

# Selective injection and fractionation of phosphopeptides by capillary electrophoresis for MALDI and ESI mass spectrometry

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## Overview

- Protein phosphorylation is an extremely important cellular event and its understanding is critical to gain further knowledge of signal transduction, gene expression, and numerous other cellular processes.
- Phosphorylation is difficult to detect and its MS signal is often suppressed because phosphorylation generally occurs in very low levels relative to the rest of the sample.
- To avoid sample adsorption issues that plague enrichment procedures based on solid sorptive phases we utilize the fact that phosphopeptides have characteristically low isoelectric points (pI) and draw them out of solution by the application of voltage.
- The sample can then be further fractionated/separated along the length of the capillary to reduce sample complexity, thus reducing the possibility of ion suppression and further increasing sensitivity.
- The capillary contents can be analyzed by MALDI MS as low-nanoliter sized spots or by ESI MS via direct infusion.
- This technique coupled with ESI MS has the added advantage of being able to monitor the separation profile from the capillary in real time. This can also be useful for maximizing signal because different areas of the separation profile can be summed up according to the species of interest.

## Introduction

- Our group has previously described a method to isolate phosphopeptides from protein digests thereby enhancing the MALDI MS detection of phosphopeptides.<sup>1</sup>
- Due to their highly acidic phosphate groups, many phosphopeptides have a net negative charge even at a pH as low as 3. When a positive potential difference is applied to the capillary the anionic peptides migrate into the capillary while most non-phosphorylated (cationic) peptides remain in sample vial.



Figure 1: Sample in buffer is placed at the cathode and 20kV is applied for 10 minutes to selectively draw the anionic species into the capillary. Peptides with 2, 4, and 5 phosphorylations are represented by 2P, 4P, and 5P respectively. Their location within the capillary is meant to be indicative of their relative mobilities.

- In this work, we extended this methodology by performing a separation of the selectively injected phosphopeptides, thus providing the capacity to handle more complex mixtures

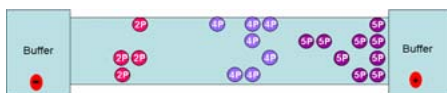


Figure 2: The sample vial is replaced with buffer and 20kV is applied for an additional 18 minutes.

- Once separated the capillary contents are pushed out onto a MALDI plate or directly infused into a nano ESI source

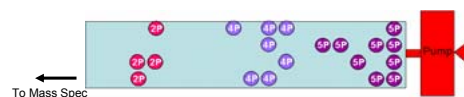


Figure 3: For ESI MS analysis the sample is pushed at 500nL/min into a nano ESI source. For MALDI MS analysis the sample is spotted onto a MALDI plate directly from the CE in 35nL droplets.

## Methods

### Instrumentation

Agilent <sup>30</sup>CE Capillary Electrophoresis System, Micromass Q-TOF Global Ultima mass spectrometer, and Bruker Daltonics Reflex IV MALDI TOF mass spectrometer.

### Materials

Fused silica capillaries with an OD 360µm and an ID 50 µm were used for all experiments. Capillaries for ESI experiments were 48.5 cm in length whereas capillaries for the MALDI experiments were 39 cm long.

For MALDI, capillaries were treated with a phospholipid, 0.1mM 1,2-Dilauroyl-sn-Glycero-3-Phosphocholine (DLPC) in 20mM Tris-HCl pH 7.4, to eliminate electroosmotic flow (EOF) and prevent sample adsorption.<sup>2</sup>

For ESI experiments EOF suppression and prevention of sample adsorption were achieved by derivatizing the capillaries with cross-linked polyacrylamide as described previously.<sup>3</sup>

### Methods

Selective sampling was performed using 20mM ammonium-formate buffer in 25% methanol at pH 3.0 for ESI MS. A 0.1M ammonium-acetate buffer at pH 5.0 was used for MALDI MS experiments.

## Experimental Challenges

### Adaptation of selective sampling technique for MALDI MS to ESI MS

The phospholipid capillary coating was found to disassemble in the presence of acetonitrile and methanol, which are commonly used in ESI to enhance sample ionization.

Our first attempt to solve this was to introduce the organic solvent subsequent to the selective injection step, directly upstream of the ESI emitter tip using a sheath flow or a 3-way junction.

Unfortunately, trace amounts of phospholipids still desorbed from the coating during the run. Although the phospholipid did not cause any problem previously with MALDI MS, it was detected by ESI MS and caused significant suppression of the phosphopeptide signals.

