



Watch

In this month's "Column Watch," Ron Majors discusses some of the scientific highlights of the 27th International Symposium on High Performance Liquid Phase Separations and Related Techniques — HPLC 2003 — which was held in Nice, France, 15–19 June 2003.

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Highlights of HPLC 2003

The 27th International Symposium on High Performance Liquid Phase Separations and Related Techniques — HPLC 2003 — which alternates between Europe and North America, was held in the beautiful city of Nice, France, on the Cote d'Azur 15–19 June 2003. This symposium is the premier scientific event for bringing together the myriad techniques related to separations in liquid and supercritical fluid media. Chaired by Antoine-Michel (Tony) Siouffi of the University of Law, Economics, and Science of Aix-Marseille (France), HPLC 2003 assembled 1255 scientists from 52 countries. This number included 240 vendor representatives from 76 exhibitors for the four-day instrument, software, and consumables exhibition. A large contingent of 163 students was evident, especially in the poster sessions.

With 687 symposium papers, including 107 oral presentations (many of which were presented during simultaneous sessions), two two-day poster sessions (34 in all) with a total of 580 posters, and five vendor seminars, attendees had their hands full deciding how to allocate their time. Furthermore, with three short courses held on Sunday, 15 June, most attendees who lasted through the whole week were ready for some weekend relaxation. The short course topics reflected areas of current interest and included high-efficiency enantiomeric separations, the always-popular high performance liquid chromatography (HPLC) troubleshooting, and capillary electrophoresis (CE) method validation. Normally, these symposia end on Friday at noon, but this year's event was held for four days only and ended on Thursday afternoon, so the organizers packed a lot of events into four days.

Obviously, HPLC was the predominant technology used by presenters in the technical sessions at the symposium. Table I provides a rough breakdown of the coverage of liquid-phase technology and techniques in separation sciences. Table II is a similar breakdown for application areas. Table III lists the detection principles used by the presenters. I based the assignments

into these three tables upon the main emphasis of individual papers as well as the separation and detection techniques used.

In this installment of "Column Watch," I will present some of the scientific highlights of HPLC 2003. Because it was impossible for one person to adequately cover all oral and poster papers, to a degree my coverage will reflect a personal bias, although I was able to get some notes from other colleagues, who are acknowledged at the end of this column. Because space is limited, I will cite few individual papers but will provide overviews of major sessions. I have elected to omit new sample preparation techniques and multidimensional chromatography methodology, but I will

Table I: Papers presented by technology or technique

Technology or Technique	Papers Presented (%)
HPLC column technology, phases, and design*	22.0
CE and electrophoresis†	19.7
Theory and retention mechanisms	13.4
Sample preparation	9.8
Multidimensional chromatography and column switching	4.8
High-throughput and fast liquid chromatography	4.1
Preparative and process chromatography	3.9
Chemometrics	3.7
Miniaturization, microfluidics, and chips	3.1
Validation	3.1
Affinity and immunoassay	3.1
Instrumentation and software development	2.7
Capillary electrochromatography	2.3
Supercritical fluid chromatography	1.7
Field-flow fractionation	1.7
Method development	0.8

* Includes monolithic phases (36% of all column presentations) and capillary and nano columns (14% of all column presentations).

† Includes micellar kinetic chromatography, capillary zone electrophoresis, isoelectric focusing, etc.

cover these topics more thoroughly in an installment of "Sample Prep Perspectives."

Poster Sessions

The mainstay of HPLC 2003 was the poster sessions in which detailed applications and methodology studies were reported and face-to-face discussions with the authors, often about very specific topics, were conducted. As judged by the note-taking at many of the poster boards, many attendees were able to extract specific experimental details that they could use back home in their daily laboratory activities. Fortunately, many of the poster authors were kind enough to provide small reproductions of their poster papers that could be taken for later perusal. Some collected business cards and addresses for sending poster reprints by mail or e-mail.

This year I had the privilege of chairing the poster committee for the awarding of the top three posters at the symposium. Examining 580 posters in four days with lecture sessions, social activities, lunch breaks, and so on was a challenging task. I want to individually thank the 19 members of the poster selection panel (see accompanying sidebar) who worked very hard to narrow down the huge collection of posters and to select the three winners by the end of the symposium. Overall, the committee felt that the quality of posters, both on technical and presentation bases, was outstanding this year and showed a definite improvement over previous symposia in this series.

Table II: Papers presented by application area

Application Areas	Papers Presented (%)
Pharmaceuticals	19.7
Proteins and proteomics	13.8
Biological samples*	12.1
Food and beverages	10.1
Chiral compounds	7.6
Environmental and toxins	6.2
Amino and organic acids	6.0
Natural products	4.7
Organic chemicals	3.5
Other†	3.5
Genomics and DNA	3.1
Polymers	2.7
Pesticides and herbicides	2.7
Inorganic ions	1.6
Amines and biogenic amines	1.6
Vitamins	1.2

*Blood, urine, cerebrospinal fluid, tissue, etc.
†Surfactants, organometallics, lipids, etc.

