Development of a Targeted MS-based Method for Measurement of Insulin, Cortisol, Glucagon-like peptide 1, acyl and desacyl Ghrelin, and Carboxylated Osteocalcin in Porcine Serum

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Abstract

Hormones are chemical messengers that travel through blood and tissue to regulate millions of metabolic pathways. Accurate measurement of hormones is important for reliable diagnostics and health research. Current methods for hormone quantification rely on antibodies to capture and detect hormones. These forms of assays suffer from poor specificity and reproducibility and can involve large money and time commitments. Liquid chromatography (LC) coupled tandem mass spectrometry (MS/MS) is an alternative quantitation method already widely used for the quantification of small metabolites. Although advances in mass spectrometry have allowed for the quantification of larger peptides and steroid hormones, there exist no methods specific to pig hormones. Pigs are considered an excellent model system for the study of human metabolism as the two species share many similarities in digestive anatomy and physiology. Additionally, despite the great potential of LC-MS/MS for simultaneous quantitation, multiplex methods are scarce.

In this study, we developed and validated an LC-MS/MS-based method to simultaneously quantify several post-prandial hormones including cortisol, GLP-1 (7-37), GLP-1 (7-36), acyl and desacyl ghrelin, and carboxylated osteocalcin. Post-prandial hormones are those that respond to feeding and are essential in understanding the impact of diet on appetite regulation, glycemic control, and body composition. Although not a post-prandial hormone, cortisol was included since stress is known to affect post-prandial hormonal response. Hormones were isolated from 100 uL of pig serum using an optimized acetonitrile-based protein precipitation extraction. The extracted sample was analyzed by LC-MS/MS. The final method had excellent recovery and reproducibility. The validated detection range encompasses the typical ranges for the target hormones in adult pigs and piglets. Although calibration curves for all analytes demonstrated R^2

> 0.9, additional work should be done to further improve linearity and sensitivity for more precise and accurate measurement. Overall, our method meets the threshold as a bioanalytical method for the measurement of seven hormones from a small sample size.

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Chapter 1 – Introduction to Immunoassays and LC-MS for Hormone Quantification

1.1 Introduction to Hormones and Hormone Analysis

Hormones are signaling molecules that regulate bodily functions. Most mammals house over a hundred unique hormones which work together to control millions of intricate and overlapping metabolic pathways (Hiller-Sturmhöfel & Bartke, 1998). Hormones and signaling molecules can range from proteins and peptides to lipids and other small molecules. Subtle shifts in hormone levels are often indicative of a disruption from normal function (Kulkarni et al., 2016). Thus, the accurate measurement of hormone levels is an important tool for clinical diagnostics, treatment monitoring, and health research. Currently, immunoassays are the most common analytical technique to measure hormones although there has been a gradual shift towards more sensitive and robust techniques involving liquid chromatography-mass spectrometry (LC-MS) based methods.

1.2 Immunoassays

Immunoassays are the most common method to measure hormone levels and rely on antigen-antibody interactions. An immunoassay consists of three components: the target molecule known as the antigen, the antigen-specific antibody, and a label. The antibody is introduced to the sample matrix and is expected to selectively bind to the target molecule. Unbound antibody is eliminated from the reaction and a label is introduced which binds to the antigen-antibody complex. The quantity of label can be quantified through a variety of detection methods as discussed below. Immunoassays may differ by reaction method and label types (Darwish, 2006).

The reaction method of an immunoassay can be classified as competitive or non-competitive. In a competitive immunoassay, the analyte competes with a labeled antigen for binding to the antibody, so the intensity of label is inversely related to the amount of analyte present. In a non-competitive immunoassay, only the analyte is expected to bind to the antigen and the amount of analyte present is directly related to label intensity (Darwish, 2006).

Quantitation in an immunoassay requires the use of labels. Types of labels include enzymes, radioisotopes, and fluorophores, and binding can be measured using colorimetry, radioactivity, or fluorometry. In all forms of quantitative immunoassay, a serial dilution of standard will be required to create a standard curve from which concentrations of the analyte can be determined. (Slagle & Ghosn, 1996).

1.2.1 Advantages and disadvantages of immunoassays

Immunoassays are a convenient choice due to the variety of commercial kits available. Commercial kits generally do not require expensive equipment or instruments. Due to the variety of detection methods, immunoassays can provide a wide and dynamic detection range and can be customized to suit unique matrices and analytes. Despite their popularity, many doubts have been raised regarding the use of immunoassays, particularly for hormone measurement (Pinho et al., 2019).

A major disadvantage of immunoassays is poor selectivity, as it is not uncommon for the selected antibody to bind non-specifically to matrix components or target analogs. Additionally, many hormones undergo post-translational modifications to activate their function. Immunoassays typically cannot differentiate proteins with or without post-translational modifications. In some instances, antibodies have been raised to recombinant or unfolded

proteins or peptides, and questions as to the similarity in structure to the actual protein must be considered. There is a growing body evidence that demonstrates large variation in measurements between manufacturers and even lots of the same kit, which brings into question reproducibility and precision of immunoassays. At least some of the differences between kits and lots can be attributed to differences in antibodies and reagents used (Nandi et al., 2014; Pinho et al., 2019). There is also potential for large inter-analyst variation within the same immunoassay kit. This variation can be due to small changes in temperature and humidity, which can impact reaction kinetics. Extra care must be taken for immunoassays that require disposal of unbound components after each step, since the thoroughness with which an analyst removes these components can also affect reproducibility (Hu et al., 2020; Webster et al., 1990).

The time-consuming nature of some immunoassay techniques represents a distinct disadvantage. The use of competitive immunoassays implies multiple long incubation periods required to reach equilibrium between the competing antigens. The ability to rapidly detect several hormones via immunoassay is further hindered by their narrow multiplexing ability. Few multiplex kits have been validated and little development has been made in recent years to address this deficiency. Development of multiplex immunoassays and their antibodies presents greater challenges and costs than even their single-analyte counterparts (Pinho et al., 2019; Tighe et al., 2015).

Immunoassays are also susceptible to matrix interference. Matrix components may bind to and block the binding site of hormones, leading to artificially low or high measurements. Improper handling of the matrix, as in the case of hemolyzed blood, can render a sample unfit for an immunoassay (Hughes et al., 2009).

Finally, the scarcity of well-validated kits for less studied hormones and species presents another challenge. The development and sourcing of antibodies and reagents for in-house development of an immunoassay can be expensive and time consuming.

1.3 Mass Spectrometry for Hormone Quantification

The disadvantages of immunoassays and the rising need for more accurate hormone quantification prompted the search for alternative and more robust measures of hormone analysis. Mass Spectrometry (MS) has historically been used to characterize small molecules based on their mass to charge ratio. With recent advancements in ionization methods, the detection and quantitation of larger molecules, including proteins and peptides can be accomplished (Kushnir et al., 2010; Sabbagh et al., 2016).

MS-based analysis of biomolecules can follow either a top-down or bottom-up approach. A bottom-up method is used for larger molecules, such as proteins, and involves digestion of the parent compound prior to analysis. The smaller digestates are used to characterize the initial compound. The bottom-up approach is in direct contrast to the top-down method which involves direct analysis of the intact compound. Small peptide hormones are typically quantified using the top-down method. Similarly, steroid hormones are also analyzed in a top-down manner since they are smaller and do not fragment as extensively as larger macromolecules (Catherman et al., 2014).

1.3.1 Targeted vs. untargeted analysis

MS-based targeted analysis refers to the analysis of a small number of compounds for which chemical standards are available and absolute quantification can be achieved. Targeted MS-based quantification of hormones has been widely studied to replace traditional immunoassay-

based methods (Chen et al., 2013; Pinho et al., 2019; Rauh et al., 2007). The general principle for targeted mass spectrometry enlists the use of an external standard, typically a purified version of the analyte, to generate a standard concentration curve. An internal standard of known concentration is also added to each sample to account for sample-to-sample variation that may occur due to matrix effects. The internal standard is a similar, but distinct, compound from the analyte to be measured. Common internal standards include isotope-labelled or chemically tagged compounds. Targeted analyses require optimization and prior testing of the analyte to optimize the assay parameters for the target matrix and analytes (Liebler & Zimmerman, 2013; Manes & Nita-Lazar, 2018).

In contrast, untargeted MS methods are simpler and allow for identification of a greater number of compounds in a sample at the expense of absolute quantitation. Typically, the compounds of interest are extracted from the sample and the peak area of each compound is measured. Identification is based on mass spectral databases, although often peaks are of unknown origin. Generally, with untargeted MS methods, relative quantification may be achieved; however, there are no MS databases containing information pertaining to hormones.

Given the drawbacks of untargeted MS for hormone analysis, the method described in this thesis is a targeted method.

1.3.2 Instrumentation

A mass spectrometer is comprised of three main components: the ionization source, the mass analyzer, and the detector (Karpievitch et al., 2010). In the ionization source, a vaporized sample is bombarded into smaller charged particles using an electron beam source. Electrospray ionization (ESI) is a “soft” ionization method widely used for high molecular weight

biomolecules such as proteins and large peptides (Wang et al., 2018). Soft ionization techniques results in fewer molecular fragments and are ideal for hormone analysis. In ESI, charged droplets are produced by the potential difference between the high voltage capillary, through which the sample is sprayed. A second spray of heated inert gas evaporates solvent to form individual gas phase analyte ions (Karpievitch et al., 2010; Wang et al., 2018).

Following ionization, ions are accelerated through electric plates as they enter the mass analyzer, where they are sorted by their mass to charge ratio. There are several types of mass analyzers including quadrupole (Q), ion trap (IT), time-of-flight (TOF), and fourier transform (FT). The type of analyzer can impact the sensitivity and resolution of the spectrometer (Haag, 2016). The choice of mass analyzer depends on the mass of the analyte, ability of the analyzer to interact with the chosen ion source, limit of detection, and desired resolving power.

In all mass analyzers, the ions are deflected differentially by their mass to charge ratio. The reflected ions eventually reach the detector, which generates a signal spectrum of mass-to-charge ratio vs. relative abundance (Haag, 2016). For small molecules, in cases where the charge is one, the m/z ratio equals the mass. However, bigger molecules, like peptides, will carry multiple charges, making them detectable in a restricted m/z range (Khoo, 2010).

Mass analyzers may also be combined (such as tandem MS (MS/MS)). Tandem MS involves fragmentation of "parent" or "precursor" ions generated at the ionization source which can be further fragmented in a collision cell and analyzed in a second mass analyzer. The resulting "daughter" or "product" ions reveal additional information on structure of the analyte, and the monitoring of these products is an important technique in targeted peptidomics and proteomics. In quantitative analyses that use multiple reaction monitoring, the additional information

provided by the precursor to product ion transition increases the selectivity of the method for the target analyte (Haag, 2016).

The major advantage of MS/MS is the use of selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes. In an SRM experiment, the precursor ions can be filtered by abundance, allowing only the desired precursor ion to continue to MS2. The result is increased selectivity and improved data quality. MRM mode allows to select multiple precursor ions, allowing for multiplex assays of complex matrices (Field, 2013).

1.3.3 Sample preparation

Unlike an immunoassay, MS-based assays with ESI require sample cleanup and chromatography to make the analysis possible. The coupling of chromatographic separation prior to MS expands the instrument's potential to analyze pre-separated complex matrices. Liquid chromatography (LC) uses a liquid mobile phase to transport a sample through a coated column. Gas chromatography (GC) enlists a gaseous mobile phase and requires the sample to be volatile. LC flow rate is slower than GC and subject to greater band-broadening. However, LC limits the formation of new products and the degradation of sample that may occur with the high temperature conditions of GC. For non-volatile compounds, LC is the preferred technique for analysis of peptide hormones (Rauh, 2012). For small molecules in aqueous environments, reversed phase LC using a hydrophobic stationary phase and combination of aqueous and organic mobile phases is most common. The retention time for each unique compound will depend on its interaction with the between the stationary phase and mobile phase. Differentially eluted compounds then enter the ionization source of the mass spectrometer. Ultimately, the results yield a chromatogram of the abundance of each compound eluted over a set period of

time, and for each time point on the chromatogram, a m/z ratio and relative abundance of the compound(s) eluted (Perez et al., 2016; Vinaixa et al., 2016).

Sample extraction and preparation prior to chromatography can further enhance MS-based assays by concentrating the analyte and removing interfering compounds. Removal of large proteins and salts is especially important before chromatography to ensure longevity of chromatographic columns. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are highly cited as pre-LC-MS extraction methods. While LLE relies on the compound's affinity for the solvents to separate the analyte(s), SPE is a chromatographic technique. Protein precipitation is a faster extraction method that aims to remove large proteins from the matrix. Any of these extraction methods may be combined with filtration to further concentrate the analyte(s) and eliminate compounds with masses outside the range of interest (Field, 2013; Kushnir et al., 2010).

1.3.4 Advantages and disadvantages of LC-MS/MS

Compared with immunoassays, MS assays have greater specificity for the analyte since they rely on the mass and structure of the molecules rather than its interaction with an antibody. As such, MS assays have less variation and greater reproducibility when compared to immunoassay kits, which can vary widely depending on the antibody used (Pinho et al., 2019). The use of internal standards allows the analyst to account for matrix interference and extraction loss, which can be especially useful when samples are not handled optimally or contain drugs and/or preservatives. The specificity of LC-MS/MS also allows for the differentiation between analogs and separation between active versus inactive forms of hormones. Many hormones require post-translational modifications for activation, and it is often the active form of the hormone that carries biological significance (Rauh et al., 2007; Wolf et al., 2004).

