A previous article surveyed the separation technologies currently used in proteomics studies.¹ I noted that two-dimensional (2D) gel electrophoresis is the core separation technique for proteomics. However, the importance of proteomics studies in drug discovery necessitates the use of techniques that can process large numbers of samples in a reasonable time frame. 2D gel electrophoresis does not lend itself easily to this application. Even with the improvements in 2D gel electrophoresis during the past several years, it is still labour-intensive and requires at least two days to process a set of gels. Although some steps in gel handling can be partially or completely automated, loading the first-dimension isoelectric focusing gel, transferring the immobilized pH gradient strip to the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, and staining and destaining requires considerable hands-on time. Finally, the sample must be electroeluted or blotted from the gel for subsequent steps. A more fundamental limitation of 2D gel electrophoresis is its failure to display the entire protein complement of the sample on the gel surface. Large proteins and hydrophobic proteins such as membrane proteins have difficulty entering the gel. Acidic proteins (pI <4) and basic proteins (pI >9) are poorly resolved. Most importantly, the levels of low-copy-number proteins present in low abundance are below the detection limit of silver staining techniques. This limitation is probably the most critical because many of the regulatory proteins that might be involved in the disease process and are candidates for therapeutic intervention are present in the cell in low concentrations. The gel’s modest sample capacity prevents the loading of sufficient protein to be able to visualize low-abundance proteins without having them masked by high-abundance proteins.

Liquid Chromatography: Many Advantages but Some Limitations

Chromatographic techniques have several advantages that make them desirable for sample prefraction before one-dimensional (1D) or 2D gel electrophoresis, or to replace gel electrophoresis altogether. The high capacities of modern high performance liquid chromatography (HPLC) packings are compatible with high sample loads for preparative isolation. If samples are introduced in a weak solvent relative to the initial mobile-phase conditions, analysts can concentrate large volumes on-column with no loss in resolution. HPLC is an instrumental technique with full automation of sample injection, separation, detection and fraction collection.

The flexibility in selecting from a variety of separation chemistries improves the success rate for recovering classes of proteins that are difficult to handle in gel electrophoresis. Reversed-phase, hydrophobic interaction and hydrophilic interaction chromatography are general techniques for separating polypeptides based upon their hydrophobic or polar characteristics. Cation-exchange chromatography can be used for basic proteins and anion-exchange chromatography is useful for analysing acidic proteins. Size-exclusion chromatography can serve as a prefractionation technique before any interactive chromatographic mode. Immobilized metal affinity chromatography can isolate polypeptides based upon the presence of histidine residues or phosphate groups. Affinity chromatography can isolate a specific protein or class of proteins or remove high-abundance housekeeping proteins from a sample (e.g., to remove immunoglobulins using a protein A column or albumins using a Cibacron blue column).
In gel electrophoresis, users can analyse multiple samples in parallel on a single 1D gel and can process multiple 2D gels in parallel in multiple-position electrophoresis tanks and destaining stations. In contrast, a serial technique such as column chromatography is restricted to one-at-a-time sample analysis. Chromatography’s throughput limitation is somewhat offset by the ability to automate the entire process. However, if analysts use chromatography as the sole separation technique to isolate proteins before mass spectrometry (MS) analysis, then the pI and molecular weight information obtained in the course of 1D or 2D gel separations is unavailable for use in database searches.

**Why Multidimensional Liquid Chromatography?**

The initial characterization of the human genome sequence revealed that it contains no more than 30 000–70 000 open reading frames. However, the human proteome is estimated to contain from 100 000 to 2 million proteins, of which 10 000–20 000 are expressed at any given time. In a typical experiment in expression proteomics, the intention is to characterize the entire complement of proteins expressed in a cell or tissue under a defined metabolic state. To do this task, analysts resolve proteins from each other and cleave them with a proteolytic enzyme such as trypsin. The cleavage products can be analysed directly by MS or, more typically, separated chromatographically and identified by MS and MS–MS (Figure 1). Because trypsin typically yields approximately 20 peptides per protein, the resulting mixture can contain 200 000–500 000 cleavage products. The separation power of a chromatographic system can be characterized by its peak capacity; for example, the number of peaks that can be resolved to the baseline. The most powerful chromatography column technique — gradient reversed-phase HPLC — has a theoretical peak capacity of approximately 200. The actual separation performance will be much more modest, approximately 50–100 components. Even the best single-mode chromatographic separation cannot provide sufficient resolution for proteomic studies of this type; therefore, many protein chemists are coupling two or more separation modes to achieve adequate resolution of complex peptide mixtures.

