Tips & Tricks to Achieve Fast, Sensitive, and Reproducible Separation of Amino Acids

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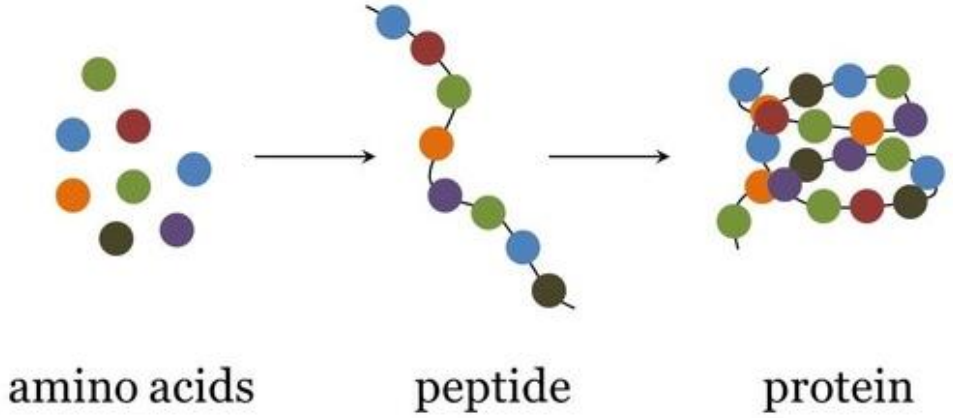
Some Basics



Amino Acids are the building blocks of proteins

Amino Acids require derivatization to be detected by UV or FL

- OPA/FMOC, Ninhydrin, Dansyl chloride, and PITC are common reagents used

Derivatization can be done pre-column or post column

- OPA/FMOC, Dansyl chloride, and PITC are common reagents used for pre-column
- Ninhydrin is common for post column methods

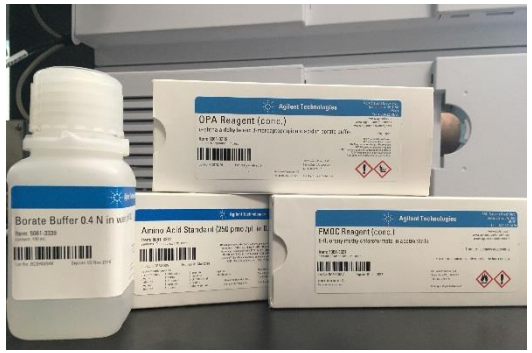
Analysis of AA can be done by several methods:

- GC, CE, HPAE-PAD
- LC/UV/FL, LC/MS

Why is Amino acid analysis important?

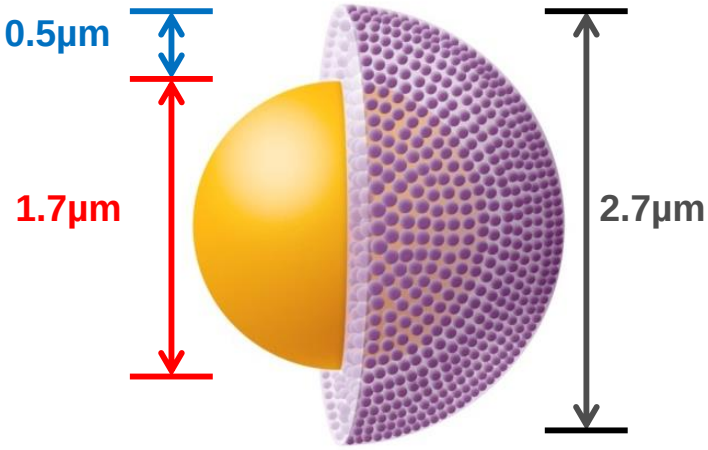
- Important for protein and peptide identification and quantitation
- Part of reverse-phase characterization in biopharma
- Required by the FDA
- Important for monitoring cell culture media
- Used for the analysis of metabolic intermediates - “Bound vs. Free”

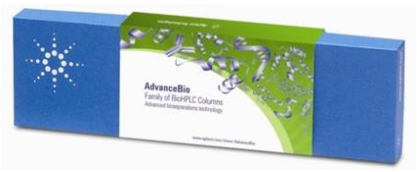
The Agilent Amino Acid Analysis solution



Ready to use AdvanceBio AAA kit (Standards and Reagents)

All Agilent LC systems including Infinity II systems





AdvanceBio AAA Columns
Fast and rugged

Agilent AdvanceBio AAA

Previous Agilent AAA Method

Agilent has a well established solution for Amino Acid Analysis

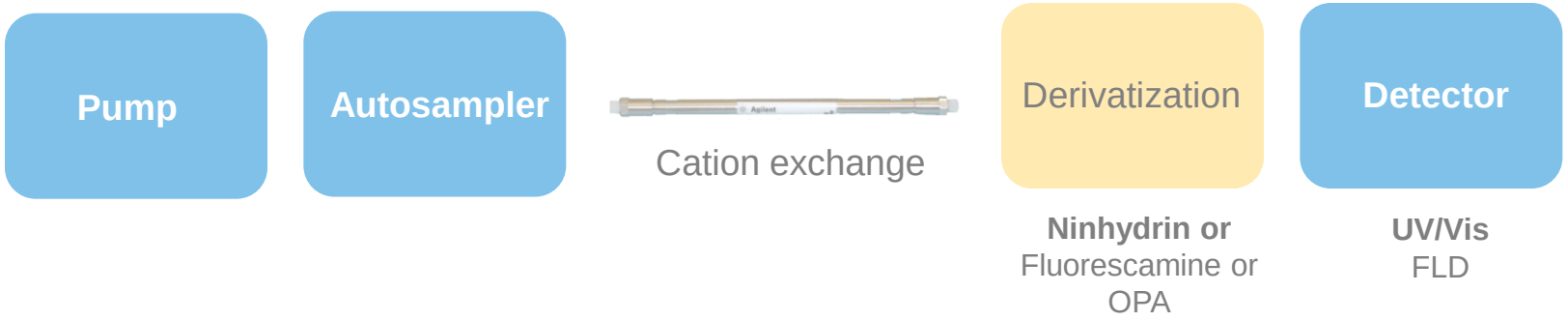
- based on automated pre-column derivatization capabilities of Agilent Autosamplers
- Uses ZORBAX Eclipse AAA column
- Well established method using reagents and standards from Agilent

What's New?

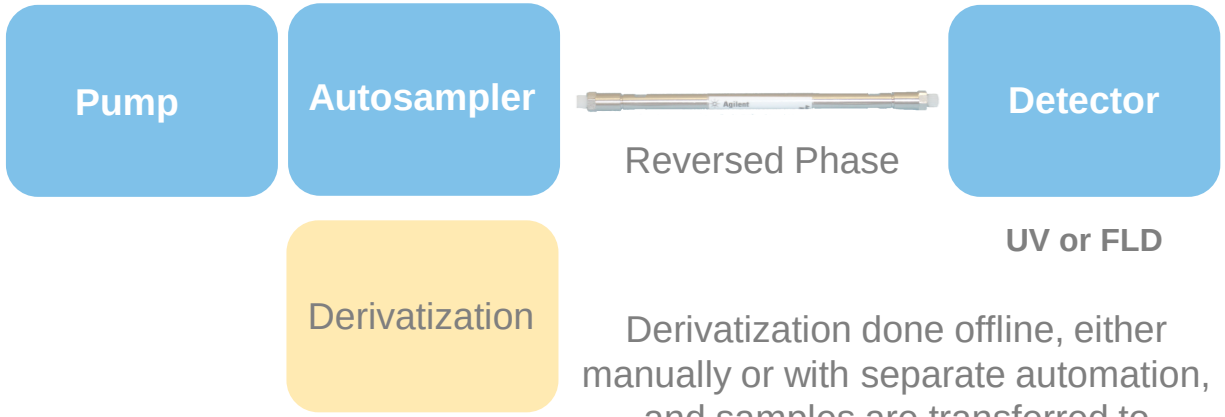
- All reagents conveniently kitted together under a single part number
- Introduced an HpH chemistry on a Poroshell particle for improved column lifetime
  - Traditional silica columns dissolve above neutral pH, but HpH chemistry stabilizes column
    - AA derivatization and separation are most efficient at higher pH
  - Poroshell column with 2 µm frits is less susceptible to clogging

Pre- vs Post-Column Derivatization

Post Column Derivatization - The historic Gold Standard of dedicated Amino Acid Analyzers

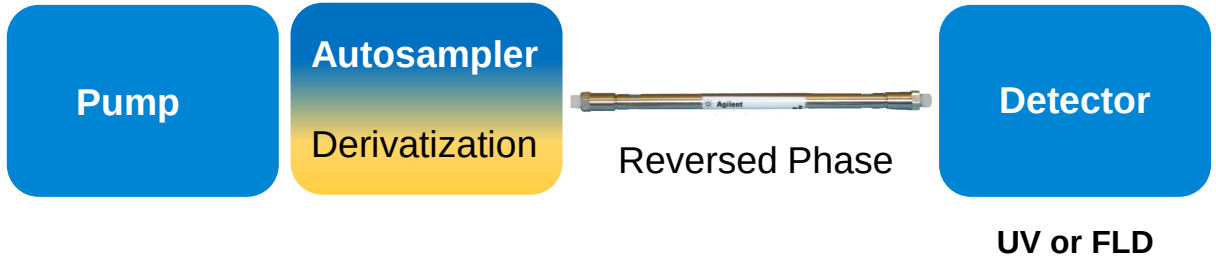


Pre Column Derivatization - Offline:



Derivatization done offline, either manually or with separate automation, and samples are transferred to autosampler

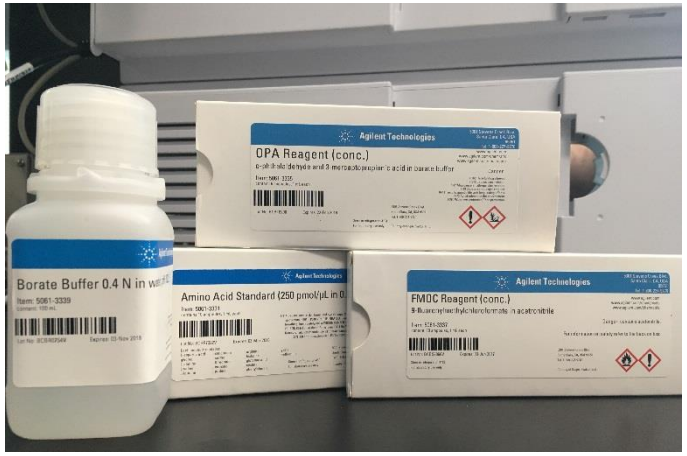
Pre Column Derivatization - Online:



Derivatization done online, in the autosampler – eliminates error associated with manual sample handling for highly consistent, reproducible results

AdvanceBio AAA Reagent Kit

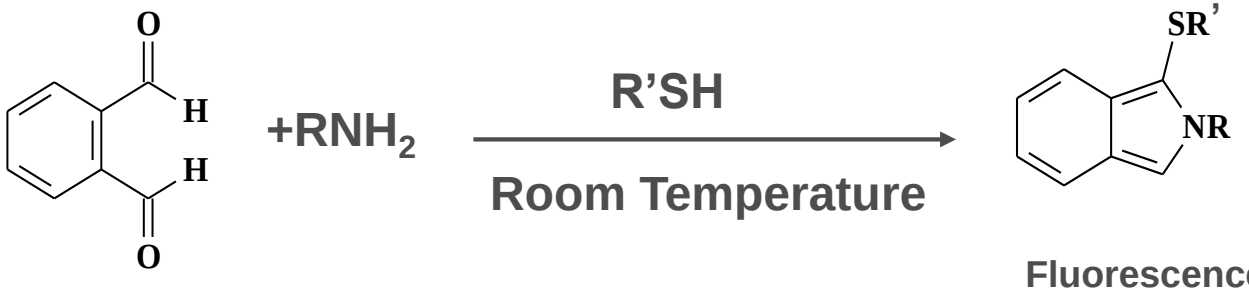
Part Number	Material
5061-3339	100mL Borate Buffer
5061-3337	FMOC reagent - 10 ampoules, 1 mL each
5061-3335	OPA reagent, 10 mg/mL, 6 ampoules
5062-2479	Dithiodiproprionic acid (DTDPA)
5061-3330	AA, standard 1nmol 10/PK
5061-3331	AA standards, 250 pmol 10/PK
5061-3332	AA standards, 100 pmol 10/PK
5061-3333	AA standards, 25 pmol 10/PK
5061-3334	AA standards, 10 pmol 10/PK
5062-2478	AA supplements, 1g each



Order components individually, or together as part of a kit with a single part number (5190-9426)

Automated Derivatization in the Autosampler

Ortho Phthalaldehyde (OPA)

