Supplemental Information

Expanding the Limits of Human Blood Metabolite Quantitation using NMR Spectroscopy

G. A. Nagana Gowda,[1*] Yashas N. Gowda[1] and Daniel Raftery[1,2,3*]

[1]*Northwest Metabolomics Research Center, Anesthesiology and Pain Medicine, and*

[2]*Department of Chemistry, University of Washington, Seattle, WA 98109,* [3]*Fred Hutchinson Cancer Research Center, Seattle, WA 98109*

This file contains 10 pages 1 to 10. The first page is the Title page; page 2 contains Table S1; page 3 contains Table S2 and Table S3; pages 4 and 5 contain Table S4; pages 6 and 7 contains Table S5; and pages 8, 9 and 10 contain Supplemental Figures S1, S2 and S3, respectively.

Table S1: List of metabolites used for spiking experiments to confirm their presence in the pooled human serum NMR spectra.

Metabolites confirmed to be present in serum	Metabolites not confirmed to be present in serum
1. 1-Methylhistidine	36. Cis-Aconitic acid
2. 3-Methylhistidine	37. Acetoacetic acid
3. 4-Methyl-2-oxovaleric acid (2-Oxoisocaproic acid)	38. Pyruvic acid
	39. Pyridoxine
4. 3-Methyl-2-oxobutanoic acid (2-Oxoisovaleric acid)	40. 2-Hydroxyisocaproic acid
	41. Malonic acid
5. 2-Hydroxybutyric acid	42. 1-Methylguanidine
6. D-(+)-Mannose	43. Citrulline
7. Hypoxanthine	44. Acetamide
8. Sarcosine	45. Trimethyl Amine N-oxide
9. Pyroglutamic acid	46. D-(-)-Fructose
10. DL-Ornithine	47. Gamma-aminobutyric acid
11. N,N-Dimethylglycine	48. Isocaproic acid
12. 2-Hydroxy-3-methylbutyric acid (2-hydroxyisovaleric acid)	49. 2-Ketoglutaric acid
	50. 2-Methylbutyric acid
13. Arginine	51. Methylmalonic acid
14. Succinic acid	52. D-Glucose-6-phosphate
15. Betaine	53. L-Cystine
16. Carnitine	54. Adipic acid
17. Fumaric acid	55. 2-Methylglutaric acid
18. Serine	56. Monomethylsuccinic acid
19. N-Acetyl glycine	57. Propionic acid
20. Isobutyric acid	58. Oxaloacetic acid
21. 3-Hydroxyisovaleric acid	59. Allantoin
22. Xanthine	60. 4-Pyridoxic acid
23. Hippuric acid	61. L-Ascorbic acid
24. Myo-Inositol	62. L-Cysteine
25. Sucrose	63. Suberic acid
26. O-Acetylcarnitine	64. 3-Methylbutyric acid
27. 2-Aminobutyric acid	65. 2-Oxobutyric acid
28. L-Methionine	
29. Isovaleric acid	
30. Acetone	
31. 2-Propanol	
32. Dimethylamine	
33. Uridine	
34. 3-Methyl-2-oxovaleric acid	
35. 1,2-Propanediol	

Table S2: Pooled human serum samples used in this study.		
Method	**Solvent to serum ratio (v/v)**	**Number of samples**
Methanol Precipitation	1:1	4
	2:1	4
	3:1	4
	4:1	4
Acetonitrile Precipitation	1:1	4
	2:1	4
	3:1	4
	4:1	4
Ultrafiltration	-	2

Table S3: Concentration of the six volatile metabolites detected only ultrafiltered serum.	
Metabolite	**Concentration (μM)**
Acetone	1.1± 0.3
Ethanol	103.7± 2.4
Dimethylamine	0.25± 0.1
Methanol*	551±26
2-Propanol	7.7±1.2
Urea	42.0±5.0
*Higher concentration value for methanol is due to the contribution from the filter membrane (see *Anal. Chem.* **2014**, 86:5433-5440)	

Table S4: Metabolites detected in pooled human blood serum and their concentrations (in μM) determined by NMR spectra obtained after protein precipitation using different proportions of methanol or acetonitrile solvent.

