Supporting Information for

Comparative Triplex Tandem Mass Spectrometry Assays of Lysosomal Enzyme Activities in Dried Blood Spots Using Liquid-Liquid Extraction and Fast Liquid Chromatography: Application to Newborn Screening of Pompe, Fabry, and Hurler Diseases

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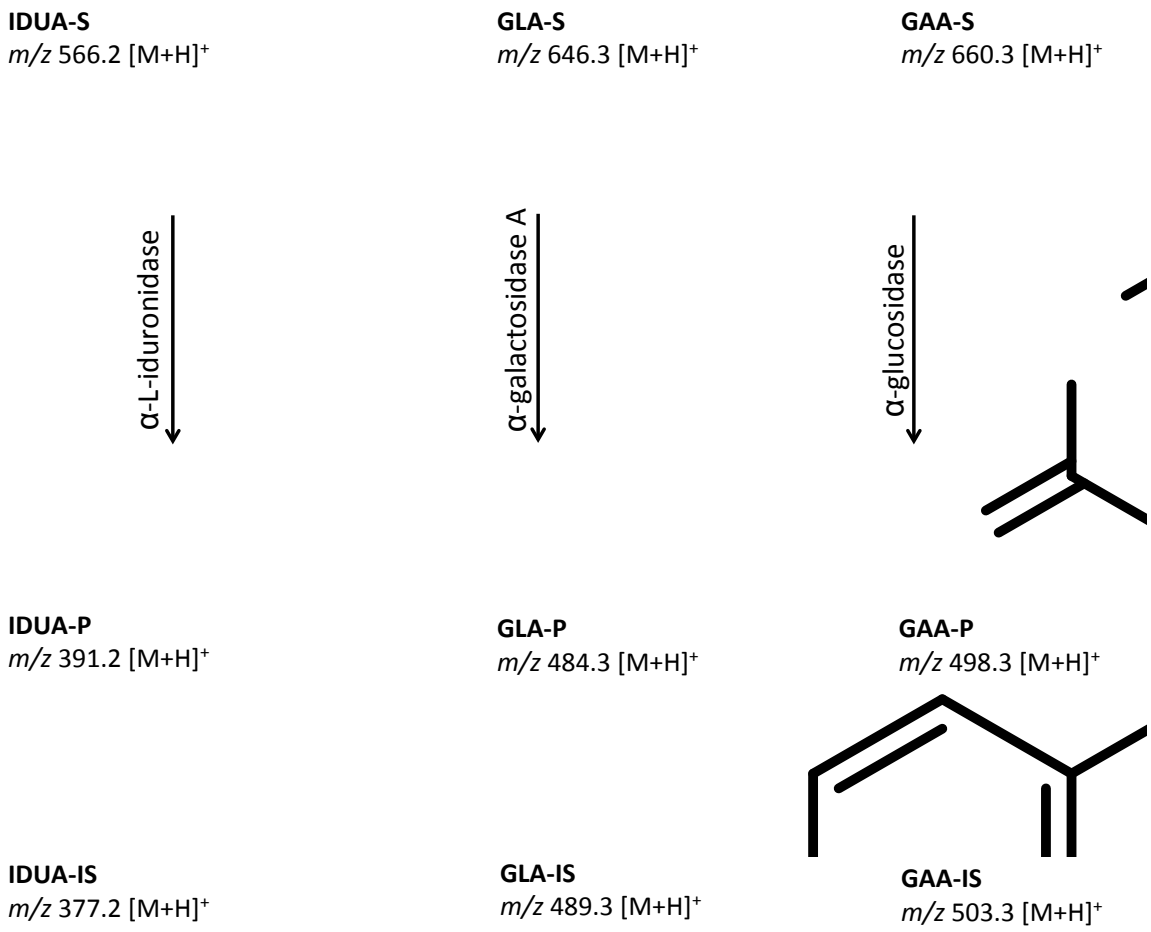
Scheme S1, Tables S1-S8, Figures S1-S9.

Materials. GLA substrate (GLA-S), GLA internal standard (GLA-IS), GAA substrate (GAA-S), and GAA internal standard(GAA-IS) were received from the Centers for Disease Control and Prevention, Atlanta. IDA substrate (IdA-S) and IdA internal standard (IS) were obtained from Drs. K. Zhang and J. Keutzer at Genzyme Corp. The structures and ion *m/z* values for substrates, products, and internal standards are shown in Scheme S1. Quality control (QC) DBS samples were obtained from the CDC and stored at -20 °C in a zip-lock plastic bag. QC DBS are prepared from pre-treated blood to differ in the enzyme activity and are denoted as base, low, medium, high, and an adult DBS.[S1] All experiments were conducted in compliance with institutional review board (IRB) guidelines. DBS from patients previously diagnosed with Fabry, Pompe and MPS-I were obtained from our clinical program as anonymous samples, in compliance with IRB requirements. DBS of Fabry patients were from affected males. DBS from Pompe patients were from both infantile and late-onset clinical forms. DBS from MPS-I samples were from patients with early childhood presentations (Hurler). The DBS were received from birthing centres and kept at ambient temperature during shipment (<10 days). For assays carried out in the Washington State Newborn Screening Laboratory, DBS were used after all routine testing was performed (i.e. leftover DBS) and were up to ~6 months old and kept at ambient laboratory temperature. All DBS samples were manually punched with a 1/8-inch diameter whole punch.

Assay Incubation. The assay buffer, quench buffer, and assay cocktail were prepared and used in a 16 h (overnight) incubation at 37°C while shaking. The Pompe assay uses acarbose to selectively inhibit the non-lysosomal enzyme maltase glucoamylase which catalyzes glycogen hydrolysis; an acarbose solution was prepared[S2] and stored at 5°C for 7 days. The standard arrangement of 96-well plate was as follows; the first and the last column contained three wells (the first two and the last one in the column) with blank samples (filter paper punch) and five wells with the QC samples: QC base, QC low, QC medium, QC high and an adult DBS placed in this order from top to bottom.

Sample Work-Up Protocols. Two different protocols were used. The standard protocol involved six steps:[S2] (1) Incubation of DBS with substrates and internal standards in a deep



Scheme S1. Structures and ion *m/z* values of enzyme substrates, products, and internal standards for IDUA, GLA, and GAA assays.

96-well plate in ammonium formate buffer (30 µL, 0.1 M, pH 4.4) followed by (2) quench with ammonium acetate buffer (100 µL, 0.1 M, pH 5.5), (3) liquid/liquid extraction with ethyl acetate (400 µL), mixing and brief centrifugation to separate solvent layers, (4) removal of the top 200 µL and transfer to a new 96 well plate, (5) dry-down under a stream of air (SPE Dry 96 Dual Argonaut sample concentrator system, Biotage) with a flow rate of 40-80 PSI of air and heating <35 °C (typically <30 min), and (6) reconstitution in the mobile phase (100 µL, 80% acetonitrile:20% water with 0.2% formic acid). This sample work-up protocol is referred to as EA (ethyl acetate liquid-liquid extraction) in the methods comparison section.

A modified protocol employed (1) 16 h incubation of DBS in a shallow 96-well plate (0.5 mL, Axygen Scientific, VWR International, cat. no. 47743-982), followed by (2) quench with acetonitrile (170 µL) added with a multichannel manual pipette (Department of Chemistry) or by using a Rainin Liquidator 96-tip pipette (Newborn Screening Laboratory). The plate was covered with sealing foil and (3) centrifuged for 5 min at 3000 rpm to pull down the precipitate. Then, the sealing foil was removed and replaced with an aluminum foil, because long exposure to acetonitrile vapors dissolves the sealant glue. The processed sample plate was directly subjected to LC or FIA MS/MS analysis. This assay protocol is further referred to as AcN (acetonitrile quench) in the methods comparison section.

Flow Injection. 10 µL of sample was injected into the mobile phase (80% acetonitrile; 20% water; 0.2% formic acid; v/v/v) using a 2777C Sample Manager (Waters, Milford, MA), and infused into the mass spectrometer at a flow rate of 0.1 mL/min maintained by a 1525 Micro Binary HPLC Pump (Waters, Milford, MA).

