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Quantification of the Hormones Progesterone and Cortisol in Whale Breath Samples Using Novel, Non-Invasive Sampling and Analysis with Highly-Sensitive ACQUITY UPLC and Xevo TQ-S

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APPLICATION BENEFITS

- Provides a novel, non-invasive sampling method to obtain sample from whale blow. These samples can then be analyzed to determine the levels of progesterone and cortisol.
- A sensitive, repeatable, quantitative LC-tandem quadrupole MS method is shown.
- Parallel acquisition of MRM and full scan MS data allows both the compounds of interest, and also the matrix background to be monitored.
- Simultaneous quantitative and investigative analysis can be performed for different whale species.
- Allows for a deeper understanding of the impact of man's activity in the oceans on these large and elusive marine mammals and for management plans to become better informed.

WATERS SOLUTIONS

ACQUITY UPLC® System,
Xevo® TQ-S Mass Spectrometer,
TargetLynx™ Application Manager

KEY WORDS

steroid hormone analysis, glucocorticoid, whale samples

INTRODUCTION

The conservation and management of large whale populations require information about all aspects of their biology, life history, and behavior. However, it is extremely difficult to determine many of the important life history parameters, such as reproductive status, without using lethal or invasive methods. As such, efforts are now focused on obtaining as much information as possible from the samples collected remotely, with a minimum of disturbance to the whales. Various excreted samples, such as sloughed skin and feces are being used to determine sex and maturity, as well as life history stage.[1,2]

Attention has recently shifted more towards what can be analyzed from samples of whale blow for health assessment[3] or steroid hormone analysis.[4] Hormone analysis is of particular interest as high levels of progesterone can be used as an indicator of pregnancy status, while other steroids, such as glucocorticoids may be markers of the short-term, acute stress response.

A recent study[5] reported that it was possible to detect both male and female reproductive hormones in whale blow samples using liquid chromatography-mass spectrometry (LC/MS). Following these findings, we have investigated improving the detection of progesterone and cortisol (the major glucocorticoid identified in many marine mammal species), with a much more sensitive LC/MS/MS quantitative method.

The blow collection, on-board storage, and clean-up method outlined by Hogg *et al.* (2009)[5] was followed in this study. Samples were collected from a variety of mysticete and odontocete whale species from Canada and Norway, including the humpback whale (*Megaptera novaeangliae*), sperm whale (*Physeter macrocephalus*), long-finned pilot whale (*Globicephala melaena*) and northern bottlenose whale (*Hyperoodon ampullatus*). In some cases the animals were individually identifiable and sex could be determined as animals were recognized by their fluke patterns, known from previous studies (largely humpback whales sampled in the St Lawrence Estuary, Canada). However, in most cases, it was not possible to determine the sex or maturity of the animal.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_8, 2.1 x 50 mm, 1.7 µm
Column temp.:	40 °C
Flow rate:	0.6 mL/min
Mobile phase A:	Water + 0.5% formic acid
Mobile phase B:	Acetonitrile

Gradient:

Time (min)	%A	%B
0.0	90	10
0.5	90	10
3.0	10	90
4.0	10	90
4.1	90	10
5.0	90	10

Injection volume:	1 µL, partial loop (pressure assisted)

MS conditions

MS system:	Xevo TQ-S
Ionization:	ESI positive
Capillary voltage:	3.3 kV
Source offset:	50 V
Source temperature:	150 °C
Desolvation gas temp.:	650 °C
Desolvation gas:	1000 L/hr
Cone gas flow:	150 L/hr
Acquisition:	RADAR [multiple reaction monitoring (MRM) with full scan]
Collision gas:	Argon at 3.5 x 10^{-3} mbar

Whale blow samples were collected using the same method as described in Hogg *et al*. (2009).[5] Briefly, blow samples were collected remotely using inert nylon stockings stretched over a five-inch embroidery ring attached to a pole, as shown in Figure 1.

Figure 1. Collection of whale blow samples that were then analyzed using ACQUITY UPLC and Xevo TQ-S.

The collection material was cleaned by sonication for 15 min in 100% acetonitrile, and then for a further 15 min in deionized water, changing the water every 5 min. Whale blow samples were stored on board the sampling vessel and during transportation to the laboratory in a 5-mL inhibitor (100 mM $MnCl_2$/100 µg/mL amoxicillin/potassium clavulanate).

