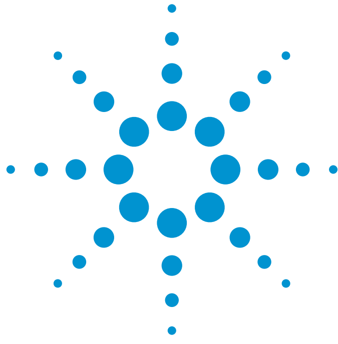

Workflow Automation for LC/MS: In-Solution Protein Digestion, Peptide Cleanup, and Strong Cation-Exchange Fractionation of Peptides Enabled by AssayMAP Technology

Application Note

Clinical Research

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Abstract

Sample preparation for LC/MS interrogation of peptides often comprises multistep workflows including in-solution protein digestion, peptide cleanup, and peptide fractionation. This process is usually tailored to a specific application based upon sample characteristics and the goal of the assay (that is, quantification or characterization). Automation of sample preparation workflows can increase sample processing capacity, reduce variability, and eliminate the need for skilled labor to perform repetitive tasks. However, automation is not typically used as a platform for primary assay development because it is rare that assay developers have the expertise required to develop complex automation protocols. Instead, assays are typically developed using wet-bench techniques and adapted to automation with the help of automation experts.

The AssayMAP Peptide Sample Prep solution eliminates the requirement for expertise in automation. Instead, assay developers can focus on the science of the assay enabled by a simple software-user interface and flexible protocols that give assay developers full control of key experimental variables. Now, the benefits of scalable, precision automation can be realized without requiring assay developers, scientists, or technicians to become automation experts. With the AssayMAP platform, entire workflows can be developed on the same hardware needed to scale when high-throughput sample processing is required. This reduces or eliminates the additional time and resources required to adapt protocols to an automation format. Here we demonstrate a typical LC/MS workflow for discovery (shotgun) proteomics demonstrating in-solution digestion, reversed-phase peptide cleanup, and strong cation-exchange fractionation (SCX) of peptides all performed using the AssayMAP Bravo liquid handler. More than 15,000 unique peptide sequences were identified from six SCX fractions generated from an *E. coli* protein lysate in which 64–67 % of those peptides were identified exclusively in one of the six fractions through the use of step-wise elution from SCX cartridges using increasing pH or ionic strength.

Agilent Technologies

Introduction

Sample preparation workflows benefit from automation by permitting the ability to scale sample processing to meet the desired throughput and by relieving the burdens of repetitive, but critical, sample handling tasks from skilled labor. For multistep workflows, this also minimizes propagation of error and maximizes reproducibility by leveraging automation platforms featuring precision liquid handling. This is especially relevant for protein and peptide analysis by LC/MS where errors in multistep sample preparation workflows are magnified by the extreme precision and sensitivity of state-of-the-art analytical instrumentation. However, a significant challenge arises in automation for sample preparation; existing workflows are often adapted to, rather than developed on an automation platform leading to additional optimization and resource allocation, which delays the benefits of workflow automation. To those not expertly trained on an automation platform, automation can be viewed as impractical for development and overly rigid. Such rigidity is prized for fully developed assays, but it can hinder development because it requires assay developers to have expertise in automation, to devote time to becoming an automation expert, or require them to rely on an automation expert to aid in assay development. True enablement, from the standpoint of the assay developer, comes from an automation platform that permits start-to-finish assay development, seamlessly integrates multistep protocols into linked workflows, and requires no expertise in automation allowing them to focus on the science of the assay rather than on automation.

Agilent has introduced the AssayMAP Peptide Sample Prep suite of software and microscale cartridges for the AssayMAP Bravo automation platform to directly address the needs of assay developers for LC/MS applications in the context of workflow automation for proteomics. At the core of this technology lies the precision liquid handling capability of the AssayMAP Bravo configured to handle microtiter plates. The AssayMAP Bravo head is equipped with an array of 96 syringe probes (250 µL) operated through liquid displacement, which enables strict flow-controlled microscale chromatography using disposable 5-µL packed-bed cartridges. Additional differentiating innovations arose when automation engineers and LC/MS assay scientists synergistically developed modular, flexible protocols for proteomics applications. These protocols are presented in a simple, easy-to-use software interface giving assay developers full control of experimental parameters without requiring an intimate knowledge of the underlying automation. This allows wet-bench type assay development on an automation platform. AssayMAP protocols were developed for in-solution protein digestion, peptide cleanup (desalting), and peptide fractionation. These protocols were designed to be operated as stand-alone modules or they can be mated to build more complex workflow architectures facilitated by a host of automation utilities to transition between protocols.

