ELSEVIER

Journal of Chromatography B, 752 (2001) 281–291

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Automated LC–LC–MS–MS platform using binary ion-exchange and gradient reversed-phase chromatography for improved proteomic analyses

Michael T. Davis[a,*], Jill Beierle[a], Edward T. Bures[a], Michael D. McGinley[a], Jessica Mort[a], John H. Robinson[a], Chris S. Spahr[a], Wen Yu[a], Roland Luethy[a], Scott D. Patterson[b]

[a]*Departments of Biochemistry and Computational Biology, Amgen, Inc., Thousand Oaks, CA 91320, USA*
[b]*Celeva Genomics, Rockville, MD 20850, USA*

Abstract

A simple multidimensional liquid chromatography system utilizing an isocratic pump and a HPLC system is described for the comprehensive proteomic analysis of complex peptide digest mixtures by coupled LC–LC–MS–MS techniques. A binary ion-exchange separation was achieved through the use of a strong cation-exchange column followed by a reversed-phase column for data-dependent LC–MS–MS analysis of the unbound analytes, and following salt elution (and concomitant column reequilibration), the bound analytes. Off-line validation of the platform showed near quantitative recovery of fractionated peptides and essentially complete ion-exchange partitioning. In comparative analyses of a highly complex peptide digest mixture a >40% increase in the number of peptide and protein identifications was achieved using this multidimensional platform compared to an unfractionated control.

Keywords: Proteomics

1. Introduction

Chromatography-based methods for the analysis of components of complex protein mixtures have provided an alternative approach for proteomic analyses [1–6]. The protein components of these biological fluids are identified following enzymatic digestion of the protein mixture that is fractionated by reversed-phase high-performance liquid chromatography (HPLC) coupled on-line to mass spectrometry (MS) capable of data-dependent MS to produce fragment ion (MS–MS) spectra from as many components as possible. These spectra are then subjected to uninterpreted database search algorithms that match the MS–MS spectra to amino acid sequence (including translated nucleotide sequences) (e.g., Refs. [7–9]). This approach utilizes the efficiency of gas-phase ion separation in conjunction with hydrophobic reversed-phase liquid chromatography (RPLC) fractionation to achieve a high-resolution separation of the components. Instrumental parameters for the subsequent LC–MS–MS analysis of these mixtures have been optimized to generate the maximum number of quality MS–MS spectra from each sample however not all candidate ions are analyzed [10]. To increase the number of precursor ions selected for fragmentation optimization of the gas-phase separation and

*Corresponding author. Tel.: +1-805-447-2353; fax: +1-805-499-7464.

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00547-8

selection of precursor ions using iterative analyses over incremental survey scan mass ranges in concert with the use of dynamic exclusion utilities has been employed [4,5]. Precursor ion complexity, defined for our purposes as the number of precursor ion candidates presented to the mass spectrometer in a given period of time, could be reduced with greater fractionation of the samples prior to digestion but only at the expense of sample throughput and with questionable efficacy. Two alternatives exist to reduce this complexity, either selective capture of specific types of peptides, such as we and others have described previously for cysteinyl peptide capture [5,6,11], or a multidimensional separation of the peptide digest mixture prior to mass spectral analysis [3,12]. Both approaches represent direct methods for reducing precursor ion complexity with minimal modification of established protocols.

Most multidimensional separations interfaced with mass spectrometry utilize first dimensional separations that complement a second dimension of reversed-phase chromatography. Thus, the sample is presented to the mass spectrometer in its most desirable form for electrospray ionization. Candidates for the primary chromatographic separation have included size-exclusion chromatography (SEC) [12] and ion-exchange chromatography (IEC) [3], although IEC is usually reserved for the analysis of proteins [13]. The requirement of an organic modifier in most IEC buffers precluded the use of coupled IEC–RPLC separations for truly comprehensive analyses [14]. Rapid and comprehensive multidimensional separations of limited peptide mixtures have also been demonstrated using RPLC coupled to capillary zone electrophoresis (CZE) [15]. The resolving power of coupled separations is maximized by using a rapid second dimensional separation that continuously samples an ongoing first dimensional separation [16]. Although these platforms have their utility, the rapid second dimensional separation placed undue time constraints on the mass spectral analysis (i.e., narrow peak widths). As such, almost all of these applications have limited the mass spectrometer to a single mass range [12,13,15] or in-source fragmentation analysis [14]. The priority of these approaches has always been the comprehensive separation of the mixed components not the structural analysis of the resolved species. In their analysis of the effect of the second dimensional sampling rate on resolution in multidimensional LC separations Murphy et al. [16] conclude that optimization must start in the second dimensional separation. If detailed structural information or peptide sequence identification is the desired outcome of the analysis, mass spectrometer parameters become paramount.

