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Development of online high-/low-pH reversed-phase–reversed-phase two-dimensional liquid chromatography for shotgun proteomics: A reversed-phase-strong cation exchange-reversed-phase approach

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ABSTRACT

Previously, we described an online high-/low-pH RP–RP LC system exhibiting high-throughput, automatability, and performance comparable with that of SCX-RP. Herein, we report a variant of the RP–RP platform, RP-SCX-RP, featuring an additional SCX trap column between the two LC dimensions. The SCX column in combination with the second-dimension RP can be used as an SCX-RP biphasic column for trapping peptides in the eluent from the first RP column. We evaluated the performance of the new platform through proteomic analysis of *Arabidopsis thaliana* chloroplast samples and mouse embryonic mouse fibroblast STO cell lysate at low-microgram levels. In general, RP-SCX-RP enhanced protein identification by allowing the detection of a larger number of hydrophilic peptides. Furthermore, the platform was useful for the quantitative analyses of crude chloroplast samples for iTRAQ applications at low-microgram levels. In addition, it allowed the online removal of sodium dodecyl sulfate and other chemicals used in excess in iTRAQ reactions, avoiding the need for time-consuming offline SCX cleanup prior to RP–RP separation. Relative to the RP–RP system, our newly developed RP-SCX-RP platform allowed the detection of a larger number of differentially expressed proteins in a crude iTRAQ-labeled chloroplast protein sample.

1. Introduction

Mass spectrometry (MS) has become a powerful tool for protein investigations in recent years. Shotgun proteomics analysis typically combined proteolytic digestion (e.g., trypsin treatment of cell lysates) with liquid chromatography (LC)/tandem mass spectrometry (MS/MS) [1]. Complex samples usually contain thousands of proteins; because their abundances can range over five orders of magnitude [2], the resulting digested peptide mixtures are even more complicated. Hence, effective separation or sub-fractionation is critical to reduce sample complexity for comprehensive proteomic analysis.

Multidimensional liquid chromatography (MD-LC) is a technique combining two or more dimensions of LC, thereby enhancing peak capacity and resolving power during peptide separation [3,4]. MD-LC is commonly used to minimize sample complexity and improve protein identification in shotgun proteomics analysis. The resulting minimized peptide co-elution and ion suppression during MS analysis increases the sensitivity and dynamic range for protein identification [5,6].

MD-LC can be performed either online or offline. Offline MD-LC is more flexible because it allows the use of incompatible buffers in the various LC dimensions. In addition, because it can be performed with larger amounts of sample, the identification of low-abundant proteins is possible. On the other hand, online MD-LC allows automation, with minimal sample loss and contamination, high-throughput operation, and the analyses of small amounts of samples [7]. At present, the combination of strong cation exchange (SCX) with reversed-phase (RP) chromatography (SCX-RP) is the most prevalent MD-LC technique for separating peptides based on charge and hydrophobicity [8]. It is compatible to both offline and online implementation [9,10]. SCX-RP can be performed conveniently online using a biphasic column configuration [8] or in a valve-switching mode [11]. While peptide separation through SCX is based mainly on electrostatic interactions, weak hydrophobic interactions might also exist between the peptides and the stationary phase, thereby diminishing its orthogonality to a certain extent [12]. Recently, two-dimensional RP chromatography (RP–RP), with the individual columns operating at high and low pH, respectively, has become a popular technique. Although separations in

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the two RP dimensions are both based on hydrophobicity, high-/low-pH RP–RP can exhibit remarkably altered selectivity because of the different charges of the amino acid side chains of the peptides at different values of pH [13,14]. Furthermore, high-/low-pH RP–RP generates peak capacities comparable with those of SCX-RP, thereby making it a promising alternative MD-LC technique for proteomics analyses [13].

Online coupling of high-/low-pH RP–RP is challenging; indeed, it is more practical to perform MD-LC offline because of incompatibilities in the solvent strengths used in the two LC dimensions. Previously, we demonstrated a fully automated online RP–RP platform for shotgun proteomic analysis. The first-dimension high-pH RP eluent was fractionated and transferred to the second-dimension low-pH RP column based on partial loop injection using a column-switching method [15,16]. Here, we present a modified set-up for RP–RP analysis: incorporating an SCX trap column between the first and second LC dimensions to form a triple RP-SCX-RP column. Theoretically, the trap column offers enhanced ability to retain the fractionated peptides, even at high organic content, because the SCX column mainly interacts with peptides based on their net charges. The presence of the SCX trap column also focuses the peptides prior to the second RP separation [17,18]. In addition, SCX is usually used to remove detergents [e.g., sodium dodecyl sulfate (SDS)] and isotopic labeling chemicals in iTRAQ proteomics applications that involve the isobaric tagging of peptides for high-throughput identification and quantification of proteins. Suppression of electrospray ionization (ESI) signals by SDS has been reported previously and its removal through an RP column might not be efficient [19]. In contrast, the SCX trap column in our modified system potentially enables online quantitative analyses of crude iTRAQ-labeled samples without prior cleaning-up procedures. In this study, we first employed a mixture of standard peptides to test the effectiveness of the trapping of fractionated peptides using the SCX trap column. Subsequently, we evaluated the performance of the RP-SCX-RP technique relative to the RP–RP method through the analysis of *Arabidopsis* (*Arabidopsis thaliana*) chloroplast proteins and the mouse embryonic fibroblast STO cell lysate. We further employed the platform in the analysis of a crude iTRAQ-labeled protein sample derived from *Arabidopsis* chloroplasts. In total, 642 proteins were successfully identified and 211 proteins were differentially expressed. Thus, our newly developed RP-SCX-RP system is compatible with the quantitative analysis of small-sized crude iTRAQ-labeled samples.

