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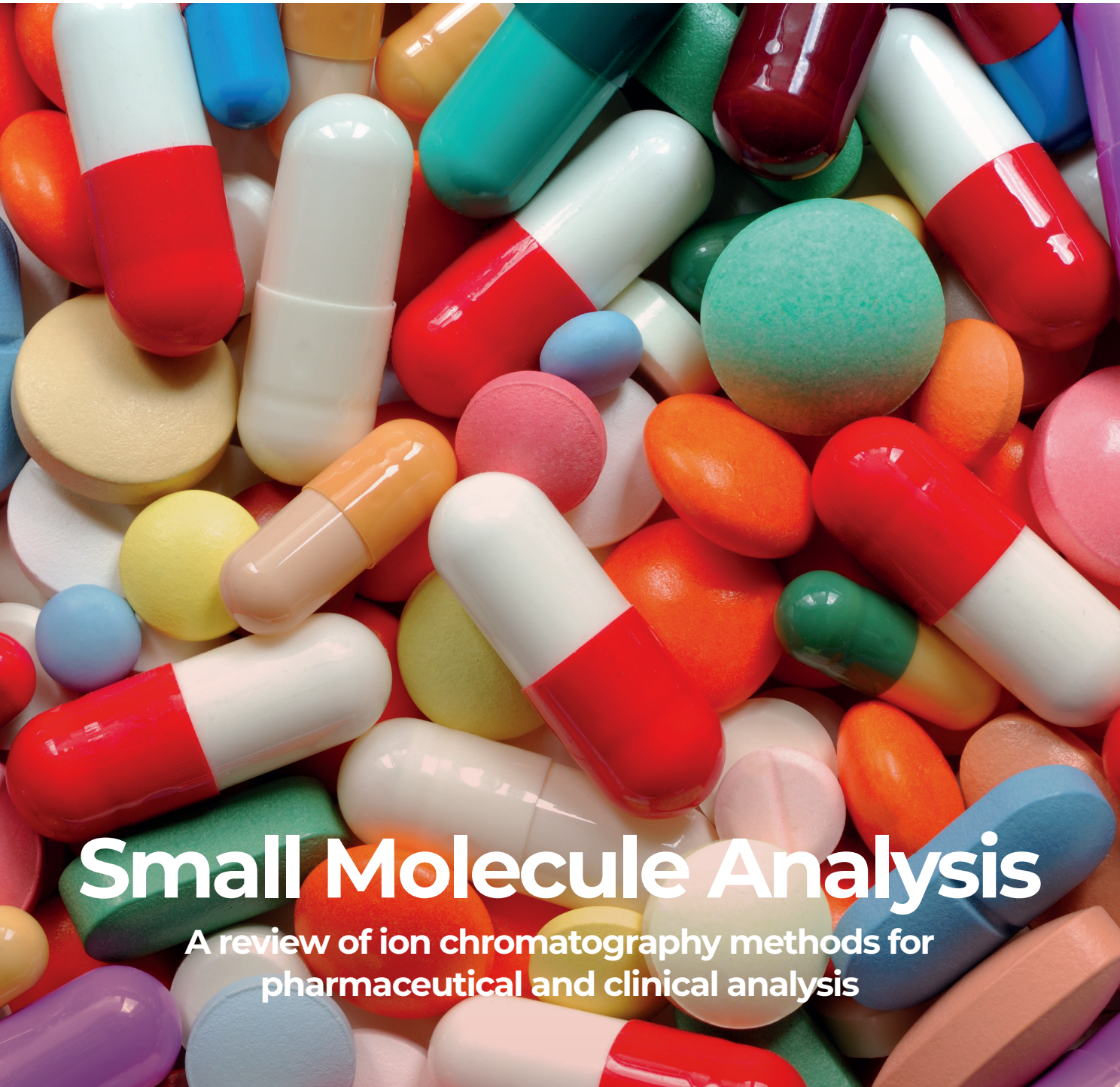

Small Molecule Analysis

A review of ion chromatography methods for pharmaceutical and clinical analysis

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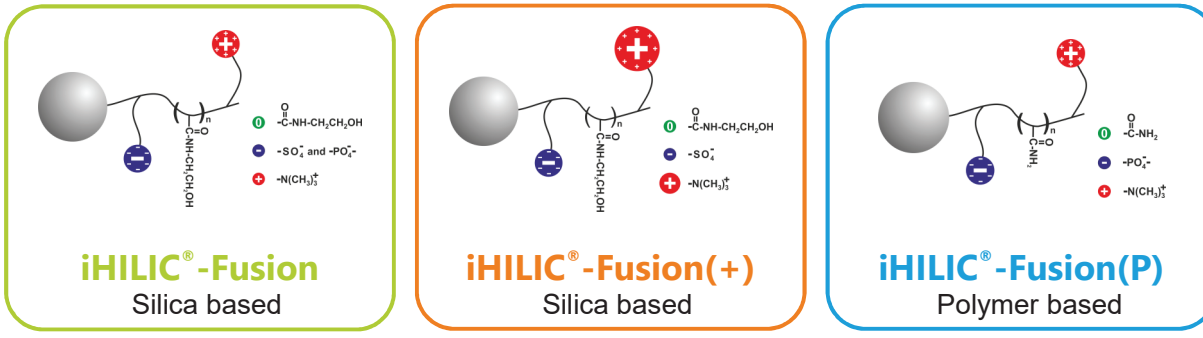

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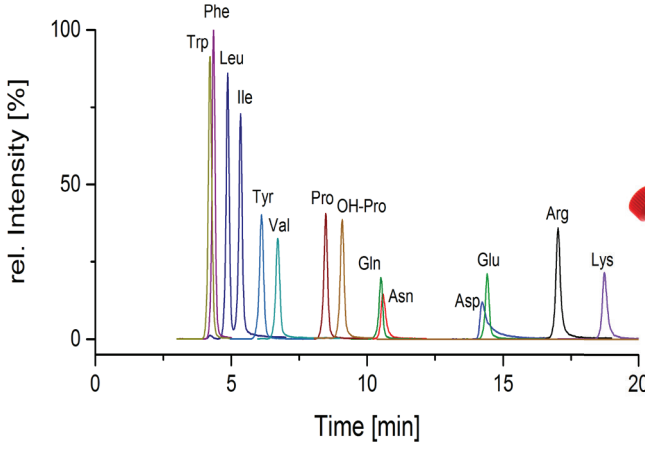

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Mike Tessalone
mtessalone@mmhgroup.com

Editorial Director
Laura Bush
lbush@mmhgroup.com

Editor-in-Chief
Alasdair Matheson
amatheson@mmhgroup.com

Managing Editor
Kate Jones
kjones@mmhgroup.com

Associate Editor
Lewis Botcherby
lbotcherby@mmhgroup.com

Associate Publisher
Oliver Waters
owaters@mmhgroup.com

Sales Executive
Liz Mclean
lmclean@mmhgroup.com

Sales Operations Executive
Sarah Darcy
sdarcy@mmhgroup.com

Senior Director, Digital Media
Michael Kushner
mkushner@mmhgroup.com

Webcast Operations Manager
Kristen Moore
kmoore@mmhgroup.com

Project Manager
Vania Oliveira
voliveira@mmhgroup.com

Digital Production Manager
Sabina Advani
sadvani@mmhgroup.com

Managing Editor, Special Projects
Kaylynn Chiarello-Ebner
kebner@mmhgroup.com

Art Director
Gwendolyn Salas
gsalas@mjhlifesciences.com

Graphic Designer
Courtney Soden
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INTERVIEW

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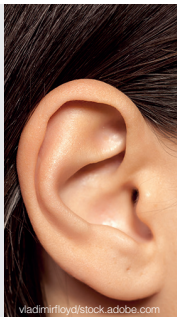

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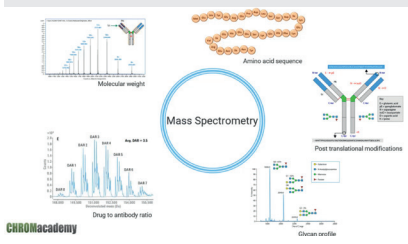

NEWS

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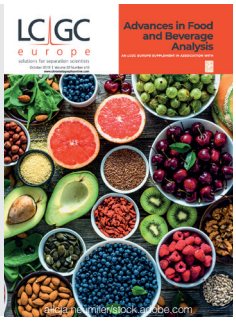


Ion Chromatography for Small Molecule Determination in Clinical and Pharmaceutical Studies

Rajmund Michalski[1], Anna Błażewicz[2], Joanna Kończyk[3], Katarzyna Krupa[2], and Przemysław Niziński[2], [1]Institute of Environmental Engineering, Polish Academy of Sciences, Zabrze, Poland, [2]Collegium Pharmaceuticum, Medical University of Lublin, Poland, [3]Institute of Chemistry, Faculty of Science & Technology, Jan Dlugosz University in Czestochowa, Czestochowa, Poland

In clinical and pharmaceutical research, the application of reliable, reproducible, and the best-available analytical methods and techniques are necessary because the quality of analysis may have a crucial effect on the health and life of patients. The role of inorganic and organic ions, as well as ionizable substances, is very important for health. A very useful instrumental method for this range is ion chromatography (IC) and related techniques. Various ion chromatography methods are used in clinical and pharmaceutical research mainly to determine inorganic anions and cations, metals and metalloids, as well as selected organic compounds. This article presents possible uses of ion chromatography and related techniques combined with various detection methods for clinical and pharmaceutical analysis of common inorganic and organic anions and cations. An overview of achievements in this area from the past 10 years is presented and the most important trends and development perspectives for ion chromatography are described.

KEY POINTS

- **Ion chromatography is a versatile method used in clinical and pharmaceutical research.**
- **The method demonstrate high selectivity, repeatability, precision, and recovery.**
- **The review of ion chromatography and related techniques in applications in small molecule determination from the last 10 years is presented.**

Elements and chemical substances, particularly those that may be analyzed using ion chromatography (IC) and related techniques, play an important role in regular human health and life quality. This mainly concerns inorganic anions (F^-, Cl^- NO_3^-, PO_4^{3-}, SO_4^{2-}) and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}), selected metals and metalloids (Fe, Cu, Al, Zn, As, Cd, Mn), and organic compounds (amines, carboxylic acids, carbohydrates, peptides, proteins). Depending on the concentration and action time, these substances have a considerable impact on living organisms. Analyses of selected ions in body fluids or tissues can help to understand the causes of various neurological, cardiological, osteoarticular, dermatological, endocrinological, or gynaecological disorders, and their determination is helpful in disease diagnosis, forensic science, and in judicial cases. The use of IC in the pharmaceutical industry is equally important and promising. In recent years, there has been a lot of verified information about the demise of patients caused by consumption of fake medications, medical products, or dietary supplements. It is estimated that half of pharmaceuticals present in the global market are forged (1). The medications and dietary supplements available in the market must be identically effective in terms of therapeutic properties and safety for consumers. Providing a reproducible composition of pharmaceuticals or dietary supplements is related to a very strict quality control of each product series (2).

Ion Chromatography and Related Techniques in Clinical and Pharmaceutical Analysis

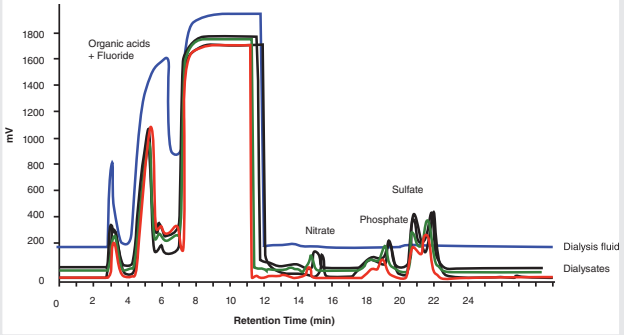
IC has been used as a reference method for analyzing anions and cations in water

and wastewater for over 40 years (3). Thanks to the introduction of more selective stationary phases in the analytical columns, as well as detection and sample preparation methods, IC applications have been extended to other types of analytes and matrices, including pharmaceutical and biomedical specimens (4). Taking into consideration the separation mechanisms and the resulting types of stationary and mobile phases, it is possible to distinguish IC with or without suppressed conductivity, high perfomance ion exclusion chromatography (HPIEC) (5), and mobile phase ion chromatography (MPIC) (6). HPIEC is mainly used for separation of weak organic and inorganic acids or for group separation of organic and inorganic compounds. MPIC has been applied for determining hydrophobic ions, such as sulphonates, alkaloids, barbiturates, and selected ionic complexes of metals and metalloids. Reversed-phase high performance liquid chromatography (HPLC) (7) and hydrophilic interaction chromatography (HILIC) (8) are also used for analyzing ionizable substances. Ultrahigh-pressure liquid chromatography (UHPLC) technology was originally developed for reversed-phase LC applications, and it is now also available for MPIC and IC due to the availability of 1.7- and 3-µm non-porous particles. It may create a new level of performance in IC, but because of fine particles, the separation quality is improved at the cost of higher pressure. The disadvantage of this technology is that currently there are only a very limited number of commercially available stationary phases.

Simultaneous determination of anions, cations, and neutral species is possible when using a trimodal stationary phase that can provide reserved-phase, anion-exchange, and cation-exchange retention mechanism. Taking into consideration problems with the selectivity of analyte separations, particularly in complex matrices samples, an interesting solution is multidimensional IC. It is based on linking two individual chromatographic systems in such a way that a section of the effluent of the first system after passing the detector is transferred into the second chromatographic system (9,10).

