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Direct Determination of Cyanate in a Urea Solution and a Urea-Containing Protein Buffer

INTRODUCTION

Urea is commonly used in protein purification, including large scale purification of recombinant proteins for commercial purposes, and in recombinant protein manufacturing to denature and solubilize proteins.^{1–3} Some proteins are readily soluble and denature at moderate urea concentrations (4–6 M), however, most solubilize and denature at higher concentrations (8–10 M).^{4–6} Urea degrades to cyanate and ammonium in aqueous solutions. The maximum rate of cyanate production occurs near neutral pH, the typical pH range of biological buffers.⁷

Cyanate can carbamylate proteins through a reaction with free amino, carboxyl, sulfhydryl, imidazole, phenolic hydroxyl, and phosphate groups.⁸ These are unwanted modifications that can alter the protein's stability, function, and efficiency. While some of these reactions can be reversed by altering the pH of the solution, cyanate-induced carbamylation reactions to N-terminal amino acids, however—such as arginine and lysine—are irreversible.⁸ Urea solutions are commonly deionized to remove cyanate for this reason. Unfortunately, high cyanate concentrations can accumulate in urea solutions regardless of prior deionization, with some urea buffers reporting cyanate concentrations as high as 20 mM.⁷ An accurate, sensitive method for measuring cyanate in high ionic strength matrices is needed to help monitor and control quality in these buffers.

Ion chromatography (IC) is an ideal method for cyanate determination. A 2004 publication showed determination of cyanate in urea solutions by IC using a Dionex IonPac® AS14 column with 3.5 mM Na₂CO₃/1.0 mM NaHCO₃ eluent, and suppressed conductivity

detection.⁹ This method was used to evaluate the efficiency of cyanate scavengers and make recommendations for protein buffers that reduced cyanate accumulation and subsequent carbamylation reactions. The authors evaluated separate 0.1 M citrate, phosphate, and borate buffers in 8 M urea across a pH range of 5–9. They reported that the citrate (pH = 6) buffered urea solution demonstrated the best suppression of cyanate accumulation (<0.2 mM cyanate). However, phosphate buffers at pH 6 and 7 (<0.5 mM cyanate) were preferred over the citrate buffers because citrate actively carboxylates proteins. (It is sometimes used as a carboxylating agent.) While this analytical method was effective, the authors believed it could be improved by using a high-capacity hydroxide-selective anion-exchange column with better chloride-cyanate resolution. Hydroxide eluent delivers better sensitivity than carbonate/bicarbonate. Improved resolution between chloride and cyanate, combined with the capability to inject more concentrated samples due to the higher column capacity also provides increased method sensitivity.

This application note shows determination of cyanate in samples of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer (pH = 8.4) using a Reagent-Free™ Ion Chromatography (RFIC™) system. This method provides improved sensitivity, allows smaller volume sample injections, lowers the required dilution, and demonstrates higher resolution of cyanate from chloride compared to the previously published method. Cyanate is separated using

a hydroxide-selective IonPac AS15 (5- μ m) column with electrolytically generated 25 mM potassium hydroxide eluent and suppressed conductivity detection. The IonPac AS15 column is a 3 x 150 mm high-capacity (60 μ Eq/column) column, with a smaller particle size, diameter, and length than the IonPac AS14 column described in a previous method. These changes improve sensitivity, reduce eluent consumption, and allow higher sample throughput. The mobile phase is electrolytically generated, which reduces labor, improves consistency, and provides the reproducibility of an RFIC system. Linearity, limit of detection (LOD), precision, recoveries, and stability of cyanate in urea as a function of temperature are discussed.

EXPERIMENTAL

Equipment

Dionex ICS-3000 RFIC-EG™ system consisting of:

SP Single gradient pump

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

CD Conductivity Detector

EG Eluent Generator

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

EluGen® EGC II KOH cartridge (P/N 058900)

Continuously Regenerated Anion Trap Column (CR-ATC, P/N 060477)

Chromeleon® 6.8 Chromatography Workstation

Virtual Column™ Separation Simulator (optional)

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

^aThis application can be performed on other Dionex RFIC-EG systems.

REAGENTS AND STANDARDS

Reagents

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

Use only ACS reagent grade chemicals for all reagents and standards.