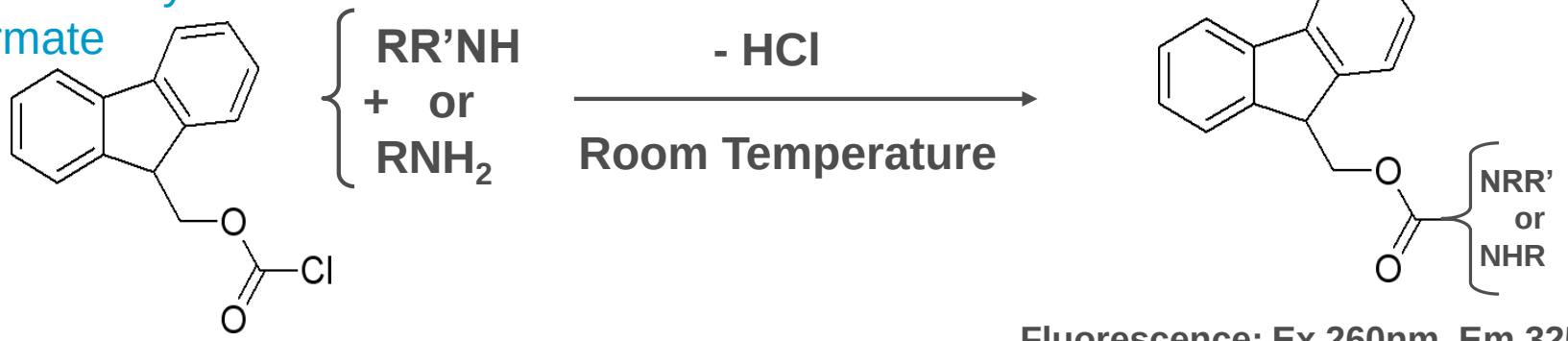


Non-fluorescent
Does not absorb at 338nm

Fluorescence: Ex 340nm, Em 450nm
DAD: 338, 10nm; Ref. 390, 20nm

1. Allows visualization by UV or FL
2. Helps retain very polar compounds

Fluorenylmethoxy chloroformate (FMOC)



Fluorescent
Absorbs at 262nm and
Fluorescences at 324nm

Fluorescence: Ex 260nm, Em 325nm
DAD: 262, 16nm; Ref. 324,8nm

Optimal pH for reaction with AA: ~10.0

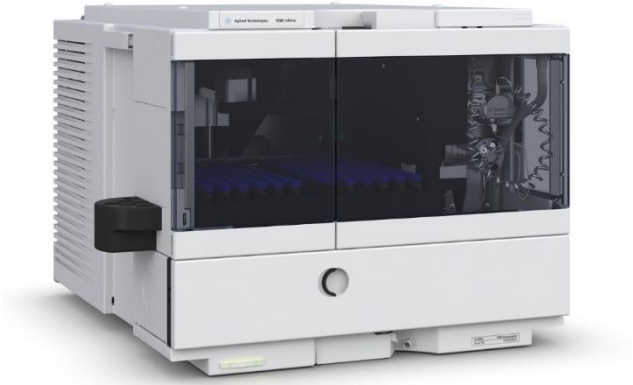
Online derivatization/Injection program

- Draw 2.5 µL from borate vial (Agilent p/n 5061-3339)
- Draw 1.0 µL from sample vial
- Mix 3.5 µL in wash port 5 times
- Wait 0.2 min
- Draw 0.5 µL from OPA vial (Agilent p/n 5061-3335)
- Mix 4.0 µL in wash port 10 times default speed
- Draw 0.4 µL from FMOC vial (Agilent p/n 5061-3337)
- Mix 4.4 µL in wash port 10 times default speed
- Draw 32 µL from injection diluent vial
- Mix 20 µL in wash port 8 times
- Inject
- Wait 0.1 min
- Valve bypass

1290 Infinity II Multisampler

Method can be programmed into ANY Agilent autosampler –

- Eliminates manual labor and variability
- Enables highly precise data



1260 Infinity II Vialsampler

Online derivatization/Injection program

OpenLab ChemStation C.01

Setup Method

Quat. Pump | HiP Sampler | HiP Sampler Injector Program | Column Comp. | DAD | FLD | Instrument Curves

Use Injector Program

Function	Parameter
Draw	Draw 2.5 µL from location "P1-D-1" with default speed using default offset
Draw	Draw 1 µL from sample with default speed using default offset
Mix	Mix 3.5 µL from air with default speed for 5 times
Wait	Wait 0.2 min
Draw	Draw 0.5 µL from location "P1-D-2" with default speed using default offset
Mix	Mix 4 µL from air with default speed for 10 times
Draw	Draw 0.4 µL from location "P1-D-3" with default speed using default offset
Mix	Mix 4.4 µL from air with default speed for 10 times
Draw	Draw 32 µL from location "P1-D-4" with default speed using default offset
Mix	Mix 20 µL from air with default speed for 8 times
Inject	Inject
Wait	Wait 0.1 min
Valve	Switch valve to "Bypass"

Append | Insert | Delete | Clear all | Move up

Cut | Copy | Paste | Move down

1290 Infinity II Multisampler

Method can be programmed into ANY Agilent autosampler –

- Eliminates manual labor and variability
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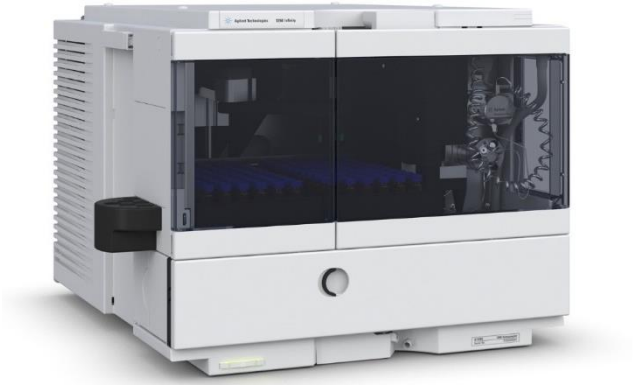
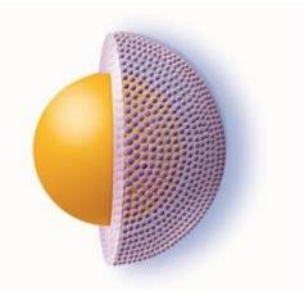


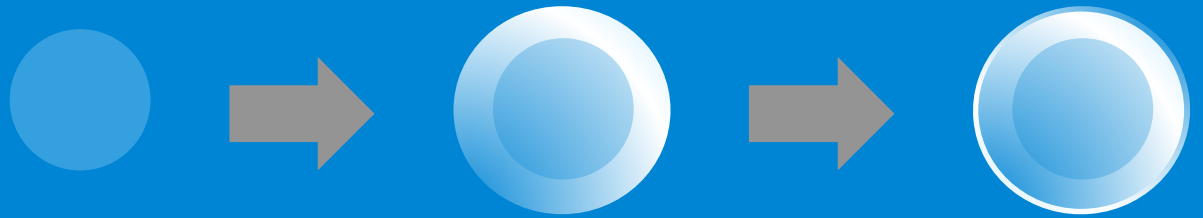
1260 Infinity II Vialsampler

Robust Columns for AAA

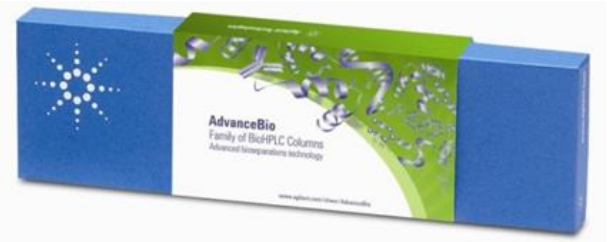


A robust, high efficiency Fast LC column with resistance to elevated pH and temperature offering users performance comparable to that of sub-2 µm alternatives but with up to 50% less back pressure.

Utilizes a proprietary technology for particle synthesis



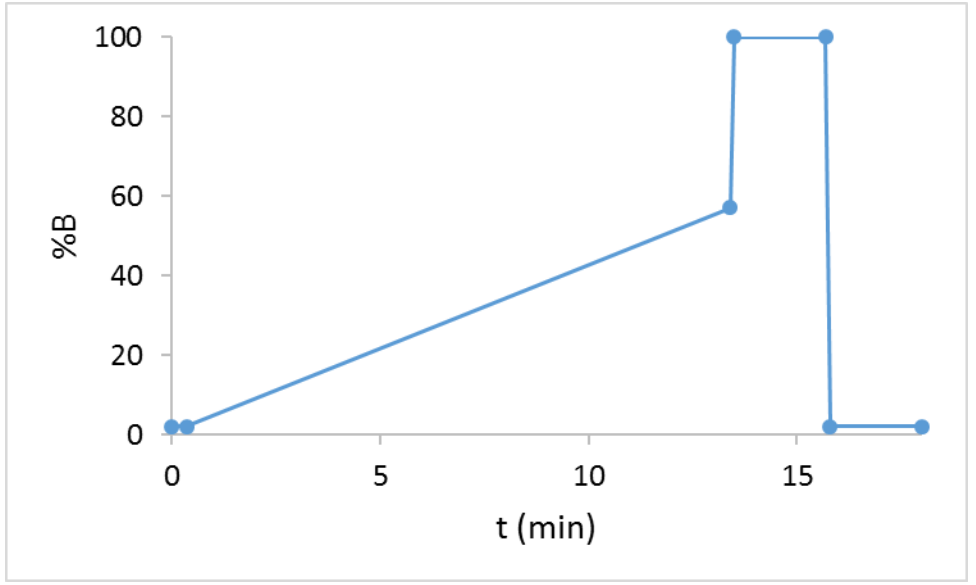
- 2.7 µm particles, 110 Å pore size
- Two dimensions available: 3.0 x 100 mm, 4.6 x 100 mm
  - Guard columns also available in each i.d.
- Each individual column is tested for efficiency
- Each batch is tested with amino acid standards to ensure performance



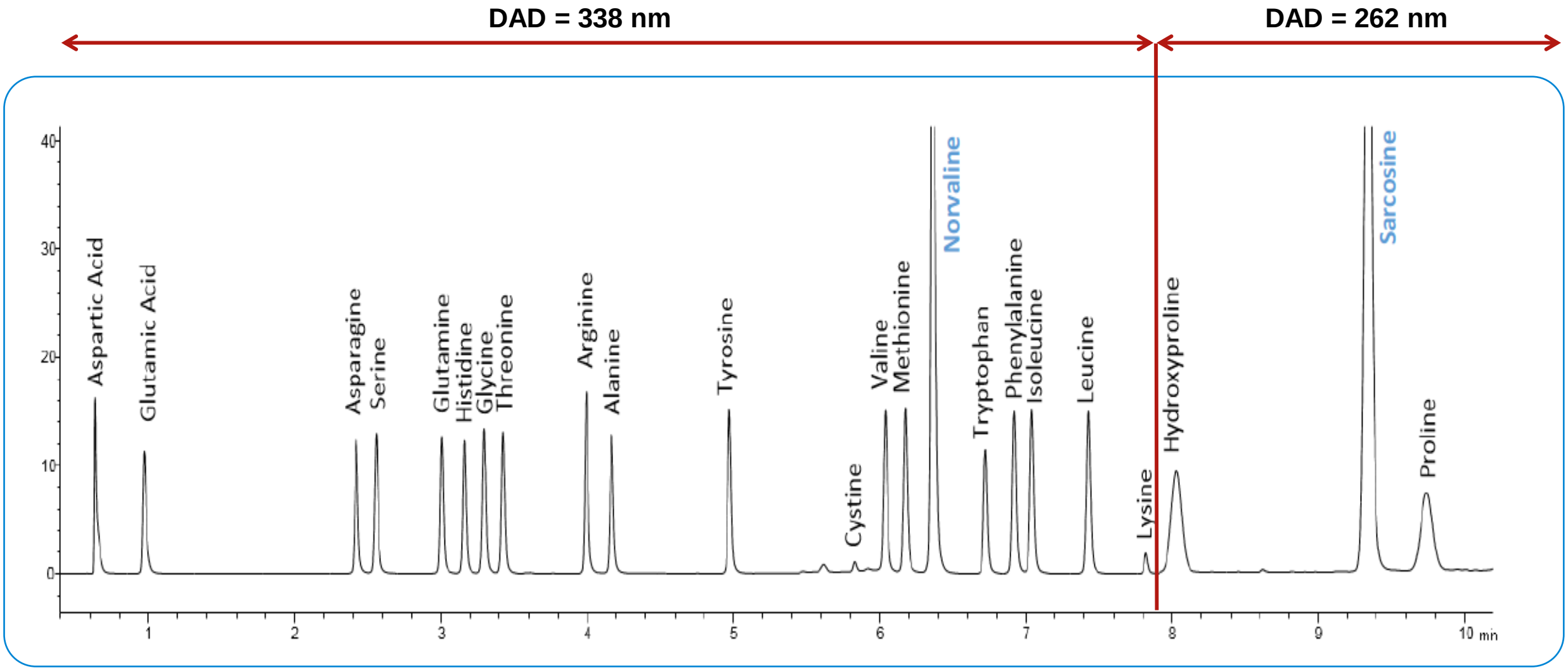
Chromatographic Method

- Flow rate – 1.5 mL/min for 4.6 mm and 0.62 mL/min for 3 mm i.d.
- Injection volume – 1µL with needle wash at the wash port for 7s
- Column temperature – 40 ºC
- Detection wavelength – 338 and 262nm
- Samples- Agilent AAA standards, media samples and protein hydrolysate standards

