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Fully automated micro- and nanoscale one- or two-dimensional high-performance liquid chromatography system for liquid chromatography–mass spectrometry compatible with non-volatile salts for ion exchange chromatography

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Abstract

A one- or two-dimensional high performance liquid chromatography system for electrospray ionization mass spectrometers has been developed that is optimized for ion exchange and reversed phase separations. A unique and simple valve configuration permits the use of a variety of non-volatile salts; ammonium sulfate was used in an example of strong cation exchange separations. The system was designed and evaluated for both micro- and nanoflow chromatography. The peptide detection limit was ∼100 fmol for micro- and 20 fmol for nanoflow, demonstrating the concentration and mass sensitivity improvements expected with nanoelectrospray ionization. The 1D/2D-HPLC MS system is fully automated for routine peptide analyses, compatible with direct injection of proteolytic digests, and exhibits chromatographic reproducibility and sensitivity. Software permits operator selection of either a 1D or 2D configuration with corresponding system parameters as required for individual samples. The hardware elements and resulting performance are described in this paper.
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Keywords: Proteomics; Two-dimensional high performance liquid chromatography (2D-HPLC); Electrospray ionization (ESI); Mass spectrometry (MS)

1. Introduction

Proteomic analyses aim to characterize multiple proteins in diverse samples as comprehensively as possible. Currently, methods combining polyacrylamide gel electrophoresis (1D or 2D PAGE) protein separations with either time of flight mass spectrometry using matrix assisted laser desorption/ionization (MALDI-TOF) or tandem mass spectrometry (MS/MS) using online electrospray ionization (ESI) are widely used [1–4]. Protein level separations in HPLC can be complicated due to constraints imposed by the diverse adsorptive and solubility properties of proteins. Complete proteolysis of proteins into peptides produces a more tractable mixture amenable to HPLC with ESI-MS/MS [5,6], although digestion greatly increases the complexity of the mixture. A complete system for peptide analyses requires an HPLC capable of high resolution separation, the use of chromatographic solvents and flow rates compatible with ESI, and MS/MS instrumentation with comprehensive software tools for sequence assignments and database collation.

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP, reversed phase; IEX, ion exchange; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; R.S.D., relative standard deviation; S/N, signal-to-noise

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We have investigated 2D-HPLC for high resolution peptide separations. The most important issues for maximizing peak capacity in 2D-HPLC are the selection of orthogonal chromatographic modes and the design for connecting the first and second dimensions. Several separation modes, including ion exchange (IEX), reversed phase (RP), and size exclusion (SEC) have been recommended for 2D-HPLC [7,8]. IEX or SEC can be used for the first dimension, because column loading capacity and sample recovery are more important than resolving power or selectivity. IEX has a larger loading capacity than other chromatographic modes, but IEX may exhibit optimal performance with salt or pH gradients containing concentrated and non-volatile salt buffers that are incompatible with ESI and MS operations. RP is a logical second dimension mode because of its high resolution and use of aqueous-organic solvents that are ESI compatible.

The design for coupling the first and the second dimension of chromatography is critical for realizing the aggregate potential of both separations. Multidimensional Protein Identification Technology (MudPIT) uses the simplest approach; a single column packed with both IEX and RP resins as described by Washburn et al. [6]. Several designs have been reported using two separate columns for the first dimension and the second dimension, individually [9–12]. Opiteck et al. [10] described mounting two collection tubes on a switching valve. Using this mechanism, eluent from the first column is loaded into one tube, the tubes interchanged, and the eluent is then loaded onto a RP column. Eluent, including salts in the SCX buffer, flows directly into the ESI and MS. Two similar configurations were reported by Opiteck et al. [11] and by Wagner et al. [12]. Both use an SEC or IEX first stage followed by matched dual RP columns mounted on multiport switching valves. Sample eluting from the first column is trapped and concentrated on alternate RP columns, with elution programs timed to synchronize the gradient separations. Sample concentration improved the separations on the chromatogram relative to using a sample loop. Neither system included desalting. M.T. Davis described a design with desalting capability [9]. Analytes in the effluent from the SCX column were collected on a trapping column, and then eluted by back-flushing in a small volume onto a RP column. Licklider described a vented column design with a multiport valve and T-joint for nanoliter sample loading from the sample loop of an autosampler [13]. However, this system did not permit fully automated 2D operation.

We have designed a new configuration using a unique valve combination for online sample trapping and desalting in order to achieve a fully automated two-dimensional HPLC system compatible with ESI-MS. The system has both microflow (1–10 μL/min) and nanoflow (200–400 nL/min) capabilities. Microflow retains the high capacity and robustness of larger diameter columns (150–300 μm i.d.) for routine analyses; nanoflow increases ESI sensitivity as a result of increased concentration [14–16]. There have been previous publications describing 2D-HPLC systems for mass spectrometry, but the operation and performance of our system allows automated direct analyses of protein digests without sacrificing the high performance of conventional HPLC for compatibility with ESI. The performance of this system is described with the resulting data presented. Additionally, the valve configuration developed for 2D has led to a robust 1D trapping injection technique useful for microscale or nanoscale HPLC using a commercial autosampler.

2. Experimental

2.1. Chemical reagents

Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (Muskegon, MI, USA). Chemicals purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA) include dithiothreitol (99%), ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), calcium chloride (96% ACS), formic acid (98%), ammonium sulfate (ultra-pure 99.999%), and ammonium formate (ultra-pure 99.995%). Water was obtained from a NANOpure ultra water purification system manufactured by Barnstead (Dubuque, IA, USA).

2.2. Sample preparation

Standard proteins for system validation were purchased from Sigma–Aldrich in the highest purity available; bovine serum albumin (BSA), equine apomyoglobin, bovine β-casein, carbonic anhydrase from bovine erythrocytes, lysozyme from chicken egg white and enolase from baker's yeast. Each protein was solubilized in 50 mM Tris–HCl, pH 8.0 (Quality Biological Inc., Gaithersburg, MD, USA) 50 mM dithiothreitol, 5 mM EDTA and 10 mM calcium chloride to yield 50 pmol/μL final concentration. Protein solutions were mixed and sequencing grade modified trypsin (Promega, Madison, WI, USA) was added into each solution at 25:1 substrate: enzyme ratio. Each solution was incubated at 37 °C overnight and stored at −20 °C. Standard samples were prepared by dilution of protein digests with mobile phase A solution for liquid chromatography analyses.

2.3. Trapping injection for 1D analyses

All major components for the HPLC system were built using LC-VP series liquid chromatograph components, including the LC-10AD_{VP} solvent delivery pump, DGU-14A online degasser, SIL-10AD_{VP} autosampler, SCL-10A_{VP} system controller, CTO-10AC_{VP} column oven and FCV-12AH two-position switching valve (Shimadzu Corporation, Kyoto, Japan) with microflow control firmware for the 300 μm column (BetaBasic-18, 0.3 mm × 100 mm, 5 μm particle size; Thermo Hypersil-Keystone, Bellefonte, PA, USA) at 10 μL/min flow rate without a flow splitting device.

