PROTEOMICS METABOLOMICS GENOMICS INFORMATICS
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Efficient peptide fractionation and improved protein identification with the Agilent 3100 OFFGEL Fractionator

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The comprehensive analysis of complex protein or peptide mixtures by LC/MS can be improved by including a prefractionation step in the workflow. This application note illustrates the effectiveness of OFFGEL electrophoresis with the Agilent 3100 OFFGEL Fractionator for the prefractionation of peptides. The 3100 was used to fractionate a tryptic digest of *E. coli* proteins into 24 fractions. Peptides were identified by reversed phase liquid chromatography with mass spectrometric detection. About ninety percent of the identified individual peptides were found in only one or two fractions. The distribution of the calculated isoelectric points for the peptides identified in each fraction was especially narrow in the acidic pH range. Standard deviations approached the size of the pH segment covered by the respective fraction.

Introduction

The success of proteome analysis projects is highly dependent on the quality of the sample fractionation employed prior to analysis by mass spectroscopy (MS). For peptide-level analysis, cation-exchange (SCX) and reversed-phase chromatography (RP) are typically combined. The use of immobilized pH gradient gel-based isoelectric focusing (IPG IEF) instead of SCX has been described before (1). Compared to cation-exchange chromatography, IPG IEF provides higher resolution separation, and experimentally derived pI information which can be used as a filter criterion for tandem mass spectral data validation (2). However, a major limitation of this method is the tedious post-IEF sample processing that requires cutting the IPG gel strip into sections, then extracting and cleaning up peptides from the gel sections.

In the present work, an *E. coli* total protein digest was fractionated by OFFGEL electrophoresis with the Agilent 3100 OFFGEL Fractionator and by reversed-phase chromatography, and then analyzed by mass detection (RP-LC/MS). OFFGEL electrophoresis is a recent advance in separation technology that fractionates proteins or peptides according to their pI. This technique achieves the same high resolution as IPG gels, but recovers the sample in the liquid phase. Therefore, it fits nicely into the LC/MS workflow. After fractionation and acidification, a portion of the sample was injected directly onto a chip-based reversed-phase column without additional sample preparation, eliminating the need for tedious and error-prone peptide isolation from the IPG gel. The distribution of the calculated isoelectric points for the identified peptides in the respective fractions was especially narrow in the acidic pH range, with standard deviations in the range of the expected fraction width. About ninety percent of the identified peptides were found in only one or two fractions, demonstrating the resolution of the technique.

Experimental

Sample preparation

An *E. coli* total protein lysate from Bio-Rad (Hercules, CA, USA) was reduced and denatured using 50% 2,2,2-trifluoroethanol with 200 mM DTT at 95°C for 20 minutes. This was followed by alkylation with iodoacetamide at room temperature for 1 hour. The reduced and alkylated sample was diluted 1:10. Trypsin (Promega, Madison, WI, USA) was added at 1:20 enzyme:substrate and the sample was incubated overnight at 37°C. The digest was aliquoted, dried, and stored frozen until use.

OFFGEL electrophoresis

For pI-based peptide separation, the Agilent 3100 OFFGEL Fractionator with a 24-well setup and a 24 cm, pH 3–10 IPG strip was used according to the protocol. The sample was focused with a maximum current of 50 µA and typical voltages ranging from 500 V to 4000 V, until 50 kVh was reached after 24 hours. The recovered fractions (volumes between 100 µL and 150 µL) were acidified with 5 µL formic acid.

HPLC-Chip/MS analysis

A 0.5 µL aliquot of each fraction was injected onto an LC/MS system consisting of an 1100 Series liquid chromatograph, HPLC-Chip Cube MS interface, and 1100 Series LC/MSD Trap XCT Ultra ion trap mass spectrometer (all Agilent Technologies). The system was equipped with an HPLC-Chip (Agilent Technologies) that incorporated a 40 nL enrichment column and a 43 mm x 75 µm analytical column packed with ZORBAX 300SB-C18 5 µm particles. Peptides were loaded onto the enrichment column with 97% solvent A (water with 0.1% formic acid) and 3% B (90% acetonitrile with 0.1% formic acid) at 4 µL/min. They were then eluted with a gradient from 3% B to 45% B in 30 minutes, followed by a steep gradient to 80% B in 5 minutes at a flow rate of 0.3 µL/min. The total run time, including column reconditioning, was 45 minutes.