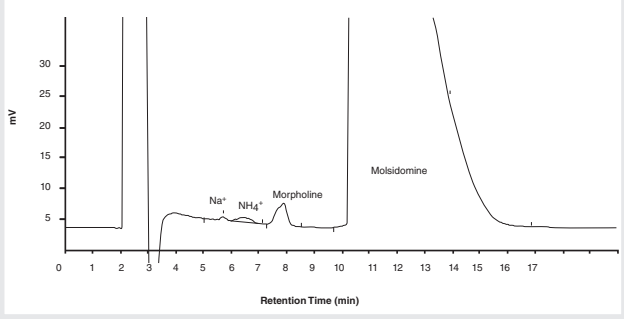
The detection methods used in IC and related techniques can be divided into direct and indirect methods, or into electrochemical (conductometric and amperometric detection) and spectroscopic (UV–vis, mass spectrometry [MS]) techniques (11). Conductometric detection is used most often, but it does not allow for determining substances with values of pK > 7. In the range of inorganic analysis, IC with conductivity detection was suggested as a reference method for determining Na, K, Ca, and Mg ions in the blood serum as early as 1997 (12). Contactless conductivity measurements are another option; they have been employed for the detection of inorganic or small organic ions in conventional capillary electrophoresis, and less often in microchip electrophoresis (13). A very useful detection method, specific

FIGURE 1: Chromatograms of anions in dialysis fluid samples and dialysates sampled from the peritoneal dialysis patient. Separation conditions: column: 250 × 4.6 mm Dionex IonPac AS14; eluent: 1.7 mM Na_2CO_3 + 1.6 mM $NaHCO_3$; detection suppressed conductivity.



for compounds, such as, carbohydrates, amino acids, alditols, glycols, and alcohols, is pulsed amperometric detection (PAD). The application of cation-exchange chromatography (CEC) coupled with integrated pulsed amperometry in clinical tests was first described by Cole and Evrovski (14) in 1997. A PAD detector can be used for the determination of iodide ions in healthy and pathological human thyroids, urine, and serum samples, in addition to the determination of common inorganic ions in serum (15). Other detectors used for organic substances determination are evaporative light scattering detectors (ELSDs) (16) and charged aerosol detectors (CADs) (17). Instead of measuring the intensity of radiation absorbed by analytes present in the eluate, an ELSD measures the radiation dispersed on the particles of the aerosol formed by the analytes (18). CAD offers high sensitivity, broad dynamic measurement range, and result repeatability (19). For metal and metalloids analysis, UV–vis detection and atomic absorption spectrometry (AAS), inductively coupled plasma optical emission spectrometry (ICP-OES), and inductively coupled plasma–mass spectrometry (ICP-MS) (20) offer advantages such as speed, good precision, and repeatability. Their disadvantages include lack of differentiation between metals occurring at different oxidation states. For that reason, different separation and detection methods are coupled in the form of hyphenated techniques (21). The most popular IC-based hyphenated techniques are IC-ICP-MS and IC-MS (22). They offer great possibilities and their main advantages include extremely low limits of detection and quantification, and very good precision and repeatability of determinations. However, they have some limitations, such as high price and complexity of the apparatus, as well as requiring an understanding of analytical methodologies and instruments. At present, most studies concern hyphenated techniques

FIGURE 2: Chromatogram of morpholine in molsidomine. Separation conditions: column: 250 × 4.6 mm Metrosep C2; eluent: 1.7 mM HNO_3 + 0.7 mM dipicolinic acid + acetone 10% (*v*/*v*); detection: non-suppressed conductivity.



based on mass spectrometry, including those dedicated to metabolite analyses in clinical and pharmaceutical samples (23).

Sample Preparation Methods

The opportunities offered by IC and its varieties for pharmaceutical and clinical research are related to progress in the development of sample preparation methods. These kinds of samples usually require time-consuming preparation procedures before the chromatographic analysis. The risk is related to various oxidation, reduction, complexation, precipitation, and bio- and photochemical processes that may occur in the sample between the sampling and the analyte determination (24). As a result of the sample state of matter, two groups of preparation methods can be distinguished. The first one concerns liquid samples and includes filtration, dilution, pH changes, derivatization, liquid–liquid extraction (LLE), solid-phase extraction (SPE), and membrane techniques (25). The other is used for solid samples and encompasses drying, homogenization, extraction/elution, etching, or incineration. Dense solid samples analyzed with IC require turning the analytes into a solution. Such analytical systems are collectively known as *combustion ion chromatography* (CIC). Its main advantages are: extension of IC applications for analyzing solid samples in an online mode; full automation and control of the incineration process; application of one software type for the entire analytical procedure; possible quick exchange of attachments for solid and liquid sample analyses; and simple calibration (26).

Examples of IC Applications in Clinical and Pharmaceutical Studies of Small Molecules

One year after the official establishment of IC, the first study on its uses in clinical analysis was published (27). Bhattacharyya and Rohrer (28) described examples of IC applications in tests of pharmaceutical products and biomedical samples. In 2011, Jenke (29) discussed IC applications for clinical research and the pharmaceutical industry in an overview study. IC is an important analytical method for the analysis of pharmacopoeia grades of water used in pharmaceutical industry (30).

Very popular IC applications in clinical analysis are determining ions and other substances in perspiration. Perspiration mainly consists of water (~ 99%) with small amounts of nitrogen compounds (such as amino acids and urea), K^+, Na^+, and Cl^- ions, and various metabolites and xenobiotics. When a disease occurs, perspiration may be a source of information on biomarkers of various diseases, including schizophrenia, cystic fibrosis, diabetes, or cancer (31). Thanks to its characteristics, perspiration analysis is a quick and simple process when compared to analyzing other body fluids. This particularly concerns blood because its collection has an invasive character and is related to a higher risk of infection. At present, progress in the miniaturization of measurement apparatus allows for perspiration or saliva tests to be undertaken without a visit to a clinic or hospital.

IC is a decisive analytical tool for solving some analytical problems in the field of nephrology. Many methods used in the past for determining oxalates in the urine of patients suffering from kidney stones, such as permanganometry (after oxalate oxidation to CO_2) or spectrophotometry (after oxalate reduction to glycoxylic or glycolic acid) are time- and work-consuming. Another interesting example is analyzing the ion composition of fluids for peritoneal dialysis and dialysates themselves. The appropriate concentrations of anions and cations and mutual concentrations determine the dialysis correctness and efficiency. Figure 1 shows chromatograms of anions in the dialysis fluids and dialysates sampled from the patient treated with peritoneal dialysis (32).

Research into the biomarkers of many conditions has contributed to the extension of IC applications for analyses of body fluids (blood, urine) (33) or tissue samples (thyroid) (34). The studies of plasma samples in patients with alcoholic liver cirrhosis showed a significant correlation between many heavy metals and the disease (35). The research into metals in hair samples with IC demonstrated a significant correlation between Zn, Pb, and depression (36). Other IC applications in clinical analysis include determinations of homocysteine (risk factor in coronary artery disease), methionine together with other amino acids present in the plasma, or catecholamines (including noradrenaline, dopamine) in urine. Smith *et al.* (37) developed a method based on IC whose advantages included the ability to determine the diversity of physiologically important anions in one cycle. Carbohydrate analyses in bodily fluid samples are more and more often conducted with high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The clinical application of this method concerns, among others, monitoring of the irregular functioning

of the intestines during the HIV infection (38). An important application is determination of deoxy-2-[18]fluoro-D-glucose, which is one of the most widely used radiopharmaceuticals for positron emission tomography studies (39).

Among many pharmaceutical products, there are some that can be determined directly by IC methods. One example is the group of bisphosphonates that are used to treat bone disorders including osteoporosis, such as clodronate or water-soluble vitamins. As a result of similar properties and low separation selectivity a serious problem in pharmaceutical analysis is the analysis of aliphatic amines. A typical example for the significance of aliphatic amines is the analysis of amyloamine and tert-butylamine. Next, for the analysis of aliphatic quaternary ammonium compounds, cation-exchange chromatography with conductivity detection can be applied. IC with conductometric detection is used for determining morpholine pollutants in molsidomine (used in prophylaxis and treatment of angina pectoris and cardiac insufficiency). An example chromatogram is presented in Figure 2.

In recent years, the number of monographs discussing research procedures based on IC has been on the rise. The *US Pharmacopeia 32–National Formulary 27* edition contained two chapters on IC (345 and 1065), and four chapters describing measurement methods based on IC (1045; 1052; 1055; 1086). At present, the document contains over 110 monographs using one or more research IC-based procedures.

Table 1 presents examples from the literature over the past 10 years concerning the applications of IC and its varieties in the clinical and pharmaceutical research grouped according to the used detection mode (40–53).

Conclusions

IC and related techniques have been extensively employed in clinical and pharmaceutical research. The most important

TABLE 1: Examples of applications of IC with different detection modes in the analysis of clinical and pharmaceutical samples

Analytes	Separation Column	Eluent	Matrices	References
Conductivity				
Monoethylsulfate	Metrohm Metrosep A Supp5	Na_2CO_3 + $NaHCO_3$	Drugs	(40)
N_3^-	Metrohm Metrosep A Supp10	5 mM $NaHCO_3$ + 5 mM Na_2CO_3	Irbesartan drug	(41)
F^-, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, oxalate	Shodex IC SI-90	1.7 mM $NaHCO_3$ + 1.8 mM Na_2CO_3	Urinary stones	(42)
UV–vis Detection				
SCN^-	Zorbax SAXC18	10 mM KH_2PO_4	Pharmaceuticals	(43)
Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}	Dionex IonPac CS5A	7 mM PDCA + 66 mM KOH + 5.6 mM K_2SO_4 + 74 mM HCOOH	Human fluids	(44)
Amperometric Detection				
I^-	Chromsep LC-Varian	0.1 M HNO_3 + 20% acetonitrile + 0.5 mM EDTA	Pharmaceuticals	(45)
Ascorbic acid	Dionex IonPac AS11-HC	1.25 mM NaOH	Drugs	(46)
MS and ICP-MS Detections				
ClO_4^-, SCN^-, NO_3^-, I^-	Dionex IonPac AS20	50 mM NaOH	Human urine	(47)
Chromium species	Shodex RSpak NN-614	90 mM $(NH_4)_2SO_4$ + 10 mM NH_4NO_3	Homeopathic drugs	(48)
ClO_4^-	Dionex IonPac AS20	50 mM NaOH	Dried Blood Spots	(49)
SeO_3^{2-}, SeO_4^{2-}, SeMe, TMSe	Hamilton PRP-X100 Phenomenex PRP-X200	Anions: 10 mM COO(NH4)2 + 2% MeOH Cations: 10 mM pyridine	Human urine	(50)
ClO^{2-}, ClO^{4-}	Dionex IonPac AS15	KOH	Blood	(51)
I^-	Dionex IonPac AG11	Buffer A: 10 mM Tris-HAc Buffer B: Tris-HAc + 500 mM NH4Ac + 5% MeOH	Serum, urine	(52)
As^{3+}, As^{5+}, DMA, arsenobetaine, MMA	Dionex IonPac AS7	20–200 mM $(NH_4)_2CO_3$	Children and pregnant woman urine	(53)

advantages in IC are: the simultaneous determination of several ions in a short time; the small sample volume necessary for analyses; the possibility of using different detectors; simultaneous separation of anions and cations, or organic and inorganic ions; separation of ions of the same element at different oxidation states (speciation analysis); full automatization; and safety and low exploitation costs (green chemistry). Like all methods, IC has some drawbacks. The most important are the limited selectivity in complex matrices and the time-consuming and labour-intensive methods for most clinical and pharmaceutical samples.

Future Trends and Perspectives

The future of clinical and pharmaceutical applications of IC and related techniques will involve interdisciplinary studies and in-depth speciation analyses suitable for evaluating the analysis of typical and non-typical analytes. Furthermore, interesting possibilities still exist in the areas of ultrahigh speed separations, tools for computer-assisted method development, multidimensional separations, and miniaturization. The challenges in IC are also related to increasing the use of these techniques in molecular biology and genetics research (genomics, proteomics, metabolomics, transcriptomics). Certainly, the interest from the clinical studies and pharmaceutical industry in IC and related techniques is growing and is expected to increase steadily in the future (54).

References

1) H. Rebiere, P. Guinot, D. Chauvey, and C. Brenier, *J. Pharm. Biomed. Anal.* **142**, 286–306 (2017).

