Analysis of *E.coli* Tryptic Digest and Intact Protein Using the Agilent 1290 Infinity 2D-LC Solution with Diode Array Detection and Q-TOF LC/MS

Application Note

Proteomics & Protein Sciences

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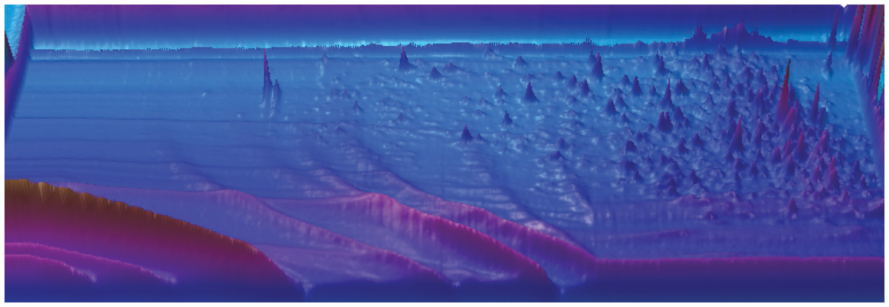
Abstract

The Agilent 1290 Infinity 2D-LC Solution was used for the comprehensive two-dimensional LC analysis of complex protein and peptide samples by combining strong cation-exchange (SCX) and reversed-phase LC (RPLC) in first and second dimensions, respectively. The SCX × RPLC separation was coupled to diode-array detection (DAD) and an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS. The features of the setup are demonstrated by the analyses of both intact proteins and tryptic digest peptides, originating from an *Escherichia coli* (*E. coli*) lysate, with the same system and column configuration. The LC × LC experimental peak capacity for the analysis of the tryptic digest ranged from 1,156 to 2,250 for 75-minute and 225-minute analysis times, respectively.



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Introduction

In the field of protein analysis, sample complexity is enormous. It is not uncommon to be confronted with samples containing hundreds of proteins, and following digestion, thousands of peptides. Unraveling this complexity demands the best separation power. This is mainly determined by efficiency and selectivity, and can be measured by peak capacity, that is, the number of peaks separated with unit resolution that can fit into a certain retention time window or analysis. Comprehensive two-dimensional LC (LC × LC) is the most drastic means of maximizing peak capacity, as long as the two dimensions are orthogonal and the separation obtained in the first dimension is maintained upon transfer to the second dimension[1,2]. Since ion-exchange chromatography (IEX) and RPLC are orthogonal techniques and are useful for the analysis of intact proteins as well as for their digests, a combination of both in one LC × LC configuration enables significant enhancement of peak capacity, thereby producing a detailed characterization of the sample.

This Application Note builds on an earlier publication[3], and describes the combination of SCX with RPLC for the analysis of *E.coli* tryptic digest and intact protein using the Agilent 1290 Infinity 2D-LC Solution. Both intact protein and peptide analyses were done with the same instrument and column configuration using diode-array detection (DAD) and an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS.

Experimental

Samples and sample preparation

The *E. coli* sample (*E. coli* protein sample, 2.7 mg, lyophilized control) was purchased from Bio-Rad Laboratories N.V., Temse, Belgium. Lysates were dissolved in 8 M urea buffered at pH 5.6 with phosphate to a final concentration of 2.7 mg/mL. The digest was prepared by desalting the lysate, followed by reductive alkylation with dithiothreitol and iodoacetamide, and by trypsin digestion in 100 mM tris-HCl pH 8. The final solution contained 8 mg/mL digested *E. coli* proteins.

Instrumentation

An Agilent 1290 Infinity 2D-LC Solution was hyphenated to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS, with the following components:

- Two Agilent 1290 Infinity Binary Pumps with seal wash option (1st and 2nd dimension) (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Autosampler Thermostat (G1330A)
- Two Agilent 1290 Infinity Thermostatted Column Compartments (1st and 2nd dimension) (G1316C)
- Agilent 1290 Infinity Diode Array Detector with standard flow cell (10 mm, 1 µL) (G4212A)
- Agilent 1290 Infinity Valve Drive (G1170A)
- Agilent 2D-LC Valve (2-position/4-port duo valve) (G4236A)

Software

- Agilent OpenLAB CDS ChemStation Revision (C.01.06) (with 2D-LC add-on software)
- Agilent MassHunter (B.05.01) (for instrument control)
- Agilent MassHunter Data Acquisition (B.06.00)
- Agilent MassHunter BioConfirm (B.06.00)
- Agilent MassHunter Spectrum Mill (B.04.00)
- GC Image LC × LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

Table 1 shows the capillaries required to set up the system, and Figure 1 shows a scheme of the setup.

Table 1. Connections for the instrument modules. Capillary numbers refer to capillary connections in Figure 1.

