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# Ion chromatographic quantification of cyanate in urea solutions: estimation of the efficiency of cyanate scavengers for use in recombinant protein manufacturing

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**Abstract**

The chaotrope urea is commonly used during recombinant protein manufacturing as a denaturant/solublizing agent. The adventitious accumulation of cyanate in urea solutions during product manufacturing can cause unwanted carbamylation of proteins, leading to alterations in drug product structure, stability and function. We have developed an ion chromatographic method to quantify cyanate production in urea solutions, suitable for analysis of samples from manufacturing process buffers. We discuss assay development, system suitability criteria and limitations on assay applicability. The assay has a linear range from 2 to 250 μM, with LOQ/LOD values of 6 and 2 μM, respectively. Assay accuracy through spike/recovery testing were established and both precision and intermediate precision were estimated. We assessed the utility of the assay by testing a variety of biological buffers and potential cyanate scavengers, which could be used during protein purification processes, for their ability to control the level of cyanate in 8 M urea solutions buffered over the range of pH 5–10. Our results demonstrate pH dependence for prevention of cyanate accumulation by these buffers/scavengers and indicate useful buffers, pH ranges, and additives for controlling cyanate accumulation during recombinant protein manufacturing. The pertinence of these approaches in preventing protein carbamylation during manufacturing are discussed.
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*Keywords:* Carbamylation; Cyanate; Urea

## 1. Introduction

Recombinant protein manufacturing processes often require buffer additives to promote protein solubilization or/and denaturation. The two most commonly utilized substances for these purposes are guanidine and urea [1]. The association of these chaotropic agents with proteins leads to changes in protein non-covalent interaction resulting in loss of protein secondary/tertiary structure; at high chaotrope concentrations (6–8 M) denatured protein solubility is enhanced [2,3]. Urea in aqueous solutions decomposes, resulting in a buildup of ammonium and cyanate ions [4,5]. Cyanate levels in solutions of urea are the product of an equilibrium accumulation from the reversible reaction of ammonium and carbonate ions yielding cyanate [6]. The effects of solution temperature and pH on cyanate generation in solutions of urea have been studied at both low and high urea concentrations [7], both high temperature and basic pH increase the equilibrium levels of cyanate [6]. Many functional groups in proteins are readily modified with residual cyanate derived from urea [8,9]. Cyanate is reactive towards amino, carboxyl, sulfhydryl, imidazole, phenolic hydroxyl, and phosphate groups in proteins yielding carbamyl derivatives [10–13]. Cyanate induced carbamylation occurs with a rate maximum near neutral pH, and the rate is unaffected by small changes in pH. Modification of amino groups of proteins by cyanate is the most stable and problematic reaction in proteins. Carbamylation of reactive groups other than amino groups may be reversed under mild conditions, assuming the protein and process are stable to the required changes in pH needed to reverse the reaction [8]. Since the reactivity of amino groups in proteins to cyanate is linearly related to their p$K_a$, the α-amino groups (p$K_a$ about 8) react

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approximately 100 times faster than the ε-amino groups of lysine (p$K_a$ about 10.7) [10].

Carbamylation of various amino acid sidechains in solutions of urea has been established for many proteins, including hemoglobin, lens crystalline, chymotrypsin, and brain proteins [14–17]. Carbamyl modification of amine sidechains results in changes in protein charge state which can lead to conformational alterations, variations in analytical profiles and modification of bioactivity [18–20].

It is obviously preferable to prevent, as much as is possible, any protein sidechain carbamylation during recombinant protein production. This implies the need for process controls to insure high efficiency removal or prevention of accumulation of cyanate in urea-containing buffers. For critical recombinant protein production work, urea can be deionized with a mixed bed ion-exchange resin. In spite of its efficiency in the removal of cyanate, de-ionization alone is inadequate for use in processes requiring significant hold times in high urea, as the equilibrium concentration of cyanate is eventually re-established. De-ionization is also time-consuming, laborious, and expensive at manufacturing scale. Control of cyanate formation through proper buffer and pH selection, and the addition of scavengers, i.e. Tris or glycine, has been suggested [10].

Several analytical methods have been developed for the quantitative and qualitative determination of cyanate. Simple spectrophotometric assays utilize colorimetric cyanate reaction with copper-pyridine [5,21] or 2-aminobenzoic acid [22]. HPLC methods include anion exchange in phosphate buffer pH 7.0 of cyanate captured from exhaust gases with UV detection at 190 nm and reverse phase analysis of radiolabelled cyanate in octylamine-based ion pairing buffer [23,24]. HPLC methods involving cyanate modification to enhance detectability include use of thionitrobenzoic acid derivatization of cyanate with UV detection [25]. Several HPLC methods exist for analyzing thiocyanates including fluorescent derivatization or complexation analysis of cyanate-like compounds in body fluids clarified by precipitation and ion chromatography (IC) with amperometric detection [26–29]. A method combining capillary electrophoresis with indirect fluorescence detection for cyanate estimation in wastewater has also been reported, using fluorescein as the fluorophore [30]. A similar fluorescent HPLC assay for cyanide/cyanate has been developed using the cerium (III)/cerium (IV) redox pair as fluorophore [31].

A method with high specificity for cyanate ion measurement is necessary for accurate analysis in heterogeneous sample backgrounds, such as recombinant protein manufacturing process intermediates varying in buffer components, pH, salt and urea concentration. We also required a simple, rapid, high-throughput assay preferably with no sample extraction or derivatization. IC with conductivity detection for analysis of cyanate has been reported in saline gold processing samples and in environmental water samples [32,33]. As an alternative approach to other HPLC methods, IC with conductivity detection offers the possibility of short analysis times and good resolution of cyanate ions from process-related contaminants with micromolar detection limits. We discuss the development of an IC assay for cyanate suitable for measurement of low levels of cyanate ion in complex urea-containing manufacturing process backgrounds. The method was qualified for use as an in process assay appropriate for process validation use in assessing manufacturing intermediate hold times. The method demonstrated a linear range from 2 to 250 μM cyanate, with LOQ/LOD values = 6/2 μM. Accuracy and precision were estimated from system suitability test data at the 10 μM test level through spike/recovery tests in process buffers.

