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Advanced proteomic liquid chromatography

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Abstract

Liquid chromatography coupled with mass spectrometry is the predominant platform used to analyze proteomics samples consisting of large numbers of proteins and their proteolytic products (e.g., truncated polypeptides) and spanning a wide range of relative concentrations. This review provides an overview of advanced capillary liquid chromatography techniques and methodologies that greatly improve separation resolving power and proteomics analysis coverage, sensitivity, and throughput.

Keywords

Liquid chromatography; Proteomics; Peptides; Proteins; Column technologies

1. Introduction

Characterization of proteins in cells, tissues, and bodily fluids presents a number of challenges for analytical techniques. Among these challenges is the analytical ability to identify and quantify as many proteins/peptides as possible from proteomics samples that can contain roughly hundreds of thousands of potentially detectable species in a single sample. A second major challenge is working with limited sizes of available samples, such as a clinical biopsy or even a single cell (e.g., in studies of cell-to-cell heterogeneity). Differing from genomics, proteomics samples are not amenable to amplification and the ability to measure analytes in small proteomic samples is largely governed by the sensitivity of the analytical technique. Compounding these issues are the heterogeneous and dynamic properties of the proteome (e.g., protein expression levels differ among cell types, as well as physiological and environmental conditions), which challenge analytical throughput as numerous proteomics sample analyses are often required to study changes in individual proteins of interest. Finally, detecting biologically 'important' proteins in complex samples (e.g., those that change significantly between conditions) can be further challenged by concentrations that span eight orders of magnitude in human cells and greater than ten orders of magnitude in human plasma [1]. Some affinity-based methods such as targeted depletion of high-abundance albumin and antibodies in human serum or plasma have been developed to enrich low-abundance components prior to the analysis. The advancement of such selective sample preparation approaches has been discussed in detail in several reviews [2-4].

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While mass spectrometry (MS) is an effective tool for sequencing protein and peptide sequences, even the most advanced mass spectrometers with fast scanning rates and high resolving power have their limits in dynamic range per spectrum. Additionally, data quality (e.g., analysis depth) can be impacted when too many different species are introduced at once. Implementation of liquid phase separation(s) prior to MS analysis helps mitigate this issue by reducing the number of analytes entering the mass spectrometer at any given time, which minimizes the potential for ionization suppression where a nominally detectable species is not detected due to detector dynamic range limitations, and under-sampling in ion selection for MS/MS analysis in 'shotgun' measurements. Furthermore, analytes can be focused within narrow zones (or peaks) during the liquid phase separation step(s), which concentrates them and benefits MS detection sensitivity. Of all the liquid phase separation techniques, liquid chromatography (LC), especially in its capillary format, has significantly advanced over the past decade to make it a prevalent technique in modern day proteomics analyses as the physicochemical properties (e.g., mass, charge, and hydrophobicity) of peptides make them amenable to efficient LC separation.

Herein, we review some of the advanced single- and multi-dimension LC techniques and methodologies that facilitate global quantitative proteomics measurements, mainly with bottom-up approaches. While many of the single-dimension techniques were developed in our laboratory to surmount the proteomics challenges above, our intent is to illustrate their role within the broader context of a state-of-the-art integrated proteomics effort.

2. Proteomic single-dimension LC

A single-dimension LC separation is one of the basic elements in LC-MS-based proteomics. Reversed phase (RP) is the most common LC format for separation of peptides, either with packed or monolithic columns, because of the compatibility of the LC mobile phases with subsequent MS detection. Over the past years, numerous efforts have focused on enhancing the separation efficiency (quality), which can be quantified by the peak capacity (the maximum number of components that can be resolved in a given separation time [5]). Below, we describe the resulting improvements for both packed and monolithic RPLC columns, and present the detailed information of some milestone examples in Table 1.

2.1. Packed columns

For a packed RPLC column, the maximum peak capacity (C_p) obtainable for gradient separation of peptides is governed by column length (L) and particle size (d_p), and the relationships can be simplified as [6]:

$$C_p \propto \sqrt{L} \quad (\text{at fixed particle size})$$

$$\text{and } C_p \propto \sqrt{1/d_p} \quad (\text{at fixed column length})$$

Based on these relationships, strategies for maximizing peak capacity have tended to focus on increasing LC column lengths and decreasing packing material particle sizes. When MS is used for detector, the chromatographic peak capacity is calculated by dividing the effective separation time window (i.e., the time zone where peptides elute) by 4σ that is measured at the chromatographic peak height of 10% (note: the chromatographic peak is profiled with use of MS single ions at a m/z range that covers the whole isotope peak width; multiple single ion chromatographic peaks along the whole separation windows with various MS intensities are needed for fair calculation of peak capacity). The chromatographic peak capacity directly affects the proteomics analysis coverage. Kocher et al. recently demonstrated that the number of peptides were linearly increased with increasing the chromatographic peak capacity (Figure 1) [7].

Single dimensional analysis has been applied for both microbial and mammalian proteomics (Table 1). Ten years ago, conventional packed LC columns were typically short (e.g., ~ 20 cm), and the obtainable peak capacity was limited to ~ 100. Shen et al. demonstrated that peak capacity could be increased by using a long capillary (80 cm $\times$ 150 μ m i.d.) column and demonstrated high efficiency ($C_p \sim 1000$) separation of peptides over the course of 3 h [8]. The column was packed with 3- μ m C18-bonded porous particles, and a pressure of 10 Kpsi was used to deliver the mobile phases. The study showed that up to 500 μ g digested protein samples could be loaded onto the long packed capillary column before separation quality started to degrade. The ability to load large samples is beneficial for detecting less abundant peptides, although the number of additional peptide identifications is not necessarily increased linearly in proportion to the amount of sample injected. Shortly thereafter, they implemented a parallel multiple capillary LC format (85 cm $\times$ 150 μ m i.d. columns, 3 μ m porous particles) that increased the analysis throughput while maintaining the separation efficiency [9]. The two-column configuration in which one column is utilized for separation while the other is being washed eliminated delays stemming from column regeneration (or equilibration). More importantly, this configuration allowed for automation and continuous MS analysis.

In a later work, the automated RPLC separations were pushed to operate at 20 Kpsi to further increase the separation peak capacity [6]. Various lengths of LC columns and particle sizes (3 μ m) were examined to obtain optimal RPLC for separating peptides [6]. Using a 200 cm $\times$ 50 μ m i.d. column containing 3 μ m porous C18 particles and operated at 20 Kpsi, a peak capacity of 1500 was obtained for an RPLC separation of a global tryptic digest of the microorganism *Shewanella oneidensis* (Figure 2). This peak capacity remains the highest reported to date for separating peptides. At constant pressure of 20 Kpsi, the use of smaller particles does not further increase peak capacities, although use of the smaller particle-packed LC columns can improve peak capacity generation rates, which benefits fast proteomics analysis (see below).

