Published in final edited form as:

J Chromatogr A. 2017 November 10; 1523: 3–16. doi:10.1016/j.chroma.2017.06.042.

Development of Capillary Liquid Chromatography: a Personal Perspective Minireview

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Abstract

This is a historical account on the development of capillary LC from its beginning to the present day. The first investigations into the viability of capillary LC date back to the late 1970s, a decade after the pioneering efforts in HPLC. The drastically reduced column dimensions were required to counter the slow solute diffusion in liquids. There were numerous instrumental difficulties with sample introduction and detection in the picoliter or even femtoliter volumes. High-efficiency separations were needed in the analysis of complex biological mixtures. Miniaturization brought distinct advantages in spectroscopic and electrochemical detection. Since the 1980s, column technologies underwent significant changes: (a) from glass-drawn microcapillaries to slurry-packed, small-diameter fused silica columns; and (b) in microcapillaries packed alternatively with sub-2- μm particles or monoliths. The viability of LC-MS combination has dramatically promoted the use of small-diameter capillaries. Through “omics technologies”, capillary LC/tandem MS accounts for most applications in proteomics, glycomics and metabolomics.

Keywords

Open tubular vs. packed capillaries; LC-LIF; Capillary LC/MS

1. Background Information

As a biochemistry graduate student during the 1960s, I could be both amazed and frustrated by the complexity of biological mixtures as demonstrated with protein concentrates showing UV absorbance over the number of test tubes collecting the eluates from long chromatographic columns packed with dextran gels or cellulose-based ion exchangers. And, after repeated separations, when I thought a targeted protein was purified to homogeneity, a planar electrophoretic medium (like a paper or starch gel used in those days) would almost invariably reveal at least several trailing zones to add to my frustration. Similarly, you could run a plant extract, for example, on a thin layer of adsorbent or a paper medium with an appropriate mobile phase and visualize a number of spots in one direction, and then turn the medium perpendicularly, develop a chromatogram again with a different solvent and subsequently multiply a number of detected solute spots. Structural elucidation of the

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molecules behind different fractions or spots was at best difficult, but mostly impossible, with the biochemist's primitive tools at the time. I thought even then that there ought to be better ways of analyzing biological samples, but needless to say, no one could even dream how different the world would become in 40–50 years with all the analytical power of today's omics technologies.

I was still sold on chromatography and, as the next step in my scientific training at the Czechoslovak Academy of Sciences in Brno, I took my chances with it to become eventually a life-long affair. The experience there represented quite a turn in my understanding as to what could be done chromatographically with very complex mixtures: there was capillary gas chromatography (GC), albeit with only petroleum samples. The chromatographic resolving power was fully there, but structural identification of mixture components was still a major problem. However, a fix was clearly on the horizon through the combination of GC with mass spectrometry (GC-MS). While learning GC-MS during the historically eventful year of 1968* at Karolinska Institute in Stockholm, I rediscovered my interest in biochemical separations, which a year later my postdoctoral mentor, Albert Zlatkis of the University of Houston, kindly encouraged me to pursue in his laboratory. At the time, the pioneering studies by Evan and Marjorie Horning in Houston [1,2] and my new friend Chuck Sweeley [3] on microscale derivatization of the seemingly nonvolatile compounds, such as steroids and carbohydrates, was setting a new and important stage for multicomponent GC separations of complex biologically important mixtures. However, all their work was done with packed GC columns and higher efficiencies were apparently needed to resolve samples. My previous work on glass capillary columns and their utilization in GC-MS seemed ideal in this task, which I pursued at a full speed during my 2-year appointment at the University of Houston [4–7].

After spending several years of scientific training in good analytical laboratories, I was fortunate to interview for several academic positions and ultimately landed on the chemistry faculty at Indiana University. The faculty here liked my research on developing glass capillary columns for biochemical and environmentally-oriented separations as well as my earlier experience with the GC-MS combination [8]. I was soon fortunate to attract several talented graduate students, most notably among them Milton Lee and Jim Jorgenson, two future leaders in analytical separation science. While basking in the “newly found glory” of capillary GC and GC-MS as a young academician, I was also aware that I needed to broaden my range of research topics. The rapidly emerging field of HPLC in the early 1970s just provided the opportunity to engage in the design of new stationary phases and some attempts to improve the detection capabilities in HPLC. While, from my own historical perspective, I consider my early activities in HPLC somewhat incremental, these contributions opened the door for me to participate in the first HPLC symposia and meet the key scientists in that field. I felt already then that there were some dichotomies between the HPLC and GC fields and philosophical differences as to “what was important to do”. While some of the

*Footnote: I left Czechoslovakia at the beginning of 1968 during the period now historically known as the “Prague Spring” for its modest regime liberalization attempts. Any hopes for liberalization were however dashed by Soviet-led invasion in August 1968; for sure, this changed my plans to return to my homeland. The year 1968 was a very eventful year in many other respects: student revolts in the European cities, including Stockholm; antiwar demonstrations; and several turbulent events in the U.S. as well.

discrepancies between LC and GC analytical practice were already in the 1960s noted by Giddings in his authoritative reviews on chromatography [9,10], it is remarkable how long some of his suggestions lay dormant.

The institution of tenure at most U.S. universities remains a controversial subject for many academicians. It is viewed by many as a brutal “swim or sink” process, which leaves those denied a tenure embittered for many years. Others who pass the test may naively assume that they have received a license of doing any reckless or foolish research they may wish to pursue and their institutions would not fire them. I must have belonged to the latter category, as the tenure promise cemented my wishes to enter two risky avenues: capillary LC and supercritical fluid chromatography (SFC). Both research areas were largely inspired by Giddings’ papers pointing to the discrepancies between the theoretical potential of LC and its practice.

