Analysis of Amino Acids by HPLC

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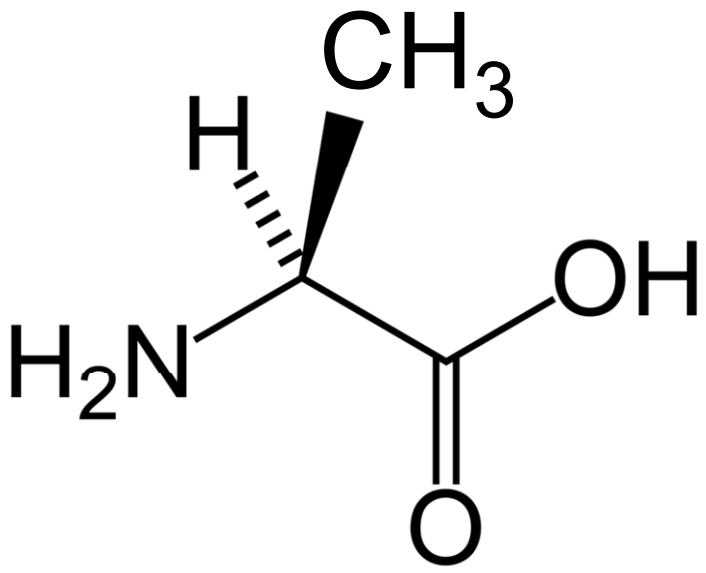
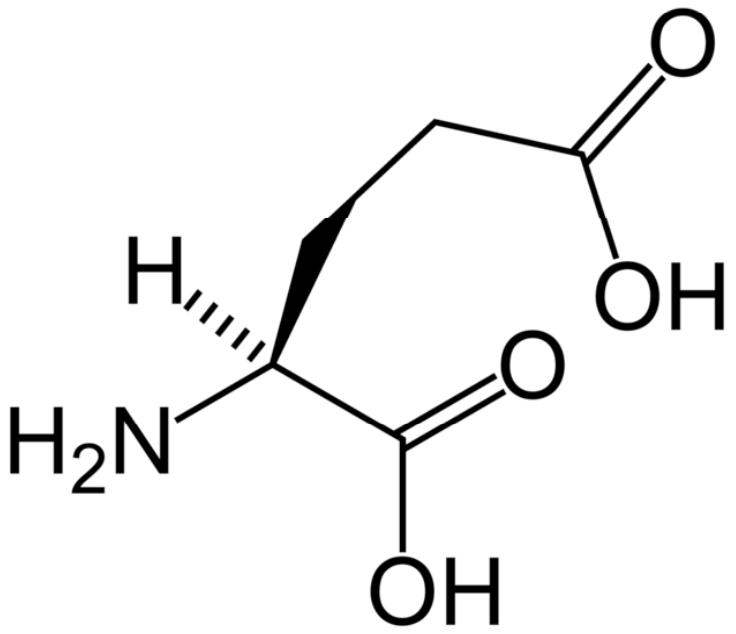
Outline

- Amino Acids – Structure, Chemistry
- Separation Considerations
  - Challenges
  - Instrumentation
- Derivatization – OPA, FMOC
- Overview of Separations
- Examples

Amino Acids – Structure, Chemistry

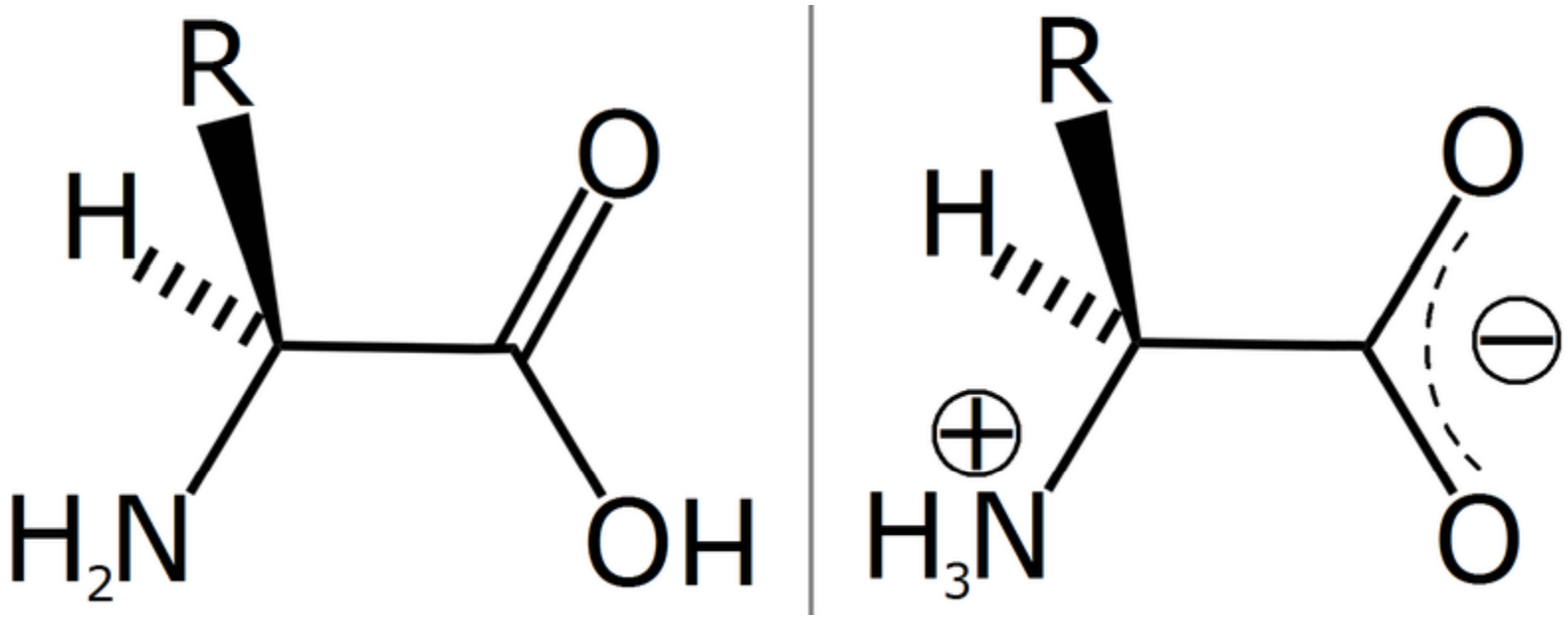


Alanine (Ala)



Glutamic Acid (Glu)

Amino Acids – Zwitterionic

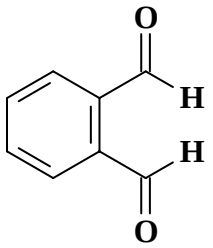


Separation Considerations

- Zwitterions - poor solubility near iso-electric point
- Most have poor UV absorbance
- Derivatization – OPA, FMOC
  - •Reduce polarity – increases retention in reversed-phase chromatography
  - •Improve sensitivity – UV, Fluorescence
- Detector; DAD, FLD, MS, ELSD

Ortho Phthalaldehyde (OPA) and Fluorenylmethoxy chloroformate (FMOC) Reactions with Amines

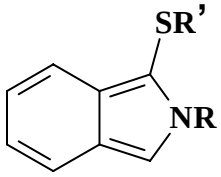
OPA



+RNH_2

R'SH

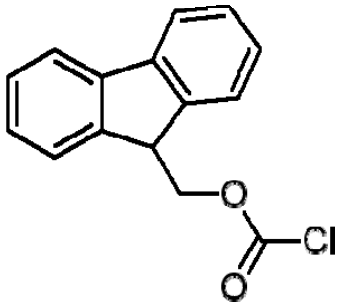
Room Temperature



Non-fluorescent
Does not absorb at 338nm

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

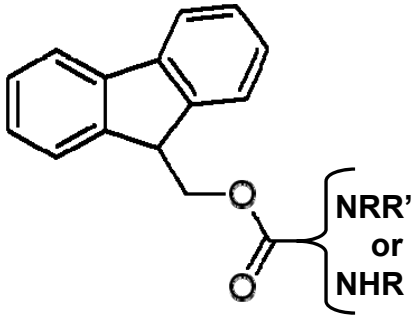
FMOC



+ { RR'NH or RNH_2

- HCl

Room Temperature



Fluorescent
Absorbs at 262nm and
Fluorescences at 324nm

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

Names and Order of Elution for OPA and FMOC Derivatives of Amino Acids

Peak #	AA Name	AA Abbreviation	Derivative Type
1	Aspartic Acid	ASP	OPA
2	Glutamic Acid	GLU	OPA
3	Asparagine	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	Cystine	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	Sarcosine*	SAR	FMOC
23	Proline	PRO	FMOC

* Internal Standard

Agilent AAA Methods - They've Evolved

- Automated Amino Acid Analysis – AminoQuant I & II (1987)
  - 1090
  - 1100, Pub. No. 5968-5658E
- Eclipse AAA
  - Columns
    - 993400-902, 4.6x150mm, 5um
    - 963400-902, 4.6x150mm, 3.5um
    - 966400-902, 3.0x150mm, 3.5um
  - Pub. No. 5980-1193E
- Eclipse Plus
  - Column Options
  - Application Notes
    - 5989-6279EN
    - 5990-4547EN
    - 5989-6297EN
    - 5990-3283EN

Agilent's Solutions for Amino Acid Analysis A Closer Look

Agilent has one basic type of chemistry for amino acid analysis – OPA/FMOC derivatization.

We have 3 columns and methods that can be used to achieve separations of amino acids.

- AminoQuant – this is our oldest methodology
  - It will go out of support due to supply inconsistencies
- Eclipse AAA – this method uses a specially tested Eclipse XDB-C18 column and modified mobile phase for improved resolution
  - This method is not set-up for anything other than 4.6mm ID columns
  - Guard columns are needed for maximum lifetime
- Eclipse Plus AAA
  - Has solutions for all column dimensions and works on multiple LC's
  - Kits have not been created yet

AminoQuant Amino Acid Method

Based on Hypersil ODS material that is specially treated.

Currently we have to adjust the mobile phase and provide directions to customers with each batch.

This is not good for regulated methods.

These columns will be made obsolete in the future.

AminoQuant Method

AminoQuant
serial no.: USOE007668
part no.: 79916AA-572
batch no.: 51205964
L x i.d. = 200 x 2.1 mm; dp = 5 µm

Eluent:A: 500 ml 20 mM Sodium acetate + 2 mg EDTA + 0.018% Triethylamine(v/v) adjusted to pH 7.2 with Acetic acid + 0.3% Tetrahydrofuran
B:100 ml 20 mM Sodiumacetate adjusted to pH7.2 with Acetic acid + 200 ml Methanol + 200 ml Acetonitrile
Gradient:

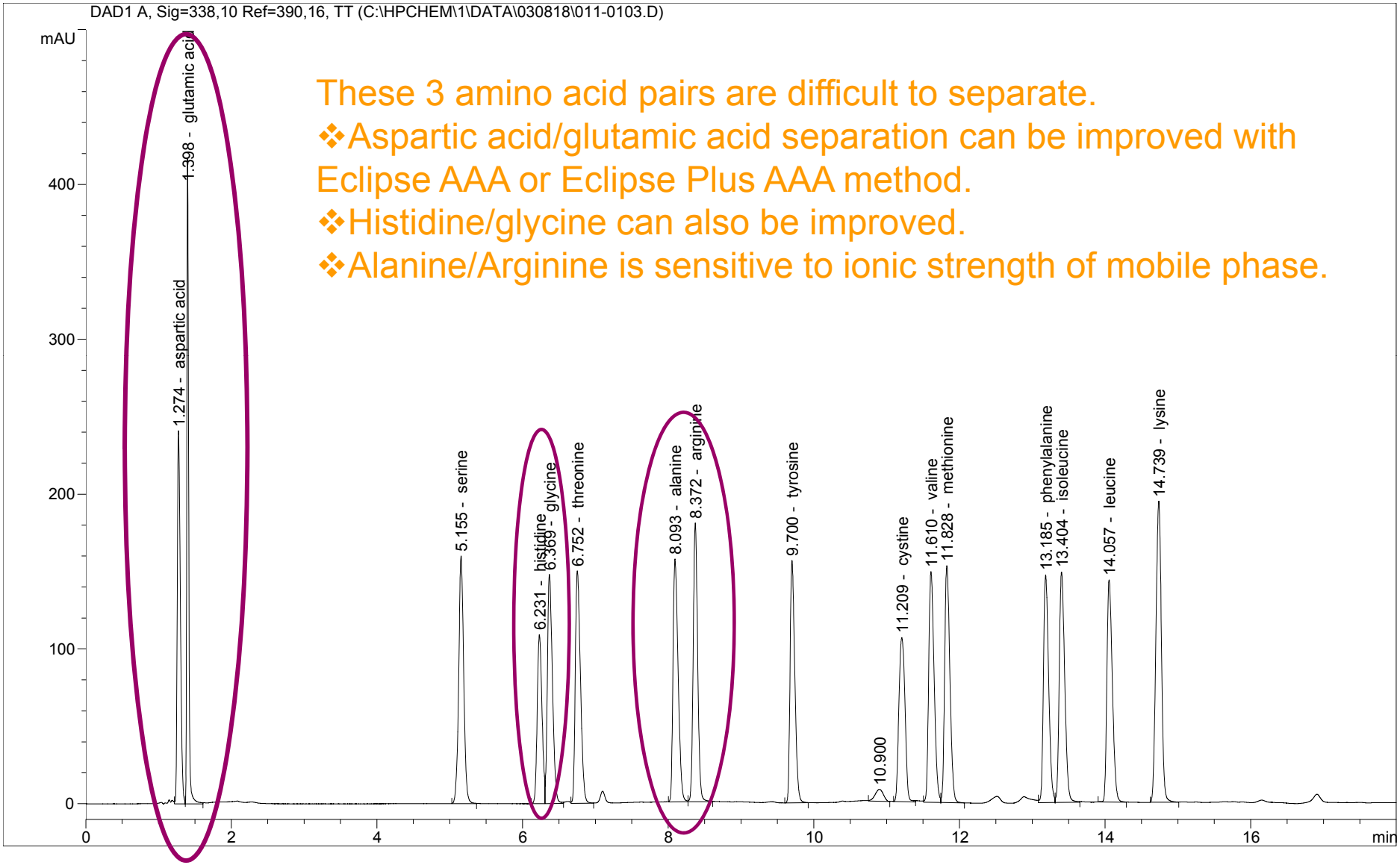
Time (min)	% A	% B	flow
0	100	0	0.45
17.0	40	60	
18.0	0	100	0.45
18.1	0	100	0.8
23.9	0	100	
24.0	0	100	0.8
25.0	100	0	0.45