In this application note, we describe a multistep AssayMAP workflow for a typical discovery (shotgun) proteomics experiment whereby *E. coli* protein lysate was digested, subjected to reversed-phase cleanup, and fractionated using strong cation-exchange fractionation with step-wise elution by increasing ionic strength or pH. LC/MS analysis using an Agilent 6550 iFunnel Q-TOF LC/MS system with an Agilent 1290 Infinity LC System and AdvanceBio Peptide Mapping column identified more than 15,000 unique peptide sequences from *E. coli* using either fractionation technique. Of those 15,000 peptides, approximately 10,000 peptides were identified exclusively in 1-of-the-6 fractions demonstrating the utility of step-wise fractionation from AssayMAP SCX cartridges. Replicate analyses revealed the highly reproducible LC/MS workflow for proteome characterization by precision Agilent analytics.

Experimental

In-solution digestion

Lyophilized *E. coli* protein lysate was reconstituted to 10 µg/µL with a urea-based Denaturation Mixture (Table 1). Ten-microliter aliquots were made into each of four wells of two U-bottom sample plates. The In-Solution Digestion protocol can accommodate up to four 96-well microplates. In this case, all samples could have easily fit onto one column of a sample plate, but because the samples were going to be further processed post-digestion on different days, it was advantageous to split the samples onto two plates. Reagent plates were prepared to match the well configuration of the sample plates. The In-Solution Digestion protocol was launched and all plates were placed on the deck of the AssayMAP Bravo.

Table 1. On-deck samples and reagents for AssayMAP In-Solution Digestion, Peptide Cleanup, and Fractionation protocols.

On-deck samples and reagents	
In-solution digestion v1.0	
Sample	*E. coli* protein lysate (Bio-Rad Laboratores) reconstituted in denaturation mixture to 10 µg/µL
Denaturation mixture	8 M urea with 5 mM TCEP and 150 mM Tris (pH 8)
Alkylant	70 mM iodoacetamide
Diluent mixture	50 mM Tris (pH 8)
Protease	Agilent proteomics grade trypsin (0.33 µg/µL in 50 mM acetic acid)
Wash station	Deionized water
Peptide cleanup v1.1	
Sample	TFA-acidified tryptic digests of *E. coli* (pH ~ 2.6)
Stringent syringe wash buffer (priming)	99.9% ACN/0.1% TFA
Utility buffer (equilibration/wash)	0.1% TFA
Elution buffer	70% ACN/0.1% formic acid
Wash station	Deionized water
Fractionation v1.0	
Sample	Tryptic digests of *E. coli* in SCX fractionation equilibration buffer
Fractionation method	SCX: Step-wise elution by increasing ionic strength
Priming buffer	5 mM KH_2PO_4, 350 mM KCl; pH 2.6/25% ACN
Equilibration buffer	5 mM KH_2PO_4, pH 2.6/25% ACN
Elution Buffer 1	5 mM KH_2PO_4, 30 mM KCl; pH 2.6/25% ACN
Elution Buffer 2	5 mM KH_2PO_4, 50 mM KCl; pH 2.6/25% ACN
Elution Buffer 3	5 mM KH_2PO_4, 85 mM KCl; pH 2.6/25% ACN
Elution Buffer 4	5 mM KH_2PO_4, 115 mM KCl; pH 2.6/25% ACN
Elution Buffer 5	5 mM KH_2PO_4, 150 mM KCl; pH 2.6/25% ACN
Elution Buffer 6	5 mM KH_2PO_4, 350 mM KCl; pH 2.6/25% ACN
Fractionation method	SCX: Step-wise elution by increasing pH
Priming buffer	400 mM ammonium formate, 1% formic acid/25% ACN
Equilibration buffer	1% formic acid/25% ACN
Elution Buffer 1	40 mM ammonium formate, pH 3.5/25% ACN
Elution Buffer 2	40 mM ammonium formate, pH 4.0/25% ACN
Elution Buffer 3	40 mM ammonium acetate, pH 4.5/25% ACN
Elution Buffer 4	40 mM ammonium acetate, pH 5.0/25% ACN
Elution Buffer 5	40 mM ammonium acetate, pH 5.5/25% ACN
Elution Buffer 6	100 mM ammonium hydroxide, pH 9.5/25% ACN
Wash station	Deionized water

Note: All buffers were made as v/v mixtures. The pH of the aqueous component of each buffer was measured and adjusted before the addition of ACN

Application Settings were entered in the In-Solution Digest user interface using the values indicated in Figure 1 and the protocol was initiated. After the Denaturation Mixture was added, the two sample plates were removed from the deck, sealed with a removable plastic seal using a PlateLoc, and placed in a 60 °C incubator for 1 hour for protein denaturation and reduction of disulfide bonds. The sample plate was briefly centrifuged, the seal removed, and the plate was placed back on the deck.

