1.0 Purpose

The purpose of this procedure is to define the method for the determination of amino acids and similar compounds in raw materials and finished products by LC-MS.

2.0 Scope

This procedure applies to the determination of glycine, proline, methionine, hydroxyproline, glutamine, γ-aminobutyric acid, lysine, and ornithine in the QC laboratory at Ion Labs.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **LC-MS** – Liquid chromatography – mass spectrometry

4.2 **QC** – Quality control

4.3 **ACN** – Acetonitrile

4.4 **PVDF** – Polyvinylidene fluoride

4.5 **H_2O** – Deionized water (>18MΩ·cm)

4.6 **GLY** – Glycine

4.7 **PRO** – Proline

4.8 **MET** – Methionine

4.9 **HPR** – Hydroxyproline

4.10 **GABA** – γ-Aminobutyric acid

4.11 **GLUT** – Glutamine

4.12 **LYS** – Lysine

4.13 **ORN** – Ornithine

4.14 **SER** – Serine

5.0 References

5.1 PRTCL-21-0017, Protocol, Validation of an Analytical Method for the Determination of Glycine, Proline, Hydroxyproline, and Methionine

5.2 PRTCL-21-0055, Protocol, Validation of a Method for the Determination of GABA by LC-MS

5.3 PRTCL-22-0023, Protocol, Validation of a Method for the Determination of Glutamine by LC-MS

5.4 PRTCL-23-0018, Protocol, Validation of a Method for the Determination of Amino Acids and Similar Compounds by LC-MS

6.0 Supplies

6.1 Chemicals

6.1.1 Reference standards for the target analytes

6.1.2 Isotopically labelled amino acid standards

6.1.2.1 L-glutamine-$^{13}C_5$,$^{15}N_2$; Glycine-$^{13}C_2$,^{15}N; L-lysine-$^{13}C_6$,$^{15}N_2$; L-methionine-$^{13}C_5$,^{15}N; L-proline-$^{13}C_5$,^{15}N; L-serine-$^{13}C_3$,^{15}N

6.1.3 ACN (LC-MS grade)

6.1.4 Ammonium formate (LC-MS grade)

6.1.5 Formic acid (LC-MS grade)

6.1.6 Formic acid (HPLC grade)

6.1.7 Methanol (HPLC grade)

6.2 Glassware and Disposables

6.2.1 Volumetric glassware as required for standard and sample preparations

6.2.2 HPLC vials, 2mL with screw-cap enclosures and septa

6.2.3 Tips for adjustable pipettes

6.2.4 0.45 μm nylon or PVDF syringe filters

6.2.5 10-mL plastic syringes

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, and column oven

6.3.2 Agilent Ultivo mass spectrometer using MassHunter software

6.3.3 Analytical balance

6.3.4 Sonicator

6.3.5 Wrist action shaker

6.3.6 Adjustable pipette and tips

7.0 Preparation of Mobile Phase, Extraction Solvent, Standards, and Samples

7.1 Mobile Phase A (15 mM ammonium formate + 15 mM formic acid in ACN-H_2O 82-18)

7.1.1 100% Mobile Phase A is used for GABA, GLUT, GLY, HPR, MET, and PRO.

7.1.2 Transfer 180 mL of H_2O to a suitable container.

7.1.3 Add 0.95 g of ammonium formate (LC-MS grade).

7.1.4 Add 0.57 mL of formic acid.

7.1.5 Add 820 mL of ACN, and mix well.

7.2 Mobile Phase B (15 mM ammonium formate + 80 mM formic acid in ACN-H_2O 75-25)

7.2.1 100% Mobile Phase B is used for LYS and ORN.

