DIONEX

Application Note 150

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Determination of Amino Acids in Cell Cultures and Fermentation Broths

INTRODUCTION

Cell cultures and fermentation broths are used in the manufacture of biotherapeutics and many other biological materials produced using recombinant genetic technology. Broths are also used for production of methanol and ethanol as alternative energy sources to fossil fuels. Fermentation broths are complex mixtures of nutrients, waste products, cells and cell debris, and desired products, such as protein biotherapeutics (cytokines, monoclonal antibodies, etc). Recently, there is more interest in characterizing the amino acids and their metabolic by-products in fermentation broths because these components affect the yield of the desired products. Essential amino acids (for humans: Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val) are those that the organism is incapable of synthesizing from other nutrients (other amino acids, carbohydrates, etc), and therefore must obtain through diet. Furthermore, when other carbon sources (typically carbohydrates) are unavailable, amino acids are used for energy production through the urea cycle and gluconeogenic pathways. By-products of amino acid metabolism, including ammonia, urea, α-keto acids, fumarate, citrulline, ornithine, malate, oxaloacetic acid, and others, can reduce desired yields or alter final product quality.

Most amino acids are poor chromophores and require high concentrations to be detected by UV absorbance. Many of the ingredients of fermentation broths or cell cultures are chromophoric and can interfere with the direct detection of amino acids by absorbance. Amino acids, carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and therefore directly detected by amperometry. This detection method is specific for those analytes that can be oxidized at a selected potential, leaving all other compounds undetected. Integrated pulsed amperometric detection (IPAD) is a powerful detection technique with a broad linear range and very low detection limits. *AAA-Direct*™ is a combination of anion-exchange chromatography (AE) and IPAD. The high-performance anion-exchange column, AminoPac® PA10, is capable of separating all common amino acids.[1–3] For complex samples containing mixtures of both carbohydrates and amino acids—such as cell cultures and fermentation broths—*AAA Direct* allows the simultaneous determination of carbohydrates, glycols, sugar alcohols (alditols), and amino acids, with little interference from other broth ingredients.[4] Although pre- and postcolumn derivatization-based methods are commonly used to evaluate samples for amino acid content, these techniques suffer from higher operating costs and are unable to detect carbohydrates simultaneously with amino acids. *AAA-Direct* can simultaneously monitor, by direct injection, all common amino acids found in cell culture and fermentation broths using a single chromatographic method.

This application note describes the use of *AAA-Direct* to analyze common amino acids in the presence of simple sugars, sugar alcohols, alcohols, and glycols in yeast and bacterial fermentation broths. The yeast extract-peptone-dextrose (YPD) broth and the Luria-Bertani (LB) broths are common media for eukaryotic and prokaryotic cell cultures. Serum-free protein-free hybridoma medium, minimal essential medium (MEM), and fetal bovine serum are common mammialian culture media or additives. All are complex and contain undefined media ingredients. Thus they present a great challenge for most chromatographic methods. The YPD and LB broth formulations contain inorganic and organic anionic ingredients that have been analyzed using the IonPac® AS11 anion-exchange column with suppressed conductivity detection,[5–7] and contain carbohydrates and alditols that have been analyzed using the CarboPac™ PA1 and MA1 columns.[8–11] In this application note, the AminoPac PA10 anion-exchange column is used to separate amino acid and carbohydrate ingredients in cell culture and fermentation broth media. Typical cell culture and fermentation broth samples contain relatively high levels (100 mM) of glucose or other carbohydrates as carbon sources. High carbohydrate concentrations can hamper the determination of some amino acids due to coelution. The simultaneous determination of carbohydrate and all common amino acids depends on the type of carbohydrate(s) present. This application note presents alternative strategies that enable the determination of all amino acids in the presence of the most common fermentation and cell culture carbohydrates.

Also in this application note, disposable gold (Au) electrodes are evaluated for use in amino acid determination in cell culture and fermentation broths. Replacing or reconditioning the conventional Au working electrode increases the cost of analysis. A variability in electrochemical response from electrode to electrode, or from reconditioning to reconditioning is also possible. Dionex designed disposable Au working electrodes to provide greater electrode-to-electrode reproducibility and convenient replacement when working with difficult samples. These electrodes are guaranteed not to lose more than 20% of response for one week of continuous use.

EQUIPMENT

Dionex BioLC® system consisting of:

- GP50 Gradient Pump, microbore (2 mm) with degas option
- ED50 Electrochemical Detector with combination pH/Ag/AgCl reference electrode and:
  - *AAA-Direct* Certified Disposable Au Working Electrode (P/N 060082)
  - *AAA-Direct* Certified Au Working Electrode (Conventional; P/N 055832)
- AS50 Autosampler
- AS50TC Thermal Compartment
- Chromeleon® Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium hydroxide, 50% (w/w) (Fisher Scientific and J. T. Baker)

Deionized water, 18 MΩ-cm resistance or higher

Sodium acetate, anhydrous (*AAA-Direct* Certified, Dionex Corp., P/N 059326)

Standards

Acetyl-D-glucosamine, N- (Sigma Chemical Co.)

Alanine (Sigma Chemical Co.)

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

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

Arabitol, L- (Aldrich Chemical Co.)

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.)

Deoxy-D-glucose, 2-; reference-grade (Pfanstiehl Laboratories)

Erythritol (Pfanstiehl Laboratories)

Fructose, D-; reference-grade (Pfanstiehl Laboratories)

Fucose; reference-grade (Pfanstiehl Laboratories)

Galactitol; reference-grade (Pfanstiehl Laboratories)
Galactosamine; reference-grade (Pfanstiehl Laboratories)
Galactose, D-; reference-grade (Pfanstiehl Laboratories)
Gentiobiose (Sigma Chemical Co)
Glucopyranoside, *n*-Octyl-ß-D-; (Sigma Chemical Co)
Glucosamine, D-; reference-grade (Pfanstiehl Laboratories)
Glucose, ß-D-; reference-grade (Pfanstiehl Laboratories)
Glucuronic acid (Sigma Chemical Co)
Glutamate (Sigma Chemical Co)
Glutamine (Sigma Chemical Co.)
Glycerol (EM Science)
Glycine (Sigma Chemical Co.)
Histidine (Sigma Chemical Co)
Hydroxylysine (Sigma Chemical Co.)
Hydroxyproline (Sigma Chemical Co.)
Inositol (myo-) (Sigma Chemical Co.)
Isoleucine (Sigma Chemical Co)
Lactose, α-, monohydrate (Sigma Chemical Co.)
Lactulose (Sigma Chemical Co)
Leucine (Sigma Chemical Co)
Lysine (Sigma Chemical Co.)
Lyxose (Sigma Chemical Co.)
Maltitol (Aldrich Chemical Co.)
Maltose, monohydrate; reference-grade (Pfanstiehl Laboratories)
Maltohexaose (Sigma Chemical Co)
Maltotetraose (Sigma Chemical Co)
Maltotriose, hydrate (Aldrich Chemical Co.)
Mannitol, ACS-grade (J. T. Baker Incorporated)
Mannosamine (Sigma Chemical Co.)
Mannose (Sigma Chemical Co.)
Melibiose (Sigma Chemical Co)
Methanol (Fisher Scientific)
Methionine (Sigma Chemical Co)
Methionine sulfoxide (Sigma Chemical Co.)
Norleucine (Sigma Chemical Co.)
Ornithine (Sigma Chemical Co.)
Palatinose (Sigma Chemical Co)
Phenylalanine (Sigma Chemical Co)
Proline (Sigma Chemical Co)
Raffinose, pentahydrate; reference-grade (Pfanstiehl Laboratories)
Rhamnose, L-, monohydrate (Pfanstiehl Laboratories)
Ribitol; reference-grade (Pfanstiehl Laboratories)
Ribose, D-; reference-grade (Pfanstiehl Laboratories)
Serine (Sigma Chemical Co)
Sodium sulfite (Sigma Chemical Co.)
Sorbitol (Eastman Chemical Co.)
Sorbose (Sigma Chemical Co)
Sucralose (McNeil Nutritionals, Inc.)
Sucrose (Fisher Scientific)
Taurine (Sigma Chemical Co.)
Threonine (Sigma Chemical Co)
Thioglucose, ß-D; (Sigma Chemical Co)
Trehalose, α-α-, dihydrate; reference-grade (Pfanstiehl Laboratories)
Tryptophan (Sigma Chemical Co)
Turanose (Sigma Chemical Co)
Tyrosine (Sigma Chemical Co)
Urea (Sigma Chemical Co.)
Urocanic acid (Sigma Chemical Co.)
Valine (Sigma Chemical Co.)
Xylose, D-; anhydrous (Sigma Chemical Co.)