Another advantage of LC-MS assays is the ability to multiplex. The ability to multiplex with LC-MS means sample volume requirements are much lower and total analysis can be completed rapidly (Field, 2013). However, multiplexing with LC-MS is not without limitations since the process of integrating compounds of different physiochemical properties into a single extraction method and LC-MS protocol can be rigorous (Christians et al., 2012).

With an MS-based assay, the analyst can measure compounds for kits and/or antibodies that may not be commercially available. The availability of advanced optimizer software allows those with minimal experience with MS to optimize MS parameters and select precursor and product ions rapidly. Development of an MS assay requires only the purified analyte and an internal standard. While procurement of pure hormones may be expensive, the burden of cost is comparatively lower than that of antibodies for a custom immunoassay (Rauh, 2012).

As with immunoassays, one common area for error with MS assays is the potential for human error during manual sample preparation. This can be minimized with the use of automated sample extraction instruments and development of simple extraction techniques (Piehowski et al., 2013). Another potential area of error with MS assays is ion suppression. Ion suppression occurs when compounds in the matrix or environment interfere with detection or elution of the analyte, influencing the extent of ionization. Ion suppression is also a concern when the interfering compound(s) are co-eluted with the analyte and compete for ionization, which can affect sensitivity and linearity of a targeted assay. However, measures can be taken during sample preparation and instrument optimization to account for such effects (Annesley, 2003).

1.4 Development of LC-MS/MS Multiplex Assays for Post-prandial Hormones

Post-prandial hormones involved in appetite regulation and body composition are of general interest. As food intake and energy expenditure must be balanced to maintain a healthy body

weight, hormones are required to communicate anorexigenic and orexigenic signals to influence appetite, satiety, body mass, digestion, and absorption (Miller, 2017; Woods & D'Alessio, 2008). The study of these hormones is vital for understanding the impact of diet on physiology. The development of multiplex assays to quantify such hormones has major potential for nutrition research.

For our method development, we chose to analyze several related post-prandial hormones involved in appetite control, body composition, and glucose homeostasis. The selected hormones include insulin, cortisol, Glucagon-like peptide 1 (GLP-1) (7-37), GLP-1 (7-36), acyl and desacyl ghrelin, and carboxylated osteocalcin. Below, a brief description of each is provided.

Ghrelin is best known as an appetite stimulating hormone which rises during periods of fasting and drops immediately after feeding (Akalu et al., 2020). There are two major forms of ghrelin in mammals including the active acylated ghrelin and the inactive desacyl ghrelin. Acylated ghrelin binds to ghrelin receptors in the hypothalamus and pituitary gland to increase appetite. Acylated ghrelin is also responsible for several non-appetite related functions. Acylated ghrelin is a regulator of glucose homeostasis through inhibition of insulin secretion and is also involved in modification of body composition indirectly through its interaction with growth factors and directly through myocytes, adipocytes, and osteoblasts (Pradhan et al., 2013). Although desacyl ghrelin is considered the inactive form of the hormone, accumulating evidence suggests it functions as an agonist to acylated ghrelin. Desacyl ghrelin may reduce appetite and promote insulin secretion (Ibrahim Abdalla, 2015; Pacifico et al., 2009).

GLP-1 is an appetite suppressant which rises following a meal. In contrast to acylated ghrelin, GLP-1 decreases appetite and promotes secretion of insulin. The two active forms of GLP-1 in the blood are GLP-1 (7-37) and the GLP-1 (7-36) amide (Müller et al., 2019). Low levels of

active GLP-1 after consumption of a meal are associated with increased risk of obesity and diabetes (Breij et al., 2017).

The glycemic control hormone insulin is also involved in signaling cascades involving appetite and body composition. Insulin is inversely associated with appetite through suppression of ghrelin activation and secretion (Koliaki et al., 2010). Additionally, insulin has also been found to directly communicate with the hypothalamus to promote an anorexigenic neural response (Salehi et al., 2012; Stevenson & Allerton, 2018).

Osteocalcin (OC), although most well known as a measure of osteoblast activity, is also a post-prandial hormone (Wei & Karsenty, 2015). Osteocalcin circulates the blood as either carboxylated or decarboxylated OC. A post-prandial rise in insulin stimulates the release of decarboxylated OC which participates in a feed-forward loop to promote insulin secretion. Decreases in insulin, such as before a meal, shifts the system in favor of carboxylated OC. Carboxylated OC inhibits bone mineralization (Zoch et al., 2016). Interestingly, both carboxylated and decarboxylated OC were found to interact directly with myocytes and adipocytes to promote glucose uptake and increase insulin sensitivity (Hill et al., 2014).

Although it is not considered a post-prandial hormone, the steroid hormone cortisol is an important mediator of appetite, body composition and glucose homeostasis. Physical and neurologic stressors increase circulating cortisol, and cortisol has been shown to directly impact insulin secretion (Schernthaner-Reiter et al., 2021). Indeed, it has been shown that cortisol suppresses insulin resulting in shifting of metabolism to towards gluconeogenesis. A prospective longitudinal analysis on humans found chronic stress to be associated with higher levels of cortisol and insulin and greater weight gain (Chao et al., 2017). High levels of cortisol can indirectly increase food cravings and overall calorie consumption by mediation of ghrelin

(Azzam et al., 2017; Epel et al., 2001). Since feeding studies may induce stress on subjects, stress-induced perturbations in appetite regulation should be accounted for when examining the effect of any intervention on appetite and body composition.

The highly interconnected relationship between appetite control, body composition, stress, and glycemic control reinforces the importance of diet in prevention and progression of chronic diseases. The interrelation of these hormones also represents an interesting opportunity for the development of a multiplex LC-MS/MS assay.

Several methods have already been published on multiplex analysis of human steroid hormones (Broccardo et al., 2013; Nadarajah et al., 2017; Snaterse et al., 2020). Steroid hormones consist of small, cholesterol-derived compounds (Holst et al., 2004). As discussed in earlier sections, small molecule LC-MS is straightforward since the molecules generally do not fragment during soft ionization in ESI or MALDI. This means that mass and charge values will be consistent across instruments and protocols, and there is no loss of sensitivity secondary to extensive fragmentation. LC-MS based analysis of proteins and peptides is distinct from small molecule analysis in several ways. Sensitivity and selectivity of protein analysis is highly dependent on the fragment of the protein being monitored. Fragmentation can vary largely by sample preparation and instrument parameters. Additionally, extraction and enrichment of peptides from biological samples is complicated by the complexity and variety of peptides (van de Merbel, 2019).

Despite the challenges, considerable progress has been made in peptide hormone analysis. Several papers have published validated protocols to extract and quantify peptide hormones (Chen et al., 2013; Lapko et al., 2013; Rauh et al., 2007). Most methods have focused on human forms of insulinotropic peptide hormones including insulin (Chen et al., 2013), insulin-like

growth factor (Barton et al., 2010), GLP-1 (Wolf et al., 2004) and glucagon (Delinsky et al., 2004; Lapko et al., 2013; Miyachi et al., 2017).

The work in this thesis aims to develop a multiplex LC-MS/MS based method to quantify ghrelin, osteocalcin, GLP-1, insulin, and cortisol in serum. Additional postprandial hormones, including leptin, CCK, and IGF-1 were considered but ultimately deemed unsuitable due to the difficulties obtaining custom standards for large peptides. Decarboxylated OC was also initially included in the method but removed due to issues with the custom synthesized hormone standard.

For this study, we chose to develop a method to analyze these hormones in pig serum. Pigs are considered an excellent model system for the study of human metabolism as the two species share many similarities in digestive anatomy and physiology (Ziegler et al., 2016). To our knowledge this is the first method validated to quantify this combination of hormones and the first LC-MS method specific to pig hormones.

1.5 Framework for Bioanalytical Method Validation

After development of the multiplex biomarker assay, we sought to validate the assay. Although the desired parameters and validation process may differ by the assay purpose, the goal of validation will be to formally demonstrate the suitability of the method for the intended analysis and its ability to provide reproducible data within the method's limits. Method validation of biological samples should establish guidelines and parameters for proper sample handling, sample preparation, and interpretation of results (Tiwari & Tiwari, 2010). The validation parameters that were used in this study are discussed below.

1.5.1 Specificity

Specificity refers to the ability of the method to isolate the analyte of interest from compounds normally present in the matrix. In initial testing, the property of the analyte intended to be used for quantification must be established to be specific to the analyte. In the case of LC-MS, the property is the retention time and transition of the analyte obtained from optimization experiments performed with a pure standard. Specificity can be established by yielding a positive result when the analyte is present and a negative result when the analyte is known to be absent (Tiwari & Tiwari, 2010). Analyte analogs may be spiked or assayed in isolation to assess whether they are distinguishable from the analyte itself (Thomas et al., 2020).

1.5.2 Precision and repeatability

Precision refers the closeness in agreement between measurements of samples. Typically, this is evaluated by running several replicates of a sample. Precision can be represented numerically through calculation of the standard deviation and coefficient of variation (%CV). While standard deviation represents the spread of the replicates, %CV represents the spread of the replicates from the mean. A %CV cutoff lower than 15% is expected for concentration level tested, with the exception of LLOQ (lower limit of quantification) for which values as high as 20% CV can be regarded as acceptable (Tiwari & Tiwari, 2010).

The method of measuring precision differs by the type of precision being determined. Instrument and method precision involve measurement of variation between replicates run within in a single series. Repeatability or intra-assay precision involves determination of precision between series, with each series performed under identical laboratory and analyst conditions. Intermediate precision may also be measured by comparing identical samples run on different days or under

different instrument conditions. Generally, this includes a measurement of inter-day and inter-week precision (Tiwari & Tiwari, 2010).

Variations and error introduced by instrument can also be accounted for by the running quality control samples prior to each run. A quality control (QC) sample will be a sample of known composition and concentration that is run prior to the analytes. Instrument consistency can be determined by assessing whether there is a consistent response of the QC samples (Božović & Kulasingam, 2013).

1.5.3 Linearity and range

The range of an assay refers to the upper and lower detection limits that for which the method has been validated. All concentration values within the range should be tested for precision and accuracy. The range is determined via calibration curve. An analyte is spiked in known quantities into an analyte-free matrix. Since biological matrices can greatly affect instrument response in LC-MS, the use of an analyte-free matrix ensures that the calibration curve corresponds to the samples being analyzed (Khamis et al., 2021). A calibration curve is generated by serial dilution of the spiked analyte-free matrix to generate a concentration gradient. The concentration gradient should be at a range appropriate to the expected concentration of the analyte in the matrix. The calibration curve is assessed for linearity. Statistical interpretation of analyte in samples is often dependent on a linear relationship between concentration and response. Generally, an R^2 of > 0.90 is considered acceptable (Araujo, 2009; Tiwari & Tiwari, 2010).

1.5.4 Limits of detection and quantitation

The limits of detection and quantitation refer to the lower limits of the method. While limit of detection is defined as the lowest concentration that can be distinguished from zero concentration, limit of quantitation is the lowest concentration that can be confidently quantified. Experimentally, these parameters are determined by running replicates of low concentration samples with the lowest concentration being a blank analyte-free matrix. The LOD will be the concentration that can be differentiated from 0 concentration with a %CV below 20, while the LOQ will be the concentration at which a measurement can be made with %CV below 20. In LC-MS, the LOQ and LOD may also be decided based on the signal-to-noise ratio (s/n). Often with complex matrices, there will be some baseline noise. A s/n greater than 5:1 is generally considered acceptable for quantification. The concentration at which the s/n reaches goes below 3:1 can be considered the LOD. The LOQ may equal the LOD if concentration at the LOD meets the precision and accuracy requirements (Božović & Kulasingam, 2013).

1.5.5 Recovery

Recovery is defined as the amount of analyte retained after sample preparation. A poor recovery will lead to poor sensitivity and suggests that the sample preparation and extraction is not suitable. Recovery is assessed by running a set of samples spiked with analyte before extraction (A) and another set spiked after extraction (B). Recovery equals the measurement of signal in samples spiked before extraction divided by the measurement of signal in samples spiked after extraction x 100% (A/B x 100%). The cut off for recovery depends on the purpose of assay and type of analyte. Generally, a recovery between 80-120% is considered acceptable (Božović & Kulasingam, 2013; Zhou et al., 2017).

1.5.6 Method comparison

Finally, after assessing the above parameters using the MS instrument, it is important benchmark the assay by comparing the new method to a previously validated and accepted method. Published LC-MS methods have compared measurements to previously cited immunoassay kits. Since immunoassay kits do not have perfect accuracy in themselves, the goal of the comparison is not to match the values exactly but rather to ensure that there is a linear relation and consistency between the measurement obtained. This is done by running identical samples with a range of concentrations on both the method being validated and the already validated immunoassay. Measurements obtained on both methods are plotted as a correlation curve (Marin et al., 2021).