The effects of flow dynamics and diffusion-controlled solute mass transfer had to be taken carefully into consideration if efficient separations with the condensed mobile phases were to be realized. The large differences in solute diffusivities in the gas phase and the liquid phase, with the values encountered in a dense gas or supercritical fluid being intermediate [11], dictated small dimensions for any future columns to be considered: the inner column diameter; particle size, if a capillary was packed; and a thickness and geometry of the sorptive layer. This all clearly called for miniaturization of the overall systems. But how do we design such microcolumns and what type of equipment do we optimally need to introduce tiny samples, generate mobile-phase gradients, and ultimately, detect or identify the separated compounds at the column outlet? Most importantly, we were entering a technically demanding area, which needed talented co-workers, excellent experimentalists and dedicated scientists. In the area of capillary LC, I found an outstanding coworker in Dr. Takao Tsuda, my first Japanese postdoctoral associate, who joined my laboratory in 1976 for one year. His activities resulted in two key publications in Analytical Chemistry in early 1978 [12,13].

2. Biochemical Separations in the 1970s

Before discussing further our capillary LC research, I wish to return to some of my thoughts on the initial rationale for developing capillary LC and other micro- and nanoscale separation techniques. In retrospect, throughout my entire research career, I have found utmost satisfaction in developing new analytical tools and techniques that could be directly applied to what I viewed as biochemically and biologically important problems. During my first years as a “chromatography apprentice” in Brno, I was fascinated by capillary GC resolving dozens and even hundreds of components from a complex sample, albeit only for petroleum hydrocarbons, or at best, mixtures of fatty acid methyl esters. Thanks to glass capillary column technologies, a few years later, we could also analyze steroids, sugars and some other biochemical compounds converted to volatile derivatives [14], but what about all the large and nonvolatile substances that one would surely encounter in a complex biological sample? It may not be widely appreciated today that the concept of “metabolic profiling”, a term introduced by Dalglish et al. in 1966 [15], long preceded what is now called “metabolomics”. Their work represented a significant departure from performing merely a

set of isolated determinations describing the composition of a biological material as practiced until then. While using one particular measurement method, such as GC or GC-MS, the whole series of metabolically related substances could be at once displayed qualitatively and quantitatively in a single chromatographic run. For example, it became feasible to profile most metabolites of the steroid hormones extracted from a physiological fluid sample and quantitatively evaluate them [14,16] under different conditions of health or disease.

Increasing interest in analyzing complex mixtures by capillary GC and GC-MS during the 1970s is attributable to both major improvements in technology of glass capillary columns and the advances in GC instrumentation. The columns featuring efficiencies in the excess of 100,000 theoretical plates became readily available for resolving up to several hundred components in a single run, with typical analysis times of 1–2 hours. A state-of-the-art chromatogram demonstrated by our laboratory in 1974 [17] is shown in Fig. 1 (A), featuring a concentrate of urinary volatile compounds as an example. Comparable efficiencies could also be obtained for the multicomponent separations of steroids, urinary acidic metabolites and polyols, all seemingly polar compounds, paving the way to metabolic profiling with the applications to human genetic disorders and metabolic abnormalities of human diseases [reviewed in ref 14 and 16]. In contrast, Fig. 1 (B) shows an HPLC run of the type we practiced at about the same time in the same laboratory [18]. Even with elaborate explanations on my part, what did really my students think about the differences between the resolving power of GC versus HPLC? Were we going to do something about it?

While attending the early HPLC international conferences, I could not find whole lot of interest among the participants in analyzing complex mixtures. A strong emphasis at these meetings was placed on column selectivities, mobile-phase effects, and generation of gradients. On the positive side, the previously intractable (not “GC-able”) compounds, such as pharmaceuticals could then be successfully analyzed through HPLC. Most were present in relatively uncomplicated mixtures and contained a UV chromophore in their molecules for the sake of detection. No equivalent of a universal and sensitive GC detector, such as the flame ionization detector, was available with HPLC, and a viable LC-MS combination still had to wait for decades to be developed. Among the notably exceptional earlier studies using high-pressure LC, C.D. Scott at the Oak Ridge National Laboratory emphasized the potential of LC in profiling UV-absorbing components of physiological fluids[19].

When considering electrophoresis, the field now widely viewed as highly complementary to LC, there was little sign of any idea crossfertilization between electrophoresis and LC during the 1970s. While the electrophoresis in gels and other media had developed into a set of very useful techniques to a biochemist, most analytical chemists considered them “artsy”, perhaps even unsophisticated and irrelevant. However, the introduction of 2D-gel electrophoresis by O’Farrell [20] in 1975 was clearly a major advance, demonstrating hundreds of proteins resolved as the result of combining two complementary separation principles. Evidently, at that stage, HPLC of proteins seemed a long way to catch up. Another result of the previous research in electrophoresis which fascinated me was a description of the microdisc electrophoresis of proteins on a 215 μm gel, representing separation of a protein extract from just 60 isolated neurons [21]. Isolation of minute biological samples, resolution of complex

mixtures and their sensitive detection – could we somehow put all these together? A monumental move in this direction was started through the work of Jorgenson and Lukacs on capillary zone electrophoresis reported in 1981 [22]. Their spectacular results were quite rapidly followed by commercial developments in the area of capillary electrophoresis (CE), albeit initially with the relatively small molecules. In retrospect, it appears that these developments helped to turn attention of the relatively conservative HPLC audiences to the prospects of capillary LC as well. There was a distinct overlap in various instrumental aspects of both techniques; most importantly, in detection requirements and parameters. I have already expressed my tribute to Jim Jorgenson for his CE pioneering work some time ago [23] in a special journal issue.

