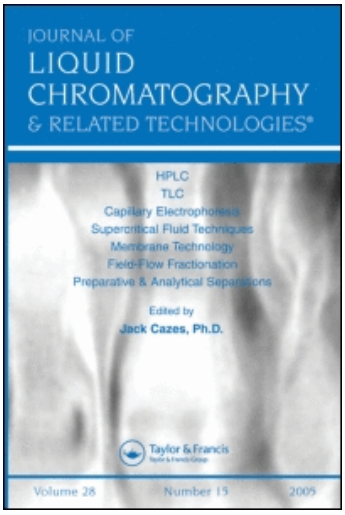

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ANALYSIS OF PHOSPHOENKEPHALINS BY COMBINED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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ABSTRACT

A method based upon a combination of high performance liquid chromatography (HPLC) and electrospray ionization (ESI) mass spectrometry (MS) for the analysis of phosphorylated peptides in a complex mixture of 14 phosphoenkephalins and their nonphosphorylated analogues is described. A narrow-bore column is directly connected to the ESI interface without the need of a solvent flow-splitter. A full-scan mass analysis in the positive-ion mode is used to provide the total ion current chromatogram and the molecular mass of each eluting component. Selective detection of phosphorylated peptides is achieved by selected-ion recording of the phosphate group marker ion (PO_3^-) in the negative-ion mode. These two scans are acquired simultaneously on a single injection of the sample. The influence of water clusters on background ion current is also discussed.

INTRODUCTION

To determine the state of phosphorylation of peptides and proteins a highly sensitive and specific experimental approach is needed. The traditional approach of incubation of cells and tissues with ^{32}P-labeled phosphate followed by isolation of the labeled protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) or chromatographic techniques and identification of the modified amino acids by peptide mapping and Edman degradation techniques has several weak points.[1,2] Beside being a potential radioactive hazard, the procedure is labor-intensive, prone to sample losses, and often fails to recognize the exact site of phosphorylation. In addition, the phosphate ester bonds to serine and threonine are less stable during the chemical degradation step of the Edman procedure and recoveries of PTH derivatives of phosphotyrosine are often compromised.

Because of exceptional molecular specificity, high detection sensitivity, and versatility in determining structures of unknown compounds, several mass spectrometric (MS) techniques are playing increasingly important roles in characterization of phosphopeptides.[3] Earlier work in this field used fast atom bombardment (FAB) with a varying degree of success.[4,5] Liquid secondary ionization mass spectrometry (liquid-SIMS, a variation of FAB) was used by us for sequence determination of phosphopeptides.[6] Several applications of electrospray ionization (ESI)-MS for the detection of phosphopeptides have been presented.[7-11] Matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-MS has been also used for the analysis of phosphopeptides.[12,13]

In this study, we present an HPLC/ESI-MS method for the analysis of a mixture of phosphorylated and nonphosphorylated enkephalin peptides. The objective is to develop a technique that can be used to separate and characterize phosphoenkephalins from body tissues and fluids. The method capitalizes on the combined strength of HPLC to purify, concentrate (into a plug), and resolve complex mixture of analytes and ESI-MS to provide the compound-specific information (mass and structure). Unlike the traditional approach, no radiolabeling is required in this method.

Two MS techniques are used in this study to monitor the HPLC eluents. In one, the ion current is monitored in a wide mass range to generate a total ion current (TIC) chromatogram. This full-scan technique provides the knowledge of the molecular mass of each eluting component. A benefit of full-scan is that the presence of phosphopeptides in a mixture may be established quickly from the measured molecular mass. The mass of a phosphorylated peptide is increased by 80 Da for each phospho unit from the corresponding nonphosphorylated analogue. In the second technique, the ion current due to the phosphate-specific marker ion PO_3^- at *m/z* 79 is monitored in the selected-ion

recording (SIR) mode. This scan is useful for selective detection of phosphopeptides. The marker ion is generated in the ES interface region via collision-induced dissociation (CID) of the target phosphopeptide by stepping up the voltage of the sampling cone. Both these scans are combined to allow simultaneous measurement of the mass of all eluting components and selective detection of phosphopeptides in a single HPLC injection.

