DIONEX

Application Update 152

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An Improved Gradient Method for the *AAA-Direct*™ Separation of Amino Acids and Carbohydrates in Complex Sample Matrices

INTRODUCTION

AAA-Direct is an established technique for amino acid analysis. This technique has proven capabilities for the separation of amino acids and carbohydrates in complex samples containing a large number of ingredients such as fermentation broths and cell culture media.[1-4] The highly sensitive direct detection capability of amperometry used in *AAA-Direct* eliminates the need for pre- or post-column derivatization. Chemical derivatization techniques complicate analysis, add cost for expensive reagents, introduce safety hazards to lab personnel exposed to toxic solvents, and add a hazardous waste stream that must be safely disposed. *AAA-Direct* eliminates these complications. The high sensitivity of amperometric detection enables determinations of amino acids and carbohydrates down to the femtomole level.[5,6]

In this Application Update, we combine into a single method individual improvements made to the standard *AAA-Direct* gradient program. These individual improvements are now in the *AAA-Direct* manual.[7] The long-term stability of the *AAA-Direct* system is improved by adding 10 mM NaOH to the water (typically Channel A), and by adding 25 mM NaOH to the 1 M sodium acetate (Channel C). The standard *AAA-Direct* gradient program has two eluent channels that are pH-neutral. Over time microorganisms may contaminate the system and can cause the appearance of undesirable system peaks during the acetate gradient. The addition of NaOH to these formerly pH-neutral eluent channels reduces the growth of microorganisms in the eluent bottles and tubing delivering eluent to the pump, significantly reducing system peaks.

Using the standard *AAA-Direct* method, trace histidine (His), aspartate (Asp), and glutamate (Glu) may appear as carryover peaks, a result of their interaction with the analytical column. Improved methodology also prescribes the use of a fourth channel containing 100 mM acetic acid for additional column washing. The addition of a brief automated acidic column wash effectively removes residual His, Asp, and Glu prior to the next injection. We modified the acetate gradient program to improve the resolution of amino acids and carbohydrates from a greater number of ingredient peaks typically found in fermentation and cell culture media, and from unknown system peaks.

This update shows that such combined changes to the *AAA-Direct* method improve the performance of our previously published method.[1,2,7] This new method successfully determines amino acids in yeast extract-peptone-dextrose yeast culture medium (YPD Broth), and Dulbecco's modified Eagle's (with F-12), M199, L-15 (Leibovitz), and McCoy's 5A media for mammalian cell culture.

EQUIPMENT

Dionex ICS-3000 system consisting of:

- Gradient Pump (optimized for 2-mm i.d. columns), with degas option.
- DC Detector Chromatography Module with Dual Temperature and Electrochemical Detector with:
  - Combination pH/Ag/AgCl reference electrode
  - *AAA-Certified*™ Disposable Au Working Electrode (P/N 060082 for pack of 6; P/N 060140 for 4 bundled packages)
  - *AAA-Certified* Au Working Electrode (Conventional, P/N 061749)
- AS Autosampler
- Chromeleon® Chromatography Management Software

CONDITIONS

AAA-Direct Method:

Columns:	AminoPac® PA10 Analytical (P/N 55406)
	AminoPac PA10 Guard (P/N 55407)
Flow Rate:	0.25 mL/min
Eluent:	(A) 10 mM NaOH
	(B) 250 mM NaOH
	(C) 1 M Sodium acetate, 25 mM NaOH
	(D) 100 mM Acetic acid
Inj. Volume:	10 µL or 25 µL
Temperature:	30 ˚C
Detection:	Integrated pulsed amperometry, disposable or conventional Au working electrodes

AAA-Direct Waveform:

	Potential (V)			
Time (s)	*vs. pH*	*Gain Region**	*Ramp**	*Integration*
0.00	+0.13	Off	On	Off
0.04	+0.13	Off	On	Off
0.05	+0.33	Off	On	Off
0.21	+0.33	On	On	On
0.22	+0.55	On	On	On
0.46	+0.55	On	On	On
0.47	+0.33	On	On	On
0.56	+0.33	Off	On	Off
0.57	-1.67	Off	On	Off
0.58	-1.67	Off	On	Off
0.59	+0.93	Off	On	Off
0.60	+0.13	Off	On	Off

* Parameters not used in the ICS-2500, DX-500, DX-600, or BioLC System programs.

Table 1. *AAA-Direct* Gradient Methods

METHOD 1
Standard *AAA-Direct* Gradient Method

Initial NaOH Eluent Concentration: 60 mM
Isocratic Time (min): 2 min
***AAA-Direct* Method Name: 60/2**

Event Time (min)	Curve Type	%A Water	%B 250 mM NaOH	%C 1 M Sodium Acetate
0.0	5	76	24	0
2.0	5	76	24	0
8.0	8	64	36	0
11.0	8	64	36	0
18.0	8	40	20	40
21.0	5	44	16	40
23.0	8	14	16	70
42.0	5	14	16	70
42.1	5	20	80	0
44.1	5	20	80	0
44.2	5	76	24	0
75.0	5	76	24	0

METHOD 2
Improved Standard *AAA-Direct* Gradient Method

Initial NaOH Eluent Concentration: 60 mM
Isocratic Time (min): 2 min
***AAA-Direct* Method Name: 60/2**