	Methanol to Serum ratio (v/v)			Acetonitrile to Serum ratio (v/v)		
Metabolite	2:1	3:1	4:1	2:1	3:1	4:1
1-Methylhistidine	4.5±0.1	4.2 ± 0.0	4.4 ± 0.0	3.3 ± 0.1	2.1 ± 0.1	2.0 ± 0.0
1,2-Propanediol[$]	7.5 ± 0.1	6.6 ± 0.0	6.0 ± 0.1	6.1 ± 0.1	4.5 ± 0.1	3.3 ± 0.1
2-Hydroxybutyric acid	37.7 ± 0.4	37.4 ± 0.0	34.8 ± 0.6	36.4 ± 0.6	36.1 ± 1.0	32.6 ± 0.3
2-Hydroxyisovaleric acid	13 ± 0.0	12.7 ± 0.4	11.3 ± 0.1	11.9 ± 0.7	11.1 ± 0.1	10.9 ± 0.1
2-Aminobutyric acid	16.0 ±0.0	16.2 ±0.3	16.1 ±0.1	15.3 ±0.1	15.1 ±0.1	12.5 ±0.1
2-Propanol	NM	NM	NM	NM	NM	NM
2-Oxoisocaproic acid	13.7 ± 0.1	13.4 ± 0.3	11.7 ± 1.0	13.7 ± 0.1	13.4 ± 0.3	13.6 ± 0.0
2-Oxoisovaleric acid	2.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.0	2.2 ± 0.0	2.1 ± 0.0
3-Hydroxybutyric acid	89 ± 0.8	82.2 ± 0.3	82.1 ± 0.1	89.0 ± 0.3	85.7 ± 2.1	73.2 ± 0.6
3-Hydroxyisovaleric acid	2.0 ± 0.0	2.2 ± 0.0	1.7 ± 0.1	1.8 ± 0.0	1.5 ± 0.1	1.7 ± 0.1
3-Methylhistidine	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.1 ± 0.1	2.4 ± 0.0	1.8 ± 0.0
3-Methyl-2-oxovaleric acid	9.6 ± 0.3	10.1 ± 0.1	8.3 ± 0.1	11.1 ± 0.1	10.6 ± 0.6	10.4 ± 0.3
Acetic acid	271.3 ± 0.1	269.0 ± 1.1	246.1 ± 0.1	271.0 ± 0.6	270.7 ± 0.1	254.2 ± 2.8
Acetone	NM	NM	NM	NM	NM	NM
Acetylcarnitine	4.0± 0.0	4.0± 0.0	4.0± 0.0	4.0± 0.0	4.0± 0.0	3.8± 0.0
N-Acetylglycine	57.2 ± 1.9	59.0 ± 1.9	55.4 ± 0.6	50.9 ± 1.9	46.4 ± 0.6	43.1 ± 0.1
Alanine	353.1 ± 5.8	342.4 ± 2.3	341.0 ± 1.4	335.3 ± 4.7	270.0 ± 2.8	251.0 ± 1.4
Arginine	138.2 ± 0.3	138.7 ± 1.0	132.2 ± 0.3	136.2 ± 0.3	120.7 ± 1.0	103.0 ± 1.4
Asparagine	44.4 ± 0.3	44.1 ± 0.1	38.7 ± 0.1	35.2 ± 0.8	24.1 ± 0.1	20.3 ± 0.1
Aspartic acid	35.6 ± 0.6	33.5 ± 0.7	28.9 ± 0.1	28.2 ± 0.3	15.6 ± 0.3	11.9 ± 0.1
Benzoic acid	36.3 ± 0.1	36.2 ± 0.0	35.8 ± 0.3	35.9 ± 0.1	34.2 ± 0.3	34.7 ± 0.4
Betaine	48.4 ± 0.3	49.5 ± 0.7	47.8 ± 0.3	48.8 ± 0.6	44.2 ± 1.1	41.1 ± 0.1
Carnitine	35.0 ± 0.3	34.5 ± 0.7	32.2 ± 0.3	28.3 ± 0.4	28.4 ± 1.1	26.7 ± 0.1
Choline	117.9 ± 0.1	116.2 ± 0.3	111.4 ± 0.3	113.5 ± 0.7	114.0 ± 1.4	115.5 ± 0.7
Citric acid	18.6 ± 0.0	19.1 ± 0.1	18.7 ± 0.1	6.5 ± 0.1	0	0
Creatine	28.4 ± 0.6	28.9 ± 0.1	27.3 ± 0.1	25.4 ± 0.3	22.1 ± 0.1	18.4 ± 0.3
Creatinine	77.9 ± 0.7	76.2 ± 0.3	69.3 ± 0.1	76.1 ± 0.1	74.6 ± 0.3	74.3 ± 0.1
Dimethylamine	NM	NM	NM	NM	NM	NM
Dimethylglycine	3.0 ± 0.0	3.0 ± 0.0	2.8 ± 0.0	2.7 ± 0.1	2.5 ± 0.1	2.4 ± 0.0
Ethanol	NM	NM	NM	NM	NM	NM
Formic acid	30.3 ± 1.8	27.1 ± 0.4	22.3 ± 0.1	17.5 ± 0.7	17.1 ± 0.1	17.2 ± 0.0
Fumaric acid	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0	0
Glucose[&]	3456 ± 90.5	3407 ± 1.4	3282.0 ± 2.8	3348.0 ± 11.3	2941.0 ± 24.0	2650.0 ± 14.1
Glutamic acid	257.1 ± 0.1	255.6 ± 0.6	237.0 ± 1.4	200.5 ± 0.1	148.0 ± 4.8	101.0 ± 1.4
Glutamine	170.2 ± 3.1	173.3 ± 1.0	167.5 ± 0.7	167.5 ± 0.1	116.5 ± 0.1	89.5 ± 0.1
Glycerol	184.9 ± 3.5	182.2 ± 0.3	165.7 ± 0.4	184.4 ± 0.3	176.4 ± 9.5	165.0 ± 1.4
Glycine	407.8 ± 0.0	407.8 ± 0.0	381.0 ± 1.4	374.7 ± 1.3	279.4 ± 3.7	228.3 ± 1.0
Hippuric acid	NM	NM	NM	NM	NM	NM
Histidine	62.0 ± 2.3	62.9 ± 2.4	63.0 ± 0.8	63.0 ± 0.8	47.7 ± 0.1	39.3 ± 2.4
Hypoxanthine	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.3	2.1 ± 0.1	2.2±0.0