The beauty of 2D gel electrophoresis as a separation technique is the orthogonality of the two separation dimensions — separation by charge (isoelectric point) in the first dimension and separation by size in the second dimension. For a chromatographic approach to provide comparable separation power, users should select two modes that have different separation selectivities. Coupling ion-exchange and reversed-phase chromatography as the first and second dimensions, respectively, has three appealing advantages. The first advantage is that the two modes have complementary selectivities that are similar to those of 2D gel electrophoresis. Ion-exchange separation is roughly equivalent to isoelectric focusing. Reversed-phase chromatography provides a quasi-molecular size separation in which retention tends to increase with increasing molecular weight. Therefore, the resolving power of the coupled modes will approximate the product of the peak capacities of the individual modes. The second advantage of this approach to multidimensional liquid chromatography (LC) is the solvent compatibility of the two modes when ion-exchange chromatography is used as the first dimension. The elution solvents for ion-exchange chromatography (aqueous buffers with varying concentrations of a neutral salt) are weak solvents for reversed-phase chromatography. The third advantage is the compatibility of reversed-phase chromatography elution solvents (water and organic solvents with volatile organic acid additives) with electrospray and matrix-assisted laser-desorption ionization (MALDI) systems.

**Approaches to Multidimensional LC**

The literature abounds with descriptions of multidimensional LC methods. Special considerations in designing a proteomics multidimensional LC system include the requirement for gradient elution in all dimensions, the huge complexity of samples, the fact that virtually every component in the sample is an analyte and the requirement for MS-compatible solvents in the final dimension. In contrast, using MS detection relaxes the constraints on LC resolution because additional separation occurs in the mass domain.

Researchers have used several strategies in multidimensional LC separations for proteomics studies. These strategies include discontinuous off-line or on-line multidimensional LC using fraction collection, on-line direct coupling of two dimensions and on-line multidimensional LC using column switching. In the following discussion, I will provide an example of each of these approaches to proteomics.

**Discontinuous multidimensional LC using fraction collection:** The simplest and most straightforward approach to multidimensional LC is fraction collection followed by the injection of the individual fractions onto the second-dimension column. Aebersold’s group has used this approach for analysing the yeast proteome using isotope-coded affinity tags. In this technique, the authors reacted cysteine residues with a reagent that bore a sulphhydril-specific group, a biotin affinity group and a linker that could be labelled isotopically for differential detection by MS. They grew cell populations using galactose or ethanol as the carbon source. They harvested proteins from each cell population and tagged their cysteinyl residues with heavy or light isotope-coded affinity tag reagents, respectively. The two tagged protein extracts were mixed and subjected to tryptic digestion and the tagged peptide cleavage products were isolated using avidin affinity chromatography. They chromatographed the recovered peptides on a strong-cation-exchange column using a potassium chloride gradient with a constant 25% acetonitrile concentration. They collected 30 fractions, of which four were selected for analysis by capillary reversed-phase LC–electrospray ionization MS and MS–MS. The incorporation of the affinity step reduced the complexity of the peptide mixture by approximately one order of magnitude but because most proteins contain at least one cysteine residue, the peptide population represented approximately 92% of the yeast proteome. The off-line multidimensional LC approach has several advantages. The strong-cation-exchange column has sufficient loading capacity to permit the recovery of both high- and low-abundance proteins. This capability was evident by the identification of many proteins that are known to be expressed at low levels in yeast. In addition, the off-line strong-cation-
exchange separation effectively removes the detergents and chaotropic agents that are used in the reduction and alkylation steps and would cause contamination and ion suppression in the capillary LC–electrospray ionization MS system.