This problem was finally solved by the use of a covalently bonded cross-linked polyacrylamide coating.<sup>3</sup>

This coating was stable in the presence of organic solvent so the need for a sheath flow or a 3-way junction was abolished and 25% methanol was incorporated into the running buffer.

Another advantage of the cross-linked polyacrylamide coating is that it reproducibly gives a very low ( $10^{-6}$  –  $10^{-7}$  cm<sup>2</sup>·V<sup>-1</sup>·s<sup>-1</sup>) EOF at pH 3.0. This was not possible with phospholipid coatings.

### Salt adducts

Throughout this work sodium and potassium adducts on the phosphopeptides of α-casein have caused a major decrease in signal.

This salt contamination is always most prevalent on the multiply phosphorylated species presumably because of increased electrostatic interactions.

The strong pH dependence of this method prevents changes to be made to reduce the effects of salt (e.g., acidify sample, use negative ion mode).

## Results

Peptide	Sequence	Observed m/z	pI	Charge	Selective Injection + Fractionation
α1	MSKELK	1003.4	4.5	2	++
α2	MSKELK	1003.4	4.5	2	++
α3	MSKELK	1003.4	4.5	2	++
α4	MSKELK	1003.4	4.5	2	++
α5	MSKELK	1003.4	4.5	2	++
α6	MSKELK	1003.4	4.5	2	++
α7	MSKELK	1003.4	4.5	2	++
α8	MSKELK	1003.4	4.5	2	++
α9	MSKELK	1003.4	4.5	2	++
α10	MSKELK	1003.4	4.5	2	++
α11	MSKELK	1003.4	4.5	2	++
α12	MSKELK	1003.4	4.5	2	++
α13	MSKELK	1003.4	4.5	2	++
α14	MSKELK	1003.4	4.5	2	++
α15	MSKELK	1003.4	4.5	2	++
α16	MSKELK	1003.4	4.5	2	++
α17	MSKELK	1003.4	4.5	2	++
α18	MSKELK	1003.4	4.5	2	++
α19	MSKELK	1003.4	4.5	2	++
α20	MSKELK	1003.4	4.5	2	++
α21	MSKELK	1003.4	4.5	2	++
α22	MSKELK	1003.4	4.5	2	++
α23	MSKELK	1003.4	4.5	2	++
α24	MSKELK	1003.4	4.5	2	++
α25	MSKELK	1003.4	4.5	2	++
α26	MSKELK	1003.4	4.5	2	++
α27	MSKELK	1003.4	4.5	2	++
α28	MSKELK	1003.4	4.5	2	++
α29	MSKELK	1003.4	4.5	2	++
α30	MSKELK	1003.4	4.5	2	++
α31	MSKELK	1003.4	4.5	2	++
α32	MSKELK	1003.4	4.5	2	++
α33	MSKELK	1003.4	4.5	2	++
α34	MSKELK	1003.4	4.5	2	++
α35	MSKELK	1003.4	4.5	2	++
α36	MSKELK	1003.4	4.5	2	++
α37	MSKELK	1003.4	4.5	2	++
α38	MSKELK	1003.4	4.5	2	++
α39	MSKELK	1003.4	4.5	2	++
α40	MSKELK	1003.4	4.5	2	++
α41	MSKELK	1003.4	4.5	2	++
α42	MSKELK	1003.4	4.5	2	++
α43	MSKELK	1003.4	4.5	2	++
α44	MSKELK	1003.4	4.5	2	++
α45	MSKELK	1003.4	4.5	2	++
α46	MSKELK	1003.4	4.5	2	++
α47	MSKELK	1003.4	4.5	2	++
α48	MSKELK	1003.4	4.5	2	++
α49	MSKELK	1003.4	4.5	2	++
α50	MSKELK	1003.4	4.5	2	++
α51	MSKELK	1003.4	4.5	2	++
α52	MSKELK	1003.4	4.5	2	++
α53	MSKELK	1003.4	4.5	2	++
α54	MSKELK	1003.4	4.5	2	++
α55	MSKELK	1003.4	4.5	2	++
α56	MSKELK	1003.4	4.5	2	++
α57	MSKELK	1003.4	4.5	2	++
α58	MSKELK	1003.4	4.5	2	++
α59	MSKELK	1003.4	4.5	2	++
α60	MSKELK	1003.4	4.5	2	++
α61	MSKELK	1003.4	4.5	2	++
α62	MSKELK	1003.4	4.5	2	++
α63	MSKELK	1003.4	4.5	2	++
α64	MSKELK	1003.4	4.5	2	++
α65	MSKELK	1003.4	4.5	2	++
α66	MSKELK	1003.4	4.5	2	++
α67	MSKELK	1003.4	4.5	2	++
α68	MSKELK	1003.4	4.5	2	++
α69	MSKELK	1003.4	4.5	2	++
α70	MSKELK	1003.4	4.5	2	++
α71	MSKELK	1003.4	4.5	2	++
α72	MSKELK	1003.4	4.5	2	++
α73	MSKELK	1003.4	4.5	2	++
α74	MSKELK	1003.4	4.5	2	++
α75	MSKELK	1003.4	4.5	2	++
α76	MSKELK	1003.4	4.5	2	++
α77	MSKELK	1003.4	4.5	2	++
α78	MSKELK	1003.4	4.5	2	++
α79	MSKELK	1003.4	4.5	2	++
α80	MSKELK	1003.4	4.5	2	++
α81	MSKELK	1003.4	4.5	2	++
α82	MSKELK	1003.4	4.5	2	++
α83	MSKELK	1003.4	4.5	2	++
α84	MSKELK	1003.4	4.5	2	++
α85	MSKELK	1003.4	4.5	2	++
α86	MSKELK	1003.4	4.5	2	++
α87	MSKELK	1003.4	4.5	2	++
α88	MSKELK	1003.4	4.5	2	++
α89	MSKELK	1003.4	4.5	2	++
α90	MSKELK	1003.4	4.5	2	++
α91	MSKELK	1003.4	4.5	2	++
α92	MSKELK	1003.4	4.5	2	++
α93	MSKELK	1003.4	4.5	2	++
α94	MSKELK	1003.4	4.5	2	++
α95	MSKELK	1003.4	4.5	2	++
α96	MSKELK	1003.4	4.5	2	++
α97	MSKELK	1003.4	4.5	2	++
α98	MSKELK	1003.4	4.5	2	++
α99	MSKELK	1003.4	4.5	2	++
α100	MSKELK	1003.4	4.5	2	++

