DIONEX

Application Note 179

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Carbohydrate and Amino Acid Analysis Using 3-D Amperometry

INTRODUCTION

AAA-Direct™ is an established technique for amino acid analysis. This technique has proven capabilities for 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 precolumn or postcolumn derivatization. Chemical derivatization techniques complicate analysis, add costs for expensive reagents, introduce safety hazards to lab personnel exposed to toxic solvents, and add a hazardous waste stream that requires safe disposal. *AAA-Direct* eliminates these complications. Amperometric detection is sensitive, allowing the detection of <1 pmol of a carbohydrate or amino acid.[5,6]

Amperometry is an electrochemical detection method that uses a flow cell consisting of three electrodes [working electrode, reference electrode, and counter (auxiliary) electrode]. Varying voltage potentials are applied between the working electrode and the reference electrode by means of a waveform program stored in the detector software. The waveform period is typically less than one second, and is repeated throughout the chromatographic period. Applying defined voltages oxidizes specific analytes separated by the analytical column. Analyte oxidation results in a flow of electrons (current). The current is measured between the working and counter electrodes, integrated during a defined time period within the waveform to yield charge (coulombs), and then recorded by the data management software (i.e., Chromeleon®). Only certain compounds are oxidized at a given applied voltage potential, therefore amperometry can impart a degree of specificity by adjusting the applied voltage.[6]

3-D Amperometry is an extension of conventional amperometry, enabling the continuous acquisition of current throughout the entire waveform period rather than only during a predefined period within the waveform when current is integrated. The complete data set enables, among other things, postchromatographic current integration of the amperometric data. Because different chemical compounds oxidize differently at given applied voltages, subtle differences in the amount of current generated through a waveform can provide additional information about the identity and purity of the substances being analyzed. These differences can be measured by comparing the peak areas obtained for current integrated through different time periods within the waveform. The ratio of these peak areas provides a convenient analytical approach for comparing the identity between unknown and standard peaks. Similarly, a measure for the extent of purity is the extent of deviation from the ratio determined for the standard.[7] In addition, a waveform integration range selective to carbohydrates or specific amino acids can reduce or eliminate peak interferences and improve their peak integrations. The relationship of 3-D amperometry to conventional amperometry is in some ways similar to the relationship of diode array detection to single wavelength UV absorbance detection.

3-D amperometry improves the resolution of specific carbohydrate and amino acid peaks in samples where these compounds partially coelute. Published research[6] has demonstrated that alternative waveform integration ranges can enhance the detection of carbohydrates and hydroxyl-containing amino acids (e.g., serine, threonine, hydroxylysine, hydroxyproline) while reducing the response of other amino acids. This produced additional evidence that certain peaks were carbohydrates and allowed for easier integration of some peaks. Prior to the introduction of 3-D amperometry, this required two separate injections with each run using separate waveform integration periods. 3-D amperometry enables this same selective technique to be performed on a single injection, post-chromatographically. 3-D amperometry can also be used to adjust detector sensitivity to match desired peak area response levels. Improvements in Dionex electrochemical detection have produced higher detection sensitivity and therefore, for a given compound, older instruments may have lower response. The adjustment of waveform integration ranges can be used to quickly determine an integration range that produces peak areas matching preexisting response specifications established in official methods or standard operating procedures (SOPs).

EQUIPMENT

Dionex ICS-3000 system consisting of:

- DP Gradient Pump (optimized for 2-mm i.d. columns) with degas option.
- DP Electrochemical Detector with combination pH/Ag/AgCl reference electrode and *AAA-Certified*™ Disposable Au Working Electrode (P/N 060082 for pack of 6; P/N 060140 for 4 bundled packages) or *AAA-Certified* Au Working Electrode (Conventional, P/N 079850)
- 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: 25 mM NaOH, 1 M Sodium acetate
	D: 100 mM Acetic acid
Inj. Volume:	10 or 25 µL
Temperature:	30 °C
Separation Method:	See Table 1, Description of *AAA-Direct* Gradient Methods
Detection:	Integrated pulsed amperometry, disposable or conventional Au working electrodes

AAA-Direct Waveform:

Times (s)	Potential (V) 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

*Reference electrode in the pH mode

Table 1. Standard *AAA-Direct* Gradient Method

Initial NaOH Eluent Concentration: 60 mM
Initial Isocratic Time: 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	0.0
21.0	5	50.0	10.0	40.0
23.0	8	21.9	8.1	70.0
42.0	5	21.9	79.2	0.0
42.1	5	20.8	79.2	0.0
44.1	5	20.8	20.8	0.0
44.2	5	79.2	20.8	0.0
75.0	5	79.2	20.8	0.0

REAGENTS AND STANDARDS

Reagents

Sodium hydroxide, 50% (w/w)

Sodium acetate, anhydrous (electrochemical grade, Dionex P/N 059326)

Glacial acetic acid, HPLC grade, 17.5 M

Deionized water, 18.2 MΩ-cm resistance or higher

Standards

All standards, except where noted, were supplied by Sigma Chemical Company

Alanine
Amino acid standard mix (NIST, Standard Reference Material 2389)
Asparagine
Aspartate
Cystine
Fructose, D-; reference grade (Pfanstiehl Laboratories)
Glucose, ß-D-; reference grade (Pfanstiehl Laboratories)
Glutamate
Glutamine
Glycine
Histidine
Hydroxylysine
Hydroxyproline
Inositol (myo-)
Isoleucine
Leucine
Lysine
Methionine
Methionine sulfoxide
Phenylalanine
Proline
Serine
Sucrose (Fisher Scientific)
Threonine
Tryptophan
Tyrosine
Valine

Culture Media

Luria-Bertani (LB) Broth (BD Diagnostics, DIFCO Cat# 0447-17-3)

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 (18.2 MΩ-cm or higher). Filter all water through a 0.2-µm nylon filter (Nalgene® 90-mm Media-Plus, P/N 500-118; Nalge Nunc International) under vacuum to degas. Biological contamination should be absent. It is important to minimize contamination by 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 at this pH) and is the required source for NaOH.

Dilute 26 mL of 50% (w/w) NaOH solution into 1974 ± 0.5 g of thoroughly degassed water to yield 250 mM NaOH. Dilute 1.05 mL 50% NaOH into 1999 ± 0.5 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 two bottles each containing 82 g of the electrochemical grade anhydrous sodium acetate in ~800 mL purified water. Gravimetrically adjust the total volume from each bottle to 1081.0 ± 0.5 g (999 mL water + 82 g sodium acetate) with additional water. Filter the solution through a 0.2-µm nylon 1-L filter unit (see comments above), combine, and add 2.62 mL 50% NaOH to the 1998 mL sodium acetate solution. Immediately place the solution under 4–5 psi helium or nitrogen to reduce carbonate contamination.

Table 2. Modified *AAA-Direct* Gradient Method

Inital NaOH Eluent Concentration: Generic
Initial Isocratic Time: 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	40.0	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	0.0
66.2	5	20.0	80.0	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 2 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.

100 mM Acetic Acid

To prepare 2 L of eluent, dissolve 11.5 mL of HPLC grade glacial acetic acid (17.5 M) in 1988 ± 0.5 g purified filter-degassed water. 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.

SYSTEM PREPARATION AND SETUP

The preparation and setup of the *AAA-Direct* system is described in the Product Manual for *AAA-Direct* Amino Acid Analysis System.[8] 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 the command *3D_Amp.AcqOn* at the beginning of 3-D amperometry data collection, and *3D_Amp.AcqOff* at the end. Dionex Technical Note 63 provides detailed instructions for selecting waveform integration ranges for post-chromatographic data extraction.[7]

STANDARD AND SAMPLE PREPARATION

Standard

Solid standards were maintained desiccated under vacuum prior to use. They were dissolve 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 (nominal concentration 2.5 mM) SRM 2389 from NIST, was diluted in water to produce known concentrations of each amino acid ranging from 9.5 to 11.75 µM (except cystine, 4.6 µM). When needed, additional amino acids (e.g., tryptophan) and carbohydrates were added to the NIST amino acid standard mix during dilution.

LB Broth Medium

LB Broth (250 mg) was dissolved in 10.0 mL aseptically filtered (0.2 µm, nylon) water. An aliquot was centrifuged at 16,000 x *g* for 10 min, then the supernatant was removed and diluted 100-fold in purified water. Diluted supernatant was analyzed directly.