2. Experimental

2.1. Materials and reagents

Polyimide-coated fused-silica capillary (FSC) tubing was obtained from Polymicro Technologies (Phoenix, AZ). Electrically actuated four- and six-port, two-position switching valves, 2-μm stainless-steel screens, and zero-dead-volume unions were acquired from Valco Instruments (Houston, TX). PEEK tubing and microtees were obtained from Upchurch Scientific (Oak Harbor, WA). Jupiter C_{18} packing materials (3-μm particles, 300-Å pores) were purchased from Phenomenex (Torrance, CA). Strong cation exchange (SCX) packing materials (Polysulfoethyl, 5-μm particles, 300-Å pores) were purchased from PolyLC (Columbia, MD, USA). Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and standard proteins were acquired from Sigma–Aldrich (St. Louis, MO). Modified sequencing-grade trypsin was purchased from Promega (Madison, WI). The iTRAQ labeling kit, tris(2-carboxyethyl)phosphine (TECP), and methyl methanethiosulfonate (MMTS) were purchased from Applied Biosystems. Bradford assay reagent was purchased from Bio-Rad (Hercules, CA). Formic acid (>98%) and ammonium hydroxide (ca. 28%) were obtained from Fluka (St. Louis, MO). A Miili-Q system (Millipore, Bedford, MA) was used for water purification. Acetonitrile (ACN) was acquired from Tedia (Fairfield, OH).

2.2. Sample preparation

2.2.1. Standard protein

Proteins (100 μg) were dissolved in 100 mM ammonium bicarbonate (100 μL) and incubated with 50 mM DTT (6 μL) to reduce disulfide bridges for 30 min at 60 °C. Each sample was then cooled to room temperature prior to alkylation with 100 mM IAA (6 μL) for 60 min at room temperature in the dark, followed by digestion with modified trypsin (4 μg) at a 1:25 (w/w) trypsin to protein ratio at 37 °C overnight.

2.2.2. Mouse embryonic fibroblasts

The mouse embryonic fibroblast STO cell line (American Type Culture Collection) was cultured as previously described [20]. Cells were harvested and washed with phosphate-buffered saline (PBS) and then lysed using RIPA lysis buffer at 4 °C overnight. The lysate was then centrifuged (14,000 × *g*, 5 min). The protein concentration of the supernatant was measured using the Bradford assay.

2.2.3. Chloroplast protein

Chloroplasts were isolated essentially as described previously [21]. Briefly, *Arabidopsis* seeds (ca. 160 μL) were germinated on 10-mm × 150-mm plates and grown under 16-h/8-h light/dark cycles for 16 days. *Arabidopsis* wild-type (Col) and its three mutants-*thylakoid formation 1* (*thf1*), *Clp protease regulatory subunits 4* (*clpr4*), and *thf1 clpr4* double mutant-were used for investigation. Leaves from the harvested seedlings were ground in SHE buffer (330 mM sorbitol, 50 mM HEPES–KOH, 2 mM EDTA–2Na and 5 mM ascorbic acid, pH 7.8; 20 mL). After centrifugation (2550 rpm), chloroplasts were extracted at the intermediate layer of a 40%/70% Percoll step-gradient, and then resuspended in SHE buffer (0.5 mL). Proteins were released by adding 0.7% SDS and heating at 95 °C for 5 min. The protein concentration was determined using the Bradford assay. Proteins (100 μg) were dried, re-dissolved in 100 mM ammonium bicarbonate (100 μL) and incubated with 50 mM DTT (6 μL) to reduce disulfide bridges for 30 min at 60 °C. Each sample was then cooled to room temperature prior to alkylation with 100 mM IAA (6 μL) for 60 min at room temperature in the dark, followed by digestion with modified trypsin (4 μg) at a 1:25 (w/w) trypsin-to-protein ratio at 37 °C overnight. For iTRAQ-labeled samples, protein samples (100 μg) were reduced in volume to 50 μL, followed by the addition of SDS, TECP, and MMTS for cysteine-blocking using the reagents provided in the iTRAQ kit. Samples were digested by 1:33 sequencing-grade trypsin at 37 °C overnight. The trypsin-treated samples were labeled with four different iTRAQ tags (wild type: 113; *thf1*: 117; *clpr4*: 115; *thf1 clpr4*: 119) at room temperature for 2 h following the manufacturer's instructions. After completing the reactions, the labeled samples were dried for LC–MS/MS analysis.

2.3. Liquid chromatography

All capillary-flow LC experiments were performed using two Agilent 1100 series capillary pumps with a 1 well-plate autosampler (Agilent Technologies, Wilmington, DE). Column flow switching and injections were conducted with two six-port and one four-port, two-position electrically actuated switching valves. The RP capillary columns for the high- and low-pH separations (150 μm i.d. × 150 mm length) and SCX trap column (150 μm i.d. × 30 mm length) were packed in-house using Jupiter C_{18} packing materials (3-μm particle size, 300-Å pore size) and Polysulfoethyl packing materials (5-μm particles, 300-Å pore size), respectively, on

