DIONEX

Application Note 210

Determination of the Phosphate Content of Phosphorylated Proteins

INTRODUCTION

Protein phosphorylation is one of the most investigated post translational modifications, because phosphoproteins are vital to many cellular activities. Phosphorylation regulates protein activity, marks proteins for destruction, acts as a biomarker,[1–3] is responsible for cell recognition,[4,5] and provides critical energy needed for metabolism.[1–5] Often phosphorylation acts as a switch, by turning the target substrate or cellular pathway to active or inactive.[6] Phosphorylation is a dynamic process where 33% of all proteins are phosphorylated at least once during their lifetime.[6] Phosphorylation processes are reversible and controlled by the combined action of two classes of phosphoprotein enzymes, kinases and phosphatases.[3,6] Kinases remove phosphate or transfer phosphate to other compounds, and switch the target substrate to active or inactive.[3,6] Thus kinases are actively researched for drug design and localized cancer treatment.[2,3] Some researchers study the role of phosphoproteins in the more complex signaling networks.[3] The determination of active phosphorylation sites is important to understanding the phosphorylation mechanisms and pathways and for the development of future therapeutic pharmaceuticals. Protein quantities are often limited, and obtained after labor intensive and lengthy processes from either purification or recombinant methods. Thus, a sensitive and reliable analysis method is needed for phosphate that consumes minimal protein.

Ion chromatography (IC) with suppressed conductivity detection is a widely used technique to determine inorganic anions, such as phosphate. A Reagent-Free™ IC (RFIC™) system improves the ease-of-use of IC by requiring only deionized water as a source to electrolytically generate the hydroxide mobile phase in line, and produces lower background signal and baseline noise to achieve a greater S/N. This allows for greater sensitivity, smaller sample injections, and lower protein consumption.

In this Application Note, we describe the determination of phosphate with an RFIC-EG system and suppressed conductivity detection. This method directly determines phosphate without any derivatization in 5 min, saving sample and time. We used this method to determine phosphate in six lots of ovalbumin, and single lots of phosvitin and β-casein. The RFIC-EG system delivers fast, accurate, and reproducible determinations of the phosphate contents of phosphorylated proteins.

EXPERIMENTAL

EQUIPMENT

Dionex ICS-2000 RFIC-EG Ion Chromatography system[a]

AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray

Chromeleon® 6.8 Chromatography Workstation

Sample Vial kit, 0.3 mL polypropylene with caps and septa (P/N 055428)

Centrifuge

Thermolyne™ Dri-Bath heater with heating block and thermometer

UV/Vis spectrophotometer

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter

[a] This application can also be performed on other Dionex RFIC-EG systems.

REAGENTS AND STANDARDS

Reagents

Deionized water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity, freshly degassed by ultrasonic agitation and applied vacuum

Use only ACS reagent grade chemicals for all reagents and standards unless otherwise noted

Alkaline phosphatase, bovine intestinal mucosa, lyophilized, 35% protein, 3436 units/mg protein (Aldrich, P/N P6772-2KU)

Micro BCA™ Protein Assay kit (Pierce Biotechnology, P/N 23250)

Sodium phosphate, dibasic anhydrous (Na_2HPO_4, JT Baker, BioReagent, ultrapure, P/N JT4062-1, FW 141.96)

Tris (tris-hydroxymethylaminomethane, $(NH_2C(CH_2OH)_3$, Aldrich, P/N 252859, FW 121.14)

Samples

β-Casein, bovine milk, >90% (Aldrich, P/N C6905, 24 kDa molecular weight)

Ovalbumin from chicken hen egg white, Grade V, VI, >98%, lyophilized powder (Aldrich, P/N A5503, P/N A2512, 45 kDa),

Lot 18C-80351, Grade V (assigned as Lot 1)

Lot 40K7070, Grade V (assigned as Lot 2), 2000 QC report

Lot 37H7010, Grade VI (assigned as Lot 3), 1997 QC report

Lot 51H7085, Grade VI (assigned as Lot 4), 1991 QC report

Lot 79H7003, Grade VI (assigned as Lot 5), 1999 QC report

Lot 087k7004, Grade V (assigned as Lot 6), 2007 QC report

Phosvitin from chicken hen egg yolk (Aldrich, P/N P1253, 34 kDa molecular weight)

