Alternate methods to detect and quantify urocanic acid in domesticated pig (*Sus domesticus*) blood serum

Investigating UCA detection by HPLC analysis

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Abstract
The project aims to explore alternate methods to detect and quantify cis- and trans- Urocanic Acid (UCA), a chemical associated mechanisms of seasonal reproductive control. The UROC1 gene, known to be present in canine and ovine species, codes for urocanate, which acts on trans- UCA. This is suspected to influence breeding patterns. The project aims to determine if UCA, in either or both of its isomers, is identifiable in domesticated pig (Sus domesticus) blood serum. We hypothesise that remnants of seasonality will be indicated by differing isoform ratios between periods of optimal and suboptimal fertility. This may provide insight into the functional effects of the proposed locus and porcine seasonal subfertility. If present, high performance liquid chromatography (HPLC) will enable quantification of cis- and trans-UCA (mAU) in serum samples and will be presented through chromatograms, box plots and tables. We were successful in the development of a selective HPLC method of cis- and trans- UCA isomers in pig blood serum. However, the extraction processes used for the isolation of these isomers were unsuccessful and require further research. The implications experimental results will enable the identification of UCA in future studies.

Keywords
Animal Reproduction, genetics, pig

Introduction

Global Food Security and Porcine Subfertility

The global population is estimated to reach 9.7 billion by 2050 thus making it crucial to develop strategies to improve sustainability and capability within the swine industry to better ensure food security [1]. With population pressure and a growing middle class demanding meat source protein, it is essential to gain a more robust understanding of genetic factors contributing to seasonal subfertility [2,3].

Increased reliability in production volume could be achieved through maximising genetic yield from stock [4]. Various factors including ovarian production, sperm quality and heat stress have been investigated in relation to domestic pig (*Sus* domesticus) seasonal subfertility, but this lapse remains unexplained [5]. Consequences of seasonal subfertility include reduced fecundity and fertility manifesting as difficulty coming into oestrus, higher anoestrus rates in sows and gilts, higher rates of early pregnancy failure, and lower litter sizes [6].Practically, this subfertility restricts the ability to provide consistent supply to market and meet consumer demand [7]. If the genetic factors limiting reproductive efficiency were better understood, this may facilitate the development of technologies or management practices to decrease genetic wastage and improve production consistency to better meet demand [8].

Seasonal breeding limits reproductive output and is affected by global latitude and genes influencing melatonin synthesis and catabolism [9]. However, evidence suggests this melatonin pathway does not exclusively control seasonality [10]. Canids exhibit varied breeding patterns where certain breeds are strictly seasonal breeders whilst others are not [11]. Ongoing research exploring reproductive seasonality in canids has utilised Urocanic acid (UCA) quantification. Results suggest the UCA pathway effects reproduction rate, illustrated by a strong association mapping signal.

Preliminary Investigations into the Histidine Deaminase Pathway and UCA

Unpublished research by the Claire Wade group at The University of Sydney explored the genome of domesticated and wild canids through performing a genome wide association study (GAWS). Whilst, wild groups are strictly seasonal breeders, domesticated breeds, with the exception of the Tibetan Mastiff, are non-seasonal breeders [11]. It is the high degree of relatedness between these two groups of canids that makes them an ideal model to investigate genetic mechanisms of seasonal breeding. GAWS results illustrated segregation between wild and domestic and domesticated candids at a locus containing 10 genes, where 2 genes were highly associated with reproductive physiological pathways. Of these 2 genes identified, urocanase and plexin A1, the urocanase gene was strongly segregated between the groups with domesticated canids possessing the mutant allele.

Urocanase converts trans-UCA to isoglutamine, which is then subject to multiple changes via the histamine-deaminase pathway [12]. UCA exists in two isomers, trans-UCA within the epidermis, and cis-UCA. Following ultraviolet B exposure, trans-UCA isomerises to cis-UCA. Trans-UCA has previously been identified in various tissues including the brain, skin and liver [13, 14].UCA exists in the epidermis and research has research has reflected immunosuppressive and photoreceptive properties in human and murine models [15].

Further unpublished research by the Claire Wade group investigated UCA concentrations in merino sheep over a 6-month period and provides evidence of this histidine deaminase pathway in production animals. In this study, increased trans-UCA concentrations in blood serum were correlated with a decreased percentage of cycling ewes.

Due to the role of urocanase and UCA in the histidine deaminase pathway and the GAWS results, potential exists in extending this research to other animal species to explore genetic reproductive biochemical pathways which exist that may influence fertility [16]. It is hypothesised that the observed association in canid and ovine models could be detectable in swine, where an observable quantitative change in UCA concentration in over 12-months may explain subfertility as residual seasonality.

Methods of UCA Detection

Various studies have investigated UCA detection and utilised various methods including enzyme-linked immunosorbent assays (ELISAs), mass spectography and high-performance liquid chromatography (HPLC) [17].

HPLC methods were selected for this study as it is the most readily accessible. HPLC separates analytes from liquid samples via the column, analytes are identified against known standards and hence, if present, enable quantification and observe relative concentration changes of UCA isomers [18]. Chromatography is based on mobile and stationary phases. When a solution is inserted for analysis, impurities present in solution have a greater attraction to the mobile phase and hence removes impurities from solution [19]. Differences in hydrophobicity, the principle of hydrophobicity- based chromatography, enables protein separation without inhibiting biological activity [20]. The absorption spectrum of the resulting solution is measured, analysed to detect and quantify concentrations of the target substrate, and illustrated in a chromatogram. Cis- and tran-UCA have been identified in animal blood serum using these principles in both published and unpublished research [18].

Pham *et al.* (2017) utilised ultra-performance liquid chromatography (UPLC) to detect and quantify UCA in human skin samples collected via sticky-tape impression methods [21]. UPLC utilises the same principles as HPLC but is often used to detect particle sizes less than 2µm in diameter to achieve improved speed, resolution, and sensitivity. This method utilises potassium hydroxide (KOH) to deprotonate samples and hydrochloric acid (HCl) to protonate samples prior to UPLC analysis. Both the addition of KOH and HCl cause UCA to become an ion in solution, hence making it more soluble in water. Hence, impurities and non-target molecules will precipitate out of solution and the remaining supernatant will be purer in preparation for HLC analysis [21].

Solid-phase extraction is the most common method utilised for the extraction, concentration and fractionation of organic compounds, especially in methods utilising analysis of liquid solutes, to facilitate more accurate detection of target molecules [22]. Solid-phase extraction methods are precise, low cost and highly selective, hence are appropriate for sample preparation [23]. The principle behind utilising solid- phase extraction methods for this study is to increase the relative concentration of UCA in samples prior to HPLC analysis. This method removes non-target molecules by dissolving them in methanol, which is then left to evaporate from the sample. Theoretically, solid phase extraction methods dissolve impurities and non-target molecules into methanol, which then is left to evaporate leaving only UCA. Therefore, the relative concentration of UCA should increase, improving the ability to detect UCA by HPLC analysis. Solid-phase extraction methods are often automated, utilising a variety of common cartridge types (e.g ion- exchange, reverse- phase, normal-phase) and mobile phases (e.g. methanol, water, buffer e.g. phosphate, KOH, HCl and organic solvents e.g acetonitrile) [24].