2) R. Michalski, *Mini Rev. Med. Chem.* **14**(10), 862–872 (2014).
3) R. Michalski, *Crit. Rev. Anal. Chem.* **36**(2), 107–127 (2006).
4) J. Weiss, *Handbook of Ion Chromatography*, Third, completely revised and updated edition (Wiley-VCH Verlag GmBH & Co. KGaA, Weinheim, Germany, 2008).
5) J.S. Fritz, *J. Chromatogr. A* **546**, 111–118 (1991).
6) M.T.W. Hearn, *Ion-pair chromatography: theory and biological and pharmaceutical applications* (Marcel Dekker Inc, New York, New York, USA,1985).
7) M.C. Gennaro and S. Angelino, *J. Chromatogr. A* **789**(1), 181–194 (1997).
8) B. Buszewski and S. Noga, *Anal. Bioanal. Chem.* **402**(1), 231–247 (2012).
9) C.J. Venkatramani, *Advances in Pharmaceutical Analysis, LCGC Europe* Special Issue **31**(s10), 22–29 (2018).
10) M. Iguiniz and S. Heinisch, *J. Pharm. Biomed. Anal.* **145**, 482–503 (2017).
11) P.R. Haddad, P.N. Nesterenko, and W. Buchberger, *J. Chromatogr. A* **1184**(1), 456–473 (2008).
12) L. Thienpont, J.E Van Nuwenborg, and D. Stöckl, *J. Chromatogr. A* **789**, 557–568 (1997).
13) P. Kubáň and P.C. Hauser, *Electrophoresis* **40**(1), 124–139 (2019).
14) D.E.C. Cole and J. Evrovski, *J. Chromatogr. A* **789**(1), 221–232 (1997).
15) A. Błażewicz *et al., J. Chromatogr. B* **879**(9), 573–578 (2011).
16) N. Vervoort, D. Daemen, and G. Torok, *J. Chromatogr. A* **1189**(1–2), 92–100 (2008).
17) L.-E. Magnusson, D.S. Risley, and J.A. Koropchak, *J. Chromatogr. A* **1421**, 68–81 (2015).
18) E. Dvorackova, M. Snoblova, and P. Hrdlicka, *J. Sep. Sci.* **37**(4), 323–337 (2014).
19) Z. Huang *et al., J. Pharm. Biomed. Anal.* **50**(5), 809–814 (2009).
20) R. Michalski, *Crit. Rev. Anal. Chem.* **39**(4), 230–250 (2009).
21) R. Michalski, M. Jabłonska, S. Szopa, and A. Łyko, *Crit. Rev. Anal. Chem.* **41**(2), 133–150 (2011).
22) R. Michalski, *Application of IC-MS and IC-ICP-MS in Environmental Research* (John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2016).
23) N. Iwamoto and T. Shimada, *Pharmacol. Ther.* **185**, 147–154 (2018).
24) P.R. Haddad, P. Doble, and M. Macka, *J. Chromatogr. A* **856**(1–2), 145–177 (1999).
25) W. Frenzel and R. Michalski, in *Application of IC-MS and IC-ICP-MS in Environmental Research*, R. Michalski, Ed. (John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2016), pp. 210–266
26) J.-M. Liu, C. Liu, G.-Z. Fang, and S. Wang, *RSC Adv.* **5**, 58713–58726 (2015).
27) C. Anderson, *Clin. Chem.* **22**(9), 1424–1426 (1976).
28) L. Bhattacharyya, in *Applications of Ion Chromatography for Pharmaceutical and Biological Products*, L. Bhattacharyya, Ed. (John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2012), pp. 135–157.
29) D. Jenke, *J. Chromatogr. Sci.* **49**(7), 524–539 (2011).
30) J.P. Waterworth, *J. Chromatogr. A* **770**(1–2), 99–104 (1997).
31) A. Mena-Bravo and M.D.L. de Castro, *J. Pharm. Biomed. Anal.* **90**, 139–147 (2014).
32) R. Michalski and A. Lyko, *J. Liq. Chromatogr. Relat. Technol.* **39**, 96–103 (2015).
33) A. Blazewicz, M. Klatka, A. Astel, M. Partyka, and R. Kocjan, *Biol. Trace Elem. Res.* **155**(2), 190–200 (2013).
34) A. Blazewicz *et al., J. Chromatogr. B, Anal. Technol. Biomed. Life Sci.* **878**(1), 34–38 (2010).
35) A. Błażewicz *et al., Biomed Res. Int.* **2017**, 1–10 (2017).
36) A. Prystupa *et al., Int. J. Environ. Res. Public Health* **13**(6), 582–592 (2016).
37) R.E. Smith *et al., J. Chromatogr. A* **439**(1), 83–92 (1988).
38) D. Rojo, C. Barbas, and F.J. Ruperez, *Bioanalysis* **4**(10), 1235–1243 (2012).
39) P. Hajos, D. Lukacs, E. Farsang, and K. Horvath, *J. Chromatogr. Sci.* **54**(10), 1752–1760 (2016).
40) S.J. Prasanna, H.K. Sharma, K. Mukkanti, M. Sivakumaran, K.S.R.P. Kumar, and V.J. Kumar, *J. Pharm. Biomed. Anal.* **50**, 1065–1069 (2009).
41) N.H. Subramanian, P. Manigandan, R.G. Jeevan, and G. Radhakrishnan, *J. Chromatogr. Sci.* **47**(7), 540–544 (2009).
42) E. Saussereau, J.-P. Goulle, and C. Lacroix, *J. Anal. Toxicol.* **31**(7), 383–387 (2007).
43) C. Fernandes, R.S. Leite, and F.M. Lancas, *J. Chromatogr. Sci.* **45**(5), 236–241 (2007).
44) P. Zakaria *et al., J. Chromatogr. A* **1216**(38), 6600–6610 (2009).
45) T.K. Malongo *et al., Talanta* **76**(3), 540–547 (2008).
46) M. Sahin, L. Ozcan, B. Usta, and Y. Sahin, *Biosens. Bioelectron.* **24**(12), 3492–3497 (2009).
47) L. Valentin-Blasini, B.C. Blount, and A. Delinsky, *J. Chromatogr. A* **1155**(1), 40–46 (2007).
48) H. Hagendorfer and W. Goessler, *Talanta* **76**(3), 656–661 (2008).
49) S.M. Otero-Santos, A.D. Delinsky, L. Valentin-Blasini, J. Schiffer, and B.C. Blount, *Anal. Chem.* **81**(5), 1931–1936 (2009).
50) T. Jäger, H. Drexler, and T. Göen, *J. Anal. At. Spectrom.* **28**, 1402–1409 (2013).
51) A. Schwan, R. Martin, and W. Goessler, *Anal. Methods* **7**, 9198–9205 (2015).
52) B. Michalke and H. Witte, *J. Trace Elem. Med. Biol.* **29**, 63–68 (2015).
53) A.J. Signes-Pastor *et al., Expo. Heal.* **9**(2), 105–111 (2017).
54) G. Cudiama, *LCGC North America* **36**(1), 12 (2018).

Rajmund Michalski works in the Institute of Environmental Engineering at the Polish Academy of Science in Zabrze (Poland) since 1988. His research interests are ion chromatography (sample preparation, columns, eluents, detectors, and applications) and application of hyphenated techniques (IC-ICP-MS, IC-MS) in species analysis (inorganic disinfection byproducts, metal/metalloids ions). He has authored books, manuscripts, and over 170 publications about the applications of ion chromatography in environmental and food samples.

Anna Błażewicz is an associate professor at the Medical University of Lublin where she is the head of the Laboratory of Instrumental Analysis and currently coordinates eight interdisciplinary, international, non-commercial research projects. Her research interests include mainly new applications of chromatographic techniques, clinical, food and environmental chemistry, bioanalytics, and pathobiochemistry.

Joanna Kończyk is a researcher and academic teacher in the Institute of Chemistry of Jan Dlugosz University in Czestochowa. Her scientific interests include application of chromatographic (HPLC and IC) and spectroscopic (AAS, UV–vis) methods for quantitative and qualitative analysis of environmental, pharmaceutical, and food samples.

Katarzyna Krupa is a doctoral candidate in the Department of Analytical Chemistry at the Medical University of Lublin. Her primary research focus is the determination of heavy metals in various clinical samples, ion chromatography, and mass spectrometry.

Przemysław Niziński is a doctoral candidate in the Department of Analytical Chemistry at the Medical University of Lublin. His research interest include environmental toxicology, especially endocrine disruptors and heavy metals pollution, ion chromatography, and sample preparation techniques.

Effects of Flow Rate on UV Detection in Liquid Chromatography

Dwight R. Stoll, LC Troubleshooting Editor

If I increase the flow rate of my separation when using UV absorbance detection, should I expect peak area to change?

In my personal experience with troubleshooting my equipment in my laboratory, and in thinking about topics for this column, I have found that effective troubleshooting skills and techniques are built on a solid foundational understanding of how the system under study (which is broken, if we are troubleshooting) is supposed to work. On a number of occasions I have found myself thinking about and discussing with students and liquid chromatography (LC) practitioners the impact of flow rate on characteristics of chromatograms and peaks. For this month's "LC Troubleshooting" I've decided to dig into this basic, but very important, topic, with the intention that a deeper theoretical understanding of what should happen will help diagnose problems that may be related to flow rate when something does not look right.

Fundamentals

It is instructive to start a discussion of the effect of flow rate on LC separations with a kind of inventory of possible effects, along with a comparison of the predictions of simple theory and observations from real experiments (supported by more elaborate theories).

In this article I am going to focus on the last two rows of Table 1 because I have found through discussions with a variety of people that some confusion originates from these topics. Readers interested in the topics addressed in the second and third rows are referred to the references cited there for more information.

Relevant Background on Principles of Detection by Absorption of UV-Visible Light (UV Detection)

When thinking about the effects of flow rate on UV detection, it is critically important to recognize that we refer to UV detection as a type of "concentration-sensitive" detection. Concentration-sensitive detection is fundamentally different from "mass-sensitive" detection. Readers interested in the differences between these types of detection, and which LC detectors fall into which category, are referred to a recent educational article focused on this topic by Pavel Urban (4). Briefly, concentration-sensitive detectors respond to changes in analyte concentration presented to the detector (that is, moles/L, or mg/mL), whereas mass-sensitive detectors usually respond to changes in the mass of analyte presented to the detector over time (for example, pg/s). In the case of UV detection in particular, the detector reports absorbance values (A) in response to changes in analyte concentration (c) arriving at the detector. These absorbance values can be related to analyte concentration using the Beer-Lambert law:

$$A = \varepsilon bc \qquad [1]$$

where ε is a measure of the absorptivity of the analyte, and b is a measure of the length of the light path through the detector flow cell. Readers interested in more details associated with the inner workings of UV detectors are referred to a recent article by Michael Dong and Jedrzej Wysocki in *LCGC* (5).

Details Related to the Effect of Flow Rate on Peak Height (UV Detection)

To understand the effects of flow rate on peak height and area, we need to start with a model of chromatographic peaks. In the simplest case, we use a Gaussian distribution as a model of the peak shape, which expresses the dependence of analyte concentration in the LC column effluent arriving at the UV detector on time.

$$C_{\text{detected,i}} = \frac{\text{moles of } A}{\sqrt{2\pi}\sigma_v} \exp\left(\frac{-(t_i - t_R)^2}{2\sigma_t^2}\right) \qquad [2]$$

Here, $C_{\text{detected,i}}$ is the concentration of the analyte arriving at the detector at time i, "moles of A" is the number of moles of the analyte injected into the column, t_i is a time point in the chromatogram, t_R is the retention time of the analyte, and σ_v and σ_t are the standard deviations of the distribution

FIGURE 1: General shape of the dependence of plate height on flow rate that results from a van Deemter-type relationship $(H = A + \frac{B}{F} + C * F)$.

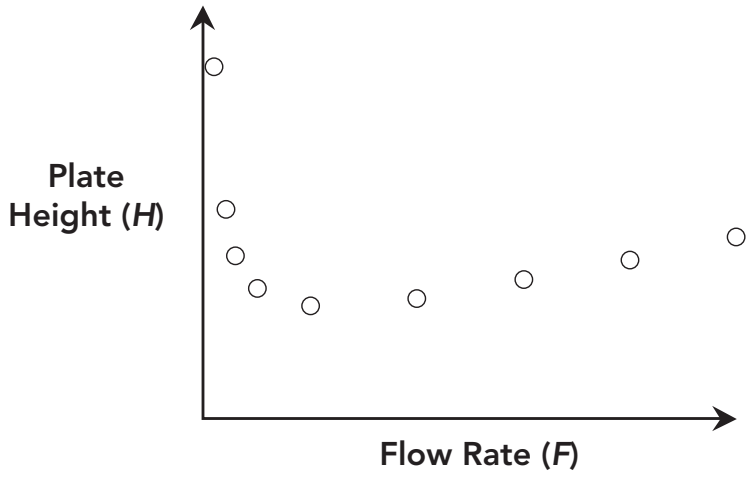
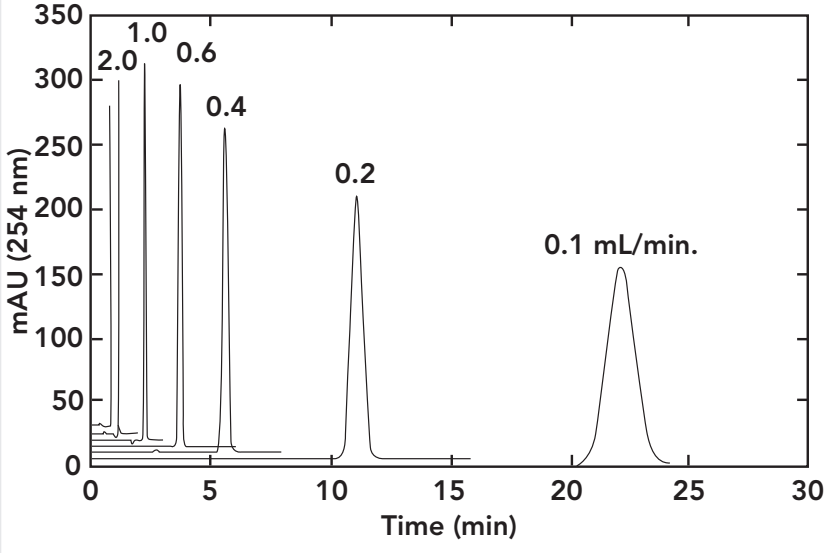


FIGURE 2: Effect of flow rate (indicated at the top of each peak; the abel for 3 mL/min is not shown for clarity). Starting from 0.1 mL min the baseline of each chromatogram is offset by 5 mAU to facilitate visualization. Chromatographic conditions: column, 50 mm × 4.6 mm, 5.0-µm Agilent SB-C18; mobile phase, 50:50 acetonitrile–water; temperature, 40 °C; injection volume, 1 µL; data acquisition rate, 40 Hz; analyte, acetophenone at 0.5 mg/mL in acetonitrile. The retention factor of acetophenone is about 2 under these conditions.



(that is, a measure of the peak width) in volume and time units, respectively. At the apex of a chromatographic peak, $t_i = t_R$ and we have exp(0) = 1. Thus, the concentration of the analyte at the peak apex, and therefore the peak height, is entirely determined by the pre-exponential term:

$$\frac{\text{moles of } A}{\sqrt{2\pi}\sigma_v} \quad [3]$$

Now, the moles of analyte injected are not affected by the flow rate, nor is $\sqrt{2\pi}$. Although there is no explicit dependence of σ_v on flow rate, the flow rate will affect the peak height whenever the flow rate affects the plate height (*H*) of the column in use, which is almost always the case. The relationship between plate height and σ_v is shown in equations 3 and 4, where *N* is the column efficiency or plate number for the column, and V_R is the retention volume of the analyte ($V_R = t_R * F$).

$$H = \frac{L}{N} \quad [4]$$

$$N = \left(\frac{V_R}{\sigma_v}\right)^2 = \left(\frac{t_R}{\sigma_t}\right)^2 \quad [5]$$

From a theoretical point of view, we know quite a bit about the dependence of plate height on flow rate through relationships such as the van Deemter equation (6). The general shape of this type of dependence is shown in Figure 1.

