

Fully Automated Off-line Multidimensional LC Methods in Proteomics

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INTRODUCTION

Multidimensional liquid chromatography has matured as a separation technique for complex sample analysis in proteomics. Two-dimensional LC, often a combination of ion-exchange and reversed phase chromatography, has been applied for peptide and protein separations, in either on-line or off-line approaches. Off-line 2-D LC methods have the advantage that the method is more flexible and easier to optimize with the drawback of being more laborious.

Here we present an LC instrument designed for fully automated multidimensional LC. The autosampler is equipped with an injector that allows both injection and fractionation. The pump module comprises two gradient pumps for the first and second dimension separation. The instrument injection and fractionation performance is discussed. An application of fully automated 2-D LC of peptides is presented.



Figure 1A. UltiMate™ 3000 Capillary/Nano LC System with fractionation option and dual-gradient pump.

INSTRUMENTAL SET-UP

The system for fully automated off-line multidimensional LC consists of a UltiMate 3000 Capillary/Nano LC with an 8-port injector as shown in Figure 1B.

For sample loading the valve is in position 1-2, connecting the syringe with the sample loop and the 15 μ L injection needle. After filling the loop the sample is injected by switching the valve to position 8-1. The column (pos 4) is now connected with the injection needle through pos 6, allowing the mobile phase to be fractionated. A dual needle design (pre-puncture and injection needle) makes fractionation of the LC run in open and closed vials possible. When no fractionation is required the needle is moved to a drain position.

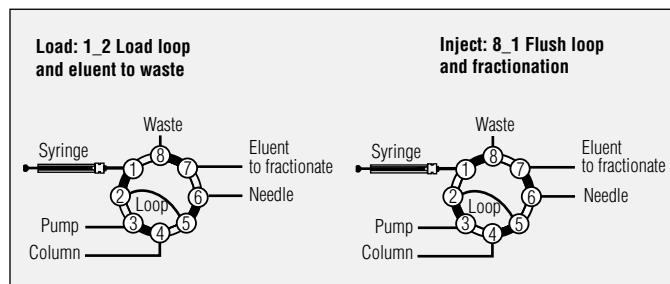


Figure 1B. Autosampler 8-port injection valve for sample injection and fractionation.

INJECTION AND FRACTIONATION PERFORMANCE

The injection performance of the autosampler was studied by flow injection analysis and in RP-HPLC. For experimental conditions see Table 1.

Table 1. Experimental Conditions for Injection and Fractionation Measurements		
	FIA	HPLC
Column	n.a.	Acclaim C18 120, 2.1 mm x 10 cm
Mobile Phase	Water + 0.05% TFA	Water/ACN 40:60 v/v%
Sample	Caffeine, 10 µg/mL water	Methyl-, propyl-, and butylparabene 10 µg/mL
Injection Volume	5 µL	5 µL
UV Detection	272 nm (180 nL flow cell)	254 nm (500 nL flow cell)
Oven Temperature	25 °C	20 °C
Flow Rate	100 µL/min	250 µL/min
Fractionation	n.a.	5 x 30s fraction collected, 1/25 th reinjected

Both in FIA and RP-HPLC mode the injection reproducibility and the carryover are excellent as shown in Table 2.

Table 2. Injection Performance		
	FIA (caffeine)	RP-HPLC (propylparabene)
Repeatability (Peak Area, n=10)	0.1% RSD	0.34% RSD
Carryover (n=3)	0.005%	0.026%

The fractionation performance was evaluated by an isocratic separation of three alkylparabenes. The sample was fractionated in 30 s intervals and collected in sample vials (see Figure 2). Out of each 125 µL fraction, 5 µL was reinjected to assess the reproducibility of the procedure. The RSD and carryover for three fractionation experiments is shown in Table 2 for peak #2 and compared to a caffeine standard sample.

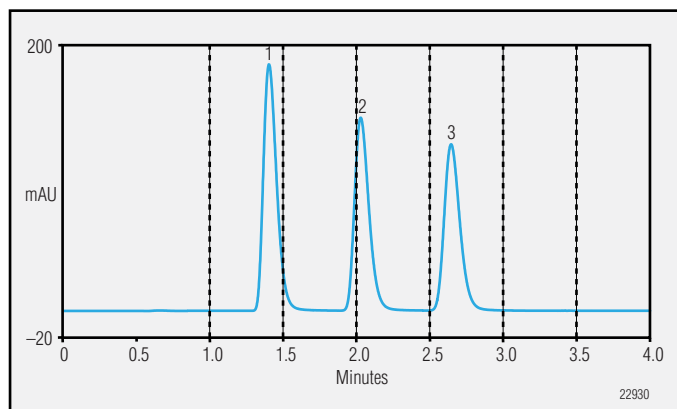


Figure 2. Isocratic separation of alkylparabenes with indicated fractionation events. Peaks: (1) methyl-, (2) propyl-, and (3) butylparabene.

