

# Recent Developments in Column Technologies for the Analysis of Proteins and Peptides

Column developments in HPLC have benefited protein and peptide separations in a number of ways. More silica based phases and column formats have become available, expanding the use of HPLC for the separation of proteins by different modes of chromatography as well as improving resolution and throughput. Hydrophobic interaction chromatography, immobilized metal affinity chromatography as well as ion exchange and size exclusion are now practical analytical tools for proteins and peptides. New enrichment columns are benefiting proteomic research.

**H**igh performance liquid chromatography (HPLC) has become the workhorse of the biotechnology and pharmaceutical industries where it is used to identify, characterize and purify molecules at all stages of a process from research and development to quality control and validation. The progress in HPLC since the early 1970s has been partially responsible for the many advances in biochemistry and biotechnology. With a variety of separation modes to choose from which exploits the different chemical parameters of biomolecules, HPLC has established itself as indispensable tool for the separation of native amino acids, peptides, and proteins as well as an array of other biomolecules such as carbohydrates, nucleic acids, and lipids. In particular, the modes of reversed-phase, ion-exchange, size-exclusion (gel filtration), affinity, and hydrophobic interaction chromatography have been applied for biomolecules separations. Continual improvements in HPLC column technology have provided better chromatographic resolution, faster separations, higher analyte recovery, and greater sensitivity for these molecules. With a high level of interest in the separations of proteins and peptides, particularly in the proteomics area, the development of specialty columns has accelerated. The explosive growth in liquid chromatography–mass spectrometry (LC–MS) also has led to new separation columns and mobile phases well suited to meet the optimized needs of this powerful detection and identification tech-

nique. The purpose of the article is to update my earlier paper (1) and review some of the recent advances in columns for proteins and peptides.

I will focus on two main areas: stationary phase chemistry and new column formats.

## New Stationary Phases

### Reversed-phase silica-based columns:

Although reversed-phase HPLC was used for the separation of proteins and peptides before the 1980s, it was in 1982 that a milestone paper was published (2) that demonstrated how large pore silica (300 Å) could separate and resolve proteins much better than the typical small (100 Å) pore silica in common use. This discovery greatly accelerated the use of reversed-phase HPLC for protein separations. It also coincided with the rise of the biotechnology industry that has led to widespread use of reversed-phase HPLC for protein and peptide separations. This, in turn, was a major factor in changes to the regulation of protein therapeutic drugs by the FDA that began in 1995 and culminated in the recent transfer of regulatory responsibility for recombinant protein drugs from CBER to CDER.

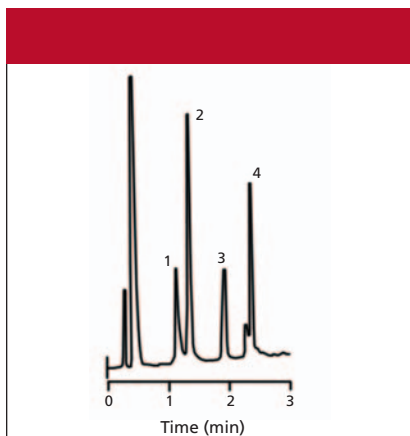
A second significant event in the field of reversed-phase HPLC was the development of high purity silica in the mid-1990s. Earlier silica gels (known as Type A) had traces of metal and higher surface acidity which gave problems with peak tailing and low recovery. This advance in silica quality has led to two developments that greatly have affected the use of reversed-phase HPLC for

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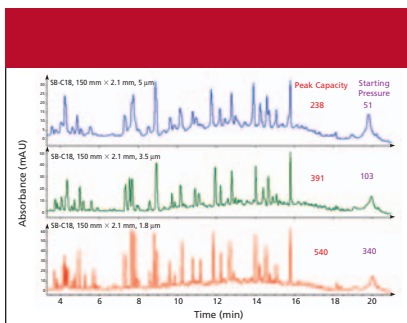


**Figure 1:** Separation of proteins using a small-particle silica column. Column: Alltech Prosphere C18, 1.5  $\mu\text{m}$ ; elution gradient: 25–75% acetonitrile with 0.1% trifluoroacetic acid in 4 min. Peaks: 1 = cytochrome c, 2 = lysozyme, 3 = b-lactoglobulin, 4 = ovalbumin. (Data courtesy of Alltech.)

protein–peptide separations: the use of small particle columns for rapid separations and improvement in resolution in critical applications, and the development of new phases with different selectivity.

