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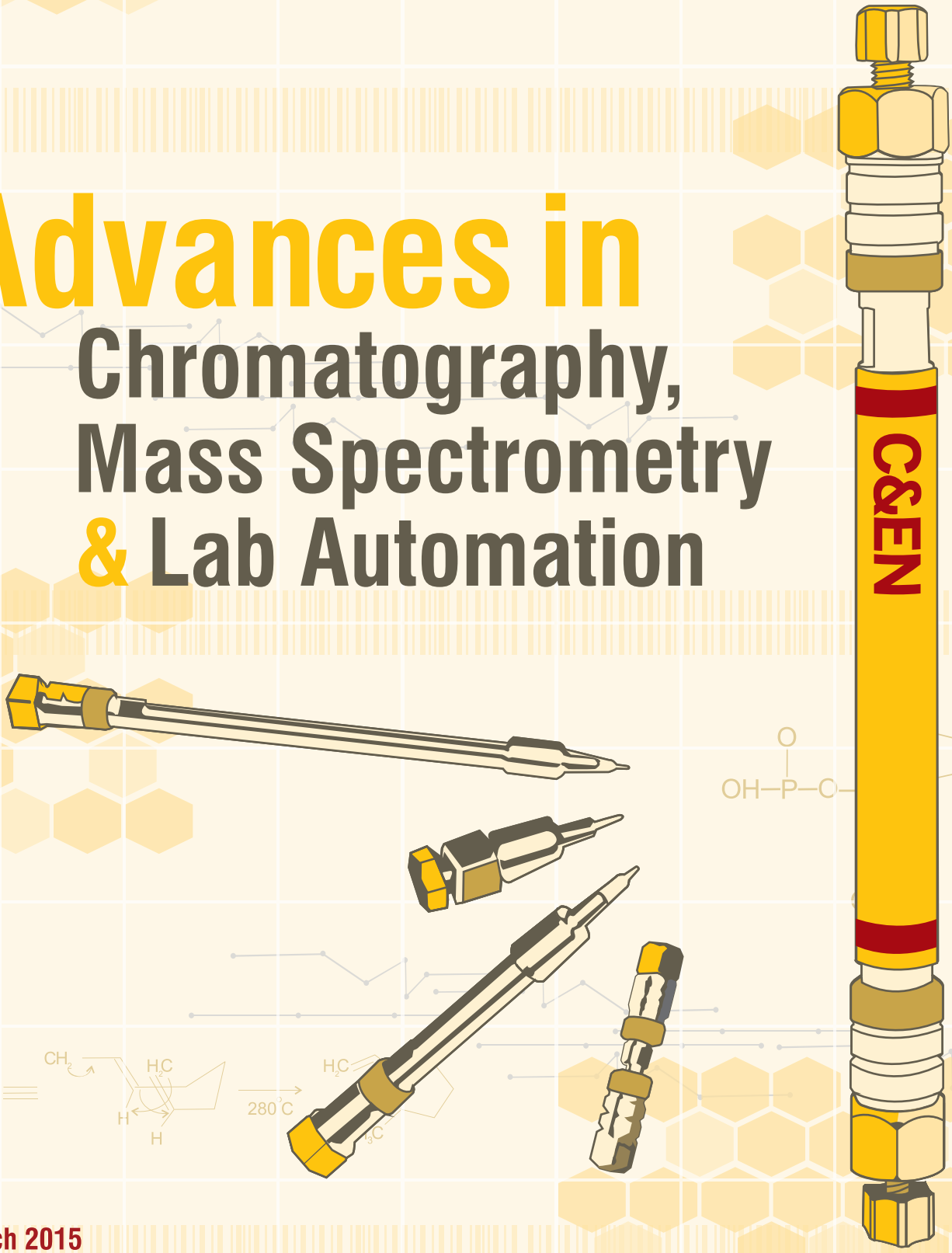

March 2015

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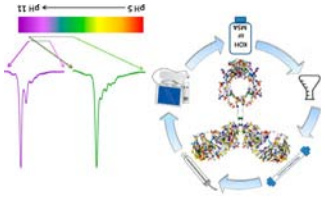
A SUPPLEMENT TO C&EN
CHEMICAL & ENGINEERING NEWS

Advances in Chromatography, Mass Spectrometry & Lab Automation



High-Throughput Chromatography Profiles Monoclonal Antibody Charge Heterogeneity

ADVERTISER INDEX

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PUBLISHER'S NOTE

Equipped to Succeed

WE ARE DELIGHTED to present the first C&EN Supplement of 2015: "Advances in Chromatography, Mass Spectrometry, and Lab Automation." This supplement includes a selection of application notes contributed by leading instrumentation companies. We have also included abstracts from ten of the most popular and cited research articles featuring groundbreaking technologies in "separation science" published in 2014 in the prestigious journal Analytical Chemistry. Finally, in case you missed it, we reproduce a fascinating feature article by C&EN's Celia Arnaud, originally published in May 2014, on one of the most exciting technology developments in the field, namely supercritical fluid chromatography.

Following the popularity of the C&EN Supplements introduced last year, we are planning six diverse supplements for 2015. Four, including this issue, will follow the time-trusted "app note" model. We'll also publish two special supplements later in the year – a special report on technology development in the BRICS countries, and the "Top 20 Drugs in the Pipeline."

We welcome our new contributing editor, Dr. Victoria Mountain. (Vicki is the former managing editor of the journal Chemistry & Biology and assisted ACS in the launch of ACS Chemical Biology.) Our thanks also to Analytical Chemistry managing editor Antonella Mazur and the companies that have supported this C&EN Supplement.

Finally, we welcome some important new faces at C&EN, beginning with the magazine's new editor-in-chief, Dr. Bibiana Campos Seijo. Bibi is the former editor and publisher of Chemistry World, and we're excited to help implement her vision for the publication.

We're also excited to introduce a new dedicated North American ad sales team, located in New York City, Chicago, and Los Angeles, and look forward to forging new and prosperous partnerships with our advertising community.

Best wishes,

Kevin Davies, PhD
Publisher, C&EN

For the record: The editorial content in this supplement was created without direct involvement of C&EN reporters or editors, with the exception of the article reprinted from C&EN.

BREAKTHROUGHS IN BREAKING UP – INNOVATIONS IN THE ART OF SEPARATION

By Vicki Mountain, PhD

In March 2014, we launched a new and exciting series of special supplements to Chemical & Engineering News magazine, kicking off with the Separation Science issue featuring Advances in Chromatography and Mass Spectrometry. One year later, once again, there is much to say about these rapidly developing technologies that are increasingly adapted to support novel research across the full spectrum of chemistry, biology, as well as biotechnology. To keep you, our readers, up-to-date with these latest cutting-edge innovations, we are pleased present a snapshot of the most significant examples with the first special supplement of 2015, Advances in Chromatography, Mass Spectrometry, and Lab Automation.

Some particularly exciting innovations in the mass spectrometry space were showcased in a special symposium held at the 248th American Chemical Society's National Meeting, in August 2014 in San Francisco[1]. Oral presentations covered innovations broadly in three areas – "Thermochemistry, Reactivity and Elemental Analyses", "Probing Ion Structure", and "Novel Methodologies". The breadth of new methods for examining complex molecules by mass spectrometry was highlighted in presentations describing the expansion of mass spectrometry into new environments such as the air-water interface, facilitated by Field Induced Droplet Ionization (Beauchamp, J.E., California Institute of Technology). This particular development will support deep investigation of chemical reactions within the possibly unique microenvironments at the surfaces of lakes and oceans, as well as the human respiratory system. Other advances reported system adaptations that permit bioanalytical mass spectrometry of samples under ambient conditions using a simple device with a single multifunctional probe. This device will allow non-expert users to assess the chemical composition of living systems, including singles cells and tissues, as well post-translational modification of proteins without the need for extraction methods (Yang, Z. et al., University of Oklahoma).

