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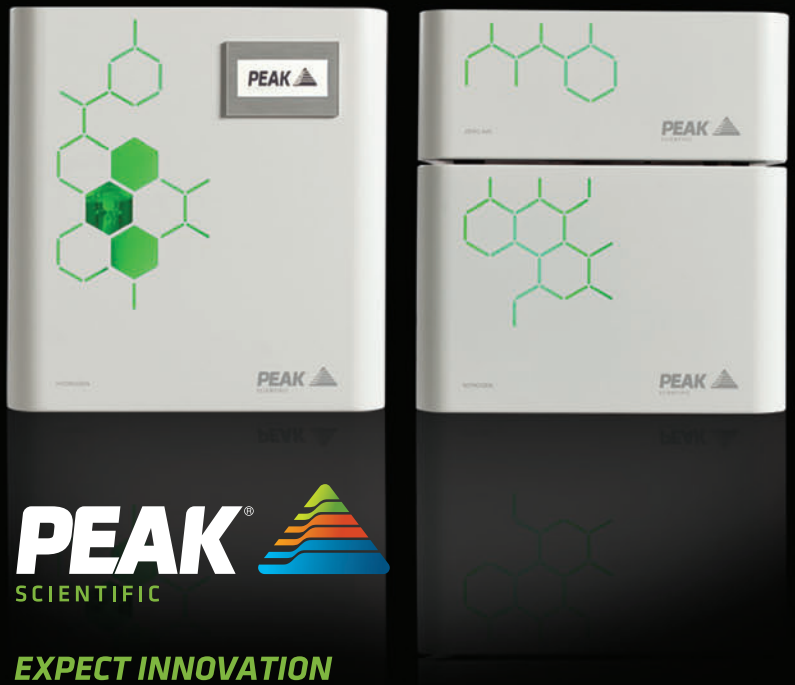

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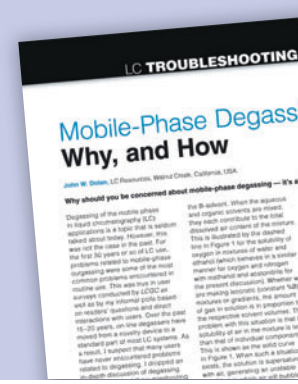

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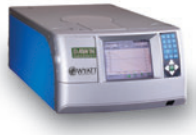

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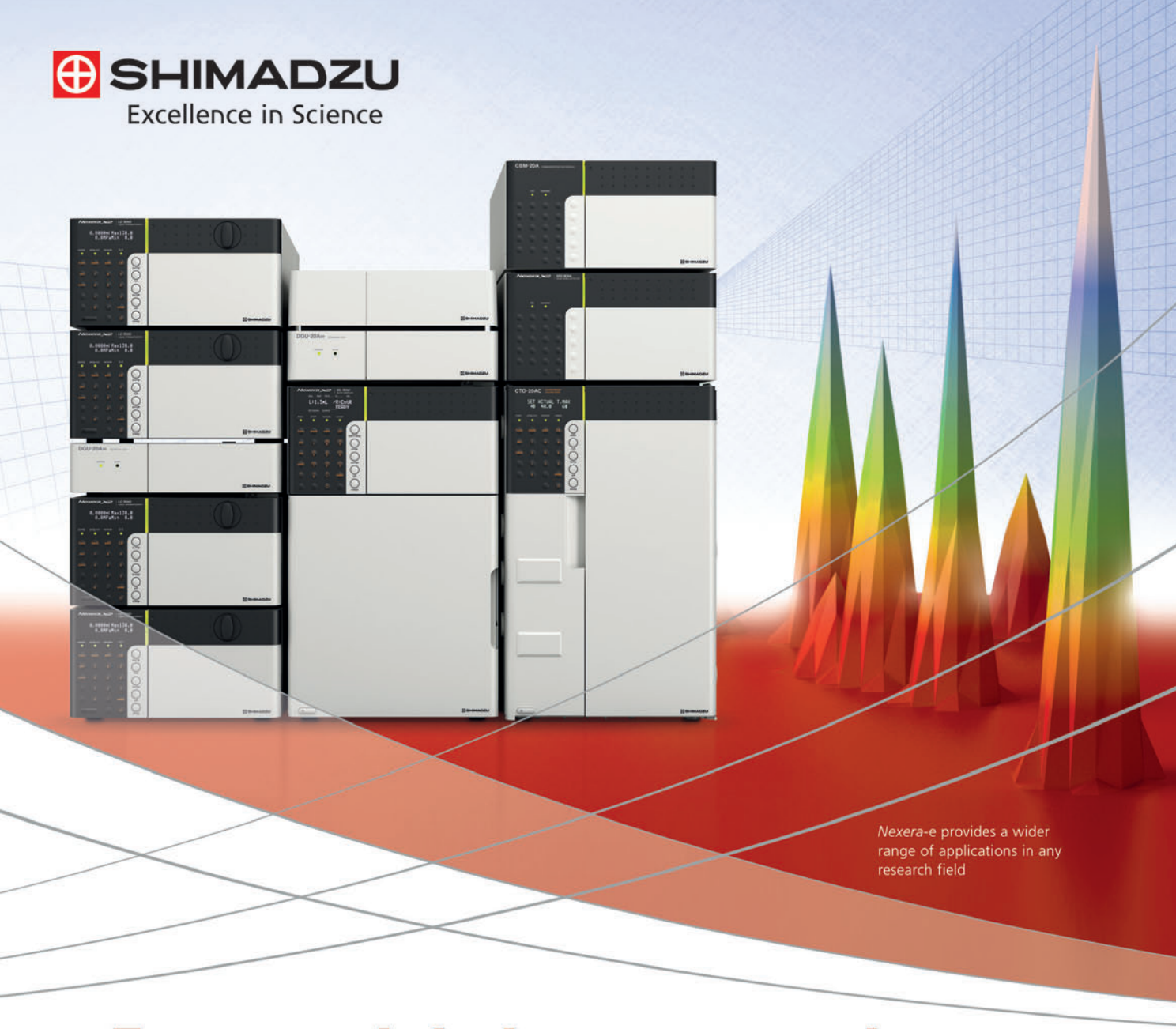

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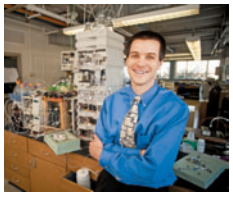
Is Two-Dimensional LC Worth the Effort?

Two-dimensional liquid chromatography (2D LC) is a powerful technique, but method development is very involved and time-consuming. So, for what types of analyses does it make sense to invest the extra effort to develop a 2D LC method instead of a 1D method? Dwight Stoll explains.



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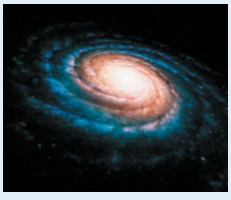
ASTROBIOLOGY INTERVIEW

A New Frontier: NanoLC–NanoESI–MS in Astrobiology

The Column interviewed Mike Callahan, an analytical chemist at NASA's Goddard Space Flight Center (Maryland, USA), who led a team to develop a new nano LC–nano ESI–MS method to detect life's building blocks in "spacedust". Read it in full here: **goo.gl/tIMz5L**



RESEARCH NEWS

Steroid Analysis Without Derivatization

A new method using high-temperature liquid chromatography with photodiode detection and isotope ratio mass spectrometry (HT–LC–PDA–IRMS) has been developed by scientists at the University of Duisberg-Essen in Essen, Germany.

Full story here: **goo.gl/hKXZsR**

LCGC BLOG

Basics, Applications, and Innovation in SPE

Solid-phase extraction (SPE) is a versatile and reliable technique that is often used for sample cleanup and concentration. Kevin Schug offers some insight into SPE basics and how to achieve successful extractions.

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Myths in Ultrahigh-Pressure Liquid Chromatography

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We are proud to announce that the 2014 Lifetime Achievement in Chromatography Award is granted to Fred E. Regnier and the 2014 Emerging Leader in Chromatography Award is presented to André de Villiers. Read their profiles here: **goo.gl/PLRjxf**

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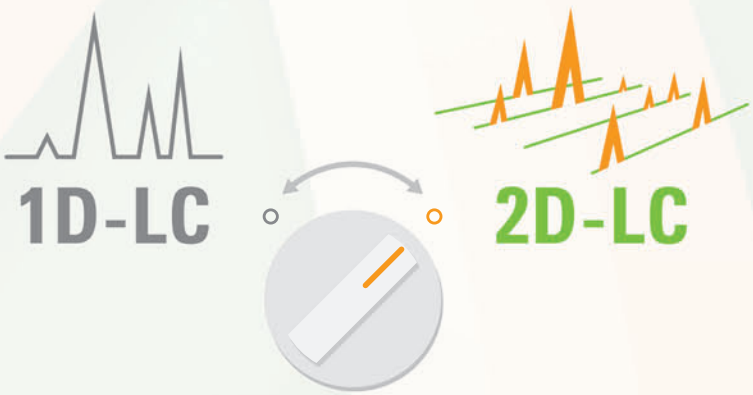

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Analysis of Four Phthalate Monoesters in Human Urine Using Liquid Chromatography Tandem Mass Spectrometry

Jianhua Cheng, Haijing Li, Shengming Wu, Yu Li, Kunpeng Ma, Junjian Fang, Rong Gao, Jiexin Liu, Xianzhong Yan, and Fangting Dong, National Center of Biomedical Analysis, Beijing, China.

A method was developed using high performance liquid chromatography with tandem mass spectrometry (HPLC–MS–MS) detection for the analysis of phthalate metabolites in urine samples. The urine samples were treated using solid-phase extraction columns. Multiple reaction monitoring models were used for quantitative detection with high sensitivity and selectivity. In this work, a fast, sensitive, and accurate quantitative method is provided to detect toxic phthalate esters in urine and assess environmental toxicants.

Phthalate esters are widely used as plasticizers in manufacturing. Phthalate esters are also used as raw materials of pesticide carriers, paint, adhesives, cosmetics, lubricants, and so on (1). Because phthalate esters are noncovalently bound to the plastic and maintain relatively independent chemical properties, they can leach out of these products and regularly release into the environment as time goes on (2,3). Globally, more than six million tonnes of phthalates are used each year (4). Presently, phthalate esters have been detected in many different areas in the ecological environment of the world's major industrial countries; they have become one of the most popular global pollutants. This has severely threatened the environment and human health, despite the fact that phthalate esters play an important role in the production and life of mankind (5).

Diet, skin, and inhalation are considered the major exposure routes for phthalate esters (6). When phthalate esters enter the human body they are rapidly metabolized and hydrolyzed to the corresponding phthalate monoesters before absorption, which are the biologically active molecules and recognized testicular toxicant (7,8), and then further oxidized into various metabolites (9). General metabolic pathways of phthalates in humans are shown in Figure 1 (10). Most phthalate esters and their metabolites are excreted in urine or faeces. The total concentrations of phthalate metabolites in urine are normally used as biomarkers of individual intake of phthalate esters (11). The monoesters contain a free reactive carboxylic acid that can conjugate with α-D-glucuronic acid to produce more hydrophilic compounds. Phthalate esters, which mimic oestrogen, have been reported to affect multiple biochemical processes as environmental hormones in humans and wildlife. It is one of the major reasons for male reproductive problems (12–14). Excessive exposure to phthalate esters can increase the risk of breast cancer for women. Many phthalate esters can damage the reproductive systems of offspring as well (15–18). For instance, studies showed that some toxicologically active metabolites, such as MEHP, MBPA, and MBzP, are capable of crossing rodent placenta, which causes adverse effects on the development of the male reproductive system and induces early embryonic death, respectively (19,20). Animal experiments have shown that the toxicities of some monoesters are greater than those of corresponding diesters (21).

Phthalate monoesters are considered to be valuable biomarkers for exposure to phthalate esters. Measuring phthalate monoesters investigates the contamination status, absorption, and metabolism of phthalate esters in the body better than phthalate diesters. Several developed methods have been used to quantify phthalate monoesters, including gas chromatography–mass spectrometry (GC–MS) (22,23). Calafat developed a sensitive method for measuring 13 phthalate metabolites in breast milk using isotope dilution high performance liquid chromatography (HPLC) with negative ion electrospray ionization tandem mass spectrometry (24). This method showed good reproducibility and accuracy. Liquid chromatography–tandem mass spectrometry (LC–MS–MS) methods with high selectivity and sensitivity show an advantage compared to GC–MS methods because of simple sample pretreatment without derivatization.

Some methods, such as on-line solid-phase extraction (SPE) coupled to HPLC–MS–MS, are considered the most advanced

KEY POINTS

- An LC–MS–MS method has been developed for the simultaneous analysis of five phthalate ester metabolites in human urine.
- The method has proven to be sensitive, accurate, and precise with a short run time of 12 min.
- The method can help to assess human exposure to phthalates or other environmental toxicants in environmental toxicology studies.

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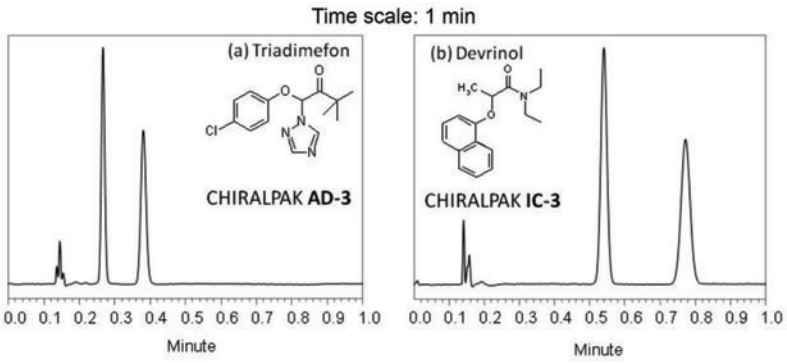

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Figure 1: General metabolic pathways of phthalates in humans. R = H; phthalic acid = PA, C_2H_5; monoethyl phthalate = MEP, $(CH_2)_3CH_3$; monobutyl phthalate = MBP, $CH_2C_6H_5$; monobenzyl phthalate = MBzP, CH_2CH (C_2H_5) $(CH_2)_3CH_3$; mono (2-ethylhexyl) phthalate = MEHP.

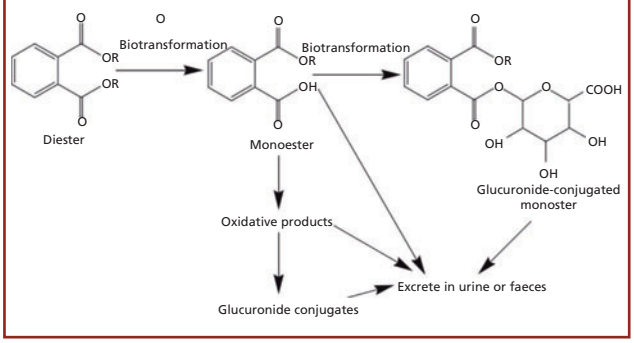
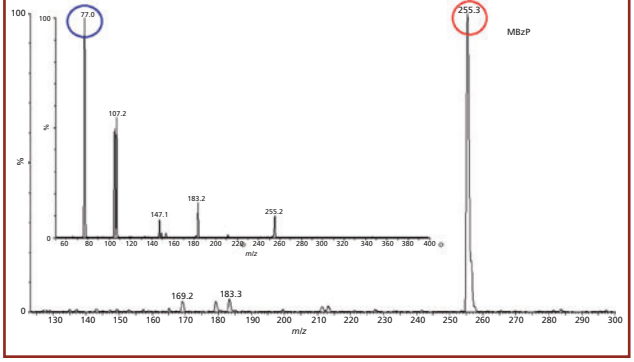


Figure 2: MS and MS–MS optimization of the representative target analyte: MBzP.



method to investigate phthalate esters and their metabolites, but the high cost limited their widespread application. To develop a sensitive, selective, low cost, and efficient analytical method to quantify these chemicals appears essential for understanding how human health might be affected by exposure to phthalates under general laboratory conditions. In this article, we report a method for the quantitative detection of four phthalate monoesters including MEP, MBP, MBzP, and MEHP simultaneously in human urine samples. The method involves the enzymatic dissociation of conjugated monoesters, SPE, and separation and detection using HPLC with negative ion electrospray ionization tandem mass spectrometry. The method was used to analyze human urine samples collected in the same area for evaluating potential phthalate ester exposure.

Table 1: The mobile-phase gradient.

Time (min)	A%	B%	Curve
0.00	70.0	30.0	1
0.20	70.0	30.0	1
2.00	52.0	48.0	6
7.00	42.0	58.0	6
7.10	0.0	100.0	1
8.30	0.0	100.0	1
8.40	70.0	30.0	1
12.00	70.0	30.0	1

Experimental

Materials: PA, MEP, MBP, MBzP, MEHP (>99.9%), and their isotope-labelled internal standards (>99.9%) were purchased from AccuStandard, Inc. Methanol, acetonitrile, and ethyl acetate were purchased from Sigma Aldrich Laboratories, Inc. β-glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical. Ammonium acetate (1 M, pH 6.5), formic acid solution (0.1 M), acetic acid solution (0.1%, v/v), and phosphate buffer solution (pH 2.0) were prepared. Water was purified using a Milli-Q gradient A10 system (Millipore). SPE columns were purchased from Varian, Inc. (Nexus ABS Elut, 60 mg/3 mL). Human urine samples (pooled from 84 individuals) were collected in one area and stored at -80 °C. In this study, plastic equipment was not used in any sampling or experimental processes to avoid contamination, and all glass apparatus were washed with chromic acid solution and rinsed with deionized water and methanol before drying.

HPLC Conditions: Chromatographic separations were performed on a Waters Quattro Micro LC–MS–MS system (Waters Corporation) with a 150 mm × 2.1 mm Kromasil 100-5C_{18} column (Akzo Nobel) set at 40 °C. Mobile-phase A was 0.1% acetic acid in water, and mobile-phase B was 0.1% acetic in acetonitrile. The mobile-phase gradient is shown in Table 1. The sample injection volume was 20 µL. The flow rate was 0.3 mL/min.

Mass Spectrometer Conditions: The MS detector was used in the negative electrospray ionization (ESI) mode. The optimum operation conditions were as follows: ionization voltage: -2.85 kV; cone voltage: 20 V; ion source temperature: 100 °C; drying gas temperature: 300 °C; electron multiplier voltage: 650 V; and collision energy: 16–19 eV. A multiple reaction monitoring (MRM) model was used. The detection channels are shown in Table 2.

Sample Preparation: Urine samples were thawed and vortexed homogeneously. Each 950-µL urine sample was transferred into a glass tube and mixed with 50 µL of internal

Table 2: Linearity of phthalate ester analytes.

Compound	Retention Time (min)	Standard Compound	Internal Standard	Linearity (ng/mL)	Correlation Coefficient (r^2)	LOD	LOQ
		Parent→Daughter (*m/z*)				(ng/mL)	
PA	1.27	165→77		2.5–2000	0.9987	0.87	2.90
MEP	3.63	193→77	197→79	5–2000	0.9990	5.33	17.76
MBP	6.44	221→77	225→79	5–2000	0.9993	2.12	7.07
MBzP	6.62	255→77	259→79	5–2000	0.9978	1.93	6.44
MEHP	10.97	277→134	281→137	2.5–2000	0.9968	0.85	2.82

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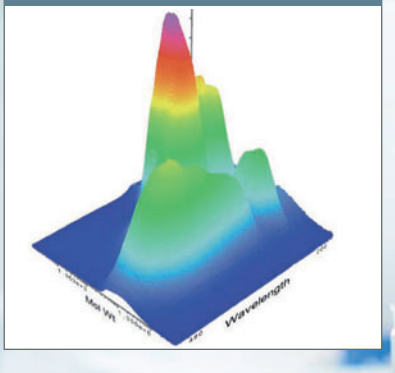

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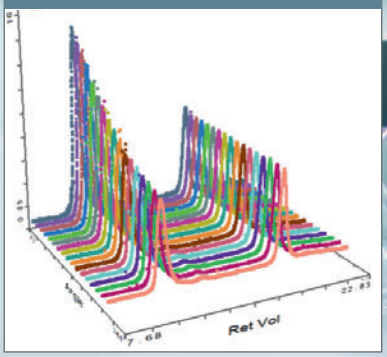

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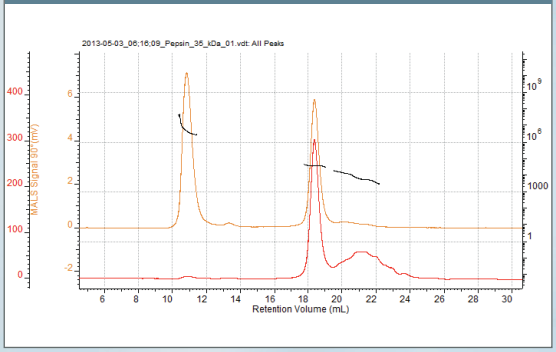

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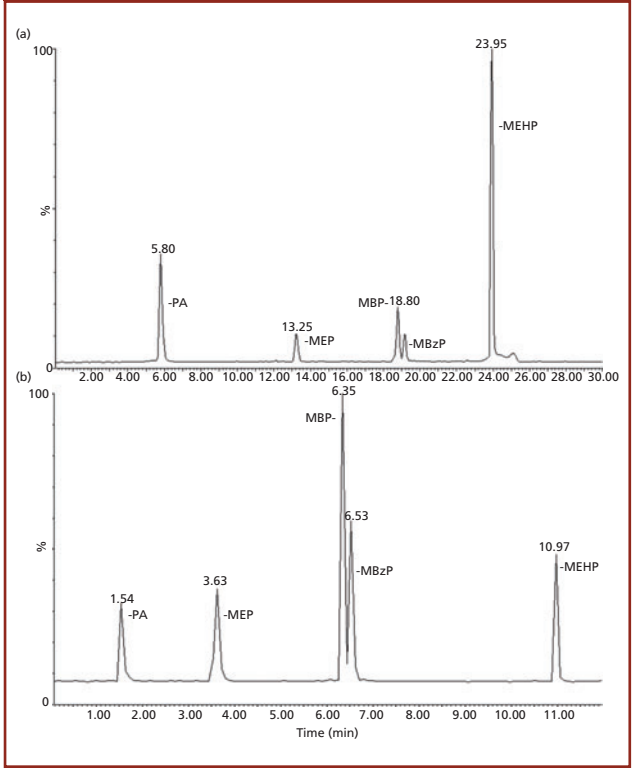
Table 3: The intra- and interday precision of the method.

	Intraday Precision			Interday Precision		
	Spiked Levels (n = 3, RSD, %)			Spiked Levels (n = 3, RSD, %)		
	5 ng/mL	10 ng/mL	50 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL
PA	10.83	8.98	2.94	2.15	15.61	4.37
MEP	5.40	8.58	3.89	2.86	6.35	4.35
MBP	9.52	4.66	4.13	13.69	18.84	5.81
MBzP	5.55	12.18	4.45	5.85	5.27	1.74
MEHP	13.22	8.99	3.83	5.21	11.05	14.24

Table 4: Recoveries of five phthalate esters in human urine samples.

	Spiked Levels (%)					
	3.91 ng/mL		7.81 ng/mL		15.63 ng/mL	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
PA	117.65	4.09	78.10	3.51	95.33	0.33
MEP	92.07	2.28	112.68	1.43	101.09	0.07
MBP	125.32	10.20	101.15	0.16	122.20	1.16
MBzP	109.97	2.40	93.47	0.81	96.61	0.23
MEHP	81.84	5.38	107.55	0.92	119.00	1.04

Figure 3: Optimization of chromatographic conditions: (a) 30-min and (b) 12-min elution times.



standard mixture solution (1 µg/mL $^{13}C_4$-MEP, $^{13}C_4$-MBP, $^{13}C_4$- MBzP, and $^{13}C_4$-MEHP). Then, 5 µL of β-glucuronidase (200 U/mL) and 245 µL ammonium acetate buffer (1 M, pH 6.5) were added to the tube and vortexed in turn. Next, the samples were incubated at 37 °C for 90 min in a shaking water bath and treated with an SPE column. The SPE cartridges were conditioned with 1 mL of methanol, 1 mL of acetonitrile, and 1 mL of phosphate buffer solution (pH 2.0) added successively. Then, 1 mL of urine sample was diluted with 1 mL of phosphate buffer solution (pH 2.0) and added to the SPE column. The cartridges were then washed with 2 mL of formic acid solution (0.1 M) and 1 mL of water. The cartridges were dried under negative pressure. The target analytes were eluted sequentially with 1 mL of acetonitrile and 1 mL of ethyl acetate. The eluent was collected together and concentrated to dry under nitrogen at 55 °C. The dry residue was reconstituted with 200 µL of 1:9 (v/v) acetonitrile–water for the next step of the analysis.

Table 5: Concentrations of five phthalate esters detected in human urine samples (n = 84).

	PA	MEP	MBP	MBzP	MEHP
Mean concentration (ng/mL)	5.31	16.45	96.76	0.07	3.83
Concentration range (ng/mL)	1.4–31.3	0–398.7	0–663.7	0–3.1	0–184.1
Detection rate (%)	100	32	99	1	21

Method Validation: The analytical method was validated to demonstrate the linear range, recovery, limit of detection (LOD), accuracy, and precision. All samples were prepared as described above using blank (or spiked) urine samples. Urine samples with a series of concentrations (2.5, 5, 10, 50, 100, 200, 500, 1000, and 2000 ng/mL standard mixture solutions, including PA, MEP, MBP, MBzP, and MEHP) were prepared to investigate the linear range, recovery, and LOD. The LOD and limit of quantification (LOQ) were calculated using signal-to-noise of 3 and 10 times based on the blank samples, respectively. Samples spiked at three concentration levels (3.91, 7.81, and 15.63 ng/mL) were used to determine the accuracy of the method. Three repetitions at concentrations of 5, 10, and 50 ng/mL were used to determine the intraday and interday precision in one day and the successive three days, respectively.

Application of the Proposed Method: In this study, 84 individual human urine samples were collected in the same area and stored at -80 °C until analysis. The pretreatment and HPLC–MS–MS methods used to analyze these human urine samples were described above.

Results and Discussion

Optimization of HPLC–MS–MS Conditions: To obtain specific, accurate, and sensitive results for quantification during method development, the MS conditions were first optimized by direct infusion. Collision energies were optimized to give the greatest fragmentation with the maximum response for each target analyte. The range of the collision energy was fine tuned from 13–16 eV to 16–19 eV because too much collision energy can cause a loss of valuable stable fragment peaks. The best response of each phthalate ester was about 10^6 for MS spectra and 10^5 for MS–MS spectra (see Figure 2). The parent ion and daughter ion for each phthalate in the MRM detection mode was chosen and is shown in Table 2.