Figure 1 shows the separation of several proteins in less than 3 min using bonded silica particles of 1.5  $\mu\text{m}$  in diameter. It is now possible with small particle columns to significantly reduce the time required for protein and peptide separations. The increased efficiency of sub-2- $\mu\text{m}$  particles allows the use of shorter columns and higher flow rates. On the other hand, smaller particles also can lead to higher backpressure that places more constraints on the instrumentation. Fortunately, these high throughput separations require columns of 50 mm and shorter so that pressure is not a major concern.

Although small particles are most often used to reduce analysis times and increase analytical throughput, when used in conventional size columns, small particles offer the potential for higher resolution of complex samples. Perhaps the most complex chromatographic analysis encountered in the life sciences is the separation of the many peptides resulting from protease digestion of entire proteomes before analysis by MS and identification by database searching. Because of the great complexity of these peptide mixtures, the procedure most commonly used, multidimensional protein identification technology (MudPIT) (3), results in the identification of only a small portion of the peptides and the proteins from which they come. Typically no



**Figure 2:** Separation of a peptide mixture using very small particle columns. Columns: Zorbax Stablebond SB-C18, particle size as indicated. 3.5- $\mu\text{m}$  Rapid Resolution; 1.8- $\mu\text{m}$  Rapid Resolution HT; gradient: 30 min, water–acetonitrile; temperature: 80  $^{\circ}\text{C}$ . (Data courtesy of Agilent Technologies, Santa Clara, California.) (Ref. 4)

more than 2000–3000 proteins are identified out of a total of 30,000 plus. Figure 2 shows that using these small-particle reversed-phase columns results in much higher peak capacity, which in turn results in more separated peptides, more peptides identified by MS, and more proteins identified. Using a column with 1.8- $\mu\text{m}$  particles, the peak capacity of a 30-min gradient was 540 compared to a peak capacity of 238, less than half that number, using a conventional 5- $\mu\text{m}$  particle reversed-phase column. Increased peak capacity will increase the number of identifiable peptides by at least that factor, resulting in a significant increase in the number of proteins identified. The likelihood of identifying proteins present at lower abundance also is increased because of an increased probability of finding and identifying the lower level peptides from such proteins.

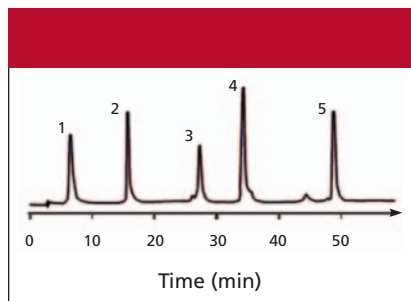
Other approaches also have contributed to further interest in high-throughput HPLC. The use of superficially porous reversed-phase columns with particles reminiscent of the 40- $\mu\text{m}$  pellicular packings of the late 1960s to the early 1970s but with dramatically reduced particle diameters (5  $\mu\text{m}$ ) have allowed the rapid separations of proteins (4). The reduced diffusion path length into the superficially porous layer permits the rapid mass transfer of large proteins into and out of the packing particle. Bonded nonporous silicas where the particles are much smaller (1.5  $\mu\text{m}$ ) received limited acceptance partly due to their limited surface coverage and resultant low capacity. Polymeric and silica-based monoliths are the latest innovation for high-speed separations and they will be covered later under new column formats.