Time (min)	%B
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	stop



Fast and Rugged Amino Acids Separation



Order of Elution for OPA and FMOC derivatives

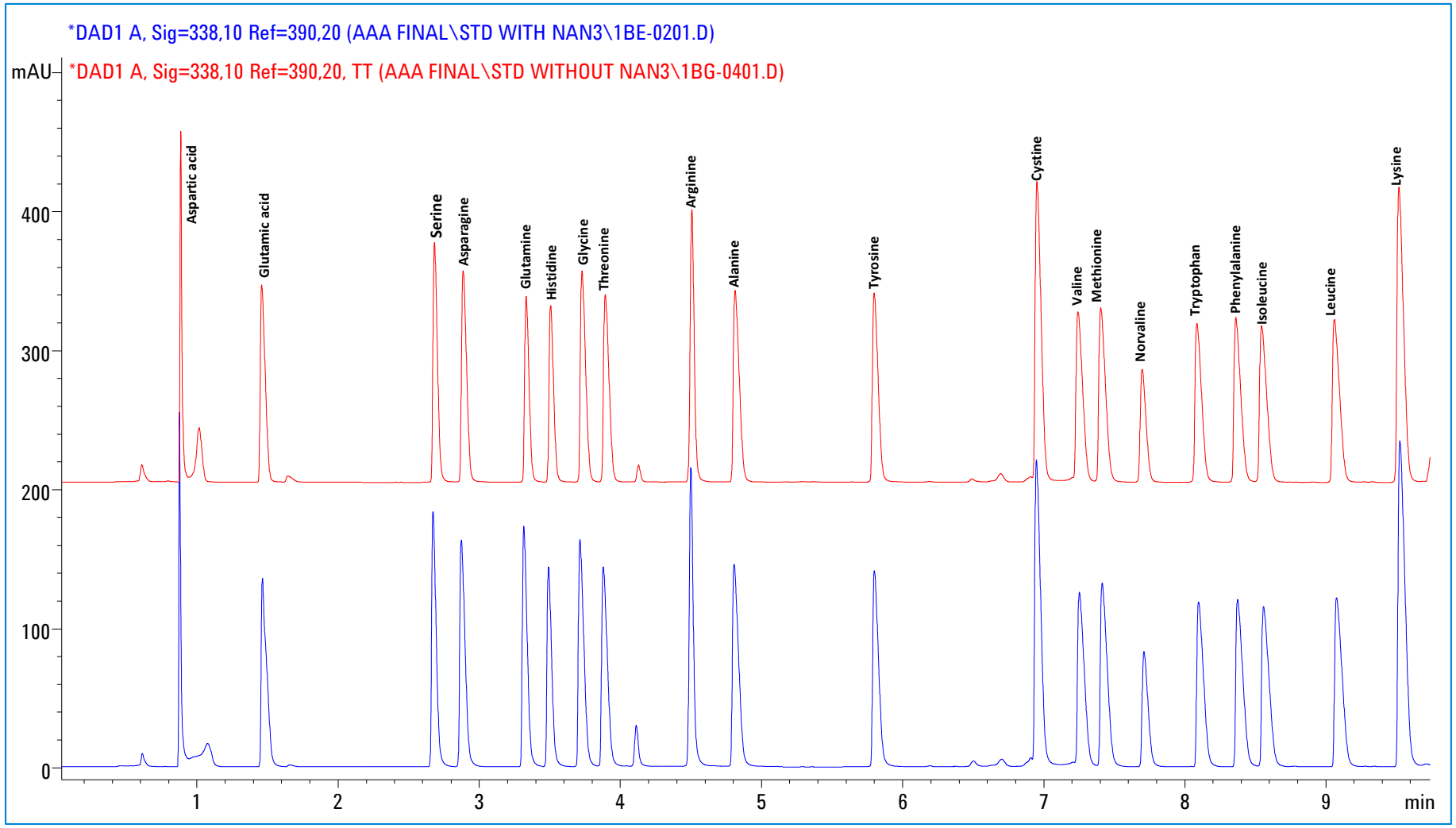
Peak #	AA Name	AA Abbreviation	Derivative Type
1	Asparic Acid	ASP	OPA
2	Glutamic Acid	GLU	OPA
3	Aspraragine	ASN	OPA
4	Serine	SER	OPA
5	Glutamine	GLN	OPA
6	Histidine	HIS	OPA
7	Glycine	GLY	OPA
8	Threonine	THR	OPA
9	Arginine	ARG	OPA
10	Alanine	ALA	OPA
11	Tyrosine	TYR	OPA
12	Cysteine	CYS-CYS	OPA
13	Valine	VAL	OPA
14	Methionine	MET	OPA
15	Norvaline*	NVA	OPA
16	Tryptophan	TRP	OPA
17	Phenylalanine	PHE	OPA
18	Isoleucine	ILE	OPA
19	Leucine	LEU	OPA
20	Lysine	LYS	OPA
21	Hydroxyproline	HYP	FMOC
22	Sacrosine (IS)	SAR	FMOC
23	Proline	PRO	FMOC

Primary AA

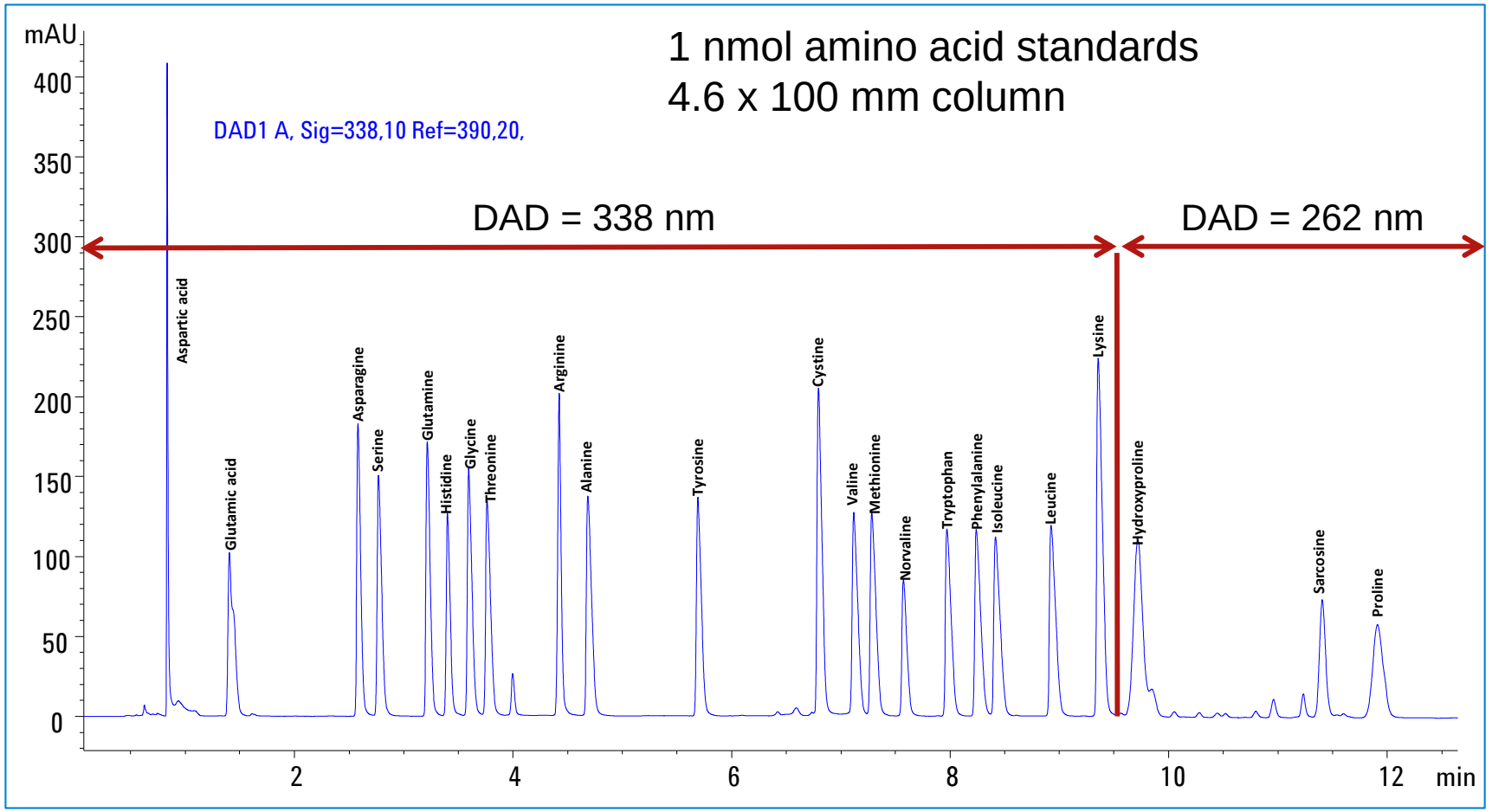
Secondary AA

Elution Profile with and without Sodium Azide

- Historically NaN_3 has been added to aqueous mobile phase to reduce bacterial growth.
- NaN_3 is highly toxic.
- No effect on the separation.
- Highly recommend filtering mobile phases (0.45 or 0.2 µm) to reduce bacterial growth.



Reproducible Separations



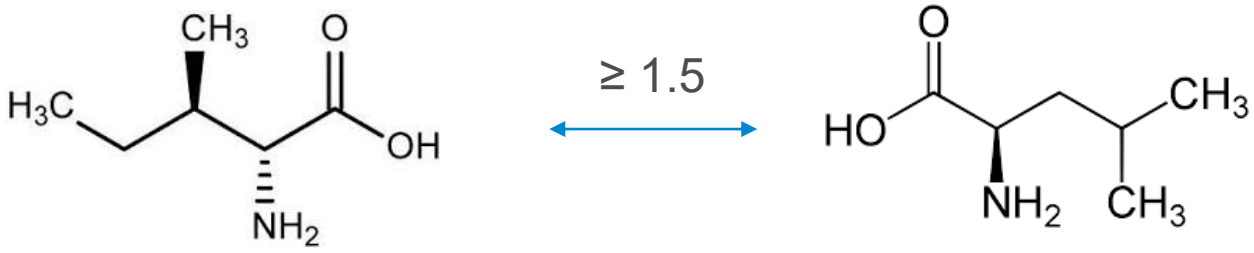
- Retention time %RSD mostly under 1%
- Peak area %RSD mostly under 3%

Amino Acids	RT RSD (%)	Area RSD (%)
1. Aspartic acid	1.270	1.066
2. Glutamic acid	0.973	1.85
3. Asparagine	0.605	1.79
4. Serine	0.629	1.82
5. Glutamine	0.470	1.56
6. Histidine	0.430	1.22
7. Glycine	0.477	1.92
8. Threonine	0.440	1.95
9. Arginine	0.251	2.15
10. Alanine	0.280	3.06
11. Tyrosine	0.128	1.65
12. Cystine	0.067	1.9
13. Valine	0.084	2.47
14. Methionine	0.073	1.82
15. Norvaline	0.073	1.72
16. Tryptophan	0.054	1.57
17. Phenylalanine	0.051	1.66
18. Isoleucine	0.047	1.72
19. Leucine	0.03	1.7
20. Lysine	0.028	1.66
21. Hydroxyproline	0.021	4.13
22. Sarcosine	0.026	1.15
23. Proline	0.021	4.36

System suitability as per European Pharmacopoeia (Ph.Eur)

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid and is produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of not less than 1.5