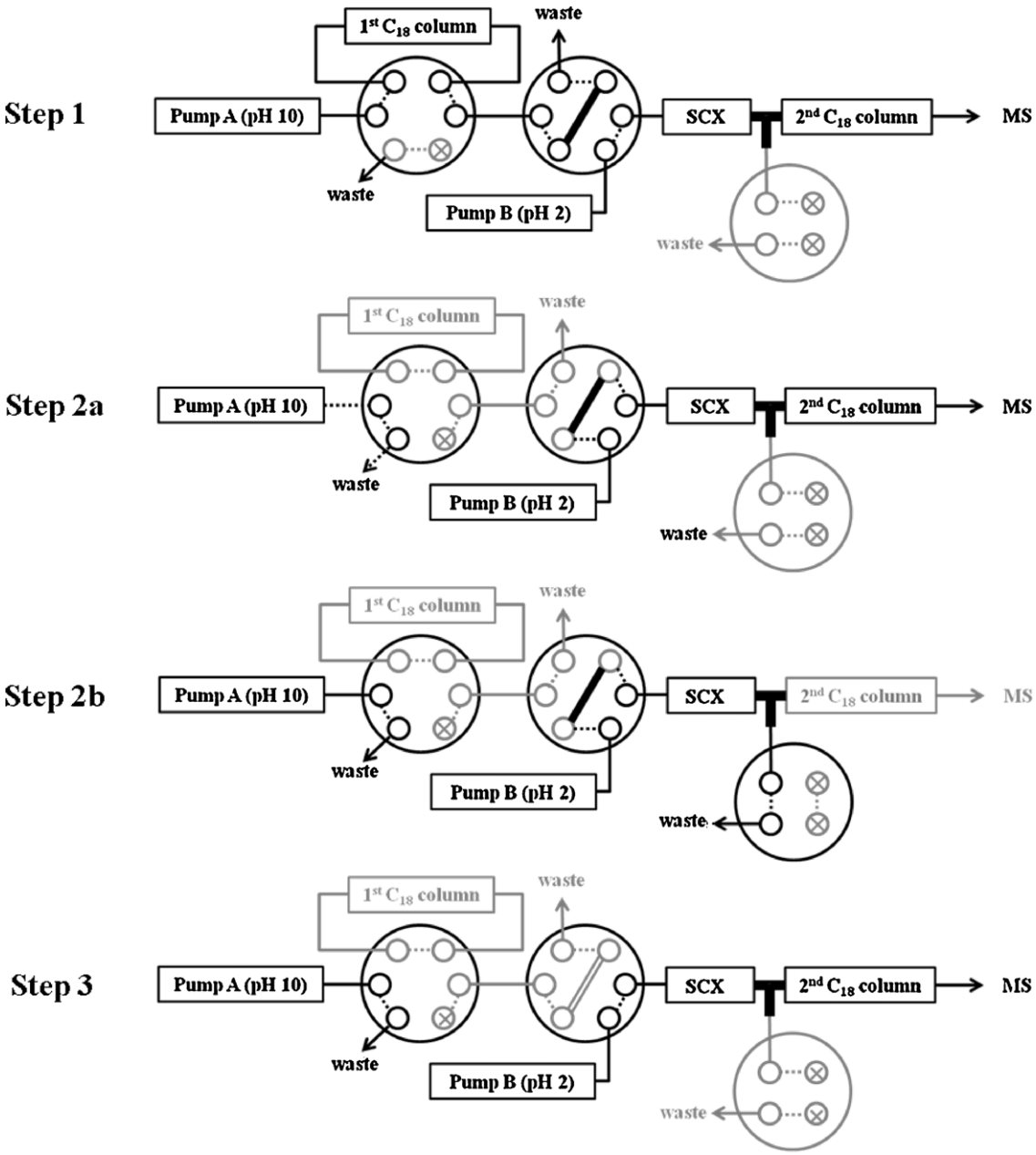


Fig. 1. Schematic representation and workflow of the RP-SCX-RP platform. **Step 1** First-dimension high-pH RP gradient separation; **Step 2a**, online solvent mixing followed by SCX-RP dual-trapping of fractionated peptides; **Step 2b**, online solvent mixing followed by SCX clean-up and peptide trapping for iTRAQ experiment; **Step 3**, second-dimension low-pH RP gradient separation.

an ultrahigh-pressure syringe pump operating under constant-pressure mode (up to 6000 psi). For high-pH RP separation, solvents A and B were prepared as previously described [13]. Briefly, a stock solution of 200 mM ammonium formate was prepared by adjusting a 200 mM solution of NH_4OH to pH 10 by adding formic acid (≥98%). Solvents A and B were then prepared by diluting the stock solution with water and ACN, respectively, to obtain the respective 20 mM ammonium formate buffers. For low-pH RP separation, solvent C (2% ACN, 0.5% formic acid in water) and (98% ACN, 0.5% formic in water) solvent D were used.

2.4. Online solvent adjustment experiments

A flow injection model experiment mimicking the online second-dimension RP sample loading was used to evaluate the performance of the SCX column for peptide trapping upstream of the low-pH RP separation. For each experiment, pulses of a standard protein digest (2 μg each of alpha-casein, beta-casein, beta-lactoglobin, BSA, lysozyme c, and myoglobin, dissolved in 5 μL of 40% solvent B) were introduced onto the mixing loop, which had been pre-filled with solvent C, using a syringe pump operated at 1 μL/min for 5 min. The collected eluent was then treated in three different configurations (Supplementary Fig. 1). Briefly, in configuration I, a 5-μL sample loop was used without online solvent mixing (control experiment). In configuration II, a 30-μL sample loop was used with offered online solvent mixing, mimicking the conditions in the original RP–RP platform. In configuration III, a 30-μL sample loop was used as well as an SCX column, mimicking the condition in the modified RP-SCX-RP platform. Peptides trapped by the SCX column were then eluted with a buffer (500 mM ammonium acetate, pH 7) to the low-pH RP column. For all experiments, the LC gradient was programmed as follows: 0–30 min, 0% D, 2 μL/min; 30–35 min,

0–5% D, 1 μL/min; 35–95 min, 5–35% D; 1 μL/min; 95–105 min, 35–80% D, 1 μL/min; 105–120 min, 2 μL/min, 0% D.

