Multi-class steroid profiling in short-finned pilot whale blubber using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Jillian Wisse[1], Douglas P. Nowacek[2], and Ashley Boggs[3]

[1]Duke University Department of Medicine
[2]Duke University Nicholas School of the Environment
[3]National Institute of Standards and Technology

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Abstract

Rationale: Wildlife scientists are quantifying steroid hormones in a growing number of tissue types and employing novel methods which must undergo validation before application. This study tested the accuracy and precision of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for use on blubber samples from short-finned pilot whales (*Globicephala macrorhynchus*). We expanded upon a method for corticosteroid quantification by increasing the number of analytes and optimizing internal standards application. Methods: We optimized a method for the quantification of seven steroid hormones using LC-MS/MS with a C18 column. We assessed the accuracy and precision of this updated C18 method and an existing Biphenyl method for use with short-finned pilot whale blubber tissue by conducting a spike-recovery experiment and calculating percent recovery and relative standard deviation (RSD) for each analyte. To explore the potential for running this method more cost-effectively with fewer matched internal standards (IS), we compared the performance of multiple internal standards for each analyte. Results: The C18 method produced reliable quantitation for the seven target adrenal steroids. The measurement of all 11 adrenal and gonadal analytes was both accurate and precise, with percent recoveries between 82 % to 110 % and RSDs below 10 %. IS comparisons showed 10 of 11 analytes could be calculated accurately and precisely with at least one of the IS substitutes. Though many internal standard substitutions met percent recovery and RSD requirements, some of these substitutions significantly altered the analyte concentrations calculated. Discussion: The methods developed and tested in this study provide reliable detection and quantification of 11 steroid hormones, including DHEA, which has not been previously quantified in blubber. These methods can be used for more comprehensive assessments of adrenal and gonadal steroid hormones from whales. Laboratories can reduce costs through IS substitution but should consider how these substitutions might affect results.

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Short title: Steroid profiling in pilot whale blubber using LC-MS/MS

Jillian H. Wisse[1], Douglas P. Nowacek[2,3], Ashley S.P. Boggs[4]

1. Duke University, Department of Medicine
2. Duke University, Nicholas School of the Environment
3. Duke University, Pratt School of Engineering
4. National Institute of Standards and Technology

Contact: Jillian H. Wisse, jillian.wisse@duke.edu, (919)389-0083, 905 S. Lasalle St., Durham, North Carolina 27710

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Methods: We optimized a method for the quantification of seven steroid hormones using LC-MS/MS with a C18 column. We assessed the accuracy and precision of this updated C18 method and an existing Biphenyl method for use with short-finned pilot whale blubber tissue by conducting a spike-recovery experiment and calculating percent recovery and relative standard deviation (RSD) for each analyte. To explore the potential for running this method more cost-effectively with fewer matched internal standards (IS), we compared the performance of multiple internal standards for each analyte.

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Discussion: The methods developed and tested in this study provide reliable detection and quantification of 11 steroid hormones, including DHEA, which has not been previously quantified in blubber. These methods can be used for more comprehensive assessments of adrenal and gonadal steroid hormones from whales. Laboratories can reduce costs through IS substitution but should consider how these substitutions might affect results.

Keywords

Steroid hormones, Liquid chromatography-tandem mass spectrometry, Corticosteroids, Androgens, Progestogens

Introduction

Steroid hormones mediate physiology and behavior; without these compounds, animals would be unable to reproduce or survive long enough to do so. The lipophilic nature of these small molecules aids in their transport into many tissues, enabling scientists to conduct wildlife studies through the collection of feces, hair, blubber, and other methods less invasive than capture. Whale and dolphin (i.e., cetacean) blubber, can be collected at sea by remote blubber biopsy and contains adrenal and gonadal steroids (1, 2).

Scientists often use enzyme immunoassay (EIA) to quantify steroid hormones in wildlife samples. EIAs are sensitive, affordable, and simple to use; however, they are subject to antibody cross-reactivity (3, 4) and are limited to one hormone per analysis. In contrast, mass spectrometry offers high specificity and the ability to concurrently measure multiple compounds (5). Enabled by efficient blubber extraction methods, e.g., Boggs et al. (6), liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been developed for quantifying steroid hormones in cetacean blubber (6, 7).

LC-MS/MS methods have been employed to reliably quantify corticosteroids, androgens, progestogens, and estrogens in blubber from dolphins (6, 8) and baleen whales (7, 9, 10). Good chromatographic separation has been achieved by running corticosteroids on a C18 column and separately running androgens and progestogens on a Biphenyl column (6). Because many steroids have identical precursor and product ions, using different chromatographic sorbents can reliably separate multiple classes of steroids from one sample extraction.

Though studies have successfully employed these methods for assessments of stress and reproductive hormones in blubber from free-ranging populations, the inter-specific variability of blubber tissue (11) makes it necessary to test for matrix effects before applying existing methods to new species. In this study, we tested and adapted methods established in Boggs et al. (6) for use with blubber from short-finned pilot whales (*Globicephala macrorhynchus*). We conducted a spike recovery experiment with blubber samples from stranded individuals to assess this method's accuracy and precision for application in this species' blubber tissue.

This study aimed to expand existing methods by adding three steroid hormones connected to stress in cetaceans: aldosterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone-sulfate (DHEAS). Aldosterone, a mineralocorticoid, regulates sodium balance and increases in response to stressors in marine mammal species (12, 13). Aldosterone has been measured in cetacean serum and feces using immunoassay (14) and recently detected in the blubber of a stranded gray whale (10). DHEA and its sulfonated version, DHEAS are prohormones primarily synthesized in the adrenal cortex and secreted in response to adrenocorticotropic hormone (ACTH). However, they are also synthesized in the gonads and are categorized as androgens. Changes in DHEAS and DHEA concentrations have been connected to chronic stress (15) and disease (16), but neither of these hormones have been measured in cetacean blubber.

We added these steroid hormones through the optimization of an LC-MS/MS method using a C18 column. Isotopically labeled internal standards (IS) were added to samples to enable accurate analyte quantification by controlling for sample loss throughout processing. Ideally, methods include a matched IS for each analyte which helps verify peak identity and reduce matrix interference in measurements, but the expense of these compounds impedes universal use. To guide decisions around which ISs should be used in future methods, we assessed and compared the performance of 10 isotopically labeled ISs for 11 analytes.