Table 3. Fractionation Performance			
	Methyl-parabene	Propyl-parabene	Butyl-parabene
Recovery (%)	110	104	104
Repeatability (%RSD)	6	2	2

The fractionation performance of the autosampler shows acceptable recovery and good precision.

FULLY AUTOMATED OFF-LINE MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY

The fractionation option of the autosampler allows for fully automated off-line 2-D LC when the injection/fractionation valve is combined with one or two 2-position switching valves.

Figure 3 shows the experimental setup for fully automated off-line multidimensional LC of peptides. Peptides are separated on a SCX column, fractionated through the injection needle in vials, reinjected onto a monolithic trap and separated on a capillary PS-DVB monolithic column. Two independent gradient pumps supply the mobile phases for the ion-exchange and reversed-phase separations.

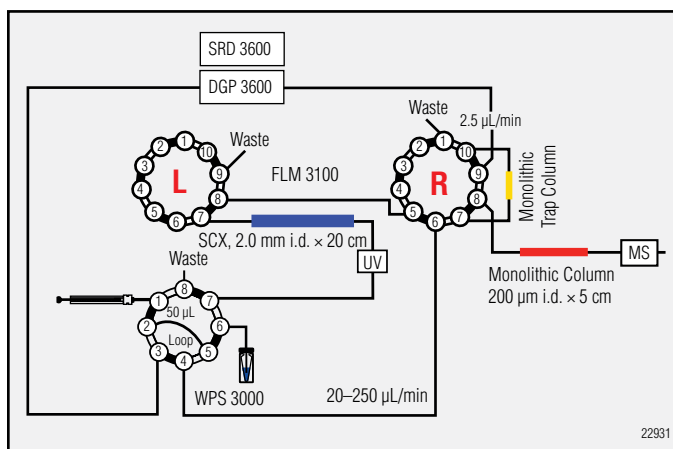


Figure 3. LC setup for fully automated off-line 2-D LC of peptides.

Table 4. Experimental Conditions for Fully Automated Off-line 2-D LC		
	First dimension – SCX	Second dimension – RP
Column	PolySULFOETHYL, 2.1 mm x 20 cm, 5 µm, 200 Å	PS-DVB Monolith, 200 µm i.d. x 5 cm PS-DVB Monolithic trap, 200 µm i.d. x 5 mm
Mobile Phase A	95/5 Water/ACN, 5 mMol phosphate, pH = 3	Water + 0.05% TFA
Mobile Phase B	95/5 Water/ACN, 5 mMol phosphate, pH = 3, 1 M NaCl	20/80 Water/ACN + 0.04% TFA
Loading Solvent		Water + 0.05% TFA at 20 µL/min
Flow Rate	250 µL/min	2.5 µL/min
Gradient	0% to 60% B in 20 min 5 min wash at 100% B 20 min equilibration	0% to 35% B in 10 min 90% B for 2 min 6 min equilibration
UV Detection	214 nm (500 nL flow cell)	214 nm (3 nL flow cell)
Sample	Digest of 6 proteins (4 pmol/µL)	SCX fractions
Injection Volume	25 µL	25 µL
Fractionation	20 fractions of 1 min	
Oven Temperature	Ambient	40 °C

The first and second dimension separations of the protein digest sample are shown in Figure 4. The chromatograms show good chromatographic resolution and efficient peptide elution between IEX fractions. The total analysis time of the 2-D LC method is approximately 11 h for 20 RP fractions.

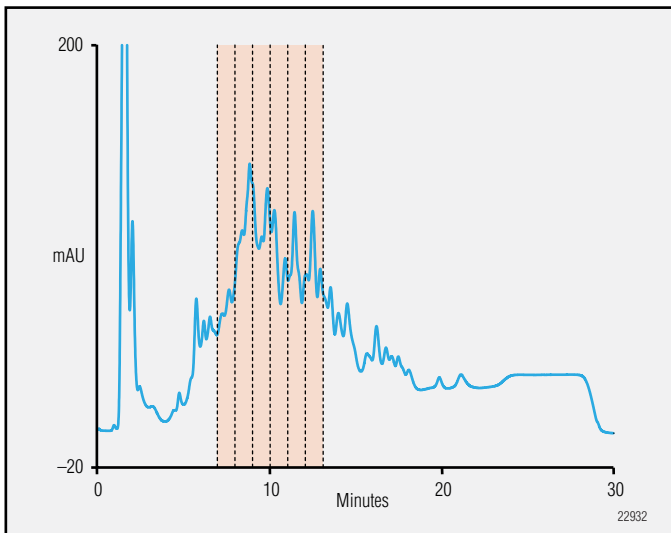


Figure 4A. First dimension separation of peptides on SCX column.

