Journal of Chromatography A, 1218 (2011) 8760–8776

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Review

Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics

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ARTICLE INFO

Article history:
Available online 6 June 2011

Keywords:
Liquid chromatography
Electrophoresis
High accuracy mass spectrometry
Proteins
Top-down proteomics

ABSTRACT

Mass spectrometry used in combination with a wide variety of separation methods is the principal methodology for proteomics. In bottom-up approach, proteins are cleaved with a specific proteolytic enzyme, followed by peptide separation and MS identification. In top-down approach intact proteins are introduced into the mass spectrometer. The ions generated by electrospray ionization are then subjected to gas-phase separation, fragmentation, fragment separation, and automated interpretation of mass spectrometric and chromatographic data yielding both the molecular weight of the intact protein and the protein fragmentation pattern. This approach requires high accuracy mass measurement analysers capable of separating the multi-charged isotopic cluster of proteins, such as hybrid ion trap-Fourier transform instruments (LTQ-FTICR, LTQ-Orbitrap). Front-end separation technologies tailored for proteins are of primary importance to implement top-down proteomics. This review intends to provide the state of art of protein chromatographic and electrophoretic separation methods suitable for MS coupling, and to illustrate both monodimensional and multidimensional approaches used for LC–MS top-down proteomics. In addition, some recent progresses in protein chromatography that may provide an alternative to those currently employed are also discussed.

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1. Introduction

Nowadays, proteomics is a well-established science, with applications in different fields, such as environmental [1], agricultural [2], biotechnology, and mainly medicine. In particular, proteomics studies play a central role in the discovery of disease biomarkers and drug targets.

Mass spectrometry used in combination with a wide variety of separation methods is the principal methodology for proteomics. Two fundamental strategies for protein identification and characterization by MS are currently employed in proteomics. In bottom-up approaches, purified proteins, or complex protein mixtures, are subjected to proteolytic cleavage, and the peptide products are analyzed by MS. In top-down proteomics, intact protein ions or large protein fragments are subjected to gas-phase fragmentation for MS analysis [3].

There is some ambiguity about the employment of the terms "top-down" and "bottom-up" in proteomics. In one lexicon, they designate the entity that is subjected to the primary separation technique. In this regard, separation of proteins by gel

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doi:10.1016/j.chroma.2011.05.094

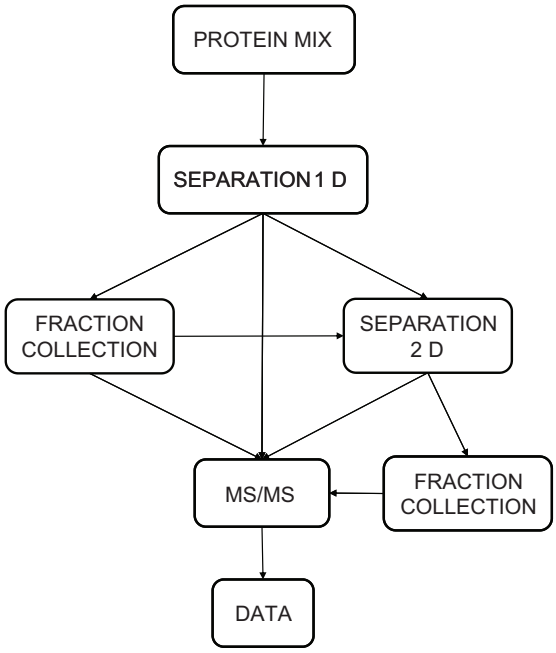


Fig. 1. Schematic diagram of top-down proteomics strategy. Protein mixture is fractionated (on-line or off-line) and directly analyzed by high resolution (HR) MS and MS/MS or fractionated in a second dimension before HR MS and MS/MS.

electrophoresis followed by in-gel proteolytic digestion and LC–MS analysis of the resulting peptide mixture can be considered a top-down approach, because the initial entities separated are proteins. In another lexicon, adopted by Reid and McLuckey [4] in their review of top-down protein characterization, top-down and bottom-up refer to the entities introduced into the mass spectrometer. As the majority of the scientists involved in proteomics research agree with the second definition, this terminology will be the one adopted in the present review.

Bottom-up strategy is the most widely used one for protein identification and characterization, and generally RP nanoLC/electrospray ionization (ESI)-MS/MS is employed to analyze the proteolytic mixtures. However, this approach, even if mature from the point of view of methodologies, instrumentations and bioinformatic tools, presents some limitations. In fact, information on protein sequence is only partially obtained, with potential loss of data regarding isoforms and post-translational modifications (PTMs).

Top-down proteomics experiments consist in introducing intact proteins into the mass spectrometer: the ions generated by ESI (rarely by matrix assisted laser desorption ionization, MALDI) are then subjected to gas-phase separation, fragmentation, fragment separation, and automated interpretation of mass spectrometric and chromatographic data yielding both the molecular weight (MW) of the intact protein and the protein fragmentation ladders. A schematic diagram of generic top-down experiment is shown in Fig. 1. The protein-containing sample can be submitted, depending on its complexity and information request to one dimension (1D) or 2D separation before MS and MS/MS. In turn, separations can operate on-line or off-line with MS and between them.

The top-down strategy can integrate and support information obtained from bottom-up analysis, in particular regarding complete protein sequence and PTM localization as well as any combination possibly existing between modifications on distinct parts of the protein sequence. The main limitations of this approach are that it requires high accuracy mass measurement analysers such as Fourier transform ion cyclotron resonance (FTICR), hybrid ion trap-FTICR instruments, capable of separating the multi-charged isotopic cluster of proteins as large as 229 kDa [5], or the more recently introduced LTQ-Orbitrap [6]; the difficulty in the determination of product ion masses from multiply charged product ions (with possible ambiguity in the interpretation of top-down MS/MS spectra) [3]; the lack of suitable software for reconstructing protein identity. Moreover, differently from bottom-up experiments, full automation in data treatment is possible only depending on instrumentation capability.

Due to the problems of coupling on-line LC to a FTICR instrument still maintaining its very high resolution, especially when electron transfer dissociation (ETD) and/or electron capture dissociation are used as fragmentation techniques, much of the early top-down work has been done with infusion of isolated proteins or simple protein mixtures [3]. This difficulty is mainly due to the relatively long duty cycle needed for a sufficiently sensitive MS/MS spectra acquisition as the LTQ-FTICR instrument require greater ion population than the simpler LTQ instrument. The introduction of devices able in enhancing ion transmission and/or ion desolvation [7,8] alleviates the problem reducing the filling time of LTQ; in addition, the automatic gain control (AGC) of the filling time allows the instrument to work at the best condition even as ion flow rate changes. Recently, also less resolution instruments with a faster duty cycle such as a quadrupole-time of flight (Q-TOF) [9], and MALDI-TOF/TOF [10] have been used in infusion mode with promising results.

Despite the availability of high-performance mass spectrometers, methods for MS-compatible separation and high-throughput identification of intact proteins are yet underdeveloped. Therefore, top-down analysis is often implemented as a technique for precise characterization of one or a few protein targets. Because the complexity of most samples is usually not resolved by MS and MS/MS, for top-down proteomics front-end separation technologies tailored for proteins should be implemented. Protein separation by LC and electrophoretic methods is an important issue, whose theoretical aspects are outside the purpose of this review and have been discussed elsewhere [11–13].

The current LC methods for protein separation employ a wide variety of techniques and stationary phases, i.e. ion exchange (IEC), size exclusion (SEC), RPLC, hydrophylic interaction (HILIC), hydrophobic interaction, mixed mode, immobilized metal on affinity, dye–ligand affinity, immobilized artificial membrane, liquid–liquid partition, and displacement, as well as chromatofocusing [14]. In addition, also electrophoresis, in the form of isoelectrofocusing (IEF), gel electrophoresis, capillary electrophoresis (CE), and capillary electrochromatography (CEC) are currently used [15]. Some of these methods cannot be connected on-line with ESI-MS, but may be used as a first step in an off-line/on-line 2D separation method.

During the two last decades, the task of biomolecule isolation and purification has achieved increasing progress [16]. The evolution in both column and packing designs devote strong efforts in accelerating the separation of the large proteins, mainly in order to miniaturize the process and avoid analyte losses [12]. Enormous progress has been made in column chromatography with both porous and non-porous supports. Intact proteome analysis by MS intrinsically possesses lower sensitivity, due to signal distribution formed through multiple charged states and strong affinity to adducts. Thus, separation of a larger quantity of starting material represents the simplest strategy to overcome sensitivity limitations of the mass spectrometer. To overcome the low capacity of non-porous supports, which instead allow fast separation, different materials had been tested, however, the most suitable appeared to be the monolithic materials, emerged at the end of the 1980s. The monolith in fact was shown to possess the advantages of both membranes (low pressure-drop and ease of handling) and conventional columns (high efficiency and resolution). In this respect, IEF has the

advantage that the entire column is filled by the sample with proteins focalized in narrow bands, thus realizing high capacity and sample concentration. Many reviews have been published regarding technology and applications of chromatography and related techniques to protein separation [17–27]. The main purpose of this review is to provide the state of art in coupling separation, both chromatographic and electrophoretic, to on-line MS for top-down proteomics. Off-line coupling will be also briefly discussed. Finally, some recent progresses in protein chromatography that may provide an alternative to those currently employed are also illustrated and briefly discussed.

2. Separation techniques

2.1. Chromatography

In contrast to peptide separation, the effective fractionation of proteins at the intact level continues to present a challenge in solution-based platforms of proteomics and still remains heavily reliant on gel-based technology. Nevertheless, the sample bias, irreproducibility, poor recovery for intact proteins, and poor throughput of this platform gave rise to exploration of liquid phase-based alternatives. However, protein recovery is a great concern also in solution platforms. Irreversible adsorption has been repeatedly reported as a significant problem in intact protein chromatography [28,29]. Although the phenomena of ghost peak, carry-over and irreversible adsorption have been recognized early in chromatography of proteins, and despite tens of years of improvement in column technology, it still represents a problem. Proteins may interact very strongly with physical and chemical active sites, which may be constitutive of the column packing or induced by aging. Controlled porosity supports, injection of clean samples (i.e. lipid-free), and high quality materials (i.e. connections) may alleviate the impact of these problems. Recently, with the introduction of heat resistant packings, also relatively elevate temperatures (50–70 °C) may improve protein recoveries by lowering strong interactions. Raising the column temperature from 25 to 60 °C increased also efficiency by reducing interdiffusion coefficients, protein refolding and protein–protein interaction. With alkylated, silica-based RP stationary phases, irreversible protein adsorption has been found to depend on alkyl chain length, whereby shorter alkyl groups facilitated higher recoveries [29]. Thus, the less retentive, shorter C4, C5 alkyl chains are typically employed for intact protein separations. However, given the touted benefits of polymeric media such as uniform hydrophobicity and increased mechanical strength [30–34], also this kind of packing is widely used for the chromatographic separation of intact proteins. Although IEC has questionable recovery for certain proteins, also when weak anion or cation exchange are employed [35,36], it may be used as first step for 2D protein fractionation.

HILIC is a form of direct phase LC which is performed using a water miscible organic solvent such as acetonitrile as weak solvent and water as strong solvent. HILIC is particularly suitable for analyzing very polar compounds by LC/ESI-MS, as the organic phase-rich solvent produces a very efficient electrospray events. Moreover, the quasi-total orthogonality respect to RPLC makes the use of HILIC in 2D separation useful. Recently, an exhaustive review of this topic has been published [37].

Another very important issue is efficiency, expressed both as resolution and peak capacity. Particle shape, diameter and porosity, column length and gradient form play a fundamental part in determining efficiency [11,38]. Longer columns, small spherical particles and slow gradients increase peak capacity but, especially for proteins, porosity is of comparable importance. A theoretical analysis of conditions influencing the efficiency of a column in the separation of macromolecules with MW exceeding 10^5 (very small interdiffusion coefficient) revealed that the optimum size of porous particles in the packing medium is about 1 μm [11]. When this size is reached, the slow diffusion of solutes within the pores does not restrict the separation quality. While even smaller sizes would theoretically be desirable, current limitations in technology such as packing problems, elevated back pressure, etc., render the concept impracticable.

Finally, column diameter is of paramount importance in determining capacity and sensitivity. For top-down proteomics, nano-RPLC plays a major role in both single and multidimensional protein separations in an effort to increase the overall peak capacity for the resolution of complex protein mixtures prior to MS analysis and increase sensitivity and dynamic range.

Wang et al. [39] examined the effects of various chromatography conditions, including alkyl chain length in the stationary phase, capillary column temperature and ion-pairing agent, on the resolution of intact proteins. Fused-silica capillaries 130 mm × 50 μm i.d. home-packed with 5 μm C4 and C18 bonded silica particles were tested. Pore dimension of packing was not reported, but from results we can suppose a 300 Å packing. The flow rate was 200 nL min^{-1}, phase A water and phase B acetonitrile, both containing 0.1% (v/v) trifluoroacetic acid (TFA) or 0.02% (v/v) heptafluorobutyric acid (HFBA). A 30 min linear gradient from 10% to 65% B was utilized. Four model proteins, including bovine serum albumin, cytochrome *c*, myoglobin, and ribonuclease A, prepared in 10 mmol L^{-1} Tris at pH 7.0 with a final concentration of 0.1 μmol L^{-1} each were used for tests, and were monitored using a Q-TOF mass spectrometer.

Both separation efficiency and resolution of model proteins achieved with the C18 column were considerably better than those obtained from the C4 column, particularly for early eluted proteins. Still, potential sample loss due to irreversible protein adsorption onto the C18 stationary phase may outweigh the benefits of better separation performance and may adversely impact the ability to perform comprehensive proteome analysis, particularly towards the identification of low abundance proteins. The use of 0.02% (v/v) HFBA as ion-pairing agent instead of 0.1% (v/v) TFA resulted in significant deterioration of separation. Further increase in HFBA concentration contributed to a dramatic reduction in protein ion intensities. Raising the column temperature from 25 to 60 °C increased efficiency and decreased irreversible adsorption. The soluble fraction of intact proteins from *Saccharomyces cerevisiae* was then analyzed and a peak capacity of 360 over a gradient run time of 60 min was obtained (see Fig. 2). Such a peak capacity is noteworthy, but still insufficient for extended top-down proteomics, for which a 2D separation method may be mandatory.

