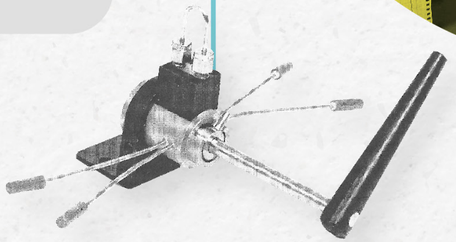
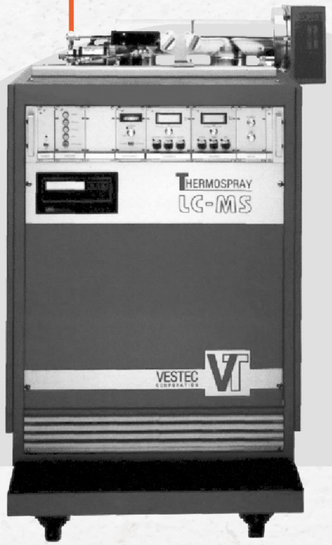
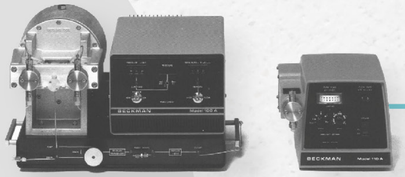
the Analytical Scientist



SPECIAL SERIES: *HPLC*





IN MY VIEW

HPLC Doctors and Nurses

Why spend time and money bringing in specialists to fix HPLC maladies, if we can learn the skills to remedy most ills ourselves?

"All HPLC practitioners will be aware of the need to overcome issues in both method development and routine analysis. Even the most sophisticated equipment can present unexpected chromatographic behavior. And though such issues certainly plague modern instruments less frequently, sooner or later, a problem will inevitably occur.

There are some preventative measures that can be taken to increase the chance of seamless operation and performance of HPLC instrumentation – the most common of these being:

- filtering of the mobile phase solvents,
- filtering of the samples prior to injection,
- using the right buffers and following instructions to remove or avoid precipitation of salts in the system due to organic solvent,
- preparing fresh buffer or aqueous solutions instead of storing in the fridge,
- proper washing of the column after use.

But issues occur – and recur – regardless of these measures, and chromatographic abnormalities can be difficult to spot. It is easier during analysis of standards than of unknown samples for sure, though, and injection of control samples can help us to spot aberrant outcomes.

Record keeping is a requirement in any analytical procedure, and it should be considered absolutely essential in HPLC. (It was actually one of the first lessons I learned in practicing HPLC, and it is one of the most important lessons I teach my students as they begin using the technique…). We should know how the system works when functioning properly; only then can we recognize any irregular signs and symptoms – allowing us to subsequently resolve the problem. Recording pressure and keeping typical chromatograms for comparison enable the recognition of a non-proper function.

Knowing how to confront the problems and solve them could be considered a prerequisite for efficient HPLC operators. HPLC is incredibly useful, but also highly complex. Analytical chemists must know the fundamental theory behind the simple act of sample injection. We are "analysts," not "analyzers," and – as practicing scientists – we must all have a firm grasp of this knowledge.

It is not easy to have the right answers to all problems in chromatographic analysis. HPLC troubleshooting manuals outline a vast number of potential irregular functions, and also many corrective actions – but not all lead to the right solution. And that's not to mention the fact that, although we all know the rule that we have to change one thing at a time, when we are in hurry, we sometimes ignore it and change many more...

But what happens when more "invasive therapy" is required? Are all HPLC practitioners able to proceed and fix the most common problems? Of course, we can call for assistance and technical support, but this is often time-consuming and associated with financial cost – nevertheless, we often find ourselves seeking an expert technician. But shouldn't chromatographers themselves be the expert technicians? Being "doctors" to our own HPLC systems – healing its symptoms when sick – could save us both time and money, allowing us to inject both of these resources back into our research, perhaps learning valuable lessons in the process.

I suggest that analytical scientists – especially novices – using HPLC instrumentation should attend hands-on workshops to learn how to confront the problems arising in routine operation. And, at the very least, surely it would be better to invest time in actually reading the HPLC system's instruction manual rather than blindly paying an external technician to fix the problem.

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REFERENCES AVAILIABLE ONLINE

IN MY VIEW

Look What You Missed… With Gert Desmet

The pandemic had Gert Desmet missing long-haul economy flights… But he did find the time to hike, run and, crucially, work on his favorite ideas in the theory of chromatography.

On March 6, 2020, I was teaching a class on computer-aided modeling when I received the news that campus would close down for three weeks to "grab the virus by the throat and make a speedy end to its spreading." Two years later, and I still haven't attended a single scientific meeting in person or visited a colleague to discuss collaborative work or learn a new technique.

What initially only seemed a brief interlude eventually turned our lives upside-down. In academia, we all sorely missed eye-contact with the students and their feedback when teaching. And I also dearly missed the little conference corridor gatherings where the real latest research results are discussed (most presentations in the lecture halls and on Zoom are about yesterday's research) and where new collaborations are forged. I even started to miss long-haul economy flights, where fate always places you between a crying baby and a heavy snorer… The pandemic also caused problematic delays for PhD students (especially during the first lockdown) and a great deal of stress for those of us trying to secure research funding.

But every cloud has its silver lining. The pandemic drastically slowed down life, allowing me to discover the most beautiful hiking trails (surprisingly close to home) and to drastically increase my running mileage. Another great joy came with the new electronic meeting format; suddenly and magically, all meetings started and ended perfectly on time – unprecedented in academic media! The pandemic also gave me the rest and quiet to work on some of my favorite ideas in the theory of chromatography, such as the velocity- and retention-factor dependence of the eddy-dispersion term or the establishment of an analytical expression for the retention factor dependency of the mobile phase mass transfer. This theoretical work helped me keep up publishing pace in a period where the data stream from the lab was drying up.