Other new technologies included Laserspray Imaging Mass Spectrometry, which was developed for analysis of the chemical composition of brain tissues, as well as identifying temporal changes in brain chemistry (Trimpin, S. et al., Wayne State University, and Indiana University). The technique, in combination with MRI and microscopy, has been successfully used to pinpoint the location of chemical tracers in the brains of mice. Hilkka Kenttämaa (Purdue University) described a multi-port valve interface for a linear ion trap mass spectrometer that supports automated, high-throughput screening of complex mixtures, and in addition also allows rapid testing of new neutral reagents to to identify useful functionalities.

An interesting application was described by Christopher Silva, who along with colleagues at United States Department of Agriculture, adopted mass spectrometry as the foundation for a protocol to detect, quantify and distinguish among subtypes of Shiga toxins in the low attomole range. Shiga Toxin-producing Escherichia coli (STEC) are the cause of many serious food-borne illnesses, making simple and rapid detection paramount for food safety. Crucially, the researchers reported that, as the method inactivates the toxins present in the sample, it is both safe and effective.

This C&EN supplement continues to spotlight scientific breakthroughs by highlighting new technology from leading companies in these fields -- EMD-Millipore, Thermo, and Advion. We also include a selection of the top ten research articles published in Analytical Chemistry over the past year, as selected by the journal's editors, in our Top 10 Articles section. Finally, we spotlight another exciting aspect of chromatography – supercritical fluid chromatography – as described by C&EN reporter Celia Arnaud in a May 2014 cover story.

With that, I invite you to dive in and enjoy the 2015 collection of Advances in Chromatography, Mass Spectrometry, and Lab Automation. We have five other themed supplements planned for publication throughout this year, including:

- Advances in Spectroscopy & Material Analysis
- Water Analysis & the Environment
- Chemical & Laboratory Opportunities in the "BRICS" Countries
- Top 20 Pharmaceuticals in the Pipeline
- Trends in "Omics" Technologies

As always, we hope that you find this special collection useful and look forward to hearing from you with your valuable feedback and comments.

References

1. http://abstracts.acs.org/chem/248nm/program/divisionindex.php?nl=1&act=presentations&val=Innovations+in+Mass+Spectrometry&ses=Innovations+in+Mass+Spectrometry&prog=234231

Vicki Mountain, Ph.D. is a contributing editor on this C&EN Supplement and is a freelance science editor and writer based in Medford, MA.

TOP TEN CHROMATOGRAPHY, MASS SPECTROMETRY, AND LAB AUTOMATION PAPERS

***Analytical Chemistry's* Most Popular Papers of 2014**

PRESENTING THE ten most notable advances in separation science published in *Analytical Chemistry* in 2014: Hand-selected by the Editors, these reports include technical and conceptual innovations that must not be missed. Browse the article abstracts below, and keep up with the latest developments in this field by visiting http://pubs.acs.org/journal/ancham

Chromatographic Resolution of Closely Related Species in Pharmaceutical Chemistry: Dehalogenation Impurities and Mixtures of Halogen Isomers

Erik L. Regalado, Ping Zhuang, Yadan Chen, Alexey A. Makarov, Wes A. Schafer, Neil McGachy, and Christopher J. Welch
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Anal. Chem., **2014,** 86 (1), pp 805–813
DOI: 10.1021/ac403376h

In recent years, the use of halogen-containing molecules has proliferated in the pharmaceutical industry, where the incorporation of halogens, especially fluorine, has become vitally important for blocking metabolism and enhancing the biological activity of pharmaceuticals. The chromatographic separation of halogen-containing pharmaceuticals from associated isomers or dehalogenation impurities can sometimes be quite difficult. In an attempt to identify the best current tools available for addressing this important problem, a survey of the suitability of four chromatographic method development platforms (ultra high-performance liquid chromatography (UHPLC), core shell HPLC, achiral supercritical fluid chromatography (SFC) and chiral SFC) for separating closely related mixtures of halogen-containing pharmaceuticals and their dehalogenated isosteres is described. Of the 132 column and mobile phase combinations examined for each mixture, a small subset of conditions were found to afford the best overall performance, with a single UHPLC method (2.1 × 50 mm, 1.9 μm Hypersil Gold PFP, acetonitrile/methanol based aqueous eluents containing either phosphoric or perchloric acid with 150 mM sodium perchlorate) affording excellent separation for all samples. Similarly, a survey of several families of closely related halogen-containing small molecules representing the diversity of impurities that can sometimes be found in purchased starting materials for synthesis revealed chiral SFC (Chiralcel OJ-3 and Chiralpak IB, isopropanol or ethanol with 25 mM isobutylamine/carbon dioxide) as well as the UHPLC (2.1 × 50 mm, 1.8 μm ZORBAX RRHD Eclipse Plus C18 and the Gold PFP, acetonitrile/methanol based aqueous eluents containing phosphoric acid) as preferred methods. ■

Simultaneous Analysis of 22 Volatile Organic Compounds in Cigarette Smoke Using Gas Sampling Bags for High-Throughput Solid-Phase Microextraction

Maureen M. Sampson, David M. Chambers, Daniel Y. Pazo, Fallon Moliere, Benjamin C. Blount, and Clifford H. Watson
Centers for Disease Control and Prevention, 4770 Buford Highway, Atlanta, Georgia 30341,
Anal. Chem., **2014,** 86 (14), pp 7088–7095
DOI: 10.1021/ac5015518

Quantifying volatile organic compounds (VOCs) in cigarette smoke is necessary to establish smoke-related exposure estimates and evaluate emerging products and potential reduced-exposure products. In response to this need, we developed an automated, multi-VOC quantification method for machine-generated, mainstream cigarette smoke using solid-phase microextraction gas chromatography–mass spectrometry (SPME-GC–MS). This method was developed to simultaneously quantify a broad range of smoke VOCs (i.e., carbonyls and volatiles, which historically have been measured by separate assays) for large exposure assessment studies. Our approach collects and maintains vapor-phase smoke in a gas sampling bag, where it is homogenized with isotopically

labeled analogue internal standards and sampled using gas-phase SPME. High throughput is achieved by SPME automation using a CTC Analytics platform and custom bag tray. This method has successfully quantified 22 structurally diverse VOCs (e.g., benzene and associated monoaromatics, aldehydes and ketones, furans, acrylonitrile, 1,3-butadiene, vinyl chloride, and nitromethane) in the microgram range in mainstream smoke from 1R5F and 3R4F research cigarettes smoked under ISO (Cambridge Filter or FTC) and Intense (Health Canada or Canadian Intense) conditions. Our results are comparable to previous studies with few exceptions. Method accuracy was evaluated with third-party reference samples (≤15% error). Short-term diffusion losses from the gas sampling bag were minimal, with a 10% decrease in absolute response after 24 h. For most analytes, research cigarette inter- and intrarun precisions were ≤20% relative standard deviation (RSD). This method provides an accurate and robust means to quantify VOCs in cigarette smoke spanning a range of yields that is sufficient to characterize smoke exposure estimates. ■

Tuning the Selectivity of Ionic Liquid Stationary Phases for Enhanced Separation of Nonpolar Analytes in Kerosene Using Multidimensional Gas Chromatography

Leandro W. Hantao †§, Ali Najafi †, Cheng Zhang †, Fabio Augusto §, and Jared L. Anderson†‡
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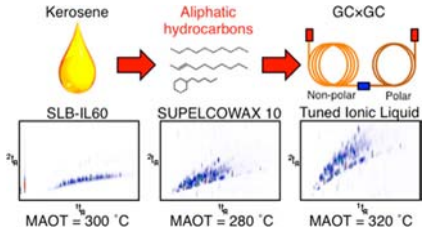
Anal. Chem., **2014,** 86 (8), pp 3717–3721
DOI: 10.1021/ac5004129

