon Kirchhoff and Daniel Gatch for providing the lake and tap water samples, respectively. Acknowledgements are also due to Rachel Avina, Elijah Long, and Tharuka Ubayasena for their assistance in the early stages of this work.

References

18) J.W. Munch, P. Grimmet, EPA Method 522, Determination of 1,4-Dioxane in Drinking Water by Solid Phase Extraction (SPE) and Gas Chromatography Mass Spectrometry (GC/MS) with Selected Ion Monitoring (SIM), 2008.

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Control or Chaos: How Can We Run a Gas Chromatograph from Anywhere?

Nicholas H. Snow, Department of Chemistry and Biochemistry, Seton Hall University, New Jersey, USA

This instalment of “GC Connections” is a follow up to September’s column “From Detector to Decision: How Does the GC Instrument Generate Your Data?” This time, we explore the other side of the instrument-data system relationship—how the data system controls the functions of the instrument. Drawing on classical electronics and instrument designs, we see the evolution of instrument controls from knobs and gauges on the front panel of the instrument to computer control and, finally, to today’s web-based systems that allow instrument control and monitoring from anywhere. We see that the same fundamental electronic principles used to manually control gas chromatographs in the 1970s are still at the centre of today’s modern electronically controlled systems.

In our previous column, we explained that the controls for today’s gas chromatographs include an on-board computer that provides the low voltage signals that actuate and direct analog devices that control functions such as starting, stopping, and data collecting (1). Taking this idea one step further, we see that this computer controls all aspects of instrument operation, including temperature and flow control and valve or switch actuation. As seen in the September column, the basic controls (valves opening and closing, heaters heating, and switches moving) for data collection and analysis are still analog operations that are performed by mechanical devices even though these devices have been miniaturized. Digital control of these analog devices is what allows a gas chromatograph (GC) instrument to be operated from an external data system or even from off-site as long as the requisite software and networking are available.

Figure 1 shows the flow of data or instructions between an external control system and an electronic component on a GC instrument, such as the inlet heater. As the user, you want to instruct the instrument to heat the inlet to 250 °C. First, you enter the value into the data system (or on the front panel of the instrument if it has a keypad or touch screen). Second, the data system transfers the request to the instrument using one of the communication protocols (typically USB or ethernet) where the microprocessor on the instrument receives the signal and, through its on-board software, sends a low-voltage signal to a relay, which closes a mechanical switch to activate the heater. A second circuit within the inlet block includes a temperature sensor (usually a temperature-sensitive resistor) that provides a signal back to the on board software when the desired temperature is reached so that the heater circuit can be opened (in other words, turned off).

With digital electronics and software providing the interface between the operator and the device (in this case the inlet heater), we can see that, if the software can be operated through a data system separated from the GC instrument over a company intranet or over the internet, then the instrument can as well. Even though software provides the user with convenient and powerful controls, the instrument itself still relies on mechanical devices to operate. Although these devices have been miniaturized, they still fundamentally perform the same tasks that the first GC instruments did. In our previous instalment, we discussed the basic principles of digital and analog electronics in gas chromatographs. We saw that, although instrument control and data collection are now digital, the underlying chemistry that occurs at the detector to generate signals and the underlying mechanics that allow control of temperatures, gas flows, and instrument functions, are analog. We noted that analog and digital signals can be interconverted and that digital signals also provide the capability for instruments to have friendly and easy-to-use controls and user interfaces.
In this instalment, we look more closely at the processes by which modern GC instruments are controlled on the front end, with functions such as starting and stopping, actuating valves, such as the split purge vent, controlling temperature zones, and gas flows. As we did in September, we see how these operations work in a modern GC instrument by looking back at some older systems in which these operations were performed manually or with mechanical controls.

All of the control functions on early GC instruments were manually operated, as seen in many pictures of early systems available on the Internet, such as a photograph of an F&M Scientific Model 700 dual column gas chromatograph of the early 1960s, available from the Science History Institute website (2). Several knobs and switches and gas outlet ports can be seen. Needle valves and manual regulators controlled the gas flows. The column oven was accessed from the top. Clearly, this instrument required the operator to be present to perform every function, including injecting the sample; there was no auto-sampler.