Another breakthrough that probably would not have happened without the pandemic, and the insight of a brilliant young PhD student called Bram Huygens, was the extension of the Taylor-Aris theory to complex generic chromatographic media, which has opened the door to establishing analytical forms for the van Deemter-equation in a whole series of geometries – this was unthinkable previously. I am convinced this will bring our understanding of packing quality and its effect on column performance to the next level, which will help us design new types of chromatographic supports.

Most of all, the peace and quiet also allowed me to develop some ideas for new experimental approaches and for new column designs. Until now, this was still in its infancy, but will hopefully soon be tested in the lab. A lot of attention in our group also went to finding new ways to pack particles in much more ordered and open configurations than the randomly packed columns we have to live with today.

I spoke about about some of these ideas in San Diego for HPLC 2022 – a celebratory 50th edition by the way and the first HPLC for three years. We must be grateful to Frank Svec for his courage to start up the engine again in a period when the future course of the pandemic was still very uncertain. Due to his efforts, we can now again offer the youngest generation the opportunity to present their work, discuss it with other scientists, and establish their networks. These are crucial aspects in their development as scientists.

In conclusion, I think the pandemic has forced us to rethink our way of working and living. And I do believe that if we could retain some of the positive aspects, such as a more efficient way of organizing our working days and less unnecessary travel, we will be living in a better world than before March, 2020.

Gert Desmet is Professor of Chemical Engineering at Vrije Universiteit Brussel, Belgium.

REFERENCES AVAILIABLE ONLINE

IN MY VIEW

Look What You Missed… With Adam Woolley

Adam Woolley considers what we've missed due to COVID-19 and where we go from here

As I write this in mid-March, it's almost exactly two years since my university announced the cancellation of in-person classes at the start of the pandemic, and almost exactly one year since I received my first vaccine dose. I am astounded at the significant biomedical advances that have taken us from worrisome uncertainty two years ago, to hope one year ago, and now on to optimism for a promising future. I am fortunate to have not lost close family or friends to the coronavirus, but I mourn with those whose loved ones have passed on.

The pandemic has negatively affected scientific productivity, with students isolating due to a positive test or quarantining as a result of a close contact with a positive case. Laboratory research definitely slowed for a time, and some students initially refocused their efforts to write review papers. We experienced significant delays with laboratory supplies (who didn't run out of gloves at some point?) and with obtaining clinical samples from medical collaborators. For the most part, those logistical challenges are now subsiding.

On the bright side, four of my PhD students graduated in the middle of the pandemic, and they're all doing great things. One started a company, another is a postdoc at a university, one is working for the government, and the last is teaching at a university. Despite the challenges, or maybe even because of them, these recent graduates have succeeded in important ways. It feels like we finally might be on the tail end of this pandemic, but, if I've learned anything, it's that predicting this pandemic's trajectory is impossible.

A key take-home message from the pandemic has been the power of science and medicine to solve problems. We've certainly seen that in chemical analysis, particularly with COVID-19 testing, which received strong support from the separation science community. In my own lab, despite various slowdowns, we have been able to move research forward in several important ways. For example, we focused on using 3D printed microfluidics in assessing risk for preterm birth with maternal blood serum samples; we made significant progress in multiplexed immunoaffinity extraction, solid-phase extraction and fluorescence labeling, and microchip electrophoresis of these biomarkers. We're also making strides to combine those three processes together in a miniaturized platform.

I am looking forward to attending in-person scientific meetings once again this year – I'm grateful that virtual gatherings have been a possibility over the past two years, but the quality and nature of interactions in those meetings have not been fully satisfactory. That said, virtual meetings, lockdowns, shortages, and so on, have helped my students develop resilience – and I believe that the pandemic will result in a new generation of scientists who are nimbler and more adaptable.

Looking further down the road, I still see a significant need for advancing miniaturized systems for chemical analysis, and we hope to be part of that. One new branch of work that my lab started during the pandemic is in droplet microfluidics, and we're excited about the potential to use the approach to study antibiotic susceptibility.

Personally, my career took an unexpected turn into administration during the pandemic: I applied, interviewed, and was appointed Dean of Graduate Studies at my university. One of my conditions for accepting the position was for me to be able to continue mentoring students in my lab, so although I'm pausing undergraduate teaching, I remain committed to continuing research. The future looks bright indeed for bioanalytical separation science and its practitioners.

Adam Woolley is Dean of Graduate Studies and a Professor in the Department of Chemistry and Biochemistry at Brigham Young University, USA

REFERENCES AVAILIABLE ONLINE

FEATURE

The Top 10 Game Changers in HPLC History

Progress in any field is expected if not inevitable; as technology advances and as new knowledge is gained, iterative improvements feed a baseline of linear evolution. But there are also often leaps forward – true innovations. Here, we look back on the first 50 years of HPLC instrumentation and select the Top 10 (okay, you got me, a tie made it a Top 11) breakthroughs that paved the way for the technique we know today.

By Ron Majors and John Baltrus

When HPLC was "discovered" over 50 years ago, it revolutionized the field of analytical chemistry. Major developments in the technique – which seemed to occur almost yearly in the 1970s and the early 1980s – ranged from revolutionary to evolutionary. During that period, the technique quickly shifted from a large-particle, gravity-fed, large-bore glass column technique to an automated, small-particle, high-pressure, narrow-bore stainless steel column technique. In the story of HPLC development, the column and the instrument are intertwined – and both have played pivotal roles in increasing separation speed, boosting efficiency, and improving quantitation.

In this article, we attempt to identify – with the help of a panel of LC, data systems and mass spectrometry experts (see "We Couldn't Have Done It Without You") – the developments that truly "made a difference." We believe the resulting Top 10 allowed HPLC to surpass most other techniques in terms of application range and its ability to answer analytical questions. And so, without further ado...