In this study, a series of ionic liquids (ILs) are evaluated as stationary phases in comprehensive two-dimensional gas chromatography (GC × GC) for the separation of aliphatic hydrocarbons from kerosene. IL-based stationary phases were carefully designed to evaluate the role of cavity formation/dispersive interaction on the chromatographic retention of nonpolar analytes by GC × GC. The maximum allowable operating temperature (MAOT) of the IL-based columns was compared to that of commercial IL-based columns. Evaluation of the solvation characteristics of GC columns guided the selection of the best performing IL-based stationary phases for the resolution of aliphatic hydrocarbons, namely, trihexyl(tetradecyl)phosphonium tetrachloroferrate ([P66614][FeCl4]) and trihexyl(tetradecyl)phosphonium tris(pentafluoroethyl)trifluorophosphate ([P66614][FAP]) ILs. The best performing [P66614][FeCl4] IL-based column exhibited a MAOT of 320 °C, higher than the commercial SUPELCOWAX 10 (MAOT of 280 °C) and commercial IL-based columns (MAOT up to 300 °C). The structurally tuned [P66614][FeCl4] IL stationary phase exhibited improved separation of aliphatic hydrocarbons by GC × GC compared to the commercial columns examined (e.g., OV-1701, SUPELCOWAX 10, SLB-IL60, SLB-IL100, and SLB-IL111). ■



Automated Dispersive Liquid–Liquid Microextraction–Gas Chromatography–Mass Spectrometry

Liang Guo and Hian Kee Lee
Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore
Anal. Chem., **2014,** 86 (8), pp 3743–3749
DOI: 10.1021/ac404088c

An innovative automated procedure, low-density solvent based/solvent demulsification dispersive liquid–liquid microextraction (automated DLLME) coupled to gas chromatography–mass spectrometry (GC/MS) analysis, has been developed. The most significant innovation of the method is the automation. The entire procedure, including the extraction of the model analytes (phthalate esters) by DLLME from the aqueous sample solution, breaking up of the emulsion after extraction, collection of the extract, and analysis of the extract by GC/MS, was completely automated. The applications of low-density solvent as extraction solvent and the solvent demulsification technique to break up the emulsion simplified the procedure and facilitated its automation. Orthogonal array design (OAD) as an efficient optimization strategy was employed to optimize the extraction parameters, with all the experiments conducted auotmatically. An OA16 (41 × 212) matrix was initially employed for the identification of optimized extraction parameters (type and volume of extraction solvent, type and volume of dispersive solvent and demulsification solvent, demulsification time, and injection speed). Then, on the basis of the results, more levels (values) of five extraction parameters were investigated by an OA16 (45) matrix and quantitatively assessed by the analysis of variance (ANOVA). Enrichment factors of between 178- and 272-fold were obtained

for the phthalate esters. The linearities were in the range of 0.1 and 50 μg/L and 0.2 and 50 μg/L, depending on the analytes. Good limits of detection (in the range of 0.01 to 0.02 μg/L) and satisfactory repeatability (relative standard deviations of below 5.9%) were obtained. The proposed method demonstrates for the first time integrated sample preparation by DLLME and analysis by GC/MS that can be operated automatically across multiple experiments. ■

Semiautomated pH Gradient Ion-Exchange Chromatography of Monoclonal Antibody Charge Variants

Mohammad Talebi †, Robert A. Shellie †, Emily F. Hilder †, Nathan A. Lacher ‡, and Paul R. Haddad †
†Australian Centre for Research on Separation Science (ACROSS), School of Physical Sciences, University of Tasmania, Hobart, Tasmania 7005, Australia
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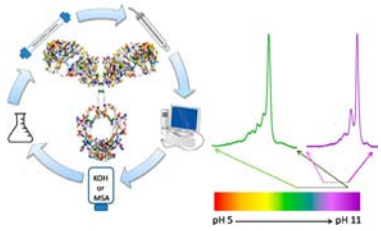
Anal. Chem., **2014,** 86 (19), pp 9794–9799
DOI: 10.1021/ac502372r

A new approach using a chromatography system equipped with isocratic pumps and an electrolytic eluent generator (EG) is introduced, replacing external pH gradient delivery using conventional gradient systems, in which bottled buffers with preadjusted pH are mixed using a gradient pump. The EG is capable of generating high purity base or acid required for online preparation of the buffer at the point of use, utilizing deionized water as the only carrier stream. Typically, the buffer was generated from online titration of a reagent composed of



low molecular weight amines. The reagent was delivered isocratically into a static mixing tee, where it was titrated to the required pH with electrolytically generated base or acid. The required pH gradient was thus conveniently generated by electrically controlling the concentration of titrant. Also, since the pH was adjusted at the point of use, this approach offered enhanced throughput in terms of eluent preparation time and labor, and with a more reproducible pH profile. The performance of the system was demonstrated by running pH gradients ranging from pH 8.2 to 10.9 on a polymer monolith cation-exchange column for high throughput profiling of charge heterogeneity of intact, basic therapeutic monoclonal antibodies. A high degree of flexibility in modulating the key parameters of the pH gradient, including the buffer concentration, the pH gradient slope and the operating pH range was demonstrated. This enabled fine-tuning of the separation conditions for each individual antibody in order to enhance the chromatographic resolution. ■

High-Throughput Cell and Tissue Analysis with Enhanced Molecular Coverage by Laser Ablation Electrospray Ionization Mass Spectrometry Using Ion Mobility Separation

Bindesh Shrestha and Akos Vertes
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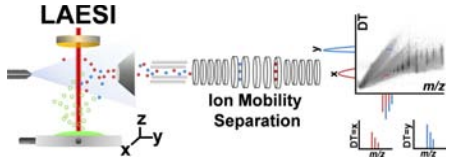
Anal. Chem., **2014,** 86 (9), pp 4308–4315
DOI: 10.1021/ac500007t

Ambient ionization methods, such as laser ablation electrospray ionization (LAESI), facilitate the direct analysis of unperturbed cells and tissues in their native states. However, the lack of a separation step in these ionization techniques results in limited molecular coverage due to interferences, ion suppression effects, and the lack of ability to differentiate between structural isomers and isobaric species. In this contribution, LAESI mass spectrometry (MS) coupled with ion mobility separation (IMS) is utilized for the direct analysis of protein mixtures, megakaryoblast cell pellets, mouse brain sections, and Arabidopsis



thaliana leaves. We demonstrate that the collision cross sections of ions generated by LAESI are similar to the ones obtained by ESI. In various applications, LAESI-IMS-MS allows for the high-throughput separation and mass spectrometric detection of biomolecules on the millisecond time scale with enhanced molecular coverage. For example, direct analysis of mouse brain tissue without IMS had yielded ~300 ionic species, whereas with IMS over 1 100 different ions were detected. Differentiating between ions of similar mass-to-charge ratios with dissimilar drift times in complex biological samples removes some systematic distortions in isotope distribution patterns and improves the fidelity of molecular identification. Coupling IMS with LAESI-MS also expands the dynamic range by increasing the signal-to-noise ratio due to the separation of isobaric or other interfering ionic species. We have also shown that identification of potential biomarkers by LAESI can be enhanced by using the drift times of individual ions as an additional parameter in supervised

orthogonal projections to latent structures discriminant analysis. Comparative analysis of drift time versus mass-to-charge ratio plots was performed for similar tissue samples to pinpoint significant metabolic differences. ■

Site-Specific Characterization of $_D$-Amino Acid Containing Peptide Epimers by Ion Mobility Spectrometry

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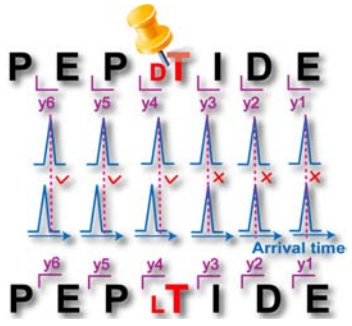
Anal. Chem., **2014,** 86 (6), pp 2972–2981
DOI: 10.1021/ac4033824

