



# Variation in the extraction efficiency of estradiol and progesterone in moist and lyophilized feces of the black howler monkey (*Alouatta pigra*): alternative methods

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Several fecal steroid extraction techniques have been developed to measure the ovary function in different species of mammals. However, regardless of the method of extraction and the sample type chosen, it has been observed that they can yield results with different percentages of recuperation. The objective of this study was to determine whether the type of substratum, solvent and extraction method used have any influence on the extraction efficiency in the feces of *Alouatta pigra* (black howler monkey). For this purpose we used two methods: agitation and ebullition. With each method, we utilized moist and lyophilized feces. The validation of radioimmunoassay method was accurate and precise for quantify estradiol and progesterone in lyophilized feces of *A. pigra*. To both of which ethanol and methanol, absolute and at 80%, were added, besides the hormones <sup>125</sup>I-Estradiol and <sup>125</sup>I-Progesterone. The extraction efficiency for <sup>125</sup>I-Estradiol was from 87.72 ± 3.97 to 41.24 ± 2.67%, and for <sup>125</sup>I-Progesterone from 71.15 ± 4.24 to 42.30 ± 1.19% when we used the agitation method. Whereas with the ebullition method, the extraction efficiency for <sup>125</sup>I-Estradiol ranged from 86.89 ± 2.66 to 71.68 ± 3.02% and for <sup>125</sup>I-Progesterone from 98.31 ± 1.26 to 85.40 ± 1.98%. Due to the differences found in these assays, which depend on the method used, the type of feces employed and the type of solvent added to them, we recommend the ebullition method and the lyophilized feces of *A. pigra* for extracting the hormones, since in moist feces there may exist variables which might interfere in the quantification of <sup>125</sup>I-Estradiol and <sup>125</sup>I-Progesterone.

**Keywords:** extraction method, estradiol, progesterone, feces, *Alouatta pigra*

## INTRODUCTION

Fecal hormone analysis has provided a non-invasive tool for monitoring the endocrine condition in wild animals (Mateo and Cavigelli, 2005) and has been extensively employed in the field endocrinology of non-human primates (Brockman and Whitten, 1996; Ziegler et al., 1996). This contribution is important when the physiological events to be studied are infrequent or continue for long periods (Lasley and Kirkpatrick, 1991; Harper and Austad, 2000; Wasser et al., 2000; Millspaugh et al., 2001, 2002; Möstl and Palme, 2002; Touma et al., 2003). Therefore the acknowledged value of monitoring the reproductive function through the analysis of hormones in excreta has created interest in the choice of an appropriate biological sample, because the feces of diverse vertebrates contain a mixture of metabolized hormones such as progestins, estrogens, androgens, glucocorticoids, and mineral corticoids (Palme and Möstl, 1997; Hunt et al., 2004) which, in some cases, are mixed with metabolites of different polarities (Goymann, 2005; Möstl and Palme, 2005; Palme, 2005). Adlercreutz and Martin (1980) propose that the excretion rate and the metabolism of steroid hormones and their metabolites vary from one species to another, which must be considered for the procedures of steroid extraction from feces (Möstl and Palme, 2005; Palme, 2005; Ziegler and Wittwer, 2005).

Since the techniques of fecal hormone extraction have facilitated hormone analysis, it becomes necessary to concentrate the substance of interest and, at the same time, to eliminate those compounds that may interfere in said analysis (Wasser et al., 1993; Möstl and Palme, 2005), such as water, urine, and inert matter, which can mask the quantification of steroid hormones. Some research methods used for hormone extraction are based on the selection of a solvent, the application of temperature (Gao and Liu, 2005), the types and time of agitation, using moist feces (Heistermann et al., 1996; Wasser et al., 2000; Hunt and Wasser, 2003; Hunt et al., 2004; Cristóbal-Azkarate et al., 2007) nor lyophilized feces (Wasser et al., 1997, 2004; Goymann et al., 1999; Khan et al., 2002; Hunt et al., 2004) from diverse species of mammals. Also in these studies include the evaluation of free ligand activity added to the substratum and validation of the assay (Buchanan and Goldsmith, 2004; Palme et al., 2005).

In order to quantify the steroid metabolites in primate feces, has been necessary determine the efficacy of the hormone recovery to be studied in moist or lyophilized fecal samples (Risler et al., 1987; Wasser et al., 1988, 1992; Ziegler et al., 1996), and also what type of solvent can be used to improve the efficiency of hormonal recovery from feces. For the moment, there are few if any standardized techniques for evaluating the extraction efficacy in both samples for

the same species of primates, as is the case of the species *Alouatta pigra* (black howler monkey).

