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Steroid extraction: Get the best out of faecal samples

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Summary

Faecal steroid hormone metabolites are becoming increasingly popular as parameters for reproductive functions and stress. The extraction of the steroids from the faecal matrix represents the initial step before quantification can be performed. The steroid metabolites present in the faecal matrix are of varying polarity and composition, so selection of a proper extraction procedure is essential.

There have been some studies to address this complex but often neglected point. Radiolabelled steroids (e.g. cortisol or progesterone) have frequently been added to faecal samples to estimate the efficiency of the extraction procedures used. However, native, unmetabolized steroids are normally not present in the faeces and therefore the results are artificial and do not accurately reflect the actual recoveries of the substances of interest. In this respect, recovery experiments based on faecal samples from radiometabolism studies are more informative. In these samples, the metabolite content accurately reflects the mixture of metabolites present in the given species. As a result, it is possible to evaluate different extraction methods for use with faecal samples. We present studies on sheep, horses, pigs, hares and dogs that utilized samples containing naturally metabolized, ^{14}C-labelled steroids.

Zusammenfassung

Extraktion von Steroiden aus Kotproben

Die Messung von Steroiden im Kot zur Erhebung von Reproduktionsstatus oder Belastungen wurde in letzter Zeit zusehends populär. Die Extraktion dieser Steroide aus dem Kot stellt dabei den ersten Schritt für eine Bestimmung dar. Die im Kot enthaltenen Steroidmetaboliten sind von wechselnder Polarität und Zusammensetzung, die Wahl der richtigen Extraktionsmethode ist daher entscheidend.

Mittlerweile haben sich einige Studien mit dieser komplexen, aber weitgehend unbeachteten Thematik auseinandergesetzt. Dabei wurden oft radioaktiv markierte Steroidhormone (z.B. Kortisol oder Progesteron) dem abgesetzten Kot zugefügt, um die Extraktionseffizienz zu testen. Allerdings kommen die zugegebenen Steroidhormone in dieser, unveränderten Form im Kot nicht vor. Die Ergebnisse spiegeln daher nicht die tatsächliche Wiederfindungsrate wider. Diesbezüglich sind Experimente, die Kotproben aus Radiometabolismusstudien verwenden, wesentlich aufschlussreicher. In diesen Proben entsprechen die radioaktiven Metaboliten den tatsächlichen Steroidmetaboliten in der untersuchten Spezies. Die hier vorgestellten Studien, die solches Probenmaterial zur Evaluierung verschiedener Extraktionsmethoden von Steroiden in Kotproben von Schafen, Pferden, Schweinen, Hasen und Hunden einsetzten, enthalten somit die natürlich metabolisierten ^{14}C-markierten Steroide. Basierend auf unseren Ergebnissen, empfehlen wir die Extraktion von Steroiden mittels simpler Suspension der Kotproben in einem hochprozentigen, einfachen Alkohol (für Glukokortikoidmetaboliten erwies sich 80%iges Methanol für alle getesteten Säugetierarten als am besten geeignet). Dadurch ließ sich nicht nur die Extraktionsausbeute deutlich steigern, sondern auch der Anteil an nicht konjugierten Metaboliten, die von den meisten verwendeten Antikörpern besser erkannt werden. Die Vorteile dieser Methode liegen auf der Hand: eine sehr leichte Handhabung (Eindampfen ist nicht erforderlich), eine hohe Extraktionsausbeute und eine hohe Präzision.

We recommend extracting faecal steroids by simply suspending the faeces in a high percentage of a primary alcohol (for glucocorticoid metabolites 80% aqueous methanol proved best suited for virtually all mammalian species tested so far). Not only does the procedure significantly increase the total amount of recovered radioactivity, it also increases the percentage of unconjugated metabolites, which are more likely to be recognized by the antibodies used in various immunoassays. The advantages of this extraction procedure are clear: it is very easy to use (no evaporation step is needed), it yields high recoveries and variation based on the extraction procedure is reduced to a minimum.

