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LC-MS analysis of intact amino acids on a novel mixed-mode HPLC column

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PURPOSE

There are four established methods for analyzing amino acids: prelabeled, post-labeled, ion-pairing reversed-phase, and normal-phase, but each of these methods has disadvantages.
The pre-labeled method has problems with derivitization efficiency and cost, while the post-labeled method is usually not compatible with LC-MS due to non-volatile mobile phases. The ion-pairing reversed-phase method has difficulty separating polar amino acids; on the other hand, the normal-phase mode has problems separating all the compounds, especially the Leu and Ile isomers.
We have developed a novel amino acid separation column for LC-MS(/MS) which can separate all 20 amino acids in protein using a mixed-mode stationary phase structure. We have also estimated separation and detection characteristics using LC-MS instruments.

METHOD

We used a novel amino acid analysis column for LC-MS. This stationary phase consists of 3um silica particles modified with ion ligands which provide IEX and normal phase mixed-mode separation.

LC-MS instrument:
A mass spectrometer, LCMS-2020 with LC-30A binary pumps (Shimadzu Corporation, Kyoto)

Reagents:
All of reagents including standard amino acids and bovine serum were purchased as commercial reagents.

RESULTS

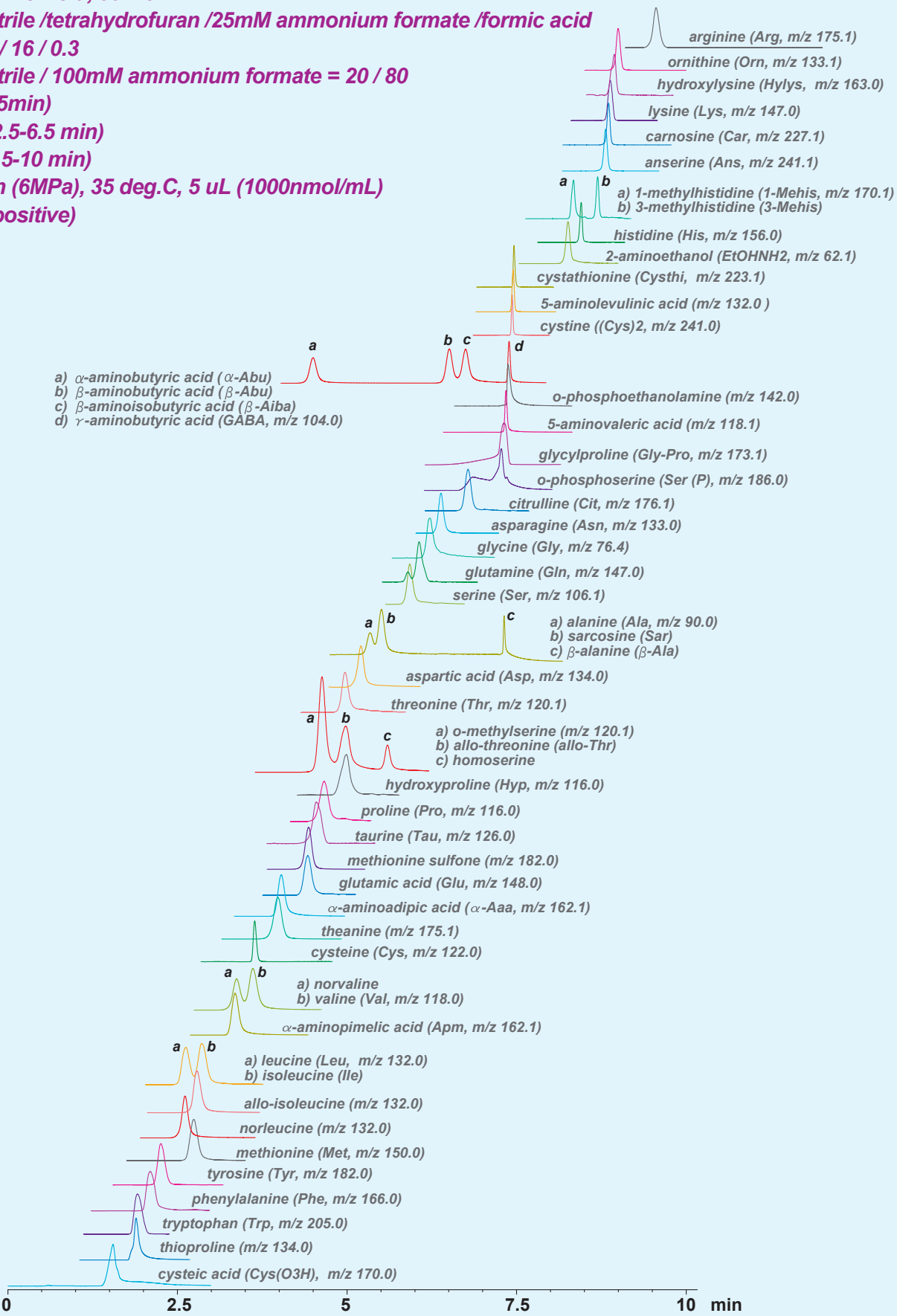
1) Achieved to separate and detect or non-label amino acids
2) High throughput separation with Leu/Ile separation in 5min
3) Simple gradient separation
4) Both single and triple MS system is applicable
5) Applicable to dipeptides analysis
6) Extensibility to various amino acid related compounds
7) Ultra high throughput analysis in one minute

CONCLUSION

This novel HPLC method will be a powerful tool for amino acid LC-MS(/MS) analysis in many different biochemistry applications.

