Supplementary Information

An LC-MS/MS Method for the Simultaneous Quantification of Insulin, Cortisol, Glucagon-like peptide 1, Ghrelin, and Osteocalcin

Zhichao Zhang[1] ‡, Hareem Siddiqi[1] ‡, Yu-Ping Huang[2], Shannon M[c]Clorry[1], Peng Ji[1], Daniela Barile[2] and Carolyn M. Slupsky[1&2*]

[1]Department of Nutrition, University of California, Davis, Davis, CA, USA 95616

[2]Department of Food Science & Technology, University of California, Davis, Davis, CA, USA, 95616

[*]Corresponding author: Carolyn M. Slupsky, cslupsky@ucdavis.edu; tel: 530-752-6804; fax: 530-752-8966.

‡ These authors contributed equally.

Table S1. Diluents used for hormones standards.

Hormone	Diluent	Hormone	Diluent
Porcine insulin	0.2% formic acid in ultrapure water	Human insulin	0.2% formic acid in ultrapure water
Porcine osteocalcin	0.2% formic acid in ultrapure water	Human osteocalcin	3% ammonia water
Porcine ghrelin	0.3% acetic acid in ultrapure water	Human ghrelin	Ultrapure water
Porcine des-acyl ghrelin	0.3% acetic acid in ultrapure water	Human des-acyl ghrelin	Ultrapure water
GLP-1 (7-36)	80:20:0.1 H2O:MeOH: formic acid	Methyl GLP-1 (7-36)	Ultrapure water
GLP-1 (7-37)	DMSO	Methyl GLP-1 (7-37)	3% ammonia water
Cortisol	MeOH	d4-Cortisol	MeOH

Table S2. Expanded parameters for external and internal standard ions monitored using dynamic MRM.

	Precursor ion	Product ion	Retention time (min)	Fragmentor voltage (V)	Collison Energy (eV)	Cell Accelerator (V)
External standard ions						
Cortisol	363.2	121.0	20.1	160	30	4
Pig des-acyl ghrelin	639.1	84.0	10.1	160	100	4
Pig ghrelin	553.9	84.0	13.5	140	90	4
Pig carboxylated osteocalcin	1145.4	1115.3	21.2	160	100	4
GLP-1 (7-36)	660.6	660.6	19.7	135	0	4
GLP-1 (7-37)	672.1	84.1	20.1	140	120	4
Pig insulin	1155.7	86.0	20.2	160	30	4
Internal standard ions						
d4-Cortisol	367.2	121.0	20.1	140	26	4
Human des-acyl ghrelin	541.5	70.0	10.6	120	40	4
Human ghrelin	562.5	70.0	13.9	120	40	4
Human carboxylated osteocalcin	1186.9	1186.9	19.9	135	0	4
Methyl GLP-1 (7-36)	663.5	72.1	19.7	135	100	4
Methyl GLP-1 (7-37)	674.9	84.1	20.1	140	120	4
Human insulin	1161.7	1158.5	19.7	160	30	4

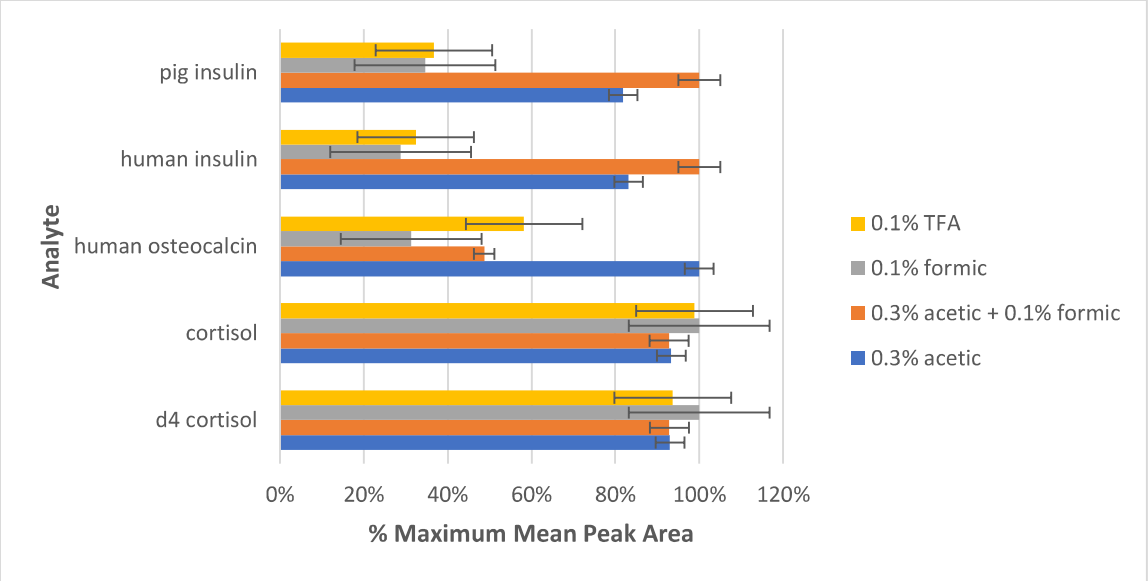


Figure S1. Relative signal intensity of hormones extracted with four types of acids. Pig serum was spiked with QC1 concentrations of the available analytes and extracted using 75% ACN with the four following acids: 0.1% TFA, 0.1% formic acid, a combination of 0.3% acetic acid and 0.1% formic acid, or 0.3% acetic acid. Analysis was performed in triplicate and the average peak area is visualized in the figure. No single pH modifier provided maximum signal intensity for all analytes. While cortisol was largely unaffected by acid type, signal intensity of des-acyl and acyl ghrelin and osteocalcin were much lower with 0.1% TFA or 0.1% formic acid. The combination of acetic and formic acid was also not suitable for osteocalcin, thus acetic acid was considered for future trials.

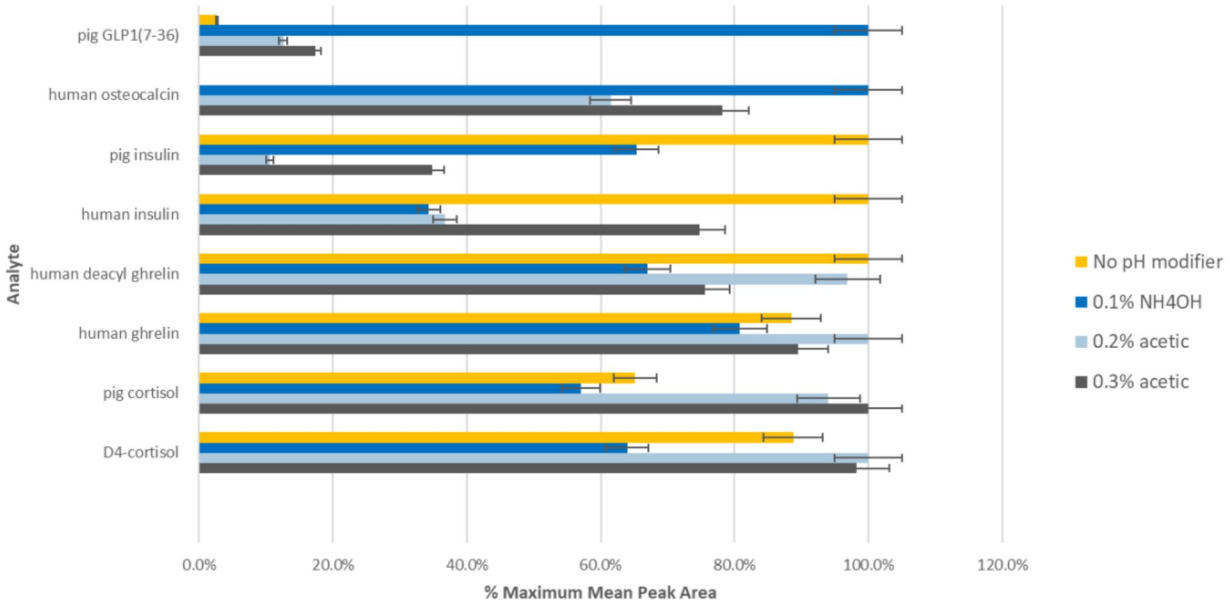


Figure S2. Relative signal intensity of spiked serum samples extracted with either no pH modifier, 0.1% NH_4OH, 0.2% acetic acid, or 0.3% acetic acid. Pig serum was spiked with QC1 concentrations of the available analytes and extracted using the four different protein precipitation solvents. Analysis was performed in triplicate and the average peak area with SD was visualized in the figure. No single pH modifier provided maximum signal intensity for all analytes.

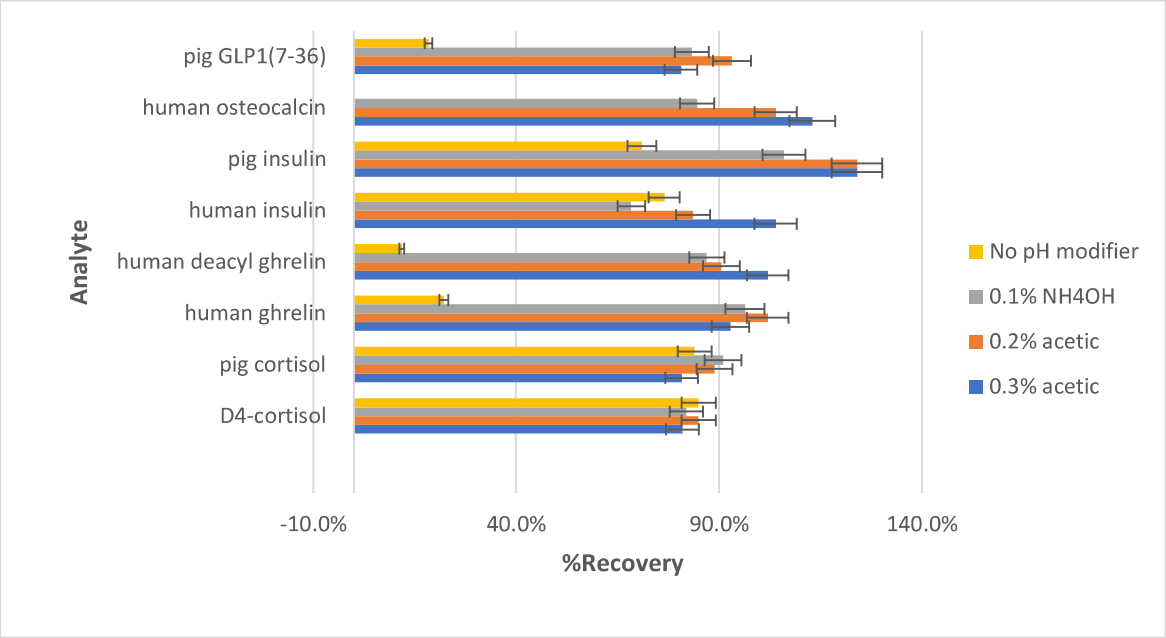


Figure S3. Recovery of spiked serum samples extracted with different pH modifiers. Pig serum was spiked with QC1 concentrations of the available analytes either before or after extraction. Samples were extracted using the four different pH modified protein precipitation solvents. %Recovery was calculated as the ratio of the signal intensity of samples spiked before extraction versus after extraction. All conditions were run in triplicate and the average %recovery with SD was visualized in the figure. No single pH modifier provided optimal recovery for all analytes.