A recent publication described the use of a stepped IEC separation (with reduced organic content) coupled with a secondary RPLC separation for the extensive MS–MS analysis of peptide digest mixtures for the purpose of identifying the components of intracellular protein complexes [3]. The use of a discontinuous separation sacrifices some of the resolving power of the coupled technique, however, it is a small sacrifice in favor of the mass spectrometer. We have adopted a similar approach in the design of our multidimensional system, but have further simplified the primary dimension to a ‘‘binary mobility’’ (for definition see Ref. [17]) IEC separation [18]. This system was modeled after an affinity capture–RPLC platform recently described for the selected enrichment of metabolites of interest from complex matrices (serum) [17] and shares its inherent simplicity. The ‘‘binary mobility’’ design affords greater flexibility as the multidimensional system can be reverted to its base RPLC system in a matter of minutes. Data is presented from both off-line validations and fully automated analyses of highly complex peptide digest mixtures derived from conditioned media leading to the identification of hundreds of protein components in a single sample.

2. Experimental

2.1. Reagents and supplies

Unless otherwise stated, all reagents were of the highest quality available. All solvents used were obtained from Burdick and Jackson (Muskegon, MI, USA).

2.2. Sample preparation

All samples employed in the following analyses were complex protein digest mixtures derived from fractionated conditioned media from either human

lung fibroblasts (CCD 34Lu, ATCC: CRL-1491; American Type Culture Collection, Rockville, MD, USA) or human brain glioma cells (Hs 683, ATCC: HTB-138; American Type Culture Collection). The proteins were reduced and alkylated (iodoacetamide) and enzymatically digested (trypsin; Boehringer Mannheim, Indianapolis, IN, USA) by conventional methods. The total number of components present in these samples is unknown but is expected to be high (hundreds). The enzymatically-digested samples were subjected to solid-phase extraction (SepPak; Waters, Milford, MA, USA) followed by methanol elution and vacuum concentration. The samples to be analyzed by the one-dimensional platform were resuspended in 0.1% formic acid while samples for the multidimensional system were resuspended in water.

2.3. LC instrumentation – single dimensional analyses

The standard platform for all LC–MS analyses was a Hewlett-Packard (HP) 1100 liquid chromatograph (Palo Alto, CA, USA) optimized for a 1.0 mm column format [6]. Samples were injected onto a 250×1.0 mm C_{18} column (Metachem Technologies, Torrance, CA, USA) equilibrated in 3% buffer B at 60 μl/min [buffer A: 0.1% (v/v) formic acid; buffer B: 90% (v/v) aqueous acetonitrile–0.09% (v/v) formic acid]. Following a 15-min isocratic elution, the column was developed using a linear gradient to 50% B over 185 min before rising to 90% B in 5 min. Column washing at 90% B was maintained for 15 min and then ramped down to the starting conditions over 5 min. A 40 min reequilibration was employed to restore the column to initial conditions. Sample injections were performed using the HP1100 autoinjector set to deliver 100 μl injections.

2.4. LC instrumentation – multidimensional analyses

Minimal modifications were required to convert the HP1100 LC described above to a multidimensional platform (refer to Fig. 1). An additional pump (ABI 140D syringe pump operated at 100 μl/min, continuously) was added for the isocratic delivery of a volatile, low-ionic-strength IEC loading buffer (5 m*M* ammonium formate, 5% acetonitrile, 0.01% NaN_3, pH 3.8) to the autoinjector. The IEC loading buffer was delivered to the HP autoinjector in place of the reversed-phase gradient and thus drove the delivery of all samples. An automated divert valve (panel mounted on the mass spectrometer) was employed to automatically recycle the IEC buffer while the LC–MS–MS analyses were underway (IEC idle) to minimize waste. The intuitive requirement of synchronized operation of the secondary pump is eliminated by the selective delivery of the solvent (autoinjector or reservoir, Fig. 1). Samples prepared for IEC analyses were loaded onto a 50× 2.0 mm strong cation-exchange column (SCX, PolySULFOETHYL A; Poly LC, Columbia, MD, USA) with the effluent passing through the column selection valve to a 50×2.0 mm C_{18} trapping column (BetaBasic-18; Keystone Scientific, Bellefonte, PA, USA) plumbed in the standard ''loop'' position. As samples were loaded, the unretained analytes (Flow-through, F) were concentrated onto the trapping column with the balance of the buffer flowing to waste; away from the mass spectrometer. The LC–MS–MS analysis of the trapped analytes was initiated by switching the column selection valve on-line, at 20 min post injection, and back flushing the trapping column onto the analytical column described above using the organic gradient from the HP1100 pump module. The flow of IEC buffer through the SCX column was maintained for an additional 10 min before being recycled to the reservoir by the LCQ divert valve. Gradient parameters were as described with the inclusion of an additional 20 min of isocratic delivery in the beginning of the run coincident with the performance of the IEC separation. Following the completion of the analysis of the F sample and the reequilibration of the columns, the analysis of the IEC retained analytes (Bound, B) was initiated by the injection of 250 μl of 0.5 *M* elution buffer (0.2 *M* sodium phosphate, 0.3 *M* sodium acetate) to simultaneously elute the bound components and recondition the IEC column. The released components were trapped and analyzed as described above for the IEC F components while the salt was diverted away from the mass spectrometer.

