Partial Analytic Validation of Determination of Cortisol in Dog Hair using a Commercial EIA Kit

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Abstract: The quantification of cortisol in hair samples is a relatively recent method that has been increasingly used for the evaluation of long-term cortisol secretion and chronic stress levels, in both humans and non-human animals. The use of hair presents many advantages in relation to the use of other biological matrices, e.g. a very low invasiveness of the sample collection. Besides, cortisol analysis in hair samples provides measurements over a long period of time, which can be used as a chronic stress indicator. Nevertheless, the physiology of cortisol in hair and the evaluation of reliable methods for its quantification in hair samples need to be further investigated. The aim of this study was to perform a partial analytical validation for the quantification of cortisol in domestic dog hair samples using a commercially available high sensitivity salivary cortisol enzyme immunoassay kit. Results on both precision and linearity tests were overall favorable, supporting that the kit can be used to reliably measure hair cortisol concentrations in dogs.

Keywords: cortisol; dog; EIA; hair; partial validation; stress

1. Introduction

The use of hair to quantify different substances is relatively recent, with the first findings being in drugs detection [1] and subsequent studies showing that the evaluation of drugs in the hair reflected changes in drug exposure over time [2]. Consequently, methods of quantification of endogenous compounds, such as cortisol, in hair became of great interest [3].

In recent years, hair cortisol concentrations (HCC) have been shown to be a reflection of the long-term activity of the hypothalamic-pituitary-adrenal (HPA) axis [4]. In particular, hair has been increasingly used to evaluate cortisol concentrations in both human and non-human animal species, since HCC provide a reliable reflection of both long-term cortisol secretion and stress, and they can be altered by numerous stressors and pathological conditions in many species [5–14].

When quantifying cortisol for the evaluation of stress, it is important that the process of sampling does not interfere with the stress marker [5]. This can be difficult for some biological matrices, due to the invasiveness of the collection [15], and due to the proximity to people for those animals not domesticated or not accustomed to humans [5].For instance, adrenocortical production has traditionally been quantified in blood, and for this reason the measurement of plasmatic cortisol is considered the “gold standard”, both to assess stress response and to establish comparative relationships with cortisol levels obtained with other types of samples [16]. Although it is true that circulating cortisol increased in dogs when displaying stress-related behaviours [17], when entering the shelter [18,19], and under a social spatial restriction [17,20], it may also rise for reasons other than a stress experience, such as increased HPA activity after a positive excitation or physical activity [21,22]. In this type of situations, cortisol levels may reflect psychological arousal, instead of providing unequivocal indicator of psychological stress [19], suggesting that blood cortisol is not always reliable for the assessment of animal welfare. What is more, for some animals, restriction and handling during blood collection may represent a stressor itself, increasing peripheral glucocorticoid levels in a few minutes[23–26].

In dogs, salivary cortisol seems to follow the circadian rhythm of serum cortisol, thus making saliva sampling another valid and non-invasive technique useful in chronomedicine to estimate free cortisol [27]. Saliva is a less invasive matrix than blood to be collected, thus explaining its common use. However, saliva collection has some disadvantages that may sometimes prevent it from being a reliable indicator of animal welfare. For example, previous feeding or drinking may interfere with cortisol determination[16].

Urine and faeces sampling are also frequently used to quantify cortisol levels and, normally, they imply no or very low invasiveness for the individual. Urinary cortisol-creatinine ratio (C/Cr) is one of the most commonly reported non-invasive indicator of stress in published studies about canine welfare[28]. Urinary cortisol reflects a stress response of the preceding 4-8 hours. Therefore, measuring urinary cortisol to assess the stress response has the potential advantage of a time-integrated assessment of the free hormone [16], being a reliable indicator of acute stress in dogs [18,26].In dogs, urine can be easily collected during the walk using a dog urine collector and transferred it to a sterile cup. However, urinary cortisol measures present some disadvantages that make them inadequate for assessing welfare in sheltered dogs, e.g.: difficulties in obtaining the total urine volume over a prolonged period [16], individual variability in cortisol response to kenneling [29], increases of the levels after exercise [30,31] or excitement[32] instead of after exposure to stress.

As for faeces, advantages and challenges of non-invasive measurement of glucocorticoids have been thoroughly reviewedrecently [33–35]. However, it may be difficult to confirm correspondence between the sample and the individual [5], and both diet and changes in diet may be a confounding factor, affecting both the results[33] or even plasma glucocorticoids levels [39].