The details of these relationships are not important here. The important fact is that, for relatively small changes in flow rate, the changes in plate height and σ_v, and therefore peak height, will be relatively minor, as shown by the experimental data discussed below. Readers interested in

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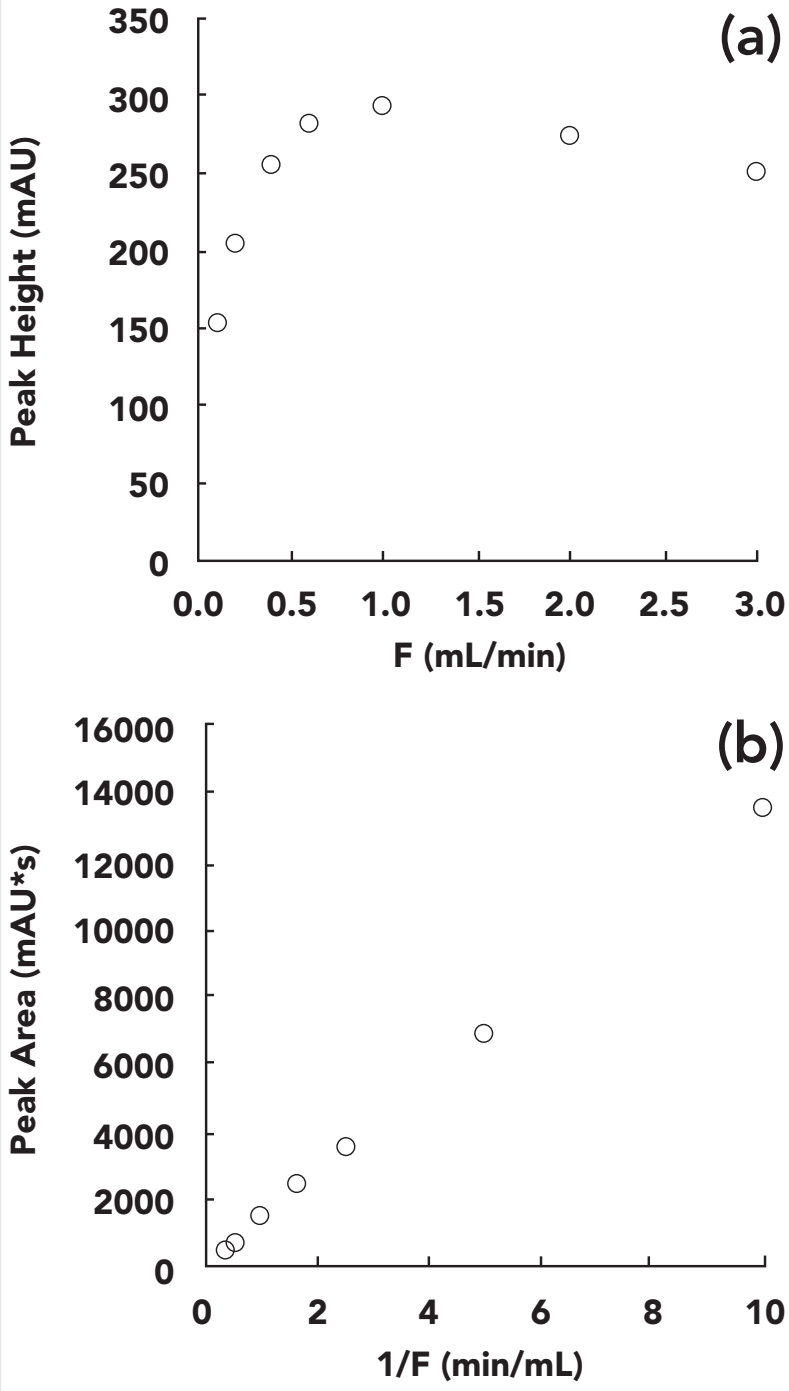
FIGURE 3: Quantitative view of the dependence of peak height (a) and peak area (b) on flow rate for the separations shown in Figure 2.



learning more about the dependence of plate height on flow rate are referred to the literature, which is a rich source of material on this topic (7).

Details Related to the Effect of Flow Rate on Peak Area(UV Detection)

Whereas the peak height is determined entirely by the pre-exponential term in equation 1, the peak area is determined by the integral of this equation, where the limits of integration are the time points that define the "start" and "end" of the peak. Indeed, when we talk about peak area we sometimes refer to the "area under the curve". Now, if we consider a chromatographic peak obtained with a specific set of conditions and think about what happens when we double the flow rate, we will observe that the width of the peak decreases by about a factor of two. The degree of decrease would be exactly a factor of two in a case where the plate number is not affected by flow rate because the ratio of t_R and σ_t is dictated by the plate number, as in equation 5. However, in most real situations, the plate number is affected by flow rate as discussed above, and the degree of change in width will be slightly different accordingly.

The net effect of flow rate on peak area in the case of UV detection is a consequence of two things happening at the same time: 1) the peak width changes in time units, expanding or contracting the integration window; and 2) the peak height is independent of flow rate, such that even if the peak becomes wider, time is added to the window over which the analyte is detected at a high concentration. In other words, the analyte flows through the UV detection cell at a finite velocity. The time over which the analyte can absorb photons

TABLE 1: Inventory of some expected effects of flow rate on LC chromatograms and peaks

Effect of Flow Rate (*F*) on…	Prediction of Simple LC Theory	Observations from Experiments and More Detailed Theory
Retention Time	Retention time increases in proportion to 1/*F*	-----
Retention Factor	No Effect	Use of high flow rates and pressures can lead to conditions where retention factors appear to depend on flow rate; this is more likely an outcome of a change in column temperature due to viscous heating (1), or a dependence of retention factors on pressure (2).
Column Inlet Pressure (P)	Pressure increases in proportion to *F*	Deviations from our expectations will occur if column temperature changes due to viscous heating (1), or if turbulent flow develops in connecting capillaries, or both (3). Both of these effects could lead to an apparent nonlinear dependence of *P* on *F*.
Peak Height (UV Detection)	No Effect	If the variation in flow rate is sufficient to have a measurable effect on the plate height of the column, then the peak height will change as a result of a change in peak variance.
Peak Area (UV Detection)	Area increases in proportion to 1/*F*	-----

is determined by the length of the light path the analyte travels through, and the velocity through that path. As the flow rate is reduced, the velocity through the detection path decreases, the residence time increases, and there are more opportunities for photons to be absorbed. Following this logic, we would expect to observe that peak area will increase in proportion to the inverse of the flow rate (that is, $A \propto \frac{1}{F}$).

Let's Look at Some Data

To illustrate the key points made above, I've made some experimental measurements of peak height and area at different flow rates, all under isocratic conditions. Figure 2 shows a series of chromatograms obtained at different flow rates in the range of 0.1 to 3.0 mL/min for the analyte acetophenone. From these chromatograms we see two clear trends: 1) the peak height varies slightly across these flow rates, but not in a simple linear way; and 2) the area under each peak obviously increases dramatically as flow rate is reduced.

Figure 3 shows a more quantitative view of peak height (a) and area (b) results from the chromatograms shown in Figure 2. We see that the shape of the dependence of the peak height on flow rate is the inverse of the shape of the plate height versus flow rate curve shown in Figure 1. Whereas there is a minimum in the *H* versus *F* curve in Figure 1, there is a maximum in the dependence of peak height on *F* around 1.0 mL/min in Figure 3(a). This is expected because of the inverse relationship between $C_{detected}$ and σ_v.

On the other hand, the dependence of the peak area on flow rate (Figure 3[b]) is very different. We see that the peak area increases in direct proportion to the inverse of the flow rate. This is because each part of the peak moves through the detection flow cell more slowly at a lower flow rate, the residence time in the detection zone is longer, and each analyte molecule contributes more to the measured absorbance.

Closing Thoughts

Our effectiveness in troubleshooting problems with LC separations improves as we deepen our basic understanding of how the separations work. In this article we have examined the dependence of peak height and area on flow rate when using UV detection. Whereas peak height is only weakly dependent on flow rate, the peak area is strongly dependent on *F*, and decreases significantly as flow rate is increased. The extent of the expected decrease is important to know when troubleshooting problems with quantitation. For example, a leak between the injector and detector could also lead to decreases in peak area at higher flow rates (and consequently higher pressures).

References

1) F. Gritti, M. Martin, and G. Guiochon, *Anal. Chem.* **81**, 3365–3384 (2009). doi.org/10.1021/ac802632x.
2) S. Fekete, J.-L. Veuthey, D.V. McCalley, and D. Guillarme, *J. Chromatogr. A* **1270**, 127–138 (2012). doi.org/10.1016/j.chroma.2012.10.056.
3) J. Halvorson, A.M. Lenhoff, M. Dittmann, and D.R. Stoll, *J. Chromatogr. A* **1536**, 185–194 (2016). doi.org/10.1016/j.chroma.2016.12.084.
4) P.L. Urban, *J. Chem. Educ.* **93**, 984–987 (2016). doi.org/10.1021/acs.jchemed.5b00986.
5) M. Dong and J. Wysocki, *LCGC North Am.* **37**, 750–759 (2019).
6) J.J. van Deemter and F.J. Zuiderweg, *Chem. Eng. Sci.* **5**, 271–289 (1956). doi.org/10.1016/0009-2509(56)80003-1.
7) G. Desmet, *Anal. Chem.* **87**, 8593–8602 (2015). doi.org/10.1021/ac504473p.

Dwight R. Stoll is the editor of "LC Troubleshooting." Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota, USA. 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

Can We Continue to Draw the Line?

Heather Longden[1] and R.D. McDowall[2], [1]Waters Corporation, Milford, Massachusetts, USA, [2]R.D. McDowall Ltd, Bromley, Kent, UK

Chromatographic peak integration continues to be a major regulatory issue and was first discussed in this column in 2015. Is the approach to manual intervention and manual integration outlined still acceptable in the light of regulatory citations and guidance documents published since then?

Peak integration is at the heart of chromatographic analysis. Understanding how data are acquired by a chromatography data system (CDS), how peaks are integrated, and how each integration parameter operates is essential to effective analysis. The key reference on chromatographic integration was written over 20 years ago by Normal Dyson (1). Chromatographic integration in regulated laboratories was discussed in this column in 2015 (2) where a structured approach to first manual intervention (change of peak windows and processing parameters with automatic baseline placement) and then manual integration (manual repositioning baselines) was presented and discussed. The aim was to take a scientifically sound approach to integration in a regulated laboratory. Manual intervention can be applied to any analysis, but should be applied consistently to all injections in a sequence because chromatography is a comparative analytical technique. Newton and McDowall also discussed peak integration in the third part of a six-part series on data integrity, which included an outline of the order of processing of files in a sequence and the contents of an integration standard operating procedure (SOP) (3). Manual integration needs to be carefully controlled and managed because this is an area where an inspector will focus. Before we look in detail at integration, let us step back and look at the applicable regulations.

Laboratory Controls and Record Requirements

It is important to understand the regulatory requirements for laboratory controls and records as these provide a major input to any discussion. The US good manufacturing practice (GMP) regulations for laboratory controls include this requirement (4):

21 CFR 211.160(b) *Laboratory controls shall include the establishment of scientifically sound and appropriate specifications, standards, sampling plans, and test procedures designed to assure that components, drug product containers, closures, in-process materials, labeling, and drug products conform to appropriate standards of identity, strength, quality, and purity.*

This is a relatively simple regulation to understand: everything done in the laboratory including peak integration must be scientifically sound. A similar requirement for scientific soundness in the laboratory is found in section 11.12 of EU GMP Part 2 for active pharmaceutical ingredients (5).

As chromatography is a comparative analytical technique all injections should be integrated in the same way as much as possible. This generalization is tested near the limits of quantification and where complex mixtures are separated. Next, we have:

21 CFR 211.194(a) *Laboratory records shall include complete data derived from all tests necessary to assure compliance with established specifications and standards, including examinations and assays,.....* (4).

It is important to understand the regulatory requirements for laboratory controls and records as these provide a major input to any discussion.

The requirement for laboratory records is also simple: complete data has been discussed in this

column earlier (6) and other articles (7,8). The EU GMP regulations are not as simple to interpret; chapter 4 refers to raw data that is not defined (9). A discussion of the meaning of raw data has been presented earlier and is equivalent to complete data in the US GMP regulations (6).

Don't Do This in Your Laboratory

How are these regulations interpreted by inspectors? Here are some examples involving scientifically unsound practices and uncontrolled integration from the FDA:

- *Integration of chromatograms for method STM-0076 <redacted> has been performed inconsistently. The <redacted> chromatograms exhibit tailing. Prior to March 2017 <redacted> was generally integrated as an impurity. In March 2017 a recommendation was made in STM- 0076 to <redacted> at this retention time.*
  The recommendation is often but not always followed and results in this area being integrated <redacted>. There is a lack of scientific justification to support if the tailing portion should be integrated. The change ... was implemented without fully evaluating the impact on previously processed data (10).
- *Your test methods were not capable of demonstrating the purity of your drugs... analysts reprocessed data up to 12 times, and only included the final result in the report for review by Quality Assurance. Your Deputy Manager, Quality Control stated that it is common practice to "play with parameters" to get the proper integration (11).*
- *Failure to ensure that all test procedures are scientifically sound and appropriate to ensure that your API conform to established standards of quality and purity. You failed to establish adequate test procedures. For example, your analyst manually integrated a HPLC test for <redacted> API despite the fact that the chromatogram lacked peak resolution. ..... You lacked an approved protocol for manual integration or quality oversight of the practice (12).*
- *Method QC/STP/I2252-04 was not followed in analysis if <redacted> tablets for <redacted> by gas chromatography. The method requires the standards be prepared with <redacted>. During the preparation of standards for sequences QC863VEN1606A, the standards were prepared with both <redacted> and <redacted>, so the same standards could be used to evaluate <redacted> tablets for <redacted> and <redacted> tablets for <redacted>. The <redacted> and <redacted> peaks coelute, potentially reducing the accuracy of the standard area count compared to the approved method (13).*

Defining Manual Integration

In an earlier "Questions of Quality" column on integration (2) it was noted that there was no definition of manual integration. This column implicitly defined manual integration as manual placement of the baseline by a chromatographer. The Parenteral Drug Association's (PDA's) *Technical Report No. 80* defines manual integration as a:

> *Process used by a person to modify the integration of a peak area by modifying the baseline, splitting peaks or dropping a baseline as assigned by the chromatography software to overrule the pre-established integration parameters within the chromatographic software* (14).