7.2.2 Transfer 250 mL of H_2O to a suitable container.

7.2.3 Add 0.95 g of ammonium formate (LC-MS grade).

7.2.4 Add 3.0 mL of formic acid.

7.2.5 Add 750 mL of ACN, and mix well.

7.3 Extraction Solvent

7.3.1 Transfer 100 mL methanol to a suitable container.

7.3.2 Add 10.0 mL of formic acid (HPLC grade).

7.3.3 Add 900 mL of H_2O, and mix well.

7.4 Internal Standard Stock Solution (0.1 mM)

7.4.1 Prepare 40% methanol solution (scale as necessary).

7.4.1.1 Transfer 300 mL of H_2O to a suitably-sized beaker.

7.4.1.2 Add 200 mL of methanol, and mix well.

7.4.2 Prepare a solution containing a mixture of each isotopically labelled internal standard at about 0.1 mM in 40% methanol.

7.4.2.1 If using Cambridge Isotope Laboratories part number MSK-CAA-1, prepare the Internal Standard Stock Solution as follows:

7.4.2.1.1 Use several 5 mL portions of 40% methanol solution to dissolve and completely transfer the contents of the Canonical Amino Acid Mix standard into a 25-mL volumetric flask.

7.4.2.1.2 Dilute to volume using 40% methanol solution.

7.4.3 Store the solution at -20°C.

7.4.4 Equilibrate to room temperature, and mix well prior to use.

7.5 Working Internal Standard

7.5.1 Transfer 0.625mL of the Internal Standard Stock Solution to a 25-mL volumetric flask.

7.5.2 Dilute to volume using Mobile Phase, and mix well.

7.5.3 Store the solution at -20°C.

7.5.4 Equilibrate to room temperature, and mix well prior to use.

Note: Only prepare standards for analytes to be quantified.

7.6 Stock Standard A (250 mcg/mL PRO, HPR, MET, and GABA)

7.6.1 Accurately weigh and transfer about 25 mg each of PRO, HPR, MET, and GABA reference standards into a 100-mL volumetric flask.

7.6.2 Dissolve in and dilute to volume with H_2O.

7.7 Stock Standard B (500 mcg/mL GLUT, LYS, and ORN)

7.7.1 Accurately weigh and transfer about 25 mg each of GLUT, LYS, and ORN reference standards into a 50-mL volumetric flask.

7.7.2 Dissolve in and dilute to volume with H_2O.

7.8 Stock Standard C (500 mcg/mL GLY)

7.8.1 Accurately weigh and transfer about 25 mg of GLY into a 50-mL volumetric flask.

7.8.2 Dissolve in and dilute to volume with H_2O.

7.9 Intermediate Standard (2 mcg/mL PRO, HPR, MET, and GABA; 6 mcg/mL GLUT, LYS, and ORN; 20 mcg/mL GLY)

7.9.1 Transfer 2.0 mL of Stock Standard A to a 250-mL volumetric flask.

7.9.2 Add 3.0 mL of Stock Standard B.

7.9.3 Add 10.0 mL of Stock Standard C.

7.9.4 Dilute to volume with H_2O.

7.10 Working Standard (80 ng/mL PRO, HPR, MET, and GABA; 240 ng/mL GLUT, LYS, and ORN; 800 ng/mL GLY)

7.10.1 Transfer 2.0 mL of Intermediate Standard to a 50-mL volumetric flask.

7.10.2 Dilute to volume with Mobile Phase.

7.10.3 Combine 0.4 mL of the Working Standard with 0.4 mL of the Working Internal Standard in an HPLC vial, and vortex to mix.

7.11 Sample Preparation (5 mg/mL)

7.11.1 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, then follow preparation procedure as described below, maintaining concentration within the linear range of this method.

7.11.2 The default sample weight is 500 mg. Finished products with large dosage units and/or very small amount of target analyte may require larger sample size. For raw materials or finished products with a very large amount of target analyte, the sample size may be decreased with a minimum recommended weight of 50 mg.

7.11.3 Ensure that the sample is homogeneous prior to weighing.

7.11.3.1 For capsules, combine the fill material from at least 10 dosage units and homogenize in a mortar and pestle if necessary.

