Short Communication

Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay

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Abstract

It is a common practice to extract steroids from plasma, serum, or tissue samples prior to steroid measurement by radioimmunoassay (RIA) or enzyme immunoassay (EIA). Steroid extraction is critical because it can remove substances that interfere with the RIA or EIA. Steroid extraction is commonly achieved using organic solvents, such as diethyl ether or dichloromethane. However, organic solvent extractions can suffer from low recovery, imprecise recovery, or incomplete removal of assay interference. Here, we describe validations of a simple protocol to extract steroids (e.g., dehydroepiandrosterone, corticosterone, and estradiol) from avian plasma, serum, and brain tissue using solid phase extraction (SPE) with commercially available C18 columns. We compare various methods for (1) eluting steroids from columns, (2) drying eluates, and (3) resuspending dried eluates prior to RIA. The SPE method yields high and consistent recoveries. The SPE method also effectively separates steroids from interfering substances, even when extracting steroids from lipid-rich plasma and brain tissue. These data indicate that SPE is superior to organic solvent extraction on several measures. SPE should be broadly useful for extracting steroids from plasma or tissue samples.

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Keywords: Corticosterone; Dehydroepiandrosterone; DHEA; Estradiol; Neurosteroid; Songbird; RIA; EIA; Song sparrow; European starling; Zebra finch; Avian; Yolk; Bird; Chicken; Stress; Hormone; C18; Sample preparation; Steroid extraction; Lipid

1. Introduction

Analyzing steroid levels via immunoassay is a very common method to determine endocrine status. For example, plasma levels of corticosterone and sex steroids are often measured to evaluate stress physiology (Newman and Soma, 2006; Romero et al., 2006) or reproductive state in songbirds and other species (Williams et al., 2005). In addition to circulating steroids in the plasma, there is accumulating evidence of local steroid synthesis in the brain and other organs (Baulieu, 1997; Soma et al., 2004; Soma et al., 2005, 2006; Demas et al., 2007). Thus it is critical to measure steroid levels within target tissues.

When measuring steroids, especially in tissues, sample preparation prior to immunoassays is a crucial step in the analytical process and has a direct impact on the accuracy and precision of the assay (Chard, 1995). A major obstacle in analyzing steroids in tissues is that lipids, such as free fatty acids and triglycerides, can interfere with immunoassays (Rash et al., 1980; Jawad et al., 1981). In particular, brain tissue is rich in lipids, and measuring steroids in egg yolk is also complicated by the high lipid content (Schwabl, 1995). Extremely lipemic plasma samples can also be a problem, especially in animals with a lipid-rich diet, such as seabirds (Speake et al., 1999), carnivores, and nesting birds. Female birds that are depositing yolk into eggs can also have high levels of plasma lipids. Thus,
separating steroids in plasma and tissue samples from lip- 
ids and other sources of assay interference can be impor-
tant for accurate and reliable measurements.

Traditionally, organic solvents (e.g., dichloromethane 
(DCM), diethyl ether, hexane) have been used to extract 
steroids from samples (Samuels, 1949). However, many 
variables must be considered when choosing an appropri-
ate extraction technique; these include the steroid being 
measured, study species, sex, season, reproductive state, 
and sample type (tissue, plasma and serum). Organic sol-
vent extraction may be appropriate for plasma samples 
with low lipid content (Soma and Wingfield, 2001) or for 
immunoassays that require very small amounts of plasma. 
However, for plasma samples or tissue samples with high 
lipid content, organic solvent extraction can yield low 
recoveries (O’Grady, 1968), variable recoveries (Fuqua 
et al., 1995), or incomplete removal of interfering sub-
stances. Importantly, the effectiveness of organic solvent 
extraction should be determined empirically on a case-by-
case basis. Another consideration is that organic solvents 
used for steroid extraction are often highly reactive or 
evertheless flammable. For example, DCM is an organo-
chlorine with carcinogenic properties, and diethyl ether 
has high volatility and a low ignition point.

Solid phase extraction (SPE) is an alternative to tradi-
tional organic solvent extractions (Thurman and Mills, 
1998; Telepchak et al., 2004). Previous studies using SPE 
report high and reliable analyte recovery and more effective 
SPE is relatively straightforward to set up in a laboratory 
and the materials are commercially available. In SPE, a solid 
sorbent material, typically an alkyl bonded silica, is packed 
into a column. Extracting relatively non-polar compounds 
such as steroids) from a polar matrix (such as water) requires 
a solid sorbent containing non-polar functional 
groups of octadecyl (C18) bonded silicas. Steroids bond with 
C18 groups on the sorbent and are extracted from the sample 
matrix. Steroids are then eluted from the column.

