BIOPROCESSING

Evolution and Revolution in Chromatography

User-Friendliness, Automation, Integration, and Consistency in HPLC

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High-performance liquid chromatography's (HPLC) evolution into an essential life science analyzer has been accompanied by a familiar paradox: As developers added more functionality, systems became easier to use.

Make no mistake. Most improvements in HPLC instrumentation have been incremental, as can be expected from a mature field. By constantly improving and upgrading the "little things," instrument vendors have charted a slow but steady upward course in what L.P. Raman, technical marketing manager at **Whatman** (www.whatman.com), identifies as modern HPLC's hallmarks: user-friendliness, automation, integration, and consistency.

And the Living Is Easy...

One of the incidentals that has made HPLC users' lives so much easier has been improved sample prep. In addition to its HPLC columns and media, Whatman has been pushing and upgrading its Mini-UniPrep™ syringeless filter line as a productivity tool for busy HPLC labs.

According to the company, Mini-UniPrep reduces sample processing time by one third while reducing the need for other consumables like syringes, sample cups, and transfer containers. Whatman's Slit Septa Mini-UniPrep facilitates sample prep through HPLC robotics; Amber Mini-UniPrep protects light-sensitive samples.

Whatman is also working with an unnamed German company on what Raman calls "end-to-end sample prep" for research and manufacturing markets.

Tom Jupille, president of **LC Resources** (www.lcresources.com), envisions tomorrow's HPLC instruments as laboratory "appliances." To a large degree this has already come to pass: gone are the days when operators spent more time priming pumps, unclogging injectors, and looking for wrenches than designing experiments and injecting samples.

"In the past, new HPLCs were like toasters that came with a 350-page thesis on Ohm's law and the theory of starch," he observes wryly. "Instruments are a means to an end. The less involvement users have with the nuts and bolts of HPLC systems, the happier they'll be."

Jupille spends much of his time teaching open-enrollment courses on HPLC methods development and troubleshooting, while sister company Separation Science Associates provides HPLC consulting, writing, and communications services.

His vision of HPLC parallels the evolution of laboratory instrumentation, which these days rely as much on computing technology and component integration as on fluidics or analyte detection.

HPLC training reflects instrumentation's evolution. Ten years ago, says Jupille, much of his *Practical HPLC* course covered nuts-and-bolts problem solving.

"If you had an air bubble, we taught students to disconnect the outlet fitting, lift up the solvent reservoir, turn up the flow rate, hit the pump head with the handle of a screwdriver to get the air bubbles out, and repeat the sequence until everything stabilized.

"Today, if you're using a **Waters** Alliance, for example, you go to the menu screen and click 'prime,' and the machine opens the valve for you, turns the pump up to full steam for a few seconds to purge the air bubbles, closes the valve, repressurizes the pump, and automatically repeats the process if there's any air left in the pump head.

Aimed at proteomics markets, Agilent's HPLC Chip provides fully functional modular HPLC capability.

"I don't think HPLC is ever going to be as simple as using a toaster," he says, "and it always helps to know what's going on. However, like with today's automobiles, drivers will no longer need to know what's going on underneath the hood to go out for a drive."

It's the Proteome, Stupid!

Proteomics continues to drive HPLC innovation, especially with respect to analyzing ever smaller samples, sometimes from a single cell. Columns are getting narrower, media particles smaller, and pumps more powerful. Two-dimensional techniques, including LC/LC, gel/LC, LC/MS, and LC/MS^n, have become as routine for proteomics work as 2-D NMR was for small-molecule experiments two decades ago.

Multidimensional LC typically employs ion exchange as a first dimension followed by reversed-phase separation, fed into a mass spectrometer. Commercial systems rely on coupled columns with automated fraction collection and reinjection onto the second-dimension column.

LC Packings' (www.lcpackings.com; a **Dionex** company) UltiMate™ dual-gradient pumping system, for example, uses a capillary LC pump for the first dimension and a nano-LC pump for the second dimension. UltiMate also operates in parallel HPLC mode for side-by-side separations.

Shimadzu Scientific Instruments (www.ssi.shimadzu.com) claims its 2-D Micro-HPLC System analyzes peptide digests that cannot be resolved through gel electrophoresis.