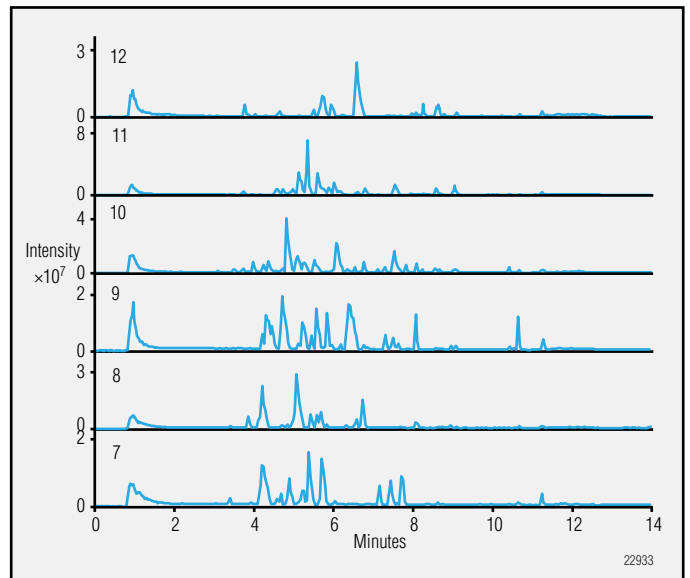


Figure 4B. BPC-MS of second dimension separation on PS-DVB monolithic column of peptide fractions 7–12.

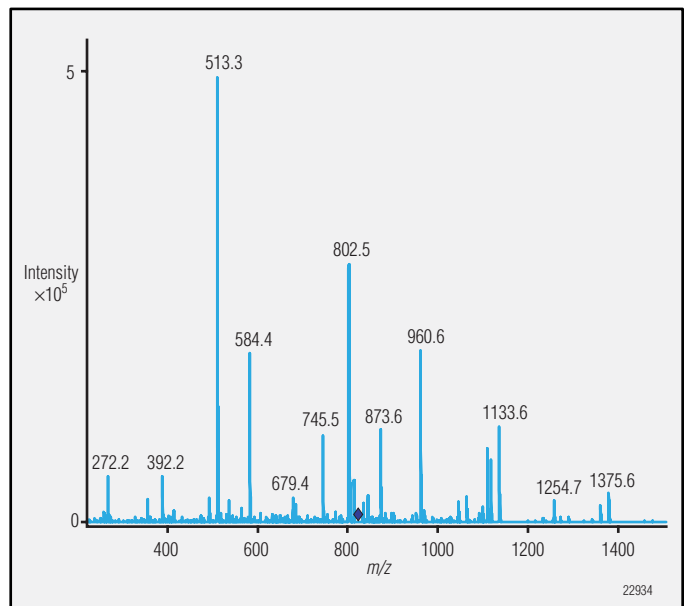


Figure 4C. MS/MS spectrum of Serotransferrin peptide FDEFFSAGCAPGSPR.

**Table 5. Protein Sequence Coverage (%)
1-D LC vs. 2-D LC (Total MASCOT Ion Score)**

Protein, (Mw, kDa)	1-D LC	2-D LC
Cytochrome c (11.7)	17 (102)	61 (492)
Lysozyme (14.8)	30 (249)	81 (668)
Alcohol dehydrogenase (37.3)	28 (368)	53 (832)
Bovine serum albumin (71.3)	31 (863)	70 (2389)
Serotransferrin (80.0)	36 (1380)	66 (2850)
β -galactosidase (117.4)	41 (1675)	54 (2533)

The separated peptides from the protein digest mixture were identified with tandem MS. The off-line 2-D LC method was compared to a RP separation of 1 h on the 200- μ m i.d. monolithic column. Significantly improved sequence coverage and MASCOT ion score were obtained for all proteins, indicating the power of the multidimensional LC method (Table 4). In addition it was confirmed that all peptides eluted in one or two SCX fractions only.

CONCLUSIONS

- An autosampler with combined injection and fractionation capabilities has been developed.
- The injection and fractionation performance is excellent in terms of repeatability, carryover, and recovery.
- The dead volume of the fraction collector is as small as 2.4–15 μ L depending on the size of the injection needle.
- The instrument allows for fully automated off-line 2-D LC.
- Off-line 2-D LC applications for peptides and proteins can be run automatically, that is, sample injection, fraction collection, and reinjection of collected fractions on a second column. A second fractionation step is optional.

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