According to theory [10,11], the peak capacity should exceed the number of components in a sample by a factor of 100 to resolve 98% of them. However, with the sophisticated MS instrumentation available today, not all components have to be individually separated to obtain high proteome coverage. For example, with the separation power achieved with the 40 cm $\times$ 50 μ m i.d. column and 1.4 μ m porous C18 particles, a 12-h single LC-tandem MS (MS/MS) analysis of a *S. oneidensis* tryptic digest sample enabled identification of >12,000 peptides and >2000 proteins that covered ~40% of all protein database entries [6]. This approach has gained recognition and has been applied in recent years to analyze mammalian proteomes, leading to identification of >4000 proteins from a single LC-MS/MS analysis [12,13].

Other efforts to enhance analytical sensitivity have centered on preparing extremely narrow packed capillary RPLC columns [14]. For example, long (87 cm) capillary columns with i.d.'s down to 15 μ m have been packed successfully with 3 μ m C18 particles [14]. Note that packing such columns requires small particles that are typically highly uniform, which makes column preparation increasingly difficult as column i.d. decreases. Figure 3 shows base peak chromatograms that highlight LC-MS performance for long (>85 cm) packed capillary columns with i.d.'s ranging from 15 to 75 μ m. The number of species detected in 100 ng of a yeast tryptic digest (MS intensities of > 40 counts/s) increased ~ 200-fold upon decreasing the capillary i.d. from 75 to 15 μ m. This increase is likely due to the increased concentration of analytes eluting from the small column to the electrospray emitter, which is particularly advantageous for proteomic applications in which available sample sizes are limited. Although the use of extremely narrow columns in combination with low flow rates can improve MS detection sensitivity, their use has practical limitations in terms of how to

accurately load extremely small (e.g., ~ng) samples onto the analytical system. Current proteomics sample processing methods still require a relatively large volume (e.g., >5 μ L) to minimize sample loss from the surfaces and “dead corners” of the containers. With a column operated at nano-flow rates (e.g., 15 μ m i.d. column operated at 20 nL/min), it can require hundreds of minutes to load the sample.

To address the problem of long loading times, Shen et al. incorporated online micro-SPE into their nanoLC system and demonstrated substantially (~400-fold) faster sample loading could be accomplished without compromising overall system performance [15]. This study offers a useful guideline of appropriate on-line SPE parameters for different sizes of LC columns in order to achieve optimal performance (Table 2). In a later study, they utilized a system comprised of a 50 μ m i.d. SPE and 15 μ m i.d. packed LC column (operated at a flow rate of ~20 nL/min and an optimal LC linear velocity of ~0.2 cm/s) in combination with an FTICR MS to identify proteins present at zeptomole levels [16]. Using this system, ~6000 molecules of peptides were detected. When applied to analyze proteomics samples, the SPE-LC-FTICR MS platform enabled identification of 106 proteins from just 5 pg of a tryptic digest of *Deinococcus radiodurans* (Figure 4). Currently, this technique is being implemented in our laboratory to enable proteomics analysis of a limited number of mammalian cells (e.g., 1-500 cells). An added benefit of coupling nanoLC with micro-SPE is the robustness; for example, a 50 μ m i.d. SPE - 15 μ m i.d. LC system is equivalent to that of a 50 μ m i.d. LC [15].

Packed columns containing small particles also have been explored for fast LC separations to attain ultra-high-throughput proteomics analyses [17]. Application of short (20 cm $\times$ 50 μ m i.d.) capillary columns packed with ~0.8 μ m C18-bonded porous particles [19,18] resulted in identification of ~4000 *S. oneidensis* tryptic peptides (originating from ~1000 proteins) in a single 50 min ion-trap MS/MS analysis. An 8-min LC-MS/MS analysis enabled identification of ~700 peptides (~250 proteins) [18]. Under such fast LC separation conditions, proteome coverage is largely controlled by the scanning rate of the MS instrument (e.g., MS/MS data acquisition speed). A more efficient approach for extending coverage in ultra-high proteomics is to acquire high mass measurement accuracy MS spectra (i.e., the accurate mass and time (AMT) tag approach [20]) rather than MS/MS spectra. Fast separations in combination with a high mass measurement accuracy MS approach resulted in identification of ~2000 different peptides from ~600 proteins in only 2-3 min (Fig 5) [19]. Figure 6 illustrates the relationship between proteome coverage and analysis time for three different RPLC-MS platforms. Based on this study, TOF MS would be more effective for proteomics analyses ~120 s because of its fast MS acquisition speed (0.2 s), while FTICR MS would be more effective for analyses ~150 s because of improved mass accuracies.

Specialized instrumentation with ultra-high pressures is usually required to deliver the mobile phase through long packed capillary columns. However, conventional LC instrumentation with lower pump pressures are more widely used in most proteomics laboratories. One solution for delivering mobile phases through long packed columns with a conventional HPLC pumping system is to elevate the column temperature to reduce mobile phase viscosity and thus decrease the back pressure [21]. A disadvantage of high temperature RPLC is the increased potential for damaging the bonded reversed phases (e.g., Si-O-Si bonds). An alternative approach is to reduce the mobile phase linear flow rate for low pressure operation (e.g., <6000 psi), although potentially with a trade-off in separation efficiency. New types of stationary phases may also benefit operating pressures required for long packed LC columns. For example, pellicular (superficially porous) particles operated under low pressure have been reported to yield ~50% higher peak capacities [22]. A few researchers have devoted their efforts to the development and evaluation of HPLC columns

packed with such pellicular particles for protein/peptide separations [23-26]. Sandra and co-workers obtained peak capacities of > 400 at 60°C using a one-piece $120\text{ cm} \times 100\text{ }\mu\text{m}$ i.d. column packed with $5\text{ }\mu\text{m}$ Zorbax 300SB C_{18} particles while analyzing a depleted human serum tryptic digest using conventional instrumentation [27].

2.2. Monolithic columns

Another solution for operating long columns under moderate pressures is to use a different type of column such as a monolithic column. These columns contain porous channels rather than beads and have two advantages over packed columns. The first is their inherent high permeability that produces low resistance for mobile phase delivery and the second is the ease in which these narrow columns can be manufactured. Application of a long (3.5 m) monolithic column at $<3000\text{ psi}$ operating pressure proved successful for identification of 2602 proteins from *Escherichia coli* cells [28]. In another work, Luo et al. prepared $70\text{ cm} \times 20\text{ }\mu\text{m}$ i.d. silica-based monolithic columns for proteomic analysis, which they operated at a flow rate of 40 nL/min [29] and demonstrated detection of low attomole peptides. Later, they decreased the size to $25\text{ cm} \times 10\text{ }\mu\text{m}$ i.d. and operated the monolithic columns at 10 nL/min [30]. In this study, they identified 5510 unique peptides from 1443 distinct *S. oneidensis* proteins in a single 4-h LC-MS/MS analysis of a 300-ng tryptic digest sample.