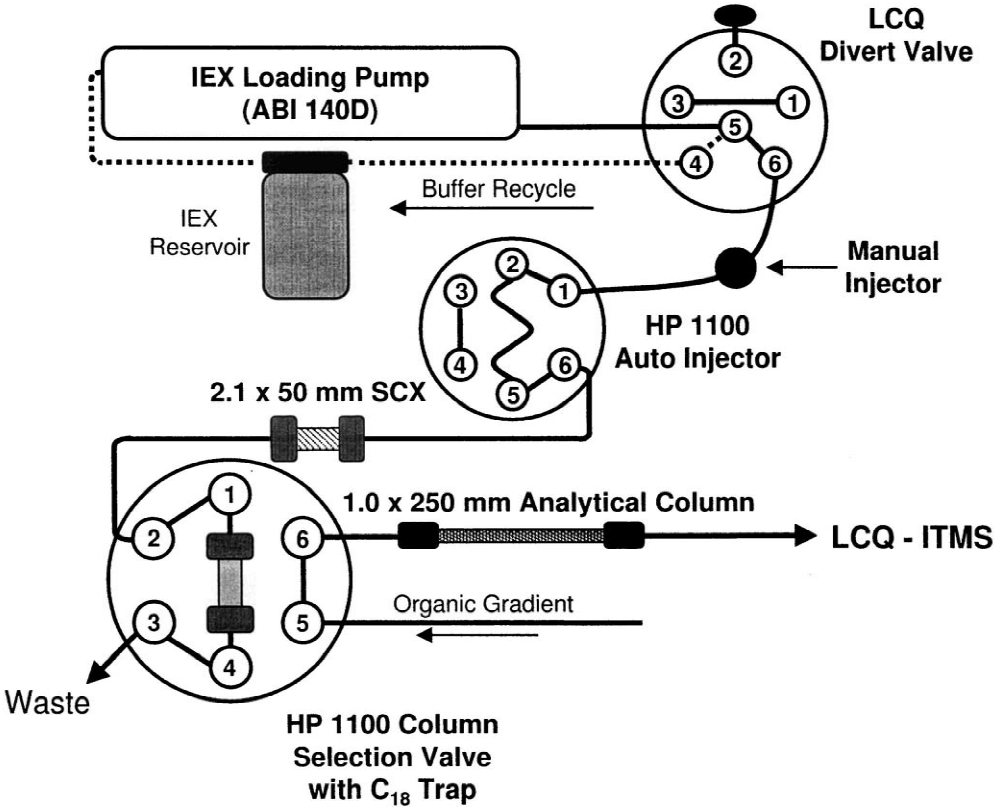

Fig. 1. Multidimensional LC–MS platform. Flow schematic is illustrated in the sample loading mode. Continuous, manual, operation of the ABI 140D syringe pump is afforded by the recycling of unused IEC loading buffer (flow returned to reservoir at 30 min post injection). A manual injector (1 ml loop) is included for the initial conditioning of the SCX column (two filled loop injections, 15 min apart).

2.5. Off-line validation of the multidimensional platform

Off-line IEC separations were performed and compared to an unfractionated control to validate the proposed multidimensional platform. The off-line system consisted of the ABI 140 pump plumbed directly to a Rheodyne 7125 sample injector (Cotati, CA, USA; 500 μl loop) for the delivery of the IEC loading buffer. The effluent from the injector passed through the SCX column to a Rheodyne 7000 switching valve with the peptide trapping column plumbed in the loop position. The configuration of the switching valve allowed switchable inputs from the IEC column and a HP1090 liquid chromatograph for equilibration of the RP column. A second injector was placed in-line between the HP1090 and the column switching valve to allow bulk elution of the trapped peptides in a reduced volume using a bolus injection of 70% aqueous methanol. An aliquot of a complex mixture was fractionated over the IEC system as described above using a 30 min collection onto the RP column. Trapped peptides were bulk eluted using a filled loop (1 ml) of 70% aqueous methanol with sample collected for 7 min at 100 μl/min. The F and B fractions were taken to dryness and resuspended in 0.1% formic acid for injection onto the single dimension system described above. An equivalent volume of the starting material was also taken to dryness, reconstituted in a similar manner and subjected to the same analysis to allow direct comparison with the fractionated material. This approach established a constant system architecture to facilitate retention time-based comparisons of the LC–MS–MS data. Chromatographic parameters are described in Fig. 2.

