

Direct Determination of Cyanate in a Urea Solution and a Urea-Containing Protein Buffer Using a Reagent-Free Ion Chromatography System

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ABSTRACT

Urea is commonly used in protein purification, including the large scale purification of recombinant proteins for commercial purposes, and in recombinant protein manufacturing to denature and solubilize proteins.¹⁻³ In aqueous solutions, urea degrades to cyanate and ammonium. Cyanate can carbamylate proteins, an unwanted modification that can alter their stability, function, and efficiency. Therefore, an accurate and sensitive method is needed to measure cyanate in urea-containing protein buffers.

This poster describes the use of a Reagent-Free™ IC system with eluent generation (RFIC-EG™ system) to determine cyanate in urea and urea-containing protein buffers. This method improves upon a previous IC method that uses a manually prepared carbonate eluent to effectively evaluate the efficiency of cyanate scavengers and make recommendations for protein buffers that reduce cyanate accumulation and subsequent carbamylation.⁴ The linearity, accuracy, and precision are discussed in this presentation.

EXPERIMENTAL

Equipment

Dionex ICS-3000 RFIC-EG system consisting of:

SP Single gradient pump

DC Detector/Chromatography module, single or dual temperature zone configuration

CD Conductivity Detector with an ASRS® 300 (2 mm) suppressor

EG Eluent Generator with EluGen® EGC II KOH cartridge and Continuously Regenerating Anion Trap Column (CR-ATC)

AS Autosampler with Sample Tray Temperature Controlling option, 1.5 mL sample tray, and 0.3 mL polypropylene sample vials

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Samples

Urea solutions were prepared from solid compounds in 18.2 MΩ-cm resistivity deionized water and diluted 50-fold prior to cyanate determinations:

8 M urea

8 M urea with 1 M chloride

8 M urea with 1 M chloride and 50 mM phosphate (pH = 8.4)

All solutions containing urea were prepared the same day of the experiments unless otherwise stated.

Conditions

Columns: IonPac® AS15-5μm Analytical (3 × 150 mm)

Eluent: 25 mM Potassium hydroxide

Eluent Source: EGC II KOH with CR-ATC

Flow Rate: 0.5 mL/min

Column/Tray

Temperatures: 30 °C / 4 °C

Injection Volume: 5 μL

Detection: Suppressed Conductivity (ASRS 300, 2 mm, recycle mode 31 mA)

RESULTS AND DISCUSSION

To minimize the time required to select the hydroxide-selective, high-capacity column with the highest resolution for cyanate and to optimize conditions, the Dionex Virtual Column™ Separation Simulator was used to simulate the separation by modeling cyanate as nitrite. The simulator results demonstrated that the IonPac AS15 column with 20 mM potassium hydroxide provided the optimum chloride/nitrite and nitrite/carbonate resolution with $R_s > 3$. The Virtual Column simulator accelerated method development by eliminating the time required to evaluate several columns and eluents that might have been suitable for this application.

The confirmation experiments of the Virtual Column results showed that cyanate was well resolved from chloride and eluted from the column in <9 min using 25 mM potassium hydroxide.

Figure 1 demonstrates good peak response and peak asymmetry for 2 μM cyanate separated on the IonPac AS15-5 μm column using an electrolytically generated 25 mM potassium hydroxide at 0.5 mL/min.

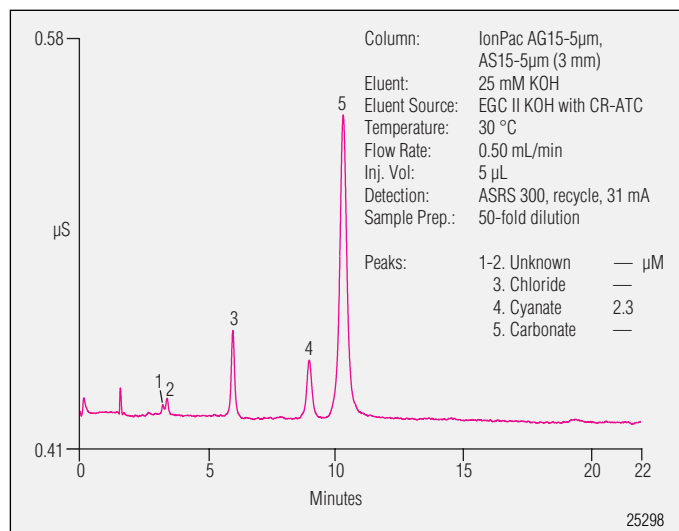


Figure 1. Determination of 2 μM cyanate using ion chromatography.

