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COVER STORY

Application of Liquid- and Supercritical Fluid Chromatography Coupled with High-Resolution Mass Spectrometry for the Analysis of Short-, Medium-, and Long-Chain Chlorinated Paraffins in Dietary Supplements
Jakub Tomasko, David Maxa, Klara Navratilova, Tomas Kourensky, Vojtech Hrbek, Jana Hajsova, and Jana Pulkrabova
This article presents SFC and UHPLC—both coupled with HRMS—methods for the analysis of short-, medium-, and long-chain CPs in fish oil-based dietary supplements.

COLUMNS

LIQUID CHROMATOGRAPHY

Essentials of LC Troubleshooting, Part 4: What is Going On with the Baseline?
Dwight R. Stoll
Knowing the likely cause of baseline-related problems will help you solve them.

QUESTIONS OF QUALITY

What Are Orphan Data?
R.D. McDowall
The term orphans is used frequently in the context of data integrity. What does it mean for chromatography data systems? How can we prevent or detect orphan data?

GAS CHROMATOGRAPHY

Application of Porous Layer Open Tubular Columns: Beyond Permanent Gases
Lina Mikaliunaite, David S. Bell, and Robert E. Synovec
The application of PLOT columns beyond their original primary use for permanent gaseous sample separations is discussed.

DEPARTMENTS

MULTIMEDIA HIGHLIGHTS

A snapshot of recent multimedia content from LCGC Europe

CEO’S NOTE

An update from the CEO

PRODUCTS

A compilation of the latest products for separation scientists from leading vendors

EVENTS

NTS Workshop on Analytical Techniques and Implementation event preview and other important events for chromatographers
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October Update

Welcome to the October issue of LCGC Europe! Our cover story this month focuses on food analysis and presents supercritical fluid chromatography (SFC) and ultrahigh-pressure liquid chromatography (UHPLC)—both coupled with high-resolution mass spectrometry (HRMS)—methods for the analysis of short-, medium-, and long-chain chlorinated paraffins (CPs) in fish oil-based dietary supplements. CPs are an emerging group of environmental pollutants associated with adverse effects on human health, and in this article the authors seek to use their findings to assess the CP levels in food and the environment, in particular for long-chain chlorinated paraffins (LCCPs).

In LC Troubleshooting, Dwight Stoll continues his essentials of LC troubleshooting series by investigating detector baselines that do not look right.

Questions of Quality focuses on orphan chromatography data system (CDS) data and the ways that they can be generated to falsify results. How can we prevent or detect orphan data?

In the latest instalment of Column Watch, the application of porous layer open tubular (PLOT) columns beyond their original primary use for permanent gaseous sample separations is discussed.

As we move back into a world of face-to-face meetings, we take a look at upcoming conferences, with a particular focus on the NTS Workshop on Analytical Techniques and Implementation, which will take place 29–30 November 2022 in Odense, Denmark.

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Mike Hennessy Jr,
President and CEO, MJH Life Sciences®

NOTE FROM THE CEO

MULTIMEDIA HIGHLIGHTS

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INTERVIEW

Rising Stars of Separation Science
The Column spoke to Natalia Manousi, from the Aristotle University of Thessaloniki in Thessaloniki, Greece, about her work in the development and application of novel sample preparation techniques. Read more: https://bit.ly/3rm39x2

VIRTUAL SYMPOSIUM

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In this virtual symposium, leading specialists in GC revealed how they apply theory and the latest advances in technology to solve very difficult challenges using gas chromatography in “the real world”. Catch up here: https://bit.ly/3pNmKWm

FOOD SUPPLEMENT

RAFA 2022 Companion: Hot Topics in Food Analysis
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E-BOOK

Pharma 4.0 and the Digital Regulated Laboratory Part 2
This sponsored e-book reveals why you should digitalize your regulated laboratory. Read more: https://bit.ly/3UQratG
Chlorinated paraffins (CPs) are an emerging and ubiquitous group of environmental pollutants associated with adverse effects on human health, including endocrine disruption and possible carcinogenicity. In this study, supercritical fluid chromatography (SFC) and ultrahigh-performance liquid chromatography (UHPLC)—both coupled with high-resolution mass spectrometry (HRMS)—methods for the analysis of short-, medium-, and long-chain CPs in fish oil-based dietary supplements were developed and validated at concentration levels of 0.6 and 3.0 µg/g lipid weight (lw). The recoveries were in the range of 80–96% and repeatabilities, expressed as relative standard deviations, were <19%. The limits of detection for the UHPLC–HRMS method (from 0.03 to 0.05 µg/g lw) were 5 to 10 times lower than those obtained by SFC–HRMS (from 0.13 to 0.50 µg/lw).

**KEY POINTS**

- Chlorinated paraffins (CPs) are an emerging group of environmental pollutants associated with adverse effects on human health.
- SFC and UHPLC-based methods for the analysis of SCCPs, MCCPs, and LCCPs in fish oil-based dietary supplements were developed and validated.

Chlorinated paraffins (CPs) are widespread environmental pollutants, and are used predominantly as additives (such as plasticizers and flame retardants) in plastic materials (1,2), high temperature and pressure-resistant lubricants, metal-working fluids, and flame retardants in adhesives and textiles (3).

CPs are divided into three main groups by their carbon chain lengths: (i) short- (SCCPs; C_{10}–C_{13}), (ii) medium- (MCCPs; C_{14}–C_{17}), and (iii) long-chain chlorinated paraffins (LCCPs: C_{18}–C_{30}) (4).

SCCPs are endocrine disruptors (5) and possible carcinogens to humans (6), therefore, they were added to the Stockholm Convention (Annex A) in 2017 as persistent organic pollutants (POPs) (7). The data on both MCCPs and LCCPs adverse effects on human health are very limited, and so they are not currently regulated (8). On the other hand, MCCPs are under evaluation for listing in the Stockholm Convention, and the risk assessment and risk profile are being performed (7).

The instrumental analysis of CPs in food and biological samples (usually at trace levels) (9,10) is a demanding task. In recent years, several gas chromatography (GC) or liquid chromatography (LC) -based methods have been published (9–12). Nevertheless,
CPs cannot be completely separated to individual compounds, which has led (together with the lack of well-characterized standard mixtures) to the need for unconventional quantification approaches (13–15). The LCCP standards have the least available options on the market.

The main goal of this study was to develop and validate supercritical fluid chromatography (SFC) and ultrahigh-performance liquid chromatography (UHPLC)—both coupled with high-resolution mass spectrometry (HRMS) with electrospray ionization operated in negative mode (ESI−)—methods for the determination of CPs (particularly LCCPs as those cannot be analyzed by GC–HRMS due to low vapour pressure). Fish oil dietary supplements were selected as the sample matrix, as an example of an adequately complex type of sample. The validated methods are necessary for future studies to assess the CPs levels in food and the environment, particularly for LCCPs, which are the least studied among the major CP groups.

Materials and Methods
Standard Solutions and Chemicals:
Thirty-one standard mixtures of SCCPs, MCCPs, and LCCPs of 10 µg/mL or 100 µg/mL cyclohexane with various chlorine contents (from 36.0% to 65.3%) were obtained from LGC Standards. The detailed list is documented in our previous paper (16). Isotopically labelled internal standard of β-hexabromocyclododecane ([13C12]-β-HBCD; 50 µg/mL toluene) was purchased from Wellington Laboratories.

Acetonitrile, methanol, n-hexane, ethyl acetate, and isopropanol were obtained from Honeywell. Dichloromethane, isoctane, sulfuric acid, ammonium formate, and silica gel 60 (particle size 0.063–0.200 mm) were purchased from Merck, while sodium sulfate (anhydrous) was bought from Lach-Ner.

Deionized water was made by a water purification system Milli-Q by Merck. Technical gases (carbon dioxide 4.8 and nitrogen 4.0) were supplied by SIAD.

