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Advances in Chromatography and Mass Spectrometry

March 3, 2014

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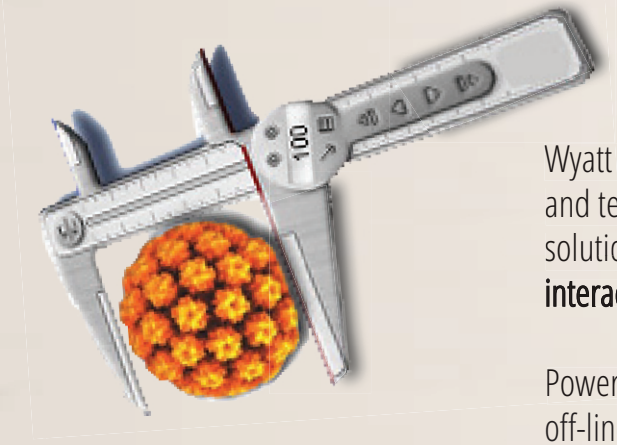

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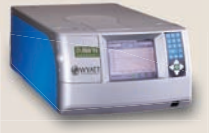

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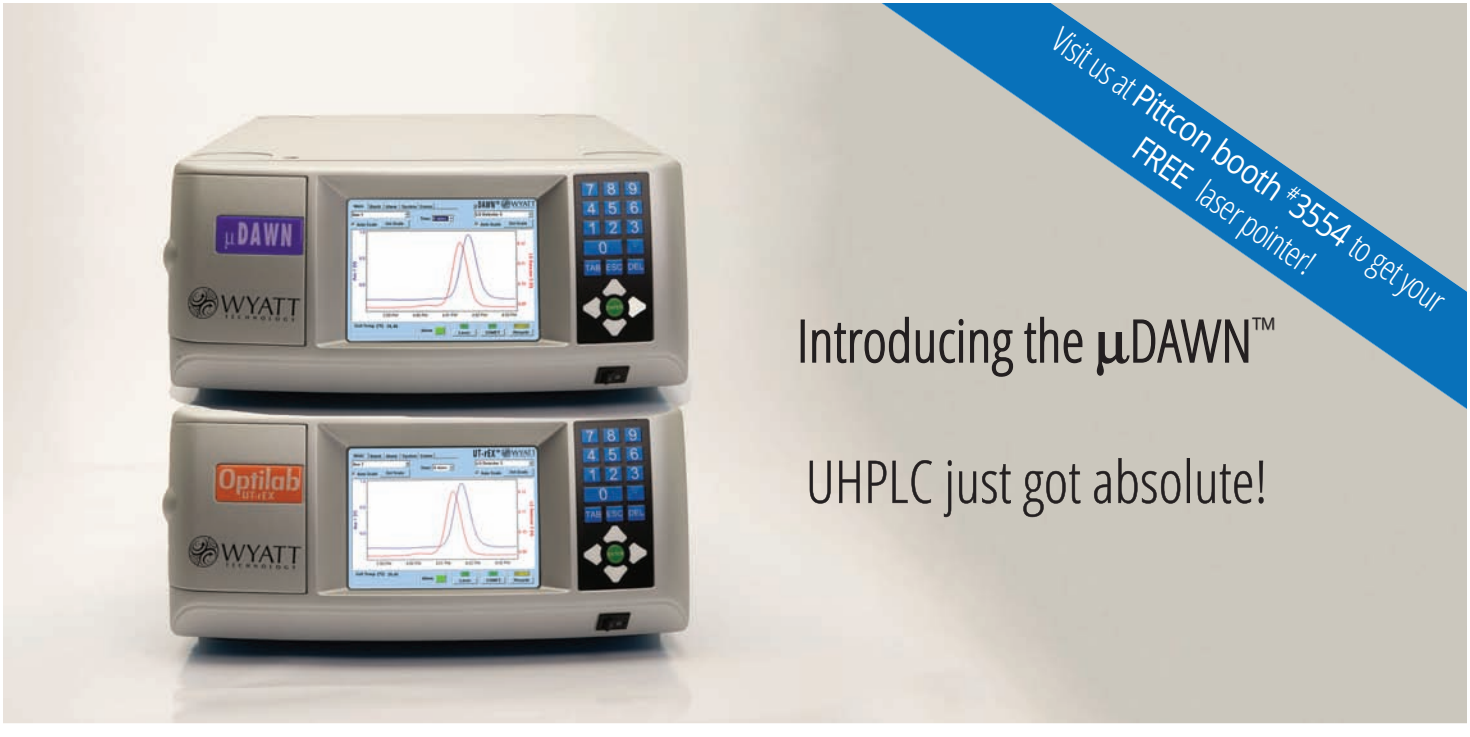

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PUBLISHER'S NOTE

Introducing C&EN Supplements

WE ARE EXCITED to present *"Advances in Chromatography and Mass Spectrometry,"* a special supplement to *Chemical & Engineering News* magazine.

This is the first in a new quarterly series of supplements produced by the C&EN Media Group that will provide a deeper dive into areas of intense interest to C&EN readers. This supplement includes 19 application notes contributed by a total of 10 leading instrumentation companies. We have also included abstracts from 10 of the most popular and cited research articles on these themes published in 2013 in the journal *Analytical Chemistry,* published (as is C&EN) by the American Chemical Society.

Later this year, we look forward to publishing additional supplements on a range of interesting topics. Our next supplement in June will showcase advances in spectroscopy, again inviting application notes from leading technology suppliers in that arena. And in September, we eagerly anticipate publishing a special report on the "Top 50 Drugs"—an in-depth report on the stories and takeaways of the drugs that are both on the market and in the pipeline. We will close out the year with our fourth supplement on a topic to be announced shortly.

Our thanks go to contributing editor Malorye Allison Branca, *Analytical Chemistry* managing editor Antonella Mazur, and the companies and organizations that have supported this inaugural C&EN supplement.

Kevin Davies, PhD
Publisher, C&EN

The editorial content in this supplement was created without direct involvement of C&EN reporters or editors.

NEW HORIZONS IN SEPARATION SCIENCE

Malorye Allison Branca

WELCOME to this special supplement to C&EN—the first in a new, quarterly series of exciting supplements to *Chemical & Engineering News* magazine—showcasing important examples of technology innovation and implementation in "separation science," specifically chromatography and mass spectrometry.

A vibrant field that each year brings technological improvements and new insights, chromatography, often importantly paired with mass spectrometry, is rapidly improving abilities to conduct more comprehensive and efficient separation and detection of an ever-expanding range of samples. Separations are carried out for purposes as varied as pharmaceutical research, quality assurance and process development for a huge variety of manufactured products and forensic investigations.

High-performance liquid chromatography (HPLC) is "one of the most often-used analysis technique and spans nearly every chemical application," writes Thomas L. Chester, of the University of Cincinnati, in a 2013 review article in the prestigious ACS journal Analytical Chemistry.[1] Experts and suppliers in this field continue to make advances in the application of theory, capabilities, and practice of both assay and screening methods. Advances in mass spectrometry have also spurred the field, allowing more precise detection of a wider range of substances.

One of the key challenges in chromatography is the trade-off bet ween greater separation efficiency of smaller particles and the pressure required to pump liquids through a column. Smaller particles require greater pressure, which in turn causes frictional heating of the mobile phase. Nonetheless, over time a decrease in the diameters of the totally porous stationary particles are observed. In the 1990s, commercial particles reached approximately 2.5 μm—the practical lower limit with conventional pumps. Pumps have also improved, and the combination of stronger pumps, smaller particles and smaller extra-column volumes was dubbed ultra-high performance liquid chromatography (UHPLC).

A few years ago, commercial versions of core-shell particles also became widely available, spurring great interest. These particles are not completely porous but rather comprise a porous layer surrounding a solid spherical core. These can provide multiple benefits: better reduced plate heights, requiring one-half to one-third of the pressure required to operate columns with completely porous particles and improved thermal conductivity. Today, a range of core-shell columns with different bonded-phase chemistries are commercially available and new versions will continue to be developed. Other key developments have been monolithic columns and hydrophilic interaction liquid chromatography (HILIC).

Gas chromatography also continues to progress. This technique has been practiced for more than a half-century; today the vast majority of such analyses employ two columns (2-D GC), and use either heart-cutting or comprehensive techniques. New hybrid instruments are testing novel approaches, such as using combinations of capillary columns, cryogenic trapping region and flow switching devices. Smart interfaces, high-speed and high-temperature approaches are

also in development. As a result, "each year brings improvements and insights that allow a wider range of samples to be analyzed with more informative, higher resolution separations,"[2] according to John V. Seeley and Stacy K. Seeley, of Oakland University and Kettering University, respectively, also writing in last year in Analytical Chemistry.

This special supplement to C&EN covers a wide range of techniques and applications from leading companies in this field and their customers. The scientists working on these techniques are all seeking better, faster and more efficient separation methods. Among the application notes in this issue are descriptions on how to more efficiently detect pesticides in liquid samples, analyze drug conjugates and identify and isolate poorly resolved compounds. The technologies used include supercritical fluid chromatography, size-exclusion chromatography with light scattering detection, LC/ and GC/MS, MS/MS and more.

As a bonus, we've also reproduced the abstracts from 10 of the most popular and cited research and review articles published in Analytical Chemistry during 2013. These also cover a wide range of applications and technologies, as well as some of the most intriguing new techniques in development.

We hope you enjoy this collection of articles and find them valuable. We welcome your feedback and look forward to producing additional C&EN supplements throughout the course of the year.

References

1 Chester, Thomas L. Recent Developments in High-Performance Liquid Chromatography Stationary Phases. *Analytical Chemistry,* 2013, **85,** 579–589.

2 Seeley, J.V. and Seeley, S.K. Multidimensional Gas Chromatography: Fundamental Advances and New Applications. *Analytical Chemistry,* 2013, **85,** 557–578.

Malorye Allison Branca is a contributing editor on this C&EN Supplement and freelance science writer based in Acton, Mass. ■

TOP TEN CHROMATOGRAPHY PAPERS

***Analytical Chemistry's* Most Popular Papers of 2013**

WHAT CAUGHT your and your colleagues' attention in 2013? Below are listed the ten most popular chromatography-related articles that appeared in *Analytical Chemistry* that year. Browse through them, make your own picks, and please visit http://pubs.acs.org/journal/ancham to see the most recent reports.

Perspectives on the Evolution of the Column Efficiency in Liquid Chromatography

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Anal. Chem., **2013,** 85 (6), pp 3017–3035
DOI: 10.1021/ac3033307

When analyses of mixtures of small molecules are carried out at mobile phase velocities close to (for isocratic runs) or somewhat above (for gradient runs) the optimum velocity, the eddy diffusion term contributes to at least 75% of the band broadening. Future improvements in column performance may come only from a reduction of the eddy diffusion term. The classical models of axial dispersion of Gunn and Giddings are revisited and their predictions compared to recently reported eddy dispersion data obtained by solving numerically the Navier–Stokes equations and simulating advective-diffusive transport in the bulk region and in confined geometries of reconstructed and computer-generated random sphere packings. The Gunn model fails to describe these data. In contrast, the Giddings model succeeds, provided that his original guesses regarding the values of two parameters of his model are adjusted. Accurate measurements of real eddy dispersion data in modern high-pressure liquid chromatography (HPLC) columns were performed by applying a well established experimental protocol. Their results demonstrate that the other contribution to band broadening, sample dispersion in the homogeneous bulk region of these packed beds, accounts for less than 30% of the total eddy dispersion at velocities larger than the optimum velocity. This shows that the resolution power of modern HPLC columns is essentially controlled by wall and/or border layer trans-column eddy dispersion effects, depending on whether the column is radially equilibrated or not. Under a preasymptotic dispersion regime, the performance of short and wide HPLC columns is controlled by the border effects. As the bed aspect ratio (D/d_p) increases, the column performance tends toward that of the infinite diameter column. Further improvement appears possible using radial segmentation of the outlet flow. Under an asymptotic dispersion regime, the reduced column plate height of long and thin HPLC columns is controlled by the wall effects and can be optimized only by improving the packing procedures, keeping as low as possible the bed aspect ratio and maximizing the transverse dispersion coefficient.

Multidimensional Gas Chromatography: Fundamental Advances and New Applications

John V. Seeley *† and Stacy K. Seeley ‡
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Anal. Chem., **2013,** 85 (2), pp 557–578
DOI: 10.1021/ac303195u

Multidimensional gas chromatography (MDGC) is a technique for isolating and identifying volatile and semi-volatile organic compounds present in complex mixtures. MDGC separations employ two or more gas chromatographic separations in a sequential fashion. The separation produced by each stage is maintained, at least in part, so that the resolving power of the composite separation exceeds that of the individual stages. Although MDGC has been in existence for more than 50 years, each year brings improvements and insights that allow a wider range of samples to be analyzed with more informative, higher-resolution separations. This review summarizes the advances and applications of MDGC that have been described in nearly 200 articles published between January 2011 and November 2012. The vast majority of MDGC separations use two columns, and so, they are classified as two-dimensional gas chromatography (2-D GC). These separations frequently fall into one of two categories: heartcutting 2-D GC or comprehensive two-dimensional gas chromatography (GC × GC). While these two techniques use similar hardware, they are implemented in very different ways. Heartcutting 2-D GC is a more mature technology than GC × GC, and as a result, there have been far fewer research articles in the past two years devoted to heart cutting 2-D GC. This review examines studies aimed at improving the materials and methods used for conducting MDGC separations. Recent applications of MDGC in a wide range of fields are also considered.

Smart Three-Dimensional Gas Chromatography

Di Chen †‡, Jung Hwan Seo ‡§, Jing Liu †‡, Katsuo Kurabayashi ‡§, and Xudong Fan *†‡
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Department of Mechanical and System Design Engineering, Hongik University, 94 Wausan-ro, Mapo-gu, Seoul, 121-791, South Korea
Anal. Chem., **2013,** 85 (14), pp 6871–6875
DOI: 10.1021/ac401152v

We developed a complete computer-controlled smart 3-dimensional gas chromatography (3-D GC) system with an automation algorithm. This smart 3-D GC architecture enabled independent optimization of and control over each dimension of separation and allowed for much longer separation time for the second- and third-dimensional columns than the conventional comprehensive 3-D GC could normally achieve. Therefore, it can potentially be employed to construct a novel GC system that exploits the multidimensional separation capability to a greater extent. In this Article, we introduced the smart 3-D GC concept, described its operation, and demonstrated its feasibility by separating 22 vapor analytes.

Serial Affinity Chromatography as a Selection Tool in Glycoproteomics

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Anal. Chem., **2013,** 85 (15), pp 7125–7132
DOI: 10.1021/ac400653z

Glycan-targeting affinity chromatography systems are becoming increasingly important as tools in the purification, enrichment, and identification of glycoproteins. The great advantage of this strategy is that immobilized lectin and antibody selectors allow specific glycan structures to be matched with a particular

protein. It is also possible to show that a glycan seen at one site in a glycoprotein may not be present at another glycosylation site in the same glycoprotein. A problem with single-column affinity chromatography is how to obtain information on glycan diversity within the oligosaccharide portions of captured glycoproteins. Although all the glycoprotein species bearing a particular glycan feature will be captured by an affinity column, there is no way of knowing whether the ligand being targeted appears alone or coresides with a series of other glycan features in the same oligosaccharide conjugate. The work being described here examines the utility of serial affinity columns in determining whether individual glycan structures appear alone or together with other glycans in specific proteins. Four different types of affinity columns were examined in two series; the LEL → HPA → anti-Le^{x}Ab → anti-sLe^{x}Ab series and the anti-sLe^{x}Ab → anti-Le^{x}Ab → HPA → LEL series. Patterns in protein capture from these two series were very different. Thus, serial affinity chromatography (SAC) can be a valuable tool in recognizing diversity in protein glycosylation, especially when the order of columns in the SAC series is varied. Two clear types of diversity were recognized. One is the independent occurrence of different affinity-targetable glycan features in the same glycoprotein. The other is that multiple targetable glycan features were coresident in the same glycoprotein. The great advantage of this method is that it couples easily with current methods used in glycoproteomics.

