Intact Casein - A Model System for the Separation of Intact Phosphorylated Proteins by CESI-MS

Stephen Lock
SCIEX UK

Overview

Who Should Read This: Senior Scientists and Lab Directors with an interest in Proteomics Research.

Focus: Intact phosphorylated protein separation by CESI-MS.

Goals: To improve the detection and characterization of intact protein phosphorylation by mass spectrometry.

Problem: Understanding the degree of phosphorylation of intact proteins provides important insights into the regulation of many biological processes. However, intact protein phosphorylation is challenging to analyse by LC/MS. This is because the phosphorylation isoforms do not separate well from other species on the front-end LC, leading to loss of data through signal suppression in the mass spectrometer. This reduced separation also limits data dependent acquisition inhibiting the top down sequencing of low abundant co-eluting protein isoforms.

Results: The Sciex CESI 8000 Plus system was able to separate charge isoforms of phosphoproteins at which could not be separated by LC/MS. Ultra-low flow CESI-MS with 1000 fold less material injected produces similar sensitivities to LC/MS, making it an ideal technique for the analysis of limited quantity samples.

Key Challenges:

- Separation and detection of intact phosphorylated proteins.
- Analysis of limited amounts of sample.

Key Features:

- CESI-MS was able to separate charge isoforms of phosphoproteins at room temperature which co-eluted in LC-MS.
- Sample consumption by CESI-MS was 0.1% of LC-MS but provided the same sensitivity.

CESI 8000 Plus High Performance Separation – ESI Module

Experimental Design

Sample Preparation for CESI-MS

α Casein dephosphorylated (a protein which 20% phosphorylated containing several phosphor-isoforms) was obtained from Sigma Aldrich and dissolved in 1% formic acid before injecting onto the CESI 8000 Plus system (CESI-MS) or a standard LC-MS system.

CESI-MS Analysis

Samples were injected by pressure onto a 30 µm ID, 91 cm long Polyethylenimine (positively) coated capillary.[1] The CE separation used reverse polarity (cathode at the sample injection side) with a background electrolyte (BGE) consisting of acetic acid with water/organic solvent mixtures (Table 1). The TripleTOF® 5600 mass spectrometer, fitted with a NanoSpray® III source, was used in full scan mode with an ionspray voltage set in the range of 1600 – 2200 V which was optimized for the methanol content of the back ground electrolyte (BGE). Due to the low flow rates of CESI-MS, no source gas 1 was used and the curtain gas was set to 4.2 psi.

LC-MS Analysis

The TripleTOF® 5600 system used was fitted with a DuoSpray™ Ion Source and used in full scan mode with the ionspray voltage and source settings optimized for a 300 µl/min flow rate. Apart from the source conditions, the MS method was identical to the one used for CESI-MS. A C4 reverse phase column (Acquity UPLC BEH C4 1.7 µm 1.0 x 50mm column) was used for the LC-MS analysis. A sample injection volume of 1 µl was used to produce a sharp peak on a Shimadzu Nexera HPLC system. The gradient conditions employed are shown in Table 2.

Action	Time (min)	Pressure (psi)	Pressure Direction	Voltage (kV)	Solution
Rinse	3	100	Forward	0	0.1 Molar HCl
Rinse	3	100	Forward	0	BGE ϒ
Rinse	1	75	Reverse	0	10% Acetic acid
Injection	10 sec	0.5 ¥	Forward	0	Sample Vial
BGE push	5 sec	0.5	Forward	0	BGE ϒ
Separation	30	2	Forward and reverse	30	BGE ϒ
Separation	2	100	Forward and reverse	10	BGE ϒ
Voltage Ramp down	2	10	Forward	1	BGE ϒ
	¥ = Injection pressure was varied, ϒ = 2.5% Acetic acid containing various % Methanol				

Table 1. CESI method parameters used for separations using electrokinetic injections at 25° C.

Time	Flow rate (µl/min)	% Water Containing 0.1% Formic Acid	% Acetonitrile Containing 0.1% Formic Acid
0.1	300	90	10
2	300	90	10
20.0	300	40	60
21.0	300	40	60
21.5	300	10	90
23.0	300	10	90
23.2	300	90	10
25	300	90	10

Table 2. LC-MS gradient method used for the separation of samples at 40° C.

The Effect of Organic Content of the BGE on the CESI-MS Separation

For this study the maximum separation voltage of (30 kV) was used for the separation. One factor that improves resolution is increasing the separation time by keeping the sample plug longer in the capillary. A way to accomplish this is to adjust the BGE and in Figure 1 the amount of methanol was varied. By increasing the methanol content of the BGE the electroosmotic flow was reduced[2] and the migration times were extended and the protein isoform separation improved.

