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# Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate-derivatized amino acid mixtures

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**Abstract**

Recent improvements in HPLC-based methods for analyzing amino acids have made it feasible to analyze accurately a wide variety of sample types. The compatibility of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in the presence of salts and lipids makes the reagent an ideal choice for the analysis of complex samples. Method development was accomplished through the use of a quaternary HPLC eluent system with optimized pH and ionic strength. One chromatographic system was developed to achieve high resolution for a collagen sample containing hydroxyproline (Hyp) and hydroxylysine (Hyl) in addition to the hydrolyzate amino acids. A second procedure optimized resolution for a sample mixture of 24 amino acids. The quaternary HPLC streamlined routine operation and improved reproducibility as the multi-solvent eluent blends allowed precise, rapid changes in pH during the gradient separation. The resulting optimized methods blend the buffered eluents to achieve the accurate pH control required. Run times were reduced to less than 1 h by employing an increased flow-rate during the final third of the run. For the 24-amino acid mixture, high resolution was achieved for a number of important amino acids including Asn, Gln, Trp, γ-aminobutyric acid, ornithine, Hyp, and Hyl, as well as the 16 hydrolyzate amino acids in a single analysis. Examples of collagen hydrolyzate analysis and cell culture media samples are given to illustrate the utility of the methods.

*Keywords:* Separation optimization; Cell culture media; Amino acids; Collagen

## 1. Introduction

The separation and quantitation of amino acids in protein and peptide hydrolyzates is a standard technique in many biochemistry laboratories. Methods based on the separation of free amino acids using ion-exchange columns followed by post-column derivatization with ninhydrin were introduced by Moore and Stein [1,2] nearly 50 years ago. Despite significant improvements in instrumentation and column technology that have increased sensitivity, reduced analysis times and automated the operation, the fundamental procedure remains largely unchanged. In the past 20 years, however, there has been a rapid increase in the use of HPLC systems in conjunction with reversed-phase chromatography. These developments have largely been driven by the need for faster analyses and increased ability to analyze limited sample amounts. First *ortho*-phthalaldehyde [3,4] (OPA) and then phenylisothiocyanate [5–8] (Edman's reagent, PITC) derivatization chemistries became accepted alternatives to ion-exchange analysis. Other techniques based on pre-column derivatization chemistries worth noting include 9-fluorenylmethyl chloroformate [9] (FMOC-Cl), a hybrid of OPA and FMOC-Cl [10] and dansyl

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[11] and dabsyl chloride [12]. Recent collaborative studies have indicated that among protein chemists the PITC-based technique has grown to equal the ion-exchange method in usage [13].

Several years ago we introduced a new method for amino acid analysis [14] using the novel reagent AQC. Notable advantages of this method include rapid, quantitative and linear reactions, highly stable substituted urea adducts, good reaction yields in the presence of salts and detergents, and highly sensitive fluorescence detection. The unique fluorescence properties of the reagent also allow for direct injection of the reaction mixture without sample cleanup. The response from excess hydrolyzed reagent is very small and the peak is easily resolved from the derivatized amino acid analytes. Initial reports [14–16] demonstrated excellent compositional analysis of purified, hydrolyzed peptides and proteins. More recently AQC has been used for a wide variety of samples including feed grain hydrolyzates [17,18], and non-amino acid analytes such as polyamines [19] and intact, unhydrolyzed peptides [20–22].

One of the key strengths of reversed-phase HPLC-based analyses is the tremendous flexibility in developing separations of complex mixtures. An example of this resolving power has been the subject of several papers using PITC derivatization for the analysis of physiologic amino acids [23–25]. These papers noted that successful resolution of such a complex mixture requires stringent control over mobile phase composition, especially buffer pH and organic solvent concentration. It is also necessary to optimize the HPLC system performance to ensure reproducible chromatography. Small changes in mobile phase composition and gradient pump performance can have an adverse effect on key peak pair resolution, and separations development and implementation of the method on multiple HPLC systems can only be successful if operational details are well-controlled.

A recent publication using the AQC method showed that by optimizing mobile phase pH and a complex gradient in organic solvent, several important non-hydrolyzate amino acids could be separated [17]. These additional components included the amide amino acids, Asn and Gln, cysteine derivatives carboxymethyl cysteine and pyridylethyl cysteine, and the hydroxylated amino acids hydroxyproline (Hyp) and hydroxylysine (Hyl). However, this particular separation system demanded precise control of the buffer pH and could be difficult to reproduce in different laboratories using different pH meters. The current study shows how quaternary eluent blending of gradient HPLC systems can be used to speed methods development and simplify routine eluent preparation with exquisite pH control. The eluent ionic strength is simultaneously optimized. In addition we have used a new HPLC system to improve the reproducibility of analyte retention times, particularly in the first half of the separation where the extremely shallow nature of the gradient profile puts heavy demands on the tolerances of the solvent delivery system.

## 2. Materials and methods

### 2.1. Chemicals

AccQ·Fluor reagent kit (AQC, borate buffer) and AccQ·Tag Eluent A concentrate were acquired from Waters (Milford, MA, USA). Acetonitrile (MeCN), disodium ethylenediaminetetraacetic acid (EDTA), phosphoric acid, sodium acetate trihydrate, and sodium azide were from Baker (Phillipsburg, PA, USA); triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI, USA). Amino acid standards were from Pierce (Rockford, IL, USA) or Sigma (St. Louis, MO, USA). Collagen Type III was from Sigma. Cell culture media, and cell culture supernatants were from collaborative studies with Life Technologies (Grand Island, NY, USA) and Repligen (Cambridge MA, USA) respectively.