Therefore, the aims of this manuscript are to (1) report an optimized method for simultaneous quantification of eight adrenal hormones, (2) assess the accuracy and precision of two LC-MS/MS methods applied to pilot whale blubber, and (3) compare the performances of isotopically labeled ISs for each analyte.

Materials and Methods

LC-MS/MS Method Optimization

We divided target analytes into two groups for liquid chromatography, referred to as the 'gonadal steroids' (17α-hydroxyprogesterone (17OHP$_4$), androstenedione (AE), testosterone (T), and progesterone(P$_4$)) and 'adrenal steroids' (aldosterone (ALD), cortisol (F), cortisone (E), corticosterone (B), 11-deoxycortisol (S), 11-decoxycorticosterone (11DOC), DHEA, and DHEAS) (Table 1). We sourced standards and internal standards from multiple suppliers (Supplementary Table 1). We assessed the four gonadal steroids using the LC-MS/MS method developed previously (6) and detailed in the methods section. Previous studies have described the separation and quantitation of five corticosteroids of interest (F, E, B, S, and 11DOC) in blubber (9, 17). We adapted these methods to incorporate ALD, DHEA, and DHEAS, for the concurrent measurement of all eight adrenal steroids of interest.

We conducted optimization using direct injection of individual analytes (Supp. Table 1) onto an AB Sciex (Framingham, MA) API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer. The Boggs et al. method (6) used positive mode electrospray ionization (ESI) to identify mass transitions for corticosteroids (F, E, B, S, and 11DOC). Therefore, we used matched ionization methods to optimize ALD, DHEA, and DHEAS. We evaluated fragmentation patterns at varying collision energies until we identified at least two candidate product ions for each analyte. We used tuning mode to optimize source (curtain gas, temperature, ion source gas, interface heater, collision gas, ion spray voltage) and compound parameters in positive and negative ionization mode (Table 1).

Using a mixture of the eight adrenal analytes, we created a chromatography method on a Zorbax Eclipse Plus C18 column (150 mm x 2.1 mm, 5 μm particle size) from Agilent with the 1200 Series HPLC system with a binary pump and autosampler from Agilent (Santa Clara, CA). We determined compound retention

times through multiple-reaction monitoring (MRM), by monitoring two mass transitions per compound. We selected the peak with the largest area as the quantitative ion and the second largest peak as the qualitative ion. The resulting scheduled multiple reaction monitoring (sMRM) method used a 240-second detection window for each mass transition, including six internal matched standards (Table 1, Figure 1).

Spike-retrieval experiment

Samples

We collected blubber samples post-mortem from two short-finned pilot whales, live-stranded in North Carolina, USA. Both carcasses were in fresh-dead condition (Code 1, i.e., meaning stranded alive then died or euthanized) when samples were collected. One sample came from an adult male (ID: RJM009), 480 cm in length, that stranded in 2009, hereafter referred to as the 'male sample'. The 'female sample' was obtained from a pregnant adult female (ID: RT48), 352 cm in length, that stranded in 2005.

Sample extraction

For each of the whales, we processed ten replicates, approximately 0.4 g of blubber each: five endogenous (un-spiked) and five spiked with 400 μL of a calibrant mixture of the 11 target analytes (masses in Supplementary Table 2). In addition to three blanks, we ran three un-spiked replicates from a standard reference material (NIST SRM 1945), taken from a pregnant female short-finned pilot whale. For quantification, we diluted neat standards in methanol to create ten calibration standards covering a physiologically relevant range of concentrations (Supplementary Table 2). We created an IS mix with ten isotopically labeled matched ISs (Supplementary Table 2), which we added to all samples (blanks, SRMs, calibrants, and blubber) before extraction. We prepared all stocks and samples gravimetrically and extracted all samples, including blanks and calibrants, using a bead homogenization and QuEChERS extraction protocol previously detailed (6).

LC-MS/MS

To analyze gonadal steroids, we reconstituted samples in HPLC grade methanol and used the biphenyl LC-MS/MS method (6) (Table 1). We injected 10 μL of each sample onto the Restek (Bellefonte, PA) Ultra Biphenyl column (250 mm x 4.6 mm, 5 μm particle size) column and conducted separation using a gradient of acetonitrile and methanol (both with 0.1 % volume formic acid) over 36 minutes at a flow rate of 500 μL/min.

We analyzed adrenal steroids using the C18 LC-MS/MS method developed in this study and described above. Before injecting 10 μL of each sample onto a C18 column for adrenal steroid analysis, we conducted a solvent exchange, bringing samples to a 50:50 volume fraction of Milli-Q water and methanol (described in Galligan et al. (8). Once injected, we separated steroids using a gradient of methanol and Milli-Q water (both with 0.1 % volume acetic acid) throughout the 52-minute method at a 250 μL/min flow rate (Table 2).

Quantification

After mass spectrometry, we used Sciex Analyst software (Version: 1.6; Framingham, MA) to integrate peaks manually. To determine analyte concentrations, we first calculated peak area ratios between the target compound and the matched IS. We then used the peak area ratios of calibrants to interpolate linear regressions, establishing calibration curves (Supplementary Tables 3 and 4). For the biphenyl data, where there were high analyte concentrations in spiked and pregnant samples, we created separate high and low calibration curves to target relevant concentrations.

We defined each analyte's observed reporting limit (RL_{obs}) as the lowest calibrant used in the calibration curve. We calculated the limit of detection (LOD) by adding the mean of the three blanks with three times the standard deviation of the blanks. We compared the RL_{obs} and LOD and used the higher of the two as the reporting limit (RL). For calculations using analyte concentrations, we substituted values below RL with RL/2.

We determined accuracy with the percent analyte recovered in the spike-recovery experiment and used relative standard deviation (RSD) to assess precision. We calculated percent recovery by comparing recovered analyte mass in spiked samples with expected analyte mass, using the following equation:

$$Percent\ Recovery = \frac{\mathrm{ab}}{cb + de} \times 100\%$$

where 'a' is the analyte mass fraction (ng/g) measured in a spiked sample, 'b' is the mass (g) of that spiked sample, 'c' is the mean analyte mass fraction (ng/g) measured in endogenous (i.e., un-spiked) samples, 'd' is the analyte mass fraction (ng/g) in the calibrant used to spike the sample, 'e' is the mass of the spike (g). We considered percent recovery between 70 % and 120 % and relative standard deviation (RSD) values below 15 % acceptable.

