DIONEX

Technical Note 705

Automated Enrichment and Determination of Phosphopeptides Using Immobilized Metal Affinity and Reversed-Phase Chromatography with Column Switching

INTRODUCTION

Protein phosphorylation is the most common known biochemical means of regulating cellular functions. Kinase and phosphatase enzymes continually add and remove phosphates on target proteins to modulate their activity. These proteins may have multiple sites for phosphorylation that affect different aspects of their function. The most common phosphorylation sites are at serine, threonine, or tyrosine residues.

A very general assay for phosphoproteins first cleaves them into peptides with a proteolytic enzyme such as trypsin, then analyzes for the phosphopeptides. This can provide information on the degree and location of phosphorylation. Because the resulting chromatograms are very complex, the data analysis can be daunting even with the aid of mass spectrometry. Immobilized metal affinity chromatography (IMAC) in the ferric form is a popular technique for selectively isolating and enriching the phosphopeptides from a peptide digest.

Here we present an automated method combining IMAC with reversed-phase chromatography and UV detection to determine phosphopeptides. Due to its engineered surface structure, the Dionex ProPac® IMAC-10 stationary phase has unique features that permit this application. Unlike gel-based media, it is mechanically rugged and prepared in a reusable packed column. In addition, the binding and release kinetics are more rapid and complete than with gel-based resins. The ProPac IMAC may be cleaned and regenerated easily. In favorable cases, recoveries are quantitative and carryover is below the detection limit.

The Acclaim® 300 C18 columns are designed and specified for applications in peptide mapping and analysis of small proteins. The high efficiency of the 3-µm particle size in a 4.6 x 150 mm format allows fast separations.

The Summit® x2 Dual-Gradient System for HPLC features a pair of ternary pumps in the same cabinet controlled by the same instrument program. This integration provides for simpler installation of both the hardware and software. In combination with the 10-port switching valve in the column compartment, the Summit x2 package is an elegant implementation for 2-D liquid chromatography.

EQUIPMENT

Dionex Summit x2 Dual-Gradient System consisting of:

- Summit P680 DGP6 dual ternary gradient pump
- TCC-100 Thermostatted Column Compartment with 2-position, 10-port switching valve
- ASI-100T™ Automated Sample Injector (temperature-controlled sample tray is recommended)
- UVD 340U diode array detector equipped with micro flow cell (P/N 5065.1810M or 160235)
- Chromeleon® Chromatography Management Software, version 6.7

Centrifuge, Eppendorf® Model 5402 or similar

REAGENTS AND STANDARDS

Acetonitrile, UV grade (B & J)

Deionized water, 18MΩ-cm resistance

Trifluoroacetic acid (TFA), HPLC/Spectro grade (Pierce Chemical, P/N 28901)

Tetrasodium pyrophosphate decahydrate (TSPP), $Na_4P_2O_7 \cdot 10H_2O$, (Sigma Chemical, P/N S6422)

Formic acid, 98% (EM Science, P/N FX0440-6)

Acetic acid, glacial (J.T. Baker, P/N 9524)

Ammonium hydroxide (Fisher Scientific, P/N A669)

Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$ (Aldrich Chemical, P/N 207926)

Monophosphopeptide standard (Sigma Chemical, P/N M-8689)

Tetraphosphopeptide standard (Sigma Chemical, P/N T-4946)

ß-Casein (Sigma Chemical, P/N C8905)

Tributylphosphine solution, 0.2 M in *N*-methylpyrollidine (Sigma Chemical, P/N T7567)

Iodoacetamide (Sigma Chemical, P/N A3221)

Dithiothreitol (Sigma Chemical, P/N D9779)

Trypsin (Pierce Chemical, P/N 20230, immobilized TPCK-treated trypsin)

Ultrafilters, 10000 MWCO centrifugal filters (Millipore, P/N UFC3 LGC00)

CONDITIONS

Columns: ProPac IMAC-10, 2 x 50 mm; Acclaim 300 C18, reversed phase, 4.6 x 150 mm

Inj. Volume: variable, up to 30 µL

Temperature: 30 °C

Detector: UV 214 nm, 5 nm bandwidth

One experiment is executed in three instrument programs in the following order:

PROGRAM 1, LOAD IMAC

Time (min)	IMAC			RP			
	%A	%B	Flow	%A	%B	Flow	Events
–9.0	100	0	0.20	93	7	1.00	valve position A
0.0	100	0		93	7		inject; acquire data
2.6	100	0	OFF	93	7		