The disadvantage of discontinuous multidimensional LC with fraction collection is the necessity of operator intervention to collect and reinject fractions. This limitation can be circumvented by coupling the two dimensions using a multiple-solvent delivery HPLC system equipped with an automatic fraction collector and a column-switching valve. With this modification, fractions are collected across the elution gradient of the first-dimension separation. The column-switching valve is rotated to put the second-dimension column in line and collected fractions are analysed in sequence. Several HPLC instrument manufacturers now make automated 2D systems using ion exchange (or chromato-focusing, in one instance) as the first dimension and reversed-phase chromatography as the second dimension.

**Directly coupled–column multidimensional LC:** In this approach, analysts couple two columns with orthogonal separation selectivities in tandem and fractions are eluted from the first column with a series of pulsed steps of increasing eluent strength. Each step is followed by a continuous gradient elution from the second column, which can be interfaced directly with an electrospray ionization MS–MS system. An example of this approach is the multidimensional protein identification technology (MuDPT) developed by Yates’ group. In this system, the researchers packed a 140 × 0.1 mm fused-silica capillary with both ion-exchange and reversed-phase particles. A 40 mm segment at the capillary inlet was packed with 5 µm particles that carried a strong-cation-exchange phase and the distal 100 mm was packed with 5 µm C18 particles. After sample introduction, analytes were eluted from the tandem system with a complex 15-step elution protocol (Figure 2). The protocol included an initial reversed-phase gradient elution followed by a series of salt step elutions with increasing ionic strength, each followed by a reversed-phase gradient. They identified peptides by on-line electrospray ionization–ion-trap MS–MS using shotgun sequence analysis.

Washburn and co-workers evaluated the multidimensional protein identification technology approach by analysing a lysate of the yeast *Saccharomyces cerevisiae*. A database search of the multidimensional protein identification technology–generated mass spectra enabled the assignment of 5540 peptides to 1484 proteins in the yeast proteome (this number represents approximately 24% of the 6139 open reading frames in the yeast genome). Analysis of the identified proteins indicated that they provided a representative sampling of the yeast proteome (Figure 3), including classes of proteins that are difficult to detect using 2D gels. As demonstrated in this distribution, identified proteins included those with extremes of isoelectric points (pI < 4.3 and pI > 10), low molecular weight (MW < 10 kDa), high molecular weight (MW > 180 kDa) and both peripheral and integral membrane proteins. The codon adaptation index is a measure of the level of protein expression and proteins with low codon adaptation index values are low-abundance species. The significant proportion of low codon adaptation index proteins identified in this study indicated that the multidimensional protein identification technology method, unlike 2D gel electrophoresis, has no inherent bias against low-copy-number proteins.

Instrument vendors have demonstrated directly coupled–column multidimensional LC on commercial systems. A typical approach uses on-line coupled strong-cation-exchange and reversed-phase columns. After loading a sample on the strong-cation-exchange column, users can elute peptides with incremental injections of a volatile salt of increasing ionic strength using an injector programme. At each salt pulse, the eluted peptides are captured on an in-line capillary reversed-phase column and resolved with a water–acetonitrile and 0.1% formic acid gradient.

**Multidimensional LC using column switching:** Multidimensional LC systems using column-switching techniques expand the flexibility of multidimensional LC at the expense of greater complexity.

Davis and colleagues developed a relatively simple 2D LC–MS system using binary ion-exchange separation coupled with gradient reversed-phase chromatography. This system comprised a strong-cation-exchange column, a reversed-phase trapping column and a reversed-phase analytical column (Figure 4). Samples injected onto the 50 × 2 mm strong-cation-exchange column were resolved into unbound flowthrough and bound fractions. The flowthrough fraction was eluted using a low-ionic-strength loading buffer (5 mM ammonium formate and 5% acetonitrile [pH 3.8]) delivered by a syringe pump. The unbound peptides were captured on the 50 × 2 mm C18 reversed-phase trapping column. These unbound peptides were backflushed from the trapping column onto the 250 × 1 mm C18 analytical column and separated using a slow 185 min water–acetonitrile and formic acid gradient. Bound peptides were then eluted from the strong-cation-exchange column with a 250 µL injection of 0.5 M salt. The bound fraction peptides were captured on the trapping column and eluted onto the analytical column with the same regime used for the flowthrough fraction. The simple binary first-dimension fractionation relaxed the time constraints on...
data-dependent MS–MS analysis of the peptides that were resolved in the second dimension and it was the preferred approach for the authors’ high-throughput environment.