Steroids from the moist feces of black howler monkey (*A. pigra*) have been extracted, and the efficacy of the extraction of steroid metabolites radio marked with tritium have been evaluated (Van Belle et al., 2009). However, due to factors which may or may not modify the measurement of efficiency of hormone extraction in feces from the species (Wasser et al., 1988; Brown et al., 1994; Ziegler et al., 1996; Terio et al., 2002; Palme, 2005), we consider that is necessary to add radio marked hormones to the fecal samples in order to measure the quantity of said tracer at the time of implementing a new method of extraction in such a way that the recovered percentage of the tracer makes it possible to evaluate the recovery efficacy in the biological sample. The objectives of this study were: (1) to determine whether the physical condition of the sample (moist or lyophilized feces) and the type of solvent may influence the extraction efficacy of the hormones  $^{125}\text{I}$ -estradiol and  $^{125}\text{I}$ -progesterone added to the stratum (feces); (2) to compare the protocol for extraction in this study, agitation vs. ebullition, in order to obtain and value the percentage of increased extraction efficiency of the radio marked hormone added to moist and lyophilized feces of howler monkeys; and (3) to validate the hormonal extraction technique in the lyophilized feces of *A. pigra*.

## METHODS

### STUDY SITE AND SUBJECTS

In this study, we collected fecal samples from 16 free-living adult female black howler monkeys (*A. pigra*) belonging to different groups (Office No. SGPA/DGVS/04949/07). These were collected during November and December, 2007 in fragmented forest on the private property of the Alamo (18°48'15.1"N and 90°58'59.8"W) and Calax Ranches (18°50'37.6"N and 91°18'26.4"W) and the Tormento Natural Reserve (18°16'25"N and 90°43'55"W), all areas belonging to Campeche, Campeche, México.

### FECAL SAMPLE COLLECTION

Fecal samples were collected immediately after the first individual excretions (between 0700 and 0900 hours) and transferred into a commercial plastic container (50 ml) with a lid. These fresh fecal samples were collected if they were clean of urine or any other pollutant (e.g., rain and rubbish). All fecal samples from different females were pooled and homogenized together, then put into a container for transport and storage in a freezer at  $-20^{\circ}\text{C}$  until their analysis in the Laboratory of Steroid Hormones, National Institute of Medical Sciences and Nutrition "Salvador Zubiran," Mexico D.F. All the samples were stored and transported under the same conditions.

### SAMPLE PREPARATION

From the previously frozen fecal samples, 42 were defrosted gradually to room temperature and later weighed ( $\sim 0.600\text{ g}$ ); then they were placed in a (50 ml) tube of polypropylene previously labeled. The other frozen samples ( $n = 35$ ) were lyophilized to dryness during 3 days in a freeze dry system (LABCONCO, Freezone 6, EQUIPAR S.A. de C.V. México) and pulverized in a mortar to remove the inert material, as well as leaves and hard seeds, as described by Heistermann et al. (1996). Each sample was thawed

and weighed ( $\sim 0.200\text{ g}$ ), then placed in a previously labeled 50-ml polypropylene screw-top tube, which was stored at  $-20^{\circ}\text{C}$  until hormonal analysis.

### FECAL ESTRADIOL AND PROGESTERONE EXTRACTION

For fecal estradiol and progesterone extraction we employed two methods, (1) agitation and (2) ebullition (called boiling by Wasser). We employed said methods with some modifications to the method described by Wasser et al. (1991, 2000), Palme et al. (2000), Möstl and Palme (2002), and Cristóbal-Azkarate et al. (2006) for the extraction of steroids. In both methods we used moist and dry samples, with two organic solvents at different concentrations (absolute methanol, 80% methanol, and 80% ethanol).

### AGITATION METHODS I

#### ***Moist feces treated with absolute methanol and added $^{125}\text{I}$ -estradiol and $^{125}\text{I}$ -progesterone***

Initially we used  $\sim 0.600\text{ g}$  of moist homogenized fecal sample ( $n = 7$ ), which was placed in 15-ml polypropylene tubes. Subsequently, 4.0 ml of absolute methanol ( $\text{CH}_3\text{OH}$ ) and 1 ml of radioactive tracer  $^{125}\text{I}$ -Estradiol radioimmunoassay (RIA) kit (Coat-A-Count Estradiol, DPC®, Diagnostic Products Corporation, Los Angeles, CA, USA) were added directly to each tube.

Other tubes containing the same amount of moist feces ( $n = 7$ ) received 4.0 ml of absolute methanol and 1.0 ml of radioactive tracer  $^{125}\text{I}$ -Progesterone RIA kit (Coat-A-Count Progesterone, DPC®, Diagnostic Products Corporation, Los Angeles, CA, USA) to determine the recovery efficiency of each hormone, estradiol and progesterone. After that, all the tubes were shaken in a Vortex (Shaker model "Vortex") for 1 min in order to homogenize the samples with the methanol, and then they were shaken continuously for 18 h. After this lapse of time, they were centrifuged at 3500 rpm at a temperature of  $4^{\circ}\text{C}$  for 15 min; then the supernatant was poured into other clean tubes and these were placed in a double boiler at  $75^{\circ}\text{C}$  within an extraction bell until their total evaporation. Later on, the tubes were re-suspended with 3.0 ml of buffer (standard zero calibrator, DHEA, DPC, Los Angeles, CA, USA). Once again these tubes were placed in the double boiler at  $37^{\circ}\text{C}$  for 30 min in order to homogenize the samples and were shaken for 1 min in the vortex. Finally, all the samples were counted in duplicate in the gamma counter for RIA (Packard® Cobra™II Auto-Gamma® counter, A Canberra Company, CT, USA).