A second benefit of using high purity silica is that new phases with distinct selectivity can be successfully prepared and used on high purity silica. Earlier attempts to affect selectivity by bonding polar groups to silica met with limited success because of the impure and variable nature of the silica surface. Since the advent of high purity silica, there has been a proliferation of stationary phases offering different selectivity characteristics that has broadened the range of selectivity for HPLC.

#### **Ion-exchange columns for protein separations:**

Although ion-exchange chromatography long has been used for protein separations, it generally has not had the resolution capabilities of reversed-phase chromatography. Ion exchange traditionally has been used for general-purpose purification of proteins. Although silica-based ion-exchange packings have been developed for the HPLC separation of ionic and ionizable compounds, polymeric phases appear to be in favor due to their ruggedness, high pH capability for analysis and cleaning, and variety of chemistries. Dionex (Sunnyvale, California) has developed several new ion-exchange materials based upon pellicular, hydrophilic polymer-based materials that offer much better resolution than previous columns. With these newer columns the use of ion-exchange chromatography has expanded and it is now widely used to monitor charge variants in biotechnology-derived and natural proteins. With these columns carboxy-terminal processing of monoclonal antibodies can easily be determined (1), as well as determining deamidation and monitoring the sialic acid content of glycoproteins.

Polymeric packings based upon polyhydroxymethacrylate offer a “biofriendly” material. Showa Denko (Kawasaki, Japan) has developed a series of strong and weak cation- and anion-exchange capillary columns (0.3-mm i.d.) called the Shodex MS-IEC series while their Japanese rival, Tosoh Bioscience (Montgomeryville, Pennsylvania) has developed similar macroporous cation and anion resins (BioAssist S and Q, respectively) with 1200  $\text{\AA}$  (cation) or 4000  $\text{\AA}$  (anion) pores that allow access to the binding sites for very large biomolecules resulting in improved capacity and resolution. Supelco’s (Bellefonte, Pennsylvania) wide pore (1000  $\text{\AA}$ ) polymethacrylate offerings (Discovery Bio PolyMS-SCX and -WAX) provides 0.3-mequiv/g ion-exchange



**Figure 3:** Hydrophobic interaction separation of five proteins. Column: Dionex HIC; gradient: 2 M ammonium sulfate to 0 M ammonium sulfate in 0.1 M monobasic sodium phosphate (pH 7.0). Peaks: 1 = cytochrome c, 2 = myoglobin, 3 = ribonuclease A, 4 = lysozyme, 5 = chymotrypsinogen. (Data courtesy of Dionex Corporation.)

capacity and a pH range of 1–13 for the strong cation column and 2–10 for the anion column.

Most recently, the use of capillary and nano-LC cation-exchange columns have found widespread use as a selective column for the first stage of the multidimensional chromatographic fractionation of complex protein digests in proteomics studies.

**Size-exclusion chromatography:** Size-exclusion columns are different from other chromatographic columns in that the goal is for the molecules to have no interactions with the surface of the particle. In this way the separation is based exclusively on the hydrodynamic size of the molecule determining the degree of penetration into the particle pores. Consequently size-exclusion columns, often called gel filtration when used with aqueous mobile phases, have been developed with highly hydrophilic surfaces to eliminate interactions with proteins. The few advances that have been made in recent years in size-exclusion materials rely on surface modifications for their uniqueness and in a reduction in particle size and column dimensions for improved speed. In this Special Supplement, Barth and Saunders (6) have done a good job of covering the newer aqueous-compatible size-exclusion columns suitable for protein separation and they will not be covered here.