Event Time (min)	Curve Type	%A 10 mM NaOH	%B 250 mM NaOH	%C 25 mM NaOH +1 M Sodium Acetate
0.0	5	79.2	20.8	0.0
2.0	5	79.2	20.8	0.0
8.0	8	66.7	33.3	0.0
11.0	8	66.7	33.3	0.0
18.0	8	45.8	14.2	40.0
21.0	5	50.0	10.0	40.0
23.0	8	21.9	8.1	70.0
42.0	5	21.9	8.1	70.0
42.1	5	20.8	79.2	0.0
44.1	5	20.8	79.2	0.0
44.2	5	79.2	20.8	0.0
75.0	5	79.2	20.8	0.0

METHOD 3
Modified *AAA-Direct* Gradient Method

Initial NaOH Eluent Concentratration: Generic
Isocratic Time (min): 8 min
***AAA-Direct* Method Name: X/8**

Event Time (min)	Curve Type	%A 10 mM NaOH	%B 250 mM NaOH	%C 25 mM NaOH + 1 M Sodium Acetate	%D 100 mM Acetic Acid
0.0	5	100-%B	*	0.0	0.0
8.0	5	100-%B	*	0.0	0.0
14.0	8	66.7	33.3	0.0	0.0
17.0	5	66.7	33.3	0.0	0.0
24.0	8	1.0	89.0	10.0	0.0
27.0	5	1.0	89.0	10.0	0.0
30.0	8	0.0	80.0	20.0	0.0
32.0	5	0.0	80.0	20.0	0.0
34.0	8	40.0	30.0	30.0	0.0
36.0	5	30.0	30.0	30.0	0.0
38.0	8	30.0	30.0	40.0	0.0
40.0	5	30.0	30.0	40.0	0.0
42.0	8	20.0	30.0	50.0	0.0
44.0	5	20.0	30.0	50.0	0.0
46.0	8	10.0	30.0	60.0	0.0
48.0	5	10.0	30.0	60.0	0.0
50.0	8	0.0	30.0	70.0	0.0
62.0	5	0.0	30.0	70.0	0.0
62.1	8	0.0	0.0	0.0	100.0
64.1	5	0.0	0.0	0.0	100.0
64.2	8	20.0	80	0.0	0.0
66.2	5	20.0	80	0.0	0.0
66.3	5	100-%B	*	0.0	0.0
92.0	5	100-%B	*	0.0	0.0

* To obtain the following initial concentrations of NaOH (mM), substitute the following %B at Event Times 0, 8, 66.3, and 92 min in Method 3 above:

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		

Note: In this document, conditions are described as x/y, where x is the initial NaOH eluent concentration, and y is the isocratic time for this eluent. For example, method 20/8 refers to the program method using 20 mM NaOH as the starting eluent concentration, and it is held for 8 min before the start of the NaOH gradient.

REAGENTS AND STANDARDS

Standards

Alanine (Sigma Chemical Co.)

Amino acid standard mix (NIST, Standard Reference Material 2389)

Asparagine (Sigma Chemical Co.)

Aspartate (Sigma Chemical Co.)

Cellobiose, D-, anhydrous (Sigma Chemical Co.)

Citrulline (Sigma Chemical Co.)

Cysteic Acid (Sigma Chemical Co.)

Cysteine (Sigma Chemical Co.)

Cystine (Sigma Chemical Co.)

Galactose, D-; reference grade (Pfanstiehl Laboratories)

Glucose, β-D-; reference grade (Pfanstiehl Laboratories)

Glutamate (Sigma Chemical Co.)

Glutamine (Sigma Chemical Co.)

Glycine (Sigma Chemical Co.)

HEPES (Sigma Chemical Co.)

Histidine (Sigma Chemical Co.)

Hydroxylysine (Sigma Chemical Co.)

Hydroxyproline (Sigma Chemical Co.)

Isoleucine (Sigma Chemical Co.)

Leucine (Sigma Chemical Co.)

Lysine (Sigma Chemical Co.)

Maltose, monohydrate; reference grade (Pfanstiehl Laboratories)

Methionine (Sigma Chemical Co.)

Methionine sulfoxide (Sigma Chemical Co.)

Ornithine (Sigma Chemical Co.)

Phenylalanine (Sigma Chemical Co.)

Proline (Sigma Chemical Co.)

Serine (Sigma Chemical Co.)

Taurine (Sigma Chemical Co.)

Threonine (Sigma Chemical Co.)

Trehalose, α-α-, dihydrate; reference grade (Pfanstiehl Laboratories)

Tryptophan (Sigma Chemical Co.)

Tyrosine (Sigma Chemical Co.)

Valine (Sigma Chemical Co.)

Culture and Media

Bacto™ YPD Broth (BD Diagnostics, Cat# 242820)

Dulbecco's Modified Eagle's Medium F12 (Sigma-Aldrich, Cat# D6421)

Medium 199 (Sigma-Aldrich Chemical Co., Cat# M4530)

L-15 Medium Leibovitz (Sigma-Aldrich Chemical Co., Cat# L5520)

McCoy's 5A Modified Medium (Sigma-Aldrich Chemical Co., Cat# M8403)

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium Hydroxide Eluents

10 mM and 250 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ-cm). All water is filtered through 0.2-µm Nylon filter (Nalgene, P/N 164-0020; Nalge Nunc International) under vacuum to degas. Biological contamination should be absent. It is important to minimize contamination Ωby carbonate, a divalent anion at high pH that is a strong eluent causing changes in amino acid and carbohydrate retention times. Commercially available NaOH pellets are covered with a thin layer of sodium carbonate and *should not* be used. A 50% (w/w) NaOH solution is much lower in carbonate (carbonate precipitates in this solution) and is the required source for NaOH.