The protocol continued with the addition of alkylant, an incubation step to alkylate free cysteines (40 minutes at room temperature), the addition of diluent, and a trypsin addition (1:33; trypsin:protein). These were all carried out sequentially on deck without interruption or user intervention. After the protocol was complete, the sample plates were removed, sealed using a PlateLoc, and placed into a 37 °C incubator for overnight digestion. The sample plates were removed from the incubator and briefly centrifuged. Each sample plate was acidified by the addition of 20 µL of 10 % TFA. One plate of acidified *E. coli* digests was immediately processed through the Peptide Cleanup Protocol and the other plate was stored at –80 °C until needed. The same digestion experiment was repeated at a later time generating two additional sample plates.

Peptide cleanup

Buffers (Table 1) were dispensed into the first column of the 12-column plates corresponding to the reagent plates for Stringent Syringe Wash (priming) Buffer, Utility (equilibration/washing) buffer, and Elution Buffer. Empty U-bottom plates were used for Flow Through Collection and Eluate Collection plates. A total of four AssayMAP C18 cartridges and four AssayMAP Resin-Free cartridges were used to fill a single column in the Cartridge Seating Station. Although only four *E. coli* digests from a single sample plate were being processed through Peptide Cleanup, eight cartridges were required to fill out a full column for proper seating of the AssayMAP cartridges. The AssayMAP Bravo applies a differential pressure to seat AssayMAP cartridges in full-column (eight-cartridge) increments. Optimum cartridge performance depends on proper seating of AssayMAP cartridges onto the probes of the AssayMAP Bravo 96-channel head. AssayMAP Resin-Free cartridges serve as convenient, reusable, substitutes for packed cartridges when the number of samples is not a multiple of 8. The U-bottom sample plate from the digestion step containing the four wells (A1-D1) of TFA-acidified *E. coli* digest was placed at deck location 4. The Peptide Cleanup protocol was launched and Application Settings were entered on the Peptide Cleanup user interface, and labware types were selected in the Labware Table as shown in Figure 2. A volume of 245 µL of *E. coli* digest (~ 92.5 µg) was aspirated onto each AssayMAP C18 cartridge. Samples were eluted in 20 µL of elution buffer and diluted 10-fold with the appropriate SCX equilibration buffer.

Figure 1. User interface for the Agilent AssayMAP In-Solution Digestion protocol with the parameters used for *E. coli* digestion.

Figure 2. User interface for the Agilent AssayMAP Peptide Cleanup protocol with the parameters used for cleanup of TFA-acidified *E. coli* tryptic digests.

Strong cation-exchange fractionation by increasing ionic strength

Under low pH conditions (~ < pH 3), positively charged peptides can be bound and eluted from negatively charged strong-cation exchange resins using low-pH buffers of increasing ionic strength (increasing concentration of salt). In general, peptides elute from SCX stationary phases in order of increasing net positive charge (increasing number of basic residues)[1]. The presence of organic solvent in the buffers (typically acetonitrile) helps to minimize nonspecific hydrophobic interactions of peptides with the stationary phase.

SCX elution buffers for step-wise fractionation using ionic strength (Table 1) were aliquoted into the wells of U-bottom plates corresponding to the same well locations of the samples in the sample plate. The Plate Stacking protocol was launched to build a stack of six Elution Buffer plates and a stack of six Fraction Collection plates using the gripper arm of the AssayMAP Bravo. Additional reagent and waste plates were placed on the deck of the AssayMAP Bravo (Figure 3) as was the sample plate containing the four *E. coli* digests that had been processed through the Peptide Cleanup protocol and diluted in SCX equilibration buffer. Four AssayMAP SCX and four AssayMAP Resin-Free cartridges were arranged to create a full column at the Cartridge Seating Station. The fractionation protocol was initiated and upon completion, six 20-µL fractions of each *E. coli* digest sample had been collected. In addition, the flowthrough/wash volume for each sample was retained to assess peptide binding. Each set of fractions (24 in total), now containing relatively high concentrations of nonvolatile potassium chloride salt, was diluted 10-fold in 0.1 % TFA to reduce the concentration of acetonitrile, consolidated onto a single plate, and again processed through the Peptide Cleanup protocol. The relatively salt-free flow through/wash samples were lyophilized directly along with the eluates from the fractions processed through the Peptide Cleanup protocol.

This collection of 24 fractions and four flow through/wash samples represent "Day 1" samples. Day 1 samples were analyzed by LC/MS. After data collection of Day 1 samples was complete, the second sample plate from the In-Solution Digestion protocol ("Day 2" samples) was processed using the same AssayMAP workflow and analyzed.

