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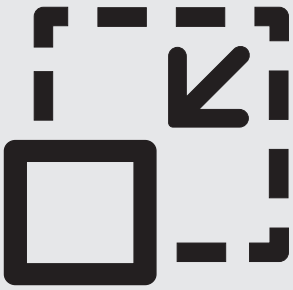

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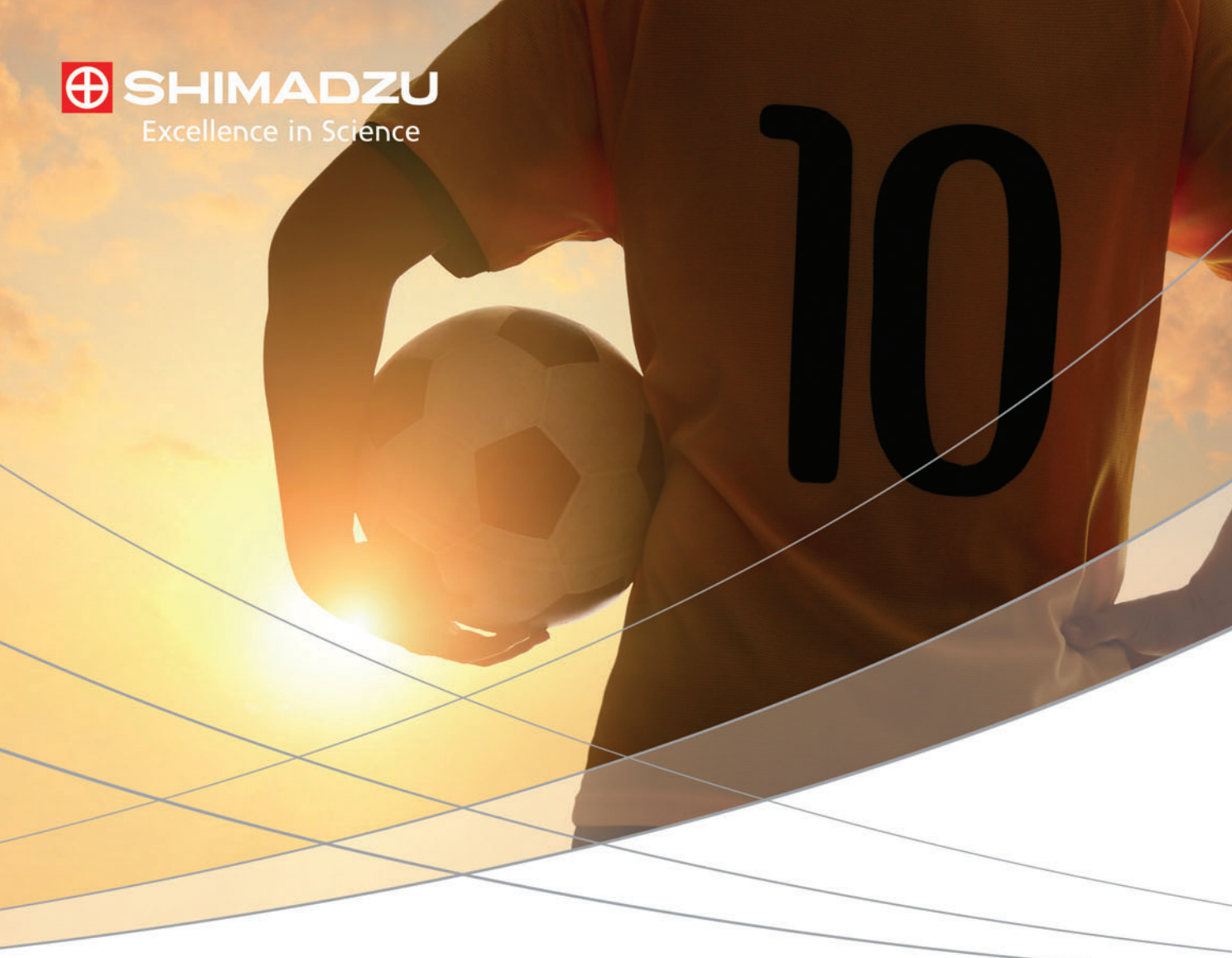

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COVER STORY

COLUMNS

DEPARTMENTS

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INTERVIEW

The Benefits of IEC to Monitor Charge Heterogeneity in Monoclonal Antibodies

The Column spoke to Richard Shannon from AstraZeneca about his work characterizing mAbs, why IEC is his technique of choice for analyzing mAbs, and offers his advice for anyone wanting to use the technique.

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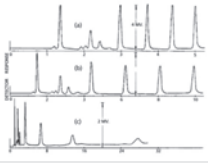

GC CONNECTIONS

Temperature Programmed GC: Why Are All Those Peaks So Sharp?

Nicholas H. Snow dives into temperature programming, examining several points, including the differences in peaks between temperature programmed and isothermal chromatograms, and its requirement in splitless injections.

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INCOGNITO

How Much Longer Can Chromatography Last?

Incognito is thinking long-term. Is there scope for hope in the future? What progress has chromatographic science made and what we might expect in the third and subsequent decades of the century.

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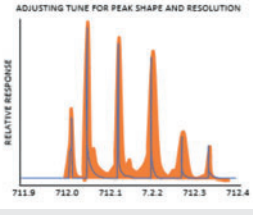

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Clinical Metabolomics: Expanding the Metabolome Coverage Using Advanced Analytical Techniques

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Metabolomics, the comprehensive analysis of all metabolites and intermediate products of reactions present within a biological system, is a promising field to enable precision medicine. Clinical metabolomics faces two main challenges at the bioanalytical level. The first is the need for high resolution to obtain maximum metabolome coverage. This is exemplified by the latest version of the Human Metabolome Database (HMDB), which reports more than 110,000 metabolites and endogenous compounds. The second is the high-throughput needed to enable the analysis of a large number of samples typically encountered in large-scale cohort studies. Reversed-phase liquid chromatography (LC)—at regular or ultrahigh pressures—combined with high-resolution mass spectrometry (HRMS) has long been considered the "gold standard" in metabolomics. However, these conventional reversed-phase LC–MS approaches are no longer sufficient to analyze the vast variety of polar compounds, as well as discriminate closely related compounds such as isomers or enantiomers. This review article discusses the novel separation and detection strategies that are considered promising in clinical metabolomics to enhance the metabolome coverage. It includes hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), multidimensional LC approaches, as well as ion-mobility mass spectrometry (IM-MS) and data-independent acquisition (DIA) analysis methods.

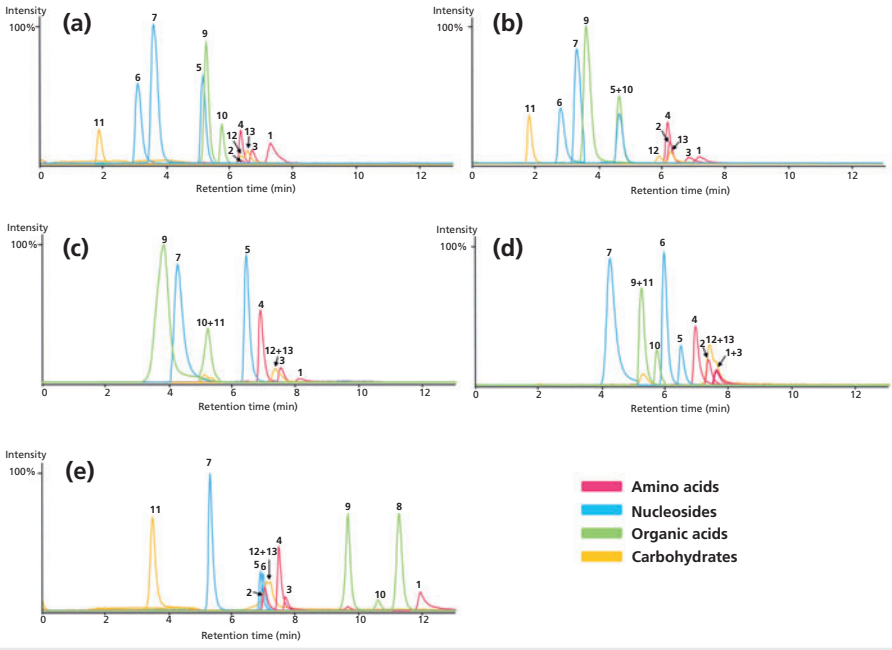
KEY POINTS

- **The analytical portfolio focusing on metabolomics is increasing.**
- **Advanced separation approaches, such as HILIC, SFC, and 2D-LC, enable metabolite separations not achievable with classical methods.**
- **New mass spectrometric approaches, such as DIA and IM-MS, add an extra dimension of separation to the analytical workflow.**
- **These relatively new technologies show clear potential to increase metabolome coverage.**

Metabolomics, first formally introduced in the early 2000s and described as the comprehensive analysis of all metabolites present within a biological system, has attracted a growing interest over the last decade in clinical research. Together with other "omics" approaches, such as genomics and proteomics, metabolomics plays a key role in the implementation of personalized medicine. Two approaches are typically considered in metabolomics. In targeted metabolomics, known metabolites from given biochemical pathway(s) are measured in a quantitative manner. Untargeted approaches, on the other hand, focus on the global and unbiased analysis of the highest number of compounds included in the metabolome leading to qualitative and semiquantitative information (relative differences between populations). Both approaches have been increasingly used over the last couple of years in personalized medicine and drug discovery, in the aim of finding new metabolite biomarker candidates for earlier and more accurate diagnosis, improve the prognosis and staging of diseases, and increase the global understanding of pathophysiological processes via the discovery of novel biomolecular pathways (1,2).

In 2017, the fourth version of the Human Metabolome Database (HMDB 4.0) covered more than 110,000 fully annotated metabolites. This is a threefold increase

FIGURE 1: Illustration of the differences in selectivity observed upon modifications of the experimental parameters, that is, stationary phase and mobile phase composition, for the separation of a representative set of metabolites belonging to different classes—amino acids, nucleosides, organic acids, and carbohydrates—using HILIC. (a) Luna HILIC (cross-linked diol groups) column, 20-mM ammonium formate at pH 3.5; (b) Luna HILIC (cross-linked diol groups) column 20-mM ammonium acetate at pH 6.0; (c) ZIC-HILIC (sulphobetaine) column, 20-mM ammonium formate at pH 3.5; (d) ZIC-HILIC (sulphobetaine) column, 20-mM ammonium acetate at pH 6.0; (e) Luna NH_2 column, 20-mM ammonium acetate at pH 9.0. Analytes: (1) aspartic acid, (2) proline, (3) threonine, (4) tyrosine, (5) guanosine, (6) inosine, (7) adenine, (8) malic acid, (9) hippuric acid, (10) nicotinic acid, (11) rhamnose, (12) trehalose, and (13) maltose. Experimental conditions: see reference 16. Adapted with permission from reference 16.



compared with the previous release of HMDB 3.0 in 2013 (3). The human metabolome is very complex and comprises a large diversity of compounds, including amino acids, organic acids, nucleosides, lipids, small peptides, carbohydrates, biogenic amines, hormones, vitamins, and minerals. Moreover, xenobiotics such as drugs, cosmetics, contaminants, pollutants, and their respective phase-I and phase-II metabolites are also part of this metabolome. The (ideally) comprehensive analysis of the metabolome is therefore linked to several analytical challenges due to (i) the large differences in physicochemical properties (polarity, solubility, pK_a values, molecular mass), (ii) the broad dynamic range needed to analyze both trace compounds and highly abundant metabolites (up to nine orders of magnitude difference), and (iii) the presence of multiple isomers with structural similarities but significant differences in their biological activities (lipid-based signalling molecules) (4).

Overall, this complexity highlights the need for state-of-the-art analytical approaches capable of tackling such challenges and enabling a qualitative and quantitative assessment of the metabolome. This should be done with the highest possible metabolic coverage via high resolving power and selectivity.

In this context, metabolomics has strongly benefitted from the latest developments in the fields of both chromatography and mass spectrometry (MS) over the last two decades. The use of reversed-phase liquid chromatography (LC) columns equipped with sub-2-µm fully porous particles (ultrahigh-pressure liquid chromatography, UHPLC) or sub-3-µm superficially porous particles (core–shell technology) are now considered well-established methods in metabolomics owing to the dramatic improvements in resolution and throughput obtained with such phases compared with conventional high performance liquid chromatography (HPLC) (5–7). On the other hand, recent liquid-based chromatographic and MS innovations, notably within hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), multidimensional LC, ion-mobility mass spectrometry (IM-MS), and data-independent acquisition (DIA) approaches, are not widely used in metabolomics, despite the significant improvement in metabolite coverage expected with such techniques. Therefore, in this review article, the latest developments in the above-mentioned fields of chromatography and MS are discussed with a focus on their ability to increase the metabolome coverage.

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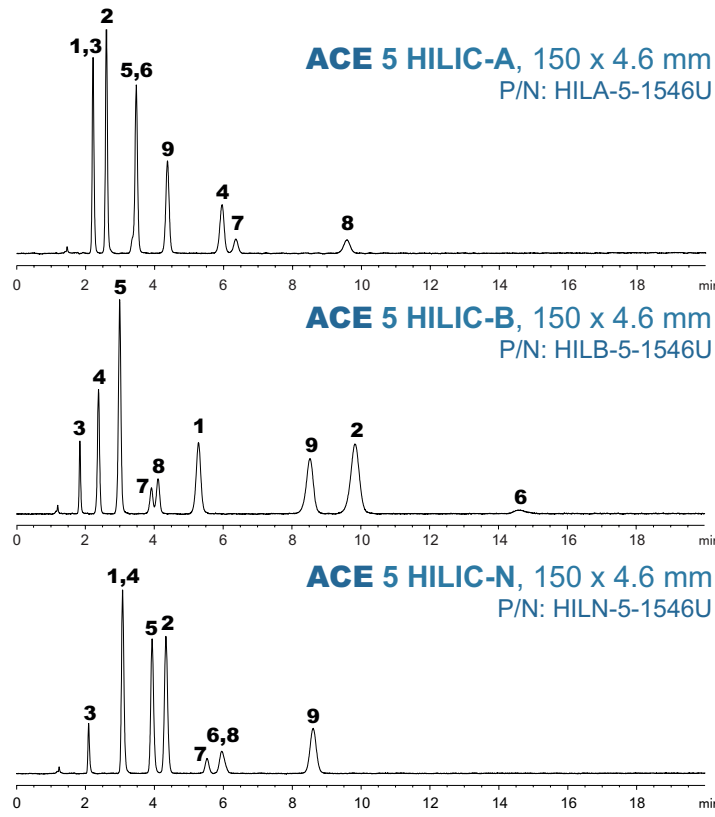

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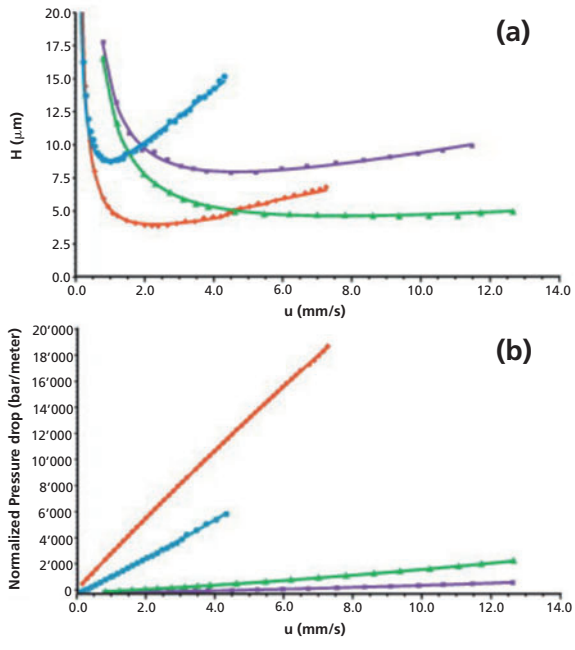
FIGURE 2: Improvements in plate height, mobile phase velocity, and pressure drop observed with supercritical fluid chromatography (SFC) compared to high performance liquid chromatography (HPLC) using columns packed with 3.5-µm particles, as well as ultrahigh-performance SFC (UHPSFC) versus ultrahigh-pressure LC (UHPLC) using columns packed with 1.7-µm particles. (a) Van Deemter curves obtained for butylparaben using a 50 mm × 4.6 mm, 3.5-µm XTerra RP18 (blue dots, HPLC), a 50 mm × 2.1 mm, 1.7-µm Acquity Shield C18 (red diamonds, UHPLC), a 100 mm × 3.0 mm, 3.5-µm Acquity UPC2 BEH 2-EP (purple squares, SFC), and a 100 mm × 3.0 mm, 1.7-µm Acquity UPC2 BEH 2-EP (green triangles, UHPSFC). (b) Corresponding generated column pressure drop normalized to 1 m of column. The optimal plate height is similar between UHPLC and UHPSFC, while the optimal velocity is 4× higher in UHPSFC vs. UHPLC (10 vs. 2.3 mm/s). Because of the low viscosity of supercritical fluids, the pressure drop is much lower in (UHP) SFC compared to (UHP)LC. This shows the benefits of (UHP) SFC in metabolomics, where excellent efficiencies can be obtained at higher throughput. Experimental conditions: see reference 23. Adapted with permission from reference 23.



Improvement of Metabolic Coverage: Chromatographic Innovations

Hydrophilic Interaction Chromatography: Reversed-phase LC-based methods have long prevailed in metabolomics because of the large variety of column chemistries available, the ease of use, and retention time reproducibility. However, a large number of polar or ionizable metabolites, such as amino acids, small organic acids, nucleosides, phosphate derivatives, or saccharides, are not well-retained using reversed-phase LC. Still, many of these polar metabolites play an essential role in multiple (patho)physiological processes, showing the need for alternative approaches. HILIC, a technique first proposed by Alpert in 1990 (8), is well-suited for the analysis of polar compounds. Retention is based on a multimodal separation mechanism between a polar stationary phase and a relatively hydrophobic mobile phase composed of an aqueous–organic mixture with a high organic proportion. With a concentration of 5–40% of water in the eluent, a water-enriched layer is formed at the surface of the stationary phase, facilitating analyte partitioning between this stagnant phase and the bulk mobile phase. The exact mechanisms involved in retention and separation are not fully understood but mostly rely on hydrophilic partitioning, dipole-dipole interaction, hydrogen bonds, and electrostatic interactions (depending on the stationary phase chemistry) (5,9).