The chromatographic conditions were optimized using the instrumentation and column described in the Experimental section of this article. The ratio between the two phases and

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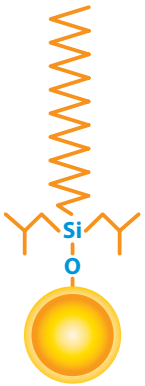

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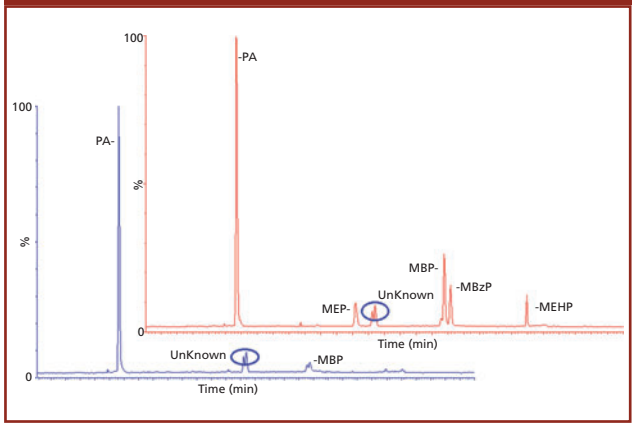
Figure 4: Representative LC–MS–MS chromatograms; the blue chromatogram is a nonspiked human urine sample; the red chromatogram is a spiked human urine sample with 200 ng/mL of the five mixture standards.



gradients was investigated using a 30-min separation time and a solvent gradient ranging from 94% mobile-phase A to the final concentration shown in Table 1. The target analytes were significantly separated. However, detection development time should be as short as possible while still meeting the resolution required. Because these phthalate esters (including PA) have a polar group such as -COOH, the proportion of organic phase (mobile-phase B) was increased to elute the chromatographic peaks faster from the C18 column. The whole elution time was shortened to 12 min, the initial ratio of the water phase was dropped to 70% with the same gradient procedure, and the five target analytes were separated significantly (Figure 3[a] and 3[b]). The chromatographic peaks of MBP and MBzP were overlapped when the water phase was below 70%.

Method Validation: The calibration curves were obtained using standard solutions of five phthalates with concentrations ranging from 5 ng/mL to 2000 ng/mL. The correlation coefficients are shown in Table 2. The results show good correlation (the correlation coefficient values were greater than 0.99) between the peak areas and the concentrations of the phthalates. The LODs of the five phthalates were 0.85–5.33 ng/mL and the LOQs were 2.82–17.76 ng/mL. The intra- and interday precision of the method are summarized in Table 3. The RSDs of those five phthalate esters ranged from 1.74% to 14.24%. Samples spiked at levels 3.91, 7.81, and 15.63 ng/mL were analyzed three times each. The method validation studies for spiked samples indicated that the present method provides good recoveries and reasonable precision for phthalates at these three levels. As can be seen from Table 4, the recoveries were 81.84% and 125.32% with RSDs between 0.07% and 10.20%. These results demonstrate that the method developed has acceptable precision.

Application of the Method: The LC–MS–MS method described above was applied to the analysis of 84 human urine samples collected in one area (Figure 4). Table 5 presents the summary of four phthalate monoester metabolites and PA in these human urine samples. Overall, the detection rates of PA, MEP, MBP, MBzP, and MEHP were 100%, 32%, 99%, 1%, and 21%, respectively. The detection mean concentrations of PA, MEP, MBP, MBzP, and MEHP in all urine samples was 5.31, 16.45, 96.76, 0.07, and 3.83 ng/mL, respectively. The detection concentration ranges of PA, MEP, MBP, MBzP, and MEHP in all urine samples were 1.4–31.3, 0–398.7, 0–663.7, 0–3.1, and 0–184.1 ng/mL, respectively. The results showed that the subjects were exposed to some phthalate esters, especially MEP, MBP, and MEHP, with high concentrations in that region. This study confirmed detection levels of four phthalate metabolites and PA in human urine samples.

Conclusions

We developed an LC–MS–MS method for the simultaneous analysis of five phthalate ester metabolites in human urine. The method has proven to be sensitive, accurate, and precise with a relatively short chromatographic run time of 12 min. The results demonstrate that this method can be of value in assessing human exposure to phthalates or other environmental toxicants in environmental toxicology studies.

Acknowledgements

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Mobile-Phase Degassing: What, Why, and How

John W. Dolan, LC Resources, Walnut Creek, California, USA.

Why should you be concerned about mobile-phase degassing — it's all done automatically, isn't it?

Degassing of the mobile phase in liquid chromatography (LC) applications is a topic that is seldom talked about today. However, this was not the case in the past. For the first 30 years or so of LC use, problems related to mobile-phase outgassing were some of the most common problems encountered in routine use. This was true in user surveys conducted by *LCGC* as well as by my informal polls based on readers' questions and direct interactions with users. Over the past 15–20 years, on-line degassers have moved from a novelty device to a standard part of most LC systems. As a result, I suspect that many users have never encountered problems related to degassing. I dropped an in-depth discussion of degassing from my popular LC troubleshooting class, but in a recent class the topic came up again. Although in-line degassing helps us avoid most solvent out-gassing problems, it does not solve all problems related to dissolved air in the mobile phase. In this month's instalment of "LC Troubleshooting" I would like to review what degassing is all about. What is it? Why do we need it? How is it accomplished? Are there times when we should be especially watchful for problems related to it?

Why Degas the Mobile Phase?

When solvents are in contact with the atmosphere, air gradually dissolves into the solvent. Air, of course, is primarily nitrogen and oxygen. In reversed-phase LC, the most common solvents are water or buffer as the A-solvent and acetonitrile or methanol as the B-solvent. When the aqueous and organic solvents are mixed, they each contribute to the total dissolved air content of the mixture. This is illustrated by the dashed line in Figure 1 for the solubility of oxygen in mixtures of water and ethanol (which behaves in a similar manner for oxygen and nitrogen with methanol and acetonitrile for the present discussion). Whether we are making isocratic (constant %B) mixtures or gradients, the amount of gas in solution is in proportion to the respective solvent volumes. The problem with this situation is that the solubility of air in the mixture is less than that of individual components. This is shown as the solid curve in Figure 1. When such a situation exists, the solution is supersaturated with air, generating an unstable condition in which air will bubble out, or outgas, from the solution.

Degassing of the mobile phase in liquid chromatography (LC) applications is a topic that is seldom talked about today.

If mobile-phase outgassing occurs within the LC system, the most common problem areas are the pumps and detector. At the extreme, air in the pump will cause the pump to stop delivering mobile phase to the column. If only an occasional bubble is present, the flow rate will be erratic, causing retention time problems. Air in an optical detector, such as an ultraviolet (UV), fluorescence, or refractive index detector, will scatter light passing through the flow cell, causing noise spikes in the chromatogram or an off-scale signal. These problems can be eliminated if the air is removed from the mobile phase.

Early Solutions

The data of Figure 1 suggest that to avoid mobile-phase outgassing we do not have to remove all the dissolved gas from solution, but only reduce the total amount of dissolved gas so that a plot for the resulting mixture would result in a line falling below the solid line in Figure 1 (1). If half of the dissolved gas is removed, we should be safe. One simple way to do this is to use a vacuum to degas the solvents. This can be done easily by placing the solvent in a vacuum flask and pulling a partial vacuum with a water aspirator or mechanical pump of similar capacity. To help facilitate the process, a stir bar or a few (clean!) boiling stones can be added to the flask. Although I don't have quantitative data on this, many workers find that sonicating the solution while vacuum degassing seems to be more effective than vacuum alone. In any event, a few minutes of vacuum degassing will remove about 60–70% of the dissolved gas (2). Sonication alone will only remove 20–25% of the gas (2), which is insufficient to avoid outgassing with most LC systems. For some LC systems, the amount of vacuum applied while filtering the mobile phase may remove enough gas to avoid problems. The most effective way to degas the

Figure 1: Solubility of oxygen in ethanol (dashed line); saturation concentration of oxygen in the mixture (solid line). See text for details. Adapted from reference 1.

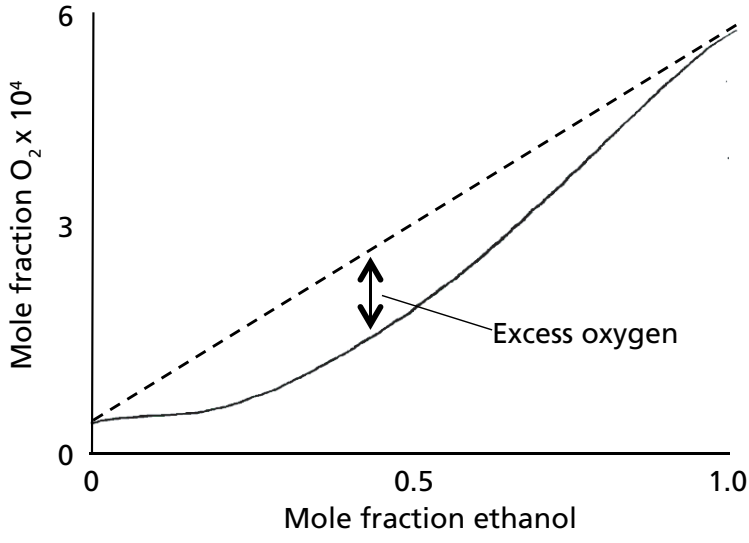
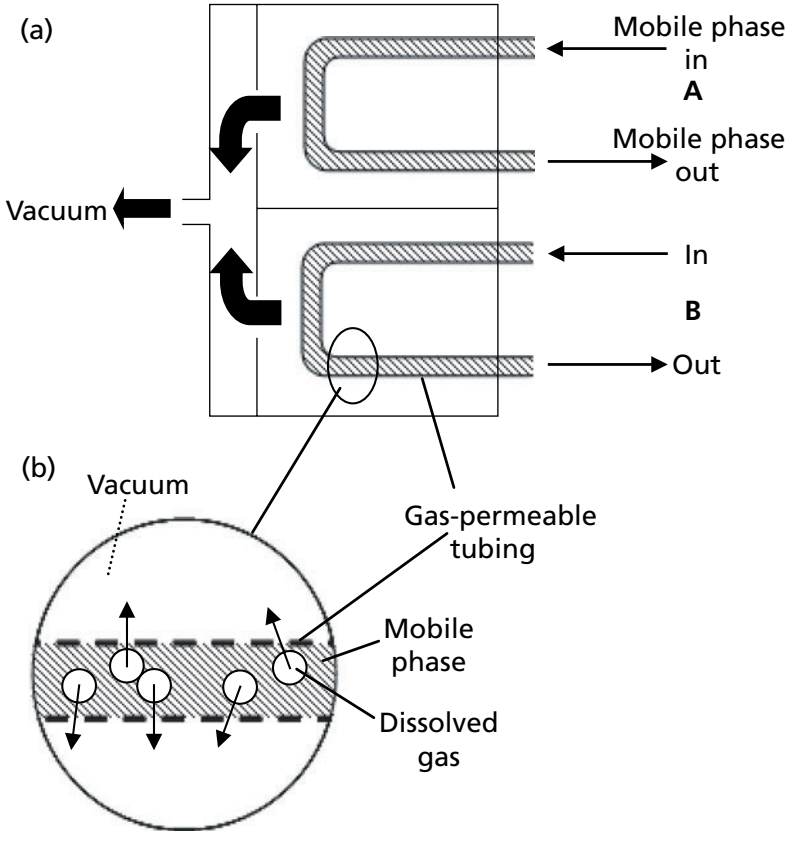


Figure 2: Schematic of in-line vacuum degasser: (a) Flow path; (b) expanded section to illustrate gas passing through semipermeable membrane. See text for discussion.



solvents is to bubble helium through the mobile phase by sparging for a few minutes, which removes approximately 80% of the dissolved air (2). It seems contradictory to use a gas to degas a solution, but the solubility characteristics of helium in the mobile phase are such that outgassing is not a problem. Helium sparging was widely used for degassing, and although it is used less today because of the ease of in-line vacuum degassing and decreased availability of helium, it is still the most effective degassing technique.

If mobile-phase outgassing occurs within the LC system, the most common problem areas are the pumps and detector.

Until the late 1970s, all LC systems were run with either premixed mobile phases in the isocratic mode or used high-pressure mixing to generate a gradient by mixing the mobile-phase components after the pumps. With these systems, the premixed mobile phase was degassed before use or the individual solvents were degassed for gradient applications. On-line mixing of isocratic mobile phases was also possible if the solvents were degassed first. The advantage of high-pressure mixing is that the pumps only pump degassed solvents or mobile-phase mixtures, and because the solvents were mixed under pressure, any bubbles that might otherwise form at atmospheric pressure would stay in solution because of the elevated pressure of the system. Often, care had to be taken to provide a small back pressure (for example, 50 psi) on the outlet of the detector to keep the mobile phase from outgassing in the detector cell.

In the late 1970s, Spectra-Physics (now a part of Thermo Fisher Scientific) introduced an LC system that incorporated low-pressure mixing. Solvents were blended in a proportioning manifold before they reached the pump, a technique that is used in low-pressure mixing systems offered by most LC manufacturers today. When low-pressure mixing is used, the solvents are mixed at atmospheric pressure (or sometimes a bit below atmospheric pressure),

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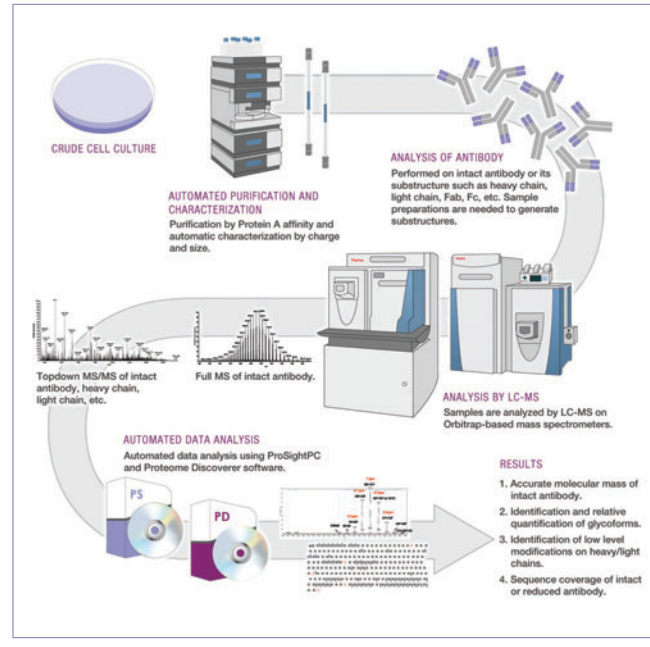

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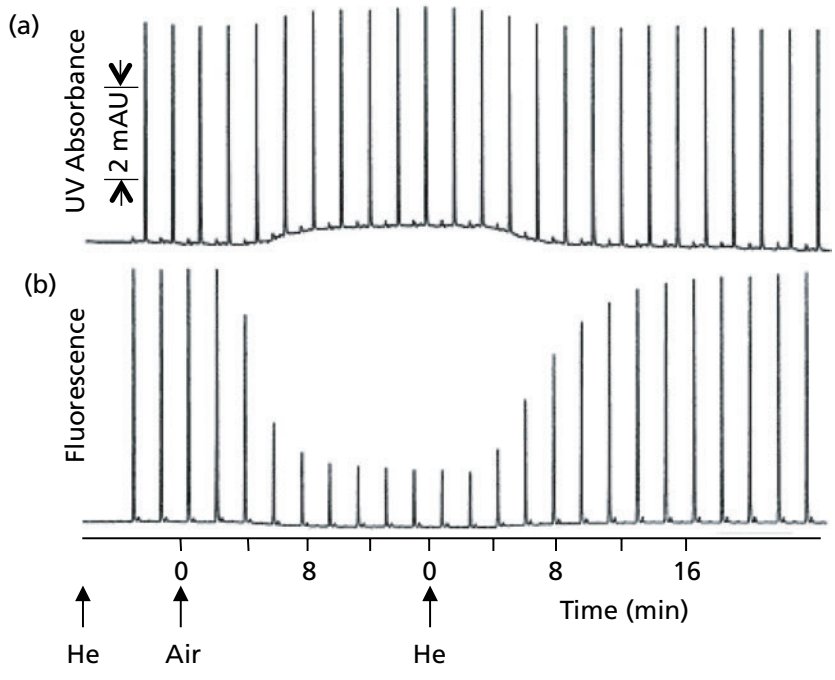
Figure 3: The effect of helium and air dissolved in the mobile phase on the response of naphthalene: (a) UV detection at 254 nm; (b) fluorescence detection with 250-nm excitation and 340-nm emission. The mobile phase was sparged with helium or air, as indicated. See text for details. Adapted from reference 1.



so outgassing of the mobile phase is a huge problem. The Spectra-Physics system included a built-in helium sparging system to degas the solvents before mixing, so outgassing was avoided. Although Spectra-Physics had a patent on helium sparging (3), which discouraged other manufacturers from offering the same technology, it was hard to prevent individual users from constructing their own helium sparging apparatus for personal use, so the technique became popular.

In-Line Degasser

Although in-line degassing was patented in 1984 (4), it was not often used in LC systems until the late 1990s. More recently, in-line degassing has become the most common degassing technique — a standard component of most new LC systems. The function of the in-line degasser is illustrated in Figure 2. The degasser comprises a gas-permeable tube or membrane, through which the mobile phase passes, and a vacuum chamber as in Figure 2(a). The membrane is similar to the semipermeable membrane used in rain jackets, which liquid water is not able to penetrate, but water vapour does pass through. When vacuum is applied on the outside of the membrane (Figure 2[b]), dissolved gas passes through the pores in the membrane and the liquid mobile phase stays inside. When the proper combination of membrane porosity, vacuum, and residence time is used, the in-line degasser removes enough dissolved gas to avoid outgassing so that the LC pump will operate reliably.

In-line degassers are quite reliable, and will operate for months or years with little or no maintenance. It is a good practice to avoid storage of the degasser with buffer or water in the tubing, because microbial growth can occur, and the pores can become blocked or general contamination can occur. A small amount of biological contamination may be removed by flushing all the mobile-phase pathways with 30% phosphoric acid and then rinsing with water (check the system maintenance manual for specific instructions and precautions). In some cases, the membranes may need to be replaced when contaminated, an expensive event, so it is best to avoid it by removing aqueous phases when not in use. Other failures can occur if the vacuum pump fails or the tubing between the vacuum manifold and the pump or pump and waste are damaged, blocked, or come loose.

It is a good practice to avoid storage of the degasser with buffer or water in the tubing, because microbial growth can occur, and the pores can become blocked or general contamination can occur.

Because in-line degasser use is almost universal, and in general requires little or no maintenance, many users are not aware of problems related to bubbles in the mobile phase. Although in-line degassers are effective in reducing the total gas burden of the mobile phase to acceptable levels, dissolved air still remains in the mobile phase. For some applications, this residual air can cause problems, as is noted below.

Problems with Dissolved Oxygen

For LC applications using UV detection at wavelengths greater than 200 nm, mass spectrometry (MS) detection, and many other LC detectors, a little residual dissolved gas in the mobile phase is not a problem. As mentioned above, for detectors that rely on passing mobile phase through an optical path, such as refractive index or UV detectors, as long as air bubbles are not present, problems are rare. A slight back pressure on the flow cell, such as that created by using a capillary tube for a waste line or installing a back-pressure regulator after the cell, will be sufficient to avoid outgassing in the flow cell. In other

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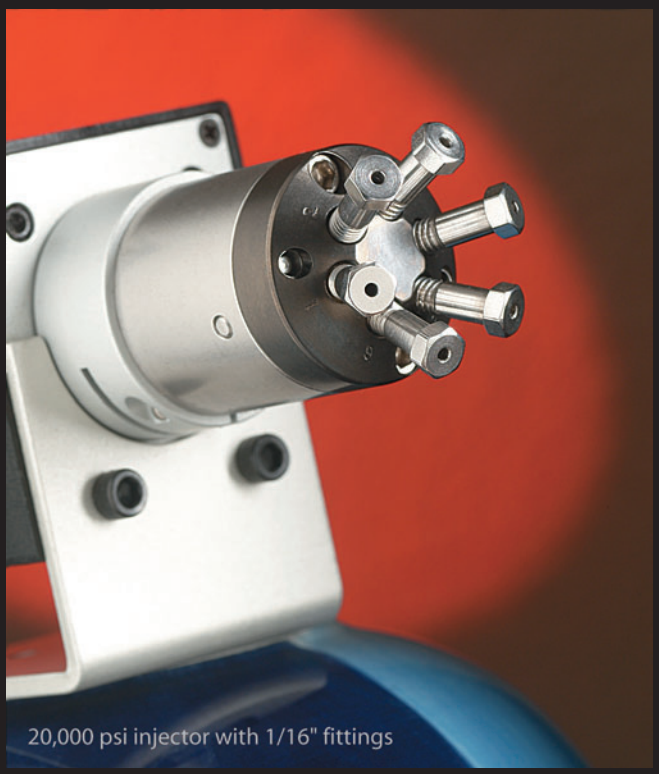

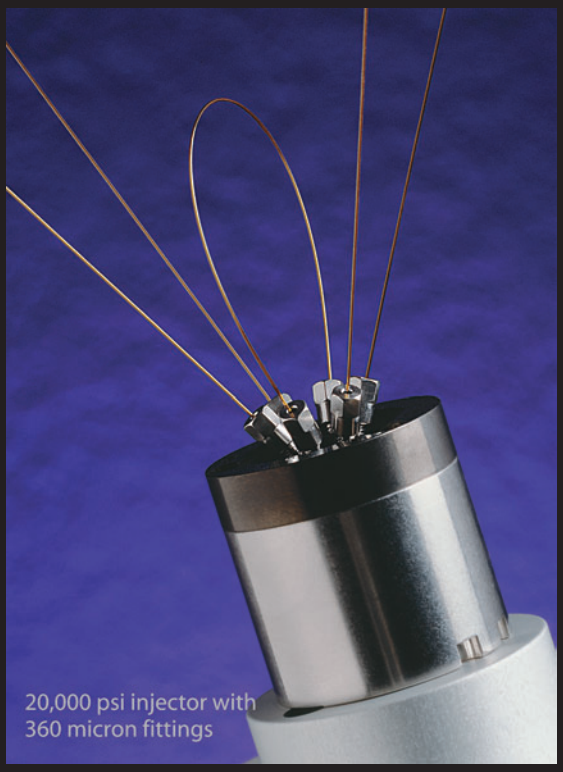

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cases, dissolved oxygen, even at low concentrations, may compromise detection.

An example of compromised fluorescence detection for naphthalene is shown in Figure 3 (1). The chromatograms start at the left with helium-sparged mobile phase, then air is bubbled through the mobile phase instead of helium; finally, helium is again used as the sparging gas. Each peak in the chromatograms represents a separate injection of naphthalene. When UV detection at 254 nm is used (Figure 3[a]), the baseline rises a bit when air is present in the mobile phase because oxygen absorbs UV light somewhat under these conditions, but the signal rises by the same amount, so no sensitivity is lost. Switching back to helium displaces the dissolved oxygen and the baseline returns to the original position. Under normal conditions, where the baseline is autozeroed before each injection, it is unlikely that this baseline shift would be noticed. Contrast this detection response to the fluorescence response for naphthalene when 250 nm is used for excitation and 340 nm for emission. The presence of oxygen (air) in the mobile phase has little effect on the baseline, but the signal drops noticeably because oxygen quenches the fluorescence of naphthalene under these conditions. As the oxygen is gradually displaced by helium at the right of Figure 3[b], the response increases. This is a case where vacuum degassing or in-line degassing is unlikely to remove sufficient oxygen for maximum sensitivity and stable operation. You can imagine the potential problems if the mobile phase were helium-sparged off-line and then placed on the LC system. Over time, air would redissolve into the mobile phase and the fluorescence signal would gradually drop, creating a problem of changing sensitivity that might be hard to track down. One possible alternative solution to the problem might be to continuously bubble nitrogen through the mobile-phase reservoirs and then use an in-line degasser to remove excess nitrogen. The nitrogen would displace the dissolved oxygen and the vacuum degasser should then be sufficient to avoid outgassing.

When water and acetonitrile are used as the mobile phase, it has been reported that UV detection down to 185 nm is possible if the optical path of the detector is purged with helium to remove oxygen from the light path.

Helium sparging, although less commonly used today, remains the gold standard for mobile phase degassing.

When water and acetonitrile are used as the mobile phase, it has been reported that UV detection down to 185 nm is possible if the optical path of the detector is purged with helium to remove oxygen from the light path (5). In this case, it would also be important to remove all oxygen from the mobile phase. Another possible detection problem can occur when an electrochemical (amperometric) detector is used in the reductive mode. In this mode, oxygen interferes with detection, so any dissolved oxygen in the mobile phase will cause problems. As with the fluorescence example above, sparging with helium or the use of nitrogen sparging in combination with in-line vacuum degassing would be expected to mitigate this problem. In some cases with reductive electrochemical detection, enough oxygen diffuses through the PTFE tubing leading from the reservoirs to the pumps to cause problems. In such cases, replacement of the transfer tubing with non-gas-permeable tubing, such as metal tubing or polyetheretherketone (PEEK) may be necessary.

Conclusions

The widespread use of in-line mobile-phase degassers in LC has greatly improved the reliability of today's LC systems. These devices are generally reliable and require little maintenance other than ensuring that they don't become contaminated. However, just because the degassing process takes place automatically does not mean that problems related to dissolved gas will never exist. There is still a possibility of outgassing in the detector as the mobile phase returns from high pressure to atmospheric pressure. Maintenance of a slight back pressure on the detector flow cell will usually avoid this problem, but care should be taken that the restrictor does not overpressure the flow cell. In some applications, such as sub-200-nm detection by UV, fluorescence, and reductive electrochemistry, the residual dissolved oxygen in vacuum-degassed mobile phases may be sufficient to compromise detection. In such cases, supplemental or alternative degassing techniques may need to be used. Helium sparging, although less commonly used today, remains the gold standard for mobile phase degassing.

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Gas Cylinder Setup and Use

John V. Hinshaw, Serveron Corporation, Beaverton, Oregon, USA.

This month's instalment discusses the laboratory deployment of gas cylinders with appropriate pressure regulators, gas filtration, and gas fittings, as well as the question of cylinder retirement.

A previous "GC Connections" instalment (1) discussed the intake and disposition of compressed gas cylinders, including verifying gas cylinders and their content upon receipt, safely moving and securing them in place, and organizing them by their current use. After cylinders are in place in the laboratory, the next steps are to attach appropriate gas regulators, tubing, filters, and fittings to bring the high-purity gas supplies to one or more gas chromatographs.

Pressure Regulators

Pressure regulators are unsung heroes in the laboratory. They accomplish the expansion of high-pressure cylinder gases from dangerous levels to what can reasonably be handled by laboratory instruments while maintaining nearly constant output pressures and — if correctly matched to the application — not contaminating the gas stream. Of course, pressure regulator selection, installation, and maintenance are essential for obtaining the best performance. But before discussing the whys and whats of regulators, here's a brief description of how they work.

In Control: Laboratory pressure regulators are designed to expand gases from cylinder pressures as high as 3000 psig (20.7 MPa) down to operating pressures as low as 10–20 psig (150–275 kPa). Gas-tank regulators are mechanical devices with springs, screws, and a counterbalancing diaphragm. Electronic pressure control (EPC) has not yet found its way into the tank realm in any significant way, although some laboratory hydrogen generators incorporate EPC. Electronic pressure control of in-instrument gases will be covered in a future "GC Connections" instalment.

Pressure regulators are unsung heroes in the laboratory. They accomplish the expansion of high-pressure cylinder gases from dangerous levels to what can reasonably be handled by laboratory instruments while maintaining nearly constant output pressures.