### 2.2. Chromatographic instrumentation

The two HPLC systems used were (A) a Waters Alliance system consisting of a 2690XE Separations Module, a 474 scanning fluorescence detector and a 486 tunable UV–visible absorbance detector, and (B) a 625 LC system equipped with a column heater, a 715 UltraWisp with sample cooling option, a 470 scanning fluorescence detector and a 486 tunable UV–visible absorbance detector (all from Waters). A typical system is shown schematically in Fig. 1. A

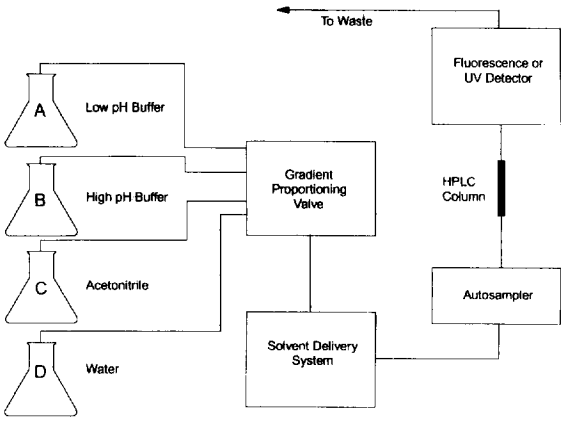


Fig. 1. Schematic of a typical quaternary HPLC system used simultaneously for pH blending and organic solvent gradient delivery.

Waters Millennium 2010 Chromatography Manager was used to control system operation, and results management.

### 2.3. Collagen hydrolysis

Samples (100 mg) were taken from stock solutions (5 mg/ml) and placed in a 6×50 mm test-tube and dried under vacuum. The procedure followed one previously published [26]. Briefly, the dried samples were placed in a larger vessel and, following the addition of 200 μl of 6 *M* HCl containing a single crystal of phenol, sealed under vacuum after several alternating cycles of vacuum and nitrogen flushing. Hydrolysis was carried out at 114°C for 24 h. After removing excess acid under reduced pressure, the samples were dissolved in 100 μl of borate buffer and 5 μl were used for derivatization and amino acid analysis.

### 2.4. Preparation of standards, cell culture media, and supernatants

Aqueous 2.5 m*M* stock solutions of α-aminobutyric acid (Aab), γ-aminobutyric acid (GABA), Asn, Gln, Hyp, Hyl, ornithine (Orn), taurine (Tau), and Trp were prepared. Mixture I consisted of 40 μl each of Hyl, Hyp and Pierce H mixed with 880 μl of water (0.1 m*M* per amino acid). Forty μl of each stock solution and Pierce H standard were mixed with water to a final volume of 1 ml to make Mixture 2 (0.1 m*M* per amino acid). A 10 μl volume of standard solution was measured by syringe into a pyrolyzed 6×50 mm tube and derivatized. 100 μl samples of media or cell free supernatants were pipetted into 1.5 ml centrifuge tubes. An equal volume of 0.4 m*M* internal standard Aab was added. Following vortexing, an equal volume of MeCN was added to precipitate proteins (total volume 400 μl). Samples were centrifuged in an Eppendorf microfuge (Model 5415) at 16 000 *g* for 5 min yielding a clear, ready to derivatize solution.

### 2.5. Derivatization of standards and samples

In a typical analysis, 5–20 μl of sample was buffered to pH 8.8 (AccQ·Flour borate buffer) to yield a total volume of 80 μl. Derivatization was initiated by the addition of 20 μl of AccQ·Fluor reagent (3 mg/ml in MeCN). Reaction of the AQC with all primary and secondary amines is rapid ($t_{1/2}<1$ s) and excess reagent is hydrolyzed within 1 min. Completion of hydrolysis of any tyrosine phenol modification is accelerated by heating for 10 min at 55°C. Alternatively the 2690XE Separations Module can be used to heat samples to 40°C for 90 min to facilitate phenolic adduct hydrolysis.

### 2.6. Methods development

Optimization of peak resolution was facilitated by blending two eluents with different pH, water and MeCN. Blending protocols allow for investigations of pH and ionic strength affects without changing eluents. The presence of multiple pH eluents also allows for pH changes during the run, which results in shorter run times and superior resolution.

### 2.7. Chromatographic analysis

Separations were carried out using a 20×3.9 mm Sentry guard column (Nova-Pak $C_{18}$ bonded silica) connected to a 150×3.9 mm AccQ·Tag column (both from Waters). Working eluents for Mixture II separations were prepared by diluting 100 ml of concentrate to 1100 ml with water. Eluent concentrates were prepared by dissolving 148 g of NaOAc trihydrate in 1.0 l of water and adding 7.06 g of

TEA. For the Mixture I separation, the concentrate at pH 5.05 was obtained from Waters (AccQ·Tag Eluent A). The concentrate consisted of 190 g NaOAc, 22.7 g TEA, 5 g of sodium azide and 1 g disodium EDTA added to 1 l of water. The resulting solution was titrated to pH 5.05 with phosphoric acid. Concentrated eluents were titrated to the indicated pH using either a 50% phosphoric acid solution, or 1 *M* NaOH. Working eluent was prepared by mixing 100 ml of the concentrate with 1 l of water.

Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm or by UV absorbance at 248 nm.

## 3. Results

### 3.1. Collagen analysis

Chromatographic conditions previously reported for hydrolyzate samples [14] failed to provide sufficient resolution for collagen standard, Mixture I (Fig. 2). Despite the low response for the reagent hydrolysis product 6-aminoquinoline (AMQ), resolution of Hyp and AMQ was poor. The two Hyl peaks, present because the commercial standard is provided as a diastereomer, eluted in a crowded portion of the chromatogram near Val, and improved resolution was also desirable. In addition, analysis of a hydrolyzed collagen sample revealed that the resolution of Gly and His needed improvement due to the enormous response from Gly. Separations development described below was designed to remedy these problems without compromising the ability to quantify the remaining amino acids.

