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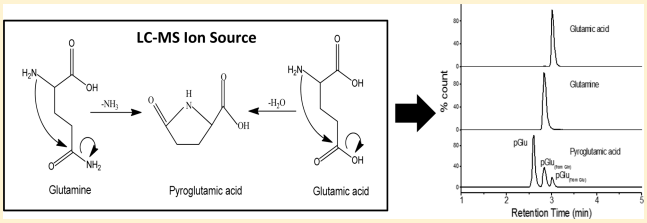
An Artifact in LC-MS/MS Measurement of Glutamine and Glutamic Acid: In-Source Cyclization to Pyroglutamic Acid

Preeti Purwaha,[†] Leslie P. Silva,[†] David H. Hawke,[‡] John N. Weinstein,[†] and Philip L. Lorenzi*[,†]

[†]Department of Bioinformatics and Computational Biology and [‡]Proteomics and Metabolomics Facility, Department of Pathology, MD Anderson Cancer Center, University of Texas, Houston, Texas 77054, United States

Supporting Information

ABSTRACT: Advances in metabolomics, particularly for research on cancer, have increased the demand for accurate, highly sensitive methods for measuring glutamine (Gln) and glutamic acid (Glu) in cell cultures and other biological samples. N-terminal Gln and Glu residues in proteins or peptides have been reported to cyclize to pyroglutamic acid (pGlu) during liquid chromatography (LC)-mass spectrometry (MS) analysis, but cyclization of free Gln and Glu to free pGlu during LC-MS analysis has not been well-characterized. Using an LC-MS/MS protocol that we developed to separate Gln, Glu, and pGlu, we found that free Gln and Glu cyclize to pGlu in the electrospray ionization source, revealing a previously uncharacterized artifact in metabolomic studies. Analysis of Gln standards over a concentration range from 0.39 to 200 μM indicated that a minimum of 33% and maximum of almost 100% of Gln was converted to pGlu in the ionization source, with the extent of conversion dependent on fragmentor voltage. We conclude that the sensitivity and accuracy of Gln, Glu, and pGlu quantitation by electrospray ionization-based mass spectrometry can be improved dramatically by using (i) chromatographic conditions that adequately separate the three metabolites, (ii) isotopic internal standards to correct for in-source pGlu formation, and (iii) user-optimized fragmentor voltage for acquisition of the MS spectra. These findings have immediate impact on metabolomics and metabolism research using LC-MS technologies.



Glutamine (Gln) is a central player in metabolic pathways and in many diseases. In the cancer research community, for example, there is renewed interest in the various important roles that Gln plays in both biology and therapy. It serves as (1) a direct building block for protein synthesis; (2) a precursor for synthesis of glutamic acid (Glu) by glutaminase;[1] (3) a precursor for synthesis of asparagine by asparagine synthetase;[2] (4) a source of α-ketoglutarate, which supports the tricarboxylic acid cycle and, therefore, energy production in proliferating cancer cells;[3−5] (5) a source of carbon for fatty acid synthesis;[6,7] (6) a precursor for the antioxidant glutathione;[8] (7) a precursor for pyrimidine synthesis;[9] (8) a positive modulator of autophagy;[10,11] and (9) a proposed antacid.[12] It is therefore important to be able to measure free Gln concentrations accurately and with high sensitivity. However, as we will describe, such measurements of Gln and Glu are challenging.

Two potential causes of inaccurate Gln and Glu measurements include chemical derivatization and cyclization to pyroglutamic acid (pGlu). Until very recently, amino acid concentrations have been measured by gas chromatography−mass spectrometry (GC/MS) following chemical derivatization, which can introduce artifacts.[13] To circumvent that problem, we and others have developed label-free, targeted approaches to amino acid analysis.[14] Nevertheless, Gln can be cyclized to pGlu enzymatically or nonenzymatically under acidic, alkaline, and/or high temperature conditions.[15−19] The process involves loss of the side-chain amide nitrogen as ammonia. Like Gln, Glu can also be cyclized to pGlu, enzymatically by glutaminyl cyclase (QPCT in humans) in a process that involves loss of the side chain hydroxyl group as water.[19−26]