RESULTS AND DISCUSSION

The *AAA-Direct* waveform program integrates current between 210 and 560 ms, and is suitable for detection of both amino acids and carbohydrates (Figure 1). The standard *AAA-Direct* gradient program (Table 1), which begins analysis using 60 mM NaOH for 2 min, does not separate amino acids from some carbohydrates. Glucose coelutes with alanine and threonine, and fructose and sucrose interfere with valine and isoleucine, respectively. Dionex Application Note 150,[1] Application Update 152,[9] and other publications[2,3] show that carbohydrates and amino acids can be resolved by altering the initial NaOH eluent concentration and duration.

Figure 2 shows the elution of glucose, fructose and sucrose in amino acids using an initial 40 mM NaOH for 8 min (Table 2, the 40/8 method) with the 210–560 ms waveform integration range. Glucose is resolved from both alanine and threonine, eluting after asparagine. Fructose and sucrose coelute with glycine and proline, respectively. The 110–210 ms waveform integration range (Figure 3) significantly reduces the asparagine peak, improving the detection of the glucose peak. The reduction of the glycine and proline peak areas improves detection of the fructose and sucrose peaks, respectively.

By the most accepted definitions of resolution between two peaks, resolution may be improved between asparagine and glucose through reduction of the peak area for asparagine, relative to glucose. The resolution factor equation relates the differences in retention times of the two peaks to the sum of both peak widths (U.S. Pharmacopeia definition), or to the peak widths at 50% peak height (European Pharmacopeia definition). Although the retention time difference between two peaks remains unchanged by integration range modifications, the relative peak widths can be reduced for the two peaks using this technique, thus increasing the resolution factor.

In Figure 4, the asparagine and glucose peaks shown in Figures 2–3 are expanded and compared using the 210–560 ms and the 110–210 ms waveform integration ranges. This figure illustrates the improvement in peak area accuracy using a valley-to-valley method of chromatographic peak integration, where the reduction of peak area for asparagine (relative to glucose) significantly improves the evaluation of glucose peak area. The resolution factor (USP definition) for asparagine and glucose increased from 1.1 to 1.2.

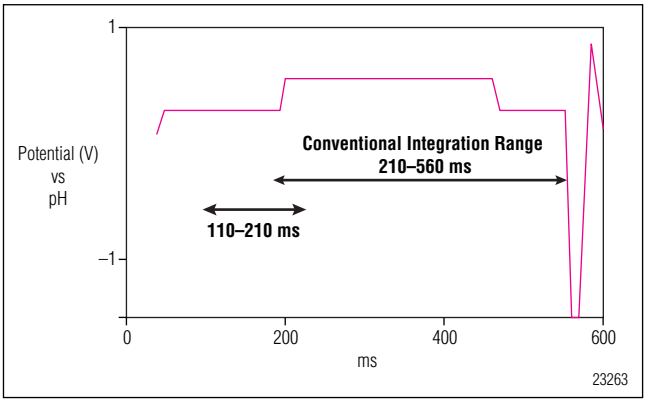

Figure 1. Comparison of the conventional AAA-Direct *waveform integration range (210–560 ms) and an alternative compound selective integration range (110–210 ms).*

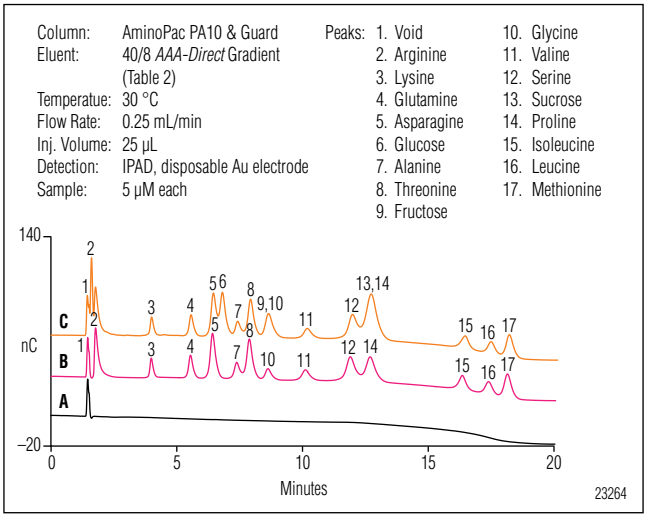

Figure 2. Comparison of (A) water blank, (B) 5 µM amino acids, and (C) 5 µM amino acids with 5 µM glucose, fructose, and sucrose using the conventional waveform integration range of 210–560 ms with the standard 40/8 AAA-Direct *gradient method (Table 2).*