Differently from small and mid-size molecules, protein physical parameters strongly affect the kinetics of the adsorption process, its thermodynamics, and the character of the interaction between the proteins and the surface. In addition to these factors, also the mutual interactions between individual protein molecules affect their adsorption.

Another typology of chromatography is the so called perfusion chromatography. In perfusion chromatography packing with very large pores of up to 1 μm have been used. These large pores allow at least part of the mobile phase to flow through the beads, rather than around them, therefore improving the kinetics of separation in the interior of the beads by reducing the diffusional path length. The perfusion concept has been further improved by using flat macroporous polymer membranes, few mm in length. The separation proceeds by gradient elution and the method is useful in the chromatographic modes based on the on-off principle. This principle is the result of the observation that when the mobile phase composition is varied for isocratic elution, it is found that the protein never migrate normally, but is either retained completely or eluted at t_0. However, the macroporous membranes have limited capacities and

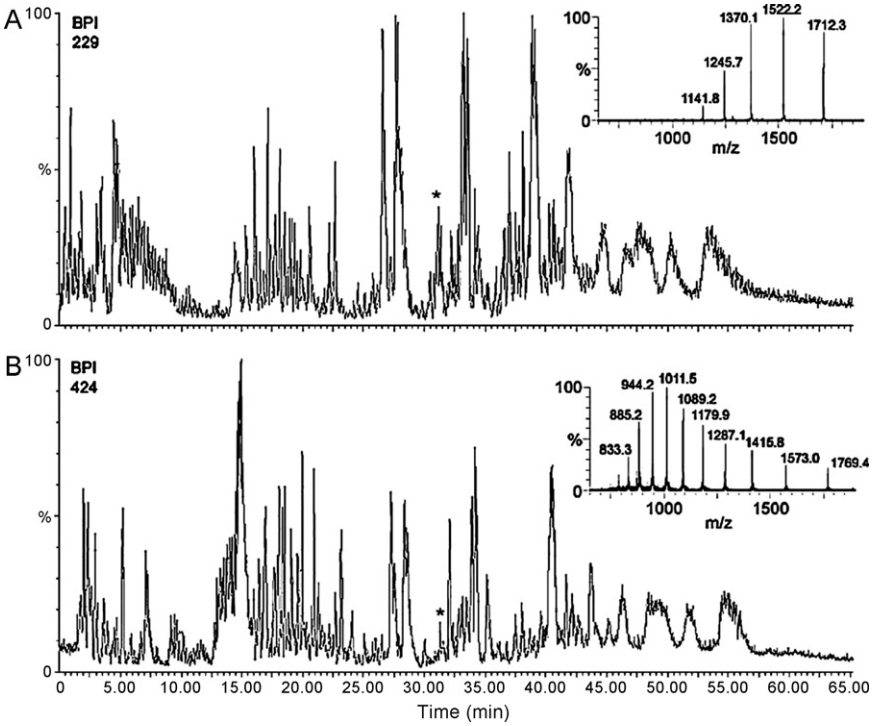


Fig. 2. Base peak chromatograms for the soluble fraction of yeast cell lysates in the (A) native and (B) denatured states using nano-RPLC–ESI-MS. C18 column temperature: 60 °C; mobile phase: a 60-min linear gradient from 10% to 65% acetonitrile (containing 0.1% TFA) at a flow rate of 200 nL min^{-1}; sample loading: 2 μg of total yeast protein (1 μL injection of 2 mg mL^{-1} yeast protein solution) spiked with ribonuclease A. Eluted ribonuclease A peaks are marked with * and the mass spectra taken from the average of scans under the peaks are shown as insets.
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can only be used in a specialized form of separation. More recently, a rigid biporous bead which presents very interesting flow hydrodynamic characteristics has been fabricated by double emulsification of glycidyl methacrylate and ethylene glycol dimethacrylate [40]. The polymer was characterized to possess micropores (20–100 nm) and superpores (300-4000 nm), and was also derivatized to act as weak anion exchanger for very fast separation of proteins. Up to now this technique has never been applied to proteomics.

Wagner et al. [41] proposed a 2D separation for proteins of MW < 20,000 Da which are not well resolved in 2D-polyacrilamide gel electrophoresis (PAGE). In the first dimension 2.5 μm, 35 mm × 4.6 mm i.d. polymeric beads bonded with diethylaminoethyl or sulfonic acid groups, were applied as ion exchangers and operated at a flow rate of 1 mL min^{-1}. To achieve very high-speed and high-resolution separations in the second dimension, short columns of 14 mm × 4.6 mm i.d. with 1.5 μm C18 bonded, non-porous silica (NPS) packing were chosen and operated at a flow rate of 2.5 mL min^{-1}. Two RP columns were used in parallel in the second dimension. The analyte fractions from the IEC column were transferred alternatively to one or the other of the two RP columns using a switching valve. Proteins which were not completely resolved in the first dimension can, in most cases, be separated in the second dimension. The total value of peak capacity was calculated to 600. Repeatability (RSD) of the retention times of proteins were less than 1%, while the RSDs of the peak areas were less than 15%. The problem of this system was the low recovery of proteins injected amount less than 100 μg.

Both silica-based and polymeric monolithic columns are at present a hot issue in protein separation. Reviews are available in the literature regarding fabrication [22,42], characterization [43], applications [24], and microscale separations [44]. Monoliths do not need to be packed into a column since they can be prepared *in situ* by polymerization. They can be considered a single large "particle" of porous material that fills entirely the column volume without any interparticular voids typical of packed columns. Because of that, all the mobile phase must flow through the stationary phase. Moreover, because of their wide through-pores, much higher separation efficiency at higher flow rate than in the case with particle-packed columns can be achieved. Modern monoliths to be used as columns for HPLC emerged in the late 1980s–mid 1990s [45–47]. After the first attempts, a rapid development started which led to rigid, macroporous polymer bed [48]. This was followed by silica monoliths that could be functionalized at the pore surface [47,49]. With the rapid development of micro- and nanoscale chromatographic separation systems hyphenated to mass spectrometers, it has become clear that monoliths were the right choice for separation of proteolytic digests in proteomics by providing the better stability, resolution and sensitivity compared to the regular scale separation systems. This large impact has not been followed by a similar one in the field of top-down proteomics. This is probably due to the difficulties of preparing in-house efficient, stable and reproducible nanobore monolithic columns for researchers not specialized to use the related technology.

Ultraperformance (UP)LC is a relatively new LC technique which makes use of columns packed with particles smaller than 2 μm. To alleviate the very high pressure needed for mobile phase flowing at a suitable linear velocity, a relatively high temperature (~50 °C) is used, with an increased lowering of the interdiffusion coefficient. These columns are now commonly employed for small molecule separation, but are not very exploited for protein separation.

Everley and Croley [50] focused on extending the separation performance improvements of UPLC for small molecules to intact proteins. The parameters of temperature, organic modifier and mobile phase gradient were optimized. Employing a combination

of high temperatures, strong solvents and relatively fast gradient, yielded optimal results, which included enhanced resolution, sensitivity and a threefold increase in throughput.

Eschelbach and Jorgenson [28] investigated the effect of the very elevated pressure that could be used in UPLC (>1500 bar) on protein recovery. The columns used for the experiment (32–36 cm × 50 μm i.d., 150 Å) were prepared in-house. They found that the elevated pressure was very favourable in reducing (for some eliminating at all) strong adsorption phenomena and incomplete recovery.

In another recent paper dealing with the evaluation of the performance of olive (*Olea europaea* L.) stone protein profiling for cultivar fingerprinting by UV detection [51], different typologies of columns such as conventional, perfusion, monolithic, superficially porous particles and two UPLC (1.8 μm particle size, 80 Å pore size; and 1.9 μm particle size, 175 Å pore size) were compared. Best resolution was obtained with the UPLC column 175 Å pore size, whereas, as could be expected, the 80 Å pore size column was clogged after few injections. These results demonstrate that by using UPLC better performances (both in terms of peak capacity and protein recovery) can be obtained. Large proteins may need a larger pore packing (300 Å), and very large pore packing must be handled carefully, as it can give mechanical strength problems. In addition, the more expensive LC system may discourage the UPLC diffusion. Nevertheless, particularly the higher protein recovery should be very beneficial in top-down quantitative proteomics.

2.2. Electrophoresis

Proteome separations are most beneficial when the elution order occurs in a predictable fashion. Such predictability can permit the isolation of a particular protein, or class of proteins. 2D-PAGE has been the traditional approach for protein characterization. IEF is a powerful technique for the separation of protein mixtures based on pI, and is currently used in most proteomics analyses coupled with SDS-PAGE, that is based on MW, as second dimension, and increasingly in conjunction with mass spectrometric analysis.

In tube IEF was introduced since 1970s [52] while capillary (C)IEF has been introduced and developed by Hjerten et al. [53,54]. The fused silica capillary is coated with a linear polymer to eliminate electroosmotic flow [55], filled with ampholytes for the creation of a pH gradient, the protein mixture is introduced inside and a high voltage is applied. The proteins migrate until they have zero net charge and are focused into discrete and narrow zones with local pHs corresponding to their pIs. Then, protein bands may be mobilized to one capillary end by electrochemical or hydraulic (gravity, pressure) methods. The zone-sharpening effect is the characteristic of IEF that makes CIEF a high-resolution method for protein separation with a pI difference as small as 0.04 pH unit [56]. Furthermore, the focusing effect of CIEF permits analysis of very dilute protein samples with a typical concentration factor of 50–100 times. CIEF has applied as a micropreparative tool for protein analysis in MALDI-TOF MS by using an automated collection interface [57] and directly to ESI MS [58]. On-line combination of CIEF in free solution, coupled with ESI FTICR MS has displayed considerable potential for the analysis of complex protein mixtures [59,60]. Since IEF bases the separation of proteins only on their pI value, which is completely orthogonal not only to MW, but also to hydrophobicity, IEF is a good candidate for the first dimension in a 2D separation system where the second dimension is RPLC. CIEF can be coupled on-line to other electrophoretic or chromatographic techniques [61].

SDS-PAGE is a powerful method for size-based protein separation. The most serious limitation of SDS-PAGE relates to the recovery of protein from the gel. In alternative to in gel protein migration, proteins can be electrophoretically eluted from SDS-PAGE (whole Gel Elution), and the ability to purify a target protein with extremely high resolution has been well established [62]. Although removing SDS from solution may be difficult [63], SDS is often needed for protein solubilization, as well as for establishing separation through SDS-PAGE. Concentration of SDS as high as 4% was shown to facilitate transmembrane protein recovery. In the LC/ESI MS experiments, however, SDS is inevitably viewed as "enemy number1". MS analysis following SDS reduction in the sample has been extensively reported. Ion exchange, HILIC, gel filtration and dialysis have been reported for SDS elimination, however simpler methods based on protein precipitation by acetone [64] or chloroform/methanol/water (CMW) [65] (proteins precipitate at interface) are preferable.

Very recently [66] alternative detergents, i.e. maltosides and glucosides, have been used for membrane proteins extraction followed by RPLC–QTOF MS, without detergent elimination. By using methanol instead of acetonitrile as organic phase the authors were able in identifying intact membrane proteins. However, shortcoming of this method were the between sample irreproducibility of t_R and the appearance of complexes protein–detergent in the chromatogram.

The use of an acid labile surfactant (ALS) [67] instead of SDS or other stable detergents seems a more appropriate solution for the problem.

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are separation technologies very suitable for the analysis and separation of proteins and were interfaced to MS until the end of 1980s [68,69]. However, CE and CEC are rarely employed in top-down proteomics. A disadvantage of these techniques may be the usually very small loadability, in addition, improvements in separation media are still needed to fully exploit their potentiality [70].

3. Off-line combination with high resolution mass spectrometry

Some of the first works in top-down proteomics deal with the capability of this approach to discover sequencing errors and PTMs and were conducted in infusion mode, for single purified protein characterization [71–73], and the MS aspects, including the fragmentation methods, predominated. These aspects, already discussed in many reviews dealing mainly with MS in top-down proteomics [4,74–77] will not be considered in detail in this review.

For multi-protein analysis, top-down proteomics sample preparation may be laborious [78] and accomplished on-line or off-line. The techniques employed for off-line prefractionation include in tube gel electrophoresis [79], SEC [80], IEC [80–82], chelate affinity chromatography or differential precipitation [83], immunoaffinity [84], and RPLC [39,85].

Off-line 2D separation has been also performed before ESI-LTQ-FTICR or MALDI-TOF MS, the first dimension consisting in continuous-elution (CE)PAGE facilitated by an ALS and followed by RPLC. ALS was decomposed before RPLC, the fractions were then injected onto a C4 column (100 mm × 4.6 mm i.d.) or non-porous silica (NPS) column (14 mm × 4.6 mm i.d.), and subfractions analyzed by MS [79,86]. Chromatofocusing as the first dimension and NPS RPLC in the second fractionation step have been also reported [86,87]. Although free-flow (FF) CIEF was coupled on-line with ESI-FTICR MS since 1993 [88], a very efficient and reproducible commercially available apparatus for FF-IEF has been used in the form of an effective off-line protein prefractionation technique only more recently [89]. After IEF, fractions were desalted by C18 zip tip and analyzed by FTICR MS. Finally, Pesavento et al. reported a 2D separation consisting in a RPLC-HILIC off-line coupling [90,91]. The major advantage of using off-line separation is that a relatively large sample is available for infusion ESI MS and

MS/MS so that many measurements in different conditions can be performed. Thus, for off-line separation, normal bore columns are usually employed.

4. On-line coupling with high resolution mass spectrometry

This section deals with top-down proteomics whose methodology include 1D separation or 2D separation, the second of which coupled on-line with high resolution MS or MS/MS.

4.1. One dimensional separation

Jensen et al. [92,93] described the development and initial application of the combination of CIEF and FTICR MS for measurements of the proteome of the model system *Escherichia coli* and *Deinococcus radiodurans*. Isotope depletion of the growth media was used to improve mass measurement accuracy, and the comparison of CIEF-FTICR results for the analysis of cell lysates harvested from *E. coli* cultured in normal and isotopically depleted media were presented. Cell cultures were grown in normal or isotopically depleted (C, O, N) media, harvested, and resuspended in phosphate buffered saline and lysed by mechanical agitation.