2.5. Workflow of the original RP–RP and RP-SCX-RP platform

Online RP–RP 2DLC was performed as described previously (Supplementary Fig. 2) [15] while the RP-SCX-RP platform was implemented as shown in Fig. 1. Briefly, pumps 1 and 2 delivered the mobile phase for the first- and second-dimension RP columns, respectively. Alkaline buffers A and B were used as the mobile phase for pump 1 in the first RP; acidic buffers C and D were used as the mobile phase for pump 2 in the second RP. The complete 2D-LC analysis of a single sample comprised 12 fractions (F1–F12) eluted from the high-pH RP column, with each fraction further separated in the low-pH RP column, followed by MS/MS analysis. A sample was introduced by an auto-sampler at 1 μL/min for 30 min and first fractionated in the first-dimension LC using pump 1 at 1 μL/min with a gradient of 0–12% B (F1), 12–14% B (F2), 14–16% B (F3), 16–18% B (F4), 18–20% B (F5), 20–22% B (F6), 22–24% B (F7), 24–26% B (F8), 26–28% B (F9), 28–30% B (F10), 30–32% B (F11), and 32–38% B (F12). Each fraction of the first-dimension LC eluent was collected using a 30-μL mixing loop prefilled with solvent C in each 5-min interval (Supplementary Fig. 2, step 1). For RP–RP, the stored fraction was then transferred directly for second-dimension LC separation (Supplementary Fig. 2, step 2) at 2 μL/min (0–27 min, 0% D). The whole gradient was performed at 1 μL/min as follows: 27–28 min, 0–5% D; 28–188 min, 5–35% D; 188–199 min, 35–80% D; 199–209 min, 80% D; 209–240 min, 0% D (Supplementary Fig. 2, step 3). For RP-SCX-RP, the stored fraction was then transferred to an SCX trap column with a stream of 100% solvent C at 2 μL/min for 25 min (Fig. 1, step 2a). Peptides trapped by the SCX column were then eluted by bypassing the mixing loop onto the second-dimension RP with buffer (500 mM ammonium acetate, pH 7.0, 8 μL) through an auto-sampler with 100% C at 2 μL/min and the column was equilibrated 100% C at 2 μL/min for 20 min (Fig. 1, step 3). The peptides on the low-pH RP column were then separated using pump 2 with solvents C and D. The gradient was performed at 1 μL/min as follows: 0–1 min, 0–5% D; 1–161 min, 5–35% D; 161–171 min, 35% D; 171–182 min, 35–80% D; 182–207 min, 0% D. This process completed the whole cycle of the first fraction analysis. For the analysis of subsequent fractions, a similar cycle was conducted, except that the isocratic flow of pump 1 in step 2 was based on the organic composition at the end of the gradient of each fraction (e.g., 12% B for F1, 14% B for F2, and 16% B for F3). For iTRAQ experiments in RP-SCX-RP, a similar operation was performed except that the stored eluent in the mixing loop was transferred to the waste by passing through the SCX column with a stream of 100% solvent C at 2 μL/min for 25 min (Fig. 1, step 2b). Peptides trapped on the SCX column were eluted by bypassing the mixing loop onto the low-pH RP column with buffer (500 mM ammonium acetate, pH 7.0, 8 μL) through an auto-sampler with 100% C at 2 μL/min (Fig. 2, step 3).

2.6. MS and peptide identification

All MS data were acquired using an AB Sciex QSTAR XL Q-ToF mass spectrometer (AB sciex; Foster City, CA) and Analyst QS 1.1 software. The optimized acquisition parameters were as follows: nanospray voltage, 3000 V; DP, 60 V; FP, 220 V; DP2, 25 V; collision gas (CG), 5; GS1, 0 psi; GS2, 0 psi; CUR, 20. Collision-induced dissociation spectra were acquired in the information dependent acquisition (IDA) mode with the scan cycles set to perform a 1-s full scan over a mass-to-charge (m/z) range of 400–1600, followed by four 1-s MS/MS scans of the four most abundant peaks that exceeded 10 counts and carries a charge state between +2 and +4 in the range m/z 100–1500. The dynamic exclusion time of

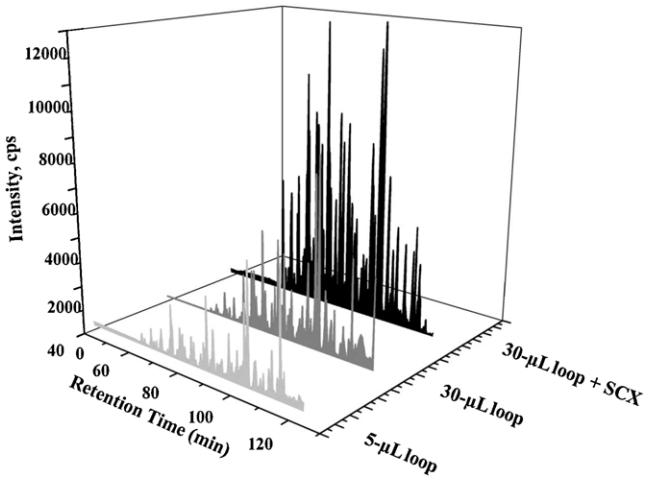


Fig. 2. Base peak chromatograms from the analyses of standard peptide digests obtained using (**i**) a 5-μL mixing loop (**configuration I**); (**ii**) a 30-μL mixing loop (**configuration II**); and (**iii**) a 30-μL mixing loop followed by an SCX trap column (**configuration III**). The highest signal intensities were obtained using **configuration III**.