Despite the suggestion that pGlu is only a degradation product, it has several important biological functions: (1) being a glutamate analogue and a potential precursor, it has been studied as an agonist of glutamate in brain-related research,[27] (2) it provides resistance to protein degradation by amino peptidases,[22] (3) it has been shown to be involved in synthesis of neurotransmitters in neuronal cells,[28] and (4) it has been shown to have an antidiabetic effect in type-2 diabetes in rats and mice.[29] When we tried to measure free Gln and Glu in enzyme kinetic reactions with L-asparaginase[30] and in cancer cells by liquid chromatography (LC)-MS,[14,31] we were surprised to detect significant levels of free pGlu. When we began monitoring pGlu more closely, we found high levels of pGlu in Gln and Glu standards, an unexpected observation that prompted us to determine the source of the pGlu.

Our investigation into the source of pGlu next led us to use Fourier Transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) to ascertain that the Gln and Glu standards were nearly 100% pure, suggesting that the pGlu was generated during the analytical process. To test whether the

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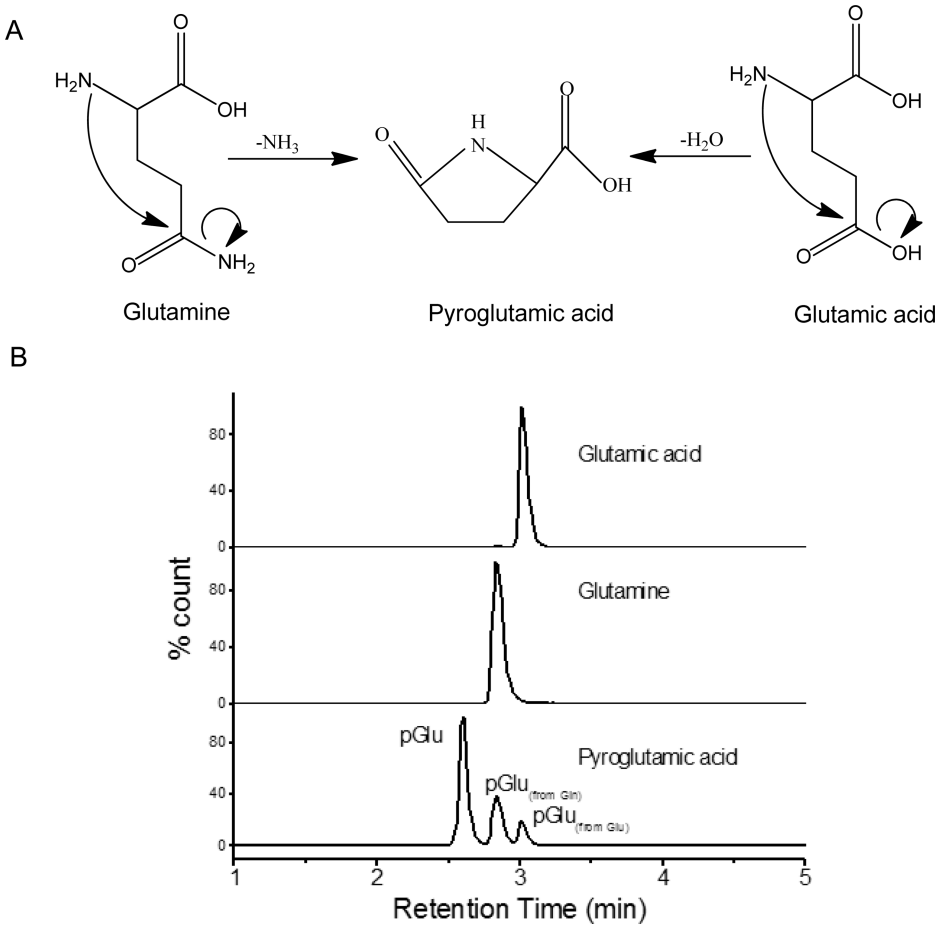

Figure 1. (A) Mechanism of Gln and Glu cyclization to pGlu after ammonia and water loss, respectively. (B) MRMs of Glu, Gln, and pGlu from an equimolar mix of Glu, Gln, and pGlu standards (1 mM) showing the "in-source" formation of pGlu from Gln and Glu. The MS spectra were acquired using the optimal conditions for each analyte as described in the Experimental Section.