The Best Poster Awards, sponsored by Agilent Technologies GmbH (Waldbronn, Germany), were announced at the closing session. This year's first-place winners were M. Bartolini, V. Cavrini, and V. Andrisano of the University of Bologna (Italy) and G. Felix of the University of Bordeaux (Talence, France) for their poster "Development of a HPLC System Based on an Immobilized Acetylcholinesterase (Ache) Monolithic Micro-Immobilized Enzyme Reactor for On-Line Inhibition Studies." The second-place winners were O. Corcoran of King's College (London, United Kingdom) in conjunction with A. Freund, W. Maas, and M. Spraul of Bruker Daltonics (Billerica, Massachusetts and Rheinstetten, Germany) for their poster "A New Cryoflowprobe for LC-NMR-MS." Figure 1 shows Olivera Corcoran receiving her award.

The third-place winners were C. Lucy of the University of Alberta (Edmonton, Alberta, Canada) and P. Hatsis of the National Research Council (Halifax, Nova Scotia, Canada) for their poster "Ion Chromatography at over 50 Times the Speed of Small" — the *Small* in the title is a reference to Hamish Small and co-workers from Dow Chemical Co. (Midland, Michigan) who did the seminal investigation on ion chromatography in 1975.

Opening Session

The opening session was highlighted by a presentation by Pascal Kintz of the Institute de Medicine Legale (Strasbourg, France) who discussed real-world applications of liquid chromatography-mass spectrometry (LC-MS) and LC-MS-MS in forensic tox-

icology. Despite the proven advantages in compound identification and sensitivity and the fact that more than 50% of the papers at HPLC 2003 reported the use of MS as a detection technique, Kintz reported that fewer than 1% of the forensic laboratories in France are using LC-MS and even fewer (<0.1%) are using LC-MS-MS. Instead, GC-MS (the gold standard), headspace GC-MS, inductively coupled plasma (ICP) MS (for metals), and immunoassays are in widespread use. Primary reasons for this situation are that LC-MS was introduced to French toxicology laboratories in 1995 and, more importantly, no useful database is available. In

Poster Selection Panel Members

Anne-Francoise Aubry, Bristol-Myers Squibb (New Brunswick, New York)
Carlo Bertucci, University of Bologna (Bologna, Italy)
Steve Cohen, Waters Corp. (Milford, Massachusetts)
Geoff Cox, Chiral Technologies (Exton, Pennsylvania)
Reg Cross, Swinburne University of Technology (Hawthorne, Victoria, Australia)
John Dolan, BASi Northwest Laboratory (McMinnville, Oregon)
Roy Eksteen, Tosoh Bioscience (Montgomeryville, Pennsylvania)
Maureen Joseph, Agilent Technologies (Wilmington, Delaware)
David Lloyd, Bristol-Myers Squibb (New Brunswick, New York)
Ronald Majors, Agilent Technologies (Wilmington, Delaware)
Maria Matyska-Pesek, San Jose State University (San Jose, California)
Joseph Pesek, San Jose State University (San Jose, California)
Matt Przybyciel, ES Industries (Berlin, New Jersey)
Lane Sander, NIST (Gaithersburg, Maryland)
Peter Schoenmakers, University of Amsterdam (Amsterdam, The Netherlands)
Frank Steiner, Saarland University (Saarbrücken, Germany)
Frantisek Svec, University of California-Berkeley
Denise Wallworth, Advanced Separation Technologies Ltd. (Astec) (Manchester, United Kingdom)
Jeff Wheatley, Pfizer Global R/D (La Jolla, California)

Table III: Types of detection techniques used in presentations at HPLC 2002*

Detection Techniques Used	Use (%)
Mass spectrometry (MS)	35.4
Tandem MS (MS-MS)	13.7
UV spectroscopy	16.1
Fluorescence and laser-induced fluorescence	11.8
Electrochemical	6.2
Nuclear magnetic resonance	5.6
Evaporative light scattering	3.7
Conductivity	2.5
Miscellaneous†	5.0

*Not every abstract indicated the detection technique used, so only those that provided this information are counted in this table.
†Includes refractive index, radioactivity, chemiluminescence, atomic absorption spectrometry, inductively coupled plasma MS, and atomic emission spectrometry detection.