the acquired ions was set at 120 s. The acquired MS/MS spectra were searched against the theoretical spectra generated from the sequences in the Uniprot15.8 Arabidopsis (31,669 entries; http://www.uniprot.org) and in the Uniport15.8 with the *Mus musculus* reviewed subset (16,313 entries; http://www.uniport.org) for Arabidopsis proteins and mouse embryonic fibroblast cells, respectively, using the Paragon algorithm in the ProteinPilot 3.0 software (Applied Biosystems, Framingham, MA). In all searches, trypsin was set as the enzyme used and IAA was chosen as the cysteine alkylation reagent. A thorough identification search with biological modification settings containing 225 built-in modifications was used. Precursor mass accuracy and product ion mass accuracy were predetermined by choosing the instrument option as QSTAR electrospray in the software. The identified peptides from the Paragon algorithm were grouped into minimal non-redundant proteins sets by the ProGroup algorithm. The peptide score was based on the statistical confidence of sequence matching specified in the software; 99% and 95% confidence translated into scores of 2.0 and 1.3, respectively. For protein identifications to be considered, a minimal unused ProtScore of 1.3 with at least one 95% confidence peptide was required. For all quantification analyses, MMTS was selected as the cysteine modification agent and iTRAQ 8-plex peptide-labeled quantification was chosen. Protein quantification was performed using ProteinPilot, based on the integrated area underneath the report ion peaks. False discovery rate (FDR) analysis was performed using the PSPEP add-on function of ProteinPilot and a decoy database of reverse sequences [22].

3. Results and discussion

3.1. Evaluation of peptide trapping efficiency of the SCX column

Solvent incompatibility between the two LC dimensions is a major challenge for effective fractionation of peptides using online high-pH RP/low-pH RP chromatography. Previously, we demonstrated the advantages of partial loop injection in which the organic composition was diluted by transferring the high-pH eluent from the first RP column as a fraction of the contents within a 30-μL sample loop prefilled with a low-pH, highly aqueous buffer (water/ACN/formic acid, 97.5:2:0.5) [15]. The decrease in the organic content of the eluent facilitated the focusing of the fractioned peptides on the second RP column. The online adjustment

of the 30-μL sample loop for focusing peptides on the second-dimension column could, however, be influenced by the organic content of the high-pH eluent. Using our previous approach, we found that increasing the organic content lowered the number of peptides identified and their signal intensities (data not shown). In this study, we attempted to minimize the flow-through problem caused by solvent incompatibility, thereby increasing the capability of the second-dimension RP column to retain fractioned peptides from the first RP column. In the modified setup, we located an additional SCX trap column upstream of the second RP column (Fig. 1). This system enhanced the trapping efficiency because interactions between the SCX resin and peptides are based mainly on net charges, with minimal influence of the organic content. The SCX flow-through was trapped by the second-dimension RP column. In addition, the SCX trap column enables peptide re-concentration and focusing prior to the second RP separation; as such, narrow sample bands are introduced [17,18,23]. The eluent must, however, be at a sufficiently low pH for SCX peptide trapping. The pH of the mixture of solvents A and C (1:5, v/v), measured offline, was ca. 2.5. Therefore, a 5-μL sample of eluent from the first-dimension RP column collected in a 30-μL sample loop should result in a pH suitable for SCX trapping, assuming that prefect mixing is achieved (as it had been offline).

To evaluate the performance of the RP-SCX-RP system versus the original RP–RP system, we introduced a mixture of standard digest dissolved in 40% solvent B to the mixing loop with configuration I–III (Supplementary Fig. 1) for comparison. We identified a total of 75 unique peptides (95% confidence) in configuration III (mimicking RP-SCX-RP), representing a 44.2% increase relative to the 52 unique peptides identified in configuration II (mimicking RP–RP). The beneficial effect was further illustrated by the highest signals for the base peak chromatogram being obtained with configuration III. This signal enhancement by the use of SCX trap column was also previously described [24]. Besides, we compared the ion intensities of 20 identified peptides detected in those experiments. In all cases, the ion intensities were normalized to those obtained in the standard run (mixture of standard digest dissolved in 2% solvent B) performed in configuration I and are described as percentage recoveries. For the analyses using configurations I and II, the recoveries were generally low for the more hydrophilic peptides, but they increased upon increasing the hydrophobicity of the peptides (Fig. 3).

RP columns generally show poor performances on the separation of more hydrophilic peptides. In our investigations, this phenomenon was evidenced by the broadening of peaks for those peptides in configuration II as compared to configuration III (data not shown), potentially leading to reduction in peptide identification. Our data also demonstrated that most of the highly hydrophilic peptide was eluted as flow-through in configuration II even under the reduced organic strength introduced by the mixing loop, such as the hydrophilic peptide SHCIAEVEK (hydrophobicity: 8.92) (Supplementary Fig. 3A). In addition, the MS signal detected was substantially lower than configuration III which incorporated the SCX trap column. This peptide was probably not hydrophobic enough to be fully re-concentrated on the second RP column and was "lost" in the flow-through as described recently [15]. On the other hand, the flow-through effect and signal reduction were not obvious for the more hydrophobic peptide YLEFISDAIIHVLHSK (hydrophobicity: 43.66) detected in configuration II (Supplementary Fig. 3B). Furthermore, as the signal for the SHCIAEVEK peptide obtained in configuration II (RP–RP) was below the selection threshold for information dependent acquisition (IDA) experiments, no MS/MS spectrum was acquired. By contrast, the same peptide detected in configuration III (RP-SCX-RP) could be identified through its MS/MS spectrum at 95% confidence. In the case for a hydrophilic peptide whose signal obtained in configuration II was strong enough to be selected for IDA-triggered fragmentation, the MS/MS spectrum acquired were in much lower quality compared to configuration III (Supplementary Fig. 4). Taken together, the use of an SCX trap column in RP–RP 2D-LC enriched the identification of hydrophilic peptides by reduction of flow-through problems and enhancement of MS signals, and offering improved second-dimension RP separation.