liquid chromatography (LC) conditions (e.g., mobile phase acidity) were responsible for pGlu formation, we performed direct injection (flow injection analysis, FIA) of Gln and Glu standards in water. Those analyses revealed significant levels of free pGlu, suggesting that the LC conditions were not responsible for the observed pGlu. Finally, we found that pGlu was generated from Gln and Glu in the ion source of the mass spectrometer. Importantly, we conclude that accurate measurement of free Gln, Glu, and pGlu by mass spectrometry requires chromatographic separation of the three species to differentiate between pGlu present naturally and that generated from Gln and Glu in the ion source. A further increase in measurement accuracy can be obtained by optimizing MS ionization source conditions to minimize in-source loss of Gln or Glu to pGlu. The LC-MS/MS method used in this study achieved those goals. We used multiple reaction monitoring (MRM) transitions specific to each metabolite and chromatographic conditions that separate them. In addition, we used isotopic internal standards and analyzed electrospray ionization (ESI) source parameters to identify conditions that minimize the cyclization reaction.

EXPERIMENTAL SECTION

Materials. Gln, Glu, pGlu, $^{13}C_5$,$^{15}N_2$-Gln, and $^{13}C_5$,^{15}N-Glu were purchased from Sigma-Aldrich (St. Louis, MO). LC/MS grade water, methanol, and acetonitrile (ACN) were purchased from Honeywell Burdick and Jackson (Muskegon, MI). Formic acid and heptafluorobutyric acid (HFBA) were obtained from Sigma-Aldrich.

Liquid Chromatography and Mass Spectrometry. Liquid chromatography was performed on an Agilent 1290 Infinity UHPLC system equipped with a Zorbax SB C-18 column (3.0 × 100 mm, 1.8 μm particle size; Agilent) at 25 °C column temperature. Mobile phase consisted of (A) water and (B) ACN each containing 0.3% HFBA and 0.5% formic acid. Gradient conditions were: 0.01 to 2 min = 2% to 30% B; 2 to 4.1 min = 30% to 40.0% B; 4.1 to 4.8 min = 40% to 45% B; 4.8 to 4.9 min = 45% to 90% B; 4.9 to 5.5 min = 90% B; 5.5 to 5.6 min = 90% to 2% B; 5.6 to 8.0 min = 2% B. Injection volume was 5.0 μL, and flow rate was 0.3 mL/min. LC-MS/MS analysis was performed on an Agilent 6460 triple quadrupole equipped with a jet stream ESI source. MRM was performed in the positive ion mode. Other MS parameters included: gas temperature at 300 °C, drying gas at 7 L/min, nebulizer pressure at 50 psi, sheath gas temperature at 325 °C, sheath gas flow at 10 L/min, capillary voltage (CV) at 3750 V, dwell time of 20 ms, and nozzle voltage (NV) at 0 V. The MRM transitions and optimized source conditions (Mass Hunter Optimizer B.04.01) used were: (i) for Glu: 148.1 > 84.1 and 148.1 > 56.1, collision energy (CE) 14 V, fragmentor voltage (FV) 74 V, and cell accelerator voltage (CAV) 7 V; (ii) for Gln: 147.1 > 84.1 and 147.1 > 56.1, CE 10 V, FV 76 V, and CAV 7 V; and (iii) for pGlu: 130.0 > 84.1 and 130.0 > 56.1, CE 10 V, FV 100 V, and CAV 7 V.

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Mass Hunter (version B.04.01) was used for data acquisition. Mass Hunter Qualitative analysis and Quantitative analysis were used for data processing. Ten μM $^{13}C_5$,$^{15}N_2$-Gln (Sigma) and $^{13}C_5$,^{15}N-Glu (Sigma) were used as internal standards. The corresponding MRM transitions and optimized source conditions were: (i) for $^{13}C_5$,$^{15}N_2$-Gln: 154.1 > 89.1, CE 10 V, FV 78 V, and CAV 7 V; and (ii) for $^{13}C_5$,^{15}N-Glu: 154.1 > 89.1, CE 10 V, FV 80 V, and CAV 7 V.