Dilute 26 mL of 50% (w/w) NaOH solution into 1974 g of thoroughly degassed water to yield 250 mM NaOH. Dilute 1.05 mL 50% NaOH into 1999 g water to yield 10 mM NaOH. Immediately blanket the NaOH eluents under 4–5 psi helium or nitrogen to reduce carbonate contamination.

25 mM NaOH in 1 M Sodium acetate

To prepare 2 L of eluent, dissolve the contents of a bottle containing 82 g of the *AAA-Direct* Certified anhydrous sodium acetate in ~800 mL purified water. Adjust the total volume to 1.0 L with additional water using a dedicated graduated cylinder.

Filter this solution through a 1-L 0.2-µm Nylon filter unit (see comments above). Repeat for a second bottle and then gently combine into a 2-L plastic eluent bottle. Add 2.62 mL 50% NaOH to the 2.0 L sodium acetate solution, and immediately place it under 4–5 psi helium or nitrogen to reduce carbonate contamination.

100 mM Acetic Acid

To prepare 2 L of eluent, add 11.5 mL of HPLC Grade (99.7%) glacial acetic acid (17.5 M) to 1.5 L purified filter-degassed water and then bring the volume to 2.0 L. Immediately place it under 4–5 psi helium or nitrogen.

Keep the eluents blanketed under 5–8 psi (34–55 kPa) of inert gas (helium or nitrogen) at all times. On-line degassing is necessary because amperometric detection is sensitive to oxygen in the eluent. For older Dionex systems (e.g., models ICS-2500, DX-500, DX-600, BioLC), set the pump to degas for 30 s every 4 min. For the ICS-3000, the degas function is always on (but can be turned off for other applications, if desired). The degas status is checked in Chromeleon through the Control/Command/Pump_1/Degasser Vacuum pathway where the display is "OK" or "Not OK".

STANDARD AND SAMPLE PREPARATION

Standards

Solid standards were maintained desiccated under vacuum prior to use. They were dissolved in purified water to 10 g/L concentrations. These were combined and further diluted with water to yield the desired stock mixture concentrations. The solutions were maintained frozen at –20 ˚C until needed. The amino acid standard mix, SRM 2389, from NIST (320 µL) was diluted in 100 mL water to produce known concentrations with each amino acid ranging from 7.7 to 9.4 µM (except cystine, 3.7 µM). When needed, additional amino acids (e.g., tryptophan) and carbohydrates were added to the NIST amino acid standard mix during dilution.

YPD Broth Media

Bacto Yeast Extract-Peptone-Dextrose (YPD) Broth (1.0 g) was dissolved in 20 mL aseptically filtered (0.2 µm, Nylon) water. An aliquot was centrifuged at 16,000 *g* for 10 min and diluted 1000-fold in purified water. Diluted supernatant was analyzed directly.

Mammalian Cell Culture Medium

Dulbecco's Modified Eagle's Medium F12, Medium 199, L-15 Medium Leibovitz, McCoy's 5A Modified Medium were sterile commercially available (Sigma-Aldrich) ready-to-use liquids, that we diluted 10-, 100-, and 1000-fold with water for analysis. Diluted samples were analyzed directly. Their respective ingredients are listed in Table 2.

SYSTEM PREPARATION AND SETUP

The preparation and setup of the *AAA-Direct* system are described in the Product Manual for *AAA-Direct* Amino Acid Analysis System.[7] For optimal performance, it is important that the guidelines provided in this manual be followed closely. Verification of performance should be performed. In an ICS-3000 using two separate systems and one AS autosampler the AS should be configured in *Sequential Mode* using a diverter valve, and each injection port volume accurately calibrated prior to use. The Chromeleon program file for the ICS-3000 should be programmed to contain an audit log command for pH, background, and backpressure at 0.00 min to assist in tracking system performance. The pH recorded by the reference electrode in the electrochemical cell should remain ±0.5 pH units from the theoretical pH for a given hydroxide eluent applied to the beginning of the gradient program (e.g., 60 mM NaOH should be pH 12.8, and 15 mM NaOH should be pH 12.2). A deviation from this range is an indication of excessive reference electrode wear, and may require its replacement (routinely required every 3–6 months for the ED40/50 reference electrode, and 6–12 months for the ICS-3000 reference electrode). Disposable electrodes should be replaced after seven days of continuous use. The background should remain within the range of 25–90 nC for the 60 mM NaOH concentration. Higher backgrounds are expected for lower NaOH concentrations. Backgrounds lower than this level at 60 mM NaOH may indicate in-line degas malfunction in the gradient pump, while higher values may be an indication of disposable electrode malfunction, or contamination of the eluents, column, or both. Variation in background is expected through the gradient program, as the NaOH concentration will change during this program. The backpressure of the combined new analytical and guard column set should be recorded ~1 h after it was first installed using 15–60 mM NaOH at 0.25 mL/min. Typical backpressure for new column sets range from 1960 to 2320 psi, thereafter the pressure should range from ±500 psi. Excessive backpressure is an indication of blockage to either the plumbing leading to the column, the frits of the guard column, or contamination of the columns. Contaminated columns may be cleaned following the instructions provided in the column manual.[7] An increase in backpressure (~500 psi) is expected through the gradient program, as the viscosity will increase with increasing NaOH and sodium acetate concentrations. Audit log values for pressure, pH, and background may be easily trended using features available in Chromeleon. Also, maximum and