Figure 1 shows a simplified diagram of a typical single-stage mechanical gas-pressure regulator such as what is found at the gas tank or sometimes in-line as part of a manifolded gas distribution setup. The regulator consists of a two-piece sealed housing with the internal volumes separated by a flexible diaphragm. The outlet gas pressure exerts force on one side of the diaphragm and a spring pushes in the opposite direction. The amount of force exerted by the tensioning spring on the diaphragm determines the output pressure level. A pressure adjustment knob turns a screw that increases or decreases the force on the spring and diaphragm.

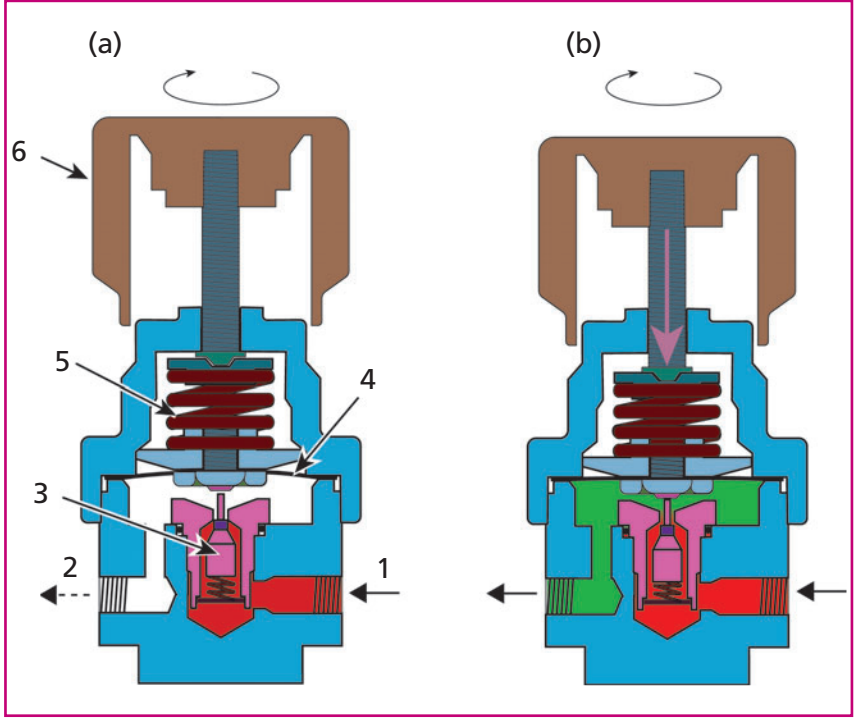
In Figure 1(a), the adjustment knob is fully turned out and the spring exerts no force on the diaphragm: The pressure on the outside of the diaphragm is equal to ambient pressure. Gas at the tank pressure has entered the regulator at the inlet connection, and the poppet valve below the diaphragm is fully seated so that no gas flows through it. The upper shaft of the poppet is not in contact with the actuating seat that is attached to the diaphragm.

In Figure 1(b), the adjustment knob has been turned clockwise and its shaft has moved downwards to exert force on the spring and diaphragm. This movement has caused the actuating seat under the diaphragm to contact the upper shaft of the poppet valve and move it downwards, which in turn unseats the poppet and allows gas to flow into the outlet chamber. As pressure builds up in the outlet chamber, the diaphragm is forced upwards against the tensioning spring. The regulator settles into a constant outlet pressure when the spring force and the pneumatic force are balanced as gas flows out of the regulator.

As the output gas flow increases, the poppet valve opens up to sustain the output pressure balance against the tensioning spring. Conversely, as the output gas flow decreases the poppet valve moves upwards and restricts gas flow, again so that the output pressure is maintained. If the outlet flow goes to zero, the poppet will seal off the inlet.

Nonlinear Effects: Mechanical pressure regulators are subject to a few side effects with which many gas chromatographers are familiar. A nonideality occurs when the outlet flow rate increases so much that the

Figure 1: Mechanical gas-pressure regulator: (a) The adjustment knob is fully withdrawn and no pressure is applied to the tensioning spring — the poppet valve is closed and no gas flows. (b) The adjustment knob is turned clockwise and advanced into the regulator. There is tension on the spring so that the poppet valve is contacted and gas flows to the outlet. 1 = inlet connection, 2 = outlet connection, 3 = poppet valve, 4 = flexible diaphragm, 5 = tensioning spring, 6 = adjustment knob. The inlet gas chamber is coloured red; the output chamber with pressure-controlled gas is shown in green.



poppet valve is forced fully open. Any additional flow demand will cause the outlet pressure to drop well below its set point. In this condition, the regulator is unable to supply enough gas and can be said to be choked off. The basic capability of a regulator to deliver steady pressure control at high flows is encapsulated in its flow coefficient, or C_v. The flow coefficient is measured with the regulator fully open, however, and does not represent a realistic situation for gas chromatography (GC) applications. Most tank regulators in GC laboratories have maximum flows of tens of litres per minute or more, but this situation rarely occurs with GC instruments that consume gas at a few hundred millilitres per minute or less. The C_v coefficient is not relevant for normal GC installations with the types of regulators that are usually supplied for GC applications.

The outlet pressure will decrease somewhat as flows increase between the minimum flow and the choke-point flow: This effect is termed *droop*. The poppet valve must move towards a more open position at higher flows, which in turn displaces the inherently nonlinear diaphragm and tensioning spring towards a lower set-point pressure. Droop causes gas pressures to change slightly at the GC instrument gas inlet connection as conditions change, such as when the inlet mode is changed from split to splitless in some instruments.

The GC instrument's internal carrier-gas control system will compensate for droop if the maximum column inlet pressure to be used is set lower than the tank pressure regulator output by at least 20%. For example, if the maximum carrier-gas pressure is 70 psig (482 kPa) then the tank regulator should be set to at least 85 psig (586 kPa). Normally, the manufacturer's recommended incoming carrier-gas pressure will be sufficient, but operators need to be aware of the necessity to increase supply pressures if higher inlet pressures are to be used — for example, for some types of high-speed chromatography.

A third nonideal effect is the dependency of the output pressure on the incoming tank pressure, sometimes called the *supply-pressure effect*. As the cylinder pressure decreases the output pressure will start to increase, roughly as a small percentage of the decrease in supply pressure. For example, if the tank pressure declines by 1000 psi and the output pressure increases by 10 psi then the supply-pressure effect is 1%.

Another unwanted effect is the dependency of the output pressure on the incoming tank pressure, sometimes called the *supply-pressure effect*.

This increase in output pressure occurs because the input gas pressure exerts some force on the poppet valve, which, while small in comparison to the tensioning spring–diaphragm, constitutes a small reduction in the overall output pressure. Lower supply pressures then cause the output pressure to increase slightly as the counter force on the poppet lessens. The degree of supply-pressure effect depends on the regulator design: A high-quality regulator will incorporate some features to minimize it.

In extreme cases the output pressure rise could exceed a safe limit. The best solution for controlling the supply-pressure effect is to use dual-stage regulation from the tank to the instrument. Usually this approach involves using a single gas-regulation device at the gas tank that incorporates two regulator stages connected in series internally. The first stage reduces the pressure to an intermediate level of 300–500 psi, and the second drops the pressure to the service level. Often when a manifolded gas supply system is used, the first regulation

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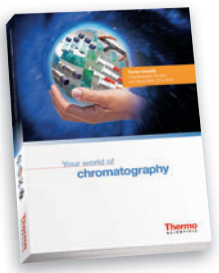

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stage is installed at the tank and a second regulator is installed in-line immediately upstream from one or more instruments.

In either case, the supply effect is now well-controlled. If the first regulator has a supply-side effect of 5%/psi and the second has a supply-side effect of 1%/psi, then the overall effect will be 5% of 1%, or only 0.05%, until the tank pressure reaches the intermediate pressure. From that point down to a final pressure of 100 psig, the supply effect will be equal to that of the second stage. So when the cylinder pressure drops by 2300 psi across its full range from 2600 psig down to the intermediate 300 psig the output pressure from a high-quality regulator would be expected to increase less than 1 psi. For the remaining 200 psi the first stage no longer functions, so the output pressure would increase by an additional 2 psi for a total pressure increase of around 3 psi as the cylinder is depleted.

Many regulators incorporate a safety relief valve on the low-pressure side. This valve will open at a predetermined high pressure and help limit the high pressures that could otherwise be applied to the downstream equipment should the internals of the regulator fail. Such failures are rare and are usually caused by misuse or contamination. To ensure long regulator lifetimes, the following practices should be employed:

- Never allow a pressure regulator to be back-pressurized through the outlet connection.
- Ensure the cleanliness of the high-pressure side tank connection. Check for the absence of dust and particulates when connecting the regulator — but never open the tank valve with no regulator attached in a misguided attempt to blow off the seat!
- Never over-compress the diaphragm and spring by turning the pressure knob fully towards higher pressure levels. Many high-quality regulators incorporate internal stops that prevent over-compression, but I have seen several instances where such damage has occurred.

Evidence of an internal failure resulting in high pressures at the outlet can be found when the outlet pressure gauge has been over-wound and shows a positive offset at zero pressure. Immediately take out of service, label as broken or defective, and dispose properly of any compromised regulators. Using such defective devices creates an unacceptable safety hazard.

Along with selecting an appropriate dual-stage regulation scheme, the correct fittings must be used at the inlet and outlet of a regulator before it can be put into use.

Keeping It Clean

In addition to delivering as constant an output pressure as possible over the life span of a gas cylinder, a pressure regulator must not add any significant amount of other gases or contaminants. The gas stream in a regulator is exposed to a variety of materials, including the internal seals, the poppet seat, the diaphragm, and the other internal areas. The types of materials and their cleanliness are crucial factors for maintaining gas purity as it passes through. Counter to intuition, even though the inside of the regulator is at elevated pressures, nitrogen, water, and oxygen can leak into the gas stream from the atmosphere. The sealing materials between the inside of the regulator and the atmosphere determine how much leakage will occur, assuming that there are no overt leaks because of defective assembly or poorly executed connections to and from the regulator.

For general GC use, high-purity models for noncorrosive service with a brass body, stainless-steel diaphragm, fluoro-polymer seats and seals, and a packless outlet valve are appropriate. For high-sensitivity applications such as GC–mass spectrometry (MS), electron-capture detection (ECD), or other high-performance applications, upgrading the body material to stainless steel can help ensure a higher level of carrier-gas purity because of more stringent cleaning during assembly along with the accompanying incremental improvements in the other internal materials. Regulators rated for corrosive service are unnecessary for GC consumable gases, but they may be appropriate for certain calibration gases and sample streams.

Regulator cost is related to both the gas purity rating as well as the pneumatic performance. Very low-cost regulators are generally not appropriate for GC use. Chromatographers should pay attention to regulator specifications and choose those that are suitable for GC applications, following the manufacturer's requirements rather than trying to save a little by opting for less-expensive gas control devices.

Filters: Proper filtration of GC gases is the stop-gap that keeps small levels of contaminants that originate in regulators, fittings, and tubing from becoming big problems. Filters are not suitable as cleanup devices for poor quality gas-cylinder contents, inexpensive contaminated tubing, or low-quality regulators. Carrier gas from a less-expensive four-nines (99.99%) helium cylinder will not be redeemed to six-nines (99.9999%) levels by the use of in-line oxygen, water, and hydrocarbon filters. Filters can restore the quality of lightly contaminated gases, but do not have the capability to clean up intrinsically contaminated gases on the fly. A recent "GC Connections" instalment addressed the specific types of filters that are recommended for various GC inlets, columns, and detectors (2).

The Right Connection

Along with selecting an appropriate dual-stage regulation scheme, the correct fittings must be used at the inlet and outlet of a regulator before it can be put into use. Pressure regulators are usually supplied with a high-pressure inlet fitting that will only connect to a matching fitting on the tank — they are ordered for a specific gas or gas type such as helium and nitrogen, air, or hydrogen. The exact fittings for carrier, detector,

and calibration gases vary by country and region, so chromatographers should refer to local regulations and to their gas suppliers for the correct information.

Changing an existing regulator from one inlet fitting to another is not recommended. The risks of not attaching the high-pressure connection correctly and damaging the regulator are not offset by the relatively small cost of procuring a set of specific regulators for individual gas use.

A gas cylinder is ready for retirement when its pressure has decayed to a level of around twice the regulated downstream pressure.

The outlet fitting of the regulator must match the size and type of downstream tubing, both diameter and material. Don't mix brass fitting nuts with stainless steel fittings, for example, and always make fresh connections by cutting the tubing and starting over if there is any doubt about reusing a connection. Be sure to follow the fitting manufacturer's exact instructions when making new or reusing tubing or pipe connections, and use available fitting-specific gauges to check for proper assembly. Over-tightening a fitting is the number one mistake that inexperienced chromatographers commit in this area.

Retirement

A gas cylinder is ready for retirement when its pressure has decayed to a level of around twice the regulated downstream pressure. For example, a helium cylinder that supplies 80 psig should be retired as soon as the internal pressure drops to 160 psig. Laboratory staff should check cylinder pressures weekly to ensure that none drop so low that the downstream pressure starts to decay, thereby compromising the attached instruments' performance.

On behalf of gas suppliers everywhere, please be sure to leave some positive pressure in a retired cylinder when it is returned. When the supplier receives a cylinder with the valve closed and significant internal pressure they gain some assurance that the remaining contents are intact and probably not seriously contaminated. Although the suppliers cannot assume zero contamination, this is a much better cylinder state than no pressure, negative pressure, or, worst of all, an open cylinder valve.

I recommend installing a purge tee fitting and valve at the outlet of carrier-gas regulators. This accessory permits clean carrier gas to be isolated in the downstream tubing while changing a cylinder, and it allows the regulator to be purged of the air that inevitably will enter during a changeover. The alternative of pushing a slug of room air into the tubing will shorten the lifetime of the carrier gas oxygen and water filters.

The Inside Story

Now that we have followed GC gases from their origins — such as the formation of helium from radioactive decay in the earth's crust — to separation, purification, and the supply chain that brings them to the laboratory, captured in high-pressure cylinders or perhaps liberated on-demand, it's time to return to the gas chromatograph itself and trace the gases' paths through the pneumatic controls, inlets, columns, and detectors. The next instalment of "GC Connections" will visit electronic pneumatic pressure control for carrier and detector gases to see how modern GC systems combine theory with practical considerations to yield optimized performance with a wide range of column dimensions and detector types.

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Highlights of HPLC 2014

Ronald E. Majors, Column Watch Editor.

HPLC 2014 was held 11–15 May in New Orleans, Louisiana, USA, for the first time and much earlier than normal. This instalment of "Column Watch" covers some of the technology and application advances presented at HPLC 2014. We review the overall liquid-phase chromatographic trends, summarize the awards presented, and discuss the column technology highlights observed at the symposium.

The 41st International Symposium on High Performance Liquid Phase Separations and Related Techniques, which alternates between Europe and North America, with occasional side meetings in Australia and Asia, was held 11–15 May in New Orleans, Louisiana — its first time in the southern part of the United States. More affectionately known as HPLC 2014, the symposium is the premier scientific event for bringing together the myriad techniques related to separations in liquid and supercritical fluid media. Chaired by Professor J. Michael Ramsey of the University of North Carolina at Chapel Hill, USA, with the able assistance of the symposium organizer Professor Edward Yeung of Iowa State University, USA, and Janet Cunningham of Barr Enterprises, HPLC 2014 assembled 770 delegates from 39 different countries around the world. This number included vendor representatives from 44 exhibitors for the three-day plus instrument, software, and consumables exhibition. The number of conferees was just over half of the attendance at HPLC 2013 in Amsterdam, The Netherlands, and in line with the HPLC 2012 symposium held in Anaheim, California, USA. For some reason, the number of delegates for the United States version of this meeting has fallen off in recent times, perhaps analogous to the falloff in other analytical symposia such as Pittcon. However, other meetings such as the American Society for Mass Spectrometry (ASMS) Conference on Mass Spectrometry and Allied Topics seem to be gathering steam, undoubtedly driven by the recent surge in the use of mass spectrometry (MS) detection in chromatography and other analytical techniques.

The venue for HPLC 2014 was the New Orleans Hilton located right on the Mississippi River close to the convention centre where Pittcon has been held many times. The five-day plus event had a total of 155 oral presentation in plenary talks and mostly in three parallel sessions, which made it a bit difficult to cover topics of interest that often ran at the same time. Fortunately, all three lecture halls were within a few feet of each other so getting from one session to another didn't pose much of a problem. At HPLC a total of 425 posters were presented in sessions with 25 different themes. Posters were up for the entire symposium so they could be viewed at almost any time of the day. With an ample social event schedule including three receptions and a symposium dinner and party, 10 vendor workshops, eight tutorial educational sessions, and five short courses (held during the previous weekend), attendees had their hands full deciding how to allocate their time. The tutorials were particularly well attended, some with standing room only, and covered current topics such as troubleshooting method development, polymeric monoliths, microfluidics, column myths, ultrahigh-pressure liquid chromatography (UHPLC) theory and practice, the effect of dwell volume, ion chromatography versus electrophoresis, and new Food and Drug Administration (FDA) regulations affecting high performance liquid chromatography (HPLC).

In this instalment, I present some scientific highlights of HPLC 2014. This report also covers the various awards and honourary sessions that took place. Since it was virtually impossible for one person to adequately cover all oral and poster papers, my coverage will reflect a somewhat personal bias.

Trends in Liquid-Phase Technology and Techniques

Obviously, HPLC was the predominant technology in the technical sessions at the symposium, but sample preparation, the use of electrophoretic techniques (mostly in a capillary format), and an increase in supercritical fluid chromatography (SFC) papers were strongly evident. From a perusal of the poster and oral presentation abstracts, I broke down some of the major areas of coverage in this year's symposium and listed them in tables. These tables are useful to spot trends in the technology and new application areas for liquid-phase separations that were introduced in this series.

Table 1 provides a rough breakdown of the coverage of liquid-phase technology and techniques in the separation sciences. Compared to HPLC 2013, some slight shifts in technology emphasis were noted. Again this year, new developments in column technology led the pack with oral presentations and poster papers dealing with many new phases

Table 1: HPLC 2014 papers presented by technology or technique.

Technology or Technique	Symposium Papers Presented (%)
HPLC column technology, phases, and design (Monoliths: 38%) (Superficially porous particles: 41%) (Sub-2-µm porous: 21%)	23
Sample preparation (solid-phase extraction)	20
2D, multidimensional, comprehensive LC×LC, column switching	12
Capillary electrophoresis (including MEKC, CZE, IEF)	10
Theory, retention mechanisms, modelling	7.9
Microfluidics, lab-on-a-chip	6.8
Method development, optimization, method transfer	5.5
Supercritical fluid chromatography (SFC)	5.3
Instrumentation, design	4.8
Preparative chromatography	1.8
Capillary electrochromatography (CEC)	0.9
Miscellaneous*	2.4

*Includes pressure-induced separations, temperature studies, field-flow fractionation, hydrodynamic chromatography, and capillary and nano LC.

and formats. However, compared to previous symposia in the series, the percentage of column-related talks actually dropped from a third of all presentations to a quarter of all presentations. Surprisingly, nearly 40% of the columns' papers dealt with monoliths, with polymeric monolith coverage nearly 2:1 over silica-based monolith talks and posters. In the future, silica monolith coverage may grow because the patents are winding down and perhaps new companies may investigate the technology. Although not yet considered a commercial success, research interest, especially in academia, in monolith technology is still running high. The polymeric monolith segment is less covered by intellectual property rights than the silica monolith segment. Silica gel-based monoliths are seeing their second generation, and maybe a third generation, of commercial products with better efficiency, but slightly higher pressure drops because of the change in the macropore–mesopore domain ratios. Still, the silica monoliths are only available from one source. However, a continuation of new developments in polymeric monoliths devoted to the separation of small molecules has shown improvements in column efficiency. Originally, silica-based monoliths were considered to be best for small molecules and polymeric monoliths were thought to be optimum for large biomolecules only. Those beliefs are beginning to change as silica monoliths are being developed for large molecules and polymeric monoliths for small-molecule separations.

Three other "hot" areas in column technology this year were:

- Continuing with the observation made in my recent Pittcon article (1), superficially porous packings (SPPs, also referred to as shell particles, poroshell, core–shell, and fused-core packings) that rival the sub-2-µm particles in terms of column efficiency, but with substantially lower pressure drops are now the hottest area in HPLC and UHPLC. The poster and oral papers referring to SPPs outnumbered those devoted to sub-2-µm totally porous particles. The availability of sub-2-µm SPPs and up to 5-µm SPPs have expanded the use of these special particles to mainstream HPLC as well as UHPLC applications. At HPLC 2014, there were reports of even smaller SPPs than those currently commercially available.
- Papers on two-dimensional (2D) and multidimensional chromatography doubled compared to last year's meeting. The technique is becoming more mainstream since major LC instrument companies now have easy-to-use accessories that provide accurate and rapid column switching and LC×LC (comprehensive LC) capabilities. For LC×LC, acceptance has also been brought about by the availability of more orthogonal stationary phases and column configurations such as short, fast SPP and monolithic columns for the second dimension. The 2D techniques are mainly useful when complex samples are encountered; food analysis was the most popular area for applications this year.
- Microfluidics, microchips, and nanochannels were all part of a continuing theme both in oral and poster sessions. Undoubtedly, the strong interests of the symposium chair in this discipline probably accounted for a slight bias in acceptance of papers looking at smaller dimension columns and instrument design to accommodate them. One could also lump in micro- and nano-LC columns into this category that I didn't segment separately. All of these approaches not only result in solvent and sample savings, lower dispersion, and higher sensitivity, but also easier interfacing into detectors such as MS detectors. One paper even showed the use of the flame ionization detector, which can cope with a low solvent flow rate.

In the columns area, I broke down the modes being used by HPLC 2014 attendees (see Table 2). As always, on a relative basis, reversed-phase LC again dominated the usage (43% of all papers) with hydrophilic-interaction chromatography (HILIC) maintaining its position at a distant number two. HILIC serves as a separation technique for polar analytes that are weakly retained by reversed-phase chromatography. The number of chiral separation papers showed a strong number three with SFC applications a driving force.

Sample preparation technologies were well represented in the poster papers, but only five oral presentations were presented that

Table 2: HPLC modes represented at HPLC 2014.

HPLC Mode	Relative Percentage of Papers (Oral and Poster) (%)
Reversed phase	43
HILIC, aqueous normal phase	14
Chiral	12
Anion exchange	7.8
Affinity	6.2
Cation exchange	4.6
Size exclusion	4.3
Normal phase	2.9
Mixed mode (such as reversed phase–ion exchange)	2.6
Other*	2.9
Capillary electrochromatography (CEC)	0.9
Miscellaneous*	2.4

*Consists of hydrophobic interaction, graphitized carbon, inverse SEC, cell membrane, C60-fullerene phases.

Table 3: Papers presented by major application area.

Application Area	Papers Presented (%)
Proteomics, proteins, peptides, biomarker	21
Food, food safety, beverages	9.6
Genomics, DNA and RNA, nucleic acid constituents	8.2
Pharmaceuticals, API, drug discovery	8.0
Consumer products, chemical products	7.6
Other life science (lipids, steroids, carbohydrates)	6.7
Endogeneous compounds in biological fluids or tissues	6.5
Natural products, traditional Chinese medicines	6.5
Environmental, industrial hygiene, pesticides	5.7
Drugs and metabolites in biological fluids or tissues	4.9
Toxins in biological fluids or tissues	4.1
Petroleum, hydrocarbons	3.5
Inorganic ions	3.1
Quality by design, validation	2.7
Drugs of abuse	1.8

had a sample preparation theme. No organized sessions were devoted to sample preparation this year. For poster presentations, the most prominent sample preparation subjects were solid-phase extraction (SPE), on-column digestion of proteins using immobilized enzymes, protein precipitation for analyzing drugs and metabolites in biological fluids, and filtration. On-line SPE, related to the column switching approach discussed above, was the subject of several posters. New instrumentation accessories have made on-line SPE easier to perform.

Electrodriven separation techniques (such as capillary electrophoresis [CE], capillary zone electrophoresis [CZE], micellar electrokinetic chromatography [MEKC], and isoelectric focusing [IEF]) grew this year with a strong showing in both oral and poster papers with many applications papers depicting great strides in interfacing to MS. A continued lack of interest in capillary electrochromatography (CEC) was noted with only four presentations at HPLC 2014.

Areas of Application

Table 3 is a brief breakdown of the most popular application areas reported at HPLC 2014. This year, I finely divided the applications areas, but again oral and poster presentations on life science topics dominated all applications. The various "omics" (for example, metabolomics, proteomics, and lipidomics) were out in front with post-translational modifications (such as glycosylated and phosphorylated proteins), monoclonal antibodies, and the search for biomarkers as the major fields of study. The techniques LC–MS and LC–MS–MS are an absolute requirement for these studies; hence, there were an overwhelming number of papers with these MS technologies presented at this meeting.

Pharmaceutical and biopharmaceutical companies are still the most prolific users of HPLC and UHPLC. If one adds up all the areas in Table 2 related to pharmaceuticals, it would be the second biggest application category, followed by food and food safety applications. Most of the other application areas were roughly the same as in previous years (2,3).

Awards and Honours at HPLC 2014

The HPLC meetings have become the venue for chromatography awards presented by various groups for best posters, best oral presentation by a young investigator, and awards from the Chromatographic Society. This year a new award for significant contributions by industrial chromatographers was added to the mix.

Poster Sessions and Best Poster Awards: The mainstay of HPLC 2014 was the poster sessions where more detailed applications and methodology studies were reported, often in very specific areas, and face-to-face discussions with the authors were conducted. Fortunately, many of the poster authors were kind enough to provide small reproductions of their poster papers that could be taken for later

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Thomas MEIER *(Bachem AG, Switzerland)*

Pharmaceutical Small Molecule Purification: Where Are We and Where Do We Need to Be?
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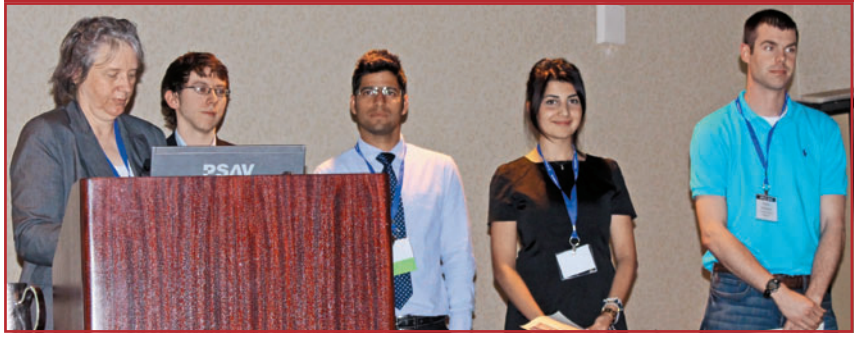
- Small and Large Molecules Purification, including Peptides, Biomolecules and Chiral
- Process Development, Scale-up, Continuous Chromatography, Multicolumn Processes
- Fundamentals and Process Simulation in Preparative Chromatography
- Latest Trends in Material and Media Development

SUBMISSION DEADLINES:
- Abstract submission for **oral communications: April 30, 2014**
- Abstracts for **posters** can be submitted after April 30 and **until August 25, 2014**
- Abstract submission is final as of **August 26, 2014**

Figure 1: Top three best poster awards, sponsored by Agilent Technologies. From left to right: Dr. Monika Dittman of Agilent Technologies, award presenter; third place winner (tied): Alexander Siegle, Ruprecht-Karls-Universität Heidelberg in Heidelberg, Germany, "Hadamard Transform Multiplexing LC: Boosting Throughput and Sensitivity of HPLC"; third place winner (tied): Suresh Kumar, Brigham Young University in Provo, Utah, USA, "An Integrated Microfluidic-based System for Complete Analysis of Preterm Birth Biomarkers"; second place winner: Dina Lakayan, VU University Amsterdam in Amsterdam, The Netherlands, "Hyphenation of Surface Plasmon Resonance optical sensing to size-exclusion chromatography for analysis of antibody preparations"; first place winner, Stephenen Groskreutz, University of Pittsburgh, Pittsburgh, Pennsylvania, USA, "Modelling Temperature Assisted Solute Focusing in Capillary High Performance Liquid Chromatography." (Photo courtesy of Martin Gilar, Waters.)



perusal. Some authors collected business cards and addresses for sending poster reprints by mail or e-mail. Compared to HPLC 2013 (1), the number of posters was greatly decreased. Only about half of the poster presenters elected to have their poster evaluated for the Best Poster Contest, which made the job a bit easier for the 31 reviewers that were assigned to the Poster Committee. The posters were up for four days, which gave viewers plenty of time to find them. It appeared that there were fewer "no shows" than had been observed in previous HPLC symposia.