Traditionally, the $_D$-amino acid containing peptide (DAACP) candidate can be discovered by observing the differences of biological activity and chromatographic retention time between the synthetic peptides and naturally occurring peptides. However, it is difficult to determine the exact position of $_D$-amino acid in the DAACP candidates. Herein, we developed a novel site-specific strategy to rapidly and precisely localize $_D$-amino acids in peptides by ion mobility spectrometry (IMS) analysis of mass spectrometry (MS)-generated epimeric fragment ions. Briefly, the $_{D/L}$-peptide epimers were separated by online reversed-phase liquid chromatography and fragmented by collision-induced dissociation (CID), followed by IMS analysis. The epimeric fragment ions resulting from $_{D/L}$-peptide epimers exhibit conformational differences, thus showing different mobilities in IMS. The arrival time shift between the epimeric fragment ions was used as criteria to localize the $_D$-amino acid substitution. The utility of this strategy was demonstrated by analysis of peptide epimers with different molecular sizes, [$_D$-Trp]-melanocyte-stimulating hormone, [$_D$-Ala]-deltorphin, [$_D$-Phe]-achatin-I, and their counterparts that contain all-$_L$ amino acids. Furthermore, the crustacean hyperglycemia hormones (CHHs, 8.5 kDa) were isolated from the American lobster Homarus americanus and identified by integration of MS-based bottom-up and top-down sequencing approaches. The IMS data acquired using our novel site-specific strategy localized the site of isomerization of $_L$- to $_D$-Phe at the third residue of the CHHs from the N-terminus. Collectively, this study demonstrates a new method for discovery of DAACPs using IMS technique with the ability to localize $_D$-amino acid residues. ■



Tryptic Digestion Coupled with Ambient Desorption Electrospray Ionization and Liquid Extraction Surface Analysis Mass Spectrometry Enabling Identification of Skeletal Muscle Proteins in Mixtures and Distinguishing between Beef, Pork, Horse, Chicken, and Turkey Meat

Magdalena Montowska †‡, Wei Rao †, Morgan R. Alexander §, Gregory A. Tucker #, and David A. Barrett †
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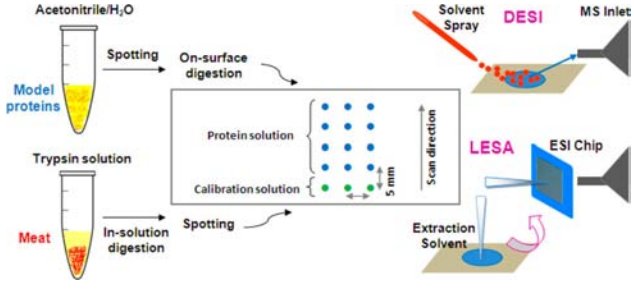
Anal. Chem., **2014,** 86 (9), pp 4479–4487
DOI: 10.1021/ac5003432

The use of ambient desorption electrospray ionization mass spectrometry (DESI-MS) and liquid extraction surface analysis mass spectrometry (LESA-MS) is explored for the first time to analyze skeletal muscle proteins obtained from a mixture of standard proteins and raw meat. Single proteins and mixtures of up to five proteins (myoglobin, troponin C, actin, bovine serum albumin (BSA), tropomyosin) were deposited onto a polymer surface, followed by in situ tryptic digestion and comparative analysis using DESI-MS and LESA-MS using tandem electrospray MS. Peptide peaks specific to individual proteins were readily distinguishable with good signal-to-noise ratio in the five-component mixture. LESA-MS gave a more stable analysis and greater sensitivity compared with DESI-MS. Meat tryptic digests were subjected to peptidomics analysis by DESI-MS and LESA-MS. Bovine, horse, pig, chicken, and



turkey muscle digests were clearly discriminated using multivariate data analysis (MVA) of the peptidomic data sets. The most abundant skeletal muscle proteins were identified and correctly classified according to the species following MS/MS analysis. The study shows, for the first time, that ambient ionization techniques such as DESI-MS and LESA-MS have great potential for species-specific analysis and differentiation of skeletal muscle proteins by direct surface desorption. ■

Mini 12, Miniature Mass Spectrometer for Clinical and Other Applications—Introduction and Characterization

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Anal. Chem., **2014,** 86 (6), pp 2909–2916
DOI: 10.1021/ac403766c

A benchtop miniature mass spectrometer system, Mini 12, with ambient ionization source and tandem mass spectrometry capabilities has been developed and characterized. This instrument was developed as a self-contained system to produce quantitative results for unprocessed samples of small volumes including nonvolatile analytes. The ion processing system, vacuum system, and control system are detailed. An integrated sample loading system facilitates automated operation. A user interface has been developed to acquire and to interpret analytical results for personnel who have limited mass spectrometry knowledge. Peak widths of Δm/z 0.6 Th (full width at half-maximum) and a mass range of up to m/z 900 are demonstrated with the rectilinear ion trap mass analyzer. Multistage experiments up to MS5 are accomplished. Consumable cartridges have been designed for use in ambient paper spray ionization, and the recently developed extraction spray ionization method has been employed to improve the quantitative performance. Monitoring of trace-levels of chemicals in therapeutic drugs, as well as in food safety and environmental protection operations is demonstrated. Dual MS/MS scans are implemented to obtain the intensities of the fragment ions from the analyte and its internal standard, and the ratio is used in quantitative analysis of complex samples. Limits of quantitation (LOQ) of 7.5 ng/mL, with relative standard deviations below 10%, have been obtained for selected therapeutic drugs in whole blood throughout their individual therapeutic ranges. ■

Nano-LC/MALDI-MS Using a Column-Integrated Spotting Probe for Analysis of Complex Biomolecule Samples

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Koichi Tanaka Laboratory of Advanced Science and Technology, Shimadzu Corporation, 1, Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan
Anal. Chem., **2014,** 86 (5), pp 2549–2558
DOI: 10.1021/ac4037069

Nanoflow liquid chromatography (nano-LC) is an essential technique for highly sensitive analysis of complex biological samples, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is advantageous for rapid identification of proteins and in-depth analysis of post-translational modifications (PTMs). A combination of nano-LC and MALDI-MS (nano-LC/MALDI-MS) is useful for highly sensitive and detailed analysis in life sciences. However, the existing system does not fully utilize the advantages of each technique, especially in the interface of eluate transfer from nano-LC to a MALDI plate. To effectively combine nano-LC with MALDI-MS, we integrated a nano-LC column and a deposition probe for the first time (column probe) and incorporated it into a nano-LC/MALDI-MS system. Spotting nanoliter eluate droplets directly from the column onto the MALDI plate prevents postcolumn diffusion and preserves the chromatographic resolution. A DHB prespotted plate was prepared to suit the fabricated column probe to concentrate the droplets of nano-LC eluate. The performance of the advanced nano-LC/MALDI-MS system was substantiated by analyzing protein digests. When the system was coupled with multidimensional liquid chromatography (MDLC), trace amounts of glycopeptides that spiked into complex samples were successfully detected. Thus, a nano-LC/MALDI-MS direct-spotting system that eliminates postcolumn diffusion was constructed, and the efficacy of the system was demonstrated through highly sensitive analysis of the protein digests or spiked glycopeptides. ■