Capillary no.	Connections	Capillary type
1	D1 pump to ALS	Calibration capillary (p/n G1312-67500)
2	ALS to D1 column (1.6-µL heat exchanger)	Stainless steel, 0.17 mm
3	D1 column to 2DLC valve	Stainless steel, 0.12 × 200 mm
4	D2 pump to 2DLC valve	Stainless steel, 0.17 mm
5	2D-LC valve to D2 column (1.6-µL heat exchanger)	Stainless steel, 0.12 × 270 mm
6	T-piece to DAD	Stainless steel, 0.12 × 270 mm
7	T-piece to MS (AJS source or DAD D2)	Stainless steel, 0.075 × 340 mm (p/n 5067-4783)

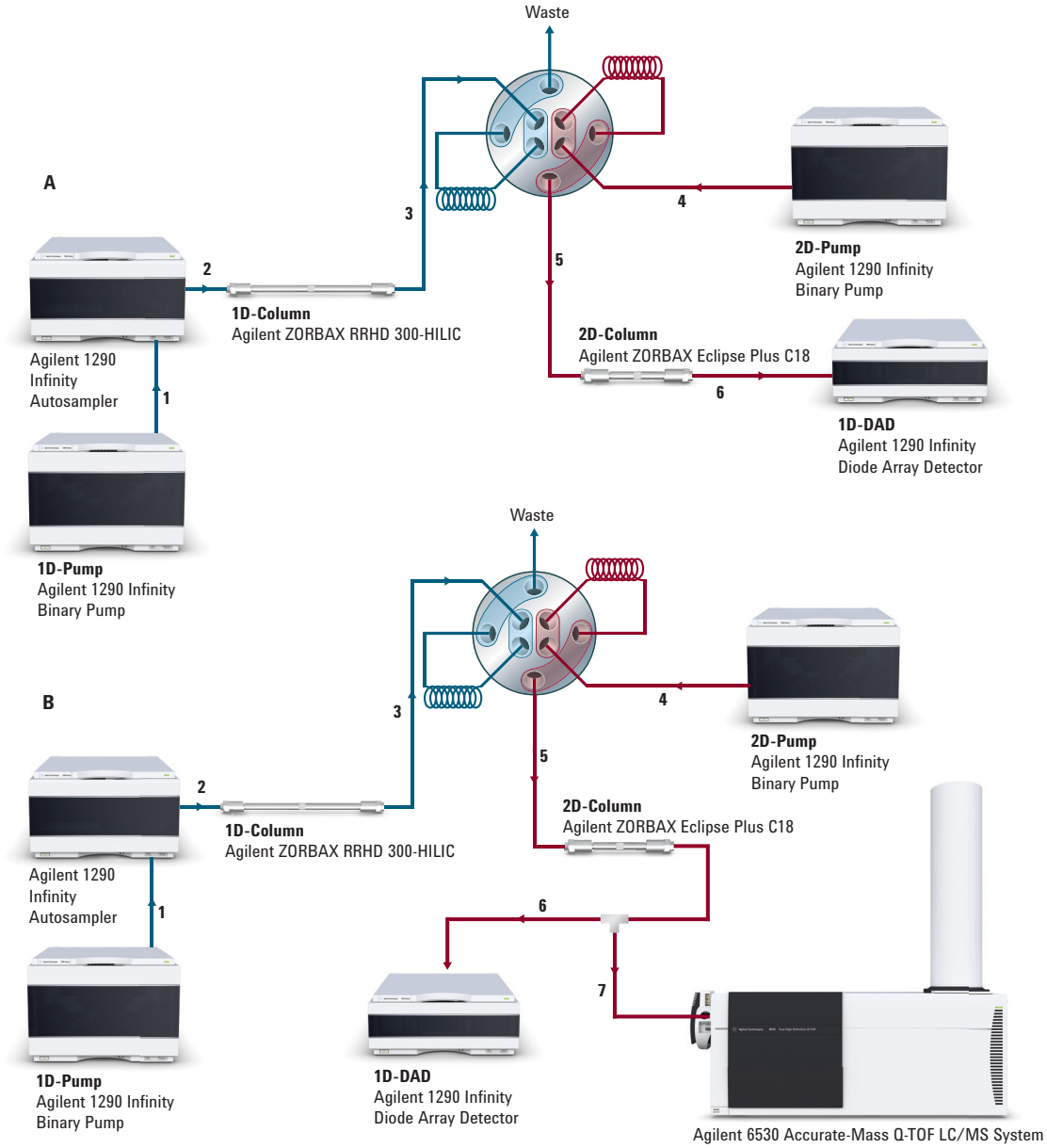

Figure 1. Scheme of the instrument configurations with DAD (A) and MS (B).

