

Improvements in the Gradient Program for Amino Acid Analysis Using Anion-Exchange Chromatography and Integrated Pulsed Amperometric Detection

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ABSTRACT

Anion-exchange chromatography with integrated pulsed amperometric detection (AE-IPAD) directly detects amino acids, carbohydrates, alditols, and glycols in the same injection without pre- or postcolumn derivatization. We previously published the successful use of this technique, also known as *AAA-Direct*[™], for the determination of free amino acids in cell culture and fermentation broth media.¹⁻³ The retention time selectivity of carbohydrates varies differently than amino acids with changes in eluent NaOH concentration, and thus unique separations are possible by varying the initial concentration and duration of eluent. In the previously published applications (see Figures 1 and 2), we were not completely satisfied with the resolution of specific amino acids with system-related peaks and unknown sample component peaks in very complex media eluting in the acetate gradient of the method. In this poster, we present significant improvements to the gradient method that improves resolution, and provides greater system stability over time.

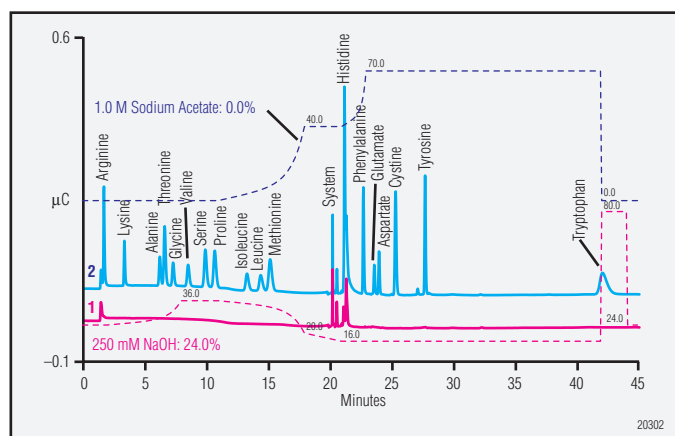


Figure 1. Common amino acids separated using the standard (unmodified) gradient method. Comparison of chromatograms for (1) a water blank and (2) 8 µM amino acid standard mix.

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Standards Analyzed

Amino acids, carbohydrates, alditols, and other compounds investigated in this poster were purchased from the National Institute for Standards and Technology (NIST, Standard Reference Material 2389, Gaithersburg, MD), Sigma-Aldrich Chemical Co. (St. Louis, MO), Pfanstiehl Laboratories, Inc. (Waukegan, IL), McNeil Nutritionals, Inc. (Fort Washington, PA), Eastman Chemical Co. (Rochester, NY), Fisher Scientific (Pittsburgh, Pennsylvania), J.T. Baker Inc. (Phillipsburg, NJ), or EM Science (Gibbstown, NJ).

Cell Culture Media Analyzed

Bacto[®] YPD Broth (BD Laboratories, Cat. No. 0428-17-5)

Dulbecco's Modified Eagle's Medium: F-12

Medium 199 (Sigma-Aldrich Chemical Co., Cat. No. M4530)

L-15 Leibovitz Medium (Sigma-Aldrich Chemical Co., Cat. No. L5520)

McCoy's 5A Medium, Modified (Sigma-Aldrich Chemical Co., Cat. No. M8403)

All solid media (YPD Broth) were reconstituted in water to their normal concentrations, centrifuged at 16,000× g for 10 min, and the supernatant diluted in water. Liquid media or reconstituted media were diluted 10-, 100-, or 1000-fold in water. These diluted media were analyzed directly.

Equipment

Dionex ICS-2500 or ICS-3000 system (configured for microbore) consisting of Gradient Pump, Electrochemical Detector with *AAA-Direct* Certified Disposable Au Electrodes and combination pH/Ag/AgCl Reference Electrode, Autosampler, Column Oven, Eluent Organizer

Chromeleon[®] Chromatography Management Software

Helium (4.5 grade, high purity 99.5%)

Filter unit, 0.2 µm nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 500-118)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)

Vial, 0.5 mL, polypropylene, microinjection, 12-32 mm screw thread cap, and preslit Teflon[®]/silicone septum (Dionex P/N 055428)