The Poster Committee Chairpersons were Monika Dittman of Agilent Technologies and Chris Pohl of Thermo Fisher Scientific. The Poster Committee devoted a great deal of time and thought to the job at hand. They worked very hard to narrow down the collection of posters by the end of the third day to 17 finalists whose posters were identified with a Finalist placard. From these 17 finalists, reviewers chose the top 10 winners by the Thursday afternoon of the symposium. The selection criteria were based on three factors: Inspiration (creativity, newness, uniqueness, originality); transpiration (experimental execution; completeness of work); and presentation (overall readability, visual impression, author's explanation). Winning posters were viewed by all of the final committee jurors.

The Best Poster Awards, sponsored by Agilent Technologies, were announced at the closing session on Thursday afternoon. For HPLC 2014, the top three Best Poster Award winners (along with their affiliation and title of their poster) are shown in Figure 1. There was a tie for third place between two posters, which was a first since poster prizes started being awarded. Each winner received a cash prize. Because of space limitations, detailed technical coverage of each of the award-winning posters cannot be provided. It suffices to say that all the winners and finalists should be proud of their accomplishments since they represent the top 1% of all posters presented at HPLC 2014.

Horváth Award: For the ninth year in a row, the Horváth Award sessions, named for the late Professor Csaba Horváth, one of the founders of this series and a mentor of young scientists, were featured. This award, supported by HPLC Inc. a nonprofit group under the guidance of the Permanent Scientific Committee was established for young scientists in the separation sciences under the age of 35. The award, based on the best oral lecture presented in the Horváth sessions, was selected by a jury named by the Permanent Scientific Committee and consists of a cash prize, an invitation for an oral presentation at HPLC 2015, and a crystal trophy. This year there were seven nominees all with strong research credentials.

The winner of the 2014 Horváth Award was William Black (Figure 2) of the University of North Carolina at Chapel Hill, North Carolina, USA, marking the second year in a row that the award winner was from this institution. The title of his oral presentation was "Integrating Solid-Phase Extraction with Microchip Capillary Electrophoresis — Electrospray Ionization". With limited preconcentration possibilities for CE and with disruptions in electroosmotic flow and band broadening introduced by conventional on-line SPE techniques, Black described a microfluidic platform that was a promising candidate for an integrated CE system. Microfluidic technology is well suited towards integrating multiple functionalities and can precisely manipulate small volumes with zero dead volume. In addition, lab-on-a-chip technologies are amenable to automation, resulting in improved reproducibility and throughput. In his study, a novel design is presented that couples microfluidic SPE with microchip CE–electrospray ionization (ESI) with the goal of a fully integrated analysis system combining the sample processing power of SPE with the speed and separation performance of CE. The design was reproducible (migration times %RSD = 0.5), required low sample consumption (25 fmole loaded on chip), and showed a 150-fold improvement in sensitivity while maintaining high separation efficiency and MS compatibility. Furthermore, this design is potentially adaptable to many different applications by utilizing alternate stationary phases, resulting in a fast, automatable, fully integrated universal platform for sample analysis. Future work will be the addition of alternate sample prep techniques such as on-line desalting,

Figure 2: William Black of the University of North Carolina, Chapel Hill, North Carolina, USA, winner of the 2014 Csaba Horvath Young Scientist Award. (Photo courtesy of Martin Gilar, Waters.)

integration with hydrogen–deuterium exchange MS, and improvement in analyte recovery, especially for low *k*' compounds.

Chromatographic Society Awards: In 1978, Nobel Prize winner Professor A.J.P. Martin gave permission for his name to be associated with the "Martin Medal." The Martin Medal is awarded to scientists who have made outstanding contributions to the advancement of separation science.

At HPLC 2014, the Martin Medal was awarded to Professor Nobuo Tanaka, retired from Kyoto Institute of Technology in 2009, GL Sciences (2009–2013) in Tokyo and, most recently, a visiting professor with the University of California, Davis, California, USA. Professor Tanaka's research is truly multidisciplinary and internationally acclaimed. His work spans research on highly selective stationary phases, isotope separation and separations based on isotopic chirality, separation mechanism elucidation, multidimensional separations, and biological separations. Other notable areas of research include his seminal work on pressure-induced retention changes in reversed-phase LC and stationary phase characterization; and he was also a major contributor in the area of monolithic silica capillary columns for LC and CEC. He contributed to the development of the monolithic silica rod column that was commercialized by Merck in Germany. Tanaka has received numerous awards reflecting his outstanding contributions to the chromatographic sciences.

Although the award ceremony will take place at another chromatography meeting, the Jubilee Medal winner for 2014 is Prof. Michael Lammerhofer of the University of Tubingen, Germany. This award is presented to an up-and-coming young separation scientist. Some of Dr. Lammerhofer's cited contributions were those in chiral stationary phases, metabolomics, and plasmid DNA analysis.

Uwe Neue Award: Uwe Neue, who passed away more than three years ago, was an industrial chemist working for Waters who gained international respect for his contributions to the field of separation science, especially for work that turned into commercial products. The Uwe Neue Award, sponsored by Waters, is directed to scientists, like Uwe, who have had great careers in industry while contributing to the further development of chromatography. The inaugural winner announced at the last HPLC 2013 meeting was Jack Kirkland, and this year's winner was Gerard Rozing, now retired from Agilent Technologies (see Figure 3). Rozing is widely known and recognized as an outstanding researcher for his work that bridges fundamental science with technical solutions in a commercial environment. He worked in industry for more than 30 years before retiring in 2012 to become a consultant for Agilent Technologies and other organizations. He has authored more than 57 publications and holds six US patents.

On his personal webpage, Rozing stated "It is always a great honour to become recognized through an award. But I feel deeply honoured by this award since it is named after Dr. Uwe Neue, a contemporary peer with whom I shared a common career pathway in many aspects. I met Uwe the first time some 25 years ago during one of the HPLC symposium series and many times since then. I have been impressed with his ability to maintain his scientific integrity in a for-profit-organization environment where unlike in academia, the science has to compromise with engineering, marketing, finance, sales, and manufacturing functions." In his address, Gerard thanked the Permanent Scientific Committee for his selection, Waters for their sponsorship, and his colleagues at Agilent for their help and support over the years. In particular, he thanked Peter Hupe, the father of Agilent's liquid-phase separations business and cofounder of this HPLC symposium series, for his part in being Rozing's role model. In addition, he also thanked his friend and career-long manager, Fred Strohmeier, currently senior vice-president of Agilent, who gave him this role and freedom to pursue his more recent career in managing university relations and external collaborations.

Rozing's Award lecture was entitled "Current and Future Perspectives on UHPLC; Requirements for Improved Abilities and Functionality". In his lecture, he first discussed the big changes that occurred a decade ago with the evolution of HPLC to UHPLC driven by the increased pressure requirements of sub-2-µm packings and the advent of high pressure solvent delivery systems that preserved the efficiency gains with the smaller particles. He then speculated as to whether increased pressure beyond current usage will deliver even more chromatographic performance. He concluded that kinetic optimization predicts that the maximum plate number achievable does not increase proportionally with pressure, but that increased pressure will result in a shorter time to obtain the required plate number. However, such inferences from kinetic plot data apply only if *H* is independent of column length; physical and chemical properties of solvent and solute, particle properties, and column dimensions are independent of pressure; and frictional heating will be negligible. However, we now know that high pressure does affect all of these parameters, but not independently. Rozing then went on to illustrate how pressure

Figure 3: The Uwe Neue Award presented by Waters. From left to right: Martin Gilar of Waters; Jack Kirkland of Advanced Materials Technology (2013 winner); and Gerard Rozing, retired, Agilent Technologies (2014 winner). (Photo courtesy of Martin Gilar, Waters.)

affects each of these parameters and presents some challenges in future system design. He presented some alternative ideas such as constant pressure operation and the increased use of multidimensional LC to address the constraints placed on systems and columns by higher pressure operation. For a copy of his complete presentation, go to his website: www.rozing.com.

New Column Technology Highlights

Many oral, poster, and tutorial sessions were devoted to stationary phases and column technology, always topics of high interest at this series of meetings.

Monoliths: As mentioned earlier, in the columns' area, monoliths received a great deal of attention at HPLC 2014. Two oral sessions and one poster session were devoted to monoliths, although monolith papers were scattered throughout the oral and poster sessions. Monolith columns have long been desirable since they exhibit high permeability and low pressure drop (because of increased bed porosity), show reasonable separation efficiencies, have the absence of frits to confine the packing material, are easy to fabricate, and nowadays can be made fairly reproducibly. Although this technology has been around for several years, as a routine tool it has yet to see widespread acceptance on the commercial side, but improvements continue to be made for both polymer- and silica-based monoliths.

In his opening keynote lecture on Monday morning, Professor Frantisek Svec of the Molecular Foundry at Lawrence Berkeley National Laboratory summarized the work of the last two decades of polymeric monolith development, much of which was performed in his own laboratories. His first publication in this area appeared in 1992 (4) and was based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths. The initial work involved single-step polymerization where the active functionalities were found throughout the monolith but some of these groups were not accessible. Each new monolith chemistry required individualized optimization. The next stage in monolith development involved chemical modification where accessible functionalities could be found throughout the monolith. Further improvement in monolith synthesis was the use of photografting to apply a monolayer of functionalities only on the pore surface. This approach required one-time optimization. A two-step (or multistep) process was used for UV-transparent monomers. An example of the chemistry applied here were the photografted poly(butylmethacrylate-*co*-ethylene dimethacrylate) monoliths. The group next explored the attachment of nanoparticles with the desired functionality to the pore surface. The surface of the monolith required prefunctionalization before adding the nanoparticles. Their initial study involved the modification of the monolith with functional latex nanoparticles. After these nanoparticles were attached, they formed a very stable, reliable ion-exchange phase.

Recently, Svec and his group began to work with metallic nanoparticles. In the first studies, gold nanoparticles (15 nm diameter) were used. These were found to be quite selective for thiol groups which allowed for a stable, universal ligand that could be used to pull out thiol-containing proteins and peptides from solution. The thiol compounds can be exchanged, thus making the gold nanoparticle on monolith a universal intermediate ligand that enables the surface chemistry to be changed via the attachment of different thiol-containing compounds with desired functionalities. He showed a separation of proteins by reversed-phase chromatography on a HSC18 functionality. The HSC18 was then replaced with an $-SO_3$ containing thiol and used in an ion-exchange mode thereby reversing the elution order. He went on to further exploit this technology by using the gold nanoparticles with two different thiols to provide a mixed-mode stationary phase, one mode with a reversed-phase moiety and the other with a carboxyl functionality. To keep the costs down, Svec is now replacing the gold nanoparticles with silver nanoparticles. The silver also interacts with the thiol groups. The silver–monolith conjugate can now be used as a scavenger for compounds such as iodine and iodide contained in iodinated organic compounds, especially useful for radioactive iodine removal from reaction mixtures, thereby generating a closed solid radioactive waste for easier disposal.

Later in the day, Emily Hilder of the University of Tasmania continued the discussion on the preparation and characterization of porous polymer monoliths for chromatography. She and her colleagues have been focusing on trying to improve the reproducibility of polymeric monoliths by investigating the degree of bed homogeneity using novel polymerization methods. She reviewed a number of the approaches to form ordered porous polymers, such as cryopolymerization using unidirectional freezing, the incorporation of nanoparticles during the polymerization process that improved structural homogeneity, polymerized high internal phase emulsions, and medium internal phase emulsions. They also investigated a range of different emulsion stabilizers including amphiphilic block co-polymers, surfactants, as well as various nanoparticles such as charged ones. The results of the various approaches were studied by scanning transmission x-ray microscopy, which provides high-resolution information about the spatial distribution of chemical components within these monolithic materials. The unidirectional freezing method gave very ordered structures which could be changed by varying the monomer concentration and immersion speed that affected pore size.

A number of other papers dealt with the synthesis of polymeric monoliths for specific tasks. Polymeric monoliths have long been thought to be best suited for macromolecules, but there has been ongoing work on the production of polymer monoliths for small molecules. One research group at the University of Pardubice in the Czech Republic has investigated short thermal polymerization times for the preparation of lauryl methacrylate-*co*-tetraethyleneglycol dimethacrylate monolithic scaffolds that underwent a secondary polymerization with a zwitterionic monomer and crosslinker to form a HILIC phase. The polymeric monolith was synthesized inside of a 0.32-mm i.d. fused-silica capillary and the column showed 55,000 plates/m for thiourea. Another poster paper studied a similar reactive polyacrylate monolith that was derivatized inside a fused silica capillary resulting in a column also used for HILIC as well as a support for CEC. A group from Saudi Arabia used an in situ polymerization procedure using itaconic anhydride as a monomer and ethylene dimethacrylate as a crosslinker. The itaconic anhydride enabled post-polymerization modification of the monolith column via amidation with a suitable amine. Using octadecylamine and benzylamine as post-polymerization reactants allowed the generation of reversed phase functionality, one with an alkyl (C18) and the other with an aromatic benzene ring.

Silica monoliths were also covered in both oral and poster presentations. Of course, these have been around as commercial products for a long time but researchers continue to work to improve them.

Merck Millipore's Karen Cabrera presented an oral talk about modifications that they have made on their silica monoliths to make them more suitable for large biomolecules. By developing a third-generation monolith with a bimodal pore structure (for example, 2-µm macropores and 30-nm mesopores), the column was found to provide good performance for proteins and antibodies. Separations could be performed within 3–4 min with no carryover. Furthermore, the monolith could be derivatized with 3-glycidyloxypropylsilane, thereby obtaining an epoxide surface enabling chemical immobilization of proteins. Enzymes such as trypsin and affinity phases such as protein A could be immobilized. The trypsin column was coupled in front of an LC column and used for on-column protein enzymatic digestion followed by separation. The protein A phase could be used to quantitatively measure IgG from mixtures. In a poster paper, the same group showed how dirty samples from the food and pharmaceutical industries can be directly injected onto a monolithic silica column and it suffers no ill effects. The reason is that the column doesn't require frits and the unique structure of the stationary phase with flow-through pores provides a high permeability compared to a typical packed LC column. Of course, samples have to be filtered to remove particulates, but spiked samples such as homogenized porcine kidney, a soy drink, and human urine were successfully analyzed by LC–MS. In an interesting poster from Kyoto University in Kyoto, Japan, the author Takuya Kubo immobilized carbon-based nanomaterials (such as graphene, fullerene, carbon nanotubes) to the walls of a fused silica column and to a silica monolithic capillary using a photothermal reactive agent, perfluorophenyl azide. The phases were used for the baseline separation of polycyclic aromatic hydrocarbons with the modified monolith providing a better separation than the wall-coated version. A titanium oxide capillary column–based monolith was developed using the sol-gel process by the Carol Collins group at the University of Campinas in Campinas, Brazil.

The 2014 Martin Gold Medalist Nobuo Tanaka, one of the world experts in silica monoliths, presented a paper in one of the monolith sessions where he spent most of his time discussing extracolumn effects with both small monolithic silica and packed silica capillary columns, the latter with a superficially porous support and a 2-µm C18 totally porous packing material. Basically, it was concluded that for a 50 mm × 1 mm column, unless one has a nanoLC or a very well optimized UHPLC system, it is very difficult to achieve the full efficiency of any column. Columns with smaller internal diameters do not pack as efficiently as columns with larger internal diameters, and some of the efficiency loss is because of the looser packing density. The effect is worst for early eluted peaks. Monolithic silica columns are less affected by the retention factor because of their higher porosity resulting in larger peak volume than particulate columns, but they are less efficient at higher flow rate because of the presence of large macropores.

High-pH Columns: Why do chromatographers want to use high-pH mobile phases? High pH will sometimes provide improved selectivity between a target compound and impurities. In

addition, basic compounds become neutral at high pH and often exhibit higher loading capacities compared to protonated species.

Silica gel has always had the limitation of dissolving at high pH and with a typical monolayer bonded phase, the upper pH limit is usually stated as pH 8. Over the years, to extend this upper limit, workers have coated the particle with polymer or a very high loading of bonded phase, used special phases to prevent hydroxide ion attack, used special organic buffers that impede the progress of attack, and so on. Silica-organic hybrids and polymer particles have been one solution to this pH limitation. The latter frequently have much lower efficiency than silica-based columns.

Recently, silica researchers started looking at other ways to extend the pH range of silica gel-based bonded phase particles. At HPLC 2014, posters of four different companies showed new proprietary silica-based products that exceed the regular pH 8 specification: AkzoNobel/Kromasil, Agilent Technologies, Advanced Chromatography Technologies (ACT), and Daiso. The Kromasil EternityXT material is based on a stable fully porous silica hybrid with organosilane reinforcement designed to work in a wider pH environment from 1 to 12. It is available in particle sizes from 1.9 to 10 µm, all with the same chemistry. Phases include C18 and phenyl-hexyl. The column can be rinsed with sodium hydroxide solution. The Agilent product is Poroshell 120 HPH and comes in C18 and C8 phases. It is a superficially porous particle with a 2.7-µm size with a recommended pH range of pH 3–11. Agilent uses a proprietary process to organically modify the Poroshell 120 silica support. The ACT UltraCore column comprises superficially porous particles with C18 and phenyl-hexyl bonded phases for reversed-phase applications. It uses Encapsulated Bonding Technology, which is designed to provide an extended pH range of 1.5–11.0. The fourth company, Daiso, a Japanese company, reported on two new base silicas featuring extended pH range: Peptisil for general peptide purification and Insusil for insulin purification. Both products are bulk silica designed for process scale LC.

Sub-2-µm Superficially Porous Particles: As reported earlier, SPP particles were still very much in vogue at HPLC 2014. Joe DeStefano of Advanced Materials Technology gave an interesting take on the future direction of UHPLC columns with a provocative title of his lecture "Are Sub-2-µm Superficially Porous Particles Needed for Small Molecule Separations?" DeStefano considered the tradeoffs between using SPPs in the 1.3–1.7-µm range (or even smaller) and the currently popular 2.5–2.7-µm sizes. As theory suggests, column efficiency is dramatically improved with smaller particles. However, the smallest SPP currently available does not provide the expected efficiency based on particle size, probably because of the difficulty of packing very small particles into homogeneous beds. In addition, most UHPLC and HPLC instruments are unable to maintain the high efficiencies of the columns because of band spreading in the extracolumn volumes of the connecting tubing and flow cells. Also, these smaller particle columns operate at extremely high pressures, require the use of more-specialized, more-expensive low-dispersion UHPLC systems, and may not be user friendly for routine operations. The smaller-particle SPP columns may be subject to more frictional heating, and columns with smaller internal diameters (≤3 mm) must be used to minimize this effect. Since smaller-porosity frits must be used to contain the packing, smaller-particle columns may be more subject to plugging. The practical answer is to have an SPP with a diameter between the smallest and those more typically being used today. DeStefano went on to recommend consideration of 2-µm SPP columns where the solid core would be 1.2 µm with the porous outer shell being 0.4 µm. He showed a series of van Deemter plots comparing sub-2-µm SPP and 1.7-µm porous particles to 2.0- and 2.7-µm SPP particles. The 2.0-µm, 50 mm × 2.1 mm SPP column gave 15,570 plates when operated with a flow rate of 0.5 mL/min and showed the best performance in terms of plates/bar compared to sub-2-µm columns.

In his keynote lecture, Professor Yukui Zhang of the Chinese Academy of Sciences in Dalian, China, showed some results on a 1.9-µm core–shell particle that was synthesized in his group's laboratory. Rather than the layer-by-layer method used in current technologies, they used a three-step method starting with a narrow particle size distribution (1.56 ± 0.06 µm) nonporous silica particle prepared by a modified, seeded growth method, a mesoporous shell formation by a one-pot template dissolution and redeposition strategy, and finally a pore size expansion via acid refluxing (HCl treatment). By such a strategy, core–shell materials with pore channels perpendicular to the particle surface, adjustable particle size, controlled shell thickness, tailored pore diameter, and high surface area could be obtained. The final material was derivatized for reversed-phase and chiral chromatography. The C18 phase displayed 211,300 plates/m for naphthalene. A wider pore version (1.5-nm pore size) was used to separate intact proteins. In the same presentation, Zhang also reported on their work on organic-silica hybrid monoliths.

Monodisperse Porous Silica Columns: Recently, with the emphasis on SPP and their narrow particle size distribution giving greatly increased performance, the idea of narrow particle size distribution totally porous particles for increased performance has arisen, especially since a new commercial product (Titan, Sigma Aldrich/Supelco) has been introduced. In a recent special issue of *LCGC North America* (8), Richard Henry addressed the issue of the impact of particle-size distribution on HPLC column performance. He found that a narrow distribution of porous particles did show better performance as evidenced with a lower reduced plate height with a lower pressure drop than a similar particle size with a wider particle size distribution. At HPLC 2014, Nagae and Tsukamoto of ChromaNik Technologies presented a poster where they too examined the effect of monodispersity on performance of reversed-phase packings prepared

from similar silica starting materials. They compared a conventional 3.19 µm totally porous particle (D_{90}/D_{10} = 1.48) to a 2.81-µm monodisperse totally porous particle (D_{90}/D_{10} = 1.09) and a 2.78 µm core–shell particle (D_{90}/D_{10} = 1.11). Reduced plate heights were 2.57, 2.22, and 1.75, respectively, indicating that the narrow distribution porous particle gave about 16% better efficiency than the conventional wider distribution porous particle. However, in their studies the core–shell particle outperformed the porous particles by nearly 50% on a reduced plate height basis.

HILIC: David McCalley of the University of the West of England can always be counted upon to deliver a clear, informative lecture and his keynote oral presentation at HPLC 2014 was no exception. He talked about new developments in HILIC. Table 2 shows the relative strength of HILIC in mode usage whose remarkable growth shown here agrees with a recent survey of *LCGC* readers (9). The mechanism of HILIC separations is quite complex, involving a mixture of partition of solutes into the water layer on the stationary phase surface, adsorption onto polar surface groups, and ionic interactions with the silica matrix for those particles based on that medium. In the present paper, David and colleagues further investigated the mechanism of HILIC, studying the effect of buffer composition on retention and peak shape of acidic, basic, and neutral solutes on a variety of columns — bare silica, zwitterionic, amide, and silica hydride. Buffers investigated included 0.1% formic acid (v/v), 5 mM ammonium formate (pH 3.0), and 0.1% phosphoric acid (v/v). Investigating a 90:10 (v/v) mixture of acetonitrile and the three buffers and measuring retention of the test compounds, he found that for the ammonium formate buffer, there was relatively low retention of neutrals and acidics but a higher retention of basic solutes, probably because of ionic interactions. For the formic acid buffer, there was an even higher retention of basic compounds compared to the ammonium formate buffer, perhaps because of the absence of competition from ammonium cations. For the phosphoric acid, ionic interactions couldn't be suppressed even at the low pH given by this acid. Formic acid gave poor efficiency for ionizable solutes with all types of silica-based HILIC columns in part because of the low ionic strength of the acid, which is exacerbated by the high concentration of acetonitrile in the mobile phase. Salt buffers like ammonium formate have a higher ionic strength and may also promote formation of the water layer on the column surface. Further evidence for ionic interactions was shown by comparing retention of the base pyridine and (neutral) uridine, which have similar hydrophilicity. However, pyridine is much more strongly retained. Consideration of the pH in the aqueous–organic mobile phase may be more appropriate in understanding retention.

Surprisingly, the silica hydride–based column gave similar selectivity to the silica column and a higher retention of cations just like bare silica columns.

As a side note, McCalley investigated the use of charged aerosol detection (CAD) and found it to be quite useful for HILIC separations, presumably because of the high content of organic solvent, and thus the ease of desolvation of solutes. The charged aerosol detector did show a nonlinear response curve for many solutes, but gave good sensitivity.

Column Packing Studies: Some researchers keep working on silica- and polymeric-monolith columns to realize their full potential, others continue to think about the next generation of conventional packed HPLC columns. For the latter group, the idea of decreased particle size immediately comes to mind, whether it is a totally porous particle or a superficially porous particle. The production techniques for such small particles, even submicrometer, are already available. The sizing could be a challenge for porous particles, although the process to make superficially porous particles is readily available. There are several obstacles facing researchers: Ensuring that the next generation of instruments will provide low enough dispersion to realize full efficiency of the columns at hand; the frictional heating that may arise from even smaller particles and higher pressures; the impact of higher pressures on physical and chemical properties of solvents and solutes; and the ability to pack particles effectively in all dimensions of tubing from microchannels to analytical scale. It should be noted in the latter case that many still consider HPLC column packing to be an art (5).

To that end, Jim Jorgenson's group at the University of North Carolina has begun a series of studies looking at optimized procedures to pack smaller particles (sub-2-µm) routinely and reproducibly. At HPLC 2014, he reported on some early results in their attempt to understand the slurry packing of capillary LC columns. Capillary columns, of course, would be best for heat dissipation, but present a bigger challenge than larger bore column formats. In their previous studies, they had success in packing nonporous reversed-phase packings as small as 0.9-µm but in the case of porous particles, column efficiency tended to decline as the particle diameter decreased, especially below 1.5-µm. They estimated that for small molecules, with operating pressures in the 1000–4000 bar range, particles of around 1-µm in size should be close to optimum.