Novel Analytical Methods for Flame Retardants and Plasticizers Based on Gas Chromatography, Comprehensive Two-Dimensional Gas Chromatography, and Direct Probe Coupled to Atmospheric Pressure Chemical Ionization-High Resolution Time-of-Flight-Mass Spectrometry

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Anal. Chem., **2013,** 85 (20), pp 9572–9580
DOI: 10.1021/ac4017314

In this study, we assess the applicability of different analytical techniques, namely, direct probe (DP), gas chromatography (GC), and comprehensive two-dimensional gas chromatography (GC × GC) coupled to atmospheric pressure chemical ionization (APCI) with a high resolution (HR)-time-of-flight (TOF)-mass spectrometry (MS) for the analysis of flame retardants and plasticizers in electronic waste and car interiors. APCI-HRTOFMS is a combination scarcely exploited yet with GC or with a direct probe for screening purposes and to the best of our knowledge, never with GC × GC to provide comprehensive information. Because of the increasing number of flame retardants and questions about their environmental fate, there is a need for the development of wider target and untargeted screening techniques to assess human exposure to these compounds. With the use of the APCI source, we took the advantage of using a soft ionization technique that provides mainly molecular ions, in addition to the accuracy of HRMS for identification. The direct probe provided a very easy and inexpensive method for the identification of flame retardants without any sample preparation. This technique seems extremely useful for the screening of solid materials such as electrical devices, electronics and other waste. GC-APCI-HRTOF-MS appeared to be more sensitive compared to liquid chromatography (LC)-APCI/atmospheric pressure photoionization (APPI)-HRTOF-MS for a wider range of flame retardants with absolute detection limits in the range of 0.5–25 pg. A variety of tri- to decabromodiphenyl ethers, phosphorus flame retardants and new flame retardants were found in the samples at levels from microgram per gram to milligram per gram levels.

Recent Developments in High-Performance Liquid Chromatography Stationary Phases

Thomas L. Chester
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Anal. Chem., **2013,** 85 (2), pp 579–589
DOI: 10.1021/ac303180y

Experts and suppliers continue to make advances in the application of theory, capabilities, and practice of high-performance liquid chromatography (HPLC). It is one of the most often-used analysis techniques and spans nearly every chemical application. Many users, even those with years of success operating an HPLC instrument and producing useful results, could benefit from recent knowledge of what is possible, what has changed, and what might be coming soon. There is little incentive to update or replace an old, reliable method when the cost and potential benefits of a replacement are uncertain. Awareness of what is available now and what is on the horizon is essential for the progressive practitioner. We will focus primarily on the last 2 years of developments but will include several older reports of significance. The citations are not exhaustive but are intended to represent recent work and advances.

Improved Synthesis of Carbon-Clad Silica Stationary Phases

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Anal. Chem., **2013,** 85 (24), pp 11765–11770
DOI: 10.1021/ac401986j

Previously, we described a novel method for cladding elemental carbon onto the surface of catalytically activated silica by a chemical vapor deposition (CVD) method using hexane as the carbon source and its use as a substitute for carbon-clad zirconia.1,2 In that method, we showed that very close to exactly one uniform monolayer of Al(III) was deposited on the silica by a process analogous to precipitation from homogeneous solution in order to preclude pore blockage. The purpose of the Al(III) monolayer is to activate the surface for subsequent CVD of carbon. In this work, we present an improved procedure for preparing the carbon-clad silica (denoted CCSi) phases along with a new column packing process. The new method yields CCSi phases having better efficiency, peak symmetry, and higher retentivity compared to carbon-clad zirconia. The enhancements were achieved by modifying the original procedure in three ways: First, the kinetics of the deposition of Al(III) were more stringently controlled. Second, the CVD chamber was flushed with a mixture of hydrogen and nitrogen gas during the carbon cladding process to minimize generation of polar sites by oxygen incorporation. Third, the fine particles generated during the CVD process were exhaustively removed by flotation in an appropriate solvent.

Identification of Dyes on Single Textile Fibers by HPLC-DAD-MS

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Anal. Chem., **2013,** 85 (23), pp 11335–11343
DOI: 10.1021/ac402173e

An HPLC-DAD-MS method is described to analyze textile dyes in different dye classes (reactive, basic, acid, direct, disperse). The described method is sensitive enough to analyze single fibers with a length of a few millimeters or less, which makes it suitable for forensic analyses. The current paper describes the information content of the acquired data as well as the results of a validation study, in which the repeatability, specificity, and limit of detection of the method were assessed by repeated measurements of nine different dyes in the mentioned dye classes. The mass accuracy (deviation generally <2 ppm) and absorbance spectra were found to be highly stable in several measurements over a period of 8 weeks. Deviation in retention times were observed and attributed to small experimental effects and a precolumn blockage. The results show that dye analysis is possible for most fibers with a minimum length of one or a few millimeters.

Online NMR and HPLC as a Reaction Monitoring Platform for Pharmaceutical Process Development

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Anal. Chem., **2013,** 85 (19), pp 8928–8932
DOI: 10.1021/ac402382d

Detector response is not always equivalent between detectors or instrument types. Factors that impact detector response include molecular structure and detection wavelength. In liquid chromatography (LC), ultraviolet (UV) is often the primary detector; however, without determination of UV response factors for each analyte, chromatographic results are reported on an area percent rather than a weight percent. In extreme cases, response factors can differ by several orders of magnitude for structurally dissimilar compounds, making the uncalibrated data useless for quantitative applications. While impurity reference standards are normally used to calculate UV relative response factors (RRFs), reference standards of reaction mixture components are typically not available during route scouting or in the early stages of process development. Here, we describe an approach to establish RRFs from a single experiment using both online nuclear magnetic resonance (NMR) and LC. NMR is used as a mass detector from which a UV response factor can be determined to correct the high performance liquid chromatography (HPLC) data. Online reaction monitoring using simultaneous NMR and HPLC provides a platform to expedite the development and understanding of pharmaceutical reaction processes. Ultimately, the knowledge provided by a structurally information rich technique such as NMR can be correlated with more prevalent and mobile instrumentation [e.g., LC, mid-infrared spectrometers (MIR)] for additional routine process understanding and optimization.

How Ternary Mobile Phases Allow Tuning of Analyte Retention in Hydrophilic Interaction Liquid Chromatography

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Anal. Chem., **2013,** 85 (18), pp 8850–8856
DOI: 10.1021/ac402123a

An attractive yet hardly explored feature of hydrophilic interaction liquid chromatography (HILIC) is the tuning of analyte retention through the addition of an alcohol to the water (W)–acetonitrile (ACN) mobile phase (MP). When retention times increase sharply between 10/90 and 5/95 (v/v) W/ACN, intermediate retention values are stepwise accessible with a ternary MP of 5/90/5 (v/v/v) W/ACN/alcohol by switching from methanol to ethanol to isopropyl alcohol. We investigate the physicochemical basis of this retention tuning by molecular dynamics simulations using a model of a 9 nm silica pore between two solvent reservoirs. Our simulations show that alcohol molecules insert themselves neatly into the retentive W-rich layer at the silica surface, without disrupting the layer's structure or altering its essential properties. With the decreasing tendency of an alcohol (methanol > ethanol > isopropyl alcohol) to move toward the silica surface, the contrast between the W-rich layer and the bulk MP sharpens as the latter becomes more organic, while the W density near the silica surface remains high. Analyte retention increases with the ratio between the W mole fraction in the diffuse part of the W-rich layer and that in the bulk MP. We predict that tuning of HILIC retention is possible over a wide range through the choice of the third solvent in a W/ACN-based ternary MP, whereby the largest retention values can be expected from W-immiscible solvents that fully remain in the bulk MP. ■

DYNAPRO PLATE READER TO EXAMINE NON-SPECIFIC SMALL MOLECULAR AGGREGATION

Ali Khaki, Katherine M. Brendza, Brian E. Schultz, and Roman Sakowicz
(Gilead Sciences) Wyatt Technology

Promiscuous non-stoichiometric inhibition can be a serious problem when screening libraries compounds in a high-throughput format. According to one widely accepted model (McGovern *et al, J. Med. Chem.* 2003) promiscuous inhibition is connected to the formation of small micelle-like aggregates of the compound. A rapid method of detecting such compound behavior would greatly facilitate screening hits selection.

In order to develop such a method, we have utilized the DynaPro Plate Reader to examine the light scattering properties of a dilution series of known aggregating inhibitors.

We created a plate with a three-fold compound dilution series in duplicate along with two DMSO (non-compound) controls. We chose a 96-well format (Greiner Bio-One 96 SensoPlate) with a sample volume of 100μl.

Each well contained 99μl of 50 mM potassium phosphate buffer, pH 7 (filtered through a 0.2 μm filter) and 1 μl of either DMSO or the small molecule dilution series. The plate was subjected to a brief centrifugation prior to reading, to remove bubbles. Because of the heterogeneous nature of the samples, we used short reads with more replicates to allow for averaging of the population.

As expected, the general trend was that the compound dilution caused a reduction in the scattering signal intensity (Fig. 1). When examining the amplitude of the autocorrelation function, we observed an increase in the amplitude with increasing concentration of small molecules. As the concentration of a compound decreased below some threshold, signals reverted to buffer-like behavior (Fig. 2). An increase in normalized intensity was correlated with increasing small molecule concentration (Fig. 3).

In summary, we have successfully utilized the DynaPro Plate reader to examine the light scattering behavior of known promiscuous inhibitors. This approach can now be extended to a wider range of

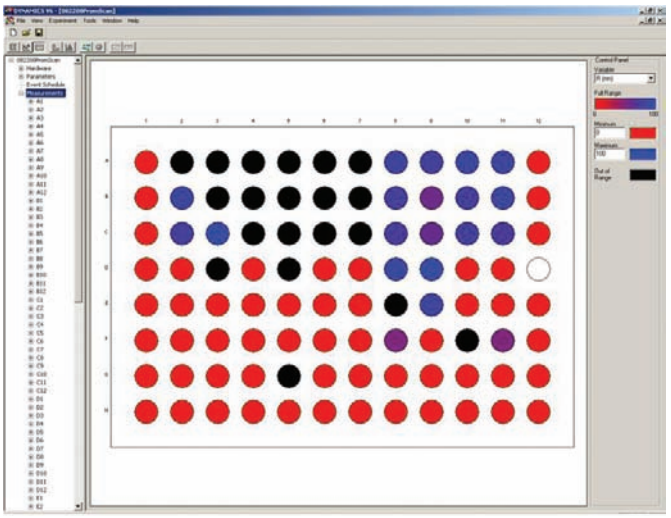

Figure 1
Plate layout and representative results. Lanes A-H are 3-fold serial dilutions of compounds starting at 100mM (row A). Lanes 1,12 DMSO controls; 2,3 Quercetin: 4, 5 Miconazole; 6,7 Clolrimazole, 8,9 Rottlerin; and 10, 11 I4PTH. Measurement of R, hydrodynamic radius in nm. Colors black, out of range; blue, fully saturated signal, graded to red, minimum signal; white, no sample/undetectable scattering signal.

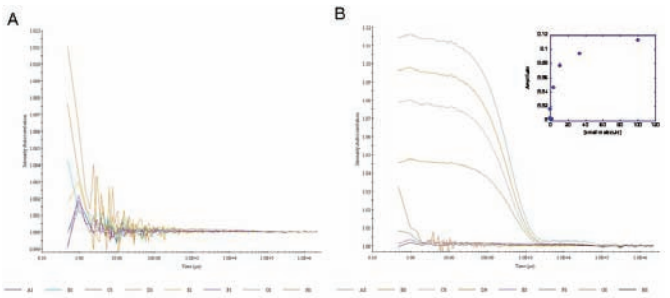

Figure 2
Comparison of Buffer to Rottlerin. A) Light scattering intensity autocorrelation for buffer with DMSO alone. B) Light scattering intensity autocorrelation for the dilution series of Rottlerin. The amplitude of the signal decreases with decreasing compound concentration (inset plot).

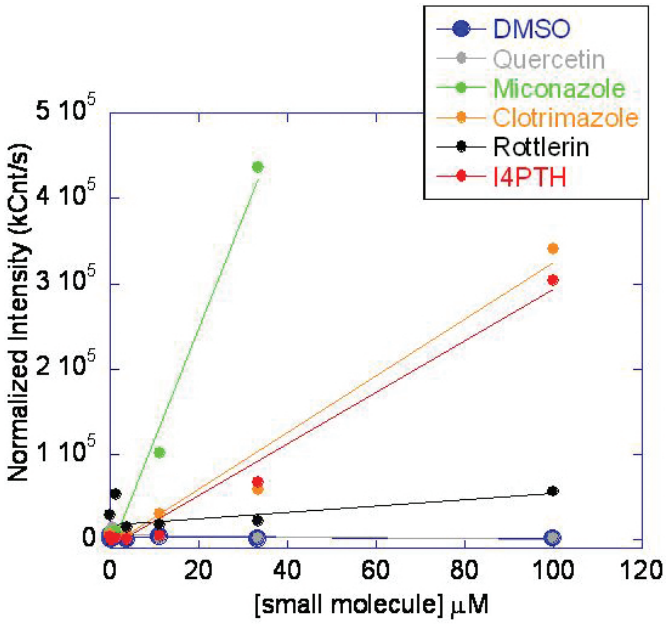

Figure 3
Correlation of Normalized Intensity and promiscuous inhibitor concentration.

compounds and to study the effect of enzymes on aggregate formation. ■

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IDENTIFICATION AND ISOLATION OF POORLY RESOLVED COMPOUNDS USING ISOLERA™ DALTON

Biotage

In this application note we demonstrate the power of mass detection in Isolera™ Dalton by investigating the incomplete separation of two dyes, Butter yellow (Mass 225.29 g/mol) and Sudan red (Mass 278.31 g/mol), by normal phase flash purification.

With flash purification several distinct compounds may co-elute in the same peak. To achieve good results, users need confidence that each peak corresponds to only a single compound.

When using Isolera Dalton, m/z values of parent ions can be entered manually or detected from screening a direct injection of the mixture to be purified. Up to four ions can be monitored in this way, with the option to trigger fraction collection by mass. In this application note we demonstrate the power of mass detection in Isolera Dalton by investigating the incomplete separation of two dyes, Butter yellow (Mass 225.29 g/mol) and Sudan red (Mass 278.31 g/mol), by normal-phase flash purification.

Results

The purification elutes the two dyes as two partially resolved peaks, as shown by the UV data (black and red traces). From the UV data it is challenging to fractionate each dye into separate test tubes. However, the Isolera Dalton mass detector's sensitivity to changes in each eluting compound's MW clearly identifies and fractionates the overlapping peaks (labeled as 226 and 279) into three fractions—pure Sudan red (green fraction), pure Butter yellow (yellow fraction) and a mix of both (blue fraction). UV fractionation would, at best, provide two impure fractions. ■

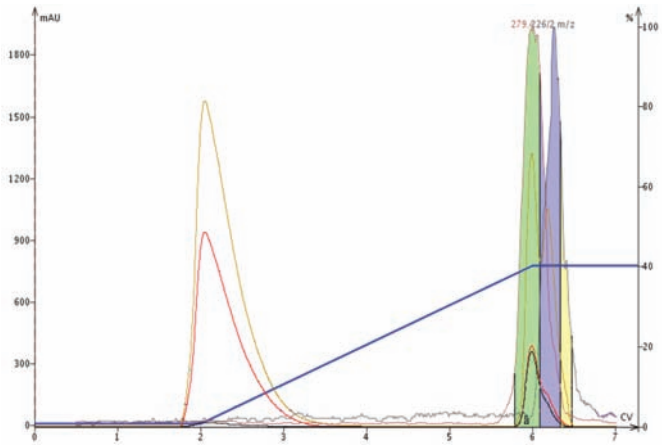

Figure 1

Chromatogram of the purification. The first broad peak at 2 CV:s is cyclohexane, the highlighted region is where the compounds were detected by mass (collection on one signal only).