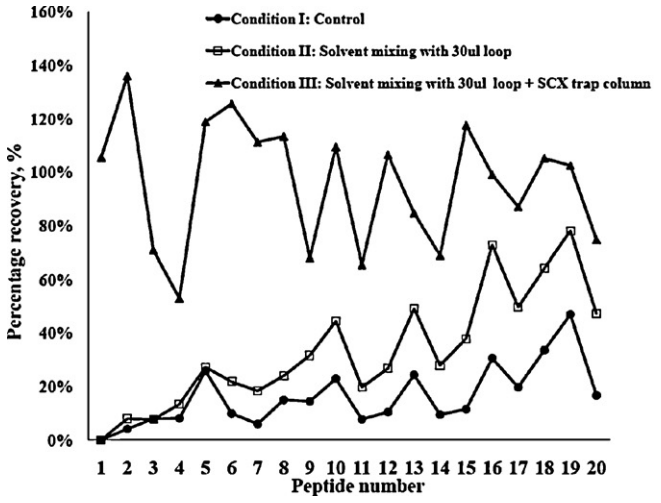
3.2. Performance of RP-SCX-RP platform for analysis of complex samples

To examine the performance of RP-SCX-RP versus RP–RP in proteomics analysis, we analyzed a trypsin-digested complex sample derived from *Arabidopsis* chloroplast proteins (15 μg). For fair comparison, we used the same number of fractions (12) for the first-dimension RP column and an identical gradient for both the high- and low-pH RP columns for both platforms. In RP–RP analysis, 685 proteins and 5911 unique peptides were identified after duplicate analyses. Among them, 422 proteins (61.6%) and 1924 (32.5%) unique peptides were common in both technical replicates (Fig. 4). In the RP-SCX-RP system, the numbers of proteins and unique peptides identified from duplicate analyses were 862 and 6757, respectively. Among them, 557 proteins (64.6%) and 2502 (37.0%) unique peptides were common in both technical replicates (Fig. 4; a list of the proteins and peptides is provided in the Supporting information). Relative to the RP–RP system, RP-SCX-RP analysis resulted in the identification of 25.8% more proteins and 14.3% more unique peptides. Overall, 88.3% of the proteins (604 of 685) and 61.0% of the peptides (3605 of 5911) identified in the RP–RP analysis were also detected in the RP-SCX-RP analysis. Hence, the RP-SCX-RP platform exhibited improved performance over the RP–RP system for proteome analysis, as measured by the identification of additional peptides and proteins.

We also analyzed the mouse embryonic fibroblast STO cell lysate (ca. 17 μg of protein) using the two platforms. Notably, however, the percentage increases in protein/peptide identification in the RP-SCX-RP platform over the RP–RP system were not as substantial as those obtained for the for chloroplast sample. In the RP–RP analysis, the numbers of proteins and peptides were 2145 and 8810, respectively; we identified 2201 proteins and 9352 peptides in a single RP-SCX-RP analysis (Supplementary Fig. 5), only modest increases of 2.6 and 6.2%, respectively, relative to the RP–RP system.

3.3. Peptide hydrophobicity distribution

We compared the distributions of identified peptides from the RP-SCX-RP and RP–RP platforms in different hydrophobicity ranges. Fig. 5A reveals that RP-SCX-RP analysis resulted in the detection of more peptides in most hydrophobicity ranges. Interestingly, the magnitude of the percentage increase was related to the peptide hydrophobicity. In general, larger increases occurred for the identification of peptides with lower hydrophobicity (Fig. 5B). For example, the RP-SCX-RP platform detected 68 and 71.4% more unique peptides in the hydrophobicity ranges of less than 15 and 15–20, respectively, relative to the RP–RP system. In fact, the SCX trap column was demonstrated to identify more hydrophilic peptides than the RP trap column [24]. It has been found that the magnitude of the increase in the number of detected peptides gradually declined upon increasing hydrophobicity. This trend is consistent with the one we observed in the solvent online adjustment experiment (see Section 3.1), which revealed that the recovery of hydrophilic peptides in the RP–RP platform was lower than that of hydrophobic peptides. Hydrophilic peptides are less likely to be retained on the second RP column at a high organic content, leading to a flow-through problem that decreases the intensity of the peptide signals through ion suppression during MS/MS



No.	Peptide Sequence	Protein	m/z	charge	Relative Hydrophobicity[b]
1	SHCIAEVEK	BSA	531.2	2	8.92
2	DLGEEHFK	BSA	487.7	2	14.77
3	FESNFNTQATNR	Lysozyme C	714.8	2	17.11
4	SSGTSYPDVLK	Trypsin	577.3	2	18.23
5	IIVTQTMK	Beta-lactoglobulin	467.3	2	18.39
6	HLVDEPQNLIK	BSA	653.4	2	18.84
7	VEADIAGHGQEVLIR	Myoglobin	536.3	3	23.26
8	VLVLDTDYK	Beta-lactoglobulin	533.2	2	24.41
9	LYQEPVLGPVR	Beta-casein	635.9	2	26.49
10	HQGLPQEVLNENLLR	Alpha-S1-casein	587.3	3	27.24
11	NTDGSTDYGILQINSR	Lysozyme C	877.4	2	27.47
12	LVNELTEFAK	BSA	582.3	2	28.92
13	LLYQEPVLGPVR	Beta-casein	692.4	2	31.24
14	LGEYGFQNALIVR	BSA	740.4	2	34.17
15	SIVHPSYNSNTLNNDIMLIK	Trypsin	758.4	3	34.52
16	IHPFAQTQSLVYPFPGPIPN	Beta-casein	1112.6	2	37.65
17	FFVAPFPEVFGK	Alpha-S1-casein	692.9	2	41.31
18	YLGYLEQLLR	Alpha-S1-casein	634.4	2	42.44
19	YLEFISDAIIHVLHSK	Myoglobin	629.3	3	43.66
20	SLAMAASDISLLDAQSAPLR	Beta-lactoglobulin	1015.5	2	43.85