Chloride standard (1000 mg/L; Dionex, P/N 037159)

Sodium carbonate, anhydrous (Na₂CO₃) (VWR, JT3602)

Sodium chloride (NaCl) (VWR, JT3624)

Sodium cyanate (NaOCN) (Aldrich, 185086)

Sodium phosphate, dibasic anhydrous (Na₂HPO₄) (VWR, JT3828)

Sodium phosphate, monobasic monohydrate (NaH₂PO₄·H₂O) (VWR, JT3818)

Urea (H₂NCONH₂) (VWR, JT4204-1)

Urea Matrix Sample Solutions

Urea solutions were prepared from solid compounds in 18.2 M Ω -cm deionized water and diluted 50 \times 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 5- μ m Analytical (3 x 150 mm, P/N 057594) IonPac AG15 5- μ m Guard, (3 x 30 mm, P/N 057597)
Eluent ^a :	25 mM KOH
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.5 mL/min
Column Temperature:	30 °C
Tray Temperature:	4 °C ^b
Injection Volume:	5 μ L
Detection:	Suppressed conductivity, ASRS® 300 (2 mm, P/N 064555), recycle mode, 31 mA
Background Conductivity:	<1 μ S
Baseline Noise:	<2 nS
System Backpressure:	~2200 psi
Run Time:	22 min

^a Add a step change at 12 min to 65 mM KOH when determining cyanate in samples containing phosphate. The eluent conditions are: 25 mM KOH for 0 to 12 min, and 65 mM KOH from 12 to 20 min, recycle mode, 81 mA.

^b Temperature was maintained at 4 °C using the temperature-controlled autosampler tray to minimize changes in cyanate concentration.

PREPARATION OF SOLUTIONS AND REAGENTS

It is essential to use high quality, Type 1 water, with a resistivity of 18.2 M Ω -cm or greater, and it must be relatively free of dissolved carbon dioxide. Degas the deionized water using ultrasonic agitation with applied vacuum.

1 M Stock Cyanate Standard Solution

To prepare a 1 M stock cyanate standard solution, dissolve 6.50 g of sodium cyanate (NaOCN, FW 65.01 g/mol) with deionized water in a 100 mL volumetric flask and bring to volume. Gently shake the flask to thoroughly mix the solution.

Primary and Working Cyanate Standard Solutions

To prepare a 1.00 mM cyanate primary standard, pipette 100 μ L of the 1 M cyanate stock standard solution into a 100-mL volumetric flask, bring to volume with deionized water, and shake the flask gently to mix.

To prepare 1, 2, 4, 10, 20, 50, and 100 μ M cyanate individual working standards, pipette 50, 100, 200, 500, 1000, 2500, and 5000 μ L, respectively of the 1.00 mM cyanate primary standard solution into separate 50 mL volumetric flasks. Bring to volume with deionized water and shake gently to mix.

Prepare the 0.13, 0.25, and 0.50 μ M cyanate detection limit standards by diluting the 1.0 μ M cyanate working standard using serial dilutions. For example, a portion of the 1.0 μ M cyanate standard is diluted 50% to 0.50 μ M cyanate. The 0.13 and 0.25 μ M cyanate standards are prepared in a similar way from the 0.25 and 0.50 μ M cyanate standards, respectively.

Store all standards at 4 $^{\circ}$ C. Prepare the 1–20 μ M standard solutions weekly and the primary and stock standard solutions monthly.

Urea Matrix Sample Solutions

To prepare 8 M urea (H₂NCONH₂, FW 60.06 g/mole) matrix sample solution, dissolve 48.05 g urea with deionized water in a 100 mL volumetric flask, dilute to volume, and mix thoroughly.

Prepare the combined 8 M urea, 1 M sodium chloride (NaCl, FW 58.44 g/mole) matrix sample solution in a similar manner using 48.05 g urea, 5.84 g sodium chloride, and deionized water in a 100 mL volumetric flask.

Prepare the combined 8 M urea, 1 M sodium chloride, and 50 mM phosphate (49.5 mM sodium phosphate monobasic (NaH₂PO₄·H₂O), 0.5 mM sodium phosphate dibasic (Na₂HPO₄) matrix solution using 48.05 g urea, 5.84 g sodium chloride, 0.683 g sodium phosphate monobasic (NaH₂PO₄·H₂O, FW 58.44 g/mole), 0.007 g sodium phosphate dibasic (Na₂HPO₄, FW 141.96 g/mole), and deionized water in a 100 mL volumetric flask. Bring to volume, and mix thoroughly (pH = 8.4).

