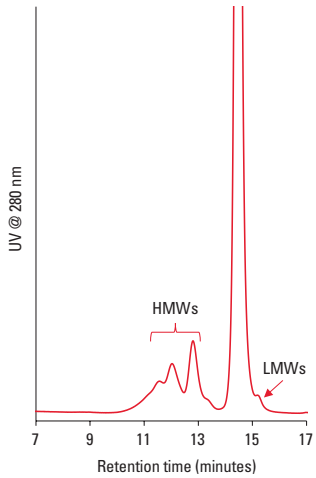
LC GC®
north america
solutions for separation scientists

Solutions for Monoclonal Antibody Analysis and Characterization

TSKgel UP-SW Aggregate:

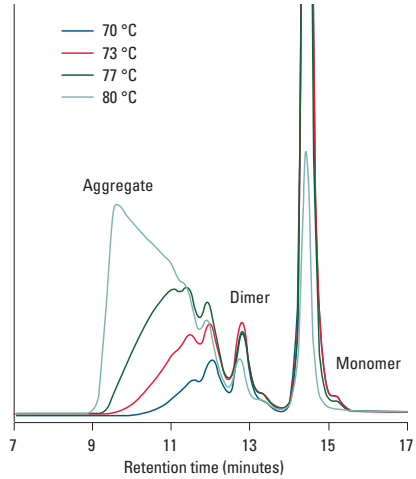
Providing Superior Separation for High Order Aggregates and Macromolecules by Size Exclusion Chromatography

Superior Resolution Analysis of mAb Aggregates



TSKgel UP-SW Aggregate column allows a more detailed view on the higher order aggregates.

Analysis of Heat Forced mAb Aggregation



Changes in the aggregate peak profile at four different temperature points are easily discerned between 70 - 80 °C using the TSKgel UP-SW Aggregate column.

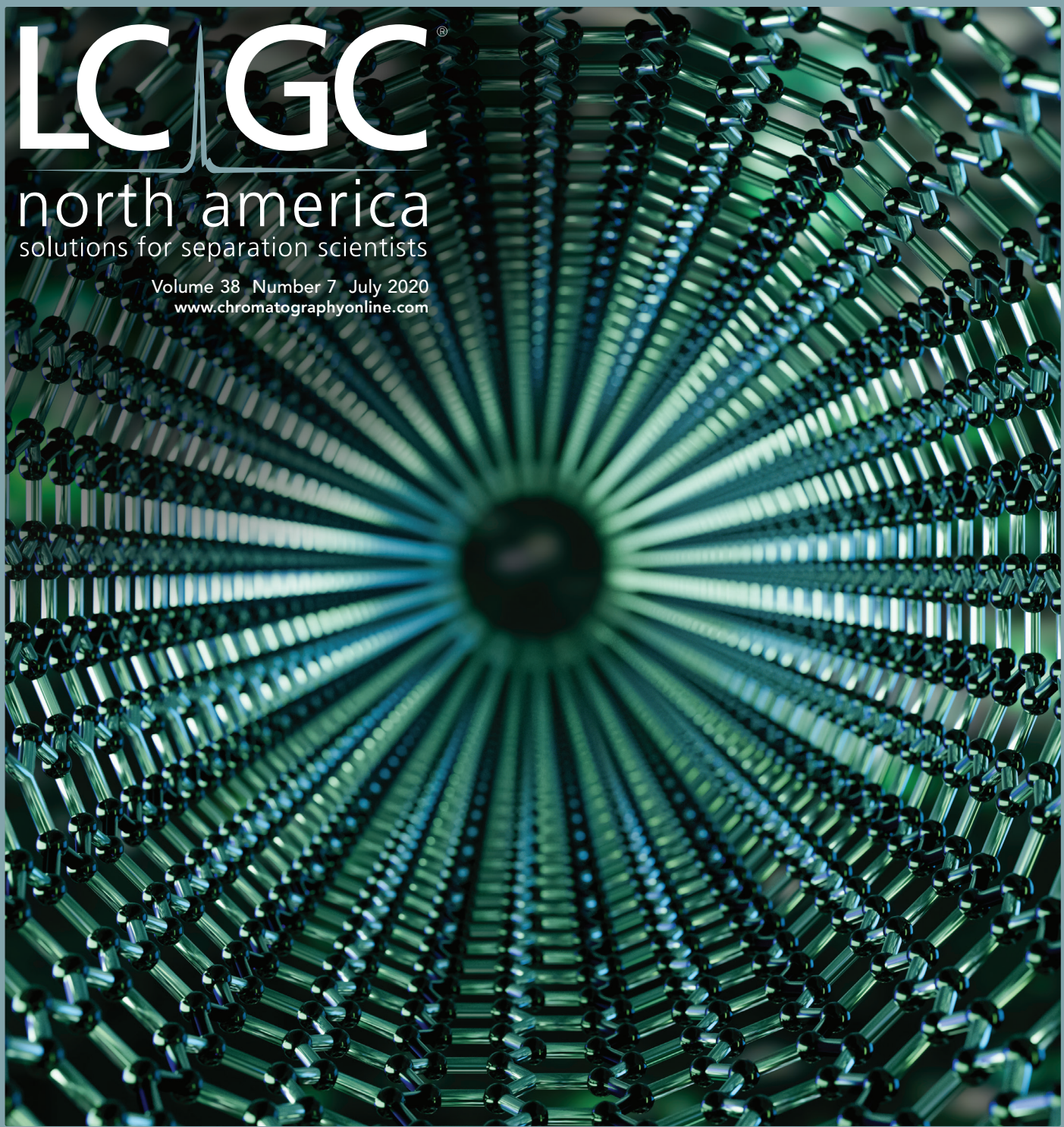
TOSOH BIOSCIENCE
www.tosohbioscience.com

TOSOH

GC
Cold Injections in Gas Chromatography

THEORY & FUNDAMENTALS
Better Reversed-Phase Separations of mAbs Using Organic Alcohols

APPLICATIONS
Assessing Compound Migration from Food Can Coatings



LC GC north america

solutions for separation scientists

Volume 38 Number 7 July 2020
www.chromatographyonline.com

LC
Starting Up Your LC System After the COVID-19 Shutdown

SAMPLE PREPARATION
ORGANOPHOSPHORUS PESTICIDE ANALYSIS BY HPLC

DATA HANDLING
Are You Invalidating OOS Results into Compliance?

HAMILTON®

Reversed-Phase Separation of Isoflavones and Mycotoxins in Soy Beans

Estrogen Receptors (ER) are found in tissues throughout the body including but not limited to bone, brain, heart, lung, and breast.[1,2] ER binding is primarily activated by the cyclic diol, estradiol.[3] Estrogenic molecules derived from plants (phytoestrogens) can also bind to ER's in mammals. Plants produce phytoestrogenic compounds like isoflavones and coumestans for varying functions such as serving as deterrents against herbivores, attractants for bees or other pollinators, or recruitment signals for symbiotic soil bacteria.[4,5]

In humans, as omnivores, effects from the consumption of certain plant foods leading to disease is of particular interest. Soy beans for example contain important phytoestrogenic isoflavones, Genistein and Diadzein, which can beneficially bind to ER's and have been shown to reduce the risk of certain cancers.[6] Soy beans, however, can also contain a fungal metabolite and mycotoxin, Zearalenone (ZEA), which has been shown to preferentially bind to ER's and cause an increase in the proliferation of certain tumor cells.[7] Both the mycotoxin and the isoflavones are observed as strong binders to ER's.[8]

Analyzing soy beans and other crops for beneficial and harmful compounds is an indispensable tool for assessing the viability of harvests for consumption. With this in mind, we have developed a streamlined and reliable HPLC method to separate some known phytoestrogenic and mycotoxic ER binders using the Hamilton PRP-C18 column.

(1) Yaghmaie F, Saeed O, Garan SA, Freitag W, Timiras PS, Sternberg H (2005). "Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice." Neuro Endocrinology Letters. 26 (3): 197–203.

(2) Babiker FA, De Windt LJ, van Eickels M, Grohe C, Meyer R, Doevendans PA,. (2002). "Estrogenic hormone action in the heart: regulatory network and function." Cardiovascular Research. 53 (3): 709–19.

(3) Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, Maggi A, Muramatsu M, Parker MG, Gustafsson JA,. (2006). "International Union of Pharmacology. LXIV. Estrogen receptors." Pharmacological Reviews. 58 (4): 773–81.

(4) Koes RE, Quattrocchio F, Mol JNM,. (1994). "The flavonoid bio-synthetic pathway in plants: function and evolution." Bio Essays. 16: 123–132.

(5) Bladergroen MR, Spaink HP,. (1998). "Genes and signal molecules involved in the rhizobia-leguminoseae symbiosis." Curr Opin Plant Biol. 1: 353–359.

(6) Yu X, Zhu J, Mi M. et al,. (2012). "Anti-angiogenic genistein inhibits VEGF-induced endothelial cell activation by decreasing PTK activity and MAPK activation." Med Oncol, 29: 349-57.

(7) Hueza IM, Raspantini, PCF, Raspantini, LER, Latorre, AO, Gorniak SL,. (2014). "Zearalenone, an Estrogenic Mycotoxin, is an Immunotoxic Compound." Toxins (Basel). 6 (3): 1080–1095.

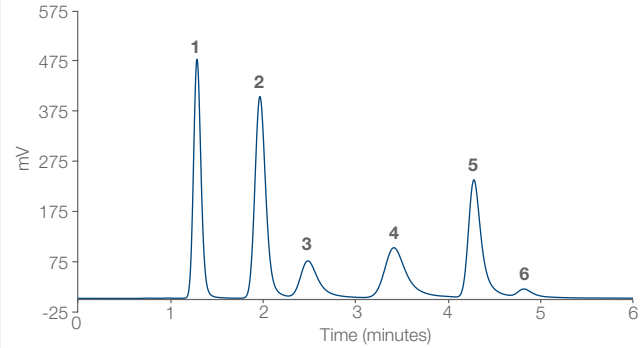
(8) Fox J, Starcevic M, Jones P. Burow M, McLachlan J,. (2004). "Phytoestrogen Signaling and Symbiotic Gene Activation are Disrupted by Endocrine-Disrupting Chemicals." Environmental Health Perspectives. 112: 672-7.

Column Information

Packing Material	Dimensions	Part Number
PRP-C18 (5 µm)	150 x 4.6 mm	79676

Chromatographic Conditions

Gradient	0.00–2.3 min, 50% B 2.3–6 min, 50–99% B 6–7 min, 99% B 7–10 min, 50% B
Temperature	40°C
Injection Volume	10 µL
Detection	UV at 236 nm
Eluent A	Phosphoric Acid 0.2%
Eluent B	Acetonitrile
Flow Rate	2 mL/min



Compounds:

1: Daidzein	0.017 µg/µL	**4:** alpha-Zearalenol	0.087 µg/µL
2: Genistein	0.017 µg/µL	**5:** Ochratoxin A	0.350 µg/µL
3: beta-Zearalenol	0.087 µg/µL	**6:** Zearalenone	0.008 µg/µL

Author: *Adam L. Moore, PhD, Hamilton Company*

Lit. No. L80101 — 11/2019

Web: www.hamiltoncompany.com
USA: 800-648-5950
Europe: +40-356-635-055

To find a representative in your area, please visit hamiltoncompany.com/contacts.

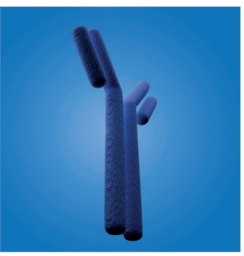
Hamilton Americas & Pacific Rim
Hamilton Company Inc.
4970 Energy Way
Reno, Nevada 89502 USA
Tel: +1-775-858-3000
Fax: +1-775-856-7259
sales@hamiltoncompany.com

Hamilton Europe, Asia & Africa
Hamilton Central Europe S.R.L.
str. Hamilton no. 2-4
307210 Giarmata, Romania
Tel: +40-356-635-055
Fax: +40-356-635-060
contact.lab.ro@hamilton-ce.com

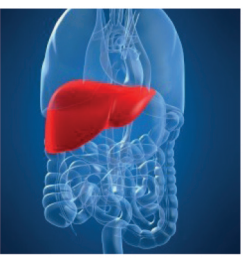
NEW

Roxy Exceed

Electrochemistry System for EC-MS



Proteomics



Metabolism

Drug Stability

Environmental

Synthesis

www.AntecScientific.com

LCGC north america

MANUSCRIPTS: For manuscript preparation guidelines, see chromatographyonline.com/lcgc-author-guidelines, or call The Editor, (732) 596-0276. *LCGC* welcomes unsolicited articles, manuscripts, photographs, illustrations, and other materials but cannot be held responsible for their safekeeping or return. Every precaution is taken to ensure accuracy, but *LCGC* cannot accept responsibility for the accuracy of information supplied herein or for any opinion expressed.

SUBSCRIPTIONS: For subscription and circulation information: *LCGC*, P.O. Box 457, Cranbury, NJ 08512-0457, or email mmhinfo@mmhgroup.com. Delivery of *LCGC* outside the United States is 14 days after printing. (*LCGC Europe* and *LCGC Asia Pacific* are available free of charge to users and specifiers of chromatographic equipment in Western Europe and Asia and Australia, respectively.)

CHANGE OF ADDRESS: Send change of address to *LCGC*, P.O. Box 457, Cranbury, NJ 08512-0457; alternately, send change via e-mail to fulfill@mjhassoc.com or go to the following URL: http://mmhpubs.mmhgroup.com/Welcome.aspx?pubid=LCGC Allow four to six weeks for change. PUBLICATIONS MAIL AGREEMENT No. 40612608. Return all undeliverable Canadian addresses to: IMEX Global Solutions, P.O. Box 25542, London, ON, N6C 6B2, CANADA. Canadian GST number: R-124213133RT001.

C.A.S.T. DATA AND LIST INFORMATION: Contact Melissa Stillwell, tel. (218) 740-6831, e-mail MStillwell@mmhgroup.com.
REPRINTS: Contact Michael J. Tessalone, e-mail: MTessalone@mjhlifesciences.com
INTERNATIONAL LICENSING: Contact Kim Scaffidi, e-mail: kscaffidi@mjhassoc.com

CUSTOMER INQUIRIES: Customer inquiries can be forwarded directly to MJH Life Sciences, Attn: Subscriptions, 2 Clarke Drive, Suite 100, Cranbury, NJ 08512; e-mail: mmhinfo@mmhgroup.com

Alliance for Audited Media

50% Recycled Paper 10-20% Post Consumer Waste

485F US Highway One South,
Suite 210
Iselin, NJ 08830
(732) 596-0276
Fax: (732) 647-1235

CONTENTS

LC GC north america
solutions for separation scientists

COVER IMAGERY BY PRODUCTION PERIG-stock.adobe.com

COLUMNS

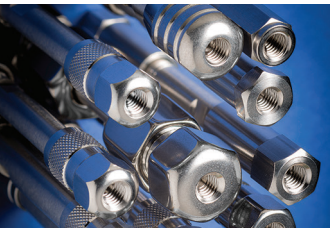
FEATURE ARTICLE

PEER-REVIEWED ARTICLES

DEPARTMENTS

LCGC North America (ISSN 1527-5949 print) (ISSN 1939-1889 digital) is published monthly by MultiMedia Healthcare, LLC., 2 Clarke Drive, Suite 100, Cranbury, NJ 08512, and is distributed free of charge to users and specifiers of chromatographic equipment in the United States and Canada. LCGC is available on a paid subscription basis to nonqualified readers in the United States and its possessions at the rate of: 1 year (13 issues), $83.95; 2 years (26 issues), $151.20; in Canada and Mexico: 1 year (13 issues), $107.10; 2 years (26 issues), $168.53; in all other countries: 1 year (13 issues), $157.50; 2 years (26 issues), $281.40. Periodicals postage paid at Trenton, NJ 08650 and additional mailing offices. POSTMASTER: Please send address changes to LCGC, P.O. Box 457, Cranbury NJ 08512-0457. PUBLICATIONS MAIL AGREEMENT NO. 40612608, Return Undeliverable Canadian Addresses to: IMEX Global Solutions, P. O. Box 25542, London, ON N6C 6B2, CANADA Canadian GST number: R-124213133RT001. Printed in the USA.

Editorial Advisory Board



LC TROUBLESHOOTING

Recovering from a COVID-19 Shutdown: Tips and Tricks for Starting Up, Part II

COVID-19-related laboratory shutdowns are sure to cause a myriad of problems with liquid chromatography (LC) instrumentation across the globe. Taking a systematic approach to restarting these systems will save money and time in the long run by preventing problems that may otherwise appear in days or weeks following startup.

Dwight R. Stoll and Tony Taylor

In March of this year, many organizations took unprecedented steps to halt the spread of COVID-19, including severely restricting work in laboratories, or even shutting down entire laboratories, buildings, and worksites for weeks at a time. While some of these shutdowns were planned days in advance and executed well, I have heard many stories from scientists indicating that the shutdowns were sudden, and did not allow time to properly prepare their analytical instrumentation to be idle for weeks or months at a time. Unfortunately, this means that these scientists are going to encounter many challenges when they return to the laboratory that will necessarily include a lot of troubleshooting to figure out why their systems are not working properly before they can return to their normal experience of producing high quality data. For last month's installment of "LC Troubleshooting," I asked Tony Taylor to join me in pulling together advice for starting up liquid chromatography (LC) systems after they have been idled for weeks or months at a time (1). In that installment, we mainly addressed challenges with microbial growth in solvent bottles and different parts of the LC system itself, and obstruction of the LC flow path that can occur as a result of precipitation of buffer salts or other debris. For this month's installment, I've asked Tony to join me again, this time sharing advice related to the health of columns, qualifying system performance, and a little about restarting work with LC–mass spectrometry (MS) systems in particular. I hope that these suggestions are helpful as you return to the laboratory, but I am sure many strange things will be observed after so many LC users have been away from the laboratory for prolonged periods. If you've encountered a problem and gained some troubleshooting experience that you think others might be able to learn from, please don't hesitate to send your story my way.

- Dwight Stoll

The Column: Is It Still Okay?

Last month, we noted that the column is the heart of any chromatography system, and, as such, we need to ensure that our columns are in a healthy condition prior to performing analyses. It is possible that the shutdown period may lead to mechanical or chemical problems, and the solutions to these problems will be different. Until you know that the column has been properly flushed, the column outlet should not be connected to the rest of the system. Disconnecting the column outlet from the flow path will avoid any unwanted compounds or debris that may come out of the column from causing problems with any other components in the system. You can either attach a waste line to the column outlet to collect the effluent, or simply let it drip into a beaker or similar container.

Mechanical Problems

If your column was left on the system when the laboratory was shut down, there is a possibility that it will now be full of air, because the mobile phase solvent may have evaporated over time. Similarly, if the column was removed from the system, but not plugged at both ends, it will again be full of air, which can lead to problems for many types of columns. We need to carefully guard against applying high pressures to columns that are dry, as this may cause mechanical reorganization of the packed bed of particles, leading to unwanted voids, channeling, and significantly reduced column performance. In the following discussion, please bear in mind that the pressure applied to the column inlet should be increased gradually when first turning the flow back on (in steps of <10 bar where possible); this can be achieved by starting the pump at a very low flow rate (for example, 10 µL/min. for 2.1 to 4.6 mm i.d. columns), and increasing the flow rate in steps of 10 µL/min (larger steps can be used provided the corresponding pressure increase is not much more than 10 bar per step). Some newer systems also provide the option to specify a flow rate ramp rate that is used when the pump is turned on.

It may be possible that, upon starting the column flushing procedure, a high-back pressure is encountered due to blockages in either the column inlet frit (pressure increase will be immediately noticeable) or the outlet frit (pressure will build more gradually over time). In the case of the former, it may be possible to reverse the direction of the column prior to turning on the flow, in order to back-flush the debris from the frit. Overall,

this may have the longer-term effect of slightly reducing the efficiency of the packed bed, but the column should be usable for your application, unless it was heavily voided prior to the instrument shutdown.

Chemical Problems

In most cases, the following column flushing routine can be applied to reversed-phase, silica-based stationary phases as a kind of generic column cleanup step. Approximate column volumes for several different dimensions of columns in common use are given in Table I.

- Set the column thermostat compartment to 60 °C.
- Flush with the following solvents in the order shown. For 2.1 mm i.d. columns use a flow rate of about 0.1 mL/min; for 4.6 mm i.d. columns use a flow rate of about 0.5 mL/min.
- 10:90 methanol:water for 20 column volumes (to remove any precipitated buffers)
- Increase organic composition to 100% methanol, and flush for 20 column volumes.
- Flush with 20 column volumes of 75:25 acetonitrile:isopropyl alcohol (IPA).
- Flush with 20 column volumes of the starting mobile phase of your method.

The rationale behind this series of flushing solvents is to cover a wide range of solvent polarities that give the best chance of dissolving and eluting anything that has adsorbed to the stationary phase. Only after these flushing steps should the column be reconnected to the detector. If the column flow direction has been reversed as discussed above, switch back to the original orientation prior to reconnecting the column to the system.

In this brief discussion, we have focused on suggestions for restarting work with reversed-phase columns, and in a very generic way. Readers interested in a more detailed discussion of cleaning procedures for reversed-phase columns are referred to the excellent *LCGC North America* article on this topic by Ron Majors (2).

Is It Safe to Collect Data Again?

At a minimum, before collecting important data again, one should run the system suitability test that is appropriate to the analysis at hand. The topics of system suitability and operational qualification (OQ) have been discussed in prior installments of "LC Troubleshooting" by John Dolan, and readers interested in more detail are referred to these articles (3,4).

In general terms, system suitability tests are designed to provide data that indicate an LC system is functioning in a way that it can be expected to produce reliable data for a particular analysis. In other words, it is application- or method-specific. In many situations, these data are sufficient to give the user confidence that the system is "ready to go." However, in other situations and under certain regulatory frameworks, it may be necessary to carry out a more extensive OQ of the instrument that will verify instrument performance, regardless of the methods being run. While doing OQ tests will undoubtedly take more time when restarting an LC system, we feel strongly that this time spent at startup can save a lot of time in the long term by catching problems early, and addressing them before data acquisition resumes. In other words, a little extra effort now will decrease the likelihood that we encounter unwelcome surprises down the line!

A typical OQ verification routine will include some or all of the following tests that we have annotated briefly to give an explanation of what the test does, and the information it provides toward an assessment of operational performance. Wherever a "reference compound" is referred to in these descriptions, caffeine is very commonly used, particularly for systems with ultraviolet (UV) detectors.

Pump Flow Rate Accuracy and Precision

Typically, a number of flow rate measurements are made at different flow rates using a digital flow meter. Flow rate accuracy is important for transfer of methods between systems and retention time agreement with standard methods of test. Flow rate precision has a direct impact on the repeatability of peak height and area.

Column Temperature Accuracy and Stability

Typically, a temperature sensor is used to measure either the column compartment temperature, or the temperature of the column effluent. Temperature is measured over time at two different setpoints, and the absolute difference between them, as well as the variability, are compared to manufacturers or regulatory criteria. Temperature accuracy can be critical for chromatographic selectivity, especially when separating ionizable analytes (5), and temperature stability has a strong influence on the repeatability of retention times.

UV Wavelength Accuracy

Typically, a caffeine or holmium oxide standard is used under conditions of no mobile phase flow, and the absorbance is recorded at several wavelengths across the range of the detector. The measured positions for the peaks (maxima) and troughs (minima) of the standard are compared to the known (expected) values for the standard. The difference between the measured and expected wavelengths of maximum and minimum absorption is compared to manufacturers specifications. Wavelength accuracy is vital for both qualitative and quantitative work, and transferability of methods between systems.

Detector Noise and Drift

Short- and long-term variation in the detector signal is determined with a fixed eluent composition (typically water). The detector signal is measured over a certain period of time and at a particular frequency to determine the short-term variation in the signal, referred to as the *noise*. The slope of the detector signal over a longer time period (tens of minutes) is also measured, and this is referred to as the *detector drift*. The magnitudes of the noise and drift directly influence the ability to differentiate between real peaks for low concentration analytes and random variation in the detector signal.

Signal-to-Noise Ratio (S/N)

The detector sensitivity is also evaluated using a reference compound under specific conditions and compared to a target specification. There are many different manufacturer and regulatory recommendations on the topic of signal-to-noise ratio (S/N), and you should follow the guidance most appropriate for your situation.

Injection Precision

Using a reference standard, the peak height and area are measured for several replicate injections, sometimes for both small and large injection volumes. The absolute values for peak height and area, as well as the relative standard deviation of each value, are compared to manufacturers specifications.

TABLE I: Approximate void volumes of some column sizes in common use (2).

