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Review

Multidimensional separation of peptides for effective proteomic analysis

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Abstract

Current solution based proteomic analysis methods are generally based on enzymatic digestion of a protein mixture followed by separation using multidimensional liquid chromatography and/or electrophoresis where peptide identification is typically accomplished by tandem mass spectrometry (MS/MS). It is generally accepted that no single chromatographic or electrophoretic procedure is capable of resolving the complex mixture of peptides that results from a global proteolytic digest of a proteome. Therefore, combining two or more orthogonal (multimodal) separation procedures dramatically improves the overall resolution and results in a larger number of peptides being identified from complex proteome digests. Separation of a proteome digest is a particularly challenging analytical problem due to the large number of peptides and the wide concentration dynamic range. While it has been demonstrated that increasing the number of dimensions of separation prior to MS analysis increases the number of peptides that may be identified, a balance between the time invested and the overall results obtained must be carefully considered. This manuscript provides a review of two- and three-dimensional peptide separation strategies combined with MS for the analysis of complex peptide mixtures.
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Keywords: Multidimensional separation; Peptides; Proteomic analysis

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Abbreviations: 2-D, two-dimensional; 3-D, three-dimensional; IEX, ion exchange chromatography; SCX, strong cation exchange chromatography; RP, reversed-phase; IEF, isoelectric focusing; ICAT, isotope coded affinity tag; MS/MS, tandem mass spectrometry; CID, collision induced dissociation; PAGE, polyacrylamide gel electrophoresis; LIF, laser-induced fluorescence; MudPIT, multidimensional protein identification technology; ESI, electrospray ionization; CGE, capillary gel electrophoresis

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1. Introduction

The proteome describes the entire compliment of proteins expressed by a cell at a point in time and includes systematic approaches for the study of the distribution, abundance, modification, identification, interactions, structure, and function of large sets of proteins as well as their involvement in different diseases such as cancer. Proteome analysis rather allows investigation of normal and diseased states. To discover the constituents of a proteome, typically intact proteins are digested into peptides and fractionated using a variety of chromatographic and/or electrophoretic techniques prior to their actual identification using some form of mass spectrometric technique. In the last few years, significant research has been carried out to develop electrophoretic and chromatographic methods to enable the resolution and identification of all peptides (proteins) in a given proteome. This task is not a trivial separation exercise because, for example, a serum proteome may contain up to 20,000 proteins with a concentration dynamic range of 10^{10} which when digested may result in 400,000–600,000 peptides [1,2]. No single chromatographic or electrophoretic procedure to date possesses the peak capacity required to resolve such a complex mixture into its individual components. Many attempts have been made using different electrophoretic and chromatographic modes and a combination of both techniques employing multidimensional procedures, off-line and on-line, to separate such complex protein/peptide mixtures. However, no method presently exists that can be used to separate, detect and quantify *all* proteins within a given proteome. According to Giddings [3], the peak capacity of a multidimensional separation is the product of the peak capacities of its component one-dimensional methods. Fractionation is a critical aspect of mass spectral identification of peptides. A mass spectrometer can perform mass measurements on several but not many co-eluting peptides. Conventional MS/MS cycle times for most instruments typically enable only a limited number of these peptides to be selected for collision induced dissociation (CID). Therefore, limiting the number of co-eluting peptides helps to increase the percentage of species that may be identified. The aim of this review is to highlight the different liquid phase multidimensional separation strategies that have been employed for fractionation and separation of complex mixtures of peptides.

2. Separation science

The two main fields of separation science that apply to the analysis of peptides and proteins in the new science of proteomics are chromatography and electrophoresis. Procedures applicable to the separation of peptides are mainly high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Different modes of HPLC (reversed-phase, ion exchange, size exclusion, affinity, hydrophobic interaction) and CE (capillary zone, isoelectric focusing, isotachophoresis, affinity, micellar) and a combination of both techniques in hyphenated multidimensional formats have been used for the fractionation and/or separation of peptides. In the following sections, two- and three-dimensional (2-D, 3-D) separation strategies that have been used for the separation of peptides will be discussed.

3. Fractionation

Fractionation of a proteome can be conducted at the subcellular, protein or peptide levels. The cell can be divided into its subcellular components, such as mitochondria or nuclei; after which, the protein content of each compartment is analyzed. Also, the proteins within a cell may be divided into fractions based on their solubility properties, i.e., soluble or cytosolic and insoluble or membrane proteins. Fractionation may be carried out at the peptide level by chromatography or electrophoresis as described in the following sections or by the MS with the inherent resolving capabilities of the instrument employing gas phase fractionation [4].

Fractionation of complex peptide mixtures by chromatography or electrophoresis (Table 1) is achieved by manipulating either (a) the mobile phase properties or (b) stationary phase properties (column packing material). The principle of chromatographic fractionation of peptides is based on their interaction with the stationary phase and the mobile phase. The interaction may be adsorption on silica surfaces, par-

Table 1
List of various methods used to fractionate peptides based on their physical or chemical property

Fractionation method	Physical/chemical property
Ultracentrifugation	Density
Size exclusion chromatography	Stoke's radius
Isoelectric focusing	Isoelectric point
Hydrophobic interaction chromatography	Hydrophobicity
Reversed-phase chromatography	Hydrophobicity
Ion-exchange chromatography	Charge
Affinity chromatography	Specific biomolecular interaction
Capillary electrophoresis	Size/charge

titioning on reversed-phase (RP) materials, size exclusion, affinity or ion exchange (IEX) based on the effective charge of the peptides under specific mobile phase conditions. Separations may in many cases is the result of a combination of modes such as partitioning, adsorption, etc.

Chromatographic fractionation is achieved by using mobile phase gradients whereby proteins or peptides are differentially eluted by changing the organic modifier concentration (RP) or the ionic strength of the buffer as a function of time (hydrophobic interaction chromatography and IEX) or the pH of the mobile phase (IEX). Fractionation of proteins/peptides by isoelectric focusing (IEF) is based on their isoelectric point (p*I*). The principle of IEF is very simple. Peptides are mixed with the desired pH range carrier ampholyte mixture, or a carrier without ampholyte (exploiting the amphoteric nature of peptides) in a focusing cell. When an electric potential is applied to the focusing cell, peptides/proteins migrate to a position in the established pH gradient to a point in which their net charge is zero. The result is the "focusing" of peptides/proteins into *narrow* bands at their p*I* values. Fractions can be collected and further resolved by electrophoresis or chromatography. An advantage of liquid phase IEF is the ability to fractionate a complex mixture of peptides or proteins according to their p*I*s in a non-gel medium.