The Top 10

Some of the chosen products in the Top 10, though not necessarily the best sellers or performers, were the first to be introduced into the market and being first gave other instrument developers a target to beat, driving further advancements in the technology ending with today's UHPLC.

1. LCS-1000
Year: 1967
Company: Picker Nuclear

The first commercial integrated instrument to qualify as a modern LC instrument, including a UV 254nm detector, acquired by Varian Associates in 1968.

2. 6-Port Injection Valve
Year: 1968
Company: Valco

Did away with septum injector and stop-flow techniques, allowed high-pressure injections, improved retention time, reproducibility, automation and quantitation.

3. Modular Components
Year: 1968
Company: LDC/Milton Roy

Components like the standalone 254 UV detector, LDC RI detector and Milton Roy minipump were used quickly by modular chromatographers. The first instruments were integrated, with all internal parts in a single box. The modular market sprung up to allow researchers to get the best component (for example, pump, injector, column holder/oven, detector). LDC was the first component company to step up and supply affordable and functional modules.

=4. Autolab System IV Computing Integrator
Year: 1969
Company: Autolab

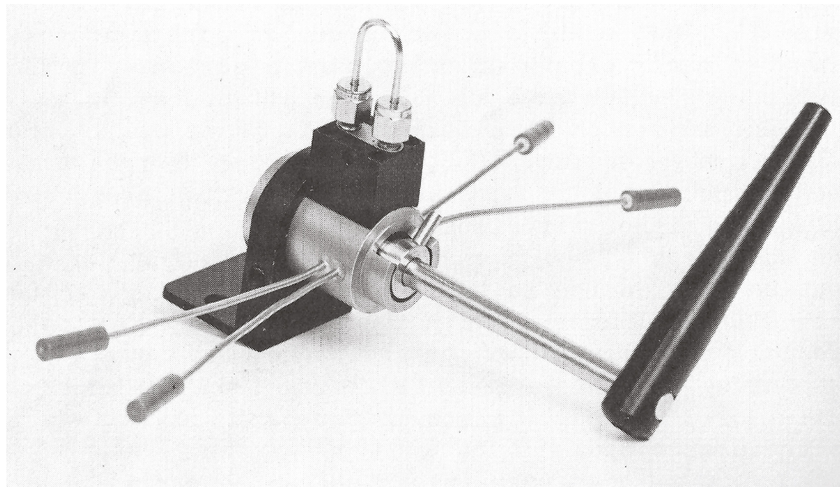
The first automatic integrator for chromatography replaced cut and weigh, planimeters and recorders with built-in mechanical integration. This data system was first used in GC and adapted to LC. Features included tangent peak detection, baseline correction and normalized peak areas, and allowed the use of response factors and internal standards. Autolab acquired by SpectraPhysics in 1969.

=4. M-6000 pump
Year: 1972
Company: Waters Associates

First pump designed specifically for HPLC. Key features: dual reciprocating, less need for pulse damper, 6,000 psi output, and a low-volume chamber.

5. Model 708AL Autosampler
Year: 1974
Company: Micromeritics

The first automated sampling device for HPLC; improved throughput and quantitation ability. Future products improved on this unit.



The Top 10 (cont)

6. HP 1084

Year: 1976
Company: Hewlett-Packard

Integrated HPLC system with automated sample injection, flow control, UV detector control, recording and reporting. First LC with digital processor control, with a builtin keyboard with push button control. It became the gold standard for quality and performance for many years.

7. 8450 Diode Array Detector

Year: 1977
Company: Hewlett-Packard

The diode array detector allowed on-the-fly UV-VIS spectra during the chromatographic process, with excellent signal-to-noise performance. Productivity increased compared with stop-flow spectral scanning. Other companies soon followed with their own diode arrays.

8. Moving Belt LC-MS Interface

Year: 1976
Company: Finnigan MAT

The moving belt was introduced by McFadden, Schwartz & Bradford of Finnigan MAT (6). Despite some drawbacks, the moving belt provided true chromatographic interfacing, the first successful LC-MS interface on the market, and was the best of many approaches – until electrospray came along.

9. Charged Aerosol Detector (CAD)

Year: 2005
Company: ESA Biosciences

Sometimes described as “the poor man’s mass spectrometer,” the CAD is a universal detector. It has much greater sensitivity than the RI detector (also a universal detector). It is still in widespread use today.

10. Acquity UPLC

Year: 2004
Company: Waters Associates

For the introduction of sub-two micron particles, new higher pressure instruments were required. The Waters Acquity UPLC system was the first system designed to meet the needs of the new small particle columns with a pressure output of 12,000 psi. Lower extra column

The Top 10 narrative

To simply rank the Top 10 does not do justice to their impact. Over the following pages, we tell the story behind the Top 10 (and the honorable mentions), giving much needed context – and a history lesson to all but the most seasoned LC users!

The mighty column

Column technology was one of the main drivers in instrumental development. Figure 1 shows a historical chart of commercial HPLC particle development. The work of Csaba Horváth and coworkers from Yale University in New Haven, CT (1) in 1967 on pellicular packings (now referred to as superficially porous packings, SPP) represented a breakthrough in HPLC column and instrument technology. Fifty-micrometer spherical glass beads were coated with a thin polymeric layer (1-3µm) of polystyrene resin, derivatized to form anion exchange functionality and used for the separation of nucleotides. The particles were packed into stainless-steel columns (1 mm ID, 3 m length), which gave rise to high back pressure and the need to use pumps to push solvents through the column. Notably, we see the beginning of a trend where smaller particles increase column efficiency (plates) to provide better separation performance, while also increasing column back pressure with the inverse square of the average particle diameter; thus, pump output pressure would become an important factor over the following years. But it was not the only driver; as we will see, parameters like flow rate, extra column effects (system dead volume), decreased peak widths, speed of elution, sample throughput and detector capability all became drivers to improve all parts of the LC system.