A large diversity of phase chemistries based on silica or polymer material modified with polar functional groups, for example, aminopropyl and amine, amide, diol, triazole, sulfobetaine, phosphorylcholine, hydroxyethyl, and sulfoethyl are nowadays commercially available for HILIC analysis. Whereas for reversed-phase LC analyte retention can be easily predicted, helping to facilitate method development, this remains difficult in HILIC. The chromatographic selectivity is also strongly dependent on the stationary phase chemistry and composition of the mobile phase, as illustrated in Figure 1. A careful and extensive screening of different conditions during method development using a large set of representative metabolites is therefore recommended to obtain an adequate metabolite coverage. The help of modern computer-assisted method development strategies, such as the predictive elution window shifting and stretching (PEWS^2) approach (10), could be useful here to speed up method development. Numerous studies comparing the different stationary phases for metabolomics applications showed that diol, amide, and zwitterionic phases usually give the best results in terms of metabolite coverage, therefore representing a good starting point in the method development process (11,12). Small organic acids, sugar phosphates, and nucleosides are difficult to analyze with reversed-phase LC. Using HILIC mode these compounds are better retained, especially with polymeric zwitterionic phases, which allows analysis at high pH (pH 9–10) thanks to the polymeric nature of the stationary phase (13). Adding phosphate at micromolar concentrations to the mobile phase has also shown to further improve the peak shape and sensitivity when analyzing such metabolites with a

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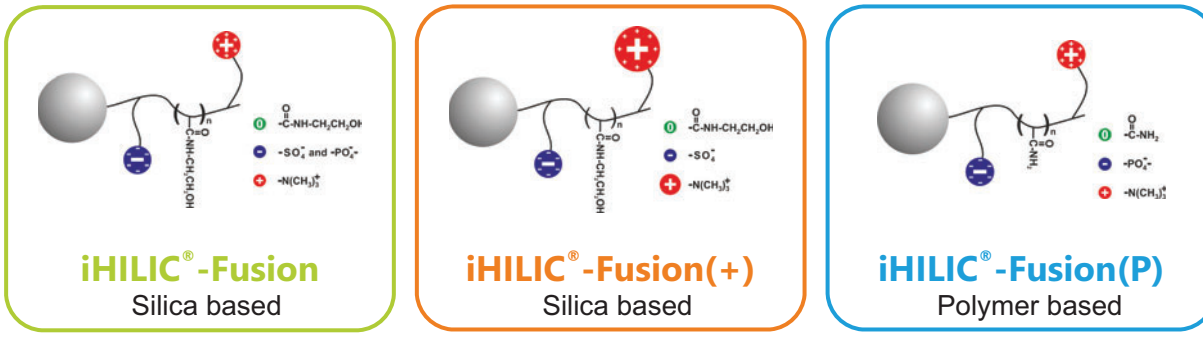

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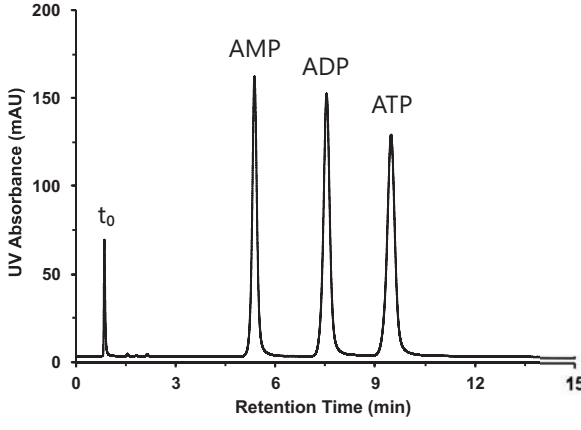

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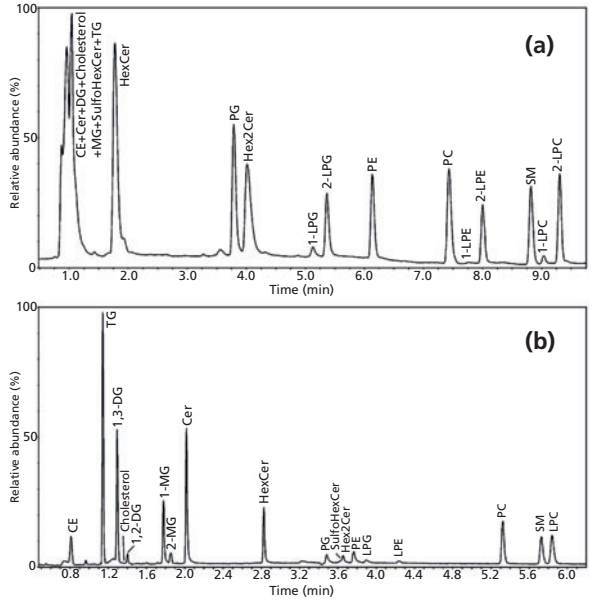
FIGURE 3: Comparison of lipidome coverage obtained with hydrophilic interaction chromatography (HILIC) and ultrahigh-performance supercritical fluid chromatography (UHPSFC). (a) HILIC analysis of lipid internal standards using a 150 mm × 2.1 mm, 1.7-µm Acquity UPLC BEH HILIC column. (b) UHPSFC analysis of lipid internal standards using a 100 × 3 mm, 1.7-µm Acquity BEH UPC^2 column. Peak annotation: CE, cholesteryl ester; Cer, ceramide; DG, diacylglycerol; Hex2Cer, dihexosylceramide; HexCer, hexosylceramide; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; MG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin; SulfoHexCer, sulfohexosylceramide; and TG, triacylglycerol. Adapted with permission from reference 25.



zwitterionic phase (14). Next to the stationary phase chemistry, the composition of the mobile phase strongly influences the selectivity and quality of the separation. Acetonitrile is the optimal organic solvent because it is water-soluble and aprotic. Protic solvents such as methanol, isopropanol, and ethanol are not recommended due to competition with water for the solvation of the stationary phase, which may lead to lower analyte retention (15,16). In HILIC, the high proportion of acetonitrile in the mobile phase decreases its viscosity compared with the mobile phase mixtures used in reversed-phase LC, which offers additional advantages such as the possibility to use longer columns (leading to higher efficiencies), a higher electrospray ionization (ESI) sensitivity, and higher volatility (15). The buffer composition (that is, salts concentration and pH) has a strong impact on both selectivity (Figure 1) and retention time reproducibility. The buffer concentration (commonly ≤50 mM to avoid salt precipitation in acetonitrile) influences the thickness of the water layer and thus the hydrophilic interaction, and plays an essential role in electrostatic interactions. Ammonium formate and acetate buffers are commonly used because of their MS compatibility. They also give better peak shapes than the corresponding acid solutions (16). Trifluoroacetic acid is not recommended in HILIC–MS because it leads to strong ion suppression in the range of compounds studied. Finally, an adequate and repeatable buffer pH is crucial in HILIC to ensure reproducible analyses. Changes in buffer pH will lead to a higher retention variability, showing the importance of repeatable procedures when preparing the buffer solutions.

Despite all the above mentioned advantages and the improved metabolite coverage that can potentially be obtained using the technique, HILIC remains sparsely used in metabolomics, mostly confined to untargeted studies (16,17). The complex mechanism of HILIC separation, the longer equilibration times, the attention required to ensure reproducibly prepared mobile-phase buffers, and the challenges in finding an adequate sample injection solvent might explain why this technique has not been widely adopted yet. However, there are now numerous excellent reviews available discussing these challenges, offering solutions and providing guidelines for state-of-the-art HILIC analysis (1,9,15,16,18). This will hopefully foster the use of HILIC in routine metabolomics.

Supercritical Fluid Chromatography: Although not new—the use of fluids in their supercritical state was first reported in the 1960s—SFC has shown a spectacular comeback in the last decade. This is due to the introduction of a new generation of instruments capable of performing robust, reproducible, reliable, and quantitative analysis. Similar to what has been observed in conventional LC, these new instruments have also fostered the development of columns packed with sub-2-µm fully porous (ultrahigh-performance SFC, UHPSFC) and sub-3-µm superficially porous particles specially designed for SFC analysis. Moreover, the new source designs recently developed for interfacing SFC with MS have also strongly contributed to developing the use of SFC in bioanalysis, including metabolomics (19,20). The metamorphosis of the technique has transformed UHPSFC–MS into a very competitive separation approach, complementary to UHPLC–MS, as underlined in the first ever inter-laboratory study. Between the 19 participating laboratories, similar or even better repeatability and reproducibility using SFC was

shown for the determination of impurities in pharmaceutical formulations compared with conventional LC methods (21).

Supercritical fluids have unique properties that take advantage of both gas and liquids, with viscosity and diffusivity very close to those of a gas, while their density and solvating power is close to those of a liquid. Overall, these inherent characteristics enable high separation efficiency at high mobile phase velocity with a low back pressure generated, and good solvation and fast transportation of the analytes (22). Carbon dioxide has been considered the solvent of choice as a result of the low critical temperature and critical pressure (31 °C and 74 bar, respectively) as well as its low toxicity, low flammability, and environmentally friendly properties. However, the low polarity of pure CO_2 limits its application to the analysis of rather nonpolar or hydrophobic compounds such as lipids. The addition of a miscible co-solvent (referred to as *modifier*, typically methanol) to the mobile phase is an adequate strategy to enable the retention of polar compounds. The use of an organic co-solvent influences the solvating power of the mobile phase, its hydrogen-bonding donor and acceptor properties, its density, the interaction between analytes and mobile phase, as well as the adsorption of analytes on the stationary phase (20,22). Yet, adding a modifier to this supercritical fluid increases the critical temperature and pressure of the fluid. In current applications, the pressure is commonly maintained over its critical point while the temperature is below its critical value. In this case, the fluid is in a subcritical state, showing a chromatographic behaviour close to LC.

The addition of acids (formic acid, citric acid), bases (trimethylamine, isopropylamine), or salts (ammonium acetate, ammonium fluoride) at low concentrations in the modifier also increases the range of compounds that can be analyzed using SFC, especially ionizable compounds such as polyacids, aliphatic amines, and other polar metabolites (20,22). These additives also increase the separation efficiency and peak shape by acting as ion-pairing agents and by covering active sites on the stationary phase, leading to less tailing and better elution of polar compounds. The latest trend in SFC is the use of water as an additive in a CO_2–methanol mobile phase to improve peak shape (at a proportion of 1–5%, miscible

in the mobile phase) or to enable the elution of very polar compounds (up to 30%, forming a ternary mixture) (20,22).

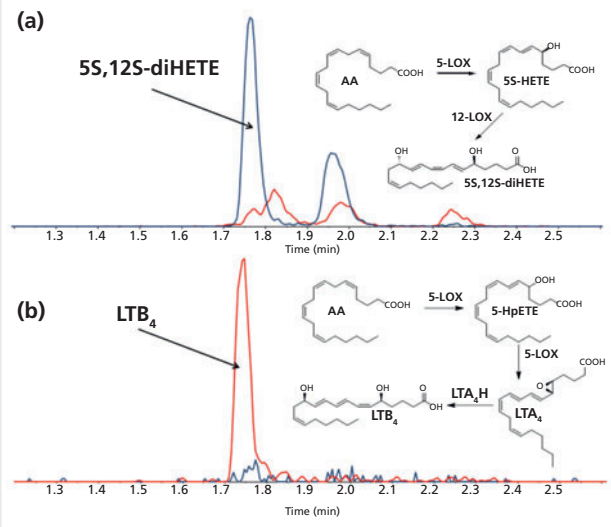
A number of SFC-specific stationary phases have been developed and commercialized in the past years, including 2-ethylpyridine, 4-ethylpyridine, pyridine amide, amino phenyl, 2-picolylamine, diethylamine, diol, and 1-aminoanthrocene. In addition to SFC-specific columns, reversed-phase LC- and HILIC-type stationary phases (ethylene-bridged silica, C18, fluorophenyl, amide) can also be used in SFC.

Most of the SFC-specific columns are also available in sub-2-µm format. However, the extracolumn band broadening of the state-of-the-art UHPSFC instruments currently on the market are still higher than the corresponding values obtained on UHPLC systems (namely, 85 μL^2 vs. 2 to 20 μL^2), hindering the use of typical UHPLC column dimensions (50 mm × 2.1 mm, 1.7-µm) with these systems. On the other hand, 4.6-mm internal diameter (i.d.) columns require flow rates above the system limits (22). Therefore, most of the current state-of-the-art SFC applications are performed using 100 mm × 3.0 mm columns packed with sub-2-µm fully porous and sub-3-µm superficially porous particles, which represents an adequate compromise and can lead to excellent kinetic performance with a low pressure drop, as illustrated in Figure 2 (23).

Modern UHPSFC–MS analysis has recently started to gain more attention from the metabolomics community, not only in the fields of lipidomics but also as a complementary technique to UHPLC–MS to increase the metabolome coverage. Multiple metabolite classes, including amino acids, bile acids, cannabinoids, fatty acids, saccharides, steroids, and tocopherols, have been successfully analyzed using UHPSFC–MS (24). A good example of the potential of UHPSFC was described by Holcapek and co-workers, who demonstrated the comprehensive and quantitative analysis of different lipid classes (25). Figure 3 shows the chromatogram obtained for the analysis of lipid internal standards with UHPLC–HILIC–MS (Figure 3[a]) and with UHPSFC (Figure 3[b]), both coupled to a quadrupole–travelling-wave ion mobility–time-of-flight (TOF) mass analyzer. Both HILIC and UHPSFC enable the separation of lipid classes without the typical overlap that is seen when using conventional reversed-phase LC approaches. Nonpolar lipids (cholesterol esters and triglycerides) as well as species with one hydroxyl group (ceramides, diglycerides, monoglycerides, and cholesterol) show poor retention in HILIC and elute in the void volume (Figure 3[a]). On the other hand, all lipids are retained using UHPSFC (Figure 3[b]). Whereas the positional isomers 1,2-DG/1,3-DG and 1,2-MG/1,3-MG are well resolved in UHPSFC (Figure 3[b]), the positional isomers of the more polar lysolipids 1-LPG/2-LPG, 1-LPE/2-LPE, and 1-LPC/2-LPC are well resolved using HILIC (Figure 3[a]) (25). This example illustrates the complementary nature of the different chromatographic modes, where no single analytical technique currently enables a comprehensive coverage of the metabolome.

Despite the excellent performance that can be reached with modern UHPSFC–MS, it remains little used in metabolomics—and only by a limited number of research groups. This reluctance might be explained by the large diversity of stationary phases currently available, together with the flexibility offered

FIGURE 4: Additional selectivity obtained with differential mobility spectrometry (DMS) combined with micro-LC–MS/MS to separate the two lipid diastereomers 5S,12S-diHETE and LTB_4 in murine peritoneal cell ethanol extracts. Both compounds coelute when using conventional C18 stationary phases, but can be separated when using a different compensation voltage in DMS. (a) Signal observed for 5S,12S-diHETE in the control mice population, (b) Signal observed for LTB_4 in the challenged mice. Red trace, signal obtained at a compensation voltage of 17.9 V, corresponding to LTB_4; blue trace, signal obtained at a compensation voltage of 20.3 V, corresponding to 5S,12S-diHETE. AA, arachidonic acid; 5S-HETE, 5(S)-hydroxyeicosatetraenoic acid; 12-LOX, 12-lipoxygenase; 5HpETE, 5-hydroperoxy-eicosatetraenoic acid; LTA_4, leukotriene A4. Unknown: undefined isomer in the trace of 5S,12S-diHETE. Adapted with permission from reference 37.



in the composition of the mobile phase (modifier, additives, gradient composition). Indeed, the method development step *a priori* may be seen as very cumbersome and time-consuming. However, it can be facilitated by using column classification maps to help select adequate column chemistries (26) and by using method optimization work (27).

Overall, there is no doubt that the multiple advantages of modern SFC will foster its use in metabolomics in the coming years as a complementary chromatographic approach to expand the metabolome coverage. The advantages of SFC in metabolomics include (i) its application range versatility, with a large range of metabolites with very diverse physicochemical properties that can be analyzed within a single run (as shown in reference 27), (ii) the sample compatibility with the mobile phase used in SFC, (iii) the excellent sensitivity of UHPSFC–MS, comparable or superior to that of UHPLC–MS, and (iv) the flexibility offered with the state-of-the-art instruments, which allows for both UHPLC and UHPSFC analysis within one single system and an unlimited combination of solvent and stationary phases. SFC technology has faced the same reluctance as HILIC a decade earlier, but both techniques are promised to rise further in the field of metabolomics.

Multidimensional Chromatographic Separations: A straightforward approach to increase the metabolome coverage of very complex samples or closely related metabolites is to add another separation dimension to provide additional selectivity.

Similar to what has been observed in the field of SFC, on-line two-dimensional liquid chromatography (2D-LC) is far from being a new concept but has seen a significant breakthrough in the last couple of years thanks to significant advances in theory and instrumentation. In on-line 2D-LC, two individual LC separations are combined, typically using a four-port duo valve or 10-port valve with two sampling loops, or connected to special valves with multiple sample parking loops.

Capturing all peaks—or a large number of fractions—from the first dimension into the second dimension is referred to as *comprehensive 2D-LC* (also called LC×LC), while multiple heart-cutting 2D-LC (also called *LC-LC*) is used when one or few distinct fractions are collected from the first dimension and are subjected to a high-resolution analysis in the second dimension. Selective comprehensive 2D-LC (sLC×LC) is an intermediate approach where a series of fractions across one or more regions in the first dimension chromatogram are transferred to the second dimension (28,29). The comprehensive LC×LC approach appears particularly interesting in untargeted metabolomics, where hundreds of features can be profiled during one single analysis.

A large diversity of chromatographic modes can be combined in 2D-LC, including reversed-phase LC, HILIC, normal-phase LC, ion-exchange chromatography (IEX), ion-pairing chromatography, and porous graphitized carbon (PGC) columns. Different stationary phase chemistries and mobile-phase compositions can be employed, aiming for the highest orthogonality of separation between the two dimensions. The selection of the two separation dimensions depends on the analytes, the compatibility and miscibility of the mobile-phase solvents, the compatibility with the detector, and the selection of a faster technique (that is, based on UHPLC conditions) for the second dimension (4,28).

With the recent advent of state-of-the-art instruments for 2D-LC analysis, the number of experimental parameters that can be optimized during method development has dramatically increased. Indeed, setting up a complete 2D-LC method requires optimization of multiple parameters, including column dimension, stationary phase, particle sizes, mobile-phase composition, gradient conditions, sample loop volume, injection volumes, flow rates, and modulation times. This can lead to a rather cumbersome and lengthy method development. Moreover, state-of-the-art 2D-LC analyses usually require a dedicated instrument (even though one-dimensional [1D]-LC systems can be upgraded to 2D-LC with only minor investment). Finally, hyphenating 2D-LC to MS adds another challenge, since the insertion of an additional LC dimension may induce a significant dilution of the effluent injected to the MS system (28,30).

Overall, this might explain the reluctance in using this technique in metabolomics, despite the remarkable promises 2D-LC holds in significantly expanding the metabolome coverage. This reluctance is similar to the one observed for HILIC and SFC in metabolomics, where inexperienced users are struggling to get reproducible data. Moreover, they might lack sufficient theoretical and practical knowledge to get the best out of those techniques. However, 2D-LC is currently a very dynamic field and a number of excellent guidelines and tutorials have recently been published by experts in the field, guiding the inexperienced user through this method development (28–31). The recent developments in instrumentation, including the use of active-modulation techniques to alleviate the MS detector sensitivity problems and minimize effects from poorly compatible mobile phases, software tools to support method development, as well as continuous improvements in the algorithm available for processing 2D chromatograms, will certainly foster its use in clinical metabolomics. Most of the applications reported so far have been mostly based on heart-cutting approaches and proof-of-concept studies rather than clinical applications. However, the results presented highlighted the potential of 2D-LC in metabolomics, showing for instance, a twofold increased coverage of intracellular energy metabolites using a combination of reversed-phase LC with PGC (32) and the acquisition of both metabolomic and lipidomic information in a single analysis using heart-cutting 2D-LC (33).

Improvement of Metabolic Coverage: MS Developments

Ion-Mobility Mass Spectrometry: Among all recent developments discussed here, IM-MS is probably the one that has already been largely accepted by the metabolomics community even though it remains a relatively young technique. IM-MS adds an orthogonal separation dimension between chromatographic separation and MS detection without impacting the analysis time. IM-MS separation occurs in a timescale of milliseconds, which makes this technique fully compatible with both fast LC and high-throughput MS approaches (especially TOF mass analyzers, which offer fast duty cycles) (34).

IM-MS is a gas-phase technique separating ions driven through an ion mobility cell under an electric field in the presence of an inert buffer gas. Ions are separated according to their mobility or drift time, which is intrinsically linked to their size, shape, and charge. Assuming that the experimental parameters (for example, drift-tube length, gas pressure, temperature, electric field) are constant, the ion drift time is proportional to the rotationally averaged collision cross-section (CCS) value, which represents the effective area involved in the interaction between an ion and the gas present in the ion mobility cell. The CSS value is not only highly reproducible but also unique for each analyte, and reflects its chemical structure and three-dimensional configuration. This shows the power of IM-MS in metabolomics, especially in untargeted metabolomics, where CCS values can be used in addition to conventional parameters typically

reported in libraries (retention time, mass-to-charge ratio, fragmentation pattern) for metabolite characterization and to increase the confidence in metabolite identification (4,35).

