Separations Combined with Mass Spectrometry

Kenneth B. Tomer*

Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, North Carolina 27709

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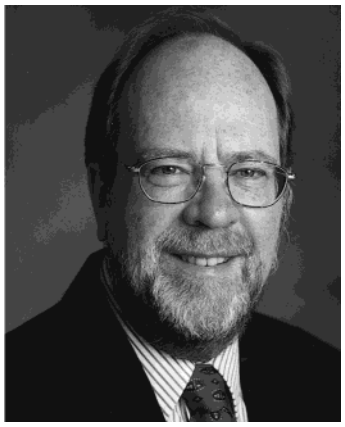

Kenneth B. Tomer received his B. S. degree in Chemistry at Ohio State University. He earned his Ph.D. in 1970 at the University of Colorado in organic mass spectrometry under the direction of Robert H. Shapiro. After postdoctoral appointments at the H. C. Ørsted Institute, University of Copenhagen (with Professor Ole Buchardt) and Stanford University (with Professor Carl Djerassi), he was Assistant Professor at Brooklyn College of the City University of New York followed by Assistant Research Professor in the Pediatrics Department of the University of Pennsylvania Medical School. He then spent four years at the Research Triangle Institute in North Carolina before taking a position in the Midwest Center for Mass Spectrometry (Assistant/Associate Director, Research Associate Professor) with Professor Michael Gross in 1981. He returned to North Carolina in 1986 as Leader of the Mass Spectrometry Workgroup, Laboratory of Structural Biology, at the National Institute of Environmental Health Sciences/National Institutes of Health. His research areas include development and application of nanoscale capillary LC and CE coupled with mass spectrometry; application of MS/MS techniques to biomolecule structure elucidation; development of affinity techniques combined with MS, especially for mapping epitopes on HIV-related proteins and for phosphopeptide determination; development and application of MS-based techniques for probing the tertiary structures of proteins and protein complexes; and functional proteomics.

I. Introduction

The combination of separation techniques with mass spectrometry has had a tremendous impact on mass spectrometry over the years with significant time and effort being expended on improving the mating of the two techniques. Similarly, developments in mass spectrometry have also had a dramatic effect on the direction of research in the separation sciences. Thus, comprehensive reviews of the current state of separations combined with mass spectrometry have considerable timeliness.

Several very fundamental developments in separations and in mass spectrometry occurred around 1990. These have been reviewed elsewhere in depth, but the details are worth summarizing briefly here. The primary focus of this review, however, will be on technical developments in separations and in mass spectrometry, particularly from the last 5 years, especially with reference to novel applications that

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are driving developments in the combined techniques. In many cases, the separations are performed off-line rather than on-line with the mass spectrometer. For the most part, these off-line applications will not be discussed except where there is potential for an on-line combination that has not been achieved in practicality. In the early stages of some areas, technical developments have occurred using flowing liquid streams, but not necessarily with separations. Discussions of these areas will be included. The reader may also note that there are two general themes underlying much of the development in separations combined with MS. These are "smaller (columns) and lower (detection levels)" and "more (samples) and faster (analysis times)." The ability of MS to provide mass information specific to each analyte is important in each theme as it decreases the need for optimal separation of components of a mixture.

II. Mass Spectrometry

A. Ionization Modes

Two significant developments in ionization techniques occurred in the late 1980s and early 1990s that have greatly affected the field of mass spectrometry. These were the popularization of atmospheric pressure chemical ionization (APCI), the closely related electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI). Throughout this review the term ESI will be used for both APCI and ESI unless APCI is specifically being discussed. The similarities and differences between APCI and ESI will not be discussed in more detail as these are being covered in another review in this issue. MALDI will be discussed in a later section.

1. ESI

The use of the term "popularization" with ESI in the above paragraph is intentional, because the ionization technique and commercial instruments based on APCI ionization had been available for quite some time. The true capabilities of this technique, however, were not initially realized. The impact the technique had on mass spectrometry in general and separations/MS can be likened to the effect the meteor impact in the Cretaceous Era had on dinosaurs. The cutting-edge of bioanalytical MS instrumentation was commonly accepted as bigger magnets, bigger instruments. The ability of ESI to form multiply charged ions from large biomolecules allowed these analyses to be performed on relatively low-cost analyzers, such as quadrupoles and, thus, abruptly ended the era of heavy metal in mass spectrometry. (This should not be taken as implying that magnetic sector-based instruments no longer have a use, but the number of applications to which they are most suited has diminished.)

The impact of ESI on separations/MS has also been dramatic. The ESI source involves flowing a liquid stream through a charged needle (or through an electrical discharge). Previously, one of the major problems to overcome in interfacing separations to

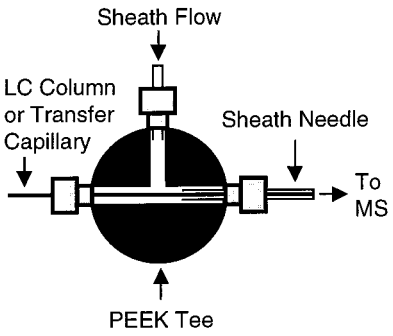

Figure 1. Generic diagram of a sheath flow LC−ESI interface.

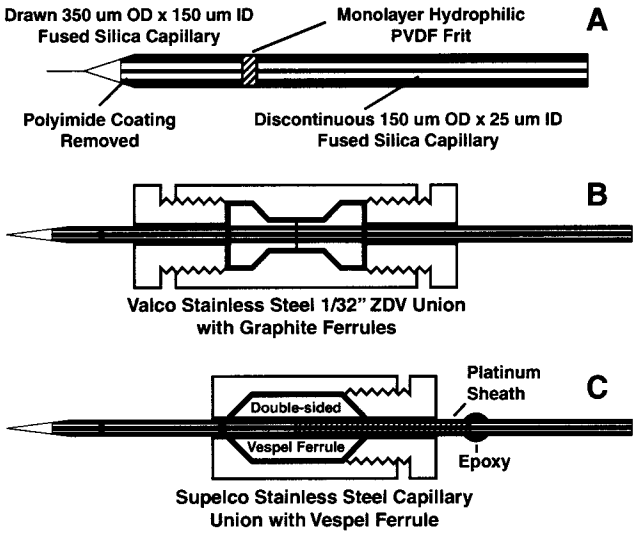

Figure 2. Sheathless microelectrospray interface. The microspray interface is a coaxial arrangement of a drawn 350 μm × 150 μm fused silica capillary outer tip containing an internal 150 μm × 25 μm transfer line with an integrated membrane filter to help prevent plugging of the spray needle. (A) Fused silica microspray tip. (B) Valco union with graphite ferrules, potential applied directly union. (C) Supelco union with Vespel ferrule, potential applied to platinum sheath on the transfer line. (Reprinted with permission from ref 7. Copyright 1995 American Chemical Society.)

MS had been to move a liquid at atmospheric pressure to an ionization source/analyzer at high vacuum. With ESI, this now became routine. HPLC columns with flow rates of ca. 5 μL/min or more can be directly interfaced to an ESI or APCI source, with higher flow rates being accommodated by nebulization and/or heating. For lower flow rate separation systems, capillary LC and capillary electrophoresis, designs for interfaces between the separation systems and ESI sources have fallen into two major categories: the sheath-flow interface[1−6] (Figure 1) and the sheathless interface[7] (Figure 2). The sheath-flow interface can either employ a transfer line from the end of the separation capillary to the source or have the separation capillary end at the source.

2. Particle Beam Interface

One of the major advantages of soft ionization techniques such as ESI and APCI is that relatively abundant molecular ion species are formed. This feature can at times, however, also be a disadvantage.

Both the structural information that is available from EI spectra, and the ability to compare spectra with standard library spectra for identification of unknowns are unavailable. In 1984, Willoughby and Browner introduced the particle beam (PB) interface.[8] In this interface, the LC effluent was first nebulized, generally by a coaxial He flow, desolvated to yield a high velocity particle beam, plus volatile liquid vapors, and He, which was then passed through a momentum (jet) separator. The solute particles impact a heated source, flash evaporate, and ionize. Library searchable EI type spectra result. PB interfaces have generally lost favor since the popularization of ESI type interfaces due to nonlinear analyte response, compound-specific optimization conditions, relatively poor detection limits (especially at high water concentrations in the mobile phase), and loss of chromatographic resolution during the flash evaporation process.[9−13] Because of the attraction of obtaining library-searchable EI spectra and the ease of use of normal-phase LC solvents, some efforts have been made to improve the PB interface, primarily by Cappiello and co-workers who have developed a capillary scale PB interface,[14−17] and through development of a radio frequency inductively heated eluent jet interface.[18,19] Although improvements in performance have been made, the authors note that the capillary PB interface needs more commercial development to be competitive.[13]

B. Mass Analyzers

Mass analyzers are covered in detail in another review in this issue. Certain aspects of recent analyzer designs, however, are critical to separations combined with MS, and those developments in analyzer design that relate to separations will be discussed.

1. Duty Cycle

One continuing problem with interfacing separation techniques with MS that was recognized initially in GC−MS was that, as separation efficiency increased, the number of MS scans across a chromatographic peak decreased. That is, the duty cycle of the instrument was long relative to the chromatographic peak width in time. Even with scan rates on the order of 500 or 1000 Da/s, only one or two scans could be acquired during the elution of a sharp GC, CE, or capillary LC peak into the ion source. Because the typical analyzer, e.g., quadrupole or sector, spent relatively little time on individual ions within a scan, significant amounts of data could be lost, leading to reduced sensitivity and reduced information content. The combination of reduced information content and reduced sensitivity could be very unfortunate when an analyte produced a molecular ion of low relative abundance that was at the threshold of the instruments detection limit or was not eluting when the MS scanned across that mass (Figure 3).

2. Time-Of-Flight-MS

The obvious potential of time-of-flight analyzers to address this scan speed/peak elution time problem

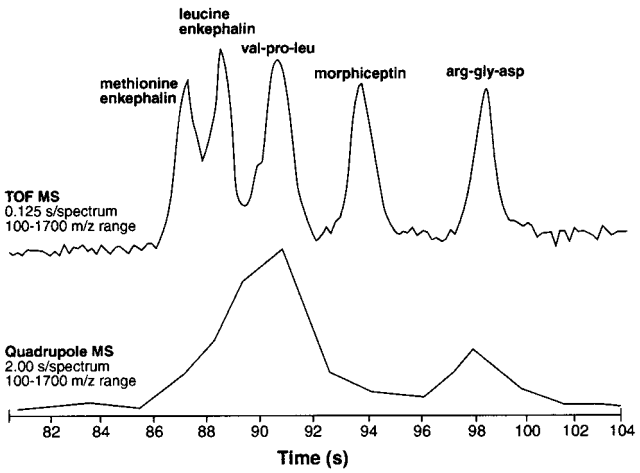

Figure 3. Comparison of the TICs from a peptide separation using the TOF with a 0.125 s/spectrum acquisition rate and the same separation obtained from a quadrupole MS scanned at a 2.00s/spectrum acquisition rate. Note that morphiceptin was not observed in the quadrupole TIC. (Reprinted with permission from ref 26. Copyright 1995 American Chemical Society.)

was quickly recognized. The time-of-flight analyzer was the basis of one of the early commercially available instruments (the Bendix time-of-flight). This instrument, however, did not possess long-term viability, primarily due to relatively poor mass resolution (ca. 250)[20] compared to other available analyzers. The popularity and success of time-of-flight analyzers in MALDI/MS fostered rapid improvements in the technology, however. The mass resolution of the early commercial MALDI instruments had been improved to 400−500 for ions under 10 000 Da.[21] MALDI data obtained at this resolution proved to be quite useful in peptide and protein analysis. A combination of the renaissance in TOF instrumentation brought about by the commercial development and application of MALDI/TOF, and the increasing scan speed and duty cycle problems caused by increased separation efficiencies led a number of researchers to examine the applicability of TOF analyzers for separations combined with MS.

In 1991, Lee and co-workers reported on the development of a time-of-flight analyzer with an API source.[22] Ions were formed externally to the analyzer and passed through a nozzle into the vacuum which caused a supersonic expansion of the ion beam. The ion beam was then repelled orthogonally into the analyzer. The supersonic expansion narrowed the velocity distribution of the ion beam prior to entry into the analyzer, leading to improved mass resolution (∼500).

In the following year, Boyle and Whitehouse published the coupling of capillary electrophoresis with ESI/TOF.[23] This group incorporated ion storage similar to that of Lee[22] and of Dodonov and co-workers,[24,25] as well as reflectron technology, into the basic orthogonal ion insertion design. A reflectron is an ion mirror that provides improved focusing of the ion packet, as well as reversing the direction of the ion beam. Ion storage in this device was provided by a decelerating field. Mass resolution of 1190 could be obtained at *m*/*z* 174 and 260 for the cytochrome *c*

$(M + 13H)^{+13}$ ion of *m/z* 951. The authors also noted that the addition of ion storage capabilities improved the duty cycle of the mass spectrometer to $(2.3-2.6) \times 10^{-2}$ with a pulse rate of 2−4 kHz. The Analytica of Branford group in 1996 published a version of this instrument for coupling to separations (capillary electrophoresis) in which improved electronics eliminated the requirement for the reflectron.[26] Spectral integration times of 0.125 s per spectrum (each spectrum a sum of 1024 scans) and a mass resolution of 1000 (fwhm) at *m/z* 556 were reported.

Several other approaches to ion storage devices in conjunction with TOF analyzers have been reported, including a quadrupole ion trap.[27,28] In these studies, 4 spectra/s were acquired. High sensitivity was achieved because the ions formed by electrospray from the CE eluent could be stored during acquisition. Although theoretically with sufficiently complex separations, storage may lead to remixing of separated analytes, this has not yet been found to be a problem.

In 1996, Morris et al., described a novel quadrupole/orthogonal time-of-flight tandem mass spectrometer.[29] In this design, for operation in the MS mode, electrospray produced ions are shaped by the quadrupole, operating in the rf-only wide band-pass mode, and hexapoles so that spatially and energetically well-defined ions can be pulsed orthogonally into a reflectron time-of-flight analyzer. The pulse rate was either 16 kHz for a 1500 Da mass range or 8 kHz for 6000 Da mass range. High mass resolution, over 5000, and high sensitivity, MS−MS spectra from 500 attomol, were demonstrated for this instrument. The LC−MS−MS analysis of a neuroendocrine peptide mixture was used to demonstrate the instruments capabilities. In 1997, Lazar et al., also published a design of an orthogonal TOF/MS which used rf-only quadrupoles to steer and shape the ions, similar to that reported by Morris.[30] The quadrupoles are operated at relatively high pressures ($10^{-1}-10^{-3}$ Torr), and the combination of collisional damping and the rf field confined the ions close to the quadrupoles' axis which resulted in reduced velocity and reduced velocity distributions. Spectra resulting from summation of 100 scans at a 10 kHz pulse rate (0.01 s acquisition time) were shown, while spectra from capillary electrophoretic separations were acquired at a rate of 5 kHz with 1000 summed scans/spectrum. Lee noted that data acquisition and file size were the weak links at this point. The Lazar instrument was not designed for MS−MS capability. Both of these instruments became the basis for commercial instruments, the MicroMass Q-Tof,[31] and the Sensar[32] (now marketed as the Jaguar), respectively.

The rapid acceptance of the Micromass hybrid Q-Tof mass spectrometer for ESI analyses rapidly led to the appearance of dedicated ESI/TOF instruments. Micromass,[31] Leco,[32] Analytica of Branford,[33] Brucker,[34] and PE Biosystems[35] have all introduced LC−ESI/TOF mass spectrometers with some variation in ion optics.

3. Ion Traps and FT-MS

The ion trap mass analyzer has been extensively exploited for separations combined with MS. The development of this analyzer, however, has not been as directly tied to its capabilities *vis a vis* separations and, therefore, this topic will be left to other reviews in this issue. Fourier Transform Ion Cyclotron Resonance (FT-ICR) analyzers have also not been developed primarily for use with separations, although capillary HPLC[36,37] and CE[38−46] have been interfaced with ESI−FT−ICR MS.

III. Separations

Among the forces driving developments in the separation sciences have been increased resolving power, miniaturization, decreased sample consumption, improved detection limits, and increased speed of analysis. There is, of course, significant overlap among these factors. The 1980s and early 1990s witnessed the development of new technologies, capillary HPLC, capillary electrophoresis and capillary electrochromatography, that addressed these forces in different ways.

A. HPLC

1. Miniaturization

The trend toward miniaturization has had significant impact in the field of HPLC. Two underlying goals were and are still the primary impetuses behind column miniaturization: improved separation efficiencies and the desire to interface HPLC with MS. Giddings calculated that the theoretical limits to separation efficiencies, N_{lim}, in both gas and liquid chromatography were proportional to $1/D_{mp}\eta$ where D_{mp} is the diffusion coefficient of the analyte in the mobile phase and the mobile phase viscosity is η. This led to the observation that the ratio of limiting theoretical plates in LC to that in GC was 1000. This advantage of LC over GC was not observed with conventional i.d. columns (4.6−1.0 mm). Jorgenson and Guthrie showed that the maximum separation efficiency for open tubular columns was 30 million for a column of about 3 μm i.d.[47] For a number of practical reasons, such as loading capacity, open tubular capillary LC columns have not been widely used.