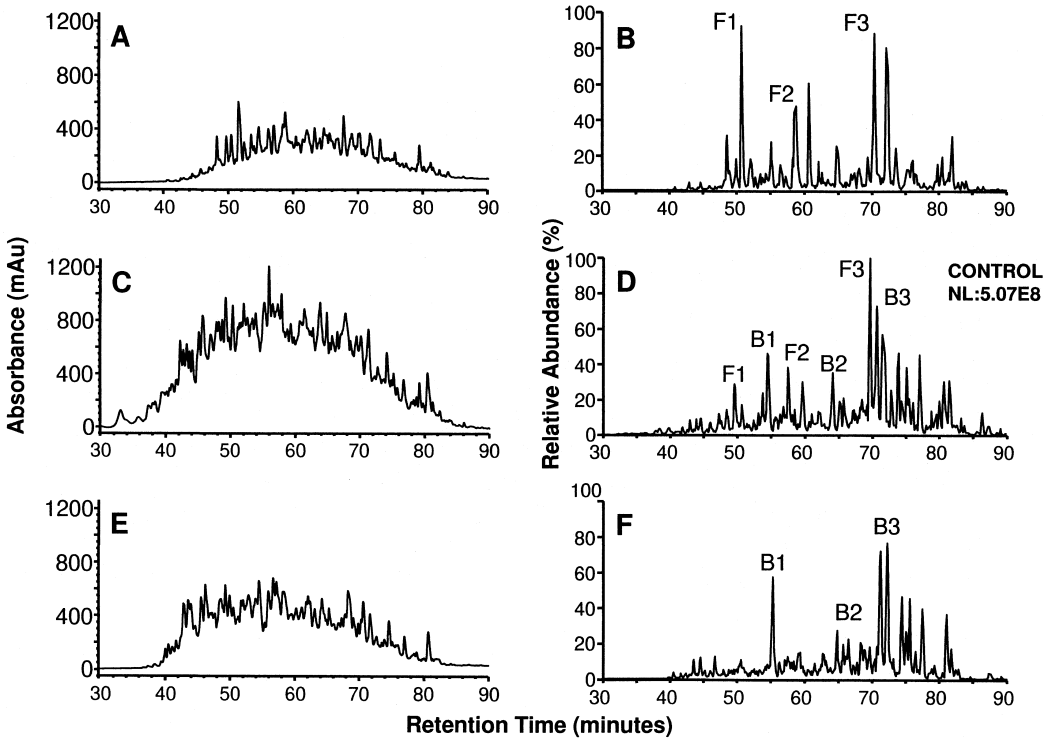

Fig. 2. Off-line validation of binary IEC fractionation. UV traces and base peak chromatograms of fractionated CCD 34Lu conditioned media sample (A and B, IEC flowthrough, C and D, starting load, E and F, IEC bound, respectively). Reference ions in the base peak chromatograms are labeled (F) for Flowthrough and (B) for Bound. Normalization is relative to the control intensity. Column development was from 3 to 50% buffer B over 120 min at 60 μl/min with UV detection of the eluent at 214 nm.

2.6. Mass spectral analysis

All mass spectral analyses were performed using a Finnigan LCQ ion trap mass spectrometer (LCQ-IT-MS; Thermoquest, San Jose, CA, USA) optimized for 1.0 mm column-based data-dependent LC–MS–MS analyses as previously described [10]. The column effluent was reduced using a simple stream splitter (HPLC tee) to 30 μl/min prior to connection with the LCQ interface. Data-dependent LC–MS–MS analyses were performed using the standard ''Triple-play'' format of a survey scan followed by a data-dependent ''Zoom'' (higher resolution, narrow range) and MS–MS scans. The ''Triple-play'' cycle is completed with a return to the survey scan mode for acquisition of a new precursor ion. Iterative analyses over incremental survey scan mass ranges were employed to exploit the gas-phase fractionation afforded by the mass spectrometer as described previously [4,5]. Six survey scan mass ranges, 400–550, 535–635, 720–720, 705–865, 850–1065 and 1050–2000 m/z were utilized. However, only the four low mass ranges were compared in these analyses as the complexity of the high mass analysis was determined to be too low to warrant fractionation of the sample. Dynamic exclusion was enabled with a 5 u exclusion window and a 60-s duration.

2.7. Data analysis

All mass spectral analyses were reduced using automated uninterpreted database search algorithms developed in the laboratory with visual confirmation of all returned matches [8,10]. A non-redundant database comprised of Swiss-Prot, Genpept and PIR entries and a six-way translation of dbEST was searched. Criteria for match acceptance were based upon a scoring filter with contributions from pre-

cursor and fragment ion tolerances (1.5 u), ratio of matched-to-unmatched ions, and the visual confirmation of a prominently labeled *b* and/or *y* type ion series. Relative intensities were expected to be increased for proline-initiated fragments and following large hydrophobic and acidic residues.

3. Results

3.1. Preliminary validation

The simplicity of the multidimensional system presented in Fig. 1 is maximized through the use of a binary IEC fractionation and long RPLC–MS analyses. The binary fractionation should represent a complete separation of the complex digest mixtures based upon the selectivity of the loading conditions as some peptides will be retained and others will not. In addition, the use of the single high salt elution step simultaneously reconditions the SCX column for subsequent analyses. Although greater fractionation could be achieved with intermediate salt elutions, there are limits to the feasibility of increasing run numbers/sample in a high throughput environment. A binary fractionation merely doubles the demand for instrument time. The extended RPLC–MS analysis obviates the need for precise synchronization of the multidimensional platform since the addition of a 20 min IEC loading cycle is insignificant compared to the total run time (>4 h injection cycle).

Validation of the proposed multidimensional system was provided through off-line experiments using a manual model system described in the Experimental section. The only significant deviation from the design of the automated platform was the bolus elution of the trapped analytes from the reversed-phase column. This was necessary so that the fractionated sample could be collected in a reduced volume and directly compared with an unfractionated aliquot using the single dimension platform. Given the constant system architecture of the standard LC–MS platform, retention time-based comparisons were now feasible. The creation of even a simple multidimensional system unavoidably increases the overall system dead volume thereby precluding time-based analyses.