Fig. 3. Percentage recoveries of peptides with different theoretical hydrophobicities obtained using (**i**) a 5-μL mixing loop (**configuration I**); (**ii**) a 30-μL mixing loop (**configuration II**); and (**iii**) a 30-μL mixing loop followed by an SCX trap column (**configuration III**). The percentage recovery of peptides corresponds to the ion intensity of the selected peptides in all runs normalized to the intensity in a standard run (standard peptide digests dissolved in 2% solvent B) obtained in **configuration I**. The hydrophobicities were determined using a sequence-specific retention calculator (SSRC) [30,31].

analysis. Additionally, peak broadening for the more hydrophilic peptides is likely to further decrease the resolution of separation. For the RP-SCX-RP platform, peptide trapping is based mainly on charge, thereby minimizing the flow-through problem resulting from solvent incompatibility. This behavior is consistent with the greater number of hydrophilic (lower number of hydrophobic) peptides identified by the RP-SCX-RP platform relative to the RP–RP system. We observed a similar trend for the analysis of the STO cell lysate. The percentage increase in identification was greater for peptides exhibiting lower hydrophobicity than it was for those of higher hydrophobicity (Supplementary Fig. 6). We observed mild decreases, however, in the numbers of identified peptides with high hydrophobicity (>35) for the RP-SCX-RP platform's analyses of both the chloroplast and STO cell samples. This phenomenon was probably related to hydrophobic interactions between the SCX sorbent and the peptides. It is a common problem for online SCX-RP to be unable to identify some hydrophobic peptides [25].

The performance of RP-SCX-RP platform relative to the RP–RP system for the analysis of the STO lysate was not as promising as that for the chloroplast protein in terms of increases in the number of identified proteins and peptides, presumably because of differences in the distributions of peptide hydrophobicity between the two samples. The percentages of identified peptides with hydrophobicities of less than 30 were 49.9 and 24.9% in the chloroplast and STO samples, respectively. As mentioned above, the effect of the organic content on the peptide signal intensity and identification was related to the peptide hydrophobicity. Thus, although the RP-SCX-RP platform mainly enhanced the detection of the less-hydrophobic peptides, this feature might not be obvious for samples featuring predominantly hydrophobic peptides (e.g., the STO cell lysate).

3.4. Application of iTRAQ samples in RP-SCX-RP platform

The use of iTRAQ technology for simultaneous identification and quantification of proteins in different biological samples has been emerging rapidly in recent years [26]. SDS detergent (ca. 0.02%) and the excess of labeling reagents used in the iTRAQ reactions are usually removed prior to MS/MS analysis to minimize the effects of ion suppression. In fact, samples incorporating 0.02% SDS have exhibited strong suppression of peptide signals and decreased peptide identification in 1D reversed-phase LC/MS/MS experiments [19].

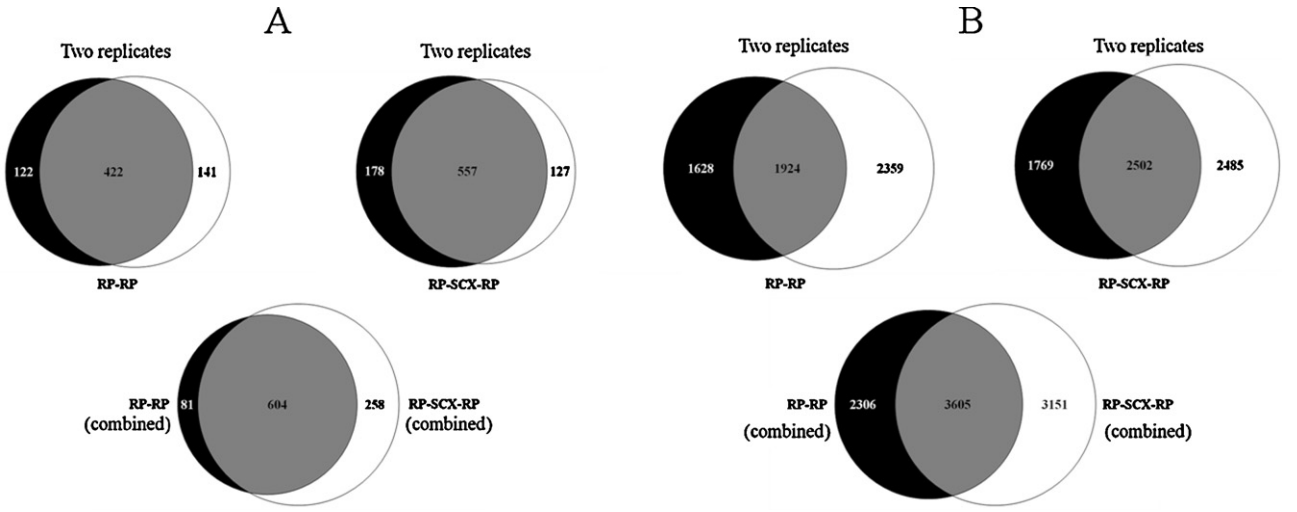


Fig. 4. (**A**) Protein and (**B**) peptide identifications using the RP–RP and RP-SCX-RP platforms. Total unique proteins (95% confidence) and unique peptides (95% confidence) in duplicate runs for chloroplast sample were used to represent the protein and peptide overlap in the RP–RP and RP-SCX-RP platforms.