contrast, GC–MS has myriad compounds in published and on-line databases that enable rapid identification of possible compounds of forensic interest. The standard tests performed in forensic laboratories are blood alcohol, cause of death, analysis of street drugs, and analysis of food, especially poisoning cases. Some of the newer tests demanded of forensic laboratories are for cases involving driving under the influence of drugs, impairment evaluation, chemical-assisted rape, history of drug abuse, and doping control. Although blood, urine, gastric content, bile, and vitreous humor are the typical sample types for forensic work, more recently, less-invasive approaches for sampling substances such as hair (a reliable method for long-term history of drug abuse), saliva (especially for parent drug), and sweat (can be used but smaller amounts of drug are present). The potential of LC–MS–MS analysis was demonstrated by an analysis of buprenorphine, a heroin substitute used instead of methadone. HPLC with diode-array detection can measure this compound only at the 40-ng/mL level in blood, GC–MS at the 0.5-ng/mL level, and HPLC with electrochemical detection at the 0.2-ng/mL level; however, HPLC–MS–MS easily can measure at levels as low as 0.02 ng/mL. Because of sensitivity and selectivity advantages with a better database of forensic compounds, Kintz foresees a great future for this hyphenated technique.

J. Michael Ramsey of the Oak Ridge National Laboratory (Oak Ridge, Tennessee) presented the opening session's second lecture, which was about molecular transport through nanoscale channels. Ramsey, one of the pioneers in microfluidics and applications of chip-based tech-

nology, gave an overview of the work being performed in his laboratory and others. As researchers proceed to use channel dimensions as small as 1 nm — so-called nanofluidics — new issues evolve. As channels shrink to smaller than micrometer scale, analysts face questions about how to fabricate such small features, and the dimensional stability of these features is a concern. The characteristics of electrokinetic fluid transport at molecular dimensions have not been probed experimentally in great detail, because making molecular-scale measurements brings many challenges. Solid–liquid interfacial characteristics will become even more important to control at nanometer dimensions because fluids no longer exhibit bulk characteristics. These questions are among the many issues pondered by Ramsey and co-workers. They recently have performed the first direct experimental benchmarking of electroosmotic transport under double-layer overlap conditions and found that continuum mechanics appear to describe this transport accurately to 80-nm scale. He and his colleagues expect continuum descriptions of this transport to break down at length scales of approximately 10 nm. Ramsey's group also has shown that new separation mechanisms for separating polyelectrolytes in free solution become possible when channel dimensions approach the hydrodynamic radius of the molecules. They demonstrated an electrohydrodynamic type of separation of deoxyribonucleic acid (DNA) base pairs in 320-nm channels, in which the hydrodynamic radius of the molecules was similar to but smaller than the channel lateral dimensions. They observed a sieving type of separation in 100-nm channels, in which the average size of the molecules was greater than the channel size. As channels become even smaller, especially in two dimensions, (two-dimensional [2-D] nanoscale confinement), Ramsey foresees more-intriguing possibilities such as a molecular version of a Beckman Coulter Counter (Fullerton, California) or structural characterization of single molecules of biopolymers.

New Column Technology Highlights

As Table I shows, the development and study of columns and stationary phases dominates the new technologies, but investigations using CE are a close second. This observation is consistent with the last three years' HPLC symposia (1–3).

Monolith column technology: At HPLC 2003, monolithic phases dominated column technology and application papers. Two oral sessions were devoted to presentations about silica- and polymeric-based monoliths and more than one-third of all column and sample preparation posters were devoted to this area. The monolith column sessions were very well attended. In an oral presentation, Nobuo Tanaka and co-workers from the Kyoto Institute of Technology, Kyoto Monotech Co., and Kyoto University (all in Kyoto, Japan) and the Max Planck Institute (Potsdam, Germany) demonstrated the advantages of preparing silica monolithic capillary columns with lengths as long as 2.5 m and inner diameters as small as 0.05 mm. The longest capillary columns can generate more than 200,000 theoretical plates in 20 min with a pressure drop of only 20 MPa. In the ultrahigh-pressure mode with the capillary operating at 200 MPa, the capillary columns were able to generate 125,000 plates in 2 min.

Surprisingly, the porosity of these columns is 95%, which is much greater than that of 4.6-mm i.d. silica-rod columns. The phase ratios are 0.02–0.04 for the capillary columns, and those of the silica-rod columns are approximately 0.06. The permeability of the monolithic capillary columns is 0.08–0.09 μm^2 versus 0.02–0.04 μm^2 for a packed capillary column of the same dimensions. Thus, the capillary monoliths also show great potential for high-speed separations at lower pressure drops, as was demonstrated for the traditional silica-rod columns. In fact, these monolith capillaries nearly match the efficiencies demonstrated for electrodriven capillaries packed with 1.8- μm particles.