Different IM-MS technologies are currently commercially available, namely, (i) drift-tube ion-mobility spectrometry (DTIMS), (ii) travelling-wave ion-mobility spectrometry (TWIMS), (iii) field-asymmetric ion-mobility (FAIMS), also called *differential-mobility spectrometry* (DMS), (iv) differential mobility analyzer (DMA), and (v) confinement-and-selective-release ion mobility, also called *trapped ion mobility spectrometry* (TIMS). They differ amongst each other in terms of applied electric field and state of the buffer gas. DTIMS and TWIMS belong to the time-dispersive methods, where all ions drift along the same pathway and have a different drift time. FAIMS and DMA are space-dispersive methods that separate ions following different drift paths, based on their mobility difference. In TIMS, the ions are first trapped in a pressurized region before being selectively released based on their mobility differences. By using DTIMS instruments, CCS values can be directly derived from the drift time while other approaches require the use of calibrants with known CCS values to calculate the CCS value from the drift time of an unknown (35,36).

IM-MS is able to improve the metabolome coverage by enhancing the selectivity and resolution between metabolites, but one of its major impactful applications probably lies in the field of lipidomics. Indeed, lipid analysis remains exceptionally challenging because of their structural diversity and the multiple lipid isomers that can be present in a biological sample. Contrary to conventional MS/MS approaches, IM-MS enables the discrimination between lipid isomers that differ only in the position of the acyl chain or the double bond, or with a different double bond geometry. An example is shown in Figure 4 with the two lipids 5S,12S-diHETE and LTB4 , both of which arise from different pathways and have different biological activities. 5S,12S-diHETE and LTB4 are diastereomers and geometrical isomers. Therefore, they show identical mass spectra and similar retention behaviour using conventional LC–MS/MS analysis. However, adding IM-MS (in this example DMS) enables a baseline separation of these two compounds using two different compensation voltages (37).

Beside lipid analysis, IM-MS has also demonstrated its usefulness for the analysis of polar metabolites in

various body fluids. Most of these applications were untargeted, as discussed in references 34 and 36.

Despite its promising contribution to improve the metabolome coverage and metabolite annotation using the CCS value, IM-MS still faces important challenges linked to data interpretation. Indeed, in an LC–IM-MS workflow, the potential in-source fragments, dimers, and adducts will also be separated in the ion mobility cell. A correct regrouping and assignment of these signal features adds another layer of complexity, which is currently not completely tackled by the software available, especially in untargeted metabolomics workflows (35).

Data-Independent Acquisition: Another MS-based strategy used to improve the metabolic coverage is data-independent acquisition (DIA), which allows for the detection and identification of lower abundant metabolites otherwise not recorded with conventional data-dependent acquisition methods. DIA approaches are not new but they have gained more attention since the advent of SWATH-MS approaches. In DIA, precursors selection windows are defined in the first quadrupole (MS1) of a tandem mass spectrometer; all ions are then fragmented in the collision cell and collected into a composite spectrum in the third quadrupole (MS2). Several DIA techniques have been reported so far, including MSEverything (MS^E), all ion fragmentation (AIF), MSX, and SWATH (38). In MS^E and AIF, all coeluted precursor ions in the whole selected mass range are fragmented to acquire MS2 spectra. MS^E alternatively acquires the full MS1 scan with low collision energy (full MS spectrum) and MS2 scan from all precursor ions with high collision energy (MS/MS spectrum). In AIF, all precursor ions are transmitted into a higher energy collisional dissociation cell for fragmentation. Both AIF and MS^E acquisitions generate highly complex multiplexed MS2 spectra. SWATH, which stands for *Sequential Window Acquisition of all THeoretical fragment ion spectra* and was first described in 2012, has been developed to reduce this data complexity by using a narrow isolation window (39). In SWATH-based DIA techniques, implemented on quadrupole-time-of-flight (QTOF) or less frequently Q-Orbital trap instruments, all precursors ions are sequentially fragmented in a serial of quadrupole isolation windows (Q1 windows). The complete "snapshots" of all metabolite ions and their product ions in MS2 are recorded through the whole chromatogram. The full mass range can be covered in one cycle depending on the selected MS1 scan range and the width of the isolation window. The SWATH windows can be both fixed (typically 25 Da) or variable (that is, the window width is not uniform), and are selected depending on the selectivity required and the cycle time (as short as possible if combined with UHPLC). The complexity of the multiplexed MS2 spectra is therefore decreased by reducing the number of simultaneously fragmented precursor ions, which also improves the overall quantitative performance (37,38).

SWATH-MS is now widely used in proteomics and has emerged as a powerful technique in other clinical applications because of its reproducibility, speed, compound coverage, and quantitation accuracy. The great performance observed in proteomics fields has also attracted the attention of the metabolomics community looking to expand the information gathered on the metabolome within a single run. A number of metabolomics and lipidomics applications of LC–SWATH-MS have already been reported in the literature. For example, UHPLC–SWATH-MS was used to investigate the changes in the urinary metabolome of rat models upon administration of vinpocetine. Information on both drug metabolism and endogenous metabolite expression changes were gathered, with the simultaneous detection of 28 drug metabolites as well as altered endogenous compounds (40). Using a combination of SWATH-MS and selected reaction monitoring (SRM), Zha *et al.* developed a two-step workflow to discover potential biomarkers for colorectal cancer. In this method, SWATH-MS was first used to acquire the MS2 spectra for all metabolites in one pooled biological sample. In the second step, a large set of SRM transitions was acquired, targeting both known and unknown compounds (around 1000–2000 metabolites). This approach increased the coverage in targeted metabolomics analysis, where more than 1300 metabolite were profiled in one run in colorectal cancer tissues (41).

Further developments of SWATH technology are still required, particularly in the data analysis pipeline. Indeed, in a DIA-based dataset, the direct connections between precursor and product ions are missing, rendering the metabolite identification very challenging. Chromatographic ion profiles can be used to reconstruct these connections, but coelution and co-fragmentation of precursor ions makes it complicated. Several software tools have been recently developed to overcome the challenges related to DIA-based data analysis (39). The open-source software MS-DIAL, for example, uses a mathematical deconvolution of fragment ions to extract the original spectra and reconstruct the link between precursor and product ion, allowing for compound identification, annotation, and quantitation. It also implements additional functions typically used in untargeted data processing, namely, peak alignment, filtering, and missing value interpolation (42).

Overall, SWATH-MS represents a great tool to expand the metabolome coverage and obtain both qualitative and

quantitative information within a single run. The complexity of the generated data remains a challenge since the reconstructed spectral quality impacts both the confidence in metabolite annotation and quantitation accuracy. The addition of IM-MS in LC–SWATH-MS workflows might help to decrease the spectral complexity by adding an additional separation of the precursor ion to help facilitate the spectral deconvolution, as well as providing CCS values to help in metabolite identification, but also increase the need for adequate data processing software tools.

Conclusions

The last decade has seen a tremendous amount of technological developments in liquid-phase chromatography and MS techniques, developments initially for other applications but showing a considerable potential in metabolomics. Modern clinical metabolomics applications rely on two essential aspects, namely, high-throughput analysis and comprehensive metabolome coverage. The latter is crucial in the quest for the Holy Grail, that is, the discovery of new biomarkers that could ultimately lead to a better understanding of (patho)physiological conditions, an earlier disease diagnosis, a better prognosis evaluation, and an individualized prediction of treatment response. The more comprehensive the metabolome coverage is, the higher the chances are of finding specific metabolites or metabolite fingerprints.

The chromatographic and mass spectrometric innovations presented here have also largely demonstrated their relevance in expanding the metabolome coverage. Most of those techniques, however, are still in their infancy in the field of clinical metabolomics and are rarely used for large-scale studies, where reversed-phase LC–MS and gas chromatography (GC)–MS remain the gold standard techniques. Very few studies have reported the use of HILIC–MS for the analysis of hundreds of samples, while the robustness of SFC and 2D-LC needs to be further investigated, as well as the potential of these two novel techniques in large-scale metabolomics applications. One of the main obvious reasons is the lack of practical background knowledge of non-experienced users, who struggle to get repeatable and reproducible results. In this context, leading experts in these fields and professors play a crucial role and are strongly encouraged to share their knowledge with the younger generation of scientists. Moreover, further technological improvements are needed to ensure the batch-to-batch reproducibility of SFC and HILIC chromatographic columns, which currently remains a clear bottleneck in metabolomics. As an example,

acceptable repeatabilities can be obtained in HILIC with >1000 injections (depending on the stationary phase chemistry) of pretreated biological samples using standard procedures (including adequate column re-equilibration time). However, it is much more difficult to reach such repeatability when using HILIC columns from different batches, rendering the use of HILIC in large-scale studies much more challenging than reversed-phase LC.

Increasing the metabolome coverage does not stop at discriminating metabolites with close physicochemical properties. An important aspect often overlooked in clinical metabolomics is the distinction of optical isomers (stereoisomers such as enantiomers). An excellent example is 2-hydroxyglutarate (2-HG), the first oncometabolite (cancer-causing metabolite) ever reported. Both D- and L- stereoisomers of hydroxyglutaric acid are normal endogenous metabolites found in human body fluids. D-2-HG—not L-2-HG—is produced in the presence of gain-of-function mutations of isocitrate dehydrogenase, causing a cascading effect in the cell that leads to genetic perturbations and malignant transformation. Typical routine analytical techniques only measure 2-HG, which strictly speaking corresponds to the sum of both D- and L-forms. Since the endogenous serum levels of L-2-HG have shown to be equal or even exceed the levels of D-2-HG in healthy individuals, it is essential to use state-of-the-art analytical techniques to discriminate between the two stereoisomers (43). Some of the advanced techniques discussed here, mostly SFC, are applicable to chiral analysis and are therefore expected to play a crucial role in next-generation metabolomics.

Overall, despite the technological improvements within each of the discussed techniques, none of the state-of-the-art analytical techniques is currently capable of exhaustively assessing the metabolome. SFC will certainly become a gold standard chromatographic technique complementary to reversed-phase LC and HILIC because of the versatility and flexibility offered (convergence chromatography) and the experimental conditions, where a large diversity of metabolites can be analyzed without strong variations of the operating parameters. Moreover, the higher throughput obtained with UHPSFC is also a clear advantage in clinical metabolomics, notably with the next generation of instruments, allowing for higher back pressure to be generated, which is still a limitation in the instruments currently on the market.

The future of metabolomics probably relies on the combination of different separation dimensions in an on-line format, as demonstrated with 2D-LC approaches. The first 2D-LC-SFC application has been reported in the literature for simultaneous achiral-chiral analysis of pharmaceutical compounds (44), a multidimensional approach that might be further investigated for metabolomics-based applications. The combination of multidimensional LC with IM-MS has a promising future in metabolomics, showing the remarkable advantage of improving the metabolome coverage while keeping similar throughput. Alternative approaches based on miniaturization of conventional LC techniques and the use of micro-pillar array columns instead of columns packed with porous particles will also probably help to further expand the metabolome coverage, as already shown in lipidomics where structural lipid isomers were chromatographically baseline resolved using micro-pillar array columns (45).

One should also keep in mind the challenges associated with a substantial improvement of the metabolome coverage. First, the development of cutting-edge analytical instruments should not forget the importance of sample preparation, which should be as simple and generic as possible while providing sufficient clean-up to lower the occurrence of matrix effects. Moreover, an increased number of metabolites in quantitative targeted metabolomics means an increased number of internal standards, which raises the overall costs. Finally, enhancing the number of metabolite features measured in a studied population requests a much higher number of samples and subjects included in the study design to keep a sufficient statistical power, which in turn substantially increases the costs and the number of samples analyzed. A compromise between all aspects is definitely needed to achieve successful results in the field of clinical metabolomics.

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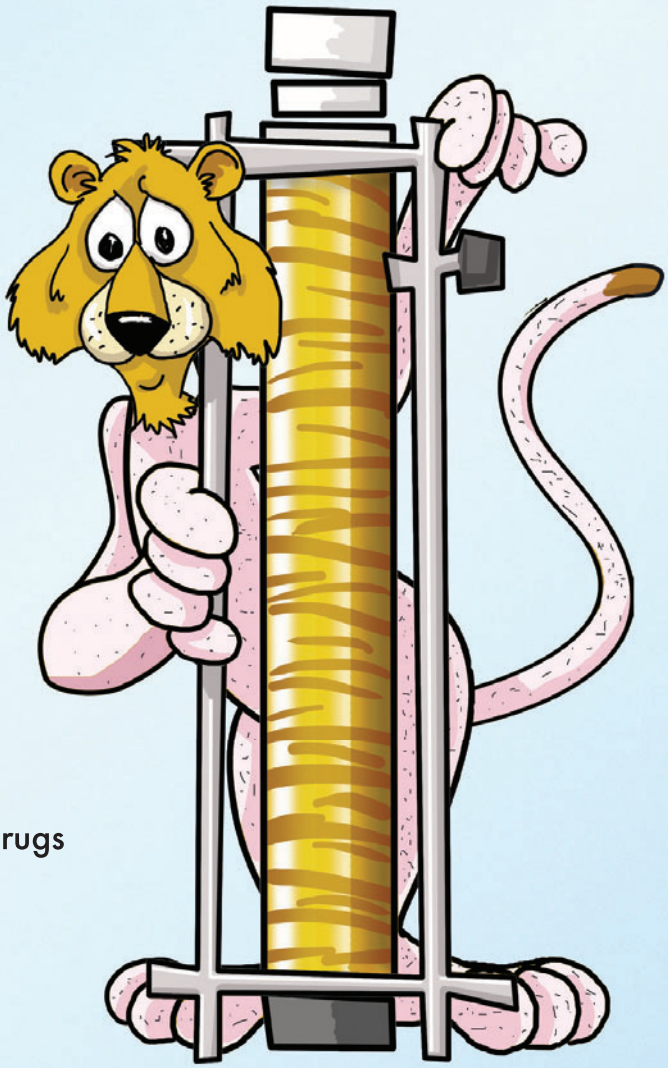

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A Q&A

Next-Gen Multi-Angle Light Scattering

Daniel Some, PhD
Principal Scientist
Wyatt Technology

New HPLC/UHPLC product line offers more robust measurements and increased uptime.

In March 2019, Wyatt Technology Corporation launched its next-generation of online multi-angle light scattering (MALS), refractive index, and differential viscometry detectors for high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC) systems. LCGC recently asked Dan Some, PhD, Principal Scientist at Wyatt Technology, about the advancements made in Wyatt's product line for absolute macromolecular characterization.

LCGC: Can you explain what is size-exclusion chromatography (SEC)-MALS and why it is of interest to protein and polymer scientists?

Some: SEC-MALS couples online multi-angle light scattering detection and other online detectors (such as refractive index and differential viscometry) to size-exclusion chromatography. With this technique, the only purpose of the SEC column is to separate the different molecules from each other. The actual characterization of the molecules takes place solely within the detectors, which allows absolute characterization to be performed. This method does not depend on the retention time within the column, the conformation of the molecule, or a molecule's interactions with the column. Thus, in SEC-MALS we do not encounter the errors of typical analytical SEC where reference molecules are run even though they might (and often do) behave differently on the column than your molecules.

This technique allows us to analyze monodispersed molecules, such as proteins, or polydispersed macromolecules, such as heterogeneous polymers, to determine their molecular weight, size, conformation, and branching ratio. The oligomeric state of proteins in native solution can be determined, resulting in a much better understanding of the essential biophysical properties of the macromolecules than can be obtained from analytical SEC.

LCGC: What would you say is new and improved in Wyatt's DAWN, Optilab, and ViscoStar products launched in March 2019?

Some: In March, we launched a re-envisioned product line of the DAWN, Optilab, and ViscoStar online detectors for SEC with multi-angle light scattering. While these detectors offer the same industry-leading sensitivity, range of measurements, and other features

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"Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions."

that our customers are used to for maximum characterization of their macromolecules, the new products have a sexy, new modern look and feel. For example, there is a large capacitive touchscreen that allows users to interact more intuitively with the instrument and access the information that they need from the front panel. The instruments also have improvements in serviceability and maintainability, achieved by making them more modular. In fact, individual modules can be swapped out on-site. In addition, CheckPlus software performs a full diagnosis and sends those diagnostics to an engineer at Wyatt for a more in-depth look. Depending on what the engineer decides, a technician can come on-site and swap out the modules with very little downtime.

LCGC: What are some of the newest innovations in the DAWN line, which has been Wyatt's flagship product for 37 years?

Some: In previous generations, we worked on improving the technical specifications, getting higher sensitivity, expanding the range of measurements, and adding user interface improvements. Key in the new generation of DAWN detectors is the built-in intelligence that assists users in knowing when their SEC-MALS system is ready for optimal measurements, when the noise level is low enough, and when the system is fully equilibrated. In addition, swappable flow cells allow for a new flow cell to be swapped in without the need for laser alignment. Opto-mechanics are more robust, and modifications to the optical design further reduce stray light. Dedicated slots for the WyattQELS dynamic light scattering module have been added so that, rather than sacrificing one of the MALS angles as with the previous models, WyattQELS gets its own slot, and the software automatically identifies into which angle the user has placed the WyattQELS optical fiber.

LCGC: What do you see as the main value to customers in the updated product line?

Some: Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions. The platform's self-diagnostics and CheckPlus instrument log application permit remote evaluation by our service team, and full on-site repair service.

LCGC: What can Wyatt offer to those who use UHPLC?

Some: microDAWN is the multi-angle light scattering online product for use with UHPLC. microOptilab is the refractive index detector UHPLC, and microViscoStar is the differential viscometer for UHPLC. Users can get the complete range of characterization of molecular weight, size, and conformation, with all the benefits of UHPLC, which means faster runs, lower sample consumption, lower mobile phase consumption, and enhanced productivity.

LCGC: Where can readers go to learn more about SEC-MALS technology and applications?

Some: The best place to start is our website, which is www.wyatt.com, and there we have information about the theory of SEC-MALS, light scattering, and other technologies. Folks can learn about the various solutions that the instruments offer, the applications they provide, the different types of analytes that can be analyzed, the industries served, and the products' features and benefits. There is also an extensive library of webinars that can be viewed to learn more.

With a long history in scientific instrumentation, Wyatt Technology is a leader in light scattering instruments, accessories, software and services for determining the properties of macromolecules and nanoparticles in solution.

Mind the Diluent: Effects of Sample Diluent on Analyte Recovery in Reversed-Phase and HILIC Separations

Dwight R. Stoll[1] and Anne E. Mack[2], [1]LC Troubleshooting Editor, [2]Agilent Technologies, Wilmington, Delaware, USA

The sample solvent can have a big impact on peak shape in both reversed-phase and hydrophilic interaction liquid chromatography (HILIC) separations, especially when large volumes are injected. Diluting the sample with weak solvent can be an effective solution to mitigate this problem, but we have to be careful to not lose analytes of interest to precipitation or phase separation.

In the August 2019 instalment of "LC Troubleshooting", I wrote about one of the hot topics that was discussed by several speakers at the HPLC meeting in Milan, Italy, in June—coupling of ion-exchange separations to mass spectrometric (MS) detection (1). This month, I'd like to discuss another topic addressed at the meeting. Anne Mack gave a talk that highlighted some things to consider when deciding whether to use reversed-phase or hydrophilic interaction liquid chromatography, (HILIC) especially when some of the components of the sample mixture at hand are hydrophilic. These considerations included the relative retention of the analytes of interest under reversed-phase and HILIC conditions, the effect of the sample diluent and injection volume and peak shape, and the effect of the sample diluent on apparent recovery of the analyte. The recovery aspect nicely complements the LC Troubleshooting article I wrote in January 2019 on the effects of the diluent on peak shape (2), so I've asked Anne to join me in writing this month's instalment of "LC Troubleshooting".