1 55 Amino acids (standard)

Intrada Amino Acid, 50 x 3 mm
A: acetonitrile /tetrahydrofuran /25mM ammonium formate /formic acid = 9 / 75 / 16 / 0.3
B: acetonitrile / 100mM ammonium formate = 20 / 80
0 %B (0-2.5min)
0-17 %B (2.5-6.5 min)
100 %B (6.5-10 min)
0.6 mL/min (6MPa), 35 deg.C, 5 uL (1000nmol/mL)
ESI (SIM, positive)



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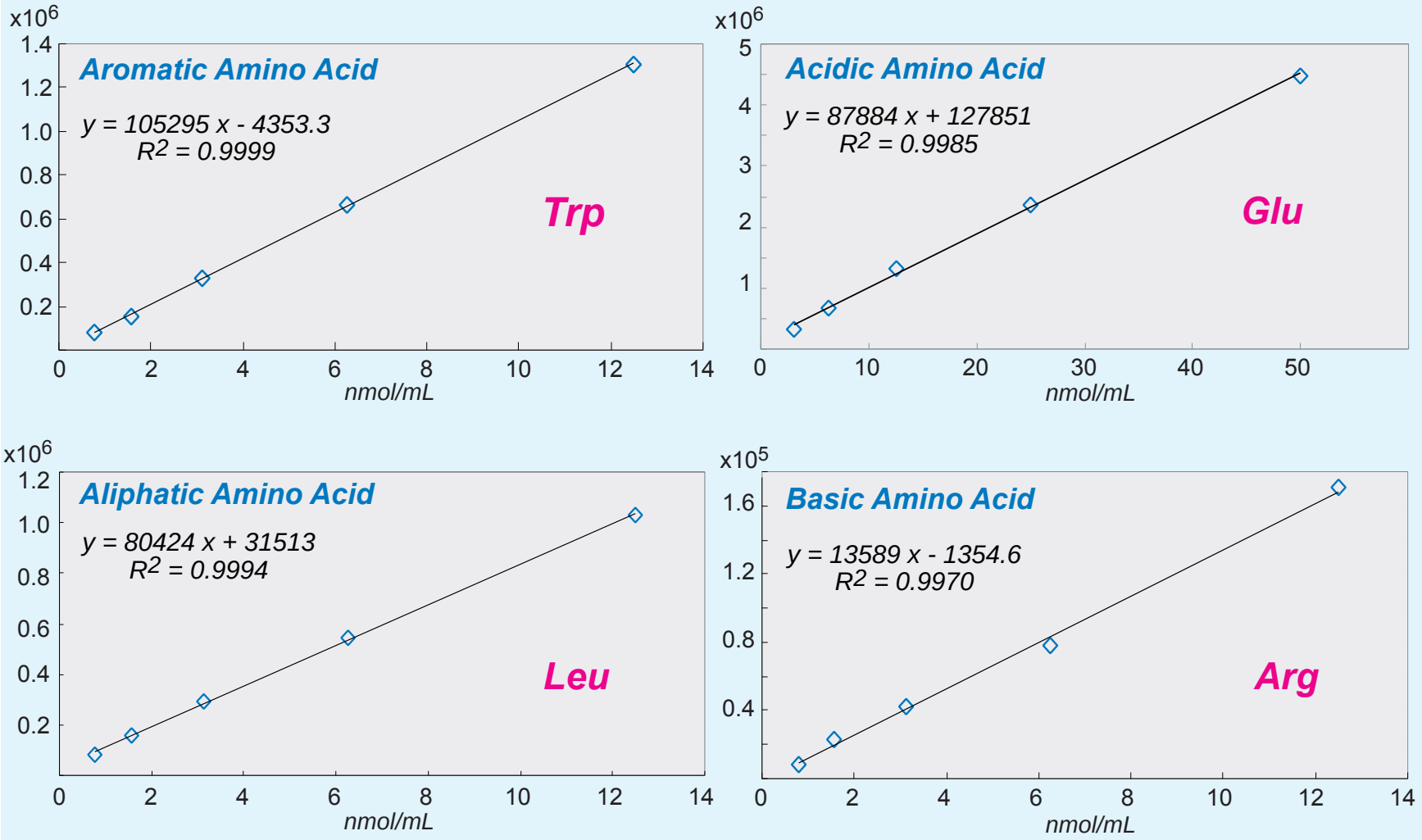
55 standard amino acids were separated and detected on LC-MS. Leucine and Isoleucine isomers were also separated in several minutes.