#### ***Moist and lyophilized feces treated with methanol and ethanol at 80% and added $^{125}\text{I}$ -estradiol and $^{125}\text{I}$ -progesterone***

About 4.0 ml of 80% methanol and 1 ml of radioactive tracer  $^{125}\text{I}$ -Estradiol RIA kit were added to the tubes with moist ( $\sim 0.600\text{ g}$ ,  $n = 7$ ) and lyophilized ( $\sim 0.200\text{ g}$ ,  $n = 7$ ) previously weighed feces. Other tubes containing the same amount of moist and lyophilized feces also received the addition of 4.0 ml of 80% methanol and 1.0 ml of radioactive tracer  $^{125}\text{I}$ -Progesterone RIA kit, in order to monitor the extraction efficiency of each hormone. We used the same procedure previously described for hormonal extraction.

## EBULLITION METHODS II

### **Moist and lyophilized feces treated with absolute methanol and 80% methanol and added $^{125}\text{I}$ -estradiol and $^{125}\text{I}$ -progesterone**

About 4.0 ml of 80% methanol and 1.0 ml of radioactive tracer  $^{125}\text{I}$ -Estradiol RIA kit were added to the tubes containing previously weighed ( $\sim 0.600$  g) moist feces ( $n = 7$ ). Other tubes containing previously weighed ( $\sim 0.200$  g) lyophilized feces ( $n = 7$ ) received the addition of 4.0 ml of absolute methanol and 1.0 ml of radioactive tracer  $^{125}\text{I}$ -Estradiol RIA kit to determine the extraction efficiency of these tests.

To determine the extraction efficiency of progesterone, other tubes with moist ( $n = 7$ ) and lyophilized ( $n = 7$ ) fecal samples received the addition of 4.0 ml of absolute methanol and 1.0 ml of radioactive tracer  $^{125}\text{I}$ -Progesterone RIA kit; whereas the remaining tubes with lyophilized feces ( $n = 8$ ) received 4.0 ml of 80% methanol and 1.0 ml of the same radioactive tracer. Subsequently, all capped tubes were vortexed for 3 min in a pulsating vortexer (agitator model "vortex") and then placed in a hot water bath at  $75^\circ\text{C}$  for 20 min. The tubes were removed and mixed in a pulsating vortexer during 1 min; then all were centrifuged for 15 min at 3500 rpm at  $4^\circ\text{C}$ . The supernatant was poured off into a clean glass tube. The tubes with sediment were re-suspended with 1.0 ml of absolute methanol and/or 80% methanol (only for those samples that required 80% methanol), then they were centrifuged again at 3500 rpm for 15 min at  $4^\circ\text{C}$ . The supernatant was transferred to the tubes that already contained the first supernatant.

Subsequently, these tubes were placed in a hot water bath at  $75^\circ\text{C}$  until evaporation occurred. Later on, they were re-suspended with 3.0 ml of buffer (albumin 4%) and placed again in a hot water bath at  $37^\circ\text{C}$  for 30 min, then shaken in the vortex for 1 min. Finally, an aliquot of 200  $\mu\text{l}$  was obtained from each independent fecal sample and transferred to tubes which were counted in duplicate in the gamma counter for RIA to obtain the percentage of recovery by the radioactive tracer  $^{125}\text{I}$ -estradiol and  $^{125}\text{I}$ -progesterone added to each sample in the various tests in this study.

## ANALYTIC VALIDATION

### **Accuracy**

Accuracy is the degree to which the measured concentration corresponds to the true concentration of a substance. This is related in part to the specificity of the assay. We used the dose–response curve and parallelism to determine whether the hormone in the pooled fecal extracts was interacting with the antibody in a manner similar to the standard curve; the calculations were made through parallelism. This also required an analysis of the sample pool without any added standard to determine the amount of endogenous (or background) hormone present. The concentration in all of the samples was expressed in nanogram per gram for progesterone and picogram per gram for estradiol.

### **Dose–response study**

Extracts from dried fecal samples, randomly selected, were divided into five portions. To each portion of extracted fecal samples, a known value of the standard curve of estradiol was added, while five other portions of extracted fecal sample received a standard curve of progesterone; in both cases the first extract functioned as the control. Estradiol was added in doses of 0, 150, 500, and

1800 pg/ml in each of the tubes, and 0, 5, 10, and 20 ng/ml of progesterone were added in the other tubes. Subsequently, each aliquot was analyzed as an individual sample and counted in duplicate in the gamma counter for RIA.

### **Parallelism**

In this study we showed the parallelism of the pool samples with the standard curve. Samples to be tested should reflect the range of normal concentrations expected. To test the parallelism between fecal extracts and standard, the pooled samples were serially diluted four times (1:2, 1:4, 1:8, 1:16, and 1:32) by using  $\text{E}_2$  and  $\text{P}_4$  of the standard curve provided by the RIA kit for estradiol and progesterone. Data from each of the serially diluted samples made it possible to obtain the percentage of union of the antibody (% B/TB) of the standard curve and the fecal extracts. Then each aliquot was analyzed as an individual sample and counted in duplicate in the gamma counter for RIA.