Instrumental conditions

1st Dimension	Tryptic digest analysis	Intact protein analysis
Column	Agilent Bio SCX, NP 10, 2.1 × 250 mm, 10 µm, PEEK (p/n 5190-2439)	
Mobile phase	A) 10 mM phosphate pH 3.0 B) 10 mM phosphate pH 3.0 + 1 M NaCl	A) 10 mM phosphate pH 5.4 B) 10 mM phosphate pH 5.4 + 1 M NaCl
Flow rate	65 µL/min	65 µL/min
Gradient	0 to 35 minutes, 0 to 5 % B (0 to 70 minutes[1], 0 to 105 minutes[2]) 35 to 0 minutes, 15 to 80 % B (70 to 140 minutes[1], 105 to 210 minutes[2]) 70 to 75 minutes, 80 % B (140 to 150 minutes[1], 210 to 225 minutes[2]) Post time, 10 minutes at 0 % B	0 to 5 minutes, 0 % B 5 to 45 minutes, 0 to 100 % B 45 to 55 minutes, 100 % B Post time, 10 minutes at 0 % B
Temperature	23 °C	23 °C
2nd Dimension		
Column	Agilent ZORBAX 300SB-C18, 4.6 × 50 mm, 3.5 µm (p/n 865973-902)	
Mobile phase	A) 0.1 % phosphoric acid in water (for DAD) A) 0.1 % formic acid in water (for MS) B) Acetonitrile	A) 0.1 % phosphoric acid in water (for DAD) A) 0.1 % formic acid in water (for MS) B) Acetonitrile
Flow rate	3.5 mL/min	3 mL/min
Idle flow rate	0.4 mL/min	0.4 mL/min
Initial gradient	0 to 0.35 minutes, 2 to 41 % B 0.35 to 0.4 minutes, 41 % B 0.4 to 0.5 minutes, 2 % B	0 to 0.40 minutes, 2 to 62 % B 0.4 to 0.5 minutes, 2 % B
Gradient modulation	2 to 41 % B at 25 minutes (50 minutes[1], 75 minutes[2]) 2 to 60 % B at 65 minutes (130 minutes[1], 195 minutes[2])	15 to 75 % B at 40 minutes
Temperature	50 °C	60 °C
Modulation		
Modulation on	5 to 70 minutes (5 to 140 minutes[1], 5 to 210 minutes[2])	5 to 50 minutes
Loops	Two 40-µL loops, cocurrent configuration	Two 40-µL loops, cocurrent configuration
Modulation time	0.5 minutes	0.5 minutes
Injection		
Volume	2 µL	20 µL
Temperature	8 °C	8 °C
Needle wash	5 seconds flush port (water:acetonitrile 50:50)	5 seconds flush port (water:acetonitrile 50:50)
Detection DAD		
Wavelength	Signal 214/4 nm, ref. 360/100 nm	Signal 214/4 nm, ref. 360/100 nm
Data rate	80 Hz	80 Hz

[1] Gradient/analysis time 140/150 min
[2] Gradient/analysis time 210/225 min

Detection MS	MS and MS/MS	MS Centroid and Profile
	Agilent JetStream Technology source, positive ionization	Agilent JetStream Technology source, positive ionization
Drying gas temperature	340 °C	340 °C
Drying gas flow	10 L/min	10 L/min
Nebulizer pressure	45 psig	45 psig
Sheath gas temperature	400 °C	400 °C
Sheath gas flow	11 L/min	11 L/min
Capillary voltage	3,500 V	3,500 V
Nozzle voltage	1,000 V	1,000 V
Fragmentor	175 V	200 V
	Centroid data at 8 spectra/s	Centroid or Profile data at 8 spectra/s
Mass range	MS, 200 to 3,000 *m/z* MS/MS, 100 to 3,000 *m/z*	400 to 3,200 *m/z*
Extended dynamic range (2 GHz)	Resolution 10,000 for *m/z* 1,000	Resolution 10,000 for *m/z* 1,000
MS/MS settings		
	Auto MS/MS based on precursor abundance	
	Three precursors/cycle	
	Priority 2+ and 3+ ions	
	Isolation width, medium	
	Collision energy, 4 × (*m/z*/100) – 4.8	

Results and Discussion

Comments on the methods

To enable good detection of the peptides and proteins with diode-array detection, phosphoric acid was chosen as the RPLC additive (instead of TFA or formic acid) because of its transparency at 214 nm. When using MS for detection, the inorganic acid was replaced with formic acid. Adding the same amount (0.1 %) of acid to the aqueous component of the RPLC mobile phase gave similar selectivity, as shown below.