Chapter 2 - Development of a method for measurement of insulin, cortisol, GLP-1 (7-37), GLP-1 (7-36), acyl and desacyl ghrelin, and carboxylated osteocalcin from 100 uL of serum

2.1 Introduction

Accurate measurement of blood hormones is important for reliable diagnostics and health research. Current methods for hormone quantification rely largely on antibodies to capture and detect hormones. Procurement of antibodies can be expensive and custom synthesis can be time intensive. LC-MS/MS is widely used for the quantification of small molecules (Pinho et al., 2019). Although advances in mass spectrometry have allowed for quantification of larger peptides and steroid hormones, there exist no methods specific to pig hormones. Pigs are an advantageous model for human research due to their short gestation period and larger size. Pig digestive anatomy and physiology carry a high degree of similarity to that of humans, making pigs an ideal model for studies that examine the effects of diet on metabolism (Gonzalez et al., 2015). The effect of diet on metabolism is mediated by hormones that control appetite, body composition, and stress. Here, for the first time we describe the development, optimization, and validation of an LC-MS/MS based method to simultaneously quantify insulin, cortisol, GLP-1 (7-37) GLP-1 (7-36), acyl and desacyl ghrelin, and carboxylated osteocalcin from 100 µL of pig serum.

2.2 Materials and Methods

2.2.1 Instrumentation

Unless otherwise specified, all experiments were performed on an Agilent 1260 Infinity II LC system and auto-sampler utilizing an Agilent AdvanceBio Peptide C18 column (120Å, 2.1 x 150 mm, 2.7 µm) connected to an Agilent 6470 Triple Quad LC/MS equipped with an electrospray

ionization (ESI) source. The Agilent MassHunter software suite (version 10.1) was used to monitor and optimize output in real-time and perform data analysis.

2.2.2 Reagents and Chemicals

LC-MS grade acetonitrile (ACN), methanol (MeOH), formic acid, and acetic acid were purchased from Sigma Aldrich. Porcine serum used for method development was purchased from ThermoFisher.

Human ghrelin, and des-acyl ghrelin internal standards were purchased from R&D Systems. Human and porcine insulin, deuterated cortisol, and cortisol were purchased from Millipore Sigma. Human carboxylated osteocalcin was purchased from AnaSpec. Methylated GLP-1 (7-36) and GLP-1 (7-37) internal standards were custom synthesized and provided by GeneScript. Porcine GLP-1 (7-36), GLP-1 (7-37), y-carboxy osteocalcin, ghrelin, and deacylated ghrelin were custom synthesized by GeneScript. All peptides were purchased at purity of $\geq$ 95%.

A porcine insulin ELISA kit (Mercodia) and cortisol ELISA kit (Cayman Scientific) were obtained for the purpose of method comparison.

2.2.3 Prevention of non-specific binding

Non-specific binding occurs when compounds, such as proteins and peptides, adhere to unintended surfaces (Fruitger et al., 2021). Non-specific binding can result in a loss of analyte and can impact the precision and accuracy of a method. To prevent non-specific binding of target hormones, all sample preparation was done using 2 mL Pierce low protein binding microcentrifuge tubes (ThermoFisher). LC-MS analysis was completed by transferring filtered samples to Waters QuanRecovery LC-MS vials.

2.2.4 Preparation of Standards and Calibrants

All hormones were reconstituted according to manufacturer specifications in order to ensure stability of hormones during storage. The solvents used are described in **Table 1.** All peptides were diluted to the appropriate concentration and aliquoted before storage at -20 °C. Each aliquot was used only once after thawing to avoid multiple freeze–thaw cycles.

Internal standard stocks were prepared such that 1 µL of stock into 100 µL of sample would result in the desired final concentration (**Table 2**). The concentration of internal standard was calculated relative to the mid-range endogenous concentration of the hormones in pig serum. External standard stock aliquots were prepared at the desired upper limit of quantitation.

For generation of the calibration curve, 20 µL of the external standard stock was combined with 40 µL of 0.2% acetic acid to a achieve 3-fold dilution. The three-fold diluted spike mixture was then diluted three-fold and this process was repeated to generate a series of seven spike solutions. One µL of each spike solution was combined with a separate 100 µL aliquot of calibration curve matrix to generate seven calibrants. An internal standard spike solution was added in equal amounts across all calibrants. An un-spiked calibration curve matrix (described in **Section 2.2.5**) served as the lower end of the curve. The concentration of analyte in calibrants is shown in **Table 3**.

Table 1. Solvents used for peptide standards.

Peptide	Molecular Weight (kDa)	Diluent	Peptide	Molecular weight (kDa)	Diluent
Porcine insulin	5778	0.2% formic acid	Human insulin	5734	0.2% formic acid
Porcine osteocalcin	5721	0.2% formic acid	Human osteocalcin	5879	3% ammonia water
Porcine ghrelin	3317	0.3% acetic acid	Human ghrelin	3371	Ultrapure water
Porcine desacyl ghrelin	3191	0.3% acetic acid	Human desacyl ghrelin	3245	Ultrapure water
GLP-1 (7-36)	3298	80:20:0.1 H2O:MeOH: formic acid	Methyl GLP-1 (7-36)	3313	Ultrapure water
GLP-1 (7-37)	3356	DMSO	Methyl GLP-1 (7-37)	3371	3% ammonia water
Cortisol	363	MeOH	d4-Cortisol	367	MeOH

Table 2. Concentration of internal standards used in all samples.

	Unit	Concentration
Insulin	ng/mL	0.125
Me-GLP-1 (7-36)	ng/mL	0.187
Me-GLP-1 (7-37)	ng/mL	0.125
Ghrelin	ug/mL	0.125
Desacyl Ghrelin	ug/mL	0.125
Osteocalcin	ng/mL	6.25
Cortisol	ng/mL	12.5

Table 3. Concentration of external standards in calibration samples.

		Calibration Level							
	Unit	8	7	6	5	4	3	2	1
Pig Insulin	ng/mL	0.625	0.21	0.07	0.02	0.01	0.003	0.001	0.00
GLP-1 (7-36)	ng/mL	0.625	0.21	0.07	0.02	0.01	0.003	0.001	0.00
GLP-1 (7-37)	ng/mL	0.625	0.21	0.07	0.02	0.01	0.003	0.001	0.00
Pig Ghrelin	ug/mL	0.625	0.21	0.07	0.02	0.01	0.003	0.001	0.00
Pig desacyl Ghrelin	ug/mL	0.625	0.21	0.07	0.02	0.01	0.003	0.001	0.00
Pig y-carboxy Osteocalcin	ng/mL	62.5	20.83	6.94	2.31	0.77	0.26	0.086	0.00
Cortisol	ng/mL	62.5	20.83	6.94	2.31	0.77	0.26	0.086	0.00

2.2.5 Preparation of quality control samples

For method validation, quality control (QC) samples were prepared by spiking pig serum with different concentration levels of pig hormones: high (QC1), medium (QC2), and low (QC3). In total, 1 µL of each stock solution was added to 100 µL of either pig serum or calibration curve matrix (40% filtered pig serum with 60% pig serum). Concentrations of hormones in QC samples is shown in **Table 4**. All QC samples included internal standards at a final concentration shown in **Table 2.**

Table 4. Concentration of pig hormones in QC samples.

	Unit	QC1	QC2	QC3
Insulin	ng/mL	0.625	0.0625	0.0125
GLP-1 (7-36)	ng/mL	0.625	0.0625	0.0125
GLP-1 (7-37)	ng/mL	0.625	0.0625	0.0125
Ghrelin	ug/mL	0.625	0.0625	0.0125
Desacyl Ghrelin	ug/mL	0.625	0.0625	0.0125
Osteocalcin	ng/mL	62.5	6.25	1.25
Cortisol	ng/mL	62.5	6.25	1.25

2.2.6 Preparation of analyte-free matrix for calibration curve

A calibration curve for LC-MS base quantitation was generated by spiking internal and external standards into a calibration curve matrix. The ideal calibration curve matrix had little to no endogenous analyte and included the matrix components necessary for ionization and stabilization of the desired analyte. Preparation of all tested calibration curve matrices is detailed below.

Charcoal is commonly used to strip biological fluids of lipophilic compounds and proteins (Sikora et al., 2016). For our calibration curve, charcoal stripped serum was prepared using a method adapted from Dixit & Chang, 1984. Briefly 800 mg of activated carbon was combined

with 2 mL of pig serum and vortexed for 10 min. The solution was then centrifuged for 30 min at 16000 rcf and the supernatant collected and stored at 4 °C.

Many targeted LC-MS assays use non-serum surrogate matrices for generation of the calibration curve (Chen et al., 2013; Howard, 2018; Rauh et al., 2007). Four non-serum surrogate matrices were prepared for analysis (**Table 5**).

Table 5. Description and composition of non-serum matrices

Description	Composition
Surrogate matrix adapted from Howard, 2018	20:80:0.2:0.1 MeOH: H_2O: acetic acid: bovine serum albumin (BSA)
Surrogate matrix with glycerin	20:77.5:0.2:0.1:2.5 MeOH: H_2O: acetic acid: BSA: glycerin
Phosphate buffered saline (PBS)-based surrogate matrix	2% BSA in PBS with 0.2% acetic acid
Extraction solvent	CAN: H_2O: acetic acid 75:25:0.2

Chicken serum was investigated as a surrogate serum since avian GLP-1 is distinct from mammalian GLP-1 (Zhang et al., 2019). One milliliter of commercial chicken serum extract was prepared by addition of four parts 0.2% acetic acid in 75% ACN.

Finally, a partially filtered piglet serum was examined for its effectiveness as a calibration curve matrix. Filtration of serum through a 30kDa centrifugal filters (Millipore-Sigma) depletes the matrix of some peptide and steroid hormones due to non-specific binding of these compounds to the polyether sulfone material of the filter. We used combined filtered and unfiltered serum in a 40:60 ratio for generation of the calibration curve. Five 300 µL aliquots of piglet serum from a previous study were combined and vortexed. Six hundred microliters of serum composite were filtered using a 30 kDa centrifugal filter. Two hundred and forty microliters of the serum filtrate

were combined with 360 µL of unfiltered pig serum to achieve a 40:60 ratio of filtered to unfiltered serum. The serum was then extracted by addition of four parts 0.2% acetic acid in 75% ACN in order to remove large proteins.

2.3 Method Validation & Statistical Analysis

2.3.1 Specificity

Specificity of the retention time and transition selected for each hormone was established by observing for analyte signal in spiked and solvent blank samples. Specificity of the transition and retention time selected was established by an absence of signal in solvent blank samples, including chicken serum and protein precipitation solvent. Furthermore, the suitability of the transition and retention time for the analyte was established by presence of a peak in spiked samples and pig serum.

2.3.2 Precision

Precision is expressed as %CV and was determined by analysis of triplicate running QC samples. The concentration of QC samples corresponds to the highest concentration (QC1) as described in **Table 4**. QC samples were prepared in commercial pig serum. Analysis was done within a set of samples run the same day for intra-day precision and on two different days for inter-day precision.

2.3.3 Recovery

For calculation of recovery, analysis was performed in triplicate. All samples (n=6) were spiked to the high QC concentration (QC1) as described in **Table 4**. Three samples were spiked prior to extraction and three were spiked after extraction. Recovery was expressed as percentage of analyte recovered when spiked before extraction over recovery when spiked after extraction.

2.3.4 Linearity

Linearity was established by analysis of the calibration curve (**Table 3**). The strength of correlation between the expected concentrations and peak area is expressed as R^2. A correlation of $R^2 \geq 0.9$ indicated a positive relation wherein detected analyte response increased with concentration.

2.3.5 LOQ

LOQ was defined as the lowest concentration calibrant that was distinguishable from zero concentration as evidenced by the lowest point on the calibration curve that had %CV $\leq$ 20 when analyzed in duplicate.

2.3.6 Method comparison

Pig insulin and cortisol measurements obtained from the final LC-MS method was compared to results from commercial porcine insulin ELISA (Mercodia) and cortisol ELISA (Caymen Scientific) kits. QC high, QC medium, QC low, commercial pig serum, piglet serum and commercial chicken serum were run in duplicate using both LC-MS and ELISA. Each sample was plotted based on the concentration yielded from both methods.

2.4 Results and Discussion

The work in this thesis consists of two stages: method development and optimization, and method validation. LC-MS multiplex analysis of hormones represents a technical challenge due to the variety of structures and chemical properties of the hormones. The goal of method development and optimization was to select for sample preparation and analysis conditions that were suitable for all the target hormones. The final method was validated by calculation of parameters relating to method reproducibility, accuracy, and precision.

2.4.1 Optimization of sample extraction

Protein precipitation with 75% acetonitrile was selected as the ideal extraction method due to its speed and efficacy as evidenced by a previous LC-MS multiplex peptide assay (Howard, 2018). Acetonitrile allows for the rapid and effective extraction of cationic peptides and low molecular weight compounds (Chertov et al., 2004). When ACN is added to serum, large proteins will precipitate out of solution. The supernatant contained low molecular weight proteins, peptides, and other small biomolecules. pH modification of acetonitrile by the addition of acids or bases can further improve extraction efficacy although the best pH modifier depends heavily on the target analyte(s). Experiments were performed to evaluate the impact of acid type, incubation, and temperature on recovery.