Culture and Media

Bacto® YPD Broth (BD Laboratories, Cat #0428-17-5)
LB Broth (BD Laboratories, Cat #0446-17-3)
Serum-Free Protein-Free Hybridoma Medium (Sigma-Aldrich Chemical Co., Cat #S-2897)
Minimal Essential Medium (MEM; Sigma-Aldrich Chemical Co., Cat #M-0643)

CONDITIONS

AminoPac PA10 Method:

Columns:	AminoPac PA10 Analytical (P/N 55406)
	AminoPac PA10 Guard (P/N 55407)
Flow Rates:	0.25 mL/min
Eluent:	A: Water
	B: 250 mM NaOH
	C: 1 M sodium acetate
Injection Volume:	10 or 25 µL
Temperature:	30 °C
Detection (ED50):	Integrated pulsed amperometry, disposable or conventional Au working electrodes; reference electrode in pH mode
Gradient:	See Table 1 below

Waveform for the ED50:

Time(s)	*Potential (V)*	*Integration*
0.00	+0.13	
0.04	+0.13	
0.05	+0.33	
0.21	+0.33	Begin
0.22	+0.55	
0.46	+0.55	
0.47	+0.33	
0.56	+0.33	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

Table 1. *AAA-Direct* Gradient Methods

Initial NaOH Eluent Concentration:				60 mM	60 mM	50 mM	40 mM	30 mM	25 mM	20 mM	15 mM	10 mM
Isocratic Time (min):				2	8	8	8	8	8	8	8	8
***AAA-Direct* Condition Name:**				60/2	60/8	50/8	40/8	30/8	25/8	20/8	15/8	10/8
***Initial %B (250 mM NaOH) at T = 0 min:**				**24%**	**24%**	**20%**	**16%**	**12%**	**10%**	**8%**	**6%**	**4%**
Event	**%B (NaOH)**	**%C (Acetate)**	**Curve Type**	**Event Time (min)**								
Start Initial, Start Isocratic	Initial %B*	0	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
End Initial, Start Gradient	Initial %B*	0	5	2.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Hold Isocratic NaOH	36	0	8	8.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Begin First Acetate Gradient	36	0	8	11.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0
Begin Reverse NaOH/Hold Acetate	20	40	8	18.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Begin Second Acetate Gradient	16	40	5	21.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0
Hold Isocratic NaOH/Acetate	16	70	8	23.0	29.0	29.0	29.0	29.0	29.0	29.0	29.0	29.0
End Isocratic NaOH/Acetate	16	70	5	42.0	48.0	48.0	48.0	48.0	48.0	48.0	48.0	48.0
Begin High NaOH Column Wash	80	0	5	42.1	48.1	48.1	48.1	48.1	48.1	48.1	48.1	48.1
End High NaOH Column Wash	80	0	5	44.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1
Begin Column Reequilibration	Initial %B*	0	5	44.2	50.2	50.2	50.2	50.2	50.2	50.2	50.2	50.2
End of Run	Initial %B*	0	5	75.0	81.0	81.0	81.0	81.0	81.0	81.0	81.0	81.0

*Initial % B is defined in the header of each column.

Note: In this document, eluent conditions (see Table 1) are described as x/y, where x is the initial NaOH eluent concentration, and y is the isocratic time for this eluent. For example, condition 20/8 refers to the program method using 20 mM NaOH as the starting eluent concentration, and this concentration is held for 8 min before the start of the NaOH gradient. The gradients that follow the isocratic NaOH portion of the separation are the same for all methods.

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium Hydroxide Eluents

250 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ-cm). Biological contamination should be absent. It is important to minimize contamination by carbonate, a divalent anion at high pH that is a strongly eluting species that causes changes in amino acid and carbohydrate retention times. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate (carbonate precipitates at this pH) and is the preferred source for sodium hydroxide.

Dilute 26 mL of 50% (w/w) sodium hydroxide solution into 1974 mL of thoroughly degassed water to yield 250 mM sodium hydroxide. Immediately blanket the NaOH eluent with inert gas.

1 M Sodium Acetate

To prepare 1 L of eluent, dissolve 82 g of *AAA-Direct* Certified anhydrous sodium acetate in ~800 mL purified water. Adjust the total volume to 1000 mL with additional water. Filter the solution through a 0.2-µm nylon filter unit (see comments above) and place it under 4–5 psi helium or nitrogen to reduce microbial contamination and carbonate buildup. Note that the use of a sodium acetate that is not *AAA-Direct* Certified can lead to a loss of detector response from contamination of the electrode by impurities.

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. Set the pump to degas for 30 s every 4 min.

STOCK STANDARDS

Solid standards were maintained desiccated under vacuum prior to use. They were dissolved in purified water to 10 g/L concentrations. These solutions were combined and further diluted with the same water used for eluent preparation 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 water to 100 mL to produce known concentrations, each amino acid ranging from 7.6 to 9.4 µM (except cystine, 3.7 µM). Aliquots of the amino acid standard mix are maintained frozen.

SAMPLE PREPARATION

YPD Broth Media

Bacto Yeast Extract-Peptone-Dextrose (YPD) broth (1.0 g) was dissolved in 20.0 mL asceptically 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.

LB Broth Media

Luria-Bertani (LB) broth was prepared at a concentration of 25 mg/mL with asceptically filtered (0.2 µm, nylon) water. The media was prepared as described for the YPD broth, but only diluted 100-fold. Diluted supernatant was analyzed directly.