Linearity, Limit of Detection, Limit of Quantification

To qualify the cyanate method, the linearity, system noise, limit of quantification (LOQ), and limit of detection (LOD) were evaluated. The linearity of cyanate peak area responses from 1 to 100 μM was determined using a least squares regression fit. The resulting correlation coefficient (r^2) was 0.9993.

The peak-to-peak baseline noise was measured in 1 min segments from 20 to 60 min without injecting a sample. The noise was 0.95 ± 0.13 nS ($n = 3$). The LOD was determined to be 0.25 μM cyanate (S/N 3.01).

The detection limits were significantly lower than previously reported (2 μM) using an IonPac AS14 column with bicarbonate/carbonate eluents,⁴ mostly due to the advantages of using an electrolytically-generated hydroxide eluent with suppressed conductivity detection. The LOQ is 0.8 μM (S/N 10).

SAMPLE ANALYSIS

Accuracy and Precision

We applied the method to 50-fold dilutions of urea buffer samples.

Sample A: 8 M urea,

Sample B: 8 M urea with 1 M chloride, and

Sample C: 8 M urea with 1 M chloride and 50 mM phosphate (pH = 8.4).

Figure 2 compares an unspiked urea sample (Sample A, diluted 50-fold) to the same sample with 2.2 μM cyanate added (Figure 2B). The cyanate peaks had good peak symmetry and were well resolved from carbonate and other ionic components in the urea buffer. The recovery of the 2.2 μM added cyanate was 99.7%, indicating the method is accurate.

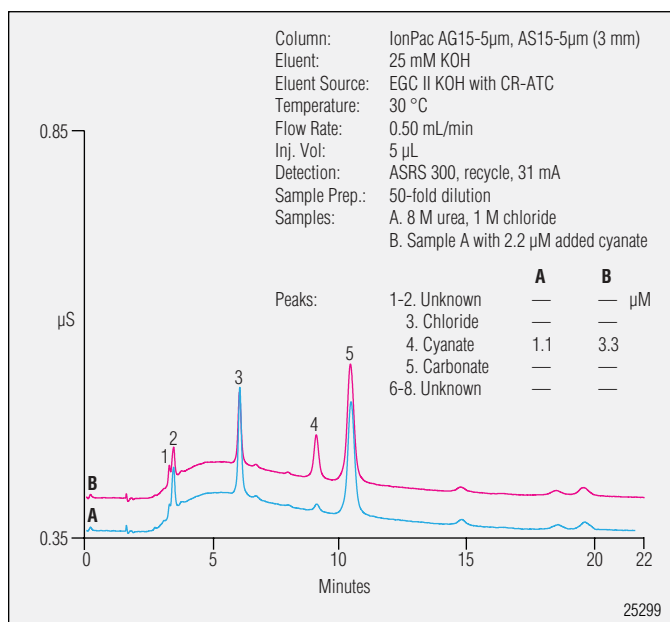


Figure 2. Comparison of A) 8 M urea to B) 8 M urea spiked with 2.2 μM cyanate.

We found that the urea and urea-containing solutions, Samples A–C, had similar cyanate concentrations of $1.1 \pm 0.1 \mu\text{M}$ in freshly prepared 50-fold diluted solutions (Table 1). The calculated recoveries were 89–104.4% for 0.8–4.9 μM additions of cyanate. Table 1 shows the results of 1.2–2.2 μM additions of cyanate with 99.7–104.4% recoveries.