### **Precision**

Precision refers to the repeatability of a measured value or the consistency of results. It is a measure of random error defined as the variation among replicate measurements of a defined sample. The precision was assessed with the intra- and inter-assay coefficients of variation and is expressed as the coefficient of variation (%CV). The coefficient of variation was obtained by pooling the control samples, low, medium, and high (quality controls), all samples being analyzed in duplicate.

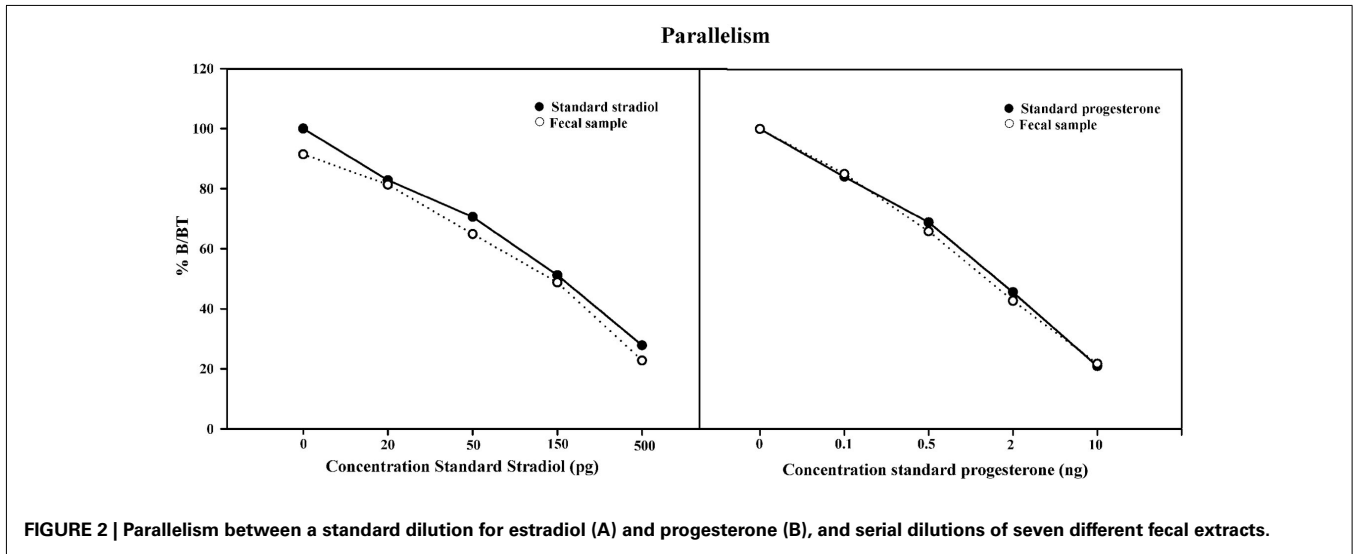
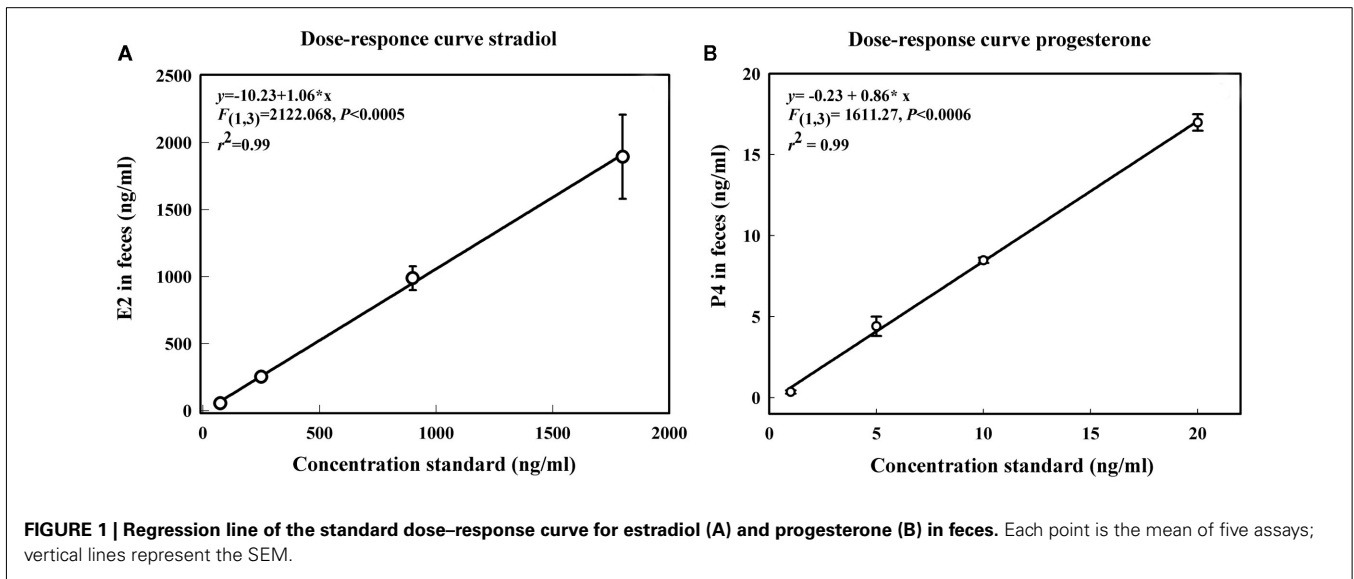
### **Data analysis**