CONDITIONS

Column:	IonPac® Fast Anion IIIA guard, 3 × 50 mm, P/N 062966, IonPac Fast Anion IIIA analytical, 3 × 250 mm, P/N 062964
Eluent:	25 mM Potassium hydroxide (KOH)
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	1.0 mL/min
Column Temperature:	30 ºC
Tray Temperature:	10 ºC
Inj. Volume:	5 µL (full loop injection)
Detection:	Suppressed conductivity, ASRS® 300 (2 mm, P/N 064555), recycle mode, 62 mA
Background Conductance:	<1 µS
Baseline Noise:	<2 nS
System Backpressure:	~2200 psi
Run Time:	5 min

PREPARATION OF SOLUTIONS AND REAGENTS

It is essential to use high quality, Type 1 water, 18.2 MΩ-cm resistivity and it should contain as little dissolved carbon dioxide as possible for the preparation of eluents, standards, and samples. Degas the deionized water using the Nalgene filter unit (0.2 µm, nylon) with applied vacuum and ultrasonic agitation.

Preparation of Eluent A, Degassed Type 1 Deionized Water

Prepare 2 L of freshly degassed Type 1 deionized water using the Nalgene filter unit (0.2 µm, nylon) with applied vacuum and ultrasonic agitation.

10 mM Stock Phosphate Standard Solution

To prepare a 10 mM stock phosphate standard solution, dissolve 142 mg of anhydrous Na_2HPO_4 with Type 1 deionized water in a 100 mL volumetric flask. Dilute to the mark and shake the flask gently to mix.

Working Phosphate Standard Solutions

To prepare 1, 5, 10, 20, 100, 150, and 300 µM phosphate individual working standards, pipette 10, 50, 100, 200, 1000, 1500, 3000 µL, respectively of the 10 mM phosphate stock standard solution into separate 100 mL HDPE bottles. Dilute with Type 1 deionized water to 100.00 g total weight and shake gently to mix. Store all standards at 4 °C. Prepare the 1–300 µM standard solutions weekly and the stock standard solutions monthly.

Preparation of 50 mM Tris Buffer (pH 9) and Alkaline Phosphatase Working Solutions

To prepare 50 mM Tris buffer (pH 9), dissolve 606 mg of Tris in 100 mL of aseptic (deionized, filtered, 0.2 µm) Type 1 deionized water. Adjust to pH 9 with dilute HCl, if needed. Store the Tris buffer solution at 4 ºC.

To prepare 700 units/mL (0.7 units/µL) working solution of alkaline phosphatase, pipette 3000 µL of 50 mM Tris buffer into the bottle containing 1.76 mg (entire bottle) of 35% alkaline phosphatase, bovine intestinal mucosa (3436 units/mg protein). Swirl gently to dissolve. Dispense 200 µL aliquots into separate 1.5 mL micro-centrifuge tubes. Store at –20 ºC or below. Defrost the necessary number of aliquots prior to use.

PREPARATION OF SAMPLES

Phosphoprotein Samples

To prepare 5 mg/mL ovalbumin samples, dissolve 20 mg of solid with 4 mL of deionized Type 1 water in a 20 mL scintillation vial. Gently swirl the solution until thoroughly mixed. Dispense 200 µL aliquots of the 5 mg/mL ovalbumin into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 ºC or below and defrost the aliquots as needed. Prepare individual solutions of 5 mg/mL phosvitin using the same procedure as the 5 mg/L ovalbumin. To prevent the formation of casein micelles, prepare a 0.5 mg/mL β-casein solution in 50 mM Tris buffer (pH 8).[7]

Dephosphorylation with Alkaline Phosphatase

To prepare chicken hen ovalbumin (45 kDa) samples (1–2 moles phosphate per mole of protein) for phosphate determinations, digest 19.2 µL of 5 mg/mL (96 µg) ovalbumin plus 120 µL of 50 mM Tris buffer (pH 9), 134 µL of deionized water, and 27 µL of 0.7 units/µL alkaline phosphatase in Tris buffer to a total volume of 300 µL at 37 ºC for 5 h. Centrifuge the digested protein at 14,000 × g for 5 min, and pipette the supernatant into 1.5 mL sample vials. To prepare chicken hen phosvitin (34 kDa), and bovine milk β-casein (24 kDa), the amounts of protein and alkaline phosphatase were proportioned relative to the ovalbumin conditions according to the molecular weight and the expected phosphate/protein mole ratio. Control samples of Tris buffer, alkaline phosphatase, and proteins in buffer were prepared, heated, and centrifuged in the same manner as the protein samples. Total protein was determined on all proteins according to the instructions on the Micro-BCA Protein assay kit.