Strong cation-exchange fractionation by increasing pH

SCX fractionation of peptides can also be accomplished using AssayMAP SCX cartridges with step-wise elution using increasing pH. As with SCX fractionation using increasing ionic strength, peptides bind to the stationary phase under low pH conditions. However, elution is achieved by increasing the pH of the elution buffers rather than increasing ionic strength. As the pH is raised during elution, the net positive charge of peptides decreases. When the pH of the elution buffer approaches the isoelectric point (pI) of peptides, the electrostatic interactions with the negatively charged stationary phase weakens facilitating elution[2]. Peptides are expected to elute in order of increasing pI, which also generally correlates to an increasing number of basic residues.

SCX fractionation of peptides using step-wise pH elution was carried out in the same manner as elution by ionic strength except that a different buffer system was used (Table 1). However, cleanup of these fractions was not required because of the use of volatile buffers that allowed the samples to be lyophilized directly. This process resulted in the collection of 24 fractions and four flow through/wash samples for each "Day 1" and "Day 2" sample set.

Figure 3. User interface for the Agilent AssayMAP Fractionation protocol with the parameters used for SCX fractionation of *E. coli* tryptic digests.

LC/MS

All LC/MS analyses were conducted on an Agilent iFunnel 6550 Q-TOF LC/MS equipped with a Dual Agilent Jet Stream ESI source (Table 2). The mass spectrometer was coupled to a 1290 Infinity Binary LC System housing an AdvanceBio Peptide Mapping C18 column (2.1 × 250 mm, 2.7 µm, 120 Å pore size) maintained at 55 °C. Mobile phase A was 0.1 % formic acid in water, and mobile phase B was 99.9 % acetonitrile with 0.1% formic acid. *E. coli* fractions were reconstituted in 30 µL of mobile phase A. Twenty microliters of each fraction was injected and chromatographed over a 60-minute linear gradient from 2.5 %–35 % B at a flow rate of 400 µL/min. The Q-TOF was operated in Auto MS/MS mode in which the 10 most abundant precursors were selected for MS/MS per cycle using the narrow isolation parameter and an active exclusion time of 1 minute. The acquisition rate was set at 8 Hz for MS and 3.5 Hz for MS/MS scans. An internal reference mass was not used, but mass calibration was performed daily. Data analysis was performed using a combination of Agilent MassHunter (Version B.05.00, Build 5.0.519.0), Spectrum Mill (Rev B.04.00.127), Morpheus (64-bit, for Agilent .d files) [3], and custom scripts.

Table 2. Analytical instrumentation, consumables, and accessories

Analytical instrumentation and consumables	
Automation	
Core automation platform	Agilent AssayMAP Bravo (G5542A)
AssayMAP starter kits	96 C18 cartridges and labware for digestion and cleanup (G5496-60013) 96 SCX cartridges and labware for fractionation (G5496-60014) 96 RP-S cartridges and labware for digestion and cleanup (G6596-60034)
AssayMAP cartridges (Qty. 96 cartridges/rack)	96 C18 cartridge rack (5190-6532) 96 SCX cartridge rack (5190-6536) 96 Resin-Free cartridge rack (G5496-60009) 96 RP-S cartridge rack (G5496-60033)
AssayMAP Bravo accessories	Risers, 146 mm (G5498B#055) Peltier Thermal Station with STC controller (G5498B#035) Custom Plate Nest (G5498B#017) PCR Plate Insert (G5498B#013)
Additional accessories	Agilent PlateLoc Thermal Microplate Sealer (G5402A)
LC/MS	
Mass spectrometer	Agilent 6550 iFunnel Q-TOF LC/MS Dual Agilent Jet Stream ESI
LC System and columns	Agilent 1290 Infinity LC System **Agilent AdvanceBio Pepide Mapping Columns (C18)** Analytical: 2.1 × 250 mm, 2.7 µm (651750-902) Guard: 2.1 mm Fast Guard (851725-911)

Results and Discussion

Large-scale SCX fractionation of *E. coli* digests with an all AssayMAP workflow

LC/MS results of the multiday experiments in which *E. coli* protein lysates were digested, cleaned up/desalted, and fractionated using AssayMAP protocols and cartridges are shown in Table 3. For both step-wise elution by increasing ionic strength or increasing pH, an average of more than 15,000 distinct peptide sequences were identified for each sample replicate (n = 8) after filtering to a 1 % false discovery rate (FDR) for each fractionation experiment. Very few peptides were identified in the sample flow through/wash demonstrating both the effectiveness of the Peptide Cleanup protocol for removing salts and the near quantitative binding characteristics of tryptic peptides on AssayMAP SCX cartridges under the described experimental conditions.

For SCX by increasing ionic strength, more than 10,000 distinct peptides were fractionated in a manner to bin them exclusively into one of the six fractions. For example, for SCX Fraction 2 (50 mM KCl), there was an average of ~25,750 MS/MS spectra acquired per 60-minute gradient leading to 9,035 peptide-spectrum matches (PSMs) at a 1 % FDR. Those PSMs reduced to 6,424 distinct peptide sequences within Fraction 2. Of those 6,424 distinct peptide sequences, 3,471 were found exclusively in Fraction 2.