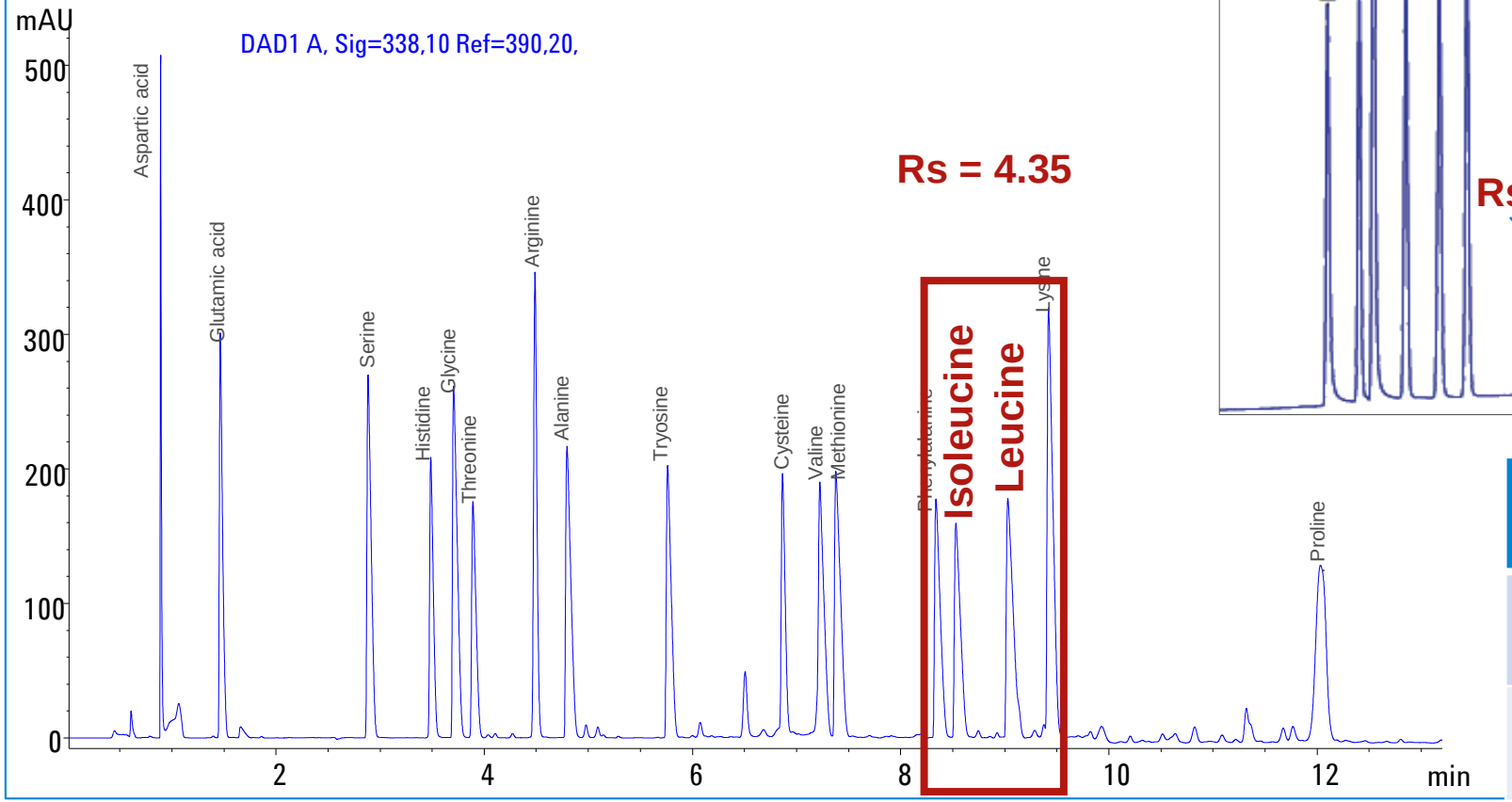
Isoleucine

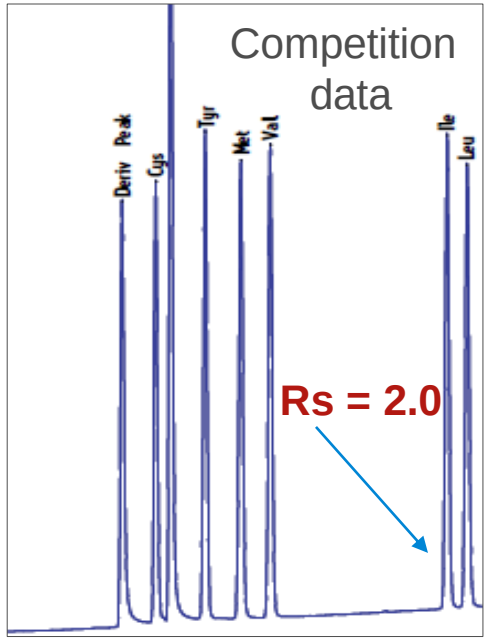
Leucine

Ref: Ph.Eur.9.0 (2.2.56) Amino Acid Analysis

Ample Resolution of Leucine & Isoleucine

Baseline resolution of isoleucine and leucine (Rs = 4.35) meeting the regulatory requirements for these components.

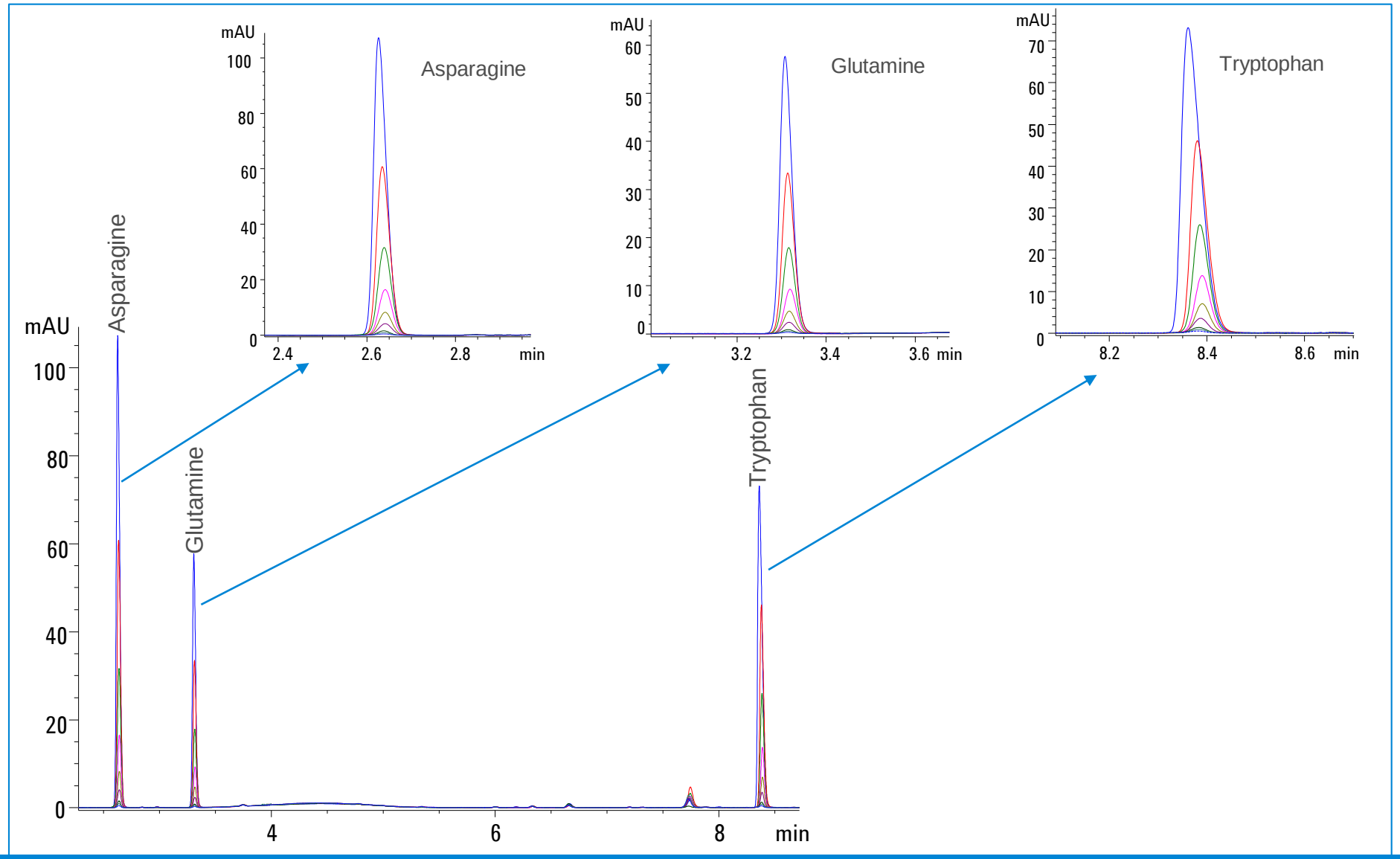




Protein hydrolysate sample

Column	Leu/Ile Rs (≥ 1.5)
Agilent AdvanceBio AAA 4.6 × 100 mm	4.5
Agilent AdvanceBio AAA 3 × 100 mm	4.6

Linearity and Limits of Detection & Quantitation



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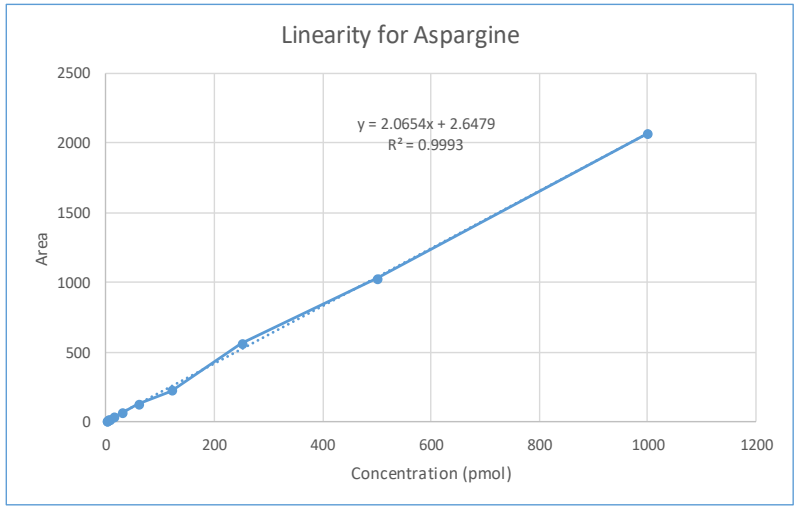
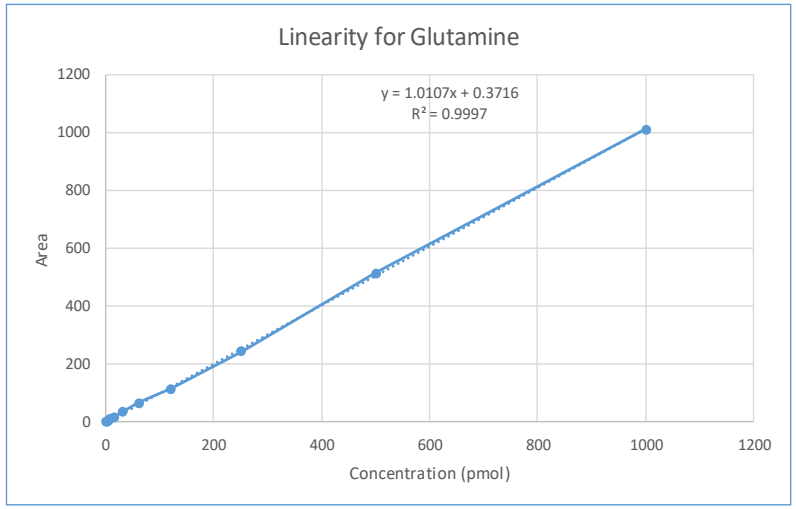
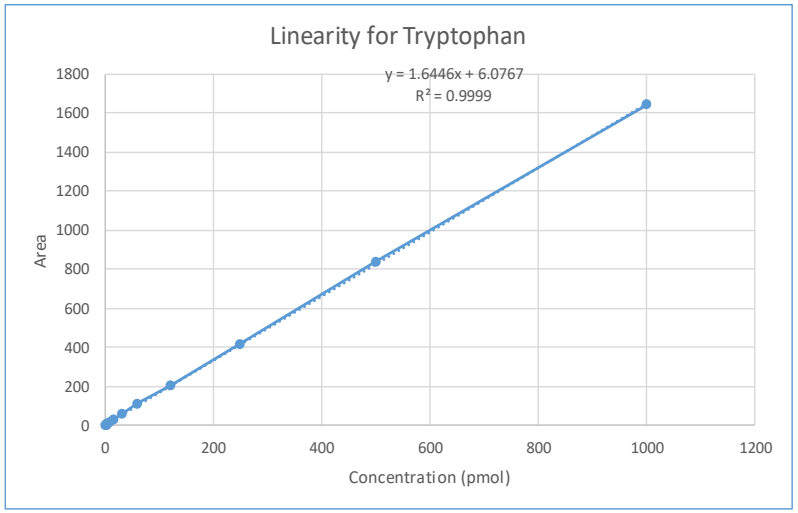