The several knobs on the front of this and other early and simple gas chromatographs provided the familiar temperature controls for the column oven, detector, and injector port. Additional knobs provided control functions for a thermal conductivity detector, including filament current, with coarse and fine adjustments and signal attenuation. A chart recorder (not shown) would have plotted the analog detector signal versus time on a roll of paper. Compared to today’s instruments, this system was also very bulky, had high power consumption, and required the user to be present for every analysis. Following the analysis, data processing was done by hand, with the chromatogram being printed out on a paper roll. Instrumental conditions were entered by hand directly on the paper or entered into a lab notebook.

The knobs for the temperature controllers are called potentiometers, which provide a convenient means for regulating the voltage by changing the...
important control functions on any gas chromatograph: the remote start–stop and valve actuators, the inlet (and detector) heaters, and the split vent (purge) valve. Although we now control these through the keypad, software, or an app, and even though the some of the devices themselves have been miniaturized, they still perform the same functions as on past instruments.

In September, we examined the remote-control port on a 1990s-era GC instrument and saw several control functions. Each of these functions works using a simple relay called a contact closure, essentially an electrically operated switch that can leave the circuit open (no current is flowing) or closed (current flows). The flowing current in a closed contact closure can direct the actuation of a switch or valve or perform other tasks. The contact closure is directed to open or close by the application of a small voltage to the switch.

Figure 3 shows a simplified circuit diagram including a contact closure relay. A common relay for a single switch includes four contacts: Two are for the circuit being opened or closed, and the other two are for controlling the relay itself, as seen by the four leads shown in Figure 3. In the case of the start–stop function, when the GC instrument is not performing a run, the circuit is open (the switch is off). A low-voltage signal from a data system is sent to the relay, which mechanically closes the circuit, causing current to flow and starting the GC instrument. The relay is much like turning on the switch for a light bulb. When the instrument starts, the relay usually switches “on”, then resets quickly to the “off” position, generating a start “pulse” that instructs the instrument to begin the run. A similar relay instruction is then used at the end of the run for the GC instrument to signal to the data system and other devices that the run is over.

The zone heaters are a second set of analog devices that are now controlled digitally. All gas chromatographs have three heated zones: the inlet, the column oven, and the detector. The inlet is heated to assist in vaporizing the injected sample, the column oven is heated to control the separation, and the detector is heated to keep it clean. Each of these heaters is a direct current device that operates according to Ohm’s Law and Joule heating, often called ohmic or resistive heating. The relationships between voltage (V), current (I), resistance (R), and heat (J) are given by:

\[ V = IR \quad [1] \]

\[ P = Jh = R^2I^2 \quad [2] \]

In short, heat is generated in a resistor proportionally to the square of the input current and proportionally to the resistance. The heaters in the oven, inlet, and detector are simple resistors to which current is applied to produce heat, much in the same way as the oven in your kitchen. On older GC instruments, these heaters were controlled using the potentiometers on the front of the instrument. On newer, digitally controlled GC instruments, the heaters are turned on and off by low voltage signals sent to relays, as described above and the temperature is monitored by a second circuit using a temperature-sensitive resistor.

Gas flow control valves are a third necessary control in a GC instrument. A typical solenoid valve classically used in split–splitless inlets to switch between split and splitless mode is shown in...
The fundamentals of split and splitless injection modes were discussed in a recent “GC Connections” installment (2). In short, the solenoid valve is used to redirect most of the carrier gas flow from the glass sleeve to the split purge vent during splitless operation, without changing the overall flow into the column.

The valve is actuated by a simple contact closure that directs the valve to be either “open” in split mode or “closed” in splitless mode. When the valve is open in a split injection, flow enters through both the septum purge and split vent lines and exits to the septum purge vent and to the total flow controller and back pressure regulator ultimately to the split vent. When the valve is closed for a splitless injection, the split vent inlet line from the inlet is closed, forcing all of the flow to enter the valve through the septum purge line and still exit through both exit lines. With the inlet still pressurized this has the effect in the inlet of reducing the flow through the inlet liner for the splitless injection. With these older valves, an audible “click” could be heard whenever the valve was opened or closed. For many old-school chromatographers, this sound was routine and comforting.