Replacing the nonvolatile phosphoric acid with an MS-compatible acid is only one of the adjustments that need to be made to enable MS detection. The significant amount of salt used in the first dimension will be transferred to the second dimension on each modulation, and will elute as a salt plug in every second dimension gradient. It is not possible to use the MS diverter valve to send the salt plug to the waste for a short time, as this would add too much dead volume, and would adversely affect second dimension efficiency. All NaCl will thus enter the MS source and will accumulate mainly on the spray shield (Figure 2). The salt can be removed by simply rinsing the surface with pure water without removing the source from the system. No significant loss in sensitivity was observed. To keep the amount of salt that enters the source with each analysis as low as possible it is, however, advisable to disable the modulation when no compounds of interest are eluting. This is especially beneficial at the end of the run when the NaCl concentration is highest (1 M). When modulation is switched off, the first dimension effluent passes through one of the modulator valve loops to waste. The second dimension flow rate can also be reduced during this period to reduce solvent consumption. However, more frequent cleaning of ion optics and replacement of the inlet capillary might be required if higher salt concentrations are used over a longer time period. Further, sensitivity might be reduced.

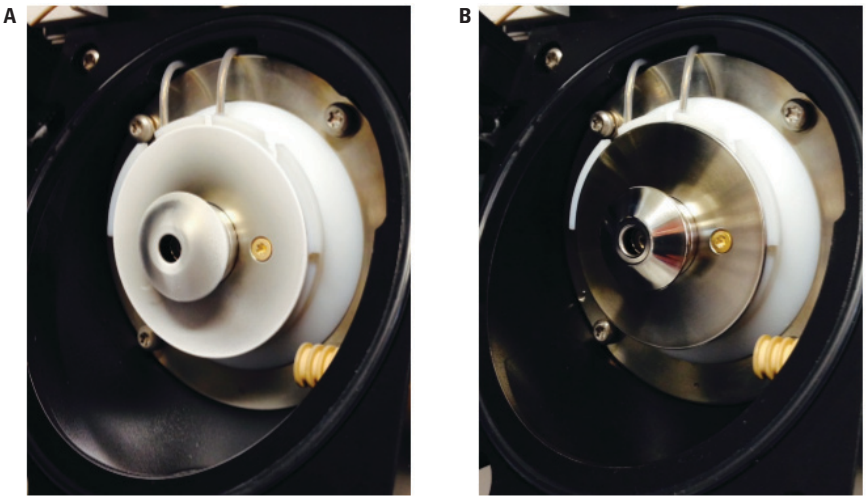

Figure 2. MS source spray shield at the end of a sequence (A) and after cleaning (B). The accumulated salt can easily be removed by rinsing with pure water.

With diode-array detection, the complete second dimension flow is directed through the detector cell with a 0.12-mm id stainless steel capillary, but this is not possible with MS. The flow rate of 3 to 4 mL/min is too high for the system, and the flow needs to be split prior to entering the source. This is done with a zero-dead-volume T-piece after the second dimension column, which splits the flow to a 75-µm id capillary that connects to the MS inlet and a 0.12-mm id capillary connected to the DAD. The 75-µm id capillary creates sufficient restriction to split the flow approximately 1/10 with minimal band broadening because of its low internal volume. This configuration is shown in Figure 1.

Analysis of tryptic digest

The main goal of 2D-LC analysis is to have a significantly increased peak capacity compared to 1D-LC. The combination of SCX at pH 3 and RPLC results in excellent orthogonality. Compounds that remain unresolved on SCX are typically separated in the second dimension RPLC analyses. Since SCX cannot compete with RPLC in terms of efficiency, this orthogonality is of the utmost importance.

Peak capacity can be maximized by increasing the first dimension gradient time, that is, lowering the slope of the salt gradient. This approach was reported for bovine serum albumin (BSA) digests using RPLC[4]. In this work, we evaluated gradient/analysis times of 70 to 75 minutes, 140 to 150 minutes (original analysis time x2), and 210 to 225 minutes (original analysis time x3).

The results for the analyses of the *E. coli* digest with DAD at 214 nm are shown in Figure 3. From the contour plots, it is clear that the peak capacity increased as the gradient time increased. This was solely due to the increased peak capacity in the first dimension, as the second dimension parameters (and peak capacity) were not changed.

Peak capacity in 1D-LC can be calculated by dividing the elution time window with the average peak width at the base (4σ). In theory, the total peak capacity in LC × LC is the product of the peak capacities of each of the dimensions. In the best case, this value needs to be corrected with a factor of approximately 0.5 to 0.65 for completely orthogonal systems[5,6]. The average peak width was approximately 1.1, 1.3, and 1.6 minutes in the first dimension for a 75, 150, and 225-minute gradient, respectively, and 0.5 seconds in the second dimension. Taking into account that all peptides eluted between 5 and 25 seconds in the second dimension, the peak capacities can be calculated for a fully orthogonal system, as shown in Table 1.