Abbreviations: EIA = enzyme immunoassay; FCM = faecal cortisol/corticosterone metabolites; SPE = solid phase extraction

Introduction

Measurement of steroid metabolites in faecal samples has become a frequently used non-invasive tool in various research fields such as wildlife endocrinology, animal welfare, ecology and reproduction (SHERIFF et al., 2011; PALME, 2012; DANTZER et al., 2013; SCHWARZENBERGER and BROWN, 2013). Metabolism and excretion of steroids differ widely between species and even between sexes (TOUMA et al. 2003; PALME et al., 2005) and faecal steroid metabolites are a mixture of several metabolites with different structures and polarities. It is thus necessary to validate these non-invasive methods extensively for each species (TOUMA and PALME, 2005). The extraction of the steroids from the faecal matrix represents the initial step before quantification can be undertaken (Fig. 1), regardless of the technique (MÖSTL et al., 2005; MURTAGH et al., 2013). Although of great importance, this step hardly ever forms part of the evaluation process (PALME, 2005) and selection of the appropriate protocol is sometimes based more on vague experiences or traditions than on a sound validation. This review highlights the main points of concern and deals with the most common pitfalls concerning extraction procedures of faecal steroids.

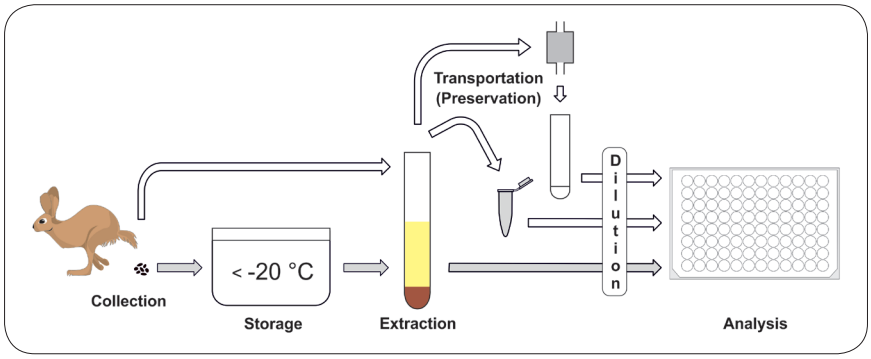

Fig. 1: Scheme illustrating important steps from defaecation to the analysis of faecal steroid hormone metabolites

Evaluation of the extraction procedure – the importance of naturally occurring steroids

There have been some studies to address this complex but often neglected point (e.g. BROWN et al., 1994; PALME and MÖSTL, 1997). However, in most cases radiolabelled steroid hormones (e.g. $^{3}H/^{14}C$-cortisol/-corticosterone or -progesterone) have been added to faecal samples to estimate the efficiency of extraction procedures (YOUNG et al., 2004; ZIEGLER and WITTWER, 2005). The results do not reflect the actual recoveries (MÖSTL et al., 2005; PALME, 2005). Steroids are heavily metabolized by the liver and in the gut (MÖSTL and PALME, 2002) and although they are present in the blood, native, unmetabolized glucocorticoids such as cortisol or corticosterone are virtually absent in the faeces. The same is true for other steroids such as progesterone and testosterone (PALME et al., 1997; DANTZER et al., 2011) as verified by almost all radiometabolism studies conducted so far (for a review see PALME et al., 2005), which report only very small amounts, if any, of radioactive substances with chromatographic properties similar to the parent steroids in the blood. Even worse than $^{3}H/^{14}C$-labelled steroid hormones are iodine (^{125}I)-labelled steroids, which have been employed in a few studies. Such labels (iodine has to be coupled to the steroid via a bridge) greatly alter the chemical properties of the steroid, while ^{14}C- or ^{3}H-labelled substances show unchanged molecular structures. Regardless of the label chosen, an evaluation of the extraction protocol based on parent steroid hormones is highly artificial and results are of only limited value. BROWN et al. (1994) compared the recovery rates of ^{14}C-progesterone and ^{14}C-oestradiol directly added to faecal samples immediately before the extraction procedure with i.v. administered, metabolized and excreted ^{14}C-progesterone and ^{14}C-oestradiol. Recovery was above 90% in samples where radioactivity was directly added to the faeces and did not change when different extraction procedures were used. However, recovery of metabolized steroids ranged from 56.9±0.7% (using 100% ethanol) to 90.1±0.8% (using 90% ethanol). Similarly, PALME et al. (1996) demonstrated that adding ^{14}C-progesterone overestimates the recovery of the extraction procedure (Fig. 2). The results underline the importance of using naturally occurring steroid metabolites (best gained from radiometabolism studies) for recovery studies instead of unmetabolized steroids directly added to faecal samples (MÖSTL et al., 2005; PALME, 2005).