To demonstrate the utility of the ion chromatographic method for cyanate quantification, we used the assay to examine the ability of a series of buffers and cyanate scavengers to reduce cyanate buildup in recombinant protein intermediates containing high levels of non-deionized urea (8 M). We tested a group of commonly used biological buffers, including phosphate, citrate, borate, HEPES, MOPS, and MES at appropriate pH values for their ability to control cyanate accumulation. Cyanate scavengers assessed included ethylenediamine, glycinamide, glycylglycine, trans-4-hydroxyl-proline, taurine, hydralazine, and diethanolamine, and a set of 21 amino acids, at concentrations ranging from 5 to 25 mM. Control of free cyanate ion in process buffers stored at ambient temperature was assessed over a 2 week period. Our results indicate appropriate buffers/scavengers and pH values useful in urea solutions for control of cyanate accumulation during recombinant protein manufacturing.

## 2. Experimental

### 2.1. Materials

Biological buffers: pyridine, citric acid, acetic acid, imidazole, ethylenediamine, MES, Bis–Tris, MOPS, HEPES, Tricine, Tris, glycylglycine, glycinamide, Bicine, 2-amino-2-methyl-propanediol (Ammediol), taurine, ethanolamine, diethanolamine, triethanolamine, ACES, and CHES were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) and Sigma Chemical Co. (St. Louis, MO).

Amino acids: alanine (Ala), glycine (Gly), valine, leucine, isoleucine, proline, threonine (Thr), serine, tyrosine, cysteine (Cys), methionine (Met), phenylalanine, tryptophan, aspartic acid, asparagine, glutamic acid, glutamine, arginine (Arg), histidine (His), lysine (Lys), and trans-4-hydroxyproline (OH-Pro) were obtained from Sigma Chemical Co. (St. Louis, MO).

Other reagents: β-phenylethylamine, methylamine, hydralazine, histamine, His–Gly, and triglycine, were obtained from Sigma Chemical Co. (St. Louis, MO), sodium cyanate and semicarbazide hydrochloride from Aldrich (Milwaukee, WI) and glycinamide and aminomethansulfonic acid from Fluka (Milwaukee, WI). Urea, Tris, sodium chloride,

glycylglycine, sodium carbonate, sodium bicarbonate, histidine, and hydrochloric acid were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Pyridine and Bis–Tris were obtained from Spectrum (Gardena, CA) and AG 501-X8 resin from BioRad (Hercules, CA).

### 2.2. Instrumentation

IC methods were run on a Dionex BioLC system consisting of a GS50 gradient pump, ED50 electrochemical detector, an AS50 chromatography compartment, and autosampler using nitrogen degassing with a variable injection capacity from 1 to 100 μl. An ASRS®-Ultra (4 mm) suppressor was used. Chromatograms were recorded using PeakNet chromatography workstation software version 6.40.

### 2.3. Abbreviations

IC, ion chromatography; S.D., standard deviation; CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantitation; MES, morpholino ethanesulfonic acid; HEPES, hydroxyethyl piperazine ethanesulfonic acid; Tris, Tris hydroxymethylaminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ACES, *N*-2-Acetamido-2-hydroxyethanesulfonic acid; CHES, cyclohexylaminoethanesulfonic acid; Ammediol, 2-amino-2-methyl-1,3-propanediol.

### 2.4. IC method

The IC method is based upon isocratic anion exchange chromatographic resolution of cyanate ions from buffer/process components in a carbonate/bicarbonate buffer using a Dionex AS14 (4 mm × 250 mm) column with an AG14 (4 mm × 50 mm) guard column and conductivity detection at 35 °C. An ASRS®-Ultra (4 mm) suppressor was used. The AEX column was maintained at ambient laboratory temperature. Autosampler storage temperature was set at 8 °C. The chromatographic eluant buffer was prepared by dissolving 37.1 g $Na_2CO_3$ (350 mM $Na_2CO_3$) and 8.4 g of $NaHCO_3$ (100 mM $NaHCO_3$) in 1 l Milli-Q water to obtain a concentrated stock solution. The concentrate was filtered through 0.22 μm filtration unit and stored at ambient temperature for up to 3 months before use. A 100-fold dilution of the carbonate stock solution was performed on the day of analysis with Milli-Q water. The chromatographic separation was isocratic at 1.2 ml/min flow rate with a 15 min run time. Signals were detected by suppressed conductivity using the system auto-suppression recycle mode at a 24 mA power setting. Peak areas were calculated and reported as μS × min. A 100 mM sodium cyanate stock solution for use in system calibration was prepared in Milli-Q water. Serial dilutions of the cyanate stock solution from 250 to 2 μM were made using Milli-Q water for linearity determinations. A bracketing system suitability standard at the 10 μM cyanate concentration was utilized for each analytical sequence. No internal control for retention was used; retention time values in minutes are reported from time of injection. Sample injection volumes were 100 μM. Samples at pH values <7.4 were neutralized by addition of sodium hydroxide prior to analysis.

### 2.5. Deionized urea buffers

A HR 10 × 10 column (Amersham Biosciences) was packed with 7.6 ml AG 501-X8 mixed bed ion exchange resin from BioRad. The freshly packed column was washed and equilibrated prior to use with Milli-Q water. A 70 ml volume of each process buffer containing 8 M urea held at ambient temperature for either 4 or 50 days was passed over the mixed bed ion exchanger at a flow rate of 1.0 ml/min using an ÄKTA explorer 100 FPLC system (Amersham Biosciences). The de-ionized buffers were collected after the buffers had passed through the column and stored at ambient temperature for a maximum of 3 days before use.