The Kolmogorov–Smirnov normality test was used in order to verify the type of data distribution. Once they passed the normality test, the data were analyzed through the One-Way ANOVA test for repeated samples, and thereafter through the Tukey test ( $P < 0.05$ ). When the normality test was not passed, they were evaluated by using the Kruskal–Wallis test. The median coefficient of variation (%CV) was calculated from the internal and external variation of the fecal pool. The results are expressed as the mean  $\pm$  SE of each variable evaluated.

## RESULTS

### ANALYTIC VALIDATION

Assay validation results showed that in the dose–response curve there was a correlation coefficient of  $r^2 = 0.996$  (Figure 1A) for estradiol and  $r^2 = 0.999$  (Figure 1B) for progesterone. The dilutions of the fecal extracts showed parallelism with those of the standard curve for estradiol and progesterone (Figures 2A,B), with no difference in their slope (value of  $P > 0.05$ ). This sample curve parallels the standard curve; the sample hormone is immunologically similar to the standard and can be measured proportionately. The inter-assay variation coefficient was 9.21% for estradiol and 8.97% for progesterone; whereas the intra-assay values were 8.69% for estradiol and 8.03% for progesterone. These results enable us to evaluate the accuracy and precision of the radioimmunoassay in estimating its internal deviation and in identifying the errors made during the process. The respective cross-reactivity is provided by the manufacturer. The degree of sensitivity for the estradiol and progesterone assays in the feces was detected with a counter from



Packard® Cobra™II Auto-Gamma® (A Canberra Company, CT, USA), and was found to be 0.00272 pg/ml for E<sub>2</sub> and 0.01 ng/ml for P<sub>4</sub> (minimum concentration detectable), respectively.

#### PERCENTAGE OF EXTRACTION EFFICIENCY

In this study, we observed the recuperation percentage of extraction of the tracer <sup>125</sup>I-Estradiol and <sup>125</sup>I-Progesterone added to the moist and lyophilized feces of *A. pigra*, tested with two types of methods and treated with two solvents at different concentrations (see Tables 1 and 2).

#### COMPARISON OF EXTRACTION EFFICIENCY OF TWO RADIOACTIVE TRACERS ADDED TO MOIST AND LYOPHILIZED FECES TREATED WITH TWO SOLVENTS

##### Agitation method for estradiol

It was found that the extraction efficiency of the radioactive tracer <sup>125</sup>I-Estradiol added to moist feces treated with ethanol

at 80% ( $87.72 \pm 3.97\%$ ) showed significant differences [one-way ANOVA:  $F(2,12) = 32.514, P < 0.001$ ] when compared with that obtained from moist feces treated with absolute methanol and lyophilized feces with ethanol at 80%. No significant differences [ $F(1,9) = 2.658, P < 0.137$ ] were found between the extraction efficiency of the radioactive tracer <sup>125</sup>I-Estradiol added to moist feces treated with absolute methanol and that of the same tracer added to lyophilized feces treated with ethanol at 80% (Figure 3A).

##### Ebullition method for estradiol

Using the ebullition method, we found significant differences [ $F(1,12) = 14.220, P < 0.003$ ] between the extraction efficiency of the radioactive tracer <sup>125</sup>I-Estradiol added to moist feces and to lyophilized ones, both treated with absolute methanol. The extraction efficiency with moist feces treated with absolute methanol turned out to be greater than with lyophilized feces treated with absolute methanol (Figure 3B).

**Table 1 | Shows the percentage of extraction of the tracer  $^{125}\text{I}$ -estradiol added in wet and lyophilized feces of *Alouatta pigra* tested with two types of methods.**

Percentage of estradiol extraction				
Shaking methods			Ebullition methods	
Feces wet		Feces lyophilized	Feces wet	Feces lyophilized
Methanol absolute	Ethanol 80%	Ethanol 80%	Methanol absolute	Methanol absolute
51.18 ± 6.78%	87.72 ± 3.97%	41.24 ± 2.67%	86.89 ± 2.66%	71.68 ± 3.02%

**Table 2 | Shows the percentage of extraction of the tracer  $^{125}\text{I}$ -progesterone added in wet and lyophilized feces of *Alouatta pigra* tested with two types of methods.**

Percentage of progesterone extraction					
Shaking methods			Ebullition methods		
Feces moist		Feces lyophilized	Feces moist	Feces lyophilized	
Methanol absolute	Methanol 80%	Methanol 80%	Methanol absolute	Methanol absolute	Methanol 80%
71.15 ± 4.24%	42.30 ± 1.19%	67.13 ± 1.88%	98.31 ± 1.26%	87.82 ± 2.84%	85.40 ± 1.98%

### Agitation method for progesterone

We found significant differences [ $F_{(2,15)} = 52.776$ ,  $P < 0.001$ ] between the extraction efficiency of  $^{125}\text{I}$ -Progesterone added to moist feces treated with absolute methanol and to lyophilized feces treated with methanol at 80%. However, no significant differences [ $F_{(1,9)} = 1.010$ ,  $P < 0.341$ ] were noted between the extraction efficiency of  $^{125}\text{I}$ -progesterone added to moist feces treated with absolute methanol and to lyophilized feces treated with methanol at 80% (Figure 3C).