Aims, Hypotheses and Potential Research Applications

This research **aims** to develop an selective HPLC protocol to detect and quantify both cis- and trans-UCA in porcine (*Sus domesticus*) blood serum. We **hypothesise** that cis- and trans- UCA will be detectable in pig blood serum utilising HPLC analysis given the previous success that has reported by Zare *et al.* (2012) and Barresi *et al.* (2011) detecting UCA by these methods.

This research may provide insight into the functional effects of UROC1 activity, the effects of UCA and the histidine deaminase pathway contribute to better understanding porcine seasonal subfertility [18]. Acquiring a more robust understanding of seasonal reproductive control mechanisms and effects in food species may facilitate artificial manipulation of reproductive rates; for example, through artificial lighting schemes, seasonally tailored nutrition or CRISPR genome editing [25, 26]. Improving production output via genetic yield from farrowing periods without the need for additional animals or environmental resources, such as land and grain, would be highly impactful as global population increases [27].

Materials and Methods

Ethics Statement

The work in this study was approved by The University of Sydney Animal Ethics Committee under approval number 2021/2005.

Sample Collection

Venous blood samples were obtained from 25 sows located at May Farm piggery monthly for 12-months.

Pigs were restrained with a rope snare over the maxilla, behind the canine teeth (Figure 1) that was tethered to a fix point (Figure 2). Constant tension was placed on the snare until bleeding procedures (Figure 3) were completed and then the pigs were released.

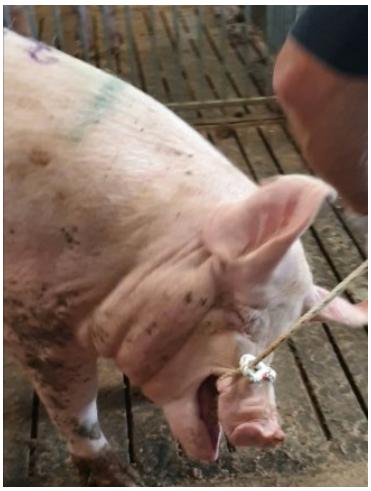

Figure 1: Correct snare placement

Figure 2: Correct tethering position

Following the snaring procedures, blood was collected via the jugular vein using a 1.5 inch, 18G needle, vacutainer holder and serum vacutainers (Figure 3) [28]. Tubes were labelled using a simple numerical system citing pig tag numbers, collection time and date.

Figure 3: Correct positioning of pig and operator for jugular vein blood collection

Vacutainer tubes were immediately covered with aluminium foil and placed in a covered ice box. Samples were then centrifuged (Sigma 4k15 Centrifuge) at 800g for 15 minutes at 21ºC. Blood serum (the supernatant) was then extracted using glass disposable Pasteur pipettes into labelled 5mL serum storage tubes which were then wrapped in aluminium foil and placed back into the covered ice box for storage in a -20ºC freezer until preparation.

Purchase of Materials for Subsequent Stages

Refer to Table 1.

Table 1: Source locations for materials utilised for sample preparation and HPLC analysis.

Material	**Source Location**
Filters	Merck Millipore
Syringes	Terumo Australia
HPLC Guard Filters	Security Guard
Chemicals (HCl, cis- and trans-UCA, sodium citrate, citric acid, KOH, acetonitrile, methanol)	Thermo Fisher Scientific
HPLC Column; Solid-Phase Extraction Cartridges	Agilent

Sample Preparation I

Standard Preparation

Standards were prepared for cis- and trans-UCA. Both were separately dissolved in a 1:1 ratio solution with 0.05M HCl (0.001%w/v (g/mL)). Tubes containing 50mL of this concentration were placed into a 55°C oven for 20 minutes to ensure each UCA was entirely dissolved. The respective solutions were then filtered through 0.22µl membrane syringe filters into 10mL tubes. Three aliquots were prepared following dilution of the original concentration with 0.05M HCl resulting in concentrations of 0.01mg/mL, 0.001mg/mL, and 0.0001mg/mL in addition to the original 1mg/mL. The baseline was adjusted so it was 0 (+/-0.5) mAU from 13-15 minutes as this was the most stable period.

Sample Preparation

Methods were adapted from Zare *et al.* (2012) [18].

To inactivate enzymatic activity, serum samples were originally combined with 0.05M HCl at a 1:1 ratio, placed in an 80°C water bath for 10 minutes and centrifuged (Sigma 4k15 Centrifuge, Germany) at 15,700g for 10 minutes.

Of the resulting supernatant, 0.5mL was pipetted into 1.5mL Eppendorf tubes and 300µl of 10%(w/v) sodium citrate solution (+/- 1%) was added and vortexed. For each mL of supernatant, 0.5 mL of 10%(w/v) citric acid (+/- 1%) was added to the supernatant-sodium citrate solution, and vortexed again. This solution was left to sit at room temperature (25°C) for 15 minutes and then centrifuged again. Remaining supernatant was filtered through a 0.2µm nylon membrane filter and prepared for HPLC analysis.

HPLC Detection of UCA in Standards and Samples

An Agilent Technologies 1200 infinity series HPLC unit comprising of a G1379B degasser, G1212A binary pump, G1315D diode-array detector, a variable wavelength detector, autosampler and injector and a stainless-steel Reverse Phase ProteCol HPLC column (C18Gx5µm particle size x 120 Å pore size, 250mmx4.6mm ID) was utilised to detect cis- and trans-UCA in the standards and samples [29]. Agilent Chemstation online was used to initiate and control this process. The mobile phase comprised of 25mM citrate buffer (pH 3.2) and acetonitrile in an 88:12 ratio, with the addition of 3mM sodium octane sulphonate. Column temperature was maintained at room temperature (25°C), isocratic elution with a flow rate of 1.0 mL/min, over 30 minutes was

utilised with detection of cis- and trans-UCA was at a wavelength of 267nm. Data was collected, analysed, and graphed using Agilent Chemstation offline (Agilent Australia, 2022).

Sample Preparation II

New standard samples of both cis-UCA were prepared for spiking samples and HPLC calibration at concentrations of 0.1mg/mL, 0.01mg/mL and 0.001mg/mL. All standards to be used for HPLC calibration were filtered according to the method described in Stage 1.

The standards were prepared using the following method. 2mg of cis-UCA and 2mg of trans-UCA was added to separate tubes containing 2mL of 0.05M HCl. The cis- and trans-UCA solutions were then heated in an oven at 55°C for 10 minutes, removed and then agitated until the UCA was completely dissolved.

New standard samples were prepared each day that HPLC analysis was performed.

Blood Serum Sample Preparation

An alternate sample was prepared using 0.1M HCl. Within a system check, the water bath temperature was found to be below the required temperature (70°C). Using an electric thermometer, the thermostat on the bath was adjusted until a temperature of 80°C was maintained for 10 minutes. For all subsequent preparations, an electric thermometer was used to verify the water temperature was at 80°C prior to samples being placed into the bath.