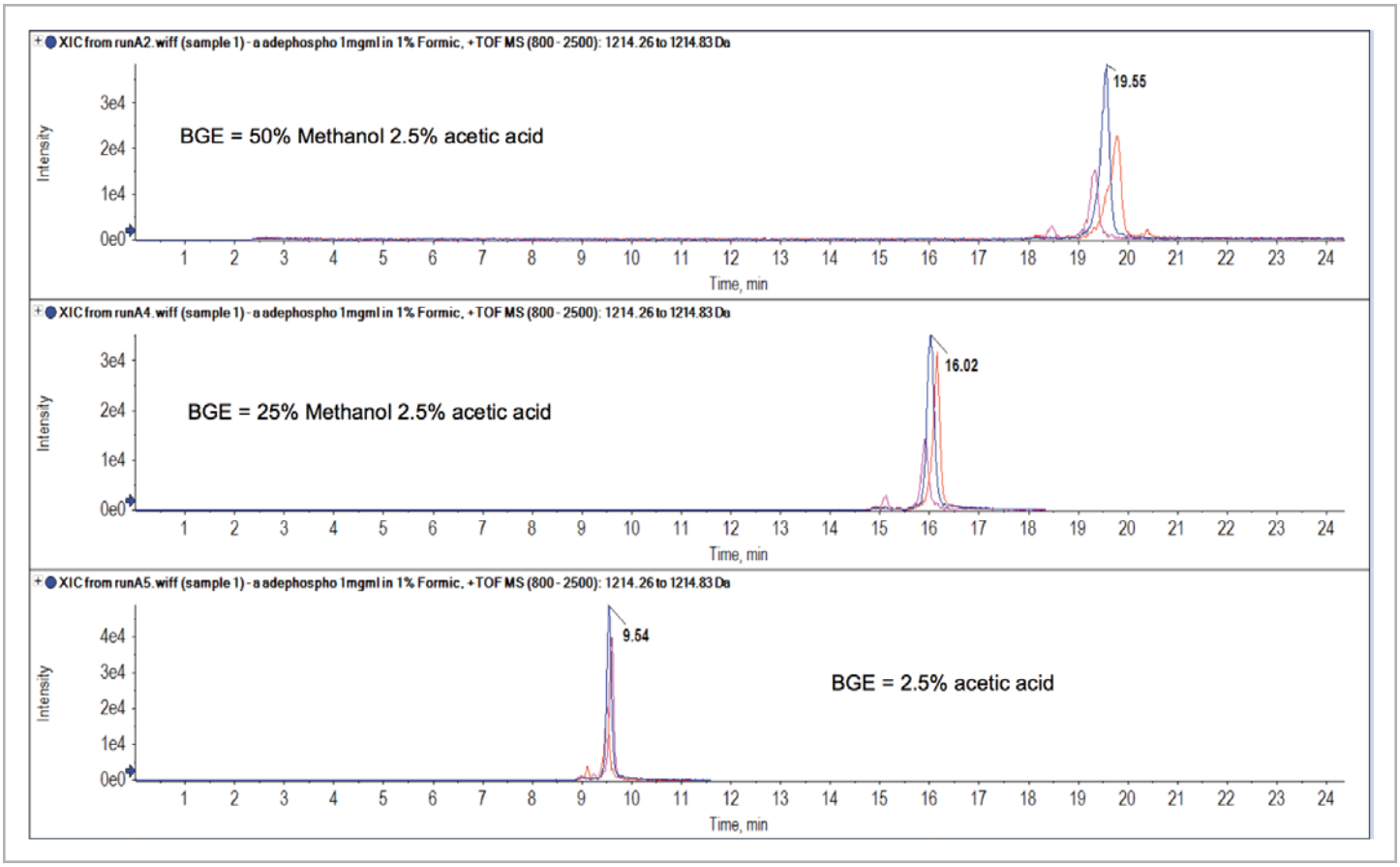

Figure 1. The effect of organic content in the BGE on the analysis of α casein dephosphorylated. Each color trace is the extracted ion chromatogram for the major multiply charged species for an isoform.

The Effect of Injection Volume on the CESI-MS Separation

One of the other factors which effects resolution is the injection conditions used in CESI-MS. In Figure 2 the separation conditions remained constant but injection volume was decreased by decreasing the pressure of the injection. In this experiment the volume loaded onto the capillary was reduced by reducing the injection pressure. The reduction in injection volume improved the separation of the isoforms but did not produce the corresponding drop in sensitivity with a 10 fold drop in capillary injection volume only producing a 3 fold drop in peak intensity.

