AAA-Direct System

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thermo scientific

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Product Manual

for

Dionex *AAA-Direct*, Amino Acid Analysis System

Dionex *AAA-Direct*,

SPECIFIED COLUMNS
AminoPac PA10 Analytical Column (2x250mm) P/N 055406
AminoPac PA10 Guard Column (2x50 mm) P/N 055407

RECOMMENDED ACCESSORIES
Dionex AAA-Certified Disposable Gold Electrodes
Pack of 6, P/N 060082

ALSO RECOMMENDED
Dionex AAA-Direct Start Up Kit P/N 059539

Revision History:

Revision 14, September, 2018, content reformatted to meet current branding and ISO standards. Minor corrections made to specification data.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

SAFETY	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
WARNING	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
CAUTION	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
NOTE	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

1.1 Dionex *AAA-Direct* Amino Acid Analysis System

The Thermo Scientific™ Dionex™ *AAA-Direct*™ Amino Acid Analysis System is specifically designed to separate a wide range of amino acids by gradient anion exchange with Pulsed Electrochemical Detection (PED). Amino sugars and carbohydrates can be separated and detected simultaneously with amino acids, if they are present in the sample. Additional capabilities include separation and detection of the wide range of sugars, phosphorylated amino acids and common oxidation products of sulfur-containing amino acids (e.g. cysteic acid, methionine sulfone or methionine sulfoxide).

1.2 Dionex AminoPac PA10 Anion Exchange Column

The Thermo Scientific™ Dionex™ AminoPac™ PA10 columns are packed with a hydrophobic, polymeric, pellicular anion exchange resin stable over the range of pH 0–14. This unique pH-stability of the packing material allows the use of eluent compositions that are conducive to anodic oxidation of amino acids at gold electrodes.

Resin Characteristics:

Particle Size:	8.5 µm
Pore Size:	Microporous (<10 Å)
Cross-linking:	55% DVB
Ion Exchange Capacity:	60 µ Equivalents/Column (2 x 250 mm)

Latex Characteristics:

Functional Group:	Alkyl Quaternary Ammonium Ions
Latex Diameter:	80 nm
Latex Cross-linking:	1%

Typical Operating Parameters:

pH Range:	pH = 0 - 14
Temperature Limit:5	0 °C
Pressure Limit:	4,000 psi
Organic Solvent Limit:	100% Acetonitrile, Methanol, (Acetone if required for cleaning)
Typical Eluents:	High Purity Water (18 megohm-cm), Sodium Hydroxide, Sodium Acetate

1.3 Dionex AAA-Certified Gold Working Electrodes

Thermo Scientific currently offers two types of Thermo Scientific™ Dionex™ AAA-Certified™ Gold Working Electrodes; disposable electrodes, and non-disposable or conventional electrodes. All Dionex AAA-Certified Gold Electrodes are optimized to enable gold oxide catalyzed oxidation of amino acids. This mode of detection differs from the Au hydroxide catalyzed oxidation of carbohydrates at lower potentials. In principle, it is feasible to convert a gold electrode from one mode of detection to another; however, this may require time and is thus not recommended whenever large numbers of samples need to be processed.

1.3.1 Dionex AAA-Certified Disposable Gold Electrodes

The Disposable Electrodes are especially useful for laboratories with high sample throughput requirements. The Thermo Scientific Dionex AAA-Certified Disposable Gold Electrodes are optimized for high electrode-to-electrode reproducibility and can be expected to deliver a stable detection for up to one week of continuous use; provided only the recommended waveforms are applied and all system operating instructions are closely followed. If the detection performance of a Disposable Electrode is affected, it is simply replaced, and the laborious and time-consuming electrode regeneration is thus avoided.

Dionex AAA-Certified Disposable Gold Electrodes can be ordered as a pack of six disposable electrodes with two cell gaskets (P/N 060082) or as four bundled packages of 6 electrodes and 2 gaskets (24 electrodes and 8 gaskets, P/N 060140). The ED cells come equipped with a machined polyethylene block (P/N 062158) that is used to mount the disposable electrode. Please note: you can also use any Thermo Scientific Dionex non-disposable working electrode to hold the Disposable Electrodes in place and obtain good detection performance (see Section 12, Step 1).

NOTE

Throughout this manual, we discuss the 80 nC Rule for non-disposable electrodes. When working with disposable electrodes, however, please apply the 20 nC Rule instead. The observed background should be within ± 20 nC of the actual background value in the Lot Validation sheet. The Lot Validation sheet is included with every shipment of disposable electrodes. In addition, the peak height of histidine should be equal to, or greater than that shown in the Lot Validation sheet, under the test conditions specified. The Lot Validation sheet is included with every shipment of disposable electrodes.

CAUTION

Never polish a disposable electrode.

1.3.2 Dionex AAA-Certified Conventional Gold Electrodes

Dionex AAA-Certified Conventional Gold Electrodes are sold as a single unit (P/N 063722).

CAUTION

Do not polish a new Dionex AAA-Certified Conventional Gold Electrode.

Conventional electrodes continue to be useful for research, such as in waveform optimization or when trying out new eluent compositions and sample pretreatment procedures. Damaged working electrodes can be restored using the procedures from Section 10.8 of this manual.

1.4 Dionex *AAA-Direct* System (Dionex ICS-3000/5000/5000$^+$)

- DP Pump, Gradient - Isocratic Configuration
- EO Organizer and Eluent Bottles
- DC with Dual Temperature Zones, One Injection Valve
- ED Detector
- ED Cell with Reference Electrode
- ED Electrode, Au (Conventional or Disposable) - AAA Cert.
- Columns (Guard and Analytical)
- Standards / Concentrates
- CHROMELEON Chromatography Workstation / CMXpress
- AS-AP or Equivalent Autosampler

1.5 Replacement Parts for Dionex AAA-Certified Electrochemical Gold Cells

Part Number	Product Description
060141	Gasket for Disposable Electrode (Pack of 4), 2 mil (0.002 in.), Teflon®
045972	Gasket for Non-Disposable Electrode, 1 mil (0.001 in.), Ultem
061879	Combination pH-Ag/AgCl Reference Electrode

Please note that in this manual, ED can stand for ED/ED40/ED50/ED50A/ED3000/ED5000/ED5000+.

Complete listing of all spare parts for the Thermo Scientific Dionex electrochemical detectors can be found in the User's Compendium P/N 065340.

1.6 Dionex AminoPac PA10 Anion Exchange Columns

Part Number	Product Description
055406	Dionex AminoPac PA10 Analytical Column, 2 mm
055407	Dionex AminoPac PA10 Guard Column, 2 mm

For assistance, contact Technical Support for Thermo Scientific Dionex Products. In the U.S., call 1-800-346-6390. Outside the U.S., call the nearest Thermo Fisher Scientific office.

2. Operation and System Requirements

2.1 Requirements

The amino acid separations with Dionex AminoPac PA10 columns are optimized for use with the Thermo Scientific Dionex ICS-3000/5000/5000^{+}. Please note that the system consists of metal-free components, the key module of which is the DP pump configured for pumping gradients.

Tubing anywhere between the injection valve and detector should be < 0.005 in. i.d. PEEK tubing. Minimize the length of all liquid lines, but especially that of the tubing between the column and the detector cell. The use of larger diameter and/or longer tubing may decrease peak resolution.

Each of the possible configurations offers multiple sampling options; however, consistently reproducible quantification and an absence of disturbing artifacts are achieved only by using the "full loop mode" and in conjunction with a **25 µL sample loop P/N 042857**. Good reproducibility of retention times requires the use of temperature-control modules from Thermo Scientific Dionex and application of the exact settings described in the following sections of this manual.

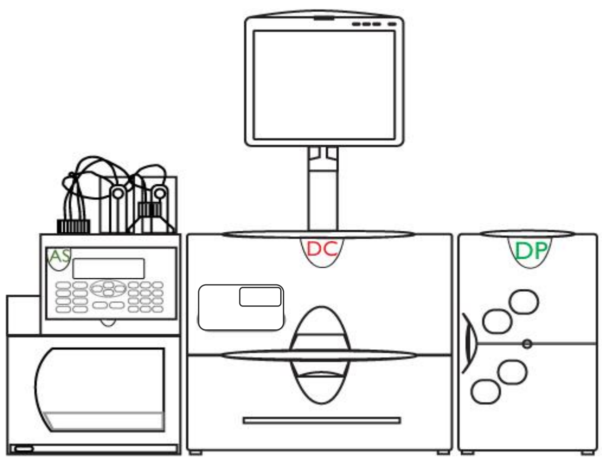


Figure 1
Automated Dionex *AAA-Direct* System

2.2 System Operation Requirements

The Dionex *AAA-Direct* Amino Acid Systems are configured to fulfill the following key requirements:

A. Mobile phase components are kept under helium or nitrogen at all times.
B. On-line degassing of eluents.
C. Accurate and precise flow rates at 0.25 mL/min.
D. Choice between pH and Ag/AgCl reference electrodes.
E. Programmable Integrated Pulsed Amperometry Detection (IPAD) waveforms with frequencies of 1 Hz or higher.
F. Minimized contribution to the background signal by contaminants from the system and reagents.
G. Column oven for constant temperature control of the guard column, separation column and detection cell.

2.3 Dionex AminoPac PA10 Column Operational Parameters

pH Range:	pH = 0–14
Temperature limit:5	0 °C
Pressure limit:	4,000 psi
Organic Solvent Limit:	100% acetonitrile, methanol acetone if required for cleaning. The column effluent containing a high content of organic solvent should go directly to waste and not be directed through the ED cell.
Typical eluents:	High purity water (18.2 megohm-cm), sodium hydroxide, sodium acetate

3. Purity Requirements for Chemicals

Obtaining reliable, reproducible and accurate results requires eluents that are free of impurities and prepared only from the chemicals recommended below. Thermo Scientific cannot guarantee proper column performance when alternate suppliers of chemicals or lower purity water are utilized.

3.1 Deionized Water

The deionized water used to prepare eluents should be Type I reagent Grade Water with a specific resistance of 18.2 megohm-cm. The deionized water should be free of ionized impurities, organics, microorganisms and particulate matter larger than 0.2 µm. The availability of UV treatment as a part of the water purification unit is recommended. Follow the manufacturer's instructions regarding the replacement of ion exchange and adsorbent cartridges. Expanding their period of use beyond the recommended time may lead to bacterial contamination and as a result, a laborious cleanup may be required. Use of contaminated water for eluents can lead to high background signals and gradient artifacts.

3.2 Sodium Hydroxide

Use diluted 50% w/w sodium hydroxide (Certified Grade, Fisher Scientific P/N UN 1824) for preparation.

3.3 Sodium Acetate

Thermo Scientific highly recommends the use of Thermo Scientific Dionex Sodium Acetate Reagent (P/N 059326) with Dionex *AAA-Direct* systems. Failure to use the Dionex Sodium Acetate Reagent can result in contamination of your Dionex *AAA-Direct* system and fouling of your Dionex AAA-Certified Gold Electrode. The symptoms of this contamination include an up to 80% decrease in peak response over time, and considerable time cleaning the system. Thermo Scientific cannot guarantee proper detection performance when different grades or alternate suppliers of sodium acetate are utilized.

4. Getting Started

4.1 The Most Important Rules

ALWAYS use gloves (non-powder) when handling eluent bottles, samples or electrode cell parts. Don't touch these with your bare hands.

ALWAYS use 50% NaOH solution rather than NaOH pellets to make eluent.

ALWAYS use dedicated glassware and disposable glass or plastic ware for volume adjustments.

ALWAYS keep your NaOH eluent blanketed by inert gas. Prepare new NaOH eluent if left unblanketed for more than 30 minutes.

ALWAYS pull at least 40 mL of new eluent through the lines when changing eluent or adding fresh eluent. This will ensure that your fresh eluent is primed through the lines up to the pump heads.

ALWAYS use pre-slit septa with the injection vials.

ALWAYS use 25 µL loop size; larger loops will cause loss of resolution.

ALWAYS install and use the piston wash option.

NEVER go to the next step of the procedure if the previous has failed.

NEVER start an installation with any of the check list items below missing.

NEVER use bottled HPLC water. Do not store 18.2 megohm-cm water; always use freshly drawn water for any preparation of eluents.

NEVER use 'communal' filtration units or filters made of unknown or unsuitable (cellulose derivatives, polysulfone) materials.

NEVER use inlet filters.

NEVER use MeOH or other organic solvent as rinse fluid in the autosampler. Use only 20 ppm sodium azide, or water if replaced daily.

NEVER run above 50 °C or 3,500 psi.

4.2 Initial Check List

These items MUST be available in your lab. The absence of any of these may compromise your analysis.

- ☐ Laboratory water unit delivering 18.2 megohm-cm water at the installation site.
- ☐ Vacuum pump available for use with the vacuum filtration units.
- ☐ Sterile-packed Nylon Nalgene Filtration Units, Funnel Size 1.0 L (VWR 28198-514, Fisher. 09-740-46 or. 164-0020).
- ☐ Inert gas cylinder (helium or nitrogen) with a regulator valve (ca 0–200 psi at the low pressure side) and the appropriate size adaptors plus tubing.
- ☐ NIST Amino Acid standards (SRM 2389A, 2.5 mM solution) or equivalent (NIST, Gaithersburg, MD, USA).
- ☐ One spare Dionex AAA-Certified Conventional Gold Electrode P/N 063722
- ☐ One spare Dionex pH-Ag/AgCl reference electrode P/N 061879.
- ☐ Sterile-packed, 10 mL (Fisher 13-650L) and 25 mL (Fisher 13-650P) pipettes and suitable pipeting bulbs (Fisher 13-681-50) or pumps.
- ☐ Sodium azide solid (FS26628-22-8NaN3) for preparation of diluent solution.
- ☐ Powder-free, disposable gloves (Fisher 19-041-171, at least 1 box).

5. Preparation of Eluents and Standards

NOTE

Always sanitize the entire analyzer with 2 M NaOH prior to initial start-up (see SECTION 6.1.2), after idle periods, or whenever the detection background exceeds 80 nC under initial gradient conditions.

Follow these precautions rigorously when preparing eluents:

A. Minimize any extraneous contamination of eluents. For example, a trace of an ion pairing agent introduced into the eluent from a "shared" filtration apparatus will cause an interference with some of the amino acid peaks. Dedicate glassware, pipettes, and filtration apparatus for exclusive use in preparation of Dionex *AAA-Direct* eluents only. Wear disposable, powder-free gloves whenever preparing or refilling eluents.

B. Minimize the level of carbonate introduced into the eluents during preparation.

C. Avoid bacterial contamination of eluent bottles and tubing. The bacterial contamination is minimized by wearing gloves, keeping containers closed whenever possible and by ultrafiltration (filter pore size < 0.2 µm). Use ultrafiltration as indicated in the instructions for preparing each of the three mobile phases. Microorganisms, if present in the system, produce amino acids thus causing elevated background levels and spurious peaks.

D. The system wash with 2 M NaOH, described in Section 10.5, is the only reliable technique to remove bacteria once they enter into the system.

5.1 Eluent E1: Deionized Water

Filter the pure deionized water through 0.2 µm Nylon filters, then transfer it into bottle E1 of the system. Thermo Scientific recommends the use of the sterilized, sterile packed, 1 liter-funnel, vacuum-filtration units from Nalgene which are ideal for filtration of all eluents (Fisher 09-740-46).

Seal the filtered water immediately. Remember, that atmospheric carbon dioxide adsorbs even into pure water, albeit at much lower levels than in alkaline solutions. Minimize the contact time of water surface with the atmosphere.

5.2 Eluent E2: 0.250 M Sodium Hydroxide

The first step in the preparation of sodium hydroxide eluent is filtration of a water aliquot (typically 1.0 L), using the sterilized Nalgene filtration unit described above. Seal the filtered water immediately after filtration, while preparing a disposable glass pipette (25.0 mL pipettes, Fisher 13-650P) and a pipette filler. Using a pipette filler, draw an aliquot of 50% sodium hydroxide into the pipette. Unseal the filtered water and insert the full pipette approximately 1 inch below the water surface and release the sodium hydroxide. If done properly, and without stirring, most of the concentrated sodium hydroxide stays at the lower half of the container and the rate of carbon dioxide adsorption is much lower than that of a homogeneous 250 mM sodium hydroxide solution. Seal the container immediately after the sodium hydroxide transfer is complete. Remember to put the screw cap back on the 50% hydroxide bottle immediately as well. Mix the contents of the tightly sealed container holding the 250 mM hydroxide.

Unscrew the cap of the eluent bottle E2 attached to the system. Allow the helium or nitrogen gas to blow out of the cap. Unseal the bottle holding 250 mM hydroxide and immediately, without delay, start the transfer into the eluent bottle E2. Try to minimize the carbon dioxide absorption by holding the gas orifice of the bottle cap as close as possible to the 250 mM hydroxide during the transfer. With the inert gas still blowing, put the cap back on the eluent bottle. Allow the pressure to build up inside the bottle and reopen the cap briefly several times, to allow trapped air to be gradually replaced by the inert gas.

5.3 Eluent E3: 1.0 M Sodium Acetate

A. Using 18.2 megohm-cm water, add approximately 450 mL deionized water to one of the Dionex sodium acetate containers.

B. Replace the top and shake until the contents are completely dissolved.

C. Transfer the sodium acetate solution to a 1 L container, such as a dedicated Nalgene flask from the vacuum filtration unit.

D. Rinse the 500 mL sodium acetate container with approximately 100 mL water, transferring the rinse water into the 1 L dedicated Nalgene flask.

E. After the rinse, fill the contents of the 1 L container to the 1 L mark with water.

F. Thoroughly mix the eluent solution, then filter it through a 0.2 µm Nylon filter, using a sterile Nalgene vacuum filtration unit.

G. Transfer the filtered sodium acetate eluent into the Eluent E3 bottle, making sure to minimize the exposure time to atmospheric carbon dioxide.

NOTE

Dionex recommends the use of dedicated glassware, pipettes and filtration apparatus for exclusive use in the preparation of Dionex AAA-Direct eluents.

5.4 Diluent Containing Norleucine and Sodium Azide

SAFETY

Sodium azide (NaN_3) should be handled and disposed of according to the guide lines provided by the manufacturer.

Prepare 4 mM stock solution of norleucine (524.8 mg/L, Fisher 327-57-1) in 0.1 M HCl. Dilute 1:500 with a deionized water solution containing ca. 20 mg of NaN_3/L. The resulting diluent solution is stable for months if stored in a refrigerator. Use it to prepare final dilutions from standard stock solution and to redissolve hydrolysate samples after evaporation to dryness. If sodium azide is not used, samples must be stored frozen.

5.5 Amino Acid Standards

Dilute aliquots of Standard Reference Material 2389A (NIST, Gaithersburg, MD) either 1:500 or 1:250 x with the diluent (see Section 5.4) to obtain 5 µM or 10 µM standard solutions. The prepared standard solutions will remain stable for weeks, if stored in a refrigerator. The trace of sodium azide introduced with the diluent solution stabilizes standards for up to 48 hours at room temperature.

6. System Installation and Start-Up

There are four distinct stages during an installation of new Dionex *AAA-Direct* systems.

A. System configuration and start-up
B. Verification of system cleanliness
C. Verification of system response
D. Verification of system functionality

Make sure that each section passes before moving onto the next. If you are having problems, check the troubleshooting guide at the end of this procedure. If you are still having problems, **, contact Technical Support for Thermo Scientific Dionex Products. In the U.S., call 1-800-346-6390. Outside the U.S., call the nearest Thermo Fisher Scientific office.**

6.1 System Configuration and Start-up

Configure the system with the Thermo Scientific Dionex DC on the left and the AS-AP (or equivalent) autosampler on the op, the injection module (the DC) on the right and the pump on the right. Nitrogen or helium should be delivered to the eluent organizer with about 5-6 psi at each bottle. Make sure that extra care is taken to minimize dead volume between the injector valve and detection cell. Use 0.005" i.d. tubing from the injection valve to the column and from the column to the detector cell inlet. Make all fluidic and electrical connections, but do not install the column yet. Instead install the yellow tubing (0.003" i.d.) from the Installation Kit between the injector and detector cell inlet. Assemble the electrochemical cell with the Dionex AAA-Certified Conventional Gold working electrode. Verify that the modules are communicating.

CAUTION

Do not polish or touch the gold surface prior to installation

6.1.1 Creating a Sequence and an Instrument Method

Create a sequence "HisNIST" and two Instrument Methods by following the example in Appendix B. The waveform can be programed into the Instrument Method by selecting "pH" as "Reference Electrode" and "Gold, pH-Ag-AgCl, RE, AAA" as "Waveform" in the "EDet1 Options for Options (ED:EDet1)" during the creation of Instrument Method in the Chromeleon (CM) software. Please refer to the manual of CM for more detail about it (Quick Start Guide Chromeleon 7.2, P/N: 7, rev 1.0, 2013). The waveform is also shown in Table 1 (page 26) and the gradient profile is from table 4 (page 49) in this manual.

6.1.2 System Rinse

NOTE

RINSE a new system with 2 M NaOH prior to use.
DO NOT polish new Dionex AAA-Certified Conventional electrodes.
DO NOT install Dionex AminoPac PA10 column before confirming background < 80 nC.