To ensure integrity of the chemicals, new sodium citrate and citric acid solutions were purchased and used to make new solutions. New citric acid buffer was made by adding 9.40mg of sodium citrate, 3.62mg of citric acid and 0.65g of sodium octane sulfonate to 700mL of MilliQ water. MilliQ water was then added until a volume of 880mL was achieved. To this solution, 220mL of acetonitrile was added before use.

HPLC Detection of UCA in Standards and Samples

The absorbance was set at 280nm, 215nm and 267nm. Whilst 267nm is specific to the detection of cis- and trans-UCA, 280nm and 215nm is to detect salts and other impurities within the column which might accumulate.

For each run, 50µL of sample was injected to ensure the entire length of the 20µL loop was full of sample, with excess sample ejected before commencing the run. On the 6/5/22, the following samples were run with the above conditions: anonymous cis and trans- UCA standards, Sample 217, Sample 276, Sample 224, Sample 206, Sample 266, Sample 272. Performing HPLC analysis on the anonymous cis- and trans- UCA was done to ensure the retention time was consistent with previous runs.

Following review of these sample results and identification of artifacts suspected to be the result of calculus accumulation within the guard column hence was replaced before next runs. This was done to reduce any possible interference with HPLC analysis.

Sample Preparation III: Spiking Blood Serum Samples

Skin sample preparation methods were adapted from Pham *et al.* (2017) [21]. To 0.45mL of thawed serum sample, 0.45mL of 0.1M KOH was added and then shaken on an automatic shaker for 2 hours on high speed. Subsequently, 0.15mL of 0.38M HCl was added and shaken again for 2 hours on high speed. A precipitate formed and the solution was then filtered through a 0.22µl membrane syringe filters and frozen until HPLC analysis.

To ensure that the HPLC set up was correct and that buffers and standards were adequate, samples were analysed with the addition of a spiked standards. Serum sample 288 was prepared according to the above methods with the addition of 0.25mL of 0.001mg/mL trans-UCA and CIS- UCA standards, respectively.

HPLC configuration and analysis was performed per methods outlined in Stage 2 methods.

Sample Preparation IV: De-protonation Prior to Normal Processing

Under a fume hood, 1000µL of 99% acetonitrile was added to 500µL of serum sample 217. Upon addition of the acetonitrile, a white precipitate formed, and the suspension was agitated on an orbital shaker for 2 hours. After allowing the solution to rest for 10 minutes, the samples were centrifuged at 16, 100g for 10 minutes. The resulting supernatant was removed and frozen for later processing and labelled '217 + Ace'.

Sample and HPLC configuration and analysis was performed per methods in Stage 2 methods.

Sample Preparation V: Solid-Phase Extraction

The solid-phase extraction cartridge and 2mL collection tube were placed into the slot below the extraction cartridge insertion point. All insertion points were tightened and the air vacuum was pressure tested. Into the solid phase-extraction cartridge, 1mL of pure methanol was placed. The air vacuum was turned on to a pressure of 20Pa and turned off once all of the MilliQ water had passed through the cartridge. The 2mL collection tube was emptied.

0.5mL of processed and filtered blood plasma was pipetted into the solid-phase extraction cartridge. The air vacuum was turned on to a pressure of 20Pa and turned off once all of the entire processed and filtered blood plasma sample had passed through the cartridge. The 2mL collection tube was replaced with a clean, labelled and foil wrapped 2mL tube.

0.5mL of pure methanol was pipetted into the solid-phase extraction cartridge. The air vacuum was turned on to a pressure of 20Pa and turned off once all of the methanol had passed through the cartridge.

All equipment was turned off, the solid-phase extraction cartridge was disposed of and all samples were collected. The methanol was evaporated from the samples by leaving the foil wrapped tubes open for three days. After the three days the samples were gently heated to ensure complete evaporation of methanol. Samples were then eluted with a 3:5 solution of sodium citrate to citric acid.

Results

Sample Preparation I
Analysis of Standards

Chromatograms were produced following HPLC analysis of the 0.01 mg/mL (Figures 4i, ii), 0.001mg/mL (Figures 5i, ii) and 0.0001mg/mL (Figures 6i, ii) trans- (green) and cis- (purple) standards combined. Only the 267nm signal is displayed as it is the most appropriate for the detection and measurement of UCA concentration. There was no detection of additional impurities at 280 and 215nm.

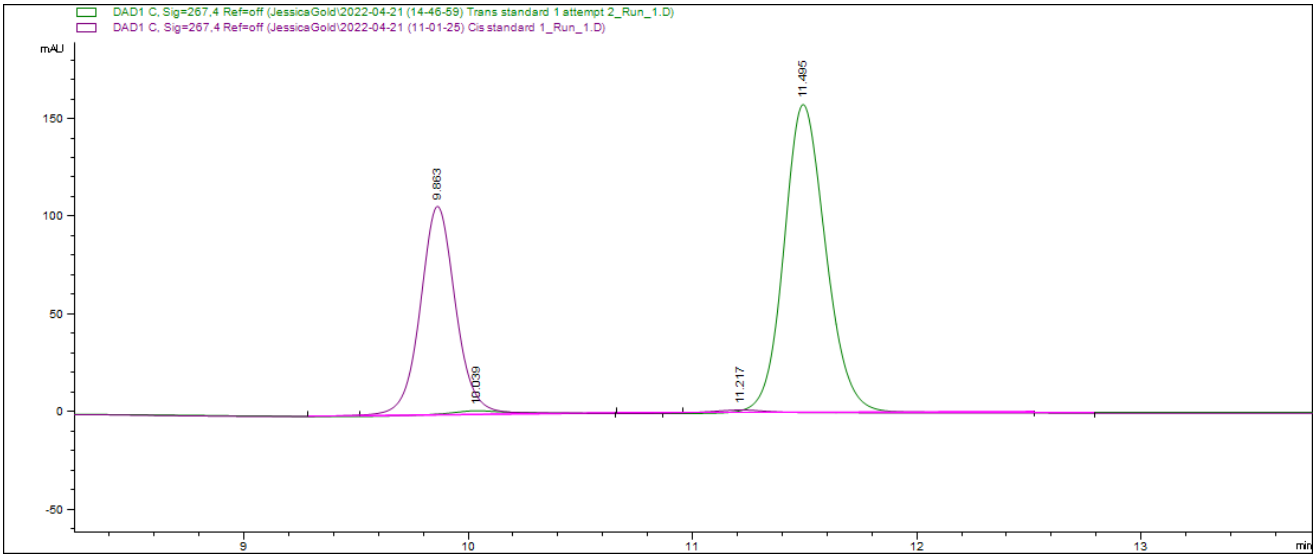

Figure 4: HPLC chromatogram of 0.01 mg/mL trans- (green) and cis-UCA (purple) standards overlayed with retention time peaks of 11.495 and 9.863 minutes, respectively.