Here we describe validations of SPE to isolate steroids 
from avian plasma and brain. We address two primary 
issues: (1) optimization of steroid recovery using radiola-
beled and radioinert steroids and (2) removal of substances 
that interfere with radioimmunoassay (RIA). Our experimen-
tal focus is on songbirds, which are widely studied for a 
variety of research questions about hormonal and environ-
mental influences on physiology and behavior (Goodson 
et al., 2005b). To our knowledge, measurement of steroid 
levels in songbird brain has not been previously described.

2. Materials and methods

2.1. Subjects

Plasma pools were created from (1) wild nesting European starlings 
(Sturnus vulgaris), (2) captive nesting zebra finches (Taeniopygia guttata), 
and (3) wild adult song sparrows (Melospiza melodia) in non-breeding con-
dition. Plasma from nesting European starlings appeared lipemic upon 
visual inspection. Brain tissue was collected from wild nesting European

starlings, captive adult zebra finches, and wild adult male song sparrows 
in non-breeding condition. Commercially available chicken serum (Sigma 
C-540) was used for preliminary validations. Animal use protocols were 
approved by the Animal Care Committee at the University of British 
Columbia and conform to the guidelines promulgated by the Canadian 
Council on Animal Care.

2.2. Reagents

Radioinert dehydroepiandrosterone (DHEA), corticosterone (CORT), 
and 17β-estradiol (E2) were purchased from Steraloids (Newport, Rhode 
Island). Radiolabeled steroids were purchased from Perkin-Elmer (3H-
DHEA, NET814; 3H-E2, NET517). HPLC-grade methanol (MeOH) and 
HPLC-grade dichloromethane (DCM) were purchased from Fisher Scien-
tific. Absolute ethanol (EtOH) was obtained from the UBC Chemistry 
Department.

2.3. SPE protocol

The SPE protocol involves seven major steps: (1) Solvation, columns 
are primed with ethanol; (2) Equilibration, water is passed through col-
umns to prepare sorbent for sample loading; (3) Sample loading, samples 
in aqueous matrix are passed through the columns; (4) Interference elu-
ation, water is passed through columns to wash out interfering polar 
substances; (5) Sample elution, steroids are eluted from columns with a small 
amount of eluant into 7 mL glass vials; (6) Drying, eluates are dried; and 
(7) Resuspension, dried eluates are resuspended in assay buffer.

For SPE, we used a 24-place vacuum manifold (United Chemical 
Technologies, UCTVMF024GL, approx $930 cdn), non-endcapped C18 
columns (500 mg C18 material, 6 mL column volume, UCTCUC18156, 
approx. $2 cdn/column), and polytetrafluoroethylene tips. We systemati-
cally manipulated several variables after sample loading to evaluate effects 
on (1) steroid recovery and (2) removal of assay interference.

To measure steroid recovery, plasma and brain samples were spiked with 
known quantities of tritiated steroids. A known amount of tritiated steroid 
was added to samples prior to loading samples onto columns. To evaluate 
loss of tritiated steroids during the process, aliquots were counted in a liquid 
scintillation counter (Beckman Coulter LS-6500) for 2 min. After initial 
studies using chicken serum spiked with 3H-DHEA or 3H-E2, the protocol 
was applied to song sparrow brain tissue spiked with either 3H-DHEA or 
3H-E2. In addition, steroid recovery was evaluated by spiking samples with 
radioinert steroids prior to extraction and measuring extracted samples 
using RIA. By comparing unspiked and spiked samples, we were able to 
calculate recovery of exogenous radioinert steroids.

2.4. Eluting solvent

Choosing the appropriate solvent for elution is important in maximiz-
ing extraction efficiency. We compared several solvents (5 mL, all HPLC-
grade): methanol, 90% MeOH (9:1 MeOH:dH2O), and DCM. These sol-
vents are common in SPE (MeOH) or traditional organic solvent extrac-
tion methods (DCM).

2.5. Drying method

Following sample elution, the eluates are dried prior to resuspension in 
assay buffer. We compared recovery efficiency when eluates were dried 
under air or nitrogen (N2). To dry eluates under air, the vacuum manifold 
was used to pull a steady stream of air through the columns and over the 
eluates at room temperature. To dry eluates under N2, samples were 
placed in a water bath at 38 °C under a steady stream of medical-grade N2.

