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Liquid Chromatography

Liquid Chromatography—Its Development and Key Role in Life Science Applications

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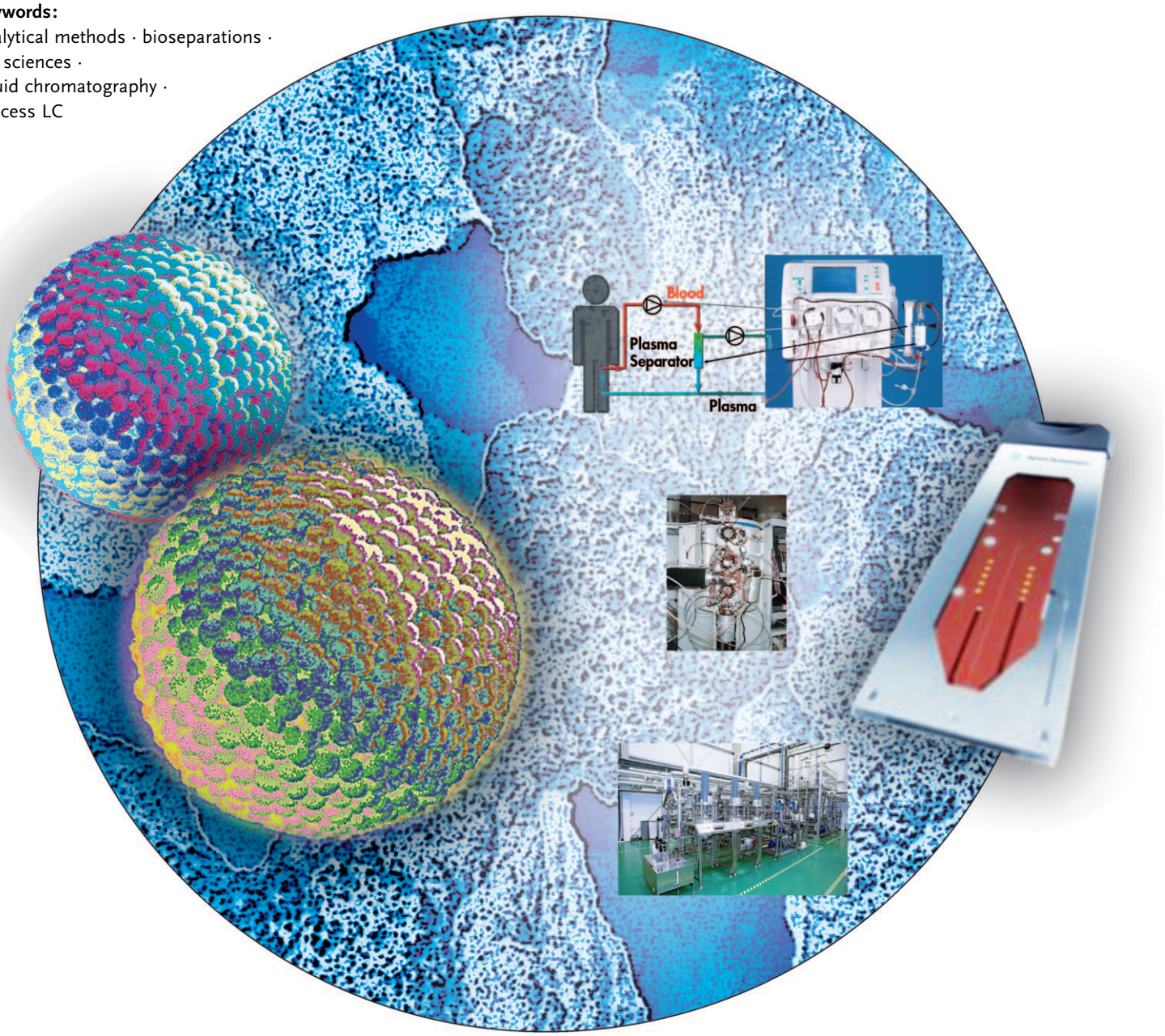

Liquid chromatographic methods cover the broadest range of applications imaginable today. Nowhere is this more evident and relevant than in the life sciences, where identification of target substances relevant in disease mechanisms is performed down to the femtomole level. On the other hand, purification of therapeutic drugs on a multiton scale is performed by process LC. The complexity and abundance range of biological systems in combination with the extreme purity requirements for drug manufacturing are the challenges that can be mastered today by chromatography, after more than a century of research and development. However, significant improvement is still required for a better understanding of the scientific fundamentals of the underlying phenomena and exploiting those for an enhanced quality of live.

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1. Introduction

This Review attempts to follow the path from the discovery of liquid chromatography (LC) through a stochastic evolution to the current state of development—in particular, in the life sciences.

Liquid chromatography has developed into the most widely practiced analytical method today. While there are various alternatives, including spectroscopic and other techniques, for the analysis of small molecules and materials, LC is playing an increasingly important role in unraveling the secrets of the modern life sciences. Furthermore, LC is the only method that offers equal performance in analytical as well as in preparative and process applications. The production of modern biotherapeutics, such as recombinant proteins and antibodies, would be virtually impossible without several chromatographic recovery and purification steps.

The objective of this Review is to show the development of the various branches of LC from a historical perspective, and to highlight the achievements and advancements of LC in the life sciences in particular. In this context, we understand "life sciences" in a broader sense as being the science concerned with plants, animals, humans, and other organisms. The life sciences can be grouped into endogenous applications (clinical diagnostics, therapeutic applications, and metabolism) and exogenous applications (of nutrients, contaminants, pollutants, and allergenes). Drug discovery, development, and production are also considered, and even the first isolation of plant constituents by Tswett as well as by Kuhn and Lederer was on life science samples.[1,2] The separation and isolation of proteins began in the 1950s, and has been improved continuously over the following decades—predominantly in the preparative field—and has resulted in the first successful production-scale purification of recombinant proteins for therapeutic applications.[3] The application of LC in the life sciences suffered more than other areas from a lack of selective detection. Refractive index (RI) and ultraviolet (UV) detectors, both single and multiple wavelength systems, did not provide the required selectivity and sensitivity, while fluorescence and electrochemical detectors lacked universality. A breakthrough occurred when mass spectrometry (MS) was applied to the characterization of peptides and proteins, thus enabling their identification and purity control. The coupling of LC to MS provided the basis for an analytical platform able to support the demands of the postgenomic era.