Although fast LC separations can be achieved with monolithic columns by flowing mobile phases at high linear velocities, the high (volume) flow rate will dramatically decrease MS performance (sensitivity) when electrospray is used for ionization. Additionally, monolithic columns produce relative low separation efficiency (peak capacity) compared with packed columns, although new generations of long monolithic columns have been developed to increase their chromatographic performance [31,32]. The unique value of monolithic columns stems from the relative ease of fabrication of ultra narrow columns (e.g., $<10\text{ }\mu\text{m}$ i.d.) of long length that can achieve relatively high resolution with proteomic samples.

3. Proteomic multidimensional LC

3.1. General considerations

Multidimensional LC in proteomics analyses is typically based on RPLC in combination with another LC mechanism(s) for fractionating samples prior to RPLC-MS analysis. For example, strong-cation exchange (SCX) $\times$ RP LC is one of the most common combinations of a multidimensional LC system; the first dimension (i.e., SCX LC) fractionates peptides/proteins in the sample based on the charge, and then the second dimension (i.e., RPLC) further separates the analytes in each fraction of SCX LC based on the analyte hydrophobicity. The success of a multidimensional separation system is mainly determined by factors that include orthogonality of the separation elements, and separation efficiency and sample recovery in each of the separation elements. Orthogonality is largely determined by the analyte retention mechanisms provided by different separation elements. Although a variety of physicochemical properties that attribute to peptide/protein retention on LC columns can be exploited to design different multidimensional LC systems, true orthogonality is difficult to achieve, even with SCX $\times$ RP LC[33]. Potential correlations among molecular weight/size, hydrophobicity/polarity, charge, and isoelectric point (pI) of peptides/proteins may reduce the orthogonality for any two different separation elements. Gilar et al. thoroughly investigated the orthogonality of peptide separations in two-dimensional (2D) LC systems and developed a geometric approach to define orthogonality [34]. This study concluded that no separation mode in currently available 2D LC systems is likely to offer a completely orthogonal separation, although SCX $\times$ RP, hydrophilic interaction chromatography (HILIC) $\times$ RP, and high pH RP $\times$ low pH RP 2D LC systems provided suitable orthogonality (Figure 6).

Theoretically, the peak capacity of a multidimensional separation system is defined as the product of individual peak capacities provided by individual separation elements (dimension): $C_{p(total)} = C_{p1} \times C_{p2} \times \dots$ [35]. In reality, the total peak capacity obtained from multidimensional LC in high throughput proteomics analyses is usually much lower than the theoretical one. The reason for the observed reduction is that only a limited number of fractions (e.g., <50) from the first dimension are transferred to the next dimension, regardless of peak capacity, in the interest of maintaining analytical throughput for advanced MS analysis of complex mixtures. Therefore, the overall peak capacity is more appropriately estimated as the product of the number of fractions in the first dimension and the C_p in the second dimension (assuming the peak capacity of the first dimension $\gg$ the number of fractions). Partial orthogonality among different dimensions can be further corrected by using the geometric approach developed by Gilar et al. [34].

Proteomic multidimensional LC can be realized in online or offline modes of operation, and each approach has its own advantages and disadvantages [36,37]. In offline multidimensional LC, the fractions of a proteomics sample separated in the first dimension are collected via a collector for the subsequent dimension separation. (It is noted that offline operation can also be automated.) Offline fractionation allows for more flexible analysis; for example, a large amount of initial samples to be injected to increase the overall coverage of proteins/peptides, multiple runs of each fraction to overcome MS/MS under-sampling, additional processing of samples, and more choices of mobile phases for a variety of processes (e.g., evaporation, buffer exchange, and chemical reaction). The collected fractions also can be measured in parallel on different mass spectrometers, which can provide different analytical information and/or improve sample analysis throughput. The major disadvantage of off-line systems is the potential sample loss in the collector, a limitation for analyses of small sizes of proteomics samples. The online mode achieves multidimensional LC separations in a closed system without losing sample on system collector surfaces. Disadvantages of online separations include limited flexibility in subsequent analysis of the fractions and stringent compatibility of the mobile phases applied in the different dimensions. The offline mode is useful for analyzing highly complex samples (e.g., non-depleted human serum tryptic digests) with high proteomic analysis coverage that is the major advantage of multi-dimensional proteomics, while the online mode is more convenient when dealing with relatively limited sample sizes or unstable compounds.

In the following sections, we describe the major multidimensional LC systems and methodologies applicable to proteomics. Table 3 lists the detailed information for some representative proteomics applications.

3.2. SCX×RPLC

SCX coupled with RPLC is the most widely applied 2D LC system in proteomics, especially for separation of peptides in the bottom-up approach. Yates and coworkers were the first to demonstrate SCX×RPLC (Figure 8a) in conjunction with MS/MS (MudPIT) as a viable protocol for proteome analysis [38,39]. In the original MudPIT approach, online 2D LC separations are performed using a single column that is partially packed with both SCX and RP stationary phases. Subsequent variants of MudPIT, such as the use of triphasic and tetraphasic columns have been developed to improve sample loading, desalting, and chromatographic efficiency [40-42]. Another approach is the use of individual SCX and RP columns and switching valves to accomplish online SCX×RPLC separations.

Under SCX conditions (pH<3), tryptic peptides are positively charged. The N-termini (pKa 7.6) and side chains of arginine (pKa 12.5), histidine (pKa 6.45), and lysine (pKa 10.2) are protonated, while the C-termini (pKa 3.6) and side chains of aspartic (pKa 3.95) and

glutamic acid (pKa 4.45) are neutral [43]. The positively charged peptides bind to the SCX sorbents and are eluted into fractions via mobile phase gradients that can be applied step-wise or continuously to change mobile phase salt concentrations and/or pH values. A stepwise gradient can be achieved simply by loading different salt and/or eluents, while a continuous gradient requires use of an additional SCX mobile phase delivery pumping system (Figure 8b) [44-50]. Compared to a step-wise gradient, a semi-continuous or linear salt gradient is reported to improve the chromatographic efficiency and reduce the distribution of peptides over multiple fractions [45,46,51].