Packed capillary columns with 100−300 μm i.d.s were reported by Takeuchi and Ishii[48,49] in the early 1980s. Knox and Parcher[50] calculated that for packed capillaries and small bore packed columns the reduced plate height curve should be similar to wide-bore columns, but that packed capillaries could not be operated in a practical manner in which the column would develop over 10 000 plates. As the i.d. of the columns decreased, however, it was observed that efficiencies increased, which Karlsson and Novotny attributed to the "wall-effect" in which the entire packing bed is affected by the column wall.[51] Thus, Karlsson and Novotny concluded that improved resolution and separation efficiency should be obtained with small i.d. columns. The decrease in column i.d. from 4.6 mm to 320 μm (packed capillary columns) reduces the flow rate from 1 mL/min to 4.9 μL/min. This flow rate is within the 1−10 μL/min flow

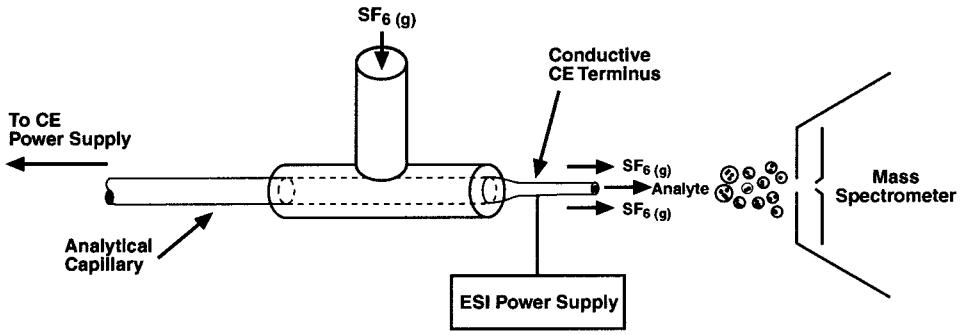

Figure 4. Sheathless CE−ESI interface. (Reprinted with permission from ref 69. Copyright 1994 Elsevier Science B. V.)

rates that early LC−MS interfaces were compatible with. Further reductions in column i.d. to 100 μm or less (nanoscale) reduced the optimal flow rates to ca. 120 nL/min, while open tubular LC columns with 3−10 μm i.d. have optimal flow rates of ca. 1.2 nL/min. Concomitant with the reduced column size is reduced relative loading capacity (from 8469 for 4.6 mm columns to 1 for nanoscale columns). On the other hand, the relative concentration at the detector is inversely related to the amount injected and, for equal amounts injected, increases from 1 for a 4.6 mm column to 8459 for a nanoscale column.[52] Thus, the reduced loading capacity is compensated for by the increased relative concentration. This is an especially important consideration for ESI-MS, which often behaves as a concentration dependent detector.[53−55]

(a) ESI Source Designs for Use with Capillary/Nanoscale LC. Although most of the initial reports of capillary LC−MS used continuous flow fast-atom bombardment sources, there were several early reports in 1990−91 of use with ESI, primarily with the sheath-flow interface.[2−6] With the advent of microspray/ESI, direct coupling of the separation capillary without use of a sheath flow became possible.[7] Although capillary and nanoscale LC combined with ESI−MS can now be considered standard operating systems, there have continued to be improvements or alternative concepts reported in interface designs[7,56−60] and in optimization of the capillary LC systems.[61,62] Several reviews on the subject have appeared.[52,55,63,64] Early commercial sources were primarily based on the sheath-flow interface. As a consequence of the trend to further miniaturization, liquid flows have become more compatible with MS. The lower flow rates have, in turn, spurred development of MS sources/interfaces that can take advantage of the lower flow rates. In 1994, several groups reported ESI sources designed to work with nanoliter/minutes flow rates (Figure 4).[65−69] These sources were based on the use of a tapered glass needle as the liquid introduction device. The high voltage necessary to initiate and maintain electrospray was provided by a connection external to the source,[66] by a wire through the capillary,[69] or by a metallic-coated tip.[68] The Caprioli et al. source[66] was designed to operate with a bed of column packing, while the Smith et al. source[69] was designed for use with capillary electrophoresis. The absence of a makeup flow and closer positioning of the spray needle to the ESI sampling

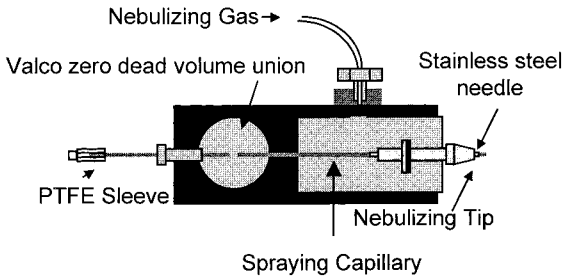

Figure 5. Nanoflow LC−ESI interface. (Reprinted with permission from ref 73. Copyright 1994 Elsevier Science B. V.)

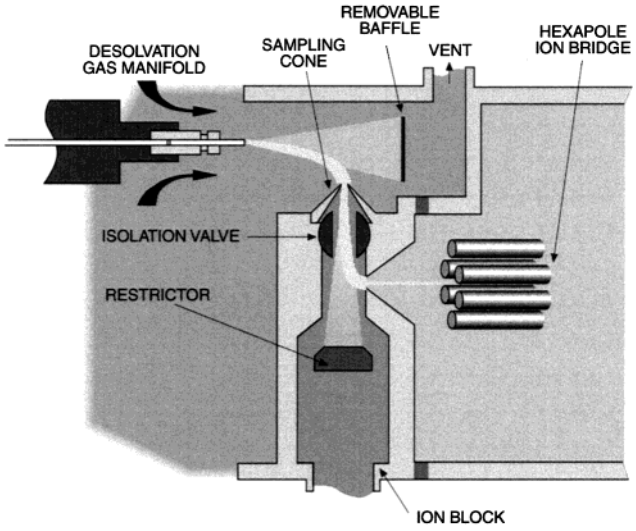

Figure 6. Z-spray ESI source. (Reprinted with permission from Micromass U.K., Ltd.)

orifice improved sensitivity such that attomole sensitivities were reported.[67] Commercial variations of these sources are now routinely available.[31,70−72] Currently, most manufacturers offer nanoelectrospray sources based on electrospray-induced low nanoliter/min flows from glass capillaries and/or sources based on pressurized flows at the several hundred nanoliter/min flow range and above (nanoflow electrospray, Figure 5[73]). The latter sources are routinely coupled with capillary and nanoscale HPLC, and, less routinely, with capillary electrophoresis. Orthogonal spray designs have also become increasingly popular. In orthogonal spray sources, the ions formed in the spray are electrically pulled into the sampling cone at right angles to the direction of the spray (Figure 6). Nonionized background species are

not introduced into the mass spectrometer where they potentially could hit the detector, thereby reducing the background level.

Although tandem mass spectrometry is not a theme of this review, separations on-line with tandem MS analyses play a critical role in the determination of unknowns. As the amount of substance available for analysis decreases (an acute problem in many bioanalytical analyses), and as the number of analytes that can overlap in a chromatographic peak increase, e.g., from a complex proteolytic digest, the more difficult it is to get meaningful MS−MS spectra from all the coeluting analytes, even on time-of-flight based analyzers. CE has faced a similar problem in that peak widths may be too narrow to obtain a good MS−MS spectrum from the analyte, especially on a quadrupole based instrument. Smith and co-workers at Pacific Northwest Laboratories have addressed this problem in CE by reducing the voltage, and thus, the electroosmotic flow when an analyte elutes from the column (based on in-line UV detection).[74] Lee[75] proposed a means of slowing elution in capillary LC by releasing the pressure at the head of the column when the analyte starts to elute. In this approach, there is a lag between pressure release and reduction in the elution rate of the analyte because the pressure must be reduced across the length of the column. Moseley and collaborators have devised a peak trapping approach for use with capillary LC−ESI−MS−MS on a hybrid Q-Tof mass spectrometer that releases the pressure at the end of the column by switching flow to a lower flow rate pump, thereby transporting the "trapped" peak to the ESI source at a lower flow rate. The flow through the column is switched to a plugged outlet in the valve and is thus stopped. The pressure on the head of the column does not increase because there is a ~1000:1 split in the solvent flow from the pumps to the column. In the analysis of a protein isolated from a weak SDS−PAGE band and then digested in-gel, MS−MS spectra for 159 peaks were obtained that would have been missed under normal operating conditions.[76]

b. Applications. With 12 chapters in this issue to be devoted to MS applications, it would be an unnecessary duplication to try to tabulate or describe the numerous applications of capillary/nanoscale HPLC−ESI−MS. It is possibly useful, however, to point out that the development of capillary and nanoscale HPLC has significantly impacted the practical levels of substances, especially analytes from biological tissues and fluids, that can be analyzed due to improved separation efficiency, relative loading capacity, improved source efficiency at low flow rates, and ability to pass the entire effluent from the column into the mass spectrometer. These features have certainly been major factors in making MS a critical tool in the study of biological systems. The identification of major histocompatibility class II peptides by Hunt's group[77] based on the nanoscale capillary HPLC−ESI−MS−MS methodology[3,78] could be considered the initial breakthrough application in this area.[79] Another area that has been greatly impacted by the development of capillary/nanoscale HPLC−ESI−MS is protein identification and identification of posttranslational modifications, especially phosphorylation, in relation to proteomics.

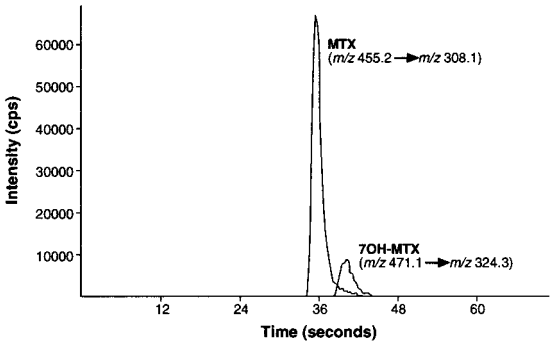

Figure 7. Fast LC−ESI−MS−MS reconstructed selected reaction chromatogram show separation of methotrexate (MTX) and 7-hydroxymethotrexte (7OH-MTX) from plasma in under 60 s. (Reprinted with permission from ref 82. Copyright 1999 American Chemical Society.)

2. High-Speed LC Separations

As indicated above, two major themes in separations combined with MS have been miniaturization and improved separation efficiency. Another major theme has been faster separations, especially in pharmaceutical metabolic studies and in toxicological quantitative studies. The driving force in these studies is to accurately and precisely analyze as many samples as possible in as short a time as possible. Instead of improving separation efficiency, the goal here is to validate minimal separation efficiencies that can still yield accurate results. Kennedy et al., have recently reviewed fast LC from the chromatographic perspective.[80]

Henion and co-workers have emphasized automated workup procedures combined with short minibore (2 mm i.d.) or microbore (1 mm i.d.) columns with flow rates of 150 μL/min to 2.0 mL/min as an answer to more rapid sample throughput.[81−86] All analyses used selected reaction monitoring to provide specificity in analyte detection and TurboIonSpray[87] (a heated source/interface tolerant of high flows) to handle the high flow rates. Using 96 well extraction techniques (liquid−liquid or solid phase) and HPLC flow rates of 150 μL/min, 1.75 min cycle times (including column reequilibration) for the analysis of methotrexates were possible (Figure 7).[82] Precisions and accuracies of better than ±15% were reported. Increasing the flow rate to 1.0 mL/min and coupling four autosamplers to one instrument, 30 s run times for the analysis of bezodiazepines and 1152 samples in 12 h were reported.[83] With higher flow rates, up to 2 mL/min, run-times of 15−30 s were achieved with good precision (8.9%) and accuracy (97.7%) for samples from blood and urine.[86] Similar experimental conditions, high mobile phase flow rates, were employed by Knebel et al. to analyze the β_2 agonist reproterol in 20−30 s,[88] by Wolf et al. for biotin analyses in 4 min,[89] by Neubecker et al. for norepinephrin analyses,[90] by Jemal et al. for a carboxylic acid drug in plasma in under 2 min run time,[91] by Volmer et al. for the analysis of corticosteroids using gradient flow rates from 0.2−1.0 mL/min,[92] by Ding and Neue for method development[93] and by Watt et

al. for drug analysis in serum.[94] Matuszewski et al., however, noted that, in the absence of sample cleanup, analyte suppression due to "unseen" interferences can be observed when very fast LC separations are used.[95]

In 1997, Ayrton et al., at Glaxo-Wellcome Research and Development demonstrated the application of turbulent flow chromatography with MS detection as an alternative method for high throughput analyses.[96] Turbulent flow (vs laminar flow) chromatography occurs at high linear flow rates (0.5 m/s) where the solvent front profile becomes plug-like rather than parabolic.[97] Using standard packing particle sizes, the pressures required to obtain this linear velocity are considered prohibitive. Recently large diameter particles, 50−150 μm, became available which can be eluted with a mobile phase flow rate of 35 mL/min and a linear velocity of 7.6 cm/s. By switching the column effluent to waste for the first minute, the column also acted as a cleanup device. Thus, plasma or serum samples could be directly injected onto the column, followed by a 1 min elution of low retentate to waste, and analyte eluted by a rapid gradient (over 0.6 min) at a flow rate of 4 mL/min on a 1 mm i.d. column. The eluent was split such that 400 μL/min were directed to the MS. Standard errors in precision and accuracy were found to be typically less than 10%. In a more recent paper, Ayerton et al. observed that the chromatographic conditions used in their "turbulent flow chromatography" were not severe enough to initiate turbulent flow, and they now call the technique "ultrahigh flow rate" liquid chromatography.[98] Zimmer et al.,[99] Brignol et al.,[100] Wu et al.,[101] and Jemal et al.[102] have also applied this technique to the analysis of drugs in plasma with results equally precise and accurate as those reported by Ayerton et al. Jemal et al., also have used a combination of the large particle column and a standard analytical column,[103] as well as using the large particle column under relatively low flow rates (500−800 μL/min)[104] for the analysis of plasma samples with no prior sample workup.

Another approach to increasing the throughput in LC−MS has been to use multiple LC columns combined with ESI−MS. In 1998, Zeng and Kassel reported the design of an automated HPLC−ESI−MS system in which two columns with two sprayers were operated in parallel on one instrument.[105] Flows from both LC columns passed into the MS simultaneously. This system was designed for combined analysis and sample purification of entries from a combinatorial library. In 1999, Korfmacher et al. demonstrated that a similar design, in which the effluents from parallel LC columns were combined and presented to the ESI−MS,[106] was applicable to the to pharmacokinetic studies.

A similar approach to interfacing multiple LC columns to MS was first reported by de Biasi et al. in 1999.[107] In this design, the effluents from four parallel LC columns were presented to the MS via four independent sprayers. A rotating aperture was used to admit the effluent from each column in rapid succession. All four effluents could be sampled in less than 1 s. Thus, four analyses could be obtained in the time it normally took to run a single analysis. A commercial version with this design is currently available from Micromass and can be fitted to a quadrupole or a TOF based analyzer.[31] Also in 1999, Kassel and co-workers reported the design of an eight column system which incorporated a rotating aperture.[108]

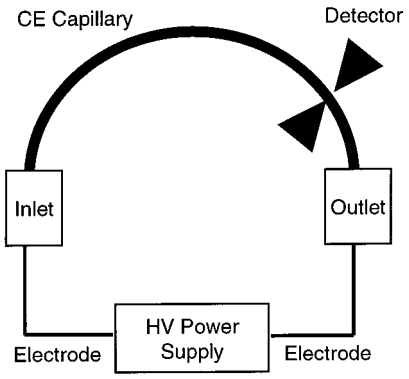

Figure 8. Basic CE instrumentation.

B. Capillary Electrophoresis

Capillary electrophoresis was first reported by Jorgenson and Lukas in 1981.[109] Briefly, capillary electrophoresis (CE) or capillary zone electrophoresis (CZE) is the separation of charged analytes dissolved in a buffer by differential migration in an electric field placed across the ends of a capillary column. Typical CE instrumentation is shown in Figure 8. Electric fields of 300−400 V/cm with 75 μm i.d. and 50−100 cm long capillaries are commonly used. Bulk flow of the buffer also occurs, leading to flow rates in the low nanoliters per minute range. The flow profile is flat and piston-like rather than parabolic shaped as found in laminar flow, pressure-driven regimes. The flat profile leads to very high separation efficiencies with over 1 000 000 plates reported.[110] Because the separation mechanism in CE is different than that of LC, CE, and LC should not be thought of as competitive separation techniques but should be thought of as complementary.

1. Interfaces

Several groups began to explore interfacing CE with MS in the late 1980s.[111−115] In general, the techniques used to interface CE and ESI have fallen into one of three approaches: direct coupling, sheath flow interfaces, and liquid−junction interfaces.

The direct coupling type of interface is conceptually the simplest, although, possibly, the most difficult experimentally. In these interfaces, the CE "ground" and/or the electrospray initiating voltage can be applied via coating the tip of the CE capillary with a conductive metal,[116−120] inserting an electrode into the end of the CE capillary,[116,121,122] or using the CE voltage to drive spray formation.[123] In some cases, the CE column is directly connected to a separate spraying tip.[124−126] A related design applies the spray voltage via a stainless steel liner made from syringe needle stock through which the separation capillary is placed, with electrical connection being maintained by a natural liquid film that builds up at the outer surface of the tip of the separation capillary.[127] These

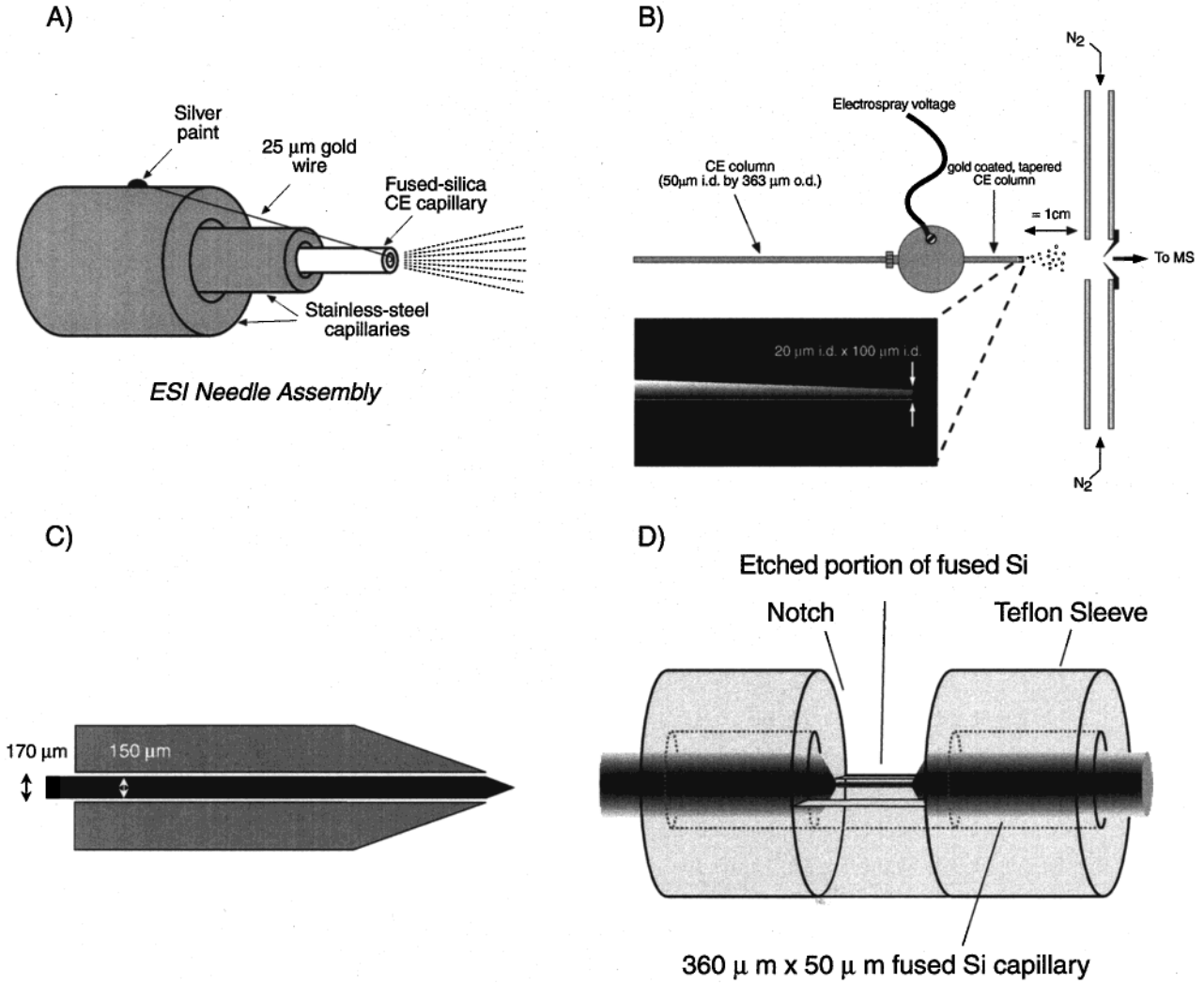

Figure 9. Interface designs for the direct coupling of CE to ESI−MS. (A) wire in-capillary design (Reprinted with permission from ref 122. Copyright 1994 American Chemical Society); (B) tapered metallized capillary tip design in which the CE capillary end is tapered and the sputter-coated with gold (Reprinted with permission from ref 118. Copyright 1997 American Chemical Society); (C) liner interface in which the outlet of the CE capillary was led through a stainless steel liner the tip of which had been ground to a sharp end and polished. The end of the fused silica column was also ground to a sharp point. The gap between the fused silica column and the liner was approximately 10 μm. (Reprinted with permission from ref 127. Copyright 1999 Elsevier B. V.) (D) porous glass joint design. The center of the fused silica is etched so that the capillary walls are <20 μm thick and is center in the notch of a Teflon sleeve. This joint is immersed in a reservoir of 1% acetic acid. The CE termination/micro ESI voltage is applied to the acid solution. (Reprinted with permission from ref 135. Copyright 1998 John Wiley & Sons.)

interfaces have become more popular since the development of nanoESI sources. (See Figure 9.)