The microflow system employed a two-position six-port switching valve, FCV-12AH, as a switching valve and a 0.5 mm × 2 mm RP trapping column (Peptide CapTrap;

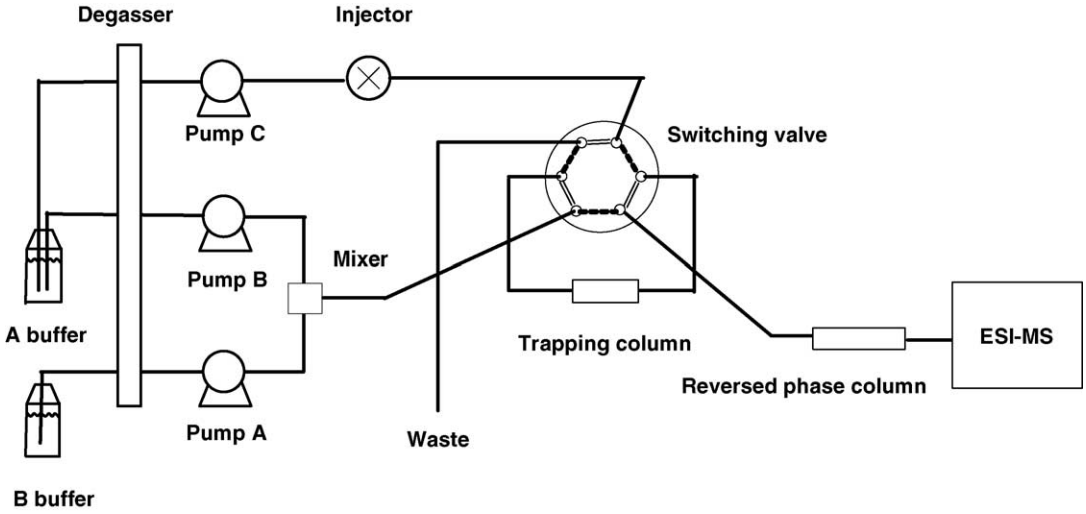


Fig. 1. Schematic flow diagram of 1D trapping injection system. The switching valve is alternated between sample loading (dotted line) or analysis (double line) positions.

Michrom BioResources Inc., Auburn, CA, USA) mounted on the FCV-12AH. A schematic diagram of the trapping injection system is shown in Fig. 1. RP mobile phase A and sample loading buffer contains acetonitrile:water:formic acid = 5:95:0.1 (v/v/v) and RP mobile phase B contains acetonitrile:water:formic acid = 80:20:0.1.

Sample solutions were injected using the SIL-10AD autosampler. For sample loading, mobile phase A was pumped at 50 μL/min, and after a 3 min loading, trapping, and desalting period, the position of the switching valve was changed to an analysis position to elute the trapped target materials onto a 0.3 mm × 100 mm C18 RP column, operated at room temperature.

The nanoscale system substituted a Cheminert CN2 6-port two-position switching valve (Valco Instrument Co. Inc., Houston, TX, USA) for the FCV-12AH used on the microsystem. The column was a 0.075 mm × 100 mm BetaBasic-18 RP column mounted on the PicoView nanospray ESI interface (New Objective, Woburn, MA, USA) and was operated at a flow rate of 400 nL/min, which was achieved using a flow-splitting device consisting of a pressure regulator (Upchurch Scientific, Oak Harbor, WA, USA) and a T-joint with the pump flow rate at 15 μL/min. Sample loading and trapping was performed as described for microscale chromatography.

2.4. Microscale automated 1D/2D system configuration

By expanding the trapping column concept, the HPLC system was converted to an automated 2D system with dual binary gradient elution capabilities for the first (SCX) and second (RP) dimensions, and a single pump for desalting. This system has a unique valve configuration utilizing a combination of two six-port two-position valves (both FCV-12AH) for solvent switching and one 14-port rotary valve (Valco ST six-position valve) on which six individual CapTraps were mounted. The configuration enabled trapping onto individual CapTraps, followed by desalting and back flush loading onto a RP column. The process was automated using two controllers as shown in Fig. 2(a–c) (schematic flow diagram) and Fig. 3 (time chart of the control program). The first controller manages the time sequence and synchronizes data acquisition, injection and SCX gradient elution; the second controller directs the RP analysis cycle and is subservient to the first. A CTO-10AC$_{VP}$ column oven was used to thermostat both the SCX and RP columns at 40 °C.

Sample was loaded onto a 1.0 mm × 50 mm SCX column (PolySulfoethyl A; Poly LC Inc., Columbia, MD, USA) operating 80 μL/min total flow rate. SCX mobile phase A was ammonium formate buffer which contained 5 mM ammonium formate and 5 mM formic acid. SCX mobile phase B was the same as mobile phase A plus 100 mM ammonium sulfate. Samples were eluted using a six-step salt gradient. Each SCX salt step used a 5 min trapping period, longer than the 3 min used for 1D, in order to allow elution with 10 SCX column bed volumes with each of the six selectable steps of B buffer, 0–100%. Peptides eluted in a given SCX step were trapped onto individual CapTraps mounted on the 14-port rotary valve. After trapping of peptides from all six step elution events was completed, the position of one six-port two-position switching valve (valve A) was changed to connect the flow of the desalting solution through the CapTrap. Desalting was performed for 3 min with 0.1% formic acid at 80 μL/min, and then the six-port two-position valve (valve B) was rotated to the RP analysis position. The sample trapped on the CapTrap was back-flushed and eluted onto a 0.3 mm × 100 mm C18 RP column directly coupled to an ESI mass spectrometer. Gradient elution with RP mobile phases A and B was programmed to flow 10% B for 3 min, linearly increase to 60% B over 30 min, then to 80% over 5 min, and hold at 80% B for 5 min before returning to 10% B to re-equilibrate for 17 min. The duration of the re-equilibration step was determined empirically based on