An aggregate analysis of all of the fractions in a sample set shows that, on average, 67.1 % of the distinct peptides were binned into a specific fraction. This has to be considered within the context of typical data-dependent MS/MS experiment relying on automated peptide-spectrum matching. However, these data reveal highly efficient step-wise fractionation from AssayMAP SCX cartridges. Similar results were achieved with step-wise pH elution in which 64.2 % of the distinct peptides were binned into a specific fraction.

Table 3. Peptide identifications from LC/MS analyses of SCX-fractionated *E. coli* tryptic digests.

E. coli (n = 8) SCX fraction no.	Avg. no. of PSMs*	Avg. # of distinct peptides found in specified fraction	Avg. # of distinct peptides exclusive to specified fraction	Avg. % of distinct peptides exclusive to specified fraction
Increasing ionic strength				
Flow through/wash	117	83	39	47.0 %
Fraction 1 (30 mM KCl)	2,853	1,544	471	30.5 %
Fraction 2 (50 mM KCl)	9,035	6,424	3,471	54.0 %
Fraction 3 (85 mM KCl)	7,692	5,245	1,774	33.8 %
Fraction 4 (115 mM KCl)	9,011	5,424	2,221	40.9 %
Fraction 5 (150 mM KCl)	5,858	3,538	1,055	29.8 %
Fraction 6 (350 mM KCl)	5,711	3,195	1,200	37.6 %
Summary	40,277	15,241‡	10,231	67.1 %
Increasing pH				
Flow through/wash	178	109	33	40.4 %
Fraction 1 (pH 3.5)	7,157	3,678	1,686	45.8 %
Fraction 2 (pH 4.0)	11,001	6,544	2,933	44.8 %
Fraction 3 (pH 4.5)	8,020	5,083	1,502	29.5 %
Fraction 4 (pH 5.0)	8,977	4,997	1,371	27.4 %
Fraction 5 (pH 5.5)	7,503	4,214	730	17.3 %
Fraction 6 (pH 9.5)	9,023	4,709	1,580	33.6 %
Summary	51,859	15,335‡	9,846	64.2 %

* Results filtered to a 1% false discovery rate (FDR), PSM = peptide-spectrum match

‡ This value considers the overall number of distinct peptides across all fractions and is not a simple summation of the number of distinct peptides found within each fraction.

Elution of peptides based their physiochemical properties and mode of elution trended with predictions. The average number of basic residues (H, K, R) per peptide increased with each fraction from 0.99 (Fraction 1) to 2.39 (Fraction 6) for elution with increasing ionic strength (Table 4). A smaller increase was observed for elution with increasing pH from 1.01 (Fraction 1) to 1.87 (Fraction 2). Similarly, the average pI trended upward with increasing fraction number for both elution conditions.

In Figures 4 and 5, the total ion chromatograms (TICs) generated for each fraction from each of the eight sample replicates are overlaid showing the high degree of similarity for inter- and intraday analyses. Extracted ion chromatograms (EICs) of select peptides are displayed at the bottom of Figures 4 and 5. All eight replicates were overlaid and the peak area % CVs and mass errors were tabulated. For example, the peak area % CV for the peptide HVAILGDLQGPK was 2.8 % with an average mass error of –5.6 ppm without the use of an internal standard or reference mass correction (Figure 4, bottom). Importantly, this level of reproducibility was achieved after going through an AssayMAP workflow involving in-solution digestion, C18 cleanup, SCX fractionation (ionic strength), and another round of C18 cleanup. Similarly, the peptide VVDAAVEK had a peak area % CV of 5.3 % with an average mass error of 4.9 ppm having gone through a similar workflow using SCX fractionation by pH and a single round of cleanup (Figure 5, bottom). Peak area % CVs ranged from 2.3–8.1 % with mass errors less than 8 ppm for the 12 peptides shown at the bottom of Figures 4 and 5. In addition to low % CV and mass error, the average retention time variation for the 12 peptides across all eight replicates was less than 0.085 % (< 3 s).

Table 4. The average number of basic amino acid amino residues and estimated isoelectric point (pI) of distinct peptides identified in *E. coli* fractions.