The sheath flow interface[1] has been the most widely used interface design. Because CE flow rates are in the low nanoliter per minute range and standard ESI sources operate optimally with flow rates around 5 μL/min, additional liquid flow needs to be added to the CE flow. In sheath flow designs, the makeup liquid is introduced coaxially via a capillary concentric with the separation capillary. An advantage of this design is that the makeup fluid can be optimized independently of the separation buffer. It is also relatively simple to implement. Nebulizing gas can be introduced through a third concentric capillary. Newer designs using significantly lower sheath flows have also been reported.[128]

The use of a sheath flow can, however, affect the separation. Foret et al., observed that counterions in the liquid sheath could migrate into the separation capillary, forming a moving ionic boundary inside the capillary counter to the flow of analyte.[129] Experimentally, this effect could be observed as delays in migration time, changes in migration order, and loss of resolution compared to offline CE. These disturbances tended to be most noticeable for columns with low electroosmotic flows. The authors noted that use of a common counterion in the sheath and background electrolytes, use of sheath counterion with electrophoretic mobility and pK_a similar to the background electrolyte, or use of a pressure difference between the capillary ends is sufficient to induce a hydrodynamic flow greater than the rate of propagation of the ionic boundary in the capillary, thus minimizing such effects. Tang and co-workers also noted this effect in a study comparing CE−MS with capillary isoelectric focusing MS.[130]

In the liquid junction interface for CE−ESI−MS,[131] the separation capillary terminates in a reservoir

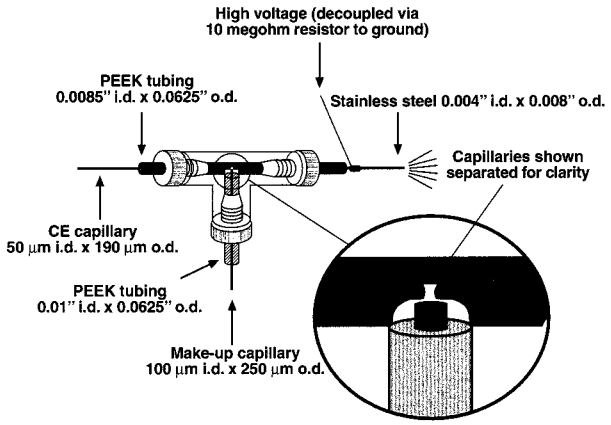

Figure 10. Self-aligning liquid junction interface. See description in the text. (Reprinted with permission from ref 134. Copyright 1996 Elsevier B. V.)

filled with makeup fluid. A transfer capillary is positioned opposite the end of the separation capillary with a very narrow gap between the two. The effluent from the separation column is carried in to the transfer column by the flow of the makeup fluid. Electrical contact is made through the liquid reservoir. There have been a number of disadvantages noted with this type of interface, e.g., reproducibility of positioning the transfer capillary, and potential loss of separation efficiency in passage through the transfer capillary.[132,133] A recent version developed by Henion's group incorporates a self-aligning junction (Figure 10).[134] In this interface, the separation capillary and spray needle (either stainless steel or fused silica) are held together in a piece of PEEK tubing in which a window has been cut. The makeup flow is regulated by a syringe pump. Results using this design with either a stainless steel sprayer or a fused silica sprayer were compared with results from a sheath flow interface. Using the fused silica sprayer, baseline peak widths of, on average, 9.6 s were obtained in comparison to 9.0 s for the sheath flow. The stainless steel sprayer gave significantly broader peaks (peak resolution decreased by up to a factor of 10). Sensitivities with the fused silica sprayer are also about the same as for the sheath flow interface and about two to three times better than with a stainless steel sprayer.

There have been several reports of interface designs incorporating aspects of sheathless interfaces and of liquid junction interfaces which utilize a liquid junction for electrical connection, but do not incorporate a makeup fluid. Smith's laboratory connected the CE separation capillary to a short spray capillary using polysulfone microdialysis tubing.[133] This column was then placed inside a 250 μL Eppendorf pipet tip which contained a background electrolyte. High voltage was applied to the background electrolyte with electrical contact to the end of the separation column being made through the microdialysis tubing. Advantages claimed for this interface include avoidance of addition of makeup fluid and avoidance of the need to coat the capillary tip with a conducting metal. Hunt's group has similarly used a porous glass joint to provide electrical connectivity.[135] Combined with membrane preconcentration transient isotachophoresis CE, they observed attomole detection limits for tumor peptides.

2. CE-MS Sensitivity

Routine sensitivities in the femtomole to attomole range (amount injected) have been reported for CE−ESI−MS analysis of a number of analytes, especially peptides.[136] The volume of analyte solution that is typically injected into a CE column, however, is on the order of low nanoliters. The concentration limits-of-detection (cLODs), therefore, are in the micromolar range. This relatively high concentration level is not nearly as attractive. The high cLODs have certainly presented a significant barrier to the routine application of CE−MS. This has generated considerable interest and effort in improving the cLODs. Although some ways to increase sensitivity are to increase the sensitivity of the MS detector or to increase the efficiency of analyte transfer to the gas phase and ionization, increases in instrument sensitivity will probably not readily lead in the near-term to the orders-of-magnitude increase in cLOD that are desirable. Most effort within the field has, therefore, concentrated (so to speak) on increasing the amount of sample that can be loaded onto the column without significant detriment to the separation efficiency.

a. Isotachophoresis. Isotachophoretic (ITP) focusing has been one electrophoretic technique that has been used to increase the concentration of an analyte. In ITP, the sample is injected onto the column between a leading and a tailing electrolyte. The leading electrolyte has a high mobility and the tailing electrolyte is of low mobility. In the presence of the high voltage field, the analytes separate into individual bands based on their mobility. The analyte in each band becomes concentrated so that the concentration in each band is constant and is determined by the concentration of the leading electrolyte. Thus, very dilute samples can be concentrated. Karger and co-workers introduced transient ITP (tITP) for analyte concentrating in CE−MS.[137] In the tITP experiment, the column was first filled with a background electrolyte, and then a large volume (e.g., 750 nL) of analyte solution in ammonium acetate buffer was loaded onto the column. The end of the column was placed in the background electrolyte reservoir and voltage applied. The ammonium ions have high relative mobility and move ahead of the sample ions, and the sample ions then stack in narrow bands behind the ammonium ion band. As the ammonium ions move through the slower background electrolyte, the ammonium ion concentration rapidly decreases to the point that ITP migration no longer occurs, and standard free zone electrophoresis commences. One 100-fold increases in the concentrations of analytes was observed using this approach. Similar improvements in cLODs using tITP have been reported by Locke and Thibault and co-workers for shellfish toxins,[138] by van der Greef and co-workers for recombinant human IL-6 fragments,[139] and by Andren and co-workers for the measurement of endogenous neurotransmitters and neuropeptides.[140] Isoelectric focusing followed by mobilization and ESI−MS analysis has also been successfully

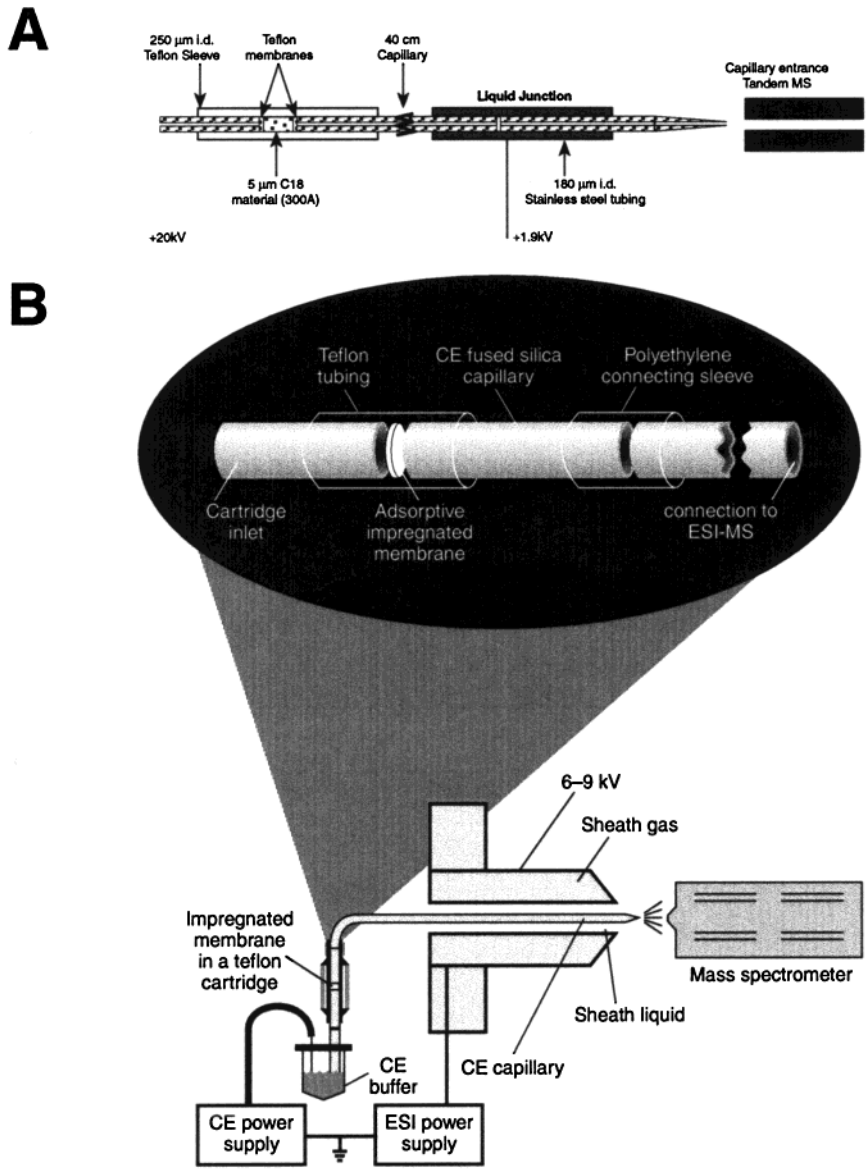

Figure 11. Schematic of (A) cPC−CE (Reprinted with permission from ref 153. Copyright 1996 Nature America, Inc.); (B) mPC−CE using a coaxial liquid sheath configuration (Reprinted with permission from ref 150. Copyright 1999 American Chemical Society.)

employed by Tang et al. to improve cLODs for proteins by 2 orders of magnitude.[141]

b. Preconcentration. Naylor and Tomlinson and co-workers at the Mayo clinic have developed two approaches to preconcentration of the sample on-line with CE−MS, chromatographic preconcentration (cPC),[142,143] and membrane preconcentration (mPC).[144−150] In cPC, a short C_{18} packed precolumn is used on-line with the CE column in an approach similar to those of Swartz and Merion[151] and of Morita and Sawada.[152] Analytes are adsorbed onto the packing, then eluted into the separation column. Figeys et al. have also demonstrated the application of cPC for analyte concentration in CE−MS analysis (Figure 11A).[153,154]

In mPC, a membrane impregnated with an adsorptive material, such as C18 or styrene-divinyl benzene, is placed between a transfer capillary and the separation column (Figure 11B). The analyte was washed off the mPC into the CE column with a small volume of methanol or methanol:water:TFA, followed by CE buffer. Optimum separation efficiency was observed using transient ITP to focus the analytes.

The Mayo group recently reported a study on the optimization of conditions for protein analysis using mPC−CE and Polybrene coated capillaries.[155] This study did not use MS detection, but buffer systems, etc., were chosen for their MS compatibility. Optimum conditions were found to incorporate a C_2 impregnated PTFE membrane, a background electrolyte of 5% acetic acid and 2 mM ammonium acetate. The optimal elution solvent was determined to be 80% acetonitrile:water and the optimal elution volume was 60 nL. Recoveries were found to be relatively poor with protein standards, ca. 25%, but much better, ≥90%, with physiological samples, probably due to sample matrix affects reducing irreversible absorption of the protein onto the membrane. Focusing of the desorbed analytes was achieved by tITP using the background electrolyte as the acidic leading stacking buffer and 0.5% ammonium hydroxide in water as the trailing stacking buffer. Higher

concentrations of ammonium hydroxide gave improved peak shape, but resolution of the proteins was lost. These optimized conditions have been used to determine proteins in human aqueous humor from patients with different pathologies using a 1 μL injection onto the mPC cartridge.[156]

Lower molecular weight analytes have been found to elute from a styrene divinylbenzene mPC cartridge with ca. 100 nL of 80% methanol with refocusing of analytes by tITP. These conditions were used to separate major histocompatibility complex class I peptides with detection and identification by ESI−MS and MS−MS.[157,158] Recently, Hunt and co-workers applied mPC−CE to the analysis of major histocompatibility complex class I peptides isolated from a melanoma cell line.[135]

Thibault and co-workers have recently compared preconcentration using C_{18} cPC and styrenedivinylbenzene mPC for the analysis of lipopolysaccharides by CE-negative ion ESI−MS analysis.[159] They noted that the overall electropherograms obtained using the two preconcentration techniques were qualitatively similar, but that the mPC showed an overall improvement in sensitivity by up to a factor of 5 in concentration.

It was pointed out in a review in 1998, that few applications of mPC/cPC had been reported, other than by the developers.[136] This was also true to a great extent for other preconcentration techniques, such as tITP. This situation is slowly changing, in that a handful of other laboratories have reported applications. Considering, however, that one of the major hindrances to widespread application of CE−MS has been poor cLODs, this situation is still surprising. It may be that, given the state-of-the-art in capillary HPLC, the better absolute sensitivities associated with the higher resolution of CE peaks compared to HPLC do not compensate for the better cLODs associated with HPLC even compared to PC−CE−MS. Given the ability of MS to more readily distinguish overlapping components under less than ideal separation conditions than other detection methods, acceptance of some reduced separation efficiency compared to more involved experimental procedures may be a rational choice in some situations.

3. Micellar Electrokinetic Chromatography and Sieving CE Combined with MS

Micellar electrokinetic chromatography (MEKC) [often also referred to as micellar electrokinetic capillary chromatography (MECC)] is often used to separate uncharged particles. In MEKC, surfactants are added to the buffer at concentrations above their critical micelle concentration so that the analyte can be solvated by the micelles. Separation of uncharged analytes is achieved due to their differential partitioning between the micelles and buffer.[160] Although MEKC is a popular technique in CE, MEKC has rarely been used in combination with MS. This has primarily been due to the potential for source contamination by the surfactants and due to suppression effects on the analyte signal due to competition between the charged surfactant and the analyte for the available charges.[161] There have been several approaches attempted for combining MEKC with MS:[162] the use of high molecular weight surfactants whose electrosprayed ions are above the mass range scanned by the MS;[163] the use of surfactants that migrate toward the anode with the micellar velocity being controlled by adjustment of the electroosmotic flow;[164] partial filling in which the surfactant was introduced only into part of the capillary with the analytes passing through the surfactant where separation occurs and then migrates into the surfactant free buffer which carries them to the MS;[165−167] and elimination of the entrance of the surfactant into the MS by heart cutting techniques.[168] Although these approaches have demonstrated some success, they have not been widely applied.

The use of sieving buffers, or gel-filled CE, has proven instrumental in DNA sequencing using CE analysis (see for example ref 169). There have been very few reports, however, of coupling CE using sieving buffers with MS, primarily because of some of the same difficulties encountered with surfactants, especially source contamination. Garcia and Henion first reported the use of gel-filled capillaries in CE−MS in 1992.[170] The next report was by Barry et al. in 1996 in which a poly(vinylpyrrolidone) (PVP) matrix (14% (w/v) in 20 mM ammonium acetate at pH 9) was used for the analysis of short modified oligonucleotides.[171] In a later report by Harsch and Vouros, isomeric modified oligonucleotides at the micromolar concentration level were separated using the PVP matrix and analysis on an ion trap MS.[172]

4. CE-MS Outlook

In writing this review, it became obvious that the amount of verbiage needed to describe the current state of a technique is inversely proportional to the state of development of the technique. An example is the longer section devoted to CE than to capillary/nanoscale HPLC. The high separation efficiency of CE makes it extremely attractive for the separation of complex mixtures, especially biological or environmental, but low concentration limits of detection are still a drawback. None the less, CE−ESI−MS seems well-suited for a number of applications. One specific example is the separation of glycoforms of glycosylated peptides from protein digests. Bateman et al. showed the separation of the microheterogeneic glycoforms from the mild hydrolysis of the α-chain from α-amylase inhibitor 1 analyzed by CE−MS. Three distinct isoforms of each of the amino acid sequences 1−20, 10−21, 10−20, 54−76, and 64−76, due to the presence of 3−6 mannose units, were apparent.[173] (Figure 12).