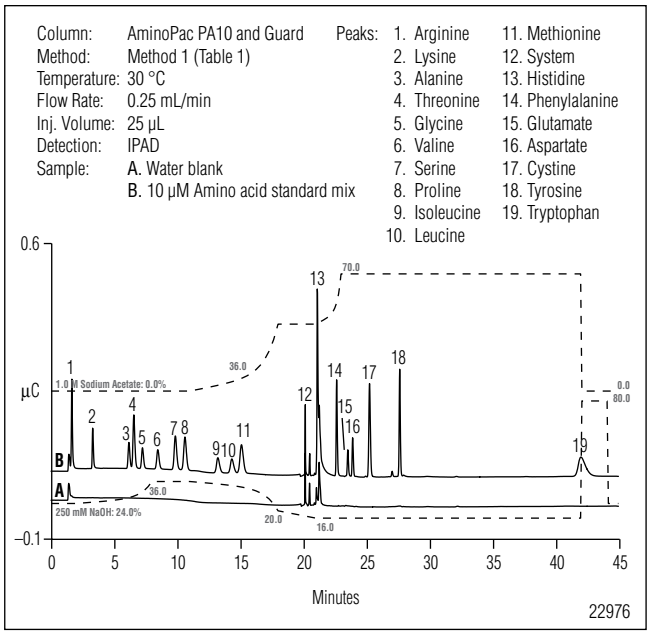

Figure 1. Separation of amino acid standards (NIST, supplemented with tryptophan) using the Standard AAA-Direct *Method (Table 1, Method 1). (A) Water, (B) amino acid standard mix (8 µm, 25-µL injection). Chromatography was performed on an ICS-2500.*

minimum threshold values may be programmed directly into the software so that an alarm is activated in Chromeleon when a value exceeds these limits.

RESULTS AND DISCUSSION

The standard *AAA-Direct* gradient method (Table 1, Method 1)[7] is suitable for most amino acid separations (Figure 1). However,

(1) His coelutes at times with minor system peaks resulting from impurities in the eluent and from sample matrix components collecting on the column and eluting during the acetate gradient.

(2) There is a tendency for these system impurities to increase over time from biological contamination of water and acetate eluent lines, and when working with complex biological samples.

(3) There is some His peak tailing.

(4) Unless a suitable rinsing procedure is incorporated in the gradient, there is 1–4% carryover of His, Asp, Glu, and Tyr from previous injections due to adsorption on the column.

(5) There is incomplete resolution of ingredient peaks present in complex culture media eluting during the acetate gradient. Figure 2 shows these deficiencies as they may appear after a prolonged period of use with complex biological samples.

To correct these deficiencies, we developed a method that includes NaOH in both the water (channel A) and 1 M sodium acetate (channel C) eluents to maintain sterility of the eluent lines (Table 1, Method 2). Method 2 has exactly the same gradient and eluent compositions as the standard *AAA-Direct* gradient program, but with adjustments to the percentages of channel A and B to reflect the presence of extra NaOH in these channels. The sizes of minor system peaks were reduced during the acetate gradient, which increased system ruggedness by reducing the need to perform system sanitization. We further modified the method (Table 1, Method 2) by adjusting the initial NaOH concentration and duration of the isocratic phase to better resolve amino acids from excessive amounts of carbohydrates typically present in fermentation broth and cell culture media. These gradient modifications are described in previous publications.[1,2] Method 3 (Table1) resolves the remaining small system peaks and the cell culture media ingredients from the His, Phe, Asp, Glu, and Cystine peaks. This improved resolution results from the addition of multiple acetate gradient steps. The addition of a 2-min 100 mM acetic acid column wash in Method 3 (Table 1) eliminated minor carryover of His, Phe, Asp, Glu, and Tyr, and thus increased the quantitative accuracy for these peaks near their lower limits of detection. Previous approaches to solving some of these deficiencies were described as separate method modules.[7] Here we incorporate all these improvements and present a single method, adaptable to varying initial sodium hydroxide eluent concentrations that are useful for altering carbohydrate retention time selectivities.[1,2] The new method reduces system peaks, resolves the remaining minor system peaks from the peaks of interest, increases long-term stability of the *AAA-Direct* system, eliminates trace carryover from previous injections, and improves resolution of media ingredient peaks. Good resolution of all amino acids is maintained without frequent system sanitization using 2 M NaOH as described in the *AAA-Direct* Manual.[7] Comparing Figures 2 and 3 shows the improvements realized with the new method. For example, the unknown peak eluting just after His in the water blank chromatogram using the standard gradient (Figure 2, Trace A) is absent in the water blank using the new gradient (Figure 3, Trace A). The unknown ingredient peak in YPD broth on the trailing edge of the Phe peak using the standard gradient (Figure 2, Trace C) is resolved into multiple small peaks eluting between Phe and Glu using the new method (Figure 3, Trace C).

The modified standard *AAA-Direct* methods (Table 1, Methods 2 and 3) were tested for compatibility with changes in initial eluent sodium hydroxide concentration and changes in duration of the initial sodium hydroxide concentration. We found that including NaOH in channels A and B did not alter the chromatography from what was typically observed, except that many system-related peaks were reduced or eliminated, and the chromatography was more reproducible from day-to-day. We also found that the method changes did not interfere with the selectivity changes achieved by varying initial sodium hydroxide concentration and duration of that eluent. These selectivity changes can be used to optimize separations of amino acids from carbohydrates in a variety of cell culture media. The previously published retention times of 30 amino acids, and 42 carbohydrates using varying initial eluent sodium hydroxide concentration[1,2] can still be used to design specific separations. Although the absolute values of most retention times will differ with the new methods, the order of elution remains the same.

