Journal of Visualized Experiments

Cortisol Measurement in Koala (Phascolartos Cinereus) Fur

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59216R3
Full Title:	Cortisol Measurement in Koala (Phascolartos Cinereus) Fur
Keywords:	Chronic Stress; Marsupials; Koala; Glucocorticoids; Fur; extraction
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Additional Information:	
Question	**Response**
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Sydney, NSW, Australia

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Xiaoyan Cao, PhD.
Review Editor
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617.674.1888

3/06/2019

Dear Prof Cao,

I am kindly submitting my revision R2 for our manuscript entitled: Cortisol measurement in koala (*Phascolartos cinereus*) fur.

The review process and the reviewers' comments successfully guided us to correct the remaining major issues related to the methods including the sample washing step (which should come before sample grinding) and to complete dry the sample extracts under N2 gas before reconstituting in assay buffer. We revised the volume % of the solvents from 90% to 100% to suit the assay requirements.

Thus by following the suggestions provided by the reviewers we have been able to come to the conclusion that 100% methanol rather than ethanol or isopropanol is the most suitable solvent for hormone extraction.

With this resubmission, we hope for your kind positive consideration of our original manuscript for publication in JoVE.

Sincerely,

Edward

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Editorial Board
Frontiers in Endocrinology

TITLE:

Cortisol Measurement in Koala (*Phascolartos Cinereus*) Fur

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KEYWORDS:

chronic stress, koala, glucocorticoids, fur, methanol, ethanol, isopropanol

SUMMARY:

We present a protocol to determine the optimal extraction solvent to measure cortisol from koala fur. The solvents used in this protocol are methanol, ethanol and isopropanol. Determining an optimal extraction solvent will aid in reliably measuring fur to determine the impact of chronic stress on koalas.

ABSTRACT:

Optimal methods of hormone extraction used to measure stress in animals across sample types are not always the same. Australia's iconic marsupial species, the koala (*Phascolarctos cinereus*), faces prolonged exposure to anthropogenic-induced stressors and assessment of chronic stress in wild populations is urgently warranted. One of the most effective ways to measure chronic stress is through analyzing the glucocorticoid hormone cortisol in hair or fur, as it supports physiological and behavioral responses. This laboratory validation study aims to test current techniques to validate an optimal hormone extraction method to be used as a non-invasive measure of cortisol in koala fur. It is recognized that using non-invasive techniques to measure stress hormones is preferred over traditional, invasive techniques due to their ideal practical and ethical standpoints. Additionally, it is comparatively easier to acquire fur from koalas than it is to acquire samples of their blood. This study used samples of koala fur acquired from the Adelaide Koala and Wildlife Hospital to run a number of hormone extraction techniques in an attempt to validate an optimal cortisol extraction method. Results showed that 100% methanol provided the most optimal solvent extraction compared to 100% ethanol or 100% isopropanol based on parallelism results. In conclusion, this method of cortisol extraction from koala fur provided a reliable non-invasive assay that could be used to study chronic stress in koalas.

INTRODUCTION:

Australian ecosystems sustain human life through the provision of services including food and fiber among many other dynamic interactions[1]. Ironically, it is human activity that operates as the dominant driver of ecosystem disruption through biodiversity change[2]. Habitat fragmentation, known as the process of dividing large continuous habitats into small patches

of land, isolated from each other, is the major anthropogenic biodiversity change threatening Australian ecosystems[2]. Habitat fragmentation modifies the structure and diversity of species composition in any given area, thus reducing the area of habitat necessary for these species to maintain viable populations[2]. The result of this is increased competition between species for resources including food, fuel, fiber, and water[3]. The destruction of Australian ecosystems through biodiversity change is having catastrophic consequences on many Australian native species[1].

Australia's most iconic marsupial species, the koala (*Phascolarctos cinereus*), depends on Australian ecosystems remaining healthy for their survival[4]. The introduction of the European Settlement caused a rapid decline in Australian populations of koalas, as they were slaughtered for their pelts in pursuit of profit in a large export trade[5]. This practice was banned in the 1980's and populations of koalas were then able to stabilize[5]. However, exponential growth of the human population has resulted in this species competing for much of their habitat, and their survival is again under threat[6]. According to the International Union for the Conservation of Nature (IUCN), all populations of Australian koalas are listed as vulnerable to extinction with a decreasing population trend[7]. This listing is attributed to the uncertainty around relevant population parameters and marked variation in population trends for this species[7]. As the most iconic and endemic animals, koalas largely benefit the Australian economy through tourism (NSW Office of Environment and Heritage 2018). An estimation suggests that koala related tourism has generated approximately 9,000 jobs and contributes between $1.1 and $2.5 billion to the economy (NSW Office of Environment and Heritage 2018). The removal of any one species has the potential to be catastrophic, and can be seen in the steady decline of native Australian wildlife[6]. Additionally, the Australia economy will feel the ramifications if populations of Australian koalas continue to decline at the rate they are[6].