A 300 mm × 50 μm i.d. fused-silica capillaries were used for CIEF. The capillary was filled with a sample solution (~0.5 mg mL^{-1} total proteins) containing 0.5% Pharmalyte 3-10. CIEF of cell lysate was performed at 8 kV for 10 min. Once the focusing was complete, the capillary tip (emitter) was fixed to protrude ~1 mm outside the ESI sheath needle. A sheath liquid composed of 50% methanol, 49% water, and 1% acetic acid (v/v/v) at pH 2.6 was delivered at a flow rate of 2 μL min^{-1} to maintain the optimum condition for ESI, and mobilization by gravity was applied concurrently with cathodic mobilization. The ESI mass spectra were acquired with a 7 T ESI-FTICR MS. Isotopically depleted medium produced a simpler isotopic peak distribution that improved sensitivity and facilitated interpretation. The initial studies revealed 400–1000 putative proteins in the mass range of 2–100 kDa from total injections of ~300 ng of *E. coli* proteins in a single CIEF-FTICR analysis.

The same technique, with only minor modifications, was used by Martinović et al. [94] to separate and detect intact non covalent protein complexes. 50 mm × 50 μm i.d. fused-silica capillaries were filled with a standard protein complex samples containing 0.5% Pharmalyte 3-10. IEF was performed at 13 kV for 15 min, then the capillary was interfaced to ESI by the sheath capillary and the sheath liquid was delivered. When an acidified sheath liquid and high voltage in the inlet-skimmer region were used, the CIEF-separated native complexes were detected dissociated, while when a neutral sheath liquid and a lower voltage were used, the intact complexes were detected by MS.

Capillary RPLC coupled to FTICR MS was used by Lee et al. [95] to characterize intact proteins from previously isolated large subunits of the yeast ribosome. Capillary LC was described in a previous work for peptide separation [96] and consisted in a 800 mm × 150 μm i.d. column packed with C5 stationary phase (5 μm, 300 Å). An isocratic/slow/fast gradient of acetonitrile/water, both 0.1% TFA for a total run time of 150 min was performed for separation. The study identified 42 of the 43 core large ribosomal subunit proteins and 58 (of 64 possible) core large subunit protein isoforms having unique masses in a single run, demonstrating the ability of top-down in identifying PTMs and isoforms.

In the work by Hernández et al. [97] non-covalent complexes of proteins and RNA were studied by an integrated procedure. The studied structure was U1 snRNP, which is an essential component of the spliceosome in human, consisting of RNA and ten proteins, several of which are post-translationally modified and exist as multiple isoforms. Sample was submitted to various treatments (extraction, complex reconstitution, buffer exchange). Intact complexes were detected by infusion on a modified ESI Q-TOF MS for high m/z acquisition of non-covalent complexes, while the masses of individual subunits were measured by a normal ESI Q-TOF MS following LC separation of the proteins by a capillary polystyrene-divinylbenzene (PS-DVB) monolithic column (50 mm × 200 μm i.d.). The column was held at 60 °C, flow rate was 3 μL min^{-1}. A 20 min linear gradient of acetonitrile/water, both containing TFA (0.04 and 0.05%, respectively) was used. The same chromatographic conditions were also used to collect fraction for bottom-up analysis. Detailed information about incorporation into the structure of different isoforms, dynamic interaction, and structure stability were obtained.

In the work of Wenger et al. [98], a dual on-line/off-line approach that improves upon standard LC–MS/MS strategies was presented. HeLa human cells were used. Top-down or middle-down samples (obtained by endoprotease enzymatic hydrolysis) were fractionated with a PLRP-S 1000 Å, 5 μm, 150 mm × 1.0 mm i.d. polymeric column at flow rate of 100 μL min^{-1}. Mobile phases were water and acetonitrile:isopropanol 90:10 (v/v), both containing 0.2% (v/v) formic acid (FA) and 0.01% (v/v) TFA. A three-step gradient (isocratic/fast/slow) for a total time of 111 min was used. 300 nL min^{-1} was infused into the mass spectrometer via the chip-based nanoESI source and the remaining 99.7 μL min^{-1} were collected for subsequent off-line analyses. In performing the bulk of the data acquisition off-line, the increased spray time can be used to average multiple scans with more ions accumulated before detection. An off-line mode of operation also allows the prior information of the entire chromatographic run to be available in determining what to fragment; therefore, more intelligent decisions can be made in terms of when precursors should be fragmented and which charge state should be selected for MS/MS. Nearly 4 times as many protein forms are identified with the off-line platform compared to the only on-line approach.

The same dual on-line/off-line approach of Wenger was employed for *Methanosarcina acetivorans* proteome characterization [99]. Some sample pretreatments, like acid precipitation, were performed to reduce the complexity. Thereafter, using both automated nanospray and on-line LC–MS strategies, 99 proteins were newly identified by top-down MS/MS respect to a previous work [86]. Off-line separation was performed on a 150 mm × 4.6 mm i.d. C4 column, employing as mobile phases water with 0.1% (v/v) TFA and acetonitrile with 0.08% (v/v) TFA, at 1 mL min^{-1} flow rate. A 60 min gradient was used and 1 min fraction were collected. For nanoLC–MS, 75 μm columns, 80–120 mm in length packed with 10 μm C4 material were used. Twenty μg of total protein was loaded onto each column. Via an ESI source, samples were introduced into a 12 T LTQ-FTICR mass spectrometer at 300 nL min^{-1}. Gradients were the same as previously published [98].

Very recently, Wu et al. [100] proposed a top-down/bottom-up integrated procedure that, for protein separation, makes use of a LC column very similar to that of Lee [95], but 200 μm instead of 150 μm. This apparently little difference permitted to have sample enough to make: (i) on-line nanoESI MS of proteins; (ii) a target MS/MS in infusion mode by using both CID and ECD fragmentation; a triptic peptide analysis by nanoLC/nanoESI-MS/MS. A 12 T FTICR mass spectrometer was used. The methodology was applied to yeast proteosome and 15 proteins were fully characterized.

By means of top-down proteomics combined with metabolic labeling techniques such as stable isotope labeling by amino acids in cell culture (SILAC), quantitative comparison of intact protein expression under differing experimental conditions is possible. In the work of Collier et al. [101], using the SILAC technique, cultures of the filamentous fungus *Aspergillus flavus* were grown under identi-

cal conditions incorporating either ^{12}C6- or ^{13}C6-labeled arginine. Intact proteins from a 1:1 mixture of cell lysates from both cultures were then separated.

RPLC was performed using a 150 mm × 75 μm i.d. capillary column packed in-house with 5 μm-C18 stationary phase. Mobile phases were water:acetonitrile 95:5 (v/v), water:acetonitrile 5:95 (v/v), both containing 0.2% (v/v) FA. Samples (1 μg of total protein) were injected over the course of 10 min, trapped and washed on a C18 trap cartridge at 1 μL min^{-1} then, a switching valve was triggered to move the sample in line with the gradient. Elution was carried out at 250 nL min^{-1}, the gradient was isocratic/fast/slow, for a total time of 85 min.

In all, 1318 intact or fragment protein masses were detected corresponding to 659 SILAC pairs, of which 22 were confidently identified. Broadband precursor ion spectra provided accurate intact mass and allowed for arginine counting, which aided in the identification of proteins combined with the sequence information from data-dependent MS/MS spectra. Quantification of proteins with few arginines was consistent with the 1:1 mixture ratio. With greater numbers of arginine in a protein, increasingly complex heavy isotope spectra were observed, resulting in incorrect quantification. Endogenously produced arginine limited the labeling efficiency of the heavy culture to between 72 and 85% as determined using isotope distribution modeling. The authors would further develop methods to improve top-down quantification in SILAC experiments and reduce endogenous amino acid incorporation through use of an arginine auxotroph in *A. flavus*.

The same research group [102] demonstrated the possibility of quantitative comparison of intact protein expression of human embryonic stem cells (HESCs) under differing experimental conditions. Quantitative top-down proteomics of HESCs was performed using the SILAC method reported above [101] and nanoLC–LTQ-FTICR MS.

Ten μL samples containing ~1 μg of total protein were injected, desalted on a 50 mm × 75 μm i.d. C18 trap, and eluted onto a capillary column 200 mm × 75 μm i.d. The packed column was operated at 80 °C, mobile phases and gradient were very similar to that used in the previous work [101] and MS analyses were performed on a LTQ-FTICR Ultra.

After data inspection, the authors concluded that quantifying intact proteins using the SILAC method remains a challenging task, even with the relatively high incorporation of stable isotope labeled lysine and arginine ~98%. While the use of dual labeling strategies is beneficial in the case of bottom-up quantification by SILAC, even the small amount of light amino acid incorporated in the place of the stable isotope label is compounded. This effect is magnified further when a labeling amino acid is converted by the biological system into a different residue, as is the case with arginine–proline conversion. Many of these effects can be taken into account easily with quantification software with bottom-up experiments, but remain unaddressed from the top-down perspective.

In the paper by Chi et al. [68,103], direct analysis of intact proteins on a chromatographic time scale was demonstrated on a modified linear ion trap mass spectrometer. Reaction of multiply charged protein ions with fluoranthene radical anions allowed electron transfer and promoted random fragmentation of amide bonds along the protein backbone. Multiply charged fragment ions were then deprotonated in a second ion/ion reaction with even-electron benzoate anions. Fragmentation spectra were simplified and m/z values for the resulting singly and doubly charged ions were used to read a sequence of 15–40 amino acids at both the N-terminus and the C-terminus of the protein. This information, along with the measured mass of the intact protein, were employed to identify known proteins and to detect the presence of PTMs. The time scale for recording ETD/proton transfer reaction-MS/MS spectra on intact proteins is identical to that currently employed for acquiring CID spectra on mixtures of tryptic peptides.

An aliquot of reduced, alkylated and acidified *E. coli* ribosomal proteins (~5 pmol) was loaded onto a C8 pre-column (40 mm × 75 μm i.d.). After washing, the pre-column was connected to the analytical C8 column (100 mm × 50 μm i.d.), interfaced with the modified LTQ mass spectrometer. Mobile phases were 0.1 mol L^{-1} acetic acid, and 70% acetonitrile in 0.1 mol L^{-1} acetic acid. Proteins were eluted at a flow rate of 60 nL min^{-1} using a 75 min linear gradient.

The decreasing in charge state in the ESI source enabled, in a single automated LC–MS experiment, 46 of the 55 known unique proteins identified and a number of PTMs detected. This results did not improve the previous one in terms of identification power [95], but it has been obtained by a largely different technology.

The differential expression of histone isoforms may serve as a potential marker in the classification of diseases affected by chromatin abnormalities. Four papers have recently published concerning the top-down proteomics analysis of histone isoforms [104–107]. The histone extraction from nuclei of cell cultures was performed by using little variation of an established procedure, thereafter histone isoform qualitative and semi-quantitative label free analysis was performed by RPLC–MS.

Li et al. [104] performed the separation by a C18 column, 250 mm × 1.0 mm i.d., at a flow rate of 50 μL min^{-1}. Mobile phases were 0.05% TFA in water and 0.05% TFA in acetonitrile. A fast/slow gradient was used for a total time of 120 min. All LC–MS experiments were performed on an LTQ-FTICR MS operating at 7 T. The LTQ-FT method consisted of two scan events: a full scan in the ion trap followed by a broadband high-resolution scan in the trapped ion cell of the FTICR MS portion of the instrument.

Naldi et al. [105] used two different methods and instruments for histone LC–MS analysis. In the first case they used for separation a C4 column, 5 μm, 300 Å, 150 mm × 2.0 mm i.d., equipped with a guard column at a flow rate of 100 μL min^{-1}. The injection volume was 5 mL. Mobile phases were water:HFBA:FA 100:0.04:0.2, and acetonitrile:HFBA:FA 100:0.04:0.2, and a linear gradient of 100 min was used. Histones were detected by an ion trap mass spectrometer.

In the second procedure histone samples were fractionated by a C18 capillary column (150 mm × 300 μm i.d.) at a flow rate of 5 μL min^{-1} using as mobile phases water and acetonitrile both containing 0.06% (v/v) HFBA with a 70 min linear gradient. The hybrid Q-TOF mass analyser was employed for obtaining mass spectra in a m/z range spanning from 600 to 2000.

Su et al. [106] separated histone extracts by a C18 column, 5 μm, 300 Å, 250 mm × 1.0 mm i.d., at a flow rate of 50 μL min^{-1}. Mobile phases were water and acetonitrile, both 0.01% in TFA. A fast/slow gradient for a total time of 25 min was used. Proteins were detected by an ESI-TOF mass spectrometer.

In a successive article [108], the same research group applied the developed method to explore differences in histone composition in primary chronic lymphocytic leukemia cells. In particular by developing a profiling strategy for its potential to assess histone isoform distributions in primary tumor cells, demonstrating that this approach is highly reproducible.

Finally, in a more recent paper, Contrepois et al. [107] developed an improved approach based on UPLC–LTQ-Orbitrap MS. The column was a C18 Hypersil GOLD 1.9 μm, 175 Å, 150 mm × 2.1 mm i.d., operated at a flow rate of 300 μL min^{-1}. Mobile phases were the same as in ref. [106]. The oven temperature was set at 50 °C. Phase B was linearly increased from 0 to 50% in 12 min. The whole analysis was performed within 19 min, which is ~2–4-fold faster than previously published procedures. High mass accuracy measurements combined with MS/MS experiments enabled accurate histone identification. Experimental and biological variations were

thoroughly assessed and were 8% and 16% on average, respectively.

In the work by Mohr et al. [109], the potential of interfacing Orbitrap MS to RPLC for intact protein analysis was investigated. Commercially available monolithic column 50 mm × 1.0 mm i.d. PS-DVB, and home-made 150 mm × 0.1 mm i.d. PS-DVB capillary column were used. A mix of 9 standard proteins was used for testing. MS analysis was performed with an LTQ Orbitrap XL.