## 3. Results and discussion

### 3.1. IC method development/optimization

Initial IC method parameters investigated were based upon published analyses of cyanate in gold-processing wastewater using a carbonate/bicarbonate buffer system [32]. Initial ion chromatograms indicated that resolution of chloride from the cyanate peak would be critical for accurate analysis of process buffers containing varying levels of NaCl in addition to low levels of cyanate. When eluting with a buffer mixture of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate, a 0.60 min separation of $Cl^-$ and cyanate ions was observed using a Dionex AS4A anion exchange column (data not shown). We studied a range of concentrations for carbonate/bicarbonate from 18/1.7 to 1.8/17 mM to improve the separation of $Cl^-$ and cyanate ions in the isocratic method. Increasing the carbonate concentration 10-fold from 1.8 to 18 with bicarbonate remaining at 1.7 mM yielded a maximum 0.64 min difference in retention time for $Cl^-$ and cyanate. Increasing the bicarbonate concentration 10-fold from 1.7 to 17 with carbonate remaining at 1.8 mM yielded a 0.53 min difference in retention time (data not shown). Since varying the ratio and concentration of the carbonate/bicarbonate buffer did not improve resolution of $Cl^-$ and cyanate, we selected a Dionex AS14 anion exchange column with smaller particle diameter, higher capacity, and different quaternary amine for our studies. The application of AS14 column in the method yielded a superior separation, with a 1.3 min retention time difference between $Cl^-$ and cyanate at low carbonate/bicarbonate concentrations of 3.5 mM/1.0 mM, respectively (Fig. 1), which could not be achieved with any carbonate/bicarbonate ratio tested using the AS4A.

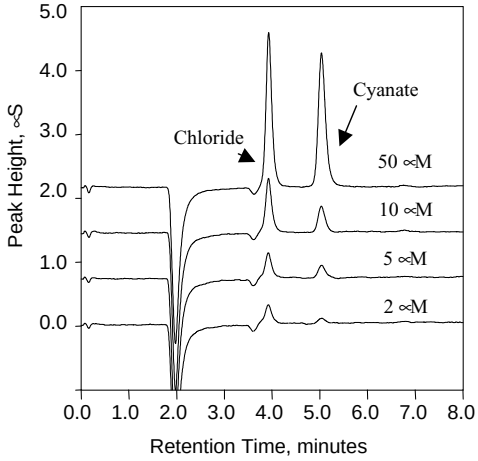


Fig. 1. Ion chromatograms, overlay of equimolar mixtures of chloride and cyanate at 2, 5, 10 and 50 μM, 100 μl injections.

Initial method testing of cyanate spike recoveries in a series of manufacturing process buffers varying in pH from 3.8 to 8.0 and NaCl from 0.0 to 1 M indicated poor recoveries correlated with low sample pH (data not shown). For efficient cyanate retention on the anion exchanger, we determined that the pH of test samples must be adjusted to 7.5 or above by addition of sodium hydroxide before testing in order to obtain spike recoveries >90%. Injection of samples containing high concentrations of urea had a minor effect on the shape of the ion chromatographic baseline, but no effect on the peak shape or retention time of the cyanate ion (Fig. 2). Integration parameters for the ion chromatograms were adjusted to yield reproducible peak areas in chromatograms derived from high urea samples. In addition, spike/recovery tests for cyanate quantitation in process buffers containing varying levels of NaCl demonstrated that as expected, dilutions are necessary in buffers containing high salt concentration to achieve reproducible cyanate ion recoveries by eliminating chromatographic interference from high chloride ion levels (Fig. 3). At least a 100 fold dilution of 8 M urea, 50 mM Tris, 1 M NaCl was required for adequate resolution of $Cl^-$ and cyanate ions and cyanate recoveries over 80% at the 10 μM cyanate level. As an alternative to sample dilution to eliminate $Cl^-$ interference, the direct removal of $Cl^-$ from samples with disposable cartridges containing a cation-exchange resin in the $Ag^+$ form [35] was tried. However, the cartridge effluent of a 10 μM cyanate sample was found only to be 3 μM. The poor cyanate recovery indicated that cyanate was captured by the Ag cartridge. No sample dilutions were found necessary for samples containing 100 mM or less of buffer salts (Tris) and 10 mM or less of NaCl.

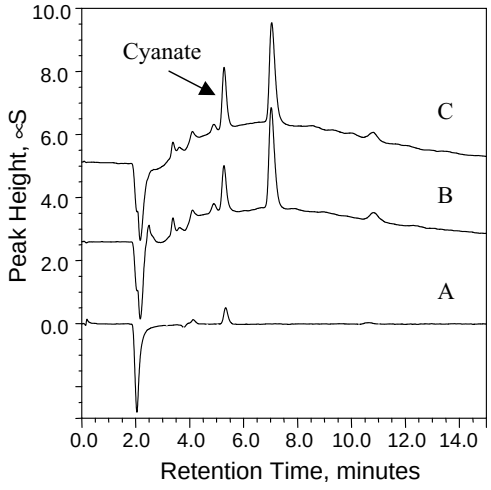


Fig. 2. Ion chromatograms, A: 0 M urea, cyanate standard, B: 8 M urea solution, non-deionized, C: 8 M urea solution, non-deionized with 10 μM cyanate spike.

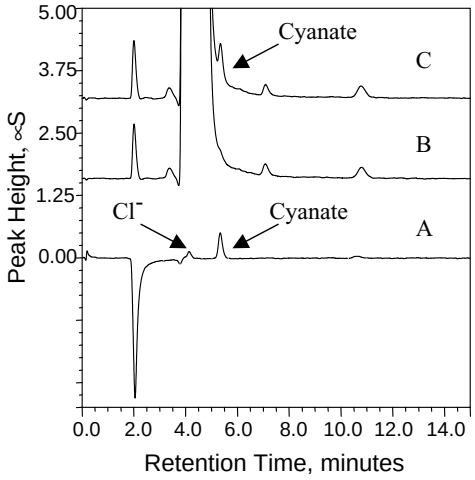


Fig. 3. Ion chromatograms, A: 0 M urea, cyanate standard, B: 8 M Urea, 1 M NaCl, diluted 1:100 with water prior to injection, C: 8 M Urea, 1 M NaCl, diluted 1:100 with water prior to injection, spiked with 10 μM cyanate.

