

Tracing Nitrogen Metabolism in Mouse Tissues with Gas Chromatography-Mass Spectrometry

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[Abstract] Nitrogen-containing metabolites including ammonia, amino acids, and nucleotides, are essential for cell metabolism, growth, and neural transmission. Nitrogen metabolism is tightly coordinated with carbon metabolism in the breakdown and biosynthesis of amino acids and nucleotides. Both nuclear magnetic resonance spectroscopy and mass spectrometry including gas chromatography-mass spectrometry (GC MS) and liquid chromatography (LC MS) have been used to measure nitrogen metabolism. Here we describe a protocol to trace nitrogen metabolism in multiple mouse tissues using ¹⁵N-ammonia coupled with GC MS. This protocol includes detailed procedures in tracer injection, tissue preparation, metabolite extraction, GC MS analysis and natural abundance corrections. This protocol will provide a useful tool to study tissue-specific nitrogen in metabolically active tissues such as the retina, brain, liver, and tumor.

Keywords: GC MS, Mass spectrometry, Nitrogen metabolism, ¹⁵N tracing, Ammonia metabolism, Amino acids, Ammonia, Stable isotope tracer

[Background] Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry including mass spectrometry (GC MS) and liquid chromatography (LC MS) have been successfully used for system-wide metabolite measurements in various organisms (Fiehn, 2002 and 2016; Chokkathukalam *et al.*, 2014). However, each method has its limitations depending on the type of studies, which include absolute quantification, metabolite properties, sensitivity, robustness, isotope analysis and cost-effectiveness (Chokkathukalam *et al.*, 2014). Compared to NMR, MS-based approaches are more commonly used as a result of their higher sensitivity and metabolite coverage. Stable isotope labeling coupled with LC MS or GC MS allows for sensitively quantifying dynamic metabolic changes in healthy and diseased tissue or cells (Jang *et al.*, 2018). LC MS coupled with stable nitrogen isotope reveals how glutamine nitrogen metabolism coordinates with carbon metabolism in cancer cells (Wang *et al.*, 2019). GC MS has superior chromatographic resolution and cost-effectiveness (Jang *et al.*, 2018). Here we have developed a robust method to quantify nitrogen-derived metabolites using stable nitrogen isotope coupled with GC MS. We identified several key metabolic features in the retina with this method, including the metabolic communications within the neural retina and between the retina and retinal pigment epithelium (RPE) (Albertino *et al.*, 1989; Du *et al.*, 2015 and 2016; Grenell *et al.*, 2019; Yam *et al.*, 2019). Recently, we used ¹⁵N-ammonia to trace nitrogen metabolism *in vivo* in mice and revealed tissue-specific metabolic pathways (Xu *et al.*, 2020). In this protocol, we described procedures in

nitrogen tracer injection in mice, tissue preparation, metabolite extraction, GC MS sample preparation, instrument analysis, and natural abundance corrections.