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ISOLERA™ DALTON: MASS-DIRECTED FLASH PURIFICATION OF A PEPTIDIC OPIOID NEUROTRANSMITTER

Biotage

Reversed-phase flash chromatography coupled to fully automated, mass-directed fractionation was employed to purify a 5-residue opioid peptide (MW= 555 g/mol) following solid-phase peptide synthesis. The purification process was streamlined by avoiding the need to scout fractions for the desired product since the fractions were collected based on the desired mass. The peptide was purified in 8 min with an isolated product mass recovery of 65%.

There are real advantages to using mass-directed fractionation over UV-based fraction collection that apply to synthesized peptides and small molecules alike. These advantages include increased fractionation accuracy and the ability to avoid fraction scouting. In addition, mass-directed fractionation in peptide purification offers the potential to remove contaminants that may have UV overlap with the desired peptide product. ■

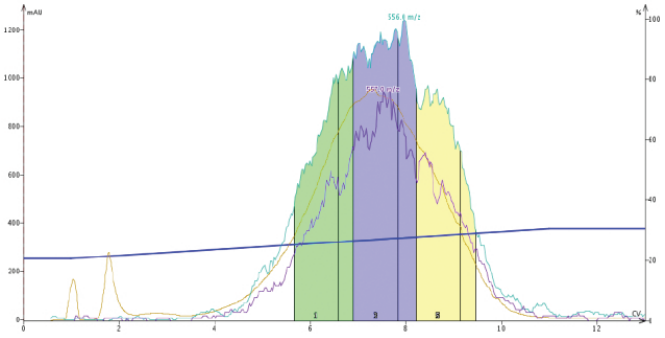

Figure 1
Mass-directed flash purification of Leu-enkephalin-NH2.

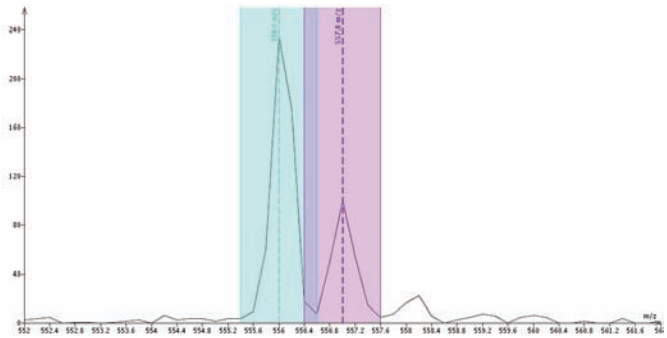

Figure 2
Isolera™ Dalton automatically selected peaks to trigger mass direction.

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MASS-DIRECTED FRACTION COLLECTION FOR PREPARATIVE SFC: RECOVERY AND PURITY DETERMINATION USING A JASCO PREP SFC COUPLED WITH THE EXPRESSION COMPACT MASS SPECTROMETER USING A SIMPLE PASSIVE SPLIT

Mark Hardink, Frank Riley, and Qi Yan (Pfizer); Simon Prosser (Advion); John Burchell and DJ Tognarelli (JASCO) Advion

Introduction

Supercritical fluid chromatography (SFC) is a rapidly growing technique. Initially used almost exclusively only for thermally labile and chiral compounds, it is now accepted as a versatile analytical and purification technique applied across a broad range of compounds. It is higher-throughput than regular HPLC and Prep-LC and offers the "green" advantages of much lower solvent consumption, consumable and disposal costs. SFC is compatible with a wide range of detection methods including UV, electrospray (ESI) and atmospheric pressure chemical ionization (APCI) mass spectrometry.

Methods/Experimental

The initial tests explored a wide range of flow rates, both isocratic and gradient, to test performance and reliability of the passive splitter, coincident timing of MS and UV signal and the clearance of the source with highly concentrated samples. The system was further evaluated by testing the recovery and purity of compounds in a three-component mixture.

Test #1: Three component standard mixture, 700 µL injections, gradient elution, mass-directed collection using TIC of each peak per injection starting with Peak A, then B & C (Flavone, Carbamazepine and Sulfamethazine). Repeat for a total of 5 injections. Measure the purity and recovery of the three collected fractions.

Test #2: Three component standard mixture, 700 µL injections, gradient elution, UV-directed collection using UV of each peaks per injection starting with Peak A, then B & C (Flavone, Carbamazepine, and Sulfamethazine). Repeat for a total of 5 injections. Measure the purity and recovery of the three collected fractions.

Mass spectrometer:	Advion expression CMS
SFC:	Jasco Prep SFC System
Total flow:	70 ml/min
BPR:	120 bar
Column:	Waters Viridis Silica 2-Ethyl-pyridine OBD Prep Column, 100Å, 5 µm, 19 mm x 250 mm
Co-solvent:	Methanol
Gradient:	5-25% in 7 min.
Splitter configuration:	100 cm (2 x 50 cm) PEEK-Sil - 0.025 mm ID x 1/16' OD coupled with ZDV union
Makeup pump flow:	8 mL/min MeOH (constant flow for 30% modifier and below)
Interface:	Jasco ChromNav set up to read two analog outputs from expression CMS mapped to the total ion chromatogram (TIC) and one selected extracted ion chromatogram (XIC)mg/mL
Sample:	Flavone 9.56 mg/mL Carbamazepine 9.56 mg/mL Sulfamethazine 9.48 mg/mL

Results

See Figures and Tables overleaf.

Discussion

- The expression CMS can be simply interfaced to both analytical scale and prep scale SFC using a simple passive split before the UV detector.
- Timing delay between UV and MS signals was determined to be 3.6 seconds.

Still Waiting?

Don't wait for someone else to give you the answer.

> "There is no substitute for having a mass spectrometer a few steps away from your bench."
>
> — *John Matson, PhD, Virginia Tech*

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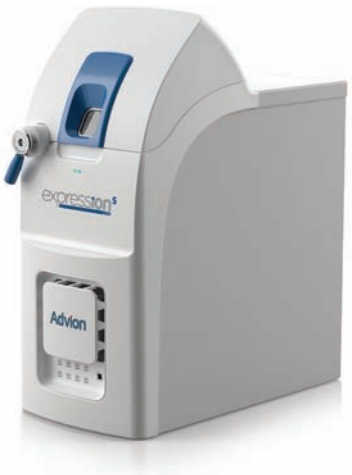

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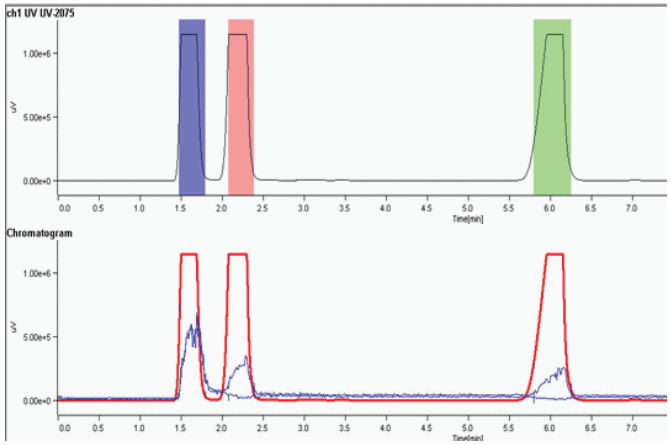

Figure 1
TIC triggered Fraction Collection (5 injections).

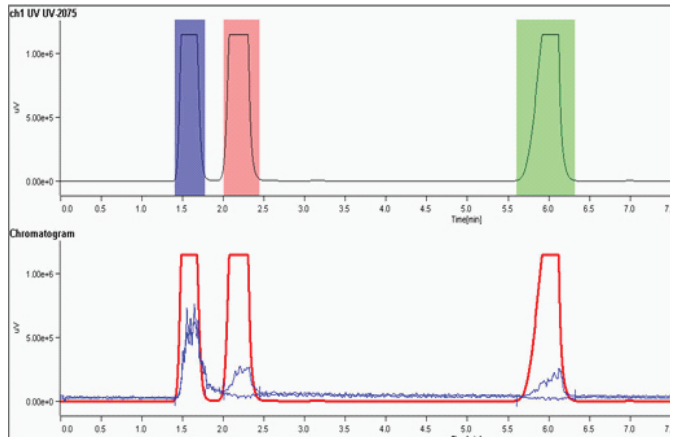

Figure 2
UV Triggered fraction Collection (5 injections).

JASCO/Advion SFC PREP recovery

For Figure 1

	Weight of Analyte mg	Sample Volume mL	Sample Concentration mg/ mL	Injection Volume µL	# of Injections	Theory Sample Injected on Column mg	Sample Recovered mg	Recovery %
TIC Trigger								
Flavone*	477.8	50	9.56	700	7	46.8244	44.4	94.8
Carbamazepine	478.2	50	9.56	700	5	33.474	31.4	93.8
Sulfamethazine	473.9	50	9.48	700	5	33.173	31.8	95.9

***** Two **additional fractions of Flavone were collected during instrument set-up and not disposed, showing a larger collection sample.**

JASCO/Advion SFC PREP recovery

For Figure 2

	Weight of Analyte mg	Sample Volume mL	Sample Concentration mg/ mL	Injection Volume µL	# of Injections	Theory Sample Injected on Column mg	Sample Recovered mg	Recovery %	Difference vs. TIC Triggering %
UV Trigger									
Flavone	477.8	50	9.56	700	5	33.446	31.7	94.8	0.0
Carbamazepine	478.2	50	9.56	700	5	33.474	33.5	100.1	6.3
Sulfamethazine	473.9	50	9.48	700	5	33.173	31.3	94.4	-1.6

- All fractions collected of each analyte were tested analytically and showed that each was 99.9% pure or greater.
- Difference between the recoveries of that were triggered by UV verses the fractions triggered by MS-TIC were less than 2% for the flavone and sulfamethazine peak and 6% for the carbamazepine. ■

Additional information: http://www.advion.com/applications/expression-applications/supercritical-fluid-chromatography-sfc/

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MICROWAVE REACTIONS GENERATING A GASEOUS BYPRODUCT

Michael J. Karney CEM Corp.

Abstract

The pressure monitoring devices on most microwave reactors which seal the reaction vessel are designed for high temperature/high pressure reactions and cannot remove undesired gaseous by-products that can stall a reaction. CEM's ActiVent® pressure device for the Discover® SP microwave synthesizer provides the capability to successfully manage both types of reactions easily.

Introduction

The Wolff-Kishner reduction offers a mild method to deoxygenate carbonyl compounds to the corresponding alkane, as opposed to harsh methods requiring the use of dangerous metal reagents (Clemmensen reduction, etc). By proceeding through the hydrazone, the compound is reduced through a loss of N_2 to generate the desired alkane. Conventionally, this reaction must be performed in an open vessel as nitrogen build up in a closed vessel will stall the reaction and lead to low yields. The ActiVent pressure device found on the Discover SP has the unique capability to vent gaseous reaction by-products and reseal the reaction vessel, allowing chemistry to continue above the boiling point of the solution without any loss of reagents.

Methods/Experimental Conditions

Benzophenone (1.82 g, 10 mmol), hydrazine hydrate (50%; 1.87 mL, 30 mmol), and potassium hydroxide (1.12g, 20 mmol) were added to a 35-mL microwave reaction vial containing a magnetic stir bar and 13.3 mL of diethylene glycol. The vial was capped and heated in a CEM Discover SP microwave synthesizer using a two-stage heating program (Dynamic method; Temperature Stage 1: 80 °C, 1-minute hold time, 300 psi pressure limit, 300 Watts power; Temperature Stage 2: 225 °C, 10-minute hold time, 300 psi pressure limit, 300 Watts power) and a single stage pressure venting program (Pressure Stage 1: Delta Pressure = 25, PressureSP = 150, Times At SP = 100). The solution was cooled to room temperature, diluted with 20 mL of diethyl ether, washed with a saturated solution of ammonium chloride, washed with brine, dried over magnesium sulfate, and concentrated *in vacuo* to furnish the benzylbenzene product as a colorless oil in a 96% yield. The product was analyzed on an Agilent 7890A/5975 GC/MS.

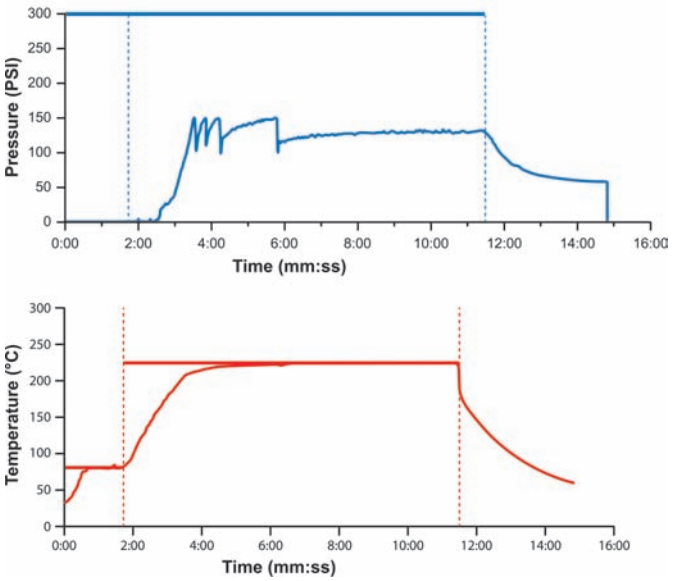

Figure 1

Pressure (top) and temperature (bottom) profiles of the Wolff-Kishner reduction.

Results

The reaction was started and heated according to the programmed method. Around 2:20 total time (m:ss; 150 °C) the reaction began generating pressure due to reflux, while within 30-40 seconds (~175 °C) a more rapid pressure increase resulting from nitrogen evolution was noted (Figure 1). The ActiVent pressure device relieved the internal vessel pressure four times, venting only gas (25-50 psi per vent) while maintaining original reaction volume despite a reaction tempera-

ture above reflux for the solution. Additionally, after each vent the reaction vessel resealed and maintained pressure as if the cap had never opened. After six minutes total reaction time, rapid pressure evolution is seen to cease and a constant pressure is maintained until the reaction end.

Discussion

Using the ActiVent pressure management system, microwave reactions that would otherwise stall due to over-pressurization or exceeding instrument limits can be performed with ease. The result is cleaner, faster chemistry that can take advantage of microwave heating. ■

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MOLECULAR WEIGHT DETERMINATION OF LMWH SEC/MALS VS. SEC/UV-RI

Lin Rao and John Beirne (Scientific Protein Labs, LLC) Wyatt Technology

Low-molecular-weight heparins (LMWHs) are obtained by fractionation or depolymerization of natural heparins. They are defined as having a mass-average molecular weight of *less* than 8000 and for which at least 60% of the total weight has a molecular mass *less* than 8000.

Size-exclusion chromatography (SEC) has been the most common way of measuring the molecular weight and molecular weight distributions of LMWHs by using the two most common detection technologies: ultraviolet (UV) coupled with refractive index (RI) detection. However, these detectors embody a relative method in order to determine molecular weights, requiring calibration standards. A newer, absolute method involves the use of multi-angle light scattering (MALS), which does not require any standards. The European Pharmacopeia (EP) monograph for LMWH specifies the use of the UV/RI detection method and provides a known calibration standard. Many laboratories around the world have adopted this method.

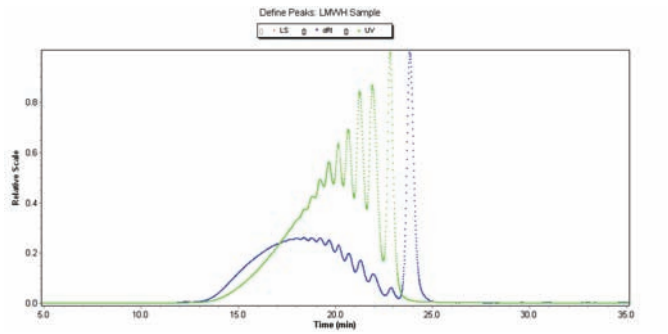

Figure 1
Examples of UV and RI traces for an LMWH sample.