Considering full orthogonality in our approach (SCX × RPLC), correcting these values with a factor of 0.6 is more realistic. This means that practical peak capacities will be approximately 1,156, 1,883, and 2,250 for 75, 150, and 225-minute analysis times, respectively.

To identify the peptides eluting from the second dimension column with peak widths of 0.5 seconds, a fast mass spectrometer is required. An Agilent 6530 Accurate-Mass Q-TOF LC/MS was used, operated at an acquisition speed

Table 1. Peak capacities calculated for a fully orthogonal system.

Gradient/analysis time (min)	Elution window (min)	Peak capacity
70 to 75	10 to 63	1,927
140 to 150	10 to 112	3,138
210 to 225	10 to 158	3,750

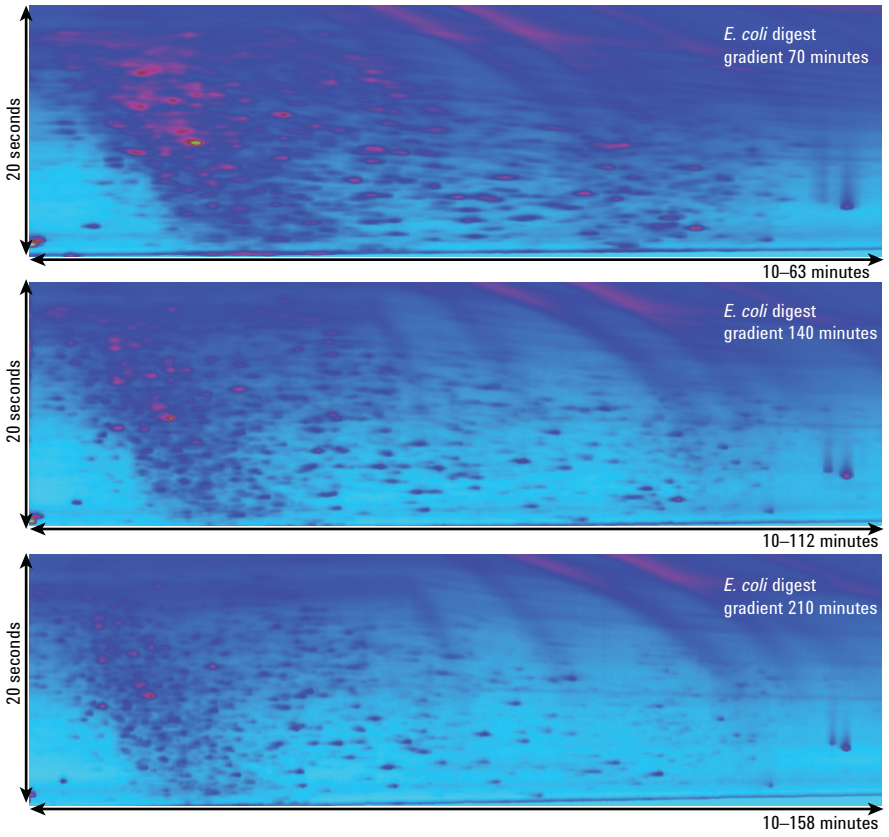

Figure 3. UV 214 nm contour plots of an *E. coli* digest run with various SCX gradient times.

of eight spectra/s in both MS and MS/MS modes. The exit of the second dimension column was coupled to a T-piece that split the flow before the MS inlet. This was required because the second-dimension flow rates of 3.5 and 3.0 mL/min respectively would have been too high for the ion source. However, this reduces sensitivity because the ion source with Jet Stream technology is mass-flow dependent. Care was taken to minimize peak broadening due to additional dead volume and connections. The result for the 150-minute gradient is shown in Figure 4 with a comparison to a UV plot. As can be seen, selectivity was largely maintained, although phosphoric acid (DAD) has been replaced by formic acid (MS) in the second dimension.

The identity of the spots was determined by searching the MS/MS spectra against the *E. coli* protein database (SWISS-PROT) using Agilent MassHunter Spectrum Mill software. To provide an example, some identifications are summarized in Table 2. Figure 5 shows MS/MS spectra of some selected peptides.

