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Technical Report

On-line Comprehensive RP-LC×RP-LC/IT-TOF for the Analysis of Proteome Isoforms

A meaningful evaluation tool for native and recombinant proteins

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Abstract:

This bottom-up approach relies on both the power of 2D-LC separation techniques, and the sensitivity of MS (ESI-IT-TOF) detection. After separation by RP-LC×RP-LC is performed at the peptide level, both PDA and MS 2D plots are obtained by means of a dedicated software, which further allows qualitative and quantitative data analysis, as well as spectral comparison/subtraction. Tandem MS data obtained by collision-induced dissociation (CID) of the peptides were used for database search for α-casein and dephosphorylated α-casein, with high sequence coverage.

Keywords: RP-LC×RP-LC–PDA–IT-TOF, tryptic digest, proteome analysis, protein isoforms

1. Introduction

The analysis of complex biochemical systems, such as proteins and peptides isolated from tissues, cells, and body fluids has always represented a major task for analytical chemistry. A high demand is in fact placed both on the power of separation techniques, given the extremely high complexity of the proteome samples, and on the sensitivity of detection methods, to enable probing of low abundant proteins or peptides. Different strategies have been developed attempting to address the needs of modern proteomics, increase the overall throughput of proteomics experiments, and facilitate researchers to investigate into the complicated biological networks in which proteins are involved, at different levels.

For many years, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by mass spectrometry (MS) has been the workhorse in analytical proteomics, its major drawbacks consisting in difficulty of automation, low accessibility of membrane-bound proteins, problematic detection of proteins with large molecular weight, high pI, strong hydrophobicity, or low abundance [1–4]. Over the last decade, considerable effort has been put in the development of ultra-high efficiency liquid chromatographic (LC) methodologies, pushing gel-free separation techniques to evolve beside the more troublesome and tedious 2D-PAGE experimental designs. 2D separation set-ups based on RP-LC are characterized by superior resolution and peak capacity, more homogeneous distribution of peptides elution in the separation window, robustness and easy handling [5, 6].

This technical report describes the first comprehensive 2D LC system, in which the on-line coupling of RP-LC×RP-LC to MS (IT-TOF) is investigated (Fig. 1). The two dimensions consisted both of a novel fused-core stationary phase specifically designed for peptide separation, interfaced through an electronically activated 2-position, ten-port valve. The performance of the system was assessed by means of tryptic mapping (protein unfolding, trypsin digestion, and reversed-phase chromatography of the peptide samples), followed by ESI MS characterization of α-casein and dephosphorylated α-casein (Fig. 2).

Fig. 1 LC×LC instrumentation with PDA and LCMS-IT-TOF detection

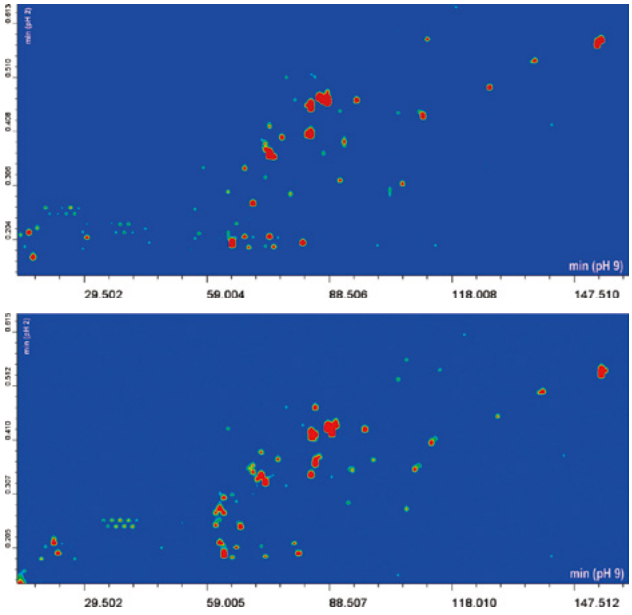

Fig. 2 Plots: α-casein (top) and dephosphorylated α-casein (bottom) tryptic digests