Microbore columns optimally fit to the very robust and easy to operate standard ESI sources that can be utilized with metal needles, while miniaturized or nanospray source designs, employing small diameter fused silica tips, must be utilized with column i.d.s smaller than 0.50 mm in order to minimize post-column dead volume and avoid additional peak dispersion. However, the latter source designs are less robust and prone to clogging. Finally, much less sample can be analyzed with the small i.d. column, alternatively, much more sensitivity can be obtained. The authors measured a peak capacities of 131 and 120 for the 0.10 and 1.0 mm i.d. columns, respectively, within 20–25 min separation time. Considering that the former column is 3 times longer than the latter one, this is a small difference, confirming that column length has a small effect in protein separation. This leveled peak capacity for different column length could be due to the fact that the same gradient was used, whereas longer column usually need longer gradient to exploit their higher efficiency. The polymeric stationary phase, lacking any functional groups capable of hydrophilic or ionic interactions such as silanols present in silica-based materials, facilitates high recoveries of most proteins, but ovalbumin, and eliminate carryover during intact protein chromatography. The scanning speed of the mass spectrometer at the highest resolution of 100,000 is incapable of fully preserving the chromatographic profile of proteins eluting with peak widths of 30 s and less, resulting in moderate instrumental band dispersion.

The work of Wynne et al. [110] was an application of top-down proteomics to study *Erwinia herbicola* and *Enterobacter cloacae* bacteria, for which the necessary protein sequences are not available in protein sequence repositories. RPLC-LTQ-Orbitrap XL was used. An aliquot of bacteria lysate was injected onto a 150 mm × 0.1 mm i.d., 5 μm particles, C8 column on-line to the mass spectrometer ESI source. Mobile phases were water and acetonitrile, 0.1% TFA and flow rate was set to 500 nL min^{-1}. The gradient program used a 15 min sample load, followed by a 50 min gradient from 10% B to 70% B.

Experimental data were matched with homologous proteins from related organisms with sequenced genomes, demonstrating considerable shared protein sequence between closely related bacteria. Using this homology-based approach, they were not only able to identify representative proteins, but were also able to place the two target bacteria in their correct phylogeny. For *E. herbicola* 14 proteins were identified from 72 intact molecular masses, while for *E. cloacae* identified proteins were 15 from 129 intact molecular masses that allowed a phylogenetic positioning of the microorganisms studied.

The capability of the top-down approach in performing nonlabeled quantitative differential proteomics was demonstrated by Mazur et al. [111]. Differential MS was applied to the determination of apolipoprotein CIII from HDL_3. The protein was isolated from plasma by ultracentrifugation and samples were analyzed by nano-RPLC-LTQ Orbitrap. Column was a POROS R1, 60 mm × 75 μm i.d., a linear gradient of acidified (acetic acid) acetonitrile/water for a run time of 30 min was applied. The standard addition method was used for accurate quantitation. The main advantage of the top-down MS was the detection of quantitative differences in abundance of the protein isoforms.

4.2. Multidimensional separation

Because of the complexity of proteomic samples, a single dimension separation normally cannot provide sufficient resolution. After the first system which coupled SCX to RP LC before MS analysis [112], several multidimensional separation methods have been used for intact protein profiling. The MW and pI are arguably the most accessible intrinsic properties of a protein, and have been used for years in the solid form of 2D-PAGE, thus size-based and pI-based protein separation in solution can be a highly valued asset in protein studies. Size-based proteome separation as in tube SDS-PAGE and solution IEF would certainly complement other promising solution platforms such as IEC and RPLC, by affording the potential for multidimensional separation of intact proteins. Newly developed 2D strategies for intact protein separation before MS are reviewed in this section.

In the work by Wall et al. [113], a miniaturized Rotofor apparatus for liquid-phase IEF [114] was used to fractionate proteins from a cell lysate of human erythroleukemia. The fractions were then analyzed using on-line NPS-RPLC/ESI-TOF MS, and the same conditions were also used to perform fraction collection followed by bottom-up analysis by MALDI-TOF. A 33 mm × 3 mm i.d. column, packed with 1.5 μm diameter non porous ODS was used in both cases. Flow rate was 200 μL min^{-1}, mobile phase were 0.1% (v/v) TFA and 0.1% TFA in acetonitrile. A binary fast/slow gradient of 10 min was used.

Overall, the method provided a means for mapping hundreds of proteins and could be considered as complementary to 2D-PAGE. The use of intact protein MW along with the pI and peptides mapping provided not only protein identification, but also PTMs, like acetylation, phosphorylation, N-terminal methionine cleavage and characterization. The authors concluded that improvements of databases is a pivotal issue to take full advantage of top-down approach.

Kachman et al. [115] use a very similar system for mapping the protein content of ovarian surface epithelial cells and an ovarian carcinoma-derived cell line. The NPS analytical column was longer (55 mm) and a different column with larger i.d. (8 mm) was employed to collect fractions for bottom-up proteomics. Bovine insulin was used as an IS. Numerous differentially expressed proteins were visualized, and many of the proteins were identified with high confidence. Some of the identified proteins were overexpressed in the ovarian carcinoma cell line.

In the work by Zhou et al. [116] a CIEF system used for pI fractionation, with on-line coupling to RPLC and ESI-Q-TOF MS were used. CIEF was performed with a microdialysis membrane-based cathodic cell mounted inside the commercial automated CE system with a UV detector. A 700 mm × 75 μm i.d. capillary was used. The CIEF capillary was filled with ampholyte solution, and protein test mixtures and seven IEF markers were injected from the anodic side. Focusing was performed in an electric field of 300 V cm^{-1}; after a focusing period of 6 min, the focused bands were hydrodynamically pushed through a UV detector at 280 nm or into a 100 mm long 150 μm i.d. PEEKsil tubing sampling loop attached to the microselection valve. The valve position was then switched, and proteins were transferred to a 200 mm PEEK storage loop attached to a 10-port loop selection system. During the sampling and storage steps, another sampling loop was used to collect the next fraction without any sample losses. The microselection valve position changed to sequentially load fractions into the nine storage loops.

Each stored sample was then passed through a C4 trap column attached to a six-port valve located on a capillary LC system. Proteins in the stored sample were captured on the trap column and washed with a solution of 89.9% water, 0.1% TFA, and 10% acetonitrile. The protein fraction was then eluted from the trap column and separated on a C18 column (150 mm × 75 μm i.d.). Mobile

phases were 94.4% water, 0.5% FA, 0.1% TFA and 5% acetonitrile and 94.4% acetonitrile, 5% H_2O, 0.5% FA, and 0.1% TFA at a flow rate of 200 nL min^{-1}. A linear gradient of 40 min was used. Proteins were analyzed with a Q-TOF mass spectrometer.

The system was able to detect 100 pg (3 fmol) of carbonic anhydrase spiked into a complex mixture (1 μg of total protein). Proteins under 50 kDa from wild type *Chlorobium tepidum* were profiled by CIEF-RPLC–MS in an experiment lasting less than 8 h. Around 1200 individual protein masses were detected, some appearing to be subtly altered forms of the same protein.

In the work by Millea et al. [117], strong anion exchange chromatography (SAX) 50 mm × 4.6 mm i.d. (10 μm 1000 Å pore) was used for first separation dimension. The mobile phase was produced by solvent lines A (100 mmol L^{-1} Tris–HCl) and B (100 mmol L^{-1} Tris Base) with controlled system pH at a constant buffer concentration, while solvent lines C (1.25 mol L^{-1} NaCl) and D (MilliQ water) generated the salt step gradient. The gradient consisted of eight NaCl steps (0, 50, 100, 150, 200, 250, 300, 500, 1000 mmol L^{-1}) in a background of 20 mmol L^{-1} Tris Buffer (pH 7.8). Then, fractions from the SAX column were trapped and desalted by using two C4, 10 mm × 2.1 mm i.d., (3.5 μm, 300 Å) columns, and 5 min washing with aqueous acidic solution. A Symmetry 300 C4, 100 mm × 2.1 mm i.d., 3.5 μm analytical column was used for the second dimension RP separation. All columns were maintained at 30 °C. Mobile phase were water and acetonitrile, both containing 0.1% FA and 0.001% TFA (v/v). A fast/slow gradient for a total gradient time of 24 min, at 500 μL min^{-1} flow rate was used.

Second dimension effluent exiting the analytical column was split with 90% of the effluent delivered through a dual wavelength absorbance detector (λ 214 and 280 nm) to a fraction collector, while the remaining effluent was delivered to a LC/ESI-TOF MS system. Fraction collection was achieved using a 30 s collection time with a total of 42 fractions (during min 2–23 of each RP gradient cycle) collected during each of the nine RP cycles analyzed. The collected fractions were analyzed by a bottom-up protocol.

The summary of results from this study indicated that roughly one-third of all detected components generated corresponding data from both top-down and bottom-up analyses, and that significant and novel information can be derived from this application of the hybrid analytical methodology. Bottom-up recognized more proteins, while top-down more isoforms.

Simpson et al. [118] compared the combination of SEC fractionation with RPLC–FTICR MS separation to the combination of RPLC fractionation with CIEF-FTICR MS for the analysis of the *Shewanella oneidensis* proteome.

SEC and RPLC preparative fractionations of the cell lysate were performed using a conventional HPLC system equipped with an UV detector (set at 280 nm) and a fraction collector. For SEC, a BioSep-SEC-S2000, 600 mm × 21.2 mm i.d. column, preceded by a guard column, was used; the mobile phase was 100 mmol L^{-1} ammonium bicarbonate (adjusted to pH 6.8) and the flow rate was 5 mL min^{-1}. For RPLC, a Jupiter C4 stationary phase (10 μm particles; 300 Å pore size) 250 mm × 10 mm i.d. column, preceded by a guard column, was used; mobile phase were water/acetonitrile 95/5 (v/v) with 0.1% TFA, and acetonitrile/water 90/10 (v/v) with 0.1% TFA, and the flow rate was 4 mL min^{-1}. An isocratic/fast/slow gradient for a total time of 60 min was used. Fractions were collected inspecting the chromatographic profile during the SEC run while fixed-duration (6 min) fractions were collected during the RPLC run. Protein concentrations for each fraction were determined after evaporation to 100–200 μL; a comparison of these measurements with the initial protein concentration determined for the cell lysate was used to calculate fractionation recoveries. The amounts of protein injected were 22.5 mg for the SEC fractionation and 4 mg for the RPLC fractionation; the difference between the injected amounts reflects the sample requirements of the second separation dimension.

Protein RPLC after SEC separation was very similar to that reported in ref. [94] with some differences in the mobile phases composition and gradient time (110 min instead of 150 min). Prior to loading, protein samples were adjusted to 1.5 mg mL^{-1} in mobile phase A.

CIEF was performed using 800 mm × 50 μm i.d. fused-silica capillaries coated with hydroxypropyl cellulose. Carrier ampholytes (3–10) were mixed (0.5%) with the sample and the capillary was filled with the solution (~0.5 μg of proteins). The duration of focusing was 30 min. After that, swapping sheath liquid resulted in chemical mobilization.

Protein recovery for SEC fractionation was 25% while that for RPLC fractionation was 13%. This very low recovery may be due to precipitation and adsorption phenomena during processes. SEC-RPLC–FTICR MS allowed the detection of 297 proteins, as opposed to 166 using RPLC–CIEF-FTICR MS, indicating that approaches based on LC–MS provide better coverage. However, there were significant differences in the sets of proteins detected and both approaches provide a basis for accurately quantifying changes in protein and modified protein abundances.

The paper by Parks et al. [119] described a method for high-resolution MS/MS of intact proteins on a chromatographic time scale, using off-line weak anion exchange chromatography (WAX) and on-line RPLC–MS/MS. Mass spectrometric analyses were carried out using LTQ-FTICR mass spectrometer. To maintain analysis speed, CID was used to produce fragment ions in the linear ion trap before transmission to the ICR cell.

Prefractionation was performed off-line using a 250 mm × 4.6 mm i.d. WAX column to separate 15 mg of total protein from a yeast whole cell lysate. A 95 min gradient run at 600 μL min^{-1} was used to elute proteins into a 96-well plate starting with an initial mobile phase of 50 mmol L^{-1} Tris–HCl (pH 8). Ionic strength was increased linearly throughout the run using NaCl to a final concentration of 750 mmol L^{-1}. Forty-five 1.2 mL fractions were collected and stored at 4 °C.

All RPLC separations were accomplished using a C5 column, 150 × 1 mm i.d. The mobile phases were 95/5 (v/v) water:organic modifier and 90/10 (v/v) acetonitrile:2-propanol; 0.2% FA and 0.1% (v/v) TFA were added to both mobile phases. The column was kept at a temperature of 55 °C to enhance mass transfer during the separation. The chromatographic gradient was isocratic/fast/slow for a total run time of 95 min.

In a single LC–MS/MS run, 22 proteins with MWs from 14 to 35 kDa have been identified on a yeast whole cell lysate. When this experiment was extended to metabolically labeled proteins mixed at a ratio of 1:1, 231 protein pairs were readily detectable from *S. cerevisiae*, of which 38 proteins were unambiguously identified from high-resolution MS/MS data. The diacetylated form of histone H4 was also fully characterized in an automated LC–MS/MS experiment. Thirty-nine additional proteins were identified and characterized from LC–MS/MS of selected anion exchange fractions. Automated localization of multiple acetylations on histone H4 was also accomplished on an LC time scale from a complex protein mixture. This was the first demonstration of top-down proteomics (i.e. many identifications) on LTQ-FTICR systems using high-resolution MS/MS data obtained on a chromatographic time scale.

Future prospects for this platform at MWs higher than 35 kDa have also been shown by utilizing ion trap full scan mass spectra. Before, top-down proteomics with high-resolution MS/MS fragmentation was limited mainly to off-line data acquisition. Development of this work would include efforts to reduce the duty cycle of the experiment by using other fragmentation techniques or tandem MS^n schemes.

Also Sharma et al. [120] investigated the combination of WAX fractionation and on-line RPLC separation using a FTICR mass spectrometer for the detection of intact proteins from a *S. onei-*

densis MR-1 cell lysate. WAX fractionation was performed with a PolyWAX LP column (5 μm particles, 1000 Å pore size). The column (100 mm × 4.6 mm i.d.) was preceded by a guard column (mobile phases were 20 mmol L^{-1} potassium phosphate (pH 7.0) with 5% (v/v) acetonitrile, and 20 mmol L^{-1} potassium phosphate and 600 mmol L^{-1} potassium chloride (pH 7.0) with 5% (v/v) acetonitrile). The fractionation started with a 30 min hold at 100% A, continued with a linear gradient to 100% B over 90 min, and finished with a 30 min hold at 100% B. The mobile phase flow rate was 0.5 mL min^{-1}, and the injection volume was 5 mL (4.35 mg of protein); UV detection was performed at 230 nm, and only 12 fractions (6 mL each) were collected in the time frame from 1 to 145 min. After protein samples concentration the 12 fractions were combined into six unequal fractions to obtain a sufficient amount of protein in each sample for multiple analyses. The recovery of proteins was about 45%. The RPLC system used for on-line intact protein separations was largely the same as that used for peptide separations by Shen et al. [96] that employed a column 800 mm × 75 μm i.d. packed with C5 stationary phase (5 μm, 300 Å). Mobile phases were the same as in ref. [94], while the high operating pressure, and the reduced i.d. for the column required a loading time for 45 min, which was reduced to ~5 min by adding a SPE column. ESI using a pulled fused-silica needle was used to interface the RPLC separation to a modified APEX-Q FTICR mass spectrometer. Bottom up analysis by an ion trap MS was also performed.