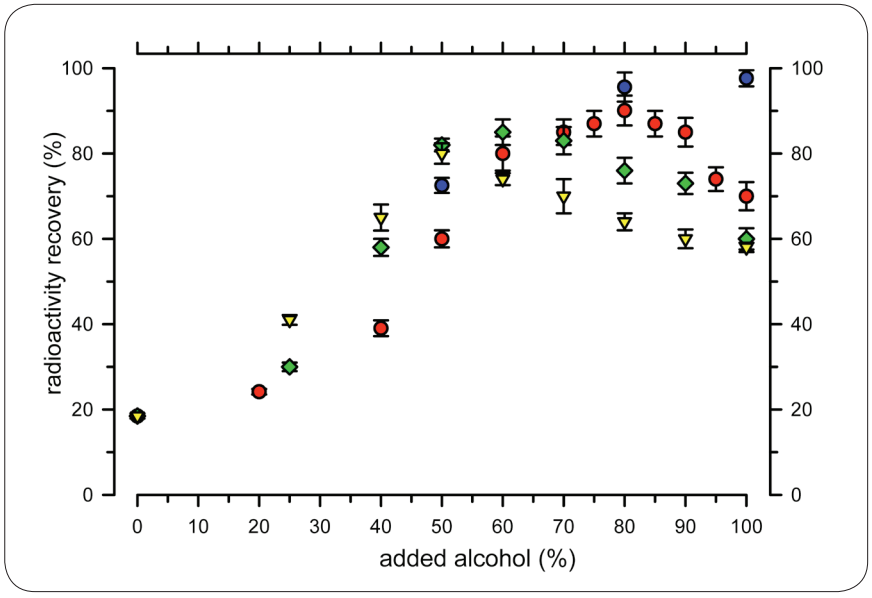

Fig. 2: Recovered amounts (%) of radiolabelled metabolites (mean±SD) in faeces of a ewe infused with ^{14}C-progesterone after extraction with different percentages of methanol (red filled circles), ethanol (diamonds) and isopropanol (triangles) compared to recovered amounts of unmetabolized ^{14}C-progesterone added to the faeces before extraction with methanol (blue filled circles; after PALME et al., 1997).

Choosing the right solvent

Currently, the most commonly used extraction procedure is probably to vortex a portion of (wet) faeces suspended in a primary alcohol/water mixture (high percentage for mammalian faeces). For example, in the case of FCM (see PALME, 2013, for a comprehensive list of published papers), most studies utilize methanol (57% of 680 papers; reviews not included) or ethanol (30%) for extraction, with percentages of 80% or higher dominating in studies dealing with mammals (~75% of all papers), whereas in bird studies 60% methanol prevails (used in about 50% of all papers published).