C. Capillary Electrochromatography-MS

Capillary electrochromatography (CEC) is a combination of HPLC and CE in which an electric field is used to create bulk liquid flow through a packed capillary LC column.[174] The flow through the column is more piston-like than parabolic, resulting in improved efficiencies compared to capillary LC. Another advantage associated with CEC, in comparison to capillary HPLC, is a shorter analysis time. Addition-

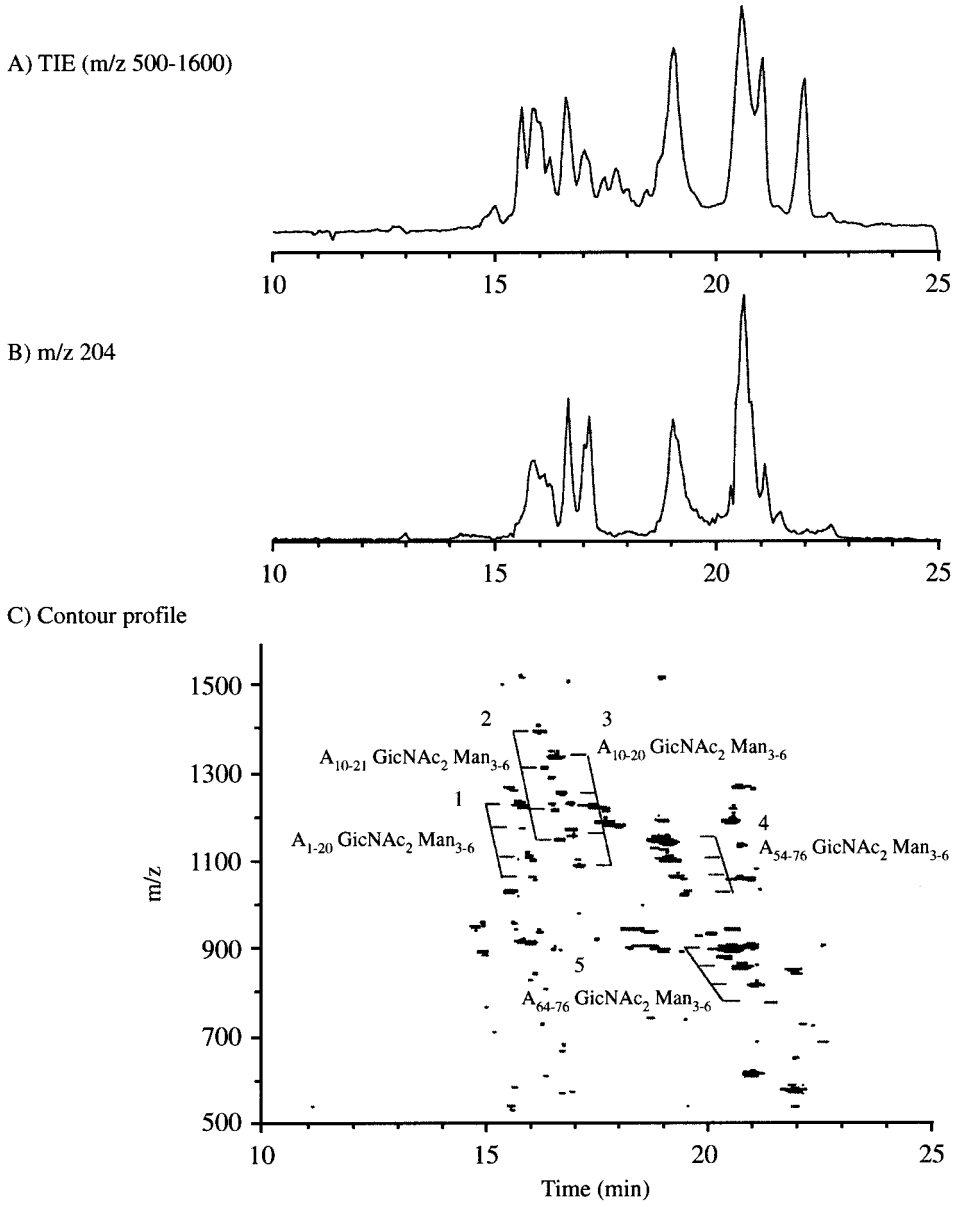

Figure 12. Example of the application of CE−ESI−MS to the determination of glycan heterogeneity in the α-chain of α-amylase inhibitor. (A) Total ion electropherogram (*m*/*z* 500−1600); (B) extracted ion for *m*/*z* 204 (characteristic fragment ion coming from N-linked glycans); (C) contour profile of *m*/*z* vs time. (Reprinted with permission from ref 173. Copyright 1998 Elsevier B. V.)

ally, it has been noted that CEC is a good alternative to micellar electrokinetic chromatography (MEKC) for the separation of neutral compounds, especially for use with mass spectrometric detection, because detection is not hampered by interference by the micellular matrix.[175,176] It has been proposed that the combination of the high efficiency characteristic of CE with the significantly greater versatility of chromatography leads to a superior chromatographic system.[177] Although this assessment has not been met with universal agreement, interest in this technique has grown tremendously, with several reviews appearing.[178−183]

1. Interface Design

Reports of coupling CEC and variants with MS began to appear in the early 1990s,[52,184−186] but the majority have appeared in the last five years. Lord et al.,[187] and Bayer and co-workers using a sheathless interface,[188] and Lane and co-workers using a sheath flow interface[189] reported results of the coupling of CEC with ESI in 1995. Several groups have recently developed novel approaches to the CEC instrumentation and interface with ESI−MS. Lane et al., have developed an automated system which includes an integrated autosampler and microprocessor-based controller.[190] Apffel and co-workers at Hewlett-Packard and collaborators at Genentech and Scios Nova designed a system based on the HP1600A HP^{3D} CE that permitted capillary electrophoresis, isocratic and gradient CEC, isocratic and gradient capillary LC, and electrically assisted gradient capillary LC (pressurized CEC) (Figure 13).[191] As noted by others, the combination of pneumatically driven and electrically driven flows offers the best opportunity to fine-tune the separation. Bubble formation at the frit at the column end due to degassing has been a serious problem that has been observed with CEC. This has

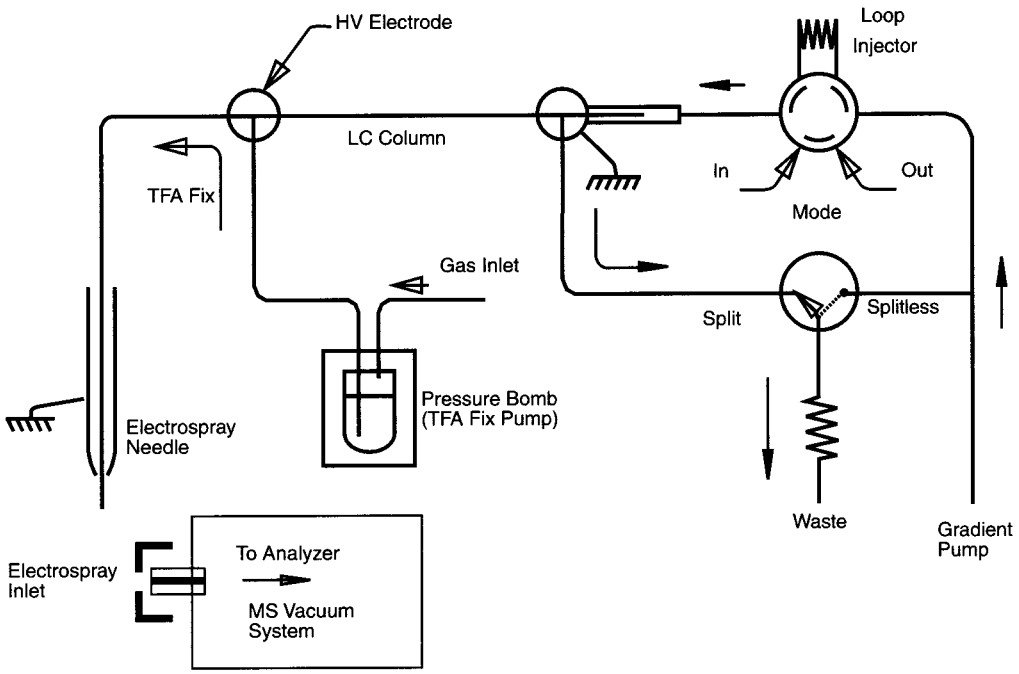

Figure 13. Schematic of electrically assisted capillary LC−MS system. TFA Fix[332] is a makeup flow of 50%acetic acid used to counteract the signal suppression effects of the trifluoracetic acid used in the mobile phase. The electrospray needle was a fused silica transfer line that was beveled and painted with gold paint to provide electrical connection to ground. (Reprinted with permission from ref 191. Copyright 1999 Elsevier B. V.)

been usually solved by applying pressure at the outlet of the CEC column. As the outlet in CEC−ESI−MS is usually at the spray tip, pressurization is difficult to achieve. It has been observed, however, that the use of tapered columns or sprayers reduces bubble formation without pressurization.[192,193] Alternatively, the use of supplemental pressure appears to reduce bubble formation at frits.[194,195]

2. Applications

Applications of CEC−ESI−MS that have been reported include the separation of textile dyes,[187] corticosteroids,[190,196−198] dansylated secondary amine tags from an encoded combinatorial library,[199] drug mixtures,[194] and the biotransformation products of thalidomide.[200] Ding and Vouros have applied CEC−ESI−MS to the analysis of polycyclic aromatic hydrocarbons and their products from the in vitro reaction with deoxynucleotides (Figure 14).[175,176] In the Ding and Vouros experiments, picomole levels of analytes were focused at the front of the column using typical liquid chromatographic techniques (analyte dissolved in a low percentage organic solvent). They also pointed out that, as a peak elutes from the column, the voltage can be reduced so that the electroosmotic flow is reduced, increasing the time during which the analyte is presented to the mass spectrometer.

3. Comparisons of CEC-MS with HPLC-MS and CE-MS

There have been relatively few direct comparisons of the same analysis on the same column under only pressurized flow and under only electroosmotic flow. Lubman and co-workers compared the separation of a tryptic digest of bovine cytochrome *c* on the identical column using pressure only and voltage plus pressure (pressure less than in the pressure only case).[201,202] By maintaining positive pressure on the gradient to help maintain a constant flow rate, the analysis times were found to be reduced (25%) and increased separation efficiency was observed with application of 1000 V. It was also noted that the order of elution varied as a function of the combination of voltage and pressure, permitting optimization of the separation (Figure 15). In a later paper, the use of a mixed-mode stationary phase containing both reversed-phase packing and anion-exchange packing was investigated.[203] The anion−exchange packing helped to maintain stable electroosmotic flow at low pH while the reversed-phase packing provided the chromatographic interaction. Alexander and collaborators have recently published an evaluation of nanoscale capillary LC and CEC using UV detection.[204] Under isocratic conditions, CEC separations showed about 57% higher efficiency than did the nanoscale capillary. Under gradient conditions, the separations showed little difference.

Spikmans et al. observed nanomolar to subnanomolar sensitivity for salbutamol analyzed by CEC.[205] This same group also reported low nanomolar sensitivity by CE−ESI−MS using ITP for preconcentrating the analytes,[206] while Cai and Henion reported subnanomolar sensitivity for related compounds isolated by immunoaffinity chromatography on-line with LC (1 mm i.d. columns)−ESI−MS−MS.[207] For these types of compounds, the superiority of CEC separations in terms of efficiency over HPLC or CE is not striking.

From the few comparative MS studies available, CEC can be used to fine-tune a separation of compounds that cannot be separated under LC conditions, with a combination of both pressure and

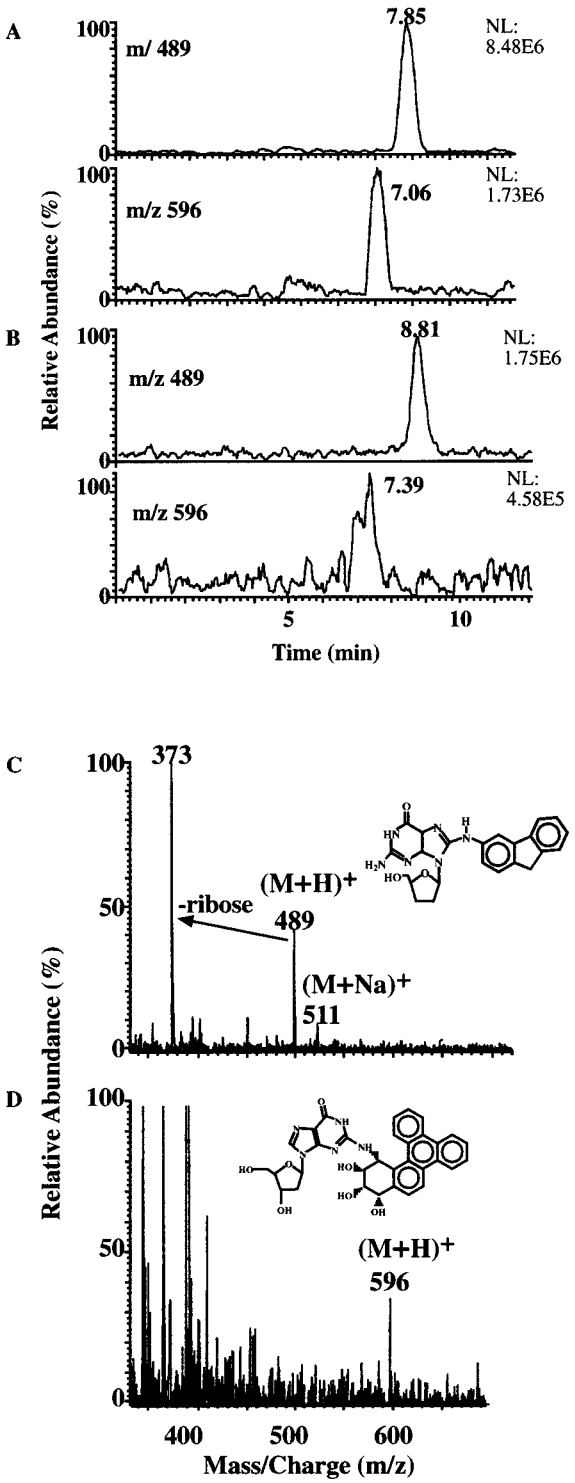

Figure 14. Extracted ion electrochromatograms of (A) *m*/*z* 489.5 (acetylaminofluorene deoxyguanosine, AAF-dG) and (B) 596.5 (benzo[*g*]chrysene-dG from the CEC-MS analysis of two mixtures at different concentration levels): (A) AAF-dG at 6.6×10^{-5} M and benzo[*g*]chrysene-dG at 2.2×10^{-5} M; (B) AAF-dG at 6.6×10^{-5} M and benzo[*g*]chrysene-dG at 2.2×10^{-6} M; (C) Mass spectrum of AAF-dG acquired from the separation in panel B; (D) Mass spectrum of benzo[*g*]chrysene-dG acquired from the separation in panel B. (Reprinted with permission from ref 175. Copyright 1997 American Chemical Society.)

electrically driven flow being employed. With the mass resolving power of MS detection, obtaining an optimal separation may not be as important, however, as when other detectors are employed. One situation where CEC may be very useful is as part of microfluidic devices (see below).

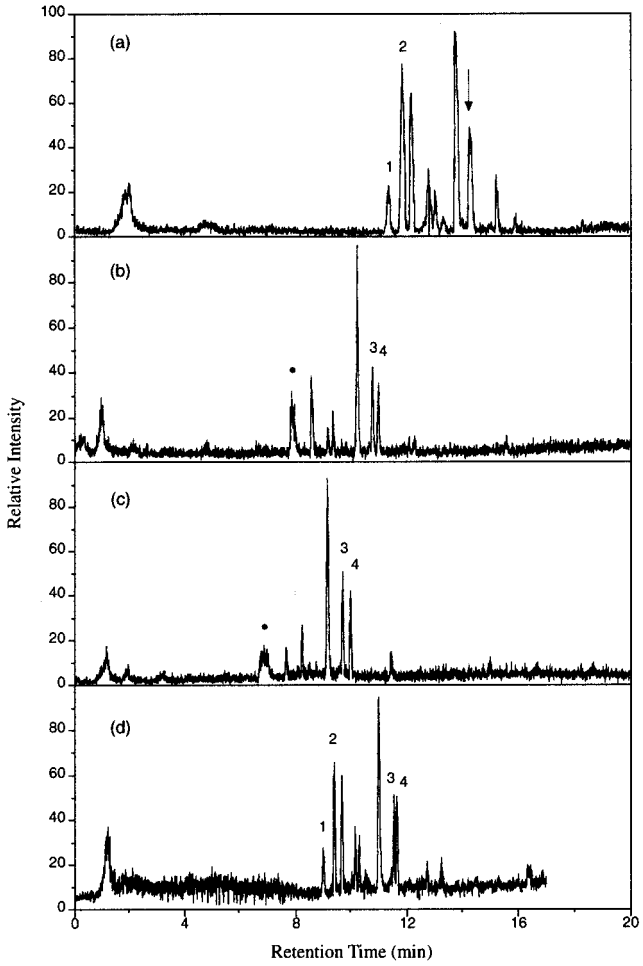

Figure 15. Reconstructed total ion chromatograms from a separation of a bovine cytochrome *c* digest using a 20 M 0 to 50% acetonitrile gradient with sample injections of 8 pmol corresponding to the original protein. Column lengths, 6 cm. Column operating conditions: (a) HPLC mode with a back-pressure of 90 bar; (b) 1000 V applied voltage with 50 bar supplementary pressure; (c) 1400 V applied voltage with 50 bar supplementary pressure; and (d) 600 F applied voltage with 70 bar supplementary pressure. (Reprinted with permission from ref 201. Copyright 1997 American Chemical Society.)

D. Multidimensional Chromatography with MS

For some complex samples, even the combination of a high resolution separation technique, such as CE or capillary LC, with the mass discrimination capability of MS can be insufficient for adequate resolution of components. One way to address this problem is to add a second, orthogonal separation technique. Column switching techniques in which a selected fraction from an LC column is directed to a second column is one approach to adding a second separation dimension. To comprehensively analyze a sample using two dimensions of separation, however, the second dimension must be fast relative to the first dimension, such that all fractions from the first dimension are adequately sampled. Bushey and Jorgenson pioneered such a comprehensive two-

dimensional HPLC separation using CE in combination with HPLC in the late 1980s.[208−210] In this combination, the CE-based separation is rapid compared to the HPLC separation, and thus, the HPLC fractions can be interrogated profusely by the CE separation. With a 15 cm CE column, CE run times of 30 s. were used.