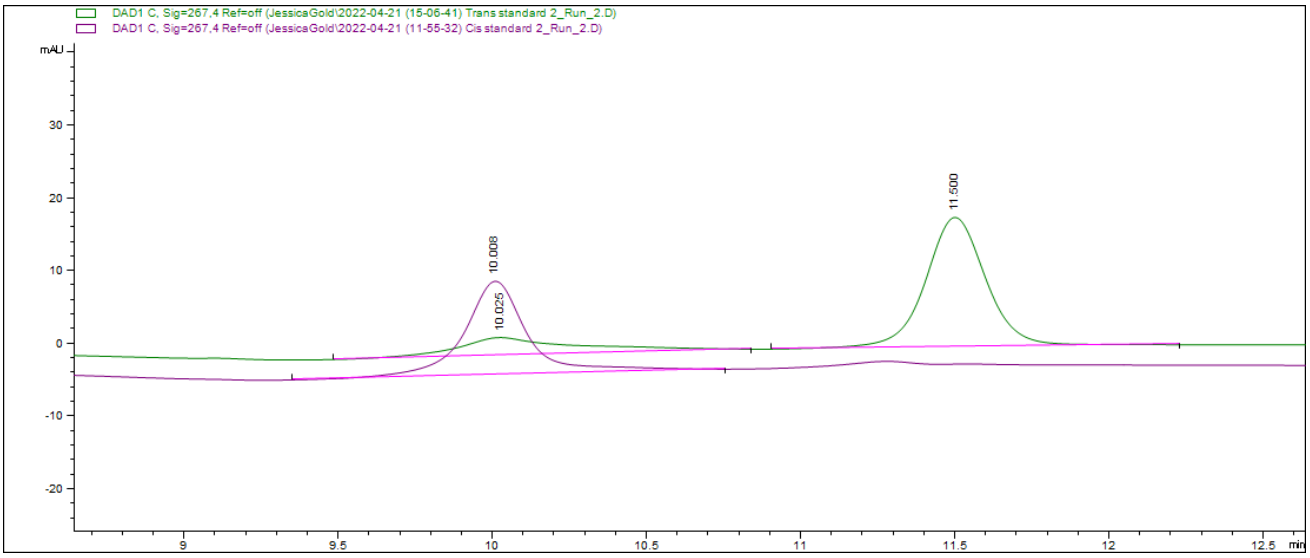

Figure 5: HPLC chromatogram of 0.001 mg/mL trans- (green) and cis-UCA (purple) standards overlayed. The retention time of the trans-UCA peaks of interest is at 10.025 and 11.500 minutes. Cis-UCA has a peak of interest with a retention time of 10.008 minutes. This chromatogram shows that peaks for both trans- and cis-UCA are flattening out and retention time for both peaks is increasing. This may be suggestive of problems detecting 0.001mg/mL of both standards.

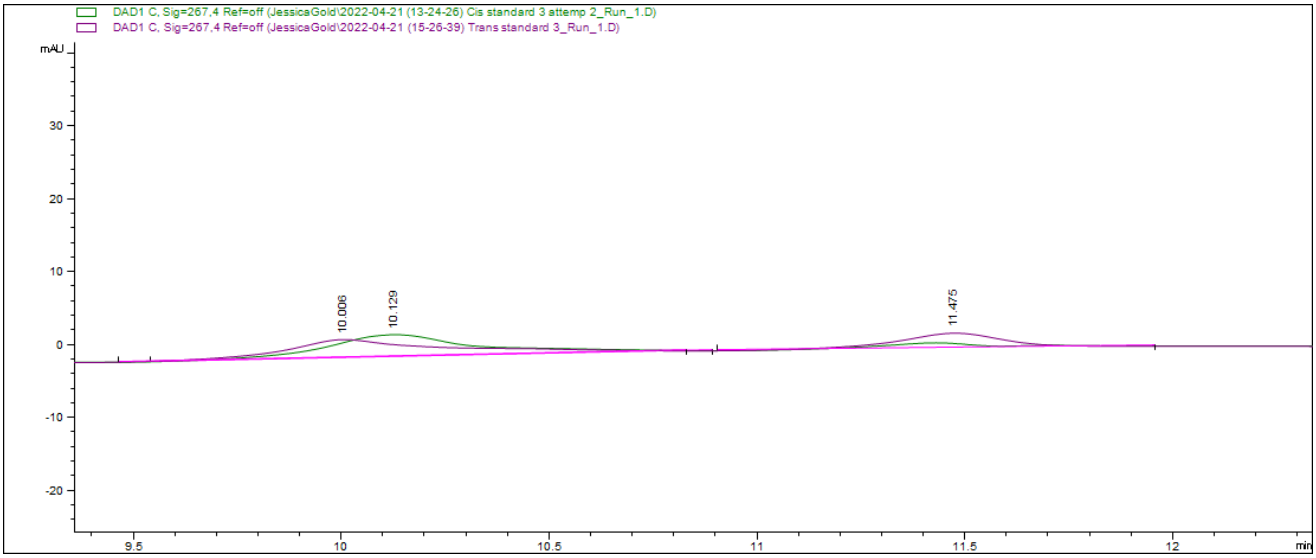

Figure 6: HPLC chromatogram of 0.0001 mg/mL trans- (green) and cis- UCA (purple) standards overlayed. The retention time of the cis-UCA peaks of interest is at 10.006 and 11.375 minutes. Trans-UCA has a peak of interest with a retention time of 10.129 minutes.

The chromatogram figures 4-6 have been focussed into the areas of the peaks. Only molecules that adhere to the column, like UCA, are retained and elute at a later point.

As shown by the difference peak shapes of 0.1, 0.001 and 0.0001 mg/mL cis- and trans- enantiomers, it is important to note that peaks are sharp, narrow, and have the same retention time at each concentration. The chromatograms also showed that the cis- and trans-UCA enantiomers eluted from the HPLC column at ~10 and ~11.5 minutes, respectively. Standardised sample chromatograms were tabulated for future comparison with analysis of pig blood serum samples (Table 2).

Table 2: HPLC analysis of cis- and trans-UCA standard samples

Sample	**Concentration (mg/mL)**	**Peak Height (mAU)**
Cis-UCA	0.01	105.9
Cis-UCA	0.001	11.6
Cis-UCA	0.0001	1.4
Trans-UCA	0.01	157.6
Trans-UCA	0.001	17.5
Trans-UCA	0.0001	1.8

Sample Preparation II

The chromatogram resulting from running the anonymous cis-UCA (Figure 7) standard showed the most appropriate measurement of cis-UCA concentration through a peak at 267nm. The cis-UCA peak should be expected to occur consistently at the same time, close to 9.8 minutes.