Internal Standard Comparison

Initially, we used matched ISs to determine peak area ratios and calculate analyte concentrations. We then compared the suitability of IS substitutions by calculating analyte concentrations from peak area ratios created with other ISs in the method. For example, cortisol (F) concentrations were calculated using F-$^{13}C_3$ for ratios, then compared with concentrations calculated using each IS monitored in the C18 method (Ald-d_4, E-$^{13}C_3$, B-d_4, S-$^{13}C_3$, 11DOC-$^{13}C_3$). We repeated this for each analyte, resulting in six comparisons for each adrenal steroid and four for the gonadal steroids. To assess the performance of these substitutions, we used the equation above to calculate percent recovery and RSD for each substitution. While we considered substitutions with calibration curve slopes near 1.0 to represent better performance, that did not always mean they performed similarly to the matched IS. We compared endogenous concentrations between IS substitutions to determine whether substitutions substantially altered analyte concentrations calculated. Substitutions with calibration curves close to 1.0 were considered to perform more similarly to the matched IS and prioritized in identifying suitable IS substitutions.

Results

LC-MS/MS Method Optimization

We optimized all eight adrenal steroids in positive and negative ionization mode; however, positive mode performed better for all analytes and therefore negative mode data will not be discussed. Using tuning mode, we optimized source (curtain gas = 276 kPa temperature = 600 ^{o}C, ion source gas 1 = 209 kPa, ion source gas 2 = 414 kPa, interface heater = Off, collision gas- High, ion spray voltage = 4000 V) and compound parameters (Table 1). DHEAS did not ionize well in positive mode and was later removed from the final positive ionization method.

Spike-retrieval experiment

When using matched internal standards, mean percent recoveries ranged from 83 % to 110 % (Figure 2). RSDs were between 1.4 % to 9.4 % for all compounds, except corticosterone (18.8 %). Injection of one SRM sample onto the C18 column failed; therefore, we determined the mean SRM concentrations for adrenal steroids from two samples. One un-spiked replicate from the male sample and two spiked replicates from the female sample were improperly extracted and excluded from analyses.

For un-spiked samples, several mean analyte concentrations fell below the RL. Mean endogenous ALD concentrations fell below the RL in all three samples and endogenous B and 11DOC could only be quantified in the SRM, which had higher concentrations of all adrenal steroids. All biphenyl analytes were quantified above the RL in un-spiked samples, except for T, which was not quantifiable in the female sample or the SRM (also female).

Internal Standard Comparison

In the biphenyl separation method, all analytes performed best with their matched ISs, though RSD and percent recovery remained in the acceptable range for several substitutions (Table 3). For 17OHP$_4$, the T-$^{13}C_3$ and AE-$^{13}C_3$ ISs yielded good percent recoveries (98.0 % and 108.9 % respectively) and RSDs (4.3 % and 1.4 % respectively), though measurements of endogenous concentrations decreased, especially at lower concentrations. For calculating T, the nearby 17OHP$_4$-$^{13}C_3$ IS peak performed well as an IS substitute (percent recovery = 110 % RSD = 3.7 %). For AE, the T-$^{13}C_3$ and 17OHP$_4$-$^{13}C_3$substitutions showed good recovery (99.4 % and 107 % respectively) and consistent endogenous AE measurements (Figure 3), but RSDs were not considered acceptable (18.9 % and 21.0 % respectively). For quantifying P$_4$, AE-$^{13}C_3$ was a good IS substitute, with acceptable percent recovery (109 %), RSD (5.3 %), and P$_4$ measurements consistent with concentrations determined with the matched IS. When quantified with T-$^{13}C_3$ and 17OHP$_4$-$^{13}C_3$, P$_4$ concentrations were 28.1 to 67.0 % lower than the concentration determined with P$_4$-$^{13}C_3$ (Figure 3).

In the C18 method, the four $^{13}C_3$ ISs outperformed the two d_4 standards for quantification of every adrenal steroid, except ALD (Figure 4). The B-d_4 peaks had small peak areas compared to the other analytes, which likely caused higher variability in the IS ratios used to calculate concentrations. This is likely reflected by high RSD values (18.5 % - 23.0 %) when using B-d_4 as an IS for other adrenal steroids (Table 4). As a target compound, B was best quantified with $^{13}C_3$ ISs, which were characterized by larger, more distinct peaks than B-d_4. In comparison to an RSD of 18.8 % when calculated with B-d_4, RSDs for B fell below 4.5 % when calculated with S-$^{13}C_3$, F-$^{13}C_3$, E-$^{13}C_3$, and 11DOC-$^{13}C_3$. Though ALD performed best with ALD-d_4, this IS also had a relatively small and broad peak, leading to more variability when used as an IS substitute, with analyte RSDs ranging from 14.8 % to 16.5 %. Except for quantifying 11DOC with S-$^{13}C_3$, the accuracies and RSDs calculated for E, F, B, S, 11DOC, and DHEA using$^{13}C_3$ ISs all fell within acceptable ranges.

For the most part, individual differences were the primary drivers in analyte concentration. However, even when percent recovery and RSD fell within acceptable thresholds, IS substitutions altered observed analyte concentrations (Figure 4). In the female sample, concentrations of B were close to the RL, 0.869 ng/g. When calculated with B-d_4, concentrations fell below RL but exceeded RL when calculated with S-$^{13}C_3$, 11DOC-$^{13}C_3$, and E-$^{13}C_3$. In both methods, substitutions that failed to meet acceptable RSD and/or percent recovery thresholds altered concentrations substantially. In the C18 method, concentrations varied from those calculated with matched ISs by as much as 63.3 %. On average, good substitutions varied much less from matched IS analyte concentrations (mean = 15.5 % median = 11.3 %) than poor substitutions (mean = 31.7 % median = 30.1 %).