ESI-MS/MS Selected Reaction Monitoring. Mass spectrometry analyses were performed in positive ion mode on Waters Quattro Micro and Acquity TQD tandem quadrupole mass spectrometers (Waters, Milford, MA). Data were acquired and evaluated using Mass Lynx software version 4.1. The following specific ion transitions were selected for each substrate, product and internal standard resulting in simultaneous record of nine selected reaction monitoring (SRM) ion channels, specifically, *m/z* 660.3 → *m/z* 560.3, *m/z* 498.3 → *m/z* 398.2, and *m/z* 503.3 → *m/z* 403.2 for GAA-S, GAA-P, and GAA-IS, respectively; *m/z* 646.3 → *m/z* 546.3, *m/z* 484.3 → *m/z* 384.2, and *m/z* 489.3 → *m/z* 389.2 for GLA-S, GLA-P and GLA-IS, respectively, and *m/z* 567.2→ *m/z* 467.2, *m/z* 391.2→ *m/z* 291.1, and *m/z* 377.2→ *m/z* 277.1 for IdA-S, IdA-P, and IdA-IS, respectively. The instrument settings were as follows:

Capillary voltage	3000 V
Extractor	2
RF	0.2
Source temp	120 °C
Desolvation temp	350 °C
Cone Gas Flow	50 L/h
Desolvation Gas Flow	800 L/h
Collision gas flow	0.20 mL/min
LM 1 Resolution	15
HM 1 Resolution	15
Ion Energy	0.2
MSMS Mode Entrance	2
MSMS mode Exit	2
LM 2 Resolution	15
HM 2 Resolution	15
Ion Energy 2	0.2
Multiplier	650
Collision Cell Pressure	2.2 e-3 mbar
Collision Gas	Argon

Analyte	SRM transition	Cone Voltage (V)	Collision Energy (eV)
IDUA-IS	*m/z* 377.2 → *m/z* 277.1	19.0	9.0
IDUA-P	*m/z* 391.2 → *m/z* 291.2	19.0	10.0
GLA-P	*m/z* 484.2 → *m/z* 384.3	23.0	13.0
GLA-IS	*m/z* 489.3 → *m/z* 489.3	23.0	13.0
GAA-P	*m/z* 498.3 → *m/z* 398.4	27.0	17.0
GAA-IS	*m/z* 503.3 → *m/z* 403.4	27.0	17.0

All transitions were conducted with the following settings: dwell time, 0.1 s; delay, 0.02 s.

Phospholipids present in the DBS were detected using the trimethylammonium-ethyl phosphate ion (*m/z* 184) characteristic for phosphatidylcholines (PC), lysoPC and sphingomyelins (SM).[S3] Additional SRMs[S4] were *m/z* 454.3→ *m/z* 313.3, *m/z* 496.1→ *m/z* 184.1, and *m/z* 524.4→ *m/z* 184.1 for lysoPE (C16:0), lysoPC (C16:0), and lysoPC (C18:0), respectively (cone voltage 30 eV; collision energy 25 eV).

Enzyme Activity Calculations. Enzyme activities were quantified from the amount of product formed, which was calculated from the product to internal standard ion intensity ratios after correction for response factors.[S5] The enzyme activity in units of μmol h^{-1} (L of blood)$^{-1}$ was calculated from the measured amount of product assuming that a 3-mm (1/8 inch) DBS punch contained 3.2 μL of blood.

LC Method Performance. A system suitability test (SST) was performed on the LC system by injecting a mixture of standards (10 μL) consisting of GAA-S (50.0 μM); GAA-P and IS (0.5 μM each); GLA-S (150.0 μM); GLA-P and IS (0.3 μM each); IdA-S (125.0 μM) and IdA-P and IS (1.0 μM each) and the results are given in Table S2. The data demonstrate excellent reproducibility of both retention times and peak areas. Despite the short time per run (1.5 min), the intra- and inter-column coefficients of variation (CV) for retention times were typically≤1%. The peak areas showed intra- and inter-column CV values at 8-12% but mostly stayed below 10%. Table S6 shows that lower detection limits were obtained for GAA and GLA products and internal standards (20.4-29.9 fmol injected on column) than for IdA-P/IS (380.0-387.5 fmol). This is presumably due to higher electrospray ionization efficiencies for GAA-P/IS and GLA-P/IS compared to those of IdA-P/IS. The injection was performed in 20 replicates (10 injections per each column), and the LC system reproducibility within one column (intra-column, n = 10) and between both columns (inter-column; n = 20) was evaluated as discussed below. Limits of detection were evaluated according to USP 34.[S6] Matrix effects and ion suppression by substrates were studied for the acetonitrile quenched samples using post-column infusion.[S7] The standard solutions of GAA-IS andP, GLA-IS and P (1 μM each), and IdA-IS and P (3 μM each) were steadily infused at a flow rate of 30 μL/min and combined with LC eluent through a T-piece connector during the separation of a sample (10 μL). The overall matrix effects were evaluated using a punch of filter paper (blank sample) and a DBS punch (regular assay) which were incubated in the assay buffer for 16 hours, then quenched with acetonitrile, and spiked with substrates, internal standards, and products at concentrations corresponding to a typical assay. The protein concentration was measured spectrophotometrically with a standard Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, San Jose, CA).

Protein Separation. The efficiency of protein precipitation was compared for water/CH_3CN 70:30 and 15:85 (v/v) solvent systems, which respectively correspond to the optimized LC elution solvent and to the sample composition after assay quenching with acetonitrile (170 μL). The addition of formic acid 0.1% (v) was used to study the influence of pH. The data in Table S1 (Supporting Information) show the amount of total blood protein extracted from a 3-mm DBS punch retained in the solution after quenching with four different solvents. Quenching with the optimized LC elution solvent mixture (water : CH_3CN : formic acid 70:30:0.1 v/v/v) resulted in the retention of most blood proteins in the solution. In contrast, quenching with acetonitrile reduced the amount of soluble proteins 100-fold to levels that were readily handled by the guard LC columns.

Ion Suppression. The separation of both IdA-IS and IdA-P from GLA-S was complete regardless of sample solvent, as illustrated by an expanded view of the pertinent interval on the LC-MS/MS trace (Figure S1, Supporting Information). Table S1 shows that about 99% of total blood proteins are removed from solution by acetonitrile mediated quenching, and highly polar matrix components elute in the void volume. Hence, proteins and highly polar matrix components are unlikely to interfere with the LC analysis.

DBS contain phospholipids that have been frequently indentified as a major cause of LC matrix effects. The phospholipid elution profile of a typical acetonitrile-quenched enzyme assay was acquired by monitoring the trimethylammonium-ethyl phosphate ion (*m*/*z* 184), which accounts for the overall signal of the PC, lysoPC and sphingomyelins (SM) structural classes.[S3] For more detailed information, selected compound-specific SRM transitions were recorded as well.[S4] Figure S1 shows that GAA-P/IS and lysoPC (C 18:0) partly co-eluted, whereas the other phospholipids lysoPC (C 16:0) and lysoPE (C 16:0) were sufficiently separated from the analytes. A quantitative assessment of the overall matrix effects was carried out according to Matuszewski et al,[S8] (Table S3).

Robustness Tests. As in the case of LC/AcN, the protein precipitate is removed from solution by brief centrifugation, but the sediment pellet is present in a microtiter plate during analysis. We considered the possibility that a portion of the pellet could float close to the top of the well and cause clogging of the injection needle. To address this issue, we checked the reproducibility of the FIA/AcN method. The instrument was run in continuous operation for nearly 30 h with 960 injections, and no clogging was observed. A 5-fold increase of the system back-pressure, compared to the initial conditions, was observed and attributed to a deposit build-up in the on-line filter frit (results not shown). There was no evidence that the deposit originated from the sample. It may be due to multiple reasons, including the autosampler needle penetration of the alumina foil that covers the sample plate, or from particulates originating from the HPLC pump seals.

Method Validation

General: Because of a limited sample volume, partial validation of intra-assay accuracy and precision, inter-day precision, recovery, sensitivity, and reproducibility was performed as described below.