Based on recovery experiments using faeces containing naturally occurring radiolabelled glucocorticoid metabolites, 80% aqueous methanol proved best suited for extraction in virtually all mammalian species tested so far (PALME and MÖSTL, 1997; MÖSTL and PALME, 2002; MÖSTL et al., 2005; PALME et al., 2005). It significantly increased total radioactivity recovered as well as the percentage of unconjugated (diethyl ether-soluble) metabolites, which are more likely to be recognized by the antibodies used in various immunoassays (Fig. 3). Besides methanol, other alcohols such as ethanol (methanol and ethanol have different properties, e.g. methanol being more polar) or propanol are used in various concentrations. Depending on the polarity of the mixture of steroid metabolites in the faecal samples, recovery is considerably affected by the nature and concentration of the solvent used. Fig. 2 shows the amounts of ^{14}C-progesterone metabolites recovered from the faeces of a ewe after extraction with different percentages of methanol, ethanol and isopropanol. Here again, 80% (in the case of mares and pigs a higher percentage) methanol yielded the highest portion of progesterone metabolites (88±2%; PALME et al., 1997). The authors also showed that adding unmetabolized ^{14}C-progesterone to faecal samples resulted in recoveries of between 90.5 and 99.6% for either alcohol (Fig. 2). This result again demonstrates that the commonly used method of adding unmetabolized steroids (whether radiolabelled or not) to faecal samples leads to artificially high recoveries and is therefore not appropriate. Nevertheless, it might be useful for evaluating the precision of the person carrying out the extraction.

Multi-step extractions

Faecal steroids are normally present in relatively high concentrations. Thus, extracts can be (or might have to be) diluted before use in a sensitive immunoassay. Dilution is also important because a higher percentage (>10%) of an alcohol will interfere with the immunoassay (influencing steroid-antibody binding or activity of the enzyme; MÖSTL et al., 2005). The presence of several interfering substances in the extracts might cause blank values in the EIA. The problem can be avoided by EIAs utilizing biotinylated steroids as label, as in this case the enzyme is never in direct contact with the extract because it is added after a washing step (MÖSTL et al., 2005).

Nevertheless, it might be useful to combine two or more extraction steps to increase recovery or remove disturbing substances. In the case of low levels of steroid metabolites, such a system can enable measurement when a single-step extraction fails. MERL et al. (2000) described a two-phase extraction in faecal samples of horses combining 80% methanol and diethyl ether. First, faecal samples were vortexed with 80% methanol for 30 minutes. After centrifugation, an aliquot of the supernatant was briefly mixed with diethyl ether and 5% sodium hydrogen carbonate. Water was added and the tube turned upside down four times before the aqueous phase was frozen at -24 °C. The ether phase was decanted and dried down before the extracts were re-dissolved in assay buffer. The combination of the two solvents enabled measurement of the small amounts of unconjugated cortisol metabolites (11,17-dioxoandrostanes) in horse faeces.

To extract oestrogens (especially in the case of low concentrations and in the presence of many disturbing substances) it might also be useful to

include an additional extraction step based on the ability of oestrogens to form (polar) salts. Potassium hydroxide (1.5 ml, 1 mol/l) and 0.5 ml of a mixture of chloroform:n-hexane (6:4) is added to 0.5 g wet faeces. The polar oestrogen salts are water-soluble and dissolve in the potassium hydroxide phase while unwanted substances (especially fats) remain in the chloroform:n-hexane phase and can easily be removed. After shaking (30 min) and centrifugation, an aliquot (0.5 ml) of the hydroxide phase is transferred to a new vial and a few µl of glacial ethanoic acid is added. Afterwards oestrogens (no longer present as salt) can be extracted with diethyl ether which is dried down before the oestrogens are re-dissolved in assay buffer for analysis (MÖSTL et al., 1983).

Combining two solvents must be done very carefully and one has to keep an eye that the steroids of interest are not lost. MATSUMORO et al. (1999) described a two-step extraction method for faecal steroid hormones in samples of female Cynomolgus monkeys. Faecal samples were suspended in distilled water and an aliquot transferred to a new vial and extracted with a hexane:ether mixture. In distilled water, primarily polar metabolites are extracted while the hexane:ether solution is highly non-polar and therefore extracts only very non-polar metabolites. Most metabolites were lost during the extraction as a result of the combination of a very polar with a non-polar solvent, as non-polar metabolites are largely lost in the first extraction step. This was evidenced by rather low recovery rates of the added steroids (<30% for oestradiol and <20% for progesterone) in the experiment (MATSUMORO et al., 1999).