The first HPLC generation

In an earlier publication (2), a case was made that the work of Horvath and colleaguesbreakthrough work was the beginning of HPLC (at the time referring to high pressure LC). A schematic of their gradient elution instrument is depicted in Figure 2. Their home-made system used two Milton Roy Minipumps (Riviera Beach, Florida). The gradient was formed by starting with a stirred reservoir containing the weak solvent, and then having a pump deliver a controlled but increasing amount of the strong solvent to the weak solvent reservoir. A second high-pressure pump pushed the solvent mixture of increasing strength from the reservoir to the column. For detection, a modified spectrophotometer with an 8-µL flow cell was used, indicative of future developments. In the late 1960s, they teamed up with a local instrument company, Picker Nuclear (White Plains, NY), to construct the LCS-1000 – the first true commercial HPLC instrument. The design was spearheaded by engineer Emmett Watson, who had left Waters Associates to become a consultant. The final instrument had a high-pressure pump (up to 4000 psi), a sampling loop valve, an oven for temperature control, the column with the pellicular packing mentioned above, and a fixed wavelength detector (254-nm) based on a low-pressure mercury vapor lamp. The instrument did not fit into Picker Nuclear's range of non-chromatographic products and, shortly after, it was acquired by Varian Associates (Walnut Creek, CA).

Almost simultaneously, Waters Associates – a leader in size-exclusion chromatography at the time – modified its integrated GPC-100 instrument to perform regular HPLC. Changes such as reduced dead-volume, a higher-pressure Milton Roy pump, as well as a flow-through UV detector and (optional) refractive index (RI) detector were incorporated into the existing instrument. The ALC-100 (see Figure 3) was introduced at the 1968 Pittsburgh Conference (ALC was an

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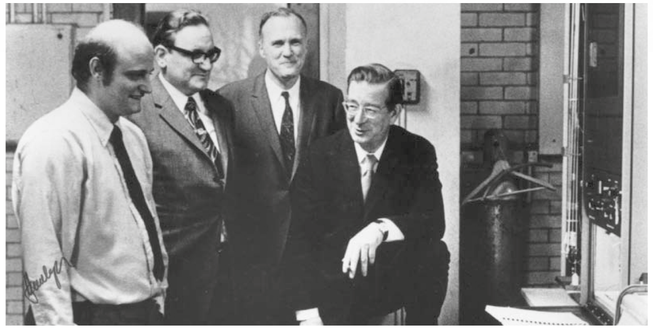
Honorable Mentions

Choosing a Top 10 was not easy. To enrich the story (and to help us sleep better at night), we also decided to highlight those technologies that strongly influenced modern HPLC technology but didn't quite make the cut.



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History in the Making

Within the Science History Institute, near Independence Hall in Philadelphia, you will find a permanent exhibit titled Making Modernity, which places historical analytical instrumentation in the context of the great human adventure of discovery in the chemical and molecular sciences.

The Top 10 narrative (cont)

acronym for analytical liquid chromatograph). Waters immediately became "The Liquid Chromatography People" and remains a leader in HPLC today.

Dupont, the Delaware-based chemical giant, also joined the unfolding HPLC (now high performance LC) market. Introduced in 1969, the Model 820 integrated chromatograph (see 'Honorable Mention' 1b) had a constant pressure pump, a home-grown UV 254-nm detector (Model 410) and their own ZIPAX SPP. They introduced the first chemically-bonded phases that revolutionized the practice of gradient HPLC. Their ZORBAX column products remain and are now manufactured and sold by Agilent Technologies. (Dupont left the HPLC instrument market in 1986 followed by IBM Instruments, who briefly entered this market.)

Injecting some sense

Fortunately, when packing the new SPP packing materials (37-50-µm particle diameter range) introduced by Dupont and Waters (3) into standard columns (2.1 mm ID, 50- or 100-cm length) the back pressure was modest, so the first commercial liquid chromatographs employed an on-line GC septum injector. As the packing material particles became smaller, the pressure capability of septum injectors was exceeded, spurring the use of stop-flow techniques. Such manual injection was cumbersome, limiting sample throughput, so Stan Stearns, founder of Valco (Houston, TX), adapted his GC valve to allow injection pressures up to 4500 psi. The six-port injection valve (see rank No.4) was a real breakthrough in productivity and reproducibility and allowed flexibility in sample volumes by changing the sample loop size. It also allowed later automation with autosamplers.

Waters introduced their U6K Injector (1973) after using a six-port injection valve in their integrated LC series (see 'Honorable Mention' 2a). It offered convenient, variable volume and reliable injection and could be automated. The injector also possessed an innovative bypass channel that reduced the pressure shock when the valve was cycled, which protected the column against collapse after repeated injections. Columns were less robust in the early days but as column packing methods improved it became less of a problem.

Later, Rheodyne's (Berkeley, CA) Model 7125 Injection Valve (1976), modified the Valco approach such that the syringe loaded the sample into the center of the valve allowing variable injection volumes using a single loop (see 'Honorable Mention' 2b).

The rise of the module

In the early days, many chromatographers reasoned that each manufacturer had different strengths and therefore wanted to couple the best pump with the best injector, and the best detector, and so on. The aim was to build a superior system with quickly interchangeable (or upgradable) modules. To meet the demand, some manufacturers (especially those in the OEM business) decided to develop standalone modules that could be optimized for performance. The first of these companies was Laboratory Data Control (LDC, Riviera Beach, Florida) who also employed the services of Emmett Watson to

ONLINE

What About the Next 50 Years?

We asked our expert panel to gaze into their crystal balls and predict at least one game changer they'd expect to see in the next 50 years of HPLC

We Couldn't Have Done It Without You

The authors sincerely thank experts in the field of HPLC, mass spectrometry, and data systems for lending their time to help identify the real breakthrough instruments, supplying dates of product introductions, and helping to run down information that has long been "lost," especially in the commercial sector.