Column Dimensions (mm x mm i.d.)	Column (Void) Volume (mL)
250 x 4.6	2.5
150 x 4.6	1.5
150 x 3.0	0.64
150 x 2.1	0.28
50 x 4.6	0.50
30 x 4.6	0.30
15 x 4.6	0.15

Injection precision is particularly important for quantitative analysis; the better the injection precision is (that is, smaller standard deviation), the better is the ability of the method to differentiate between samples having similar analyte concentrations.

Detector Response Linearity

Typically a reference standard is injected multiple times in a range of concentrations

A Q&A

Automated On-line microSPE Clean-up of QuEChERS Extracts for Pesticides Analysis

Daniela Cavagnino
Product Marketing Manager for Gas Chromatographs and GC Autosamplers in the Applied Analytical Technologies Group
Thermo Fisher Scientific

Cristina Jacob
Product Manager of LC TriPlus Autosamplers
Thermo Fisher Scientific

Laboratories are increasingly interested in doing faster, simpler multi-residue pesticides analysis with minimal clean-up in an effort to achieve higher sample throughput. The limitation of this approach is that matrix coextractives can interfere with the chromatographic analysis and contaminate liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography-tandem mass spectrometry (GC-MS/MS) systems. The addition of a solid-phase extraction clean-up step can help reduce these issues but laboratories are reluctant to do so because of the time required for such manual procedures.

In a recent *LCGC* webcast, Daniela Cavagnino, product marketing manager for gas chromatographs and GC autosamplers in the Applied Analytical Technologies Group at Thermo Fisher Scientific, and Cristina Jacob, product manager of LC TriPlus Autosamplers™ at Thermo Fisher Scientific, discussed this issue. They explored an optimized, online, miniaturized cartridge approach that provides effective clean-up of QuEChERS extracts through unattended automated workflows. Here, they answer questions posed by the audience during the webcast.

Your presentation explored µSPE cleanup of QuEChERS extracts for on-line GC-MS/MS and LC-MS/MS analysis of pesticides. You included data for pesticides analysis in dry food matrices (e.g., rice, wheat), tea, grapes, and avocado. Have you also evaluated other classes of analytes like polar pesticides, veterinary drugs, or mycotoxins?

Jacob: We have not tested polar pesticides because we prefer to use a workflow based on metal-free ion chromatography systems rather than liquid chromatography. The clean-up of polar pesticides is extremely difficult. Many publications in the literature have reported most clean-up options evaluated are ineffective. Either the clean-up results in losses of polar analytes or if the analytes are recovered, there is minimal removal of the matrix. It is the classic clean-up conundrum. However, one off-line clean-up that is effective when used in combination with ion chromatography is the use of Thermo Scientific™ Dionex™ OnGuard™ II RP cartridges as described in Application Note 73204. Regarding veterinary drugs, our colleagues have been testing this application and the results are very promising. Hopefully, we will have more information to share in the near future. We did not evaluate mycotoxins, but I agree it is an interesting possibility and has potential.

For GC analysis, is it possible to automatically add an analyte protectant before injection?

Cavagnino: Yes, the automated workflow includes the optional step for the addition of internal standards or analyte protectants. In fact, analyte protectants are particularly useful in GC analysis of pesticides because they help to mask the active sites in the GC inlet and in the column, improving the peak shape, signal response and repeatability for target analytes, as well as reducing possible decomposition of more labile compounds. Typical analyte protectants are those compounds like sorbitol or ethyl glycerol that are capable of interacting with the active sites with their hydroxyl groups. This effect is known as “matrix-induced signal enhancement.”

SPONSORED BY

ThermoFisher SCIENTIFIC

LCGC north america

Can you explain how you prepared the standards? Did you clean up the matrix for preparation of the standards offline or online?

Jacob: In the case of GC analysis, the standards were prepared off-line and then the individual standards were cleaned up automatically, a kind of procedural standard approach, thus any losses in the cartridge are automatically corrected by the standards. The recovery and precision were excellent using this approach, which is permitted by the EU SANTE Guideline Document N° SANTE/12682/2019 for Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed.

The matrix-match approach was used in the case of the LC, where the blank matrix extract was cleaned up using µSPE and aliquots of the cleaned-up extract were spiked with the standard mixture at different concentration levels.

> If you take into account the labor cost savings, µSPE can be very competitive and convenient.

Is it possible to reuse the µSPE cartridge?

Cavagnino: This question comes up quite often, but it is not recommended to reuse the same cartridge for another sample. One key benefit of the µSPE cartridges is they are disposable, which avoids any risk of cross contamination between samples. In addition, the disposability ensures the maximum repeatability of the cleanup process. For all these reasons, it is not recommended to reuse the µSPE cartridge.

Can the system automatically prepare the standards from a bulk volume of blank sample extract and a standard solution if placed somewhere on the tray? That would save a lot of time and also reduce errors.

Jacob: Yes, this is possible and is something that we are evaluating. Again, initial results look extremely promising, and we hope to share more information very soon.

What is the cost of the cartridge compared to the cost of d-SPE sorbent?

Cavagnino: This is an important point for the user. We need to consider that the cost of the reagents for the dispersive SPE is variable according to the numbers and types of sorbents that are included in the tube or pouch. Cost also varies depending on the different countries, so it is difficult to make a direct cost comparison. On average, the cost of the µSPE cartridge and the additional vials required in the workflow is likely to be slightly higher. If you take into account the labor cost savings, however, µSPE can be very competitive and convenient. Also, µSPE has been demonstrated to be more effective, delivering high-quality results.

If required, is it possible to reinject a cleaned-up extract if there is a reason for reanalysis? In other words, is the seal in the vial good enough to prevent evaporation and or could the system automatically dilute the sample and rerun if the response in the first injection is higher than the calibration range?

Jacob: Yes, it is possible to rerun the sample. The eluate vials are well sealed, which prevents evaporation. In addition, in the case of the LC, the eluate tray can be kept at a controlled temperature. Also, it is possible to automate a dilution step by the system, if required before re-analysis of the sample. I would also like to add that although the workflow presented in this webcast does not include the dilution steps, we are currently working on another QuEChERS workflow where the dilution is included. We will share more information about it in the near future.

How long does the cleanup take?

Cavagnino: It is quite quick; it takes 10–12 minutes to complete the cleanup process. But what is more important is that the cleanup workflows are executed during the chromatographic separation of the previous sample. Thus, users will benefit from the best sample throughput because both sample preparation and analysis times are optimized.

Is it a requirement to add an internal standard in the case of GC-MS/MS?

Cavagnino: It is a good practice in pesticide residues analysis to use isotope-labeled internal standards along with matrix-matched calibration standards to compensate for possible matrix effects. In our testing of rice and wheat samples, however, we just added the triphenyl phosphate after the cleanup for GC analysis to check for the injection errors. Even though we did not use any isotopically labeled internal standards, we still achieved excellent results.

that cover the normal operating range of the detector (for example, up to 1.5 AU for a UV detector). Statistical assessment of linearity is performed using a combination of linear regression, residuals analysis, *F*-tests, and relative standard deviation of detector response for each analyte concentration, as well as ratios of signals. Linearity of detector response is critical for quantitative analysis, and has a direct impact on the accuracy of analyte concentrations reported based on use of calibration curves.

Solvent Gradient Composition

Typically, a tracer compound such as acetone is added to one of the mobile phase solvents (usually the "B solvent"), and a method is used that steps through different mixtures of two solvents, one of which contains the tracer compound that can be observed by the detector (for example, acetone absorbs well at 265 nm). For example, a method may start at 0% B, and increase in steps of 5% B until 100% B is reached. The signal due the presence of the tracer compound is used as an indirect measure of the ratio of the volumes of the A and B solvent that are combined by the pump to make the mobile phase mixture of A and B. The average detector signal at each % B level is compared to the expected value, and the short-term variation at each step may also be evaluated. Finally, some OQ routines call for the analysis of a linear gradient profile using the same tracer compound. The accuracy and repeatability of the gradient profile are critical for both qualitative and quantitative analysis, transfer of methods between instruments, and repeatability of retention times.

Suggestions for LC Systems with Mass Spectrometric (MS) Detectors

MS detectors can be particularly susceptible to problems on startup following extended periods in standby mode or shutdown, and one needs to pay particular attention to these detectors prior to restarting work with them. As there is a lot of variation in maintenance protocols for different MS manufacturers and instrument types, it is essential that you carefully follow the manufacturers guidance when considering the following steps.

First, thoroughly clean the ionization source, preferably when the instrument is not under vacuum. However, it is not necessary to vent the instrument if it is under vacuum at the time of cleaning. In any case, follow the manufacturers recommended procedure for cleaning the source. The emitter (that is, sprayer or nebulizer) should also be checked carefully prior to re-establishing flow from the LC system, as residual eluent solvents evaporate during extended storage periods, often leaving residues or even blocking the flow path entirely, and can be difficult to remove. If significant residue or an obstruction is observed, remove the nebulizer and sonicate for 10 min (first in water, and then in IPA). Be careful to suspend the nebulizer tip in the cleaning solvent in such a way that it does not contact the bottom or walls of the container, as this could damage the tip itself, and affect spray performance.

If the instrument has been vented, carefully monitor the vacuum levels in the instrument when pumping it back down, and check the vacuum levels against the manufacturers specifications.

Before using the MS for data acquisition a verification of its performance will be required. At a minimum this will include a full tune (autotune), and this is typically achieved using the manufacturer's recommended tuning compound or solution and performance criteria. If your instrument includes an on-board tuning solution that can be activated using the control software, make sure there is enough tuning solution in the reservoir prior to starting the autotune routine. The autotune routine will tune the electrostatic lenses within the instrument as well as the voltages applied to the mass filtering device in order to optimize and verify, amongst other things, mass accuracy across a wide range of values, instrument sensitivity, and response profile.

While the on-board autotune is very useful to set the detector parameters and check them against the manufacturer's performance requirements, one may also need to carry out further "whole-system" performance checks with a typical set of performance criteria involving: 1) response linearity (or response profile if it is expected to be non-linear based on previous experience); 2) injection precision; 3) carryover; 4) signal-to-noise ratio; and 5) minimum detection limits.

Summary

Given the variety of ways different laboratories were shutdown early on in the global COVID-19 outbreak, it is likely that LC users will encounter a wide variety of problems when they return to their laboratories and resume work with their instruments. In this installment of "LC Troubleshooting," we have provided suggestions specific to handling LC columns and LC systems with MS detectors when restarting work after a long time away. We have also discussed the value of running system suitability and OQ tests before starting to collect important data again. These tests will be helpful for identifying problems that may have been caused by the shutdown, so that they can be resolved before causing trouble later on.

References

(1) D.R. Stoll and T. Taylor, *LCGC North Am.* **38**(6), 320–324 (2020).

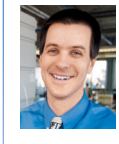
(2) R.E. Majors, *LCGC North Am.* **21**(1),19–26 (2003).

(3) J.W. Dolan, *LCGC North Am.* **22**(5), 430–435 (2004).

(4) J.W. Dolan and G. Hall, *LCGC North Am.* **20**(9), 842–848 (2002).

(5) D.R. Stoll, *LCGC North Am.* **38**(5), 261–268 (2020).

ABOUT THE COLUMN EDITOR



Dwight R. Stoll is the editor of "LC Troubleshooting." Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 60 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC's editorial advisory board. Direct correspondence to: LCGCedit@mmhgroup.com

ABOUT THE CO-AUTHOR

Tony Taylor is the Chief Science Officer of Arch Sciences Group and the Technical Director of CHROMacademy. Direct correspondence to: LCGCedit@mmhgroup.com.

COLUMN WATCH

High-Throughput and High-Efficiency Separations of Antibodies by Reversed-Phase Chromatography Using Organic Alcohols

Biomacromolecules, especially monoclonal antibodies, are complex molecular species that have a myriad of functional groups. This complexity has led to much discussion on strategies for improving efficiency and analyte recovery of chromatographic methods used to characterize these molecules. This edition of "Column Watch" will focus on the roles of temperature and organic modifier for yielding improved efficiency in analyzing biomacromolecules. It is shown that these variables can be used together to achieve higher throughput, resolution, and recovery when analyzing monoclonal antibodies and antibody-drug conjugates.

Cory E. Muraco and Hillel K. Brandes

Monoclonal antibodies (mAbs) are a promising class of biologics for the treatment of several autoimmune diseases and cancers. An additional application of mAbs, however, is when a cytotoxic payload (such as a drug) is attached to the mAb, allowing for the mAb to target a certain cell type or tissue and deliver the payload to a specific target. This combination of mAb plus cytotoxic drug, connected through an organic linker, is known as an *antibody-drug conjugate* (ADC). As of January 2020, there are 89 ADCs in the pharmaceutical pipeline (1).

One downside of mAb-based drugs, however, is that, due to their structural complexity, there is significant heterogeneity. This heterogeneity can arise due to the presence of charge variants, glycosylation variants, or phosphorylation variants; these arise by nature of the biological production process.

Several different chromatographic strategies are applied to investigate and resolve the structural and chemical variants of mAbs. Size-exclusion chromatography is used as one method to assess the aggregation of a mAb sample. Ion-exchange chromatography is a suitable method to conduct charge variant analysis. Both chromatographic modes can present issues of compatibility with electrospray ionization mass spectrometry (ESI-MS), which is routinely used for protein characterization. Hydrophobic interaction chromatography (HIC) is routinely employed for analysis and characterization of protein glycans. Reversed-phase chromatography has long been a method of choice for analyzing proteins due to its high resolution and compatibility with mass spectroscopy (MS). Reversed-phase chromatography of proteins, however, has its own issues. Of primary significance is that protein structures can be flexible in comparison to structures of small organic molecules. This fact may present a chromatographic challenge, as various structural conformations may differentially interact with the stationary phase. With proteins, peak shape in reversed-phase chromatography is generally enhanced by parameters that stabilize a single denatured state (2–4). Temperature is one parameter that can dramatically affect the tertiary and quaternary structure of proteins, and could lead to a "denatured state."

Another aspect of protein and peptide reversed-phase chromatography is that, for most applications, elution must be by utilization of a solvent strength gradient. This requirement is due to at least two reasons: 1) polypeptides are generally polyionic, and, therefore, can present problems of secondary interactions with the silica surface, potentially causing issues of peak tailing; and 2) partitioning of polypeptide analytes between the mobile and stationary phase occurs over a narrow window of solvent strengths (as compared to most small molecules), therefore exhibiting much more of an on-off adsorption phenomenon. With the requirement for gradient elution comes the requirement for column re-equilibration prior to injection of a sample. Column re-equilibration can be sped up by reducing changes to the solvation state of the silica surface, as has been shown by inclusion of low levels of small, primary alcohols in the mobile phase (5,6). How this mechanistically takes place has not been defined, but computer modeling of short, primary alcohols in binary mobile phase systems is consistent with intercalation of the alcohol into the stationary phase, with the hydroxyl hydrogen-bonding to the surface silanols or an adsorbed water layer (7). This phenomenon may have additional benefits in masking silanols, therefore improving peak shape. Scott and Simpson reported that 1-butanol can form a simple monolayer on a C18-bonded silica surface; this also fits with a model of a small, primary alcohol hydrogen bonding to the surface silanols (8).

High temperature has been shown to be necessary in achieving optimal analyte recov-

ery and peak shape of mAbs in reversed-phase chromatography (9). This fact has been confirmed to be the case, irrespective of the column used or the specific mAb sample (10). Additionally, Fekete and associates have shown that inclusion of low levels of 1-butanol reduced the temperature optimum for the reversed-phase chromatography of the mAb (9). Thus, the mechanism of any conferred benefits of inclusion of low levels of primary alcohols in the mobile phase is not entirely clear. These previously published data suggest a primary mechanism of masking of the silica surface; another possibility might be imagined to explain the effects on antibody chromatography in which the intercalated 1-butanol is oriented with the hydroxyl facing the bulk mobile phase, thus lending some polarity to the environment at the antibody-stationary phase interface. Such models might be elucidated by inspecting results with analogs of 1-butanol.

Effect of Organic Alcohol on Monoclonal Antibody Characterization

These ideas were investigated further using a commercially available mAb reference material. Initially, the goal was to at least confirm previous reports on the chromatographic effects of 1-butanol on the reversed-phase chromatography of mAbs. Two primary alcohols, 1-propanol and 1-butanol, were investigated. Figure 1 shows chromatographic results of the recovery of the mAb at varying percentages of 1-butanol at 55 °C.

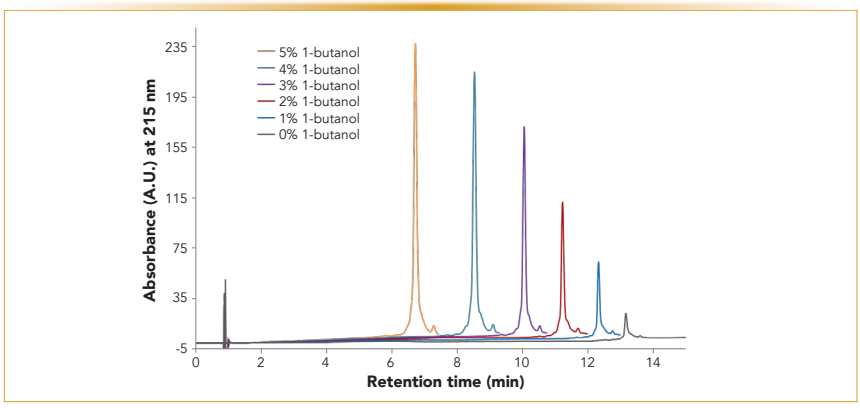


FIGURE 1: Analysis of mAb reference material by reversed-phase chromatography with varying amounts of 1-butanol. Conditions: Column: BIOshell A400 Protein C4, 10 cm x 4.6 mm i.d., 3.4-µm; Mobile Phase: [A] 70:30 0.1% TFA in water: 0.1% TFA in acetonitrile; [B] 60:40 0.1% TFA in water: 0.1% TFA in acetonitrile; [C] 70:25:5 0.1% TFA in water: 0.1% TFA in acetonitrile: 0.1% TFA in alcohol; [D] 60:35:5 0.1% TFA in water: 0.1% TFA in acetonitrile: 0.1% TFA in alcohol; Gradient: For x = 0, 20, 40, 60, 80, or 100; [(100-x)% A, 0% B, x% C, 0% D] to [0% A, (100-x% B), 0% C, x% D] in 15 min; Flow Rate: 1.0 mL/min; Column Temp.: 55 °C; Detector: UV, 215 nm; Injection: 3.0 µL; Sample: mAb reference material, 1 g/L, 0.05% TFA in water.

As noted in Figure 1, the peak area and height of the analyte increased as the concentration of 1-butanol increased. In addition, as the concentration of 1-butanol increased, one can begin to resolve impurities from the main analyte peak. Finally, the retention time of the mAb decreased with increasing 1-butanol concentration. This phenomenon was further investigated by looking at how temperature played a role in the recovery of the mAb. Figure 2 displays the results of this analysis.

As can be seen in Figure 2, it should become obvious that one of the main advantages of including 1-butanol in the mobile phase is the much lower temperature required to achieve maximum recovery of the analyte. The data, however, cannot differentiate effects due to possible mitigation against thermal degradation or impacts on the actual chromatography of the mAb. The experiment was repeated, this time with 1-propanol as the mobile phase modifier. Figure 3 shows the results of this analysis.

As shown in Figure 3, the temperature required to achieve the maximum recovery of the analyte was much higher (around 80 °C) in comparison to 1-butanol. While 1-propanol has been shown to have similar benefits as 1-butanol for keeping the silica surface solvated, and shielding surface silanols, it clearly did not provide the same chromatographic benefits in this case, as compared to 1-butanol (5,6).

Continuing the investigation, the effect of type of alcohol (primary, secondary, and so forth) on analyte recovery was examined. As noted in Figure 4, the secondary alcohol elicits good recovery of the antibody, albeit not as good as 1-butanol. It could be inferred that, due to steric effects, 2-butanol should be not as effective as primary alcohols in hydrogen-bonding with surface silanols or an adsorbed water layer. However, 2-butanol achieved higher recovery of the analyte at low to moderate temperatures than with

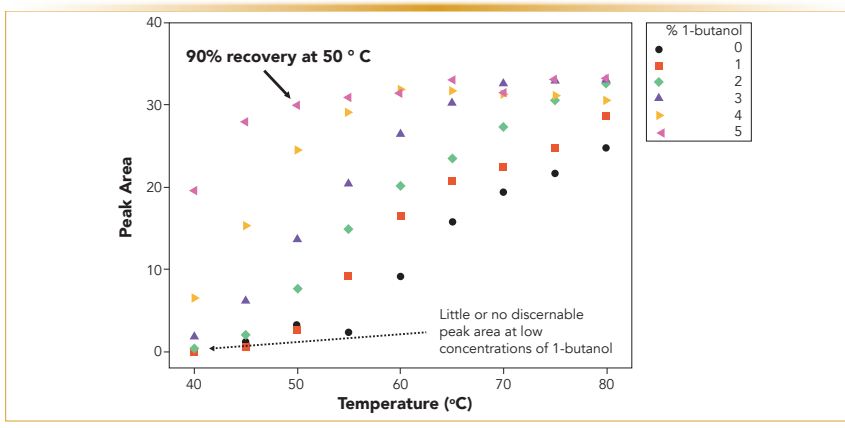


FIGURE 2: Recovery, measured by peak area, as a function of temperature and 1-butanol concentration. Note that the maximum recovery of the analyte occurs at much lower temperatures as the concentration of 1-butanol increases. Chromatographic conditions are the same as those described in Figure 1 except for temperature, which was varied as indicated in the figure.

1-propanol, suggesting that the mechanistic explanation is not as simple as hydrogen-bonding to the silica surface.

A final test was to examine the use of 1,4-butanediol. The idea is that this alcohol could possibly play a dual role of hydrogen-bonding to the silica surface, as well contributing additional polarity to the interface where protein adsorption takes place on the stationary phase. The results are shown in Figure 5.

As can be deduced from Figure 5, there appears to be no advantage in adding 1,4-butanediol to the mobile phase. Perhaps the two terminal hydroxyls render it sufficiently polar such that it no longer readily distributes within the stationary phase to provide any performance benefit to the reversed-phase chromatography. In fact, the collective results beg the question, could the difference in the effects simply be due to the solubility of the alcohol within the stationary phase?

It also suggested to the authors that it would be most interesting to do a comparative study with an identical column or particle, but with a bonded butanol phase (the terminal hydroxyl exposed to the bulk solvent). Such an experiment could shed light on this mechanistic question regarding the conferred impacts of added butanol in the mobile phase. As mentioned earlier, perhaps the hydroxyl faces the bulk mobile phase to lend polarity to the environment at the antibody-stationary phase interface.

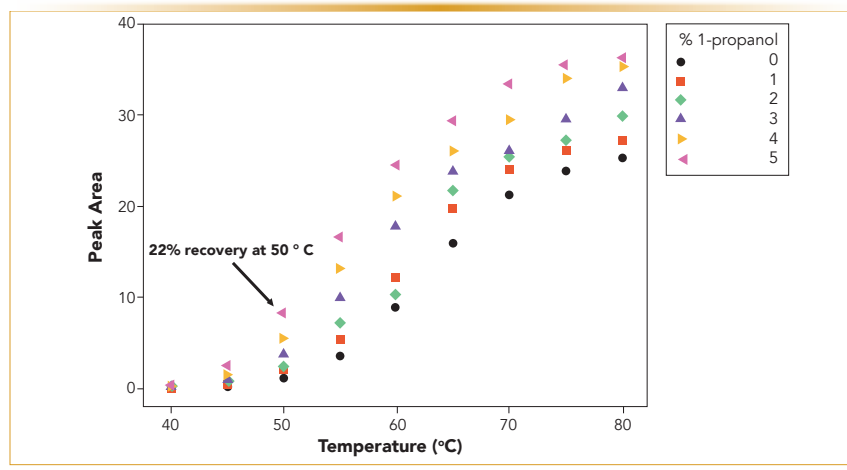


FIGURE 3: Recovery, measured by peak area, as a function of temperature and 1-propanol concentration. Note the much-reduced recovery at 50 °C with 1-propanol versus 1-butanol. Chromatographic conditions were the same as those described in Figure 1 except for temperature which was varied as indicated in the figure.