Isobutyric acid	5.9 ± 0.4	5.9 ± 0.4	5.6 ± 0.0	5.7 ± 0.1	5.1 ± 0.4	5.5 ± 0.1
Isoleucine	68.6 ± 0.0	62.4 ± 2.3	64.1 ± 0.1	63.2±2.0	55.5 ± 0.1	47.0 ± 1.4
Isovaleric acid	10.2 ±0.3	10.8 ±0.0	11.2 ±0.6	9.9 ±0.4	9.2 ±0.3	9.5 ±0.1
Lactic acid	2870.6 ± 15.0	2668.0 ± 50.9	2452.0 ± 17.0	2350.0 ± 70.7	1826.0 ± 48.0	1747.0 ± 38.2
Leucine	118.9 ± 2.4	117.7 ± 0.1	116.0 ± 0.0	119.6 ± 1.4	108.4 ± 0.3	101.0 ± 0.6
Lysine	119.8 ± 0.0	124.6 ± 2.0	121.5 ± 0.1	99.5 ± 3.5	53.5 ± 0.4	36.6 ± 0.6
Mannose[@]	64.0 ± 0.0	64.0 ± 0.0	62.5 ± 0.7	57.8 ± 1.7	51.1 ± 0.1	41.5 ± 0.4
Methanol	NM	NM	NM	NM	NM	NM
Methionine	27.1 ± 0.4	26.9 ± 0.1	25.4 ± 0.3	25.7 ± 0.4	22.5 ± 0.1	17.7 ± 0.1
Myoinositol	22.4 ± 0.0	21.8 ± 0.0	18.9 ± 0.7	17.2 ± 0.6	12.3 ± 1.5	9.5 ± 0.4
Ornithine	32.6 ± 0.0	32.5 ± 0.1	32.2 ± 0.0	27.8 ± 0.3	18.0 ± 0.0	18.0 ± 0.0
Phenylalanine	133.3 ± 0.1	134 ± 0.0	120.9 ± 0.4	126.3 ± 0.1	120.7 ± 3.8	114.7 ± 0.4
Proline	231.9 ± 2.1	230 ± 0.0	211.5 ± 0.7	211.8 ± 0.3	209.0 ± 4.2	189.0 ± 1.4
Pyroglutamic acid	85.4 ± 1.7	87 ± 0.6	85.5 ± 0.7	84.5 ± 0.7	83.5 ± 0.7	74.5 ± 0.7
Sarcosine	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.4 ± 0.0	1.0 ± 0.0
Serine	133.6 ± 0.0	129.6 ± 0.6	118.0 ± 0.0	119.8 ± 0.3	109.5 ± 0.0	71.0 ± 1.4
Succinic acid	18.9 ± 2.4	18.8 ± 0.6	16.3 ± 0.1	12.8 ± 0.3	7.7 ± 0.7	3.2 ± 0.3
Sucrose	NM	NM	NM	NM	NM	NM
Threonine	160.4 ± 1.7	157.2 ± 0.8	145.0 ± 1.4	126.3 ± 0.1	94.8 ± 0.6	77.3 ± 0.1
Tryptophan	47.5 ± 0.7	47.8 ± 0.3	47.9 ± 0.1	48.4 ± 0.3	48.4 ± 0.3	48.3 ± 0.1
Tyrosine	70.3 ± 0.7	71.1 ± 1.0	62.2 ± 0.3	68.9 ± 0.1	61.8 ± 1.4	56.5 ± 0.4
Urea	NM	NM	NM	NM	NM	NM
Uridine	3.3 ± 0.1	3.3 ± 0.1	3.0 ± 0.0	3.4 ± 0.0	3.4 ± 0.0	3.5 ± 0.1
Valine	153.8 ± 2.0	151.9 ± 0.4	151.8 ± 0.3	151.9 ± 0.1	135.2 ± 0.3	121.7 ± 0.4
Xanthine	4.3 ± 0.4	4.6 ± 0.3	4.6 ± 0.0	4.2 ± 0.0	2.7 ± 0.1	1.9 ± 0.1