John Dolan (retired, LC Resources), Bob Stevenson (columnist emeritus, American Laboratory) and Dick Henry (founder, Keystone Scientific) spent countless hours digging through their files (and basements) to come up with some key contributions.

Tom Jupille (retired, LC Resources), Jane Gale (Historian at the American Society of Mass Spectrometry), Jack Henion (Emeritus Professor at Cornell University and Founder of Advion Biosciences), Dieter Hoehn (retired VP, Hewlett Packard), Geoff Cox (retired HPLC expert) and Glenn Ouchi (retired data handling expert) deserve thanks for helping to run down some critical information and provide sources of materials.

The Top 10 narrative (cont)

build reasonably priced standalone modules, including a 254-nm UV detector (see rank No.3). This detector became the workhorse OEM component for several chromatography companies. The much larger Milton Roy Company acquired LDC and became a supplier of modules to many HPLC companies and individual chromatographers. Component companies like Valco and Rheodyne specialized in injection and switching valves. Detector companies like Schoeffel, Cecil Instruments (honorable mention) and Pye Unicam developed specialized detectors. Many smaller companies developed other peripherals that could be quickly exchanged as the need arose.

Pump it up

Waters, hopping on the module bandwagon, developed a standalone pump called the M-6000 – the first expressly developed for HPLC (see rank No.4). Early pumps based on a one piston design delivered pulsating flow, resulting in the need for pulse-dampening systems that could reduce noise in the flow-sensitive detectors. However, the large volume of pulse dampers delayed gradient mobile phases from reaching the column, increasing analysis times. The M-6000 used two reciprocating pistons for smoother flow to the column. And its pressure rating of 6000 psi was sufficient for the 10-µm microparticulate packings in 25 cm columns (4.6 mm ID) introduced in the early 1970s. Altex (Berkeley, CA) developed an entirely new concept using a variable piston speed with fast refill (see 'Honorable Mention' 4b); the flow from the resulting Model 110 pump was smoother than most low-cost reciprocating pumps of the day.

To provide higher pressures with non-pulsating flow, Varian Associates (Palo Alto, CA) (see 'Honorable Mention' 4a), Isco (Lincoln, Nebraska) and Nester-Faust (Norwalk, CT, later Perkin Elmer) chose to develop syringe pumps. Here, large volume (250 mL) pistons were driven by a precise stepping motor. These pumps were basically pulse-free and could reach high pressures, up to 6000 psi and later 8500 psi. For binary gradients, two syringe pumps were required. But chromatographers ran into problems during gradient elution because of solvent bulk compressibility; the actual flows of each solvent to the mixer were not as programmed on the controller, generating compositional errors – especially when each pump had a different starting volume. Syringe pumps, though unique and novel, eventually vanished. Nevertheless, syringe pumps deserve an honorable mention since this novel technology attempted to think outside of the box in providing pulseless, high pressure flow.

Hello, Industry 3.0

In the late 1960s and early 1970s, strip chart recorders were the main data output. For quantitation, manual methods, such as cutting and weighing the chart paper or using a mechanical planimeter, were the norm. But this all changed when Autolab, later a division of SpectraPhysics (Sunnyvale, CA), introduced their System IV computing integrator, which provided digital readout. Output could be presented as simple area percent or based on calibration factors stored in the method. Because of its large dynamic range, chromatographers no longer had to make multiple injections while adjusting the signal attenuation, which increased laboratory productivity. The microprocessor-controlled Hewlett Packard HP 3380A integrator (1974) (see 'Honorable Mention' 5a) went further and served both as a recorder and alpha-numeric printer-plotter so all information was on a single piece of chart paper. After that, many manufacturers introduced their own data systems to supplement the HPLC hardware. Finally, Nelson Analytical (1979) developed data analysis software based on personal computers. They took advantage of new-large scale integrated (LSI) circuitry to construct analog-to-digital converters and provided instrument control and data acquisition with powerful calculation ability. The Nelson products became the standards in systems of many manufacturers. When the IBM PC was introduced, Nelson adapted their software to allow both instrument control and data handling – a concept that quickly replaced the standalone integrator in future instruments.

About the same time, in the mid-1970s, HPLC was increasingly being used in pharmaceutical and other industries, where users needed to analyze many samples per day. The first HPLC autosampler to reach the market was from Georgia-based Micromeritics, a company specialized in particle size measurement. The Model 708A LC Autosampler (1974, see rank No.5) used tubular vials in a rotating tray. A needle was lowered into the vial, puncturing its cap, while a collar simultaneously pushed the cap down into the vial displacing the sample into the sample loop. The system allowed 1-3 injections from the vial. By the end of the 1970s, most of the major suppliers had introduced their own autosampler.

The Top 10 narrative (cont)

Revenge of the integrated system

As HPLC was experiencing widespread acceptance, central control of instrument peripherals made better sense; the component LC concept was losing favor as modules couldn't all talk to each other. Users also discovered that gradient elution was a must for analyzing samples with a wide variety of components, which acted as another driving factor towards central control. Two-pump gradient systems for binary gradient production were the first type designed (the two pumps were plumbed together on the high-pressure side and a mixer was required).

Hewlett-Packard's Analytical Division (now part of Agilent Technologies) improved on the Hupe-Busch system that they acquired two years earlier. The HP 1084 (see rank No.6) was the first microprocessor-controlled LC with unique flow control plus an autosampler, UV detector, and an external fluorescence detector. The precise flow control of the 1084 was a great selling feature and gave highly reproducible retention times.