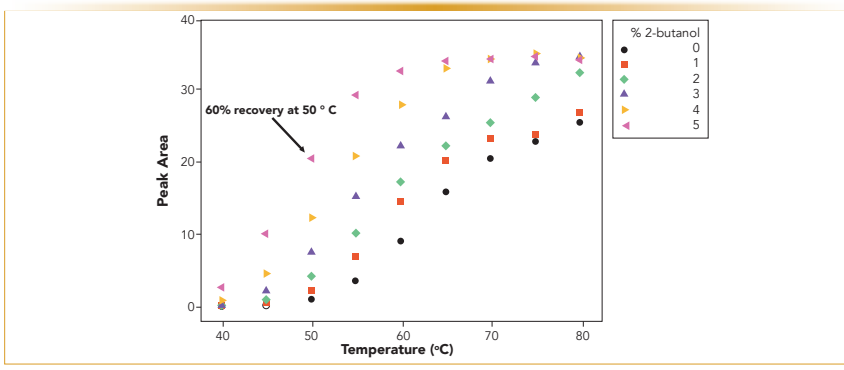


FIGURE 4: Recovery, measured by peak area, as a function of temperature and 2-butanol concentration. Chromatographic conditions are the same as those described in Figure 1 except for temperature which was varied, as indicated, in the figure.

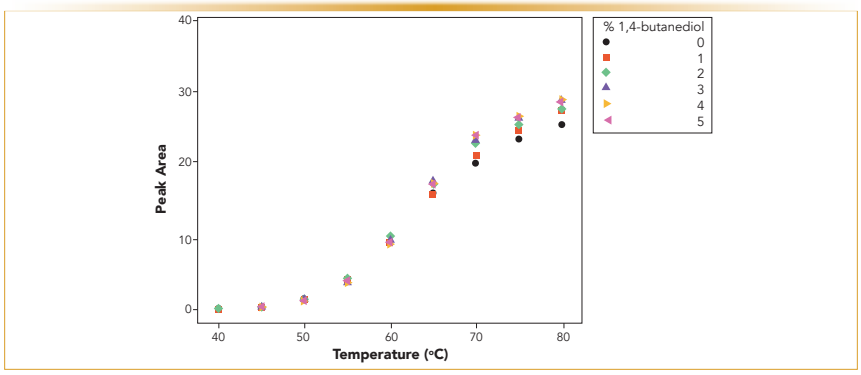


FIGURE 5: Recovery, measured by peak area, as a function of temperature and 1,4-butanediol concentration. Note that the concentration of 1,4-butanediol does not appear to influence recovery. Chromatographic conditions are the same as those described in Figure 1, except for temperature which was varied, as indicated, in the figure.

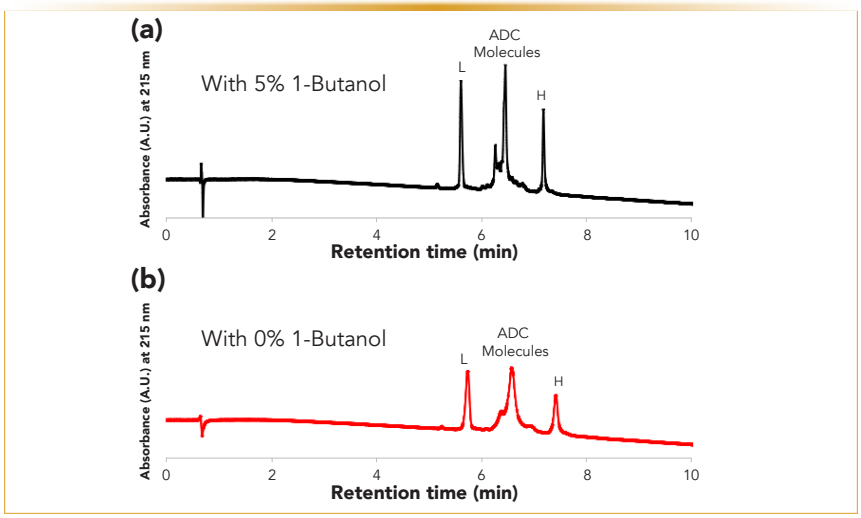


FIGURE 6: Analysis of an ADC by reversed-phase chromatography (a) with or (b) without 1-butanol. Conditions: Column: BIOshell A400 Protein C4, 10 cm x 4.6 mm I.D., 3.4-µm; Mobile Phase: [A] 0.1% DFA in water; [B] 0.1% DFA in acetonitrile; [C] 95:5 0.1% DFA in acetonitrile: 0.1% DFA in 1-butanol; Gradient: 25% B to 45% B in 10 min (no butanol method) OR 25% C to 45% C in 10 min (with butanol); Flow rate: 0.5 mL/min; Column temp.: 55 °C; Detector: UV, 215 nm; Injection: 2.5 µL; Sample: ADC SR-388, 1 mg/mL, 0.1% DFA in water.

Effects of 1-Butanol on Analysis of an ADC

In addition to all the inherent complexity associated with a mAb, characterization of an ADC introduces further complexity to the separation challenge due to the addition of the cytotoxic drug (payload) and organic linker. Creation of a cysteine-linked ADC, where the payload is attached to cysteine amino acid residues, leads to a mixture of ADC molecules with different amounts of payload attached to the mAb vehicle. Commonly seen permutations of a cysteine-linked ADC molecule include the native mAb molecule (no payload attached to the mAb), followed by ADCs with two, four, six, or eight payloads attached. In addition, impurities from the mAb production process and unbound cytotoxic drug are also present in ADC samples.

As was discussed in the previous section, incorporating 1-butanol into the mobile phase yielded improved recoveries, higher throughput, and higher efficiency than with mobile phases without 1-butanol. To see if this observation can be translated to a more complex protein molecule, 1-butanol was incorporated into the mobile phase used for the analysis of an ADC by reversed-phase chromatography. Figure 6 displays the chromatographic results of this analysis. Note the improved resolution and recovery of some of the peaks corresponding to labeled heavy and light chains of the ADC. Interestingly, there does not appear to be a reduction in retention time as was seen with the mAb analyte.

However, there is an effect on the separation as the efficiency of the method with 1-butanol was higher (as indicated by more narrow peak widths) than without the 1-butanol. An explanation for this result may possibly be tied with the tertiary structure of the protein. The structural integrity of cysteine-linked ADCs is less than in native mAbs, due to some of the cysteines used in disulfide bonds between the light and heavy chains of the mAb being reduced to add the payload molecules. Since the method conditions would permit denaturing of the protein, less energy would be required to cause denaturation. Denatured proteins expose more hydrophobic amino acids to the stationary phase, and thus could lead to peak broadening due to a stronger

interaction between the stationary phase and the protein. Perhaps the 1-butanol is solvating the protein molecule and minimizing protein denaturation (or minimizing the number of denatured forms of the protein) leading to sharper peaks. Additional investigations are needed to fully test these possible explanations.

Conclusions

Despite these seemingly conflicting results, what emerges is a picture in which the role of the alcohol in conferring a chromatographic benefit to the reversed-phase chromatography of mAbs and ADCs (or maybe most any other immunoglobulin G molecule) is not as simple as has been suggested from other chemical and computer modeling studies—that something other than masking the silica surface is at play here. Perhaps there is a separate effect on the thermal stability of the mAb in this common mobile phase. Nevertheless, as shown in Figures 1 and 2, the addition of 1-butanol to the mobile phase can elicit excellent recovery of an antibody standard at far lower temperatures than in its absence, and can serve as a general method for reversed-phase chromatography of antibodies, or perhaps any other proteins that exhibit poor peak shape at moderate temperatures.

References

(1) A. Keown, *Biospace* https://www.biospace.com/article/with-3-approvals-in-2019-companies-see-great-future-for-adc-therapies/ Accessed June 6, 2020.

(2) K.D. Nugent, W.G. Burton, T.K. Slattery, B.F. Johnson, and L.R. Snyder, *J. Chromatogr. A* **443,** 381–397 (1988).

(3) K. Benedek, S. Dong, and B.L. Karger, *J. Chromatogr.* **317,** 227–243 (1984).

(4) X.M. Lu, K. Benedek, and B.L. Karger, *J. Chromatogr. A* **359,** 19–29 (1986).

(5) L.A. Cole and J.G. Dorsey, *Anal. Chem.* **62,** 16–21 (1990).

(6) A.P. Schellinger, D.R. Stoll, and P W. Carr, *J. Chromatogr. A* **1192**(1), 54–61 (2008).

(7) J.L. Rafferty, J. Ling Zhang, I. Siepmann, and M.R. Schure, *Anal. Chem.* **79**(17), 6551–6558 (2007).

(8) R.P.W. Scott and C.F. Simpson, *Faraday Symp. Chem. Soc.* **15,** 69–82 (1980).

(9) S. Fekete, S. Rudaz, J.-L. Veuthey, and D. Guillarme, *J. Sep. Sci.* **35**(22), 3113–3123 (2012).

(10) R. Eksteen, D. Bell, and H. Brandes, "Using Temperature to Improve Peak Shape of Hydrophobic Proteins in Reversed-Phase HPLC." HPLC Conference Poster (2014).

ABOUT THE AUTHORS

Cory E. Muraco is the Global Franchise Product Manager, Liquid Chromatography Technology, at MilliporeSigma in Bellefonte, Pennsylvania. Cory completed his graduate studies in 2013 at Youngstown State University where he focused on using analytical chemistry to deduce the role of protein primary and tertiary structure on protein oxidation. Cory joined Merck KGaA in 2013, where he assumed the role of R&D Scientist working in the Chemical Standards division developing certified reference materials (CRMs) for multiple industries. In 2015, Cory joined the HPLC Application and R&D division of Merck KGaA where he researched and developed new column technology and applications. In 2019, Cory was promoted to his current role, where he is responsible for developing and executing the marketing and R&D strategy surrounding new HPLC column technology.

Hillel K. Brandes is an Analytical Technology Specialist with MilliporeSigma in Salt lake City, Utah. In his prior role, he was a Principal R&D Scientist, focusing on HPLC product development and application support.

ABOUT THE COLUMN EDITOR

David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for LCGC and is the Editor for "Column Watch." Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: LCGCedit@mmhgroup.com

A Q&A

A New Approach to Light Scattering Detection for the Characterization of Proteins and Polymers

Dr. Sébastien Rouzeau
Product Manager of GPC/SEC Systems and Columns
Tosoh Bioscience LLC

Traditional light scattering detectors have their own innate limitations. However, an updated instrument design can help alleviate measurement setbacks. *LCGC* spoke with Dr. Sébastien Rouzeau, Product Manager for Gel Permeation / Size Exclusion Chromatography (GPC/SEC) Systems and Columns at Tosoh Bioscience, about a new approach to light scattering and how it can benefit protein and polymer characterization.

LCGC: What is light scattering?

Rouzeau: The basis of light scattering can be described using a situation familiar to most people: driving in the fog at night. A car's headlights illuminate droplets in the fog, which re-emits light in all directions. As a result, we can see the fog from wherever we stand, even from behind the car.

In analytical instrumentation, a laser beam is used to illuminate a vial or a cell that contains a sample in solution. The theory and equations of light scattering were established back in the 19th century by Lord Rayleigh and are still used today by all light scattering instruments. According to the Rayleigh equation, the intensity of scattered light is related to the molecular weight of the sample. However, that intensity is not equal in all directions; it changes with the angle of observation, and this scattering pattern is related to the size of the sample. So essentially, light scattering relates to both molecular weight and size of the molecules.

LCGC: Why has light scattering become so popular for polymer and protein characterization?

Rouzeau: The light scattering phenomenon is primarily used to determine the true or absolute molecular weight of a sample. And in some cases, size information can be obtained in the form of the sample's radius of gyration (R_g), which is truly related to the shape and the structure of the molecule.

Light scattering detection is most often coupled with SEC so that different species in the sample are separated by size first before being measured by light scattering in order to obtain their molecular weight and size. This is known as SEC-multiangle light scattering (SEC-MALS).

Typically for protein applications, monomers, oligomers, or higher aggregates and fragments can be easily identified by their molecular weight both in their native or denatured state, regardless of their shape or whether or not they are globular. So in that regard, SEC-MALS differs from high-performance liquid chromatography (HPLC) with mass spectrometry in that the analysis will denature the protein during the process. With liquid chromatography–mass spectrometry (LC–MS), it is actually impossible to identify the protein oligomers that are present in the sample.

For polymer characterization, light scattering is mostly used to look into the molecular weight distribution of polymers without referring to standards, which is a major advantage over conventional SEC. And it is also possible to obtain valuable information on the structure or the confirmation of the sample (e.g., branching).

LCGC: How do traditional light scattering detectors work?

Rouzeau: Essentially, light scattering instruments either measure as close to the incident beam as possible to obtain molecular weight, or measure at multiple angles to observe how the intensity changes and extrapolate back to 0°

SPONSORED BY

TOSOH BIOSCIENCE

LC|GC north america

to estimate molecular weight. This is the well-known MALS approach.

Typically, a MALS detector would consist of multiple photodiodes that are arranged around a circular or cylindrical flow cell that is usually made of quartz glass. These photodiodes collect the scattered light simultaneously and continuously while the sample is flowing through the cell. All the signals are recorded, and the intensity of the scattered light is plotted against the angle of observation to obtain molecular weight and R_g via an extrapolation model.

LCGC: Are there any limitations to MALS measurements?

Rouzeau: Like any analytical technique, MALS has its limitations, some of which are because of the instrumentation itself. Traditional MALS detectors have one major and purely technical limitation due to their lowest and highest measurable angles. This is a result of the geometry of the flow cell as well as because of the limited space available to physically fit the detectors around it. The lowest and highest positions that can be measured are relatively far from 0° and 180°.

Additionally, because of the proximity of the incident laser beam and stray light, those angles are subject to higher background noise. This affects the quality of the signal to the point where the highest and lowest angles sometimes become unusable and must be discarded for data processing.

One might ask why this is an issue considering the presence of multiple angles. The issue arises from the fact that the lowest angle is extremely critical to obtain an accurate molecular weight from the extrapolation. The highest angle is also very important when it comes to detecting very small differences in scattering for small samples in size. This is basically the reason why the lower limit for R_g measurements by a traditional MALS detector is approximately 10–12 nm, because below that, no angular dissymmetry can be accurately detected.

LCGC: How does the LenS3 MALS from Tosoh address such limitations?

Rouzeau: The LenS3 MALS was specifically designed to address the previously mentioned limitations. In this device, the traditional flow cell is replaced with a completely new concept of "flow channel". This new channel is an elongated flow path with a dual-cone shape that is machined directly in a black polymeric material. The block is sealed with two lenses—one at each end—which will let the laser beam go through the channel to illuminate the sample while ensuring the collection of the scattered light at both an ultralow and ultrahigh angle. There is also a third angle that collects light at 90° to form a three-angle measurement.

This new design features two major benefits over traditional flow cells. The first is that the black polymeric material eliminates any stray light that could interfere with the scattered light and generate noise. And second, the elongated flow path maximizes the interaction with the sample and significantly increases the amount of light that can be collected.

In addition to the new flow channel, the LenS3 also uses a green laser that produces about three times higher intensity of scattered light than a regular red laser, which increases the signal. And lastly, the device's optical bench eliminates the incident beam at the low and high angle position so that both provide a clean signal.

Overall, what sets this instrument apart from others is twofold: the position of the angles—with a true usable low angle for direct molecular weight measurement, combined with an extreme high angle for R_g measurement—and the new flow channel and optics that maximize the signal-to-noise to increase the overall sensitivity of the detector.

LCGC: From a practical standpoint, what does all this mean for SEC-MALS users?

Rouzeau: The instrument's higher sensitivity brings about various benefits for users. For example, the LenS3 requires smaller quantities to characterize samples, which is critical for protein applications where sample quantity can be a major limiting factor when it comes to physico-chemical characterization.

Another notable benefit is the ability to detect the presence of aggregates and fragments of an antibody down to a much lower level when compared with conventional detectors. For polymers, a higher sensitivity means samples can be measured with a very low molecular weight, a very low dn/dc, and a broad distribution, or all of the above.

Additionally, the high sensitivity combined with the position of the extreme angles opens a whole new field of application for small macromolecules with an R_g below 10 nm because the angular dissymmetry can be measured for such small sizes, which was not possible before.

LCGC: Can you give us a few concrete examples of what users will gain from this technology?

Rouzeau: For instance, because the LenS3 is fully compatible with UHPLC systems, we injected decreasing concentrations and volumes of a monoclonal antibody (mAb) in our applications laboratory. We were able to detect the mAb down to as low as only 2 ng of sample loading, which is extremely low.

We also achieved the complete molecular weight profiling of an unpurified and purified oligonucleotide despite the relatively low molecular weight and sample loading used. For polymers, we successfully determined the R_g of a series of polystyrene standards down to only 2 nm in radius. To our surprise, the R_g values agreed perfectly with the R_g measured by small angle X-ray scattering reported in the literature (1).

Reference

1. F. Abe, Y. Fumiaki, T. Yoshizaki, and H. Yamakawa, Macromolecules, 26(8), 1884-1890 (1994)

GC CONNECTIONS

Beat the Heat: Cold Injections in Gas Chromatography

In capillary gas chromatography (GC), we heat the most common inlets (split and splitless) to vaporize the samples and transfer them to the column. While heating and vaporizing the sample in the inlet presents the most convenient means for transferring it to the capillary column head, several problems that result in sample losses and quantitative reproducibility problems occur. We will briefly discuss the problems with sample heating in classical split and splitless inlets, followed by an introduction to cold inlets, which have been available since the 1970s, but are underutilized. Next, we will discuss modes of cold sample introduction, including cold on-column, cold split and splitless, cold splitless with solvent venting, and large volume injection. We will close with key points and considerations, and argue that users should consider cold injection as a purchase option or upgrade for any gas chromatograph.

Nicholas H. Snow

This installment of "GC Connections" follows up and builds on the basic discussion presented in LCGC North America *in May, 2018 (1).*

Many gas chromatographers would agree that inlets and sample introduction are the most challenging aspects of capillary gas chromatography, and we see that most gas chromatographers are still using the same inlets (split and splitless) and techniques, especially injecting with a 10 µL syringe, developed in the 1950s and 1960s (2). We make a fundamental, yet incorrect, assumption that the inlet must be heated in order to rapidly vaporize the sample, mix it with the carrier gas, and transfer it to the column. This assumption led to the development of both the split and splitless techniques in common use today. It also leads to several of the main causes of reproducibility problems and the need for troubleshooting in gas chromatography.

Figure 1, a photograph first published in the May, 2018 installment of "GC Connections," illustrates some of the key problems. It shows the result when a few milliliters of water (like a sample solvent) is transferred using a spoon to a hot cast iron skillet on a stove (like a heated inlet liner). The picture was taken a few seconds after the water was introduced to the skillet. There are several take-home points about hot sample injections from this photo.

- No matter how hot and massive the inlet, the solvent does not evaporate instantly. This is a major cause of *discrimination*, or the preferential loss of some analytes.
- The layer of gas between the solvent and the surface also causes some of the analytes to be exposed directly to the hot surface, causing analyte decomposition.
- In splitless inlets, the sample requires up to a minute or more to transfer to the column, leading to band broadening.
- Note the discoloration of the water on the surface. The solvent can actually reintroduce contaminants from a dirty inlet. This causes contamination or *ghost peaks*.
- While not shown here, some of the water remained in the spoon, analogous to a syringe. The syringe needle is also a leading cause of discrimination and apparent sample losses.

Although they are convenient to use, heated split and splitless inlets can clearly cause many problems and challenges for method development and analysis by gas chromatography (GC). This is enough of a challenge that the book by Grob, the classic text on split and splitless inlets, is nearly 500 pages long (3)!

Figure 2 shows diagrams of the pressure, temperature, and flows in classical split (Figure 2a) and splitless (Figure 2b) injections. These show the relative simplicity and convenience of the two techniques, and provide the main reason why there has been resistance in the community to others. In split injections (Figure 2a), once the split ratio (total flow) and column flow rate are set, none of the parameters change during the run. The inlet temperature is constant and hot throughout the injection, and the analytical run (often 250 °C) and the purge flow through the inlet liner is constant and high (often on the order of 100 mL/min). To save carrier gas, the gas saver feature on modern inlets can be used to reduce the purge flow once the injection process is complete. In splitless injections (Figure 2b), the purge flow through the inlet liner is low at the beginning of the run and at the injection. It stays low for a specified time, and then is rapidly raised by switching a solenoid valve. During the "purge-off" time, vapors in the inlet liner are transferred slowly to the column, with no other outlet. The oven temperature starts low (this is a requirement in splitless injections) and a temperature program is then performed. As in split injections, the purge flow can be reduced later in the run to save carrier gas. The added parameter of the purge valve starting "off" and coming "on" after a specified time is still relatively straightforward to optimize.

A solution to the problems with split and splitless injections has existed since the late 1970s with the invention of programmed temperature vaporization inlets, termed PTV by Vogt (4,5). Major instrument vendors have offered PTV and cool-on column inlets as an add-on to new gas chromatographs, but they have never become popular. In the rest of this installment, we will discuss the fundamentals of programmed temperature injections using PTV and cool on-column inlets, and follow up with key points and discussion that should lead you to consider cold inlets with your next GC.

Most of the instrument vendors and a few specialty vendors offer solutions for cold inlets in GC. Some vendors have their own terminology for the classical PTV inlet, some sticking with PTV, while others use "multi-mode inlet" or "cooled injection system." Nearly all vendors use cool or cold on-column to describe their on-column inlet offerings. With varying capabilities, they all allow a user to inject a sample into a cooled inlet, and then rapidly heat the inlet to transfer the sample to the column. Following the injection and heated transfer, and before the next injection, the inlet is cooled by a jet of air, carbon dioxide or liquid nitrogen.

FIGURE 1: A few mL of water on a hot cast-iron skillet shows how injected samples evaporate in a heated inlet.

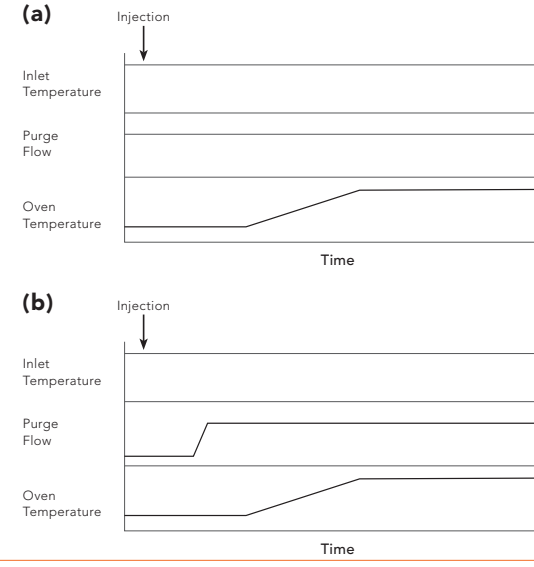


FIGURE 2: Diagrams of temperatures and flows in (a) classical split, and (b) splitless injections.

Cool On-Column

The simplest and least expensive means for cold injections is to add a cool on-column inlet to the GC. This inlet, as the name implies, is cooled during injection, and allows sample to be injected directly onto the column. Following injection, the inlet (which includes the column head) is heated along with the column via temperature programming. The cool on-column inlet is the best of all inlets for ensuring that the entire sample that leaves the syringe needle reaches the column. However, it is the worst of all inlets for keeping the column clean; the entire sample, including matrix components that cause glass sleeves in other inlets to need replacement, reaches the column. Cool on-column is especially useful for "clean" samples in non-polar solvents, or solvents whose polarity closely matches that of the column. A retention gap, a short (typically 5 m or so) length of uncoated fused-silica tubing is often used between the inlet and analytical column, to provide a polar surface to allow the use of polar solvents, and an easily replaced guard column to protect the analytical column. If used with columns smaller than 0.53 mm inside diameter, cool on-column requires a special tapered syringe needle, which is both more costly and more fragile than traditional syringes. Large volume injections can be performed with cool on-column using a solvent vapor exit (6). Several vendors commercialized this process in the 1990s, so solvent vapor exit upgrade solutions may be available for cool on-column equipped systems. Check with the vendor.

Programmed Temperature Vaporization (PTV)

The most important difference between a PTV inlet and a typical split or splitless inlet is a much lower thermal mass to allow rapid heating and cooling. A glass liner, much like the glass liners used in split and splitless inlets, is used, as is a septum or septum-less head. Some PTV inlets have both a split vent, like the split or splitless inlet, and a solvent vent, used for purging larger volumes of injected solvent commonly used in large volume injections. They also have a septum purge if a septum is used. A PTV inlet can be installed at instrument purchase, or it can be retrofitted. The inlets offered by some specialty vendors are designed to be easily retrofitted onto existing systems, as are inlets offered by the major vendors.