Sample:
Amino acid standard [part no. 5061-3330]

name	abbr.
L-Aspartaic acid	ASP
L-Glutamic acid	GLU
LSerine	SER
L-Histidine	HIS
Glycine	GLY
L-Threonine	THR
LArginine	ARG
L-Alanine	ALA
L-Tyrosine	TYR
L-Cystine	CY2
L-Valine	VAL
L-Methionine	MET
L-Phenylalanine	PHE
L-Isoleucine	ILE
L-Leucine	LEU
I-Lysine	LYS
L-Proline	PRO

Flow: 0.45 ml/min
Temperature: 40 °C
Injection: injection program, including derivatisation steps with OPA and FMOC.
injected mixture contains 1 µl AA sample.

Example of Amino Acid Separation on AminoQuant



These 3 amino acid pairs are difficult to separate.

- Aspartic acid/glutamic acid separation can be improved with Eclipse AAA or Eclipse Plus AAA method.
- Histidine/glycine can also be improved.
- Alanine/Arginine is sensitive to ionic strength of mobile phase.

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Eclipse AAA Method

Primary goal of Eclipse AAA method was to provide better resolution of the critical pairs in the AminoQuant method.

Secondary goal was to provide options for a faster separation.

An application/technical note (Pub no. 5980-1193) and detailed instructions (Agilent ZORBAX Eclipse AAA Instructions for Use, Pub no. 5980-3088EN, June 2008) are available.

The method has been in place for a number of years and is reliable.

It was originally written for an 1100 and the autosampler derivatization protocol has been rewritten for the 1200.

Assumes a binary instrument for optimum results.

Eclipse AAA Method

Column: ZORBAX Eclipse-AAA; 3.5µm; L x i.d.=150 x 4.6 mm [USXH001289]
Eluent: A: 40 mM Phosphate buffer **pH 7.8** B: Methanol /Acetonitrile/Water = 45/45/10
Gradient:

Time (min)	%B
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
26	0

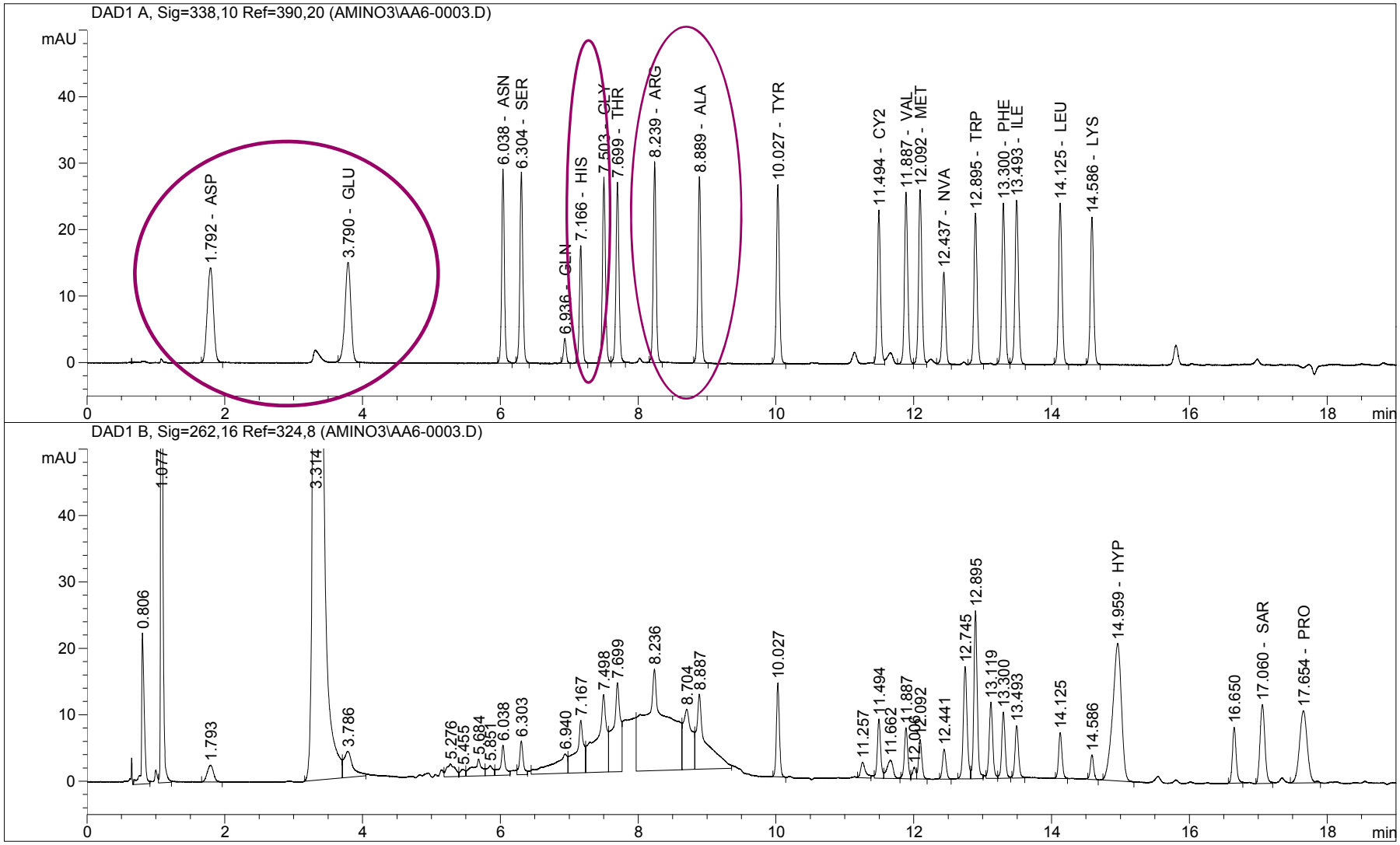
Flow: 2.0 ml/min
Temperature: 40 °C
Injection: injection program, including derivatisation steps with OPA and FMOC, injected mixture contains 0.5 µl AA sample.
Detection: DAD
signal A: λ = 338 nm, bw = 10 nm; reference = 390 nm, bw = 20 nm (for OPA-amino acids)
signal B: λ = 262 nm, bw = 16 nm; reference = 324 nm, bw = 8 nm(for FMOC-amino acids)
FLD time = 0 min: Ex/Em = 340/450 nm, gain = 10
time = 15 min: Ex/Em = 266/305 nm, gain = 9

Amino Acids in Eclipse AAA Method

Amino acid standard [part no. 5061-3331] + supplement amino acid kit [part no. 5062-2478]

name	abbr.	Concentration (nmol/µl)
Aspartate	ASP	0.9
Glutamate	GLU	0.9
Asparagine	ASN	1.8
Serine	SER	0.9
Glutamine	GLN	1.8
Histidine	HIS	0.9
Glycine	GLY	0.9
Threonine	THR	0.9
Arginine	ARG	0.9
Alanine	ALA	0.9
Tyrosine	TYR	0.9
Cystine	CY2	0.9
Valine	VAL	0.9
Methionine	MET	0.9
Norvaline	NVA	0.5
Tryptophan	TRP	1.8
Phenylalanine	PHE	0.9
Isoleusine	ILE	0.9
Leusine	LEU	0.9
Lysine	LYS	0.9
Hydroxiproline	HYP	1.8
Sarcosine	SAR	0.5
Proline	PRO	0.9

Eclipse AAA Method Chromatograms (UV and FLD)



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Eclipse AAA Method

The same 3 pairs of amino acids that are poorly separated or have problems due to mobile phase ionic strength on the AminoQuant column are better separated by the Eclipse AAA method.

The pH of the mobile phase is higher for this separation.

Therefore a guard column is strongly recommended for maximum lifetime with this method.

It should be changed every 100 injections and a total expected lifetime will be about 500 injections.

Eclipse Plus AAA Method

Goal of the Eclipse Plus AAA method is to offer more column options.

This includes sub 2um column choices.

Gives more options to choose between resolution and speed.

This is more critical with more complex samples (possibly more amino acids) and matrices.

Amino Acid Analysis on Eclipse Plus-C18, 2.1, 3.0 or 4.6 x 50mm, 1.8µ Column: Experimental Conditions

AAA on production 1200SL using Eclipse Plus-C18, 2.1 or 4.6 x 50mm, 1.8u
Mobile phase A: 10mM Na_2HPO_4 – 10mM $Na_2B_4O_7$, pH 8.2
5.6gm anhydrous Na_2HPO_4 + 15.2gm $Na_2B_4O_7 \cdot 10H_2O$ in 4L water + 32mg NaN_3
Adjust to ~pH 9 with 6ml concentrated HCl and then small drops until pH 8.2. **Be cautious with strong acids!**
Filter through 0.45µregenerated cellulose membranes (Agilent P/N 3150-0576)
Stable for ~ 2 weeks at room temperature
Mobile phase B: ACN: MeOH: H_2O 45:45:10 by volume
Injection diluent: 1ml Mobile phase A + 15µl concentrated H_3PO_4 in a 1 ml vial. Make this in 100ml batches.
Instrument config:
Pump: no mixer, no pulse dampener, bypass used (at 0.1min after inject command), compressibility settings used: A= 35, B= 80
Flow rate: 0.420ml/min for 2.1mm ID; 0.85ml/min for 3.0mmID; 2.00ml/min for 4.6mm ID

Gradient Timetable:	Time (min)	%B
	0.0	2.0
	1.0	2.0
	7.0	57.0
	7.1	100.0
	8.4	100.0
	8.6	2.0
	Stop time 8.7	

DAD: PW 0.01min; slit 4nm; Stop time 7min (adjust as needed) Cell = 5µl, 6mm flow path (Agilent P/N G1315-60025
338, 10nm; Ref 390, 20nm
262, 16nm; Ref 324, 8nm
338, 10nm; Ref 390, 20nm
230, 16nm; Ref 360,100nm
Timetable Signal C): 0.00 min 338, 10nm; Ref 390, 20nm
5.53 min 262, 16nm; Ref 324, 8nm (adjust as needed; 4.6mm ID transition at ~5.4min)
FLD: PW 0.01min, Stop time 7 min (adjust as needed), never use this detector before another due to fragility of flow cell
Ex 340nm; **Em** 450nm; Filter 390nm (Default filter)
Timetable Signal: 0.00 min **Ex** 340nm, **Em** 450nm; PMT Gain 10 (as needed)
5.53 min **Ex** 260nm, **Em** 325nm; PMT Gain 10 (as needed; 4.6mm ID transition at ~5.4min)

Amino Acid Analysis on Eclipse Plus-C18, 2.1, 3.0 or 4.6 x 50mm, 1.8µ Column: Conditions cont'd