The capillary monoliths have some practical limitations that must be overcome. For example, because the flow rates are quite low (less than 2 $\mu\text{L}/\text{min}$), flow rate and gradient compositional control could be difficult, and sample injection issues must be resolved. Using a conventional six-port sample valve for direct injections causes a loss of efficiency, so Tanaka developed a split injection system and advocated the use of a weaker injection solvent to help focus analytes at the column head. Additional work with a 2-D LC system using a fluorinated phase in the first dimension and a silica-rod column in the second dimension provided a peak capacity of approximately 1000. Tanaka and colleagues applied this comprehensive system to a crude lead extract and by using



Figure 1: Photograph of second poster prize winner Olivera Corcoran of King's College (London, United Kingdom) receiving her prize from Ron Majors (Agilent Technologies).

LC–MS–MS were able to separate 350 compounds, classify 250 compounds, and identify 100 compounds.

In the same session, Dieter Lubda and co-workers from Merck KGaA (Darmstadt, Germany), Kyoto Monoliths, and Kyoto University introduced their CapRod columns that have lengths as long as 112 cm and inner diameters of 0.1 and 0.2 mm. Using a 112 cm \times 0.2 mm column, they generated 120,000 plates under isocratic conditions. The flow rates they used were in the 0.5–2.5 μ L/min range. These silica-based monolith columns were used to separate macromolecules. They investigated the separation of a cytochrome C digest using an endcapped C18 reversed-phase CapRod column with a formic acid buffer, which provided better sensitivity in LC–MS compared with other ion-pairing reagents such as trifluoroacetic acid.

Michael Buchmeister and co-workers from the University of Innsbruck (Austria) and Saarland University (Saarbrücken, Germany) synthesized polymeric monoliths using transition metal-based (metathesis-based) catalyzed polymerization (4). Using the ruthenium compound as a catalyst and the reaction depicted in Figure 2, they synthesized monolithic materials whose functionality could be varied by surface derivatization through the R group or by in situ grafting of a functional monomer. They were able to generate carboxylic, amine, alcohol, phenolic, and chiral selector surface functionality. With their *living polymerization* synthesis procedure, they also were able to control the specific surface and porosity, including pore-size distribution that could be bimodal, that was measured by inverse size-exclusion chromatography and mercury intrusion. Buchmeister's group has synthesized monolith phases inside capillaries with inner diameters as small as 15 μ m.

Tatiana Tennikova of the Russian Academy of Sciences (St. Petersburg) investi-

gated hybridization between complementary polyribonucleotides (molecular weight of 100,000 Da) by covalently bonding one of them to a monolithic disk using various types of coupling chemistry. Then, she demonstrated affinity chromatography on these monoliths whose open, unified structure enabled flow rates as high as 10 mL/min with no diffusion limitations. Tennikova investigated the effect of flow rate, concentration, and temperature on the formation of these biospecific complexes.

Frantisek Svec of the University of California–Berkeley is one of the pioneers in the field of polymeric monolith technology. He presented a historical introduction of the various approaches to prepare silica and polymeric monoliths and then proceeded to describe two approaches used in his laboratory. The first molding procedure used thermally initiated free radical polymerization that was best suited for macroporous monoliths with a variety of functional groups for the fast chromatographic separations of biomolecules. The preparation time was 12–24 h, and temperatures in the range of 50–70 $^{\circ}$ C were required. The more recent approach used a photo-initiated polymerization method (similar to photolithography) to prepare monoliths within capillaries and channels of microfabricated devices. Using a masking procedure, the monolith could be located on a specific place on the microfabricated device. Ultraviolet (UV) light was used to irradiate the polymerization mixture forming the monolith. Svec could control the pore size and the type of free radical initiator by modifying the polymerization mixture and could control the polymerization time by adjusting the UV intensity. With this method, no heating was required because the polymerization occurred at room temperature. The polymerization time generally was less than 10 min. He found the best approach for incorporating different chemistries was to use a generic monolith that could be functionalized by UV-initiated grafting of functional monomers. In this manner, several chemistries could be incorporated into the same device, which was more difficult when direct polymerization techniques were used.

Svec also had interest in using capillary electrochromatography (CEC) to separate basic peptides and proteins. Ionized functionalities are required in a monolith to achieve electroosmotic flow. Unfortunately, proteins interact with ionized surface

chemistries, so he used a multilayer grafting approach in which layers of grafted phases were built up so that the changes in ionic character could be controlled in the various layers and the electroosmotic flow–protein interaction environment was optimized.

He used another interesting grafting approach for proteomics studies for LC–MS work. In these studies, the monolith was formed inside of an electrospray ionization emitter. In an initial segment, a monolithic enzymatic reactor was constructed by immobilizing trypsin; in a second segment, a preconcentration monolith was formed; and in the third segment, a separating monolith was formed. Svec injected a myoglobin mixture into the segmented monolith. In the enzyme reactor segment, the myoglobin was digested to form a series of peptides that were concentrated in the preconcentration zone. Finally, the peptides were released by a slug of 100% acetonitrile, separated in the separation zone, and subjected to MS–MS analysis. Various configurations, including other series of derivatized monolith segments, could be constructed.

