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But My Peaks Are Not Gaussian! Part 3: Physicochemical Causes of Peak Tailing

David McCalley and Dwight R. Stoll

In this third part in the series, we discuss chemical causes of peak asymmetry, including effects from mass overload and slow desorption kinetics.

INTERVIEW

ANALYSIS FOCUS: FOOD ANALYSIS

Combating Olive Oil Fraud Using GC–IMS and FGC-Enose

Lewis Botcherby

The adulteration of high-quality foods is big business. Typical adulteration of olive oil involves the use of other types of oil, such as seed oils or pomace oils, the introduction of re-esterified oils, or the creation of mixtures with refined oils to create a lower quality product that can still be sold at a premium price. Fortunately, these processes can be easily detected using standard methods. However, fraudsters now seek more advanced methods using soft refined oils or oils with a tailored composition, making detection with existing procedures difficult. LCGC spoke to Michele Suman about novel screening and confirmatory analytical strategies he has investigated to regain the upper hand in the fight against olive oil adulteration.

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Daniel W. Armstrong
University of Texas, Arlington, Texas, USA

Günther K. Born
Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck, Austria

Deirdre Cabooter
Department of Pharmaceutical and Pharmacological Sciences, University of Leuven, Belgium

Peter Carr
Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, USA

Jean-Pierre Chervet
Antec Scientific, Zoeterwoude, The Netherlands

Jan H. Christensen
Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark

Adrian Clarke
Novartis, Switzerland

Danilo Corradini
Istituto di Cromatografia del CNR, Rome, Italy

Gert Desmet
Transport Modelling and Analytical Separation Science, Vrije Universiteit, Brussels, Belgium

John W. Dolan
LC Resources, McMinnville, Oregon, USA

Anthony F. Fell
Pharmaceutical Chemistry, University of Bradford, Bradford, UK

Attila Felinger
Professor of Chemistry, Department of Analytical and Environmental Chemistry, University of Pecs, Hungary

Paul Ferguson
AstraZeneca, UK

Francesco Gasparri
Dipartimento di Studi di Chimica e Tecnologia delle Substance Biologicamente Attive, Universita’ La Sapienza, Rome, Italy

Joseph L. Glajch
Molecular Pharmaceutics, Cambridge, Massachusetts, USA

Davy Guillaume
School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland

Jin Haginaka
School of Pharmaceutical and Pharmaceutical Sciences, Mokogawa Women’s University, Nishinomiya, Japan

Javier Hernández-Borges
Department of Chemistry (Analytical Chemistry Division), University of La Laguna Canary Islands, Spain

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Serenity Corp, Beaverton, Oregon, USA

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VTT Technical Research of Finland, Finland

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Kiyokatsu Jinno
School of Materials Sciences, Toyohashi University of Technology, Japan

Huba Kalász
Sommeliers University of Medicine, Budapest, Hungary

Hian Kee Lee
National University of Singapore, Singapore

Wolfgang Lindner
Institute of Analytical Chemistry, University of Vienna, Austria

Henk Lingenman
Faculteit der Scheikunde, Free University, Amsterdam, The Netherlands

Tom Lynch
Analytical consultant, Newbury, UK

Ronald E. Majors
Analytical consultant, West Chester, Pennsylvania, USA

Debby Mangelings
Department of Analytical Chemistry and Pharmaceutical Technology, Vrije Universiteit, Brussels, Belgium

Phillip Marrist
Monash University, School of Chemistry, Victoria, Australia

David Mccalley
Department of Applied Sciences, University of West of England, Bristol, UK

Robert D. McDowall
McDowall Consulting, Bromley, Kent, UK

Mary Ellen McNally
DuPont Crop Protection, Newark, Delaware, USA

Imre Molnár
Mohr Research Institute, Berlin, Germany

Luigi Mondello
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Department of Chemistry, University of Liverpool, Liverpool, UK

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Wayne State University, Detroit, Michigan, USA

Fred E. Regnier
Department of Biochemistry, Purdue University, West Lafayette, Indiana, USA

Harald Ritchie
Advanced Materials Technology, Chestor, UK

Koen Sandra
Research Institute for Chromatography, Kontich, Belgium

Pat Sandra
Research Institute for Chromatography, Kontich, Belgium

Peter Schoenmakers
Department of Chemical Engineering, Universiteit van Amsterdam, Amsterdam, The Netherlands

Robert Shellie
Deakin University, Melbourne, Australia

Yvan Vander Heyden
Vrije Universiteit Brussel, Brussels, Belgium

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Executive Director
Laura Bush
Ibush@mjhlifesciences.com

Editor-in-Chief
Mike Tessalone
mtessalone@mjhlifesciences.com

Managing Editor
Kate Jones
kjones@mjhlifesciences.com

Senior Graphic Designer
Senior Art Director
Gwendolyn Salas
gsalas@mjhlifesciences.com

Anita Bali
abali@mjhlifesciences.com

Senior Virtual Program Manager
Lindsay Glade
Lglade@mhn.com

Senior Project Manager
Amites Bakshi
abakshi@mjhlifesciences.com

Digital Production Manager
Sabina Abraham
sabrina@mjhlifesciences.com

Managing Editor, Special Projects
Kaylinn Chiarlo-Elter
kclier@mhn.com

Creative Director, Publishing
Melisa Ferinni
mferinni@mmigroup.com

Senior Art Director
Gwendolyn Salas
gsalas@mjhlifesciences.com

Graphic Designer
Courtney Soden
csoden@mjhlifesciences.com

Corporate
President & CEO
Mike Hennessy Jr
mhennessy@mjhlifesciences.com

Vice Chairman
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RISING STARS OF SEPARATION SCIENCE
Jelle De Vos
The Column interviewed Jelle De Vos about his recent work to develop a chromatographic method to characterize nucleocapsid proteins from SARS-CoV-2, and the development of 3D-LC as a separation concept.
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QUESTIONS OF QUALITY
It’s Qualification, But Not As We Know It?
Qualification and calibration of high performance liquid chromatography (HPLC) chromatographs is a regulatory requirement, but how prescriptive should guidance be?
Read more: https://bit.ly/3cDwWKu

NEWS
Analyzing PFAS in Human Breast Milk Using Lipidomics
Lipidomics researchers from Örebro University have developed a method to analyze levels of PFAS in human breast milk using UHPLC-QTOF-MS.
Read more: https://bit.ly/3r84jxg

December Update
In this issue of LCGC Europe, we turn our attention to groundwater contamination. This issue has a range of potential sources and it can be difficult to distinguish the true cause. Compounds can leak into groundwater via old or damaged wastewater treatment plant infrastructure. Similar contaminant impacts can also arise from other sources, such as agriculture, and it can be difficult to distinguish the true source of such contamination, especially where wastewater treatment plants are located in agricultural areas. Liquid chromatography triple quadrupole mass spectrometry (LC–QQQ–MS) was used to generate unique fingerprints of pollution to differentiate groundwater contamination from wastewater and agriculture sources in sites where both sources may be present.

This month’s LC Troubleshooting presents the final part of our series on peak asymmetry. Asymmetric peak shapes can be caused by several physicochemical phenomena, such as analyte adsorption to different types of sites within the stationary phase, and overload tailing. Understanding these phenomena can help identify the cause of asymmetry, which in turn dictates the troubleshooting approach.

The adulteration of high-quality foods is big business and fraudsters are constantly seeking out ways to create a lower quality product that can still be sold at a premium price. We spoke to Michele Suman about a novel screening and confirmatory analytical strategies he has investigated to regain the upper hand in the fight against olive oil fraudsters.