2. Major Achievements in LC and Their Implications.

Before focusing the discussion on the specific contributions and potentials of LC in life sciences, the general development of LC merits some consideration. Adsorptive separations were already practiced in the 19th century.[1,2] Tswett, however, was the first to adopt the term "chromatography" in 1903 for the isolation of chlorophyll constituents.[1,2] The practical use of this method was strongly dependent on finding suitable and sufficiently well characterized adsorbents. This finding provided an early indication that mastering liquid chromatography requires a mix of skills, including proper material selection and characterization, as well as a good understanding of liquid–solid interactions.

Kuhn and Lederer in Heidelberg, Germany, returned to the isolation of plant constituents two decades later.[4] In the course of this study Kuhn and Brockmann recognized the need for more reproducible and also more selective adsorbents that were specially tuned for specific separation problems.[1] This recognized demand for standardized and reproducible stationary phases later led to the first materials standardized in terms of adsorption strength, and describes the first attempt towards reproducible separations.

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After these early approaches, applications of liquid chromatography appeared more rapidly between 1960 and 1970 when high-performance liquid chromatography (HPLC) was developed as an analytical tool in addition to gas chromatography.[5]

At this time, gas chromatography (GC) dominated the analytical field, being driven by the needs of the petrochemical industry.[5] However, demands also grew for the efficient separation of polar and charged compound mixtures in the field of pharmaceutical and environmental analysis, as well as for purity control of industrial chemical products. Classical LC involved the use of coarse silica or alumina particles packed in large-bore glass columns. The gravity-operated separations were performed with organic eluents at linear velocities of about 0.01 mm s^{-1}, thus the separation times were very long.

The investigation of mass-transport processes and the distribution equilibria of analytes in a LC column as well as consideration of the hydrodynamics led to equations that gave clear directions of how to substantially improve classical column liquid chromatography into a more efficient, modernized version: reduce the particle size of the adsorbent to about 10 μm, pack these particles into pressure-stable columns, and operate the separations at high and constant linear velocities of about 1 mm s^{-1}.[6]

Mechanically stable microparticulate silica adsorbents were later manufactured, cut into narrow size fractions, and densely packed into stainless-steel columns.[7] These columns needed to be operated by pulsation-free pumps andfitted with proper injection systems and flow-through cells for continuous detection. Thus, material development drove the design and construction of chromatographic instruments.

This interplay between improvements in the adsorbents, proper column design, and corresponding system development became the typical innovation cycle during the exploration phase of HPLC, and has continued as such over the last 50 years.

When discussing the development of LC as an analytical method it is often overlooked that liquid chromatography was first practiced as a purification method, and thus started as a preparative method. It is probably fair to state that liquid chromatography is the only technique that enables the separation and identification of both femtomolar amounts of compounds from complex matrices in the life sciences, but also allows the purification and isolation of synthetic industrial products in ton amounts.

A critical assessment of LC reveals that the development of modern LC methods and the corresponding technologies are based on three main pillars, which have not only developed largely independently from each other, but also on different time scales (Figure 1).

It is not really surprising that development within those pillars has not seen a lot of "cross-fertilization". For example,

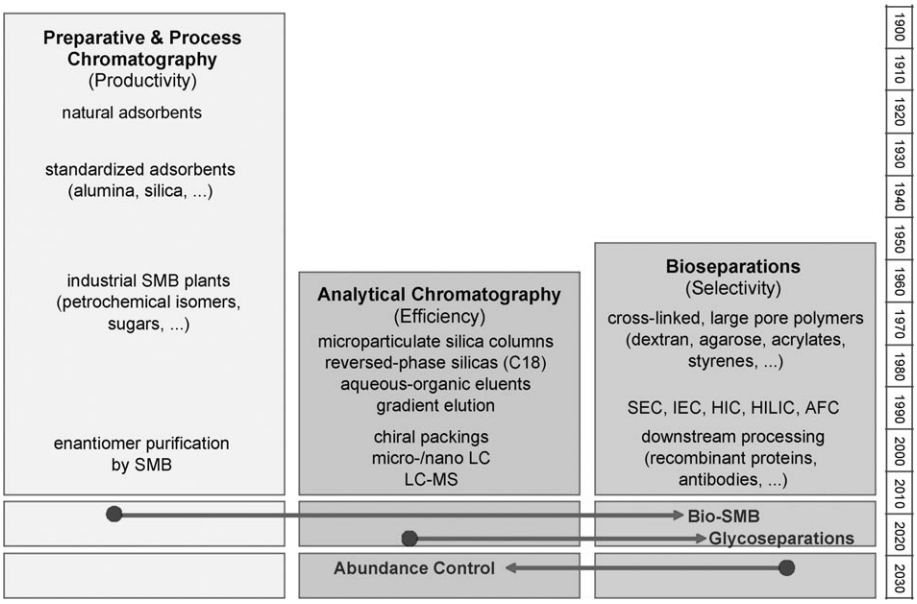

Figure 1. The three pillars of liquid chromatography: historical development, potential, and cross-fertilization.

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the "restart" in the field of preparative and process chromatography after the dormant period between the 1930s and the middle of the 1960s was not induced by the parallel emergence of HPLC, but by engineers searching for more-effective purification technologies in the petrochemical and food processing fields through the development of continuous countercurrent concepts, such as simulated moving-bed (SMB) separations.[8,9]

While preparative as well as analytical liquid chromatography relied heavily on equipment, engineering, and on the physical aspects of their systems for advancements, the category of "bioseparations" (right in Figure 1) was based around a different key aspect, namely selective materials which allowed the processing of biopolymers (recombinant

proteins) under nondegrading conditions that enabled their bioactivity to be maintained. There was much less focus in this area on process engineering aspects, which led to the interesting phenomenon that large-scale production concepts of proteins were designed around the mechanical instability of soft gels.[3]

2.1. Preparative and Process LC

Engineering of continuous process chromatography, such as the simulated moving-bed technology, combined with the optimization of materials for specific separation tasks led to very efficient large-scale processes in the petrochemical area and in food processing pioneered by Union Oil Products Inc.[8,9] The concept of SMB chromatography was adapted in the 1990s to transfer the technology to the production of pharmaceuticals (by the development of smaller instruments).[10,11] Several types of highly selective adsorbents were developed during the 1980s for the resolution of racemates into enantiomers, mainly for analytical HPLC applications.[12–14] The availability of enantioselective materials in bulk quantities and packed in large-bore stainless-steel columns under dynamic axial compression (DAC)[15,16] enabled the production of pure enantiomers in the multi-ton range by the SMB technology. Productivities larger than 10 kg of pure product per kilogram of packing per day were achieved in the following years (Figure 2).