An aliquot of a complex mixture was fractionated as described and directly compared via data-dependent MS–MS analysis (triple play, see Experimental) against an equal aliquot of the starting load (SL) using the single dimensional LC–MS platform. A steeper organic gradient was utilized along with a single survey scan mass range (400–2000 m/z) to facilitate comparisons between the three runs. Fig. 2 presents the respective UV detector trace and base peak chromatograms of the IEC F (A and B), SL (C and D) and IEC B samples (E and F). The base peak chromatograms of the three analyses are normalized relative to the control. The complete fractionation of the most abundant components is readily confirmed upon comparison of the IEC F and IEC B base peak chromatograms (peaks labeled F 1–3 and B 1–3 in Fig. 2A and C). The near quantitative recovery of the fractionated peptides can be seen by comparison of the intensities of the labeled peaks in the base peak chromatograms of the fractionated analyses against the control (Fig. 2A and C, vs. Fig. 2B, respectively). No immediate explanation is apparent for the increase in the intensity of the peak labeled F1 in Fig. 2A relative to the control.

A finer analysis of the IEC partitioning and relative peptide recoveries was obtained by the detailed analysis of a discreet retention time window. Fig. 3 presents the average of all survey scans collected through the time period from 70 to 75 min from the IEC F, IEC B and SL analyses (Fig. 3A–C, respectively; limited to 800–1600 m/z for simplicity). This time frame was chosen on the basis of high complexity and good retention time reproducibility of the landmark components in the base peak chromatograms. The partitioning of the labeled ions in the SL analysis (Fig. 3B) to their respective IEC fractions (F to Fig. 3A and B to Fig. 3C) confirms the near complete binary fractionation of the sample. Excellent sample recovery is evident in the comparable relative intensities of the labeled ions across the three averaged spectra. The intense ion labeled F* in Fig. 3A (1055 m/z) eluted at 69.6 min in the SL analysis and is therefore not represented in Fig. 3B. Ions common to all three analyses are labeled C (common) and represent peptide sequences with ambivalent IEC character rather than evidence for column overload. The sequence of the common ion at 943 m/z was determined from its MS–MS spectrum to be VSVQLEASPAFLAVPVEK (α-2-macro-

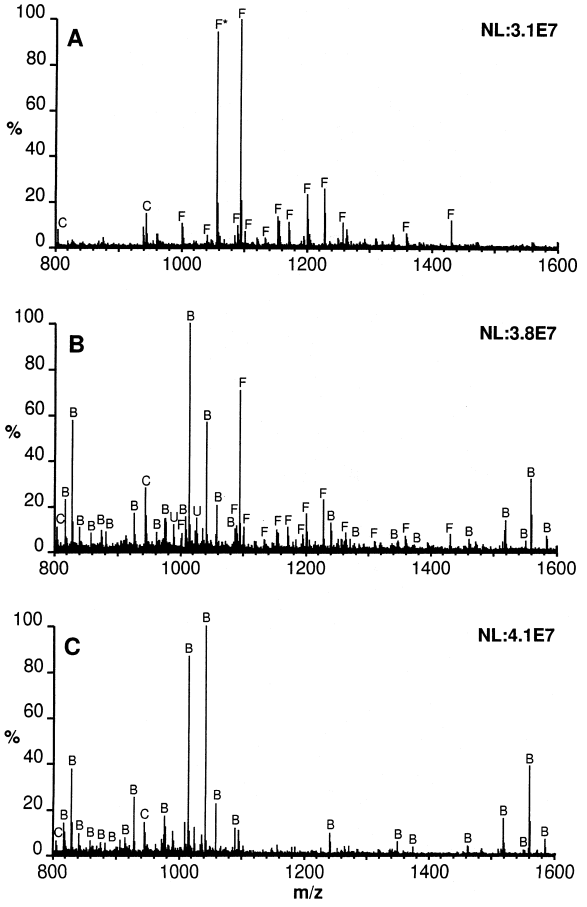

Fig. 3. Detailed mass spectral analysis of IEC partitioned ions. Averaged survey scan mass spectrum over the time range from 70 to 75 min of the IEC F, SL and IEC B analyses presented in Fig. 2 (A, B and C, respectively). Labeled ions are: (F) IEC F partitioned ions, (B) IEC B partitioned ions, (C) ions common to both, and (U) Ions that were unique to the SL analysis. Relative quantification is impaired by the discontinuous sampling rate of the mass spectrometer during triple play analyses and retention time variances of ions eluting near the boundaries of the defined time window.

globulin). The net charge of this sequence would be near neutral at pH 3.8 as the penultimate position of the second Glu residue would be expected to have a semi-negating effect on the C-terminal Lys in terms of its affinity for the SCX resin.