In 1997, Jorgenson and co-workers published three papers demonstrating the potential of 2-D combinations of LC with LC,[211] size-exclusion chromatography (SEC) with LC[212] and LC with CE[213] combined with MS detection. In the LC−LC−MS system, the first column was a 750 μm i.d. × 12.5 cm long cation-exchange column and the second was a 500 μm i.d. × 10 cm long POROS reversed-phase column. A 2 h gradient run was used for the cation-exchange column, while a 1.25 min gradient was used for the RPLC column. On-line UV detection showed that most protein peaks were about 6 min wide while the peak widths in the second dimension are 5 s wide. The calculated peak capacity of the separation system was 512, compared to 45 for a 90 min LC separation on a 2.1 mm i.d. × 25 cm long column with 2.0 min wide peaks. Peak capacity, an estimate of the resolving power of a separation system, is the number of peaks that could theoretically fit and be resolved in the available separation space, assuming an equal distribution of peaks.[214] A separation of an *Escherichia coli* cell lysate was shown. Separations of tryptic digests of ovalbumin and bovine serum albumin (BSA) using a combination of six 7.8 mm × 300 mm G2000SW$_{XL}$ size-exclusion columns in series with a 4.6 mm id C_{18} column were shown to illustrate the SEC−RPLC−MS experiments. The SEC separation took 160 min and each RPLC separation took 4 min. Peak capacity was 495. Coverage of the protein sequences was approximately 90% which was stated as a typical coverage level at the sample loads employed (1 nmol ovalbumin, 750 pmol for BSA). Opticek et al., have more recently described an SEC−LC−MS system using a microcolumn with a 20-fold lower flow rate which increases the concentration of the analytes in the chromatographic peaks. High-resolution proteolytic maps of 15 pmol protein were shown.[215]

The instrumental design required to combine LC with CE on-line with MS was more complicated than that needed for the LC−LC or SEC−LC combinations (Figure 16). The LC effluent (15 μL/min) was split with 14 μL/min going to a UV detector and 1 μL/min going to the CE−MS interface. This interface was continuously flushed with CE buffer at a flow rate of 100 μL/m. To make a CE injection, the flush flow is diverted, allowing the LC effluent to flow across the ~75 μm interface gap onto the CE column. The separation capillary terminated near the end of a sheath capillary that carried makeup flow. CE separations were carried out on an APS-derivatized[216] fused-silica capillary (29 μm i.d. × 15 cm) at pH 11. Voltage drop across the capillary was 22 kV. At pH 11, all peptides should be negatively charged and migrate against the electroosmotic flow. Thus, no peptide should elute before the neutral marker.

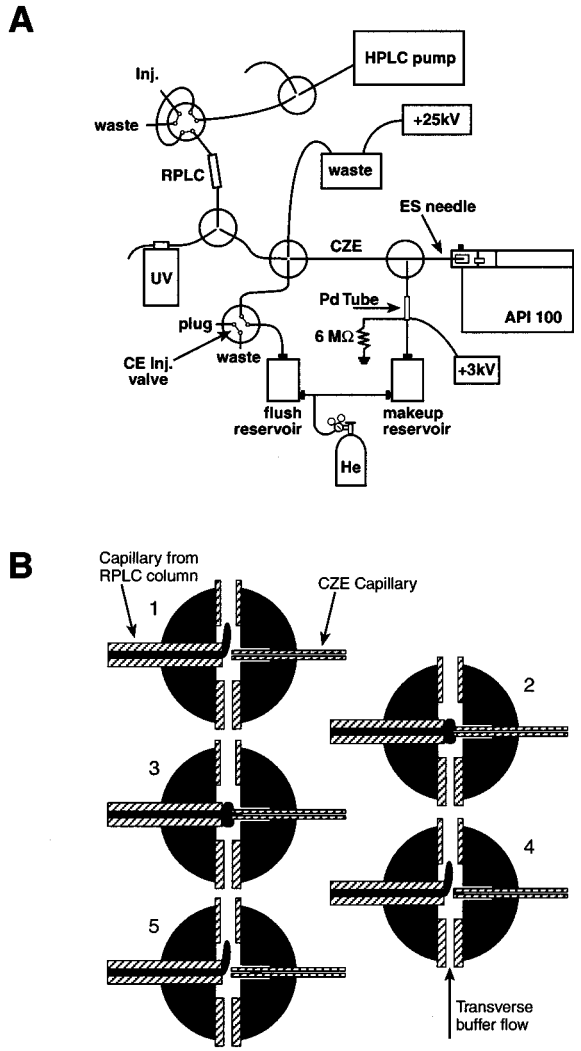

Figure 16. (A) Schematic of the LC−CE−ESI−MS system. (B) Schematic representation of an injection made using a flow gated interface. (1) CE running condition with flush flow on and high voltage on. (2) Flush flow off and CE voltage off. The analyte diffuses across the gap. (3) Flush flow off and Ce injection voltage on. (4) Flush flow on and CE voltage off. (5) Flush flow on and CE voltage on. (Reprinted with permission from ref 213. Copyright 1997 American Society for Mass Spectrometry.)

Under conditions, for example, where the neutral maker elutes in 15 s and all peptides elute within 30 s, injections can be made every 15 s. The widths of the peaks eluting from the CE column were less than 400 ms, which could not be adequately sampled by the quadrupole MS used. To alleviate this problem to some extent, relatively long injection times were used to increase the average peak width to 1.2 s. The capability of the system was demonstrated by the separation of synthetic peptides and of a tryptic digest of ribonuclease B (Figure 17). All nine tryptic peptides within the scanned mass range were separated, including two glycoforms of the glycosylated tryptic fragment 6. Peak capacity for the LC−CE system is greater than 20 000.[217]

These papers have been proof-of-principle publications, rather than applications to unknown systems or necessarily very difficult systems, and have not been compared directly to the corresponding 1-D separations. Thus, it is difficult to tell if these 2-D

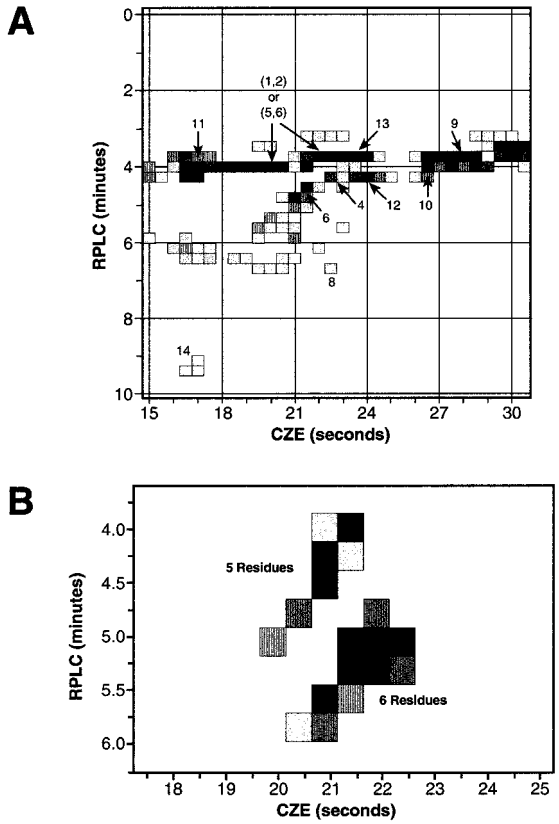

Figure 17. (A) Base-peak chromatogram of the two-dimensional view of the LC−CE separation of a tryptic digest of ribonuclease B; (B) Selected ion chromatogram from figure a for the multiple forms of the glycopeptide in ribonuclease B. (Reprinted with permission from ref 213. Copyright 1997 American Society for Mass Spectrometry.)

separations will ultimately provide additional analytical detail that was not provided by a corresponding 1-D-MS separation. With MS data acquisition being the limiting factor, especially for LC−CE, the recently available ESI-TOF MS instruments should make such data intensive experiments practical, and possibly lead to "real" applications.

Two alternative approaches to two-dimensional separations were recently reported by Link et al.[218] in which either a strong cation-exchange capillary column was placed in series with a reversed-phase capillary column or a biphasic column containing both the reversed-phase packing and the cation-exchange packing (not intermixed) was used. Analytes from a digest of a denatured ribosomal protein complex were absorbed onto the cation-exchange packing and step eluted (salt gradient) onto the reversed-phase column. The analytes trapped on the reversed-phase column were then eluted into the MS. Over 100 proteins were identified in a single run.

E. Microfabricated Microfluidic Devices (Microchips)

The current edge of the envelope in the trend toward miniaturization in separations is microfabricated microfluidic devices, commonly called microchips. Miniaturization has had a tremendous (even that can be viewed as an understatement) impact on electronics, which has led to a commensurate impact on society in general. During the past decade, the technologies involved in electronic chip fabrication have begun to be applied to handling liquids instead of electrons. Most microfluidic devices have been fabricated using photolithographic techniques from the microelectronics industry, and thus, the basic fabrication technology is well developed.

In 1992, Harrison et al. reported the fabrication of a capillary electrophoretic separation system micromachined on a planar glass chip (~15 × 4 cm).[219] They obtained a separation of fluorescein isothiocyanate-labeled amino acids with a separation efficiency of over 100 000 plates. A separation distance of 10 cm and an electric field of 592 V/cm were used. Analyte solutions were 10 μM with 30 pL (300 amol) being injected and detected.[220] With decreasing chip size, the complexity of the chip design has increased. For example, Moore et al. reported a chip design in which the chip was ca. 50 mm × 30 mm and had a serpentine separation channel 171 mm long etched in it.[221] This chip was used for demonstration of the micellar electrokinetic capillary chromatographic (MECC or MEKC) separation of coumarin dyes.

Initial examples of analytical applications of chip-based technologies have been primarily electrophoresis-based.[221−230] Capillary electrophoresis was an attractive separation technique for several reasons. First was that a simple open channel on the chip could be used for the separation, without additional concerns such as how to pack the microchannels, which permitted attention to be focused on the basics of the technique. A second reason is that the motive force in the separation system, high voltage potential, was more easily incorporated on the chip than was high pressure differential. Additionally, capillary electrophoresis was already noted for its use in separating very low total amounts of analytes, and detection systems, electrochemical and laser-induced fluorescence, that could detect the low levels of analytes separated on chips were available. A number of the application papers focused on DNA separations, and the first commercial microchip separations-based analytical instrument is designed for DNA separations.[231,232]

There have, however, been relatively few applications of microchip-based separations of the types of samples that have typically confronted analytical chemists, (e.g., complex biological, environmental, or reaction mixtures) with the exception of DNA sequencing or PCR reactions. Among the applications reported are the separation of FITC-derivatized urine and immunoassay of theophylline in serum reported by Thormann and co-workers,[222] and the separation of FITC-derivatized biogenic amines from soy.[233] The lack of these types of applications may be attributed, in part, to the very low concentrations of the analytes of interest, difficulties in handling "real samples",[234] and difficulties in identifying unknown peaks in the complex separations (unless there is a specific derivative formed for detection).

The first reports of the coupling of microfabricated microfluidic devices with mass spectrometry were on the use of microchips in the direct infusion of analyte

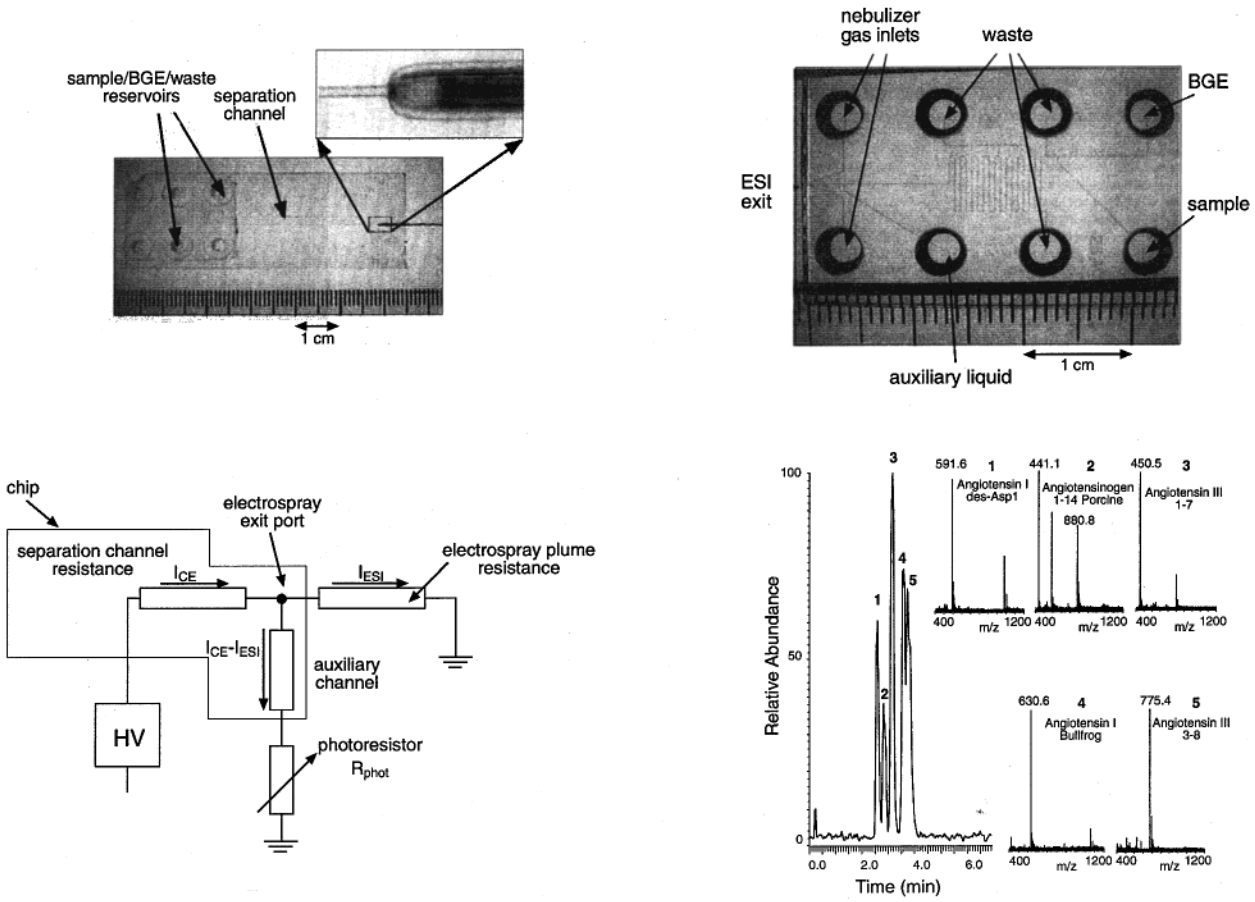

Figure 18. (A) Photograph of the microdevice using an attached transfer capillary. (B) Photograph of the microdevice using a pneumatic nebulizer. (C) Electrical scheme of the microdevice in operation. The electrical current I_{CE}, delivered by the HV power supply transports the ions in the separation channel. The potential of the ES exit port can be controlled by an incandescent lamp illuminating the photoresistor, R_{phot}. A small fraction of the electrophoresis current I_{CE} is transported to ground by the electrosprayed ions while the remaining current returns to ground through the auxiliary channel and the photoresister. (D) CE−ESI−MS analysis of a peptide mixture in the microdevice with a pneumatic nebulizer (B) using transient isotachophoretic sample preconcentration. Sample concentration: 20 μg/mL of each peptide dissolved in 100 mM ammonium acetate. Injection size: 6 mm plug (~11 nL). BGE: 1% (v/v) formic acid in water. Auxiliary liquid: 1% (v/v) formic acid in 50% (v/v) methanol/water. Electric field strength: 400 V/cm. ESI voltage: 4 kV. Nebulizer gas flow rate: 0.3 L/m, 141 kPa. (Reprinted with permission from ref 239. Copyright 1999 American Chemical Society.)

solutions in combination with electrospray ionization by Figeys et al.[235] and Karger's group.[236] The microchip fabricated by the Karger group consisted of a chip with nine parallel channels,[236,237] each channel connected to two wells. One well was connected to high voltage, while the second was connected to a syringe pump to deliver the analyte solution. The chip was mounted on a three-dimensional stage to align the chip with the ESI source and to sequentially align each channel with the source. The electrospray was initiated by the potential difference between the solution existing at the edge of the chip and the electrospray source that was held at ground potential. To prevent wetting of the chip edge and mixing of the eluents from adjacent channels, the edge of the chip was coated with either Imunopen or *n*-octyltriacetoxysilane. Although these measures reduced wetting of the chip edge, there was still the potential for wetting and mixing problems. The authors noted that, without external pressure to generate a flow rate of 100−200 nL/min, the spray was unstable due to low electroosmotic flow. In the second paper from this group, proteins were digested in the sample well on the chip and then delivered directly to the mass spectrometer.[237] Shortly after the original Karger article appeared, Ramsey and Ramsey reported on the use of electroosmotic pumping to induce flow sufficient to sustain electrospray.[238]

Recently, the Karger lab described two new microfabricated devices that could be interfaced to MS (Figure 18).[239] Both chips contained sample inlet ports, preconcentration sample loops, a separation channel, and a port for ESI coupling. In the first design, the chip could be coupled to the ESI source using a fused silica transfer capillary. The important feature of the second design was that a miniaturized pneumatic nebulizer was fabricated as part of the chip. The sample reservoir volume on the chip was 15 μL which was sufficient to spray for 20 min (some evaporation of the solvent was noted). The efficiency of the chip with the nebulizer was less than that of the chip using a transfer line because the nebulizer chip used a serpentine separation channel which contributed to band-broadening and because of some hydrodynamic flow in the channel between the ESI exit and the auxiliary liquid channel junction. The capability for transient isotachophoretic sample concentration was demonstrated on this chip.