Figure 2. View of a production-scale SMB plant. Reproduced with permission from R.-M. Nicoud (Novasep, Vandeuvre-les-Nancy, France).

2.2. Analytical HPLC

Analytical HPLC was significantly influenced at the start by GC, and was applied to the analysis of pharmaceuticals and synthetic chemicals. The most important developments in the 1970s were the manufacture of microparticulate spherical silica adsorbents with a narrow particle-size distribution and particle diameters smaller than 10 μm, their transformation into *n*-octadecyl- and *n*-octyl-bonded derivatives (reversed-phase (RP) silicas), and efficient and reproducible packing into stainless-steel columns.[7,17] Over the course of the last decades the particle diameter of packings was continuously reduced from 5 to 3–4, and more recently to less than 2 μm to maximize the column efficiency, to achieve fast analysis, and to enhance the sensitivity.[18] HPLC systems with columns packed with ethyl-bridged sub-2 μm hybrid silica particles were recently designed and operated at pressures of up to 1500 bar; such systems are called ultra-high-performance liquid chromatography (UPLC) systems.[18,19]

For research applications, the inner diameter of the column was subsequently reduced from the traditional 4.6 and 4.0 mm to 2.0 and 1.0 mm; the aim of this development was to increase the sensitivity of detection because of the reduced dilution of the samples. Such so-called microbore columns can still be operated with conventional LC equipment at flow rates between 0.1 and 5.0 mL min^{-1}.

The real breakthrough in miniaturization occurred around 1980 with the introduction of fused-silica capillary columns coated with a polyethyleneimine layer.[20,21] The inner diameters of the columns varied between 300 and 20 μm. The operation of such columns required special micro- and nano-LC instruments, which generated flow rates in the range of μl min^{-1} to nl min^{-1}.[22] The capillaries were packed with microparticulate silica and resulted in plate numbers in excess of 100000 plates m^{-1}.[23] Fused-silica capillaries were also manufactured as continuous beds with monolithic silica and applied in the gradient-elution reversed-phase mode.[24]

Although LC systems with a high reproducibility have been on the market for a long time, the corresponding micro- and nano-LC instruments only became available in the last decade. This development has had a strong influence on driving miniaturized LC to become a routine analysis in bioscience research. The advantages of such systems are: small sample amounts are needed, the solvent consumption is very low, and higher sensitivity is achieved due to minimal sample dilution. However, the handling and operating of such systems is somewhat more complicated than conventional LC systems and requires special knowledge and skills of the operator.

A milestone in the advancement of HPLC was its coupling to a mass spectrometer, which acted as a detector and a further separator, during the 1990s.[25]

Similar to LC, the first studies on MS date back to 1912.[25] Since then the technique has been improved continuously. The coupling of MS to gas chromatography has had a significant impact on the analysis of volatile compounds. Another advancement occurred in the 1980s when MS was applied to the study of nonvolatile compounds such as peptides, oligosaccharides, phospholipids, and bile salts. A milestone in the development of MS was its application to the analysis of high-molecular-weight compounds such as synthetic polymers, proteins, glycans, and polynucleotides by using electrospray ionization (ESI) and matrix-assisted laser-desorption/ionization (MALDI) techniques. Applications in environmental, food safety, and clinical analysis for the detection of small molecules are perhaps the most striking examples for LC-MS. Improved accuracy in low-mass determination, even at low resolution, improvements in sensitivity, better detection limits, and more-efficient tandem mass spectrometry, even for high-molecular-mass compounds, are

some of the main achievements. It should be emphasized that the development of LC-MS methods is still continuing.

Micro- and nano-LC systems are ideally suited to be coupled with MS because of the low flow rates. Another important feature is the ability to position the outlet of the capillary into the ion source of the mass spectrometer. Microchips have been introduced to facilitate the connection and to achieve high reproducibility (Figure 3).[26]

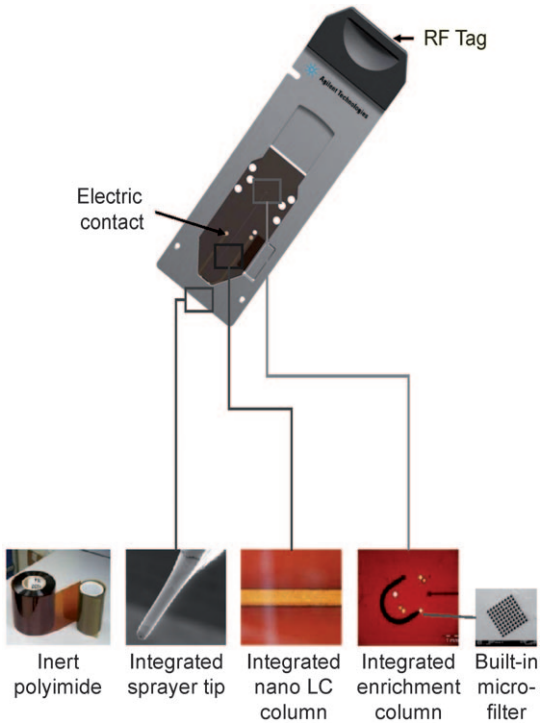

Figure 3. Features of a HPLC chip. RF tag = radiofrequency tag. Reproduced with permission from G. Rozing (Agilent Technologies GmbH, Waldbronn, Germany).