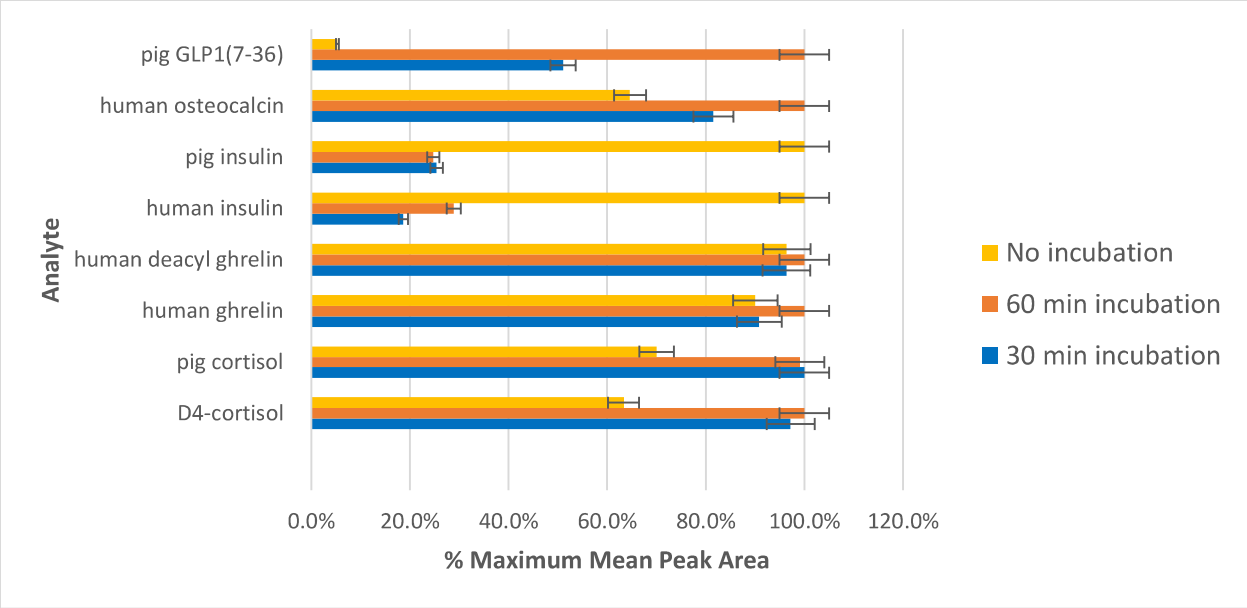
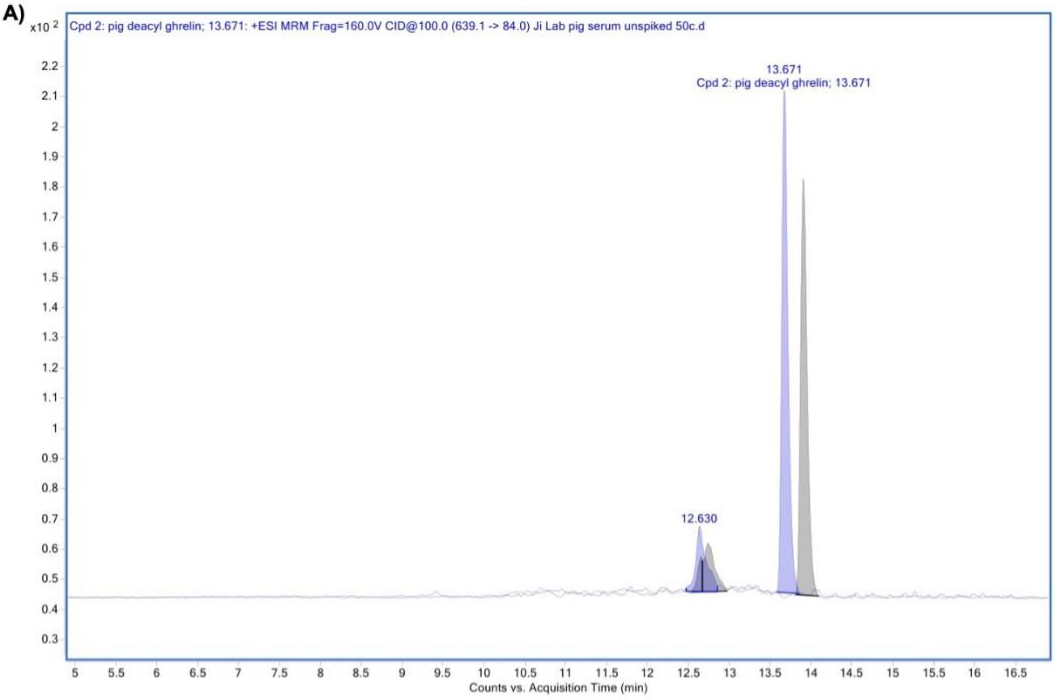
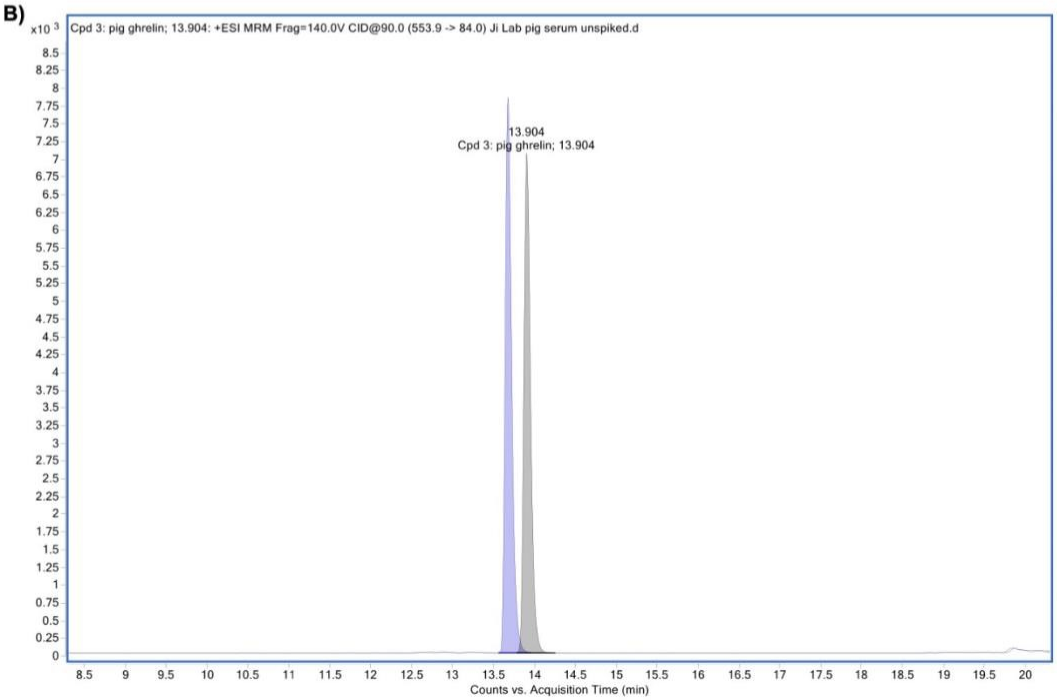


Figure S4. Relative signal intensity of samples incubated with acetonitrile for 0, 30, or 60 minutes. Pig serum was spiked with QC1 concentrations of the available analytes and extracted using 0.2% acetic acid in 75% ACN. The serum and protein precipitation solvent mixtures were incubated at 4°C for 0, 30, or 60 minutes. All conditions were performed in triplicate and the average peak area with SD was visualized in the figure.

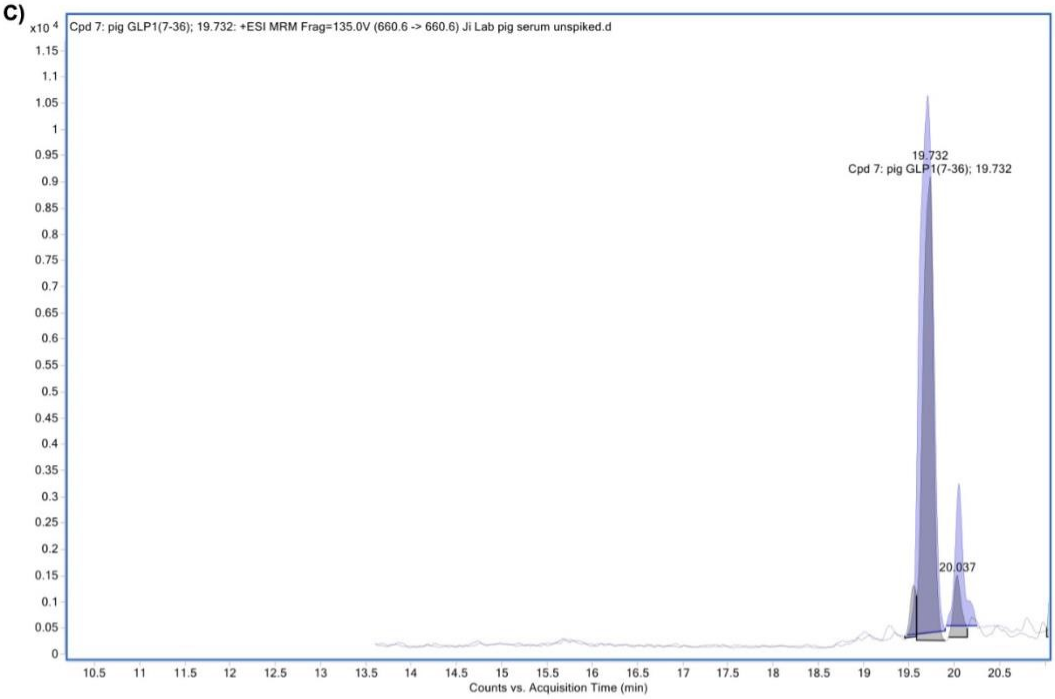
A)


Cpd 2: pig deacyl ghrelin; 13.671: +ESI MRM Frag=160.0V CID@100.0 (639.1 -> 84.0) Ji Lab pig serum unspiked 50c.d
x10 2
13.671
Cpd 2: pig deacyl ghrelin; 13.671
12.630
Counts vs. Acquisition Time (min)

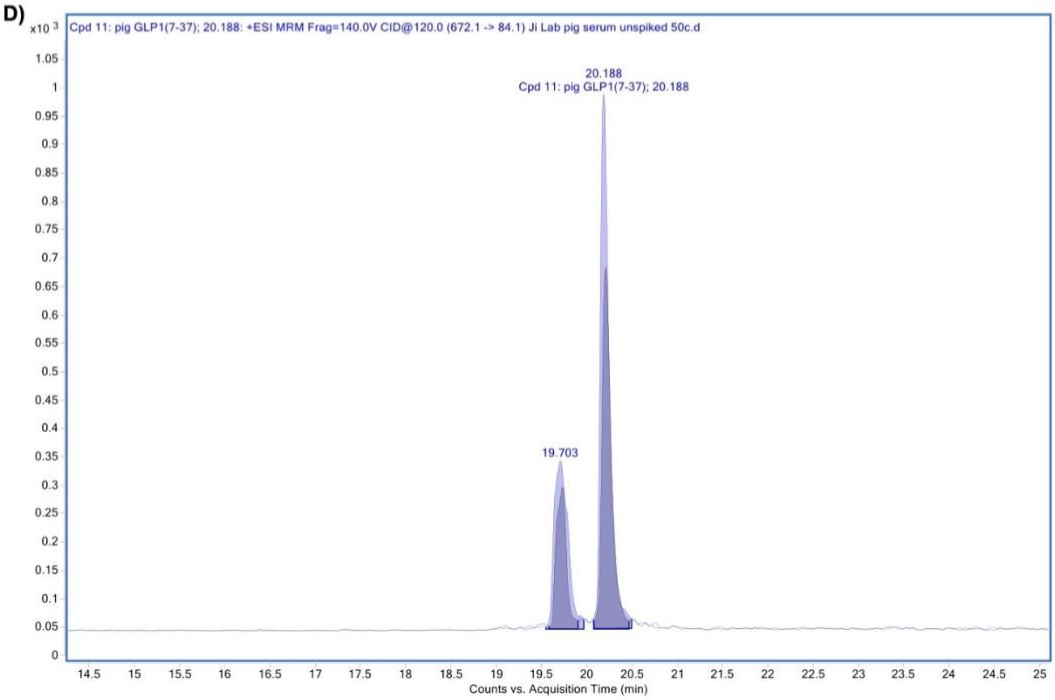
B)


Cpd 3: pig ghrelin; 13.904: +ESI MRM Frag=140.0V CID@90.0 (553.9 -> 84.0) Ji Lab pig serum unspiked.d
x10 3
13.904
Cpd 3: pig ghrelin; 13.904
Counts vs. Acquisition Time (min)

C)


Cpd 7: pig GLP1(7-36); 19.732: +ESI MRM Frag=135.0V (660.6 -> 660.6) Ji Lab pig serum unspiked.d
x10 4
19.732
Cpd 7: pig GLP1(7-36); 19.732
20.037
Counts vs. Acquisition Time (min)

D)


Cpd 11: pig GLP1(7-37); 20.188: +ESI MRM Frag=140.0V CID@120.0 (672.1 -> 84.1) Ji Lab pig serum unspiked 50c.d
x10 3
20.188
Cpd 11: pig GLP1(7-37); 20.188
19.703
Counts vs. Acquisition Time (min)