Discussion

The Boggs et al. (2017) methods for quantifying steroid hormones in whale blubber can be applied to blubber samples from short-finned pilot whales. As the spike-recovery experiment demonstrated, this tissue matrix does not interfere with the reliable measurement of hormones when assessed with appropriate internal standards. When using matched ISs, accuracy and RSD were acceptable for all 11 hormones measured, except B. This is rectified by substituting B-d_4 with an appropriate IS, like 11DOC-$^{13}C_3$. Accuracy was similar to Boggs 2017 (84 % -112 %) and showed increased precision for some analytes when compared with Boggs et al. 2017 and Dalle Luche et al. 2019 (6, 9). This improvement was likely due to the inclusion of additional matched ISs for adrenal analytes. Differences in T and P$_4$ concentrations between adult male and known-pregnant female samples demonstrate the biological validity of this method, which is promising for the future application of this method to blubber biopsy samples from free-swimming individuals.

The C18 method optimized in this study is among the first to validate the measurement of aldosterone and DHEA in whale blubber. Though endogenous aldosterone was not present in measurable quantities in the pilot whale blubber assessed, this is the first LC-MS/MS method to quantify DHEA in cetacean blubber. We expect adrenal steroids to be significantly higher in blubber from live-stranded whales than free-swimming individuals. Even so, analyte concentrations were below RL in some samples. What this method gains from specificity, it loses in sensitivity. This is especially true for ALD, which had a relatively high RL of 3.54 ng/g, which is similar to the LOD published in Wittmaack et al. 2022 (10), 3.72 ng/g. Future studies should explore this further to determine whether resolution can be improved and whether ALD metabolites are

abundant and measurable in this tissue.

The IS assessment conducted in this study highlights the necessity of careful IS selection when developing LC-MS/MS methods. Not all analytes from this study required matched ISs. In most cases, nearby ^{13}C-labeled IS peaks could be used as substitutes without sacrificing significant accuracy and precision. However, chromatographic proximity is not the sole indicator of a suitable IS substitution. Even using IS substitutions of nearby ^{13}C-labeled peaks affected performance and concentrations measured (see S/11DOC-$^{13}C_3$ in Table 4). Matrix interferences at the retention time of the analyte could be responsible for this effect. ALD-d_4 and B-d_4 standards performed poorly compared to ^{13}C-labeled standards. This could stem from hydrogen/deuterium exchange occurring during extraction or measurement, or may reflect the relatively low sensitivity of the method to these analytes. If using ALD-d_4 or B-d_4 as ISs, researchers should consider using concentrations well above endogenous levels or reliable IS substitutions. Further studies should assess the use of ^{13}C-labeled ISs for ALD and B.

This is the first study to validate the measurement of multi-class steroid hormone profiling in short-finned pilot whales. Where prior studies used immunoassay to measure progesterone in only females (18) or testosterone in only males (19), we present a comprehensive method to concurrently measure hormones regardless of sex. Comprehensive studies like this can maximize the information gleaned from small and difficult-to-obtain samples, such as blubber biopsies. Aside from testosterone, this is the first study to quantify steroid hormones in male short-finned pilot whales. This method did not prove effective in the measurement of aldosterone but was able to reliably detect DHEA, which has not been previously measured in short-finned pilot whales. While corticosterone is the dominant glucocorticoid in some smaller animals, like mice and birds, cortisol was the most abundant glucocorticoid observed in these samples. The predominance of cortisol echoes the patterns seen in cetaceans and other large mammals. The use of LC-MS/MS for highly-specific multiclass steroid profiling can inform which analytes researchers select for studies with single-target methods, like immunoassay.

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Ethics

Work was carried out under NMFS Permit #14809. Samples were collected under Stranding Agreements from NOAA and shared from UNC Wilmington. Funding was provided by Duke University and the US Navy's Marine Species Monitoring Program. We do not claim any conflicts of interest.

Disclaimer: Commercial equipment, instruments, or materials are identified to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Tables and Figures

Table 1. C18 Compound Optimization Parameters, including Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE), and Cell Exit Potential (CXP).

Steroid Common Name	Retention Time (min)
Adrenal Steroids (C18 Method)	Adrenal Steroids (C18 Method)
Aldosterone	16.5
Cortisone	18.0
Cortisol	19.6
Corticosterone	23.7
11-Deoxycortisol	24.4
11-Deoxycorticosterone	28.2
Dehydroepiandrosterone	30.0
Gonadal Steroids (Biphenyl Method)	Gonadal Steroids (Biphenyl Method)
Androstenedione	10.8
Testosterone	8.7
Progesterone	12.8
17α-hydroxyprogesterone	8.5

Figure 1. Example of C18 SMRM Peaks in short-finned pilot whale blubber using LC-MS/MS. This particular chromatogram was created for an adult female (ID: RT48).

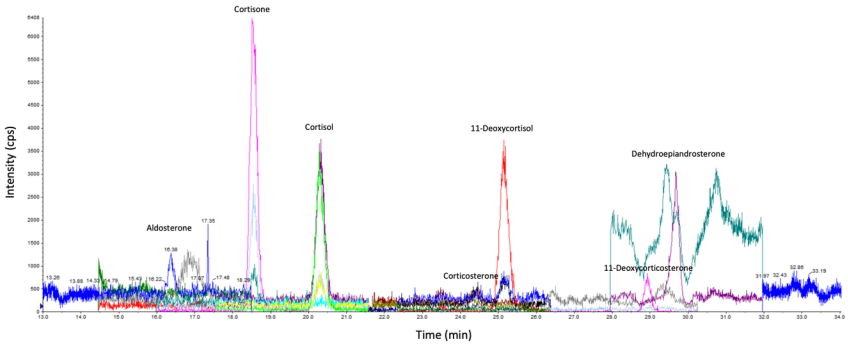


Table 2. C18 method mobile phase gradient

Step	**Time (min)**	**Water**	**Methanol**
0	0.0	80 %	20 %
1	2.0	80 %	20 %
2	7.0	52 %	48 %
3	10.0	50 %	50 %
4	15.0	47 %	53 %
5	20.0	42 %	58 %
6	24.0	35 %	65 %

Step	Time (min)	Water	Methanol
7	32.0	25 %	75 %
8	35.0	10 %	90 %
9	35.1	0 %	100 %
10	40.0	0 %	100 %
11	40.1	80 %	20 %
12	52.0	80 %	20 %

Figure 2. Mean extraction efficiencies for target adrenal and gonadal steroids in pilot whale blubber, determined by a spike-retrieval experiment with blubber samples from two individuals (RJM009 and RT48). Analytes were quantified using isotopically matched internal standards. DHEA, which did not have a matched internal standard, was quantified using S-$^{13}C_3$. Error bars represent standard deviation and red lines delineate the acceptable percent recovery range (70% - 120%). RSDs are shown in the shaded bar along the bottom.