Comparisons of similar windows at the beginning of the runs were not as favorable. This was observed in the lack of components with retention times <40 min in the UV traces of the IEC fractionated analyses relative to the starting material (Fig. 2A and C, relative to Fig. 2B). A detailed examination of the averaged spectra suggest that quantitative recoveries were not obtained until after 45–50 min had elapsed (data not shown). Since these samples were all taken to dryness and reconstituted in like fashion, the loss of peptides in the IEC fractionated samples must be due to lack of binding to the trapping column in the presence of the 5% organic modifier. These losses would be of considerable concern if the goal of the experiment was the comprehensive analysis of a single protein (i.e., characterization). However, since our interest is the comprehensive identification of the protein components of a mixture, which can be obtained from the MS–MS analysis of any one of a number of potential tryptic peptides, these small losses were acceptable.

3.2. Automated analyses

Having validated the design and methods for the use of a multidimensional LC platform for the analysis of complex mixtures the platform was put into production for the selected analysis of highly abundant sample mixtures in a high throughput environment. In a single dimensional analysis, six iterative LC–MS analyses would be performed over incremental survey scan mass ranges as detailed in the Experimental section. Those samples selected for analysis using the multidimensional approach would be analyzed over the first four mass ranges as both IEC F and IEC B runs while the two higher mass range analyses would be performed using the single dimension approach on another LCQ-IT-MS system for a total of 10 runs per fraction. A single fraction of Hs 683 conditioned media is presented below for illustration of the utility of the automated multidimensional platform.

Fig. 4 presents comparable UV traces and base peak chromatograms from a single survey scan mass range (620–720 m/z) from the IEC F, SL control and IEC B analyses (A and B, C and D and E and F, respectively). These control analyses were conducted for the purpose of these evaluations and are not part of our normal protocol. The approximately 25 min retention time shift apparent from the comparison of the labeled peaks in an IEC fractionated analysis and that of the SL control is due to the altered system

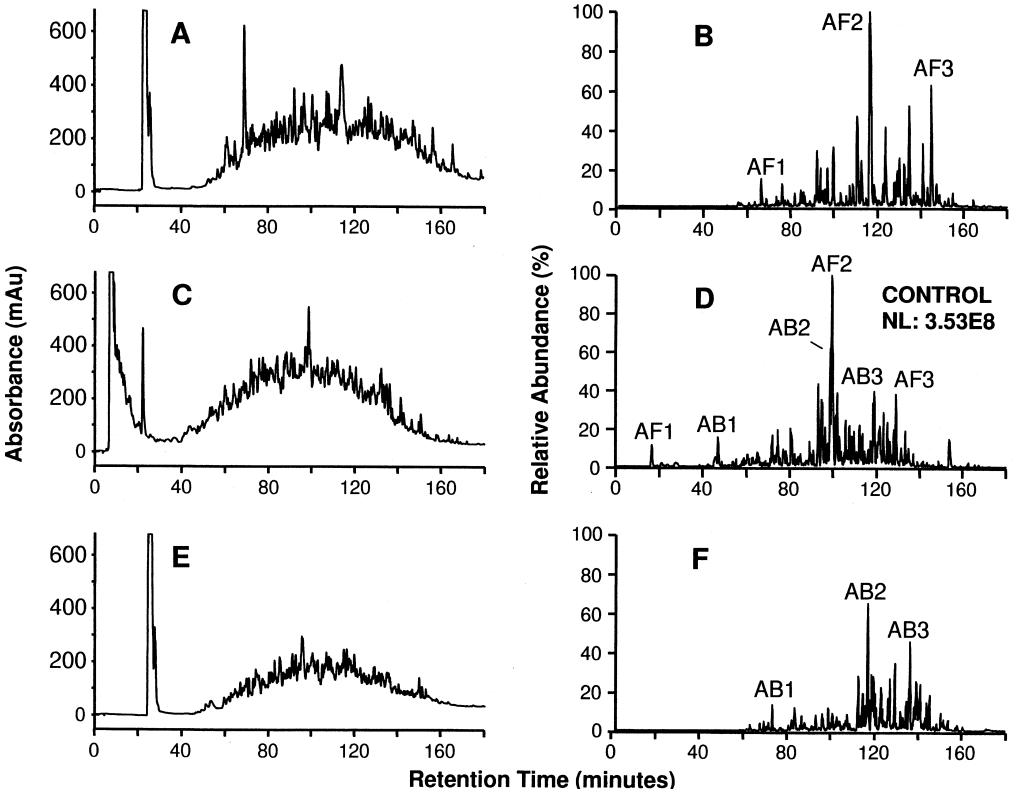

Fig. 4. Comparative analyses of fractionated Hs 683 conditioned media by single and multidimensional LC–MS platforms. UV traces and base peak chromatograms of IEC F (A and B), starting load (C and D) and IEC bound (E and F) conditioned media samples. Base peak chromatograms are presented normalized to the intensity of the control. Landmark ions are labeled AF and AB for automated Flowthrough and Bound analyses, respectively. The rotation of the column selection valve is evident in the appearance of a breakthrough artifact at 22 min in the IEC partitioned analyses (A and E).

architecture (and illustrates the value of the off-line comparison performed above). The reduction in the retention time shift in the latter portion of the IEC analyses suggests the exaggerated shift observed in the beginning of these analyses may be an effect of the organic component of the IEC loading buffer (5% acetonitrile). Although the peak intensities of comparable ions detected in the IEC analyses and the SL control (refer to labeled peaks, Fig. 4) do vary, the IEC fractionation is grossly complete with good recovery of the fractionated analytes (as judged from the UV data, Fig. 4A and E, relative to Fig. 4B). A full accounting of the IEC fractionated and control analyses across the first four survey scan mass ranges is presented in Table 1.