Is this definition acceptable? It is wordy, repetitious, and could be better phrased. The use of the word *overrule* is contentious. As noted above, CDS software is not perfect and an application can struggle to separate overlapping peaks obvious to a trained eye. The biggest issue with this definition is that there is no mention of scientific soundness as defined in the FDA GMP regulations presented earlier (4).

A simpler, more concise, and better definition of manual integration could be "manual repositioning of peak baselines with scientific justification for their positioning".

Implicit within this definition is the use of CDS software—otherwise you'd be drawing baselines on paper. However, this also requires that the chromatographer is trained, and ideally software technical controls should prevent manual repositioning of baselines where this is not justified by the type of the analysis. In addition, the audit trail should record the actions of the analyst optimizing the peak integration, and the system should provide a means to recall the original automated integrated chromatogram.

Should Manual Integration Be Banned?

From the citations above, would it be reasonable to ban manual integration in regulated laboratories? Let's think this through. Experienced analysts know that chromatographic analysis can be affected by temperature, humidity, column history, as well as

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mobile phase preparation, so that one day's analysis often varies slightly from the previous day's run. To achieve consistent output and measurement, it is critical to adapt and optimize factors such as peak detection threshold or retention time windows to ensure consistent, correct, and accurate integration. But how can reviewers, approvers, quality, or outside auditors recognize the legitimate vs. egregious use of manual integration?

Banning the use of manual integration is a common response to avoid questions about data integrity. However, there are three outcomes to this crude action:

- Laboratories will have to accept poor and inconsistent integration.
- Analysts will find a workaround that permits them to integrate each chromatogram with a different set of integration parameters (typically involves performing quantification in a laboratory information management system [LIMS], or worse, a spreadsheet, without traceability back to the integration methods).
- Analysts will be forced to spend hours of their day developing complex and manipulative methods to address variations between chromatograms with a single processing method. Typically, this will require many "integration events" that could even include placing peak starts and ends at specific time points; in effect, performing manual integration to satisfy the "no manual integration" rule and deceive the reviewer.

In the wrong hands, with the wrong intent, and without a robust training and review process, altering chromatographic peak processing parameters has been misused by analysts to falsify results. How can this be managed?

The Changing Regulatory Landscape

Since the earlier "Questions of Quality" column on integration (2) there have been many publications on data integrity from regulatory authorities such as Medicines and Healthcare products Regulatory Agency (MHRA), World Health Organization (WHO), European Medicines Agency (EMA), Pharmaceutical Inspection Co-operation Scheme (PIC/S), and Food and Drug Administration (FDA) (15–20), as well as industry bodies such as GAMP, PDA, and European Compliance Academy (ECA) (14,21,22). There is also the recent publication of the draft of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) M10 on bioanalytical method validation (23) that combines the current views outlined in earlier guidance documents issued by the EMA and FDA (24,25). However, only the PDA and ICH guidance documents have specific sections on chromatographic integration (14,23).

In the wrong hands, with the wrong intent, and without a robust training and review process, altering chromatographic peak processing parameters has been misused by analysts to falsify results.

Guidance for Integration

The PDA's *Technical Report No. 80* has a large section on chromatographic integration, and the guidance document illustrates both acceptable and unacceptable peak integration practices (14). ICH M10 section 3.3.6 outlines the current thinking for integration of chromatograms in bioanalysis (23):

- Chromatogram integration and reintegration should be described in a study plan, protocol, or SOP.
- Any deviation from the procedures described *a priori* should be discussed in the Bioanalytical Report.
- The list of chromatograms that required reintegration, including any manual integrations, and the reasons for reintegration should be included in the Bioanalytical Report. Original and reintegrated chromatograms and initial and repeat integration results should be kept for future reference and submitted in the Bioanalytical Report for comparative BA/BE (bioanalytical or bioequivalence) studies.

Gone is the burdensome FDA requirement for a manager to preapprove any manual integration (25), to be replaced by a plan or SOP for controlling the integration process with the before and after chromatograms included in the study report showing the impact of the changes of manual integration.

Why Manually Integrate Peaks?

We need to consider why we need to integrate peaks manually and the reason is that there are situations where a CDS cannot integrate peaks correctly. Some examples are:

- Split peaks
- Shoulder peaks
- Tailing peaks
- Baseline noise
- Negative peaks
- Coeluting peaks
- Rising, falling, or excessively noisy baselines
- Slowly eluting peaks (where the CDS has difficulty identifying the peak end).

The reasons for the inability of the integration method may be due to:

- Poor method development and validation where the analytical procedure or the integration method is not optimized or robust
- Requirements for quantitation of very small peaks, especially new, unexpected peaks resulting from impurities or excipients
- Complex sample matrices resulting in interfering peaks that may still be present after sample preparation, for example, biological samples, contrast media
- Analysis of complex mixtures may result in a heavy manual integration workload as the CDS method is not able to integrate all peaks automatically, for example, contrast media, fermentation samples.

There must be a scientifically sound (4) justification for manual intervention and manual integration as outlined in the earlier "Questions of Quality" column (2). Remember that manual integration slows an analytical process and is inefficient (3).

The more that peak integration is automated, the faster it is, with the bonus of lower regulatory scrutiny. Otherwise the review could take longer than the actual analysis.

This places responsibility on the laboratory to develop robust analytical chromatographic procedures with reliable separations that are fit for use. A key component of this approach is that the resultant peak integration must be consistent, not the use of consistent parameters or settings to achieve that peak integration. This is a subtle but vital difference that is not always appreciated.

Quality-by-Design for Robust Methods

The regulatory world is changing for analytical procedures and this should have a positive impact on peak integration. There is an ICH final concept paper (26) for the development of ICH Q14 on analytical procedure development guidelines and a revision of ICH Q2(R1) on validation. The current problem with ICH Q2(R1) (27) is that it is focused on method validation with nothing mentioned about the most important phase of the life cycle: method development. Understanding how a method works and what are the key variables is often overlooked due to time pressures. Failure to plan is planning to fail.

A life cycle approach has been addressed by a proposed *USP* general chapter <1220> on analytical procedure life cycle management (28). The *USP* life cycle begins by defining the analytical target profile (ATP), that is, the start of the process to define and validate a design space for each analytical procedure. It ensures that critical parameters are managed and controlled, and changes within the design space are known and predictable, for example, organic modifier changes of the mobile phase. Knowing the design space of an analytical procedure should result in better peak shape and resolution and hence accurate automated peak integration. Outcomes of this analytical procedure life cycle management (APLM) or method life cycle management (MLCM) approach should be more robust methods, reproducible chromatography, scientifically consistent peak integration, and, hopefully, reduced out of specification (OOS) results.

Is the FDA Banning the Use of Inhibit Integration Events?

The use of the inhibit integration function is a hot regulatory topic now, as can be seen from this FDA regulatory citation:

1. Failure to ensure that test procedures are scientifically sound .... Our investigators observed that the software you use to conduct high performance liquid chromatography (HPLC) analyses of API for unknown impurities is configured to permit extensive use of the "inhibit integration" function without scientific justification.

For example, our investigator reviewed the integration parameters you used for HPLC identification of impurities in release testing for <redacted>. These parameters demonstrated that your software was set to inhibit peak integration at four different time periods throughout the analysis.

Inhibiting integration at various points during release testing for commercial batches is not scientifically justified. It can mask identification and quantitation of impurities in your API, which may result in releasing API that do not conform to specifications (29).

This citation is based on 21 CFR 211.160(b) (4), which was presented earlier in this column, and the key questions to ask are if, where, and when can integrate inhibit be used? It has been said in some audits and inspections that this function cannot be used. This is an untenable situation and there is no explicit or implicit statement in the GXP regulations for this attitude. However, it comes back to scientific soundness and a laboratory must be able to justify the use of the function. Let us consider the following scenario:

- There is a baseline perturbation with a large negative peak after an injection. A peak of interest elutes shortly after the negative peak. The use of integrate inhibit is fully justified from the start of the injection until the baseline has returned to normal and before elution of the peak of interest. Otherwise there is a large probability that baseline placement of the analyte could be adversely influenced by the negative peak.
- Similar scenarios occur when extraneous peaks are washed from the column, or baseline perturbations from mobile phase changes during a gradient elution or wash at the end of a chromatogram.

What is more problematic is the use of integrate inhibit in the middle of a run as cited above (29). If system, blank, or other non-sample peaks occur in the middle of a chromatogram, traditionally those peaks were not integrated. Because of suspicions that the excluded peaks might be real impurities, excluding these system peaks needs to be carefully documented and justified in the method development and validation reports, otherwise they should be integrated and marked clearly as system peaks.

System Evaluation Injections

Trial injections using actual samples feature in many warning letters (30) and question 13 of the FDA data integrity guidance (20) states that:

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LGC

FDA prohibits sampling and testing with the goal of achieving a specific result or to overcome an unacceptable result (e.g., testing different samples until the desired passing result is obtained).

Only system evaluation injections prepared from a suitable reference standard can be used to evaluate if the chromatographic system is ready.

This is correct and should never be acceptable in a GXP laboratory SOP.

However, consider the following situation: you are analyzing low volume samples from a nonclinical study. There is a total volume of 20 µL plasma sample that is extracted and there is only enough for a single injection from each sample. Ask yourself the question; are you going to commit an analytical run of samples without checking that the system is ready? The cost of repeating the study is a high six figure sum if a run does not work. Therefore, from a practical perspective, we need a way of checking that a system is ready for analysis, but one that does not involve testing into compliance with samples. Ah, somebody says use system suitability test (SST) injections. The problem is that you may need several replicates to determine if the system is ready, and SST injections should never be started until you are confident that the system is equilibrated.

We propose the following approach for system evaluation, readiness injections, or equilibration checks:

- The ability to use system evaluation injections must be documented in an applicable SOP or analytical procedure.
- The minimum column equilibration time needs to be documented in the method to avoid excessive system readiness injections.
- Only system evaluation injections prepared from a suitable reference standard can be used to evaluate if the chromatographic system is ready. Records of the solution preparation must be available. Ideally, a test mixture that mimics the separation characteristics, but is easily distinguishable from real samples should be used.
- Should the maximum number of system evaluation injections that can be made be documented in the procedure before a problem with the chromatographic system needs to be investigated? If the cause is thought be an equilibration issue, waiting and injecting again should be sufficient. If the system continues to not behave, then an investigation is needed; the cause should be found, remediated, and documented in the instrument logbook before checking the system evaluation again. If the problem requires maintenance to resolve it, for example, pump seal replacement, then requalification of the pump should be conducted and documented before beginning the analysis.
- Using sample preparations as equilibration injections or "system readiness" checks must be clearly prohibited. The FDA guidance offers suggestions about the use of a well-characterized secondary standard for such a purpose.
- System evaluation injections are part of the complete data for the analytical run and must be included in the instrument logbook entries along with any investigation and remediation work on the instrument. A common practice is to store the data from these tests in a separate folder or location to the real analyses. This practice needs careful management and documentation as it becomes difficult to connect those injections to the official laboratory work. Ideally, all work including system evaluation injections should be stored in the same location.

Five Rules of Integration

An integration SOP was discussed earlier (3,25) to help understand what should be in it and the associated training. There are five rules to consider:

- *Rule 1: The main function of a CDS is not to correct your poor chromatography.*
  This places greater emphasis on the development of robust chromatographic procedures so that the factors involved in the separation are known and controlled adequately. Whenever possible, separations should be developed such that automatic integration is the norm not the exception. Management need to understand that adequate time must be given to method development and validation. This is especially true for pharmacopoeial methods that never work as written.
- *Rule 2: Never use default integration parameters, always configure specific integration for each method.*
  Without exception, peak integration and result processing must be defined and validated for each method so that all peak windows and names are defined and if

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necessary any system peaks are identified. Using a default or generic method results in excessive need for manual integration to name and calculate peaks.

- *Rule 3: Always use automatic integration as a first option and control manual integration practices.* Remember that the use of manual integration is a regulatory concern and use needs to be scientifically sound. Also be aware that, as discussed earlier, manual integration slows down a process, so see Rule 1 to get the right method depending on the sample matrix and peaks of interest.
- *Rule 4: Understand how the CDS works and how the numbers are generated.* This requires basic training in the principles of peak integration and how a CDS works. The problem is that with mergers, acquisitions, and encouraging experienced analysts to retire and employ younger workers, skills are being eroded and a CDS can be looked at as a black box that always gives the right answers.
- *Rule 5: Use your brain—think.* This rule is sometimes difficult to follow but follows on from Rule 4. You can have what appears to be a perfect separation and peak integration, but look at peak start and end placement—do they look right? Use the zoom and overlay functions of the CDS to see if standards and samples have the right peak shape. The analyst is responsible for executing applicable procedures correctly, which includes correct peak integration. The reviewer, however, also has a role to ensure that all integration (whether automated, optimized, or manually placed) follows the method guidance for placing baselines as the SOP describes, especially when the representative area for unresolved peaks are being estimated. Significant peak area manipulation should be easily noticed by an experienced reviewer.