7.11.3.2 For tablets, combine at least 10 dosage units and homogenize in a mortar and pestle.

7.11.3.3 For chewable gels (gummies), homogenize at least 10 dosage units as outlined in D-793.

7.11.4 Transfer 500 mg of sample to a 100-mL volumetric flask.

7.11.5 Add about 65 mL of Extraction Solvent.

7.11.6 Sonicate 5 min.

7.11.7 Shake on a wrist-action shaker for 20 min.

7.11.8 Dilute to volume with Extraction Solvent.

7.11.9 If the sample is laden with particulate matter, filtration through a 0.45 µm membrane (discarding 2 – 3 mL before collecting the sample for further dilution) may be necessary prior to further dilution of the sample.

7.12 Working Sample Preparation

7.12.1 The linear range of the method for each analyte is listed below. The working sample preparation must be within the linear range of the method.

7.12.1.1 PRO, HPR, MET, and GABA: 25 – 125 ng/mL.

7.12.1.2 GLUT, LYS, and ORN: 80 – 400 ng/mL.

7.12.1.3 GLY: 250 -1250 ng/mL.

7.12.2 Dilute the *Stock Sample* to target concentration for each amino acid. Perform the final dilution using Mobile Phase. If multiple dilutions are required, perform intermediate dilutions using H_2O.

7.12.3 Filter through a 0.45 µm membrane (discarding 2 – 3 mL before collecting the sample for analysis). If the stock sample was filtered, filtration of the working sample is not required.

7.12.4 Combine 0.4 mL of the Working Sample with 0.4 mL of the Working Internal Standard in an HPLC vial, and vortex to mix.

7.13 Instrument Method Parameters

7.13.1 Place an HPLC vial containing a blank (diluent) in position P1-A1.

7.13.2 Place an HPLC vial containing the working standard in position P1-A2

7.13.3 Analyte that require different Source Parameters (Section 7.13.8.1.4) must be analyzed with separate acquisition methods

7.13.4 Column: Kinetex HILIC, 2.6 µm, 2.1 mm x 100 mm or equivalent

7.13.5 Sampler

7.13.5.1 Injection Volume: 4 µL

7.13.5.2 Enable Needle Wash: Selected

7.13.5.3 Mode: Flush Port

7.13.5.4 Time: 3 sec

7.13.5.5 Sample Flush-Out Factor: 5.0 times injection volume

7.13.5.6 Overlapped Injection Mode: Off

7.13.6 Binary Pump

7.13.6.1 Flow Rate: 0.4 mL/min

7.13.6.2 Mobile Phase: Isocratic

7.13.6.3 Stoptime: 5.5 min

7.13.7 Column Oven

7.13.7.1 Temperature: 35 °C

7.13.8 QQQ

7.13.8.1 Acquisition

7.13.8.1.1 Scan type: MRM

7.13.8.1.2 Polarity: Positive

7.13.8.1.3 Acquisition Parameters

Analyte	Int Std	MS1 Res	Precursor (m/z)	MS2 Res	Product (m/z)	Dwell (ms)	Frag (V)	CE (V)
GLY	GLY-IS	Unit	76.0	Unit	48.0	200	41	0
GLY-IS	N/A	Unit	79.0	Unit	50.0	200	41	0
PRO	PRO-IS	Unit	116.0	Unit	70.0	75	70	10
PRO-IS	N/A	Unit	122.0	Unit	75.0	75	70	10
HPR	SER-IS	Unit	132.0	Unit	68.0	180	80	14
SER-IS	N/A	Unit	110.0	Unit	63.0	75	60	7
MET	MET-IS	Unit	150.0	Unit	56.0	110	88	12
MET-IS	N/A	Unit	156.0	Unit	60.0	110	88	12
GABA	GLUT-IS	Unit	104.0	Unit	87.0	60	42	6
GLUT	GLUT-IS	Unit	147.1	Unit	84.0	160	60	15
GLUT-IS	N/A	Unit	154.4	Unit	89.0	160	60	15
LYS	LYS-IS	Unit	147.0	Unit	84.0	360	65	15
LYS-IS	N/A	Unit	155.0	Unit	90.0	360	65	15
ORN	LYS-IS	Unit	133.0	Unit	70.0	460	60	18