EXPERIMENTAL

Materials

Protein sequencing grade trifluoroacetic acid (TFA) was obtained from Sigma Chemical Co (St. Louis, MO, USA) and HPLC grade acetonitrile from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade water was prepared in-house with a Millipore Milli-Q (Bedford, MA, USA) water purification system. The non-phosphopeptides were purchased from Sigma Chemical Co. All phosphopeptides were synthesized in our laboratory using Fmoc solid-phase peptide synthesis protocol and purified by RP-HPLC before use. Each peptide was dissolved in deionized water and mixed together to provide a mixture that contained 69–81 pmol/μL of each peptide.

High-Performance Liquid Chromatography

Chromatography separation was carried out by using an HP 1050 quaternary pumping system (Hewlett Packard, Palo Alto, CA, USA) outfitted with a photodiode array UV-visible detector and a PC-based ChemStation data system. A narrow-bore (250x2.1 mm) Vydac 201HS52 C-18 column packed with 5 μm and 90 Å pore size particles was used for separation of the peptide mixture. A solvent mixture of HPLC grade acetonitrile in deionized water containing 0.1% TFA was used as the mobile phase at a flow rate of 0.20 mL/min. The non-linear gradient consisted of 14–16% acetonitrile in 0–10 min, 16–22% in 10–20 min, 22–24% in 20–30 min, and 24–32% from 30–40 min.

High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry

A schematic diagram of the HPLC/ESI-MS system is shown in Figure 1. In this system, the 1050 liquid chromatograph was interfaced to a single quadrupole mass spectrometer (model Platform II, Micromass, Manchester, UK) via a megaflow ESI probe. The outlet of the UV-visible diode array detector

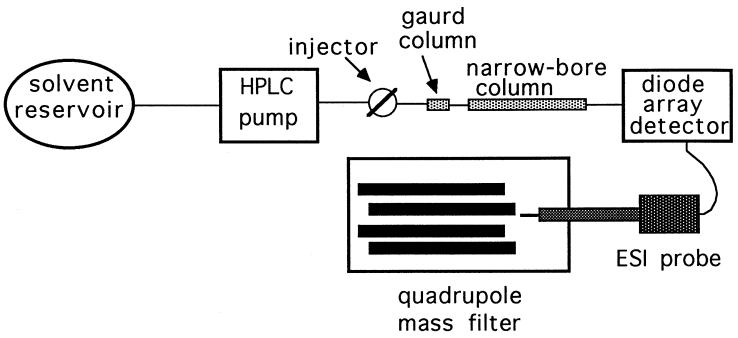


Figure 1. Schematic diagram of the HPLC/ESI-MS system.

was connected directly to the ESI probe. The chromatography conditions were the same as described above. The ES ion source temperature was set to 180°C. Nitrogen was used both as nebulizing and drying gases at flow rates of 15 L/h and 350 L/h, respectively. Full-scan and SIR scans were acquired sequentially on a single injection of the sample. First, a full scan was acquired in the positive-ion mode by scanning the quadrupole in 545–990 Da mass range at a scan rate of 1.2 s/scan, interscan time of 0.1 s, and the sampling cone voltage of 30 V. The instrument was then switched to the negative-ion mode to record the phosphate group marker ion *m/z* 79 in the SIR mode at a dwell time of 0.1 s and the sampling cone voltage of –200 V. This cycle was repeated throughout the HPLC run. PC-based MassLynx and ChemStation data systems controlled the mass spectrometer and HPLC, respectively.