The modified gradient method (Table 1, Method 3) was tested for suitability using complex undefined media, such as YPD Broth supernatant. Figure 4A shows a separation of YPD Broth supernatant (1000-fold dilution) using the previously published 40/8 method[1,2] where incomplete resolution of some amino acids and unidentified ingredient peaks are observed in the acetate gradient region of the chromatogram. Figure 4B shows the improved resolution of amino acids and unidentified ingredient peaks using the new modified 40/8 method (Table 1, Method 3). For example, the improved separation resolves maltose from the main system peak, which was not previously attained using either the standard *AAA-Direct* gradient program (Methods 1 and 2), or the previously published gradient programs using varying initial [NaOH].[1,2] Figure 5 shows the same broth analyzed at 100-fold dilution using a second gradient method (Method 3, 40/8), and demonstrates good resolution even at a higher sample load.

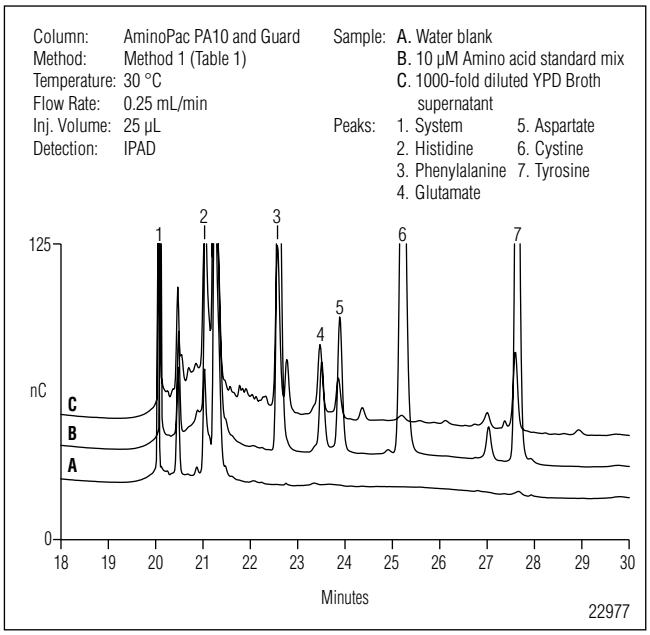

Figure 2. Separation of amino acids in the acetate gradient region of the chromatogram for a nonsanitized system using the Standard AAA-Direct *method (Method 1, Table 1, 25-µL injection). Comparison of the (A) water blank, (B) 10 µM NIST amino acid standard mix, and (C) 1000-fold diluted YPD Broth supernatant. Chromatography was performed on an ICS-2500.*

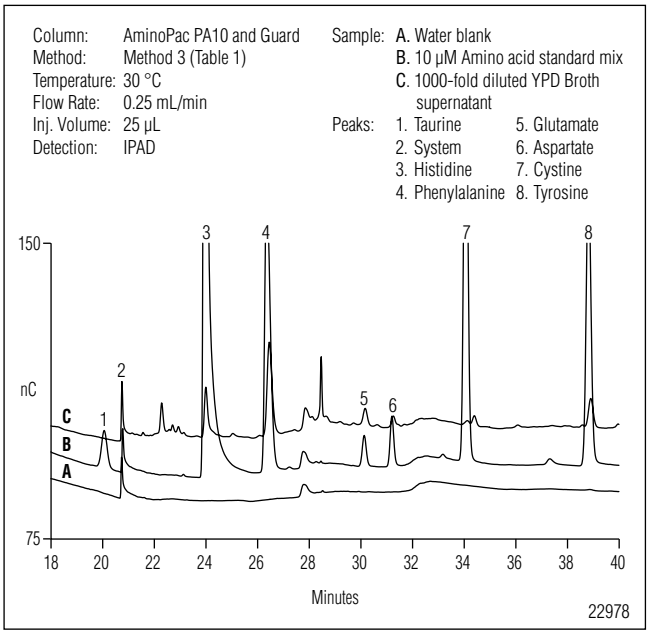

Figure 3. Separation of amino acids in the acetate gradient region of the chromatogram using a modified AAA-Direct *method (Method 3, Table 1, 25-µL injection). Comparison of the (A) water blank, (B) 10 µM amino acid standard mix, and (C) 1000-fold diluted YPD Broth supernatant. Chromatography was performed on an ICS-2500.*

In Figure 6, using method 15/8 (Table 1, Method 3) we identified all amino acid and carbohydrate peaks expected to be present in Medium 199[8] (Table 2) except cysteine, which is converted to cystine under the alkaline conditions used for separation, and deoxyribose, which was not analyzed. At a tenfold dilution, the presence of phenol red (pH indicator), sodium bicarbonate (buffer), and the many other ingredients (Table 2) did not appear to interfere with analyte quantification. Trace amounts of fructose and sucrose, both common impurities of dextrose, were detected. Their elution positions are marked, but their peaks are not observed using the scale of Figure 6. When the baseline is zoomed to higher magnification (not shown), trace peaks are visible. These results observed using the ICS-3000 are identical to those previously published using the ICS-2500.[3]