Table 1. Gradient Method

	Method 1: Previously Published	Method 2: Improved	Method 3: Improved																																																																																																																																																																																																																																																																																	
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<p>*To obtain the following mM NaOH initial concentrations, substitute X with the following %B at Event Times 0, 8, 66.3, and 92 min:</p> <table border="1"> <thead> <tr> <th>mM NaOH</th> <th>%B</th> <th>mM NaOH</th> <th>%B</th> </tr> </thead> <tbody> <tr><td>10</td><td>0.00</td><td>40</td><td>12.50</td></tr> <tr><td>15</td><td>2.08</td><td>45</td><td>14.58</td></tr> <tr><td>20</td><td>4.17</td><td>50</td><td>16.67</td></tr> <tr><td>25</td><td>6.25</td><td>55</td><td>18.75</td></tr> <tr><td>30</td><td>8.33</td><td>60</td><td>20.83</td></tr> <tr><td>35</td><td>10.42</td><td></td><td></td></tr> </tbody> </table>				mM NaOH	%B	mM NaOH	%B	10	0.00	40	12.50	15	2.08	45	14.58	20	4.17	50	16.67	25	6.25	55	18.75	30	8.33	60	20.83	35	10.42																																																																																																																																																																																																																																																							
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Chromatography

Columns: AminoPac® PA10 Analytical Column, 2 × 250 mm (P/N 55406, Dionex)
AminoPac Guard Column, 2 × 50 mm (P/N 55407, Dionex).

Flow Rate: 0.25 mL/min

Temperature: 30 °C.

In. Volume: 25 µL (full-loop injection mode).

Reference Electrode Mode: pH

Waveform for ECD Detector: See references 2, 4

Eluent: A: 10 mM NaOH
B: 250 mM NaOH
C: 25 mM NaOH in 1 M sodium acetate
D: 100 mM acetic acid

Deionized Water: 18 MΩ-cm resistance or better, free of biological or chemical contamination, prefiltered through 0.2 µm nylon filter to degas.

Gradient Method: See Table 1

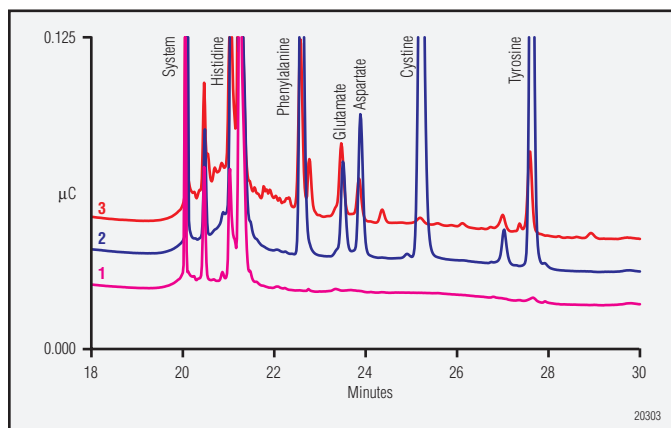


Figure 2. Typical acetate gradient region of previously published method for (1) water blank injections, (2) amino acid standards, and (3) 1000-fold diluted YPD broth supernatant using a non-sanitized system. Incomplete resolution of histidine, phenylalanine, glutamate, aspartate, and unknown broth ingredient peaks is observed.

RESULTS AND DISCUSSION

The AE-IPAD system using previously published methods^{1,2} is well suited for most amino acid, carbohydrate, alditol, and glycol separations. However, (1) histidine (His) coelutes with minor system peaks and (2) there is some His peak distortion; (3) there is a 1–4% carryover of His, aspartate (Asp), glutamate (Glu), and tyrosine (Tyr) from previous injections; (4) there is a tendency for system impurities to increase over time from biological contamination of water and acetate eluent lines; and (5) ingredient peaks present in complex culture media and eluting during the acetate gradient are incompletely resolved. Figure 2 shows these deficiencies.

To correct these deficiencies, a new method was developed that included NaOH in both the water (channel A) and 1 M sodium acetate (channel C) eluents to maintain sterility of the eluent lines. This method reduced the appearance of minor system peaks during the acetate gradient, and increased system ruggedness by reducing the need to perform system sanitization. The gradient program was modified (see Table 1) to accommodate the NaOH in channels A and C, and to resolve the small system peaks that remain and unknown cell culture media ingredients from His, phenylalanine (Phe), Asp, Glu, and cystine. Finally, the column was washed with 100 mM acetic acid for 2 min to eliminate minor carryover of His, Phe, Asp, Glu, and Tyr, and increase the quantitative accuracy for these peaks near their lower limit of detection. Figure 3 shows these improvements.

