PROTOCOL MSMC-002 version 1.1
PROTOCOL: FREE AMINO ACID EXTRACTION
Contributed by staff of MSU Mass Spectrometry and Metabolomics Core
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Purpose: for quantification of free amino acids in biological samples. Extraction method is suitable for GC/MS or LC/MS/MS analysis.

Materials and equipment:

- Milli-Q water
- QIAGEN TissueLyser or another method for tissue disruption (e.g. 3 mm stainless steel ball bearings, VXB.com, Cat#Kit12064)
- Benchtop centrifuge with rotor for 1.7 ml microfuge tubes or 96 well plates
- Hot water bath (with heating capability to 90°C)
- Centrifugal low-binding hydrophilic PTFE 0.2 μM filters (individual sample filters: Millipore, Cat# UFC30LG25; 96-well plate filters, Millipore Cat# MSRLN0410 (0.5 ml) or MDRLN0410 (1.9 ml)
- Unlabeled amino acid standards (available from the MSMC)
- $^{13}C,^{15}N$ stable isotope-labeled amino acid internal standards (available from the MSMC or from Sigma, Cat# 767964)
- Autosampler vials, caps and inserts (available in BMB stores); large sample sets can be prepared in skirted PCR microtiter plates (BMB Stores: Denville, C18080)(sealing covers: Analytical Sales and Service, Cat# 961801)
- Analytical balance
- Labels (pre-printed or barcoded) for vials
- PFHA = perfluoroheptanoic acid (Sigma cat# 342041)

Extraction solvent preparation:
Water containing labeled amino acid standards will be used as the extraction solvent. The Sigma labeled amino acid internal standard (IS) mix contains all 20 amino acids at various concentrations that differ according to batch. For the current MSMC amino acid IS stocks, redissolve the ~ 0.2 μmol dried aliquots in 1 ml of Milli-Q water to give a 100X stock. Dilute to 1X in Milli-Q water for use in preparing samples.

[*The 0.2 μmol amount is based on assuming 20 mM starting concentration in the mix from Sigma. Some amino acids are at higher concentration than this and some lower but all should be detectable once diluted to give an approx. 2 μM working concentration.*]

- If cysteine measurement is important, addition of a reducing agent (20 μM dithiothreitol, DTT) to reduce cysteine disulfides can be added but only after protein is removed (to prevent release of protein-cysteine disulfides).

Tissue collection:
For plant tissue (leaf), determine the fresh weight of the sample then flash freeze tissue in liquid nitrogen. Typically collect approximately 20 mg of fresh tissue.

[*Amino acid levels can change quickly with changes in light conditions and can fluctuate depending on time of day, so be sure to be consistent with handling of plants prior to freezing of tissue*.]

For other types of samples, will need to determine empirically or from literature the amount of sample needed for extraction to give free amino acid concentrations in the range needed for the analysis.

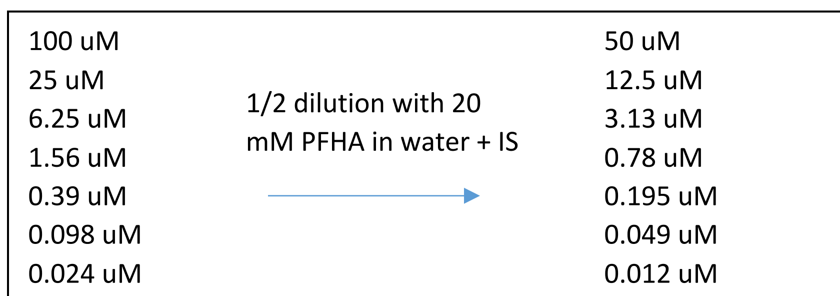
- For bacteria samples from a growing culture, after spinning down cells it is best to wash the cells with 150 mM aqueous ammonium formate before extraction to remove residual growth media which contains salts and possibly amino acids that will interfere with the analysis.

Sample preparation:

1) Homogenize the frozen tissue using whatever means available (Qiagen TissueLyser, paint shaker with stainless steel bead in the tube, polypropylene pestle, etc).
   a. For analysis of bacteria cells, this step is skipped and hot water extraction step #2 is sufficient.
2) Add 400. µL of extraction solvent containing labeled standards using a 1000-µL pipetter. Incubate promptly in hot water bath at 90°C for 5 min. Cool on ice.
3) Centrifuge 10 min, 13,000 x *g* at 4°C
4) Filter supernatant through low-binding hydrophilic PTFE filter units
5) Mix an equal volume of sample and 20mM PFHA in water (i.e. 100 µl sample + 100 µl of 20 mM PFHA in water) – this is to have a final concentration of 10 mM PFHA in the extract for best chromatographic retention and separation of early eluting amino acids.
   a. Do not include any internal standards in the 20 mM PFHA solution. If 2 µM internal standards are included in the 20 mM PFHA, then a correction factor (multiply X2) has to be applied to the final concentration data from Quanlynx.
6) Transfer sample to LC autosampler vials (available at BMB stores)
7) Store samples at -20˚ C (or -80˚ C) until ready to analyze by LC/MS/MS

Amino acid standard curve

1) Prepare a stock solution of each unlabeled amino acid at 5 mM in water (amino acid standards available at MSMC)
   [*Some amino acids are more difficult to dissolve than others and require some heating or formic acid to help to dissolve*]
2) Combine 100 µl of each 5 mM amino acid stock and then add Milli-Q water to bring final volume to 5 ml to give a final concentration of 100 µM
3) Make serial 1/4 dilutions starting from 100 µM, then make 1/2 dilutions of each sample using 20 mM PFHA in water containing 4 µM labeled internal standards (final concentration of internal standards is then 2 µM)



Notes:

To avoid multiple freeze/thaw cycles of standards, aliquot 100 µl aliquots of the standard curve samples and use only once.

Other non-protein amino acids (i.e. hydroxyproline, ornithine, homocysteine, etc.) can also be analyzed in the samples provided that standards are available.

Amino Acid masses

1-letter code	3-letter code	Chemical formula	Avg MW	mg to make 20 ml of 5 mM stock
A	Ala	$C_3H_7NO_2$	89.09	8.9
R	Arg	$C_6H_{14}N_4O_2$	174.2	17.4
N	Asn	$C_4H_8N_2O_3$	132.12	13.2
D	Asp	$C_4H_7NO_4$	133.10	13.3
C	Cys	$C_3H_7NO_2S$	121.16	12.1
E	Glu	$C_5H_9NO_4$	147.13	14.7
Q	Gln	$C_5H_{10}N_2O_3$	146.14	14.6
G	Gly	$C_2H_5NO_2$	75.07	7.5
H	His	$C_6H_9N_3O_2$	155.15	15.5
I	Ile	$C_6H_{13}NO_2$	131.17	13.1
L	Leu	$C_6H_{13}NO_2$	131.17	13.1
K	Lys	$C_6H_{14}N_2O_2$	146.19	14.6
M	Met	$C_5H_{11}NO_2S$	149.21	14.9
F	Phe	$C_9H_{11}NO_2$	165.19	16.5
P	Pro	$C_5H_9NO_2$	115.13	11.5
S	Ser	$C_3H_7NO_3$	105.09	10.5
T	Thr	$C_4H_9NO_3$	119.12	11.9
W	Trp	$C_{11}H_{12}N_2O_2$	204.23	20.4
Y	Tyr	$C_9H_{11}NO_3$	181.19	18.1
V	Val	$C_5H_{11}NO_2$	117.15	11.7