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2. Experimental

2-1. Instrument

- Shimadzu CBM-20A controller
- Shimadzu LC-20AD dual-plunger parallel-flow pumps (D1-LC)
- Shimadzu LC-20AB solvent delivery module (D2-LC)
- Shimadzu DGU-20A5 degassing unit
- Shimadzu CTO-20A column oven
- Shimadzu SIL-20A autosampler
- Shimadzu SPD-M20A photo diode array detector (8 µL flow cell)
- Shimadzu LCMS-IT-TOF (ESI source)

For connecting the two dimensions: electronically-controlled 2-position, ten-port high pressure switching valve (with two 100 µL sampling loops), Fig. 3.

2-2. Software

- Shimadzu LCMSsolution (Version 3.50.346)

2-3. 2D Software

- ChromSquare (Version 2.0) from Chromaleont, Messina, Italy

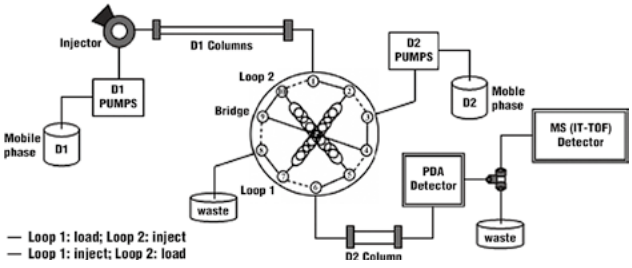

Fig. 3 Schematic of the 2D system and the switching valve

2-4. Chromatographic Methods

First dimension (Reversed-phase)	
Column	: Ascentis Express Peptide ES-C18, 150 mmL. × 2.1 mmI.D., 2.7 µm d.p. (Sigma-Aldrich/Supelco, Bellefonte, PA, USA)
Mobile phase	: (A) 10 mM CH_3COONH_4 in H_2O (pH 9) (B) 10 mM CH_3COONH_4 in H_2O/ACN 10:90 (pH 9)
Gradient	: 0–40 min, 0 to 10% B, 40–60 min, to 20% B, 60–200 min, to 50%, 200–220 min, to 100% B (hold for 20 min)
Flow rate	: 100 µL/min
Column oven	: 35 °C
Injection vol.	: 20 µL

Second dimension (Reversed-phase)	
Column	: Ascentis Express Peptide ES-C18, 30 mmL. × 4.6 mmI.D., 2.7 µm d.p. (Sigma-Aldrich/Supelco, Bellefonte, PA, USA)
Mobile phase	: (A) 0.1% TFA in H_2O (pH 2) (B) 0.1% TFA in H_2O/ACN 10:90 (pH 2)
Gradient	: 0–0.05 min, 0 to 20% B, 0.05–0.40 min, to 40% B, 0.40–0.50 min, to 50% B, 0.50–0.69 min, to 90% B, 0.69–0.70 min, to 0% B, 0.70–1.00 min, to 0% B
Flow rate	: 4 mL/min
Column oven	: 35 °C
Modulation time	: 1 min
Loop size	: 100 µL

2-5. Detection

PDA wavelength: 215 nm; sampling rate 12.5 Hz; time constant 0.080 sec. LCMS-IT-TOF: ESI positive mode; flow from the LC system 180 µL/min; detector voltage 1.60 kV; CDL temperature 200 °C; block heater temperature 200 °C; nebulizing gas flow (N_2) 1.5 L/min; ion accumulation time 40 msec; full scan 200–2000 *m/z*; repeat 3; ASC 70%.

2-6. Sample Preparation

Tryptic digestion was made according to Bushey and Jorgeson [7]: one-tenth gram of α-casein or dephosphorylated α-casein were dissolved in 10 mL of 0.01 M $HCOONH_4$ buffer, and the pH adjusted to 8.0 with NH_4OH; the solution was heated in a boiling water bath for 6 min. After the solution cooled, 2.0 mg of trypsin from bovine pancreas was added, and the mixture was allowed to react for 4 h at +37 °C; the reaction was quenched by adding 0.1% TFA to pH 2. The digests were stored at +4 °C, and filtered prior to injection through 0.45 µm nylon membrane (Whatman).