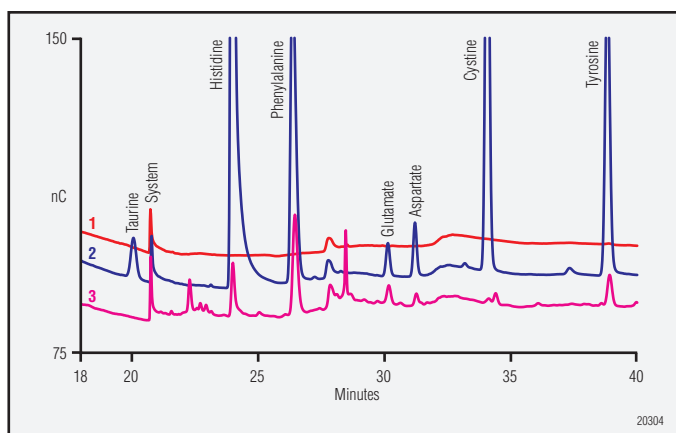


Figure 3. Typical acetate gradient region of a recently developed AAA-Direct method for (1) water blank injections, (2) standards, and (3) 1000-fold diluted YPD broth supernatant injections. Amino acid peaks and unknown broth ingredient peaks are better resolved.

The new method reduces system peaks, moves remaining minor system peaks into non-coeluting regions, increases long-term stability of the AAA-Direct system, eliminates trace carryover from previous injections, and improves resolution of unknown broth-related peaks. Good chromatographic separation of all amino acids is maintained without frequent system sanitization.

The effect of lowering initial NaOH eluent concentration and extending the isocratic time from 2 min to 8 min as previously described¹⁻³ was investigated for amino acids, carbohydrates, alditols, and glycols commonly found in cell culture and fermentation broths. We found our improvements did not interfere with the known selectivity effects. Figure 4A shows the effect of lowering initial NaOH concentration on carbohydrate retention times, and Figure 4B shows the effect on amino acids. These selectivity changes can be used to optimize cell culture and fermentation broth analysis.