Between 1990 and 1995 developments in materials in the field of ceramics through the use of templates enabled the synthesis of defined porous silica bodies of given morphology, for example, as cylinders.[27] Tanaka and co-workers used this approach in their application of such continuous beds as columns in LC.[24] In this way, so-called monolithic silica columns were introduced as an alternative to particle-packed columns.[7,27] The primary goals were to achieve a high column performance with a small drop in the column pressure. Monolithic silica creates a pressure-stable continuous bed with a bimodal pore-size distribution, and was first synthesized with flow-through pores of 2 μm and diffusional pores of 12 nm. The monolith is clad with a leak-tight polymer (polyether ether ketone, PEEK) to form the chromatographic column for conventional HPLC systems. The first products showed a plate number equivalent to particle packed columns of 3–4 μm and a drop in column pressure corresponding to a column packed with 13 μm particles. The synthesis of monolithic silica provides some unique opportunities to control the pore size and porosity: Compared to the metastable interstitial column porosity of particle packed beds, the porosity resulting from the flow-through pores in monolithic silica columns is much larger than in particle-packed beds and can be adjusted and controlled by the synthesis. The porosity of the flow-through pores remains constant over the lifetime of the column.

Furthermore, the two pore systems can be varied in terms of pore size and porosity independently from each other. The pore structure can be modeled and simulated, for example, by the pore network model to achieve optimum mass-transfer kinetics of analytes of a given size by linking the structural properties of the pores with the chromatographic performance parameters.[28] In this way, columns can be tailored for specific applications.

In this context it should be emphasized that continuous columns based on organic polymers were introduced much earlier than monolithic silica. Pioneers in this field were Hjerten[29] as well as Svec and Tennikova.[30] Organic polymer monoliths are made as discs, cylinders, and membranes, and possess a broad pore-size distribution that ranges from macropores to mesopores. The preferred field of application is the analysis, isolation, and purification of biopolymers.[30]

Recently, the concept of porous layer beads advocated in the 1970s was revitalized with the synthesis of particles with a diameter of approximately 3 μm and composed of a solid core and a porous shell. These materials have the same efficiency as columns packed with sub-2 μm particles, but show a smaller drop in column pressure.[31]

In summary, analytical HPLC offers a wide variety of options in terms of the variety of efficient reversed-phase silica columns, but also in terms of phases for affinity and mixed-mode separations. These columns provide an effective platform for pharmaceutical analysis, including for drug discovery and drug development as well as for bioscience applications.

2.3. Bioseparations

The separation of proteins and other biopolymers has some distinctly different challenges compared to the separation of low-molecular-weight analytes from synthetic products. Biopolymers have a molecular weight ranging from several thousand to several million Daltons. Proteins consist of chains of amino acids linked by a peptide bond, and depending on the pH value can be negatively or positively charged. Their isoelectric point is where the two charges are equivalent. More importantly, they can undergo changes in their tertiary structure (change in conformation), which determines their biological activity.

Biopolymers are separated in aqueous buffered eluents, usually at pH 4—9, under conditions that enable their bioactivity to be maintained, or under denaturing conditions.

The high molecular mass of the molecules results in them having much lower diffusion coefficients (a factor of 100 or more) than small molecules. Since the chromatographic separation occurs under equilibrium conditions through selective solute–surface interactions in the chromatographic column, the proteins must have sufficient time to diffuse in

and out of the pores. To achieve this, the linear velocity of the eluent at optimum conditions also has to be 100 times lower to resolve these species. Correspondingly, the analysis time increases by the same factor. This holds strictly true in size-exclusion chromatography (SEC) which is performed under isocratic conditions. In gradient elution, biopolymers are resolved under controlled near-equilibrium conditions.

However, interaction of the biopolymeric solutes with the functional groups on the pore surface (stationary phase) is mandatory. While LC of small molecules is performed on adsorbents with a pore diameter of 6–10 nm, peptides require packing materials with pores that have diameters of approximately 30 nm, and proteins need diameters in excess of 50 nm.

Bioseparation started around 1950 with the introduction of cross-linked dextrans as size-exclusion packing materials by researchers at the University of Uppsala.[32,33] These gels were not stable to pressure; compared to rigid silicas, such hydrophilic polymers showed distinct swelling properties and were termed soft gels. The limited pressure stability was improved substantially by cross-linking, for example, in agarose gels. More importantly, different surface functional groups were introduced by chemical reactions. The derivatized products enhanced the selectivity range for biomolecules substantially. Subsequently, the agarose gels were also manufactured as beads, and the rigidity was raised by further cross-linking reactions.

Around 1980, initiatives were started to functionalize microparticulate macroporous silicas to achieve more-efficient and faster separations than before.[34] Between 1990 and 1995, so-called perfusive particles were brought into the discussion. These were aimed at overcoming the intrinsic disadvantage of LC packing materials with respect to hindered diffusion in the pores.[35] Such particles had large pores in which a convective flow could be generated, thereby improving considerably the mass-transfer kinetics.

Another concept used to overcome pore-diffusion effects of large molecules was to employ surface-functionalized nonporous silica particles or nonporous cross-linked polymer particles of approximately 1–2 μm diameter in hydrophobic interaction chromatography (HIC) as well as ion-exchange and affinity chromatography.[36] Here, retention took place at the outer surface of the microparticles. Although the back pressure of such materials packed in short columns smaller than 50 mm length was below 400 bar, columns with nonporous sub-2 μm particles found no application in routine separations of biopolymers.

Reversed-phase HPLC has played a key role as an analytical method in the development and control of biopharmaceuticals.[37,38] However, the applicability of reversed-phase HPLC for protein separation on an industrial scale is limited, because of the need for organic solvents as eluents, and the associated consequences, as described at the end of Section 2. The notable exception is its use in the purification of biosynthetic human insulin (as it was called at the time) by Eli Lilly.[39]

Since the late 1960s, increasingly robust adsorbents have been developed for process-scale chromatography. The challenge was, and still is, overcoming mass-transfer limitations arising from diffusion, which is in part limited by residence time, bead porosity, bead size, and, in the case of continuous stationary phases, matrix morphology.