RESULTS AND DISCUSSION

In our previous work, we studied the effect of two homologous series of hydrophobic ion-pairing reagents, consisting of perfluorinated carboxylic (trifluoroacetic, pentafluoropropionic, and hexafluorobutyric) acids and sodium alkyl (butane, hexane, and heptane) sulfonates on RP-HPLC separation of the same mixture of 14 enkephalin peptides.[14] In that study, a conventional wide-bore (4.6 mm) C-18 column with a nonlinear gradient of acetonitrile in water at a flow rate of 1.5 mL/min was used to separate the peptide mixture.

It was found that the retention time of each peptide increased in proportion with the counter ion hydrophobicity. All of the seven ion-pairing reagents were effective in resolving the individual components of this mixture. This method,

however, can not be transferred to an LC/ESI-MS setup primarily because of high solvent flow rate. Also, not all of the above ion-pairing reagents can be used; only volatile additives must be used in mass spectrometry detection of the HPLC eluents.

The combination of HPLC with ESI-MS is one of the most exciting developments of recent times in analytical methodology.[15] The choice of an HPLC column is very critical in the success of HPLC/ESI-MS in biochemical applications. Although small-bore columns have the advantage of small sample requirement,[16,17] they require special HPLC equipment to maintain reliable and reproducible solvent gradients at low flow rates used in these columns. To overcome this problem, a combination of pre-column flow-splitter and solvent delivery from the conventional HPLC equipment is used. A 4.6-mm analytical column can be coupled to ESI-MS provided post-column flow splitting is used. Post- or pre-column flow splitting, however, has one major disadvantage in that a large portion of the sample is wasted.

The system described in the present study is aimed at providing a routine and analytically rugged operation using normal diameter analytical columns. A 2.1-mm column was chosen (over 4.6-mm column) as a compromise to achieve greater sensitivity while making use of a conventional HPLC instrumentation. In addition, this column can be operated at a flow rate, which permits the separation and detection of peptides with minimal background interference (see below). A high background ion current was observed at 0.5−1.0 mL/min flow rates commonly used with the 4.6 mm-id column.

Background Ion Current

Because background ion current effects detection sensitivity, we first optimized conditions that produce minimal background. The main contribution to the background was found to arise from two types of water clusters, $H^+(H_2O)_n$ and $CH_3CNH^+(H_2O)_n$. Several parameters were found to affect the formation of these clusters. The background increased with the flow rate and the aqueous content of the mobile phase. The extent of cluster formation decreases with increases in the sampling cone voltage, drying gas flow, and the source temperature.

Because the flow rate and composition of the solvent are determined by the gradient required for optimum separation, these parameters can not be changed for reducing the background. The sampling cone voltage can not be increased beyond a certain limit (~ 30 V) because of the possibility of fragmentation of peptides.

