"The Frog—that arch-martyr to science—affords the most convenient subject."

Rudiments of Pathological Histology by Carl Wedl (1855)

Thesis

Setting up a non-invasive LC-MS platform for semi- quantification of steroid hormones in anurans

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Foreword

Thank you for taking the time and effort in reading this report.

Due to unforeseen circumstances regarding the COVID-19 crisis in 2020, this study was severely set back. Not enough time in the laboratory was available to run enough iterations of the methods to completely refine them. Therefore, the method has not yet been implemented on actual anuran water samples. To compensate, the recommendations section of this report provides well thought out suggestions on how to get to the point of being able to implement this platform on real world samples. This report was written to provide a comprehensive starting point for the next student or researcher wishing to follow up on this study.

Although the study itself is far from concluded, I (the author) have gained valuable knowledge and experience regarding LC-MS method development, data management, teamwork and general laboratory practices. For this, I am thankful to everyone involved. A special thanks goes out to the following people:

Peter Lindenburg for leading our team,
Sonja Kaal for theoretical advice,
Evelyne Burgwal for practical assistance,
Marianne Salters for love and support.

No frogs were harmed during the course of this study.

Samenvatting

Amfibieën, en specifiek kikkers, behoren tot de meest bedreigde diersoorten op aarde. Het vergelijken van de fysiologie tussen kikkers uit stedelijke en landelijk leefgebieden levert belangrijke informatie over het effect van verstedelijking op kikkers. Deze informatie kan gebruikt worden om te helpen bij het behouden en beschermen van deze diersoorten. Het doel van dit onderzoek was om een vloeistofchromatografie-massa spectrometrie platform op te zetten, inclusief bemonstering, monstervoorbewerking en analyse methoden, dat gebruikt kon worden om meer te leren over de gevolgen van verstedelijking op de fysiologie van kikkers.

De steroïde hormonen testosteron en corticosteron zijn uitgekozen als primaire analieten voor dit onderzoek omdat er een hoge kans is dat deze meetbaar uitgescheden worden door kikkers. Testosteron is betrokken bij aggressie en mannelijke sexuele ontwikkeling. Corticosteron is gerelateerd aan energie regulatie en stress reacties.

De bemonsteringsmethode waar dit onderzoek op is gebaseerd maakte gebruik van waterbaden spotten op gedroogd filter papier om kikkers relatief makkelijk en op niet-invasieve wijze te bemonsteren. Een bijbehorende monstervoorbewerkingsmethode en analysemethode op een vloeistofchromatografie-massa spectrometrie systeem zijn opgezet en gevalideerd.

De analysemethode slaagde voor de validatie op basis van een gevonden herhaalbaarheid van tussen de 3.83% en 10.34%, een minimale selectiviteit van 1.516 en geen tekenen van carry-over. Voor testosteron was het meetbereik 0.05 tot 5 µg/mL, met een detectielimiet van 9.22 (± 2.93) * 10^{-3} µg/mL. Voor corticosteron was het meetbereik 0.06 tot 6 µg/mL, met een detectielimiet van 9.65 (± 3.07) * 10^{-3} µg/mL. Het platform was niet geslaagd voor de validatie op basis van nog onbepaalde reproduceerbaarheid en te lage terugvinding. Op basis van de resultaten van de validatie was het platform nog niet geschikt om toe te passen. Een sterk fundament is neergezet om het platform in de toekomst op echte monsters toe te passen met een paar kleine aanpassingen.

Abstract

Amphibians, and in particular anurans, are amongst the most endangered species on earth. Comparing the physiology between urban and rural anurans provides key information on the effect of urbanization on anurans. In turn, this information can help with preservation and conservation of the species as a whole. The goal of this study was to set up a liquid chromatograhy-mass spectrometry platform, including sampling, sample preparation and analysis methods, that can be used to learn more about impact of urbanization on the physiology of anurans.

The steroid hormones testosterone and corticosterone were chosen as main analytes for this study, because there is a high probability they are measurably excreted by anurans. Testosterone is principally involved in aggression and male sexual development. Corticosterone is related to energy regulation and stress response.

The sampling method on which this study was based utilized water-bath spotting on dried filter papers to sample anurans non-invasively and with relative ease. A complementary sample preparation method and an analysis method on a liquid chromatography-mass spectrometry system were set up and validated.

The analysis method passed validation based on a determined repeatability between 3.83% and 10.34%, a minimal selectivity of 1.516 and no signs of carry-over. For testosterone, the measuring range was 0.05 to 5 µg/mL with a limit of detection of 9.22 (± 2.93) * 10^{-3} µg/mL. For corticosterone, the measuring range was 0.06 to 6 µg/mL with a limit of detection of 9.65 (± 3.07) * 10^{-3} µg/mL. The platform did not pass validation based on undetermined reproducibility and low recovery. Based on the results of the validation, the analysis method was not yet deemed fit for the purpose of analyzing and comparing anuran water samples. A strong foundation was laid out to implement the platform in the future on real world samples following minor adjustments.

Index

Abbreviations

In order of appearance:

IUCN	- International Union for Conservation of Nature
TS	- Testosterone
CS	- Corticosterone
LC-MS	- Liquid chromatography-mass spectrometry
DFP	- Dried filter paper
CHO	- Cholesterol
IUPAC	- International Union of Pure and Applied Chemistry
EIA	- Enzyme immunoassay
RP-HPLC	- Reversed phase high performance liquid chromatography
QTOF	- Quadrupole time-of-flight MS
ESI	- Electrospray ionization
QMS	- Quadrupole mass filter
m/z	- Mass to charge ratio
TOF	- Time-of-flight mass analyser
LOD	- Limit of detection
MS/MS	- Tandem MS
QqQ	- Triple quadrupole MS system
SRM	- Selective reaction monitoring
MRM	- Multiple reaction monitoring
MeOH	- Methanol
FA	- Formic acid
IPA	- Isopropyl alcohol
EIC	- Extracted ion chromatogram
LOB	- Limit of blank
RSD	- Relative standard deviation
SPE	- Solid phase extraction
PMB	- Paramagnetic beads

Introduction

Research justification

Figure 1. IUCN Red List of threatened species in percentage by class.[1]

During the inception of this study, over 40% of all amphibian species were threatened with extinction and this percentage is rising. In fact, amphibians are ranked the most threatened class on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species as of 2019.[1] The main order of the amphibia is the anuran, most commonly known as frogs and toads. Panama is home to over 200 different species of anurans, yet many of those species are in immediate danger of extinction.[2] Knowledge about the inner mechanisms of reproduction is key to ensuring a species survival. Urbanization has shown to have a sizeable impact on the breeding patterns of anurans.[3][4] A recent study has shown that urban frogs have adapted their mating system as a reaction to changes in their environment when compared to their rural counterparts. Specifically, the study presents that urban male túngara frogs (*Physalaemus pustulosus*) have increased the salience of their mating calls in response to greater competition from other males and relative absence of predators.[5]

Essential to understanding the biology behind the process of reproduction, is understanding the chemistry. Acoustic communication in anurans is a key part of their mating process. This process is modulated by their neuromodulatory and endocrine systems. For example, female túngara frogs have shown increased circulating levels of oestrogen and progesterone in response to phonotactic reception of male mating calls.[6] This illustrates that biological systems have underlying chemical mechanisms. The endocrine system communicates, in part, through prolactin and steroid hormone regulation.[7] In this study the initial focus is on the steroid hormone regulatory process of the endocrine system of anurans, namely the secretion of testosterone (TS) and corticosterone (CS).

The exact mechanisms of the endocrine system of anurans, and specifically the metabolism of steroid hormones in anurans, have not yet been fully explored. However, for humans many studies have been performed on the pathways of steroid hormones, mainly for medical purposes.[8][9] Inter-species comparative studies can help lay the foundation to examining and ultimately understanding how certain systems work.[10] Although interchangeability is not a given[11], certain assumptions have to be made to take the first step in exploration. For this reason, most background research taken into consideration for this particular study has humans as the prime focal point. Hormones are the key coordinators for developmental, physiological and behavioural mechanisms in all living organisms. TS and CS have been selected because they play an essential role in communication, regulation of sexual behaviour and mediation of organismal responses to environmental change.[10] Looking into the levels of TS and CS in anurans has an added value for environmental conservation and preservation purposes.

Research goal

To examine the effect of urbanization on the physiology of anurans, biologists travelled to Panama where a discrepancy was found between the behaviour of urban and rural anurans. Normally, anurans are either killed or at least harmed during the sampling process. The main goal of analysing anurans for this study was to preserve them, so harming them was undesirable. For this reason, a new sampling method that would cause minimum harm to the sample subjects was used. This sampling method had never been used for the sampling of anurans, so a standardized and accurate method for analysing the acquired samples was requested.

82 Túngara frogs (*Physalaemus pustulosus*) were captured, sampled and subsequently set free in their natural habitat. This was done at two different sites at Pipeline Road near Gamboa, a small town in the Republic of Panama (Central America). One site was considered relatively urbanized and the other relatively rural. A new analysis method using liquid chromatography-mass spectrometry (LC-MS) was set up by the author to accurately examine and compare the collected samples on content of steroid hormone levels.

The primary aim of this study was to set up and validate a full LC-MS platform. The platform would include robust imitation of the non-invasive sampling method for the purpose of optimization of the sampling process of anurans for future studies. The second part of the platform would include a newly set up sample preparation method to prepare the dried filter paper (DFP) for analysis on an LC-MS system. Finally, the platform included a new LC-MS analysis method specifically tuned to measure the original concentration of TS and CS in the study samples as accurately as possible.

To reach this goal, several objectives were set up. Firstly, TS and CS were to be made detectable and quantifiable on the LC-MS and the approximate linear measuring range of the analysis method had to be determined. Secondly, a sample preparation method for extracting TS and CS from DFP and preparing the extract for analysis was set up and optimized. Finally, the sample preparation method and LC-MS analysis method were to be validated.

If validation proves the LC-MS platform is fit for the purpose of analysing TS and CS in anuran water samples stored on DFP, the platform can be used to compare the physiology of anurans living in urban areas to those living in lesser urbanized habitats. The method could also be extended to include other analytes following the same principles, setup and objectives designed in this study, providing increasingly more information on the physiology of anurans.

Theory

Chemical properties of steroids

The chemical structures of TS and CS are shown in figures 2A and 2B, respectively. All steroids are derived from cholesterol (CHO; shown in figure 2C). The nomenclature and positioning of the carbons and possible functional groups of CHO-derived molecules is explained through the International Union of Pure and Applied Chemistry (IUPAC) steroid ring system displayed in figure 2D.[12]

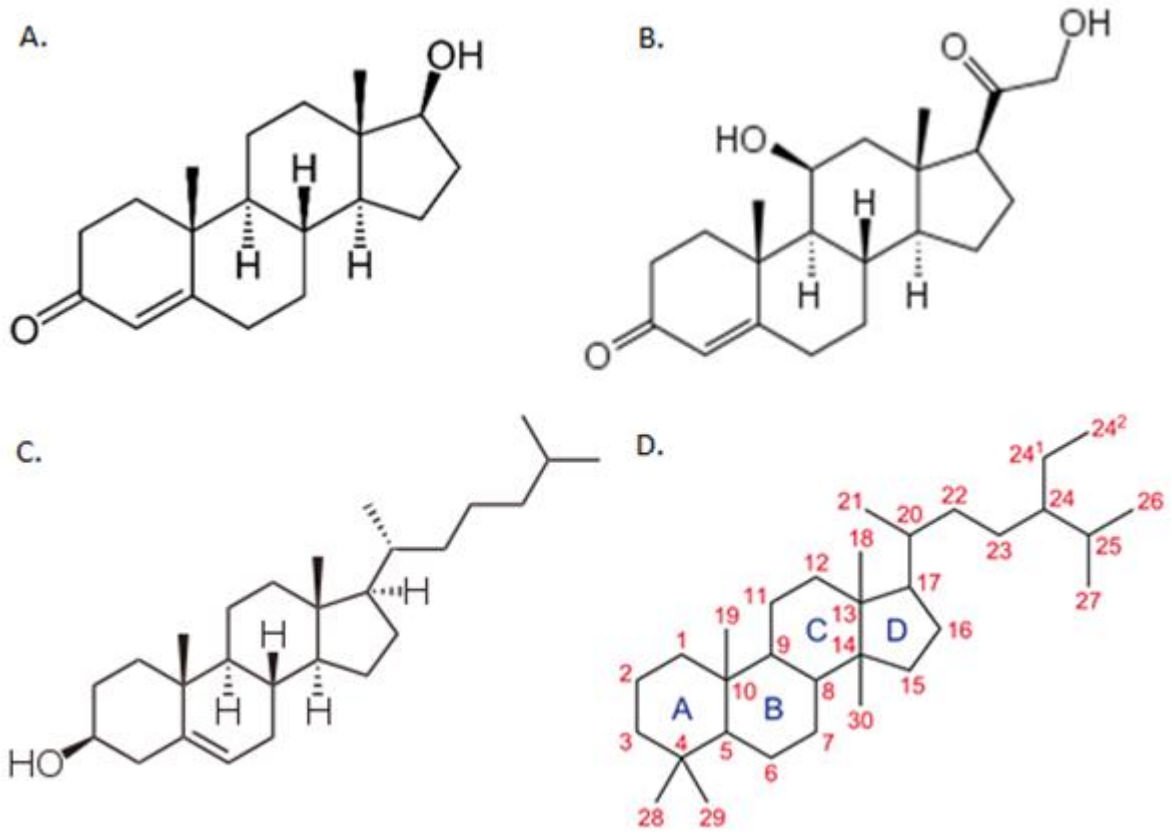


Figure 2. A: Chemical structure of testosterone (17β-Hydroxyandrost-4-en-3-one; $C_{19}H_{28}O_2$); B: chemical structure of corticosterone ((11β)-11,21-Dihydroxypregn-4-ene-3,20-dione; $C_{21}H_{30}O_4$); C: chemical structure of cholesterol (cholest-5-en-3β-ol; $C_{27}H_{46}O$); D: IUPAC steroid ring system.[12]

CHO is a relatively flat molecule, consisting of a polycyclic hydrocarbon backbone made up of three conjoined hexagonal rings and one pentagonal ring. CHO is characterized by a sidechain connected to the 17th carbon of the main structure and two angular methyl groups at positions 18 and 19. From the structure of CHO, three principal types of steroids can be derived. In all cases, the double bond between carbons five and six will be converted in the double bond between carbons four and five. Partial removal of the side chain transmutes to the pregnane type under which corticosteroids such as CS fall. Complete removal of the side chain yields the androstane type under which androgens such as TS fall. Lastly, loss of the methyl group on the 19th carbon, along with an aromatization of the first hexagonal ring presents us with the estrane type under which estrogens fall.

TS, the main androgen, is principally involved in aggression and male sexual development.[13][14] While TS is partly excreted in its original form, in the body TS is partly converted into estradiol (Estra-1,3,5(10)-triene-3,17β-diol; $C_{18}H_{24}O_2$) by Sertoli cell-derived aromatase enzyme and into dihydrotestosterone (5α-Androstan-17β-ol-3-one; $C_{19}H_{30}O_2$) by 5α-reductase type 2 enzyme.[15]

Furthermore, TS is neutralized during transportation through the body through esterification with a sulfate-ion on the hydroxy group located at carbon seventeen. This reversible reaction transforms the hormone into an inert organic anion, ideal for transportation. Before excretion, the sulfate-group is usually de-esterified. Most steroids can be sulfated, including TS and CS.[16][17]

Glucocorticoids manage all sorts of energy regulation, mostly through the release of glucose in the bloodstream. This release or inhibition of energy is associated with multiple sorts of stress-related behavioural responses such as the fight-or-flight reaction to stressful events or providing energy to combat abiding emotional stress.[14] CS is the main glucocorticoid for amphibians, reptiles and birds. Unlike humans and fish where cortisol (11β,17α,21-Trihydroxypregn-4-ene-3,20-dione; $C_{21}H_{30}O_5$) is the main glucocorticoid. CS is the precursor to the mineralocorticoid aldosterone (11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al; $C_{21}H_{28}O_5$) which is the primary steroid hormone that regulates homeostasis.[18]

The documentation listed in the above three paragraphs illustrates that all steroid hormones have a certain level of similarity and interconnectedness regarding biosynthesis and chemical structure. TS and CS were chosen for this study, because of the high probability that they are measurably excreted in their underivatized form by anurans. Excretion of bodily compounds in anurans happens mostly through diaphoresis. Water-baths have proven to capture physiologically relevant changes in the concentration of certain steroid hormones.[19] The assumption was made this is also true for TS and CS. If the platform devised in this study proves successful in identifying and quantifying the levels of TS and CS in anuran water-bath samples, the platform can further be generalized to other steroid hormones and possibly other kinds of analytes, such as biogenic amines or proteins, using only minor adjustments.

Sampling and storage

Multiple studies have been performed on the stability of steroid hormones during storage. Depending on the storage method, results regarding degradation vary. In complex matrices, steroids are stable for a maximum of 14 to 28 days.[20][21] Some steroid hormones have shown to be stable during long-term storage in water, for up to three to twelve months without introducing a major storage effect.[22][23] Extracted hormones dissolved in MeOH or absorbed on DFP are the preferred methods of sample storage based on stability. The sampling method using DFP is considered superior because of the ease of handling, low level of invasiveness for the organism and low labour costs.[23] To the author's knowledge, sampling water-baths on DFP has never before been used in the context of sampling anurans.