The type of acid or based used, and the pH of the sample can impact ionization of compounds during the MS run and can also impact extraction efficiency. The ideal acid or base type depends largely on the isoelectric point of the peptides of interest and other proteins and peptides in the matrix. Thus, we performed experiments to compare the effectiveness of several pH modifiers on recovery and ionization of peptides. The acids and bases examined included acetic acid, formic acid, trifluoroacetic acid (TFA), and ammonium hydroxide (NH□OH). Due to the heterogeneity in structural and chemical properties of our analytes, there was no acid type that was optimal for all hormones, so the pH modifier that was selected provided the best recovery and signal intensity for all analytes in our multiplex assay.

An initial trial compared 0.3% acetic acid, 0.1% formic acid, a mixture of 0.3% acetic acid and 0.1% formic acid, and 0.1% TFA on signal intensity. Results from this experiment are shown in **Figure 1.** While the external and internal standards for cortisol had consistent signal intensity across all four acid mixtures, 0.3% acetic acid provided the best signal intensity across all six

peptides tested. While the acetic and formic acid mixture also provided good signal intensity, the chromatograms for samples extracted with this mixture exhibited increased noise (data not shown).

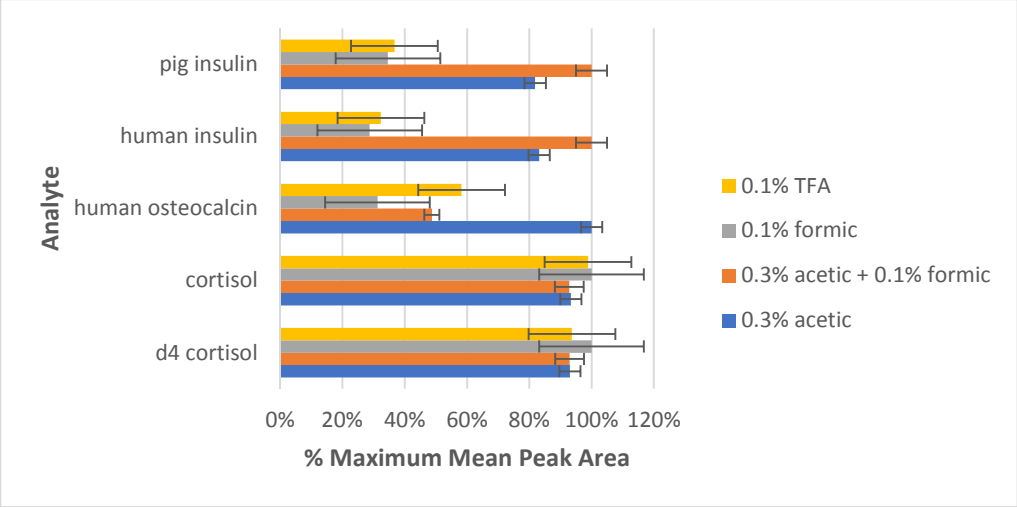


Figure 1. Relative signal intensity of hormones extracted with four types of acids

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 desacyl and acyl ghrelin, and osteocalcin was 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.

A subsequent trial examined additional pH modifiers on signal intensity (**Figure 2**) and analyte recovery (**Figure 3**). We compared the effects of 0.3% acetic acid, 0.2% acetic acid, 0.1% NH_4OH, and no acid/base. Protein precipitation with acetic acid provided the highest signal intensity for cortisol, ghrelin, and IGF-1 while NH_4OH worked best for GLP-1 and osteocalcin. Insulin displayed superior signal intensity when no pH modifier was present. While the lack of pH modifier dramatically reduced recovery of GLP-1 (7-36) and both forms of ghrelin, pH modifier type did not drastically affect recovery of any other hormone. Due to the poor recovery

of ghrelin when no pH modifier was present, this extraction was deemed unsuitable. The optimal pH modifier was 0.2 % acetic acid since it provided better recovery for most hormones compared to 0.3% acetic acid.

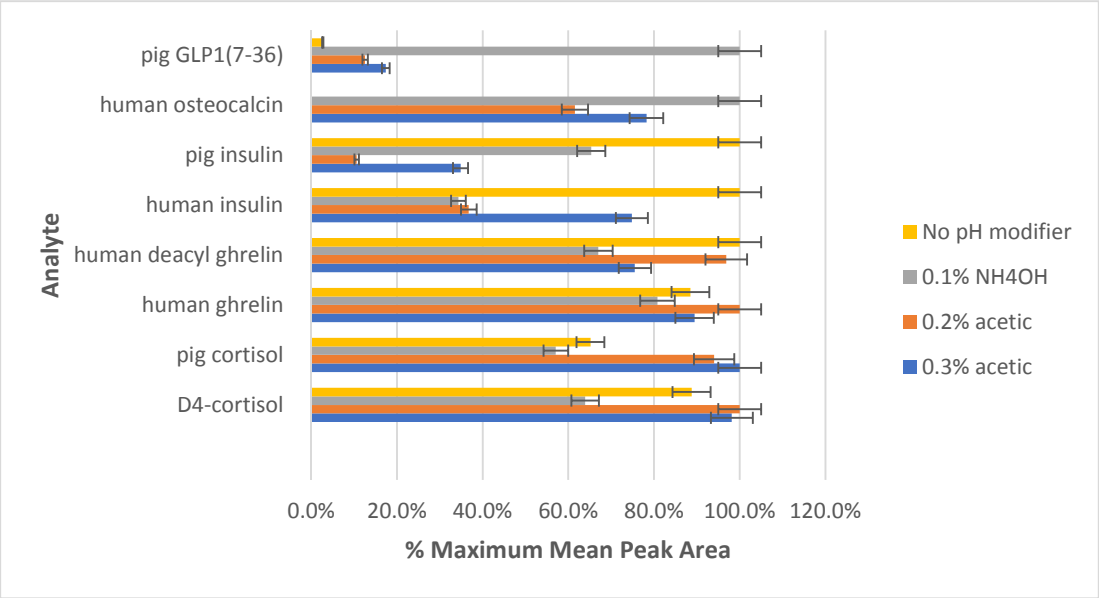


Figure 2. Relative signal intensity of spiked serum samples extracted with either no pH modifier, 0.1% NH_4OH, 0.2% acetic acid, or 0.3% acetic acid.

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 is visualized in the figure. No single pH modifier provided maximum signal intensity for all analytes.

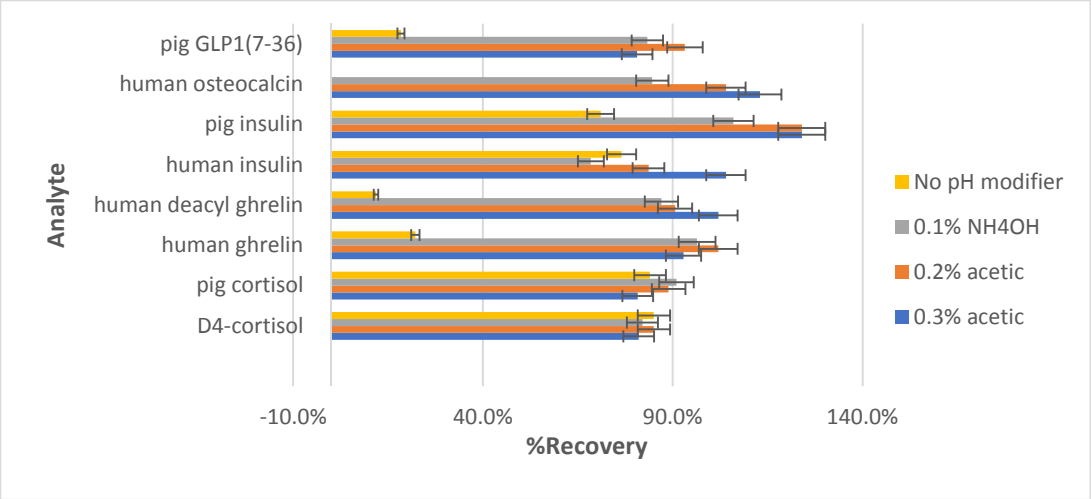


Figure 3. Recovery of spiked serum samples extracted with four different either no pH modifier, 0.1% NH_4OH, 0.2% acetic acid, or 0.3% acetic acid.

Pig serum was spiked with QC1 concentrations of the available analytes 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 is visualized in the figure. No single pH modifier provided optimal recovery for all analytes.

Next, we explored the addition of a cold temperature incubation step on protein precipitation with our optimized solvent. Cold temperature incubation of serum with protein precipitation solvent prior to centrifugation has been shown to improve signal intensity by ensuring more complete removal of large proteins. Previous methods have demonstrated successful enrichment and extraction of peptides from serum when incubating the serum with acetonitrile at 4 °C (Li et al., 2020). We compared the effects of a 0-, 30-, and 60-minute incubation on signal intensity of select peptides (**Figure 4**). With the exceptions of both pig and human insulin, incubation increased signal intensity of hormones. Incubation for either 30 or 60 minutes was not found to greatly affect recovery of hormones except insulin (data not shown). Recovery of pig insulin dropped from 91% to 59% between a 30- and 60-minute incubation. Thus, a 30-minute incubation was selected for further analyses.

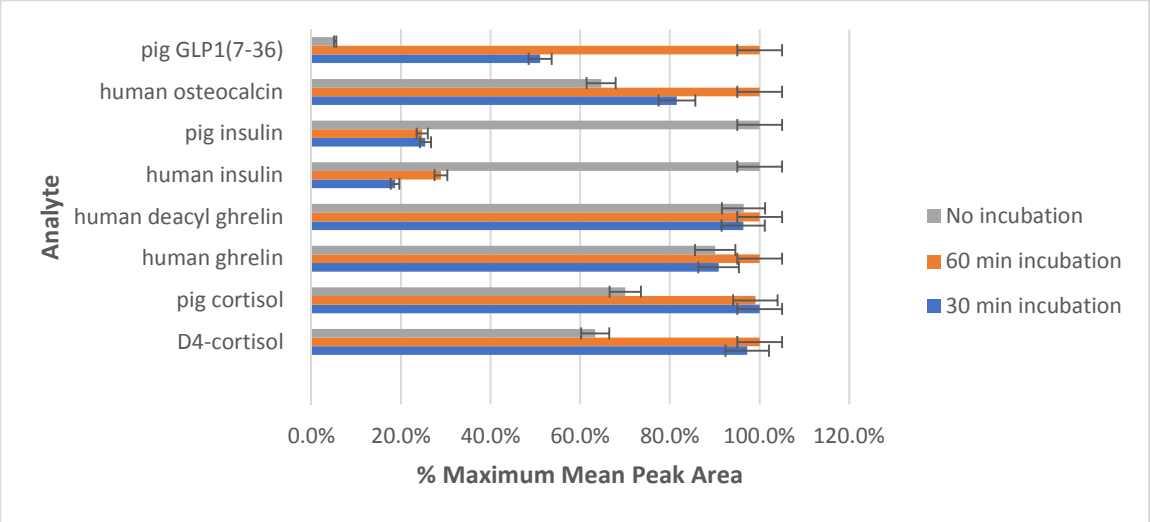


Figure 4. Relative signal intensity of sample incubated with acetonitrile for 0, 30, or 60 minutes.

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 is visualized in the figure.

Temperature was also found to be important during sample preparation. Initially, sample extraction was performed without temperature control and demonstrated large intra- and inter-day variation. Due to degradation of hormones at room temperature, samples and solvents were kept on ice throughout extraction.

The final sample extraction procedure is illustrated in **Figure 5**. The workflow involves the addition of four parts 75% ACN in water with 0.2% acetic acid to one part serum. The serum and protein precipitation solvent was thoroughly mixed and incubated for 30 minutes at 4°C. Next, the mixture was centrifuged at 10,000 rpm for 5 minutes, with the supernatant collected and filtered through a 0.2 µm syringe filter prior to loading into MS vials. The use of a 0.2 µm syringe filter was necessary for longevity of the HPLC column.

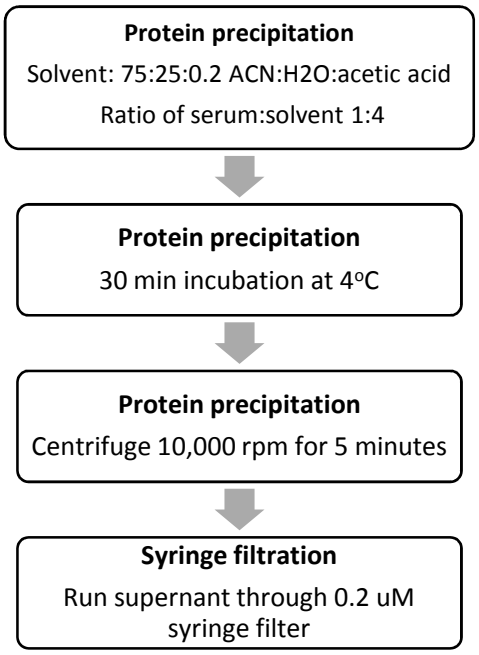


Figure 5. Final sample extraction workflow.