3. Open Tubular Columns or Packed Capillaries?

Open-tubular LC received a minor consideration in the HPLC pioneering work by Horvath, Preiss and Lipsky [24] where uncoated metal capillaries with the diameters typical of GC columns were evaluated in terms of chromatographic band-spreading. Although the measured plate heights were found more favorable than those predicted by the Taylor [25] and Golay theories [26], presumably due to some wall roughness and the effects of column coiling, the authors did not find this approach sufficiently attractive to pursue it further. Clearly, the column diameters were a way too large. Could the wall roughness and the coil effect be different in the capillaries of much smaller diameters? This was certainly one of the questions that Dr. Tsuda and I tried to answer in 1976, but most importantly, we wished to address a more fundamental issue in defining the instrumental limitations of working with the columns featuring 1,000-times or lesser flow-rates [12,13]. In 1970, Giorgio Nota and coworkers [27] reported the use of alkali-etched glass open tubular columns to fractionate a mixture of fluorescently-labeled amino acids. In the absence of a suitable detector, they sequentially deposited the capillary eluent on thin-layer plates to demonstrate the fractionation pattern. The wide band dispersion was observed due to the relatively large capillary diameters (200–300 μm).

My previous experience with the preparation of glass capillary columns for GC separations, dating back to my time in Brno a decade before, led to a simple idea of preparing microcolumns with small inner diameters, whereby small adsorbent particles could be drawn inside thick-walled tubes by means of the glass drawing machine [28]. Through varying the initial tube diameter, particle size and type, and adjustment of glass drawing conditions, we could prepare various column types to experiment with. While this approach gave us a great flexibility in varying the column dimensions, how were we able to design a system that could introduce such tiny amounts of sample and then detect them not in microliter, but rather nanoliter, or even picoliter volumes, without large contributions of the extra-column band- broadening? No existing commercial HPLC equipment was conducive to deal with such small columns without drastic modifications. In our first rather primitive instrumental setup [12], we resorted to some simple instrumental modifications that I had known from my capillary GC experience: (a) a split sample introduction; and (b) the use of makeup flow to reduce the dead-volume problems with a conventional UV detector. Through the great experimental skills of Dr. Tsuda and his enormous patience, we were able to assess the limits of equipment in working with open tubular microcapillaries [13] and also produce first

promising chromatograms indicating the efficiency potential of packed capillaries [12]. The first such successful recording is shown in Fig. 2, demonstrating 85,000 theoretical plates achieved in 100 min; as we thought then, not too bad for the initial result. The submitted papers generated enthusiastic reviews, however, the first symposia presentations met with either neutral or even a hostile response: “How can you detect anything in such small volumes?” or “Who cares how many theoretical plates you can generate when HPLC already works so well”. Only later, over the years of research, I have learned that almost any new direction you try to come up with may initially be met with hostility.

Miniaturization of scientific measurements had been a distinct trend in science and technology for a long time. As an example, I watched during my early years at Indiana University my close faculty colleague Mark Wightman to develop carbon fiber microelectrodes for the distinct benefits of neurochemical experiments. So why not miniaturize chromatography or electrophoresis? While I felt that our initial work on LC microcapillaries was a step, however uncertain at the time, in this direction, any reservations now seem nearly irrelevant in the view of today’s proliferation of capillary systems (nowadays often termed as “microflow LC” or “nanoflow LC”) and even the separations done on microchips.

I met Professor Daido Ishii (from Nagoya University in Japan), a pioneer in LC miniaturization, during the 1977 Pittsburgh Conference for the first time. We established a productive relationship for the following number of years to come. Upon returning to Japan, Dr. Tsuda actually joined Professor Ishii’s group and continued working with both open tubular and packed capillaries. In collaboration with the Ishii group, we published one paper on a continuation of some column technologies in packed microcapillaries drawn from prepacked glass tubes [29]. During my first visit to Japan in 1978, I was pleasantly surprised by the keen interest of both the academic and industrial scientists in LC miniaturization.

The early 1980s were the time when several new directions were coming together, all centered around the use of small columns and miniaturized detection: besides capillary LC, there was capillary electrophoresis [22], and capillary supercritical chromatography [30]. Another important innovation coming from Japan was micellar electrokinetic capillary chromatography (MECC) originated by Shigeru Terabe [31]. This was clearly an exciting period which could lead to new important directions in science. During one of the scientific meetings, Professor Ishii and I discussed a desirability to organize a joint U.S. –Japanese seminar on microcolumn separations and their ancillary techniques. Under the joint sponsorship of the U.S. National Science Foundation and the Japanese Society for Promotion of Science, this meeting took place on the campus of the University of Hawaii at Manoa during August 1982. This seminar was limited to roughly 50 participants including the observers from industry and several participants from other countries (Australia, Israel, Sweden and Switzerland). A group picture from the meeting is shown in Fig. 3. By any measure, this small meeting was a great success in defining the needs for novel column designs and important directions in detection technologies. For example, ultra-microelectrodes and the possibilities to study single biological cells were discussed, as were different laser-based and other miniaturized detectors. The field of capillary separations

apparently started to attract scientists from other fields, including spectroscopists and electrochemists.