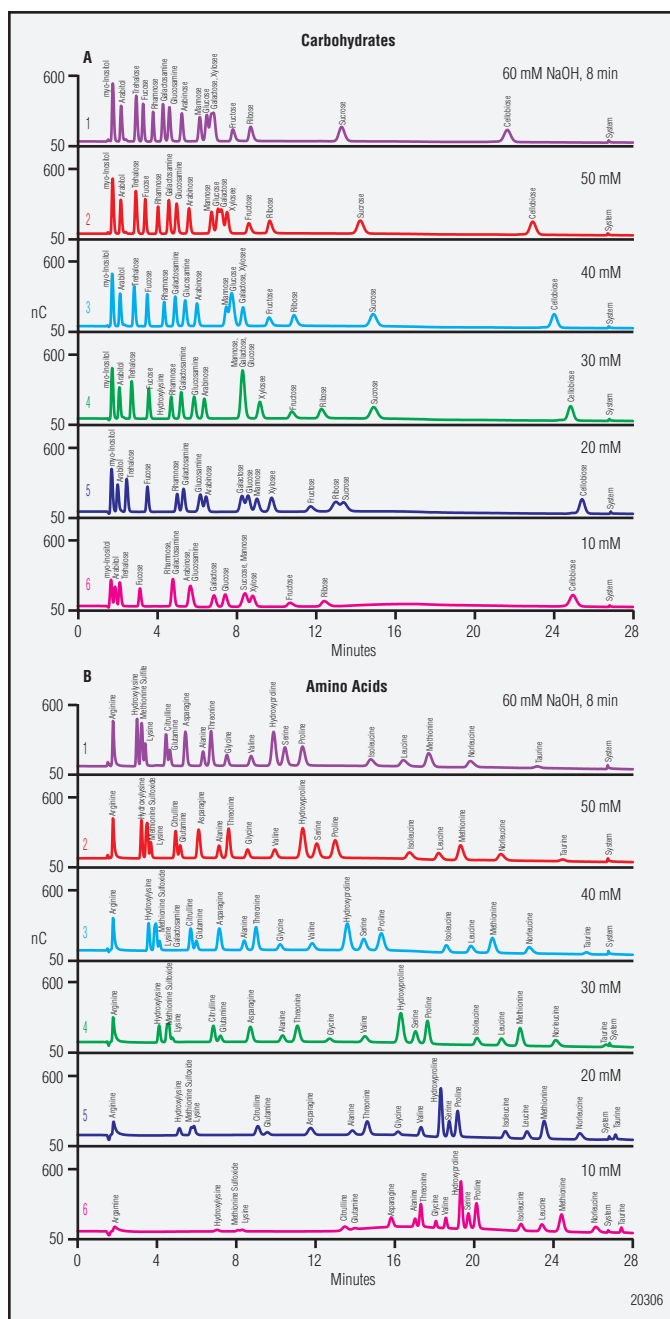


Figure 4. The effect of varying initial sodium hydroxide eluent concentration on (A) carbohydrate retention times and (B) amino acid retention times.

Measured retention times of 30 amino acids and 42 carbohydrates using varying initial eluent (NaOH) were published for the previous gradient method,¹⁻³ and can be used to design specific separations; however, exact retention times may differ.

Our improved gradient methods were tested for suitability using complex undefined media, such as YPD broth supernatant, where good resolution of amino acids, carbohydrates, and many unidentified ingredient peaks was observed (Figure 5). Maltose was resolved from the main system peak, not previously accomplished.

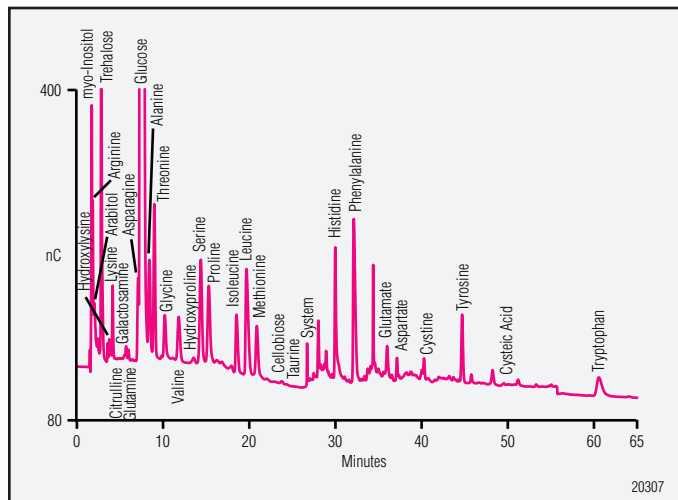


Figure 5. Separation of amino acids, carbohydrates, alditols in 100-fold dilution of YPD broth supernatant (25 μ L) using an improved AAA-Direct 40/8 gradient method.

In Figure 6, we could identify all amino acid and carbohydrate peaks expected to be present in Medium 199⁵; except cysteine, which converted to cystine under the alkaline conditions used for separation, and deoxy-ribose for which standards were unavailable at the time of this work. The presence of phenol red (pH indicator), sodium bicarbonate (buffer), and the many other ingredients (Table 2) did not appear to cause interference at a 10-fold dilution. Trace amounts of fructose and sucrose, both common impurities of dextrose, were detected.

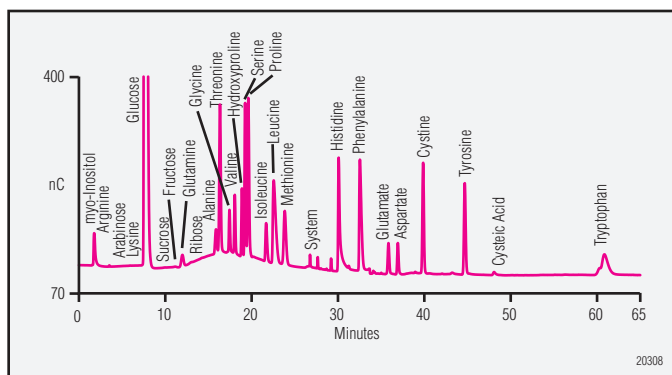


Figure 6. Separation of amino acids in Medium 199 (10-fold dilution, 25 μ L) using an improved AAA-Direct 15/8 gradient method.

In Figure 7, we could identify all amino acid and carbohydrate peaks expected to be present in Dulbecco's Modified Eagle's: F-12 Ham Mixture⁵ (Table 2), except cysteine for the reason described above. Leucine and HEPES buffer were found to coelute. Peak identities and estimations of their relative concentrations for the ingredients of this coeluting peak were successfully determined using 3-D amperometry.⁶ The amino acids were measured close to their expected levels.