The PTV inlet is often termed a "multi-mode" inlet because it can perform several injection and sampling techniques on a single platform, using a single inlet. In addition to the traditional hot split and splitless tech-

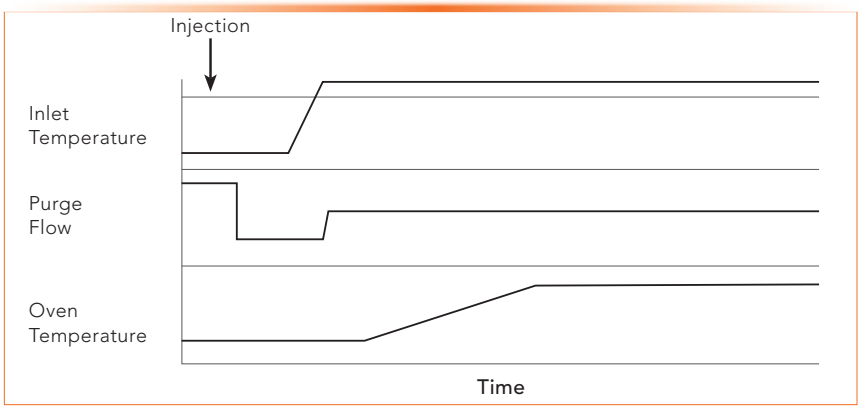


FIGURE 3: Diagram of temperatures and flows in a PTV large volume injection.

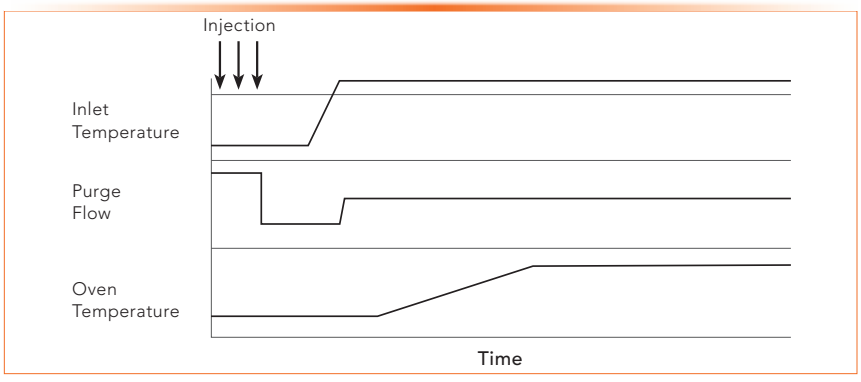


FIGURE 4: Diagram of temperatures and flows in a PTV large volume injection process with multiple injections to generate a very large volume injection.

niques familiar to all of us, it can perform cold split and splitless, and cold splitless with solvent venting (large volume injection). Detailed summaries of inlet capabilities and a thorough description of large volume injection can be found online (7,8). As the heart of a comprehensive online sampling system and GC, a PTV inlet can serve as an interface for thermal desorption, pyrolysis, headspace, SPME and sorptive extractions, and even online HPLC-GC (9).

The original PTV inlets were little more than split or splitless inlets with the large thermal block removed and heating tape installed. This simple fundamental lies at the heart of PTV inlets today. When selecting a PTV inlet, there are a few instrumental options to consider. First, a PTV inlet can be ordered with or mounted on a new GC; it can also be retrofitted onto an existing system. The most important factors are how the inlet is heated and cooled. Is the heating rate and maximum temperature sufficient for the needed application? A typical PTV inlet can be heated up to temperatures in excess of 500 °C. The final temperature should be high enough to desorb all analytes of interest into the column. The high temperatures, often well above the stated column maximum temperature, do not damage the column, as the highest temperatures are not maintained for more than a few seconds before the inlet is cooled. The second choice is about cooling. Air cooling is simpler and less expensive, but requires more time than cryogenic cooling. Cryogenic cooling also allows rapid cooling to below room temperature for more effective use of highly volatile solvents or analytes.

PTV inlets can perform the same roles as traditional split and splitless inlets, hence the common term *multi-mode* now used to describe them. The lower thermal mass of the inlet causes a compromise in heating for the traditional hot split and splitless techniques. It is well known that, when a liquid evaporates, heat from the surroundings is needed to force the evaporation, causing the surroundings to cool. Limiting cooling during evaporation is the reason for the high thermal mass (heavy metal block) that is used for the housing of traditional inlets. Even with this high thermal mass, there is some cooling as the solvent evaporates, especially for polar solvents such as methanol, with high enthalpies of vaporization. The cooling is uncontrolled and usually non-reproducible, and is one of the causes of inlet discrimination and mysterious reproducibility problems in traditional split and splitless injections. The PTV inlet does not have the large thermal mass, so in some cases it may be subject to additional cooling as the solvent evaporates, when traditional hot split and splitless injections are performed.

To limit this problem, and control solvent evaporation, split and splitless injections can be performed cold. The sample is injected with the inlet cooled, and the inlet is then heated rapidly to control the evaporation process. Cold injections also reduce or usually eliminate syringe needle discrimination, often seen in hot split and splitless injections. Since a traditional split or splitless inlet comes standard on new GCs, and since you are already using traditional split and splitless injections, a new GC with a PTV can easily be mounted with the traditional split or splitless inlet as well, and parallel studies can be performed to validate the multi-mode inlet.

Have you ever wanted to simply inject a larger volume of sample? Perhaps the most important limitation of classical splitless injection is the limitation of injection volume to 1 (or a few) µL. The ability to inject larger sample volumes, up to hundreds of microliters or even milliliters, is the most important aspect of using a PTV inlet. Injecting the sample cold, into a packed inlet liner at a temperature below the boiling point of the solvent, venting most of the solvent to waste and then rapidly heating the inlet to transfer the remaining sample and solvent to the column allows the inlet to accept much larger sample volumes.

Large volume injection is a lot like splitless on steroids. Figure 3 shows a simplified schematic of the process. Prior to the injection, the inlet is cooled and the purge flow through the inlet liner is high. The oven is also cooled in preparation for temperature programming. A large volume, say 100 µL, is injected using a standard syringe and an autoinjector into an inlet liner packed with

an adsorbent to provide a large surface area, and possibly some selectivity. The open purge valve removes about 95 to 99% of the solvent. Analytes remain sorbed on the packing, and are concentrated in the solvent that remains in the inlet. Timing this purging period to remove most, but not all, of the solvent is one of the most crucial steps in large volume injection. Inlet vendors provide calculators to assist with determining the correct timing. Once the solvent is purged, the purge valve is closed and the inlet is rapidly heated, to transfer the remaining solvent and now-concentrated sample into the column. Like splitless, the purge vent is then opened again to clean the inlet and the inlet is cooled following the run. In short, the inlet acts as a sample concentrator and splitless inlet all in one.

If a PTV inlet can handle a single injection of 100 µL, then why not inject more? It is actually possible to inject samples up to milliliters (yes, mL) using a PTV inlet by taking advantage of the packed liner, the cooled inlet and the vapor pressure of the solvent. A diagram of the process is shown in Figure 4. Consider each injected sample as an aliquot. Prior to the run, the inlet and column oven are cooled, and the purge flow through the inlet liner is high. With the inlet cooled and in its solvent vent mode, the solvent evaporates out the vent once the sample is injected. Once most of the solvent has evaporated, another aliquot can be injected. The solvent evaporates, leaving the analytes adsorbed in the packed liner. If this process is repeated 10 times using a 100 µL syringe, a 1000 µL sample has been injected. After the final injection and solvent venting, the solvent vent valve is closed, flow goes through the column, the inlet is heated, and the analytes are transferred to the column as in a splitless injection. In method development, the timing of the injections with the solvent vent flow rate must be carefully optimized. If all of the solvent is evaporated at any point, the vapor pressure of the analytes and the high solvent vent flow rate may cause some of them to desorb out through the solvent vent and be lost. This capability allows a PTV inlet to be the transfer device in online LC-GC (9).

A cooled inlet with large volume injection capability, combined with a robotic autosampler (a "rail"), offers many intriguing possibilities for automated online sample preparation and injection. Since the inlet is cooled, exchanging liners and performing maintenance is much faster than with heated inlets. Simply remove the top nut, and replace the septum and liner. No extra cooling and subsequent heating and equilibration, which are time consuming, are required. This is especially useful for laboratories that run "dirty" samples requiring inlet maintenance often.

Further, the top nut and seal can be configured to allow the inlet to be opened, and the inlet liner exchanged automatically. This is termed *automatic liner exchange*, and it opens up several opportunities. A PTV inlet with automatic liner exchange can double as a pyrolysis or thermal desorption unit. Samples can be loaded directly into the inlet liners either on a rail-type autosampler, or on a separate instrument. Automated liner exchange is then used to load each inlet liner into the inlet, one at a time, for analysis. The PTV inlet is then heated to desorb analytes into the column. Most PTV inlets can rapidly heat to 500 °C or more, providing some capability for pyrolysis as well as thermal desorption.

We close with some questions and discussion points that you should discuss with your vendor when considering a cold inlet.

- How are they equipped to support and provide method development assistance? Especially with PTV inlets, there are method development considerations that most gas chromatographers have not learned. Who will be providing technical support and how available will they be?
- Is this their own inlet, or is it provided by a third party? If a third party vendor provides the inlet, you may wish to work with the third party directly for purchasing the inlet and associated sampling devices, such as a rail auto-sampler.
- It is common to pair a PTV inlet with a rail-type autosampler. Be sure to discuss technical support and maintenance for the rail and the inlet in addition to the GC. Most likely, these will be additional operating costs.

In my opinion, if a cold inlet is not a "must have," it is a "must consider" for any GC purchase today, and is worth considering for a retrofit. By reducing the possibilities for discrimination and other problems associated with traditional heated inlets, the extra upfront cost and learning time is more than offset by the greater capabilities and reduced troubleshooting.

References

(1) N.H. Snow, *LCGC North Am.* **36**(7), 448–454. (2018). http://www.chromatographyonline.com/split-splitless-and-beyond-getting-most-your-inlet-0

(2) J.V. Hinshaw, *LCGC Europe*, **20**(12), 626–631 (2002). http://www.chromatographyonline.com/split-decision-1

(3) K. Grob, *Split and Splitless Injection for Quantitative Gas Chromatography: Concepts, Processes, Practical Guidelines, Sources of Error* (John Wiley & Sons, New York, New York, 2007)

(4) W. Vogt, K. Jacob, and H. W. Obwexer, *J. Chromatogr. A* **174,** 437–439 (1979).

(5) W, Vogt, K. Jacob, A-B. Ohnesorge, and H.W. Obwexer, *J. Chromatogr. A* **186,** 197–205 (1979).

(6) K. Grob, H-G. Schmarr, and A. Mosandl, *J. High Resolut. Chromatogr.* (now *Journal of Separation Science*) **12**(6) 375–382 (1989).

(7) "Sample Introduction in Capillary Gas Chromatography" GL Sciences (accessed June, 2020). https://www.glsciences.eu/optic/gc-injections-manual.pdf

(8) "Large Volume Injection in Capillary Gas Chromatography" GL Sciences (accessed June, 2020). https://www.glsciences.eu/optic/LVI-training-manual.pdf

(9) A. Apedo and N.H. Snow, *LCGC North Am.* **37**(11), 802–809 (2019). http://www.chromatographyonline.com/online-lc-gc-ultimate-gc-connection-0

ABOUT THE AUTHOR

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

FOCUS ON FOOD ANALYSIS

Investigations into the Migration of Packaging Components into Food

In recent years, concern has arisen about the potential for compounds in food packaging, such as bisphenyl A (BPA), to migrate into food. Rafael Paseiro-Cerrato of the U.S. Food and Drug Administration (FDA) has conducted studies to investigate this concern. Specifically, those studies have addressed various types of can coatings in both short-term and long-term studies, as well as the question of whether short-term study protocols accurately simulate migration during longer-term storage. He used a range of analytical techniques, including gas chromatography (GC), high performance liquid chromatography (HPLC), and ultrahigh pressure liquid chromatography (UHPLC) combined with a diode array detector (DAD), charged aerosol detection (CAD), mass spectrometry (MS), and high-resolution MS (HRMS). He also investigated direct analysis in real time–MS (DART MS). Here, he talks to us about that work.

Laura Bush

You have done a lot of research on the migration of polymeric compounds from food packaging, particularly can coatings, into food. You note that cross-linked polyester resins are being introduced on the market as alternatives to epoxy resins, which typically contain BPA as coatings for metal food cans. In one study, you looked at methods for identifying unknown compounds from polyester can coatings that could potentially migrate into food (1). Going into the study, how much a priori information did you have about the potential compounds in the liners? Are food manufacturers not required to disclose the compounds they use in can liners?

In the United States, any food contact material (FCM) must be approved by the Food and Drug Administration (FDA) before being placed into the market. Manufacturers must comply with the current legislation regarding pre-market approval of food contact materials. In the case of food can coatings approved prior to 2000, they are mainly represented by listings in *Title 21 Code of Federal Regulations (CFR) Parts 170–199* as the result of agency actions on food additive petitions. In the CFR, lists of substances authorized to be used in the manufacturing of FCM are displayed, and it is available for public access. Since 2000, the way to introduce a FCM into the market is through the food contact notification (FCN) process. Manufacturers must file information to the FDA related to the FCM, including administrative, chemical, environmental, and toxicological information on substances. Migration of oligomers, residuals, and impurities must be determined, and estimated exposure is evaluated against toxicology information. A database that lists effective premarket notifications can be found on the FDA website (www.fda.gov). This information, as well as the information that can be accessed in the published literature, was used as a starting point for the identification studies.

How many different can coatings did you analyze? Do you think you have identified any compounds of major health concern? How large was the potential pool of compounds?

First, I would like to point out that the idea behind this study was to identify potential migrants and to track them in migration studies. In the real world, cans are processed and shipped to distributors. They may be sold immediately, or they may be warehoused for varying periods of time. In a modern distribution system, the storage time is very short. But some unpopular products can linger for months, or longer, in warehouses. FDA has designed a migration test to allow notifiers to develop migration data in a short (10 d) time period in the laboratory.

The goal of these experiments was to evaluate if current FDA migration tests accurately predict migration results into food and food simulants after a long-term storage. To perform these migration experiments, we needed first to identify potential migrants in the coatings, and, once identified, track them through migration experiments. In this first manuscript, we analyzed and identified potential migrants in polyester coatings. But we extended the food cans study to other coatings, including epoxy, acrylic-phenolic, and vinyl coatings. Related to the second question, as explained above, because any FCM needs to be approved by the FDA before being placed into the market, we

did not expect any of these migrants to be a health concern. On the other hand, for the identification of potential migrants, we used internal data bases that contains hundreds of compounds.

Some of the samples in your study were subjected to a retort step. For those not familiar with food processing, could you briefly explain the retort process, and when it is used? In your estimation, how closely does the retort step in the analytical test mimic real-world conditions, and how did you arrive at that opinion?

A retort step, as we think of it, is a high-temperature sterilization step (typically around 121 °C for up to 2 h) to ensure the safety of canned foods, particularly those of low acidity. Dangerous microorganisms are eliminated, and the shelf-life of the canned food is extended. To mimic this common practice in food cans, to simulate real world conditions in our experiments, some of the employed cans were retorted in a commercial food retorting machine. In addition, the FDA guidelines for industry (see www.fda.gov) specifies that if cans are going to be subjected to a retort step, a thermal treatment at 121 °C for 2 h, followed by a migration test for 10 d at 40 °C, should be performed. Therefore, by retorting the food cans, we are simulating real food can processing conditions as well as complying with the FDA guidelines.

Following extraction with acetonitrile, you used a variety of analytical techniques, including gas chromatography–mass spectrometry (GC–MS), high performance liquid chromatography (HPLC) with a diode array detector (DAD) and MS (HPLC–DAD/MS), HPLC with DAD and charged aerosol detection (CAD), and ultrahigh-pressure LC with high-resolution MS (UHPLC–HRMS). Why did you need to use so many techniques, and how did you determine the optimum technique for each of your experiments?

The aim of this experiment was to identify a large variety of potential migrants in the coatings. Migrants can be very diverse and can have different molecular weights (MW), polarities, belong to different chemical families (acids and esters, for example), and have different chemical properties (such as, for example, their boiling point). Therefore, migrants may have different responses depending on the analytical techniques. To capture a wide range of potential migrants present in the coating, the most logical way is to use a large battery of analytical techniques. We used GC for detection of volatile and semi-volatile compounds, and LC for non-volatile compounds. The various detectors captured migrants with different physicochemical properties. For example, DADs detects substances with chromophore groups, while charged-aerosols-detectors (CADs) may detect substances without chromophore groups. By using both detectors, we ensure that we are capturing both compounds with chromophore and those with no chromophore groups. In addition, each detector gives relevant information about migrants. For example, the HRMS supplies accurate mass of compounds, while the DAD gives spectrometric information. If during the migrant's identification we obtain the accurate mass of an oligomer with a good ppm agreement (≤ 5 ppm) and an appropriate retention time, the technique gives enough confidence that this is likely the proposed compound. However, polyester oligomers may be formed with different isomeric monomers, such as isophthalic and terephthalic acid, which have the same exact mass, but completely different absorption in the ultraviolet (UV) spectra. The additional spectral data supplies valuable information on which one is the principal monomer present in that oligomer. The DAD detector will give this type of information, and we will know if the tentatively identified oligomers are mainly based on isophthalic or terephthalic acid based on the UV spectra. The use of several detectors complements each other. Data obtained using several analytical techniques also guides the researcher on which would be the best strategy to determine migrants for future analysis.

What compounds did you identify, and what did you find in terms of which techniques were best to identify which compounds?

Most of the identified compounds were polyester oligomers, but we also identified commonly used monomers used in polyester coatings. Each employed technique has relevance in the identification process, but probably the technique that supplied the most relevant information for the identification was, in my opinion, the UHPLC-HRMS, since it allows to separate compounds in the analytical column, and gives accurate mass and supply information about the fragments of a migrant. In any identification process, it is important to state that compounds remain tentatively identified until analytical standards are obtained and analyzed to experimentally prove that the identification was correct. In this study, to give confidence in the identification results, we were able to obtain standards of some tentatively identified migrants that supported that our identification was appropriate.

You also tested whether rapid, direct detection of the identified oligomers was practical, using direct analysis in real time–MS (DART–MS). What did you find? Could future studies be limited to a small set of techniques?

Using DART–MS, we tentatively identified 23 compounds directly from the coating (no sample preparation) in just a few minutes and with minimal method optimization. The DART–MS analysis was as simple as placing the coating between the DART source and the HRMS for a few seconds. In the end, we obtained in minutes very similar results as in the UHPLC-HRMS, where the analysis may take days. This means that the employment of DART–MS represents an improvement in the sample throughput for identification of substances, and it is a technique that has a lot of potential for food packaging applications.

Do you think you were not able to detect any important compounds?

When analyzing a matrix with unknown chemical composition, no results are 100% definitive. We could potentially miss information during the process. When developing strategies for the identification of

Front & Center

Benefits of High-Resolution Mass Spectrometry in a Forensic Toxicology Laboratory

Analytical techniques in forensic toxicology are constantly challenged by the need to detect an increasing number of substances and their metabolites, from recognized illicit and prescription drugs to emerging novel psychoactive substances (or so-called designer drugs). The ability to routinely screen for a large number and wide range of compounds has, therefore, become an essential need for forensic toxicology laboratories. In addition, while many pharmaceuticals, controlled substances, and drugs of abuse previously encountered in criminal case work are well-characterized, investigators face the regular emergence of new agents and analogs about which little, if anything, is known. High-resolution mass spectrometry (HRMS) is now providing forensic laboratories with comprehensive screening capabilities for an increased number and variety of substances as well as the capacity to identify unknown compounds.

Michael Toms, supervising criminalist at the Sacramento District Attorney's Office Laboratory of Forensic Services (Sacramento, CA), explains what type of testing takes place in their forensic toxicology laboratory: "Our services are primarily for criminal casework, the majority involving driving under the influence of drugs (DUID), although we also handle drug-facilitated sexual assault (DFSA), probation and coroner work. The number of drugs we need to look for is increasing all the time and we are in the final stages of moving from enzyme immunoassay (EIA) for the initial drug screening to high-resolution mass spectrometry. Most importantly, for our DUID caseload high-resolution mass spectrometry will enable us to screen each sample for more drugs than is possible with the EIA system. We will also be able to create libraries specific to other types of casework where we know the particular drugs of interest."

The toxicology laboratory processes 4,000–5,000 DUID cases annually. However, samples with a high alcohol content are not usually screened for drugs unless it is specifically requested, a distinct drawback that limits access to the full understanding of impairment and something that high-resolution MS screening addresses. Says Toms, "If the officer indicates it's a DUI for alcohol, then we typically don't go forward with the toxicology. However, newer instrumentation would allow us to test those samples as well so we can see if drivers who are consuming alcohol are also consuming drugs."

Current EIA workflows in Toms's laboratory only allow screening of each sample for around 50 drugs covering the common classes including opiates, amphetamines, benzodiazepines, cocaine and cannabinoids. The EIA will typically indicate the presence of a class of drugs, with confirmatory testing carried out using two tandem-quadrupole MS systems (Waters Xevo TQ-S), which are used to confirm and quantify specific drugs from the classes indicated by the EIA. The toxicology laboratory has been running this protocol very successfully for several years. The National Safety Council's Alcohol, Drugs, and Impairment Division's recommendations for the toxicological investigation of suspected alcohol- and drug-impaired driving cases and motor vehicle fatalities (1) request considerably more than this. At present, if a new drug becomes prevalent in the county, something must drop out of the EIA testing to make way for it. "Previously, we would run barbiturates, for example, which although now less widespread, are still prescribed. But, we took that plate out of the assay system in order to test for Ambien [zolpidem], something we started seeing more," says Toms. "With high-resolution MS screening, we won't need to forego other drug classes to add in new ones as we can simply keep on increasing."

Facing these limitations with the EIA approach, the laboratory had been looking for some time to move to a high-resolution MS workflow for drug screening. "Our experience with our Xevo TQ-S systems has been extremely good, and we worked closely with our vendor to fully examine our screening needs before deciding on the Waters Xevo G2-XS QTof Quadrupole Time-of-Flight MS equipped with UNIFI software, which will completely replace EIA for this application," confirms Toms. "All the elements in this system are integrated and it's robust, which is really important to us, as is the phenomenal support that the company provides. When you have one instrument dedicated to a specific high-workload task, service responsiveness is essential."

The Xevo G2-XS QTof Quadrupole Time-of-Flight MS

In combination with the UNIFI Scientific Information System, the Xevo G2-XS QTof provides a versatile high-resolution MS system for forensic toxicology applications that enables both targeted and non-targeted screening, and, therefore, enabling identification of "expected" and "unexpected" compounds. The Xevo G2-XS QTof system acquires data in a non-targeted mode utilizing patented MSe technology. No ions or compounds are excluded as they would be in a traditional MRM acquisition mode using tandem quadrupole-based mass spectrometer systems. Accurate mass is acquired on both precursor and fragment ions in addition to the retention time. The acquired data is then searched against a well-characterized library of over 1600 compounds. Identification is based on the comparison of acquired precursor ion, fragment ion, retention time and isotope ratio information. Compounds not present in the library can be further investigated using a number of tools built into UNIFI including: common fragment search, MOL files, and elucidation using elemental composition derivation. The need to develop and optimize chromatography, MS and data processing methods is eliminated as complete acquisition and processing methods are supplied.

The UNIFI platform combines data acquisition, processing, visualization and reporting in a secure compliant environment. It includes powerful investigative tools for the identification of novel substances or unexpected compounds. For example, the elemental composition of the component, or components, under investigation is automatically calculated and searched against on-line libraries and databases using both precursor and fragment ion data. *In-silico* fragmentation is also utilized to help with the investigation and identification of compounds for which standards are not available or are difficult to obtain. By using molecular structure information, UNIFI automatically uses theoretical fragments and assigns plausible structures to the observed ions. These structures can then be added to the library for future investigations.

"Using high resolution MS, we can identify more drugs in less time, and get a great deal more information."

A striking illustration of the Xevo G2-XS QTof system's ability to dig deeper into a sample occurred before the laboratory's adoption of the system during the investigation of a death suspected to be the result of suicide by aconitine poisoning. Aconitine is an alkaloid toxin produced by the *Aconitum napellus* plant (common names include monkshood and wolfs-bane). Tandem quadrupole MS analysis of the residue in a bottle associated with the case, and suspected to contain the poison resulted in the generation of a peak that could not be elucidated. Toms explains, "We were able to get one pretty solid peak, but even though we had some standards, it did not match any of them. We asked Waters to help and their analysis of the residue using the Xevo G2-XS QTof revealed a series of 13 aconitine-type norditerpenoid alkaloid compounds that were presumptively identified through a Chemspider (Royal Society of Chemistry) database search. The QTof system also identified six of these compounds in a blood sample from the subject, together with 12 other drugs that were positively identified by the UNIFI toxicology database. Using the high-resolution MS data, the UNIFI process makes it possible to go out and elucidate compounds by searching against on-line libraries and databases."