The microcolumn developments advanced substantially during the early 1980s, but largely due to the research done in academic laboratories. The major companies manufacturing HPLC instrumentation showed only a limited interest in capillary LC until the breakthrough of electrospray ionization and LC-MS roughly a decade later. Paradoxically, during the same period there was a very high commercial interest in capillary electrophoresis, even though some instrumental difficulties (e.g., those associated with detection) were shared by both techniques. This was later also demonstrated through a short-lived enthusiasm about capillary electrochromatography, which was supposed to “combine the best part of the two worlds”, i.e., those of CE and HPLC.

Let us now return to the type of microcolumns and technologies to produce them during the 1980s. In our initial assessment of open tubular microcolumns [13], the results showed a rough agreement with the Golay theory and only slight benefits of the surface roughness effect. Under the laminar flow conditions, a solute mass transfer in the radial direction could only be achieved through diffusion, while the benefits of turbulent flow chromatography, as hypothesized by Pretorius and Smuts [32], would require unrealistically high pressures. In order to capitalize on open tubular LC, it was clearly necessary to go to much smaller column diameters. This fact, combined with instrumental contributions to band-broadening, made me lose interest in open tubular LC already at that time. However, more optimistic predictions were reached by Knox and Gilbert [33] through their kinetic optimization study. These were further reinforced by Jorgenson and Guthrie [34]. As seen in their graph of efficiency (number of theoretical plates) against the column diameter (Fig. 4), very high column efficiencies could be theoretically reachable with the column diameters of less than 10 μm . While this represented considerable challenges in instrumentation (difficult sample introduction and small-volume detectors with extremely fast response), column fabrication (surface treatment, stationary phase immobilization and surface deactivation), and operation (e.g., column clogging problems), numerous investigators continued to pursue open tubular LC for roughly a decade. Some respectable separations were achieved with variously treated capillaries of the inner diameters between 30–50 μm , starting with the pioneering studies by the Ishii group in Japan [35, 36] and finally reaching efficiencies on the order of 10^5 theoretical plates [37] with around 10 μm i.d. columns. It is of historical interest only to show physical dimensions of the microcolumns drawn out of glass (Fig. 5) at these times.

It is important to note that some studies merely tried to utilize small capillaries as a convenient means for the unprecedented detection in femtoliter volumes with very small electrodes [38, 39] and laser beams [40]. In retrospect, what was a daunting task due to various experimental difficulties during the 1980s is now readily achievable with microchip separation channels. It seems not entirely coincidental that one of the pioneers of the separations on microchips, Andreas Manz, did his doctoral research with small diameter capillary columns under Professor Willy Simon [38], one of the key participants of our small meeting in Hawaii.

Meanwhile, the development of additional column technologies continued throughout the 1980s, giving researchers different options, as reflected in Fig. 6. Besides the open tubular types, partially or fully packed capillaries were investigated. The partially packed capillaries, originating from drawing out thick-walled glass tubes packed with different particles [12, 29] were soon replaced by fused silica capillaries packed with small particles [41, 42]. Clearly, the flexible fused silica columns were much easier to handle than the fragile glass capillaries. Packing procedures for such column with slurries of particles suspended in appropriate solvents had to be developed [42]. Initially, such microcolumns featured the inner diameters around 200 μm and were packed with 5 μm or even 3 μm particles [43]. One- to 2-meter long columns could be prepared to yield efficiencies on the order of 10^5 theoretical plates and separate complex mixtures of biological or environmental interest [43–46]. Given the particle diameter as the characteristic dimension of these columns, already in mid-1980s, the capillaries packed with 5 μm silica particles were reaching their theoretical potential, but typical separations of complex mixtures could take several hours.

Very high reproducibility of packed capillaries could be demonstrated already in mid-1980s [47], but a most surprising result of a study by Karlsson and Novotny [48] was that a reduced plate height of slurry-packed capillaries decreased substantially as a function of the column radius. We were then able to prepare a 1.95 meter column, with the inner diameter of 44 μm and 5 μm particles, featuring 226,000 theoretical plates in 33 min. While this represented at the time a “kinetic record” for LC, just a year later Kennedy and Jorgenson [49] demonstrated that even smaller capillaries could be packed efficiently with particle slurries. Very similar packed capillaries represent the bulk of separation columns used today in thousands of laboratories in proteomic, glycomic and metabolomic investigations. Their suitability for combination with mass spectrometry has further enhanced the scope of scientific research unimaginable at the onset of capillary LC decades ago.

As it turned out, the small diameter (40–80 μm) fused silica capillaries were easier to pack than the larger ones (200 μm and above). A typical reproducibility of column performance is shown in Fig. 7 (K.E. Karlsson and M.V. Novotny, unpublished experiments, 1987) with a set of five consecutively prepared columns packed with 5 μm C18 silica particles. The results could be quite easily reproduced from one laboratory to another, and many research groups started to set up their own slurry-packing devices since those times. The area was ripe for commercialization, which was further accelerated by the advances in LC-MS coupling via electrospray ionization (ESI) a few years later.

A decade later, a major advance in capillary LC occurred with the description of small diameter columns packed with 1.5 μm particles at the pressures as high as 60,000 psi (4,100 bar) and also operated at very high pressures [50]. This seminal contribution by the Jorgenson group at the University of North Carolina was the beginning of the LC subfield, now called the ultrahigh-pressure LC (UHPLC). While decreasing the particle size has been a trend throughout the entire history of HPLC, the effective combination of a very small particle size and small capillary diameters is the key technological gain, since the small column dimensions dissipate the deleterious frictional heat generated by very large pressures and mobile-phase velocities, while also reducing the safety hazards. As seen in Fig. 8 reproduced from the 1997 paper [50], a chromatogram that could be obtained during a

previous decade under gradient elution in several hours, at best, is now recorded under isocratic condition in a mere 35-min time interval. The column efficiencies in the excess of 300,000 theoretical plates were recorded. Many following studies from the Jorgenson laboratory featuring the separations of complex peptide mixtures and proteins at very high resolution were subsequently reported together with further advances in small particle technologies and improvements in understanding the column packing procedures. These advances will be described in more detail in an article by the other authors honoring Professor Jorgenson in this journal issue.