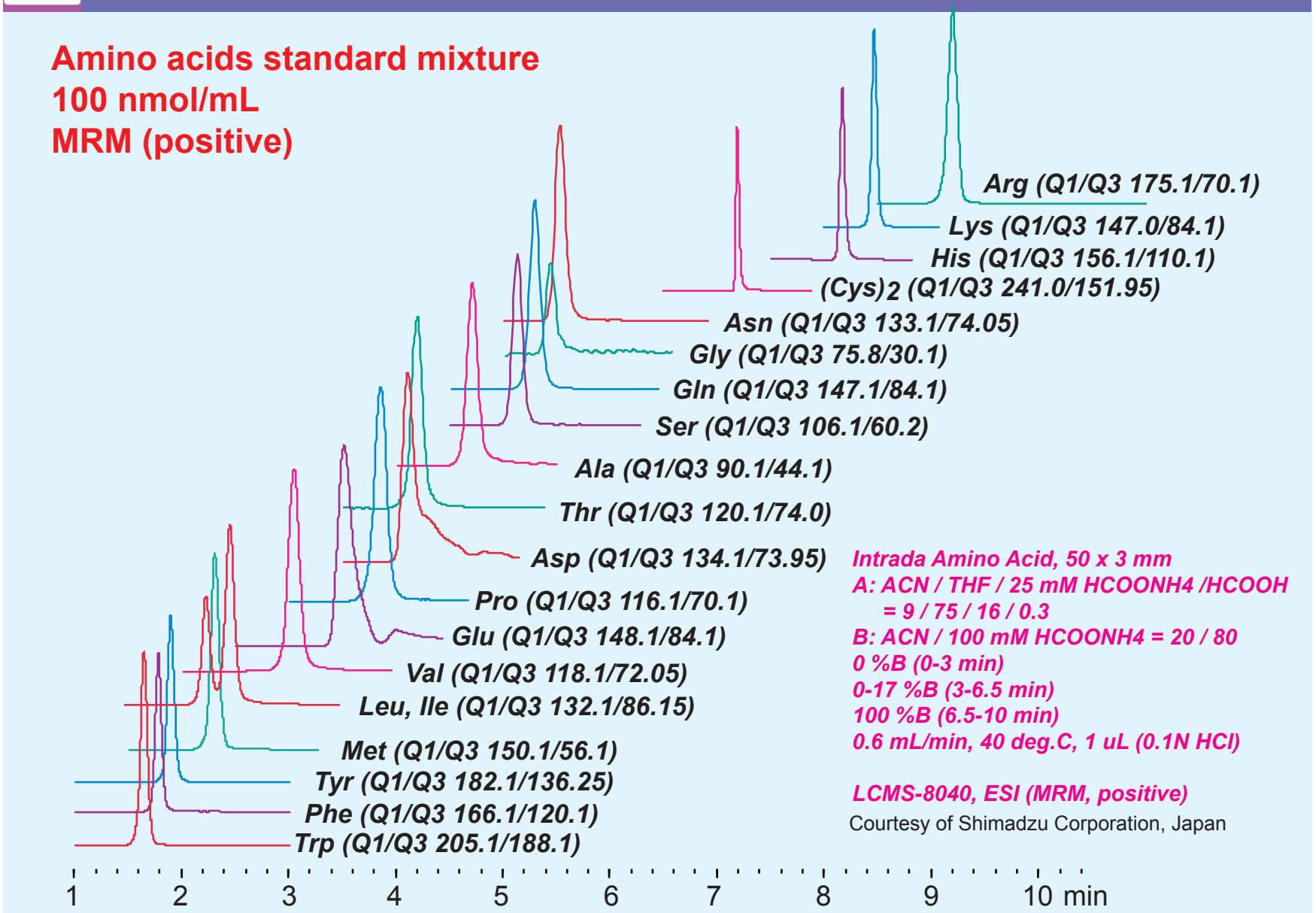
2 Calibration Standard Curve for Various Amino Acids



Typical standard (aromatic, aliphatic, acidic and basic) amino acids showed good results for linearity and sensitivity.