To prepare 100-, 50-, and 10-fold dilutions of the matrix sample solutions for the dilution experiments, pipette 200, 400, and 2000 μ L, respectively, of the matrix sample solution into a (tared) 20 mL polypropylene scintillation vial and add deionized water until a total weight of 20.00 g is reached.

PRECAUTIONS

The urea solutions must be stored frozen at -20 or -40 $^{\circ}$ C and defrosted before use or prepared fresh daily as the cyanate concentrations increase over time and with increased temperature.

SYSTEM SETUP

Refer to the instructions in the ICS-3000 Installation¹⁰ and Operator's¹¹ manuals, AS Autosampler Operator's manual,¹² and column,¹³ and suppressor¹⁴ product manuals for system setup and configuration.

RESULTS AND DISCUSSION

Method Development and Optimization

The challenge in this application is to accurately determine low concentrations of cyanate in a matrix with high concentrations of salts (up to 20,000× higher) and to resolve a cyanate peak eluting near a significantly larger chloride peak. In a previous study, cyanate was separated on a Dionex IonPac AS14 column using carbonate/bicarbonate eluent at a flow rate of 1.2 mL/min, with suppressed conductivity detection.⁹ The authors reported a 1.3 min retention time difference between chloride and cyanate. However, cyanate is not fully resolved from chloride in urea solutions containing 1 M chloride. The authors also reported that the chromatogram's baseline was affected when 8 M urea solutions were injected. As urea eluted through the column, the baseline of the chromatogram increased >2 μS at approximately 3 min, then slowly drifted downward to the original baseline. The cyanate detection limit corresponding to the undiluted urea solution is high (200 μM). An RFIC system with a hydroxide-selective column and electrolytically generated hydroxide eluent can be easily optimized to resolve low concentrations of cyanate from high concentrations of chloride in urea samples. Electrolytically generated hydroxide eluent improves sensitivity and reduces baseline noise.

In order to optimize conditions and to minimize the time required to select the hydroxide-selective, high-capacity column with the highest resolution for cyanate, the authors used the Chromeleon Virtual Column simulator to model separation conditions. Nitrite was used to model cyanate because it is not currently part of the Virtual Column database and was chosen after reviewing chromatograms in column manuals for various high-capacity hydroxide-selective columns. (Only these types of columns were considered because some urea-containing solutions contain molar concentrations of chloride and other anions that may overload low-capacity columns). Virtual Column simulator was used to evaluate the separation of chloride, nitrite, carbonate, and phosphate with isocratic hydroxide eluents at 30 °C. The simulator results demonstrated that the IonPac AS15 column using 20 mM KOH provided the optimum chloride/nitrite and nitrite/carbonate resolution with $R_s > 3$. The Virtual Column simulator proved to be a valuable tool in helping accelerate method development by eliminating the time required to select a column and eluent suitable for this application.

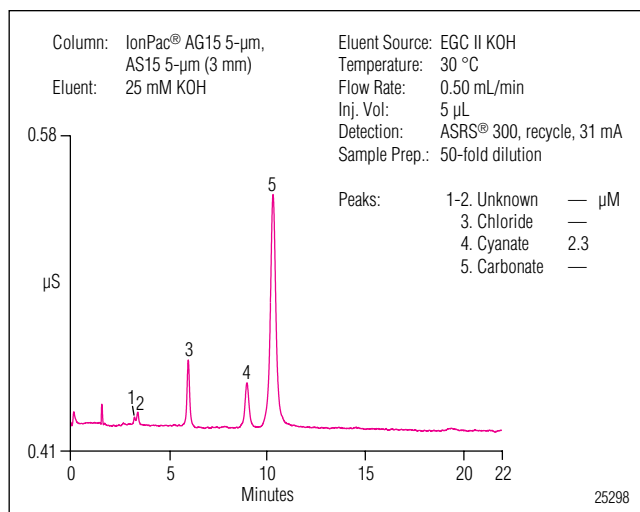


Figure 1. Determination of 2 μM cyanate by ion chromatography.