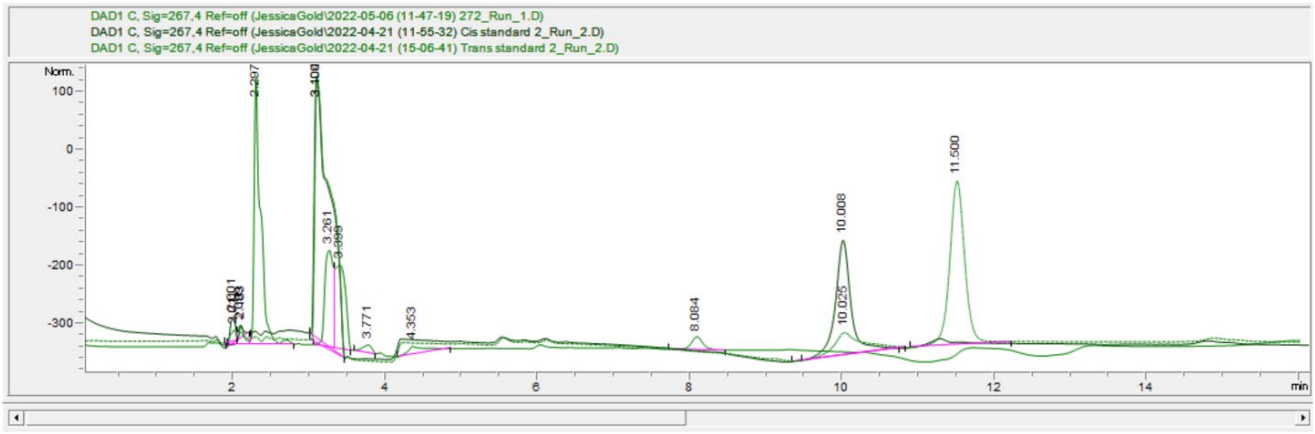

Figure 7: HPLC Chromatogram of the anonymous cis-UCA standards, illustrating results from 0-18 minutes run time. The green and purple traces correspond to trans- and cis- UCA, respectively.

The chromatograms resulting from running of samples 217, 276, 224, 206, 266 and 272 were compared (Figure 8). Only the 267nm signal is displayed as it is the most appropriate for the measurement of UCA concentration. The chromatogram shows the samples yielded extremely similar results. The peaks at approximately 8 minutes of run time are of an unknown origin (i.e not UCA) but were also identified by Zare *et al.* 2012 [18]. However, the artifactual peaks identifiable from 11-14 minutes have not been previously reported in the literature.

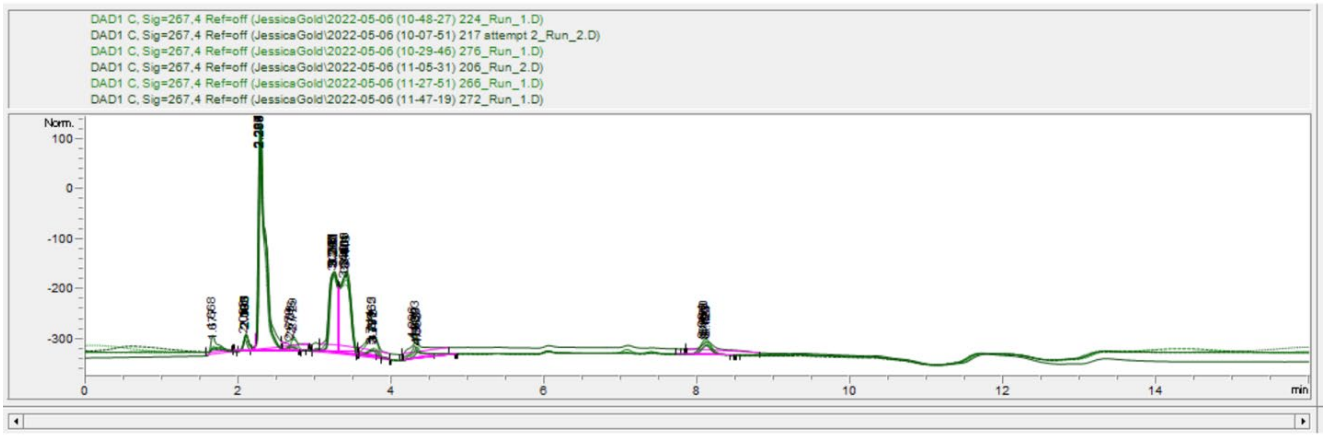

Figure 8: HPLC chromatogram resulting from running and comparing HPLC results of samples 217, 276, 224, 206, 266 and 272 from 0-18 minutes of run time. The green and purple traces correspond to trans- and cis- UCA.

The resulting chromatogram from the combined sample 217 (Figure 9) runs show only the 267nm signal displayed as it is the most appropriate for the measurement of UCA concentration. The second run of sample 217 (purple) was balanced and adjusted whilst the first run (green) was not.

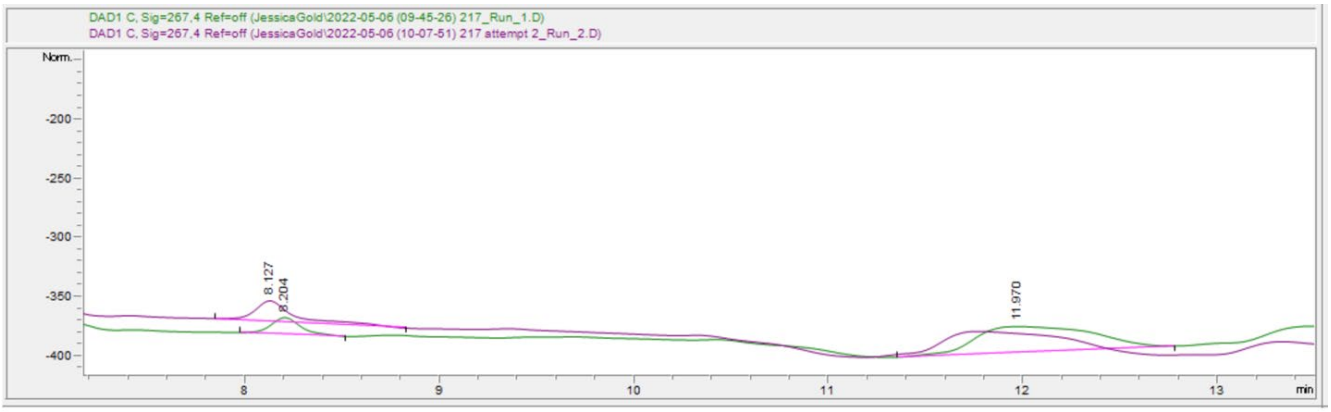

Figure 9: The combined chromatogram resulting from HPLC analysis of sample 217 runs from 0-14 minutes of run time showing a cis-UCA (purple) peak of interest at 8.127 minutes. There are also peaks of interest corresponding to trans-UCA (green) at 8.204 minutes and 11.970 minutes.

The resulting chromatograms from the HPLC analysis of samples 276 (Figure 10), 224 (Figure 11), 206 (Figure 12), 266 (Figure 13) and 272 (Figure 14) runs display only the 267nm signal is displayed as it is the most appropriate for the measurement of UCA concentration. Unlike sample 217, there is no peak at 11.970 minutes but all samples illustrate a similar peak at roughly 8 minutes, unable to be attributed to either cis- or trans-UCA. The chromatograms of all aforementioned samples illustrate unidentifiable artifacts from 11-14 minutes (Figures 10-14).