Along with blood and urine cortisol, salivary cortisol levels correspond to real-time levels, representing just a short time period [5,40], being useful markers for the assessment of daily fluctuations in dogs [27]. This can be an advantage when the response to an acute stressor must be evaluated. However, when assessing an integrated cortisol response, repetitive sampling for these kind of matrixes is required [6], which can be difficult to do, or it can even lead to confounding effects of the collection procedures [16]. Hair cortisol level may eliminate the confounding effect that natural daily fluctuations have in these other types of samples (i.e., blood, saliva, urine), and appear to be significantly less variable than in saliva or faeces [41].Therefore, it results to be a more practical approach in monitoring the effects of long-term stressors such as social or physical environments and disease progression [41,42].

HCC analysis present complementary ways of monitoring stress through a long-term systematic cortisol exposure evaluation [16].Besides, collecting hair has many advantages: it is not painful nor invasive (or, at least, less than other types of collections), it does not require the presence of a professional, and it can facilitate many studies in wild animals[5], since sometimes can be collected from nests [43], faeces [44], etc. Hair can be easily preserved, it is not affected by variations in water content, nor by the acute stress impact, including that caused by handling of the sampling[45]. Centrifugation, refrigeration or freezing of hair is not necessary, and cortisol levels can remain stable for years in hair stored at room temperature [6,46–48].

However, some challenges exist in the HCC analysis. For instance, it has been reported that local cortisol production in the hair follicle may also add to HCC [49]. This was demonstrated for guinea pigs, in which only limited amounts of systemically administered radioactive cortisol were deposited in the hair, whereas large quantities of unlabeled glucocorticoids were found [50]. In humans, hair follicle (in vitro) itself produced cortisol following CRH stimulation, and thus was equivalent to the hypothalamic-pituitary-adrenal axis [49]. Nonetheless, it must be mentioned that studies showing a close correspondence between HCC and conditions with well-defined changes in classical HPA axis components evidenced the fact that HCC can accurately reflect systemic cortisol levels and it may just be marginally influenced by local cortisol production[51].When measuring cortisol in hair, there are other factors that can affect the results:

(a) Hair growth. The time delay between cortisol incorporation into the hair and the time point at which this hair section arrives at the skin surface depends on hair growth velocity [5]. Hair growth has an approximate rate of 1 cm/month [2,6], and it occurs in 3-phases cycles (anagen, catagen and telogen) [52]. Cortisol is incorporated into the growing hair shaft from blood vessels via passive diffusion only during anagen [40]. Since this incorporation occurs in the hair follicle, some millimetres below the skin surface [52,53], there is a time delay between cortisol incorporation into the hair and arrival of the hair to the skin surface [54,55]. This delay varies according to the species, body region and hair growth velocity [56,57]. Besides the fact that hair generally grows faster during summer than winter [58,59], differences in hair growth rates depending on species, age, sex or body region, or possible confounding effects (e.g., variation in skin temperature, hair colour) have been also found [60,61], and sometimes a large individual variability has been reported as well [62,63]. In any case, enough actively growing hairs must be present in the sample when using HCC as stress indicator, which can be easily obtained by a shave-reshave method[40,64].

(b) Incorporation of cortisol to hair through sources other than bloodstream, such as skin secretions and external environmental contamination [2].

(c) Individual factors such as age, sex, species, and hair colour [5].

(d) Body region. HCC have been found to vary depending on body region in several species [8,14,43,56,65–73]. This could be a consequence of the variation in the production of hair follicles in the three hair growth phases, and the differences in hair shedding depending on the area of the animal's body. Since cortisol is incorporated into hair in the anagen phase, higher HCC would be expected in the body regions with more follicles in the anagen phase [74].

(e) Hair length. HCC have been shown to decline from the first proximal hair segments to the distal ones in chimpanzees [43], horses [66], and women [75], which might be due to the UV radiation [42], influence of grooming [71], or washing frequency and hair treatments [2,76]. However, this declination has not been reported for dogs [63], grizzly bears [77], and orangutans [47].In addition, recent reviews emphasized against sub-segmenting hair samples, and questioned that a stressor in specific periods in the past can be uncovered in a specific segment of hair [78].

Taken together, considering the usefulness of HCC for evaluating stress, mechanisms of cortisol incorporation into the hair shaft, potential sources of hair contamination, and reliable HCC quantification methods need to be further investigated[5].