### 3.2. Assay qualification linearity studies

The assay was qualified for use in validating cyanate limits for manufacturing process intermediate hold times according to ICH guidelines [34]. The linear range of the assay was determined by analysis of cyanate prepared in Milli-Q water at concentrations of 0, 2, 5, 10, 50, and 250 μM (0–25 nmoles). All cyanate concentrations were analyzed in duplicate. The method showed very good linearity at low concentrations of cyanate with correlation coefficient values ≥0.999 (Fig. 1). The mean slope ($S$) of the linear cyanate calibration was 0.0105 with a standard deviation of 0.0009 and the mean $y$-intercept was −0.016 with a standard deviation of 0.0061 ($n = 29$).

### 3.3. Assay limits of quantitation and detection

Mean LOQ and LOD values, determined from the residual standard deviation ($s$) in the $y$-intercepts from the

Table 1
Chromatographic system suitability study

| Chromatographic parameter | Mean value ($n = 36$) | S.D. | %CV |
|---|---|---|---|
| Retention time | 5.33 min | 0.07 | 1.3 |
| Peak area | 0.103 μS min | 0.005 | 4.9 |
| Peak width | 0.311 | 0.009 | 2.9 |
| Peak asymmetry | 1.298 | 0.059 | 4.5 |
| Column efficiency | 4760 | 186 | 3.9 |

Peak width determined by the half-width method. Column plate number estimated by the tangent method. Peak asymmetry determined by US/EU standard method.

linearity data and slopes ($m$) from 29 linearity calibration analyses, using equations 1 and 2 were ≤6 and 2 μM (0.2 and 0.6 nmoles), respectively. These limits of quantitation and detection are based upon use of a 0.1 ml sample injection.

$$\mathrm{LOQ} = \frac{10s}{m} \quad (1)$$

$$\mathrm{LOD} = \frac{3.3s}{m} \quad (2)$$

Freshly prepared low salt process buffers containing non-deionized 8 M urea were found to routinely contain levels of cyanate of approximately 75 μM, although some batches of urea were found to yield concentrations of cyanate as high as 250 μM (data not shown). These initial cyanate levels are within the linear range of the assay. Buffers containing the highest levels of NaCl tested (1 M), necessitating 100-fold sample dilution in the IC assay, would require cyanate levels of at least 600 μM for accurate quantitation with an IC assay LOQ at 6 μM cyanate after dilution.

### 3.4. Assay qualification system suitability criteria

A bracketing 10 μM cyanate system suitability reference standard was analyzed with each sequence of samples tested during assay qualification. The mean data from the qualification system suitability tests was used to set system suitability acceptance criteria for subsequent assay testing ($n = 36$). Table 1 summarizes the statistical analysis of the system suitability data for peak retention, column efficiency, peak asymmetry, peak area, and peak width. Based upon this data, system suitability acceptance criteria were initially set at three standard deviations from the mean values listed in Table 1. Since assay robustness testing with multiple IC systems and analysts has not yet occurred, it is likely that assay acceptance criteria will need to be refined when additional test results are available, particularly for performance parameters, such as peak area, which can be affected by system variations, such as detector lamp life or analyst sample preparation variation.

### 3.5. Assay qualification, accuracy by spike recovery

Six purification process buffers from a recombinant protein manufacturing process were chosen to study the accuracy and precision of the cyanate test method using spike/recovery analysis. Low level spike/recovery samples were prepared by adding concentrated cyanate to process intermediate buffers to yield a final 10 μM spike concentration. An 8 M aqueous urea solution was used as a control to gauge the background generation of cyanate over the buffer hold times of 1 and 2 days at 2–8 °C for samples stored in the HPLC system autosampler during the analytical sequence. Three process buffers at pH 8 consisted of 8 M urea and 50 mM Tris, with 0 mM, 140 mM, or 1 M sodium chloride. Two other process buffers contained 8 M Urea and 100 mM acetic acid with pH adjusted to either 3.8 or 5.5, the latter containing 25 mM sodium chloride (Table 2). These buffers, from an actual manufacturing process, were chosen as they represent a typical range of buffer pH values and salt concentrations encountered in recombinant protein production.

Cyanate was spiked into each of the buffers at the 10 μM level and analyzed by IC in twelve replicates (Table 2). The data in Table 2 represents the cyanate level in spiked samples after subtraction of the residual cyanate level quantitated in the respective unspiked process buffer. Background cyanate measured in the freshly prepared 8 M urea solutions was approximately 75 μM in the six buffers tested, so actual cyanate test levels in the spiked samples were in the range of 85 μM (8.5 nmole). Spike recoveries in five low salt buffers where no sample dilution was necessary exceeded 90%, with an overall mean recovery at the 10 μM level of 96% with a S.D. of 13.2 and a %CV of 13.8 ($n = 72$). The buffer containing 1 M NaCl with a mean 80% recovery required 100-fold dilution to accurately quantify cyanate in the presence of high levels of the interfering $Cl^-$.

Table 2
Percent cyanate spike recovery in 8 M urea process buffers

| Process buffer | Mean % recovery | S.D. | %CV |
|---|---|---|---|
| A, $n = 12$ | 113.5 | 15.0 | 13.2 |
| B, $n = 12$ | 94.3 | 4.8 | 5.1 |
| C, $n = 12$ | 99.8 | 7.0 | 7.0 |
| D, $n = 12$ | 97.1 | 9.5 | 9.8 |
| E, $n = 12$ | 91.7 | 5.5 | 6.0 |
| F, $n = 12$ | 79.6 | 6.2 | 7.8 |
| Mean, $n = 72$[a] | 96.0 | 13.2 | 13.8 |

All test at a 10 μM cyanate spike concentration; Buffer A, 8 M urea, pH 7.6; Buffer B, 8 M urea, 100 mM acetate, pH 3.8; Buffer C, 8 M urea, 100 mM acetate, 25 mM NaCl, pH 5.5; Buffer D, 8 M urea, 50 mM Tris, pH 8.0; Buffer E, 8 M urea, 50 mM Tris, 140 mM NaCl, pH 8.0; Buffer F, 8 M urea, 50 mM Tris, 1 M NaCl, pH 8.0.

[a] Overall spike recovery, mean for analyses A–F.