In addition to elucidation tools, both fragment ion and precursor ion data can be added to your library and are always available for retrospective analysis, something that may be especially important when new substances emerge. "With the UNIFI platform, we can detect a broader range of drugs and also it allows us to go back into cases and search for new compounds. We can implement those compounds into our libraries and then re-search or re-scan previous cases," Toms says.

According to Toms, the biggest benefit to bringing high-resolution MS into his toxicology laboratory is identifying, in routine screening, those samples that contain specific drugs, not simply a particular class of drugs, enabling analysts to quickly eliminate negatives. Secondary confirmation testing and quantitation for particular drugs using the Xevo TQ-S tandem quadrupole systems will continue in order to meet good forensic practice requirements to analyze samples with two different methods. Commenting on the impact of the new system on the laboratory and its resources, Toms says, "Using a high-resolution MS system, we can identify more drugs in less time. We can also use off time to run the analyses, giving us 24-hour operation, something that is not possible with EIA because someone needs to be around to deal with any problems and to manage labile reagents. If I need to make sure the new MS system is still running, I can log into it from home and potentially address any issues that might arise. So, we can run more samples in less time and get a great deal more information than before."

Reference

1. BK Logan, et al., *J. Anal. Toxicol.* 42 (2) 63-68 (2018).

unknowns, there are different approaches to try to capture most of the compounds of interest. In this study, we used different techniques for identification (GC, LC), several databases, instrument settings that can capture large variety of molecules (extended chromatographic gradients), and detectors in full scan mode. To obtain confidence in the identification, and to prove that the employed identification methods work, we used a wide variety of standards with different MW, polarity, and chemical groups. We typically use a QC mixture to qualitatively calibrate the instruments (for example, a Grob mixture) to help ensure we get all compounds through the instrument and to the detector. However, that does not mean that we are detecting all compounds in the extracts. Migrants maybe extracted at a low concentration, have less sensitivity in a specific detector, or may simply not be included in any database. However, the identified compounds in this study were expected, and with the identified migrants, we were able to perform the long-term migration studies that were the main goal of this investigation.

In a subsequent study, you evaluated long-term migration testing from polyester can coatings into food simulants (2). In that study, you examined the short-term protocols for such tests that are intended to mimic processing and long-term storage conditions. Why did you undertake this study in this way?

Current migration tests are intended to model what happens over those long storage times. After the retort step, migration tests usually go up to 10 d at a lower temperature. However, can coatings may be in contact with food for several years (approximately 2 to 5 years). Therefore, we wanted to evaluate if the current testing adequately simulates what may happen in food cans over the years, to ensure that food cans are tested appropriately.

What were the food simulants you used, and why did you use simulants instead of real food samples? What are the challenges or caveats in extrapolating from your method to real food samples?

We used five types of food simulants: water, 3% acetic acid, 10% ethanol, 50% ethanol, and isooctane. These simulants either mimic or exaggerate migration that could occur in a wide range of foods, from aqueous to fatty foods. Food simulants are approved to be used under several national legislations, including the United States. Food simulants are usually simple solvent mixtures that facilitate the analysis of migrants (analyzing food is quite challenging), and tend to overestimate the migration that would occur in real foods. Depending on the type food intended to be in contact with a food contact material, there are specifically recommended food simulants. For aqueous foods, for example, a recommended food simulant is 10% ethanol. When extrapolating the data obtained in the food simulants to food, we will generally have worst case scenario estimates of how much mass transfer occurs into the real food. In addition, for this study, migration will supply relevant information on what may happen after a long-term exposure when the coating is in contact with food.

How did you determine what compounds to study? How did you handle the lack of standards for many of the oligomers studied?

For the monomers of interest (isophthalic acid, terephthalic acid, and nadic acid), we had standards available, and therefore they were selected for the analysis. For the oligomers, we did not. Selection of compounds with no available standards was based on their different polarities, structures, and concentrations in the samples. To monitor these substances throughout the experiment, we used some readily available oligomer standards that belonged to the same family of polyester oligomers as proxies. This approach has been used in the past by other authors. By using these available standards, we could track the relative changes in intensity during the migration experiment. In addition, the use of standards facilitates the method validation and gives confidence in the instrument performance.

What approach did you take to sample pretreatment?

We used a sample concentrator and an C18 SPE to achieve appropriate sensitivity for the analysis. In the case of monomers, I would point out the need of adding formic acid to the water simulant before loading the SPE C18 cartridge. This acidic condition allows the monomers to be retained in the cartridge, and then be eluted using acetonitrile. For some oligomer analysis, food simulants were injected in the HPLC without any sample pretreatment. This is a good example of the advantages of using food simulants instead of real food.

In this study, you monitored the migration of monomers and identified oligomers of polyester can coatings into food simulants during short-term and long-term migration experiments, from one day to roughly 1.5 years (515 d). How did you choose the time frame of the migration periods? What would happen after several years of storage?

Food cans are designed to have a shelf life between 2 to 5 years. Considering that traditional migration test consists in real time thermal processing followed by 10 d at 40 °C, we decided a that a migration test at 40 °C for 1.5 years monitoring multiple time points from 1 d to 1.5 years could model migration over long-term storage times.

Regarding the second question, the experimental data obtained in the study up to 1.5 years suggest that increases in concentration may occur beyond the 10 d at 40 °C during long-term migration experiment, particularly in ethanol-based food simulants.

What did you find in terms of the migration of the compounds into the food simulants? What do these results indicate about the accuracy of short-term studies?

As specified in the previous question, increases in concentration may occur during the long-term experiment in food simulants. However, even in the worst-case migration scenario, the monomer concentrations in the simulants were well below the limits of concerns. This means that, even

though the migration concentrations do not represent a concern, the short-term studies may underestimate the exposure of some compounds. That is why the experimental data obtained from this study suggest that changes in concentration may occur beyond the 10-d test. Therefore, migration protocols may need to be reviewed or modified to accurately predict migration after a long-term storage.

You also conducted a similar study analyzing short-term and long-term migration from epoxy resins and acrylic-phenolic coatings (3). Did the coatings addressed in this study present any analytical challenges different from the previous study of polyester coatings?

The analytical approach for the analysis of the coatings was similar. This is, in part, because food simulants simplify the analysis when compared with real food. In the method validation results, when comparing recoveries and stability in the food simulants, it can be observed that epoxy derivatives are more stable, and recoveries are better and more robust. These results are consistent with the chemistry of the analyzed compounds. This gives useful information about the challenges of analyzing different can coatings.

In both of these studies, did you investigate not only compounds present in the coatings, but also byproducts that might be formed by interaction of the coatings and the food simulants or real food samples?

During both studies, we tracked several migrants in the simulants over the experiment. Comparison of the migration tests and an acetonitrile extract of the coating showed that the migrant concentration in the extract was lower for certain compounds than in the simulants. This was particularly true for polyesters, but also for acrylic-phenolic coatings. In the case of epoxies, we found two compounds, probably non-epoxy derivatives, that also fit to this pattern. It was hypothesized that hydrolysis of certain compounds could occur during the migration experiments. In the case of polyesters, we observed similar trends in the stability study using available standards, which support this hypothesis. There is another explanation for this experimental observation, which is related with the simulant interacting with the coatings.

What did you find in this study in terms of the migration of the various compounds? Were there significant differences in the migration into the different types of food simulants?

We observed that in the water food simulant after the retort steps, the migrant concentration remained stable over the long migration experiments. In the case of 50% ethanol food simulant using non-retorted cans, the migrant concentration increased beyond the 10-d test until it reached a plateau and then remained stable. We have hypothesized that migration test at higher temperatures could give better estimates of migration. In terms of concentrations, they were higher in 50% ethanol as expected. This is because mass transfer rates of these compounds are usually higher in less polar food and food simulants.

Do you have any serious concerns about what you found?

No. As obtained in our previous studies related to can coatings, obtained results show that, even in the worst migration scenario, concentrations in the food simulants were below the limits of concern.

Based on the results of the study, how well do standard short-term study protocols simulate real conditions over longer-term storage?

Results obtained in this study align with our previous migration studies on can coatings, which suggest there might be a need for revision of the long-term storage simulation protocols to more accurately represent what would occur after actual long-term storage. Based on the result of these simulant studies, we are continuing the investigation by performing accelerated migration tests in food simulants, and studying the influence of long-term storage in real food in order to guide decisions on what migration test modifications might be necessary.

You followed up on your long-term migration studies of can coatings with an assessment of accelerated migration testing (4). What was the aim of this study? What did you find in this study?

The aim of this study was to evaluate if accelerated migration testing conditions (60 °C for shorter time periods) could simulate migration we observed in previous long-term migration studies in can coatings at 40 °C for 1.5 years, because it is not practical for those hoping to submit Food Contact Notifications to routinely conduct long term migration tests.

To perform the experiment, we placed the previously analyzed coatings, including vinyl, polyester, epoxy-resins, and acrylic-phenolic coatings, in contact with food simulants. We conducted a migration test from 4 h to 30 d at 60 °C. We sampled at multiple time points during the experiment. The accelerated migration testing condition in food simulants showed that migration at 60 °C for shorter times (days) produced similar results to the long-term study (up to 1.5 years) at 40 °C. This suggests how we might adjust our simulation protocol sometime in the future.

In another study, you examined the migration of three compounds from polyester food cans into actual food samples (5). How did you determine the compounds to study, and what food samples to test?

In this study, we analyzed food cans that can be purchased in the U.S. market. We selected food cans lined with polyester coatings. We wanted to collect data on migrant concentrations in food. In addition, we also wanted to use this study to investigate the influence storage time on migrant concentrations. To perform the experiment, we selected two of the previously tentatively identified oligomers and monomers used in the manufacture of polyester that was found in the food simulant migration studies. We selected a wide variety of canned foods, such as coconut milk, chicken noodle soup, and mushrooms, among others.

• Continued on Page 421

Analysis of Organophosphorus Pesticides by HPLC Using Magnetic SPE with Nitrogen-Doped Reduced Graphene Oxide/Fe_3O_4 Nanocomposite as the Adsorbent

In this study, a method was developed for extraction and preconcentration of trace amounts of organophosphorus pesticides (OPPs), including fenitrothion, chlorpyrifos-methyl, and chlorpyrifos in environmental water. The method uses a new magnetic solid-phase extraction technique, followed by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Nitrogen-doped reduced graphene oxide-iron oxide (Fe_3O_4) nanocomposite, used as the adsorbent of organophosphorus pesticides, was successfully prepared using an easy hydrolysis process. The nanocomposite was synthesized and characterized by X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FT-IR) spectroscopy, and X-ray diffraction (XRD). The effects of type and ratio of eluents, amount of the graphene oxide (GO)/Fe_3O_4 nanocomposite, pH, ionic strength, equilibrium time, and ultrasound time on the quantitative recoveries of fenitrothion, chlorpyrifos-methyl, and chlorpyrifos were investigated. On the basis of the best condition, the recoveries of the target analytes in real water samples were between 84.40% and 105.05%. The relative standard deviations varied from 1.21% to 4.22%. Finally, the method was successfully applied to the determination of three organophosphorus pesticides in real environmental water samples.

Yi Gao and Qingqing Pan

Organophosphorus pesticides (OPPs) have been widely used for preventing or decreasing damage caused by pests, weeds, and plant disease, and are very important for high-yield production in agriculture (1,2). However, as a result of extensive use, these OPPs enter the environmental water system by various sources, and, as a result, both human and animal populations are exposed to OPPs through drinking water. The most harmful outcome, however, may be the formation of mutagenic compounds during conventional oxidation processes (3). Therefore, it is necessary to develop an effective method for monitoring OPPs in real environmental water, which is useful for ensuring environmental and health safety.

The determination of OPPs has been carried out with a variety of techniques, such as gas chromatography with a flame photometric detector (GC-FPD) (4,5), liquid chromatography–mass spectrometry (LC-MS) (6), gas chromatography–mass spectrometry (GC-MS) (7), and gas chromatography with nitrogen–phosphorus detector (GC-NPD) (8). Even though most OPPs are analyzed by GC, high performance liquid chromatography (HPLC) is preferred to GC for analysis of OPPs because of the thermal lability of these compounds. In addition, HPLC is one of the most sensitive and selective analytical methods for the determination of organic pollutants in environmental water.

However, there are challenges in using HPLC for such analyses, because OPPs in environmental water samples, such as tap water, underground water, farmland water, lake water, and river water, are often present at low concentrations, and these matrices are considered to be very complex. Therefore, for HPLC analysis of OPPs in environmental water samples, an appropriate sample preparation technique is required to isolate and enrich the target analytes (9–11). To address this challenge, various sample preparation methods for analysis of OPPs have been researched, such as liquid–liquid extraction (LLE) (12), ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) (13), liquid–liquid microextraction based on solidification of floating organic droplets (DLLME-SFO) (14), and other traditional sample pretreatment methods.

In recent years, there has been much interest in the study of the magnetic materials, such as iron oxide (Fe_3O_4) nanoparticles, which have been widely used for enrichment of organic pollutants in water, owing to various advantages, including their stability, good separation characteristics, availability, and avoidance of secondary contamination of samples (15–19). However, Fe_3O_4 nanoparticles, have a tendency to agglomerate into larger particles because of the magnetic tendencies of this compound (20–24). The most effective technique is to load Fe_3O_4 onto supporting materials, such as activated carbon or graphene. There are many researchers who load Fe_3O_4 onto graphene to analyze organic contaminants in water (21,25,26). However, in recent studies, the structure of the activated carbon was changed after treatment by strong oxidation (18,27). In another study, therefore, a new magnetic material, a nitrogen-doped reduced graphene oxide-Fe_3O_4 nanocomposite (N-RGO/Fe_3O_4), which was based on Fe_3O_4, was synthesized using an easy method. N-RGO/Fe_3O_4 has high adsorption capacity and strong catalytic ability for removing OPPs at low concentrations from environmental water, and the N-RGO incorporated with magnetic Fe_3O_4 nanoparticles is able to effectively overcome the challenge of the separation of adsorbent from aqueous solution (28).

TABLE I: Regression equations, linearity, the detection limits, and repeatability of the developed HPLC method for the analysis of water

Compound	Intercepts	Linear Range (µg/mL)	R^2	LOD (µg/mL)	RSD (n = 5, %)
Fenitrothion	Y = 4015.98+9.80*10^6X	0.6–600	0.9993	0.04	2.21
Chlorpyrifos	Y = 10708.56+1.71*10^7X	0.6–600	0.9995	0.05	1.53
Chlorpyrifos	Y = 17788.43+3.2*10^6X	0.8–800	0.9999	0.06	2.90

TABLE II: Results of determination and recoveries of real water spiked with three OPPs (n = 5)

Analyte	Spiked (mg/L)	Laboratory Water			Lake Water		
		Found (mg/L)	RECOVERY (%)	RSD (%)	Found (mg/L)	RECOVERY (%)	RSD (%)
Fenitrothion	0	ND	–	–	ND	–	–
	5	4.32	86.40	2.18	4.22	84.40	3.72
	10	9.38	93.80	2.06	9.12	91.20	2.29
	20	18.19	90.05	1.80	18.01	90.05	2.10
Chlorpyrifos	0	ND	–	–	ND	–	–
	5	4.66	93.20	4.22	5.12	102.40	3.25
	10	9.98	99.80	2.87	9.87	98.70	2.90
	20	20.11	100.60	1.81	19.21	96.05	2.01
Chlorpyrifos methyl	0	ND	–	–	ND	ND	–
	5	5.11	102.20	2.45	4.79	95.80	2.19
	10	10.08	100.80	3.21	10.32	103.20	3.91
	20	19.21	96.05	1.21	21.01	105.05	2.62

ND: Not detected.

However, N-RGO/Fe_3O_4 has long been used for electrochemical super capacitors and electrochemical catalysis (29–32). The aim of the present study, therefore, is to establish a new method of magnetic solid-phase extraction (MSPE) for enriching and separating OPPs in water samples. This is the first time that the novel N-RGO/Fe_3O_4 has been used as a solid-phase extraction agent for the enrichment of OPPs including fenitrothion, chlorpyrifos methyl, and chlorpyrifos in real water samples. This separation process can be performed directly in crude samples containing suspended solid materials without the need for filtration or additional centrifugation, which makes separation faster and easier. Furthermore, the recoveries of OPPs and reusability of the N-RGO/Fe_3O_4 were also evaluated. This method using sample pretreatment with magnetic SPE followed by HPLC analysis offers several advantages, including good precision and recoveries, low cost, and good reproducibility. Given these advantages, this new method may be broadly useful in many other fields.

Materials and Methods

Chemicals

Fenitrothion (purity >98%), chlorpyrifos methyl (purity >98%), chlorpyrifos (purity >98%), GO (purity >98%, layers <3), $FeSO_4 \cdot 7H_2O$, NaCl, acetic acid, and $NH_3 \cdot H_2O$ were obtained from Aladdin Ltd. The reagents used for the elution, including methanol, ethanol, acetone, acetonitrile, and ethyl acetate, were purchased from Taixin Ltd. Deionized water was provided using a laboratory system. All chemicals were used as received,

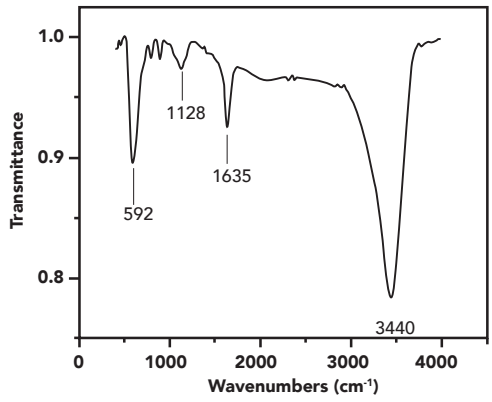


FIGURE 1: FT-IR spectrum of N-RGO/Fe_3O_4.

without further purification. A stock solution containing fenitrothion, chlorpyrifos methyl, and chlorpyrifos at 100, 430, and 500 mg/L concentration, respectively, was prepared in methanol, and stored at 4 °C.

Instruments

An LC-20AT series HPLC system equipped with a solvent delivery pump, an SPD-20A UV-vis detector (Shimadzu), and an LC solution workstation were used for the analyses. An HY-5 mechanical shaker (Jintan Etong Electric Corp.) was used in this work. An SZ-2 system (Shanghai Lu West Analytical Instruments) was used to prepare double deionized water. A high-speed centrifuge was employed to centrifuge the sample solutions (Model 800).

Preparation of N-RGO/Fe_3O_4

The N-RGO was prepared by adding 260 mg graphene oxide (GO), 100 mL water, and 4 mL aqueous ammonia into a 250 mL round-bottom flask, and then heating at 180 °C for 24 h, followed by adding 5.2 mL of $NH_3 \cdot H_2O$ with sonication for 7 min to thoroughly mix the solution. Finally, 8 mL of 0.35 g/mL freshly prepared $FeSO_4 \cdot 7H_2O$ was added to the solution, and heated in a water bath at 85 °C for 6 h to form N-RGO/Fe_3O_4. After being cooled to room temperature, negative-pressure filtration was used, and then N-RGO/Fe_3O_4 solids were washed with deionized water to neutralize them. Finally, N-RGO/Fe_3O_4 solids were placed in an oven to dry, and the dry solids were placed in storage for the next phase of the experiment.

HPLC Conditions

The HPLC separation was performed on a C18 column (150 mm × 4.6 mm, 5 µm, Beijing Jingkerida Technology Co., Ltd.) with equivalent elution using methanol and water at a rate of 1 mL/min and detection at 285 nm. The composition of equivalent elution was 85% methanol and water (0.1% acetic acid). The injection volume was 20 µL, and the column temperature was 35 °C.

Sample Preparation

We added 30 mg of N-RGO/Fe_3O_4 into 150 mL water to which was added 0.075 mL fenitrothion, 0.015 mL chlorpyrifos methyl, and 0.015ml chlorpyrifos, with a concentration of 5.0 mg/L for each analyte. The mixture was sonicated for 1 min to disperse the graphene, and then the conical flask was shaken on the platform of an orbital incubator for 30 min for adsorption equilibrium. Subsequently, an external magnet was placed on the bottom of the tube. After the solid phase was aggregated, the water phase was discarded. Simultaneously, the collected sorbents adsorbing the target analytes were eluted with 0.5 mL methanol and 0.5 mL ethyl acetate to desorb the analytes, which was sonicated for 5 min. Separation of solid and liquid phases was the same as above. The eluted solution was collected, then dried under a stream of nitrogen at 55 °C, and dissolved with 1 mL methanol. Finally, after filtration through 0.22 µm membrane, 20 µL of the solution was analyzed by HPLC.

Results and Discussion

Characterization of N-RGO/Fe_3O_4

In this study, the N-RGO/Fe_3O_4 was synthesized and characterized by Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and X-ray photoelectron spectroscopy (XPS).

FT-IR was used to identify the presence of functional groups and chemical bonds in material. Figure 1 shows the FT-IR spectra of N-RGO/Fe_3O_4 powders. The intense band at 592 cm^{-1} is assigned to the Fe-O bonds stretching of the magnetite, and the intense band at 3440 cm^{-1} is attributed to stretching of the amino group, which can provide the evidence to demonstrate that amino group were successfully grafted onto the surface of the RGO/Fe_3O_4 during preparation. The band at 1635 and 1128 cm^{-1} could be assigned to the aromatic skeleton C=C and C-C stretching vibration of the autoxidized graphitic domains and stretching vibrations of the epoxy, further confirming the above results.

XRD is a common technology to determine the crystal structure of the superparamagnetic nanoparticles. The XRD pattern of the N-RGO/Fe_3O_4 is depicted in Figure 2. It shows diffraction peaks at 2θ = 18.10°, 30.13°, 35.69°, 36.36°, 43.04°, 53.51°, 57.07°, and 62.64°, which correspond to crystal indexes of (220), (311), (400), (222), (422), (511), and (440), respectively (JCPDS no. 19–0629) (30,33). For N-RGO/Fe_3O_4, the disappearance of the diffraction peaks corresponding to GO (24.6°) and N-RGO (43.1°) is attributed to inhibition of the restacking of graphene layers by the crystal growth of Fe_3O_4, leading to decreasing integrity of graphene oxide crystal structure (28).

XPS was employed to further investigate the chemical composition of N-RGO/Fe_3O_4. As shown in Figure 3a, the XPS wide scan spectra of N-RGO/Fe_3O_4 clearly exhibited that the magnetic material mainly consisted of Fe, C, N, and O. Figure 3a also shows that the observation of Fe 2p3/2 and Fe 2p1/2 signals at 711 and 725 eV arose from the magnetic Fe_3O_4 core of prepared material, and from Figure 3a, and binding energy of N 1s was about 400 eV, which

was attributed to the amino group. Thus, the presence of the peak for N 1s suggested the formation of amino group on surface of Fe_3O_4. Figures 3b and 3c show the high-resolution XPS of C 1s and O 1s. As can be seen from C 1s, the appearance of peaks at the binding energies of 284.7, 286.4, and 288.8 eV were corresponding to C-C in aromatic rings, C-OH, and C=O, respectively, which confirmed that RGO was successfully immobilized on N-Fe_3O_4 by acylation reaction. For O 1s, the typical peak at 530.3 and 531.8 eV were corresponding to O-H and C=O, respectively, which suggest there were different oxygen-containing groups. Thus, these indicated the successful preparation of N-RGO/Fe_3O_4.

Optimized MSPE Procedures

In the present study, a 150 mL sample to which was added 0.075 mL fenitrothion, 0.015 mL chlorpyrifos methyl, and 0.015 mL chlorpyrifos for each analyte, was used to study the extraction performance of OPPs under different experimental conditions. During optimization, the experiments were analyzed them at least in duplicate.

Selection of Elution Solvents and Ratios

In this study, four elution solvents, including methanol, acetone, acetonitrile, and ethyl acetate, were used. Methanol has a better elution effect on chlorpyrifos and chlorpyrifos methyl than acetone, acetonitrile, or ethyl acetate. However, in addition to ethyl acetate, other elution solvents have little elution capacity for fenitrothion. This may be accounted for by their better solvation capabilities for the target analytes. Based on the above considerations, a mixed solvent of methanol and ethyl acetate was selected as the elution solvent.

The rate of the elution solvent was also an important factor affecting the extraction and recoveries efficiency. To achieve good extraction and recoveries for the target analytes, different ratios of methanol and ethyl acetate (for example, $V_{methanol}$: $V_{ethyl\ acetate}$=5:1; 3:1; 1:1; 1:3; and 1:5, respectively) were compared at these study. As shown in Figure 4a, the results showed that the

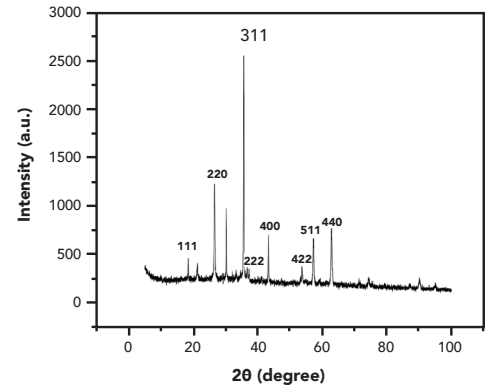


FIGURE 2: XRD spectrum of N-RGO/Fe_3O_4.