Methodology

Liquid chromatography-mass spectrometry and enzyme immunoassay

Detection and quantification of steroid hormones is primarily done using a technique called enzyme immunoassay (EIA).[24] EIA involves binding specific antibodies to the antigen of a specific analyte. This antibody is usually either a chromogen or a fluorogen which makes the analyte visible by eye or microscope and quantifiable by instruments such as spectrophotometers. EIA suffers from several structural issues[25], the issues relevant to this study are presented below:

- Ability to only measure one analyte per processed sample.[25]
- In complex matrices the antibodies can bind to other molecules besides the target analyte causing poor accuracy.[16]
- Limited sensitivity and range compared to other analysis methods.[26]
- High variability and low repeatability due to lack of standardization across EIA kits.[25]

Another technique used for steroid hormone analyses is LC-MS. LC-MS has been chosen over EIA in this study because it directly tackles the issues mentioned above. Another reason LC-MS is preferred, is because it is easier to generalize the analysis method to other steroid hormones besides TS and CS.

Reversed phase-high performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) was used to chromatographically separate analytes because steroids are relatively non-polar and non-volatile molecules. The stationary phase was C18-based and the mobile phase was a gradient mix of MS-grade water as a polar solvent and methanol (MeOH) as a non-polar solvent. Since CS is a more polar molecule than TS, while using RP-HPLC, CS elutes first and TS follows afterwards.

Mass spectrometry principles

After elution, MS was used to further separate the analytes based on their mass-to-charge ratio (m/z). Unless indicated otherwise, the MS system used in this study was an electrospray ionization high resolution quadrupole time-of-flight MS (QTOF). A schematic overview of the system is shown in figure 3.[27]

The most relevant parts of the QTOF were the electrospray ionization source, the hexapole and analytical quadrupole mass filters and the time-of-flight mass analyzer. The principles behind these parts of the QTOF will briefly be explained in the following sections of the report.

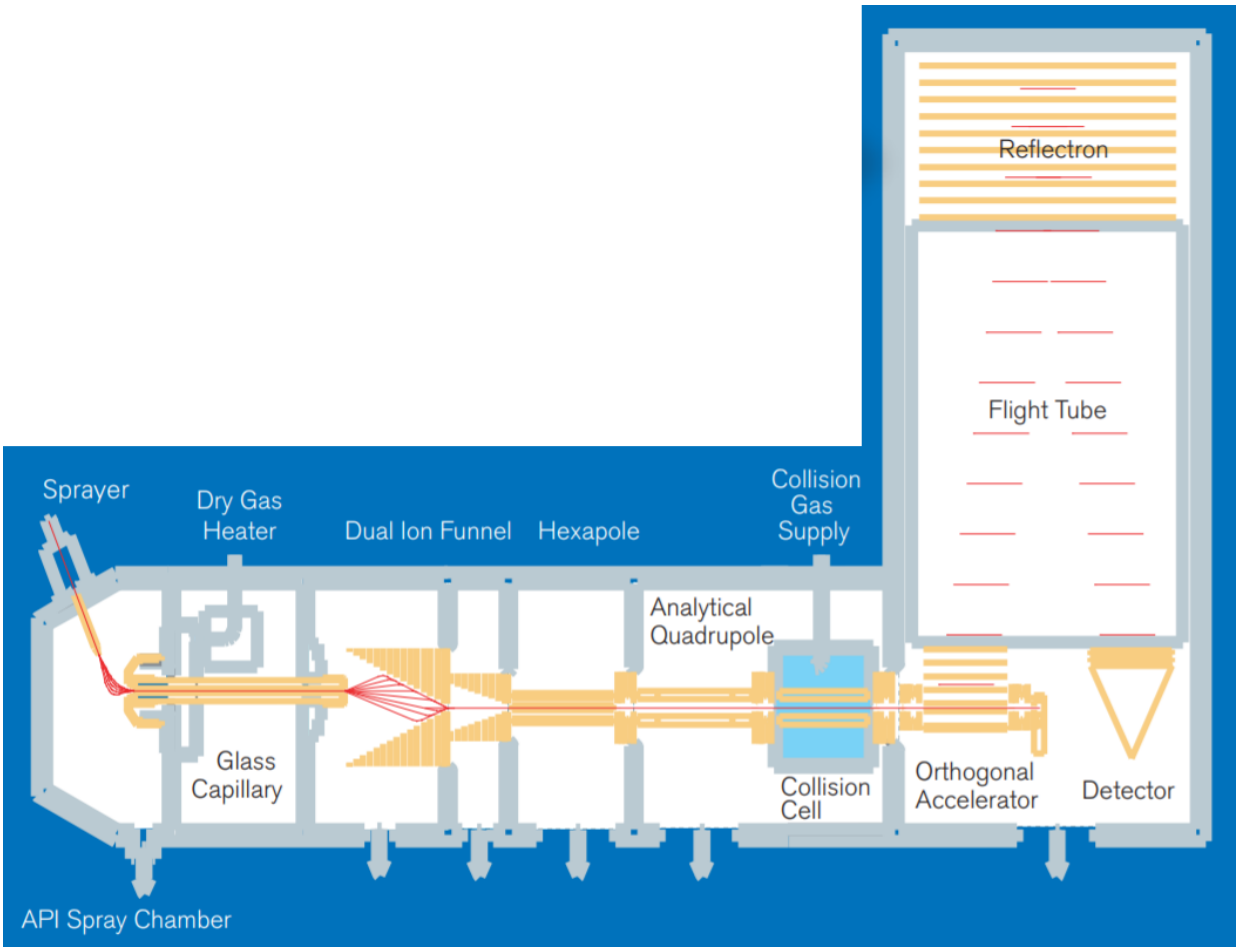


Figure 3: A schematic overview of the QTOF MS used in this study.[27]

Electrospray ionization source

In the electrospray ionization source, a technique called electrospray ionization (ESI) creates ions from molecules that are in liquid state. A nebulizer sprays the solvent containing the analytes into the source as tiny droplets, surrounded by inert nitrogen gas (nebulizing gas). The nebulizer contains a needle with an electrode on which a high voltage is set. This creates an electric field which ionizes the molecules contained in the solvent. The source ends with a glass capillary which is also set on high voltage, attracting nearby positive or negative ions depending on the mode of the QTOF. A current of heated nitrogen (drying gas) runs alongside the capillary, causing the solvent to evaporate. Through this process the ions contained in the solvent enter a gaseous state. Figure 4 illustrates the mechanism taking place in the source. Finally, the ions are carried through the capillary in a vacuum to the rest of the QTOF. ESI is a soft form of ionization, ideal for the analysis of metabolites because relatively small molecules (<900 Da) stay largely intact.[28]

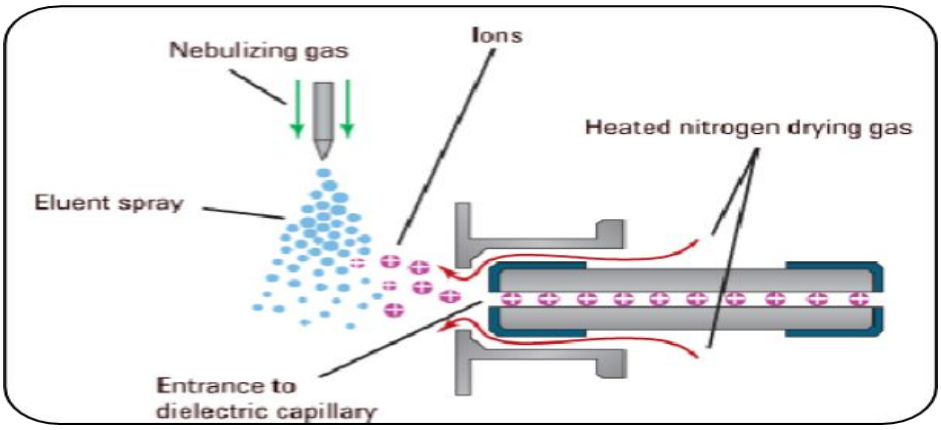


Figure 4: A schematic overview of the mechanism of electrospray ionization.[28]

Quadrupole and hexapole mass filters

Mass filters can be used to filter analytes from undesirable ions based on their m/z value. A quadrupole mass filter (QMS) consists of four cylindrical rods which create an oscillating electric field capable of selectively separating ions, based on the stability of their trajectory when moving between the rods. This trajectory is directly correlated to a specific m/z. Figure 5 shows a schematic overview of a QMS.[29] The hexapole mass filter is like a QMS, one key difference being that it consists of six rods instead of four which causes higher ion transmission.

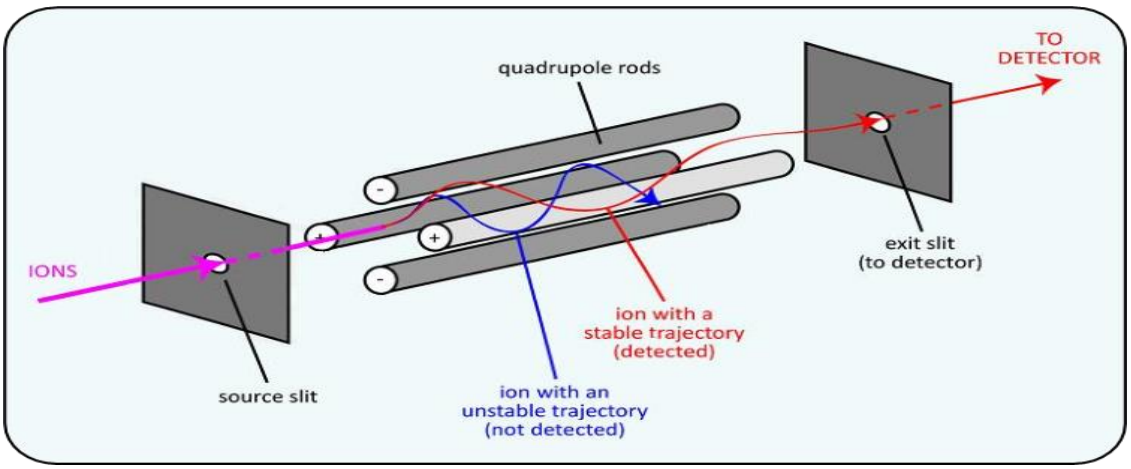


Figure 5: Schematic overview of a QMS.[29]

Time-of-flight mass analyser

To derive the mass of ions, a time-of-flight mass analyser (TOF) uses an ion accelerator, a field-free flight tube and an ion detector, depicted on the right in figure 3.[27] Before entering the flight tube, ions are orthogonally accelerated with an exact amount of energy. The time required for an ion to pass through the entirety of the field-free region of the flight tube is dependent on its mass. Since the energy received by the ions during acceleration is the same, heavier ions will have a lower speed exiting the accelerator compared to lighter ions. Inside the flight tube is a reflectron which is comprised of a single stage ion mirror that reflects ions back through the flight tube. A big advantage of the use of a reflectron, is that it reduces the spread of the time of arrival of ions that have the same m/z, thus increasing the sensitivity of the instrument.[30] After passing through the flight tube, the ions arrive at an electron multiplier detector. Inside the detector, the electrical signal given off by the ion at the detector is multiplied to a measurable level through a process called secondary emission.[29] After multiple steps of secondary emission the final electrical signal is converted and summed up in a count which is presented in an MS spectrum by a computer.

Direct Infusion and tandem mass spectrometry

Direct infusion is a technique where a sample is directly injected in a MS system, without chromatographical separation beforehand. Direct infusion is mostly used for identifying analytes during untargeted analyses and calibrating a system for target analytes. It is not usually used for quantification. A disadvantage of this technique is that pollutants in the sample are simultaneously infused. Pollutants can distort the signal of the analytes of interest, through a process called ion suppression. Detectors have a maximum number of ions they can measure simultaneously. Once this maximum is exceeded, not all ions can be detected anymore, reducing the accuracy and precision of the analysis. Internal standards can be used to quantify ion suppression, at the ironic expense of possibly causing more ion suppression.

Besides being used for calibration, analysis through direct infusion was explored on a tandem MS (MS/MS) system. MS/MS offers vastly superior sensitivity and specificity compared to singular MS detection techniques.[31] The MS/MS system used for direct infusion exploration was a triple quadrupole MS system (QqQ). The QqQ consisted of a QMS, a collision cell and another QMS, with these parts set in tandem. A collision cell is used for fragmentation. A schematic overview of a collision cell is shown in figure 6.[29] The collision cell is filled with an inert collision gas which collides with ions entering the cell. The original ions that enter are called precursor ions. The collision of the precursor ions with the collision gas causes fragmentation which produces specific product ions. Each ion fragmentates in a unique way, which is why fragmentation increases specificity.

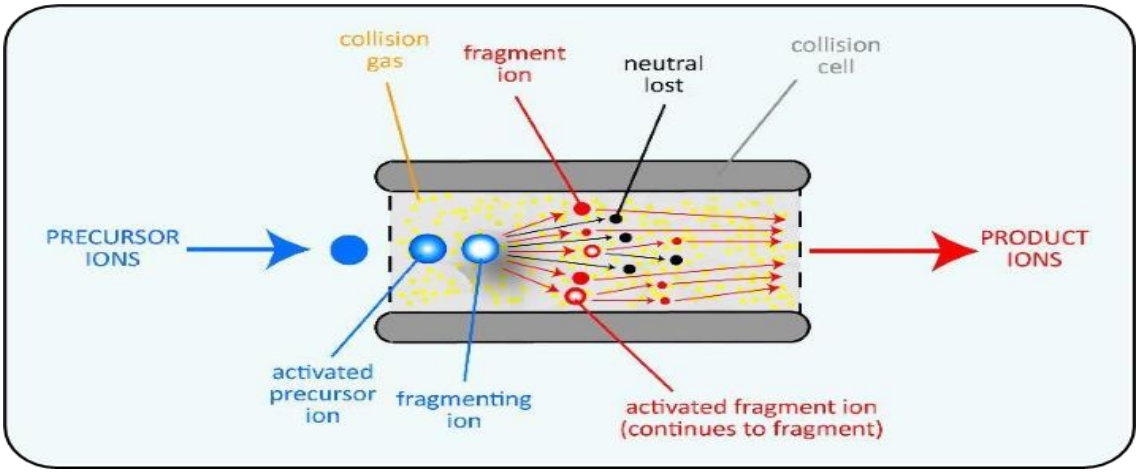


Figure 6: Schematic overview of a collision cell.[29]

Fragmentation makes a detection technique called selective reaction monitoring (SRM) available. During SRM, both precursor and product ions are monitored. The mass filters of the MS system are set to the specific m/z values of the precursor and product ions. This way, the voltage inside the mass filters no longer has to be switched as often, letting through more ions of interest. This drastically increases the sensitivity and specificity of the analysis method, which explains why SRM is popular in targeted analytics.[32] If multiple analytes are concerned, multiple SRM reactions can be monitored at the same time. In this case, the detection technique is appropriately called multiple reaction monitoring (MRM). The m/z values of several precursor or parent ions can be selected in the first mass filter. Afterwards, multiple product ions can be selected for each of the precursor ions. The sensitivity and specificity provided by MRM make it an ideal candidate for use with direct infusion.

For this study, the QTOF was used in full-scan mode, which means no fragmentation took place. In case the QTOF is not sensitive enough for the determination of TS and CS in the study samples, the method can be adjusted to include fragmentation and the use of MRM. In this case, the method is transformed from an LC-MS method to an LC-MS/MS method.

Validation

Validation of an analytical procedure is essential to determine if the method is fit for purpose. Fit for purpose regarding the analysis method of this study is defined as the ability to accurately measure TS and CS in anuran water-bath samples spotted on DFP. A validation plan was set up according to the NEN-EN-ISO 17025 guidelines.[33] The method developed for this study was newly created, this means that the relevant performance characteristics that were to be determined for this study were as follows:

- Limit of detection
- Measuring range
- Repeatability
- Reproducibility
- Selectivity
- Recovery
- Carry-over

An overview of these performance characteristics and how they were calculated is given in the following segments of the report.

Limit of detection

To derive concentrations from the MS signals, the signals were first divided by the slope of the corresponding calibration curve. The traditional method to calculating the limit of detection (LOD) uses the following formula:

$$LOD = 3.3 * S_r$$

Where:

LOD	= lowest analyte concentration likely (>95% chance) to be detectable in measured samples (in µg/mL)
S_r	= within-laboratory repeatability standard deviation, in this case the standard deviation of low concentration samples measured during validation (in µg/mL)

Although the LOD is relatively easy to calculate via this method, it suffers from the structural weakness that no evidence is given to prove that a low concentration of analyte will produce a signal distinguishable from a sample in which there are no analytes present (blank).[34] Therefore, another method was taken into account to calculate the LOD, if necessary.

Officially, the LOD is dependent on the signal an instrument gives off when measuring blanks and the signal of a sample containing a low concentration of analytes.[34] To accurately determine the LOD respecting this dependancy, the limit of blank (LOB) should be calculated. The LOB is defined as the highest expected concentration found in a pre-determined set of blanks.[34] The LOB can be ascertained through the formula presented on the following page:

$$LOB = mean_{blank} + 1.645 * SD_{blank}$$

Where:

LOB	= The highest expected concentration of an analyte found in blanks under reproducible conditions (in µg/mL)
$mean_{blank}$	= The average concentration of analyte in blanks (in µg/mL)
SD_{blank}	= The standard deviation of analyte concentration in blanks (in µg/mL)

Once the LOB has been determined, the following formula can be used to determine the LOD:

$$LOD = LOB + 1.645 * SD_{low\ concentration}$$

Where:

LOD	= The lowest detectable analyte concentration distinguishable from the LOB (in µg/mL)
LOB	= The highest expected concentration of an analyte found in blanks under reproducible conditions (in µg/mL)
$SD_{low\ concentration}$	= The standard deviation of analyte concentration in low concentration sample (in µg/mL)

When using an MS detector and EICs for calculation, the signal of a blank sample could be zero. If this is the case, the traditional method for calculating the LOD will be used.

Measuring range

Inside the measuring range, a linear relation can be found between the intensity of MS counts and concentration of analytes. This means that the concentration in a sample can directly be derived from the given MS counts.