Prepare a solution of 2 M NaOH to rinse each bottle, by diluting 104 mL of 50% sodium hydroxide to 1 L with deionized water. Place the 2 M NaOH in a pre-rinsed bottle and place all 4 eluent lines in it. Withdraw at least 40 mL of sodium hydroxide from each line, using the priming feature of the DP. Close the solvent draw-off valve and leave the pump proportioning at 25/25/25/25 for 15 minutes. Make sure all surfaces come into contact with the sodium hydroxide; rotate the injection valve. Repeat the above process with 18.2 megohm-cm water.

6.2 Verification of System Cleanliness

Prepare a new set of eluents as described in Sections 5.1, 5.2, and 5.3.

Set the eluent composition to 100% for each eluent and pump out at least 40 mL of eluent from each eluent line after filling the eluent bottles using the priming feature of the DP.

6.2.1 System Background Check

Verify the system background using the initial conditions of the program from the Sequence as described in Section 6.1.1, which uses Waveform Table 1 and gradient Table 4 for protein hydrolysates in this manual. Make sure that

A. the detector is set to pH mode (not AgCl mode) and the cell is not yet on,
B. the pump is pumping 76% E1 (DI water) and 24% E2 (0.25 M NaOH), at 0.25 mL/min,
C. a length of yellow tubing is installed between the injector and the detector cell to generate 1000–2300 psi backpressure
D. the columns are still not installed.

Confirm that the pH readout is between 12.1 and 13.0 with a freshly calibrated reference electrode. With pH within range, turn on the cell and begin monitoring the background signal from the control panel for at least 30 min. Confirm that the baseline is < 80 nC. If the background exceeds 80 nC or the pH is out of range, see the Troubleshooting section (section 10).

6.3 Verification of Column Cleanliness

NOTE: If installing a new column set on a new system, proceed directly to 6.4

Install the Dionex AminoPac PA10 column set only after the Initial System Test (6.1.2 and 6.2.1) determines a background level within the specified range. A premature installation on a contaminated system will cause delays during the column equilibration.

Flush the column with 60 mM NaOH into waste for 10 minutes prior to connecting it to the ED cell.

To equilibrate a column which has been stored long term, conduct a gradient run defined by Method STD_AAA, injecting 25 µL of deionized water.

Figure 2 demonstrates the typical appearance of a blank gradient chromatogram. Note: The appearance of various small artifacts is strongly magnified by the narrow range of 0 to 100 nC chosen for this plot. Evaluate the magnitude of gradient rise as indicated by the two horizontal lines. The large, sharp peak, appearing at approximately 23 minutes, is due to a narrow zone of hydroxide ions being displaced from the column by the increasing concentration of the acetate eluent.

Should the background shift exceed 50 nC, perform the 2 M sodium hydroxide (NaOH) wash as described in Section 6.1.2, "System Rinse." Alternative, but also somewhat more time consuming, methods for decreasing the magnitude of the gradient step baseline shift include storing the system in 250 mM sodium hydroxide (100% E2) overnight (suitable for discontinuous manual injector systems) or pumping 100 mM NaOH/ 600 mM NaOAc at 40 °C for 2–3 hours followed by a long series of blank gradients at 30 °C (suitable with automatic systems overnight or over a weekend).

Generally, a system running continuously, 24 hours a day, delivers a more consistent performance with background shifts due to the gradient being as low as 5 nC. A system turned off every night or a system running for a long period of time at the low-concentration starting eluent conditions exhibits higher levels of gradient rise, frequently exceeding the target value of 50–80 nC.

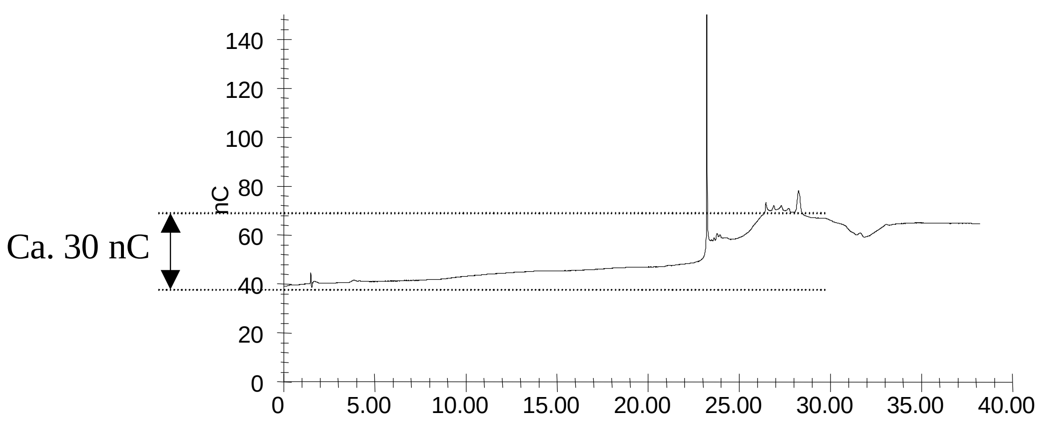


Figure 2
Typical Appearance of a Blank Gradient

6.4 Verification of System Response

6.4.1 Adjusting the Eluent Composition

Change eluent composition to 36% E1 (DI water): 24% E2 (0.25 M NaOH): 40% E3 (1.0 M NaOAc) at 0.25 mL/min. Wait 10 minutes until the background is stable and < 130 nC. If it is drifting down, wait as long as it takes to stabilize below 130 nC. If the background exceeds 130 nC, see Section 10, Troubleshooting.

6.4.2 Column Installation

Stop the flow, turn off the cell voltage and remove the yellow restrictor tubing. Install the Dionex AminoPac PA10 guard and analytical columns, but DO NOT connect the column outlet to the cell inlet. Turn the pump back on at 0.25 mL/min and pump 36% E1 (DI water): 24% E2 (0.25 M NaOH): 40% E3 (1.0 M NaOAc) through the column and into a waste container for 10 min. Connect the column tubing to the cell and verify the background is still < 130 nC. If it is not, see the troubleshooting section at the end of this manual.

6.4.3 Histidine Injection

Make an 8 µM solution of histidine by adding 1 mL of water to the dry residue in the micro vial shipped with the Dionex *AAA-Direct* Start Up Kit (P/N 059539). Place a vial with DI water in position RA1 of the autosampler and the histidine quality solution in position RA2. Run lines 1 and 2 in the Installation sequence created as a copy of the HisNIST sequence from the Installation Disk (36% E1:24% E2:40% E3, isocratic, with waveform from Table 1). Confirm that the peak height for histidine is >200 nC (Figure 3) and the %RSD for His peak height is < 5%. If this is not the case, see the troubleshooting section at the end of this manual.

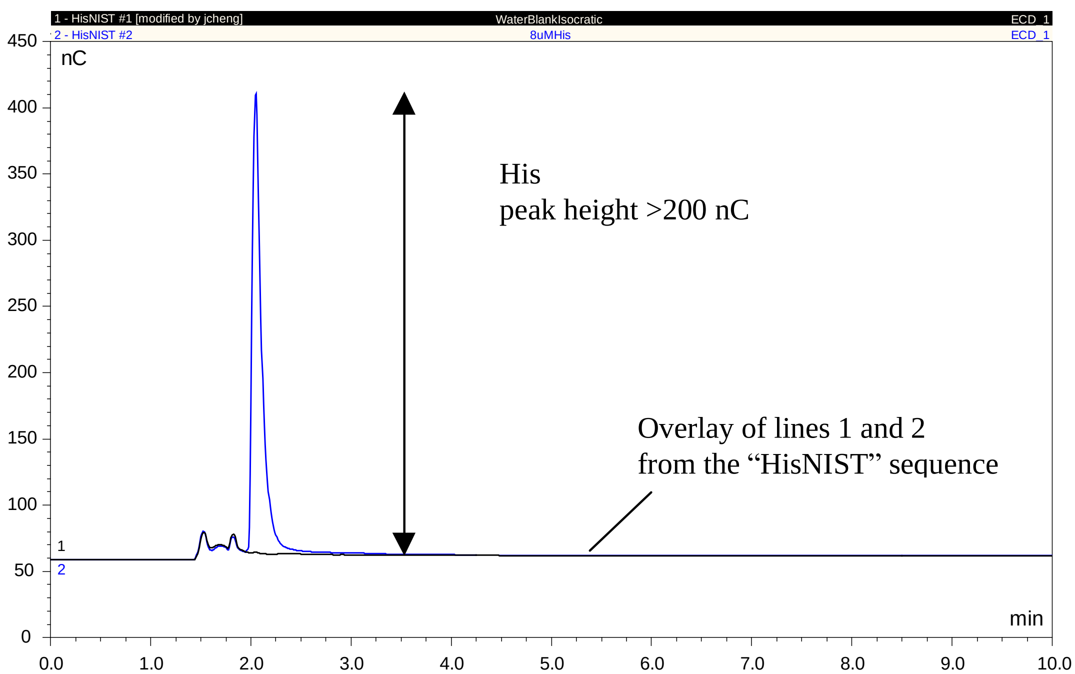


Figure 3
Testing the Detection Response

6.5 Verification of System Functionality

6.5.1 Injection of NIST SRM 2389A Standard

Program the pump to deliver 76% E1 (DI water): 24% E2 (0.25 M NaOH) (initial conditions of line 5 of the installation sequence) and let the system equilibrate. Set the column oven to 30 °C. Verify that the background level returns to <80 nC. If it does not, see the troubleshooting section at the end of this manual. Prepare 1 L of 20 mg/L of sodium azide in water. Prepare 100.00 mL of 8 µM NIST standard by pipeting exactly 320.0 µL of NIST SRM 2389ATable 9 concentrate into a clean 100 mL volumetric flask and filling up to 100 mL with the 20 mg/L azide solution. Make sure that there is still a water-blank vial in position RA1 of the autosampler and place the 8 µM NIST standard into position RA3. Execute lines 5 and 4 of the sequence in Section 6.1.1. Confirm that the baseline rise from the start of the run to the top of the acetate gradient does not exceed 50 nC. If it does, see the troubleshooting section at the end of this manual. Confirm that the Arginine peak is >120 nC/235 pmol (Figure 4). Overlay your separation with that from line 6 of the HisNIST sequence and confirm that the resolution between alanine and threonine is comparable.

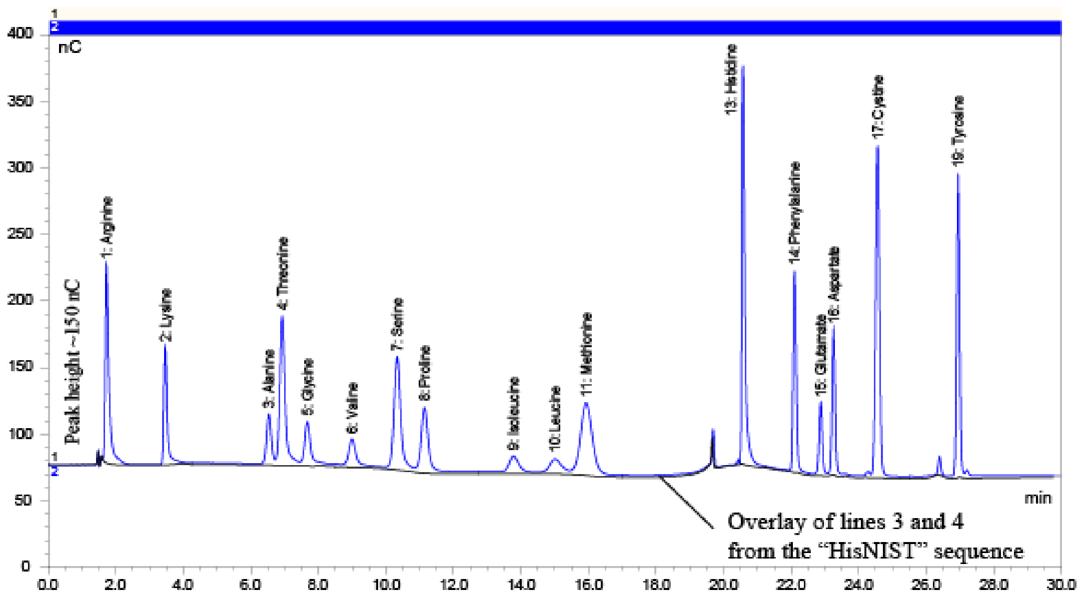


Figure 4
System Test

6.6 System Stand-by and Shutdown

As with all amino acid analyses, the best results in terms of reproducibility are obtained with continuous use. If it is not possible to keep the system in continuous use, then the system should be taken care of as described below, depending upon whether the shutdown is short-term or long-term.

6.6.1 Short-Term Stand-by

Short-term stand-by is defined as overnight, or over a weekend. If the system is to be shutdown for longer than 2–3 days, then follow the procedure for long-term shutdown.

To put the system into short-term stand-by, eluent should be pumped continuously through the system until the system is next ready to be used. Thermo Scientific recommends pumping all three eluents through the system at 0.05 mL/min using the ratio 36% E1: 24% E2: 40% E3 (where E1 is water, E2 is 250 mM sodium hydroxide and E3 is 1.0 M sodium acetate). This can be accomplished automatically by adding an extra line to your final schedule of the day, with a new method reflecting these conditions. If the system is being run manually, then these conditions should be programmed into the computer or via the front panel of the pump after the last injection has been completed.

6.6.2 Long-Term Shutdown

Long-term shutdown is defined as longer than a weekend (2–3 days). If the system is only going to be idle overnight, or over a weekend, then follow the procedure for short-term stand-by.

To shut the system down long-term, Thermo Scientific recommends the following procedure:

A. Program the pump to deliver 60 mM sodium hydroxide. Pump this solution through the columns for 60 minutes at 0.25 mL/min. Turn off the pump, remove the columns, plug the ends with the plugs that were in place when you received the columns and store them.
B. Using a union or a piece of 0.05" i.d. tubing to replace the columns, reconnect the detector to the injection valve and rinse the entire IC system with water for 60 minutes to eliminate all traces of acetate and carbonate which could crystallize in the check valves, lines etc.
C. Turn off the pump, remove the reference electrode and immerse it in 3 M KCl. The original "soaker" bottles in which the electrode was shipped is ideal for the storage container.
D. Disassemble the rest of the ED cell, rinse the working electrode in 18.2 megohm-cm water (wear gloves to avoid contaminating the electrode), allow it to dry and then place the electrode in a clean bag or other suitable clean, enclosed container. The titanium body can be stored in a drawer placed on a fresh towel or other type of clean surface.
E. For storage periods longer than a week, we recommend storing the system in 95% water 5% acetonitrile. Do not use methanol because it is IPAD positive and would cause high background and other problems unless thoroughly washed out of the system at the next system startup. Remember to never use methanol in the AS rinsing solution for the same reason.

7. Selecting Detection and Gradient Methods

7.1 Introduction to Detection Method

The amino acid oxidation at gold electrodes is made possible by a rapid sequence of potentials (waveform) adjusted between the working electrode (gold) and the reference electrode (pH-Ag/AgCl). Resulting currents are measured by integration during a short time interval extending over several steps of the detection waveform. The standard, recommended amino acid waveform is shown in Table 1.

Table 1
Dionex *AAA-Direct* Waveform Potentials

Time (sec)	Potential (V) vs. Ag/AgCl	Potential (V) vs. pH	Integration
0.000	-0.20	+0.13	
0.040	-0.20	+0.13	
0.050	0.00	+0.33	
0.210	0.00	+0.33	Begin
0.220	+0.22	+0.55*	
0.460	+0.22	+0.55*	
0.470	0.00	+0.33	
0.560	0.00	+0.33	End
0.570	-2.00	-1.67	
0.580	-2.00	-1.67	
0.590	+0.60	+0.93	
0.600	-0.20	+0.13	

* In the older editions of this manual the potential was +0.60 for this portion of the waveform. The lower potential increases the length of useful performance by preventing an excessive gold oxide formation in certain situations (i.e., positive shifts of reference potential).

CAUTION

Do not polish a new Dionex AAA-Certified Conventional Gold Electrode.
Never polish a Dionex AAA-Certified Disposable Gold Electrode.

Refer to Section 10 - Troubleshooting of this manual for an overview of reconditioning techniques for gold working electrodes.

The reference electrode for the Dionex ED is a pH - Ag/AgCl combination electrode (P/N 061879). There are advantages to using the pH reference electrode. In particular, the gradient induced baseline shifts are better suppressed when the pH electrode is used. Always verify the correct selection of reference electrode prior to turning the cell voltage on. The reference electrode selection is made/checked in Chromeleon.

It is advantageous to always have available at least one unused "known good" combination reference electrode. If stored in saturated KCl, a combination electrode can be kept for years with its reference potential virtually unchanged. In contrast, the reference electrodes mounted inside the ED cell and exposed to flowing sodium hydroxide have only a limited lifetime of ca. 3 to 6 months. As a result of prolonged exposure to alkaline solutions, the 0.1 M KCl solution inside the reference electrode gradually becomes alkaline and the silver chloride layer on the Ag wire immersed into that solution either dissolves or converts to a mixture of silver oxide and silver hydroxide. As that happens, the reference potential shifts and becomes increasingly unstable. Shifting reference potential is experienced by the user either as an unusually high background or a decrease in sensitivity of detection. A combination of both effects is also possible.

CAUTION

Never leave a reference electrode inside a disconnected ED cell.

Furthermore, a combination reference electrode can be irreversibly damaged by drying out. This happens most frequently by leaving the reference electrode inside a disconnected ED cell. Always remove the reference cell from the ED cell, when the system is not in use (i.e. cell inlet and outlet are not connected to a flowing eluent). After the removal from the ED cell, keep the reference electrode immersed in 3 M KCl solution (224 g KCl/L) at all times.

With a “known good” reference electrode, it is possible to carry out one of the following checks of the combination reference electrode being used in the ED cell (See Dionex PIU_ED_1 for additional details).

A. Immerse the “known good” reference electrode and the tested electrode into the same 0.1 M KCl solution. Using a voltmeter, measure the potential between the two electrodes. We recommend to discard and replace any tested electrode differing by more than 30 mV from a “known good” Ag/AgCl reference.
B. Use the procedure in the ED manual to measure the potential difference between two reference electrodes immersed in the same 0.1 M KCl solution. See a detailed description on www.Dionex.com (search: “electrochemical detector 001”).
C. Simply replace the electrode you wish to check by a “known good” reference electrode inside the ED cell. Apply the voltage to the cell. Discontinue using the checked electrode if the insertion of the “known good” electrode decreases the background from > 80 nC to < 80 nC.
D. Immediately remove the “known good” electrode and store it properly. This referencing procedure will work as long as you do not leave your “known good” electrode inside the ED cell for more than a few hours at a time and store it properly (immersed in 3 M KCl) in the intervening periods of time.

7.2 Dionex AminoPac PA10 Column Test Chromatogram

A representative test chromatogram for the Dionex AminoPac PA10 column is shown in Figure 5. Each Dionex AminoPac PA10 column is tested using this test protocol. The test chromatogram was generated using a Dionex *AAA-Direct* Analyzer and the gradient in Table 2C. Similar separations can be obtained by performing a fully automatic gradient illustrated in Table 2A.

Injection Volume:	25 µL
Standard:	NIST=2.5 µmol/mL in 0.1 M HCl solution
Diluted Standard (with DI water):	20 nmol/mL*
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System	
Operating Backpressure:	<3,000 psi
Eluent:	
E1:	Deionized water
E2:	250 mM NaOH
E3:	1 M Sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED Operating Parameters:	AAA Au, pH reference, waveform in Table 1
Gradient:	Table 2C

*Note: Approximate concentration. Refer to the NIST SRM Certificate of Analysis for the exact value of standard components.