To refine the results obtained by Virtual Column simulator, the retention times of a 5 μL injection of 1 mM cyanate, chloride, and carbonate were determined on an IonPac AS15 5- μm , 3 x 150 mm column, using 20, 25, and 30 mM potassium hydroxide at 0.5 mL/min and 30 °C. The experiments showed cyanate well resolved from chloride and carbonate by 3.2 min ($R_s > 3$) using either 20 or 25 mM potassium hydroxide, and, as expected, retention times decreased with increasing eluent strength. 25 mM potassium hydroxide eluent was selected for this assay. Figure 1 demonstrates good peak response and peak asymmetry for 2 μM cyanate separated on the IonPac AS15 5- μm column using electrolytically generated 25 mM potassium hydroxide at 0.5 mL/min.

During the initial method evaluation, the effects of column overload using 8 M urea samples with and without spiked concentrations of 1 M sodium chloride were tested. The chromatograms of undiluted 8 M urea showed a similar baseline disturbance (3 μS) from urea as reported in the literature⁹ (not shown). As urea elutes from the column, the baseline shifts upward at approximately 3 min then slowly back to the original baseline at 15 min. All tested urea-containing solutions demonstrated similar results. The magnitude of the baseline shifts reduced with dilution (Figures 2–4). Urea contains minor unidentified ions that do not interfere with the cyanate peak and elute from the column in less than 20 min. 8 M urea samples did not overload the column. To determine whether the molar concentrations of chloride expected in the samples would cause column overloading, 20-, 50-, and 100-fold dilutions of 8 M urea solutions containing 1 M chloride were injected. The chromatography of the 20-fold dilution

samples (50 mM chloride) showed distorted chloride peaks typical of column overloading. At least 50× dilutions of 8 M urea with 1 M chloride are required to avoid this phenomenon and to obtain adequate resolution of chloride and cyanate.

Method Qualification

To qualify the cyanate method, linearity, system noise, limit of quantification, and LOD were evaluated. The peak area response of cyanate was determined from 1 to 100 μM, using triplicate injections of the calibration standards. The linearity of cyanate peak area responses was determined with Chromeleon software 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 acceptably low (0.95 ± 0.13 nS ($n = 3$)). To determine the limit of detection, seven replicates of 0.13, 0.25, and 0.50 μM cyanate were injected. The peak responses of cyanate were compared against the baseline noise using $3 \times S/N$. The LOD was 0.25 μM cyanate (S/N 3.01). These detection limits are significantly lower than previously reported (2 μM) using an IonPac AS14 column with bicarbonate/carbonate eluents.⁹ This improvement is likely due to the advantages of using electrolytically generated hydroxide eluent with suppressed conductivity detection. The IonPac AS15 column has similar capacity per column but 30% less

volume than the AS14 column. These differences provide improvement in peak response and sensitivity. This allows for a smaller sample (5 μL) to be injected, resulting in less column overload and longer column life. The limit of quantification, using $10 \times S/N$, is 0.8 μM (1.0 μM, S/N 11.6).

Determination of Cyanate in Urea and Urea-Containing Solutions

The method was applied to 50-fold dilutions of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer (pH 8.4). The authors determined the urea and urea-containing solutions had similar cyanate concentrations of 1.1 ± 0.1 μM in 50× diluted solutions (Table 1). The cyanate peak was well resolved from chloride and carbonate, and had good peak shape (Figure 2A). An acceptably small baseline drift (<0.15 μS) from urea was observed starting at 2.4 min and ending at about 15 min. In urea-containing solutions with molar concentrations of chloride, cyanate elutes on the base of the large chloride peak and is not fully resolved from chloride (Figure 3A). The chromatography is similar for urea-containing solutions with chloride and phosphate (Figure 4A).

To determine the method accuracy, multiple additions of cyanate (1.2, 2.2, and 3.6 μM) were added to 50× dilutions of 8 M urea samples. In addition, 50× dilutions of 8 M urea with 1M chloride and 8 M urea with 1 M chloride, and 50 mM phosphate buffer (pH = 8.4) were

Table 1. Recoveries of Cyanate in Urea Solutions (Dilution Factor: 50×)