2.4.2 Determination and selection of hormone identifiers

Detection and quantitation of hormones with LC-MS is achieved through each compound's unique retention time and transition. Retention time refers to the time at which the compound is eluted from the LC column. Retention time is dependent on the compound's interaction with the stationary and mobile phase. The transition refers to size and charge (m/z) of the precursor and product ions produced during MS/MS. The transition selected will be the most abundant precursor ion and its most abundant product ion. The most abundant precursor and product ion can be optimized by increasing or decreasing the nozzle voltage/declustering voltage (produces the precursor ion) and collision energy (produces the product ion). Together, the retention time and transition provide a unique fingerprint for identification of each target analyte.

For determination of retention time, solutions of each external and internal standard at a concentration of 0.1 mg/mL were introduced to the LC-MS/MS. Water containing 0.2% formic acid (solvent A) and acetonitrile containing 0.2% formic acid (solvent B) were used as eluents. The total ion chromatogram was monitored for the peak indicating sample elution. The elution gradient described in **Table 6** provided excellent separation of analyte hormones.

Table 6. Gradient elution timetable.

Time (min)	A (%)	B (%)	Flow (mL/min)
0.00	99.0	1.0	0.200
5.00	95.0	5.0	0.200
10.00	79.0	21.0	0.200
15.00	75.5	24.5	0.200
18.00	46.5	53.5	0.200
21.00	26.0	74.0	0.200
25.00	1.0	99.0	0.350
26.00	0.0	100.0	0.350
28.00	0.0	100.0	0.350
35.00	99.0	1.0	0.200
37.00	99.0	1.0	0.200

Next, the transition for each internal and external standard was assessed using the Agilent MassHunter Optimizer software. After injection of a 0.1 mg/mL solution of each standard, the software automatically optimized for collision energy and declustering potential and scanned for the most abundant precursor and product ions. The selected transitions are indicated in **Table 7.** The second most abundant transitions were selected as qualifiers to increase method robustness. Identifiers for qualifiers are listed in **Table 8.** Additional details on declustering potential, collision energy, and cell accelerator voltage can be found in **Supplementary Tables 1** and **2.**

Table 7. Retention time and transition for quantifiers.

	External Standards		Internal Standards	
	Transition	Retention time (min)	Transition	Retention time (min)
Cortisol	363.2 → 121.0	20.1	367.2 → 121.0	20.1
deacyl Ghrelin	639.1 → 84.0	10.1	541.5 → 70.0	10.6
Ghrelin	553.9 → 84.0	13.5	562.5 → 70.0	13.9
Carboxylated osteocalcin	1145.4 → 1115.3	21.2	1186.9 → 1186.9	19.9
GLP-1 (7-36)	660.6 → 660.6	19.7	663.5 → 72.1	19.7
GLP-1 (7-37)	672.1 → 84.1	20.1	674.9 → 84.1	20.1
Insulin	1155.7 → 86.0	20.2	1161.7 → 1158.5	19.7

Table 8. Retention time and transitions for qualifiers.

	External Standards		Internal Standards	
	Transition	Retention time (min)	Transition	Retention time (min)
Cortisol	363.2 → 327.2	20.1	367.2 → 331.2	20.1
deacyl Ghrelin	639.1 → 129.1	10.1	541.5 → 620.9	10.6
Ghrelin	553.9 → 129.1	13.5	562.5 → 689.0	13.9
Carboxylated osteocalcin	1145.4 → 1118.9	21.2	--	19.9
GLP-1 (7-36)	--	--	663.5 → 72.1	19.7
GLP-1 (7-37)	672.1 → 136.1	20.1	674.9 → 110.0	20.1
Insulin	1155.7 → 1152.4	20.2	1161.7 → 86.0	19.7

Because ionization of compounds is dependent on matrix, we confirmed the suitability of the selected transitions for detection in pig serum. Chromatograms for external standards (**Figure 6**) and internal standards (**Figure 7**) produced by QC1, QC2, and QC3 in pig serum were observed for peak shape and s/n. Samples were extracted using the extraction method shown in **Figure 5.** Peak shape of analyte in pig serum exhibited the ideal narrow and sharp peak shapes. Very little to no background was observed for most hormones despite the presence of the serum matrix. Peak broadening was observed for pig insulin and the quantifier peak had a s/n ratio of 1:4. Additional transitions were tested for quantification of insulin in pig serum, however, the initial transition selected yielded the greatest peak area and highest s/n.

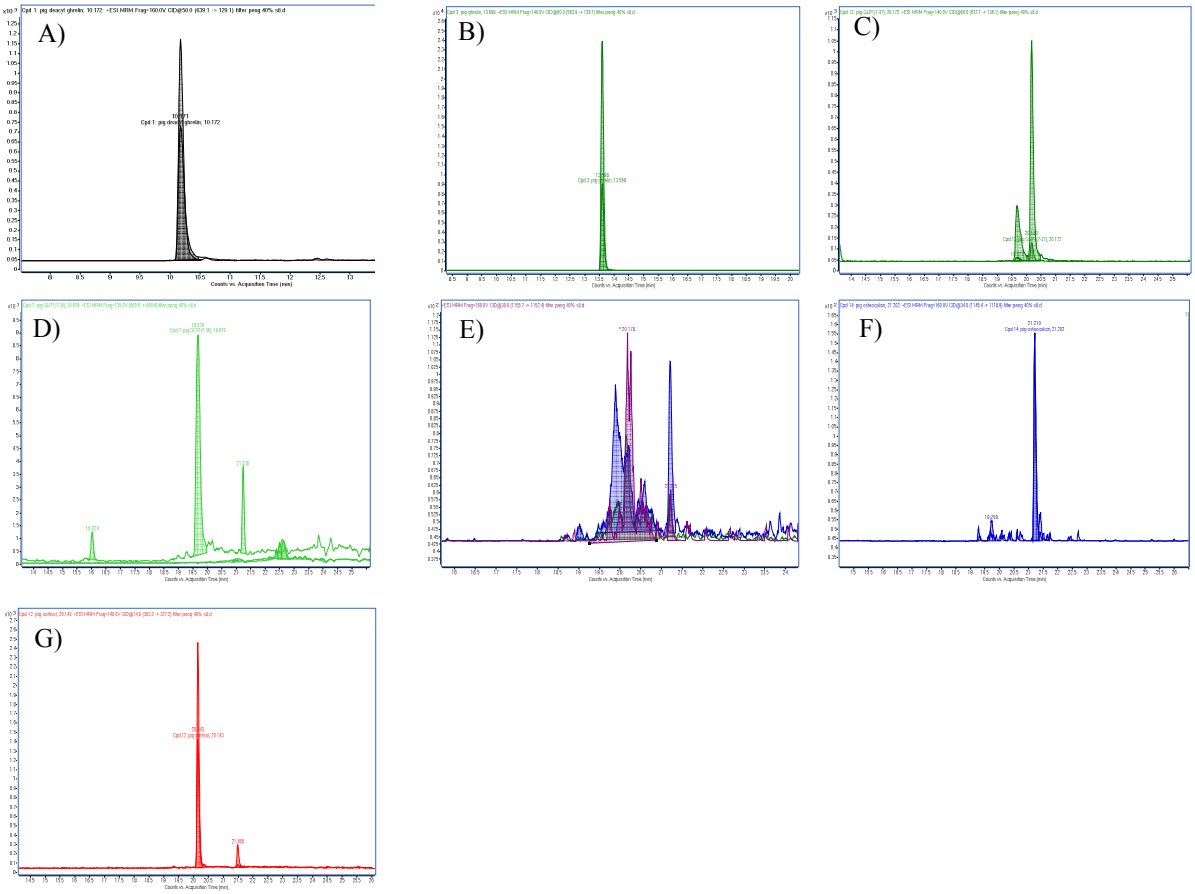


Figure 6. External standard quantifier chromatograms.

External standard chromatograms for pig desacyl (A) and acyl ghrelin (B), GLP-1 (7-37) (C), GLP-1 (7-36) (D), insulin (E), osteocalcin (F), and cortisol (G) from QC1 pig serum using default MS parameters.

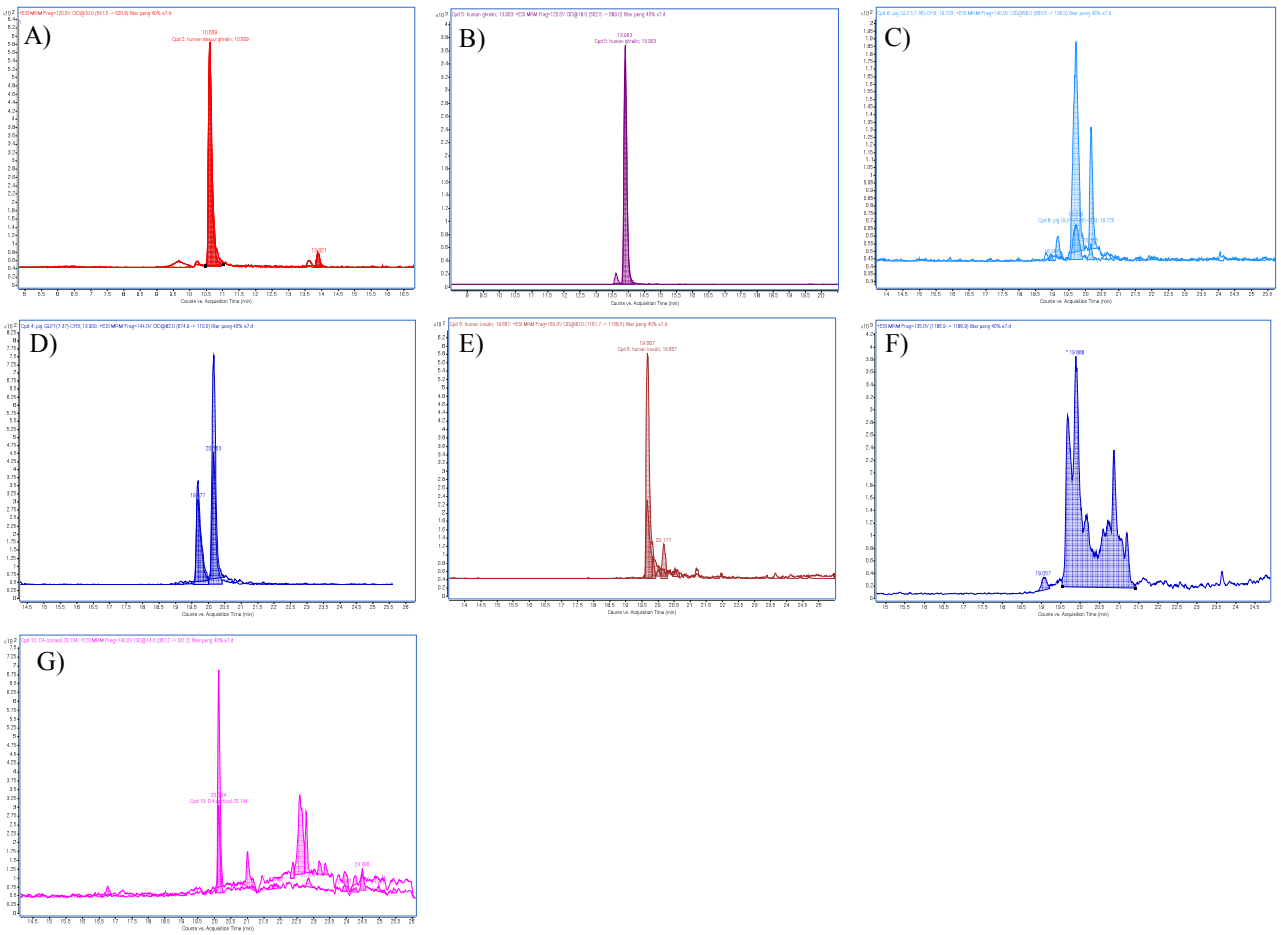


Figure 7. Internal standard quantifier and qualifier chromatograms.

Internal standard chromatograms for pig desacyl (A) and acyl ghrelin (B), GLP-1 (7-37) (C), GLP-1 (7-36) (D), insulin (E), osteocalcin (F), and cortisol (G) from QC1 pig serum using default MS parameters.

2.4.3 Optimization of LC and MS conditions

Following selection of hormone identifier, LC-MS conditions were optimized to determine the conditions that provided sharp and narrow peaks, and greatest resolution of the analytes. MS parameters from previous LC-MS/MS methods for quantitative analysis of peptide hormones (Rauh et al., 2007; Thomas et al., 2020) were selected initially. The effects of each parameter on

analyte resolution were individually assessed. All samples were analyzed in positive ionization mode.

While an increase in capillary voltage from 3500V to 4000V reduced peak height for pig and human osteocalcin, no other hormones were affected. Increase in nebulizer pressure from 30 psi to 40 psi resulted in decreased signal intensity for all insulins and GLP-1s. A decrease in nebulizer pressure below 30 psi had no impact on analyte chromatograms. Finally, a nozzle voltage of 500V was concluded as ideal for all compounds since a decrease to 0V greatly decreased signal intensity for osteocalcins, ghrelins, and insulins. An increase past 500V had no impact on analyte chromatograms. A comparison of initial versus final MS conditions is shown below (**Table 9**).