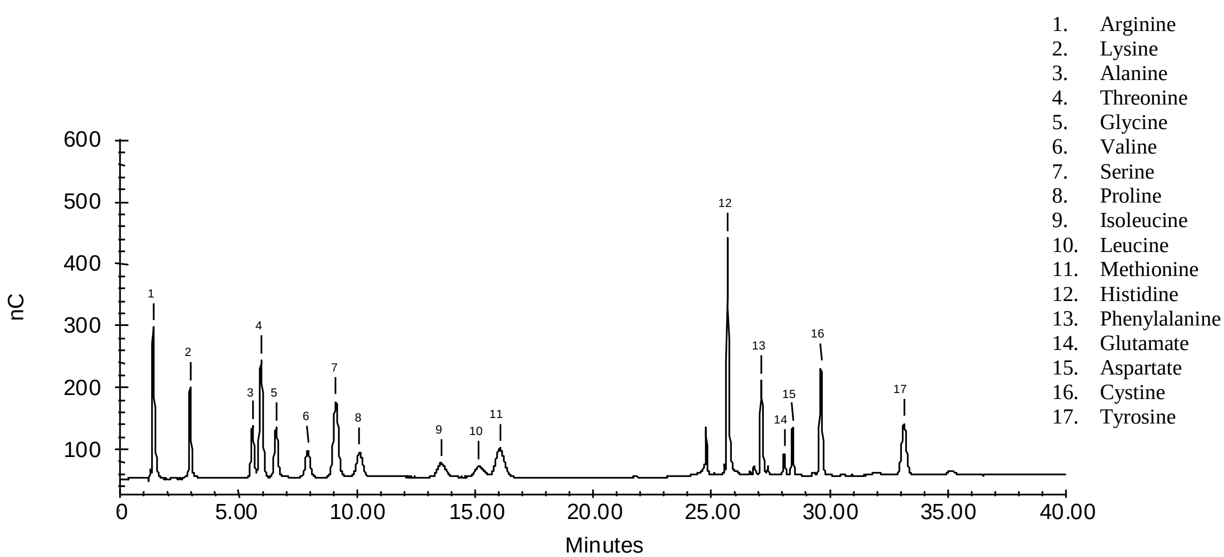


Figure 5
Dionex AminoPac PA10 Column Test Chromatogram

7.3 Selection of Gradient Method

Table 2
Overview of Gradient Methods

	Initial E1/E2/E3	Interim E1/E2/E3	Final E1/E2/E3	Purpose	Notes and Recommendations
Table 2A	80/20/0	68/32/0	36/24/40	Column testing by Thermo Scientific	Do not use for actual samples. Final composition is not strong enough to elute Trp and other strongly retained analytes E1 (DI water); E2 (0.25 M NaOH); E3 (1 M NaOAc) for all gradients except Table 7.
Table 2B	80/20/0	68/32/0	36/24/40	Example of run time optimization	Same as above
Table 2C	0/50/50	80/20	36/24/40	Adaptation of Table 2A for manual systems. Rinsing is performed before the injection. There is no need for precise timing of consecutive injections.	Do not use with automated systems. Always run a water blank as the first injection of the day and use gradients with rinsing steps at the end.
Table 3	84/16/0	68/32	36/24/40	Initial E1/E2 lowered to improve separation of glucose	Final E1/E2/E3 not strong enough. Always insert an acetic acid rinsing step (See Table 5)
Table 4	76/24/0	64/36/0	14/16/70	Analysis of hydrolysates. Good starting point for unknown samples	Long term experience indicates a need for additional rinsing step (see Table 5). Small traces of His, Phe, Glu,Asp, Tyr can sometime be carried over into the next separation.
Table 5	76/24/0	64/36/0	14/16/70	Universal “workhorse” gradient. Ideal for hydrolysates and as a starting point for unknown samples.	E4: 0.1 M acetic acid, 100%E4 45-47 min. The rinsing can also be done ‘on the fly,’ see footnote to Table 5.
Table 6	76/24/0	0/90/10	14/16/70	Improves peak shape of His	Includes the 0.1M acetic acid rinse
Table 7	79.2/20.8	66.7/33.3	21.9/8.1/70	Keeps eluents E1 and E3 sterile at all times	E1: 10 mM NaOH, E2: 250 mM NaOH, E3: 1 M NaOAc, 25 mM NaOH
Table 8	97.92/2.08	0/90/0	0/30/70	Separation of complex mixtures of carbohydrates and amino acids, e.g., cell culture media.	Same composition of E1, E2, and E3, as in Table 7. Includes the 0.1 M acetic acid rinse.

Table 9	76/24/0	64/36/0	0/16/84	Fast separation of 17 amino acids plus tryptophan. Separation and re equilibration completed under 40 min	Includes the 0.1M acetic acid rinse
Table 10	76/24/0	40/20/40	14/16/70	17 amino acid separation. Total run time 25 min	Tryptophan if present is removed during the 0.1 M acetic acid rinse. This method is used in 2D separations of AA and carbohydrates.

7.3.1 Gradient Methods for Continuously Operating Automatic Systems

Fully automatic Dionex *AAA-Direct* systems (see Figure 1) are the preferred systems for routine, high-throughput analysis. Experience shows a constant series of blank gradient runs to be the most efficient way of maintaining low backgrounds and minimizing the size of baseline rise during gradients. For optimum retention time reproducibility, each series of standard and sample injections should be preceded by at least one blank gradient run. In other words, precise timing of column re-equilibration and maintaining constant intervals between injections are essential for an acceptable reproducibility of all retention times.

Table 2A
Test Gradient Conditions, Automated

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	80	20	0		
0.0	80	20	0		Inject
2.0	80	20	0		Inject valve to load position
12.0	80	20	0		Begin hydroxide gradient
16.0	68	32	0	8	Begin acetate gradient
24.0	36	24	40	8	
40.0	36	24	40		
40.1	20	80	0	5	Column wash with hydroxide
42.1	20	80	0		
42.2	80	20	0	5	Equilibrate to starting conditions
62	80	20	0		

E1: water E2: 250 mM NaOH E3: 1.0 M NaOAc

We recommend the gradient method in Table 2A, "Test Gradient Conditions, Automated," for initial runs on a new system and for evaluation of columns. Please note that the flow rate is 0.25 mL/min in all steps of the gradient table.

For standard mixtures and samples known not to contain tryptophan or any other strongly retained analytes, it is possible to cut short the length of the elution at 40% of E3 from 40 minutes to 30 minutes. The hydroxide column wash then starts and begins at 30.1 and 32.1 minutes respectively with the last segment of the gradient table changing from 62 to only 52 minutes. These conditions are shown in Table 2B, "Fast Gradient Conditions, Automated."

Table 2B
Fast Gradient Conditions, Automated

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	80	20	0		
0.0	80	20	0		Inject
2.0	80	20	0		Inject valve to load position
12.0	80	20	0		Begin hydroxide gradient
16.0	68	32	0	8	Begin acetate gradient
24.0	36	24	40	8	
30.0	36	24	40		
30.1	20	80	0	5	Column wash with hydroxide
32.1	20	80	0		
32.2	80	20	0	5	Equilibrate to starting conditions
52	80	20	0		

E1: water; E2: 250 mM NaOH; E3: 1.0 M NaOAc.

Please note that the flow rate is 0.25 mL/min in all steps of the gradient table.

Watch for unexpected, poorly-shaped, peaks in the region between histidine and tyrosine when cutting short the duration of the strong eluent segment. Whenever this occurs, return to the original timing in Table 2A, "Test Gradient Conditions, Automated," or use even stronger gradient conditions discussed in the Applications in Section 8.

7.3.2 Gradients for Manual, Discontinuously Operating Systems

The Gradient Conditions in Table 2C, "Gradient Conditions - Manual, Discontinuous Operation," make it possible to obtain an identical chromatogram as in Figure 5 with acceptable constancy of retention time starting with the first run. Non-constant time intervals between two injections, typical for manually operated injectors, do not have any effect on the reproducibility of retention times. The gradient method described in Table 2C achieves all that by a column wash executed at the beginning of the gradient program and by a longer re-equilibration time preceding the injection. The user has up to 39 minutes to fill the sample loop during the pre-injection period of each run.

Table 2C
Gradient Conditions - Manual, Discontinuous Operation

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	0	50	50		Strong wash begins
0.0	0	50	50		
10.0	0	50	50		
10.1	80	20	0	5	Start of re-equilibration to starting conditions
40.0	80	20	0		
40.1	80	20	0		Valve from Load to Inject, start data acquisition
42.0	80	20	0		Valve from Inject to Load
52.0	80	20	0		Begin hydroxide gradient
56.0	68	32	0	8	Begin acetate gradient
64.0	36	24	40	8	
80.0	36	24	40		

Please note that the flow rate is 0.25 mL/min in all steps of the gradient table.

E1: water; E2: 250 mM NaOH; E3: 1.0 M NaOAc.

8. Applications

The detection waveform in Table 1 has been found useful for all applications developed to date.

NOTE

Use the detection waveform from Section 7.1 for all applications

All the gradient conditions used in these applications and listed under experimental conditions and are tabulated in Section 9, Specialized Gradient Methods. Depending on your system, you may need to make small adjustments to your gradient conditions or operating temperature to achieve resolution of all analytes. Usually, the method adjustments will be to the gradient conditions (tryptophan, presence or absence of carbohydrates) or the column temperature (oxidation products of S-amino acids).

All gradient conditions used in these applications (and tabulated in Section 9) are presented in a form suitable for continuously operated, fully automated systems. Please refer to Table 2A, "Test Gradient Conditions, Automated," and 2C, "Gradient Conditions - Manual, Discontinuous Operation," in the preceding section, if you need to convert any of the gradient conditions to those suitable for discontinuously operated, manual systems.

8.1 Simultaneous Monitoring of Amino Acids and Carbohydrates in Fermentation Broths

We recommend the use of a special gradient for the separation of amino acids typically found in fermentation broth samples. The gradient modification (see Table 3, “Gradient Conditions for Amino Acids and Carbohydrates”) is necessary in order to separate the glucose and alanine peaks. These two peaks co-elute using the conditions recommended for the Standard Chromatogram Gradient (Section 7.3.2). Use the Amino Acids Waveform as listed in Table 1.

Sample Volume:	25 µL of broth after filtration (0.4 µm filter) and 1000x dilution
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	Deionized water
E2:	250 mM NaOH
E3:	1 M Sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED Waveform:	See Table 1
Gradient Conditions:	See Table 3

1. Arginine
2. Lysine
3. Glutamine
4. Glucose
5. Alanine
6. Threonine
7. Glycine
8. Valine
9. Serine
10. Proline
11. Isoleucine
12. Leucine
13. Methionine
14. Histidine
15. Phenylalanine
16. Glutamate
17. Aspartate
18. Cystine
19. Tyrosine

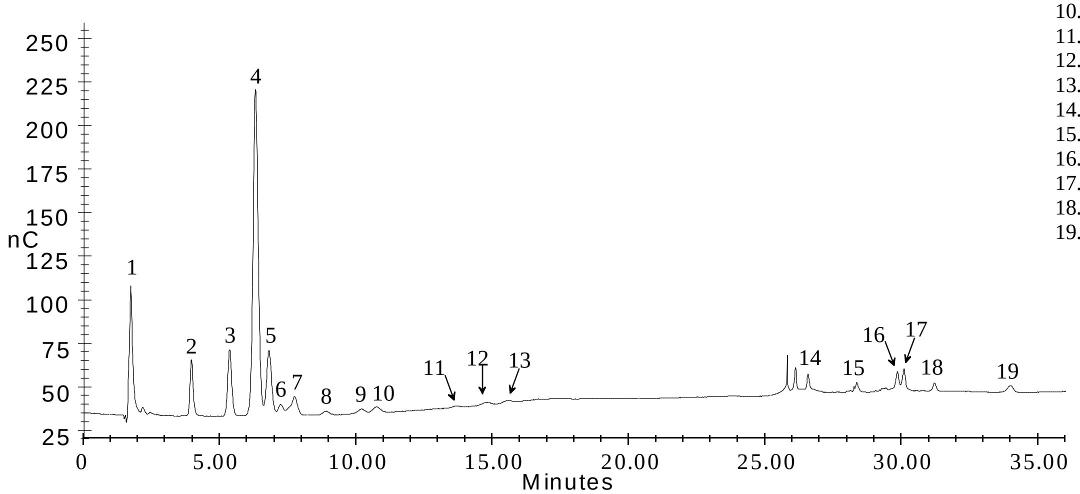


Figure 6
Simultaneous Monitoring of Amino Acids and Glucose in Fermentation Broths

NOTE

Simultaneous separations of sugars and amino acids are possible at equimolar levels or in some cases up to a 100:1 molar ratio. See References 13 and 17 in Section 8.10 for examples of gradient development. Samples containing excessive concentrations of carbohydrates (100:1 and higher) must be pretreated to make possible an interference-free analysis of all amino acids.

Thermo Scientific offers an accessory to the Dionex AAA-Direct system (Carbohydrate Removal Accessory P/N 065244) that makes possible a fully automatic on-line removal of carbohydrates from amino acid containing samples. Off-line removal of carbohydrates has also been described in the literature (Reference 15, Section 8.10)

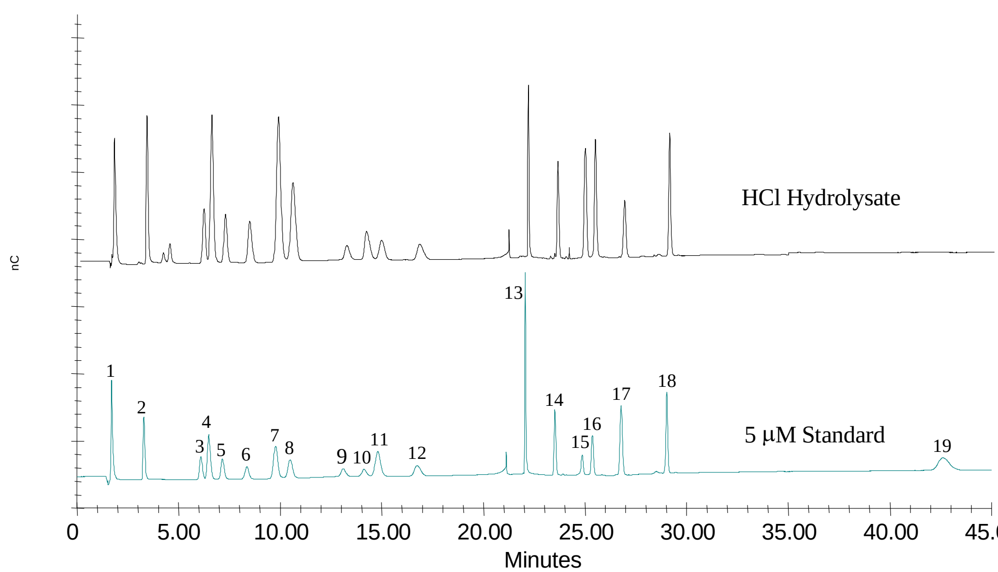
8.2 Analysis of Amino Acids in Hydrolysates

The present technique based on anion exchange separations with IPAD detection can be utilized for samples from all common types of protein hydrolysis protocols. For a detailed description and discussion of currently utilized hydrolytic techniques, refer for example to “Hydrolysis of Samples for Amino Acid Analysis,” by G. B. Irvine in Protein Sequencing Protocols, edited by B. J. Smith, Humana Press, 1997.

The relative value of different hydrolytic procedures is explained in the literature reference quoted above. As illustrated in Figure 7, “Analysis of Amino Acids in Hydrolysates,” the most informative separations are usually those from HCl hydrolysis. Because of its volatility, HCl can be removed completely by an evaporation step and the original matrix acidity does not interfere with the chromatography. Tryptophan usually does not survive the HCl hydrolysis and although it is included in the standard mixture, it does not appear in the sample chromatogram.

Sample preparation:	Hydrolyze 0.1 mg sample in 1.0 mL of 6 M HCl . Evaporate to dryness and reconstitute to the same volume with norleucine/azide diluent from Section 4.4. Dilute an aliquot 1,000–2,000x with the norleucine/azide diluent from Section 5.4.
Injection Volume:	25 µL
Standard:	NIST SRM 2389, 500x dilution using norleucine/azide diluent from Section 5.4.
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	18.2 megohm water
E2:	250 mM NaOH
E3:	1 M Sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 4

1. Arginine
2. Lysine
3. Alanine
4. Threonine
5. Glycine
6. Valine
7. Serine
8. Proline
9. Isoleucine
10. Leucine
11. Methionine
12. Norleucine
13. Histidine
14. Phenylalanine
15. Glutamate
16. Aspartate
17. Cystine
18. Tyrosine
19. Tryptophan



*Note: Approximate concentration. Refer to the NIST SRM Certificate of Analysis for the exact value of standard components.

Figure 7
Analysis of Amino Acids in Hydrolysates

8.3 Analysis of Tryptophan

Under certain conditions, it is possible to obtain a peak for tryptophan in MSA hydrolysates. This requires special conditions, discussed in “Hydrolysis of Samples for Amino Acid Analysis,” by G. B. Irvine in Protein Sequencing Protocols, edited by B. J. Smith, Humana Press, 1997. The easiest approach to tryptophan analysis is, however, by NaOH hydrolysis. It should be noted that the sodium hydroxide matrix is very compatible with the Dionex *AAA-Direct* method. The same is not true for some other amino acid methods (e.g. Ninhydrin, PITC). Although probably feasible, the hydrolysis method for the chromatogram in Figure 8, “Analysis of Tryptophan” was not optimized for all amino acids. The sample hydrolysed by NaOH to obtain the separation in Figure 8, “Analysis of Tryptophan,” is identical to the sample hydrolyzed by HCl for Figure 7, “Analysis of Amino Acids in Hydrolysates.” Note, for example, that the peak of hydroxyproline is not present in the NaOH hydrolysate. Also missing in the NaOH chromatogram are peaks for cystine and threonine.

Sample preparation:	Hydrolyze 0.1– 0.2 mg sample in 400 µL of 4.2 M NaOH. Dilute an aliquot 100x with the norleucine/azide diluent from Section 5.4.
Injection Volume:	25 µL
Standard:	NIST SRM 2389, 500x dilution using norleucine/azide diluent from Section 5.4 with tryptophan added.
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	18.2 megohm-cm water
E2:	250 mM NaOH
E3:	1 M sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 4

1. Arginine
2. Lysine
3. Alanine
4. Threonine
5. Glycine
6. Valine
7. Serine
8. Proline
9. Isoleucine
10. Leucine
11. Methionine
12. Norleucine
13. Histidine
14. Phenylalanine
15. Glutamate
16. Aspartate
17. Cystine
18. Tyrosine
19. Tryptophan

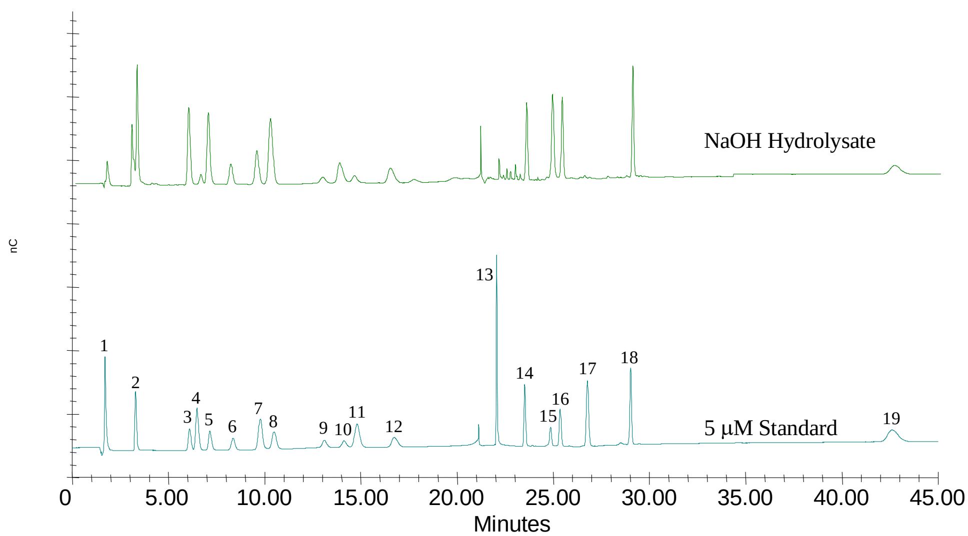


Figure 8
Analysis of Tryptophan

NOTE

See also Thermo Scientific Dionex Application Note 142 (Fast method for tryptophan analysis).

8.4 Analysis of Oxidation Products of Methionine, Cystine, and Cysteine

Using the gradient conditions of the Dionex *AAA-Direct* method, all cysteine converts (dimerizes) on column to cystine. The cystine peak in the chromatogram is thus always a sum of all cysteine and cystine originally present in the sample. In this context, another technique should be mentioned, which utilizes Dionex ED detector and Au working electrode in conjunction with either the Thermo Scientific Dionex OmniPac PCX-500 or Dionex OmniPac PCX-100 cation exchange column with acidic eluent conditions; P. J. Vandeberg and D. C. Johnson, Anal. Chem. 65 (1993), p. 2713. That technique has been shown to be very selective for sulfur amino acids and is capable of separating not only cysteine from cystine, but also methionine, homocysteine and homocystine in a single run. A successful application of that technique for the analysis of homocysteine in blood plasma has been reported in the literature, J. Evrovski, M. Callaghan and D. E. C. Cole, Clin. Chem. 41 (1995), p. 757.

For protein and peptide analysis, most users, however, perform an oxidative step in conjunction with methanesulfonic acid (MSA) or HCl hydrolysis to obtain reliable results for cysteine/cystine and methionine. The “performic acid/HCl” procedure (for a detailed description see “Hydrolysis of Samples for Amino Acid Analysis,” by G. B. Irvine in Protein Sequencing Protocols, edited by B. J. Smith, Humana Press, 1997), yields cysteic acid for cystine/cysteine and methionine sulfone for methionine. Under MSA hydrolysis conditions, it is possible for oxidation of methionine to go partially or completely to methionine sulfoxide. A suitable separation technique must be able to account for both oxidation products of methionine in addition to the cysteic acid.

The chromatogram in Figure 9, “Analysis of Oxydation Products of Methionine, Cystine, and Cysteine,” shows a standard mixture of all possible oxidation products (upper trace) together with methionine sulfone and cysteic acid peaks in a hydrolysate sample.