Aqueous solutions of the peptide standard mixture were prepared at 100 ppm.

3. Results and Discussion

Parameters for MS detection were optimized using a mixture of five standard peptides, and the results in terms of mass accuracy are reported in Table 1. The chromatographic separation was first optimized in the two dimensions, separately. In order to enhance the separation power, four narrow-bore columns have been serially coupled in the D1, achieving a theoretical peak capacity of 402. Peptides were eluted from the first dimension with basic mobile phase (pH 9).

Table 1 Mass accuracy for the LCMS-IT-TOF analysis of a standard peptide mixture

AA sequence	Monoisotopic mass	Predicted $[M+H]^+$	Measured $[M+H]^+$	Error (amu)	Error (ppm)
GLY-TYR (1)	238.0954	239.10333	239.1023	–0.00103	4.30
VAL-TYR-VAL (2)	370.21079	380.2186	380.2164	–0.00229	6.02
TYR-GLY-GLY-PHE-MET (3)	573.22579	574.23369	574.23340	+0.00031	0.53
TYR-GLY-GLY-PHE-LEU (4)	555.26936	556.27726	556.2767	–0.0056	1.00
ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE (5)	1045.53457	1046.54247	1046.5457	+0.00323	3.08

The D2 column consisted of RP-LC, due to its straightforward linkage to MS detection, and was operated at low pH, attempting to deliver a certain degree of orthogonality to D1. In the D2 separation, all the peaks eluted within 0.6 min, allowing enough time space for re-conditioning (Fig. 4).

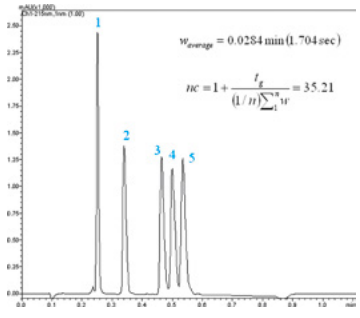

Fig. 4 One-min D2 separation of a standard peptide mixture

3-1. Tryptic Mapping and MS Characterization

The contour plot obtained for the comprehensive RP-LC×RP-LC–PDA–IT-TOF analysis of α-casein and dephosphorylated α-casein is shown in Fig. 2. Replicate analyses ($n = 3$) of the digests were run on the 2D system, and reproducibility of the retention times was calculated for five selected peaks spread throughout the elution window, yielding an average CV% of 0.555 (averaged CV% in the two chromatographic dimensions).

The overall peak capacity of the comprehensive separation was calculated as 8540, being multiplicative of the individual values obtained for the two dimensions ($n_1 \times n_2$). These values are merely theoretical, however, any effect of the first dimension undersampling (about 1 fraction per peak capacity), nor the selectivity correlation (orthogonality), or the retention window in both dimensions, which does not cover the whole gradient duration. Therefore, some adjustments were made, in the calculation, which are fundamental for realistic peak capacity calculations.

First, the practical peak capacity of the separation, which accounts for orthogonality between the two dimensions (retention correlation derived from solute retention vectors), was calculated using the equation developed by Liu *et al.* [8]. This calculation is based on solute retention parameters and, therefore, is more accurate in describing resolving power than those calculated by the multiplicative rule. A value of 3982 was obtained.

For the quantitative estimation of the undersampling effect, a very recent approach developed by Carr's research group was employed [9], which also accounts for the effective retention time window and the second dimension gradient time. By applying such a calculation, the peak capacity calculated for the 2D-LC system was further halved, yielding a value of 1802.