7.13.8.1.4 Source Parameters

Analytes	Source	Gas Temp (°C)	Gas Flow (L/min)	Nebulizer Pressure (psi)	Sheath Temp (°C)	Sheath Flow (L/min)	Capillary Voltage (V)	Nozzle Voltage (V)
GLY, GLY-IS, GLUT, GLUT-IS, GABA	AJS ESI	300	8	50	350	12	2500	100
PRO, PRO-IS, MET, MET-IS, HPR, SER-IS	AJS ESI	300	8	25	350	12	4500	500
ORN, LYS, LYS-IS	AJS ESI	300	8	25	350	12	3500	350

7.14 Retention Times

Analyte	Retention Time (min)
MET, MET-IS	1.8
HPR	2.9
SER-IS	2.9
PRO, PRO-IS	3.0
GLY, GLY-IS	3.2
ORN	3.3
GLUT, GLUT-IS	3.5
LYS, LYS-IS	3.6
GABA	4.0

7.15 Recommended Sequence

7.15.1 Make at least 3 injections of Diluent.

7.15.2 Make 5 injections of the Working Standard.

7.15.3 Make a single injection of each Working Sample.

7.15.4 Make a single injection of the Working Standard after every six samples and at the end of the run.

7.16 System Suitability Requirements

7.16.1 The %RSD for five consecutive injections of Working Standard is NMT 3%.

7.16.2 The %RSD for all injections of Working Standard is NMT 5%.

7.16.3 No significant (>0.5%) interference is present in the diluent injection.