Fractionation by chromatography is not new, as a literature search can reveal, being first used in 1960 by Moore and Lee to fractionate water soluble proteins from liver by IEX chromatography on a diethylaminoethyl (DEAE) cellulose column [5]. Since then, many different chromatographic and electrophoretic methods have been used for fractionating proteins and peptides [6,7]. The obvious need for fractionation is to minimize the number of peptides being introduced to the MS at any given point in the analysis, which allows for detection of a greater number of peptides across a greater dynamic concentration range. The advantages of an on-line approach are speed and high throughput, however, such approaches have their limitations. There are stringent requirements, such as the second dimension must be faster than the first, the columns used are restricted in dimensions that limit the amount of sample that can be efficiently loaded, it does not allow re-analysis of fractions, and may require special instrumental modifications. An off-line approach, although slower, and may lead to sample loss, has several advantages. It is simple and easy to perform and allows larger analytical columns (up to 4.6 mm i.d.) packed with different materials to be used, thereby enabling injection of larger amounts of sample. In addition, off-line fractionation most often utilizes commercially available equipment that does not require instrumental modifications, and allows the re-analysis of collected fractions. However, recovery using an on-line approach is higher and sample loss is minimized.

4. Multidimensional separations

4.1. Historical perspective

Although the initial implementation of high-resolution 2-D is attributed to O'Farrell in 1975 [8], the original concept of two-dimensional protein separation by electrophoresis was proposed by Smithies and Poulik in 1956 [9]. They recognized that "a combination of the two electrophoretic processes on a gel at right angles should therefore give a much greater degree of resolution than is possible with either separately." The two modes of resolution are (a) molecular size and (b) free solution mobility on a starch gel. Five years later, Raymond [10] who named his 2-D experiments "orthogonal gel electrophoresis or Orthacryl" realized that the combination of electrophoretic and molecular filtration effects produce new and startling resolutions that could not be obtained by other electrophoretic procedures. Laurell, in 1965 [11] devised a 2-D procedure for the separation of serum proteins whereby electrophoresis on an agarose gel was used in the first dimension, and affinity electrophoresis in the second dimension. Two-dimensional PAGE became the method of choice for biologists after its introduction by O'Farrell [8] for the separation of cellular proteins under denaturing conditions. The principle of 2-D PAGE is resolution of proteins based on their p*I* in the first dimension and molecular mass in the second dimension. Despite its limitations, 2-D PAGE is still the most widely used multidimensional separation technique for the analysis of complex protein mixtures. One of its key strengths is its ability to allow investigators to compare protein expression levels in cells under different environmental conditions or treatments.

An advantage of chromatographic and electrophoretic techniques is that they allow the freedom to resolve a complex mixture by different routes employing different separation mechanisms based on physical and chemical properties of the solute mixture [12]. A requirement of any successful 2-D procedure is that the two selected dimensions possess different, but compatible, separation mechanisms (orthogonal), especially true in cases when an on-line format is selected. In addition to 2-D PAGE, many multidimensional separation approaches have been published, employing HPLC and/or CE, for the separation of peptide mixtures. The advantages of HPLC and CE multidimensional procedures are that they are automatable, sensitive, reproducible, fast, and quantitative. Unlike 2-D PAGE, separations of proteins/peptides by HPLC

and CE can be coupled with different detection systems, such as ultra-violet (UV), MS or laser-induced fluorescence (LIF). In addition, HPLC and CE allow the use of mobile phase gradient elution for improved resolution. Indeed, the concept of gradient elution is not new, its advantages were realized in 1949 by two different groups [13,14].

4.2. General comments regarding separation and detection of peptides

Multidimensional separation of complex peptide mixtures involves the use of two or more separation procedures that have to be orthogonal and compatible with each other. Multidimensional IEX and RP separations are carried out by linear or step-gradients, each having its advantages and limitations. It has been suggested by Wagner et al. that linear gradients instead of step-gradients result in peak broadening and lower peptide recovery [15]. Unfortunately, no data was presented comparing both approaches. Fractions can be collected from a gradient procedure as they elute from the column by time, drop or by inflexion point using an autosampler. Collection of fractions based on inflexion points eliminates the splitting of broad peaks between two fractions.

Most RP-HPLC separations are carried out using acetonitrile gradients; improvements in selectivity can be accomplished by addition of an ion pairing agent, such as formic or acetic acid. Ion exchange chromatography is conducted using salts such as sodium chloride, potassium chloride, ammonium acetate or ammonium formate at different concentrations in a gradient format. The salt selected, its concentration and the buffer composition used in the first dimension should not deleteriously affect the second dimension of separation in terms of the peak shape (resolution). For example, typically ammonium salts are used to replace mineral salts (KCl and NaCl) when downstream detection is by ESI-MS [16]. Also, if CE is the second separation mode, too much salt from the first dimension will generate excess Joule's heating thereby diminishing the resolution. Finally, in global proteomic studies, the multidimensional system selected should allow for as much proteome coverage as possible, and not only selected fractions (peaks) of the first dimension, as in heart cutting procedures used in HPLC [17] and gas chromatography [18] where only regions (peaks) of interest are subjected to further analysis.

4.3. Multidimensional separation strategies

In most current HPLC and CE multidimensional approaches, the proteins are digested into peptides prior to separation. The advantage of such an approach is that peptides possess greater solubility in a wider range of solvents and are hence easier to separate than proteins. This fact is especially true for membrane proteins that are typically insoluble in aqueous buffers. The disadvantage in working with peptides, however, is the increased number of species that must be resolved. Multidimensional separation methods are necessary to simplify such complex mixtures to allow for the greatest number of species to be successfully identified by subsequent MS analysis. While MS technology has and will continue to rapidly improve, even the best dynamic range measurement capabilities are still six to seven orders of magnitude smaller than the dynamic range of protein concentration of proteins present within a serum sample. The ability to decrease the number of co-eluting peptides introduced to the spectrometer at any one time is directly proportional to the successful and comprehensive characterization of a proteome sample. Table 1 shows the different separation mechanisms that can be used in 1-D, 2-D or 3-D format for the resolution of a simple or complex mixture of peptides. There have been many multidimensional approaches, employing chromatography and electrophoresis for the separation of peptide mixtures. For example, two different HPLC separation mechanisms, or two different CE separation mechanisms, or a combination of HPLC and CE mechanisms can be employed to resolve complex mixtures. Two- and three-dimensional strategies, as will be discussed later, allowed the mass spectral detection of thousands of peptides and resulted in the identification of hundreds of proteins from complex proteome digests, for example, the serum proteome. Here, we will discuss selected examples of these strategies as they apply to the separation of complex peptide mixtures (e.g., protein digests).