Since 2000, the purification of monoclonal antibodies—with their improved expression levels—has dominated the development of affinity chromatography. Process affinity chromatography using Protein A adsorbents has received much attention through the introduction of new products manufactured without using animal-derived raw materials, improved robustness and resistance to alkaline cleaning, and binding capacities in the 20–30 $g\,L^{-1}$ range, with short residence times, and at flow rates between 100 and 500 $cm\,h^{-1}$. Driven by the need for increasing product titers,[40] the bottleneck in biopharmaceutical development has moved to downstream processing[41] and will require even more innovation and improvement. The development of high-flow ion exchangers operating at over 700 $cm\,h^{-1}$ in 20 cm columns and capacities of about 100 $g\,L^{-1}$ at residence times of 2–6 minutes to cope with 40 kg bioreactor batches and a product output of 1 tonne per year is projected.[42] Current perception is that process chromatography is the single largest cost center in downstream processing.[43] Of the current alternatives, few technologies are likely to have a major impact on downstream processes, and process chromatography will remain the workhorse of the industry. Perfusion chromatography perhaps never lived up to its expectations, but the struggle to overcome mass-transfer limitations in chromatography is currently being addressed by the use of monoliths, which still suffer from low capacities for bulk protein purification.[35] Membrane chromatography is becoming a more widespread technique for removing impurities. However, because of its selectivity, affinity chromatography still shows promise for the future, particularly when alkaline-resistant, synthetic ligands are used instead of protein or peptide ligands. Various engineering solutions have been attempted over the years to address the various challenges of unit operations. Recently, continuous multicolumn countercurrent solvent gradient purification processes have been developed for the production of biotherapeutics, such as recombinant proteins, which are starting to be implemented in the isolation and purification of proteins and antibodies.[44]

To conclude, in contrast to low-molecular-weight analytes, which are resolved on microparticulate reversed-phase silica columns, columns packed with cross-linked dextrans, agaroses, polymethacrylates, and others are preferred for protein separations.[45] An arsenal of selective modes is available to tune the selectivity: hydrophobic interaction chromatography (HIC), hydrophilic interaction chromatography (HILIC), ion-exchange chromatography (IEC), the variants of affinity chromatography (AC), and size-exclusion chromatography (SEC).[46–48] The first three modes are operated as gradient-elution techniques, in AC a step gradient is employed, while SEC is performed under isocratic conditions.[46–48] Reversed-phase LC bears a high risk of conformational changes in proteins and the associated loss of bioactivity. This is caused either by the hydrophobic nature of the surface of the reversed-phase silica packing materials or by the use of a hydrophobic organic solvent such as acetonitrile. The strength of RPLC is more in the area of peptide separations.

3. LC in the Postgenomic Era

A quantum leap in life science separations occurred in the course of the human genome project during the 1990s, when Venter et al. used several hundred sequencers in a brute-force approach to sequence the human genome within several months.[49] The fact that this accomplishment was based on multiparallel capillary electrophoresis (an elution-based separation process) was never really appreciated by the scientific community in regard to the logical consequences. While the human genome project was dealing with a static system composed of only four building blocks, the following battle to conquer the human proteome was of significantly higher complexity: dealing with a dynamically changing system of 20 amino acids (in humans), with a substantial range of posttranslational modifications (which are about 100 variations), and huge concentration differences of 10^8 to 10^{10} (abundance). Thus, proteomics bears a much higher degree of complexity than genomics, a fact which was highly underestimated at the outset.

The most characteristic features in the field of proteomics are:

- The chemical constituents are of widely different structure: sugars, carbohydrates, nucleosides, nucleotides, polynucleotides, DNA, RNA, amino acids, peptides, proteins, glycoproteins.
- There are an extremely large number of constituents (>1 million).
- There is a high abundance ratio of $1{:}10^9$.
- The molecular weight ranges from 100 to several million Daltons.
- There are constituents with relatively small differences in their chemical structure, for example, glycosylated and phosphorylated derivatives, which are biologically highly relevant.
- The number of detected constituents increases exponentially while their concentration decreases.

A logical choice for the separation tasks involved in proteome research would be a focus on automated LC approaches. However, 2D gel electrophoresis on gel slabs combined with high-resolution staining techniques and consecutive mass spectrometry of the spots became the separation strategy of choice for mid-size and large proteins. This technique reached a sort of perfection within its natural limitations. Progress was made in reproducible gels, advanced multiple dye staining, spot-cutting robotics, protein digestion, and extraction, etc. Digested proteins are often separated using reversed-phase gradient elution on fused-silica capillaries coupled directly to MS systems or to the MALDI detector. Data analysis is easy and Mascot search engine scores are amazingly high, identifications can be made with confidence, and high sequence coverage is achieved. However, this technique has disadvantages and limitations:

- Small proteins and peptides are generally missed, even when dense gels are used.
- The loadability of gels lies in a very narrow range.
- The low-abundance components are missed, because of the loading limitation.
- The separation capacity is limited to about 50 000 components.
- Various posttranslational modifications disperse the same protein over the entire gel, thus reducing the sensitivity and increasing the complexity of the method.
- The procedures are time consuming, require skilled personnel, and full automation is difficult.

Considering the limitations, LC appears to offer an excellent advantage when gels perform poorly. In any case, reversed-phase LC is used after digestion of the excised gel spot for further sample handling.