3

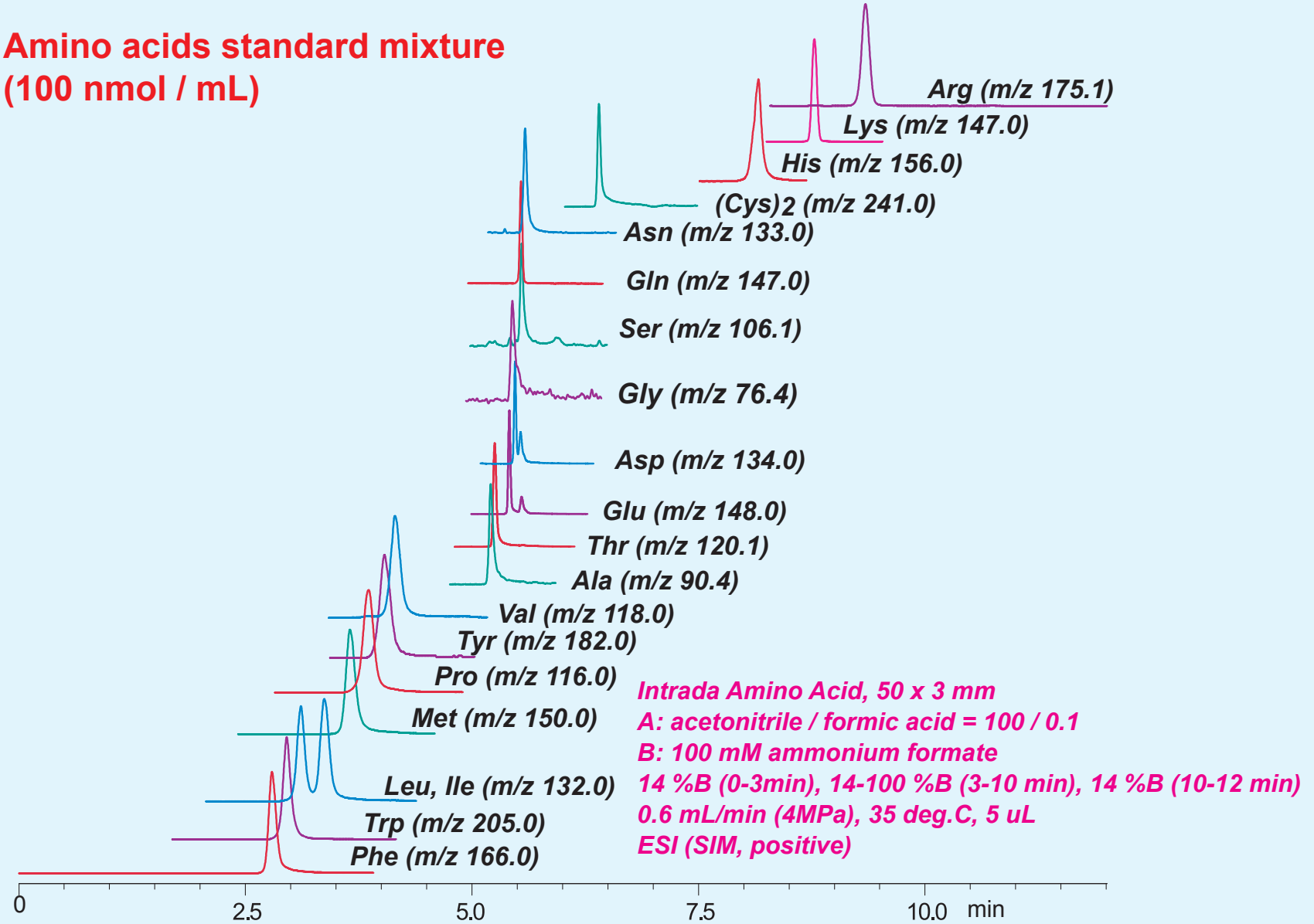
LC-MS/MS (MRM) Analysis



20 standard amino acids of protein were separated and detected on LC-MS/MS (MRM).

4 Simple Gradient Conditions

Amino acids standard mixture
(100 nmol / mL)



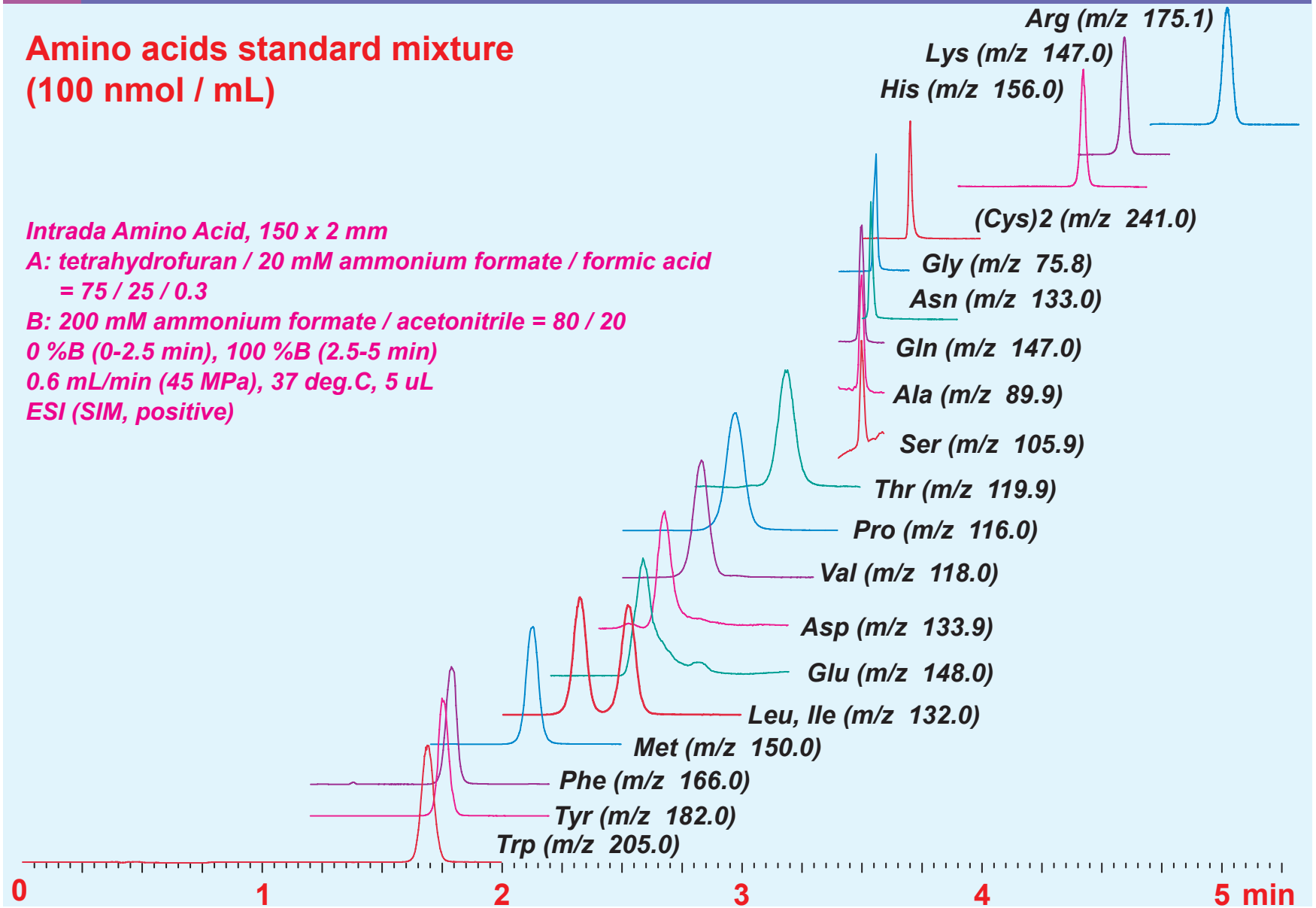
Intrada Amino Acid, 50 x 3 mm
A: acetonitrile / formic acid = 100 / 0.1
B: 100 mM ammonium formate
14 %B (0-3min), 14-100 %B (3-10 min), 14 %B (10-12 min)
0.6 mL/min (4MPa), 35 deg.C, 5 uL
ESI (SIM, positive)