5. Two-dimensional approaches [19–61]

5.1. HPLC-IEX/HPLC-RP

The combination of IEX liquid chromatography (mainly strong cation exchange chromatography (SCX)) in the first dimension and RP chromatography in the second dimension, are the dominant procedures for the separation of proteome digests today. Yates and coworkers [19–21] developed an on-line multidimensional protein identification technology (MudPIT) where the SCXLC and RPLC materials are sequencially packed into a single microcapillary column (Fig. 1). An acidified tryptic digest of 80S ribosomes isolated from yeast was loaded onto the MudPIT column. Discrete fractions of peptides can be displaced from the SCX stationary phase directly onto the RP section of the column by salt

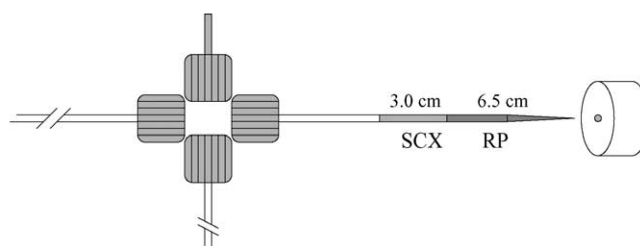
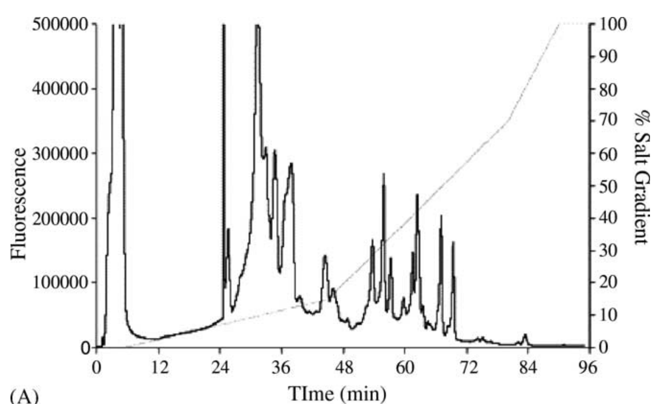


Fig. 1. Two-phase SCX/RP-HPLC column (reprinted with permission from [19]).

pulses, followed by RP separation of each subset of peptides. This iterative process was repeated 12 times using increasing salt pulses [19]. An advantage of MudPIT is that the entire system is coupled directly on-line with MS. This group followed up their initial demonstration by applying the MudPIT strategy for the identification of almost 1500 proteins within the yeast proteome [21] and for quantitation of proteins from cultures of *Saccharomyces cerevisiea* [22]. Similar off-line procedures were reported for the separation of peptides (protein digests) in human plasma filtrate [23], plasma peptides [24], blood ultrafiltrate [25], human urine [26], and yeast proteome [27]. In our laboratory, off-line SCX fractionation followed by microcapillary (μ) RPLC-MS/MS was applied to a global proteomic analysis (i.e., protein digests) of the low molecular weight human serum proteome [28], membrane proteins isolated from mouse natural killer cells [29], plasma membrane proteins [30], lipid raft proteins [31], the *E. coli* proteome [32], and the mouse cortical neuron proteome [33]. An example of the SCX chromatogram of a serum protein digest is given in Fig. 2A and B is the RPLC chromatogram of fraction 31. While most multidimensional peptide separations published to date have used SCX as the first dimension, Wagner et al. [15] employed IEX (anion and cation) chromatography as the first dimension and RPLC as the second dimension. Tryptic digested ribosomes were first fractionated using both anion and cation exchange chromatography and subsequently separated by RP-HPLC coupled online with ESI-MS. The authors reported a few interesting observations: (a) every fraction derived from IEX mainly provided peptides that could not be detected in preceding or following fractions; (b) one-dimensional RP chromatography gave better resolution than IEX of the same peptide mixture. This lower resolution is why IEX is normally the first dimension in multidimensional separations since subsequent separation steps should possess higher resolving capability; and (c) an off-line 2-D HPLC system allows the application of salt gradients with the addition of organic modifiers instead of salt step-gradients that cause peak broadening and lower peptide recovery [15].



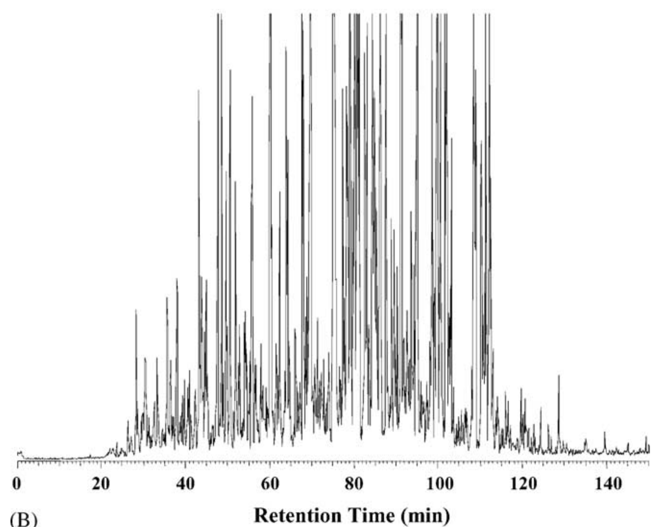


Fig. 2. Strong cation exchange chromatogram of the separation of a serum protein digest (A) and RPLC chromatogram of fraction 31 resolved by SCX (B).

When SCX is used as the first dimension for fractionation, a dilemma is faced for deciding on how many successive pulses with increasing salt concentration should be used or how many fractions should be collected in an off-line salt gradient fractionation. The answer depends on a cost/benefit analysis; factors such as the complexity of the peptide mixture, sample size, gradient time, available time, MS mode, and the purpose of the proteome analysis must be weighed. It is not unusual to collect as many as 96 SCX fractions or more from a complex protein digest. Another aspect of IEX that the analyst has to decide upon is the buffer type, concentration, and pH. High salt concentrations result in sharp peaks, short elution times, and a large number of co-eluting peptides. On the other hand, low salt concentration results in broad peaks, long elution times, and reduced number of co-eluting peptides [15].

5.2. HPLC/CZE

Jorgenson and coworkers [35–37] have developed many elegant on-line multicolumn approaches with various combinations of size exclusion chromatography, RP chromatography, IEX chromatography and CZE for the separation of peptides and proteins. Moore and Jorgenson used on-line RPLC/CZE [35] separations to resolve complex mixtures of peptides. In these comprehensive procedures, the second dimension separation was designed to be much faster than the first dimension. This order enables the second dimension (CZE) to analyze many fractions eluting from the HPLC column without creating a bottleneck at the final separation stage. For example, the effluent from a 15 min RPLC run is sampled 60 times by the CZE system employing a high speed optical injection system which may be thought of as an "inverse" injection method [35].