The SwissProt database was searched with the restriction to *E. coli,* using the Agilent Spectrum Mill software. An iterative searching strategy was employed for all searches. Autovalidation flagged the high-confidence identifications so that no false positives were included.

Result and Discussion

In OFFGEL electrophoresis, protein or peptide separation takes place in a two-phase system, with an upper liquid phase that is divided into compartments and a lower phase being a conventional rehydrated IPG gel strip. Typically, the sample is diluted into the focusing buffer and loaded into all wells. Because there is no fluidic connection between the wells, proteins or peptides are forced to migrate through the IPG gel where the actual separation takes place. After IEF, the proteins or peptides are present in the liquid phase and can be recovered conveniently from the wells for further processing (Figure 1).

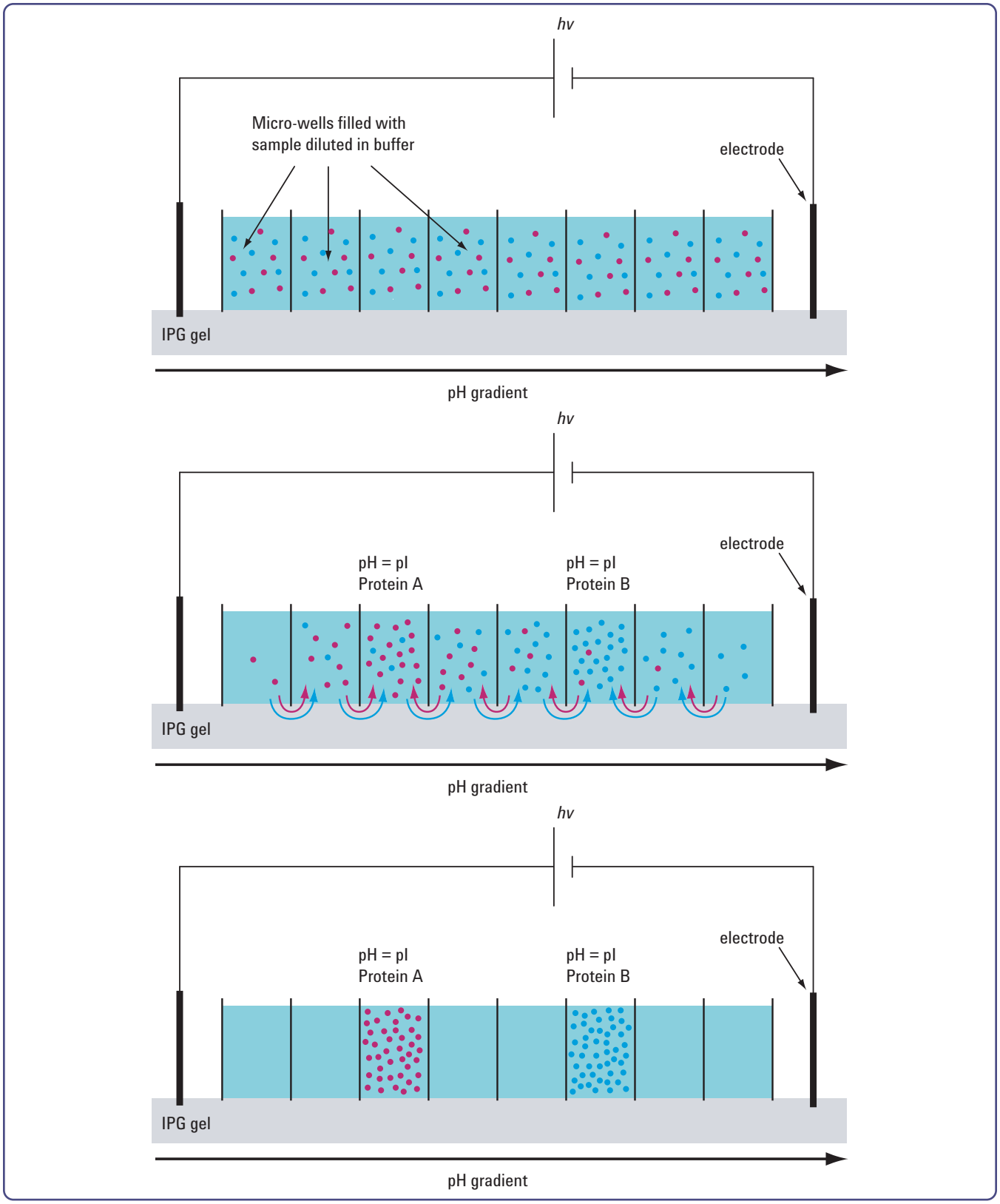

Figure 1. Principle of OFFGEL electrophoresis

A useful fractionation technique should combine ease of use and good recovery of the sample with excellent resolution (individual peptides predominantly in one fraction) and good distribution over the fractions for maximum complexity reduction. The HPLC-Chip/MS analysis of the *E. coli* peptide sample fractionated by OFFGEL electrophoresis revealed that 74% of the identified individual peptides were found in only one fraction and about 90% in one or two fractions (Figure 2). Furthermore, peptides showed a good distribution across the fractions (Figure 3a and Table 1), in line with results of an *in silicio* tryptic digest of the whole *E. coli* proteome (4).