Remember that the use of manual integration is a regulatory concern and use needs to be scientifically sound.

Quo Vadis Peak Integration?

If you think that peak integration is a regulatory issue now, what will it be like in the future? The May 2019 supplement to *LCGC Europe* gives an interesting glimpse via an article from Wahab *et al.* (31), who discuss advanced signal processing techniques that could be used in chromatographic integration. The techniques listed are:

- Deconvolution of extracolumn effects by Fourier transformation for removing band broadening
- Peak area extraction by iterative curve fitting for partial overlapping peaks in a chromatogram
- Model-free approaches for peak information extraction is another approach for extracting peak areas from overlapping peaks in complex matrices
- Direct resolution by power law increases resolution by reducing peak width and trailing
- Direct resolution enhancement by even derivative peak sharpening also increases resolution by reducing peak width.

It is beyond the scope of this column to present and discuss what is already in this paper (31), but if any of these techniques are integrated into a chromatography data system, then their use needs to be justified scientifically. This means from development through validation to use of a method.

If regulators are worried by peak integration now, they could be paranoid in the future!

Summary

At the start of this column we asked the question: is the approach to manual intervention and manual integration still acceptable in the light of regulatory citations and guidance documents published since 2015? Yes is the answer, but the integration parameters used in each method need to be scientifically sound and justified on a method by method basis. This means that more attention to detail must be made when developing each method, understanding and controlling the factors that influence chromatographic separation and peak shape. Good peak integration requires good chromatography. The bottom line is—are you in control of the analytical procedure and peak integration?

References

1) N. Dyson, *Chromatographic Integration Methods 2nd Edition* (Royal Society of Chemistry, Cambridge, UK, 1998).
2) R.D. McDowall, *LCGC Europe* **28**(6), 336–342 (2015).
3) M.E. Newton and R.D. McDowall, *LCGC North America* **36**(5), 330–335 (2018).
4) 21 CFR 211, *Current Good Manufacturing Practice for Finished Pharmaceutical Products* (Food and Drug Administration, Silver Spring, Maryland, USA, 2008).
5) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) guidelines, Part 2 - Basic Requirements for Active Substances used as Starting Materials* (European Commission, Brussels Belgium, 2014).
6) R.D. McDowall, *LCGC North America* **37**(4), 265–268 (2019).

7) R.D. McDowall, *Spectroscopy* **31**(11), 18–21 (2016).
8) R.D. McDowall, *Data Integrity and Data Governance: Practical Implementation in Regulated Laboratories* (Royal Society of Chemistry, Cambridge, UK, 2019).
9) EudraLex, *Volume 4 Good Manufacturing Practice (GMP) Guidelines, Chapter 4 Documentation* (European Commision, Brussels, Belgium, 2011).
10) US Food and Drug Administration, 483 Observations: Adello Biosciences (Food and Drug Administration, Silver Spring, Maryland, USA, 2018).
11) US Food and Drug Administration, Warning Letter: Megafine Pharma Ltd (Food and Drug Administration, Silver Spring, Maryland, USA, 2017).
12) US Food and Drug Administration, Warning Letter: Hubei Danjiangkou Danao Pharmaceutical Co., Ltd (Food and Drug Administration, Silver Spring, Maryland, USA, 2017).
13) US Food and Drug Administration, 483 Observations: Cadila Healthcare Limited (Food and Drug Administration, Silver Spring, Maryland, USA, 2019).
14) PDA, *Technical Report 80: Data Integrity Management System for Pharmaceutical Laboratories* (Parenteral Drug Association [PDA], Bethesda, Maryland, USA, 2018).
15) MHRA, *MHRA expectation regarding self inspection and data integrity* 2013 [cited 1 Jan. 2016]. Available from: http://webarchive.nationalarchives.gov.uk/20141205150130/http://www.mhra.gov.uk/Howweregulate/Medicines/Inspectionandstandards/GoodManufacturingPractice/News/CON355490.
16) MHRA, *GMP Data Integrity Definitions and Guidance for Industry 2nd Edition* (Medicines and Healthcare products Regulatory Agency, London, UK, 2015).
17) MHRA, *GXP Data Integrity Guidance and Definitions* (Medicines and Healthcare products Regulatory Agency, London, UK 2018).
18) WHO, *Technical Report Series No.996 Annex 5 Guidance on Good Data and Records Management Practices* (World Health Organization, Geneva, Switzerland, 2016).
19) Pharmaceutical Inspection Cooperation Scheme, *PIC/S PI-041-3 Good Practices for Data Management and Integrity in Regulated GMP / GDP Environments Draft* (Geneva, Switzerland, 2018).
20) Food and Drug Administration, *FDA Guidance for Industry Data Integrity and Compliance With Drug CGMP Questions and Answers* (Food and Drug Administration, Silver Spring, Maryland, USA, 2018).
21) *GAMP Guide Records and Data Integrity* (International Society for Pharmaceutical Engineering, Tampa, Florida, USA, 2017).
22) *GAMP Good Practice Guide: Data Integrity - Key Concepts* (International Society for Pharmaceutical Engineering, Tampa, Florida, USA, 2018).
23) International Conference on Harmonization, *ICH M10 Bioanalytical Method Validation Stage 2 Draft* (ICH, Geneva, Switzerland, 2019).
24) *EMA Guideline on Bioanalytical Method Validation* (European Medicines Agency, London, UK, 2011).
25) US Food and Drug Administration, *FDA Guidance for Industry: Bioanalytical Methods Validation* (Food and Drug Administration, Silver Spring, Maryland, USA, 2018).
26) International Conference on Harmonization, *Final Concept Paper ICH Q14: Analytical Procedure Development and Revision of Q2(R1) Analytical Validation* (ICH, Geneva, Switzerland, 2018).
27) International Conference on Harmonization, *ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology* (ICH, Geneva, Switzerland, 2005).
28) G.P. Martin *et al., Pharmacopoeial Forum* **43**(1), (2017).
29) US Food and Drug Administration Warning Letter: Divi's Laboratories Ltd. (Unit II) (Warning Letter 320-17-34) (Food and Drug Administration, Silver Spring, Maryland, USA, 2017).
30) R.D. McDowall, *LCGC Europe* **27**(9), 486–492 (2014).
31) M.F. Wahab, G. Hellinghausen, and D.W. Armstrong, *Recent Developments in HPLC and UHPLC, LCGC Europe* Supplement **32**(s5), 22–28 (2019).

Heather Longden is a Senior Marketing Manager, Pharmaceutical Regulatory Compliance at Waters Corporation in Milford, Massachusetts, USA. "Questions of Quality" editor **Bob McDowall** is Director of R.D. McDowall Limited, Bromley, Kent, UK. He is also a member of *LCGC Europe*'s editorial advisory board. Direct correspondence about this column to the editor-in-chief, Alasdair Matheson, amatheson@mmhgroup.com

Exploring Energetic Materials Using Nontargeted Analysis

LCGC Europe **spoke to Leon Barron and Matteo Gallidabino to discuss novel nontargeted approaches to analyze explosive materials using ion chromatography (IC) with high resolution mass spectrometry (HRMS), and the challenges and solutions analysts can encounter when developing nontargeted methods.**

Interview by Alasdair Matheson, Editor-in-Chief, *LCGC Europe*

Leon Barron is a senior lecturer in forensic science at King's College London, UK. He received his Ph.D. in analytical chemistry from Dublin City University, Ireland, in 2005. His expertise lies in analytical chemistry, particularly in separation science, mass spectrometry, and machine learning for targeted and nontargeted applications in environmental, forensic, and biological systems analysis.

Matteo D. Gallidabino is currently a senior lecturer in forensic science at Northumbria University in Newcastle, UK. He has a comprehensive background in criminalistics that he obtained at the School of Criminal Justice of the University of Lausanne, Switzerland. His work focuses on next-generation analytical techniques, successfully combining separation methods, mass spectrometry, and advanced data analytics to provide enhanced information in a forensic context and better support the court in the decision-making process.

Q. Nontargeted analysis (NTA) is currently gaining wider acceptance. What is NTA and where is it being used in your area of research?

Leon Barron: Most analyses are targeted in nature. That is, a number of specific compounds are selected before the analysis occurs. Nontargeted analysis (NTA) refers to applications where no specific analytes are shortlisted beforehand and the instrument captures everything it can detect, so that the data can be reviewed in a flexible way later. There are several ways to perform NTA including: (a) using all of the data generated by the instrument to classify or differentiate samples as a whole from each other by, for example, principal component analysis (PCA); (b) identifying specific "features" in the data that change significantly following exposure to a toxic substance, for example, and, whilst still not necessarily knowing its identity. As an extension of NTA, suspect screening is the identification of new compounds in the sample by matching measured data to one or more databases or by manual search using theoretical ion accurate *m/z* . For liquid chromatography (LC)- and gas chromatography (GC)-based techniques, NTA has been most useful when coupled to high resolution mass spectrometry (HRMS) instruments, which comprise of either time-of-flight (TOF) or orbital ion trap-based mass analyzers. The use of HRMS helps immensely to resolve significantly larger numbers of features and arguably represents the best means to most rapidly identify these afterwards too. In my area of environmental and forensic chemistry, NTA is becoming more commonly used. For example, environmental metabolomics is now emerging more to identify any endogenous metabolite features that change in aquatic species as a result of exposure to contamination or specific environmental conditions in rivers (1–4). We have also extensively used suspect screening to identify new organic contaminants, including pharmaceuticals, illicit drugs, explosives, their metabolites, precursors, and transformation products in complex samples such as wastewater and river water to monitor community-scale activities (5–7). In forensic science, the ability to retrospectively mine such large datasets is very useful to go back and assess whether an analyte might have been present (8).

Q. Have there been any major technological breakthroughs nontargeted analysis?

LB: Arguably the most significant technological breakthrough that has pushed NTA forwards is the increased commercial availability of HRMS instruments that can be coupled to separation techniques such as LC or GC, for example. Mass accuracies of <1 ppm are now readily achievable

with resolutions up to 140,000 full-width at half peak maximum (FWHM), providing elemental composition-level information in many cases. Similarly, the ability to perform different modes of data independent analysis (DIA) offers extra flexibility for NTA including "all ion" fragmentation and sequential window acquisition of all theoretical mass spectra analysis (SWATH), for example. Along with the ability to perform traditional targeted analysis too (data-dependent analysis [DDA]), it has become possible to perform targeted, NTA, and suspect screening using the same instrument and, in certain cases, simultaneously. As a result, published targeted methods are generally growing with respect to the number of analytes they include as new compounds are discovered or added continually (9,10). This has presented analysts with a new challenge scale and treatment of data. Given the amount of data these instruments acquire, data analysis is now the bottleneck. It can take significantly longer to review and interpret the results generated for a single sample than it takes to run it in the laboratory! Whilst excellent processing tools and databases exist for MS data, there is less focus on separations data in my opinion and machine learning has recently proved useful here (10,11). I see massive potential in the use of machine learning generally moving forwards, not only for suspect screening but also for NTA, for example, for prediction of changes in 'omics datasets or linking these to effects following toxicant exposure.

Q. You recently developed a method using both targeted and nontargeted gradient ion chromatography (IC) with HRMS to profile black powder substitutes and gunpowder residues. What were the aims of this research?

LB: The aim of this work was to develop and validate a new gradient IC-HRMS method that would be broadly applicable to quantitative determination of trace concentrations of low-molecular-weight inorganic and organic anions, but primarily that would be suitable for forensic casework in energetic materials analysis, including ammunition and explosives (12). We also wanted to exploit HRMS to potentially offer us more information about the sample. First, we focused on identifying a black powder substitute in fingermarks and sweat deposits from a donor using IC-HRMS. Using a mixture of targeted analysis and NTA with PCA, we investigated the time since the materials were handled, which was very exciting! Following this, we identified features in the data that drove any temporal trends to potentially serve as a new way to include or exclude similar residues of such materials found at a crime scene that were relevant to the case. We also aimed at gunshot residue to see whether we could use NTA to classify by the original ammunition used.

Matteo Gallidabino: Samples submitted to forensic analysis are usually characterized by a higher number of species than those usually targeted by traditional methods. This means that they also potentially contain complementary information, which may be helpful to track back the trace origin and deposition mechanism. Hence, a complementary aim of this project was to assess if enhanced intelligence could actually be extracted from forensic samples through the judicious combination of NTA with modern data analytics.

Q. What were the main obstacles you encountered in this project and how did you overcome them?

LB: I have been working with IC-MS since around 2001 and coupling the two techniques has often been cumbersome in comparison to LC–MS in my experience (13). Thankfully, modern IC-MS systems are now available with integrated instrument control and data analysis. However, in this particular application there were two main challenges because IC generally operates using purely aqueous eluents. First, IC eluates are not very volatile and auxiliary pumps are often needed to deliver organic solvent into the eluate to aid gas phase transfer in electrospray ionization (ESI), especially for

trace analysis. Second, extraction of complex samples typically encountered in forensic casework normally use organic solvents not always compatible with IC separations. Therefore, we have been trying to find ways to circumvent these two issues by using organic solvent in the eluent itself, which presents its own challenges (14). So far, we have tried a number of additives, including methanol, acetonitrile, and ethanol, which have each removed the need for auxiliary pumps making the coupling process much simpler, and keeps the system cleaner at the same time. The trade-off is IC selectivity, which changes markedly and, in some cases, unpredictably. Furthermore, some organic solvents transform under alkaline conditions, which leads to interference; for example, acetonitrile can hydrolyze in hydroxide-based eluents to yield acetate ions in the background signals. In this work, we aligned the IC eluent with that of the sample extraction solvent directly, that is, 50:50 (*v/v*) ethanol–water. We also improved the selectivity over previous IC methods by introducing a gradient separation using carbonate–bicarbonate as an eluent and this worked very well.