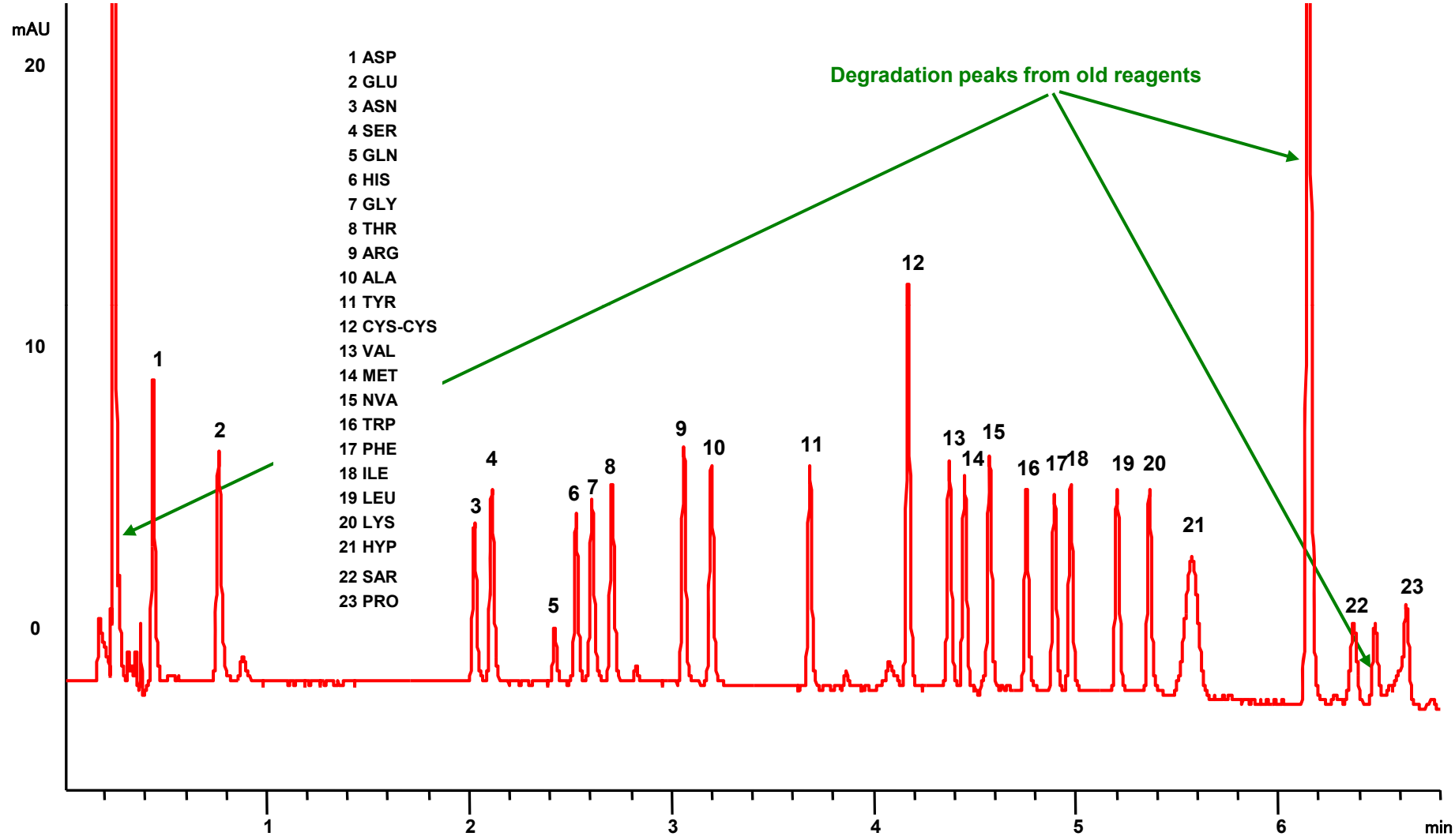
TCC: used with low dispersion kit installed; T = 40°C for column side, 35°C for exit side. Low dispersion kit used on both sides.
WPS: Def. vol set to 0.5ul, def speed used throughout injector program is 200ul/min

Injector program:

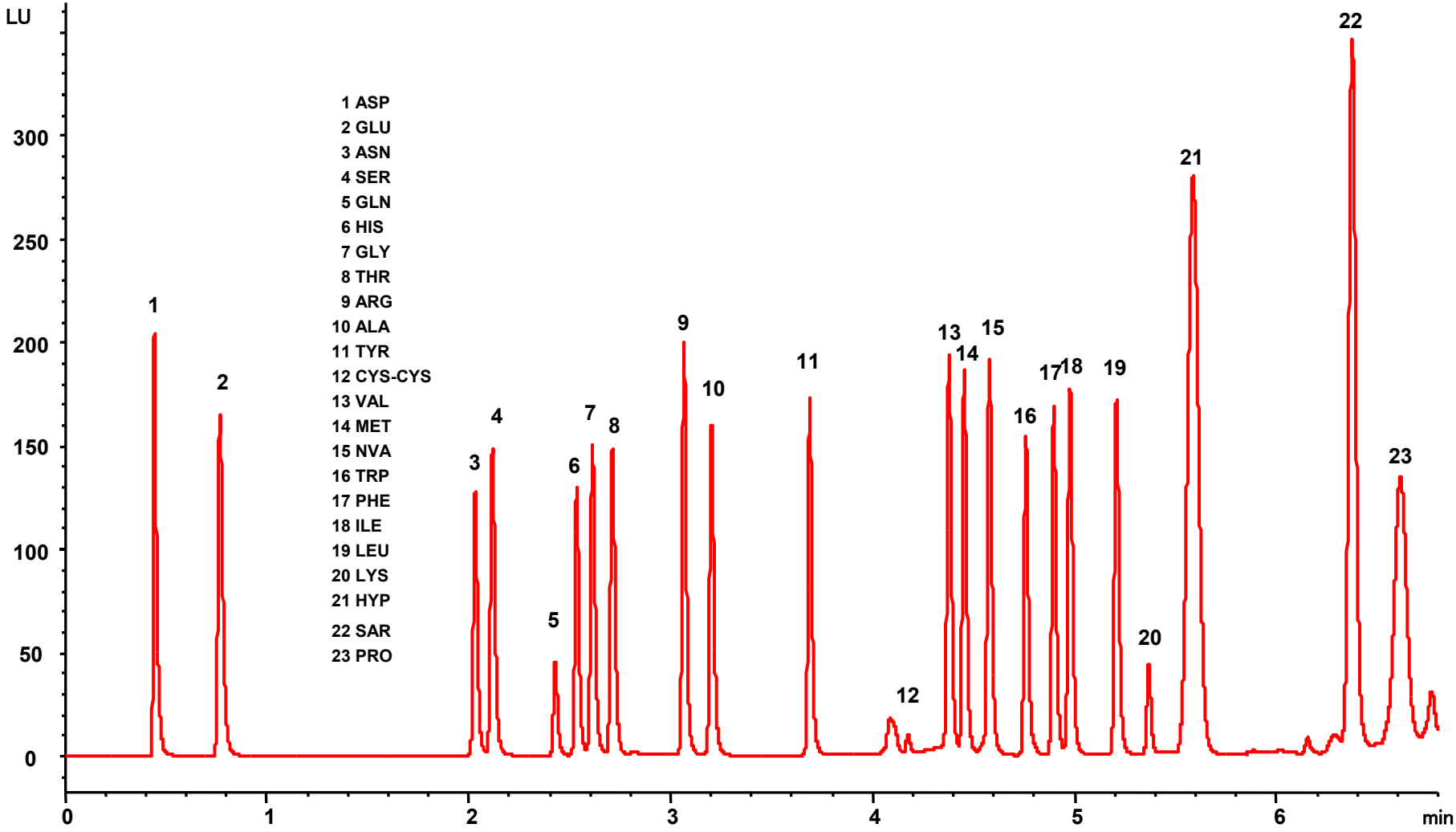
1) Draw 2.5µl from Borate vial 1(Agilent P/N 5061-3339)
2) Draw 0.5µl from Sample vial
3) Mix 3µl in washport 5X
4) Wait 0.2min
5) Draw 0.5µl from OPA vial 2 (Agilent P/N 5061-3335)
6) Mix 3.5µl in washport 6X
7) Draw 0.4µl from FMOC vial 3 (Agilent P/N 5061-3337)
8) Mix in 3.9µl in washport 10X
9) Draw 32µl from Diluent vial 4
10) Mix 20µl in seat 8X
11) Inject
12) Wait 0.10 min
13) Valve bypass

Tubing is 0.12mm ID throughout. Maximum sensitivity is obtained with 2.1mm columns. To properly integrate the first peak, set the integrator to detect negative peaks. If you wish to minimize the degradants in the DAD chromatogram use new reagents and mobile phase. OPA and FMOC are not stable left open to the atmosphere at room temperature. All modules are 1200SL where available; the autosampler is a standard 1200 SL WPS.

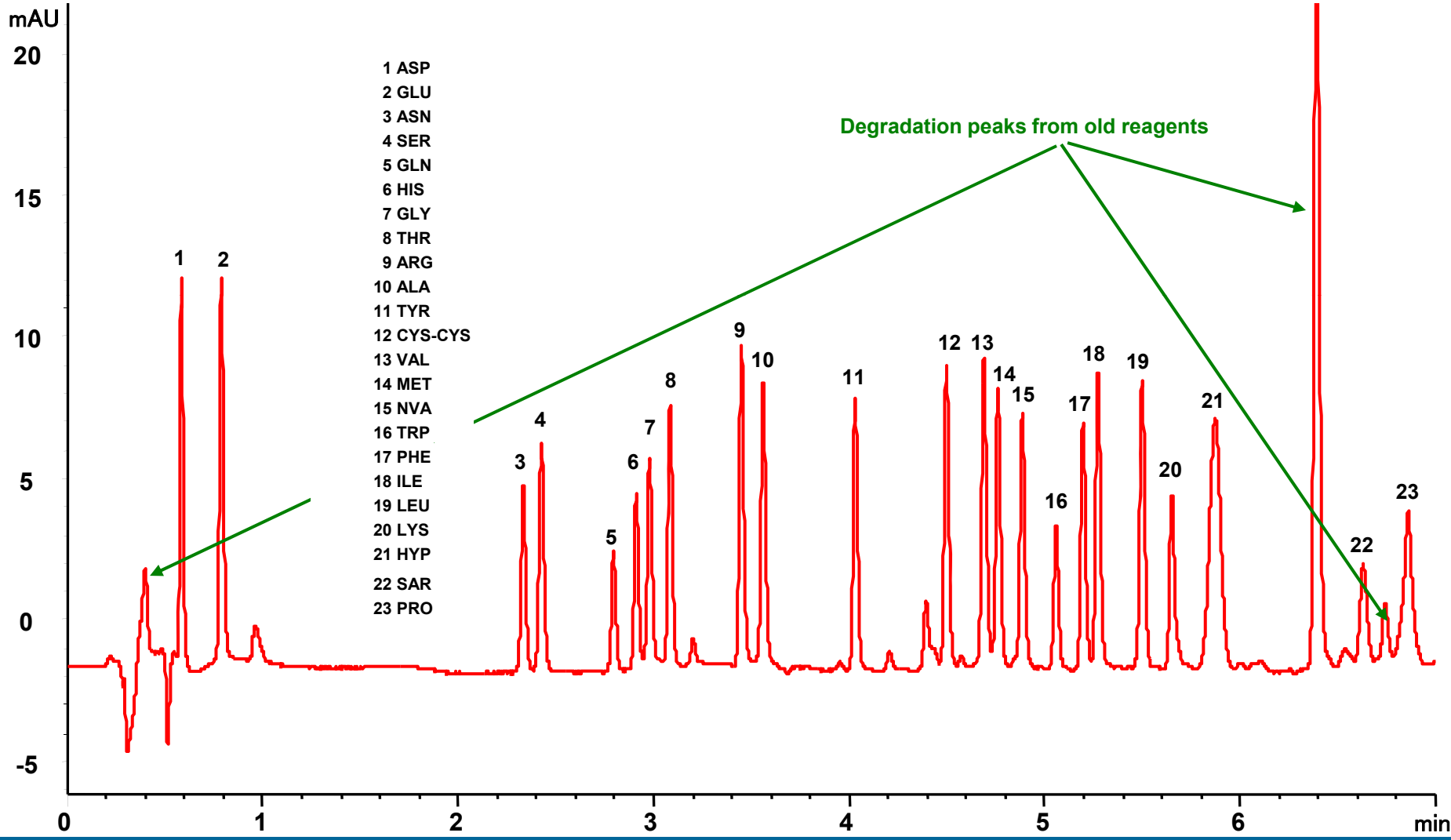
Amino Acid Analysis on Eclipse Plus-C18, 4.6 x 50mm, 1.8µ Column: DAD 125pMole on column