The measuring range is defined as the interval between the lowest and highest concentration measurable at which pre-determined performance characteristics are still valid. For the lower range either the LOD or lowest validated measurand is chosen based on the results of the validation. If no repeated measurements were performed outside the concentration of the calibration standards, the concentration of the lowest and highest standard of the calibration curve were chosen for the lower and upper limit, respectively. In the unlikely case a sample containing an analyte concentration higher than the upper limit of the measuring range is found, the sample can be diluted down to a concentration where it will fall inside of the measuring range.

Repeatability

For this study only the within-day and within-analist repeatability has been taken into account. This means that the repeatability is a performance characteristic that describes the variation in concentration of samples measured by the same instrument and the same researcher on the same day. It is expressed as a percentage related to the spread of the results of a certain set of samples, compared to their average results. Following this description, the repeatability is measured by the relative standard deviation (RSD), also known as the coefficient of variation. Calculated in this manner, the repeatability covers the within-day precision of the analysis method. The repeatability was calculated using the following formula:

$$RSD = 100\% * \frac{s}{\bar{x}}$$

Where:

RSD	= The relative standard deviation, in this case it describes the average spread of the results relative to their average result (in percentage)
s	= The standard deviation of the concentrations of a sample set (in µg/mL)
$\bar{x}$	= The average concentration of a sample set (in µg/mL)

Reproducibility

Reproducibility is a performance characteristic similar to the repeatability, barring a few varied factors. For a new non-normalised analysis method the between-day reproducibility is relevant to be taken into account. The between-day reproducibility describes the variation in results using the same instrument, by the same researcher, on different days of measurement. It is calculated the same way the repeatability is calculated, using the results of a sample set that was taken over multiple days. Other reproducibilities such as between-researcher- or between-instrument reproducibilities could provide additional insight into the overal robustness of the analysis method.

Selectivity

Analytical selectivity is a notoriously hard to define performance characteristic.[35] When chromatography is concerned, it is usually described by the resolution between peaks of interest. A good resolution is considered as complete baseline separation and no interference between eluting analytes. It is calculated through dividing the difference in retention time between two peaks by the sum of the width of both peaks, as such:

$$Resolution = 2 * \frac{t_{r2} - t_{r1}}{w_{b1} + w_{b2}}$$

Where:

Resolution	= Chromatographic peak resolution between two peaks.
t_{rx}	= Retention time of peak x, peak 1 should belong to the peak with the shortest retention time. (in minutes)
w_{bx}	= Peak width at baseline for peak x. (in minutes)

Recovery

The recovery is a determination of how much analytes are recovered during an extraction, compared to the original amount of analytes that are actually present in the sample. Officially, the recovery can be calculated through the following formula:

$$Recovery = 100\% * \frac{\frac{\sum (x_i - x_{blank})}{n}}{x_{theoretical}}$$

Where:

Recovery	= The average recovery of analytes (in percentage)
x_i	= The determined concentration of an analyte in a certain sample (in µg/mL)
x_{blank}	= The determined concentration of an analyte in a blank sample (in µg/mL)
n	= Total number of analysed samples
$x_{theoretical}$	= The theoretical concentration of an analyte in a certain sample (in µg/mL)

As mentioned before, this study used a matrix-matched calibration. Because of this, instead of taking the determined and theoretical concentrations of certain samples, the direct signal given off by the MS of the samples were compared to the signals given off by samples that did not go through the sample preparation process. Therefore, the formula was transformed to:

$$Recovery = 100\% * \frac{\frac{\sum (Signal_i - Signal_{blank})}{n}}{Signal_{np}}$$

Where:

Recovery	= The average recovery of analytes (in percentage)
$Signal_i$	= The signal given off by the detector of an analyte in a certain sample (in MS counts)
$Signal_{blank}$	= The signal given off by the detector of an analyte in a blank sample (in MS counts)
n	= Total number of analysed samples
$Signal_{np}$	= The signal given off by the detector of an analyte in a sample that did not go through the sample preparation process (in MS counts)

The spread of the recovery between samples was an important factor. This was calculated by determining the RSD of multiple singular recovery determinations, in the same way the repeatability was measured.

Carry-over

A carry-over effect occurs when the results of a sample analysis impacts the results of a following sample. It is determined by first measuring a high concentration sample or standard, followed by one or multiple blanks. The percentual concentration found in these blanks compared to the original high concentration sample is called the carry-over effect.

Validation benchmarks

Benchmarks had been set for the validation to be deemed successful. The repeatability and reproducibility could not exceed 15%. The resolution could not be lower than 1.5. The recovery should lie between 80 and 120%, with an RSD of under 15%. Carry-over effects could not exceed 5%. The recommendations for validation of a non-normalized small molecule LC-MS platform listed in this study[36] by Jenkins et al. were used as a reference.

Statistics

Confidence intervals

When depicting a mean value derived from a sample set, a confidence interval shows the interval where the true mean of the population would lie between, with a certainty based on a pre-determined significant chance level.[37] Whenever a confidence interval, also known as the margin of error, is shown, a significance level of 95% was used (α = 0.05). Confidence intervals were calculated using the following formula:

$$Margin\ of\ error = t * \frac{s}{\sqrt{n}}$$

Where:

Margin of error	= 95% confidence interval between which the true value of the calculated mean can be found.
t	= Statistical t-value dependent on sample size and significance level*
s	= Sample standard deviation
n	= Total number of samples

*Values of t for n-1 degrees of freedom and a 95% significance level can be found in appendix 1.[37]

Bartlett's test for homoscedasticity

Homoscedasticity is defined as homogeneity of variances between different populations or sample sets. Assuming a normal distribution of variance across sample sets, Bartlett's test can be used to see if the variance of the sample sets is statistically significantly different or not.[37] The test constructs two hypotheses and tests these against eachother:

Null hypothesis (H_0):	All data sets tested have equal variance.
Alternate hypothesis (H_a):	At least two data sets tested have different variance.

Bartlett's test statistic (X^2) is used to test for these hypotheses, it can be calculated through the following formula:

$$\chi^2 = \frac{(N-k)\ln(S_p^2) - \sum_{i=1}^{k}(n_i - 1)\ln(S_i^2)}{1 + \frac{1}{3(k-1)}\left(\sum_{i=1}^{k}\left(\frac{1}{n_i - 1}\right) - \frac{1}{N-k}\right)}$$

With sample sets 1 through i; where:

X^2	= Bartlett's test statistic
k	= Number of sample sets tested
n_i	= Size of sample set i
S^2_i	= Variance of sample set i
N	= Sum of the sample sizes
S^2_p	= Pooled estimate of variances

Bartlett's test statistic has an approximate Chi-squared distribution, which means it can be directly compared against the upper tail critical value for the Chi-squared distribution for k – 1 degrees of freedom (df), a significance level of 95% (α = 0.05) was used. The critical value can be looked up in table 1:

Table 1: Chi-squared table of critical values.[37]

df (k - 1)	α					
	0.100	0.050	0.025	0.010	0.005	0.001
1	2.7055	3.8415	5.0239	6.6349	7.8794	10.8276
2	4.6052	5.9915	7.3778	9.2103	10.5966	13.8155
3	6.2514	7.8147	9.3484	11.3449	12.8382	16.2662
4	7.7794	9.4877	11.1433	13.2767	14.8603	18.4668
5	9.2364	11.0705	12.8325	15.0863	16.7496	20.5150
6	10.6446	12.5916	14.4494	16.8119	18.5476	22.4577
7	12.0170	14.0671	16.0128	18.4753	20.2777	24.3219
8	13.3616	15.5073	17.5345	20.0902	21.9550	26.1245
9	14.6837	16.9190	19.0228	21.6660	23.5894	27.8772
10	15.9872	18.3070	20.4832	23.2093	25.1882	29.5883

The null hypothesis is rejected if Bartlett's test statistic exceeds the critical value ($X^2 > X^2_{k-1,\alpha}$). If the null hypothesis is accepted, it can be said with 95% certainty, that the sample sets that were tested have equal variances amongst eachother.

Outliers

The acquired data was frequently checked for outliers. This was done using the Grubbs' test for outliers.[38] This test is used to detect single outliers in univariate data sets that are expected to follow a normal distribution. None of the data sets used for analysis indicated the possibility of containing more than one outlier, so there was no need for any other tests for outliers. The Grubbs' test is defined by testing for two hypotheses, these are:

Null hypothesis (H_0): The data set contains no outliers.
Alternate hypothesis (H_a): The data set contains one outlier.

To test for these hypotheses the Grubbs' test statistic is calculated and compared to a critical value based on a certain significance level. The two-sided test statistic is calculated as follows:

$$G = \frac{max\ |y_i - \bar{y}|}{s}$$

Where:
G = Grubbs' test statistic, otherwise denoted as the largest absolute deviation from the sample mean in relation to the standard deviation.
y_i = Value of a certain sample in the data set.
$\bar{y}$ = Mean of the values of the data set.
s = Standard deviation of the values of the data set.

The found test statistic is then compared to a critical value in the following way:

$$G > \frac{(N-1)}{\sqrt{N}} \sqrt{\frac{(t_{\alpha/(2N),N-2})^2}{N-2+(t_{\alpha/(2N),N-2})^2}}$$

Where:

G	= Grubbs' test statistic
N	= Number of samples in the data set.
$t_{\alpha/(2N),N-2}$	= Critical value related to the t-distribution with N-2 degrees of freedom and a certain significance level.

A significance level of 95% was used (α = 0.05). In case the Grubbs' test statistic is found to be larger than the critical value, the null hypothesis is rejected and the value with the highest deviation from the mean is considered an outlier. In case an outlier was found, the outlier was omitted from the data set and was not used for further calculations.

Methods and procedures

Materials

All chemicals used in this study were purchased from VWR in the Netherlands. Liquids were produced by Merck in Germany. These include methanol (MeOH; CAS number: 67-56-1; MS-grade (>99%)), MS-grade water (CAS number: 7732-18-5; LC-MS grade (100%)), formic acid (FA; CAS number: 64-18-6; for analysis (100%)) for the preparation of solvents and solutions. Isopropyl alcohol (IPA; CAS number: 67-63-0; MS-grade (>99.9%)) was used for cleaning the LC and MS systems. Pure analytical standards of TS (CAS number: 58-22-0; >99% pure) and CS (CAS number: 50-22-6; >99% pure) were produced by Acros Organics and Cayman Chemical, respectively. 903 Protein Saver Snap Apart Card Whatman 903™ were used as DFP for the study samples and method development.

Each time samples were to be analysed a set of calibration standards was prepared simultaneously, undergoing the same sample preparation as the samples that were to be measured for a fully matrix-matched calibration line. Calibration standards were prepared on-site using stock solutions prepared during the start of the study. The first stock solution being TS at a concentration of 1000 µg/mL in MeOH and the second solution CS at a concentration of 400 µg/mL in MeOH. Stock solutions were stored at -20 °C throughout the study.

From the stock solutions, a mixed analytical standard containing 250 µg/mL TS and 300 µg/mL CS was prepared. This mixed analytical standard was referred to as Cal8. Cal8 was diluted down by a factor of two for each calibration level, including a hundred-fold dilution level as the final standard. The concentrations and corresponding dilution factors are presented below in table 2:

Table 2: Concentration of the eight calibration solutions when spotted and when analysed, including dilution factor.

Calibration level	Concentration TS during spotting (µg/mL)	Concentration TS when analysed (µg/mL)	Concentration CS (µg/mL)	Concentration CS when analysed (µg/mL)	Dilution factor
Cal8	250	5	300	6	na
Cal7	125	2.5	150	3	2
Cal6	62.5	1.25	75	1.5	4
Cal5	31.3	0.625	37.5	0.75	8
Cal4	15.6	0.313	18.8	0.375	16
Cal3	7.8	0.156	9.4	0.188	32
Cal2	3.9	0.078	4.7	0.094	64
Cal1	2.5	0.050	3	0.060	100
Blank	0	0	0	0	0

MeOH was used for blanks. Since solvent evaporates after spotting, this was considered a similar matrix to that of the anuran water samples. Once prepared, 100 µL of the calibration solutions were spotted on individual spots on marked DFPs and stored in a plastic zip lock bag at room temperature.

Apparatus

The HPLC system used in this study was an Agilent 1200 series HPLC system, including a 1200 series quaternary pump, high performance autosampler, vacuum degasser and column oven (shown left in figure 7).[39] The column used was a Kinetix® 2.6 µm C18 100 Å column with a length and diameter of 100 x 2.1 mm supplied by Phenomenex.[40] The HPLC was connected to a Bruker micrOTOF-Q™ ESI-Qq-TOF mass spectrometer (shown right in figure 7).[27] It was operated through Compass Hystar software.

Figure 7: (left) the Agilent 1200 series HPLC system used for this study[39], (right) the Bruker micrOTOF-Q™ ESI-Qq-TOF mass spectrometer used for this study.[27]

A Finnigan TSQ Quantum Ultra Triple Quadrupole MS, manufactured by Thermo Scientific, was used for direct infusion experiments. It was controlled through Xcalibur software (version 4.0) and calibrated through TSQ Tune.

A 14-part hollow pipe set designed by HBM Machine[41] was used to cut out spots from the DFPs during the extraction process.

HPLC settings

Solvents were prepared on-site using measuring cylinders. The polar solvent (solvent A) was comprised of 95% water, 5% MeOH and 0.1% FA. The non-polar solvent (solvent B) was comprised of 5% water, 95% MeOH and 0.1% FA. The column oven was set to a constant 40 °C, the flowrate was 0.2 mL/min and injection volume was 5 µL.

Sampling

82 Túngara frogs (*Physalaemus pustulosus*) were sampled at Pipeline Road near Gamboa, a small town in the Republic of Panama. After being captured in their natural habitat, the frogs were dried off and placed in 50 mL falcon tubes. 1.5 mL of purified water was added to the tubes. After a five-hour waiting period, 20 to 320 µL of the water in the tubes was spotted on each of the spots on the DFP, the exact volume was noted. The frogs were then released back into the wild, unharmed. The spotted DFPs were stored in a plastic zip lock bag at room temperature and taken back to the Netherlands for further analysis.

Extraction

To extract the hormones from the DFPs the spots were individually cut-out using a hollow pipe set.[41] Unless noted otherwise, a 13 mm attachment part was used to cut the spots out of the DFP. The cut-out spot was then placed in a 2 mL marked container and 1 mL MeOH was added, making sure the spot was completely submerged. The container was then held stationary for twenty minutes to allow the analytes to migrate from the DFP to the solvent. Exactly 200 µL of the extract was pipetted into a marked LC-vial and diluted with 800 µL MS-grade water, finishing the extraction.

MS settings

The QTOF was set to full scan mode. Scanning was done using a prefabricated scanning method designed for the analysis of small ions, such as metabolites. This method scanned 120 times per minute with a range of 50 to 1000 m/z in positive ionization mode. The source was cleaned with MS-grade water and IPA between each 24-hour period of accumulative runtime, after which the system was recalibrated using a generic MS tuning mix. A calibration report using direct fusion of an analytical solution of TS and CS on a QqQ MS system is listed in the results section of this report.

TS has a monoisotopic molecular weight of 288.2 g·mol^{-1}. The extracted ion chromatogram (EIC) for TS was set to 289.2 (± 0.05) m/z, taking into account the expected m/z corresponding to $[M+H]^+$ in positive ionization mode. CS has a monoisotopic molecular weight of 346.2 g·mol^{-1}. The EIC for CS was set to 347.2 (± 0.05) m/z, also taking the corresponding expected m/z into account. All calculations were made using the EICs corresponding to the $[M+H]^+$ values.

Due to the presence of sodium ions (Na^+, 23 g·mol^{-1}) in the system, EICs corresponding to $[M+Na]^+$ were also taken into consideration, although eventually no calculations were performed using these data. The m/z values for these ions correspond to 311.2 (± 0.05) for TS and 369.2 (± 0.05) for CS.

An example of an MS spectrum of TS is given in figure 8. The two largest fragments are 289.2213 m/z which corresponds with the expected $[M+H]^+$ value and 311.2023 m/z which corresponds with the expected value for $[M+Na]^+$.

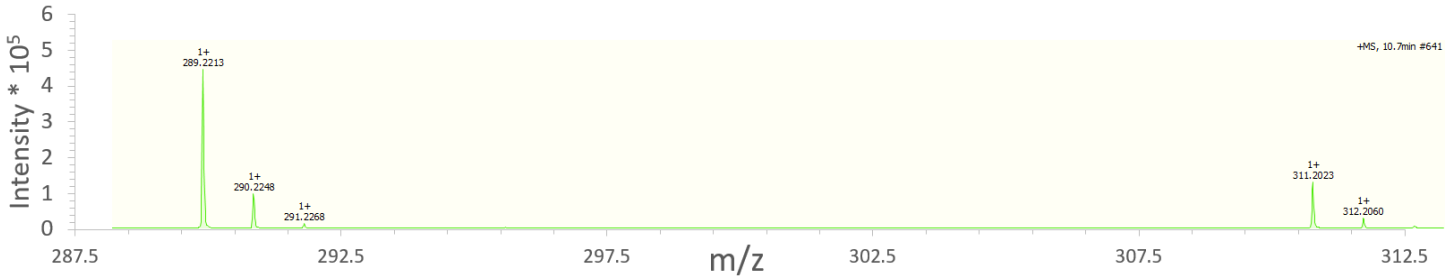


Figure 8: MS spectrum of a single analytical standard of testosterone, eluting at 10.7 minutes in the corresponding chromatogram. m/z values are listed on the x-axis and intensity of MS counts is shown on the y-axis.