We previously developed an SEC/MALS method and found it to be very suitable for the analysis of LMWHs. We have recently adopted the UV-RI method described in the EP monograph and compared the molecular weight results generated for LMWH using each detection type. The adopted method uses an Agilent LC-1200 series HPLC, 0.2M Sodium Sulfate pH 5.0 mobile phase, Tosoh TSK-gel

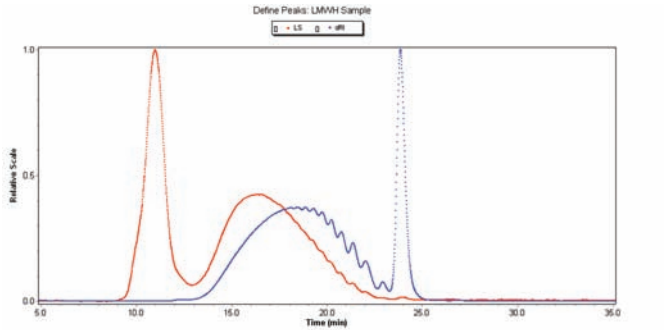

Figure 2
Examples of LS and RI traces for an LMWH sample.

G2000 SWxl column with Tosoh TSK-gel Guard SWxl, Waters 2487 dual wavelength UV detector, and Wyatt Optilab rEX refractive index detector. For MALS analysis the UV detector was replaced with a Wyatt miniDAWN TREOS detector; all other methods aspects remained the same.

The results indicated that both detection types are suitable and acceptable for the analysis of LMWHs. The molecular weight and distribution results generated using each detection type are comparable. This indicates that a SEC/MALS method could be adopted in place of the SEC/UV-RI method currently required by the EP monograph, and that it would result in less time because it obviates the need for calibration standards. ■

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RAPID PREPARATION OF A CEREBELLIN POSITIONAL SCAN LIBRARY

Michael J. Karney CEM Corp.

Abstract

Peptide synthesis often suffers from long reaction times and low yields due to problems such as aggregation. Microwave energy can help break up these aggregates improving the synthesis quality while, in combination with advanced fluidics, speeding up the synthesis rate.

Introduction

In order to evaluate the role of specific residues in peptide activity and binding interactions, an alanine scan library can be prepared. Alanine scanning is used to identify sites of protein-protein interactions by systematically replacing the functional groups along the peptide with a single methyl group while maintaining the same backbone confirmation.

Cerebellin, a 16-mer peptide, is found in the Purkinje cells of the brain. Cerebellin has been shown to play a role in the maintenance of parallel fiber-Purkinje cell synapses. It has also been shown to increase catecholamine release from the human adrenal gland.

The next generation microwave peptide synthesizers incorporate significant hardware and method improvements allowing for cycle times of only 4 minutes. With these rapid methods, the synthesis of peptide libraries can be performed in an iterative fashion in a timeframe that rivals parallel synthesizers, but with superior peptide quality. Solid-phase peptide synthesis of a fourteen-peptide alanine scanning library of cerebellin was performed using the CEM Liberty Blue™ automated microwave peptide synthesizer.

Methods/Experimental Conditions

The peptides were prepared using the Liberty Blue automated microwave peptide synthesizer on 0.277 g of Fmoc-Rink Amide MBHA resin (0.36 meq/g substitution). Deprotection (20% piperidine with 0.1 M HOBt in DMF) was performed for 1 minute at 90 °C. Coupling reactions were performed with 5 fold excess Fmoc-AA-OH with 1:1:1 AA/DIC/HOBt for 2 minutes at 90 °C (5 minutes at 50 °C for His; 2x2 minutes at 90 °C for Arg). Cleavage was performed using 92.5:2.5:2.5:2.5 TFA/H2O/TIS/DODT for 30 minutes at 38 °C. Following cleavage, the peptide was precipitated and washed with diethyl ether. Peptides were analyzed on a Waters Atlantis C18 column (2.1 ×150 mm) at 214 nm with a gradient of 5 – 70% MeCN (0.1% trifluoroacetic acid), 0 – 20 min. Mass analysis was performed using an LCQ Advantage ion trap mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA).

Results

Peptide	Sequence	Purity
Cerebellin	SGSAKVAFSAIRSTNH	71%
H16A	SGSAKVAFSAIRSTNA	67%
N15A	SGSAKVAFSAIRSTAH	57%
T14A	SGSAKVAFSAIRSANH	68%
S13A	SGSAKVAFSAIRATNH	78%
R12A	SGSAKVAFSAIASTNH	70%
I11A	SGSAKVAFSAARSTNH	66%
S9A	SGSAKVAFAAIRSTNH	60%
F8A	SGSAKVAASAIRSTNH	70%
V6A	SGSAKAAFSAIRSTNH	60%
K5A	SGSAAVAFSAIRSTNH	52%
S3A	SGAAKVAFSAIRSTNH	72%
G2A	SASAKVAFSAIRSTNH	62%
S1A	AGSAKVAFSAIRSTNH	59%

Table
Cerebellin Alanine scan.

An Alanine scan library of fourteen peptides based on the neuropeptide cerebellin was synthesized at 0.1 mmol scale. All peptides were obtained at greater than 50% purity (Table 1). Each synthesis was accomplished in less than 70 minutes, and the entire library was generated in less than a day.

Discussion

The speed and efficiency of next generation microwave peptide synthesizers is changing the way library peptide synthesis is performed. Traditionally, parallel synthesis was required to generate libraries of peptides in a timely fashion, but now with 4-minute cycle times, peptides can be generated sequentially in the same amount of time or faster than the parallel methods. In addition, preparing a library of peptides in a sequential manner provides complete control of the synthesis of each and every peptide and the peptides are available immediately after synthesis, eliminating the cleavage and purification bottlenecks typically experienced with parallel synthesizers. ■

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SEPARATION OF DIASTEREOMERIC CHIRAL METABOLITES USING UPC2 MS/MS

Chester L. Bowen, Hermes Licea-Perez, and Christopher Evans (Bioanalytical Science and Toxicokinetics, PTS DMPK, GlaxoSmithKline); Paul D. Rainville, Tom DiPillipo, Denise Heyburn, and Robert S. Plumb (Waters Corp.) Waters Corp.

Abstract

This application note shows how the combination of UPC2® and tandem quadrupole mass spectrometry provides for a means to produce fast, high resolution separations of chiral compounds in the DMPK laboratory.

Introduction

The separation and detection of GSK1322322, a novel antibacterial agent, and three associated stereoisomer metabolites was investigated utilizing UltraPerformance Convergence Chromatography™ (UPC^{2}®) coupled with tandem quadrupole mass spectrometry. This separation was attempted on various other platforms with no success (UV, chiral HPLC, mass spec).

There have been numerous examples of metabolism creating new centers of chirality in new chemical entities (NCEs). This chiral inversion can have pharmacokinetic, pharmacodynamics, and safety consequences.[1-3]

Therefore because of the heightened awareness of stereoselective metabolism, there are growing government requirements and regulations in chiral metabolite identification and quantification during drug development.

Recent advances have potentially opened the door for the use of UPC^{2}/MS/MS in routine bioanalysis with chiral entities, in a regulated environment. UPC^{2} applies the performance advantages of UPLC to supercritical fluid chromatography, using supercritical carbon dioxide as the major mobile phase. The results presented here explore the robustness of this technique and its application to clinical study samples in the quest to investigate *in vivo* chiral inversion.

Experimental Conditions

Chromatography Conditions

System:	ACQUITY UPC^{2}
Column:	Chiral Pak AD-H, 5-μm, 4.6 x 150 mm
ABPR pressure:	2750 PSI
Column temp.:	40 °C
Sample temp.:	Ambient
Injection vol.:	5 μL
Flow rate:	3 mL/min
Mobile phase:	CO_2/isopropanol with 0.4% diethylamine (80/20)
Run time:	10 minutes

MS Conditions

MS system:	Xevo® TQ-S
Ionization mode:	ESI +
Acquisition mode:	MRM
Capillary voltage:	4 kV
Collision energy:	30 V
Cone voltage:	25 V

Data Management

MassLynx® 4.1 Software

Sample Preparation

GSK1322322, GSK1343981, GSK1785312, and GSK1784667 were extracted from 100 μL human plasma by protein precipitation using acetonitrile containing [2H2 13C2] –GSK1322322 as an internal standard, followed by derivatization with camphanic chloride (1 mg/mL in acetontrile) for 15 minutes at 37 °C. Extracts were analyzed by UPC^{2}/MS/MS using an electrospray interface and multiple reaction monitoring (MRM).

The ACQUITY UPC².

Results And Discussion

As stated in the introduction, the separation of GSK1322322 and associated stereoisomer metabolites was attempted on a variety of other platforms. One such method that was produced was a 45-minute chiral HPLC-UV method using heptane/ethanol/DEA and formic acid. However this method was neither compatible with nor ideal in a mass spectrometer-based DMPK bioanalytical environment due to the long run time and the organic mobile phase solvents required for the separation. The separation further produced wide peak widths on the order of 2- 5 minutes at the peak base.

Conclusions

UPC^2 coupled with tandem quadrupole mass spectrometry was successfully applied in the investigation of stereoselective metabolism in a DMPK environment.

The UPC^2/MS/MS method was successfully validated over a three-day period for the parent compound GSK1322322 and the three diastereomeric metabolites from pooled clinical and preclinical dog plasma samples.

References

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SEPARATION OF SCHIZANDRIN, SCHIZANDRIN A, AND SCHIZANDRIN B IN A TABLET SAMPLE

Xu Qun, Huang Xiongfeng, and Jeff Rohrer Thermo Fisher Scientific

Abstract

The work shown here describes an efficient UHPLC method to determine schizandrin, schizandrin A, and schizandrin B in Hugan tablets for product quality control.

Introduction

Schisandra chinensis (Turcz.) Baill is an important traditional Chinese medicine believed to be an anticarcinogen and provide hepatoprotection, among other attributes. Its major active components are lignanoids, and the three major lignanoids are schizandrin, schizandrin A, and schizandrin B[1]. Hugan tablets, which contain Schisandra chinensis (Turcz.) Baill, are a traditional Chinese medicine for hepatoprotection. The Pharmacopoeia of the People's Republic of China (PPRC) 2010 regulates its quality control with a UHPLC method for the determination of schizandrin, schizandrin A and schizandrin B[2].

Methods and Experimental Conditions

The separation for this application was performed on a Thermo Scientific™ Acclaim™ Rapid Separation Liquid Chromatography (RSLC) 120 C18, 2.2 µm (2.1 × 100 mm) column based on the chromatographic conditions in the PPRC monograph. The chromatograms of schizandrin, schizandrin A, and schizandrin B in a Hugan tablet sample (Suzhong Pharmaceuticals Co., Ltd., Jiangsu, China).

Equipment

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system, including:

- HPG-3400RS Binary Pump with Solvent Selector Valves
- WPS 3000RS Autosampler
- TCC-3000RS Thermostatted Column Compartment
- DAD-3000RS Diode Array Detector

Thermo Scientific™ Dionex™ Chromeleon™ 6.80 SR9 Chromatography Data System software or higher.

Sample Preparation[2]

Put 0.7 g of sample powder to 25 mL of water-saturated ethyl acetate, and weigh the mixture. Ultrasonically

Column: Acclaim RSLC 120 C18 (2.1 × 100 mm, 2.1 µm)
Mobile phase: CH_3CN/H_2O, in gradient: CH_3CN: 0–3 min, 45%; 3–5 min, 45–80%; 15.1 min, 80–100%; 17 min, 100%
Flow Rate: 0.4 mL/min
Injection Volume: 2 µL
Temperature: 40 °C
Detection: UV at 250 nm
Chromatograms: A) Standards
B) Tablet sample

Peaks: 1. Schizandrin
2. Schizandrin A
3. Schizandrin B

UV spectra: A1 peak 1 of standard
A2 peak 2 of standard
A3 peak 3 of standard
B1 peak 1 of sample
B2 peak 2 of sample
B3 peak 3 of sample

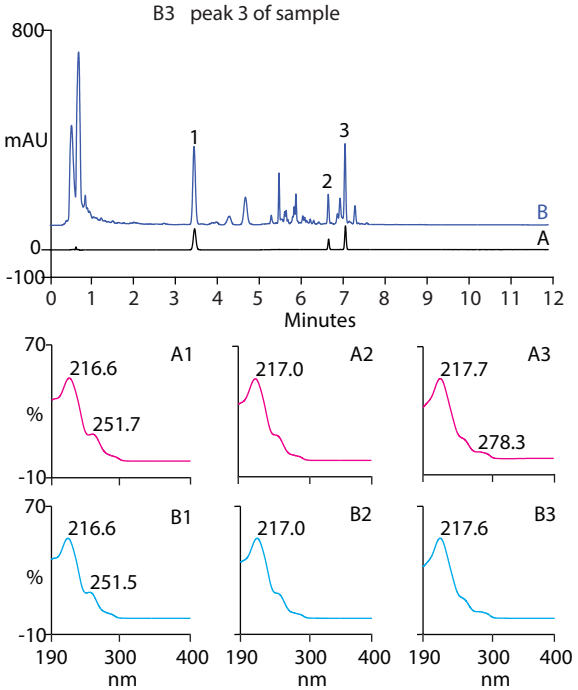

Figure 1

Chromatograms of schizandrin, schizandrin A, and schizandrin B mixed standard and a Hugan tablet sample.

extract (500 W and 60 KHz) for 30 min. After the solution cools to room temperature, replace the lost weight with ethyl acetate. After filtering, dry 15 mL of filtrate using a rotary evaporator. Dissolve the residue in 5 mL methanol.

Results and Discussion

The UV spectra of the three analytes collected in the standard and tablet sample are highly consistent. The calculated peak purity match factors for schizandrin, schizandrin A, and schizandrin B separated from the tablet sample extract are all 1000 (the corresponding value for 100% purity). Good separations between the analytes and other compounds were achieved with resolution *(Rs)* ≥ 1.9. These results demonstrate that the Acclaim RSLC 120 C18 column provides good selectivity and suitability for determination of schizandrin, schizandrin A, and schizandrin B in the Hugan tablet sample.

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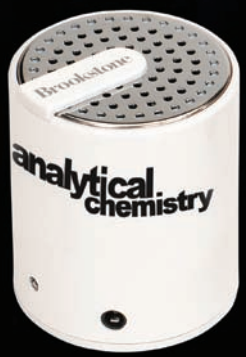

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ANTIBODY DRUG CONJUGATE (ADC) ANALYSIS WITH SEC-MALS

Wyatt Technology

There has been a significant resurgence in the development of antibody-drug conjugates (ADC) as target-directed therapeutic agents for cancer treatment. Among the factors critical to effective ADC design is the Drug Antibody Ratio (DAR). The DAR describes the degree of drug addition which directly impacts both potency and potential toxicity of the therapeutic, and can have significant effects on properties such as stability and aggregation. Determination of DAR is, therefore, of critical importance in the development of novel ADC therapeutics.

DAR is typically assessed by mass spectrometry (MALDI-TOF or ESI-MS) or UV spectroscopy. Calculations based on UV absorption are often complicated by similarities in extinction coefficients of the antibody and small molecule. Mass spectrometry, though a powerful tool for M_w determination, depends on uniform ionization and recovery between compounds-which is not always the case for ADCs.

We present here a method for DAR determination based on SEC-MALS in conjunction with UV absorption and differential refractive index detection. Figure 1 shows UV traces for two model ADCs; molecular weights of the entire ADC complexes are determined directly from light scattering data.