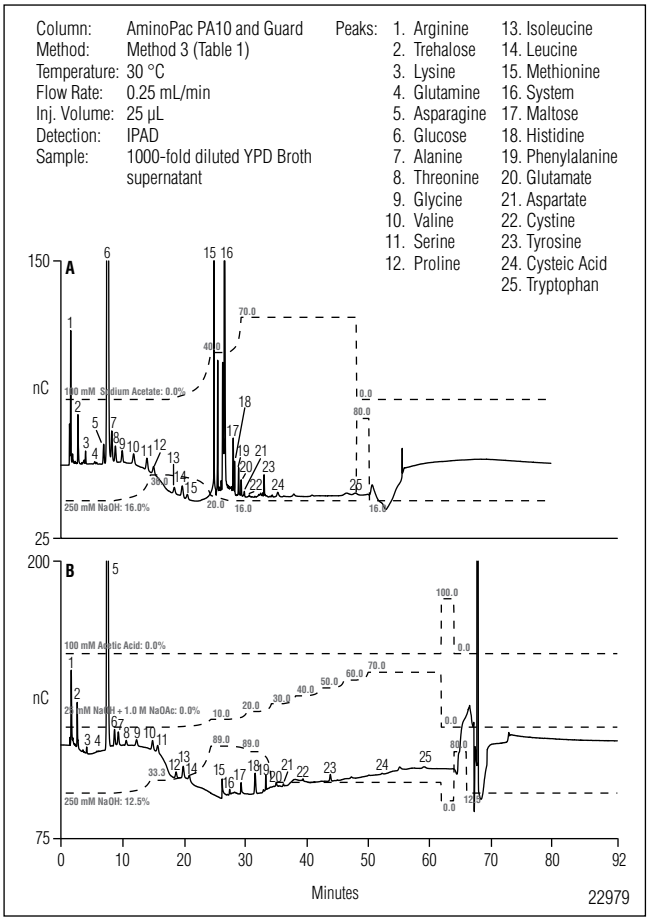

Figure 4. Separation of amino acids, carbohydrates, alditols in 1000-fold dilution of YPD Broth supernatant (10 µL) using (A) the previously published[1,2] AAA-Direct *40/8 gradient method, and (B) the new modified 40/8 gradient method (Table 1, Method 3). Chromatography was performed on an ICS-2500.*

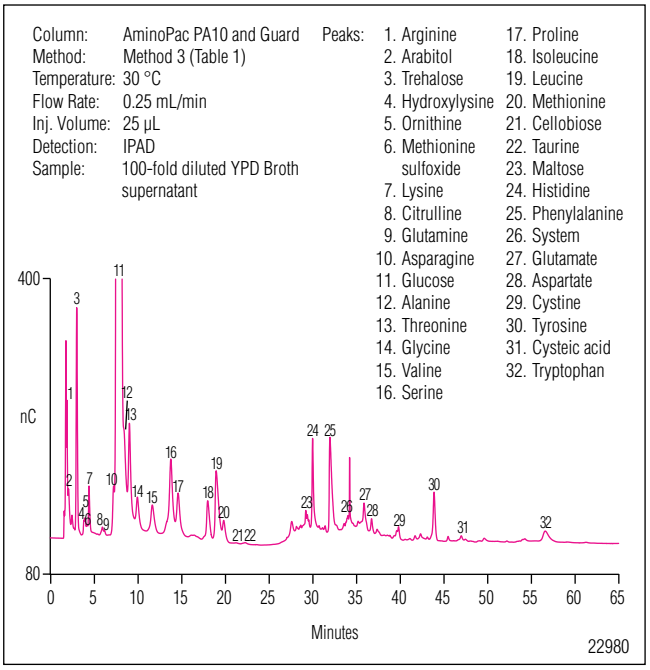

Figure 5. YPD broth supernatant (100-fold dilution, 25-µL injection) using the 40/8 gradient method (Table 1, Method 3).

Figure 7 shows that a 15/8 method (Method 3), separates all amino acid and carbohydrate peaks expected to be present in Dulbecco's Modified Eagle's: F-12 Ham Mixture (Table 2); except cysteine for the reason explained above. A higher than expected level of leucine (Leu) was observed in this media. The concentration stated by the manufacturer was 59 µg/mL, and the measured concentration was 990 µg/mL. The other amino acids were measured close to their expected levels. Under specific circumstances, 3-D Amperometry can be used for peak identification and for estimation of concentration for two coeluting peaks. Using 3-D amperometric techniques described in Technical Note 63,[9] the presumed Leu peak in this medium was determined to be HEPES coeluting with Leu and Met, and its estimated concentration corresponded closely with the manufacturer's expected value.

All the expected amino acids in L-15 (Leibovitz) medium are identified (Table 2). In Figure 8, asparagine (Asn) coelutes with galactose at 30 mM NaOH (Method 3, condition 30/8), but is resolved at either 25 mM NaOH (Method 3, condition 25/8) or at 35 mM NaOH (Method 3, condition 35/8). L-15 contains galactose instead of the commonly used glucose (dextrose) as a carbon source for cell culture. Glutamine is a common supplement to cell culture media, but was omitted from this medium.