PROGRAM 2, HPLC ANALYSIS OF FIRST FRACTION

Time (min)	IMAC			RP			
	%A	%B	Flow	%A	%B	Flow	Events
0.0				93	7	1.00	valve position B
0.25							autozero detector
1.0							acquire data
15.0			ON	31	69	1.00	
16.0				0	100	1.00	
20.0				0	100	1.00	

PROGRAM 3, ELUTE IMAC

Time (min)	IMAC			RP			
	%A	%B	Flow	%A	%B	Flow	Events
–7.0	100	0	0.20	93	7	1.00	valve position A
–6.0	0	100	0.20				
0.0	0	100					inject; acquire data
2.6	0	100	OFF	93	7		

PROGRAM 2, HPLC ANALYSIS OF SECOND FRACTION

Time (min)	IMAC			RP			
	%A	%B	Flow	%A	%B	Flow	Events
0.0				93	7	1.00	valve position B
0.25							autozero detector
1.0							acquire data
15.0			ON	31	69	1.00	
16.0				0	100	1.00	
20.0				0	100	1.00	

In Program 2, it is important not to specify the IMAC eluent composition. At fifteen minutes is an instruction to turn on the pump but leave the composition unchanged from the previous program.

Fraction collection loop, 420 µL (PEEK tubing, 0.5 mm i.d., 130 cm length)