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Mike Hennessy Jr,
President and CEO, MJH Life Sciences™
The water industry in most countries must comply with strict legislative controls on what they can and cannot discharge into the environment. Such legal requirements apply not only to the final effluent but also include potential impacts on the local environment from the plant itself. Such impacts could occur from leakage of untreated or partially treated wastewater from treatment infrastructure (for example, from old or damaged pipework and/or improper or damaged liners), lined treatment lagoons, and/or discharge of untreated effluents during periods of high-water flow, for example, after heavy rain. However it gets there, insufficiently treated wastewater can be a source of several differing types of pollutants to the environment, including synthetic chemicals, such as Contaminants of Emerging Concern (CECs) and natural inorganic substances, such as nutrients, primarily nitrogen and phosphorous.

Groundwater CSI: Unravelling Pollution Sources in Complex Environments with Liquid Chromatography Triple Quadrupole Mass Spectrometry

Oliver A.H. Jones¹, Matthew Currell², and William McCance²,³ ¹Australian Centre for Research on Separation Science, School of Science, RMIT University, Bundoora West Campus, Victoria, Australia, ²School of Engineering, RMIT University, Melbourne, Victoria, Australia, ³BlueSphere Environmental Pty Ltd, Southbank, Victoria, Australia

Wastewater treatment plants (WWTP) are used worldwide to purify domestic and industrial wastewater before it is returned to the environment. Even after treatment, wastewater may still contain a variety of contaminants, including nutrients (usually nitrogen and phosphorus) and organic pollutants, such as endocrine disruptors and pesticides. These compounds can leak into groundwater via old and/or damaged infrastructure, leaching from biosolids storage areas and/or release of effluents. However, similar contaminant impacts can come from other sources, such as agriculture. It is often very difficult to distinguish the true source of such contamination, especially where wastewater treatment plants are located in agricultural areas. Robust and sensitive techniques are needed to characterize impacts where there are multiple potential sources. This study analyzed a variety of synthetic chemicals using liquid chromatography triple quadrupole mass spectrometry (LC–QQQ–MS) to generate unique fingerprints of pollution. These were used to distinguish the impact on the local groundwater of a WWTP in southeast Victoria (Australia) from that of local agriculture. The use of such novel tracers could become a valuable tool in environmental monitoring, management, and remediation in the future.

KEY POINTS

- Groundwater contamination is a worldwide issue with a range of potential sources.
- Standard tracer compounds often cannot distinguish between wastewater and agricultural sources of contamination.
- CECs have been proposed as novel groundwater tracers but may not always be effective in complex environments.
- Specific CEC ratios can give more information than raw concentrations for contaminant source differentiation.

The water industry in most countries must comply with strict legislative controls on what they can and cannot discharge into the environment. Such legal requirements apply not only to the final effluent but also include potential impacts on the local environment from the plant itself. Such impacts could occur from leakage of untreated or partially treated wastewater from treatment infrastructure (for example, from old or damaged pipework and/or improper or damaged liners), lined treatment lagoons, and/or discharge of untreated effluents during periods of high-water flow, for example, after heavy rain. However it gets there, insufficiently treated wastewater can be a source of several differing types of pollutants to the environment, including synthetic chemicals, such as Contaminants of Emerging Concern (CECs) and natural inorganic substances, such as nutrients, primarily nitrogen and phosphorous.

CECs is a general term covering a broad class of compound classes, including pharmaceuticals, personal care and/or household cleaning products, flame retardants, and agricultural chemicals. They are classified as “emerging” because they have often, though not always, only recently been identified in various environmental matrices (usually at the ng/L to ug/L levels) through advances in analytical methodology, or
in many cases are not yet regulated or monitored. Many are
calcitrant and, since wastewater treatment plants (WWTPs)
are generally not specifically designed to remove them,
they can pass through WWTPs and into the environment.

Despite many CECs being predominantly unregulated, a number
of these compounds are beginning to attract attention due to their
potential impacts on both human health and the environment,
pertinent examples being per- and polyfluorinated substances
(PFAS) (1) and disinfection by-products (2). Some CECs are
new, such as the recently identified 6PPD-quinone (reported to
be the cause of major die-offs of U.S. Pacific Northwest coho
salmon (Oncorhynchus kisutch)) (3). Others are perhaps better
classed as contaminants of re-emerging concern since their
presence has been known about for many years but they have
only recently come to mainstream attention. For example, the
presence of pharmaceutical substances in the environment has
been reported since the 1970s (4). CECs are consistently found
in groundwater, surface water, municipal wastewater, drinking
water, soil, and food sources. The persistence of these chemicals
is a threat because they can potentially bioaccumulate.

Although it does not get as much press attention as CECs,
nutrient pollution is a widespread, costly, and challenging
environmental problem that can have diverse and far-reaching
impacts on public health, the environment, and the economy.

Nutrients such as nitrates and phosphates are naturally
occurring compounds, but the way we concentrate and use
them isn’t and this is what leads to problems. Excess nutrients
in water bodies can cause algal blooms that severely reduce or
eliminate oxygen in the water, potentially leading to the deaths
of large numbers of aquatic organisms. Some algal blooms
can produce toxins that can cause serious illness in humans
and wildlife. High levels of nitrate in drinking water can also be
dangerous to health, especially for infants and pregnant women.

Nutrient pollution can come from agriculture, a significant
source of pollution in many countries (5). CECs may also enter
the environment from farming if recycled water is used for

![Figure 1](image1.png)

**FIGURE 1**: Groundwater monitoring bore with possible sources of N and P contamination in (a) background and (b) groundwater samples ready for solid-phase extraction in the laboratory. Image credit W. McCance.

![Figure 2](image2.png)

**FIGURE 2**: Hydrological overview of the study site showing total nitrogen concentration. It is impossible to differentiate nitrogen from the WWTP (outlined in red in the upper centre of the figure) from that coming from nearby market gardens (outlined in yellow to the right of the image). The dashed white arrow shows the direction of groundwater flow. Image credit W. McCance.

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irrigation and/or biosolids are used without adequate precautions (biosolid regulations usually don’t regulate CECs so the presence and concentration of these compounds in biosolids often isn’t known). Conventional monitoring methods such as major ions (analyzed via ion chromatography [IC]) and stable isotopes (analyzed via isotope ratio mass spectrometry or cavity ring-down spectrometry) are therefore often unable to distinguish between individual contaminant sources. In complex environments with multiple potential sources, this can be a particularly challenging task. This is a problem, as constraining the nature and extent of contamination is essential if remediation actions are to be fair and effective. This is particularly relevant for WWTPs located in urban, peri-urban, or intensively cultivated agricultural areas. In such areas, multiple potential contamination sources, such as livestock, fertilizers, wastewater irrigation, and domestic septic systems, may all contribute similar contaminants and so make the identification of the origins and pathways of contamination—and hence, determining suitable management and/or remediation strategies—exceedingly difficult (6). What is needed to overcome current limitations is a way to fingerprint pollution more definitively in order to identify the source. This is the objective of the current work.

Recent advances in separation science techniques have enabled the potential use of CECs to provide a more definitive assessment of contaminant sources, plume delineation, and even (potentially) indicating the age of contamination (recent versus legacy), even when present at trace levels (7–10).

FIGURE 3: Plotting the ratio of carbamazepine to simazine resulted in the successful identification of three contaminated hotspots distinct from background agricultural input. Circles show groundwater bore sampling sites. Triangles show infrastructure sample sites. Image credit W. McCance.