Component analysis is automated within the ASTRA 6 software package by using the differential refractive index increments *(dn/dc)* and extinction coefficients, which are empirically determined for each species or mined from the literature, to calculate the molar mass of the entire complex as well as for each component of the complex.

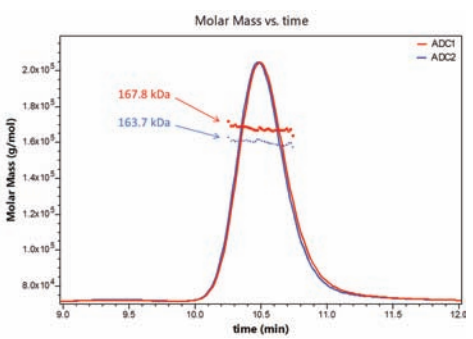

Figure 1
Molar masses for two distinct ADC formulations are determined using SEC-MALS analysis.

In this example an antibody has been alkylated with a compound having a nominal molecular weight of 1250 Da (Figure 2). Molar masses of the antibody fractions are similar, which indicates that the overall differences between the two formulations reflect distinct average DARs which are consistent with values obtained by orthogonal techniques. Note that the molar mass traces for the conjugated moiety represent the *total* amount of attached pendant groups; the horizontal trends indicate that modification is uniform throughout the population eluting in that peak. ■

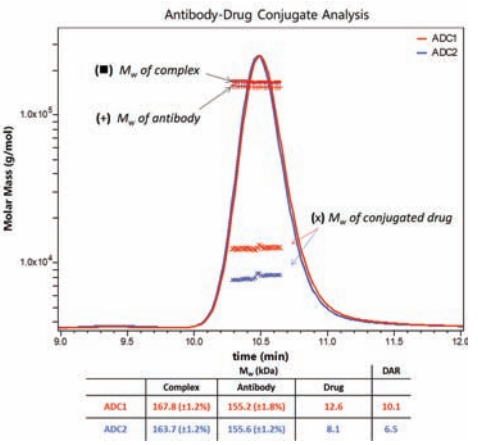

Figure 2
Molar Masses for the antibody and total appended drug are calculated in the ASTRA software package based on prior knowledge of each component's extinction coefficent and dn/dc, allowing determination of DAR based on a nominal Mw of 1250 Da for an individual drug.

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CHARACTERIZATION OF PLGA USING SEC-MALS-VIS

Wyatt Technology

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer based on glycolic acid and lactic acid. The two monomer units are linked together by ester linkages form linear polyester chains. The obtained product is biodegradable and biocompatible, and it is approved by the Food and Drug Administration (FDA) for production of various therapeutic devices as well as for drug delivery applications. The properties of PLGA can be tuned by the ratio of the two monomers and by its molar mass distribution.

The characterization of PLGA by means of conventional size exclusion chromatography (SEC) is problematic because of the lack of suitable calibration standards. In addition, the linear polyester structure can be modified by the addition of small amounts of polyfunctional monomer to obtain branched chains of differing degrees of branching. The degree of branching becomes an additional parameter that can be used to adjust PLGA properties—all of which renders conventional column calibration an inadequate analytical technique.

In this application note, two commercially available samples were analyzed by SEC coupled to a Multi-Angle Light Scattering (MALS) detector (HELEOS), a refractive index detector (Optilab rEX), and a viscosity (VIS) detector (ViscoStar). The ViscoStar was used in order to discover additional information about the molecular structure of the analyzed polymers. In addition to molar mass distributions, the SEC-MALS-VIS system yields the relationship between intrinsic viscosity and molar mass (Mark-Houwink plot) that can provide deep insight into the molecular structure of the polymers being analyzed.

In Figure 1 the molar mass distributions are given as differential distribution plots. As seen from the plots, the two samples span markedly different molar mass ranges. The Mark-Houwink plots of the two samples are shown in Figure 2 together with the plot of linear polystyrene that is shown simply for the sake of comparison. The slope of the Mark-Houwink plot of the linear polystyrene is 0.71, a typical value for linear random coils in thermodynamically good solvents. The slope of the red sample roughly corresponds to a linear structure as well. However, there is a slight indication of deviation from linearity at the region of high molar masses that may indicate the presence of branched molecules. The Mark-Houwink plot of the

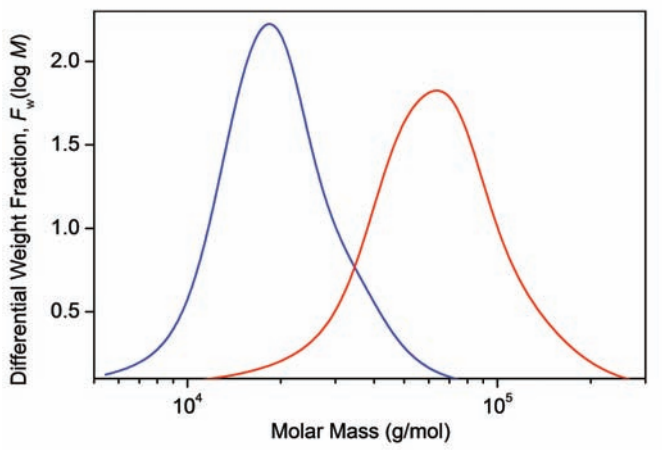

Figure 1
Differential molar mass distribution curves of two PLGA samples.

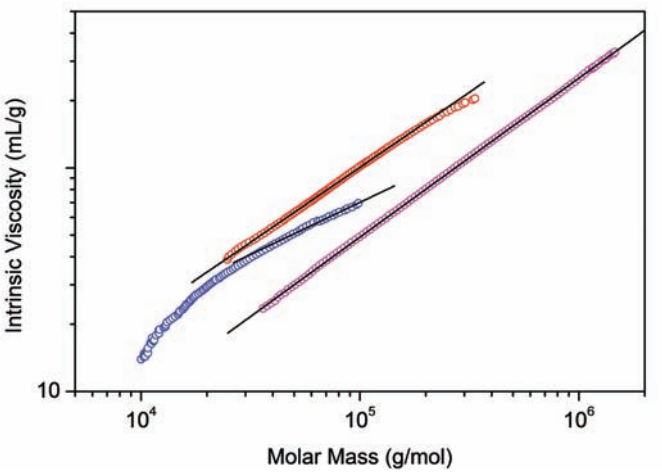

Figure 2
Mark-Houwink plots of two samples of PLGA (red and blue) and linear polystyrene (magenta). The lines are linear extrapolations of the data.

blue sample is curved. Curvature of the Mark-Houwink plot generally reveals branching. In addition, the slope of the higher molar mass portion of the Mark-Houwink plot of 0.48 suggests significant branching.

SEC-MALS-VIS is an excellent method for the characterization of PLGA polyesters as it has the ability to determine not only the molar mass distribution, but to reveal subtle differences in PLGA's molecular structure. ■

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FORMIC ACID–AMMONIUM FORMATE BUFFER SYSTEM FOR LC/MS ANALYSIS OF VITAMIN D

Stephen C. Roemer, Subhra Bhattacharya, and Deva Puranam Fisher Chemical – Thermo Fisher Scientific

Abstract

Acidic mobile phase solutions consisting of 0.05% formic acid and 10 mM ammonium formate in methanol and water were developed for analysis of vitamin D using LC/MS. Proper retention/elution of 25-OH vitamin D_2 through reverse phase columns and trace level MS detection was achieved with these solutions due to their optimal ionic strength, low pH, and interference-free baseline.

Introduction

A challenge for clinical research laboratories has been the variability within different methodologies for accurate measurement of vitamin D in biological fluids, although LC/MS is positioned best to standardize the process by providing high specificity and sensitivity via suitable chromatography and MS detection (1). In the present work, an acidic buffer system for mobile phase solutions was evaluated for trace level analysis of 25-OH vitamin D_2 using ESI-LC/MS.

Methods

Stock solutions of 25-OH vitamin D_2 (NIST, Cat. # SRM 2972) were obtained in ethanol and stored at -20°C. Fisher Chemical mobile phases had the following formulations: 10 mM ammonium formate and 0.05% formic acid in water (MB123) and in methanol (MB122). An Agilent 1100 series LC equipped with the Model SL single quadrupole mass spectrometry detector (MSD) was used for the vitamin D assay. Zorbax SB-C8 column (2.1 mm x 150 mm, 3.5 micron pore size) was selected. The sample elution was performed with column temperature of 50°C at a flow rate of 0.25 ml/min for 30 min using the gradient profile in Table 1. ESI-MS data was generated in positive mode with capillary voltage 3500V and fragmentation voltage 70V. Approximately two nanogram of 25-OH D_2 standard was loaded on column per injection.

Time (min)	% MB123	% MB122
2	60	40
10	15	85
12	15	85
20	0	100
30	0	100

Table 1
LC gradient for vitamin D_2 assay.

Results and Discussion

The selected ion monitoring (SIM) chromatogram of 25-OH vitamin D_2 and the corresponding mass spectra (Figs. 1, 2) show that the mass baseline (noise level) contributed by the acidic (pH 3.5) mobile phases is very low in positive mode of MSD. Also, no tailing of m/z 395 peak was observed using this method. An earlier LC/MS study indicated that a formic acid–ammonium formate buffer system contributed to repression of peak tailing of amines resulting in improved signal intensity (2). Ongoing research shows this buffer system to be suitable for other compounds such as various hormones and immunosuppressive drugs which require acidic conditions for enhancing LC/MS detection.

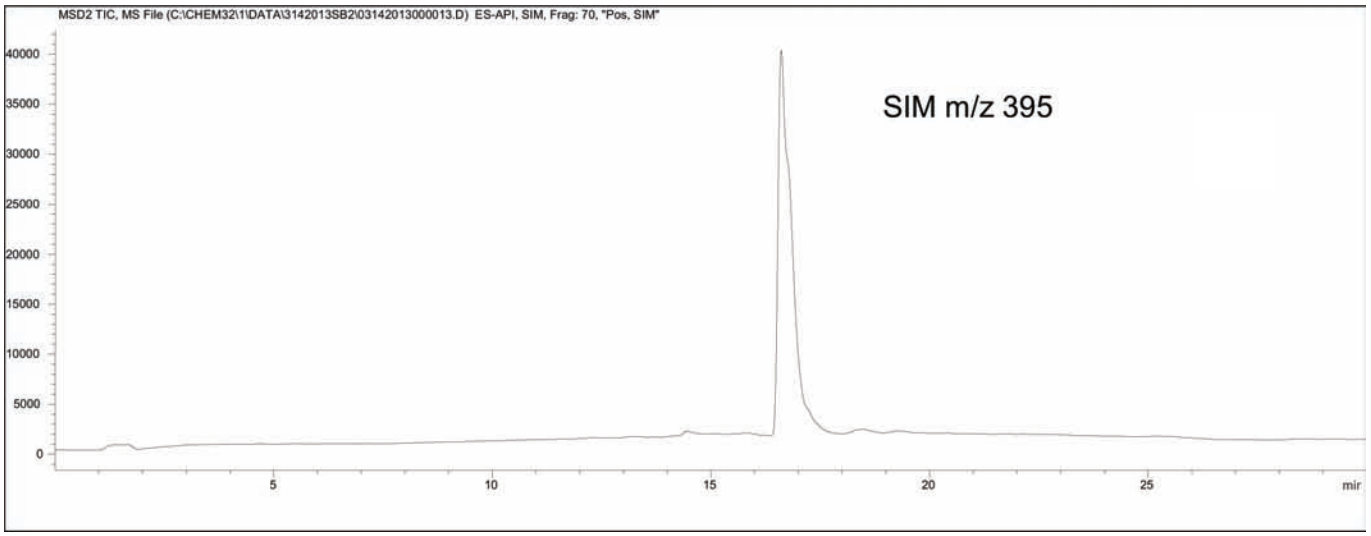

Figure 1
SIM of m/z 395 (25-OH vitamin D_2, 2.29 ng on column) using acidic mobile phases.

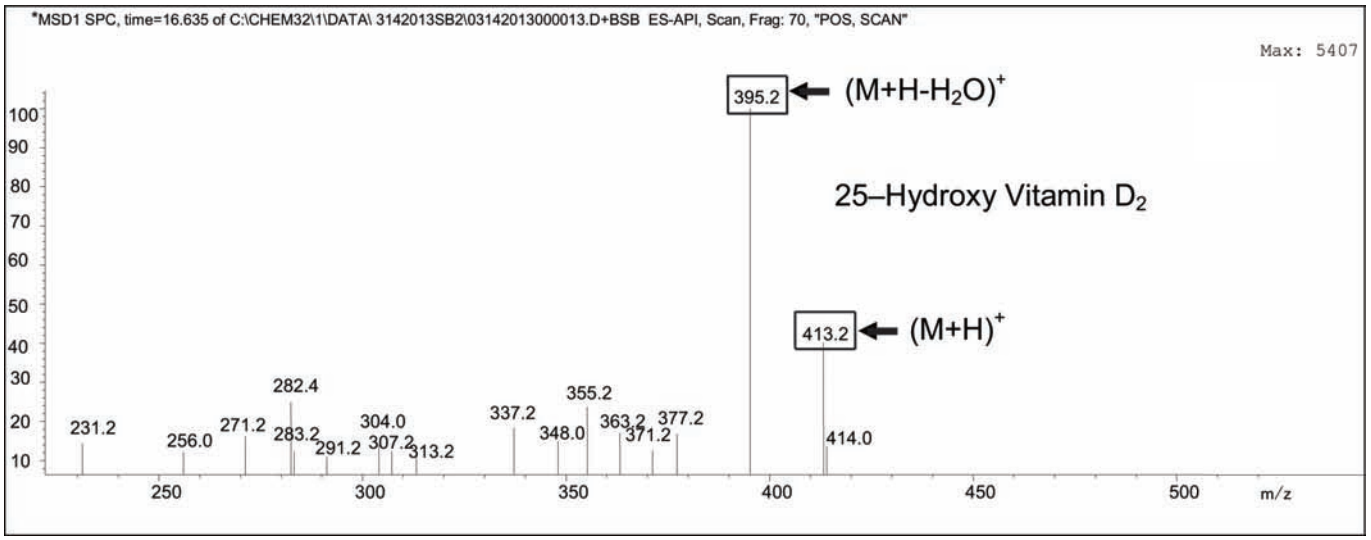

Figure 2
Corresponding mass spectra of the characteristic ions m/z 395 and m/z 413.

References

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KINETICS OF ENZYME-INHIBITOR ASSOCIATIONS WITH CG-MALS

Wyatt Technology

The kinetics of association or dissociation is of paramount importance in many biological systems. For example, antithrombin III forms a covalent bond with the catalytic serine of several proteases involved in the coagulation cascade, and the rate of this reaction can increase up to 1000x in the presence of certain macromolecules. Here, we determine the second-order rate constant for the covalent association of thrombin *a* (Thr) and antithrombin III (AT) by Composition Gradient Multi-Angle Light Scattering (CG-MALS).

Human thrombin *a* and antithrombin III (Haematologic Technologies, Inc.) were diluted to 60 μg/mL and 120 μg/mL, respectively, in phosphate buffered saline (PBS, pH 7.4) and filtered to 0.02 μm. Composition gradients were created using a Calypso II and delivered to an online UV/Vis concentration detector and DAWN HELEOS. The method consisted of six injections at constant AT concentration of 60 μg/mL and Thr concentrations from 0 to 30 μg/mL. After each injection into the UV and MALS detectors, the flow was stopped for 2000 s to allow the reaction to come to completion. Calypso software was used to run the method, acquire MALS and UV signals, and calculate the apparent weight-averaged molar mass as a function of time.

The light scattering data for all five measurements with nonzero thrombin concentrations were fit simultaneously to a model of 1:1 irreversible association between thrombin and antithrombin (Figure 2). Using this model, the second order rate constant was calculated as $k_{on} = 6.09x^{103}$ $M^{-1}s^{-1}$, similar to results measured by other methods (e.g., $k_a = 5.8x10^3$ $M^{-1}s^{-1}$ measured 25°C by fluorescence)[1]. The analysis simultaneously determined the fraction of thrombin capable of binding antithrombin. This calculated fraction, 77%, compared favorably with the manufacturer's reported specific activity of the antithrombin (0.79 mol Thr/mol AT).