Mammalian Cell Culture Media

Minimal essential medium (MEM) was prepared at a concentration of 9.66 mg/mL with aseptically filtered (0.2 µm, nylon) water. This media was prepared according to their recommended lot-specific concentrations, centrifuged at 16,000 × g for 10 min, and the supernatant was diluted 100-fold in water. The Serum-Free Protein-Free Hybridoma Medium[12,13] was a sterile, commercially available, ready-to-use liquid that we diluted 100-fold with water for analysis. Diluted samples were analyzed directly.

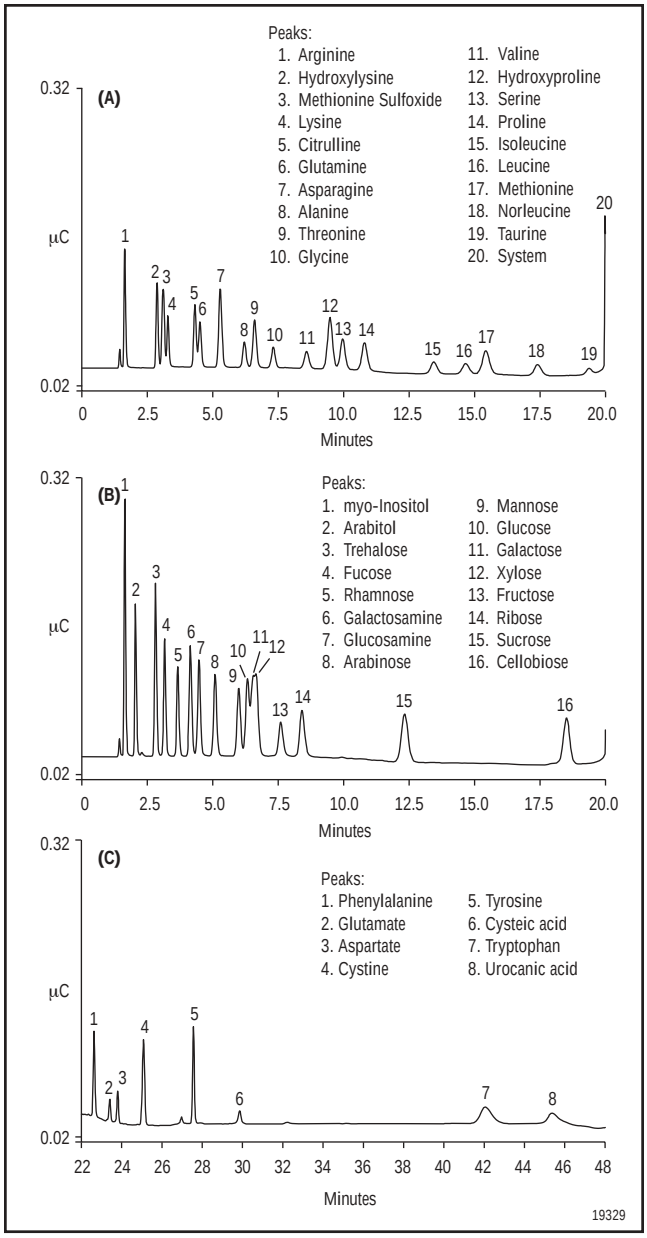

Figure 1. *Common amino acids (A and C) and carbohydrates (B) found in fermentation broths analyzed using the standard gradient* AAA-Direct *method. Glucose coelutes with alanine and threonine.*

RESULTS AND DISCUSSION

Separation

Figure 1 shows the separation of the common cell culture and fermentation broth amino acids (Panels A and C) and carbohydrates (Panel B) using an AminoPac PA10 column set with the published standard *AAA-Direct* gradient method.[14] The standard gradient method uses an initial NaOH concentration of 60 mM, which is held for 2 min (see Table 1, condition 60/2). Using the standard method, sugar alcohols (alditols) and glycols coelute with the first eluting amino acid, Arg. Some monosaccharides also coelute with the early eluting amino acids. For example, glucose coelutes with Ala and Thr, and in amounts exceeding 100–1000 μM, the tail of glucose peak interferes with Gly quantification. Table 2 lists the retention times of common cell culture and fermentation broth amino acids and carbohydrates under this condition (60/2). The extension of this isocratic phase beyond 2 min, before the beginning of the NaOH gradient, does not improve the chromatography in the first 10 min. Peaks eluting after 10 min elute slightly later, and peaks eluting after the acetate gradient begins had increased retention times by approximately the amount of increase in the isocratic section.

The greatest impact on the selectivity of early eluting amino acids and carbohydrates is achieved through changing the initial NaOH concentration. Table 2 shows the effect of decreasing NaOH eluent concentrations. Amino acids eluted later as the [NaOH] decreased, with the exceptions of Arg, which did not change, and Lys, with a slight retention decrease. Carbohydrates initially elute later with decreasing [NaOH], but eventually elute earlier below a [NaOH] specific to each carbohydrate. For example, trehalose begins to elute earlier below 40 mM NaOH, whereas fructose elutes earlier below 15 mM NaOH. The combined use of an extended isocratic phase and the lower [NaOH] improves the separation of monosaccharides from amino acids. When excessive amounts of carbohydrates—typically glucose—are present in cell culture media or fermentation broths, an extended lower [NaOH] makes determinations of both amino acids and carbohydrates possible with a single injection.

When culture media contains carbohydrates other than glucose, it is also possible to select optimal conditions from Table 2 that allow amino acid determinations. Figure 2 shows the effect of varying initial [NaOH] on the separation of glucose from amino acids present in YPD broth supernatant. Ala and Thr coelute with glucose at 60 mM NaOH. When the [NaOH] is lowered to 50 mM, Thr is resolved from glucose and Ala. At 40 mM NaOH, both Ala and Thr are resolved from glucose.

Table 2. Retention Times of Amino Acids, Carbohydrates, Alditols, and Glycols with Different Initial Isocratic NaOH Eluent Concentrations