Materials and Reagents

1. 2 ml microtube
2. 1.5 ml tube
3. Syringe with gauge 26 needle (BD, catalog number: 9304308)
4. Syringe for 0.2 µm syringe filter (BD, catalog number: 7212651)
5. ALS Syringe for GC injection (Agilent Technologies, catalog number: 5181-3354)
6. Microtubes (Axygen Scientific, catalog number: 02820037 for 1.5 ml and catalog number: 12018028 for 2 ml)
7. 0.2 µm syringe filter (Thermo Scientific, catalog number: 00293112)
8. Cap for GC MS vial (Agilent Technologies, catalog number: 5182-0717)
9. Glass inserts (Agilent Technologies, catalog number: 5181-2085, specification: 250 µl)
10. Disposable pestle (Argos Technologies, catalog number: 7339-901)
11. C57 B6/J mouse (Jackson Lab, catalog number: 664)
12. Ethanol (Fisher Chemical, catalog number: 172382, LOT-specific concentration: 96%)
13. Liquid nitrogen
14. Methanol (Fisher Chemical, Optima™ LC/MS Grade, catalog number: 164905)
15. HPLC Water (Fisher Chemical, Optima™ LC/MS Grade, catalog number: 7732-18-5)
16. Hexane (Sigma-Aldrich, HPLC Grade, catalog number: MKCF5755)
17. Methylene Chloride (Fisher Chemical, catalog number: 163938)
18. Helium Gas (MATHESON, catalog number: HE UHP1A)
19. Pyridine (Sigma-Aldrich, catalog number: SHBK1583)
20. N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (TBDMS) (Sigma-Aldrich, catalog number: 394882)
21. Ammonium-¹⁵N Chloride (¹⁵NH₄Cl) (Sigma-Aldrich, catalog number: 229251)
22. Amino Acid Standard mix (Sigma-Aldrich, catalog number: AAS18-5 ml)
23. 10× Phosphate Buffered Saline (PBS) (Fisher Chemical, catalog number: 10010049)
24. Hank's Balanced Salt Solutions (HBSS) (Fisher Chemical, catalog number: 14170112)
25. EDTA (Fisher Chemical, catalog number: 6N011324)
26. ¹⁵NH₄Cl- solution (see Recipes)
27. Extraction buffer (see Recipes)
28. Internal Standard (see Recipes)
29. Methoxyamine mix (see Recipes)

Equipment

1. Dumont Tweezer, Style 5 (Electron Microscopy Sciences, catalog number: 0108-5-PO)
2. -20 °C refrigerator
3. Dissecting microscope (Zeiss, model: Stemi 2000-C)
4. FirstHand Surgical Instrument Kits for Mice and Rats (Kent Scientific Corporation, model: INSMOUSEKIT)
5. Battery-Operated Pestle Motor Mixer (Argos Technologies, catalog number: EW-44468-25)
6. Omni Tissue Homogenizer (115V 125W) (OMNI International the Homogenizer Company, THB-01)
7. Gas chromatograph-mass spectrometer (Agilent Technologies, model: 7890B/5977B GC-MS)
8. DB-5ms GC Column (length 30 m, id 0.25 mm, film thickness 0.25 µm) (Agilent Technologies, catalog number: 122-5532)
9. Gel Pump (Savant Instruments, GP110)
10. Speed vac Plus (Savant Instruments, SC110A)
11. Centrifuge (Eppendorf, catalog number: 5424)
12. Thermomixer (Eppendorf ThermoMixer C, 5382000015)

Software

1. MassHunter Workstation Software (Agilent Version B.07.01)
2. MS Quantitation software (Agilent Version B.07.01/Build 7.1.524.0)
3. Python 2.6+ (<http://www.pythonxy.com>)
4. IsoCor Software (<http://metasys.insa-toulouse.fr/software/isocor>)

Procedure

Figure 1 is an overview of the procedures in this protocol.

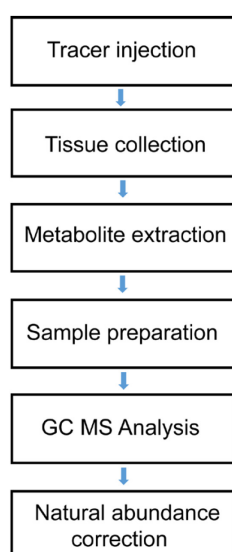


Figure 1. The flow chart for this protocol. Animals are injected with the ^{15}N tracer, and metabolites are extracted to analyze for ^{15}N -labeled metabolites using GC MS.

A. Tracer Injection

1. Freshly prepare $^{15}\text{NH}_4\text{Cl}$ in PBS (see Recipe 1).
2. Weigh mice and calculate the volume for the injection.
3. Intraperitoneal injection (IP) of $^{15}\text{NH}_4\text{Cl}$ at 167 mg/kg or the same volume of PBS.

B. Tissue collection

1. Mice were quickly sacrificed with cervical dislocation at different time points after injection. For example, 0 min, 5 min, 15 min, 30 min and 60 min.

Note: Different metabolites can reach their peak enrichment at different time points depending on the tissue.