Solid phase extraction (SPE) is a special extraction method utilizing small columns (e.g. Sep-Pak C_{18} cartridges from Waters, Milford, MA). The method can be used both as a clean-up procedure before chromatographic separations (PALME et al. 1997; ZIEGLER and WITTWER, 2005; MURTAGH et al., 2013) and as a way to stabilize and preserve steroids during storage and/or transportation (Fig. 1; e.g. SHUTT et al., 2012). However, as cartridges are expensive and loading the steroid extracts onto the columns is very time consuming, the latter use is not practical for a large number of samples.

Extraction or suspension

By definition, extractions (liquid/liquid) use two immiscible phases to separate a solute from one phase into the other (e.g. diethyl ether extraction of unconjugated steroids from blood plasma samples). This is not the case when solvents such as methanol or other alcohols are used to 'extract' steroid metabolites from faecal samples. To be precise, they form a suspension (a heterogeneous mixture containing solid particles that are sufficiently large for sedimentation) with the faecal samples. This may be recognized as a sort of SPE, with parts of the faeces forming the solid

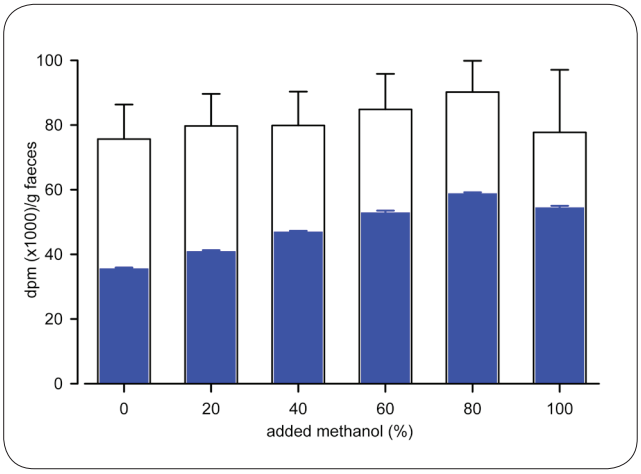

Fig. 3: Absolute amounts (mean±SD) of total (black framed white box) and diethyl ether-extractable (blue box) radioactivity (dpm=disintegrations per min) in portions (0.5 g; n=5 each) of a well homogenized faecal sample from a dog injected with ^{14}C-cortisol after extraction with different percentages of methanol (for details see SCHATZ and PALME, 2001)

phase. Distribution equilibrium is relatively quickly established, so shaking on a multi-vortex for 30 min (or intense hand-shaking for ~1 min) is sufficient. Longer periods (some researchers extract their samples for hours and even overnight) will not increase recovery. Extracting a second time (after decanting) with a newly added solvent does (e.g. BROWN et al., 1994; PALME et al., 1996) but this is time consuming and it might not be possible to re-dissolve the dried down (combined) extracts completely (especially when using a small volume of assay buffer). Alternatively, taking the portion remaining in the faeces (e.g. by adding the volume of the faecal pellet) into account when calculating concentrations will yield an accurate estimate (Fig. 4). This is nicely demonstrated by a small experiment with samples (containing the naturally occurring metabolites) collected after injection of different ^{14}C-steroids (oestrone, progesterone, testosterone and cortisol) in sheep, pigs and ponies (PALME et al., 1996) and only the latter in dogs, cats and hares (SCHATZ and PALME, 2001; TESKEY-GERSTL et al., 2000). Portions (0.5 g; n=5) of well homogenized faecal samples (n=16 in total) were extracted with 5 ml of 80% methanol (for details of the radioactivity measurement see PALME et al., 1996). Overall recovered radioactivity (mean±SD; included vs excluding the amounts of faeces for calculation; Fig. 4) was 94.9±3.9% vs 86.2±3.5% (in the case of sheep only: 96.8±3.9% vs 88.0±3.6%) of total metabolites present, indicating that it is favourable to add the amount of faeces for calculation.