Table 1 summarizes the results obtained [120]. While 447 unique proteins in the 5–40 kDa mass range were identified in the bottom-up analysis, tentative identifications were made for 80 of the 715 intact proteins detected in the top-down analysis, and overall, the results reflect better proteome coverage in the context of identified proteins. In addition to the 80 intact protein identifications, four additional intact proteins with less than 100 amino acids that may not be identified by MS/MS bottom-up were identified. Finally, protein identifications and PTMs were assigned for ~10% of the detected proteins by comparing intact protein mass measurements to proteins identified in peptide MS/MS analysis of an aliquot of the same fraction.

Lee et al. [121] reported the intact protein separation scheme based on gel-eluted liquid fraction entrapment electrophoresis (GELFrEE) [122] as first dimensional protein fractionation. This MW-based separation involves continuous elution SDS-PAGE in a tube format, in which proteins are constantly eluted from the gel column and collected in a solution phase, providing broad mass range separation with high-resolution, reproducibility, and recovery. This GELFrEE technique generally provides rapid partitioning of a proteome into about 20 samples containing proteins in discrete mass ranges from ~10 kDa to 200 kDa in 1 h. In this study, 5 GELFrEE fractions containing up to 25-kDa proteins of human HeLa cell lysate were analyzed repeatedly by nano-RPLC on-line with a LTQ-FTICR MS for MS/MS on a chromatographic time scale. Because a protein mass predicts the fraction in which it will elute, this general approach should be applicable to a wide variety of problems in protein chemistry in both targeted and proteomics modes of operation. Sixteen GELFrEE fractions with a volume of 150 μL each were collected over 1 h after elution of the dye front from a gel 6 mm × 10 mm, and GELFrEE fractions containing proteins from 5 to 25 kDa were cleaned to remove SDS by CMW precipitation. Protein pellets were resuspended with 40 μL of LC starting buffer with protein recovery from the solubilization of the pellets at roughly 50%. Ten μL of the sample was injected onto a 20 mm × 150 μm i.d. C4 or PLRP-S trap column. Samples were then transferred onto the respective analytical column, 100 mm × 75 μm i.d.; mobile phases were 95% water and 5% acetonitrile (A) and 5% water, 95% acetonitrile, both 0.2% FA, and separation was performed with either a short or long gradient. The short gradient lasted 55 min, while the long one lasted 70 min, and flow rate was 300 nL min^{-1}. Samples were analyzed with either a commercial 12 T or modified 14.5 T LTQ-FT Ultra mass spectrometer. Each GELFrEE fraction <25 kDa was subjected to nanoLC–MS/MS. Each of the 4–8 GELFrEE fractions contained about10 μg total protein per fraction before sample cleanup.

Fig. 3 shows representative data and ProSight software output from an LC–MS/MS injection of fraction #3 of a collection at 14.5 T. Fig. 3a shows a base-peak chromatogram for this GELFrEE fraction, with representative MS and MS/MS spectra (with identifications and *E*-values) illustrated in Fig. 3b and c, respectively. Highlighted in Fig. 3d is an N-terminal acetylated form of nucleoside diphosphate kinase B. In total, 37 distinct protein forms were detected in this run. There were 71 redundant protein identifications corresponding to 13 unique accession numbers. These data were typical for this study and highlight one challenge of top-down MS: the same protein form was repeatedly targeted for fragmentation within a single injection. Thus, a simple but significant improvement would be to prevent the instrument from fragmenting different charge states of the same protein.

Chromatographic peak full width at half-maximum fell in the 15- to 45-s range. Such chromatography is favourable from a separation perspective, but places a temporal constraint on MS/MS data acquisition. For top-down MS, high mass accuracy in MS/MS is highly advantageous for precise characterization of PTMs, but is acquired at the expense of duty cycle. The cycle time achieved during the top 2 or 3 data-dependent MS/MS acquisitions is roughly 4–5 cycles min^{-1} (~2–6 s $scan^{-1}$). For that cycle time, each abundant protein is targeted for fragmentation at least once. Within any given elution peak, whole protein charge state distributions are observed routinely with isotopic resolution and may be sampled more than once. Approximately one quarter of the MS/MS events led to an identification, with the proteins ranging from 5 to 12.5 kDa. Surprisingly, comparison between the two RPLC gradients used are not reported. The described GELFrEE, using only an aliquot of fraction, enables a variety of post-separation measurements can be made beyond or besides top-down MS, including bottom-up MS/MS, Western blotting, etc.

In the work by Botelho et al. [123], methods for reduction of SDS in protein solutions were evaluated, demonstrating that a simple modification of the well-known acetone and CMW precipitation method can reduce SDS present in GELFrEE to permit LC–MS analysis.

Six standard proteins (cytochrome *c*, myoglobin, ubiquitin, carbonic anhydrase, lysozyme, and bovine serum albumin), prepared in solution with varying concentrations of SDS, were subjected to intact protein analysis. A C4 column 100 mm × 200 μm i.d., 5 μm × 300 Å at flow rate of 2 μL min^{-1} was used, and 10 μL of sample (corresponding to 1 μg of total protein) was injected. Mobile phases were water and acetonitrile, both 0.1% FA, and gradient was run for 40 min. The column was connected to an ESI-TOF mass spectrometer.

Experiment results showed that MS tolerance to SDS is surprisingly high relative to a direct infusion ESI experiment, as 0.01% has little affect on both a top-down and bottom-up proteome workflow. By incorporating a simple washing step, two organic solvent precipitation protocols (acetone and CMW) will reliably reduce the level of SDS in a protein sample to well below threshold values. GELFrEE fractionation followed by LC/ESI-TOF MS revealed results comparable to a widely used bottom up approach (SDS-PAGE, band excision, trypsin digestion, and peptide analysis by nanoLC–MS), both in terms of the number and type of identified proteins for bottom-up MS. Sample loss due to incomplete protein resolubilization following detergent removal may represent a possible disadvantage.

Table 1
Summary of results for proteins identified in RPLC–IT MS/MS peptide analysis and intact proteins detected in RPLC–FTICR MS analysis of *S. oneidensis* unfractionated cell lysate and WAX fractions 1–6.

Protein sample	Proteins in provisional bottom-up database	Putative intact proteins detected	Tentative intact proteins identified (including modified proteins)
Whole cell lysate	257	195	27
WAX fraction 1	129	345	29
WAX fraction 2	161	288	29
WAX fraction 3	139	39	5
WAX fraction 4	166	125	11
WAX fraction 5	99	78	7
WAX fraction 6	140	164	12
Total	447	715	84

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To overcome the limitations of off-line multi dimensional LC and enable sensitive characterization of protein isoforms using top-down MS, Tian et al. [124] developed a new online 2D-weak cation exchange (WCX)-HILIC/RPLC platform. RPLC was used for the second dimension due to its compatibility with MS, and WCX-HILIC was implemented as the first dimension. WCX-HILIC, a mixed-mode chromatography introduced by Alpert in 1990 [125], features the simultaneous presence of hydrophilic interaction (due to the use of a higher percentage of organic solvent in the mobile phase than traditional WCX) and electrostatic interaction (due to an ionic stationary phase). The first mechanism enables separation of differentially methylated isoforms, while the second one enables separation of differentially acetylated isoforms [126]. Because WCX-HILIC normally uses a salt ($NaClO_4$) gradient that

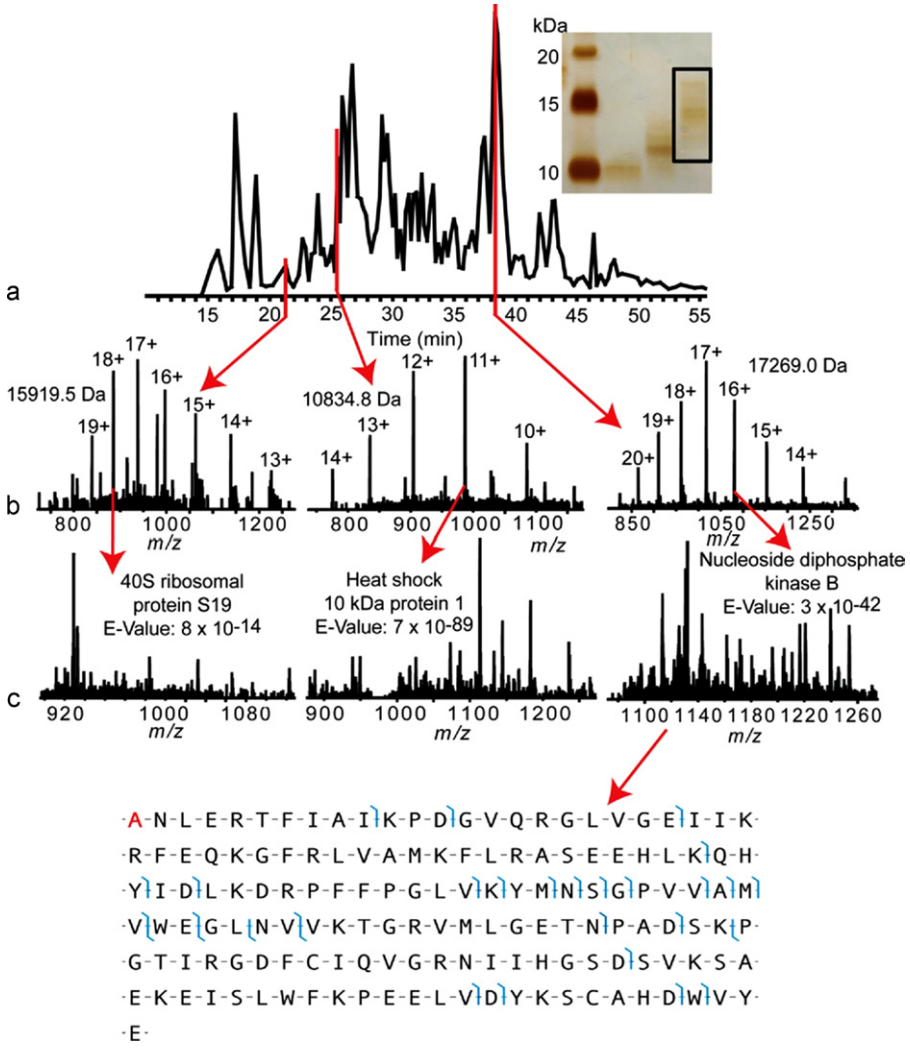


Fig. 3. Examples selected from an LC–MS/MS injection of fraction #3 from a Tris–glycine GE run. (a) A base-peak chromatogram is shown, with (b) charge state distributions from selected retention times. (c) Abundant charge states (above the arrows) were targeted for fragmentation. Fragmentation mass spectra for each protein are shown along with the corresponding identifications and *E*-values. A fragmentation map (d) results from the matching fragment ions of nucleoside diphosphate kinase B. The protein is N-terminally acetylated.
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is not compatible with MS, fractions have to be collected, recovered, and desalted before they are individually introduced into the mass spectrometer using direct infusion ESI. In this work, fractions from the first dimension were desalted and transferred to the second dimension on-line to achieve higher sensitivity and throughput. The 2D-LC platform was coupled with LTQ-Orbitrap MS and tested by analyzing histones purified from human fibroblasts.

The core histone mixture was first separated on a PolyLC PolyCAT A 800 mm × 200 μm i.d. (5 μm particles; 1000 Å pore size) column. The separation was carried out under the constant pressure of 3000 psi. Mobile phase A consisted of 15 mmol L^{-1} triethylammonium phosphate containing 70% (v/v) acetonitrile, and mobile phase B consisted of mobile phase A plus 0.68 mol L^{-1} $NaClO_4$. A 20 μL aliquot of the core histone mixture (1.2 mg mL^{-1}) was loaded into the sample loop. The sample was then transferred to the first dimension SPE trap and, after loading, the exponential separation gradient was started. The column flow rate was ~1 μL min^{-1} at the beginning of the gradient. An online UV detector at 214 nm was used to monitor the elution profile of proteins (see Fig. 4). On-line fractions from this separation were collected in a series of 20 capillary loops with the start and stop time of each fraction guided by the chromatographic profile. Each stored fraction from the first dimension was further separated in the second dimension using a C5 600 mm × 75 μm i.d. column, 5 μm particles, 300 Å pore size. As the first dimension fractions have a high percentage of organic solvent, they cannot be loaded into the second dimension hydrophobic SPE column directly. To overcome this problem, a tee was used to split the buffer A stream into two sub-streams with one stream (about 1 μL min^{-1}) pushing the stored fraction out and the other stream (about 4 μL min^{-1}) diluting the fraction before it enters the SPE2 trap. Once one fraction was loaded, RPLC buffer B was switched on to initiate the analytical gradient and separate the loaded proteins through the capillary RPLC column.