Previous studies [14,17] had demonstrated the effects of pH and gradient slope on the retention behavior of AMQ. Separations shown in Figs. 3 and 4 illustrate these effects. Unfortunately, the lower pH system decreased the resolution of Gly and His. In contrast, decreasing the gradient steepness had the positive effect of improving both the resolution of AMQ and Hyp as well as that of Gly and His. As shown in Fig. 3 the higher pH eluent could effect baseline resolution of AMQ and Hyp if the shallower gradient slope were used. Additional improvement in the resolution of Gly and His as well as the AMQ/

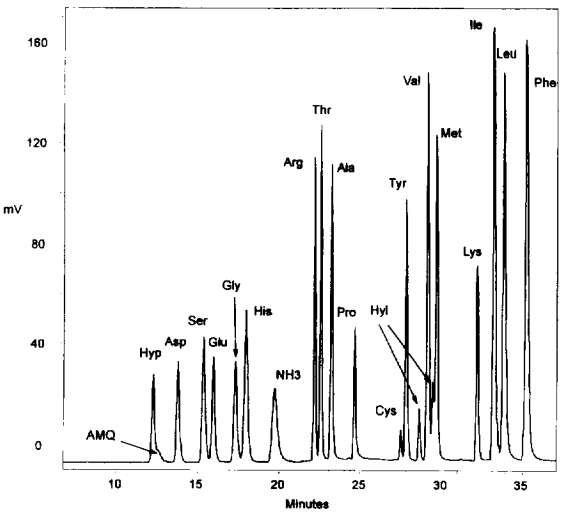


Fig. 2. Chromatographic separation of Mixture 1. The derivatized amino acid Mixture I (50 pmol each amino acid in 10 μl) was chromatographed using the conditions indicated. Detection was by fluorescence. The column temperature was 37°C. Eluent A= sodium acetate 140 m*M*, TEA 17 m*M* as described in the methods section, pH 5.05, Eluent B=MeCN, Eluent C=water. Gradient: Initial=0% B, 0.5=1% B, 18=5% B, 19=9% B, 29.5=17% B, hold for 5.5 min, wash with 60% MeCN in water for 3 min, equilibrate with 100% A for 9 min before subsequent injection, step gradient at 0.5 min, wash and equilibration steps, all other steps linear.

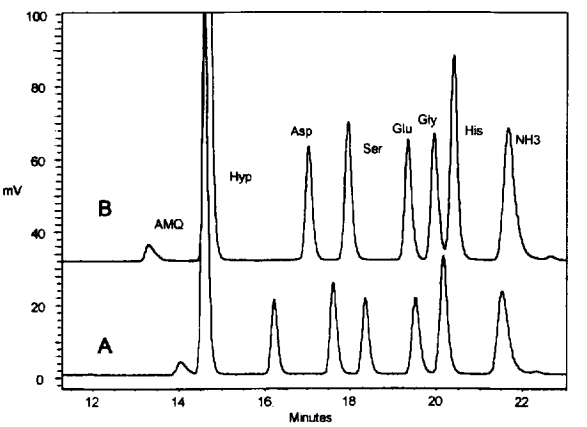


Fig. 3. Influence of eluent pH on resolution of hydrophilic amino acids in Mixture I, Hyp=250 pmol injected, all others 50 pmol. The gradient was initial=0% B, 20=5% B, 24=9% B, 34=17% B hold for 6 min, all other conditions were identical to those used in Fig. 2, except in (B) Eluent A pH=4.95.

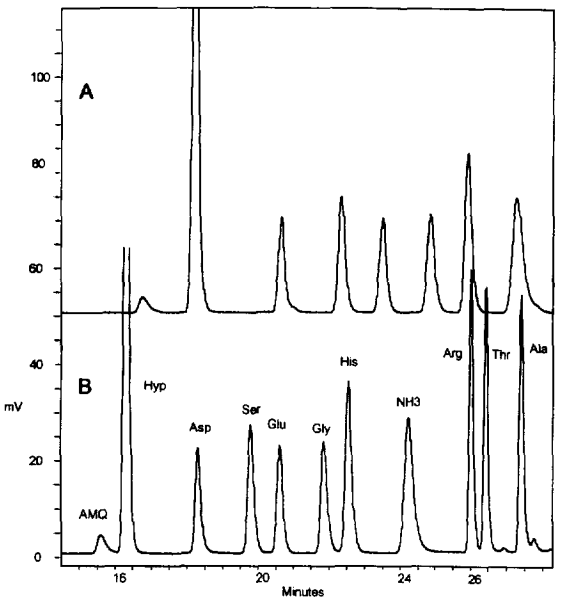


Fig. 4. Influence of gradient slope on resolution of hydrophilic amino acids in Mixture I, Hyp=250 pmol injected, all others 50 pmol. Column temperature=34°C. Gradient: (A) Initial=0% B, 25=5% B, 29=9% B, 39=17% B hold for 6 min (B) Initial=0% B, 20=5% B, 24=9% B, 34=17% B hold for 6 min; all other conditions were identical to those used in Fig. 2.