Hormone extraction

Hormones were extracted using solid phase extraction (SPE) where samples were centrifuged at 3,000 rpm for 15 min to remove the sample and inhibitor from the stocking. Extracts were loaded onto the SPE cartridges that had been preconditioned with 20.0-mL acetonitrile followed by 5.0 mL deionized water. Samples were loaded at 5.0 mL/min and washed with 7.5 mL deionized water to remove salts. Elution of the hormones was then carried out with 5.0 mL 100% acetonitrile, and the eluent was dried under compressed air. Samples were reconstituted in 60 µL 60% acetonitrile in preparation for LC/MS analysis. Since the LC/MS/MS method can be used to investigate the presence of many different proteins simultaneously, both progesterone and cortisol (the latter being a potential stress marker) were analyzed. Standards of progesterone and cortisol from 2 ng/µL to 100 ng/µL in 60% acetonitrile were also included.

Table 1 shows the different species of whales used, along with their sex (if known).

Sample no.	Species	Sex	Quality code
S1	Humpback whale	UK	3
S2	Humpback whale	M	3
S3	Humpback whale	UK	3
S4	Humpback whale	F	2
S5	Humpback whale	CALF, UK	3
S6	Humpback whale	F	2
S7	Humpback whale	UK	4
S8	Humpback whale	F	2
S9	Humpback whale	M	3
S10	Humpback whale	M	3
S11	Humpback whale	M	2
S12	Humpback whale	UK	4
S13	Humpback whale	M	3
S14	Humpback whale	M	3
S15	Humpback whale	UK	3
S16	Humpback whale	UK	4
S17	Sperm whale	M	4
S18	Sperm whale	UK	2
S19	Long finned pilot whale	UK	2
S20	Long finned pilot whale	F	3
S21	Long finned pilot whale	CALF, UK	NR
S22	Unknown	UK	NR
S23	Unknown	UK	NR
S24	Northern bottlenose whale	UK	3
S25	Northern bottlenose whale	UK	3

Table 1. Whale species used during the experiments.

Waters® IntelliStart™ Technology (combining internal calibration fluidics and diagnostics software) integrated into MassLynx 4.1 Software, was used to automatically tune, calibrate, and conduct the systems performance checks before the analytical experiments. IntelliStart was also used to optimize all the MRM transitions for the hormones of interest automatically. For these analyses, two MRM transitions were used for each compound and the MRMs monitored are summarized in Table 2.

Compound	Precursor (*m/z*)	Product (*m/z*)	Cone voltage (V)	Collision energy (eV)
Cortisol	315.2	97.1	40	20
	315.3	109.1		22
Progesterone	363.2	121.1	2	20
	363.2	327.3		14

Table 2. ESI positive MRM conditions for the hormones of interest.

The RADAR Technology of the Xevo TQ-S was also used, which allows both full scan data and MRM data to be acquired in parallel during the same sample analysis. The benefit of this technology is that it enables both targeted quantification and research-based experiments to be performed in the same injection.

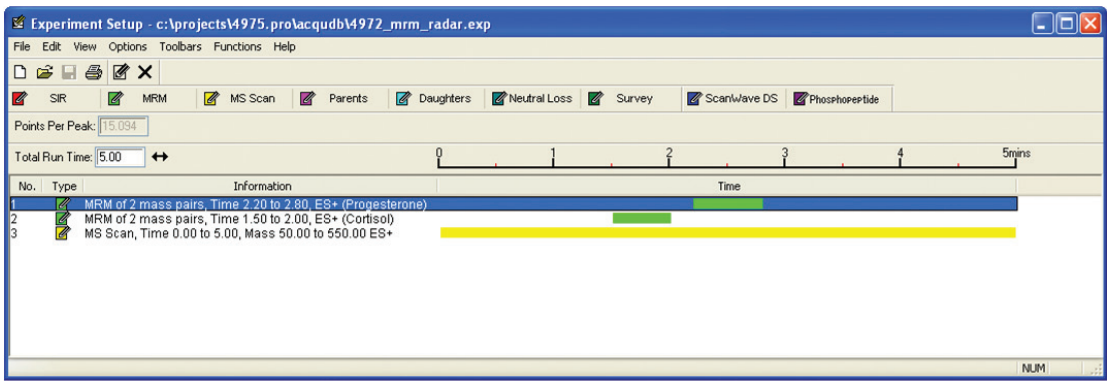

Figure 2. Screenshot of RADAR activated on the Xevo TQ-S: the first two functions (green) show the MRMs that have been set up; and the third function (yellow) enables full scan ESI positive data to be collected.

The data were processed using the TargetLynx quantification application manager. TargetLynx automates data acquisition, processing, and reporting for quantitative results. It incorporates a range of confirmatory checks to identify samples that fall outside user-specified or regulatory thresholds. For this experiment, the confirmatory checks were not used, as it is the first time these two compounds had been quantified in whale blow samples, hence the thresholds were not yet known.