As we do not usually know the identity of an energetic material in forensic science, it is necessary to perform both organic and inorganic screening. This extract would therefore normally be analyzed directly by an LC–HRMS method for a large suite of organic high-order explosives, precursors, and transformation products and then, following solvent exchange to remove ethanol, by IC-MS. By developing the separation in ethanolic eluents, our aim was that the sample extract could be analyzed directly, which could increase throughput and robustness.

MG: Challenges to overcome during this project were numerous, and not just limited to the decision of which strategy to adopt. A priority for the IC-HRMS method was that it could easily be aligned with current forensic practices. As 50:50 (*v/v*) ethanol–water is often used to extract explosive samples in casework, we decided to adopt this as the eluent. Preliminary tests on ESI performance supported the choice because they showed that this mixture led to the same or better signals than conventional eluents used in liquid-based chromatographic techniques. That was promising, but the viscosity of the mixture obviously also brought some challenges with the column back pressure that had to be addressed by increasing the column temperature. Also, ESI using 50:50 (*v/v*) ethanol–water has been rarely investigated before, so the best conditions were essentially unknown. Therefore, the implementation of the method basically became a problem of fine-tuning all the parameters involved in the chromatographic, ionization, and detection steps! We eventually used a statistical-based, design-of-experiments (DOE) approach to deconvolute this complexity and properly investigate analyte separation and responses to find optimal conditions. The use of predictive modelling methods was therefore not just limited to the evaluation of the new approach in an operational context, but also to its optimization. Thanks to this, in any case, we were able to achieve excellent analytical performance.

Q. What is novel about your approach and what benefits does it offer over previous techniques?

LB: The use of IC-MS is not new, but its use in forensic science is really only emerging now. The benefits of this particular method were that a larger number of anions could be detected (n = 19) than previously possible as a result of the optimization of gradient conditions. The lower limit of detection lies in the low µg/L range, making it suitable for direct trace analysis across a range of applications if needed. One of the main benefits this method offered is obviously its direct integration into standard workflows, making the analytical process far simpler and practical for the analysis of organic–solvent-based extracts. As well as keeping the system clean, it also enables elution of hydrophobic/non-charged species, which would otherwise be fully retained by IC, thereby widening its scope.

While several applications have been reported in environmental science using IC-MS, for example (15), the use of IC-HRMS is quite rare. This technique offers obvious advantages in forensic science, especially for NTA and suspect screening. Here, our approach allowed us to show how both the targeted anions and the NTA profile of the rest of the contaminated sweat sample changed over a period of hours following contact with the black powder substitute, even after washing their hands! Similarly, for a range of different gunshot residues collected after firing a gun, we were able to link these with three original ammunition brands that were used. Using NTA, we were able to tentatively identify several new potential compounds afterwards that could provide additional linkages between different evidence types together. The ability to provide some degree of source apportionment is a major advantage of any technique in forensic science and we established this proof of principle here.

MG: This novel approach is quite revolutionary and has all the characteristics to have a large impact on forensic practice. We proved that the combination of NTA-based techniques and advanced data analysis could actually provide enhanced intelligence for use in crime investigation. Not only does the method allow the main

components in the submitted samples to be rapidly identified, but it can also extract additional information such as the time since handling and potential origin of the analyzed traces. Some supplementary work is still needed to truly implement these possibilities in actual casework, but our method is an effective step forwards. In this regard, it has the potential to unlock a range of new possibilities in forensic profiling, and also to further highlight the value of forensic science in crime investigation.

Q. Are you planning to develop this research further?

LB: Yes, we have just started a new project that will combine LC and IC-HRMS analysis together to identify precursors and indicative reactant species related to threat agent manufacture, including explosives and drugs, for example. In 2017, we were the first laboratory to identify residues of high-order explosives in municipal sewage using LC–HRMS, having performed drug-based wastewater epidemiology for many years (7,16). This project seems like an obvious way to integrate both techniques for application to a very complex matrix, such as wastewater, using NTA. Lastly, confirmatory analysis even of simple anions and cations is very much needed in other areas of forensic science, for example, in support of "acid attack" investigations. I also plan to extend my previous research into the analysis of disinfectants and their by-products. We have already made some progress recently using IC-HRMS for drinking water (17), but it will be good to extend our knowledge on the breadth of toxic species formed following disinfection processes in several other areas too.

MG: We previously showed how machine learning could help to associate different gunshot traces found at the scene of a gun crime (18). The approach worked well, but it could be further improved if coupled with NTA data and, thus, integrated in an 'omics workflow. We are working on that and going to test this hypothesis, and also for the analysis of arson accelerants. The final objective is to develop a transversal profiling approach that can be applied across different fields, and better support the criminal judicial system in the decision-making.

Q. Do you have any practical advice for chromatographers who are embarking on developing a nontargeted analysis method?

LB: First, try to make your NTA method as generic as possible so that it can capture a wide chemical space. This may mean developing a longer, shallower gradient to separate as many features as possible. Also, be aware that a single NTA method will not cover everything. You may need to identify species that might fall outside the scope and whether you need multiple separation modes to cover what you need, for example. This is exactly why we put LC and IC-HRMS together for NTA of explosives-related evidence. Lastly, when setting up your sequences, make sure to randomize your samples, controls, and quality controls (QCs). NTA can often produce deceptively nice trends or classifications, but it is important that groupings or observed changes in the data are actually real, and not just a product of instrument performance drift. You may also want to think about your mass analyzer because you may not always get very high mass accuracy, resolution, and data acquisition speed all in one HRMS instrument.

In my opinion, visualizing, manipulating, and interpreting the data are actually the hardest parts, rather than the laboratory science. Vendor-licensed and open-source software is available and helps with data normalization and chromatogram alignment, and a range of online databases support new compound identification if needed later on. Also, to give you added flexibility, try to learn a coding language such as R or Python. In many cases, freely available codes have already been written for complex tasks and these can be a very useful resource, not only for NTA but also other areas such as PCA and machine learning. It is not as hard as you think! as been properly validated and assessed!

References

1) C. Bedia *et al.*, in *Comprehensive Analytical Chemistry* (Elsevier 2018), pp. 533–582.
2) R.B.D. Sotto *et al., J. Hazard. Mater.* **339**, 63–72 (2017).
3) M.R. Viant and U. Sommer, *Metabolomics* **9**,144–158 (2013).
4) C. Gómez-Canela *et al., Sci. Total Environ.* **562**, 777–788 (2016).
5) K. Munro *et al., Sci. Total Environ.* **657**, 1099–1111 (2019).
6) K. Munro *et al., J. Chromatogr. A* **1396**, 34–44 (2015).
7) H. Rapp-Wright *et al., J. Hazard. Mater.* **329**, 11–21 (2017).
8) T.H. Miller *et al., Anal. Chem.* **85**,10330–10337 (2013).
9) R. Bade *et al., Sci. Total Environ.* **538**, 934–941 (2015).
10) C.B. Mollerup *et al., J. Chromatogr. A* **1542**, 82–88 (2018).
11) L.P. Barron and G.L. McEneff, *Talanta* **147**, 261–270 (2016).
12) M.D. Gallidabino *et al., Anal. Chim. Acta.* **1072**, 1–14 (2019).
13) L. Barron and B. Paull, *Talanta* **69**, 621–630 (2006).
14) E.S. Gilchrist *et al., Anal. Chim. Acta.* **865**, 83–91 (2015).
15) L. Barron and E. Gilchrist, *Anal. Chim. Acta.* **806**, 27–54 (2014).
16) I. González-Mariño *et al., Addiction* (2019). doi:10.1111/add.14767
17) M.D. Gallidabino *et al., Talanta* **178**, 57–68 (2018).
18) M.D. Gallidabino *et al., Analyst* **144**,1128–1139 (2019).

Pyrolysis System

The Gerstel Pyro performs automated pyrolysis at up to 1000 °C of 40, 80, or 120 samples in combination with the Gerstel MultiPurpose Sampler (MPS) robotic. Thermal desorption before pyrolysis-GC–MS removes interfering volatiles ensuring a clean pyrogram. Smart Ramped Pyrolysis (SRP) reportedly provides additional information on polymer mixtures without prior method development.

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

Pulsation Pump

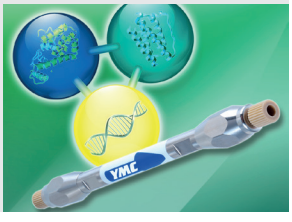
At low flow rates (0.5 mL/min or lower), check valves become a variable factor for flow volume and pulsation. The new GL Sciences' MP-22 No-Pulsation pump does not use any check valves, instead it uses a fast switching valve. Together with the linear-driven pistons, this results in stable flows and pressure, according to the company, even with low flows.

www.glsciences.eu
GL Sciences B.V., Eindhoven, The Netherlands.

(U)HPLC Column

Similar to the YMC-Triart Bio C4 material, YMC-Triart Bio C18 offers 300 Å pore size and is designed with peptides, proteins, and oligonucleotides in mind. The hybrid-silica base (1.9, 3, or 5 µm) and polymeric bonding provide a robust C18 phase with a wide temperature (up to 90 °C) and pH range (1–12) that separates mid-MW biomolecules.

https://ymc.de/files/imported/publications/293/documents/YMC-Triart%20Bio%20C18%20Flyer_1119.pdf
YMC Europe GmbH, Dinslaken, Germany.



HILIC Columns

Hilicon offers a broad range of HILIC products to separate polar compounds. Three column chemistries in UHPLC and HPLC, iHILIC-Fusion, iHILIC-Fusion(+), and iHILIC-Fusion(P), provide customized and complementary selectivity, excellent durability, and very low column bleeding, according to the company. The columns are suitable for the analysis of polar compounds in "omics" research, food and beverage analysis, pharma discovery, and clinical diagnostics.

www.hilicon.com
Hilicon AB, Umeå, Sweden.

Ion Chromatography System

The Thermo Scientific Dionex ICS-6000 ion chromatography system is designed to run any ion chromatography (IC) application. Whether the need is to perform very complex ion chromatography analyses, routine QA/QC methods, cutting-edge research, or obtaining trace levels, the ICS-6000 will reportedly produce unmatched results. According to the company, the system can be used for high throughput needs yet is flexible enough for any new challenges.

www.thermofisher.com
Thermo Fisher Scientific, San Jose, California, USA.

Leak Detector

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

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

Micro-Pillar Array Columns

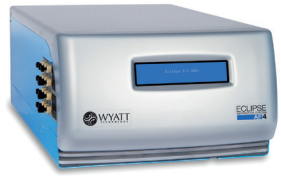
For comprehensive proteomics the 200 cm µPAC micro-pillar array columns are a good choice, whereas the 50 cm µPAC columns are better suited to perform higher throughput analyses with shorter gradient solvent times, according to the company. In addition, the µPAC Trapping columns were developed with identical morphology as the analytical columns to ensure optimal chromatographic performance.

www.pharmafluidics.com
PharmaFluidics, Ghent, Belgium.

AF4 System

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

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



Sample Preparation

LCTech has introduced an automated system designed to clean up samples that need to remain melted in PCB and dioxin analysis. Three specifically designed heating zones keep the sample liquid from sample vial to the first column. The DEXTech Heat, which is based on the established DEXTech Pure system, processes difficult samples, such as stearin or PFADs. Excellent automated, reliable results, without clogging, are produced, according to the company.

www.LCTech.de
LCTech GmbH, Obertaufkirchen, Germany.

GPC/SEC Validation Kit

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

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

Hydrogen Generator

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

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

Sample Automation

Markes' new Centri multitechnique platform is an advance in sample automation and concentration for GC–MS, according to the company, and offers four sampling modes: HiSorb high-capacity sorptive extraction, headspace, SPME, and thermal desorption. The company reports analyte focusing allows increased sensitivity in all modes, state-of-the-art robotics increases sample throughput, and sample re-collection allows repeat analysis without having to repeat lengthy sample extraction procedures.

http://chem.markes.com/Centri
Markes International Ltd., Llantrisant, UK.

44th International Symposium On Capillary Chromatography and the 17th GC×GC Symposium


Credit: stefanotermanini/stock.adobe.com

The **44th International Symposium On Capillary Chromatography (ISCC) and 17th GC×GC Symposium** will be held at the **Palazzo dei Congressi**, in **Riva del Garda, Italy,** from **24–29 May 2020**. Over the years, the ISCC conference has established its reputation as a forum for microcolumn separation techniques. Since the first meeting in Hindelang in 1975, the most important developments in capillary gas chromatography (GC), microcolumn liquid chromatography (LC), and electromigration techniques have been presented in this symposium series. The format and the atmosphere of the 44th meeting will be similar to the previous meetings, with a particular emphasis on mass spectrometry (MS) this year.

Past meetings have been held in Hindelang, Riva del Garda, Monterey, Baltimore, Gifu, Kobe, Wintergreen, Park City, Las Vegas, Dalian, Albuquerque, Portland, and San Diego. This year the "Palazzo dei Congressi" in Riva del Garda, Italy, will accommodate the 44th meeting. The six-day event will feature recent findings from leading academic and industrial experts in the form of lectures and posters. Apart from the most recent advances in the fields of pressure and electrodriven microcolumn separation techniques and comprehensive two-dimensional (2D)-GC, this year will again have particular emphasis on comprehensive separation technologies combined with capillary chromatography and 2D-GC with various forms of mass spectrometry, from unit-mass to high resolution, and from single- to hybrid analyzers.

The conference also offers sessions on capillary GC, microcolumn LC, electromigration methods, and microfabricated analytical systems, which are expected to cover lab-on-a-chip, column technology, coupled and multidimensional techniques, comprehensive techniques, hyphenated techniques, sampling and sample preparation, trace analysis, and automation. Application sessions include environmental; energy, petrochemical, industrial; biomedical, pharmaceutical; and the analysis of natural products, food, flavours, and fragrances. Workshop seminars from instrument manufacturers and an extensive exhibition of instrumentation, accessories, and supplies will run in parallel to the scientific programme.