In earlier work in their laboratories along with that of Ulrich Tallarek at Philipps-Universitat in Marburg, Germany, they considered packing morphology and separation efficiency of low-aspect-ratio capillary UHPLC columns. They combined chromatographic studies with confocal laser scanning microscopy to examine packing structure, including transcolumn porosity heterogeneity (6). The studies correlated structural features of the packing, such as an increased number of packing voids, with decreased efficiency. They investigated the effect of slurry concentration: 5, 10, 20 mg/mL all the way up to 100 mg/mL and found that higher slurry concentrations and rapid packing gave them higher numbers of packed bed voids while low slurry concentrations and slower packing gave more particle size

segregation. They figured that a balance in concentration must be achieved to minimize the two types of bed heterogeneities. In considering bridged ethyl hybrid (BEH) particles between 1.4- and 1.9-µm, they found that for a given length of column they needed a different intermediate slurry concentration to produce the best performance. For example, they found that for a 34 cm × 75 µm capillary packed with fully porous 1.4-µm BEH particles that an optimum slurry concentration (lowest *H* value) was 20 mg/mL. When they tried to pack a 1-m capillary column with a 20-mg/mL slurry of 1.4-µm BEH particles, the results weren't conclusive. However, when they broke the column into three approximately 30-cm sections, the outlet section (that is, the first packed segment) of the column performed not only better than the other two sections, but also as expected given the initial slurry concentration study. Work is continuing on optimum slurry packing techniques for small particles and capillary columns.

Method Translation and Related Parameters

Since the advent of UHPLC and the myriad instruments with high pressure capability have been on the market, many laboratories are now looking to convert older methods to the newer instrument platforms. Method transfer should be a relatively painless exercise since all one has to do is to get a column with the same chemistry but with a different column particle size and perhaps a different column length and internal diameter. Wrong! There are a number of parameters that have to be addressed. Some aspects of method translation were covered in a tutorial and poster papers. A poster paper by Karim Kassam of Advanced Chemistry Development entitled "Translations Between Differing Formats of Liquid Chromatography: Advantages, Principles, and Possible Pitfalls" gave some food for thought in making these translations. Assuming one has a column with the same chemistry hopefully by the same manufacturer, this eliminates one overwhelming parameter that has to be overcome. The paper outlined the key factors to LC method translation whether it is HPLC to UHPLC, HPLC to HPLC, or UHPLC to HPLC. They are: Dwell volume differences, incorrect dead volume estimations, differences in instrument design, differences in efficiency caused by extracolumn band broadening effects and peak overloading, and retention time shifts as a result of heat of friction and pressure. Dwell volume is the volume from where the solvents A and B first meet to the head of the column. Without going into a lot of detail, there are methods available to determine dwell volume for a given instrument, but the value must be stated for any method because application of a fixed gradient for two instruments with different dwell volumes will result in a totally different chromatogram. The dead volume of a column is the volume to elute an unretained peak. When using different types of packed or monolithic columns, one must determine the dead volume by the injection of an unretained peak. In reversed-phase chromatography, something like uracil is frequently used. For more details, consult reference 7. Eduard Rogatsky of the Albert Einstein College of Medicine, New York, USA, gave a tutorial entitled "Dwell Volume: Hidden Aspects of UPLC Method Transfer". Rogatsky contends that different manufacturers measure dwell volume using different methodologies. He further states that there is no standard method to use. The physical volume of the system is not equal to the dwell volume (also called the gradient delay volume). The dwell volume will change with pressure if a pulse damper is part of the system. He recommends to measure the delay volume by using a water–acetone mixture in one pump and a UV detector to measure the absorbance change of the baseline. Make a step change (or linear gradient) and then take the midpoint (one-half the maximum absorbance) of the absorbance change. It all goes back to making sure that you measure the dwell volume the same way for both instruments when making a method translation and make sure you always state the dwell volume as part of the method.

Future HPLC Symposia

The next major symposium in this series, the 42nd Symposium, will be held 21–25 June 2015, in Geneva, Switzerland. The chairman of this meeting will be Professor Gérard Hopfgartner of the University of Geneva. For more information, go to www.hplc2015.org. On 21–25 September 2015, the series (#43) returns to China, this time being held in Beijing. The symposium chair will be Professor Guibin Jiang of the Chinese Academy of Sciences. You can learn about this meeting at the following website: www.hplc2015-beijing.org. If you can't make it to Switzerland or China, HPLC 2016 will return to San Francisco, California, USA, as it has every 10th year since HPLC 1986. The dates will be 19–24 June 2016, and the chair will be Professor Robert T. Kennedy of the University of Michigan. Bookmark all of these websites so that you can keep up on the latest happenings.

Acknowledgements

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Radical Mass Spectrometry as a New Frontier for Bioanalysis

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In this instalment, we discuss radical ion chemistry as an increasingly important area of mass spectrometry (MS) development and the application to bioanalysis. At the current stage, most of the research is performed by a small set of academic groups. Given the unique capability offered by radical MS compared to the traditional studies on even-electron ions of analytes, it is likely that these types of fundamental studies will attract more attention and even be commercialized in the near future.

Radicals are atoms, molecules, or ions that contain one or more unpaired valence electrons or an open electron shell. They are, generally, highly reactive because of their need to convert themselves to more stable, even-electron species. Well-controlled radical chemistry can provide the means for conducting some of the most difficult and delicate chemical transformations, like converting RNA to DNA by ribonucleotide reductase, an enzyme with a radical as its catalysis centre. Radical chemistry can be coupled with mass spectrometry (MS) to tackle traditionally challenging problems such as sequencing disulphide proteins and determining C=C location in lipids. Here, we discuss this increasingly important area of MS development and its application to bioanalysis.

Background

MS has been established as a powerful tool for qualitative and quantitative chemical analysis. Its principle of analysis involves at least three crucial steps for extracting as much information as possible from an analyte: Forming ions from the analyte molecule of interest, performing mass-to-charge ratio (*m/z*) analysis (so-called *mass analysis*), and perturbing ions to induce *m/z* changes. It typically relies on *tandem mass spectrometry* (MS–MS), a process in which ions are allowed to interact with neutral molecules, electrons, ions, electromagnetic waves, and so on.

During the past several decades, these three steps have reflected significant advances. The development of various soft-ionization methods permits the transformation of almost any molecule or chemical entity from its original physical state to gas-phase ions with the least change to its structure. (Nevertheless, it is frequently necessary to add a charge carrier to the analyte, facilitating MS analysis.)

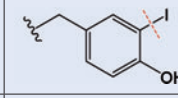
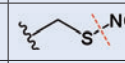
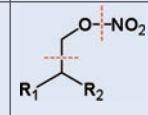
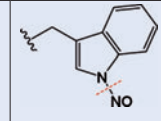
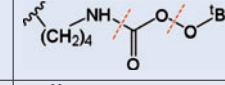
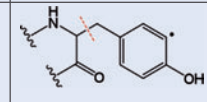
Several types of mass analyzers, the component of mass spectrometers that separate ions according to their mass-to-charge ratios, have been developed and commercialized. Instruments with time-of-flight (TOF) analyzers offer a fast speed of analysis. Fourier transform ion-cyclotron resonance (FT-ICR) and orbital ion trap instruments offer high-performance mass measurement and achieve a resolution in millions and sub-parts-per-million mass accuracy. Accelerator MS (AMS) instruments offer superb dynamic range (10^8–10^{15}) for isotope analysis. Finally, some instruments could even be made portable, miniaturized for performing field analyses.

For tandem MS, many activation and dissociation options are available, including collision-induced dissociation (CID) and electron-based excitation or dissociation methods. Clearly, MS has entered into a golden age of advanced development and can be readily applied to solving complicated problems, whether in the areas of drug discovery, cancer research, environmental monitoring, or exploring Mars.

Some of our most pressing questions about MS concern whether its usefulness has been fully exploited and, if not, what its next major advance will be. Its history shows that innovation in instrumentation and inquiries in new gas-phase ion chemistry often went side-by-side, providing the impetus for additional MS development. We believe the scope of gas-phase ion chemistry is unlimited, that its exploration so far has been only marginal, and that it may therefore give rise to the next major MS development.

An area of increasing attention is the development of gas-phase, radical-ion chemistry for bioanalysis. Radical ions, which consist of unpaired electrons, derived from biomolecules (peptides, proteins, lipids, and carbohydrates) are significantly less investigated than even-electron ions. The available ionization methods do not lend themselves to directly forming the radical ions of biomolecules, so additional reaction steps are required to effect the transformation from even-electron ions. Several research groups have invested a major effort in developing methods to produce radical ions of biomolecules. Given the coexistence of two reactive functional groups within one ion, a charge and a radical site, the chemistry of radical ions is rich. It differs significantly from the chemistry of even-electron ions. This difference can be exploited to provide structural information that complements the structural information gleaned from even-electron ions. A well-known example is the development of electron-capture dissociation (ECD) and electron-transfer dissociation (ETD). These techniques provide rich structural information for protein analysis that complements the information from CID. Thus, it substantially improves the

Table 1: Representative list of peptide radical ions studied in gas phase; type of radical ions, methods of formation, and precursors.

Radical Ion Type	Method of Formation	Precursors	Structure	References
$[Pep + (n+1)H]^{n\bullet+}$ (*,‡)	ECD/ETD/MAD	$[Pep + (n+1)H]^{(n+1)+}$		1–3
$[Pep + (n-1)H]^{n\bullet+}$(*,§)	CID EID/FsLID	$[Pep+ M+L]^{n+}$ $[Pep + H]n+$		4,5 6–8
a_n+1 or x_n+1 radical(†,‡)	UVPD, 157 nm	$[Pep + nH]n+$		9
Tyrosinyl radical(†,§)	UVPD, 266 nm	Iodine-substituted tyrosine		10,11
Thiyl radical(†,§)	CID	S-Nitrosated cysteine		12,13
Histidine radical(†,§)	CID	$[Histidine +M+L]^{n}$		14
Ser/Thr side-chain radical(†,§)	CID	Nitrate esters of serine/threonine		15
Trp-aminyl radical(†,§)	CID	N-Nitrosated tryptophan		13
Aminyl radical(†,§)	CID	Lysine peroxycarbamate		16
Glycyl radical(†,§)	MS3 CID	3-Tyrosinyl free radical		17
$[Pep - nH]^{(n-1)\bullet-}$(*,§)	EDD/NETD	$[Pep - nH]^{n-}$		18,19
$[Pep - nH]^{(n+1)\bullet-}$ (*,‡)	niECD	$[Pep - nH]^{n-}$		20

Pep = peptide; M = metal ion; dashed line = hemolytic bond cleavage; * = radical site is not defined; † = initial radical site is defined; ‡ = hydrogen rich radicals; § = hydrogen deficient radicals

capability of protein identification and characterization.

Bio-radicals have been implicated as important intermediates in a wide variety of biochemical processes. At the molecular level, they are associated with oxidative damage to proteins, DNAs, and lipids, which have been shown to be related to ageing, neurodegenerative diseases, and cancers. Several classes of enzymes also use radicals for catalysis. For example, ribonucleotide reductase uses a free radical (thiyl radical) for its catalytic activity. Interestingly, the radical storage centre is tens of amino acid residues away from the catalytic centre. It is not clear, at this point, how the enzyme tightly regulates radical reactivity along this long-range radical transfer process. In general, knowledge of the bio-radical species is constrained by the limited techniques available to analyze these reactive intermediates. Studying the gas-phase chemistry of bio-radicals, therefore, provides direct experimental evidence of their intrinsic chemical properties (reactivity, energetics, and structure), which can help explain the fate, including intra molecular and intermolecular radical transfer, of bio-radical species after they initially form.

The following discussions provide examples of methods of forming bio-radical ions of different classes of biomolecules and how radical chemistry and MS can generate useful structural information.

Peptides and Proteins

Table 1 summarizes the major types of amino acid or peptide radical ions that have been studied, together with their methods of formation. They are categorized as follows, according to their underlying mechanisms of radical-ion formation:

- Electronic state change of even-electron species via electron capture, electron transfer, electron detachment, interactions with high-energy photons, or collisions with meta-stable neutrals.
- Intramolecular electron transfer in peptide-metal-ligand complexes.
- Homolytic cleavage of labile bonds (either by CID or photolysis).
- Radical reactions of disulphide-linked peptides in the plume of electrospray ionization (ESI).

When an electron adds to an even-electron peptide cation (multiply protonated), it results in more hydrogen than the corresponding even-electron species. Subsequent dissociation of the hydrogen-rich radical ions produces N-Cα backbone fragments, as compared with amide-bond cleavages of even-electron ions from vibrational activation (21–23). Analytical advantages of fragmenting hydrogen-rich radical ions include less dependence on peptide sequences and preservation of the labile modification on amino acid residues. ECD (1,24) and ETD (2,19) are the two major methods using fragmentation

Figure 1: Radical reactions in the nanoESI plume involving disulphide-linked peptides to form sulphur-centred peptide radical ions.

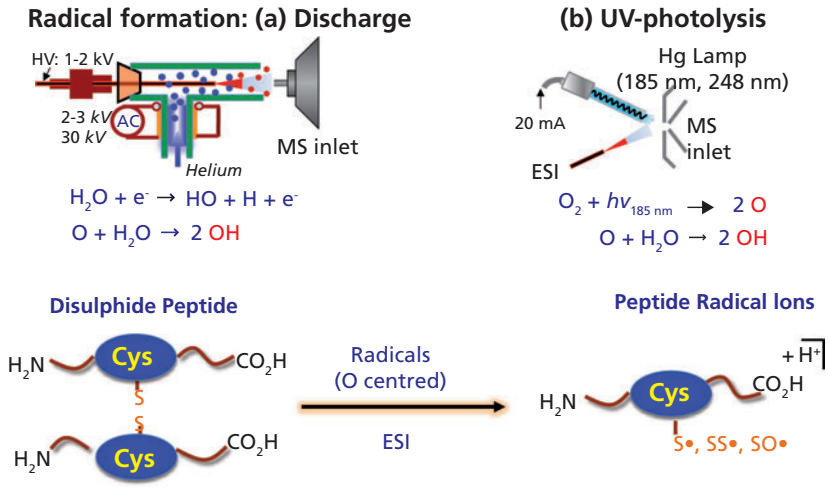
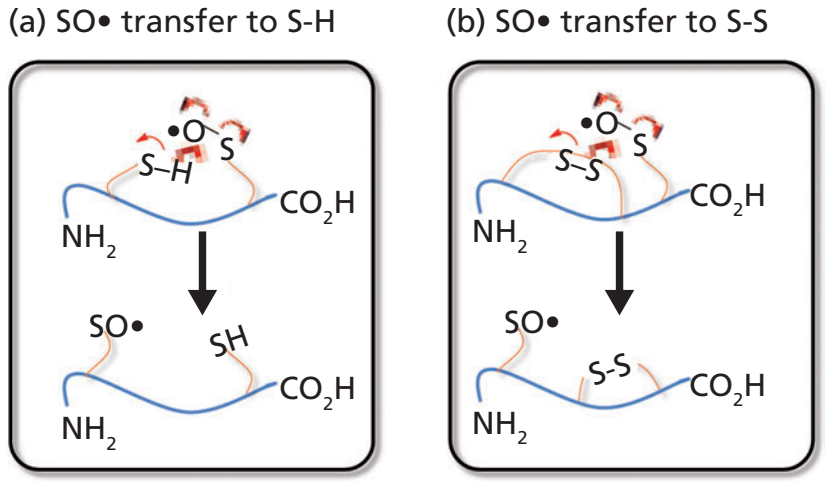


Figure 2: Intramolecular cysteine sulphinyl radical (SO•Cys) transfer with (a) a free thiol and (b) a disulphide bond.



of the hydrogen-rich radical ions and have been shown to be effective for protein analysis by providing structural information complementary to that available by the CID of even-electron ions.

Fragmentation of hydrogen-deficient radical ions, which (when compared with even-electron peptide ions) evidence fewer hydrogen atoms, has also been studied. Their dissociation chemistry differs from that of hydrogen-rich species and is sensitive to the nature of radicals (25). Several research groups have contributed significantly to this area by developing unique methods of forming hydrogen-deficient radical ions and studying their gas-phase ion chemistry (Table 1). It has been shown that radical-dissociation chemistry can be analytically useful in protein analysis by inducing site-specific fragmentation (26,27) differentiating structural isomers or stereoisomers (17,28), and probing labile post-translational modifications (PTMs) (29).

An alternative approach to generating the peptide radical ions used by our group involves a radical addition to a peptide ion, to form peptide radical ions, as shown in equation 1. Except for proton-transfer reactions, other possible reaction routes, such as electron-transfer and functional-group abstraction by radical species, could lead to the formation of peptide radical ions:

$$[Pep + nH]^{n+} + R\bullet \rightarrow [Pep + nH+R]^{n\bullet+} \quad [1]$$

This approach also gives rise to the formation of hydrogen-deficient radical ions, which facilitates choosing the proper radical-reaction chemistry for target peptides, thus conferring the flexibility to produce different types of radicals (such as carbon-centred versus heteroatom centred radicals) with highly specific radical sites. Moreover, because the charging process of the peptide ions can be separated from the radical formation step, this approach makes it possible to manipulate the charge properties of radical ions — the charge states, charge carriers (proton or metal ions), and charge polarities.

The capability of forming site-specific radicals is essential for studying radical migration in biomolecule systems, whereas manipulation of the charge property is useful in investigating its role in peptide radical chemistry.

Our group recently applied radical reactions in the nanoelectrospray ionization (nanoESI) plume region for the formation of sulphur-centred peptide radical ions. The experimental setup involves a radical-inducing source: an atmospheric-pressure, low-temperature, helium-plasma source or a low-pressure Hg lamp in the proximity of a nanoESI plume (Figure 1). A high number density of OH radicals can be generated and subsequently reacted with disulphide-linked peptides entrained in the spray plume. Given the high reactivity of the disulphide bond towards radical species, cleavage at the bond is highly favourable. This preference results in the well-controlled formation of sulphur-centered radicals such as the cysteine sulphinyl radical (-SO•), thiyl radical (-S•), and perthiyl radical (-SS•).

These peptide radical ions can be mass isolated in a mass spectrometer for further investigation. Note that these three types of radical species have also been detected in enzymatic systems, though little chemistry is known because of their transient nature in condense phases. With their formation in the gas phase, the intrinsic chemical properties lend themselves to extensive study.

Ion and molecule reactions in a quadrupole ion trap show that the reactivity of the three types of sulphur radicals follows the order of thiyl (RS•) > perthiyl (RSS•) > sulphinyl

Figure 3: Coupling radical reactions in the ESI plume region with a subsequent MS–MS scan of the disulphide-bonded, cleaved-peptide, radical ions to improve sequence information.

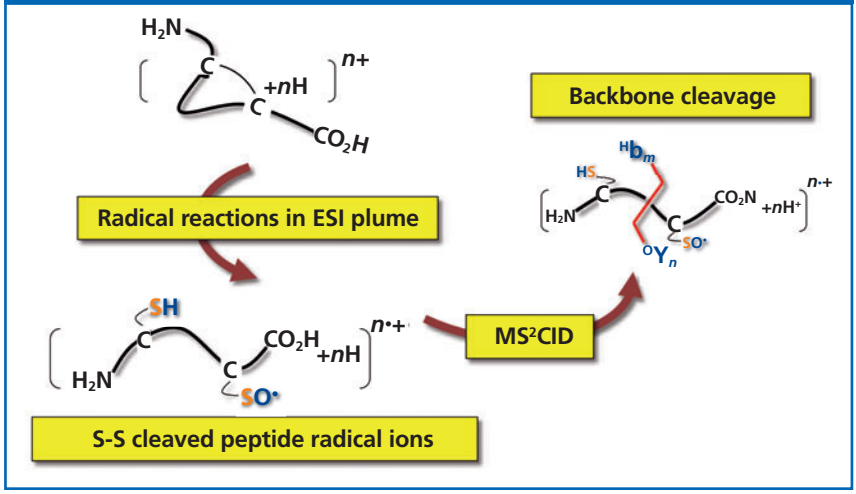
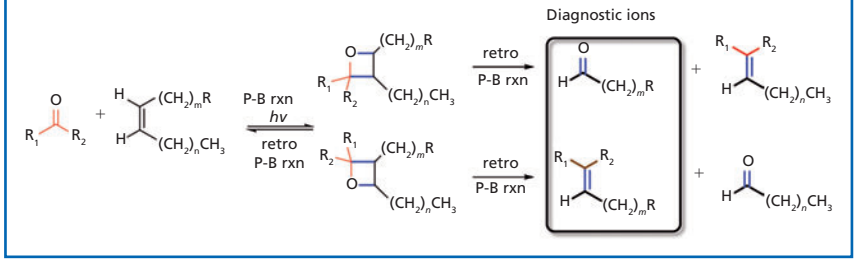


Figure 4: Paternò–Büchi (P-B) reaction between ketone, aldehyde, and olefin in lipids together with retro P-B reactions, to form diagnostic ions for the determination of C=C location.



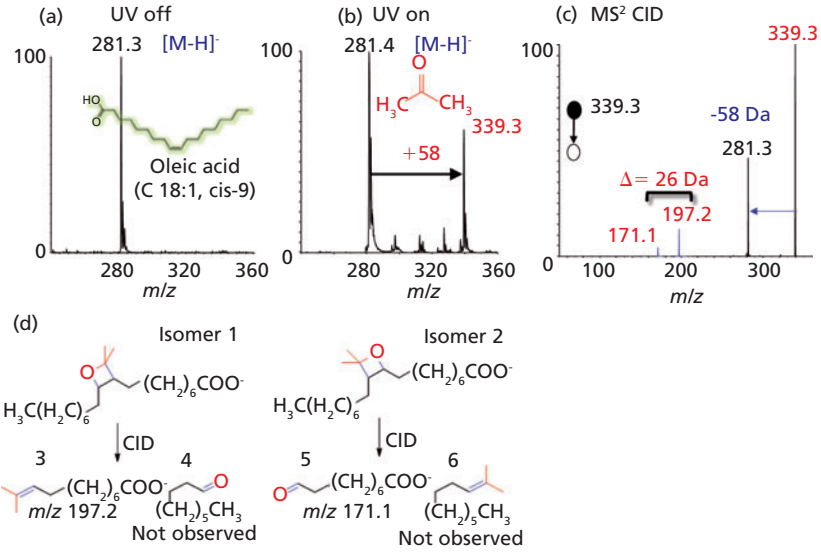
(RSO•). Although the cysteine sulphinyl radical is quite inert under conventional bimolecular reaction conditions, collisional activation facilitates intramolecular reactions. These reactions reveal novel chemistry, because the supplied external energy overcomes the energy barrier. Two newly discovered reaction channels involve the exchange of the cysteine sulphinyl radical (SO•Cys), with either a free thiol (-SH) or a disulphide bond (S-S) within a peptide ion system (reactions shown in Figure 2). Note that, in a protein system, the exchange of the sulphinyl radical with a disulphide bond leads to disulphide-bond scrambling. These reaction phenomena are significant, for they may affect how a radical intermediate migrates in a protein system and attacks other functional groups, resulting in conformational or functional changes. In addition, the reactions suggest that the *oxidative damage* — damage done by forming a sulphinyl radical — can potentially be repaired by reacting with a nearby thiol group (Figure 2[a]).

Disulphide bond formation is a critical PTM, which is highly relevant to the structural integrity of a protein and its biological function. An MS method targeting an intact, disulphide peptide or protein is desirable because it can provide linkage information about disulphide bonds and rich sequence information. It is worth noting that cleavage of the disulphide bond within an even-electron peptide ion is typically a higher-energy process under collisional activation, as compared with cleavage of the peptide backbone (amide bond) to produce protein-sequence ions. To produce a fragment ion from the peptide backbone region within a disulphide bond loop requires the cleaving of at least two bonds. Thus, few sequence fragment ions form under CID conditions, making sequence information difficult to obtain. The routine procedure in proteomics, therefore, involves disulphide reduction and alkylation, to open the disulphide bonds. Indeed, the sequence information obtained from CID-based tandem MS can be significantly improved, but only at the cost of losing information about disulphide-bond connecting patterns.

Unlike the chemistry of even-electron ions, when a radical is introduced into the peptide or protein system, the disulphide bond presents itself as an easy target. For instance, its cleavage is a favourable process where radical intermediates are produced, in ECD, ETD, electron-detached dissociation (EDD), and UV-photodissociation. Among those means of dissociation, ETD is increasingly applied for disulphide peptide or protein characterization. Facile cleavage at the disulphide bond and separation of chains originally connected by disulphide bridges have been reported from ETD of interchain, disulphide-linked peptides. For peptides consisting of intrachain disulphide bonds, ETD can produce rich-sequence information even from backbone regions covered by the disulphide bone loop. This facility of EDT is the result of radical cascades in which N-Cα bond cleavage from ETD leads to the formation of *c* and *z*• ions, and the carbon-centred radical *z*• can further react and cleave the disulphide bone to release *c*/*z*, *c* + 32/*z* - 32, *c* -33/*z* + 33 types of sequence ions, all of which are useful for peptide identification.

Radical reactions in the ESI plume (setup shown in Figure 1) also efficiently cleaves the disulphide bond. One approach to achieving enhanced sequence information for the disulphide peptide combines radical reactions in the ESI plume with subsequent MS^2-CID, to fragment peptide radical ions, thus obtaining the traditional *b*/*y* type fragment ions (Figure 3). We applied this method to a series of naturally occurring peptides that contained one or multiple disulphide bonds. In doing so, we obtained significantly enhanced sequence information, as compared with CID of the intact disulphide peptide ions. Using insulin, a polypeptide containing three disulphide bonds, as an example, rich-sequence information corresponding to 75% of backbone cleavages is obtained from radical reactions in ESI and subsequent CID, which compares favourably to the reported ECD and ETD results (~60%). Moreover, this result is significantly more favourable than the 26% backbone cleavages obtained from CID of intact, disulphide, peptide ions.

Figure 5: Paternò–Büchi (P-B) reaction between oleic acid and acetone in the nanoESI plume upon UV irradiation. MS^1 spectra of oleic acid (a) before UV irradiation, (b) upon UV irradiation, in which the P-B reaction product is located at *m/z* 339.3, and (c) MS^2-CID of the P-B reaction product. The diagnostic ions (*m/z* 171.1 and 197.2) for the C=C location are observed. (d) Structures of the diagnostics ions (structures 3 and 5). The structures explain the origin of the 26-Da mass difference.



Lipids

Lipids serve in numerous biological functions such as structural binding blocks, energy storage, and signalling transduction. The degree of a lipid's unsaturation (the number of C=C bonds) as well as the positions of the C=C bonds determine its overall structure and therefore its biochemical and biophysical properties. For instance, *n*-3 polyunsaturated fatty acids (PUFAs) (also called omega-3 fatty acids, in which 3 is the double-bond position counted from *n*, the terminal methyl group) are essential for the functional development of the brain and retina. By contrast, no such effects have been observed for *n*-6 PUFAs. The efforts to determine double-bond positions in lipids, however, are not trivial, largely because of a need to distinguish the correct structure from a large number of possible double-bond-position isomers. In the field of lipidomics, therefore, MS is the analytical method of choice, because of its high sensitivity, its selectivity, and its ability to provide detailed structural information. Tandem MS of even-electron lipid ions based on collisional activation is useful in providing the head group and acyl-chain composition information. Yet because cleavages at the C-C and C=C positions require significantly higher energies than other possible fragmentation channels, such as neutral or charged head-group losses and fragmentation at the ester bond, obtaining C=C position information cannot be achieved directly from low-energy collisional activation. When the collision energy is high enough (that is, ~ keV from sector instruments or a TOF-TOF instrument), charge-remote fragmentation is possible. Thus, the C-C bonds adjacent to the C=C bonds are cleaved, so the location of C=C is inferable. Compared with the brute-force method of simply increasing the level of collision energy, reactions targeting the unique chemistry of C=C have been used, including ozonolysis, methoxylation, and olefin cross-metathesis. When coupled with MS analysis (on-line or off-line), these chemistry types provide information about the location of C=C.