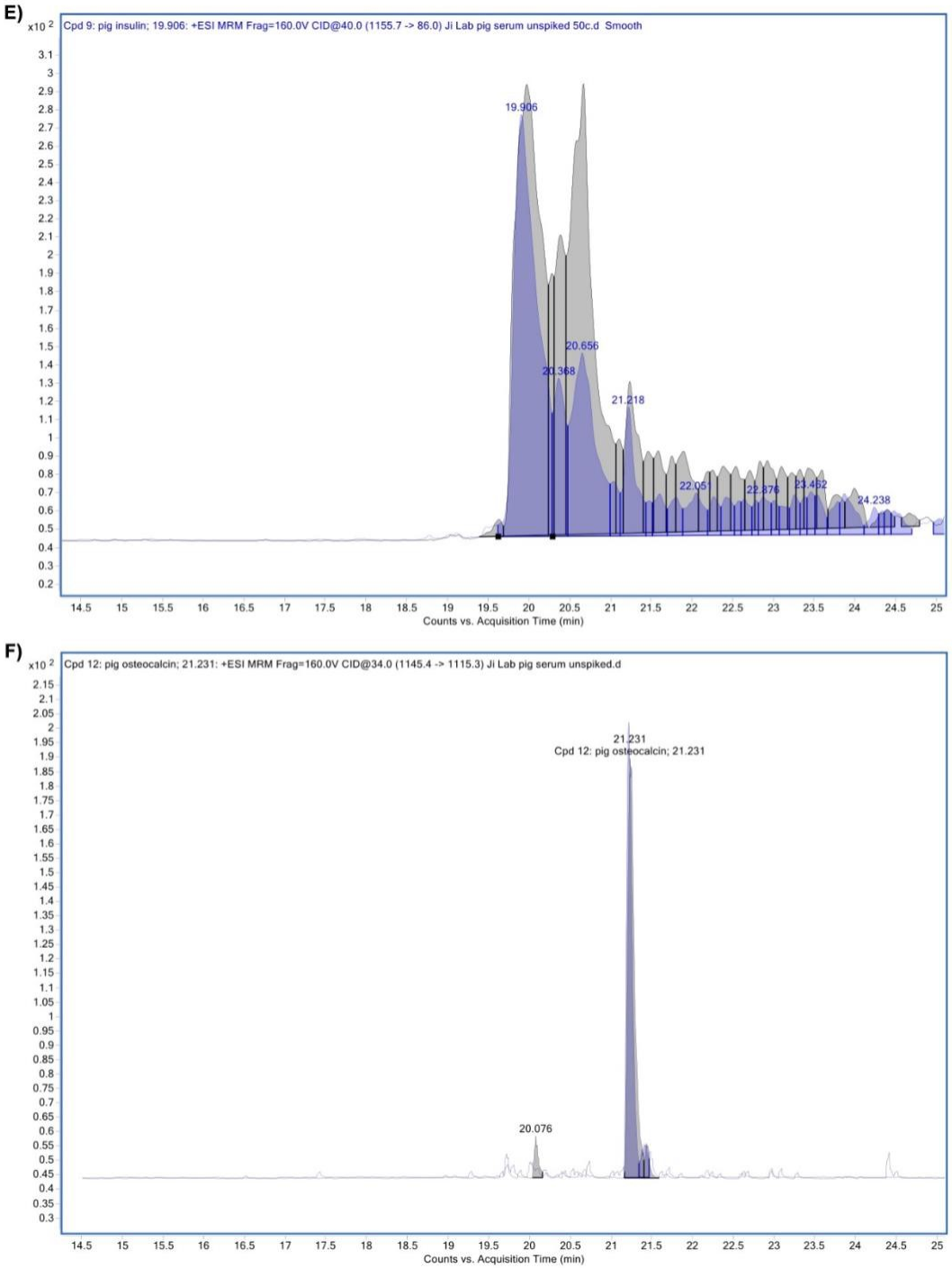


Figure S5. **Analyte chromatograms before and after column temperature change.** Chromatograms for (A) des-acyl ghrelin, (B) acyl ghrelin, (C) GLP-1 (7-36), (D) GLP-1(7-37), (E) insulin, and (F) osteocalcin in pig serum. The same aliquot of pig serum extract was run under a column temperature of 35 °C (shown in black) and 50 °C (shown in blue). Comparison of peak height showed greater signal intensity with higher column temperature. Insulin (shown in E) also demonstrated a sharper peak shape with 50 °C column temperature.

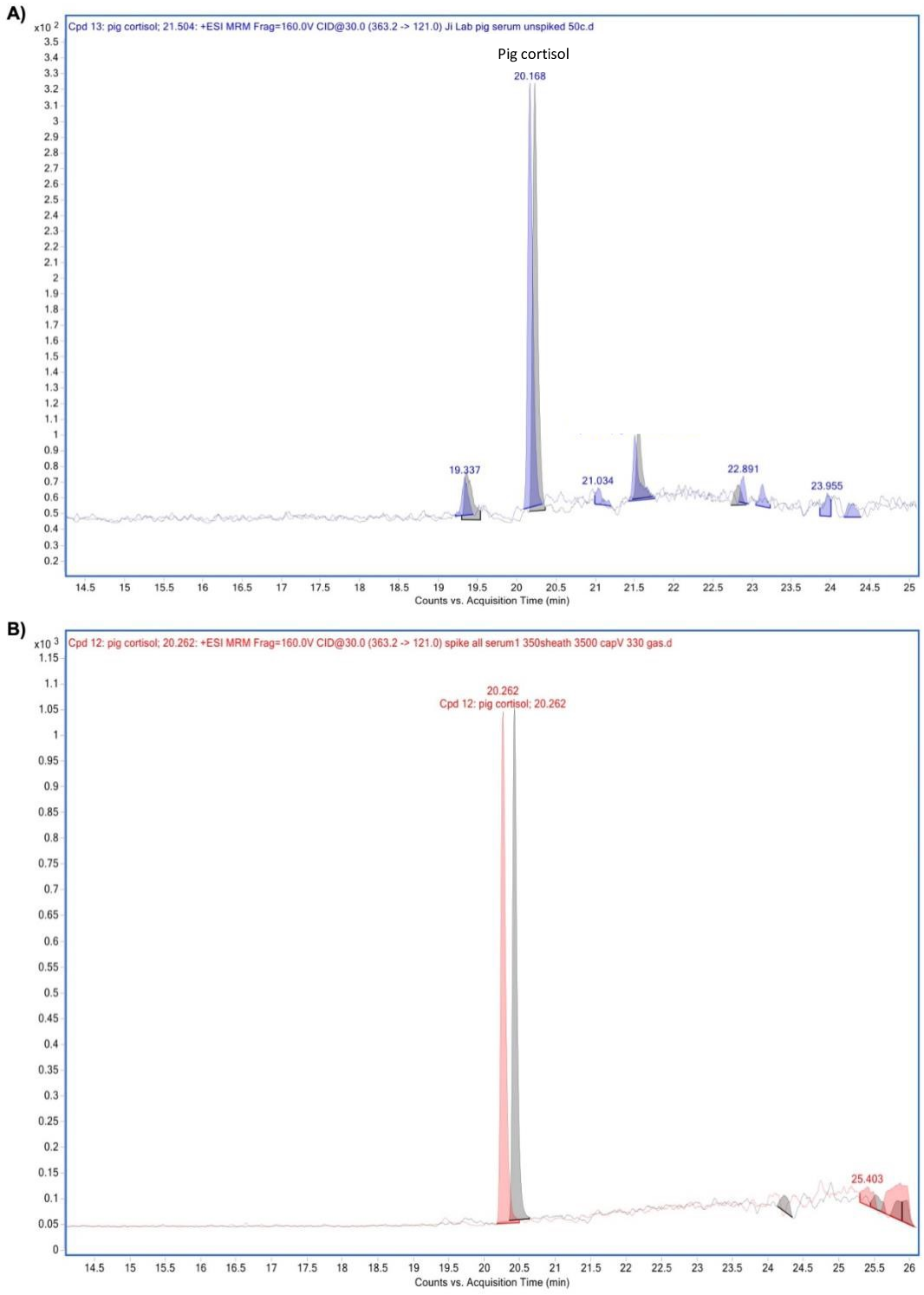
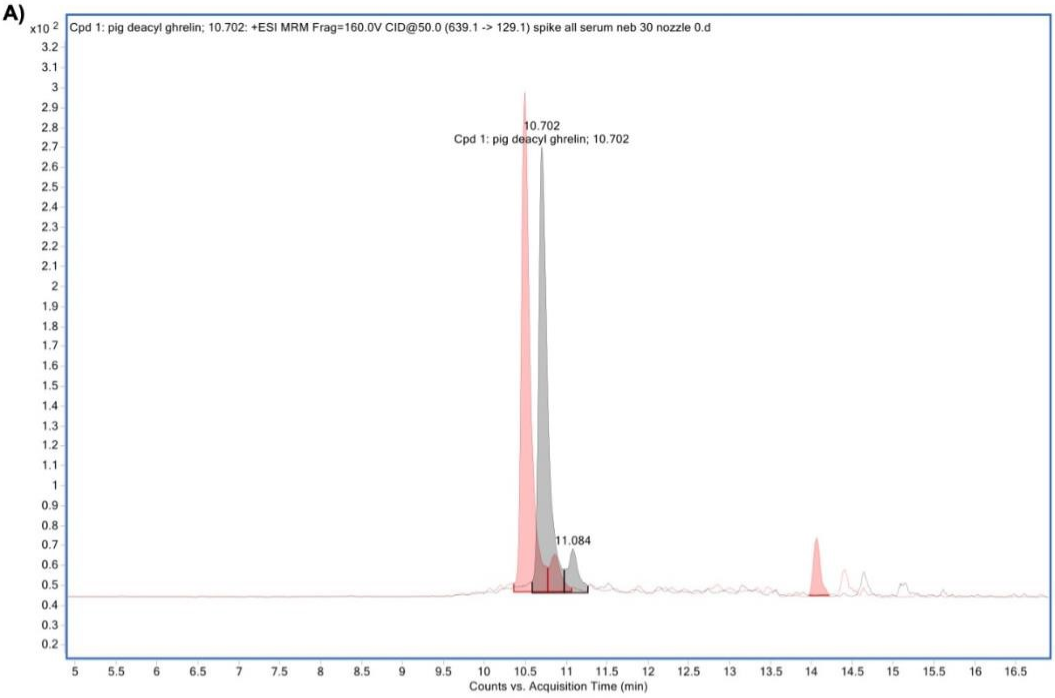
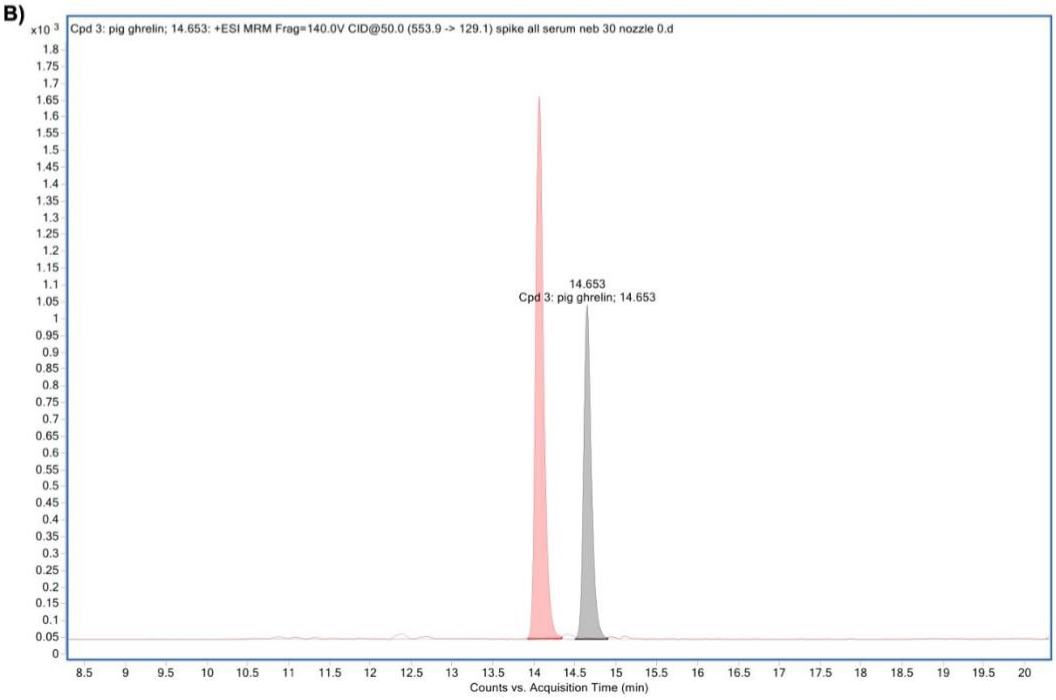


Figure S6. Cortisol chromatograms before and after LC-MS optimization. Chromatograms for cortisol (A) before and after column temperature change from 35°C to 50°C and (B) before and after increase of gas temperature and sheath gas temperature. The peaks in grey indicate cortisol signal prior to optimization. Optimization of LC-MS temperatures did not visibly impact cortisol peak shape and height.