Figure 4 shows several additional gas lines and wires seen in and around those for the split–spitless inlet, which serves as a second inlet in the pictured system. Today’s gas chromatographs include...
all of the pneumatic components and the associated electronics for operating the inlets in single pneumatic packs that can be easily inserted or removed from the main instrument. Although this provides easy installation and “plug and play” convenience, it comes at the cost that none of the components are user serviceable. In the 1990s, if the solenoid valve wore out (because it is mechanical; it eventually would), it was a simple and inexpensive matter to order a new one and replace it. If the solenoid valve equivalent or another single component in the pneumatic pack has to be replaced, the entire unit usually has to be replaced, often at a significant cost.

Within the GC instrument, all of these electronic components are controlled through the main board, which is shown in Figure 5. Most interesting to note is the Z-80 microprocessor developed in the 1970s still finds its use today in a variety of devices in the “Internet of things”. The chip with the white label is the instrument’s own firmware; the actual programming that runs the GC instrument. Between them is a static memory chip. All of the devices we have discussed so far—potentiometers, relays, valve actuators, flow controllers, and regulators—have been miniaturized and placed on chips or in solid-state cartridges in most modern gas chromatographs. They can be easily actuated using the same low-voltage pulses (binary, on and off) involved with the transistor–transistor logic (TTL) and digital communications we discussed in September.

The development of solid-state electronic controls and devices for the various valves, switches, and controllers needed on a modern GC instrument have revolutionized instrument design and control. Miniaturized GC instruments designed from the “ground up” for capillary GC and ease of operation are now commonplace and have progressed greatly from the large, fully manual systems used in the 1970s. Connecting these microprocessor-controlled instruments directly to the Internet or through a company intranet provides the possibility of operating the instrument and analyzing the data remotely. Even as the instruments have become smaller and the once bulky control devices are now contained in solid-state electronic or pneumatic packs, the same basic electronic and mechanical controls still underlie instrument operation. By looking back at these more bulky components and how they operate, we can better understand what these automated devices are doing and how they are doing it.

References
2) https://www.sciencehistory.org/model-700-dual-column-gas-chromatograph

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What’s Good About the WHO Good Chromatography Practices Guidance? Part 1

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In September the World Health Organisation (WHO) issued a new guidance document on Good Chromatography Practices. What guidance does it contain and is it useful? Has the document failed its SST acceptance criteria?

Chromatography data systems (CDSs) have been at the heart of data integrity falsification, fraud and poor data management practices for 15 years since Able Laboratories 483 Observations were published by the FDA in 2005 (1,2). Although regulatory authorities have published much guidance on data integrity (3–7) there is little detailed guidance for chromatography data systems (CDS) except for the Parenteral Drug Association (PDA) Technical Report 80 where there is a good section on peak integration (8).

In September, the World Health Organisation (WHO) published Technical Report Series (TRS) 1025 which is the 54th report from the WHO Expert Committee on Specifications for Pharmaceutical Preparations, a slimline 345 pages for download. Tucked away in Annex 4 is a section entitled Good Chromatography Practices of obvious interest to the readership of LCGC Europe (9). As I’m reading the document and writing this column, I can attest to the effectiveness of lalochezia or using swearing to relieve frustration.

The Curate’s Egg
The WHO Good Chromatography Practices (GChromP) document is best described as a curate’s egg. This expression was coined from a cartoon published in a British satirical magazine in the late 19th century that pictured a curate (assistant to a vicar) having breakfast with a Bishop and the curate is eating a rotten egg. When the Bishop points this out the curate responds politely, if rather stupidly, that the egg is good in parts. The same can be said of the WHO chromatography guidance document (9). However, there are more bad parts than good which makes this a poor guidance document for chromatography.

Dopey Document
Anything issued by a regulatory authority should be exemplary to demonstrate how document controls should be implemented. This is not the case here. The numbering of the clauses within the document does not comply with ALCOA+ criteria as clause 1.4 is missing and there are two 6.1 clauses and two 8.1 clauses (9). How can you trust a guidance that can’t get the simplest things right? Anyone for accuracy? Quality oversight appears conspicuous by its absence.