RESULTS AND DISCUSSION

The sample preparation procedure included the solid phase extraction step, in order to remove any potentially interfering endogenous salts present. (Although as it can be seen later in Figure 7, there was still quite a lot of matrix present in the final sample analyzed).

Within the sample analysis a series of calibration standards (between 0.5 and 50.0 pg/µL) were prepared for the hormones, and these were used for quantification. An example chromatogram for the lowest standard injected is shown in Figure 3.

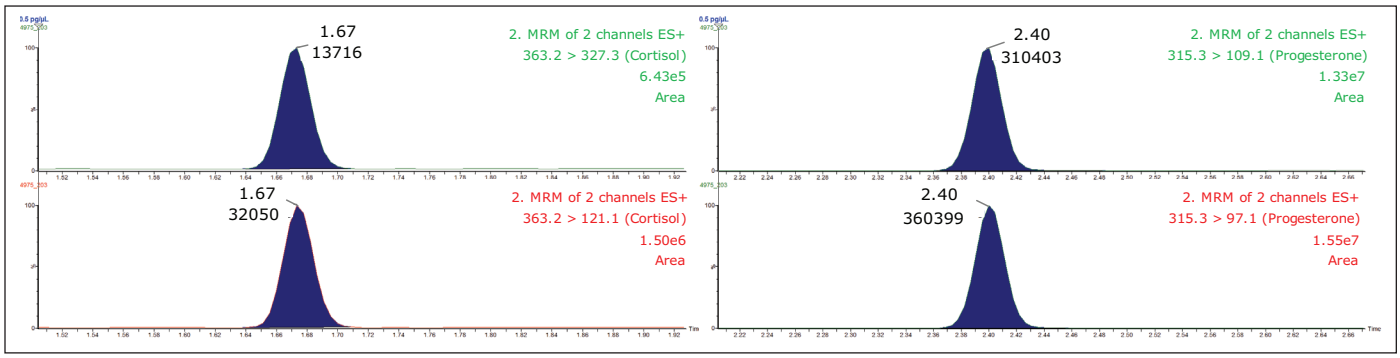

Figure 3. Chromatographic example for 0.5 pg/µL standard.

TargetLynx Application Manager was used to produce the calibration curves from these standards and automatically process the sample data. An example of TargetLynx data is shown in Figure 4.

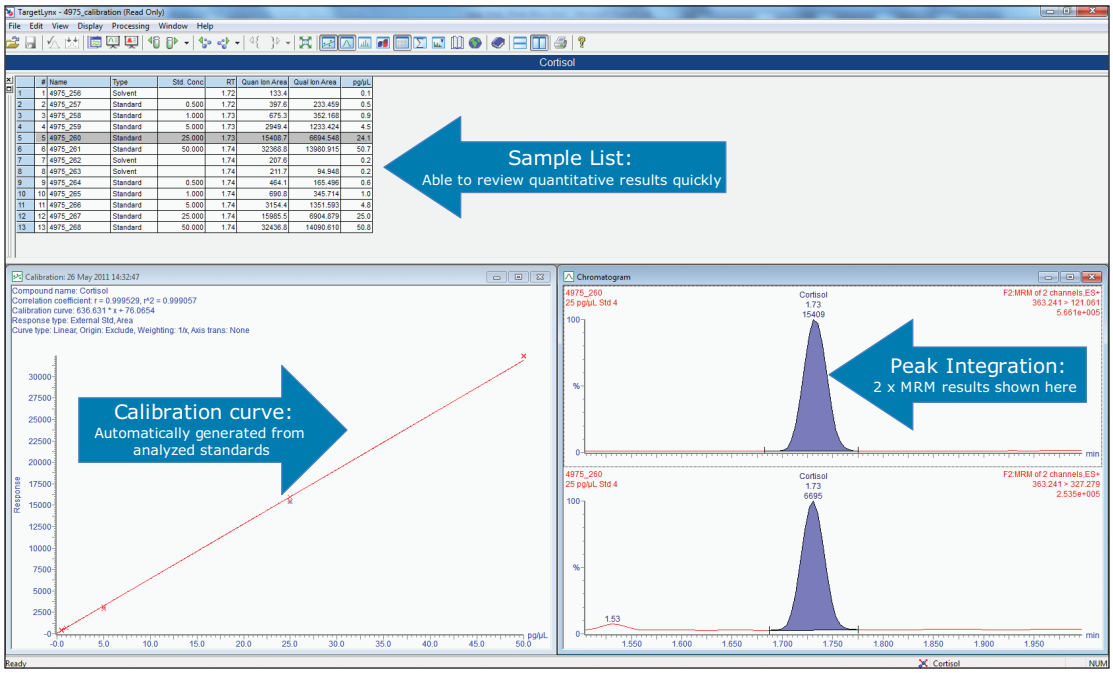

Figure 4. An example of the view within the TargetLynx summary window.