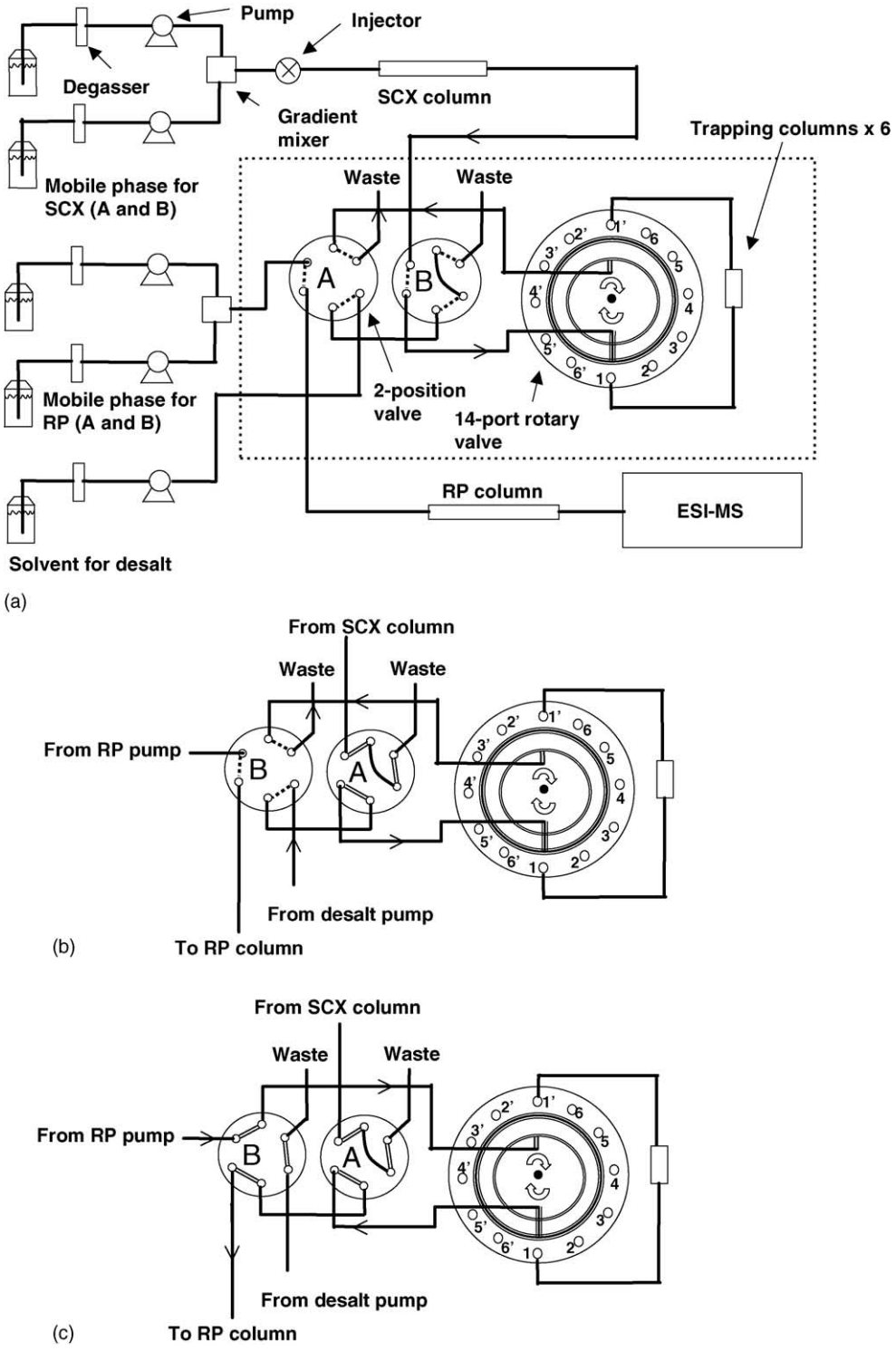


Fig. 2. (a–c) Schematic representations of the microscale 2D-HPLC system. There are six individual trapping columns mounted on the 14-port rotary valve (port 1–1′, is shown; not shown are those between 2 and 2′, 3 and 3′, etc.) valve A functions as solvent selector for the RP mobile phase or desalting. Valve B alternates between trapping and RP analyses. Valve positions for trapping function are shown in panel (a), desalting (b), analysis (c).

the time required for the total ion current to stabilize, resulting in longer re-equilibration time than conventionally used for RP.

The cycle of desalting, loading onto RP column, and separation on RP column was repeated for each CapTrap, that is, for each ion exchange fraction. During the last RP analysis, the SCX column was washed extensively with 100% SCX mobile phase B and then switched to allow 30 min re-equilibration at 0% of SCX B buffer prior to the next sample injection.

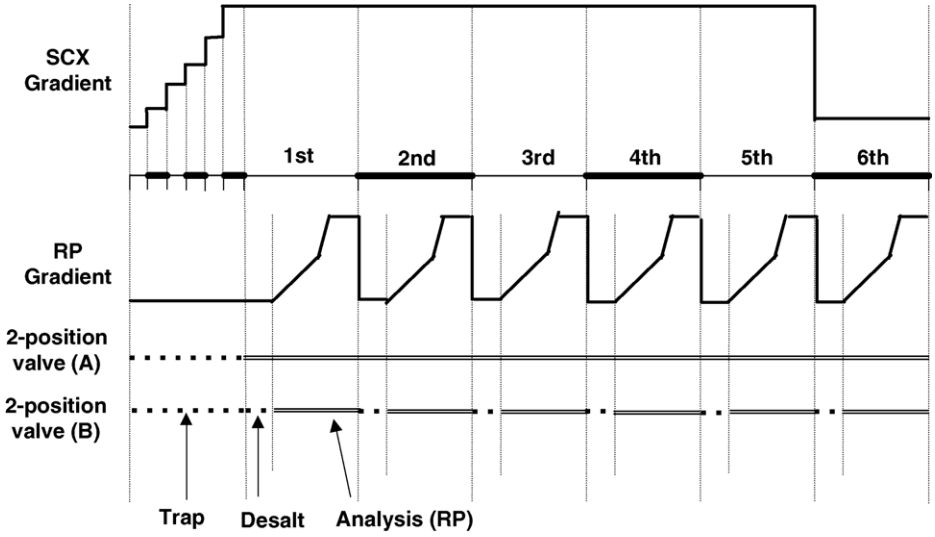


Fig. 3. Sequence event program time chart for the microscale 2D-HPLC system. SCX step gradient elution was performed in the first segment of this time chart indicated as Trap. Each RP analysis proceeds after desalting. During the six RP analyses, the SCX column is washed with a high concentration of salt solution buffer B and is equilibrated prior to the next sample analysis cycle.

2.5. Nanoscale automated 1D/2D system configuration

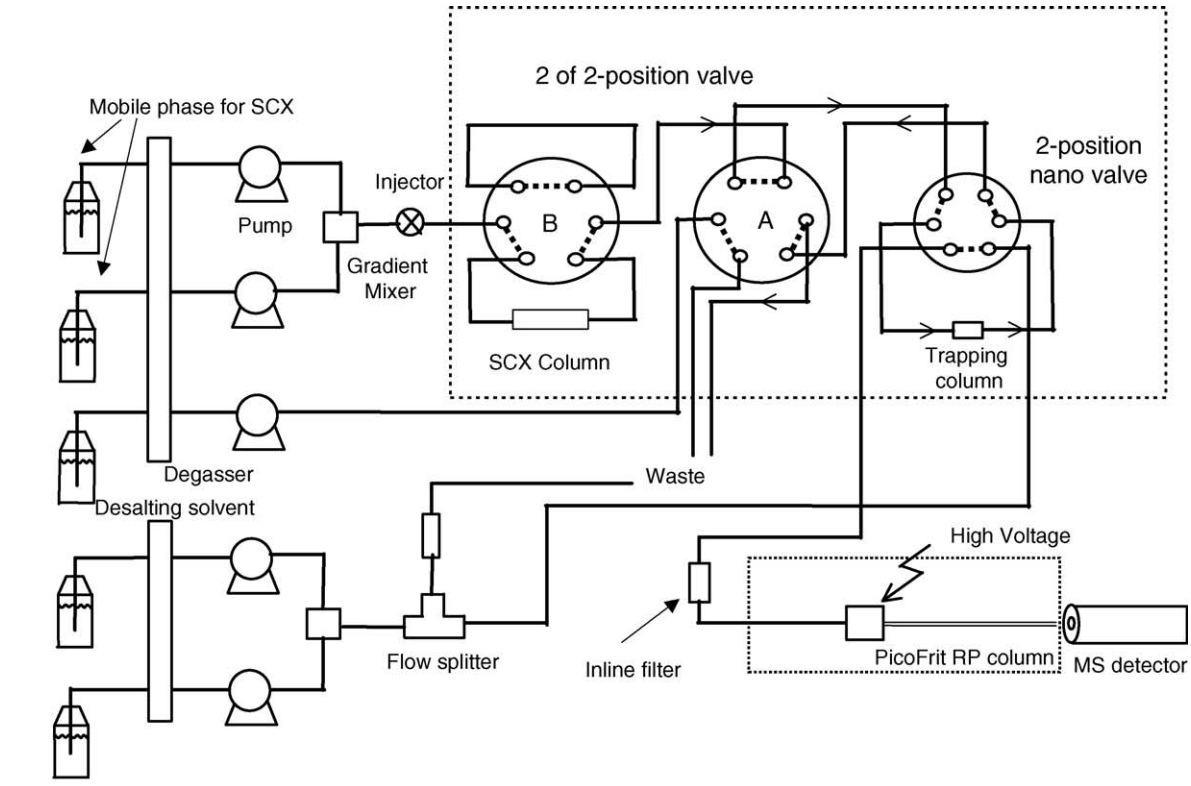
A combination of two six-port two-position valves (20 nL internal volume, port-to-port) was used because the 14-port rotary valve used for the microflow system had too large an internal volume for nanoflow rates. The nanoflow and microflow system configurations were very similar; however, the control program was different for the trapping cycle. In the nanoflow system, the sample was loaded onto a SCX column and trapping was performed for 5 min at 80 μL/min flow rate at room temperature, then the position of one valve on which the SCX column was mounted was rotated to allow desalting for 4 min with 0.1% formic acid at 80 μL/min. Finally, the position of the low internal volume six-port two-position valve was rotated into the RP analysis position and the desalted sample was eluted from the CapTrap and loaded onto a 0.075 mm × 100 mm C18 RP column at a flow rate of 400 nL/min. The RP column was operated at room temperature. The sample was eluted isocratically with 10% RP buffer B for 3 min, followed by a linear gradient programmed first to 60% B over 40 min, and then to 80% B for 5 min. The concentration of RP mobile phase B was reduced to 10% over 2 min, and then re-equilibrated over 25 min with 10% B to stabilize both the initial column conditions and the total ion current in the mass spectrometer. For the nanoflow system, the cycle of SCX elution, desalting and RP analysis was repeated six times sequentially using the same CapTrap. During RP analysis, the SCX column was switched “off-line” and the SCX pumps stopped. When the last RP step was in progress, the SCX B buffer was set at 100% to wash the SCX column for 40 min and then back to 0% B to condition the column for 40 min before the next sample cycle. Schematic flow diagrams and the timing control program are shown in Figs. 4(a–c) and 5.