The suitability of compounds to act as novel groundwater tracers is evaluated according to four key criteria (6):
1. Have a sufficient presence in raw wastewater and/or treated effluents and/or groundwater to be detected regularly;
2. Diagnostic of WWTP and/or agricultural impacts as opposed to other potential off-site contamination sources;
3. Persistent in the subsurface environment; and
4. Amenable to rapid and sensitive analysis (since fast analysis is required by industry).

Several CECs including pharmaceuticals (carbamazepine, crotamiton, primidone, atenolol sulfamethoxazole, and acesulfame), pesticides (cyclamate and simazine), and artificial sweeteners (acesulfame, sucralose, and saccharin) have been identified to meet a number of these criteria. However, in many cases these are not all the subject of regular monitoring—if they are monitored at all. Groundwater can also be surprisingly complex as an analytical matrix, resulting in matrix effects and a reduction in analytical sensitivity. The study therefore required the development of advanced analytical methods for the trace analysis of these compounds in environmental matrices, in addition to a detailed understanding of the site and the associated hydrogeology.

The aim of the work was to be able to differentiate groundwater contamination from wastewater and agriculture sources in sites where both sources may be present.

Methods

Study Site: The study was conducted at a WWTP located approximately 100 km south of the city of Melbourne, in South-Eastern Australia, that sits on an aquifer subject to numerous extractive uses, mostly associated with local agricultural activities. Groundwater within the region flows from the upper portions of the catchment—with predominantly agricultural land uses—beneath the WWTP and towards a conservation area and groundwater-dependent wetland ecosystem located adjacent to the site. Potential also exists for a local groundwater flow component towards a small stream (an ephemeral surface water feature), located to the west of the site. The area is well served with groundwater monitoring bores to enable the measurement of water flows and monitoring of possible contamination. Distinguishing potential contamination sources in this area is further complicated by the fact that treated recycled water from the plant is used for irrigation purposes in the agricultural area. This recycled water (and any contaminates within it) may therefore percolate down to the underlying groundwater.

Analytical Methods: CECs represent some of the most challenging compounds in environmental chemistry, due to their diverse occurrence, properties, fate, and concentration in different environments. These compounds are generally present in the µg/L
or ng/L concentration range or lower. This means that suitable tracer compounds may occur in groundwater at levels close to their detection limits, requiring sensitive detection methods and (often) sample pretreatment, not to mention the often-complex matrix, such as wastewater, with many competing compounds. As a result, gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS), or tandem mass spectrometry (MS/MS) are most widely used in the determination of CECs.

Samples for CEC analysis (carbamazepine, crotamiton, primidone, atenolol, sulfamethoxazole, acesulfame, sucralose, saccharin, cyclamate, and simazine) were field filtered through 0.45-μm in-line filters following the collection of samples for routine water quality analysis and collected in pre-cleaned amber glass bottles (see Figure 1). Amber glass was used to reduce the degradation of light-sensitive compounds during transport. All bottles were preconditioned with an ethanol, ultrapure water, and acetone triple rinse followed by oven baking at 150 °C for a minimum of 2 h. Once collected, samples were preserved below 4 °C and transported to RMIT University’s analytical laboratory for analysis.

The solid-phase extraction (SPE) method was based on the validated method of Van Stempvoort (11) with minor modifications. All SPE cartridges were first preconditioned using 2 × 3 mL of methanol followed by 2 × 3 mL of Milli-Q water, with samples drawn through the cartridges at a rate of ~2.5–5 mL/min. A 900-mL aliquot of each sample was then spiked with analytical surrogates (deuterated and/or 13C labelled versions of the compounds under study) prior to extraction through Oasis HLB 6 cc (500 mg, Waters) SPE cartridges using a vacuum manifold.

Once the sample was passed through, the SPE cartridges were allowed to dry under vacuum for approximately 5 min and then the target compounds were eluted with 2 × 1 mL 50:50 methanol–acetonitrile followed by 2 × 1 mL 75:25 acetonitrile–Milli-Q water source to ensure all compounds were removed. Eluents were collected in 15 mL plastic centrifuge tubes and evaporated to near dryness under nitrogen. Once dry, extracts were then reconstituted using the starting mobile phase of the liquid chromatography system, as shown in Table 1.

Once extracted, samples were analyzed using liquid chromatography hyphenated to triple quadrupole mass spectrometry (LC–QQQ–MS). This system was composed of an Agilent 1260 Infinity LC system, coupled to an Agilent 6490 Triple...
Quad MS using positive and negative ion monitoring mode. The operating and multiple reaction monitoring (MRM) conditions are outlined in Tables 1 and 2.

**Results**

It was impossible to use standard tracers such as nutrients (N and P) to distinguish wastewater-derived sources of groundwater contamination in the vicinity of the WWTP under study. This is illustrated in Figure 2, where it can clearly be seen that while there appears to be elevated nitrogen in the groundwater around the WWTP, there is also a similar level of elevated nitrogen up-gradient from the plant coming from the surrounding agricultural land.

Of the 10 compounds selected for analysis, only three were able to be quantified in the sample matrix, with signal-to-noise ratios sufficient to enable reliable detection and quantification. These were the pharmaceuticals, carbamazepine and sulfamethoxazole, and the pesticide, simazine.

Simazine was the most commonly detected compound, being identified in all samples. Carbamazepine was detected in 13 of 20 groundwater samples and sulfamethoxazole in one. Concentrations of these compounds ranged from <10 to 175 ng/L and groundwater reporting concentrations ranged from <1 to 253 ng/L (Table 3). The observed concentrations are broadly consistent with previous research on CECs in groundwater affected by WWTP sites (11).

Site effluent had an elevated concentration of all CEC compounds (carbamazepine, simazine, and sulfamethoxazole), with a median ΣCECs of 282 ng/L, compared to treatment infrastructure (median ΣCECs of 119 ng/L) and on-site groundwater (median ΣCECs of 38 ng/L).

Groundwater bores located down-gradient of the WWTP recorded elevated concentrations of carbamazepine (ranging from 20 to 252 ng/L) compared to the remaining on-site groundwater bores (ranging from <1 to 15 ng/L). Up-gradient bores reported concentrations below the limit of quantification (LOQ) in all cases. Groundwater bores upstream of the WWTP recorded elevated concentrations of simazine (ranging from 14 to 39 ng/L), compared to the remaining on-site groundwater bores (ranging from 2 to 12 ng/L). Up-gradient bores also reported detectable simazine concentrations ranging from 8 to 20 ng/L. This suggests both an up-gradient source of simazine in the region, perhaps related to agricultural land uses or potentially the use of recycled water, as well as some additional input related to the WWTP. This is consistent with previous work that suggests wastewaters can be an important source of pesticides in the aquatic environment (12). This may occur due to spills or disposal of excess pesticides into sewer networks or groundwater infiltration into shallow sewer pipes. Use of recycled water for irrigation can lead to this water, and any associated CECs, percolating down to the groundwater system. Similarly, agricultural chemicals are often present in domestic sewage; sources can include run-off from domestic gardens and housing developments, and incorrect disposal to storm drains.

The compound with the highest source specificity was carbamazepine, which was found to be more diagnostic of WWTP impacts compared to the herbicide simazine, which came from agricultural and wastewater-derived sources (12). The relatively lower detection frequency of sulfamethoxazole may have been due to the complex sample matrix (groundwater, site effluent, and partially treated effluent). In other sites, the CECs from wastewater and agriculture will likely differ depending on the catchment. For example, simazine is banned or regulated in some countries (though not Australia) so likely won’t be found in groundwater in such places, though other pesticides would be present instead.