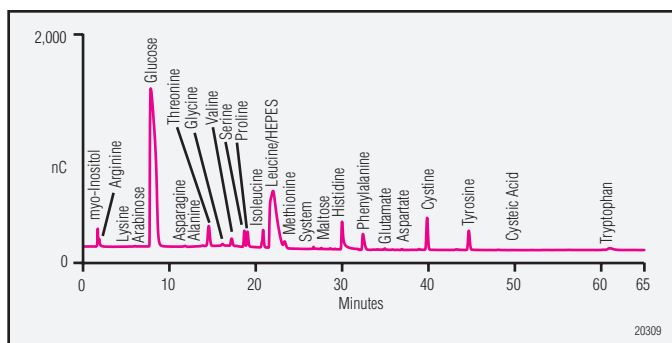


Figure 7. Separation of amino acids in Dulbecco's Modified Eagle's: F-12 Ham Mixture (10-fold dilution, 25 μ L) using an improved AAA-Direct 15/8 gradient method.

Table 2. Media Ingredients				
	Medium 199	Dulbecco's ME: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
Amino Acids				
L-Alanine	+	+	+	+
L-Arginine HCl	+	+	+	+
L-Asparagine		(+)	+	+
L-Aspartic Acid	+	(+)		+
L-Cysteine HCl	+	+	+	+
L-Cystine	+			
L-Glutamic Acid	+	(+)		(-)
L-Glutamine	+	(-)	(-)	(-)
Glycine	+	+	+	+
L-Histidine HCl	+	+	+	+
L-Hydroxyproline	+	+	+	+
L-Isoleucine	+	+	+	+
L-Leucine	+	+	+	+
L-Lysine HCl	+	+	+	+
L-Methionine	+	+	(-)	+
L-Phenylalanine	+	+	+	+
L-Proline	+	+	+	+
L-Serine	+	+	+	+
L-Threonine	+	+	+	+
L-Tryptophan	+	+	+	+
L-Tyrosine	+	+	+	+
L-Valine	+	+	+	+
Carbohydrates				
Dextrose (Glucose)	+	(+)		(+)
D (+) Galactose			+	
Deoxyribose	+			
Ribose	+			
Vitamins				
Aminobenzoic Acid (p-)	+			+
Ascorbic Acid	+			+
Biotin (D-)	+	+		+
Calciferol	+			
Choline Chloride	+	+	+	+
Flavin Mononucleotide, sodium			(+)	
Folic Acid	+	+	+	+
Inositol (-, or myo-)	+	+	+	+
Menadione	+			
Nicotinamide	+	+	+	+
Nicotinic Acid	+			
Pantothenate (D-Ca-)	+	+	+	+
Pyridoxal HCl	+	+	+	+
Pyridoxine HCl	+	(-)	+	+
Retinol Acetate	(+)			
Riboflavin	+	+		+
Riboflavin-5-phosphate, sodium			(-)	
Thiamine Monophosphate			+	
Thiamine HCl	+	+		+
Tocopherol (DL-alpha-) Phosphate	+			
Vitamin A	+			
Vitamin B ₁₂		+		+
Inorganic Salts				
CaCl ₂	+	(+)	(+)	(+)
CuSO ₄		+		
Fe(NO ₃) ₃	+	+		
FeSO ₄		(+)		
KCl	+	+	(+)	(+)
MgCl ₂	(-)	(+)	(+)	(+)
MgSO ₄	+	(+)	(+)	(+)
NaCl	+	(+)	(+)	(+)
NaHCO ₃	(+)	(+)	(+)	(+)
Na ₂ HPO ₄		(+)	(+)	
NaH ₂ PO ₄	(-)	(+)	(+)	(+)
KH ₂ PO ₄	(+)		(+)	
ZnSO ₄		+		
Other Components				
Adenine HCl	+			
AMP dihydrate	+			
ATP, tetrahydrate	+			
Bacto-Peptone			(-)	+
Cholesterol	+			
Guanine HCl	+			
HEPES		(+)		
Hypoxanthine	+	+		
L-Glutathione (reduced)	+		(-)	+
Linoleic Acid		(+)		
Phenol Red	+		(+)	(+)
Putrescine		+		
Sodium Acetate	+			
Sodium Pyruvate			(+)	
Thioctic Acid (DL-)		(+)		
Thymidine		+		
Thymine	+			
Tween 80	+			
Uracil	+			
Xanthine	+			

(+) Manufacturer's specific additions, not present in published recipe (see Reference 4).