Sample Preparation Procedure:
In this study, a sample of fish oil-based dietary supplement with no measurable CPs contamination found in a previous study (16) was chosen for method development and validation. Four other samples (with higher levels of CPs contamination previously determined by GC–HRMS) were analyzed to compare the new methods. A multilayer solid-phase extraction (SPE) was used in this study for the sample preparation. Briefly, the CPs were isolated from fish oil on an SPE column (78 × 12 mm with Luer tip) (LCTech GmbH) filled with (from bottom to top) silica gel (0.5 g; deactivated by 2% anhydrous baked for 4 h at 600 °C), and sulphuric acid-modified silica gel (1 g; 40% of H2SO4, w/w). Each column was washed with 3:1 (v/v) n-hexane–dichloromethane and then conditioned with n-hexane. A 100-mg measure of the sample diluted in n-hexane was loaded onto the SPE column and the analytes were eluted with 3:1 v/v n-hexane–dichloromethane. The sample was subsequently concentrated by a rotary vacuum evaporator followed by drying in a gentle stream of nitrogen. It was then dissolved in 500 µL of n-hexane, and the residual lipids were subsequently mineralized by a few drops of concentrated sulfuric acid. After one hour, an aliquot of 250 µL was evaporated and redissolved in 250 µL of the syringe standard (50 ng/mL; 13C12-[β-HBCD]) in acetonitrile.

Instrumental Analysis:
UHPLC–ESI(−)–HRMS Analysis:
The LC–ESI(−)–HRMS analysis of CPs was performed by Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a TripleTOF 6600 HRMS system (Sciex) with electrospray ionization operated in a negative mode. The method was developed from initial conditions published elsewhere (17). In this study, the target analytes (injection volume 5 µL acetonitrile) were separated on a 100 × 2.1 mm, 1.7-µm Acquity UPLC BEH C18 (Waters) column maintained at 40 °C. Methanol (A) and a mixture of 65:30:5 (v/v/v) isopropanol–methanol–water (B) were used as mobile phases. The initial conditions were 10% B for 1 min followed by a gradient with the following steps: to 30% B at 1.5 min, to 60% B at 2 min, to 80% at 3 min, to 90% B at 3.5 min, to 100% B at 4 min (3.5 min isocratic hold; the total run time was 11 min including the return to the initial state and equilibration). The mobile phase flow rate was 0.2 mL/min.

Regarding MS source conditions, desolvation temperature was set to 450 °C and the capillary voltage was -4.5 kV. The acquisition speed was 2 spectra/s and the mass range 100 to 1500 m/z. The [M+Cl]- ions were monitored.

SFC–ESI(−)–HRMS Analysis:
A previously published SFC-based method (16) was used in this study with several modifications (gradient of mobile phases, composition of mobile phase B, and make-up solvent were changed). The supercritical fluid chromatograph Acquity UPC2 coupled with a Synapt G2 Si high-resolution mass spectrometer (both Waters) with electrospray ionization operated in a negative mode was employed. The target analytes (injection volume 3 µL acetonitrile) were separated on a 100 × 3.0 mm, 1.8-µm Viridis HSS C18 SB (Waters) column maintained at 70 °C. Supercritical CO2 was used as mobile phase A and 5 mM ammonium formate in 99:1 (v/v) methanol–water was employed as mobile phase B. The initial conditions were 100% A for 0.5 min followed by a gradient to 35% B at 5 min (1 min isocratic hold; the total runtime was 8 min including the return to the initial state and equilibration). The mobile phase flow rate was 1.8 mL/min. After the separation
under supercritical conditions, the CO$_2$ evaporated and had to be substituted by another mobile phase—a make-up solvent—which leads the analytes into the ion source. In this study, the make-up solvent was a mixture of 45:45:10 (v/v/v) methanol–ethyl acetate–dichloromethane (flow rate 0.5 mL/min).

In the MS system, the desolvation gas temperature was 250 °C and the capillary voltage was set to -3 kV. The acquisition speed was 2 spectra/s and the mass range 250 to 1500 m/z. The [M+Cl]$^-$ ions were monitored.

**Method Validation:** The analytical method for the determination of SCCPs, MCCPs, and LCCPs was validated by the analysis of artificially contaminated samples (at two different concentration levels, each level prepared in six parallels). The selected spike levels were 0.6 and 3 µg/g lw for each of the CP groups. The standard mixtures used were C$_{10}$-C$_{13}$ 63.0% Cl, C$_{14}$–C$_{17}$ 57.0% Cl, and C$_{18}$–C$_{20}$ 49.0% Cl. The limits of detection (LODs) were determined as the lowest standard level at which any CP congener group was integrable (with S/N ≥ 10).

**Evaluation of Matrix Effects:** The matrix effects were evaluated by comparing matrix standards (prepared in duplicate) with solvent standards at a level of 0.6 µg/g lw. For a comparison, matrix effects of the extract with residual lipids (without sulfuric acid treatment) were evaluated as well.

**Results and Discussion**

**Comparison of Method Performance Characteristics of SFC and UHPLC-Based Methods for the Analysis of CPs in Fish Oil-Based Dietary Supplements:** The method performance characteristics of SFC–HRMS and UHPLC–HRMS methods for analysis...
of SCCPs, MCCPs, and LCCPs are shown in Table 1. There are no officially recommended reference values for recovery and repeatability in the analysis of CPs, therefore the acceptable values (recovery from 60 to 120% and repeatability <20%) were adopted from Commission Regulation (EU) 2017/644 concerning sampling and analysis of polychlorinated dibenzodioxins/furans and polychlorinated biphenyls (other chlorinated POPs) in foodstuffs. The recoveries varied from 84 to 95% (SFC-based instrumental method) and from 80 to 96% (UHPLC-based instrumental method), respectively.

FIGURE 1: Matrix effects evaluation (at concentration level of 0.6 µg/g lw) with significant signal suppression in samples without sulfuric acid treatment.

FIGURE 2: Comparison of SFC and UHPLC methods for the determination of CPs in dietary supplements. While the levels of SCCPs and MCCPs were comparable, the differences of LCCP concentrations might have been caused by matrix effects.

FIGURE 3: Comparison of extracted ion chromatograms of C_{13}Cl_{6}H_{22} ([M+Cl]⁺ or [M-Cl]⁻ ion formations) obtained using three instrumental systems: (a) SFC–HRMS (424.952 ± 0.025 m/z), (b) UHPLC–HRMS (424.952 ± 0.005 m/z), and (c) GC–HRMS (354.006 ± 0.007 m/z); the GC–HRMS conditions are described in our previous paper (16).

The repeatabilities (expressed as relative standard deviations, RSDs) were <19%. Both methods were therefore successfully validated on levels of 0.6 and 3 µg/g lw, with no significant differences regarding recovery and repeatability. The UHPLC instrumentation proved to yield lower LODs (5 to 10 times lower, see Table 1) than the SFC method (probably due to the presence of splitter on the interface of SFC and MS). On the other hand, the SFC proved to be a robust method regarding injection solvent, that is, the use of acetonitrile in this study and isooctane used elsewhere (16), where the samples prepared for GC–HRMS analysis (in isocane) were also measured by SFC–HRMS (as a complementary method for screening of LCCPs).

Matrix Effects Evaluation: The matrix effects are illustrated in Figure 1. The positive effect of a sulfuric acid treatment is documented there. In samples without the sulfuric acid treatment, there can be seen a strong signal suppression, which was more significant in SFC (with a response decrease of up to 69% for the MCCPs). The signal suppression was presumably caused by methyl or ethyl esters of fatty acids, as these esters are sometimes a dominant form of fatty acids in concentrated fish oil-based dietary supplements (19). The free fatty acids released from ester bonds by sulfuric acid were then separated from CPs on the chromatographic column, decreasing effectively the matrix effects, which was verified by successful validation.

SFC and UHPLC Method Comparison: The Determination of CPs in Fish Oil-Based Dietary Supplements: For the developed and validated methods final comparison, the methods were used for determination of CPs in four fish oil-based dietary supplements (Figure 2). Chromatograms illustrating the separation differences of several chromatographic systems are shown in Figure 3. The concentrations...
of SCCPs and MCCPs obtained employing SFC and UHPLC systems were comparable (considering 30% uncertainty). The concentrations were in the range of 1.01–37.22 µg/g lw (SCCPs) and 0.83–37.76 µg/g lw (MCCPs), respectively. These results were lower than concentrations obtained by GC–HRMS in our previously published study (16). The differences may have been caused by still remaining matrix effects, even after sulfuric acid treatment of the samples. Similarly, concentrations of LCCPs obtained by UHPLC–HRMS (0.10–0.36 µg/g lw) were in the samples 3 and 4 lower than those obtained by SFC–HRMS (0.20–1.59 µg/g lw). The differences might have been caused by slightly higher matrix effects (Figure 1).