Temperature of the LC-MS was also found to greatly impact analyte signal. We observed degradation of insulin and ghrelin when running the autosampler at room temperature (data not shown), and thus subsequently, the autosampler temperature was maintained at 8 °C to limit degradation of hormones during the analysis. We found that hormones were stable for at least 24 hours when stored in the autosampler at 8 °C.

Column temperature was determined to have a significant impact on peak intensity and noise. An increase of column temperature from 35 °C to 50 °C resulted in higher peak intensity for all peptide hormones (**Figures 8A-8E**) and greater s/n ratio for both human and pig insulin (**Figure 8E**). Column temperature had no visible impact on peak shape and height for cortisol (**Supplementary Figure 1A**). Thus, a column temperature of 50 °C was maintained for the final method.

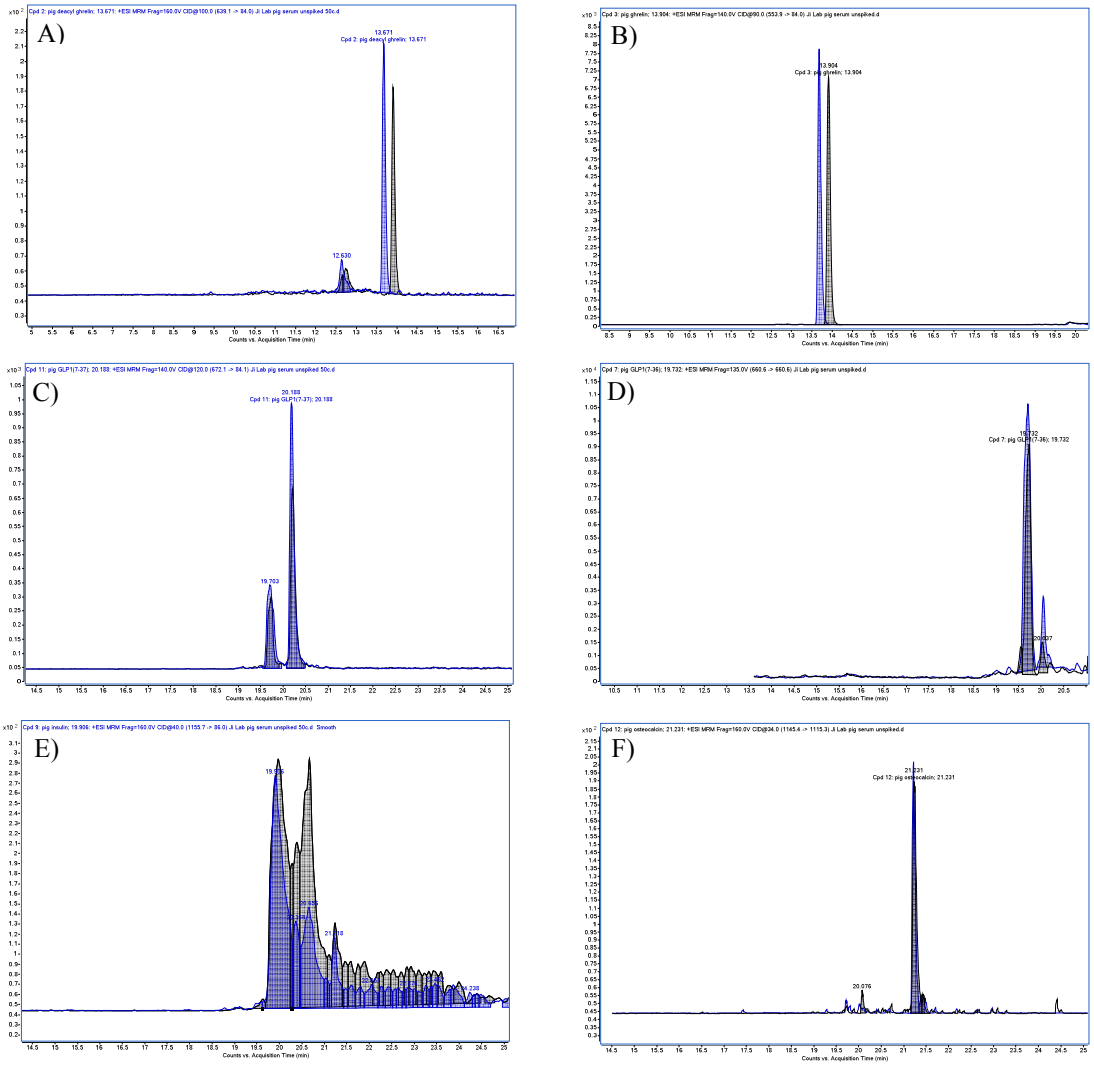


Figure 8. Analyte chromatograms before and after column temperature change.

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

Similarly, an increase in gas temp and sheath gas temperature resulted in greater signal intensity for all analytes (**Figures 9A-9E**). Cortisol peak shape was not impacted by alteration of MS conditions (**Supplementary Figure 1B**).

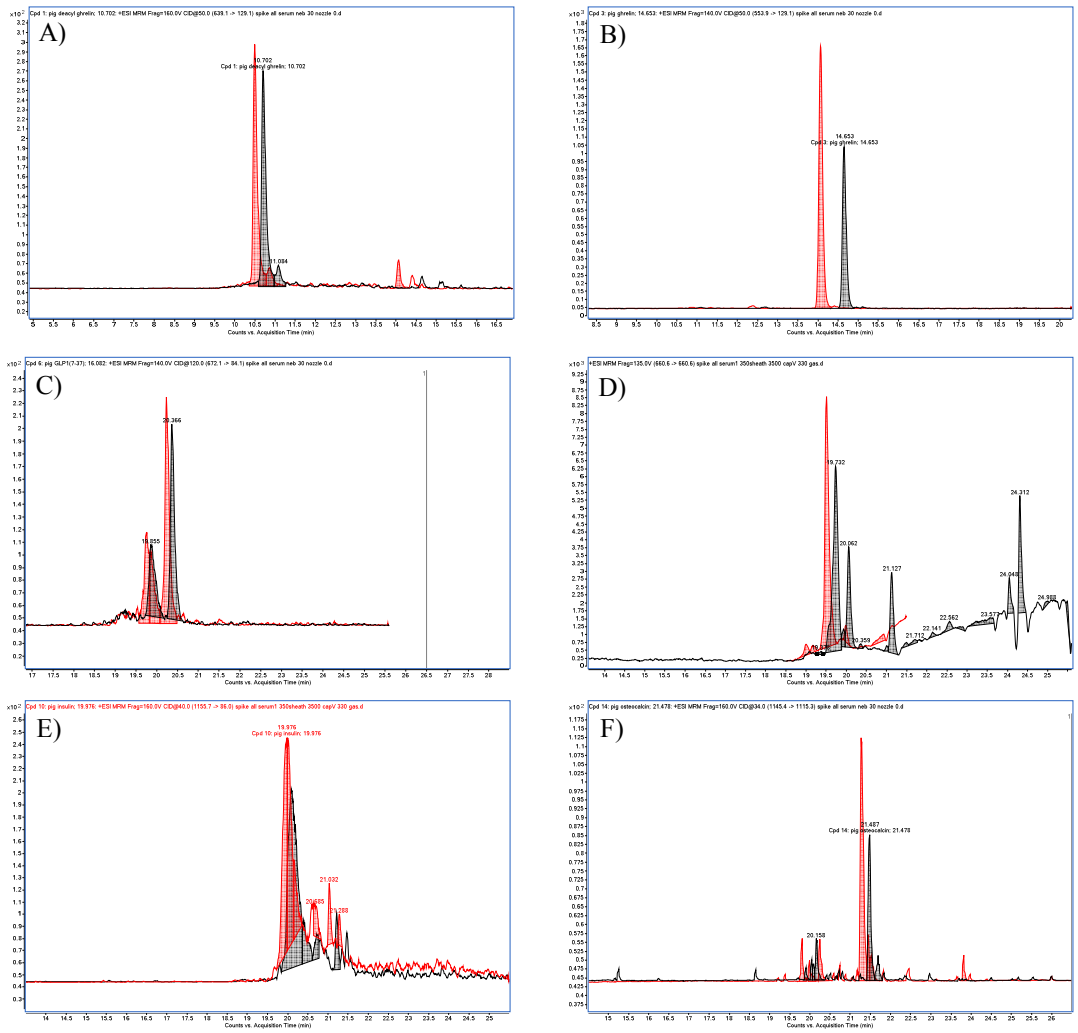


Figure 9. Analyte chromatograms before and after increase in sheath gas temperature and gas temperature.

Chromatograms for pig desacyl (A) and acyl ghrelin (B), GLP-1 (7-37) (C), GLP-1 (7-36) (D), insulin (E), and osteocalcin (F) from pig serum. The same aliquot of pig serum extract was run under conditions of sheath gas temperature of 300°C and gas temperature of 290°C (shown in black) versus sheath gas temperature of 350°C and gas temperature of 330°C (red). Comparison of peak height shows greater signal intensity with temperatures. GLP-1 (7-36) (D) demonstrated increased s/n in MS conditions with higher temperatures. The broad peak shape on insulin observed in **Figure 8E** was reduced with increase in gas temperature and sheath gas temperature (E).

Overall, modification of LC-MS parameters from default and from conditions used in previous publications significantly impacted analyte resolution. While nebulizer pressure, capillary voltage and nozzle voltage were ideal in initial conditions, increases in LC-MS temperatures resulted in greater signal intensity and sharper peaks. **Table 9** provides a comparison of initial and final MS conditions.

Table 9. Initial and final MS conditions

	Initial	Final
Gas Temp	290 °C	330 °C
Gas Flow	10 L/min	10 L/min
Nebulizer	30 psi	30 psi
Sheath Gas Temp	300 °C	350 °C
Sheath Gas Flow	11 L/min	11 L/min
Capillary	3500 V	3500 V
Nozzle Voltage	500 V	500 V
Chamber current	0.19 uA	0.19 uA

2.4.4 Optimization of Calibration Curve

The accuracy of a quantitative LC-MS/MS assay is heavily dependent on the calibration curve. The relationship between analyte concentration and detected response in a series of samples with known concentrations is used to estimate the concentration of unknown samples. Differences in analyte ionization between the calibration and sample matrix are accounted for by the internal standard. The range of the calibration curve must be representative of the expected quantitation range. As discussed in earlier sections, ionization of compounds is highly matrix-dependent and small changes in matrix can greatly impact signal intensity and background noise. Several experiments were conducted to find the most suitable calibration curve matrix. The goal was to identify a matrix that contained little to no analyte to achieve a low limit of quantitation and good accuracy. The retention time and background noise of analytes in the calibration curve should closely match the same parameters in the sample to be analyzed, and there must be a

strong positive correlation between analyte concentration and detected response. Non-pig serum matrices or heavily processed pig serum were deemed unsuitable for quantitation. A mixture of filtered pig serum and pig serum produced the best calibration curve (**Figure 12**).

2.4.4.1 Charcoal-stripped serum

Charcoal treatment of blood is used to deplete the matrix of lipids and lipophilic compounds. Dextran-coated charcoal can deplete steroid hormones while allowing for proteins and peptides to remain in solution (Sikora et al., 2016). The use of non-treated charcoal can lead to greater reductions in peptides and other small molecules (Dixit & Chang, 1984). Treatment of pig serum with activated carbon successfully depleted all hormones from pig serum as evidenced by lack of analyte signal detected in our MRM method. Although some GLP-1 (7-36) remained in solution, peak area was reduced by 100-fold. Generation of an eight-point calibration curve in charcoal stripped serum resulted in good linearity ($R^2 \geq 0.99$) for ghrelin, desacyl ghrelin, and cortisol (**Figures 10A-10C**). However, poor linearity was observed for the remaining analytes (**Figures 10D-10G**).