2.6. Resuspension volume

Resuspending dried eluates prior to radioimmunoassay is an important 
step that greatly affects recovery. Steroids have low solubility in aqueous 
buffers and may adhere to glass vials after drying. To determine whether
the volume of resuspension buffer affects recovery, two volumes of phosphate buffered saline with gelatin (PBSG) were added to dried eluates: 220 μl or 440 μl. These volumes are sufficient for one or two immunoassays depending on expected steroid concentrations and assay sensitivity.

2.7. Ethanol in resuspension

We also determined whether EtOH aids in resuspending steroids. Using high-grade absolute ethanol, we compared the effects of 0, 5, and 10% EtOH in the resuspension buffer on recovery of radiolabeled steroids from dried eluates. The absolute EtOH was added directly to the bottom of the glass vials containing dried eluates, the vials were vortexed briefly, the assay buffer was added, and the vials were vortexed (2 min).

An important concern is whether EtOH in the resuspension buffer affects the immunoassay (e.g., by affecting the primary or secondary antibodies). To address this issue, we determined whether 5 or 10% EtOH had an effect on the measured concentrations of known amounts of steroids.

2.8. Resuspension mixing method

We determined whether a manual vortexer (VWR, 58816-121) or a rack shaker (IKA Vibrax VWR basic) affected recovery of radiolabeled steroids. Resuspension buffer was added to dried eluates, vials were either vortexed (4 min) or shaken (60 min), stored at 4°C overnight, and either vortexed (4 min) or shaken (60 min) again.

2.9. Radioimmunoassays

We examined recovery of exogenous radioinert DHEA, CORT and E2 via RIA. Known quantities of radioinert DHEA, CORT or E2 were added to samples prior to SPE. We calculated recovery by comparing the quantity of steroids in unspiked and spiked samples.

To determine whether SPE effectively removed substances that interfere with the RIAs, we serially diluted plasma and brain samples after SPE. If interfering substances are removed, then serially diluted samples should be parallel to the standard curve, when comparing the percentage of tracer bound (Chard, 1995). To measure DHEA, we used a specific double antibody RIA (DSL 8900, Diagnostic Systems Laboratories, Webster, TX). The DHEA RIA was modified according to Granger et al. (1999) to increase sensitivity and has been previously used for songbird plasma (Goodson et al., 2005a; Soma, 2006). To measure CORT, we used a specific double antibody RIA (ImmuChem 07-120103, MP Biomedicals, Orangeburg, NY) which was modified according to Shircliff et al. (2000). To measure E2, we used a specific double antibody RIA (DSL 39100, Diagnostic Systems Laboratories, Webster, TX) which was modified according to Washburn et al. (2002).

2.10. Statistical analysis

To determine parallelism between serial dilutions of plasma and brain tissue with the standard curve, we tested equality of slopes using an analysis of covariance (ANCOVA). A lack of significant interaction between serial dilution and standard curve indicates that the line slopes are similar. If the interaction term is significant, then the slopes of the serially diluted sample and the standard curve are not parallel. We used JMP IN 5.1 (SAS, Cary, NC) for linear models and Sigma Stat 3.0 (Jandel Scientific, San Rafael, CA) for between group comparisons. We considered test results significant for p < 0.05. Means ± standard errors represent central tendency and variability, respectively.

3. Results

3.1. Recovery of radiolabeled steroids

The highest recoveries of radiolabeled DHEA and E2 from chicken serum were obtained when samples were eluted with 90% MeOH and then resuspended in buffer with 10% EtOH, although the difference between 5 and 10% EtOH in resuspension buffer was slight (Table 1). Resuspending dried extracts in either 220 μl or 440 μl resuspension matrix did not alter the recovery of 3H-DHEA (Table 1). Drying eluates under N2 at 38°C, particularly with 5 or 10% EtOH in the resuspension buffer, significantly increased recovery of radiolabeled DHEA and E2 (Table 2). Resuspending dried eluates with either a manual vortexer or automatic shaker did not affect recovery of radiolabeled DHEA when EtOH was added to the resuspension buffer (5% EtOH: t-test, t = −0.49, p = 0.64; 10% EtOH: t-test, t = 0.36, p = 0.72).