Conclusions
This article describes a validation of two analytical approaches (employing SFC- and UHPLC-based methods) with a single sample preparation procedure for the analysis of SCCPs, MCCPs, and LCCPs in fish oil-based dietary supplements. The recoveries ranged from 80 to 96% (with RSDs <19%). The UHPLC instrumentation showed lower LODs than SFC, but SFC demonstrated a good robustness because acetonitrile or isooctane extracts could be analyzed under the same conditions.

The methods were then used to determine CPs contamination in four samples of dietary supplements. The SCCP and MCCP concentrations obtained by both systems were comparable, while the LCCP concentrations differed (with UHPLC yielding lower results). The SCCP and MCCP levels were also compared with results obtained employing GC–HRMS (which were previously published). The results obtained by SFC and UHPLC were slightly lower than those obtained by the GC-based method. This might have been caused by matrix effects in some samples, and in the following studies further research is needed (that is, selection of a 13C-labelled CP internal standard and/or use of a more complex clean-up procedure). Finally, the methods are planned to be verified in interlaboratory studies.

Acknowledgements
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References
5) H. Li, S. Gao, M. Yang, et al., Chemosphere 244, 125393 (2020).
Some liquid chromatography (LC) troubleshooting topics never get old because there are some problems that persist in the practice of LC, even as instrument technology improves over time. There are many ways for things to go wrong in an LC system that ultimately manifest as detector baselines that do not look right. Developing a short list of the likely causes of these results can help streamline our troubleshooting experience when baseline-related problems occur.

Writing this “LC Troubleshooting” column and thinking about topics each month is interesting in the sense that there are some topics that just never get old. Whereas in the chromatography research world certain topics or ideas become obsolete as they are displaced by newer and better ideas, there are certain topics in the troubleshooting world that have remained relevant since the very first troubleshooting article appeared in this magazine in 1983 (1). Over the last few years, I focused several “LC Troubleshooting” instalments on contemporary trends (for example, the relatively recent advances in our understanding of the effects of pressure on retention [2]) in liquid chromatography (LC) that are affecting the way we approach our interpretation of LC results, and how we approach troubleshooting with modern LC instruments. In this instalment, I am continuing a series that I started back in January 2022 (3) that focuses on some of the “bread and butter” topics of LC troubleshooting—those elements that are essential for any troubleshooter, no matter the vintage of the system we are working with. The topics at the heart of this series are highly related to LCGC’s well-known “LC Troubleshooting” wall chart (4) that hangs in many laboratories. The fourth instalment in this series focuses on problems related to detector baselines that do not look right. As with all of the “essential” troubleshooting topics, problems with baselines have been discussed in “LC Troubleshooting” articles in the past (5). However, the solutions to these problems have changed over time. The causes of these anomalous baselines can have chemical or physical origins, or both. Discussing some of the most commonly observed causes empowers users to spot these problems when they occur, and it enables them to get a start on the troubleshooting effort by narrowing the list of possibilities to investigate and the range of possible solutions. I hope LC users young and old will find the tips and reminders related to this important topic in this column useful.

What Is to Be Expected?
A critical step in any troubleshooting exercise—but one that I think is not appreciated enough—is recognizing that there is a problem to be solved. Recognizing that there is a problem usually amounts to recognizing that what is happening with the instrument is different from what is expected to happen. Our expectations are formed from theories, empirical knowledge, and experience (6). “Normal” baselines can look very different, depending on the chromatographic...
conditions and detector type in use. An ideal detector baseline is flat (that is, no drift), with a low degree of noise in the signal and free of artifacts such as “ghost peaks” or other features in the signal that are not related to the analytes we are trying to separate. Figure 1 illustrates the difference between detector “drift” and “noise” in the signal. The closer our real detector baselines are to this ideal, the more powerful our methods are for quantifying analytes accurately, with confidence, and at low concentrations.

**Chemical Causes of Baseline Anomalies**

In my laboratory, we observed several different types of baseline anomalies that we ultimately attributed to problems with the reagents used to prepare mobile phases.

**Cause #1: Mobile Phase Impurities That Accumulate On-Column**

 Occasionally, mobile phase solvents (for example, water and acetonitrile) or additives (for example, formic acid and triethylamine) contain impurities that are highly retained under the conditions of a chromatographic experiment. When using isocratic methods, accumulation may not become apparent until the column is “washed” with a strong solvent (for example, a high level of acetonitrile) at the end of a series of sample injections or prior to storing the column. During the washing step, elution of the impurities that had accumulated on the column will be observed as a large, broad change in the detector signal. When using gradient elution methods, impurities are retained by the column early in the gradient, but then they are washed out in the middle of the gradient or during a washing step at the end of the gradient. Figure 2 shows an example of this occurrence from our recent work aimed at developing a reversed-phase method that involves dimethylcyclohexylamine in the A solvent used in the gradient. With the mobile phase from supplier A, a large impurity peak is observed at the end of the gradient, whereas this peak is much smaller when using the mobile phase prepared with the additive obtained from supplier B.

**Cause #2: Mobile Phase Impurities That Contribute to High and Changing Baselines**

In specific cases where the impurities are highly soluble in the mobile phase, they are not retained by the column and contribute towards a higher-than-normal detector signal throughout the chromatogram. When using gradient elution, if the impurities are only present in one of the solvents used in the gradient (for example, only in the mainly aqueous solvent used in a gradient for a reversed-phase separation), then the detector signal may change slowly and in relation to the fraction of that solvent contributing to the mobile phase at any point in time. Figure 3 shows an example of this occurring from our recent work involving mass spectrometric detection. In this case, we ultimately found that the higher-than-expected signal was because of the presence of short chain alkylamines present in the isopropanol we were using as a component of our mobile phase. This particular case was quite surprising given that the isopropanol we had sourced was sold as a “liquid chromatography–mass spectrometry (LC–MS)-grade” solvent. Changing to isopropanol

**FIGURE 1:** Illustration of detector “drift” and “noise” in the signal.

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from a different manufacturer immediately solved the problem, which is shown in the chromatograms in Figure 3.

**Cause #3: Mobile Phase Impurities That Produce “Ghost Peaks”**

In cases similar to those discussed in the preceding two sections, the mobile phase impurities do not manifest as signal features that look like typical chromatographic peaks. Therefore, they don’t directly conflict with analyte peaks per se. However, if the physicochemical properties of the impurities are similar to the analytes of interest in a separation, then the impurities can manifest as features in the baseline that look like typical peaks. These are sometimes referred to as “ghost peaks” because they persist in the baseline even when no sample is injected. This topic has been discussed many times in the history of the “LC Troubleshooting” column (11 articles use the keyword “ghost peak”), and, on several occasions, an entire instalment has been committed to the topic. Readers interested in this particular type of baseline are referred to these articles, the most recent of which was published in October 2016 (7).

**Physical and Physicochemical Causes of Baseline Anomalies**

In addition to the chemical causes of baseline problems discussed in the previous section, there are many other causes that are either purely physical in nature or can be characterized as having both chemical and physical components (that is, physicochemical).