Table 1. Recoveries of Cyanate in Urea Solutions (50-Fold Dilution Factor)						
Sample ^a	Amount Found (μM)	RSD	Amount Added (μM)	Amount Measured (μM)	RSD	Recovery (%)
A	1.13	0.50	2.20	3.32	1.38	99.7
B	1.11	1.07	2.22	3.48	0.55	104.4
C	1.00	1.41	1.24	2.27	0.69	101.1

n = 7

a Freshly prepared solutions.

A) 8 M Urea, B) 8 M Urea, 1 M Chloride, C) 8 M Urea, 1 M Chloride, 50 mM Phosphate Buffer pH = 8.4

To determine the retention time and peak area precisions, seven replicate injections of 2 μM cyanate were spiked in deionized water and 50-fold dilutions of Samples A–C. Cyanate had similar retention times for all samples, 8.99 to 9.07 min. The retention time and peak area precisions were <0.1 and <2% for all three samples.

In urea-containing solutions with 1 M concentrations of chloride, cyanate is not fully resolved from chloride, as in the 50-fold diluted sample of Sample B (Figure 3) and Sample C (Figure 4), but is quantifiable.

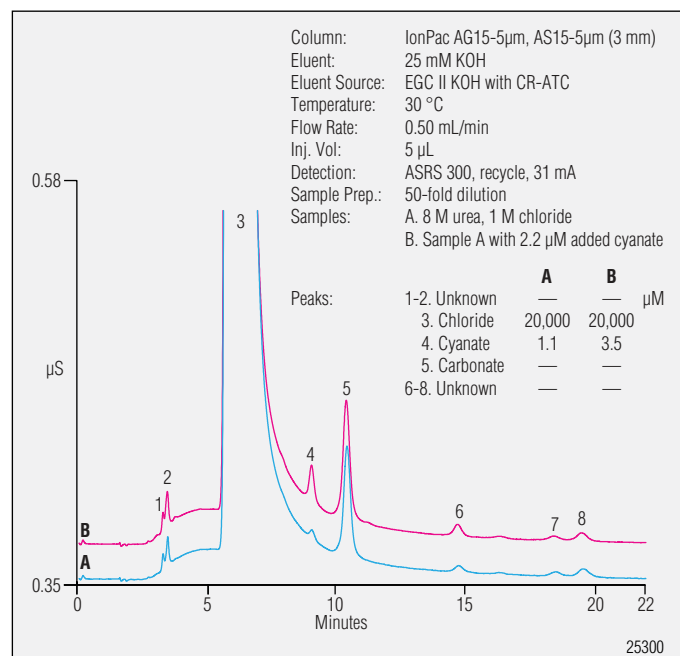


Figure 3. Comparison of A) 8 M urea, 1 M chloride to B) 8 M urea, 1 M chloride with 2.2 μM cyanate.