2. Enucleate mouse eyes and isolate neural retina and the eyecups under dissecting microscope in cold HBSS. Store the neural retina in a pre-weighed 1.5 ml microtube and the eyecup in a pre-weighed 2 ml microtube. Weigh the tissues and snap-freeze in liquid nitrogen.
3. Perform cardiac puncture to withdraw 100-300 μl blood using a syringe with a 26G needle. Gently dispense the blood to EDTA-containing tubes. Place the blood samples on ice. Centrifuge the blood at $1,008 \times g$ for 10 min at 4°C , transfer the supernatant into a new 1.5 ml tube, and store supernatant in -20°C refrigerator.
4. Quickly remove brain and liver tissue, store them in a pre-weighed 2 ml microtube and snap-freeze them in liquid nitrogen.

Note: Take out the whole brain to avoid heterogeneity from different regions. Cut a small piece of liver tissue (~40 mg) from the same lobe with scissors.

C. Metabolite Extraction

1. Tissue metabolite extraction (Figure 2)

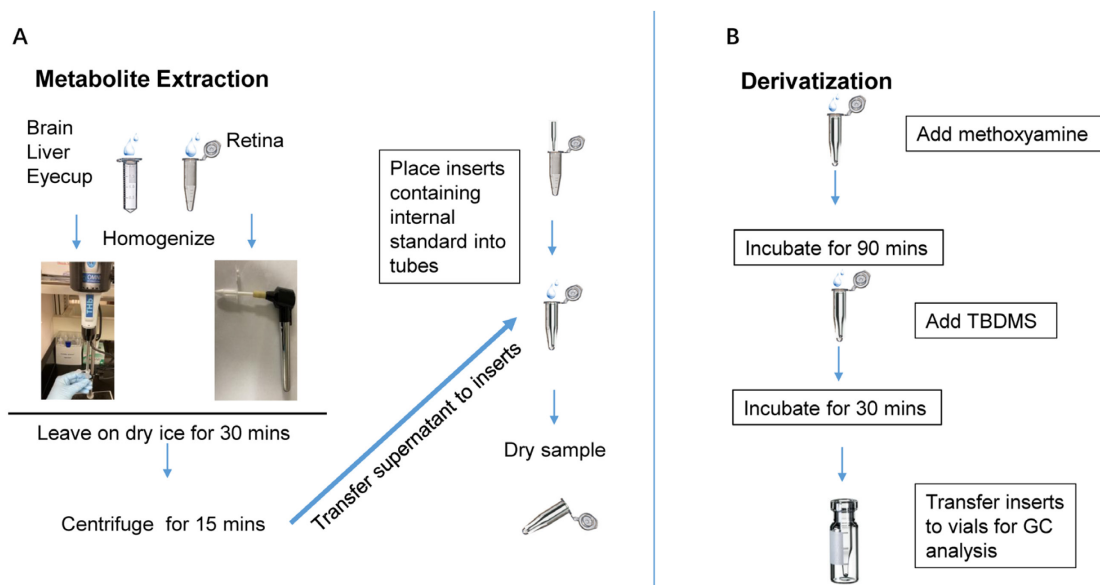


Figure 2. A schematic for metabolite extraction and derivatization. A. The brain, liver and eyecups were homogenized with an Omni THb Homogenizer, while the retina was homogenized with a pestle motor mixer. The homogenates were left on dry ice for 30 min and then centrifuged at $25,200 \times g$ at 4°C for 15 min. The supernatant was transferred to a glass insert containing 5 μl internal standard and dried with a speed vacuum. B. Add 10 μl of freshly prepared methoxyamine (20 mg/ml) into the dried samples in the insert and incubate for 90 min at 37°C in a thermomixer, followed by a 30 min incubation at 70°C after the addition of 30 μl of TBDMS. Transfer each insert into vials for GC MS analysis.