**Cause #1: Detector Response to a Major Mobile Phase Component**

Sometimes, small changes in the preparation of solvents used for a LC method can have a dramatic effect on the appearance of the baseline. A classic example of this effect is the change from a phosphate-buffered mobile phase to one involving formic acid as an additive. Whereas phosphate species are highly transparent to UV light down to approximately 200 nm, carboxylate species (such as formate, acetate) absorb much more than phosphates below approximately 230 nm. This difference is readily observed when gradient elution is used, and the additive is only present in one of the solvents used in the mobile phase gradient (for example, the majority aqueous solvent). Figure 4 shows the detector baselines observed in such a case, where we see that the absorbance at 210 nm decreases dramatically, and roughly in proportion to the composition of the A/B solvent mixture at any point during the gradient. On the other hand, the absorbance at 254 nm is relatively unchanged during the gradient because the formate additive absorbs very little at this wavelength. The most frequently used solutions to the dramatically changing baseline are to either use a higher
wavelength where the additive does not absorb so much light (as in [B]), or to add a similar concentration of the additive to the B solvent used in the gradient so that a change in the A/B solvent mixture does not significantly change the concentration of the additive arriving at the detector.

**Cause #2: Inconsistent Mobile Phase Composition Because of Pump Problems**

Recently, I’ve written about ways that things can go wrong with modern high performance LC (HPLC) pumps (3). Sticky check valves and trapped air bubbles are common causes of inconsistent flow from a high pressure pump head. When one of the two pumps in a binary pumping system fails, it can lead to extreme changes in the mobile phase composition that go through the HPLC column and eventually to the detector. If the detector in use is sensitive to these changes in composition, then an anomalous detector baseline can be a symptom of a problem that is really caused by the pump. An extreme example is a situation where the A solvent contains ammonium acetate, the B solvent is acetonitrile, and UV detection is used at 210 nm. In this case, the A solvent absorbs much more light than the B solvent, but if the composition of the A/B mixture is consistent over time, a smooth, flat detector baseline will be observed. However, if the flow from the A channel becomes inconsistent because of a check valve problem or an air bubble, then the composition of the A/B mixture will not be consistent, leading to a saw-tooth pattern in the baseline. An example of such a scenario is shown in Figure 5. This pattern in the signal is distinctly different from patterns that emerge because of other problems discussed in this instalment and can be readily recognized. Readers interested in troubleshooting pump problems that could be the ultimate cause of this type of observation are referred to previous “LC Troubleshooting” articles on this topic (for example, [3]).
**Cause #3: Temperature Effects**

Different detectors are affected by changes in temperature in different ways. The signal baseline for refractive index (RI) detection is notoriously sensitive to changes in temperature. Therefore, stabilizing the temperature of the detector itself and the laboratory environment will help minimize drift in the baseline. UV absorbance detectors are also prone to changes in temperature, which causes drift in the baseline, but to a lesser extent when compared to RI detectors. Modern UV detector designs are much less susceptible to otherwise identical conditions, but one at a relatively high acquisition rate of 160 points/s (B), and one at a much lower rate of 2.5 points/s (A). If a method is inadvertently or unintentionally set to a high acquisition rate, then simply reverting to a lower rate will yield a much smoother baseline. However, do be careful not to use an acquisition rate that is so low that it causes artificial broadening of the chromatographic peaks of interest (B).

Second, when using spectroscopic detectors, conditions that lead to a small amount of light reaching the detector can also lead to fuzzy baselines. In the case of UV absorbance detection, this phenomenon can occur when a) the lamp is not producing the correct amount of light (for example, if the lamp has exceeded its lifetime or has not been turned on); b) something in the mobile phase is absorbing a large fraction of the light at the wavelength being monitored (for example, detecting below 220 nm when using a high concentration of formic acid); or c) partial or total occlusion of the light path between the lamp and the detector because of improper installation of a flow cell or some other blockage. Most modern HPLC systems are supported with software that includes many diagnostic tests, one of which will test for “lamp intensity” for a UV detector. If weak lamp output or partial occlusion of the light path is suspected as a cause of a fuzzy baseline, running this diagnostic test is a good place to start the troubleshooting process.

**Cause #4: Baselines That Are Too “Fuzzy”**

Figure 1 illustrates the difference between “drift” in detector baseline (the slow changing, or low frequency features), and “noise” in the signal (the fast changing, or high frequency features). Many of the problems discussed so far in this instalment cause slow changes in the detector signal, but there are also many potential causes of noise that is larger than expected, which we sometimes refer to as “fuzzy” baselines. First, a data acquisition rate that is higher than normal will lead to baselines that look fuzzier than normal because the actual short-term variations in the signal are revealed when a high acquisition rate is used, which would otherwise be hidden when using a lower acquisition rate. Figure 6 shows a comparison of baselines (UV detection) obtained with otherwise identical conditions, but one at a relatively high acquisition rate of 160 points/s (B), and one at a much lower rate of 2.5 points/s (A). If a method is inadvertently or unintentionally set to a high acquisition rate, then simply reverting to a lower rate will yield a much smoother baseline. However, do be careful not to use an acquisition rate that is so low (drift vs. noise). Understanding these details provides a good place to start troubleshooting, but does not capture all possibilities. Readers interested in learning about a deeper list of causes and solutions for problems related to detector baselines are referred to the LCGC Troubleshooting Guide wall chart.

**Summary**

In this fourth instalment on essential topics in LC troubleshooting, I discussed some of the causes of anomalous baselines. Most problems fall into one of two categories—problems characterized by baseline drift (low frequency signal changes) or excessive noise (high frequency signal changes). These problems can have chemical or physical origins. Similar to other essential troubleshooting topics, effectively troubleshooting baseline problems starts with a clear expectation of what a typical baseline looks like for the application at hand, and then narrowing down the list of possible causes by the type of anomaly.

**Acknowledgements**

I’d like to thank Tina Dahlseid, Maria Sylvester, Henry Noma, and Zach Kruger for supplying some of the data shown in this article.

**References**

What Are Orphan Data?

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

The term orphan data is used frequently in the context of data integrity. What does it mean for chromatography data systems? How can we prevent or detect orphan data?

During a recent training course on data integrity, the question “What are orphan data?” was asked by one of the attendees. This gave me the excuse to write this column, but the real reason is that I forgot the column deadline. What we will cover here is understanding what orphan data means and how it applies to a chromatography data system (CDS). We will then look at ways to prevent the several types of orphan data being generated, starting with background information about the meaning and scope of raw data and complete data as they apply to chromatographic analysis.

The aim of this column is to help laboratory reviewers, quality assurance (QA), and auditors to prevent or detect orphan data to ensure the integrity of results.

Understanding Raw Data and Complete Data

Raw data and complete data are Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) terms used for records generated during a regulated activity. In an earlier “Questions of Quality” column, I discussed the definition and meaning of raw data in the context of a CDS (1). Raw data is a term that derives from FDA Good Laboratory Practice in 21 CFR 58.3(k) (2) published in 1978 and essentially means all data from original observations and activities, for example, acquisition, processing, interpretation, and calculation, to the reporting of the results. Most importantly, anyone should be able reconstruct the report of the work from the raw data (2,3). Please note, both the data integrity guidances from the MHRA (4) and PIC/S PI-041 (5) definitions of raw data are wrong as they only refer to original observations. The US GMP term complete data and the ability to equate this to raw data was discussed in my “Focus on Quality” column in Spectroscopy in 2016 (6).

Summarizing both raw data and complete data for a chromatographic analysis includes the following data: sample information, sample preparation records, acquisition method, instrument control file, CDS data files, processing method, calculations (unfortunately, I suspect, including any spreadsheets), system suitability testing (SST) parameters, audit trail entries, generation of the reportable result, plus instrument logbooks, laboratory notebooks, and worksheets. The principles outlined for CDS are applicable for other instrument systems.

As an evil auditor I want to be able to take any result and trace it back to the chromatography data, integration, and sample preparation records easily and transparently. I want to be able to trace all data from the start of the analysis to the result, including qualification data and tests performed after maintenance. In this latter review, I want to see all the data, including any problems encountered during the chromatographic work such as leaky liquid chromatography (LC) pump seals, blocked autosampler needles, clapped-out column separations, and instrument failures—plus their resolution. All these problems will generate data that may or may not be usable for generating the reportable result. However, all data must be kept as they are part of raw data (2,3,7) or 21 CFR 211.194(a) complete data (8).