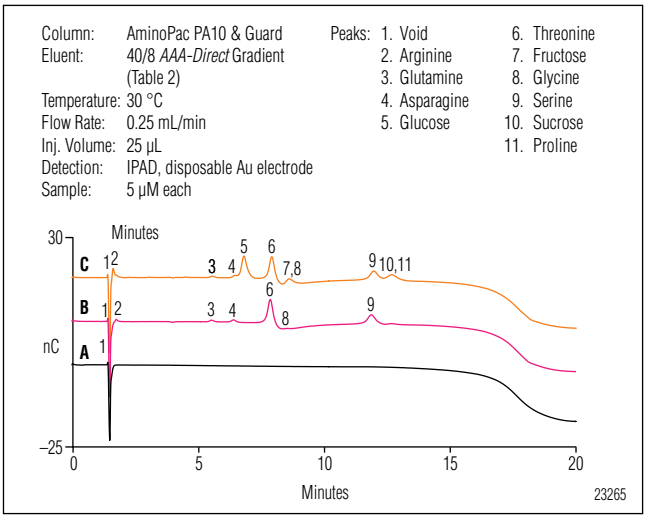

Figure 3. Comparison of (A) water blank, (B) 5 µM amino acids, and (C) 5 µM amino acids with 5 µM glucose, fructose, and sucrose using the selective waveform integration range of 110–210 ms with the standard 40/8 AAA-Direct *gradient method (Table 2).*

3-D amperometry can also improve the detection of imperfect separations of amino acids. Contaminated AminoPac columns can lose baseline resolution of serine and proline. Serine/proline resolution can be improved in the majority of cases by rinsing the column with HCl/CH_3CN.[8] Using the conventional waveform integration range of 210–560 ms, peak integration is compromised by the imperfect resolution of the two peaks (Figure 5A). The waveform integration range of 110–210 ms selectively reduces the peak area for the proline peak, enabling better peak integration of serine (Figure 5B). Similarly, threonine integration is simplified using the 110–210 ms range (Figure 6B) compared to the conventional range (Figure 6A). In some cases, the void peak can interfere with arginine determination. Figure 7 shows the effect of waveform integration range adjustments to the selective reduction of void peak interferences, and improvement in arginine peak integration.

Changes in waveform integration range alter the peak area for electrochemically active substances separated and detected using HPAE-IPAD. Although the response factors for these substances will vary depending on the nature of the substance and the waveform integration range used, a linear relationship to analyte concentration is still assured. Similar linearity for glucose peak area tested at 1–200 µM using the conventional waveform integration of 210–560 ms, and with 110–210 ms is demonstrated in Table 3. Table 3 also compares the linearity of glucose obtained from slightly reducing the conventional waveform integration range (shortened end times) to 210–540 ms, 210–520 ms, and 210–500 ms. Although the slopes and y-intercepts decrease when the conventional waveform integration interval is shortened, the linearity (r^2) remains the same. Only slight increases in the upper limit of linearity, baseline noise, lower limit of detection (LOD) and quantification (LOQ) were observed by shortening the integration end times. This demonstrates how minor adjustments in waveform integration ranges can alter detector response (slope) without significantly compromising the calibration performance. This feature

Table 3. Comparison of Linearity and Detection Limits for Varying Waveform Integration Ranges