Briefly, the system is based on an argon ion laser. The samples are tagged with fluorescein isothiocyanate (FITC). Ninety-five percent of the beam's power from the laser is split into a gating beam, focused nearer the injection end of

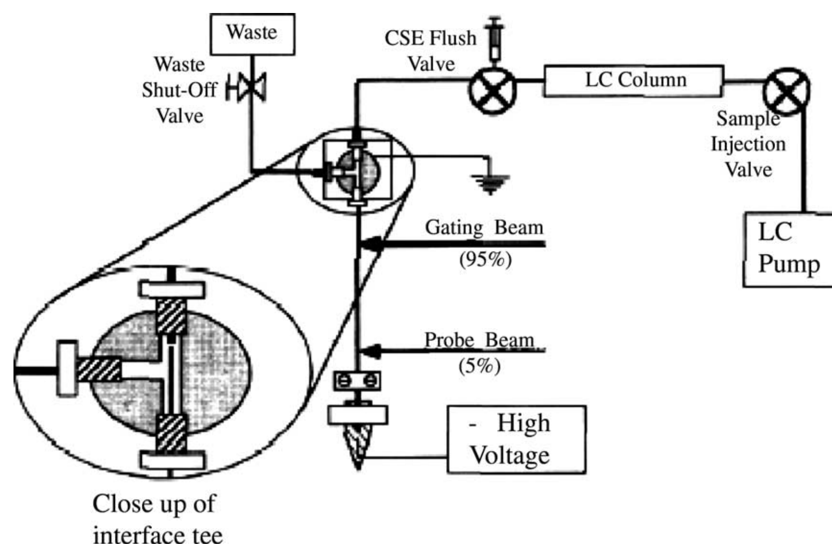


Fig. 3. Two-dimensional RPLC/fast-CZE instrumental diagram (reprinted with permission from [35]).

the capillary and a probe beam; and 5% of the laser power is focused nearer the exit end of the capillary. To make an injection, the gating beam is momentarily blocked with a computer-controlled shutter. This allows a small slug of material to pass through where its components are resolved and detected. The interface of the HPLC to the CZE is shown in (Fig. 3). This gating system was developed because mechanical injection methods are too slow for the fast CZE separation.

Issaq et al. [38,39] used a simple off-line RPLC/CZE system for separating a protein digest, whereby fractions eluting from the HPLC column were collected every 30 s, concentrated under vacuum, and analyzed by a single capillary CE instrument [38] and by a 96-array CE instrument [39] with LIF detection. The CE separation mode can be either CZE [38,39] for separation of peptides or capillary gel electrophoresis for the separation of proteins [39]. A 2-D plot of the sample concentration is then constructed, the dimensions being the LC and CZE elution times of the respective peptides. Intensity of the spots, i.e., quantification, was represented by different colors. The CZE array allowed the total HPLC/CE experiment to be completed within 1 h. He et al. [40] used a similar 2-D method to map cell extracts. In their study, the cell protein extracts were first separated, based on hydrophobicity using a C-4 RPLC column. Fractions from the RPLC system were collected into 96-well microtiter plates, dried under vacuum, and reconstituted with water. The fractions were then analyzed by multiplexed CZE and detected by UV absorption. Prior to analysis by CZE, the reconstituted fractions were concentrated on-column using large volume sample stacking with polarity switching. Recently, Janini et al. reported a simple interface for on-line CE-MS that was used as a second dimension to study peptide fractions from a SCX column [41]. Figeys et al. [42] has also coupled a solid phase extraction (SPE) device (C-18, 5 μm particles) to CE/ESI-MS-MS system.

5.3. Affinity/RP chromatography

Affinity chromatography is used to interrogate a specific subset of peptides, such as glycopeptides and phosphopeptides, from a proteome or protein digest. Regnier and coworkers [43–46] used affinity chromatography selection of peptides to reduce the complexity of the peptide mixture and showed that the identified peptides can act as analytical surrogates of their protein of origin. Fractions collected from the affinity column were separated by RPLC and identified by MS. They [43,44] used lectin affinity chromatography for the identification of glycoproteins in complex mixtures derived from either human blood serum or a cancer cell line. The process involved the following steps: (a) reduction and alkylation of the proteome sample; (b) proteolysis with trypsin; (c) affinity chromatographic selection of the glycopeptides with immobilized lectin; and (d) transfer of the glycopeptide fraction for analysis by on-line RPLC matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF)-MS. In another study from the same laboratory copper(II) immobilized affinity chromatography (Cu^{2+}-IMAC) in tandem with RPLC was applied to a yeast protein extract. After trypsin digestion, peptides rich in histidyl residues were selected, eluted from the IMAC column and resolved by RPLC. Concanavalin A affinity chromatography was used to extract glycopeptides from whole serum, the resultant mixture of glycopeptides was further analyzed by RP-HPLC/ESI-MS [47].

Recently, Hunt and coworkers [48] reported a multidimensional separation strategy that enables characterization of phosphopeptides from whole cell lysates in a single experiment. The proteins are digested with trypsin followed by esterification of carboxylic groups within the resulting peptides. Immobilized metal-affinity chromatography was used in the first separation dimension to enrich

for phosphopeptides, which were subsequently analyzed by nano-flow RPLC-MS. More than 1000 phosphopeptides were identified from the analysis of a whole-cell lysate of *Saccharomyces*.

5.4. Affinity chromatography/MEKC

Micellar electrokinetic chromatography (MEKC) is used for the separation of neutral as well as charged compounds by their partitioning into micelles. Amini et al. [49] investigated the selection and separation of histidine-containing peptides by Cu^{2+}-IMAC. Ovalbumin was chosen as a model protein for investigation of the selection and separation of histidine-containing peptides by IMAC off-line coupled with capillary electrophoresis and MALDI TOF-MS. Two of five histidine-containing peptides in addition to some non-histidine-containing peptides from tryptic digest of ovalbumin were captured by IMAC. To separate and purify the selected peptides, the IMAC sample was analyzed by CZE, however, the sample was not resolved by CZE, therefore, micellar electrokinetic chromatography (MEKC), using 10–75 mM SDS was used. Analysis of IMAC sample by MEKC using low concentrations of SDS (10 mM) was characterized by MALDI TOF-MS. When using SDS at 75 mM, the migration times of the RPLC fractions of the IMAC sample were used to identify the peaks.

5.5. IEF/RP chromatography

Isoelectric focusing is an excellent technique that has been successfully applied for the separation of proteins/peptides according to their p*I*. Xiao et al. [50] used a liquid phase IEF device in a novel ampholyte-free format for fractionation of a tryptically digested serum sample. The focusing cell, which is divided into 20 chambers by permeable membranes, was first filled with the serum sample, followed by a focusing step. After focusing, the entire peptide mixture is resolved into 20 fractions in the different chambers, which are collected into individual tubes. Following IEF, the individual peptide fractions were analyzed by μRPLC-MS/MS. The authors reported the detection of 957 peptides that corresponded to 477 proteins. In another study from our laboratory, intact proteins were separated by IEF, digested with trypsin, followed by separation using RP chromatography and the peptides identified by ESI-MS/MS as shown in Fig. 4.