**Hydrophobic interaction chromatography:** Hydrophobic interaction chromatography (HIC) is based upon the traditional method of protein purification of “salting-out” proteins using high concentrations of ammonium sulfate. In HIC, proteins are adsorbed onto a weakly hydrophobic support by removal of the shell of hydration surrounding the protein

using a strong (2 M) ammonium sulfate buffer. With the water of hydration substantially removed, proteins readily adsorb to a weakly hydrophobic surface. By slowly removing the salt in a reversed gradient (slow reduction in the ammonium sulfate concentration), proteins are rehydrated and desorb from the surface one by one and are separated. While resolution is less than that obtained with reversed-phase HPLC, HIC offers the benefit of non-denaturing conditions and has worked well for large-scale purification. Because of limited resolving power, however, it has been used much less for analytical separations. With the use of high purity silica, it is now feasible to produce much higher resolution HIC materials as evidenced in Figure 3. The resolution and peak shape in this separation of several proteins rivals that of reversed-phase and shows HIC to be a practical tool for protein analysis.

Some notable HIC columns that have surfaced are those produced by Tosoh Bioscience, Nacalai Tesque (Kyoto, Japan), and OraChrom (Woburn, Massachusetts). The ToyoPearl PPG-600M with a 750-Å pore size, a methacrylate base material and a polypropylene glycol surface chemistry has a high dynamic binding capacity (for example, for monoclonal antibody AntiLH = 38 mg/mL) and an exclusion limit of  $8 \times 10^5$  Da. It is recommended for the HIC separation of monoclonal antibodies, hydrophobic proteins and pegylated targets. The Cosmosil 5HIC silica-based column of Nacalai Tesque has a smaller pore size (300 Å) but because of its structural rigidity can take high flow rates and pressures and have no problems with swelling. OraChrom's Styros HIC column is based upon a copolymer of polystyrene-divinylbenzene and methacrylate that resembles a monolithic material and is functionalized with phenyl, butyl or ether groups. It can be cleaned in place with harsh reagents such as sodium hydroxide solution.

**Affinity chromatography:** Affinity columns are among the most selective of all chromatographic modes. Affinity chromatography encompasses a family of techniques in which an immobilized ligand is used to capture a specific molecule from solution based upon highly biospecific binding. Unlike differential migration chromatography, affinity separations could be referred to as digital chromatography because the analyte is “on” or “off” the col-

umn. The analyte is first strongly bound by an interaction such as antigen-antibody and then by either a displacement or step pH change, desorption rapidly occurs.

In this sense, affinity chromatography resembles solid-phase extraction. Affinity chromatography is often a step in the purification process.

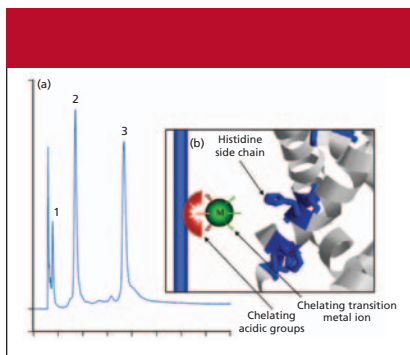
Users might wish to prepare their own affinity columns. Thus, some manufacturers supply a packed column or bulk media with a linker or spacer arm containing a reactive functional group such as epoxy, aldehyde, or amine. The user then covalently bonds the ligand of their choice. The column then becomes a very specific sorbent for the analyte to be purified out of a mixture of materials. A recent review of the isolation of antigens and antibodies by affinity chromatography was published (7).

Popular affinity phases for monoclonal antibody purifications are protein A and protein G. Quite a number of manufacturer's provide these media in low pressure or HPLC formats. Some commercial examples are Millipore's (Billerica, Massachusetts) Montage Antibody purification kit (Protein A and G), OraChrom's Styros Protein A, and Showa Denko's Shodex APG-M3T that has a protein G ligand.

Some suppliers will provide columns or media that have a specific ligand. An example would be the Styros Benzamidine (OraChrom) that has a *p*-aminobenzamidine functional group that specifically binds with a serine protease such as thrombin after tag cleavage with recombinant proteins.