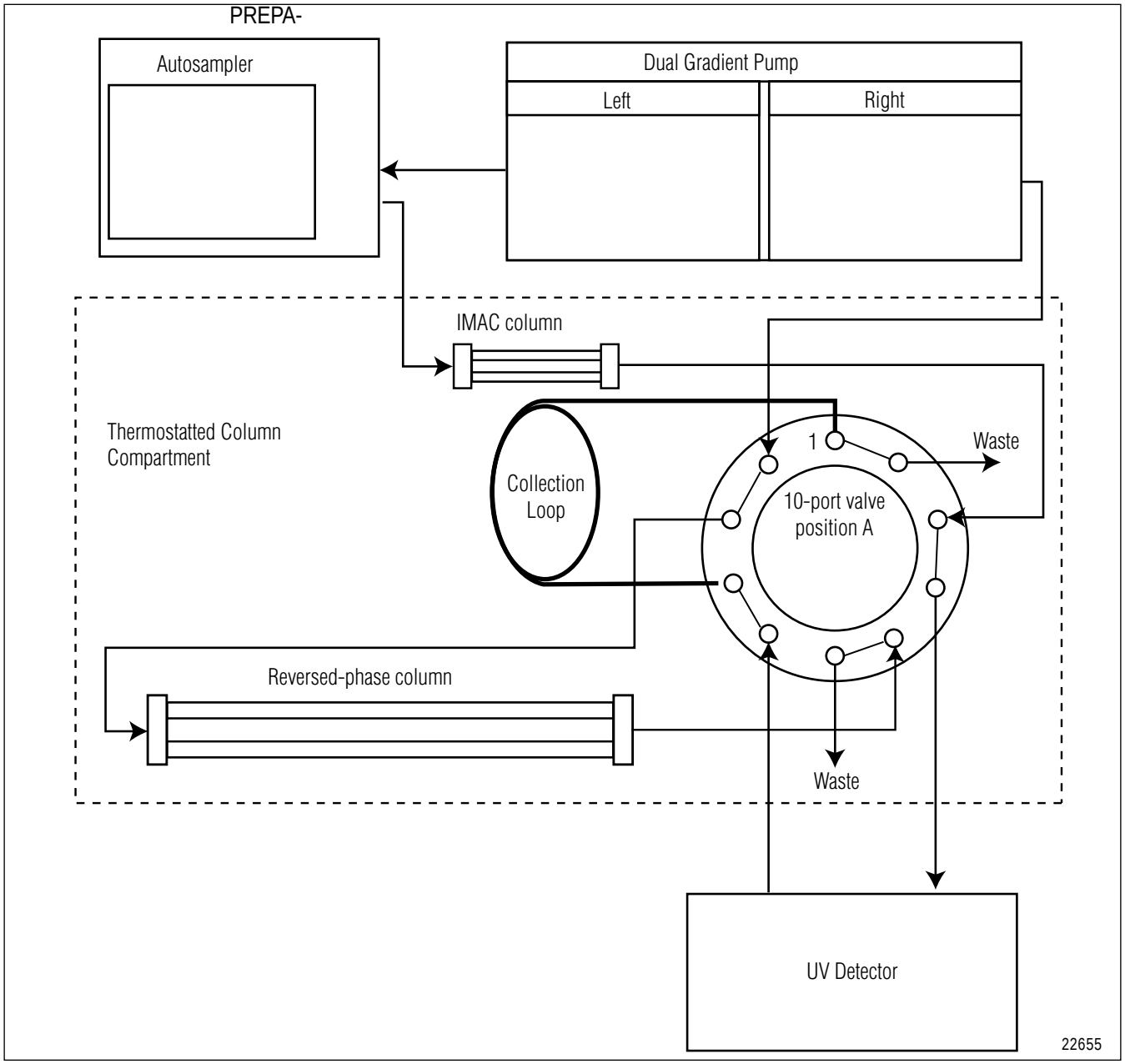

Figure 1. *Connection diagram for the HPLC system.*

RATION AND SETUP OF SYSTEM

When the switching valve is in position A (refer to Figure 1), a sample is injected onto the IMAC column, and the eluate passes through the detector and is trapped in the collection loop when the IMAC eluent flow is turned off. In valve position B, the contents of the loop are injected onto the C18 column, and the detector is switched to monitor the reversed-phase chromatography. A complete experiment performs this sequence twice; once for IMAC eluent A, and again for IMAC eluent B.

Install the Summit HPLC system in the usual way with the TCC-100 on the bottom, the ASI-100 in the middle and P680 DGP pump on top. Set the UVD 340U to the right.

All tubing connections may be made using PEEK tubing of suitable inner diameter. Connect the left pump of the P680 DGP to the inlet of the ASI-100. Using 0.12 mm i.d. (0.005 in.) tubing, connect the outlet of the ASI-100 to the IMAC column, the column outlet to port 3 of the switching valve and port 4 to the UVD 340U. Using 0.25 mm i.d. (0.010 in.) tubing, connect the outlet of the UVD 340U to port 7. Using 0.25 mm i.d. (0.010 in.) tubing, connect the reversed-phase column inlet to port 9 and outlet to port 5. Using 0.25 mm i.d. (0.010 in.) tubing, connect the right pump of the P680 DGP to port 10. Fabricate a sample collection loop from 130 cm of 0.5 mm i.d. (0.020 in.) tubing and connect it to ports 1 and 8. Connect ports 2 and 6 to waste.