The Spectrum Mill software automatically calculates the pI for every peptide identified. Using these data, average pI values with standard deviations were calculated, without any data filtering, for all peptides identified in each fraction. The average pI values fit fairly well to the expected pH range calculated according to the specifications of the IPG strips (Figure 3b). Major deviations were only observed for fractions 13–18 (pH range 6.5–8.1). These deviations very likely resulted from the low number of peptides found in this pH range. Deviations between expected and observed pI values were reported for conventional IPG IEF as well (1, 2).

Since IEF separates peptides according to their pI, consideration of the standard deviation of the pI value distribution in each fraction should provide an alternative method to judge the resolving power of OFFGEL electrophoresis with the 3100. Taking all fractions together, an average standard deviation of ± 0.42 pI units was observed. However, the standard deviations differ significantly between fractions, with the general trend that fractions with lower numbers of identified peptides had higher standard deviations (compare Figures 3a and 3b). When fractions with a low peptide content (< 60 peptide identifications: fractions 7, 8, and 18–20) were excluded, the average standard deviation dropped to ± 0.33 pI units. Very low standard deviations that approach the pH interval covered by a single well (i.e. 0.26 pH units) were observed for several fractions with high peptide content in the acidic pH range (Table 1). In total, 3506 distinct peptides and 670 distinct proteins were identified in this study.