Sample preparation:	Hydrolyze 0.1 mg sample in 400 µL of 6 M HCl, after oxidation with performic acid. Evaporate to dryness reconstitute in the same volume of norleucine/azide diluent. Dilute an aliquot 100x with the norleucine/azide diluent from Section 5.4
Injection Volume:	25 µL hydrolysate (lower trace) and standard (upper trace)
Standard:	20 µM methionine sulfoxide, methionine sulfone, and cysteic acid
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	35 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	18.2 megohm-cm water
E2:	250 mM NaOH
E3:	1 M sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 4

1. Arginine
2. Methionine sulfoxide
3. Lysine
4. Alanine
5. Threonine
6. Methionine sulfone
7. Glycine
8. Valine
9. Serine
10. Proline
11. Isoleucine
12. Leucine
13. Norleucine
14. Histidine
15. Phenylalanine
16. Glutamate
17. Aspartate
18. Cysteic acid

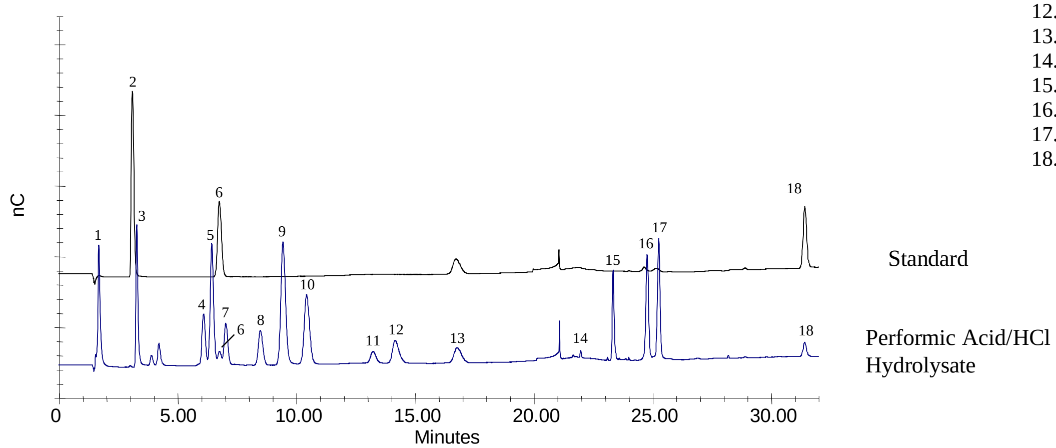


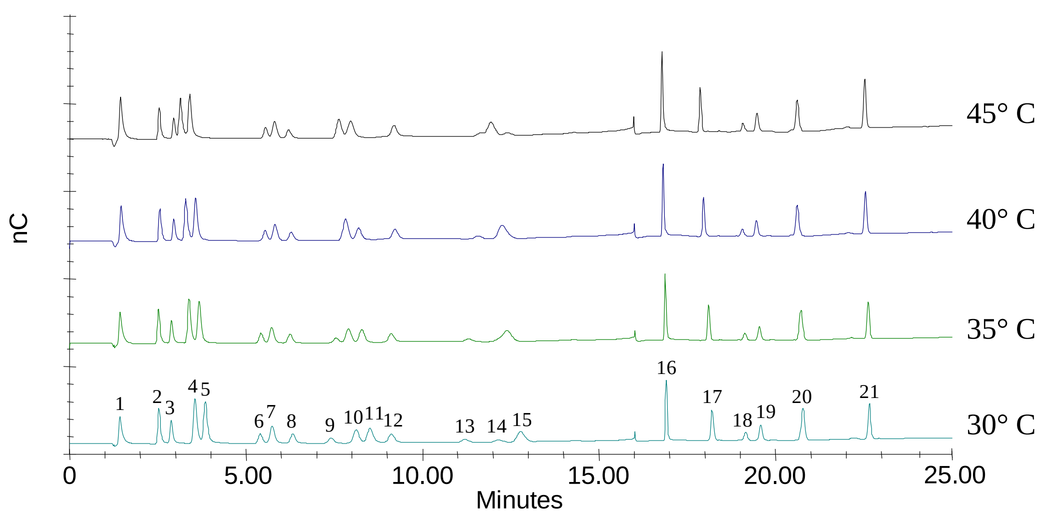
Figure 9
Analysis of Oxydation Products of Methionine, Cystine, and Cysteine

8.5 Influence of Temperature on the Separation of Amino Acids and Amino Sugars

In order to separate the methionine sulfone peak from threonine and glycine on the Dionex AminoPac PA10 column, it is necessary to use the column temperature of 35 °C instead of the more usual 30 °C. See Figure 9, “Analysis of Oxydation Products of Methionine, Cystine, and Cysteine.” The series of chromatograms presented in Figure 10, “Influence of Temperature on the Separation of Amino Acids and Amino Sugars,” illustrates the changes in retention behavior of amino acids and amino sugars occurring with temperature. Note: While the temperature-induced changes in the “acetate” region of the chromatogram are only minimal, the changes in retention occurring between 2 and 15 minutes are profound. The need for a precise temperature control is obvious. As the temperature is increased, the methionine retention time decreases while the isoleucine and leucine retention times remain essentially unchanged. As a result, methionine and leucine coelute at 35°C and 40°C. At 45°C, leucine elutes after methionine and isoleucine is a shoulder on the front of methionine. Also note the resolution of hydroxyproline and serine decreases as the temperature increases above 30°C. In case of incomplete oxidation of methionine, the results for leucine may show a considerable positive error. However, the absence or presence of the methionine peak can be easily verified by running a chromatogram at 30°C.

Injection Volume:	25 µL
Standard:	NIST SRM 2389 Amino Acid standard (8 µM* all components with hydroxylysine, galactosamine, glucosamine, and hydroxyproline added.)
Column:	Dionex AminoPac PA10 analytical column
Column temperature:	30 °C, 35 °C, 40 °C, 45 °C as indicated
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	18.2 megohm-cm water
E2:	250 mM NaOH
E3:	1 M sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 4

1. Arginine
2. Hydroxylysine
3. Lysine
4. Galactosamine
5. Glucosamine
6. Alanine
7. Threonine
8. Glycine
9. Valine
10. Hydroxyproline
11. Serine
12. Proline
13. Isoleucine
14. Leucine
15. Methionine
16. Histidine
17. Phenylalanine
18. Glutamate
19. Aspartate
20. Cystine
21. Tyrosine



*Note: Approximate concentration. Refer to the NIST SRM Certificate of Analysis for the exact value of standard components.

Figure 10
Influence of Temperature on the Separation of Amino Acids and Amino Sugars

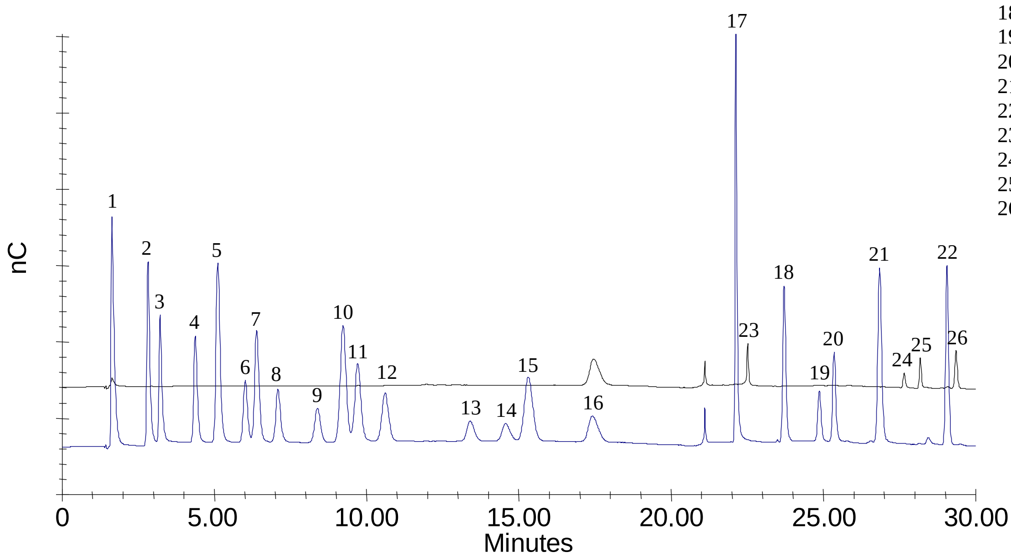
8.6 Analysis of Phospho-Amino Acids

Phospho-amino acids, being strongly anionic, elute in the acetate gradient region under the Table 4 gradient conditions. Figure 11, "Analysis of phospho-Amino Acids," shows a separation of "hydrolysate" standard and a separation of four selected phospho-amino acids overlaid. The four phospho-amino acids are separated from each other and the more common amino acids. Note: The injected amounts of the phospho-amino acids are 50 pmol. The estimated detection limits for these analytes are in the fmol range.

Samples for the analysis of phospho-amino acids are usually hydrolyzed under modified conditions. Consult literature before analyzing your samples for those compounds.

Injection Volume:	25 µL
Standard:	2 µM all P-AA (upper trace), 8 µM* all peaks (lower trace)
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	18.2 megohm-cm water
E2:	250 mM NaOH
E3:	1 M sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 4

1. Arginine
2. Hydroxylysine
3. Lysine
4. Glutamine
5. Asparagine
6. Alanine
7. Threonine
8. Glycine
9. Valine
10. Hydroxyproline
11. Serine
12. Proline
13. Isoleucine
14. Leucine
15. Methionine
16. Norleucine
17. Histidine
18. Phenylalanine
19. Glutamate
20. Aspartate
21. Cystine
22. Tyrosine
23. P-Arginine
24. P-Serine
25. P-Threonine
26. P-Tyrosine



*Note: Approximate concentration. Refer to the NIST SRM certificate of Analysis for the exact value of standard components.

Figure 11
Analysis of Phospho-Amino Acids

8.7 MSA Hydrolysis of Meat Samples

In this section, samples were hydrolyzed using 4 M methanesulfonic acid. As illustrated in Figure 12, "Analysis of Meat Hydrolysates," samples hydrolyzed by that technique may contain carbohydrates and the use of the Gradient Conditions from Table 3, "Gradient Conditions for Amino Acids and Carbohydrates," is thus recommended. Note that the two amino sugars also appearing in the chromatograms are separated by both gradient methods from Table 3 or Table 4, "Gradient Conditions for Protein Hydrolysates"; therefore the method in Table 3 is recommended for meats or other foods with high sugar content. Also note, the higher initial concentration of the Table 4 gradient would cause glucose and alanine to co-elute.

Sample preparation:	Hydrolyze 0.1 g of meat in 5.0 mL of 4.0 M MSA for 16 hours at 100 °C. Dilute 5x with water. In the next dilution step, dilute 500 fold with 8.0 µM norleucine azide diluent. (section 5.4)
Injection Volume:	25 µL
Sample Concentration:	8.0 µM, all amino acids in "standard"
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	Deionized water
E2:	250 mM NaOH
E3:	1 M Sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED waveform:	See Table 1
Gradient Conditions:	See Table 3

1. Arginine
2. Hydroxylysine
3. Lysine
4. Galactosamine
5. Glucosamine
6. Glucose
7. Alanine
8. Threonine
9. Glycine
10. Valine
11. Hydroxyproline
12. Serine
13. Proline
14. Isoleucine
15. Leucine
16. Methionine
17. Norleucine
18. Histidine
19. Phenylalanine
20. Glutamate
21. Aspartate
22. Cystine
23. Tyrosine

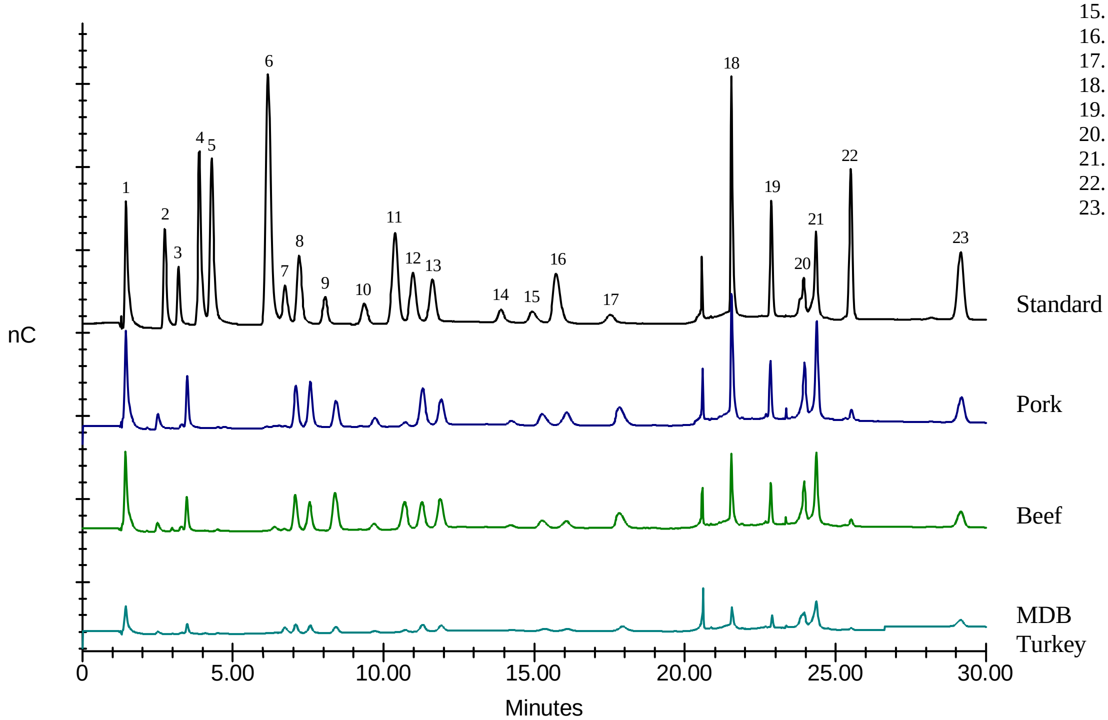


Figure 12
Analysis of Meat Hydrolysates

8.8 Free Amino Acids in Beverage Samples

The gradient conditions from Table 3, "Gradient Conditions for Amino Acids and Carbohydrates," are the preferred method for analyzing free amino acids in beverage samples. The sample preparation is relatively uncomplicated and consists only of sample filtration (0.4 µm disposable filter cartridges) and dilution (typically 500 or 1000x). Add approximately 20 mg/L sodium azide to the diluent to keep the dilute sample stable for a longer time at the room temperature. All chromatograms in this Section were generated using a 25 µL injection. The concentration of all standard components was 8.0 µM.

Injection Volume:	25 µL
Sample Concentration:	8 µM of all standard components
Column:	Dionex AminoPac PA10 analytical and guard columns
Column temperature:	30 °C
Expected System	
Operating Backpressure:	< 3,000 psi
Eluent:	
E1:	Deionized water
E2:	250 mM NaOH
E3:	1 M Sodium acetate
Eluent Flow Rate:	0.25 mL/min
ED Waveform:	See Table 1
Gradient Conditions:	See Table 3

1. Arginine
2. Hydroxylysine
3. Lysine
4. Galactosamine
5. Glucosamine
6. Glucose
7. Alanine
8. Threonine
9. Fructose
10. Glycine
11. Valine
12. Hydroxyproline
13. Serine
14. Proline
15. Isoleucine
16. Leucine
17. Methionine
18. Norleucine
19. Histidine
20. Phenylalanine
21. Glutamate
22. Aspartate
23. Cystine
24. Tyrosine

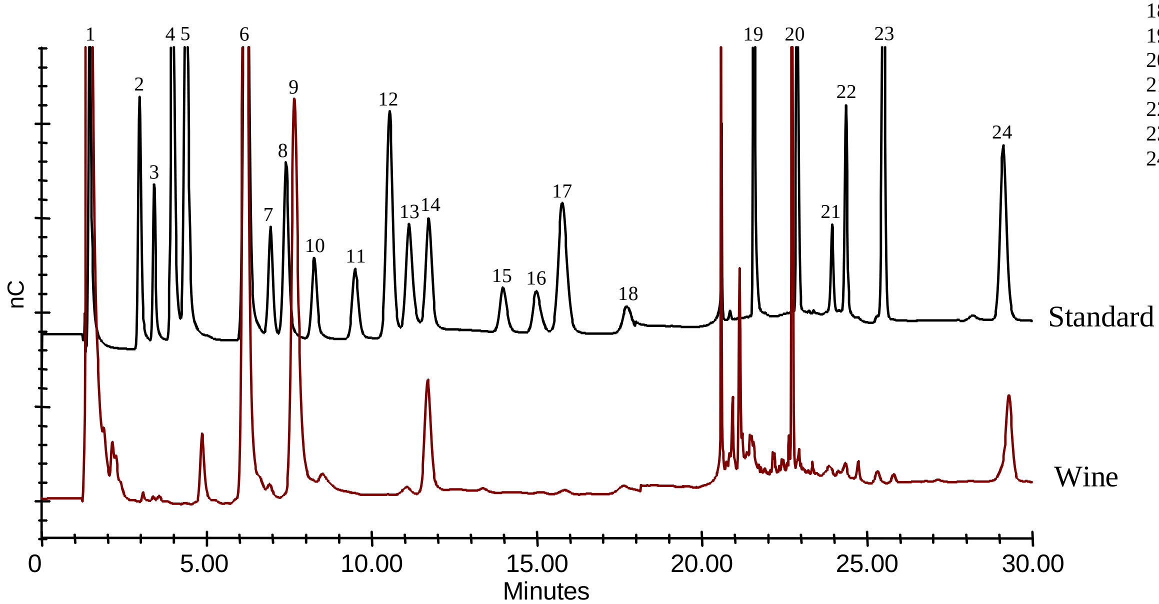


Figure 13
Amino Acids and Sugars in Red Wine

Samples containing excessive concentrations of carbohydrates (100:1 and higher) must be pretreated to make possible an interference-free analysis of all amino acids.

Thermo Scientific offers an accessory to the Dionex *AAA-Direct* system (***Carbohydrate Removal Accessory*** P/N 065244) that makes possible a fully automatic on-line removal of carbohydrates from amino acid containing samples. Off-line removal of carbohydrates has also been described in the literature (Reference 15, section 8.10)

1. Arginine
2. Hydroxylysine
3. Lysine
4. Galactosamine
5. Glucosamine
6. Glucose
7. Alanine
8. Threonine
9. Glycine
10. Valine
11. Hydroxyproline
12. Serine
13. Proline
14. Isoleucine
15. Leucine
16. Methionine
17. Norleucine
18. Histidine
19. Phenylalanine
20. Glutamate
21. Aspartate
22. Cystine
23. Tyrosine

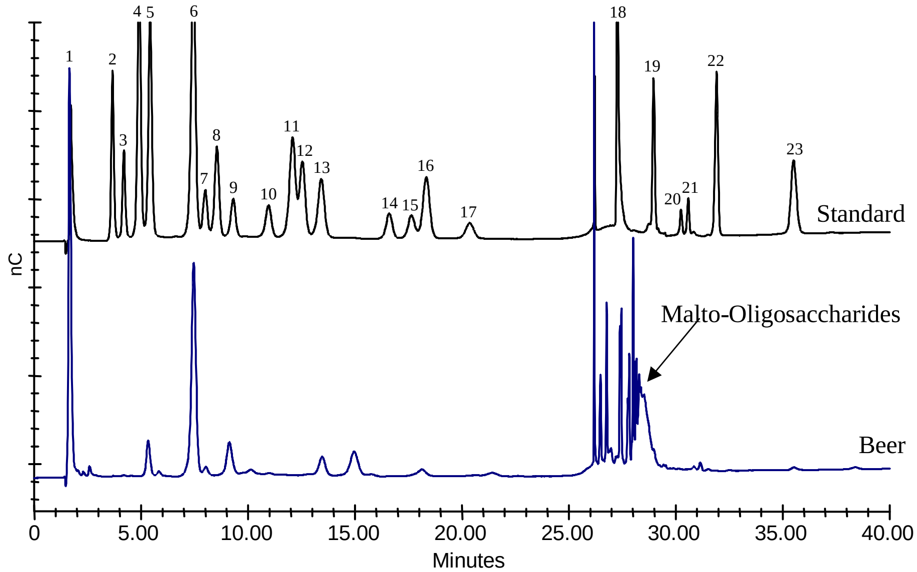


Figure 14
Amino Acids and Carbohydrates in Beer

1. Arginine
2. Hydroxylysine
3. Lysine
4. Galactosamine
5. Glucosamine
6. Glucose
7. Alanine
8. Threonine
9. Fructose
10. Glycine
11. Valine
12. Hydroxyproline
13. Serine
14. Sucrose
15. Proline
16. Isoleucine
17. Leucine
18. Methionine
19. Norleucine
20. Histidine
21. Phenylalanine
22. Glutamate
23. Aspartate
24. Cystine
25. Tyrosine

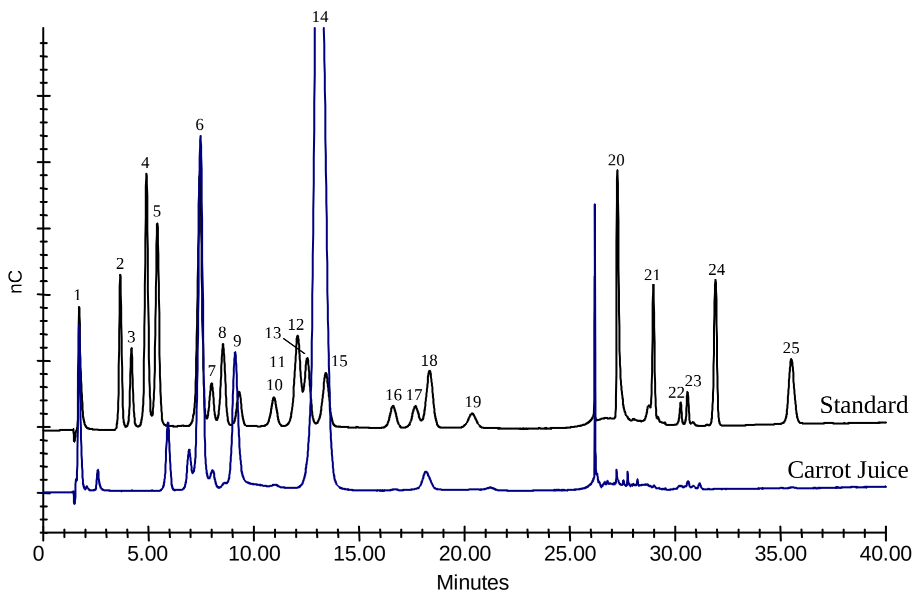


Figure 15
Amino Acids and Carbohydrates in Carrot Juice

8.9 Amino Acids and Sugars in Cell Culture Media

Figure 16A shows a separation of components of a cell culture media. The middle portion of the same chromatogram is presented in Figure 16B.