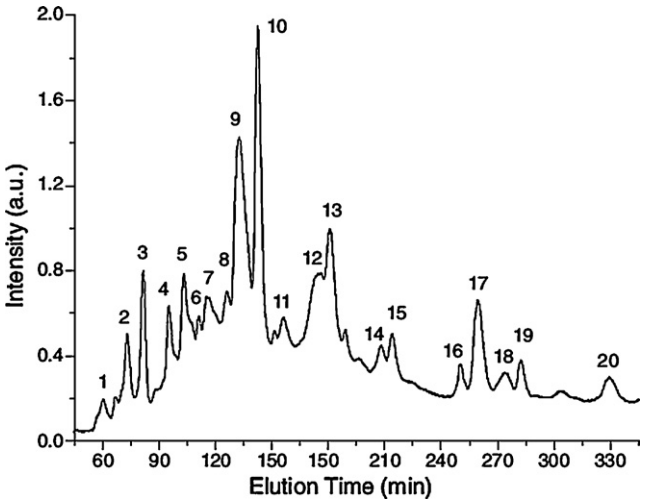


Fig. 4. UV chromatogram acquired during the separation of 24 mg core histones using WCX-HILIC. Twenty fractions for the 20 peaks as numbered were collected for subsequent RPLC–MS/MS based protein identification via protein database search using ProSightPC (a.u., arbitrary units).
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The separation was carried out using a gradient of mobile phase A (aqueous solution, 20% acetonitrile, 5% isopropanol, 0.6% acetic acid, 0.01% TFA) and mobile phase B (45% acetonitrile, 45% iso-

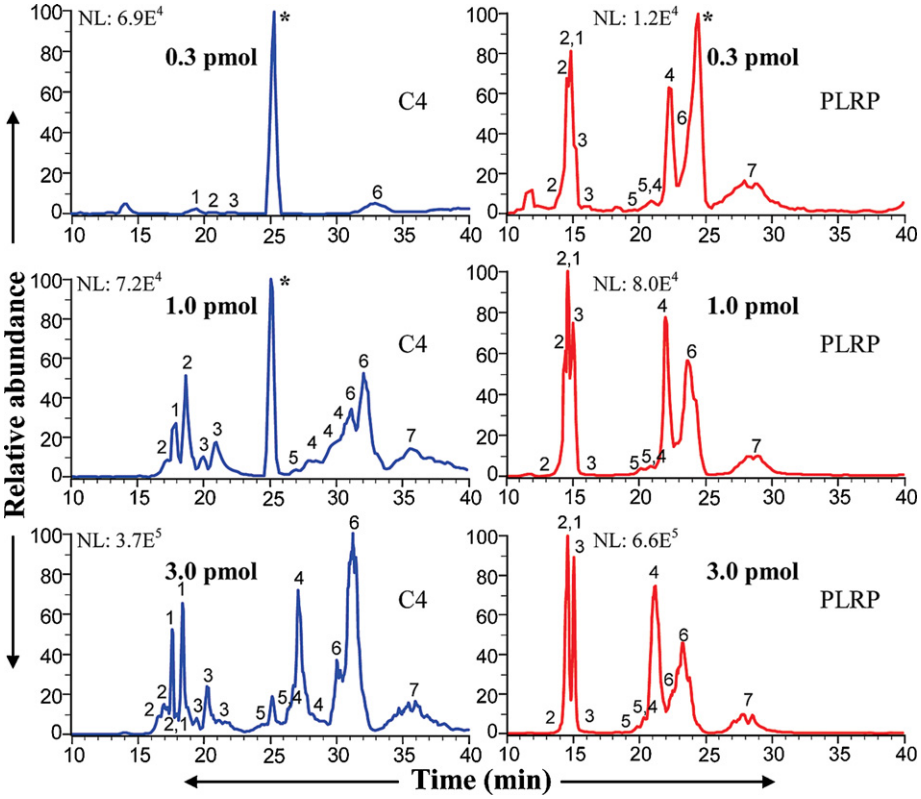


Fig. 5. Protein sensitivi ty and resolution in C4 and PLRP nanocapillary columns. (A) Chromatograms for RPLC separation of a mixture of seven protein standards using C4 (blue) and PLRP (red) (75 μm i.d. × 100 mm, 300 Å, 5 μm stationary phase) analytical columns. Standards used were (1) ubiquitin, (2) cytochrome *c*, (3) superoxide dismutase, (4) myoglobin, (5) R-casein, (6) carbonic anhydrase, and (7) ovalbumin. A contaminant peak at 25 min is indicated with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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Table 2
Summary of published chromatographic mass spectrometric methods for top-down proteomics.

Separation 1	Separation 2	MS	Sample	Ref.
CIEF 300 mm × 50 μm i.d.		FTICR	Cell lysate *E. coli*	[92]
CIEF 300 mm × 50 μm i.d.		FTICR	Cell lysate *E. coli* + *D. radiodurans*	[93]
CIEF 50 mm × 50 μm i.d.		FTICR	Non-covalent complexes	[94]
RP LC C5 silica 5 μm, 800 mm × 150 μm i.d.		FTICR	Yeast ribosome	[95]
RP poly(ST-DVB) monolith 5 μm, 50 mm × 200 μm i.d.		Q TOF	Non-covalent DNA–protein complexes	[97]
RP LC PLRP-S 5 μm, 150 mm × 1.0 mm i.d.		LTQ FTICR	Human HeLa cell pellet of proteins	[98]
RP LC C4 silica 10 μm, 80–120 mm × 75 μm i.d.		LTQ FTICR	*M. acetivorans* proteome	[99]
RP LC C5 silica 5 μm, 800 mm × 200 μm i.d.		APEX-Q FTICR	Yeast proteosome	[100]
RP LC C18 silica 5 μm, 150 mm × 75 μm i.d.		LTQ FTICR	*A. flavus* lysate Quantitative SILAC	[101]
RP LC C18 silica 5 μm, 200 mm × 75 μm i.d.		LTQ FTICR	HESC cells	[102]
RP LC C8 silica 5 μm, 100 mm × 50 μm i.d.		Modified LTQ	*E. coli* ribosomal proteins	[103]
RP LC C18 5 μm, 250 mm × 1.0 mm i.d.		LTQ FTICR	Histones	[104]
RP LC C18: 5 μm, 150 mm × 300 μm i.d. C4: 5 μm, 150 mm × 2 mm i.d.		Ion trap Q TOF	Histones	[105]
RP LC C18 5 μm, 250 mm × 1.0 mm i.d.		TOF	Histones	[106]
RP UPLC C18 1.9 μm, 150 mm × 2.1 mm i.d.		LTQ-Orbitrap	Histones	[107]
RP LC PS-DVB monolithic 50 mm × 1.0 mm i.d. 150 mm × 0.1 mm i.d.		LTQ-Orbitrap	Protein standards	[109]
RP LC C8 5 μm, 150 mm × 0.1 mm i.d.		LTQ-Orbitrap XL	*E. herbicola*, *E. cloacae* cell lysates	[110]
RP POROS R1 5 μm, 60 mm × 75 μm i.d.		FTICR-Orbitrap XL	Apolipoprotein CIII	[111]
IEF with mini-Rotofor	NPS-RP LC 1.5 μm, 33 mm × 3 mm i.d.	TOF	Human erythroleukemia cell lysate	[113]
IEF with Rotofor	NPS-RP LC 1.5 μm, 55 mm × 3 mm i.d.	TOF	Ovarian epithelial Ovarian carcinoma	[115]
IEF with Rotofor 70 cm × 75 μm i.d.	RP LC C18 5 μm, 200 mm × 75 μm i.d.	Q-TOF	*C. tepidum* cell lysate	[116]
SAX 10 μm, 50 mm × 4.6 mm i.d.	RP LC C4 3.5 μm, 100 mm × 2.1 mm i.d.	TOF	*E. coli* cell lysate	[117]
SEC 600 mm × 21.2 mm i.d. or RP LC C4 250 mm × 10 mm i.d.	RP LC C5 5 μm, 800 mm × 150 μm i.d. or CIEF 80 cm × 50 μm i.d.	FTICR	*S. oneidensis* proteome	[118]
WAX 250 mm × 4.6 mm i.d.	RP LC C5 5 μm, 150 mm × 1 mm i.d.	LTQ-FT ICR	*S. cerevisiae* yeast cell lysates	[119]
WAX 5 μm, 100 mm × 4.6 mm i.d.	RP LC C5 5 μm, 800 mm × 75 μm i.d.	APEX-Q FTICR	*S. oneidensis* MR1 cell lysate	[120]
GELFrEE 6 cm × 0.6 cm i.d.	RP LC PLRP-S 5 μm, 100 mm × 75 μm i.d.	LTQ-FT ICR	Human HeLa cell lysate	[121]
GELFrEE 1 cm i.d.	RP LC C4 5 μm, 100 mm × 200 μm i.d.	TOF	Yeast proteome	[123]
WCX-HILIC 5 μm, 800 mm × 200 μm i.d.	RP LC C5 5 μm, 600 mm × 75 μm i.d.	LTQ-Orbitrap	Histones	[124]
CIEF + GELFrEE 6 cm × 0.6 cm i.d.	RP LC PLRP-S 5 μm, 100 mm × 75 μm i.d.	LTQ FT ICR	*S. cerevisiae* proteome	[127]

propanol, and identical percentages of acetic acid and TFA as buffer A). The RPLC columns were connected to the ESI-LTQ-Orbitrap mass spectrometer.

Using ~24 mg of core histone mixture, 41 H4 isoforms were identified, and the type and locations of PTMs were unambiguously mapped for 20 of these variants. Compared to corresponding off-line studies reported previously, on-line WCX-HILIC-RPLC platform offered higher sensitivity and throughput for characterization of histones.

Vellaichamy et al. [127] developed a tridimensional separation method in which solution IEF was coupled off-line to GELFrEE, and GELFrEE also off-line to an improved RPLC system. This prefractionation method is largely based on the previous work by Tran and Doucette [128] that reported the first semipreparative 2D liquid electrophoresis experiment in a multiplexed format, which facilitates high-throughput analysis of all fractions collected from the first IEF dimension of separation. Up to 128 fraction (8 × 16) could be separated by coupling CIEF to GELFrEE.

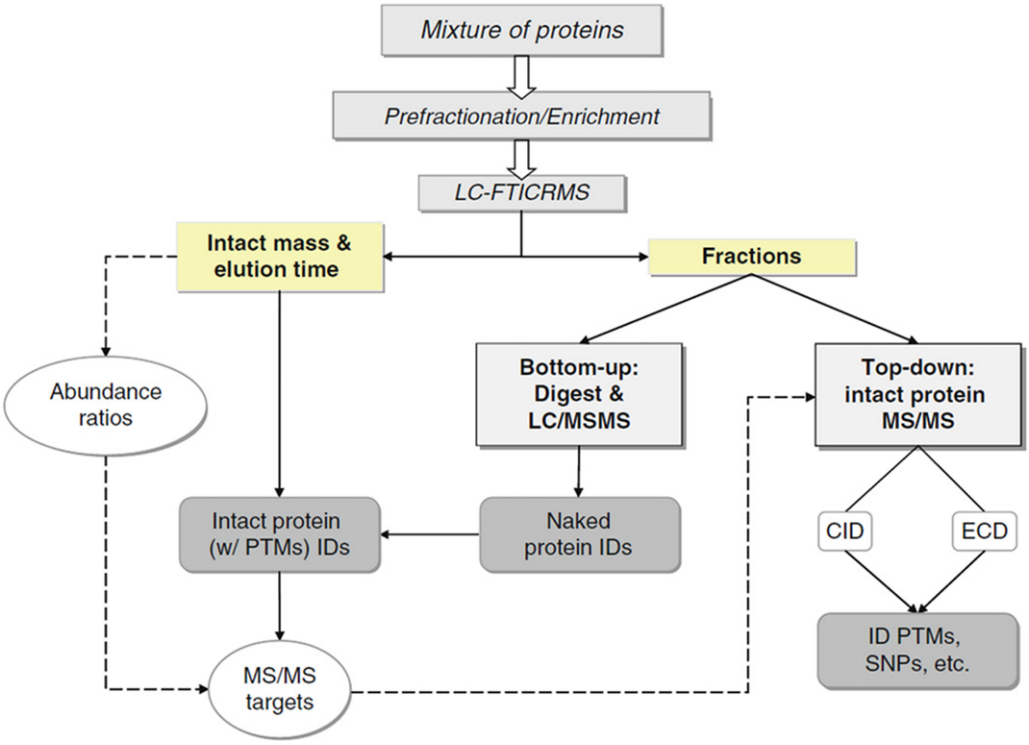


Fig. 6. Schematic diagram of the integrated top-down bottom-up proteomics strategy. Protein mixtures are fractionated to a level at which the reduced analyte complexity per fraction allows the inference of proteins from a peptide-level characterization of the same fraction. Accurate intact protein mass and time measurements facilitate protein abundance profiling that incorporates protein modifications, as well as high-throughput selection of biologically relevant targets for subsequent off-line gas-phase fragmentation using only an aliquot of the original collect RPLC fraction.
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For testing the system 3 mg *S. cerevisiae* proteins were solubilized in the IEF buffer, and focused using an in-house eight-channel IEF system; the cytosolic fraction of human cell lines ($\sim 1–3 \times 10^8$ cells) was also used. Proteins in IEF fractions were precipitated using cold acetone and subsequently separated with multiplexed GELFrEE as previously described [122]. Approximately 100 μL of sample buffer was loaded onto a GELFrEE column, and fractions (150 μL) were collected for 1.5 h starting after the elution of the dye front. Proteins were treated with the CMW system to deplete SDS.

Nano-bore analytical columns (100 mm × 75 μm i.d.) with an integral fritted nanospray emitter containing 5 μm PLRP (300, 1000, or 4000 Å pore size) stationary phase or 5 μm C4 bonded porous silica (300 Å pore size) were tested. The nanoLC system was operated at a flow rate of 300 nL min^{-1}. A 75 min gradient with buffers A (water:acetonitrile 95/5, v/v, with 0.2% FA) and B (acetonitrile:water 95/5, v/v, with 0.2% FA) was used.

Protein pellets were resuspended in 40 μL of buffer A, and 10 μL of the sample was injected. Performances of silica-based and polymeric stationary phases were compared before real sample analysis.

Ion trap base-peak chromatograms obtained from C4 and PLRP nanoLC of three different equimolar amounts (0.3, 1, and 3 pmol) of a seven-protein mix are shown in Fig. 5. Precursor ions for four of these proteins were detected from one LC–MS injection of 0.3 pmol of total protein on the C4 column. When the protein amount was increased to 1 pmol, distinct chromatographic elution of six of the proteins was observed along with a poorly resolved peak for ovalbumin (Fig. 5). As the sample amount increased from 1 to 3 pmol, an increase in signal-to-noise (S/N) ratio was observed for all proteins (except ovalbumin) on the C4 column. The PLRP stationary phase of the same (300 Å) pore size enabled all seven proteins to be detected even at the lowest loading amount (0.3 pmol). Furthermore, with the same protein amount, a higher S/N ratio was observed for all proteins tested, but ovalbumin. Overall, PLRP medium exhibited higher protein recoveries which gave rise to an increased S/N ratio of protein spectra by factors of 2–3. Further, at 1 pmol loading, a peak capacity of 21 and 30 was obtained for the C4 and PLRP media, respectively.