It is suggested that prevalence of death and disease in response to habitat fragmentation is the result of chronic stress[8]. Already, twenty-four marsupial species have been declared extinct in Australia due to habitat fragmentation, with koalas following a similar trend[8]. The complexity of habitat fragmentation and biological systems is synergistic but can be unpacked through analysis of the stress response[6]. Generally, any disturbance in an animals natural surroundings activates a complex cascade of neurohormonal events, known as a 'fight or flight' response[9,10]. This response to stress is a process that begins in the brain where the hypothalamic-pituitary-adrenal (HPA) axis is activated[11]. A component of the brain called the hypothalamus releases corticotrophin-releasing hormone (CRH), which then signals the anterior pituitary to release adrenocorticotrophic hormone (ACTH)[11]. This in turn stimulates the glucocorticoid secretion from the adrenal medulla. The body circulates glucocorticoids through the blood, which diverts the storage of glucose from glycogen and mobilizes glucose from stored glycogen[11]. This cascade of neurohormonal events is the response used by the animal to deal with unpredictable stimuli[11]. However, when glucocorticoids are being released and remain elevated for a prolonged period of time, the animal is considered to be experiencing chronic stress[12,13]. This process involves diverting energy away from other corporal bodily functions, as it is needed for ongoing glucocorticoid production[13]. As a result, chronic stress can prohibit growth, reproduction and immunity, all being key fitness traits required for survival[14].

Measuring an animal's glucocorticoid production is a common indicator used to determine whether or not the animal is experiencing physiological stress[15]. To do so, glucocorticoids can be measured in blood plasma, serum, saliva, urine or faeces[16]. However, evidence suggests that hair is a much more effective indicator of chronic stress, as opposed to the aforementioned[16]. This is because hair is thought to incorporate blood-borne hormones during its growth phase; it is relatively stable; and any cortisol detected in hair reflects physiological stress experienced over the period of hair growth, which can be weeks through to months[16]. Furthermore, any collection of cortisol should be non-invasive in order to minimise the stress associated with capture and handling[16]. However, any stress experienced during this event would not impact glucocorticoid levels in hair[16]. There have been many studies that explore the proficiency of using hair to measure long-term stress in a number of animals, and include studies on reindeer, grizzly bears, rhesus monkeys, muskoxen, and brown bears[17-21]. Hair cortisol is usually extracted by first washing the sample to ensure sweat and sebum-derived cortisol deposited on the surface of the hair is not co-extracted with cortisol and then pulverizing the sample in a bead-beater[22]. After washing, the sample needs to be dried to ensure complete evaporation[22]. Finally, using a solvent, the sample can be extracted and reconstituted to facilitate the assay of cortisol[22]. The most common solvent used to extract cortisol from fur is methanol[21,23]; however, there are some studies that use ethanol and isopropanol in their cortisol extraction techniques. For example, a study that used ethanol was successful for extracting cortisol from human amniotic fluid[24]. Additionally, a study that used isopropanol was successful for extracting cortisol from human hair and nails[25,26]. For this reason, this study tested all three solvents (methanol, ethanol, and isopropanol) to determine which was the most successful for extraction of cortisol from samples of koala fur.

The primary objective of this study was to use current techniques to validate an optimal hormone extraction technique to be used as a non-invasive measure of cortisol from koala fur. This was achieved by testing three extraction solvents (methanol, ethanol, and isopropanol). We hypothesized that methanol will be the optimal solvent used for extracting cortisol from koala fur because it is the recommended solvent of extraction by Arbor assay cortisol kits[27].

PROTOCOL:

This project was performed under strict animal and human care guidelines. Animal ethics was granted by Western Sydney University (A12373). Additionally, a lab risk assessment and biosafety and radiation form were submitted and accepted by Western Sydney University to safely undertake this research (B12366).

NOTE: Koala fur samples for this project were obtained from the Adelaide Koala and Wildlife Hospital, located at 282 Anzac Highway, Plympton South Australia. Fur was taken from one koala which had been admitted to the hospital and euthanized due to their severe injuries. The deceased koala had been stored in a freezer within a body bag soon after death. After removing the deceased koala from the body bag, 1.2 g of fur was shaved from the nape of the neck using standard animal clippers. The fur was shaved as close as possible to the skin, so as to ensure the skin was not cut. Once shaved, the deceased koala was put back into the body bag and placed in the freezer. The fur was then placed in a pouch made of aluminum

foil and stored below -20 °C. In transit, the fur was kept at ambient temperature, and on arrival to the laboratory, the fur was stored at -80 °C.

1. Koala fur cortisol extraction

1.1. Remove the fur from storage at -80 °C and allow time to thaw.

1.2. Weigh the fur on a laboratory analytical precision balance.

1.3. Place 60 mg of the fur into a pre-weighed and labelled 1.5 mL centrifuge tube and repeat until 18 tubes are filled.

NOTE: 18 fur sub-samples were used for this validation study.

1.4. Add 1 mL of 100% high-performance liquid chromatography (HPLC) grade isopropanol to each tube using a pipette.

1.5. Vortex samples for 30 s.

1.6. Strain each sample using a 0.5 mm micro precision sieve so as to achieve separation of liquid and fur.

1.7. Discard the liquid into a waste container.

1.8. Place each fur sample into a labelled plastic weighing boat, then place into a vacuum desiccator, leave the fur to dry for 3 days.

1.9. Once completely dry, place each sample into a labelled 1.5 mL microcentrifuge tube.

1.10. Place each sample in a bead mill with 3 chrome steel beads (3.2 mm) and pulverize for 2 min at 30 shakes per second.

1.11. Pipette 1.5 mL of the first extraction technique (100% analytical grade ethanol) into 6 1.5 mL microcentrifuge tubes containing the fur sample.

1.12. Perform the same for 100% analytical grade methanol and 100% analytical grade isopropanol until eighteen 1.5 mL microcentrifuge tubes are filled.

1.13. Cap each 1.5 mL microcentrifuge tube and incubate at room temperature (RT) with constant pulsating using a shaker for 3 h.