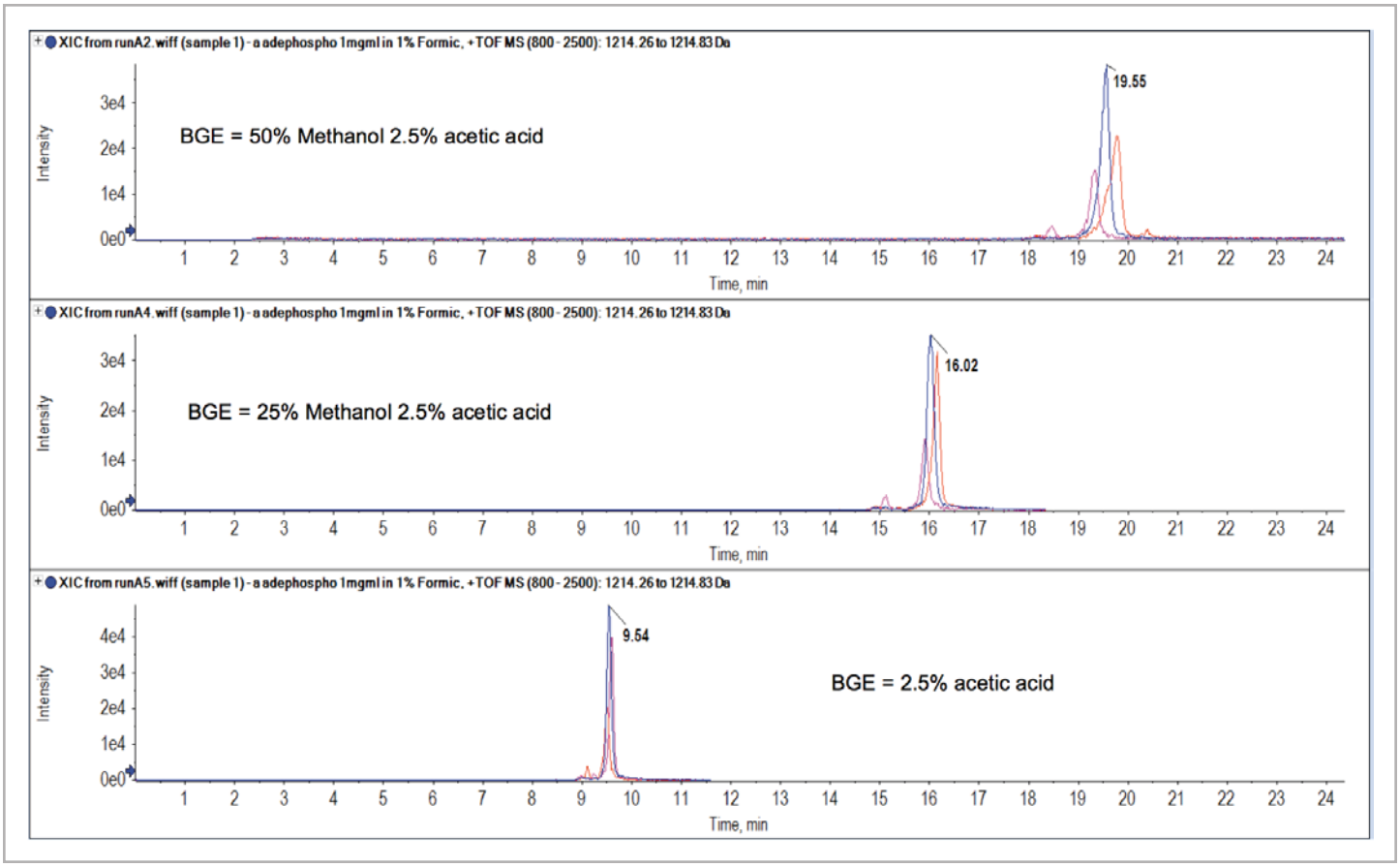

Figure 2. CESI–MS analysis of α casein dephosphorylated using a BGE of 2.5% acetic acid in 50% Methanol with a separation voltage of 30 kV. Each color trace is the extracted ion chromatogram for the major multiply charged species for an isoform.

Comparison of CESI-MS with LC-MS in the Separation of a Phosphorylated Protein Standard (Dephosphorylated α Casein)

Initially, the same on-column amount of protein (1 ng) was injected onto both the LC-MS and CESI-MS system. However, the proteins were not detected by LC-MS at this low level. Therefore, the same sample concentration (1mg/mL) was used on both systems and 0.7 ng was analyzed by CESI-MS compared with 1 µg (over a 1000 times more sample) by LC-MS (Figure 3). In this comparison both approaches gave similar protein peak intensities. However, in the LC-MS analysis the 5 major protein isoforms co-eluted while in CESI-MS the 5 major isoforms [P1 (mw = 20457 Da), P2 (mw = 20377 Da), P3 (mw = 23136.5 Da), P4 (mw = 23057 Da) and P5 (mw = 22977 Da)] were separated. The molecular weight of P3-P5 decreased (by a loss of a phosphate residue) while their CESI-MS migration time increased. This was because the loss of the phosphate decreased the isoelectric point of the protein, slowing it down in the reverse polarity field as the electrophoretic field is attracting the protein stronger to the injection side of the capillary.