The effect of pore size was also studied for PLRP by injecting 1 pmol of the protein mix onto 300, 1000, and 4000 Å PLRP media. Firstly, a change in selectivity was observed. Further, while chromatography with 1000 Å pore size medium showed little change in the peak width for smaller proteins, a noticeable reduction in peak width was observed for larger proteins. In addition, an approximately 20% delay in retention times was also observed. For the larger proteins, a further reduction in peak widths and increasing in t_R was observed when the PLRP pore size was increased to 4000 Å. Overall, these phenomena can result from restricted diffusion of higher MW proteins [31]. The authors concluded that 1000 Å PLRP medium provides better resolution without compromising on peak widths for proteins across a wide molecular mass range.

Finally, detection of proteins >50 kDa was routinely accomplished in the LTQ of a hybrid FTICR mass spectrometer. Thereafter, protein identification was enabled by nozzle-skimmer dissociation and detection of fragment ions with <10 ppm mass accuracy. This approach led to identification of proteins up to 80 kDa, with 10–60 proteins identified in single LC–MS runs of samples prefractionated by IEF and GELFrEE.

One benefit of having the intact mass of a protein is the ability to provide a precise identification from several related forms, which are otherwise ambiguous when identifications are based on a few fragment ions measured at high accuracy. As full proteome runs become more robust for top-down MS, advanced software will be required to fully realize the conceptual advantages of top-down.

The study by Durbin et al. [129] describes several improvements for processing top-down data streams by building on their previous platforms [98,130], which allowed handling most of the data idiosyncrasies obtained through high-throughput top-down proteomics platforms currently in operation [121,127].

HeLa Se cell proteins (~2 mg) were reduced and alkylated, then precipitated with cold acetone and focused using an eight-channel solution IEF system as previously described [131]. Following IEF, selected fractions were precipitated using cold acetone and subsequently separated using GELFrEE [122]. After SDS removal for selected fractions, 10 μL of the cleaned up sample were injected onto 100 mm × 75 μm i.d. PLRP-S (5 μm particle size) capillary column. Mobile phases were water and acetonitrile, both 0.2% FA; a fast/slow/fast gradient for a total time of 55 min was used at a flow rate of 300 nL min^{-1}. Samples were analyzed on a 12 T LTQ FTICR Ultra.

Software functionality was demonstrated using comparative LC–MS data from yeast strains and from human cells undergoing chemical stress. The authors concluded that a unified approach to intact protein detection algorithms for both high- and low-resolution spectra has been established with associated capacities for PTMs finding and protein/PTMs visualization. This expansive platform has been set up to provide the analysis tools necessary to assure that intact mass information is captured by high-throughput implementations of top-down MS.

In Table 2 a summary of the separation methods, both mono- and multi-dimensional, of coupling chromatography or electrophoresis to MS for top-down proteomics is reported.

5. Conclusions and perspective

Mass spectrometry of intact proteins has at present intrinsic limitations. Mono-charged protein ions, such as those produced by MALDI source, cannot be detected with high resolution, while ions produced by the ESI source spread the signal in crowded clusters of multicharged isotopic peaks.

In addition, the charge state distribution under denaturing conditions becomes wider as the protein size increases, and the different charge states may show different fragmentation patterns and dissociation efficiencies. In general, for a given set of conditions and instrumental setting, the dissociation efficiency reduces as the protein size increases. In addition, the charge state distribution under denaturing conditions becomes wider as the protein size increases, and the different charge states may show different fragmentation patterns and dissociation efficiencies [75]. Fragmentation of single charge state causes loss in sensitivity, because only a fraction of the protein molecules are in that specific charge state, whereas collective fragmentation of all charge states may produce a fragment ion spectrum with abundant and possibly overlapping of fragmentations. To overcome some of these problems, techniques that use Q-IT mass spectrometers and positive-negative ion reactions to manipulate charge states of protein ions generated by ESI have been developed. The use of nozzle-skimmer CID may, in part, attain the same purpose, as fragment charge distribution is narrower than that of the larger precursor, but the problem of the acquisition of a large number of redundant data still remains unsolved. Technology of top-down proteomics is still improving, and an indeterminable period of time will be needed to reach the maturity of bottom-up. In the mean time, as proposed by Garcia [74] the intermediate approach of middle-down proteomics, where intact proteins were proteolyzed with enzymes specific for less abundant amino acids bonds may be a useful approach.

The hyphenation of LC as a multidimensional technique coupled to high-resolution MS for top down proteomics is still in its infancy if compared to routine LC analysis in pharmaceutical, environmental or even bottom-up proteomics. The development of porous microparticles and porous monoliths are one significant achievement among other drivers in instrumentation and data handling which can provide the basis for the widespread application of top down approach. The large macropore of the monolithic column facilitate the analyte diffusion, but efficiency trend was to decrease for too large macropores.

A better tuning of chromatographic conditions may improve the productivity.

In a recent paper, Detobel et al. [132] investigated column properties and LC conditions affecting the separation performance of PS-DVB monolithic capillary columns that differ in length (50, 100, and 250 mm) and macropore size. A decrease in peak width was observed with increasing column temperature from 20 to 80 °C. Increasing the column temperature from 20 to 60 °C no significant effects on peak area were observed for the protein test mixture used (RSD <6%). At 80 °C however, the peak area declined up to 60% depending on the time of elution. Longer gradient times yielded higher peak capacities up to a certain degree; at relatively long gradient times the rate of increase was less steep and peak capacity evolved towards a maximum. Column length showed a distinct effect on peak capacity, indicating that protein elution in gradient RPLC is not purely based on an on-off mechanism. Longer columns means higher pressure: this fact may pose technical problems but, as discussed above, high pressure improves proteins recovery. Protein recovery is an important issue, especially in quantitative proteomics, and is a factor limiting 2D separation in top-down proteomics. We can conclude that, at present, "top-down" proteomics may be a very useful complement of the bottom-up approach in identifying PTMs, isoforms, and small differences in protein sequence due to identification for analogy with similar phylogenetic organisms or errors in database based on genomic data. An integrated strategy, which include selection of a sub proteome, top-down proteomics in a chromatographic time scale, MS/MS of selected fraction in infusion mode, and bottom-up proteomics (Fig. 6) seems the more robust approach.

As far as 2D separation is concerned, the second dimension should possess the greatest peak capacity as possible. Although at present MS/MS scan velocity limitation did not permit to exploit entirely this opportunity, progresses in MS technology, may change this situation rapidly. Very high peak capacity in the first dimension cannot be apparently exploited because of throughput limitations, but has the virtue of decreasing the protein overlapping in different fractions.

New forms of chromatographic media amenable for protein separation have been recently described in literature. These media include a modified super-macroporous monolithic cryogel for high protein adsorption capacity [133]; hydrophilic gigaporous polystyrene-based microspheres derivatized for anionic exchange [134]; porous layer open tubular (PLOT) columns [135,136]; novel copolymerized polar/NPS based particles [137].

Among them, the PLOT columns seem the most promising. A porous layer of PS-DVB was synthesized on the wall of a 10 μm i.d. silica capillary and columns as long as 3 m can be produced with satisfactory reproducibility. A relatively high flow rate (20 nL min^{-1}) which is very amenable for nanoESI can be used. Loadability of real samples and robustness may constitute a limitation. New silica-based stationary phases with different polarity may at least provide different selectivity compared to the hydrophobic ones and discover, in a parallel experiment, proteins that were disregarded by MS, because of overlapping with a more abundant protein.

References

[1] V.J. Nesatyy, M.J.F. Suter, Environ. Sci. Technol. 41 (2007) 6891.