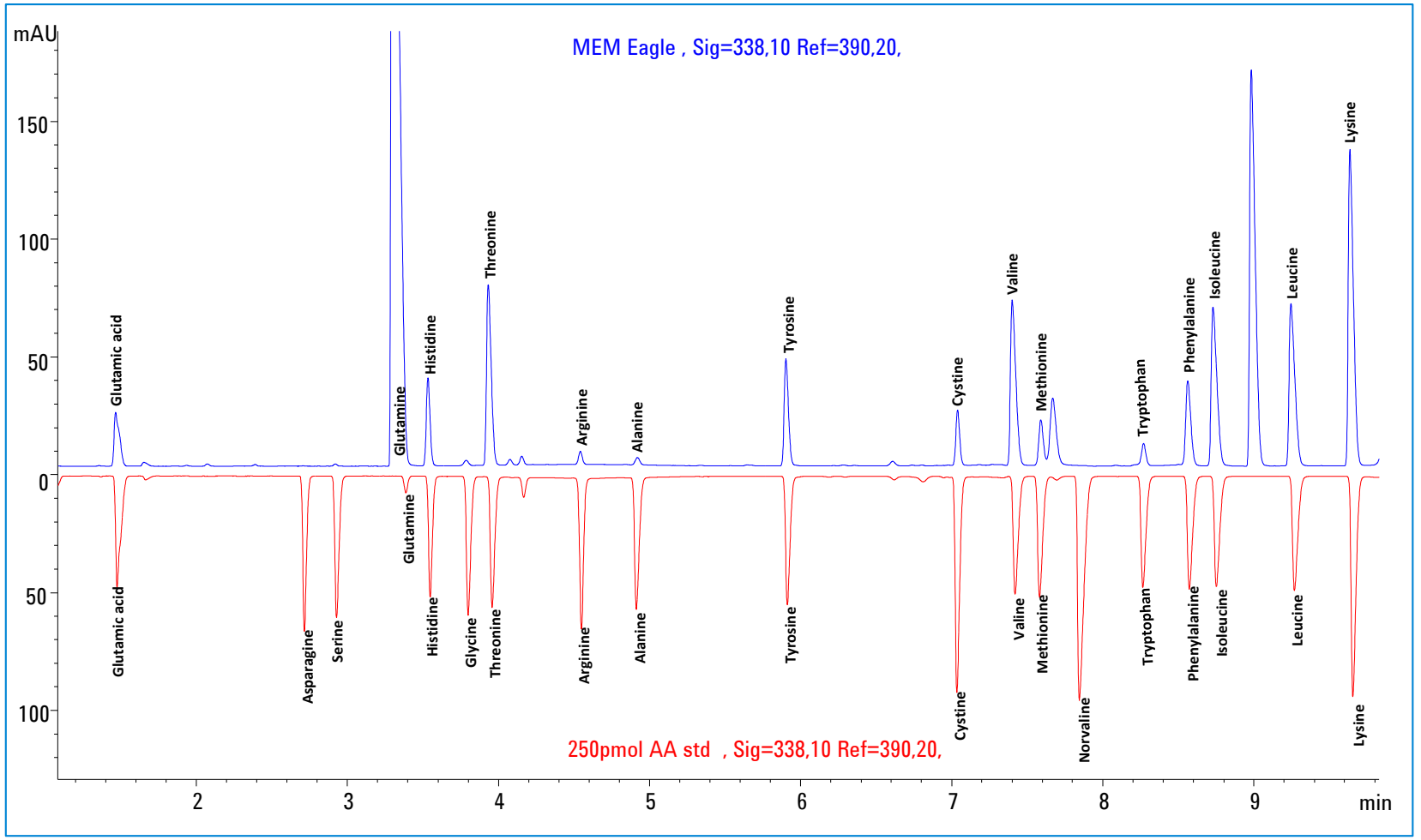


Concentration (pmol)	S/N ratio
Asparagine	
0.9 (LOD)	5.3
1.9 (LOQ)	10.8
Glutamine	
0.9 (LOD)	3.0
3.8 (LOQ)	13.8
Tryptophan	
0.9 (LOD)	4.5
3.8 (LOQ)	20.5

S/N >3.0 = LOD
S/N > 10 = LOQ

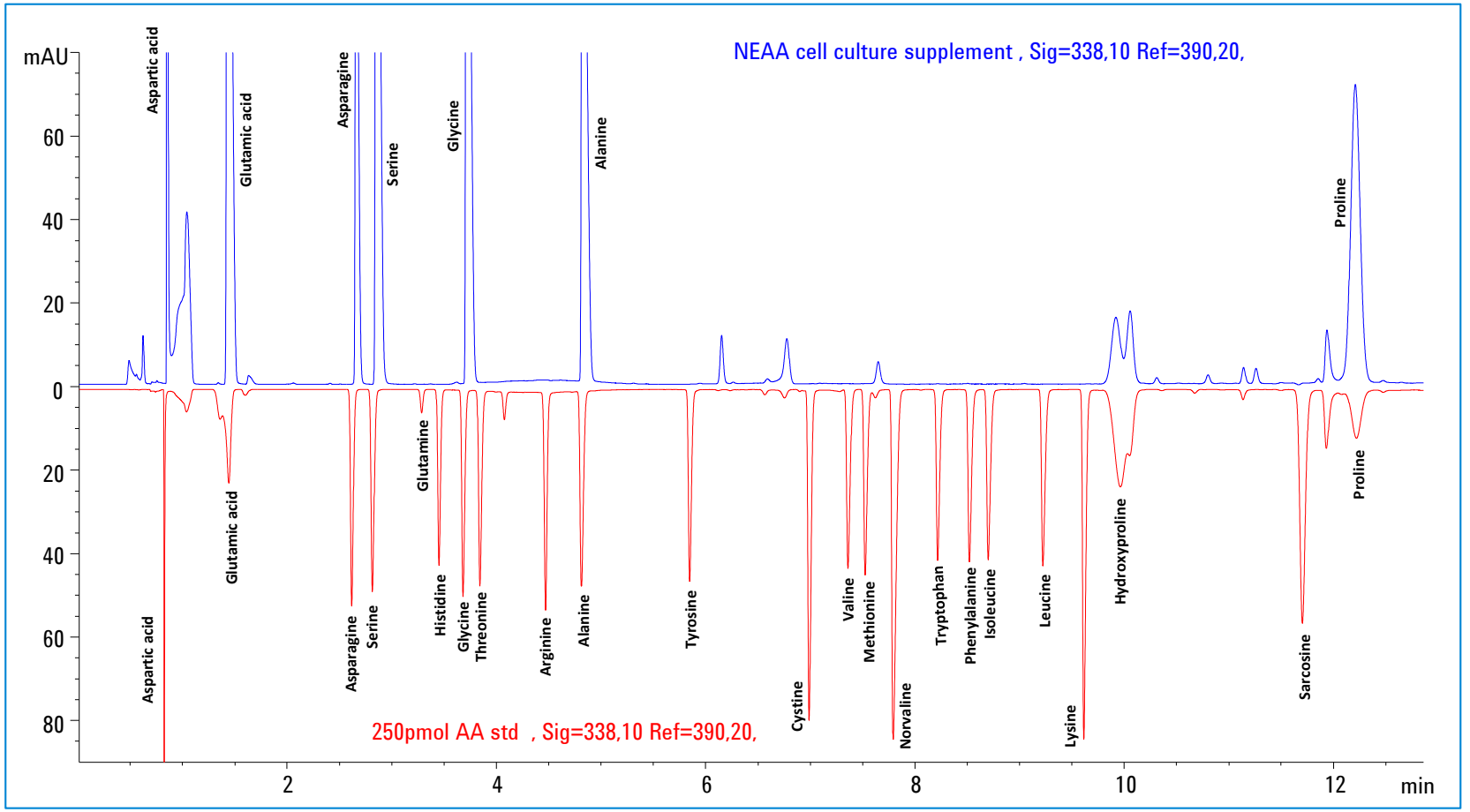
AAA of Cell Culture Media – MEM

L-Arginine, L-Cystine, L-Glutamine, L-Histidine, L-Isoleucine, L- Leucine, L-Lysine, L-Methionine, L- Phenylalanine, L-Threonine, L-Tryptophan, L- Tyrosine and L-Valine, L-Glutamic acid



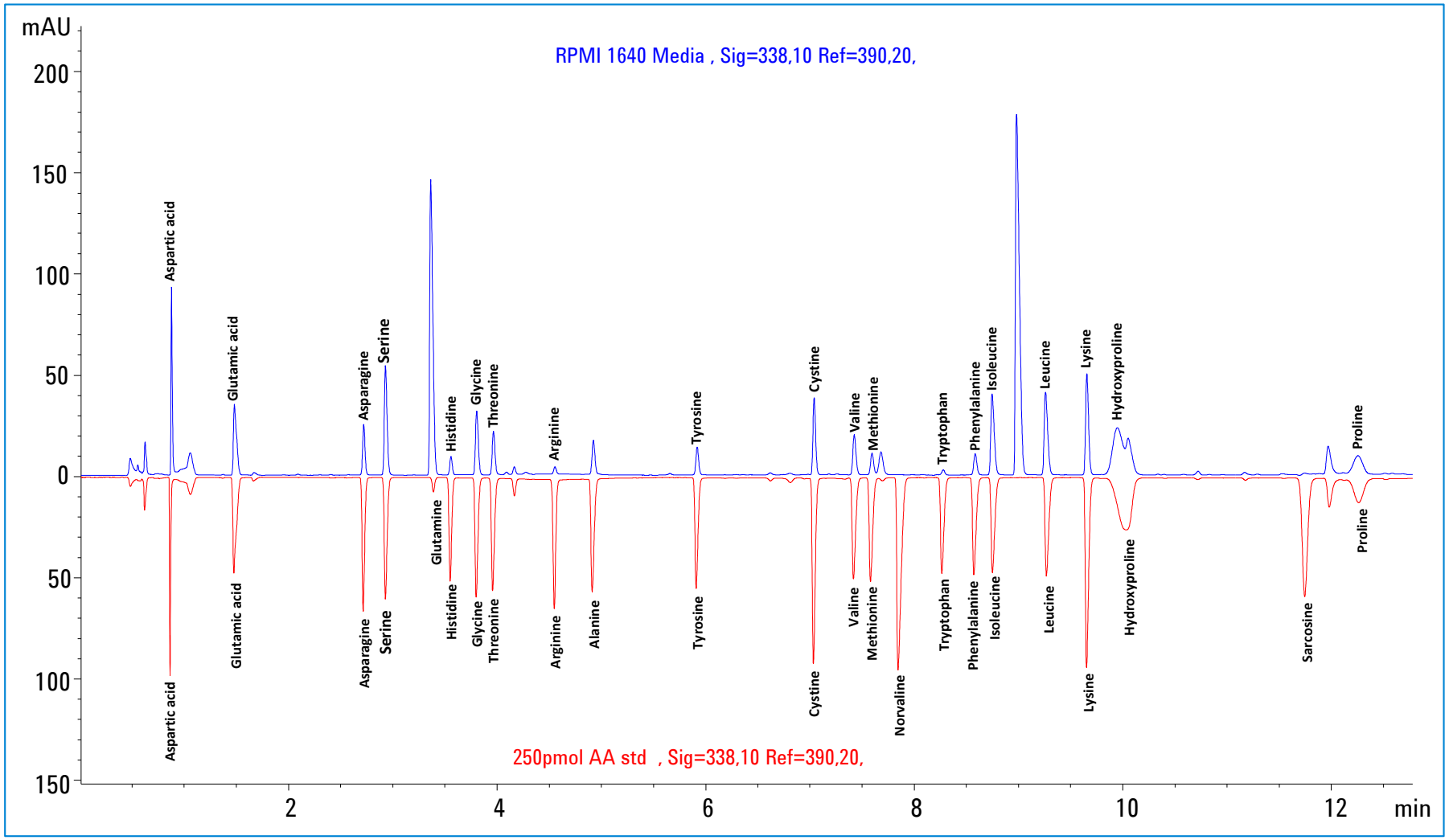
AAA of Cell Culture Media – NEAA cell culture supplement

L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, Glycine, L-Proline and L-Serine