- Pre-chill extraction buffer (Recipe 2) on dry ice for 10 min.
- Transfer pre-chilled extraction buffer into tubes with tissues. Homogenize neural retina with a handheld pestle motor mixer in 140 μl extraction buffer for 15-20 s; Homogenize eyecup with an Omni THb Homogenizer in 200 μl extraction buffer for 15-20 s; Homogenize brain or liver tissues with the Omni THb Homogenizer for 20-30 s in extraction buffer (add 200 μl extraction buffer for every 5 mg tissues).
- Centrifuge the samples at $25,200 \times g$ at 4°C for 15 min.
- Filter the supernatants with a 0.2 μm syringe filter.
- Add 5 μl internal standard (Recipe 3) to each glass insert in 1.5 ml tube.
- Transfer the supernatant to each glass insert, open the tube lid to dry in a Speed vac in the cold room.

Note: To ensure high sensitivity without overloading, transfer all the supernatant from retina or eyecup samples to dry and transfer 50 μl of brain or liver tissues to dry. Make sure the samples were fully dried. The moisture can affect the efficiency in derivatization and

ionization.

2. Plasma metabolite extraction

- Mix 10 μ l plasma sample with 40 μ l pre-chilled extraction buffer.
- Leave the mixture on ice for 15 min.
- Centrifuge the samples and transfer 10 μ l into inserts with internal standard as described in tissue metabolite extraction.

D. Sample preparation (Figure2)

- Freshly prepare methoxyamine mix (Recipe 4) and add 10 μ l to each insert with the dried sample inside a 1.5 ml microtube. Mix and close the lid, then incubate the tube at 37 °C and 300 RPM for 90 min in a thermomixer.

Note: Gently tap the tubes with inserts inside 3-5 times to mix, spin the samples down and close the lid tightly.

- After incubation, quickly spin the tubes and add 30 μ l TBDMS to each sample. Incubate at 70 °C for 30 min.
- Spin down the tubes and transfer each insert into GC MS glass vials.

E. GC-MS Analysis

1. Pre-run preparation

- Install GC MS with DB-5 MS column and syringe needle.
- Set up a flow rate of helium gas at 1 ml/min.
- Fill the needle washing solvent vials A and B with hexane and methylene chloride respectively.
- Set up the GC oven temperature gradient as **Table 1**. The total run for each sample takes 31.5 min.

Table 1. The parameters for GC oven temperature

	Rate°C/min	°C	Hold time/min	Run Time/min
Initial	0	95	2	2
Ramp1	10	270	0	19.5
Ramp2	5	300	6	31.5

- Set up the parameters for GC MS scan range, speed, frequency, cycle time and step size as **Table 2**. Choose the solvent delay for 5.4 min.

Table 2. The parameters for GC MS scan range and speeder parameters on GC-MS

Start (m/z)	Mass	End (m/z)	Mass	Threshold	Scan (u/s)	Speed	Frequency (scans/sec)	Cycle Time (ms)	Step Size (m/z)
80		600		150	1.562		2.8	355.42	0.1

- f. Set up parameters for selection monitoring (SIM) mode as **Table 3** for the ions of each metabolite that will be monitored.

Table 3. List of ions for metabolites that are monitored under SIM mode

Metabolite	SIM Time Segments (min)	Monitored ion (m/z)	RT time (Min)	Polarity
Alanine M0	8.4-11.10	260.2	10.250	Positive
Alanine M1		261.2	10.250	Positive
Valine M0	11.10-11.80	186.2	11.473	Positive
Valine M1		187.2	11.473	Positive
Urea M0		231.1	11.627	Positive
Urea M1		232.1	11.632	Positive
Leucine M0	11.80-13.90	200.2	12.081	Positive
Leucine M1		201.2	12.081	Positive
Isoleucine M0		200.2	12.435	Positive
Isoleucine M1		201.1	12.435	Positive
GABA M0		274.2	12.800	Positive
GABA M1		275.2	12.800	Positive
Proline M0		184.1	12.876	Positive
Proline M1		185.1	12.876	Positive
5-Oxoproline M0	13.90-16.90	300.2	14.774	Positive
5-Oxoproline M1		301.2	14.774	Positive
Myristic acid-D27		312.4	15.959	Positive
Aspartate M0		418.3	16.667	Positive
Aspartate M1		419.3	16.667	Positive
Glutamate M0	16.90-18.30	432.3	17.722	Positive
Glutamate M1		433.3	17.722	Positive
Asparagine M0		417.2	17.954	Positive
Asparagine M1		418.2	17.961	Positive
Asparagine M2		419.2	17.968	Positive
Glutamine M0	18.30-20.80	431.3	18.998	Positive
Glutamine M1		432.3	18.998	Positive
Glutamine M2		433.3	18.998	Positive