At the meeting, the 2020 Marcel Golay Award, sponsored by PerkinElmer, will be presented in recognition of outstanding contributions in the field of separation science. The Leslie Ettre Award, sponsored by PerkinElmer, will be presented to a young scientist for research on capillary GC applied to environmental or food analyses. The Giorgio Nota Award, sponsored by Waters, will be presented to a scientist in recognition of a lifetime of achievement in capillary LC. The John Phillips Award, sponsored by LECO and Restek, will be awarded to individuals who have made outstanding contributions to the field of GC×GC analysis. The GC×GC Lifetime Achievement Award, sponsored by LECO and Restek, honours an experienced GC×GC scientist who has made significant contributions to the field. Chromaleont, ISCC and GC×GC, Fort Worth Texas 2021, the Division of Analytical Chemistry of the Italian Chemical Society (SCI) and the Interdivisional Group of Separation Science of the Italian Chemical Society, Elsevier, Secyta, and *Separations* (MDPI) will promote scholarships for young researchers. For more information, please visit: **www.chromaleont.it/iscc**; E-mail: **iscc@chromaleont.it**

29–31 JANUARY 2020

The 16th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-16)
Ghent, Belgium
E: htc16@kuleuven.be
W: www.htc-16.com

1–5 MARCH 2020

Pittcon Conference and Expo 2020
McCormick Place, Chicago, Illinois, USA
E: info@pittcon.org
W: https://pittcon.org

31 MARCH–3 APRIL 2020

The 27th International Trade Fair for Laboratory Technology, Analysis, Biotechnology and Analytica Conference
Messe München, Munich, Germany
E: info@analytica.de
W: www.analytica.de

21–22 APRIL 2020

The 13th International Scientific Conference on Ion Chromatography and Related Techniques 2020
Zabrze, Poland
E: rajmund.michalski@ipis.zabrze.pl
W: http://ipis.pan.pl/pl/pm-konferencje/konferencje-planowane

31 MAY–3 JUNE 2020

33rd International Symposium on Preparative and Process Chromatography
Baltimore, Maryland, USA
E: janet@barrconferences.com
W: www.PREPsymposium.org

22–24 JUNE 2020

LabWorld China 2020
Shanghai, China
E: salesoperations@ubm.com
W: https://www.pmecchina.com/labworld/en

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

Efficient Separation of Polar and Nonpolar Lipid Classes Utilizing iSPE®-HILIC Material for Solid-Phase Extraction

Patrick O. Helmer[1], Wen Jiang[2], and Heiko Hayen[1], [1]Institute of Inorganic and Analytical Chemistry, University of Münster, Münster, Germany, [2]HILICON AB

Lipids are a large group of biomolecules that play an important role in all organisms. The tasks of lipids are manifold and of great relevance. By the formation of membranes, their main task is the compartmentation of cells where they interact with other biomolecules such as proteins. Furthermore, they are involved in a variety of signalling pathways and some of their representative lipid classes are important for energy storage (1). Lipids differ in their polarity. There are not only completely lipophilic representatives such as triacylglycerols (TAG) or cholesterol ester (CE) but also amphiphilic lipids such as phospholipids (PLs). The analysis of lipids is complex and challenging and is often based on liquid chromatography (LC) hyphenated with mass spectrometric (MS) detection. Due to their amphiphilic character, reversed-phase LC and hydrophilic interaction liquid chromatography (HILIC) are suitable techniques for PLs analysis. While reversed-phase LC enables a separation mainly based on their acyl moieties in lipids, HILIC can separate PL classes according to their specific hydrophilic head group as highlighted in green in Figure 1. Nonpolar lipids are not retarded by the HILIC mechanism and elute earlier from the column (2). Therefore, HILIC enables a separation of polar phospholipids and nonpolar lipids by means of HILIC solid-phase extraction (SPE).

In this application, we demonstrate a fast and efficient separation of representative polar and nonpolar lipid classes by iSPE®-HILIC cartridges. In addition to the recovery studies of eight lipid standards, the polar lipid fraction of a yeast lipid extract was also analyzed by reversed-phase LC–MS after iSPE®-HILIC SPE fractionation.

Experimental

Lipid Standards: Triacylglycerol (TAG 48:0), cholesterol (Chol), cholesteryl ester (CE 18:2), phosphatidylethanolamine (PE 32:0), phosphatidylcholine (PC 32:0), phosphatidylserine (PS 32:0), *lyso*-phosphatidylcholine (LPC 16:0), and cardiolipin (CL 72:8) are respectively from Biomol GmbH and Sigma Aldrich.

Lipids Extraction Protocol with iSPE®-HILIC:

Solvents: a) ammonium acetate buffer (20 mM, pH5.5); b) acetonitrile; c) methanol

Conditioning: 1 mL acetonitrile–buffer (90:10, *v/v*)

Equilibration: 3 mL acetonitrile–buffer (97:3, *v/v*)

Loading: ≤ 200 µL sample (for example, lipid extracts in $CHCl_3$)

Incubation: 1 min

Nonpolar lipids fraction: 4 mL acetonitrile–buffer (95:5, *v/v*)

Polar lipids fraction: 4 mL methanol–buffer (80:20, *v/v*)

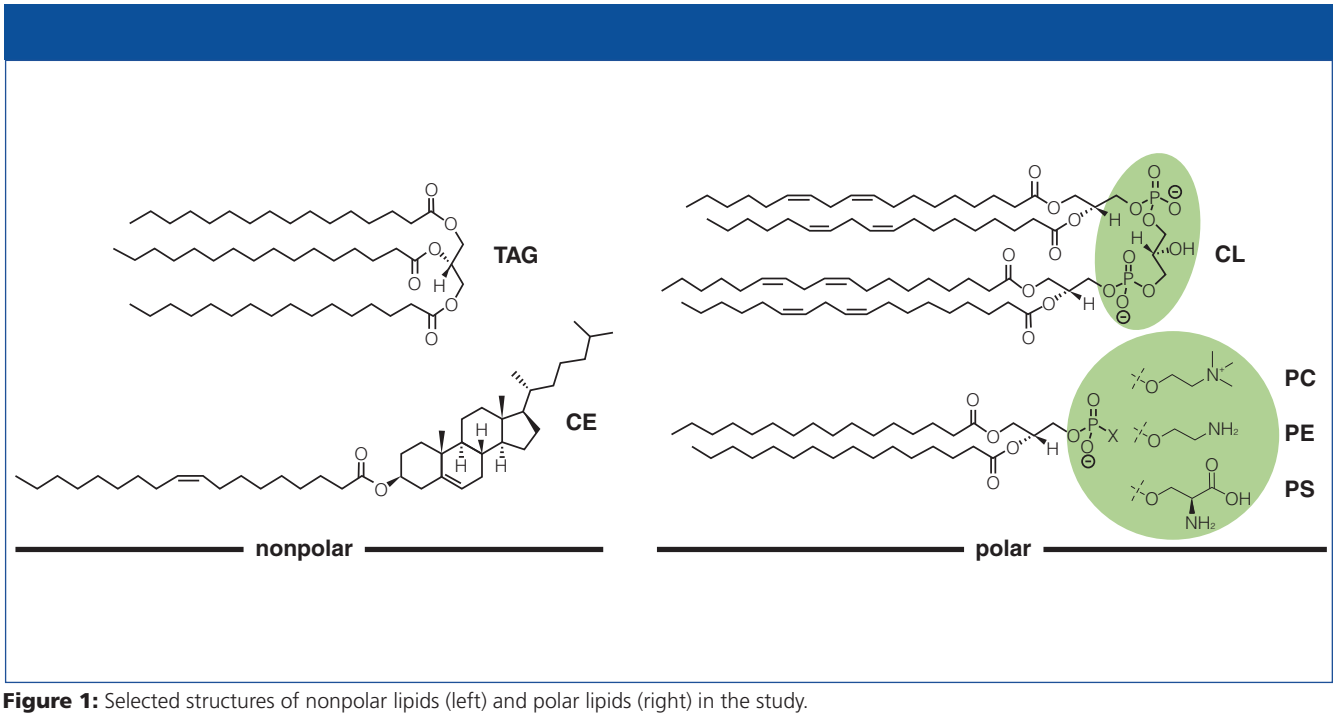


Figure 1: Selected structures of nonpolar lipids (left) and polar lipids (right) in the study.

Sample Preparation and Method Validation: The recovery of three nonpolar lipid species (TAG, Chol, and CE) and five polar phospholipids (PE, PC, PS, LPC, and CL) was determined according to Matuszewski *et al.* (3). In this work, a yeast total lipid extract (*S. cerevisiae*) was utilized as matrix as described by Helmer *et al.* (4).

The samples for the LC–MS analysis were the yeast total lipid extracts (*S. cerevisiae*) that were first cleaned up from nonpolar lipids utilizing iSPE®-HILIC cartridges (1 mL, 100 mg, 50 µm/60 Å, HILICON). After solvent evaporation, the polar lipid fraction was reconstituted in methanol and subjected to the analysis by reversed-phase LC–MS.

LC–MS Setup: A Thermo Scientific Ultimate 3000 UHPLC system was hyphenated to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. The ionization was carried out by electrospray ionization in negative ionization mode (4).

Results and Conclusion

An efficient separation of polar and nonpolar lipid representatives was achieved utilizing iSPE®-HILIC material. The lipids were separated according to their polarity and collected into polar and nonpolar lipid fractions for further LC–MS analysis. Figure 2 shows the recoveries of all eight tested lipid standards in the polar and nonpolar fraction. TAG, CE, and Chol were eluted in the nonpolar fraction, while the PLs species were in the polar fraction. Except for minor amounts of PE, no carryover into the other fraction was observed.

By fractionation of a total lipids extract into its polar and nonpolar groups, the interfering influences on chromatographic separation or mass spectrometric detection can be minimized and allows a more tailored analysis. In addition, depending on the lipid composition of tissues, low abundant lipid species such as CL can be enriched with this newly developed SPE method.

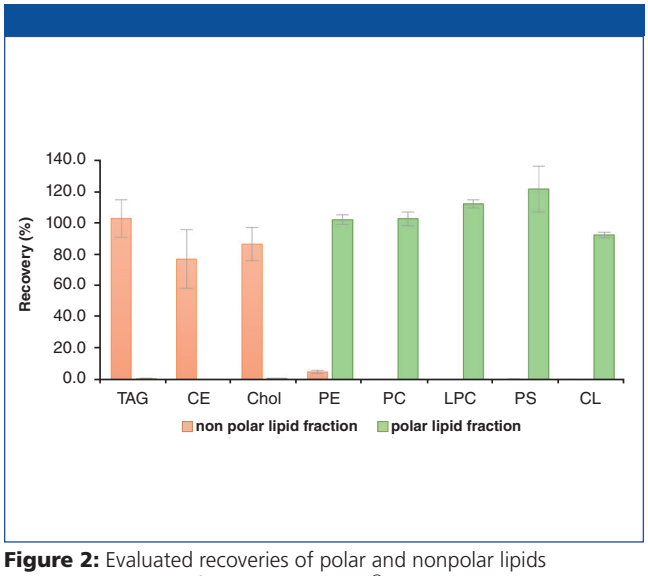


Figure 2: Evaluated recoveries of polar and nonpolar lipids in the respective SPE fractions using iSPE®-HILIC cartridge.

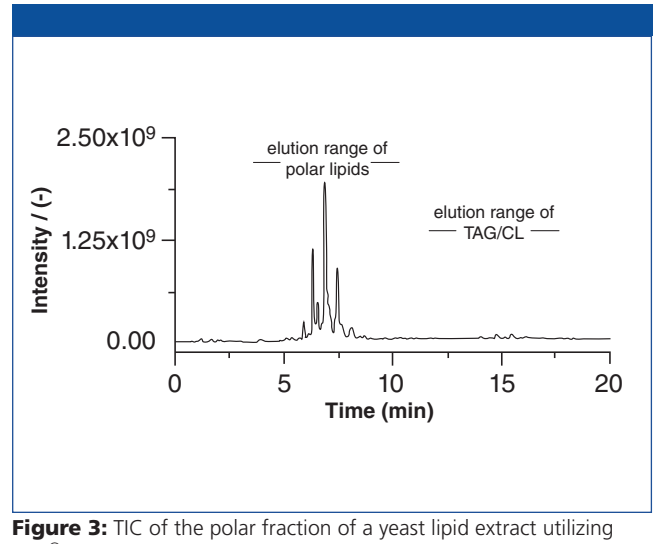


Figure 3: TIC of the polar fraction of a yeast lipid extract utilizing iSPE® HILIC and separation by reversed-phase LC–MS. The separation of polar PLs lipids and the low abundant CL species is illustrated. Interfering TAG species were excluded by cleanup.

Figure 3 shows the total ion chromatogram (TIC) of the polar fraction of a yeast lipid extract by reversed-phase LC–MS after iSPE®-HILIC sample cleanup. Polar lipids were well separated and detected in the TIC, while no interfering TAG was present in the elution range of CL. In comparison to other SPE methods for lipid separation or purification, this method does not require the use of nonpolar volatile solvents, for example, hexane. In summary, the HILIC method with iSPE®-HILIC is a reliable and robust alternative to other SPE separations such as normal-phase liquid chromatography.

References

(1) J.M. Berg *et al.*, *Stryer Biochemie*, Springer (2018).

(2) M. Lange *et al.*, *Chromatographia* **82**, 77–100 (2018).

(3) B.K. Matuszewski *et al.*, *Anal. Chem.* **75**, 3019–3030 (2003).

(4) P.O. Helmer *et al.*, *Rapid Commun. Mass Spectrom.* doi: 10.1002/rcm.8566, in press.

HILICON

HILICON AB
Tvistevägen 48A, SE-90736 Umeå, Sweden
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