Initial NaOH Eluent Concentration:	60 mM	60 mM	50 mM	40 mM	30 mM	25 mM	20 mM	15 mM	10 mM
Isocratic Time (min):	2	8	8	8	8	8	8	8	8
Urea	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Glycerol	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
myo-Inositol	1.6	1.7	1.6	1.7	1.6	1.6	1.6	1.6	1.6
Arginine	1.6	1.6	1.6	1.6	1.7	1.7	1.6	1.6	1.6
Erythritol	1.7	1.8	1.7	1.7	1.7	1.7	1.7	1.7	1.6
Methanol	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Arabitol	2.1	2.1	2.1	2.1	2.0	2.0	2.0	1.9	1.8
Galactitol	2.3	2.3	2.3	2.3	2.3	2.2	2.2	2.0	1.9
Sorbitol	2.3	2.3	2.3	2.3	2.3	2.2	2.2	2.0	1.9
Ribitol	2.3	2.3	2.3	2.3	2.3	2.2	2.2	2.1	2.4
Trehalose	2.8	2.9	2.9	2.9	2.8	2.7	2.5	2.3	2.1
Hydroxylysine	2.9	2.9	3.1	3.5	4.1	4.5	5.2	6.3	8.2
Ornithine	3.0	3.0	3.2	3.7	4.3	4.8	5.5	6.6	8.9
Methionine Sulfoxide	3.1	3.1	3.4	3.8	4.5	5.2	5.9	7.3	10.0
Fucose	3.2	3.2	3.4	3.5	3.6	3.7	3.7	3.5	3.2
Lysine	3.3	3.3	3.6	4.0	4.9	5.5	6.2	7.5	9.5
Rhamnose	3.7	3.7	3.0	4.3	4.8	5.1	5.3	5.3	5.0
Mannosamine	4.1	4.1	4.5	4.9	5.5	5.7	6.0	5.9	5.3
Galactosamine	4.2	4.2	4.5	4.9	5.4	5.6	5.7	5.6	4.9
Citrulline	4.3	4.3	4.8	5.6	6.9	7.9	9.3	11.7	14.6
Glucosamine	4.5	4.5	4.9	5.4	6.0	6.3	6.6	6.7	6.1
Glutamine	4.5	4.5	5.1	5.9	7.3	8.3	9.9	12.3	15.0
2-Deoxy-D-Glucose	4.6	4.6	4.9	5.2	5.6	5.6	5.7	5.3	4.5
N-Acetyl-D-Glucosamine	5.1	5.0	5.5	6.2	7.1	7.7	8.2	8.5	8.1
Arabinose	5.1	5.1	5.5	6.1	6.6	6.8	7.0	6.9	5.9
Asparagine	5.3	5.3	6.0	7.1	8.7	10.1	12.2	14.5	16.4
Lyxose	5.7	5.7	6.3	7.0	8.0	8.6	9.1	9.1	8.8
Maltitol	5.7	5.7	5.9	6.1	6.1	5.9	6.0	4.6	3.5
Mannose	6.1	6.0	6.7	7.5	8.6	9.2	9.8	10.0	9.4
Alanine	6.2	6.2	7.1	8.3	10.6	12.8	14.2	16.2	17.4
Glucose	6.4	6.4	7.0	7.8	8.7	9.0	9.4	9.1	8.0
Galactose	6.6	6.6	7.2	7.9	8.6	9.0	9.1	8.6	7.5
Threonine	6.6	6.6	7.6	8.9	11.4	13.7	15.0	16.7	17.6
Xylose	6.7	6.7	7.4	8.3	9.5	10.0	10.6	10.6	9.5
Glycine	7.4	7.4	8.5	10.0	12.9	15.1	16.3	17.6	18.2
Sorbose	7.6	7.7	8.5	9.7	11.3	12.0	12.8	12.4	11.6
Fructose	7.7	7.7	8.5	9.8	11.2	12.0	12.7	12.9	11.7
Ribose	8.4	8.5	9.5	10.9	12.6	13.4	14.2	14.5	13.4
Valine	8.6	8.7	10.0	11.8	14.9	16.9	17.5	18.3	18.8
Melibiose	8.8	8.9	9.9	11.4	13.0	13.7	14.3	13.6	11.0
Hydroxyproline	9.5	9.7	11.2	13.6	16.3	17.5	18.4	19.0	19.5
Serine	10.0	10.2	11.8	14.1	17.0	18.3	18.7	19.4	19.8
Proline	10.8	11.3	13.0	15.1	17.7	18.8	19.2	19.9	20.3
Lactulose	11.8	12.4	14.0	16.0	17.8	18.3	18.9	18.7	17.6
Alpha-Lactose	12.4	13.1	14.7	16.7	18.0	18.4	18.8	18.6	17.0
Sucrose	12.4	12.9	13.7	14.5	15.1	15.1	14.3	12.5	8.9
Isoleucine	13.4	14.9	17.0	18.5	20.3	21.2	21.7	22.4	22.9
Leucine	14.6	16.7	18.4	19.7	21.7	22.4	23.0	23.6	23.9
Methionine	15.4	17.7	19.3	20.6	22.5	23.2	23.8	24.3	24.6
Thioglucose (Beta-D-)	16.9	17.9	18.6	19.0	18.9	18.8	17.4	14.4	9.1
Norleucine	17.3	20.0	21.6	23.1	24.3	24.5	25.2	25.7	26.0
Gentiobiose	17.5	20.0	21.5	22.7	23.7	23.9	24.4	24.1	23.2
Turanose	17.6	20.0	21.5	22.9	23.9	24.2	24.6	25.0	25.1
Palatinose	18.5	21.5	22.8	23.8	24.5	25.4	25.4	25.5	25.4
Cellobiose	18.6	21.0	22.6	23.7	24.6	25.0	25.4	25.4	24.9
Raffinose	19.4	22.6	23.6	24.0	24.5	24.6	24.6	23.5	16.5
Taurine	19.4	22.8	24.1	25.3	25.7	26.4	26.5	26.6	26.6
Maltose	20.2	26.1	26.2	26.2	26.2	26.3	26.3	26.3	26.2
System	20.2	26.1	26.2	26.2	26.2	26.3	26.3	26.3	26.2
Maltotriose	20.3	26.3	26.3	26.3	26.3	26.3	26.7	26.3	26.7
Maltotetraose	21.0	26.8	26.8	26.8	26.8	27.0	26.8	26.9	26.8
Histidine	21.1	27.1	27.1	27.2	27.2	27.3	27.3	27.3	27.2
Maltohexaose	21.2	27.4	27.2	27.3	27.3	27.3	27.3	27.3	27.3
Glucuronic Acid	22.3	28.4	28.4	28.3	28.3	28.3	28.4	28.3	28.3
Phenylalanine	22.7	28.6	28.7	28.7	28.8	28.9	28.9	29.0	28.9
Glutamate	23.5	29.5	29.5	29.5	29.5	29.6	29.5	29.5	29.4
Aspartate	23.9	29.9	29.9	30.0	29.9	30.0	29.9	30.0	29.9
Octyl-Beta-D-Glucopyranoside (*n*-)	24.1	29.3	29.3	29.0	29.0	28.8	28.5	28.6	27.2
Sodium Sulfite	24.2	30.3	30.3	30.0	30.0	30.0	30.2	30.0	30.0
Cystine	25.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.2
Cysteine	25.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.2
Sucralose	25.4	31.4	31.9	31.9	32.0	31.9	31.4	31.9	31.9
Tyrosine	27.7	33.6	33.6	33.7	33.7	33.7	33.7	33.7	33.7
Cysteic Acid	30.0	36.0	36.2	36.0	36.1	36.1	36.0	36.2	36.2
Tryptophan	42.6	48.9	48.8	48.9	48.9	48.8	48.9	49.2	49.1
Urocanic Acid	44.9	49.9	50.0	50.2	49.7	49.9	50.2	50.1	50.0