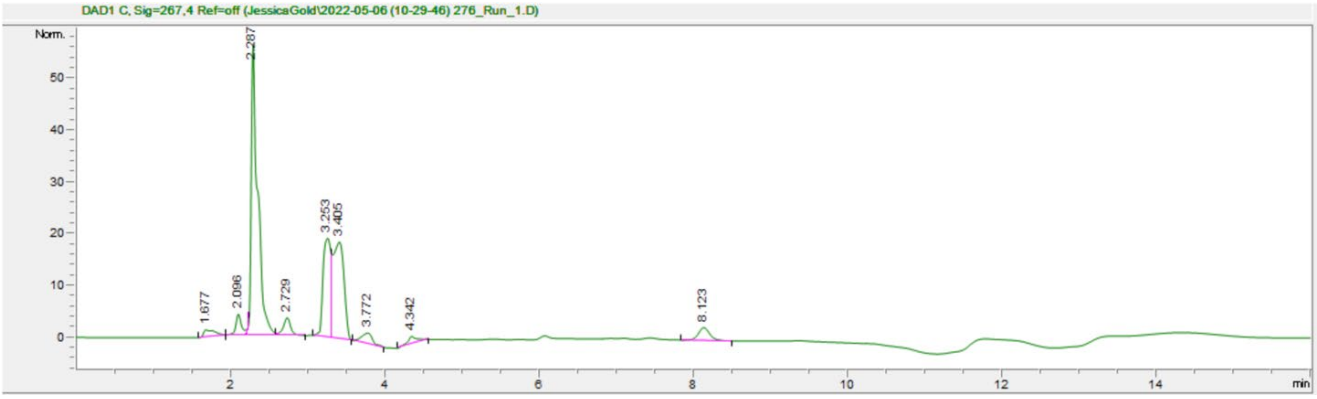

Figure 10: Chromatogram output resulting from HPLC analysis of sample 276 from 0-18 minutes of run time. Whilst there are various peaks between 1.5 and 9 minutes, these are not suggestive of cis- nor trans-UCA.

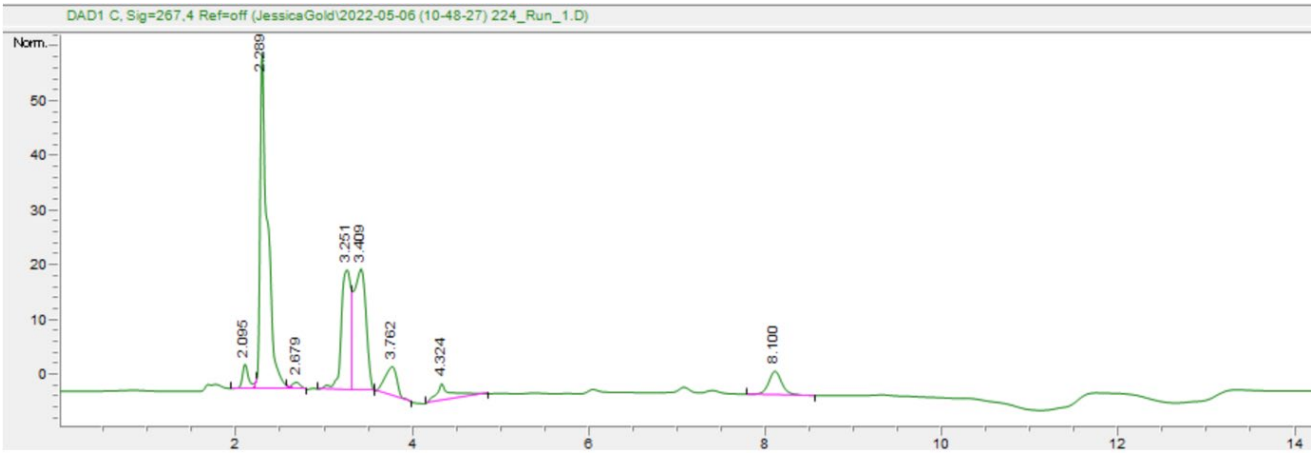

Figure 11: Chromatogram output resulting from HPLC analysis of sample 224 from 0-14 minutes of run time.

Whilst there are various peaks between 2 and 9 minutes, these are not suggestive of cis- nor trans-UCA. Further, there are also unidentifiable artifacts from 11-14 minutes of run time.

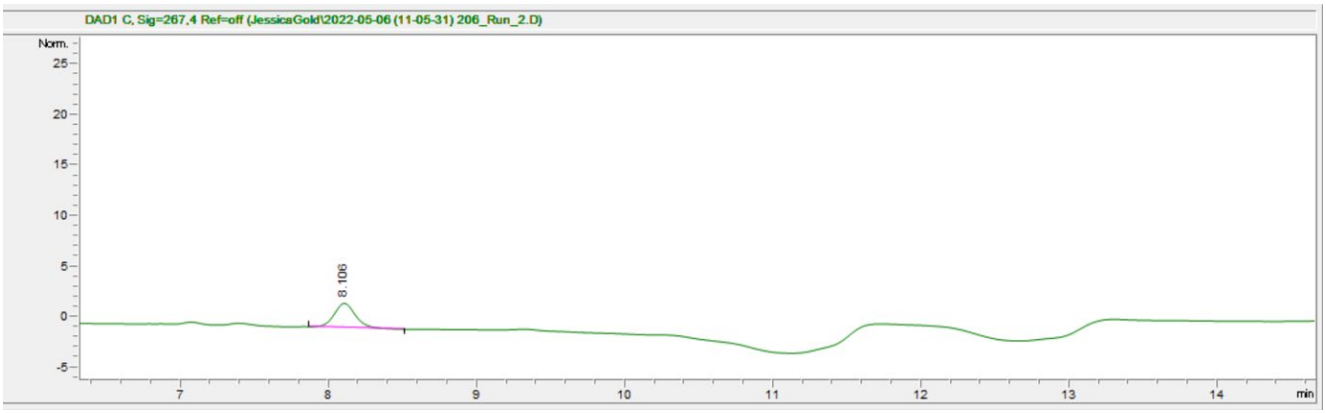

Figure 12: Chromatogram output resulting from HPLC analysis of sample 206 from 7-14 minutes of run time. Whilst there is a peak at 8.106 minutes of run time, this is not suggestive of either trans- or cis- UCA. Further, there are also unidentifiable artifacts from 11-14 minutes of run time.