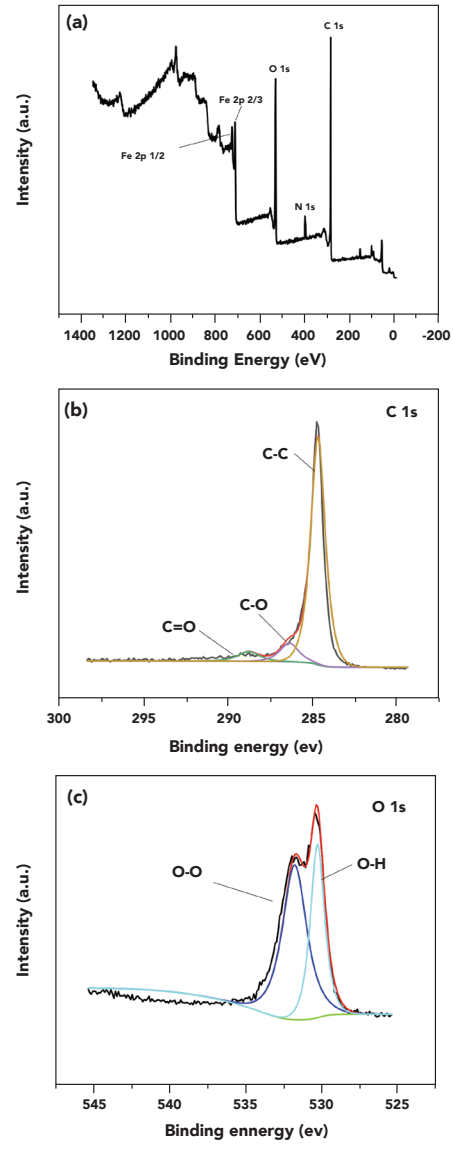


FIGURE 3: (a)–(c) Representative XPS spectra of N-RGO/Fe_3O_4.

A Q&A

Vivaspin®: Optimizing Sample Separation and Concentration Using Ultrafiltration with Chromatography

Adam Green
Product Manager
Sartorius

Select the right membrane material and molecular weight cutoffs for your target molecule.

Successful bulk separation of target and non-target molecules using ultrafiltration methods can be critical to ensuring optimal target isolation during liquid chromatography or affinity chromatography. An added benefit to ultrafiltration is the concentration of dilute samples, which ensures that required target quantities are present in the volumes needed for chromatographic systems. In addition to this, an ultrafiltration step post-chromatography can be used to polish, buffer exchange, and set a predefined concentration against a set volume. *LCGC* recently spoke with Adam Green, product manager for Sartorius, about the company's Vivaspin® range of ultrafilters and how it can be used for these purposes.

LCGC: Can you explain why ultrafiltration is important in the chromatography workflow?

Green: Ultrafiltration has three key applications within chromatography workflows. First, it provides a sample clarification, helping to separate the target molecule such as enzymes, lipoproteins, membrane proteins, and other macromolecules from unwanted molecules such as lipopolysaccharides, cell fragments, and untagged molecules. Second, it allows for target molecule concentration to a predefined concentration and set volume either upstream or downstream of the chromatography step. Finally, diafiltration with ultrafilters provides an extremely quick buffer exchange and desalting procedure. For volumes used in liquid chromatography, the Vivaspin® range is best suited and most versatile.

LCGC: What are the main factors when using Vivaspins® for sample clarification?

Green: Microfilters, such as Minisarts® from Sartorius, are great for general sample clarification of large particles. However to separate out, and help remove, unwanted smaller molecules such as albumin or immunoglobulins from plasma, that would get past a typical 0.22-µm filter, ultrafilters such as Vivaspins® are needed.

Vivaspins® work quickly, especially those from the Vivaspin® Turbo range. Vivaspins® come in eight molecular weight cutoffs (MWCOs). It is important to choose the right MWCO for your target. For true fractionation and size exclusion, there must be a 10-fold difference between the molecule you wish to retain and the molecule you would like to remove. Therefore, if you want to remove all molecules that have a molecular weight of 50 kDa or greater from a target that is 5k Da or smaller, then a 30K or 50K MWCO membrane should be used. For fractionation, use a MWCO close to the largest molecule to be retained. It prevents secondary filtration effects from the retained molecule trapping the smaller species. Finally, the Vivaspin® devices have a dedicated filtrate tube for sample collection and transfer after the process.

SPONSORED BY

SARTORIUS

LCGC north america

LCGC: What should operators consider when using Vivaspin® for sample concentration?

Green: Concentration is typically the primary function for Vivaspin® devices. This can be done before a chromatography step like high performance liquid chromatography to ensure sufficient target concentration is used, or after to provide a final polishing step and predefine the concentration. We suggest using a MWCO that is approximately one-third the size of the target molecular weight.

Not all targets work by molecular weight, such as exosomes. We have a technical selection guide that compares molecule sizes based on factors such as diameter and base pair length. For concentration, the target is retained in the retentate, whereas for clarification, it's often in the filtrate.

LCGC: Is there anything specific to consider with regard to diafiltration?

Green: Diafiltration allows for very quick buffer exchange and desalting. Buffer exchange is particularly useful after purification to ensure the isolated protein sample has the correct buffer composition and concentration to prevent denaturing or agglomeration. Vivaspins® are enabled for up to five spins in one go. Typically, this includes two or three washing steps to remove unwanted small molecules and buffer solution, one spin to concentrate with the new buffer of choice, plus about one more possible spin, if needed. Do not reuse the devices after these five spins. Ultrafilter membranes have stabilizers that maintain the pore structure and overuse will remove this and cause the pores to collapse.

LCGC: What is the process for correct ultrafiltration device selection?

Green: Sample volume will dictate the product group. For example, the Vivaspin® 500 operates with 100–500 µL and the Vivaspin® 2 operates in a 400 µL to 2 mL range, although it may be worth splitting the sample or diluting a sample to use a specific device. In addition, assess the best-suited MWCO; generally, a low MWCO for general removal of macromolecules or an MWCO one-third the size of the target for concentration applications. Finally, it is becoming more important to select the right ultrafilter membrane material. Most manufacturers just offer one type of membrane, however, many molecules react differently to different membrane properties. So, it's worth checking to see what membrane is best for the specific target. The typical go-to membrane types are polyethersulfone (PES), regenerated cellulose (RC), Hydrosart® or cellulose triacetate (CTA).

LCGC: How does one know which ultrafilter membrane is best for a molecule?

Green: It's often very molecule specific, however a PES membrane has a slight negative charge and is excellent at retaining or removing other negatively charges molecules. RC has great chemical resistance and is hydrophilic. Hydrosart® is similar to RC with very low protein binding properties, and CTA is particularly good where collecting the filtrate is of interest. We're currently developing a reference guide to benchmark which membrane is optimal for common, industry-relevant molecules.

“There are other nuances to consider when optimizing ultrafiltration processes such as sample temperature, pH, membrane absorption, reverse centrifugation, and device treatment methods.”

LCGC: What are some other applications or considerations for ultrafiltration with chromatography?

Green: Although we reference proteins a lot, ultrafiltration also provides excellent results with other molecules relevant to liquid chromatography. This includes exosomes, DNA, RNA, viruses, and virus-like particles as well as inorganics such as heavy metal aggregates. Furthermore, there are other nuances to consider when optimizing ultrafiltration processes such as sample temperature, pH, membrane absorption, reverse centrifugation, and device treatment methods.

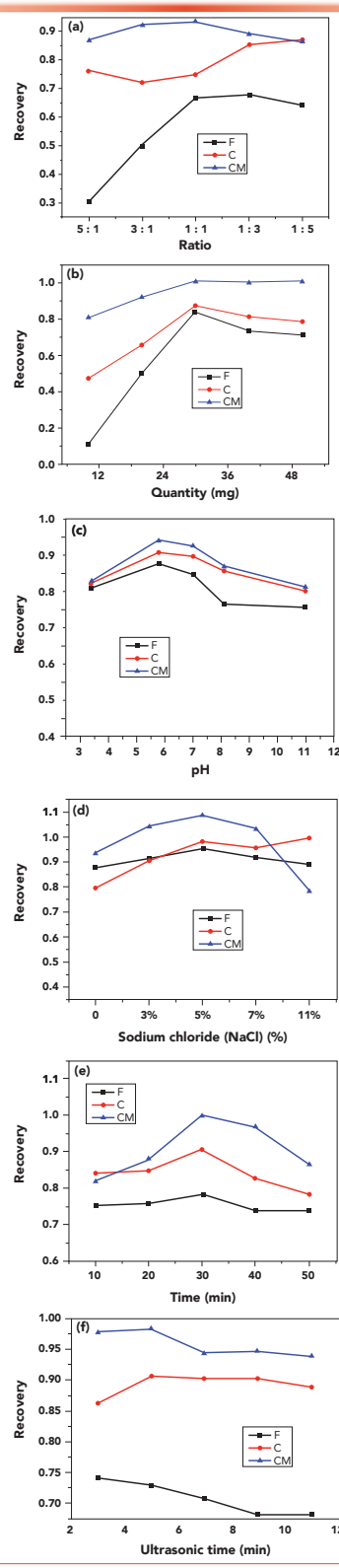


FIGURE 4: (a)–(f) Selection of different factors related to recovery and the elution solvent, solvent ratios, and sample treatment. Detailed explanations are found in the text. F is fenitrothion, C is chlorpyrifos, and C–M is chlorpyrifos methyl. Recovery of 1.0 is equivalent to 100%.

extraction recovery of fenitrothion was affected by the ratio of methanol to ethyl acetate, and when the ratio of methanol and ethyl acetate was 1:1, the recoveries of fenitrothion and chlorpyrifos methyl were highest. However, the greatest recovery of chlorpyrifos appeared at 1:3. This may be owing to the strong polarity of ethyl acetate for chlorpyrifos. Considering that the change of extraction recovery of chlorpyrifos and chlorpyrifos methyl with the ratios of methanol and ethyl acetate is less obvious than fenitrothion, $V_{methanol}$:$V_{ethyl\ acetate}$=1:1 was selected as the optimum rate for the following experiments, at a total volume equal to 1 mL.

Selection of the Amount of N-RGO/Fe_3O_4

To improve the enrichment efficiency of the extraction method, the amount of N-RGO/Fe_3O_4 was investigated, from 10 to 50 mg. As shown in Figure 4b, when the amount of N-RGO/Fe_3O_4 added was 30 mg, the recovery reached peak; this result was a result of the stronger adsorption capability of the N-RGO/Fe_3O_4. The peak areas of the three tested analytes slightly decreased when the amount of N-RGO/Fe_3O_4 was increased from 30 to 50 mg. There is one reason which can explain this phenomenon: A very high amount of adsorbent requires high amounts of desorbing solvent to elute the analytes, so the peak areas of test analytes are reduced. Therefore, an optimal sorbent amount of 30 mg was selected for further experiments.

Selection of Sample Solution pH

To examine the effect of the sample solution pH, the pH was studied ranging from 3.0 to 11.0, which was adjusted by using CH_3COOH and $NH_3{\cdot}H_2O$ solution. From the results, as shown in Figure 4c, it was found that the recoveries of OPPs increased when increasing the pH of the sample solution from 3.0 to 6.0. This is due to the electrostatic interactions that the three tested OPPs exhibit on the surface of the N-RGO/Fe_3O_4. At pH <6.0, both the analyte and the adsorbent surfaces are positively charged, and the electrostatic repulsion between two positive

charges is a robust force. Further increases in solution pH were also examined, and it was found that the peak areas of the OPPs decreased when the pH was above 6.0. This result was attributed to the fact that more oxygen-containing groups on the N-RGO/Fe_3O_4 surfaces are ionized when the pH is above 6.0, which causes lower extraction of analyte materials. To sum up, a pH of 6.0 was selected for follow-up experiments.

Selection of Ionic Strength

To investigate the effect of ionic strength on the adsorption percentage of OPPs on N-RGO/Fe_3O_4, the ionic strength was set over the range of 0–11% (adding NaCl of 0, 4.5, 7.5, 11.5, and 14.5 g), and the results are shown in Figure 4d. As can be seen, the recoveries rose rapidly with the increasing NaCl concentration at the beginning, due to the salting out effect and the higher viscosity, which may make the process of extraction between the target analytes and adsorbents more effective. Meanwhile, previous studies suggested that an excessive amount of NaCl may reduce the extraction kinetics. Thus, the optimal salt addition was determined to be 5%.

Selection of Equilibrium Time

Adsorption equilibrium time is an important factor for evaluating the adsorption capacity of the materials, because MSPE is a partition equilibrium process of analytes between adsorbents materials and sample solution. To screen out the optimum conditions, the effect of extraction time from 10 to 50 min were explored. As shown in Figure 4e, the maximum recoveries were obtained when the extraction time was 30 min for the three analytes. Thus, 30 min was selected as optimum adsorption equilibrium time for the further experiments.

Selection of Ultrasound Time

Ultrasound time is the minimum time needed for quantitative desorption of the analytes, which is another essential factor that affects the recoveries of the analytes. As shown in Figure 4f, for screening out optimum ultrasound time, 3–11 min was set for experiments. For fenitrothion, 3 min was enough to get desorption, while 5 min was needed for chlorpyrifos and chlorpyrifos methyl. In terms of total recovery for the three OPPs, 5 min was selected as the optimal ultrasound time.

Validation of the HPLC Method

To validate the developed HPLC method for the analysis of OPPs, the linearity was evaluated using a series of standard mixture solutions of the analytes. Concentrations from 0.020 to 800 µg/mL were obtained for the establishment of the calibration curve. For each level, five repetitive measurements were carried out. Relative standard deviations (RSDs), enrichment factors (EFs), and limits of detection (LODs) were examined to evaluate the HPLC method.

The recovery percentage (RSD) was calculated by following equation 1 (34):

$$RSD = (C_1\text{-}C_2)/C_1*100\% \quad [1]$$

where C_1 and C_2 are the final concentration of the analyte in the spiked sample and in the unspiked sample, respectively.

The enrichment factor was calculated with equation 2 (34):

$$EF = C_a/C_o \quad [2]$$

where C_a and C_o are the final concentration of the analyte in the organic phase (extraction solvent) and the initial concentration of the analyte in the aqueous phase, respectively.

The results are shown in Table I. Three OPPs exhibited good linearity with correlation coefficients (R^2) between 0.9993 and 0.9997, and the RSDs varied from 1.58% to 2.90%. The LODs of 0.04–0.06 µg/mL for the analytes were obtained based on the ratio of signal-to-noise (S/N = 3), with the RSDs (n = 5) lower than 3.58%. Finally, EFs were in the range of 133 to 166, which demonstrates that the HPLC method has high values and good extraction performance. Above all, it can be concluded that good sensitivity and reproducibility could be achieved with the developed HPLC method.

Application to Real Samples

The proposed N-RGO/Fe_3O_4 was applied to determine three OPPs, including fenitrothion, chlorpyrifos, and chlorpyrifos methyl residues in environmental samples under the above optimum conditions established. No OPPs were found in the real samples; the recovery was carried out by spiking known concen-

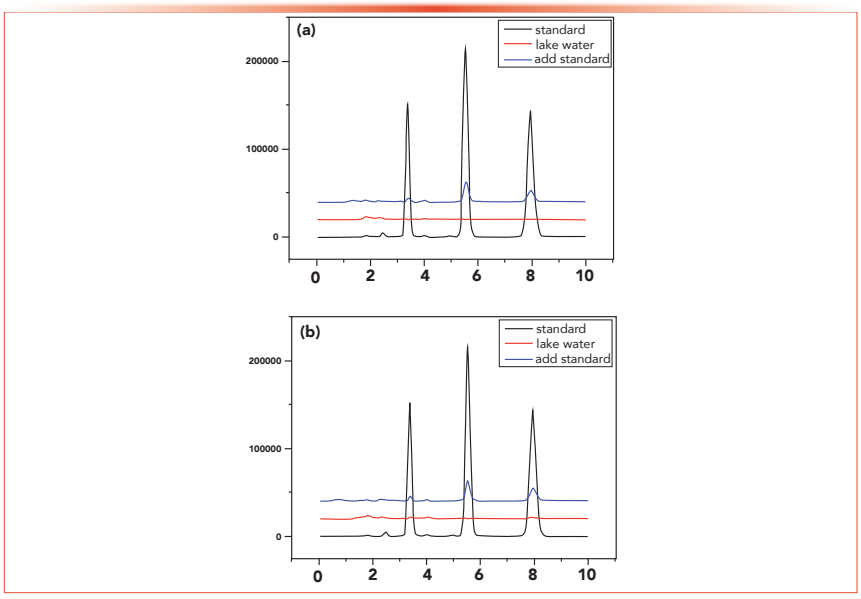


FIGURE 5: (a),(b) Replicate chromatograms of tap water and lake water.

trations of mixed standard OPPs into the samples before added the N-RGO/Fe_3O_4. For each concentration level, three replicate experiments with the whole analysis process were performed. The results are listed in Table II, and the result shows that this method has good extraction efficiency for analyzing of three OPPs in real water samples. The recoveries of the studied OPPs were between 84.40% and 105.05%. Figure 5 shows the typical chromatograms of tap water and lake water.

Conclusions

In this study, a method based on MSPE-HPLC with N-RGO/Fe_3O_4 as the adsorbent was proposed for analyzing of three organophosphorus pesticides, fenitrothion, chlorpyrifos, and chlorpyrifos methyl, in environmental water. A rapid separation of organophosphorus pesticides was achieved. The developed method provided fast analysis process, high EFs, low LODs, wide linear range, and good anti-interference ability. This method fully embodies the high speed and efficiency of HPLC. It is suitable for the speciation of OPPs in environmental studies.

Acknowledgments

We thank the Fundamental Research Funds for the Central Universities (XDJK2018C043).

References

(1) B. Jongenotter and H.G. Janssen, *LCGC Europe* **15,** 338–357 (2002)..

(2) S. Hassani, S. Momtaz, F. Vakhshiteh, A.S. Maghsoudi, M.R. Ganjali, P. Norouzi, and M. Abdollahi, *Aquat. Toxicol.* **91,** 109–130 (2017).

(3) M.I. Badawy, M.Y. Ghaly, and T.A. Gad-Allah, *Desalination* **194,** 166–175 (2006).

(4) J. Chen, C. Duan, and Y. Guan, *J. Chromatogr. B.* **878,** 1216–1225 (2010).

(5) E. Zhao, W. Zhao, L. Han, S. Jiang, and Z. Zhou, *J. Chromatogr. A* **1175,** 137–140 (2007).

(6) H. Mol, R.V. Dam, and O.M. Steijger, *J. Chromatogr. A* **1015,** 119–127 (2003).

(7) H. Chen, R. Chen, R. Feng, S. Li, *Chromatographia* **70,** 165–172 (2009).

(8) F. Tian, W. Liu, H. Fang, M. An, and S. Duan, *Chromatographia* **77,** 487–492 (2014).

(9) D. Harshit, K. Charmy, and P. Nrupesh, *Food Chem.* **230,** 448–453 (2017).

(10) T. Yaseen, H. Pu, and D.W. Sun, *Food. Addit. Contam. A* **36,** 762–778 (2019).

(11) F.K. Wan, S.A. Ong, L.N. Ho, Y.S. Wong, C.H. Voon, S.Y. Yusuf, N.A. Yusoff, and S.L. Lee, *Environ. Sci. Pollut. Res.* **23,** 16716–16721 (2016).

(12) J. Hassan and M. Sarkouhi, *Arab. J. Chem.* **9**(s1), S1–S940 (2016).

(13) J.C. Wei, J. Hu, J. Cao, J.B. Wan, C. He, Y.J. Hu, H. Hu, and P. Li, *J. Agr. Food. C* **64,** 932 (2016).

(14) G. Peng, Q. He, Y. Lu, D. Mmereki, and Z. Zhong, *Environ. Sci. Pollut. Res. Int.* **23,** 19409–19416 (2016).

(15) R. Nisticò, *Res. Chem. Intermediat.* **43,** 1–39 (2017).

(16) C. Monteil, N. Bar, A. Bee, D. Villemin, and J. Beilstein, *Nanotech.* **7,** 1447 (2016).

(17) R. Rameshbabu, N. Kumar, A. Karthigeyan, and B. Neppolian, *Mater. Chem. Phys.* **181,** 106–115 (2016).

(18) J. Wang, S. Ji, R. Ma, Q. Wu, C. Wang, J. Qiang, and W. Zhi, *Sep. Sci. Technol.* **49,** 861–867 (2014).

(19) M. Oyama and X. Chen, *Anal. Sci.* **30,** 529–532 (2014).

(20) T.F. Marinca, B.V. Neamtu, I. Chicinas, and P.J. Pascuta, *J. Therm. Anal. Calorim.* **118**(2), 1245–1251 (2014).

(21) N. Ferreirós, S. Labocha, J. El-Duweik, C. Schlecker, J. Lötsch, and G. Geisslinger, *J. Sep. Sci.* **37**(5)**,** 476–483 (2014).

(22) S.X. Gong, X.L. Wang, W. Liu, M.L. Wang, X. Wang, Z.W. Wang, and R.S. Zhao, *J. Sep. Sci.* **40**, 1755–1764 (2017).

(23) Z. Li, S. Wu, C. Yang, Y. Ma, X. Fu, P. Ling, J. Guan, and Q. Kan, *Mol. Catal.* **432,** 267–273 (2017).

(24) M.A. Yan-Hua, Y.U. Chen-Fei, and X.G. Chen, *J. Sep. Sci.* **30,** 709–712 (2014).

(25) C. Xiaoji, S. Lingxiao, Y. Xuemin, Z. Feifei, C. Jiaoyu, and M. Weimin, *Analyst* **139,** 1938–1944 (2014).

(26) J. Lu, C. Deng, X. Zhang, and P. Yang, *Acs. App.l Mater. Inter.* **5,** 7330–7334 (2013).

(27) A. Bach and R. Semiat, *Desalination* **273,** 57–63 (2011).

(28) G. Peng, M. Zhang, S. Deng, D. Shan, H. Qiang, and Y. Gang, *Hem. Eng. J.* **34,** 361–370 (2018).

(29) Z.K. Ghouri, N.A.M.Barakat, A.M. Alam, M.S. Alsoufi, T.M. Bawazeer, A.F. Mohamed, and H.Y. Kim, *Electrochimica Acta* **184,** 193–202 (2015).

(30) H.R. Naderi, P. Norouzi, M.R. Ganjali, and H. Gholipour-Ranjbar, *Powder. Technol.* **302,** 298–308 (2016).

(31) H. Heydari and M.B. Gholivand, *Appl. Phys. A.* **123,** 187–196 (2017).

(32) R. Zhou, Y. Zheng, D. Hulicovajurcakova, and S. Qiao, *J. Mater. Chem. A* **1,** 13179–13185 (2013).

(33) B. Li, H. Cao, J. Shao, M. Qu, and J.H. Warner, *J. Mater. Chem. A* **21,** 5069–5075 (2011).

(34) A.A. Nour, S. Hassan, and H. Akram, *J. Chromatography B* **1092,** 480–488 (2018).

Yi Gao is with the Department of Analytical Chemistry in the Graduate School of Chemistry and Chemical Engineering, at Southwest University, in Chongqing, in The People's Republic of China. Qingqing Pan is with the Department of Chemistry in the Graduate School of Chemistry and Chemical Engineering, at Southwest University, in Chongqing, in The People's Republic of China. Direct correspondence to: hxraining@swu.edu.cn

Are You Invalidating Out-of-Specification Results into Compliance?

Out-of-specification (OOS) results and their proper investigation is one of the current focus areas in data integrity inspections and audits. Are your OOS investigations scientifically sound and is the assignable cause correct? Or are OOS results invalidated using the ever-popular justification of analyst error?

R.D. McDowall

Life moves on, and this is reflected in the shifting focus of data integrity issues during regulatory inspections. Much of the original focus during inspections was on shared-user identities, conflicts of interest with normal users granted administrator access to an application, unofficial testing, data deletion, time traveling, and not having audit trails or turning them off (1). Very recent warning letters show that these topics are still being found such as, for example, Tismore (2), where all analysts had administrator privileges, and there were deletion of data and aborted runs, and Shriram Institute, who were told by the Food and Drug Administration (FDA) in 2016 to turn chromatography data service (CDS) audit trails on, and only did this four days before the next inspection in 2019 (3). Better late than never? Not a chance!

