

## Video Article

# One-step Metabolomics: Carbohydrates, Organic and Amino Acids Quantified in a Single Procedure

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## Abstract

Every infant born in the US is now screened for up to 42 rare genetic disorders called "inborn errors of metabolism". The screening method is based on tandem mass spectrometry and quantifies acylcarnitines as a screen for organic acidemias and also measures amino acids. All states also perform enzymatic testing for carbohydrate disorders such as galactosemia. Because the results can be non-specific, follow-up testing of positive results is required using a more definitive method. The present report describes the "urease" method of sample preparation for inborn error screening. Crystalline urease enzyme is used to remove urea from body fluids which permits most other water-soluble metabolites to be dehydrated and derivatized for gas chromatography in a single procedure. Dehydration by evaporation in a nitrogen stream is facilitated by adding acetonitrile and methylene chloride. Then, trimethylsilylation takes place in the presence of a unique catalyst, triethylammonium trifluoroacetate. Automated injection and chromatography is followed by macro-driven custom quantification of 192 metabolites and semi-quantification of every major component using specialized libraries of mass spectra of TMS derivatized biological compounds. The analysis may be performed on the widely-used Chemstation platform using the macros and libraries available from the author. In our laboratory, over 16,000 patient samples have been analyzed using the method with a diagnostic yield of about 17%—that is, 17% of the samples results reveal findings that should be acted upon by the ordering physician. Included in these are over 180 confirmed inborn errors, of which about 38% could not have been diagnosed using previous methods.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/2014/>

## Protocol

### Procedure for Processing Urine Samples

1. Thaw the urine sample in a 37° C water bath. Decant into a new container if the original is compromised.
2. Take a maximum aliquot of 13 ml of sample and store it at -20° C in a labeled conical centrifuge tube.
3. Measure and record the optical density of the sample by placing a couple of drops of urine in the refractometer.
4. Filter the sample through a 0.2 um filter.
5. Measure out the volume of sample below according to the optical density

1.000-1.009	1.00 ml
1.010-1.019	0.50 ml
1.020-1.050	0.25 ml + 0.25 ml H <sub>2</sub> O

6. The specified volume is then transferred to a Reactivial containing the following internal standards: 500 nanomoles (nmoles) <sup>3</sup> Creatine; 10 nmoles d<sub>3</sub> Methylmalonic acid; 100 nmoles each of the following <sup>13</sup>C<sub>3</sub> lactate, <sup>13</sup>C<sub>3</sub> pyruvate, <sup>13</sup>C<sub>2</sub> <sup>15</sup>N glycine, d<sub>3</sub> serine, d<sub>5</sub> phenylalanine, d<sub>11</sub> hexanoylglycine, <sup>15</sup>N<sub>2</sub> orotate, d<sub>4</sub> sebacic acid, <sup>13</sup>C<sub>6</sub> glucose, d<sub>6</sub> inositol and d<sub>5</sub> tryptophan.
7. 20 microliters (μl) of a 7.5 Units/μl solution of urease (Calzyme Laboratories catalog no. 116A0100) is added to the sample, which is then flushed and sealed under CO<sub>2</sub> through an inert septum.
8. The sample is held at 37°C for 30 minutes with more carbon dioxide gas added at 15 minute intervals to maintain pressure.
9. 20 μl more of the urease solution is added, the vial is flushed with carbon dioxide, and the sample maintained at 37°C for another 15 minutes.
10. 500 μl of 30:70 acetone : methanol is added, the rubber septum is replaced with a Teflon coated septum, and the sample is chilled at -20° C for 15 minutes.
11. Solids are removed by centrifugation at 1500 rpm x 10 minutes then decanted into a clean 2.0 cc Reactivial (Supelco/Sigma)
12. Add triethylammonium trifluoroacetate (TEA/TFA) (Sigma) as follows:
  - 20 μl for 1.00 ml samples

- 40 µl for 0.5 ml, or less, samples

13. Top off with acetonitrile and place under a nitrogen stream at 70° C until constant volume is achieved (TEA/TFA will remain) (~ 15 minutes).
14. Repeat step 13, up to 4 times until a precipitate forms (~ 10 minutes each).
15. Let cool for about 2 minutes. Top off with methylene chloride, being careful of boil over, and dry (~ 4:00 minutes).
16. Repeat step 15.
17. Add MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) (Thermal Scientific) at the following rates:
  - 150 µl for 1.00 ml samples
  - 200 µl for 0.50 ml, or less, samples
18. Cap under a nitrogen atmosphere and incubate at 70° C for 1 hour.
19. Transfer to microvials, under a nitrogen atmosphere, for analysis on gas chromatograph /mass spectrometer.
20. Place microvials for automated injection by the Agilent 5975 GC/MS: Instrument temperatures are: injector 200° C, interface 250° C, oven 80° C for 1 minute; ramp at 4° C/minute 80-130° C, ramp at 6° C/ minute 130-200° C, ramp at 12° C/ minute 200-285° C, hold for 10 minutes. Column: 25 m, 320 micron ID, 0.5 micron film thickness DB-5. Mass Spec: source 230° C, quad 150° C, Scan 50-650 amu at 2.46 scans/sec. Solvent delay 3.5 minutes.

## Representative Results

Please [click here](#) to see the representative results.

## Discussion

The urease method (1) has been cited 62 times in the medical literature with various modifications. Matsumoto's group (2,3) simplified the procedure for high-throughput neonatal screening and reported the results from 16000 patients. Kuhara and others (4-7) have reported the use of the method in several cases of inborn error diagnosis and follow-up. Rhead (8) also confirmed the method's utility for clinical diagnosis and follow-up of inborn errors. The method has been applied to urine of bears, knock-out mice, elephants and homogenates of whole fruit flies and their larvae (9). Culture media from *Cryptococcus* before and after site-directed mutagenesis were also analyzed, without the urease step (10). The method has been applied to human nutritional assessment in medical students, Down syndrome patients and demented elderly veterans after loading the subjects with oral doses of the amino acids tryptophan, methionine and isoleucine (11). All eight B-vitamins were assessed by quantifying the breakdown products of the three amino acids which, among them, require all 8 vitamins at some point in their degradation. The toxic effects of pharmaceuticals and their mitigation by vitamin supplementation has been reported (12). Amniotic fluid samples from normal and Down syndrome pregnancies were analyzed and reported (13-15).

## Disclosures

The Metabolic Screening Lab is a CLIA-licensed clinical laboratory owned by Saint Louis University, a non-profit corporation. Dr. Shoemaker does not directly profit from laboratory revenues, but may benefit indirectly from laboratory productivity. None of the methods, techniques, results, normal ranges, macros, software, libraries or conclusions are considered proprietary and are freely available to interested parties.

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