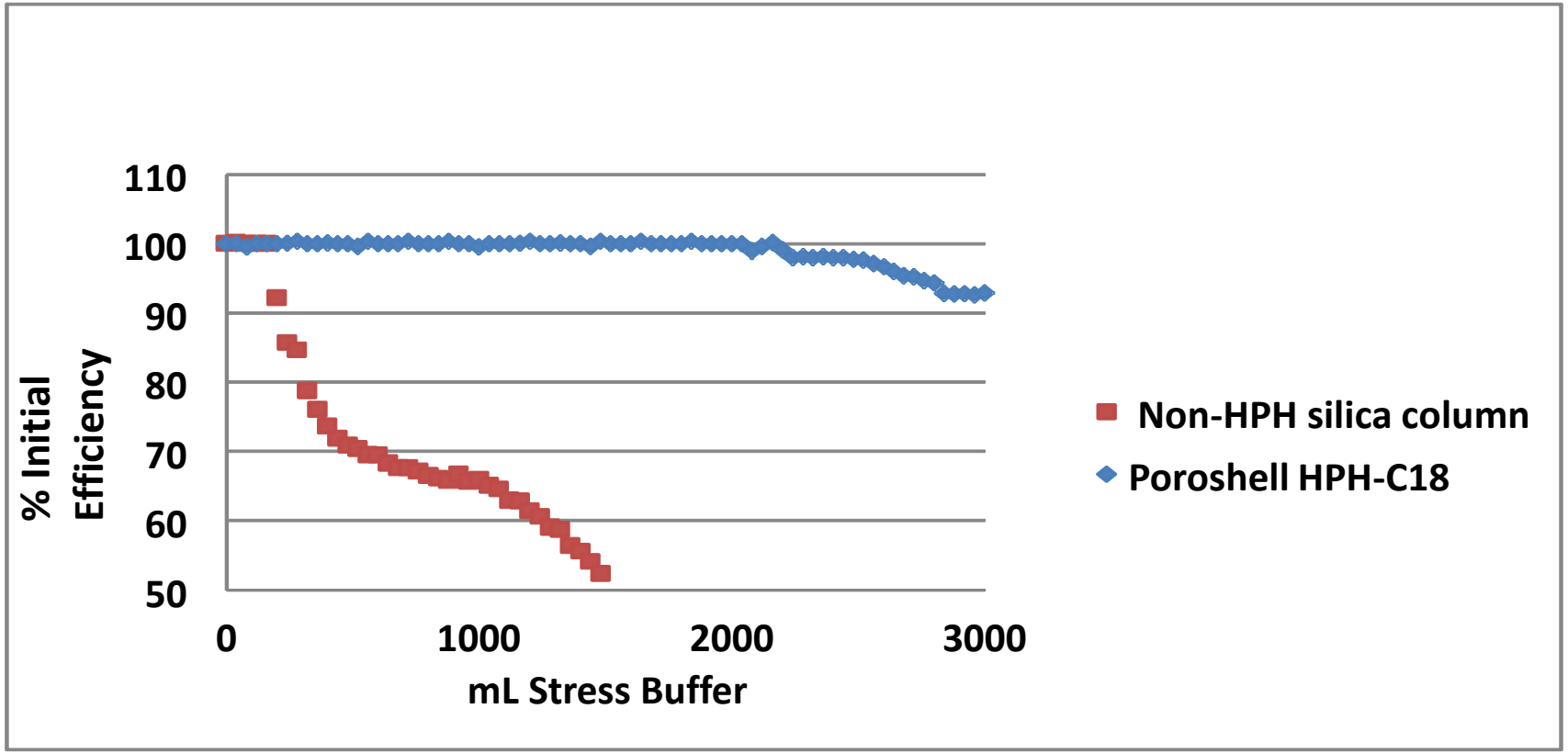


AAA of Cell Culture Media – RPMI 1640

L-Arginine, L-Glutamic acid, L-Asparagine, L-Cystine, Glycine, L-Histidine, Hydroxy-L-Proline, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine and L-Valine



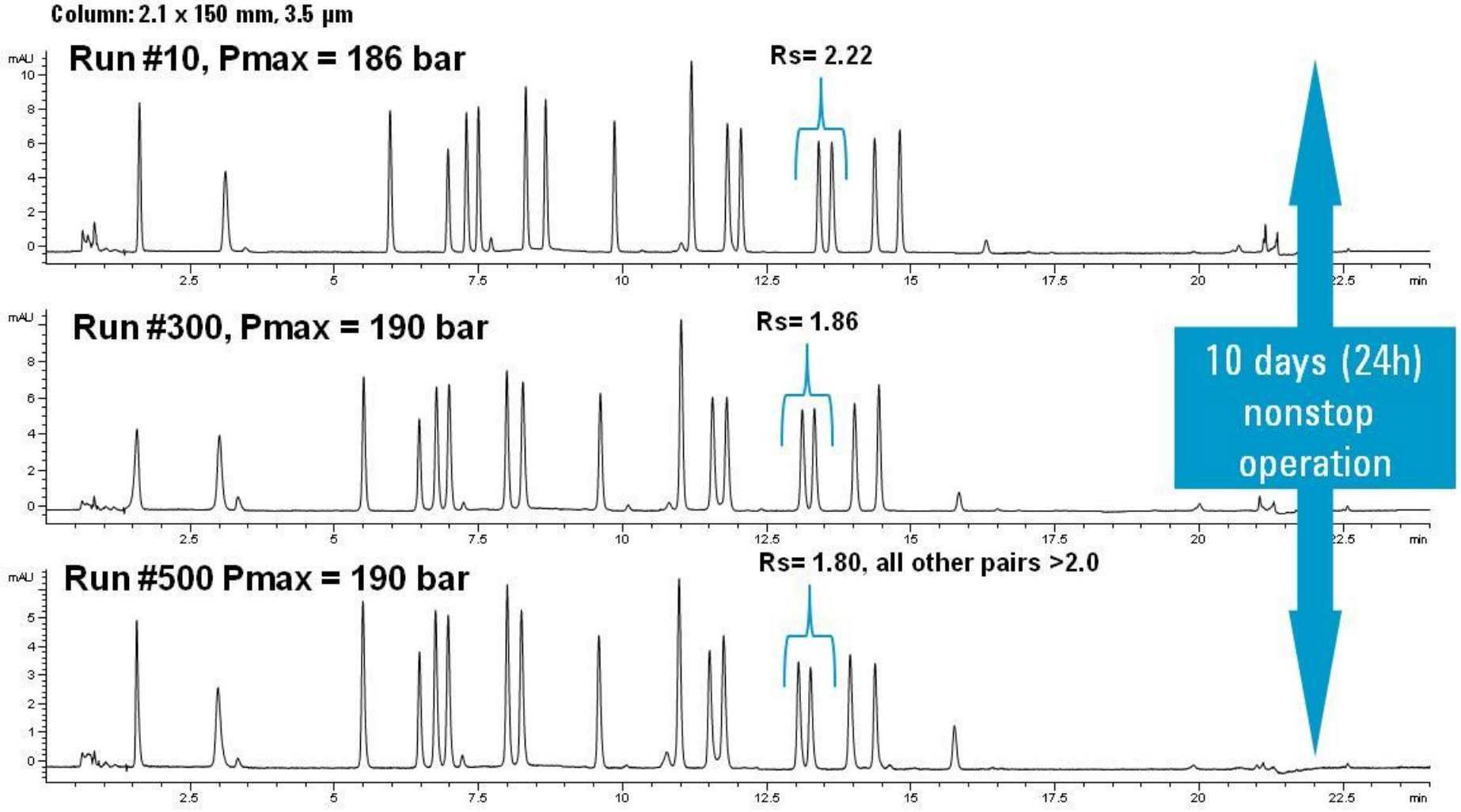
Poroshell HPH C18 last longer in phosphate buffer



Lifetime of SPP columns in phosphate buffer, **pH 8**, at elevated temperature. Mobile phase: Premixed 60% 30 mM sodium phosphate buffer at pH 8 and 40% acetonitrile; Flow rate 0.4 mL/min; UV absorbance 254 nm; 65 °C; Columns: 2.1 x 50 mm, 2.7 µm; Analyte: Naphthalene.

Traditional Lifetime Testing

Using conventional column testing Eclipse Plus C18 column test looks great and will perform well for most customers. However it is not a realistic test for most lab workflow.



Improvements in Lifetime Testing for AA Analysis

Past lifetime testing consisted of 500 injections without stopping

- This test, while it could differentiate long life columns, was not indicative of a customer workflow

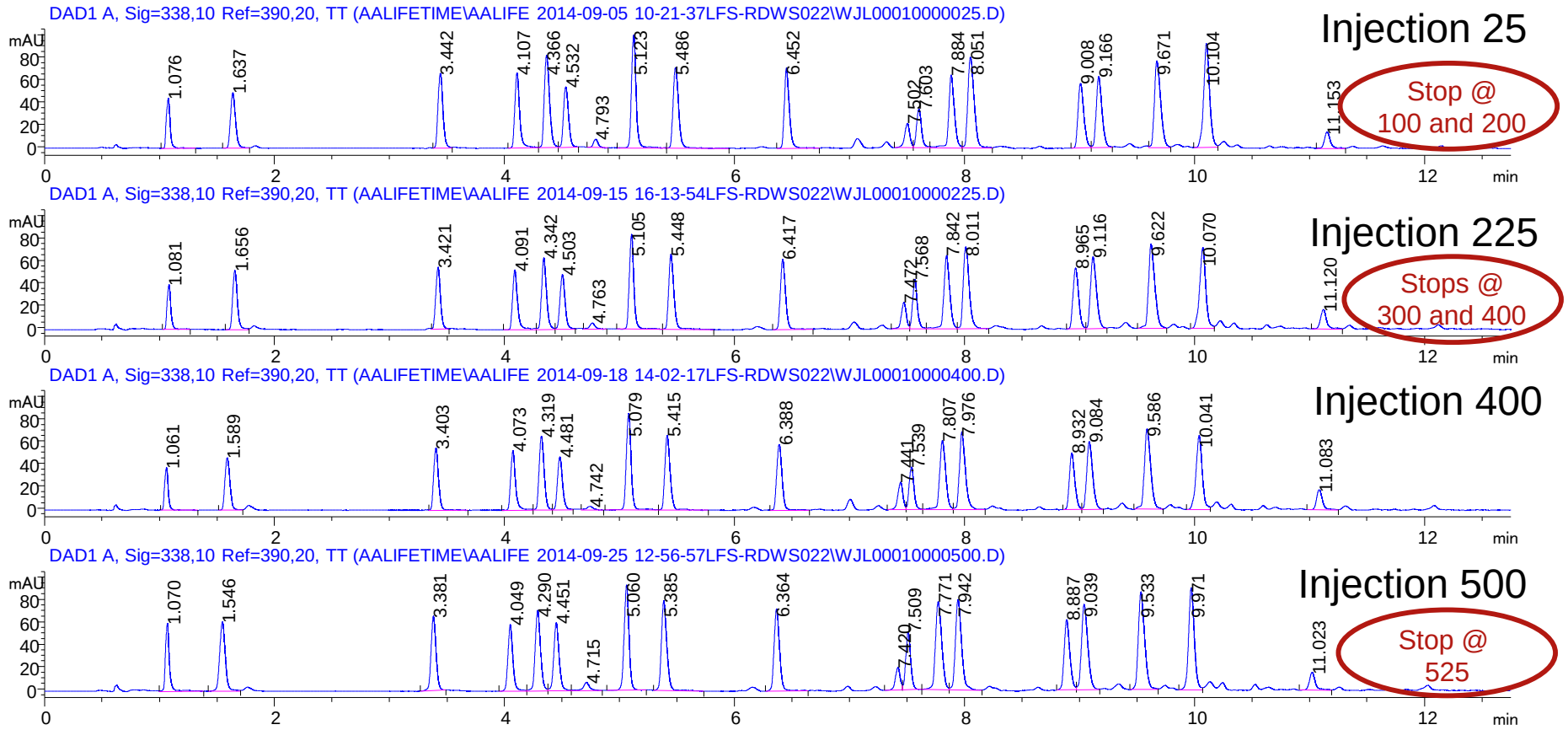
Customers will typically run ~20 to 50 or 100 samples, then stop, and resume a day or more later

- This is much more stressful on the column, and will cause columns to fail sooner versus running 500 injections straight through.

The lifetime testing for the Poroshell HPH-C18 was adapted to more customer focused workflow, with breaks between series of injections to give a true indicator of the lifetime ni the customers' hands

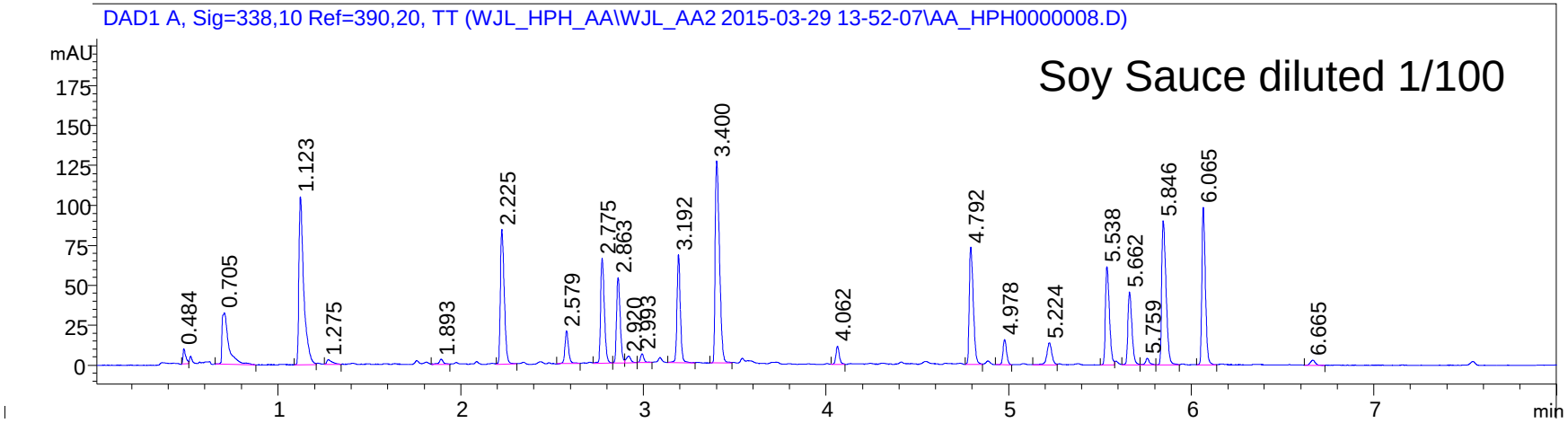
Test like a customer runs: 100 injections (3 days), Store (4 days)