Table 3
Cyanate retention time precision, column #1, day #1

| Process buffer | Mean retention time | S.D. | %CV |
|---|---|---|---|
| A, $n = 6$ | 5.37 | 0.23 | 4.3 |
| B, $n = 6$ | 5.31 | 0.01 | 0.2 |
| C, $n = 6$ | 5.31 | 0.01 | 0.2 |
| D, $n = 6$ | 5.39 | 0.01 | 0.2 |
| E, $n = 6$ | 5.38 | 0.01 | 0.2 |
| F, $n = 6$ | 5.36 | 0.01 | 0.2 |
| Mean, $n = 36$[a] | 5.35 | 0.09 | 1.7 |

All test at a 10 μM cyanate spike concentration; Buffer A, 8 M urea, pH 7.6; Buffer B, 8 M urea, 100 mM acetate, pH 3.8; Buffer C, 8 M urea, 100 mM acetate, 25 mM NaCl, pH 5.5; Buffer D, 8 M urea, 50 mM Tris, pH 8.0; Buffer E, 8 M urea, 50 mM Tris, 140 mM NaCl, pH 8.0; Buffer F, 8 M urea, 50 mM Tris, 1 M NaCl, pH 8.0.

[a] Overall retention time mean for analyses A–F.

### 3.6. Assay qualification, retention time precision/intermediate precision

Table 3 summarizes retention time precision data from six replicate analyses performed by a single analyst on a single IC system in 1 day for the six process buffers. An overall mean retention time of 5.35 min with a %CV of 1.7% was obtained from all six process buffers. Intermediate precision for cyanate retention time was estimated by averaging a total of 72 injections of 10 μM cyanate (Table 4) for these studies, performed by one analyst, on two different column lots and one IC system on two separate days. Data for peak retention time for each process buffer was pooled for analysis (Table 4). An overall mean retention time of 5.41 min with a % CV of 1.7% was determined for all 72 injections, indicating good intermediate precision for retention time. Since only a single Dionex IC system was available for use in the qualification studies detailed here, and all testing was performed by a single analyst, the method we report here cannot be considered to have been examined thoroughly to date for assay robustness according to ICH guidelines. We expect that data will become available on assay robustness after further implementation of the cyanate IC assay in our laboratories.

Table 4
Cyanate retention time intermediate precision

| Process buffer | Mean retention time $n = 12$ | S.D. | %CV |
|---|---|---|---|
| A, $n = 12$ | 5.38 | 0.15 | 2.8 |
| B, $n = 12$ | 5.37 | 0.07 | 1.3 |
| C, $n = 12$ | 5.38 | 0.07 | 1.3 |
| D, $n = 12$ | 5.45 | 0.06 | 1.1 |
| E, $n = 12$ | 5.44 | 0.07 | 1.3 |
| F, $n = 12$ | 5.41 | 0.06 | 1.1 |
| Mean, $n = 72$ | 5.41 | 0.09 | 1.7 |

Buffer A, 8 M urea, pH 7.6; Buffer B, 8 M urea, 100 mM acetate, pH 3.8; Buffer C, 8 M urea, 100 mM acetate, 25 mM NaCl, pH 5.5; Buffer D, 8 M urea, 50 mM Tris, pH 8.0; Buffer E, 8 M urea, 50 mM Tris, 140 mM NaCl, pH 8.0; Buffer F, 8 M urea, 50 mM Tris, 1 M NaCl, pH 8.0.

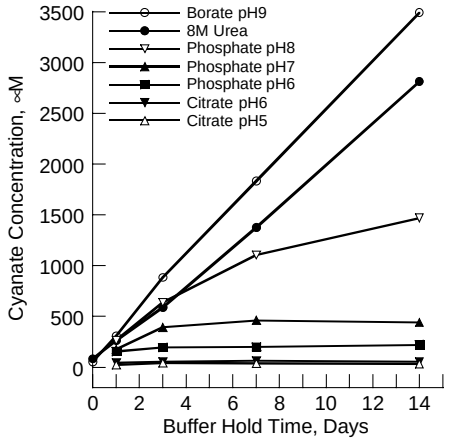


Fig. 4. Cyanate levels in unbuffered, non-deionized 8 M urea vs. non-deionized 8 M urea buffered with 0.1 M of sodium borate pH 9, sodium phosphate pH 8, 7 and 6, or sodium citrate pH 6 or 5. Solutions were held at ambient temperature during the 14 day study.

### 3.7. Accumulation of cyanate in buffered 8 M urea

The equilibrium for cyanate formation from urea decomposition is pH dependent; cyanate formation is inhibited at low pH values and can rise to an equilibrium value of approximately 10–20 mM in 8 M urea solutions at neutral or basic pH values [5]. We determined the pH dependence of cyanate levels in 8 M urea solutions buffered with 0.1 M citrate, phosphate, or borate across the pH range 5–9 (Fig. 4). Cyanate levels in borate buffer at pH 8 rose over a 2 week time course to 3.5 mM, higher than in 8 M unbuffered urea. The increase in cyanate in the absence of buffer is linear with a rate of accumulation of approximately 180 μM per day at ambient temperature. We found a similar rate of accumulation of cyanate in aqueous unbuffered urea solutions with or without initial buffer deionization over a simulated three day buffer shelf life (data not shown). Cyanate approached an equilibrium level of approximately 1.5 mM in phosphate buffer at pH 8, but was maintained below 0.5 mM at both pH 6 and 7. Citrate buffer at pH 6 maintained cyanate at levels well below those measured for phosphate buffer at pH 6. The efficiency of citrate buffer in scavenging cyanate as compared to phosphate buffer, probably through reaction to form carbamylcarboxylates, does not imply utility in preventing unwanted recombinant protein modification during manufacturing, since carbamylcarboxylates are active acylating agents [10].