A more intricate 2D LC system developed by Jorgenson’s group\(^8\)\(^9\) used size-exclusion chromatography as the first dimension and reversed-phase LC as the second dimension (Figure 5). Two versions of this system have been described, one for the separation of complex peptide mixtures\(^8\) and the other for protein separations.\(^9\) The peptide system coupled six 300 × 7.8 mm, 5 µm \(d_p\) silica-based size-exclusion columns (125 Å pores) in series for the first-dimension separation. These columns had a combined exclusion volume of 33.6 mL and a total permeation volume of 67.2 mL. The size-exclusion chromatography column set was connected by a four-port valve to one of two reversed-phase columns plumbed in parallel. The 33 × 4.6 mm reversed-phase columns were packed with 1.5 µm non-porous C18 particles. After sample injection, the size-exclusion chromatography columns were eluted at 1 mL/min for the first 40 min to elute the void volume. The flow was then reduced to 100 µL/min as the peptides were eluted from the first dimension. The researchers introduced 4 min elution segments (400 µL) in alternating fashion onto each of the two reversed-phase columns. While peptides were being trapped on one column, the previous segment was eluted from the other column using a 3 min water–acetonitrile and 0.1% trifluoroacetic acid gradient. They connected the outlets of the second-dimension columns to a second four-port valve that directed eluted peptides to in-line UV and electrospray ionization MS detectors and diverted the size-exclusion chromatography loading solvent to waste. Because the size-exclusion chromatography eluent (aqueous 0.1% trifluoroacetic acid) contained no organic modifier, the peptides eluted from the first dimension were focused on the head of the reversed-phase column during loading, which provided peak sharpening in the second dimension. They selected gradient timing in the second dimension (3 min gradient and 1 min regeneration) to obtain at least two 2D analyses for each first-dimension peak.

They evaluated the 2D peptide system with tryptic digests of ovalbumin and bovine serum albumin. In both instances, the peptides identified using 2D LC–MS accounted for 90% of the protein sequence.

To adapt the 2D size-exclusion chromatography–reversed-phase LC strategy for analysing complex protein mixtures, the researchers modified the system in both dimensions. In the first dimension, the six 125 Å-pore diameter size-exclusion chromatography columns were replaced by 12 size-exclusion chromatography columns, six with 250 Å pores and six with 450 Å pores. This column set should have an approximate fractionation range of 10–1000 KDa and a total efficiency in excess of 100 000 theoretical plates. The first-dimension column set was eluted at 150 µL/min. They used two 100 × 2.1 mm reversed-phase columns packed with poly(styrene–divinylbenzene) for the second dimension. The second-dimension valve-switching and gradient-elution protocols were essentially identical to those of the 2D peptide system. In contrast to the peptide system, the protein system used only an in-line UV detector; the fractions were collected and analysed off-line for protein mass using MALDI–time-of-flight (TOF) and electrospray ionization MS. In situations in which sequence information was required to establish protein identity, they performed N-terminal Edman sequencing. The 2D protein system was evaluated with an *E. coli* lysate; they observed approximately 250 spots in a 2D gel-like presentation of size-exclusion chromatography elution time versus reversed-phase elution time versus UV intensity. The 14 most intense of these were identified using mass analysis by MALDI-TOF MS and Edman N-terminal sequence analysis.

This size-exclusion chromatography–reversed-phase LC 2D system has two inherent limitations. First, the rapid gradient elution in the second dimension limits the amount of information that can be obtained by on-line LC–MS. Many current proteomics studies rely upon data-dependent acquisition of mass and sequence information by MS and MS–MS for protein identification. The 3 min gradient cycles

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**Figure 2: Multidimensional protein identification technology elution scheme.**

![Multidimensional protein identification technology elution scheme](image-url)
might not permit sufficient time for the multiple scanning and fragmentation cycles required by this approach. Second, a more fundamental limitation is the lack of true orthogonality of separation selectivity between the two dimensions. The second-dimension reversed-phase separation is a quasi-molecular weight dimension because small proteins tend to be hydrophilic and are eluted early, but large proteins tend to have greater hydrophobic contact area and are eluted later. This lack of full orthogonality is evident in the gel-like presentations of the separated spots — the spots are clustered along a diagonal line with most of the area devoid of signal. This result probably helps to account for the authors’ observation that approximately 30% of the theoretical peak capacity of the 2D system exhibited spot occupancy.