SCX Fraction no.	Avg. no. of basic residues (H, K, R)	Estimated* avg. pI
Increasing ionic strength		
Flow through/wash	0.21	5.63
Fraction 1 (30 mM KCl)	0.99	6.36
Fraction 2 (50 mM KCl)	1.06	6.72
Fraction 3 (85 mM KCl)	1.48	6.76
Fraction 4 (115 mM KCl)	1.83	6.87
Fraction 5 (150 mM KCl)	2.08	6.94
Fraction 6 (350 mM KCl)	2.39	6.90
Increasing pH		
Flow through/wash	0.39	5.54
Fraction 1 (pH 3.5)	1.01	6.30
Fraction 2 (pH 4.0)	1.09	6.72
Fraction 3 (pH 4.5)	1.39	6.75
Fraction 4 (pH 5.0)	1.72	6.64
Fraction 5 (pH 5.5)	1.73	6.69
Fraction 6 (pH 9.5)	1.87	6.71

Values were calculated using the “avg. no. of distinct peptides found in specifed fraction” listed in Table 3.
*The isoelectric point (pI) of peptides are simple solution-phase estimates and do not consider the effects of neighboring residues on pKa values. The pKa values for side chains of H, K, R, D, E, and Y residues and N- and C-termni were used for simple pI estimates.[4]

Peptide fractionation can serve multiple purposes depending on the specific goals of the experiment. For discovery-style experiments (such as the one described here) fractionation decreases overall sample complexity by dividing the complexity among fractions. This extends the dynamic range of the assay by permitting the identification of low-abundance peptides that would normally be masked in an unfractionated sample. In general, this increases the number of distinct peptides that can be identified aiding in characterization of the sample. For example, an unfractionated *E. coli* digest (12 µg/injection, roughly the same TIC signal as a fractionated sample) prepared on the AssayMAP Bravo was analyzed seven times using the same LC/MS method used for the fractionated samples. The amount of total LC/MS acquisition time (7 hours) was equivalent to analyzing a set of six fractions and the flow through. The unfractionated sample produced more peptide-spectrum matches (58,753), but yielded only 7,678 distinct peptide sequences compared to the ~15,000 distinct peptides found when fractionation was employed. In this case, fractionation was extremely advantageous enabling the identification of nearly twice as many distinct peptides.