**Immobilized metal affinity chromatography:** Although silica remains the preferred matrix for reversed-phase columns for protein-peptide separations, particles based upon new polymeric materials have added to the flexibility and utility of chromatography for such separations. Immobilized metal affinity chromatography (IMAC) has been used sparingly in the past for analytical and laboratory separations but is now becoming more useful for laboratory scale analysis and separations because of the availability of thin shell polymer materials. IMAC offers a different type of interactions for protein separations.

IMAC uses adsorbents with acidic groups that chelate transition metals such as copper and nickel (Figure 4). The side chain of the amino acid histidine, with a pK of about six, has a pair of electrons that coordinate with the positive charge on the



**Figure 4:** IMAC separation of several proteins based on histidine residues. Shown are (a) the resulting chromatogram and (b) a schematic of the mechanism of IMAC affinity adsorption. Acidic groups on the particles chelate transition metal ions which in turn bind histidine residues on proteins and peptides. Column: 250 mm  $\times$  4 mm Dionex ProPac IMAC-10; eluent A: 20 mM MES, 0.5 M sodium chloride (pH 6.0); eluent B: A plus 100 mM imidazole; gradient: 4–100% B, over 15 min. Peaks: 1 = ribonuclease A, 2 = myoglobin, 3 = carbonic anhydrase. (Data courtesy of Dionex Corporation.)

immobilized metals, and provide a basis for a highly selective retention of proteins and peptides containing histidine. Such specialized affinity columns can provide a new dimension of selectivity for protein separations. The mobile phase is similar to one used for ion-exchange chromatography, a buffered aqueous solution, and bound proteins are eluted with a competing compound, in this case imidazole, which resembles the side chain of histidine.

**Low trifluoroacetic acid columns:** The 2002 Nobel Prize in Chemistry recognized the development of MS techniques in the analysis of large biological molecules, particularly proteins and peptides. The electrospray ion source, developed in the 1980s by John Fenn, 2002 Nobel Laureate, has been responsible for a tremendous proliferation of LC–MS in the protein laboratory. A limitation of LC–MS in protein–peptide analysis is that the commonly used HPLC reversed-phase ion-pairing reagent for polypeptide separations, trifluoroacetic acid, binds so strongly to the polypeptide molecules that they became neutral complexes and, as such, cannot pass through the mass spectrometer and be measured. In affect, the trifluoroacetic acid reduces the ion signal obtained via the electrospray ion source.

Several solutions to the problem with trifluoroacetic acid have been investigated by the MS community including changing to a volatile and weaker reagent such as formic acid, and using very low concentrations of trifluoroacetic acid (less than 0.01%), which

do not affect the polypeptide electrospray signal very much. The problem with these solutions is that many HPLC columns give inferior peak shape (formic acid and low trifluoroacetic acid concentrations) and poor separations (low trifluoroacetic acid). In response to this problem, several companies have developed special silica reversed-phase columns that separate proteins and peptides very effectively at these low concentrations of trifluoroacetic acid. These companies remain silent on the methods they use to achieve this result, however it is believed that modification of the silica surface is at the heart of these so-called “MS compatible” columns. A detailed study on the effect of mobile phase additives on sensitivity in the analysis of proteins and peptides by HPLC–electrospray MS was recently published (8).

**Protein depletion to enhance biomarker identification:** Proteomic techniques seek to identify and quantitate expression changes in many thousands of proteins. The number of proteins is so large that more abundant proteins tend to overwhelm the chromatography, MS, and computer systems and preclude meaningful identification of lower abundance, and often important, proteins. Nowhere is this more important than in the analysis of plasma samples in the search for disease biomarkers. Highly abundant proteins such as IgG, IgA, transferrin, haptoglobin, and antitrypsin (Figure 5) represent 90% of plasma proteins and mask potential disease biomarker proteins. Protein depletion columns have become available from a number of sources that contain particles with covalently attached antibodies to the highly abundant proteins. The plasma sample is passed through the column; highly abundant proteins are retained on the column by the immobilized antibodies while most proteins containing those of interest are eluted. Proteomic analysis of the less abundant proteins that wash through the column can then proceed by conventional methods. Depletion of the sample of the most highly abundant proteins clears the way for better detection and identification of lower abundant proteins such as disease biomarkers.