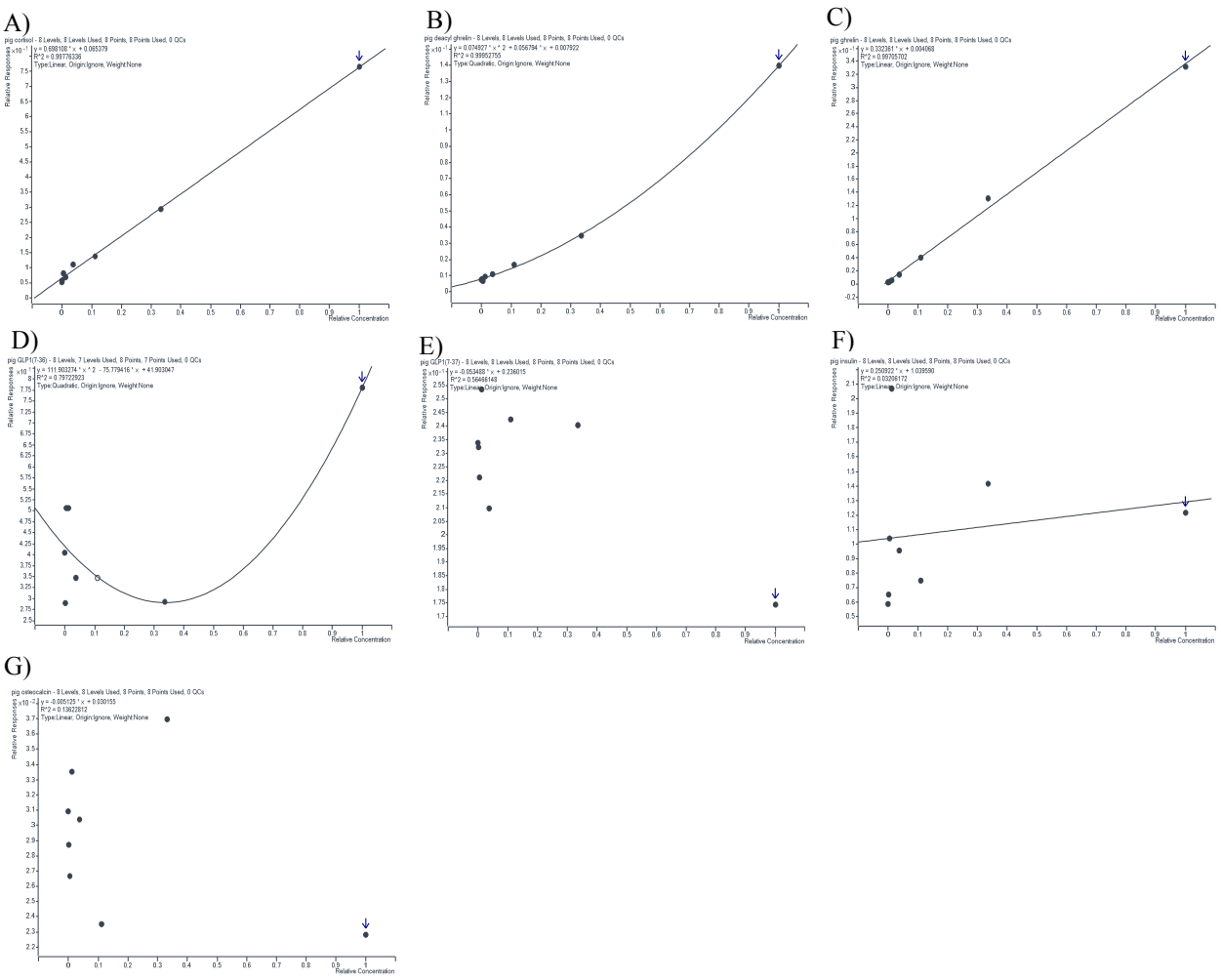


Figure 10. Charcoal-stripped serum matrix calibration lines for analytes.

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 cortisol (A), desacyl (B) and acyl ghrelin (C), GLP-1 (7-36) (D), GLP-1 (7-37) (E), insulin (F), and y-carboxy osteocalcin (G). Both GLP-1s, insulin, and osteocalcin demonstrated poor linearity in surrogate matrix.

2.4.4.2 Non-serum matrices

Previous methods have demonstrated the suitability of surrogate matrices for generation of LC-MS/MS calibration curves (Houghton et al., 2009; Howard, 2018). An ideal surrogate matrix is analyte-free and has a composition that aims to closely match the target matrix. We examined the effectiveness of five non-serum matrices. A surrogate matrix containing 20% MeOH, 80% H_2O, 1% BSA, and 0.2% acetic acid resulted in poor calibration line linearity for insulin, osteocalcin, GLP-1 (7-36), and GLP-1 (7-37). Next, we explored the addition of 2.5% glycerin to improve suspension of analyte in the surrogate matrix. The addition of glycerin did not increase surrogate matrix effectiveness.

An alternate surrogate matrix of 2% BSA and 0.2% acetic acid in PBS was additionally examined. The PBS matrix also resulted in resulted in poor calibration line linearity for insulin, osteocalcin, GLP-1 (7-36), and GLP-1 (7-37). Finally, a calibration curve produced in protein precipitation solvent resulted in poor linearity for all hormones except cortisol.

2.4.4.3 Chicken serum

Alternate species sera can provide a matrix that matches the complexity of target matrix without interference from endogenous analyte. Chicken serum represents a promising surrogate matrix for pig serum since avian GLP-1 is unique from the GLP-1 conserved across mammals. The calibration curve generated in chicken serum resulted in poor linearity for insulin, osteocalcin, GLP-1 (7-36), and GLP-1 (7-37) (**Figures 11A-11G**). Elevated background noise was observed for all hormones – except cortisol – in chicken serum compared to pig serum.

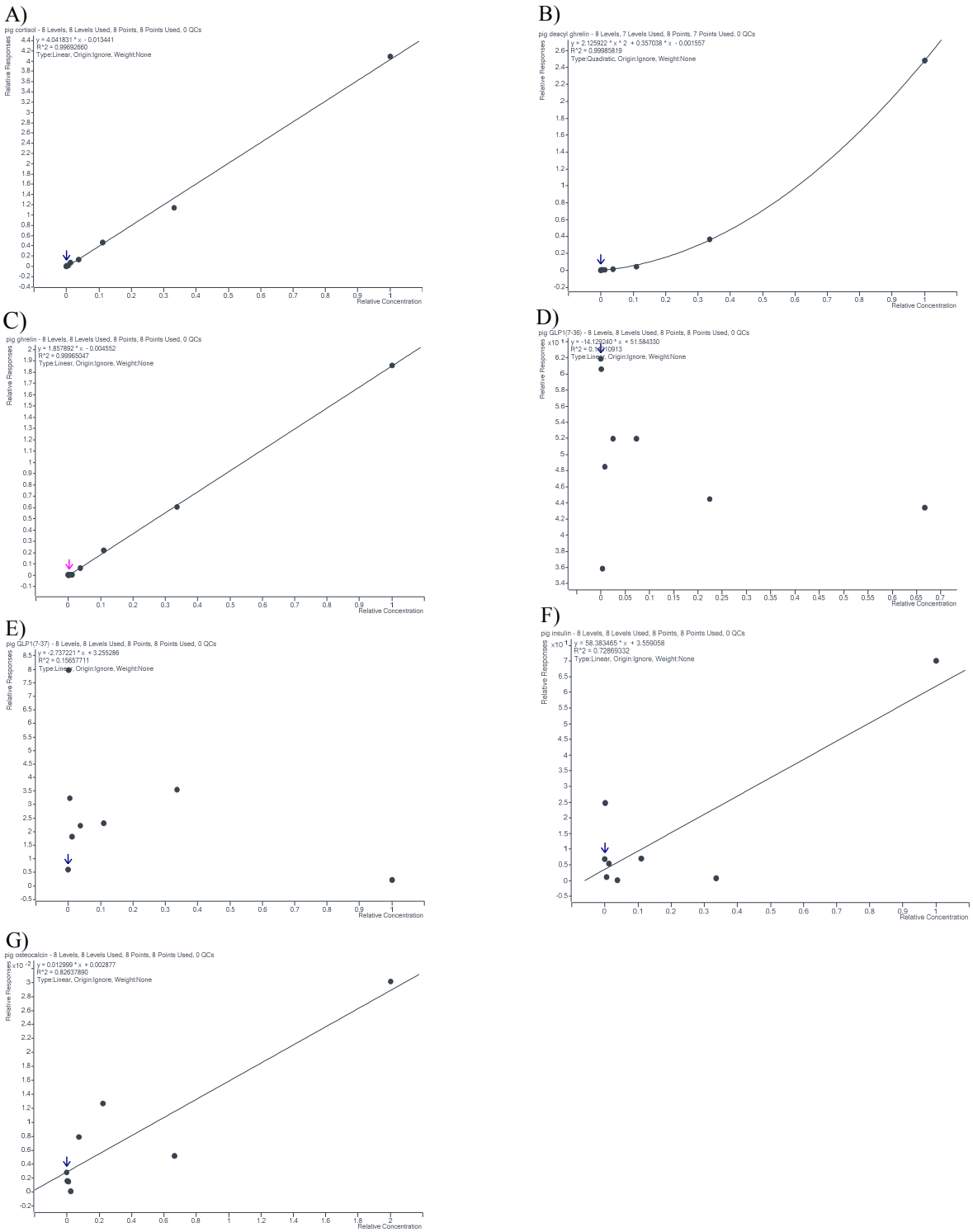


Figure 11. Chicken serum matrix calibration lines for analytes.

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 cortisol (A), desacyl (B) and acyl ghrelin (C), GLP-1 (7-36) (D), GLP-1 (7-37) (E), insulin (F), and y-carboxy osteocalcin (G). Both GLP-1s, insulin, and osteocalcin demonstrated poor linearity in chicken serum.

2.4.4.4 Piglet serum composite

During preliminary trials, it was observed that filtration of pig serum with a 30 kDa centrifugal filter resulted in near complete loss of endogenous and spiked analyte. The loss can be attributed to non-specific binding of analyte to the filter and centrifugal tube. However, filtration also resulted in loss of necessary matrix components. Thus, we examined the effectiveness of a combination of 40% filtered and 60% unfiltered serum composite as calibration curve matrix. The addition of 40% filtered serum reduced the endogenous analyte signal. A calibration curve produced in this matrix demonstrated excellent linearity ($R^2 \geq 0.98$) for cortisol, both ghrelins, osteocalcin and insulin. GLP-1 (7-36) and (7-37) had linear correlations of 0.91 and 0.94, respectively (**Figure 12A-12G**). It was decided to proceed with a matrix of 40% filtered and 60% unfiltered pig serum as a calibration curve matrix.

2.5 Results from Method Validation

The final method, with the optimized extraction method, LC-MS parameters, and calibration curve was then subjected to validation. The goal of method validation was to calculate the method parameters to assess whether our method met the threshold for precise and accurate bioanalytical quantification. To increase robustness, we compared our method to an established method for hormone quantification. Validation revealed suitability of the method for highly specific, accurate, and precise quantitation of pig ghrelin, desacyl ghrelin, cortisol, insulin, carboxylated osteocalcin, GLP-1 (7-36), and GLP-1 (7-37).

2.5.1 Linearity and Range

A positive linear or quadratic correlation was observed for all analytes (**Figure 12**). Although eight calibrants were prepared, all eight were not suitable for every analyte of interest. Poor

linearity was found when low concentration calibrants were plotted for insulin, osteocalcin, and GLP-1 (7-36). Thus, these low-level points were not included in construction of the calibration curve. The suitable quantitation range (**Table 10**) was thus defined as range with correlation $\geq$ 0.90 and s/n > 5 for each calibrant. Cortisol and ghrelin both demonstrated a strong ($R^2 \geq 0.99$) linear correlation while desacyl ghrelin had a strong quadratic correlation of 0.99. Insulin and osteocalcin had quadratic correlation $\geq$ 0.97 while GLP-1 (7-37) was also quadratic with $R^2 \geq$ 0.94. GLP-1 (7-36). GLP-1 (7-36) had a quadratic correlation of 0.94.

Table 10. Range of quantitation for all analytes.

Analyte	Quantitation range
Pig insulin	20 – 625 pg/mL
Pig desacyl ghrelin	20 – 625 ng/mL
Pig ghrelin	20 – 625 ng/mL
Pig y-carboxy osteocalcin	2.31 – 62.5 ng/mL
GLP-1 (7-36)	3.3 – 625 pg/mL
GLP-1 (7-37)	7.7 – 625 pg/mL
Cortisol	0.77 – 62.5 ng/mL

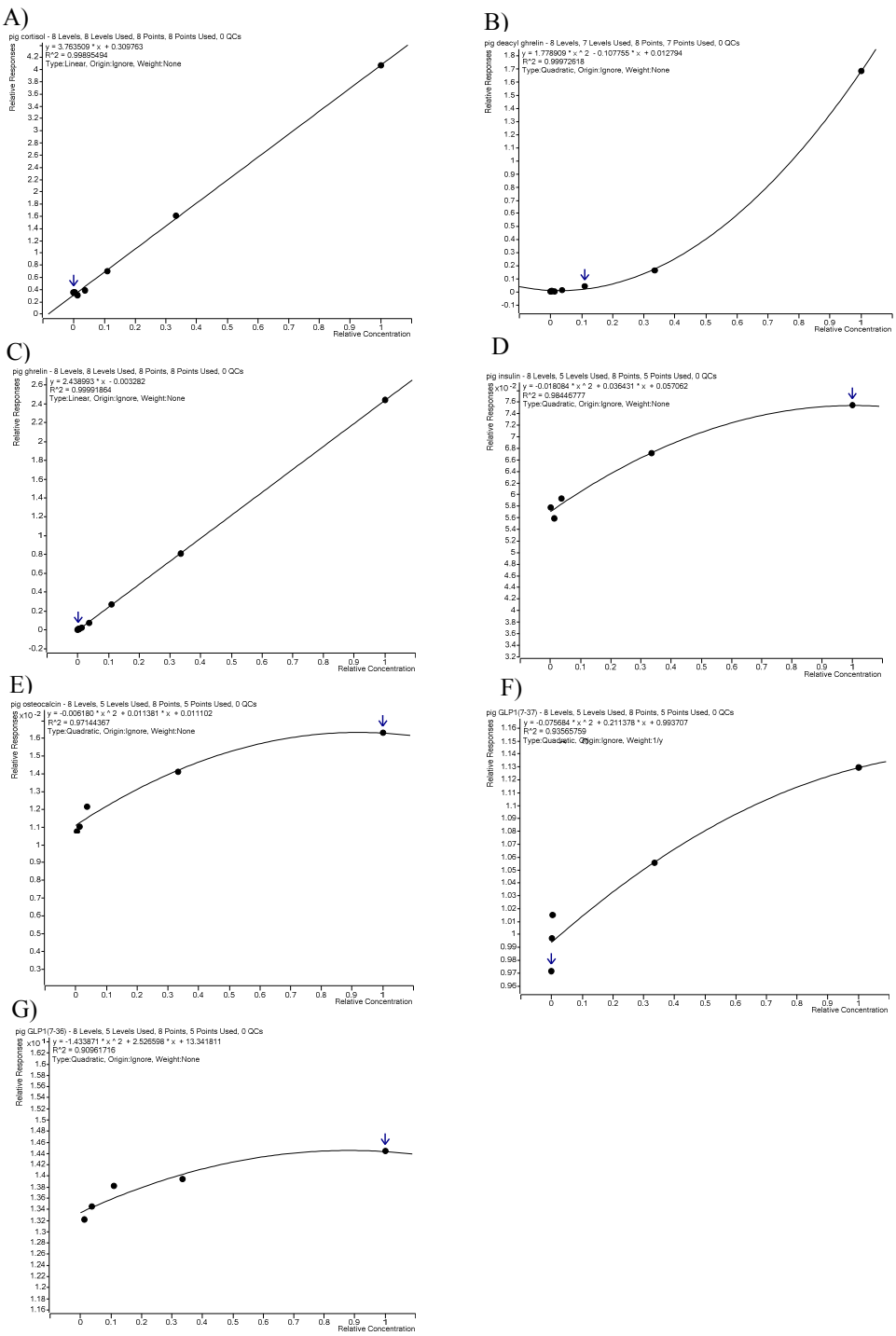


Figure 12. Composite piglet serum matrix calibration lines for analytes.