An example of an MS spectrum of CS is given in figure 9. Noteworthy fragments (from left to right) are 329.2165 m/z which corresponds with the expected value of $[M+H]^+$ minus the loss of H_2O, 347.2274 m/z which corresponds to the expected $[M+H]^+$ value and 369.2091 m/z which corresponds to the expected $[M+Na]^+$ value. All found peaks in both spectra were in line with expectations.

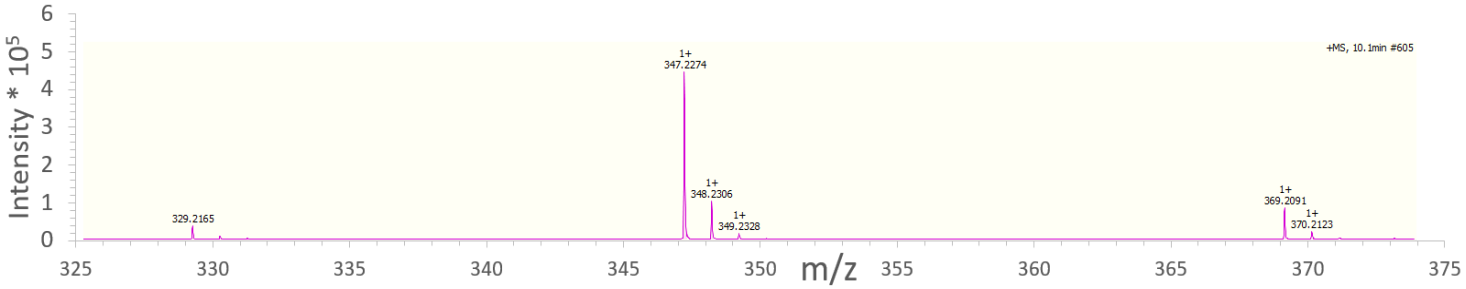


Figure 9: MS spectrum of a single analytical standard of corticosterone, eluting at 10.1 minutes in the corresponding chromatogram. m/z values are listed on the x-axis and intensity of MS counts is shown on the y-axis.

Results & discussion

An overview of the results including brief discussions will be given in the following section of the report.

HPLC Optimization

The gradient used for elution was optimized to reduce analysis time per sample as much as possible without inducing carry-over and respecting the chromatographical resolution between peaks of interest. The gradient was set to start at 20% solvent B instead of the conventional 0% start. The starting conditions were held for one minute to allow for retention and separation of the analytes. The total runtime per sample, including equilibration time, was 15 minutes. The full gradient is listed below in table 3.

Table 3: HPLC gradient used in this study. Showing time in minutes in the first column and corresponding percentage of solvent B in the second column.

Time (min)	Solvent B (%)
0	20
1	20
4	100
10	100
10.1	20
15	20

Examples of integrated and overlaid EIC chromatograms at m/z values 289.2 (± 0.05) in green and 347.2 (± 0.05) in purple that were used for the analysis of samples are presented below in figure 10 and on the following page in figure 11. Shown are the chromatograms of measured samples containing a low concentration of analytes in figure 10 and a measured sample containing a high concentration of analytes in figure 11. Both samples were measured after going through the full sample preparation process. The slight baseline drift seen in figure 10 was found to have no impact on the analyses.

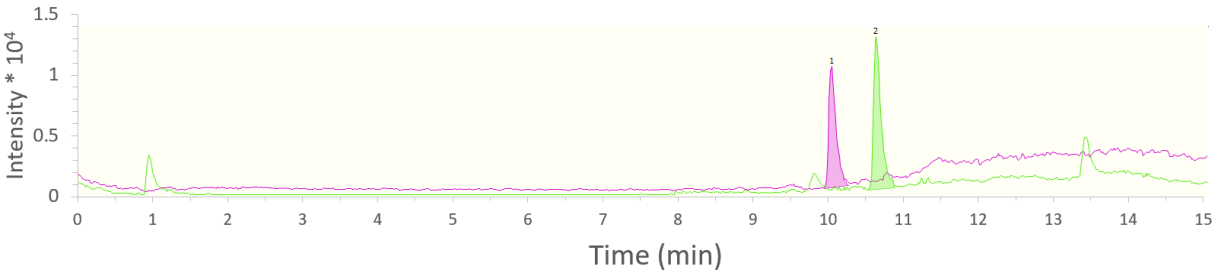


Figure 10: An integrated and overlaid chromatogram of a low concentration mixed analytical standard of 0.05 µg/mL testosterone in purple and 0.06 µg/mL corticosterone in green. On the x-axis time during the analysis run is listed in minutes, on the y-axis the intensity of MS counts is shown.

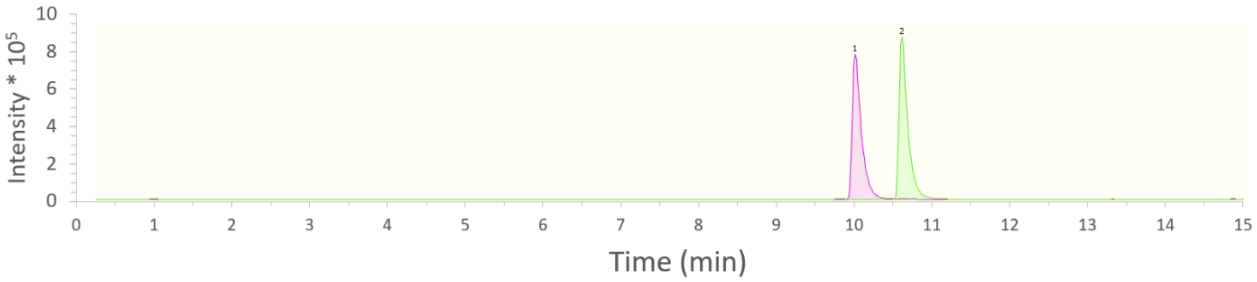


Figure 11: An overlaid chromatogram of a high concentration mixed analytical standard of 5 µg/mL testosterone in purple and 6 µg/mL corticosterone in green. On the x-axis time during the analysis run is listed in minutes, on the y-axis the intensity of MS counts is shown.

An example of a total ion chromatogram of an analysis run from which the EICs are derived is presented in figure 12. The two peaks labeled 1 and 2 (10.1 and 10.7 minutes in the chromatogram) are the peaks of corticosterone and testosterone, respectively. The baseline drift and subsequent peaks were not relevant to be examined since they had no significant effect on the aforementioned peaks of interest.

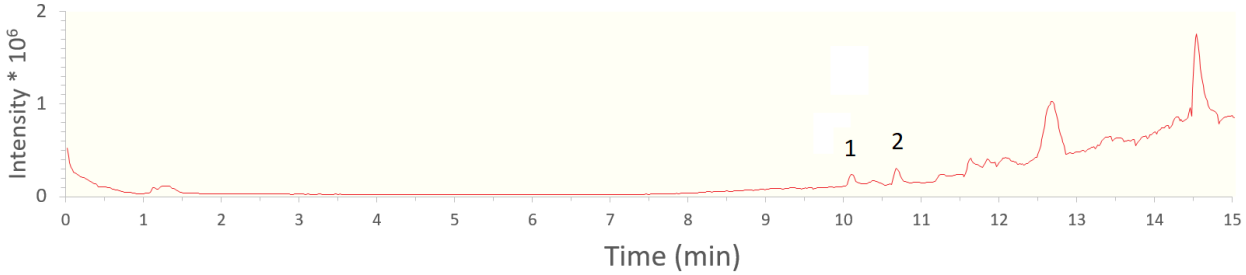


Figure 12: A total ion chromatogram of a mixed analytical standard of 0.3125 µg/mL testosterone and 0.3750 µg/mL corticosterone extracted from dried filter paper. Peaks labeled 1 and 2 are those of corticosterone and testosterone, respectively. Time is shown in minutes on the x-axis and intensity of MS counts is shown on the y-axis.

Calibration curve

For every batch of measurements a newly prepared calibration curve was measured to ensure quality of acquired data. All standards underwent the full sample preparation process, to correct for possible matrix effects. An example of the results of such a calibration curve, prepared and measured in two-fold, is presented below in table 4:

Table 4: Raw data of a set of calibration standards of testosterone and corticosterone, prepared and measured in two-fold. The concentrations and corresponding MS counts are listed for both analytes individually.

ID	Concentration testosterone (µg/mL)	MS counts 289.2 (± 0.05) m/z	Concentration corticosterone (µg/mL)	MS counts 347.2 (± 0.05) m/z
Blank	0	0	0	0
Cal1	0.05	109535	0.06	85050
		99173		76764
Cal2	0.078125	117536	0.09375	98120
		119618		88362
Cal3	0.15625	288101	0.1875	245974
		271435		245988
Cal4	0.3125	606624	0.375	487573
		606366		507817
Cal5	0.625	993895	0.75	866240
		921867		825700
Cal6	1.25	2027570	1.5	1650828
		2002792		1796160
Cal7	2.5	3225595	3	2863396
		3299198		2842977
Cal8	5	6320931	6	5937350
		6052237		5845118

Using the data from table 4, the calibration curve can be presented for both TS and CS in a visual representation. Examples of such a visual representation of both calibration curves are given in figures 13 and 14 below:

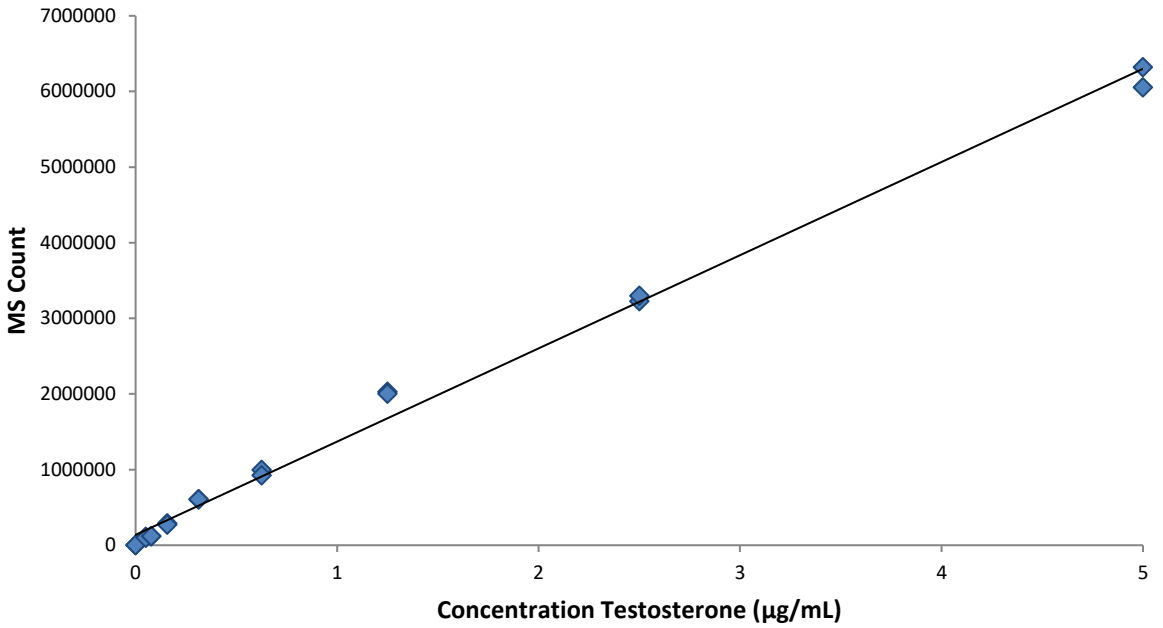


*Figure 13: Calibration line for testosterone, prepared and measured in two-fold. Formula: Concentration Testosterone (µg/mL) = 150619 (± 10220) + 1228553 (± 51865) * MS Count, α = 0.05, S_r = 160063, n = 17, R^2 = 0.9942.*

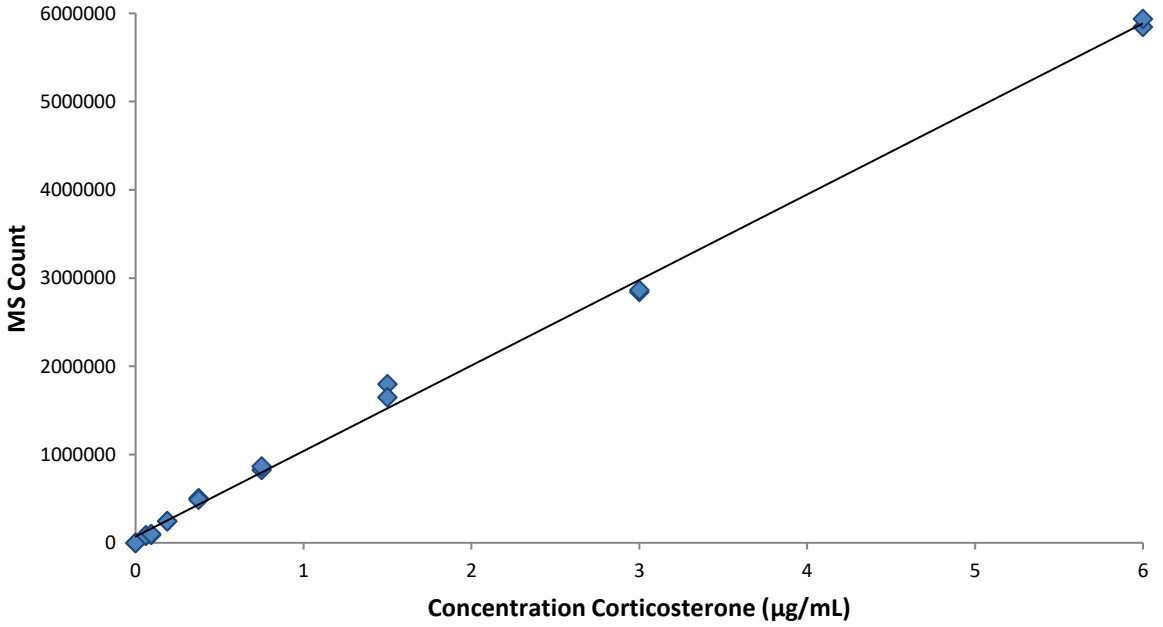


*Figure 14: Calibration line for corticosterone, prepared and measured in two-fold. Formula: Concentration Corticosterone (µg/mL) = 76479 (± 65875) + 967859 (± 27721) * MS Count, α = 0.05, S_r = 102662, n = 17, R^2 = 0.9973.*

Validation

To calculate the LOD, measuring range and repeatability standards at low, middle and high concentrations were prepared and measured in six-fold. The corresponding data is presented below in table 5.

Table 5: Data set used for the determination of the LOD, measuring range and repeatability. Six repetitions were performed at a low, middle and high concentration level. The concentration at the levels correspond with the concentration of calibration standards Cal1, Cal4 and Cal8 respectively. The concentrations and MS counts are listed per sample individually.

ID	Concentration Testosterone (µg/mL)	MS Counts 289.2 (±0.05) m/z	Concentration Corticosterone (µg/mL)	MS Counts 347.2 (±0.05) m/z
Blank	0	0	0	0
Cal1	0.05	96[illegible]	0.06	83609
		100695		90981
		97095		79862
		98520		90401
		85536		86648
		93545		87583
Mean (Cal1)	0.05	95243.7	0.06	86514.0
S_r (Cal1)	0.00279	5323.3	0.00292	4216.9
LOD	0.00922	NA	0.00965	NA
Cal4	0.3125	609450	0.375	530943
		561254		443909
		615982		546465
		544361		480196
		588641		552463
		591735		532066
Mean (Cal4)	0.3125	585237.2	0.375	514340.3
S_r (Cal4)	0.01478	27681.6	0.03128	42898.9
Cal8	5	5885317	6	6027045
		5392531		5613840
		5460779		4413172
		5715772		5137487
		5853059		5160059
		5470427		5131588
Mean (Cal8)	5	5629647.5	6	5247198.5
S_r (Cal8)	0.19164	215772.6	0.62026	542435.7

Limit of detection

The lowest calibration standard (Cal1) was sampled, extracted and measured in six-fold to determine the LOD. The signal of TS and CS in blank samples was zero, therefore the traditional method of calculating the LOD was used.

As shown in table 5, the LOD for TS was 9.22 (± 2.93) * 10^{-3} µg/mL and the LOD for CS was 9.65 (± 3.07) * 10^{-3} µg/mL.

Measuring range

The pre-determined benchmark for repeatability of the limits of the measuring range was set at a maximum of 15% RSD.

No actual measurements were performed at the LOD level of concentration. Therefore, the repeatability at this level of concentration was not determined and the LOD could not be used for the lower limit of the measuring range. For the lower limit of the measuring range, the repeatability of the lowest concentration calibration standard (Cal1) was determined. This standard was sampled, extracted and measured in six-fold to determine the repeatability at a low concentration level. Referring to table 5, the repeatabilities of TS and CS at the lower limit of the measuring range were 5.59% and 4.87%, respectively. The found repeatabilities met the benchmark requirement of <15% and the lower limit of 0.05 µg/mL for TS and 0.06 µg/mL for CS was accepted.

For the upper limit of the measuring range, the repeatability of the highest concentration calibration standard (Cal8) was determined. This standard was sampled, extracted and measured in six-fold to determine the repeatability at a high concentration level. Referring to table 5, the repeatabilities of TS and CS at the upper limit of the measuring range were 3.83% and 10.34%, respectively. The found repeatabilities met the benchmark requirement of <15% and the upper limit of 5 µg/mL for TS and 6 µg/mL for CS was accepted.

In summary, the measuring range for TS was 0.05 to 5 µg/mL. The measuring range for CS was 0.06 to 6 µg/mL.

Repeatability

Measurements performed for the determination of the repeatability were done on the same day, on the same instrument and by the same analist. This means the results represent the within-day and within-analist repeatability.