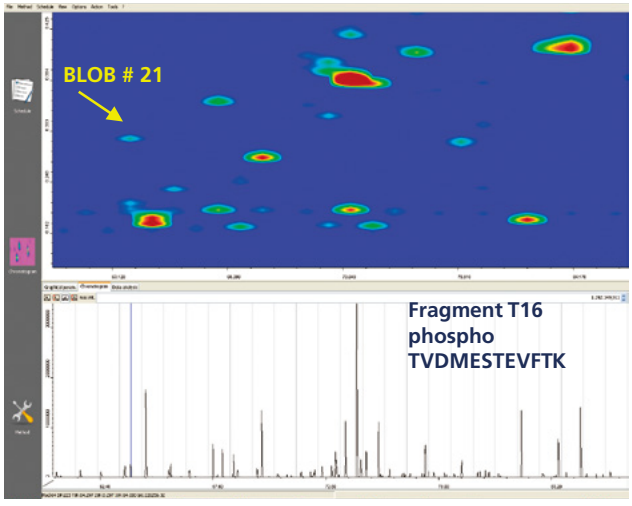

Fig. 5 ChromSquare software window showing an expansion of the α-casein 2D plot and the MS (ESI pos) spectrum of a phosphorylated peptide

In Fig. 5 is depicted the ChromSquare software window for qualitative/quantitative data analysis, showing an enlargement from the contour plot of Fig. 2. The lower window allows visualization of the whole modulation, while integration of some peaks in the selected region of interest further allows calculation of retention times in the two dimensions, as well as peak area calculation for distinctive fragments.

Fig. 6 shows a five-minute enlargement of the raw RP-LC×RP-LC–PDA–IT-TOF chromatogram corresponding to the plot in Fig. 2, and the average mass spectrum obtained for three consecutive peaks (1 min interval, corresponding to the modulation time set) are depicted in the inset. This demonstrates that at least three fractions of the peptide with *m/z* 747.3650 have been transferred from the first to the second dimension of the comprehensive system.

The acquired mass spectrometry data were manually processed, by averaging the number of scans within each chromatographic peak, and deconvolution of charge envelope was afterwards performed for $[M+H]^+$ and $[M+nH]^{n+}$ ions. The corresponding window of the LCMSsolution software is shown in Fig. 7.

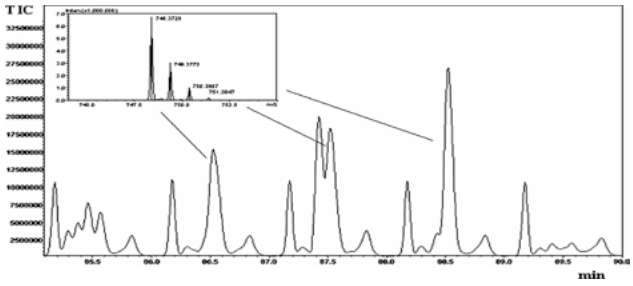

Fig. 6 Five-minute TIC from the RP-LC×RP-LC–PDA–IT-TOF separation of α-casein tryptic digest

The experimental deconvoluted molecular masses of the peptides were then compared to the theoretical values obtained by *in-silico* digestion of the proteins, to obtain the relative sequence coverage.

In-silico digestion of α-casein and dephosphorylated α-casein was performed by using PeptideMass software available at Expasy site (www.expasy.ch/tools/peptide-mass.html), selecting up to three missed cleavages (MC) for the generation of peptides.

For each identified peptide, monoisotopic molecular masses of the phosphorylated and the corresponding dephosphorylated forms are reported, together with the position of the modified aminoacidic residues (phosph. site). Many of the differences in the plots in Fig. 2 are obviously related to the presence or absence of phosphorylated peptides.

Since phosphorylation occurs on the serine residue, and a difference of (roughly) 80 Da was observed for removal of each phosphate group, it must be conclusive that only HPO_3^- is removed by dephosphorylation, leaving the serine residue intact.

By combining the results obtained from the two RP-LC dimensions, sequence coverage of 90.3% and 76.3% for α-casein and dephosphorylated α-casein, respectively, were obtained. Comparison with the corresponding values of 68.2% and 56.4% obtained for the monodimensional system (D1, four coupled columns) clearly demonstrates the usefulness of the 2D-LC system.

Identified peptides in α-casein tryptic digest are shown in the plot in Fig. 8.