Although some authors have described extraction procedures based on boiling in ethanol (sometimes performed overnight and twice), most have now shifted to protocols using a high percentage of methanol and simple shaking for extraction of faecal samples (the shift is described for example in WASSER et al., 2000).

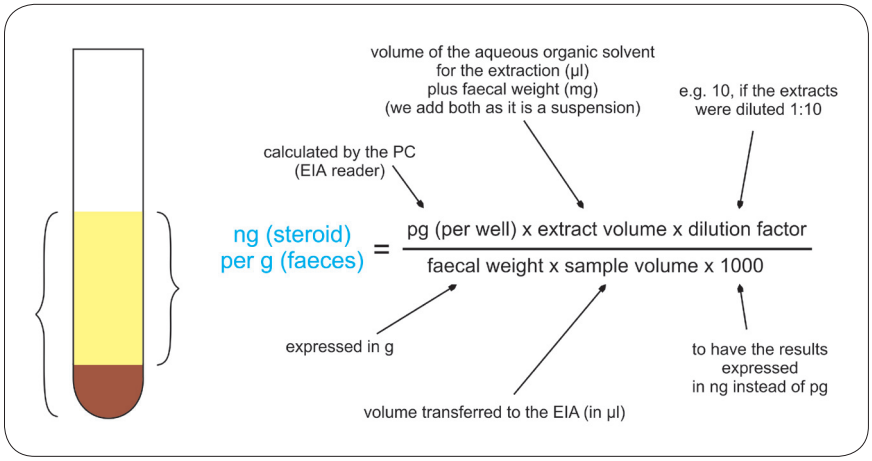

Fig. 4: Scheme of including (left bracket) vs excluding (right bracket) the amounts of faeces for calculation and the formula for calculating concentrations of faecal steroids. The 'extract' volume is 5,500 or 5,000 respectively (0.5 g faeces extracted with 5 ml solvent; standards prepared as pg/well)

Combining the right solvent with the right immunoassay

In choosing the appropriate extraction protocol, it is important to take the immunoassay to be used into consideration. Depending on the polarity of the chosen solvent, more polar or less polar substances are extracted. Immunoassays detect steroids depending on their structure and hence to a certain extent on their polarity. This point is important in species where larger amounts of conjugated metabolites are present in the faeces (e.g. in primates, carnivores or some rodents: BAHR et al., 2000; WASSER et al., 2000; SCHATZ and PALME, 2001; TOUMA et al., 2003; MONTIGLIO et al., 2012). The same phenomenon is seen in birds, where faeces and urine (with conjugates predominating) are excreted together as droppings (RETTENBACHER et al., 2004). In such cases, extracting faeces with a lower percentage of alcohol (e.g. 60% methanol) is recommended (PALME, 2005). Steroid conjugates (sulfates or glucuronides) can be assayed directly using antibodies that cross-react with them. They can also be assessed indirectly after enzymatic or acidic hydrolysis (with or without further extractions steps; e.g. HIRSCHENHAUSER et al., 2005; MURTAGH et al., 2013).

Using wet or dry samples

Both wet and dry faeces are used for analysis and measurements from both usually correlate quite well (WASSER et al., 2000; PALME, 2005; VASCONCELLOS et al., 2011). There is still no conclusive proof of the superiority of one method over the other. Unfortunately there is no 'normalizer' (such as creatinine in case of urinary samples) available to adjust steroid concentrations for the 'dilution' in the faecal matter (ELLIS et al., 2013). In general, wet samples are preferred by many researchers, as drying faecal samples is an additional, time-consuming and laborious step (MÖSTL and PALME, 2002; PALME et al., 2005; MÖSTL et al., 2005). However, when samples are small and dry quickly (in the case of small rodents; TOUMA et al., 2003, 2004; VOIGTLÄNDER et al., 2006), when they are contaminated with water (rain) or when undigested materials need to be removed, dried faeces are better suited (MILLSPAUGH and WASHBURN, 2004). In the case of floating samples collected from marine mammals, a drying step is necessary (e.g. AYRES et al., 2012). Drying should be performed at very high or very low (lyophilization) temperatures, as otherwise bacterial enzymes might find ideal conditions for further metabolism of steroids (TERIO et al., 2002; MÖSTL et al., 2005). In addition, attention must be paid to contamination with urine in mammals, which may affect the concentrations of steroid metabolites measured. If inorganic material (sand) is consumed, it may even be advantageous to determine the organic portion of the faeces and relate steroid concentrations to that (GANSWINDT et al., 2012). Especially in the case of FCM it is recommended to homogenize samples well (or to take a portion from a standardized position; e.g. within a faecal ball) before weighing an aliquot for extraction, as steroid concentrations may vary considerably within samples (PALME et al., 1996; MILLSPAUGH and WASHBURN, 2004).