1.14. Remove and strain samples using a 0.5 mm micro precision sieve.

1.15. Transfer the liquid into a new, labelled 1.5 mL microcentrifuge tube with a pipette while ensuring that the fur is discarded appropriately.

1.16. Completely dry solvent extract under a stream of N_2 vapor under a fume-cupboard.

1.17. Reconstitute the dried sample extract using 400 µL of assay buffer (composition provided in the commercial cortisol kit; see **Table of Materials**) and 100 µL of 100% analytical grade ethanol.

NOTE: Sample extracts can be stored at -80 °C.

2. Internal controls

2.1. To make controls, make a pool of extracted samples with high hormone levels. To make this pool, select samples from animals with known exposure to stressor. For example, select samples from koalas that have been rescued from environmental trauma as they will generally display high cortisol hormone levels[6].

2.1.1. To make the extract pool, take 20 µL of extract from each sample (n = 10) until a total volume of 200 µL is obtained. The extract pool can be stored at -80 °C until assays. Run the pool in each assay as low or high internal controls (see step 2.2).

2.2. For the assay, use the pool to make stocks for low and high controls that bind at 70% (C1) and 30% (C2), respectively. Obtain the dilution factor for the 30% and 70% binding points from the parallelism graph for the extract against the cortisol standard (**Figure 1**). Use the assay buffer for dilution of sample pool. For example, use 60 µL of the pool extract and 60 µL of assay buffer for 1:2 dilution.

NOTE: For the methanol extract, the 30% binding point was at neat while 70% binding point was approximately 1:2 as per **Figure 1**. Thus, these provided the dilution factor for the internal controls (C1 and C2 respectively) for running within the assay.

3. Cortisol analysis in koala fur extracts

3.1. Use a commercial cortisol kit (**Table of Materials**) and set up the 96 well strip plate including the samples, controls, cortisol standards, non-specific binding wells, and maximum binding wells following the supplier's instructions. Use the plate layout sheet provided in the kit booklet to list the positions of samples, controls, and standards on the plate map.

NOTE: It is recommended that all samples, controls, and standards are run in duplicate to allow accuracy of results.

3.2. Prepare samples. Follow the fur hormone extraction (section 1) to obtain 100% methanol extracted koala fur.

3.3. Prepare reagents. Follow the procedure described in the commercial cortisol kit to prepare the reagents including (1) assay buffer, (2) wash Buffer, and (3) standards (compositions provided in the cortisol kit, **Table of Materials**).

3.4. As per the instructions provided in the cortisol kit, pipet 50 µL of samples or standards into wells in the plate. Pipet 75 µL and 50 µL of assay buffer into the non-specific binding (NSB) wells and the maximum binding (B0 or zero standard) wells, respectively.

3.5. Add 25 µL of the cortisol conjugate to each well using a repeater pipet. Then pipet 25 µL of the cortisol antibody into each well, except the NSB wells. Gently tap the sides of the plate to ensure that the reagents are well mixed.

3.6. Cover the plate with the plate sealer and shake at room temperature for 1 h (at slow speed) using an orbital shaker.

3.7. Remove the plate sealer and aspirate the well plate by washing each well with 300 µL of wash buffer 4 times.

3.8. Dry the plate by tapping the plate on clean absorbent towels.

3.9. Pipette 100 µL of tetramethylbenzadine (TMB) substrate (composition provided in the cortisol kit, **Table of Materials**) to each well.

3.10. Place the plate sealer on the well plate and incubate at RT for 30 min.

3.11. Pipette 50 µL of stop solution to each well.

3.12. Place the well plate in a plate reader capable of reading 450 nm.

3.13. To calculate the final hormone concentration, derive the final fur cortisol concentration in ng/mg of sample by multiplying the pg/mL hormone concentration with the final extract volume (0.5 mL) and dividing by the fur sample mass (60 mg).

REPRESENTATIVE RESULTS:

Assay detection of hormone metabolites of interest is determined using parallelism. Using a parallelism curve, the 50% binding point also determines the sample dilution factor on the standard curve (**Figure 1**). As shown in the parallelism graph (**Figure 1**), the 100% ethanol and 100% Isopropanol extracts did not provide parallel displacement against the cortisol standard. However, the 100% methanol extract provided parallel displacement against the cortisol standard. Dried extracts were run neat through dilution in assay buffer (100 µL of 100% ethanol and 400 µL of assay buffer).

Intra- (within) and inter- (between) assay coefficients of variation (CV) were determined from high- (approximately 70%) and low- (approximately 30%) binding sample extracts run in all the assays. Based on the parallelism graph (**Figure 1**), the 30% (low) binding internal controls were neat koala extract pool while the 70% (high) binding internal controls were 1:2 diluted koala extract pool. CV% for internal high and low internal controls were <15%.

Error margin within the assay can be determined using quality control including the intra- and inter- assay coefficients of variation, which should be <15%. Assay sensitivity was calculated

as the value 2 standard deviations from the mean response of the blank (zero binding) samples, and expressed as 81.26 pg/well.

[Place **Figure 1** here]

Secondly, the association between each solvent extract and cortisol standard was determined using a regression plot (**Figure 2**). As shown in **Figure 2**, the 100% methanol extract provided the best line of regression with the highest R^2 value compared to the 100% ethanol and 100% isopropanol extracts.