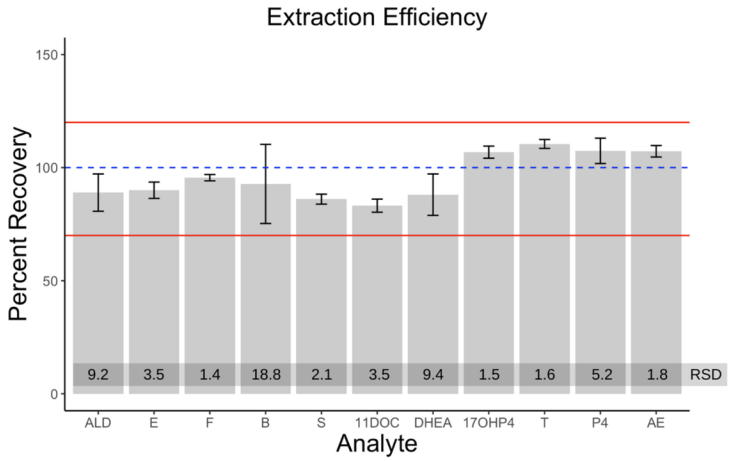


Table 3. Comparison of IS performances for each biphenyl analyte, arranged by proximity to analyte peak. RSDs and accuracies shown are averages for all male and female spiked replicates and the concentration for each sample represents the mean concentration of endogenous replicates. The best performing IS for each analyte is shown in bold. Values exceeding thresholds for RSD (> 15) and accuracy (< 70 or $> 120\%$) are in gray. The slope of the line used for each calibration curve is shown, along with the correlation (R^2) of calibrants. Separate low and high calibration curves help account for the high concentrations of gonadal steroids seen in some samples.

Analyte	IS reference	Peak distance (min)	Accuracy	RSD	RL (ng/g)	Male conc. (ng/g)	Femal
17OHP$_4$	**17OHP$_4$-$^{13}C_3$**	—	**106.83**	**1.507**	**0.2088**	**0.3508**	**0.4586**
	T-$^{13}C_3$	0.19	97.96	4.315	0.1112	0.2560	0.3208
	AE-$^{13}C_3$	2.37	108.87	1.404	0.1056	0.1600	0.2868
	P$_4$-$^{13}C_3$	3.71	222.00	59.909	0.1056	0.4575	0.8876
T	**T-$^{13}C_3$**	—	**110.49**	**1.633**	**0.1505**	**4.878**	**<LOC**

Analyte	IS reference	Peak distance (min)	Accuracy	RSD	RL (ng/g)	Male conc. (ng/g)	Femal
	17OHP$_4$-$^{13}C_3$	0.19	110.08	3.730	0.1505	5.721	<LOQ
	AE-$^{13}C_3$	2.19	120.59	26.390	0.1505	4.955	<LOQ
	P$_4$-$^{13}C_3$	3.53	231.69	55.585	0.1505	7.339	<LOQ
AE	**AE-$^{13}C_3$**	—	**107.23**	**1.818**	**1.260**	**10.514**	**12.97**
	P$_4$-C3	1.35	186.23	29.194	1.260	14.287	22.35
	T-$^{13}C_3$	2.18	99.41	18.897	1.260	9.693	10.86
	17OHP$_4$-$^{13}C_3$	2.37	106.94	21.040	1.260	11.169	13.15
P$_4$	**P$_4$-$^{13}C_3$**	—	**107.42**	**5.236**	**0.07101**	**0.3653**	**112.20**
	AE-$^{13}C_3$	1.34	108.53	5.272	0.07101	0.2320	114.40
	T-$^{13}C_3$	3.52	71.91	27.888	0.07101	0.2544	46.37
	17OHP$_4$-$^{13}C_3$	3.71	77.08	34.630	0.07101	0.2625	64.16

Figure 3. Change in mean endogenous concentrations of biphenyl (gonadal) analytes when calculated with varying IS references. Endogenous T was not quantifiable in the female sample or the SRM and is not shown on the plot.