### New Column Formats

**Monolithic columns:** In the quest for new column technologies to give faster and higher resolution separations, many researchers have reported on work with “monolithic” columns. These columns are

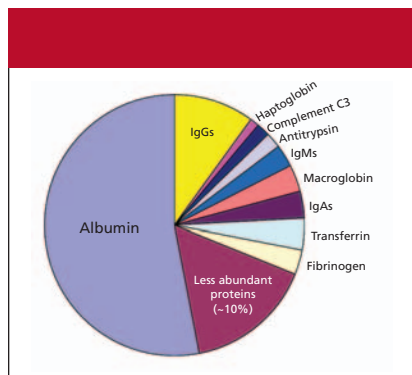
not packed with conventional small particles, but rather are formed as a single rod of very porous material that is encased in a column “package.” The materials used have been either silica gel or a variety of synthetic organic polymer materials. The advantage of the monolithic columns is high permeability, enabling these to be used at relatively high flow rates with reasonable backpressures. The final result is generally much faster separations with resolution comparable to that obtained with conventional particle columns.

There are basically two types of monoliths: silica based and polymer based. The silica-based monoliths were first described by Tanaka and colleagues (9,10). These monoliths have been commercialized by Merck KGaA (Darmstadt, Germany) as Chromolith. These columns are available in capillary (0.1 mm i.d.), analytical (4.6 mm i.d.), semipreparative (9.4 mm i.d.) and preparative (25 mm i.d.) dimensions. Phenomenex (Torrance, California) also markets a version of this product called Onyx. The silica-based monoliths seem to have the best separation properties for small molecules.

Several polymeric monolithic columns are now commercially available from Dionex/LC Packings, BIA Separations (Ljubljana, Slovenia), BioRad (Richmond, California), and Sepragen (San Leandro, California). The polymeric monoliths have large porous channels and are therefore more suitable for proteins. These monolith columns have the potential to significantly speed up protein and peptide separations. For more information on monolithic columns, see the article by Svec and Geiser in this issue (11).

**Capillary, nano, and chip columns:** The use of columns with small internal diameters (< 4 mm) has increased in use in recent years, in part because small diameter columns use much less solvent and provide higher sensitivity than traditional analytical columns and, in part, because they are more compatible with coupling to MS. LC–MS with electrospray MS today largely uses “narrow bore” columns of about 2-mm diameter because the flow rates of these columns are compatible with pneumatic-assisted electrospray ion sources (typically, 0.2–0.4 mL/min). Capillary columns (0.1–0.5 mm i.d.) also are used with conventional electrospray operating at flow rates of a few microliters per minute. Proteomic analysis, consisting of large scale sep-