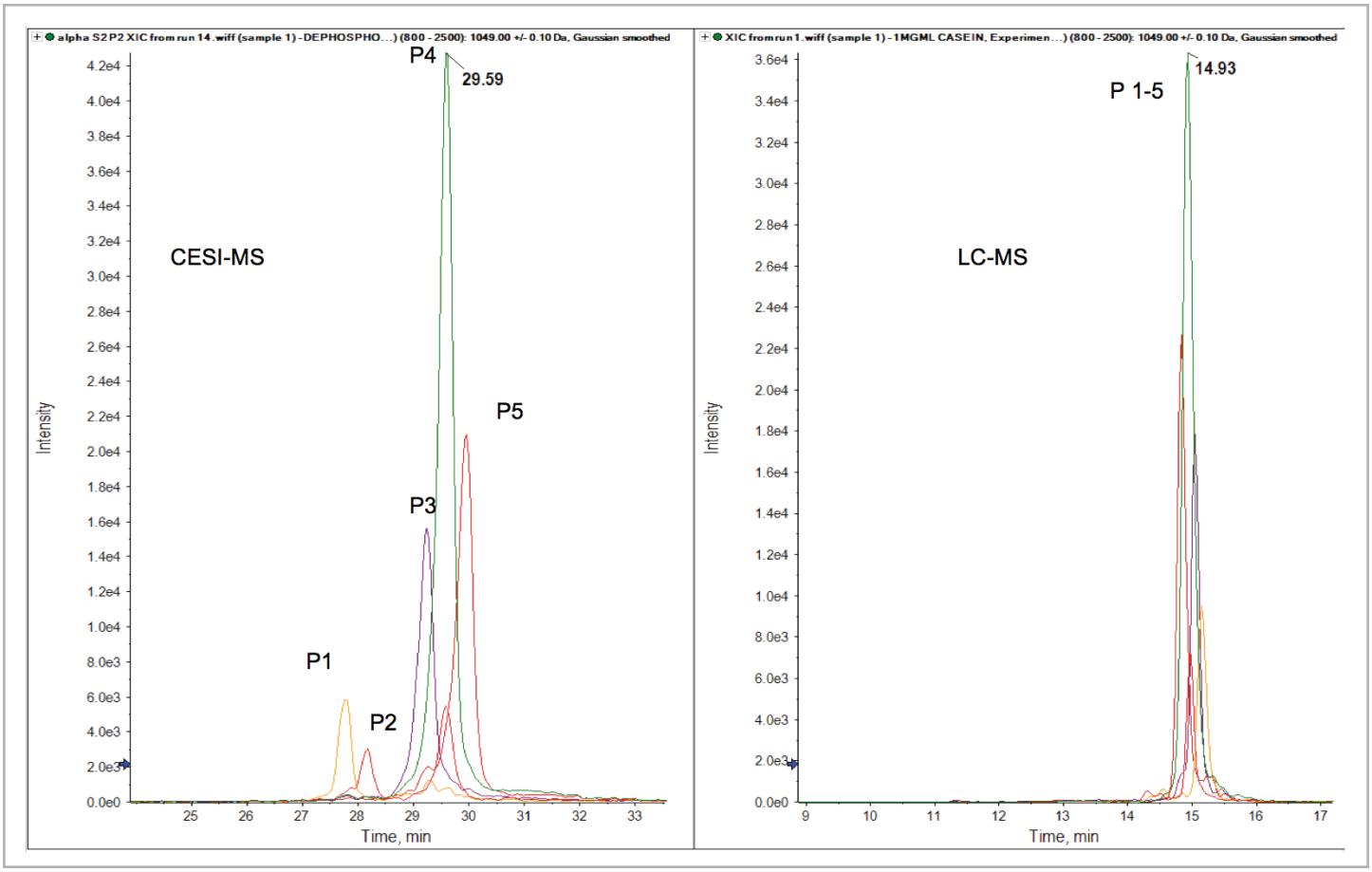

Figure 3. 1 mg/mL of α casein dephosphorylated in 1% formic acid analysed by CESI–MS (0.7 ng) vs LC-MS (1 µg). In the CESI-MS analysis the BGE was 2.5% acetic acid in 50% Methanol with a separation voltage of 20 kV.

Reference

1. Santos, M. R., et al. “A covalent, cationic polymer coating method for the CESI-MS analysis of intact proteins and polypeptides,” SCIEX Separations application note, 2015.
2. Li, S. F. Y. “Capillary Electrophoresis: Principles, Practice and Applications” 1992

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Headquarters
500 Old Connecticut Path, Framingham, MA 01701, USA
Phone 508-383-7800
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