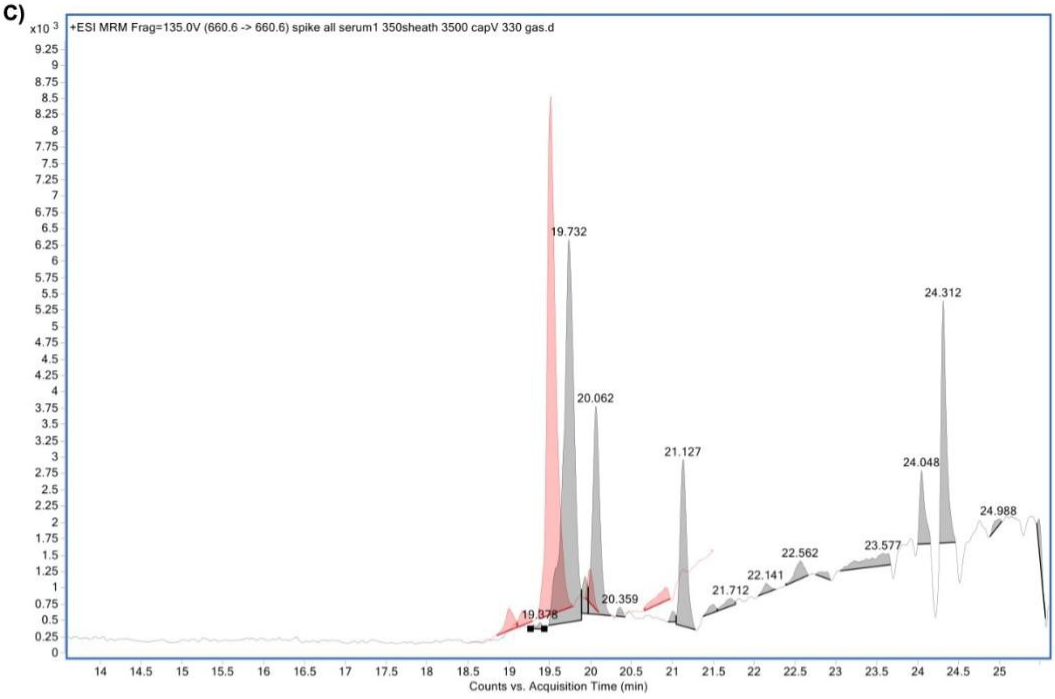
A)


x10 2
Cpd 1: pig deacyl ghrelin; 10.702: +ESI MRM Frag=160.0V CID@50.0 (639.1 -> 129.1) spike all serum neb 30 nozzle 0.d
10.702
Cpd 1: pig deacyl ghrelin; 10.702
11.084
Counts vs. Acquisition Time (min)

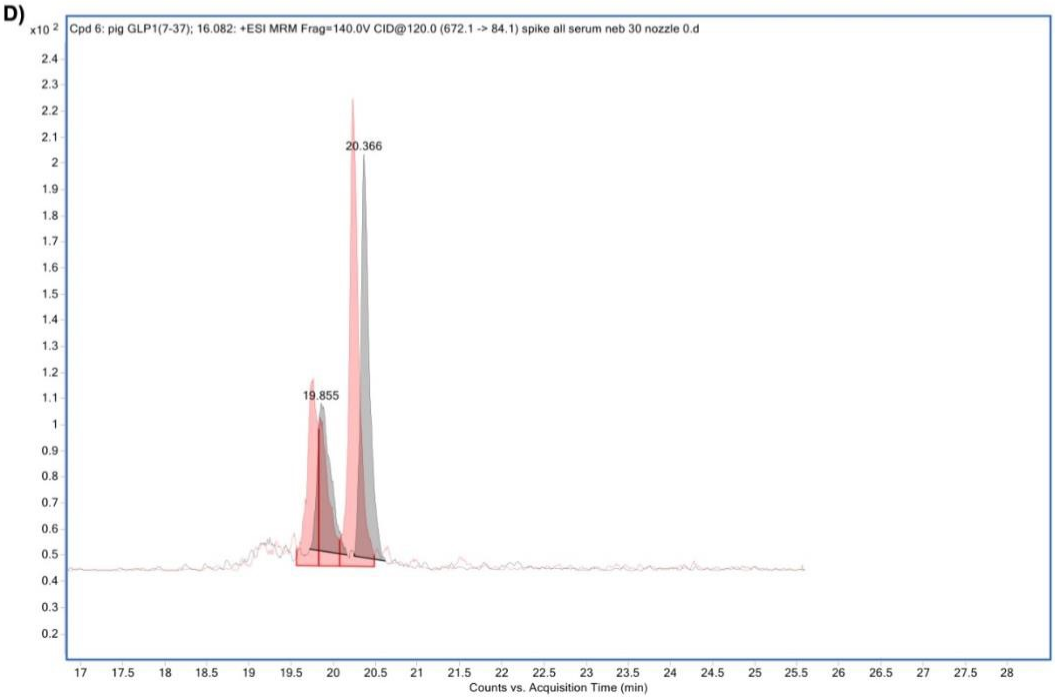
B)


x10 3
Cpd 3: pig ghrelin; 14.653: +ESI MRM Frag=140.0V CID@50.0 (553.9 -> 129.1) spike all serum neb 30 nozzle 0.d
14.653
Cpd 3: pig ghrelin; 14.653
Counts vs. Acquisition Time (min)

C)


x10 3
+ESI MRM Frag=135.0V (660.6 -> 660.6) spike all serum1 350sheath 3500 capV 330 gas.d
19.732
20.062
21.127
24.312
24.048
24.988
23.577
22.562
22.141
21.712
20.359
19.878
Counts vs. Acquisition Time (min)

D)


x10 2
Cpd 6: pig GLP1(7-37); 16.082: +ESI MRM Frag=140.0V CID@120.0 (672.1 -> 84.1) spike all serum neb 30 nozzle 0.d
20.366
19.855
Counts vs. Acquisition Time (min)

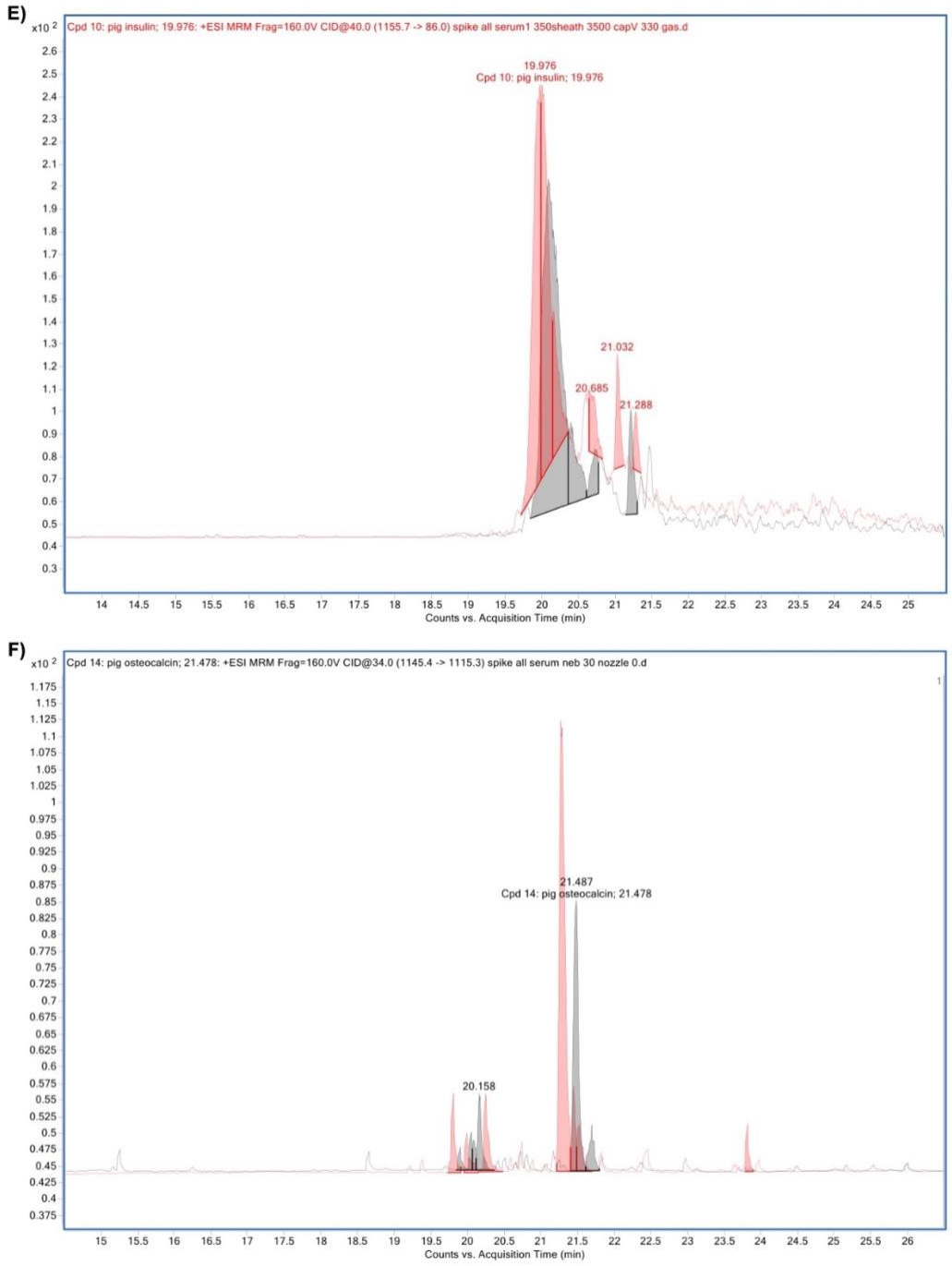
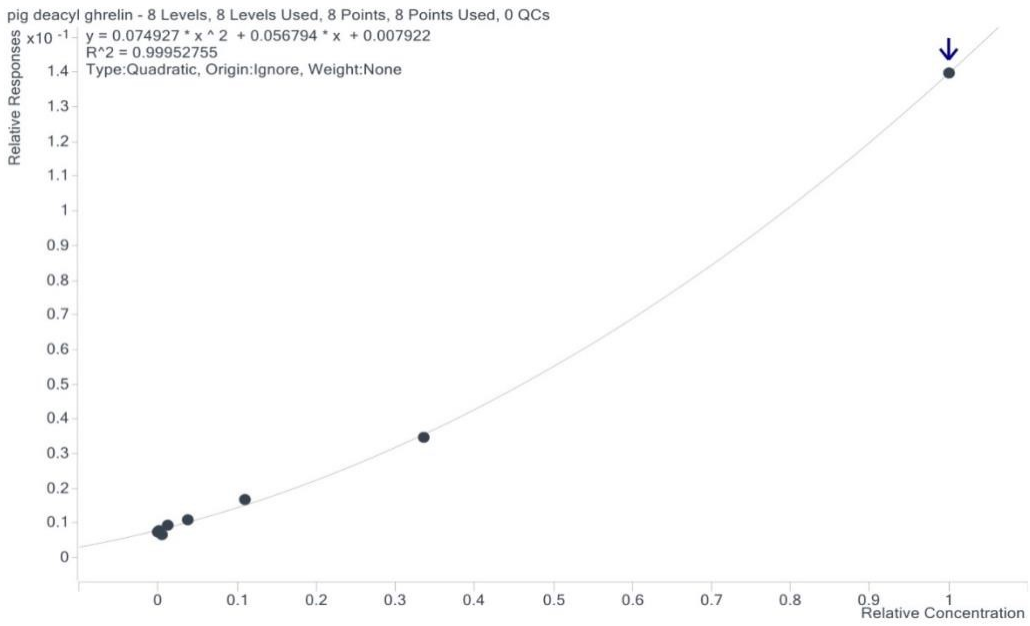
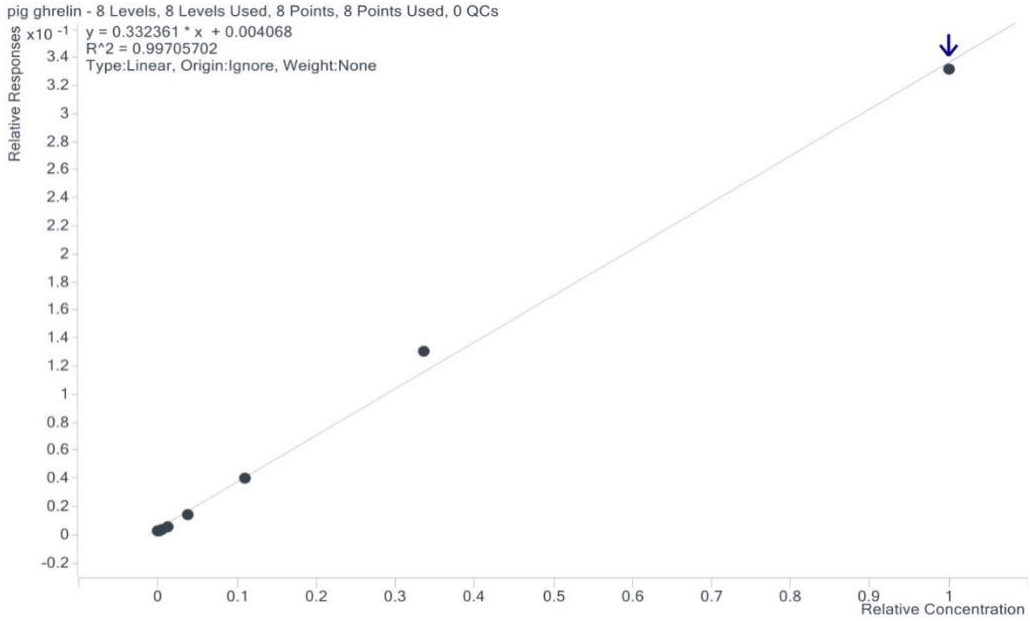