Table 1

<table>
<thead>
<tr>
<th>Steroid: eluant</th>
<th>Resuspension volume (μl)</th>
<th>% Ethanol in resuspension</th>
<th>DHEA</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA: 90% methanol</td>
<td>220</td>
<td>45.3 ± 2.2</td>
<td>50.0 ± 3.3</td>
<td>50.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>440</td>
<td>65.8 ± 1.6</td>
<td>62.1 ± 5.0</td>
<td>62.1 ± 1.7</td>
</tr>
<tr>
<td>DHEA: 100% methanol</td>
<td>220</td>
<td>70.9 ± 2.6</td>
<td>73.9 ± 2.1</td>
<td>73.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>440</td>
<td>65.5 ± 1.0</td>
<td>67.3 ± 5.0</td>
<td>67.3 ± 1.4</td>
</tr>
<tr>
<td>DHEA: dichloromethane</td>
<td>220</td>
<td>40.3 ± 2.9</td>
<td>67.3 ± 0.8</td>
<td>67.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>440</td>
<td>37.8 ± 3.6</td>
<td>62.1 ± 3.4</td>
<td>62.1 ± 1.4</td>
</tr>
<tr>
<td>Estradiol: 90% methanol</td>
<td>220</td>
<td>65.9 ± 1.6</td>
<td>73.9 ± 2.1</td>
<td>73.9 ± 0.7</td>
</tr>
<tr>
<td>Estradiol: dichloromethane</td>
<td>220</td>
<td>46.6 ± 1.3</td>
<td>52.1 ± 0.9</td>
<td>52.1 ± 1.1</td>
</tr>
</tbody>
</table>

Note: Data are means ± SEM and n = 4 for each group.
EtOH: t-test, t = −0.13, p = 0.90; data not shown). However, if no EtOH was added to the resuspension buffer, recovery increased significantly from 46 ± 1.4% to 56 ± 3.9% with the rack shaker (t-test, t = −2.51, p = 0.04).

Lastly, highest recovery of radiolabeled DHEA from songbird brain tissue was 68.9 ± 0.8% and was achieved with 5% EtOH in resuspension buffer (ANOVA, F2,11 = 4.27, p = 0.05). Highest recovery of radiolabeled E2 from songbird brain tissue was 66.6 ± 2.4% and was unaffected by percentage of EtOH in the resuspension buffer (ANOVA, F2,11 = 0.007, p = 0.9).

3.2. Recovery of radioinert steroids

We also determined recovery of known amounts of radioinert steroids that were added to samples prior to SPE and then measured via RIA (n = 4 replicates for each trial). Except where stated otherwise, 5% EtOH was used in the resuspension of dried eluates. For DHEA, recovery of 50 pg DHEA added to chicken serum was 113% with 5% EtOH in resuspension and 120% with 10% EtOH in resuspension. Recovery of 50 pg DHEA added to nestling starling plasma was 104%. Recovery of 25 pg DHEA added to 0.5 mg song sparrow brain tissue was 80% and recovery of 50 pg DHEA added to 1 mg song sparrow brain tissue was 72%.

Recovery of 0.5 pg 17β-E2 added to nestling starling plasma was 107%. Recovery of 1 pg 17β-E2 added to nestling starling brain tissue was 62%.

For CORT, recovery of 25 pg added to nestling starling plasma and brain tissue were 86% and 71%, respectively.

We also determined the effect of absolute EtOH in the resuspension buffer on RIAs. For DHEA RIA, 5% and 10% EtOH in the resuspension buffer did not affect the concentration of DHEA in known controls (Fig. 1). Similarly, for CORT or E2 RIA, when compared with 0%, 5% EtOH in the resuspension buffer did not affect the concentration of CORT or E2 in known controls (data not shown).