Dwight Stoll

Liquid chromatography (LC) is an incredibly versatile analytical tool, enabling quantitative and qualitative analysis of diverse molecule types, ranging from highly hydrophilic and water soluble (for example, inorganic metal ions) to highly lipophilic molecules that are soluble in organic solvents (for example, lipids). The reversed-phase mode of separation is arguably the most versatile single mode of LC separation, and can provide retention and separation of analytes covering a wide range of water solubility. In an extreme case, it could separate molecules as hydrophilic as small organic acids (like succinic acid [3]) and as lipophilic as fat soluble vitamins (like vitamin A [4]). However, a practical problem we run into quickly is that molecules as different in properties as these will not be highly soluble in the same sample solvent. In the worst case, choosing an inappropriate sample solvent or diluent will lead to inaccurate results, particularly for quantitation, because the analytes of interest will not be soluble in the sample solvent, and the sample that is injected into the LC instrument will not be representative of the analytes actually present in the sample vial.

The problem described here is not new by any means. But, as we push the limits of analytical methods in the reach for more speed, simplicity, and sensitivity, we inevitably run into conditions that will "break" the method, leading to inaccurate results. Therefore, we have to be careful not to break our methods, and a deeper understanding of where the limits lie and the basis for them puts us in a better position to be successful in the long run.

Review: Effects of Sample Diluent and Injection Volume on Peak Shape

As users of LC, it is common to encounter situations where the samples presented to us from some prior process or step contain a sample solvent that is quite different from the mobile phase associated with the LC method used for the analysis of the sample. For example, when using solid-phase extraction (SPE) to preconcentrate low-concentration analytes from water samples, the last step in the SPE process typically involves elution of the analytes of interest from

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the SPE adsorbent using an organic solvent like methanol. Frequently, such extracts are subsequently analyzed by reversed-phase LC, with solvent gradient elution that starts with a water-rich mobile phase. On the other hand, we might be interested in analysis of the water-soluble components from a urine sample. In this case, the sample solvent is water, while the HILIC separation will start with a mobile phase containing a high fraction of acetonitrile on the order of 90%. These mismatches between the solvent composition of the sample and the mobile phase of the LC method can lead to trouble. In January of this year, we wrote about how the combination of the volume of sample that is injected and the sample solvent composition can have a dramatic effect on peak shape. As a reminder of the data discussed in that instalment, Figure 1 shows examples of bad results obtained under reversed-phase and HILIC conditions, and how much better separations can be obtained simply changing the sample solvent. In the case of the reversed-phase separation, terrible peak shape is observed (Figure 1[a]) for some simple alkylphenones when the sample solvent contains 20% more acetonitrile than the starting mobile phase used for the solvent gradient elution program. However, simply changing the sample solvent to contain 20% less acetonitrile than the starting mobile phase in the gradient leads to a much nicer separation (Figure 1[b]). Similar effects can be observed in HILIC separations as well. Figure 1(c) shows the terrible peak shapes that are observed when a completely aqueous sample is injected into a HILIC column when the mobile phase contains 85% acetonitrile. However, this separation can also be improved dramatically by simply changing the sample solvent composition to contain 95% acetonitrile, as shown in Figure 1(d).

FIGURE 1: Examples of the effect of sample solvent on reversed-phase LC (a,b) and HILIC (c,d) separations. The analyte mixture for the reversed-phase LC separation was a series of alkylphenones, and a solvent gradient was used for elution starting at 50% acetonitrile. The analytes for the HILIC separation were cytidine and guanosine, and the mobile phase was isocratic with 90:10 acetonitrile–water buffer. Complete chromatographic conditions are given in reference 2. Figures are (a) sample solvent 70:30 acetonitrile–water, (b) sample solvent 30:70 acetonitrile–water, (c) sample solvent 0:100 acetonitrile–water, and (d) sample solvent 95:5 acetonitrile–water.

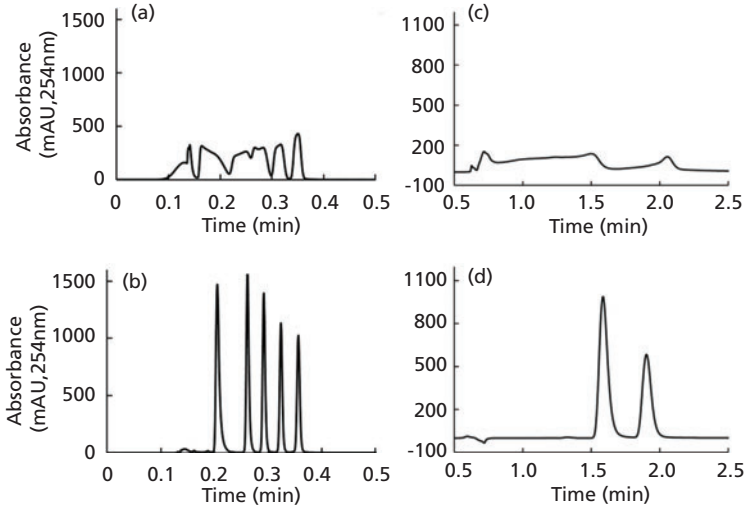
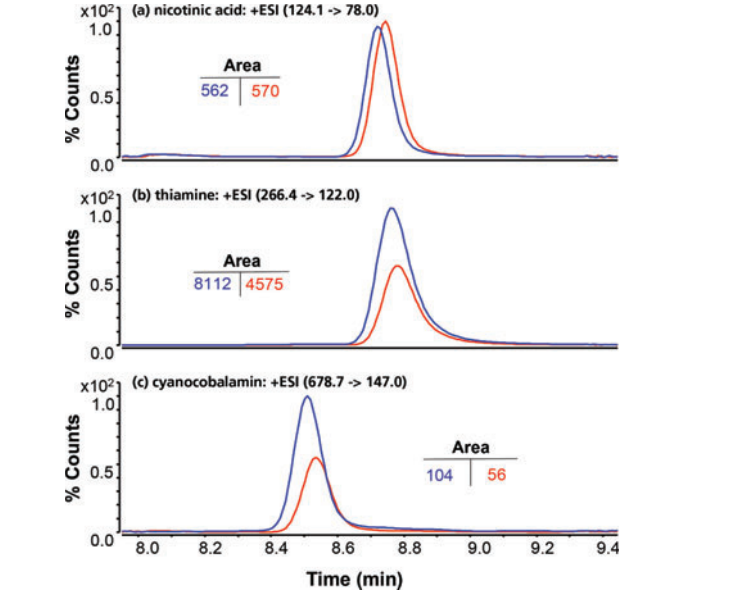


FIGURE 2: Comparison of peak areas obtained from HILIC separations injected from samples prepared in either water (blue) or 90:10 acetonitrile–water (red). Chromatographic conditions: solvent A: water, solvent B: acetonitrile; gradient elution from 95–65% B in 10 min, with 10 mM ammonium acetate (pH unadjusted) throughout; temperature, 25 °C; flow rate, 0.5 mL/min.; column, 100 mm × 2.1 mm i.d., 2.7-µm Poroshell 120 HILIC-OH5; injection volume, 0.5 µL; detection by triple quadrupole mass spectrometry.



Recognizing the importance of the sample solvent composition, particularly when the injection volume is large relative to the column volume, several different groups have developed a number of approaches to address this issue. The simplest approach is to adjust the sample solvent composition offline—that is, by addition of "weak solvent" (for example, water in reversed-phase, or acetonitrile in HILIC separations) as part of the sample preparation process prior to the LC separation. For example, in the case of the SPE extract in methanol described above, one could simply dilute this extract with some amount of water prior to analysis.

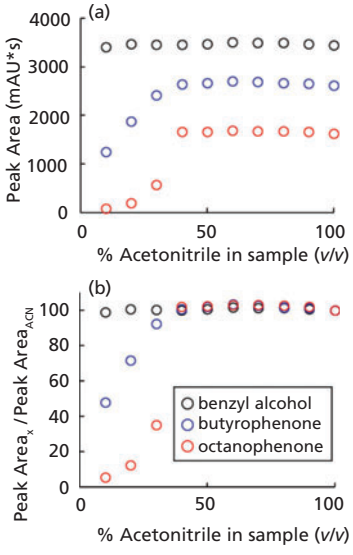
Other approaches described over the years include:

- On-line dilution of the sample with weak solvent in the autosampler needle; this is sometimes referred to as "sandwich injection" (5).
- On-line dilution of the sample with weak solvent by deliberately adding a mixer between the sample injection point and the column inlet (6).
- On-line dilution of the sample with weak solvent during injection into the LC column—this is sometimes referred to as *at-column dilution*, and requires an auxiliary pump to deliver the diluent (7).
- On-line dilution of the sample with weak solvent during injection by splitting the mobile phase flow path to achieve in-line mixing of the sample and diluent (8).

While these approaches have been used with conventional one-dimensional LC (1D-LC), this issue is also very important in two-dimensional (2D)-LC, and a variety of approaches have also been developed to address the problem in the context of 2D-LC specifically (9–12).

As with most challenges in chromatography, there is no perfect solution to this sample solvent matrix issue, and all of these approaches have advantages and disadvantages. Common to all of them, however, is the question, How much dilution is enough? This is obviously a very practical question that must be confronted in method development. Unfortunately, there is no universal answer that is suitable for all types of separation and analyte. Most often, the answer for a particular situation is determined by experiment, and typically the primary focus of such experiments

FIGURE 3: Effect of sample solvent on peak area observed for neutral compounds of varying lipophilicity under reversed-phase LC conditions. Peak areas are plotted as (a) absolute values or as (b) a percentage of the area observed when the sample solvent is 100% acetonitrile. Chromatographic conditions: solvent A: water, solvent B: acetonitrile; gradient: 10–100–100% B for 0–3.5–4.0 min; temperature, 40 °C; flow rate, 1.0 mL/min; column, 50 mm × 4.6 mm i.d., 5-µm Zorbax SB-C18; injection volume, 1.0 µL; detection by UV absorption at 210 nm. The balance of the sample solvent was water, and the concentration of each analyte was 1 mg/mL.



is the effect of the sample solvent on peak shape. In this instalment we address a secondary, but still important concern—analyte recovery.

Effect of Sample Diluent on Analyte Recovery: HILIC Separations

As discussed above, one straightforward approach to address the effect of the sample solvent mismatch relative to the mobile phase is to simply dilute the sample with weak solvent, mix, and inject. However, we have to be careful that when the diluent is added the sample solution remains homogeneous. Two mechanisms that can result in a heterogeneous solution are: 1) precipitation of some analytes or matrix components such that solids and liquids are present in the sample; and 2) phase separation of some analytes or matrix components such that two or more liquid phases are present after adding the diluent. Both of these outcomes are undesirable because the material sampled from these heterogeneous solutions will not be representative of the entire sample, and will lead to inaccurate quantitative results. For samples that contain analytes with similar physicochemical properties, these outcomes can easily be avoided by doing a few scouting experiments to see when or if precipitation or phase separation occurs. However, avoiding these outcomes can be more challenging when the components of the sample are more diverse in terms of their solubilities in the sample solvent, particularly when the diluent is added.

Figure 2 shows an example of this problem in the context of use of a HILIC method for the analysis of a sample containing water-soluble vitamins. For nicotinic acid (Figure 2[a]) the observed peak area is nominally the same whether the sample is prepared in a matrix of 100% water, or 90:10 acetonitrile–water. However, for thiamine and cyanocobalamin (Figures 2[b] and 2[c], respectively), the observed peak areas in the 90:10 acetonitrile–water matrix are roughly 50% of the area observed when the sample is prepared in 100% water and all other conditions are the same. This suggests that thiamine and cyanocobalamin are not fully soluble in 90:10 acetonitrile–water, and some of the analyte precipitates from the sample matrix before it is sampled for analysis. In a case like this, the effect of the diluent on analyte recovery must be studied, and taken into consideration when deciding what sample solvent composition will be used for the final method.

Effect of Sample Diluent on Analyte Recovery: Reversed-Phase Separations

The sample diluent can adversely affect analyte recovery in reversed-phase LC separations as well. To illustrate this effect, we prepared an analyte mixture of benzylalcohol, butyrophenone, and octanophenone—all at 1 mg/mL—in different sample solvents ranging from 10:90 acetonitrile–water to 100% acetonitrile. The predicted water solubilities for these compounds are 15, 0.34, and 0.0037 mg/mL, respectively (www.chemicalize.com). Based on this, we would expect to see consistent peak areas over the full range of sample solvent compositions for benzyl alcohol. On the other hand, we would expect to see consistent peak areas for the lipophilic butyro- and octanophenones in the samples with high levels of acetonitrile, but lower areas in the water-rich samples, because of the low water solubilities of these molecules. Figure 3(a) shows the absolute peak areas measured for the three compounds at each solvent composition; Figure 3(b) shows the same data, but normalized

to the area observed with the sample prepared in 100% acetonitrile. As expected, we see consistent peak areas for benzyl alcohol across the entire range of sample solvents. Note that the areas for intermediate sample solvent mixtures are slightly greater than 100%. This is most likely due to the volume contraction of acetonitrile–water mixtures, given the way the samples were prepared (for example, for the 50% acetonitrile sample, 500 µL of water was added to 500 µL of acetonitrile, and this results in a mixture that has a volume of about 970 µL). However, for butyro- and octanophenone we see that consistent peak areas are observed down to 40% acetonitrile, but then there is a precipitous decrease in the peak area, with the decrease more dramatic for octanophenone than for butyrophenone. This results from the formation of two phases in the samples with 30% acetonitrile or less because of the very low water solubilities of these compounds. Given that their densities are lower than that of water, there is probably a top layer of the solution in the HPLC vial that is enriched in these compounds, but not sampled by the autosampler needle, which samples from well below the liquid surface.

Closing Thoughts

In the everyday practice of LC it is common to encounter samples where the sample solvent composition is very different from the mobile phase used in the LC separation. When injecting volumes of these samples that are large relative to the volume of the column itself, this can lead to poor peak shape. Several approaches have been developed to overcome this challenge, most of which involve dilution of the sample with weak solvent prior to or during the injection step. While this can be an effective remedy for poor peak shapes, we must be careful to avoid analyte precipitation or phase separation of the sample as a result of adding too much of the weak solvent diluent. The level of diluent that is considered too much is compound dependent, but can be determined experimentally through simple screening experiments.

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Essential GC Accessories

John V. Hinshaw, GC Connections Editor

Most manufacturers ship gas chromatographs with a small collection of consumable parts and accessories, such as extra ferrules, inlet liners, or septa, and a few instrument-specific tools. In earlier times, some convenience items might have been included as well, like a bubble flow meter or a small set of tools, but now it's rare to find such things in the shipping boxes. This edition of "GC Connections" lists a number of essential items that should be on hand in every gas chromatography (GC) laboratory, their function, and how to use them effectively.

Every profession has its specialized tools. Those used in chromatography are often just as specialized as those used in computer repair or automotive work. Many of the tools and accessories that gas chromatographers keep on hand for installing, maintaining, and repairing their chromatographs are also found in plumbers', carpenters', and home toolkits. Wrenches, screwdrivers, pliers, and metal tubing cutters are some easily recognized examples. Other items such as dental instruments or paper correction fluid are familiar, but their use in the laboratory environment may not be immediately obvious. Still others, like a column flow meter, septum nut wrench, or a specialized fused-silica column cutter, aren't found outside the laboratory at all.

Here is an updated list of today's tools and accessories, with some information on their use and significance. One or more specialty manufacturers offer many of the chromatography-specific items; just look at their catalogues or on-line offerings. I scanned through several, and gleaned some new items that I have included here.

Source of Gas for Measuring Unretained Peak Times

A butane lighter is a convenient source of hydrocarbon gas for measuring an approximate unretained peak time. Butane is effectively unretained at temperatures above 75 °C on liquid-phase coated columns with phase ratios above 50. Columns at low temperatures or with lower phase ratios (thick stationary films) may retain butane, and separate the traces of ethane and propane present in the butane fuel. Use the earliest observable peak for the best estimate of unretained peak time. Natural gas is mostly methane; if your laboratory has a supply of natural gas (mine doesn't), it makes a good substitute for a lighter, and is less retained than butane—just be sure to turn off the gas once you've filled a syringe with it. A lecture bottle of methane with a suitable pressure regulator is another excellent source of the unretained substance. Concentrations in the low percent range work well. At least one chromatography supplier offers a prefilled methane bottle with a low-pressure regulator for this purpose.

Hydrocarbons won't work for unretained peak time measurements with an electron-capture detector (ECD) because it doesn't respond. Instead, try loading the syringe with a puff from a pressurized can of dust remover such as Dust-Off, which contains 100% 1,1-difluoroethane. Other halocarbons will work as well; check the label to be sure there is an ECD-sensitive compound present.

Hydrogen or helium—whichever is not the same as the carrier gas—makes good unretained peak markers for porous polymer or molecular sieve columns that retain hydrocarbons strongly, plus these two gases should be readily available in most gas chromatography (GC) laboratories. Flame ionization detection (FID) will not respond to hydrogen or helium, but other detection methods such as thermal conductivity (TCD), pulsed discharge (PDD), or helium ionization (HID), the latter with hydrogen as the unretained substance, should respond well.

Remember to use the same inlet pressure and oven temperature to remeasure unretained peak times for any specific column; that is, one with the same dimensions, film thickness, and stationary phase for comparative purposes.

Caps, Capillary Column

Polymer caps for capillary columns establish an airtight seal for storage, so that air, moisture, and airborne contaminants cannot enter. I have used septa for this purpose, but there's a risk of breaking the column and puncturing a finger instead; this only happened to me once. Another approach is to seal the end of the column by

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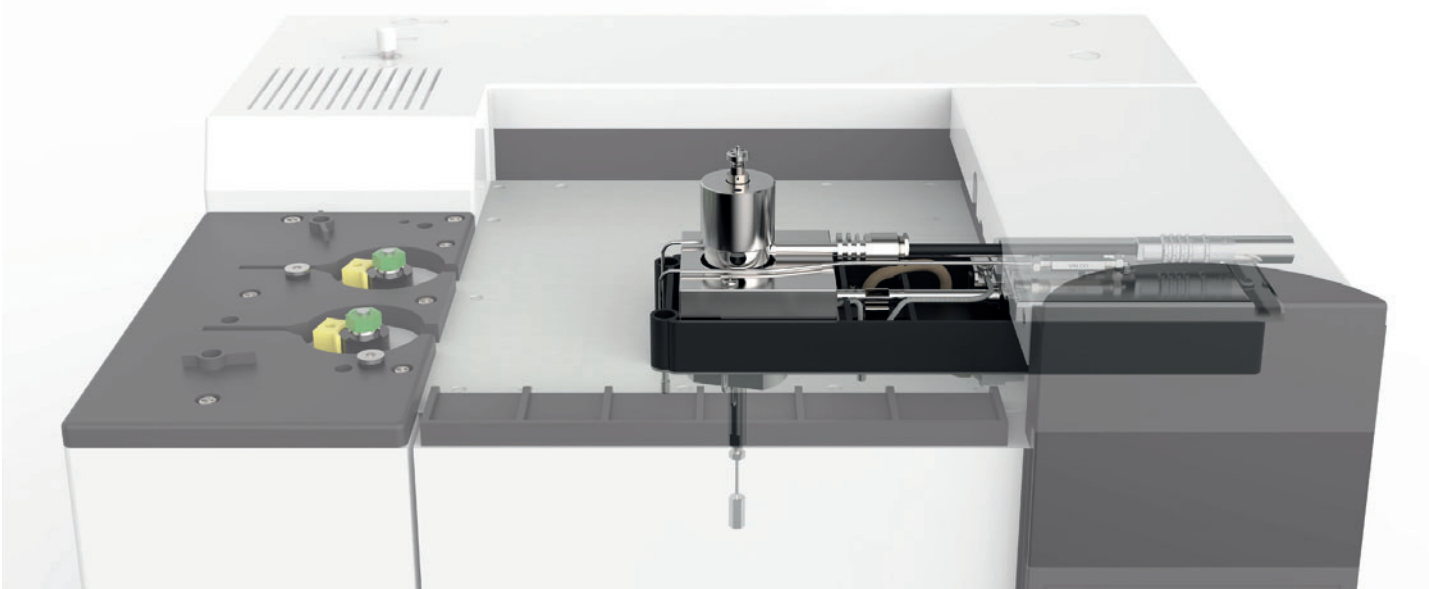

melting it. This is not recommended because it requires a hydrogen torch to achieve a high enough temperature, and it will cause stationary phase combustion by-products to migrate a significant distance into the column.