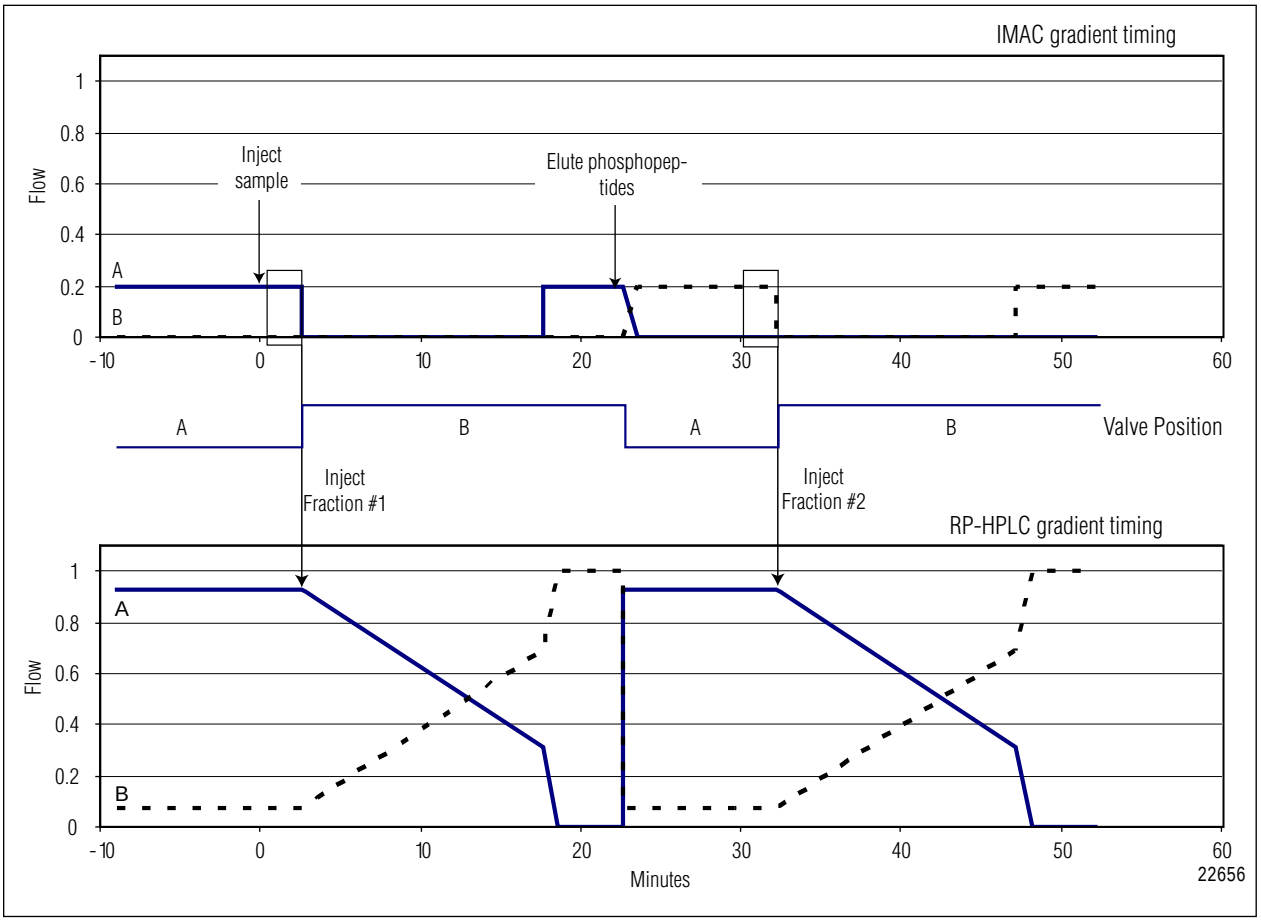

Figure 2. *Timing diagram for 2-D LC.*

PREPARATION OF REAGENTS AND STANDARDS

Phosphopeptide Standards

Add 1.0 mL of water to the contents of one vial of phosphopeptide standard to make a 100 µg/mL stock solution. Make working standards by dilution in 100 mM acetic acid.

Iron Solutions

Dissolve 0.14 g iron (III) chloride hexahydrate in 120 µL glacial acetic acid, dilute to 20 mL with water. Filter through a 0.45-µm membrane filter immediately before use.

Conversion of IMAC Column to Ferric Form

The ProPac IMAC-10 column is shipped in a metal-free form. To convert it to the ferric form, use a 1-mL disposable syringe to load 0.2 mL of filtered iron solution onto the column, followed by a rinse with 0.2 mL of deionized water. Install the column on the HPLC, and equilibrate it with IMAC mobile phase A for 1 h.

Mobile Phase A for IMAC

0.765 mL (20 mmol) of formic acid in 1000 mL of water

Mobile Phase B for IMAC

0.765 mL (20 mmol) of formic acid in 1000 mL of water titrated to pH 9.0 with ammonium hydroxide

Mobile Phase A for RP-HPLC

950 g water, 39.1 g acetonitrile, 0.44 g TSPP, 0.10 mL TFA

Mobile Phase B for RP-HPLC

546 g acetonitrile, 300 g water, 0.11 mL TFA

Dilution buffer for peptide standards

0.115 mL (2.0 mmol) of acetic acid in 20 mL of water

Dilution buffer for protein digests

0.155 mL (4.0 mmol) of formic acid in 20 mL of water

PREPARATION OF SAMPLES

Refer to the bibliography for exact protocols. Briefly, dissolve 2 mg of protein in 1 mL of pH 8, 0.1 M ammonium bicarbonate buffer, reduce with tributylphosphine for 1 h, alkylate with iodoacetamide for 1.5 h, and quench with dithiothreitol. Remove excess reagents with a 10000 MWCO centrifugal ultrafilter, and wash twice with pH 8 buffer. Wash 200 µL of immobilized trypsin slurry, and add the protein solution. Tumble at 37 °C for 24 h. For IMAC fractionation, dilute the digest 1:1 with 200 mM formic acid.

RESULTS AND DISCUSSION

ß-Casein was chosen to demonstrate this application. When digested with trypsin, it yields two phosphopeptides, one monophosphorylated (FQSpEEQQQTEDELQDK) and the other tetraphosphorylated (RELEELNVPGEIVESpLSpSpSpEESITR). These two commercially available pure peptides were used as standards.