Figure S7. Analyte chromatograms before and after an increase in sheath gas and gas temperature. Chromatograms for (A) des-acyl ghrelin, (B) acyl ghrelin, (C) GLP-1 (7-36), (D) GLP-1(7-37), (E) insulin, and (F) osteocalcin from pig serum. The same aliquot of pig serum extract was run under conditions of sheath gas temperature 300 °C and gas temperature 290 °C (shown in black) versus sheath gas temperature 350 °C and gas temperature of 330 °C (shown in red). Comparison of peak height shows greater signal intensity with higher temperature. GLP-1 (7-36) (shown in C) demonstrated increased S:N in MS conditions with higher temperatures.

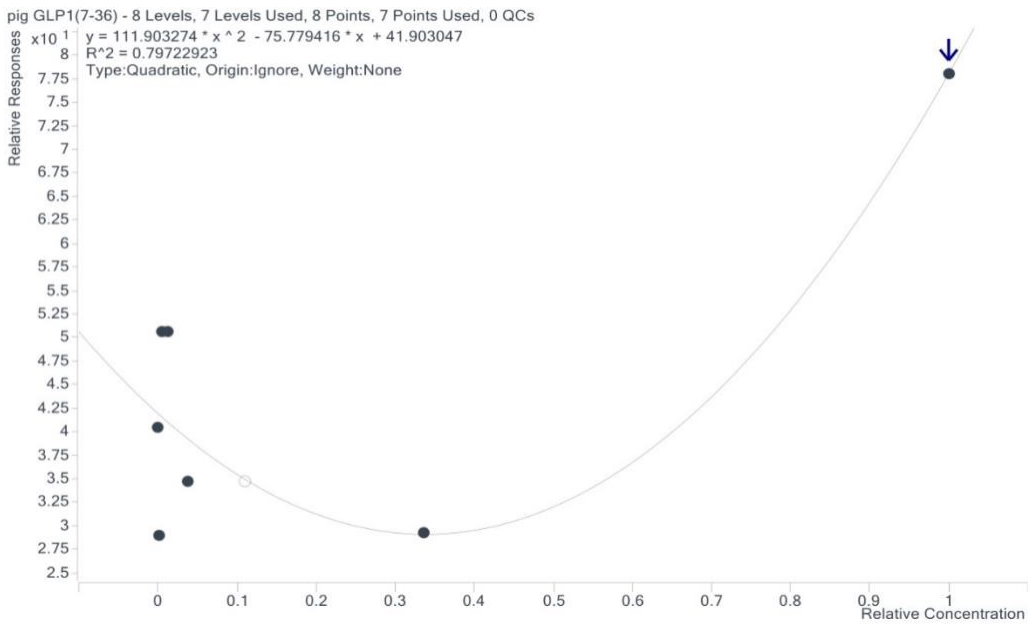
A)


pig deacyl ghrelin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 0.074927 * x ^ 2 + 0.056794 * x + 0.007922
R^2 = 0.99952755
Type:Quadratic, Origin:Ignore, Weight:None
Relative Responses
x10 -1
Relative Concentration

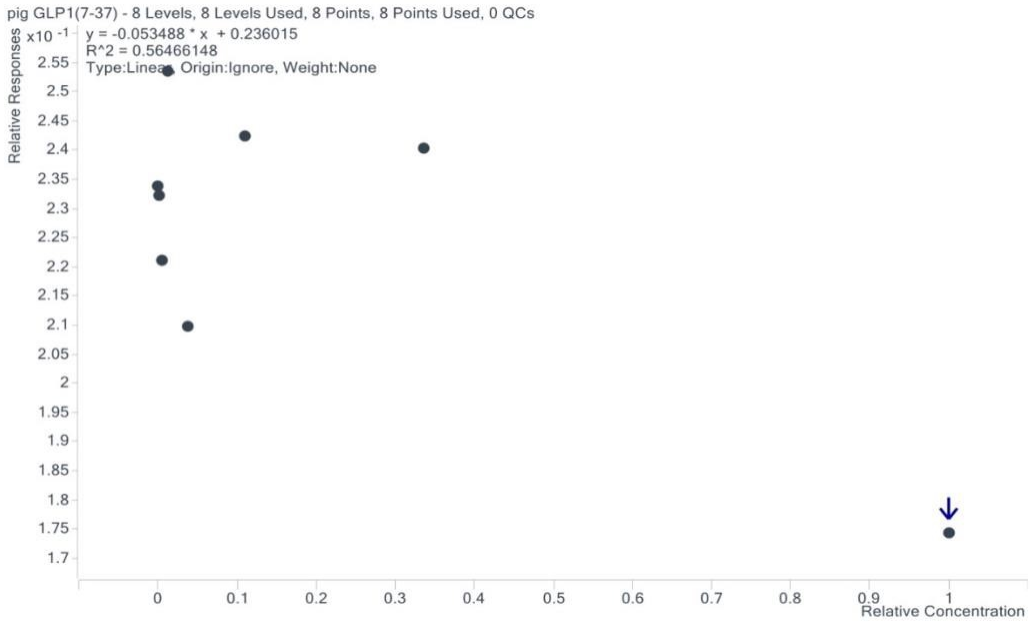
B)


pig ghrelin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 0.332361 * x + 0.004068
R^2 = 0.99705702
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
x10 -1
Relative Concentration

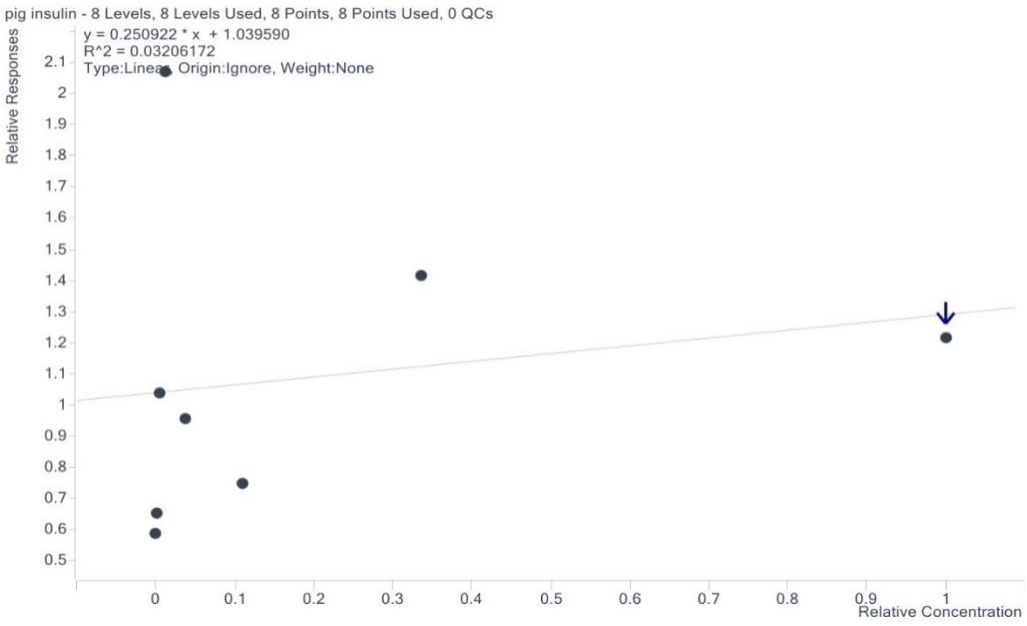
C)


pig GLP1(7-36) - 8 Levels, 7 Levels Used, 8 Points, 7 Points Used, 0 QCs
y = 111.903274 * x ^ 2 - 75.779416 * x + 41.903047
R^2 = 0.79722923
Type:Quadratic, Origin:Ignore, Weight:None
Relative Responses
x10 1
Relative Concentration

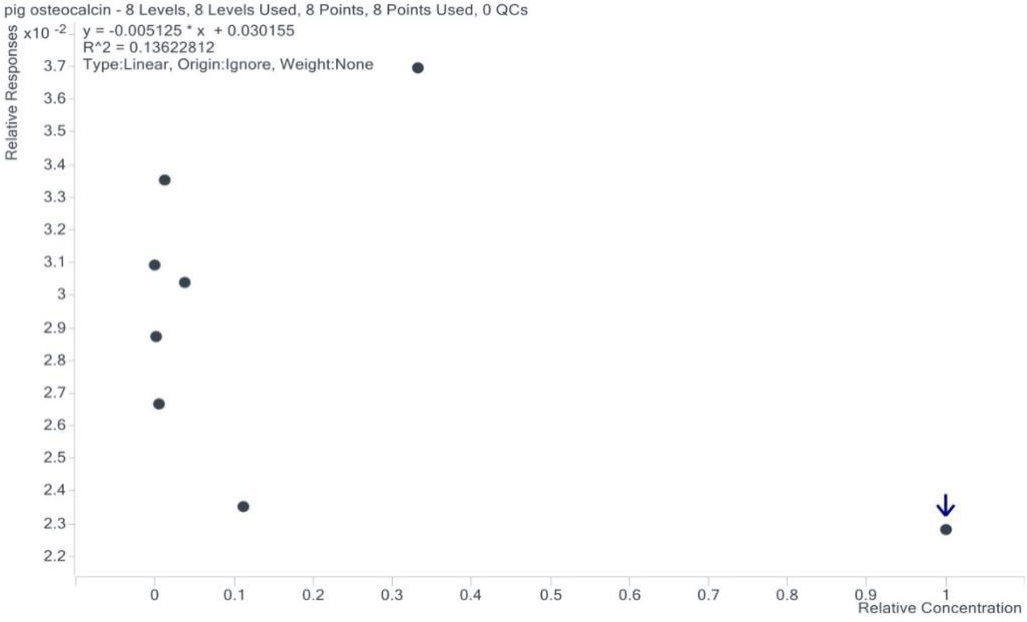
D)


pig GLP1(7-37) - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = -0.053488 * x + 0.236015
R^2 = 0.56466148
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
x10 -1
Relative Concentration

E)


pig insulin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 0.250922 * x + 1.039590
R^2 = 0.03206172
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

F)


pig osteocalcin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = -0.005125 * x + 0.030155
R^2 = 0.13622812
Type:Linear, Origin:Ignore, Weight:None
x10 -2
Relative Responses
Relative Concentration