3.3. Removal of interfering substances

To determine whether SPE effectively removed interfering substances, such as lipids, from plasma and brain samples, we compared serially diluted samples and standard curves. For DHEA, we examined plasma and brain tissue that were not extracted by either organic solvents or SPE. Without extraction, serially diluted song sparrow plasma and brain tissue yielded displacement curves that were not parallel to the standard curve (data not shown; Plasma: ANCOVA, $R^2 = 0.96$, significant interaction, $F_{2,15} = 102.31$, $p = 0.007$; Brain Tissue: ANCOVA, $R^2 = 0.98$, significant interaction, $F_{2,13} = 244.98$, $p < 0.0001$). Indeed, the serially diluted brain tissue yielded a flat displacement curve. Second, we examined the effects of DCM extraction. With DCM extraction, serial dilutions of nestling zebra finch plasma, nestling European starling plasma, and song sparrow brain tissue yielded displacement curves that were not parallel to the DHEA standard curve (Fig. 2a). Nestling zebra finch plasma: ANCOVA, $R^2 = 0.96$, significant interaction, $F_{2,13} = 28.87$, $p = 0.0002$; Nestling European starling plasma: ANCOVA, $R^2 = 0.97$, significant interaction, $F_{2,18} = 22.18$, $p < 0.0001$). Third, we evaluated the efficacy of other organic solvent extractions. Song sparrow plasma extracted with diethyl ether or hexane/ethyl acetate (9:1) also yielded displacement curves that were not parallel to the DHEA standard curve (data not shown; ANCOVA, $R^2 = 0.96$, significant interaction, $F_{2,20} = 13.87$, $p = 0.0004$).

In contrast, with SPE, serially diluted nestling European starling plasma and song sparrow brain tissue yielded displacement curves that were parallel to the DHEA standard curve (Fig. 2b). Nestling zebra finch plasma: ANCOVA, $R^2 = 0.98$, significant interaction, $F_{2,13} = 0.58$, $p = 0.46$; Nestling European starling plasma: ANCOVA, $R^2 = 0.98$, no significant interaction; $F_{2,10} = 0.04$, $p = 0.84$). Similarly, for E2 RIA, serially diluted adult zebra finch brain extracted with SPE was parallel to the E2 standard curve (Fig. 2c). ANCOVA, $R^2 = 0.56$, no significant interaction, $F_{1,8} = 4.3$, $p = 0.1$). Lastly, for CORT RIA, serially diluted adult song sparrow brain extracted with SPE was parallel to the CORT standard curve (Fig. 2d). ANCOVA, $R^2 = 0.99$, no significant interaction, $F_{1,10} = 3.84$, $p = 0.10$).

4. Discussion

Extraction of steroids from lipid-rich samples has been an obstacle for analyzing steroids in lipemic plasma and tissue. These results indicate that solid phase extraction (SPE) is a suitable method for extracting steroids from lipid-rich avian plasma and brain samples. SPE is a straightforward procedure that can easily be established in a laboratory (Fig. 3). SPE yields relatively high and consistent recovery of radiolabeled and radioinert steroids. After SPE, serially diluted plasma and brain samples yield displacement curves that are parallel to the standard curve. Interestingly, steroid levels in songbird brain have not been previously described. This method provides a reliable way to quantify steroids in small tissues or tissues that are rich in interfering substances.
Extraction techniques can be examined using radiolabeled steroids, serially diluted samples, and radioinert steroids. Adding known quantities of radiolabeled steroids to samples prior to extraction provides information on recovery, but does not indicate if the extraction removes substances that interfere with immunoassay. Examining parallelism between serially diluted samples and a standard curve reveals the ability of the extraction to remove interfering substances, but does not indicate recovery efficiency. Serial dilutions are also useful in determining an appropriate sample volume (Chard, 1995). Lastly, calculating recovery of known amounts of radioinert steroids added prior to extraction and measured with immunoassay provides information on both recovery and removal of interfering substances.

Eluting the steroids from C_{18} columns with 90% HPLC-grade MeOH in deionized water yields the highest recovery of radiolabeled steroids. Methanol forms hydrogen bonds with the silica surface and breaks the van der Waals interactions between the steroids and the C_{18} material. Thus, it is an effective solvent for eluting nonpolar analytes, such as steroids. Adjusting the solvent can affect which analytes are eluted. For example, eluting with a lower percentage of MeOH in water can specifically elute sulfated steroids, but not unconjugated steroids (Liere et al., 2004). Other non-polar solvents, such as acetonitrile and ethyl acetate, may also work well for non-polar, lipophilic analytes and should be examined in future studies.

Drying the eluates is necessary prior to resuspension in assay buffer. Drying the eluates under a steady stream of medical-grade N_{2} yields significantly higher recovery of radiolabeled steroids than drying under a stream of air. Importantly, the N_{2} is free of possible contaminating substances that may be present in air, and inert N_{2} is less likely to cause steroid oxidation. Furthermore, drying under N_{2} at 38 °C is much faster than drying at room temperature. Additionally, eluates can be dried using a vacuum concentrator, and future studies will examine this method.