Since two-pump gradient systems were expensive to build (and buy), developers such as Varian and Spectra-Physics worked on the early products that used a single pump with solvent proportioning valves on the low-pressure side. Thus, with two- or three- proportioning valves, users could generate binary or ternary gradients, offering more flexibility during method development. Low pressure gradient formation eventually became the standard for many companies. Later, even more powerful quaternary pumps were developed. The Varian LC-5000 (1978, see 'Honorable Mention' 6a) was the first integrated HPLC system that incorporated low-pressure single pump gradient capability, microprocessor control of key features, a keyboard for setting up methods, and a CRT display all in a single unit. An active inlet check valve solved the problem of sticky check valves and loss of prime. The Spectra-Physics Model 8000 launched in 1979 employed a design that is almost identical to the solvent blending designs used in today's instruments.

In 1975, Dionex was carved out as a division of Durrum Instruments to address an emerging chromatography market: ion chromatography (IC). Dionex quickly became the leader in separation of inorganic and organic ionic compounds (see 'Honorable Mention' 6b). Their patents, licensed from Dow Chemical, covered the use of an ion suppressor to remove salts from the mobile phase, which allowed conductivity detection of the separated ions. They also developed specialized IC columns that were devoted to tough separations, such as trace bromates and oxyhalides in drinking water, carbohydrate separations using pulsed amperometric detection, and ion exclusion separations. Now owned by Thermo Scientific, Dionex still controls the IC market.

Sophistication in detection

In the first 10 years of HPLC, spectroscopic detectors dominated. Stop-flow scanning spectrophotometric detectors met with little acceptance in the marketplace. With the adoption of optical diode arrays, real-time spectroscopic measurements could be performed and complete UV-VIS spectra could be obtained on-the-fly, which became the industry standard. HP's 8450 Diode Array Detector was the first product to market (1977) using this technology, and it set the gold standard for diode array detectors of the future.

Following the remarkable success of GC-MS, various interface types were developed to combine two apparently incompatible techniques – one in a liquid environment and the other in a vacuum. Interfacing technologies included direct liquid interfacing, transport devices, particle beam, continuous flow FAB, ion spray, and thermospray.

The first in the marketplace was the moving belt interface (a transport interface). The technology was borrowed from early work by R.P.W. Scott (4) and Victor Pretorius (5), who used a moving wire to transport part of the LC column effluent to a flame ionization detector (marketed by Pye Unicam in the UK). Rather than a moving wire, the first successful LC-MS interface used a moving belt which allowed more sample to be transferred for better sensitivity. Solvent was evaporated by heat prior to entering the vacuum zone of the MS. Finnigan MAT introduced this interface in 1976 (6). On the plus side, the interface offered good EI spectra and good sensitivity. But disadvantages included difficulties with non-volatile labile analytes and LC buffers, and unreliable performance.

Enter Thermospray, a more reliable soft ionization interface that passes the column effluent through a very thin heated column to

The Top 10 narrative (cont)

produce a spray of fine liquid droplets. The droplets are ionized at atmospheric pressure (API) via a low-current discharge electrode to create a solvent ion plasma. EI-type fragmentation is sometimes observed but there is significant fragmentation of protonated or deprotonated molecules. Sensitivity is low for non-volatile analytes. Developed by Marvin Vestel (7), who formed Vestec to commercialize the interface (1987), Thermospray was the most used interface until the 1990s and certainly deserves a special mention.

The electrospray interface (ESI), developed by Nobel laureate John Fenn while at Yale University (8), produces intact, high-molecular weight, multiply protonated or deprotonated ions. Jack Henion and colleagues (9) developed an ion spray interface that used nebulized nitrogen to assist electrospray operation; Sciex, using the Henion patent (10), was first to market with an ESI-type interface (1989, see 'Honorable Mention' 7b). Along with various other API techniques, ESI is now the standard LC-MS interface.

Other universal detectors were developed. The evaporative light scattering detector (ELSD) and the charged aerosol detector (CAD) both nebulize the LC mobile phase effluent into droplets, which are evaporated leaving behind small particles of non-volatile analytes. In general, the CAD, first introduced by ESA Biosciences in 2005 (see rank No.9) is more sensitive than the ELSD and can be more gradient friendly. The CAD can also detect all non-volatile and many semi-volatile analytes with a uniform response.

Age of ultra

Through most of the 1980s and 1990s, liquid chromatographers were happy to use 3–3.5 µm or 5 µm particles, 6000 psi pumps, and 4.6 mm ID columns. However, to meet the need for faster analyses, packing materials with sub-two micrometer dimensions were developed. Once again, systems were pushed to higher operating pressures. And, because the resulting viscous heating effects required smaller diameter columns, a significant reduction in extra-column volume was also necessary. The Waters Acquity UPLC system (see rank No.10) was designed to meet the needs of the new small-particle shorter columns (5 to 15 cm) with narrower bore (2.0–2.1 mm ID) in the early 2000s. The timing was right in that Acquity was a thoughtful combination of existing products/technologies resulting in a practical system with reasonably low extra-column volume. The innovation inspired other companies to make similar improvements to benefit from the increased efficiency of newer columns. Thus, a new (non-trademarked) nomenclature arose for these ultra-high-performance instruments: UHPLC. Today, over a dozen companies are in the UHPLC business.

And a few more innovations that helped the practitioner...
Dissolved air in solvents was a pain for early HPLC. Gas in the mobile phase caused pump cavitation and loss of prime. Gas in the mobile phase at the exit of the column caused bubbles in the detector flow cell. Degassing solvent was required, first attempted by boiling the solvents and trying to keep the gas out by cleverly designed reservoirs. Then along came SpectraPhysics, who showed that helium sparging of the reservoirs kept air from dissolving in the solvents and allowed low-pressure mixing to be used effectively for HPLC pumps. Later on, membrane degassers replaced helium sparging and they became a part of every HPLC/UHPLC still used today.