Selectivity: LC-MS/MS method ensures confidential level of selectivity, which is increased by the fact that artificial substrates and internal standards used for enzyme assays do not occur naturally.

Sensitivity: Limits of detection and quantification were evaluated according to USP 34[S6], the results are given in Table S6.

Linearity: The standard calibration curve and matrix-based standard curve (DBS matrix) was constructed for each product and internal standard. The mixed standard solution at the concentration level corresponding to the enzyme assay cocktail and its seven dilutions were used to construct the curves for GAA and GLA P/IS and five further dilutions were used to construct the curves for IdA P/IS. The concentration ranges are shown in Table S6.

Accuracy: The overall accuracy of the analytical procedure was determined at the concentration levels used for enzyme assays by comparing the detector response obtained from an amount of the analyte added to the biological matrix (standard solution with DBS) with the detector response obtained for the true concentration of the pure authentic standard (standard solution with filter paper).

Precision: Instrument precision or system suitability test (SST) was determined by injecting 10 µL aliquots of standard mixture consisting of GAA-S (50.0 µM); GAA-P and IS (0.5 µM each); GLA-S (150.0 µM); GLA-P and IS (0.3 µM each); IdA-S (125.0 µM) and IdA-P and IS (1.0 µM each). The injection was performed in 20 replicates (10 injections per each column), and the LC system reproducibility within one column (intra-column, $n = 10$) and between the columns (inter-column; $n = 20$) was evaluated. The results are shown in Table S2. The intra-day (batch) precision of retention time and peak area coefficients of variation (CV) was determined for two equally large batches ($n = 80$). The inter-day (batch) precision was determined for those two batches ($n = 160$) as shown in Table S4. The peak area CVs were determined for internal standards at the concentration levels used for newborn screening, because the amounts of enzyme products vary among individuals. The peak areas of GAA and GLA internal standards fulfill the levels recommended by the Bioanalytical Method Validation guidelines (<15% CV), IdA-IS shows elevated CV values since the working concentration provides low ion counts on our mass spectrometer. The intra-day precision for the identical batches ($n = 72$) was determined for all four analytical methods compared in this paper and results are displayed in Table S7.

Supporting References

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Table S1.

Protein concentration determined by spectroscopic Bradford assay in the samples quenched by different solvents.

Assay quench solvent (v/v)	Protein Concentration (μg mL^{-1})[a] $n = 6$
water:CH_3CN:formic acid, 70:30:0.1	1314.8 ± 21.1
water:CH_3CN, 70:30	1159.0 ± 18.9
water:CH_3CN:formic acid, 15:85:0.1	12.4 ± 2.6
water:CH_3CN, 15:85	15.7 ± 2.5

[a]Total dissolved proteins from a 3-mm DBS punch as determined by a standard spectroscopic Bradford assay using bovine serum albumin as a standard.

Table S2. Injection Reproducibility for the Dual Column LC System

	Column 1 (n=10)[a]				Column 2 (n=10)[a]				Total (n=20)[b]			
	RT	CV %	Area	CV %	RT	CV %	Area	CV %	RT	CV %	Area	CV %
GAA-P	1.20	0.00	19870	7.60	1.18	0.34	17320	11.70	1.19	1.00	18595	11.80
GAA-IS	1.20	0.00	19188	9.30	1.18	0.68	18330	6.61	1.19	1.12	18759	8.45
GLA-P	1.13	0.35	19987	2.32	1.11	0.00	17670	8.62	1.12	1.01	18828	8.58
GLA-IS	1.13	0.44	16754	8.20	1.11	0.44	15391	8.65	1.12	1.00	16072	9.43
IdA-P	0.79	0.62	1213	8.74	0.78	0.51	1231	4.11	0.79	1.17	1222	6.84
IdA-IS	0.75	0.00	1417	4.54	0.73	0.67	1146	10.46	0.74	1.17	1282	12.97

[a]Reproducibility of retention times and peak areas on single columns ($n = 10$ on each column).
[b]Reproducibility of retention times and peak areas between both columns ($n = 20$).
[c]Limits of detection expressed as on column amounts (fmol, fM) or nanomolar concentrations (nM/L).

Table S3. Peak areas of GAA, GLA and IdA products and internal standards added to the procedural blank (filter paper punch lacking blood; 3 mm) and in the presence of sample matrix (DBS punch; 3 mm). The recovery indicates ion suppression/enhancement.

	Blank (filter paper)	Assay (DBS)	Recovery [%]
GAA-P	20411 ± 1456	17937 ± 566	87.9
GAA-IS	20946 ± 2760	19230 ± 2267	91.8
GLA-P	18036 ± 753	17050 ± 855	94.5
GLA-IS	11624 ± 911	10827 ± 847	93.1
IdA-P	719 ± 280	862 ± 120	119.9
IdA-IS	795 ± 44	945 ± 133	119.0

Table S4. Intra-day and inter-day precision of retention times and ion intensities.

	Intra-day 1 (n=80)			Intra-day 2 (n=80)			Inter-day (n=160)		
	Mean RT	**STD**	**CV [%]**	**Mean RT**	**STD**	**CV [%]**	**Mean RT**	**STD**	**CV [%]**
IdA-IS	0.701	0.020	2.8	0.721	0.014	1.9	0.711	0.020	2.8
IdA-P	0.734	0.018	2.5	0.780	0.015	1.9	0.757	0.028	3.7
GLA-IS	1.108	0.010	0.9	1.116	0.010	0.9	1.112	0.011	0.9
GLA-P	1.113	0.009	0.8	1.120	0.009	0.8	1.116	0.010	0.9
GAA-IS	1.178	0.010	0.9	1.182	0.010	0.8	1.180	0.010	0.9
GAA-P	1.182	0.010	0.9	1.186	0.009	0.7	1.184	0.010	0.8
	Mean Area	**STD**	**CV [%]**	**Mean Area**	**STD**	**CV [%]**	**Mean Area**	**STD**	**CV [%]**
IdA-IS	614	103	16.7	875	199	22.7	737	202	27.5
GLA-IS	713	94	13.2	798	122	15.3	753	116	15.4
GAA-IS	3688	468	12.7	4091	627	15.3	3878	583	15.0

Table S5. Coefficients of variation (n = 72) for the four methods.

Peak Area CV [%]	GAA	GLA	IdA
LC/AcN	15.6	12.0	8.4
LC/EA	20.7	16.6	16.0
FIA/AcN	26.8	22.7	19.8
FIA/EA	22.5	19.7	13.7

Table S6.

	Range	Correlation	Correlation	LOD[c]		LOQ
	nM/L	Coefficient[a]	Coefficient[b]	fM	nM/L	nM/L
GAA-P	0.016-2.0	0.9978	0.9955	20	2.0	6.1
GAA-IS	0.016-2.0	0.9987	0.9983	30	3.0	9.0
GLA-P	0.009-1.2	0.9980	0.9954	27	2.7	8.1
GLA-IS	0.009-1.2	0.9989	0.9962	28	2.8	8.5
IdA-P	0.11-3.5	0.9998	0.9930	380	38.0	114.0
IdA-IS	0.11-3.6	0.9987	0.9855	387	38.7	116.2

[a] Correlation coefficient for the standard calibration curve (standard solution with filter paper)
[b] Correlation coefficient for the matrix-based standard curve (standard solution with DBS)
[c] Limits of detection expressed as on column amounts (fmol, fM) or nanomolar concentrations (nM/L)

Table S7. Coefficients of variation for retention times and peak areas.