The quest for ever smaller particles packed in narrow separation channels continues unabated, as evidenced by the industrial efforts to pack small particles into microchips as well as the use of nanosized materials under the conditions of the so-called slip flow [51]. Only time will tell how successful these recent innovations will become in the analytical practice. Will the particle-packed columns be eventually replaced by some 3D-printed structures or other microfabricated devices such as those investigated by the Desmet group [52]? It is my guess that the column design studies will occupy researchers for some years to come.

The bulk of research during the development of capillary LC and its applications has been done with the reversed-phase packings. This is understandably due to the great versatility of this type of chromatography in the separation of peptides, proteins and other biologically important mixtures. However, other small particle packings become of considerable interest in addressing more polar mixtures such as those of glycoconjugates and hydrophilic metabolites. The use of more recent packings suitable for hydrophilic interaction chromatography (HILIC) or spherical graphitized carbon particles packed into capillary dimensions will likely become more common in the near future.

4. Detection Advantages of Miniaturized Separation Techniques

During the early reports on capillary LC, a frequent question (and criticism) was: “How can you detect anything while using such tiny columns?” It was largely misunderstood that many HPLC detectors (typically, with the volumes around 10 μ L) were concentration-sensitive devices whose mass sensitivity could be substantially enhanced through proper miniaturization. This was demonstrated already with 1.0-mm i.d. columns by Scott and Kucera [53] and with open tubular LC by Ishii et al. [35] using a UV-absorbance detector. Similar considerations were certainly valid for on-column fluorescence [54, 55] and electrochemical detection [56]. Most often, an optical cell could be created through a careful removal of a polymeric coat of the fused silica capillaries, which researchers had to perform painstakingly on their modified home-made detectors. The downside was that such detectors were not commercially available, although during a rapid rise of interest in CE, similar detector arrangements had to be made. In my opinion, some conservative views of the leaders in the HPLC field, which in turn influenced the main instrument manufacturers, were not helpful to progress in capillary LC, in particular, and science, in general.

Both capillary LC and a set of electromigration techniques, broadly included under the common denominator as “HPCE”, represented a field with many exciting opportunities for

both improved separations and often dramatic improvements in sensitivity and selectivity of detection. While on the separation side, these techniques represented some of the best examples of crossfertilization of principles, all featuring “the column smallness”, not all ideas in small-volume detection survived for a long time. This is largely due to the massive success of mass spectrometers as the “ultimate detectors” since the beginning of the 1990s. Certain sophisticated devices using different spectroscopic principles, imaging detectors, flame- and plasma-based detectors have all lost their previous appeal due to the new power of LC-MS. Some of the detection approaches developed vis-à-vis capillary LC were described in my Analytical Chemistry brief review [57] a number of years ago and will not be revisited here. Perhaps electrochemical and laser-induced fluorescence (LIF) detectors best represent two areas which took utmost advantage of the drastically reduced volumes characteristic of capillary LC.

One of the most memorable presentations at the already mentioned U.S. – Japan seminar in Honolulu in 1982 was given by Professor Simon of ETH (Switzerland) describing potentiometric detection in a small liquid drop at the outlet of capillaries with the inner diameters as small as 5 μm (see Fig. 9) [38]. The use of microelectrodes as detectors for both capillary LC and CE has subsequently opened the field to studies of small biological objects such as individual biological cells. The effective volumes of a few picoliters could be attained, matching the volumes of single biological entities [58]. A scanning microvoltametric detection was subsequently described by the Jorgenson group [39] using a single fiber carbon electrode inserted into the end of a separation capillary. The following studies used this detection principle to measure neurochemically important compounds in single neuron cells of a snail [59] and in different mammalian adrenal cells [60]. For the following decades, electrochemical detection in conjunction with capillary LC was used in the analysis of this class of compounds and other metabolites in very small biological specimens, perhaps most notably by the Kennedy research group. Electrochemical detector is still being used in the Jorgenson group as a zero dead-volume device to evaluate a true efficiency of slurry-packed capillaries. With today’s emphasis on microfabricated devices, the separation channels on microchips are easily integrated with electrochemical measurements.

From the early years of capillary LC on, our group became interested in laser-induced fluorescence (LIF) detection. The lasers were a novelty to analytical chemists during the 1970s, and the first attempts to involve lasers in HPLC detection showed highly sophisticated setups, but not too impressive analytical results: “humpy” chromatograms and the detection limits at nanogram levels, not significantly better than the best HPLC detectors available at the time (reviewed by Yeung in 1981)[61]. The detection principles such as thermal lensing, light scattering, indirect polarimetry and circular dichroism were further explored in this general area, but it was laser-induced fluorescence (LIF), first reported by Diebold and Zare in 1977 [62] for the conventional HPLC columns, that appeared particularly attractive. Most of the work reported at that time [61] used conventional HPLC detector cells with roughly 10- μL volumes, obviously too large for laser beams. In capillary LC, we were dealing with picoliter volumes; in the Diebold-Zare communication, the detection limits for aflatoxins were at the subpicogram levels in a flowing drop, but estimated to be two orders of magnitude less in the actual laser beam environment. This was

clearly the way to go, but which lasers would be the “right” and convenient light sources for LIF detection in capillary LC?