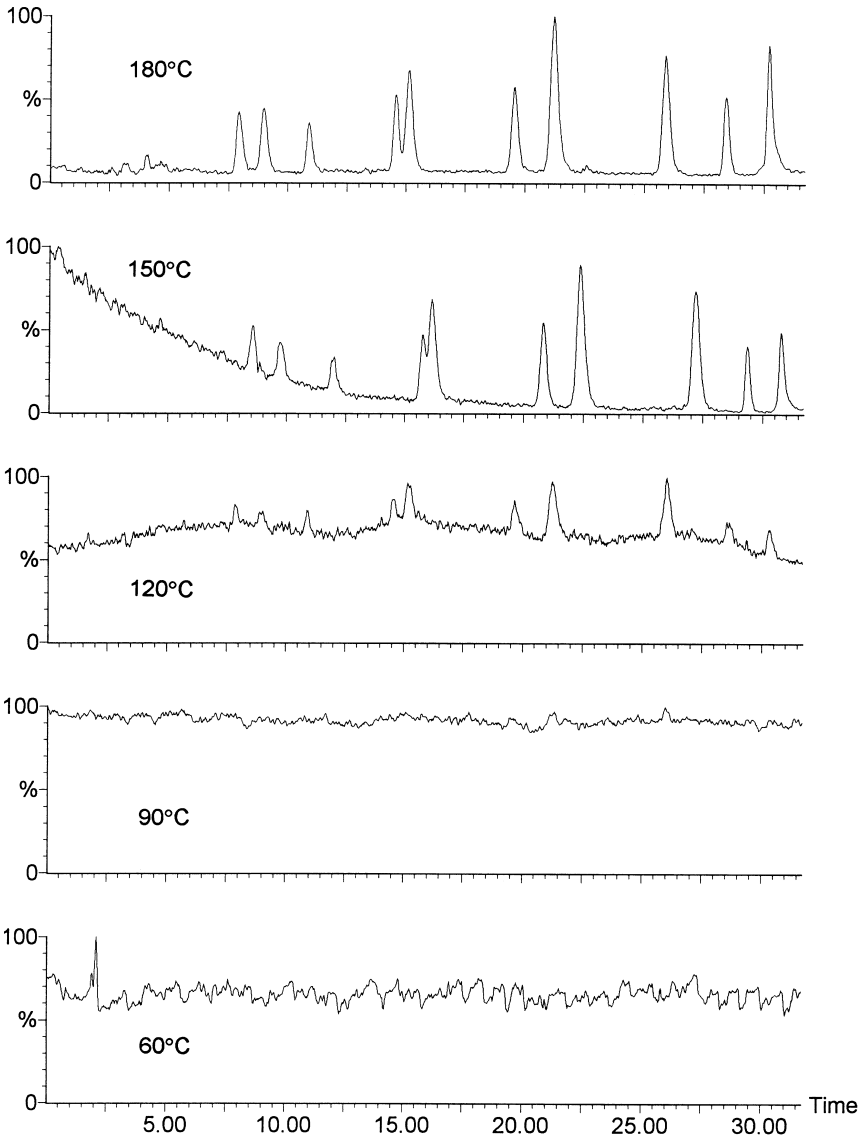


Figure 2. The effect of background ion current on the HPLC/ESI-MS separation of a mixture of 10 phosphorylated and non-phosphorylated enkephalin peptides. The TIC chromatograms were obtained at 60, 90, 120, 150, and 180°C by scanning the quadrupole in the mass range of 545-990 Da.