Flow Injection Analysis (FIA) and Selected Ion Monitoring (SIM). FIA was performed using 1.0 mM Gln in water to assess whether cyclization of Gln to pGlu occurred in the sample, during chromatography (e.g., due to acidity of the mobile phase), or inside the ion source. SIM was performed on nonlabeled and isotopically labeled Gln and Glu standards using FIA in positive ion mode to determine the extent of cyclization. Gln, Glu, and pGlu chemical standards were additionally subjected to FT-IR and NMR analysis to confirm the absence of pGlu in the original, powder-form Gln and Glu standards.

Effect of MS Parameters on Cyclization of Gln and Glu to pGlu. MS signal intensities (SI) for various concentrations of standard Gln and Glu (0.39−200 μM) were monitored using the LC-MS/MS method described above with modification of source conditions, such as CV 1500−5500 V, NV 500−1500 V, nebulizer pressure 10−50 psi, FV 10−200 V, and various sheath gas/gas temperature combinations 200−300 °C. SI of both Gln/Glu and pGlu formed in-source from Gln/Glu were measured. The ratio of peak areas (SI of pGlu from Gln: SI of Gln) was calculated for each condition.

In-Source Cyclization of Gln and Glu to pGlu in Orbitrap. The LC-MS system consisted of a Dionex Ultimate 3000 UPLC coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). Standards of Gln, Glu, and pGlu (100 μM) were injected onto a Phenomenex Kinetex C18 XB core−shell column (2.6 μm × 2.1 mm × 150 mm), and metabolites were separated using a 10 min program: 0 to 5 min = 100% A (water with 0.1% formic acid); 5 to 10 min = 0 to 100% B (ACN with 0.1% formic acid). The injection volume was 6 μL, and the flow rate was 200 μL/min. Metabolites were detected in positive ion mode with the m/z range calibrated and set at 50−1000 Da, acquisition time of 10 ms, and resolution set at 60,000. The Gln, Glu, and pGlu (100 μM) standards were also analyzed on an LC-MS system consisting of a Dionex Ultimate 3000 UPLC coupled to an Orbitrap Fusion mass spectrometer with a heated ESI source (Thermo Scientific). The source fragmentation voltage parameter for all metabolomic analyses on the Orbitrap instruments was set at zero. The source fragmentation voltage settings were also tested in the Orbitrap Elite instrument from 0 to 60 V (10 V increments).

RESULTS AND DISCUSSION

LC-MS/MS to Characterize In-Source Cyclization of Gln to pGlu. While trying to measure Gln and Glu concentrations in kinetic studies and experiments with cultured cells, we found significant pGlu and asked whether it was actually a constituent of the sample or whether it had formed by cyclization of Gln or Glu during the measurement process. The method described here enabled us to make that distinction. Central to the method is an LC protocol we developed that efficiently separates Gln, Glu, and pGlu (Figure 1). That separation allowed us to differentiate pGlu present in the sample from products of cyclization of Gln or Glu in the ESI source. Using that method, we found that pGlu measurement based on accurate mass alone can lead to erroneous results. Direct injection (flow injection analysis, FIA) performed with selected ion monitoring (SIM) of Gln in water revealed that cyclization to pGlu occurred even without a chromatographic step (Figure S-1, Supporting Information) and, notably, in the absence of the acidic LC mobile phase which was previously implicated in pGlu formation in peptides.[15] Those results suggested that ESI source conditions were responsible for Gln cyclization to pGlu. Independent FT-IR and NMR analyses confirmed that pGlu was not present in the Gln and Glu standards prior to exposure to the ESI source (data not shown).

SIM of pGlu from Unlabeled and Labeled Gln and Glu. To determine whether isotopically labeled Gln and Glu undergo the same extent of cyclization to pGlu as nonlabeled Gln and Glu, we performed selected ion monitoring (SIM) with labeled and nonlabeled Gln and Glu. SIM analysis revealed that a nearly equal fraction of Gln cyclizes to pGlu in the ion source for both the isotope-labeled and nonlabeled species. The peak area ratio for pGlu/Gln was 0.405, and the peak area ratio for labeled pGlu/labeled Gln was 0.412 (Figure S-1, Supporting Information); the same was true for Glu (data not shown). Hence, Gln and Glu labeled with stable isotope are suitable as internal standards for correction of in-source pGlu formation and accurate quantitation of Gln and Glu. Importantly, because cyclization to pGlu appears to be a first order process under constant source conditions, external and/or internal calibration allows accurate quantitation of Gln (or Glu) because the fraction of pGlu produced is constant over the range of concentrations measured. These findings are of importance to laboratories using MS-based assays for quantitating Gln, Glu, and/or pGlu.