[Place **Figure 2** here]

Furthermore, sub-set of koala fur extracted using each of the three solvents were assayed and the results are provided in **Table 1** below. As shown in **Table 1**, the observed concentration of cortisol standard was within the range of 2879.61−125.70 pg/well. Neither the ethanol or isopropanol extraction method could achieve consistency in the result as the fur extract concentrations obtained using either of the methods resulted in very high min-max range of hormone concentrations (see **Table 1** numbers marked in red), which were beyond the detection limit of the cortisol assay. However, the methanol extracts resulted in cortisol concentrations within the range of the cortisol standard (as shown in bold black numbers in **Table 1**). Furthermore, the concentrations of fur cortisol detected using methanol extraction method was highly consistent compared to the results obtained using the other two methods (see **Table 1**). Thus, we accept the null hypothesis that methanol is the most suitable solvent for koala fur hormone extraction compared to ethanol and isopropanol.

[Place **Table 1** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Parallelism of pooled koala fur extracted using 3 different solvents (100% ethanol, 100% isopropanol or 100% methanol) against cortisol standard curve under a cortisol enzyme-immunoassay. B/TB is the percentage of binding over total binding. The serial dilution factor (e.g., 1:2X mean dilution factor of 2) has been provided together with the concentration of each standard.

Figure 2: Regression plots for percentage binding of the cortisol standard against each of the 3 solvents (ethanol, methanol, and isopropanol) used to extract koala fur. The R^2 value was obtained from the line of best fit.

Figure 3: Conceptual flow diagram showing the key steps involved in the koala fur cortisol enzyme-immunoassay (EIA).

Table 1: The cortisol concentration (ng/mg) for koala fur (n = 18) extracted using 3 different solvents (ethanol, isopropanol or methanol) and run against cortisol standard curve under a cortisol enzyme-immunoassay. Bold red numbers show inconsistent concentrations for ethanol and isopropanol extracts which were beyond the assay range (pg/well). Bold black

numbers show the concentrations for fur cortisol extracted using methanol which fell within the range of the cortisol standards (pg/well).

DISCUSSION:

There are a number of studies that use a range of techniques to detect cortisol in mammalian fur. This study presents results for the detection of cortisol in fur collected from a wild koala exposed to current anthropogenic stress. This ground-breaking study used fur to test which of the three commonly used solvents are best at extracting cortisol, a measure of chronic stress, from koala fur. Results showed that 100% methanol was the recommended solvent for cortisol extraction in this type of mammalian fur.

Ethanol, methanol and isopropanol are all primary alcohols that are bonded by hydrogen molecules and are commonly used as solvents in hormone extraction experiments[28]. Generally, polar substances dissolve best in other polar substances, whereas non-polar substances dissolve best in other non-polar substances. The alcohol group containing methanol is very polar, whereas the alcohol group containing isopropanol is very non-polar. Due to its molecular build, alcohol group containing ethanol has the advantage of being both a polar and non-polar solvent. Steroid hormones such as cortisol are considered non-polar, meaning that cortisol should have a strong binding association with polar solvents.

For a more comprehensive analysis of extraction solvents used to assess physiological stress in koala fur, future research projects should attempt identical methods in that order as described in **Figure 3**. Similar studies have historically performed the wash before grinding[22], so as to ensure there is no unintended sweat and/or sebum derived cortisol deposited into the fur sample. Furthermore, it is important that measuring cortisol alone cannot guarantee a complete indication of chronic stress. Hair cortisol readings are a valuable tool when attempting to understand physiological stress experienced by an animal, but elevated HPA activity can occur under a variety of conditions including physical exercise, metabolic abnormalities and the presence of infectious disease[22]. Other important factors that should be taken into consideration to main integrity of hormone data include the following. (1) Acceptable level of random error—the coefficients of variation obtained from internal controls (CV1 and CV2) should be averaged to <15% for all assays. (2) Random error within sample assay—duplicate samples run on each plate should have a CV% of <15%; otherwise the sample will need to be re-run. (3) Assay detection limit—concentration of hormone quantified within each assay should be within the assay detection limit (between readings for highest dilution and neat standard); otherwise samples may require further dilution (if levels detected for samples are greater than the concentration of neat standard) or may not be analyzed within the assay (if levels detected for samples are less than the concentration of the highest diluted standard). (4) Assay sensitivity—this can be affected by background reading (non-specific binding), therefore it is important to maintain the highest level of quality assurance for the assay (e.g., equipment such as plate washer and plate reader must be serviced regularly). (5) Sample extract drying—this step could result in potential cross-contamination or loss of samples. It is recommended that samples be dried under steam of N_2 gas individually and to replace the Pasteur pipette used for extraction between each sample.

[Place **Figure 3** here]

The procedure outlined in this study (**Figure 3**) is one that can be easily replicated as it is relatively easy to perform, step-by-step methodology which incorporates readily available chemicals, reagents, and supplies with equipment that is likely to be found in a standard analytical laboratory. The application of this study enables a non-invasive technique to be used to assess physiological stress in both wild and captive koalas.