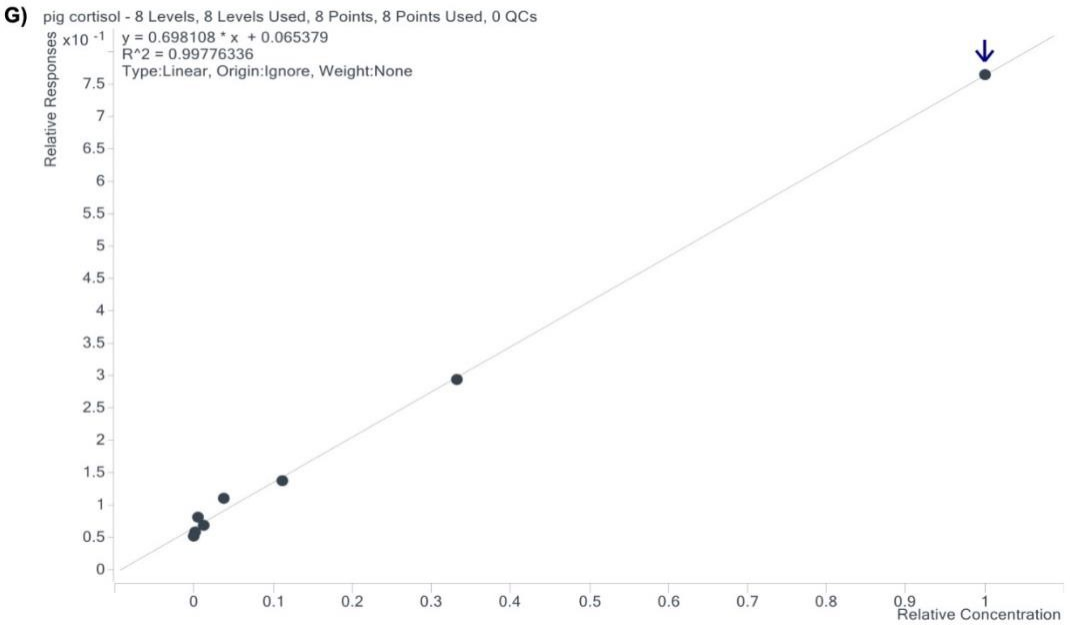
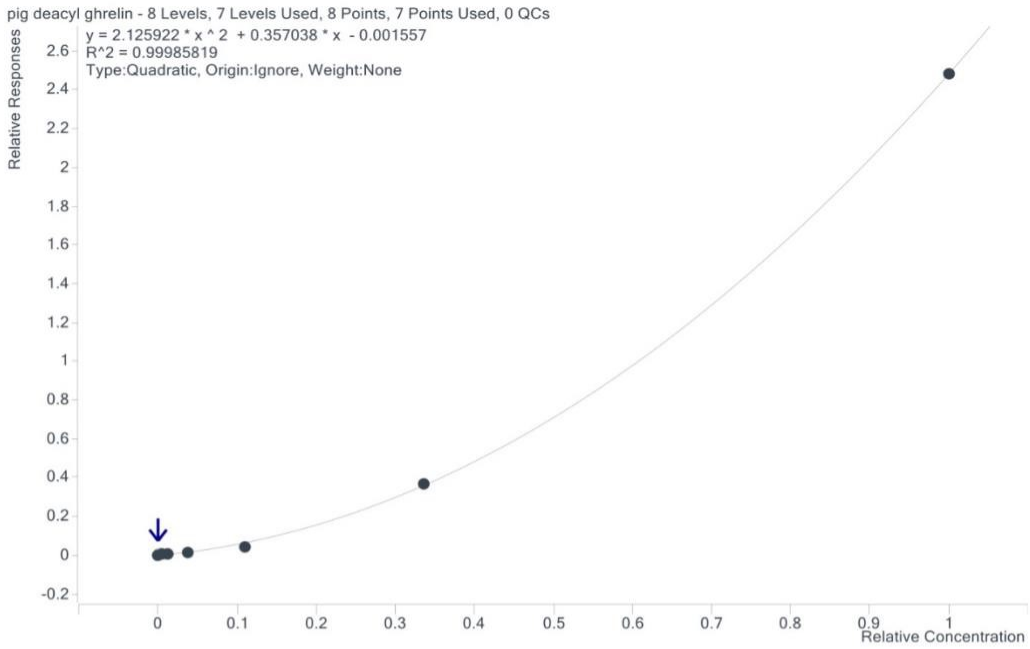
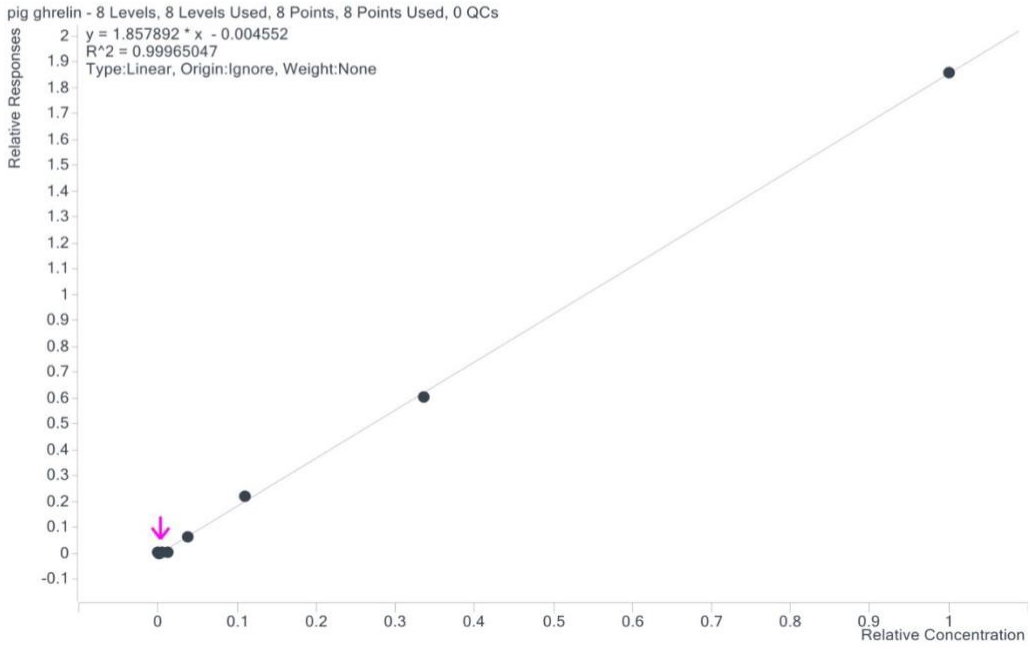


Figure S8. Charcoal-stripped serum matrix calibration curves for analytes. Eight calibrants were prepared in a charcoal stripped serum matrix. The peak area relative to internal standard was plotted against relative concentration. Plots are shown for (A) des-acyl and (B) acyl ghrelin, (C) GLP-1 (7-36), (D) GLP-1 (7-37), (E) insulin, (F) y-carboxy osteocalcin, and (G) cortisol. Both GLP-1 (7-36) and GLP-1 (7-37), as well as insulin and osteocalcin demonstrated poor linearity in surrogate matrix.

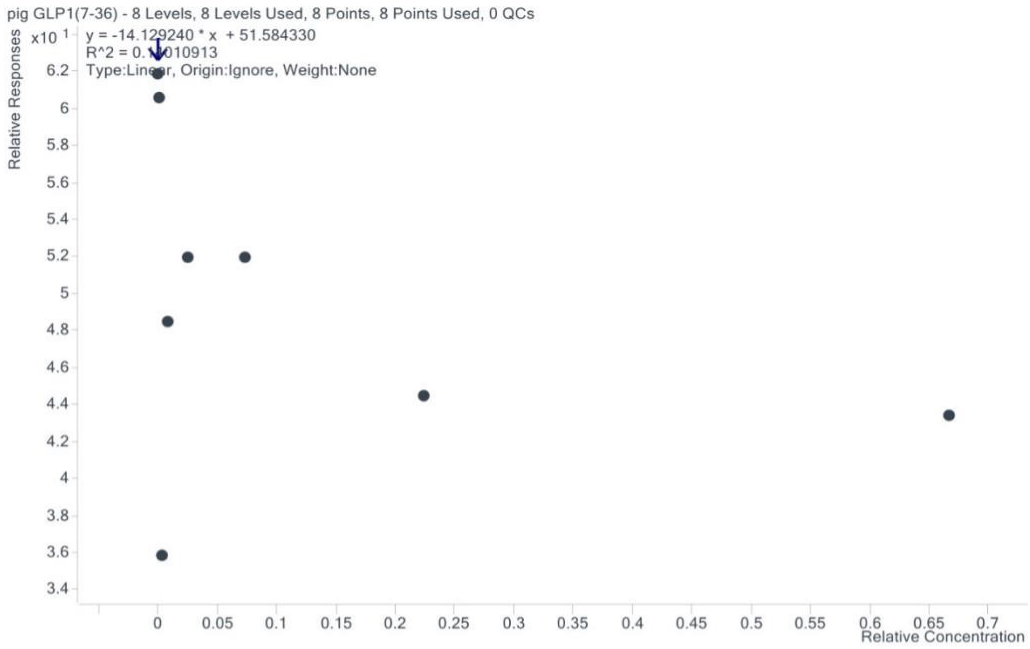
A)


pig deacyl ghrelin - 8 Levels, 7 Levels Used, 8 Points, 7 Points Used, 0 QCs
y = 2.125922 * x ^ 2 + 0.357038 * x - 0.001557
R^2 = 0.99985819
Type:Quadratic, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

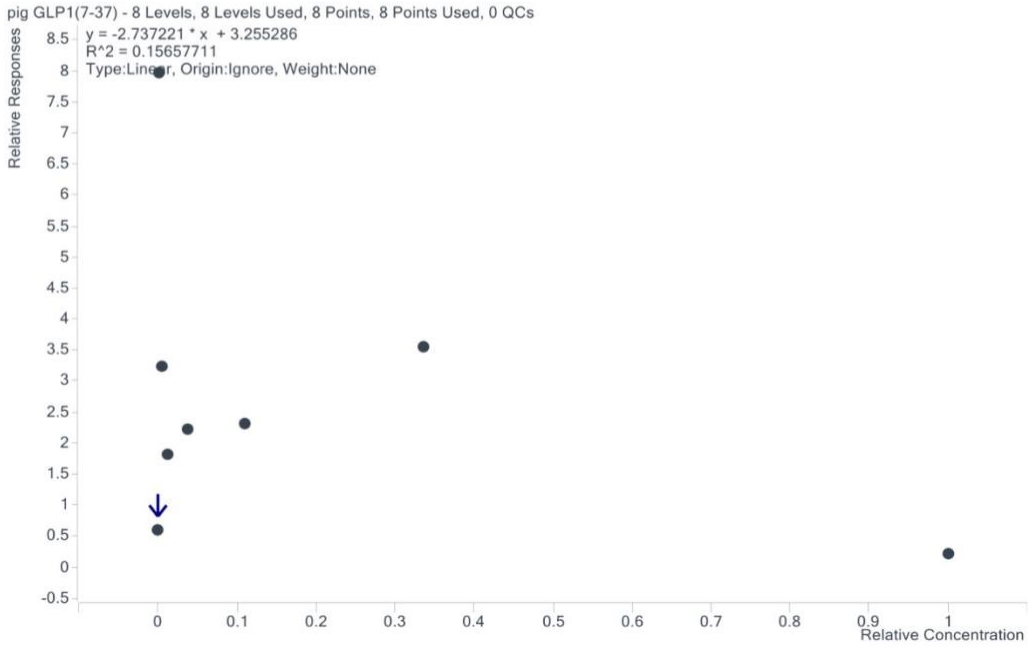
B)


pig ghrelin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 1.857892 * x - 0.004552
R^2 = 0.99965047
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