Matrix ^a	Amount Present (μM)	RSD	Amount Added (μM)	Amount Measured (μM)	RSD	Recovery (%)	Amount Added (μM)	Amount Measured (μM)	RSD	Recovery (%)	Amount Added (μM)	Amount Measured (μM)	RSD	Recovery (%)
A	1.13	0.50	1.20	2.26	0.64	96.7	2.20	3.32	1.38	99.7	3.62	4.92	0.87	103.6
B	1.11	1.07	0.91	1.88	1.03	93.1	1.31	2.43	1.44	100.4	2.22	3.48	0.55	104.4
C	1.00	1.41	0.81	1.60	1.10	89.5	1.24	2.27	0.69	101.1	1.70	2.66	1.16	98.0

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

similarly spiked with 0.9–2.2 μM , and 0.8–1.7 μM cyanate, respectively. The calculated recoveries were >89% for all solutions (Table 1). The chromatograms of the 50 \times dilution of 8 M urea and the same solution spiked with 3.6 μM cyanate are shown in Figure 2. Peaks 6–8 are unidentified ions from the urea matrix that elute from the column within 20 min, while run time was extended to 22 min to ensure that these ions were fully eluted before the next injection. The chromatograms of unspiked and spiked with 2.2 μM cyanate in 50-fold dilution of 8 M urea 1 M chloride are shown in Figure 3. The chromatograms of 8 M urea with 1 M chloride and 50 mM phosphate buffer (pH=8.4), both unspiked and spiked with 1.7 μM cyanate and diluted 50 \times are shown in Figure 4.

To determine the retention time and peak area precisions seven replicate injections of 2 μM cyanate were spiked into deionized water, 50 \times dilutions of 8M urea, 8 M urea with 1M chloride, and 8 M urea with 1M chloride and 50 mM phosphate buffer (pH 8.4). Cyanate had similar retention times for all samples—8.99 to 9.07 min (Table 2). The retention time and peak area precisions were <0.1 and <2 % for all three samples.

Sample Stability

The accumulation of cyanate in urea as a function of temperature is frequently discussed in the literature.^{1,4-9}

Table 2. Retention Time and Peak Area Precisions of 2 μM Cyanate Spiked Into 50-fold Dilution of Urea Solution

Matrix ^a	Retention Time (min)	RSD	Peak Area RSD
Deionized Water	9.07	0.02	1.06
A	9.07	0.02	1.65
B	9.03	0.04	0.65
C	8.99	0.06	1.69

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

n = 7

^a Freshly prepared solutions

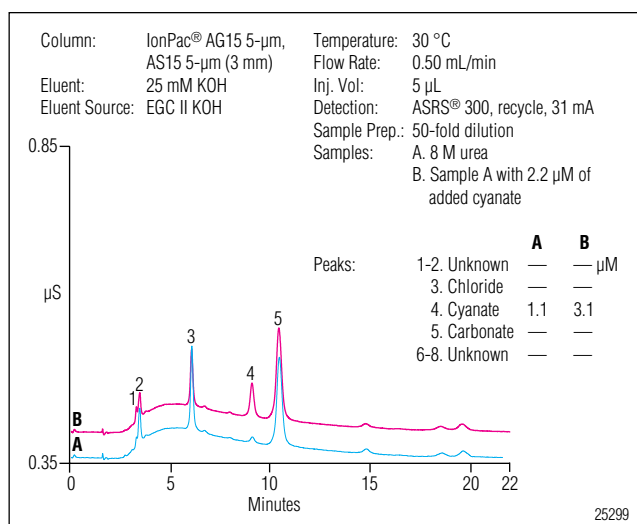


Figure 2. Comparison of A) 8 M urea B) 8 M urea with 2.2 μM cyanate.

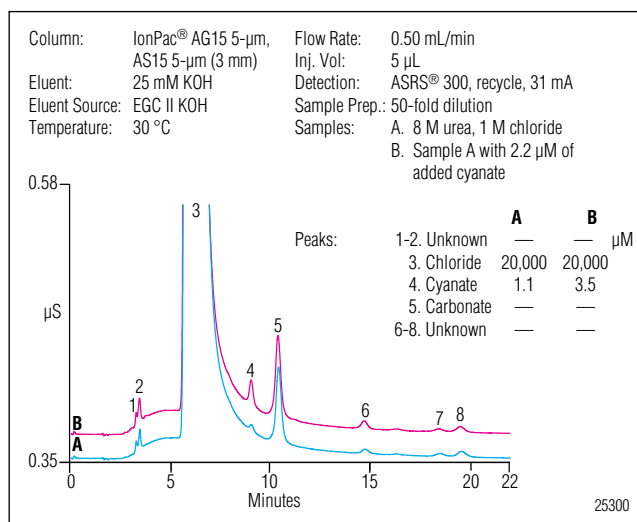


Figure 3. Comparison of A) 8 M urea 1 M chloride B) 8 M urea 1 M chloride with 2.2 μM cyanate.