Sample :	25µL of cell culture after 1:100 dilution
Standard :	25µL 10 µM hydrolysate standard
Column:	Dionex AminoPac PA10 Guard and Analytical
Column Temperature:	30 °C
Eluent	
E1:	10 mM NaOH
E2:	250 mM NaOH
E3:	25 mM NaOH, 1 M sodium acetate
E4:	0.1 M acetic acid
Eluent Flow Rate:	0.25 mL/min
ED Waveform:	See Table 1
Gradient conditions:	See Table 8

1 Arginine
2 Lysine
3 Glucose
4 Asparagine
5 Glycine
6 Threonine
7 Alanine
8 Valine
9 Serine
10 Proline
11 Isoleucine
12 Leucine
13 Hepes
14 Methionine
15 Histidine
16 Phenylalanine
17 Glutamate
18 Aspartate
19 Cystine
20 Tyrosine
21 Tryptophan

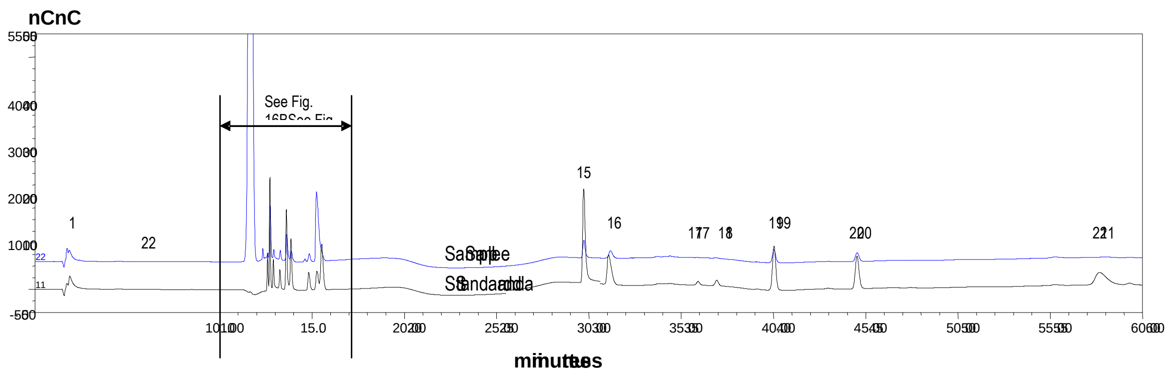


Figure 16A
Amino Acids and Sugars in Cell Culture Media

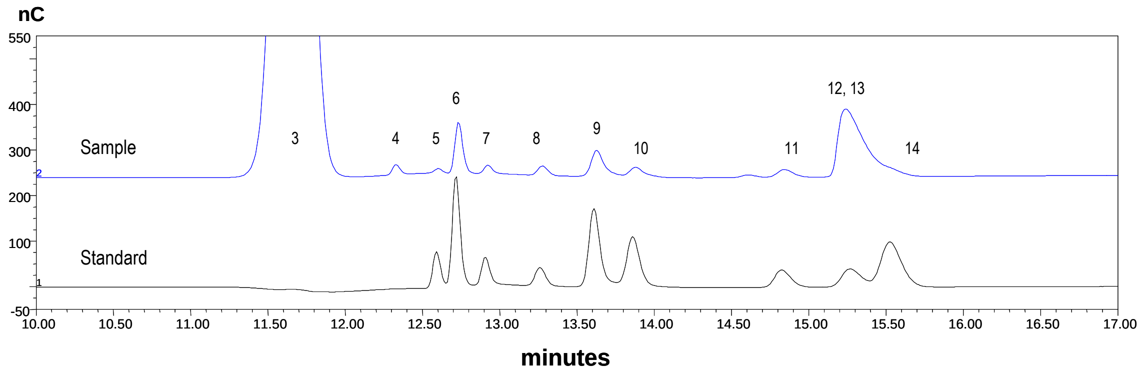


Figure 16B
Amino Acids and Sugars in Cell Culture Media (Expanded view)

8.10 Comparing Long and Short Gradient Methods for Separations of Amino Acids

Figure 17 shows a comparison of chromatograms obtained with Table 5 and Table 9 gradients. Table 5 gradient is preferable for samples of unknown amino acid composition. Once the composition is known and found suitable for a quicker method, Table 9 gradient can be used to increase the sample throughput.

Standard:	25µL 8 µM hydrolysate standard
Column:	Dionex AminoPac PA10
Column Temperature:	30 °C
Eluent	
E1:	water
E2:	250 mM NaOH
E3:	1 M sodium acetate
E4:	0.1 M acetic acid
Eluent Flow Rate:	0.25 mL/min
ED Waveform:	See Table 1
Gradient conditions: A:	Table 5; B: Table 9

1	Arginine
2	Lysine
3	Alanine
4	Threonine
5	Glycine
6	Valine
7	Serine
8	Proline
9	Isoleucine
10	Leucine
11	Methionine
12	Histidine
13	Phenylalanine
14	Glutamate
15	Aspartate
16	Cystine
17	Tyrosine
18	Tryptophan

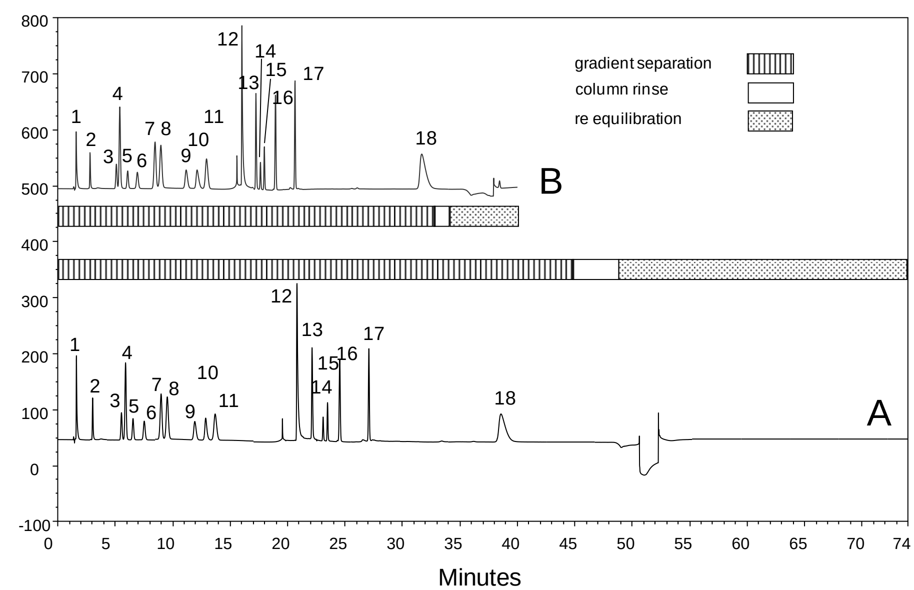


Figure 17
Two gradient methods for separating mixtures of amino acids including tryptophan

8.11 Utilization of the Quick Gradient Method in a Two-Dimensional Separation of Amino Acids and Carbohydrates

A full separation of oligosaccharides can be achieved simultaneously with a full separation of amino acids by a reduced length gradient if a Thermo Scientific Dionex CarboPac PA200 column is used as a carbohydrate column in a two-dimensional IC system. In this example, the oligosaccharides are from an inulin sample added at a relatively high concentration to a mixture of 17 hydrolyzate amino acids. Trace A in Figure 18 illustrates the interfrence of inulin oligosaccharides in a one-dimensional amino acid chromatogram on the Dionex AminoPac PA10 column. In a two-dimensional separation, carbohydrates are separated from amino acids on a precolumn installed before the Dionex AminoPac PA10 column. The oligosaccharides are sent to a Dionex CarboPac PA200 column where they are separated by an acetate gradient at a constant concentration of hydroxide (Trace B). The separation of amino acids occurs concurrently on the Dionex AminoPac PA10 column with the help of the Table 10 gradient (Trace C). For additional information on two-dimensional separations of carbohydrates and amino acids, see the Reference 25 in Section 8.12.

Sample :	25µL 8 µM hydrolysate standard plus 1.1 mg/L inulin
Columns:	A and C –Dionex AminoPac PA10; B – Dionex CarboPac PA200
Column Temperature:	30 °C
Eluent: A and C – Table 10 gradient	
E1:	water
E2:	250 mM NaOH
E3:	1 M sodium acetate
E4:	0.1 M acetic acid
Flow Rate:	0.25 mL/min
Eluent: B	
	100 mM NaOH, 120-320 mM sodium acetate
Flow Rate:	0.5 mL/min
ED Waveform: A and C:	See Table 1; B: Quadruple Pulse

1	Arginine
2	Lysine
3	Alanine
4	Threonine
5	Glycine
6	Valine
7	Serine
8	Proline
9	Isoleucine
10	Leucine
11	Methionine
12	System Peak
13	Histidine
14	Phenylalanine
15	Glutamate
16	Aspartate
17	Cystine
18	Tyrosine

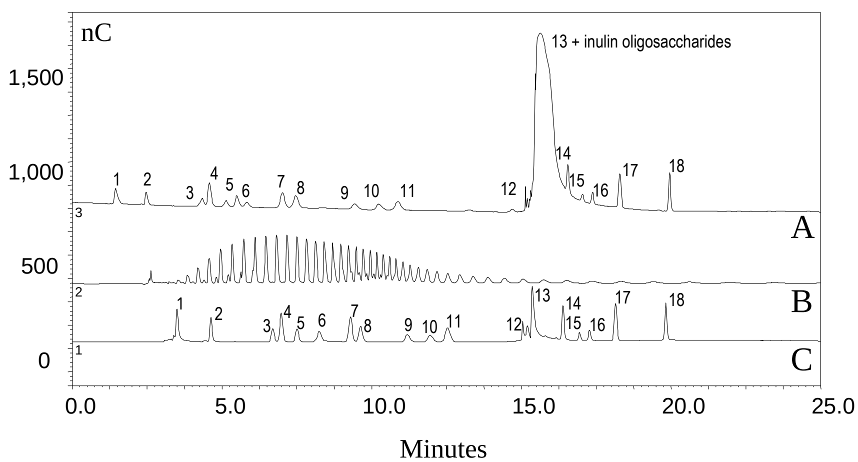


Figure 18
One- and two-dimensional separations of a mixture of oligosaccharides and amino acids

8.12 Dionex *AAA-Direct* Recommended Reading

1. Clarke, A. P., Jandik, P., Rocklin, R. D., Liu, Y. and N. Avdalovic. "An Integrated Amperometry Waveform for the Direct, Sensitive Detection of Amino Acids and Amino Sugars Following Anion-exchange Chromatography." Anal. Chem. 71, 1999, pp. 2774-2781.
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3. Dionex Corporation. "Determination of the Amino Acid Content of Peptides by *AAA-Direct*." Technical Note 50, 2000, pp. 1-20.
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5. Jandik, P., Cheng, J., Evrovski, J., and N. Avdalovic. "Simultaneous Analysis of Homocysteine and Methionine in Plasma." J. Chromatogr. B 759, 2001, pp. 145-151.
6. Jandik, P., Cheng, J., Jensen, D., Manz, S., and N. Avdalovic. "New Technique for Increasing Retention of Arginine on an Anion-exchange Column." Anal. Biochem. 287, 2000, pp. 38-44.
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8. Larson, T., Gawlitzek, M., Evans, H., Albers, U., and Cacia, J. "Chemometric Evaluation of On-Line HPLC in Mammalian Cell Cultures: Analysis of Amino Acids and Glucose." Biotechnology and Bioengineering. 77, 2002, pp. 553-563.
9. Dionex Corporation. "Technical Note 55: Screening of Sample Matrices and Individual Matrix Ingredients for Suitability in *AAA-Direct*."
10. Dionex Corporation. "Application Note 142: Determination of Tryptophan Using *AAA-Direct*."
11. Dionex Corporation. "Application Note 130: Identification of a Hydroxylysine-Containing Peptide from Its Lysine-Containing Form using *AAA-Direct*."
12. Heckenberg, A., Jandik, P., and Hanko, V., "Simple, Rapid Analysis of Carbohydrates or Amino Acids Using HPAE-PAD with Disposable Electrodes." Laboratory Equipment, September 2002, pp. 13-16.
13. Yu, H., Ding, Y.S., Mou, S., Jandik P., and Cheng, J. "Simultaneous Determination of Amino Acids and Carbohydrates by Anion Exchange Chromatography with Integrated Pulsed Amperometric Detection." J. Chromatogr. A 966, 2002, pp. 89-97.
14. Jandik, P., Cheng, J., and Avdalovic, N. "Amino Acid Analysis in Protein Hydrolysates using Anion Exchange Chromatography and IPAD Detection." Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd edition, Edited by B. J. Smith, Totowa, NJ, Humana Press, 2002.
15. Thiele, C., Gaenzle, M.G., and Vogel, R.F. " Sample Preparation for Amino Acid Determination by Integrated Pulsed Amperometric Detection in Foods." Anal. Biochem. 310, 2002, pp. 171-178.
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17. Ding, Y., Hong, Y., and Mou, S. "Direct Determination of Free Amino Acids and Sugars in Green Tea by Anion Exchange Chromatography with Integrated Pulsed Amperometric Detection." J. Chromatogr. A 982, 2002, pp. 237-244.

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25. Jandik, P., Cheng, J. “Two-Dimensional Ion Chromatography for Simultaneous Determination of Amino Acids and Carbohydrates.” Applications of Ion Chromatography for Pharmaceutical and Biological Products, Edited by L. Bhattacharyya and J.S. Rohrer. New York, NY, J. Wiley, 2012

9. Specialized Gradient Methods

The initial selection of gradient method depends on the type of system you are about to operate. We recommend composing the gradient pump methods differently for fully automated autosampler systems and for discontinuous, manual injector equipped analyzers (see Sections 7.3.1–7.3.2). Please note that the flow rate is 0.25 mL/min in all the steps of the gradient table.

9.1 Gradient Conditions for Amino Acids and Carbohydrates

It is possible, depending upon the analytes of interest, to separate and detect both amino acids and carbohydrates simultaneously, using a Dionex *AAA-Direct* system. This gradient method has been specifically developed for the separation of amino acids and carbohydrates simultaneously.

Table 3
Gradient Conditions for Amino Acids and Carbohydrates

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	84	16	0		Autosampler fills the sample loop
0.0	84	16	0		Valve from load to inject
2.0	84	16	0		Begin hydroxide gradient
12.1	68	32	0	8	
16.0	68	32	0		Begin acetate gradient
24.0	36	24	40	8	
40.0	36	24	40		
40.1	20	80	0	5	Column wash with hydroxide
42.1	20	80	0		
42.2	84	16	0	5	Equilibrate to starting conditions
65.0	84	16	0		

E1: water; E2: 250 mM NaOH; E3: 1.0 M NaOAc.

9.2 Gradient Conditions for Protein Hydrolysates

A good starting point for an unknown sample, or for any sample known to contain tryptophan and other strongly retained species, is the gradient method of Table 4. If a sample contains roughly equimolar levels of carbohydrates and amino acids then the gradient from Table 3 can be tried. The recommended approach for samples with excessive carbohydrate levels is described in Reference 7, Section 8.10.

Table 4
Gradient Conditions for Protein Hydrolysates

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	76	24	0		Autosampler fills the sample loop
0.0	76	24	0		Valve from load to inject
2.0	76	24	0		Begin hydroxide gradient, value back to Load
8.0	64	36	0	8	
11.0	64	36	0		Begin acetate gradient
18.0	40	20	40	8	
21.0	44	16	40	5	
23.0	14	16	70	8	
42.0	14	16	70		
42.1	20	80	0	5	Column wash with hydroxide
44.1	20	80	0		
44.2	76	24	0	5	Equilibrate to starting conditions
75.0	76	24	0		

E1: water; E2: 250 mM NaOH; E3: 1M NaOAc.

9.3 Gradient Conditions Including a Strong Post-Separation Rinse with 0.1M Acetic Acid for Removal of Residual Peaks in the Gradient Range between Histidine and Tyrosine.

The gradient of Table 5 makes use of a fourth, additional eluent (0.1 M acetic acid) in line E4 to eliminate the miniature carryover peaks that are sometimes observed for histidine, phenylalanine, glutamate, aspartate and tyrosine. This cleanup is performed after the last peak of interest has left the column (45.1 to 47.1 min) We refer to this part of the Table as “Post-Separation” Cleanup.

Table 5
Gradient Conditions with Strong Rinse for Residual Peaks in the Histidine/Tyrosine Region

Time (min)	%E1	%E2	%E3	%E4	Curve	Comments
Init	76	24	0	0		
0.0	76	24	0	0		
2.0	76	24	0	0		
8.0	64	36	0	0	8	
11.0	64	36	0	0		
18.0	40	20	40	0	8	
21.0	44	16	40	0	5	
23.0	14	16	70		8	
45.0	14	16	70	0		
45.1	0	0	0	100	8	This removes all strongly retained species from the column
47.1	0	0	0	100		
47.2	20	80	0	0	8	Removal of acetate from the column
49.2	20	80	0	0		
49.3	76	24	0	0	5	Equilibrate to starting conditions
74.0	76	24	0	0		

Please note that the flow rate is 0.25 mL/min in all the steps of the gradient table.
E1: water, E2: 250 mM NaOH, E3: 1M NaOAc, E4: 0.1 M HOAc (use a disposable glass pipette (10.0 mL sterile, serological pipettes, Thermo Fisher Scientific), transfer 5.72 mL acetic acid, glacial (J. T. Baker, P/N: 9522-02) to a 1 L glass eluent bottle with ~500 mL DI water, fill it up to the 1 L mark with DI water, thoroughly mix the eluent solution. Attention: it is recommended to use a glass eluent bottle for acidic eluent.)

9.4 Gradient Conditions for an Improved Peak Shape of Histidine

(Also included is the Strong Post-Separation Rinse with 0.1 M Acetic Acid)

The gradient method of Table 6 also makes use of a fourth, additional eluent (0.1 M acetic acid) in line E4 to rinse out the trace residues of strongly retained peaks after the actual separation. Additionally, the sodium hydroxide compositions are modified in Table 6 in order to minimize the tailing of histidine that is sometimes observed.

Table 6
Gradient Conditions with Strong Rinse for Residual Peaks in the Histidine/Tyrosine Region

Time (min)	%E1	%E2	%E3	%E4	Curve	Comments
Init	76	24	0	0		
0.0	76	24	0	0		
2.0	76	24	0	0		
8.0	64	36	0	0	8	
11.0	64	36	0	0		
18.0	0	90	10	0	8	Alkaline pH in this segment improves shape of His peak
21.0	0	90	10	0		
24.0	44	16	40	0	5	
26.0	14	16	70	0	5	
45.0	14	16	70	0		
45.1	0	0	0	100	8	This removes all strongly retained species from the column
47.1	0	0	0	100		
47.2	20	80	0	0	8	Removal of acetate from the column
49.2	20	80	0	0		
49.3	76	24	0	0	5	Equilibrate to starting conditions
74.0	76	24	0	0		

Please note that the flow rate is 0.25 mL/min in all the steps of the gradient table.
E1: water; E2: 250 mM NaOH; E3: 1M NaOAc; E4: 0.1 M HOAc.

An important alternative exists to the Post-Separation Cleanup. It is possible to start adding a small percentage of acetic acid in the last stages of a separation before the last peak of interest has left the column. Any possible carryover is thus eliminated during the actual separation. This approach is known as “On-the-Fly Cleanup.”

We believe that both clean up procedures are essentially equivalent in preventing distortions of quantitative results for the highly retained peaks in the region between histidine and tyrosine. The methods are easily interchangeable. However, the shape of the tryptophan peak is slightly affected when using the On-the-Fly cleanup. Regardless, a reliable quantification of tryptophan is still possible.

Note: to convert from “Post Separation Cleanup” to “On-the-fly Cleanup,” change all mobile phase compositions between 24.0 and 47.1 minutes to 30% A: 0% B: 62.5% C: 7.5% D.

9.5 Gradient Method for Improved Long-Term System Stability

The gradient method of Table 7 utilizes eluents E1 and E3 containing a low concentration of sodium hydroxide. The overall effect of this change is an improved long term stability of the system. The sterilization with 2 M sodium hydroxide (Section 10.5) has to be carried out less frequently, or not at all.

For the gradient method in Table 7, the eluents are somewhat modified from other gradient methods.