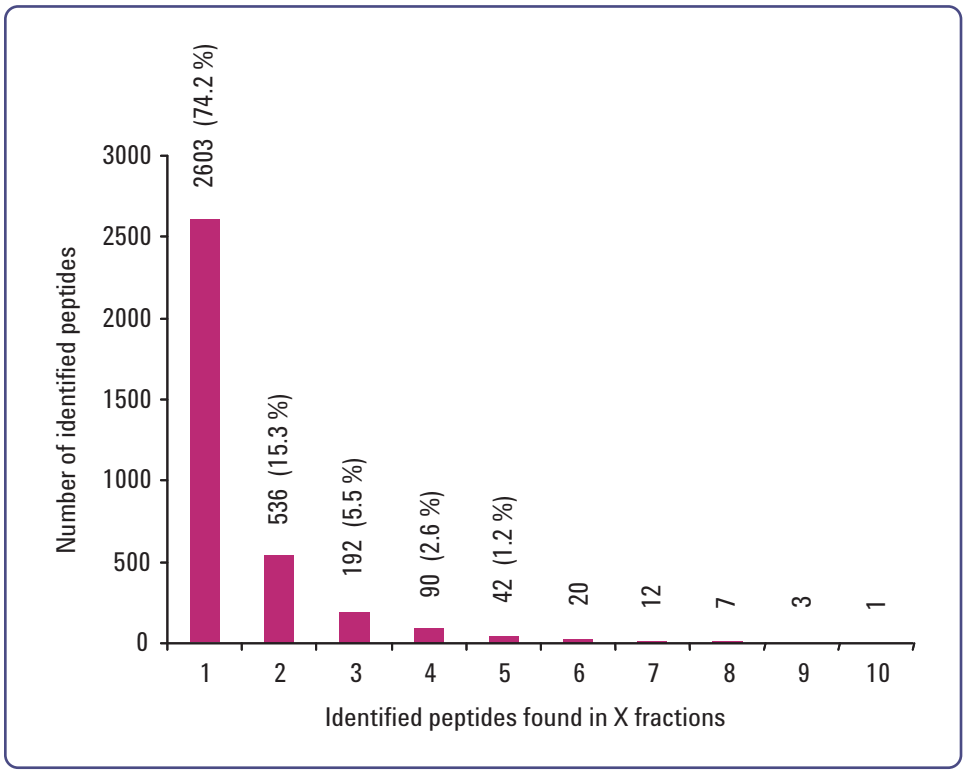

Figure 2. Fraction-wise distribution of identified *E. coli* peptides

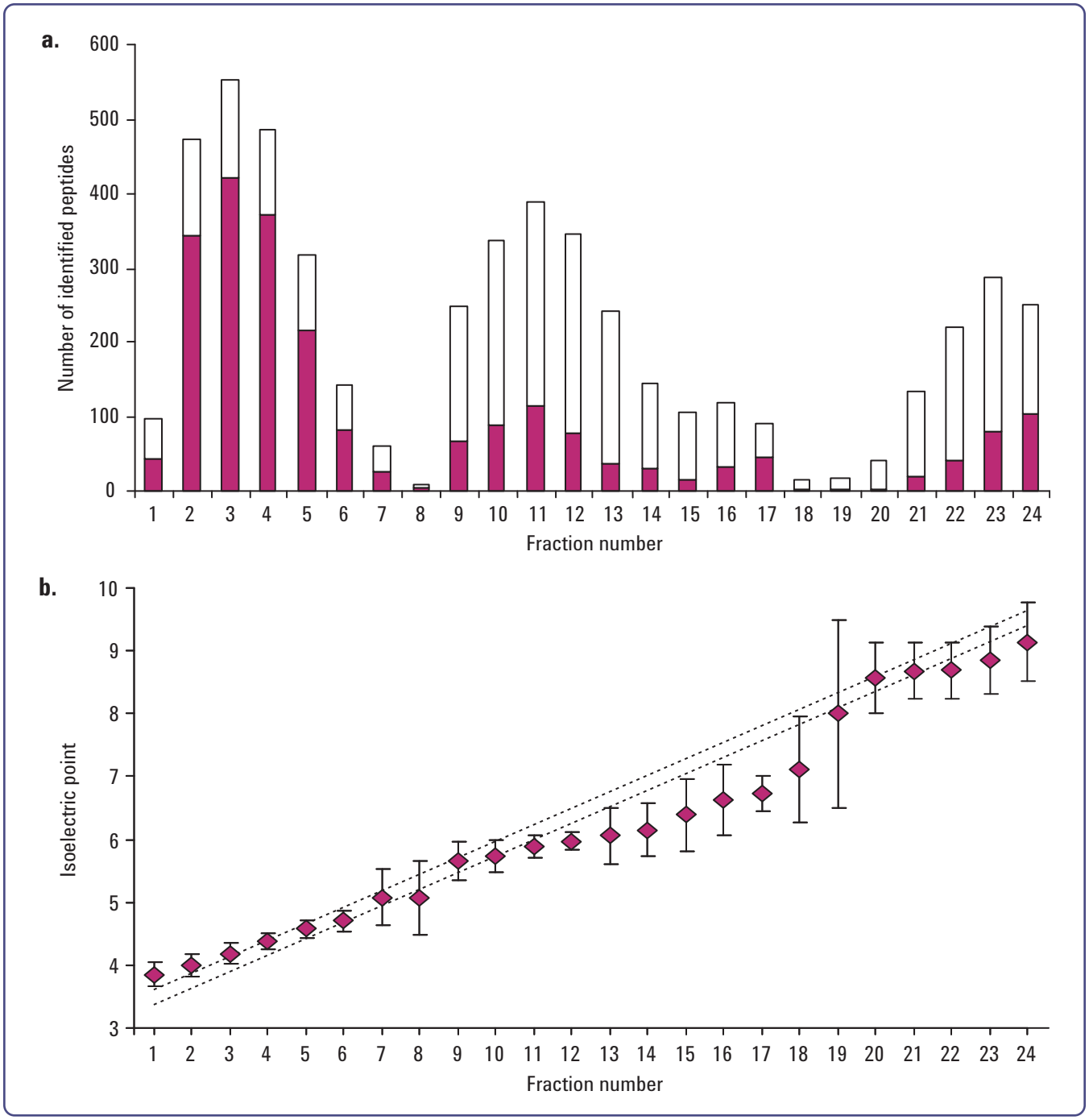

Figure 3. Analysis of *E. coli* peptides by OFFGEL electrophoresis and HPLC-Chip/MS: a) Total number of peptides identified in each fraction; the dark shaded area relates to the peptides unique to each fraction, and b) Average calculated pI values with standard deviations for all peptides identified in each fraction. The two dashed parallel lines indicate the size of the pH interval covered by each well as calculated according to the specifications of the IPG gel strip supplier.

Fraction	Expected pH range	Unique/total peptides	Average pI with standard deviation
1	3.35–3.61	43 / 97	3.85 ± 0.19
2	3.61–3.88	343 / 473	3.99 ± 0.18
3	3.88–4.14	421 / 553	4.19 ± 0.17
4	4.14–4.40	372 / 486	4.38 ± 0.13
5	4.40–4.66	216 / 316	4.58 ± 0.14
6	4.66–4.93	81 / 143	4.71 ± 0.17
7	4.93–5.19	27 / 60	5.08 ± 0.45
8	5.19–5.45	4 / 8	5.07 ± 0.58
9	5.45–5.71	67 / 248	5.66 ± 0.31
10	5.17–5.98	87 / 337	5.73 ± 0.25
11	5.98–6.24	114 / 388	5.89 ± 0.18
12	6.24–6.50	76 / 344	5.98 ± 0.15
13	6.50–6.76	37 / 241	6.06 ± 0.46
14	6.76–7.03	31 / 144	6.15 ± 0.43