The CECs likely to be present in wastewater at a specific site or area can often be identified based on the knowledge of what chemicals are used in the catchment and can be included by a water utility’s own routine on-site monitoring and/or compliance testing. The best way to find out the likely...
agricultural chemicals to be present in a specific agricultural area (assuming there is no background data available) is to look at the sales data from the local suppliers, or alternatively look at what crops are grown in the local area and determine the most commonly used pesticides for those crops. These data can save significant time undertaking untargeted analysis but are not always easy to come by.

Given the complex site history (that is, different contaminant sources over time), ubiquitous occurrence of simazine in the groundwater environment, and likely source specificity of carbamazepine, the ratio of carbamazepine to simazine (carb:sim ratio) was used as a novel way to delineate the wastewater-derived impacts from those of agriculture and/or recycled water use. The carb:sim ratio was found to vary across the site, with treatment infrastructure and site effluent reporting the highest median ratios of 2.4 and 2.3, respectively, compared to that of on-site groundwater bores (median of 0.98) and up-gradient groundwater bores (median of 0.11). Groundwater bores located adjacent to or down-gradient of the treatment assets generally exhibited elevated ratios, ranging from 0.62 to 9.3 (median of 2.8), compared to the remaining on-site groundwater bores, which ranged from 0.002 to 0.50 (median of 0.082). As such, the pharmaceutical carbamazepine was clearly elevated relative to the herbicide simazine in samples down-gradient of the WWTP, allowing for distinct source separation (where nutrient concentrations were ambiguous on their own). This is illustrated in Figure 3.

As shown in Figure 3, the carb:sim ratios suggest the existence of three contaminant “hot spots”; two located down-gradient of the site—consistent with historical impact that has since migrated a significant distance from the WWTP—and one stemming from the current treatment assets—reflecting recent impacts. Ratios in the down-gradient “hot spots” (ranging from 2.7 to 9.3) were particularly elevated relative to those directly adjacent to or down-gradient of current treatment infrastructure. This suggests differences in the relative input of these compounds over time (assuming both compounds behave relatively conservatively), which is consistent with the site development timeline and possibly a reflection of changes in effluent composition following infrastructure upgrades. The novelty of this work is that a) specific CEC ratios can be used for pollution source differentiation, and b) the data you can then acquire about groundwater impacts helps to inform management practices once sources are differentiated. The findings have significant implications for future remedial actions, if required, and provide a method with which to
further separate the regional agricultural impacts from WWTP site-derived impacts. There is also the question of whether the contaminant plume is of environmental concern as well as a useful marker of wastewater impact on groundwater. The greater Melbourne area ultimately drains into Port Philip Bay (a horse head-shaped enclosed bay on the central coast of Southern Victoria, with the city of Melbourne located on the northern tip). If we assume that all the CECs in the groundwater plume ended up in Port Philip Bay and the contribution/leakage from the plant was also constant, then given the groundwater flow rate and the concentrations of CECs we can ask what would be the contribution of the plume of each CEC per year to the bay?

Based on groundwater discharge estimates from C.J. Otto in 1992 (the most recent data), groundwater discharge to Port Phillip Bay is estimated at $5.5 \times 10^7$ m$^3$/year (13). The estimated seepage velocity of the groundwater is 13–95 m/year and there is a distance of approximately 5 km to the bay from the WWTP. This means it would take between 52 and 384 years for the contaminants to reach Port Philip Bay—assuming no degradation or other interactions are occurring (whereas in reality, the plume TABLE 2: LC–QQQ–MS MRM conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESI Mode</th>
<th>Precursor</th>
<th>Ion 1</th>
<th>Ion 2</th>
<th>Dwell (1, 2)</th>
<th>Fragmentor (V)</th>
<th>Collision Energy (1, 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>+</td>
<td>267.31</td>
<td>145.1</td>
<td>56.1</td>
<td>100, 20</td>
<td>380</td>
<td>24, 20</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>+</td>
<td>237.28</td>
<td>194.2</td>
<td>193.2</td>
<td>100, 20</td>
<td>380</td>
<td>20, 40</td>
</tr>
<tr>
<td>Primidone</td>
<td>+</td>
<td>219.26</td>
<td>91.1</td>
<td>162.1</td>
<td>100, 20</td>
<td>380</td>
<td>28, 8</td>
</tr>
<tr>
<td>Simazine</td>
<td>+</td>
<td>202.67</td>
<td>125.1</td>
<td>133.1</td>
<td>100, 20</td>
<td>380</td>
<td>17, 17</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>+</td>
<td>254.29</td>
<td>156.1</td>
<td>92.1</td>
<td>100, 20</td>
<td>380</td>
<td>12, 20</td>
</tr>
<tr>
<td>Acesulfame</td>
<td>-</td>
<td>162.00</td>
<td>82.1</td>
<td>78.1</td>
<td>100, 20</td>
<td>380</td>
<td>12, 40</td>
</tr>
<tr>
<td>Cyclamic Acid</td>
<td>-</td>
<td>178.20</td>
<td>178.0</td>
<td>80.0</td>
<td>100, 20</td>
<td>380</td>
<td>0, 28</td>
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<tr>
<td>Saccharin</td>
<td>-</td>
<td>182.20</td>
<td>42.0</td>
<td>105.8</td>
<td>100, 20</td>
<td>380</td>
<td>44, 16</td>
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<tr>
<td>Sucralose</td>
<td>-</td>
<td>395.00 / 396.60</td>
<td>359.2</td>
<td>397.0</td>
<td>100, 20</td>
<td>380</td>
<td>8, 0</td>
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</table>

Surrogates

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESI Mode</th>
<th>Precursor</th>
<th>Ion 1</th>
<th>Ion 2</th>
<th>Dwell (1, 2)</th>
<th>Fragmentor (V)</th>
<th>Collision Energy (1, 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine-\textsuperscript{13C6}</td>
<td>+</td>
<td>243.23</td>
<td>200.2</td>
<td>199.2</td>
<td>100, 20</td>
<td>380</td>
<td>16, 40</td>
</tr>
<tr>
<td>Cyclamic Acid-d11</td>
<td>-</td>
<td>189.3</td>
<td>79.9</td>
<td>100</td>
<td>380</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Simazine \textsuperscript{13C3}</td>
<td>+</td>
<td>205.61</td>
<td>206.1</td>
<td>106</td>
<td>100, 20</td>
<td>380</td>
<td>0, 12</td>
</tr>
<tr>
<td>Sulfamethoxazole-\textsuperscript{13C6}</td>
<td>+</td>
<td>260.22</td>
<td>98.2</td>
<td>114.2</td>
<td>100, 20</td>
<td>380</td>
<td>28, 24</td>
</tr>
</tbody>
</table>

TABLE 3: Summary of CEC concentrations (all concentrations reported in ng/L)