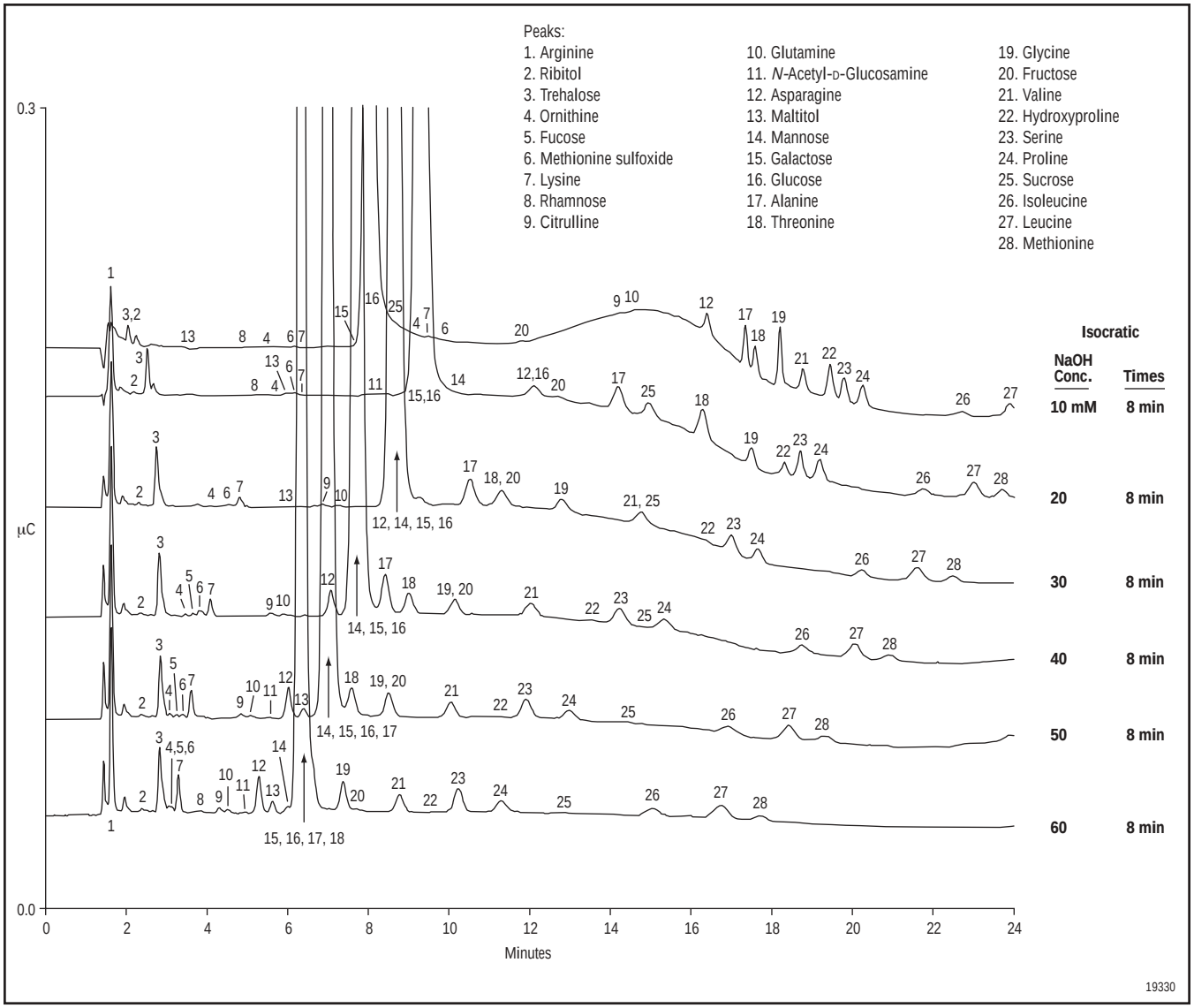

Figure 2. *YPD broth analyzed by* AAA-Direct *with varying initial isocratic NaOH concentrations and extending the isocratic elution.*

The use of low [NaOH] in a *AAA-Direct* gradient causes a baseline drift starting at about 11 min and ending at about 21 min, as shown in Figure 2 for the 10–50 mM NaOH chromatograms. This drift is related to changes in pH-dependent response for the background NaOH-eluent during the gradient, and decreases in magnitude with increasing [NaOH]. Further refinement in carbohydrate and amino acid peak selectivities is achieved by using initial [NaOH] between those listed in Table 2 (results not presented). Although not investigated in this application note, it is also possible to modify the acetate gradient region of the method to refine late-eluting carbohydrate and amino acid peak selectivities.

Detection

The detection response varies for early-eluting amino acids and carbohydrates with the initial [NaOH]. Response varies because IPAD of carbohydrates and amino acids is pH-dependent. Table 3 presents peak areas for some of the amino acids and carbohydrates investigated under varying initial eluent concentrations. Analytes that elute after the system peak and during the acetate gradient are generally unaffected because these peaks elute at essentially the same pH under the conditions of our experiment with different initial [NaOH].

Table 3. Peak Area Response for Varying Initial NaOH Eluent Concentrations Using a Disposable Au Working Electrode.

	Area Units (nC*min)*								
Initial NaOH Eluent Concentration:	**60 mM**	**60 mM**	**50 mM**	**40 mM**	**30 mM**	**25 mM**	**20 mM**	**15 mM**	**10 mM**
Isocratic Time (min):	**2**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Amino Acids									
Citrulline	8.7	8.5	8.1	7.6	6.6	5.1	4.6	3.4	3.2
Cysteic Acid	2.9	3.1	3.1	3.3	3.3	3.2	3.5	3.0	3.4
Glutamate	2.2	2.3	2.5	2.0	2.6	2.4	2.3	2.2	2.4
Glutamine	6.0	5.8	5.3	4.8	3.8	2.8	2.1	1.4	1.4
Glycine	3.3	3.3	3.0	2.9	2.1	1.9	1.6	1.5	1.4
Hydroxylysine	4.3	5.4	4.7	3.9	2.7	2.3	1.4	0.9	0.3
Lysine	4.0	3.9	3.1	2.3	1.2	0.7	0.3	0.1	0.1
Methionine	7.6	6.7	7.2	6.7	6.8	6.6	6.8	6.7	6.7
Ornithine	2.5	3.2	2.5	1.9	1.1	0.4	0.4	0.2	0.2
Proline	6.7	7.0	7.4	7.2	6.3	6.3	6.4	6.4	6.2
Tryptophan	11.9	11.2	11.1	11.1	10.9	10.7	11.4	10.7	10.9
Tyrosine	9.7	8.4	8.6	8.5	8.7	9.4	8.5	8.6	8.9
Carbohydrates									
Fructose	7.7	7.8	7.7	6.9	6.6	5.0	5.4	4.7	3.6
Galactose	14.6	14.7	14.5	14.6	14.1	12.0	13.4	12.9	11.7
Glucose	13.4	13.4	13.3	13.3	n.a.	10.1	12.9	10.4	10.7
Sucrose	16.1	17.0	17.0	17.1	16.9	17.2	15.9	13.4	9.5

* Based on standards at 10 µM concentrations with 10 µL injections.