Effect of MS Parameters on In-Source Cyclization of Gln and Glu to pGlu. No change in the extent of conversion of Gln to pGlu in-source was observed when we varied ion source conditions such as CV (1500 to 5500 V), NV (500 to 1500 V), nebulizer pressure (10 to 50 psi), or various sheath gas/gas temperature combinations (200 to 300 °C). However, FV (a parameter that controls the speed at which ions pass through a medium pressure capillary between the electrospray chamber and the mass spectrometer; 10 to 200 V) markedly influenced the extent of conversion of Gln to pGlu in-source, suggesting that collision induced dissociation (CID) may be involved in its formation. The peak ratio of pGlu (from Gln) to Gln ranged from ∼0.4 to 0.8 at FV 10 to 100 V. When we increased the FV above 100 V, that ratio increased dramatically to ∼4.5 or greater (Figure 2A,B). No quantifiable signal was observed for Gln at FV above 120 V; only pGlu was detected in that range of fragmentor voltages (Figure 2B).

In-Source Cyclization of Gln and Glu to pGlu in Orbitrap. Extracted ion chromatogram (XIC; MS1 of Orbitrap Elite) analyses of Gln, Glu, and pGlu standards also showed that pGlu was formed from Gln and Glu in the ion source due to cyclization of Gln and Glu with loss of ammonia and water, respectively, as indicated by parent mass conversion (Figure S-2, Supporting Information). Extracted ion chromatogram (XIC; MS1 of Orbitrap Fusion) analyses of Gln, Glu, and pGlu standards further confirmed that pGlu was formed from Gln and Glu in the ion source (data not shown). Source fragmentation voltage settings tested in the Orbitrap Elite instrument from 0 to 60 V (10 V increments) yielded similar conversion ratios of Gln to pGlu from 0 to 30 V (approximately 60−70% conversion). However, at 40 V and higher, the overall intensity of both Gln and pGlu dramatically declined with

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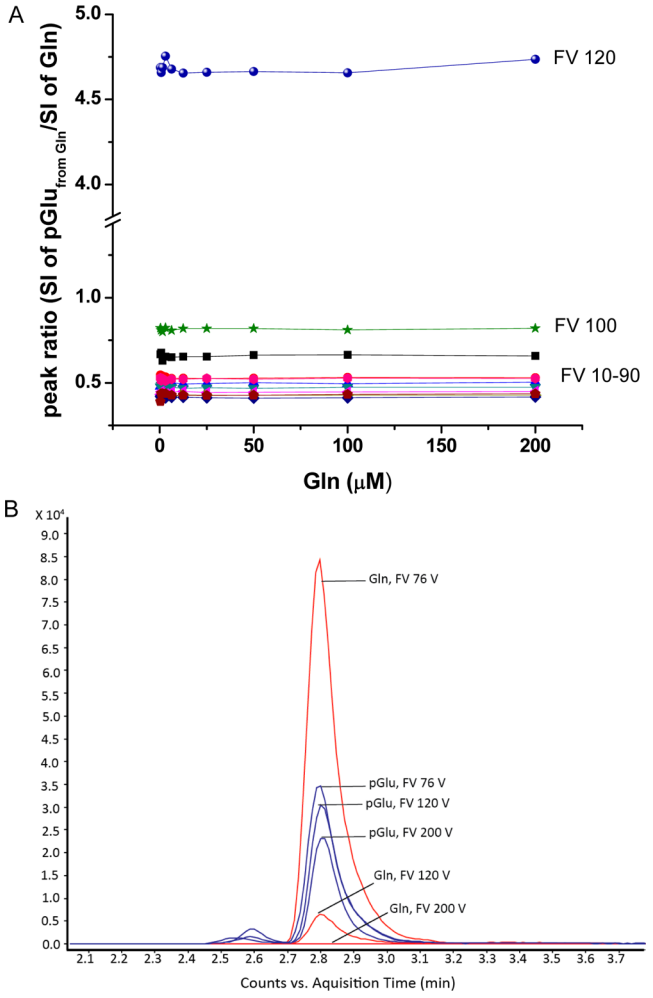