The chromatographic results of automated IMAC enrichment and RP-HPLC analysis are shown in Figure 3. In Figure 3d, the monophosphopeptide elutes at 5.8 min, and the tetraphosphopeptide at 9.2 min, and match the retention times of the standards. The best loading conditions for binding phosphopeptides to the IMAC column are at pH 3.0 ± 0.5; diluting the digested protein with 200 mM formic acid accomplishes this. Programs 1 and 3 are designed to capture the last 420 µL of eluate from the IMAC column in the loop; at 0.20 mL/min, this is the last 2.1 min of the chromatogram. The elution of phosphopeptides from the IMAC column is broad, and the relatively large collection loop is needed to collect the whole peak. The pH of the release buffer affects the timing of the fraction collection at the end of program 3. These parameters may be optimized for a particular phosphopeptide.

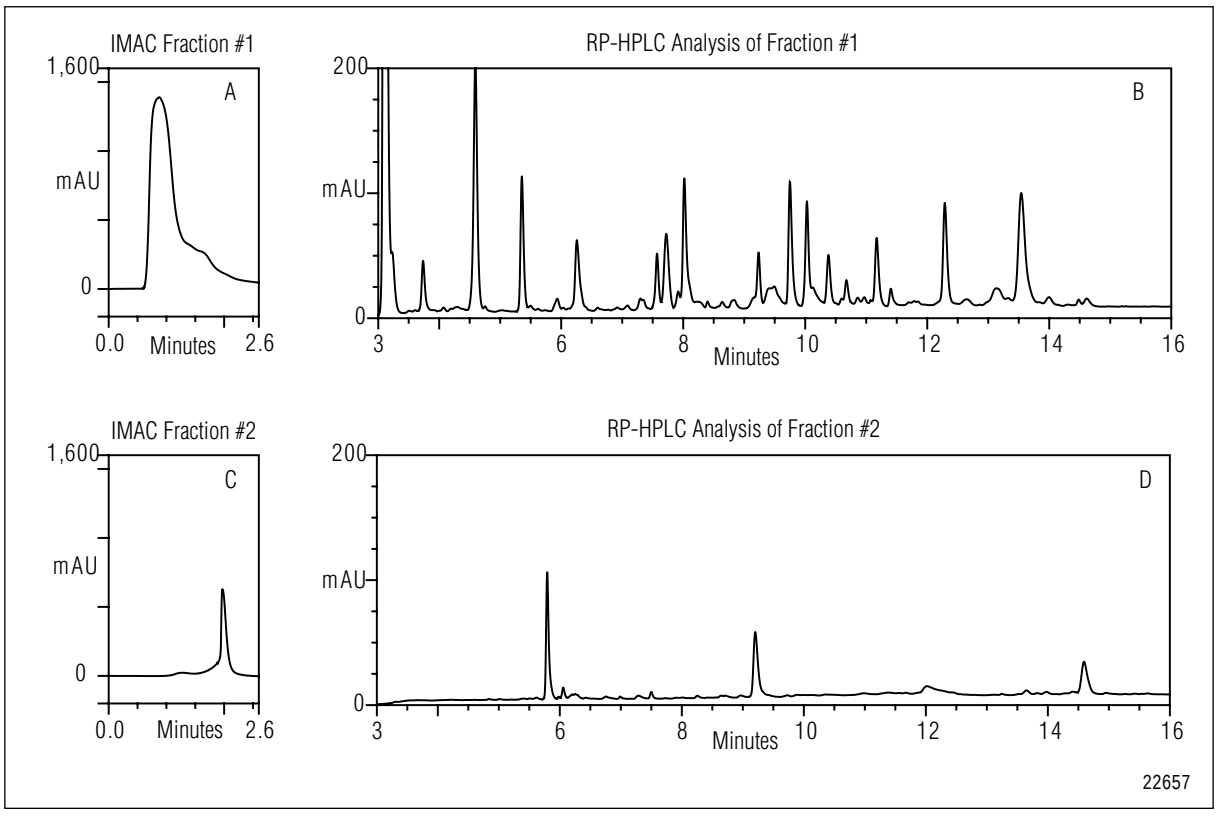

Figure 3. *A typical experiment using a tryptic digest of* β*-casein.*

The use of TSPP in the mobile phase for RP-HPLC is noteworthy. The materials of construction of the pump and Acclaim 300 column are stainless steel, and the active sites of the IMAC column are iron. Contamination of the C18 stationary phase by iron is inevitable. Pyrophosphate is an effective chelating agent at low pH and is transparent to UV light. Without it, the phosphopeptide peaks become asymmetric. An attempt to use pyrophosphate as a competitive agent in the IMAC release buffer had the effect of removing iron from the IMAC column.