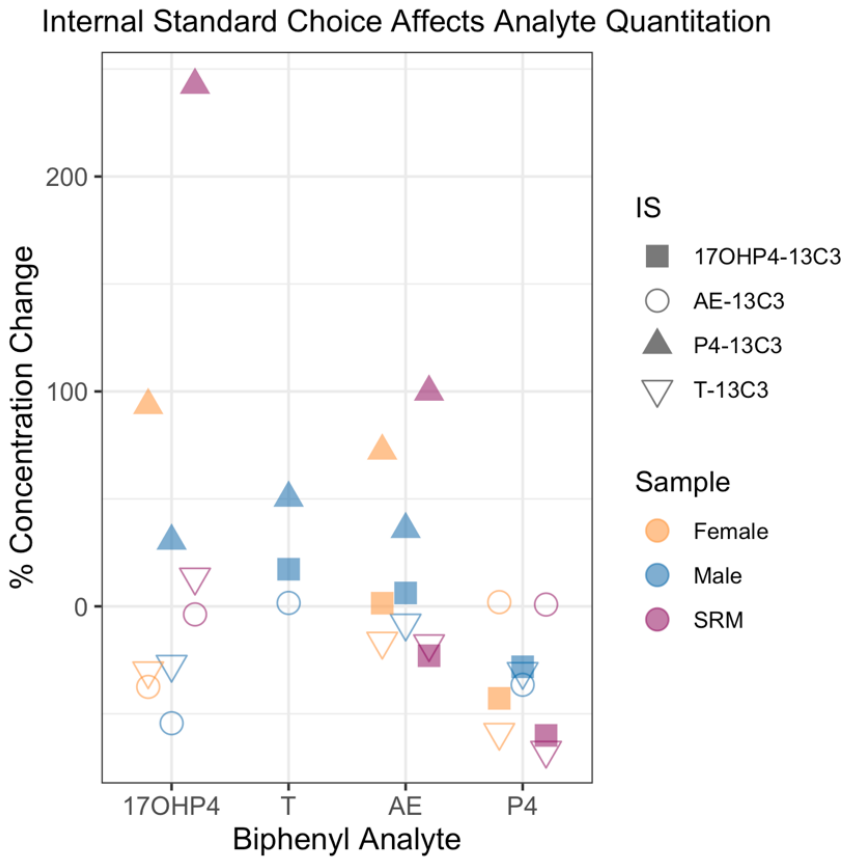
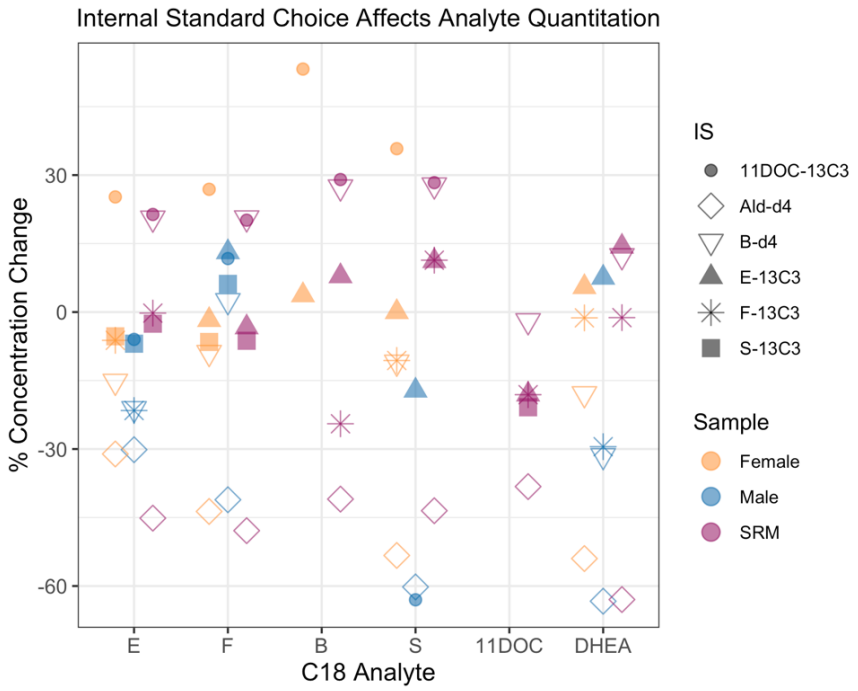


Table 4. Comparison of IS performances for each C18 analyte, arranged by proximity to analyte peak. RSDs and accuracies shown are averages for all male and female spiked replicates. Mass fractions shown for each sample (Male, Female, and SRM) represent the mean of endogenous replicates. The best performing IS for each analyte is shown in bold. Values exceeding thresholds for accuracy (< 70 or > 120 %) and RSD (> 15) are in gray.

Analyte	IS reference	Peak distance (min)	Accuracy	RSD	RL (ng/g)	Male (ng/g)	Female (ng/g
ALD	**Ald-d_4**	—	**88.96**	**9.159**	**3.539**	**<RL**	**<RL**
	E-$^{13}C_3$	1.59	69.29	12.43	3.539	<RL	<RL
	F-$^{13}C_3$	3.31	87.58	11.43	3.539	<RL	<RL
	B-d_4	7.42	64.37	21.43	3.539	<RL	<RL
	S-$^{13}C_3$	8.17	61.67	9.553	3.539	<RL	<RL
	11DOC-$^{13}C_3$	12.01	114.2	10.33	3.539	<RL	<RL
E	**E-$^{13}C_3$**	—	**90.00**	**3.534**	**0.1035**	**0.9520**	**1.378**
	F-$^{13}C_3$	1.69	94.72	4.128	0.1035	0.7467	1.292
	Ald-d_4	1.74	88.09	15.60	0.1047	0.6650	0.9494
	B-d_4	5.8	91.33	19.45	0.1035	0.7505	1.168
	S-$^{13}C_3$	6.55	89.77	3.692	0.1035	0.8860	1.304
	11DOC-$^{13}C_3$	10.39	104.3	6.807	0.1035	0.8950	1.725
F	**F-$^{13}C_3$**	—	**95.56**	**1.443**	**0.05613**	**1.494**	**3.825**
	E-$^{13}C_3$	1.74	90.06	4.115	0.05613	1.690	3.758
	Ald-d_4	3.45	93.38	15.61	0.05613	0.8798	2.154

Analyte	IS reference	Peak distance (min)	Accuracy	RSD	RL (ng/g)	Male (ng/g)	Female (ng/g
	B-d_4	4.09	92.00	18.97	0.05613	1.531	3.486
	S-$^{13}C_3$	4.84	89.09	3.344	0.05613	1.586	3.574
	11DOC-$^{13}C_3$	8.68	104.6	6.532	0.05613	1.669	4.853
B	B-d_4	—	92.79	18.85	0.8693	<RL	<RL
	S-$^{13}C_3$	0.66	89.69	2.369	0.8693	<RL	0.8997
	F-$^{13}C_3$	4.2	101.4	2.717	0.8693	<RL	<RL
	11DOC-$^{13}C_3$	**4.5**	**106.0**	**4.482**	**0.8693**	**<RL**	**1.379**
	E-$^{13}C_3$	5.92	96.74	2.540	0.8693	<RL	0.9329
	Ald-d_4	7.63	93.85	15.73	0.8693	<RL	<RL
S	**S-$^{13}C_3$**	—	**86.07**	**2.114**	**0.02738**	**0.1255**	**0.3834**
	B-d_4	0.76	89.43	18.47	0.02738	<RL	0.3413
	11DOC-$^{13}C_3$	3.83	103.4	5.938	0.02738	0.04642	0.5206
	F-$^{13}C_3$	4.87	98.57	5.013	0.02738	<RL	0.3427
	E-$^{13}C_3$	6.59	94.71	4.956	0.02738	0.1040	0.3834
	Ald-d_4	8.3	90.99	16.49	0.02738	0.04995	0.1790
11DOC	**11DOC-$^{13}C_3$**	—	**83.18**	**3.491**	**0.3664**	**<RL**	**<RL**
	S-$^{13}C_3$	3.84	69.37	3.926	0.3664	<RL	<RL
	B-d_4	4.59	71.76	20.12	0.3664	<RL	<RL
	F-$^{13}C_3$	8.7	74.12	5.565	0.3664	<RL	<RL
	E-$^{13}C_3$	10.42	70.07	4.042	0.3664	<RL	<RL
	Ald-d_4	12.13	104.4	14.81	0.3664	<RL	<RL
DHEA	11DOC-$^{13}C_3$	1.79	117.0	8.148	0.1082	<RL	<RL
	S-$^{13}C_3$	5.63	88.05	9.368	0.1423	1.618	1.194
	B-d_4	6.38	71.36	22.96	0.06617	1.109	0.9804
	F-$^{13}C_3$	**10.49**	**93.49**	**10.29**	**0.1351**	**1.141**	**1.179**
	E-$^{13}C_3$	12.21	88.57	7.898	0.2530	1.739	1.260
	Ald-d_4	13.92	83.47	15.52	0.1670	0.5932	0.5494