However, when the initial [NaOH] is varied, early-eluting compounds are contained in different pH eluents that affect electrochemical response. When an application requires low [NaOH] to separate sugars from amino acids and a lower electrochemical response is unacceptable, an in-line sample preparation method described by Jandik et al. can be used.[15] This automated method uses a short cation-exchange column to trap amino acids while allowing carbohydrates to pass through. The amino acids are then released from the trap for determination using *AAA-Direct.*

Disposable Au working electrodes usually provide a slightly higher response for amino acids, and exhibit more reproducible electrode-to-electrode performance compared to conventional gold working electrodes. The slightly improved response for disposable working electrodes is thought to be due to a more uniform gold surface resulting from the manufacturing process. Table 4 compares the amino acid peak area response factors (nC*min/nmol injected) for three different disposable electrodes. The response factor RSDs for the disposable format ranged 0–5% for each electrode, and 2–9% for the three different electrodes.

Table 4. Comparison of Representative Amino Acid Peak Area Response for Three Different Disposable Au Working Electrodes

	Peak Area Response Factor* (nC*min/nmol) Precision for Each Electrode (RSD) (n = 4 Injections Each Electrode)			Variability Between Three Separate Disposable Electrodes for Peak Area Response Factors (nC*min/nmol)		
Amino Acid	**Electrode 1**	**Electrode 2**	**Electrode 3**	**Mean**	**StDev**	**RSD**
Arginine	3.9%	2.5%	3.1%	233.7	18.4	7.9%
Lysine	2.6%	3.2%	2.8%	140.3	7.8	5.6%
Alanine	0.6%	0.5%	1.7%	133.1	1.6	1.2%
Threonine	0.8%	0.4%	0.8%	289.7	12.8	4.4%
Glycine	1.0%	1.1%	0.9%	120.2	3.8	3.2%
Valine	1.3%	0.0%	1.0%	131.6	2.8	2.1%
Serine	0.5%	1.4%	0.3%	242.5	13.1	5.4%
Proline	0.5%	1.8%	1.1%	253.4	9.4	3.7%
Isoleucine	1.0%	0.9%	1.3%	129.3	5.5	4.3%
Leucine	2.0%	2.5%	1.5%	124.0	6.8	5.5%
Methionine	1.8%	0.6%	1.7%	280.9	7.1	2.5%
Phenylalanine	1.4%	0.9%	1.8%	301.1	8.3	2.8%
Glutamate	5.1%	4.6%	3.4%	81.6	7.3	8.9%
Aspartate	1.8%	5.4%	2.6%	116.5	8.7	7.5%
Cystine	0.6%	0.3%	1.1%	847.6	46.5	5.5%
Tyrosine	0.6%	3.6%	0.7%	332.8	16.8	5.0%
Tryptophan	0.4%	1.4%	1.0%	390.9	21.3	5.5%

* Based on amino acid standards ranging in concentration between 7.3 and 9.0 µM with 10 µL injections.

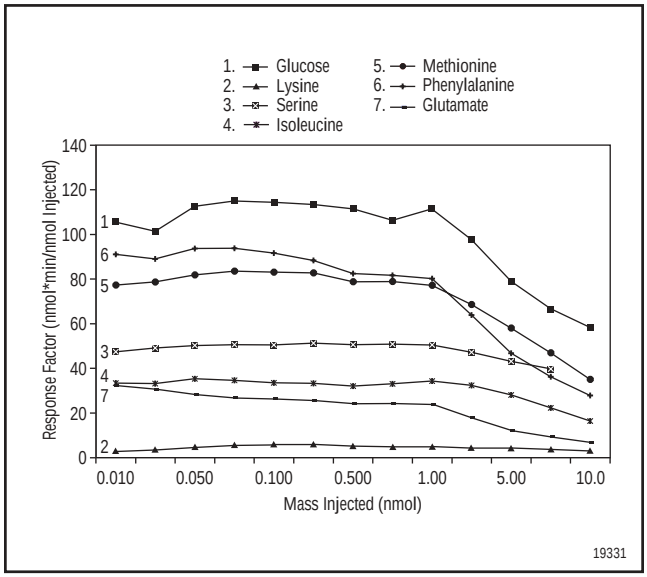

Figure 3. *Estimation of the linear range (peak area) for representative amino acids using* AAA-Direct *condition 20/8 with a disposable Au working electrode.*

Linearity

Figure 3 presents the relationship of seven representative amino acid peak area response factors (nC*min/nmol injected) to the analyte concentration injected using the disposable gold working electrode with the *AAA-Direct* condition 20/8. The figure shows concentration ranges where the response factor remains unchanged with increasing concentration. In this application note, we consider concentration ranges to be linear where the response factor remains within 20% variance from the mean of the plateau region. These results show linearity is specific to each amino acid represented in Figure 3.

The same results were found for peak height linearity (results not presented). Arg and cystine showed the lowest linear range, with response factors dropping below 80% at about 25 µM (250 pmol). Ser and Leu showed the highest level with linearity extending up to about 750 µM (7.5 nmol). The amino acids eluting in the acetate

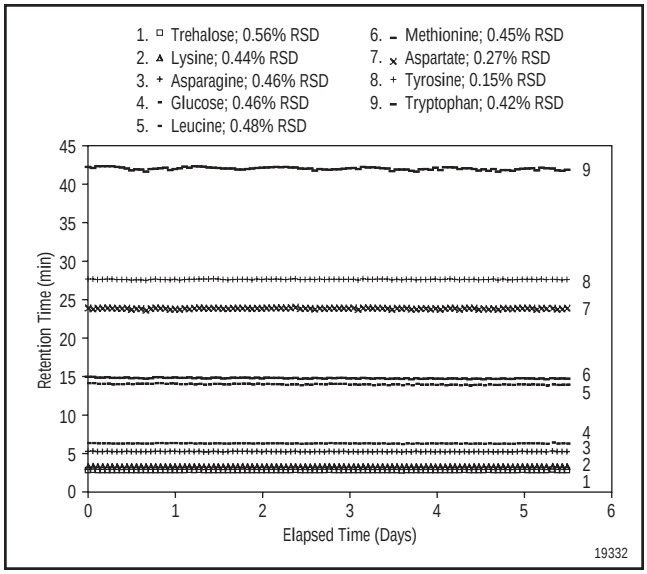

Figure 4. *Retention time reproducibility of representative amino acids and glucose for 5-day continuous analysis of YPD broth supernatant (1000-fold dilution) using* AAA Direct *condition 60/2.*