Under favorable conditions, the recovery of phosphopeptides from the IMAC column is quantitative and linear down to the detection limit of the RP-HPLC analysis. A serial dilution study using the two standard peptides was performed; the linearity and detection limits were determined with and without IMAC. The standard monophosphopeptide Sigma M-8689 was detectable at 5.7 pmol on column, with or without IMAC, and linear to at least 360 pmol (r^2 = 0.9997 without and r^2 = 0.9991 with IMAC). Recoveries were 91–113% for the range of 11–360 pmol on column.

The tetraphosphopeptide standard Sigma T-4946 was the less favorable case. With IMAC it was not detectable below 30 pmol on column. There was a clear threshold effect because without IMAC this peptide was detectable at 4 pmol. Linearity was acceptable (r^2 = 0.9995 without and r^2 = 0.998 with IMAC). Recovery was only 34–45% in the range of 30–240 pmol on column. Results were similar for a dilution study using digested ß-casein as the source of peptides.

Non-specific binding is the result of nonphosphopeptides having some affinity for the IMAC stationary phase. In particular, some highly acidic and strongly hydrophobic peptides are known to be retained this way. For example, the peak at 14.6 min (Figure 3d) is not a phosphopeptide. Genuine phosphopeptides can be abolished from the chromatogram by treatment of the sample with alkaline phosphatase, and thereby distinguished from nonspecifically-bound peptides.

Carryover between samples has not been observed even when a blank follows an injection of 40 µg of protein digest, which is about 340 times the detection limit. This is true for both the unretained and retained fractions.

PRECAUTIONS

Iodoacetamide is HIGHLY TOXIC. Handle with suitable personal protective equipment.

It has been reported that phosphate buffers cause early loss of binding capacity of the IMAC column, and are not recommended. Buffers based on formate, acetate, bicarbonate, TRIS, and HEPES have been used with the IMAC column without loss of binding capacity.

REFERENCES

1. Pierce Biotechnology. *Immobilized TPCK Trypsin*; Doc. No. 0479.
2. Sigma-Aldrich. Technical Bulletin T7567.
3. Andersson, L.; Porath, J. Isolation of Phosphorproteins by Immobilized Metal (Fe^{3+}) Affinity Chromatography. *Anal. Biochem*. **1986**, *154*, 250–254.
3. Ficarro, S. B., et al. Phosphoproteome Analysis by Mass Spectrometry and Its Application to *Saccacharomyces cerevisiae*. *Nature Biotech*. **2002**, *20*, 301–305.