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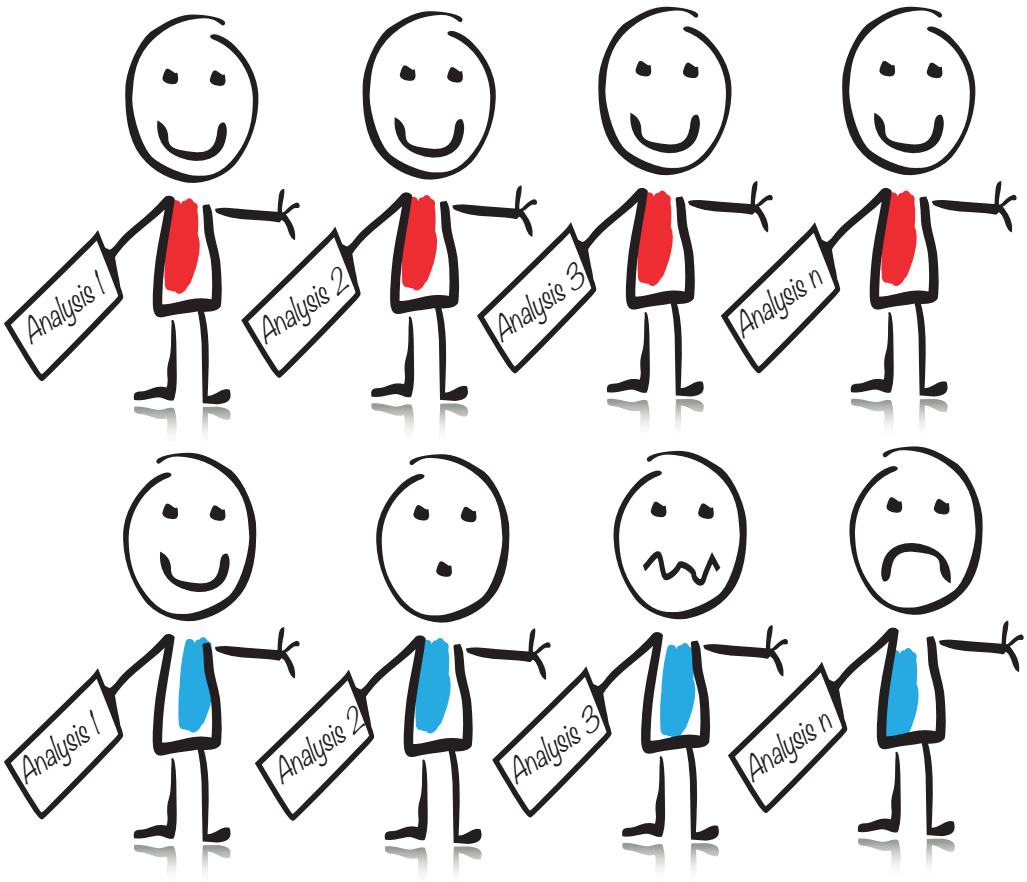
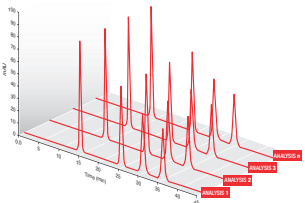


Greater sample success at low elution volumes due to high reproducibility



Providing confidence in your analytical results

DETECTION OF METHYLMALONIC ACID (MMA) IN PLASMA USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC) COUPLED WITH MASS SPECTROMETRY (MS) OR TANDEM MASS SPECTROMETRY (MS/MS)

Maricar Dube and Patrik Appelblad EMD Millipore

Abstract

Methylmalonic acid (MMA) is a biomarker for vitamin B12 deficiency. This application note describes a fast, simple, and sensitive method to detect MMA in plasma that uses a zwitterionic hydrophilic interaction chromatography (ZIC®-HILIC) column with LC-MS or LC-MS/MS.

Introduction

Methylmalonic acid (MMA) levels in serum, plasma and urine are used to monitor cobalamin (vitamin B12) deficiency[1] and methylmalonic acidemia. Different methods have been developed to quantify MMA in biological samples, including GC-MS, LC-MS/MS, HPLC, and capillary electrophoresis (CE). The main challenges that must be overcome for accurate measurement are the low physiological concentrations of MMA in human serum (100-500 nM), and the fact that MMA is a hydrophilic non-volatile compound. Retention and separation of MMA on reversed phase liquid chromatographic columns is difficult since MMA is poorly retained, and the structural isomer, succinic acid (SA), causes ion suppression because the concentration SA in serum is usually considerably higher than MMA.

Many laboratories have adopted protocols that require extraction of MMA plus steps to yield MMA-derivatives that are compatible with GC-MS or LC-MS/MS techniques using reversed phase columns[2]. This way, derivatives of MMA and SA may be differentiated due to their different fragmentation patterns. As a consequence however, the cost per MMA-test is usually considerably higher than standard immunological assays for B12.

HILIC columns efficiently separate polar hydrophilic compounds, which are not retained on reversed phase columns. The base material of HILIC columns can be either silica or polymer, and may be modified with different types of polar functionalities such as zwitterionic sulfoalkylbetaine (ZIC®-HILIC column). Because of its highly polar nature, MMA is retained on a ZIC®-HILIC column without the need to generate MMA-derivatives, making the workflow simpler, easier and faster[3]. This report describes a sensitive LC-MS/MS method to measure MMA using a ZIC®-HILIC column.

Experimental Conditions

Chromatography Conditions

Table 1

Column	SeQuant® ZIC®-HILIC (3μm, 100Å) PEEK 100× 2.1 mm
Injection	7 μL
Mobile phase	80:20 Acetonitrile/ 100 mM ammonium acetate pH 4.5*
Flow rate	400 μL/min
Temperature	40°C
Detection	(a) ESI-MS (b) MS/MS, ESI(-), MRM (m/z 117.1→73.0, 55.1)

*** There is a gradient wash process between injections**

Sample Preparation

800 μL acidified acetonitrile containing 170 nM of internal standard (D3-MMA) was used to precipitate proteins in 200 μL serum/plasma samples. Supernatant was directly injected into the column after centrifugation[3].

Results and Discussion

Isocratic separation of MMA in plasma on a ZIC®-HILIC column was achieved in less than 3 minutes. The void volume was 0.5 min, while the retention times for MMA and D3-MMA were 2.14 min and 2.13 min, respectively.

For the single stage MS detection, the limit of detection (3 x SD) and limit of quantitation (10 x SD) were 30 nM and 90 nM MMA, respectively, in plasma/serum. The method is linear up to 200 μM. Day-to-day and intra-day CVs are lower than 5%. The recovery is between 90% and 93%.

For the MS/MS detection, the limit of detection (3 × SD) was 5 nM and the limit of quantification (10 × SD) for MMA was 15 nM.

Conclusion

A fast, simple, and sensitive means to determine MMA levels in serum/plasma was developed that combined ZIC®-

HILIC separation with single stage negative ESI-MS or tandem MS. As neither MMA sample extraction nor derivatization were required, the ZIC®-HILIC-MS/MS method reported here may be an attractive alternative to existing means for measuring MMA in clinical laboratories where the existing GC-MS or reversed phase LC-MS/MS methods are tedious and laborious.

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3. Lakso, H.-A., Appelblad, A., and Schneede, J. *Quantification of methylmalonic acid in human plasma with hydrophilic interaction liquid chromatography separation and mass spectrometric detection*. **2008**. Clin. Chem. Dec. 54(12): 2028-2035

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DIRECT SAMPLE ANALYSIS WITHOUT SAMPLE PREPARATION ON A COMPACT MASS SPECTROMETER

ADVION

Introduction

Chemists must be able to quickly identify and verify the compounds they create, to evaluate and ensure product quality and safety. Techniques currently used for this purpose are adequate but may be slow, require specialized training, or result in low-quality data. Presented here is the expression Compact Mass Spectrometer, a compact and affordable single quad mass spectrometer that is easy to use and rapidly yields high quality data. Central to the speed and high-quality performance of the expression CMS is the direct analysis probe (ASAP®) that offers chemists the ability to rapidly analyze solids, liquids and powders without tedious and time-consuming sample preparation.

Method

Use is straightforward: The direct analysis probe (ASAP®) contains a glass melting point capillary insert. For analysis, dip the closed end of the capillary into the sample. Excess sample should be wiped from the tip, and the direct analysis probe inserted into the mass spectrometer (see Figures 1 and 2 for details). Results will be obtained in seconds. The results shown here in Figures 3-5 were generated using samples of carbonated drinks and paracetamol tablets.

Figure 1

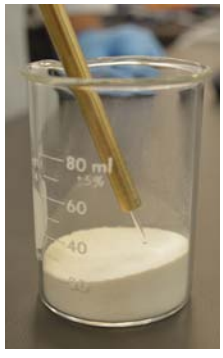


Figure 2

Summary

The expression CMS is a high-performance, affordable, single quad mass spectrometer. Its compact size allows it to fit into space-limited labs facilitating direct access and immediate results to chemists requiring mass confirmation, reaction monitoring, quality control and purity analysis. The expression CMS is therefore ideal for reaction monitoring, compound identification, assessing food safety, characterizing natural products.