	Column 1 (n=10)				Column 2 (n=10)				Total (n=20)			
	RT	CV %	Area	CV %	RT	CV %	Area	CV %	RT	CV %	Area	CV %
GAA-P	1.20	0.00	19870	7.60	1.18	0.34	17320	11.70	1.19	1.00	18595	11.80
GAA-IS	1.20	0.00	19188	9.30	1.18	0.68	18330	6.61	1.19	1.12	18759	8.45
GLA-P	1.13	0.35	19987	2.32	1.11	0.00	17670	8.62	1.12	1.01	18828	8.58
GLA-IS	1.13	0.44	16754	8.20	1.11	0.44	15391	8.65	1.12	1.00	16072	9.43
IdA-P	0.79	0.62	1213	8.74	0.78	0.51	1231	4.11	0.79	1.17	1222	6.84
IdA-IS	0.75	0.00	1417	4.54	0.73	0.67	1146	10.46	0.74	1.17	1282	12.97

Table S8. Enzyme activities measured by different sample work-up methods

	Enzyme activity [μmol/h/L of blood]											
	GAA (Pompe)				GLA (Fabry)				IDUA (MPS-IH)			
	LC/APP	LC/LLE	FIA/APP	FIA/LLE	LC/APP	LC/LLE	FIA/APP	FIA/LLE	LC/APP	LC/LLE	FIA/APP	FIA/LLE
Blank	0.10	0.24	0.07	0.11	0.06	0.14	0.13	0.16	0.03	0.14	0.11	0.05
QC base	1.53	1.30	1.06	1.40	0.63	0.56	0.63	0.76	1.47	1.58	1.76	0.97
QC low	1.89	2.14	1.58	1.82	0.78	1.06	0.98	1.12	1.86	1.88	2.15	1.14
QC med	9.70	9.08	6.76	7.80	6.20	5.75	5.57	5.32	5.60	7.51	5.80	3.34
QC high	12.63	13.66	10.27	10.58	12.63	10.52	10.21	11.27	7.62	14.61	10.16	6.04
Pompe-1	1.53	1.88	1.23	1.00	1.81	2.02	1.86	2.08	5.82	6.92	3.13	6.55
Pompe-2	1.30	1.78	0.92	1.02	2.88	2.84	2.34	2.33	4.66	6.07	3.03	4.85
Pompe-3	1.49	1.35	1.04	0.88	1.29	1.14	1.50	1.50	4.67	6.27	2.95	4.68
Pompe-4	0.66	0.66	0.47	0.41	4.70	4.87	4.46	4.41	2.91	3.73	1.78	3.12
Fabry-1	8.18	7.55	6.06	5.75	0.32	0.31	0.34	0.41	3.63	4.65	2.27	3.71
Fabry-2	6.76	6.72	5.46	4.63	0.27	0.26	0.30	0.39	2.78	3.52	2.10	3.16
Fabry-3	4.81	4.33	3.94	3.60	0.29	0.26	0.28	0.67	3.89	4.03	2.34	3.55
MPS-IH-1	5.83	5.30	4.80	4.60	2.44	2.26	2.44	2.07	1.09	1.11	1.22	0.79
MPS-IH-2	7.26	7.02	4.74	4.80	4.14	3.92	3.71	3.72	1.17	1.47	1.29	0.83
MPS-IH-3	9.05	7.36	7.13	6.98	3.55	3.52	3.73	3.59	1.15	1.66	1.41	0.88
MPS-IH-4	9.58	9.04	6.61	6.16	4.66	4.63	4.36	3.96	1.19	1.34	1.34	0.81
Healthy-1	10.08	8.87	6.51	8.45	3.72	2.74	3.03	3.71	2.47	3.66	2.81	1.72
Healthy-2	9.08	8.96	6.30	7.53	5.38	4.82	4.67	4.69	2.25	2.79	2.26	1.45
Healthy-3	19.13	18.35	15.34	14.56	12.96	11.09	11.08	10.65	4.06	5.12	4.34	2.52
Healthy-4	23.29	21.06	17.48	17.37	8.18	7.00	6.63	6.30	3.47	3.63	3.91	2.31
Healthy-5	9.38	10.41	7.16	7.64	5.08	4.52	4.76	4.72	2.91	2.61	3.02	1.77
Healthy-6	23.09	21.81	17.98	20.62	7.88	6.94	7.33	7.56	3.78	4.65	3.99	2.66
Healthy-7	27.92	24.55	20.56	22.05	6.17	5.15	5.41	5.98	2.73	2.95	2.70	1.62
Healthy-8	13.38	21.33	17.95	17.01	3.34	6.10	6.52	6.00	3.20	5.50	4.75	2.50
Healthy-9	8.17	7.85	5.72	5.36	7.40	6.30	5.40	5.67	2.60	2.92	2.34	1.38
Healthy-10	26.70	23.66	19.45	18.83	8.60	7.64	7.50	7.57	3.89	4.30	4.12	2.38
Healthy-11	12.10	11.74	8.50	9.36	4.40	3.53	3.50	4.04	2.53	2.78	2.53	1.57
Healthy-12	14.66	14.32	10.55	10.78	5.27	5.01	4.36	4.80	1.81	2.11	1.92	1.26
Healthy-13	7.49	7.58	5.85	5.72	9.77	9.70	8.25	8.42	2.81	3.50	2.80	1.52
Healthy-14	7.87	7.40	5.57	6.44	4.80	5.13	3.88	4.83	2.02	2.27	2.23	1.44
Healthy-15	20.82	16.67	15.21	16.68	7.32	7.49	6.60	7.17	5.27	5.66	4.30	2.97
Healthy-16	14.82	15.38	9.83	10.69	9.67	9.10	7.98	8.05	3.29	4.74	3.23	1.96
Healthy-17	10.92	10.28	8.35	8.90	10.63	8.58	8.77	9.07	3.27	4.55	3.35	2.17
Healthy-18	8.16	8.04	6.17	6.87	3.37	3.18	3.19	3.64	2.26	3.34	2.22	1.51
Healthy-19	9.32	9.42	7.85	8.17	8.58	7.94	7.54	7.91	3.27	3.76	3.23	2.06
Healthy-20	13.18	12.97	10.69	10.61	11.86	10.03	10.59	10.93	3.21	3.81	3.59	2.21
Healthy-21	11.95	10.23	9.32	9.66	4.58	3.73	4.12	4.16	3.67	4.08	3.34	2.06
Healthy-22	14.73	12.80	11.83	12.10	5.07	4.77	5.01	4.90	3.46	3.96	3.99	2.37
Healthy-23	9.57	8.73	7.20	6.57	6.70	6.68	5.87	5.87	2.91	4.01	3.12	1.73
Healthy-24	24.81	21.91	18.47	18.12	8.21	7.33	8.15	7.19	5.00	5.27	5.03	2.83
Healthy-25	7.99	5.58	5.77	5.98	8.57	7.92	7.90	8.02	3.94	4.72	3.78	2.23
Healthy-26	17.27	15.52	13.30	14.63	7.64	7.14	7.41	7.44	4.37	6.13	4.87	3.32
Healthy-27	17.35	17.29	13.87	15.50	10.66	8.61	8.51	9.00	3.28	3.71	3.40	2.19
Healthy-28	21.30	22.03	16.22	16.94	6.07	5.41	5.50	5.58	6.18	6.21	4.77	3.45
Healthy-29	15.81	15.43	12.13	13.05	9.13	8.34	7.62	7.78	3.68	4.82	4.03	2.84
Healthy-30	14.51	14.80	10.64	12.18	6.33	5.07	5.16	5.91	3.52	4.43	3.43	2.64
Healthy-31	10.69	8.72	7.81	8.14	9.66	6.93	8.60	8.02	3.20	3.91	3.67	2.48
$\mu_{patient}$	1.24	1.42	0.91	0.83	0.29	0.28	0.31	0.49	1.15	1.39	1.31	0.83
$\mu_{healthy}$	14.70	13.99	11.28	11.82	7.32	6.58	6.48	6.63	3.36	4.06	3.45	2.16
Δ	13.45	12.57	10.36	10.99	7.03	6.30	6.17	6.14	2.21	2.67	2.14	1.33
μ/μ	11.8	9.9	12.4	14.3	24.9	23.7	21.1	13.5	2.9	2.9	2.6	2.6