A paper by Jean-Pierre Chervet, Remco Swart, and Irina Dragan of the LC Packings division of Dionex Corp. (Amsterdam, The Netherlands) described the use of poly(styrene–divinylbenzene) (PS–DVB) monoliths in capillary and nano columns combined with MS analysis. The initial experiments were performed using both UV and electrospray ionization MS–MS detection with 50 mm \times 0.2 mm columns using formic acid gradients. The presenters discussed the optimization of instrumental considerations such as UV cell volume, connecting-tube volume, flow rate, and other important parameters. These monolithic columns run at high linear velocities and provide very rapid separations, so 100 peptides can be separated in 15 min. With peak widths at half-height being only 2–3 s wide, the cycle time of the mass spectrometers is barely sufficient to acquire sufficient data and identify the peptides. Using nano-LC coupled with matrix-assisted laser-desorption ionization (MALDI), the authors were able to deposit small increments of sample eluted from the nano column onto a target plate and acquire quantitative mass spectral data in a controlled manner.

A similar paper in the hyphenation session by C. Huber and co-workers of Saarland University also described the separation of proteins and peptides on a

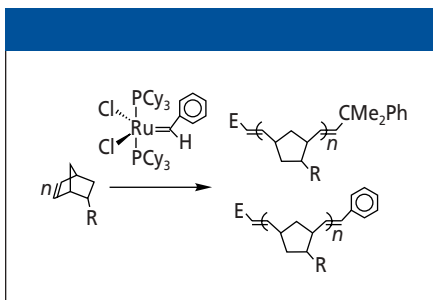


Figure 2: Typical reaction schemes for the preparation of metathesis-based synthetic monolith supports.

PS–DVB capillary column, but the presenters also extended the work to include nucleic acids in the ion-pairing mode.

Boguslaw Buszewski and Michael Szumski of Nicolas Copernicus University (Torun, Poland) presented their results of the synthesis of methacrylate monolithic

The mainstay of HPLC 2003 was the poster sessions in which detailed applications and methodology studies were reported and face-to-face discussions with the authors were conducted.

columns with various nonpolar functional groups for use in micro-LC and CEC. By cutting capillary columns into sections, they were able to show that methacrylate monoliths could be constructed very homogeneously. In addition, the strong binding of the monolith to the capillary wall resulted in mechanically stable column beds.

The generation of chip-based monoliths was an area of active interest at the symposium. Because small channels are difficult to pack efficiently with conventional particles and the use of frits is problematic, the in situ generation of monoliths appears to be an ideal application. Several papers reported the use of this approach.

Many workers believe that monoliths are the answer to their high-throughput needs. On the contrary, Diane Diehl and co-workers of Waters Corp. (Milford, Massachusetts) offered an interesting comparison of microparticulate columns and monoliths in a poster. They showed that small porous particles (3- μm d_p or less) packed into short columns (20 mm) of conventional inner diameters (2.1 and 4.6 mm) run at nominal flow rates (0.63 and 3.0 mL/min, respectively) under gradient conditions provided nearly equivalent separation times to those of a 50 mm \times 4.6 mm commercial silica monolith and resulted in a 30% solvent savings.

Maureen Joseph and co-workers of Agilent Technologies (Wilmington, Delaware)

also demonstrated fast separations in short columns (as short as 15 mm) packed with 1.8- μm porous bonded silica particles. The van Deemter curves for these columns were very flat, which enabled reasonably high flow rates (as high as 4 mL/min, depending upon solvent viscosity) and resulted in short separation times.

Other stationary-phase and column developments: Many new HPLC stationary phases were reported at HPLC 2003, most of them by representatives of commercial column companies. Because column technology was the dominant topic at the symposia, investigations of new phases were spread out in poster sessions for stationary phases, retention mechanisms, sample preparation, chiral separations, and so on. New base material synthesis procedures, surface modifications, bonding chemistries, coating, and cladding technologies all were reported.

Of special interest was a thermal treatment process developed by Carol and Kenneth Collins' group at the State University of Campinas (Campinas, Brazil). They prepared various reversed-phase materials by using conventional heating or microwave radiation to thermally oxidize polybutadiene or immobilizing poly-(methyloctadecylsilane) on silica or coated silica supports to yield stable, efficient bonded phases that provided good peak symmetry. Fluorinated, dendritic polymer, aromatic amide, polybutadiene-coated zirconia, titania- and zirconia-coated silica, phenolic resin-coated nonporous silica, and urea-embedded C18 phases were among the more interesting phases reported. Additional studies of coating zirconia with carbon phases generated a packing with increased stability and ruggedness with comparable selectivity to the more conventional reversed-phase packings. However, small amounts (0.1–5%) of aliphatic alcohols, nitriles, and amines with various chain lengths had to be used for strongly retained analytes to prevent peak tailing and low efficiency.