Key benefits to using the expression CMS include:

- No sample preparation required
- No chromatography necessary
- Data is generated in < 1 minute

Figure 3. Cola

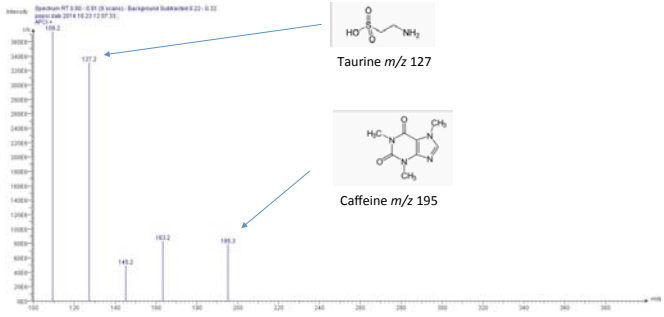


Figure 4. Non-caffeinated Drink

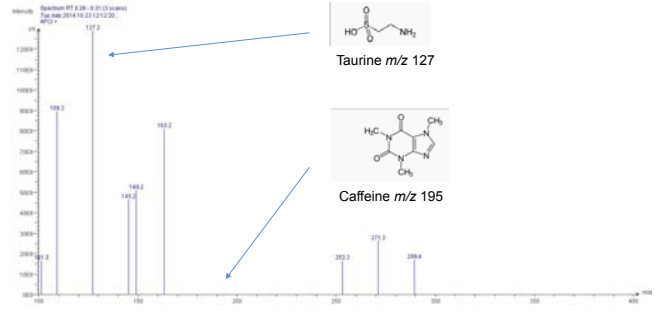
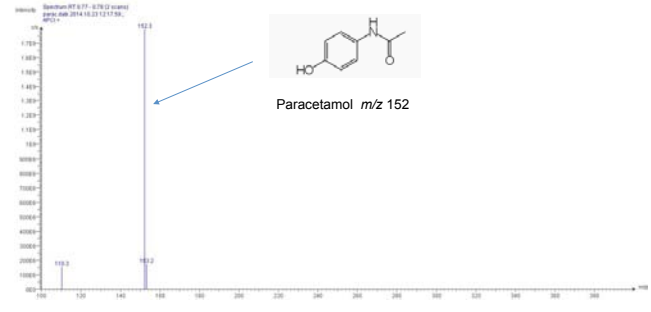


Figure 5. Paracetamol Tablet



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Introduction

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Results

Thermo Scientific™ SOLA*μ*™ plates allow users to pre-concentrate up to 20 times prior to injection, allowing greater limits of sensitivity to be achieved.

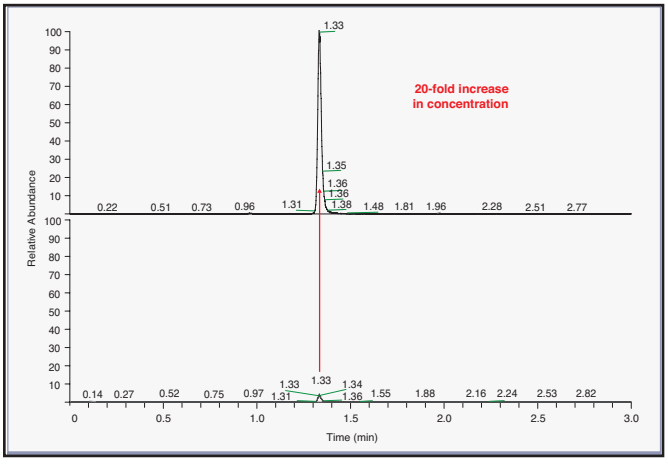

Figure 1

*Shows how SOLA*μ* allows users to pre-concentrate the sample up to 20 times prior to injection.*

Discussion

The application of award winning Thermo Scientific™ SOLA™ technology to micro-scale elution provides improved flow characteristics, which gives users the confidence in results, time after time.

SOLA*μ* provides reproducibility, robustness and ease of use at low elution volumes by utilizing the revolutionary SOLA Solid Phase Extraction (SPE) technology. This removes the need for frits, delivering a robust, reproducible format, which ensures highly consistent results at low elution volumes.

SOLA*μ* delivers:

- Robust sample processing
- Capacity for clinical scale sample volumes

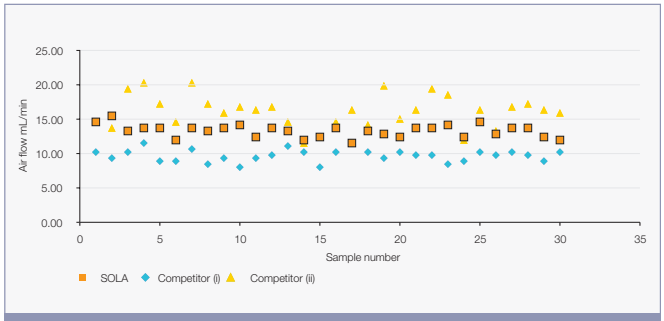

Figure 2: SOLA provides more uniform flow through characteristics compared to loose packed SPE products

Figure 2

SOLA provides more uniform flow through characteristics compared to loose packed SPE products.

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples which are limited in volume

SOLA*μ* for Pre-analysis Sample Concentration

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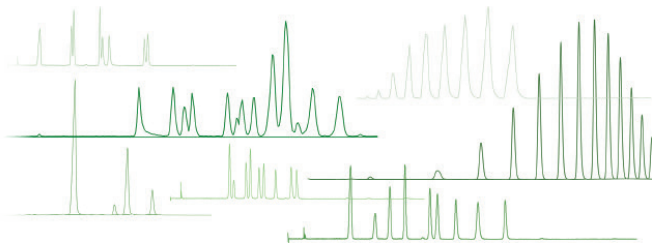
SEPARATION METHOD SEEKS NEW USERS

Celia Henry Arnaud C&EN Washington

Reprinted from C&EN, May 5, 2014.



SUPERCRITICAL FLUID chromatography, or SFC, has been around for decades. During that time, the separation technique has fallen in and out of and back in favor. But it has consistently been relegated to niche status. Could that finally be changing? Instrument manufacturers certainly hope so.

In the past several years, instrument companies Agilent Technologies and Waters Corp. have both brought out new analytical SFC instruments. The companies are optimistic that these instruments, which are more robust and more sensitive than previous generations of SFC models, will attract new users. They see SFC's potential going far beyond the large, preparative-scale purification of chiral drug compounds, where the method long ago proved its value.

Indeed, the method is gaining ground in other types of separations, including small, analytical-scale ones of achiral compounds. But other experts question whether the improvements will be enough to give SFC staying power this time.

In SFC, carbon dioxide dominates the mobile phase that carries a mixture's components through the column where they are separated. The CO_2 is usually combined with smaller concentrations of a modifier, which helps target analytes dissolve in the mobile phase. Modifiers are typically organic solvents; the most common one is methanol.

In true SFC, as the name suggests, CO_2 is supercritical, which means that it's at temperatures and pressures at which there are no phase boundaries between the liquid and gas phases. But most of the time SFC is a misnomer.

"Technically, we rarely do supercritical fluid chromatography," says SFC expert Larry T. Taylor, an emeritus chemistry professor at Virginia Tech. "Just about every application I'm aware of uses modified carbon dioxide. The temperature is usually near ambient. Under those conditions, the mobile phase is not supercritical."

SFC expert Pat Sandra, president of the Research Institute for Chromatography, located in Kortrijk, Belgium, and Lille, France, agrees. "We are exploiting the unique properties of carbon dioxide but not the unique properties of supercritical fluids," he says. "It's not SFC, but it's working."

"The terminology has probably held back the acceptance of the technology," Taylor says. "In the 1980s, people thought the word supercritical implied a dangerous situation in the laboratory. Maybe that terminology wasn't very useful. Those who work in the area just call it SFC for lack of a better term."

Nowadays, CO_2 should be considered just another mobile phase for liquid chromatographic separations, adds Martin Vollmer, Agilent's marketing director for analytical liquid chromatography and preparative LC, in Waldbronn, Germany.

The majority of LC separations come in two flavors: normal and reversed phase. In both versions, a pump is used to force a mobile phase through a column, so the methods are known as high-performance LC, or HPLC. Normal phase is the original incarnation of LC, in which compounds are separated using silica stationary phases and nonpolar solvents such as hexane or heptane. Normal phase typically separates compounds on the basis of their polar functionality. It is usually performed with an unchanging mobile-phase composition.