Figure 4. Changes in C18 (adrenal) analyte concentrations when calculated with varying IS references. Endogenous aldosterone concentrations were below the reporting limit for all three samples, regardless of IS used, and are not included below.



Supplementary Table 1. List of neat standards and manufacturers/sources.

Reference Standard	**Abbreviation**	**Company**
Androstenedione	AE	Steraloids
Androstenedione-$^{13}C_3$	AE-$^{13}C_3$	Cerilliant
Testosterone	T	Sigma-Aldrich
Testosterone-$^{13}C_3$	T-$^{13}C_3$	Cerilliant
Progesterone	P_4	Sigma-Aldrich
Progesterone-$^{13}C_3$	P_4-$^{13}C_3$	Cambridge Isotopes
17α-Hydroxyprogesterone	17OHP_4	Sigma-Aldrich
17α-Hydroxyprogesterone -$^{13}C_3$	17OHP_4-$^{13}C_3$	Cerilliant
Dehydroepiandrosterone	DHEA	Sigma
Dehydroepiandrosterone sulfate	DHEAS	Sigma
Aldosterone	ALD	Acros Organics
Aldosterone-2H4	ALD-d_4	IsoSciences
Cortisone	E	Sigma-Aldrich
Cortisone-$^{13}C_3$	E-$^{13}C_3$	Sigma-Aldrich
Cortisol	F	Sigma-Aldrich
Cortisol-$^{13}C_3$	F-$^{13}C_3$	IsoSciences
Corticosterone	B	Sigma-Aldrich
Corticosterone-$^{2}H_4$	B-d_4	IsoSciences
11-Deoxycortisol	S	Steraloids
11-Deoxycortisol-$^{13}C_3$	S-$^{13}C_3$	IsoSciences
11-Deoxycorticosterone	11DOC	Steraloids
11-Deoxycorticosterone-$^{13}C_3$	11DOC-$^{13}C_3$	IsoSciences

Supplementary Table 2. Analyte masses used in the spike-recovery experiment.

Group	**Analyte**	**Mass in High Calibrant (ng)**	**Mass in Low Calibrant (ng)**	**Av. Mass in Spik**
Adrenal (C18)	ALD	127.9	0.01114	11.70
	E	124.6	0.01085	11.40
	F	263.1	0.02290	24.07
	B	122.0	0.01062	11.16
	S	128.3	0.01117	11.74
	11DOC	150.7	0.01312	13.79
	DHEA	130.5	0.01136	11.94
Gonadal (Biphenyl)	P_4	332.8	0.02897	30.45
	$17OHP_4$	494.9	0.04309	45.28
	T	705.4	0.06141	64.53
	AE	518.4	0.04514	47.43

Supplementary Table 3. Calibration curves used to determine Biphenyl analyte concentrations with each IS reference.

Biphenyl Analyte	Curve Level	IS	R^2	Slope	Intercept	# Cals	High Cal (ng/g)	Low Cal (ng/g
$17OHP_4$	low	$17OHP_4$-$^{13}C_3$	0.9999	0.0646	0.0195	6	53.49	0.1056
		T-$^{13}C_3$	0.9999	0.4976	0.0179	6	53.49	0.1056
		AE-$^{13}C_3$	0.9999	0.7898	0.0672	4	53.49	0.1056
		P_4-$^{13}C_3$	0.9999	0.1907	0.0027	5	53.49	0.1056
	high	$17OHP_4$-$^{13}C_3$	0.9990	0.0599	0.7871	3	608.1	53.49
		T-$^{13}C_3$	0.9997	0.5613	-0.1729	4	309.5	3.527
		AE-$^{13}C_3$	1.0000	0.0639	0.0313	4	309.5	3.527
		P_4-$^{13}C_3$	1.0000	0.1976	-0.0016	4	309.5	3.527
T	low	$17OHP_4$-$^{13}C_3$	0.9999	0.1492	-0.0034	6	76.24	0.1505
		T-$^{13}C_3$	1.0000	1.1497	0.0035	6	76.24	0.1505
		AE-$^{13}C_3$	0.9998	2.1209	0.0146	4	5.027	0.1505
		P_4-$^{13}C_3$	1.0000	0.4616	0.0010	5	19.52	0.1505
	high	$17OHP_4$-$^{13}C_3$	0.9999	0.1493	-0.0098	4	76.24	1.715
		T-$^{13}C_3$	1.0000	1.1493	0.0069	4	76.24	1.715
		AE-$^{13}C_3$	0.9996	1.8151	0.3279	4	76.24	1.715
		P_4-$^{13}C_3$	0.9999	0.4398	0.0085	4	76.24	1.715
AE	low	$17OHP_4$-$^{13}C_3$	1.0000	0.0169	0.0011	5	324.2	0.4309
		T-$^{13}C_3$	0.9998	0.1381	-0.0027	5	56.04	0.4309
		AE-$^{13}C_3$	0.9999	0.2268	0.0074	3	14.34	1.260
		P_4-$^{13}C_3$	1.0000	0.0530	0.0000	4	56.03	1.260
	high	$17OHP_4$-$^{13}C_3$	0.9993	0.0178	-0.0549	5	637.0	3.695
		T-$^{13}C_3$	0.9999	0.1483	-0.0283	5	324.2	1.260
		AE-$^{13}C_3$	1.0000	0.2218	0.0049	4	324.2	3.695
		P_4-$^{13}C_3$	1.0000	0.0523	0.0006	4	324.2	3.695
P_4	low	$17OHP_4$-$^{13}C_3$	1.0000	0.1740	0.0039	4	35.97	0.07101
		T-$^{13}C_3$	1.0000	1.3282	-0.0027	4	9.208	0.07101
		AE-$^{13}C_3$	1.0000	2.2952	0.0160	4	9.208	0.07101
		P_4-$^{13}C_3$	0.9999	0.5318	-0.0001	5	9.208	0.07101
	high	$17OHP_4$-$^{13}C_3$	0.9998	0.1590	0.3676	3	208.1	9.208
		T-$^{13}C_3$	1.0000	1.4811	-0.3063	4	408.9	2.372
		AE-$^{13}C_3$	1.0000	0.4725	0.0293	4	408.9	2.372