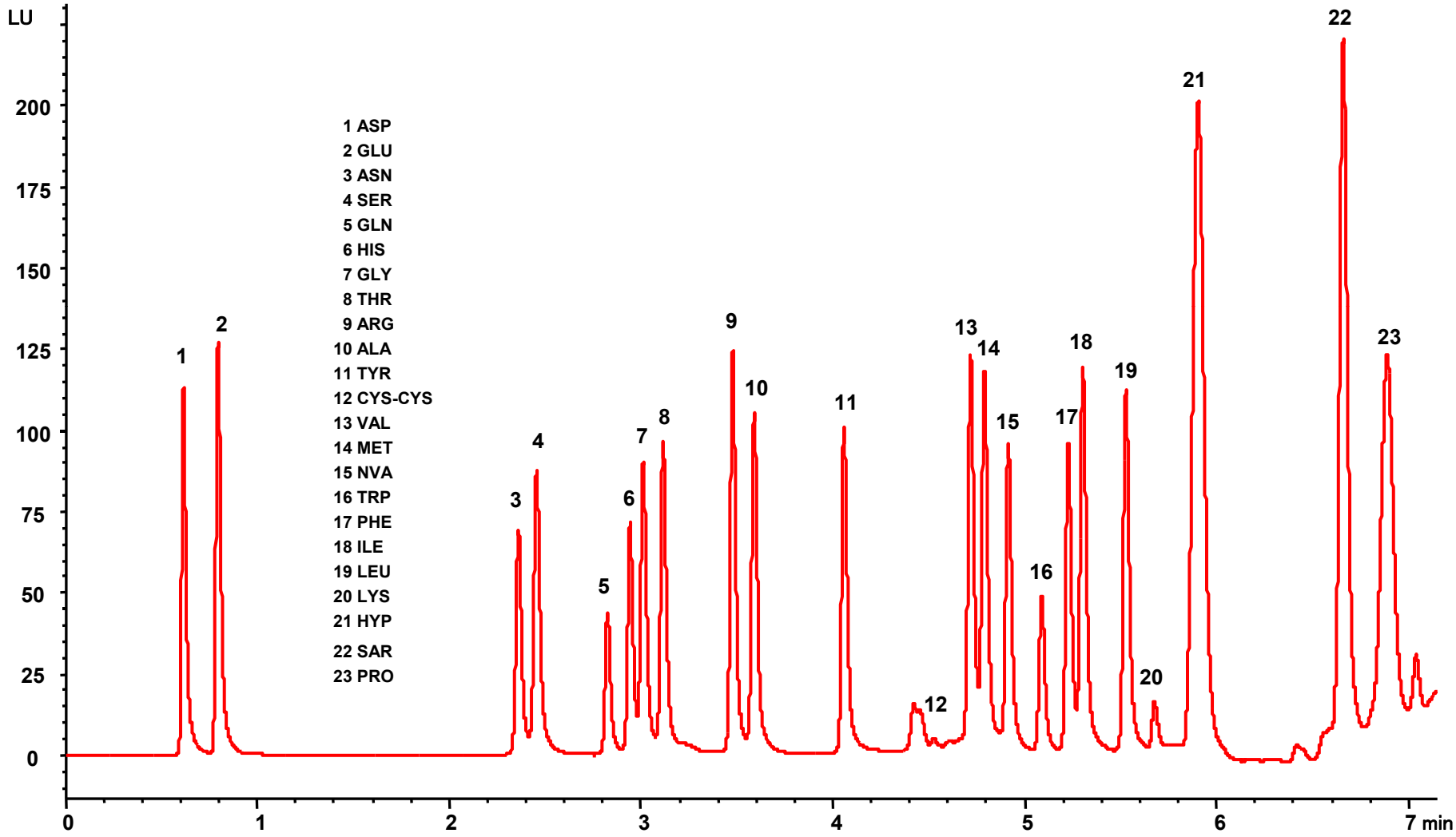
Amino Acid Analysis on Eclipse Plus-C18, 4.6 x 50mm, 1.8µ Column: FLD 125pMole on column



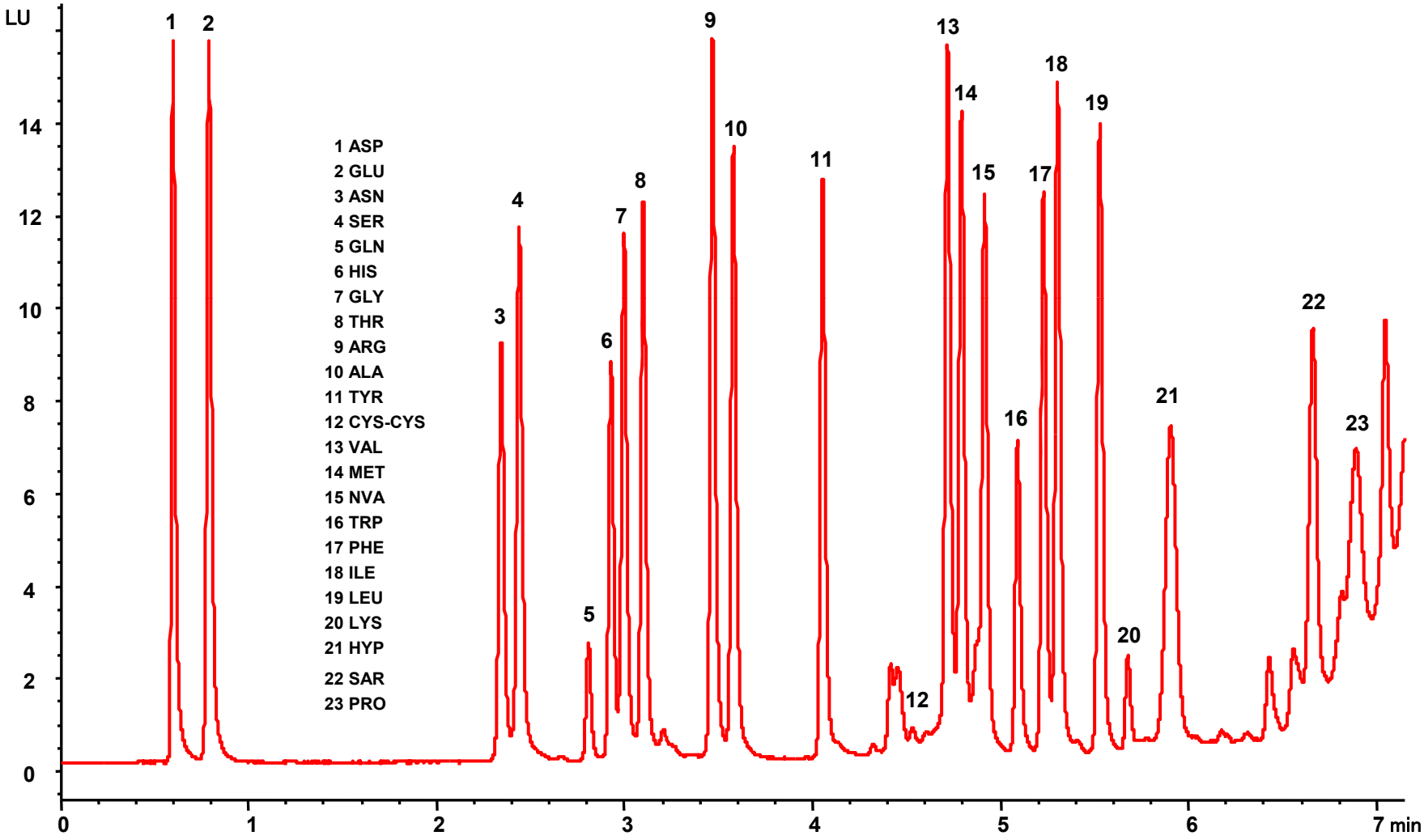
Amino Acid Analysis on Eclipse Plus-C18, 2.1 x 50mm, 1.8µ Column: DAD 50pMole on column



Amino Acid Analysis on Eclipse Plus-C18, 2.1 x 50mm, 1.8µ Column: FLD 50pMole on column



Amino Acid Analysis on Eclipse Plus-C18, 2.1 x 50mm, 1.8µ Column: FLD 5pMole on column



Many Column and Instrument Options

#	Method Category	Column/Method Name	Analysis Time w/re-equilibration	Typical Minimum Resolution Factor	Approximate mL Solvent/ Analysis*	Agilent HPLC Instrument
1	**Traditional**	**4.6 x 250, 5 µm**	40 min	2.4	64	1200 or 1200 SL
2		**3.0x 250, 5 µm**	40 min	2.4	28	1200 or 1200 SL
3	**Rapid Resolution**	**4.6x 150, 3.5 µm**	25 min	2	42	1200 or 1200 SL
4		**3.0x 150, 3.5 µm**	25 min	2	18	1200 or 1200 SL
5		**2.1x 150, 3.5 µm**	25 min	2	12	1200 or 1200 SL
6	**Rapid Res HT**	**4.6 x 100, 1.8 µm**	16 min	2.4	28	1200SL
7		**2.1 x 100, 1.8 µm**	16 min	2.4	8	1200SL
8		**4.6 x 50, 1.8 µm**	9 min	1.5	23	1200SL
9		**3.0 x 50, 1.8 µm**	9 min	1.5	10	1200SL
10		**2.1 x 50, 1.8 µm**	9 min	1.5	5	1200SL

*** includes injector program and pre-run DAD autobalancing (2.42 min), and re-equilibration time.**

Instrument Options

Online automated derivatization will change depending on your autosampler

- **G1376C well plate automatic liquid sampler (WPALS), with injection program:**
  1) **Draw 2.5 µL from Borate vial (Agilent P/N 5061-3339)**
  2) **Draw 1.0 µL from Sample vial**
  3) **Mix 3.5 µL in washport 5X**
  4) **Wait 0.2 min**
  5) **Draw 0.5 µL from OPA vial (Agilent P/N 5061-3335)**
  6) **Mix 4.0 µL in washport 10X max speed**
  7) **Draw 0.4 µL from FMOC vial (Agilent P/N 5061-3337)**
  8) **Mix 4.4 µL in washport 10X max speed**
  9) **Draw 32 µL from Injection Diluent vial**
  10) **Mix 20 µL in washport 8X**
  11) **Inject**
  12) **Wait 0.1 min**
  13) **Valve bypass**

- **G1329A automatic liquid sampler (ALS), with injection program:**
  1) **Draw 2.5 µL from Borate vial (Agilent P/N 5061-3339)**
  2) **Draw 1.0 µL from Sample vial**
  3) **Mix 3.5 µL in air, max speed 5X**
  4) **Wait 0.2 min**
  5) **Draw 0.5 µL from OPA vial (Agilent P/N 5061-3335)**
  6) **Mix 4.0 µL in air, max speed 10X max speed**
  7) **Draw 0.4 µL from FMOC vial (Agilent P/N 5061-3337)**
  8) **Mix 4.4 µL in air, max speed, 10X max speed**
  9) **Draw 32 µL from Injection Diluent vial**
  10) **Mix 20 µL in air, max speed 8X**
  11) **Inject**
  12) **Wait 0.1 min**
  13) **Valve bypass**

Linear Gradients

Traditional high resolution method gradients, 5 µm

	4.6 x 250, 5 µm	3.0 x 250, 5 µm
	PN 959990-902	PN custom
time (min.)	%B	%B
0	2	2
0.84	2	2
33.4	57	57
33.5	100	100
39.3	100	100
39.4	2	2
40	end	end
flow (mL/min.)	1.5	0.64

Rapid Resolution High Throughput method gradients, 1.8 µm, 100 mm

	4.6 x 100, 1.8 µm	2.1 x 100, 1.8 µm
	PN959964-902	PN959764-902
time (min.)	%B	%B
0	2	2
0.35	2	2
13.4	57	57
13.5	100	100
15.7	100	100
15.8	2	2
16	end	end
flow (mL/min.)	1.5	0.42

Rapid Resolution method gradients, 3.5 µm

	4.6 x 150, 3.5 µm	3.0 x 150, 3.5 µm	2.1x 150, 3.5 µm
	PN959963-902	PN959963-302	PN959763-902
time (min.)	%B	%B	%B
0	2	2	2
0.5	2	2	2
20	57	57	57
20.1	100	100	100
23.5	100	100	100
23.6	2	2	2
25	end	end	end
flow (mL/min.)	1.5	0.64	0.42

Rapid Resolution High Throughput method gradients 1.8 µm, 50 mm

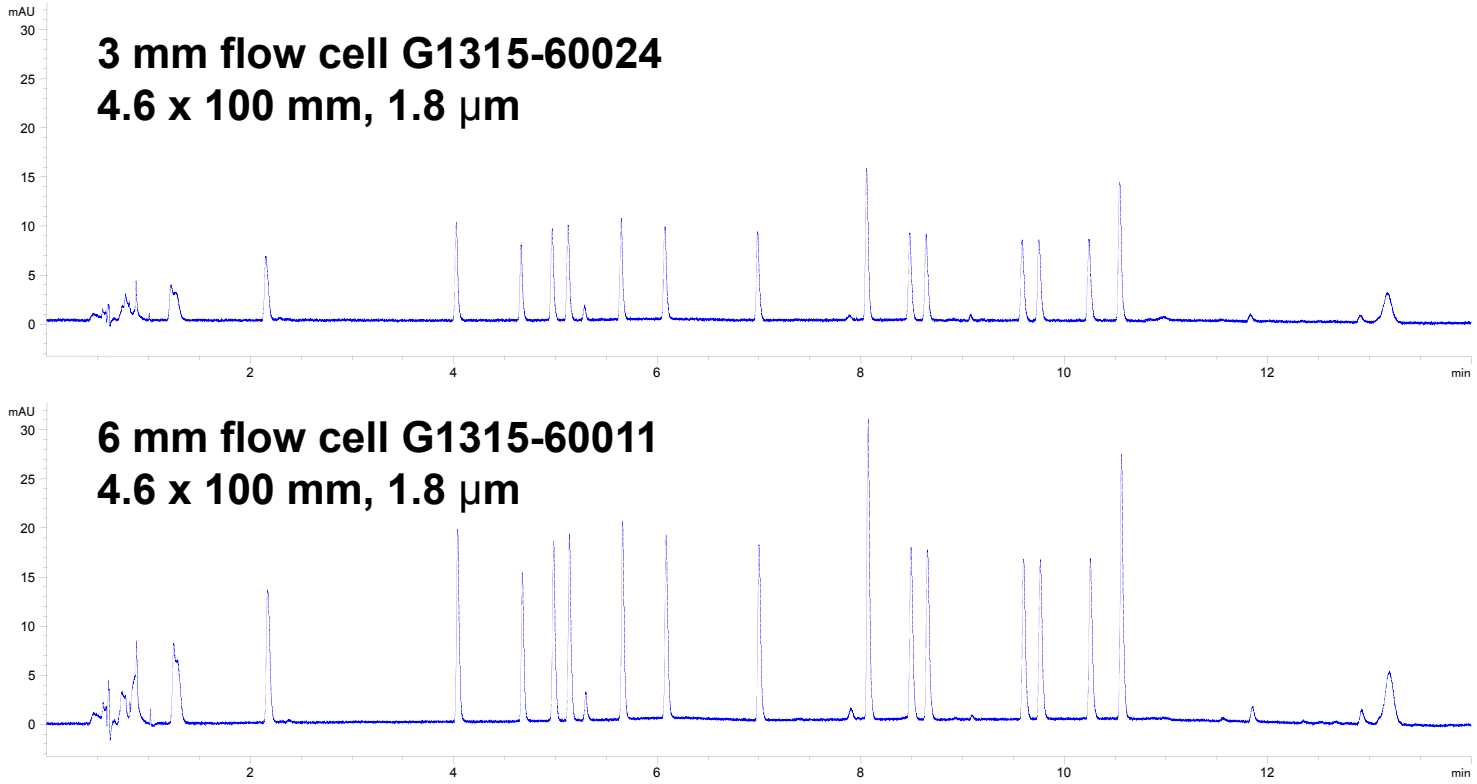
	4.6x50, 1.8 µm	3.0x50, 1.8 µm	2.1x50, 1.8 µm
	PN959941-902	PN959941-302	PN959741-902
time (min.)	%B	%B	%B
0	2	2	2
0.2	2	2	2
7.67	57	57	57
7.77	100	100	100
8.3	100	100	100
8.4	2	2	2
9	end	end	end
flow (mL/min.)	2.0	0.85	0.42