### Ebullition method for progesterone

Finally, with ebullition method we found significant differences [ $F_{(2,14)} = 6.330$ ,  $P < 0.011$ ] between the extraction efficiency of  $^{125}\text{I}$ -Progesterone added to moist feces treated with absolute methanol compared to that in lyophilized feces treated with absolute methanol and 80%. When we compared the extraction efficiency of the radioactive tracer  $^{125}\text{I}$ -Progesterone added to lyophilized feces with absolute methanol to that obtained with lyophilized feces treated with methanol at 80%, no significant differences [ $F_{(1,11)} = 0.455$ ,  $P < 0.514$ ] were found between the two groups (Figure 3D).

### COMPARISON BETWEEN METHODS OF EXTRACTION

We found statistically significant differences ( $P < 0.001$  Student–Newman–Keuls) in the mean values among the groups treated with the radioactive tracer  $^{125}\text{I}$ -estradiol and processed with two methods, agitation and ebullition. However, there were no significant differences in the percentage of extraction efficiency for  $^{125}\text{I}$ -estradiol between methods, when bars two and four of Figures 3A,B were compared. There were significant differences in the percentage of extraction efficiency when all the assays treated

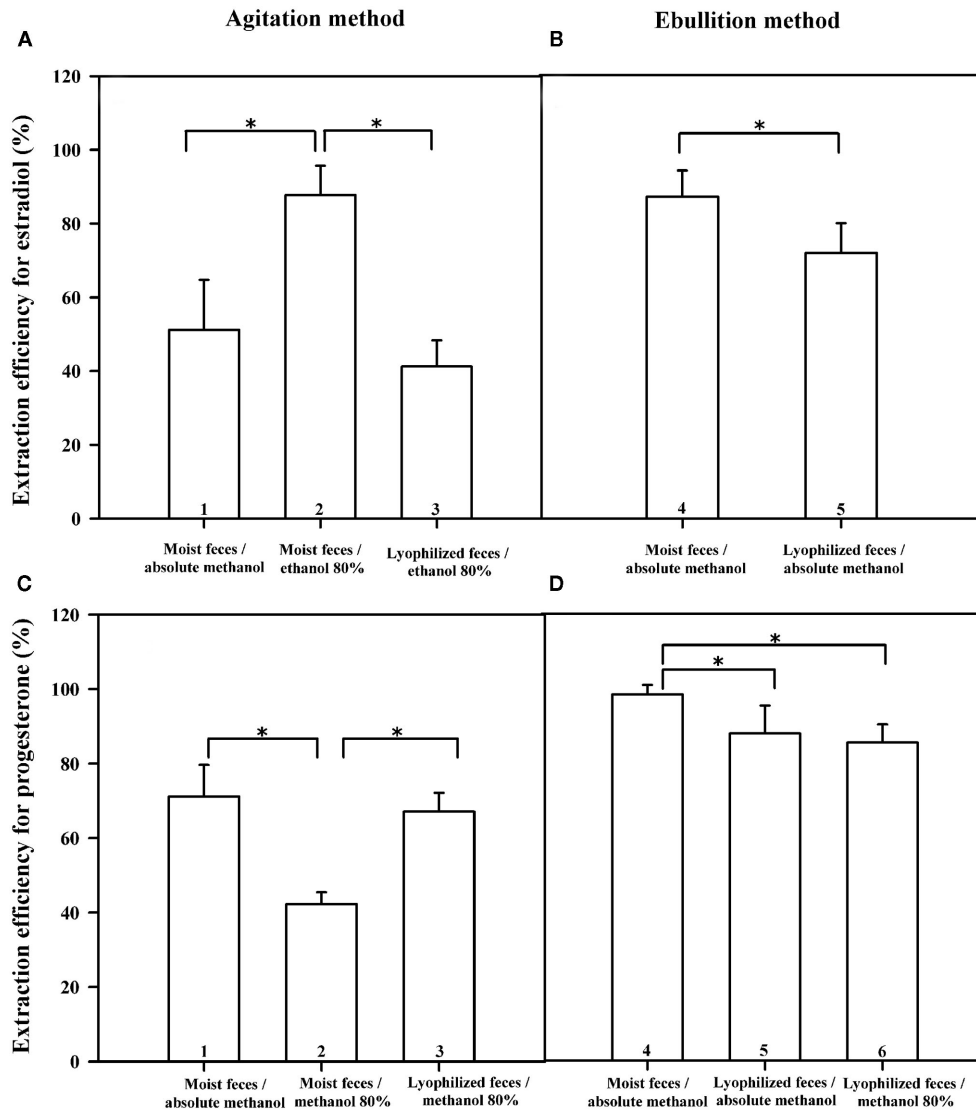
with the tracer  $^{125}\text{I}$ -progesterone and processed with the two methods were compared (Table 3).