Table 2. Media Ingredients

AMINO ACIDS

Ingredient	Medium 199	Dulbecco's Modified Eagle: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
L-Alanine	+	+	+	+
L-Arginine HCl	+	+	+	+
L-Asparagine		(+)	+	+
L-Aspartic Acid	+	(+)		+
L-Cysteine HCl	+	+	+	+
L-Cystine	+	+		
L-Glutamine Acid	+	(+)		
L-Glutamine	+	(–)	(–)	(–)
L-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

Ingredient	Medium 199	Dulbecco's Modified Eagle: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
Dextrose (Glucose)	+	(+)		+
D (+) Galactose			+	
Dexoyribose	+			
Ribose	+			
Inositol (i-, or myo-)	+	+	+	+

INORGANIC SALTS

Ingredient	Medium 199	Dulbecco's Modified Eagle: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
$CaCl_2$	+	(+)	(+)	(+)
$CuSO_4$		+		
$Fe(NO_3)_3$	+	+		
$FeSO_4$		+		
KCl	+	(+)	(+)	(+)
$MgCl_2$	(–)	(+)	(+)	
$MgSO_4$	+	(+)	(+)	(+)
NaCl	+	(+)	(+)	(+)
$NaHCO_3$	(+)	(+)		(+)
Na_2HPO_4		(+)	(+)	
NaH_2PO_4	(–)	(+)		(+)
KH_2PO_4	(+)		(+)	
$ZnSO_4$		+		

VITAMINS

Ingredient	Medium 199	Dulbecco's Modified Eagle: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
Aminobenzoic Acid (p-)	+			+
Ascorbic Acid	+			+
Biotin (D-)	+	+		+
Calciferol	+			
Choline Chloride	+	+	+	+
Flavin Mononucleotide, sodium			(+)	
Folic Acid	+	+	+	+
Menadione	+			
Nicotinamide	+	+	+	+
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}		+		+

OTHER COMPONENTS

Ingredient	Medium 199	Dulbecco's Modified Eagle: F-12 Ham Mixture	L-15 (Leibovitz) Medium	McCoy's 5A Medium
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	+			

+ indicates ingredient was part of the original recipe[8], (–) was removed by the manufacturer, (+) ingredient was not part pf the original recipe[8], but added by the manufacturer.

Figure 9 shows that 15/8 method (Method 3) separates all expected amino acids in McCoy's 5A medium, including hydroxyproline (Table 2).

In all the media studied using the improved *AAA-Direct* gradient methods, there were numerous unidentified peaks. We speculate that with additional studies, some of the unknown peaks will be identified as important consituents of media (e.g., specific vitamins). We believe the improved resolution of these peaks using the gradient methods described in this update enables future insights beyond our original scope of amino acid and carbohydrate detection.

PRECAUTIONS AND RECOMMENDATIONS

Retention time reproducibility may be affected by new preparations of the dilute NaOH used for channel A (10 mM) and the 25 mM NaOH added to channel C. The inadvertent accumulation of carbonate in these eluents either during initial preparation or as a result of leakage, the porosity of the eluent bottle, or the slight variation in the sodium hydroxide concentration from pipetting the viscous 50% NaOH, contribute to these slight variations in retention times. Adding the 50% NaOH by weight can improve retention time reproducibility. Standards should always be run after eluents have been changed to detect retention time variations.

New columns, or columns stored for periods longer than one week, or columns operated at low eluent strength for more than one day, should be washed with 20% B and 80% C for 1 h prior to reequilibration to starting conditions with the electrochemical cell turned off (ideally, removed from the flow path).

During system shutdowns for periods longer than one week, the cell should be disassembled with the reference electrode stored in its shipping container containing 3.0–3.5 M KCl. Additionally, the column should be flushed with 10 mM NaOH, removed, and its ends sealed to prevent drying. Alternatively, the system may be operated continuously with the cell turned off using 34% A, 33% B, and 33% C at a low flow of 0.05 mL/min, replenishing the eluents as needed.

The reduction of the initial NaOH concentration used to alter selectivity of carbohydrates relative to amino acids can also cause a reduction in the resolution of serine and proline for partially contaminated columns. Replacement of the first frit of the analytical column, replacement of the guard column, and following the

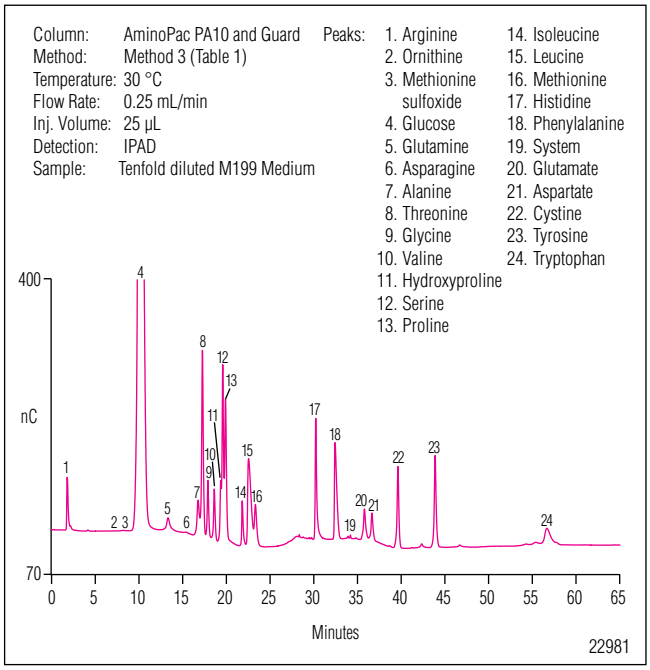

Figure 6. Separation of amino acids from glucose in M199 Medium (tenfold dilution, 25-µL injection) using the 15/8 gradient method (Table 1, Method 3).