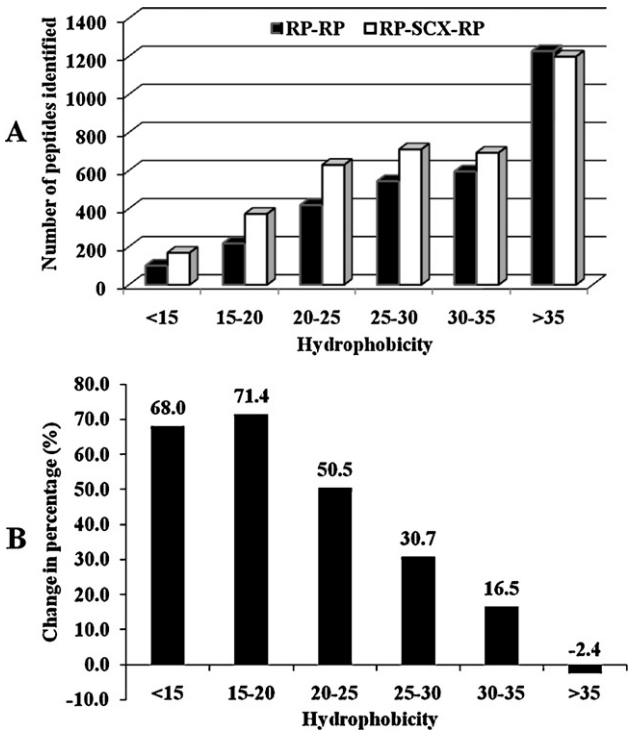


Fig. 5. (**A**) Distribution of unique peptides (95% confidence) according to their theoretical hydrophobicity (chloroplast samples); (**B**) percentage increase (RP-SCX-RP over RP–RP) in peptide (95% confidence) identification according to theoretical hydrophobicity (chloroplast samples).

Column-based removal of SDS is usually performed through ion exchange, HILIC, or gel permeation [27–29]. In iTRAQ applications, offline SDS clean-up (e.g., SCX chromatography) is recommended prior to online RP–RP-MS analysis. In our modified platform, the SCX trap column allowed both fraction-focusing and clean-up of the first-dimension eluent; the excess chemicals and detergent were removed simply by valve switching (Fig. 1, step 2b). Hence, online RP–RP analysis for iTRAQ applications is feasible when using crude labeled samples containing low amounts of protein.

To examine the performance of the RP-SCX-RP platform in iTRAQ applications, we analyzed a crude labeled sample containing approximately 15 μg of chloroplast proteins prepared from *Arabidopsis* wild type and mutants (*thf1*, *clpr4*, and *thf1clpr4*). We expected these mutants, which have different mutations affecting chloroplast development, to feature differentially expressed chloroplast proteomes. A total of 642 proteins and 3116 unique peptides (746 proteins and 5221 unique peptides at ≤1.0% global FDR). Among them, 156 proteins were up- or down-regulated (20% fold change, $p < 0.05$) in any one of the *Arabidopsis* mutants, as summarized in Supplementary Table 1. On the other hand, with the RP–RP platform we identified 540 proteins and 2151 unique peptides (645 proteins and 3634 unique peptides at ≤1.0% global FDR). Thus, the RP-SCX-RP platform identified 44.8% and 18.9% more peptides and proteins, respectively, relative to the RP–RP system. In addition, 97 proteins identified in the RP–RP platform exhibited either up- or down-regulation (20% fold change, $p < 0.05$) in any one of the mutants. Together, our results demonstrate that the RP-SCX-RP platform provides improved performance over the RP–RP system in both qualitative and quantitative analyses. The numbers of distinct peptides identified per protein in the RP-SCX-RP and RP–RP platforms were 4.85 and 3.98, respectively. Notably, a higher number of distinct peptides identified per protein is not only beneficial to the confidence of protein identification but also to protein quantification. This behavior is not surprising because if more peptides can be identified per protein then more quantitative information can be obtained per protein through the reporter ions of the iTRAQ tag in the MS/MS spectra, thereby leading to improved protein quantification (RP-SCX-RP: 156 proteins; RP–RP: 97 proteins; i.e., a 60.8% increase). In addition, the additional SCX column serves to remove the detergents and unused tagging chemicals from the crude sample (Fig. 1, step 2b), thereby minimizing ion suppression effects that would otherwise reduce the quality and quantity of peptide detection by the MS/MS system. Therefore, our results strongly indicate that the RP-SCX-RP platform is compatible with quantitative proteomics analyses using crude iTRAQ-labeled samples. Furthermore, our modified platform allows the analysis of small amounts of samples in a high-throughput manner – a feature that is not technically feasible for the routine procedure involving offline SCX clean-up followed by RP–RP analysis.

4. Conclusions

In this study, we developed a modified high-/low-pH RP–RP platform, incorporating an SCX trap column between the two dimensions, that exhibits enhanced performance for qualitative and quantitative proteomics applications. The presence of the SCX column, which trapped relative peptides from the high-organic-content eluent from the first-dimension high-pH RP column, provided enhanced signal intensity and peptide identification, especially for hydrophilic peptides. We compared the performance of the RP-SCX-RP platform directly with that of the RP–RP system over comparable instrument times (RP-SCX-RP: ca. 52 h; RP–RP: ca. 48 h). In the analysis of *Arabidopsis* chloroplast proteins, the modified platform resulted in 25.8% and 14.3% increases in protein and peptide identification, respectively. The extent of the improved performance was not as substantial, however, when using the RP-SCX-RP platform to analyze the STO lysate samples containing higher proportion of more hydrophobic peptides. The modified platform was also amenable to automated iTRAQ-based quantitative analyses without the need for offline cleaning-up procedures. Relative to our original RP–RP system, the modified system enabled the online detection of a larger number of differentially expressed proteins. Hence, the RP-SCX-RP platform appears to be suitable for further application in high-throughput qualitative and quantitative proteomics investigations at low-microgram levels with minimal sample preparation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.04.022.

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