Unger and co-workers\(^\text{10}\) have developed an on-line automated multidimensional HPLC system that includes integrated sample preparation (Figure 6). Their sample was initially fractionated by size using an on-line restricted-access media column that contained ion-exchange groups bonded to the pore system and diol groups bonded to the outer silica surface. Proteins larger than 20 kDa were excluded from the ion-exchange phase and eluted to waste. Smaller proteins were retained and enriched by interaction with the internal ion-exchange phase. These proteins were then gradient eluted onto the 2D analytical system. The first dimension was an ion-exchange column packed with 2.5 µm non-porous polymer beads that carried strong-cation-exchange (sulphonic acid) or weak-anion-exchange (diethylamino ethyl) functionalities. They accomplished elution from the first dimension using a continuous 96 min linear gradient from 0.01 to 1 M phosphate (pH 3 for cation exchange, pH 7 for anion exchange). The second dimension comprised water–acetonitrile and 0.1% trifluoroacetic acid gradients performed on four parallel 14 mm × 4.6 mm reversed-phase columns packed with 1.5 µm non-porous C18-modified silica.

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**Figure 3:** Multidimensional protein identification technology analysis of the yeast proteome. (Adapted from reference 6 with permission.)

**Figure 4:** Multidimensional LC–MS platform using a binary ion-exchange fractionation in the first dimension. The ion-exchange chromatography buffer was recycled while the LC–MS–MS analyses were being executed. (Adapted from reference 7 with permission.)

**Figure 5:** Schematic of a 2D LC system using size-exclusion chromatography columns connected in series as the first dimension and paired reversed-phase columns operating in alternating duty cycles as the second dimension. (Adapted from reference 9 with permission.)
During the first-dimension ion-exchange separation, they transferred twenty-four 4 min fractions to the second-dimension reversed-phase columns. Each of the four columns was cycled through sample introduction, elution and regeneration steps. Analytes from two of the four columns were eluted in parallel using two gradient elution systems and the remaining two columns were occupied in sample-introduction and regeneration steps, respectively. During the sample-introduction step, they focused a 2 mL fraction eluted from the first-dimension ion-exchange gradient on the head of the reversed-phase column and desalted it before initiating the second-dimension elution gradient. The solvent and sample flow in the 2D system was directed by a set of four automated 10-port valves. The researchers applied the system to the separation of proteins in a human haemofiltrate and a human foetal fibroblast cell lysate. They resolved more than 1000 components in these complex samples within the 96 min analysis time. Using high-capacity restricted-access media columns for on-line fractionation enabled the concentration of the low molecular weight components, and the non-porous materials used in the analytical columns provided fast mass transfer kinetics and high protein recoveries.

**Summary**

Scientists have proposed various two-dimensional LC systems for proteomics studies to complement or replace gel
electrophoresis techniques. Some of these systems are designed to achieve comprehensive separations of the extremely complex peptide mixtures derived from proteolysis of complete proteomes, and others are configured for resolving intact proteins. Researchers have designed systems for particular tasks such as high-throughput studies or small protein analysis. Chromatographic approaches have eliminated some of the disadvantages of gel electrophoresis. LC has the capacity to detect low-copy proteins on a background of high-abundance species. Acidic or basic proteins, hydrophobic proteins and very small and very large proteins, which are not easily observed on 2D gels, can be detected by multidimensional LC techniques.

A major advantage of multidimensional LC for proteomic studies in industrial environments is its ability to fully automate multidimensional LC–MS–MS–MS systems. However, the complete resolution of all components of a mammalian proteome and quantitative measure of protein expression remains a challenge. This task will certainly require multidimensional LC^n systems in which n is greater than 2. Scientists are currently using a strategy that combines affinity capture techniques with conventional LC modes.

References

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