In building our first optical setups for capillary LC-LIF, we learned a great deal from the reports of Dick Zare’s laboratory at Stanford and the Yeung group at Iowa State University, and used greatly the excellent services of our mechanical and electronic shops at Indiana University. My analytical graduate students and postdoctorals became rapidly experts in different aspects of this research: from setting up optical benches to signal processing, to the development of appropriate chemistries for sample derivatization. Although the optical advantages of lasers in terms of focusing and monochromaticity could be used with advantage, such as shown in the example of detecting 28 attograms (10^{-18} g) or 35,000 molecules of rhodamine 6G [63] in flow cytometry, there were only a few cases in which the available laser wavelengths coincided, or were near to, the maximum excitation of the molecules of interest. However, if we could find the means to convert the originally non-fluorescent molecules to their fluorescent derivatives, we would be in the business of performing highly sensitive measurements on many compounds. Naturally, the ideal fluorescent derivatives needed to additionally have desirable chromatographic properties, with a single peak per a mixture component. After testing different optical cells and designs, it became relatively easy to employ lasers as light sources. The helium-cadmium laser operated at 325 nm output was initially chosen by our group, while we developed simultaneously derivatization techniques for different classes of biologically important compounds such as steroids, prostaglandins and bile acids [54]. We promptly learned that high laser powers were not particularly desirable because of an increased noise due to light scattering and some photochemical reactions at the detector cell which tended to form deposits at the detection window. Through the chemical derivatization approach, we also wished to move to the blue line of the helium-cadmium laser (442 nm).

Derivatization chemistry was at the time our great strength in competition with other research groups who were entering the field of capillary separations with laser-based detection. At the time of the 1984 HPLC meeting in New York City, there were at least six different contributions on open tubular LC with a laser detection on the program, all scheduled before my lecture on Thursday afternoon. To my great relief, not a single one of these showed any practical utilization of their devices however sophisticated they were: mostly fluorescent dyes, or a single peak for riboflavin, all detected with great sensitivity, but “so what”? In my lecture, I was prepared to show some nice separations of derivatized hydroxysteroids, prostaglandins and bile acids, albeit only as standards, so I wished to demonstrate additionally some real biological sample following a solvent extraction. The “critical chromatogram” (Fig. 10) was recorded in the laboratory on Tuesday of that week while I was already at the meeting. Fortunately, the Special Delivery service did not fail: I received the wished for slide at 11:00 am at the hotel reception, and I was ready to show it to the audience in my afternoon lecture. The lecture was well-received, but the downside was that the audience already lost many participants who on Thursday afternoon flew home or went sightseeing or shopping in the City.

During the following two decades, a number of researchers (including our group) were increasingly turning to CE as the main analytical alternative. My developing interest in

glycobiology also led us to use electromigration techniques and fluorescent-tagging methodologies [64,65], some of which we continue to pursue to the present day on microchips. The advances in biomolecular mass spectrometry have modified this “one-way-trend”, and the availability of capillary LC/MS has later started to facilitate the onset of the “omics revolution”, in which CE and potentially CE-MS become additionally valuable.

Microcolumn technologies and detection techniques now continue hand-in-hand, featuring capillaries and microchannels packed with small particles in the low-micron range, and even below [51]. Monolithic columns, in their organic polymer versions as well as the silica-based matrixes, provide additional avenues in the effective column design. After many years of research, the microchip-based separations can effectively integrate with the most powerful detection techniques, be it laser-based, electrochemical, or mass-spectrometric, and then be applied to a variety of interesting problems in life sciences.

5. Developments in Coupling LC-MS

Looking at the current achievements in the ionization techniques and the great progress made in different omics technologies, it is hard to comprehend what an enormous abyss and a disconnect between the LC and MS technologies once existed. Following the success of capillary GC-MS, the pressure was on the analytical community on how to characterize seemingly complex mixtures of much less volatile components, but how could we do it? The mere idea that such solutes must be first volatilized before the ionization takes place was inconceivable, short for the substances which were just outside the GC range (e.g., some lipids) or which were volatile, but unstable to survive a passage through a GC column (e.g., some common pharmaceuticals). As a consultant to chemical and pharmaceutical companies many years ago, I could often sense a tension between the “GC folks” and “HPLC folks” centered around these very issues. For example, these people could encounter a minor, yet important drug impurity peak in an HPLC run, but it was very hard to identify that component. From the very first efforts to couple HPLC columns to a mass spectrometer in Fred McLafferty’s lab at Cornell University [66,67], it should have been clear that the LC-MS coupling was not merely a mechanical issue of how to get rid of all that gas evaporated from the milliliter volumes of a liquid. The demonstrations of spectra from that period did not include genuinely nonvolatile compounds like zwitterions, and even the moving-belt LC-MS interfaces [68] or thermospray [69], developed later, did not fare much better in that regard.

Just as even the GC-MS field fared better when capillaries replaced packed columns in the early 1970s with no need for molecular separators to remove many milliliters of the diluting and excessive carrier gas, it seemed eminently worthwhile to use the capillaries with microliter-per-minute liquid flows rather than the conventional HPLC columns with 1,000x higher flow-rates. We made this point clear in our early papers [12, 29], but hardly offered any suggestions on how to do the experiment to ionize nonvolatile substances for the benefits of MS. Yet, the demonstration that large peptide ions could be detected in the Californium-252 plasma desorption MS [70] already existed. As the chairman of the 1980 Gordon Conference on Analytical Chemistry, I gathered a group of top-level scientists from both fields for an afternoon informal brainstorming session, but there were no obvious leads

or strategies. However, we seemed to have some potentially fruitful directions discussed at the already mentioned U.S.–Japan seminar in Honolulu in 1982, as reflected in the later reviews by Tsuge [71] and Henion [72], but many years subsequently passed with only incremental improvements. One notable exception was the paper by Stenhagen and Albhorn [73] showing distinct spectra of saccharides separated with a packed capillary. In our own efforts with large lipids, we had the “right type” of capillary columns and reasonable MS instrumentation, but our results were irreproducible; we were mostly plugging the tips of our capillaries and there was not a single paper to publish!