The popularity of SCX for proteomics analysis stems from its ability to remove detergents and chaotropic agents (frequently used to extract and digest proteins) that if not removed would cause serious contamination and ion suppression issues during MS analysis. SCX chromatography also can facilitate targeted analysis of protein post-translational modifications, such as phosphorylation and N-terminal acetylation [52-54] as peptides with fewer positively charged groups (e.g., blocked N-termini) and/or more negatively charged groups (e.g., phospho-groups on the side chains of serine, threonine, or tyrosine residues) are less likely to be retained on the SCX sorbents due to the lack of net positive charges. Heck et al. achieved near complete separation of phosphorylated and acetylated peptides in Lys-N digested whole cell lysates by using slow gradient SCX LC. The N-terminal acetylated peptides and the doubly-phosphorylated peptides (0+) eluted first from the SCX column, followed by the singly-phosphorylated peptides containing one basic residue (1+) and the non-phosphorylated peptides with one basic residues (2+), and finally, the non-phosphorylated peptides containing multiple basic residues (3+) [53,54].

The use of online vs. offline SCX×RPLC depends on available sample sizes, desired proteome coverage, and throughput requirements. The offline mode of operation allows the use of higher concentrations of an organic modifier in SCX mobile phases to improve peptide recovery without influencing peptide retention on the SCX column. Moreover, the amount of materials injected onto the second dimension column and the gradient time can be optimized based on UV-absorbance monitored for the first dimension. In particular, low-abundance peptides can be detected by injecting an entire SCX fraction onto the RPLC column using a fast gradient for separation, which concentrates peptides prior to electrospray ionization [55]. A comparative study of on-line SCX×RPLC with a semi-continuous salt gradient and offline SCX×RPLC with a pumped continuous gradient [46] indicates that the online mode affords better peptide and protein identification coverage.

Sample recovery is a major issue in proteomics SCX LC. Secondary hydrophobicity interactions originating from SCX sorbents can be minimized with the addition of an organic modifier (usually 5%-25% ACN) in the mobile phase; for example, peptide recovery can be increased 3-fold by switching from 5 to 25% ACN [56]. However, high organic content can be detrimental to an online 2D LC scheme as some hydrophilic peptides may be lost; 5% ACN affords a useful compromise, while 25% ACN makes online SCX no longer compatible with the second RP LC dimension. The use of more hydrophilic SCX sorbents (e.g., 2-sulfoethyl aspartamide stationary phases) also benefits elution of hydrophobic peptides from SCX LC [57]. For example, Motoyama et al. increased peptide recovery without the need for an organic modifier by using an anion and cation mixed-bed ion-exchange resin [58].

The importance of SCX separation selectivity is increasingly being recognized. Typically, doubly-charged peptides containing one arginine or lysine are dominant in a digested protein sample; only peptides containing histidine(s) and/or internal arginine or lysine residue(s) carry more than two positive charges. Therefore, tryptic peptides of the same charge tend to elute within a relatively narrow gradient window in SCX. Winnik combined a salt gradient

with a continuous pH gradient to reduce the carryover between neighboring SCX fractions during elution in an online SCX×RPLC system [48]. In other work, Dai et al. replaced the SCX salt gradient with a stepwise pH gradient [47] and Zhou et al. further upgraded the system by using a continuous pH gradient (Figure 8c) [50]. Use of a pH gradient elution also benefits MS detection because of the absence of salts. The resulting improved separation selectivity and efficiency alleviate peptide overlapping among multiple fractions and increase overall peptide/protein identifications [47,50].

3.3. RP×RPLC

RPLC separates peptides mainly based on their hydrophobicity; however, peptide retention on the column can be manipulated through the use of different types of stationary phases, organic solvents, pHs, ion-pairing agents, and particle and pore sizes, among other conditions [59]. In particular, mobile phase pH has a significant impact on RPLC selectivity [59]. Peptide retention in RPLC at high pH (e.g., pH 10) is governed solely by solvophobic interactions between peptides and the stationary phase, while at low pH (e.g., pH 3), retention is determined by a combination of solvophobic and electrostatic interactions. The use of alkaline mobile phases for high pH RPLC raises concerns for RPLC column stability, so the RP packing materials must be resistant to elevated pH; polymer bead-based RP stationary phases tend to be optimal for this purpose. In addition, the fused silica tube typically used for manufacturing capillary LC columns should be treated to prevent erosion from the high pH mobile phases. Other concerns are that peptides may precipitate when stored at pH 10, and high pH also may induce deamidation of asparagines and glutamines and convert them into their corresponding aspartic and glutamic acids, although this was not observed in previous experiments [59]. Regardless of these concerns, RP×RPLC has gained a lot of attentions in the past few years, mainly due to a number of advantages over the traditional SCX×RP LC approach. The use of high pH mobile phase does not drastically degrade the RPLC efficiency and no obvious peptide losses have been observed in peptide fractions from a first dimension high pH RPLC. Furthermore, the salt-free mobile phases facilitate subsequent low pH RPLC-MS analysis. Lastly, peptide retention times can be accurately predicted, which provides additional information important for excluding false-positive peptide identifications.

Similar to SCX×RPLC, RP×RPLC is amenable to both online and offline setups. In offline RP×RPLC, concatenation of early and late fractions in bottom-up proteomics can minimize the overall analysis time without jeopardizing the separation efficiency [60]. Song et al. applied this method to identify phosphopeptides and demonstrated that selective pooling of short-time-interval (e.g., 1 min) fractions was better than collecting relatively long-time-interval (e.g., 2 min) fractions [61]. More recently, our group further extended this approach by pooling more short-time-interval fractions from early, middle, and late RPLC elution windows and demonstrated high protein coverage and improved throughput [62].

Compared to offline, online RP×RPLC is somewhat more difficult to implement due to the mobile phase incompatibilities. One approach for dealing with this issue is to dilute (online) the eluent from the first dimension and also use relatively large LC columns (e.g., 2.1 mm or 4.6 mm i.d.), which are used in most online RP×RPLC systems [24,63,64]. More recently developed online capillary RPLC×RPLC [65,66] also has been demonstrated for bottom-up proteomics analyses (Figure 8d) [67,68]. In this system, dilution of the organic content was realized by running the second dimension at 12 μ L/min vs. 1.5 μ L/min, i.e., an 8-fold increase in flow rate [68].