Note: *Isotopologues* are named as M0, M1, M2. M0 is the mass without labeling, and 1 to 2 represents the mass shift from the isotope labeling.

- g. Set up injection volume as 1 µl of the sample in split-less mode.
 - h. Save these parameters as “GC MS nitrogen method”.
 - i. Tune GC MS with autotune to check mass accuracy and ensure no leakage in the system.
- Note:** *Air leakage and dirty source in the system can significantly decrease the sensitivity. It is critical to tune the system weekly with regular replacement of the air trap and source maintenance.*

2. Run sample

- a. Place samples in the auto-sampler.

- b. Fill the sequence table for sample names and vial names. Select GC MS nitrogen method.
- c. Run samples with GC MS.

Note: Check the instrument that it runs properly, especially for the long run. The typical instrument running failure includes an improperly filled sequence table, defective syringe needle and malfunctional filament.

3. GC MS data processing
 - a. Set up a quantitation method using Agilent MS Quantitation software based on **Table 3**.
 - b. Click “File” in the software to set up a new batch.
 - c. Select the quantitation method and extract the peak area for each selected ion.
 - d. Export the peak area into an excel file.

F. Nature abundance correction

1. Nature abundance correction
 - a. Install IsoCor for natural abundance correction software
Download IsoCor software at <http://metasys.insa-toulouse.fr/software/isocor>; Download Python 2.6+ (<http://www.pythonxy.com>) and install modules: wxPython (v 2.8.11.0), NumPy(v1.6.0.2), SciPy(v0.9.0.1).
 - b. Set up parameters for IsoCor software
 - c. Edit “Metabolites. dat” file. Open the “Metabolites.dat” file under the IsoCor folder with Notepad and input metabolites and their elemental formula in **Table 4**. Save the file in the same folder.

Table 4. List of metabolites and their elemental formula for natural abundance correction

Metabolite	Metabolite formula
Alanine	C3H5NO2
Asparagine	C4H5N2O3
Aspartate	C4H4NO4
GABA	C4H7NO2
5-Oxoproline	C5H5NO3
Glutamine	C5H7N2O3
Glutamate	C5H6NO4
Leucine	C5H11N
Isoleucine	C5H11N
Proline	C4H7N
Valine	C4H9N
Urea	CH2N2O

- d. Edit “Derivatives. dat” file. Using Notepad to edit this file and input the chemical derivatives with their elemental formulas as **Table 5**. Set up the input data file as **Table 6** and copy the GC MS peak area (ion intensity) into this table. Save the file in the same folder.

Table 5. List of chemical derivatives with their elemental formula

OH, RH, NH	Derivative formula
TBDMS1	SiC ₃ H ₉ N
TBDMS2	Si ₂ C ₈ H ₂₁
TBDMS3	Si ₃ C ₁₄ H ₃₆

TBDMS1, 2 or 3 represents the number of TBDMS in the derivative metabolite.

2. Data correction

- Save the Excel Input data file (**Table 6**) as “xxx. txt.”
- Open IsoCor software and select Isotopic tracer as “N”.
- Select the purity of the tracer as (0.02; 0.98).

Note: The purity is dependent on the tracer you are using. For example, the purity of ¹⁵NH₄Cl is 98%. It is represented as (0.02; 0.98).

- Load the input data file using the “Load multiple means” button.
- Click on the “Process” button.
- Open a new Excel file and load Input data file_res.txt.