Fittings also proved to be a headache. More often than not, chromatographers would over-tighten fittings giving rise to potential leaks and poor performance in trying various reconnections. Fittings from different companies were incompatible and dead volumes were created. Upchurch Scientific's Fingertight fittings changed all that and made easily reused connections that, even today, can handle very high pressures.

The year 2022

(U)HPLC is now the most widely used separation technique. The early milestone developments in the initial phase of its 50 plus years of existence paved the way for the sophisticated instrumentation and columns that we have today. The technique continues to evolve with refinements that include miniaturization, LC-MS/MS, and two-dimensional separations (and beyond), leading us toward the extremely high peak capacity required for the complex samples encountered today. Separations that early workers could only dream about have come to pass and, in many cases, are routinely used in laboratories throughout the world. The sensitivity and selectivity of today's detectors blow the minds of those of us who grew up with the 254nm UV detector of the 1960s. And separation times of less than a few seconds have been achieved by researchers who used to struggle to get their separations times under an hour… What a ride!

FEATURE

Conversations About Chromatography

HPLC is a crucial technique in many application areas, but where did it all begin? And what lurks over the horizon? Sit back as we eavesdrop on a coffee break catch-up between separation experts (and long-time friends and colleagues) Peter Schoenmakers and Bob Pirok.

Peter: Hi Bob! The Analytical Scientist wants to get our thoughts on separation science - shall we talk now?

Bob: Let's do it!

Peter: Sure. I guess I'll start by saying that separation science constitutes arguable the most important group of techniques available to analytical chemists. These methods find a home in most application areas, but – as my job title indicates – I lend some focus to forensics. Separation techniques are uniquely important in this field… After all, DNA analysis also relies on these tools.

Bob: I couldn't agree more. And, with the increasing volumes of data we are producing with such methods, my research focus – the interfacing of separation science with chemometrics – becomes ever more important. HPLC is a particularly robust and reliable technique across application areas, and our equipment demonstrates an incredibly low downtime.

Peter: Less than ten percent downtime, right?

Bob: Yes, and this compares favourably with our other equipment, such as our mass spectrometers. Overall, though, what I love about

working in LC is the expertise and knowledge it requires. New challenges arise constantly, even for experts, and we are constantly provided with new puzzles to solve from industry.

Peter: Absolutely. And how do we keep our knowledge up to scratch? By visiting key conferences, like the annual HPLC meeting. I always take a number of students and postdocs to the event – it's essential to get young scientists involved, and the culture for this group is particularly strong at EU meetings. And you also attend, Bob?

Bob: Of course! I always bring the research of both myself and the wider team. In lieu of the coronavirus situation, I've implemented a chromatography journal club between our lab and further groups, and this has been great – almost better than a real conference at times! But the great thing about HPLC is the shared interests and opportunities to exchange innovative ideas. We should prevent ourselves from working on islands – so to speak – wherever possible, and bringing the community together is the best way to avoid this. This is especially the case with the crowd at HPLC, which consists of both HPLC technology specialists and specialist users, who apply the technique to specific application areas.

Peter: And these application areas are so diverse, from pharmaceuticals (where HPLC has a major role in safe drug development), to medical diagnosis, food quality and safety, industrial materials (such as polymers), and so on. In fact, our own research in multidimensional separations actually feeds right into these polymer applications; comprehensive 2D-LC contributes significantly to this field, and is now an indispensable tool.

Bob: Right, and on this front, development of retention modelling of LC separations to rapidly compute optimal method parameters for 2D-LC is surely one of the greatest breakthroughs in the field thus far?

Peter: Absolutely!

Bob: Well, that and unique couplings of different instruments. For example, using reaction modulation for nanoparticle characterization.

Peter: And let's not forget your own contributions to multidimensional separations, which now also encompass GC×GC.

Bob: Thanks, Peter – I'm flattered. Ultra-HPLC (UHPLC) has also been an indispensable tool in most of our 2D-LC research, both for high-resolution separations (dimension one) and for very fast separations (dimension two). Without UHPLC, 2D-LC would not be as powerful as it is today.

Peter: I couldn't agree more… But let's not forget where this all started some 50 years ago.

Bob: Hey I've got a question: how old would you say LC is in "human years?"

Peter: 40 years.

Bob: Really? I'd say 30 – it's mature, but there's still much more to come. And it all started with the pioneering work of Nobel Laureates Martin and Synge, who showed that HPLC required small particles to compensate for the low diffusion coefficients of liquids. This requires some pressure to give a reasonable flow rate and led to a great debate of high-pressure versus high-performance LC.

Peter: I was in high school back then, but a few years later began performing HPLC in the lab myself. We had a few Waters (M6000) pumps, and I probably didn't appreciate how great they were. I guess I was spoiled from the beginning, and you'd need to ask people (even) older than me about the early ordeals. In any case though, the 1970s were a revolutionary time in LC – gradient elution was still a new

ONLINE

Fifty Years of HPLC

My first encounter with chromatography dates back more than 30 years. My first projects focused on the application of polymers in separation science by developing a classic format of stationary phase – beads.

By Frantisek Svec, Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic

ONLINE

My Life in HPLC

I worked at Agilent Technologies in Waldbronn, Germany, from August 1988 to November 2019 as a research scientist, specializing in HPLC, capillary electrophoresis and microfluidics.

By Monika Dittmann

"Overall, packings have become much more efficient, reproducible and stable, and core-shell particles were developed to further enhance performance."

concept, and so were chemically bonded phases. LC-MS was difficult, and we had multiple manual ways to integrate peaks, including a planimeter (a fabulous instrument), followed by cutting out and weighing the peaks after photocopying the recorder trace.

Bob: I read about those! All kinds of columns (stationary phases) and mobile-phase mixtures were tried with variable success. The development of chemically bonded phases was actually a major breakthrough. This culminated in a focus on non-polar octadecyl-silica (C18 or RP-18) phases and polar (water-based) eluents. This combination of polarities was opposite to the earliest HPLC studies (polar adsorbent and apolar mobile-phase), and thus became known as reversed-phase liquid chromatography (RPLC). The conventional normal-phase (or straight-phase) LC systems soon became an anomaly.