7.17 Column Storage

7.17.1 Store the column in Mobile Phase.

7.18 Column Deep Clean and Storage

7.18.1 Cleaning Solution (100 mM ammonium formate in ACN-H_2O 10-90)

7.18.1.1 Transfer 450 mL of H_2O to a suitable container.

7.18.1.2 Add 3.854 g of ammonium formate (ACS reagent grade).

7.18.1.3 Add 50 mL of ACN, and mix well.

7.18.2 Deep clean the column at 0.2 mL/min with *Cleaning Solution* for at least 60 min.

7.18.3 Wash the column at 0.2 mL/min with H_2O/ACN (90/10) for at least 20 min.

7.18.4 Wash the column at 0.2 mL/min with Mobile Phase for at least 20 min.

7.18.5 Store the column in Mobile Phase.

7.19 System Wash

7.19.1 Perform system wash if the instrument will not be used for more than one week,

7.19.2 Remove the column.

7.19.3 Purge all lines with H_2O/ACN (50/50).

7.19.4 Open the instrument method.

7.19.5 Download the method to the instrument.

7.19.6 Pump H_2O/ACN (50/50) through the system, including the MS detector, for at least 30 min.

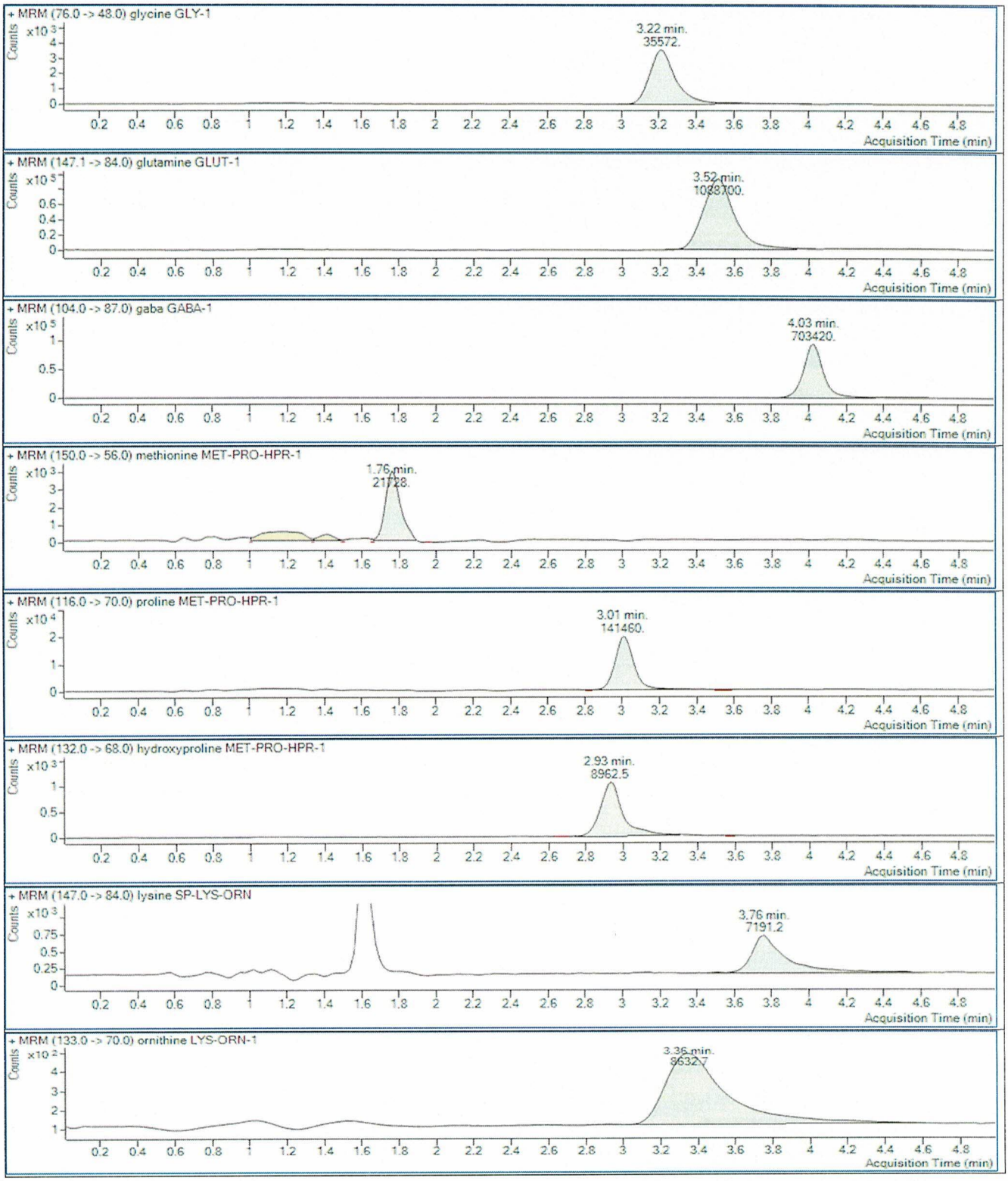
7.19.7 Put the instrument into standby mode.