		Integration Range (ms)				
		110–210	210–560	210–540	210–520	210–500
Slope (nC*min/µM)		0.217	1.343	1.286	1.225	1.154
Y-Intercept		0.529	1.471	1.248	1.067	0.790
r^2		0.9979	0.9994	0.9950	0.9996	0.9970
Upper Limit of Linearity (µM)		223	385	409	429	459
Noise (pC)	Mean	27.6	77.7	83.5	84.8	85.6
	SD	6.3	4.2	6.7	7.0	6.9
Estimated LOD (µM)	Mean	0.095	0.041	0.046	0.049	0.052
	SD	0.022	0.002	0.004	0.004	0.004
Estimated LOQ (µM)	Mean	0.316	0.137	0.153	0.162	0.173
	SD	0.072	0.007	0.012	0.013	0.014

*Upper limit of linearity is based on calculated concentration having a peak area response factor (area unit / µM injected) 20% less than the optimal peak area at 10 µM glucose. Calibration curves based on concentrations ranging from 1 to 200 µM glucose. Standard deviation (SD) based on three injections of water, noise measured at the retention time glucose would elute.

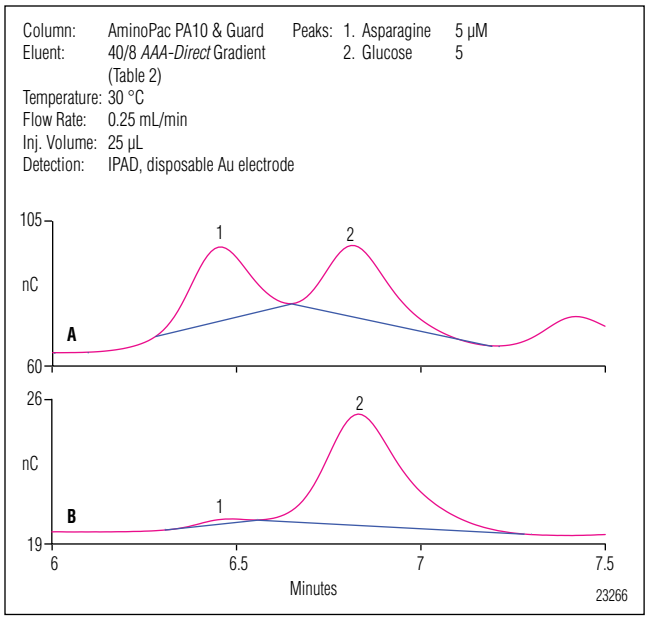

Figure 4. Comparison of 5 µM amino acids with 5 µM glucose using (A) the conventional waveform integration range of 210–560 ms, and (B) the filtering 110–210 ms integration range.

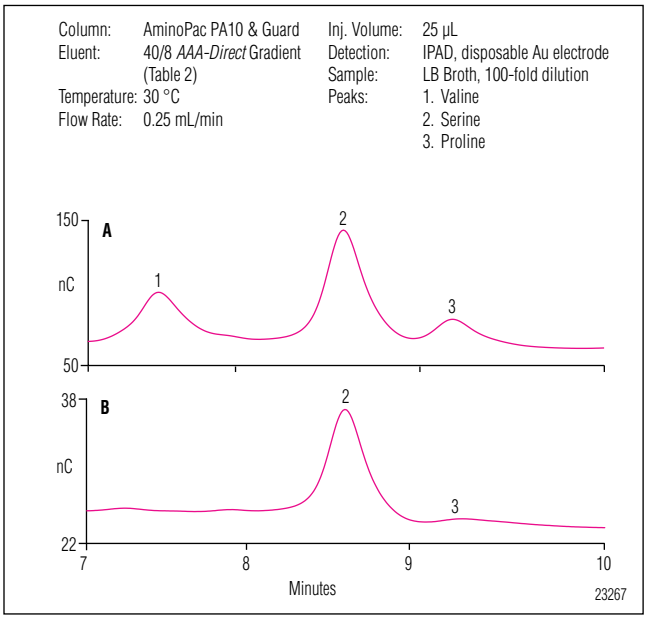

Figure 5. 3-D amperometry integration range modification for selective removal of valine and proline to improve serine detection in 100-fold diluted LB broth. (A) 210–560 ms, (B) 110–210 ms.

may allow faster transfer of applications developed using older Dionex instrumentation to the ICS-3000 when electrochemical response must meet an absolute value. This requires only a single set of calibration data that can then be evaluated in a number of ways using 3-D amperometry tools.