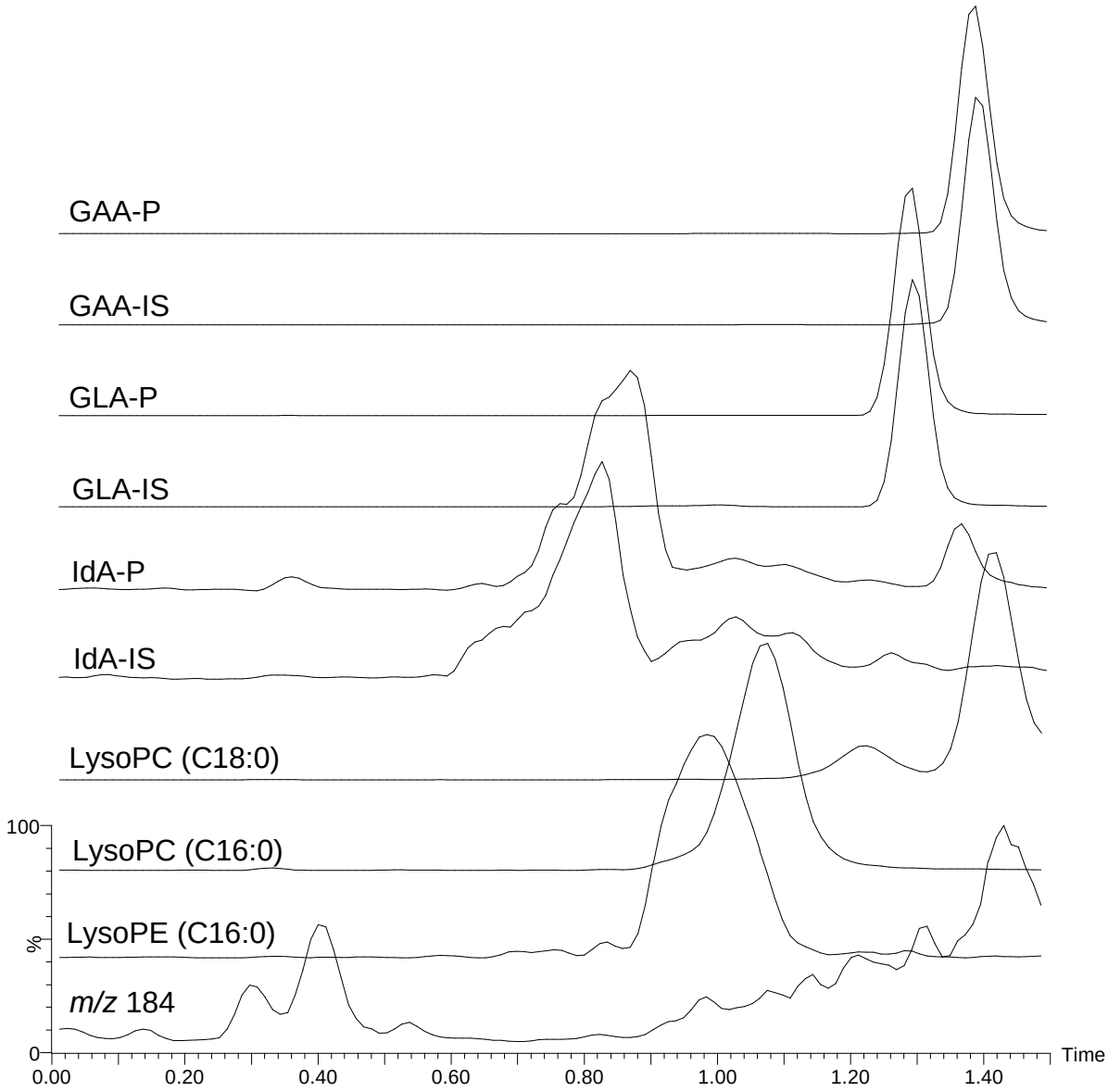


Figure S1. SRM ion traces representing GAA, GLA, and IdA products and internal standards combined with the ion trace detecting the trimethylammonium-ethyl phosphate ion (*m*/*z* 184) from phosphatidylcholines (PC), lysoPC and sphingomyelins (SM) and SRM ion traces for lysoPE (C19:0), lysoPC (C16:0) and lysoPC (C18:0).

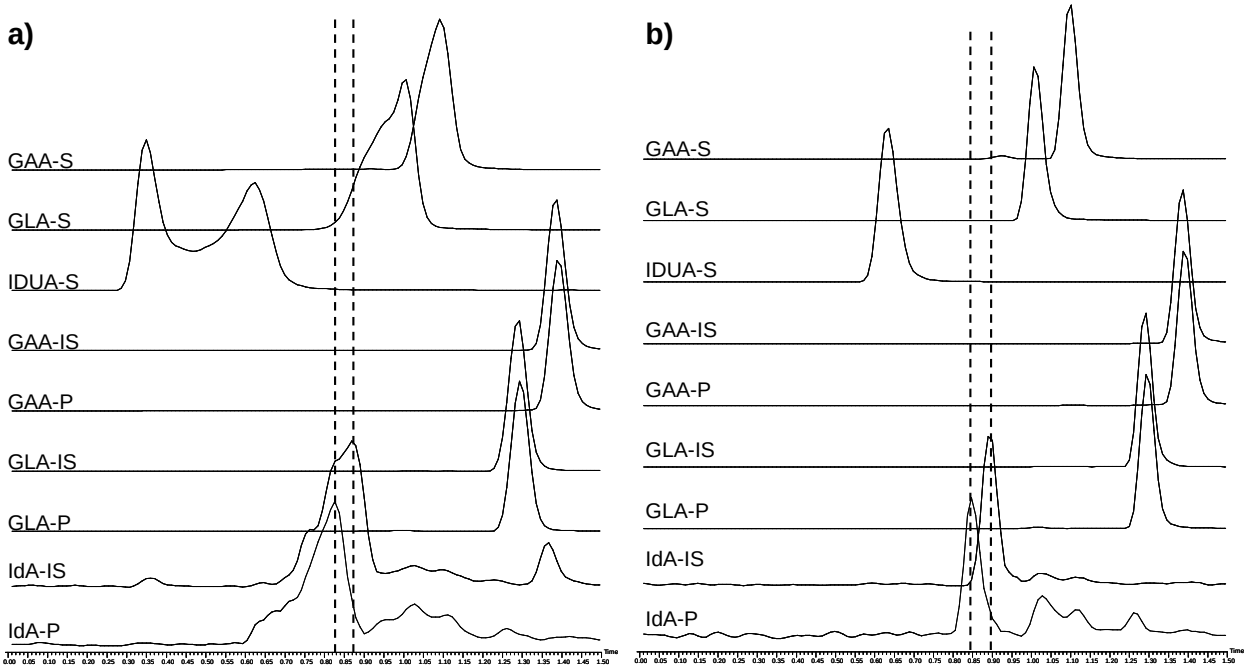


Figure S2.

Expanded view of the LC-MS/MS (SRM) chromatogram shows (a) the healthy individual specimen after acetonitrile protein precipitation and (b) the identical healthy individual specimen after acetonitrile mediated protein precipitation, but here the sample volume was diluted to the initial mobile phase solvent composition 70:30 water:acetonitrile. The peak maxima of IdA-P and IDA-IS are indicated by dashed lines. Refer to Figure 2 in the main text for the trace labeling.