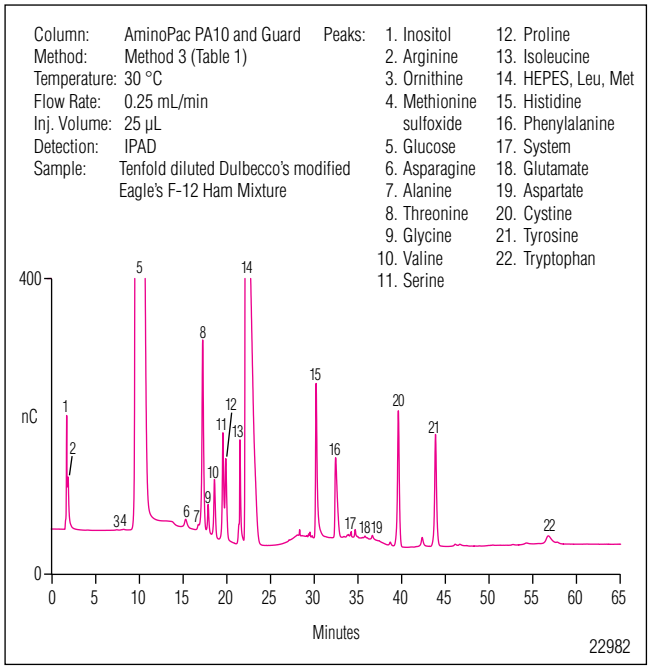

Figure 7. Separation of Dulbecco's Modified Eagle's F-12 Ham Mixture (tenfold dilution, 25-µL injection) using the 15/8 gradient method (Table 1, Method 3).

MeCN/HCl column wash procedure in the *AAA-Direct* manual[7] is normally successful in restoring this separation.

When determinations of carbohydrates are not needed, the Carbohydrate Removal Accessory (CRA) should be considered. This device will remove neutral sugars (e.g., glucose, sucrose, glactose, fructose, etc.) from samples before their injection for amino acid analysis. This device for the Dionex ICS 3000 is described in Technical Note 69.[10]

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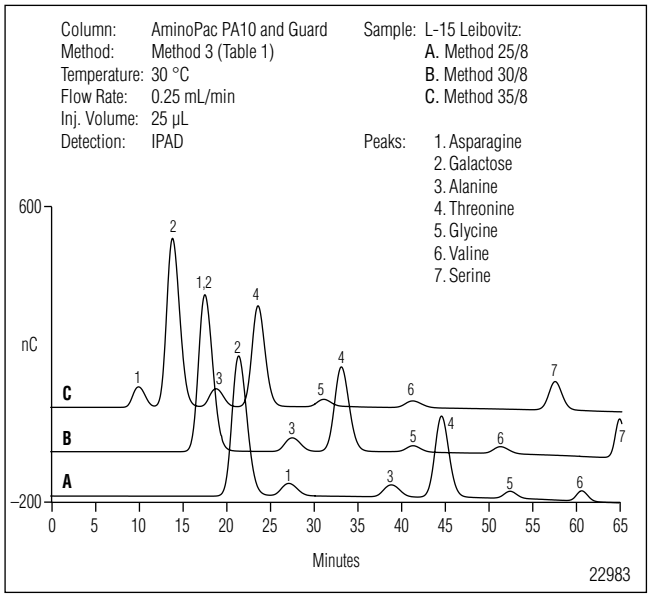

Figure 8. Coelution of asparagine and galactose in L15 Leibovitz Medium (100-fold dilution, 25-µL injection) using the method 30/8 (B), and their resolution using (A) method 25/8 and (C) method 35/8 (Table 1, Method 3).

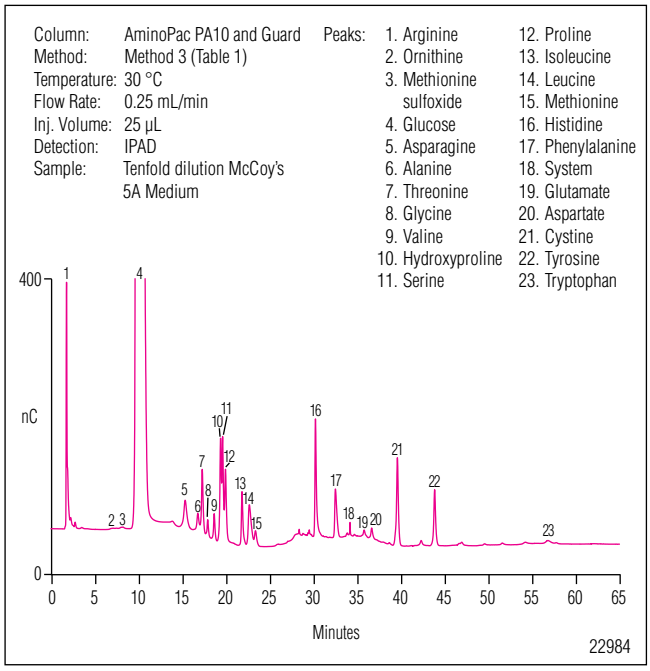

Figure 9. Separation of McCoy's 5A Medium (tenfold dilution, 25-µL injection) using the new modified 15/8 gradient method (Table 1, Method 3).

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4. Fluka Biochemika, 1001 West St. Paul Avenue, P.O. Box 2060, Milwaukee, WI 53201, USA. Tel: 800-558-9160. Web: www.sigma-aldrich.com.
5. National Institute of Standards & Technology (NIST), 100 Bureau Drive, Gaithersburg, MD 20899, USA. Tel: 301-975-2000. Web: www.nist.gov.
6. Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126, USA., Tel: 800-766-7000. Web: www.fishersci.com.
7. Gast Manufacturing Corporation, 2300 South Highway M139, P.O. Box 97, Benton Harbor, MI 49023-0097, USA. Tel: 616-926-6171. Web: www.gastmfg.com.
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