Thus, CG-MALS provided a complete picture of the association between thrombin and antithrombin in solution, including identifying a fraction of thrombin incapable of binding its inhibitor. This technique

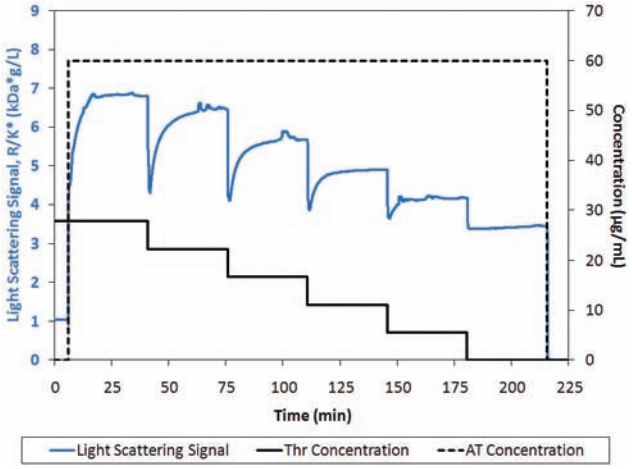

Figure 1

Light scattering and concentration data for thrombin titration at constant antithrombin concentration.

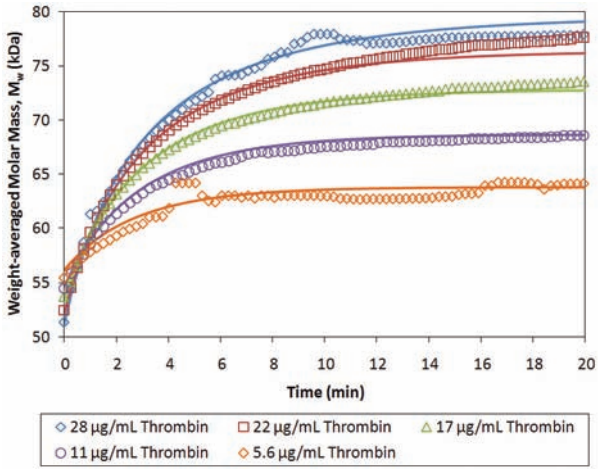

Figure 2

The increase in M_w as a function of time was fit to the appropriate association model to calculate the second order rate constant for the inhibition of Thr *by* AT, $k_{on} = 6.09x10^3$ $M^{-1}s^{-1}$. *Raw data (open symbols) and best fit curves are shown for varying Thr concentrations and constant* AT *concentration of 60 µg/mL.*

enabled observations of slow, irreversible binding and measurement of rates of 1:1 association consistent with other techniques.

References

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DETERMINATION OF CAFFEINE IN ENERGY DRINKS USING THIN-LAYER CHROMATOGRAPHY AND COMPACT MASS SPECTROMETRY (TLC/CMS)

Katerina Matheis and Hans Griesinger (Merck – Central Analytics); Susanne Minarik and Michael Schulz (Merck Millipore – Lab Essentials) Advion

Introduction

The analysis of caffeine is performed using the elution based TLC – MS Interface from CAMAG coupled with the expression compact mass spectrometer (CMS) from Advion. Because of the high matrix tolerance of TLC, no sample preparation is needed. The energy drinks are applied directly on the Merck HPTLC Si60 F254 MS-grade plate.

Methods/Experimental

Mass spectrometer:	Advion expression CMS
Interface:	CAMAG TLC-MS
Plate:	Merck HPTLC Silica gel 60 F_{254} MS-grade, 20x10 cm
Application volume:	0.5 - 3 µl
Detection:	UV 254 nm Staining with anisaldehyde acid sulphuric-acid-reagent ESI (+) mode MS (*m/z* 100-500)
Migration distance:	5 cm
Migration time:	50 min
Mobile phase:	2-Propanol/n-heptane/water 7:3:1
Extraction solvent:	Acetonitrile/water 95:5 + 0.1% formic acid
Extraction flow:	0.1 ml/min
Sample preparation:	**No sample preparation** direct application of energy drinks
Sample application:	Using the ATS4 sample applicator (CAMAG) 6 mm bandwise

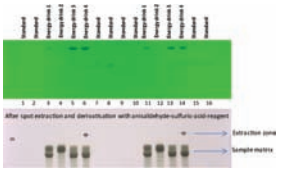

Figure 1a

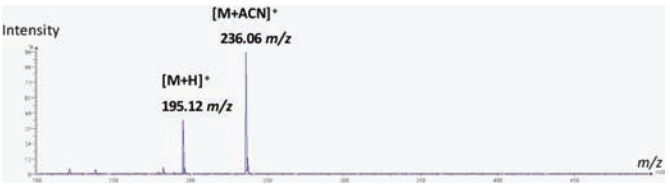

Figure 1b

Discussion

TLC is a widespread, routine, reliable tool used in nearly every chemist's laboratory. Coupling the CAMAG TLC-MS interface with Advion's expression CMS brings the specificity and sensitivity of mass spectrometry to TLC plate analysis. Now chemists can quickly, simply and unambiguously identify and quantify their compounds, even in complex mixtures, using TLC/CMS.

- Reduced sample preparation requirements
- Rapid, 30 s, mass spec. analysis
- Many other applications including mass confirmation of flash fractions and real time reaction monitoring ■

Additional information: http://www.advion.com/applications/expression-applications/tlc/

Chromatographic Data

Track	Compound	Concentration	Application Volume	hRf	Detected Mass ([M+H]+/[M+CAN]+)
1, 9	Caffeine Standard	0.10 mg/ml	0.5 µl	55	
2, 10	Caffeine Standard	0.10 mg/ml	1.0 µl	55	
3, 11	Energy Drink 1	0.17 mg/ml	0.5 µl	55	195.1/236.1 m/z
4, 12	Energy Drink 2	0.13 mg/ml	0.5 µl	56	195.1/236.1 m/z
5, 13	Energy Drink 3	0.41 mg/ml	0.5 µl	55	195.1/236.1 m/z
6, 14	Energy Drink 4	0.40 mg/ml	0.5 µl	56	
7, 15	Caffeine Standard	0.10 mg/ml	2.0 µl	55	
8, 16	Caffeine Standard	0.10 mg/ml	3.0 µl	55	

GC/MS/MS ANALYSIS OF ORGANOPHOSPHORUS PESTICIDES IN BABY FOODS

Richard R. Whitney, PhD, and Laura Chambers Shimadzu Scientific Instruments, Inc.

Introduction

Contamination of food products with pesticides is a growing global concern, particularly for baby foods. GC/MS/MS operated in the Multiple Reaction Monitoring (MRM) mode is a technique of choice for analysis of trace contaminants in complex matrices because of its high selectivity.

Experimental

Samples were prepared using the QuEChERS (Quick Easy Cheap Effective Rugged and Safe) sample preparation method[1]. Analyses were conducted using a Shimadzu GCMS-TQ8030 GC/MS/MS operated in the MRM mode, using conditions detailed in Shimadzu Application News GCMS-1304[2]. A 7-point calibration curve was conducted for 24 OP-pesticides using the matrix-matched internal standard procedure. A sample of organic blended peas was used to minimize contribution of analytes from the sample matrix.

Results and Discussion GCMSMS Operation in the MRM Mode

The reaction was started and heated according to the programmed method. Around 2:20 total time (m:ss; 150 °C) the reaction began generating pressure due to reflux, while within 30-40 seconds (~175 °C) a more rapid pressure increase resulting from nitrogen evolution was noted (Figure 1). The ActiVent pressure device relieved the internal vessel pressure four times, venting only gas (25-50 psi per vent) while maintaining original reaction volume despite a reaction temperature above reflux for the solution. Additionally, after each vent the reaction vessel resealed and maintained pressure as if the cap had never opened. After six minutes total reaction time, rapid pressure evolution is seen to cease and a constant pressure is maintained until the reaction end.

Calibration Results and Assessment of Precision

Seven calibration standards were prepared in the blended peas extract over the range of 0.5-200 ng/mL (ppb). Response factors were calculated and relative standard deviation (RSD) determined by the GCMS-solution software. The precision of the calibration was evaluated using the RSD of the response factors and the correlation coefficient (r) for each of the analytes. In general, the % RSD was < 25% and correlation coefficient values for the multi-point calibration were >0.999.

Eight replicate injections of the 1 ng/mL and 10 ng/mL standards were analyzed to assess the precision of the analytical method and the accuracy of mea-

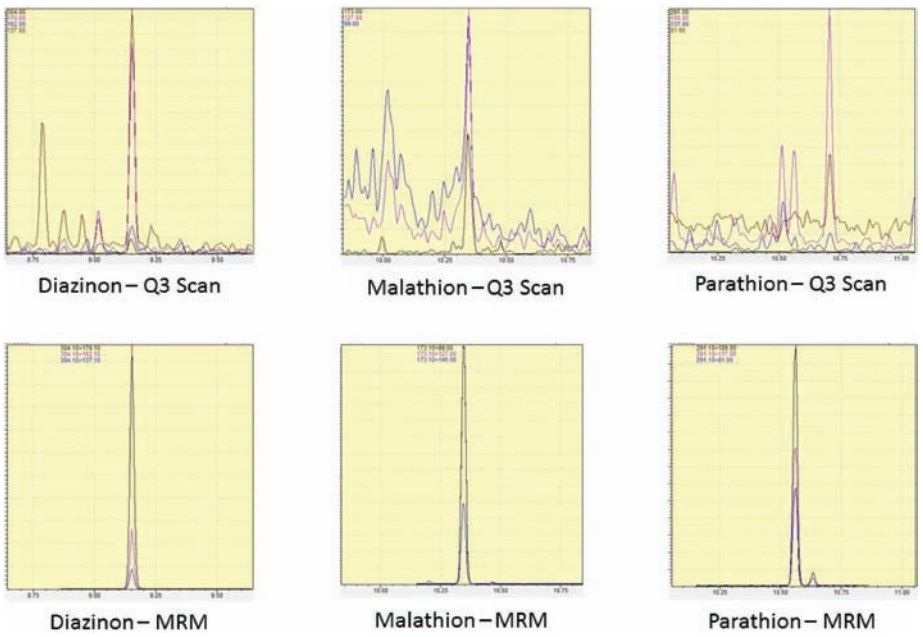

Figure 1
Extracted Ion and MRM Chromatograms for Selected Pesticides.

surement near the low end of the calibration range. The mean concentration and % RSD for the 1 ng/mL replicate analyses were 0.75-1.25 ng/mL and < 20%; mean concentration and % RSD for the 10 ng/mL replicate analyses were 8.0-11.0 ng/mL and < 10%.

Conclusion

Detection of the organophosphorus pesticides was demonstrated at low ng/mL (ppb) levels in baby food extract; linear calibration was demonstrated from 0.5-200 ng/mL. Precision and accuracy were demonstrated by replicate analyses of matrix spiked aliquots at 1 and 10 ng/mL. Calibration was conducted in the blended peas QuEChERS extract, and provided accurate, repeatable results for the sample matrix. A Shimadzu GCMS-TQ8030 system operated in the MRM mode was shown to be a rapid, sensitive, and selective technique for analysis of organophosphorus pesticides in baby foods.

References

1. AOAC Official Method 2007.01, Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate (2007).
2. Shimadzu Application News GCMS-1304. ■

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MORE EFFICIENT EXTRACTION OF MONOCROTOPHOS, DIAZINON, MALATHION, EPN, AND METHAMIDAPHOS FROM AQUEOUS SAMPLES USING SOLID PHASE EXTRACTION (SPE)

Jim Fenster and Julie McGettrick Horizon Technology, Inc.

Monocrotophos, Diazinon, Malathion, EPN, and Methamidaphos are commonly used pesticides for the control of insects and aquatic pests in rice production, other agricultural production, and fish aquaculture in parts of the world. Methamidaphos in particular is used in great quantities in rice fields in China where rice–fish culture is common as well as in many other rice-producing countries (e.g., Thailand, Malaysia, and the Philippines). Given their prevalent use throughout Asia, residues of Monocrotophos, Diazinon, Malathion, EPN, and Methamidaphos show up in many food sources and are commonly monitored in wastewater and drinking water in these regions. Methamidaphos and Monocrotophos have been either restricted or withdrawn from use in many countries, due to dangerous toxic side effects, but may be detected in any part of the world because of the global nature of food. As a result, many analytical methodologies have been created to monitor these compounds in food and the environment.

Traditional extraction methods employed use solid phase extraction (SPE) for Monocrotophos, Diazinon, Malathion, EPN and a separate liquid/liquid extraction (LLE) method for Methamidaphos. Methamidaphos is problematic to extract due to its extreme hydrophilic nature resulting in extremely low recoveries of this compound. In the specific LLE method it is necessary to add a quantity of salt (NaCl) in order to decrease Methamidaphos' affinity for water, making it partition more easily into the organic phase. This salting out technique has been employed for many years when trying to extract extremely hydrophilic polar molecules from aqueous matrices. Extraction of all five of these compounds takes time as two separate extraction methodologies must be used.

This application note was developed to demonstrate the extraction of five organophosphate compounds Monocrotophos, Diazinon, Malathion, EPN, and Methamidaphos using one solid phase extraction method with one pre-treatment step of sodium chloride.

Experimental and Results

Solid phase extraction disks (Atlantic® HLB-H, Horizon Technology, Salem, NH) and carbon cartridges (8270 Carbon Cartridge 20 CC, Kit, Horizon Technology) were used to extract the pesticides of interest from larger volumes of water (1 L), using a one-pass approach, automated using the SPE-DEX® 4790 extraction system (Horizon Technology).

Experiments were used to develop the optimized method approach and understand which adsorbent was best for each of the pesticides and how Methamidaphos was best extracted. The results using the optimized method for 4 replicates with a 10 μg/L spike are shown in Table 1. The additional step of using LLE on residual water from extract drying improved the recovery of Methamidaphos.

Recovery Data

	Total Recovery HLB Disk + Carbon Cartridge + LLE of Residual Water					
	% Rec	% Rec	% Rec	% Rec	Std Dev	% RSD
Compound						
Methamidaphos	101	77	93	90	12.45	13.83
Monocrotophos	96	104	109	103	6.72	6.50
Diazinon	83	82	85	83	1.67	2.00
Malathion	94	96	101	97	3.52	3.65
EPN	91	93	97	94	3.12	3.33

Table 1

Total Recovery Data HLB Disk, Carbon Cartridge and LLE of Residual Water.

Conclusions

A single-pass solid phase extraction method has been developed and evaluated for five organophosphate pesticides. The addition of salt and use of a carbon cartridge help to retain more polar analytes. Spike recoveries and precision for replicate preparations show excellent results. Especially hydrophilic analytes, such as Methamidaphos, can show improved recovery if the residual water after drying is re-extracted. The method development demonstrates a semi-automated procedure to speed sample preparation with excellent precision. The concepts developed in this method can be used to improve the recovery of other hydrophilic analytes.

References

1. Determination of Monocrotophos, Diazinon, Malathion, EPN, and Methamidaphos from Aqueous Samples Using Atlantic HLB SPE Disks, Jim Fenster, Julie McGettrick, Horizon Technology, Inc., Salem, NH, Application Note AN078-20120906. ■

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RAPID SEPARATION OF CATECHINS IN TEA USING CORE-SHELL COLUMNS

Pranathi P. Perati, Brian M. DeBorba, and Jeff Rohrer Thermo Fisher Scientific

Abstract

The work shown here evaluates the use of core-shell columns to determine catechins in different varieties of tea.