Correction Fluid

Use white correction fluid to mark the measured position on a column that corresponds to the correct column penetration depth into an inlet or detector. After inserting the column into the nut and ferrule and making a fresh cut on the column end, measure the depth and apply a small dab of correction fluid. For data integrity reasons, some regulated laboratories' policies don't permit correction fluid on site, although in the fully electronic laboratory this is moot. A septum into which a slot has been cut may be slid onto the column below the nut and ferrule to act as a positioning aid. Remove the septum before heating the column oven. A positioning gauge (see "Ruler" later in this column) is a good alternative. I have also used a black permanent marker for this purpose; just be careful not to get it on your fingers or clothing.

Cutters, Fused-Silica Column

The best fused-silica column cutting tool is the one that holds the column in an adjustable chuck, and cuts with a diamond chip as the operator rotates a thumb wheel. This tool also has a magnifying glass on the opposite end for inspecting the fresh cut for squareness and lack of burrs or hanging polyimide coating. A pen-like tool with a sapphire tip or ceramic scoring wafers or scribes that make a sharp cut on the column so that it may be broken cleanly in two are the best inexpensive alternatives. In any case, a fresh cut should be made and inspected just before placing the column into the inlet or detector, after sliding on the nut and ferrule.

Cutters, Diagonal

Diagonal cutters are used only for cutting electrical wires. They should never be used as a substitute for a tubing cutter. Don't even think about threatening your fused-silica column with one!

Cutters, Tubing

A large plumber's tubing cutter for 1/4-inch metal tubing, and a smaller one for 1/8-inch tubing, are used extensively during instrument installation and gas-supply setup. The correct 1/8-inch size cutters are available from some chromatography supply companies; don't try to use the mini cutters found in home supply stores—they're only good for those weekend plumbing projects. Keep a supply of new cutter blades on hand. These disks wear out rapidly, and a dull blade will distort the tubing diameter, and make it difficult to slide on the back and front ferrules. Power cutters with high-speed rotary abrasive cutting wheels are also available. These are mandatory for cutting 1/16-inch tubing for low dead-volume gas valving applications, and they work well for any tubing up to 1/4-inch diameter. After cutting and squaring the tubing (see "Tubing Reamer" and "Deburring Tools" later in this article), be sure to clean out the ends with a solvent, so that loose particles cannot get into the instrument components, or interfere with the passage of peaks through column ends.

Dental Mirror

A plastic dental mirror with a front-silvered surface makes it easy to examine the underside of an inlet fitting in the oven, or to check other inaccessible areas for loose or missing parts. The mirror also can be used to detect the flame in a flame ionization or flame photometric detector by observing condensation of emitted water vapour on the cool mirror surface. A shiny wrench is a good substitute for the mirror in this case.

Dental Pick

A dental pick is very handy for removing septa from septum nuts, and debris such as bits of graphite ferrule from fittings.

Eyedropper (Plastic)

I have a box of plastic eyedrops and they are in frequent demand. I use them to place small drops of isopropanol onto fittings for leak checks. Some types have rough volume indications more like a pipette, and I have used them to make a crude dilution of qualitative test mixtures when accuracy wasn't required.

Files, Needle

An assortment of needle files can be used to pick out ferrules from fittings, as well as to remove burrs and shape the ends of metal tubing before it is connected to a fitting. Don't forget to clean off all traces of metal before connecting.

Flexible Magnetic Pickup

A flexible 2-foot magnetic pickup comes in handy when you drop a small part inside the instrument. Another similar tool has a three-jawed "claw" operated by a plunger, and it will pick up non-magnetic items, too.

Flowmeter, Electronic

An electronic flowmeter is an expensive investment, but I believe that it will pay for itself many times over with improved accuracy and precision over bargain-priced bubble flowmeters. I prefer the type of electronic meter that senses flow directly, and that allows the

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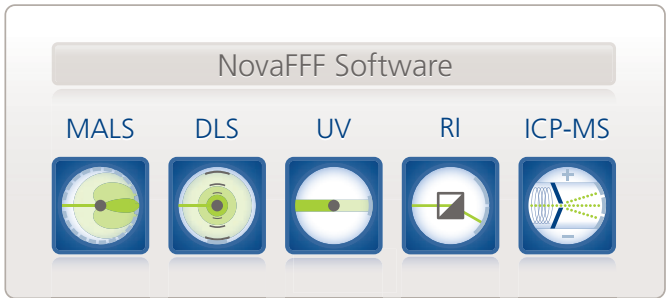

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operator to select the type of gas in use, such as air, helium, or hydrogen. The option to calculate split ratios from the measured split vent and column flows is a handy feature, but many GC systems now include this capability in their software.

Flowmeters, Bubble

If you use bubble flowmeters, keep two sizes on hand. The large size is good for measuring FID air or inlet split vent flows up to several hundred millilitres per minute. The smaller size is better for packed-column or hydrogen flame-gas flows in the 10 to 50 mL/min range. Don't try to use a bubble flowmeter to measure capillary column flows below 10 mL/min. The carrier gas will diffuse out of the bubble and you will get a low reading. Measure the unretained peak time instead, and calculate the flow rate from it. Note that this calculated flow rate or the rate displayed by electronic pressure control will only be as accurate as the column dimensions the operator uses.

Flow Measuring Adapter

Manufacturer-specific flow adapters allow a flow meter to connect to the exact dimensions of a detector's exit.

Short pieces of flexible tubing in various diameters are handy for connecting a flow meter to different size tubing (see "Tubing, Plastic and Rubber" later in this article).

Glass Wool Insertion/Removal Tool

This item is useful for those who must install glass wool in inlet liners, or for the hardy few who pack their own columns and use glass wool to hold in the packing. These days I find little use for it and I use prepacked deactivated inlet liners instead.

Gloves, Lint-free

Polymeric nitrile or vinyl lint-free gloves are essential for column installation and detector maintenance, especially for high-sensitivity detectors such as mass spectrometric, electron capture, and helium ionization types. Handling the column inlet without gloves opens the door to contamination from finger oils and dirt, while cleaning the rods of a quadrupole mass-selective detector (MSD) without gloves is asking for trouble. I recall a particular MSD that exhibited poor sensitivity. A tear-down revealed a huge thumb print on the photomultiplier tube window, perhaps deposited there by a technician who had recently finished lunch.

Hexagonal Key Set

I keep several sets of hexagonal key wrenches and drivers in the toolbox. I have sets of the traditional right-angle Allen keys in both imperial and metric sizes, as well as a collection of hex screwdrivers of both types. Although used less often, a set of star wrenches is indispensable as well. These also come in spline and security variations, some of which I've accumulated over the years. Many of these are used in laboratory instruments instead of the more traditional Phillips screw head, so they are essential for performing maintenance.

High-Temperature String

High-temperature string is a useful item for use in a GC oven to restring capillary columns, attach column connectors to column cages, or hold the column in the GC oven. The string is made of materials that can withstand temperatures of up to 400 °C, or higher.

Inlet Liner Removal Tool

A tapered high-temperature silicone rubber tool on a metal holder does a good job of grabbing glass inlet liners and removing them without cracking or chipping the liner top. Most GC instrument manufacturers will supply specific tools and instructions for a particular inlet option.

Leak Detector, Electronic

An electronic leak detector is expensive, but it indispensable for finding small leaks around hot fittings or inlets on which a liquid cannot be used (see "Leak-Checking Solution" later in the article). The most sensitive type of leak detector uses a small pump to pull air from a probe through a thermal-conductivity cell. The presence of carrier gas or hydrogen changes the thermal conductivity and causes a change in the detector's readout compared to a reference air flow. Sensitivity for nitrogen carrier is limited. I also have a small handheld battery-powered leak detector that has a series of light-emitting diodes, which indicate the detected leak rate. This detector is great for carrying around in a laboratory like mine with lots of instruments and little clear bench space.

Leak-Checking Solution

In my toolbox, the only acceptable leak-checking solution is a small bottle of pure isopropanol with an eyedropper. Other solutions may contain material such as surfactants that can leak into the gas-supply lines or columns and cause ghost peaks or other contamination.

Magnifier

A small magnifier is used to examine freshly made column or tubing cuts for burrs or uneven edges.

Manufacturer-Specific Tools

For each GC system, there are always some specialized tools. These are used, for example, to open up a split–splitless inlet, or to remove the inlet liner. Perhaps a special wrench is required for FID system flame jet replacement. Whatever the case, keep all such tools with their instrument; you will need them eventually. Some of the chromatography suppliers offer their own versions of these tools, which are often more useful than the freebie ones that come with the instruments.

Maintenance Kits

Kits consisting of a collection of the most often replaced parts and supplies are a good starting point with a new instrument. Available for inlets and detectors, these packages will get used almost immediately upon commissioning. After a while, I find it convenient to re-order only those items that are used frequently instead of purchasing additional kits. The

kits commonly include inlet liners, ferrules, flame jets, gauges, cleaning items, adapters, and common tools for their removal and replacement.

Mini Flashlight

A mini-flashlight is very handy for inspecting the interior of inlets and detectors for obstructions, as well as for illuminating the oven interior. I prefer the type with the bulb on a flexible gooseneck. A small short LED flashlight, the kind that you might get as giveaways at conference booths, also makes an excellent GC oven light. No one yet has built a GC oven with a light that comes on when the door is opened. A flashlight also makes a good dropped small part finder, to locate ferrules, for example. Just place the flashlight horizontally on the laboratory floor, sweep it around, and look for the shadow.

Paintbrush

An artist's paintbrush with handle is handy to clean out debris from small areas inside detectors or inlets. Just watch that a fibre from the brush does not dislodge. It can also be used to apply leak-checking solution to fittings, although I don't recommended this practice, due to potential contamination of the gas stream with the leak-checking solution.

Paper Clips

Jumbo-size paper clips with smooth sides are convenient for blocking off inlet or detector fittings for testing purposes. Unbend the clip and attach it to the fitting with a nut and 1-mm i.d. graphite-vespel ferrule. With the column connection blocked off, you can pressure-check an inlet. A detector check can be run in this manner without column influences on noise or stability.

Pin Vise and Drills

A small pin vise and a set of drills can be used in an emergency to drill out a used ferrule, or to enlarge one that is too small to fit a column. Sometimes the small drills can help to remove a ferrule that is stuck in a fitting, or to remove debris from inside fittings or tubing ends.

Pliers

I keep some small needlenose pliers, a pair of larger multigrip pliers, and one pair of locking pliers in my toolkit. The larger gripping pliers are useful

for holding a straight length of 1/8- or 1/4-inch metal tubing while cutting it, although I take care not to grip the tubing anywhere near a location where a connection is to be made, because the scratches from the pliers would make it impossible to get a good seal.

Press-Fit Connectors

Glass press-fit connectors make it easy to repair a broken column temporarily (until a replacement can be installed). These are available in many sizes to connect fused-silica tubing of the same or different diameter. They also connect a column with a retention gap. One manufacturer offers a vacuum–melting device that makes near-perfect connections.

Pressure Gauge, Inlet

I have a conventional 0–60 psig pressure gauge with a syringe needle attached that I can insert into an inlet through the septum. Once in a while, I need to check the inlet pressure this way, instead of relying on the instrument's gauges or electronic pressure readouts.

PTFE Tape

PTFE tape is used sparingly on tanks and interconnecting fittings where threads form the seal. Use two layers of tape, not more, and wrap them around the threads in the direction the nut tightens, so that the tape will be drawn into the fitting instead of getting pushed out. PTFE tape is never used in swage-type ferrule-sealed fittings, where it will only cause a leak, nor is it used at the high-pressure supply cylinder connection. Several types of this tape are available; be sure to select the right one.

Rinsing Reservoir for Capillary Columns

A rinsing reservoir may be useful for reviving bonded-phase capillary columns contaminated by soluble but nonvolatile sample residues. Solvent rinsing is intended for use only after bake-outs and trimming of about 0.5 m of the inlet end plus the exit portion that enters the detector heated zone have failed to restore performance. Use only the solvents that the column manufacturer recommends, and rinse only from the detector end to the inlet end to avoid depositing contaminants deeper into the column. Check and revalidate column performance after rinsing.

Ruler

A small metal ruler measures the correct column penetration depth into an inlet or detector. Don't use a plastic ruler because it might melt in contact with heated inlets or detectors. For convenience, make marks on the ruler that correspond to the correct inlet and detector depths. Several manufacturers offer capillary column installation gauges with the appropriate markings.

Scissors

A good sharp pair of scissors comes in handy for opening packages of ferrules, or for making paper stars out of waste paper that's waiting to be recycled while watching for peaks to be eluted. Scissors are never to be used to cut fused-silica columns (but you can believe that I've seen someone try it).

Screwdrivers, Phillips-Head

I have three Phillips-head screwdrivers in the toolbox: large, medium, and small. The small one is part of a set of jeweller's screwdrivers with rotating handles. These are general purpose items that any laboratory should have on hand.

Screwdrivers, Slotted-Head

I also keep three slotted-head

screwdrivers. The small one is useful for securing electrical connections to screw-type terminals.

Septum Puller

This is a specialized tool for removing septa that become stuck due to heat-adherence to the inside of the septum area of an inlet. I have also used a dental pick for this purpose. Avoid using a flat-bladed screwdriver or a knife because these may scratch the metal surfaces that seal to the septum.

Static Pad

A static pad is a grounded, conductive plastic sheet onto which it is safe to place electronic components that must be protected from damaging electrostatic discharge. Any circuit boards removed from an instrument should be placed on a grounded static pad, or in a static-proof bag.

Static Wrist Wrap

A grounded static wrist strap prevents the technician from imparting a potentially harmful static discharge into instrumentation or components. Always wear one when working inside an instrument or removing components, and in all cases be quite sure that the instrument power has been removed while the instrument itself remains grounded.

Stopwatch, Digital

A digital stopwatch times bubbles in a bubble flowmeter, and also times an unretained peak. It's often more convenient to use a stopwatch when setting up an instrument than to operate the chromatography data system for each test injection. Select a stopwatch with a readout to 0.01 s. Some GC systems include a stopwatch function on the display that includes flow, split ratio, and linear velocity calculations. These days, I just use my smartphone's stopwatch and timer functions, and then its calculator to find flow rates or average linear velocities. Good phone applications are available with additional chromatography functions.

Swabs

Recently I have used polyester swabs, mostly intended for cleanroom use, for cleaning out inlets and detector bodies. Take care that the solvents used are compatible with the swab material. Methylene chloride, for example, will dissolve polyester readily. I don't like to use cotton-tipped swabs on a wooden stick. Not only will the cotton release fibres but the wood may also release higher molecular weight compounds into cleaning solvents.

Syringe

I keep two manual syringes for setup purposes. One 10-µL gas-tight syringe is for injecting methane or butane to measure the unretained peak time and ascertain that the flame is lit and carrier gas is flowing. The other is for making liquid test-mixture injections as part of a column checkout. Sample syringes are kept separately.

Syringe-Cleaning Wires

Syringe cleaning wires may be used in an emergency to clear septum particles or other debris from syringe needles. I recommend discarding stubborn contaminated syringes; take steps to keep the syringe clean instead.

Test Mixtures

Column and detector test mixtures verify column performance and detector sensitivity. Keep a fresh vial of each type on hand, and put them in the chemical refrigerator if the label so directs. Column test mixtures are available for polar and for nonpolar capillary columns, and there are test mixtures for each detector type. Some manufacturers provide a detector test mix that combines components for testing several different detectors. Once opened, test mixtures can be kept for a while in septum-sealed vials. Their lifetime is limited because of gradual evaporation. If you keep test mix in a vial, remove the vial cap rather than puncture the septum when withdrawing liquid for injection. Some laboratories find it more convenient to keep dilute test mixtures on hand; these are more easily disposed of than the concentrated mixes. Many laboratories have their own qualification and validation standards, of course, but the manufacturer's mixtures allow easy comparison to the factory test results, and may be used for a factory-level checkout.

Tube Blender

I use this simple tool to make controlled bends of copper or stainless steel tubing for connecting the supply tanks to the filters, and then to the back of the instrument. It makes for a clean and organized looking installation. Tube benders come in sizes to fit standard tubing diameters.

Tube Reaming and Deburring Tools

These tools are used to remove burrs and irregularities from metal tubing after cutting. They are available for the standard tubing diameters, and I highly recommend using them in order to ensure leak-free connections. As always, be sure to clean off any loose metal particles or shavings.

Tubing, Plastic and Rubber

I keep several pieces of black and clear silicone rubber tubing on hand for connecting my flow meter to

column ends, split vents, and other flow sources. The narrower pieces of tubing fit inside the wider ones so that I can adapt the flow meter fitting to a variety of connections. I have some silicone tubing that seals nicely to 1/16-inch tubing, and then slides into the flow meter's 1/8-inch inner diameter inlet tubing. We ordered a small spool of this silicone tubing, because it was always disappearing into other locations in the building. Of course, I never use plastic or rubber tubing for any gas at elevated pressure, or for permanent supply or internal connections.

Tweezers and Hemostats

A pair of tweezers can hold small nuts or ferrules without risking contamination with skin oils or a burn from hot items. Some tweezers have a convenient locking feature that frees one hand for other tasks, as will a spring-loaded hemostat. Rubber tips help hold fragile capillary columns or inlet liners.

Vial, Autosampler

I keep a spare autosampler vial to check for carrier gas flow during column installation. Fill the vial halfway with distilled water, and then insert the column outlet after connecting to the inlet but before connecting to the detector. After turning on carrier gas pressure, the presence of bubbles shows positive carrier gas flow. Never heat a column until you are sure there is positive flow through it.

Vial Crimper

Vial crimpers attach aluminium crimp-top seals to autosampler vials. Several crimp-top sizes are commonly used for GC: 8-mm for 0.8-mL vials, 11-mm for 1.5 or 2.0-mL vials, and 20-mm for 5-mL and 20-mL headspace vials. Hand crimpers are the least expensive, and some are available with interchangeable jaws that accommodate different vial sizes. Automated bench-top crimpers are less mobile, but the jaws can be interchanged quickly, and they are best for laboratories with high sample throughput. I prefer the type with rechargeable batteries.

Vial Decapper

Vial decappers perform the opposite function of a crimper; they remove crimp-top seals from vials. Decappers come in the same sizes as the crimpers, and resemble a pair of pliers. Some caution is required in use, so as not to break the neck of the vial. Once caps are removed, the contents may be properly disposed. Some laboratories reuse sample vials, but I recommend a fresh vial for each sample if at all possible.

Wipes, Laboratory

I wet laboratory wipes with some isopropanol, and then clean any debris or oil off the ends of capillary columns before inserting them into inlets or detectors. They are also handy for tipping a drop of test mix off a syringe needle, if disposed of properly. Paper towels don't work as well; they may leave fibres behind, and they may deposit a chemical residue. I recently have started to use particle-free cleanroom type wipes premoistened with a mixture of 70% isopropanol and 30% deionized water.

Wire Brushes

Wire brushes can dislodge particles and debris from detector parts and some sealing surfaces. Be careful not to score polished metal surfaces, or damage ceramics. It is better to replace a severely dirty FID flame jet or collector than to clean it forcibly. The manufacturer's guidelines for cleaning will have ways to assess the reusability of these parts.

Wrenches, Adjustable

I have one large 18-inch-long adjustable wrench that looks like it belongs in an automotive garage. This is used exclusively for attaching or removing pressure regulators on gas tanks. I also have a smaller 6-inch-long adjustable wrench that I use occasionally, if someone else has walked off with the exact open-ended wrench size I need.