Liquid chromatography offers the possibility for automation and high flexibility, particularly in terms of the selectivity. This possibility allows different substances to be monitored and different quantities of substances can be separated simply by adjusting the column size. The sensitivity is boosted by reducing the internal diameter of the column. The combination of selectivity in multidimensional LC enables high separation capacities to be obtained. Quantitation is often problematic when dealing with highly complex samples. The handling of extreme differences in abundance is also quite difficult in LC-based approaches and requires experience.[50] Automated sample processing in combination with carefully chosen columns and elution sequences provides a chance to track a large number of sample components. The usefulness of multidimensional LC, including efficient sample clean up procedures, has been shown in specific applications involving various biofluids.[51]

In the following, strategies for depletion/enrichment of analytes in high abundance scenarios will be discussed. Most biofluids contain large amounts of well-known proteins such as albumin and immunoglobulins (IgGs), which overwhelm the separation system and make the detection of the low-abundant proteins and peptides close to impossible. It is thus mandatory to remove these proteins in a reproducible manner prior to digestion and separation. There are various ways of reducing the overall protein load by specific adsorption of albumin and IgGs onto affinity matrices.[52] While an affinity column is generally highly specific, the specificity exerted by the affinity ligand is limited in highly concentrated samples. A number of affinity columns based on antibodies or dye ligands have been evaluated in an effort to reduce the amount of albumin in human serum. Dye ligand chromatography, a technique used extensively in protein chromatography, was surprisingly effective,[53] in particular with regard to high binding capacities and a long column lifetime, although at the cost of selectivity. Table 1 gives an overview of depletion columns on the market, which were tested for their ability to remove albumin. The results reveal that such column kits can only be applied to deplete known high-abundance proteins to a certain level; they are certainly not useful when low-abundance targets are considered.

The detection of low-abundance peptides and proteins from biofluids is hampered by the so-called "house-keeping" proteins such as IgGs and albumin. Specific removal by affinity chromatography, for example, dye as well as immobilized metal affinity chromatography (IMAC), results in

Table 1: Amount [%] of serum proteins in the unbound protein fractions and amount of albumin after depletion of high-abundant proteins.[54]

Column (supplier)	Amount of proteins after depletion [%]	CV [%]	Minimum achieved albumin depletion [%]
aurum serum protein kit (Bio-Rad)	15.4	26.7	96.3
ProteoExtract albumin/IgG removal kit (Merck Biosciences)	31.2	7.7	97.4
multiple affinity removal column (Agilent Technologies)	12.8	8.4	99.4
POROS affinity depletion cartridges (Applied Biosystems)	19.0	9.9	96.0
albumin and IgG removal kit (Amersham Biosciences)	66.5	6.2	99.5

considerable depletion, but lacks the required reproducibility for assaying low-abundance proteins.

Most sample preparation procedures are still performed manually by solid-phase extraction, and are thus time consuming and laborious. On-line sample clean-up procedures are mandatory for highly reproducible results. For this purpose so-called restricted access materials (RAMs) were developed with a hydrophobic interior covered by a hydrophilic barrier.[54,55] The hydrophilic barrier allows passage of small molecules (also peptides) to the hydrophobic part of the stationary phase, while large molecules are excluded from the pores by a size-exclusion mechanism. There are several types of materials including internal-surface reversed phases, semipermeable surfaces, shielded hydrophobic phases, and mixed functional phases.[55]

The concept and the methods were transferred successfully to the sample clean-up of peptides and proteins from biofluids by extending the range of available materials that employed cation and anion-exchanger RAMs.[54–56]

RAM columns with strong cation-exchange functional groups on the surface (sulfonic acid groups) extracted positively charged peptides effectively from urine as well as from other biofluids. The molecular weight exclusion limit was approximately 15000 Daltons for globular proteins. As a result, complex peptide mixtures could be resolved effectively and concentrated prior to further separation in a second dimension. Strong cation exchange chromatography is generally implemented as a primary separation technique because of its potential for increased mass load capacity, while reversed-phase LC is a perfect compliment as a secondary separation technique because of its ability to remove salts and its direct compatibility with mass spectrometry through electrospray ionization. The particular advantage of this approach is that it can be integrated into a multidimensional LC system as an automated technique. Figure 4 shows an example of a multidimensional separation

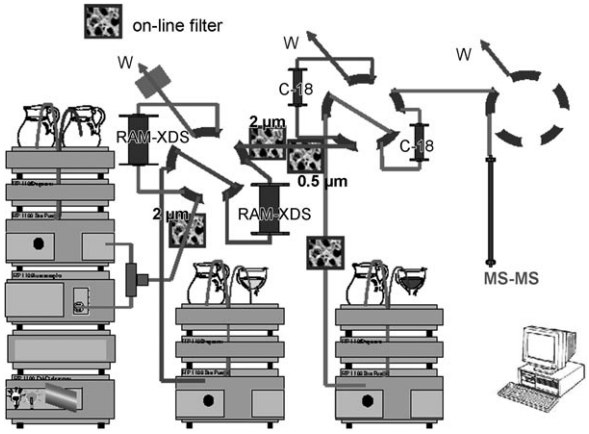

Figure 4. Schematic representation of a two-dimensional LC system for profiling endogenous peptides from biofluids.[51]

platform with on-line sample clean-up for the analysis of endogenous peptides from body fluids. The system offers several advantages: high reproducibility and ruggedness and high separation capacity (about 1500 different peptide and protein fragments were detected).[51]

In summary, LC has the potential for:

- Selective sample clean up by solid-phase extraction methods, even quantitative when run in an automated mode.
- Applying a large number of phases for tuning selectivity (RP, IEX, HIC, HILIC, SEC, AC).
- Combination into a multidimensional process, especially valuable for handling the high abundances prevalent in most applications.
- Quantitative measurements in automated operation.

4. Liquid Chromatography in 2010—Where are We Today?

4.1. Pharmaceutical Analysis

Pharmaceutical analysis (in drug discovery and development, quality assurance, and quality control) still remains the largest sector of LC applications. The goals are efficiency, fast analysis, and high sensitivity. The complexity of mixtures is relatively low compared to the analysis of biofluids in proteomics. For example, the abundance ratio is approximately 1:1000, and reversed-phase columns provide sufficient selectivity. Mass spectrometry plays a major role and helps reach enormous sensitivities. Liquid chromatography is often only considered as a sample clean-up device for MS. Despite

the dominant role of reversed-phase silica columns, columns with diol or cyano functionalities are often preferred.[57]

4.2. Metabolomics/Metabonomics

Metabolomics is the comprehensive and quantitative analysis of all the metabolites in a system.[58] Metabonomics is the process of monitoring and evaluating changes in gene expression and also from differences in life style and diet in humans and other mammals.[58] Both provide information on the status and function of a living system and identify and quantitatively measure the content of small molecules (< 1500 Da). A conservative estimate places the number of metabolic compounds in plants at 200000, which provides an extensive searching ground for new and improved medicinal and nutritional products.[59] On a related note, comparative metabolomics in humans may enable enhanced diagnostic power and individualized treatment for illness and disease.[59]

The coupling of GC and MS is the analytical platform for volatile analytes in metabolic analyses.[60] Large differences in abundance and functional group differences require multiple selectivities, which are widely offered by LC. However, the development in metabolomics relies on the improvements in mass spectrometry. Microparticulate or monolithic silica reversed-phase columns operated in the gradient-elution mode provide the required speed and resolution.