Agilent LC Flow Paths

	Traditional (5 µm) High Resolution Methods		Rapid Resolution (3.5 µm) Methods		
Method name	**4.6 x 250, 5 µm**	**3.0x 250, 5 µm**	**4.6x 150, 3.5 µm**	**3.0x 150, 3.5 µm**	**2.1x 150, 3.5 µm**
LC Model	1100	1200	1200 SL	1200 SL	1200 SL
Pump	G1312A	G1311A quat	G1312B	G1312B	G1312B
Dampener/static mixer	yes	n/a	yes	yes	bypassed
Purge valve to ALS	G1328-87600 (green 500 mm)?	G1328-87600 (green 500 mm)?	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)
ALS	G1367A	G1329A	G1367C	G1367C	G1367C
Needle seat	G1367-87101 (green)	G1313-87201 (green)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)
ALS to heat exchanger	G1313-87305 (green 180 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)
Heat exchanger	G1316 A 3 µL	G1316 A 3 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL
Heat exch. to column or guard	5021-1817 (green 150 mm)	5021-1816 (green 105 mm)	5021-1820 (red 105 mm)	5021-1820 (red 105 mm)	5021-1820 (red 105 mm)
Optional guard cartridge	820950-936 -4 pk, 4.6 id	821125-936-4pk, 2.1 id	820950-936 -4 pk, 4.6 id	821125-936-4pk, 2.1 id	821125-936-4pk, 2.1 id
Column	959990-902	custom	959963-902	959963-302	959763-902
Post column to union	5065-9931 (200 mm green)	5065-9931 (200 mm green)	n/a	n/a	n/a
ZDV union to flow cell	5022-2184	5022-2185	n/a	n/a	n/a
Detector	G1315B	G1315D	G1315C	G1315C	G1315C
Flow cell	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024
	RRHT 1.8 µm Methods (100 mm)		**RRHT 1.8 µm Methods (50 mm)**		
Method name	**4.6 x 100, 1.8 µm**	**2.1 x 100, 1.8 µm**	**4.6 x 50, 1.8 µm**	**3.0 x 50, 1.8 µm**	**2.1 x 50, 1.8 µm**
LC Model	1200 SL	1200 SL	1200 SL	1200 SL	1200 SL
Pump	G1312 B	G1312 B	G1312 B	G1312 B	G1312 B
Dampener/static mixer	yes	bypassed	yes	bypassed	bypassed
Purge valve to ALS	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)
ALS	G1367C	G1367C	G1367C	G1367C	G1367C
Needle seat	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)
ALS to heat exchanger	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)
Heat exchanger	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL
Optional guard cartridge	none	none	none	none	none
Column PN	959964-902	959764-902	959941-902	959941-302	959741-902
Column to flow cell	directly connected	directly connected	directly connected	directly connected	directly connected
Detector	G1315C	G1315C	G1315C	G1315C	G1315C
Flow cell	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024

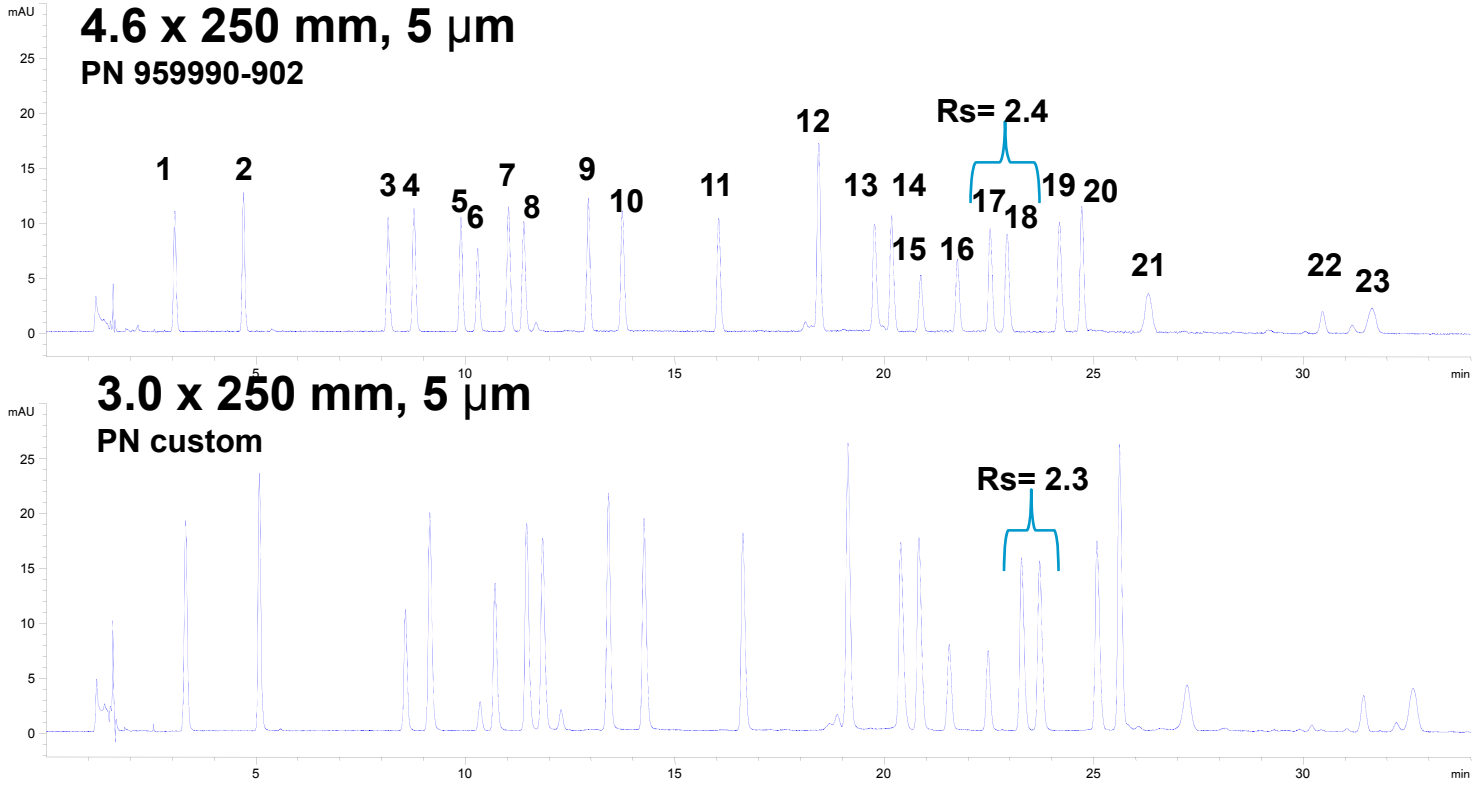
Increase Sensitivity

Sensitivity increases when a longer flow cell path is used



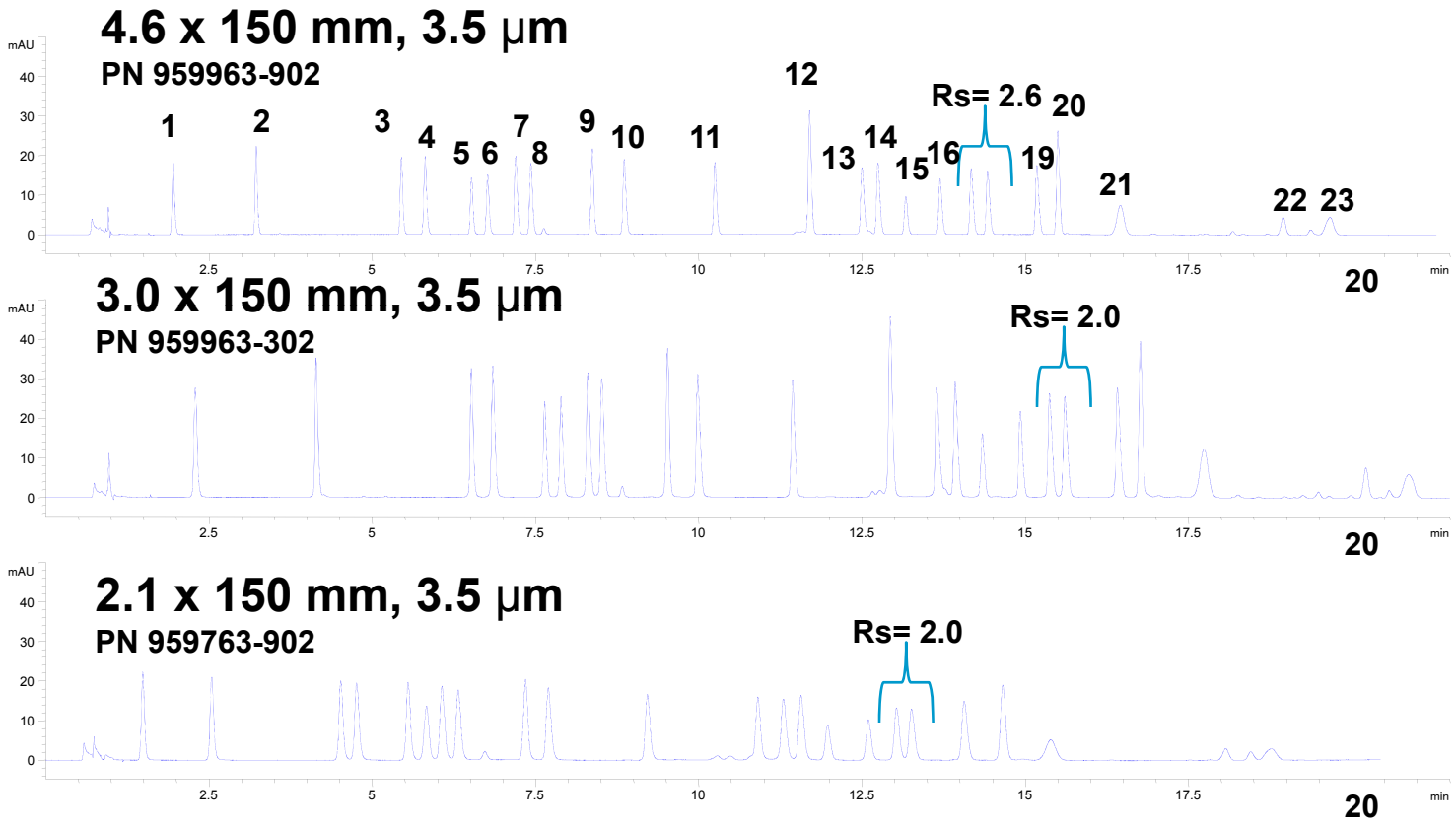
ZORBAX Eclipse Plus C18 250 mm, 5 µm Traditional High Resolution Options

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alanine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline