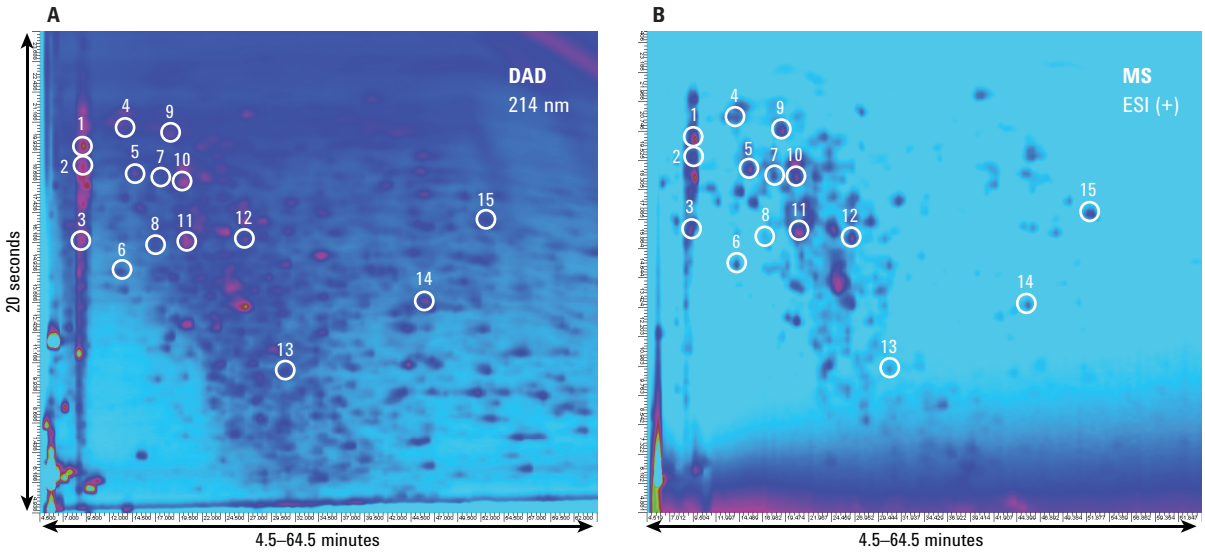

Figure 4. Comparison of 2D-LC analysis of an *E. coli* digest with UV and with MS. Gradient/analysis time was 140/150 minutes. A zoom in the region from 4.5 to 64.5 minutes is also shown.

Table 2. Identifications of spots in Figure 4.

Spot	Peptide	Protein accession	Protein description	*m/z*
1	TTDVTGTIELPEGVEMVMPGDNIK	EFTU1_ECO24	Elongation factor Tu 1	849.4184 (3+)
2	DFEAQLASTETQVGNELAPLK	ZNUA_ECOLI	High-affinity zinc uptake system protein ZnuA	754.3768 (3+)
3	DTTTIIDGVGEEAAIQGR	CH60_ECO24	60 kDa chaperonin	615.9779 (3+)
4	GFSGEDATPALEGADVVLISAGVAR	MDH_ECOBW	Malate dehydrogenase	801.4141 (3+)
5	FGVSAAAAVAVAAGPVEAAEEK	RL7_ECO24	50S ribosomal protein L7/L12	672.3513 (3+)
6	DLVESAPAALK	RL7_ECO24	50S ribosomal protein L7/L12	557.3104 (2+)
7	AVAAVNGPIAQALIGK	ENO_ECOBW	Enolase	498.2986 (3+)
8	DITADVLK	SKP_ECOLI	Chaperone protein Skp	437.7439 (2+)
9	TTLTAAITTVLAK	EFTU1_ECO24	Elongation factor Tu 1	652.3927 (2+)
10	VGEEVEIVGIK	EFTU1_ECO24	Elongation factor Tu 1	586.3307 (2+)
11	ELLSQYDFPGDDTPIVR	EFTU1_ECO24	Elongation factor Tu 1	655.6577 (3+)
12	AGENVGVLLR	EFTU1_ECO24	Elongation factor Tu 1	514.2966 (2+)
13	LLDEGR	EFTU1_ECO24	Elongation factor Tu 1	351.6919 (2+)
14	GITINTSHVEYDTPTR	EFTU1_ECO24	Elongation factor Tu 1	601.9671 (3+)
15	LGLDVLVHGEAER	METE_EODH	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	469.9215 (3+)

Intact protein analysis

The same LC × LC setup was used to analyze the intact proteins in the *E. coli* lysate. The main adaptation to the method was to increase the pH of the 1D mobile phase phosphate buffer to pH 5.4. Figure 6 shows the UV and MS contour plots of the *E. coli* lysate. The MW values associated with the labeled spots are shown in Table 3. Some representative raw and deconvoluted spectra are shown in Figure 7. Note that in some cases, substantial Na-adduct formation was evident, which was a logical consequence of the Na-phosphate buffer used in the first dimension.