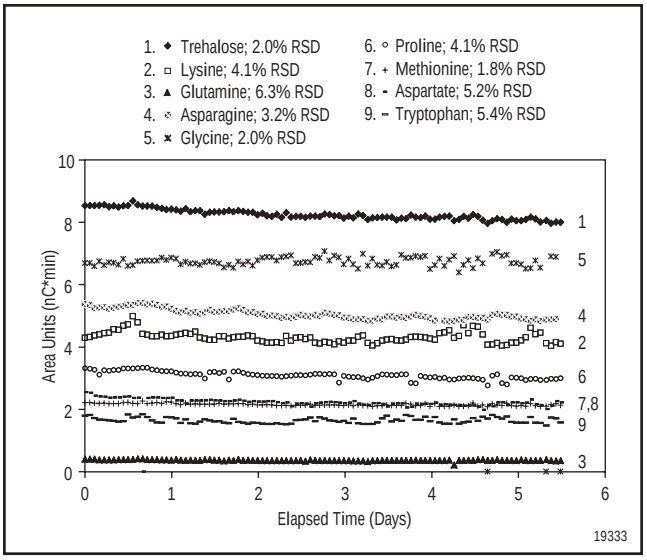

Figure 5. *Peak area reproducibility of representative amino acids and carbohydrates identified in 1000-fold diluted YPD broth supernatant over 5-day continuous analysis using* AAA-Direct *condition 60/2 with a disposable Au working electrode.*

gradient, such as His, Phe, Glu, Asp, cystine, and Tyr, tended to show slightly more abrupt drops in linearity beyond their upper limit, whereas most other amino acids showed gradual drops beyond their upper limits. Generally, amino acids showed linearity up to about 100 μM (1.0 nmol). Glucose was also evaluated, and showed linearity up to between 250 and 500 μM (2.5 and 5.0 nmol). The usable concentration ranges can be extended to higher concentrations using second-order polynomial regressions, lowering the integration potentials and using thicker cell gaskets.

Precision and Reproducibility

The peak area and retention time RSDs were determined for replicate injections of YPD broth supernatant (1000-fold dilution) over five days (100 injections) using *AAA-Direct* condition 60/2 and a disposable Au electrode. Figure 4 shows results for retention time precision. Retention time RSDs over five days ranged 0.15–0.69% for all detected analytes in the YPD broth, which included both amino acids and carbohydrates. Figure 5 shows the peak area precision for representative amino acids and carbohydrates over five days. RSD ranged 0.8–8.3% for compounds measured at concentrations above 1 μM, with the exception of His and Phe, which can have higher variance due to system-related peaks that may elute near these peaks and may affect integration. Peak area precision decreased as the measured concentration of the analyte decreased. No apparent trending in peak retention time was observed. The percent change in the mean peak area from beginning to end of the five-day study for all amino acids identified in the YPD broth that exceeded the 1 μM measured concentration ranged from –0.3% to –10%. This percent change was within the 20% guaranteed for disposable Au electrodes for the one-week period. The high retention time and response reproducibility indicates that this method is suitable for this application.

DETERMINATION OF AMINO ACIDS IN CELL CULTURE AND FERMENTATION BROTH MEDIA

YPD Broth Medium

YPD Broth contains 0.2 g yeast extract, 0.4 g peptone, and 0.4 g dextrose (glucose) per 1.0 g solid broth powder. Yeast extract is the water-soluble portion of autolyzed yeast, where autolysis is carefully controlled to preserve naturally occurring B-complex vitamins. Peptone is a water-soluble, enzymatically digested proteins. Peptone—and to a lesser extent yeast extract—are the sources of amino acids (and nitrogen) in this medium, and are not defined (complete chemical composition unknown). YPD broth contains 20 g/L (110 mM) glucose. Figure 2 shows the separation of YPD broth ingredients with varying initial [NaOH]. The adjustment of the initial [NaOH] allows resolution of the Thr, Ala, and Gly peaks from the large glucose peak. Asn and Gln are also resolved.

As discussed earlier, Figures 4 and 5 show the peak retention time and area reproducibility of representative amino acids and carbohydrates for consecutive injections of the YPD broth over five days (100 injections). In addition to the compounds listed in Figures 4 and 5, Arg, Thr, fructose, Phe, Glu, and sulfite were identified by retention time. Both amino acids and carbohydrates were determined in this medium after a 1000-fold dilution. Improvements in the measurement of trace amounts of these compounds are possible by increasing the concentration of the broth for analysis. When analyzing more concentrated broths, the glucose may be too concentrated to quantify in the same run, and more than one injection may be required to quantify all components. By selecting the suitable initial eluent conditions described in Table 2, it is possible to alter selectivity to resolve both difficult-to-measure peaks and very large glucose peaks. The carbohydrate elimination method described by Jandik et al.[15] can also be used when either trace levels of amino acids are present, or the carbohydrate determinations are not needed. The higher initial concentration of NaOH in the automated carbohydrate elimination method provides higher detector response, enabling trace amino acids to be better quantified.

Lower NaOH eluent can also lower detector response (Table 3). Lower [NaOH] conditions can provide unique selectivity, but any analysis using these conditions should use higher sample concentrations to improve accuracy and precision, and make sure the analytes of interest are within their respective linear range.

The determination of amino acids can be problematic in highly concentrated broths. The spike recovery of common amino acids from varying dilutions of YPD broth supernatant showed that broths diluted less than 500-fold had lower recovery. Full recovery was obtained at the 500-fold dilution level and higher. The recovery of desired analytes can vary with different media types and should be determined for each media to ensure accurate measurements.

LB Broth Medium

LB broth contains 10 mg of tryptone, 5 mg of yeast extract, and 10 mg of sodium chloride per 25 mg solid broth powder. Tryptone, like peptone, is a water-soluble trypsin digest of proteins, and a primary nitrogen source in the form of free amino acids. Both tryptone and yeast extract are not well defined, and contain vitamins, a variety of trace carbohydrates, and unknowns. Figure 6

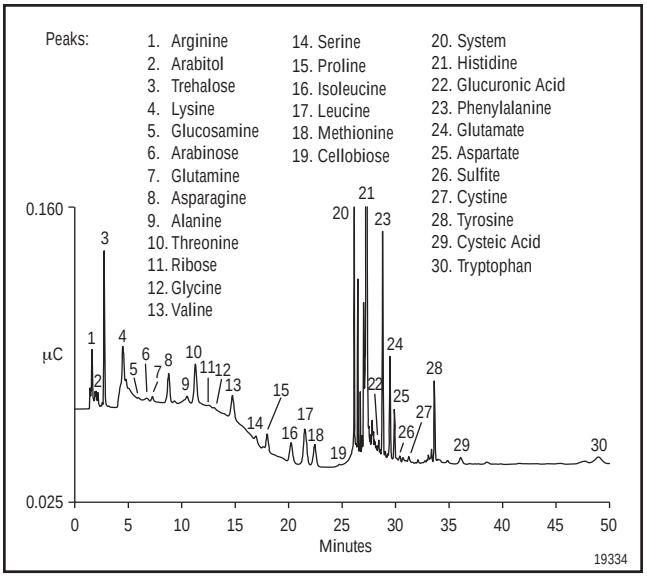

***Figure 6**. Determination of amino acids and carbohydrates in LB Broth supernatant (100-fold dilution) using* AAA-Direct *condition 30/8.*

shows the separation of amino acids found in LB broth using the *AAA-Direct* 30/8 condition. The absence of glucose from this medium[11] allows the *AAA-Direct* conditions to be run that would ordinarily cause interferences. The standard condition 60/2 may have been an acceptable method to use for this sample type, but the condition 30/8 was chosen to show changes in selectivity and resolution for this condition. *AAA-Direct* resolved 78 peaks for a 100-fold dilution of the medium; 29 peaks were identified by retention time and 21 of these were amino acids. Both Asn and Gln could be separated and determined from their Asp and Glu counterparts. Although sulfite can be determined using *AAA-Direct*, the height response (condition 60/2) is about 1.5-fold lower using this waveform compared to the method described in Application Note 54.[16] Trp can also be determined in this broth. Rapid Trp determinations (~10 min) are also possible using an isocratic method described in Application Note 142.[17,18] Erythritol, arabitol, arabinose, lactose, and maltose were identified by retention time in this media using the CarboPac columns with the carbohydrate electrochemical waveform.[10,11] Some sugars could not be successfully analyzed in this medium using the AminoPac PA10. For example, erythritol could not be resolved from other early eluting peaks near the void. Lactose was not observed, and maltose coelutes with the system peak that occurs at the beginning of the acetate gradient.