During the late 1980s, the prospects for MS ionization techniques started to improve substantially [74], so that MS-based sequencing of peptides could be considered. The introduction of “flow-FAB” (FAB = fast atom bombardment) or “dynamic FAB”, in principle a transport-type system, and its practice with capillary columns [75] looked very encouraging. However, it was really the advent of electrospray ionization (ESI) [76] that made a huge difference. Finally, a suitable ionization technique has become available for an effective coupling of capillary LC and even CE with MS!

At these early times of biomolecular MS, we became one of the first active groups to investigate the merits of ESI in relation to capillary separations. Our MS equipment (a single quadrupole) during the 1980s, used periodically in some desperate attempts for LC-MS coupling as described earlier, was not suitable for peptide sequencing work. However, we were able to establish collaboration with researchers at the Finnigan Corporation, and demonstrated in 1990 [77] the merits of capillary LC in this combination (with a triple quadrupole MS instrument). As seen in Fig. 11 (a reconstituted ion chromatogram), tryptic digest mixtures could be recorded at high sensitivity, and each peak could be subjected to tandem MS to yield a recognizable peptide sequence. With a 250- μm i.d. fused silica capillary packed with 5- μm particle, full-scan MS data became available with low picomole amounts of peptides. Since then, further substantial improvements have been made in capillary LC/MS-MS in terms of smaller column diameters (now typically practiced at about 70- μm i.d.), resulting in significantly lowered flow-rates for the benefits of better MS detection. The truly phenomenal advances in MS instrumentation over the following decades and the necessary developments in computational capabilities and handling the complex data (bioinformatics) now allow researchers to address the scientific problems which were earlier beyond our imagination and comprehension.

6. Problem-Oriented Investigations: Integration of Modern Analytical Techniques

The on-going revolution in biological sciences and modern biomedicine, now often referred as “systems biology”, has taught us many lessons on how to approach a complex scientific problem. I also think that, more than ever before, it has made scientists highly competitive with each other in the world with very expensive instruments and diminishing resources. Can hypotheses be any longer a main driver of our research efforts? Or, are technical advances in our research tools the main reason for the recent scientific achievements? While the answer probably lies somewhere in between, a very substantial role of analytical

chemists in all directions and subfields of systems biology are clearly beyond dispute. In a sharp contrast to the gone-by years emphasizing the student-mentor relationship and scientific outcomes in terms of published work, today's scientific reports in this field largely feature communications with ever greater numbers of authors. Obviously, it has become necessary to interface with many collaborators to solve an important biological problem.

The current integration of different omics technologies to solve a particular scientific problem will be the on-going process for some time to come. Additionally, I believe we have not seen the end of developing new technologies: the current emphasis on separation microchips and other microfabricated designs; smaller chromatographic particles with selectively modified surfaces; still newer ionization techniques for MS, just to mention a few directions. Just at the time of writing this minireview, I became aware of the newly developed multipass high-resolution ion mobility-MS platform with an ion transmission over 1.1 km (!) and the subsequent detection of a previously unknown sugar isomer [78]. Still a lot of amazing stuff coming in!

Whereas capillary electrophoresis with LIF detection was a key methodology in Human Genome Initiative [79], capillary LC with tandem MS accounts for the most recent progress with other omics directions: proteomics; glycoproteomics; glycomics; peptidomics; and metabolomics. The advances in bioanalytical methodologies and instrumentation now often challenge once "well-established views" on structure and function of proteins, roles of glycoconjugates in Nature, biosynthetic pathways for certain metabolites, and so on.

Capillary LC-tandem MS is perhaps most visible in the many on-going efforts to fully characterize the proteomes of different organisms. Both the "top down" and "bottom up" proteomics are highly dependent on separation technologies [80, 81] to: (a) ensure extensive proteome coverage in a given time; (b) achieve higher analytical throughput; and (c) cover large dynamic concentration range, including even trace proteins. In some the most remarkable separations of complex peptide mixtures, multidimensional LC [81], small-particle technologies [82] and monolithic capillaries [84] were used. The high-power MS instrumentation, such as FTMS or Orbitrap, is additionally important in achieving the most comprehensive results. In one example shown in Fig. 12 [85], amazingly complex data can now be displayed by the state-of-the-art capillary LC/tandem MS technologies; here, the results on the yeast proteome are compared for different mobile-phase gradient durations, identifying 5,806 peptides in 140 min and 13,682 peptides in 480 min.

During the last two decades, I too became increasingly a problem-oriented scientist as I began to be interested in the biological roles of glycosylated proteins in health and disease. At the beginning, the area needed, and still needs today, more powerful analytical approaches to solve its many problems (e.g., isomerism in glycan structures). As of today, we are still struggling with the problems that MS alone cannot solve. Consequently, we have approached the distinction of biologically important glycan isomers (potentially important disease biomarkers) in our recent studies through the use of microchip CE-LIF [86] and, with LC/MS-MS, the use of long capillaries packed with graphitized carbon particles [87]. Fig. 13 from the latter communication shows an example of a partial resolution of N-glycan

isomers isolated from human urinary exosomes, but improvements are still needed in this challenging task.