Eluent 1: 10 mM NaOH
Eluent 2: 250 mM NaOH
Eluent 3: 25 mM NaOH/1.0 M NaOAc

Table 7
Gradient Conditions for Improved Long-Term System Stability

Time (min)	%E1	%E2	%E3	Curve	Comments
Init	79.17	20.83	0.0		Autosampler fills the sample loop
0.0	79.17	20.83	0.0		Valve from load to inject
2.0	79.17	20.83	0.0		Begin hydroxide gradient, valve back to Load
8.0	66.67	33.33	0.0	8	
11.0	66.67	33.33	0.0		Begin acetate gradient
18.0	45.83	14.17	40.0	8	
21.0	50.0	10.0	40.0		
23.0	21.87	8.13	70.0		
42.0	21.87	8.13	70.0		
42.1	20.0	80.0	0.0	5	Column wash with hydroxide
44.1	20.0	80.0	0.0		
44.2	79.17	20.83	0.0	5	Equilibrate to starting conditions
75.0	79.17	20.83	0.0		

Please note that the flow rate is 0.25 mL/min in all the steps of the gradient table.
E1: 10 mM NaOH, E2: 250 mM NaOH, E3: 1 M NaOAc, 25 mM NaOH.

9.6 Gradient Method for Complex Mixtures of Amino Acids and Carbohydrates

The gradient table below defines an elution program in which the initial change of hydroxide concentration occurs in a single step between 8 and 8.1 minutes. This simple approach can be easily optimized by changing the size of the step in increments. The influence of such incremental changes on resolution of selected critical pairs of amino acids is illustrated in a footnote (**) below the gradient table. In most reports dealing with separations of sugar/amino acid mixtures (See Section 8.10: References 21-24), the initial change of hydroxide concentration is carried out gradually over a period of at least several minutes. The gradient method shown here is thus a useful new addition to existing methodology for the separation of complex samples.

Table 8
Gradient Conditions for Complex Mixtures of Amino Acids and Carbohydrates

Time (min)	%E1	%E2	%E3	%E4	Curve	Comments
Init	97.82	2.08	0	0		15 mM NaOH initial concentration*
8.0	97.82	2.08	0	0		
8.1	0	100	0	0	5	Size of this step change can be ** optimized between 35%E1/65%E2 and 0%E1/100%E2
16.0	0	100	0	0		
17.0	66.7	33.3	0	0	5	This step may be optimized by varying gradient curves numbers between 5 and 9
24.0	1.0	89.0	10.0	0	8	
27.0	1.0	89.0	10.0	0		
30.0	0	80.0	20.0	0	8	
32.0	0	80.0	20.0	0		
34.0	40.0	30.0	30.0	0	8	
36.0	40.0	30.0	30.0	0		
38.0	30.0	30.0	40.0	0	8	
40.0	30.0	30.0	40.0	0		
42.0	20.0	30.0	50.0	0	8	
44.0	20.0	30.0	50.0	0		
46.0	10.0	30.0	60.0	0	8	
48.0	10.0	30.0	60.0	0		
50.0	0	30.0	70.0	0	8	
62.0	0	30.0	70.0	0		
62.1	0	0	0	100	5	Acetic acid rinse
64.1	0	0	0	100		
64.2	20.0	80.0	0	0	5	Removal of acetate from column
66.2	20.0	80.0	0	0		
66.3	97.82	2.08	0	0	5	Equilibrate to initial conditions
92.0	97.82	2.08	0	0		

* Initial concentration may be optimized between 10 and 60 mM NaOH
** Resolution (R) of critical peak pairs of amino acids depends on the size of the step between 8.0 and 8.1 minutes in the above gradient table.
E1: 10 mM NaOH, E2: 250 mM NaOH, E3: 1 M NaOAc, 25 mM NaOH; E4: 0.1 M HOAc. Flow rate: 0.25 mL/min.

%B at 8.1 min	R Ala/Thr	R Thr/Gly	R Ser/Pro	R Ile/Leu	R Leu/Met
100	1.9	1.4	1.7	2.2	1.0
95	1.3	1.9	1.7	2.0	1.1
90	1.4	2.0	1.7	2.1	1.1
85	1.3	2.0	1.6	2.0	1.2
75	1.5	2.0	1.6	2.0	1.2
65	1.4	2.1	1.5	1.9	1.2
55	1.5	2.1	1.3	1.9	1.2
45	1.5	2.2	1.3	2.0	1.3
35	1.6	2.3	1.1	2.0	1.3

9.7 Fast Separation of 17 Amino Acids plus Tryptophan

It is possible to separate 17 hydrolysate amino acids in ca. 20 minute. The peak of tryptophan elutes in 30 minutes. The total time required for a chromatogram, including re-equilibration, is 40 minutes. Apply this approach for samples without nonproteinogenic acids such as taurin or norleucin etc. For samples where additional amino acids are present together with the 17 hydrolysate amino acids, the gradients of Tables 4 and 5 should be more suitable.

Table 9
Gradient for 40-Minutes Chromatograms

Time (min)	%E1	%E2	%E3	%E4	Curve	Comments
0.0	76	24				
2.0	76	24				
6.0	64	36			8	
8.0	64	36				
12.0	40	20	40		8	
14.0	44	16	40		5	
16.0	14	16	70			
17.9	14	16	70			
18.0		16	84		8	
32.0		16	84			
32.1				100	5	Acetic acid rinse
33.1				100		
33.2	20	80			5	
34.2	20	80				
34.3	76	24			5	Begin re equilibration to initial conditions
40.0	76	24				End re equilibration

E1: water, E2: 250 mM NaOH, E3: 1M NaOAc, E4: 0.1 M HOAc; Flow rate: 0.25 mL/min

9.8 Fast Separation of 17 Amino Acids without Tryptophan

The gradient elution method of Table 10 was developed for simultaneous 2D separations of carbohydrates and amino acids. These separations are described in detail in Reference 25 (Section 8.12). Strongly retained species such as tryptophan, if present, are removed from the column by the acetic acid rinse. Up to 0.35M acetic acid can be used if necessary.

Table 10
Gradient for 25-Minute Chromatograms

Time (min)	%E1	%E2	%E3	%E4	Curve	Comments
0.0	76	24				
2.0	76	24				
4.0	64	36			8	
5.0	64	36				
9.0	40	20	40		8	
12.0	44	16	40		5	
13.0	14	16	70		8	
17.0	14	16	70			
17.1				100	5	Acetic acid rinse
18.1				100		
18.2	20	80			8	
19.2	20	80				
19.3	76	24			5	Begin re-equilibration to initial conditions
25.0	76	24				End re-equilibration

E1: water, E2: 250 mM NaOH, E3: 1M NaAc, E4: 0.1 M HAc; Flow rate: 0.25 mL/min

10. Troubleshooting

Keep in mind that some of the problems may be related to the parts of your experimental protocol (sample contamination, imprecision during sample transfer, problems during peptide or protein hydrolysis etc.).

Make sure to follow all the rules from Section 4.1 and to recheck all of the items from Section 4.2.

The following text should help you to locate and eliminate problems traceable to the Dionex *AAA-Direct* hardware and chemistries. It also provides a selection of cleanup and reconditioning procedures that have been found effective by many users.

10.1 High Background

While it may be possible to obtain reasonable performance even with elevated background levels, high background frequently brings about an increased size of gradient artifacts and can be accompanied by a presence of ghost peaks. Detection sensitivity may also change suddenly when the background is too high.

A background > 80 nC with 60 mM sodium hydroxide at 0.25 mL/min using the waveform of Table 1 at 30 °C indicates one of the following possibilities:

A. Incorrect detection parameters.
B. Verify that "pH" is specified as a reference mode. Check all values of waveform in the instrument method against those in Table 1. If the pH reading at 76/24 (%E1/%E2 i.e., 60 mM NaOH) is above 13.2, replace the reference electrode.
C. Compromised working electrode surface.
D. Briefly install a new working electrode and check the background (as above). If the reading remains > 80 nC, remove the new electrode within 30 minutes and continue testing for column or system contamination. Otherwise continue your work with the new electrode installed.
E. Column contamination.
F. Remove the column set from the system first and replace it by the yellow tubing from installation kit or by any length of yellow PEEK tubing generating a pressure drop between 1000 and 2000 psi. If the background reading improves after the column is removed from the system, go to Section 10.3.
G. System contamination.
H. If the background remains high even without the column, carry out the 2 M sodium hydroxide rinse described in Section 10.5.

10.2 Decreased Detection Sensitivity

Always confirm the loss of response by performing at least one injection of 8 µM histidine as described in Section 6.4.3. (Make sure a decreased level of response is not being caused by system problems discussed in Section 10.4.2)

Any decrease in detection sensitivity means that the working electrode surface has been affected. The operator has to install a new working electrode. One spare gold working electrode should always be available in order to avoid unnecessary delays.

IMPORTANT

Never install a new electrode without an aggressive system cleanup (Section 10.6). The two exceptions to this rule are described below.

Exception One:

Check the pH readout. If the value is out of range or > 13.2, install a new reference electrode and then install a new gold working electrode (P/N 063722). The system cleanup is not necessary. The decrease in sensitivity was caused by a gold-oxide-buildup on the electrode surface. This was because the reference potential was too high.

A gold-oxide-covered non-disposable gold working electrode can be reconditioned by the repair polishing described in Section 10.8.1.

Exception Two:

Check the background reading while pumping 76% E1 and 24% E2 (60 mM NaOH) using the waveform of Table 1. If the background level is < 80 nC and if the sodium acetate in the mobile phase E3 is not from Thermo Scientific Dionex (P/N 059326), carry out the procedure in Section 10.7. The old working electrode can be reconditioned by the chemical treatment described in Section 10.8.3

After installing a new working electrode (with or without the complete system cleanup), confirm the normal detection sensitivity. Carry out the histidine injection test, Section 6.4.3.

Immediately remove the new working electrode from the system should the response be too low (peak height < 200 nC for 25 µL of 8 µM histidine at 36/24/40 of E1/E2/E3) and repeat the procedure in Section 10.6.

10.3 Column Problems

The Guard column protects the main column not only from contamination but also from excessive pressure fluctuations caused by the instrument or by operator errors. Have the Guard column installed at all times, disconnect it only during some of the testing described in this section.

10.3.1 Column Set Causing High Background

The column set is causing the high background if the background reading decreases after the column is replaced by a section of PEEK tubing as described in Section 10.1 F.

Disconnect the cell from the system, remove the yellow tubing and reinstall the column set. Increase the column thermostat temperature to 40 °C. Run 2 M sodium hydroxide through the column (at 0. 25 mL/min) for one hour. Reset the temperature to 30 °C, pump 60 mM sodium hydroxide through the column, connect the cell and apply waveform of Table 1. If the background remains high, remove the cell from the system again and rinse the column with 63 mM NaOH, 750 mM sodium acetate (25% E2, 75% E3) for at least four hours (preferably overnight).

10.3.2 Gradient Rise Exceeding 50 nC

The magnitude of the gradient rise can be minimized by continuously running blank gradients during the times when the system is not in use for sample or standard analysis. This will keep the column conditioned, free of carbonate buildup, and ready for analyses.

A. Make sure the gradient rise is not caused by the system and/or detector cell. Perform a gradient with the column replaced by a section of yellow PEEK tubing.
B. Increase column temperature to 40 °C and wash the guard and column with 63 mM NaOH, 750 mM sodium acetate (25% E2, 75% E3) for at least four hours (preferably overnight). Run a blank gradient at 30 °C and if necessary repeat the 25% E2, 75% E3 wash at 40 °C.

10.3.3 Peak Efficiency and Resolution Are Decreasing

Always have a spare Guard column available.

Peak deformations may sometime be caused by sample matrix. Example: undiluted MSA hydrolysates. For example, the methanesulfonate (undetected by amperometry) may overload the anion exchange column causing poor peak shapes.

A. Run a standard separation with Guard column removed from the system. Install a new Guard column should the separation improve with the old Guard removed. It is quite common to replace the Guard column several times during the lifetime of a main column.
B. Verify that only the 0.005" i.d. (Red) tubing is installed for all connections between injector and detector.

Verify that a shortest possible length of 0.010" i.d. tubing (Black) is installed between the pump and injector.

A. Check for proper installation of ferrules on all PEEK tubing starting with the injector outlet and all other connectors to the ED cell inlet.
B. Check temperature settings in your method and/or actual temperature in your column oven. Refer to Figure 10 for temperature effects.
C. The column may be overloaded. Try to inject a smaller amount of your sample or dilute the sample more.
D. Clean column with acetonitrile/ HCl:
    1. Remove main analytical and guard columns and clean each separately off-line (using a separate primed pump) at 0.25 mL/min as follows:

a) 10 min, water.

b) 60-90 min 80% acetonitrile with 200 mM HCl (160 mL HPLC grade acetonitrile + 36.7 mL water + 3.3 mL conc. HPLC grade (36%) HCl).

c) 30 min, water.

2. First reinstall the main column, and test for improved separations. If an improved separation is obtained, add the guard column and again test. If good separation is attained with the main column, but not the guard, then replace the guard.

E. If all of the above does not lead to an improved separation, the resin bed of the main column has been damaged and the main column must be replaced.

10.3.4 Decreasing Resolution of Serine and Proline

In some laboratories, the resolution of peaks of serine and proline in the chromatograms obtained with a Dionex AminoPac PA10 column can deteriorate gradually after hundreds of injections. This gradual loss of resolution occurs with all gradient programs separating the full range of amino acids. The resolution loss accompanied with a slight shortening of retention times is caused by an occasional presence of unknown trace contaminants in the sodium hydroxide and sodium acetate eluents. The loss of serine-proline resolution, once it occurs (see Figure 19), can be restored by column rinsing with 200 mM HCl in 80% aqueous acetonitrile. The details of the rinsing procedure are given in Section 10.3.3.

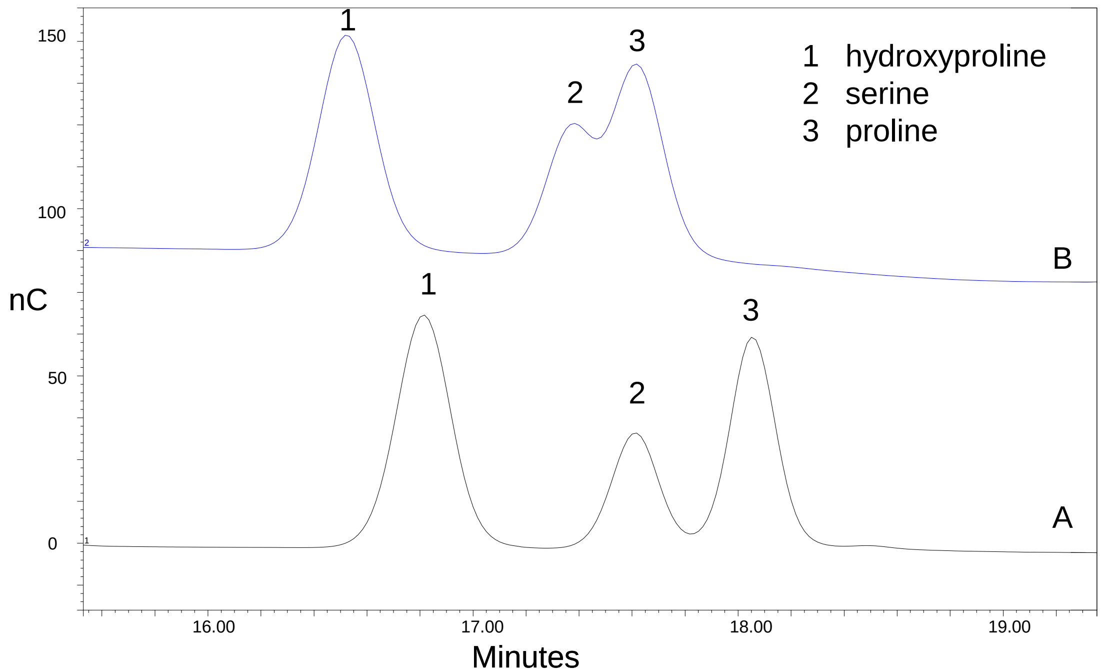


Figure 19.
Partial loss of serine-proline resolution due to trace impurities in eluents. Trace A: Initial separation; Trace B: after 323 injections (388 hours of operation).

The loss of serine-proline resolution is completely prevented by installation of one Thermo Scientific Dionex IonPac ATC-HC column (PN 059604) on the sodium hydroxide line and one

Dionex IonPac ATC-HC column on the sodium acetate line (on PTFE tubing between the eluent bottle and the pump).

All new Dionex IonPac ATC-HC columns have to be flushed with the respective eluents prior to use. Disconnect the tubing between the pump and injector valve. Connect the first of the two Dionex IonPac ATC-HC columns to the pump. Pump 50 mL of 250 mM NaOH (100% eluent B) through the first Dionex IonPac ATC-HC column into waste. Disconnect the first Dionex IonPac ATC-HC from the pump and install the second Dionex IonPac ATC-HC. Pump 50 mL of 1 M sodium acetate (100% eluent C) through it into waste. Disconnect the second Dionex IonPac ATC-HC and reconnect the tubing between the pump and injector valve. Install the first Dionex IonPac ATC-HC at the low pressure side on line B. Install the second Dionex IonPac ATC-HC at the low pressure side on line C. The Dionex IonPac ATC-HC are installed near the eluent container by replacing the 1/4/28 x 1/4/28 PEEK union located there. Use ¼-28 to 10-32 PEEK unions (PN 042627) with green tubing (PN 053305) between the union and column. Open the purge valve and prime the eluent line after the Dionex IonPac ATC- HC installation before starting gradient runs.

With the Dionex IonPac ATC-HC columns installed, the serine-proline resolution remains unchanged for thousands of injections (months of operation). It is recommended to discard the Dionex IonPac ATC-HC columns after a long term use. A new set of Dionex IonPac ATC-HC columns should be used with installation of new Dionex AminoPac PA10 columns. The Dionex IonPac ATC-HC columns will not affect an improvement once a loss of serine-proline resolution occurs. In such case, it is necessary to apply the HCl/acetonitrile rinsing procedure (See Steps G and H. in Section 10.3.3.above).

10.4 System Problems

10.4.1 High Detection Background Caused by the System

A. Verify the problem is neither detector (see Section 10.1 A, B) nor column (see Section 10.1 F) related.

B. With injector, column and detector cell installed (cell voltage off) carry out the 2 M NaOH wash as described in Section 10.5

C. Prepare new eluents.

D. Rinse all three eluent lines with the new eluents (at least 40 mL by priming speed).

10.4.2 No Peaks, Poor Peak Area Reproducibility or too Small Peak Areas

A. Check the position and filling levels of sample vials in the autosampler.

B. Check injector needle-height setting

C. Check each line of the schedule for proper injector parameters. Revert to full loop and 25 µL sample loop size if using other injection modes (push or pull).

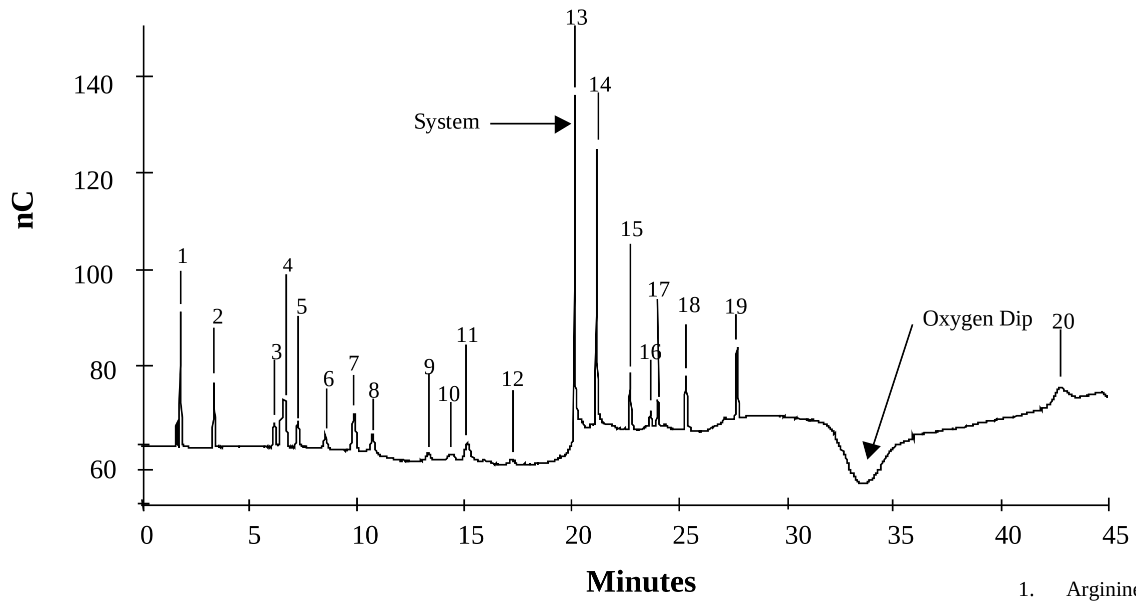
D. Service the injection valve (check for leaks, Tefzel fragments, or sediments inside the valve)

10.4.3 Large Baseline Dip in the Gradient Region of the Chromatogram

A. A large baseline dip appearing between phenylalanine and tryptophan is usually caused by co-injection of air bubbles, either by incorrect injection modes (partial loop filling) or by empty sample vials. Check your autosampler injection needle-height setting, if the problem occurs even with partially filled sample vials. Figure 20, "Effect of Coinjection of Air Bubbles," illustrates the oxygen dip resulting from using the AS "Limited Sample Mode," to inject 10 µL of sample encased with air bubbles. By using the AS "Partial Loop Mode," a 14 µL sample segment is created out of which a 10 µL segment is injected. This injection mode minimizes the oxygen dip.