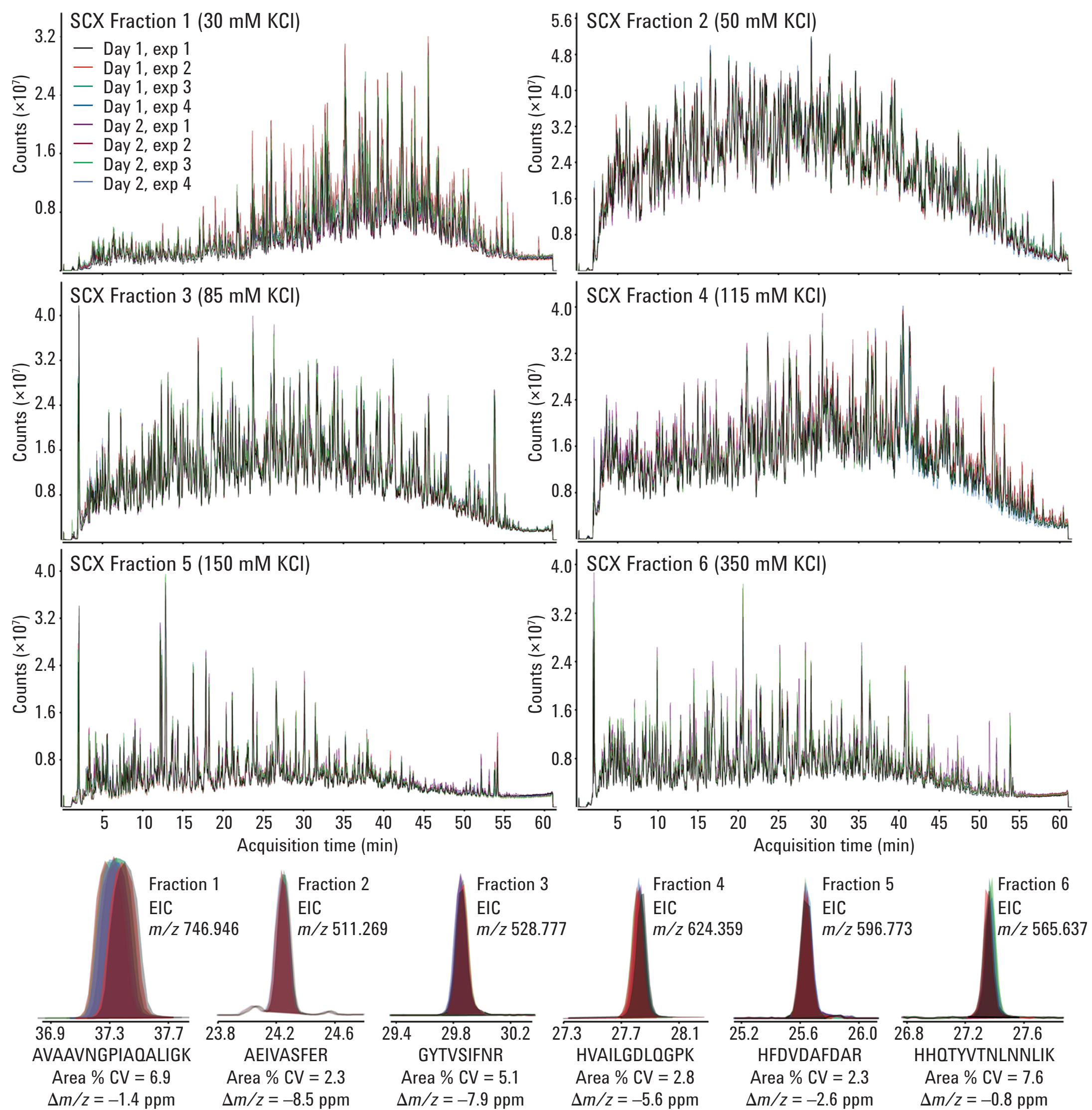

Figure 4. Overlays of eight TICs (n =4, day 1; n = 4, day 2) for each SCX fraction generated by step-wise elution with increasing ionic strength (top). Overlays of eight EICs of peptides found in the indicated fractions. Without the use of internal standards or external reference masses, peak area % CVs for these peptides are less than 7.6 % with mass errors less than 8 ppm (bottom).

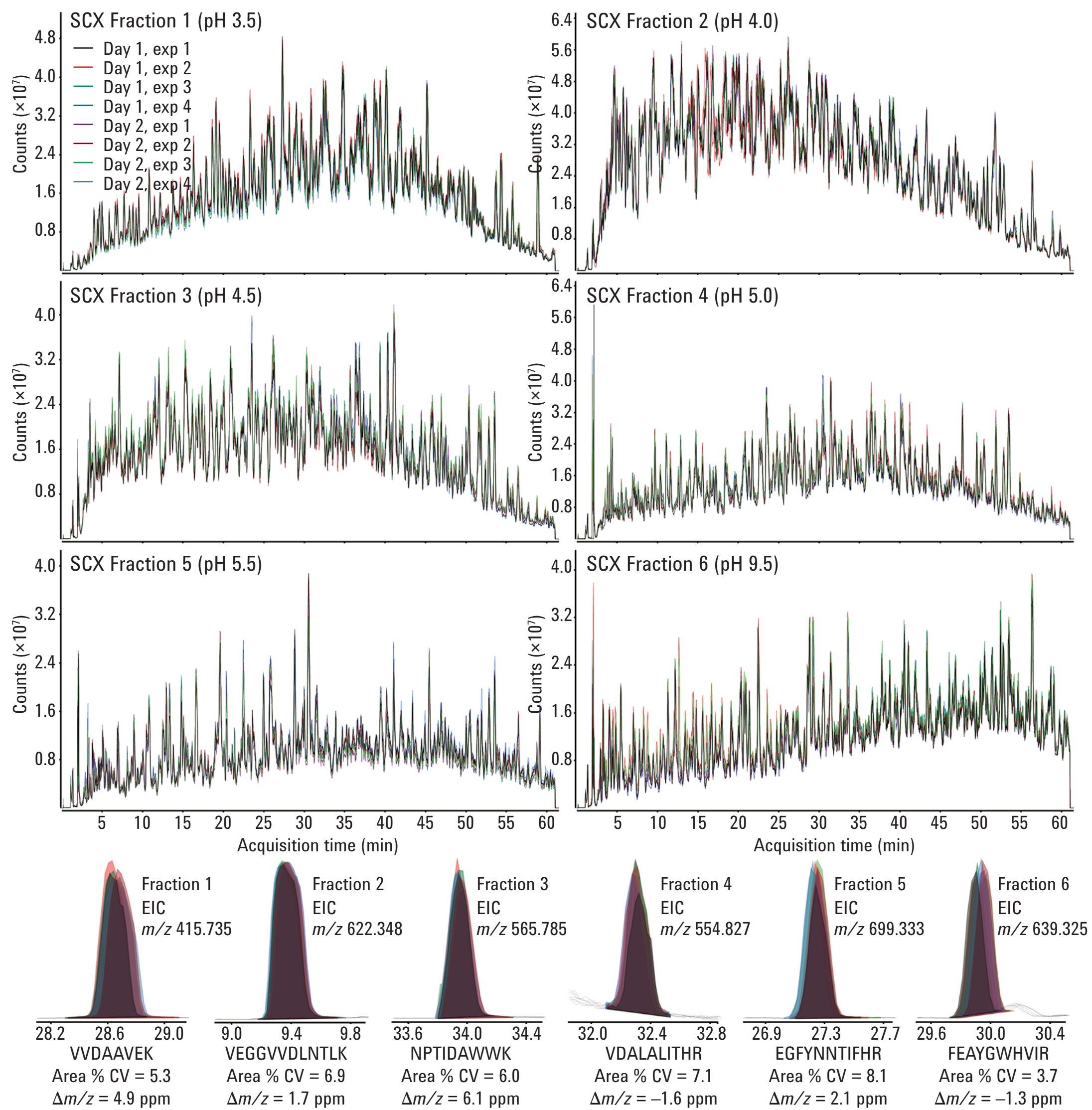

Figure 5. Overlays of eight TICs (n =4, day 1; n = 4, day 2) for each SCX fraction generated by step-wise elution with increasing pH (top). Overlays of eight EICs of peptides found in the indicated fractions. Without the use of internal standards or external reference masses, peak area % CVs for these peptides are less than 7.6 % with mass errors less than 8 ppm (bottom).