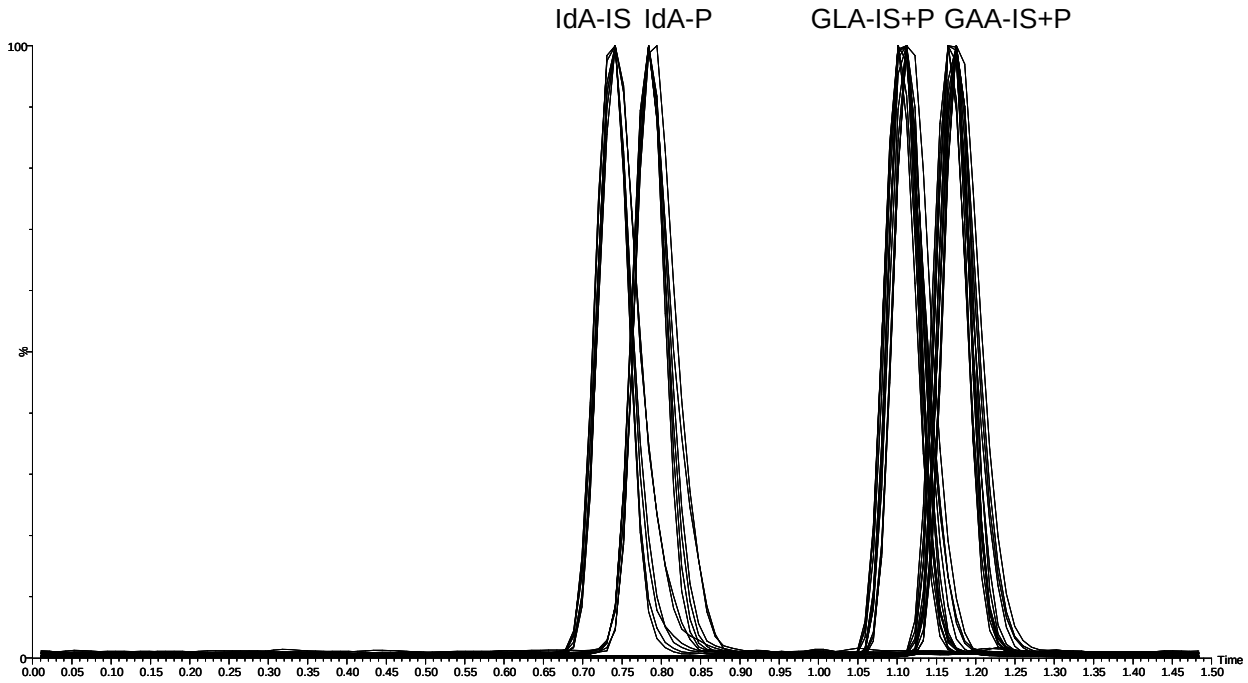
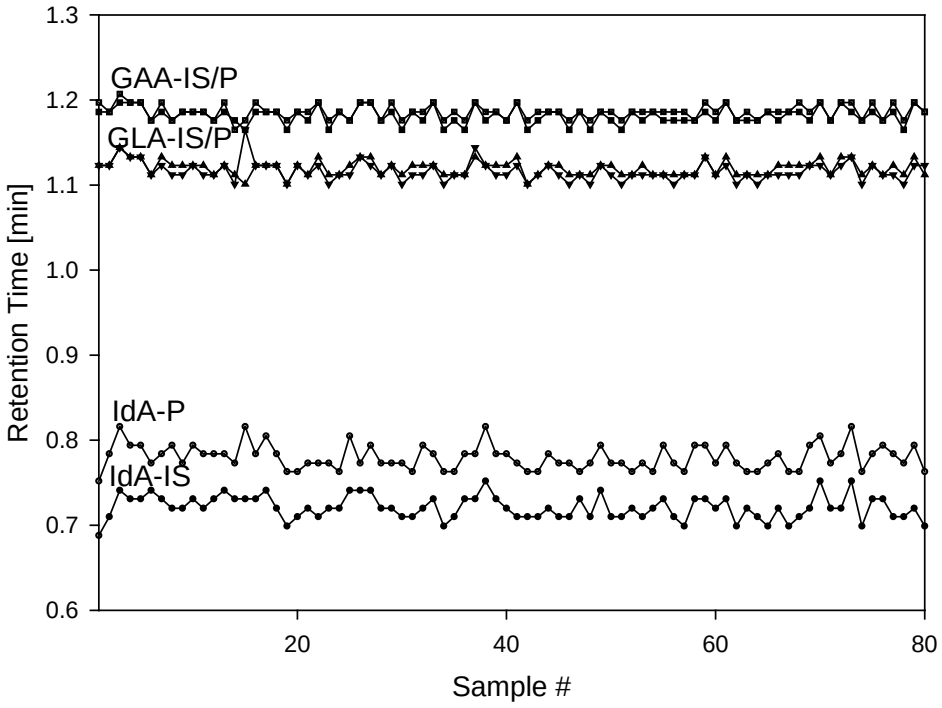
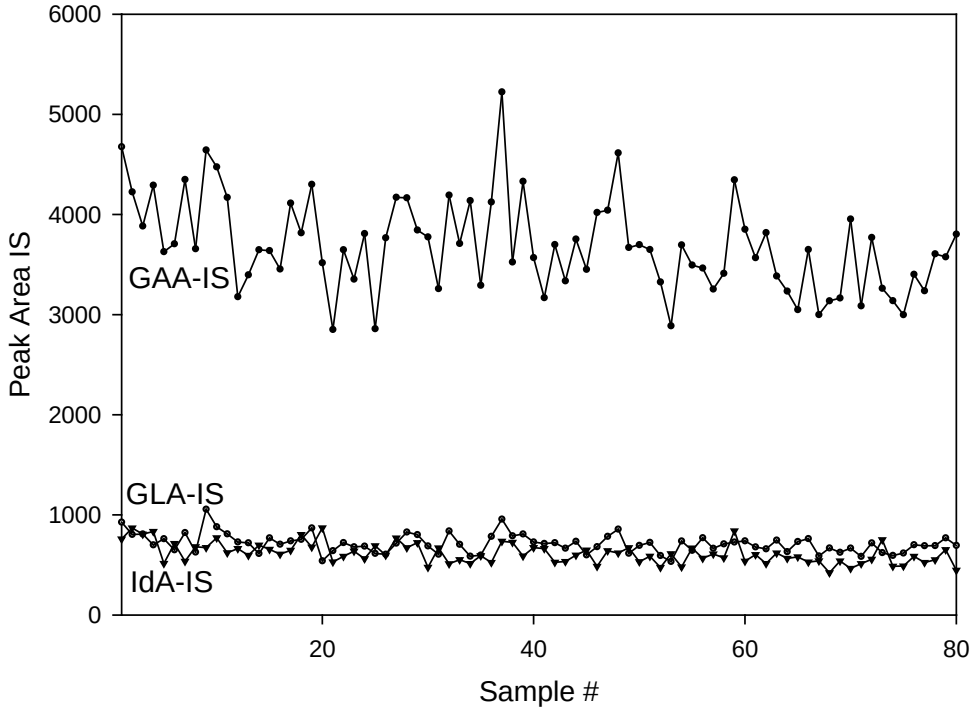


Figure S3

Six overlapping LC runs of standard compounds recorded gradually after 100, 200, 300, 400, 500 and 600 injections of acetonitrile quenched enzyme assays indicating excellent reproducibility of retention time and peak area. This single LC run consists of six SRM-based ion traces, each representing one internal standard or product.



a)



b)

Figure S4. Intra-day variations of (a) internal standards and products retention times and (b) internal standards peak areas measured for 80 individual assays previously quenched with acetonitrile.