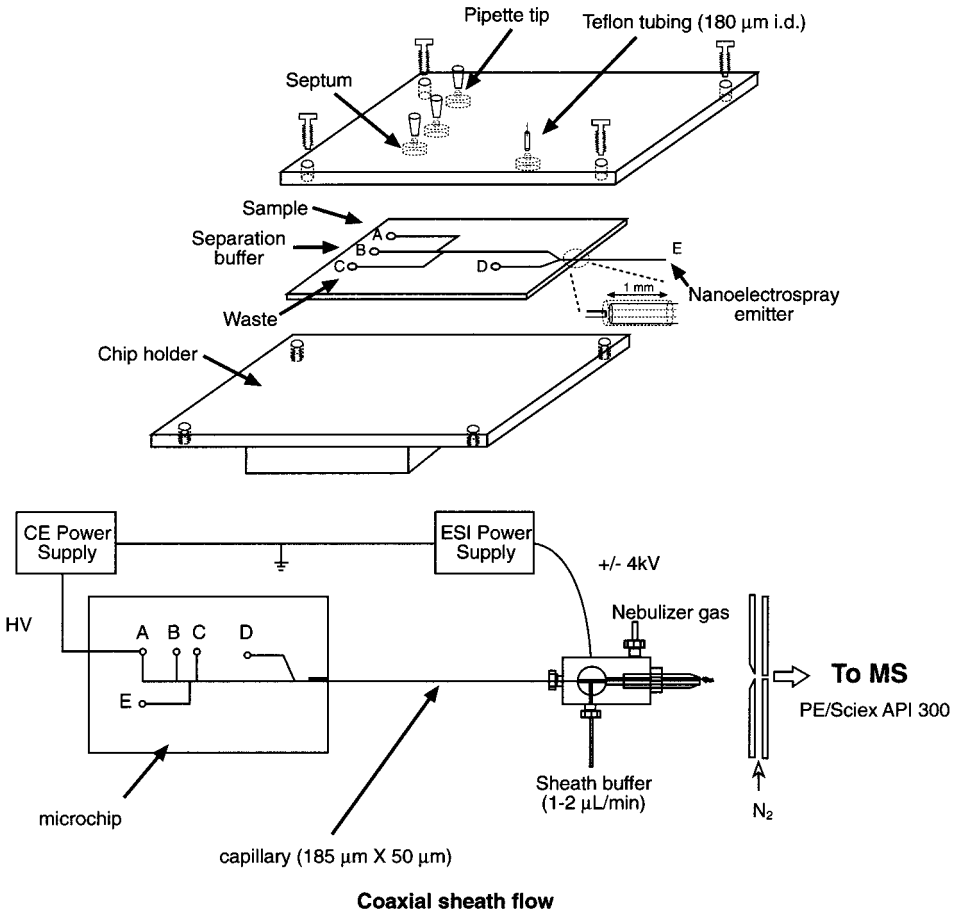

Figure 19. Schematic representation of the chip-CE configuration using (A) a disposable nanoelectrospray emitter (Reprinted with permission from ref 244, Copyright 2000 American Chemical Society) or (B) a sheath flow interface. Well A, buffer reservoir; well B, sample reservoir; well D, make up solvent at 50 nL/m; well E, waste (Reprinted with permission from ref 241. Copyright 1999 American Chemical Society.)

The early Figeys design differed from the early Karger design in that there was one outlet from the chip with multiple sample wells, rather than parallel channels.[235] In addition, the chip was not used as part of the electrospray interface itself, but, instead, a 12 cm capillary column ending in a liquid junction maintained at high voltage was used. The volume of the transfer capillary, however, was well over 50 times that of the chip channel. The comparatively large volume of the transfer capillary helped to maintain an electroosmotic flow through the device sufficient to maintain flow. Detection limits for peptide analysis were observed to be in the range of 2 fmol/μL. Minimum flow rate was approximately 0.2 μL/min. This group more recently reported fabrication from poly-(dimethylsiloxane), a soft polymer, of a device similar in design to the previous chip.[240] The use of this material was projected to lead to inexpensive microfabricated devices.

The early success of these groups in interfacing chip-based introduction/separation systems with ESI has led a number of groups to explore the potentials of chip-based MS interfaces. A chip that was designed to be used with a disposable nanospray emitter resulted from a collaboration between the Harrison lab at the University of Alberta and the Thibault lab at the National Research Council Institute for Biological Sciences in Ottawa.[241,242−244] In this design, a flat-bottomed hole was drilled into the side of the chip at the outlet of the separation channel. Tapered fused silica capillaries were inserted in the hole and bonded into place with crystal bond. Fused silica capillaries 1 cm long were used for nanospray experiments, while 10−40 cm long capillaries were used in combination with an external ESI source and a sheath flow interface (Figure 19). Capillary electrophoretic separations of standard peptides and protein digests were demonstrated using this chip. In their latest paper, two on-chip concentrating procedures were demonstrated. One technique used sample stacking where polarity switching was used to remove sample buffer prior to analysis,[245,246] while the second, for larger sample volumes, used a disposable adsorption preconcentrator external to the chip.[148] Sample stacking permitted use of volumes 20 times as large as used in normal injections (10 vs 0.5 nL), while the use of a preconcentrator permitted loading volumes ~750 times greater than the standard injection procedure. The separation and analysis of an in-gel digested protein present at less than 500 fmol from a 2-D SDS−PAGE separation of *H. influenzae* Rd$^-$ proteins was shown (Figure 20).

Licklider et al., reported the microfabrication of a chip incorporating polymeric layers of parylene.[247] These chips were machined with robust integrated emitters formed from the parylene. A reusable chip holder was designed that provided electrical and gas connections. A stable spray could be achieved with a

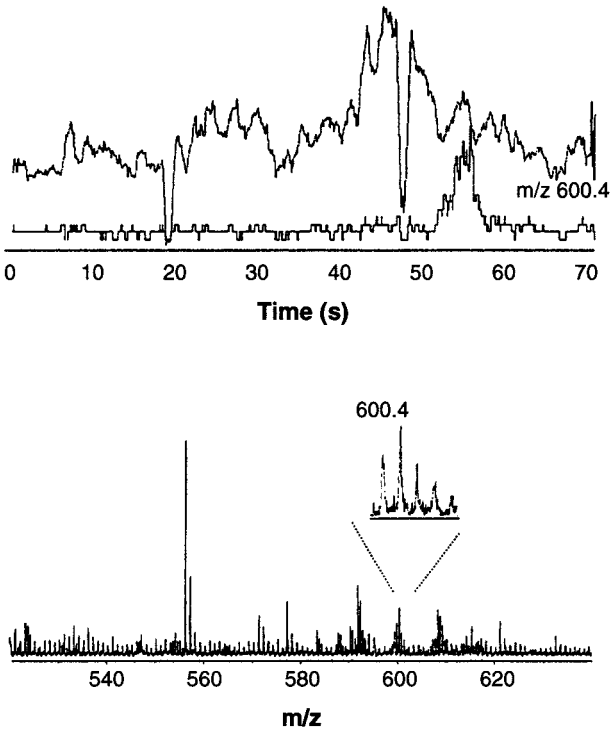

Figure 20. Chip-CE-QqTOF MS analysis of tryptic peptides from the membrane extract of *H. influenzae*; (a) total ion electropherogram and reconstructed ion electropherogram for *m/z* 600.4. (b) Mass spectrum for peak at 55 s. (Reprinted with permission from ref 244. Copyright 2000 American Chemical Society.)

low nanoliters per minute flow rate. ESI−MS data obtained using the parylene emitters was comparable to data obtained using a pulled fused silica capillary emitter.

A microfabricated device made of polycarbonate designed for use with isoelectric focusing and incorporating an integral ESI was recently reported by Wen et al.[248] The authors noted that, because the chip and emitter were machined from polycarbonate rather than silica, they were more easily machined (using laser machining) and more robust. They also reported that, using flow injection, the analyte signal obtained using the polycarbonate chip was notably stable (<∼5% deviation) for over 3 h. Unfortunately, the separation efficiency was less than that obtained with coated capillaries.[45] The authors predict that with improved micromachining capability to improve the smoothness of the machined separation channels and optimization of formation of the closed channels (which used a thermal adhesive), the separation efficiency should improve.

The incorporation of liquid chromatographic-based separations on microchip devices is still in its infancy. Because of the experimental ease of using electrically driven microchip based separations vs the more experimentally complicated application of pressure, CEC is likely to be the method of choice for initial development. Ramsey and co-workers have demonstrated the feasibility of fabrication of open tubular CEC in a chip format,[227,228] and Regnier and co-workers have fabricated chips containing collocated monolith support structures to which, after aminopropylsilylation, poly(styrene sulfate) was electrostatically bonded.[249,250] Although these chips have not been interfaced to MS, it would not be surprising to see such applications soon.

F. LC Combined with MALDI/MS

Matrix-assisted laser desorption/ionization is a second major innovation in ionization that has recently had a major impact on mass spectrometry (ESI being the first). In MALDI, the analyte is dissolved in a solution of a light-absorbing chemical, commonly called the matrix. The solution is allowed to dry and crystallize. A laser, with a wavelength that is absorbed by the matrix, is used to impart energy to the matrix, which then transfers it to the analyte, resulting in desorption and ionization of the analyte.

Because the critical step in this sample preparation technique involves crystallization, compatibility between MALDI and separations is low and difficult to achieve. Due to the high sensitivity of MALDI and its insensitivity to high concentrations of nonvolatile salt relative to ESI, however, there has been significant interest in coupling MALDI with liquid sample introduction.[251] Not unlike the pre-ESI/APCI days of coupling LC with MS, successful coupling of liquid streams on-line to MALDI have generally followed one of three approaches: continuous flow probes similar to continuous flow FAB and frit-FAB, aerosol formation, and mechanical transport devices.

Inspired by the success of continuous-flow FAB probes, in 1993, Li et al., developed a means of introducing a liquid solution via a fused-silica capillary that ended in a frit orthogonal to the analyzer.[252] The solvent contained methanol, 3-nitrobenzyl alcohol (3-NBA) as the UV light-adsorbing component and ethylene glycol. Application of this interface with LC separations was then reported in 1995.[253] Li and co-workers further reported instrumental improvements aimed at increasing resolution (parallel ion extraction and time-lag focusing).[254] A persistent problem noted in use of this interface was adduct formation resulting in poor resolution. They observed that, although the concept of CF-MALDI was viable, improved liquid matrixes would be necessary to provide useful MALDI data. Lubman and co-workers have also reported the application of CF-MALDI.[255] Utilizing an ion trap, from which unwanted solvents and matrix ions could be ejected, in combination with a TOF analyzer, picomole sensitivity and a mass range of over 8000 Da were achieved. Zhan et al., showed that a solid matrix material, such as α-cyano-4-hydroxycinnamic acid could be used in a CF-MALDI probe (Figure 21).[256] The analyte/matrix solution cocrystallizes on the probe frit. A combination of laser ablation and continuous solvent flushing regenerates the frit surface with negligible memory effect. Gel permeation chromatographic separation and on-line introduction of PEG 900 were demonstrated. Lawson and Murray approached the problems associated with using 3-NBA as a matrix by using an infrared laser.[257] The solvent used in these experiments was either 1.0% (v/v) glycerol or 0.10% glycerol and 0.1.% (v/v) TFA in ethanol. Some memory effects and adduct formation were still noted, however.

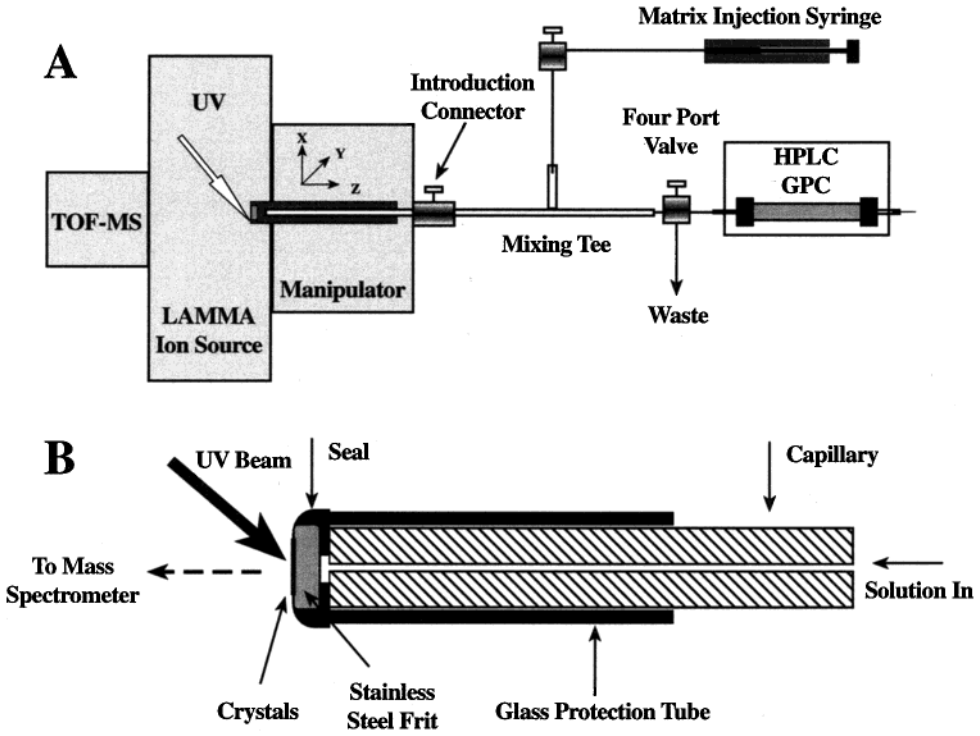

Figure 21. Schematic diagram of on-line coupling of HPLC/GPC with MALDI detection. (A) Overall schematic diagram and (B) details of the frit interface. (Reprinted with permission from ref 256. Copyright 1999 Wiley Interscience.)

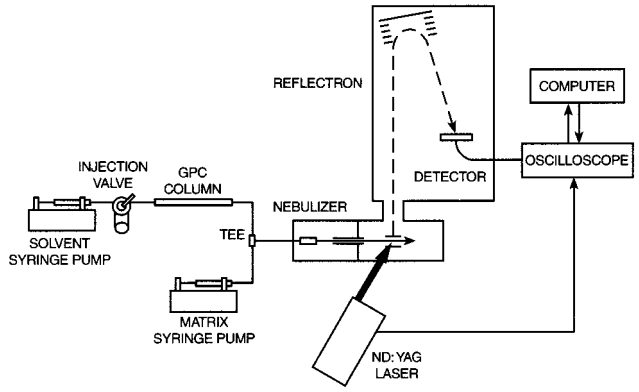

Figure 22. Aerosol MALDI apparatus configured for on-line GPC-MS. Column effluent is mixed with matrix solution prior to pneumatic nebulization. (Reprinted with permission from ref 262. Copyright 1996 American Chemical Society.)

Murray and co-workers have published a number of papers detailing aerosol sample introduction for MALDI including applications to separations.[258−263] In the latest version of the aerosol interface, the analyte solution is mixed with the matrix solution, e.g., ferulic acid, in a mixing tee and is then passed into the vacuum through a glass pneumatic nebulizer into a stainless steel laminar flow tube (Figure 22).[262] The droplets formed in the nebulizer are dried by nitrogen gas heated to 500 °C. A pulsed Nd:YAG laser is used to form ions which are introduced orthogonally into the TOF analyzer. A 3−5-fold improvement in sensitivity for a solution of 1 mg/mL peptide and 5 mg/mL matrix flowing at 0.5 mL/min was observed using this design.

In 1998, Karger et al., noting that at that time there had been limited success and no universal interface, developed a rotating quartz wheel upon which LC effluent mixed with matrix was deposited *in vacuuo* from the end of a fused-silica capillary which contacted the wheel (Figure 23).[264] The flow rate was 100−400 nL/min. Contact between the wheel and the capillary tip prevents clogging of the tip, and the wheel serves as a heat-sink to prevent freezing of the solution. As the wheel was rotated into position at the source repeller, the deposited analyte/matrix dried and crystallized. Analysis of the CE separation of a 12 peptide mix with attomole detection limits was shown. The quartz wheel rotated at 0.33 rpm, and was removed and cleaned after each analysis. Murray, in collaboration with Ørsnes, Graf, and Degn from Odense University in Denmark, modified a rotating ball inlet, developed at Odense for analysis of volatile species, for use with liquid introduction MALDI/MS.[265] The rotating ball was cleaned by laser ablation and by the rubber gasket used to isolate the instrument from atmosphere. The analysis was still plagued by poor mass resolution, especially for proteins, and adduct formation, postulated as due to the relatively high pressure in the source. The authors note that improvements are in the planning stage.

At this point, there is still no universally accepted, or even acceptable, interface for use with complex separations at low levels. The Karger design provides good sensitivity for peptides but would have limited applicability to HPLC due to the short analysis time. Mass resolution in the Karger experiments is better than that observed in the Murray instrument. This may either be due to differences in the TOF machine itself or differences in pressure in the sources ($\sim 2 \times 10^{-6}$ Torr for the Karger instrument vs 5×10^{-5} Torr for the Murray instrument) or a combination thereof. The continuous-flow interfaces may prove useful if a

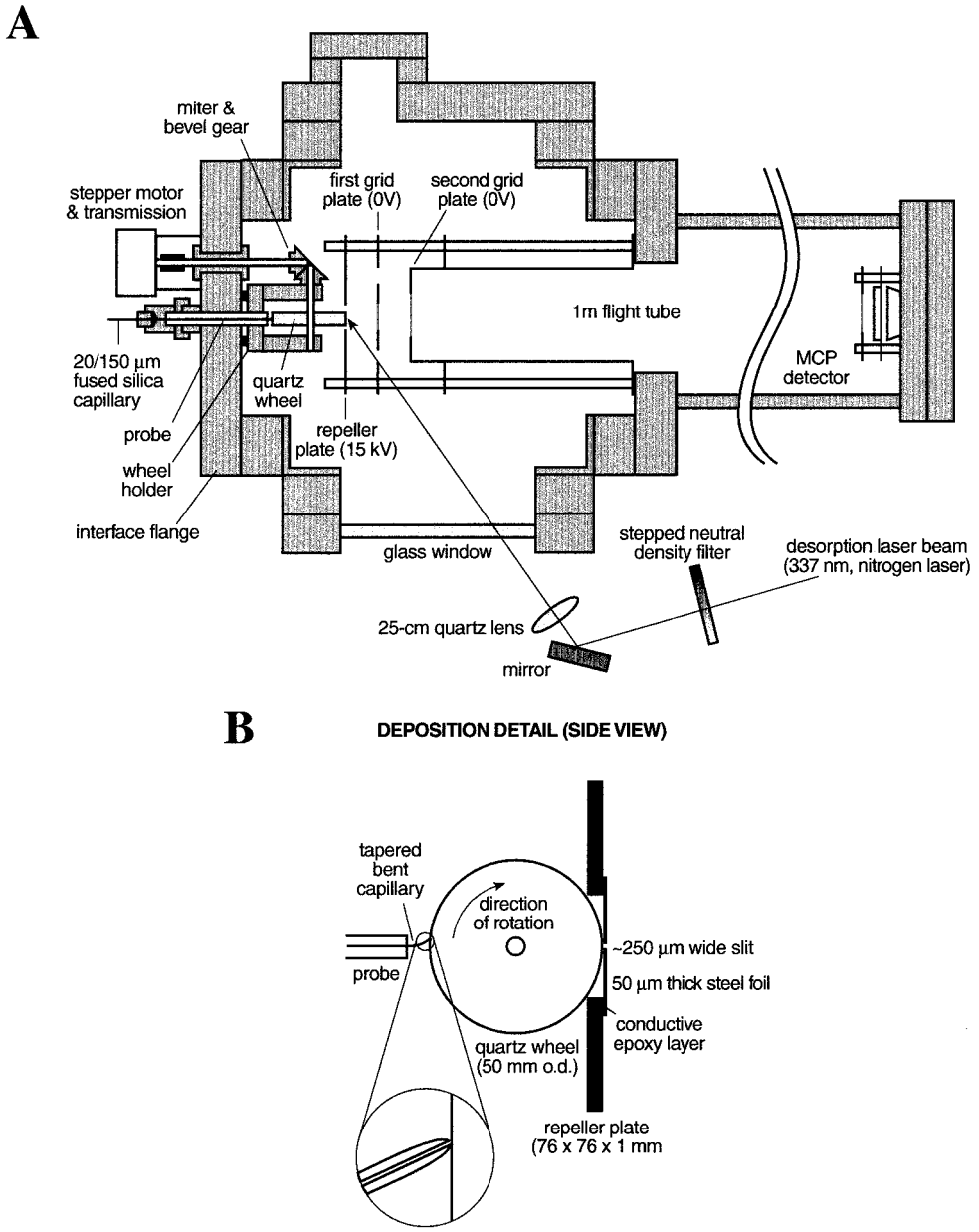

Figure 23. (A) Schematic of the on-line MALDI-TOF MS instrument (top view). (B) Detail of the design of the liquid deposition process on the rotation wheel in the vacuum of the mass spectrometer and the design of the repeller region. (Reprinted with permission from ref 264. Copyright 1998 American Chemical Society.)

better liquid matrix could be found and adduct formation reduced (also a problem with IR-MALDI). The recent appearance of papers using a continuous flow probe or mechanical analyte transport from Murray's group and the absence of recent developments of aerosol-MALDI with separations may indicate that the aerosol interface currently shows less promise. The problem of interfacing MALDI with liquid streams has not yet been solved, but it is definitely an area of intense interest.