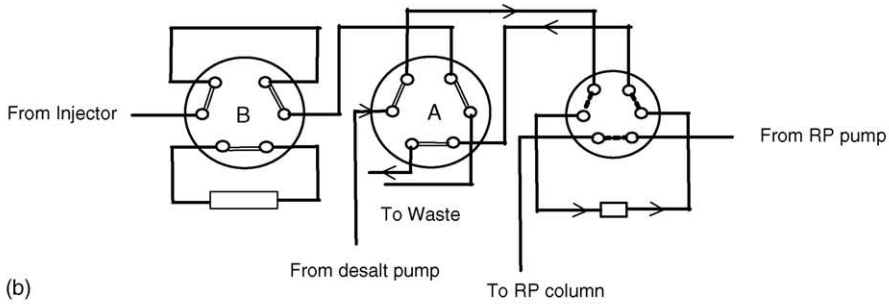
2.6. Online ESI-MS analyses

The HPLC system was directly connected to an LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). For either 1D or 2D microscale analyses, the standard ESI source optimized for a 10 μL/min flow rate was used. For nanoflow analyses, a PicoView nanoelectrospray ESI source (New Objective) optimized for a 400 nL/min flow rate, was used. Data acquisition was performed using either of two data dependent scanning sequences with dynamic exclusion. The ‘triple play’ sequence cycled between full MS scan, zoom scan, and MS/MS acquisitions. A ‘big-5’ sequence, cycled between full MS scans and MS/MS scans for the five most intense ions in the MS spectrum. In the ‘big-5’ sequence, no zoom scan is collected in order to increase the effective duty cycle of the mass spectrometer. The mass scan range was from 400 to 1800 *m/z*. The voltage for ESI was set at 5 kV for microscale LC–MS/MS at 10 μL/min flow rate, 2.1 kV for nanoscale LC–MS/MS at 400 nL/min flow rate.

2.7. Database searching and data processing

MS/MS spectral data were processed for automated interpretation using the Mascot search engine (Matrix Science Ltd., London, UK) to search the Swiss-Prot/TrEMBL database. The information contained in individual Mascot reports was parsed into a relational database using the software DBParser, as described by Yang et al. [17]. DBParser facilitated the concatenation of multiple data sets, multiple data set





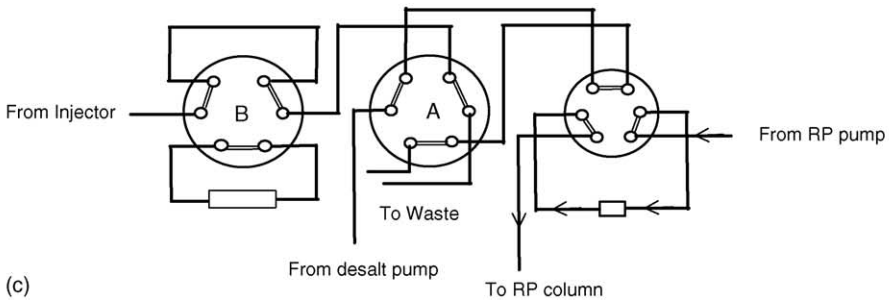


Fig. 4. (a–c) Schematic representations of the nanoscale 1D/2D-HPLC system. A single trapping column was used repeatedly in this configuration. During each RP analysis, valve B was set at the dotted position. The SCX column containing retained material was connected as a closed loop to prevent inadvertent elution or leakage. Valve A selects solvents for trapping or desalting. Valve flow paths for trapping are shown in panel (a) desalting (b) analysis (c). When used for 1D analysis, the SCX column mounted on valve B was offline (double line pathway on valve B) and the sample was injected onto the trapping column as described for the 1D trapping injection system. The 1D/2D modes are software selectable.

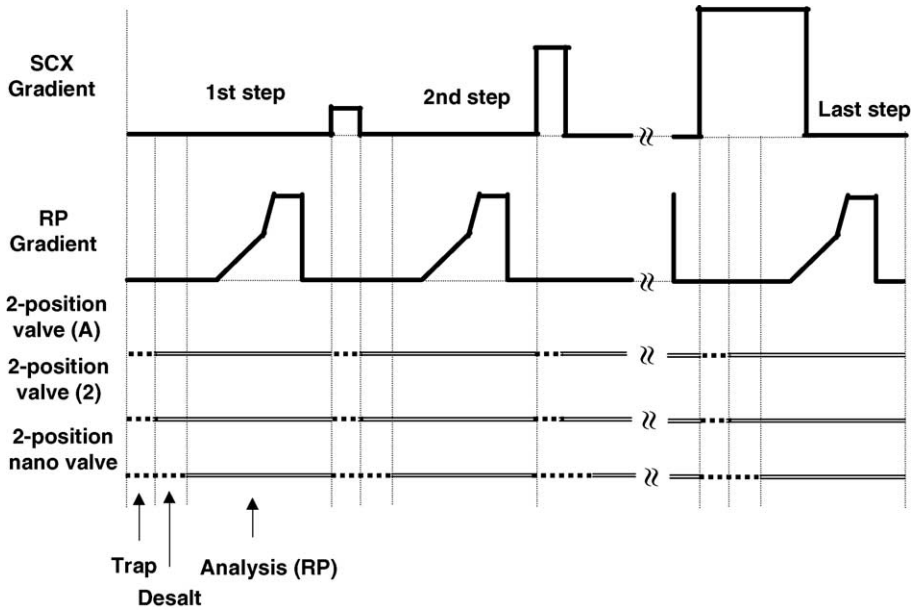


Fig. 5. Sequence event program time chart for the nanoscale 2D-HPLC system. The SCX step gradient elution proceeds independently prior to each desalting, injection and RP analysis, with cycles repeating in succession. In the final cycle, the SCX column was washed with a high concentration of salt solution B and was equilibrated prior to the next sample analysis cycle.