Simple gradient elution condition was also successful for standard amino acids separation in 10min with low pressure.

5 5min High Throughput Analysis

Amino acids standard mixture (100 nmol / mL)

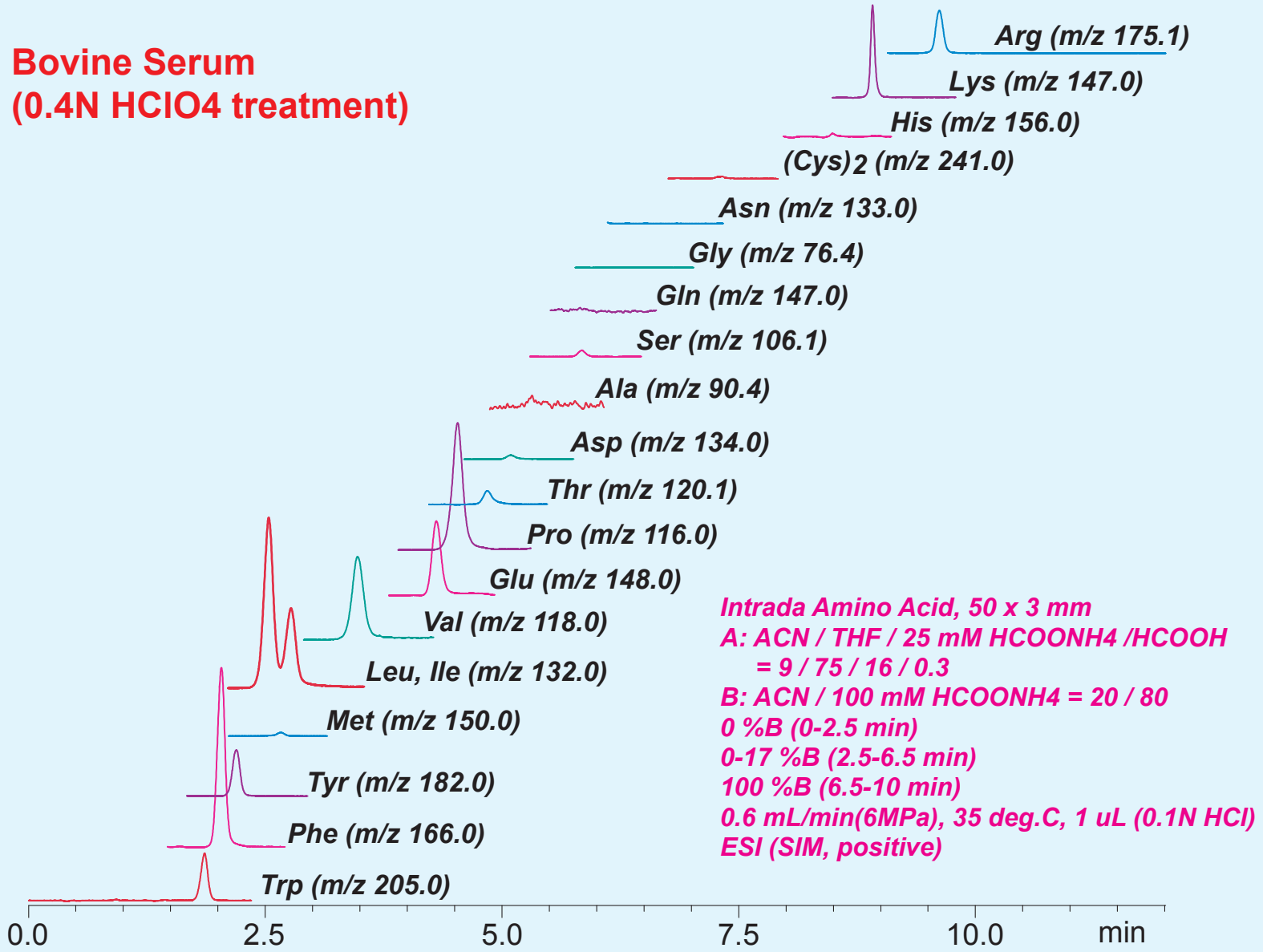
Intrada Amino Acid, 150 x 2 mm
A: tetrahydrofuran / 20 mM ammonium formate / formic acid = 75 / 25 / 0.3
B: 200 mM ammonium formate / acetonitrile = 80 / 20
0 %B (0-2.5 min), 100 %B (2.5-5 min)
0.6 mL/min (45 MPa), 37 deg.C, 5 uL
ESI (SIM, positive)



High throuput analysis using UHPLC system was achieved in 5min.