However, the Shriram Institute warning letter also contains a citation for a current inspection focus, and the subject of this article, which is the investigation and invalidation of out-of-specification (OOS) results:

> *Your Quality Unit (QU) failed to ensure that your laboratory personnel follow written procedures. For example, our investigators observed at least <redacted> samples tested between March 2019 and September 2019 in which out-of-specification (OOS) results were not investigated as required in your procedures. Your head of Quality Assurance informed our investigator during the inspection that* ***failures are investigated only upon customer request*** *(3).*

Note the highlighted text: Do the regulations state or imply you only investigate the OOS results you want? Not a chance! Unfortunately, there is not enough space to show the company response to the 483 observations and the huge amount of work that FDA required to remediate the problem, so please read the warning letter to understand why it is important to get OOS investigations right.

Lupin received a warning letter in 2017 (4), and the first citation focuses on OOS results and portions of the citation are presented below.

> *1. Your firm failed to thoroughly investigate any unexplained discrepancy or failure of a batch or any of its components to meet any of its specifications, whether or not the batch has already been distributed (21 CFR 211.192).*
>
> *Your firm* ***frequently invalidated initial out-of-specification (OOS) laboratory results without an adequate investigation*** *that addressed potential manufacturing causes.*
>
> *A. Assay Failure*
>
> *While conducting component release testing on <redacted> active pharmaceutical ingredient (API) batch (b)(4), your firm obtained a failing assay result of <redacted>% (specification range <redacted>%).*
>
> *Despite the findings of multiple values close to the original OOS value, your firm invalidated the initial failing result, stating that the* ***initial result "shall be considered an outlier and retest results shall be reported as final results."*** *Although* ***the investigation failed to identify a conclusive laboratory root cause,*** *you did not conduct an evaluation of your supplier, and reported an average result for batch release.*
>
> *B. Content Uniformity Failure*
>
> *Your investigation of content uniformity OOS results for <redacted> tablets for batch <redacted> was inadequate. Two individual units and the acceptance value (AV) were OOS for this batch, which was an exhibit batch filed in your <redacted>.*
>
> *The initial assessment of the OOS results found* ***no evidence of laboratory error*** *by the analyst. Retests from stock solution and re-sonicated samples yielded results consistent with the original OOS results, and ruled out improper sonication and dilution error as root causes. Although the investigation did not demonstrate a conclusive assignable cause, you surmised that the* ***"probable laboratory error" was inadequate cleaning of the <redacted> shaft by the analyst.*** *You then invalidated the initial OOS results and reported test results from a new set of <redacted> units that passed specifications.* ***Your firm's investigation indicated that this "confirmed" that there was a laboratory error.***

The problem in the quotations above from the warning letter is that there appears to be only a few examples of invalidation of OOS results at the company. However, you need to read the 483 Observation to see the magnitude of the OOS invalidation problem (5) that is shown in Table I. As 97% of stability OOS results were invalidated, this means that the company avoided sending the FDA field alerts that potentially could trigger a batch recall if an OOS result was confirmed. By the way, a different plant had a warning letter in 2019 and guess what? Invalidating OOS test results features in citation 1: … *You frequently closed these*

TABLE I: Percentage of OOS on investigations invalidated - Lupin Goa Plant 483 observations, April 2017 (5).

Laboratory Area	No. OOS Investigations	No. OOS Investigations Invalidated	% OOS Invalidated
Finished product	89	67	75
Stability	31	30	97
Raw material	48	34	71

OOS investigations without an assignable root cause, and released batches based on passing retest results (6).

Some companies never learn.

Method Development and Validation

Before we get into the depths of OOS investigations and the history of them, one of the potential causes of OOS results is method variability. As we discussed in an earlier article on analytical procedure lifecycle management (APLM), quality by design in method development is essential to finding and controlling critical parameters (7). A rushed development and validation can often result in an unreliable chromatographic procedure that through inherent variability can generate OOS results.

Sermon over, let's get back to OOS investigations!

Déjà vu All Over Again!

Not a lot of people know this, but we have been here before with OOS. Nearly 30 years ago, a New Jersey generic pharmaceutical company, Barr Laboratories, had some analytical testing practices that are best described as unconventional. Apart from misplaced records, test data recorded on scrap paper, the release of products not meeting their specifications, inadequate investigation of failed products, and failure to validate test methods, Barr had a QC practice that if a test was out-of-specification, then they conducted two more tests and took the best two of the three to determine batch release. The FDA was less than impressed, and issued warning letters. Barr was less than impressed, and sued the FDA with the Agency reciprocating by suing Barr (only in America!). The two law suits resulted in a single court case presided over by Judge Alfred Wolin, whose knowledge of science could be written on a very small grain of rice. However, his judgment was remarkably prescient, and the ramifications still impact the industry today (8,9).

Key Laboratory Findings of the Wolin Judgment

From the judgment, there is an impartial and reasoned interpretation of the US GMP regulations:

- Any OOS result requires a failure investigation to determine an assignable cause. The extent of the investigation depends on the nature and location of the error (laboratory or production).
- Wolin rejected Barr's two out of three testing approach as unscientific, and at the same time also rejected the unreasonable FDA request that one OOS result should result in rejection of the whole product batch.
- Good science and judgment are needed for reasonable interpretation of GMPs. Industry practice cannot be relied on as the sole interpretation of GMP: guidance from literature, seminars, textbooks and reference books, and FDA letters to manufacturers are additional sources.
- Any GMP interpretation must be "reasonable and consistent with the spirit and intent of the cGMP regulations."
- Outliers must not be rejected unless allowed by the *United States Pharmacopoeia* (*USP*) which resulted in the development and issue of *USP* <1010> on Outlier Testing (10).

GMP Regulatory Requirements

What are the GMP regulations that the FDA cited in the case of Barr Laboratories? These are found in 21 *CFR* 211.192 on production record review:

> *All drug product production and control records, including those for packaging and labelling, shall be reviewed and approved by the quality control unit to determine compliance with all established, approved written procedures before a batch is released or distributed.*
>
> *Any unexplained discrepancy …. or the failure of a batch or any of its components to meet any of its specifications shall be thoroughly investigated, whether or not the batch has already been distributed.*
>
> *The investigation shall extend to other batches of the same drug product and other drug products that may have been associated with the specific failure or discrepancy. A written record of the investigation shall be made and shall include the conclusions and follow-up (11).*

Note the first word of the second paragraph: *any*. For "any," read "all." There is not a lucky dip selection of which OOS results you wish to investigate, just as there is no selection by a customer (3). You don't have a choice. Therefore, if an unexplained discrepancy occurs, and an OOS is one, it MUST be investigated to find an assignable or root cause. If appropriate, the investigation should extend to other batches, or even other products. There also needs to be a formal report of the investigation together with corrective and preventative (CAPA) plans with monitoring to see their effectiveness.

A similar situation is found in EU *GMP*, where there are two requirements in Chapter 6 Quality Control on trending results and OOS investigations:

> *6.16. The results obtained should be recorded. Results of parameters identified as a quality attribute or as critical should be trended and checked to make sure that they are consistent with each other.*
>
> *6.35. Out-of-specification or significant atypical trends should be investigated. Any confirmed out-of-specification result, or significant negative trend, affecting product batches released on the market should be reported to the relevant competent authorities (12).*

The EU regulations were updated relatively recently and require laboratories to

trend their critical data and to investigate OOS results. Trending of results (both individual measurements and reportable results) provides a means of monitoring performance of the overall procedure and by implication the chromatographs used in the measurement of any analyte that we will discuss shortly. If there is a problem with released batches, then regulatory authorities must be informed.

How should industry interpret these regulations? Here we have the FDA to thank with two publications.

FDA Guidance for OOS Investigations

Following the Barr ruling, FDA quickly issued *Inspection of Pharmaceutical Quality Control Laboratories* in 1993 (13). Nearly half of this guidance was focused on how to inspect OOS investigations; this portion has been replaced by an FDA guidance on the subject that we will be discussing soon. However, before we do, the remainder of this 1993 guidance still is relevant today, as many processes in regulated laboratories have not changed substantially in nearly 30 years, and it contains advice on how FDA will inspect a QC laboratory and in consequence how QA can conduct internal audits. This should also be coupled with the FDA's recent update of *Compliance Policy Guide 7346.832 for Pre-Approval Inspections* (PAIs) that also outlines data integrity focus of regulatory submissions (14) that was the subject of a recent "Focus on Quality" article (15).

The second FDA publication was a draft OOS guidance for industry in 1996, with a final version released in 2006 (16). This guidance describes the various stages of an OOS laboratory investigation for chemical results, including the roles and responsibilities of those involved. This document is important as it is the only formal OOS guidance issued by a major regulatory authority. The guidance outlines a three-part, two-phase strategy for investigating an OOS chemical analysis result, as shown in Figure 1.

- Phase 1 is the laboratory investigation which is to determine if there is an assignable cause for the analytical failure. This is conducted under the auspices of Quality Control, and should be split into two parts. First, the analyst checks their work to identify any gross errors that have occurred, and correct them with appropriate documentation. If this does not identify the cause, the analyst and their supervisor initiate the OOS investigation procedure looking in more detail and determining whether the cause is within the subject of the FDA OOS guidance. If a root cause cannot be identified, then the investigation is escalated to Phase 2.
- Phase 2 is under the control of Quality Assurance to coordinate the work of both production and the laboratory; there are two elements here: Phase 2a and 2b.
- In Phase 2a, if no assignable cause is found in the laboratory, then the investigation looks to see if there is a failure in production. If there is no root cause in production, then the investigation moves back to the laboratory.
- In phase 2b, different hypotheses are formulated to try and identify an assignable cause, and a protocol is generated before any laboratory work is undertaken. Here, resampling can be undertaken if required.

Owing to space, we will only consider Phase 1 laboratory investigations in this article.

OOS Definitions

We have been talking about OOS, but we have not defined this and any associated terms, so let us see what definitions we have. Now here is where it gets interesting. You would think that, in an FDA guidance focused on OOS investigations, the term would be defined early in the document. I mean, logic would dictate this, would it not? Not a chance! We must wait until page 10 to find the definition, and then it is found, not in the main body of text, but in a small font footnote! Your tax dollars at work. Not only that, it is totally separated from the discussion about the individual results from an analysis that is found floating in the middle of page 10. Your tax dollars at work, again. There are the following definitions used in the FDA OOS guidance document:

- *Reportable Result*: The term refers to a final analytical result. This result is appropriately defined in the written approved test method, and derived from one full execution of that method, starting from the sample. It is comparable to the specification to determine pass/fail of a test (16). This is easy to understand; it is a one-for-one comparison of the analytical result with the specification and the outcome is either pass or fail. Maybes are not allowed.
- *Individual Result*: To reduce variability, two or more aliquots are often analyzed with one or two injections each, and all the results are averaged to calculate the reportable result. It may be appropriate to specify in the test method that the average of these

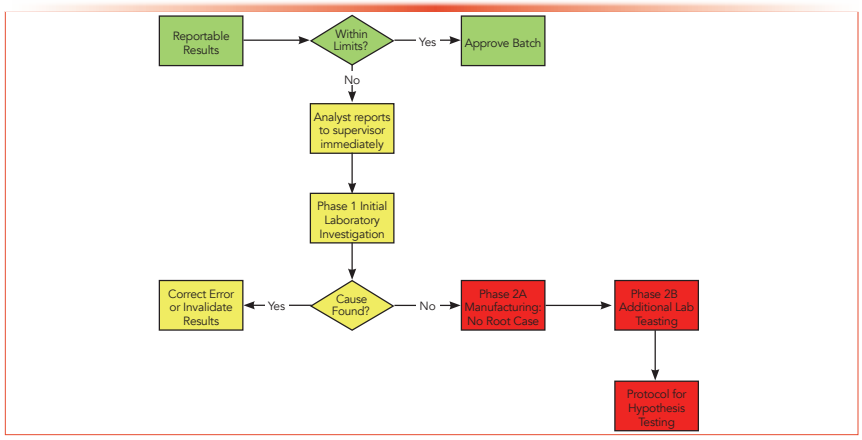
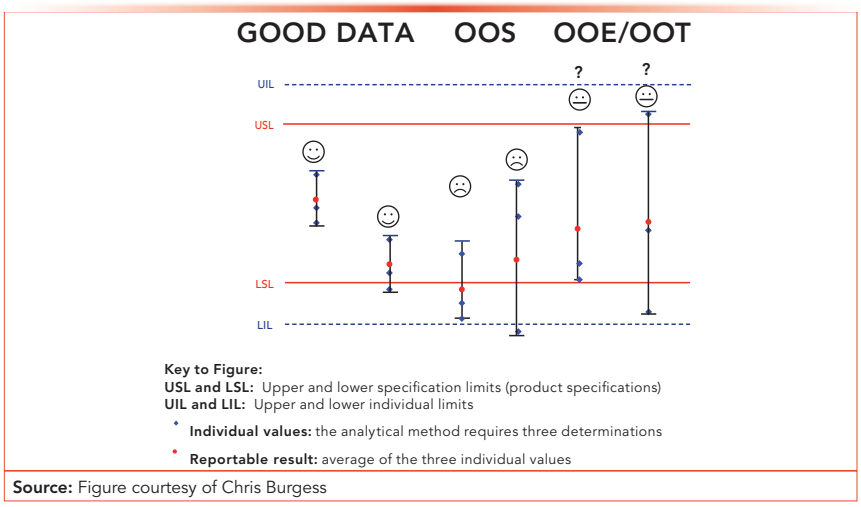


FIGURE 1: Flow chart of OOS results investigations.



Source: Figure courtesy of Chris Burgess

FIGURE 2: Understanding OOS for individual values and the reportable result.

multiple assays is considered one test, and represents one reportable result. In this case, limits on acceptable variability among the individual assay results should be based on the known variability of the method, and should also be specified in the test methodology. A set of assay results not meeting these limits should not be used (16).

- Therefore, the individual results must have their own and larger limits, due to the variance associated with a single determination. This is an addition to the product specification limits for the reportable result discussed above. Note individual results are NOT compared with the product specification.
- *Out-of-Specification (OOS) Result*: A reportable result outside of specification or acceptance criteria limits. As we are dealing with specifications, OOS results can apply to test of raw materials, starting materials, active pharmaceutical ingredients and finished products, and in-process testing. However, if a system suitability test fails, this will not generate an OOS result, as the whole run would be invalidated; however, there needs to be an investigation into the failure (16).
- *Out-of-Trend (OOT) Result*: Not an out-of-specification result, but rather the result does not fit with the expected distribution of results. An alternative definition is a time dependent result which falls outside a prediction interval or fails a statistical process control criterion (17). This can include a single result outside of acceptance limits for a replicate result used to calculate a reportable result. If investigated, the same rules as for OOS investigations apply. Think not of regulatory burden but good analytical science here. Is it better to investigate and find the reason for an OOE result, or wait until you have an OOS result that might initiate a batch recall?
- *Out of Expectation (OOE) Result*: An atypical, aberrant, or anomalous result within a series of results obtained over a short period of time, but is still within the acceptable range specification.

OOS of Individual Values and Reportable Results

To understand the relationships between the reportable result and individual values, some examples are shown in Figure 1, courtesy of Chris Burgess. The upper and lower specification and individual limits for this procedure are shown in the horizontal lines. You'll see that the individual limits are wider than the specification limits, as the variance of a single value is greater than a mean result. There are six examples shown in Figure 2, and, from the left to the right, we have the following:

1. Close individual replicates and mean in the middle of the specification range—an ideal result!
2. The individual results are closely spread, and, although one replicate is outside the specification limit, it is inside the individual limit, and therefore a good result.
3. The individual values are relatively close, and all are within the individual limits, but the reportable result is out-of-specification.
4. One of the individual results is outside of the individual result limit, which means that there an OOS result although the reportable result is within specification.

Examples 5 and 6 would be OOT or OOE results respectively, but are not OOS. Here, the variance of the individual results is wider than expected, and may indicate that there are problems with the procedure. It would therefore be prudent to investigate what are the reasons for this rather than ignore them. We will focus on OOS result only here.

Process Capability of an Analytical Procedure

Do you know how well your analytical procedures perform? If not, why not? This information provides you with valuable evidence that you can use in OOS investigations, and it is also a regulatory requirement, as mentioned earlier (12). For chromatographic methods, Individual calculated values and results should be plotted over time, with the aim being to show how a specific method performs and the variability. There are two main types of plot that can be used:

- Shewhart plots with upper and lower specification and individual results plotted over time, as illustrated in Figure 2. Both the individual values and reportable results need to be plotted; if only the latter values are used, then the true performance can be missed, as the variance is lost when averaging. Over time, this gives the process capability over time.
- Cusum or cumulative sum is a control chart that is sensitive to identifying changes in the performance of method, often before OOS results are generated. When the plot direction alters, this often indicates that a change that influences the procedure has occurred, and the reason for this should be investigated to identify the reason. This may be as subtle as a new batch of a solvent or change of column.

If your analytical data are similar to Examples 5 and 6 in Figure 2, then you could have a non-robust analytical procedure that reflects poorly on method development and validation (7); either that, or you have poorly trained staff. Prevention of OOS results is better than the investigation of them!

This Never Happens in Your Laboratory...

Even with all the automation and computerization in the world, there is still the human factor to consider. Consider the following situation. The analytical balance is qualified and has been calibrated, the reference standard is within expiry, the weight taken is within limits, and the vessel is transferred to a volumetric flask. One of three things could happen:

- The material is transferred to the flask correctly, and the solution is made up to volume as required. All is well with the world.
- During transfer, some material is dropped outside the flask, but the analyst still dissolves the material and makes the solution up to volume.
- All material is transferred to the flask correctly, but the flask is overfilled past the meniscus.

At this point, only the analyst preparing the reference solution stands between your organization and a data integrity disaster. The analyst is the only person who knows that options 2 and 3 are wrong. What happens next depends on several factors:

- Corporate data integrity policies and training
- The open culture of the laboratory that allows an individual to admit their mistakes

TABLE II: Responsibilities for the analyst and laboratory supervisor for OOS investigations (16).

Responsibilities of the Analyst	Responsibilities of the Laboratory Supervisor
• Achieve accurate laboratory testing results • Be aware of potential problems in the testing process and watch for problems that could create inaccurate results • Only use those qualified and calibrated instruments • Methods must meet SST requirements, and systems not meeting these requirements 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 • Do not discard test preparations or standard preparations before calculating the reportable result and comparing with the specification • If errors are obvious, such as the spilling of a sample solution or the incomplete transfer of a sample composite, the analyst should immediately document what happened • Analysts should not knowingly continue an analysis they expect to invalidate later for an assignable cause (that is, analyses should not be completed for the sole purpose of seeing what results can be obtained when obvious errors are known) • When unexpected results are obtained and no obvious explanation exists, the analyst should inform the supervisor • An assessment of the accuracy of the results should be started immediately	• Any OOS investigation should be objective and timely with no preconceived assumptions as to the cause. • Data should be assessed promptly to determine if a laboratory error or problems in manufacturing • Examine the actual solutions, test units, and glassware, vials, vial placement data interpretation and calculations, which might provide more credibility for laboratory error hypotheses. The following steps should be taken as part of the supervisor's assessment: 1. Discuss the test method with the analyst; confirm analyst knowledge of and performance of the correct procedure 2. Examine the raw data obtained in the analysis, including chromatograms and spectra, and identify anomalous or suspect information 3. Verify that the calculations used to convert raw data values into a final test result are scientifically sound, appropriate, and correct; also determine if unauthorized or unvalidated changes have been made to automated calculation methods 4. Confirm the performance of the instruments 5. Determine that appropriate reference standards, solvents, reagents, and other solutions were used and that they met quality control specifications 6. Evaluate the performance of the test method to ensure that it is performing according to the standard expected based on method validation data and historical data 7. Fully document and preserve records of this laboratory assessment

- The honesty of the individual analyst
- Laboratory metrics; for example, Turn Around Time (TAT) targets that can influence the actions of individuals
- The attitude of the supervisor and laboratory management to such errors.

STOP! This is the correct and only action by the analyst. Document the mistake contemporaneously and repeat the work from a suitable point (in this case, repeat the weighing). It is simpler and easier to repeat now than investigate later.

But what actually happens depends on those factors described above. Preparation of reference standard is one area where the actions of an individual analyst can compromise the integrity of data generated for one or more analytical runs. If the mistake is ignored, the possible outcomes could be an out-of-specification result or the release of an under- or over-strength batch. In the subsequent investigation, unless the mistake is mentioned, it may not be possible to have an assignable cause.

What is the FDA's View of Analyst Mistakes?

Hidden in the Responsibilities of the Analyst section in the FDA's *Guidance for Industry on Investigating OOS Results* is the following statement (16):

> *If errors are obvious, such as the spilling of a sample solution or the incomplete transfer of a sample composite, the analyst should immediately document what happened. Analysts should not knowingly continue an analysis they expect to invalidate at a later time for an assignable cause (i.e., analyses should not be completed for the sole purpose of seeing what results can be obtained when obvious errors are known).*

The only ethical option open to an analyst is to stop the work, document the error, and repeat the work from a suitable point.

Do You Know Your Laboratory OOS Rate?

According to the FDA:

> *Laboratory error should be relatively rare. Frequent errors suggest a problem that might be due to inadequate training of analysts, poorly maintained or improperly calibrated equipment, or careless work (16).*

This brings me to the first of two metrics: Do you know the percentage of OOS results across all tests that are performed in your laboratory? If not, why not?

Remember that there are three main groups of analytical procedure that can be used as release testing ranging from:

- Observation (including, but not limited to, appearance, color, and odor, for example), which is relatively simple to perform. If there is an OOS result, it is more likely to be a manufacturing issue than a laboratory one (such as particles in the sample or change in expected color). However, laboratory errors in analyses of this type will be extremely rare.
- Classical wet chemistry (including, but not limited to, melting point, titration, and loss on drying) involving a variety of analytical techniques often with manual data recording unless automated (autotitration). There is more likelihood of an error but many mistakes, such as transcription or calculation errors, should be identified and corrected during second person review.

- Instrumental analyses (including, but not limited to, identity, assay, potency, and impurity) using spectrometers and chromatographs for example. Here, the procedures can be more complex, and data analysis requires trained analysts.

As you move down this list, the likelihood of an OOS result increases with the complexity of analytical procedure and more human data interpretation that is involved. Hence the emphasis on instrumental methods, such as chromatography (1) and spectroscopy (18) in inspections.

SSTs Failure Does Not Require an OOS Investigation

Under analyst responsibilities, there is an FDA get out of jail free card for chromatographic runs where the SST injections fail to meet their predefined acceptance criteria:

> *Certain analytical methods have system suitability requirements, and systems not meeting these requirements should not be used. …. 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 (16).*

TABLE III: Some areas for checking during an OOS investigation

Topic Area	Possible Causes of an OOS
Sample management	• Incorrect sampling • Wrong container used • Wrong sample label • Poor transport conditions • Wrong storage conditions
Analytical instruments used	• Qualified or calibrated? • SST or checks on the day of use OK? • Right instrument used?
Analytical reference standards	• Wrong standard used • Contaminated standard • Expired standard or standard solution • Storage conditions not correct • Errors observed in weight, preparation and dilution
Reagents, solutions and sample preparation	• Glassware not clean • Grade B glassware used • Wrong sample weight taken • Weights of samples transposed • Incorrect pH of solution • Fixed or variable volume pipettes not calibrated or leaking • Tip of glass pipette broken or chipped • Wrong size volumetric flasks or glassware used • Correct environmental conditions used (light, temperature, and so forth)?
Chromatographic analysis	• Correct instrument, acquisition files used? • Correct column used? • Mobile phase made up correctly and degassed? • Correct gradient profile used? • Correct instrument and acquisition methods used? • Correct column and column use history and age • No unauthorized changes to files? • Typographical error with sample weights, factors, purities? • Vials placed in correct sequence or vials switched? • Peak shape the same throughout the analysis?
Data interpretation	• SST results meet acceptance criteria • Automatic integration acceptable? • Manual intervention acceptable? • Manual integration (where allowed) performed acceptably?
Calculation of the reportable result	• Typographical error (transferring data) • Formula for calculation incorrect • Rounding error

Here's where technology can come to help. Some CDS applications allow users to define acceptance criteria for SST injections. If one or more of SST injections fail these criteria, then the run stops automatically. This saves the analyst from trying to determine where there are data that could be used from the run because if the run is stopped before samples are injected, then there are no sample data available. It is important that if this function is used, it must be validated to show that it works—specified in the user requirements specification (URS) and verified that it works in the user acceptance testing (UAT). There will also be corresponding entries in the instrument log book documenting the initial problem and the steps taken to resolve the issue (11,19,20).