APPENDIX

Following is the text of the Chromeleon program used in the experiment described in this Technical Note. This text is a reference only and is not intended to be copied digitally and pasted directly into Chromeleon.

```
;  Listing 1: Program 1 for loading IMAC
   column
; 2D IMAC and RP-HPLC
; Step 1: IMAC_B non-retained fraction 1
; Step 2: RP_grad analysis of fraction 1
; Step 3: IMAC_C retained fraction 2
; Step 4: RP_grad analysis of fraction 2
   TempCtrl =     On
   Temperature.Nominal =      30.0 [°C]
   Temperature.LowerLimit =   5.0 [°C]
   Temperature.UpperLimit =   85.0 [°C]
   EquilibrationTime =  0.5 [min]
   ReadyTempDelta =     1.0 [°C]
   HumidityLeakSensor =       Standard
   GasLeakSensor =      Standard
   MsvPosition =  A
   ActiveColumn =       NotUsed
   PumpLeft.Pressure.LowerLimit =   0
   [bar]
   PumpLeft.Pressure.UpperLimit =   300
   [bar]
   PumpLeft.%A.Equate =       “MeCN”
   PumpLeft.%B.Equate =       “HCO2H”
   PumpLeft.%C.Equate =       “NH4CO2H”
   PumpRight.Pressure.LowerLimit =  0
   [bar]
   PumpRight.Pressure.UpperLimit =  300
   [bar]
   PumpRight.%A.Equate =      “70% MeCN
   0.114% TFA”
   PumpRight.%B.Equate =      “MeOH”
   PumpRight.%C.Equate =      “5% MeCN
   0.100% TFA”
   DispSpeed =    20.00 [µl/s]
   DrawSpeed =    5.00 [µl/s]
   SampleHeight =       0.50 [mm]
   SyringeDelay =       5 [s]
   UpSpeed =      10.00 [mm/s]
   DownSpeed =    10.00 [mm/s]
   RadialSpeed =  20.00 [mm/s]
   SyncWithPump =       On
   PumpDevice =   “PumpLeft”
   UV_VIS_1.Wavelength =      214 [nm]
   UV_VIS_1.Bandwidth =       5 [nm]
   UV_VIS_1.RefWavelength =   600 [nm]
   UV_VIS_1.RefBandwidth =    5 [nm]
   UV_VIS_1.Step =      Auto
   UV_VIS_1.Average =   On
   3DFIELD.Step =       0.5 [s]
-9.00
   PumpRight.Flow =     1.000 [ml/min]
   ; equilibrate RP column
   PumpRight.%B = 0.0 [%]
   PumpRight.%C = 93.0 [%]
   PumpLeft.Flow =      0.200 [ml/min]
   ; equilibrate IMAC column
   PumpLeft.%B =  100.0 [%]
   PumpLeft.%C =  0.0 [%]
 0.00
   UV.Autozero
   PumpRight.%C = 93.0 [%]
   Wait     ColumnOven.Ready and Sam-
   pler.Ready
   Inject
   UV_VIS_1.AcqOn
   PumpRight.%C = 93.0 [%]
   PumpLeft.%B =  100.0 [%]
   PumpLeft.%C =  0.0 [%]
2.60
   PumpRight.%C = 93.0 [%]    ; 0.50-
   2.60 min for 419 µL loop
   PumpLeft.Motor =     Off   ; trap
   fraction in loop
   UV_VIS_1.AcqOff
   End      ; continue with RP_grad
   method



;  Listing 2: Program 2 for RP-HPLC
   analysis of peptides
; 2D IMAC and RP-HPLC
; Step 1: IMAC_B non-retained fraction 1
; Step 2: RP_grad analysis of fraction 1
; Step 3: IMAC_C retained fraction 2
; Step 4: RP_grad analysis of fraction 2
   TempCtrl =     On
   Temperature.Nominal =      30.0 [°C]
   Temperature.LowerLimit =   5.0 [°C]
   Temperature.UpperLimit =   85.0 [°C]
   EquilibrationTime =  0.5 [min]
   ReadyTempDelta =     1.0 [°C]
   HumidityLeakSensor =       Standard
```

```
   GasLeakSensor =      Standard
;  MsvPosition =  A     ; leave it wher-
   ever it was
   ActiveColumn =       NotUsed
   PumpLeft.Pressure.LowerLimit =   0
   [bar]
   PumpLeft.Pressure.UpperLimit =   300
   [bar]
   PumpLeft.%A.Equate =       “MeCN”
   PumpLeft.%B.Equate =       “HCO2H”
   PumpLeft.%C.Equate =       “NH4CO2H”
   PumpRight.Pressure.LowerLimit =  0
   [bar]
   PumpRight.Pressure.UpperLimit =  300
   [bar]
   PumpRight.%A.Equate =      “70% MeCN
   0.114% TFA”
   PumpRight.%B.Equate =      “MeOH”
   PumpRight.%C.Equate =      “5% MeCN
   0.100% TFA”
   DispSpeed =    20.00 [µl/s]
   DrawSpeed =    5.00 [µl/s]
   SampleHeight =       0.50 [mm]
   SyringeDelay =       5 [s]
   UpSpeed =      10.00 [mm/s]
   DownSpeed =    10.00 [mm/s]
   RadialSpeed =  20.00 [mm/s]