Application for Amino Acids in Serum

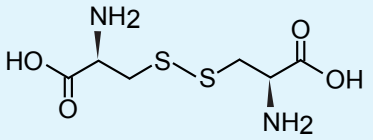
Bovine Serum
(0.4N HClO4 treatment)



Intrada Amino Acid, 50 x 3 mm
A: ACN / THF / 25 mM HCOONH4 /HCOOH
= 9 / 75 / 16 / 0.3
B: ACN / 100 mM HCOONH4 = 20 / 80
0 %B (0-2.5 min)
0-17 %B (2.5-6.5 min)
100 %B (6.5-10 min)
0.6 mL/min(6MPa), 35 deg.C, 1 uL (0.1N HCl)
ESI (SIM, positive)

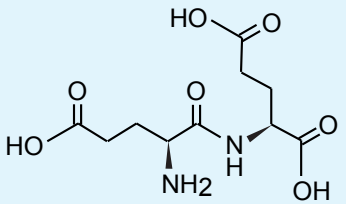
The novel amino acid analysis column was applied for amino acids in serum and several amino acids were detected.

Dipeptide Analysis

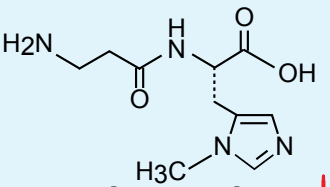


L-cystine ((Cys)2)

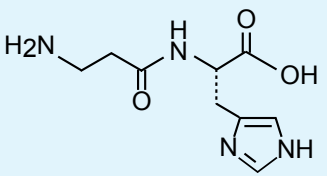
Intrada Amino Acid, 75 x 2 mm
A: acetonitrile / formic acid = 100 / 0.3
B: 100 mM ammonium formate
20-100 %B (0-10 min), 100 %B(10-12 min)
0.3 mL/min (5 MPa), 35 deg.C
2 uL (0.02-0.32 ug, 0.1N-HCl aq.)
ESI (SIM, positive)



N-(L-alpha-glutamyl)
-L-glutamic acid
(Glu-Glu)

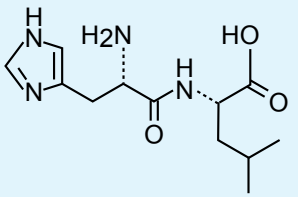


L-anserine



L-carnosine

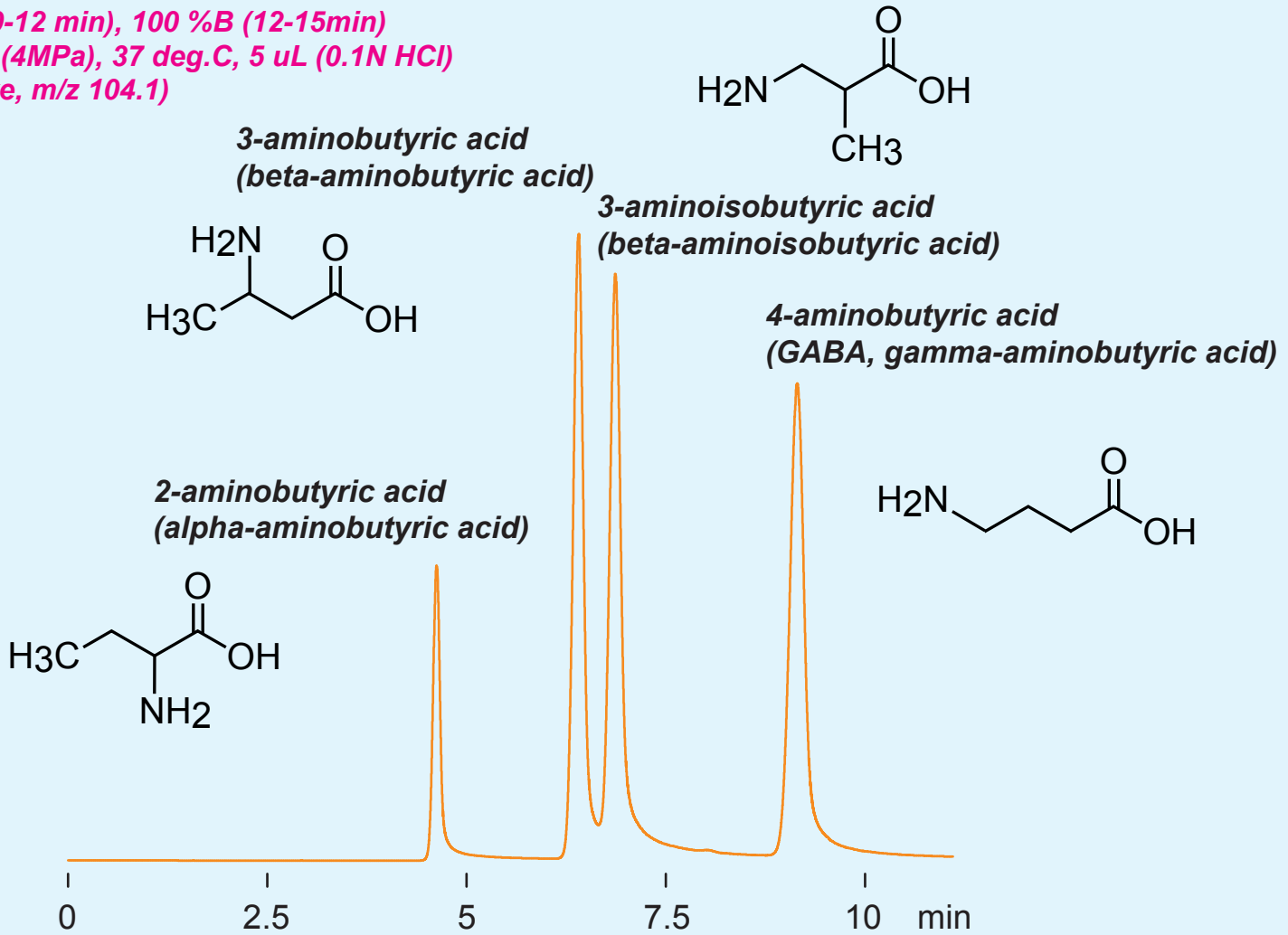
N-L-histidyl-L-leucine
(His-Leu)