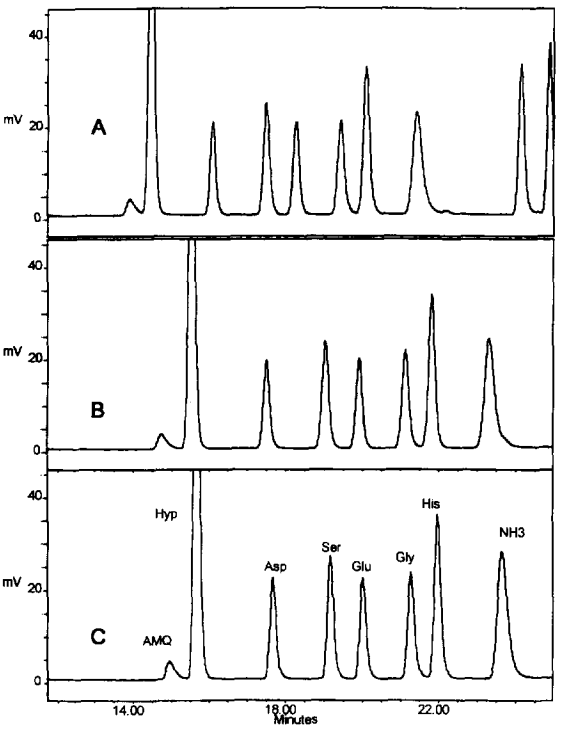


Fig. 5. Influence of column temperature on resolution of hydrophilic amino acids in Mixture I, Hyp=250 pmol injected, all others 50 pmol. (A) Temperature=37°C, (B) Temperature=34°C, (C) Temperature=32°C. The gradient was initial=0% B, 20=5% B, 24=9% B, 34=17% B hold for 6 min. All other conditions identical to those used in Fig. 2.

Hyp peak pair was achieved by reducing the column temperature from 37° to 34°C (Fig. 5). Subsequent optimization studies used the conditions described in Fig. 4A for the gradient steps before 25 min.

With the mobile phase pH near 5.0, Hyl elutes in the middle of the cluster formed by Cys, Tyr, Val and Met. A study of mobile phase pH (Fig. 6) shows that selectivity for the diderivatized amino acids (Cys, Hyl, and Lys) is dramatically altered at higher pH. However, as stated above, the more acidic mobile phase improves the critical resolution of AMQ and Hyp. Optimization of mobile phase pH in earlier studies [14] also showed that pH~5 was favorable for separating the acidic amino acids Asp and Glu from the other polar amino acids.

This conundrum was solved by adding a third mobile phase, a buffer similar in composition to Eluent A described in Fig. 2, but significantly higher in pH. The four-solvent capacity of the solvent delivery system permitted a rapid change in eluent pH, plus a final wash for system shutdown in water/acetonitrile containing no buffer. A typical gradient profile is shown in Table 1. The initial mobile phase pH was 5.05, and the step function at 29 min caused a rapid increase to pH 6.8. This resulted in large selectivity differences for the diderivatized amino acids. Cys eluted after Val, instead of before Tyr, the gap between Val and Met widened significantly, both Hyl peaks eluted between Met and Ile, and Lys eluted between Leu and Phe. The flow-rate was also increased to reduce the total analysis time. The resulting separation is shown in Fig. 7A.

These optimized conditions were used to analyze a hydrolyzed, derivatized collagen sample (Fig. 7B). Note the large excess of Gly relative to His, thus necessitating the additional resolution provided by the optimization of the early gradient slope and the column temperature. Table 2 shows the composition-

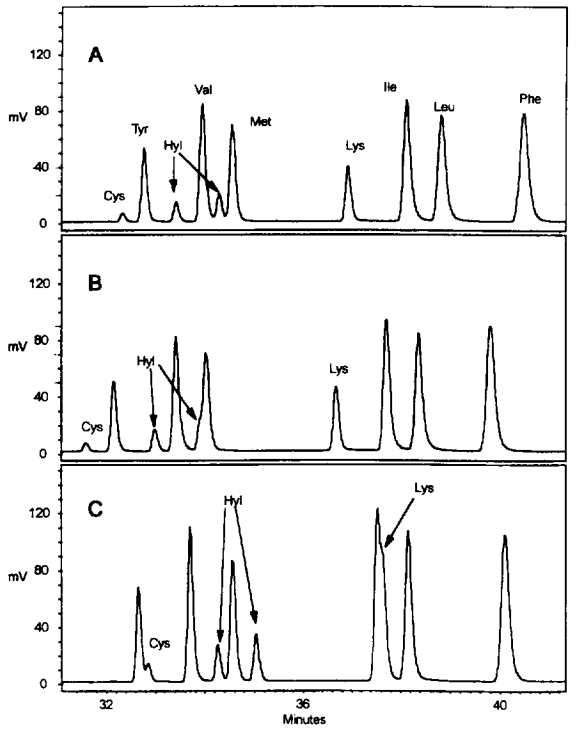


Fig. 6. Influence of pH on the elution of hydrophobic amino acids in Mixture I. The column temperature was 34°C. Buffer formulation for Eluents A and B is that described for Eluent A in the legend for Fig. 2, Eluent C=MeCN, Eluent D=water. Eluent A=pH 5.05, Eluent B=Eluent A titrated to pH 6.2 with NaOH. (A) The gradient is the same as in Fig. 3, Eluent pH 5.05, (B) same as (A) except %A=61, %B=30 at 24 min, %A=56, %B=27 at 34 min, eluent pH=5.3 after 24 min (C) same as (A) except %A=30, %B=61 at 24 min, %A=27, %B=56 at 34 min, eluent pH=5.6 after 24 min.