Biphenyl Analyte	Curve Level	IS	R^2	Slope	Intercept	# Cals	High Cal (ng/g)	Low Cal (ng/g
		P_4-$^{13}C_3$	0.9995	0.4732	0.0739	4	408.9	9.208

Supplementary Table 4. Calibration curves used to determine C18 analyte concentrations with each IS reference.

C18 Analyte	**IS**	**R^2**	**Slope**	**Intercept**	**# Cals**	**High Cal (ng/g)**	**Low Cal (ng/g)**	**LOD**
ALD	ALD-d_4	0.9994	1.1725	-0.0782	3	40.43	3.539	0.7282
	E-$^{13}C_3$	0.9337	0.1715	0.2976	4	80.00	3.539	-1.215
	F-$^{13}C_3$	0.9997	0.2846	0.2251	3	80.00	3.539	-0.183
	B-d_4	0.9954	7.4969	-1.9959	3	40.43	3.539	0.8031
	S-$^{13}C_3$	0.9950	0.1848	-0.1933	3	40.43	3.539	1.044
	11DOC-$^{13}C_3$	0.9996	0.9914	0.6806	3	80.00	3.539	-0.278
E	ALD-d_4	0.9998	0.9696	0.0056	7	77.91	0.1035	0.0663
	E-$^{13}C_3$	1.0000	1.6618	0.0664	9	153.1	0.02658	0.0188
	F-$^{13}C_3$	0.9998	7.1236	-0.2867	5	77.91	0.1035	0.1047
	B-d_4	1.0000	29.4480	0.1203	6	153.1	0.1035	0.0290
	S-$^{13}C_3$	1.0000	0.6868	0.0172	6	77.91	0.1035	0.0752
	11DOC-$^{13}C_3$	1.0000	6.1497	0.0943	4	39.38	0.1035	0.0722
F	ALD-d_4	0.9999	0.9108	0.0255	7	83.16	0.05613	-0.013
	E-$^{13}C_3$	1.0000	0.5455	0.0052	5	83.16	0.05613	0.0101
	F-$^{13}C_3$	1.0000	3.7853	0.0588	5	164.5	0.05613	-0.013
	B-d_4	0.9999	15.9360	0.2493	7	83.16	0.05613	-0.010
	S-$^{13}C_3$	1.0000	0.3895	0.0085	5	83.16	0.05613	0.0048
	11DOC-$^{13}C_3$	1.0000	3.3828	0.067	4	83.16	0.05613	-0.011
B	ALD-d_4	1.0000	3.7159	0.3096	3	38.55	0.8693	-0.085
	E-$^{13}C_3$	0.9997	0.0905	0.0148	5	38.55	0.2965	-0.051
	F-$^{13}C_3$	0.9999	0.2058	0.0531	6	149.9	0.8693	-0.717
	B-d_4	0.9999	0.7883	0.0734	4	38.55	0.2965	-0.058
	S-$^{13}C_3$	1.0000	0.1185	0.0143	4	76.28	0.8693	-0.102
	11DOC-$^{13}C_3$	0.9998	0.8768	0.0053	3	76.28	0.8693	0.0278
S	ALD-d_4	1.0000	0.7621	0.0109	4	40.56	0.02738	-0.003
	E-$^{13}C_3$	1.0000	31.2410	0.4487	5	40.56	0.02738	-0.017
	F-$^{13}C_3$	1.0000	6.6189	0.0655	4	40.56	0.02738	-0.006
	B-d_4	1.0000	1.6906	0.0732	5	80.26	0.02738	-0.042
	S-$^{13}C_3$	0.9999	0.9812	0.0276	5	80.26	0.02738	-0.024
	11DOC-$^{13}C_3$	1.0000	7.2227	0.1232	4	80.26	0.02738	-0.027
11DOC	ALD-d_4	1.0000	1.6794	0.0226	3	47.65	0.3664	0.0452
	E-$^{13}C_3$	0.9998	0.1899	0.0053	5	94.27	0.3664	0.0460
	F-$^{13}C_3$	0.9999	7.9127	0.1956	5	47.65	0.3664	0.0158
	B-d_4	1.0000	0.4575	0.0124	6	370.5	0.3664	0.0163
	S-$^{13}C_3$	0.9999	0.2668	0.0023	5	94.27	0.3664	0.0434
	11DOC-$^{13}C_3$	1.0000	1.2503	0.0628	3	47.65	0.3664	-0.043
DHEA	ALD-d_4	1.0000	2.7035	0.5443	6	81.60	0.02784	0.1082
	E-$^{13}C_3$	1.0000	0.3218	0.1334	6	81.60	0.02784	0.1423
	F-$^{13}C_3$	0.9997	17.3940	1.5849	6	41.24	0.02784	0.0661
	B-d_4	1.0000	0.7788	0.1865	6	81.60	0.02784	0.1351
	S-$^{13}C_3$	1.0000	0.4518	0.0940	4	81.60	0.02784	0.2530
	11DOC-$^{13}C_3$	1.0000	3.4153	0.3328	4	160.3	0.02784	0.1670