What is the Aim of this Guide?
Here’s where the problems start. The “Introduction” and “Scope” section has five clauses (even though it finishes at 1.6) that make general statements about the use of chromatography in QC laboratories, the sample types that can be analysed and the types of the methods that can be used. Intermingled with this are statements:
• Observations during inspections have shown that there was a need to a specific good practices (GXP) document (clause 1.1)
• Owing to the criticality of results obtained through chromatography, it must be ensured that data acquired meet ALCOA+ principles … (clause 1.3)
• This document provides information on GXP to be considered in the analysis of samples when chromatographic methods and systems are used (clause 1.4) (9).

Here and throughout the document, there are very general and non-
specific statements. Statements are often clarified with conditioning words and phrases such as “where appropriate”, “it is preferable”, “may”, “should” (there are 121 “shoulds” vs. one “must” in the document). There is no reference to the WHO Good Records Management guidance and Annex 1 containing the best description of the ALCOA criteria (5) except in clause 14.1 at the end of the document. A focused introduction with emphasis on good record keeping and data integrity practices would have been preferable.

**Structure of the GChromP Guidance**

The document structure is curious as sections appear to have been arranged as if pulled at random from a hat with one or two options left behind. The two main sections dealing with set up and system are sections 3 and 8. Now I fail to understand why section 8 on an electronic system that includes important information on system architecture (standalone versus network) is stuck in the middle of the document when it should be next to or integrated with section 3 that discusses supplier and system selection.

What is equally baffling is the total omission of the phrase chromatography data system from the entirety of the document. Instead readers are treated to a comedy of errors in calling a CDS an Electronic Data Management System (EDMS) (8.1) or computerised chromatographic data-capturing and processing system (13.2). What would have been much better is to have sections 3, 4, and 8 together with sections 5–7 on set up and configuration of the application software and interfacing of the chromatographs.

Look at the chromatography process, shown in green in Figure 1, in the words of that great analyst, Commander Spock, it is a chromatography process, but not as we know it. Presented in sections 9–14 is a selection of tasks but when seen objectively cannot be considered a chromatography process. Emmental anyone? After more lalochezia, I will continue.

**Sins of Omission**

Apart from the wacky order of sections, what is missing from this document? Here’s a list at the system level:

- A CDS with a database is preferable to one that stores data in operating system directories
- There is no discussion of how to avoid hybrid systems and the problem of linking paper printouts with the CDS electronic records (10)
- Avoid using spreadsheets as discussed in a recent Questions of Quality column (11) as all SST calculations are standard in CDS software and using custom calculations means that all records are in one location
- Cybersecurity to protect all chromatographic data is conspicuous by its absence
- Avoid using USB sticks to transfer data
- Leveraging supplier’s software development work to reduce the amount of in-house computer validation is not mentioned (12)
- There is no mention of using the CDS settings to protect e-records or use electronic signatures which are documented in a configuration specification
- There is no mention of the advantages of using the technical controls that are validated once and used multiple times versus inefficient, error prone and manual procedural controls as discussed in the WHO’s own data integrity guidance (5).

**Goofy Glossary**

Section 2 is the glossary with 12 definitions not defined in existing WHO sources (9) plus a cross reference to national pharmacopoeias. However, the terms for audit trail, backup, (computerised system) validation, data, data integrity and metadata have been published already in WHO TRS 1019, Annex 3, Appendix 5, a year earlier (13). There are three definitions that need to be discussed now:
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• **Sample set**: This term should be sequence file as sample set is used in a commercial CDS application and should not be used in any guidance document. The same is true for system and project audit trails later in the document.

• **Validation**: This is defined as The action of proving and documenting that any process, procedure or method actually and consistently leads to expected results (9). This definition is wrong as it does not include software within its scope and does not mention the specification of the system to be validated. Moreover, there is a much better definition in WHO’s TRS 1019 where the definition of computerised system validation is Confirmation by examination and provision of objective and documented evidence that a computerised system’s predetermined specifications conform to user needs and intended use and that all requirements can be consistently fulfilled (13).