The novel amino acid analysis column could be applied for dipeptides.

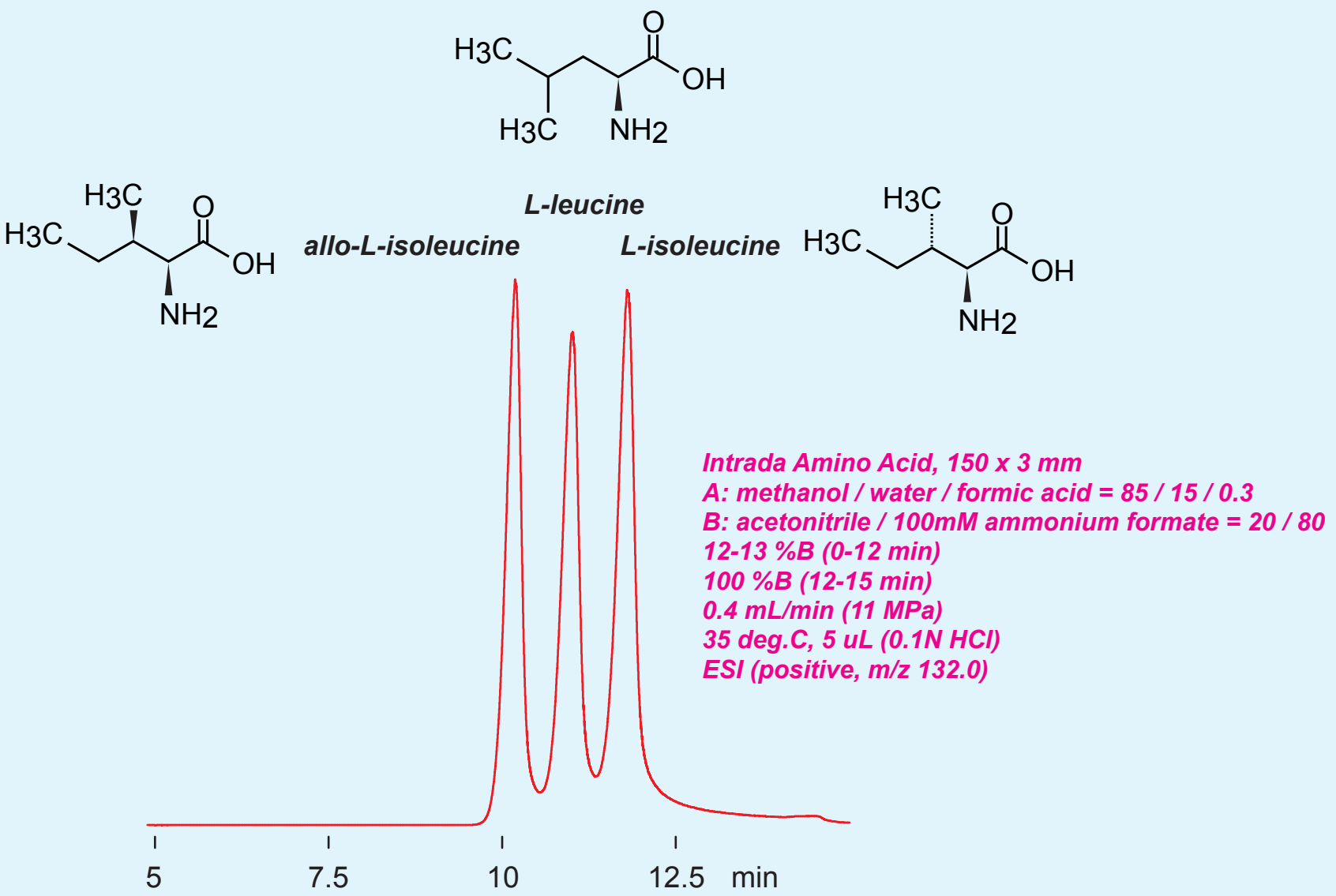
8 Alpha, Beta, Gamma Amino Acid (103Da) Isomers

Intrada Amino Acid, 100 x 3 mm
A: acetonitrile / formic acid = 100 / 0.3
B: 100mM ammonium formate
25-30 %B (0-12 min), 100 %B (12-15min)
0.4 mL/min (4MPa), 37 deg.C, 5 uL (0.1N HCl)
ESI (positive, m/z 104.1)



Not only alpha-amino acids but also beta- and gamma-amino acids isomers were well separated.