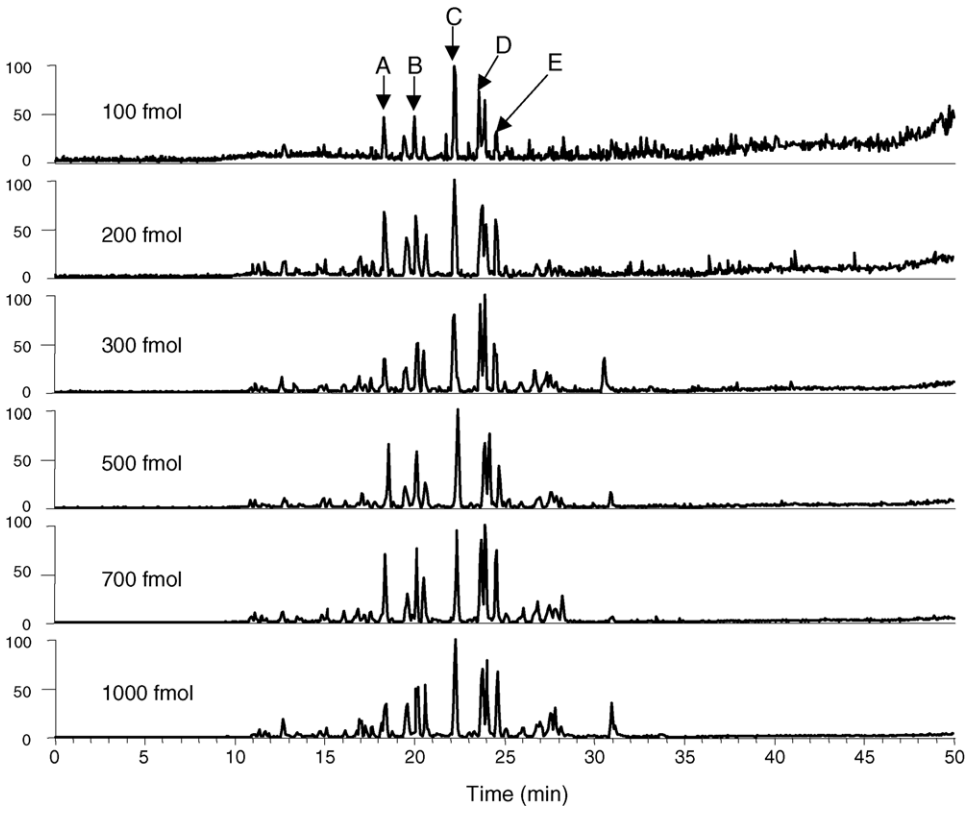


Fig. 6. Plotted re-constructed ion chromatograms in a stacked format showing increasing concentrations of standards using 1D microscale system, containing 100–1000 fmol of tryptic digests of BSA, β-casein and apomyoglobin.The retention times of the five major peaks (A–E) varied by 0.72, 0.33, 0.45, 0.51, 0.46 %R.S.D., respectively ($n = 5$). The S/N calculated for the 100 fmol sample for peaks A and B is 3.8; peak E 2.5; and for peaks C and D, 8.5 (noise level was averaged at three points on the chromatogram).

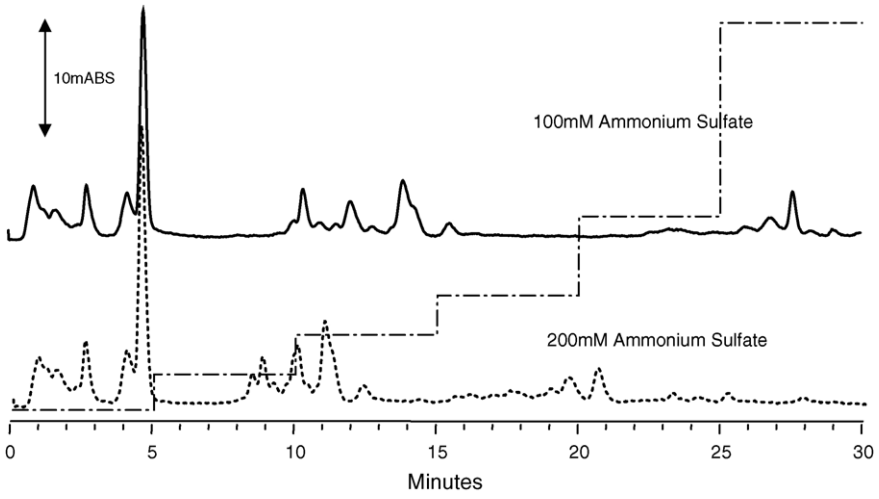


Fig. 7. Chromatographic patterns for SCX elution. Sample contained 50 pmol of apomyoglobin and BSA and 100 pmol of β-casein. The step gradient contained 0, 10, 20, 50, and 100% of B solution for 5 min intervals. Chromatogram is UV absorbance at 280 nm. The solid line chromatogram was obtained with 100 mM ammonium sulfate in B; the dotted line is 200 mM ammonium sulfate. The SCX step gradient profile is indicated by alternate long and short dashed lines.

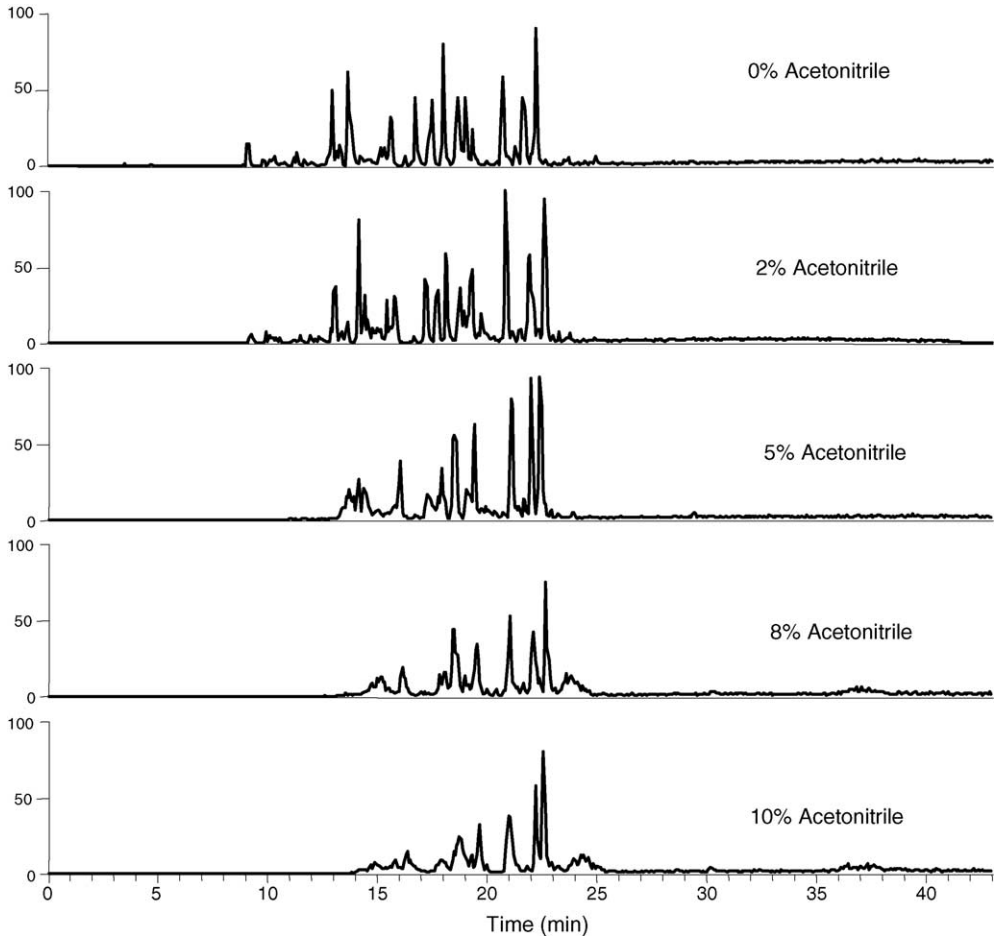


Fig. 8. Organic modifier in SCX buffer solution decreases trapping of peptides. Increasing concentrations of acetonitrile were used with 50% B concentration, 50 mM ammonium sulfate at 80 μL/min flow rate. The reconstructed ion chromatographic pattern changed markedly with greater than 5% acetonitrile concentrations due to reduced sample trapping recovery.