4.3. LC in Clinical-Chemical Analysis and in the Therapy of Acute and Chronic Illneses

Nearly unnoticed by the chromatographic community, LC has found applications in clinical-chemical analysis and has evolved into versatile and efficient platforms which are applied in monitoring in vivo the therapy for acute and chronic illneses.

The objective in clinical analysis is to extract, selectively enrich, and analyze target compounds in blood, plasma, urine, and other biofluids. The goal of therapy is to deplete or remove target pathogens and toxins. Adsorbents comprise large-diameter, biocompatible adsorbents with size-exclusion properties to exclude high-molecular-weight constituents (for example, plasma proteins) and have tailored surface functionalities which selectively enrich target compounds (such as drugs). An example is the use of restricted access materials (RAMs), which were already introduced in Section 3. The advantage of small RAM solid-phase extraction columns with dimensions of 20×2 mm are 1) direct injection of biofluids such as serum and urine, 2) direct coupling with LC systems, and most importantly 3) the option to set up automated coupled-column systems with loading, washing, desorption, and regeneration steps.

In contrast to the widespread use of chromatographic supports for analytical and preparative purposes, their application as a medical device for the selective extracorporal removal of pathogenic compounds present in blood, or selective intestinal adsorption and elimination of unwanted food ingredients, is still in its infancy. In the technique of hemoperfusion or plasmapheresis, the patients blood or plasma is circulated extracorporally over a LC column, the packing material of which selectively binds and extracts endogenous and/or exogenous noxious compounds. While on-line adsorption apheresis is quite simple in concept, the greatest demands are placed on the porous adsorbents. Parameters such as specificity, capacity, particle size, flow geometry, toxicity, sterility, pyrogenicity, and ligand stability have to be evaluated and optimized to ensure a safe and effective clinical application. Furthermore, the surface characteristics which determine the bio- and hemocompatibility have to be optimized so that cellular and humoral responses to the chromatographic support are minimal and so that no significant activation of the fibrinolytic, complement, and coagulation system occurs during acute or chronic extracorporeal treatment (Figure 5).

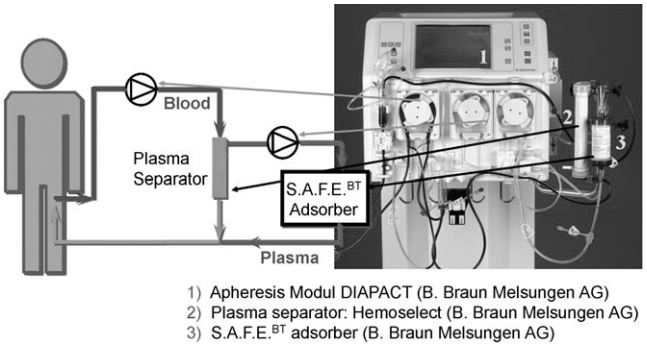

Figure 5. Instrument set up in extra corporal treatment systems. Reproduced with permission from K.-S. Boos (Laboratorium für BioSeparation, Institut für Klinische Chemie, Klinikum der Universität München).[60]

For a peroral application, such materials should have high pH stability and binding capacity as well as a neutral taste. Furthermore, they should not be adsorbed in the gastrointestinal tract.

As indicated in Table 2, a wide variety of diseases can be treated with selective chromatographic adsorbents, and requires comprehensive clinical studies. While currently implemented in a few hospitals by scientists equally skilled in medical and separation science, these methods show future potential for LC-based technologies with clinical applications.

4.4. Proteomics

Liquid chromatography has gained some acceptance as a front-end technique in sample clean up and in reversed-phase LC using fused-silica capillaries to monitor peptides from protein digests before MS detection. Other modes and even fewer combinations thereof have not made it into the arsenal of the "-omics" strategies, maybe apart from the field of glycomics. Therefore, it can be summarized, that the potential of LC is under-utilized. Linear-sequence approaches to sample processing, such as the multidimensional protein

Table 2: Methods for acute and chronic therapy that rely on chromatrographic adsorbents.[a]

Disease	Selective removal	System	Development stage
hypercholesterolemia	low-density lipoprotein (LDL), fibrinogen, heparin	H.E.L.P. (**h**eparin-mediated **e**limination of low-density **l**ipo**p**roteins)	approved
sepsis and septic shock	lipopolysaccharides (LPS), toxins of Gram-negative bacteria, lipoteichoic acids (LTA), toxins of Gram-positive bacteria	S.A.F.E.^{BT} (**s**elective **a**dsorber **f**or **e**limination of bacterial toxins; Figure 5	phase II
acute liver failure	bilirubin bile acids aromatic amino acids, bacterial toxins	PROMETHEUS (fractionated plasma separation and adsorption apheresis) MARS (**m**olecular **a**dsorbent **r**ecycling **s**ystem) M.I.D.A.S. (**m**ultiselective **i**n-line **d**etoxifying **a**dsorption **s**ystem)	pilot pilot preclinical
hyperphosphatemia (dialysis patients)	phosphate (gastrointestinal tract)	renagel SBR 759	approved phase III

identification technology (MudPit) approach are still in use, despite their inability to provide reproducible and quantifiable results.[61]

Although quite a number of sample clean-up methods have been developed and even validated in part on the basis of multidimensional LC, no major breakthrough in application has so far been observed.

Digestion-based approaches (offline and online) based on trypsin and pepsin dominate the direct analysis of proteins (Figure 6a,b). The direct strategy in Figure 6c focuses on the extraction of endogenous peptides. All the existing chromatographic columns, except a few based on the RAM principle and affinity-type columns (Figure 6d), cannot deal with multiple direct injections of biological fluids.