*Note: The output of the calculations is automatically saved in a .txt file as (Input File_res.txt) and (Input File_log.txt). Save them in the same folder. **Table 7** is a representative output file after natural abundance correction.*

Table 6. Template of Input data file for IsoCor

Sample	Metabolite	Derivative	Intensity
sample_1	Alanine M0	TBDMS1	4821208.225
	Alanine M1		5773531.695
sample_2	Asparagine M0	TBDMS3	89770.35027
	Asparagine M1		33267.51246
	Asparagine M2		15328.33784
sample_3	Aspartate M0	TBDMS3	865396.4189
	Aspartate M1		741854.1983
sample_4	GABA M0	TBDMS2	25107.35886
	GABA M1		8231.653447
sample_5	5oxoproline M0	TBDMS2	2196180.185
	5oxoproline M1		1400557.312
sample_6	Glutamine M0	TBDMS3	537705.6402
	Glutamine M1		1067577.981
	Glutamine M2		913543.2505
sample_7	Glutamate M0	TBDMS3	2152451.52
	Glutamate M1		1812623.576
sample_8	Leucine M0	TBDMS2	786491.624
	Leucine M1		143679.7564
sample_9	Isoleucine M0	TBDMS2	264237.4641
	Isoleucine M1		51663.9062
sample_10	Proline M0	TBDMS1	728178.3335
	Proline M1		133343.8743
sample_11	Valine M0	TBDMS1	512845.4033
	Valine M1		86070.23479
sample_12	Urea M0	TBDMS2	21840799.71
	Urea M1		11696209.27
	Urea M2		8180923.957

The intensity is representative data from the extracted peak area for specifically monitored ion.

Data analysis

Representative data

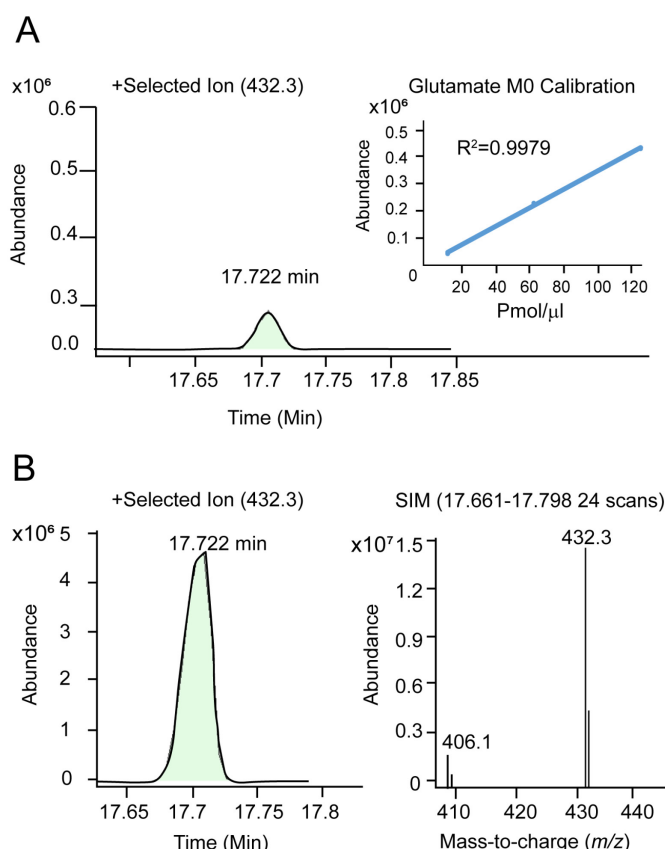


Figure 3. GC MS chromatogram from standards and mouse tissue samples. A. GC MS chromatogram for M0 glutamate (m/z 432.3) from an amino acid standard mix. The calibration curve for M0 glutamate was calculated using the standard mix. B. The chromatogram of M0 glutamate (m/z 432.3) was extracted from the TIC in mouse retina sample received PBS injection.