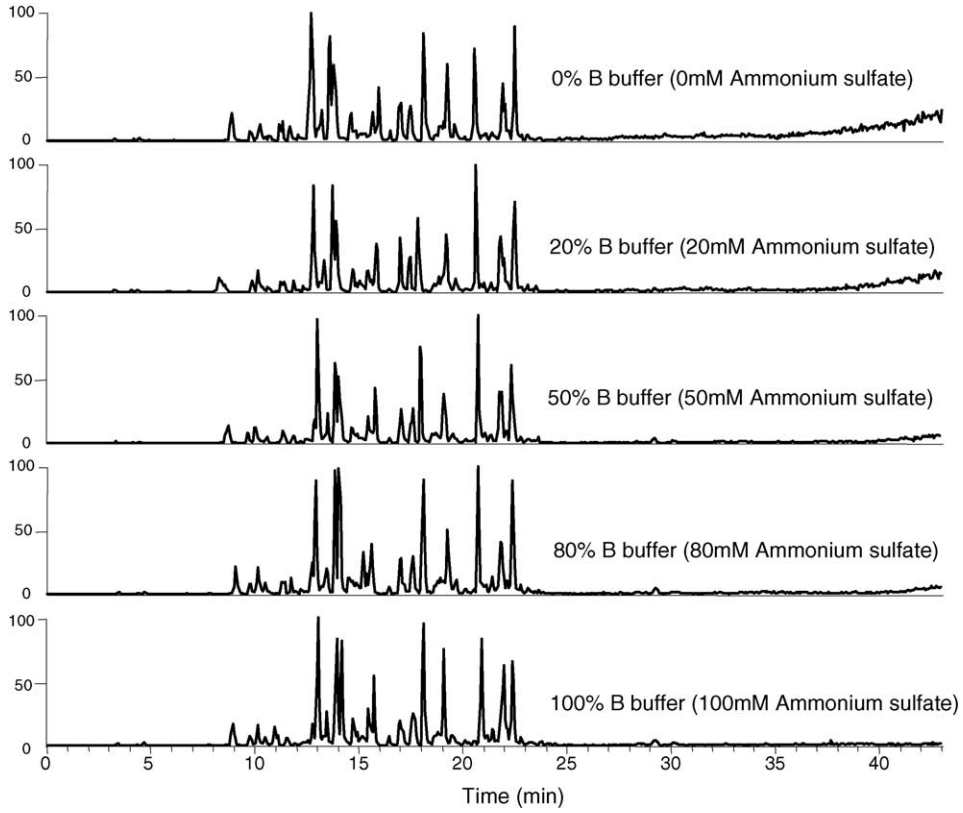


Fig. 9. The effect of ammonium sulfate concentration on trapping efficiency. Re-constructed ion chromatograms indicate minimal salt concentration effects on the trapping recovery.

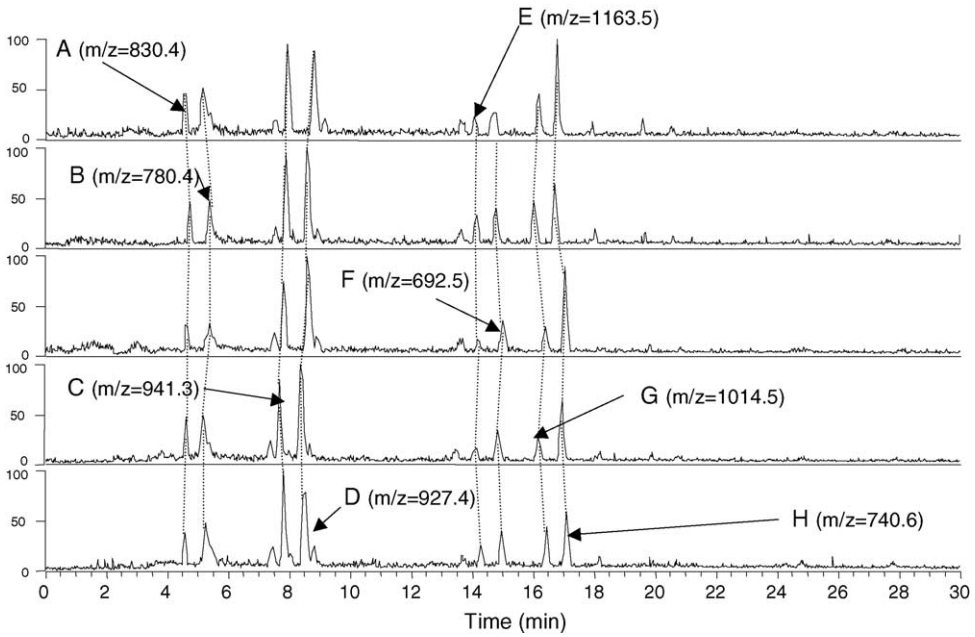


Fig. 10. Chromatograms demonstrating retention time reproducibility of the 2D microscale HPLC system. The chromatograms shown were from the third of six SCX fractions from analyses of 500 fmol of tryptic digests of BSA, β-casein, and apomyoglobin repeated five times. The retention times of the eight major peaks (A–H) varied by 1.51, 2.26, 1.31, 2.06, 0.60, 0.66, 1.05, 0.90 %R.S.D., respectively ($n = 5$).

comparisons, and the production of context-specific reports, e.g., a list of unique peptide and proteins in each data set.

3. Results and discussion

3.1. Trapping injections

Low column flow rates are required to improve mass sensitivity for ESI. However, sample loop injections are problematic at microflow rates (and especially nanoflow rates) because the relatively large sample loop or sample injection volume necessitates excessively lengthy loading periods and can cause peak broadening. Consequently, a trapping column offers a mechanism to separate sample injection flow from the analytical column flow. A trapping column should be matched to have similar, but slightly weaker retention properties, than the subsequent analytical column. Ideally, trapped analytes will be released by a lower eluotropic strength mobile phase, and will be re-concentrated at the head of the analytical column, a process resulting in sharper peaks. The choice of a polymeric C18 trapping column and a silica based C18 analytical column meets these requirements. Secondarily, the trapping, release and re-concentration cycle produces another stage of desalting, a desirable side-benefit for ESI.

The evaluation of trapping column configuration shown in Fig. 1 was performed using serial dilutions of tryptic digests of protein standards. Samples were prepared from the stock solution of each digest in 100 fmol increments, i.e., 100 fmol (10 μL of 10 fmol/μL), 200 fmol (10 μL of 20 fmol/μL) to 1 pmol (10 μL of 100 fmol/μL), and were injected sequentially into the automated 1D microflow system using an autosampler. The reproducibility of retention times of major peaks was 0.3–0.8 %R.S.D.; the limit of detection, as determined from reconstructed ion chromatograms, was approximately 100 fmol based on the representative signal to noise ratio of each peak (S/N ≥ 3) as shown in Fig. 6.

3.2. Microscale automated 2D configuration

A microscale automated 2D configuration was built with a unique valve configuration selected to meet the requirements of first dimension SCX and second dimension RP mode. Desalting was performed using a combination of switching valves that allowed the use non-volatile strong salts such as ammonium sulfate without resulting in salt deposition on the inlet surfaces of the mass spectrometer. The chromatographic elution pattern from an SCX column (1.0 mm × 50 mm Poly sulfoethyl A) is shown in Fig. 7 for a mixture of 50 pmol tryptic digests of apomyoglobin and BSA with 100 pmol of β-casein using a UV detector (SPD-10A_{VP}, Shimadzu) with semi-micro flow cell at 280 nm. Optimal SCX column performance would be attained if acetonitrile was added [18], but organic modifiers preclude efficient trapping and reduce recovery as shown in Fig. 8. The effect of ammonium sulfate concentration on the second dimension RP was evaluated. Increasing salt concentration did not affect the recovery or RP performance as shown in Fig. 9.