## DISCUSSION

### RIA VALIDATION

Our results showed that fecal estradiol and progesterone from *A. pigra* can be measured with accuracy and precision in lyophilized feces with a radioimmuno assay kit for commercial estradiol and progesterone. The dose–response curve in our study indicated the accuracy of the method, because nearly 100% of the steroid in the pre-processed fecal sample was recovered without chromatographic separation, that is, it allowed us to evaluate the exactness of the antibody, since both hormones showed a high correlation coefficient. This suggests that there is little interference for the substance that might bind with the antibody and produce altered results. The study in parallel for both estradiol and progesterone showed the content of the steroid extracted from the fecal samples. The dilutions of hormone contained in the feces presented a curve similar to that of standard dilutions (Figure 2), which means that the antibody immunologically recognized the structure of the standard (the radiomarked hormone), as well as that of the native hormone contained in the fecal sample. This suggests that lyophilized fecal steroids are valid as a useful tool for monitoring the reproductive status in captive *A. pigra*, just as they have been for other primate species (Stavisky, 1994; Stavisky et al., 1995; Palme, 2005; Touma and Palme, 2005).

The precision of the assay was generally low (<10%) for both hormones analyzed from extracts of lyophilized feces. Similarly, other studies have reported a coefficient of variation averaging from 2.1 and 2.2% to 9.4 and 18.4% (Wasser et al., 1988, 1991; Ziegler et al., 1996; Maheshwari et al., 2010). The proper validation and standardization of these assays is only the first step



**FIGURE 3 | Shows a comparison of the accuracy of extraction assessed through the quantitative recovery of exogenous  $^{125}\text{I}$ -Estradiol from fecal extracts with different solvents and methods.**

(A,C) Agitation methods, means and SE of  $^{125}\text{I}$ -Estradiol and

$^{125}\text{I}$ -Progesterone extracted from fecal extracts; and (B,D) Ebullition methods II, means and SE of  $^{125}\text{I}$ -Estradiol and  $^{125}\text{I}$ -Progesterone extracted from fecal extracts (significant comparisons indicated by connecting bars on the abscissa, \* $P < 0.001$ ).

toward establishing a reliable endocrine monitoring program in lyophilized feces of *A. pigra*. Subsequent assessment of assay quality and consistency is absolutely necessary to ensure the biological relevance of the results. We conclude that this technique of solid phase radioimmuno assay was reliable and precise for evaluating hormonal levels in the lyophilized feces of *A. pigra*.

#### PERCENTAGE OF EXTRACTION EFFICIENCY

In the present study we found statistically significant differences in the percentage of extraction efficiency for  $^{125}\text{I}$ -estradiol and  $^{125}\text{I}$ -Progesterone added to moist and lyophilized feces from the black howler monkey *A. pigra*, by using two extraction methods, agitation and ebullition. Both methods proved to be efficient for recovering the added ligand and confirmed that, quantitatively,

the assay detects the amount of hormone added to each fecal sample.

Generally, the extraction efficiency of the tracer  $^{125}\text{I}$ -Estradiol and  $^{125}\text{I}$ -Progesterone was higher in moist feces treated with different solvents than that obtained in lyophilized feces. But by eliminating the moisture and inert matter (e.g., stones or hard seeds) from the lyophilized fecal sample and processing it with the ebullition method, we found that the efficiency of extraction for both hormones was greater than that obtained with the agitation method, which indicates that the method influences the hormonal extraction efficiency. Some studies quantifying steroid hormones in mammalian feces recommend eliminating the moisture and inert matter, because they may interfere in the quantification of steroids (Martin et al., 1975; Goldin et al., 1981, 1982; Wasser et al.,

1988; Bamberg et al., 1991). However, other studies indicate that eliminating the water from the feces, for example, those of the common marmoset and the cotton-top tamarin, did not alter the profiles of estradiol concentration. The recovery of the radioactive steroid added to the feces was >80% in both species and produced minimal effects on the quantification of steroids (Ziegler et al., 1996).

On the other hand, we found that the percentage of extraction efficiency varied when two different concentrations of organic solvent were added to the same sample type. The explanation as to why the ebullition method proved to be more effective than the agitation method for extracting hormones might be that, in the former, the application of temperature furthers the solubility of the components; in addition, the solvent is able to solvate with the radioactively marked hormone, while methanol, absolute or at 80%, is better able to solvate with  $^{125}\text{I}$ -Progesterone. In the case of  $^{125}\text{I}$ -Estradiol, the use of absolute methanol favored the extraction. Many methods of fecal steroid extraction recommend the use of a vortex to optimize steroid recuperation during the extraction with methanol or ethanol (Beehner and Whitten, 2004). It seems that agitation time and temperature could influence the recuperation efficiency of the marked hormone; with mechanical agitation, the extraction of steroids from feces was from 60 to 90%, similar to that reported by other researchers (Ziegler and Wittwer, 2005).