The repeatability of the analysis method was determined at three different levels of concentration. For the low level of concentration the lower limit of the measuring range was used, this was at 0.05 µg/mL for TS and 0.06 µg/mL for CS. For the medium level of concentration one of the middle calibration standards (Cal4) was used at 0.3125 µg/mL for TS and 0.375 µg/mL for CS. For the high level of concentration the upper limit of the measuring range was used, this was at 5 µg/mL for TS and 6 µg/mL for CS. All standards were sampled, extracted and measured in six-fold. Using the data from table 5, the calculated repeatabilities are presented below in table 6.

Table 6: Within-day & within-analist repeatability at a low, medium and high concentration level of the measuring range (n = 6).

Repeatability	Testosterone	Corticosterone
Low level	5.59%	4.87%
Medium level	4.73%	8.34%
High level	3.83%	10.34%

As seen in table 6, the repeatability for the analysis method was 3.83% to 5.59% for TS and 4.87% to 10.34% for CS, depending on the concentration level. Noteworthy is the increasing repeatability for CS at higher concentration levels. Normally, repeatability and concentration have a negative correlation which would mean that repeatability would decrease at higher concentration levels. Since the found repeatability of 10.34% for CS at a high concentration level is lower than the validation benchmark of 15%, the cause for these unexpected results has not been determined.

The assumption was made that the repeatability of the instruments that were used was insignificantly low, therefore this repeatability was not determined during method development. In case the actual study samples are to be measured, the instrument repeatability should be determined beforehand. The instrument repeatability can relatively easily be determined by measuring a certain sample multiple times (a minimum of six times is recommended) in succession.

Reproducibility

The reproducibility was not yet determined. Several measurements were done on different days during validation. Variation between the results of these measurements was found, but a confounding factor made these results invalid for the calculation of the reproducibility.

In an article by Newman et al.[23], estradiol and progesterone showed no signs of degradation for a maximum of three months when stored on DFP similar to the DFP used for this study, at room temperature. The assumption was made this was also true for TS and CS. Results gained during the validation of the reproducibility showed signs of decreasing analyte signal when measured over the course of several days. Variation in results related to the between-day reproducibility of the analysis method were expected to have a normal distribution. The decrease in analyte signal could possibly have been caused by degradation, this was not tested. To test whether the found variation was caused due to degradation, and to eventually determine the between-day reproducibility, a suggestion for a follow-up experiment has been listed in the recommendations section of this report.

Selectivity

The validation benchmark for selectivity was a minimum resolution of 1.5. To see if this benchmark was reached the selectivity for one of each of the calibration levels was determined. The full data set can be found in appendix 2.

The standards containing the highest concentration of analytes (Cal8) had the broadest peaks. Therefore, these samples had the lowest selectivity and were the critical samples to determine the selectivity for. For Cal8, the peak belonging to CS which had a retention time of 10.1 minutes had a peak width at base of 0.400 minutes. The peak belonging to TS which had a retention time of 10.7 minutes had a peak width at base of 0.392 minutes. Using the formula presented in the validation theory section of this report, the selectivity of Cal8 was 1.516.

Since the found minimum selectivity of 1.516 was higher than the benchmark of 1.5, combined with the fact that the selectivity for other samples was higher than that of Cal8, the selectivity of the analysis method was deemed fit for purpose.

Recovery

To determine the recovery of the extraction method, four samples that went through the full sample preparation process were compared to three samples that were measured without going through the sample preparation process. All samples measured for the recovery determination contained 1 µg/mL TS and CS. As mentioned in the procedures section of this report, the sample preparation process is defined as spotting and afterwards extracting TS and CS from DFP before measurement. The full data set used to calculate recovery can be found in appendix 3. A summary of the relevant results regarding the initial recovery determination is presented below in table 7.

Table 7: Determined recovery of the four samples that were compared to three samples that did not go through the sample preparation process, all of the samples contained 1 µg/mL TS and CS. Listed are the total MS counts for both analytes, the calculated recovery per sample and average recovery of the sample set including 95% confidence interval.

ID	MS Counts 289.2 (±0.05) m/z	Recovery testosterone (%)	MS Counts 347.2 (±0.05) m/z	Recovery corticosterone (%)
Sample 1	1506982	31.81	1202805	33.90
Sample 2	1631408	34.44	1272200	35.86
Sample 3	1332916	28.14	1013289	28.56
Sample 4	1410290	29.77	1129284	31.83
Mean	1470399	31.04 (± 4.33)	1154395	32.54 (± 4.96)

The recovery for TS was 31.04% (± 4.33%). The recovery for CS was 32.54% (± 4.96%). The found recoveries for both analytes was too low to pass the validation.

The results of this determination inspired an experiment to see if the low recovery was caused due to cutting out a relatively small portion of the DFP. In the original extraction, a 13 mm part was used to cut out a circle out of the DFP. To test whether the recovery was related to the size of the cut-outs or not, several extractions were performed using 6, 10, 16 and 19 mm parts. The rest of the sampling and extraction process was unchanged. Six repetitions were performed for each of the cut-out sizes. Using the data listed in appendix 3, figures 15 and 16 show the results of this recovery experiment. As shown in table 8, an increase of recovery is seen when the cut-out size increases. To see whether the recovery and cut-out size are linearly correlated, the total area of the cut-outs was compared to the recovery, the results are presented on the following page in figure 15 for TS and in figure 16 for CS:

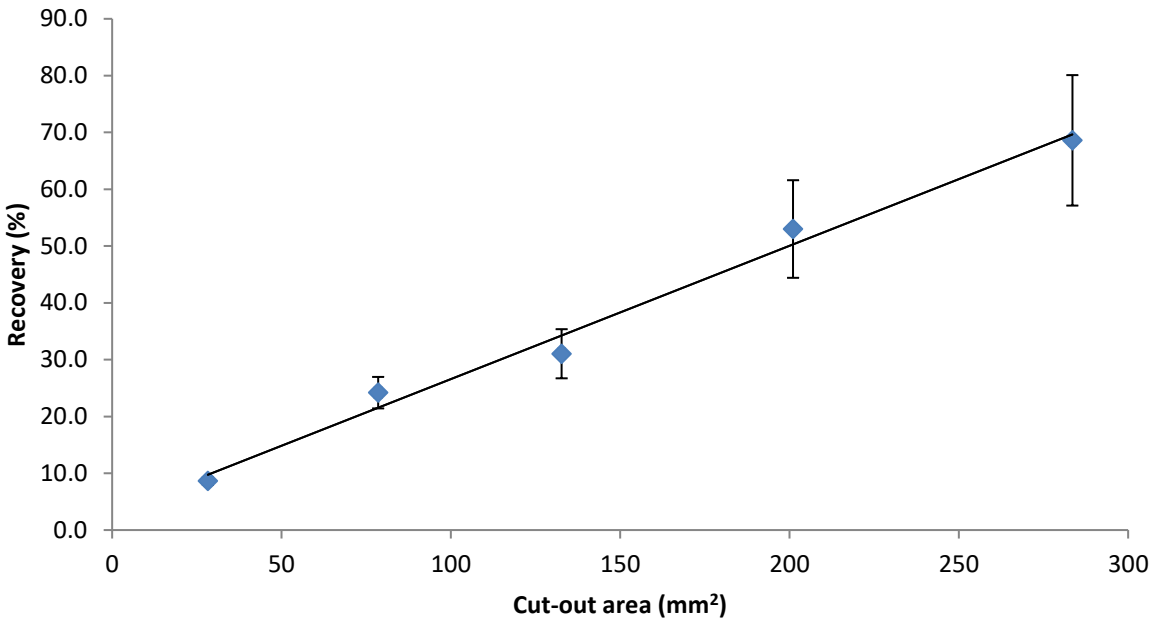


Figure 15: Scatter plot including 95% confidence intervals displaying the relation between the recovery of testosterone in percentages on the y-axis compared to the area of cut-out DFP spots in mm^2 on the x-axis. The R^2 of the correlation is 0.9881.

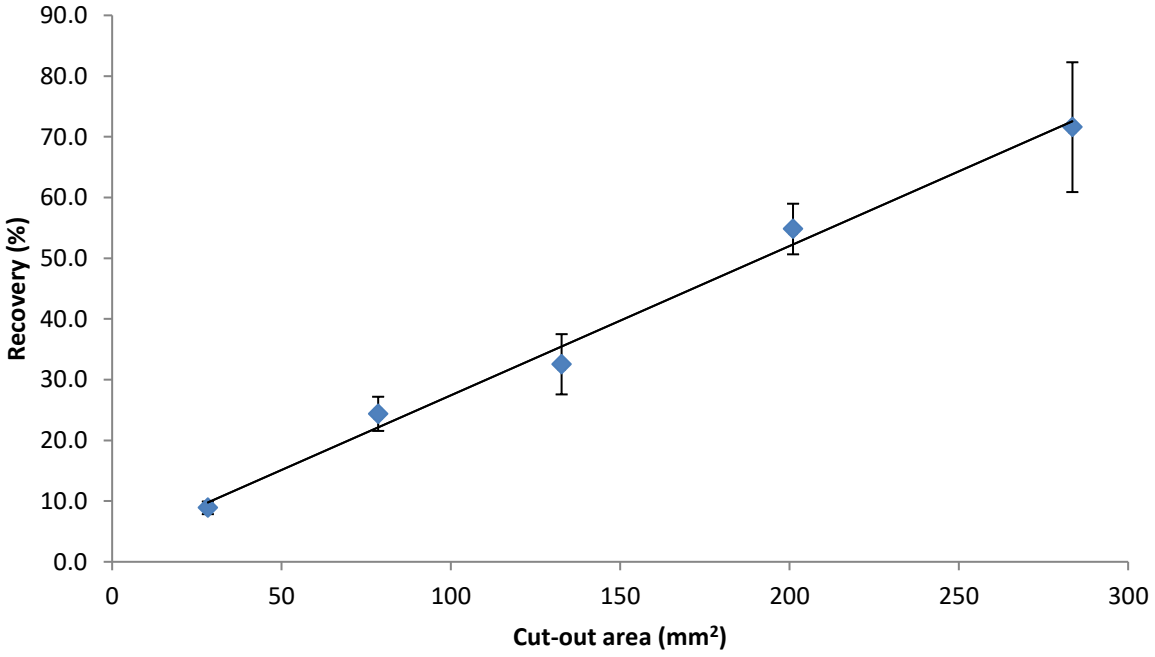


Figure 16: Scatter plot including 95% confidence intervals displaying the relation between the recovery of corticosterone in percentages on the y-axis compared to the area of cut-out DFP spots in mm^2 on the x-axis. The R^2 of the correlation is 0.9912.

For CS, a statistically significant difference is found between the recoveries at each of the different cut-out sizes. For TS, there was no statistically significant difference between 10 and 13 mm cut-outs and 16 and 19 mm cut-outs. A strong correlation between cut-out area and recovery for both analytes was found, namely an R^2 of 0.9881 for TS and 0.9912 for CS. The means, including 95% confidence intervals, and RSD of the found recoveries for both analytes is presented on the following page in table 9 to see if the diffferences between the sample sets is statistically significant:

Table 9: Results of the recovery experiment with varying sample preparation processes. The mean recovery, including 95% confidence interval, along with the RSD for each of the sample sets per analyte is listed.

ID	Mean recovery TS (%)	95% confidence interval recovery TS (%)	RSD recovery TS (%)	Mean recovery CS (%)	95% confidence interval recovery CS (%)	RSD recovery CS (%)
6 mm	8.64	0.70	7.86	8.89	1.04	10.48
10 mm	24.19	2.77	11.07	24.37	2.81	6.60
13 mm	31.04	4.33	8.76	32.54	4.96	9.59
16 mm	53.00	8.59	5.36	54.81	4.17	1.66
19 mm	68.61	11.48	11.04	71.59	10.69	11.91

A relatively large difference is seen between the variance of the different sample sets. To test whether the variance of the groups is statistically significantly different a Bartlett's test for homoscedasticity was performed, assuming that the variance amongst groups is normally distributed. No difference amongst the variance of the five groups was found for either TS and CS. This means to achieve a higher recovery, without significantly affecting the repeatability of the analysis method, bigger cut-outs could be made. An example of an adjusted sample preparation method that uses a larger portion of the DFP for extraction is listed in the recommendations section of the study.

Carry-over

Blanks measured after measuring the highest concentration standards showed zero signs of carry-over for TS and CS. So in short, the carry-over effect for both analytes was 0%.

Validation summary

A summary of the full validation of the analysis method, including whether the pre-determined benchmarks for validation were met or not, is listed below in table 10.

Table 10: Summary of the full validation of the analysis method devised during the course of this study. Included is the color-coded comparison to the benchmark values set to determine whether the method is deemed fit for purpose or not regarding certain parts of the validation.

Performance characteristic	Value testosterone	Value corticosterone	Fit for purpose (Yes/No)
Limit of detection	9.22 (± 2.93) * 10^{-3} µg/mL	9.65 (± 3.07) * 10^{-3} µg/mL	Yes*
Measuring range	0.05 to 5 µg/mL	0.06 to 6 µg/mL	Yes*
Repeatability	Low: 5.59%	Low: 4.87%	Yes
	Medium: 4.73%	Medium: 8.34%	Yes
	High: 3.83%	High: 10.34%	Yes
Reproducibility	Not yet determined.		No
Selectivity	≥1.516		Yes
Recovery	31.04% (± 4.33%)	32.54% (± 4.96%)	No
Carry-over	0%	0%	Yes

*Whether the LOD and measuring range are fit for purpose is dependent on the expected concentration of TS and CS in the anuran water samples. In case the anuran water samples contain much lower concentrations of TS and CS than the lower limit of the measuring range, several adjusted sample preparation methods that allow for preconcentration listed in the recommendations section of this report should be revised and the analysis method would need to be re-validated.

As seen in table 10, the analysis method is not yet fully deemed fit for purpose based on the pre-determined validation benchmarks set prior to the validation, namely the undetermined between-day reproducibility and too low recovery.

Direct Infusion

MS calibration

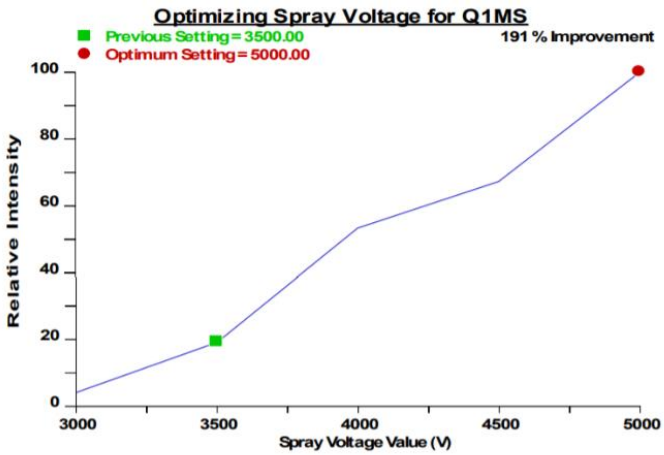
Since the QTOF was calibrated and tuned using generic settings, including a generic tuning mix for calibration and a relatively broad scanning window (50-1000 m/z), calibration specifically tuned to the target analytes could improve future results. To investigate the possibility of improving the instrument sensitivity to TS and CS, a direct infusion experiment was performed on a QqQ. For this experiment, the MS settings were optimized using TSQ Tune, to achieve the highest possible relative signal for the analytes. The experiment was performed through directly injecting mixed analytical standards of TS and CS at a concentration of approximately 1 µg/mL in 100% MeOH, steady at 5 µL/min. Initially, the two highest signal peaks were chosen as precurser ions. These corresponded to 288.7 m/z for TS and 346.8 m/z for CS. The relative intensity of ions passing by the first QMS (Q1MS) was monitored. Per setting, a relative increase in signal of 5% or more was considered significant. In total, a 1209% improvement in relative intensity was achieved. The results of the optimization process are presented below in table 11.

Table 11: Results of the optimization of the QqQ used for direct infusion. The percentual improvement was based on the relative intensity of precurser ions for TS and CS passing through Q1MS.

Parameter	Default setting	Optimum setting	Improvement (%)
Spray voltage (V)	3500	5000	191
Vaporizer temperature (°C)	38	193	73
Sheath gas pressure (psi)	35	30	Insignificant*
Capillary temperature (°C)	270	259	12
Tube lens voltage (V)	115	140	80
Skimmer offset energy (V)	0	12	29

*The improvement gained through the optimization of the sheath gas pressure setting was insignificant (<5%), therefore the default setting of 35 psi was used for further analyses.

The optimization curves are presented in figures 17A through 17F (continued on the following page), and briefly individually discussed in the following section:



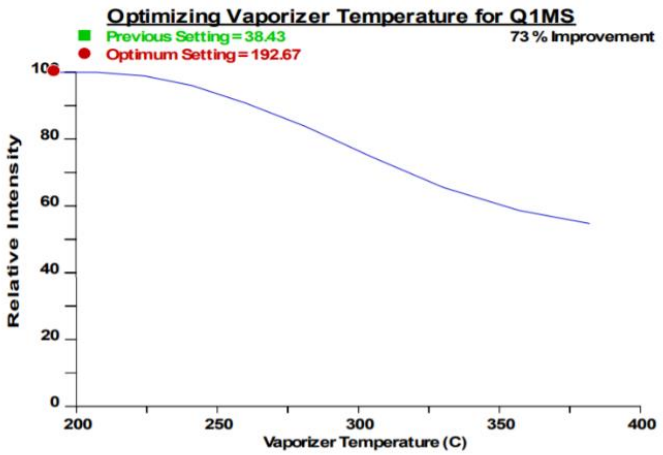


Figure 17A – 17F (top left to bottom right; continued on the following page): Optimization curves for direct infusion on the QqQ MS. The Y-axis, on all curves, shows the relative intensity of signal given off by the first QMS in percentages. For the X-axes: (A): Spray voltage. (B): Vaporizer temperature in °C.