G. Affinity Separations and Mass Spectrometry

Affinity-based chromatography has been a common tool in biology and biochemistry for some time. Once mass spectrometric instrumentation began to be applied to biological problems in earnest, it was inevitable that researchers would begin to find ways of integrating affinity techniques with mass spectrometry. There are two aspects of combining affinity techniques with MS that will be covered in this review: on-line affinity chromatography, and biomolecular interaction analysis/MS.

1. Affinity Chromatography-MS

The first report of the on-line coupling of immobilized metal ion affinity chromatography (IMAC) with MS was by Nuwaysir and Stults in 1993.[266] They used an in-laboratory fabricated column, made from 1 mm i.d. × 10 cm Teflon tubing, which was connected to the mass spectrometer via a fused silica capillary. The column was packed with chelating Sepharose and activated by pumping a 30 mM $FeCl_3$ solution through the column. The column was used to isolate phosphopeptides from tryptic digests of proteins separated by SDS−PAGE and electroblotting. A step gradient of (A) 0.1 M acetic acid (to

remove nonphosphorylated peptides), (B) distilled water, (C) 0.1% ammonium acetate/ammonium hydroxide (pH 8.0), and (D) 0.1% ammonium acetate/ammonium hydroxide (pH 9.5) was used to fractionate the phosphopeptides, which eluted in buffer D, from the nonphosphorylated peptides. Alternatively, 2% acetonitrile/0.1% ammonium acetate/ammonium hydroxide (pH 10.5) could be used in place of buffers C and D. Good quality spectra were obtained from synthetic phosphopeptides at the 10 pmol level using this technique. Analyses of tryptic digests of β-casein by standard HPLC−ESI−MS and IMAC−ESI−MS were compared. The highly phosphorylated peptides were not observed or were of very low abundance by HPLC−ESI−MS, while these phosphopeptides were readily observed by IMAC−ESI−MS.

Because of the importance of phosphorylation in biological signaling, the report of a technique that would allow specific isolation and analysis of phosphopeptides from protein digests would have been expected to generate considerable interest and follow-up. This, however, was not the case. Stults later pointed out that the simultaneous elution of all phosphorylated species from the IMAC column into the ESI−MS resulted in poor detection limits.[267] One approach to addressing the sensitivity problem has been to elute the IMAC-bound peptides off-line followed by on-line introduction.[266,267,268] The second chromatographic step is used to remove salts and reconcentrate the analyte as well as separating individual analytes. Alternatively, the IMAC media have been directly applied to MALDI targets with the MALDI matrix eluting the analytes from the media.[271,272] The tolerance of MALDI to salts and its high sensitivity make this approach viable.

A third approach taken by several laboratories has been to combine IMAC on-line with a second separation technique. Watts et al. in 1994 reported a microIMAC column (250 μm i.d.) in series with a capillary HPLC column (320 μm i.d.) directly interfaced with an ESI−MS−MS triple quadrupole instrument (Figure 24a).[273] In this design, the column could be charged with $FeCl_3$ in place using excess $FeCl_3$ solution, while the wash solutions were shunted to waste. The effective lower sensitivity limit for this combination was reported to be ca. 125 fmol. The authors also applied this combination to the determination of phosphorylation sites induced on the protein ZAP-70 in Jurkat cells and identified autophosphorylation sites and sites phosphorylated in response to specific stimuli (Figure 24b).

Cao and Stults have subsequently combined IMAC in series with capillary electrophoresis−ESI−MS.[267] In this approach, the IMAC column is used as a preconcentrator for the CE separation and was fabricated from a 150 μm i.d. × 5 cm long fused silica capillary that had been derivatized with aminopropylsilane and used a PVDF membrane as a frit. The inlet end of the CE capillary (75 μm i.d. × 75 cm long) was inserted into the IMAC capillary. The phosphopeptide samples were eluted from the IMAC column with 30% methanol/0.1% ammonium acetate/ammonium hydroxide (pH 9.6), then separated by CE using a 1% acetic acid/10% methanol running buffer and a field strength of −133 V/cm. Limits of detection of 28 fmol were reported, but this was for a nonphosphorylated peptide. Successful analysis of phosphopeptides from 240 fmol of a tryptic digest of β-casein was reported, while the successful analysis of 20 fmol of the digest was reported but not shown.

Other types of affinity-based separations have also been interfaced on-line with ESI−MS. As with metal ion affinity based approaches, there have been few reports of directly coupling the affinity column to the mass spectrometer. This is most likely due to incompatibility between elution buffers and ESI and/or the need to further separate the affinity-bound analytes.

The use of a mixed affinity/adsorption chromatographic bed was shown by van Breeman and co-workers. In this work, a column containing immobilized thymine [3-(1-thymidyl)propanoic acid chemically bound to aminopropyl silica] was used for separating oligonucleotides with different selectivity than standard reversed-phase chromatography.[274] The authors, however, noted that the high ionic strength of the mobile phase used in the separation resulted in ESI signal suppression. Lowering the ionic strength did not improve the signal-to-noise ratio.

As in the case of IMAC−ESI−MS, serial column systems have been developed to ameliorate the problems associated with direct coupling of immobilized affinity chromatography (IAC) and ESI−MS. Lombardo et al. reported the use of a two-dimensional system using a custom-packed src-homology 2 (SH2) affinity column based on streptavidin-Poros resin incubated with SH2-biotin attachment domain.[275] The second column was a reversed-phase Poros R2/H capillary column. A 200 pmol aliquot of a mixture of eight synthetic phosphopeptides corresponding to eight putative epidermal growth factor receptor phosphopeptides was analyzed. The affinity-bound analytes were displaced by a 200 nmol solution of a competitive phosphopeptide and eluted onto the reversed-phase column where separation occurred. The relatively high level of the competitive phosphopeptide used to elute the peptides of interest may help to explain the relatively high level (200 pmol) sample load used, although the reconstructed total ion chromatogram showed a good signal-to-noise ratio (100:1 or better).

The use of a immunoaffinity extraction (IAE, identical to IAC above) column (antibody affinity-bound to a Protein G column), a restricted access media LC column (RAM), and a C_8 reversed-phase column in series combined with ESI−MS (IAE/LC−LC−MS) to characterize a combinatorial library was reported by Henion and co-workers in 1996 (Figure 25).[276] In the initial elution step the antibody and the bound analytes are eluted from the affinity column onto the RAM column. A hydrophobic outer surface of the RAM column media excludes the antibody from the internal reversed-phase surface, so that the antibody is flushed through the column while the analytes are retained. The analytes were then back-flushed onto the C_8 column, separated, and analyzed by MS. Hsieh et al. have also described the combination of affinity capture of target molecules from a combinatorial library combined with subsequent

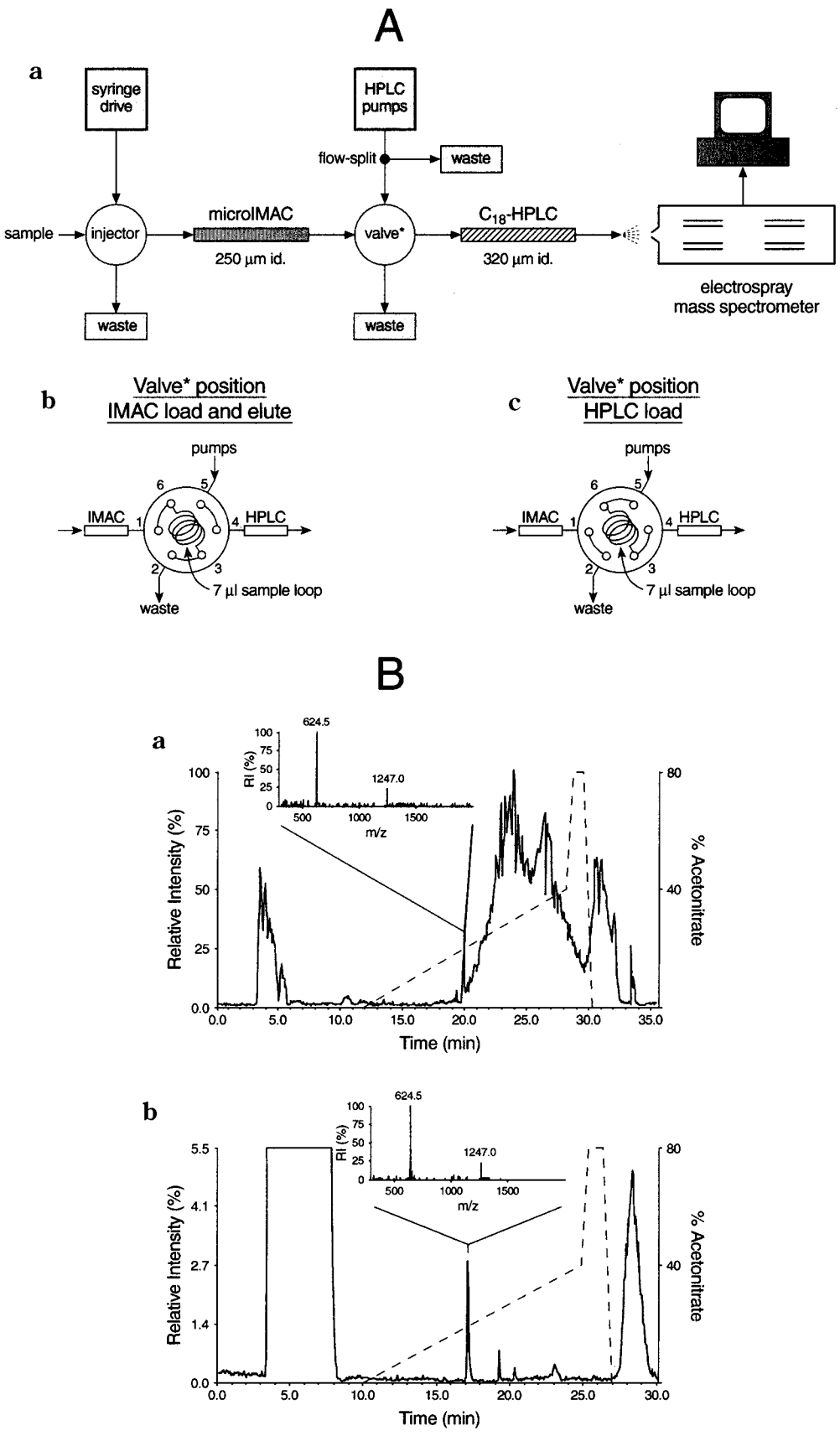

Figure 24. (A) Schematic representation of (a) IMAC−HPLC−ESI-MS instrumentation and expanded views of the switching valve used to link the microIMAC column to the HPLC system, (b) in the position for IMAC column loading, washing, and subsequent elution of bound phosphopeptides into the sample loop, and (C) in the position for loading the contents of the sample loop onto the HPLC system and subsequent development of the HPLC column. (B) Comparison of (a) HPLC−ESI-MS and (b) IMAC−HPLC−ESI-MS. Reconstructed total ion chromatogram (solid line) and spectrum of a single phosphopeptide from ZAP-70. Dashed lines are the acetonitrile concentration at a given point during the separations. (Reprinted with permission from ref 273. Copyright 1994 by the American Society for Biochemistry and Molecular Biology.)

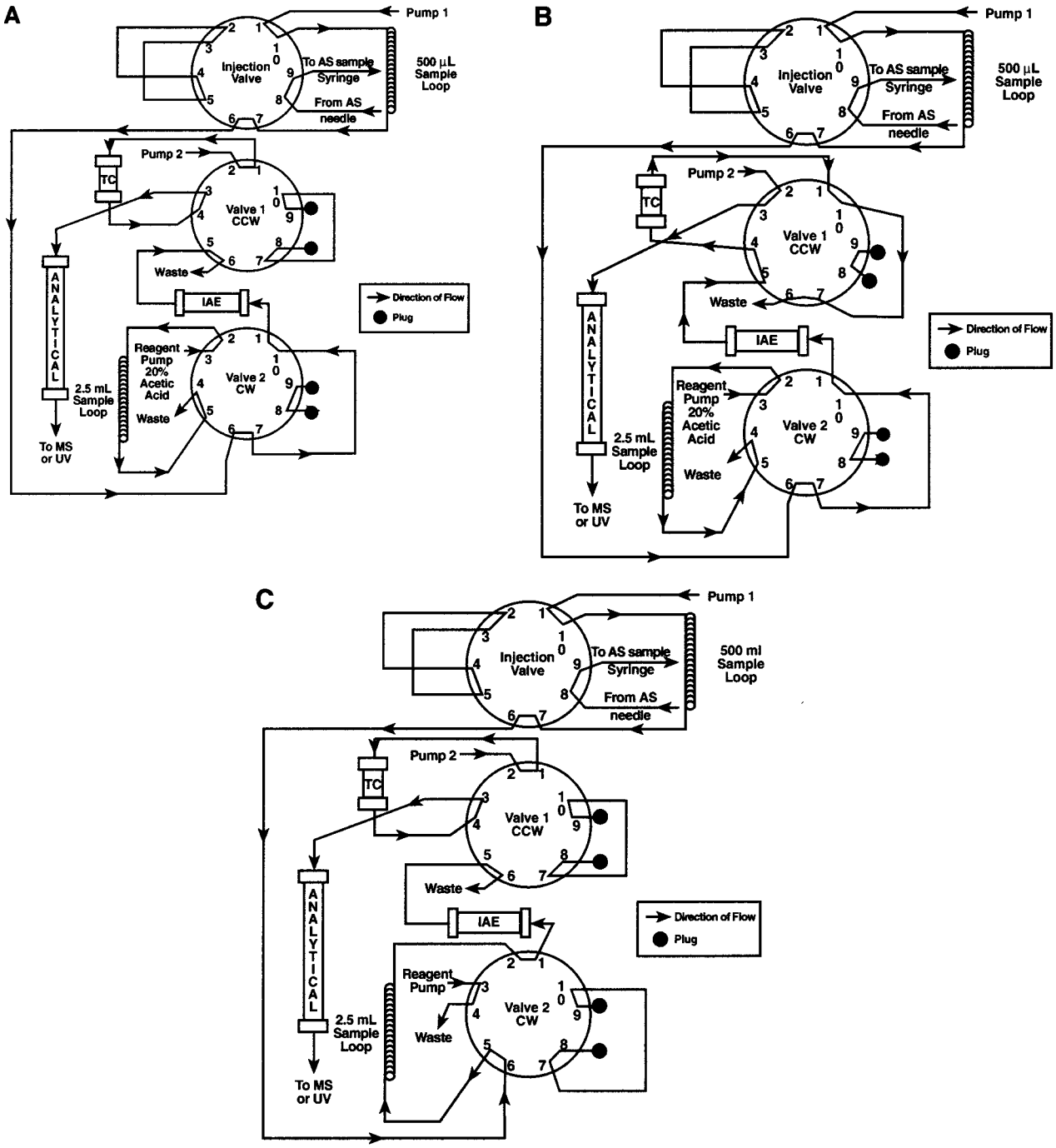

Figure 25. Schematic representation of the IAE-LC−MS instrumentation. Valve positions for (A) loading antibody and chemical library, (B) desorbing benzodiazepine-antibody complexes, and (C) backflushing trap column onto C-8 column while reconditioning IAE column. (Reprinted with permission from ref 276. Copyright 1996 American Chemical Society.)

release and high-resolution separation with ESI−MS detection.[277] Newkirk et al. used a similar apparatus, but with a Poros R-10 trapping column rather than the RAM column, to analyze fumonisins.[278] Holtzapple et al. modified Henion's setup by cross-linking the antibody to the Protein G column with pimelimidate to eliminate the necessity of the RAM column.[277,278] Limits of quantitation of 1 ng/mL were reported for the analysis of fluoroquinolones in chicken liver and in milk using this technique.

It is clear from the studies discussed above that the use of affinity chromatography on-line with ESI−MS is not only feasible, but potentially quite useful, if used in conjunction with an additional cleanup/separation column. Given the extensive use of affinity techniques in biochemistry and biology, major efforts will likely be in expanding such applications.