15	7.03–7.29	16 / 105	6.39 ± 0.58
16	7.29–7.55	32 / 118	6.62 ± 0.55
17	7.55–7.81	46 / 91	6.74 ± 0.28
18	7.81–8.08	3 / 16	7.12 ± 0.84
19	8.08–8.34	2 / 17	8.00 ± 1.49
20	8.34–8.60	3 / 42	8.56 ± 0.56
21	8.60–8.86	20 / 133	8.68 ± 0.45
22	8.86–9.13	42 / 220	8.69 ± 0.45
23	9.13–9.39	79 / 289	8.85 ± 0.53
24	9.39–9.65	104 / 251	9.14 ± 0.63

Table 1. Results of the analysis of *E. coli* peptides by OFFGEL electrophoresis and HPLC-Chip/MS. The expected pH ranges per fraction were calculated taking into account the OFFGEL well dimensions and the specifications of the IPG gel strip. Average pI values were calculated from all peptides identified in each well.

Conclusions

Off-gel electrophoresis with the Agilent 3100 OFFGEL Fractionator offers easy recovery of the sample with excellent resolution and a good distribution of the peptides across the fractions. This flexible technique for the separation of peptides as well as proteins can play a role in various steps of multidimensional separation schemes. Furthermore, the experimentally derived pI information can be used to increase the reliability of peptide identification procedures.

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