Figure 4: List of phosphopeptides detected by MALDI MS from a tryptic digest of α-casein. Selective injection was at 10kV for 5 minutes and the subsequent separation was at the same voltage for 8.5 minutes. Sample was a 1pmol/µL tryptic digest of α-casein in a 0.1 mM acetate buffer at pH 5.0. The number of positive signs denotes the relative peak intensity in the MS spectra: + for intensities < 30, ++ for intensities between 30-100, +++ for intensities > 100, and blank for no signal detected. S1 and S2 are two variants present in the commercial sample.

## Results

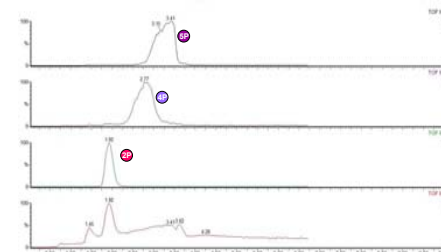


Figure 5: Selected ion chromatograms (SICs) and total ion chromatogram (TIC) of an α-casein tryptic digest analyzed by ESI MS after selective injection and separation. Sample concentration subjected to electro-injection is 10pmol/µL.

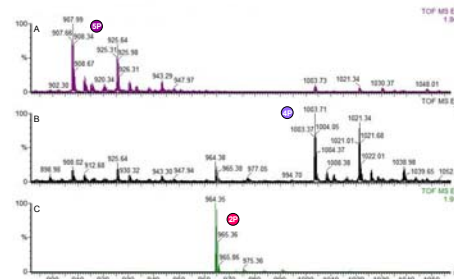


Figure 6: Mass spectra of corresponding SICs from figure 5. (A) Sum of the SIC of m/z 907.6 for phosphopeptide α1S1. (B) Sum of the SIC of m/z 1003.4 for phosphopeptide α2S2. (C) Sum of the SIC of m/z 964.4 for phosphopeptide α4S1.

- Peptides α3 and α5 of the S2 variant were also detected at lower intensities, data not shown.

## Discussion and Conclusion

With this technique we have shown how multiply phosphorylated peptides can be selectively extracted and separated from a mixture, like an α-casein digest.

This allows for the detection of multiply phosphorylated peptides which would otherwise be suppressed due to their poor ionization efficiencies.

Although ESI MS data is only shown here for multiply phosphorylated peptides, one can raise the sampling pH, for example to pH 5, to include the singly phosphorylated peptides in the analysis.

Peptides α3S2 and α5S2 were detected in low amounts most likely owing to the lower abundance of S2.

## References & Acknowledgements

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