We extended our studies on the ability of buffers to control cyanate accumulation in 8 M urea in four pH ranges: 6–6.5, 7, 8 and 9–10, using buffers with appropriate $pK_a$ at these pH values. Buffers were prepared at 100 mM concentration in 8 M urea with pH adjusted to their $pK_a \pm 1.0$; cyanate levels were monitored over a course of 14 days. 8 M urea prepared with Milli-Q water with no added organic scavenger was utilized as a baseline control for cyanate generation during the study. At pH 6, we found citrate buffer most effective in

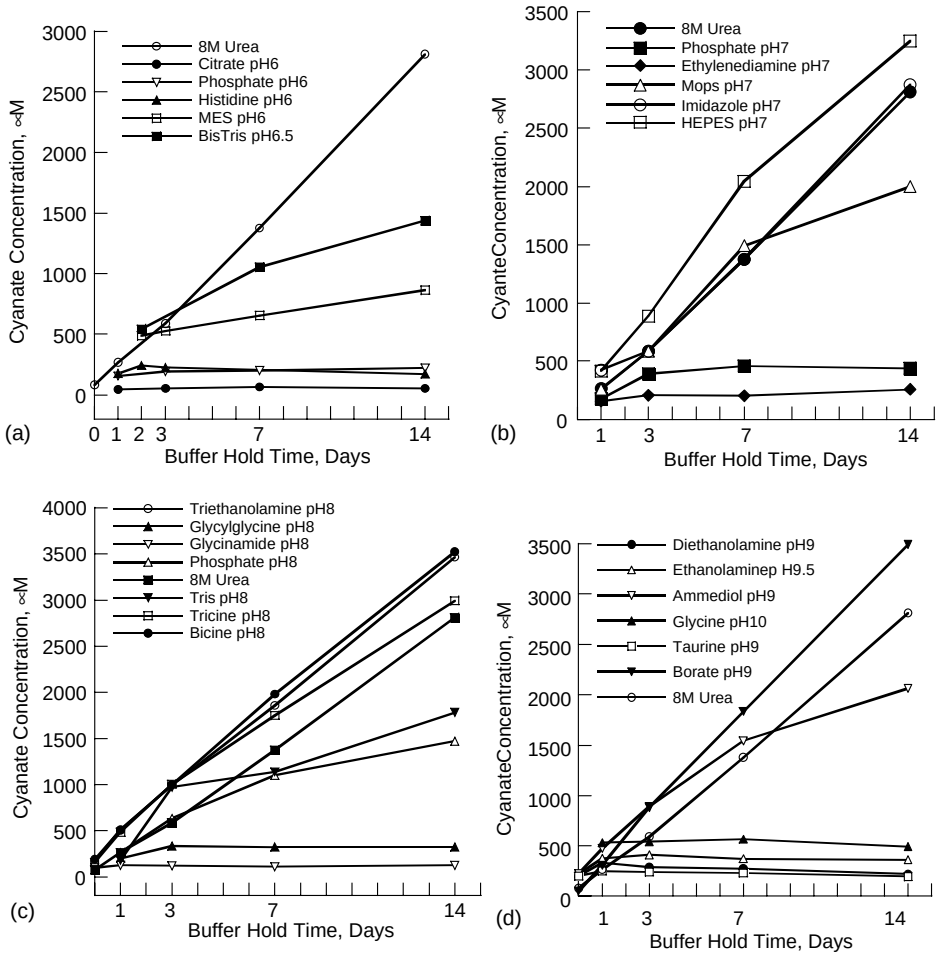


Fig. 5. (a) Cyanate levels in unbuffered, non-deionized 8 M urea vs. non-deionized 8 M urea buffered with 0.1 M of sodium citrate pH 6, sodium phosphate pH 6, histidine pH 6, MES pH 6 or Bis–Tris pH 6.5. Solutions were held at ambient temperature during the 14 day study. (b) Cyanate levels in unbuffered, non-deionized 8 M urea vs. non-deionized 8 M urea buffered with 0.1 M of sodium phosphate, ethylenediamine, MOPS, imidazole, and HEPES, all at pH 7.0. Solutions were held at ambient temperature during the 14 day study. (c) Cyanate levels in unbuffered, non-deionized 8 M urea vs. non-deionized 8 M urea buffered with 0.1 M of sodium phosphate, triethanolamine, glycylglycine, glycinamide, Tris, Tricine and Bicine, all at pH 8.0. Solutions were held at ambient temperature during the 14 day study. (d) Cyanate levels in unbuffered, non-deionized 8 M urea vs. non-deionized 8 M urea buffered with 0.1 M of sodium borate, diethanolamine, ammediol, and taurine at pH 9.0, ethanolamine at pH 9.5 and glycine at pH 10.0. Solutions were held at ambient temperature during the 14 day study.

controlling cyanate accumulation, with histidine and phosphate yielding similar results, maintaining cyanate below approximately 0.2 mM (Fig. 5a). Neither MES nor Bis–Tris at pH 6 could maintain cyanate levels below 0.5 mM over the 14 day time course. At pH 7.0, ethylenediamine proved highly effective in controlling cyanate (Fig. 5b). Phosphate buffer, as demonstrated previously, maintained cyanate below 0.5 mM over 14 days, but imidazole, MOPS and HEPES were essentially ineffective. None of the ineffective buffers at pH 7 contain a primary amine capable of reacting with free cyanate. At pH 8 where the equilibrium production of cyanate from urea will be favored, only glycylglycine and the closely related glycinamide demonstrated good control over equilibrium levels of cyanate (Fig. 5c). The tertiary amines triethanolamine and Bicine were ineffective cyanate regulators, as was the secondary amine Tricine at pH 8. The primary amine Tris allowed cyanate levels to rise to equilibrium values approaching 1.5 mM at 14 days, as did phosphate buffer at pH 8. At pH 9–10, diethanolamine and taurine were the most effective in maintaining low equilibrium cyanate levels, with both ethanolamine and glycine yielding equilibrium levels of cyanate below 0.5 mM (Fig. 5d). Ammediol and borate were ineffective at pH 9.