From Regulations to Procedures

GXP laboratories must interpret the applicable regulations and generate written instructions to perform tasks that generate records and reports (7). This includes any work performed by service engineers and the associated agreements under EU GMP Chapter 7 (9). These instructions will include the requirements for recording or collecting data, processing them, and calculating the reportable results. They must be followed or a deviation must be raised. However, many of these standard operating procedures (SOPs) can be major works of artistic fiction as laboratory procedures must describe all working practices. Often procedures do not cover all activities, leading to poor records management practices or data falsification, or they can disguise activities that generate orphan data. If data integrity is compromised, the credibility of any laboratory is also compromised.
Definition of Terms
Now we need to focus on orphan data and we start with definitions of the two words and make the connection:
Orphan: A child whose parents have died
Data: Facts, figures, and statistics collected together for reference or analysis. All original records and true copies of original records, including source data and metadata and all subsequent transformations and reports of these data, that are generated or recorded at the time of the GXP activity and allow full and complete reconstruction and evaluation of the GXP activity (4).

At first sight, these two words make unlikely bedfellows. However, put on your chromatography glasses and look in more detail. The definition of data requires everything to be collected, while orphan indicates a separation or a break from the parents, in this case the main collection of data. Therefore, orphan data are separated from the data and results that are reported and purportedly represent the complete GXP record of the activity.

Orphan CDS Data
For a CDS, orphan data refer to:
• Data generated during a qualification or requalification of an instrument that is stored on a service engineer’s PC, or, if work has been repeated, the selective reporting of passing results
• During a chromatographic process, there are several areas where orphan data could be generated, for example, unofficial injections, multiple integrations, calculations, and short or aborted runs that are not reported or included as part of an official GXP record
• A run that has been excluded by dubious means, for example, unscientific invalidation of out-of-specification (OOS) results, or invalidation by manipulating SST injections to fall outside acceptance limits.

We will look at some of these types of orphan data and look at ways to identify or prevent them from occurring. Excluded from this discussion are copying data from one location to another to deliberately falsify a record and data deletions, for the reason outlined in the next section.

The reasons for orphan data may vary, from an analyst exercising “scientific judgement” (without reference to the regulations or laboratory SOPs) to deliberate falsification of data. Regardless of the reason, the dominoes fall thus: data integrity is compromised, followed by the quality of the decision made, and ultimately patients suffer.

Configuration of the CDS Application
One of the ways to restrict opportunities for generation of orphan data lies in the architecture and configuration of the CDS used by the laboratory. This will be an integral part of the validation of the system so that a reviewer’s or QA auditor’s job are made easier.

• The right system architecture: a CDS should be a networked system with an intelligently designed database. See the McDowall and Burgess four-part series on the ideal CDS for Regulated Laboratories for more information on this topic (10–13).
• Audit trails that cannot be turned off and are designed to monitor all changes to GMP records, including recording and highlighting all integration attempts automatically.
• A CDS application that saves all data automatically without having to prompt a user to do so.
• User roles and access privileges that avoid conflicts of interest. Here it is important that no user has any privilege to delete data.
• Single predefined data storage areas: To avoid hiding orphan data and unofficial testing, it is critical that when performing an analysis that there is one, and only one, predefined data repository.
• Use the system electronically and not as a hybrid: As many of you will ignore this, you will be left with more work to do.

Orphan Data: Divorced, Hidden, or in Plain Sight?
We will look at the main means that service engineers and analysts use to generate orphan data and the ways that you can prevent or detect them in the sections
below and in Figure 1. There are three types of orphan data that reviewers, QA, and auditors need to be aware of:

- **Divorced data:**
  Your qualification data are stored on a service engineer’s laptop. Repeating qualification work and selectively reporting the passing result

- **Hidden in the CDS:**
  Test or prep injections
  Titrating test results
  Short or aborted runs
  Integrating into compliance

- **Hidden in plain sight:**
  Invalidated SST injections
  Invalidated OOS results.

We will start with divorced examples of CDS orphan data.

**The Angel of Death Calls**

Our first port of call focuses on a visit from the Angel of Death, sorry, the service engineer, who typically will perform a preventative maintenance visit and requalify your chromatographs. It depends on the level of paranoia in a company as to whether a service engineer has access to the CDS. If a laboratory is sensible, there is a user role called service or engineer where the access privileges for performing service and qualification are configured. The engineer(s) must be named CDS user(s) and the account enabled and disabled before and after the qualification visit. All qualification data are within the CDS or linked to any external tests such as temperature or flow using calibrated instruments so that an authorized laboratory user can access and review the data and work is attributed to a named individual.

If the engineer is denied CDS access, Pandora’s box is opened and the evils of the world unleashed. Actually, it is the engineer’s PC that is opened, but the end result is the same. After disconnecting the chromatograph from the CDS, the engineer connects their PC with the CDS software (Note: this is not testing the system under actual conditions of use). Depending on whether the original equipment manufacturer or a third party is used for service and requalification, you may or may not have qualification data in the same CDS as the one you use. The engineer will also be an administrator of this CDS, with an obvious conflict of interest and the right to hide and delete data or even change the PC clock. If data are stored on the engineer’s PC, how many attempts have there been to get a chromatograph to pass a test?
After qualification is complete, you will get a printed report of work performed. However, there are a few potential problems:

- Have you approved the work prior to execution and do you understand it?
- Do you get sight of the electronic records generated by the qualification? If yes, have you reviewed them?
- Do you have copies of the electronic data? If yes, are they legible and can you understand them?
- If you don’t have copies of the data, the engineer certainly does! But the individual has just ridden off into the sunset. How long do you think your data will survive before deletion?

Remember 21 CFR 211.194(d): Complete records shall be maintained of the periodic calibration of laboratory instruments, apparatus, gauges, and recording devices required by § 211.160(b) (4)(8). Complete records include the data from all attempts at qualification, any explanation of reasons for failure, how they were resolved, and all should be included in the qualification report.

If this is not achieved, your qualification data have divorced you. Let’s move on to the greener pastures of orphan data in sample testing.

**Test and Prep Samples**

One classic example of orphan data are injections stored in directories called Test and Prep, or in one example a directory called Not To Be Shown To FDA. These directories contained sample injections that were used to see if a batch would pass or not. Restricting where data files are stored will make unofficial testing more visible, but this results in the rise of the short, aborted, or incomplete runs that we will see in the next section.

Before we move on, we need to distinguish unofficial testing from system evaluation or column conditioning injections. The latter is allowed but only if you use reference standard injections and never sample solutions. You can see a discussion on this topic on the FDA website (14) and in a “Questions of Quality” column on peak integration (15). FDA require the practice of column conditioning to be traceable back to a method validation report and to be scientifically justified (14).

It is critical that if you use evaluation or conditioning injections that the practice must be documented in a SOP, and must make it clear that these injections are part of complete/raw data of the sequence and are evaluated for each run. I suggest having acceptance criteria for these injections to show that the system is ready. Also, be very careful in how you name these injections.

**Titrating CDS Results**

One way of generating passing results is to titrate the data to obtain a passing result by changing values in the sequence file, such as sample weight, reference standard purity, or water content. This was identified in one of the 2005 Able Laboratories 483 observations (16):

Observation 5: …. The substitution of data was performed by cutting and pasting of chromatograms, substituting vials, changing sample weights, and changing processing methods……

Sample weights were changed by the analyst until a passing result was obtained.

One way to prevent the generation of this type of orphan data is through effective searching of audit trail entries, but to do this effectively requires a CDS with functions to help. Ideally, a CDS should identify where modifications in a data set have been made so that the reviewer or auditor does not have to trawl through all entries. Instead, they can focus their effort where data are changed to meet the requirements of Annex 11 (17) and PIC/S PI-041 (5).

**Short or Aborted Runs**

Houston, we have a problem is another way of looking at this topic. A short run consisting of one or two injections that is then stopped or aborted, which could be seen as the updated version of Test and Prep injections. As there are no deletion rights in the CDS, what should an analyst do to explain these unofficial test data? Instrument failure—easy-peasy!

To highlight the issue, a recent citation from Aurobindo issued in August 2022 (18) showed the message centre of a CDS had logged 6337 (yes, really!) error messages
from 1 July to 1 August 2022. Analysis of these identified the following entries:

- 411 instrument failure messages
- 13 messages of sequence stopped because of error or sequence stopped by user
- 20 Failed to get the newest information of the batch queue because of the communication failure messages.