In contrast, reversed-phase chromatography uses water mixed with a polar solvent and separates compounds on the basis of their nonpolar functionality. The ratio of the water and polar solvent changes with time, resulting in a solvent gradient. Such gradients speed up overall separation time.

SFC BRINGS the power of gradient separations to normal-phase separations. "In the same way that water is a universal solvent that makes gradient separations work in reversed-phase HPLC, CO_2 is a universal solvent that makes gradient separations work in normal phase," says Mark Baynham, program director of UPC^2 separations technology at Waters.

"Normal phase is much more powerful than reversed phase," Baynham says. "For structurally similar compounds, it's better than reversed phase. For a wider range of compounds, it's better than reversed phase."

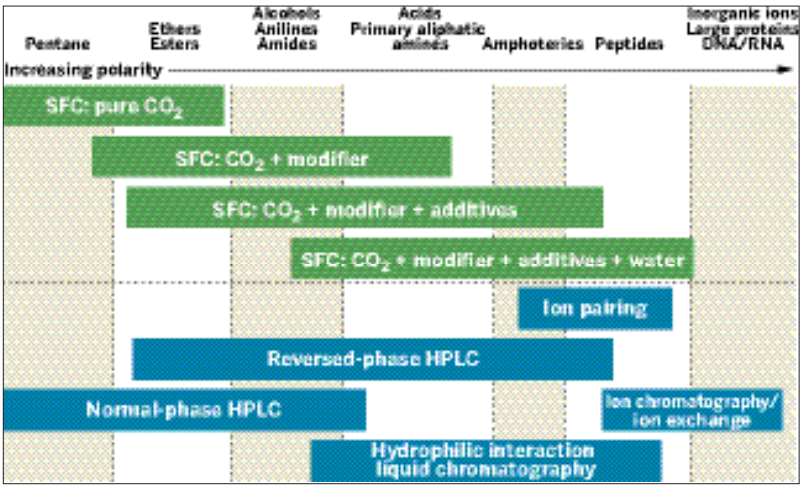
And SFC is "normal phase on steroids," says SFC guru Terry Berger of SFC Solutions. SFC separations can be much faster than reversed-phase HPLC. They also require lower pressures.

In ultra-high-performance LC, stationary phases with particle diameters less than 2 μm require high pressure to force the mobile phase through the column. By contrast, in SFC with similar particle sizes, pressure higher than 400 bar—a pressure associated with conventional HPLC rather than UHPLC—is rarely needed.

"With just a little bit of training, anybody who does LC nowadays will also be able to successfully do SFC," Vollmer says.

But those same folks might find SFC requires a little more method development than the reversed-phase LC they're used to. Most reversed-phase separations are done on variations of the octadecylsiloxane-bonded—called C18—column with a simple gradient of water and a polar organic solvent such as acetonitrile, methanol, or tetrahydrofuran. Although this combination doesn't work for everything, it works well enough for most compounds that it's the default method.

There is no such one-size-fits-most solution in SFC. "With SFC there are more options" to test, says Geoffrey Cox, presi-



FITTING IN

With just a few tweaks to its CO_2 mobile phase, supercritical fluid chromatography (SFC) can resolve a far wider range of analytes than can other common chromatography techniques.

dent of PIC Solution, an SFC company based in Media, Pa., and Avignon, France. "You need to run a sample through a screen of several columns and several solvents in order to be comfortable that you'll actually have a separation you can use."

"We need the universal stationary phase for SFC," says Christopher J. Welch, senior principal scientist in process and analytical chemistry at Merck & Co., Rahway, N.J. "The stationary phase that rivals C18 is not there yet." Researchers at Merck are collaborating with Myung Ho Hyun of Pusan National University in South Korea to develop SFC stationary phases.

ONE APPLICATION for which the power of SFC has long been recognized is the separation of racemic mixtures into their enantiomers. When Berger started his own SFC company in the mid-1990s, the eponymous Berger Instruments (which was later bought by Mettler-Toledo, Thar Instruments, and ultimately Waters) focused its efforts "on the one niche where it was absolutely obvious that SFC just killed LC—chiral separations," Berger says.

The company quickly realized that SFC-based chiral separations could be scaled up, and SFC has since come to dominate such preparative-scale separations and purifications in the pharmaceutical industry.

"Chiral separations were always normal phase, so it was easy to switch to SFC," says Holger Gumm, managing director of Sepiatec, a separations company in Berlin. "If you work in chiral separations, you must have SFC," says Sandra of the Research Institute for Chromatography.

"I think normal-phase LC has had its time," Baynham says. "To continue using it for chiral separations when the alternative is so compelling is ludicrous."

But even in chiral separations, normal-phase LC has been surprisingly tenacious. "We amazingly find customers still doing normal-phase LC for chiral work," says D. J. Tognarelli, a chromatography product specialist at Jasco, another SFC manufacturer. But, he adds, those users of normal-phase LC are becoming increasingly rare.

Researchers at pharma companies and instrument manufacturers are examining whether SFC can make similar inroads with achiral separations and purifications. Such separations are typically done with reversed-phase chromatography, Welch says. "To get your sample back, you have to dry it down, usually overnight. There's half a day for drydown. In SFC with just CO_2 and methanol, you can dry down in 30 minutes or less.

"All other things being equal, if SFC could do those jobs, the game would go to SFC," Welch continues. But the game has not yet been won. Even those who already use SFC for achiral separations continue to use conventional reversed-phase HPLC as well because SFC separations don't work for everything, Welch says.

SFC's inability to separate all compounds equally is not the only problem that has hindered its wider adoption. A major barrier to acceptance of analytical SFC in the past was poor instrument sensitivity. That problem could be attributed to the compressibility of CO_2 and to the pumps, Berger says.

In previous generations of instruments, the pumps caused noise in the chromatogram baseline that Berger describes as awful. "It was all due to the compressibility of the fluid. The fluid was being pumped accurately, but you were getting pressure and flow spikes on every stroke," he explains. Those spikes caused refractive index changes that wreaked havoc on the ultraviolet detector and translated into noisy chromatograms.

This noise has been greatly reduced in the newest analytical SFC instruments. Though they vary in the details, Agilent and Waters both use multiple pumps to separate the compression of the CO_2 from the mixing with the modifier solvent.

Agilent's instruments—the 1260 Infinity Analytical SFC system and the 1260 Infinity Hybrid SFC/UHPLC system—use an add-on component originally developed by Aurora SFC, a company started by Berger and subsequently purchased by Agilent. "Our box has its own pump that precompresses the fluid to just below the pressure of the metering pump," Berger says. "We've separated metering from compression. That eliminates all that noise."

In the Waters instrument, the Acquity UltraPerformance Convergence Chromatography (or UPC^2) system, the compressible CO_2 and the noncompressible modifier each has its own pump. The two are mixed only after the CO_2 has been compressed and both have been individually metered. The strategy allows the instrument to achieve precise gradients, Baynham says.

But it remains to be seen whether these improvements in sensitivity and precision are going to translate into new quantitative applications. For example, an area in which SFC has never been used is quality control. QC is an area where U.S. Pharmacopeia methods dominate.

Many USP methods are based on normal-phase LC, which would seem to make them ripe for conversion to SFC methods.

The LC methods can take as long as two hours, depending on the compounds and the conditions. With SFC, "every single one is usually done in two minutes with a generic gradient," Baynham says. "The fact that you can take QC from 90 minutes to two minutes means that you can do more batches."

Berger is skeptical, however, that SFC will find its way into QC quickly. Because the existing methods work, many organizations may not find it worth the effort and expense to validate new methods. "If they've got an awful method that's really tough by LC and it works well by SFC, they might switch over, but I don't see any other compelling reason."

Some people hope that SFC's reputation for being environmentally friendly will give it the boost it needs to garner more use in QC and other quantitative applications. "When you look at it as a normal-phase replacement, the green bit really stands out," Baynham says. Because it obviates the need for solvents such as heptane or hexane, SFC results in a significantly smaller carbon footprint. Plus, it eliminates other toxic solvents such as dichloromethane and tetrahydrofuran, which are used in reversed-phase HPLC.