B. Baseline dip appearing concurrently with the acetate gradient may be caused by the higher oxygen (and/or carbonate) content of the acetate solution relative to that of the other two eluents. Note: acetate eluent is moving much more slowly through the pump than either the water or sodium hydroxide eluents. Increase the duration of the pump degas time and/or cut the interval between degas times. Check the gas supply to the acetate bottle and tighten the bottle cap.

Dionex AS Autosampler
Partial (Limited Sample Mode)



1. Arginine
2. Lysine
3. Alanine
4. Threonine
5. Glycine
6. Valine
7. Serine
8. Proline
9. Isoleucine
10. Leucine
11. Methionine
12. Norleucine
13. System
14. Histidine
15. Phenylalanine
16. Glutamate
17. Aspartate
18. Cystine
19. Tyrosine
20. Tryptophan

Dionex AS Autosampler
Partial Loop Injection Mode
Injection volume + "cut volume"
(2µL + 10µL + 2µL)

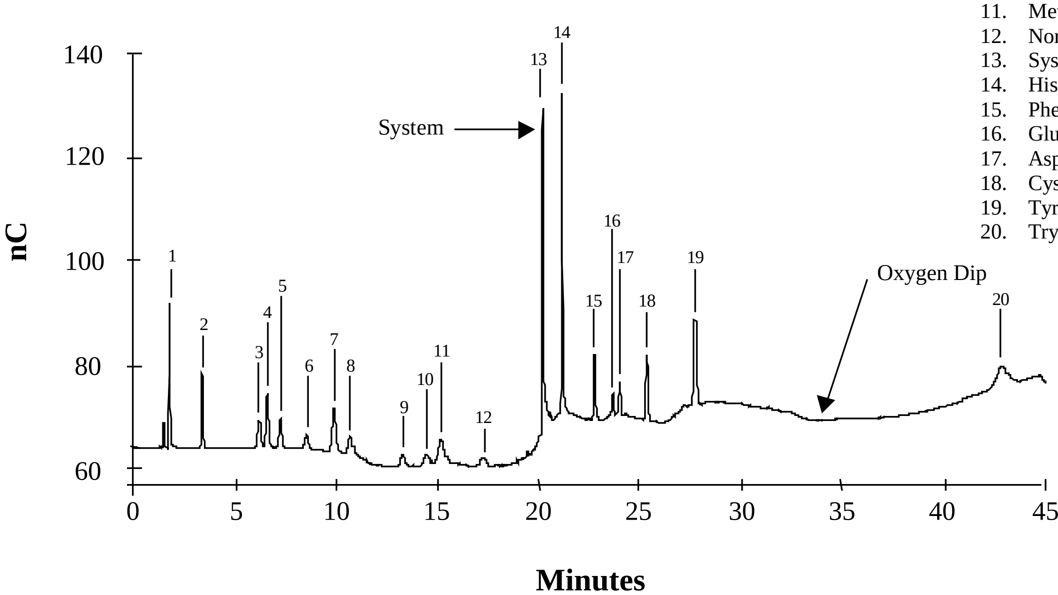


Figure 20
Effect of Co-injection of Air Bubbles

10.4.4 Incorrect or Variable Retention Times

A. Check your eluent preparation procedure for possible errors.

B. Prime the pump if necessary.

C. Measure the flow rate by weighing out the eluent collected during exactly five minutes. Recalibrate the pump if necessary.

D. The sodium hydroxide eluent contains too much carbonate and/or the re-equilibration period at the end of the gradient method is too short.

E. Set the eluent composition for 100% for each eluent and draw out at least 40 mL of eluent from each eluent line.

F. Samples containing high salt content (> 50 mM) will decrease the retention times.

G. Check and/or service the pump's proportioning valve. With the pumping turned off, the flow through the pump outlet tubing (disconnected from the injector) should be zero in all three eluent positions. Check this separately for each eluent line at the 100% setting.

10.4.5 Unidentified Peaks Appear Alongside the Expected Analyte Peaks

During the acetate gradient a number of small peaks may appear (See Figure 2). These peaks are usually due to trace contaminants in the water supply. The contaminants accumulate on the column during the isocratic section of the chromatogram and are released, frequently as irregular baseline deformations or sharp spikes, with the increasing eluent strength.

Some trace contaminants can co-elute with glutamate and aspartate compromising accuracy of quantitation of these amino acids at lower concentrations. If extraneous peaks are observed even after the water supply is excluded as a possible cause, clean the autosampler lines and sample loop. The autosampler should be cleaned using the following protocol:

A. Disconnect column and detector cell from the autosampler.

B. Set the pump to 100% deionized water.

C. Place the following solutions in the autosampler in autosampler vials and inject in sequence. Use 25 µL full loop injections:
   1. 1 M NaOH
   2. Deionized water
   3. 2-propanol
   4. Deionized water
   5. 1 M HCl
   6. Deionized water

10.5 Sodium Hydroxide Cleanup

The sodium hydroxide (2 M) rinse used to decrease column or system-related elevated background is essentially identical with the rinse performed during an installation of a new system, Section 6.1.2. Following the rinse, check the background again while pumping the 60 mM sodium hydroxide and repeat the rinse at least once if necessary. Leave the old gold working electrode in place during the first and the second checking of the detection background. Use a new or reconditioned electrode only if the background remains high even after the second rinse. Should the new electrode also produce a reading of > 80 nC, remove it from the system within 30 minutes, rinse it with water and reinstall the old electrode. In case the repeated rinse does not lower the background, perform the EDTA cleanup described in Section 10.6. Then try the background with the old electrode first and if necessary only briefly with the new electrode again. In case the new electrode delivers < 80 nC, leave it in the system and recondition the old electrode using chemical cleanup described in Section 10.8.3.

NOTE

Make sure that all internal surfaces (for example: entire length of Teflon tubing inside the eluent bottles) come in contact with 2 M NaOH. Turn the inject valve several times (inject-load) while pumping the 2 M NaOH through it. Make several injections of 2 M NaOH from sample vials.

10.6 EDTA Clean-up Procedure for Dionex AAA Direct System

1. Install new Pump Seals (P/N 064946)
2. Install micro bore tubing (red, 0.005”) between injector and column, and between column and cell.
3. Remove GM-3 gradient mixer (if applicable) and discard.
4. Wash System with 6.5 mM (2.4 g/L) Na_2EDTA (must be disodium EDTA) MW 372 g/mol
   a) Remove the column.
   b) Remove gold electrode from the cell, close the cell again using an empty holder block over a gasket.
   c) Restore liquid connection between injector valve and detection cell (column has been removed)
   d) Empty the contents of eluent container E1, rinse it with 1 L of 18 megohm water and discard the water.
   e) Filter 1 L of 18 megohm water through 0.2 um Nylon filter. Note: Do not use any other material than Nylon for eluent filtration.
   f) Transfer filtered water into eluent container E1. Pump at least 30 mL of water into pump waste at priming flow rate.
   g) Stop the pump. Close the priming valve. Pump at least 50 mL of water from E1 through the system into detector waste at a low rate 2.0-3.0 mL/min.
   h) Toward the end of the water rinse, turn the injection valve at least 3 times.
   i) Prepare 1L of 6.5 mM Na_2EDTA (must be the disodium form) and filter it through a 0.2 um Nylon filter. Discard water from E1 and replace it by the filtered aliquot of 6.5 mM EDTA.
   j) Pump at least 30 mL of EDTA into pump waste at priming flow rate.
   k) Stop the pump. Close the priming valve. Pump (3 mL/min) at least 300 mL of EDTA from E1 through the system into detector waste.
   l) Toward the end of the EDTA rinse, turn the injection valve at least 3 times.
   m) Carry out steps d to h again. Replace EDTA solution by ultrapure water in E1. Prime the PTFE tubing with the water.
   n) Rinse the system (column remains out of the system) with water from E1.
   o) Replace the rinse water in E1 by another aliquot of freshly filtered ultrapure water. Pump at least 30 mL of the water into pump waste.
   p) Install a new GM3 mixer. Re-install the column set and rinse with initial gradient composition (ca. 10 mL into detector waste)
   q) Reassemble cell with a new disposable electrode and a new 2 mil PTFE gasket (P/N 060141).

10.7 Acetate Line Cleanup

Should the system be contaminated with poor quality sodium acetate, the acetate reservoir is rinsed and filled with a new acetate solution. The old gold electrode remains in place until the entire system including the Dionex AminoPac PA10 column set is rinsed with the new acetate mobile phase. The gold electrode is then replaced and the detector response is tested by injecting a histidine solution (Section 6.4.3).

A. Turn off the detector cell, stop the pump.

B. Disconnect the Dionex IonPac ATC-HC column if it is installed in line C of the Dionex *AAA-Direct* system and replace it by a union (Note: Thermo Scientific no longer recommends the use of Dionex IonPac ATC-HC columns on all eluent lines).

C. Discard the contaminated sodium acetate and rinse the reservoir with deionized water (filtered through a 0.2 µm Nylon filter) at least three times.

D. Prepare 1 M sodium acetate solution. Dissolve 82.0 ± 0.5 grams of anhydrous sodium acetate from Thermo Scientific in a 500 mL of bottle (P/N 59326) with ca. 450 mL of deionized water, transfer the content to a larger, clean container (Nalgene bottle recommended), rinse the 500 mL of bottle with ca. 100 mL deionized water twice and transfer to the container, finally dilute it to 1 L with deionized water. Filter through a 0.2 µm Nylon filter.

E. Transfer the freshly prepared pure sodium acetate solution into the clean reservoir at line C.

F. Disconnect the pump from the Direct Control of Chromeleon. Open the priming valve to bypass the injector and column.

G. Set the pump to 100% C, start the pump and activate the “priming” button on the pump.

H. Attach a 20 mL syringe to the priming valve located below the pump heads. Open the valve and draw at least 40 mL from line C using the syringe.

I. Close the priming valve.

J. Pump 40% B (250 mM NaOH)/60% C (1.0 M NaOAc) with a flow rate of 0.25 mL/min. at 40 °C for 2–3 hours without turning the cell on to rinse out the residual contaminated sodium acetate from the Dionex *AAA-Direct* system.

K. Slide off the cover of electrochemical cell and disconnect the cable. Unscrew the working electrode from the cell body and remove the gasket carefully. Clean up the fluidic channel with wet tissue and wipe it dry with dry tissue.

L. Rinse the gasket and put it back in place. Install a new working electrode by sliding it onto the two poles protruding from the cell body and by fastening the two wing screws. Connect the pump and cell in the monitor screen panel.

M. Run a sequence of several 25 µL injections of a 8 µM histidine quality solution, using the isocratic eluent composition of 36% water (A), 24% 0.25 M NaOH (B) and 40% 1 M sodium acetate (C). The flow rate should be set at 0.25 mL/min. The standard waveform from Table 1 should be used.

N. A successful outcome is indicated by a peak height of histidine > 200 nC.

10.8 Reconditioning of Gold Electrodes

IMPORTANT ***The following procedures apply only to P/N 063772 Dionex*** AAA-Certified ***Conventional working electrodes.***

10.8.1 Mechanical Polishing

Mechanical polishing of Dionex AAA-Certified Conventional Gold Electrodes has to be more thorough than that of gold electrodes for carbohydrates. The Dionex AAA-Certified electrodes have to be polished harder and longer to achieve good results. Also the time interval required for re-equilibration of polished Dionex AAA-Certified electrodes is considerably longer in comparison with carbohydrate electrodes. It may take up to 12 hours for a freshly polished electrode to return to background values under 80 nC (at 76/26 E1/E2, Table 1 waveform and 30 °C). However, once the background reading is back at 80 nC, the electrode performance is completely and reliably restored.

A. Polish with coarse polishing compound (P/N 36319) as described in the ED manual. Polish for at least 10 minutes with as much strength as you can sustain for 10 minutes.
B. Apply several mL of water to a fresh polishing pad and polish for one minute. This step removes the coarse polishing powder particles imbedded in the gold material.
C. Polish with fine polishing compound (P/N 36318) as described in the ED manual. Polish for at least 20 minutes with as much strength as you can sustain during the entire interval of time.
D. Apply several mL of water to a fresh polishing pad and polish for one minute. This step removes the fine polishing powder particles imbedded in the gold material.
E. Reassemble the ED cell and apply the Table 1 waveform under initial gradient conditions. If necessary, wait for at least 24 hours for the response to stabilize. In many cases, it is useful to wait overnight.

Repeat the entire polishing procedure until the background drops below 80 nC under initial gradient conditions. The column should be removed from the system (or bypassed) during any detector cell testing.

10.8.2 Sanding of Receded Gold Working Electrodes

IMPORTANT ***This entire procedure should be used only for seriously damaged or receded electrodes.***

A. Sanding off of the gold electrodes is always done with subsequent coarse and fine polishing as described above.
B. The only reason to sand off an electrode is to make the gold electrode flush with the polymer block surface.
C. Use a fresh 600-grit sand paper. Make sure that the polymer block surface remains planar. If the surface is not planar, the ED cell will leak. The cell gasket will not have the required uniform seal around the entire flow path inside the assembled cell.
D. Sand for less than 1 minute (continue sanding only to bring the polymer to the same level as gold), rinse off the powder residue with deionized water. Polish the rinsed electrode on a clean polishing pad (P/N 36121) with deionized water to remove last traces of the powder residue. Rinse with water again.

10.8.3 Chemical Reconditioning of Dionex AAA-Certified Conventional Gold Working Electrodes

The chemical method of reconditioning removes chemical contamination from the working electrode surface and restores the electrode performance. If the electrode has been passivated by excessive gold oxide formation (see Section 10.1 for example), too high reference potential), the chemical cleaning will not restore the electrode performance.

SAFETY

Wear gloves and safety glasses whenever handling chromic acid solutions.

Chemical Reconditioning of Electrodes with Chromic Acid

A. **Preparation of Chromic Acid**
Dissolve/suspend 1 gram of sodium chromate in 1 mL water in a 100 mL glass beaker, slowly add 10 mL of concentrated sulfuric acid with constant stirring. Store the solution in a suitable closed glass vessel. When used for the first time, transfer about 10 mL of chromic acid from the glass vessel into a 20 mL glass scintillation vial, then screw the cap on. After that, the chromic acid solution can be returned to the closed glass vessel and stored for future use.

WARNING

Chromic acid is corrosive and carcinogenic.
Follow all usual precautions and proper disposal procedures.

B. **Reconditioning of Electrodes**

NOTE

Before, during and after the reconditioning, use proper safety equipment and avoid any skin contact with the gold electrodes.

Put the working electrode on a clean filter resting on a horizontal surface. Using a fresh glass transfer pipette, apply one or two droplets of chromic acid to the electrode surface. The chromic acid should form a hemisphere (approximately 2–3 mm in diameter) covering the entire gold surface and surrounding polymeric material.
Leave the reagent in place for 10 minutes.
Rinse the chromic acid off with DI water, then rinse the entire electrode with water again and dry it with clean air.

11. IPAD Positive Compounds Separated on Dionex AminoPac PA10 Column

Gradient: Table 4, Waveform: Table 1

Compound	RT (Min)	Coelution with	Resolving Method	MW	pKa	pKb	pKx	S
AAIBA	5.76			103.1				181
ABA	26.1			137.14				
ACA	24.16			159.23				
ACES	18.22			182.2				
Acetyl-L-cysteine, N-	26.84			163.2				
AEC	4.84			164.2				
AGA	5.06			221.2				
AIBA	5.6			103.1				
ALA	19.94			131.3				
Alanine	6.1			89.09	2.34	9.69		6
Alanine, b-	5.7			89.09	3.53	10.1		
AlloIsoleucine	12.89	Leucine		131.17				
AMCHCA	12.48	Isoleucine		157.2				
Aminoadipic acid, 2-	26.32			161.16				
Aminobutyric acid, 2-	6.72			103.12				
Amino-L-tyrosine, 3-	25.68			195.13				42
AMPA	21.66			111				
APA	19.34			180.2				
APBA	6.74			179.2				
APSA	16.4			139.2				
Arabinose	4.98			150.13				
Arginine	1.68			174.2	2.17	9.04	12.48	25
Argininosuccinic acid	22.72	Phenylalanine	1	290.2				
Asparagine	5.28			132.12	2.02	8.8		
Aspartate	23.78			133.1	1.88	9.6	3.65	
Bicine	10.42	Proline	1	163.17				
CAPS	30.56			221.3				
CAPSO	30.94			237.3				
Carnosine	20.22			226.24				
CHES	28.58			207.3				
Citrulline	4.36			175.19	2.43	9.41		167
CMC	25.52			179.2				
CPA	33.08			199.6				

Compound	RT (Min)	Coelution with	Resolving Method	MW	pKa	pKb	pKx	S
Creatinine	2.85			113.12				
Cystathionine	22.54	Phenylalanine	1	222.3				
Cystine	25			240.3		8.8		97
DAHDA	22.16			190.2				
DAPA	3.63			104.07		9.4		
DASA	22.77			148.1				
DHPA	7.24	Glycine	1	197.2				
EACA	8.72			131.2				
EGTA	24.36			380.4				
EPPS	13.24			268.3				251
Fructose	7.3			180.16				
Fucose	3.2	Lysine	1	164.16				
GABA	5.48			103.1		10.31		
Galactose	6.34	Threonine	2	180.16				
Galacturonic acid	22.16			194.14				58
Glucosamine	4.55			179.14				
Glucose	6.22	Alanine	1	180.16				
Glucuronic acid	22.46	Phenylalanine	1	194.14				
Glutamate	23.46			147.13	2.19	9.67	4.25	422
Glutamine	4.56			146.15	2.17	9.13		
Glycine	7.08			75.07	2.34	9.6		1622
Glycylleucine	11.88			188.23		7.9		
Glycyllysine	3.03			132.12				
Gly-Gly	6.22	Alanine		132.12	3.13	8.07		
Gly-Gly-Ala	4.69			203.2	3.18	7.9		
Gly-Gly-Gly	6.48	Threonine	1	189.19	3.2	7.89		
Gly-Gly-Gly-Gly	6.86	Glycine		246.23	3.18	7.87		
Gly-Gly-Phe-Ala	20.32			367.39				
Gly-Hydroxy-Pro	9.56	Serine		188.2				34
Gly-Pro	4.19			172.2	2.85	8.37		
Gly-Ser	10.6			162.1				
Histidine	21.02			155.16	1.82	9.17	6	23
Homoarginine	1.7	Arginine		188.2				56
Homocitrulline	4.84			189.19				
Homocysteine	25.88			135.19				
Homocystine	26.74			268.3				
Homoserine	6.72			119.09	2.27	9.28		
HPG	27.48	Tyrosine		167.2				
HT	34.66			220.23	2.51	9.49		

Compound	RT (Min)	Coelution with	Resolving Method	MW	pKa	pKb	pKx	S
Hydroxylysine, d-	2.97			162.15				
Hydroxyproline	9.1			131.13	1.8	9.46		
Hypotaurine	6.62	Threonine	1	109.1				
Isoleucine	12.68			131.17	2.36	9.6		43
Isomaltose	11.84			342.3				
Kynurenine	26.66			208.2				
Lactose	12.14			342.3				
Leucine	13.58			131.17	2.36	9.6		
Lysine	3.37			146.19	2.18	8.95	10.53	
Mannitol	2.57			182.17				29
Mannose	5.94	Alanine	1 or 2	180.16				
Methionine	14.36			149.21	2.28	9.21		
Methioninesulfone	7.1	Glycine		181.2				
Methioninesulfoxide	3.24	Lysine	2	165.21				
Methylhistidine, 1-	5.92	Alanine	1	169.19				5
Methyl-histidine, 3-	5.94	Alanine	1	169.19				
MM	14.4	Methionine		163.2				
NAGA	5.7			221.2				
Norleucine	17.58			131.17	2.3	9.67		
Norvaline	9.74	Serine	1	17.15	2.31	9.65		
Ornithine	3.02			132.12	2.11	8.58	10.46	
Phenylalanine	22.5			115.13	1.99	10.6		
Phospho-serine	26.36			185.1				
PMG	27.38	Tyrosine		263.1				
Proline	10.26			115.13	1.99	10.6		
Rhamnose	3.75			164.16				
Ribose	8.02	Valine	1	150.13				
Serine	9.58			105.09	2.21	9.15		
Sucrose	11.6			342.3				
Taurine	19.18			125.15				
TES	21.86			229.2				0.5
THIQCA	27.08			177.2				
Threonine	6.5			119.12	2.09	9.1		
Tricine	19.08			179.2				
Tryptophan	40.78			204.23	2.83	9.39		
Tyrosine	27.4			181.19	2.2	9.11	10.07	
Valine	8.28			117.15	2.32	9.62		
Xylose	6.54	Threonine	2	150.13				

Notes:

RT: Retention Time.