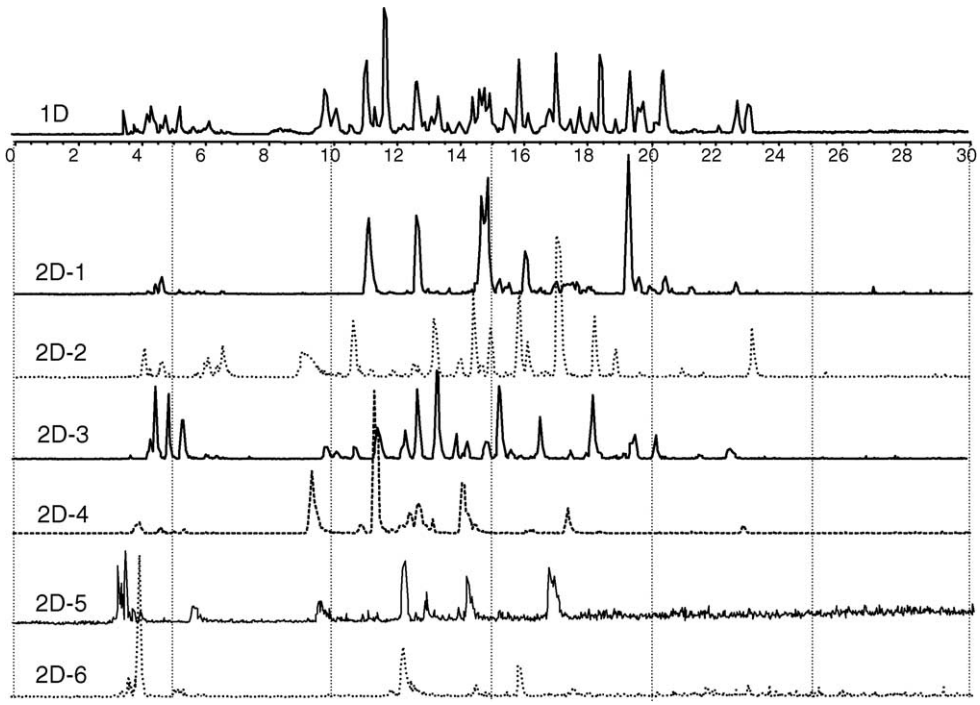


Fig. 11. Reconstructed ion chromatograms of analyses using 1D RP (top panel) and 2D SCX-RP (2D-1 through 2D-6) are displayed. SCX fractions were eluted with 0, 10, 20, 30, 50 and 100% of B buffer containing 100 mM of ammonium sulfate, respectively. Relative intensity is not same for each chromatogram.

3.3. Reproducibility and comparison of chromatographic elution patterns between 1D and 2D

Chromatographic reproducibility was evaluated by measuring relative standard deviation of retention times for eight major peaks in the third SCX fraction on the RP chromatogram of 500 fmol a mixture of three protein digests (BSA, β-casein and apomyoglobin) ($n = 5$) as shown in Fig. 10. The measured %R.S.D. of each major peak was 0.5–2%. Chromatographic elution patterns using trapping column injection and 1D RP and 2D SCX/RP analyses are stack-plotted in Fig. 11 for a mixture of tryptic digests of six proteins. Components appearing on the 1D RP chromatogram are dispersed into each of the 2D SCX fractions.

3.4. Nanoflow 1D/2D configuration

Both microflow and nanoflow configurations used the same trapping injection concept. However, nanoflow injection requires that volumes be lower than for microflow due to the reduced capacity of the 0.075 mm × 100 mm RP column. Trapping injection was evaluated for the nanoflow configuration at an optimized flow rate of 400 nL/min and substituting a six-port two-position switching valve with lower internal port-to-port volume. The resulting sensitivity gain over the

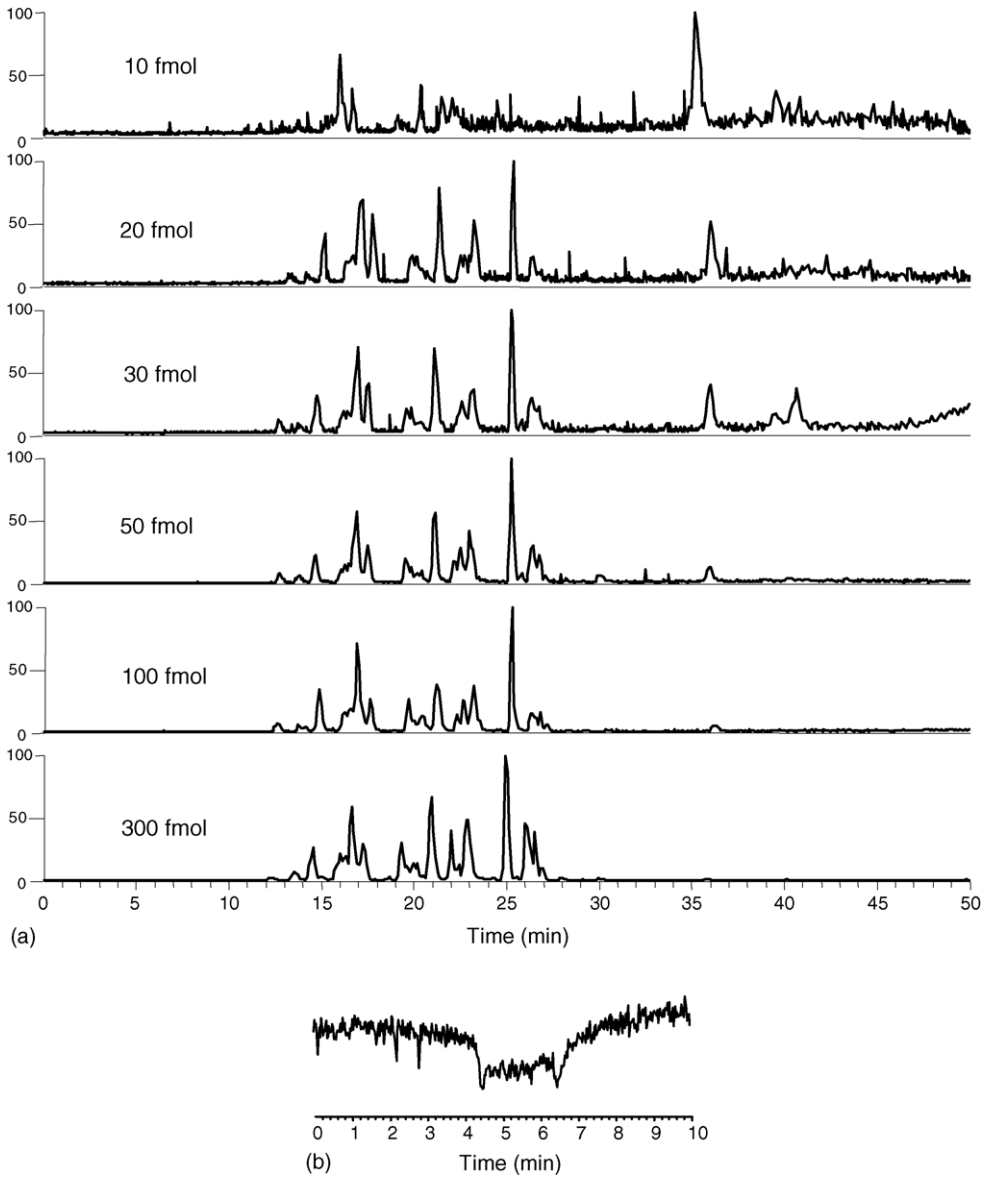


Fig. 12. (a) Plotted re-constructed ion chromatograms in a stacked format for the same protein digest demonstrating approximately 10-fold higher sensitivity using the 1D nano- vs. the microscale system. Samples contain from 10 to 300 fmol of the three tryptic digests. (b) An expanded portion of the total ion chromatogram from 0 to 10 min from one analysis shown in (a). The base line fluctuation from 4 to 7 min reflects elution of desalting solution from the trapping column, indicating that the void volume displacement is approximately 7 min.