These could be the start of potential instances of orphan data that must be investigated further. Enter stage left the unsung hero of data integrity—the instrument logbook! See 21 CFR 211.182 (8) and Chapter 4.31 (7) for the regulatory requirements that most people don’t understand and can’t implement.

If there is an instrument failure, the first place to look is the instrument logbook to see what sort of failure and then at the chromatograms (both electronic and printout) to see how the failure is seen (see Figure 2). For example, to support a justification for an instrument failure with a pump seal leak, you should see the peak shape get worse and retention times get longer as mobile phase is no longer being pumped consistently at the set rate. You should then assess what was done to fix the problem; back to the instrument logbook to see the maintenance entries. What about any requalification work? Is there a logbook entry for any testing that the leak was fixed? And where are the data or records associated with this work?

**Integrating into Compliance**

A more subtle type of orphan data is an analyst carrying out multiple peak integration attempts. In some CDS, they will be prompted to save each reintegration, meaning you only have the audit trail entries to assess. In other CDS applications, each attempt will be automatically saved, but only the “correct” result will be reported.

As indicated in multiple warning letters and 483 observations, peak integration must be controlled, otherwise the wrath of an inspector will descend. Peak integration has been covered in two “Focus on Quality” articles (15,19), where the sins of others were discussed extensively; however, peak integration 483 citations are still found, as seen in August 2022 with Sun Pharmaceuticals (20):

**Observation 4:** When an analyst processing chromatography data determines the existing processing method is not appropriate or chooses to use a different processing method for samples within the same sample set during impurities analysis, the original chromatogram is...

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not saved. The analyst can integrate the chromatogram and see the results within the software, but does not save the result. To change the processing method the analyst must document that the integration was inappropriate and get approval on Form 000505. But the original chromatogram is not saved to justify the changes to the processing method were necessary.

This observation shows that the procedural controls are totally ineffective, as the CDS lacks the technical controls and it should have been replaced with a compliant application. This is not so much orphan data as no data.

Observation 5: Procedures to ensure accurate and consistent integration of chromatographic peaks have not been established. The analysts can choose the integration algorithm and manually enter timed integration events into the processing methods. Procedures have not been established to ensure the appropriate and consistent use of these timed integration events.

Welcome to the Wild West where anything goes with peak integration and you can generate large volumes of orphan data under the guise of scientific judgement. Regardless of the analysis, all integration must be automatic first time, every time. Then you can perform manual intervention for all injections, but only in specified cases can manual integration be used and the chromatographer can reposition the baselines (19).

SST Failures
FDA’s Christmas present for chromatographers is found in the guidance on investigating OOS test results issued in 2006 and 2022 (21,22). Requirements for SST results are found in <621> and 2.2.46 of the United States Pharmacopeia and European Pharmacopoeia (23,24), respectively, to ensure that a chromatographic system is fit for use. FDA states in Section IIIA (22):

Certain analytical methods have system suitability requirements, and systems not meeting these requirements should not be used. For example, in chromatographic systems, reference standard solutions may be injected at intervals throughout chromatographic runs to measure drift, noise, and repeatability. If reference standard responses indicate that the system is not functioning properly, all of the data collected during the suspect time period should be properly identified and should not be used. The cause of the malfunction should be identified and, if possible, corrected before a decision is made whether to use any data prior to the suspect period.

This is good scientific advice by FDA. However, it is also a gilt-edged invitation to unscrupulous chromatographers to adjust the SST integration to be out of acceptance limits with a failing batch. Thus, where a sequence fails due to SST failure examine the peak integration and audit trail entries of the run and the SSTs injections specifically. This should be accompanied by a critical review of the laboratory investigation to ensure a scientifically sound reason for the invalidation. Don’t forget to review the instrument logbook as well, especially if the investigation cites instrument failure (see Figure 2).

Invalidating OOS Results
The last example of orphan data hiding in plain sight is invalidating an OOS result with an inadequate laboratory investigation. The problem with many laboratory investigations is that the outcome is “analyst error”, in which the data are invalidated, the analyst has their ears boxed, they are retrained, and all is well with the world. Until the next laboratory investigation…

Lupin received a 483 observation for invalidating 97% of stability OOS results (25) to avoid the need to file a field alert with FDA. As described above, critically evaluate the investigation and find what the assignable cause is and if it is scientifically sound. FDA is on the case with the quality metrics guidance where the only laboratory metric is percentage invalidated OOS results (26). Additionally, even where scientific judgement supports the decision, repeat failures for the same reason need to be investigated.

Common Issues
All of the orphan data examples discussed above are failures of one or more ALCOA+ or ++ criteria (27,28). Moreover, a laboratory has failed to put in place either the technical controls in the CDS and/or procedural controls and training to prevent orphan data being generated and detected by the second person reviewer, coupled with QA data integrity audits for oversight. For qualification of chromatographs, it is important that laboratories allow service engineers access to a CDS to avoid divorced data. One question arises: Do the current CDS applications have the functions available to help reviewers detect and examine cases of potential orphan data?

CDS Functions to Identify Orphan Data
To help reviewers, what new functions are required for a CDS?
Two areas spring to mind:
• Automatic identification of short or aborted runs. This would be used in second-person review of data by a supervisor, but also during data integrity audits by QA.
• SST failures is another area where automatic identification would greatly assist review and audit.

Summary
We have discussed orphan CDS data and the ways that they can be generated to falsify results. A CDS with appropriate technical controls implemented and validated can prevent some of them occurring, but for the remainder reviewers and auditors need to be trained and remain vigilant to detect them. Further functions in CDS applications would also greatly help detection of orphan data.

Acknowledgements
I would like to thank Mahboubeh Lotfinia and Paul Smith for their helpful comments during the writing of this column.

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Application of Porous Layer Open Tubular Columns: Beyond Permanent Gases

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¹Department of Chemistry, University of Washington, Seattle, Washington, USA;
²Column Watch Editor

Porous layer open tubular (PLOT) columns are traditionally built with particles that are adhered to the tubing walls. These columns have unique selectivity and provide a great alternative when gaseous samples need to be separated, but these columns have also been used to separate higher boiling point analytes. There are many different commercially available stationary phases of PLOT columns, including alumina-based columns, molecular sieves, and porous polymers. Alumina-based columns have an aluminium oxide stationary phase that is then deactivated with different salts. These columns have high capacity, superior loading ability, and produce symmetrical peaks. Molecular sieve columns are designed specifically for permanent gas separations because the columns have high retention. Porous polymer columns are highly hydrophobic, making them more applicable to analyzing a wider range of samples.

There are a wide variety of commercially available stationary phases of porous layer open tubular (PLOT) columns (Table 1) such as alumina, molecular sieve, carbon, silica, and a variety of porous polymers. In general, PLOT columns are traditionally built with particles that are adhered to the tubing walls.

Alumina columns have an aluminium oxide stationary phase that is then deactivated with various salts. These columns have high capacity and produce symmetrical peaks. Molecular sieve columns are designed specifically for permanent gas separations. Carbon-layer columns provide selectivity for inorganic and organic gases, like separating carbon monoxide and carbon dioxide. Silica columns are a great alternative to packed columns because they are made to be selective for C1–C4 compounds. Porous polymer columns are hydrophobic, which makes them more applicable to the analysis of a wider range of samples. DVB/vinylpyridine polymers are moderately polar and DVB/ethylene glycol/dimethacrylate are the most polar polymer columns. These commercially available PLOT columns (Table 1) are extensively tested to produce superior separations and are made to not shed particles, which was previously a concern with PLOT columns (1). Although PLOT columns have been primarily applied in gas chromatography (GC) with the main focus on separating low boiling point analytes (2,3), there is evidence of PLOT column use beyond light samples (4–6). The versatility of PLOT columns has been exploited even further by their use in dynamic vapour microextraction (DVME) (7–9) and even in liquid chromatography (LC) (10–12). Here, we discuss the application of PLOT columns beyond their original primary use for permanent gaseous sample separations.