8.0 Example Calculation

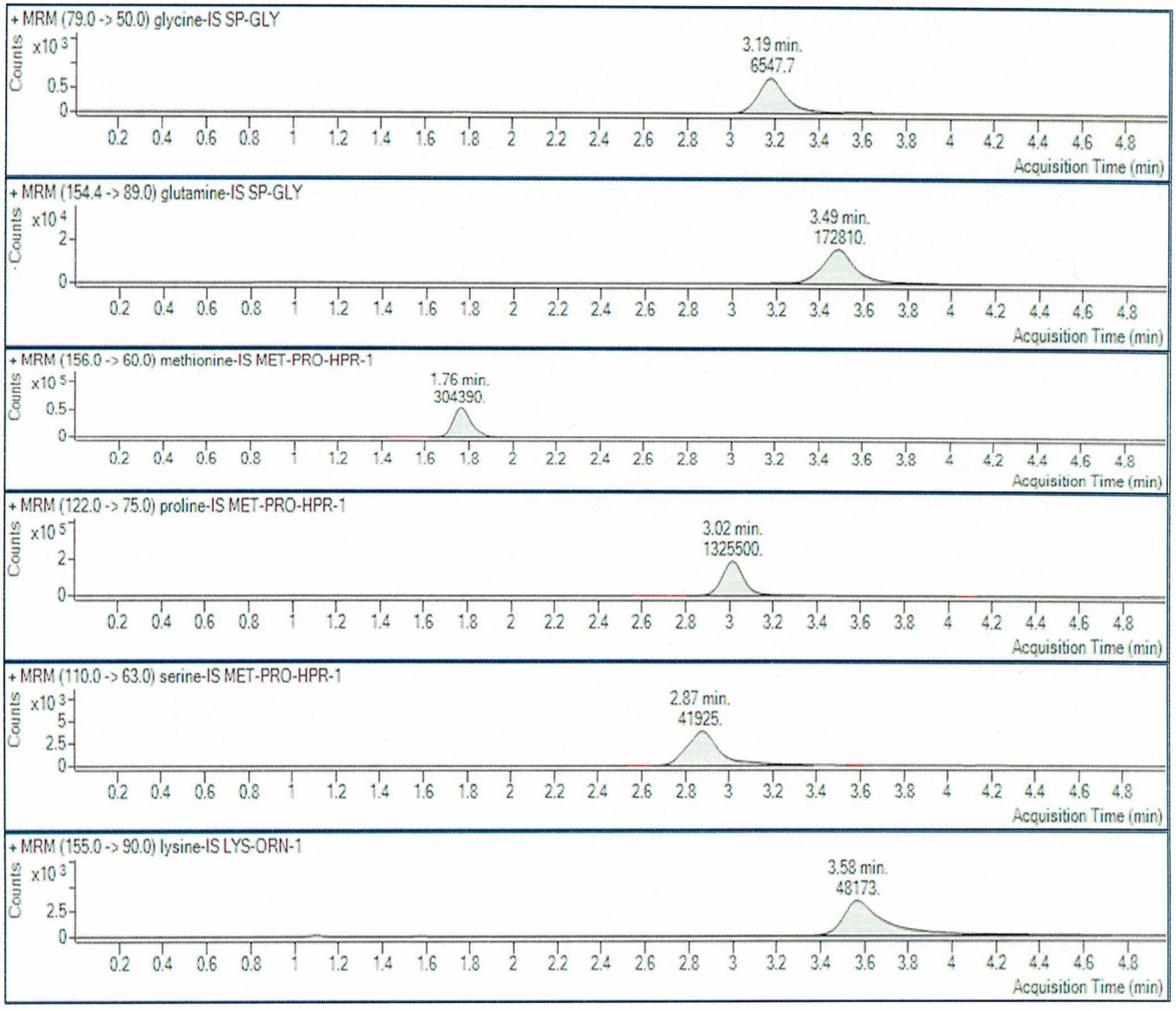
$$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{Spl_{wt}} \times \frac{V_{spl}}{LA} \times 100$$

R_u	Sample relative response
R_s	Mean standard relative response
Wt_{std}	Weight of reference standard in mg
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SS	Serving size: Weight of a single dosage unit in mg or 1 for raw materials.
Spl_{wt}	Sample weight in mg
V_{spl}	Volume of the sample preparation accounting for dilutions in mL
LA	Label amount in mg per dose or 1 for raw materials

9.0 Example Chromatograms for Target Analytes



10.0 Example Chromatograms for Internal Standards



10.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	09/28/21	New procedure.	N/A	S. Sassman
1	12/06/21	Add GABA as a target analyte. Adjust source parameters for proline, methionine, and hydroxyproline to improve linearity. Minor edits for clarity.	CC-21-0480	S. Sassman
2	01/19/23	Add glutamine as a target analyte.	CC-23-0034	S. Sassman
3	10/26/23	Change calibration approach from standard additions to internal standard, add lysine as an analyte, and combine with D-1005 (add ornithine as an analyte), change extraction solvent.	CC-23-0527	S. Sassman