The Finnigan ProteomeX™ workstation from **Thermo Electron** (www.thermo.com) offers complete 2-D LC capability plus MS.

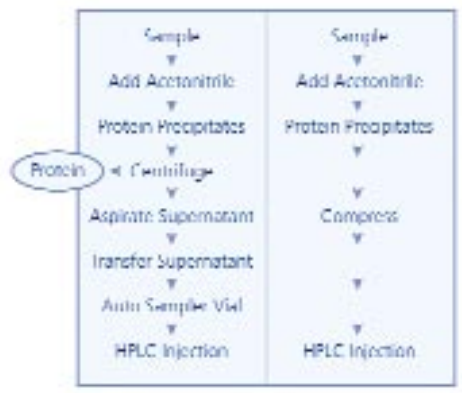
Thermo has integrated several workhorse products into the ProteomeX, including the Finnigan LCQ Deca XP MAX ion trap mass spectrometer, an orthogonal electron-spray ionization source, the Surveyor HPLC, a 10-Port Switching Valve, Thermo Hypersil-Keystone ion-exchange and C18 columns, plus ProteomeX and BioWorks software.

John Yates, Ph.D., at Scripps (La Jolla, CA) offers a possible hint into the future of 2-D HPLC with MuDPIT (multidimensional protein-identification technology).

MuDPIT employs single columns, packed sequentially with cationic exchangers and reversed-phase materials, and pulsed with salt eluent of increasing ionic strength. Effluent is introduced directly into a ThermoFinnigan mass analyzer.

According to Tim Wehr, a consultant with Separation Science Associates, MuDPIT's major drawback is that the first dimension separation is run "blind," which makes second-dimension separation less than optimal.

"No 2-D system will work perfectly for complex mixtures," states Wehr, who believes the technique might be improved through an initial affinity step rather than ion exchange.



Spin method vs. Mini-UniPrep method Whatman

Affinity methods that target either peptide sequences or specific residues (amino acids or carbohydrates) have the potential for sharper elution from the first dimension and, subsequently, narrower loading onto the second-dimension column.

Small on the Rise

Wehr, who like his boss Jupille has a bird's-eye view of the HPLC marketplace, sees columns increasingly using smaller particles and shorter lengths to accommodate high-throughput applications for drug discovery, combinatorial chemistry, and pharmacokinetic studies.

Another trend he notes is the return of normal-phase silica stationary phases, with or without a bonded polar phase.

"Silica has been eclipsed by reversed-phase separations, but with normal-phase type solvents popular in LC/MS, look for normal phase to make something of a comeback."

Wehr also mentions polar embedded reverse-phase media, for example the Acclaim® PolarAdvantage column from Dionex (www.dionex.com). Acclaim's "polar-enhanced stationary phase" consists of a C16 functional group attached to the silica backbone through a sulfonamide coupled to an ether linkage. Polar-enhanced separations offer wide pH-tolerance, and elution with purely aqueous mobile phases.

Something Completely Different

Although advances in HPLC tend to be evolutionary rather than revolutionary, two recent system introductions show there is still plenty of opportunity for innovation.

Agilent Technologies (www.agilent.com) made a flurry of HPLC-related announcements at this year's "Pittcon" conference. Of more-than-passing interest was the company's announcement that it had shipped more than 50,000 of its workhorse 1100 Series LC systems since the instrument's introduction in 1995. Almost simultaneously, the company introduced a microcollection/spotting system for the 1100 and a new proteomics workstation that incorporates the 1100.

Then in May, Agilent introduced a reusable, polymer, credit card-sized HPLC "chip" that replaces traditional LC columns for proteomics research. Formats for other applications are in the works. The chips integrate sample enrichment and nanoflow LC with connectors and spray tips for electrospray MS in true LC format, as opposed to more-common electrophoresis chips.

According to Agilent, the chip's construction eliminates half of the fittings required for an LC/MS system, thereby reducing potential for leaks and dead volumes while improving ease of use and sensitivity.

HPLC chips are manufactured using a laser that creates channels, columns, and fluid-access ports by etching the surfaces of polyimide film layers, which are then laminated to form the three-dimensional structure and trimmed to fabricate the electrospray tip and other details. The HPLC chip actually consists of two columns: one for trapping peptides from a protein digest; the other, the analysis column with the MS interface.