ACKNOWLEDGMENTS:

This work was supported through start-up research funding for Edward Narayan through the Western Sydney University, School of Science and Health. The authors thank Jack Nakhoul for assistance with sample processing.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

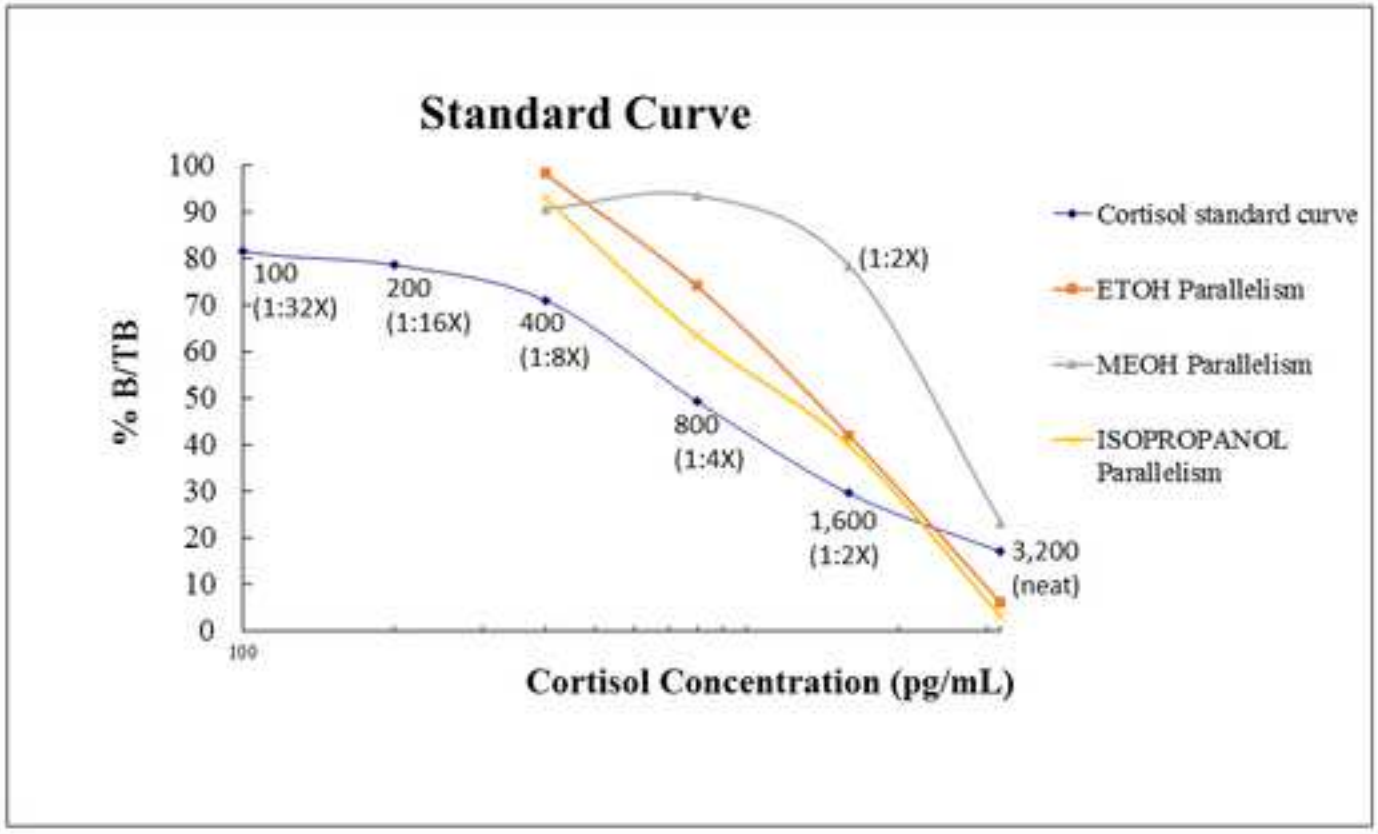

Figure 2

Click here to access/download;Figure;Fig 2.tif

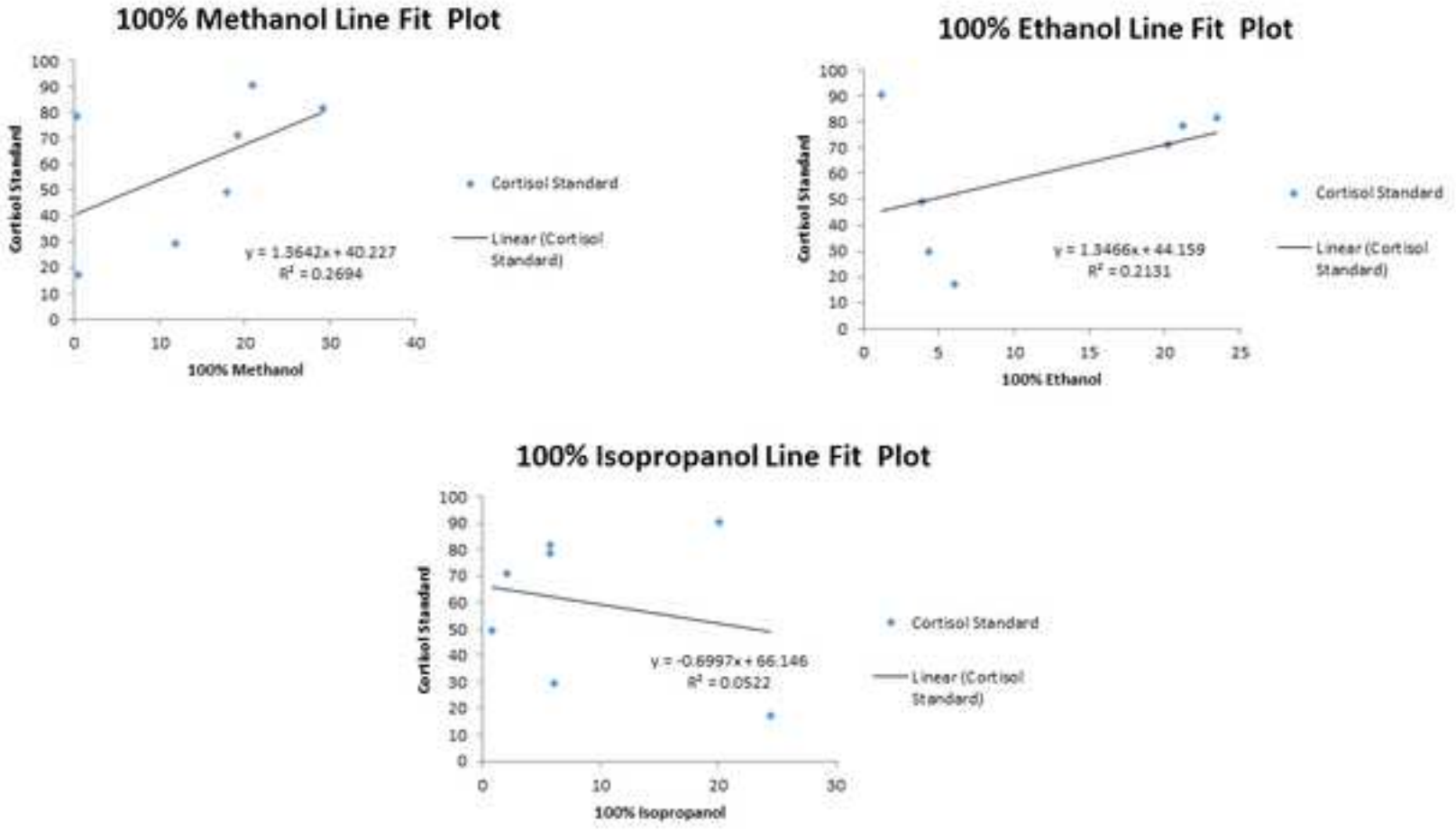

Figure 3

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Table 1 Click here to access/download;Table;59216_Table 1.xlsx

Standards	Conc. (pg/mL)	OD 1	OD 2
1	100	0.29	0.28
2	200	0.25	0.30
3	400	0.23	0.23
4	800	0.14	0.13
5	1600	0.09	0.10
6	3200	0.05	0.07
Extract solvent (100%)	**Samples**	**OD 1**	**OD 2**
Ethanol	1	0.03	0.03
	2	0.03	0.03
	5	0.03	0.03
	6	0.04	0.03
Methanol	7	0.25	0.13
	8	0.15	0.13
	9	0.17	0.12
	11	0.12	0.09
	12	0.15	0.10
Isopropanol	13	0.03	0.03
	14	0.03	0.03
	15	0.02	0.02
	16	0.03	0.04
	17	0.03	0.03
	18	0.03	0.02

OD 3	%B/TB 1	%B/TB 2	%B/TB 3
0.30	76.55	72.85	78.14
0.28	65.98	78.93	73.38
0.19	59.64	61.76	51.19
0.17	35.87	34.81	
0.13	24.77	25.83	
0.09	13.94	18.69	24.77
OD 3	**%B/TB 1**	**%B/TB 2**	
	8.65	8.12	
	8.12	6.80	
	8.92	7.33	
	9.71	8.65	
	67.04	34.81	
	39.83	34.28	