"SFC took off in the pharmaceutical development labs," Cox says. "People were able to do their purifications more quickly than with HPLC and using much less solvent. In the early days, that meant that you had less solvent to get rid of when you evaporated it. These days, with the emphasis on green chemistry, you get lots of 'greenie points' if you're using CO_2 rather than hexane as your solvent."

PROPONENTS OF SFC now see application areas outside the pharmaceutical industry. For example, Taylor has collaborated with scientists at Waters to use SFC to separate components of biodiesel, particularly fatty acid alkyl esters and impurities such as tri-, di-, and monoacylglycerols, free fatty acids, and glycerol itself. Other applications are in food and vitamin analysis.

But some applications are not practical for SFC. Sandra notes that most biomolecules, which are often polar or ionic in nature, aren't easily solubilized in SFC's CO_2-based mobile phases. Peptide separations with SFC have been reported in the literature, but such separations are limited to mixtures containing just a handful of different peptides.

For example, Tognarelli separated a five-peptide mixture for a customer who was already using SFC for other applications. "It worked pretty well and didn't take a lot of effort, but the SFC didn't really offer any more speed than reversed-phase HPLC," he says. "I told the customer: 'You're going to want to stick with HPLC for peptides.' "

That conclusion doesn't surprise Sandra. "You will never convince people in life sciences, in proteomics, in genomics, not even in metabolomics, to prefer SFC over other techniques," he says.

Moving forward, the biggest hurdle to SFC adoption is education, Berger says. "Who teaches it?" he asks. "Nobody. In the U.S., I doubt there are more than two dozen—and I bet it's less than one dozen—SFCs operating in academic laboratories."

Taylor also worries about academia ignoring SFC. "Academic labs have not embraced SFC for the last 20 years," he says. "It's disturbing to me that nobody is being trained at the undergraduate and graduate level in this type of technology. In order for it to really take off, we're going to have to have considerable training so that people will have the same comfort with SFC as exists with LC. It's depressing that there are companies who want to hire in this area, but people aren't available."

Even if the new generation of SFC instruments is adopted, it's possible that SFC will remain a niche technique. "Eighty percent of the chromatography market is reversed phase," Sandra says. Of the other 20%, "normal phase can only be about 5%, and maybe half of that will be SFC. The market will be small. I'm not pessimistic. I'm just realistic."

Berger, however, thinks SFC has the potential for a much bigger market share. The improved sensitivity of UV detection with SFC will have a significant impact on the broader QC market over time, he says.

But he still worries that this wave of the technique could recede like the others before it. The large companies have shown their willingness to jump in and out of the field.

Still, company reps remain optimistic. Waters is already seeing major accounts at global pharmaceutical companies purchase additional instruments. "If it's good technology, they're the ones who should be buying multiple units the fastest," Baynham says. Quite a few companies have bought in the double digits. "In two years, that's not bad," he adds.

"I'm convinced that it will stay because we really see uptake and interest," Agilent's Vollmer says. "It's perhaps a little slower than anybody expected, but we see a lot of interest even from fields or markets where we didn't expect it." ■

SFC Milestones

1958 Supercritical fluid chromatography first proposed by James E. Lovelock at Yale University

1962 Supercritical fluid chromatography First SFC separation of metalloporphyrins performed by Ernst Klesper at Johns Hopkins University (method called high-pressure gas chromatography)

1980s Capillary SFC (also called open tubular) developed and commercialized as an alternative to GC but abandoned in early 1990s

1983 Hewlett-Packard introduces first commercial SFC instrument, discontinues it in 1985

1986 First chiral separations by SFC of phosphine oxides and of enantiomeric amides

1992 Hewlett-Packard reenters SFC instrument market

1995 Berger Instruments, founded with technology acquired from Hewlett-Packard, introduces first preparative-scale SFC instruments (Berger later sold to Mettler-Toledo, then Thar Instruments, then Waters)

2010 to present Agilent introduces the 1260 Infinity Analytical SFC system and the 1260 Infinity Hybrid SFC/UHPLC system, which can perform either SFC or ultra-high-performance liquid chromatography on the same instrument; **Waters introduces** the Acquity UltraPerformance Convergence Chromatography, or UPC^2, system, its analytical SFC instrument

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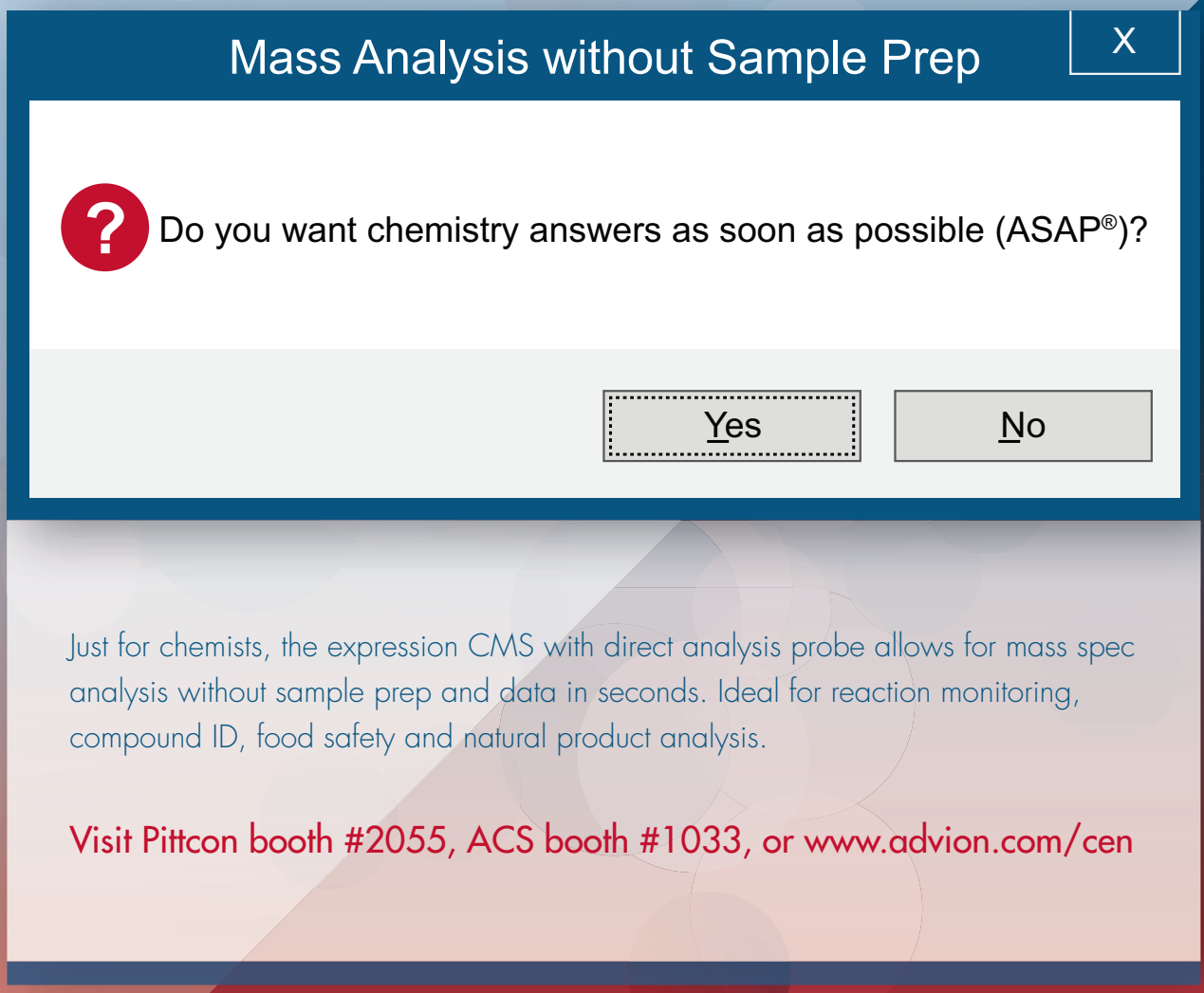

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