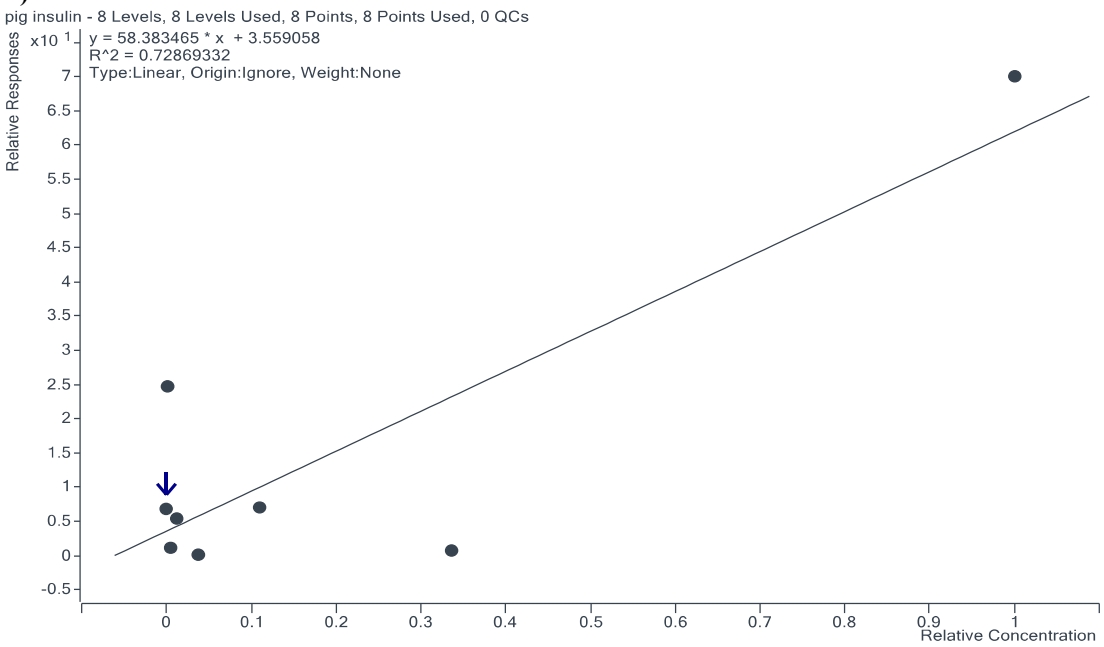
C)


pig GLP1(7-36) - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = -14.129240 * x + 51.584330
R^2 = 0.
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
x10 1
Relative Concentration

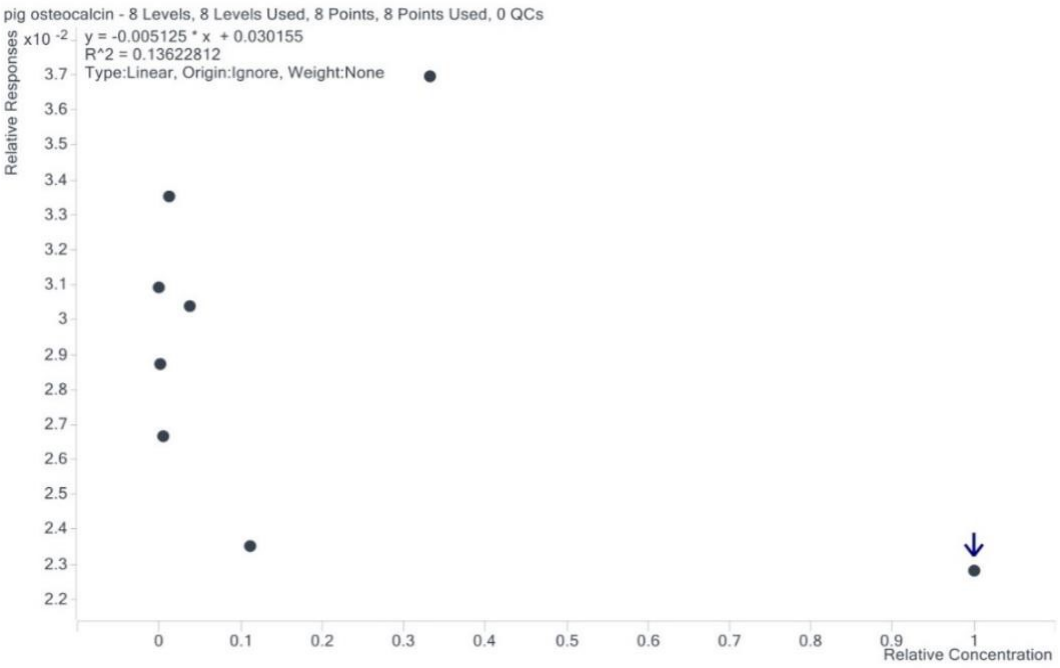
D)


pig GLP1(7-37) - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = -2.737221 * x + 3.255286
R^2 = 0.15657711
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

E)


pig insulin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 58.383465 * x + 3.559058
R^2 = 0.72869332
Type:Linear, Origin:Ignore, Weight:None
x10 1
Relative Responses
Relative Concentration

F)


pig osteocalcin - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = -0.005125 * x + 0.030155
R^2 = 0.13622812
Type:Linear, Origin:Ignore, Weight:None
x10 -2
Relative Responses
Relative Concentration

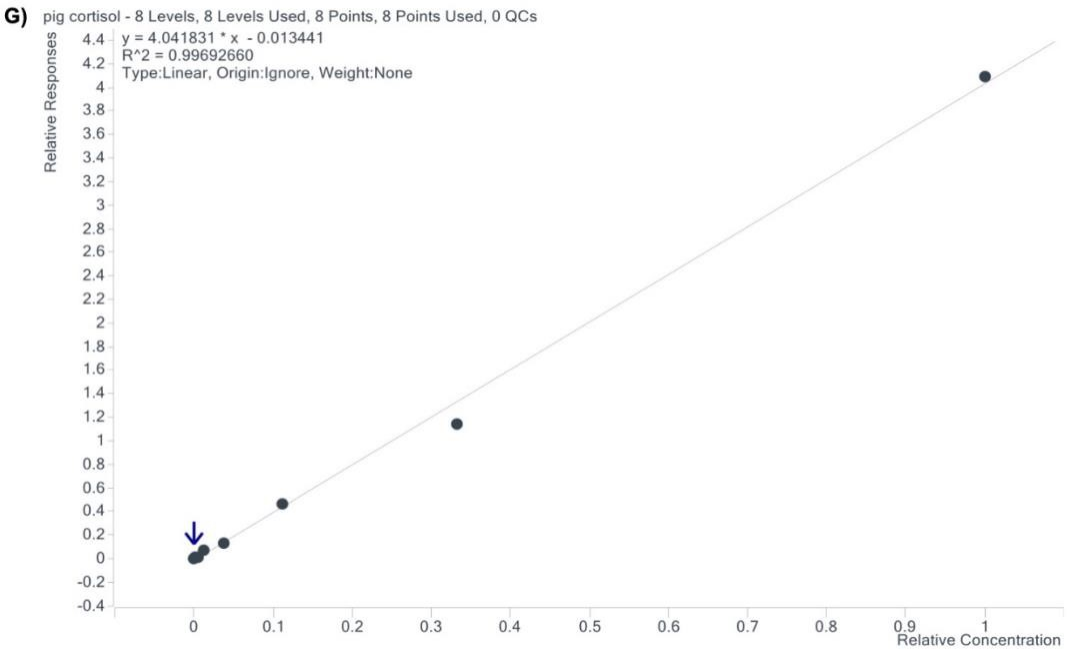
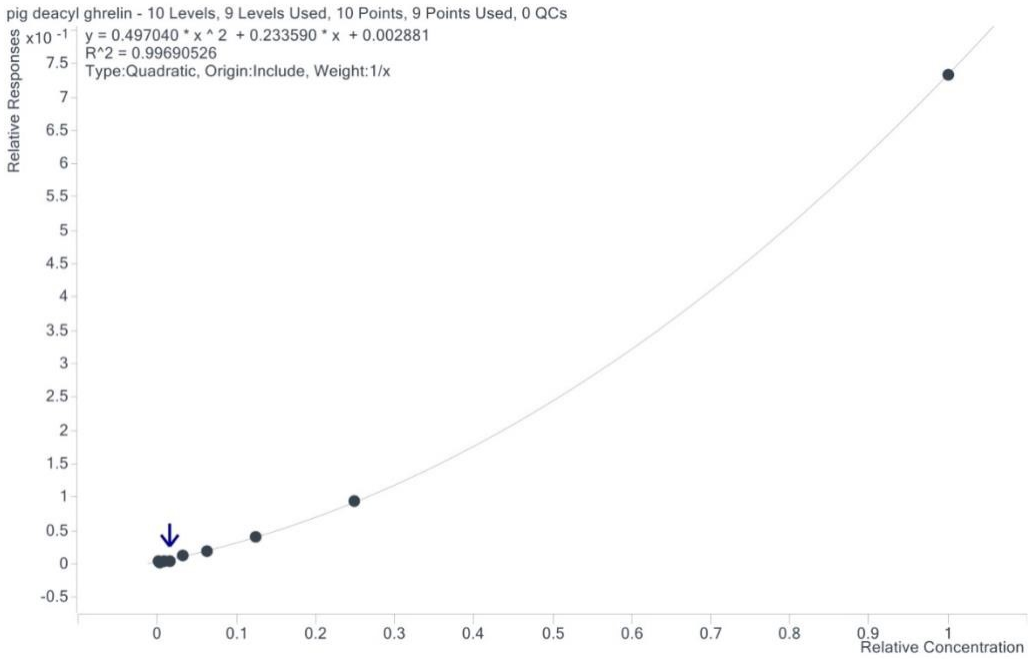
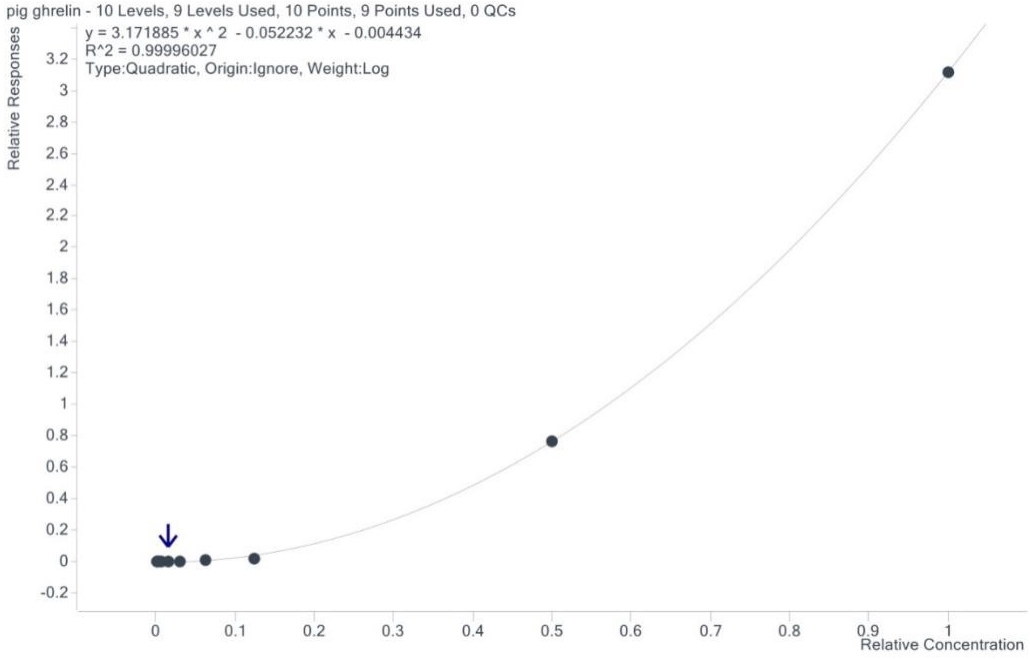


Figure S9. Chicken serum matrix calibration lines for analytes. Eight calibrants were prepared in a surrogate matrix of chicken serum extract. The peak area relative to internal standard was plotted against relative concentration. Plots are shown for (A) des-acyl and (B) acyl ghrelin, (C) GLP-1 (7-36), (D) GLP-1 (7-37), (E) insulin, (F) y-carboxy osteocalcin, and (G) cortisol. GLP-1 (7-36), GLP-1 (7-37), insulin, and osteocalcin each demonstrated poor linearity in chicken serum.