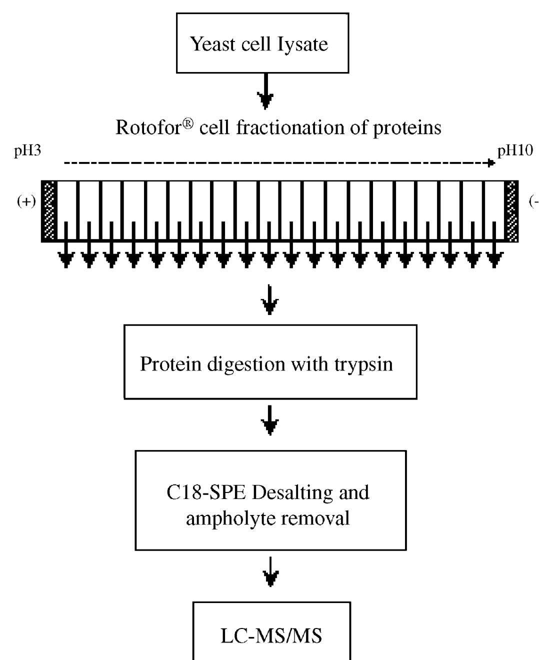


Fig. 4. Flow chart of a 2-D IEF/RP-HPLC strategy (reprinted with permission from [51]).

5.6. CIEF/CRP-HPLC

An integrated protein/peptide separation approach involving on-line combination of CIEF with μRPLC was developed to provide a means to significantly concentrate analytes (by a factor of ∼240) while having high resolving power [52,53]. The authors reported that CIEF in the first separation dimension offers a greater resolving power than that achieved using SCX chromatography, however, no comparative data was presented to support their claim. A schematic overview for the on-line integration of CIEF with CRP-HPLC as a concentrating and multidimensional separation platform is shown in Fig. 5. A 60-cm CIEF capillary (100 μm i.d./200 μm o.d.) was coated with hydroxypropyl cellulose for the elimination of electroosmotic flow and protein/peptide adsorption onto

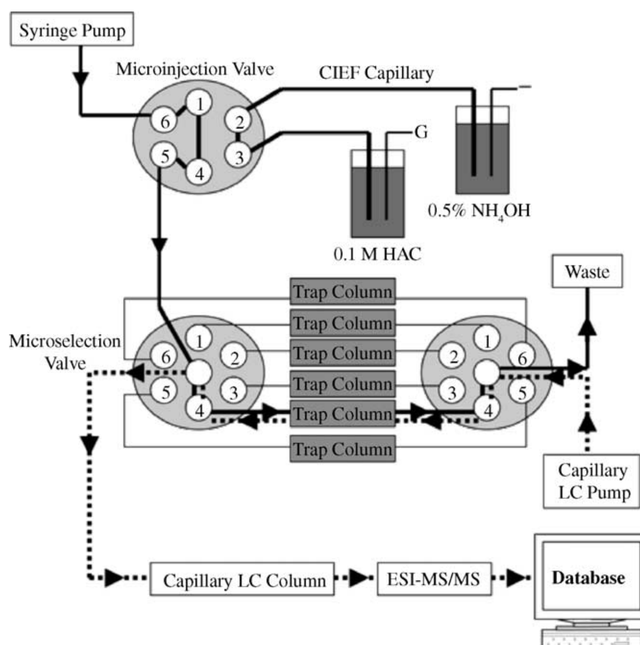


Fig. 5. Schematic of on-line integration of CIEF with CRPLC as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of CIEF fractions and the injection of fractions into a CRPLC column, respectively (reprinted with permission from [53]).

the capillary wall. The capillary was initially filled with a solution containing 2% Pharmalyte 3–10 and 2 mg/ml tryptic peptides obtained from the soluble fraction of yeast cell lysates. Ammonium hydroxide (0.5%, pH 10.5) and acetic acid (0.1 M, pH 2.5) were employed as the catholyte and the anolyte, respectively. Focusing electric field was 300 V/cm over the entire length of the CIEF capillary. The current decreased continuously as the result of analyte focusing. The focusing was considered to be complete when the current reached ~10% of the original value, usually within 30 min. The focused peptides were sequentially and hydrodynamically loaded into a 0.4 μl injection loop in a six-port microinjection valve. The loaded peptides were then injected into a C_{18} RP trap column through a six-port valve. Repeated peptide loadings and injections into various trap columns were carried out until the entire CIEF capillary content was sampled into a total of 12 unique fractions. A constant electric field of 300 V/cm was applied across the CIEF capillary for maintaining analyte band focusing in the capillary throughout the loading and injection procedures. Mass spectrometry was used for peptide detection.

5.7. CHPLC/CIEF

In the above section, CIEF was used as the first dimension to focus the peptides and CRPLC was used to resolve the peptides in each fraction. In a recent study [54], a comprehensive 2-D separation system, coupling capillary reverse-phase liquid chromatography to capillary isoelectric focusing is described for protein and peptide mapping. Since all sample fractions in CRPLC effluents could be transferred to the CIEF dimensions, the combination of the two high-efficiency separations resulted in maximal separation capabilities of each dimension. Separation effectiveness of this approach was demonstrated using complex protein/peptide samples, such as yeast cytosol and a BSA tryptic digest. A peak capacity of more than 10,000 had been achieved. A laser-induced fluorescence (LIF) detector, developed for this system, allowed for high-sensitive detection, with a fmol level of peptide detection for the BSA digest. The reason for employing RP-HPLC first, according to the authors, is that the RP material can act as a pretreatment and purification system for CIEF.

5.8. Gel filtration/HPLC

Small peptides, which are present in wine with other non-peptidic compounds, were first isolated by hollow fiber ultrafiltration by circulating the wine through a hollow fibre cartridge with a molecular mass cut-off of 3000. The wine permeate was then recirculated through eight organic membranes with molecular mass cut-off of 200–300. An 8 ml volume of each was applied to a glass column packed with Sephadex LH20 and eluted with 0.3 M sodium acetate, pH 4 at room temperature. Five-millilitre fractions were collected. Each of the sub-fractions was further resolved by HPLC using

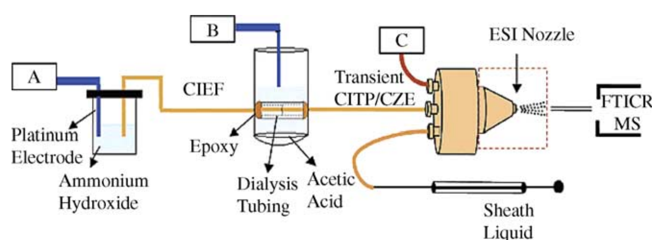


Fig. 6. Schematic of online coupling of CIEF-transient CITP/CZE with ESI-FTICR-MS using a microdialysis junction. Points A–C are either connected to ground or a high voltage power supply (reprinted with permission from Anal Chem. 75 (2003) 4432 [57]).

a porous graphitic carbon (Hypercarb) column using a 0.1% TFA/acetonitrile step-gradient. The authors reported the detection by UV absorption of nearly 60 peaks, which may contain one or several peptides. Fractions were collected and analyzed by capillary electrophoresis to determine their purity. Optimum CE resolution was achieved on a 50 cm long × 75 μm i.d. fused silica column, a 25 mM phosphate buffer, pH 2.5 and an applied voltage of 30 kV [55].