Selected Abbreviations Used in Compound :

AAIBA: a-AminoIsoButyric Acid;
ABA: p-AminoBenzoic Acid;
ACA: 2-AminoCaprylic Acid;
ACES: N-[2-ACetamido]-2-aminoEthaneSulfonic acid;
AEC: S-,2-AminoEthyl-L-Cysteine;
AGA: N-Acetyl-D-GlucosAmine;
AIBA: b-AminoIsoButyric Acid;
ALA: d-AminoLevulinic Acid;
AMCHCA: trans-4-AminoMethyl-CycloHexane Carboxylic Acid;
AMPA: AminoMethylPhosphonic Acid;
APA: p-Amino-PhenylAlanine;
APBA: 3-AminoPhenylBoronic Acid;
APSA: 3-AminoPropane Sulfonic Acid;
CAPS: -CyclohexylAmino-1-PropaneSulfonic acid;
CAPSO: 3-CyclohexylAmino-2-hydroxy-1-PropaneSulfOnic acid;
CHES: 2-[N-CycloHexylamino]EthaneSulfonic acid;
CMC: S-Carboxy Methyl-Cysteine;
CPA: p-ChloroPhenylAlanine;
DAHDA: 2,6-DiAminoHeptaneDioic Acid;
DAPA: 2,3-DiAmino-Propionic Acid;
DASA: a,b-DiAminoSuccinic Acid;
DHPA: 3,4-DiHydroxyPhenylAlanine;
EACA: e-Amino-n-Caproic Acid;
EGTA: EthyleneGlycol-bis-(b-amino ethyl ether)N,N-TetraAcetic acid;
EPPS: N-(2-hydroxyEthyl)Piperazine-N'-3-PropaneSulfonic acid;
GABA: Gamma-AminoButyric Acid;
HPG: p-HydroxyPhenyl Glycine;
HT: 5-Hydroxy-Tryptophan;
MM: a-MethylMethionine;
NAGA: N-Acetyl-D-GalactosAmine;
PMG: N,N-bis-(PhosphonoMethyl)Glycine;
TES: N-Tris[hydroxylmethyl]methyl-2-aminoEthaneSulfonic acid;
THIQCA: 1,2,3,4-TetraHydroIsoQuinoline-3-Carboxylic Acid.

Coelution with: Lists possible coelution with common amino acids.
Resolving Method: 1. Hydroxy waveform: see P. Jandik et al. J. Chromatogr. B 732 (1999) pp. 193 -201; 2. Different Gradient.

MW: Molecular weight.

pKa: Negative logarithm of the dissociation constant for a -COOH group.

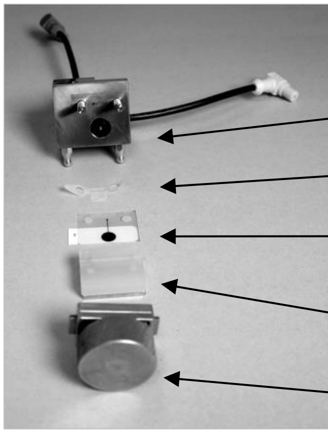
pKb: Negative logarithm of the dissociation constant for a -NH3+ group.

pKx: Negative logarithm of the dissociation constant for any other group present in the molecule.

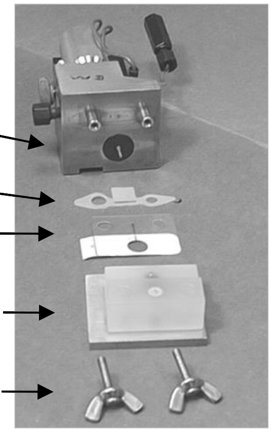
S: Solubility in water at 25°C in units of grams per kilogram of water.

12. Installation of Disposable Electrode

ED (ICS3000)



ED40 / ED50 / ED50A



STEP 1
Check availability of all parts

Cell body with reference electrode installed

PTFE gasket for disposable electrodes

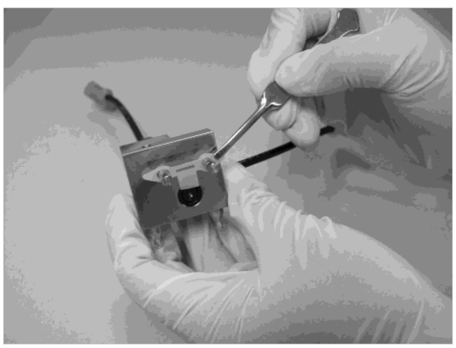
Disposable Electrode

Spacer block (P/N 062158)

Spacer block (P/N 060297)

Yoke-knob Assembly

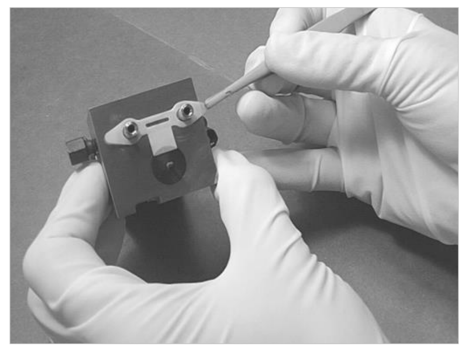
Wing nuts

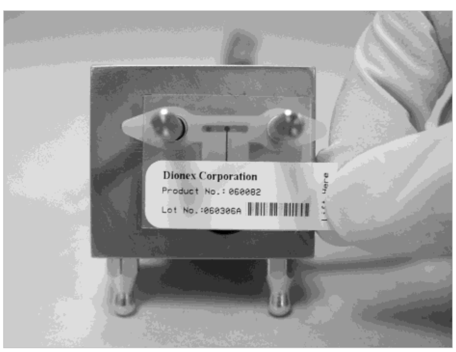


STEP 2
Install the gasket

Check for correct gasket orientation.

Avoid any wrinkles inside the sealing area of the gasket.

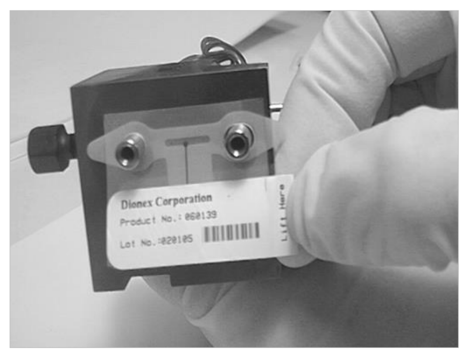


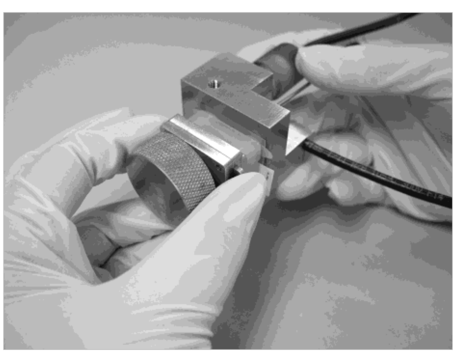


STEP 3
Install the disposable electrode

Make sure the disposable electrode is oriented correctly.

The gold electrode surface must face ED cell body





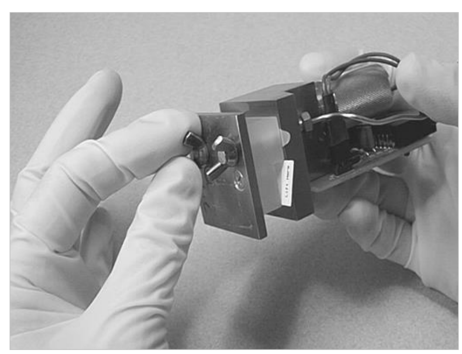
STEP 4
Place spacer block over the disposable electrode

Tighten the yoke-knob till it clicks.

Tighten the wing nuts evenly.

Finger Tight only.

Do not use tools such as pliers.



Appendix A – Column Care

A.1 New Column Equilibration

The columns are shipped in 50 mM NaOH containing 0.1% sodium azide. Before use, the column must be washed with approximately 20 mL of the starting eluent (80 min. at 0.25 mL/min).

A.2 Column Cleanup

NOTE

When cleaning an analytical and guard column in series, move the guard column after the analytical column in the eluent flow path. Otherwise contaminants that have accumulated on the guard column will be eluted onto the analytical column.

A.2.1 Mild Contamination

For mild cleaning try consecutive gradient runs, using the gradient from Table 5 in section 9.3, "Gradient Conditions Including a Strong Post-Separation Rinse with 0.1M Acetic Acid for Removal of Residual Peaks in the Gradient Range between Histidine and Tyrosine."

A.2.2 Moderate Contamination

For more stubborn contamination, inject larger amount (100–500 µL or more) of 0.1–1 M NaOH consecutively.

A.2.3 Severe Contamination

If necessary, the column can be washed with 50 mM NaOH/200 mM acetate (20% B/80% C) or 1.0 M NaOH. Usually cleaning for 2–3 hours at 0.25 mL/min is sufficient. Increase the column temperature to 40 °C during the wash. After the wash, return to 30 °°C, rinse the column with at least 20 mL of the starting gradient composition.

A.3 Column Storage

Program the pump to deliver 60 mM sodium hydroxide. Pump this solution through the columns for 60 minutes at 0.25 mL/min. Turn off the pump, remove the columns, plug the ends with the plugs that were in place when you received the columns and store them.

A.4 Replacing Column Bed Support Assemblies

NOTE

Replace the inlet bed support ONLY if the column is determined to be the cause of high system back pressure, AND cleaning of the column does not solve the problem.

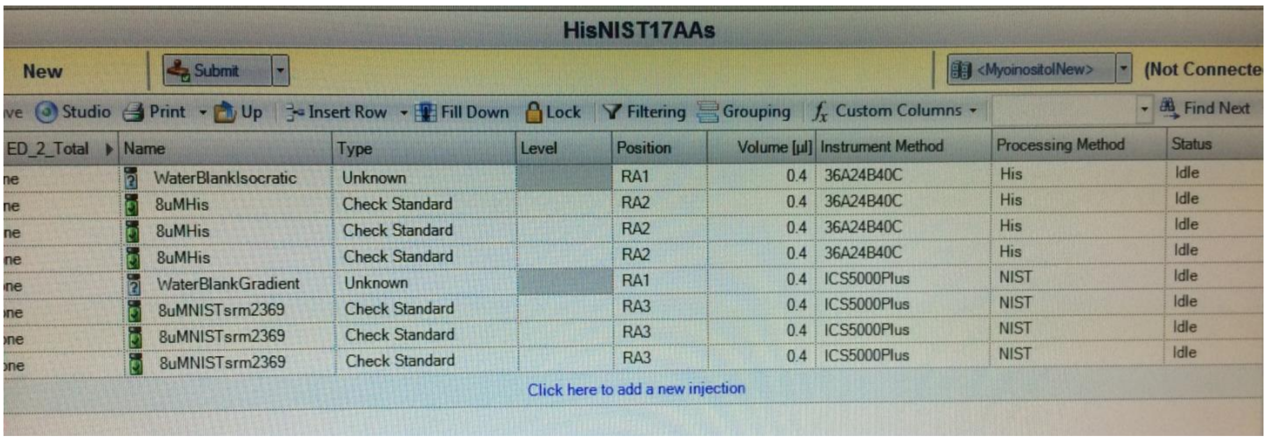
1. Carefully unscrew the inlet (top) column fitting. Use two open end wrenches.
2. Remove the bed support. Tap the end fitting against a hard, flat surface to remove the bed support and seal assembly. Do not scratch the wall or threads of the end fitting. Discard the old bed support assembly.
3. Removal of the bed support may permit a small amount of resin to extrude from the column. Carefully remove this with a flat surface such as a razor blade. Make sure the end of the column is clean and free of any particulate matter. Any resin on the end of the column tube will prevent a proper seal. Insert a new bed support assembly into the end fitting and carefully thread the end fitting and bed support assembly onto the supported column.
4. Tighten the end fitting finger tight, then an additional ¼ turn (25 in x lb.). Tighten further only if leaks are observed.

CAUTION

If the end of the column tube is not clean when inserted into the end fitting, particulate matter may prevent a proper seal between the end of the column tube and the bed support assembly. If this is the case, additional tightening may not seal the column but instead damage the column tube or break the end fitting.

Appendix B – The Sequence and the Instrument Methods

B.1 The Sequence



B.2 The Instrument Methods (Channel 2 is used)

B.2.1 The Instrument Method for His (histidine)

```
{Initial Time}    Instrument Setup
        DC.Compartment_TC.AcquireExclusiveAccess
        DC.Column_TC.AcquireExclusiveAccess
        Wait    Sampler.Ready, Run=Hold, Timeout=Infinite
        EDet2.Mode    IntAmp
        DP.Pump_1.Pressure.UpperLimit    5000 [psi]
        DP.Pump_1.Pressure.LowerLimit    200 [psi]
        DP.Pump_2.Pressure.UpperLimit    5000 [psi]
        DP.Pump_2.Pressure.LowerLimit    200 [psi]
        DP.Pump_2.%A.Equate    "%A Water"
        DP.Pump_2.%B.Equate    "%B 0.25 M NaOH"
        DP.Pump_2.%C.Equate    "%C 1 M NaAc"
        DP.Pump_2.%D.Equate    "%D"

        Sampler.CycleTime    0 [min]
        Sampler.LoopOverfill    5.000
        Sampler.InjectMode    PushFull
        Sampler.BufferWashFactor    2.000
        Sampler.WashDispSpeed    20.0 [µl/s]
        Sampler.InjectWash    AfterInj
        Sampler.WashSpeed    20.0 [µl/s]
        Sampler.WashVolume    250.0 [µl]
        Sampler.SampleHeight    2.000 [mm]
        Sampler.WasteSpeed    20.0 [µl/s]
        Sampler.DispenseDelay    2.0 [s]
        Sampler.DispSpeed    5.0 [µl/s]
        Sampler.DrawSpeed    10.0 [µl/s]
        Sampler.DrawDelay    2.0 [s]
        Sampler.DilutionMixDispenseSpeed    60.0 [µl/s]
        Sampler.DilutionMixIterations    3
        Sampler.DilutionMixSpeed    30.0 [µl/s]

        EDet2.Data_Collection_Rate    1.67 [Hz]
        EDet2.pH.UpperLimit    13.00
        EDet2.pH.LowerLimit    10.00
        DC.Column_TC.Mode    On
        DC.Column_TC.TemperatureSet    30.00 [°C]
        DC.Compartment_TC.Mode    On
        DC.Compartment_TC.TemperatureSet    30.00 [°C]
```

```
        DP.Pump_2.Flow0.25
        DP.Pump_2.%B.Value      24
        DP.Pump_2.%C.Value      40
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         5
        Sampler.PunctureOffset  0 [mm]
        EDet2.CellControl       On
        EDet2.WaveformName      "Gold, pH-Ag-AgCl RE, AAA"
        EDet2.WaveformDescription       "Amino Acids, Carbohydrates, Water Soluble Vitamins"
        EDet2.Electrode  pH

        EDet2.Waveform 0, 0.13, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.04, 0.13, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.05, 0.33, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.21, 0.33, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
        EDet2.Waveform 0.22, 0.55, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
        EDet2.Waveform 0.46, 0.55, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
        EDet2.Waveform 0.47, 0.33, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
        EDet2.Waveform 0.56, 0.33, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.57, -1.67, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.58, -1.67, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.59, 0.93, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
        EDet2.Waveform 0.6, 0.13, LastStep=On, Ramp=On, GainRegion=Off, Integration=Off

0.000   Inject
        Wait    Sampler.CycleTimeState, Run=Hold, Timeout=Infinite
        Sampler.Inject
0.000   Start Run
        EDet2.ED_2_Total.AcqOn
0.000   Run     Duration = 20.000 [min]
20.000  Stop Run
        EDet2.ED_2_Total.AcqOff
20.000  Post Run        Duration = 0.000 [min]
        DC.Column_TC.ReleaseExclusiveAccess
        DC.Compartment_TC.ReleaseExclusiveAccess
        End
```

B.2.2 The Instrument Method for NIST AAs (Amino Acids from NIST)

```
{Initial Time}    Instrument Setup
        DC.Compartment_TC.AcquireExclusiveAccess
        DC.Column_TC.AcquireExclusiveAccess
        Wait    Sampler.Ready, Run=Hold, Timeout=Infinite
        EDet2.Mode    IntAmp
        DP.Pump_1.Pressure.UpperLimit    5000 [psi]
        DP.Pump_1.Pressure.LowerLimit    200 [psi]
        DP.Pump_2.Pressure.UpperLimit    5000 [psi]
        DP.Pump_2.Pressure.LowerLimit    200 [psi]
        DP.Pump_2.%A.Equate    "%A Water"
        DP.Pump_2.%B.Equate    "%B 0.25 M NaOH"
        DP.Pump_2.%C.Equate    "%C 1 M NaAc"
        DP.Pump_2.%D.Equate    "%D"

        Sampler.CycleTime    0 [min]
        Sampler.LoopOverfill    5.000
        Sampler.InjectMode    PushFull
        Sampler.BufferWashFactor    2.000
        Sampler.WashDispSpeed    20.0 [µl/s]
        Sampler.InjectWash    AfterInj
        Sampler.WashSpeed    20.0 [µl/s]
        Sampler.WashVolume    250.0 [µl]
        Sampler.SampleHeight    2.000 [mm]
        Sampler.WasteSpeed    20.0 [µl/s]
        Sampler.DispenseDelay    2.0 [s]
        Sampler.DispSpeed    5.0 [µl/s]
        Sampler.DrawSpeed    10.0 [µl/s]
        Sampler.DrawDelay    2.0 [s]
        Sampler.DilutionMixDispenseSpeed    60.0 [µl/s]
        Sampler.DilutionMixIterations    3
        Sampler.DilutionMixSpeed    30.0 [µl/s]

        EDet2.Data_Collection_Rate    1.67 [Hz]
        EDet2.pH.UpperLimit    13.00
        EDet2.pH.LowerLimit    10.00
        DC.Column_TC.Mode    On
        DC.Column_TC.TemperatureSet    30.00 [°C]
        DC.Compartment_TC.Mode    On
        DC.Compartment_TC.TemperatureSet    30.00 [°C]
        DP.Pump_2.Flow    0.25
        DP.Pump_2.%B.Value    24
        DP.Pump_2.%C.Value    40
        DP.Pump_2.%D.Value    0
        DP.Pump_2.Curve    5
        Sampler.PunctureOffset    0 [mm]

        EDet2.CellControl    On
        EDet2.WaveformName    "Gold, pH-Ag-AgCl RE, AAA"
        EDet2.WaveformDescription    "Amino Acids, Carbohydrates, Water Soluble Vitamins"
        EDet2.Electrode  pH
```

EDet2.Waveform 0, 0.13, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.04, 0.13, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.05, 0.33, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.21, 0.33, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
EDet2.Waveform 0.22, 0.55, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
EDet2.Waveform 0.46, 0.55, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
EDet2.Waveform 0.47, 0.33, LastStep=Off, Ramp=On, GainRegion=On, Integration=On
EDet2.Waveform 0.56, 0.33, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.57, -1.67, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.58, -1.67, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.59, 0.93, LastStep=Off, Ramp=On, GainRegion=Off, Integration=Off
EDet2.Waveform 0.6, 0.13, LastStep=On, Ramp=On, GainRegion=Off, Integration=Off

0.000 Inject
Wait Sampler.CycleTimeState, Run=Hold, Timeout=Infinite
Sampler.Inject
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 24
DP.Pump_2.%C.Value 0
DP.Pump_2.%D.Value 0
DP.Pump_2.Curve 5
0.000 Start Run
EDet2.ED_2_Total.AcqOn
0.000 Run Duration = 74.000 [min]

2.000
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 24
DP.Pump_2.%C.Value 0
DP.Pump_2.%D.Value 0
DP.Pump_2.Curve 5
8.000
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 36
DP.Pump_2.%C.Value 0
DP.Pump_2.%D.Value 0
DP.Pump_2.Curve 8
11.000
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 36
DP.Pump_2.%C.Value 0
DP.Pump_2.%D.Value 0
DP.Pump_2.Curve 5
18.000
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 20
DP.Pump_2.%C.Value 40
DP.Pump_2.%D.Value 0
DP.Pump_2.Curve 8
21.000
DP.Pump_2.Flow 0.25
DP.Pump_2.%B.Value 16
DP.Pump_2.%C.Value 40
DP.Pump_2.%D.Value 0

```
        DP.Pump_2.Curve         5
23.000
        DP.Pump_2.Flow          0.25
        DP.Pump_2.%B.Value      16
        DP.Pump_2.%C.Value      70
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         8
42.000
        DP.Pump_2.Flow          0.25
        DP.Pump_2.%B.Value      16
        DP.Pump_2.%C.Value      70
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         8
42.100
        DP.Pump_2.Flow          0.25
        DP.Pump_2.%B.Value      80
        DP.Pump_2.%C.Value      0
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         8
44.100
        DP.Pump_2.Flow          0.25
        DP.Pump_2.%B.Value      80
        DP.Pump_2.%C.Value      0
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         5
44.200
        DP.Pump_2.Flow          0.25
        DP.Pump_2.%B.Value      24
        DP.Pump_2.%C.Value      0
        DP.Pump_2.%D.Value      0
        DP.Pump_2.Curve         5
75.000  Stop Run
        EDet2.ED_2_Total.AcqOff
75.000  Post Run        Duration = 0.000 [min]
        DC.Column_TC.ReleaseExclusiveAccess
        DC.Compartment_TC.ReleaseExclusiveAccess
End
```