NM-Detected, but not measured since the signal intensity was too weak or not detected in protein precipitated serum samples. Error is expressed as standard deviation. [$]1,2-propanediol NMR peak intensity is attenuated due to sample drying after protein precipitation and hence the shown values are anticipated to be lower than actual concentration in blood. [&]Sum of α- and β-Glucose. [@] Sum of α- and β-Mannose. See Figures 1 and Figure 2.

Table S5: Attenuation of metabolite concentrations (in %) relative to methanol or acetonitrile precipitated serum (2:1 v/v) determined using high resolution ^{1}H 1D NMR spectroscopy at 800 MHz.

	Relative to 2:1 MeOH			Relative to 2:1 ACN	
Metabolite	**2:1 ACN**	**3:1 MeOH**	**4:1 MeOH**	**3:1 ACN**	**4:1 ACN**
1-Methylhistidine	26.7	6.7	2.2	36.4	39.4
2-Hydroxybutyric acid	3.4	0.8	7.7	0.8	10.4
2-Hydroxyisovaleric acid	8.5	2.3	13.1	6.7	8.4
2-Aminobutyric acid	4.3	-1.3	-0.6	2.3	18.3
2-Propanol	NM	NM	NM	NM	NM
2-Oxoisocaproic acid	0	2.2	14.6	2.2	0.7
2-Oxoisovaleric acid	0.7	2.2	2.2	-6.5	-2.2
3-Hydroxybutyric acid	0	7.6	7.8	3.7	17.8
3-Hydroxyisovaleric acid	10.0	-10.0	15.0	16.7	5.6
3-Methylhistidine	-2.0	0	0	52.9	64.7
3-Methyl-2-oxovaleric acid	-15.6	-5.2	13.5	4.5	6.3
Acetic acid	0.1	0.9	9.3	0.0	6.2
Acetone	NM	NM	NM	NM	NM
Acetylcarnitine	0.0	0.0	0.0	0.0	5.0
N-Acetylglycine	11.0	-3.1	3.1	8.8	15.2
Alanine	5.0	3.0	3.4	19.5	25.1
Arginine	1.4	-0.4	4.3	11.4	24.4
Asparagine	20.7	0.8	12.8	31.5	42.3
Aspartic acid	20.8	5.9	18.8	44.7	57.8
Benzoic acid	1.1	0.3	1.4	4.7	3.3
Betaine	-0.8	-2.3	1.2	9.4	15.8
Carnitine	19.1	1.4	8.0	-0.4	5.7
Choline	3.7	1.4	5.5	-0.4	-1.8
Citric acid	65.1	-2.7	-0.5	100	100
Creatine	10.6	-1.8	3.9	13.0	27.6
Creatinine	2.3	2.2	11.0	2.0	2.4
Dimethylamine	NM	NM	NM	NM	NM
Ethanol	NM	NM	NM	NM	NM
Formic acid	42.2	10.6	26.4	2.3	1.7
Fumaric acid	66.7	0	33.3	100.0	100
Glucose[&]	3.1	1.4	5.0	12.2	20.8
Glutamic acid	22.0	0.6	7.8	26.2	49.6
Glutamine	1.6	-1.8	1.6	30.4	46.6
Glycerol	0.3	1.5	10.4	4.3	10.5
Glycine	8.1	0	6.6	25.4	39.1
Hippuric acid	NM	NM	NM	NM	NM
Histidine	-1.6	-1.5	-1.6	24.3	37.6