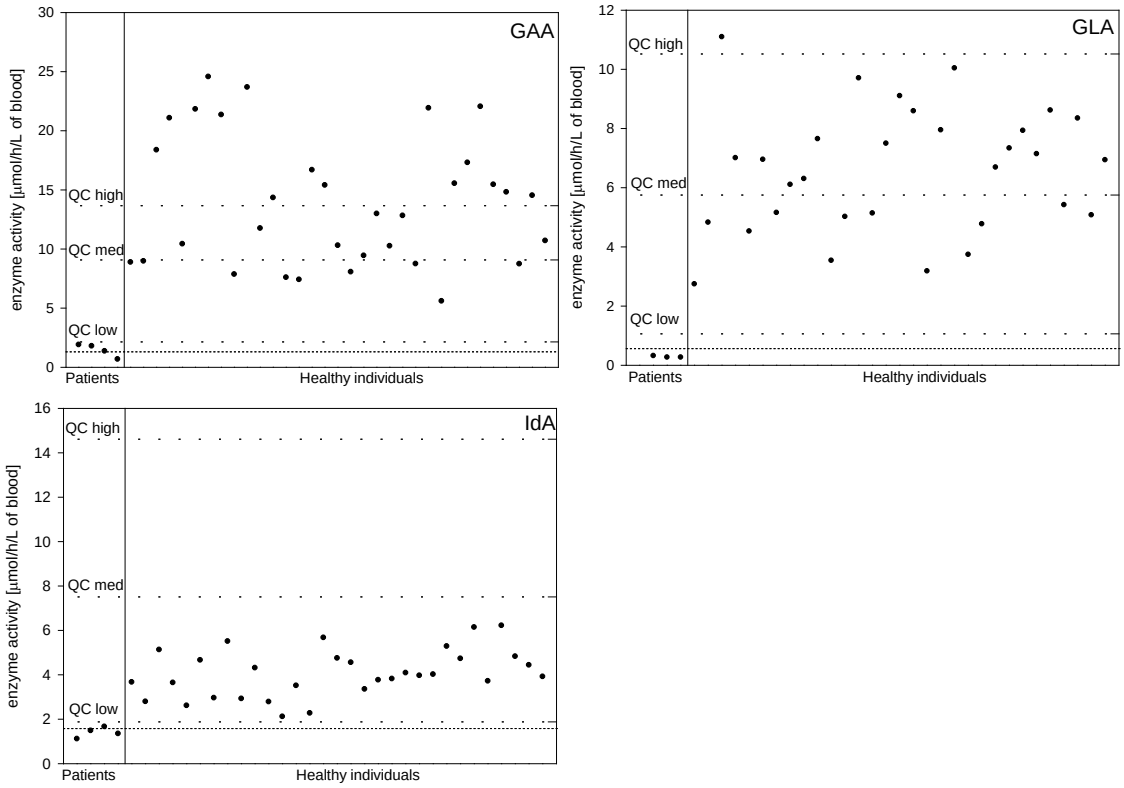


Figure S5. Enzyme activity distribution as determined by LC-ESI-MS/MS after the liquid-liquid extraction with ethyl acetate for individual sample measurements (particular values given in Table 4). The dotted lines correspond to QC base activity, and the dashed lines indicate QC low, QC medium and QC high levels.

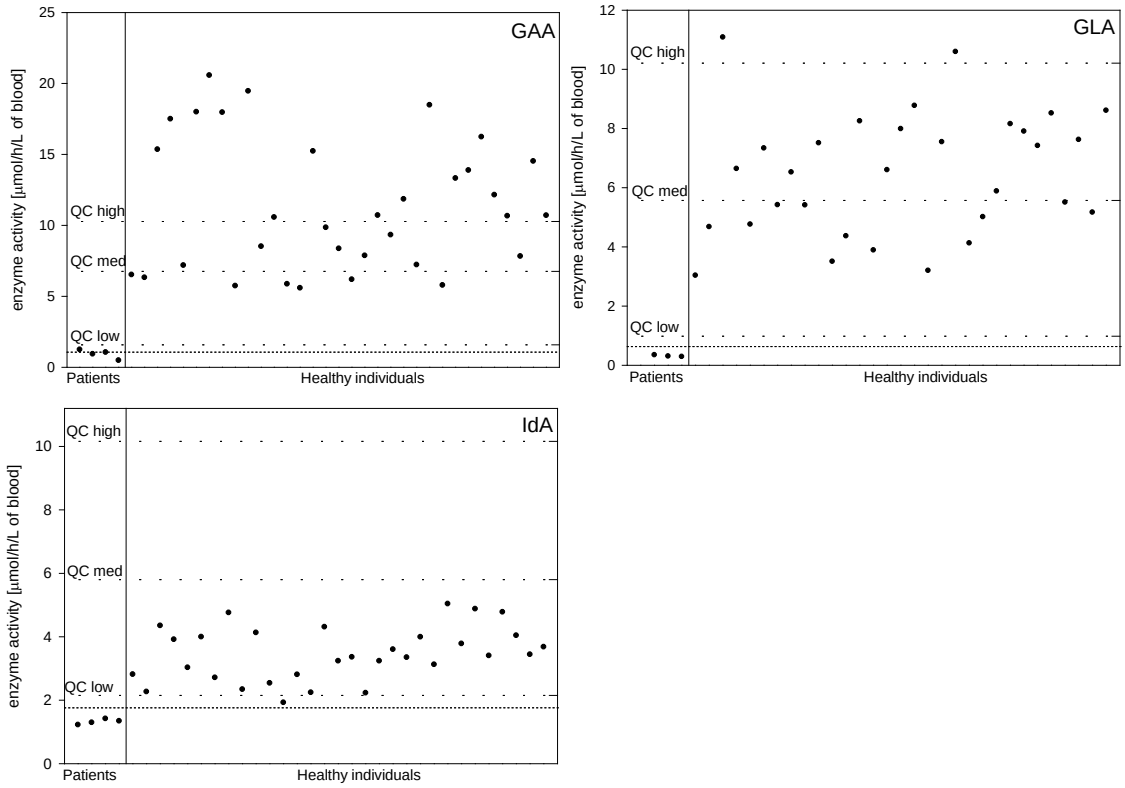


Figure S6. Enzyme activity distribution as determined by FIA-ESI-MS/MS after the acetonitrile mediated protein precipitation for individual sample measurements (particular values given in Table 4). The dotted lines correspond to QC base activity, and the dashed lines indicate QC low, QC medium and QC high levels.

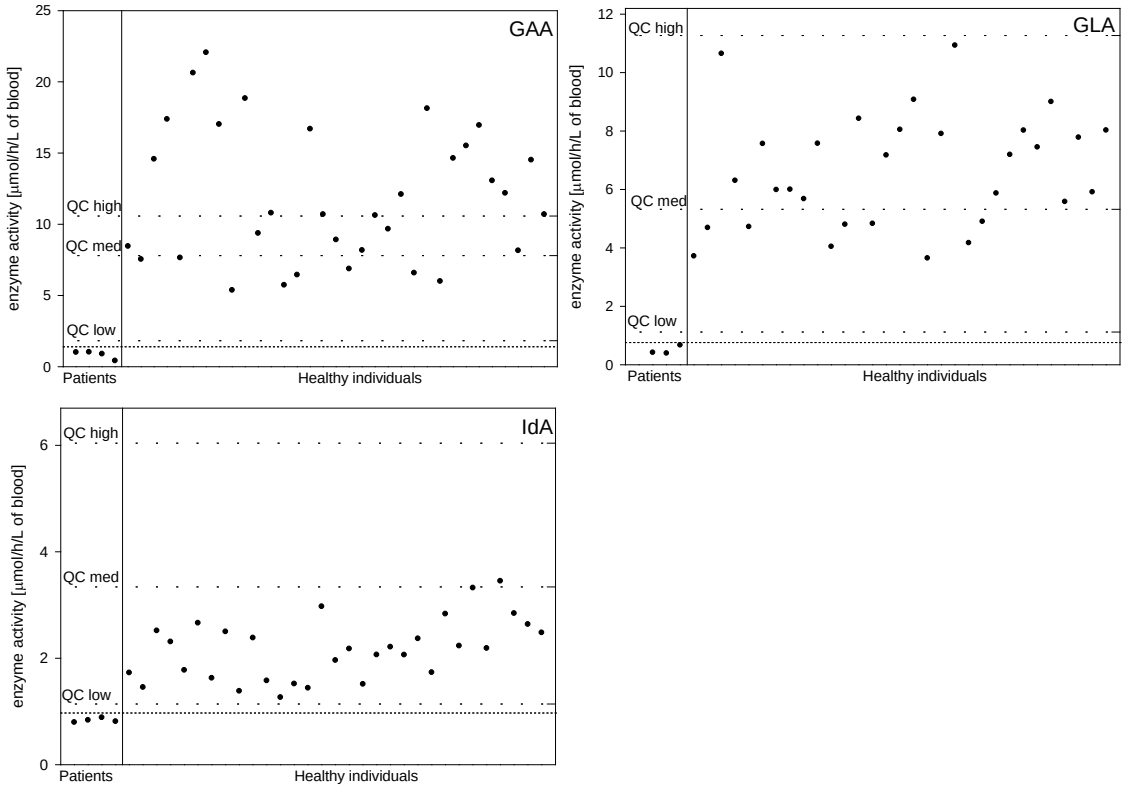


Figure S7. Enzyme activity distribution as determined by FIA-ESI-MS/MS after the liquid-liquid extraction to ethyl acetate for individual sample measurements (particular values given in Table 4). The dotted lines correspond to QC base activity, and the dashed lines indicate QC low, QC medium and QC high levels.