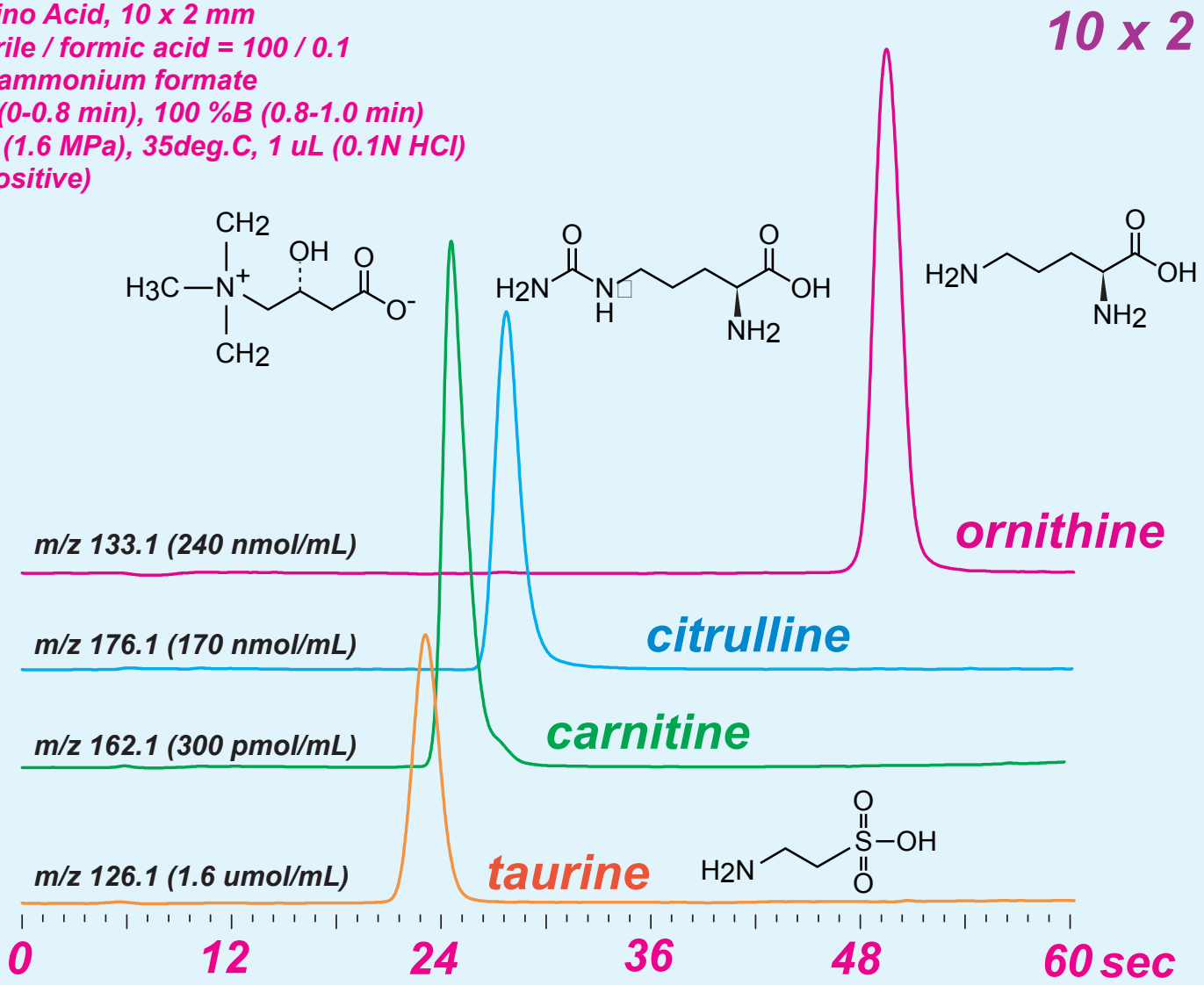
Leucine (131Da) isomers



Leucine isomers were well separated on 150mm length column.

One-Minute High Throughput Analysis

Intrada Amino Acid, 10 x 2 mm
A: acetonitrile / formic acid = 100 / 0.1
B: 100 mM ammonium formate
15-100 %B (0-0.8 min), 100 %B (0.8-1.0 min)
0.4 mL/min (1.6 MPa), 35deg.C, 1 uL (0.1N HCl)
ESI (SIM, positive)



Amino acid related compounds were analyzed by one-munite high throuhput separation with 10mm length small column.

Analytical conditions protocol

Mobile phase A:	*acetonitrile /HCOOH = 100 / (0.1 - 0.5), v/v*
Mobile phase B:	*(50-200mM) $HCOONH_4$*
Gradient:	*Initial - Final %B (Gradient Time)*
Flow rate:	*depends on column I.D.*
Temperature:	*30-40°C (up to 65°C)*
Injection solvent:	*0.1N HCl or 0.1 - 2% HCOOH*
MS detection:	*ESI, positive*