<table>
<thead>
<tr>
<th>Monitoring Group</th>
<th>Result Type</th>
<th>Carbamazepine</th>
<th>Simazine</th>
<th>Sulfamethoxazole</th>
<th>Carb:Sim Ratio</th>
<th>CECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Infrastructure</td>
<td>Min</td>
<td>45</td>
<td>12</td>
<td>41</td>
<td>1.0</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>50</td>
<td>48</td>
<td>41</td>
<td>3.7</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>48</td>
<td>30</td>
<td>41</td>
<td>2.4</td>
<td>119</td>
</tr>
<tr>
<td>Site Effluent</td>
<td>Min</td>
<td>146</td>
<td>68</td>
<td>28</td>
<td>2.2</td>
<td>242</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>175</td>
<td>70</td>
<td>78</td>
<td>2.5</td>
<td>323</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>161</td>
<td>69</td>
<td>53</td>
<td>2.3</td>
<td>282</td>
</tr>
<tr>
<td>Up-Gradient Bores</td>
<td>Min</td>
<td>&lt;1</td>
<td>8</td>
<td>&lt;10</td>
<td>0.030</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>&lt;1</td>
<td>20</td>
<td>&lt;10</td>
<td>0.18</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>&lt;1</td>
<td>12</td>
<td>&lt;10</td>
<td>0.11</td>
<td>23</td>
</tr>
<tr>
<td>On-Site Groundwater Bores</td>
<td>Min</td>
<td>&lt;1</td>
<td>2</td>
<td>&lt;10</td>
<td>0.0020</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>253</td>
<td>39</td>
<td>12</td>
<td>9.3</td>
<td>304</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>15</td>
<td>11</td>
<td>12</td>
<td>0.98</td>
<td>38</td>
</tr>
</tbody>
</table>
The contamination of land and groundwater resources creates a potentially major public health and environmental burden on current and future generations. Accurately identifying the sources, pathways, and impacts of groundwater contamination can be highly challenging due to complex geology, land use, and biogeochemical processes in aquifers. By working with Victoria’s water industry and other partners, this project developed new techniques to identify and separate groundwater contamination arising from different sources within complex environments. A method to distinguish contamination from wastewater treatment plants and intensive agriculture was developed, combining the analysis of trace micropollutants with groundwater monitoring and knowledge of background hydrology.

The use of novel groundwater tracers, particularly the carbamazepine:simazine ratio, was found to provide greater insight into the nature and extent of a groundwater contamination plume. This was particularly useful in constraining potential historic (“legacy”) impacts from more recent site-derived impacts, which was not possible using conventional methods and/or environmental tracers alone.

This approach not only assisted in discriminating between up-gradient and site-derived impacts but also provided insights into historic (“legacy”) and more recent contamination sources and their changing characteristics. The results were consistent with the existence of multiple on-site sources, with differing timescales and source concentrations. This work has enhanced the outcomes of routine monitoring and enabled a better understanding of the nature and extent of groundwater impacts, enabling ongoing monitoring, management, and remedial actions to be suitably targeted. The project has also provided wastewater treatment plant operators and practitioners with a set of tools to help to better identify contamination in complex environments, and demonstrated the essential contribution that high-end chromatography and mass spectrometry make to modern environmental science and management.

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References
Although symmetric peaks with Gaussian shapes are predicted by models of the chromatographic process, “perfect peaks” are not often observed outside of textbooks. Several physicochemical phenomena can lead to asymmetric peak shapes, including analyte adsorption to different types of sites within the stationary phase and overload tailing, which may involve a variety of factors. Understanding these phenomena can help identify whether the cause of asymmetry is most likely to have a physical or chemical origin, which in turn dictates which troubleshooting steps to start with when dealing with poor peak shapes.

In the first two parts of this series of “LC Troubleshooting” articles, I’ve written about basic concepts in peak asymmetry (1) and physical problems that can lead to fronting or tailing peaks (2). Although there are many ways things can go wrong in a purely physical sense that will lead to asymmetric peaks, addressing these problems, or even preventing them altogether, is generally more straightforward than dealing with causes of asymmetry that have chemical components. As a separation science community, we understand a lot about chemical causes of peak asymmetry, but there are some observations for which we don’t have clear explanations, and this is an open area of research in both academic and industrial laboratories. For this third part of this series, I’ve asked Professor David McCalley to join me to address some causes of peak asymmetry that have chemical components, discussing both the aspects we understand, and those where there is less clarity. David has studied chemical causes of poor peak shape in both reversed-phase and HILIC separations, and is one of the world’s foremost experts on the topic.

Dwight Stoll

A large majority of the literature describing studies of physicochemical causes of peak asymmetry in liquid chromatography (LC) has been focused on reversed-phase columns prepared with stationary phases built upon silica-based substrates. This focus on reversed-phase columns does not mean these problems are not important for other separation modes or stationary phases built upon other substrates. However, the primary focus of this instalment is on reversed-phase separation conditions and stationary phases involving silica particles because of their predominant use in LC.

In Part 1 of this series, we focused mainly on the type of peak tailing we refer to as exponential tailing, which is where the observed peak shape exhibits a kind of mixture of Gaussian and exponential distribution shapes that can be modelled nicely using a convolution of the two distributions. Some physicochemical causes of peak tailing lead to this type of exponential tailing. However, other causes lead to a different type of peak shape, which is referred to here as overload tailing. This shape is also sometimes referred to as a “shark fin” or “sailboat”. A comparison of the two shapes is shown in Figure 1. The distinct character of these peak shapes can be quite helpful for diagnosing the cause of peak tailing in many cases.

In Part 1, we discussed two metrics used to quantify the extent of peak asymmetry—the asymmetry factor (A_s), and the tailing factor (TF). The apparent column efficiency (that is, plate number N) can also be used...
to quantify the effect that peak asymmetry has on making the peak broader. This is also illustrated in Figure 1. In the case of gradient elution separations, the peak capacity—roughly a measure of how many compounds could be separated in a given analysis if the peaks are neatly arranged side-by-side without any wasted space or peak overlap—can also be used to quantify the deterioration in separation in performance because of peak asymmetry.

**Exponential Tailing: Causes and Remedies**

The exponential type of peak tailing illustrated in Figure 1 is most commonly observed when working with the protonated and positively charged form (BH⁺) of amine-containing analytes, and silica-based stationary phases for reversed-phase LC. Although this particular situation has been discussed several times in prior “LC Troubleshooting” articles (3,4), it is useful to briefly review the main points again because the chromatographic behaviour and remedies for this cause of peak tailing are different from other causes. In other words, one has to properly diagnose the cause of the tailing before selecting a remedy that is appropriate. Most silica-based stationary phases for reversed-phase LC are prepared by covalently bonding an organosilane carrying the stationary phase ligand (such as C18) to silanol (⁻Si-OH) groups at the surface of the silica particle. In spite of advances in methods over the years to convert as many of the surface silanols to siloxanes carrying the stationary phase ligand as possible, it is practically very difficult to convert all of them, which means that after the bonding step, a significant population of unreacted, free silanols
will remain. There may also be another population of unreacted silanols inaccessible to analytes that do not take part in retention or tailing processes. Silanol groups are Bronsted acids and can donate a proton to the mobile phase to produce an anionic \(\mathrm{SiO^-}\) group. A typical \(\mathrm{pK_a}\) for this dissociation reaction is approximately five, but can be greatly affected by the type of silanol group (for example, the local bonding of isolated, geminal, or vicinal silanols) and the purity of the bulk silica. Most notably, metal impurities in the silica can significantly depress the \(\mathrm{pK_a}\), leading to substantial ionization of silanol groups in mobile phases buffered as low as pH 3 or less. Readers interested in learning more about the chemistry of silica substrates can find additional material in the literature (5).

Analytes that have both some lipophilic character and a positive charge (for example, an ionized amine, \(\mathrm{BH}^+\)) can then interact with the stationary phase in very different ways. The electrostatic interaction between \(\mathrm{BH}^+\) and \(\mathrm{SiO^-}\) will be energetically strong, but in most cases the surface concentration of accessible \(\mathrm{SiO^-}\) sites will be low compared to the concentration of lipophilic ligands that give the material its reversed-phase character. On the other hand, the dispersive interaction between the lipophilic parts of the analyte and the stationary phase ligand is energetically relatively weak. These differences in interaction strengths and site concentrations can lead to exponential tailing like that shown in Figure 1.

The depression of silanol \(\mathrm{pK_a}\) by metal impurities in the silica is most serious with older “Type A” silicas. Modern manufacturing methods used to make purer “Type B” silicas have reduced the seriousness of the problem with modern reversed-phase LC columns, however the mitigation of this problem has been accompanied by a loss of diversity in the selectivity of C18 phases. In other words, as the silica substrates used for making reversed-phase LC phases have become purer, the selectivities of the resulting phases have also become more homogeneous (6).