SYSTEM PREPARATION AND SETUP

The setups for the ICS-2000 RFIC-EG system, AS Autosampler, and consumables are thoroughly described in the ICS-2000 Operator's Manual,[8] AS Autosampler Operator's Manual,[9] and the column manual.[10] Install a backpressure loop between the pump and the injection valve to increase the system backpressure to an optimum system pressure of 2200 psi (2200–2500 psi). Do not exceed the maximum system backpressure 3000 psi, as this can damage the degas module. Hydrate and install the ASRS 300 anion self-regenerating suppressor, EGC II KOH cartridge, and the CR-ATC continuous regenerating anion trap column according to the suppressor manual,[11] and the QuickStart instructions in the ICS-2000 Operator's Manual.

This application note uses a full loop injection of a 5 µL sample loop. The AS Autosampler will withdraw 35 µL (2× the sample loop plus 25 µL) for each 5 µL injection. To conserve samples, use the partial loop limited sample injection mode to withdraw only the sample injection volume. To inject a 5 µL partial loop limited sample injection, install a calibrated 10–25 µL sample loop, enter the sample loop volume in AS Autosampler module, set the injection mode on the AS Autosampler module to partial loop (menu, detail menu), enter 5 (µL) injection volume in the sequence, and select zero for the cut loop volume in the program. In this mode it is very important that the injection port tubing volume is accurate. More information on full loop, partial loop limited sample injection modes, and calibrating the volume of the injection port tubing can be found in the AS Autosampler Operator's manual.

Column Wash

To wash the column and return it to its original state using the eluent generator, disconnect the column from the cell, install a temporary waste line from the end of the column to a waste container, set the flow rate to 0.5 mL/min, and the eluent generator to 65 mM KOH. Verify that the ASRS 300 suppressor is off. Install a temporary backpressure loop between the pump and Inj. Valve 1 to bring the system backpressure to ~2200 psi. Wash the column overnight, then remove the temporary backpressure tubing, reset the original eluent and suppressor conditions, and allow the previous eluent (65 mM KOH) to flush to waste for one min. Remove the temporary waste line tubing and re-install the tubing from the column to the cell. Allow 30–60 min for the column to re-equilibrate before starting phosphate determinations.

If there is evidence that sample analysis has fouled the column and the 65 mM KOH is an inadequate wash, it may be necessary to use 10× the eluent concentration to wash the column. Prepare 250 mM NaOH eluent according to the column manual, and install temporary tubing directly from the pump to the column and then to waste. Do not run 10× eluent through the cell, eluent generator, or suppressor. Wash the column overnight at 0.2 mL/min, and then re-install and flush the column as described in the previous section. Re-equilibrate the column 30–60 min before starting phosphate determinations.

RESULTS AND DISCUSSION

One of the challenges of this analysis is that citrate, present in some samples and in the alkaline phosphatase preparation, is typically a late eluting anion and the goal is to have a fast analysis. An optimized separation on the IonPac Fast Anion IIIA eluted citrate and phosphate within 5 min and other anions, such as chloride, nitrate, carbonate, and sulfate eluted before 2.5 min and prior to phosphate.

Separation

The IonPac Fast Anion IIIA is an ultra low hydrophobic hydroxide-selective anion exchange column that was specifically developed for a fast separation of phosphate and citrate. The purchased alkaline phosphatase was buffered in sodium citrate prior to the manufacturer's lyophilization. Therefore, most of the dephosphorylated samples in this study contained both phosphate and citrate. The IonPac Fast Anion IIIA with a 25 mM KOH mobile phase enables the elution of phosphate and citrate in 2.9 and 3.9 min ($R_s > 5$), respectively without interference from other common anions. Figure 1 shows the chromatogram of 105 pmol phosphate and 1300 pmol citrate in water and Figure 2 shows the application of the method to the determination of the phosphate content of chicken hen ovalbumin. Note that no peaks interfere with the phosphate eluting at about 3 min.