• **Source data**: This definition is wrong as it fails to understand the term. Source data or raw data has been copied from the MHRA’s GXP data integrity guidance (4) which is also wrong (14). Source data is defined as Original data obtained as the first-capture of information, whether recorded on paper or electronically (9). This is wrong. Source data is a Good Clinical Practice term and is defined in ICH E6(R2) 1.51 as All information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents (original records or certified copies) (15).

This ICH definition is equivalent to GLP definitions of raw data in FDA and OECD regulations (16,17) and also EU GMP Chapter 4 raw data (18) and FDA GMP complete data in 21 CFR 211.194(a) (19). The scope of complete data and raw data have been described in earlier Questions of Quality (20) and Data Integrity Focus publications (21) and must consist of ALL data and records generated during analysis. Incidentally, a recent draft OECD GLP guidance on data integrity (22), based on MHRA guidance documents (3,4) now has a correct definition of raw data that is consistent with source data and GLP raw data definitions discussed above.

An omission from the glossary is a definition of dynamic and static data. This is necessary as chromatography data are dynamic as they must be interpreted with the requirement to retain them in dynamic format throughout the record retention period. This is stated in the WHO data integrity guidance issued in 2016 (5) as well as the MHRA (4) and FDA guidance documents (6) but there is no cross reference in the GChromP guidance. Please excuse me for a short break to shout some words from the Anglo-Saxon portion of the English language.

**Chromatographic Systems and Electronic Systems**

These are the titles of sections 3 and 8 respectively and there are two main questions.

1. Why are they separated?
2. Why is there no specific mention of chromatography data system software?

The former section presents clauses requiring the system to meet regulatory and GXP requirements (is there a difference?) and meet ALCOA+ principles. Summarising the requirements here, there need to be agreements outlining responsibilities between the supplier and the purchaser, supplier selection and qualification should ensure that hardware and software are suitable for intended use and suitably sited with the correct environmental conditions. However what hardware are we talking about: chromatographs, application servers, data storage, IT infrastructure or all of the above? This section appears to suggest that qualification by the supplier is acceptable but a supplier has no control over a laboratory’s intended use.

The problems mount when we consider section 8, strangely entitled electronic systems. No mention of CDS software at all but bizarrely the controlling application is now called an Electronic Data Management System (EDMS)! The consideration of standalone and networked systems is discussed here with the preference for the latter and the former to be risk assessed and appropriately managed, whatever that means. In the four-part series by Burgess and McDowall on the ideal chromatography data system, we stated that standalone systems are inadequate and inappropriate for a regulated laboratory (23–26). We make the point in the series that even for a single chromatograph a networked virtual server is possible to avoid storing data on a standalone PC. However, the major issue is why is this point not integrated with section 3 as this is a key consideration for selection of the CDS application? It should not be dumped in the middle of the document like an island in an ocean of stupidity.
Omitted from either section is the need for a database to organise chromatographic data rather than files stored in directories in the operating system where they are more vulnerable and audit trails are ineffective. Nowhere in the document is the need for documenting the configuration of the software to protect electronic records. The document also lacks a discussion of technical versus procedural controls and why technical controls are preferable, as discussed in several places in the WHO’s own guidance from 2016 (5), yet there is no mention of them here or a specific reference this guidance.

There is also the requirement in 13.2 that chromatographs should be interfaced to a CDS (rather than detector output being fed to a chart recorder). I appreciate that in some countries where WHO operates there are equipment limitations, but this statement would be much better placed in section 3 or 8 and not at the back of the document. Specification of a chromatograph and CDS software was discussed in a recent Questions of Quality column to give an idea of what is entailed in this process that should be done before selecting the supplier (27).

**Qualification, Validation, Maintenance and Calibration**

Although this section covers the four items in the heading I want to focus on the initially on validation of the software. Incidentally, software is not mentioned anywhere in this section which is a critical failing. The problem I have is the approach to the validation of a CDS does not match reality and I doubt the technical and practical expertise of the writers of this section as well as the quality oversight which is either woefully inadequate or non-existent.