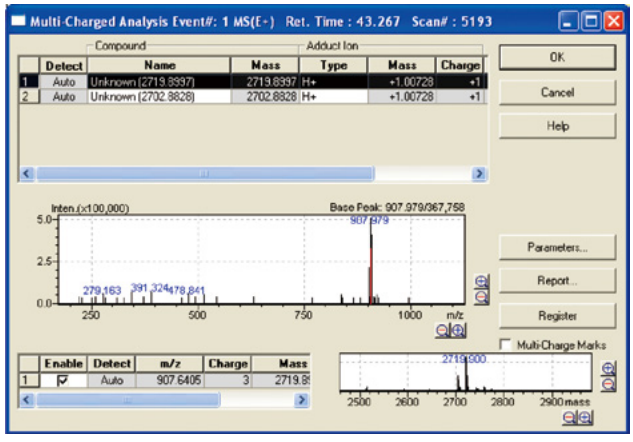

Fig. 7 LCMSsolution software window for peak deconvolution

4. Conclusions

A fully automated 2D RP-LC×RP-LC system, coupled to PDA and LCMS-IT-TOF detection was successfully employed for the analysis of *α*-casein and dephosphorylated *α*-casein tryptic digests.

Due to the ionic nature of peptides, the use of different pH values ensured enough separation selectivity between the two dimensions, consisting of the same stationary phase. Furthermore, such a combination addresses compatibility issues, thus allowing straightforward interfacing in on-line 2D LC configuration, as well as direct linkage to a mass spectrometer.

The results achieved so far hold promise for further optimization of the technique, as a valuable tool to investigate the nature of native and recombinant proteins of clinical relevance.

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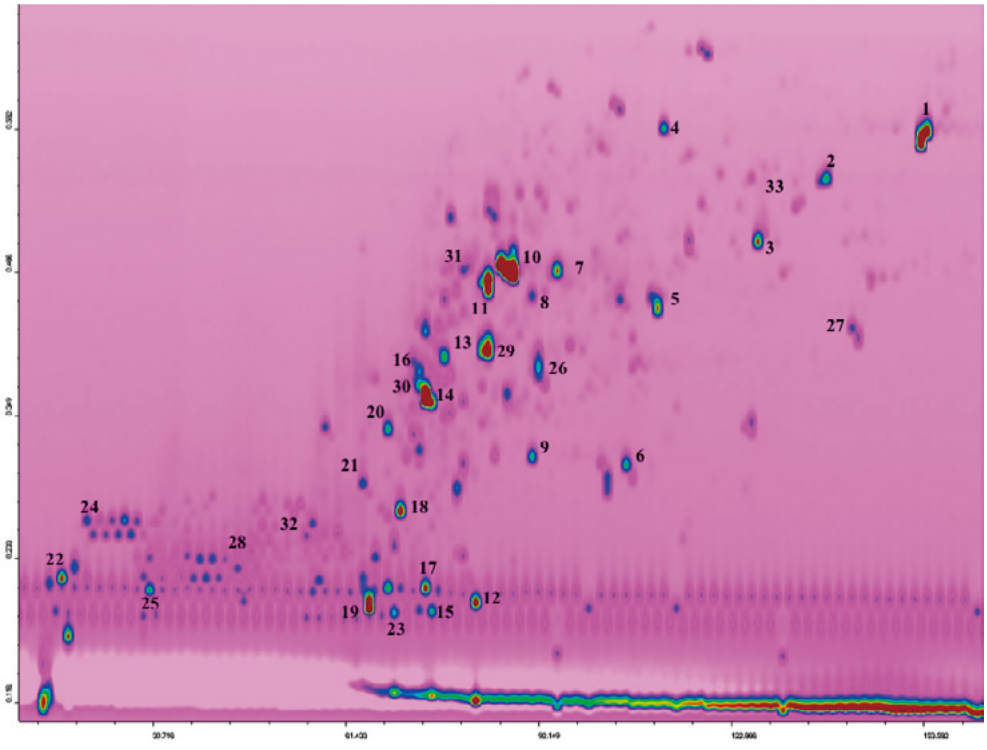

Fig. 8 Identified peptides in the 2D RP-LC×RP-LC plot of *α*-casein tryptic digest

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