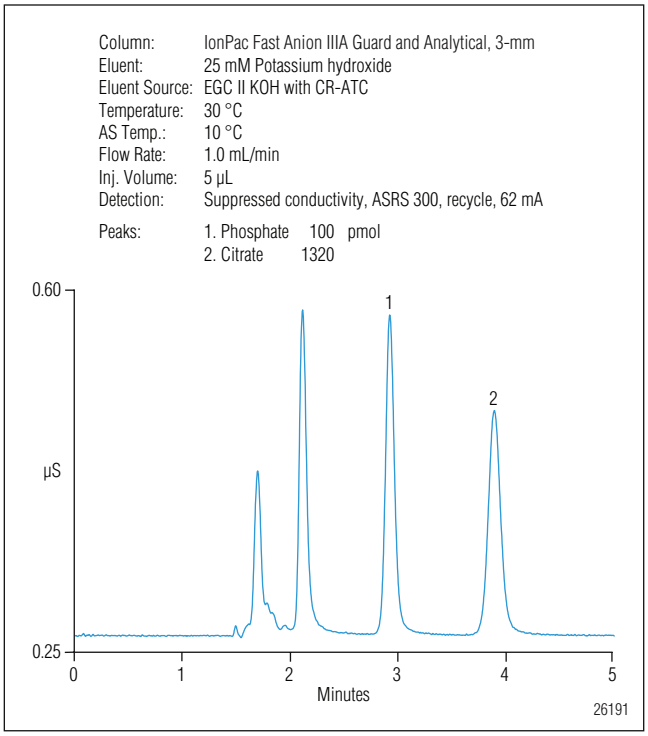

Figure 1. Determination of phosphate and citrate by IC on an IonPac Fast Anion IIIA column.

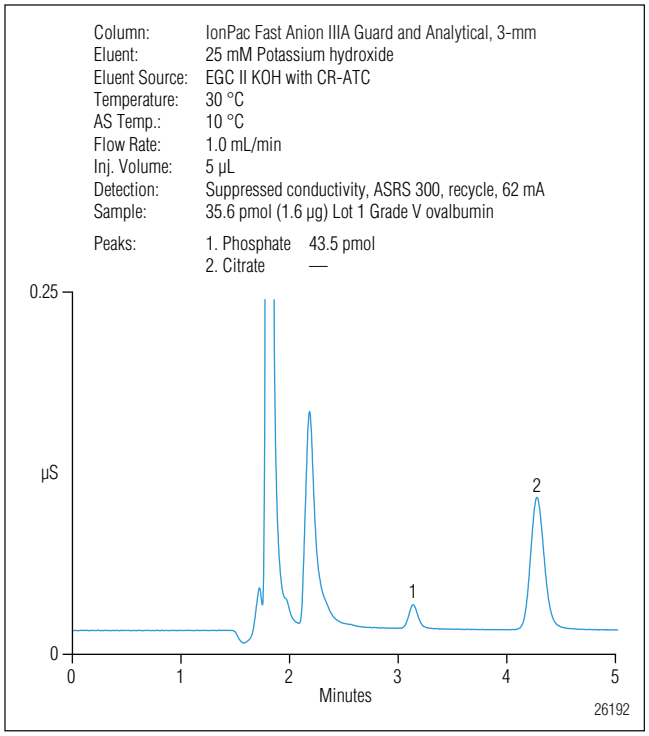

Figure 2. Determination of phosphate from dephosphorylated hen egg white ovalbumin.

Method Qualification

To qualify the method for phosphate determinations, the relationship of phosphate peak area to concentration, and the limit of detection were determined. The peak area response was determined from 5 to 1600 pmol (1–300 µM) phosphate using duplicate injections of seven standards (5, 25, 50, 100, 525, 800, and 1600 pmol or ~1, 5, 10, 20, 100, 150, and 300 µM). The response was linear with a correlation coefficient (r^2) of 0.9991. To determine the peak-to-peak noise, the baseline noise was measured in one-minute intervals from 20 to 60 min over three system blanks without injecting a sample. The noise averaged 1.63 ± 0.03 nS (n = 3).

It is well known that proteins have phosphate heterogeneity, and thus, phosphorylation is seldom stoichiometric.[1–3] To determine the limit of detection (LOD) we estimated the amount of chicken hen ovalbumin, as a protein with 1–2 moles phosphate per mole of protein, required to produce a phosphate peak response with 3× S/N. A 35 pmol sample of ovalbumin (Lot 1) was dephosphorylated with alkaline phosphatase as described in the sample preparation section. The phosphate peak had ~85× S/N, therefore the phosphate determinations were repeated with 3.5 pmol of dephosphorylated ovalbumin. The results showed that the estimated LOD for a 5 µL injection was 1.5 pmol protein for a 1–2 mole phosphate per mole protein.