5.9. CE/CE methods

Transient isotachophoresis/zone electrophoresis coupled with nano-ESI-MS was used for the selective enrichment and identification of trace peptides in proteome analysis [56]. The speed of CITP/CZE separation and the lack of column equilibration make this a high throughput method. The CITP improves the detection of low abundance peptides by concentrating them into narrow bands. In another study, Lee and coworkers [57] developed a multidimensional

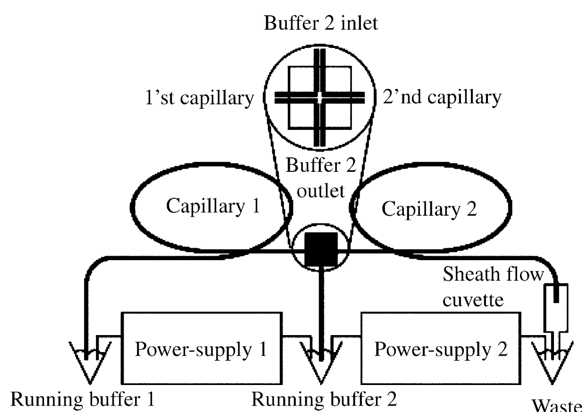


Fig. 7. Two-dimensional separation by capillary electrophoresis. The inlet of capillary 1 and buffer 2 outlet are placed in microcentrifuge tubes, which are filled with appropriate buffers. The outlet of capillary 1 and the inlet of capillary 2 are placed in an interface made from a piece of Lexan. Buffer 2 inlet is connected to a wash bottle held several cm above the waste vials. Flow from buffer 2 inlet is controlled with a low pressure valve (not shown). The outlet of capillary 2 is placed within a sheath-flow cuvette for post-column fluorescence detection. The waste outlet of the cuvette is placed at the same height as the running buffer 1 vial and the running buffer 2 outlet vial to prevent formation of a siphon through the separation capillaries (reprinted with permission from [58]).

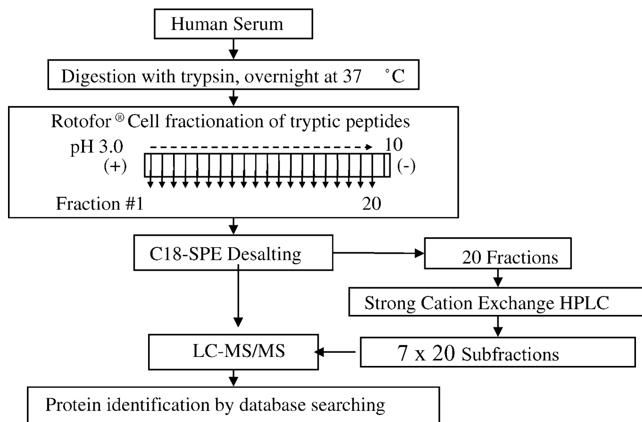


Fig. 8. Schematic of a global analysis procedure of serum proteome (reprinted with permission from [63]).

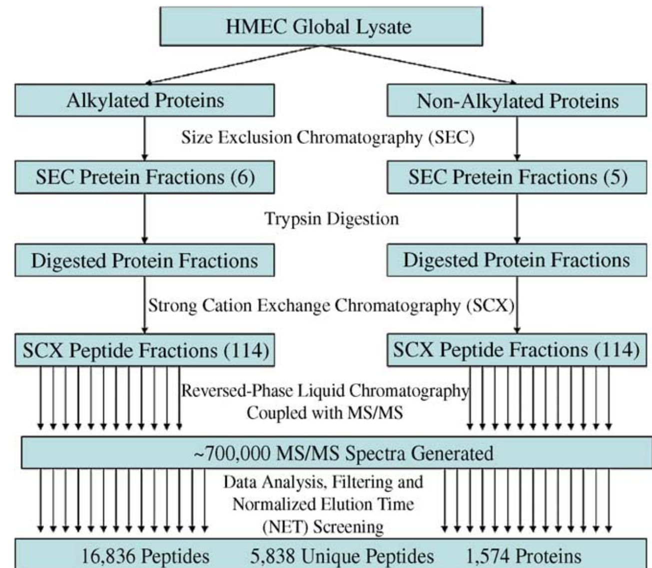


Fig. 10. Schematic diagram of sample separation and analysis. Shown is the flow of experimental information starting from cell lysate through data analysis. Two HMEC global protein samples were prepared, either alkylated or nonalkylated, and were subjected to size exclusion chromatography, separate tryptic digestion of each SEC fraction, and strong cation exchange chromatography. The subsequent 114 peptide fractions were then analyzed via reversed-phase liquid chromatography coupled with MS/MS, resulting in spectra that were analyzed using SEQUEST (reprinted with permission from [66]).

electrokinetic-based separation/concentration platform coupled with electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) for achieving the high resolution and ultrasensitive analysis of complex protein/peptide mixtures (Fig. 6). A microdialysis junction is employed as the interface for on-line combination of capillary isoelectric focusing (CIEF) with transient capillary isotachophoresis/zone electrophoresis (CITP/CZE) in an integrated platform. The excellent resolving power afforded by both CIEF and CZE separations, the electrokinetic focusing/stacking effects of CIEF and CITP greatly enhanced the dynamic range and detection sensitivity of MS for protein identification. The constructed multidimensional separation/concentration platform was used for the analysis of *Shewanella oneidensis* proteome. The electrokinetic-based platform offered the overall peak capacity comparable to those obtained using multidimensional chromatography systems, but with a much shorter run time and no need for column regeneration. A total of 1174 unique proteins were identified using this 2-D approach.