Most labs run a batch of samples (25-100) and then shut off for a few days so we did too



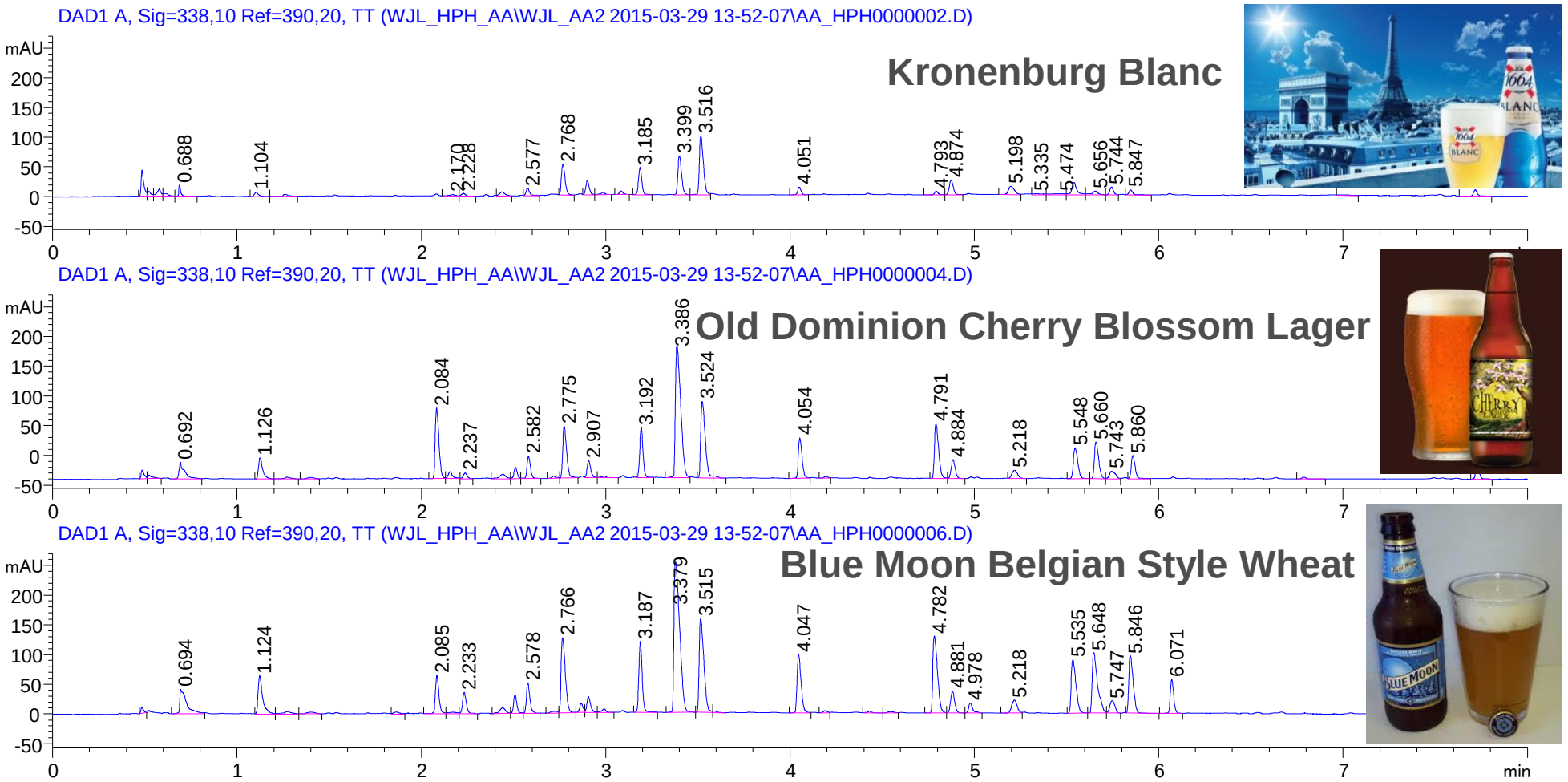
Looks great after 500 injections run 100 injections shut down 4 days repeat

Amino Acid Analysis in Fermentation Applications



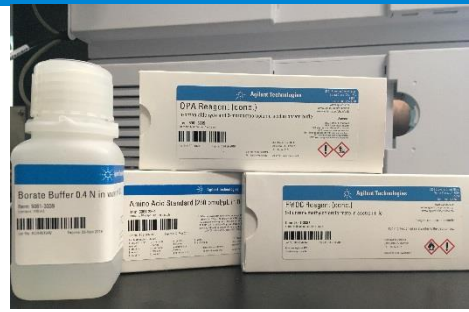
Many other foods (such as soy sauce) and pharmaceuticals that are produced using fermentation processes are monitored by AAA

Amino Acids Analysis for Batch Comparison



Quantity and diversity of amino acids is evident, can be used to monitor reactions and compare batches

Tips & Tricks - Maintenance



- Replace derivatization reagent, borate buffer, amino acid standard daily
- Recalibrate for retention times and response factors daily
- Check column and guard column performance by following specs (Rs for 2 pairs of AA)
- Replace mobile phase A and B with fresh ones every other day
- Exchange guard column if high back pressure develops
- Avoid using MAX mixing speed during sample derivatization
  - The max speed on newer LCs is much faster than older LCs (1100s, 1200s), and can cause excessive wear on the autosampler.

Tips & Tricks - Troubleshooting

Poor chromatographic resolution?

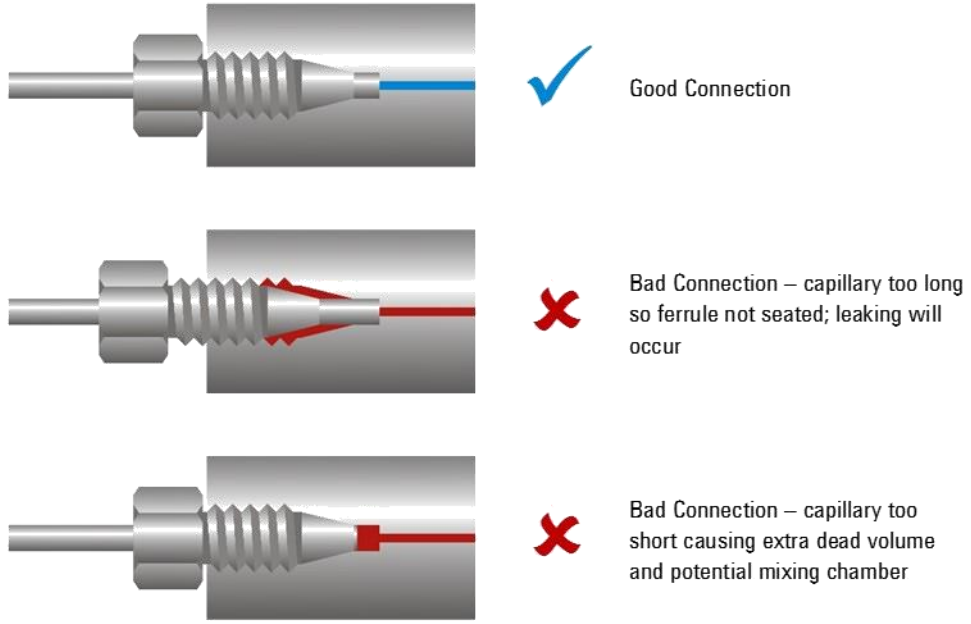
- Cell culture media does not require any sample preparation, however appropriate dilutions have to be made to suit detector response
- In all cases, use the low-volume heat exchanger with short red tubing to minimize extra column volume
- Ensure proper connections
- Damaged guard or analytical column

Low intensity chromatogram?

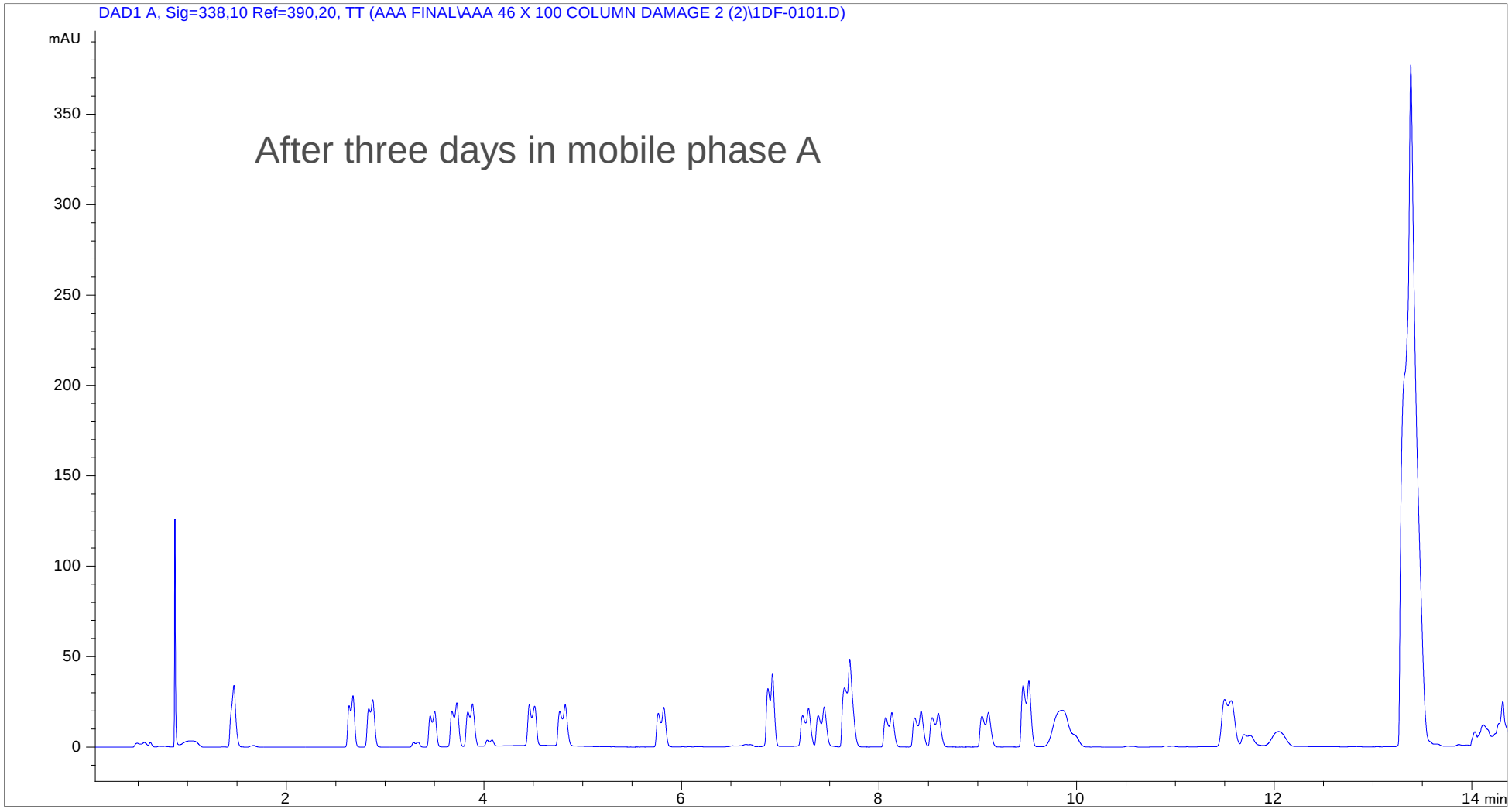
- OPA/FMOC reagent deteriorated
- Air bubble in vial insert

Column storage?