The protein sample matrix had little effect on the analysis, as indicated by only a small loss of phosphate retention time, -0.2 min over 160 injections of dephosphorylated and native protein samples. However, it is likely that the column will require periodic cleaning to remove the proteins and return the column to its original state (System Preparation and Setup section).

Phosphate Determinations in Protein Samples

The method was applied to five lots of commercially available Grade V and Grade VI chicken hen ovalbumin, and single lots of chicken hen phosvitin, and bovine milk β-casein. The phosphoproteins tested were expected to have a large range of phosphorylated sites from <2 for ovalbumin to 123 for phosvitin.

Table 1. Phosphate Determinations in Proteins					
Protein	**Lot**	**Amount (pmol)**	**Predicted Phosphate (pmol)**	**Found Phosphate (pmol)**	**Mol Ratio (Phosphate/ Protein)**
β-Casein	1	66.7	267–333[7]	277.3	4.16
Ovalbumin, Grade V	1	35.6	36–71[1–3]	58.44	1.64
Ovalbumin, Grade V	2	35.6	36–71[1–3]	40.49	1.14
Ovalbumin, Grade VI	3	35.6	36–71[1–3]	13.01	0.37
Ovalbumin, Grade VI	4	35.6	36–71[1–3]	27.92	0.78
Ovalbumin, Grade VI	5	35.6	36–71[1–3]	43.36	1.22
Ovalbumin, Grade V, new lot	6	35.6	36–71[1–3]	58.44	1.64
Phosvitin	1	22.2	955–27001[4–16]	1918	86.1

Ovalbumin, the major protein in chicken hen egg white, belongs to the serine protease inhibitor (Serpin) compound family, and is a possible storage protein.[12] Ovalbumin is used to research other serpins, calibrate electrophoresis gels, and as a carrier protein to conjugate to other proteins when researching immunological responses.[13] We evaluated three lots each of Grade V and Grade VI ovalbumin of differing ages. Lot 6 of Grade V ovalbumin, the most recently purchased lot, had similar concentrations of phosphate to Lot 1 evaluated in the method qualification, 1.64 ± 0.04 moles of phosphate / mole protein, n = 2. The older lots (2–5) had lower concentrations of phosphate, from 0.37 ± 0.02 (lot 3) to 1.20 ± 0.02 (lot 5) moles phosphate / mole protein (Table 1). The results demonstrate the heterogeneity of phosphate content of commercially available ovalbumin. Most of the lots (1–2, 5–6) of ovalbumin had 1–2 moles of phosphate per mole of protein as described in the literature.[1-3] However, ovalbumin Lots 3–4 had much less phosphorylation.

The other phosphoproteins had measurable phosphate concentrations prior to digestion with alkaline phosphatase, therefore the results of the untreated proteins were subtracted from the treated proteins (Table 1). Tris buffer and alkaline phosphatase solutions were tested as possible sources of phosphate in the untreated proteins. However, phosphate was not detected in either solution. These experiments suggest that the proteins have trace phosphate buffer from their preparation.

Phosvitin is a highly phosphorylated protein from chicken hen egg yolk containing 50% phosphoserines that are used to provide phosphate for the growing chick.[14,15] Historically, this protein has been studied for its unusually high phosphorylation and used as a protein standard for phosphate calibrations. More recently, it has been studied for its emulsifying and iron binding characteristics.[14,16] In this study, we determined high concentrations of phosphate (86.1 ± 0.0 moles phosphate / mole protein) as expected (Figure 3). The results were close to the reported values of 95–123 moles phosphate / mole protein.[14–16]