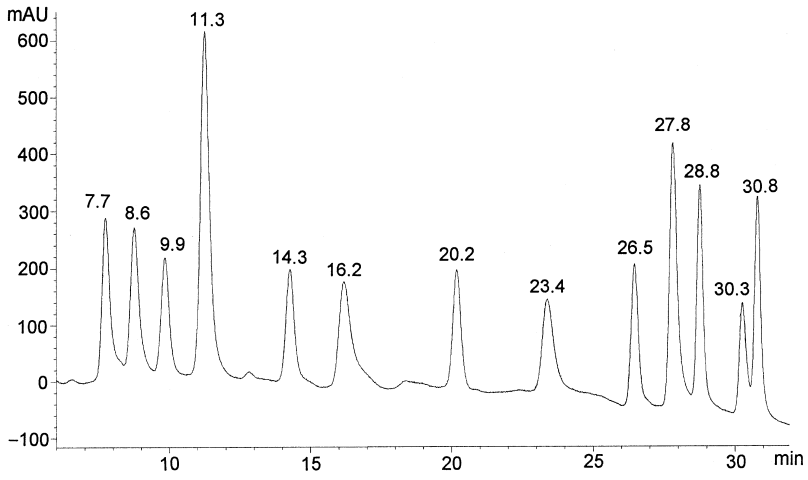


Figure 3. The UV trace (200 nm) of a mixture of 14 phosphorylated and nonphosphorylated enkephalin peptides. The mobile phase: acetonitrile in deionized water containing 0.1% TFA; the non-linear gradient: 14–16% acetonitrile in 0–10 min, 16–22% in 10–20 min, 22–24% in 20–30 min, and 24–32% from 30–40 min. (7.7=Y_pGGFMK, 8.6=Y_pGGFMR, 9.9=YGGFMK, 11.3=YGGFMR and Y_pGGFLK, 14.3=YGGFLK, 16.2=Y_pGGFM, 20.2=YGGFM, 23.4=Y_pGGFL, 26.5=YGGFL, 27.8=Y_pGGFMRGL, 28.8=YGGFMRGL, 30.3=Y_pGGFMRF, and 30.8=YGGFMRF).

A more realistic approach to minimize the cluster formation is to increase the source temperature. Comparisons of TIC chromatograms at various source temperatures show that the setting of 180°C yields highest signal-to-noise ratio (see Figure 2).

In view of the above observations, the following conditions were chosen for the analysis of the peptide mixture: mobile-phase flow, 0.2 mL/min; source temperature, 180°C; drying gas flow, 350 L/h; and sampling cone voltage, 30 V (for full-scan analysis). The mass range chosen was 545–990 Da because below 545 Da the background becomes substantial even at 180°C. To conserve nitrogen, a flow of 350 L/h was used.

UV Chromatogram

The separation of the peptide mixture was first optimized at the chosen flow rate of 0.2 mL/min without connecting the HPLC column to the ESI probe. Because we are dealing with small hydrophilic peptides, a high purity, high