Figure 2. (A) Effect of fragmentor voltage (FV) on peak ratio (signal intensity of $pGlu_{from\ Gln}$/signal intensity of Gln) at different concentrations of Gln (0.39–200 μM). Peak ratio increased on increasing FV above 100 V. Gln signal was not quantifiable above FV 120 V. (B) MRM traces acquired using 200 μM Gln at FV 76 V (optimal for Gln), FV 120 V, and FV 200 V, illustrating the loss of Gln signal as FV is increased above 100 V.

subsequent appearance of a fragment at 84.01 *m/z*, which corresponded to in-source fragmentation of the molecules. Therefore, we recommend that the source fragmentation voltage be set at 0–10 V for metabolomic analyses of Gln or Glu by Orbitrap instruments and isotopic correction for the pGlu formed from Gln or Glu in the MS.

CONCLUSIONS

We have identified and characterized a problem associated with MS-based quantitation of the metabolically central amino acids Gln and Glu. Free Gln and Glu can cyclize to pGlu inside the MS, causing erroneous measurement of Gln, Glu, and pGlu. The in-source cyclization to pGlu can be almost 100% for Gln, depending on the ionization source conditions of the instrument. Achieving adequate chromatographic separation of Gln, Glu, and pGlu allowed us to distinguish in-solution (i.e., endogenous) pGlu from that formed from Gln and Glu inside the ion source. We propose that quantitation of Gln, Glu, and/or pGlu in biological samples should be performed using (i) chromatographic conditions that adequately separate the three metabolites, (ii) isotopic internal standards to correct for in-source pGlu formation, and (iii) user-optimized fragmentor voltage for use in acquiring the MS spectra. These findings have immediate impact on metabolomics and metabolism research using MS technologies.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Address: MD Anderson Cancer Center, 7435 Fannin Street, Room 2SCR3.3027, Unit 0950, Houston, TX 77054. Phone: 713-792-9999. Fax: 713-563-4235. E-mail: PLLorenzi@mdanderson.org.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Curthoys, N. P.; Watford, M. *Annu. Rev. Nutr.* **1995**, *15*, 133–159.
(2) Richards, N. G.; Kilberg, M. S. *Annu. Rev. Biochem.* **2006**, *75*, 629–654.
(3) Le, A.; Lane, A. N.; Hamaker, M.; Bose, S.; Gouw, A.; Barbi, J.; Tsukamoto, T.; Rojas, C. J.; Slusher, B. S.; Zhang, H.; Zimmerman, L. J.; Liebler, D. C.; Slebos, R. J.; Lorkiewicz, P. K.; Higashi, R. M.; Fan, T. W.; Dang, C. V. *Cell Metab.* **2012**, *15*, 110–121.
(4) El Hage, M.; Conjard-Duplany, A.; Baverel, G.; Martin, G. *Neurochem. Int.* **2011**, *58*, 896–903.
(5) Weinberg, F.; Hamanaka, R.; Wheaton, W. W.; Weinberg, S.; Joseph, J.; Lopez, M.; Kalyanaraman, B.; Mutlu, G. M.; Budinger, G. R.; Chandel, N. S. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 8788–8793.
(6) DeBerardinis, R. J.; Mancuso, A.; Daikhin, E.; Nissim, I.; Yudkoff, M.; Wehrli, S.; Thompson, C. B. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 19345–19350.
(7) Wise, D. R.; DeBerardinis, R. J.; Mancuso, A.; Sayed, N.; Zhang, X. Y.; Pfeiffer, H. K.; Nissim, I.; Daikhin, E.; Yudkoff, M.; McMahon, S. B.; Thompson, C. B. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 18782–18787.
(8) Whillier, S.; Garcia, B.; Chapman, B. E.; Kuchel, P. W.; Raftos, J. E. *FEBS J.* **2011**, *278*, 3152–3163.
(9) Cory, J. G.; Cory, A. H. *In Vivo* **2006**, *20*, 587–589.
(10) Duran, R. V.; Oppliger, W.; Robitaille, A. M.; Heiserich, L.; Skendaj, R.; Gottlieb, E.; Hall, M. N. *Mol. Cell* **2012**, *47*, 349–358.
(11) Nicklin, P.; Bergman, P.; Zhang, B.; Triantafellow, E.; Wang, H.; Nyfeler, B.; Yang, H.; Hild, M.; Kung, C.; Wilson, C.; Myer, V. E.; MacKeigan, J. P.; Porter, J. A.; Wang, Y. K.; Cantley, L. C.; Finan, P. M.; Murphy, L. O. *Cell* **2009**, *136*, 521–534.
(12) Huang, W.; Choi, W.; Chen, Y.; Zhang, Q.; Deng, H.; He, W.; Shi, Y. *Cell Res.* **2013**, *23*, 724–727.