An important characteristic of exponential tailing caused by the interaction of cationic analytes with anionic silanol sites is that the peak shape may improve as more analyte mass is injected. When a very low mass of analyte is injected, the anionic silanol sites play a major role in the observed retention of the analyte. However, as more mass is injected, these sites become saturated, and the less energetic but more abundant lipophilic interaction sites play a more important role in determining the peak shape, which appears to improve. In cases where the observed tailing appears to be the exponential type, and the analyte is likely positively charged in the mobile phase, decreasing the mobile phase pH may help improve the peak shape. The extent to which it must be decreased to make a difference will depend on the silica type. With Type B silicas, going down to pH 3 is often sufficient, but with Type A silicas further decreasing to pH 2 may help.

**Overload Tail**: **Causes and Remedies**

The type of peak tailing referred to as overload tailing—also illustrated in Figure 1—is characterized by behaviour quite different from exponential tailing. Whereas with exponential tailing better peaks are observed when more mass is injected, with overload tailing better peaks are observed when less mass is injected. And whereas with exponential tailing injecting more mass generally causes the peak height to increase without significantly changing the retention time at the peak apex, with overload tailing injecting more mass usually leads to a significant decrease in retention time measured at the peak apex, and the peak shapes themselves are distinctive with a “shark fin” like appearance. It is important to recognize that both exponential and
overload tailing may occur together for a specific solute in a given separation, with the resulting peak shape being a mixture of those shown in Figures 1(a) and 1(b).

Figure 2(a) shows that the peak shape for propranolol—a drug molecule with a strongly basic secondary amine functional group (pKₐ for the protonated form is approximately 9)—is not too bad when 0.05 µg are injected, but just doubling the mass injected to 0.10 µg leads to a significant shift of the peak apex to the left, and a clear appearance of the characteristic "shark fin" peak shape. A useful way of quantifying the deterioration in the peak shape with increasing injected analyte mass is to plot the apparent plate number (N) vs. the injected mass, as shown in Figure 3. Here, we see that the decrease in plate number is less than 10% for propranolol when moving from 0.01 to 0.05 µg injected mass. However, injecting any more mass results in dramatic losses in efficiency, and when 3 µg is injected, only about 10% of the original efficiency remains (that is, 90% has been lost). Similar results were obtained with the protonated base nortriptyline. On the other hand, injecting increasing masses of the non-ionogenic compounds caffeine, 3-phenylpropanol, and phenol over the range of 0.01 to 3 µg does not result in decreased efficiencies; measurable losses in the plate number are not observed until approximately 7 µg are injected (7).

Up to this point, these results appear to be consistent with a mechanism similar to that described above that involves two different sites of interaction between the analyte and stationary phase, characterized by very different interaction energies; indeed, such an overloading mechanism was proposed by Guiochon in a comprehensive series of papers (8), although the physical identity of these sites was not exactly specified. However, the same type of phenomenon observed with propranolol is also observed experimentally with the strongly acidic analyte 2-naphthalenesulphonic acid—as shown in Figure 2(b)—which is deprotonated and anionic at most pH values in the mobile phase. The mechanism described above where the anionic silanol site plays a central role in the tailing peak shapes observed for cationic amine-containing analytes cannot easily be used to explain the observation of overload tailing for the sulfonic acid, nor the similarities in overloading behaviour obtained when organic polymer columns were used instead of silica-octadecysilsyl (ODS). A different mechanism has been proposed that involves mutual repulsion (or partial ionic exclusion from the stationary phase)
phase pores) of analytes of the same charge that leads to peak broadening and the types of peaks shapes shown in Figure 2 (9). The central idea is that the first analyte molecules that adsorb to the stationary phase create a kind of island of immobilized charge. In the absence of a significant concentration of buffer ions in the mobile phase, additional analytes of the same charge travelling downstream from the column inlet are repelled by the analyte ions already adsorbed to the stationary phase, and will continue travelling downstream until they encounter a stationary phase zone that does not already have analyte ions bound. This event broadens the peak and gives rise to the peak shapes shown in Figure 2. This type of mechanism can be used to explain results observed for both cationic and anionic analytes, and stationary phases based either on silica substrates or other materials.

Further study of the conditions that lead to overload tailing has also revealed some potential remedies to the problem. If the mutual repulsion mechanism described above is correct, then we would expect that loss of efficiency would occur as the injected mass is increased when a greater fraction of the analyte is ionized. This idea can be examined by varying the effect of mobile phase pH on peak shape over a range that will lead to variation in the fraction of the analyte that is ionized. It was indeed shown that much smaller overload effects for the basic drug amitriptyline were obtained at high pH where it is mostly uncharged compared with low pH where it is mostly charged (10). This suggests that adjusting the mobile phase pH can be a powerful tool for managing overload tailing when it is observed. There are limitations to this approach—most silica-based columns are not very stable above a pH of 8 (5), and not all analytes will change their ionization state in response to a change in pH (for example, sulphonates and phosphates are almost always anionic, and quaternary amines will always be cationic).

In addition to using the mobile phase pH as a tool to manage overload tailing, adjusting the composition of the mobile phase buffer can also be very effective. From the concept of the mutual repulsion mechanism, we would also expect that increasing the ionic strength of the mobile phase buffer should improve peak shape in cases where overload tailing is observed because the buffer ions can shield analyte ions entering the column from those already adsorbed to the stationary phase. The results in Figure 4 and Table 1 provide some evidence for this effect (11). Figure 4 shows a comparison of peak shapes obtained for a mixture of basic peptides in mobile phases containing either 20 mM formic acid (FA) or 8 mM trifluoroacetic acid (TFA). In this case the concentration was adjusted so that the pH of the two mobile phases would be about the same, thereby eliminating pH as a variable in the experiment. From the chromatograms we can clearly see that the peak shapes are qualitatively much better in the TFA mobile phase, and that they overload much more quickly in the FA mobile phase compared to the TFA mobile phase. These effects are quantified in Table 1 for both the FA and TFA mobile phases, as well as two other mobile phases—one with ammonia added to FA to increase the ionic strength as ammonia is protonated to give ammonium ions, and another with potassium chloride added to FA. Here we see that—using peak asymmetry as a metric—simply adding ammonia to the FA mobile phase improves the peak significantly (compare $A_s$ of 1.5 to $A_s$ of 1.9), and that the benefit increases as the mass of peptide injected increases (compare $A_s$ of 1.7 to $A_s$ of 3.5). Adding potassium chloride to the FA mobile phase improves the peak shape further, to the point where the performance is practically indistinguishable from the TFA mobile phase. Whereas plate number or efficiency is a convenient measure of the change in peak width under isocratic conditions, peak capacity can be used as a similarly convenient

### TABLE 1: Peptide separation performance with different mobile phase additives

<table>
<thead>
<tr>
<th>Buffer Composition</th>
<th>pH</th>
<th>Ionic Strength (mM)</th>
<th>$A_s$</th>
<th>$n_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM formic acid</td>
<td>2.7</td>
<td>1.9</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>20 mM Formic acid + 7 mM ammonium</td>
<td>3.3</td>
<td>7.4</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>20 mM formic acid + 20 mM KCl</td>
<td>2.7</td>
<td>22</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>8 mM trifluoroacetic acid</td>
<td>2.3</td>
<td>7.8</td>
<td>1.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Peptide P4 Concentration

$1\times$  $10\times$  $1\times$  $10\times$
measure of changes in peak width when gradient elution is used. By this metric as well, the biggest change is observed when additional ionic strength is added to the FA mobile phase, especially when a larger mass of peptide is injected.