(-) Manufacturer's specific omissions, originally present in published recipe (see Reference 4).

In Figure 8, all the expected amino acids in L-15 (Leibovitz) medium are identified (Table 2). Lysine (Lys), which coelutes with galactose at 15 mM NaOH (15/8) gradient conditions, is resolved at 20 mM NaOH (method 20/8). L-15 contains galactose instead of the commonly used glucose (dextrose) as a carbon source during cell culture. Glutamine was omitted from this media, aspartate and glutamate are not normal ingredients of this formulation, and as expected, none were detected (results not shown).

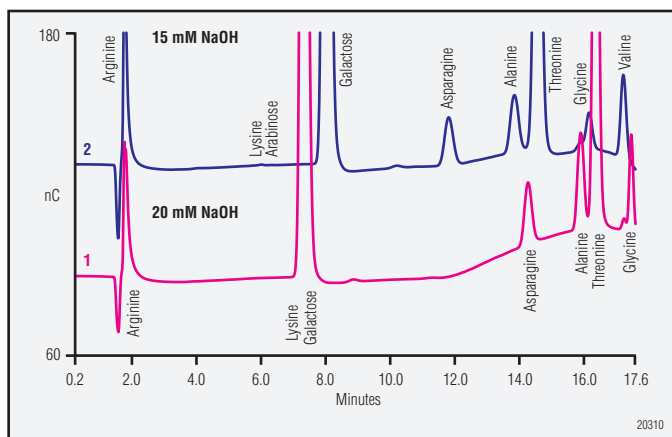


Figure 8. Separation of Lys from galactose in L-15 (Leibovitz) medium (100-fold dilution, 25 μ L) by adjusting the gradient method from (1) 15/8 to (2) 20/8. Galactose and Lys coelute using the 15/8 method.

In Figure 9, all the expected amino acids in McCoy's 5A medium were identified including hydroxyproline (Table 2), except cysteine.

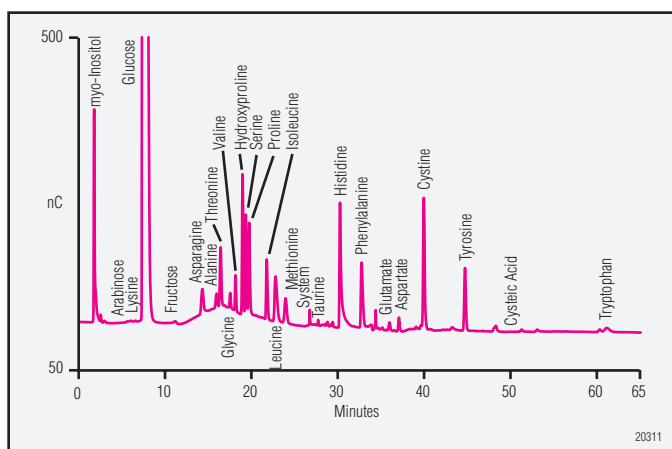


Figure 9. Separation of amino acids and carbohydrates in McCoy's 5A medium (10-fold dilution, 25 μ L) using an improved AAA-Direct 15/8 gradient method.

In all the media studied using the improved *AAA-Direct* gradient methods, numerous unidentified peaks were observed. We speculate that with additional studies, many of these peaks may be identified as important constituents of media (e.g., specific vitamins, or other components). We believe the improved resolution of these peaks using the gradient methods described in this paper makes future discoveries possible.

SUMMARY

- The revised gradient methods improved resolution of His, Phe, Asp, Glu, cystine from minor system peaks, and maltose from the major system peak (compare Figures 2 and 3).
- Addition of NaOH to eluent channels sensitive to bioburden improved system robustness by maintaining sterility, reducing minor system peaks.
- Addition of acetic acid column wash significantly reduced carryover of His, Asp, and Glu.
- These changes were compatible with and improved determinations of amino acids in cell culture and fermentation broth media (Figures 5–9) even those with sodium bicarbonate, phenol red additives.

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6. Peak Identification and Estimation of Percent Purity using HPAE with 3-D Amperometry. Technical Note 163; LPN 1757. Dionex Corporation, Sunnyvale, CA, 2005.

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