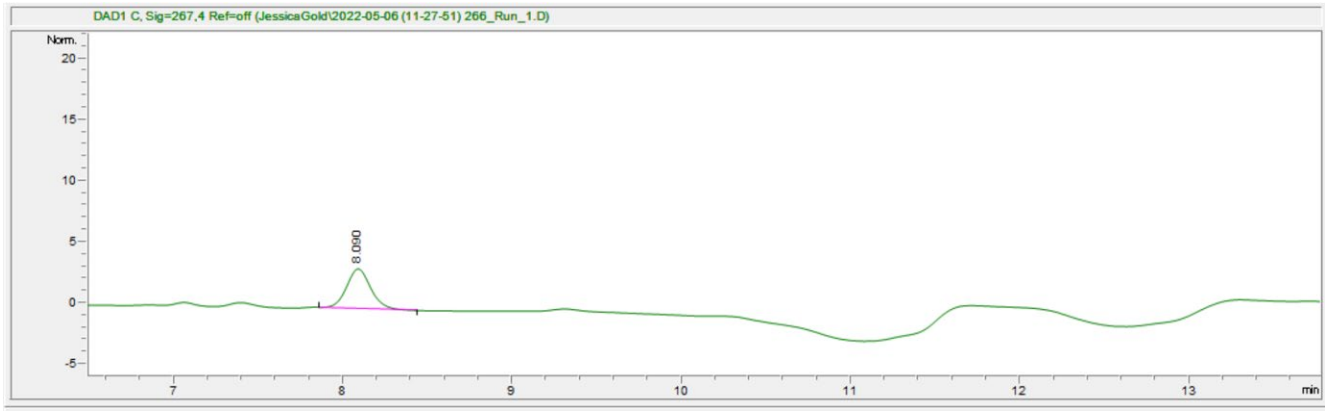

Figure 13: Chromatogram output resulting from HPLC analysis of sample 266 from 7-14 minutes of run time. Whilst there is a peak at 8.090 minutes of run time, this is not suggestive of either trans- or cis- UCA Further, there are also unidentifiable artifacts from 11-13 minutes of run time.

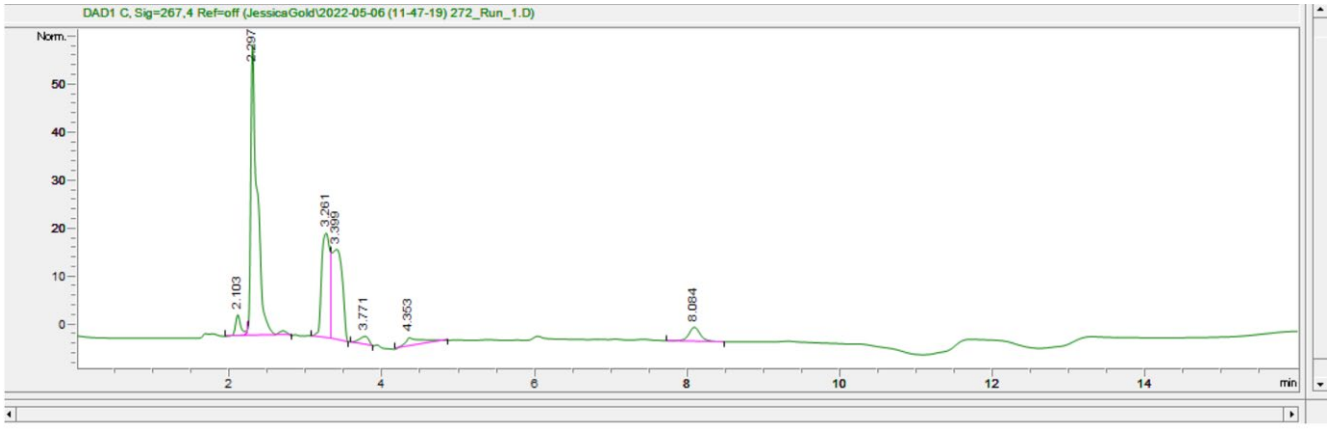

Figure 14: Chromatogram output resulting from HPLC analysis of sample 272 from 0-18 minutes of run time. Whilst there are multiple peaks from 2-5 minutes and at 8.064 minutes of run time, none of these are suggestive of either trans- or cis- UCA. Further, there are also unidentifiable artifacts from 11-14 minutes of run time.

Sample Preparation III: Spiking Blood Serum Samples

The chromatogram resulting from HPLC analysis of a cis- and trans-UCA spiked sample 288 prepared according to skin sample methods (Figure 15) displays only the 267nm signal as it is the most appropriate for the measurement of UCA concentration. There is a peak at roughly 10.25 minutes, attributed to the detection of cis- UCA. However, no corresponding trans peak was visible.

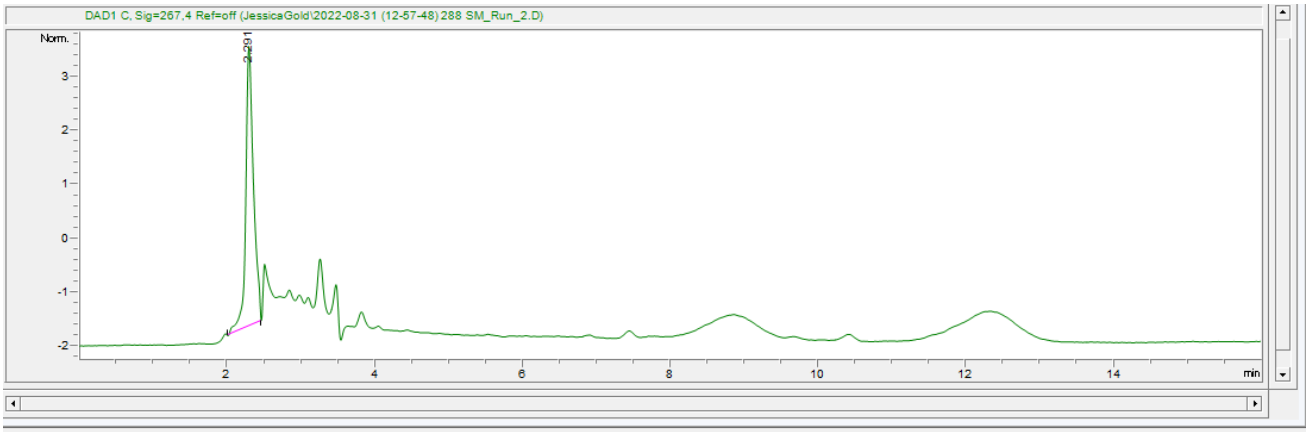

Figure 15: Chromatogram output resulting from HPLC analysis of spiked sample 288 prepared following skin sample methods showing 0-18 minutes of run time. Whilst there are multiple peaks visible between 2-4 minutes, none of which can reasonably be attributed to UCA detection.

Sample Preparation IV: De-protonation Prior to Normal Processing

The resulting chromatogram from sample 217 + Ace displays only the 267nm signal as it is the most appropriate for the measurement of UCA concentration (Figure 16). Whilst multiple small peaks are evident, none of which can reasonably be considered to result from UCA detection.