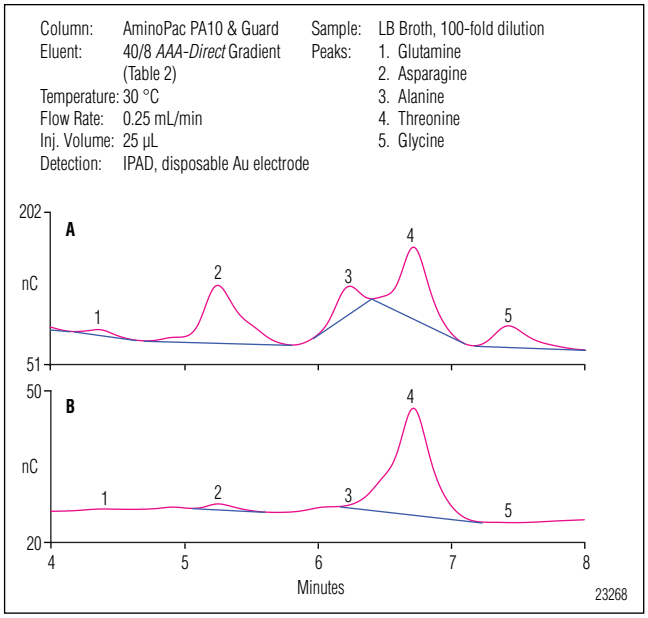

Figure 6. 3-D amperometry integration range modification for selective removal of alanine and glycine to improve threonine detection in 100-fold dilution LB broth. (A) 210–560 ms, (B) 110–210 ms.

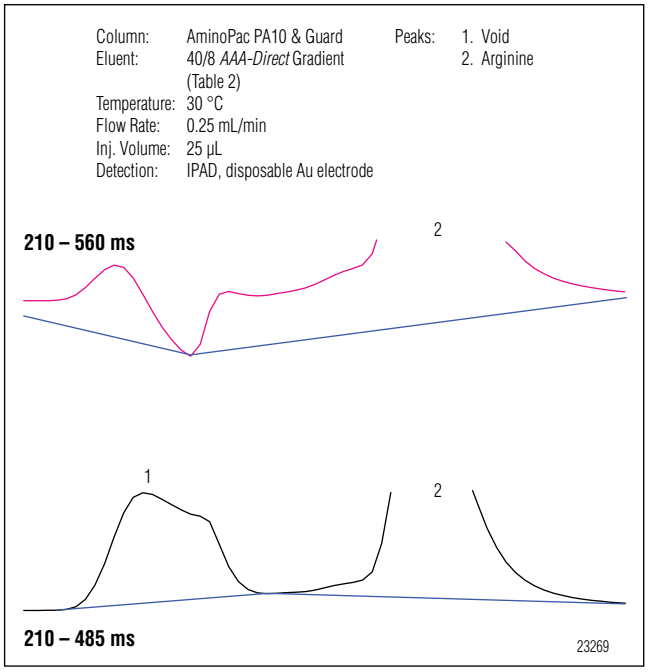

Figure 7. Decreasing the waveform integration end time to 485 ms improves the chromatographic peak integration of arginine (10 µM) by altering the void peak.

SUMMARY

Specific amperometric waveform integration ranges can provide a tool to selectively detect specific compounds. 3-D amperometry enables postchromatographic modification of waveform integration ranges, simplifying the procedure by eliminating the need to perform multiple injections. This technique can enhance the detection of carbohydrates in the presence of some coeluting amino acids, or can reduce the interferences of some amino acids coeluting with other amino acids. Additionally, it can minimize some baseline disturbances to improve integration of peaks. Peak area response can conveniently be adjusted to match values from older HPAE-IPAD chromatography systems where fixed response specifications have already been established in standard operating procedures.

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LIST OF SUPPLIERS

1. VWR Scientific Products, 3745 Bayshore Blvd., Brisbane, CA 94005, U.S.A. Tel: 800-932-5000, Web: www.vwrsp.com.
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5. National Institute of Standards & Technology (NIST), 100 Bureau Drive, Gaithersburg, MD 20899, U.S.A. Tel: 301-975-2000, Web: www.nist.gov.
6. Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126, U.S.A., Tel: 800-766-7000, Web: www.fishersci.com.
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