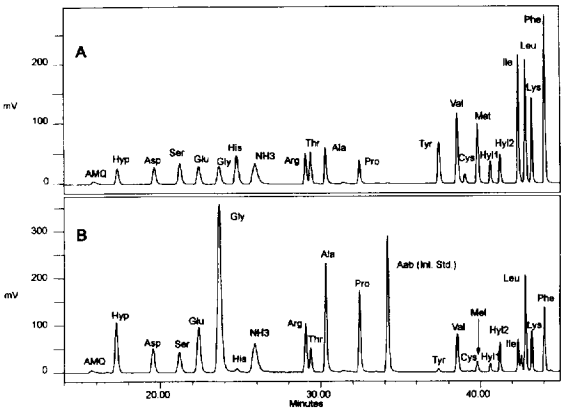


Fig. 7. Separations using optimized conditions for Mixture I separation. Eluent B=Eluent A titrated to 6.80 with NaOH, eluent C=acetonitrile. The gradient profile is given in Table 1. The column temperature was 34°C. (A) Mixture I standard, 50 pmol each amino acid. (B) Collagen hydrolyzate sample, prepared as described in Section 2. Total run time was 54 min.

al analysis of this sample, agreeing well with previously published data [27].

### 3.2. Optimization of Mixture II resolution

A number of relevant amino acid analyses require the quantification of unhydrolyzed samples. These will often contain the amides Asn and Gln, as well as other non-hydrolyzate components such as Hyp, Hyl, Tau, GABA and Orn. Effective analysis thus requires adequate resolution of these amino acids as well as those present in protein hydrolyzates. A previous publication described the separation of the amide amino acids using a buffered eluent at pH 5.80 [17], but the chromatography was complicated by the

Table 1
Gradient profile for optimized separations of Mixture 1

| Time (min) | Flow (ml/min) | %A pH 5.05 | %B pH 6.80 | %C acetonitrile | %D water | Curve[a] |
|---|---|---|---|---|---|---|
| Initial | 1.00 | 100 | 0 | 0 | 0 | * |
| 25.0 | 1.00 | 95 | 0 | 5 | 0 | 6 |
| 28.0 | 1.00 | 92 | 0 | 8 | 0 | 6 |
| 28.5 | 1.50 | 0 | 92 | 8 | 0 | 6 |
| 36.5 | 1.50 | 0 | 88 | 12 | 0 | 6 |
| 43.0 | 1.50 | 0 | 80 | 20 | 0 | 6 |
| 43.5 | 1.50 | 0 | 0 | 60 | 40 | 11 |
| 45.0 | 1.00 | 100 | 0 | 0 | 0 | 11 |

[a] Curve 6 is a linear segment; curve 11 is a step function.

Table 2
Amino acid composition of collagen

| Amino acid | Experimental data[a] | Data from Ref. [29] |
|---|---|---|
| Hyp | 107 | 97 |
| Asp | 44 | 43 |
| Ser | 34 | 25 |
| Glu | 85 | 89 |
| Gly | 316 | 333 |
| His | 3 | 2 |
| Arg | 54 | 50 |
| Thr | 26 | 23 |
| Ala | 99 | 103 |
| Pro | 113 | 120 |
| Tyr | 3 | 2 |
| Val | 19 | 18 |
| Cys | 2 | ND[b] |
| Met | 6 | 10 |
| Hyl | 24[c] | 20 |
| Ile | 9 | 9 |
| Leu | 26 | 26 |
| Lys | 16 | 15 |
| Phe | 13 | 13 |

[a] Data are expressed in residues per 1000 residues.
[b] Not determined.
[c] Value is the sum of the two Hyl isomers.

difficulty in reproducing the precise eluent pH required for good resolution, particularly for the peak cluster consisting of Ser, Asn, AMQ and Gly. There was also no provision for resolution of Orn, which was insufficiently separated from the Ile/Leu pair.

The concept of eluent blending for pH control has been a common practice in optimizing ion-exchange separations, particularly in methods development studies for protein purification [28]. In the collagen optimization studies described above, we used a step change in eluent pH to change the selectivity in two distinct regions of the chromatogram. This is analogous to the step changes in pH and/or ionic strength that form the basis for most ion-exchange amino acid analysis systems [1].

A more sophisticated use of the multi-solvent delivery capability of a quaternary eluent blending HPLC system was employed to optimize the separation of the complex mixture described above. The procedure involved varying the ratio of two buffers differing only in pH to effect small changes in total blended eluent pH. Because the relevant window of chromatographic separation is largely aqueous in composition (acetonitrile concentration<20%), there is a wide range in available buffer ratios, increasing the operational control for small but predictable changes in overall pH. This fine control has several significant practical and operational benefits. First, small errors in eluent pH adjustment, typically due to difficulty in manually calibrating and measuring pH within 0.02 units, can be corrected with a minor adjustment in gradient without modifying either buffer. Second, separations that rely on precise blending to achieve reproducible pH are actually simpler to effect than through precise titration of a single buffer. In addition, rapid changes in eluent pH can be implemented, similar to those used for the collagen separation. Control of pH through eluent blending for two eluent systems used in this study is illustrated in Figs. 8 and 9.

One of the key regions of the Mixture II separation involves the four component group Ser, Asn, AMQ and Gly. The sensitivity of AMQ retention to eluent pH has been previously noted [17]. At pH 5.80 it no longer elutes before all of the other amino acids, but is retained longer than Asp, Glu, Hyp, Ser and Asn. This sensitivity is illustrated in Fig. 8. Note that a change in pH as small as 0.05 units can modify the separation from one that is satisfactory to one that is unacceptable. The resolution in this region is also influenced by the organic solvent concentration. The key resolutions affected by the gradient slope were the Ser/Asn peak pair and the position of AMQ between Asn and Gly. The best separation was effected with a very shallow gradient as shown in Table 3.