New second-generation bridged hybrid chemistries are being investigated for selectivity and stability reasons, the latter in high pH applications. A 3-hydroxypropyl hybrid allowed further chemical modifications to yield nonsilane bonded phases. In chiral applications, mechanistic studies of macrocyclic glycopeptides stationary phases showed improved selectivity using temperature as a variable. Liquid-crystal bonded phases showed interesting phase transitions that could be used to show shape selectiv-

ity for a series of polynuclear hydrocarbons in the normal-phase mode. Another similar paper based upon cholesteric acid liquid-crystal phase showed enhanced shape selectivity and less hydrophobicity compared with monomeric and polymeric C18 reversed phases.

Hydrophilic interaction chromatography generated some interest because it is an alternative technique for the separation of very polar analytes that sometimes prove difficult to separate by reversed-phase chromatography. In this mode, a high organic low aqueous mobile phase (greater than 80% organic content) is used with a polar stationary phase. Very polar analytes can be retained in this environment. An advantage of hydrophilic interaction chromatography is that these highly organic mobile phases are compatible with MS and provide increased sensitivity compared with aqueous mobile phases. In addition, plasma samples cleaned up by protein precipitation or mixed-mode solid-phase extraction can be injected directly without additional solvent evaporation and reconstitution.

New column formats: The use of micro-LC and capillary columns (for example, 100–1000 μm i.d.) and short fast columns (15–30 mm long) has gained further momentum, driven by the availability of instrumentation, the need for high-throughput separations, and smaller sample sizes. Now commercial nano-LC columns with inner diameters smaller than 100 μm have been added to the mix, because their increased sensitivity is required to investigate tiny amounts of peptides in protein digests. New columns for the high-speed separation of proteins and other biomolecules will greatly aid the proteomics and genomics fields.

Column testing and classification: The testing of columns, defining the test criteria, test substances, and classification of HPLC columns using tools such as chemometrics (principal component analysis) was another area of interest. Test mixtures suggested in the literature — the Engelhardt mix, the Tanaka test, the Neue test, the National Institute of Standards and Technology (NIST), McCalley test, and others — were discussed ad infinitum. On the opening day, Heinz Engelhardt of Saarland University reviewed these myriad tests in a lecture titled “Sense and Common Sense of Column Tests.”

It is commonly understood that most of the approaches agree for hydrophobic test compounds because retention is proportional to the amount of bonded carbon,

and methylene selectivity is similar; however, the agreements end there. Selected compounds for shape factors, differing basic and acidic test compounds, and compounds that are metal-complexing agents usually are different for different test mixtures and often provide different results in terms of column *goodness*. The basic reason is that the silanol activity at different pH values, even on endcapped columns, plays a major role in the peak shape, efficiency, and retention of polar analytes, especially for strongly basic compounds. Some of the test compounds are more sensitive to temperature variations than others. Ion exclusion comes into play for some test compounds. For example, benzylamine sometimes yields two peaks at low pH. It is an extremely complex issue that can involve parameters such as injection volume, buffer versus no buffer, buffer concentration, sample size, ion-exchange capacity.

Melvin Euerby and Patrick Peterson of AstraZeneca Pharmaceuticals (Loughborough, United Kingdom) questioned the United States Pharmacopeia (USP) classification scheme and suggested that reversed-phase columns should be characterized by a simple procedure using principal component analysis. They compiled a database of 60 reversed-phase column characteristics based upon the Tanaka classification procedure (5). They found that many of the L1 columns (C18) overlapped with L7 (C8) and L28 (silica bonded with amine functionality) columns. By classifying the columns based upon six variables and 74 objects, they were able to create a scheme for classifying columns that made more sense. John Dolan of BASi Northwest Laboratory (McMinnville, Oregon) has extended their column classification work and refined their older equations and test procedures (6). He also has studied more compounds. They have now classified more than 200 columns, and their database enables easy selection of an appropriate backup column. Several other presenters described their testing schemes.

Roger Smith of Loughborough University's Department of Chemistry (Loughborough, United Kingdom) has been a proponent of certified reference material columns. These columns are the result of collaboration by a number of European Union (EU) laboratories. The basic idea is to have a standard packing material in standard dimensions with a standard test mixture, standard mobile phase, and other

standard operating conditions that can be used by chromatographers to calibrate their instruments. Their testing procedure is based upon the Tanaka approach. A round-robin multilaboratory test conducted in 1999 yielded very poor results in terms of standard tests and the ability to transfer a method from one laboratory to another. Much time has been spent determining the various laboratory differences that contributed to the poor results. Some of the important parameters were

- the type of heating device — water bath, heater block, or air oven;
- the inlet temperature of mobile phase;
- the grade of water used;
- the method of peak integration — width at half-height, integrator, computer, or hand integration; and
- the data rate.