Dovichi and coworkers reported on two-dimensional CE system for automated protein analysis [58]. In the system (Fig. 7), the labeled proteins are analyzed by submicellar capillary electrophoresis at pH 7.5 to perform a first dimension separation. Once the first components migrate from the capillary, a fraction is transferred to a second dimension capillary, where electrophoresis is performed at pH 11.1 to further separate the proteins. Successive fractions are transferred from the first dimension capillary to the second dimension capillary for further separation to generate, in serial fashion, a two-dimensional electropherogram. The transfer of fractions is computer-controlled. Zeptomoles of labeled proteins were detected [58]. An on-line 2-D CE system consisting of CIEF

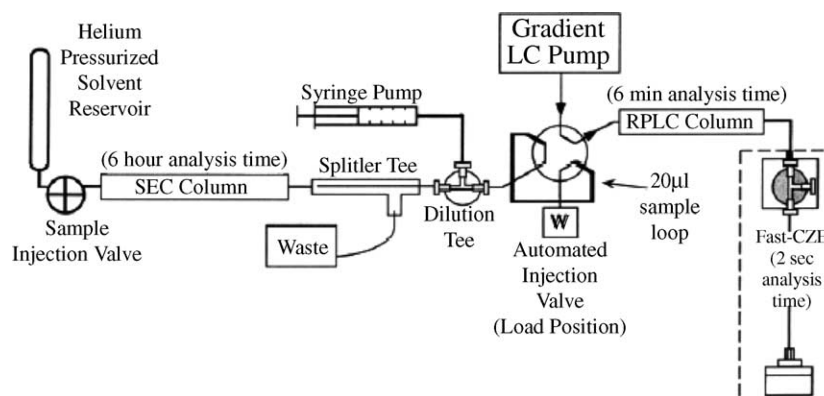


Fig. 9. Schematic diagram of 3-D SEC/RPLC/fast-CZE (reprinted with permission from [36]).

and capillary gel electrophoresis (CGE) was introduced. A dialysis interface was developed by mounting a hollow fiber on a methacrylate resin plate to hyphenate the two CE modes [59]. The two dimensions of capillary shared a cathode fixated into a reservoir in the methacrylate plate; thus, with three electrodes and only one high-voltage source, a 2-D CE framework was successfully established. A practical 2-D CIEF-CGE experiment was carried out with a target protein, hemoglobin (Hb). After the Hb variants with different isoelectric points (p*I*s) were focused in separate bands in the first-dimension capillary, they were each chemically mobilized to the second-dimension capillary for further separation in polyacrylamide gel where a single CIEF band was resolved into several peaks. Two-dimensional CE on a chip studies were recently reported [60,61].

6. Three-dimensional approaches [62–69]

In cases where 2-D approaches do not give satisfactory results, a third orthogonal separation procedure is used which would result in the resolution of a larger number of peptides. For example, comparison of results from our laboratory for the separation of human serum protein digest resulted in 951 peptides detected using 2-D IEF/RP [50] while a 3-D IEF/SCX/RP procedure of the same serum resulted

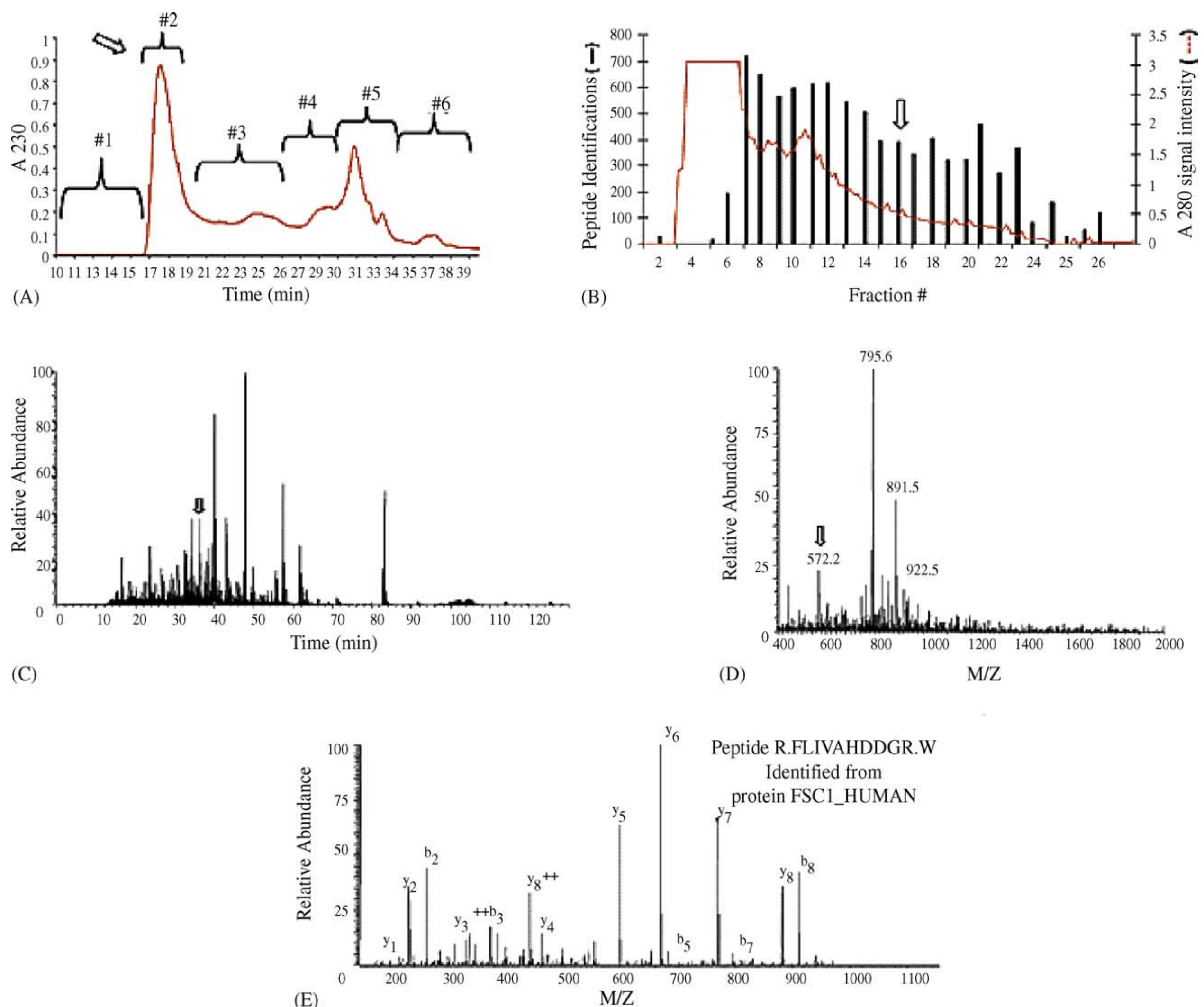


Fig. 11. Separation chromatograms and LC-MS/MS analysis. (A) Size exclusion chromatography (SEC) separation of nonalkylated HMEC cell lysate. Isocratic elution of the proteins was observed using A_{280} with brackets representing the collected fractions. A total of six fractions were collected. Fraction 1 was discarded due to lack of protein detected. Protein separation of the alkylated sample was similar, but with a total of seven fractions collected. (B) Strong cation exchange (SCX) chromatography of tryptically digested SEC fraction 2. The total number of peptide identifications for each peptide fraction is shown as a bar superimposed over the SCX absorbance. (C) Reversed-phase LC-MS/MS analysis of SCX peptide fraction 16. The base peak chromatogram represents gradient elution of 10%–60% acetonitrile over time. (D) MS scan taken at time point 35.62 min during RP LC-MS/MS analysis. Three peaks are selected for each MS scan for further identification via collision induced dissociation. (E) MS/MS scan of parent ion *m*/*z* 572.2. All major peaks have been labeled as either b- or y-ions indicating that the parent ion is the fully tryptic peptide R.FLIVAHDDGR.W, originating from protein FSC1_HUMAN (reprinted with permission from [66]).

in the identification of 2071 peptides, as described below [62].