Gas Chromatography

The first laboratory-made PLOT columns were introduced in 1963 (13,14). Since that time, these columns have been mostly used for separating light mixtures such as permanent gases (2,15,16) or light hydrocarbons (3,17,18). It has been shown that PLOT columns are very retentive for light compounds. For example, a 10-m molecular sieve column has been used to separate permanent gases in the presence of hydrocarbons and nitriles (2). With the exception of helium and neon (which coeluted), all the other permanent gases (argon, dinitrogen, krypton, xenon), along with methane and carbon monoxide, were baseline separated (2). Alumina columns have been evaluated by Ji and others, where they showed that these columns are highly retentive for C1–C6 compounds (3). The baseline separation of methane, ethane, ethylene, propane, and higher carbon content compounds was achieved using a 30-m alumina column.
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TABLE 1: Commercially available PLOT columns of different stationary phases (porous layers) compared between Restek, Supelco, Agilent, and Quadrex. Table remade and updated from Restek’s PLOT family document from 2020.

<table>
<thead>
<tr>
<th>Porous Layer</th>
<th>Restek</th>
<th>Supelco</th>
<th>Agilent</th>
<th>Quadrex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonded silica</td>
<td>------</td>
<td>------</td>
<td>CP Silica PLOT, GS-Q</td>
<td>------</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Alumina BOND/Na₂SO₄</td>
<td>Alumina-sulfate</td>
<td>GS-Alumina, CP-Al₂O₃/Na₂SO₄</td>
<td>------</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Alumina BOND/KCl</td>
<td>Alumina-chloride</td>
<td>GS-Alumina KCl, HP PLOT Al₂O₃</td>
<td>CP-Al₂O₃/KCl</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Alumina BOND/CFC</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Alumina BOND/ MAPD</td>
<td>------</td>
<td>------</td>
<td>Select Al₂O₃ MAPD</td>
</tr>
<tr>
<td>Molecular sieve 5A</td>
<td>Msieve 5A</td>
<td>Molsieve 5A</td>
<td>HP PLOT Molsieve, CP-Molesieve 5A</td>
<td>PLT-5A</td>
</tr>
<tr>
<td>Carbon</td>
<td>------</td>
<td>Carboxen-1010 PLOT, Carboxen-1006 PLOT</td>
<td>CarboBOND, CarboPLOT P7, GS-CarbonPLOT</td>
<td>------</td>
</tr>
<tr>
<td>100% Divinylbenzene</td>
<td>Q-BOND</td>
<td>Supel-Q-PLOT</td>
<td>HP PLOT Q, CP-PoraPLOT Q, CP-PoraBOND Q</td>
<td>PLT-Q</td>
</tr>
<tr>
<td>Intermediate polarity porous polymer</td>
<td>QS-BOND</td>
<td>------</td>
<td>GS-Q</td>
<td>------</td>
</tr>
<tr>
<td>DVB vinylpyridine polymer</td>
<td>S-BOND</td>
<td>------</td>
<td>CP-PoraPLOT S</td>
<td>------</td>
</tr>
<tr>
<td>DVB ethylene glycol-dimethylacrylate polymer</td>
<td>U-BOND</td>
<td>------</td>
<td>HP PLOT U, CP-PoraPLOT U, CP-PoraBOND U</td>
<td>PLT-U</td>
</tr>
</tbody>
</table>

3. Retentiveness of PLOT columns that was obtained with light compounds could potentially be applied to higher boiling point analytes and there has been some work done throughout the years to broaden the use of PLOT columns.

In 1974, fast, high temperature separations were performed using a molecular sieve column by Soulages and others where the paraffin and naphthalene content was investigated in saturated hydrocarbon distillates (19). Arpino and others in 1977 presented a novel PLOT column that was made with a graphitized thermal carbon-black polar stationary phase that could be used for GC and LC (20). This column was used to investigate two oxidation reactions, where citral (boiling point: 225 °C) was produced from geraniol (boiling point: 230 °C) (20). Reaction products were separated and more than 30 by-products were identified (20). Another application, reported in 1980 by Uden and others, involved separating volatile metal chelates and organometallic compounds (such as chromium trifluoroacetylacetonate, chromium hexafluoroacetylacetonate-trifluoroacetylacetonate chelates, copper [II] tetradentate p-ketooamine chelates, cyclopentadienylmanganese tricarbonyl compounds) using carbon-carbon-silicone Dextil PLOT stationary phases in a stainless steel column (21). Following this, in 1983, Nijs and others implemented alumina-coated PLOT columns to separate hydrocarbons up to C10 (4). In the same study, the authors demonstrated a beautiful separation of naphtha, with p-xylene (boiling point: 138 °C) being the heaviest compound present (4). Another report, where some higher boiling point analytes were separated in conjunction with permanent gases, was published by Greally and others in 1998 (5).

In this study, a novel PLOT column with a hydrophobic silica layer coated on a fused-silica capillary was used (5). Various halogenated hydrocarbons were separated in this study; although most analytes had a boiling point < 0 °C, they also separated heavier halogenated hydrocarbons such as chloroform (boiling point: 61 °C) (5). Another more recent work involving hydrocarbon mixture separations was reported by Wawrzyniak and others while evaluating a novel stationary phase of 4,4,4-trifluoro-1-phenyl-1,3-ketoiminepropyl silane used as a porous layer bonded on to 5-μm nucleosil silica (22). Halogenated hydrocarbons up to C6 were analyzed, which facilitated the separation of the heaviest compound in this study of 1,2-dichlorobenzene (BP: 180 °C) (22). Another unique application of PLOT columns includes isotopic separations where a commercially available porous polymer PLOT column has been used to separate acetaldehyde and methanol from their deuterated isotopologues, performed using headspace sampling (6).

Implementation of PLOT columns has also emerged in the field of comprehensive two-dimensional (2D) gas chromatography (GC×GC), where the complementary separation mechanisms provided by two different PLOT columns can be leveraged to produce intriguing 2D separations. Light hydrocarbons and some solvents were separated using different commercially available PLOT columns by Patrushev and others in 2018, with the heaviest compound separated being methylcyclohexane (boiling point: 101 °C) (23). GC×GC has also been shown to provide informative 2D separations for gasoline, which we described in our recent report, where a distinct commercially available PLOT column was applied for each dimension. On the first dimension (1D), a silica PLOT was applied, while on
the second dimension (2D), a porous polymer PLOT was used (24).

PLOT columns have unique selectivity and provide a great alternative to wall-coated open tubular (WCOT) columns or packed columns for separations of light samples (25–27). Environmental Protection Agency (EPA) Appendix IX volatiles calibration megamix was separated by GC using the same temperature program and chromatographic conditions on a WCOT column (Rtx-200) in Figure 1(a), and on a PLOT-Q column in Figure 1(b). Most of the mix eluted at the beginning of the chromatogram using a WCOT column; while using the PLOT-Q column, the entire mixture was well retained and separated—everything from carbon disulfide (BP: 46 °C) to naphthalene (BP: 218 °C).

In this example, even a very short length PLOT column provides unique selectivity for low boiling point analytes relative to the selectivity that is provided using a WCOT column. A heavier sample of gasoline was used to further show the unique separation abilities of PLOT columns. Gasoline is one of the heaviest “common” samples that can be run on PLOT columns. Figure 2 presents optimized gasoline separations on a porous polymer (PLOT-S) column (Figure 2(a)) and an alumina oxide (Rt-Alumina BOND/ MAPD) column (Figure 2(b)). The heaviest compound that elutes from both columns is 1-methyl naphthalene, which has a boiling point of 244 °C. Although both columns provide intriguing separations of gasoline with relatively short run times, Rt-Alumina BOND/MAPD provides a better separation of very similar molecules. As shown in Figures 2(c) and 2(d), neopentane, 1-pentene, isopentane, and pentane are separated into four peaks on Rt-Alumina BOND/MAPD (Figure 2(d)), while on PLOT-S column (Figure 2(c)), these compounds overlap into two peaks. Rt-Alumina BOND/MAPD also provides a mix of sharper peaks and broader peaks. These separations show the ability to apply PLOT columns for samples containing compounds with a wide variety of boiling points—for example, isobutane (BP: -11 °C) to 1-methyl naphthalene (BP: 244 °C).