Throughout my scientific career, I have been amazed at the substantial methodological advances that have been made in addressing the enormous complexity of biological materials. I have been gratified to participate in providing some solutions.

Acknowledgments

Preparation of this article was in part supported by grants R01GM 106084 and R21GM118340 from the National Institute of General Medical Sciences, the U.S. Department of Health and Human Services. I greatly appreciate the help of Helena A. Soini in preparation of this manuscript.

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Highlights

- History of the development of capillary separation techniques is described.
- Remarkable changes in microcolumn technology aspects during three decades.
- Capillary LC plays a crucial role in LC/tandem MS.
- The method is a cornerstone of most omics technologies.

Fig. 1.

A: Capillary GC recording of a complex mixture isolated from human urine. Reproduced with permission from Ref. [17]. B: HPLC separation of aromatic test compounds, 1: naphthoquinone; 2: o-nitroaniline; 3: m-nitroaniline; 4: p-nitroaniline. Reproduced with permission from Novotny, M., Bektesh, S.L., Denson, K.B., Grohmann, K., Parr, W., *Anal. Chem.* 45(1973) 971–974. Ref. [18]. Copyright 1973 American Chemical Society. A rough comparison of the state-of-the-art runs by the two methods at approximately same period.

Fig. 2. Separation of the model compounds on the alumina-packed microcapillary LC column. The solutes are benzene, methyl benzoate, and quinoline (in the order of elution). Reprinted with permission from Tsuda, T., Novotny, M., *Anal. Chem.* 50(1978) 271–275. Ref.[12]. Copyright 1978 American Chemical Society.

Fig.3.
Group photo of the Hawaii Symposium participants.

Fig.4. Number of theoretical plates predicted as a function of column diameter for five different analysis times. $P= 3000$ p.s.i.g. (210 bar). 1=0.5 h; 2= 1.0 h; 3=2.0 h; 4= 4.0 h; 5=8h. Reproduced with permission from Ref.[34].

Fig. 5. Two examples of the glass column cross-sections. A: Electron micrograph of the end of a 15- μm i.d. glass column. Reproduced with permission from Ref.[34]; B: cross-section micrograph of a glass capillary with 10- μm particles inside. Courtesy of Dr. Takao Tsuda, Nagoya Institute of Technology, Nagoya, Japan.

Fig 6.

Capillary column technologies explored during the 1980s. Reprinted with permission from Novotny, M., *Anal.Chem.* 60(1988) 500A-510A. Ref.[57]. Copyright 1988 American Chemical Society.

Fig 7.
Reproducibility of five successively prepared capillaries (reduced plate height vs. reduced velocity). K.-E. Karlsson, M. Novotny, unpublished results (1987).

Fig. 8. UHPLC separation of a test mixture under 19 000 psi (1300 bar) inlet pressure using a 66-cm long column packed with very small particles. Reproduced with permission from MacNair, J.E., Lewis, K.C., Jorgenson, J.W., *Anal.Chem.* 69(1997) 983–989. Ref.[50]. Copyright 1997 American Chemical Society.

Fig 9.

Ion-selective microelectrode detection in a liquid drop at the end of a separation capillary.

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Fig. 10.

Capillary LC/LIF detection of fluorescently tagged human plasma steroids. Tentatively identified compounds: 1: 5 α -androstan-3 α ,11 β -diol-17-one; 2: 5 β -androstan-3 α ,11 β -diol-17-one; 3: 5 β -pregnane-3 α ,11 β ,17 α -20 β , 21-tetrol-20-one; 4: 5 β -pregnane-3 α ,17 α , 20 β , 21-tetrol-11-one; 5: 5 β -pregnane-3 α ,11 β ,17 α -20 β , 21-pentol; 6: 5 β -pregnane-3 α ,17 α , 20 α , 21-tetrol-11-one; 7: 5 β -pregnane-3 α ,11 β ,17 α ,20 α -, 21-pentol; 8: 5 α -androstan-3 α -ol-17-one; 9: 5 α -androstene-3 α -ol-17-one; 10: 5 β -pregnane-3 α ,20 α , 21-triol; 11: 5 α -androstan-3 α ,17 β -diol. Reproduced with permission from Ref.[54].

Fig. 11. Selected-ion reconstructed chromatogram of the tryptic digest of lactoglobulin using the electrospray capillary LC-MS. Reprinted with permission from Wiley, Ref.[77]. Copyright 1990 Aster Publishing Company.

Fig. 12.

Contour plots from MaxQuant for yeast peptides separated with different chromatographic gradient times: A=480 min; B=140 min. Peptides are separated uniformly along the retention time and m/z range with both gradients. Adapted with permission: Thakur, S.S., Geiger, T., Chatterjee, B., Bandilla, P., Fröhlich, F., Cox, J., Mann, M., *Mol. Cell. Proteomics* (2011) 10(8): M110.003699. DOI:10.1074/mcp.M110.003699 from Ref.[85]. Copyright 2011 American Chemical Society for Biochemistry and Molecular Biology.

Fig. 13.

Base-peak capillary LC chromatogram of underivatized N-glycans on a long porous graphitized carbon (PGC) capillary column, featuring approximately 100,000 plates. The traces in green and purple feature isomeric structures that could be positively identified through MS/MS. Reprinted with permission from Zou, G., Benktander, J.D., Gizaw, S.T., Gaunitz, S., Novotny, M.V., *Anal. Chem.* DOI: 10.1021/acs.analchem.7b000629(2017.Ref. [87]. Copyright 2017 American Chemical Society.