### 3.8. Addition of cyanate scavengers to buffered 8 M urea

To assess the utility of chemical scavengers for use in process chromatographic buffers, which do not yield adequate inherent control of cyanate in 8 M urea, we tested a series of organic compounds including various small amines and amino acids. We analyzed the effects of cyanate scavengers in both 50 mM HEPES buffer pH 7.0 and 50 mM Tris buffer pH 8.0 containing 8 M urea using the IC assay. The cyanate scavengers were tested at 5 or 25 mM, and

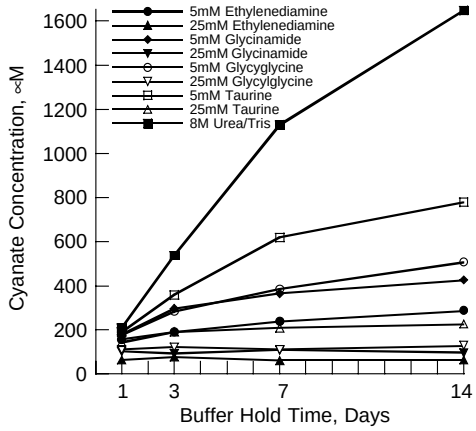


Fig. 6. Cyanate levels in 8 M urea, 50 mM Tris buffer, pH 8.0 with 5 or 25 mM of ethylenediamine, glycinamide, glycylglycine or taurine over a 2 week time course. Buffers were held at ambient temperature for the 14 day study.

buffer pH was adjusted to 7.0–8.0 after scavenger addition. The buffers were held at ambient temperature for 2 weeks and analyzed for cyanate at 1, 3, 7 and 14 days.

A comparison of residual cyanate levels over the 2 week time course at pH 8.0 in Tris buffer for ethylenediamine, glycinamide, glycylglycine and taurine is shown in Fig. 6. The only compound capable of maintaining cyanate at 0.2 mM at 5 mM concentration was ethylenediamine. Taurine, the poorest cyanate scavenger in this set, was similarly effective at the higher 25 mM concentration to ethylenediamine at 5 mM. Although less effective at the lower 5 mM concentration, both glycylglycine and glycinamide proved nearly as effective as ethylenediamine at 25 mM, maintaining cyanate levels at 14 days close to 0.1 mM. Ethylenediamine at 25 mM lowered residual cyanate levels to below 0.1 mM.

Testing the same set of compounds in HEPES buffered 8 M urea at pH 7, yielded similar results to those obtained at pH 8 (data not shown). Ethylenediamine in this case maintained cyanate below 0.1 mM at the 25 mM test level, and at approximately 0.2 mM at the 5 mM level. Glycylglycine and glycinamide were similarly effective at the higher 25 mM concentration, but at lower concentration failed to keep cyanate levels below approximately 0.2 mM. Taurine was again the least effective at either concentration. Tests at pH 8.0 for tri-glycine, His–Gly, and hydralazine at 25 mM yielded residual cyanate levels of 90, 172 and 195 μM, respectively, at 14 days (data not shown).

We also screened 21 amino acids including trans-4-hydroxyl-proline as potential cyanate scavengers prepared in 8 M Urea, 50 mM Tris pH 8.0 at 5 or 25 mM using the IC assay. Addition of amino acids as cyanate scavengers at these levels proved to have only a modest effect at pH 8 on lowering cyanate accumulation, with cyanate levels at 7 days approximately 70% of the unbuffered 8 M urea control. Of the amino acids, trans-4-hydroxyl-proline yielded the highest level of cyanate reduction at approximately 10% of the unbuffered control value, with cysteine and the basic amino acids the next most effective cyanate scavengers (Table 5). Additionally, a variety of other small organic compounds at the 25 mM concentration tested for their ability to control cyanate levels in Tris buffered 8 M urea at pH 8 proved relatively ineffective. These included histamine, diethanolamine, phenylethylamine, ethanolamine, ACES, semicarbazide, aniline, CHES, benzamide, guanidoacetic acid, 2,4,6-tri-amino-pyridimine, acetanilide and pyroglutamic acid (Table 5).

Table 5
Efficiency of 25 mM cyanate scavengers in 50 mM Tris 8 M urea, pH 8.0

| Scavenger | Residual cyanate (%) | Scavenger | Residual cyanate (%) |
|---|---|---|---|
| (a) Day 14–19 | | | |
| None | 100 | ACES | 32 |
| 1,2-Ethylenediamine | 4 | Semicarbazide | 36 |
| Gly–Gly–Gly | 5 | CHES | 42 |
| Glycinamide | 5 | Aniline | 44 |
| Gly–Gly | 9 | Aminomethane-sulfonic acid | 71 |
| His–Gly | 10 | Benzamide | 91 |
| Hydarlazine | 11 | Guanidio-acetic acid | 92 |
| Taurine | 15 | Triaminio-pyrimidine | 95 |
| L-Histidine | 20 | Acetanilide | 96 |
| Histamine | 32 | Pyro-glutamic acid | 103 |
| Phenyl-ethylamine | 32 | | |
| (b) Day 28–35 | | | |
| None | 100 | L-Threonine | 21 |
| 1,2-Ethylenediamine | 4 | L-Arginine | 21 |
| Gly–Gly–Gly | 6 | L-Histidine | 22 |
| Glycinamide | 6 | Diethanolamine | 23 |
| Gly–Gly | 8 | L-Glycine | 24 |
| HO-proline | 10 | L-Methionine | 25 |
| Hydralazine | 14 | Histamine | 27 |
| Taurine | 13 | Ethanolamine | 28 |
| L-Cysteine | 20 | L-Alanine | 42 |
| L-Lysine | 20 | Methylamine | 53 |

## 4. Conclusions

The ion chromatographic assay for cyanate presented here was designed to yield rapid, sensitive analyses in the low micromolar concentration range in a variety of potential recombinant protein manufacturing buffers containing high concentrations of urea. Our assay validation studies indicate good retention time reproducibility in buffers with or without 8 M urea at pH values ranging from 3.8 to 8.0. The isocratic assay was validated using sample injection volumes of 0.1 ml or less. We have not investigated the effect of larger sample volume injection on assay sensitivity and peak parameters. The chromatographic resolution achieved between $Cl^-$ and cyanate ions in the assay is a significant limiting factor effecting assay sensitivity which we maximized by choice of column packing and buffer composition. At sample test chloride levels of 10 mM or more the signal from

cyanate in the micromolar concentration range becomes difficult to quantify accurately due to peak overlap. All samples for analysis must be diluted if necessary to lower the $Cl^-$ peak to preclude interference with the cyanate peak. Little change in cyanate peak width and tailing after several hundred column injection/cleaning cycles with samples containing various buffers, urea and organic additives was noted, suggesting the column/buffer system chosen for the analysis will exhibit robust performance. Complete robustness analysis involving pooled data analysis after assay transfer to a second test site, IC system and analyst has not yet occurred.