Another unique property of C=C is its susceptibility to radical attack, which prompts investigation by radical-involved MS analysis. For example, Blanksby and colleagues explored radical-directed dissociation (RDD) of lipids by attaching a photo-caged radical precursor to the target analyte (30). They found that irradiation of the precursor in the mass spectrometer releases the previously caged radical, and CID of the nascent radical and lipid complex ion yields fragmentation information. Moreover, analyzing the fragment ions and fragmentation patterns can lead to ascertaining the number of unsaturation and the position of double bonds.

Our group recently explored coupling a Paternò–Büchi (P-B) reaction of C=C in lipids in the ESI plume with subsequent tandem MS, for fast and sensitive determination of the C=C location (31). The P-B reaction is a classic [2+2] photochemical reaction. In organic synthesis, it is widely used to form compounds containing an oxetane ring. The reaction mechanism involves UV excitation of the carbonyl group within an aldehyde or ketone to a ketyl diradical, which subsequently reacts with the C=C in an olefin. Depending on the relative positions of the carbonyl and the C=C bond, two position isomers of the oxtanes can be formed, as shown in Figure 4. When energy (such as heat) is applied to the P-B reaction products, retro P-B reactions proceed through two possible pathways. One route leads to the original reactants. The other leads to cleavage of the C-C bond at the initial C=C bond position and the C-O bond of the initial carbonyl group, forming a new olefin and ketone or aldehyde. The latter pathway is of special interest in this study because the reaction products carry the C=C bond's position information.

The P-B reaction can be simply executed in the nanoESI plume region by means of the setup shown in Figure 1, a UV lamp with the irradiation wavelength centred at 254 nm. The lipid analyte is dissolved in 1:1 (water:acetone), where acetone functions as the P-B reaction reagent. A reasonable reaction yield of 40% is possible for different classes of lipids, and no reaction selectivity towards specific C=C locations or configurations is found. The P-B reaction product is stable and can be mass isolated and subjected to CID. Figure 5 shows an example of the P-B reaction of oleic acid (C18:1 9Z) prepared at 10 µM in 1:1 water–acetone, with 1% ammonium hydroxide, for nanoESI. Abundant P-B reaction product (*m/z* 339.3), because of the addition of acetone to the C=C, was observed upon UV irradiation. MS^2-CID

of the P-B reaction product promotes the retro P-B reactions and provides a pair of "diagnostic ions" (*m/z* 171 and 197) with a mass difference of 26 Da, for confident determination of the C=C bond location. The structures of the diagnostic ions are shown in Figure 5(d). The method is sensitive (~femtomolar [fmol] for fatty acid) and can be readily coupled with "shotgun" lipidomics, for complex lipid-mixture analysis. An in-solution P-B reaction has been developed to couple with on-line liquid chromatographic separation methods.

In general, the P-B reaction method for lipid C=C location determination has analytical advantages. They include simple experimental setup for reactions, no need to modify the mass spectrometer, easy-to-interpret mass spectra, and inexpensive derivatizing reagents. These characteristics should make this method accessible and attractive to many laboratories.

Oligosaccharides

Saccharides are highly susceptible to decomposition by free-radical processes. In consideration of this fact, Beauchamp and colleagues (32) proposed using biomimetic synthetic reagent and free-radical chemistry to analyze saccharides. By introducing a radical at the reducing end of the saccharides, both glycosidic bond cleavage and cross-ring cleavages are observed upon CID of radical-precursor ions. Radical-driven fragmentation pathways are proposed as responsible for fragments arising from both types of cleavages. This method for the analysis of saccharides, based on free radicals, also represents a novel method for understanding the reactions between free radicals and saccharides.

Summary and Future Directions

Radical-involved chemical and biochemical reactions are attracting increased interest in the area of MS. This interest is driven by the distinct chemistry of radicals versus even-electron species, and the potential for new analytic capabilities. This chemistry can be successfully applied for the structural analysis of proteins or peptides, lipids, and saccharides. The extent of structural information obtained from MS analysis of these molecules can be greatly improved relative to conventional methods. The development of radical MS approaches and strategies has only just started. We anticipate its continued growth, both in fundamental ion chemistry and method and instrument development. We are confident that radical MS can serve as one of the new forces advancing the future development of MS in chemical analysis.

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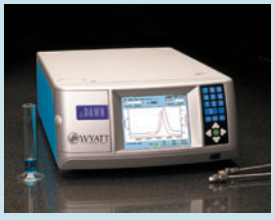
Kate Yu, "MS — The Practical Art" Editor, joined Waters in Milford, Massachusetts, USA, in 1998. She has a wealth of experience in applying LC–MS technologies to various application fields such as metabolite identification, metabolomics, quantitative bioanalysis, natural products, and environmental applications. Direct correspondence about this column should be addressed to the editor-in-chief, Alasdair Matheson, at amatheson@advanstar.com

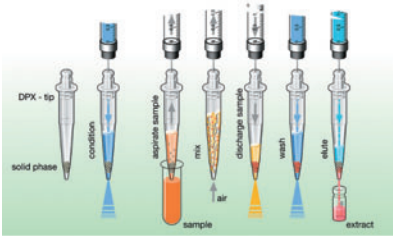
UHPLC SEC–MALS detector



Wyatt has launched µDAWN, a multi-angle light scattering (MALS) detector that can reportedly be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, proteins, or other biopolymers directly. To accommodate narrow peaks in UHPLC, the light scattering flow cell volume has been reduced from 63 µL to 10 µL. To minimize interdetector mixing, band broadening is under 7 µL.

www.wyatt.com
Wyatt Technology, California, USA.

Dispersive SPE



Dispersive SPE (DPX) based on pipette tips with loosely contained sorbent material is reported to enable efficient mixing with the sample, high recovery, and fast extraction. DPX requires very little eluent, effectively providing a concentration step. Applications include drugs of abuse and therapeutic drug monitoring, using less than 250 µL of sample — in addition to pesticides in fruit and vegetables.

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

2D–LC system

Agilent has introduced the Agilent 1290 Infinity 2D–LC instrument, which reportedly gives the separation performance you need, in a single system. Agilent report that it is easy to switch between one-dimensional UHPLC and heart-cutting, multiple heart-cutting, and comprehensive 2D–LC. It boosts performance by achieving ultrahigh peak capacity with a single 2D–LC analysis; it saves time with a quick-start kit; and reduces cost.

www.agilent.com/chem/infinity-2d-lc
Agilent Technologies, California, USA.

Pressurized liquid extraction

The PLE system from FMS is an automated pressurized liquid extraction system for the rapid extraction of a variety of compounds from environmental, biological, and food samples. Applications include a full range of EPA methods as well as testing for semi-volatile organic compounds in soils, foods, pharmaceutical, and polymeric products. It is a modular system and can extract up to six samples simultaneously.

www.fms-inc.com
FMS, Inc., Watertown, Massachusetts, USA.

SPE micro elution plates

Thermo Scientific SOLAµ solid-phase extraction (SPE) micro elution plates are designed to deliver robust, reproducible processing at elution volumes as low as 25 µL. In company-conducted experiments, the SPE plates have reportedly improved sensitivity over comparable SPE products as much as 20-fold as a result of improved pre-concentration while maintaining high levels of reproducibility.

www.thermoscientific.com/sola-spe
Thermo Fisher Scientific, Massachusetts, USA.

Fluidic products catalogue

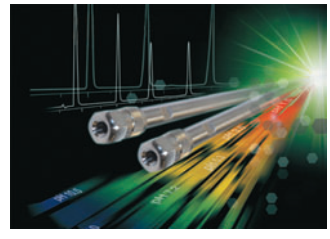
The IDEX Health & Science Catalogue 2014 presents a broad portfolio of fluidic products for laboratory applications. The product portfolio ranges from fittings, connectors, tubing, laboratory pumps, valves, frits, filters, and column hardware to debubblers and degassers. Parallel to product information you will find “How-to-Tutorials”, application guides, and overview charts.

www.idex-hs.com/catalog
IDEX Health & Science GmbH, Wertheim, Germany.

GC–MS system

Shimadzu has introduced the GC–MS–TQ8040, an accurate, cost-effective, and easy-to-use triple quadrupole GC–MS system, according to the company. The system combines ultra-fast mass spectrometry (UFMS) and multiple reaction monitoring (MRM) based on Shimadzu's patented UFsweeper technology. Smart MRM streamlines the entire process of adjusting the loop and dwell times for optimum sensitivity.
www.shimadzu.eu
Shimadzu Europa GmbH, Duisburg, Germany.

HPLC columns



ACE SuperC18 and SuperPhenylHexyl UHPLC/HPLC columns from ACT offer good stability across an extended pH range. The columns can be used with MeOH and MeCN mobile phases and offer rapid column equilibration without memory effects. ACE SuperC18 columns are available with fully porous 2-, 3-, 5-, and 10-µm particles and core–shell (superficially porous) 2.5- and 5-µm particles. ACE SuperPhenylHexyl columns are available with core–shell (superficially porous) 2.5- and 5-µm particles.
www.ace-hplc.com
Advanced Chromatography Technologies Ltd, UK.

Gas generators

The Peak Scientific Precision range of gas generators are modular designed systems, which reportedly offers a GC gas supply solution specific to laboratory needs. They have a stylish space-saving stackable design to make the most of your valuable lab space. Combinations are also available for single and multiple GC applications.
www.peakscientific.com
Peak Scientific Instruments Ltd, Inchinnan, Scotland.

Light scattering system

The Zetasizer µV from Malvern Instruments is a dual capability light scattering system that uses both dynamic light scattering (DLS) for the measurement of molecular size and static light scattering (SLS) for the measurement of absolute molecular weight. It can be added to any protein SEC system using standard chromatography tubing to boost experimental productivity by maximizing information flow from a single experiment.
www.malvern.com/secdls
Malvern Instruments Ltd., Malvern, UK.

Sorbent sampling tubes

Markes' sorbent tubes are reportedly manufactured to the highest quality and are stringently quality-controlled to provide optimum sampling and analytical performance. Metal, glass, or inert-coated tubes are available with a wide range of packing materials, and are compatible with instruments manufactured by Markes, PerkinElmer, and Shimadzu. They are also permanently labelled with a unique serial number, barcode, and sampling flow arrow, so that they are easy to use and track.
www.markes.com
Markes International, Llantrisant, Wales.

Ion chromatography

The combustion ion chromatography (CIC) system from Metrohm enables straightforward, simultaneous determination of halogens and sulphur in complex solid and liquid matrices. It is reported by the company to be ideal for routine analysis, because it integrates sample digestion by pyrolysis and subsequent analysis by ion chromatography in a single, fully automated system. Whereas other methods provide results for the total amount of halogens in the sample, CIC provides discrete results for each of the different halogens, according to the company.
www.metrohm.com
Metrohm AG, Herisau, Switzerland.

Enantiomer separation

Chiral Technologies has introduced two new products based on Daicel immobilzed chiral stationary phases for the separation of enantiomers. Chiralflash (MPLC) semi-preparative columns are reusable and compatible with commercially available flash chromatography systems. Also available are 2L-ChiralTLC, chiral thin layer chromatography plates. These plates can be used for easy method development and reaction monitoring in a synthesis laboratory.
www.chiral.fr
Chiral Technologies Europe, Cedex, France.

Safety exhaust filters

VICI's exhaust filter prevents the pollution of the lab air with VOCs and other toxic or hazardous compounds. It includes a detector which indicates the filter saturation. The filter works with all common safety caps (adapters available) for laboratory bottles and canisters and has a long lifetime as a result of its high capacity filter material.
www.vici-jour.com
VICI AG International, Schenkon, Switzerland.

Silica-based columns

Waters Corporation has introduced a line of 2.7-µm silica-based, solid-core particle columns for high performance liquid chromatography (HPLC). The company report that the columns run at lower pressures with high efficiency, giving flexibility to use longer column lengths to improve resolution or higher flow rates to improve instrument analysis time and increase throughput. The columns are available in C18+, C18, and HILIC chemistries.
www.waters.com
Waters Corporation, Massachusetts, USA.

Automated mycotoxin analysis

The newest module for the Freestyle robotic system from LC Tech is ThermELUTE that enables handling from raw extract to chromatogram without manual working steps. Thermal denaturation and large volume water based elution with a quantitative transfer directly to the analytical HPLC allow sensitive — ppq instead of ppb — and fully automated processing of sample clean-up. The throughput increases to 500 samples/week.
www.LCTech.de
LCTech GmbH, Dorfen, Germany.

Gas chromatograph

The NovaCHROM 2000 GC from AGC Instruments analyses the impurities in an argon matrix gas to ppb levels. According to the company. the latest advanced technologies provide customers with the precise results required with ease of use and support features. Using the Argon Discharge Detector (ADD), the NovaCHROM 2000 provides excellent stability and sensitivity to achieve guaranteed applications.
www.agc-instruments.com
AGC Instruments Ltd. Shannon, Ireland.

Superficially porous columns

The Raptor ARC-18 column is made specifically for high-throughput, low-sample prep LC–MS–MS applications. A well-balanced retention profile offers better detection and integration of large, multiclass analyte lists, and steric protection helps this SPP column endure the harsh, low-pH mobile phases needed for mass spec without sacrificing retention or peak quality.
www.restek.com/raptor
Restek Corporation, Pennsylvannia, USA.

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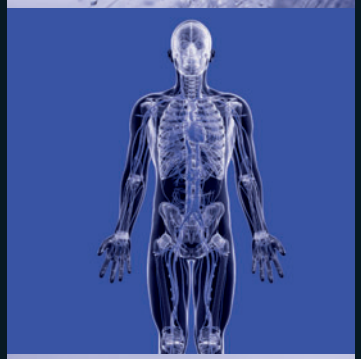

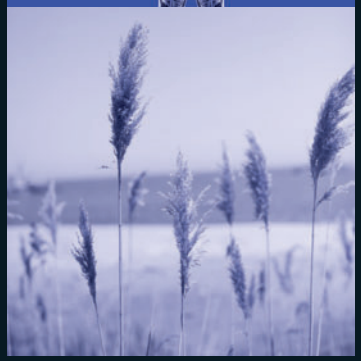

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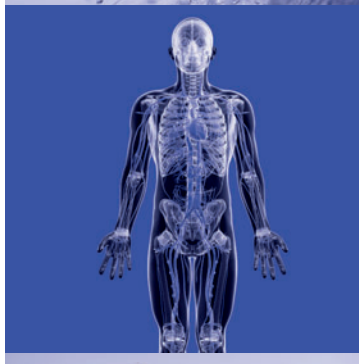

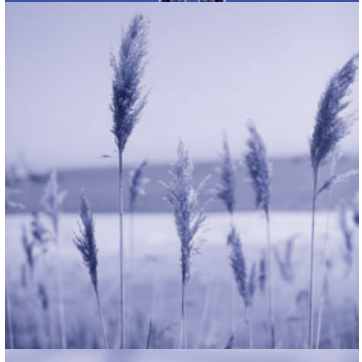

THE APPLICATIONS BOOK

Environmental

Food and Beverage

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Keeping Water Safe: Detecting Pharmaceutical and Personal Care Products in Water Using Liquid Chromatography–Mass Spectrometry

Joe Anacleto, Zicheng Yang, Helen (Qingyu) Sun, and Kefei Wang, Bruker Daltonics

The Problem with PPCPs

Pharmaceutical and personal care products (PPCPs) are products used for personal health or cosmetic reasons. This category includes a broad group of chemical substances such as human and veterinary medicines and cosmetics. The presence of PPCPs in environmental and potable water is a widespread concern due to the potentially harmful environmental effects. Evidence suggests PPCPs are linked to some ecological damage such as delayed development in fish (1).

To ensure the safety of water, PPCP concentrations are stringently monitored by environmental regulatory bodies, including the United States Environmental Protection Agency (US EPA) (2). Detection of PPCPs is traditionally a complicated process due to the range of substances potentially present. Here we explore a simple, more convenient method than traditional solid-phase extraction (SPE)-based methods for highly sensitive PPCP detection, using triple quadrupole liquid chromatography–mass spectrometry (LC–MS–MS).

Tables 1a & 1b: Instrumentation set-up for analysis of PPCPs in clean water.

Mass Spectrometer Parameters (EVOQ Elite)	
HV	4000 V
Cone gas flow	15 units
Cone gas temperature	300 °C
Heated probe gas flow	40 units
Heated probe temperature	450 °C
Nebulizer gas flow	50 units
Exhaust gas	On
Q2 pressure	1.5 mTorr (Argon)
Chromatography Parameters (Advance UHPLC)	
Trap column	YMC-Pack ODS-AQ, 3-µm, 35 mm × 2.0 mm I.D.
Column temperature	40 °C
Injection volume	400 µL
Flow rate	400 µL/min
Solvent A	2 mM ammonium formate, 0.1% FA in water
Solvent B	2 mM ammonium formate, 0.1% FA in MeOH
Solvent C	2 mM ammonium formate, 0.1% FA in water
Gradient conditions	0.0 min, 10% B 0.2 min, 10% B 0.8 min, 25% B 8.0 min, 95% B 9.0 min, 95% B 9.1 min, 10% B 12.0 min, 10% B

Figure 1: PPCPs in environmental water and nearby soil is a widespread concern.

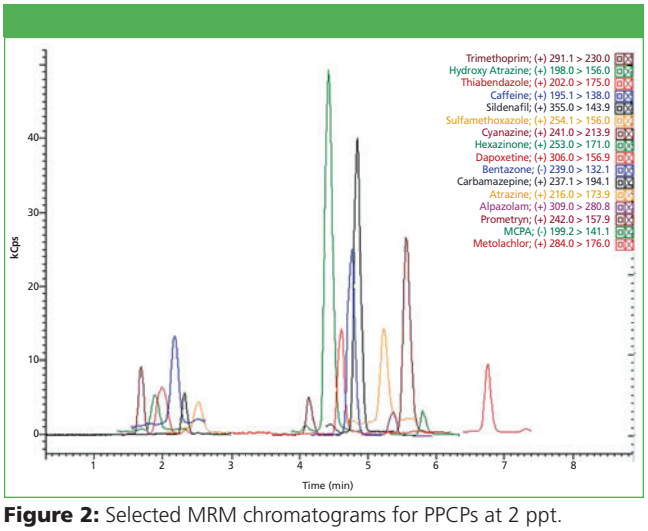

Figure 2: Selected MRM chromatograms for PPCPs at 2 ppt.

Detecting PPCPs

Conventional methods of PPCP detection in clean water have followed the defined EPA 1694 "template" for analysis, which requires the pre-concentration of large volume water samples and tedious solid-phase extraction cleanup, followed by liquid chromatography–mass spectrometry analysis to achieve the low ng/L (ppt) level detection necessary to comply with regulations (3).

Table 2: Test results for selected PPCPs in real water samples.

Compound Name	Tap Water 1	Tap Water 2	Creek Water	Bottle Water
Trimethoprim	<2	<2	5	<2
Hydroxyatrazine	4	<2	7	<2
Thiabendazole	ND	<2	<2	<2
Ciproxacin	ND	ND	ND	ND
Caffeine	ND	<2	<2	10
Sildenafil	ND	ND	ND	<2
Sulphamethoxazole	<2	<2	ND	<2
Cyanazine	ND	ND	ND	<2
Simazine	3	<2	5	ND
Metribuzin	ND	ND	ND	ND
Hexazinone	17	3	3	ND
Dapoxetine	ND	ND	ND	ND
Bentazone	ND	ND	ND	ND
Ametryn	ND	ND	<2	ND
Carboxine	ND	ND	ND	ND
Carbamazepine	<2	<2	<2	ND
Atrazine	<2	ND	ND	ND
Alpazolam	ND	ND	ND	ND
Diuron	9	<2	6.2	ND
Prometryn	ND	ND	ND	<2
2,4-D	9	<2	13	<2
MCPA	<2	<2	<2	ND
Mecoprop	<2	<2	11	2
Metolachlor	22	<2	<2	<2
Pyriproxifen	ND	<2	ND	<2

Bruker has explored how LC–MS–MS can be employed specifically for the analysis of PPCPs in clean water. PPCPs were detected at 1–2 ppt with a linear response up to 200 or 500 ppt. Excellent system robustness was obtained throughout the extended method development and sample analysis period.

Case Study: Using LC–MS–MS to Analyze PPCPs in Clean Water

The study was carried out using ultrahigh-performance liquid chromatography (UHPLC) with an integrated on-line extraction (OLE) option coupled to a triple quadrupole mass spectrometer. The OLE module enables convenient method-driven on-line sample cleanup or sample pre-concentration.

Several water samples were analyzed for a range of PPCP species, including tap water samples along with bottled water and creek water. Samples were analyzed targeting a wide range of PPCP species representing compounds displaying varied properties and concentrations. Tables 1a and 1b illustrate the Advance UHPLC and EVOQ instrumentation set up respectively.

All of the PPCPs were detected at 2 ppt or better with the injection of 0.4 mL samples with a linear response range up to 200 or 500 ppt.

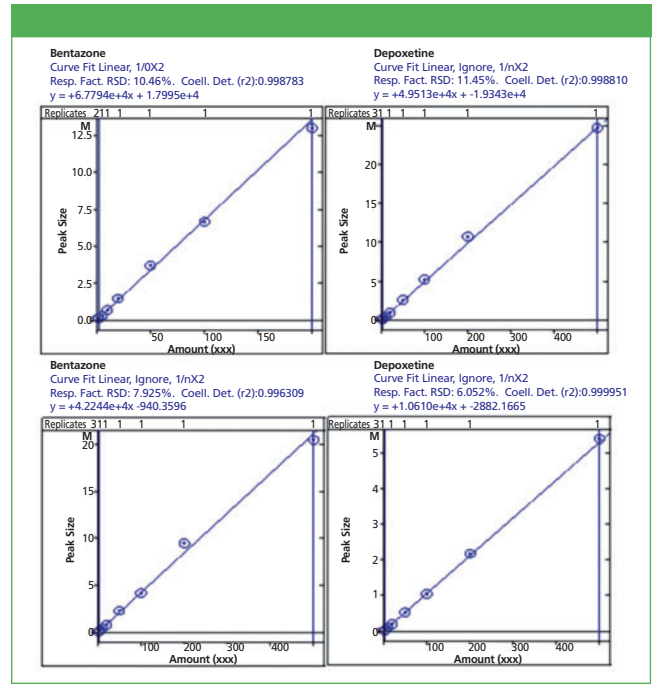

Figure 3: Selected calibration curves.

The fast polarity switch can analyze positive and negative PPCPs in the same analytical segment with excellent linear response for both polarities (Figures 2 and 3). The results for the analysis of tap, creek, and bottled waters are shown in Table 2.

Conclusion

The Bruker Advance UHPLC with OLE coupled to EVOQ LC–MS–MS detected PPCP samples at 2 ppt or better within 0.4 mL samples. Excellent linearity, sensitivity, and robustness were achieved throughout. The technique presents a more convenient and simpler approach to PPCP analysis than traditional SPE-based methods.

References

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(2) US EPA, "PPCPs Basic Information," (2010). Available at: http://www.epa.gov/ppcp/basic2.html.

(3) US EPA, "EPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS," (2007).

Bruker Daltonics Inc.
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Determination of Pesticide Residues in Soil Using a QuEChERS Approach

UCT, LLC

Soil is a complex matrix consisting of organic and inorganic material. It possesses many active sites that can retain pesticides and other residues. Compared to other matrices, soil samples are more difficult to extract and often require longer extraction times. Certain compounds may be covalently bound to the soil. These can only be removed using acid or base hydrolysis. If a hydrolysis step is employed, this may have a detrimental effect on pH sensitive analytes. This technique was not evaluated in the course of this study.

The aim of this study was to evaluate the effectiveness of the QuEChERS extraction and cleanup approach for pesticides in soil. A group of 21 LC–MS–MS amenable pesticides, comprising various chemical properties, were used for the study.

QuEChERS Procedure

Sample Extraction

1. Weigh 10 g soil sample (≥70% H_2O content) into a 50 mL centrifuge tube. Alternatively, weigh 3 g air-dried soil sample into a 50 mL tube and add 7 mL H_2O, vortex briefly, and allow to hydrate for 30 min.
2. Add 10 mL of acetonitrile to each sample.
3. Shake (manually or mechanically) or vortex samples for 5 min to extract pesticides. (In this study a Spex SamplePrep Geno/Grinder 2010 operated at 1500 rpm was used).
4. Add the contents of an **ECQUEU750CT-MP** (citrate salts) Mylar pouch to each centrifuge tube.
5. Immediately shake samples for at least 2 min.
6. Centrifuge for 5 min at ≥ 3000 rcf.

Sample Cleanup

7. Transfer a 1 mL aliquot of supernatant to a 2 mL **CUMPSC18CT** ($MgSO_4$, PSA, C18) dSPE tube.
8. Vortex samples for 0.5–1 min.
9. Centrifuge for 2 min at high rcf (e.g. ≥ 5000).
10. Filter purified supernatant through a 0.2 µm syringe filter directly into a sample vial.

Results and Discussion

In the dSPE step, a combination of PSA/C18 gives cleaner extracts than PSA alone. In this study, no major variation in results was observed between PSA and PSA/C18. In fact, the PSA/C18 gave slightly better results overall.

Thiabendazole gave low, though reproducible recovery throughout the study. Thiabendazole is a planar pesticide and could potentially be retained by strong hydrophobic interactions on the soil. In addition, it is a basic compound that is positively charged at low pH and is capable of being retained on the soil through ionic interactions, particularly by humic/fulvic acids.

LC–MS–MS Conditions	
MS instrument	Thermo Scientific™ TSQ Vantage™
MS mode	ESI^+ in MRM mode
HPLC system	Thermo Scientific™ Dionex® Ultimate® 3000
HPLC column	UCT Selectra® C18, 100 × 2.1 mm, 3-µm (p/n: SLC-18100ID21-3UM)
Guard column	UCT Selectra® C18, 10 × 2.0 mm, 3-µm, (p/n: SLC-18GDC20-3UM)
Mobile phase A	0.1% ammonium formate + 0.3% formic acid
Mobile phase B	methanol + 0.1% formic acid
Flow rate	300 µL/min
Column temperature	40 °C
Run time	25 min (including 5 min re-equilibration)
Injection volume	3 µL

Table 1: Accuracy and Precision Data.

Analyte	20 ng/g (n = 6)		100 ng/g (n = 6)	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Abamectin	74.9	11.17	71.8	6.28
Acetochlor	93.9	7.32	97.5	3.19
Atrazine	95.3	5.16	98.1	1.30
Bifenthrin	94.9	12.90	90.9	10.32
Carbaryl	95.2	7.13	93.9	3.53
Carbendazim	69.6	8.55	81.6	5.06
Chlorpyrifos	89.5	6.36	93.1	3.96
Cyprodinil	93.2	9.12	94.1	1.78
DEET	107.3	6.75	101.1	0.67
Diazinon	94.4	7.53	98.2	1.36
Dicrotophos	91.0	6.61	99.1	3.35
DIMP	82.5	6.74	88.1	1.47
Malathion	52.3	9.29	78.1	1.78
Profenofos	79.5	8.76	88.6	2.75
Pyrazophos	80.5	8.01	93.9	2.63
Pyrimethanil	90.2	4.88	92.2	2.36
Simazine	92.4	7.74	98.9	2.77
Tebuconazole	88.5	6.69	93.1	3.08
Tebuthiuron	100.7	7.39	101.1	2.14
Thiabendazole	52.8	5.61	63.1	6.80
Zoxamide	92.4	7.92	99.4	2.11

Note: triphenylphosphate (TPP) was used as an internal standard. Matrix-matched calibration curves were used for quantification.