[2] G. Caruso, C. Cavaliere, C. Guarino, R. Gubbiotti, P. Foglia, A. Laganà, Anal. Bioanal. Chem. 391 (2008) 381.
[3] N.L. Kelleher, Anal. Chem. 76 (2004) 196A.
[4] G.E. Reid, S.A. McLuckey, J. Mass Spectrom. 37 (2002) 663.
[5] X. Han, M. Jin, K. Breuker, F.W. McLafferty, Science 314 (2006) 109.
[6] B. Macek, L.F. Waanders, J.V. Olsen, M. Mann, Mol. Cell. Proteomics 5 (2006) 949.
[7] J.S. Page, K. Tang, R.D. Smith, Int. J. Mass Spectrom. 265 (2007) 244.
[8] R.B. Dixon, D.C. Muddiman, A.M. Hawkridge, A.G. Fedorov, J. Am. Soc. Mass Spectrom. 18 (2007) 1909.
[9] J. Liu, T.Y. Huang, S.A. McLuckey, Anal. Chem. 81 (2009) 1433.
[10] C.K. Fagerquist, B.R. Garbus, W.G. Miller, K.E. Williams, E. Yee, A.H. Bates, S. Boyle, L.A. Harden, M.B. Cooley, R.E. Mandrell, Anal. Chem. 82 (2010) 2717.
[11] L.R. Snyder, M.A. Stadalius, in: Cs. Horvath (Ed.), High Performance Liquid Chromatography. Advances and Perspectives, Academic Press, New York, 1986, 195 pp.
[12] F. Švec, T.B. Tennikova, Z. Deyl, Monolithic Materials: Preparation, Properties and Applications, Elsevier, Amsterdam, 2003.
[13] M.G. Kaledi, High Performance Capillary Electrophoresis, John Wiley & Sons, NY, 1998.
[14] M. Kastner, Protein Liquid Chromatography, Elsevier, Amsterdam, 2000.
[15] X. Fang, B.M. Balgley, C.S. Lee, Electrophoresis 30 (2009) 3998.
[16] D. Walls, S.T. Loughran, Protein Chromatography: Methods and Protocols, Methods in Molecular Biology, Springer, New York, 2011.
[17] W.C. Lee, J. Chromatogr. B 699 (1997) 29.
[18] A.E. Rodrigues, J. Chromatogr. B 699 (1997) 47.
[19] L.R. Snyder, J.W. Dolan, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, 2nd ed., Dekker, New York, 2002, 397 pp.
[20] H.J. Issaq, T.P. Conrads, G.M. Janini, T.D. Veenstra, Electrophoresis 23 (2002) 3048.
[21] J. Tang, M. Gao, C. Deng, X. Zhang, J. Chromatogr. B 866 (2008) 123.
[22] O. Núñez, K. Nakanishi, N. Tanaka, J. Chromatogr. A 1191 (2008) 231.
[23] M. Stastna, J. Van Eyk, in: J.E. Van Eyk, M.J. Dunn (Eds.), Clinical Proteomics, Wiley, Weinheim, 2008, 31 pp.
[24] E.G. Vlakh, T.B. Tennikova, J. Chromatogr. A 1216 (2009) 2637.
[25] D. Tao, L. Zhang, Y. Shan, Z. Liang, Y. Zhang, Anal. Bioanal. Chem. 399 (2011) 229.
[26] M. Islinger, C. Eckerskorn, A. Voelkl, Electrophoresis 31 (2010) 1754.
[27] M. Gao, D. Qi, P. Zhang, C. Deng, X. Zhang, Expert Rev. Proteomics 7 (2010) 665.
[28] J.W. Eschelbach, J.W. Jorgenson, Anal. Chem. 78 (2006) 1697.
[29] J.D. Pearson, F.E. Regnier, J. Liq. Chromatogr. 6 (1983) 497.
[30] K.A. Tweeten, T.N. Tweeten, J. Chromatogr. 359 (1986) 111.
[31] L.L. Lloyd, J. Chromatogr. 544 (1991) 201.
[32] N.Z. Zhelev, M.J. Barratt, L.C. Mahadevan, J. Chromatogr. A 763 (1997) 65.
[33] D.F. Elgar, C.S. Norris, J.S. Ayers, M. Pritchard, D.E. Otter, K.P. Palmano, J. Chromatogr. A 878 (2000) 183.
[34] L.L. Lloyd, M.I. Millichip, J.M. Watkins, J. Chromatogr. A 944 (2002) 169.
[35] T. Bruch, H. Graalfs, L. Jacob, C. Frech, J. Chromatogr. A 1216 (2009) 919.
[36] A. Staby, R. Holm Jensen, M. Bensch, J. Hubbuch, D.L. Dünweber, J. Krarup, J. Nielsen, M. Lund, S. Kidal, T. Budde Hansen, I. Holm Jensen, J. Chromatogr. A 1164 (2007) 82.
[37] P.J. Boersema, S. Mohammed, A.J.R. Heck, Anal. Bioanal. Chem. 391 (2008) 151.
[38] L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to Modern Liquid Chromatography, 3rd ed., Wiley, Hoboken (NJ), 2010.
[39] Y. Wang, B.M. Balgley, P.A. Rudnick, C.S. Lee, J. Chromatogr. A 1073 (2005) 3.
[40] G.Y. Sun, Q.H. Shi, Y. Sun, J. Chromatogr. A 1061 (2004) 159.
[41] K. Wagner, K. Racaityte, K.K. Unger, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, J. Chromatogr. A 893 (2000) 293.
[42] K.F. Du, D. Yang, Y. Sun, J. Chromatogr. A 1163 (2007) 212.
[43] K. Yao, J. Yun, S. Shen, L. Wang, X. He, X. Yu, J. Chromatogr. A 1109 (2006) 103.
[44] R. Wu, L. Hu, F. Wang, M. Ye, H. Zou, J. Chromatogr. A 1184 (2008) 369.
[45] S. Hjertén, J.L. Liao, R. Zhang, J. Chromatogr. 473 (1989) 273.
[46] T.B. Tennikova, F. Švec, B.G. Belenkii, J. Liq. Chromatogr. 13 (1990) 63.
[47] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[48] F. Švec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[49] S.M. Fields, Anal. Chem. 68 (1996) 2709.
[50] R.A. Everley, T.R. Croley, J. Chromatogr. A 1192 (2008) 239.
[51] C. Esteve, C. Del Rio, M.L. Marina, M.C. García, J. Agric. Food Chem. 58 (2010) 8176.
[52] S.H. Love, J. Chromatogr. 138 (1977) 25.
[53] S. Hjerten, M.D. Zhu, J. Chromatogr. 346 (1985) 265.
[54] S. Hjerten, J.L. Liao, J. Yao, J. Chromatogr. 387 (1987) 127.
[55] F. Kilar, S. Hjerten, Electrophoresis 10 (1989) 23.
[56] Y. Shen, F. Xiang, T.D. Veenstra, E.N. Fung, R.D. Smith, Anal. Chem. 71 (1999) 5348.
[57] F. Foret, O. Muller, J. Thorne, W. Gotzinger, B.L. Karger, J. Chromatogr. A 716 (1995) 157.
[58] Q. Tang, A.K. Harrata, C.S. Lee, Anal. Chem. 67 (1995) 3515.
[59] L. Yang, C.S. Lee, S.A. Hofstadler, L. Pasă-Tolić, R.D. Smith, Anal. Chem. 70 (1998) 3235.
[60] P.K. Jensen, L. Pasă-Tolić, G.A. Anderson, J.A. Horner, M.S. Lipton, J.E. Bruce, R.D. Smith, Anal. Chem. 71 (1999) 2076.
[61] Y.-C. Wang, M. Ho Choi, J. Han, Anal. Chem. 76 (2004) 4426.
[62] P. Davidsson, C.L. Nilsson, Biochim. Biophys. Acta 1473 (1999) 391.
[63] C.L. Nilsson, T. Larsson, E. Gustafsson, K.-A. Karlsson, P. Davidsson, Anal. Chem. 72 (2000) 2148.
[64] M. Puchades, A. Westman, K. Blennow, P. Davidsson, Rapid Commun. Mass Spectrom. 13 (1999) 344.
[65] D. Wessel, U.I.A. Flügge, Anal. Biochem. 138 (1984) 141.
[66] G. Berridge, R. Chalk, N. D'Avanzo, L. Dong, D. Doyle, J.I. Kim, X. Xia, N. Burgess-Brown, A. de Riso, E.P. Carpenter, O. Gileadi, Anal. Biochem. 410 (2011) 272.
[67] S. Konig, O. Schmidt, K. Rose, S. Thanos, M. Besselmann, M. Zeller, Electrophoresis 24 (2003) 751.
[68] R.D. Smith, J.A. Loo, C.J. Barinaga, C.D. Edmonds, H.R. Udseth, J. Chromatogr. 480 (1989) 211.
[69] R.A. Wallingford, A.G. Ewing, Adv. Chromatogr. 29 (1989) 1.
[70] Z. El Rassi, Electrophoresis 31 (2010) 174.
[71] S.K. Sze, Y. Ge, H.B. Oh, F.W. McLafferty, PNAS 99 (2002) 1774.
[72] N.L. Kelleher, H.Y. Lin, G.A. Valaskovic, D.J. Aaserud, E.K. Fridriksson, F.W. McLafferty, J. Am. Chem. Soc. 121 (1999) 806.
[73] G.H. Kruppa, J. Schoeniger, M.M. Young, Rapid Commun. Mass Spectrom. 17 (2003) 155.
[74] B.A. Garcia, J. Am. Soc. Mass Spectrom. 21 (2010) 193.
[75] B. Bogdanov, R.D. Smith, Mass Spectrom. Rev. 24 (2005) 168.
[76] A. Armirotti, G. Damonte, Proteomics 10 (2010) 3566.
[77] T.Y. Huang, S.A. McLuckey, Proteomics 10 (2010) 3577.
[78] E.C. Nice, J. Rothacker, J. Weinstock, L. Lim, B. Catimel, J. Chromatogr. A 1168 (2007) 190.
[79] F. Meng, B.J. Cargile, S.M. Patrie, J.R. Johnson, S.M. McLoughlin, N.L. Kelleher, Anal. Chem. 74 (2002) 2923.
[80] V. Zabrouskov, L. Giacomelli, K.J. van Wijk, F.W. McLafferty, Mol. Cell. Proteomics 2 (2003) 1253.
[81] M.K. Bunger, B.J. Cargile, A. Ngunjiri, J.L. Bundy, J.L. Stephenson, Anal. Chem. 80 (2008) 1459.
[82] N.C. VerBerkmoes, J.L. Bundy, L. Hauser, K.G. Asano, J. Razumovskaya, F. Larimer, R.L. Hettich, J.L. Stephenson Jr., J. Proteome Res. 1 (2002) 239.
[83] Y. Ge, B.G. Lawhorn, M. El Naggar, E. Strauss, J.H. Park, T.P. Begley, F.W. McLafferty, J. Am. Chem. Soc. 124 (2002) 672.
[84] S. Ayaz-Guner, J. Zhang, L. Li, J.W. Walker, Y. Ge, Biochemistry 48 (2009) 8161.
[85] C.M. Ryan, P. Souda, S. Bassilian, R. Ujwal, J. Zhang, J. Abramson, P. Ping, A. Durazo, J.U. Bowie, S.S. Hasan, D. Baniulis, W.A. Cramer, K.F. Faull, J.P. Whitelegge, Mol. Cell. Proteomics 9 (2010) 791.
[86] S.M. Patrie, J.T. Ferguson, D.E. Robinson, D. Whipple, M. Rother, W.W. Metcalf, N.L. Kelleher, Mol. Cell. Proteomics 5 (2006) 14.
[87] B.E. Chong, F. Yan, D.M. Lubman, F.R. Miller, Rapid Commun. Mass Spectrom. 15 (2001) 291.
[88] S.A. Hofstadler, E. Schmidt, Z. Guan, D.A. Laude Jr., J. Am. Soc. Mass Spectrom. 4 (1993) 168.
[89] S.A. Ouvry-Patat, M.P. Torres, H.H. Quek, C.A. Gelfand, P. O'Mullan, M. Nissum, G.K. Schroeder, J.H.M. Elliott, D. Dryhurst, J. Ausio, R. Wolfenden, C.H. Borchers, Proteomics 8 (2008) 2798.
[90] J.J. Pesavento, C.A. Mizzen, N.L. Kelleher, Anal. Chem. 78 (2006) 4271.
[91] J.J. Pesavento, B.A. Garcia, J.A. Streeky, N.L. Kelleher, C.A. Mizzen, Mol. Cell. Proteomics 6 (2007) 1510.
[92] P.K. Jensen, L. Paša-Tolić, G.A. Anderson, J.A. Horner, M.S. Lipton, J.E. Bruce, R.D. Smith, Anal. Chem. 71 (1999) 2076.
[93] P.K. Jensen, L. Paša-Tolić, K.K. Peden, S. Martinović, M.S. Lipton, G.A. Anderson, N. Tolić, K.K. Wong, R.D. Smith, Electrophoresis 21 (2000) 1372.
[94] S. Martinović, S.J. Berger, L. Paša-Tolić, R.D. Smith, Anal. Chem. 72 (2000) 5356.
[95] S.W. Lee, S.J. Berger, S. Martinović, L. Paša-Tolić, G.A. Anderson, Y. Shen, R. Zhao, R.D. Smith, PNAS 99 (2002) 5942.
[96] Y. Shen, R. Zhao, M.E. Belov, T.P. Conrads, G.A. Anderson, K. Tang, L. Paša-Tolić, T.D. Veenstra, M.S. Lipton, R.D. Smith, Anal. Chem. 73 (2001) 1766.
[97] H. Hernández, O.V. Makarova, E.M. Makarov, N. Morgner, Y. Muto, D.P. Krummel, C.V. Robinson, PLoS ONE 4 (9) (2009) e7202.
[98] C.D. Wenger, M.T. Boyne, J.T. Ferguson, D.E. Robinson, N.L. Kelleher, Anal. Chem. 80 (2008) 8055.
[99] J.T. Ferguson, C.D. Wenger, W.W. Metcalf, N.L. Kelleher, J. Am. Soc. Mass Spectrom. 20 (2009) 1743.
[100] S. Wu, N. Tolić, Z. Tian, E.W. Robinson, L. Paša-Tolić, in: C.H. Wu, C. Chen (Eds.), Bioinformatics for Comparative Proteomics: Methods in Molecular Biology, vol. 694, Springer, New York, 2011, 291 pp.
[101] T.S. Collier, A.M. Hawkridge, D.R. Georgianna, G.A. Payne, D.C. Muddiman, Anal. Chem. 80 (2008) 4994.
[102] T.S. Collier, P. Sarkar, B. Rao, D.C. Muddimana, J. Am. Soc. Mass Spectrom. 21 (2010) 879.
[103] A. Chi, D.L. Bai, L.Y. Geer, J. Shabanowitz, D.F. Hunt, Int. J. Mass Spectrom. 259 (2007) 197.
[104] M. Li, L. Jiang, N.L. Kelleher, J. Chromatogr. B 877 (2009) 3885.
[105] M. Naldi, N. Calonghi, L. Masotti, C. Parolin, S. Valente, A. Mai, V. Andrisano, Proteomics 9 (2009) 5437.
[106] X. Su, N.K. Jacob, R. Amunugama, D.M. Lucas, A.R. Knapp, C. Ren, M.E. Davis, G. Marcucci, M.R. Parthun, J.C. Byrd, R. Fishel, M.A. Freitas, J. Chromatogr. B 850 (2007) 440.
[107] E. Contrepois, F. Mann, J. Proteome Res. 9 (2010) 5501.
[108] X. Su, D.M. Lucas, L. Zhang, H. Xu, V. Zabrouskov, M.E. Davis, A.R. Knapp, D.C. Young, P.O. Payne, M.R. Parthun, G. Marcucci, M.R. Grever, J.C. Byrd, M.A. Freitas, Proteomics 9 (2009) 1197.

[109] J. Mohr, R. Swart, M. Samonig, G. Böhm, C.G. Huber, Proteomics 10 (2010) 3598.
[110] C. Wynne, N.J. Edwards, C. Fenselau, Proteomics 10 (2010) 3631.
[111] M.T. Mazur, H.L. Cardasis, D.S. Spellman, A. Liaw, N.A. Yates, R.C. Hendrickson, PNAS 107 (2010) 7728.
[112] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 1518.
[113] D.B. Wall, M.T. Kachman, S.S. Gong, S.J. Parus, M.W. Long, D.M. Lubman, Rapid Commun. Mass Spectrom. 15 (2001) 1649.
[114] D.B. Wall, M.T. Kachman, S. Gong, R. Hinderer, S. Parus, D.E. Misek, S.M. Hanash, D.M. Lubman, Anal. Chem. 72 (2000) 1099.
[115] M.T. Kachman, H. Wang, D.R. Schwartz, K.R. Cho, D.M. Lubman, Anal. Chem. 74 (2002) 1779.
[116] F. Zhou, T.E. Hanson, M.V. Johnston, Anal. Chem. 79 (2007) 7145.
[117] K.M. Millea, I.S. Krull, S.A. Cohen, J.C. Gebler, S.J. Berge, J. Proteome Res. 5 (2006) 135.
[118] D.C. Simpson, S. Ahn, L. Paša-Tolić, B. Bogdanov, H.M. Mottaz, A.N. Vilkov, G.A. Anderson, M.S. Lipton, R.D. Smith, Electrophoresis 27 (2006) 2722.
[119] B.A. Parks, L. Jiang, P.M. Thomas, C.D. Wenger, M.J. Roth, M.T. Boyne, P.V. Burke, K.E. Kwast, N.L. Kelleher, Anal. Chem. 79 (2007) 7984.
[120] S. Sharma, D.C. Simpson, N. Tolić, N. Jaitly, A.M. Mayampurath, R.D. Smith, L. Paša-Tolić, J. Proteome Res. 6 (2007) 602.
[121] J.E. Lee, J.F. Kellie, J.C. Tran, J.D. Tipton, A.D. Catherman, H.M. Thomas, D.R. Ahlf, K.R. Durbin, A. Vellaichamy, I. Ntai, A.G. Marshall, N.L. Kelleher, J. Am. Soc. Mass Spectrom. 20 (2009) 2183.
[122] J.C. Tran, A.A. Doucette, Anal. Chem. 80 (2008) 1568.
[123] D. Botelho, M.J. Wall, D.B. Vieira, S. Fitzsimmons, F. Liu, A. Doucette, J. Proteome Res. 9 (2010) 2863.
[124] Z. Tian, R. Zhao, N. Tolić, R.J. Moore, D.L. Stenoien, E.W. Robinson, R.D. Smith, L. Paša-Tolić, Proteomics 10 (2010) 3610.
[125] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[126] C.T. Mant, R.S. Hodges, J. Sep. Sci. 31 (2008) 2754.
[127] A. Vellaichamy, J.C. Tran, A.D. Catherman, J.E. Lee, J.F. Kellie, S.M.M. Sweet, L. Zamdborg, P.M. Thomas, D.R. Ahlf, K.R. Durbin, G.A. Valaskovic, N.L. Kelleher, Anal. Chem. 82 (2010) 1234.
[128] J.C. Tran, A.A. Doucette, Anal. Chem. 81 (2009) 6201.
[129] K.R. Durbin, J.C. Tran, L. Zamdborg, S.M.M. Sweet, A.D. Catherman, J.E. Lee, M. Li, J.F. Kellie, N.L. Kelleher, Proteomics 10 (2010) 3589.
[130] L. Zamdborg, R.D. LeDuc, K.J. Glowacz, Y.B. Kim, V. Viswanathan, I.T. Spaulding, B.P. Early, E.J. Bluhm, S. Babai, N.L. Kelleher, Nucleic Acids Res. 35 (2007) 701.
[131] J.C. Tran, A.A. Doucette, J. Proteome Res. 7 (2008) 1761.
[132] F. Detobel, K. Broeckhoven, J. Wellens, B. Wouters, R. Swart, M. Ursem, G. Desmet, S. Eeltink, J. Chromatogr. A 1217 (2010) 3085.
[133] M. Erzengin, N. Ünlü, M. Odabaş, J. Chromatogr. A 1218 (2011) 484.
[134] J.B. Qu, X.Z. Wan, Y.Q. Zhai, W.Q. Zhou, Z.G. Su, G.H. Ma, J. Chromatogr. A 1216 (2009) 6511.
[135] G. Yue, Q. Luo, J. Zhang, S.L. Wu, B.L. Karger, Anal. Chem. 79 (2007) 938.
[136] M. Rogeberg, S.R. Wilson, T. Greibrokk, E. Lundanes, J. Chromatogr. A 1217 (2010) 2782.
[137] J. Dong, L. Yu, X. Zhang, X. Xue, Z. Guo, X. Liang, Anal. Bioanal. Chem. 399 (2011) 3415.