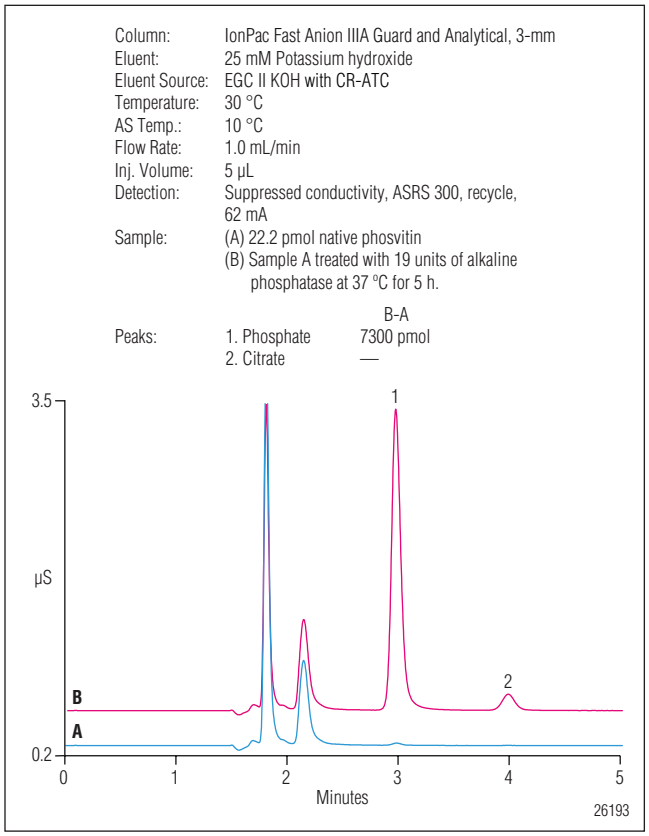

Figure 3. Determination of phosphate in alkaline phosphatase treated and untreated phosvitin from hen egg yolk.

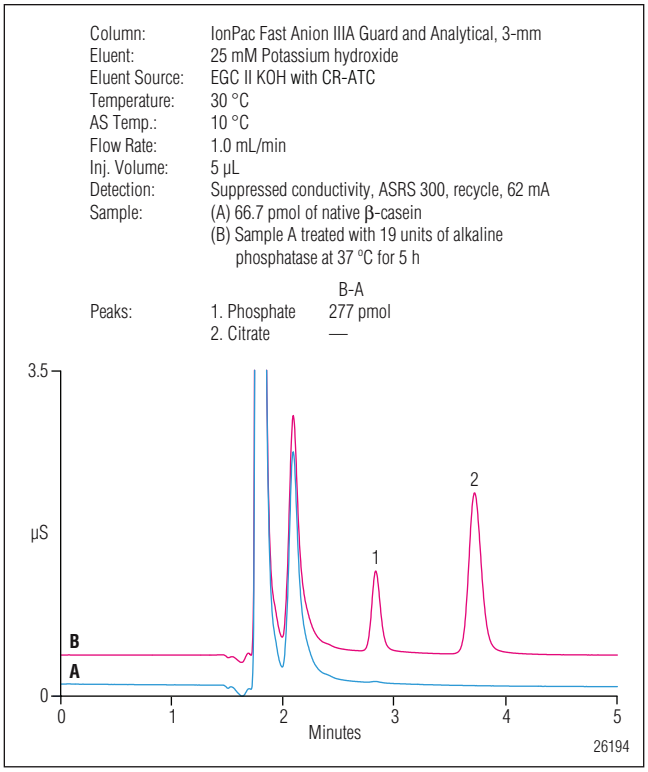

Figure 4: Determination of phosphate in alkaline phosphatase treated and untreated β-casein from bovine milk.

β-Casein is the dominant protein in milk and cheese, the carrier of calcium in milk,[7] and widely used in the manufacturer of adhesives, coatings, and food products. Bovine milk β-casein was prepared in 50 mM Tris buffer (pH 8) at 0.5 mg/mL to prevent the formation of casein micelles.[7] In these experiments, micelle formation was observed when solutions were prepared at higher concentrations and when using water as the diluent. Dephosphorylated β-casein from bovine milk had 4.16 moles of phosphate / mole protein (Figure 4). These results agreed with values reported in the literature, 4–5 moles of phosphate / mole protein.[7]

CONCLUSION

This application note demonstrates a fast, accurate, rugged, and sensitive method for determining the phosphate content of a protein without sample derivatization.

PRECAUTIONS

Do not exceed the maximum system backpressure 3000 psi, as this can damage the degas module.

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SUPPLIERS

Pierce Biotechnology, PO Box 117, Rockford, IL 61105 USA. 1-800-874-3723 www.piercenet.com

Sarstedt, Inc., 1025 St. James Church Road, P.O. Box 468, Newton, NC 28658 USA 1-828-465-4000, www.sarstedt.com

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