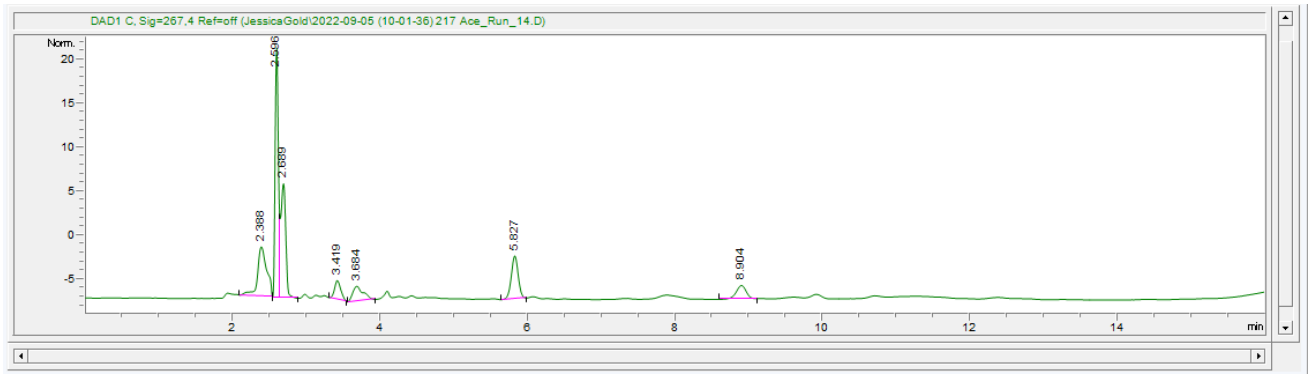

Figure 16: Chromatogram output resulting from HPLC analysis of sample 217 + Ace from 0-18 minutes of run time. Whilst there are multiple small peaks visible, none of which can reasonably be attributed to UCA detection.

Sample Preparation V: Solid-Phase Extraction

These samples were unable to be analysed using HPLC methods as they did not have enough liquid in them to filter as a hard precipitate had formed. Upon attempting to refilter these samples, the liquid could not be removed from the filter and hence samples prepared with these methods were unsuitable and discarded.

Discussion

Sample Preparation I
Detection and quantification of cis- and trans- UCA enantiomers

HPLC analysis of 0.0001mg/mL of cis- UCA and 0.0001mg/mL trans- UCA was performed. Both cis- and trans-UCA enantiomers were detected. The mAU of the 0.0001mg/mL sample was an appropriate absorbance. As such, subsequent standards were made at 0.001 and 0.01mg/mL concentrations.

Sample Preparation II
Attempting to Analyse Pig Blood Serum Samples for cis- and trans- UCA per Zare et al. (2012)

Zare *et al.* (2012) discusses the retention time of trans- and cis- UCA in fish muscle samples with retention times of 10 and 12 minutes respectively. Similarly, our spiked sample of the pig blood serum extraction solution showed a retention time between 10 and 11.5 minutes. Additionally, per Zare et al. (2012), we further confirmed from that there is no interference from other free amino acids or biogenic amines in the extracted pig cell matrix [18]. The closest amino acid or biogenic amines eluted at ~8 minutes, with the majority of amino acids and biogenic amines eluting in the first 5 minutes of the run. This confirms the selective appropriateness of this method for the identification and quantification of cis- and trans-UCA in pig blood serum.

However, when running the samples without a spike we observed the absence of both and cis- and trans-UCA peaks. While there is a peak present at 11.970 minutes in Sample 217 (Figure 11), it is unlikely to be trans-UCA as it is 0.5 minutes later than peaks evident in standard runs. Additionally, the peaks at 8.127 and 8.204 minutes do not indicate the presence of any UCA as they are approximately 1.5 minutes premature of the cis-UCA standard peak. Given this result we hypothesised that either UCA was not present in pig blood serum samples, or the extraction process was inefficient in extracting and isolating the UCA for analysis.

For Stage 3, we explored another published method of extracting UCA for analysis in biological samples. This method was published by Pham *et al.* (2017) and details the extraction of UCA in human skin samples [21]. Although, this was completed on human skin samples we hypothesised that this method may work on the extraction of human blood serum samples.

Sample Preparation III: Spiking Blood Serum Samples
Pig Blood Serum Samples Prepared using KOH and HCl, and spiked per Pham et al. (2017)

Per human skin extraction methods outlined by Pham et al. (2017), KOH and HCl were used to deprotonate and protonate the samples, respectively, to increase the purity of samples prior to HPLC analysis [21]. However, KOH was likely an ineffective base to deprotonate.

It was hypothesised that possible matrix interference within the samples was responsible for peaks not appearing on chromatograms following HPLC analysis. As such, a spike of cis- and trans- UCA was added to pig blood serum sample 228 to assess for interreference. The spiked sample showed clear cis- and trans-spiked peaks, thus matrix interference is unlikely.

Sample Preparation IV: De-protonation Prior to Normal Processing
Changing of base to Acetonitrile

Given the lack of success using KOH to deprotonate samples, the base was changed to acetonitrile to as it is a common solvent that can change the solubility profiles of target molecules. The addition of acetonitrile to samples resulted in the immediate formation of a white precipitate, in line with what has been previously reported by Pham *et al.* (2017) [21].

Similar to the action of KOH, acetonitrile ionises UCA, making it more soluble. The formed precipitate removes impurities and non-target molecules, making the supernatant purer and theoretically UCA more

readily detectable with HPLC analysis if present. However, upon analysis of the final supernatant, UCA was unidentifiable utilising HPLC methods. This could potentially result from the degradation of UCA by the acetonitrile, or UCA precipitating out of solution.

Sample Preparation V: Solid-Phase Extraction

Pig blood serum sample was dissolved in methanol and passed through a solid-phase extraction cartridge to sequester impurities and potentially isolate the free cis- and trans- UCA molecules for analysis. We opted for a solid-phase extraction process as we hypothesised potential loss of UCA in the prior extraction processes. However, this method was unsuccessful as a precipitate formed after the blood serum samples were passed through the solid-phase cartridge. This could have been for a variety of reasons, such as: the solid-phase extraction being inappropriate for the sequestration of impurities; side reactions occurring within the extraction cartridge; or the cartridge degrading and the solid phase coeluting with the sample, all resulting in a contaminated sample.

Conclusion

Despite being unable to successfully detect cis- or trans- UCA in biological samples, this study developed a HPLC method for the detection of cis- and trans- UCA in pig blood serum samples, showing good selectivity with spiked samples.

Future work will detail how the extraction process can be further refined to ensure the successful extraction of cis- and trans- UCA in pig samples. Past research has noted difficulties in isolating the cis- and trans- UCA in biological samples, as cell tissue have complex matrices and have been the major challenges in past research [18].

However, given the abundance of successful UCA isolation in various animal tissue cells discussed in the literature, the hypothesis that UCA is present in porcine cells remains. Specifically, next steps for this project will involve further refinement of sample preparation, the creation of a calibration curve, refinement of extraction methods and the use of more sensitive analysis tools, such as mass spectrometry to detect trace amounts of cis- and trans-UCA in pig blood serum samples.

Following the successful detection and quantification of cis- and trans-UCA in pig blood and discovery of evidence linking this to seasonal subfertility, further research will be required to develop methods to overcome said subfertility. These may include genetic modification, development of individually or seasonally tailored nutrition or changed housing conditions [30, 31, 32]. Application of future research will work to better improve the ability of industry to meet market demand, especially during peak periods, and ensure global food security through maximising genetic yield from each sow on-farm.

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