The quaternary system also simplified resolution of the late eluting peaks. As with the collagen separation, the key to this separation was the ability to rapidly modify eluent pH to influence the retention of the diderivatized amino acids relative to the retention of the monoderivatized analytes. However, the separation was further complicated by the need to separate an additional amino acid, namely Orn. In all systems studied, increasing the pH in the range of 5.0–6.8 resulted in increased retention of the diderivatized amino acids relative to the monoderivatized ones. This selectivity was likely due to the extra quinoline tag which would be protonated at the lower pH. This pH selectivity was also observed for the AMQ peak. Fig. 9 shows the effect of pH for the late eluting peaks. The diderivatized components also

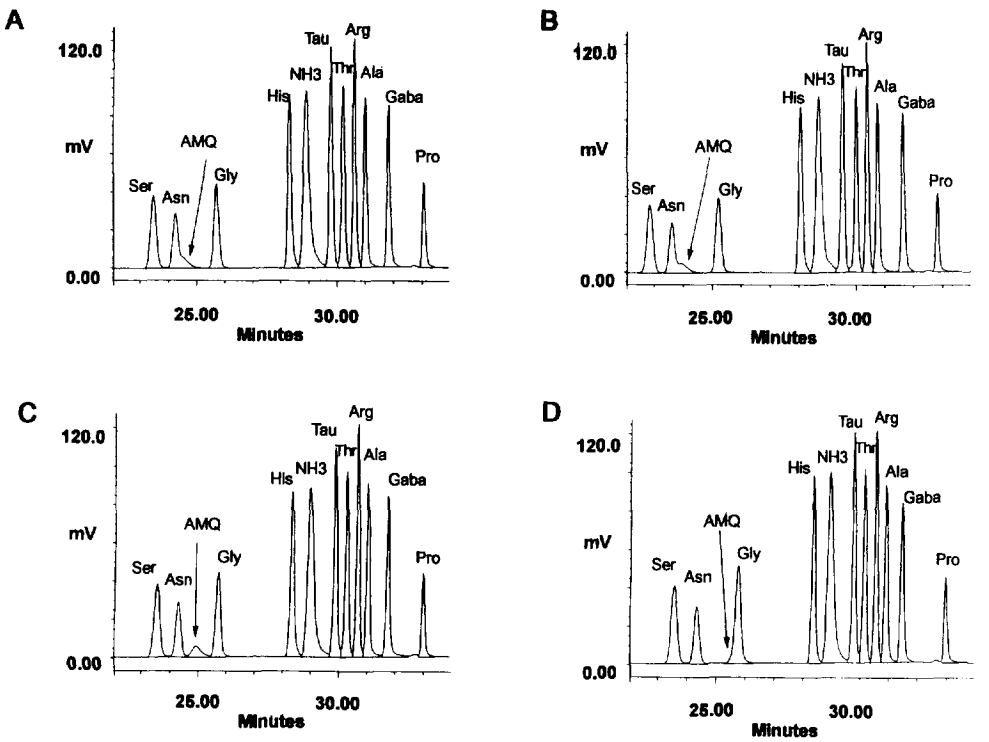


Fig. 8. Influence of pH on the resolution of hydrophilic amino acids in Mixture II. The separations were generated with eluent A and B at pH 5.70 and 5.90 respectively. The initial eluents were blended in the following ratio; (A) 100% A, 0.0% B, blended pH 5.70 (B) 75% A, 25% B, blended pH 5.75 (C) 50% A, 50% B, blended pH 5.80 and (D) 0.0% A 100% B, blended pH 5.90. The gradient was that shown in Table 3 with the following three modifications: (i) step at 33.5 min was changed to 36.7 min, flow=1.0 ml/min, the total of %A plus %B=87%, where the ratio of the eluents is given above, %C=13, %D=0; (ii) step at 33.8 min was changed to 37.0 min, flow=1.3 ml/min, %A=0, %B=87, %C=13, %D=0; (iii) step at 37.0 min was changed to 49 min, flow=1.3 ml/min, %A=0, %B=0, %C=60, %D=40. The column temperature was 39°C. Total run time was 60 min.

exhibited different selectivity as a function of organic solvent gradient slope. Shallower gradients result in longer retention relative to the monoderivatized analytes. A shallower slope can also be effected by increasing the flow-rate, which has the further benefit of reducing the analysis time.

The optimized conditions (Fig. 10) relied on careful control of pH, gradient slope and flow-rate. The quaternary solvent system made it possible to simultaneously manipulate the organic solvent concentration and the eluent pH. The shallow initial slope was essential for the separation of Ser and Asn, and combined with the correct eluent pH resolved AMQ from both Asn and Gly. The pH was increased to 6.8 to place Cys between Tyr and Val, and immediately after the elution of Cys lowered to approximately pH 6.3 to place Orn in the middle of Ile and Leu. Despite the complexity of the gradient profile, the data in Table 4 shows that modern HPLC equipment can provide extremely reproducible separations. As observed in Fig. 10B, all of the amino acids, including Asn and Gln, were well-resolved in less than 50 min. The separation of the amides is often problematic in both reversed-phase and ion-exchange systems, the latter requiring special Li-based columns [29] that typically require analysis times exceeding 2 h.