Using the UPLC® method conditions, it can be seen that the two compounds of interest were chromatographically well separated from one another. For cortisol and progesterone, the observed retention times were 1.67 and 2.40 min respectively. Both cortisol and progesterone were detected easily in the lowest calibration standard (0.5 pg/µL). Further research around this subject is needed in order to determine the exact levels of detection required, but these preliminary results suggest it is possible to achieve even lower limits of detection with more method development.

For all the standards analyzed, excellent linearity was observed for cortisol and progesterone: the $r^2 \geq 0.999$ for the two hormones, as shown in Figure 5.

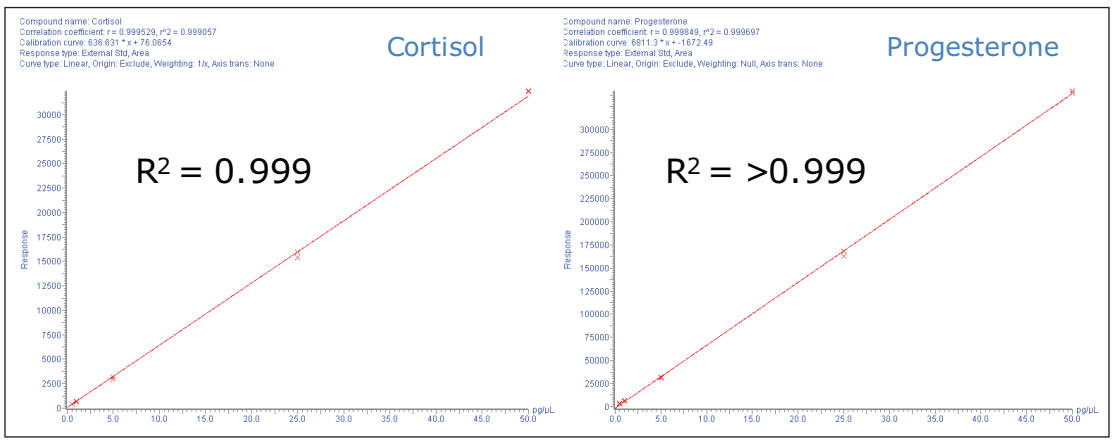

Figure 5. Calibration curves for the two hormones, cortisol and progesterone: excellent linearity can be observed for both compounds.

Typical relative standard deviations (%RSDs) for electrospray ionization were observed – this is generally around 5%. For cortisol and progesterone, the data repeatability seen was excellent, with the %RSD being <2%, as shown in Table 3.

	Cortisol		Progesterone	
Sample code	Area	pg/µL	Area	pg/µL
4975_244	3429	5.4	36408	5.6
4975_245	3348	5.3	35529	5.5
4975_246	3332	5.3	35025	5.4
4975_247	3354	5.3	35474	5.5
4975_248	3290	5.2	35758	5.5
%RSD	1.5	%RSD	1.4	

Table 3. Repeatability data for cortisol and progesterone at 5 pg/uL.

During the experiments, 25 whale blow samples were analyzed and included a variety of whale species and sex. Cortisol was detected in the majority of samples (22 of 25) at a level higher than the lowest calibration point. Progesterone was detected in 13 samples higher than the lowest calibration point. These experiments represent the first time that the levels of the two compounds have been analyzed. The maximum levels observed were 8.2 pg/µL of progesterone in a female humpback species (S14). The highest levels of cortisol (5.6 pg/µL) were also found in the female humpback species (S14).

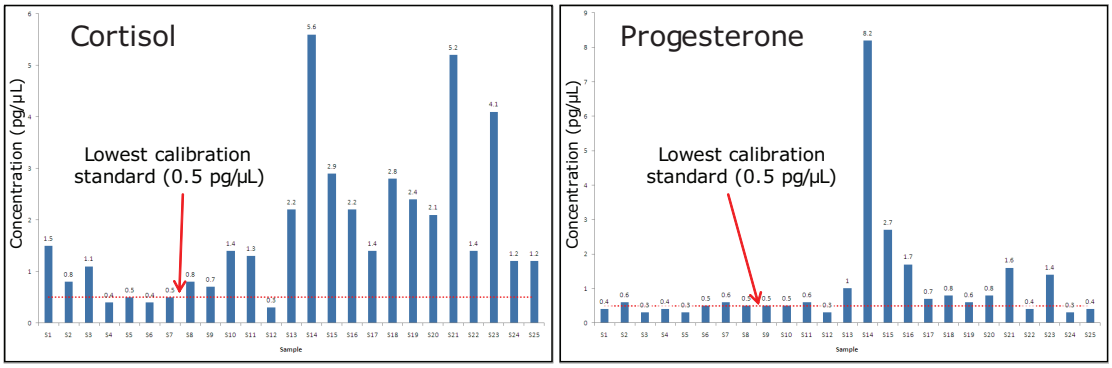

Figure 6. Cortisol and progesterone levels in the whale blow samples.