Amount of solvent used for extraction

In most studies a rate of 10:1 or higher is used, i.e. the alcohol is added at a proportion of 1 ml per 0.1 g (in the standard procedure 0.5 g faeces is suspended in 5 ml 80% methanol) or 1 ml per 0.05 g in rodent faeces containing high steroid concentrations. To assess whether this is appropriate, we performed an experiment with samples derived from radiometabolism studies (PALME et al., 1996) and evaluated the efficiency of extraction in relation to the amount of solvent added. Faecal samples containing naturally occurring metabolites following infusion of ^{14}C-cortisol and ^{14}C-progesterone in sheep were used and extracted (0.5 g; n=5 each) with 80% methanol with increasing amounts of solvent. Using a rate of 5:1 yielded a total recovery of 85.3±8.7% (mean±SD) of ^{14}C-cortisol metabolites and 85.8±11.1% of ^{14}C-progesterone metabolites. Using a rate of 10:1 increased recovery of FCM to 93.8% and reduced the SD to 6.9%. Recovery of ^{14}C-progesterone metabolites was increased to 96.2% (with the SD reduced to 6.2%). Increasing the rate further (up to 30:1) did not

substantially increase the total recovery nor did it decrease the variance. From the results we conclude that solvent rates (ml) should be at least ten times the weight of the dry faecal sample (e.g. 0.1 g faeces plus 1 ml 80% methanol).

Questions related to the stability of steroids in faecal samples – field-friendly extraction methods

It is critically important the steroids remain stable (FCM > gestagen/androgen metabolites > oestrogens, which are most stable) during all steps of the analysis (Fig. 1). Before (depending upon environmental conditions following defaecation) and after sample collection, microbial enzymes may metabolize steroids (MÖSTL and PALME, 2002). It is advisable to test the stability of faecal steroids under the conditions expected during the experiment and with samples from several individuals (of both sexes) of the species under investigation, particularly if samples cannot be frozen immediately (MÖSTL et al., 2005; PALME, 2005). Preserving samples with alcohol directly after collection (in the field) hinders bacterial metabolism and might represent a possibility for stabilizing metabolites before measurement can be done. However, adding alcohol to the sample starts the extraction procedure and thus carries the risk of losing the solvent and therefore steroid metabolites. Tightly sealed vials are mandatory. In wildlife studies it might thus be useful to perform the complete extraction procedure in the field, including evaporation of the organic solvent. Several authors have tested extraction and storage protocols under such special conditions where even electricity might not be continuously available (BEEHNER and WHITTEN, 2004; PAPPANO et al., 2010; SANTYMIRE and ARMSTRONG, 2010; MURRAY et al., 2013). SHUTT et al. (2012) tested an extraction method for gorilla faeces and compared the results with those from their standard laboratory procedure. They suspended faecal samples with 90% ethanol for 5 min by shaking the suspension horizontally by hand. They allowed the faecal sediment to settle for 30–40 min on a bench (for better and quicker separation a manually operated centrifuge from Hettich GmbH & Co. KG Tuttlingen, Germany, may be used) and transferred part of the supernatant to a new vial until shipment to the laboratory. Results from the field method strongly correlated with those obtained with the standard laboratory method of extraction. In addition, they tested completely drying down an aliquot of the extracts (which might be best suited for field studies) and proved that metabolites are stable under this condition at room temperature. Using dried down samples also reduces potential problems associated with the transport or export of faecal samples from the field. Similarly, KALBITZER and HEISTERMANN (2013) found that androgen and cortisol metabolites in baboon faeces remained relatively stable in dried down extracts. However, they found storage in dried SPE cartridges best suited for longer periods. As re-dissolving dried down extracts (e.g. 0.5 ml 80% methanol) in the vials with the same percentage of solvent may cause problems (the solubility product is exceeded and coloured deposits remain on the wall), it is recommended first to add the pure alcohol (here 0.4 ml methanol), shake and add the distilled water (here 0.1 ml) afterwards. Having the vials in a heated vortex during this step also helps.