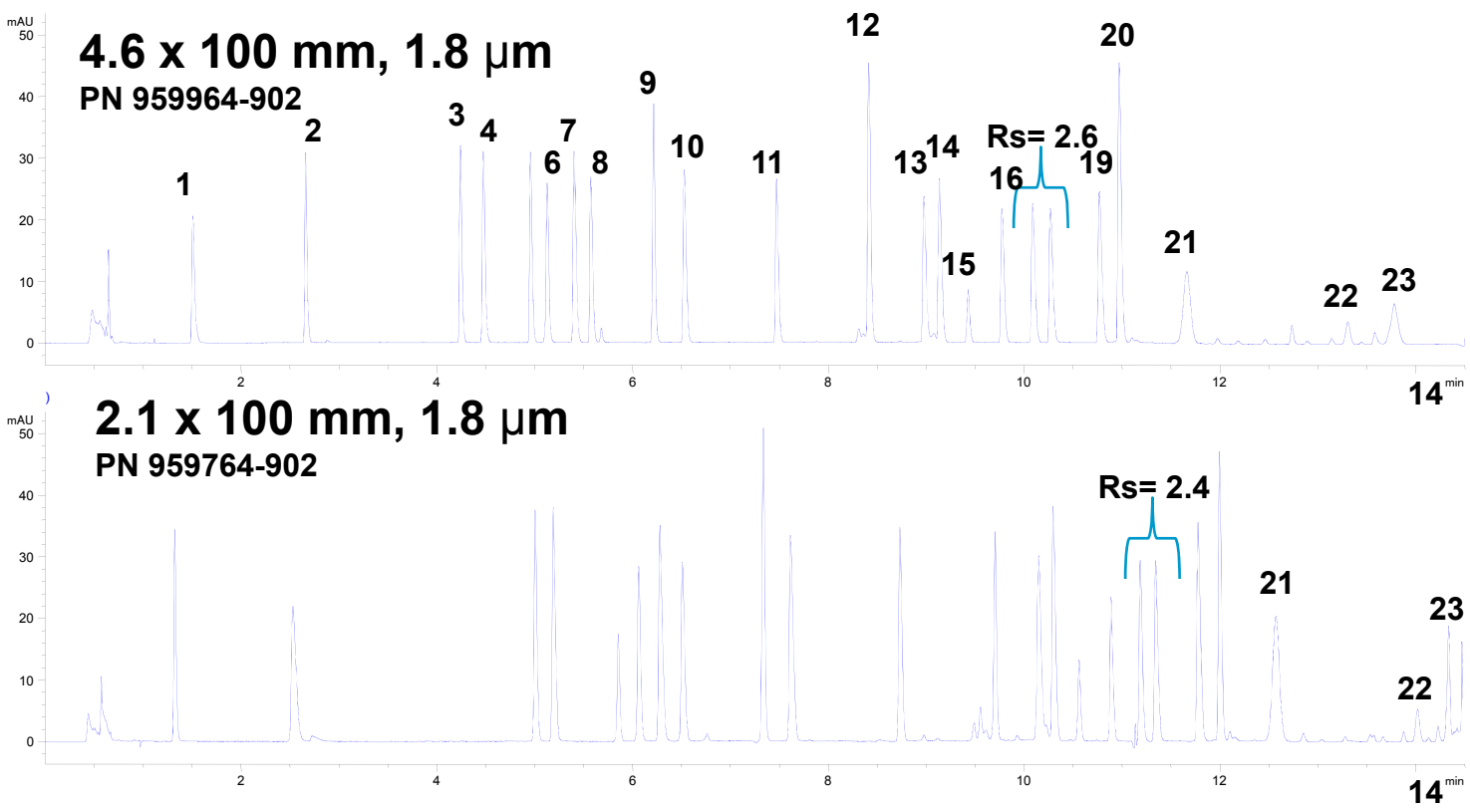
ZORBAX Eclipse Plus C18 150 mm, 3.5 µm Rapid Resolution (RR) Options

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alanine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline



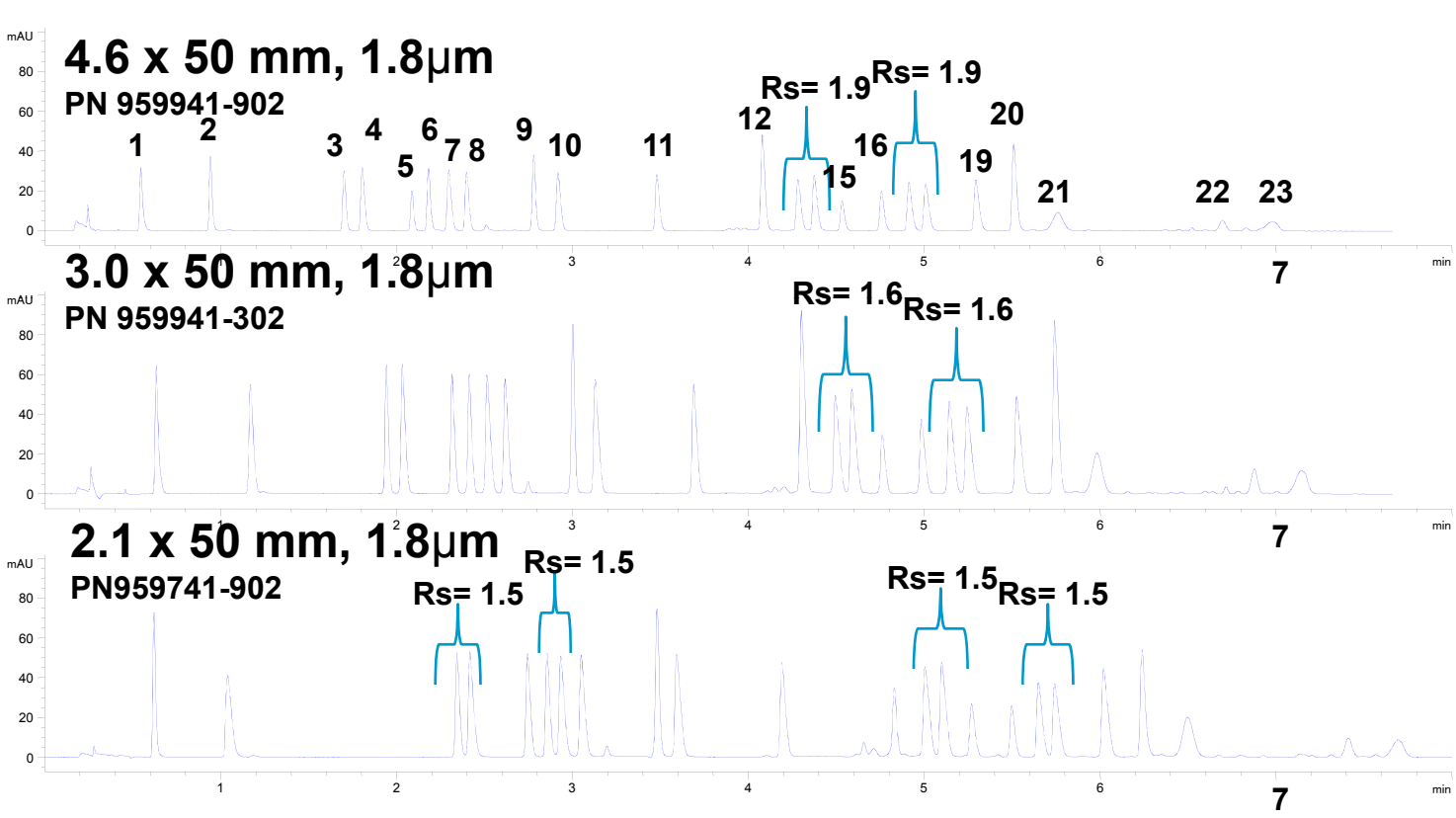
ZORBAX Eclipse Plus C18 100 mm, 1.8 µm Rapid Resolution High Throughput (RRHT) Options

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alinine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline



ZORBAX Eclipse Plus C18 50 mm, 1.8 µm Rapid Resolution High Throughput (RRHT) Options

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alinine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline



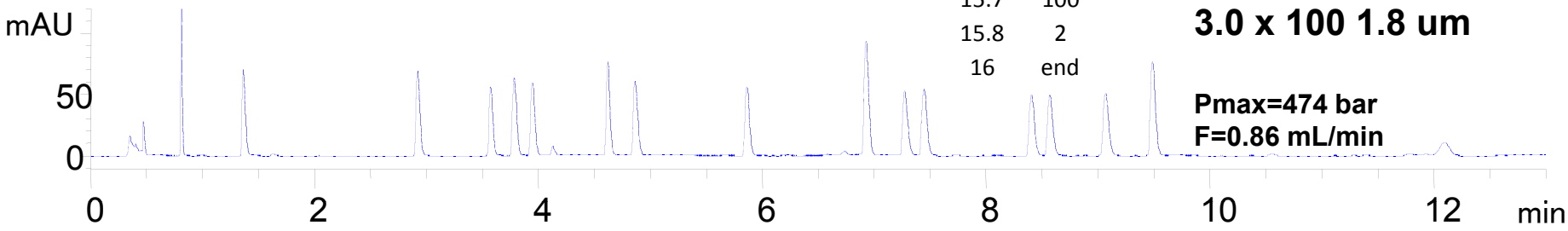
Amino Acid Analysis on 1290 Infinity UHPLC

1200SL

1290 Infinity

17 Amino Acid Analysis on 1290 Infinity

time (min.)	%B
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
16	end



RRHT Eclipse Plus C18
3.0 x 100 1.8 um

Pmax=474 bar
F=0.86 mL/min

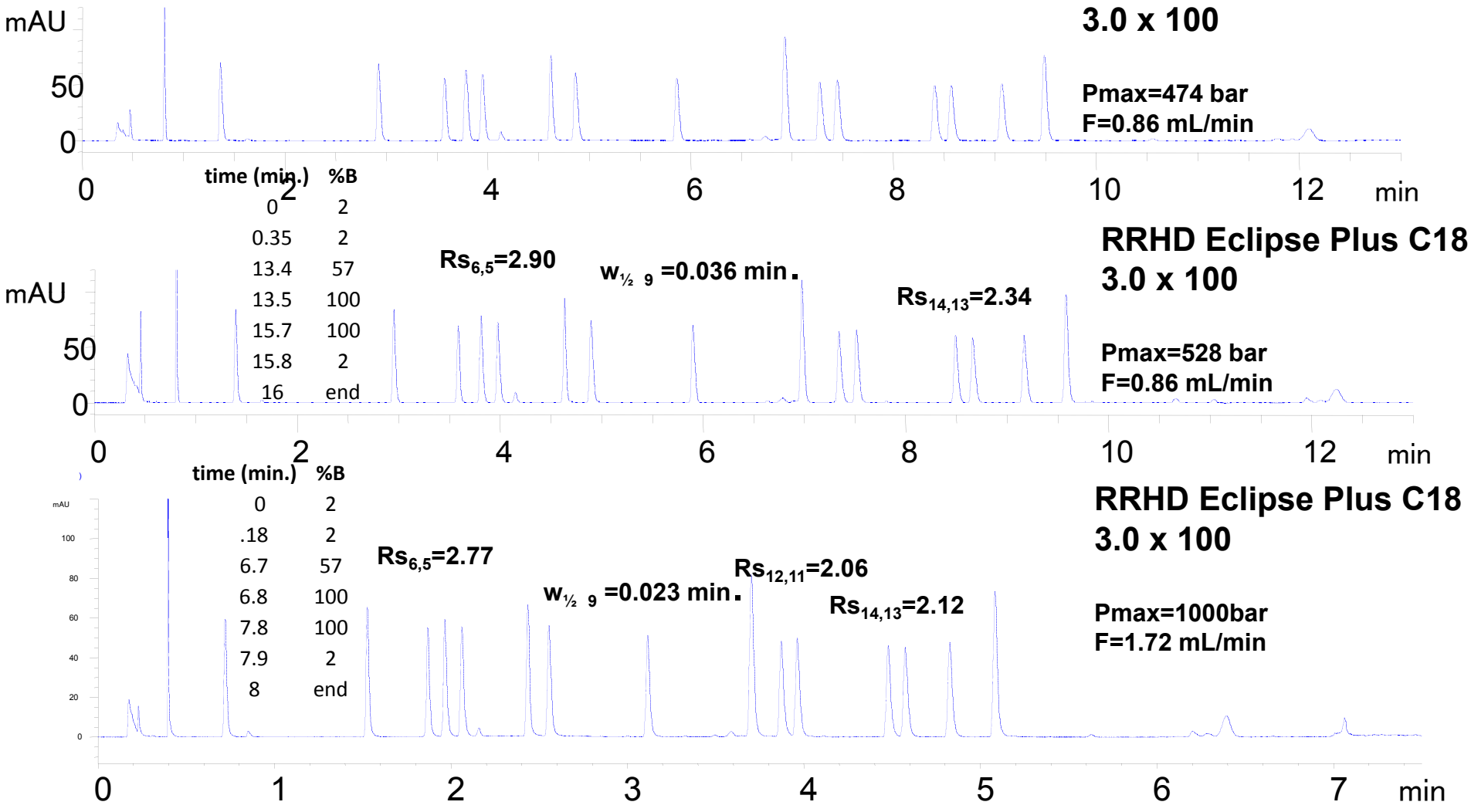
Original 1200 SL injection program 100 uL sample loop

1) Draw 2.5 µL from *Borate* vial (Agilent P/N 5061-3339)
2) Draw 1.0 µL from *Sample* vial
3) Mix 3.5 µL in washport 5X
4) Draw 0.5 µL from *OPA* vial (Agilent P/N 5061-3335)
5) Mix 4.0 µL in washport 10X max speed
6) Draw 0.4 µL from *FMOC* vial (Agilent P/N 5061-3337)
7) Mix 4.4 µL in washport 10X max speed
8) Draw 32 µL from *Injection Diluent* vial
9) Mix 20 µL in washport 8X
10) Inject