3.4. HILIC×RPLC

Among all the combinations depicted in Figure 7, HILIC×RPLC (Figure 7e) exhibits the best orthogonality [34]. The retention mechanism in first dimension HILIC is based mainly on the differential partitioning of the solutes between the mobile phase and the water-enriched solvent layer adsorbed onto the surface of the packing material, although ionic interactions may also exist depending on the stationary phase, mobile phase, and pH employed [69]. In fact, the separation selectivity of HILIC partially resembles that of SCX, although the clustering of identically-charged peptides is less prevalent in HILIC due to its primary retention partitioning mechanism [34]. For example, an off-line HILIC×RPLC system developed by Boersema et al., which utilizes a zwitterionic (ZIC)-HILIC column as the first dimension [70], produced a separation that resembled an SCX separation. However, HILIC afforded better orthogonality with RP at pH 3 and better separation power at pH 6.8, especially for doubly- and triply-charged peptides. Similar to SCX, HILIC at low pH can be employed for discovery of post translational modifications, such as phosphorylated and acetylated peptides/proteins [69]. HILIC also has the same selectivity as SCX for separating N-terminally blocked peptides (e.g., acetylation, formylation), but provides better retention towards phosphorylated peptides than SCX does. Differing from SCX sorbents that contain only negatively-charged functional groups, ZIC-HILIC sorbents contain both negative groups as well as positive groups that can attract the phospho-groups on peptides, which is important for retention of hydrophilic phosphorylated peptides.

Similar to RP×RPLC, high organic content in the HILIC mobile phases cause concerns for HILIC×RPLC systems. A high concentration of ACN (70-90%) is needed to dissolve samples for HILIC separation, and some peptides may precipitate in solvents containing such high concentrations of ACN. Wilson et al. solved the precipitation problem by loading a large volume of aqueous sample onto a short RP SPE column, and then back-flushing the peptides onto the inline HILIC column with solution containing a high percentage of ACN, which diluted the HILIC eluent (Figure 8e) [71].

4. Proteomic single-dimension vs. multidimensional LC

Multidimensional LC is widely applied in proteomics analyses and single-dimension LC is increasingly being recognized for its simple, universal, robust, and sensitive properties. However, the biggest concern that remains for single-dimension LC is the limited proteome coverage and dynamic range compared to multidimensional LC.

Shen et al. evaluated several advanced 1D RPLC-MS and offline 2D SCX×RPLC-MS platforms for proteomics analyses [6,33,72]. The proteome coverage provided by 1D RPLC-MS/MS for a microbial proteome was equivalent to that obtained with SCX×RPLC-MS/MS measurements of 10-15 fractions for microbial proteomics. However, when the number of SCX fractions was increased to 75-100, 1D LC-MS/MS proteomic analysis coverage was only ~80% of the 2D method. The proteins missed with the 1D approach were mainly theoretical and predicted proteins [72]. When applied to human blood plasma, which has one of the largest dynamic ranges of all proteomics samples, a 12h 1D RPLC (40 cm × 50 μ m i.d., 1.4 μ m d_p)-MS/MS analysis of 5 μ g of non-depleted plasma tryptic digest led to 853 protein identifications [6], which is better than the analytical coverage (593 protein identifications) obtained from 150 μ g of non-depleted plasma tryptic digest analyzed by offline SCX (80 cm × 320 μ m i.d., 3 μ m d_p , 15 fractions) ×RPLC (85 cm × 30 μ m i.d., 3 μ m d_p)-MS/MS [33]. However, the large initial amount of the SCX×RPLC-MS/MS analysis allows sufficient amount of each SCX fraction to be analyzed multiple times by RPLC-MS/MS, and a total of 39 runs of the 15 SCX fractions resulted in the identification of 881 unique proteins. In the SCX×RPLC study [33], the same RPLC column (85 cm × 30 μ m i.d., 3 μ m d_p) was used in both RPLC-MS/MS and SCX×RPLC-MS/MS to compare

performance. By using the same high-efficiency LC as one would use in single dimension analysis to construct SCX×RPLC, a proteomic analysis dynamic range of >8 orders of magnitude was obtained from 150 µg of non-depleted human plasma tryptic digest with a total of 39 RPLC-MS/MS runs of 15 SCX fractions, compared to the 4-5 orders of magnitude obtained from a total of 365 µg of the same sample consumed in 105 1D RPLC-MS/MS runs. Therefore, if a study wants to pursue extremely broad proteome coverage and large dynamic range (e.g., some mammalian proteomics such as human blood proteomic analysis), 2D LC with more fractions (e.g., >20) is the best choice.

More recently, Thakur et al. applied 1D RPLC-MS/MS to analyze mammalian cells and identified >4000 proteins [12]. Our laboratory is also evaluating the performance of 1D RPLC-MS/MS and online SCX×RPLC-MS/MS platforms for analyzing various sizes of mammalian proteomics samples, and our initial results are consistent with those reported by Thakur et al. Briefly, we identified ~26,000 peptides/~4200 proteins using RPLC-MS/MS and ~40,000 peptides/~5800 proteins using online 2D SCX×RPLC-MS/MS from 2.5 µg of sample; the protein overlap between the two approaches was ~70%. When the sample size was decreased to low-ng levels (e.g., 100 ng), our preliminary data showed that RPLC-MS/MS resulted in more peptide/protein identifications than online SCX×RPLC-MS/MS. It is worthwhile to point out that the protein overlap between the two platforms is about 70-80%, which is lower than the reproducibility of each platform (i.e., ~90%) [72], so if sample quantity and analysis time are not a concern, both approaches can be applied to complement the coverage obtained from each approach alone.

The unique benefit of a 1D RPLC-MS approach is a highly sensitive, high-throughput proteomics analysis. For example, we have demonstrated identification of 3 most abundant proteins from as little as 0.5 pg and 106 proteins from 5 pg of a microbial proteomic sample [16]. This sensitive detection is difficult to achieve with a 2D LC approach that typically requires low µg to high ng levels of proteomic samples. If available sample quantities are limited, then a high efficiency 1D LC approach is definitely the best choice. Moreover, 1D RPLC holds great potential for label-free quantitative proteomics in that all peptides would be measured in the same run, thereby avoiding run-to-run variations. Further experiments are needed to explore this concept.

5. Concluding remarks

Advanced LC techniques and methodologies coupled with MS improve coverage, sensitivity, and throughput and help address many key needs for proteomics research. Multiple LC techniques and their continuous improvements in individual separation elements as well as their hyphenated strategies are providing further advances and enabling increasingly effective large-scale proteomics applications. However, as requirements for analytical sensitivity and throughput increase, the need for even more sensitive and faster LC techniques continues. For example, studying proteome heterogeneity requires analytical tools capable of analyzing limited number of cells (or even a single cell). Another example is in the area of clinical biomarker discovery, where the need to analyze a substantial number of samples to obtain statistically relevant results demands high-throughput analytical platforms where LC separations must achieve maximal peak capacity in a short time period. Elsewhere, a combined bottom-up and top-down approach for analyzing intact protein targets has also gained interest to meet the needs encountered in the study of posttranslational modifications, intact protein truncates, and endogenous “natural” polypeptides. Further developments in LC for proteomics would likely have significant benefits for broad areas of application.