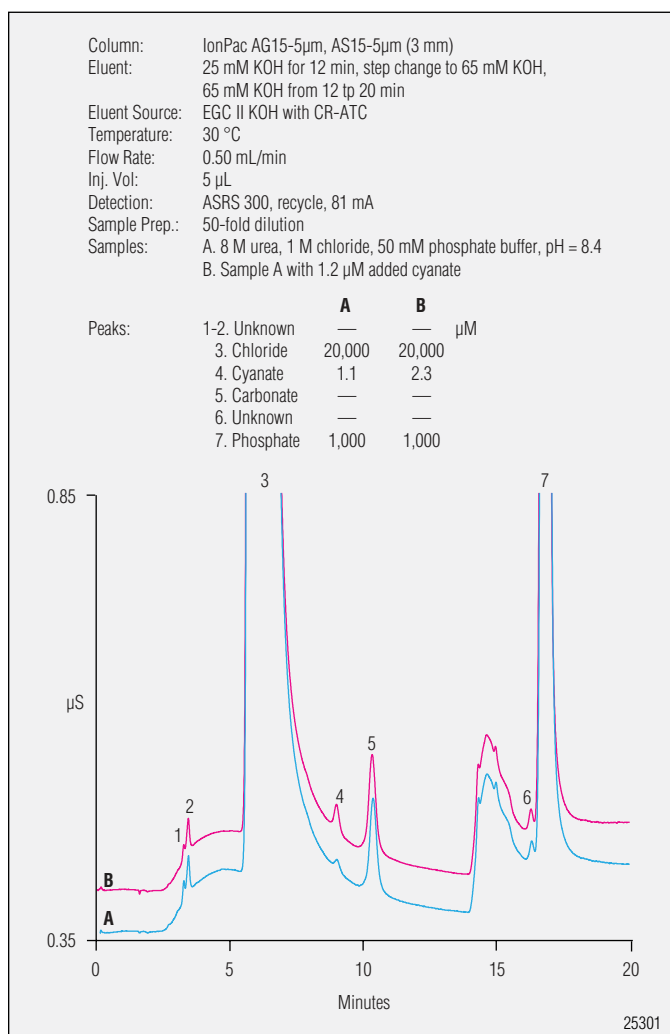


Figure 4. Comparison of A) 8 M urea, 1 M chloride, and 50 mM phosphate buffer (pH = 8.4) to B) the same sample spiked with 1.2 μM cyanate.

Fifty-fold diluted solutions of Sample B and Sample C were spiked with 2.2 and 1.2 μM cyanate, respectively. The calculated recoveries were 104.4 and 101.1%, respectively. These excellent recoveries demonstrate the high concentration of chloride has a minimal influence on the determination of cyanate.

A step change to 65 mM potassium hydroxide was added to elute the phosphate from the column in 20 min.

SAMPLE STABILITY

To study the accumulation of cyanate in urea as a function of temperature, 50-fold dilutions of Samples A–C were stored at $-40\text{ }^{\circ}\text{C}$, $4\text{ }^{\circ}\text{C}$ (AS autosampler tray), and at $25\text{ }^{\circ}\text{C}$ for four consecutive days.

The experiments demonstrated that the total cyanate concentrations were stable in 8 M urea solutions when stored at $-40\text{ }^{\circ}\text{C}$. However, cyanate increased more than 10-fold over four days when stored at $4\text{ }^{\circ}\text{C}$ (from 6 to $75\text{ }\mu\text{M}$) and increased significantly when stored at $25\text{ }^{\circ}\text{C}$ (from 24 to $886\text{ }\mu\text{M}$) (Figure 5).

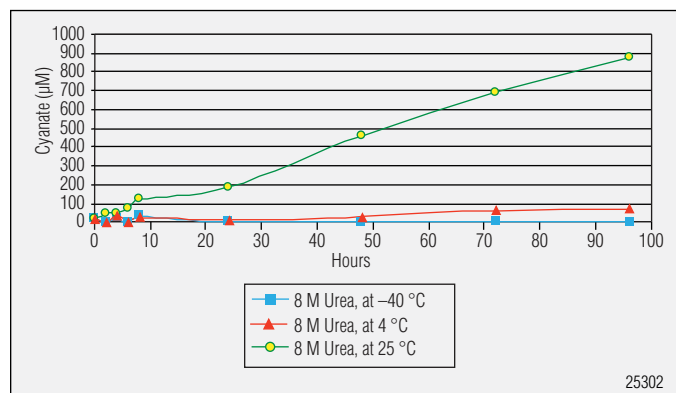


Figure 5. Effect of temperature on cyanate generation from urea solutions.

CONCLUSION

Urea degrades to cyanate, which is unwanted in urea-containing buffers used for protein purification.

Using a high-capacity anion-exchange column and suppressed conductivity detection, we accurately determined low (μM) concentrations of cyanate in 50-fold dilutions of 8 M urea and urea solutions containing molar concentrations of chloride and mM concentrations of phosphate.

This method allows a fast and accurate assay of cyanate in urea-containing solutions. A Reagent-Free IC system ensures the highest precision, eliminates the need to prepare eluents, and eliminates eluent preparation errors.

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