To determine the stability of cyanate in urea over 4 days, cyanate concentrations were determined from 50-fold dilutions of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer solutions (pH = 8.4). The solutions were stored at -40 $^{\circ}\text{C}$, 4 $^{\circ}\text{C}$ (AS autosampler tray), and 25 $^{\circ}\text{C}$ during the four-day experiment. To elute phosphate in the phosphate buffered urea solution, the method was modified with a step change to 65 mM KOH after the carbonate peak at 12 min.

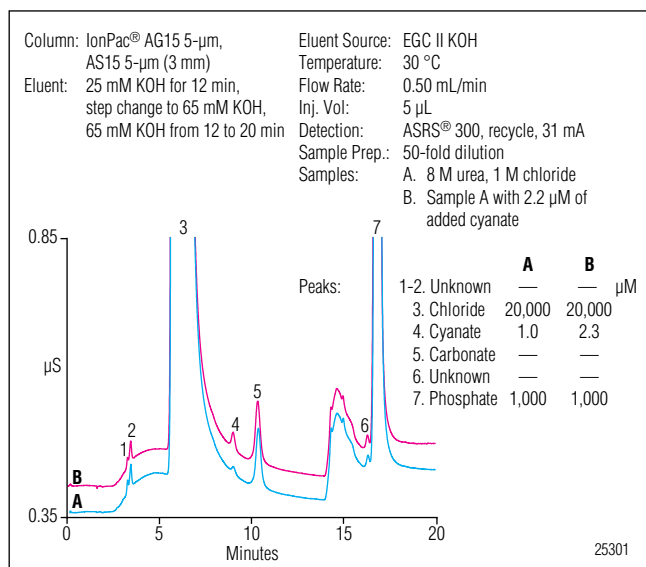


Figure 4. Comparison of A) 8 M urea 1 M chloride and 50 mM phosphate buffer (pH = 8.4) to B) Sample A spiked with 1.2 μ M cyanate.

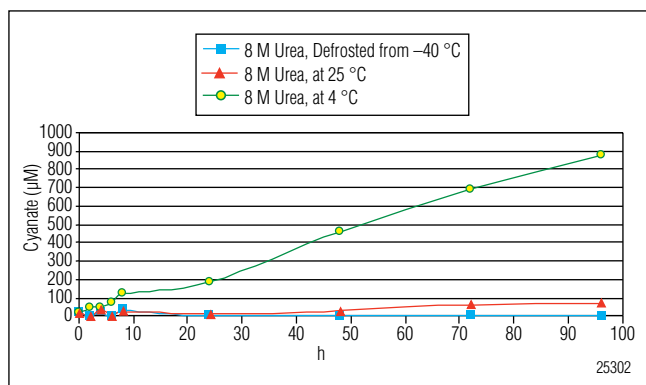


Figure 5. Effect of temperature on cyanate from urea solutions.

The experiments showed the total cyanate concentrations were stable in 8 M urea when stored at -40 °C. However, cyanate concentration increased more than 10-fold over four days when stored at 4 °C (from 6 to 75 μ M) and increased significantly when stored at 25 °C (from 24 to 886 μ M) (Figure 5). Cyanate had similar stability in 8 M urea with 1 M chloride and 8 M urea with 1 M chloride and 50 mM phosphate buffer as in the 8 M urea solutions. Cyanate accumulation in urea was not inhibited by the phosphate buffer. In the previous study, the authors reported that scavengers—in addition to phosphate and other buffers—were needed to effectively suppress the accumulation of cyanate in urea.⁹ These results agree with the previous study.

CONCLUSION

Urea degrades to cyanate, an unwanted contaminant in urea-containing buffers used for protein purification. Using a high-capacity anion-exchange column with suppressed conductivity detection, the authors accurately determined low (μ M) 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 fast, accurate determination of cyanate in urea-containing solutions. A Reagent-Free IC system ensures the highest precision, eliminates the need to prepare eluents, and eliminates possible eluent preparation errors.

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