The IC studies we performed on the effect of pH, buffer components and organic additives on cyanate control not only demonstrated the utility of the IC assay for validating residual cyanate levels in process intermediate buffers, but also provide useful information for process design in systems utilizing urea as a chaotropic agent. The data are in general consistent with the body of earlier work by Stark examining the reactivity of cyanate with functional groups in proteins [10]. Our data confirm the expected pH dependence of the urea/cyanate equilibrium, with levels of cyanate reaching the low millimolar range after 1 week at high pH. The lowered levels of cyanate observed at lower pH values must be considered not only a reflection of the effect of pH on the equilibrium but also of the ability of buffer components to react with cyanate. Stark has reported that both phosphate and carboxyl groups are reactive with cyanate; we observe a significantly lower level of cyanate in citrate buffers than in phosphate buffers at pH 6.0 (Stark, 1964). The value of citrate buffer in protecting protein from sidechain modification by controlling cyanate is unfortunately counterbalanced by the acylating potential of cyanylcarboxylates.

Other than citrate buffers, phosphate or histidine buffers at relatively high concentrations (0.1 M) are useful for cyanate control at low pH. The utility of histidine in cyanate control is apparently not directly related to reaction with the imidazole sidechain, as at pH 7 imidazole itself is a poor cyanate controller. Imidazole, like sulfhydryl amino acid sidechains, reacts rapidly with cyanate at low pH, but rapidly reverses the reaction at high pH values [13]. Stark reported that cyanate reactivity for amino acids is linearly related to p$K_a$ and is relatively unaffected by pH change near neutral pH [10]. The unprotonated amine and cyanic acid are considered the reactive species in carbamylation. At pH 7 where phosphate again provides useful cyanate control, ethylenediamine used as a buffer at 0.1 M yielded the lowest residual cyanate levels. DiMarchi has discussed the utility of ethylenediamine in cyanate reactions due to the unsterically hindered nature of the reagent and the p$K_a$ values of its two amines at 7.5 and 10.7, similar to the α and ε-amino groups of proteins [36]. At pH 8, where phosphate losses much of its ability to control cyanate, the cyanate scavengers glycylglycine and glycinamide were shown to be most effective when used as buffering agents. The expense of substances like glycylglycine or histidine are a consideration in large scale manufacturing where use of lower cost buffers like phosphate or ethylenediamine may provide adequate or superior cyanate control. At high pH Tris was shown to have poor utility in cyanate control.

We also tested a wide variety of other reagents often used in process chemistry for their ability to control cyanate in buffered 8 M urea solutions at the higher pH values of 7 and 8, using two buffers with little cyanate scavenging ability, HEPES and Tris. The reagents tested were primarily small amines and amino acids (Table 5). In most cases these compounds were found to be ineffective in lowering cyanate levels below the 0.2 mM level. Only ethylenediamine, taurine, glycylglycine, glycinamide, Gly–Gly–Gly, His–Gly and hydralazine were useful in maintaining cyanate below 0.2 mM. Of these compounds, hydralazine, His–Gly and Gly–Gly–Gly would ordinarily be considered poor choices as process additives because of cost.

It is well established that the essentially irreversible reaction of cyanate with α or ε-amino groups of proteins can have deleterious effects on protein folding, solubility, activity and purification recovery [37–39]. Our data indicate that in aqueous buffered 8 M urea solutions cyanate concentrations can reach the 3–4 mM level over 14 days at ambient temperature. The equilibrium concentration of cyanate in high concentration urea solutions above pH 6.0 can reach 20 mM [9]. Cyanate levels in freshly prepared non-deionized 8 M urea in our laboratory have been measured as high as 0.5 mM. The concentration of α-amino groups in a recombinant protein solution at a manufacturing concentration of 1 mg/ml for proteins with molecular weights ranging from 10 to 100 kDa will be 0.1 to 0.01 mM. This concentration of reactive α-amino groups is within the range of cyanate values we obtained with the most efficient combination of buffers and added cyanate scavengers. At a reaction rate constant of $K_I = 2 \times 10^{-1}$ to $3 \times 10^{-1}\ M^{-1}\ min^{-1}$ at neutral pH, a molar equivalent or excess of cyanate versus α-amino groups would be expected to lead to significant levels of carbamylation [10]. Cole reported that insulin exposed to urea under conditions that can lead to formation of cyanate was carbamylated at approximately 14% on both α-amino groups [40]. Since the carbamylation reaction is linearly dependent upon amino p$K_a$, at neutral pH modification of the α-amino groups would be expected to predominate over reaction with ε-amino groups by as much as 100-fold, although ε-amino concentrations can be much higher than α-amino, depending upon protein amino acid composition [10]. At higher pH values closer to the ε-amino p$K_a$ of 10 the higher concentration of ε-amino groups in a typical protein would be expected to lead to carbamylation at both α and ε sites.

To prevent significant degradation of process yield, and simplify purification and product analysis, manufacturing process design must incorporate controls to minimize product modification in urea-containing buffers. As an alternative to use of cyanate scavengers, many recombinant protein purification processes utilizing urea depend upon de-ionization of buffers or operation at low temperature to preclude product degradation by carbamylation. Although

these procedures may be time-consuming, costly and sometimes inefficient, yielding lowered chromatographic performance, our data on the efficiency of cyanate scavengers suggests protein purification process designers may need to consider a multi-faceted approach to cyanate control. Initial cyanate removal by de-ionization of freshly prepared buffers, use of low pH and temperature when possible, minimized hold times in urea, combined with choice of effective buffers and additives for cyanate control would appear to be necessary to yield maximum assurance of low levels of product modification. Our data indicate that dependence upon added cyanate scavengers alone will not be sufficient to lower equilibrium cyanate levels to molar values well below the typical concentration of reactive protein amino groups in process intermediates.

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