### 3.3. Analysis of Mixture II type samples

To further illustrate the applicability of this chro-

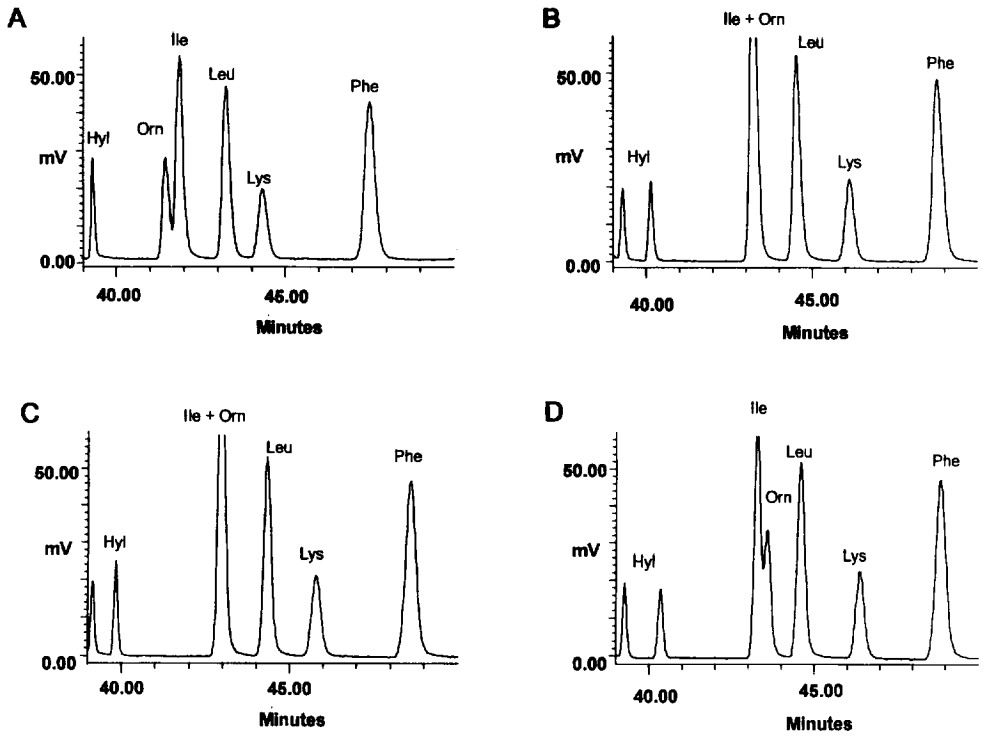


Fig. 9. Influence of pH on the resolution of hydrophobic amino acids in Mixture II. Conditions were the same as in Fig. 8.

Table 3
Gradient table for optimized separations of Mixture II

| Time (min) | Flow (ml/min) | %A pH 5.70 | %B pH 6.80 | %C acetonitrile | %D water | Curve[1] |
|---|---|---|---|---|---|---|
| Initial | 1.00 | 90.0 | 10.0 | 0.0 | 0.0 | * |
| 0.50 | 1.00 | 89.0 | 10.0 | 1.0 | 0.0 | 11 |
| 17.00 | 1.00 | 88.0 | 10.0 | 2.0 | 0.0 | 6 |
| 24.00 | 1.00 | 86.0 | 9.0 | 5.0 | 0.0 | 6 |
| 32.00 | 1.00 | 63.0 | 25.0 | 12.0 | 0.0 | 6 |
| 33.50 | 1.00 | 0.0 | 87.5 | 12.5 | 0.0 | 6 |
| 33.80 | 1.30 | 22.0 | 65.5 | 12.5 | 0.0 | 6 |
| 37.00 | 1.30 | 22.0 | 65.0 | 13.0 | 0.0 | 6 |
| 48.00 | 1.30 | 22.0 | 63.0 | 15.0 | 0.0 | 6 |
| 48.10 | 1.30 | 0.0 | 0.0 | 60.0 | 40.0 | 6 |
| 51.00 | 1.00 | 90.0 | 10.0 | 0.0 | 0.0 | 11 |
| 75.00[b] | 1.00 | 0.0 | 0.0 | 60.0 | 40.0 | 11 |
| 110.00[b] | 0.0 | 0.0 | 0.0 | 60.0 | 40.0 | 6 |

[a] Curve 6 is a linear segment; curve 11 is a step function.
[b] These steps provide an automated shutdown procedure.

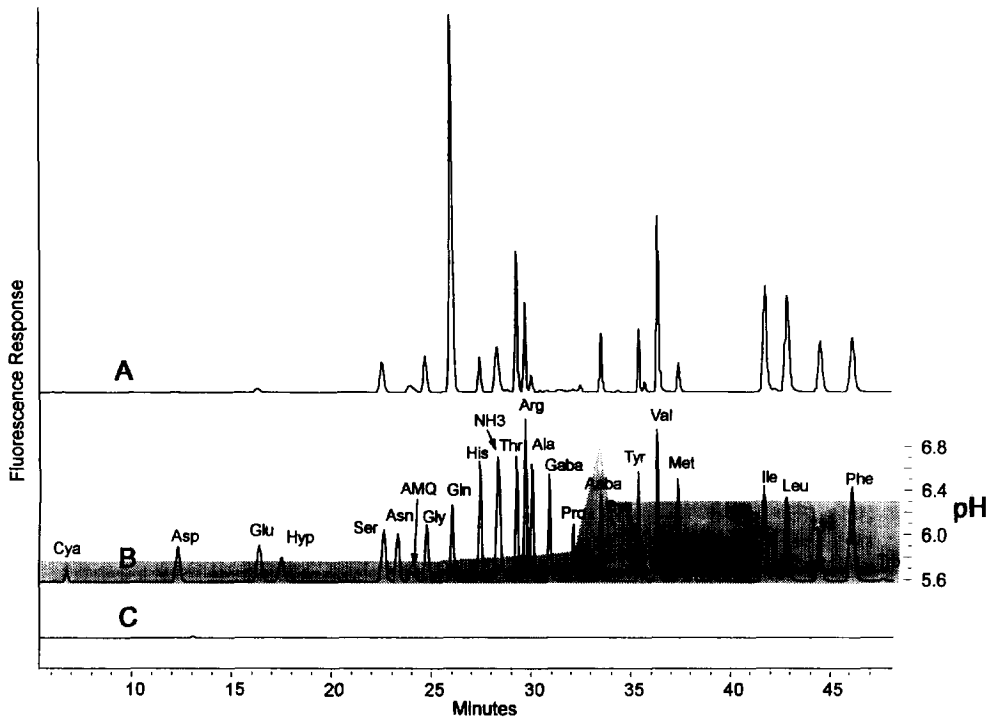


Fig. 10. Separations for free amino acid mixtures. Eluent A=pH 5.70, Eluent B=pH 6.80. Other conditions used are described in the text, Table 3 and the legend for Fig. 8. (A) DMEM plus serum, (B) Standard Mixture II, and (C) non-derivatized insect cell media. The shaded area of the standard chromatogram (B) represents the pH of the aqueous portion of the eluent formed by blending Eluents A and B as given in Table 3.