In conclusion, the use of LC approaches in academic "-omics" research has only found its way marginally into "industrial" research, for example, within diagnostic and pharmaceutical companies. The reason for this may be that the subject is still too complex and because of the necessary intensive interplay between instrument and material chemistry.

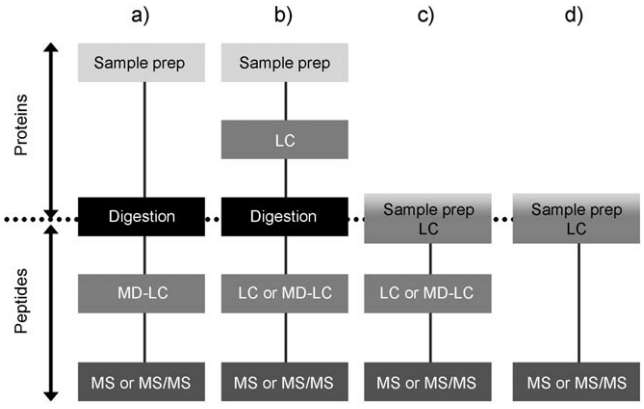

Figure 6. Workflows in proteomics.[51] a) Bottom-up approach; b) top-down approach; c) selective sample clean up directly combined with chromatographic separation (digestion-free strategy); d) sample clean up performed with affinity columns directly coupled to MS.

5. Conclusion and Outlook

The development of liquid chromatography during the last century shows the tremendous breadth of the method. At the same time it shows a certain fragmentation, with few cohesive approaches shared by all the disciplines to solve some basic questions—be it traditional isolation and purification of natural products, analytical LC of small molecules in pharmaceutical analysis, food, environmental, and materials applications, bioanalytical work in modern life science research, finally preparative and process-scale applications.

The application of conventional HPLC of small molecules in pharmaceutical analysis and metabolomics has reached a high level of maturity; the standard applications can be carried out on a routine basis.

Chromatographic separations on a preparative and large scale are in use in the form of the SMB technology for the separation of *m*- and *p*-xylene isomers for use as building blocks for the preparation of polyethylene terephthalate, for enantiomer resolution, as well as in a multistep sequence for the manufacture of monoclonal antibodies and recombinant proteins etc. Here, the costs and production capacities will have to be addressed, with a need for more integrated and efficient approaches.

The largest area in need of stimulation is the field of life science chromatography, where dealing with unstable and sometimes transient target molecules present in extremely wide concentration ranges stretches the available technology to the limit. Bringing this field forward will require significant progress in materials and systems design. Such efforts,

however, will remain largely ineffective, if they are not accompanied by much more cohesive and integrated education and training efforts in academia as well as in industry.

Multidimensional liquid chromatography (MD-LC) has proven its ability to master high abundance scenarios in analytical fields when automated and optimized. It may turn out to be indispensable for proteomics concepts that can be quantitated and vailidated, for example, in the biomarker area.

Another technology trend can be observed for the evaluation of continuous process operations in the downstream processing of antibodies, the so-called Bio-SMB. Development activities from a number of system providers coincided with growing attention from the biopharmaceutical industry for this topic.

Interestingly, this is one of the very few positive examples of links and cross-over activities between the three categories of chromatography (see Figure 1). The engineering-driven search in the 1960s for more-effective separation processes for petrochemicals led to the development of SMB technology, which was also applied to food processing. The development of chiral stationary phases for the enantioselective separation of enantiomers in analytical chromatography during the 1980s combined with a downscaled version of SMB equipment in the 1990s provided cost-effective process technology for making enantiopure pharmaceuticals. Adapting the same SMB concepts for the isolation of antibodies from complex fermentation broths will probably now allow for more cost-effective downstream processing of biopharmaceuticals within the next couple of years.

A similar path might be useful when dealing with the "glyco" issue. Since glycosilation plays a significant role in therapeutic drug efficacy, the analytical approaches developed around mixed-mode separation methods might be transferred to the process scale.

The validation of methods and assays will become a key issue in the discussion on how to improve the support to the bioscience community for the development of future diagnostic and therapeutic concepts. In particular, the development of biomarkers for "stratified medicine" approaches will make validation a precondition.

At the same time, this fits directly with the process analytical technology (PAT) initiative launched several years ago by the food and drug administration (FDA), which called for a better understanding of the process. This requires, amongst other things, a much deeper insight into the underlying interactions by using model-based approaches, which should finally allow "predictable" process design and monitoring strategies in the future so as to enhance process robustness and safety.

Abbreviations

AC	affinity chromatography
Bio-SMB	simulated moving-bed technology applied to the separation of bioproducts
C18	*n*-octadecyl-bonded chains
CV	coefficient of variation
Da	Daltons
DAC	dynamic axial compression
DNA	deoxyribonucleic acid
ESI	electrospray ionization
GC	gas chromatography
H.E.L.P.	heparin-mediated elimination of low-density lipoproteins
HIC	hydrophobic interaction chromatography
HILIC	hydrophilic interaction chromatography
HPLC	high-performance liquid chromatography
IEC	ion-exchange chromatography
IgG	immunoglobulin
LC	liquid chromatography
LDL	low-density lipoproteins
LPS	lipopolysaccharides
LTA	lipoteichoic acids
Mab	monoclonal antibodies
MALDI	matrix-assisted laser-desorption ionization
MARS	molecular adsorbent recirculation system
MD-LC	multidimensional liquid chromatography
M.I.D.A.S.	multiselective in-line detoxifying adsorption system
MS	mass spectrometry
MudPiT	multidimensional protein identification technology
PAT	process analytical technology
PEEK	polyether ether ketone
PROMETHEUS	fractionated plasma separation and adsorption apheresis
PTM	posttranslational modifications
RAM	restricted access material
RAM-XDS	RAM ion exchanger
RNA	ribonucleic acid
RP	reversed phase
RPLC	reversed-phase liquid chromatography
SAFE	selective adsorber for elimination of bacterial toxins
SEC	size-exclusion chromatography
SMB	simulated moving bed
SPE	solid-phase extraction
UPLC	ultra-high-performance liquid chromatography

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