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Determination of Phenolic Compounds in Virgin Olive Oil Using Comprehensive 2D-LC

Sonja Krieger and Sonja Schneider, Agilent Technologies, Inc.

This application note demonstrates how comprehensive 2D-LC can be used to resolve the complex mixture of hydrophilic phenols found in virgin olive oil and investigates differences in the phenol composition of several olive oils.

Virgin olive oil is associated with the health and nutritional benefits of the Mediterranean diet. In this respect, the presence of antioxidants, which are represented by hydrophilic phenols among others, plays an important role. Hydrophilic phenols contained in virgin olive oil include phenolic acids and alcohols, flavonoids, secoiridoids, and lignans (1,2).

One-dimensional liquid chromatography is not able to completely resolve the complex mixture of hydrophilic phenols present in virgin olive oil (3). Due to its high separation capability, comprehensive 2D-LC can be deployed to improve the separation.

Experimental Conditions

Comprehensive 2D-LC analysis was achieved with the Agilent 1290 Infinity 2D-LC solution. The first dimension separation used an Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl column (2.1 × 150 mm, 1.8-µm) with a gradient of water and methanol, each with 0.1% formic acid, at a flow rate of 0.05 mL/min. In the second dimension, an Agilent ZORBAX RRHD Eclipse Plus C18 column (3.0 × 50 mm, 1.8-µm) was used with shifted gradients of water and acetonitrile, each with 0.1% formic acid, at a flow rate of 3.0 mL/min. Modulation was realized using the Agilent 2-position/4-port duo-valve, equipped with two 60 µL loops and with a modulation time of 30 s. Detection was performed at 260 nm and by ESI–TOF–MS in negative ionization mode. Preparation of olive oil samples was carried out according to the protocol from the International Olive Council (4).

Results

Four different olive oil samples with high phenol content (3) purchased from Italian olive oil farms were analyzed by comprehensive 2D-LC. Figure 1 (top) exemplarily shows the 2D-LC chromatogram of one olive oil.

MS detection showed that the main hydrophilic phenols present in the analyzed olive oils are aglycons of oleuropein, ligstroside, and their derivatives. Further, elenolic acid, luteolin, apigenin, hydroxytyrosol, and hydroxytyrosol acetate were identified in all olive oils analyzed. Compared to the olive oils A–C (from the same farm), olive oil D (from another farm) showed higher percent responses of the flavonoids apigenin and luteolin (Figure 1, bottom).

Conclusions

The Agilent 1290 Infinity 2D-LC solution can be used to significantly improve the separation of hydrophilic phenols contained in virgin olive oil. This enables the investigation of differences between the compositions of hydrophilic phenols in olive oils.

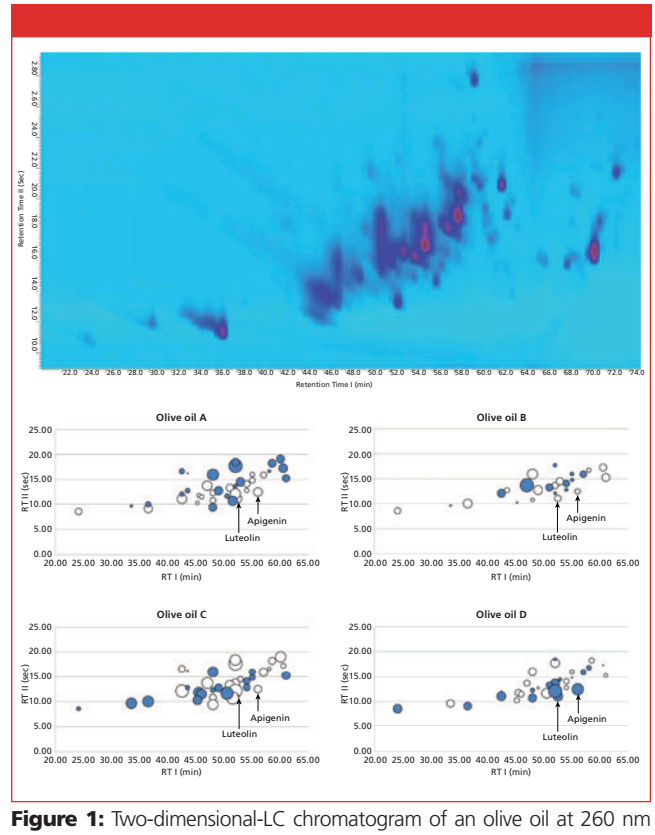

Figure 1: Two-dimensional-LC chromatogram of an olive oil at 260 nm (top) and visualization of differences between the olive oils analyzed (bottom); blue circles indicate higher percent responses and white circles lower percent responses of substances detected, areas indicate differences.

References

(1) M. El Riachy et al., *Eur. J. Lipid Sci. Technol.* **113**, 678–691 (2011).

(2) Y. Ouni et al., *Food Chemistry* **127**, 1263–1267 (2011).

(3) S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 6," Agilent Application Note, publication number 5991-3801EN, (2014).

(4) "Determination of biophenols in olive oils by HPLC," International Olive Council: COI/T.20/DOC. 29, (2009).

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Detection and Quantification of Protein Aggregates by SEC–MALS

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Proteins have a tendency to aggregate over time and the risk for drugs in the biopharmaceutical industry is that the presence of aggregates will stimulate an immune response. Size-exclusion chromatography (SEC) is a powerful tool that is commonly used to look at the aggregation of proteins.

SEC separates proteins by size, and is commonly used to measure molecular weight and characterize aggregation. By adding a light scattering detector to the system, the molecular weight of the protein monomer, oligomers, and aggregates in a sample can be measured independent of their elution volume. In addition, multi-angle light scattering (MALS) can also be used to measure the radius of gyration (Rg) of large aggregates that scatter light anisotropically.

Materials and Methods

Pepsin was characterized using a Viscotek TDAmax system connected to a Viscotek SEC-MALS 20 detector. Two Viscotek protein columns were coupled together for the separation. The detectors and columns were all held at 30 °C to ensure a good separation and to maximize baseline stability of the detectors.

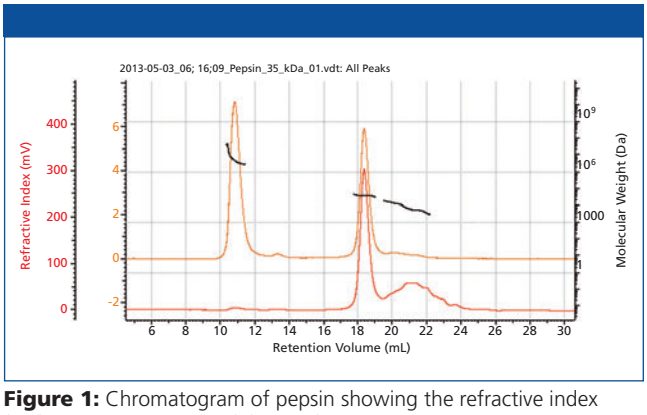

Figure 1: Chromatogram of pepsin showing the refractive index (red) and SEC–MALS (90°) (orange) detector signals.

Table 1: Measured molecular weights of the different peaks of the pepsin sample.

	Aggregates	Monomer	Digestion products
Peak RV - (mL)	10.84	18.33	20.92
Mn - (kDa)	3892.0	34.4	4.7
Mw - (kDa)	4431.0	34.7	6.4
Mw / Mn	1.138	1.008	1.364
Rg(w) - (nm)	69.9	N/C	N/C
WtFr (Peak)	0.8%	56.9%	42.3%

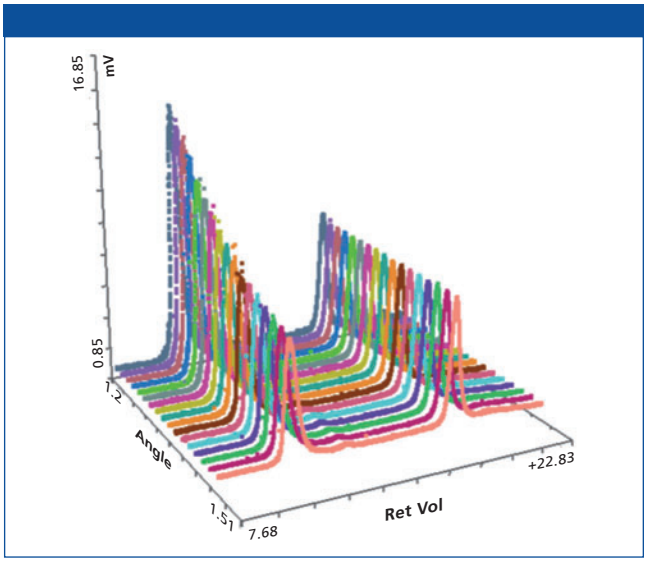

Figure 2: SEC-MALS plot showing different angular response for pepsin monomer and aggregates.

Results

Figure 1 shows that the pepsin sample contains 2 main components. The molecular weight of the second peak (18.5 mL) is measured at 34.7 kDa, which is very close to the known molecular weight of pepsin (35 kDa). The larger light scattering peak at 11 mL has a much higher and more variable molecular weight, clearly identifying it as some disordered aggregates, which are unlikely to be active. The MALS plot in Figure 2 shows that these aggregates are strongly anisotropic scatterers, and therefore have a large size compared to the protein.

As pepsin is a digestive enzyme, the broad peak at 20.9 mL is most likely to be digestion products.

Conclusion

The molecular weight and aggregate content of pepsin was successfully measured using the Viscotek SEC-MALS 20 system, and their amounts quantified. Where the aggregates are large enough, their size (Rg) can also be measured.

Reference

(1) Measuring protein aggregation with the Viscotek SEC-MALS 20, Malvern Instruments application note, www.malvern.com/MRK1927

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Molecular Weight Determination of LMWH SEC–MALS vs. SEC–UV–RI

Wyatt Technology Corporation

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.

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, 0.2 M sodium sulphate pH 5.0 mobile phase, Tosoh TSK-gel 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 method aspects remained the same.

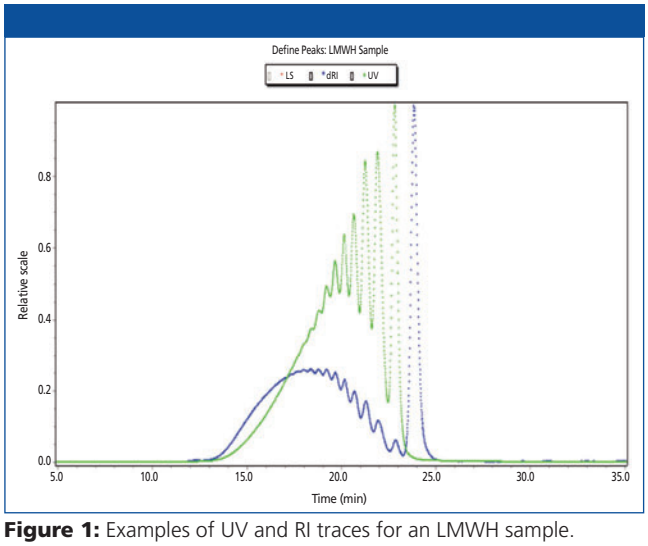

Figure 1: Examples of UV and RI traces for an LMWH sample.

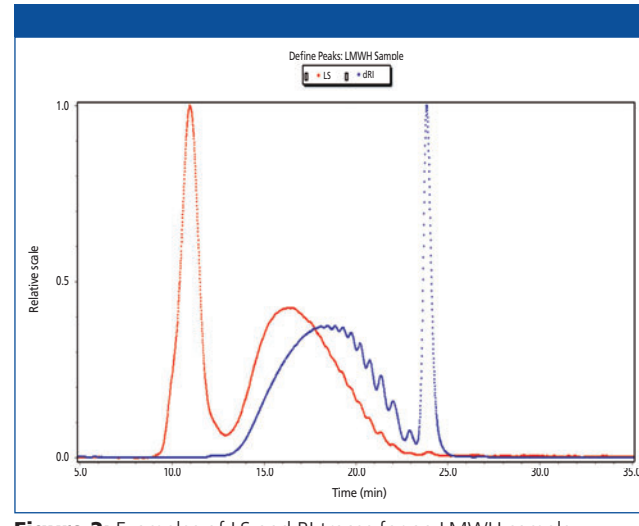

Figure 2: Examples of LS and RI traces for an LMWH sample.

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.

This note was graciously submitted by Lin Rao and John Beirne of Scientific Protein Laboratories LLC.

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Fast Screening Methods for Analgesics and Non-Steroidal Anti-Inflammatory (NSAIDS) Drugs by HPLC with Agilent Poroshell 120 Columns

William Long, Agilent Technologies, Inc.

Using Selectivity to Enhance Separation of Analgesics

Selectivity is the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, polar embedded, phenyl bonded phases and perflorophenyl, or by changing the mobile phase. In this work, 4.6 × 50 mm Poroshell 120 columns are used to quickly evaluate method development choices for the analysis of non-steroidal anti-inflammatory drugs (NSAIDS). The short column length and high efficiency provide short analysis times and rapid equilibration, leading to fast investigations of selectivity.

Experimental Conditions

Instrument: Agilent 1260 Infinity Binary LC System
Columns: Noted below
Flow rate: 2 mL/min
Mobile phase: A: 20 mM NH_4HCO_2 pH 3.0 B: Acetonitrile
Temperature: 40 °C
Detection: UV, 254 nm
Gradient:

Time	% Organic
0	8
6	100
7	100
8	8

The Agilent 1260 Infinity Binary LC System was configured as follows:

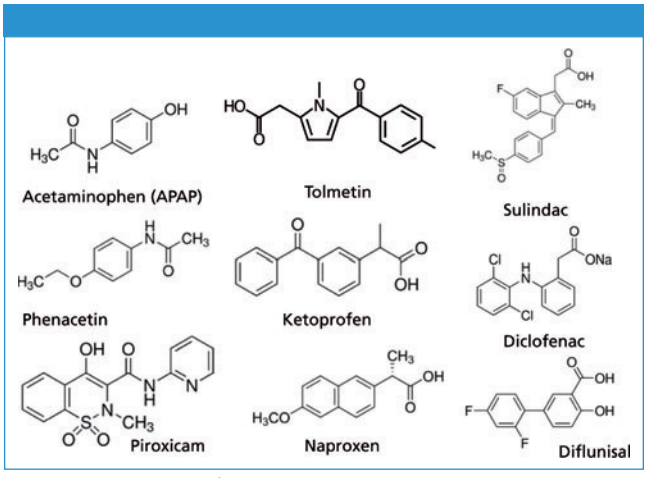

Figure 1: Structures of selected analgesics.

- G1312B Binary Pump SL, capable of delivering up to 600 bar
- G1316C Thermostatted Column Compartment (TCC)
- G1376D High Performance Autosampler SL Plus
- G4212A Diode Array Detector equipped with a G4212-60008 10 mm path length, 1 µL volume flow cell

The following columns were used in this study.

- Agilent Poroshell 120 PFP, 4.6 × 50 mm, 2.7-µm (p/n 699975-408)

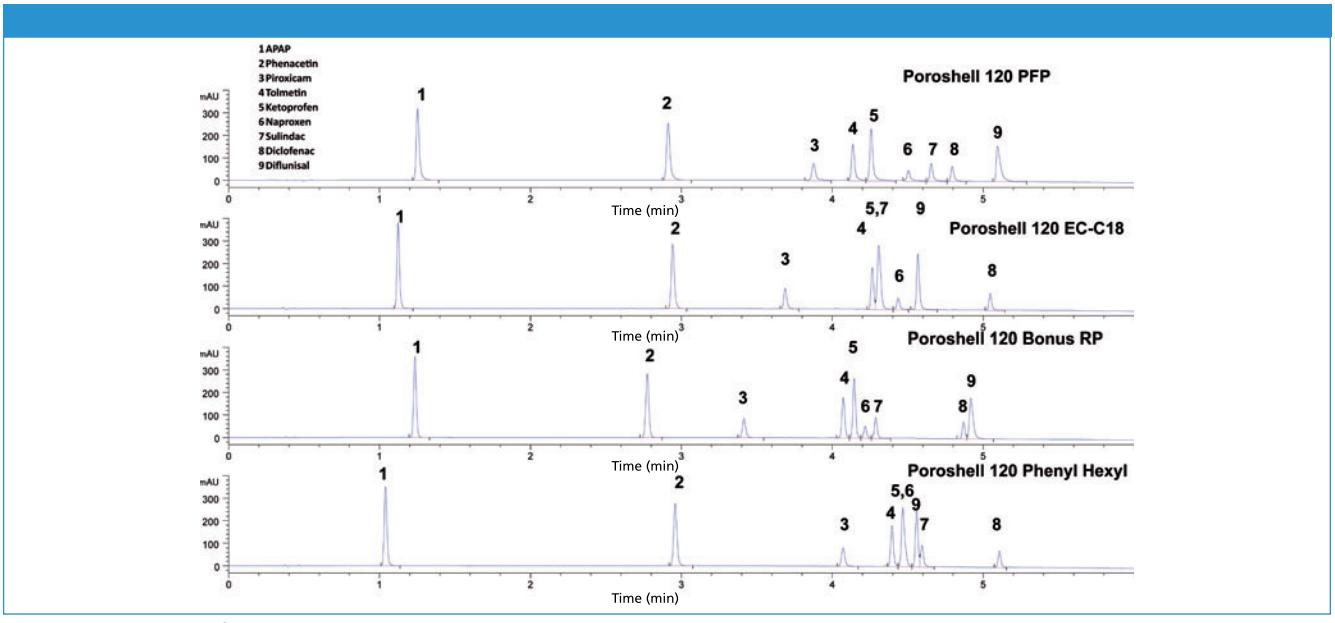

Figure 2: Separation of analgesics using Agilent Poroshell 120 columns using methanol.

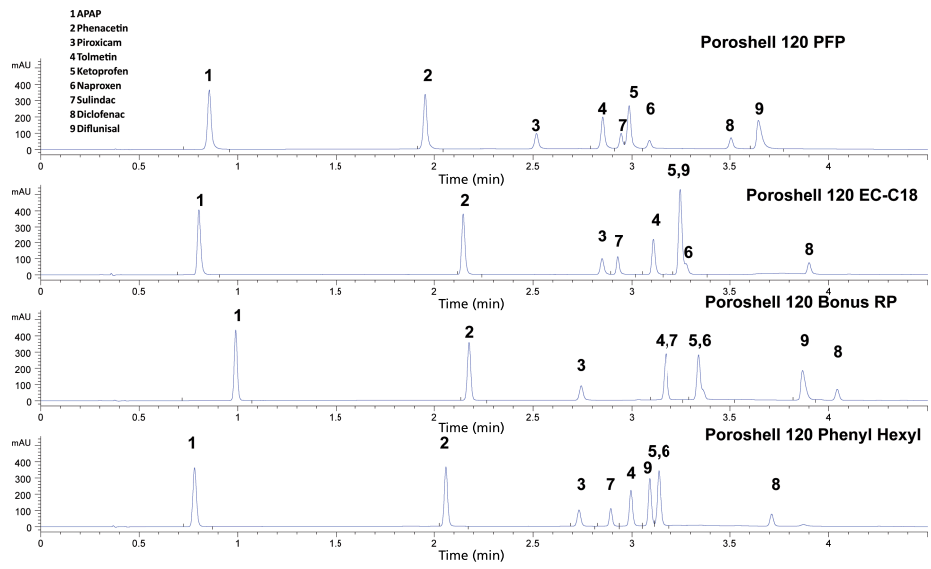

Figure 3: Separation of analgesics using Agilent Poroshell 120 columns with acetonitrile.

Table 1: Retention time, Log P, and pKa data for selected analgesics.

Compound	log P	pKa	tr PFP MeCN	trPFP MeOH	tr C18 MeCN	trC18 MeOH	trBrp MeCN	trBrP MeOH	trPH MeCN	trPH MeOH
Acetominophen	0.46	9.38	0.863	1.252	0.803	1.123	0.99	1.235	0.781	1.039
Phenacetin	1.58	2.2	1.966	2.912	2.147	2.943	2.176	2.774	20.59	2.959
Piroxicam	3.06	6.3	2.536	3.876	2.849	3.688	2.744	3.415	2.732	4.027
Tolemetin	2.79	3.5	2.868	4.137	2.928	4.265	3.173	4.073	2.893	4.395
Ketoprofen	3.12	4.45	3.008	4.258	3.109	4.308	3.342	4.146	3.137	4.468
Naproxen	3.18	4.15	3.112	4.505	3.249	4.436	3.342	4.218	3.167	4.468
Sulindac	3.42	4.7	2.934	4.656	3.249	4.308	3.173	4.288	2.995	4.594
Diclofenac	4.51	4.15	3.53	4.795	3.9	5.046	4.043	4.87	3.711	5.106
Diflunisal	4.41	2.69	3.659	5.094	3.249	4.567	3.867	4.919	3.091	4.559

- Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7-µm (p/n 699975-902)
- Agilent Poroshell 120 Bonus-RP, 4.6 × 50 mm, 2.7-µm (p/n 699968-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 4.6 × 50 mm, 2.7-µm (p/n 699975-912)

A generic gradient separation was used to evaluate these columns consisting of ammonium formate (20 mM NH_4HCO_3 pH 3.0) using either methanol or acetonitrile.

The analgesic materials all possess a wide variety of functional groups including fluorine (sulindac and diflunisal) and chlorine (diclofenac). The structures of the compounds examined are shown in Figure 1 and Table 1. All samples were prepared at 10 mg/mL in acetonitrile and were diluted in water to a final concentration of 0.1 mg/mL.

Column Choice to Enhance Selectivity

The columns were chosen to improve selectivity in the separation. They included a highly end capped C18 column recommended as a first choice in method development (Poroshell120 EC-C18).

Poroshell 120 Bonus-RP can be used for many of the same separations as a C18 column while avoiding some of the disadvantages of C18, such as poor wettability in high aqueous mobile phases. In addition, it is much more retentive for those molecules that can interact by hydrophobic interactions and also by H-bonding with the amide group. Compared to alkyl only phases, Bonus-RP has enhanced retention and selectivity for phenols, organic acids, and other polar solutes due to strong H-bonding between polar group (H-bond acceptor) and H-bond donors, like phenols and acids. Bonus-RP gives retention slightly less than a C18 allows, for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity than C18 for polar compounds. It is also compatible with 100% water.

Poroshell 120 Phenyl-Hexyl columns deliver unique selectivity for compounds with aromatic groups, providing superior resolution for these samples. Poroshell 120 Phenyl-Hexyl can also provide optimum separations of moderately polar compounds where typical alkyl phases (C18 and C8) do not provide adequate resolution. Acetonitrile tends

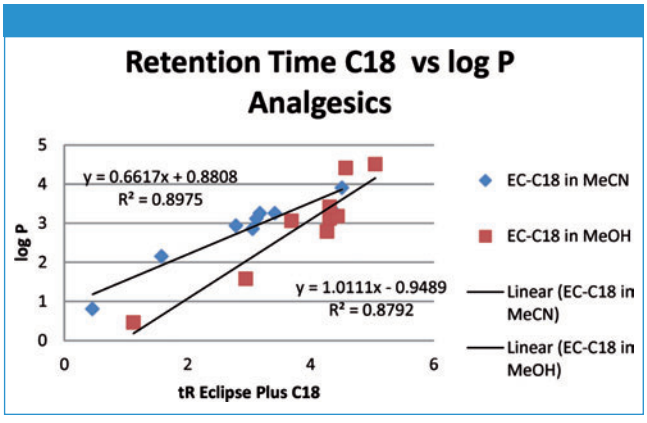

Figure 4: Poroshell 120 EC-C18 retention time vs. log P values.

to decrease the π–π interactions between aromatic and polarizable analytes and the phenyl-hexyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the different selectivity that is desired from a phenyl phase.

Poroshell 120 PFP columns possess a pentafluorophenyl ligand. This can provide an orthogonal separation mechanism to traditional reverse phase columns. By specifically targeting many polar retention mechanisms, PFP phases can separate analytes based on small differences in structure, substitution, and steric access to polar moieties. The resulting selectivity for positional isomers, halogenated compounds, and polar analytes is particularly useful in the analysis of complex mixtures, and small molecule pharmaceuticals

Results and Discussion

The separation of all nine compounds was attempted on all columns surveyed. The Poroshell 120 PFP and Poroshell 120 Bonus RP columns both fully resolved all compounds in the same order, although the spacing of the peaks is more even on PFP. The Poroshell 120 Phenyl Hexyl column does not yield the same elution order as the Poroshell 120 PFP column. This means that the PFP column is not just a stronger phenyl column, other interactions beside π-π and hydrophobic interactions are in play. All four columns elute acetominophen (APAP) and phenacetin first. The Poroshell 120 EC-C18 column did not fully separate three compounds (Tolmetin, Ketoprofen, and Sulindac).

Figure 3 shows the separation on all four columns using acetonitrile. In this case, only Poroshell 120 PFP resolves all compounds, and Poroshell 120 EC-C18 and Poroshell Phenyl Hexyl columns elute all compounds in the same order. Typically π-π interactions with Phenyl Hexyl columns are overwhelmed in acetonitrile. Again, the PFP and Bonus RP columns have very similar elution orders (with the exception of the last two peaks).

Since the Poroshell 120 PFP phase almost separates all nine compounds when using methanol or acetonitrile, it provides the best method development option for further development.

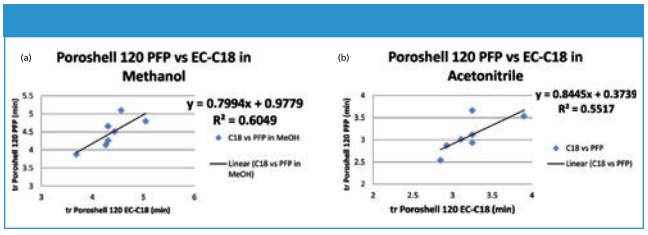

Figure 5: Poroshell 120 EC-C18 retention time vs. Poroshell 120 PFP retention time.

Table 1 lists the retention time of all nine analytes on the four columns using both methanol and acetonitrile. Log P and pKa data are also listed. The Log P refers to the equilibrium distribution of a single substance between two solvent phases separated by a boundary. It was discovered that the narcotic action of many simple organic solutes was reflected rather closely by their oil-water partition coefficients. The oil was later replaced by octanol (5). In Figure 4, Log P values correspond to retention time on the Poroshell 120 EC-C18 column using methanol or acetonitrile. Figure 5 shows the correlation of Poroshell EC-C18 and PFP retention time data in methanol and acetonitrile for the last seven analgesics. The first two analgesics, acetominophen and phenacetin, remain in the same elution order in all solvent column combinations. With these compounds removed, the correlation between EC-C18 and PFP retention in methanol (5a) and acetonitrile (5b) is poor and highly indicative of orthogonality.