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Highlights (for review)

- Reviewed the advances in separation power, sensitivity and throughput in 1D LC
- Reviewed the different combinations of 2D LC
- Compared the pros and cons of 1D and 2D LC in proteomics

Figure 1. Linear relation ($R^2=0.84$) between the peak capacity achieved for the 25 (blue) and 50 cm (pink) column and the respective number of identified peptides. Reprints from Ref [7] with permission from the publisher.

Figure 2.

Achieving a chromatographic separation peak capacity of 1500 using a 200 cm $\times$ 50 μ m i.d. capillary containing 3- μ m porous C18 particles operated at 20 Kpsi for a cellular global tryptic digest of *S. oneidensis*. Conditions: 2 μ g *S. oneidensis* global tryptic digest was loaded onto a microSPE column and then transferred to an RPLC column prior to 11.4-T FTICR MS was used for detection (data were collected after 80-min gradient, scan speed of 6 s/scan); a linear velocity of 0.12 cm/s (measured with the RP mobile phase A) at 20 Kpsi was obtained for this 200-cm-length column and the gradient was selected with reference to a conventional 10-cm column for a 100-min gradient RPLC separation (simply referred to as “100 min/10 cm”). For detailed experimental conditions, see Ref [6].

Figure 3. NanoLC/ESI-MS base peak chromatograms for 100 ng of a yeast soluble protein tryptic digest on 74.5-, 47.1-, 29.7-, and 14.9- μm -i.d. fused-silica packed capillaries. Conditions: all columns had a length of 87 cm, and the flow rates for various inner diameter columns were given in Ref [14]. Reprints from Ref [14] with permission from the publisher.

Figure 4.

Ultrahigh-resolution mass spectrometry (FTICR MS). Results from a proteomic analysis from 5 pg of a *D. radiodurans* lysate tryptic digest. Peptide assignments used the mass and LC elution time tag methodology described in the [16] (MMA values used for these assignments are indicated). The location of the peptides according to their mass and relative elution times is indicated by “spots” in the 2D display. Reprints from Ref [16] with permission from the publisher.

Figure 5.

The 140-s RPLC-FTICR MS analysis of 1 μg of a *S. oneidensis* global tryptic digest with the MS scan speed of 0.3 s/spectrum. Conditions: a 10 cm $\times$ 50 μm i.d. capillary packed with 0.8- μm porous C18-bonded silica particles was used as the RPLC column with a linear ion trap-FTICR MS for detection under conditions as described in Ref [19]. (A) Base peak chromatogram (m/z 400-2000); (B) RPLC-MS 2-D display of the detected species; (C) MMA and (D) TMA histograms obtained when the detected species (shown in the 2-D display) were searched against a reference peptide database established in a 50-min RPLC-MS/MS analysis. Reprints from Ref [19] with permission from the publisher.

Figure 6.

Proteome analysis throughput and coverage for three RPLC-accurate MS platforms and RPLC-linear ion trap MS/MS method in terms of (A) different molecular species detected, (B) different peptides identified, and (C) different proteins identified using (●)RPLC-TOF MS, (◆)RPLC-FTICR MS (11.4 T without AGC), and (◇)RPLC-linear ion trap-FTICR MS (with AGC). The dashed lines show the corresponding results for the total number of MS/MS spectra obtained for the indicated time (A), the number of peptides identified using MS/MS (B), and number of proteins identified (C), which were estimated using the results reported in Ref [18]. Conditions: a 10 cm $\times$ 50 μ m i.d. capillary packed with 0.8- μ m porous C18-bonded silica particles was used as the RPLC column with different mass spectrometers. Reprints from Ref [19] with permission from the publisher.

Figure 7.

Normalized retention time plots for selected 2D LC systems with RPLC at pH 2.6 in the second dimension. First dimension separations were RPLC on phenyl silica at pH 2.6 (a), on perfluorophenyl silica at pH 2.6 (b), on hybrid silica C18 at pH 10 (c), SEC on diol silica at pH 4.5 (d), HILIC on silica at pH 4.5 (e) and SCX on poly(2-sulfoethyl aspartamide) silica at pH 3.25 (f). For detailed experimental conditions, the reader is referred to [34]. Reprints from Ref [34] with permission from the publisher.

Figure 8.

Selected online 2D LC configurations: (a) biphasic MudPIT (SCX x RPLC) [39], (b) online SCX x RPLC setup using stepwise salt gradient [44], (c) online SCX x RPLC setup using linear pH gradient [50], (d) online high pH RP x low pH RPLC setup [68], and (e) online HILIC x RPLC setup [71]. Reprints from Ref [39,44,50,68,71] with permission from the publisher.

Table 1

The condition of 1D capillary LC-MS applied for proteomic analysis.*

L (cm)	d_c (μm)	d_p (μm)	F (nL/min)	P (Kpsi)	Sample	Sample size (μg)	T (min)	C_p	Mass spectrometer	MS/MS	MS (AMT)	# of peptide/protein
87 (Ref [14])	15	3	20	10	yeast	0.1	200	~1000	QSTAR Q-TOF			NA
87 (Ref [14])	20	3	33	10	yeast	0.1	200	~1000	QSTAR Q-TOF			NA
87 (Ref [14])	30	3	76	10	yeast	0.1	200	~1000	QSTAR Q-TOF			NA
87 (Ref [14])	50	3	155	10	yeast	0.1	200	~1000	QSTAR Q-TOF			NA
87 (Ref [14])	75	3	393	10	yeast	0.1	200	~1000	QSTAR Q-TOF			NA
200 (Ref [6])	50	3	150	20	<i>S. onesidensis</i>	15	600	~1500	LCQ ion trap			4623/1019
90 (Ref [6])	50	2	150	20	<i>S. onesidensis</i>	15	900	~1200	LCQ ion trap			4676/993 ^a
40 (Ref [6])	50	1.4	150	20	<i>S. onesidensis</i>	15	400	~1000	LCQ ion trap			4542/1012 ^a
40 (Ref [6])	50	1.4	150	20	<i>S. onesidensis</i>	10	120	~1000	LTQ ion trap			5944/1200 ^a
40 (Ref [6])	50	1.4	150	20	<i>S. onesidensis</i>	10	240	~1000	LTQ ion trap			8709/1750
40 (Ref [6])	50	1.4	150	20	<i>S. onesidensis</i>	10	600	~1000	LTQ ion trap			~12000/~2000
40 (Ref [6])	50	1.4	150	20	<i>S. onesidensis</i>	10	720	~1000	LTQ ion trap			>12000/>2000
40 (Ref [6])	50	1.4	150	20	human plasma	5	720	~1000	LTQ ion trap			2244/572 ^b
10 (Ref [19])	50	0.8	500	8.5	<i>S. onesidensis</i>	1	2	~100	Agilent TOF			1656/492
10 (Ref [19])	50	0.8	500	8.5	<i>S. onesidensis</i>	1	2.3	~100	LTQ ion trap-FTICR			991/288
20 (Ref [18])	50	0.8	500	20	<i>S. onesidensis</i>	2.5	8	~130	LTQ ion trap			666/257
20 (Ref [18])	50	0.8	500	20	<i>S. onesidensis</i>	2.5	20	~220	LTQ ion trap			1848/558
20 (Ref [18])	50	0.8	500	20	<i>S. onesidensis</i>	2.5	50	~420	LTQ ion trap			3848/949
25 (Ref [7])	75	2	NA	~10	HeLa	1	60	~220	LTQ Orbitrap Velos			~5200/~1100
25 (Ref [7])	75	2	NA	~10	HeLa	1	120	~280	LTQ Orbitrap Velos			~7200/~1400
25 (Ref [7])	75	2	NA	~10	HeLa	1	180	~320	LTQ Orbitrap Velos			~8200/~1550
25 (Ref [7])	75	2	NA	~10	HeLa	1	240	~330	LTQ Orbitrap Velos			~9300/~1650
25 (Ref [7])	75	2	NA	~10	HeLa	1	300	~350	LTQ Orbitrap Velos			~9900/~1750