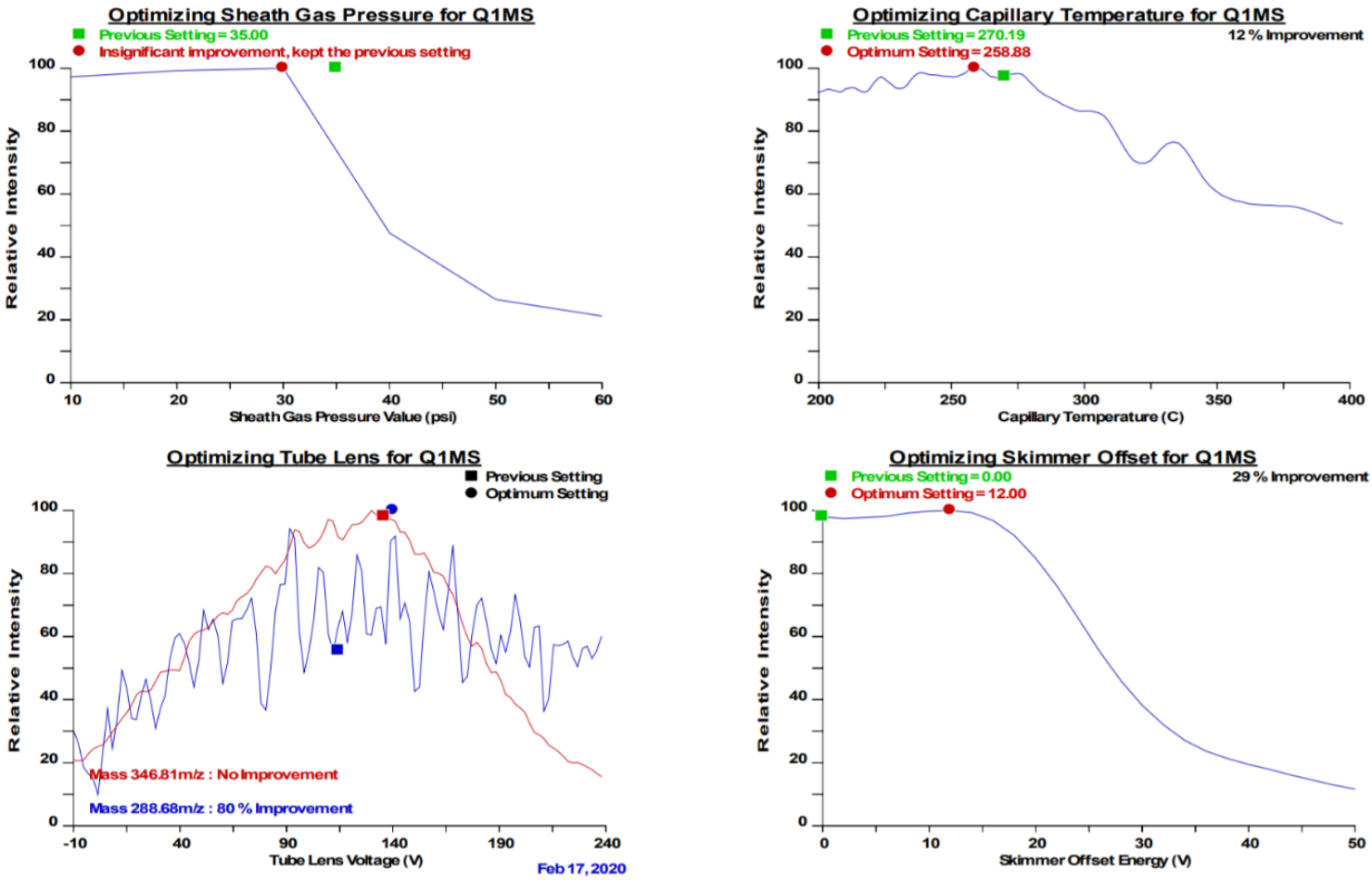


Figure 17C – 17F: Continuation of optimization curves for parameter optimization on the QqQ MS. The Y-axes show the relative intensity in percentages. For the X-axes: (C): Sheath gas pressure in psi. (D): Capillary temperature in °C. (E): Tube lens voltage. (F): Skimmer offset energy in voltage.

Brief description of the optimization process:

In figure 17A, a positive correlation between spray voltage and relative intensity can be deduced. 5000V was the maximum available setting for a 191% improvement in relative intensity. For vaporizer temperature in figure 17B, the vaporizer was set to the highest possible setting, 400 °C. Afterwards, the vaporizer was set to cool off. When the derivative function of the temperature vs relative intensity hit zero, this was deemed the optimum setting. This point was reached at 193 °C for a 73% improvement in relative intensity. For the sheath gas pressure in figure 17C, the software indicated that no improvement was found. This does not seem clear from the optimization curve provided, this is because only increments of 10 psi were plotted on the curve. For the capillary temperature in figure 17D, only a minor increase of 12% in relative intensity was found at 259 °C. For the tube lens voltage in figure 17E, the voltage was first optimized looking at the mass of the precurser ion for TS (288.7 m/z). An 80% improvement was found at 140V. Afterwards, the software looked at the mass corresponding to the precurser ion for CS (346.8 m/z), using the new default setting of 140V. No improvement was found over the previous setting of 140V. Finally, as seen in figure 17F, the skimmer offset energy was found to have a clear maximum at 12V for a 29% improvement in relative intensit

Once the settings for the precurser ions were optimized, the optimum pressure (in mTorr) in the collision cell was ascertained. The most abundant product ion was chosen, this was the ion corresponding to an m/z of 97.0, originating from the 288.7 m/z precurser ion. The optimization curve shown in figure 18A shows the results of the collision pressure optimization process. The collision pressure in millitorr pressure units was set against the relative intensity of the product ions. An optimum collision pressure of 1.50 mTorr was found.

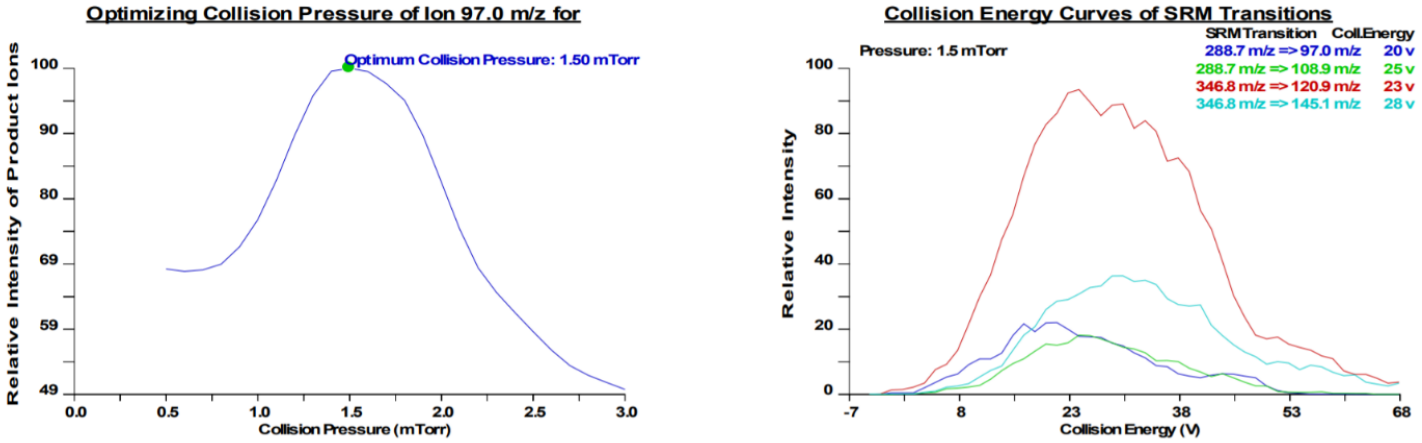


Figure 18: (A; left): Optimization curve for the collision pressure inside the hexapole collision cell. The Y-axis shows relative intensity of the product ions monitored in Q3MS. The X-axis shows the collision pressure in millitorr pressure units (mTorr). (B; right): Collision energy curves for the SRM transition

For each precurser ion, the two product ions with the highest relative intensity were chosen for further monitoring. For the precursor ion at 288.7 m/z, the two most abundant product ions were measured at 97.0 and 108.9 m/z. For the precursor ion at 346.8 m/z, the two most abundant product ions were measured at 120.9 and 145.1 m/z. Figure 18B shows the optimization curves for the collision energy for each of the ion transitions. This lead to the following four SRM transitions for the most optimum MRM measurement of TS and CS, shown in table 12:

Table 12: Optimum MRM settings for the measurement of TS and CS.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
Testosterone	288.7	97.0	20
		108.9	25
Corticosterone	346.8	120.9	23
		145.1	28

The masses of the precursor ions were slightly off the expected $[M+H]^+$ values. Due to this reason, combined with the unavailability of a suitable internal standard, for which the reason is listed in the recommendations section, no useful direct infusion analyses have yet been performed. The information provided in this section can be used as a basis to see if direction infusion can be used during continuation of this study to analyze TS and CS in anuran water samples.

Conclusion

Reflecting on the goals of this study, the platform examined in this study shows indefinite yet promising results for examining the physiology of anurans in a non-invasive manner. The sample preparation method, coupled with the LC-MS analysis method made to compare anuran water-bath samples stored on DFP by their levels of TS and CS concentration, was fully validated, but did not pass validation based on several aspects.

The LOD of the analysis method was 9.22 (± 2.93) * 10^{-3} µg/mL for TS and 9.65 (± 3.07) * 10^{-3} µg/mL for CS. The measuring range of the analysis method was 0.05 to 5 µg/mL for TS and 0.06 to 6 µg/mL for CS. The within-day and within-analist repeatability of the analysis method at a low concentration level was 5.59% and 4.87% for TS and CS, respectively. The within-day and within-analist repeatability of the analysis method at a medium concentration level was 4.73% and 8.34% for TS and CS, respectively. The within-day and within-analist repeatability of the analysis method at a high concentration level was 3.83% and 10.34% for TS and CS, respectively. The lowest selectivity of the analysis method for samples with the broadest peaks was at a resolution of 1.516. There were zero signs of carry-over effects. The analysis method passed validation based on repeatability, selectivity and carry-over effects.

The reproducibility of the analysis method could not be determined because of degradation of the analytes when stored on DFP being a possible confounding factor. The recovery of TS and CS after extraction was 31.04% (± 4.33%) and 32.54% (± 4.96%), respectively. The platform did not pass the validation on basis of reproducibility and recovery, and was deemed not fit for purpose based on these performance characteristics. Based on the results of the validation, the method can not be used to analyze anuran water samples.

Direct infusion, along with using MRM detection on a QqQ system, shows promising results for analyzing anuran water samples as long as ion suppression is no significant confounding factor. This analysis and detection technique has not yet been validated.

A strong foundation has been laid out for analyzing anuran water samples using DFP as a sample medium and LC-MS as an analysis technique. Minor adjustments will be listed in the recommendations section to finalize the platform as a complete product.

Recommendations

Internal standard

No internal standards were used during analyses for this study. Although most steroids are similar to TS and CS, regarding chemical structure and chromatographic response, other steroids are no good candidates for being used as internal standards. This is because there is a high probability they are also present in the study samples. For the continuation of this study, the use of deuterated or synthetic analytical standards of steroid hormones as internal standards is recommended. Due to high costs and lack of availability, the development of the analysis method for this study was done without the use of deuterated or synthetic analytical standards of steroid hormones as possible internal standards.

Steroid hormones are relatively hard to come by. Most are only available by ordering them through Steraloids (www.steraloids.com), a steroid hormone production company based in Newport, Rhode Island (USA). The international transport of steroid hormones is heavily regulated and requires filling out several judicial forms to justify importing them.

Between-day reproducibility

A confounding factor was found when the reproducibility was being assessed. Unexpectedly, signs of degradation of TS and CS were found while storing DFP samples at room temperature. To correct for this effect, an experiment was devised which should be executed before analyzing the anuran water samples. To correct for degradation while determining the between-day reproducibility, several similar samples should be measured over the course of atleast a few days (preferably weeks or even months, if possible) while keeping the time between spotting the DFP and time of measurement constant. The results of this experiment should give an accurate representation of the between-day reproducibility of the analysis method.

Adjusted sample preparation methods

Cleaning

In case the anuran water samples contain a significant amount of pollutions which affect the quality of results, a cleaning step can be added to the sample preparation process. Possible pollutants might be removed during the cleaning process, reducing signal noise. Besides removing pollutants, this step could allow for preconcentration.

Two possible sample preparation cleaning methods that are worth exploring are presented below:

- Solid Phase Extraction:

Solid phase extraction (SPE) is a widely used method of sample preparation. The principles of SPE are similar to those of liquid chromatography. The samples are first loaded onto a preconditioned column or cartridge. The components that have greater affinity with the packing material, compared to the original solvent, are trapped onto the column or cartridge. The solvent, including pollutants, is washed away. The columns are then left to dry. Finally, the column is washed with a similar solvent as the original solvent with which the analytes had a good affinity, eluting the compounds of interest. Afterwards, the solvent containing the analytes could be evaporated and the analytes could be

resuspended in a significantly smaller volume, greatly increasing the sensitivity of the analysis method. Although some of the analytes could be lost during SPE, reducing recovery, SPE can prove to be valuable if the original extract contained (in this case polar) pollutants. Based on several studies, SPE with C18 columns and MeOH for column activation and elution should suffice for the analysis of steroid hormones.[8][19][25][23]

- Paramagnetic beads:

Extraction using paramagnetic beads (PMB) or smart polymer beads is a relatively new extraction method which has been undergoing rapid development over the last two decades.[42] The beads are synthesized from polymers with embedded magnetic particles, thus combining the features of the magnetic and polymer particles. Analyte specific functional groups are synthesized onto the polymer chains. Extraction using PMB makes it possible to use an external magnetic field to separate the beads and bonded analytes from the rest of the sample matrix with relative ease.[42]

Full dried filter paper

Instead of cutting out parts of the DFPs when sampling, the full DFP could be used for extraction to achieve a higher recovery rate. As seen in the results of the recovery experiment on pages 40 and 41 of this report, using a larger part of the DFP for extraction directly increases recovery without affecting repeatability. In theory, using all of the DFP for extraction would achieve maximum recovery. Due to practical reasons, a few parts of the extraction process should be adjusted to be able to extract the full DFP.

First of all, the full DFP does not fit in a 2 mL container. A larger container together with a larger volume of solvent (5 mL for example) should be used to be able to fully submerge the DFP. When using such a large volume of solvent for the extract, an evaporation step is recommended. Once the solvent of the extract is evaporated the remaining analytes could be resuspended in a smaller volume of 20% MeOH / 80% water (the LC starting conditions). This could increase the concentration of the analytes in the final extract solution up to several times over, immediately readying the final extract for injection in the LC-MS system.

A downside of this adjusted sample preparation method is that instead of retrieving four samples from one DFP, only one is retrieved. This should be taken into consideration because it could have an impact on the accuracy of the final results.

Quality control: batch design

Once the anuran water samples are to be analysed, a batch design should be set up. In such a design, samples are randomly distributed into one or multiple batches, depending on the number of samples. A randomly selected sample pool should be collected for quality control and system suitability tests. Every batch should include blanks, a calibration line, study samples (preferably in duplicate or triplicate) and quality control samples. A standardized system suitability test should be performed before each batch analysis to assure quality of acquired data.

Other steroid hormones of interest

Other steroid hormones besides TS and CS have been taken into consideration since the start of this study. As stated in the introduction of this report, TS and CS were chosen because there is a high probability they are measurably excreted by anurans in their underivatized form. Controversely, other steroids also have a good chance they are excreted by anurans. A major part of steroids are excreted in the form of metabolites. A target list of immediate metabolites of TS and CS was set up to provide a starting point for which metabolites are of key interest, also taking into account the availability of analytical standards. It is recommended, when looking into other steroids besides TS and CS, to start by examining the steroids contained in this list. The full list is presented below in table 13.

Table 13: Target list for the metabolites of testosterone and corticosterone.

Metabolite	CAS nr.	IUPAC Name
5α-dihydro-testosterone	521-18-6	5α-androstan-17β-ol-3-one
17β-estradiol	50-28-2	1,3,5(10)-estratrien-3,17β-diol
Testosterone sulfate	651-45-6	17β-(sulfooxy)androst-4-en-3-one
Testosterone glucuronide	1180-25-2	17β-hydroxyandrost-4-en-3-one-3-D-glucuronide
Cortexone	64-85-7	4-pregnen-21-ol-3,20-dione
11-dehydrocorticosterone	72-23-1	4-pregnen-21-ol-3,11,20-trione
20-dihydrocorticosterone	298-25-9	5α-pregnan-11β,21-diol-3,20-dione

Data processing

When an untargeted approach is used to determine relevant metabolites in samples, or when large data sets are to be processed, Metalign can be used to automate and simplify data processing. Metalign is software designed by Dr. Ir. Arjen Lommen of RIKILT Wageningen UR. The algorithms deployed by Metalign were designed to act the same way a trained professional manually handles MS data.[43]

Metalign can be used to perform baseline correction and noise filtering. Furthermore, it can be used for the binning of accurate mass data into easier comparable nominal mass data. Finally, Metalign can prove to be most valuable when used as an exporting tool to reduce the results of multiple analyses to a single Microsoft Excel file.

Metalign uses unprocessed MS data of any format as input. The MS systems used during this study can produce multiple types of output files. When using the QTOF, computable document format (.cdf extension), and when using the QqQ RAW data files (.raw extension) proved most useful when used to export and load the acquired data into Metalign. Intermediate manual checks should be performed to make sure no essential data is lost during processing. For a complete overview of how Metalign processes data, this review[43] by A. Lommen provides additional insight.