These results teach us that increasing the ionic strength of the mobile phase can be a powerful tool for mitigating overload tailing for ionogenic compounds. The simplest means for doing this without changing the mobile phase pH, which can affect retention and selectivity, is to add an inorganic salt such as potassium chloride. Unfortunately, adding inorganic salt is not desirable when using certain detectors such as mass spectrometry (MS) or light scattering, because these additives are not volatile and will lead to contamination of the detector. Some salts may also be corrosive towards LC systems built from stainless steel parts. When using these detectors, use of additives such as ammonium formate or ammonium acetate is preferred, though this is more complicated because such additions will also affect the mobile phase pH.

Summary
In this instalment of “LC Troubleshooting”, we discussed two major physicochemical causes of peak tailing in reversed-phase LC, and some potential remedies for them. These problems often manifest with different chromatographic behaviours, which can be useful for identifying which of them is a major problem when troubleshooting poor peak shapes. When exponential tailing is observed for basic compounds (such that they are protonated and positively charged in the mobile phase), increasing the injected mass of analyte may improve the peak shape, with little effect on the retention time. In some cases, decreasing the mobile phase pH (to pH 3 for Type B silicas, or pH 2 for Type A silicas) may improve the peak shape. When overload tailing is observed (for either anionic or cationic analytes), peaks will have a distinctive “shark fin” shape, and increasing the injected mass of the analyte will usually cause a significant shift in the peak apex to shorter times. In this case, adjusting the mobile phase pH to decrease the fraction of analyte that is ionized in the mobile phase may decrease the degree of overloading, and improve the peak shape (that is, increasing the pH for bases, and decreasing the pH for acids). Finally, increasing the ionic strength of the mobile phase buffer may also help through the addition of inorganic salts or MS-friendly salts such as ammonium formate or acetate.

References
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2) D.R. Stoll, LC GC Europe 34(9), 372–375 (2021).
Combating Olive Oil Fraud Using GC–IMS and FGC-Enose

The adulteration of high-quality foods is big business. Typical adulteration of olive oil involves the use of other types of oil, such as seed oils or pomace oils, the introduction of re-esterified oils, or the creation of mixtures with refined oils to create a lower quality product that can still be sold at a premium price. Fortunately, these processes can be easily detected using standard methods. However, fraudsters now seek more advanced methods using soft refined oils or oils with a tailored composition, making detection with existing procedures difficult. LCGC spoke to Michele Suman about novel screening and confirmatory analytical strategies he has investigated to regain the upper hand in the fight against olive oil adulteration.

Q. How common is olive oil adulteration and how large a problem is it in monetary terms?
A: Olive oils are basically grouped into three quality grades, namely “extra-virgin olive oil” (EVOO), “virgin olive oil” (VOO), and “lampante olive oil” (LOO), on the basis of specific chemical and organoleptic criteria (1) from the Commission Delegated Regulation (EU) 2019/1604 of 27 September 2019 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis (2).

Referring to the European Regulation No. 2568/91 and subsequent amendments, VOO must be obtained using solely mechanical or other physical means, under conditions that do not alter the product’s integrity. In contrast, the LOO category includes those oils that do not fulfil the minimum VOO quality criteria and, therefore, are not intended for direct human consumption.

Finally, EVOO represents the top-quality grade because of its superior sensory attributes and the claimed health-promoting effects. EVOO is one of the most important and expensive edible oils, and is therefore also one of the most adulterated food commodities in the global market: limited production, higher price, and growing consumer demand represent an explicit fraud driver for EVOO.

The International Olive Council has clearly defined the limits of the specific chemical parameters able to protect EVOO against potential adulterations with other edible oils (3). Spain is the world’s largest producer of olive oil, with more than 40% of the world’s production originating from there, particularly from Andalusia. Another 20% comes from Italy, 18% from Tunisia, and approximately 12% from Greece. These four countries, together with Portugal, are also the largest exporters of olive oil.

Producing one litre of olive oil requires four to five kilos of olives on average; furthermore, processing methods also significantly affect olive oil yield.
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In 2019, European import volume surpassed 11 million tonnes, at a value of €1.6 billion, a decrease in comparison with previous years, caused by an overproduction in Europe that led to a significant drop in prices. In the long term, the European market for olive oil is expected to show stable growth of 3–5% over the next five years (4).

Q. What exactly are soft refined oils? And why do they represent a challenge to scientists to detect?
A: The term soft-deodorization refers to a deodorization process performed at a lower temperature with respect to the conventional process, for example, 100 °C instead of 180–200 °C. It basically consists of a vacuum steam distillation using nitrogen to strip out all the volatile compounds responsible for the undesirable odours. Deacidification is instead designed to remove the free fatty acids (FFA), which speed up oxidation processes and are involved in the development of rancid flavour. This is normally achieved through the addition of alkali, such as sodium hydroxide, to the oil; this results in the precipitation of the FFA as an insoluble soap dreg, subsequently removed by centrifugation and/or filtration. Since the use of lower temperatures does not seem to produce substantial compositional and structural modification in the bulk, the resulting soft-refined olive oils (SROO) are best used to blend EVOO and create illicit mixtures no longer detectable by current methods (5). Typical fraud, such as adding other types of oils like seed oils or pomace oils, the presence of re-esterified oils, or the preparation of blends with refined oils, can be detected with standard methods. For this reason, fraudsters are now focused on developing more sophisticated adulterations, such as the use of soft refined oils, that would allow for the creation of blends that cannot be discovered with regular methods. Furthermore, another relevant issue is the misrepresentation of the geographical origin of the oils. While there are currently no recognized official methods for detecting these frauds, the literature suggests different approaches with promising results: gas chromatography–mass spectrometry (GC–MS), nuclear magnetic resonance (NMR), isotopic fingerprint studies, or liquid chromatography–mass spectrometry (LC–MS) coupled with chemometrics (6).

Q. You first approached the issue with a 2020 publication where a non-targeted high-resolution mass spectrometry (HRMS) method was used. Could you discuss your approach to selecting a method, and the positives and negatives of the selected approach?
A: We developed a non-targeted LC–HRMS study for the detection of new chemical markers able to identify the addition of soft deodorized and soft deacidified low-quality virgin or lampante olive oils to EVOO (7). This approach has definite advantages in terms of effectiveness in detecting anomalies, even those not foreseen among those under examination, but it should be kept in mind that it entails high instrumental costs, technical staff of high competence, and is difficult to be directly implemented by an industrial control laboratory. The industry in this case is rather oriented to subcontract these analytical investigations to specialized external laboratories.

In this specific case, “in-house” soft refined oils were created and analyzed together with a group of pure EVOOs. In addition, different mixtures of pure EVOO and adulterated oils were included in the sample set. The markers selected through the study via robust data elaboration—12 molecules, seven of them were selected as discriminative in both the laboratories—were compared between two laboratories equipped with two different types of mass spectrometers, a quadrupole (Q)–orbital trap and a quadrupole time-of-flight (QTOF), with the successful aim to assess the reproducibility of the proposed analytical approach.

Q. Your most recent publication took a slightly different approach to the issue and evaluated LC–MS, GC–ion mobility...
spectrometry (IMS), and flash gas chromatography electronic nose (FGC-Enose) for their suitability to detect olive oil fraud. What were your findings regarding their suitability?