Fig. 2. Solid phase extraction (SPE), but not dichloromethane (DCM) extraction, effectively removed substances that interfere with RIA. (a) Serially diluted samples of nestling European starling plasma, nestling zebra finch plasma and song sparrow brain tissue extracted with DCM are not parallel to DHEA standard curve. For DCM extraction, steroids were extracted with 3 mL of HPLC-grade DCM (twice) and extracts were dried under N_{2} and resuspended in assay buffer. (b) Serially diluted samples of nestling European starling plasma and song sparrow brain tissue extracted with SPE are parallel to the DHEA standard curve. (c) Serially diluted zebra finch brain tissue extracted with SPE is parallel to the E_{2} standard curve. (d) Serially diluted song sparrow brain tissue extracted with SPE is parallel to the CORT standard curve.
After drying, dried eluates can be stored at −20 °C prior to resuspension in assay buffer. An addition of absolute EtOH in the resuspension, either 5 or 10%, significantly improves recovery of radiolabeled steroids. The EtOH is added directly to the dried eluates at the bottom of the vials and then brought up to final volume with assay buffer. In our laboratories, we routinely use 5% EtOH in resuspension buffer, which does not affect the DHEA, E2 or CORT RIAs in our laboratories. However, the effect of EtOH should be determined for each RIA or EIA used.

These data also demonstrate that SPE effectively reduces assay interference in songbird samples for measurement of DHEA, E2, and CORT. For other analytes, it is possible that a second interference elution with dilute MeOH or another eluant will be important to further reduce assay interference (e.g., Vallee et al., 2000). Further work on the interference elution(s) is a goal for future experiments.

While SPE can separate unconjugated steroids from sulfated steroids (Liere et al., 2004), SPE has typically not been used to separate various unconjugated steroids from...
each other. In this regard, SPE C18 columns generally have been used differently than Celite chromatography columns, which can separate several unconjugated steroids (Wingfield and Farner, 1975; Soma et al., 1999). Note that C18 columns can be used with HPLC to separate unconjugated steroids, but HPLC C18 columns are much longer than SPE C18 columns and thus have greater separating ability. Whether SPE C18 columns can be used to separate unconjugated steroids from each other is an important question for future studies.

These data demonstrate that SPE is useful prior to RIA, but SPE is also useful prior to EIA (Acosta et al., 2000; Love et al., 2005; Williams et al., 2005), high-performance liquid chromatography (Hojo et al., 2004; Ziegler and Wittwer, 2005), and gas chromatograph–mass spectrometry (Mensah-Nyagan et al., 1998; Vallee et al., 2000). SPE is an attractive alternative to traditional organic solvent extractions, because recoveries are high and consistent, and removal of assay interference is more complete. The equipment and columns are commercially available, and set-up is straightforward. Also, it may be possible to reduce the cost of SPE by washing and reusing the C18 columns.

These data highlight the importance of empirically determining how to prepare samples prior to steroid measurement. This should be done on a case-by-case basis. Many variables can affect the choice of sample preparation technique: the steroid measured, study species, sex, season, reproductive state, and sample type (e.g., tissue, plasma, serum). First, in some cases, no extraction may be needed. For example, Washburn et al. (2002) demonstrate that CORT can be measured in avian plasma without extraction, when using a particular CORT RIA. Second, in other cases, an organic solvent extraction may be sufficient. For example, Wingfield and Farner (1975) demonstrate that T can be measured in avian plasma after DCM extraction, especially when DCM extraction is followed by Celite column chromatography. In addition, Tsutsui and Yamazaki (1995) measured pregnenolone and DHEA in Japanese quail brain after ethyl acetate extraction. Third, in other cases, SPE may be required. The present results indicate that SPE is more effective than organic solvent extractions for removal of interfering substances from songbird brain and lipemic plasma. Migues et al. (2002) measured DHEA in chicken brain after SPE, but to our knowledge, the present report is the first examination of steroid levels in the brain of songbirds, an important model system in neuroendocrinology. Also, to compare steroid levels in tissues and plasma, both types of samples should be prepared similarly. That is, if SPE is required for tissue but not plasma samples, both types of samples should be prepared using SPE to avoid a confound. SPE should prove useful for a variety of researchers interested in measuring steroids in plasma, serum, brain, yolk, feces, urine, and whole body homogenates from a wide range of organisms.

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