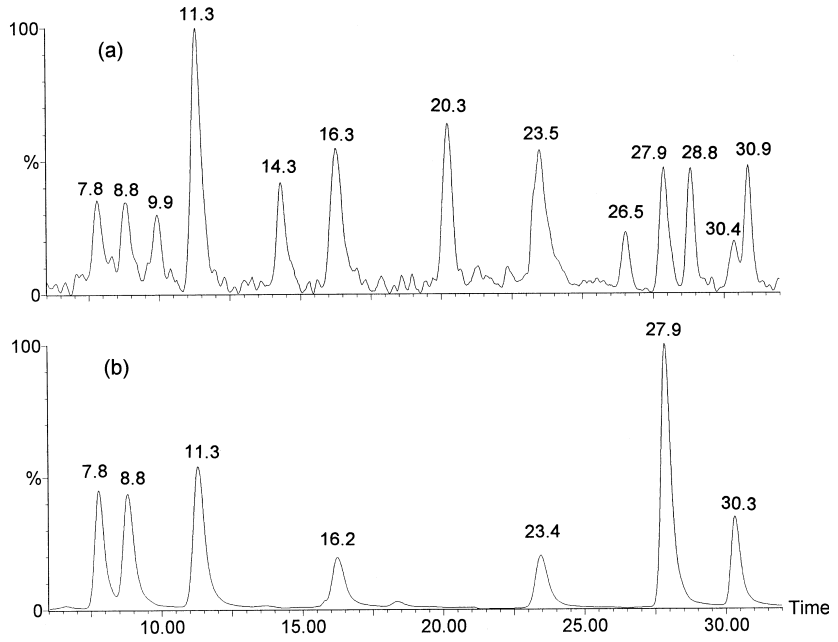
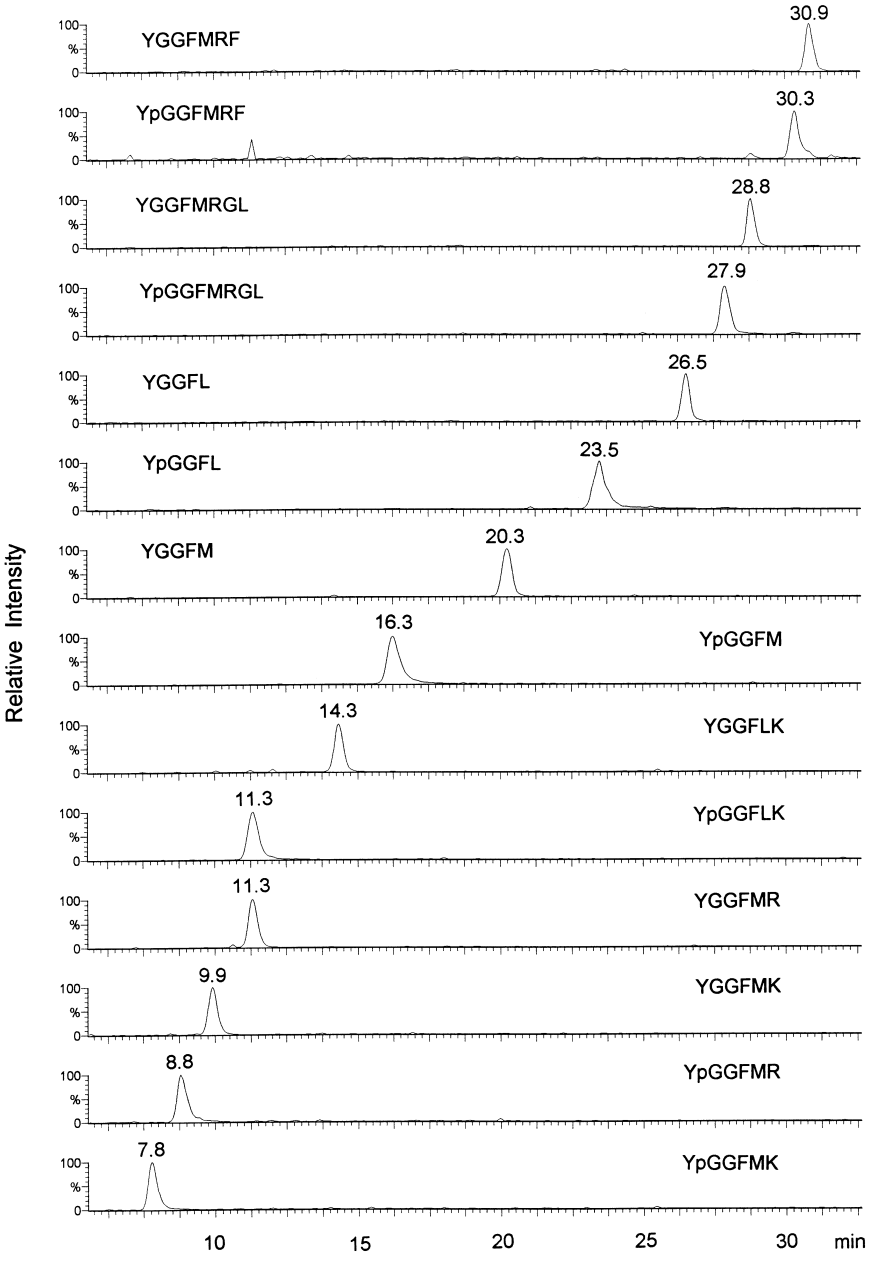


Figure 4. (a) The positive-ion TIC trace (545–990 Da) from an injection of a mixture of 14 phosphorylated and non-phosphorylated enkephalin peptides containing 345–405 pmol of each peptide. The gradient was same as in Figure 3. (b) The negative-ion SIR trace of the same mixture obtained by monitoring the PO_3^- anion. For identification of peaks, see Figures 3 and 5.

surface area, and small pore C-18 silica-based packing was selected to resolve the complex mixture of 14 peptides. Several gradients of acetonitrile in water containing 0.1% TFA were examined. The gradient shown in the experimental section was found to provide optimum separation of all of the 14 peptides within a total analysis time of 31 min.

The UV chromatogram is shown Figure 3. All peptides, except YGGFMR and Y_pGGFLK, are completely resolved. YGGFMR and Y_pGGFLK coelute at 11.3 min.