Peter: It's no wonder – RPLC has many advantages. It offers immense flexibility (fully miscible solvents ranging from water to tetrahydrofuran), high selectivity and efficiency, rapid column equilibration, compatibility with aqueous samples (including biofluids) and MS, and many more. RPLC is there to stay; it is very unlikely that LC will ever return to normality.

Bob: Indeed, and decades of research have gone into positioning (RP)LC as the reliable and robust technique we know today. Even before your first dive into HPLC in the 70s, great scientists like Huber, Kirkland, Knox, Giddings and Horvath first laid the foundations for the technique.

Peter: And let's not forget Guiochon and Snyder!

Bob: Of course! Today, alternate retention mechanisms steal only a small portion of the limelight. Hydrophilic-interaction liquid chromatography is fashionable, but it is useful only for very polar analytes. Ion-exchange chromatography remains important for ionic compounds, size-exclusion chromatography for polymers, hydrophobic-interaction chromatography for the separation of intact proteins, and supercritical-fluid chromatography has made a bit of a comeback, especially for the separation of chiral compounds. All these techniques have their niches, but RPLC occupies most of the playing field.

Peter: Very true – and likely because of the continued input given to improving the various aspects of HPLC technology. An impressive development on this front has been open-tubular LC (OTLC). Fundamentally, OTLC is attractive, provided efficient columns can be made with diameters of 10 mm or (preferably) less. Poppe's group were among those that showed it was feasible, but the dynamic working range was grossly inadequate...

Bob: Which is why efforts in the field then mainly focused on effective, alternative packing materials, like monolithic columns and – eventually – pillar-array columns. Overall, packings have become much more efficient, reproducible and stable, and core-shell particles were developed to further enhance performance.

Peter: On the topic of enhancing performance, the advent of UHPLC spurred a major jump in technology and applications. With higher pressures, UHPLC allowed smaller (sub-2 µm) particles to be used for very fast analysis, and also forced overall improvements in instrumentation. Like you said before, Bob, the technique is now...

Bob: Indispensable!

Peter: Yes, indispensable – and found in virtually every analytical lab today.

Bob: And what about the future?

Peter: First, we need to open the door to LC for non-specialists.

Bob: You said it. But this is – obviously – a major challenge for instrument and software manufacturers alike. We need intelligent software to combat the fact that the number of LC instruments is growing much faster than the number of trained specialists.

Peter: Plus, the expertise required is becoming increasingly complex. On one hand we require more expertise, but we cannot train experts at the rate at which they are needed. This dilemma we have to address with very smart artificial intelligence.

Bob: Smaller systems are also a priority. The volumes of organic solvents we use right now are too large, so that movement towards miniaturization is inevitable. Simpler, automated systems are also desirable, as you say, but in this case, we must sacrifice some efficiency for a selectivity benefit. Separation science must also move out of the lab to help protect against environmental issues and improve society.

Peter: I agree – just look at the coronavirus pandemic, for example. Separation science will surely play a pivotal role in finding a solution, just as it does in the monitoring of environmental pollutants.

Bob: Well, it's been great chatting, Peter. But it looks like it's time to get back to the lab!

Peter: Is that the time already? We should do this again sometime.

Bob: Absolutely.

SITTING DOWN WITH

Biologics Explorer

Sitting Down With… Davy Guillarme, Senior Lecturer and Research Associate, School of Pharmaceutical Sciences, University of Geneva, Switzerland

What's the focus of your research?

My main area of research has always been analytical chemistry, and in particular HPLC – a technique that has evolved rapidly since the turn of the millennium. UHPLC systems, core-shell column technology, and MS hyphenation are great examples of this evolution. Today, research in analytical chemistry is more often driven by applications than techniques, which is unfortunate; fundamental instrument research is important to make significant progress. I used to mix fundamental and applied aspects of HPLC, but now I apply HPLC (often coupled with MS) to the characterization of biopharmaceutical products, such as monoclonal antibodies, fusion proteins, and antibody-drug conjugates. More specifically, I focus on the development of innovative analytical strategies to improve speed, selectivity, and sensitivity.

What role does analytical chemistry play in the (bio)pharma industry?

In fact, analytical chemistry plays a critical role in almost every aspect of the drug development process, from discovery to development and commercialization, by providing assurances regarding medicine quality, safety, and efficacy. Constant improvements in analytical methods (for example, through improved selectivity and enhanced sensitivity to detect levels of impurities as low as 0.01 percent) are key to that mission. And that's why there is also a constant drive to develop new analytical tools for the rapid and accurate assessment of the safety of protein-based products. Ultimately, analytical science exists to protect patients.

What are the greatest challenges facing your field of research right now?

Limited selectivity and insufficient separation between biopharmaceutical isoforms – which can have differing toxicity profiles – (especially with HPLC-MS) is the greatest challenge we face. We can improve the characterization of complex drug products by increasing the number of dimensions in our analytical setup. Multidimensional LC and the addition of IMS before MS are fantastic ways through which we can achieve this; however, each is associated with shortcomings – the former can be difficult to use and the latter suffers from limited resolution. IM-MS instruments with increased resolution for reasonable costs could be transformative for the field!

What breakthroughs are you particularly proud of?

Working with colleagues from Genentech (Cinzia Stella and Julien Camperi) over the past two years, we have developed an automated, multidimensional LC approach capable of separating charge variants in ion-exchange chromatography for subsequent chemical reduction, trypsin digestion, peptide separation, and detection by Orbitrap MS. Our approach, involving four chromatographic dimensions and MS, allows us to rapidly identify and localize chemical modifications on proteins in biopharma and beyond.

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