Figure 3 is the representative GC MS chromatogram from standards and mouse tissue samples. Table 7 is the representative GC MS data that after natural abundance correction from liver tissue after injection with $^{15}\text{NH}_4\text{Cl}$. Isotopologue distribution is the enrichment of ^{15}N from the tracer. Except for branch chain amino acids including leucine, isoleucine, and valine, all the other metabolites reach their peak enrichment at 5 min after single tracer injection. The enrichment drops at 15 min due to metabolic degradation.

Table 7. The representative GC MS data of isotopologue distribution of liver tissue at 5 min and 15 min after natural abundance correction Isotopologue distribution

Compound	m/z	Ion Intensity (5 min after injection)	Isotopologue distribution (5 min after injection)	Ion Intensity (15 min after injection)	Isotopologue distribution (15 min after injection)
Alanine M0	260.2	4821208.225	49.88%	2257842.271	80.45%
Alanine M1	261.2	5773531.695	50.12%	1053703.993	19.55%
Asparagine M0	417.2	89770.35027	99.06%	37073.63075	99.78%
Asparagine M1	418.2	33267.51246	0.89%	13418.86764	0.02%
Asparagine M2	419.2	15328.33784	0.05%	6269.716755	0.20%
Aspartate M0	418.3	865396.4189	66.11%	270761.2367	86.29%
Aspartate M1	419.3	741854.1983	33.89%	139189.8465	13.72%
GABA M0	274.2	25107.35886	91.79%	13133.96644	93.02%
GABA M1	275.2	8231.653447	8.21%	4119.768265	6.98%
5-Oxoproline M0	300.2	2196180.185	71.61%	1466756.09	88.43%
5-Oxoproline M1	301.2	1400557.312	28.39%	556339.8502	11.57%
Glutamine M0	431.3	537705.6402	27.39%	416561.225	56.54%
Glutamine M1	432.3	1067577.981	45.32%	423609.1074	37.27%
Glutamine M2	433.3	913543.2505	27.29%	213795.4126	6.18%
Glutamate M0	432.3	2152451.52	67.32%	2396114.521	86.99%
Glutamate M1	433.3	1812623.576	32.68%	1236353.483	13.01%
Leucine M0	200.2	786491.624	100.00%	352411.3588	100.00%
Leucine M1	201.2	143679.7564	0.00%	64354.7646	0.00%
Isoleucine M0	200.2	264237.4641	100.00%	128111.7529	100.00%
Isoleucine M1	201.1	51663.9062	0.00%	24144.7874	0.00%
Proline M0	184.1	728178.3335	95.45%	277198.2798	93.28%
Proline M1	185.1	133343.8743	4.55%	57384.5729	6.72%
Valine M0	186.2	512845.4033	96.92%	241972.5894	96.78%
Valine M1	187.2	86070.23479	3.08%	40968.99562	3.22%
Urea M0	231.1	21840799.71	64.42%	12633751.17	73.00%
Urea M1	232.1	11696209.27	20.87%	6130869.441	20.27%
Urea M2	233.1	8180923.957	14.71%	2949020.391	6.73%

Recipes

- ¹⁵NH₄Cl- solution
Weigh ammonium-¹⁵N Chloride and dissolve in filtered 1× PBS at 33 mg/ml
- Extraction buffer
Mix methanol and HPLC water at 80:20 (Vol:Vol)
- Internal Standard
Weigh myristic acid-D27 powder and dissolve in Isopropanol: methanol: HPLC water mixture at 2:5:2 ratio (Vol:Vol:Vol)
- Methoxyamine mix
Dissolve methoxyamine hydrochloride in pyridine solution at 20 mg/ml

Note: Take the pyridine solution with a syringe needle through the sealed rubber lid to avoid moisture.

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Competing interests

The authors declare no conflicts of interest.

Ethics

Mouse experiments were performed in accordance with the National Institutes of Health guidelines and the protocol (#1611004455, 01/18/2020-01/17/2023) was approved by the Institutional Animal Care and Use Committee of West Virginia University.

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