	45.90	31.37	
	31.90	24.24	
	38.51	26.35	
	7.07	8.39	
	7.33	7.07	
	4.95	6.54	
	6.80	9.71	
	7.07	7.07	
	8.65	6.01	

Mean	SD	CV%	Logit
75.85	2.71	3.57	1.14
72.77	6.49	8.93	0.98
57.53	5.59	9.72	0.30
35.34	0.75	2.11	-0.60
25.30	0.75	2.95	-1.08
19.13	5.43	28.38	-1.44
Mean	**SD**	**CV%**	**Logit**
8.39	0.37	4.45	-2.39
7.46	0.93	12.52	-2.52
8.12	1.12	13.80	-2.43
9.18	0.75	8.14	-2.29
50.92	22.79	44.76	0.04
37.05	3.92	10.59	-0.53
38.64	10.27	26.59	-0.46
28.07	5.42	19.30	-0.94
32.43	8.59	26.50	-0.73
7.73	0.93	12.09	-2.48
7.20	0.19	2.59	-2.56
5.75	1.12	19.51	-2.80
8.26	2.05	24.89	-2.41
7.07	0.00	0.00	-2.58
7.33	1.87	25.48	-2.54

pg/well		
125.71		
152.88		
348.02		
1044.80		
1865.22		
2879.61		
pg/well	**pg/mL**	**pg/g**
9091.00	363639.83	2424.27
10600.44	2120088.17	14133.92
9483.26	1896652.73	12644.35
8064.09	1612817.03	10752.11
480.58	96116.08	640.77
954.81	190962.75	1273.09
879.98	175995.40	1173.30
1570.72	314144.90	2094.30
1222.63	244525.64	1630.17
10128.02	2025603.59	13504.02
11111.65	2222329.97	14815.53
14877.15	2975430.15	19836.20
9283.63	1856726.12	12378.17
11383.26	2276652.48	15177.68
10850.92	2170183.11	14467.89

Koala fur cortisol conc.
ng/mg
3.03
17.67
15.81
13.44
0.80
1.59
1.47
2.62
2.04
16.88
18.52
24.80
15.47
18.97
18.08