Introduction

Catechins are flavonoid phytochemical compounds found primarily in green tea and—in smaller amounts—in grapes, black tea, chocolate, and wine. Catechins are considered potent antioxidants that provide protection against certain diseases, such as cardiovascular disease and cancer. In North America, the consumption of green tea products has increased due to the reported health benefits associated with it. Next to water, tea is the most widely consumed beverage in the world, and can be found in almost 80% of all U.S. households, according to the Tea Association of the USA.[1] However, commercially available teas show a high variability in catechin content; therefore, simple and rapid methods are needed to evaluate product quality.[2]

This study evaluates a Themo Scientific™ Accucore™ C18 High-Performance LC (HPLC) column to rapidly (<6 min) determine catechins in three different types of tea. Core-shell particles improve mass transfer kinetics, and therefore separation efficiency, by restricting intraparticle diffusion to the thin, porous shell while maintaining the hydraulic permeability associated with the total particle diameter.[3-6] This work demonstrates how core-shell columns can be used to increase separation efficiency with improved mass transfer kinetics without significantly increasing pressure.

Methods and Experimental Conditions

Equipment

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system, including:

- SRD-3600 Integrated Solvent and Degasser Rack
- EO Eluent Organizer including pressure regulator and 2 L glass bottles for each pump, eluents maintained under helium or nitrogen headspace (5–8 psi)
- HPG-3400RS Pump with Solvent Selector Valves
- WPS-3000TRS Well Plate Sampler, Thermostatted
- Sample Loop, 25 µL
- TCC-3000RS Thermostatted Column Compartment
- DAD-3000RS Diode Array Detector
- Semi-Micro Flow Cell for DAD-3000 and MWD-3000 Series, SST, 2.5 µL Volume, 7 mm Path Length.

Sample Preparation

Prepare each of the tea samples by weighing 60 mg of solid and adding 7 mL of 0.05% formic acid in 70% methanol. Vortex the mixture, sonicate for 90 min, and centrifuge at 5000 RPM for 10 min. Collect the supernatant in a glass vial and add an additional 7 mL of solvent to the pellet. Vortex the mixture again to mix, sonicate for 90 min, and centrifuge at 5000 RPM for 10 min. Add the supernatant to the first 7 mL to make a total volume of 14 mL. Filter the samples using 0.2 µm cellulose acetate sterile syringe filters and dilute 1:5 with 0.05% formic acid in 70% methanol prior to analysis.

Results and Discussion

Figure 1 shows the standard with the predominant catechins in tea. In addition, free gallic acid and caffeine are naturally present in tea, and therefore were included in the mixed standard. The retention times are 0.61 min for gallic acid, 1.2 min for gallocatechin, 2.6 min for epigallocatechin (EGC), 2.7 min for catechin, 2.9 min for caffeine, 3.2 min for epicatechin (EC), 3.3 min for epigallocatechin gallate (EGCG), 3.5 min for gallocatechin gallate, and 5.0 min for epicatechin gallate, with all analytes at a concentration of 100 µg/mL with

the exception of gallic acid at 50 μg/mL and caffeine at 35 μg/mL.

All catechins with the exception of EC and EGCG were baseline resolved in <6 min.

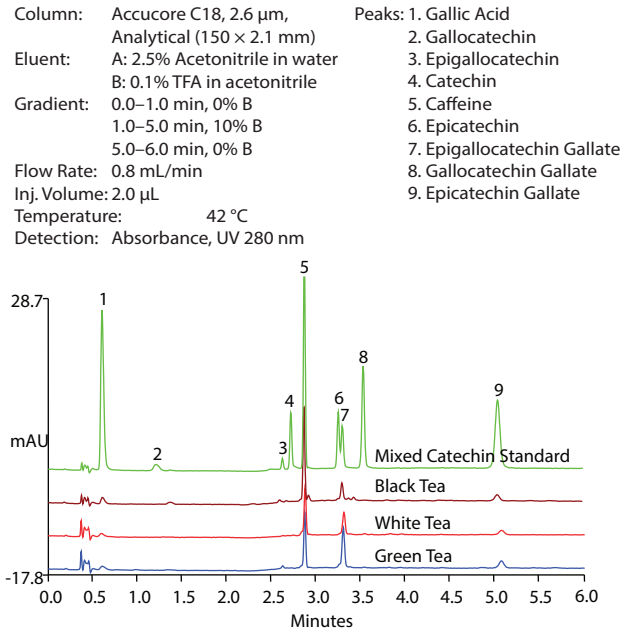

Figure 1

Separation of catechins in a mixed standard and three different commercially available teas using an Accucore C18 HPLC column.

Discussion

The samples investigated in this study included green, white, and black teas. White tea is minimally processed and is expected to be very high in catechin content. However, green tea showed the highest catechin content, suggesting that the quality of the white tea was poor and the tea was possibly adulterated with more processed varieties of teas.

This work describes a simple and rapid method to determine catechins in different commercially available teas with a simple solvent extraction. The method uses an Accucore C18 HPLC column and absorbance detection at a wavelength of 280 nm to separate and detect catechins in <6 min. The method described in this study is ideal for routine and rapid screening of catechins in different tea products.

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SAVE THE FLAVOR: ROBUST ISO-α-ACIDS ASSAYING IN BEER WITHIN TEN MINUTES

Michael Heidorn Thermo Fisher Scientific

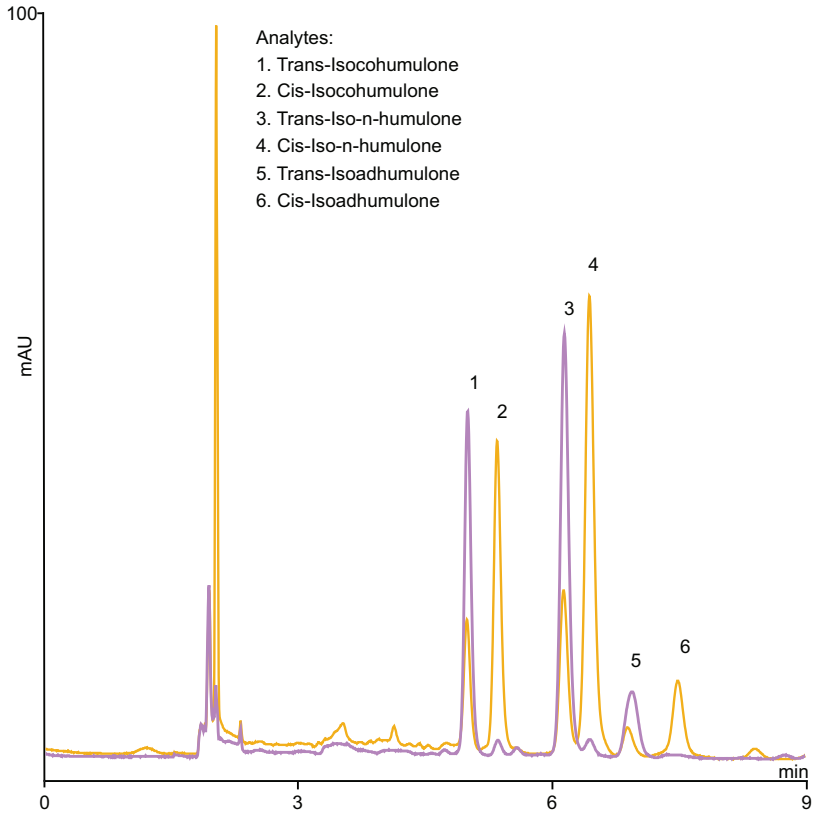
Abstract

Determination of the beer bitterness by measuring the contents of isohumulones in untreated beer—as fast as possible, including sample preparation, HPLC separation, and result evaluation. Furthermore, the application has to be robust and must provide reproducible results, increased lifetime of the analytical column, and provide a fingerprint or characteristic pattern of the cis- and trans-ratios.

Introduction

Isohumulones (iso-α-acids) are derived by humulones (α-acids), essential constituents of hop resins. The poorly water-soluble α-acids are isomerized to the better water-soluble iso-α-acids during wort-boiling. Iso-α-acids form approximately eighty percent of the typical bitterness of beer. Their antimicrobial effect leads to a sterile beverage, their tensioactive character stabilizes the foam, and they have a major influence on the general flavor, smell, and smoothness of beer.[1] The three major iso-α-acid variants which are basically present in beer only differ in their acyl side chain and comprise iso-n-humulone, isocohumulone, and isoadhumulone. Due to the stereochemistry of iso-α-acids, all of them occur as cis- and trans-isomer.

Each iso-α-acid variant provides different contributions to beer taste and foam stability. Recent investigations have shown that these differences are even true between both cis- and trans-isomers of the same iso-α-acid.[1] Furthermore, the lifetimes of cis- and trans-isomers significantly differ from each other. Degradation products of iso-α-acids sensitively influence the impor-



System:	Thermo Scientific™ Dionex™ UltiMate™ 3000 System with On-Line SPE RS Configuration
Mobile Phase:	A – water with 1% formic acid and 100 mg/L ethylenediaminetetraacetic acid disodium salt dihydrate B – acetonitrile
Pressure:	720 bar (max.)
Temperature:	35ºc
Injection:	15 µL beer or 5 µL isohumulone standard
Analytical Flow Path Parameters	
Column:	Thermo Scientific™ Hypersil GOLD™ column, 1.9 µm, 100 × 2.1 mm
Isocratic:	50% B
Flow rate:	650 µL/min
Detection:	Thermo Scientific Dionex UltiMate VWD-3400RS Variable Wavelenght Detector , 2.5µL flow cell, 270 nm
Automated On-Line SPE Parameters	
Column:	Hypersil Gold C8 column, 5 µm, 20 × 2.1 mm
Gradient:	0-2 min 25% B at 2000 µL/min, 2-4 min 100% B at 2000 µL/min, 4-7 min 25% B at 200 µL/min, 7-9 min 25% B at 2000 µL/min
Valve Position:	0 min 6_1, 1.5 min 1_2, 2 min 6_1

Figure 1
Chromatogram of Isohumulones in Beer and Isohumulones Standard (Overlay).

tant beer attributes mentioned above and the avoidance of less stable iso-α-acid variants is beneficial.[2]

Precise as well as comparable information about the genuine beer bitterness is only achievable by specific quantitation of bitter substances (isohumulones) in beer. Furthermore, the fingerprint or characteristic pattern of the cis- and trans-ratios is very important information due to the reasons mentioned above. High-Performance Liquid Chromatography (HPLC) is the only analytical method that provides these results. If injected untreated, beer causes reproducibility issues and compromises its lifetime of the column as beer does not only consist of isohumulones but also of a very complex matrix. Manual beer sample pretreatment steps, like off-line solid phase extraction (SPE) are commonly used but are very time consuming. Furthermore, traditional HPLC analyses last about half an hour.

Experiment

Equipment: System package with on-line SPE RS configuration (P/N 5200.0500) and isohumulones starter kit for on-line SPE RS system (P/N TS-MKIT0012).

Samples: Beer (German Pilsener, purchased from a local grocery store) and certified isohumulones standard DCHA-Iso, ICS-I3 (part of P/N TS-MKIT0012).

Conditions: Experimental data: listed in Figure 1.

Results and Discussion

This application provides an instant result about the content of isohumulones in untreated beer within less than ten minutes, including sample preparation, HPLC separation, and result evaluation. Sample preparation runs automatically and is not prone to manual errors resulting in highest reproducibility. Since no manual, time-consuming labor is needed, potential health risks are reduced. Samples can be run unattended, for instance, overnight or over the weekend which results in increased workload per system and, therefore, higher returns on investment. Furthermore, the HPLC separation is very robust and provides very reproducible results next to an increased lifetime of the analytical column. Being the most important benefit, the specific fingerprint or characteristic pattern of the cis- and trans-ratios is fully explored at each time for each beer.

Conclusion

The application shown here provides specific determination and quantitation of each cis- and trans-isomer of the isohumulones (iso-α-acids) within a single run. Since isocratic conditions are applied, the HPLC system is always in steady state and the analytical run is finished within ten minutes – from sampling to result. By using online SPE, an untreated beer sample is injected directly, all SPE-steps are performed automatically, and the entire analysis lasts only nine minutes. Thereby, the application represents perfect and easy-to-use beer quality monitoring. The UltiMate 3000 RS System Package with On-Line SPE in combination with the Isohumulones Starter Kit for On-Line SPE RS System provide all instrument hardware, software, and consumables needed to run this application. The certified isohumulones standard DCHA-Iso, ICS-I3 can be used for identification or calibration of the individual isohumulones.

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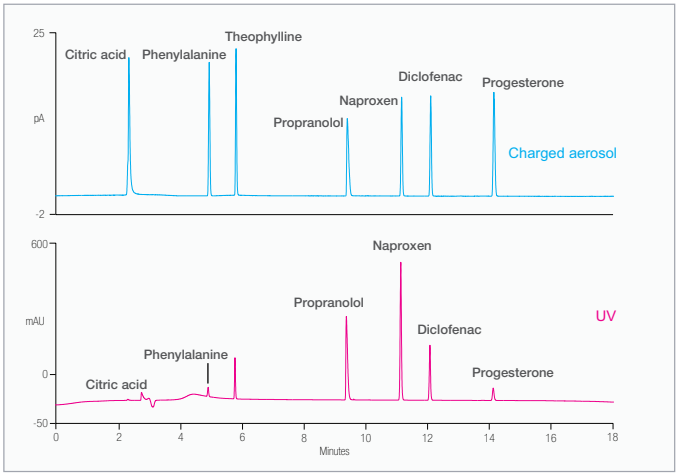

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ANALYSIS OF IMPURITIES IN MODEL BIODIESEL USING ULTRAPERFORMANCE CONVERGENCE CHROMATOGRAPHY (UPC^2)

Mehdi Ashraf-Khorassani and Larry T. Taylor (Department of Chemistry, Virginia Tech); Giorgis Isaac (Waters Corp.) Waters Corp.

Introduction

The development of new technologies that enable the production of fuels obtained from renewable resources is facilitated by both environmental concerns and a lack of fossil fuels. Biofuels, such as the biodiesel produced via trans-esterification of vegetable oils using either ethanol or methanol, are a promising alternative fuel source, particularly in countries with large territories and propitious weather for the desired agricultural activity, (*e.g.,* Brazil). Compared to diesel, biodiesel can reduce 78% of CO_2 emissions. Fatty acid alkyl esters (referred to here as commercial B100 biodiesel) that are used as automotive fuel for diesel engines should be free from lipid contaminants such as glycerol and acylglycerols.[1] Residues in biodiesel that are less than 1% are anticipated to be triacylglycerols, diacylglycerols, monoacylglycerols, and free glycerol.

The current technique for analysis of acylglycerols and glycerol involves either high-temperature GC or pre-derivatization, followed by conventional GC analysis. In this application note, the fast determination of residual glycerol and acylglycerols in model biodiesel prepared in-house using UltraPerformance Convergence Chromatography™ (UPC$^{2®}$) is described. Combining the use of supercritical CO_2 with sub-2-μm particle columns, UPC^2 represents an analysis technique that is orthogonal to reversed-phase LC and can be used to solve many troublesome separations that challenge conventional LC or GC analyses. With UPC^2 no derivatization is required, resulting in easier and faster sample preparation and eliminating artifact formation. Unlike GC/MS, UPC^2 allows for the analysis of compounds that can experience thermal degradation due to their low volatility

Experimental

Sample description: Pure fatty acid ethyl esters (C_{16}, C_{18}, C_{18}:1, C_{18}:2, C_{18}:3) were purchased from Sigma-Aldrich (St. Louis, MO) and mixed to form a model biodiesel. Pure C_{18} mono-acylglycerol, di-acylglycerol, and tri-acylglycerol plus glycerol and soybean oil were also obtained from Sigma Aldrich.