In the automated data processing scheme developed for the reduction of high volumes of LC–MS–MS data, the software removes spectra which fail criteria for minimum ion count and intensity and merges spectra derived from the same precursor ion as determined over a 6 min moving window [19]. Therefore, the number of imported spectra presented in Table 1 represents slightly less than the total number of peptide collision-induced dissociation (CID) spectra acquired and certainly more than the number of interpretable spectra. As these filters are applied evenly for all imported data, the results of the import routine can serve as a relative measure of the number of spectra obtained. In the category of total number of imported spectra, a 51.7% increase was achieved using the multidimensional approach. That this value is not simply twice that acquired from the single dimensional analyses suggests that the sample complexity was the rate-limiting factor

Table 1
Comparison of single and multidimensional LC–MS platforms for the comprehensive analysis of Hs 683 conditioned media

	One-dimensional LC–MS[a]	Two-dimensional LC–MS[b]	Relative increase (%)
Spectra imported	4028	6109	51.7
Annotated spectra	731	1036	41.7
NR[c] annotated spectra	642	923	43.7
NR peptide sequences	609	869	42.7
NR protein sequences	148	213	43.9

[a] Four runs, four survey ranges.
[b] Eight runs, four survey ranges.
[c] Non-redundant.

throughout most of the IEC runs, not the mass spectrometer, indicating that the desired result was achieved. However, more relevant metrics are the number of annotated spectra (i.e., identified), non-redundant precursor ions (removal of redundant spectra) and non-redundant peptide sequences. In each of these comparisons, multidimensional analyses consistently produced >40% more data from within the same sample source (see Table 1). In the final reduction to the number of proteins identified (defined as unique accession codes for this analysis), 213 proteins were identified from the eight LC–MS analyses using the multidimensional platform vs. 148 identifications from the four single dimension controls. Simply stated, as many as 44% more proteins were identified using the multidimensional platform. Another uniformity that presents itself in Table 1 is the annotation rate, i.e., the % of imported spectra that are identified. The roughly 18% and 17% annotation rates achieved for the single and multidimensional analyses, respectively, are anecdotally consistent with the field in general. The consistent ratio of non-redundant precursor ions to total annotated spectra (88% and 89%, combined IEC and SL, respectively) suggests the benefits obtained from the use of dynamic exclusion applied evenly across these comparisons. These numerous internal consistencies attest to the validity of these platform comparisons.

As always happens in LC–MS–MS analyses of highly complex peptide mixtures of even the same sample, no two runs are identical. The random variable in precursor ion selection will always play an important role in the acquisition of CID spectra from complex mixtures whenever sample complexity overpowers the duty cycle of the mass spectrometer. For this reason, 128 unique peptide sequences were identified in the single dimensional analyses compared to the combined multidimensional data set. Conversely though, 401 unique sequences were obtained from the combined IEC analyses. When reduced to the level of protein identifications 96 unique components were found in the IEC analyses over 23 in the control data set.

An analysis of the distribution of peptide annotations relative to retention time for the single dimensional control and the combined IEC peptides is presented in Fig. 5. The overlaid profiles of the IEC non-redundant peptides and those of the control illustrate the retention time shift observed in Fig. 4. The distribution of those peptide sequences that were

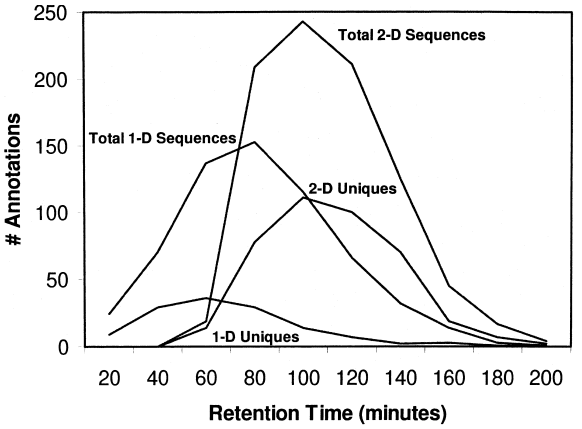

Fig. 5. Temporal distribution of annotated peptides in single and multidimensional LC–MS–MS analyses. All sequence annotations are non-redundant for occurrence and precursor ion charge state. Sequence assignments which were limited to the single (1-D) or combined multidimensional analyses (2-D) are termed as unique for this comparison.

unique to the two analyses strengthens the earlier suggestion that the presence of the 5% organic modifier in the IEC loading buffer interferes with the recovery and/or chromatography of the earliest eluting peptides. This is evident in the left shift of the unique sequences identified in the control analyses compared with the combined IEC where unique sequences are more evenly distributed throughout the time range of the analyses. Further evidence is also apparent in the rapid rise (left shift) in IEC non-redundant peptides indicating the loss or perturbation of earlier eluting components. Conventional IEC peptide separations incorporate a higher level of organic modifier, typically 10 to 20%, which is incompatible with direct coupling to a reversed-phase trapping column [14]. Other investigators have reported the use of reduced levels of organic [3] (5% as is the case here) without commenting on the potential negative affects. In our view, this is a minor problem and easily accepted as part of the price of obtaining more total information from within a single sample source.