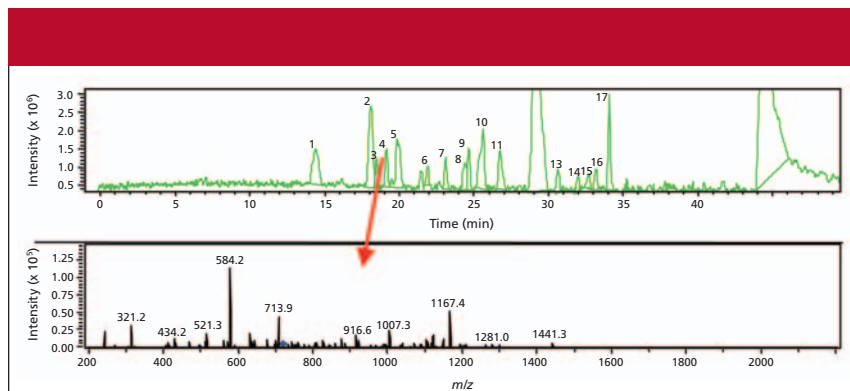


**Figure 5:** Proteins in plasma. (Data courtesy of Sigma-Aldrich, St. Louis, Missouri.)

ation and identification of proteins, has moved in the direction of using “nanoelectrospray” which requires submicroliter-per-minute flow rates. Nano columns are generally of 50 or 75  $\mu\text{m}$  in internal diameter and flow rates are typically in the range of several hundred nanoliters per minute. The great deal of work going on in proteomics greatly has expanded the use of capillary and nanocolumns. These are available from a variety of suppliers, packed with the same materials as used in larger diameter columns. The drawback is that specially adapted capillary- and nano-compatible HPLC instruments must be used because of the very low flow rates. Such instruments are now available from several suppliers.

The primary benefit of using capillary and nanocolumns in proteomic analysis is sensitivity — the ability to detect extremely low levels of proteins. Because of the complexity of proteomic samples, often containing thousands of peptides, high resolution is required together with very high sensitivity. These smaller diameter reversed-phase columns coupled with electrospray MS enable separation and identification of very large numbers of peptides in a matter of 2–3 h.

To chromatographically resolve such complex peptide mixtures and to identify individual peptides as needed by MS, researchers have resorted to multiple chromatography modes. MudPIT uses two chromatography steps interfaced back-to-back with various. The first step is to trap the tryptic digest peptides on a cation-exchange column and then by step or continuous salt gradients groups of peptides are serially injected into a reversed-phase column and further separated by gradient elution. The peptides are detected by tandem MS and attempts are made to identify them using vast spectral libraries.



**Figure 6:** Example of use of LC-Chip-MS sensitivity in the analysis of 540 amol of human serum albumin. (Data courtesy of Agilent Technologies.)

From the peptide identification, some of the proteins can be identified.

One of the problems of successfully performing nano-LC–MS is the ability to minimize system dead volumes so that the full potential of the nano-LC column and the high sensitivity of the mass spectrometer can be used to separate and analyze the tens of thousands of peptides in a typical digest. A typical nano-LC column has the dimension of 150 mm  $\times$  50 or 75  $\mu\text{m}$  with total volume in the microliter range. With the typical LC connections, just about any extracolumn volume can destroy the high resolution separation. An approach that appears to be gaining strength in the proteomics community is the use of microfluidics where the entire column and MS interface is built onto a polyamide chip. The packing is held in the chip by a lamination process and there are no traditional fittings and connections required in LC–MS. The LC-Chip–MS interface (available from Agilent Technologies) mounts on inlet of a mass spectrometer. The chip is inserted into the interface, and the electrospray outlet of the column, which is part of the chip, is optimally positioned in the source. An example of a separation and the sensitivity that can be achieved is depicted in Figure 6. The figure shows a separation of a human serum albumin tryptic digest using a reversed-phase C18 chip column with electrospray MS detection. The sample was run under typical gradient conditions using a water–acetonitrile–formic acid gradient.

## Conclusion

HPLC has become a standard in the separation, analysis and purification of proteins and peptides. These uses now range from isolation of natural peptides and proteins, to characterization of therapeutic,

biotechnology-derived proteins, to purification of large quantities of protein therapeutics to separation of complex proteomic samples. Although conventional columns packed with 5- $\mu\text{m}$  spherical silica-based particles continue to dominate the field, several recent technical innovations in column length and particle size reductions and monoliths offer faster and/or high-resolution separations, often in less time than conventional columns. Greater compatibility with MS is also an important part of the benefits of much new column technology. To resolve complex peptide mixtures, multidimensional chromatography is quickly becoming an alternative to 2D gel electrophoresis.

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