Wrenches, Open-Ended

I have an assortment of open-end wrenches in inch sizes, as well as a metric set. I keep two or three with the following sizes: 1/4-, 5/16-, 3/8-, 7/16-, 1/2-, and 9/16-inch, as well as 11/16-, 3/4-, and 1-inch, although these latter sizes are used only rarely. Chromatographers located outside the United States will find the standard metric wrenches to be a bit more useful. I apply two wrenches at once to prevent counter-rotation while tightening or loosening fittings.

Conclusion

Chromatographers, like all craftspeople, use a variety of tools to practice their craft. In a pinch, tools that are somewhat inappropriate can be used to make do, but the rapidity and ease with which the right tool gets the job done makes it well worth the expense of obtaining what's needed for the job.

"GC Connections" editor **John V. Hinshaw** is a Senior Scientist at Serveron Corporation in Beaverton, Oregon, USA, and a member of *LCGC Europe*'s editorial advisory board. Direct correspondence about this column to the author via e-mail: LCGCedit@mmhgroup.com

Biphenyl Phases

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

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

Olfactory Detection Port for GC

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

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

HPLC Phases

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

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

Hydrogen Generator

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

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

Chip-Based Chromatography

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

www.pharmafluidics.com
Pharmafluidics, Ghent, Belgium.

GPC/SEC Validation Kit

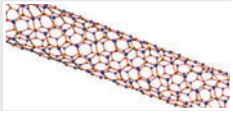
PSS EasyValid is a system suitability test that evaluates the entire GPC/SEC/GFC system, equipment, electronics, and analytical operations, to ensure that "true" molar mass results are obtained. According to the company, it is ideal for various aspects of quality assurance qualification, whether mandated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

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

Boron Nitride Nanotubes

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

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



AF4 instrument

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

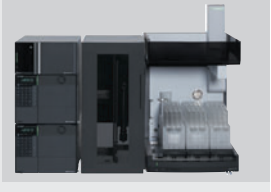
www.wyatt.com/eclipse
Wyatt Technology, Goleta, California, USA.

Leak Detector

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

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

Liquid Chromatograph



Shimadzu has announced its new Nexera Prep Series Preparative Purification Liquid Chromatograph (LC). According to the company, it provides better prep processes for extraction of target compounds and impurities during drug discovery in the pharmaceutical industry and purification of functional components in the chemical and food industries.

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

eCatalogue

The YMC Classics eCatalogue is available for download. According to the company, it is the ideal resource for all kind of specific information such as phase specifications for classic YMC columns. The eCatalogue covers multiple YMC product lines in detail.

https://ymc.de/files/imported/publications/282/documents/YMC_Classics_eCatalogue_0819.pdf
YMC Europe GmbH, Dinslaken, Germany.

Air Sampling and Thermal Desorption

Used for air sampling, the VocStar pump features a ready-to-use polymer cartridge. After the sampling the cartridge is placed in the thermal desorber or extracted with organic solvent. A direct connection to GC or GC–MS allows the detection of different volatile organic compounds (VOCs) present in the sample.

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

The 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019)

The 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019) will take place in **Prague, Czech Republic, on 5–8 November 2019.**

The **RAFA 2019** symposium will provide an overview of contemporary trends in analytical and bioanalytical strategies in food quality and safety control and will discuss challenges and novel approaches in food and natural products analysis. The following areas related to recent and emerging issues will be addressed within the **RAFA 2019** sessions: mycotoxins, marine, and plant toxins; migrants from food contact materials; processing contaminants; pesticide and veterinary drug residues; industrial contaminants; allergens, antinutrients; metals, metalloids, and speciation; healthy nutrients and vitamins; flavour-significant compounds; quality assurance/quality control (QA/QC), chemometrics, and big data handling; authenticity and food fraud; omics in food analysis; food forensics; microplastics in food; novel food bioactives and supplements; organic crops and foodstuffs; human biomonitoring; portable on-site food analysis.

The conference programme will be accompanied by several satellite events alongside workshops, seminars, and tutorials on novel analytical strategies, including workshops on "Vibrational Spectroscopy and Chemometrics for the Monitoring of Food and Feed Products and Contaminants Detection", "Human Biomonitoring in Food Quality and Safety", "Microplastics in the Food Chain", and "Portable Food Analysis and Citizen Science". Other sessions include "Data Quality and Smart Data Handling in Food Analysis" and "METROFOOD-RI: Workshop on Metrology in Food and Nutrition", among many others.

An open day from the EU-China-Safe, Delivering an Effective, Resilient, and Sustainable EU-China Food Safety Partnership, will focus on the latest developments and strategies in food safety and authenticity control and demonstrate the best practices for a joint EU-China food safety control system. Showcasing the FoodSmartphone and PhasmaFood projects, RAFA Smart Lab will demonstrate smart analyzers and applications for on-site testing of food quality and safety. Another session will discuss the collaboration challenges within the EU framework programme for research and innovation.

Vendor seminars provide the opportunity for leading companies to introduce recent instrumentation and analytical strategies for advanced food quality and safety control. An exhibition of modern instruments, laboratory equipment, reference materials, and consumables used in food analysis will be an important part of the symposium.

A platform for young scientists will be offered to present their scientific work, including Student Travel Grants and the prestigious RAFA Poster Award and sponsored poster award(s).

In summary, **RAFA 2019** will offer a high-quality scientific programme with top-quality presentations followed by stimulating discussions, a series of satellite events, a large state-of-the-art exhibition, and an attractive social programme. Scientific contributions will be presented by leading scientists through keynote lectures and by contributed oral and poster presentations. The **RAFA 2019** programme will be tailored to provide a lot of opportunities for networking as well as exploration of the latest results of the food analysis community.

E: RAFA2019@vscht.cz **W:** www.rafa2019.eu

29 SEPTEMBER–1 OCTOBER 2019

SFC 2019
Philadelphia, Pennsylvania, USA
E: register@greenchemistrygroup.org
W: www.greenchemistrygroup.org/current-conference/sfc-2019

4–9 OCTOBER 2019

Grass Roots 4: Understanding and Application of Biopharmaceutical Workflows
Longmynd Hotel, Church Stretton, UK
E: anthony.edge@agilent.com
W: https://chromsoc.com/event/grass-roots-2019

14–16 OCTOBER 2019

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

21–23 OCTOBER 2019

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

28–29 NOVEMBER 2019

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

29–31 JANUARY 2020

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

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

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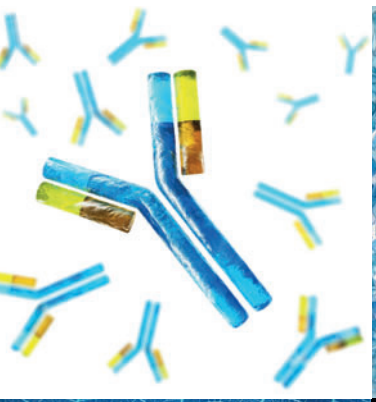
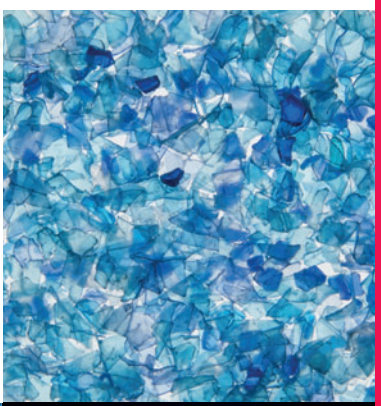


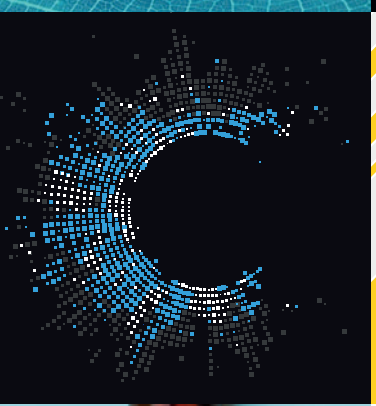


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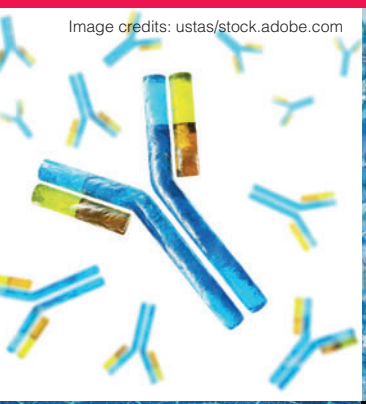
The Applications Book

LC GC europe
solutions for separation scientists

September | 2019
Volume 32 Number 9

The Applications Book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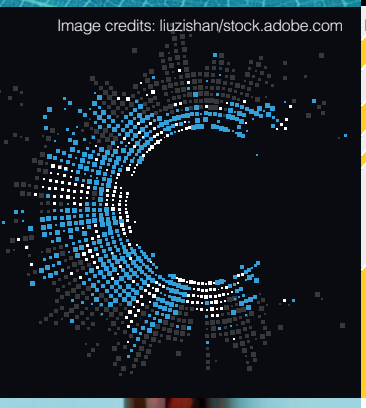
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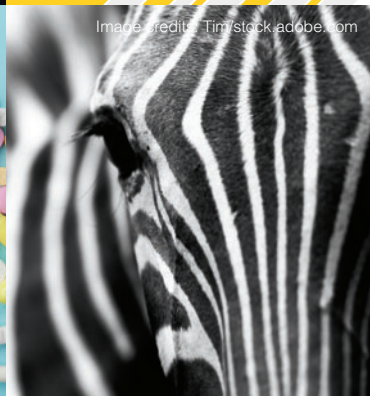
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FOOD AND BEVERAGE

MEDICAL/BIOLOGICAL

PHARMACEUTICAL/DRUG DISCOVERY

Determination of Triphenylmethane Dyes from Aquaculture Samples

Hans Rainer Wollseifen, Torsten Kretschmer, Johannes Brand, and Detlef Lambrecht, MACHEREY-NAGEL GmbH & Co. KG

This application note describes the determination of the triphenylmethane dyes Malachite Green and Crystal Violet and their metabolites from the aquaculture samples brown trout, shrimp, and tuna using dispersive SPE (dSPE) with CHROMABOND® QuEChERS mixes for sample clean-up. The eluates from dSPE cleaning are finally analyzed by HPLC–MS/MS using the biphenyl phase NUCLEODUR® π².

Motivated by various potential health benefits, human feeding behaviour is changing, leading to an increasing demand for aquatic products. Farming aquatic species is becoming an important way of producing large amounts of aquatic products all over the world. One challenge of farming aquatic species is the control of infectious diseases.

Triphenylmethane dyes (TPM) are organic compounds originally used as textile and paper dyes and have been found to be effective as antibacterial, antifungal, and antiparasitic agents in fisheries. They accumulate in fish and metabolize to the equivalent, colourless leuco-forms, which are also known as *mutagenic* (1). To protect human health, triphenylmethane dyes have been banned in many countries according to the recommendations of national and international related agencies. For example, the European Union has implemented a minimum required performance limit (MRPL) for the sum of Malachite Green and Leucomalachite Green of 2 µg/kg (2).

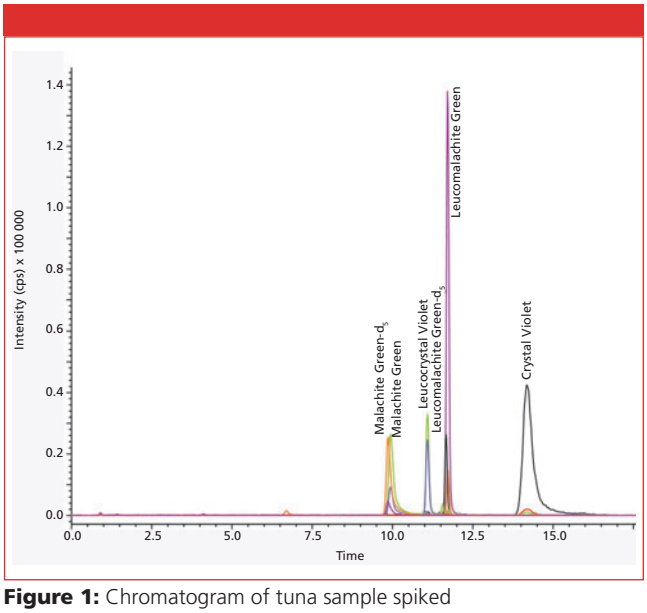

Figure 1: Chromatogram of tuna sample spiked with 1.0 µg/kg sample for each analyte.

Table 1: MRM transitions for the analysis of triphenylmethane dyes

Analyte	$[M+H]^+$	Q_1 (Quantifier)	Q_2 (Qualifier)
Crystal Violet	372.2	356.3	251.1
Leucocrystal Violet	373.9	358.0	238.2
Leucomalachite Green	330.9	316.2	239.1
Leucomalachite Green-d_5	335.9	321.1	243.1
Malachite Green	328.9	313.1	207.9
Malachite Green-d_5	333.9	318.3	213.1

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) methodology is known as a common sample preparation technique in modern analysis of pesticides in food, and offers a quick and cost-efficient determination of different analytes in strongly matrix-contaminated samples by GC–MS and LC–MS (3).

Biphenyl-modified HPLC phases feature a better retention of aromatic analytes compared to classical C_{18} phases due to additional π-π interactions.

Dispersive Solid-Phase Extraction (4)

Extraction:

- Weigh out 5 g of homogenized sample into a 50 mL brown centrifuge tube
- Add 5 µL of internal standard solution (β = 1 µg/mL Malachite Green-d_5 and Leucomalachite Green-d_5 each in acetonitrile)
- Add 25 µL of standard solution (β = 200 ng/mL Malachite Green, Leucomalachite Green, Crystal Violet, Leucocrystal Violet each in acetonitrile) for determining recovery rate
- Add 5 mL water and shake
- Add 10 mL 1% acetic acid in acetonitrile and shake
- Add the CHROMABOND® QuEChERS extraction Mix II (Macherey-Nagel REF 730971)
- Shake vigorously for 30 s and cool the mixture down in an ice bath
- Centrifuge the mixture at 4500 rpm for 5 min at 4 °C

Clean-up:

- Put 5 mL acetonitrile supernatant in a 15 mL brown centrifuge tube
- Add the CHROMABOND® QuEChERS clean-up Mix III (Macherey-Nagel REF 730972) (for samples with high fat content add 1 mL hexane)
- Shake vigorously for 30 s
- Centrifuge the mixture at 4500 rpm for 5 min at 4 °C

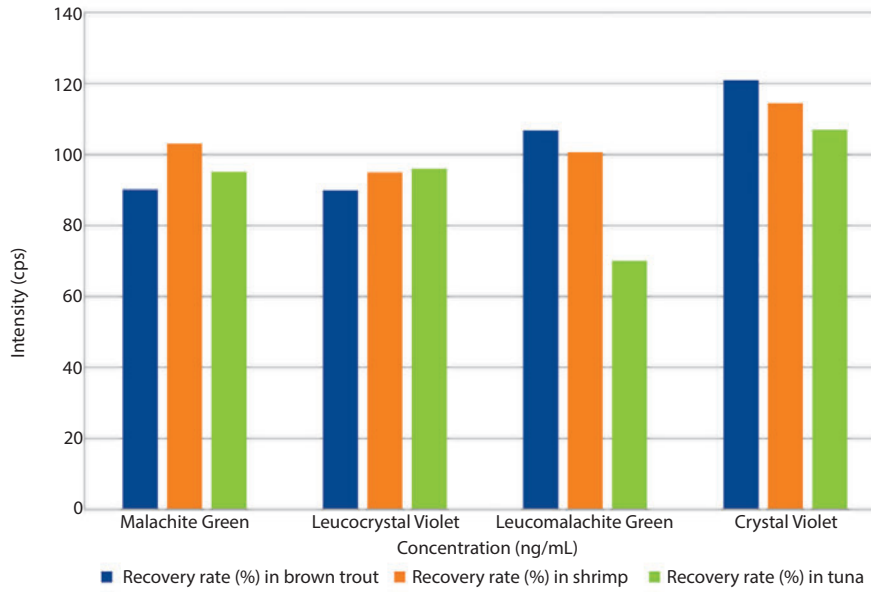

Figure 2: Recovery rates for presented dispersive solid-phase extraction method.

- Dilute the extract 1:1 with an aqueous solution of 5 mmol/L ammonium acetate + 1 mL/L formic acid and filter through a syringe filter (CHROMAFIL® Xtra PET-20/13, Macherey-Nagel REF 729208)

Subsequent Analysis: HPLC–MS/MS (5)

HPLC column: EC 100/3 NUCLEODUR® π^2, 3 µm (Macherey-Nagel REF 760636.30)
Eluent A: 5 mmol/L ammonium acetate + 0.1% formic acid in water
Eluent B: acetonitrile
Gradient: 20–85% B in 10 min, 85% B for 5 min, 85–20% B in 1 min, 20% B for 5 min
Flow rate: 0.4 mL/min
Temperature: 25 °C
Injection volume: 5 µL
MS/MS detection: LCMS 8050 (Shimadzu); ion source: ESI, positive ionization mode; scan type: MRM; interface heater on; interface current: 4000 V; interface temperature: 300 °C; DL temperature: 250 °C; nebulizing gas flow: 3.00 L/min; heating gas on; heating gas flow: 10.00 L/min; heat block: 400 °C; drying gas on; drying gas flow: 10.00 L/min

Results

The identification and quantification of TPM dyes in the cleaned sample extracts of brown trout, shrimp, and tuna were successfully performed by ESI mass spectrometry. Calibration curves were in the concentration range between 0.5 ng/mL and 50 ng/mL for each analyte. Figure 1 shows the chromatogram of QuEChERS extract from the tuna sample spiked with 1 µg/kg with high signal response and less matrix interferences.

By using the QuEChERS methodology it was possible to recover more than 70% of TPM dyes from the different matrices (see Figure 2). The use of brown centrifuge tubes protects the analytes from light-induced degradation and leads to better sensitivities and recovery rates.

Conclusion

In summary, the presented application describes a quick and convenient method for the determination of TPM dyes from aquatic samples.

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(5) HPLC Application No. 128430, MACHEREY-NAGEL, available from www.mn-net.com/apps.

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Determination of Pesticides in Coffee with QuEChERS Extraction and Silica Gel SPE Cleanup

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Coffee is one of the most widely consumed beverages in the world, partly due to the stimulating effect of its caffeine content. Like most crops, the application of pesticides in coffee cultivation is a common practice to increase production yields. This application note details an optimized method for the extraction and cleanup of pesticide residues from coffee using a QuEChERS extraction procedure followed by a silica gel SPE cleanup.

Table 1: Extraction/analytical materials	
ECMSSC50CT-MP	50-mL centrifuge tube and mylar pouch containing 4000 mg MgSO4 and 1000 mg NaCl
CUSIL156	Clean-Up® silica gel 500 mg / 6 mL column
GCLGN4MM-5	GC liner 4 mm splitless gooseneck 4 mm i.d. × 6.5 mm o.d. × 78.5 mm

Procedure

Sample Extraction:

a) Add 10 mL brewed coffee (pH adjusted to about 8 with 1 N NaOH) and 10 mL acetonitrile (MeCN) to a 50-mL centrifuge tube.
b) Add the QuEChERS extraction salts from the Mylar pouch (ECMSSC50CT-MP) to the 50-mL tube, and shake vigorously for 1 min manually or using a Spex 2010 Geno-Grinder at 1000 strokes/min.
c) Centrifuge at ≥3000 rcf for 5 min.
d) Transfer 5 mL supernatant to a clean test tube, add 1.5 mL toluene, and evaporate to about 1 mL.

Sample Cleanup of Extract:

a) Add about ½ inch of anhydrous sodium sulfate to a silica gel SPE cartridge (CUSIL156), and attach the SPE cartridge to a glass block or positive pressure manifold.
b) Wash the SPE cartridge with 6 mL dichloromethane, soak for 1 min, drain to waste, and dry the SPE cartridge for 1 min under full vacuum or pressure.
c) Condition the SPE cartridge with 2 × 6 mL hexane by gravity.
d) Insert glass collection container into the manifold, load the 1 mL concentrated sample onto the SPE cartridge, rinse the test tube with 6 mL of 15% acetone in n-hexane and apply the rinsate to the SPE cartridge, and collect.
e) Continue to elute with 3 × 6 mL of 15% acetone in n-hexane by gravity.
f) Add 1.5 mL ethyl acetate to the eluate container and evaporate to 1 mL.
g) Add internal standard, vortex for 30 s, and inject 1 µL into the GC–MS system for analysis.