4. Discussion

That multidimensional analyses of complex peptide digest mixtures yield significantly more data from within a defined sample source is obvious and has been demonstrated by many others. More elegant and sensitive techniques have been published and deserve serious consideration when sample amounts are extremely limited [3]. The novelty of this report is the simplicity of design, the facile inter-conversion between a single and multidimensional platform and general applicability of the approach to users of a variety of LC–MS platforms. In its simplest design, the addition of a single metering pump to a HP1100/Finnigan LCQ-IT-MS platform is the sole requirement for the creation of a multidimensional system. In its most elaborate form, if there is a lack of incorporated column selection (HP1100) and divert valves (LCQ), these can be easily substituted with simple automated valves controlled through contact closures. Additionally, almost every liquid chromatography system supports numerous programmable analog outputs. For less abundant, but not necessarily the rarest of samples, this methodology is easily scaleable to smaller, yet still conventional, column formats. Such systems need not be dedicated to the analysis of highly complex protein mixtures, but rather the comprehensive analysis of less complex samples. The observed effects of the 5% organic modifier in the IEC loading buffer may warrant consideration in these analyses. We have followed a rule of column format selection that is dictated by the demand for a robust, high throughput system; i.e., use the largest column that provides the level of sensitivity necessary to achieve the goal of the analysis. Although the resolving power of our system is only enhanced by a factor of two, the minimal demand for greater instrument time to perform these analyses is compatible with our high throughput environment.

In the past decade, the goal of most comprehensive analyses has been the complete characterization of a single protein through the mass spectral analysis of its peptide digest fragments. With the concomitant maturation of uninterpreted database search algorithms, higher duty cycle mass spectrometers capable of data-dependent MS–MS analysis and the anticipated completion of the Human Genome Project, this emphasis will evolve away from the analysis of a single protein to the elucidation of the protein components of intra- and extracellular complexes and compartments.

Acknowledgements

We would like to thank Geri Trail, Shue-Yuan Wang, Alison Moore and their groups for excellent cell culture optimization and conditioned media production.

References

[1] A.L. McCormack, D.M. Schieltz, B. Goode, S. Yang, G. Barnes, D. Drubin, J.R. Yates III, Anal. Chem. 69 (1997) 767.
[2] A.J. Link, E. Carmack, J.R. Yates III, Int. J. Mass Spectrom. Ion Proc. 160 (1997) 303.
[3] A.J. Link, J. Eng, D.M. Schieltz, E. Carmack, G.J. Mize, D.R. Morris, B.M. Garvik, J.R. Yates III, Nat. Biotechnol. 17 (1999) 676.

[4] P.J. Mintz, S.D. Patterson, A.F. Neuwald, C.S. Spahr, D.L. Spector, EMBO J. 18 (1999) 4308.
[5] S.D. Patterson, C.S. Spahr, E. Daugas, S.A. Susin, T. Irinopoulou, C. Koehler, G. Kroemer, Cell Death Diff. 7 (2000) 137.
[6] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[7] J.K. Eng, A.L. McCormack, J.R. Yates III, J. Am. Soc. Mass Spectrom. 5 (1994) 976.
[8] P.L. Courchesne, R. Luethy, S.D. Patterson, Electrophoresis 18 (1997) 369.
[9] S.D. Patterson, in: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology, Wiley, New York, 1998, p. 10.22.11.
[10] P.L. Courchesne, M.D. Jones, J.R. Robinson, C.S. Spahr, S. McCracken, D.L. Bentley, R. Luethy, S.D. Patterson, Electrophoresis 19 (1998) 956.
[11] S.D. Patterson, in: L.M. Savage (Ed.), Proteomics, Integrating Protein-Based Tools and Applications for Drug Discovery, International Business Communications, Southborough, MA, 1998, p. 127.
[12] G.J. Opiteck, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 2283.
[13] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 1518.
[14] G.J. Opiteck, J.W. Jorgenson, M.A. Moseley, R.J. Anderegg, J. Microcol. Sep. 10 (1998) 365.
[15] K.C. Lewis, G.J. Opiteck, J.W. Jorgenson, D.M. Sheeley, J. Am. Soc. Mass Spectrom. 8 (1997) 495.
[16] R.E. Murphy, M.R. Schure, J.P. Foley, Anal. Chem. 70 (1998) 1585.
[17] F. Regnier, G. Huang, J. Chromatogr. A 750 (1996) 3.
[18] J.Y. Cai, J. Henion, Anal. Chem. 68 (1996) 72.
[19] W. Yu, R. Luethy, S.D. Patterson, in: I.H. Kenttamaa (Ed.), Proceedings of the 47th ASMS Conference on Mass Spectromtery and Allied Topics, Dallas, TX, June 1999, p. 2799.