Supplementary text S1

Validation of Quantification

The method was validated by determining the linearity, inter- and intra-assay precision, recoveries, sensitivity, and stability.

Standard solutions of AAs were prepared by further dilution of the stock solutions in the starting mobile phase, and the final calibration standards were prepared by spiking standard stock solutions with the I.S. (final concentration, 1000 ng·mL⁻¹). Calibration was performed by a serial dilution in which different concentrations of a mixture of AA standard solutions were added to 20 μL of the internal standard solution.

The sensitivity was evaluated with the limit of detection (LOD) and the limit of quantification (LOQ) of the two transitions of each compound. The LOD and LOQ were obtained at the concentration that provided signal-to-noise ratios of 3 and 10, respectively.

Quality control (QC) samples were prepared with known concentrations of standard solution of analytes. The precision was quantified by the intra-day repeatability (one QC sample prepared six replicates of spiked samples at three different concentrations on a single day) and the inter-day repeatability (QC samples were prepared six replicates of spiked samples at three concentrations on three different days). These values were expressed as the relative standard deviations (RSDs) and were determined over 3 weeks.

The stability of the serum samples was determined under different conditions, maintained at 4 °C or room temperature for 0, 6, or 24 h after preparation. Another batch of prepared samples was kept and reanalyzed on subsequent days (1, 4, and 7 days) after interim storage at -80 °C. The freeze-thaw stability was tested after three freeze/thaw cycles (room temperature to -80 °C). The repeatability was determined by preparing and analyzing 6 replicates of one sample in parallel.

To determine the recovery of the developed method, mixed standard solutions with three different concentrations (50, 500 and 2000 ng·mL⁻¹) were added to a serum sample with a known concentration. Because of the large differences in the levels of AAs among samples, the recovery values were calculated using the concentration closest to the concentration in the sample. The recoveries were estimated by the following formula:

\[
\text{Recovery (\%)} = \left( \frac{(M1-M0)}{\text{addition}} \right) \times 100
\]

M1 is the amount of AA in serum sample to which mixed standard solutions were added.

M0 is the original amount of AA in sample. Addition is the amount of the mixed standard solutions that was added to the sample.