With regard to the stability of steroids (storage experiments), it is important to be aware of pronounced differences between steroids (TERIO et al., 2002; LYNCH et al., 2003) and species (MILLSPAUGH et al., 2003). In addition, depending upon the EIA used and thus the group of metabolites measured, FCM concentrations in faecal samples stored at room temperature can increase, decrease or remain unchanged (MÖSTL et al., 1999; MORROW et al. 2002; LEXEN et al., 2008).

Further degradation of steroids may occur when samples are defrosted before extraction. For example, MÖSTL et al. (2005) demonstrated that defrosting samples quickly, e.g. by heating (95 °C; FCM proved stable when treated with heat and/or acids), is better than thawing them slowly at room temperature. Taking the faecal samples out of the freezer in the evening and starting to process them the following day should be avoided, at least for FCM analysis.

Further considerations

Other important issues related to the extraction of faecal samples include the identification of individual samples in group-housed or wildlife animals (e.g. by special housing conditions – FRYNTA et al., 2008; VOIGTLÄNDER et al., 2006; the use of coloured food additives – FULLER et al., 2011; or genotyping – HUBER et al., 2003), the effects of diet (DANTZER et al., 2011; GOYMANN, 2012), the best expression of measured steroid concentrations (LEPSCHY et al., 2010) and selection of the most suitable method for analysis, including sound validation of the entire procedure (MÖSTL, et al., 2005; TOUMA and PALME, 2005; MURTAGH et al., 2013). Such issues are beyond the scope of this review and the interested reader is directed to the literature cited and to other reviews (e.g. MILLSPAUGH and WASHBURN, 2004; GOYMANN, 2005; PALME, 2005). It is advisable to seek help from experienced researchers in the field during the planning phase of an experiment, as analytical skills generally fail to compensate for problems arising during sample collection. In addition, it should be borne in mind that faeces may contain pathogens (such as microorganisms, viruses or prions) with zoonotic potential that require safety precautions (e.g. wearing gloves and a mask) due to the potential health risk (MÖSTL et al., 2005).

Recommendations: keep it (as) simple (as possible)

The quotation from Albert Einstein 'Make everything as simple as possible, but not simpler.' may serve as a guideline. Extraction should be kept as simple as possible. Additional steps increase the extent of variation. However, low amounts of faecal metabolites or high amounts of substances that interfere in an assay might demand more sophisticated extraction procedures. Above all, it is important to treat all samples of a given experiment in the same way (same extraction procedure, same antibodies and/or kits used) to produce comparable data.

Conclusion

Analyzing steroid metabolites in faecal samples represents a powerful and non-invasive tool for a broad range of research fields. Although measurement may appear straightforward and easy, there is a strong need to validate a newly established method in each species (and for each steroid) of interest. Validation includes the selection of a proper extraction protocol. In this review, we have pointed out the differences, strengths and weaknesses of common extraction protocols used in combination with various immunoassays. Based on our results, we recommend extracting faecal steroids by suspending the faeces in a high percentage of a primary alcohol (for glucocorticoid metabolites 80% aqueous methanol proved best for virtually all mammalian species tested). The advantages of this extraction procedure are clear: it is versatile and very easy to use (no evaporation step is needed) and it yields high recoveries and precision (i.e. variation based on the extraction procedure itself is reduced to a minimum).

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