For the samples, the RADAR mode was used during the analysis. RADAR mode is a full scan function used to assess background components during a standard MRM analysis.

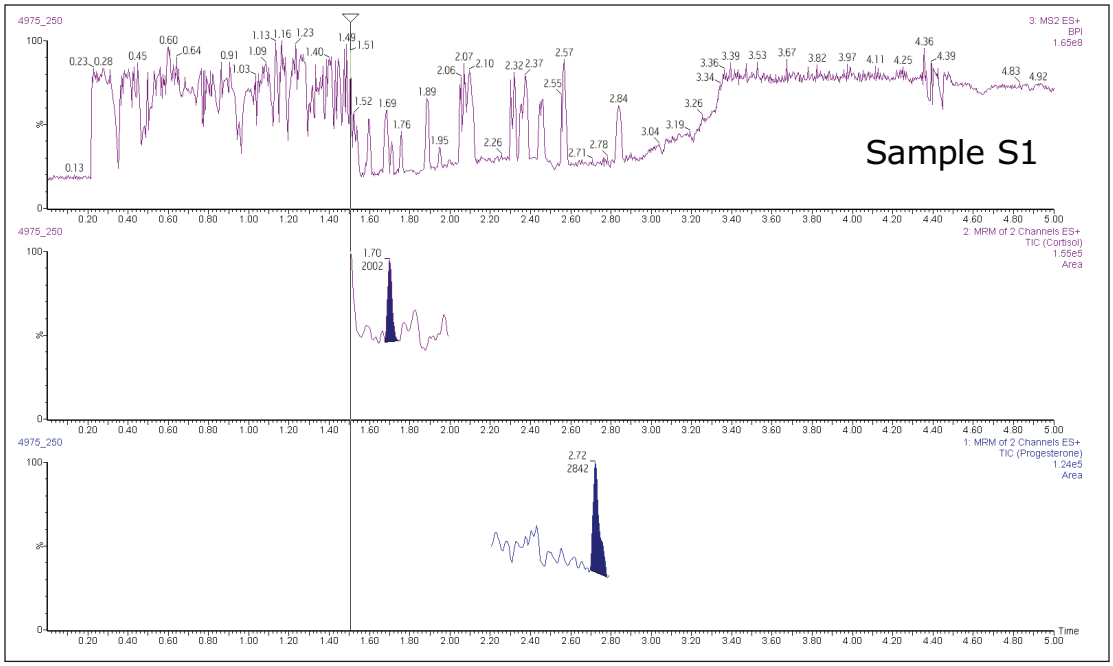

Figure 7. RADAR – parallel acquisition of targeted MRM analysis and full scan monitoring.

The additional functionality of RADAR acquisition was acquired for each of the species types. This allowed us to search for other compounds, while monitoring the matrix background. Figure 7 shows that the matrix background is quite high at the start and at the end of the run, but relatively low at the point where the hormones elute.

CONCLUSIONS

- The concentration of steroid hormones were determined at very low levels in exhaled breath (blow) samples from living whales using a remote and non-invasive method. The levels of these hormones will, for the first time, enable an assessment of the health and reproductive status of the animals to be determined.
- The ACQUITY UPLC System, combined with Xevo TQ-S showed excellent sensitivity. Both cortisol and progesterone were easily detected in the lowest calibration standard (0.5 pg/µL). This suggests that lower detection limits, if required, are possible with further method development.
- The repeatability observed for both compounds was excellent, achieving <2% RSDs for five replicates of a 5 pg/µL standard.
- Linearity was demonstrated for both compounds between 0.5 and 50.0 pg/µL; $r^2 = 0.999$ for cortisol and $r^2 > 0.999$ for progesterone.
- The additional functionality of RADAR Technology (parallel MRM and MS scan) was utilized for each of the species types, which allowed for searching of other compounds, and the monitoring of matrix background.
- Relationships between levels of stress hormones and thus physiological reactions of these endangered animals to human disturbance (*e.g.* boat traffic and noise), can thus be investigated using this remote technique.

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