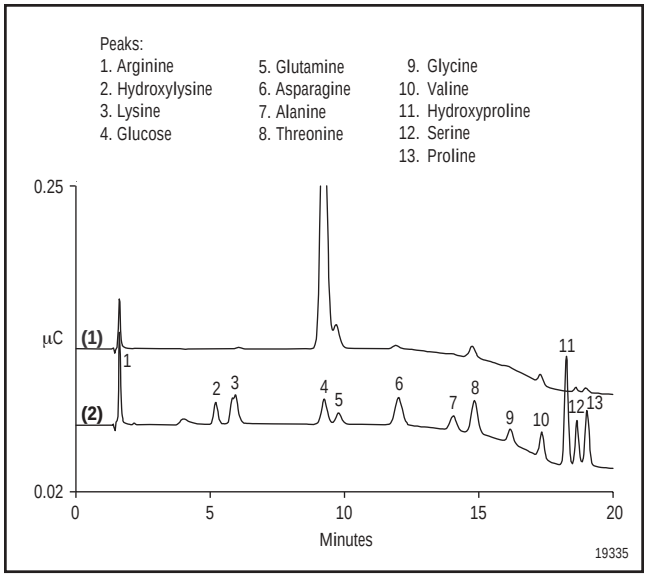

Figure 7. *Determination of amino acids and carbohydrates in Minimal Essential Medium (MEM) Eagle supernatant (100-fold dilution, 10-μL injection) using* AAA-Direct *condition 20/8. Overlay of MEM (1) with amino acid standard mix (2).*

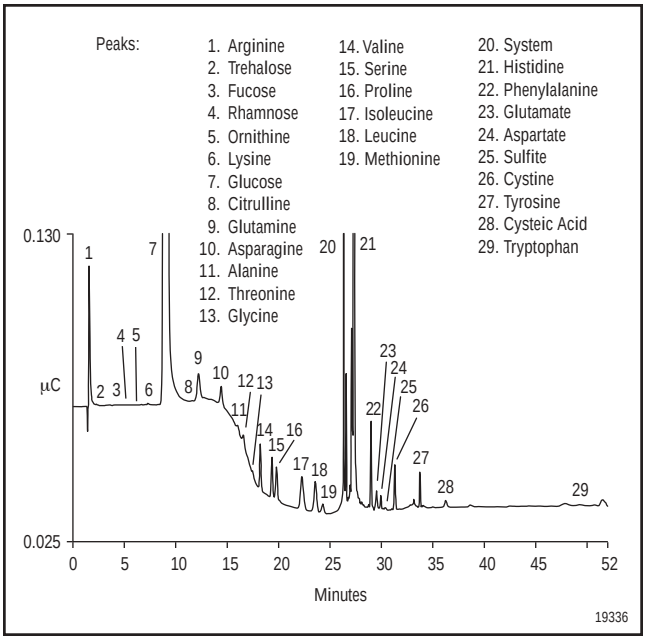

Figure 8. *Determination of amino acids and carbohydrates in Serum-Free Protein-Free Hybridoma Media supernatant (100-fold dilution) using* AAA-Direct *condition 15/8.*

Mammalian Cell Culture Media

Minimal Essential Medium (MEM)

Mammalian cell culture media are complex, consisting of a large number of ingredients, but usually less complex and more defined than media used for bacterial or yeast cultures. MEM contains 20 amino acids, Earle's salts, vitamins, phenol red (pH indicator), myo-inositol, and glucose. A chromatogram (Figure 7) for the separation of MEM Eagle reflects this simplicity by the presence of fewer peaks (33 total for 100-fold dilution) than YPD or LB broth. Nearly all amino acids and carbohydrates in this medium are separated using the *AAA-Direct* condition 20/8. The goals of the analysis dictate the choice of separation conditions. In this example of condition 20/8, glutamine is not completely resolved from glucose (Figure 7), but can be completely resolved using the 30/8 or 15/8 methods.

Serum-Free Protein-Free Hybridoma Medium

Hybridoma medium (100-fold dilution) was analyzed using the *AAA-Direct* condition 15/8 (Figure 8). Hybridoma medium is a defined medium consisting of inorganic salts, vitamins, 22 amino acids, sodium bicarbonate and MOPS buffers, and 20 other miscellaneous compounds necessary for cell growth and maintenance. The glucose concentration is 5 g/L. The presence of bicarbonate and MOPS buffers did not cause any apparent interferences at a 100-fold dilution. Phenol red, used to indicate pH, also did not cause any apparent interference. Twenty-three amino acids were identified in the hydridoma medium. All amino acids specified as present in this medium were identified (Figure 8) except for L-cysteine, which was converted to cystine under our analytical conditions. Cysteic acid is not mentioned in the recipe for this medium, but was identified by retention time. The uncommon amino acids present in this recipe, citrulline and ornithine, were also identified. Trace levels of trehalose, fucose, rhamnose, and sulfite were observed. These compounds are not listed in the medium recipe. Taurine was not determined using the *AAA-Direct* condition 15/8 presented in Figure 8, but was detected using conditions that used a higher initial NaOH concentration (Table 2).

PRECAUTIONS

Fetal Bovine Serum (FBS)

Serum is a complex sample matrix used to condition media for mammalian cell culture use. It is typically used in media at concentrations ranging 1–10%. We analyzed FBS with *AAA-Direct*. Although most amino acids can be determined in FBS, any exposure of FBS to the AminoPac PA10 column conditions the column with adsorbed compounds. These compounds cause conversion of cystine to another substance (possibly sulfenic or sulfinic acid) that elutes just before Tyr. Although the new peak could possibly be used for cystine determinations (using a serum-conditioned column), this possibility has not been fully investigated.

CONCLUSION

These results show that *AAA-Direct* can be used to determine amino acids in media used for yeast and bacterial culture (fermentation broths) and mammalian cell culture. Alterations in peak selectivity are possible by adjusting the initial eluent strength and isocratic phase for nearly any amino acid separation where excess carbohydrates exist in the sample matrix. The linear range of electrochemical response varies for each amino acid and carbohydrate, depending on the *AAA-Direct* condition used. High precision, method ruggedness, and high spike recovery are possible for these complex sample matrices. The disposable gold working electrode provides improved performance, enabling the best sensitivity and reproducibility. Complex mixtures of amino acids and carbohydrates can be monitored simultaneously during cell culture and fermentation, providing the analyst with information needed for process optimization.

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LPN 1538 PDF 7/03