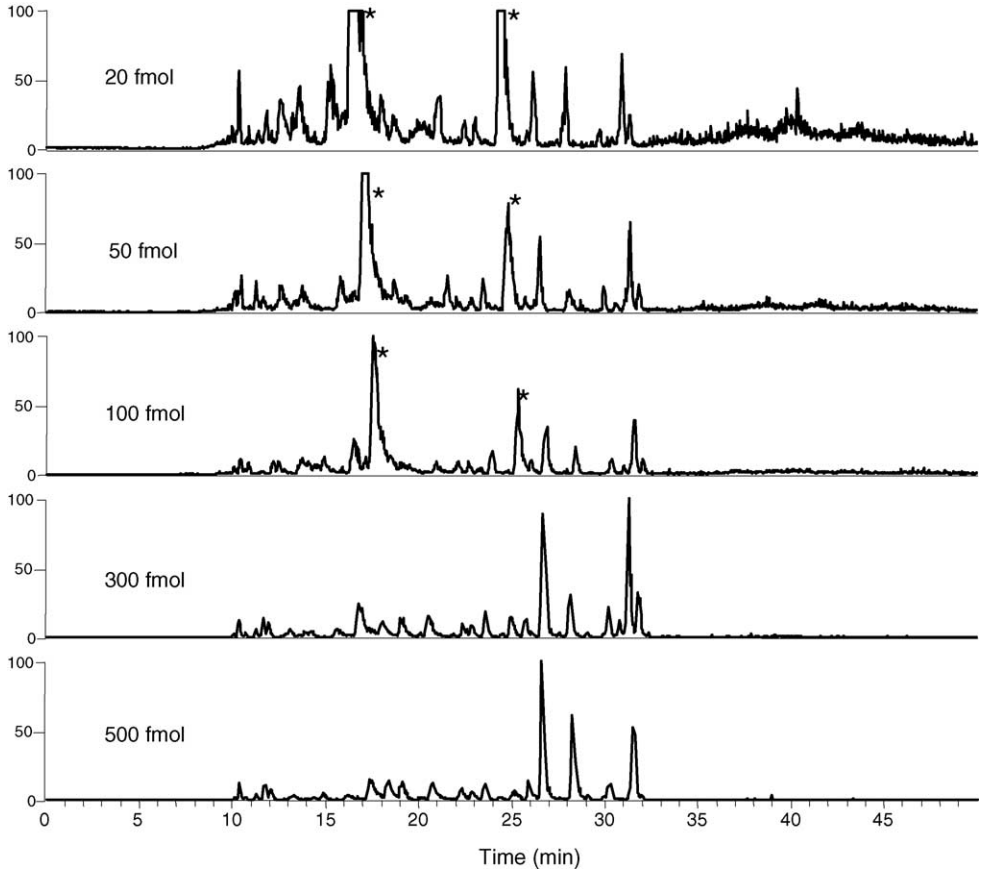


Fig. 13. Reconstructed ion chromatogram from injections varying from 20 to 500 fmol of tryptic digests of BSA, β-casein and apomyoglobin demonstrating increased sensitivity for nanoscale chromatography with ESI MS. These chromatograms were the second SCX fractions of each sample analysis. Peaks with (*) are due to contamination.

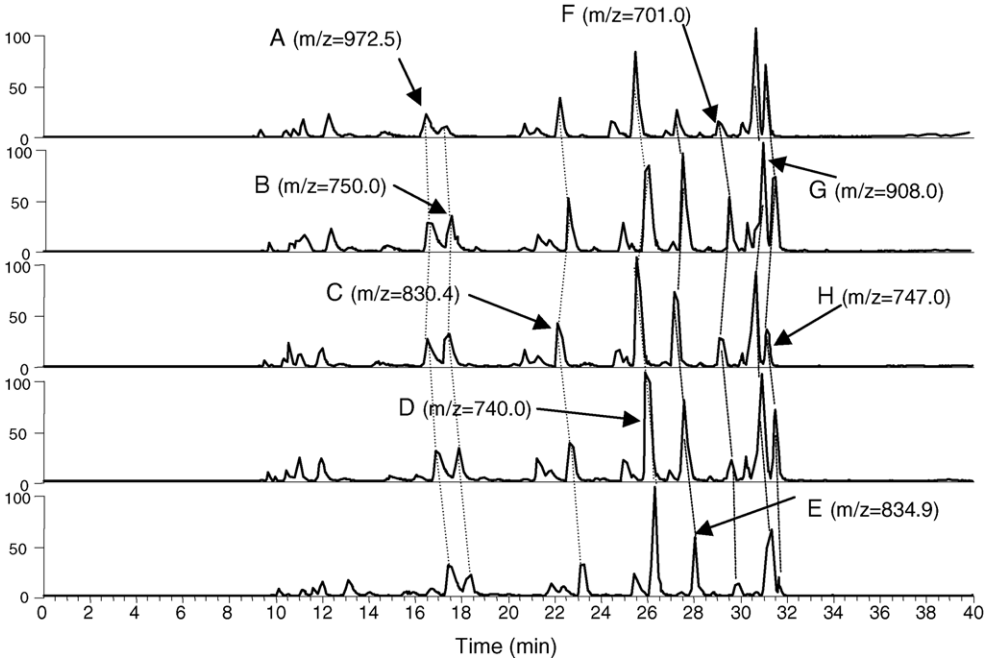


Fig. 14. Chromatograms demonstrating reproducibility of the 2D nanoscale HPLC system. The chromatograms shown were the second of six SCX fractions from analyses of 500 fmol of tryptic digests of BSA, β-casein, and apomyoglobin repeated five times. The retention times of the eight major peaks (A–H) varied by 2.35, 2.38, 1.97, 1.40, 1.29, 1.29, 0.91, 0.77 %R.S.D., respectively ($n = 5$).

microflow system (Fig. 6) is seen in the serial dilutions from 10 fmol to 1 pmol of protein digest mixtures in Fig. 12(a). These data indicate that nanoflow sensitivity was about 10 times higher than microflow, consistent with the predicted concentration increase. The internal volume of the trapping column and the switching valve were acceptable for nanoflow as the void volume retention time associated with the loading time from the trapping column to RP column was determined to be about 7 min shown as Fig. 12(b).

For the nanoflow configuration, it was also necessary to substitute lower internal volume rotary valves. For mechanical reliability and longevity, three two-position valves were used as shown in Fig. 4(a–c). Because the SCX column was mounted on a two-position valve, it could be switched offline from the flow path between steps to prevent inadvertent elution. Valve A in Fig. 4(a) and (b) serves for SCX solvent selection or desalting. We found that 4 min of desalt wash was necessary and sufficient to avoid deposition of any salt residue on the surface of the mass spectrometer heated capillary inlet. The results of injecting a concentration ladder from 20 fmol to 1 pmol indicated that 1D and 2D systems exhibited the same sensitivity (Fig. 13).

Alternatively, the valve configuration shown Fig. 4(a–c) can be used for 1D RP analysis by software selection of valve position selections. For 1D analyses, sample injection is performed with the SCX column in the offline position described in Fig. 4(a).

3.5. Performance of 1D/2D nanoflow automated system

The performance of the 1D/2D nanoflow configuration was evaluated with regard to sensitivity, reproducibility, and reliability. To illustrate reproducibility in the 2D mode, chromatograms of the second of the six SCX fractions from five repeat runs are plotted in Fig. 14. The measured retention time variation of major peaks was 0.8–2.4 %R.S.D. The results of automated Mascot database searches were evaluated for 20–200 fmol concentrations of BSA and apomyoglobin. Table 1 shows the number of identified peptides from these analyses and demonstrates that the 2D nanoHPLC system exhibits performance attainable with non-automated systems, requisite for routine high sensitivity peptide and protein identification.

Table 1
Number of identified peptides from BSA and apomyoglobin using automated Mascot MS/MS ion search for data acquired using the automated 2D nano HPLC system

Amount injected (fmol)	Number of identified peptides	
	BSA	Apomyoglobin
20	1	1
50	5	1
100	3	1
300	8	4
500	11	6

All peptides exhibited ion scores greater than identity scores. The DBParser program extracted the summary peptide lists [17].

4. Conclusions and perspectives

Employing a unique valve configuration permits routine microflow and nanoflow automated software controlled 1D or 2D-HPLC. Software controlled valves achieve efficient online desalting, permitting ion exchange chromatography with non-volatile salts in the first dimension. The automated injection trapping and desalting are compatible with repetitive, routine direct injections of peptide digests for high sensitivity nanoflow 1D or 2D-HPLC ESI-MS for proteomics research.

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