Despite the setbacks, the certified reference material column project is proceeding with a better understanding of the important evaluation parameters, and the columns, two suggested test mixtures, and the protocol soon will be available.

With so much disagreement among chromatographers about the ideal test mixture, the debate could continue. The use of chemometrics for classification of stationary phases seems to differentiate general phase types, but many of the results presented were inconclusive. The use of chemometrics for predicting the proper choice of column for a particular application seems to be far away.

Capillary Electrophoresis

I was surprised by the large representation of CE and related techniques (for example, isotachopheresis, isoelectric focusing, and micellar kinetic chromatography [MEKC]) reported at HPLC 2003. These techniques have become primary tools of separation scientists. Most of the CE applications were in aqueous-phase systems, some of which used MEKC for added hydrophobic selectivity.

Pierre Gariel and co-workers from the Centre National de la Recherche Scientifique (CNRS, Paris, France) provided insight into the phenomena controlling nonaqueous-phase systems. Nonaqueous CE has many advantages, especially for separating analytes that have little or no solubility in aqueous environments or for compounds that are difficult to separate in aqueous buffers. The phenomena involved in optimizing selectivity are ionization rates, ion-pair formation, and solvation. The authors showed how each of these

parameters could be estimated by appropriate electrophoretic or conductometric measurements or by application of theoretical models. Also in nonaqueous CE, Jacques Crommen from the University of Liege (Belgium) expanded on his work on the separation of basic pharmaceuticals using anionic cyclodextrins in combination with ion-pairing reagents.

An interesting poster by Merike Vaher and co-workers from Tallin Technical University (Tallin, Estonia) used room-temperature molten salts as background electrolytes in nonaqueous CE. The applicability of 1-butyl-3-methylimidazolium ionic liquids for the separation of uncharged acidic analytes made electrophoretic tuning possible and allowed the study of compound heteroconjugation in various solvents.

Adrian Clarke and Nicholas Pedge of AstraZeneca Pharmaceuticals studied non-UV-absorbing cations such as alkaline earth metals and organic amines commonly found in pharmaceutical preparations. They used indirect photometric detection with imidazole or *p*-aminopyridine as the absorbing displacement ions in acidic polar organic solvents. They found that the type and concentration of organic solvent could be used to manipulate migration order, and the type of absorbing displacement ion could control peak shape and sensitivity.

In an interesting twist to the CE experiment, Dan Armstrong of Iowa State University (Ames) applied the technique to the separation of intact microorganisms in the same manner as molecules are separated. In a direct current electric field, these microorganisms can be focused individually into single peaks and then separated rapidly from other microbes and chemicals. The presence of neutral polymer additives such as polyethylene glycol (0.00125% level) added to the biologically compatible buffers accentuated the differential focusing effect of these cells. These studies could prove valuable in various practical applications such as disease diagnosis and quality control of consumer products.

Chiral separations are still the most widespread analyses in the CE applications area, although applications to proteins, peptides, and amino acids; natural products; and drugs and endogenous compounds in biological fluids also appeared in the poster sessions. A very popular topic for chiral separations was the use of highly sulfonated cyclodextrins and calixarenes that seemed to provide additional selectiv-

ity for polar enantiomers compared with the traditional cyclodextrin additives.

Fewer papers about CEC were presented this year compared with previous symposia of this series, perhaps indicating waning interest in this hybrid technique. An oral presentation by Volker Schurig and co-workers from the University of Tübingen (Germany) focused on the use of CEC for cyclodextrin-mediated chiral separations. They compared enantiomer separations obtained by open-tubular capillary chromatography, packed CEC, and monolithic CEC with MS detection. Another CEC paper by Paul Haddad and co-workers from the University of Tasmania (Hobart, Tasmania, Australia) and Dionex used packed and pseudo-ion-exchange phases to control the selectivity and enhancement of small anions. To increase overall sensitivity, they used an in-line preconcentration column wall-coated with anion-exchange latex particles that enabled sub-parts-per-billion anion determinations.

Applications and Proteomics Highlights

The analysis of pharmaceuticals and their impurities, pharmaceutical formulations, dosage forms, quality-control applications, and applications to drug discovery and combinatorial chemistry dominated liquid-phase separations, as verified by Table II and similar to previous years' observations (1–3). I split the analysis of drugs and endogenous compounds in biological samples (for example, blood, urine, saliva, tissue, and cerebrospinal fluid) from the pharmaceutical applications in Table II, but the total number of pharmaceutical analysis presentations still represented nearly one-third (31.8%) of all applications, just as they did last year (2). Protein and proteomics applications had three full oral sessions and many poster papers.