2. Biomolecular Interaction Analysis (BIA)-MS

Instrumentation for the analysis of biomolecular interactions based on surface plasmon resonance (SPR) became available commercially in 1990.[281−284] A surface plasmon is the oscillation of free electrons propagating across the surface of a conductor, such as a thin layer of gold. When the surface is illuminated with light, photons of a specific wavelength couple with and transfer energy to the free electrons in the metal. Changes in the chemical

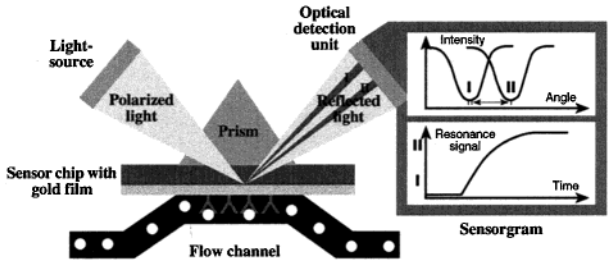

Figure 26. Diagram illustrating surface plasmon resonance. (Reprinted with permission from ref 284. Copyright 1999 Biacore, AB.)

environment of the metal will change the wavelength of light that interacts with the plasmon and lead to a reduction in the intensity of the reflected light measured at a specific combination of wavelength and angle (Figure 26). The magnitude of the shift is quantitatively related to the magnitude of the changes in the chemical environment, especially increases in mass close to the surface, and is measured as a change in the refractive index of the reflected light. BIA has been applied to studies of protein:protein interactions (e.g., antibody:antigen, protein:DNA interactions) and to the analysis of small combinatorial libraries.[282,285]

The levels of analytes bound to the BIA chips is approximately the same as needed for MS analysis. This makes the concept of interfacing a BIA instrument with mass spectrometry attractive for both researchers using BIA instrumentation and for mass spectrometrists. BIA instruments have been combined with mass spectrometers in several ways. Nelson and co-workers have used direct MALDI analysis from the sensor-chip surface.[281,282] After the SPR experiments were performed, the flow cells on the chip were washed rigorously to remove extraneous material. The flow cells were removed, MALDI matrix was added, and the chip was placed in a holder that served as a target for the laser. A ternary interaction complex between polyclonal antihuman myoglobin IgG bound to the flow cell surface, human myoglobin, and a monoclonal anti-human antibody was investigated with femtomole levels of myoglobin and the monoclonal myoglobin antibody being detected in the MALDI spectrum.[287] Nelson, Krone, and Jansson also performed an identical experiment which used a fiber optic-based BIA instrument where the affinity agent was immobilized on the fiber that was subsequently dipped into a solution containing the analyte.[288] In similar experiments using the chip-based system, a MALDI spectrum of 20 fmol of myotoxin α isolated from prairie rattlesnake venom by interaction with a bound polyclonal antibody was obtained with a signal-to-noise ratio of 19:1.[286,287] The authors have also demonstrated the ability to perform isolation of a protein by affinity capture and digestion by immobilized enzyme in different cells of the same chip. The direct MALDI/MS analysis from the chip provided good sequence coverage from 100 fmol of protein.[289] Sönksen et al. have eluted the analyte from the chips for subsequent MALDI analysis rather than using direct MALDI analysis with similar results.[290] MALDI spectra from femtomole levels of myoglobin and ParR were reported. These authors pointed out that elution of the analyte provides for more versatility in the types of MS analyses finally utilized.

To date, there have been no reports of the direct coupling of BIA instrumentation to MS or to LC−MS. The microfluidics used in BIA instrumentation are compatible with ESI−MS flow rates, as are the analyte levels. It should not be extremely difficult to devise a means of coupling the flow from the BIA instrument directly to an LC column interfaced to an MS either directly or through a trapping/desalting column in a manner similar to that discussed above for IMAC and IAC. This research area is potentially ripe for exploitation with existing instrumentation and methods.

3. Affinity Capillary Electrophoresis–MS

Affinity capillary electrophoresis (ACE) is a means of probing molecular interactions by measuring differences in the electrophoretic mobility of a compound in the absence vs the presence of a potential ligand due to complex formation, and this technique has been recently reviewed.[291−296] Although MS characterization of ligands that are found to undergo changes in mobility in the presence of a receptor should be attractive, especially from complex mixtures such as combinatorial libraries, there have been relatively few reported applications, possibly due to competition with experiments based on noncovalent complex formation. The reported applications include several examples of on-line MS detection of candidate ligands for vancomycin and an example of epitope mapping.[297−300]

H. Supercritical Fluid Chromatography–Mass Spectrometry

Supercritical fluid chromatography (SFC), in which a gas at a pressure above its critical pressure is used as the mobile phase, has also been combined with mass spectrometric detection. SFC has been most widely applied in the separation of low to moderate polarity analytes and generally has a wider range of optimum linear velocity with a 3-fold increase in the number of theoretical plates per unit time compared to HPLC. The recent interest in small molecule combinatorial library screening for potential drug candidates is beginning to rekindle interest in this area.[301,302]

The most commonly used mobile phase in SFC for the analysis of low-to-medium-polarity analytes is CO_2.[303] Polar mobile phase modifiers, such as methanol or water, and a weak base or acid additive are often added to the supercritical fluid mobile phase for the separation of more polar analytes.[304] Pressure and temperature gradients are often employed to improve the separation, as well as modifier gradients.

1. Interface Design

Because of the large expansion volume of the supercritical fluid introduced at the end of the column, most early research using chemical ionization or electron impact based MS sources focused on the use of capillary columns.[305] As in the case of

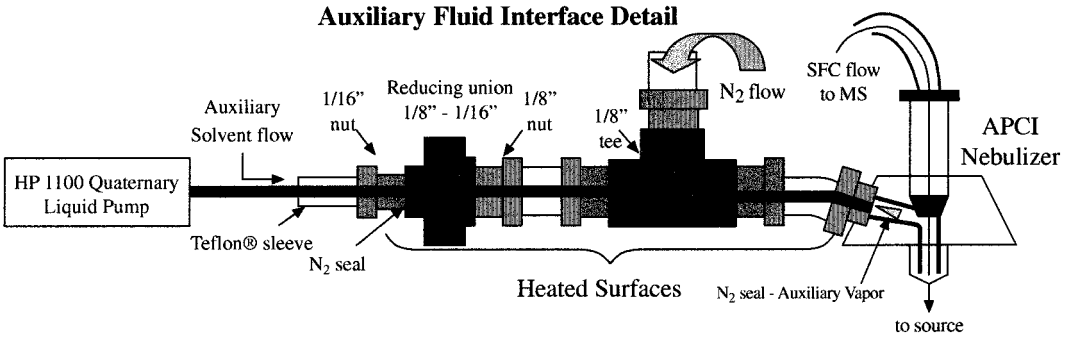

Figure 27. Block diagram of an SFC−MS system. The auxiliary fluid interface, shown in detail below, is designed to allow the quaternary liquid pump to deliver APCI-enhancing solvent to the source in vapor form using heated stainless steel surfaces. This setup delivers the auxiliary fluid vapor through the nebulizer nitrogen line into the APCI source. (Reprinted with permission from ref 308 Copyright 1999, American Chemical Society.)

coupling HPLC with MS, the commercialization of atmospheric pressure ionization techniques resulted in SFC becoming significantly more compatible with MS analysis. One important feature of most SFC−MS interfaces is a heated transfer line to compensate for cooling due to the expansion of the supercritical fluid and to vaporize any modifiers (Figure 27).[301,306−308] The majority of reported interfaces are based on the standard instrument probes with nebulizing gas and makeup flow capabilities. It has been noted that heating is more important for APCI while the use of a makeup flow to transfer the analytes from the expanding CO_2 gas to the liquid phase is important in ESI.[309]

Pinkston and co-workers have also developed an interface between packed column SFC and APCI/MS using a TurboIonSpray source. The high flow rates accommodated by the TurboIonSpray source are compatible with the high volume of mobile phase delivered by the packed column, e.g., 1.5 L/m at standard temperature and pressure.[304] It was noted, however, that as the mobile phase transitions to the gas phase in the transfer line leading from the end of the column to the spray needle, the solutes may no longer be soluble and phase separation of the mobile phase into liquid and vapor phases may occur. This can result in peak broadening, poor peak shape, detector noise, and poor quantitation. To eliminate these problems, a pump was incorporated in place of the back-pressure regulator normally used in SFC.[310] The pump maintains the mobile phase at a supercritical pressure until the spray needle. It was also observed that use of methanol as the fluid in the pressure control pump was essential for obtaining good ion abundances.

Morgan et al. found that, for lower molecular weight aromatic compounds (e.g., nitrobenzene and 4-fluorophenyl sulfone) separated by SFC without the use of an organic modifier, SFC could be directly connected to APCI/MS without heating the transfer line when a 75 μm id line is used.[311] Baker and Pinkston, however, found that this design led to unacceptable band-broadening for the compounds they studied.[304]

2. Applications

Although the majority of the papers reporting on SFC−MS since 1995 have focused on optimizing interface designs using commercially obtained standards, there have been several applications reported. Broadbent et al. have used SFC−MS in the analysis of photoproduct formation from the sunscreen component 2-ethylhexyl-*p*-methoxy cinnamate,[312] SFC−MS has been employed in the analysis of phenolic Mannich bases used in epoxy resins by Fuchslueger et al.,[313] and the separation of thiohydantoin derivatives prepared as part of a combinatorial library has been reported by Ventura et al.[301] Although several combinatorial library applications have been reported at conferences, few have appeared in the refereed literature, possibly due to patent concerns.

I. Gas Chromatography–Mass Spectrometry

Although gas chromatography−mass spectrometry (GC−MS) is often considered a mature field, there continue to be developments in the area, especially in terms of increasing speed of analyses. The concepts behind high-speed GC and current methods of minimizing GC analysis time have recently been reviewed by Cramers and colleagues at the Eindhoven University of Technology.[314,315] High-speed GC analysis, with peak widths that may be less than 100 ms,[316,317] presents problems for mass spectrometric detection similar to that presented by fast LC and by CE. The narrower in time the peak being presented to the mass spectrometer, the more difficult it is for the mass spectrometer, especially scanning mass spectrometers, to acquire unbiased mass spectra. The term "unbiased" alludes to obtaining a mass spectrum in which the relative abundances of the masses within the spectrum are not skewed due to rapidly changing analyte concentration on the mass spectral data acquisition time scale. In GC−MS, electron impact (EI) is a primary ionization mode and, under EI conditions, significant fragmentation of the analyte ion often occurs. The observed fragmentation pattern, including relative abundances as well as fragment masses, are often used for analyte identification by (computerized) comparison with "standard" spectra. If the spectrum is skewed, the correct identification may not be made. This aspect of narrow (in time) peak presentation is more acute for GC−MS than LC−ESI−MS, because for the most part, molecular ion species are the dominant species in ESI spectra, with relatively little fragmentation. Deconvolution of overlapping components during high-

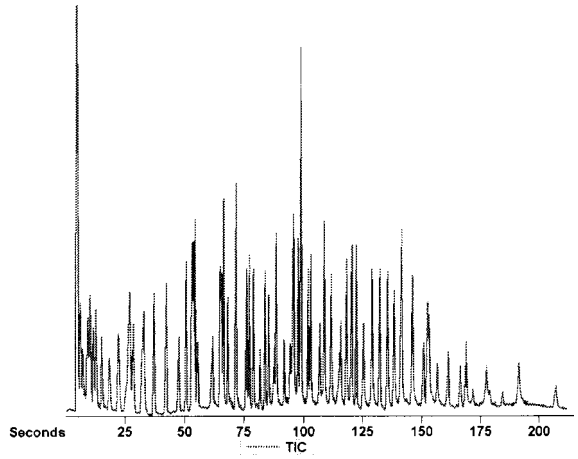

Figure 28. Fast GC−TOF-MS of an 85 component flavor and fragrance standard in ca. 200 s. (Reprinted with permission from ref 325. Copyright 1997.)

speed analyses is, as in fast LC− and CE−ESI−MS analysis, another problem exacerbated by rapid separations.

Quadrupole-based mass spectrometers typically acquire less than 40 scans/s (25−50 ms/scan) over a 100 Da mass range, which is insufficient for fast GC. Grimm and co-workers, however, have operated a linear quadrupole MS in region II of the Mathieu a/q stability diagram (a proportional to the applied dc voltage and q being proportional to the applied RF voltage), where $a \cong 0$ and $7.514 < q < 7.580$.[318] Operating in this region results in fewer rf cycles being required for unit mass resolution and higher kinetic energies can be applied to the ions to pass them into the quadrupole. This results in faster scan times, with 5 ms (1000 scans/s) scan over an 80 Da mass range being demonstrated. Operating in this stability region, however, leads to decreased sensitivity as well as more stringent requirements for the electronics.

The early Bendix TOF MS, with a 10 kHz acquisition rate, has been used as a GC detector at an acquisition rate of 1−2 scans/s.[319,320] As for mass spectral detection of narrow in-time CE peaks, time-of-flight mass spectrometers have again attracted significant attention for use with fast GC. Holland et al. and Wollnik et al. reported the development of time-of-flight detection for fast GC in the early 1990s.[321−323] A GC−TOF−MS from LECO Corp.,[324] the Pegasus II GC/MS developed from the work of Holland, is commercially available. This instrument incorporates orthogonal injection of the ions into the time-of-flight analyzer and reflectron technology for improved mass resolution. A spectral acquisition rate of 500 spectra/s over 5−1000 Da is specified. Brichford and Parry from LECO demonstrated the separation of flavor mixtures by Fast GC−TOF−MS 20 times faster than by traditional GC−MS (Figure 28).[320,321] Peak deconvolution of overlapping components was essential in the identification of the analytes.

Amirav and co-workers at Tel Aviv University have developed and applied a supersonic molecular beam (SMB) interface for fast GC−MS analysis. This interface is based on the expansion of the GC effluent

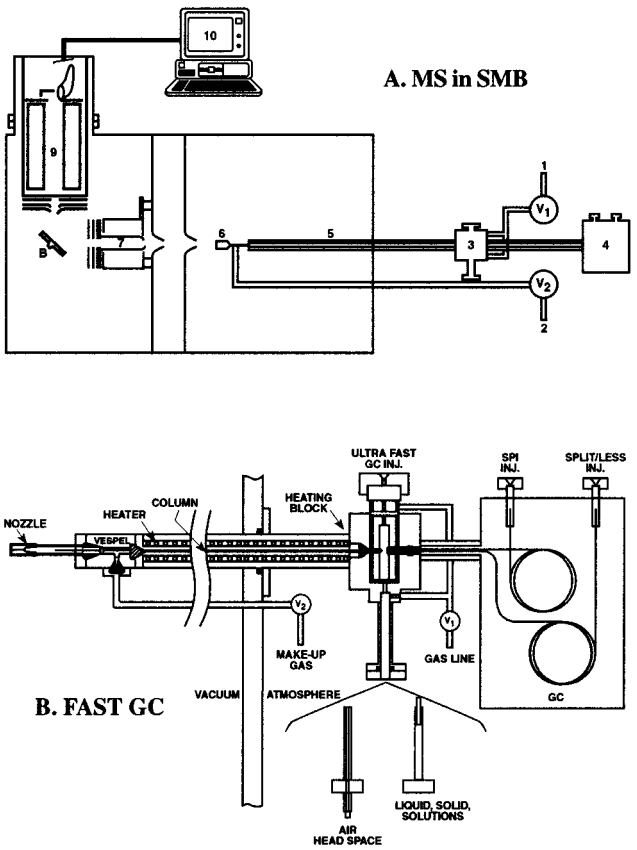

Figure 29. Schematic of the supersonic molecular beam fast GC−MS interface. The carrier gas flow is controlled by needle valves (1) and (2). The sample is injected or introduced into the vaporizing oven (3) and flows through the temperature controlled capillary column (4). The gas mixture expands through a supersonic nozzle (5) and is doubly differentially pumped. The sample molecules are ionized in the EI source (6) or by the surface (7). The EI produced ions are deflected by the ion deflector (7) and mass analyzed by the quadrupole mass spectrometer (8). (Reprinted with permission from ref 329. Copyright 1996 American Society for Mass Spectrometry.)

flowing at about 2000 cm/s from atmospheric pressure through a ca. 100 μm diameter pinhole into a vacuum.[327] A skimmer in the path of the jet forms a supersonic molecular beam with most of the carrier gas not being collected (jet separator) (Figure 29). During the supersonic expansion, the molecules are vibrationally supercooled which minimizes dissociation under EI conditions and leads to abundant molecular ions, although fragment ions are often also formed. These authors noted that the SMB approach is useful for thermolabile substances. The authors have reported a number of examples, ranging from octacosane, whose 18 eV SMB−EI MS spectrum shows only a molecular ion while fragment ions can be formed at 70 eV (molecular ion is still the base peak),[328] to thermally labile retinol in under 1 min,[329] to ovalene.[322,325,326]

IV. Concluding Remarks

Given the continuing interest in and continued advances in the development and application of separation techniques coupled with mass spectrometry, it is probably more appropriate to title these

remarks as interim rather than as concluding remarks. In the past decade, mass spectrometry has attained a position as a standard analytical methodology for a wide variety of chemical and biological problems, from small molecule structure identification to large protein characterization, and the improved ease of coupling separations with mass spectrometers has played a significant role in this attainment.

Developments in separations−mass spectrometry continue at a significant pace. The trend toward miniaturization in separations significantly influenced improvements in MS source and analyzer designs. Conversely, the increasing sensitivity and speed of mass spectrometers have encouraged continuation of the miniaturization trend. Especially critical for the wide-spread acceptance of small-scale separations−MS has been the development of commercial, turn-key systems by instrument manufacturers. These trends in miniaturization are continuing with capillary and nanoscale capillary HPLC−MS and capillary electrophoresis−MS becoming standard laboratory techniques, as well as rapid developments are occurring in capillary electrochromatography, and microfluidic device-based separation systems. The next 4 or 5 years should prove whether these latter newer techniques fulfill their promise and become widely accepted with significant applications appearing based on them. Improved separations schemes for very complex mixtures have been developed based on in-series or orthogonal separation schemes coupled with MS, and such approaches should see wider application, especially in the analysis of biological samples.

The trend toward faster separations, particularly for quantitative analyses, has generated widespread interest, especially in pharmaceutical and contract laboratory settings. If the results from high speed analyses continue to show reasonable detection limits, reproducibility, and accuracy, the development of fast LC−MS and fast GC−MS methodologies will become increasingly important in drug monitoring and metabolism studies, and trace environmental analysis.

The development of new separation technologies that can be hyphenated (interfaced) with mass spectrometry, e.g., surface plasmon resonance, and new means of interfacing separation techniques with MS, e.g., LC-MALDI, should continue. These may well lead to new applications that are especially suited to the new instrumental capabilities and the potential for interfacing new separation technologies with mass spectrometry may lead to more rapid instrumental developments.

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