Instrumental

GC–MS/MS: Agilent 6890N GC coupled to a 5975C MSD
Column: Restek Rxi®-5Sil MS (30 m × 0.25 mm, 0.25-µm)
Carrier gas: Helium (1.2 mL/min)
GC inlet temp.: 250 °C
Injection volume: 1 µL (splitless)
Temp gradient: 60 °C for 1 min, 10 °C/min to 310 °C, hold for 2 min; 28 min total
Ion source temp.: 250 °C
Ionization mode: EI (70 eV)
Acquisition mode: SIM

Results

Table 2: Recovery and RSD% from spiked coffee samples

Compound Name	Spiked at 20 ng/mL		Spiked at 200 ng/mL	
	Recovery %	RSD% (n = 5)	Recovery %	RSD% (n = 5)
Carbaryl	100.2	5.0	98.7	1.6
Tebuthiuron	95.3	6.3	99.9	2.4
DEET	102.4	5.3	99.1	2.5
Simazine	103.5	5.4	98.6	1.2
Atrazine	103.6	6.5	97.9	2.4
Diazinon	124.4	9.9	99.6	2.2
Pyrimethanil	106.4	6.3	101.6	1.2
Disulfoton	88.1	7.1	92.5	2.2
Acetochlor	103.3	5.6	98.7	1.6
Methyl parathion	91.3	6.3	97.9	1.9
Malathion	103.0	7.7	99.9	3.6
Chlorpyrifos	103.6	6.9	99.4	1.3
Triadimefon	109.3	5.1	101.5	1.6
Cyprodinil	106.4	6.8	102.4	1.0
Endosulfan I	114.0	6.2	98.2	1.7
Flutriafol	74.5	11.6	87.9	4.7
Endosulfan II	103.7	6.1	99.5	1.3
Tebuconazole	92.7	8.5	101.8	1.5
Pyrazophos	98.0	7.5	101.4	1.4
Cypermethrin (sum)	97.0	5.1	101.7	1.0

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Effective Enrichment of Glycopeptides in Drop-HILIC Approach Using iSPE®-HILIC Material

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Glycoproteomics aims at the concomitant identification of not only the composition of the glycan but also the sites of glycosylation and the determination of the protein attached with glycans. However, glycopeptide analysis is challenging because their microheterogeneity results in the reduced concentration of each individual glycopeptide molecule compared to unmodified peptides, even if they are obtained from the very same digest sample (1). Also, glycopeptides exhibit poor ionization efficiency compared to their nonglycosylated counterparts (2). Therefore, it is essential to perform selective and efficient glycopeptide enrichment by solid-phase extraction (SPE) prior to detection and identification by mass spectrometry (MS) analysis (3–10). Hydrophilic interaction liquid chromatography (HILIC) SPE has been extensively applied in the past decade because of its low bias towards different glycan types (9,10). In contrast to normal-phase liquid chromatography, the HILIC retention mechanism is mainly based on the hydrophilic partitioning of the analyte to the enriched superficial water layer surrounding the surface of the polar stationary phase (11). Ionic interaction and hydrogen bonding may also be involved in the separation depending on the sample properties and the character of HILIC stationary phase. Here, we demonstrate the glycopeptide enrichment efficiency of iSPE®-HILIC material for glycoproteomics applications using a well characterized glycoprotein standard.

Experimental

Sample Preparation and HILIC SPE Enrichment: Three micrograms of standard glycoprotein (human IgG) were subjected to trypsin digestion. Glycopeptides were enriched using 25 mg iSPE®-HILIC material (50 µm, 60 Å, HILICON) by a drop-HILIC

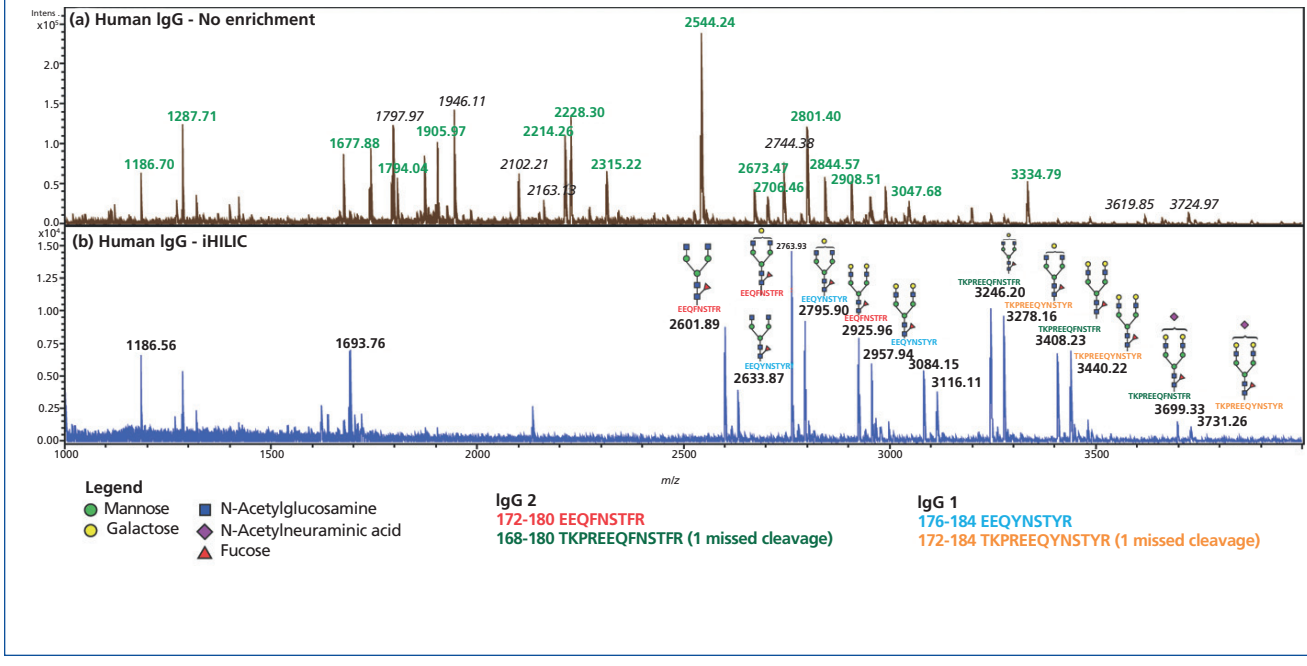

Figure 1: MALDI-TOF-MS spectra of tryptic IgG (glyco)peptides co-crystallized with DHB and analyzed in reflectron-positive ion mode. (a) No enrichment; *m/z* values highlighted in green correspond to the tryptic peptides derived from Human IgG 1-4 subclasses. (b) Enrichment using iSPE®-HILIC material; the annotated exemplary glycopeptides with the most likely structures in the same sample after enrichment. The lists of (glyco) peptides are available on request.

approach as described earlier (9). The dried samples, which contained about an aliquot of 30 ng enriched glycopeptides in each sample, were reconstituted with 100 µL of 0.1% TFA and used for the MALDI-TOF-MS analyses. Equal volumes of sample and matrix (20 mg/mL 2,5-dihydroxybenzoic acid [DHB] in 30% acetonitrile/0.1% TFA) were spotted onto a MTP 384 target plate ground steel (Bruker Daltonics).

MS System: MALDI-TOF-MS analysis was performed on a spectrometer equipped with Smartbeam™ 3D laser optics running at 5000 Hz and controlled by FlexControl 4.0 software (Bruker Daltonics). MS analysis was performed in reflector-positive mode, and the spectra were acquired within the mass range of *m/z* 1000 to 4000. In total, 20,000 shots were accumulated per spot, baseline corrected, and smoothed using Gauss algorithm with *m/z* 0.2 width and 1 cycle.

Results and Conclusion

The efficiency and selectivity of the iSPE®-HILIC material for glycopeptide enrichment were evaluated using a human Immunoglobulin G (IgG) mix. The tryptic (glyco)peptides mixture was analyzed using MALDI-TOF-MS before and after HILIC enrichment. As shown in Figure 1(a) with non-enriched samples, no signals corresponding to the IgG glycopeptides could be detected. Nevertheless, after glycopeptide enrichment using iSPE®-HILIC material, a total of 14 individual glycopeptides were identified from 30 ng of glycoprotein by MALDI-TOF-MS, shown in Figure 1(b). Next to these glycopeptides, a few unmodified polar peptides (in the lower *m/z* region as shown in Figure 1[b]) were also coenriched, but they did not interfere with glycopeptide detection. Considering the fact that HILIC enriches glycopeptides based on their hydrophilicity, it does not come as a surprise other hydrophilic compounds are coenriched (10). Such an unavoidable coenrichment of smaller hydrophilic peptides usually does not interfere with the ionization and detection of the target glycopeptides in the higher *m/z* range. For detailed information on the detected peptides, please contact us to retrieve the list of identified peptides corresponding to the different IgG subclass by MALDI-TOF-MS analysis in the nonenriched sample and the list of tryptic IgG Fc N-glycopeptides detected in the samples after iSPE®-HILIC enrichment.

In summary, iSPE®-HILIC offers a robust, reliable, and easily implemented solution for effective glycopeptides enrichment in glycoproteomics. In combination with optimized sample digestion protocols (for example, salt removal), iSPE®-HILIC-based drop-HILIC glycopeptides enrichment opens a wide range of opportunities for site-specific and in-depth glycoproteomics research.

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Characterization of Metal-Containing Nanoparticles in Tattoo Ink Using Asymmetrical Flow Field-Flow Fractionation Coupled with Multi-Angle Light Scattering and ICP-MS

Postnova

Nowadays, tattoos have become increasingly popular with both men and women, and particularly with younger people. However, an increase in popularity has meant that more and more adverse effects, such as infections and allergies, have been reported. This is due to the fact that tattoo inks can be complex in their formulation, containing several out of more than 100 pigments and additives, and also a significant quantity of impurities. The pigments are normally of particularly low purity and a majority of the ingredients are not even approved for application in cosmetic products in the European Union. Despite this, harmonized and well-established analytical methods for the comprehensive characterization of tattoo ink ingredients are still lacking (1).

A powerful technology for the investigation of the particulate content in tattoo inks is asymmetrical flow field-flow fractionation (AF4) coupled with multi-angle light scattering (MALS) and inductively-coupled plasma mass spectrometry (ICP-MS) (2). In this study, AF4-MALS-ICP-MS was applied to characterize four commercial tattoo inks: outlining black, purple deep violet, green grasshopper, and blue ice.

Sizing of Particulate Tattoo Ink Ingredients Using AF4-MALS

Using AF4-MALS, the particle size distribution in all four investigated tattoo inks could be assessed and all were found to have a significant nanoparticulate content. While outlining black, green grasshopper, and blue ice revealed monomodal particle size distributions ranging from 30–350 nm, 35–550 nm, and 25–450 nm radii of gyration (Rg), respectively, deep purple violet showed a bimodal distribution with particle sizes ranging from 20–550 nm Rg (Figure 1).

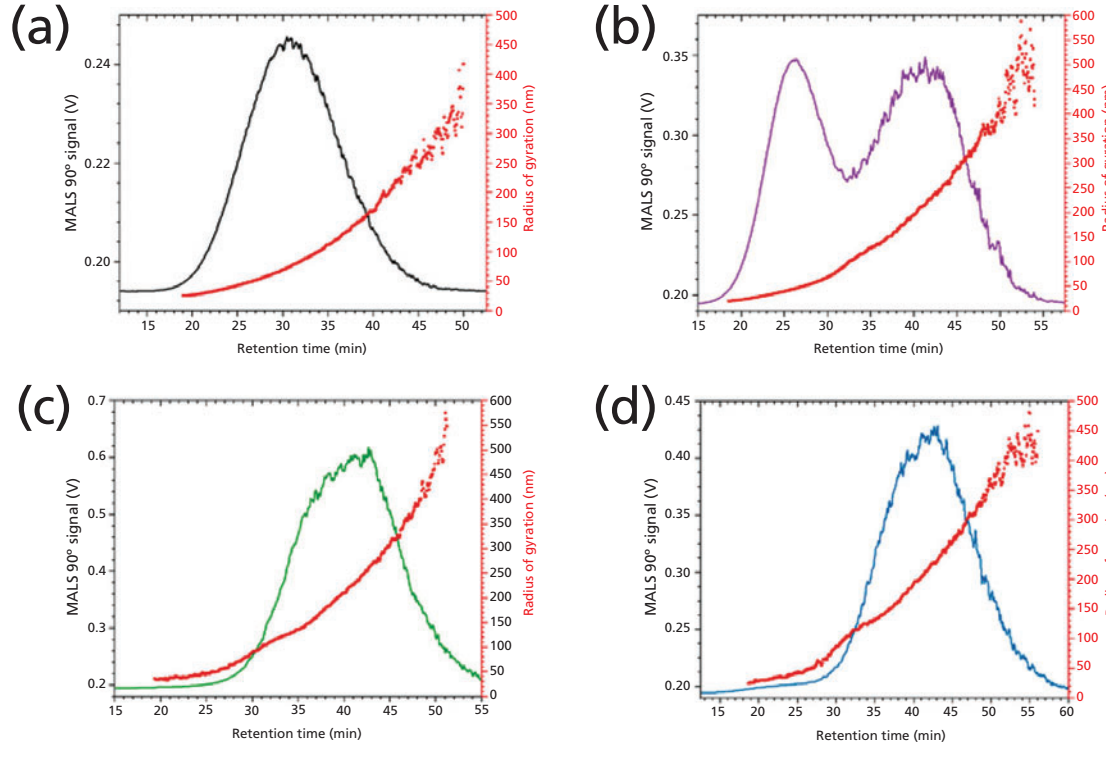

Figure 1: AF4-MALS fractograms with overlaid size (Rg) in red of (a) outlining black, (b) purple deep violet, (c) green grasshopper, and (d) blue ice.

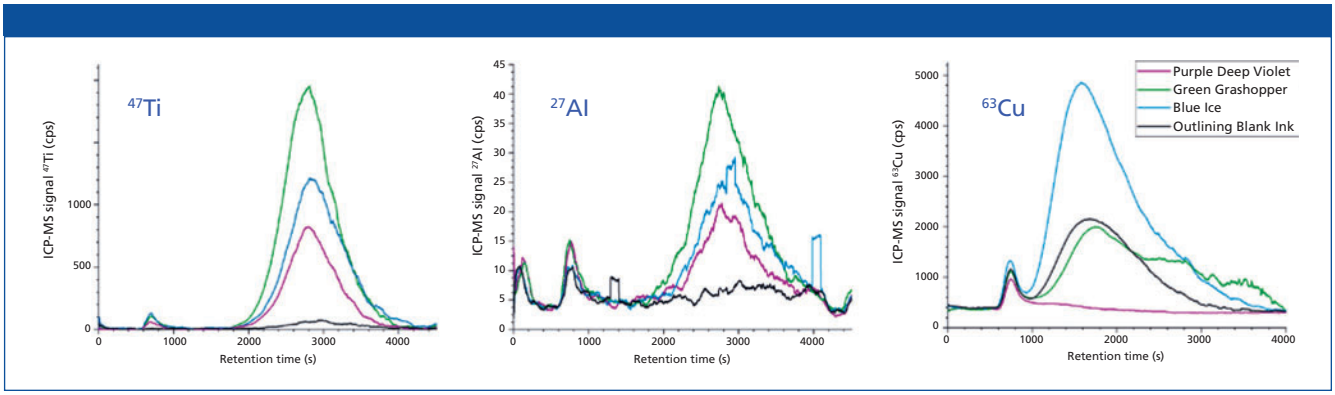

Figure 2: AF4-ICP-MS-fractograms of the four investigated tattoo inks showing the distribution of titanium (left), aluminium (middle), and copper (right).

Table 1: Summary of the obtained results from AF4-ICP-MS and batch ICP-MS

Sample	^{27}Al (mg/mL) (AF4)	^{27}Al (mg/mL) (Batch)	^{47}Ti (mg/mL) (AF4)	^{47}Ti (mg/mL) (Batch)	^{63}Cu (mg/mL) (AF4)	^{63}Cu (mg/mL) (Batch)
Outlining Ink Black	< LOD	< LOD	7.28	< LOD	0.18	0.18
Purple Deep Violet	0.3	2.7	65.2	114.0	< LOD	0.001
Green Grasshopper	0.8	5.9	160.2	195.3	2.2	2.0
Blue Ice	0.4	5.9	109.6	196.9	4.6	4.2

Chemical Identification of Particulate Tattoo Ink Ingredients Using AF4-ICP-MS

By coupling to ICP-MS, AF4 enabled size-resolved chemical identification of particulate tattoo ink ingredients. By this means, it could be demonstrated that outlining black contains particulate titanium (Ti) and copper (Cu), but no aluminium (Al), while green grasshopper and blue ice contain all three elements in particulate form. Purple deep violet, however, contains particulate Ti and Al, but no Cu (Figure 2). All investigated metals were most likely present as pigments in their oxidic state as TiO_2, Al_2O_3, and CuO.

Comparison of AF4-ICP-MS with Batch ICP-MS Results

By comparing the results obtained from AF4-ICP-MS with batch ICP-MS measurements, a quantitative differentiation between particulate and dissolved metals in the investigated tattoo inks could be achieved. While Al was predominantly dissolved, most of the Ti and Cu was present in particulate form (Table 1).

Conclusion

This study demonstrates the excellent suitability of AF4-MALS-ICP-MS for the comprehensive characterization of tattoo inks. Besides the particle size distributions obtained from the MALS data, ICP-MS also provides statements on the elemental distributions of various metals as a function of the respective particle sizes, offering valuable insight into the composition of the inks. This can not only facilitate the reliable identification and quantification of possible allergenic ingredients (and thus a better risk assessment) but may also help tattoo ink manufacturers to improve their formulations, eventually leading to safer products.

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Analysis of Fentanyl and Its Analogues in Human Urine by LC–MS/MS

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Abuse of synthetic opioid prescription painkillers such as fentanyl, along with a rapidly growing list of illicit analogues, is a significant public health problem. In this study, we developed a simple dilute-and-shoot method that provides a fast 3.5-min analysis of fentanyl and related compounds (norfentanyl, acetyl fentanyl, alfentanil, butyryl fentanyl, carfentanil, remifentanil, and sufentanil) in human urine by LC–MS/MS using a Raptor Biphenyl column.

In recent years, the illicit use of synthetic opioids has skyrocketed, and communities worldwide are now dealing with an ongoing epidemic. Of the thousands of synthetic opioid overdose deaths per year, most are related to fentanyl and its analogues. With their very high analgesic properties, synthetic opioid drugs such as fentanyl, alfentanil, remifentanil, and sufentanil are potent painkillers that have valid medical applications; however, they are also extremely addictive and are targets for abuse. In addition to abuse of these prescription drugs, the current opioid crisis is fueled by a growing number of illicit analogues, such as acetyl fentanyl and butyryl fentanyl, which have been designed specifically to evade prosecution by drug enforcement agencies.