Hypoxanthine	0	0	0	-5.0	-10.0
Isobutyric acid	3.4	0	5.1	10.5	3.5
Isoleucine	7.9	9.0	6.6	12.2	25.6
Isovaleric acid	2.9	-5.8	-9.8	7.7	4.0
Lactic acid	18.1	7.1	14.6	22.3	25.7
Leucine	-0.6	1.0	2.4	9.4	15.6
Lysine	16.9	-4.0	-1.4	46.2	63.2
Mannose[@]	9.7	0	2.3	11.6	28.2
Methanol	NM	NM	NM	NM	NM
Methionine	5.2	0.7	6.3	12.5	31.1
Myoinositol	23.2	2.7	15.6	28.5	44.8
N,N-Dimethylglycine	10	0	6.7	7.4	11.1
Ornithine	14.7	0.3	1.2	35.3	35.3
Phenylalanine	5.3	-0.5	9.3	4.4	9.2
Proline	8.7	0.8	8.8	1.3	10.8
Pyroglutamic acid	1.1	-1.9	-0.1	1.2	11.8
Sarcosine	0	0	0	12.5	37.5
Serine	10.3	3.0	11.7	8.6	40.7
Succinic acid	32.3	0.5	13.8	39.8	75.0
Sucrose	NM	NM	NM	NM	NM
Threonine	21.3	2.0	9.6	24.9	38.8
Tryptophan	-1.9	-0.6	-0.8	0	0.2
Tyrosine	2.0	-1.1	11.5	10.3	18.0
Urea	NM	NM	NM	NM	NM
Uridine	-3.0	0	9.1	0	-2.9
Valine	1.2	1.2	1.3	11.0	19.9
Xanthine	2.3	-7.0	-7.0	35.7	54.8

NM-Not measured; positive value indicates decrease and negative value indicates increase; [&]Sum of α- and β-Glucose; [@]Sum of α- and β-Mannose.