matographic system, a study of cell culture amino acid content was carried out. As the culture grows, some amino acids are used as protein building blocks or as nitrogen or energy sources, while others are produced as metabolites. To optimize culture growth, the medium can be supplemented at regular intervals to provide essential nutrients.

The optimized quaternary eluent blending conditions were used to analyze both media and cell culture fluid supernatants. A typical result is shown in Fig. 10A. Even with the simple sample preparation procedure, separations of complex samples such as insect cell media, Vero cell culture supernatants, and Ham's media show no significant interference from non-amino acid sample components. Chromatography of a non-derivatized insect cell medium sample demonstrates how the selectivity of fluorescence detection eliminates background interference (Fig. 10C). Excellent resolution and high signal-to-noise response for the derivatized amino acids simplify peak identification, integration and quantitation. Chromatographic and quantitative reproducibilities are very good (Table 4). Total time for sample preparation and derivatization of cell free solutions is less than 20 min including the 10 min heating step prior to injection.

The precise quantitation afforded by this method allowed for both verification of media formulations and supplement quality, as well as the determination of nutrient requirements for batch cultures. Studies in our lab examined the concentration of amino acids in Vero cell batch culture supernatants over 26 days of culture growth. The medium was supplemented with a mix of amino acids several times to replace nutrients depleted during the process. Results showing consistent decrease in several key amino acids

Table 4
Reproducibilty for Standard Mixture II and Culture Media Sample

| Amino acid | Retention time reproducibility | | | | Amount reproducibility | |
|---|---|---|---|---|---|---|
| | Retention time (min) | %RSD System A | Retention time (min) | %RSD System B | mg/l | %RSD ($n$=3) |
| Asp | 10.58 | 0.62 | 13.45 | 0.87 | 27.46 | 0.17 |
| Glu | 14.21 | 0.56 | 17.70 | 0.88 | 52.20 | 0.35 |
| Hypro | 15.53 | 0.60 | 16.22 | 0.89 | 7.40 | 6.71 |
| Ser | 20.51 | 0.51 | 24.14 | 0.42 | 36.25 | 1.03 |
| Asn | 21.41 | 0.39 | 24.78 | 0.38 | 21.16 | 0.63 |
| Gly | 22.25 | 0.27 | 26.36 | 0.37 | 29.91 | 1.03 |
| Gln | 24.50 | 0.22 | 27.54 | 0.33 | 343.95 | 1.61 |
| His | 26.08 | 0.17 | 28.82 | 0.25 | 27.34 | 1.06 |
| $NH_3$ | 26.66 | 0.22 | 29.72 | 0.20 | ND | ND |
| Thr | 28.09 | 0.14 | 30.52 | 0.18 | 13.86 | 1.55 |
| Arg | 28.64 | 0.09 | 30.99 | 0.16 | 188.07 | 0.70 |
| Ala | 28.89 | 0.13 | 31.30 | 0.16 | 27.49 | 1.25 |
| Gaba | 29.80 | 0.11 | ND | ND | ND | ND |
| Pro | 31.11 | 0.09 | 33.32 | 0.11 | 33.73 | 1.78 |
| Aaba | 32.49 | 0.10 | 34.65 | 0.11 | ND | ND |
| Tyr | 34.47 | 0.06 | 36.90 | 0.12 | 24.37 | 1.68 |
| Cys | 34.87 | 0.06 | 37.52 | 0.15 | 29.25 | 0.70 |
| Val | 35.35 | 0.06 | 38.41 | 0.12 | 26.60 | 0.85 |
| Met | 36.26 | 0.06 | 39.68 | 0.13 | 16.13 | 15.36 |
| Hylys1 | 37.11 | 0.04 | ND | ND | ND | ND |
| Hylys2 | 38.06 | 0.04 | ND | ND | ND | ND |
| Ile | 40.39 | 0.04 | 45.36 | 0.24 | 20.65 | 8.02 |
| Orn | 41.14 | 0.04 | 46.06 | 0.31 | ND | ND |
| Leu | 41.47 | 0.04 | 46.85 | 0.25 | 78.51 | 1.23 |
| Lys | 43.39 | 0.06 | 49.51 | 0.29 | 88.43 | 4.86 |
| Phe | 44.62 | 0.05 | 51.48 | 0.23 | 37.53 | 0.58 |

correlated well with previously published results [30].

## 4. Conclusions

Amino acid analysis using precolumn derivatization with AQC is a versatile, reproducible method. Good results are obtained for a variety of samples even in the presence of potential interferences such as salts in the sample matrix.

Separation of complex amino acid mixtures, such as those produced by the hydrolysis of collagen or free amino acids present in cell culture fluids, is simplified through the use of a quaternary gradient system. Precise control of eluent pH and organic solvent gradient slope are essential for optimum resolution and ease of buffer preparation. Excellent reproducibility can be routinely obtained with modern HPLC systems.

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