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Appendix

Appendix 1: Statistical t-value table

Table 14: Statistical t-value table used for determining confidence intervals of means.[37]

cum. prob	$t_{.50}$	$t_{.75}$	$t_{.80}$	$t_{.85}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$	$t_{.999}$	$t_{.9995}$
one-tail	0.50	0.25	0.20	0.15	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
two-tails	1.00	0.50	0.40	0.30	0.20	0.10	0.05	0.02	0.01	0.002	0.001
df											
1	0.000	1.000	1.376	1.963	3.078	6.314	12.71	31.82	63.66	318.31	636.62
2	0.000	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327	31.599
3	0.000	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215	12.924
4	0.000	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	0.000	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032	5.893	6.869
6	0.000	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	0.000	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	0.000	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	0.000	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	0.000	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	0.000	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	0.000	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	0.000	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	0.000	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	0.000	0.691	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	0.000	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	0.000	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	0.000	0.688	0.862	1.067	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	0.000	0.688	0.861	1.066	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	0.000	0.687	0.860	1.064	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	0.000	0.686	0.859	1.063	1.323	1.721	2.080	2.518	2.831	3.527	3.819
22	0.000	0.686	0.858	1.061	1.321	1.717	2.074	2.508	2.819	3.505	3.792
23	0.000	0.685	0.858	1.060	1.319	1.714	2.069	2.500	2.807	3.485	3.768
24	0.000	0.685	0.857	1.059	1.318	1.711	2.064	2.492	2.797	3.467	3.745
25	0.000	0.684	0.856	1.058	1.316	1.708	2.060	2.485	2.787	3.450	3.725
26	0.000	0.684	0.856	1.058	1.315	1.706	2.056	2.479	2.779	3.435	3.707
27	0.000	0.684	0.855	1.057	1.314	1.703	2.052	2.473	2.771	3.421	3.690
28	0.000	0.683	0.855	1.056	1.313	1.701	2.048	2.467	2.763	3.408	3.674
29	0.000	0.683	0.854	1.055	1.311	1.699	2.045	2.462	2.756	3.396	3.659
30	0.000	0.683	0.854	1.055	1.310	1.697	2.042	2.457	2.750	3.385	3.646
40	0.000	0.681	0.851	1.050	1.303	1.684	2.021	2.423	2.704	3.307	3.551
60	0.000	0.679	0.848	1.045	1.296	1.671	2.000	2.390	2.660	3.232	3.460
80	0.000	0.678	0.846	1.043	1.292	1.664	1.990	2.374	2.639	3.195	3.416
100	0.000	0.677	0.845	1.042	1.290	1.660	1.984	2.364	2.626	3.174	3.390
1000	0.000	0.675	0.842	1.037	1.282	1.646	1.962	2.330	2.581	3.098	3.300
z	0.000	0.674	0.842	1.036	1.282	1.645	1.960	2.326	2.576	3.090	3.291
	0%	50%	60%	70%	80%	90%	95%	98%	99%	99.8%	99.9%
	Confidence Level										

Appendix 2: Data set for selectivity determination

Table 15: Full data set used for the selectivity determination. Peak 1 corresponded with a m/z value of 347.2 (± 0.05) and eluted at 10.1 minutes for all standards of the calibration curve, belonging to corticosterone. Peak 2 correponded with a m/z value of 289.2 (± 0.05) and eluted at 10.7 minutes for all standards of the calibration curve, belonging to testosterone. The width of the peaks and calculated resolution for each of the standards is listed.

	Peak 1 retention time (min)	Peak 1 width at base (min)	Peak 2 retention time (min)	Peak 2 width at base (min)	Resolution
Cal1	10.1	0.307	10.7	0.340	1.856
Cal2		0.340		0.387	1.651
Cal3		0.357		0.413	1.558
Cal4		0.373		0.370	1.614
Cal5		0.377		0.377	1.593
Cal6		0.400		0.380	1.538
Cal7		0.390		0.373	1.572
Cal8		0.400		0.392	1.516

Appendix 3: Data set for recovery experiment

Table 16: Full data set used for the determination of the recovery. Three samples were measured that did not go through the sample preparation process, named NE for 'no extraction'. For 6, 10, 16 and 19 mm DFP cut-out sizes, six samples were measured. For 13 mm cut-out size, four samples were measured. Listed are the total MS counts for both analytes, the calculated recovery per sample and average recovery of the sample sets, including standard deviations.

ID	MS Counts 289.2 (±0.05) m/z	Recovery testosterone (%)	MS Counts 347.2 (±0.05) m/z	Recovery corticosterone (%)
Sample 1 (NE)	4158852	NA	3058861	NA
Sample 2 (NE)	6267250		3749789	
Sample 3 (NE)	3785584		3835701	
Mean (NE)	4737229		3548117	
SD (NE)	1338117		425880	
Sample 1 (6 mm)	388032	8.19	316608	8.92
Sample 2 (6 mm)	376480	7.95	233616	6.58
Sample 3 (6 mm)	462336	9.76	328592	9.26
Sample 4 (6 mm)	389823	8.23	296611	8.36
Sample 5 (6 mm)	413137	8.72	302953	8.54
Sample 6 (6 mm)	424638	8.96	325562	9.18
Mean (6 mm)	409074	8.64 (± 0.70)	300657	8.47 (± 1.04)
SD (6 mm)	31532	0.67	35132	0.99
Sample 1 (10 mm)	927021	19.57	843739	23.78
Sample 2 (10 mm)	1220858	25.77	961472	27.10
Sample 3 (10 mm)	1182675	24.97	918571	25.89
Sample 4 (10 mm)	924096	19.51	765693	21.58
Sample 5 (10 mm)	1077935	22.75	813089	22.92
Sample 6 (10 mm)	1101604	23.25	705173	19.87
Mean (10 mm)	1072365	22.64 (± 2.77)	834623	23.52 (± 2.81)
SD (10 mm)	125070	2.64	95072	2.68
Sample 1 (13 mm)	1506982	31.81	1202805	33.90
Sample 2 (13 mm)	1631408	34.44	1272200	35.86
Sample 3 (13 mm)	1332916	28.14	1013289	28.56
Sample 4 (13 mm)	1410290	29.77	1129284	31.83
Mean (13 mm)	1470399	31.04 (± 4.33)	1154395	32.54 (± 4.96)
SD (13 mm)	128811	2.72	110699	3.12
Sample 1 (16 mm)	2697286	56.94	2075259	58.49
Sample 2 (16 mm)	2873978	60.67	2074576	58.47
Sample 3 (16 mm)	2970944	62.71	2058949	58.03
Sample 4 (16 mm)	2052493	43.33	1863725	52.53
Sample 5 (16 mm)	2162333	45.65	1761943	49.66
Sample 6 (16 mm)	2305994	48.68	1833903	51.69
Mean (16 mm)	2510505	53.00 (± 8.59)	1944726	54.81 (± 4.17)
SD (16 mm)	387770	8.19	140854	3.97
Sample 1 (19 mm)	3988896	84.20	2984997	84.13
Sample 2 (19 mm)	3524032	74.39	2220544	62.58
Sample 3 (19 mm)	3365235	71.04	2711621	76.42

Sample 4 (19 mm)	2604400	54.98	2083045	58.71
Sample 5 (19 mm)	2712238	57.25	2118013	59.69
Sample 6 (19 mm)	3305589	69.78	2494042	70.29
Mean (19 mm)	3250065	68.61 (± 11.48)	2435377	68.64 (± 10.69)
SD (19 mm)	518347	10.94	361343	10.18

Appendix 4: Plan of Action

"The Frog—that arch-martyr to science—affords the most convenient subject."

Rudiments of Pathological Histology by Carl Wedl (1855)

Thesis

Setting up a non-invasive LC-MS platform for semi- quantification of steroid hormones in anurans

1101526

Title page

Plan of Action

Setting up a non-invasive LC-MS platform for semi- quantification of steroid hormones in anurans

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Index

Abbreviations

In order of appearance:

IUCN	- International Union for Conservation of Nature
TS	- Testosterone
CS	- Corticosterone
CHO	- Cholesterol
IUPAC	- International Union of Pure and Applied Chemistry
EIA	- Enzyme immunoassay
LC-MS	- Liquid chromatography-mass spectrometry
RP-HPLC	- Reversed phase high performance liquid chromatography
MeOH	- Methanol
MS	- Mass spectrometry
ESI	- Electrospray ionization
QMS	- Quadrupole mass analysers
DFP	- Dried filter paper
SOP	- Standard operations procedure
SPE	- Solid phase extraction
PMB	- Paramagnetic beads

Introduction

Research justification

Figure 1. IUCN Red List of threatened species in percentage by class.[1]

During the inception of this study, over 40% of all amphibian species are threatened with extinction and this percentage is rising. In fact, amphibians are ranked the most threatened class on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species as of 2019.[1] The main order of the amphibia is the anuran, most commonly known as frogs and toads. Panama is home to over 200 different species of anurans, yet many of those species are in immediate danger of extinction.[2] Knowledge about the inner mechanisms of reproduction is key to ensuring a species survival. Urbanization has shown to have a sizeable impact on the breeding patterns of anurans.[3][4] A recent study has shown that urban frogs have adapted their mating system as a reaction to changes in their environment when compared to their forest counterparts. Specifically, the study presents that urban male túngara frogs (*Physalaemus pustulosus*) have increased the salience of their mating calls in response to greater competition from other males and relative absence of predators.[5]

Essential to understanding the biology behind the process of reproduction, is understanding the chemistry. Acoustic communication in anurans is a key part of their mating process. This process is modulated by their neuromodulatory and endocrine systems. For example, female túngara frogs have shown increased circulating levels of oestrogen and progesterone in response to phonotactic reception of male mating calls.[6] This illustrates that biological systems have underlying chemical mechanisms. The endocrine system communicates, in part, through prolactin and steroid hormone regulation.[7] In this study the initial focus will be on the steroid hormone regulatory process of the endocrine system of anurans, namely the secretion of testosterone (TS) and corticosterone (CS).

The exact mechanisms of the endocrine system of anurans and specifically the metabolism of steroid hormones in anurans have not yet been fully explored. However, for humans many studies have been performed on the pathways of steroid hormones, mainly for medical purposes.[8][9] Inter-species comparative studies can help lay the foundation to examining and ultimately understanding how certain systems work.[10] Although interchangeability is not a given[11], certain assumptions have to be made to take the first step in exploration. For this reason, most background research taken into consideration for this particular study has humans as the prime focal point. Hormones are the key coordinators for developmental, physiological and behavioural mechanisms in all living organisms. TS and CS have been selected because they play an essential role in communication, regulation of sexual behaviour and mediation of organismal responses to environmental change.[10] Looking into the levels of TS and CS in anurans has an added value for environmental conservation and preservation purposes.

Chemical properties of steroids

The chemical structures of TS and CS are shown in figures 2A and 2B, respectively. All steroids are derived from cholesterol (CHO; shown in figure 2C). The nomenclature and positioning of the carbons and possible functional groups of CHO-derived molecules is explained through the International Union of Pure and Applied Chemistry (IUPAC) steroid ring system displayed in figure 2D.[12]

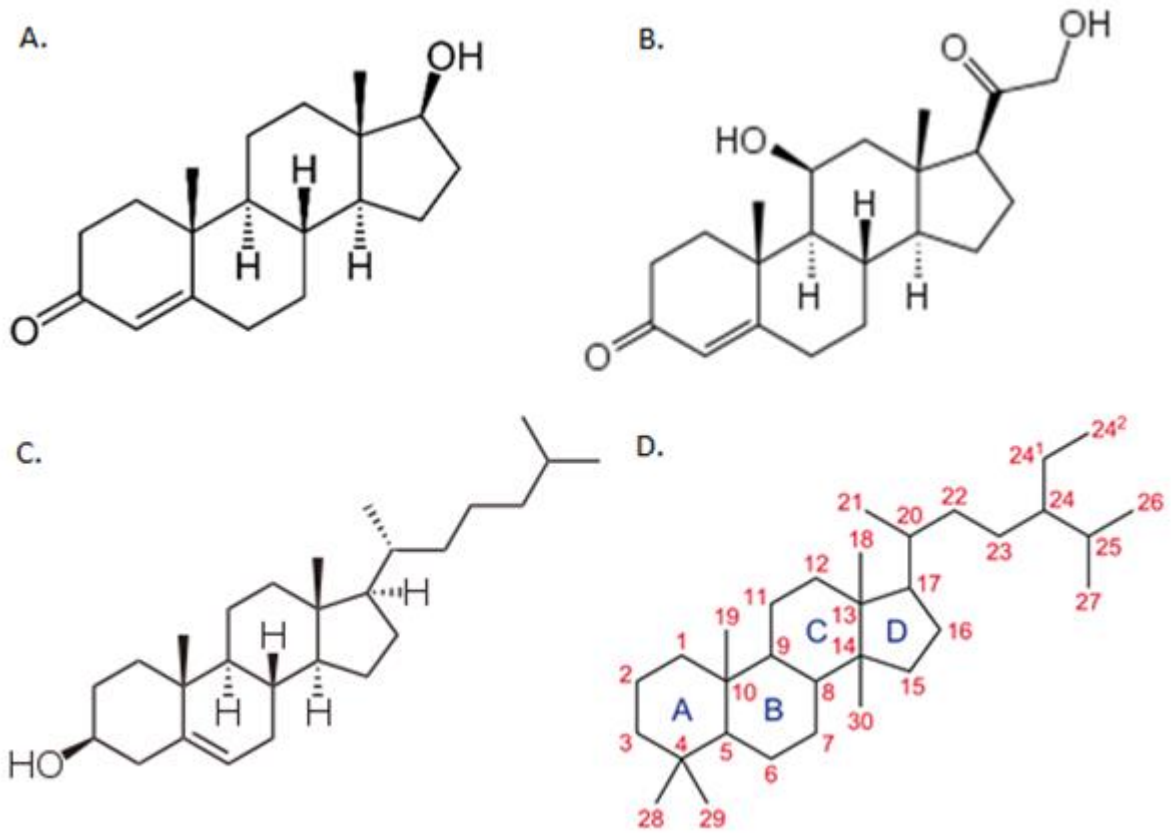

Figure 2. A: Chemical structure of testosterone (17β-Hydroxyandrost-4-en-3-one; $C_{19}H_{28}O_2$); B: chemical structure of corticosterone ((11β)-11,21-Dihydroxypregn-4-ene-3,20-dione; $C_{21}H_{30}O_4$); C: chemical structure of cholesterol (cholest-5-en-3β-ol; $C_{27}H_{46}O$); D: IUPAC steroid ring system.[12]

CHO is a relatively flat molecule, consisting of a polycyclic hydrocarbon backbone made up of three conjoined hexagonal rings and one pentagonal ring. CHO is characterized by a sidechain connected to the 17th carbon of the main structure and two angular methyl groups at positions 18 and 19. From the structure of CHO, three principal types of steroids can be derived. In all cases, the double bond between carbons five and six will be converted in the double bond between carbons four and five. Partial removal of the side chain transmutes to the pregnane type under which corticosteroids such as CS fall. Complete removal of the side chain yields the androstane type under which androgens such as TS fall. Lastly, loss of the methyl group on the 19th carbon, along with an aromatization of the first hexagonal ring presents us with the estrane type under which estrogens fall.

TS, the main androgen, is principally involved in aggression and male sexual development.[13][14] While TS is partly excreted in its original form, in the body TS is partly converted into estradiol (Estra-1,3,5(10)-triene-3,17β-diol; $C_{18}H_{24}O_2$) by Sertoli cell-derived aromatase enzyme and into dihydrotestosterone (5α-Androstan-17β-ol-3-one; $C_{19}H_{30}O_2$) by 5α-reductase type 2 enzyme.[15] Furthermore TS is neutralized during transportation through the body and eventually, but to a lesser extent, excretion through esterification with a sulfate-ion on the hydroxy group located at carbon

seventeen. This reversible reaction transforms the hormone into an inert organic anion, ideal for transportation. Most steroids can be sulfated, including TS and CS.[16][17]

Glucocorticoids manage all sorts of energy regulation, mostly through the release of glucose in the bloodstream. This release or inhibition of energy is associated with multiple sorts of stress-related behavioural responses such as the fight-or-flight reaction to stressful events or providing energy to combat abiding emotional stress.[14] CS is the main glucocorticoid for amphibians, reptiles and birds. Unlike humans and fish where cortisol (11β,17α,21-Trihydroxypregn-4-ene-3,20-dione; $C_{21}H_{30}O_5$) is the main glucocorticoid. CS is the precursor to the mineralocorticoid aldosterone (11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al; $C_{21}H_{28}O_5$) which is the primary steroid hormone that regulates homeostasis.[18]

The documentation listed in the above three paragraphs illustrates that all steroid hormones have a certain level of similarity and interconnectedness regarding biosynthesis and chemical structure. The results from this study can be used to set up a standardized non-invasive method for measuring steroid hormones in organisms. TS and CS have been chosen for this study since there is a high probability they are excreted by anurans. Water-baths have proven to capture physiologically relevant changes in the concentration of certain steroid hormones.[19] The assumption is made this is also true for TS and CS. If the method is successful in identifying and quantifying the levels of TS and CS in anuran water-bath samples, the method can further be generalized to other steroid hormones and possibly even in other organisms using only minor adjustments.