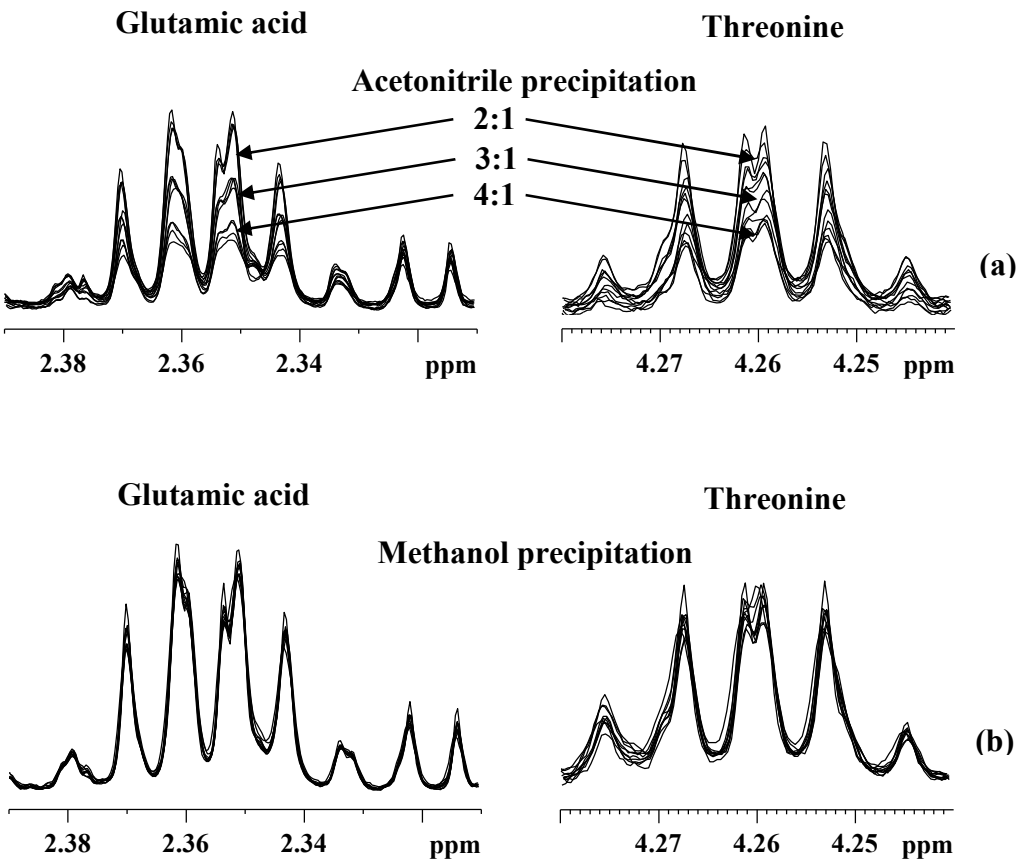

Figure S1: (a) Examples of glutamic acid and threonine NMR peaks, which depict typical reductions in peak intensity when the proportion of acetonitrile is increased from 2:1 to 4:1 for serum protein precipitation; (b) Peaks for the same metabolites indicate that methanol performs much better over a wide range of its concentration. The ratios 2:1, 3:1 and 4:1 indicate solvent to serum ratios used for protein precipitation.