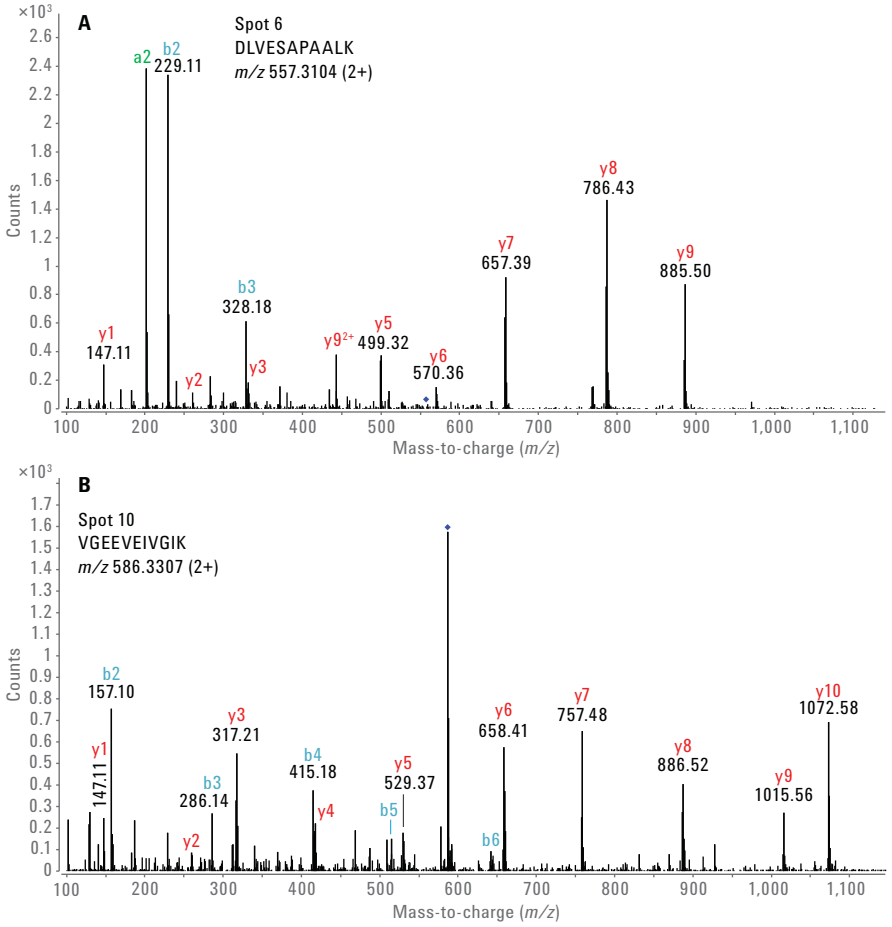

Figure 5. MS/MS spectra of spots 6 and 10 with annotation of y- and b-ions.

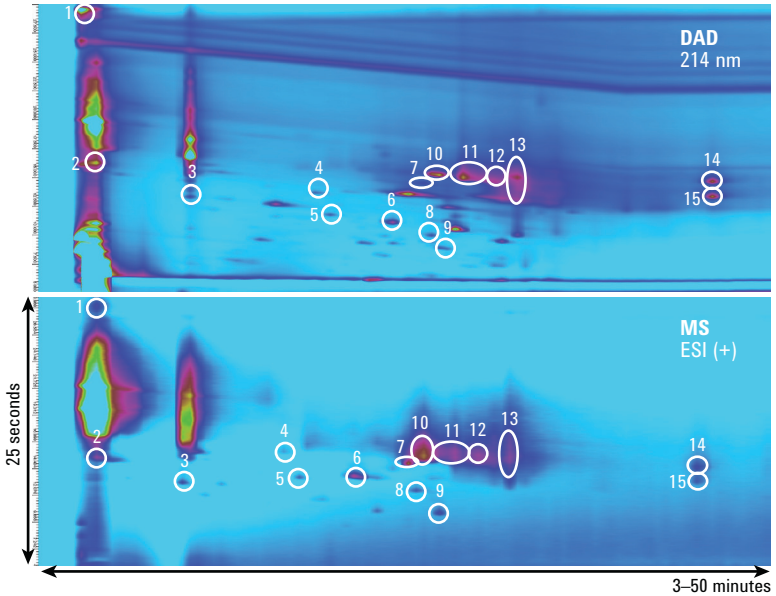

Figure 6. Comparison of 2D-LC analysis of *E. coli* lysate with UV and MS.

Table 3. Molecular weights of spots in Figure 6.

Spot number	Molceular weight
1	12,207.08
2	36,772.14
3	1,881.07
4	5,503.77
5	5,203.41
6	7,271.69
7	9,119.89
8	3,852.13
9	5,457.61
10	31,137.72
11	18,214.02; 20,640.06; 22,524.54
12	9,970.07
13	15,692.94, 15,770.05
14	9,226.56
15	9,535.85

Conclusions

The Agilent 1290 Infinity 2D-LC Solution is a valuable tool for the analysis of complex protein samples. Analysis of intact proteins as well as tryptic digests can be carried out with the same instrument and column configuration, allowing easy and automated transfer between both methods. When coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS, the identification of peptides and proteins is possible.