<i>L</i> (cm)	<i>d_c</i> (μm)	<i>d_p</i> (μm)	<i>F</i> (nL/min)	<i>P</i> (Kpsi)	Sample	Sample size (μg)	<i>T</i> (min)	<i>C_p</i>	Mass spectrometer	MS/MS	MS (AMT)	# of peptide/protein
25 (Ref [7])	75	2	NA	~10	HeLa	1	360	~380	LTQ Orbitrap Velos			~10100/~1800
25 (Ref [7])	75	2	NA	~10	HeLa	1	480	~400	LTQ Orbitrap Velos			~11000/~2000
25 (Ref [7])	75	2	NA	~10	HeLa	1	600	~430	LTQ Orbitrap Velos			~10700/~1950
50 (Ref [7])	75	2	NA	~10	HeLa	1	60	~250	LTQ Orbitrap Velos			~5200/~1200
50 (Ref [7])	75	2	NA	~10	HeLa	1	120	~350	LTQ Orbitrap Velos			~8200/~1600
50 (Ref [7])	75	2	NA	~10	HeLa	1	180	~420	LTQ Orbitrap Velos			~10100/~1900
50 (Ref [7])	75	2	NA	~10	HeLa	1	240	~495	LTQ Orbitrap Velos			~11600/~2100
50 (Ref [7])	75	2	NA	~10	HeLa	1	300	~550	LTQ Orbitrap Velos			~12400/~2250
50 (Ref [7])	75	2	NA	~10	HeLa	1	360	~580	LTQ Orbitrap Velos			~11400/~2300
50 (Ref [7])	75	2	NA	~10	HeLa	1	480	~620	LTQ Orbitrap Velos			~14100/~2550
50 (Ref [7])	75	2	NA	~10	HeLa	1	600	~700	LTQ Orbitrap Velos			~14000/~2450
50 (Ref [12])	75	1.8	75	NA	yeast	2	140	~330	LTQ Orbitrap Velos			9595/1867 ^c
50 (Ref [12])	75	1.8	75	NA	yeast	2	480	~550	LTQ Orbitrap Velos			21022/2991 ^c
50 (Ref [12])	75	1.8	75	NA	human embryonic kidney cells	2	480	~550	LTQ Orbitrap Velos			34877/5440 ^c
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	0.2	170	378	LTQ Orbitrap Velos			5833/1351
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	0.2	310	538	LTQ Orbitrap Velos			10895/2418
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	0.2	410	701	LTQ Orbitrap Velos			12752/2728
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	0.2	510	713	LTQ Orbitrap Velos			16465/3098
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	0.2	600	755	LTQ Orbitrap Velos			16948/3243
100 (Ref [13])	25	5	5	1.5	mouse embryonic stem cells	2	600	755	LTQ Orbitrap Velos			21593/3590
350 (Ref [28])	100	Mo	500	2.9	<i>E. coli</i>	4	2470	~690	LTQ Orbitrap			22196/2602 ^c

<i>L</i> (cm)	<i>d_c</i> (μm)	<i>d_p</i> (μm)	<i>F</i> (nL/min)	<i>P</i> (Kpsi)	Sample	Sample size (μg)	<i>T</i> (min)	<i>C_p</i>	Mass spectrometer	MS/MS	MS (AMT)	# of peptide/protein
70 (Ref [29])	10	Mo	40	5	<i>S. onesidensis</i>	2.5	200	~420	LCQ ion trap			1131/493
70 (Ref [29])	10	Mo	40	5	<i>S. onesidensis</i>	2.5	600	~450	LCQ ion trap			2367/855
25 (Ref [30])	10	Mo	10	~1	<i>S. onesidensis</i>	0.3	240	NA	LTQ ion trap			5510/1443

* *L*: LC column length; *d_c*: column inner diameter; *d_p*: particle size; *F*: LC flow rate; *P*: LC operation pressure; *T*: separation (analysis) time; *C_p*: LC separation peak capacity; NA: not available.

^aPeptides/proteins were identified within 24 h analysis of 15 μg of sample.

^bPeptides/proteins belonging to antibodies were excluded.

^cPeptides/proteins were identified from triplicate analyses.

Table 2

Optimized SPE-capillary and nanoscale LC arrangements at 10 Kpsi. Detailed experimental information is provided in Ref [15]. Reprints from Ref [15] with permission from the publisher.