**Dynamic Vapour Microextraction (Cryoadsorption)**

More recently, PLOT columns have also been used for cryoadsorption, which was developed at National Institute of Standards and Technology (NIST) in 2009 by Bruno and others (7). This technique is also referred to as DVME. More specifically, it is an ultra-sensitive, low volume purge-and-trap sampling method where vapour-phase analytes are concentrated on a section of the PLOT column (9). DVME is a dynamic headspace sampling technique that is especially useful when low volatility compounds are of interest.

A cryoadsorption apparatus for DVME adapted from the pioneering report by Bruno and others is shown in Figure 3(a) (7). In this work, the apparatus was equipped with a short alumina PLOT column as a purge trap (Figure 3(b)) operating at a low temperature. The apparatus was evaluated using a pure explosive compound 2,3,4-trinitrotoluene as well as a practical explosive C-4 (7). Later, the same group published a broader study using alumina-coated PLOT columns for cryoadsorption where industrial and military plastic bonded explosives (PBX) tagged C-4, Semtex-1A, Semtex-H, detonating cord (detcord), and sheet...
explosive (Detaflex) were investigated (28). DVME was adapted later by Nichols and others. In this study, short PLOT columns were used in cryoadsorption on arson fire debris samples (29).

Most recently, cryoadsorption was adapted to develop a portable vapour sampling device. The first account of such work was published in 2016 by Bruno and others, where the instrument was laboratory tested using naphthalene, kerosene, and others, producing accurate detection and quantitation after a short collection time of 3 s with a concentration limit of detection (LOD) of less than 1 ppm (8,30).

Then, this device was in-field tested by Harries and others in 2019 on an old U.S. Army communication bunker as a simulated shipping container (31). This device was able to detect naphthalene, two-explosive-related compounds, and four protein decomposition markers, as well as gasoline (31). This work was further expanded by another study that examined how long after in-field collection the samples stay preserved when collected on the PLOT column. It was determined that the integrity of the samples was fully preserved for 24 h, with losses occurring by the seventh day (9).

DVME has been shown to be a very sensitive technique. Burger and others examined the difference between natural gas sampling using multi-PLOT-cryo (Figure 3[c]) and a direct injection of the same amount of natural gas using a gas-tight syringe (Figure 3[d]). The study showed how much more sensitive and suitable for the analysis of trace components the PLOT-cryo technique is over traditional sampling methods (32). Because of the superior sensitivity of DVME, it has been used in recent years to analyze a broad range of samples: fuels (33), cannabinoids (34), and forensics (28,29,35,36).

**Liquid Chromatography**

Traditionally, LC is performed using packed columns with bonded phases, but it has been shown that open tubular columns can provide higher column efficiency (37). There have been a few studies into using open tubular columns in LC in the 1970s and 1980s (38,39), and it has been shown that to reach column efficiencies close to what is achieved in GC, the columns must be not more than 15 µm in internal diameter (i.d.) (40).

Open tubular column use was revitalized in LC after coupling to electrospray (ES) mass spectrometry (MS) (41). PLOT columns have become increasingly popular in LC in recent years. Most papers have focused on using PLOT columns for LC separations of various biological samples. In 2010, Rogeberg and others separated intact proteins in skimmed milk using polystyrene divinylbenzene PLOT columns (10); narrow peaks (0.144–0.33 min) and good within-column repeatability in retention times (0.6% RSD) were achieved, with less than 1.1% carryover injection-to-injection. In 2011, Wang and others analyzed haptoglobin from lung cancer patients with a 10 µm i.d. polystyrene-divinylbenzene PLOT column by LC coupled to collision-induced dissociation/electron transfer dissociation–MS (LC–CID/ETD-MS) (11).

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A new zwitterionic PLOT column was introduced by Peng and others in 2016 that was used to separate flavonoids from rootstock of liquorice (42). Good RSD for differences in retention time run-to-run (≤1.1%), day-to-day (≤0.78%), and column-to-column (≤1.1%) were obtained.

There have also been nano-LC and pico-LC systems that have been introduced in recent years, where a PLOT column was used for the separations. In 2009, a nano-LC system using an amine-bonded poly(vinylbenzyl chloride-divinylbenzene) hydrophilic interaction LC (HILIC) PLOT column was used for the separation of a glycan mixture, and only 50 fmol of a dextran ladder was needed to identify dextrans up to G22 (43). In 2011, Thakur and others presented a workflow where breast cancer cells were analyzed using PLOT-LC tandem MS (MS/MS), demonstrating good quantitative reproducibility between the same patient samples (44). In 2017, Li and others presented a new pico-PLOT HPLC system that was used to separate amino acid enantiomers (45). A very recent report from Vargas Medina and others in 2022 describes the development of nano-LC coupled to electron ionization (EI)–MS. Polyaromatic hydrocarbons and thermolabile pesticides were separated, and the LOD of such a system was determined to be 25 µg/L (12).

Conclusion and Future Prospectus

PLOT columns provide unique retention and selectivity for low boiling point analytes relative to WCOT columns (25–27). Although most of their use has been shown with light mixtures (2,3), there has been some work done to broaden their use throughout the years (4–6). We demonstrated their unique abilities to separate light compounds as well as heavier boiling point analytes (Figures 1 and 2), which shows a broad analyte range that can be analyzed using PLOT columns. As in 1D chromatography, GC×GC using PLOT columns has mostly been employed to separate samples containing light boiling point compounds and continues to be an underutilized application area for PLOT columns. Further applications of PLOT columns have been shown by their use in a new sampling technique of DVME, and this technique has been applied to analyze various samples, including explosives, fuels, and cannabinoids (33–35). PLOT columns have also gained attention in the LC community due to higher efficiency, where they have been adapted to be used in nano-LC systems to analyze mostly biological samples (10–12). It is important to note that because there are no commercially available PLOT-LC columns, this is a research area under development with ample opportunities. Broader use of PLOT columns is a growing research area, especially with their use for heavier samples in GC. Applying these columns for sampling using dynamic vapour microextraction and utilizing
their better column efficiency in liquid chromatography also demonstrate expanding utility of the technology.

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NTS Workshop on Analytical Techniques and Implementation

The NTS Workshop on Analytical Techniques and Implementation will take place 29–30 November 2022 in Odense, Denmark. Because of the complexity of environmental and biological samples, chemical fingerprinting methods such as nontarget screening (NTS) have become increasingly popular. However, there are still open challenges that considerably slow down the uptake of this technology by the industry and markets.

NTS methods generate large amounts of data and new types of knowledge that hardly fit into the current regulatory framework, which is based on targeted, fully quantitative analyses. Without regulations, industrial bodies have little incentive to develop and market NTS tools for screening and monitoring environmental contaminants, which also significantly constrains the transfer of knowledge and resources between academia and industry and the actual implementation of methods used in research.

The purpose of this workshop is to bring academia, industry, and regulatory bodies to the same venue to discuss the different challenging aspects, with a strong focus on implementation challenges at all levels—from how to include novel analytical platforms that show great promise to how NTS generated knowledge can be used in regulation. The workshop will close with a one-hour panel discussion on the priorities to accelerate NTS adoption.

The questions to be addressed include:
- What can existing products, commercial or otherwise, do today?
- How close are the suspect screening/NTS workflows for new and old platforms to being suitable for use outside academia/research projects?
- How can, or do, authorities use the results from suspect screening/NTS in practice (for example, in the prioritization of capillary electrochromatograph [CEC])?
- How are suspect screening/NTS results reported?
- How to set-up QA-schemes/specifications for suspect screening/NTS authorities can refer to, for example, tenders?

The workshop is organized by the University of Copenhagen under the Vandalf Project funded by Innovation Fund Denmark, and in collaboration with the Danish Environmental Protection Agency, the NORMAN network, and the Danish Society of Analytical Chemistry.

Following the workshop, the NORMAN network hosts its NORMAN general assembly meeting 1–2 December 2022 (www.norman-network.net).

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