Figure 5 (right). The RIC profile extracted from the data in Figure 4a.



Total Ion Current Chromatogram

The UV chromatogram shown in Figure 3 does not provide unambiguous identity of the HPLC peak material. Positive identification of the HPLC eluents can be obtained from the molecular mass. To obtain this information, each eluting component was analyzed in the positive-ion mode by scanning the quadrupole in a wide mass range that encompasses the molecular mass of all peptides of the mixture (i.e., 545–990 Da).

In this mass range, the ion current due to singly charged ions only is detected. Although lysine- and arginine-containing peptides also produce abundant doubly charged ions, the ion current due to these ions were not measured because they are observed at the *m/z* values (below 500 mass units) where the background ion current is substantial. Figure 4a contains the positive-ion TIC profile. The amount of each peptide used to obtained this chromatogram was 345–405 pmol. Comparison of the UV and TIC chromatograms shows that the chromatographic integrity is preserved in this LC/ESI-MS system. Similarly to the UV chromatogram, all peptides are well resolved in the TIC chromatogram (except for YGGFMR and Y_pGGFLK) and good chromatographic peak shapes are observed for all components of the mixture.

Additional security in peak identification is provided by the reconstructed ion current (RIC) profile, which is obtained by plotting the ion current at a specified mass. This type of chromatogram is useful in locating a specific peptide in a complex mixture. The RIC chromatogram shown in Figure 5 was extracted from the data of Figure 4a by plotting the ion current at the expected *m/z* value of the molecular ions of the peptides present in the mixture. The advantage of mass spectrometry detection over conventional UV detection is obvious. The coeluting components YGGFMR and Y_pGGFLK are unambiguously identified by their RIC profiles at *m/z* 730 and 764, respectively. From the full-scan data, one can also obtain a representative mass spectrum of each eluting component (not shown).

Selective Detection of Phosphopeptides

To capitalize the selectivity and sensitivity afforded by the SIR mode of analysis, the setup shown in Figure 1 was also used for selective detection of phosphopeptides in the mixture by monitoring the ion current due to the phosphite anion (PO_3^-). Cleavage of the ester bond in phosphotyrosine-containing peptides yields two phosphate marker ions PO_3^- (at *m/z* 79) and PO_2^- (at *m/z* 63).

These two ions are also produced from peptides containing phosphorylated serine and threonine.[10] Thus, monitoring the ion current at *m/z* 79 and 63 provides a method for simultaneous detection of peptides containing all three types of phosphorylated residues.

It was first necessary to optimize the sampling cone voltage needed to maximize the signal of marker ions. The intensity of *m/z* 63 and 79 signals increased with the increase in the cone voltage (from −80 to −200 V) for all seven phosphopeptides. However, the intensity of *m/z* 63 signal was roughly 7–24 times lower than that of the *m/z* 79 signal. Even at −200 V (the maximum value that can be used with the power supply of our instrument), only five peptides Y_PGGFM, Y_PGGFL, Y_PGGFMK, Y_PGGFLK, and Y_PGGFMR produced *m/z* 63 signal. Therefore, only *m/z* 79 signal was monitored. The SIR chromatogram of *m/z* 79 is shown in Figure 4b. It is clear from this figure that the peaks are observed only when phosphopeptides elute from the column. Thus, this approach offers a means of selective detection of phosphopeptides in a peptide mixture.

Comparison of the TIC and SIR chromatograms clearly shows that signal-to-noise ratio is much higher in the SIR chromatogram. Although the SIR chromatogram was obtained by injecting an aliquot of the peptide mixture that contained 345–405 pmol of each peptide, the method is applicable to the analysis of phosphopeptides in the low pmol range. The detection sensitivity can be further improved if only an SIR chromatogram was acquired. Another advantage of this technique is that once the phosphorylated peptides are identified, only those HPLC peaks need to be collected for further sequence analysis, resulting in a saving of time and labor.

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