LC column (3- μm particle packed)	SPE column (5- μm particle packed)	LC flow rate ($\mu\text{L}/\text{min}$)	SPE flow rate ($\mu\text{L}/\text{min}$)
86 cm $\times$ 15 μm i.d.	4 cm $\times$ 50 μm i.d.	~0.02	~8
86 cm $\times$ 30 μm i.d.	4 cm $\times$ 75 μm i.d.	~0.07	~13
86 cm $\times$ 50 μm i.d.	4 cm $\times$ 150 μm i.d.	~0.14	~75
86 cm $\times$ 75 μm i.d.	4 cm $\times$ 200 μm i.d.	~0.40	~120
86 cm $\times$ 150 μm i.d.	4 cm $\times$ 380 μm i.d.	~1.50	~400

Table 3

The formats and conditions of 2D LC applied for proteomic analysis.*

2D Scheme	1 st Dimension							Connection mode	
	L (cm)	d _c (μm)	Stationary phase	F (nL/min)	Buffer A	Buffer B	Elution	On-line	Off-line
SCX×RPLC (Ref [38])	4	100	5 μm SCX	300	5 mM K ₂ HPO ₄ , 5% ACN, 95% H ₂ O	250 mM KCl, 5% ACN, 95% H ₂ O	12-cycle salt steps		
SCX×RPLC (Ref [39])	4	100	5 μm SCX	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	250 mM NH ₄ Ac, 5% ACN, 0.02% HFBA	15-cycle salt steps		
SCX×RPLC (Ref [39])	4	100	5 μm SCX	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	250 mM NH ₄ Ac, 5% ACN, 0.02% HFBA	15-cycle salt steps		
SCX×RPLC (Ref [39])	4	100	5 μm SCX	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	250 mM NH ₄ Ac, 5% ACN, 0.02% HFBA	15-cycle salt steps		
SCX×RPLC (Ref [44])	1.5	500	5 μm SCX	35,000	0.1% FA, 100% H ₂ O	50, 100, 200, 500, 800, 1000, 2000 mM NH ₄ Ac in 0.1% FA	salt steps		
SCX×RPLC (Ref [47])	5	320	5 μm SCX	3000	0.1% FA, 100% H ₂ O	citric acid-NH ₄ OH buffers with different pH values (pH3-8)	pH steps		
SCX×RPLC (Ref [50])	10	320	5 μm SCX	2000	pH 2.5 buffer	pH 8.5 buffer	linear gradient		
RP×RPLC (Ref [59])	15	1000	3.5 μm C18 RP	50,000	20 mM AF in 100% H ₂ O, pH 10	20 mM AF in 10% H ₂ O and 90% ACN, pH 10	linear gradient		
RP×RPLC (Ref [67])	20	150	3 μm C18 RP	NA	20 mM AF in 98% H ₂ O, 2% ACN, pH 10	20 mM AF in 10% H ₂ O and 90% ACN, pH 10	linear gradient		
RP×RPLC (Ref [68])	15	400	2.7 μm C18 RP	1500	10 mM NH ₄ Ac in 90% H ₂ O, 10% ACN, pH 9	10 mM NH ₄ Ac in 10% H ₂ O and 90% ACN, pH 9	linear gradient		
HILIC×RPLC (Ref [70])	16	200	3 μm ZIC-HILIC	1500	0.05% FA, 20% H ₂ O, 80% ACN, pH 3	0.05% FA, 60% H ₂ O, 40% ACN, pH 3	linear gradient		
HILIC×RPLC (Ref [70])	16	200	3 μm ZIC-HILIC	1500	20 mM NH ₄ Ac, 20% H ₂ O, 80% ACN, pH 6.8	20 mM AF, 60% H ₂ O, 40% ACN, pH 6.8	linear gradient		
HILIC×RPLC (Ref [71])	15	300	5 μm ZIC-HILIC	5000	0.5 mM AF, 5% H ₂ O, 95% ACN	9.5 mM NH ₄ Ac, 95% H ₂ O, 5% ACN	linear gradient		

2D Scheme	2 nd Dimension					Mass spectrometer	Sample	Sample size (μg)	# of peptides/proteins
	L (cm)	d _c (μm)	C18 d _p (μm)	F (nL/min)	Buffer A	Buffer B			
SCX×RPLC (Ref [38])	8	100	5	300	0.5% AA	0.5% AA, 80% ACN	LCQ ion trap	yeast ribosome	>100 proteins

2D Scheme	2 nd Dimension					Mass spectrometer	Sample	Sample size (μg)	# of peptides/proteins
	L (cm)	dc (μm)	C18 d _p (μm)	F (nL/min)	Buffer A	Buffer B			
SCX×RPPLC (Ref [39])	10	100	5	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	80% ACN, 20% H ₂ O, 0.02% HFBA	yeast soluble fraction	400	1665/630
SCX×RPPLC (Ref [39])	10	100	5	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	80% ACN, 20% H ₂ O, 0.02% HFBA	yeast lightly washed insoluble fraction	400	2169/974
SCX×RPPLC (Ref [39])	10	100	5	150-250	5% ACN, 95% H ₂ O, 0.02% HFBA	80% ACN, 20% H ₂ O, 0.02% HFBA	yeast heavily washed insoluble fraction	400	2114/1036
SCX×RPPLC (Ref [44])	15	75	3	200	2% ACN, 98% H ₂ O	20% H ₂ O, 80% ACN, 0.5% FA	Jurkat cells	10	174 proteins
SCX×RPPLC (Ref [47])	10	150	5	2000	0.1% FA, 100% H ₂ O	0.1% FA, 100% ACN	mouse liver	600	5779/2067
SCX×RPPLC (Ref [50])	15	75	5	200	0.1% FA, 100% H ₂ O	0.1% FA, 100% ACN	mouse liver	300	8371/3391
RP×RPLC (Ref [59])	15	300	3.5	5000	0.1% FA, 100% H ₂ O, pH 2.6	0.1% FA, 20% H ₂ O, 80% ACN, pH 2.6	standard proteins	NA	NA
RP×RPLC (Ref [67])	20	150	3	NA	0.5% FA in 98% H ₂ O, 2% ACN, pH 2	0.5% FA in 2% H ₂ O, 98% ACN, pH 2	zebrafish embryo	12	7586/1752
RP×RPLC (Ref [68])	10	320	5	12,000	0.1% TFA in 90%	0.1% TFA in 10%	standard protein	NA	NA

2D Scheme	2 nd Dimension						Mass spectrometer	Sample	Sample size (μg)	# of peptides/proteins
	L (cm)	dc (μm)	C18 d _p (μm)	F (nL/min)	Buffer A	Buffer B				
					H ₂ O, 10% ACN, pH 2	H ₂ O, 90% ACN, pH 2				
HILIC×RPLC (Ref [70])	25.4	50	3	100	0.5% AA in 100% H ₂ O	0.5% AA in 20% H ₂ O, 80% ACN	Q-TOF	murine erythro-leukemi cells	10	4973/1040
HILIC×RPLC (Ref [70])	25.4	50	3	100	0.5% AA in 100% H ₂ O	0.5% AA in 20% H ₂ O, 80% ACN	Q-TOF	murine erythro-leukemi cells	10	6625/1284
HILIC×RPLC (Ref [71])	15	300	3	5000	0.095% FA in 95% H ₂ O, 5% ACN	0.005% FA in 5% H ₂ O, 95% ACN	TOF	rat muscle dialysate	NA	NA

* AA: acetic acid; AF: ammonium formate; others are the same as those used for Table 1.