The chip, which interfaces with the 1100 Series HPLC and the 1100 Series LC/MS ion trap mass spectrometer, is swapped in or out in seconds. Despite its small footprint, the chip holds enough room for a 20-cm HPLC column using any standard packing material.

Chip/monolith-based HPLC will promote the idea of instrument-appliances. Jupille of LC Resources likens the interchangeable separations components to computer software, which analytical scientists switch in or out depending on the type of analysis they require. "What you're really looking at is a column-on-a-chip, where you keep a library of chips sitting on a shelf that enables you to turn a protein analyzer into a DNA analyzer or aspirin analyzer."

Agilent is marketing the chip to proteomics researchers working with as little as one cell's protein output. "At that level you need a tiny column to achieve required sensitivity," observes Ron Majors, Ph.D., senior chemist at Agilent's Wilmington, DE, facility.

Chip lifetime depends on sample cleanliness. Agilent claims chips are still working fine after 300 injections, or up to 20 days of nonstop use.

According to Dr. Majors, the HPLC chip program is part of an analytics environment driven by productivity, low cost of analysis, and more rugged, reliable instrumentation.

"People are looking for faster separations

on columns that can take pH 10 or 12, or even acidic conditions. At the same time, instrumentation must match the column's technology in terms of high throughput and reliability."

UPLC

Like most cutting-edge HPLC systems, **Waters**' (www.waters.com) Acquity Ultra Performance LC™ (UPLC), which received the Pittcon Editor's Gold Award for Best New Product, is targeted for proteomics LC, LC/MS, and LC/MS/MS.

UPLC differs from conventional HPLC in two important respects: media particle size for UPLC is 1.7 µm (average), versus 5.0 µm for ordinary media, and the system runs at 15,000 psi rather than the usual 5,000. The benefits of smaller particle and higher-pressure operation are much higher speed, resolution, and sensitivity.

Radical Design

Designing an instrument that departs so radically from standard instrumentation took some doing. The most obvious upgrade is in the pump, which needs to perform reliably and uniformly at three times standard HPLC operating pressure.

In addition, the solvent-delivery system must mitigate potential solvent-compressibility across a wide range of pressures, especially for multicomponent mobile phases. Waters redesigned the injectors to handle the high pressures and protect columns from sudden pressure changes during injection.

The separation media's small size also challenged detectors, which required a sampling rate sufficiently high to capture enough data points in a short amount of time to produce a visible peak.

At the recent "American Society for Mass Spectroscopy (ASMS) Conference," in Nashville, Waters gave two presentations on UPLC, one with scientists from **AstraZeneca** (London).

Waters' data demonstrated a tenfold improvement in throughput, and between two- and threefold improvement in resolution, for detecting metabolites of performance-enhancing drugs from body fluids using UPLC.

By Waters' estimates, Acquity UPLC costs about $60,000, or about 25% more than a comparably configured HPLC system with a UV detector and PC. Acquity may be purchased alone or with a Micromass MS instrument.

Waters introduced another version of this product, NanoAcquity UPLC, at ASMS. NanoAcquity is only sold with a Micromass Q-TOF Premier MS instrument, which also debuted at the conference. Together, these products are known as the Waters Protein Expression System and are marketed to scientists performing multiplexed, quantitative proteomics.

NanoAcquity is optimized for high-resolution separations, without flow splitting, at nanoflow rates on nanoAcquity UPLC columns ranging from 75- to 320-µm internal diameters. Waters expects to ship NanoAcquity in late 2004 or early 2005.

Jupille of LC Resources likes the Waters system. "A lot of what you do, in terms of analysis time or number of samples you push through, is limited by column back-pressure. As the first significant increase in operating pressure, UPLC allows you to do a lot more than you ever could with traditional HPLC," Jupille notes.

"By increasing column pressure from 5,000 psi to 25,000 psi, for example, you can use a column that is five times as long, or you can inject five times as many samples."

HPLC has always been an exercise in trade-offs between instrument operating pressure, resolution, and run time.

"You might be able to get baseline resolution between dimethyl and trimethyl chickenwire," Jupille says, "but it might take a long time. Or you could get the compounds off the column quickly without baseline resolution. By raising the pressure, you don't need to compromise as much." GEN