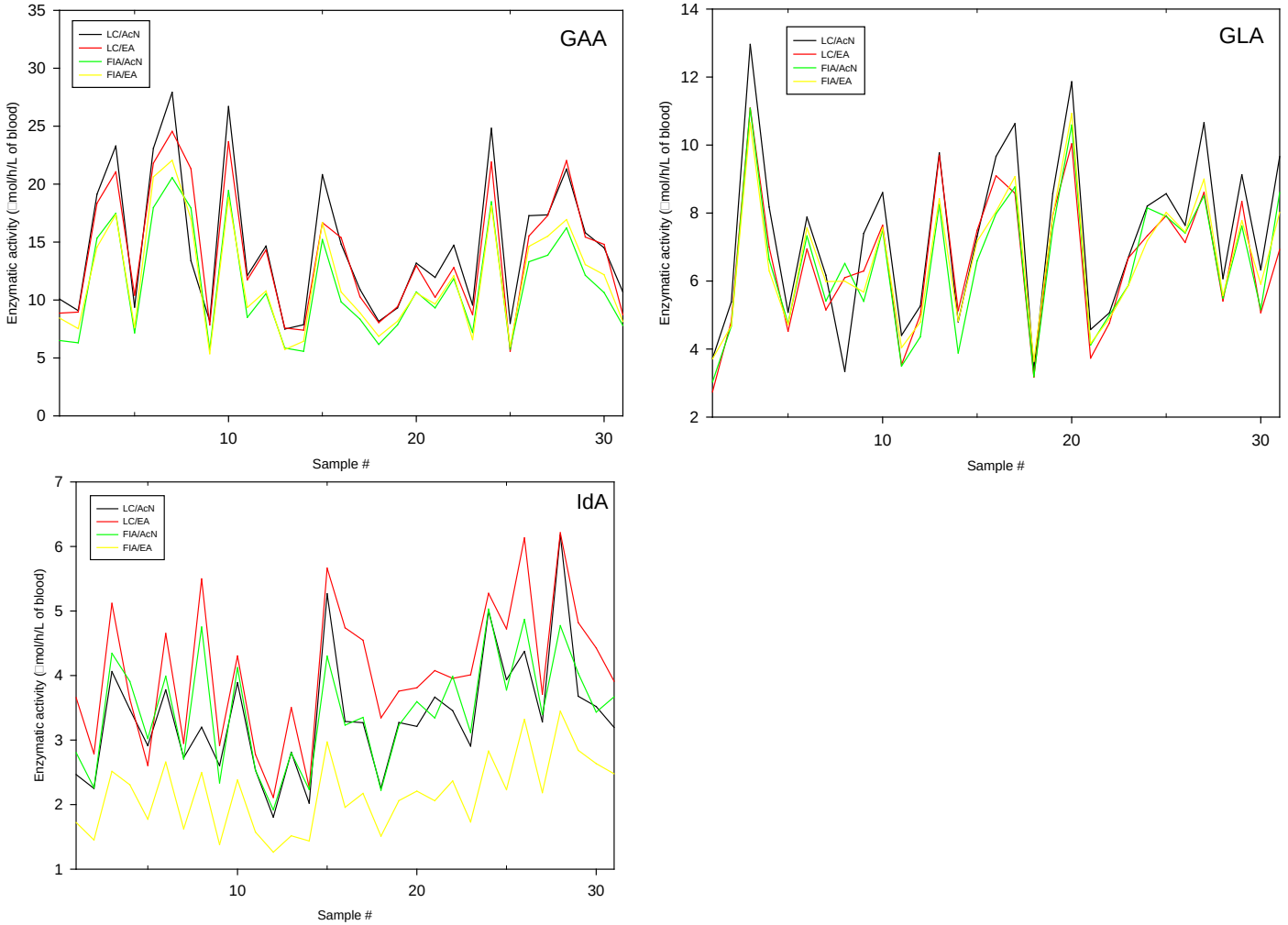


Figure S8. Correlation of the enzyme activity measured by four methods; permutations of acetonitrile mediated assay quench and liquid-liquid extraction to ethyl acetate sample work-ups with LC and FIA instrumental analysis.

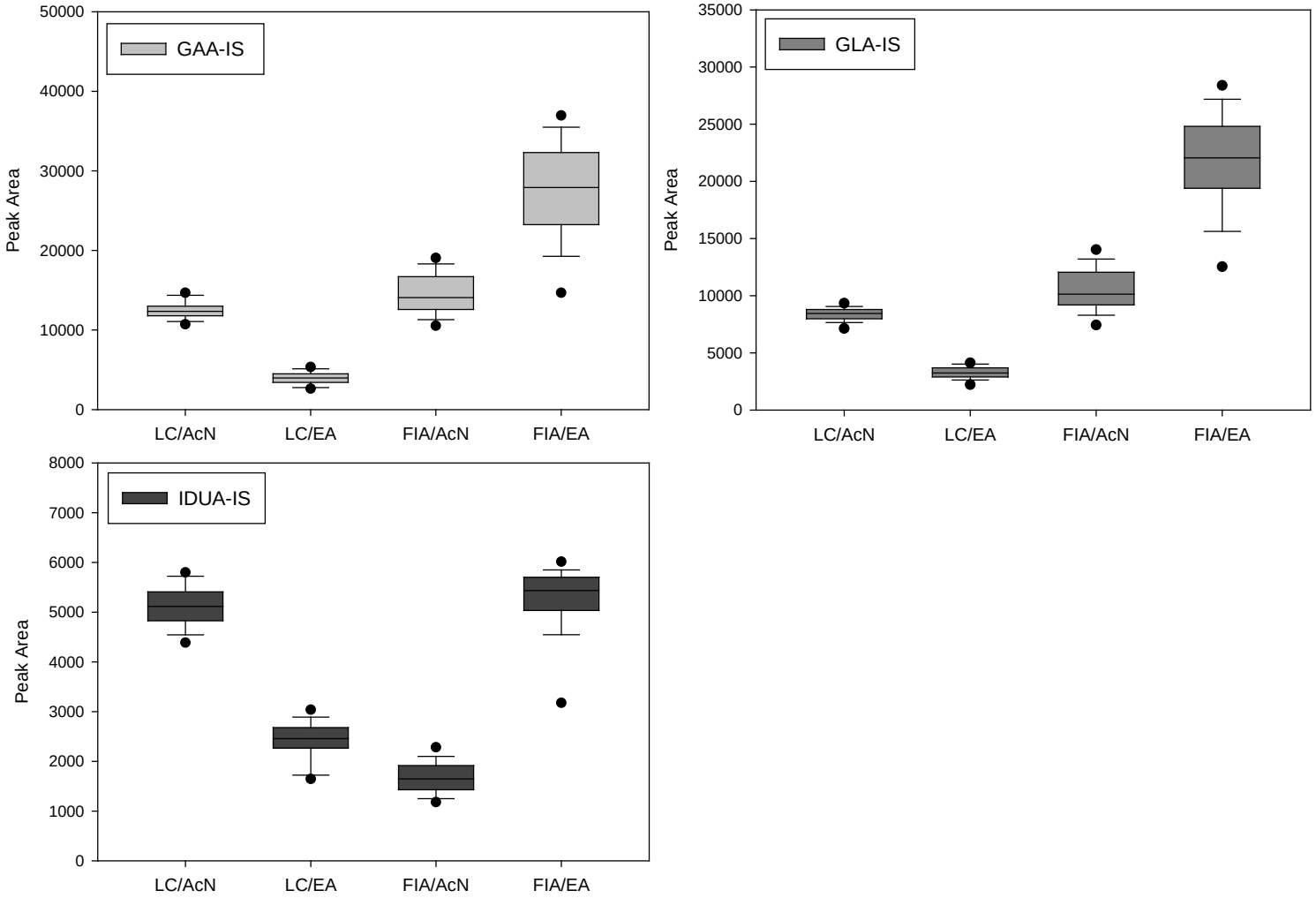


Figure S9. Reproducibilities of internal standard peak areas, represented by the 90th percentile (n = 72) plotted in the vertical boxes with median (solid line in the box) and error bars. Outliers were handled as 5th/95th percentile and are indicated by circles.