Methodology justification

Detection and quantification of steroid hormones is primarily done using a technique called enzyme immunoassay (EIA).[24] EIA involves binding specific antibodies to the antigen of a specific analyte. This antibody is usually either a chromogen or a fluorogen which makes the analyte visible by eye or microscope and quantifiable by instruments such as spectrophotometers. EIA suffers from several structural issues[25], the issues relevant to this study are presented below:

- Ability to only measure one analyte per processed sample.[25]
- In complex matrices the antibodies can bind to other molecules besides the target analyte causing poor accuracy.[16]
- Limited sensitivity and range compared to other analysis methods.[26]
- High variability and low repeatability due to lack of standardization across EIA kits.[25]

Another technique used for steroid hormone analyses is liquid chromatography-mass spectrometry (LC-MS). LC-MS has been chosen over EIA in this study because it gives better results and directly tackles the issues mentioned above. Another reason LC-MS is preferred, is because it is easier to generalize the analysis method to other steroid hormones besides TS and CS.

Reversed phase high performance liquid chromatography (RP-HPLC) will be used to chromatographically separate analytes because steroids are relatively non-polar molecules. Since CS is a more polar molecule than TS, CS will elute first and TS will follow afterwards. The stationary phase will be C18-based and the mobile phase is based on a gradient mix between purified water as a polar solvent and methanol (MeOH) as a non-polar solvent.

After elution, MS is used to further separate the analytes based on their mass. The solvent containing the analytes is vaporized. The vapor is carried to the MS via a flow of inert carrier gas and subsequently bombarded with electrons, ionizing the analytes. Ionization of steroid hormones can be done both negatively by adding an electron or positively by taking an electron away. The MS systems used in this study use electrospray ionization (ESI). ESI is a soft form of ionization, ideal for the analysis of metabolites because the relatively small molecules (<900 Da) stay largely intact.[28] Once ionized, the analytes are transported to the mass analyser through a vacuum. In the mass analyser the analytes are further separated via their mass-to-charge ratio. This study will focus on the use of quadrupole mass analysers (QMS). A QMS consists of four cylindrical rods which create an oscillating electric field capable of selectively separating ions based on the stability of their trajectory when moving between the rods. Multiple QMS systems can be used in tandem, further increasing the specificity and sensitivity of the method. A popular method of tandem MS is triple quadrupole MS where the analytes pass through three serially connected QMS in one system. The first and third QMS are used as mass filters while the second QMS acts as a collision cell to fragmentate the initial ions released from the first QMS.[29]

Goal analysis

The primary aim of this study will be to compare subsets of anuran hormone samples by levels of TS and CS using a newly created and validated LC-MS method. To reach this goal several objectives have been set up. Firstly, TS and CS will have to be detectable on the LC-MS and the linear range of the method will be determined. Secondly, TS and CS will be spiked on dried filter paper (DFP), the sample medium. The extraction method will be optimized and the recovery will be determined. Subsequently the method will be validated and a standard operations procedure (SOP) will be set up. When the SOP is finished, TS and CS in the anuran hormone samples will be quantified. If quantification is not possible, the samples will be semi-quantified so the subsets of the samples can at least be compared with each other. The setup of the study is summarized in table 1.

Table 1: Summary of the study setup.

Primary aim	Compare subsets of anuran hormone samples by levels of TS and CS using a validated LC-MS method.
First objective	Detect TS and CS using LC-MS. Determine linear range.
Second objective	Spike TS and CS on DFP. Optimize extraction method and determine recovery.
Third objective	Validate method and create SOP.
Fourth objective	Apply validated method on samples, (semi-) quantify TS and CS levels in anuran hormone samples.

Methods and procedures

A rough draft of the methods, procedures and equipment to be utilized in this study will be depicted below. Please note that the methods are subject to change based on the availability of equipment and based on the intermediate results. Unless otherwise indicated, all chemicals that will be used are of pro analysis (MS or better) grade.

Preparatory: Sampling and storage

Multiple studies have been performed on the stability of steroid hormones during storage. Depending on the storage method, results regarding degradation vary. In complex matrices steroids are stable for a maximum of 14 to 28 days.[27][30] Some steroid hormones have shown to be stable during long-term storage in water, for up to three to twelve months without introducing a major storage effect.[31][32] Extracted hormones dissolved in MeOH or absorbed on DFP are the preferred methods of sample storage based on stability. The sampling method using DFP is considered superior because of the ease of handling, low level of invasiveness for the organism and low labour costs.[32]

All sampling was done on the third and fourth day of September 2019 at four different sites at Pipeline Road near Gamboa, a small town in the Republic of Panama (Central America). 82 Túngara frogs (*Physalaemus pustulosus*) were captured in their natural habitat. After capture the frogs were dried off and placed in 50 mL falcon tubes. 1.5 mL of purified water was added to the tubes, after which followed a five-hour waiting period. Once the waiting period had passed, 40 to 400 µL of the water in the tubes was spotted on 903 Protein Saver Snap Apart Card Whatman 903™ DFP. The spotted DFPs were stored in a plastic zip lock bag at room temperature and taken back to the Netherlands for further analysis.

Step 1: Extraction

For extraction the DFP will be cut up into several pieces using a clean pair of scissors. The size of the cuts will be decided upon based on the recovery of the extraction method and the sensitivity of the analysis method. The pieces will be submerged in MeOH and vortexed and/or held stationary for a fixed amount of time to ensure maximum extraction of the analytes. After the vortex step the extract will be filtered if necessary. The recovery of the extraction method will be determined by spiking the DFP with the target analytes.

Step 2: Preparation

If necessary, a preconcentration step can be added to prepare the extract for analysis. Preconcentrating could elevate the analyte signal during analysis if the initial detection signal from the MS is too low for quantification. If the sample matrix is polluted, a cleaning step can be added to reduce signal noise. In order of increasing labour costs, three possible preparation methods are presented below:

- Evaporation:

Evaporating the extract prior to analysis should leave a higher concentration of the analytes in the extract, thus elevating the signal when passing by the MS. After evaporation the analytes can be resuspended in a fixed volume to allow for quantification.

- Solid Phase Extraction (SPE):

Besides providing a similar method of preconcentration as evaporating the extract, SPE can prove to be valuable if the extract contains (polar) pollutions. Based on several studies, SPE with C18 columns and MeOH for column activation and elution should suffice.[8][19][25][32]

- Paramagnetic beads (PMB):

Extraction using PMB or smart polymer beads is a relatively new extraction method which has been undergoing rapid development over the last two decades. The beads are synthesized from polymers with embedded magnetic particles, thus combining the features of the magnetic and polymer particles. Analyte specific functional groups are synthesized onto the polymer chains. Extraction using PMB makes it possible to use an external magnetic field to separate the beads and bonded analytes from the rest of the sample matrix with relative ease.[20]

Step 3: Chromatography and mass separation

The separation of the analytes in the samples will be done on two different LC-MS systems based on their sensitivity and availability. The parameters of the LC and MS during analysis will be based on a non-normalized steroid hormone platform and is subject to change throughout the study.

The first LC-MS system is an Agilent 1200 series HPLC system. The column initially used is a Kinetix® 2.6 µm C18 100 Å RP LC column with a length and diameter of 100 x 2.1 mm. The LC is connected to a Bruker micrOTOF ESI high resolution quadrupole time-of-flight MS detector. The second LC-MS system is a Shimadzu Ultra-HPLC system. This system uses an Acquity UPLC® BEH C18 1.7 µm RP LC column with a length and diameter of 50 x 2.1 mm supplied by Waters. The LC is connected to a Finnigan TSQ Quantum Ultra TQMS manufactured by Thermo Scientific. Both LC systems utilize binary pumps, high performance autosamplers, vacuum degassers and column ovens (35°C).

Preparation of calibrants and stock solutions will be done using an Eppendorf multipette. Absolute or relative concentrations are determined by using deuterated internal standards of TS and CS.

Step 4: Validation and quality control

After the analysis method has been set up the entire method will be validated. This will be done according to the NEN-EN-ISO 17025 guidelines[21], or as closely possible as time and available resources permit. The method developed for this study is new and non-normalised, this means that the relevant performance characteristics to be determined for this study are as follows:

- Calibration curve and range (including limits of detection and quantification)
- Recovery
- Reproducibility
- Accuracy and precision
- Selectivity and specificity (including matrix effects)
- Carryover effects

For the validation to be successful, the spread of most of the performance characteristics cannot exceed 15%. For the lower limits of detection and quantification a maximum spread of 20% is acceptable. Variation in signal due to matrix effects and carryover effects cannot exceed 20% of the lower limit of quantification and 5% of internal standard signals. For a complete overview of the recommendations for validation of a non-normalized small molecule LC-MS/MS platform this study[22] by Jenkins et al. has been used as a reference.

Before the study samples will be measured, a batch design will be set up. Samples are randomly distributed into multiple batches depending on the sample volume. A randomly selected sample pool will be collected for quality control and system suitability tests. Every batch will include blanks, a calibration line, study samples preferably in duplicate or triplicate and quality control samples. A standardized system suitability test will be performed before each batch analysis to assure quality of acquired data.

Step 5: Data analysis and processing

Acquired data will be processed using Metalign software and R version 3.6.2.[23][42] These software tools will help automate the data analysis process. Once optimized, the settings and scripts used in this study will be listed in the appendices of the final report.

Metalign will be used as a pre-processing, comparison and exporting tool. Metalign is software designed by Dr. Ir. Arjen Lommen of RIKILT Wageningen UR. It is a software tool that uses unprocessed LC-MS data as input, usually in the form of .cdf format. The software can calculate peak areas, align shifted peaks and compare multiple data sets between each other. After processing the data, Metalign can export the results into a Microsoft Excel or .csv file which can be used for further analysis.[23]

R will be used for data visualization and statistical analysis. R is a popular programming language for handling data, especially among academia. R is designed for complex statistical analyses. Furthermore, it has excellent options for clear data visualizations. The excel files produced by Metalign are in similar format, making a programming tool such as R a great choice for automated processing.[42]

Planning

The estimated schedule of planning for this study has been depicted below in a visual format (figure 3) and table format on the following page (table 2).

ACTIVITY	PLAN START	PLAN DURATION	PERCENT COMPLETE
Plan of Action	1	9	100%
Set up LC-QTOF method	1	10	100%
Set up LC-TQ method	9	6	40%
Get to know LC-TQ	10	5	10%
Optimize LC-TQ method	13	6	0%
Set up extraction method	8	4	75%
Optimize extraction method	11	4	0%
Set up preconcentration method	15	2	0%
Optimize preconcentration method	16	4	0%
Validate full method	19	4	0%
Apply method on study samples	15	11	0%
Statistical analysis	22	5	0%
Report	22	8	0%
Final presentation	27	3	0%

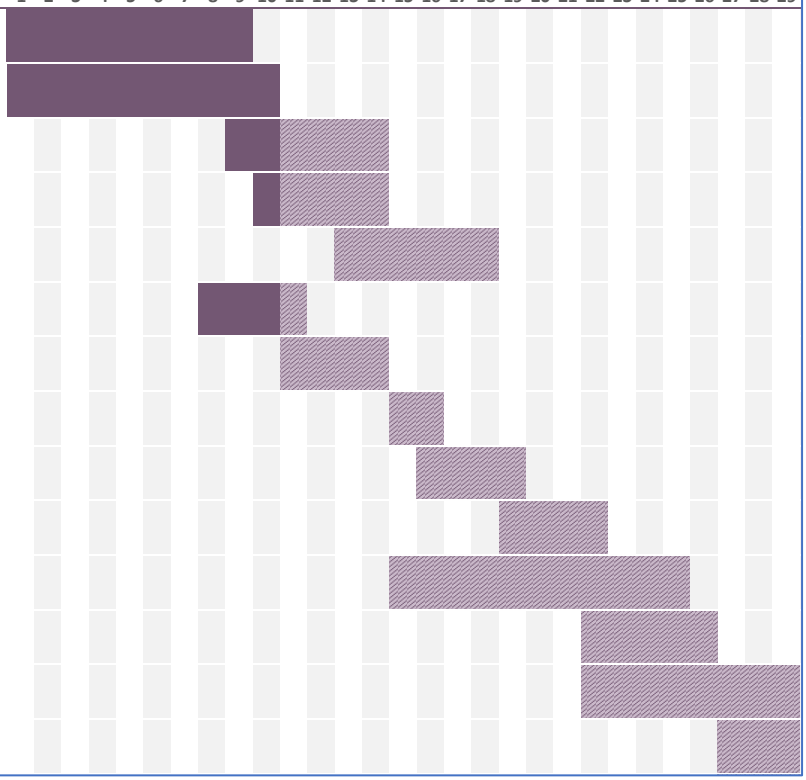

Figure 3: Visual representation for estimated planning in the form of a Gantt chart.

Table 2: Planning overview.

Week	What
1 (02-12-2019)	Start working on plan of action (PoA) and start setting up analysis method on LC-QTOF.
2 - 7 (09-12-2019 to 27-01-2020)	Order chemicals; continue work on PoA and LC-QTOF.
8 (03-02-2020)	Start setting up extraction method; continue work on PoA and LC-QTOF; start setting up extraction method.
9 (10-02-2020)	Finish PoA; start setting up LC-TQ analysis method.
10 (17-02-2020)	Finish work on LC-QTOF; start getting to know LC-TQ.
11 (24-02-2020)	Finish setting up and start optimizing extraction method.
12 (02-03-2020)	Continue working on LC-TQ and optimizing extraction method.
13 (09-03-2020)	Start optimizing LC-TQ analysis method; continue optimizing extraction method.
14 (16-03-2020)	Finish setting up LC-TQ analysis method and finish optimizing the extraction method; work on optimization of the LC-TQ method.
15 (23-03-2020)	If necessary, start setting up a preconcentration method; otherwise continue optimization of the LC-TQ method. Start applying full analysis method to study samples.
16 (30-03-2020)	Finish setting up preconcentration method and start optimization; continue optimization of LC-TQ method and analysis of study samples.
17 (06-04-2020)	Work on optimizing LC-TQ method and preconcentration method; continue analysis of study samples.
18 (13-04-2020)	Finish LC-TQ method development and optimization; continue analysis of study samples.
19 (20-04-2020)	Finish preconcentration optimization; start full analysis method validation; continue analysis of samples.
20 - 21 (27-04-2020 to 04-05-2020)	Continue validation and analysis of study samples.
22 (11-05-2020)	Start working on final report; start statistical analysis; finish validation; continue analysis of study samples.
23 - 24 (18-05-2020 to 25-05-2020)	Work on report; continue statistical analysis and analysis of study samples.
25 (01-06-2020)	Finish analysis of study samples; continue work on statistical analysis and final report.
26 (08-06-2020)	Finish statistical analysis; continue working on final report.
27 (15-06-2020)	Start work on final presentation; continue work on final report.
28 (22-06-2020)	Continue work on report and presentation.
29 (27-06-2020)	Finish report; finish final presentation.

Risk analysis

General guidelines

For this study several substances will be used which should be handled with care. For a detailed description please refer to Appendix I. Lab coats and safety glasses should be worn as appropriate in the lab. All handling of substances, samples and materials will be done whilst wearing disposable gloves for personal protection and to prevent contamination. Preparation of samples, calibrants and organic solvents will be done under the fume hood. All waste will be deposited through the appropriated disposal containers.

Methanol toxicology

Most preparation steps require the handling of MeOH. This should be taken into account at all times because MeOH can be toxic when absorbed through the skin or orally ingested. When coming into contact with a small amount of MeOH, the affected surface should be washed thoroughly with soap and water to avoid permanent damage. In case of ingestion or absorption, MeOH metabolizes into formic acid. Build up of formic acid in the blood stream can cause permanent visual damage at a minimal ingestion of 30 mL and in extreme cases lead to mortality. If a significant amount of MeOH is ingested or there is a significant amount of topical exposure, contacting a toxicologist or visiting an emergency centre is recommended.[41]

Steroid hormone risk assessment

TS has been known to have minor carcinogenic effects following long term exposure, alongside suspected damage to fertility. For this reason, all handling of TS should be done with care. CS is known to cause minor allergic skin reactions in response to topical exposure but is otherwise safe to handle. Since the quantity of steroid hormones used for this study is relatively low, the use and handling are considered safe.

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Appendix

Appendix 1: Safety documentation

CAS-No.	58-22-0
Product name	Testosterone
Synonyms	trans-Testosterone 17β-Hydroxy-3-oxo-4-androstene 17β-Hydroxy-4-androsten-3-one 4-Androsten-17β-ol-3-one
Formula	$C_{19}H_{28}O_2$
Molecular weight	288.42 g/mol
Hazard labels	
First aid measures	In case of skin contact wash thoroughly with soap and water. In case of eye contact flush with water. If swallowed rinse mouth with water and contact a physician.
Precaution/ Spillage	Do not let product enter drains. Wipe spills with tissues, dispose in solid waste container.

CAS-No.	50-22-6
Product name	Corticosterone
Synonyms	11β,21-Dihydroxy-4-pregnene-3,20-dione Reichstein's Substance H 11β,21-Dihydroxyprogesterone Kendall's Compound B 4-Pregnene-11β,21-diol-3,20-dione
Formula	$C_{21}H_{30}O_4$
Molecular weight	346.46 g/mol
Hazard labels	
First aid measures	In case of skin contact wash thoroughly with soap and water. In case of eye contact flush with water. If continuous irritation occurs contact a physician.
Precaution/ Spillage	Wipe spills with tissues, dispose in solid waste container.

CAS-No.	67-56-1
Product name	Methanol
Synonyms	Methyl alcohol
Formula	CH_4O
Molecular weight	32.04 g/mol
Hazard labels	
First aid measures	If breathed in, move person into fresh air, give artificial respiration if not breathing. In case of skin contact wash thoroughly with soap and water, if contact area is large bring victim to a hospital. In case of swallowing, bring victim into fresh air. Contact doctor and mention methanol poisoning. Make victim drink ethanol (0.3 ml per kg body weight of 40% alcoholic beverage). Symptoms from methanol poisoning include dizziness, drowsiness, blurred vision and in extreme cases seizures, coma and death.
Precaution/ Spillage	Handle under fume hood. Avoid contact with skin. Keep away from hot surfaces. Dispose in organic waste container.