Eight 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 cortisol (A), desacyl (B) and acyl ghrelin (C), insulin (D), osteocalcin (E), GLP-1 (7-37) (F), and GLP-1 (7-36) (G). All analytes demonstrated linear or quadratic $R^2 \geq 0.90$.

2.5.2 Precision

All analytes demonstrated an intra-assay variability of ≤ 10% when analyzed in triplicate (**Table 11**). Inter-assay precision, determined by comparison of n=6 samples across two days, demonstrated variability of ≤ 15% for both ghrelins, GLP-1s, osteocalcin, and cortisol. Inter-day precision for insulin was 23%. Since inter-day precision for insulin is above the 20% threshold generally accepted for bioanalytical methods, assay date should be factored into analysis when running samples over multiple days.

Table 11. Inter-day and intraday precision data.

	Intra-assay (n=3)	Inter-assay (n=6)
Analyte	%CV	%CV
Pig insulin	10.1	23.1
Pig desacyl ghrelin	1.0	15.0
Pig acyl ghrelin	7.2	7.2
Pig y-carboxy osteocalcin	5.0	13.0
GLP-1 (7-37)	10.4	9.1
GLP-1 (7-36)	4.6	8.3
Cortisol	5.2	11.0

2.5.3 Recovery

Recovery of external (**Table 12**) and internal standards (**Table 13**) largely fell within the range of 80-120%. Recovery of pig insulin and Me-GLP-1 (7-37) were 124% and 123%, respectively, suggesting matrix enhancement. Matrix enhancement occurs when matrix components increase signal of an analyte. Matrix enhancement is a matrix effect that can affect precision and accuracy

of bioanalytical methods, and as such it is important to ensure recovery remains within the 80-120% threshold.

Table 12. Recovery of external standards.

Analyte	%Recovery (n=3)
Pig insulin	124
Pig desacyl ghrelin	88
Pig acyl ghrelin	93
Pig y-carboxy osteocalcin	104
GLP-1 (7-37)	101
GLP-1 (7-36)	87
Cortisol	91

Table 13. Recovery of internal standards.

Analyte	%Recovery (n=3)
Human insulin	105
Human desacyl ghrelin	88
Human acyl ghrelin	93
Human y-carboxy osteocalcin	104
Me-GLP-1 (7-37)	123
Me-GLP-1 (7-36)	116
d4-Cortisol	91

2.5.4 Specificity and selectivity

Specificity of our MRM method was confirmed by the absence of signal in blank samples. No peaks were observed in analyte-free matrix or solvent. Signals for internal standards were absent in pig serum.

2.5.5 Limit of quantitation (LOQ)

An acceptable sensitivity can be defined as the lowest concentration that is able to be distinguished from zero with precision $\leq$ 20%. The LC-MS method demonstrated excellent sensitivity (**Table 14**) with LOQ well below the expected hormone ranges in pigs and piglets. Signal-to-noise was greater than five for all calibrants at the LOQ.

Table 14. LOQ

Analyte	LOQ
Cortisol	0.77 ng/mL
Pig insulin	20 pg/mL
Pig desacyl ghrelin	20 ng/mL
Pig acyl ghrelin	20 ng/mL
Pig y-carboxy osteocalcin	2.31 ng/mL
GLP-1 (7-37)	7.7 pg/mL
GLP-1 (7-36)	3.3 pg/mL

2.5.6 Method comparison

Due to the lack of commercial ELISA kits for pig hormones, method comparison was limited to insulin and cortisol. Insulin (**Figure 13**) and cortisol (**Figure 14**) concentration yields between LC-MS/MS and ELISA were compared to gauge method robustness and accuracy. A linear

relation was observed for the differentially measured concentrations for both insulin and cortisol, suggesting both methods are suitable for quantitation. For both hormones, the LC-MS/MS method had greater accuracy in estimating concentration of QC low, medium, and high concentration samples.

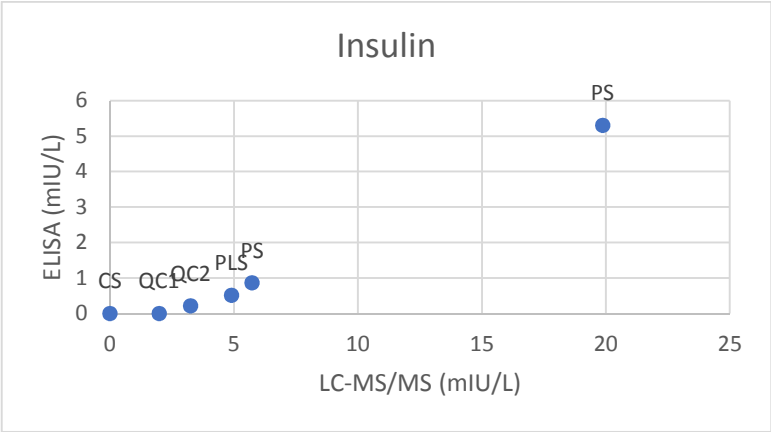


Figure 13. Graph of insulin concentrations from ELISA vs. LC-MS/MS

Insulin quantification of six samples: QC low (QC1), QC medium (QC2), QC high (QC3), piglet serum (PLS), commercial pig serum (PG), and chicken serum (CS). Quantification was performed using ELISA and LC-MS/MS. All samples were run in duplicate. LC-MS quantification for insulin is plotted on the x-axis and values from ELISA on the y-axis. QC samples are in visualized by red while serum samples are in blue.

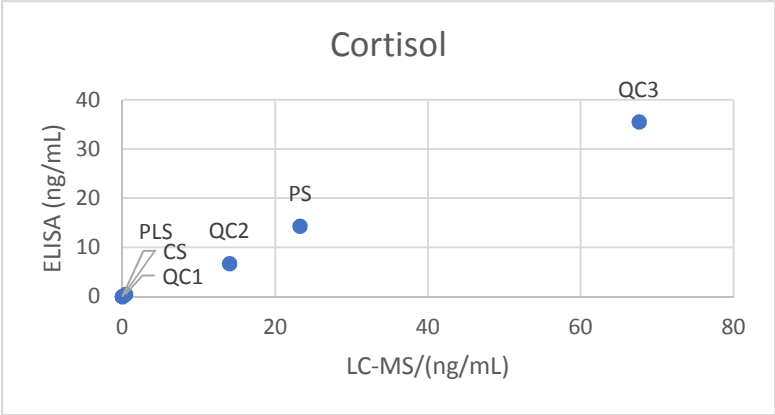


Figure 14. Graph of cortisol concentrations from ELISA vs. LC-MS/MS

Cortisol quantification of six samples: QC low (QC1), QC medium (QC2), QC high (QC3), piglet serum (PLS), commercial pig serum (PG), and chicken serum (CS). Quantification was performed using ELISA and LC-MS/MS. All samples were run in duplicate. LC-MS quantification for cortisol is plotted on the x-axis and values from ELISA on the y-axis. QC samples are in visualized by red while serum samples are in blue.

2.6 Conclusions

The work in this thesis presents a novel method for LC-MS/MS quantification of endogenous insulin, cortisol, GLP-1 (7-37), GLP-1 (7-36), acyl and desacyl ghrelin, and carboxylated osteocalcin from pig serum. Our method allows for simultaneous detection of all seven hormones in a single assay, has a shorter sample preparation period than traditional immunoassays, and has good precision. The detection range validated for each hormone is relevant to the expected endogenous levels found in both adult and infant pigs. The LOQ for many of the analytes is considerably lower than the typical sensitivity of ELISA. For example, our assay allows for quantification of GLP-1 in the picogram range whereas commercial ELISAs have LOQs in the nanogram range.

Comparison of insulin and cortisol concentrations yielded by our method versus ELISA demonstrated a discrepancy between the two methods. The hormone concentrations of QC samples estimated by LC-MS/MS better matched the expected concentrations for both insulin and cortisol. This is likely because the LC-MS/MS assay was optimized for detection of hormones in the QC matrix and does not suggest that ELISA kits are unreliable. Our method demonstrated greater sensitivity in the measurement of insulin. Since our method was optimized for detection of hormones in an acidified serum sample, the enhanced sensitivity can be partially attributed to matrix effects in the ELISA. The use of internal standards and matrix-matched calibration curves limits matrix effects in our LC-MS/MS method. In addition, incubation of serum with ACN during sample extraction prior to LC-MS/MS results in the release of albumin-bound hormones. As such, our method provides an estimate of total hormone concentrations while ELISA delivers the concentration of free hormones. Despite the differences, the ranking of

concentrations in both methods was identical resulting in a linear relationship between concentrations obtained from both methods.

Although the performance of our method meets the generally accepted criteria for bioanalytical quantitation, additional testing will be required to meet agency-specific requirements. Currently, the R^2 for GLP-1 (7-36) and GLP-1 (7-37) are 0.90 and 0.94, respectively, which is not ideal for accurate quantification of these analytes. Solid phase extraction should be explored to further improve the linearity and accuracy of the method and increase sensitivity. Future studies should additionally examine the effects of different serum storage conditions on method precision and accuracy.

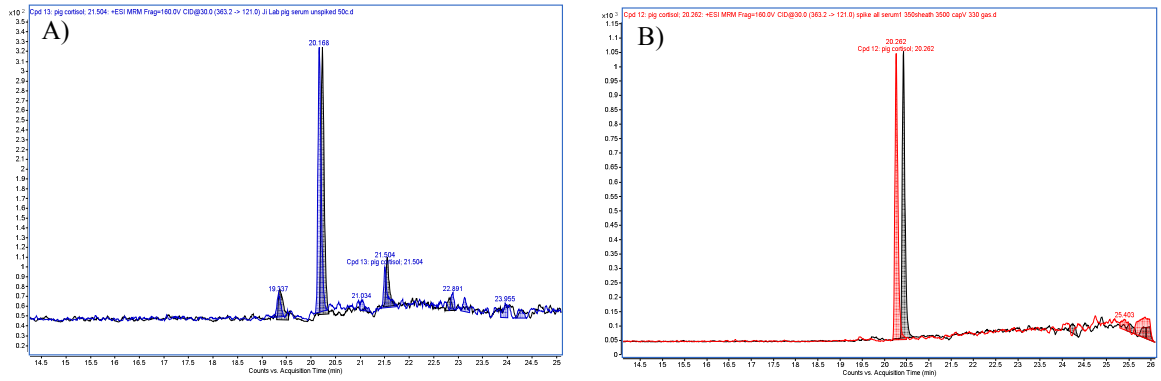
Appendix

Supplementary Table 1. Expanded parameters for external standard ions monitored by MRM.

	Precursor ion	Product ion	Retention time (min)	Declustering potential (V)	Collison Energy	Cell Accelerator (V)
Cortisol	363.2	121.0	20.1	160	30	4
Pig deacyl 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	1152.4	20.2	160	30	4

Supplementary Table 2. Expanded parameters for internal standard ions monitored by MRM.

	Precursor ion	Product ion	Retention time (min)	Declustering potential (V)	Collison Energy	Cell Accelerator (V)
d4-Cortisol	367.2	121.0	20.1	140	26	4
Human deacyl 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



Supplementary Figure 1. Cortisol chromatograms before and after LC-MS optimization

Chromatograms for cortisol before and after column temperature change from 35°C to 55°C (A) and before and after increase of gas temperature and sheath gas temperature (B). The peaks in grey indicate cortisol signa prior to optimization. Optimization of LC-MS temperatures did not visibly impact cortisol peak shape and height.

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