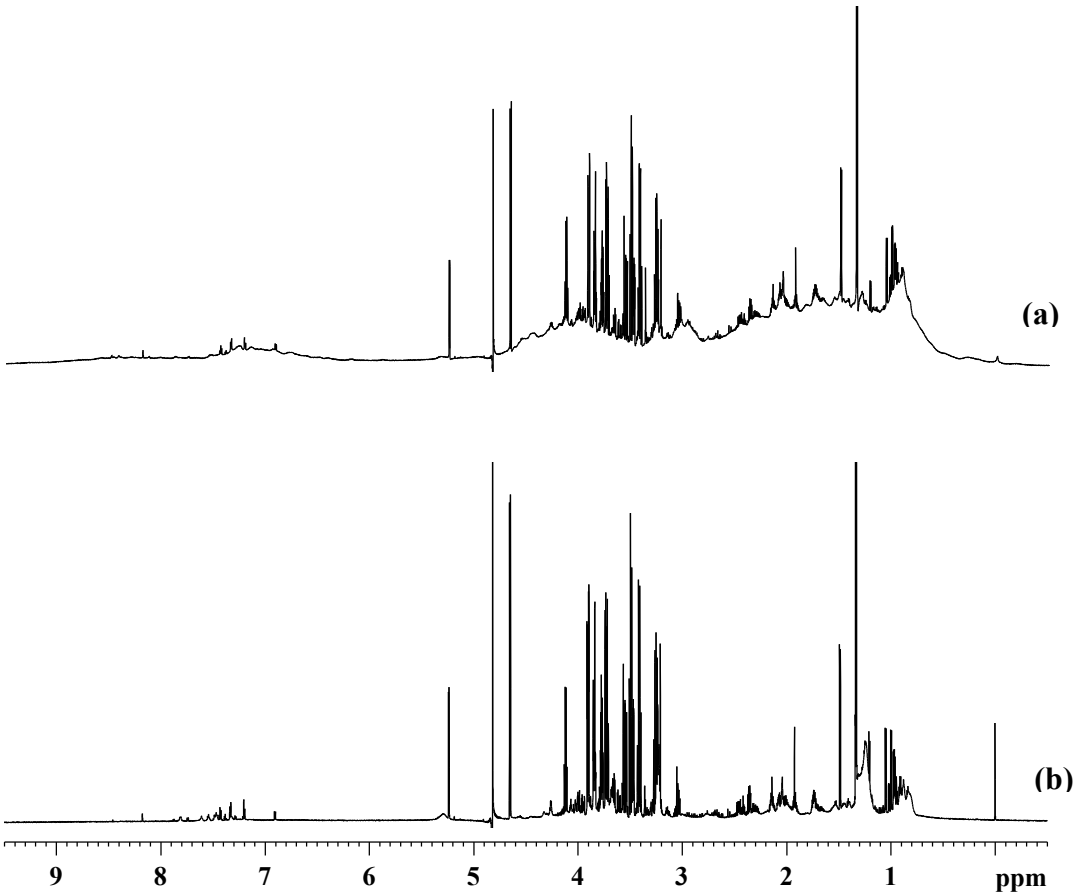

Figure S2: NMR spectra of pooled human serum obtained using the one-pulse sequence with water presaturation after protein precipitation using a methanol to serum ratio of (a) 1:1 and (b) 2:1. Note, higher level of protein background is observed in (a).

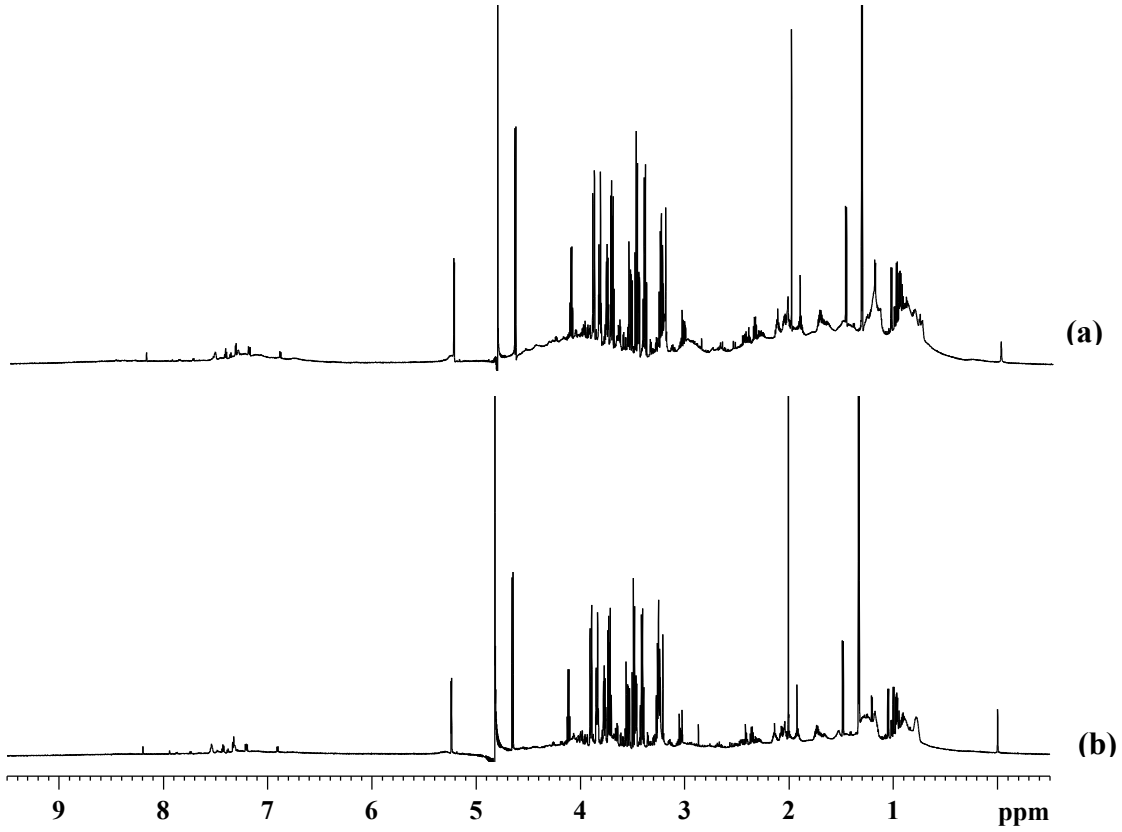

Figure S3: NMR spectra of pooled human serum obtained using the one-pulse sequence with water presaturation after protein precipitation using an acetonitrile to serum ratio of (a) 1:1 and (b) 2:1. Note, higher level of protein background is observed in (a).