These data demonstrate the utility of step-wise fractionation in a solid-phase extraction (SPE) format despite the fact that peptide fractionation is normally performed using gradient elution on a high-performance liquid chromatography (HPLC) system. The AssayMAP Bravo is an exceptional liquid handler capable of reproducibly manipulating small volumes of liquids through mounted cartridges. However, the AssayMAP Bravo is not a 96-channel HPLC and step-wise fractionation with a 5-µL packed bed cartridge does not offer the same separation performance relative to performing offline gradient fractionation using an HPLC with a comparatively large packed column.

There are fewer theoretical plates available in a small cartridge format relative to a standard-sized HPLC column and it is the number of theoretical plates that largely dictates separation efficiency[5]. Traditional SPE formats involving manual pipetting or gravity/vacuum-assisted liquid handling are useful in simple on/off assays where the goal is to bind analytes, wash away contaminants, and simultaneously elute all target analytes. In these cases, precise volume and flow control are not always needed for the experiment to be successful. By contrast, precise volume and flow control are both critical for more nuanced techniques like step-wise fractionation of chemically similar species because simple on/off chemistry does not apply. It is difficult to multiplex sample processing in traditional SPE formats such that each well, cartridge, or packed tip receives identical volumes of liquid delivered through the SPE material at the same flow rate leading to unsatisfactory reproducibility for multistep fractionation experiments.

The liquid handling properties of the AssayMAP Bravo enables reproducible step-wise fractionation in a multiplexable format stemming from the ability to precisely control both volume and flow rate through cartridges engineered to achieve optimal performance for microscale applications. The AssayMAP platform is a bridging technology enabling multiplexed, step-wise separations in an analytical SPE format for those applications not requiring HPLC performance, but still demanding highly reproducible fractionation on a scalable sample preparation platform.

Conclusions

Multistep sample preparation workflows for peptide analysis by LC/MS can be automated using the AssayMAP Bravo resulting in highly reproducible in-solution protein digestion, reversed-phase peptide cleanup, and strong cation-exchange fractionation of complex mixtures of peptides. In a proof-of-principle discovery-style shotgun experiment, 100 µg samples of protein from a cell lysate of *E. coli* was carried through multistep AssayMAP workflows featuring SCX fractionation with six elution steps using increasing ionic strength or increasing pH. Both forms of fractionation led to the identification of more than 15,000 distinct peptides empowered by an Agilent 6550 iFunnel QTOF LC/MS, an Agilent 1290 Infinity LC System, and an Agilent AdvanceBio Peptide Mapping column. Of those 15,000+ distinct peptides, 67.1 % and 64.2 % were binned exclusively into one of the six fractions using SCX by ionic strength or pH, respectively. An analysis of select peptides over the course of a multiday experiment revealed peak area % CVs ranging from 2.3–8.1 % without the use of internal standards. Compared to an analysis of unfractionated samples, nearly twice the number of distinct peptides was identified using SCX fractionating demonstrating the use of step-wise fractionation to increase assay dynamic range and improve proteome coverage for discovery-style experiments.

These experiments establish the ease in which multistep sample preparation workflows can be automated using the AssayMAP Bravo and its suite of tools engineered for precision proteomics. The AssayMAP Peptide Sample Prep automation solution gives researchers and assay developers the ability to rapidly and systematically evaluate LC/MS sample preparation conditions, chemistries, and methodologies without having expertise in automation. Concomitantly, the AssayMAP Bravo platform enables seamless throughput scaling once workflows have been finalized whether the throughput requirement is a few samples per day, or hundreds. This enablement stems from the ability to customize protocols through a simple and intuitive software interface yielding full control over important experimental variables to the user who no longer needs to be preoccupied with understanding the complexities of precision automation. For those sample preparation protocols already developed through wet-bench techniques, assay developers can readily adapt their protocols to the AssayMAP platform leveraging the investments already made in selecting and optimizing specific chemistries and experimental conditions. Overall, these data presented here demonstrates the high degree of measurement precision that can be achieved using Agilent analytics for peptide analysis by LC/MS facilitated by automated sample preparation using the AssayMAP Bravo.

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