Original 1290 Infinity injection program 20 uL sample loop (40 uL available)

1) Draw 1.3 µL from *Borate* vial (Agilent P/N 5061-3339)
2) Draw 0.5 µL from *Sample* vial
3) Mix 1.8 µL in location P1C1 5X default speed, offset
4) Draw 0.5 µL from *OPA* vial (Agilent P/N 5061-3335)
5) Mix 2.3 µL in location P1C1 5X default speed, offset
6) Draw 0.2 µL from *FMOC* vial (Agilent P/N 5061-3337)
7) Mix 2.5 µL in in location P1C1 5X default speed, offset
8) Draw 7.4 µL from *Injection Diluent* vial
9) Mix 9.9 µL in location P1C1 5X default speed, offset
10) Inject

Double Flow Rate and Halve Gradient Time to Double Throughput



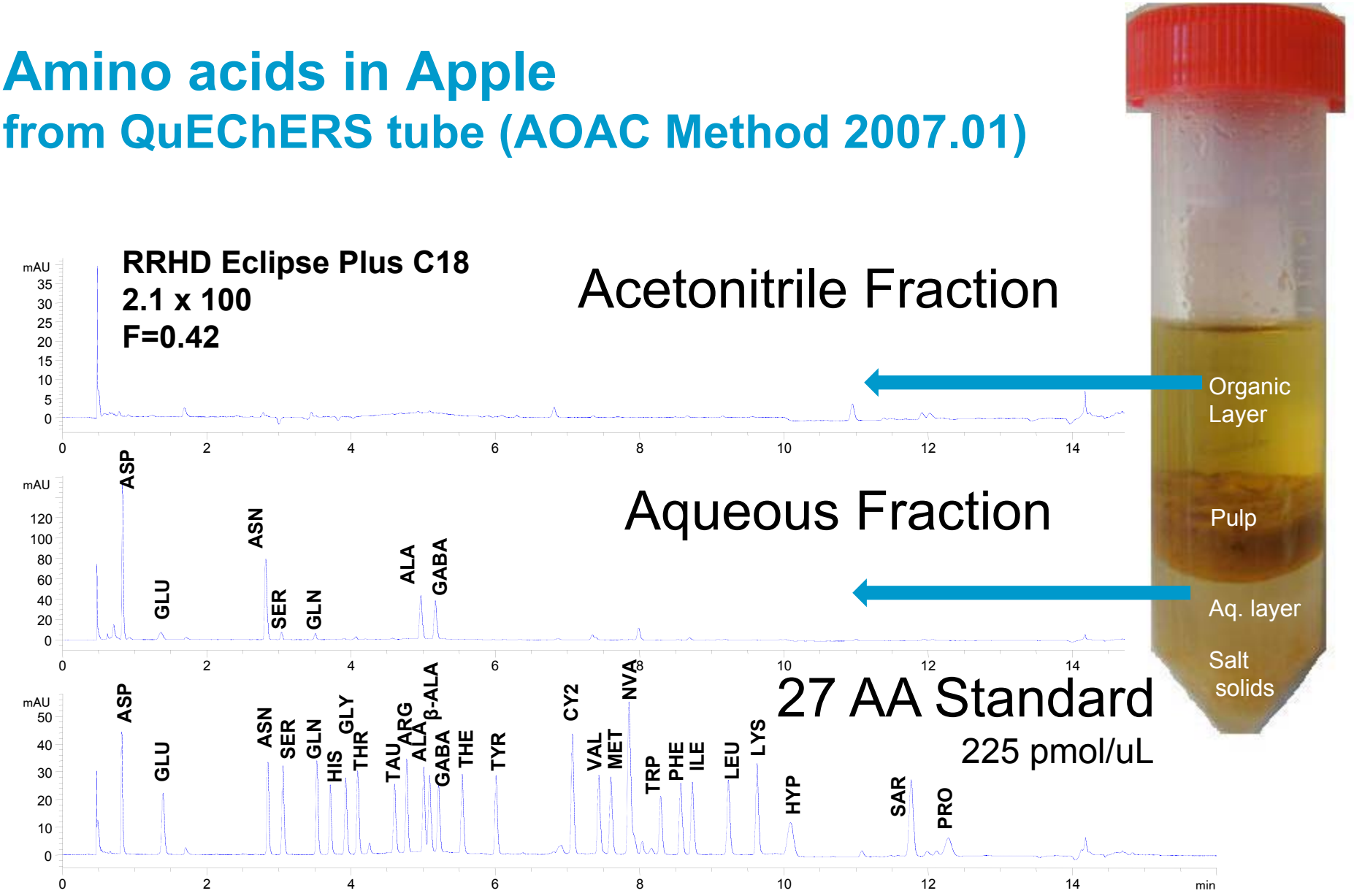
Summary of Method

- Use a programmable autosampler to do on-line derivatization
  - OPA reacts with 1° amino acids
  - FMOC reacts with 2° amino acids
- Use a gradient to separate the derivatized amino acids
- Use UV and FLD to detect derivatized amino acids

Examples

- **Samples from AOAC methods using SampliQ QuEChERS kits**
  - Fruits & Vegetables
    - Apple
    - Spinach
- **Beverages**
- **Protein Hydrolysates**

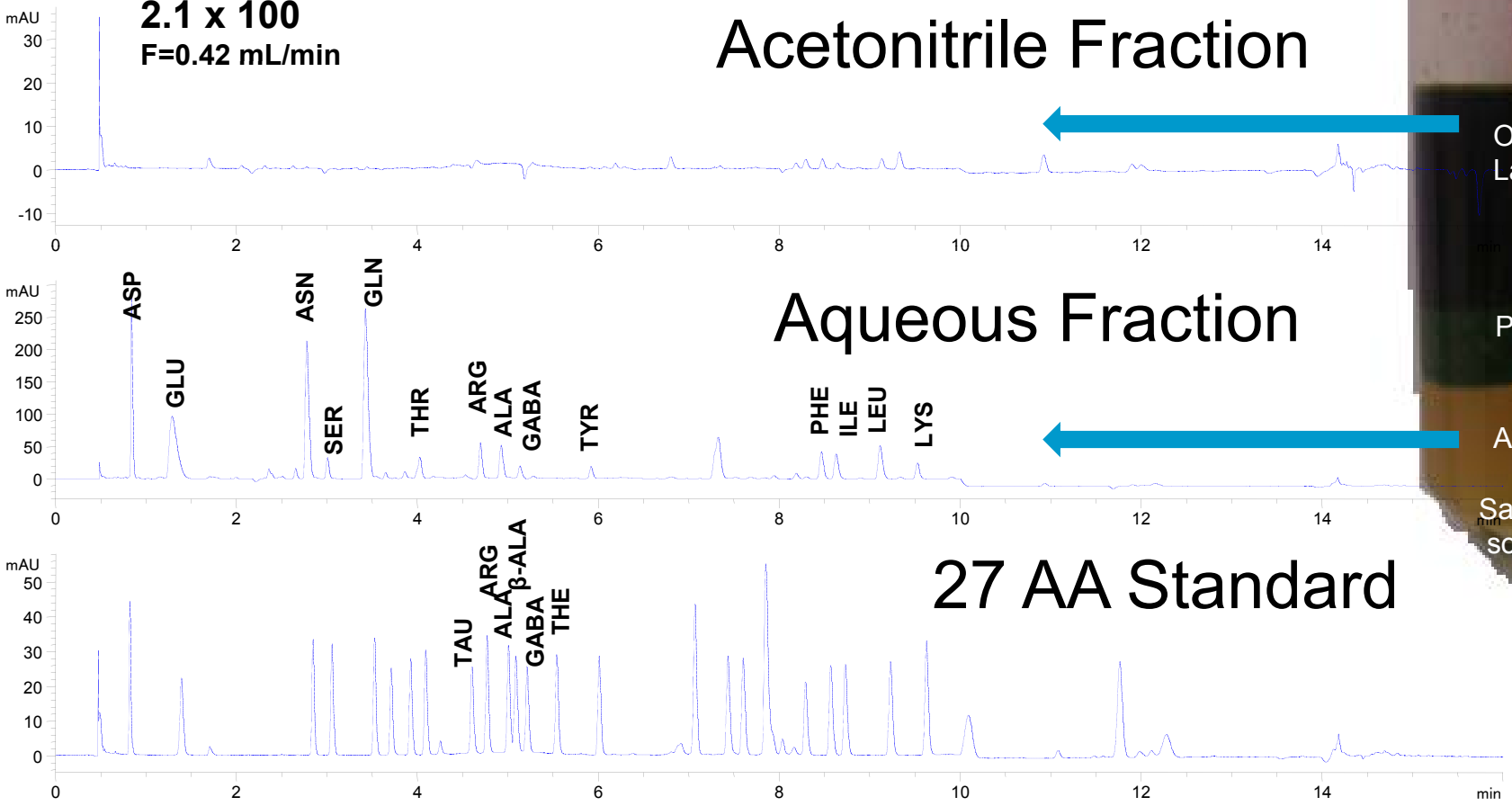
Amino acids in Apple
from QuEChERS tube (AOAC Method 2007.01)



Includes: GABA, β-alanine, taurine, theanine, norvaline, hydroxyproline, sarcosine

Amino acids in Spinach leaf
from QuEChERS tube (AOAC Method 2007.01)

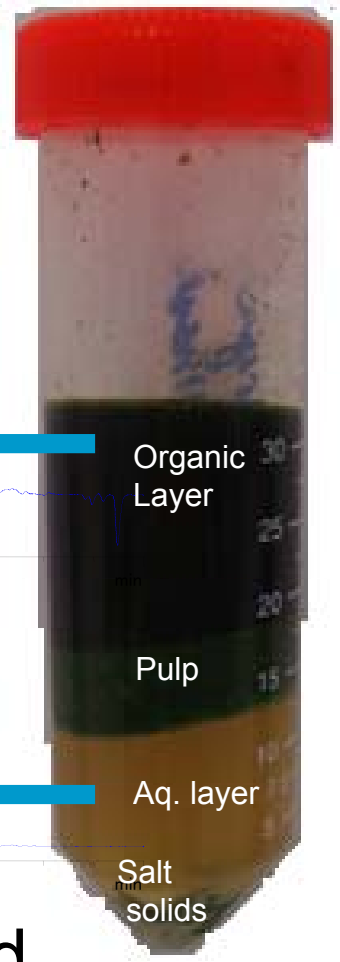
RRHD Eclipse Plus C18
2.1 x 100
F=0.42 mL/min



Acetonitrile Fraction

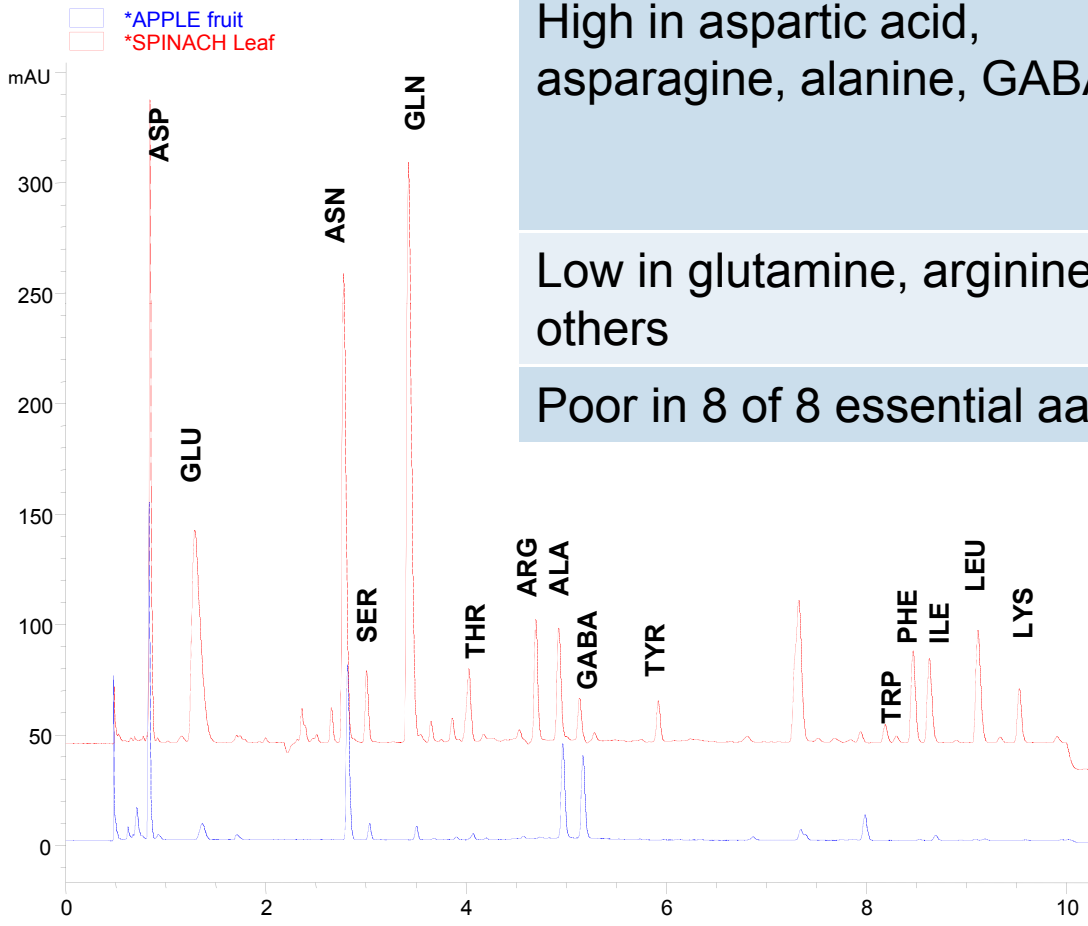
Aqueous Fraction

27 AA Standard



Amino Acid Profile Comparison

Apple	Spinach
High in aspartic acid, asparagine, alanine, GABA	High in aspartic acid, glutamic acid, asparagine, arginine, GABA
Low in glutamine, arginine others	High in glutamine, arginine others
Poor in 8 of 8 essential aa	Rich in 6 of 8 essential aa