Again this year, the topic of proteomics received a high level of attention. Although 2-D gel electrophoresis still is in widespread use, the use of LC coupled with MS is growing rapidly for the investigation of complex mixtures of proteins and peptides; therefore, a large number of contributions at HPLC 2003 were devoted to these studies. I discussed a few of the column approaches in the monolith section above.

Many papers and posters devoted to the investigations of these complex peptide digests represented applications that use MS and MS–MS as detection techniques. In these studies, multidimensional nano-LC was combined with MS–MS to pro-

vide sequence information that is measured against massive databases comparing theoretical MS values with actual MS values. Nano columns, typically in the 75–100 μm i.d. range but some with inner diameters as small as 15 μm , were reported because the low sample mass of the numerous individual peptides that result from the digests requires a higher sensitivity. CE combined with MS also was used as a tool to resolve some of the complex peptide mixtures that result from protein digestion.

Several authors presented oral papers that dealt with some of the more interesting challenges of proteomics such as protein separation and identification, the study of proteins as disease biomarkers, on-line digestions of proteins and preconcentration, posttranslational modifications, micropurifications using affinity separations and precipitation techniques, and studies of enzymatic reactions.

Joseph Pesek of San Jose State University (San Jose, California) investigated both capillary and planar electrophoretic approaches to proteomic analysis in one and two dimensions, the latter using microplates with 100 channels. By treating the internal surfaces of capillaries and 2-D channels on microplates with different types of chemical etchings and bonded-phase modifications, he was able to show that CE selectivity and resolution can be strongly affected by these treatments. In his 2-D work, he was able to successfully use size exclusion because his deactivation procedure prevented protein adsorption while isoelectric focusing was performed in the second dimension using a stable pH gradient bonded to the walls of each of the 100 channels.

The search for biomarkers to reveal disease states is one of the goals of proteomics studies, and several papers addressed this subject. R. Bischoff and co-workers from the University of Groningen and the University Hospital (Groningen, The Netherlands) were able to find squamous cell carcinoma antigen, a known marker for cervical cancer in women, in human serum tryptic digests using LC–MS. After depletion of higher-abundance proteins, human serum albumin, and IgG before digestion, they were able to load an LC column with more sample and found five expected tryptic peptide fragments of squamous cell carcinoma antigen based upon their molecular masses. These fragments were confirmed by MS–MS. David Lubman and co-workers from the University of Michigan (Ann Arbor) used 2-D separations for

profiling protein expression of cancer cell lysates with chromatofocusing as the first dimension and reversed-phase chromatography as the second dimension. The first dimension was based on pI fractionation, and the second dimension relied upon hydrophobic interactions. The effluent was directed into an electrospray time-of-flight (TOF) mass spectrometer for measurement of intact protein.

Barry Karger of Northeastern University (Boston, Massachusetts) provided an update on his progress using high-resolution reversed-phase HPLC coupled off-line with MALDI-TOF MS and MALDI-TOF MS-MS. A main advantage of these combinations is that the separation and analysis steps are decoupled. Using PS-DVB monoliths fabricated in 0.020-mm i.d. capillaries, he was able to gather a great deal of information by precise parallel streaking onto a MALDI target with this high-resolution system. Because ion suppression is no problem using the off-line approach, the system showed good dynamic range, provided high throughput, and exhibited improved signal-to-noise ratios with cross-correlation analysis algorithms and mass accuracy.

In the closing plenary session, Fred Regnier and P. Liu of Purdue University (West Lafayette, Indiana) described a new procedure for identifying single amino acid polymorphism in proteins. These polymorphisms result from single nucleotide polymorphisms that cause mutations in genes that will eventually cause subtle changes in protein conformation. For example, sickle cell anemia in humans is believed to be caused by a single amino acid polymorphism. Their method is based upon a heavy isotope coding strategy (with heavy water containing oxygen-18) that exploits differential derivatization of amine and carboxyl groups generated during proteolysis as a means of coding to reveal single amino acid polymorphisms. Their procedure involves tagging the amino acid carboxyl termini, which allows large numbers of proteins to be examined simultaneously. A novel approach to increase resolution was the insertion of an ion mobility spectrometer between the LC sprayer and the TOF mass spectrometer.

Detection Highlights

Table III shows that MS was by far the predominant detection method. Nearly half of all papers presented at HPLC 2003 used this detection technique. Of course, the UV detector probably is in more wide-

spread use in most laboratories, but the usefulness of MS undoubtedly will make mass spectrometers the detectors of choice eventually.

HPLC 2004

The next major symposium in this series, the 28th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2004), moves back to the United States and will be held in the City of Brotherly Love, Philadelphia, 12–18 June 2004. The chairman of this upcoming event will be Mark Shure of Rohm and Haas (Spring House, Pennsylvania). For more information, consult the official web site at <http://www.hplcsymposium.org>.

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