Reversed-phase liquid chromatography with double gradient elution for the separation and mass spectrometric analysis of peptides

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Abstract: Highly effective separation of complex peptide mixture is a prerequisite for protein identification with high coverage in proteomics. Currently, peptide mixture is separated by two-dimensional liquid chromatography (2D-LC) or ion exchange chromatography as the first dimension and reversed-phase chromatography as the second dimension in Shotgun proteomics. Though the 2D-LC is now widely used, its separation efficiency still needs further improvement. In this work, the first dimensional separation was performed by the pH and organic solvent double-gradient elution. And then the two fractions one from the early eluted section and the other from the later eluted section with equal time intervals were pooled and analyzed by MS/MS. The experimental results from the protein mixture of Saccharomyces cerevisiae lysate showed that the separation by pH and organic solvent double-gradient elution RP-HPLC-nanoRPLC coupled with MS/MS identified 567 more yeast protein groups 3 035 peptides over strong cation exchange chromatography-nanoRPLC coupled with MS/MS. The pI values and relative molecular masses of identified peptides ranged from 3.42 to 12.01 and from 587.67 to
3 499. 79\% respectively. The pI values and relative molecular masses of identified proteins ranged from 3. 82 to 12. 19 and from 3 446. 55 to 432 905\% respectively. These results indicated that this new 2DLC-MS method has the advantages over the conventional Shotgun method\% and were expected to be applied in the separation of complex samples for proteomic studies in the future.

**Key words** reversed-phase liquid chromatography\% RPLC\% pH gradient elution\% organic solvent gradient elution\% nano reversed-phase liquid chromatography-tandem mass spectrometry \% nanoRPLC-MS/MS\% peptides\% saccharomyces cerevisiae

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1

1.1

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<th>EC2000</th>
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<th>Thermo Fisher</th>
<th>Millipore</th>
<th>XBridge C18 250 mm \times 4. 6 mm 3. 5 µm Waters</th>
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<td>trypsin% DTT</td>
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Promega | IAA | Acros | ACN | HPLC | J. T. Baker | FA |

Fluka | | | NH\textsubscript{3}HCO\textsubscript{3} | BSA | Sigma |
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<td>Sigma</td>
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</tbody>
</table>
1.2

BSA 0.5 50 mmol/L NH₄HCO₃ 2 mg/mL 50 μL BSA

5.5 μL 100 mmol/L DTT 10 min 6.2 μL

1 mol/L IAA 1 h 1:50 BSAA 2 μg/mL BSA 37 °C 12 h

300 mg 1 mL 9.5 mol/L DTT 20 μL 3.28 μL 100 mmol/L DTT 37 °C 4 h 3.64 μL 1 mol/L IAA 1 h 50 mmol/L NH₄HCO₃ 215 μL NH₄HCO₃ 1:50 1 mol/L IAA

37 °C 23.58 mg/mL

1.3

BioBasic SCX XBridge C18 250 mm×4.6 mm 5 μm Thermo RPLC

250 mm×4.6 mm 3.5 μm Waters XBridge C18 250 mm×4.6 mm 3.5 μm Waters

1 SCX A 5 mmol/L KH₂PO₄ +30% ACN pH 2.7

2 RPLC A 2% +98% H₂O +2% H₃PO₄ pH 3

3 pH 10 A 2% +98% H₂O +2% H₃PO₄ pH 10

4 pH 3 ~10 A 2% +98% H₂O +2% H₃PO₄ pH 10

100% A ~10% B 0.8 mL/min 3 min 214 nm BSA 10 μg/mL 50 μg/mL

1.4 nanoRPLC-MS/MS

SCX 52 s 30 s nanoRPLC-MS/MS

nanoRPLC-MS/MS pH 3 ~10 min 100% A ~30% B 100% B 3 min 100% B 5 min 100% B 30 s 120 s 60 s 2 s 61 B 30 min 63...120 1 61 62...59 119 60 120 2...62...59

nanoRPLC-MS/MS A 98% H₂O +2% B +0.1% ACN B 80% +20% H₂O +0.1% ACN 0 ~60 min 5% B ~50% B 0.3 μL/min 100 mm×75 μm Thermo BioBasic-18

5 μm 30 min

MS/MS m/z 400 ~2000 35% 5 2.0 kV pBuild

1.5

Bioworks 3.2 7.1 11 081 2010 7

http://downloads.yeastgenome.org/sequence/GenBank/trypsin

1.5 FDR ≤1% ΔCn ≥0. 0.8 8

pBuild 1.5

2

2.1 SCX pH RPLC BSA

10 μg 1.3
2.2 SCX $ \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \time...
Table 1: Identified results of nanoRPLC-MS/MS of tryptic digest products of protein mixture of saccharomyces cerevisiae lysate by SCX and double gradient RPLC.

<table>
<thead>
<tr>
<th>Method</th>
<th>Identified peptide</th>
<th>Identified protein group</th>
</tr>
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<tbody>
<tr>
<td>SCX-nanoRPLC-MS/MS</td>
<td>1210</td>
<td>766</td>
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<tr>
<td>Double gradient RPLC-nanoRPLC-MS/MS</td>
<td>4245</td>
<td>1333</td>
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Fig. 2: Chromatograms of tryptic products of protein mixture of saccharomyces cerevisiae lysate by SCX and RPLC at different pHs.

The a – d chromatographic conditions are the same as in Fig. 1a – d, respectively.

Fig. 3: pI distribution of identified peptides with different separation methods.

The pH range is 3 – 10.
Fig. 4  Relative molecular mass [M₉] distribution of identified peptides with different separation methods

Fig. 5  Hydropathicity [GRAVY] distribution of identified peptides with different separation methods

Fig. 6  pI distribution of identified proteins with double gradient RPLC separation

Fig. 7  Mᵢ distribution of identified proteins with double gradient RPLC separation
第3期

马岩，等：反相高效液相色谱双梯度洗脱分离肽混合物及质谱分析

由此可看出，双梯度洗脱方法高于两维凝胶电泳分离蛋白质的等电点和相对分子质量范围（一般两维凝胶电泳分离的蛋白质的等电点为...表明双梯度洗脱方法可以克服两维凝胶电泳分离鉴定蛋白质在相对分子质量和...结论

本研究建立了梯度结合有机相梯度分离肽段混合物的方法。此方法不仅提高了峰容量，而且使色谱峰在分离鉴定的时间内均匀分布，相对而言延长了质谱捕获时间，从而增加了鉴定效率，在复杂体系蛋白质组研究中有较好的应用前景。