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Centrifuge Tubes	n/a	n/a	1.5 mL
Chrome Steel Beads	n/a	n/a	3.2 mm x 3
Cortisol Kit	Arbor Assays	K003-H1W	Manufactured in Michigan USA
DetectX Cortisol Enzyme Immunoassay Kit	Arbor Assays	K003-H5	Used first-time for cortisol testing in koala fur
Ethanol	n/a	n/a	HPLC Grade
Isopropanol	n/a	n/a	HPLC Grade
Methanol	n/a	n/a	HPLC Grade
Micro Pipette	n/a	n/a	n/a
Micro Precision Sieve	n/a	n/a	0.5 mm
Microplate Reader	Bio Radi	n/a	n/a
Microplate Washer	Bio Radi	n/a	n/a
Orbital Shaker	Bio Line	n/a	n/a
Plastic Weighing Boat	n/a	n/a	n/a
Plate Sealer	n/a	n/a	n/a
Precision Balance	n/a	n/a	n/a
Vortex Mixer	Eppendorf	n/a	n/a

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Author(s): Renae Charalambous, Edward Narayan

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CORRESPONDING AUTHOR:

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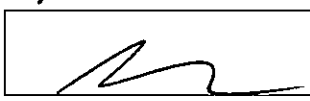
Signature:	
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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response: Manuscript has been thoroughly proof read.

2. Please revise lines 225-227, 232-234, and 240-243 to avoid previously published text.

Author response: These sentences have be re-written or omitted to avoid previously published text.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Arbor Assay DetectX Cortisol kit, Bio Red iMark, Eppendorf, etc.

Author response: Commercial language has been removed.

4. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc.

Author response: Revised as numbered list.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Author response: Protocol has been revised to contain only action items and actions have been described using imperative tense.

6. Lines 145-156: Please move the equipment information to the Table of Materials.

Author response: Moved.

7. Lines 158-220: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Author response: Discussion moved to discussion section.

8. Lines 230-234: Please include these steps in the protocol.

Author response: steps included in the protocol.

9. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Author response: A paragraph has been added to the discussion section to highlight critical steps, modifications/troubleshooting and limitations of the technique (see below).

For a more comprehensive analysis of extraction solvents used to assess physiological stress in koala fur, future research projects should attempt identical methods in that order as described in Fig. xxx. Similar studies have historically performed the wash before grinding 22, so as to ensure there is no unintended sweat and/or sebum derived cortisol deposited into the fur sample. Furthermore, it is important that measuring cortisol alone cannot guarantee a complete indication of chronic stress. Hair cortisol readings are a valuable tool when attempting to understand physiological stress experienced by an animal, but elevated HPA activity can occur under a variety of conditions including physical exercise, metabolic abnormalities and the presence of infectious disease 22. Other important factors that should be taken into consideration to main integrity of hormone data include (1) Acceptable level of random error – the coefficients of variation (CV%) obtained from internal controls (CV1 and CV2) should be averaged to < 15 % for all assays. (2) Random error within sample assay – duplicate samples run on each plate should have a CV% of < 15% otherwise the sample will need to be re-run. (3) Assay detection limit – concentration of hormone quantified within each assay should be within the assay detection limit (between readings for highest dilution and neat standard) otherwise samples may require further dilution (if levels detected for samples are greater than the concentration of neat standard) or may not be analysed within the assay (if levels detected for samples are less than the concentration of the highest diluted standard). (4) Assay sensitivity – this can be affected by background reading (non-specific binding) therefore it is important to maintain the highest level of quality assurance for the assay (e.g. equipment such as plate washer and plate reader must be serviced regularly).

10. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

Author response: acknowledgements section has been provided.

11. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Author response: Provided.

12. References: Please do not abbreviate journal titles.

Author response: corrected.

13. Figure 1: Please indicate the numbers under the x ticks. Please explain what 1:2X means in the figure legend.

Author response: information provided.

14. Table 1: Please upload it to your Editorial Manager account as an .xls or .xlsx file.

Author response: Provided.

15. Table of Materials: Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.

Author response: revised.

16. Please provide a point by point response to each of the reviewers' comments and editorial comments.

Author response: Provided.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:

Manuscript Summary:

I understand that the manuscript titled "Cortisol measurement in koala 1 (Phascolartos cinereus) fur" came to me to review the responses to reviewers. I checked the responses one by one. I think now the manuscript is fit to be accepted. However, I wish the authors could reply the comments by referring the reply in the text by LINE number. Also, changing color of edited text could make it easier for other reviewers to find out how the issues are addressed. When I see other responses to the comments, I found it hard to follow the responses in the way that authors provided a response. Anyways, in my point of view the lack of presentation in this work is covered and could be accepted for publication in the present form.

Sincerely

Author response: We have used track change this time to show the changes made.

Reviewer #2:

Manuscript Summary:

This revised manuscript details the extraction and enzyme immunoassay methodology used to measure cortisol in koala hair. This is a non-invasive option for measuring this hormone, which could be a useful approach for assessing adrenal production of cortisol over a prolonged period, such as in response to anthropogenic disturbance. Despite a number of improvements, I still have several concerns over the methodology that must be corrected before this manuscript is ready for publication, especially as a methods paper for others in the field to follow.

Major Concerns:

There are still methodological concerns with this manuscript that should not be left unchanged if this manuscript/video is to be used to demonstrate this method for readers/viewers to replicate.