Relevant literature reports that some steroids are mainly conjugated as glucuronides or sulfates; this makes the steroids hydro-soluble and promotes changes in their polarity and retention times, thus influencing their extraction (Touma and Palme, 2005). For instance, Pappano et al. (2010) found no differences in the extraction of steroids (glucocorticoids and testosterone) from *Theropithecus gelada* when using methanol and acetone (8:2), or with methanol and water (9:1). However, they did find differences upon using a solution of methanol: water at 8:2. These researchers suggest that aqueous solutions extract a greater amount of polar metabolites (like glucocorticoids or conjugated metabolites); whereas organic solutions extract a lesser amount of polar metabolites (testosterone and unconjugated metabolites).

Similar studies on other mammalian quantifying hormones in the cat (*Felis catus*), obtained a greater extraction of [ $^{14}\text{C}$ ] Estradiol ( $90.1 \pm 0.8\%$ ) when using ethanol at 90% in comparison with ethanol at 100% ( $74.7 \pm 3.3\%$ ), a similar effect having been observed with [ $^{14}\text{C}$ ] Progesterone ( $87.2 \pm 1.4$  vs.  $56.9 \pm 0.7\%$ , respectively). This seems to indicate that the percentage of solvent used in the extraction is crucial for a better recovery of the hormone (Brown et al., 1994). To this effect, Wasser et al. (1991) point out that the extraction of hormones from lyophilized feces boiled in absolute ethanol and acetone was  $88.3 \pm 0.6$  and  $90.6 \pm 0.6$  for estradiol and progesterone in feces of *Papio cynocephalus cynocephalus*, respectively. Similarly Wasser et al. (2000) point out that the extraction of hormones from lyophilized feces boiled in ethanol at 90 or 100% is most adequate for some species (Yellow Baboon, Longtailed macaque, African elephant, Gerenuk, Scimitar-horned oryx), reporting also that hormonal extraction from moist feces/methanol or dry feces/ethanol with the ebullition method gave similar results.

**Table 3 | Shows the comparison between the method of agitation and ebullition and significant differences in the percentage of extraction efficiency of all the assays treated with the tracers  $^{125}\text{I}$ -estradiol and  $^{125}\text{I}$ -progesterone.**

Method agitation vs. method ebullition	P
<b>Comparisons of the extraction efficiency for <math>^{125}\text{I}</math>-estradiol between method agitation and ebullition (Figures 3A vs. 3B)</b>	
Bar 1 vs. bar 4	<0.001
Bar 1 vs. bar 5	<0.001
Bar 2 vs. bar 4	0.877
Bar 2 vs. bar 5	0.016
Bar 3 vs. bar 4	<0.001
Bar 3 vs. bar 5	<0.001
<b>Comparisons of the extraction efficiency for <math>^{125}\text{I}</math>-progesterone between method agitation and ebullition (Figures 3C vs. 3D)</b>	
Bar 1 vs. bar 4	<0.001
Bar 1 vs. bar 5	<0.001
Bar 1 vs. bar 6	<0.001
Bar 2 vs. bar 4	<0.001
Bar 2 vs. bar 5	<0.001
Bar 2 vs. bar 6	<0.001
Bar 3 vs. bar 4	<0.001
Bar 3 vs. bar 5	<0.001
Bar 3 vs. bar 6	<0.001

The numbers who have the bars indicate the type of samples treated with different solvents used in each method (see **Figures 3A–D**).

A previous study of lyophilized fecal steroid analysis for the female Javan Gibbon by Maheshwari et al. (2010) found that the average recovery of [ $^3\text{H}$ ]-progesterone gave a mean  $\pm$  SD value of  $78.2 \pm 6.6\%$ . Ziegler et al. (1996), on the other hand, reported high recoveries (>80%) of tritiated steroids that were added directly to the feces before extraction to determine recoveries in two species of Callitrichids (*Saguinus oedipus* and *Callithrix jacchus*), utilizing methanol for both species. In consistency, Wasser and Hunt (2005) compared the extraction of steroids using the ebullition method with ethanol or methanol at 90 and 100%; this did not alter the immune reactivity of the steroid, and the recuperation was similar (>85%). Likewise, the extraction of estradiol and progesterone marked with tritium and added to moist feces of *A. pigra*, which were then treated with water/methanol and subjected to manual agitation, permitted a recuperation of about 91.8% for  $^3\text{H}$ -Estradiol and 95.0% for  $^3\text{H}$ -Progesterone (Van Belle et al., 2009).

In the foregoing context, we have several important observations to make. First, there is a considerable variation in the percentage of extraction efficiency of fecal  $\text{E}_2$  and  $\text{P}_4$  recovered from moist and lyophilized feces treated with different organic solvents. Second, the differences between methods suggest that the procedures, agitation, temperature, and time modify the efficiency of extraction in both samples. We conclude that the ebullition method is the more effective one for hormonal extraction, and that the type of sample, moist or lyophilized, interferes with the extraction efficiency in both of the methods employed.

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