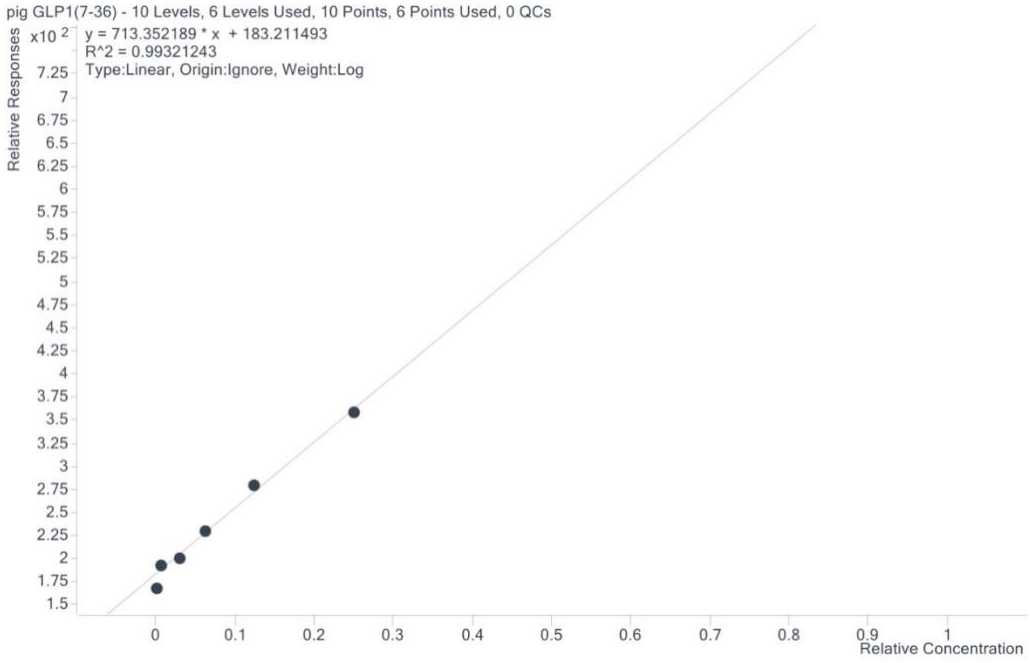
A)


pig deacyl ghrelin - 10 Levels, 9 Levels Used, 10 Points, 9 Points Used, 0 QCs
y = 0.497040 * x ^ 2 + 0.233590 * x + 0.002881
R^2 = 0.99690526
Type:Quadratic, Origin:Include, Weight:1/x
Relative Responses
x10 -1
Relative Concentration

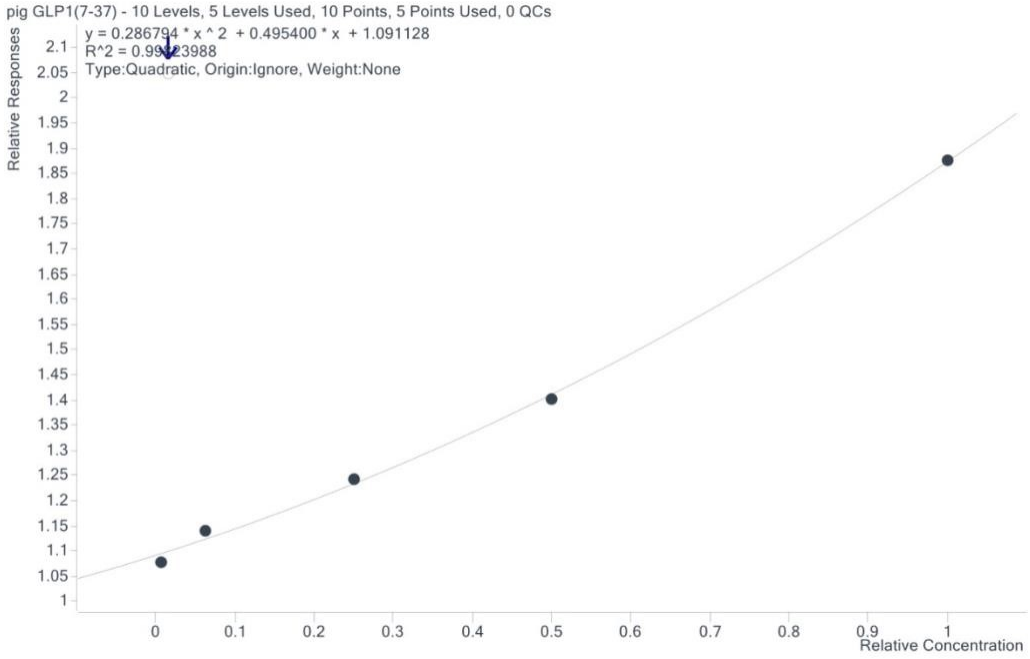
B)


pig ghrelin - 10 Levels, 9 Levels Used, 10 Points, 9 Points Used, 0 QCs
y = 3.171885 * x ^ 2 - 0.052232 * x - 0.004434
R^2 = 0.99996027
Type:Quadratic, Origin:Ignore, Weight:Log
Relative Responses
Relative Concentration

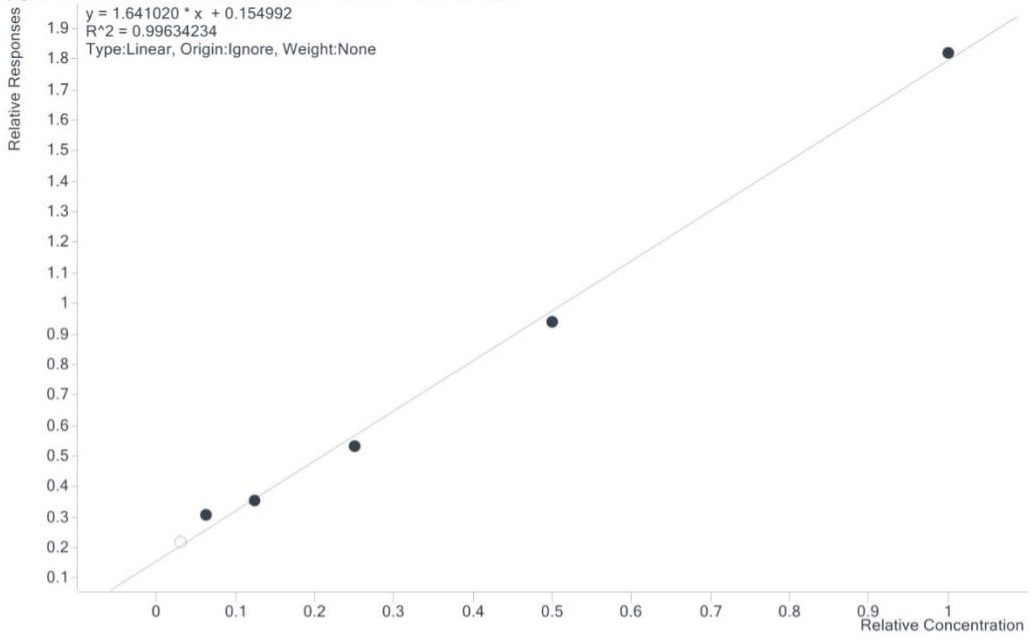
C)


pig GLP1(7-36) - 10 Levels, 6 Levels Used, 10 Points, 6 Points Used, 0 QCs
y = 713.352189 * x + 183.211493
R^2 = 0.99321243
Type:Linear, Origin:Ignore, Weight:Log
x10 2
Relative Responses
Relative Concentration

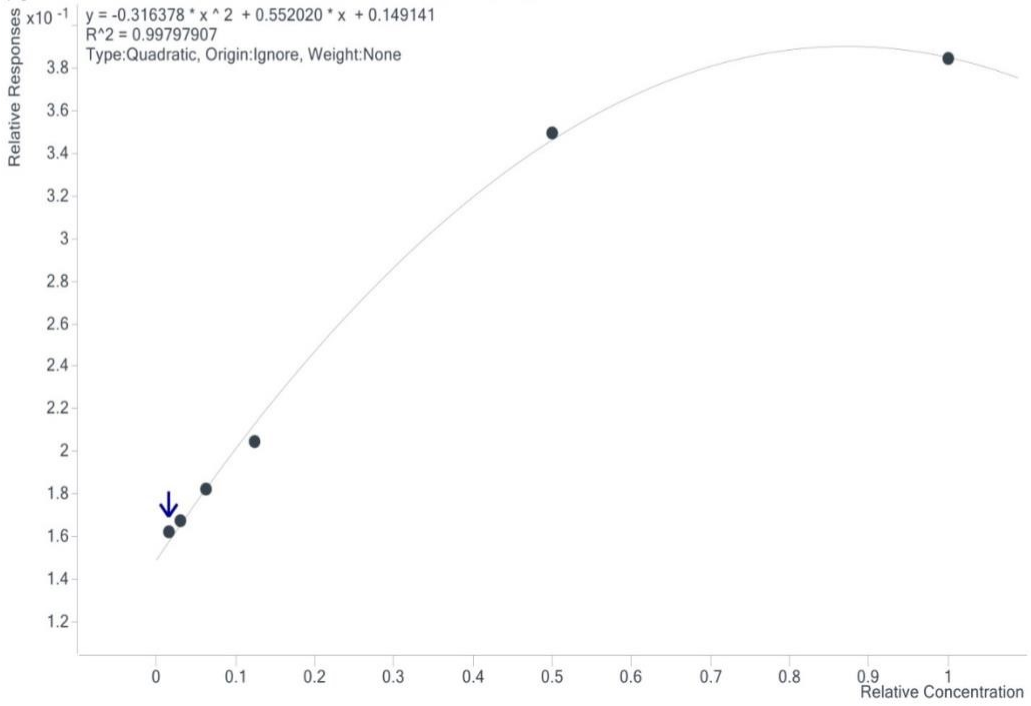
D)


pig GLP1(7-37) - 10 Levels, 5 Levels Used, 10 Points, 5 Points Used, 0 QCs
y = 0.286794 * x ^ 2 + 0.495400 * x + 1.091128
R^2 = 0.99923988
Type:Quadratic, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

E) pig insulin - 10 Levels, 5 Levels Used, 10 Points, 5 Points Used, 0 QCs



F) pig osteocalcin - 10 Levels, 6 Levels Used, 10 Points, 6 Points Used, 0 QCs



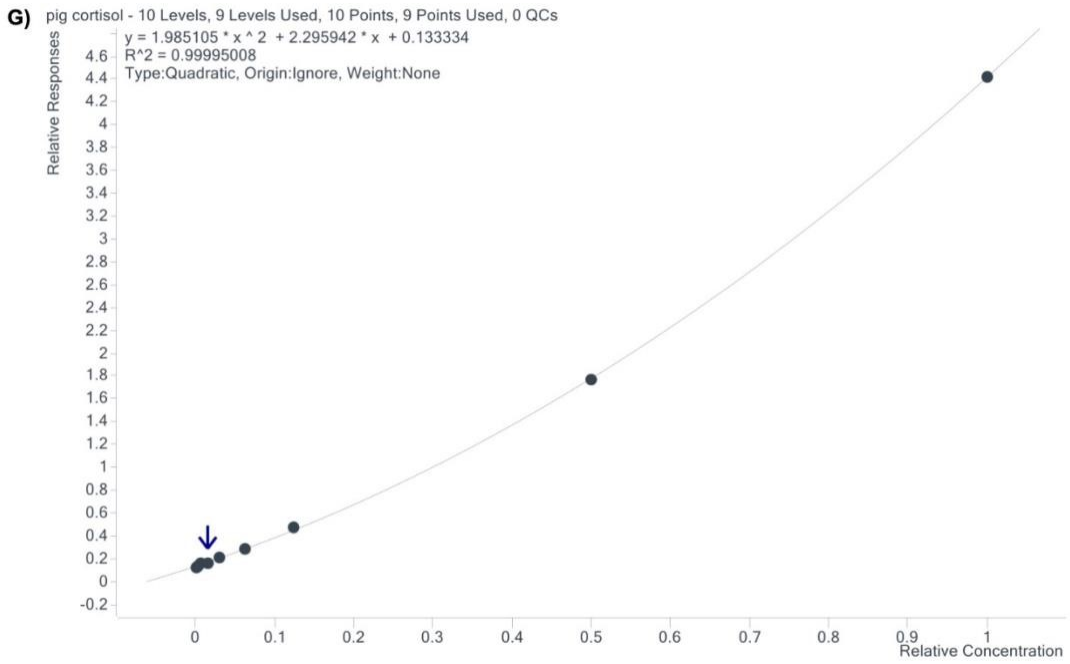


Figure S10. Composite piglet serum matrix calibration lines for analytes. Five to nine calibrants were prepared in a piglet serum extract matrix comprised of 40% filtered serum and 60% unfiltered serum. The peak area relative to internal standard was plotted against relative concentration. Plots are shown for (A) des-acyl and (B) acyl ghrelin, (C) GLP-1 (7-36), (D) GLP-1 (7-37), (E) insulin, (F) osteocalcin, and (G) cortisol. All analytes demonstrated linear or quadratic $R^2 \geq 0.993$.