1. Pulverizing the hair samples before washing is a serious issue with this method, and could result in readers compromising their data if they follow this technique. I appreciate that you have added a sentence to this effect in the discussion, but I do not think it is appropriate to present the technique this way in a methods paper. I recommend you re-extract your hair sample (wash before you pulverize and use 100% solvent to extract) and repeat your analyses.

Author response: In the revised methods, samples were firstly washed, dried and then grinded prior to extraction using 100% of each solvent.

2. The assay methodology is also still inappropriate as the solvent is being run directly (or diluted 1:2) on the assay which will likely lead to unreliable results. As the assay manufacturer states themselves, you should dry your extracts and re-suspend in the appropriate assay buffer before conducting your parallelism or analyzing your 18 extracts.

Author response: In the revised methods, extracts were firstly dried under nitrogen gas before re-suspending in the assay buffer as per the Abor assay protocol so that the final volume of alcohol in the sample is <5%. This is how we intend to present the demonstration video for this method.

3. Results - the authors no longer report the results of running their replicated extractions, only the parallelism serial dilution. Although this was a necessary addition, it should not be in place of the replicated extracts -these are needed too, so that we can assess how repeatable (and therefore useful) your methodology is.

Author response: We have showed results for the replicated extracts in new Table 1.0 and used this to demonstrate that 100% methanol rather than 100% ethanol is the most reliable extraction solvent.

Minor Concerns:

Lines 22-23: Suggest adding 'in different sample types' to this sentence - as is, this sounds like all methods are inconsistent, which is not true, I think you mean the optimal method across sample types is not always the same.

Author response: Revised.

Line 103: Suggest changing cortisol to glucocorticoids, because rarely is cortisol measured in urine or feces

Author response: Corrected

Lines 114-117: This statement is not true - hair cortisol is usually extracted by first washing and then pulverizing.

Author response: sentence has been corrected.

Line 126: You are extracting cortisol from hair, not extracting cortisol in hair

Author response: corrected.

Line 128-129: This is not the reason for using 90% methanol. The standard practice is to use 100% solvent for extraction of steroids from hair and then dry/reconstitute your extracts in an appropriate assay buffer, so please remove this statement so as not to mislead the reader.

Author response: Removed statement.

Line 131: You are not using 'current techniques'; you are using a modified technique. Please remove this statement so as not to mislead the reader.

Author response: Removed statement.

Line 136: Arbor assays recommends the use of 100% methanol, so please edit this sentence to say methanol is the recommended solvent, not 90% methanol is recommended.

Author response: Removed statement.

Lines 159-220: the methods are written in a mixture of tenses - if this is a protocol to be followed, please check consistency.

Author response: checked.

Lines 177-183: It is not standard protocol to pulverize hair samples before washing - you are likely losing a portion of the cortisol from within the hair - i.e. your sample - so I do not think this is appropriate methodology for a methods journal.

Author response: In our revised methods that we wish to present involves washing and then pulverising.

Line 204: Please change 'each solvent' to 'each extract'

Author response: corrected.

Line 207: Please change 'pools' to 'diluted samples' as this covers both your serial dilution used here, and your diluted extracts that you should also have run

Author response: corrected.

Lines 214 and 218: Now that you have the equipment list, the specific brand of your equipment is not needed here. This is now written as a protocol, so instructing the reader to wash 4 times (they don't have to use a plate washer) and to read the absorbance in a plate reader capable of plate reader capable of measuring at 450nm, would be better.

Author response: corrected.

Lines 225 - 227: Please report the results of the parallelism. Standard for this field is to do a regression of the percent binding of the standards and each pool.

Author response: Results has been provided in new Figure 2.0.

Lines 227-234: As written, this is just the method for how to run controls, not results. Do you have any CVs to report?

Author response: We have reported the CVs for high and low internal controls.

Line 247: the CV between sample replicates (duplicate wells) does not tell you how consistent the extraction protocol is. If you run all 18 extracts, each in duplicate, how similar are your values from each extraction technique? This is the information the reader needs to know to assess which is the better extraction method - all we are really seeing in table 1 is which solvent fit better within the limits of the standard curve, and how good your pipetting was.

Author response: We have replaced Table 1.0 with results including binding for the 18 extracts run on the cortisol assay. This shows that methanol extraction was the most reliable as the concentrations (pg/well) were within the range limit of the assay.

Figure 1: Isopropanol is misspelled in the legend

Author response: Corrected.

Methods and Figure 1 legend: A 1:2 dilution of solvent is not appropriate for this assay, you should dry and re-suspend in assays buffer as stated in the Arbor Assays extraction protocol.

Author response: Corrected.

Reviewer #3:

Manuscript Summary:

This study provides useful step by step guidelines for the efficient and reliable extraction of cortisol from koala fur and it should be very useful for others wishing to analyse cortisol extracted from koala fur to determine physiological status of the HPA axis. The techniques are clearly described and the efficiency of organic extraction methods are compared. Extraction methods can often be compared using a 3H spike however I see no reliable way to use this spike method to guarantee the actual efficiency of cortisol extraction from the cortisol incorporated naturally into the matrix of the hair.

Major Concerns:

No major concerns

Minor Concerns:

No minor concerns

A few minor corrections that could be altered at typesetting stage

L16 'Here' should be deleted

Author response: deleted

L154 Eppendorf MiniSpin Plus - also appears later in text for correction

Author response: removed

L174 Add 3 chrome steel beads (3.2mm) and pulverize

Author response: added

L220 'will be used' alter tense to conform to section style

Author response: corrected

L246 Isopropanol