6.1. IEF/SCX/RP

A comprehensive analysis of the serum proteome requires effective fractionation and instrumental analysis approaches. Chan et al. [63] employed a multidimensional peptide separation strategy combined with tandem mass spectrometry (MS/MS) for the identification of proteins in human serum. After enzymatically digesting serum with trypsin, the peptides were fractionated using liquid phase isoelectric focusing (IEF) in a novel ampholyte-free format. The 20 IEF fractions were separated by SCX chromatography into seven subfractions each, collected and analyzed by RP-HPLC ESI-MS/MS. It was reported that 2071 peptides were detected which resulted in the identification of 1143 unique proteins. A schematic of the procedure is presented in Fig. 8. This three-dimensional approach resulted in the identification of three times as much proteins as a 2-D IEF/HPLC procedure (1143 proteins versus 477 proteins).

6.2. RP/SCX/RP

McDonald et al. [64] compared three on-line coupled HPLC MS/MS strategies: single RP-HPLC, two-phase MudPIT (SCX-RP), and three-phase MudPIT (RP-SCX-RP), for the separation of peptides in a complex protein digest. The three-phase column is a variation of the original two-phase MudPIT column (Fig. 10) with an additional RP material behind the SCX material. This three-phase column can be used in addition to fractionation and separation for "on-line" desalting of the sample. They reported that the three-phase MudPIT column yielded a greater number, 431, of peptide identifications versus 147 and 341 for the 1-D and 2-D MudPIT systems, respectively. A similar off-line approach was employed by Atwood et al. [65] to resolve the peptides in the *Trypanosoma cruzi* proteome digest. After digestion of the proteins, peptides were resolved on a reversed-phase column based on their hydrophobicity. Nine fractions were collected using a gradient of 5–45% acetonitrile; after which, each fraction was separated by SCX into eight sub-fractions. Peptides in each sub-fraction were then resolved by RP chromatography and detected by MS/MS.

6.3. Size exclusion/RP/CZE

Moore and Jorgenson [36] developed a comprehensive three-dimensional peptide separation method based on molecular size, hydrophobicity, and electrophoretic mobility. Size exclusion chromatography (SEC) is used as the first dimension to give a rough separation of the peptides based on their molecular size. The SEC effluent is repetitively sampled on-line into a rapid 2-D RP-HPLC/CZE system with an analysis time of 7 min (Fig. 9). Analysis of the data from this system consisted of a series of 2-D "slices" of the SEC effluent, which when stacked together gave the 3-D separation "volume". The method required the coupling of three columns took 102 h and resulted in 6000 peaks. Such comprehensive approach may be used to compare protein expression levels from two different states, healthy and diseased.

6.4. SEC/SCX/RP

In this approach peptides (proteins) are resolved according to their molecular size, ionic charge and hydrophobicity. Jacobs et al. [66] fractionated intact proteins from human mammary epithelial cells (HMEC) first by SEC. The six protein fractions were separately digested using trypsin, and the resulting tryptic peptides further separated using SCX (Fig. 10), yielding a total of 114 peptide fractions. Each fraction was then analyzed by RPLC coupled with electrospray ionization tandem mass spectrometry Fig. 11. The combined approach resulted in a total of 5838 unique peptides identified covering 1574 different proteins.

6.5. Affinity/SCX/RP

Affinity chromatography is used to select a group of peptides and by doing so simplify a complex peptide mixture. Such an approach based on the use of isotope coded affinity tags (ICAT) was used not only to simplify the proteome mixture of *S. cerevisiea* [67] and human myeloid leukemia (HL-60) cells [68] but for the accurate quantification of cysteinyl residue containing proteins. In this strategy, cysteinyl residues within proteins are modified with a thiol reactive

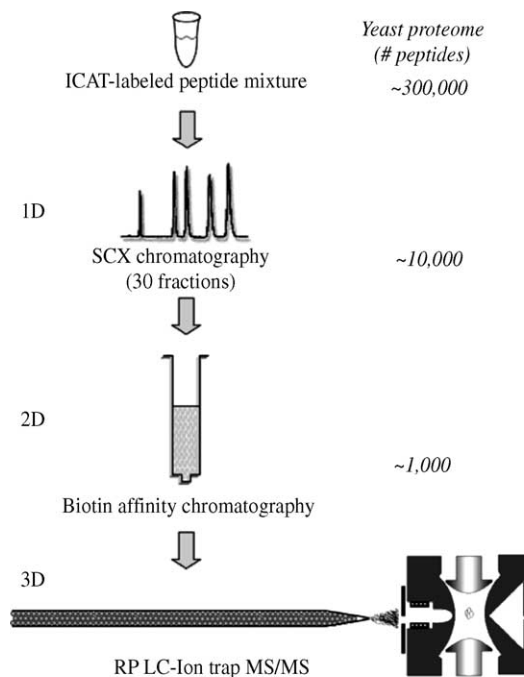


Fig. 12. Proteome analysis using a combination of the isotope coded affinity tag (ICAT) strategy and multidimensional chromatography (reprinted with permission from [69]).

reagent that contains a biotin moiety. The proteins are first denatured, reduced, labeled at cystienyl residues with the ICAT reagent, and enzymatically digested. The modified peptides are recovered using immobilized avidin chromatography. Although the main purpose of affinity-isolating only the cysteinyl-containing peptides is to reduce the complexity of the proteome sample, an ICAT labeled proteome digest sample is still quite complex. Therefore, most investigators employing ICAT reagents use a combination of SCX and RPLC-MS/MS for the identification and quantitation of the modified peptides. In a recent publication [69], whole cell proteins were first denatured, reduced, labeled at cysteine residues with ICAT strategy, and digested by trypsin. SCX was first used to fractionate the ICAT-labeled complex peptide mixture after which each collected fraction was passed over an avidin column to isolate only cysteine containing peptides. The resulting mixture of ICAT-labeled peptides was then resolved by RP chromatography and analyzed by MS/MS (Fig. 12).

7. Concluding remarks

It is clear from the above discussion and review of the scientific literature that separation science, chromatography, and electrophoresis give the analyst a wide array of options that can be used to resolve a complex mixture of peptides employing 2-D and 3-D separation strategies. It is also abundantly clear that no single chromatographic or electrophoretic procedure is capable of resolving the complex mixture of peptides that results from a global digest of a proteome. Combining two or more orthogonal separation procedures dramatically improves the overall resolution and results in a larger number of peptides being identified. In conclusion, multidimensional separation of peptides combined with mass spectrometry is an important aspect of proteomic research.

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