Methods Conditions

Column:	ACQUITY UPC^2 HSS C18 SB (3.0 x 150 mm, 1.8 μm)
Sample prep:	5% sample in DCM/MeOH
ABPR:	1500 psi
Column temp.:	25 °C
Injection volume:	2-8 μL
Sample solvent:	DCM/MeOH (50:50)
Flow rate:	1-2 mL/min
Mobile phase A:	Compressed CO_2
Mobile phase B:	Acetonitrile/methanol (90:10)
Make up solvent:	IPA
Make up flow rate:	0.2 mL/min
Gradient:	98:2 to 80:20 in 18 minutes
Detectors:	ACQUITY UPC^2 PDA 210 nm, Ref. 400-500 nm ACQUITY UPC^2 ELS

Results And Discussion

A single ACQUITY UPC^2 HSS C_{18} SB Column (3.0 x 150 mm) packed with 1.8-μm particles was used to separate glycerol, soybean oil acylglycerols, and model biodiesel components. A gradient of CO_2 and acetonitrile/MeOH (90:10) served as the optimum mobile phase. First, the separation of model biodiesel, triacylglycerols, diacylglycerols, monoacylglycerols, and free glycerol was obtained (Figure 1). Baseline separation of all compounds was observed, which is important since most biodiesels have minor components that elute early and can interfere with proper quantitation of residual acylglycerols in the sample.

Reproducibility of the separation was excellent for all analytes. RSD for all peak areas was between 1% and 4%, and RSD for retention time of all peaks was less than 0.1%. Next, chromatograms were produced by single injection of the three classes of components. Peaks from each class of compounds were resolved and no interferences were observed. A mixture prepared by spiking the model biodiesel with a known weight of soybean oil acylglycerols and glycerol was injected into the same column. Separation of triacylglycerols, diacylglycerols, monoacylglycerols, and free glycerol from model biodiesel at 5.0% to 0.2% was easily obtained. Glycerol was detected by ELS detection in model biodiesel with a S/N ratio of 10:1 at the 0.05% level with a 4-µL injection volume (2 µg mass on-column), and a S/N ratio of 40:1 for 4 µg injected on-column. A complete separation of all analytes was obtained in less than 15 minutes.

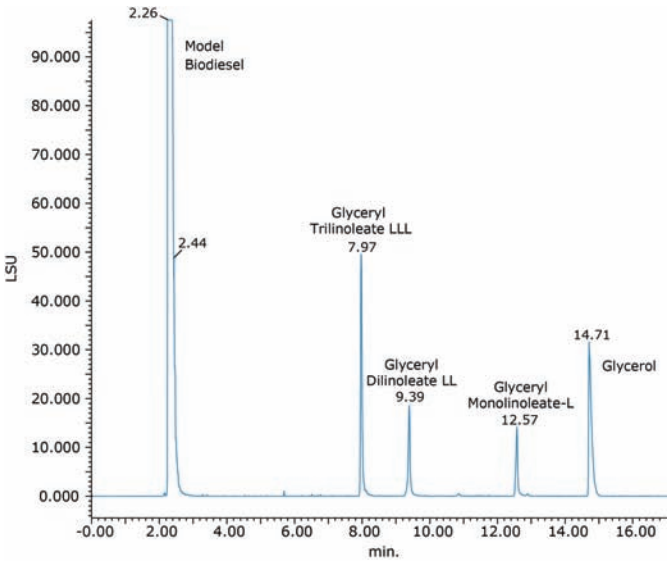

Figure 1
UPC² separation of model biodiesel spiked with glycerol and glyceryl mono-, di-, tri- linoleate. Detection performed by ELS.

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CHARACTERIZATION OF TiO_2, NANOPARTICLE DISPERSION IN CELL CULTURE MEDIA USING HIGH-THROUGHPUT DYNAMIC LIGHT SCATTERING

Zhaoxia Ji (University of California (UC) Center for Environmental Implications of Nanotechnology); Jeffrey I. Zin (UC Center for Environmental Implications of Nanotechnology and Department of Chemistry and UCLA Department of Chemistry and Biochemistry) Wyatt Technology

Characterization of nanoparticle size and distribution biological environments and understanding the parameters that affect them are imperative to accurately nanoparticle toxicity. In the case of *in vitro* studies, nanoparticles must be well-dispersed to ensure uniform dosing. Similarly, for *in vivo* studies, delivering nanoparticles in a well-dispersed form is necessary for accurate assessment of their toxicity. Based on these considerations, the goal of this investigation is to accurately evaluate the TiO_2 nanoparticle dispersion in different cell culture media using the high throughput Dynamic Light Scattering DynaProM Plate Reader. To improve the dispersion of these nanoparticles, dispersing agents such as bovine serum albumin (BSA) and fetal bovine serum (FBS) were explored.

The concentrations for TiO_2, BSA, and FBS were 50 μg/mL, 2 mg/mL, and 0.5-5 wt.%, respectively. All measurements were conducted in a 384-well plate at room temperature. To ensure reproducibility, samples were loaded in triplicate and five runs were collected for each well.

The spectral view (Figure 1) suggests BSA (2 mg/mL) as an effective dispersing agent in all media but LB and TSB contrast, 5 wt.% FBS (equivalent to 2 mg/mL BSA) resulted in highly dispersed TiO_2 in all media. No noticeable change in nanoparticle dispersion was observed with FBS concentration decreased to 1 wt.% (Figure 1), which implies FBS as an effective dispersing agent. In addition to the qualitative analysis, particle size distribution was also calculated using the

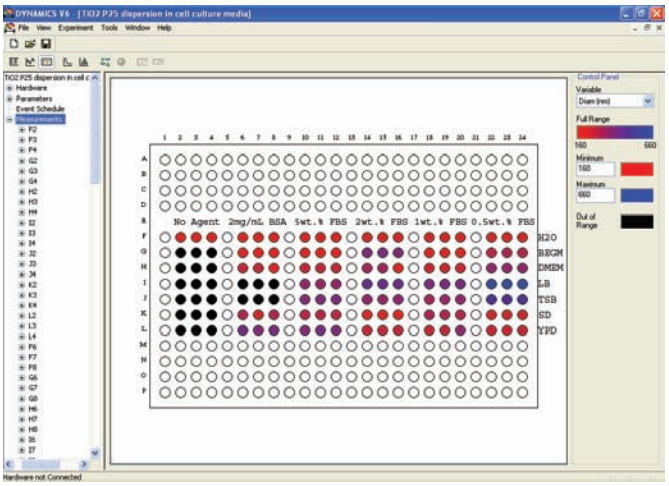

Figure 1
Spectral view of TiO_2, nanoparticle dispersion in water and six different cell culture media (BEGM, DMEM, LB, TSB, SD, and YPD) suggests severe agglomeration in all media without dispersing agents, improved dispersion in most media using 2 mg/mL BSA, and the best dispersion using ≥1 wt.% FBS.

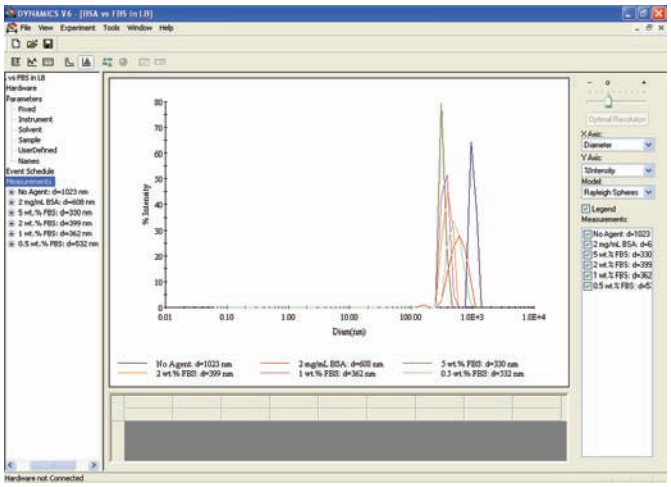

Figure 2
TiO_2 particle size distribution in LB calculated by the built-in regularization algorithm shows the effect of BSA and FBS dispersing agents. The corresponding hydrodynamic diameter (d) of TiO_2 was determined to be 1023 nm, 608 nm, 330 nm, 399 nm, 362 nm, and 532 nm for no dispersing agent, 2 mg/mL BSA, 5 wt.% FBS, 2 wt.% FBS, 1 wt.% FBS, and 0.5 wt.% FBS, respectively.

built-in regularization algorithm.

Figure 2 shows an example of TiO_2 particle size distribution in LB, which again confirmed the effectiveness of FBS.

0verall, this study demonstrated the use of the DynaProM as a convenient and effective tool for determining TiO_2 nanoparticle size and for evaluating the effects of different dispersing agents much faster than conventional "batch" DLS would have allowed. Currently, more effort is being made to understand the dispersion mechanisms. Kinetic/stability study of TiO_2 suspensions is also being performed. ■

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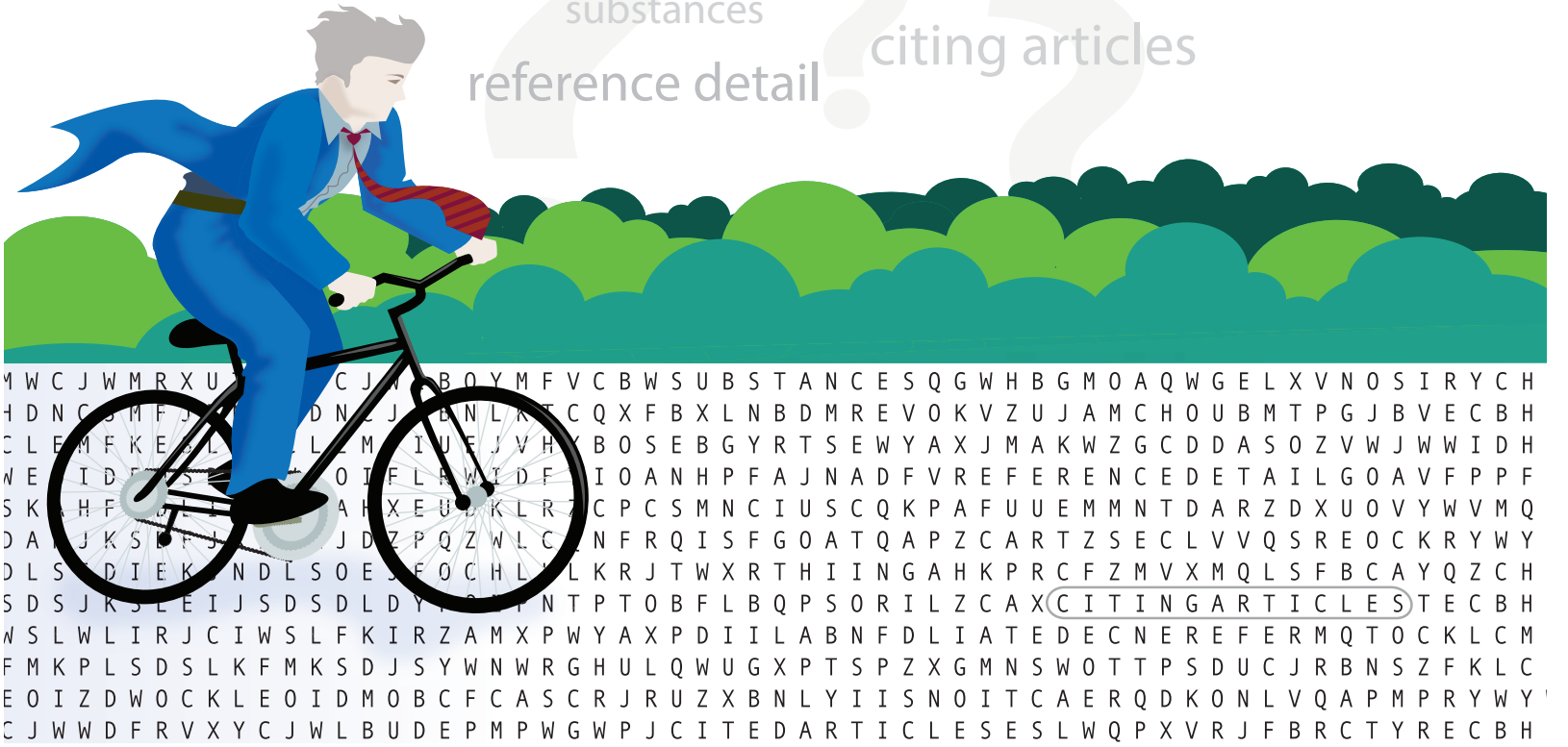

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DETERMINATION OF ORGANIC COMPOUNDS IN DRINKING WATER USING DIVINYLBENZENE (DVB) DISKS AND CARTRIDGES FOR USEPA METHOD 525.3

Robert Johnson and Brian LaBrecque Horizon Technology, Inc.

Since 1988, USEPA Method 525 has been the standard for extraction of a large suite of organic compounds with measurement using GC/MS. In June, 2012, the EPA published a method update rule allowing an additional ten drinking water methods for analysis.

One of the methods approved by this action is method 525.3 for the determination of semi-volatile organic compounds in finished drinking water. The method analytes are extracted and concentrated from the water using solid phase extraction. Extracts are injected onto a capillary GC column and analyzed using mass spectrometry. Method 525.3 is similar in many ways to its predecessor, method 525.2 (Rev 2.0 – 1995), however there are significant changes that make the newer 525.3 method vastly improved. Several of the major changes are as follows:

- The sorbent material has been changed from C18 to DVB (divinylbenzene). This yields better recoveries over a wider pH range.
- The preservation/dechlorination scheme has changed from HCl and sodium sulfite, to ascorbic acid, EDTA, and citric acid. This is safer for field sampling crews and allows bottles to be shipped with the preservatives pre-added.
- The internal standard is added to the final extract, not prior to the extraction as with 525.2.
- The use of SIM mode is an option for compounds that need additional sensitivity
- The surrogate perylene-d12 has been dropped.
- Pentachlorophenol-C13 is now used as an internal standard for pentachlorophenol.

Divinylbenzene sorbent can be used in either disk or cartridge form, and the extraction automated for additional reproducibility, with less attention. Studies were performed using the SPE-DEX® 4790 Extractor with Atlantic® DVB disks and the SmartPrep® Cartridge Extractor with DVB cartridges. Experimental details can be found in the full application notes listed in the references. Spike recoveries at two concentrations (2μg/L and 5μg/L) and precision (RSDs) are shown for the disk system in Table 1 for selected analytes. Recoveries and precision are also shown for two different flow rates for the cartridge system. In both cases, a full liter of sample was used, although it is expected that smaller samples will become more popular in the

Spike Recoveries

	Disk		Disk		Cartridge 10 mL/min		Cartridge 30 mL/min	
	2.0 μg/L Spike		5.0 μg/L Spike		5.0 μg/L Spike		5.0 μg/L Spike	
	% Rec	RSD	% Rec	RSD	% Rec	RSD	% Rec	RSD
Analyte								
Acenaphthylene	86.6	1.1	90.5	1.3	92.2	4.9	94.8	3.9
Chlordane, cis	89.2	4.9	89.7	2.5	96.5	4.4	99.9	4.3
Hexachlorobenzene	89.5	1.9	90.5	1.1	90.6	3.9	95.0	4.5
Simazine	94.2	1.8	98.7	2.9	97.2	6.7	101.0	4.1
2-chlorobiphenyl	87.2	1.8	90.0	0.5	93.9	2.1	94.3	3.7

Table 1

Spike Recoveries and Precision using USEPA Method 525.3 with DVB Disks and Cartridges for Selected Analytes.

US in the near future and is already more popular in Europe. In both cases, smaller samples can be used with the current operational set-up.

The data in Table 1 is well within the limits set by the method and was excellent for all compounds measured. The choice of disk or cartridge will depend on the laboratory sample load and variety. In either case, automation can provide additional precision and free the operator for other tasks.

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You can creatively blend normal or reversed phase solvents and even automatically incorporate additives in real time with our exclusive Nanolink interface and advanced chromatography capabilities.

Learn more about Isolera™ Dalton at
www.biotage.com/product-page/isolera-dalton

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