- Never leave the column in mobile phase A even if it's just overnight
- For short term always store the column in mobile phase B
- For long term, store column in 50/50 acetonitrile/H_2O

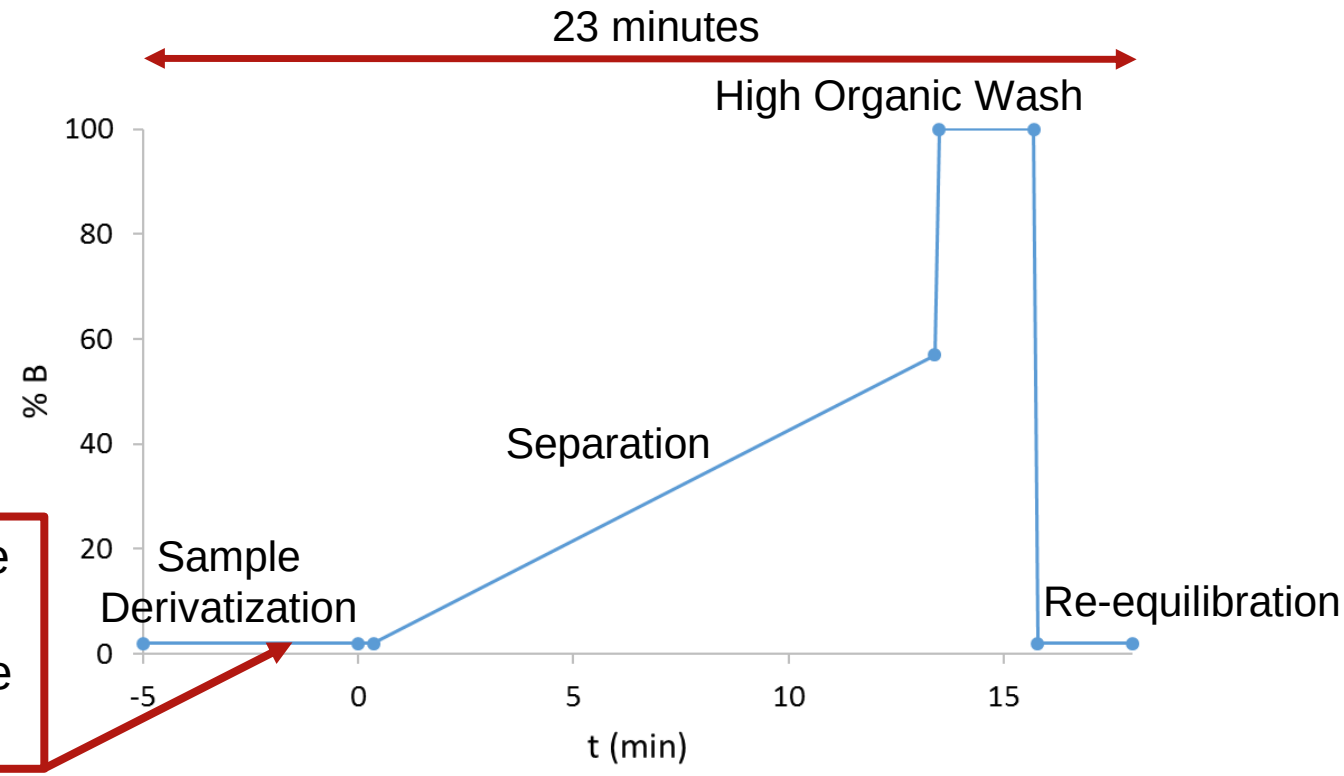


Damaged Column



Tips & Tricks – Saving Time

- After Injection 1, during the sample derivatization for Injection 2, the initial mobile phase condition is flowing through the LC and the column
- Save time by shortening the length of the re-equilibration time at the end of the method by the amount of time consumed by sample derivatization



Initial mobile phase conditions are running through the column!

Time (min)	%B
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	stop

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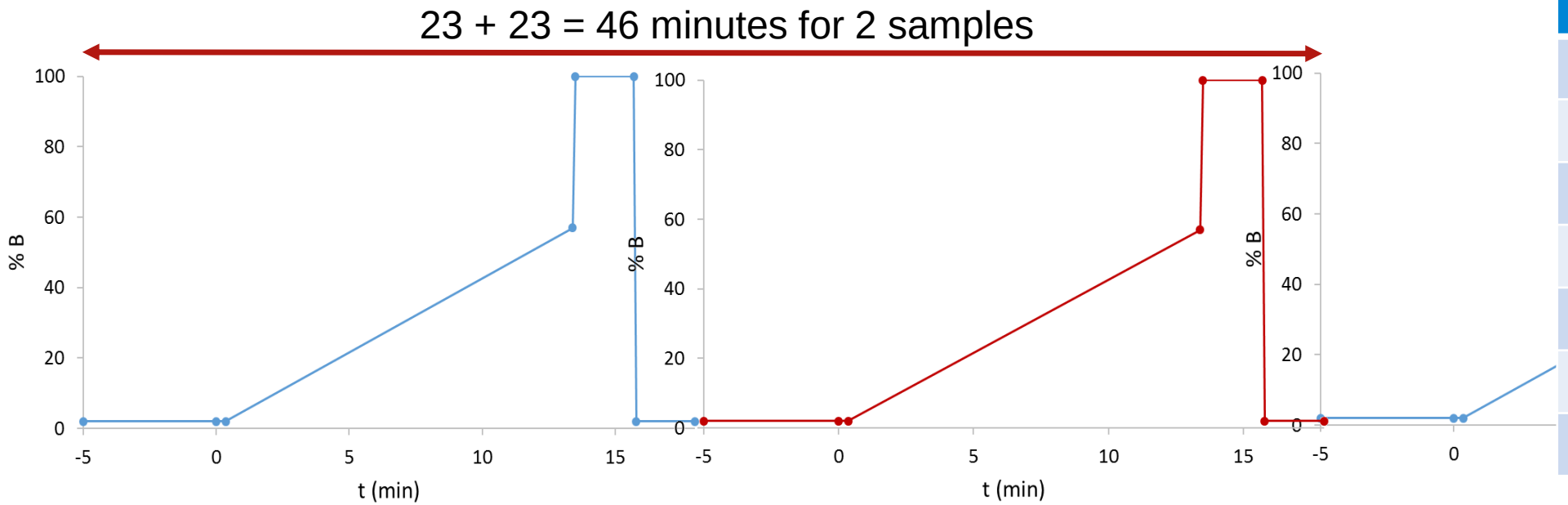

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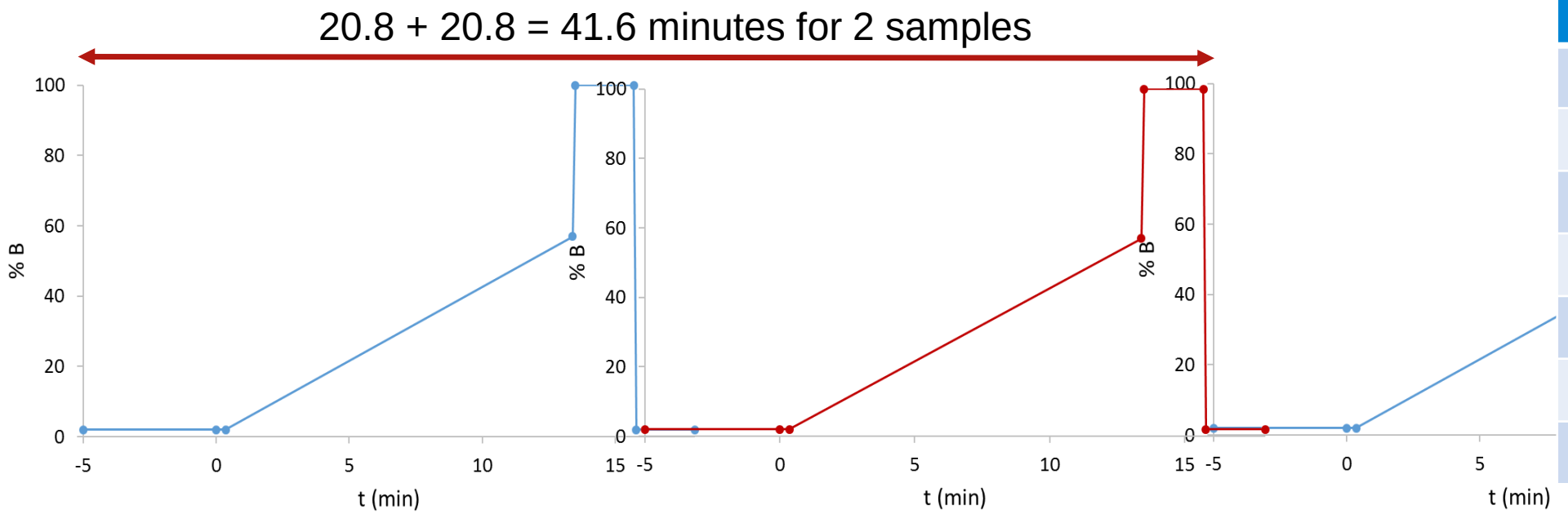

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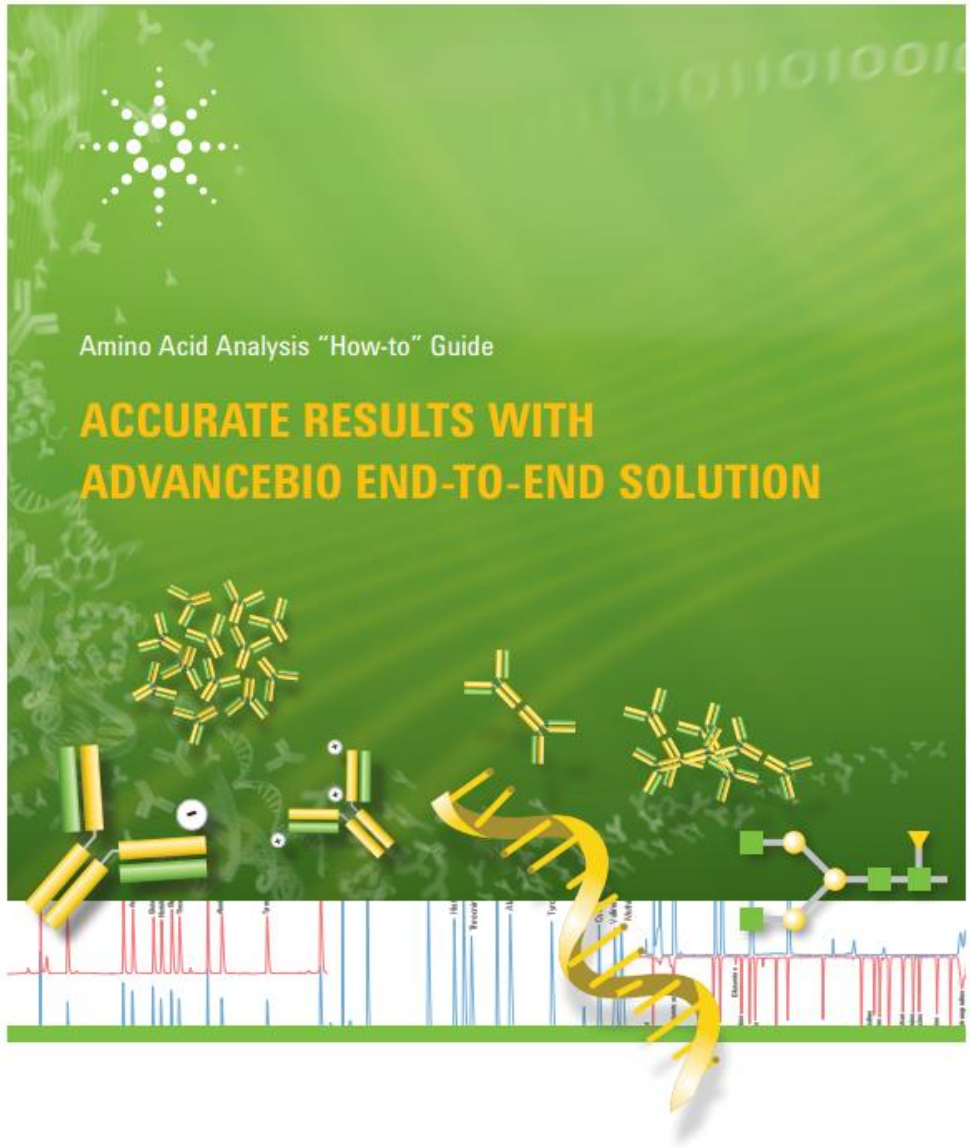
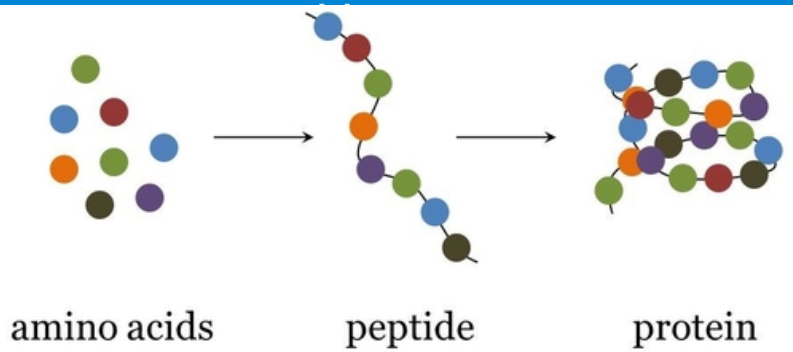
How-To Guide – Step by Step Instructions & Method Details



All the information you need

Document number 5991-7694EN

Summary



- We used the Agilent AdvanceBio AAA solution for the automated online derivatization and separation of amino acids.
- Area and RT precision of the method were excellent, and Leu/Ile resolution met the system suitability requirement.
- Linearity curves with ten standard concentrations of three amino acids, ranging from 0.9pmol to 1nmol, had excellent coefficient of linearity values, indicating that the method was quantitative and accurate.
- The LOD and LOQ for the amino acids were 0.9pmol and 3.8 pmol respectively, indicating that the method was sensitive.
- This method was able to separate and detect, amino acids from a variety of samples, including cell culture media, protein hydrolysate, and fermentation reactions.