Section 4.3 states that all stages of qualification (this should be validation) should be considered. But of what? The scope of the qualification is not stated. However, whatever we are qualifying we need a user requirements specification (URS), design qualification (DQ), factory acceptance tests (FAT), site acceptance tests (SAT), installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ). This is stupid, from a personal perspective of 35 years experience of validating laboratory informatics applications and qualification of analytical instruments I have never required FAT or SAT as this is applicable to production equipment as shown in my book on the subject (12). There is the grave omission of documenting the software configuration to protect the electronic records or implement electronic signatures and the need for user requirements to be traceable throughout the life cycle (12). Interestingly, the reference for computerised system validation (CSV) is quoted as WHO Technical Report Series 1019 Annex 3, Appendix 6 (20), but the requirements for CSV are found in Annex 3, Appendix 5 (13), referenced in further reading! Pay attention! Are you following this? In Appendix 5 on CSV there is NO reference to FAT and SAT but CSV does require a configuration specification to record the application settings. However, Appendix 6 for Guidelines on Qualification which is mainly for production equipment has requirements for FAT and SAT (28). This is not a typographical error. Strike one for quality and technical oversight of this guidance.

**Access and Privileges**

Definition of user roles and their access privileges is presented in section 5 (9). This is relatively good, requiring a procedure for user account management, a list of current and historical users, no conflicts of interest with the allocation of administrator rights. Clause 5.5. notes that the settings on paper should match those allocated electronically (presumably in the CDS software that is not mentioned, again). I disagree with one item in 5.1 that the SOP for user account management covers the creation and deletion of user groups and users. There is no mention of modification which is typical in many laboratories. For example, a new hire will be assigned a trainee role and after a period of assessment their privileges will be modified to analyst. Also, I disagree with deletion of user groups and users; best practice is to disable an account as this leaves the user identity in the system and does not allow the creation of a new user with the old identity. This is an essential requirement for ensuring attribution of action to an individual i.e. the first A in ALCOA+.

**Audit Trail**

This section has a fatal flaw in clause 6.1 that renders the whole section useless by omitting monitoring deletions of data (9). The writers may hide behind for example but guidance needs to be explicit especially when it comes to audit trail functionality. In contrast, the definition of audit trail in the glossary does include deletion. Strike two for consistency and quality oversight.

In 6.3 the clause mentions system and project audit trails which with sample set is explicit terminology for the functions for a commercial CDS
application. As such these should not be part of a non-partisan guidance document from a regulatory authority.

**Date and Time Stamps**

Date and time stamps are important for ensuring data integrity and it is good that there is a section on the subject. However, like the curate’s egg there are a few omissions. Section 7.2 requires the clock to be locked which is a bit stupid if the CDS is installed on a standalone PC with no option to correct time. A better phrasing would be to state that access to and changes of the clock should be restricted to authorised individuals and documented. For networked CDS applications, there is no mention of a trusted time source to ensure the time is correct within a minute as stated in a withdrawn draft FDA guidance on time stamps (2002). There is no mention that the time and date format should be unambiguous and documented in specifications for the system.

In addition, what happens with summer and winter time changes? The only guidance on this topic comes from an FDA Guidance for Industry on Computerised Systems in Clinical Investigations: "Controls should be established to ensure that the system’s date and time are correct. The ability to change the date or time should be limited to authorized personnel, and such personnel should be notified if a system date or time discrepancy is detected. Any changes to date or time should always be documented. We do not expect documentation of time changes that systems make automatically to adjust to daylight savings time conventions (29)."

However, the configuration of the operating system to adjust time automatically should be included in the specification documents as well a statement that the change will not be recorded.

**To Be Continued…**

In the second part of this review of the WHO’s *Good Chromatography Practices* guidance document we will look at what the guidance says for performing chromatographic analysis.

**References**


20) R.D. McDowall, 31(1) 18–21 (2016).


22) OECD Draft Advisory Document of the Working Group on Good Laboratory Practice on GLP Data Integrity, Organisation for Economic Cooperation and Development: Geneva (2020)


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