ug/mL	Apple	Spinach
ASP	99	195
ASN	77	220
ALA	31	46
GABA	43	22

Comparison of the Amino Acid Content of a Variety of Bottled Beers

Amino Acid	Typical American Beer	American Beer brewed to German purity laws	German Beer brewed for US market	English Beer brewed for US market
	µMoles /ml	µMoles /ml	µMoles /ml	µMoles /ml
ASP	1.06	0.73	0.26	1.15
GLU	1.07	0.93	0.80	1.98
ASN	1.19	0.98	0.14	0.54
SER	0.45	0.40	0.12	0.26
GLN	0.67	0.76	0.38	0.33
HIS	0.68	1.00	1.07	0.89
GLY	1.54	2.00	1.61	1.57
THR	0.30	0.34	0.74	0.20
ARG	0.48	0.94	1.29	1.83
ALA	4.28	5.14	4.56	4.60
TYR	1.36	2.86	1.65	1.77
CYS-CYS	0.08	0.10	0.08	0.05
VAL	1.92	3.35	1.93	2.83
MET	0.17	0.27	0.11	0.16
NVA	IS	IS	IS	IS
TRP	0.58	1.07	0.80	0.67
PHE	1.33	2.21	1.23	2.07
ILE	0.54	0.97	0.34	0.94
LEU	1.02	1.71	0.55	1.75
LYS	0.30	0.32	0.08	0.90
HYP	0.20	1.74	1.17	1.26
SAR	IS	IS	IS	IS
PRO	8.65	34.84	15.88	10.29
Total =	27.9	62.7	34.8	36.0

Underivatized AAs

- Reversed-Phase
- Ion Exchange
- Ion pair reversed-phase LC
- Detectors
  - ELSD
  - MS
  - CLND
  - UV-Vis

Underivatized Amino Acids on Bonus-RP

Method

LC Conditions

Column:	ZORBAX Bonus-RP, narrow-bore (100 mm × 2.1 mm, 3.5 µm)
Flow rate:	0.2 mL/min
Mobile phase:	0.01 mM acetic acid in 0.2% aqueous solution of formic acid
Injection:	20 µL out of 1000 µL

MS Conditions

Ionization mode:	Positive APCI
Nebulizer pressure:	55 psi
Drying gas flow:	4 L/min
Drying gas temperature:	320 °C
Vaporizer temperature:	425 °C
Skimmer:	20 V
Capillary voltage:	3 kV
Fragmentor voltage:	55 V
Dwell time:	27 ms

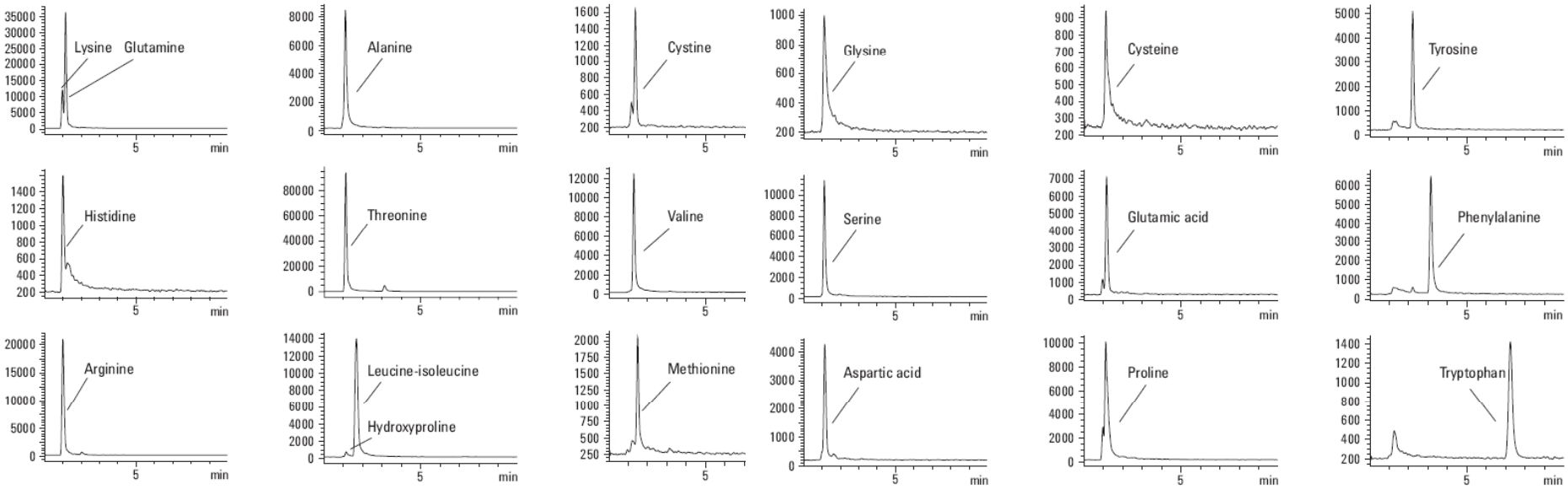
Pub. No. 5989-5838EN

Characteristic Fragments of 22 AAs and Ions Used in SIM Mode for Quantification

ID	Fragment ions *m/z*	Selected ion *m/z*
Alanine (ala)	90, 73	90
Arginine (arg)	175, 129	175
Asparagine (asn)	133, 116, 87, 74	133
Aspartic acid (asp)	134, 116, 88	134
Cysteine (cys)	122, 105, 87, 73	122
Cystine(cys-cys)	241, 122	241
Glutamic acid (glu)	148, 130, 102	148
Glutamine (gln)	147, 130, 101	147
Glycine (gly)	76, 59	76
Histidine (his)	156, 110, 96, 73, 59	156
Hydroxyproline (hyp)	132, 86	132
Leucine-isoleucine (leu-ile)	132, 86	132
Lysine (lys)	147, 130, 84	147
Methionine (met)	150, 133, 104	150
Phenylalanine (phe)	166, 149, 120	166
Proline (pro)	116, 70	116
Serine (ser)	106, 88, 60	106
Threonine (thr)	120, 102, 74	120
Tyrosine (tyr)	182, 165, 136, 123	182
Tryptophan (trp)	205, 188, 130	188
Valine (val)	118, 72	118

Determination of Underivatized Free Amino Acids using LC/APCI-MS

MS signals for specific *m/z* of 22 amino acids in green peas using a single injection



22 Free Amino Acids in Various Foods*

Food Types	Ala	Arg	Asn	Asp	Cys	Cys-Cys	Glu	Gln	Gly	His	Hyp
Baby food	3.59	1.25	1.07	0.50	0.17	9.77	0.37	1.06	0.52	0.30	1.42
Tomato	10.07	3.94	11.02	36.86	1.31	1.52	85.94	49.64	2.03	0.34	0.73
Pea	69.84	37.26	112.10	30.22	6.96	3.62	15.78	57.49	7.92	3.27	1.17
Pear	< LOD	1.81	45.27	17.01	0.60	13.58	8.19	86.33	1.14	< LOD	< LOD
Apple paste	< LOD	1.40	3.80	3.56	0.30	16.04	2.18	23.46	< LOD	< LOD	< LOD
Apple juice	2.03	1.79	12.80	10.58	0.98	12.52	4.82	39.89	< LOD	0.13	0.43
Sour cherry juice	1.53	1.62	24.10	7.30	0.38	10.95	7.11	52.45	< LOD	1.01	0.32
Orange juice	3.95	8.30	8.08	15.15	0.70	9.99	5.10	3.36	1.38	1.11	0.53
Pomegranate juice	15.66	1.99	13.58	17.33	0.27	16.53	17.23	42.35	< LOD	0.17	0.44
Peach juice	3.38	1.64	78.57	21.52	0.84	12.26	3.38	10.05	1.44	0.14	0.35
White grape juice	6.54	2.18	10.50	16.08	0.50	16.06	8.34	41.50	1.26	0.11	0.43
Red grape juice	8.58	0.60	5.32	5.54	0.74	17.60	7.98	4.17	1.52	1.46	0.52
Beer	4.00	0.88	2.98	1.15	< LOD	5.33	1.89	1.54	1.48	0.26	8.59
Milk	7.02	16.50	8.99	4.00	0.36	16.12	9.06	4.85	1.38	0.75	0.36
Wine	4.49	1.35	0.94	0.55	0.66	0.92	0.73	0.42	1.69	0.46	0.24
Honey	12.23	< LOD	4.16	8.43	< LOD	203.22	7.93	18.90	2.93	1.26	0.76
Green coffee	41.06	2.38	31.36	70.26	43.19	14.82	57.18	3.89	3.43	3.27	< LOD
Hazelnut	12.98	1.28	2.21	13.05	< LOD	6.02	21.01	0.51	2.57	< LOD	< LOD
Walnut	6.56	2.71	0.71	7.42	< LOD	4.90	46.80	3.55	1.72	0.38	0.74
Almond	11.39	4.14	57.51	44.92	0.73	4.50	3100	6.31	3.76	0.34	0.46
Pistachio	25.15	7.58	14.45	27.40	1.36	5.82	35.64	39.28	2.88	< LOD	7.26

Food Types	Leu-ile	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Trp	Val
Baby food	0.30	< LOD	0.20	0.26	0.78	0.40	1.66	0.50	0.12	1.21
Tomato	0.96	< LOD	0.63	3.25	6.30	3.38	2.70	2.05	0.74	2.43
Pea	26.84	17.41	4.44	13.52	21.50	55.52	0.03	15.45	3.31	38.60
Pear	1.38	< LOD	< LOD	0.44	17.92	3.86	1.23	0.68	0.48	5.25
Apple paste	< LOD	< LOD	< LOD	< LOD	< LOD	0.57	< LOD	< LOD	< LOD	< LOD
Apple juice	0.32	< LOD	< LOD	< LOD	4.48	0.78	0.33	< LOD	< LOD	1.70
Sour cherry juice	0.23	< LOD	4.52	0.14	6.52	1.29	0.53	0.25	< LOD	2.62
Orange juice	0.37	1.76	4.72	0.99	30.55	6.07	1.13	0.99	0.58	3.64
Pomegranate juice	1.37	< LOD	0.53	0.94	8.52	3.01	1.94	1.76	0.64	4.62
Peach juice	< LOD	< LOD	< LOD	0.45	12.89	2.48	1.17	0.33	< LOD	2.18
White grape juice	1.54	1.21	0.89	0.81	8.94	2.80	1.75	1.80	0.64	5.10
Red grape juice	1.80	1.39	0.64	1.88	14.05	4.30	5.33	3.90	0.75	5.03
Beer	< LOD	1.17	0.30	0.15	0.84	< LOD	0.32	0.27	< LOD	3.84
Milk	2.04	1.43	1.80	0.61	11.61	3.14	3.12	2.33	0.65	5.40
Wine	0.23	1.28	0.25	0.25	30.50	0.36	0.31	2.37	1.86	0.88
Honey	2.06	4.80	< LOD	11.18	81.32	6.31	2.65	8.63	< LOD	6.34
Green coffee	6.37	4.35	1.66	14.31	32.30	20.48	3.63	13.58	7.66	8.61
Hazelnut	3.24	2.06	< LOD	2.41	5.38	3.84	2.38	3.69	2.16	4.08
Walnut	1.81	1.64	0.52	1.09	5.50	1.44	1.12	1.71	1.75	3.01
Almond	7.21	4.93	1.93	6.08	33.65	7.82	3.34	4.07	2.28	10.33
Pistachio	2.75	3.64	1.43	381	55.98	3.80	3.71	6.00	1.51	5.73

*as mg/100g FW

Conclusions

•There are a number of separation challenges when analyzing amino acids

• A flexible, automated online derivatization method for amino acids using ZORBAX Eclipse Plus C18 can be customized and optimized

• The variety of column choices with Eclipse Plus lets you choose between high resolution, high speed, reduced solvent consumption, or a combination that bests suits your needs

• Amino acids can be analyzed without derivatization

• Instrumentation plays a key role in successful amino acid analysis