What is an OOS Investigation?

An OOS investigation is triggered by an analyst when the reportable result is outside of the specification limits as shown in Figure 2. The analyst informs their supervisor and this should begin the laboratory investigation by following the laboratory OOS procedure. The responsibilities of both individuals is presented in Table II, which is copied from the FDA OOS guidance document. The FDA is very specific in listing the responsibilities of both the analyst who performs the analysis and the laboratory supervisor who will be conducting the investigation. The guidance document will be the source for a laboratory SOP that will detail what will be done for a laboratory OOS investigation.

According to the FDA:

> *To be meaningful, the investigation should be thorough, timely, unbiased, well-documented, and scientifically sound (16).*

Note well the criteria for a laboratory investigation:

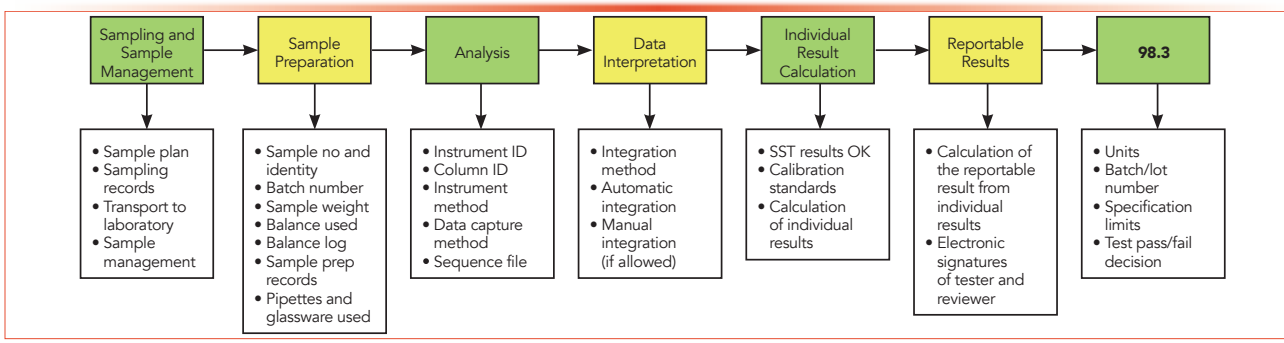


FIGURE 3: Scope of an OOS Investigation of an analytical procedure involving HPLC.

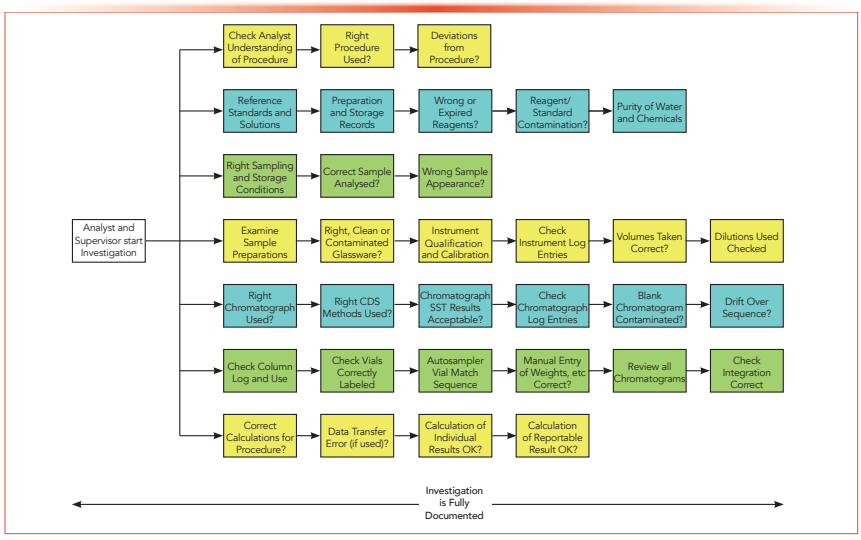


FIGURE 4: Some Areas in an Analytical Procedure to Evaluate for an Assignable Cause for an OOS Result.

- **Thorough:** The whole of the analytical process must be considered for assignable causes of the error, as shown in Figure 3. The investigation needs to go into detail.
- **Timely:** The investigation should start quickly, but also be completed as quickly as possible, and compatible with being thorough and well-document. Investigations that are still open 30, 60, or more days after the original analysis will be subject to more regulatory scrutiny, with suggestions of inadequate staffing of the laboratory.
- **Unbiased:** Start the investigation with an open mind, rather than believe that all OOS results are the result of analyst error before starting.
- **Well-documented:** There is a range of interpretations here, from a single page uncontrolled checklist with most possible causes of an OOS, to a flow chart with questions completed as the supervisor and analyst work through the investigation.
- **Scientifically sound:** Provide a rational explanation for the OOS result that is based on evidence and not supposition. From this must come the root cause to invalidate the OOS result, and any plans to prevent a reoccurrence.

The investigation begins by checking the chromatographer's knowledge of the analytical procedure, and that the right procedure was used. This is followed by ensuring the sampling was performed correctly, and that the right sample was analyzed, and so on throughout the analysis. Areas to check for potential errors and assignable causes are shown in Figure 4 and Table III, and has been derived from the FDA OOS guidance.

Don't Do This in Your Laboratory!

The Lupin plant in Nagpur is the source of this example of how not to do undertake an OOS laboratory investigation, and this is quoted from the 483 form that was issued in January 2020 (21). Citation 2 is an observation for failing to follow the investigation SOP, but we will look at citation 1, where the details of inadequate laboratory investigations are documented. Some parts of the citation are heavily redacted, so this is a best attempt at reconstructing the investigation that was carried out:

- There was an OOS result from a dissolution test.
- The OOS was hypothesized as being due to an analyst transposing samples from different time points.
- The original sample solutions were remeasured, but the results appeared to be similar to the original.
- A comment was added to the record that is redacted in the 483 that appears to document an analyst comment that some of the solution was spilled, and that this would account for the discrepancy in results. This is a very convenient spillage.
- However, the comment was not made by the analyst who performed the original work. A second analyst was told by his supervisor that the first analyst had said he had made a mistake, and the second analyst documented this, as directed by the supervisor.
- There was no documentation or corroborating evidence provided to support this, or that an interview with the original analyst ever occurred.
- A substitution of a solution was made which, when retested, passed. Well, what a surprise!

- The original test results were invalidated, and the passing results used to release the product.
- QC and QA personnel signed off the investigation, even though they knew of the substitution of the solution and potential manipulation.
- The original analyst was unavailable during the inspection.
- No deviation or CAPA was instigated.
- No definitive root cause of the OOS result was ever determined.

Any wonder that a 483 observation was raised?

FDA Guidance on Quality Metrics

The second metric that is important in OOS investigations is a topic in the FDA *Draft Guidance on Quality Metrics* (22) that emphasizes the importance of correct OOS investigations. There are three metrics covering manufacturing and quality control, but there is only one metric for QC that is the percentage of invalidated OOS rate, defined as follows:

> *Invalidated Out-of-Specification (OOS) Rate (IOOSR) as an indicator of the operation of a laboratory. IOOSR = the number of OOS test results for lot release and long-term stability testing invalidated by the covered establishment due to an aberration of the measurement process divided by the total number of lot release and long-term stability OOS test results in the current reporting timeframe (21).*

What is important is that the rate covers not only batch release but also stability testing. The rationale for using the invalidated OOS rate can be seen in Table I and the corresponding 483 observations and warning letters (4,5). An aim is that FDA can conduct risk-based inspections, and, if a firm has low regulatory risk, they will be relying on these quality metrics to extend the time between inspections. Woe betide a firm who massages these metrics.

Outsourced Analysis?

If your organization outsources manufacturing and QC analysis, how should you monitor the work? From a QC perspective, any OOS result should be notified to your organization from the contract facility. You must have oversight of any laboratory investigation on your products or analysis to ensure that the FDA criteria of an investigation are met as outlined above. In addition, if FDA are interested in a metric of the percentage of invalidated OOS results, so are you. You should have these figures for your work, but also across the whole of the outsourced laboratory. Therefore, you should review all OOS investigations on your products, either via video conference or on site during any supplier audits. In words of that great data integrity expert, Ronald Reagan, trust but verify.

Summary

Scientifically sound OOS laboratory investigations are an essential part of ensuring data integrity. Outlined here are the key requirements for an OOS investigation to find and assignable or root cause so that a result could be invalidated. Note that the FDA and other regulatory authorities take a very keen interest in invalidated OOS results, especially where analyst error is cited continually as the cause of the OOS. Your laboratory should know the OOS rate as well as the percentage of OOS results invalidated.

Acknowledgment

I would like to thank Chris Burgess for permission to use Figure 2 in this article and for comments made in preparation of this article.

References

(1) R.D. McDowall, *Data Integrity and Data Governance: Practical Implementation in Regulated Laboratories*. (Royal Society of Chemistry, Cambridge, United Kingdom, 2019).

(2) *FDA Warning Letter Tismore Health and Wellness Pty Limited* (Food and Drug Administration, Silver Spring, Maryland, 2019).

(3) *FDA Warning Letter Shriram Institute for Industrial Research* (Food and Drug Administration, Silver Spring, Maryland, 2020).

(4) *FDA Warning Letter Lupin Ltd.* (Food and Drug Administration, Silver Spring, Maryland, 2017).

(5) *FDA 483 Observations: Lupin Ltd.* (Food and Drug Administration, Silver Spring, Maryland, 2017).

(6) *FDA Warning Letter Lupin Ltd.* (Food and Drug Administration, Silver Spring, Maryland, 2019).

(7) R.D. McDowall, *LCGC North Am.* **38**(4), 233–240 (2020).

(8) R.J. Davis, *Judge Wolin's Interpretation of Current Good Manufacturing Practice Issues Contained in the Court's Ruling United States vs. Barr Laboratories in Development and Validation of Analytical Methods*, C.L. Riley and T.W. Rosanske, Editors (Pergammon Press, Oxford, United Kingdon, 1996), p. 252.

(9) Barr Laboratories: "Court Decision Strengthens FDA's Regulatory Power" (1993). Available from: https://www.fda.gov/Drugs/DevelopmentApprovalProcess/Manufacturing/ucm212214.htm.

(10) *USP General Chapter <1010> Outlier Testing* (United States Pharmacopoeia Convention Inc., Rockville, Maryland, 2012).

(11) *21 CFR 211 Current Good Manufacturing Practice for Finished Pharmaceutical Products* (Food and Drug Administration, Silver Spring, Maryland, 2008).

(12) EudraLex - Volume 4 *Good Manufacturing Practice (GMP) Guidelines, Chapter 6 Quality Control* (European Commission, Brussels, Belgium, 2014).

(13) *Inspection of Pharmaceutical Quality Control Laboratories* (Food and Drug Administration, Rockville, Maryland, 1993).

(14) *FDA Compliance Program Guide CPG 7346.832 Pre-Approval Inspections* (Food and Drug Administration, Silver Spring, Maryland, 2019).

(15) R.D. McDowall, *Spectroscopy* **34**(12), 14–19 (2019).

(16) *FDA Guidance for Industry Out-of-specification Results* (Food and Drug Administration, Silver Spring, Maryland, 2006).

(17) C. Burgess, Personal Communication.

(18) P.A. Smith and R.D. McDowall, *Spectroscopy* **34**(9), 22–28 (2019).

(19) EudraLex - Volume 4 *Good Manufacturing Practice (GMP) Guidelines, Chapter 4 Documentation* (European Commission, Brussels, Belgium, 2011).

(20) R.D. McDowall, *Spectroscopy* **32**(12), 8–12 (2017).

(21) *FDA 483 Observations Lupin Ltd.* (Food and Drug Administration, Silver Spring, Maryland, 2019).

(22) *FDA Guidance for Industry Submission of Quality Metrics Data, Revision 1* (Food and Drug Administration, Rockville, Maryland, 2016).

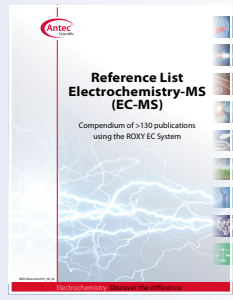
R.D. McDowall
is the director of R.D. McDowall Limited in the UK. Direct correspondence to: rdmcdowall@btconnect.com

PRODUCTS & RESOURCES

EC-MS compendium

Antec's Reference List Electrochemistry-MS (EC-MS) is a free compendium of scientific publications using the ROXY EC System. According to the company, the publication covers applications in fast prediction and mimicking of drug metabolism, drug stability testing and controlled degradation, rapid environmental degradation and persistent organic pollutants, reduction of disulfide bonds in proteins, and biopharmaceuticals including electrochemical synthesis.

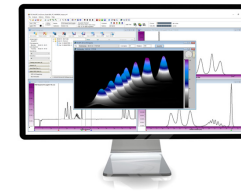
Antec Scientific, Boston, MA.
www.antecscientific.com



Compliant GPC/SEC software

WinGPC UniChrom from PSS is designed as a macromolecular chromatography data system developed for comprehensive analysis of macromolecules using liquid chromatography in one or two dimensions. According to the company, its Compliance Pack enables users to comply with regulations such as FDA 21CFR11, supporting all techniques including light scattering, viscometry, and triple detection.

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



GC capillary columns

Stationary-phase gas chromatography (GC) columns from REFLEX Analytical are designed for use in sample matrices such as food, environmental, clinical toxicology, petrochemical, flavors, and fragrances. According to the company, stationary-phase selections include polysiloxane, polyethylene glycol, and PLOTs.

REFLEX Analytical Corp.,
Ridgewood, NJ.
www.reflexusa.com

LC–MS/MS spectrometer

The LCMS-8060NX triple quadrupole (TQ) liquid chromatograph mass spectrometer (LC–MS/MS) from PSS is designed with a heat assist feature to increase desolvation efficiency and promote ionization of a range of compounds. According to the company, the system's UFsweeper technology sweeps ions from the collision cell without deceleration, maintaining high-sensitivity analysis even at high acquisition speeds.

Shimadzu Scientific,
Columbia, MD.
www.ssi.shimadzu.com

Chromatography data system

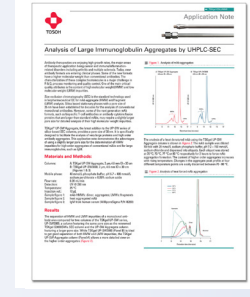
Thermo Scientific's Chromeleon 7.3 Chromatography Data System (CDS) software is designed to provide greater automation and workflow support for analytical scientists across a range of sectors. According to the company, the software leverages cloud-based technology, allowing operation from remote locations across global laboratory networks.

Thermo Fisher Scientific,
San Jose, CA.
www.thermofisher.com

Application note

An application note from Tosoh titled "Analysis of Large Immunoglobulin Aggregates by UHPLC–SEC" demonstrates the advantages of using a 30 nm pore size, as found in the TSKgel UP-SW Aggregate column. According to the company, the column is suitable for the determination of high molecular weight impurities for high-order aggregates of conventional mAbs, and for large immunoglobulins, such as IgM.

Tosoh Bioscience, LLC,
King of Prussia, PA.
www.tosohbioscience.com



Autosampler syringes

Two brand-specific syringes from Hamilton are designed for use with CTC PAL liquid chromatography autosampler systems. According to the company, the direct attachment design of the C-Line syringe needle to the barrel eliminates contact between the sample solvent and adhesive. The X-Type syringes reportedly feature near-zero carryover.

Hamilton Company,
Reno, NV.
www.hamiltoncompany.com

Ion chromatograph

Metrohm's Eco IC ion chromatograph is designed for the routine analysis of anions, cations, and polar substances in water. According to the company, the chromatograph includes a suppressor, a conductivity detector, and software, and allows for automatic analysis of up to 36 samples.

Metrohm USA,
Riverview, FL.
www.metrohmusa.com

• Continued from Page 401

What analytical techniques did you use in this study, and why?

We used Fourier transform infrared (FT-IR) spectrometry for the coating identification, allowing us to select the products manufactured with polyester coatings for the analysis. In the sample preparation process, food was homogenized, migrants were extracted with acetonitrile containing 0.1% formic acid, centrifuged, concentrated, precleaned, conditioned in appropriate solvent, filtered, and finally injected in the chromatographs. LC was selected as the analytical technique for the analyses of migrants, based on the results from the previous work. For monomer quantification, we used a LC–MS/MS detector, which is selective, robust, and provides good sensitivity. For oligomer determination, we selected LC-DAD, which, based on our previous experience, supplies good and reliable results for oligomers determination. In addition, we also used UHPLC-HRMS for the confirmation of these compounds.

What did you find overall? And what did you find specifically in terms of the influence of storage times, store, and lot in the concentrations of packaging migrants?

In this study, we measured the monomers and we estimated oligomer concentrations in different food samples. Obtained results were in the part-per-billion concentration range. We also investigated the influence of storage time. In general, we observed that, although some migrants increased in concentrations based on the best-by-date of the products, others decreased in concentration in the food samples. However, because products could not be traced back, we don't have information on multiple factors that could impact migrant concentrations. These include temperature variations during storage or transport, lot variations, and overall food consistency. As a result, because conditions were not controlled, it is not possible to attribute observed changes to storage time in the studies samples. The method we used for this study was validated in the different food samples following the FDA FVM Guidelines for Single Laboratory Validation.

Do these results suggest the need for significant additional studies of a wide range of real food samples? What are your next steps in this work?

A controlled experiment on real food samples would enhance our understanding of the impact long-term storage has on real food. However, although it would be interesting to continue investigating the effects of long-term storage in real canned foods, that would require significant help and input from the food industry. A controlled experiment like this would require a diverse set of food cans, coated with the same coating, (polyesters, vinyl, acrylic-phenolic or epoxy) and placed in contact with different types of food (from aqueous food to fatty food), in a set of controlled time-temperature conditions (20, 30, and 40 °C for times up to 1.5 years) over a long period of time.

References

(1) R. Paseiro-Cerrato, S. MacMahon, C.D. Ridge, G.O. Noonan, and T.H. Begley, *J. Chromatogr. A* **1444,** 106–113 (2016). DOI: 10.1016/j.chroma.2016.03.038

(2) R. Paseiro-Cerrato, G.O. Noonan, and T.H. Begley, *J. Agric. Food. Chem.* **64,** 2377–2385 (2016). DOI: 10.1021/acs.jafc.5b05880

(3) R. Paseiro-Cerrato, J. DeVries, and T.H. Begley, *J. Agric. Food. Chem.* **65,** 294–2602 (2017). DOI: 10.1021/acs.jafc.7b00081

(4) R. Paseiro-Cerrato, L. DeJager, and T.H. Begley, *Molecules* **24,** 3123 (2019). DOI: 10.3390/molecules24173123

(5) R. Paseiro-Cerrato, L. DeJager, and T.H. Begley, *Food Control* **101,** 69–76 (2019). DOI: 10.1016/j.foodcont.2019.02.033

ABOUT THE AUTHORS

Rafael Paseiro-Cerrato is an expert in the field of food packaging materials, with a strong background in analytical chemistry, pharmacy, nutrition and food science. He actively leads research projects involving analytical methods development, validation and data analysis resulting in numerous publications. Since 2012, he has been working at the Center for Food Safety and Applied Nutrition within the US Food and Drug Administration in College Park, Maryland.

Ad Index

VIEWPOINTS

Living the Virtual Life: Transitions in Teaching and Research

Kevin A. Schug

My initial inclination was to write about something other than the current status of life, given the threats of coronavirus. However, after a month extension to the "shelter in place" was ordered by the United States government this past March, and after various e-discussions with friends and colleagues throughout the world who are experiencing similar challenges, I felt I might have something to offer to make life easier. I have just started using virtual meeting technologies for teaching and research meetings. As a novice with the use of various virtual platforms to deliver course content or conduct meetings, some of this might be obvious to many of you. However, if my little bit of experience can be of value to just a few, then it is probably worth sharing.

Before the whole coronavirus outbreak started, I had hardly familiarized myself with the two main virtual learning and communication systems that UT Arlington prefers—Microsoft Teams and Canvas. The reason for that is because I have found the preferences of the university to change a little too often. It seems like every couple of years, the university changes some platform. At first, I was gung ho to use any new platforms that the university desired. However, after many such changes, relearning and re-entering information into new profiles and management systems lost its luster. Then, during spring break, the announcement came that all classes would be moved online for the rest of the semester (now, it looks like this might be the case for UT Arlington for summer 2020, as well).

I have never been a huge proponent of online learning, especially as it relates to learning and teaching chemistry. While I believe that lecture course content can be delivered effectively online, there is an undeniable need for extensive hands-on laboratory experimentation in a physical science curriculum. Additionally, face-to-face lectures are likely more engaging for the students, but I have found that just with a few sessions under my belt, I can deliver class in a manner quite similar to that which I do in a classroom using Microsoft Teams.

I have had the honor of being a member of the University of Texas System Academy of Distinguished Teachers (ADT) since 2016. This collection of less than 40 of the top instructional faculty from across the UT System hold a wealth of knowledge about classroom and online instruction. As I was preparing to redesign my senior-level instrumental course for online delivery, I was heartened by an ADT blog from a colleague at UT El Paso (1). The main advice was to keep it simple and to try not to do too much. It is easy to become enamored (and overwhelmed) by the vast amount of technology and possibilities available for online instruction. The blog also emphasized keeping the channels open, and to not set the stakes too high. I found this all to be extremely valuable, and I thought of this advice often as I debated how to reorganize and deliver the course.

Ultimately, I decided that lecture course instruction would be done synchronously, using Microsoft Teams. I purchased a document camera. My regular lectures in the classroom primarily use the chalkboard, so that students are encouraged to write the material, as I do. Using the document camera and setting up a live event through Microsoft Teams allows me to convey the material in a very similar fashion. The live event is also recorded, in case anyone wants to watch it later, or again. The notes and other class materials can be scanned and uploaded in a repository in Teams (or in Canvas—I have preferred Teams, to this point). During lectures, students can ask questions verbally or through chat. Even after just a couple of lectures, I am heartened by how responsive the students are to using these different interfaces. They obviously have a lot more experience than I do. I actually seem to get more questions online than I did in person—although it is not clear whether that is because the material is harder to understand online, or if people feel more comfortable behind their computer screen. Regardless, it provides more opportunities to reiterate key points to the students. I recommend Microsoft Teams to anyone struggling with delivering synchronous course content. I have not yet embarked on creating any additional asynchronous lecture videos, but that would also be possible through Teams.

I hope everyone is staying safe and healthy, as well as practicing social distancing best practices. While I do not mind this extended time at home for now, it would be nice to get back into the lab and back on a regular schedule—and to visit again with friends, colleagues, and students, in person.

References

(1) https://www.utsystem.edu/sites/academy-of-distinguished-teachers/blog/remote-teaching-faculty-tips-2020-03-13

Kevin A. Schug is a Full Professor and the Shimadzu Distinguished Professor of Analytical Chemistry in the Department of Chemistry & Biochemistry at The University of Texas at Arlington. Direct correspondence to: kschug@uta.edu

CANNABIS SCIENCE CONFERENCE

Analytical, Medical, Cultivation & Hemp Tracks!

Attend Canna Boot Camp!

Build Brand Awareness

Share Information

Network & Learn

PORTLAND, OREGON

Aug.31-Sept.2, 2020

OREGON CONVENTION CENTER

The World's Largest and Most Technical Cannabis Science Event!

CANNABIS SCIENCE CONFERENCE

Hosted by Joshua Crossney & Andrea Peraza (CSC Events, LLC)

Sponsorship and exhibition opportunities are available.
Contact Andrea at Andrea@CannabisScienceConference.com

CANNA BOOT CAMP

CANNABIS EXPERTS

EXCITING EXHIBIT FLOOR

POSTER SESSIONS

INTERACTIVE PANELS

NETWORKING MIXERS

CULTIVATION EXPERTS

MEDICAL EXPERTS

CannabisScienceConference.com

SHIMADZU
Excellence in Science

New Series of GC-MS and GC-MS/MS Instruments

Combines Exceptional Accuracy and Sensitivity with Easy Maintenance and Operation

Utilizing the most advanced gas chromatograph available, and incorporating a variety of technological advances, Shimadzu's **new NX series of single-quad and triple-quad GCMS instruments** offers the highest performance levels available. And, with outstanding functionality, a variety of Smart features, easy maintenance, and intuitive software, these systems provide more efficient workflows, greater uptime, and reduced costs for higher productivity and better ROI for any laboratory.

Learn more about Shimadzu's NX Series Mass Spectrometers
Call (800) 477-1227 or visit us online at **www.ssi.shimadzu.com**
Shimadzu Scientific Instruments Inc., 7102 Riverwood Dr., Columbia, MD 21046, USA