(13) Kanani, H. H.; Klapa, M. I. *Metab. Eng.* **2007**, *9*, 39−51.
(14) Purwaha, P.; Lorenzi, P. L.; Silva, L. P.; Hawke, D. H.; Weinstein, J. N. *Metabolomics* **2014**, DOI: 10.1007/s11306-014-0634-1.
(15) Kumar, M.; Chatterjee, A.; Khedkar, A. P.; Kusumanchi, M.; Adhikary, L. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 202−212.
(16) Ozturk, S. S.; Palsson, B. O. *Biotechnol. Prog.* **1990**, *6*, 121−128.
(17) Bray, H. G.; James, S. P.; et al. *Biochem. J.* **1949**, *44*, 625−627.
(18) Reimer, J.; Shamshurin, D.; Harder, M.; Yamchuk, A.; Spicer, V.; Krokhin, O. V. *J. Chromatogr., A* **2011**, *1218*, 5101−5107.
(19) Eckstein, J. A.; Ammerman, G. M.; Reveles, J. M.; Ackermann, B. L. *J. Neurosci. Methods* **2008**, *171*, 190−196.
(20) Schilling, S.; Hoffmann, T.; Manhart, S.; Hoffmann, M.; Demuth, H. U. *FEBS Lett.* **2004**, *563*, 191−196.
(21) Huang, K. F.; Liu, Y. L.; Cheng, W. J.; Ko, T. P.; Wang, A. H. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 13117−13122.
(22) Chelius, D.; Jing, K.; Lueras, A.; Rehder, D. S.; Dillon, T. M.; Vizel, A.; Rajan, R. S.; Li, T.; Treuheit, M. J.; Bondarenko, P. V. *Anal. Chem.* **2006**, *78*, 2370−2376.
(23) Yu, L.; Vizel, A.; Huff, M. B.; Young, M.; Remmele, R. L., Jr.; He, B. *J. Pharm. Biomed. Anal.* **2006**, *42*, 455−463.
(24) Gilbert, J. B.; Price, V. E.; Greenstein, J. P. *J. Biol. Chem.* **1949**, *180*, 209−218.
(25) Tritsch, G. L.; Moore, G. E. *Exp. Cell Res.* **1962**, *28*, 360−364.
(26) Liu, Y. D.; Goetze, A. M.; Bass, R. B.; Flynn, G. C. *J. Biol. Chem.* **2011**, *286*, 11211−11217.
(27) Moret, C.; Briley, M. *Trends Pharmacol. Sci.* **1988**, *9*, 278−279.
(28) Borg, J.; Stenger, A.; Toazara, J. *Neurochem. Int.* **1986**, *8*, 397−402.
(29) Yoshinari, O.; Igarashi, K. *Br. J. Nutr.* **2011**, *106*, 995−1004.
(30) Chan, W. K.; Lorenzi, P. L.; Anishkin, A.; Purwaha, P.; Rogers, D. M.; Sukharev, S.; Rempe, S. B.; Weinstein, J. N. *Blood* **2014**, DOI: 10.1182/blood-2013-10-535112.
(31) Silva, L. P.; Lorenzi, P. L.; Purwaha, P.; Yong, V.; Hawke, D. H.; Weinstein, J. N. *Anal. Chem.* **2013**, *85*, 9536−9542.