A: In parallel to LC–MS studies, in the last couple of years we have explored the potentialities of two emerging headspace (HS)-based techniques, namely GC–IMS and FGC-Enose for the detection of EVOO blending with SROO. These two techniques offer rapid, minimal sample preparation, high-throughput, and non-destructive solutions for quality and authenticity testing, which are highly sought by modern food industries. They provide global information about the volatile organic compound (VOC) profile in a short time. The methods exhibited both robustness and stability over time, giving promising results for screening purposes.

Additionally, the strategy of targeting the volatile profile is of interest because the main changes due to soft refining (especially for deodorization) are not expected to occur in the bulk fraction.

Very recently we also aimed our efforts into deepening the potential of the latest generation of multivariate statistical analysis: low-level data fusion of GC–IMS and the FGC-Enose datasets were performed. The merged unique fingerprints were submitted to partial least-squares discriminant analysis (PLS-DA) and the extrapolated most informative variables were used to build support vector machine (SVM) classifiers. The results of this PLS-DA-SVM strategy on the combination of datasets demonstrated that the discriminatory capability of the two merged GC-based techniques was remarkably improved compared to the individual ones, improving, for example, the sensitivity, which means that 20% or lower adulteration percentages detection could be achieved in the future.

**Laboratories need to learn to “merge” complementary data from techniques capable of providing orthogonal information.**

Q. What were the main challenges developing this approach and how did you overcome them?

A: It was, of course, rather expensive to set up in the laboratory samples appropriately adulterated to “teach machines the frauds to recognize”, as well as collect data with several instrumental systems and multiple approaches, but perhaps the most complex part was the final stage of data processing.

To face the increasingly complex analytical challenges placed by fraudsters in olive oil adulteration and other areas of food analysis, laboratories need to recruit specialized staff with cross-skills in analytical chemistry and statistics, and learn to “merge” effectively complementary data from techniques capable of providing orthogonal information and therefore capable of enhancing both selective and discriminatory potentialities.

Q. What other projects are you working on?

A: We are currently working on both rapid screening and confirmatory methods to address the geographical origin of cereals, with a particular focus on wheat.

**References**

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Restek Corporation, Bellefonte, Pennsylvania, USA.

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Wyatt Technologies, Santa Barbara, California, USA.
Sphingolipids differ from other glycerolipids in their sphingoid backbone. In eukaryotic cells, sphingomyelins (SMs) are found as a phosphorylated subclass of sphingolipids to serve as structural lipids in the outer leaflet of the cell membrane. In addition, SMs play a major role in the formation of lipid rafts (highly functionalized parts of the cell membrane) and can serve as biomarkers for diseases (1,2). SMs contain a fatty acid linked to the sphingoid base and a phosphocholine headgroup. Unlike the more common glycerophospholipids bearing an ester bond to link the fatty acid to the backbone, SMs are based on an amide bond (Figure 1). This property is often used in the conventional sample preparation for the analysis of SMs, which involves an alkaline hydrolysis of the ester bonds of glycerophospholipids such as phosphatidylglycerols (PG), phosphatidylcholines (PC), and phosphatidylethanolamines (PE). The latter are much more abundant in total lipid extracts and thus hamper the direct analysis of SMs. As we reported earlier, the laborious hydrolysis step can be replaced and automated with a two-dimensional liquid chromatography (2D-LC) approach (3). Hydrophilic interaction liquid chromatography (HILIC) is used in the first dimension (1D) to separate the lipids according to their polar headgroup (cf. Figure 1). In addition, we demonstrate that even lipid classes bearing the same headgroup (SM, PC) can be resolved chromatographically. The separated fractions are then cut out and stored in a sample loop for the second dimension (2D) reversed-phase LC via a switching valve setup. The SM fractions are further separated according to their fatty acid chain length and identified by electrospray ionization-mass spectrometry. In this application, we present a novel heart-cut 2D-LC for the shortened analysis of SMs in the model organism Caenorhabditis elegans (C. elegans).

Experimental

Lipid Extract: 65,000 Dauer C. elegans nematodes were extracted as described earlier (3). LC–MS/MS Setup: Thermo Scientific Ultimate 3000 system with dual gradient pump hyphenated to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. The ionization was carried out utilizing electrospray ionization in negative ionization mode (ESI-). The HESI-II probe was held at 250 °C and operated at -3 kV, and sweep gas at 1 AU, as described earlier (3).

1D-HILIC Separation:

Column: 20 × 2.1 mm, 5 μm, iHILIC®-Fusion(+)

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Eluents: A) ammonium formate solution (35 mM, pH 4) and acetonitrile (95:5, v/v); B) acetonitrile

Gradient Elution: 0–0.5 min, 97% B; 0.5–3.5 min, from 97–85% B; 3.5–8.5 min, 85–60%; 8.5–19.5 min, 60% B; 19.5–20 min, 60–97% B; 20–25 min, 97% B (column equilibration)

Flow Rate: 0.3 mL/min

Column Temperature: 40 °C

Injection Volume: 5–10 μL

Heart-Cut Setup for SM Transfer: The SM fraction from the 1D-HILIC was transferred onto the 2D-reversed-phase LC through a six-port valve and a 200 μL stainless-steel sample loop in a heart-cut approach, as shown in Figure 2. The valve was in Position II at the beginning of the separation. It was changed to Position I at 6.4 min and kept until 6.9 min to cut out the eluted SM fraction from the 1D-HILIC into the sample loop, whereafter the valve was switched back to Position II at 6.9 min and kept until 7.5 min to completely transfer the fraction onto the 2D-reversed-phase LC. The 2D-separation of SMs was performed after switching the valve to Position I. At 28 min, the valve was changed back to Position II to prepare for the next sample injection.

Results and Discussion

Sphingomyelins in Dauer C. elegans larvae were analyzed with a 2D HILIC-reversed-phase LC approach. The 1D-separation of lipids was achieved with a fast tailored HILIC method. As shown in Figure 3, SMs were separated from glycerophospholipid classes (PG, PE, PC). The difference in abundance of the lipid classes can be seen from the intensities of their peaks, which show the importance of

Figure 1: Exemplary structures of phospholipids in eukaryotic cells. Headgroups are highlighted in red, fatty acid linkage by ester bond in blue, and amide bond in green, respectively.

B; 3.5–8.5 min, 85–60%; 8.5–19.5 min, 60% B; 19.5–20 min, 60–97% B; 20–25 min, 97% B (column equilibration)
a reliable separation of sphingolipids from glycerophospholipids. Otherwise, ion suppression and matrix effects can hamper the identification and detection of the low concentrated SMs by MS. In addition, nonpolar lipids exhibit no retention by 1D-HILIC separation and thus were excluded from the sphingolipid analysis.

It was also demonstrated in Figure 3 that the coelution of the sphingolipids resulted in a narrow transfer window of 0.5 min (150 µL transfer volume) in the 1D-HILIC of the Dauer C. elegans extract. This transfer window was first identified with a standard substance (SM(d18:1/16:0)) and then confirmed by C. elegans samples, where accurate masses and retention time were utilized. Three identified coeluting C. elegans SMs are shown in the upper part of Figure 3.

The SM fraction in the transfer window was stored in a stainless-steel loop and injected into the 2D-reversed-phase LC via the valve setup shown in Figure 2. The SMs were subsequently separated according to their FA-acyl chain length. A reduced MS background in the 2D separation was observed. This phenomenon has also been reported in our previous work; a 1D-HILIC separation created a significant reduction in the total MS ion current and a better detection limit for low abundant sphingolipids in the 2D-LC method (3). In this application, a total of 21 SMs were detected in the Dauer C. elegans samples by this shortened 2D-LC method involving a fast HILIC separation. Sample analysis was therefore streamlined by omitting the commonly used laborious alkaline depletion step during sample preparation.

References
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