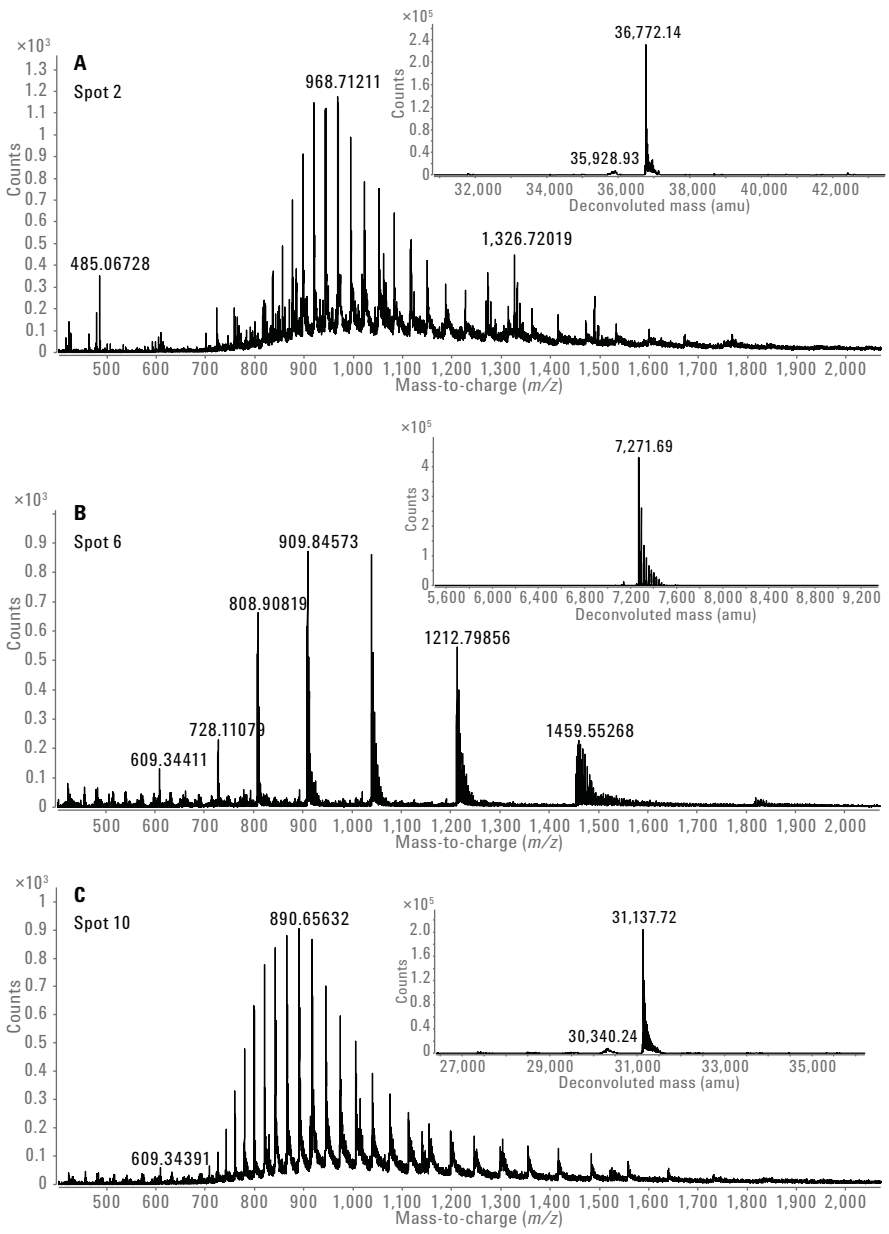

Figure 7. Raw and deconvoluted spectra of spots 2, 6, and 10.

References

1. François, I; Sandra, K; Sandra, P. Comprehensive liquid chromatography: fundamental aspects and practical considerations - a review. *Anal. Chim. Acta*. **2009**, *64*, pp 14-31.

2. Sandra, K; *et al.* Highly efficient peptide separations in proteomics. Part 2: bi- and multidimensional liquid-based separation techniques. *J. Chromatogr. B* **2009**, *877*, pp 1019-1039.

3. Vanhoenacker, G; *et al.* Analysis of monoclonal antibody digests with the Agilent 1290 Infinity 2D-LC Solution. *Agilent Technologies Application Note*, publication number 5991-2880EN, **2013**.

4. Vanhoenacker, G; *et al.*. Tryptic digest analysis using the Agilent 1290 Infinity LC System. *Agilent Technologies Application Note*, publication number 5990-4031EN, **2009**.

5. Blumberg, L. M. Linear peak capacity of a comprehensive multidimensional separation. *J. Sep. Sci.* **2008**, *31*, pp 3352-3357.

6. Blumberg, L. M. Multidimensional Gas Chromatography: Theoretical Considerations. In *Comprehensive Chromatography in Combination with Mass Spectrometry*; Mondello, L. Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 13-63.

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