   SyncWithPump =       On
   PumpDevice =   “PumpRight”
   UV_VIS_1.Wavelength =      214 [nm]
   UV_VIS_1.Bandwidth =       5 [nm]
   UV_VIS_1.RefWavelength =   600 [nm]
   UV_VIS_1.RefBandwidth =    5 [nm]
   UV_VIS_1.Step =      Auto
   UV_VIS_1.Average =   On
   3DFIELD.Step =       0.5 [s]
0.00
   PumpRight.Flow =     1.000 [ml/min]
   PumpRight.%B = 0.0 [%]
   PumpRight.%C = 93.0 [%]
   MsvPosition =  B     ; inject IMAC
   fraction
0.25
   UV.Autozero
1.00
   UV_VIS_1.AcqOn
15.00
   PumpRight.%C = 31.0 [%]
   PumpLeft.Motor =     On    ; some
   washing to reduce carryover
16.00
   PumpRight.%C = 0.0 [%]
   UV_VIS_1.AcqOff
20.00
   PumpRight.%C = 0.0 [%]
   PumpRight.Flow =     1.000 [ml/min]
   PumpRight.%B = 0.0 [%]
   End


;  Listing 3: Program 3 for elution of
   phosphopeptides from IMAC
; 2D IMAC and RP-HPLC
; Step 1: IMAC_B non-retained fraction 1
; Step 2: RP_grad analysis of fraction 1
; Step 3: IMAC_C retained fraction 2
; Step 4: RP_grad analysis of fraction 2
   TempCtrl =     On
   Temperature.Nominal =      30.0 [°C]
   Temperature.LowerLimit =   5.0 [°C]
   Temperature.UpperLimit =   85.0 [°C]
   EquilibrationTime =  0.5 [min]
   ReadyTempDelta =     1.0 [°C]
   HumidityLeakSensor =       Standard
   GasLeakSensor =      Standard
   MsvPosition =  A
   ActiveColumn =       NotUsed
   PumpLeft.Pressure.LowerLimit =   0
   [bar]
   PumpLeft.Pressure.UpperLimit =   300
   [bar]
   PumpLeft.%A.Equate =       “MeCN”
   PumpLeft.%B.Equate =       “HCO2H”
   PumpLeft.%C.Equate =       “NH4CO2H”
   PumpRight.Pressure.LowerLimit =  0
   [bar]
   PumpRight.Pressure.UpperLimit =  300
   [bar]
   PumpRight.%A.Equate =      “70% MeCN
   0.114% TFA”
   PumpRight.%B.Equate =      “MeOH”
   PumpRight.%C.Equate =      “5% MeCN
   0.100% TFA”
   DispSpeed =    20.00 [µl/s]
   DrawSpeed =    5.00 [µl/s]
   SampleHeight =       0.50 [mm]
   SyringeDelay =       5 [s]
   UpSpeed =      10.00 [mm/s]
```

```
   DownSpeed =    10.00 [mm/s]
   RadialSpeed =  20.00 [mm/s]
   SyncWithPump =       On
   PumpDevice =   “PumpLeft”
   UV_VIS_1.Wavelength =      214 [nm]
   UV_VIS_1.Bandwidth =       5 [nm]
   UV_VIS_1.RefWavelength =   600 [nm]
   UV_VIS_1.RefBandwidth =    5 [nm]
   UV_VIS_1.Step =      Auto
   UV_VIS_1.Average =   On
   3DFIELD.Step =       0.5 [s]
-7.00
   PumpRight.Flow =     1.000 [ml/min]
   ; equilibrate RP column
   PumpRight.%B = 0.0 [%]
   PumpRight.%C = 93.0 [%]

   PumpLeft.Flow =      0.200 [ml/min]
   PumpLeft.%B =  100.0 [%]
   PumpLeft.%C =  0.0 [%]
-6.00
   PumpLeft.%B =  100.0 [%]
   PumpLeft.%C =  0.0 [%]
   PumpLeft.%B =  0.0 [%]; start elution
   of IMAC column
   PumpLeft.%C =  100.0 [%]
0.00
   UV.Autozero
   PumpRight.%C = 93.0 [%]
   Wait     ColumnOven.Ready and Sam-
   pler.Ready
   Inject
   UV_VIS_1.AcqOn
   PumpRight.%C = 93.0 [%]
   PumpLeft.%B =  0.0 [%]
   PumpLeft.%C =  100.0 [%]
2.60
   PumpRight.%C = 93.0 [%]    ; 0.5--2.6
   min 419 µL loop
   PumpLeft.Motor =     Off; trap frac-
   tion in loop
   UV_VIS_1.AcqOff
   End; continue with RP_grad method
```


Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
Sunnyvale, CA (408) 737-8522 Westmont, IL (630) 789-3660
Houston, TX (281) 847-5652 Atlanta, GA (770) 432-8100
Marlton, NJ (856) 596-06009 Canada (905) 844-9650

Europe
Austria (01) 616 51 25 Belgium (03) 3-353 42 94
Denmark 36 36 90 90 France 01 39 30 01 10
Germany 06126-991-0 Italy (06) 66 51 50 52
The Netherlands (0161) 43 43 03 Switzerland (062) 205 99 66
United Kingdom (01276) 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282
India 91-22-28475235 Japan (06) 6885-1213
Korea 82 2 2653 2580

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