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This work demonstrates how different chemistries and organic modifiers such as acetonitrile and methanol can develop different selectivity and can be used to optimize the separation. In this case, using an alternative selectivity column such as Poroshell 120 PFP yielded better results than a more commonly used C18 chemistry. Fluorinated stationary phases are useful because of their enhanced interaction with halogens, and conjugated compounds.

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Characterizing Polysaccharide Structure with SEC–MALS and Intrinsic Viscosity Measurements

Malvern Instruments Ltd.

The physical properties of polymers and polysaccharides depend strongly on their molecular properties, the most important of which are the molecular weight and molecular weight distribution, and the molecular size and structure. Gel permeation chromatography (GPC), also called size-exclusion chromatography (SEC), is the most commonly used tool for assessing these parameters, and is enhanced by coupling it to light scattering and viscometer detectors. In particular, the concurrent measurement of Rg using multi-angle light scattering (MALS) and intrinsic viscosity offers exceptional insight into the structure of synthetic and natural polymer molecules.

In this application note, molecular weight Rg and IV data are combined to study the structure of different polysaccharides.

Materials and Methods

Analysis was carried out using a Viscotek TDAmax system with SEC-MALS 20 detector. Viscotek A6000M columns were used and all samples were dissolved overnight in the mobile phase to a concentration of 0.5–3 mg/mL, with an injection volume of 100 µL.

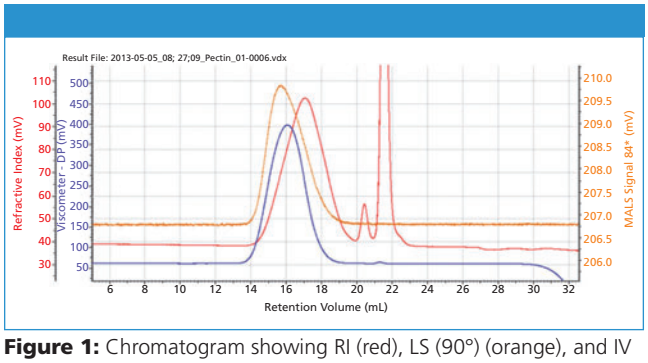

Figure 1: Chromatogram showing RI (red), LS (90°) (orange), and IV (blue) detector responses for pectin.

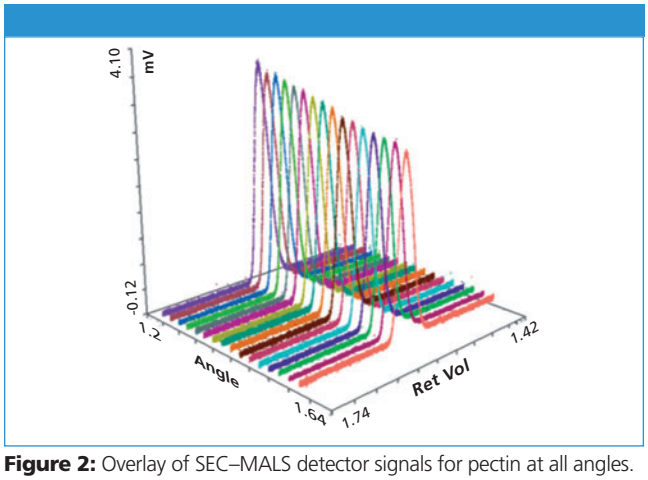

Figure 2: Overlay of SEC–MALS detector signals for pectin at all angles.

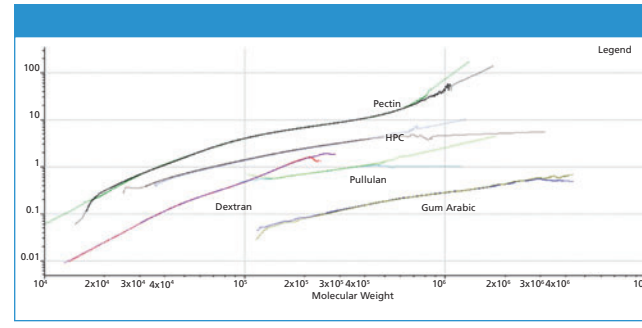

Figure 3: Mark-Houwink plot comparing pectin with other polysaccharides.

Results

Figure 1 shows an example chromatogram of pectin including the RI, LS (90°), and IV detectors, and Figure 2 shows the angular data from the SEC-MALS 20 detector. A slight angular dependence can be seen across the different angles, allowing Rg to be measured for this sample.

The results are also plotted on a Mark-Houwink (IV vs. MW) plot for a visual comparison. This allows comparison of different samples and is ideally suited to the study of branching and other structural changes.

Discussion

The Mark-Houwink plot shows that pectin is the highest and therefore the least dense molecule of those under investigation. As a comparison, gum arabic, which has the highest molecular weight, is the lowest on the plot, indicating that it is the smallest or most dense of the different samples.

In summary, the molecular weight, size, and intrinsic viscosity of pectin was measured using the Viscotek TDA and SEC-MALS 20 systems and compared with a selection of other polysaccharides. The differences in molecular structures between the different polysaccharides are very clear and easily observed using the Mark-Houwink plot.

Reference

(1) Characterizing polysaccharide structure with SEC-MALS and intrinsic viscosity measurements,
Malvern Instruments application note,
www.malvern.com/MRK1929

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Ion Chromatography – A Versatile Tool in Pharma Analysis

Stephanie Kappes, Alfred Steinbach, and Katinka Ruth,
Metrohm

High standards have to be met by the pharmaceutical industry when it comes to drug quality and safety. These standards are documented in pharmacopoeias as officially recognized pharmaceutical rules, and published as legal tools of customer protection by authorities such as governments and medical societies. The identification of a drug depends on sensitive, reliable instruments and methods — as does the determination of the drug's compliance with applicable regulations.

Ion chromatography (IC) is the method of choice to determine active ingredients, excipients, and traces of impurities, as well as metabolites in the form of organic and inorganic ions or polar substances, in a number of pharmaceuticals, pharmaceutical solutions, and even body fluids. It can determine several substances within a very short time in a single analysis — and can even distinguish chemically similar analytes. The concentration of analytes can vary from ng/L up to the per cent range. The large selection of separation columns and elution systems available makes IC useful for almost any kind of analyte. Interfering effects caused by the sample matrix can easily be avoided by using the right sample preparation or choosing a suitable detection method. In-line sample preparation is a feature of many modern IC systems, as the focus of recent advances in IC has been mainly on ease of use. However, convenience is not the only advantage brought by automation of the IC process: Reducing human interference to a minimum also means reducing the chances of mistakes and contamination.

Depending on the requirements of analyte and matrix, there is a broad range of detection methods to choose from:

- Conductivity detection with and without suppression
- Amperometric detection
- Spectrophotometric detection with and without post-column derivatization (UV–vis)
- Coupled detection methods such as IC–MS and IC–ICP–MS

Pharmaceutical samples come in many different forms which require different ion chromatographic approaches. What follows is an overview of frequent sample types with example analyses.

Pharmaceutical Solutions

The term "pharmaceutical solutions" denotes isotonic solutions, hemodialysis solutions, or infusion solutions. They contain anions, cations, carbohydrates, and organic acids, the concentrations of which frequently differ from one another by several orders of magnitude. Within the context of production monitoring and final quality control, an analysis method is required that can determine these ingredients with a high degree of precision. In addition, the analysis should be quick and require minimal effort. With its intelligent analytical procedure and automatic in-line sample preparation, IC fully accomplishes this task.

Two example analyses of hemodialysis solutions are shown in Figures 1–2. Patients suffering from renal failure require hemodialysis to compensate for the loss of the kidney's blood-cleansing function. During the process, the patient's blood exchanges solutes with a hemodialysis solution through a semipermeable membrane. The exchanged solutes include, among others, waste products such as urea and phosphate, which diffuse out of the blood and into the dialysis solution along the concentration gradient. The composition of dialysis solutions is complex because the removal of solutes from the blood changes its osmotic activity; therefore, it has to take place at a controlled rate, which is achieved by the right solute concentration. A strong change in osmotic activity can cause dialysis disequilibrium syndrome where, because of the low solute concentration in blood, solutes are washed out from other body compartments.

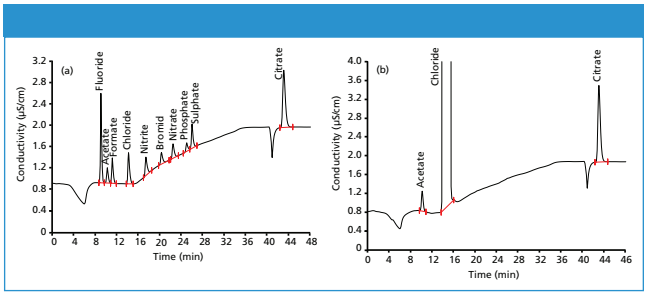

Figure 1: IC measurement on a Metrosep A Supp 7 - 250/4.0 using Na_2CO_3 gradient elution, followed by sequential suppression and conductivity detection. (a): Anion standard including acetate and citrate; (b): acetate and citrate in hemodialysis solution.

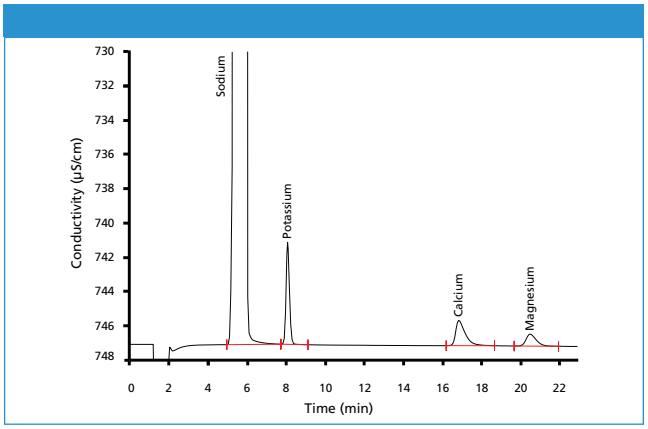

Figure 2: Cations in diluted hemodialysis concentrate using the Metrosep C 4 - 150/4.0 column and non-suppressed conductivity detection.

Figure 1 shows the simultaneous determination of citrate and acetate in diluted hemodialysis solution. In part A, an anion standard was measured; part B shows the sample determination. Citrate is added to hemodialysis solutions for its anticoagulant properties and acetate is added as a buffer substance. It is transferred to the patient's bloodstream during hemodialysis and stabilizes the blood's pH value. This is necessary because the kidneys of dialysis patients are not capable of excreting acid components – therefore, patients are often acidotic.

Besides citrate and acetate, the chromatogram reveals the presence of a close to physiological concentration of chloride. By using physiological solute concentrations, the concentration gradient is reduced to a minimum and a dynamic equilibrium is reached between the blood and dialysis solution. The loss of certain solutes — including chloride — is thereby prevented.

Figure 2 shows the determination of cations in hemodialysis concentrate after an automated in-line dilution step. Like chloride, the cations are present in close to physiological concentrations to avoid their drainage from patients' blood by osmosis.

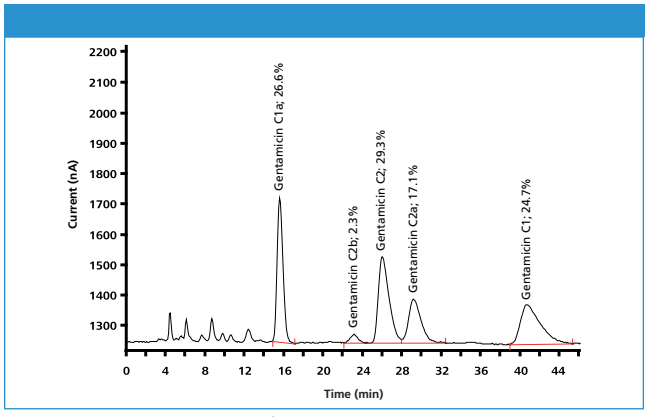

Figure 3: IC determination of the antibiotic gentamicin by pulsed amperometric detection; column: Polymer Laboratories RP-S; eluent: 60 g/L Na_2SO_4, 1.75 g/L sodium octane sulphonate, 1.34 g/L NaH_2PO_4, 8 mL/L THF (pH = 3, H_3PO_4); post-column addition: 300 mmol/L NaOH.

Table 1: Precision and recovery of azide.

	Peak area		
	Mean value (µS/cm)	RSD (%)	Recovery (%)
5 µg/L spike	0.42	1.96	101.7
30 µg/L spike	2.57	0.14	103.4
n = 3 measurements			

Active Pharmaceutical Ingredients

Active pharmaceutical ingredients (APIs) in medicines such as gentamicin, neomycin, cefadroxil, or bethanechol chloride can be determined by IC in accordance with the regulations of the U.S. Pharmacopeia and European Pharmacopoeia. The requirements regarding precision, separation, and recovery of the analytes are described in detail in the pharmacopoeias. Figure 3 depicts the ion chromatogram of an analysis of gentamicin, an antibiotic belonging to the group of aminoglycosides. Aminoglycosides are bactericidal antibiotics that block protein biosynthesis by binding to ribosomes, thereby causing mistakes in the translation from mRNA to DNA. Gentamicin consists of several closely related compounds, namely gentamicin C_1, gentamicin C_{1a}, and gentamicin C_2, C_{2a}, and C_{2b}. In spite of their structural similarity, IC achieves a good separation of the different gentamicin components.

Impurities in Pharmaceuticals

Apart from API analysis, it is also possible to determine impurities in pharmaceutical products by IC. Even small concentrations of an impurity can cause significant side effects. For example, in the synthesis of the antihypertensive irbesartan, azide can be detected in traces as an impurity in the product. Azide is strongly toxic to humans and its concentration in irbesartan is therefore subject to rigorous controls. The U.S. Pharmacopeia recommends ion chromatographic azide determination after direct injection according to USP<621>. In this method, a transfer solution consisting of the IC eluent and a suitable organic solvent is used to remove the API from the analytical column. However, this procedure is tedious, time-consuming, and cannot be automated.

Azide determination is more selective, more sensitive, and, above all, quicker with the use of in-line matrix elimination, where the interfering pharmaceutical matrix is separated from the target analyte in the course of sample preparation. The ion chromatogram in Figure 4 shows the analysis of an irbesartan sample spiked with different concentrations of azide. The signal is recorded by a conductivity detector following sequential suppression. Table 1 lists the average recovery values of azide that were achieved over three measurements, as well as the mean conductivity measured by the detector and the relative standard deviation.

The determination of azide in irbesartan with preceding matrix elimination fulfills all requirements of the regulatory authorities, which concern the selectivity of the method, its limits of detection and quantitation, precision, linearity, accuracy, and robustness. Therefore, it can be used as a quicker and more sensitive alternative to the proposed determination according to USP<621>.

Table 2: Selection of IC applications in the pharmaceutical industry.

Pharmaceutical	Analyte		Detection
Adrenaline	Adrenaline	API	Amperometric detection
…	…	…	…
Ibuprofen	Ibuprofen, Valerophenone	API	Spectrophotometric detection
…	…	…	…
Zoledronate/ Zoledronic acid	Phosphite, phosphate	Impurities	Conductivity detection

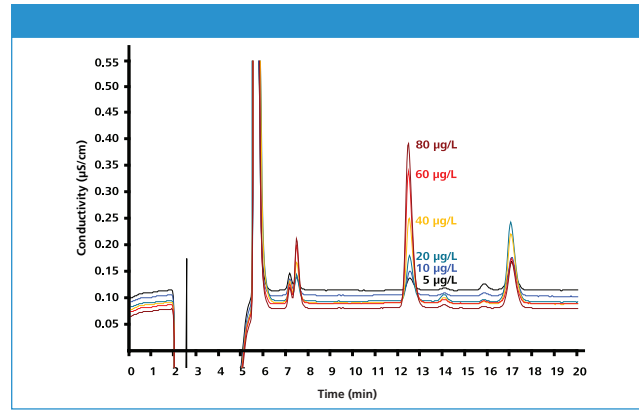

Figure 4: Irbesartan sample spiked with 5–80 µg/L azide; column: Metrosep A Supp 10 - 250/4.0; eluent: 5 mmol/L Na_2CO_3, 5 mmol/L $NaHCO_3$; inline matrix elimination with 70:30 (v/v) methanol/water.

Conclusion

Today, IC covers a diverse field of applications in the pharmaceutical industry. A selection of three out of more than 80 applications is listed in Table 2. The technique has become extremely versatile because of the large number of different columns, eluent and gradient options, sample preparation techniques, and automation possibilities that are available to the user.

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Antibody Drug Conjugate (ADC) Analysis with SEC–MALS

Wyatt Technology Corporation

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 that 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.

Here we present 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.

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 that 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.

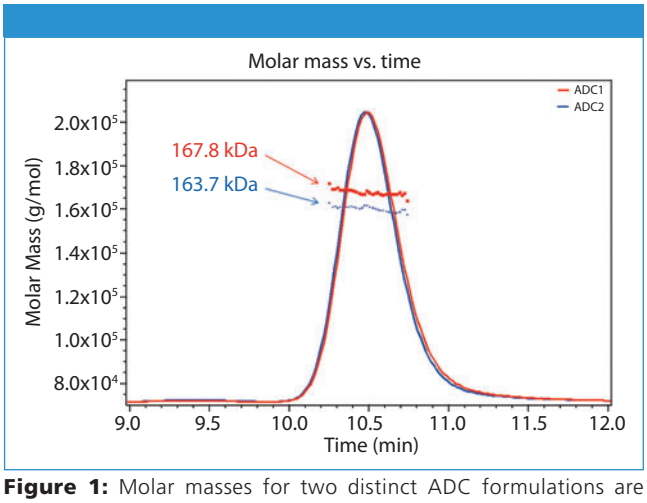
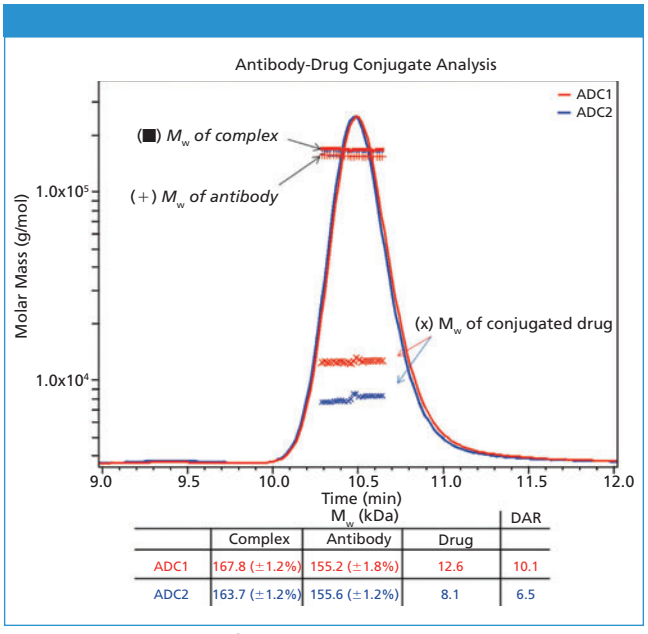


Figure 1: Molar masses for two distinct ADC formulations are determined using SEC–MALS analysis.



M_w (kDa)	Complex	Antibody	Drug	DAR
ADC1	167.8 (±1.2%)	155.2 (±1.8%)	12.6	10.1
ADC2	163.7 (±1.2%)	155.6 (±1.2%)	8.1	6.5

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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34th International Symposium on Halogenated Persistent Organic Pollutants (Dioxin 2014)

The **34th International Symposium on Halogenated Persistent Organic Pollutants (Dioxin 2014)** will be held at the **Hotel Meliá Castilla** in **Madrid, Spain,** from **31 August–5 September 2014.** This international symposium was first held in Rome, Italy, in 1980, and has been held in different cities around the world ever since. The Dioxin conference is one of the biggest international meetings and has previously attracted up to 1200 delegates from over 40 different countries, always counting on the invaluable support from multiple key sponsors and exhibitors.

Halogenated persistent organic pollutants are mostly man-made chemicals that pose a recognized threat to humans and the environment. This year, **Dioxin 2014** will continue to present recent advances in all areas dealing with these contaminants by gathering a scientific community determined to look for new scientific strategies, policies, and technological advances to protect the global environment from these and other related chemicals, and preserve human health and the ecosystem worldwide.

High-quality plenary lectures will be presented by the following leading scientists: Joan O. Grimalt (Spanish National Research Council, Spain); Antonia M. Calafat (Centers for Disease Control and Prevention, USA); Ricardo Barra (University of Concepción, Chile); Hindrik Bouwman (North-West University, South Africa); Jiang Guibing (Chinese Academy of Sciences, China); and Jacob de Boer (Institute for Environmental Studies, Netherlands). Oral presentations and posters will be presented in parallel, covering different topics pertaining to halogenated persistant organic pollutants including: Sources; environmental levels and ecosystems; food and feed; toxicology; human exposure; regulation; policy and risk assessment. A core topic will be the analytical determination of these pollutants that in turn will cover various subfields: Sampling and preparation methods; new screening methods; novel instrument techniques; non-target and emerging contaminants; multidimensional techniques; QA/QC; inter-laboratory studies; reference materials; development of bioanalytical methods; and data handling. The analytical identification and quantification of many of these halogenated pollutants can be extremely challenging, and so chromatography will hold a prominent position, particularly when hyphenated with mass spectrometry in a different array of modalities, as it is able to provide an unrivalled degree of sensitivity and selectivity.

Outstanding student presentations will be recognized with the "Otto Hutzinger Student Award" that acknowledges scientific contributions to the field of halogenated persistent organic pollutants. All students, whether undergraduate or graduate, presenting an extended abstract for either an oral or poster presentation are encouraged to apply for this award.

In short, this symposium offers a high quality scientific programme combining both traditional topics and emerging issues that will be complemented by special satellite events and exhibitors presenting their latest equipment and services. Furthermore, an exciting social programme awaits all attendees. Dioxin 2014 will provide great opportunities for all delegates from all over the world to exchange new ideas face to face and to establish future collaborations. Finally, the success of Dioxin 2014 will be enhanced by the special atmosphere of Madrid's social life, an essential element in generating synergies for scientific cooperation and friendship.

The deadline for poster contributions has now passed, but the organizers are open to late entries.

Chair Person: Dr. Begoña Jiménez
E-mail: dioxin2014@mci-group.com **Website:** www.dioxin2014.org

14–18 September 2014
30th International Symposium on Chromatography (ISC 2014)
Salzburg Congress, Salzburg, Austria
Chairpersons: Wolfgang Buchberger, Michael Laemmerhofer, and Wolfgang Lindner
Tel: +43 (0)512 575 600
E-mail: i.kaehler@cmi.at
Website: www.isc2014.at

30 September–2 October 2014
International Symposium on GPC/SEC and Related Techniques
The Westin Grand Frankfurt, Germany
Organizers: Waters Corporation and PSS Polymer Standards Service GmbH
Tel: +49 69 2981 719
E-mail: Suzanne@cosmoscience.org
Website: goo.gl/j9DCrW

12–16 October 2014
The 17th International Symposium on Field- and Flow-based Separations (FFF 2014)
The University of Utah, Salt Lake City, Utah, USA
Organizer: The Organizing and Scientific Programme Committees headed by Bruce Gale
Tel: +1(801) 585 5944
E-mail: bruce.gale@utah.edu
Website: http://fff2014.mech.utah.edu

10–12 November 2014
World Mycotoxin Forum
Vienna Marriott Hotel, Vienna, Austria
Chair: Helena B. Bastiaanse
Tel: +31 30 2294247
Fax: +31 30 2252910
E-mail: WMF@bastiaanse-communication.com
Website: www.bastiaanse-communication.com/wmf

Send any event news to Bethany Degg: bdegg@advanstar.com

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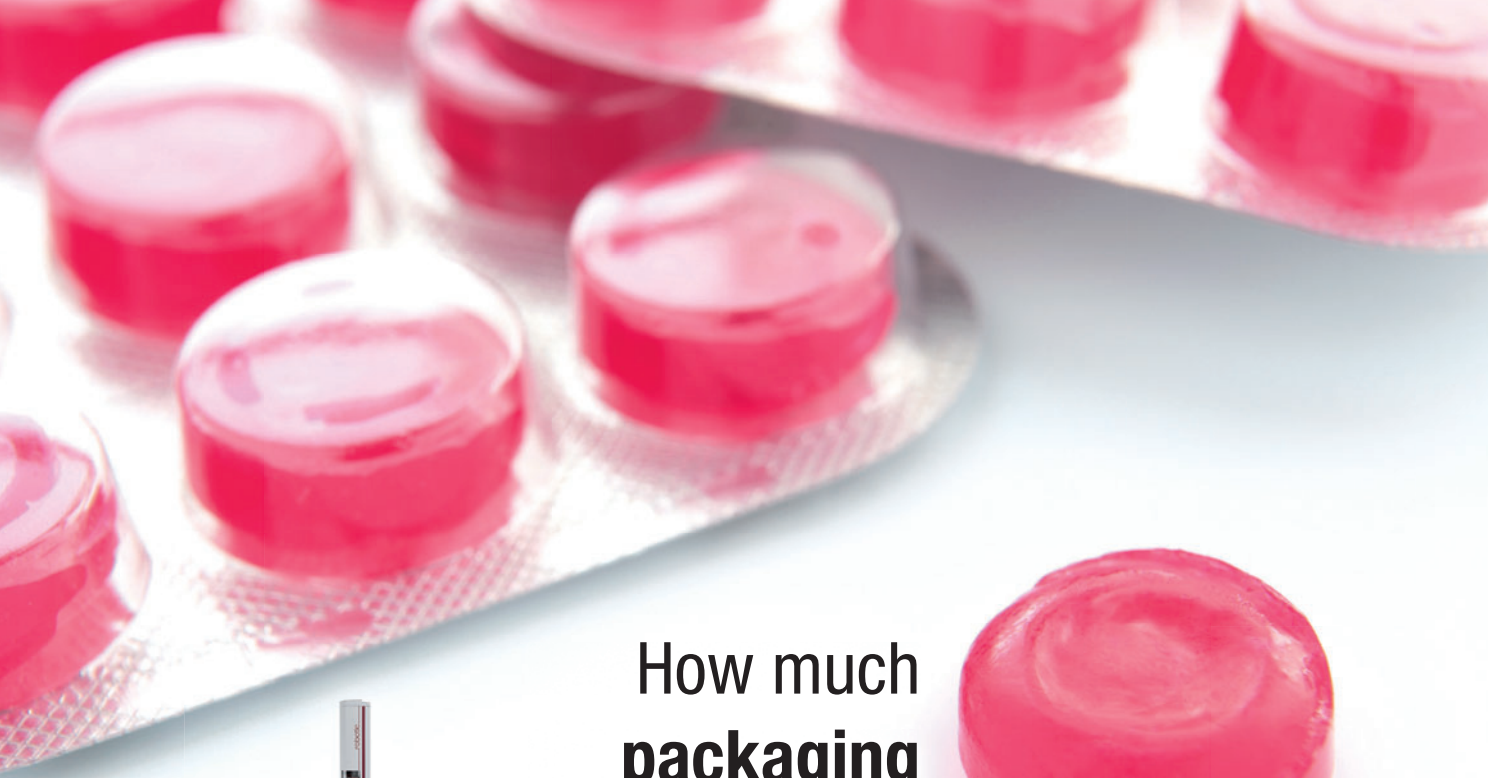

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