As the number of opioid drugs and deaths increases, so does the need for a fast, accurate method for the simultaneous analysis of fentanyl and its analogues. Therefore, we developed this LC–MS/MS method for measuring fentanyl, six analogues, and one metabolite (norfentanyl) in human urine. A simple dilute-and-shoot sample preparation procedure was coupled with a fast (3.5 min) chromatographic analysis using a Raptor Biphenyl column. This method provides accurate, precise identification and quantitation of fentanyl and related compounds, making it suitable for a variety of testing applications, including clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

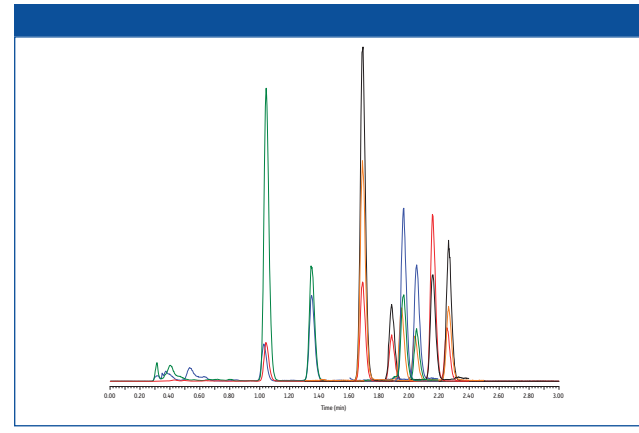

Figure 1: The Raptor Biphenyl column effectively separated all target compounds in urine with no observed matrix interferences. Peak elution order: norfentanyl-D_5, norfentanyl, remifentanil, acetyl fentanyl-$^{13}C_6$, acetyl fentanyl, alfentanil, fentanyl-D_5, fentanyl, carfentanil-D_5, carfentanil, butyryl fentanyl, sufentanil-D_5, sufentanil.

Experimental Conditions

Sample Preparation: The analytes were fortified into pooled human urine. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water–methanol solution (fivefold dilution) and 10 µL of internal

Table 1: Analyte transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier	Internal Standard
Norfentanyl	233.27	84.15	56.06	Norfentanyl-D_5
Acetyl fentanyl	323.37	188.25	105.15	Acetyl fentanyl-$^{13}C_6$
Fentanyl	337.37	188.26	105.08	Fentanyl-D_5
Butyryl fentanyl	351.43	188.20	105.15	Carfentanil-D_5
Remifentanil	377.37	113.15	317.30	Norfentanyl-D_5
Sufentanil	387.40	238.19	111.06	Sufentanil-D_5
Carfentanil	395.40	113.14	335.35	Carfentanil-D_5
Alfentanil	417.47	268.31	197.23	Acetyl fentanyl-$^{13}C_6$
Norfentanyl-D_5	238.30	84.15	—	—
Acetyl fentanyl-$^{13}C_6$	329.37	188.25	—	—
Fentanyl-D_5	342.47	188.27	—	—
Sufentanil-D_5	392.40	238.25	—	—
Carfentanil-D_5	400.40	340.41	—	—

standard (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat. #25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected into the LC–MS/MS.

Calibration Standards and Quality Control Samples: The calibration standards were prepared in pooled human urine at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL. Three levels of QC samples (0.75, 4.0, and 20 ng/mL) were prepared in urine for testing accuracy and precision with established calibration standard curves. Recovery analyses were performed on three different days. All standards and QC samples were subjected to the sample preparation procedure described.

LC–MS/MS analysis of fentanyl and its analogues was performed on an ACQUITY UPLC instrument coupled with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows, and analyte transitions are provided in Table 1.

Analytical column:	Raptor Biphenyl (5 µm, 50 mm × 2.1 mm; cat. #9309552)	
Guard column:	Raptor Biphenyl EXP guard column cartridge, (5 µm, 5 mm × 2.1 mm; cat. #930950252)	
Mobile phase A:	0.1% Formic acid in water	
Mobile phase B:	0.1% Formic acid in methanol	
Gradient	Time (min)	%B
	0.00	30
	2.50	70
	2.51	30
	3.50	30
Flow rate:	0.4 mL/min	
Injection volume:	5 µL	
Column temp.:	40 °C	
Ion mode:	Positive ESI	

Results

Chromatographic Performance: All eight analytes were well separated within a 2.5-min gradient elution (3.5-min total analysis time) on a Raptor Biphenyl column (Figure 1). No significant matrix interference was observed to negatively affect quantification of the fivefold diluted urine samples. The 5-µm particle Raptor Biphenyl column used here is a superficially porous particle (SPP) column. It was selected for this method in part because it provides similar performance to a smaller particle size fully porous particle (FPP) column, but it generates less system back pressure.

Linearity: Linear responses were obtained for all compounds and the calibration ranges encompassed typical concentration levels monitored for both research and abuse. Using 1/x weighted linear regression ($1/x^2$ for butyryl fentanyl), calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, alfentanil, acetyl fentanyl, butyryl fentanyl, and sufentanil; from 0.10 to 50 ng/mL for remifentanil; and from 0.25 to 50 ng/mL for norfentanyl and carfentanil. All analytes showed acceptable linearity with r^2 values of 0.996 or greater and deviations of <12% (<20% for the lowest concentrated standard).

Accuracy and Precision: Based on three independent experiments conducted on multiple days, method accuracy for the analysis of fentanyl and its analogues was demonstrated by the %recovery values, which were within 10% of the nominal concentration for all compounds at all QC levels. The %RSD range was 0.5–8.3% and 3.4–8.4% for intraday and interday comparisons, respectively, indicating acceptable method precision (Table 2).

Conclusions

A simple dilute-and-shoot method was developed for the quantitative analysis of fentanyl and its analogues in human urine. The analytical method was demonstrated to be fast, rugged, and sensitive with acceptable accuracy and precision for urine sample analysis. The Raptor Biphenyl column is well suited for the analysis of these synthetic opioid compounds and this method can be applied to clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

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Table 2: Accuracy and precision results for fentanyl and related compounds in urine QC samples

Analyte	QC Level 1 (0.750 ng/mL)			QC Level 2 (4.00 ng/mL)			QC Level 3 (20.0 ng/mL)		
	Average Conc. (ng/mL)	Average % Accuracy	%RSD	Average Conc. (ng/mL)	Average % Accuracy	%RSD	Average Conc. (ng/mL)	Average % Accuracy	%RSD
Acetyl fentanyl	0.761	102	1.54	3.99	99.7	2.08	19.9	99.3	0.856
Alfentanil	0.733	97.6	3.34	3.96	98.9	8.38	20.9	104	6.73
Butyryl fentanyl	0.741	98.9	6.29	3.77	94.3	6.01	20.8	104	4.95
Carfentanil	0.757	101	7.34	3.76	94.0	4.64	20.6	103	4.24
Fentanyl	0.761	102	1.98	3.96	99.1	2.31	19.9	99.6	1.04
Norfentanyl	0.768	103	6.50	4.04	101	1.84	20.1	101	2.55
Remifentanil	0.765	102	3.42	3.97	99.2	3.68	20.8	104	4.14
Sufentanil	0.752	100	1.67	3.93	98.3	1.28	20.1	100	0.943

LC–MS/MS Analysis of Sphingophospholipids Using a Metal-Free Column

YMC Europe GmbH

Sphingolipids are major components forming biological membranes, and they serve as intracellular signalling molecules. It is important to measure the amount of these molecules in biological samples because they have a massive influence on various metabolic diseases such as obesity, diabetes, and Alzheimer's disease. However, in LC analyses of sphingophospholipids, such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), a phosphate group in these molecules causes significant peak tailing, and therefore loss of sensitivity and reproducibility. Recently, an improved method using a YMC-Triart C18 metal-free column was reported by Dr. Gowda *et al.* (1).

Adsorption of Compounds with Phosphate Groups

In LC analysis, compounds containing phosphate groups tend to be adsorbed on the metallic surfaces in the flow path of the LC system. This results in peak tailing, carryover, and insufficient sensitivity.

It is important to use a column packed with a packing material containing less metal impurities to prevent these problems. Material used for the column hardware is also important. Stainless steel-free column hardware is effective to improve peak shapes, especially for highly sensitive analyses such as LC–MS.

YMC-Triart C18 metal-free columns are ideal for highly sensitive analysis of coordination compounds because their hardware consists of a PEEK-lined stainless steel tube and PEEK frits.

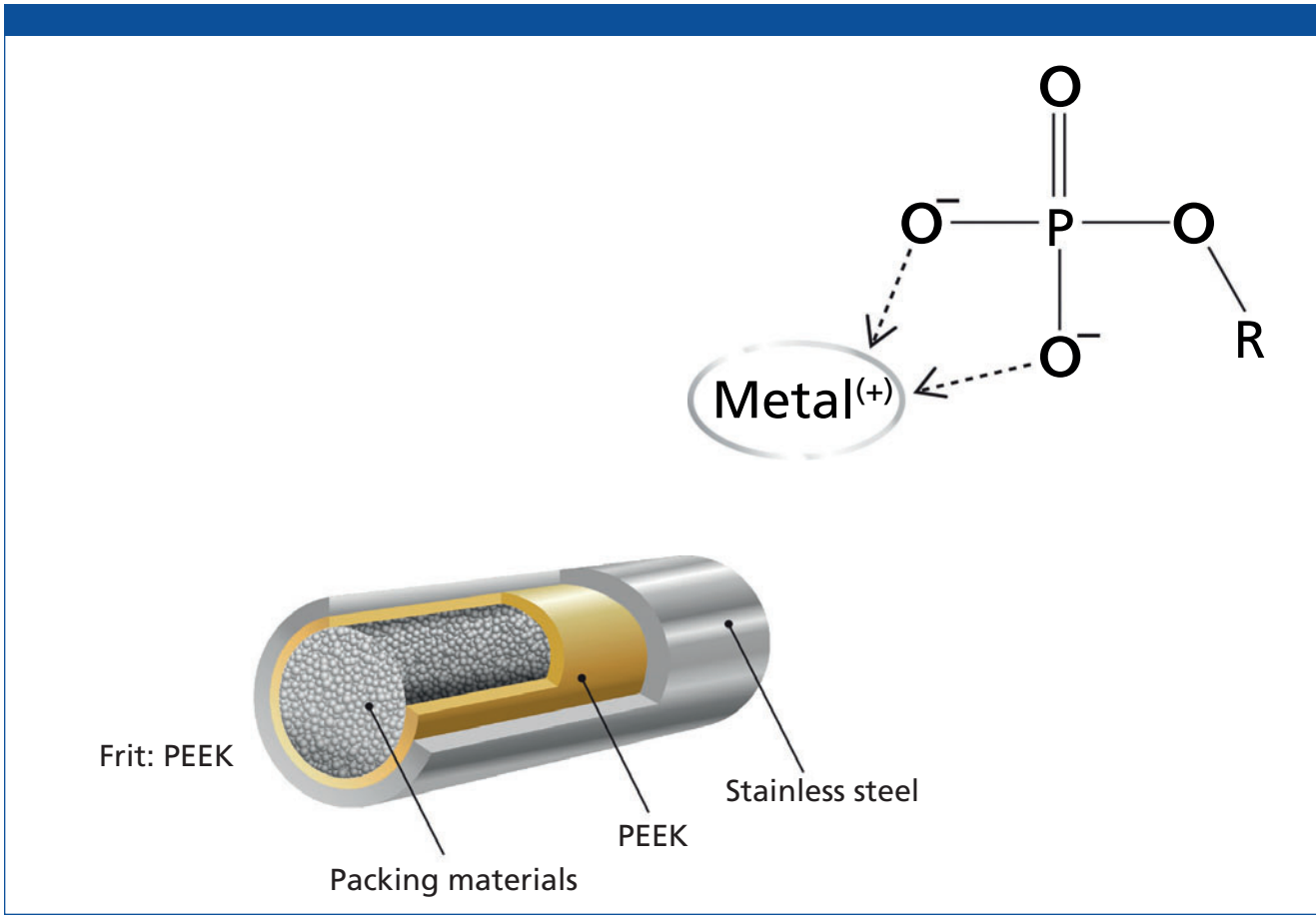
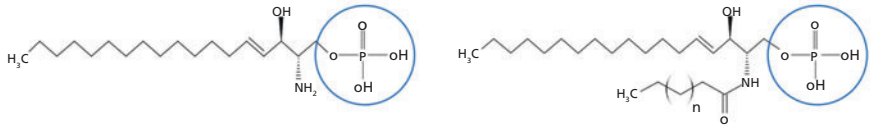


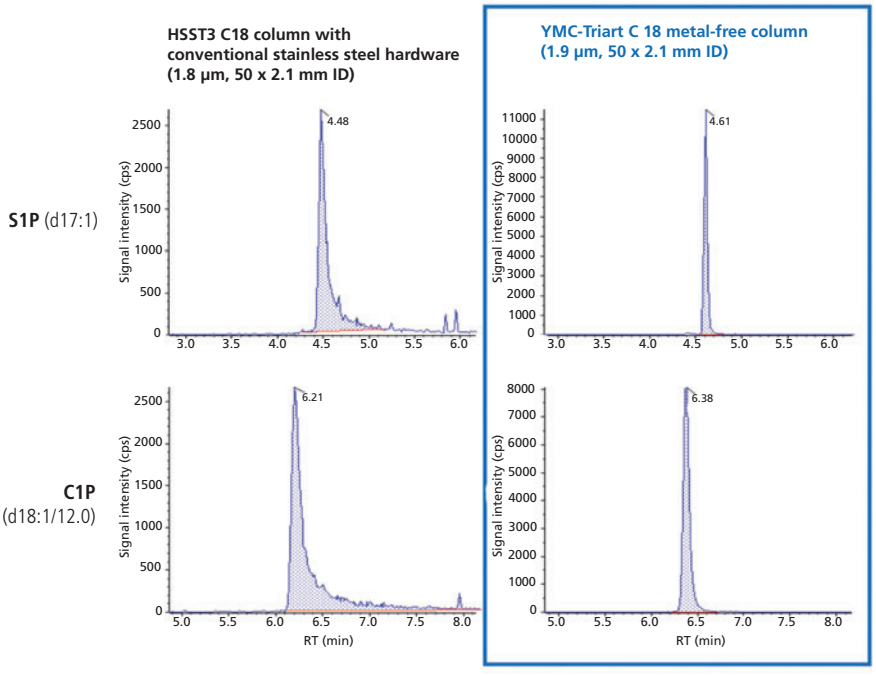
Figure 1: Example of metal coordinating compound and design of YMC-Triart metal-free columns.

Analysis of Sphingophospholipids



Sphingosine-1-phosphate (S1P)

Ceramide-1-phosphate (C1P)



Part No:	TA12SP9-05Q1PTP	Flow rate:	0.25 mL/min
Eluent:	A) methanol/acetonitrile/water (1/1/3) containing X B) 2-propanol containing X X: 5 mM ammonium acetate, 500 nM EDTA and 0.025% NH water	Temperature:	40 °C
		Detection:	ESI, positive
		Injection:	1 µL
		Instrument:	LC) Waters ACQUITY UPLC H-class system MS) AB Sciex QTRAP 6500
Gradient:	0%B (0–1 min), 0–50%B (1–5 min), 50–64%B (5–11 min), 64–95%B (11–13 min), 95%B (13–15 min), 0%B (15–20 min)		

Figure 2: Chromatograms of two sphingophospholipids analyzed using a standard stainless steel column and a YMC-Triart C18 metal-free column.

Significant peak tailing was observed for the conventional stainless steel column.

On the other hand, peak shape and intensity were improved using the YMC-Triart C18 metal-free column.

Reference

(1) S.G.B. Gowda, K. Ikeda, and M. Arita, *Analytical and Bioanalytical Chemistry* **410**(20), 4793–4803 (2018).

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Characterization of PLGA Using SEC-MALS-IV

Wyatt Technology Corp.

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer based on glycolic acid and lactic acid. The two monomer units are linked together by ester linkages and form linear polyester chains. The final product is biodegradable and biocompatible, and is approved by the Food and Drug Administration (FDA) for production of various therapeutic devices as well as for drug delivery applications. The properties of PLGA can be tuned by the ratio of the two monomers and by the overall molar mass distribution.

The characterization of PLGA by means of conventional size-exclusion chromatography (SEC) is problematic because of the lack of suitable calibration standards. In addition, the linear polyester structure can be modified by the addition of small amounts of polyfunctional monomer to obtain branched chains of differing degrees of branching. The degree of branching becomes an additional parameter that can be used to adjust PLGA properties—all of which renders conventional column calibration an inadequate analytical technique.

In this application note, two commercially available samples were analyzed in the ASTRA® software by SEC coupled to a multi-angle light scattering (MALS) detector (DAWN®), a refractive index detector (Optilab®), and a differential viscosity detector (ViscoStar®). The MALS and RI signals were combined to determine absolute molecular weight, independently of elution time or column calibration. The ViscoStar was used to measure intrinsic viscosity (IV) and uncover additional information about the molecular structure of the analyzed polymers. In addition to molar mass distributions, the SEC-MALS-IV system yields the relationship between intrinsic viscosity and molar mass (Mark–Houwink–Sakurada plot) that can provide a deep insight into the molecular structure of the polymers being analyzed.

In Figure 1 the molar mass distributions are given as differential distribution plots. As seen from the plots, the two samples span markedly different molar mass ranges. The Mark–Houwink–Sakurada plots of the two samples are shown in Figure 2 together with the plot of linear polystyrene, shown simply for the sake of comparison. The slope of the Mark–Houwink–Sakurada plot of the linear polystyrene is 0.71, a typical value for linear random coils in thermodynamically good solvents. The slope of the red sample roughly corresponds to a linear structure as well. However, there is a slight indication of deviation from linearity at the region of high molar masses that may indicate the presence of branched molecules. The Mark–Houwink–Sakurada plot of the blue sample is curved. Curvature of the Mark–Houwink–Sakurada plot generally reveals branching.

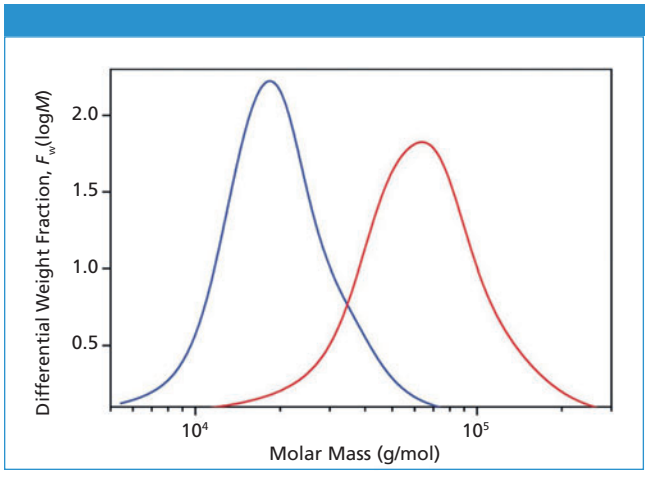

Figure 1: Differential molar mass distribution curves of two PLGA samples.

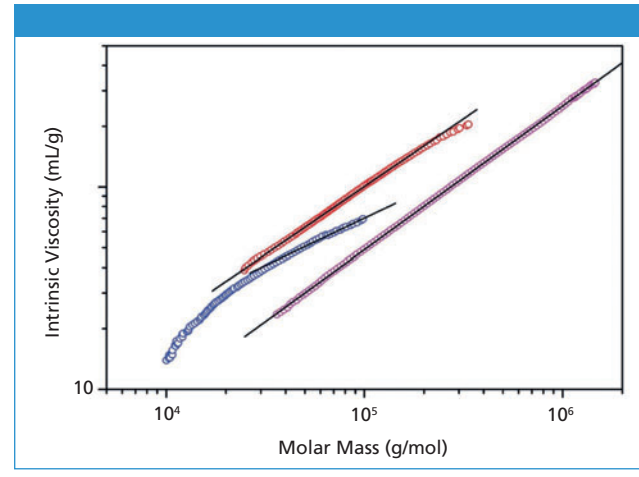

Figure 2: Mark–Houwink–Sakurada plots of two samples of PLGA (red and blue) and linear polystyrene (magenta). The lines are linear extrapolations of the data.

In addition, the slope of the higher molar mass portion of the Mark–Houwink–Sakurada plot of 0.48 suggests significant branching. SEC-MALS-IV is an excellent method for the characterization of PLGA polyesters because it has the ability to determine not only the absolute molar mass distribution but also to reveal subtle differences in PLGA's molecular structure.

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