Since the first studies began to quantify cortisol in the hair of different species, including human and non-human animals [1–3,41,45,49,63,64,79–84], the most commonly used methods have been:

(a) Enzyme immunoassays (EIA) [7,41,63,64,66,81,82,84–91]. It is a low-cost method that offers quick results and good sensitivity[3].However, this method has some limitations, such as the variation found in different studies between results among similar EIA methods, which still questions its validity and accuracy[3].

(b) Radio-immunoassays (RIA). This method is highly sensitive and specific, with low reported CVs for intra and inter-assay [45,92], and cross reactivity with other hormones less than 2.4% [92], but it is a very expensive method that requires special precautions and equipped laboratories [3].

(c) High performance–liquid chromatography–mass spectrometry (HPLC-MS).This is a highly specific and sensitive method, frequently used [1,75,79,80,93], but relatively expensive [3].

Reported CVs for all these three methods (EIA, RIA and HPLC-MS) are around or below 10%, and the three of them might be affected by an external contamination of the hair, so further studies are still necessary[3].In relation to EIAs, there have been many studies validating different kits for the detection of several substances in hair samples, such as buprenorphine [80], drugs of abuse [94], methamphetamine [95], benzodiazepines [96], and opiates [97]. Specifically, HCC quantification through EIA has been assessed in different species, such as hyraxes [89], horses [66], Rhesus macaques [64], dairy cattle [98], and humans [84], including pregnant women [7,75] and children [85].

In the particular case of dogs, the use of LC-MS/MS for the quantification of HCC has been assessed[41]. RIA method has also been used for quantifying cortisol levels in hair for healthy dogs [45]and for dogs suffering from hypercortisolism [49]. Although some studies have used EIA method for the quantification of HCC in dogs [41,63,82], slight weaknesses have been found: e.g. high variability between dogs [41, 63], a slightly high inter-assay coefficient of variation (around 15%) [63,82]. The available literature often reports the use of different kits and/or different preparation procedures of the samples. Given that the use of different EIA kits can cause differences in HCC results [6], additional studies developing a reliable protocol using the same batch of immunoassay, and using assays that have low inter-assay variability are needed [6]. Even when the use of a specific kit has been validated, modifications of the bioanalytical method need to be checked through a partial validation.

In summary, as specified by Accorsi et al. [45], although hair cortisol quantification can be an accurate marker of chronic stress, it has two fundamental limitations: (1) the incomplete information on hair physiology, and (2) the lack of laboratory validation to date [45].

The aim of this study was to perform a partial validation for the quantification of cortisol levels extracted from domestic dog hair samples using an expanded range high sensitivity EIA kit commercially available for the quantification of salivary cortisol. Previous literature has already used this kit, however changes in the procedure (especially in the preparation and extraction of the sample) suggests assessing the reliability of the method.

2. Materials and Methods

The procedure was communicated to the Ethics Committee of the University of Pisa, Italy (OPBA, Organismo Preposto per il Benessere Animale) and received a favorable opinion with Decision N.52/2018.

2.1. Hair samples collection and storage

Hair samples were collected on February and November 2019, from 5 adult, healthy, pet dogs, 3 females and 2 males, 5.0 ± 2.5 years old. Each dog had a different hair colour, ranging from white to black. Hair was collected during a routine veterinary check, in the presence of the owner, and since all the dogs were pet dogs, no anticipated stress condition was considered. Hair samples were collected from the back of the neck, as close as possible to the dog skin, using scissors in order to reduce stress in the dogs, as our previous experience was that many dogs are scared of electric trimmers. Hair was cut rather than plucked in order to avoid including follicles in the sample and to prevent potential blood contamination [99].Hair samples were individually stored in pieces of paper and cautiously stored in a dry and dark place to avoid a possible washout effect by UV radiation [42], although hair cortisol seems to have a high long-term stability over months and years, as shown in cattle [46], bears [47], and human mummies [48], although no studies regarding washing-out effects in dogs are currently available [99].

2.2. Validation parameters

The possible application of an EIA kit for salivary cortisol in canine hair samples was determined by evaluating precision and linearity. Two of the same kits, run in different days, were used.

Precision of the kit was assessed by evaluating repeatability (intra-assay or precision within an assay; inter-assay or precision between assays) using repetitions of samples collected from 4 dogs. From one of them (dog 1), one big sample was taken from the neck, and divided manually into coat and undercoat hair samples to further analysis. Each hair sample was divided into 6 smaller samples; each of them was used separately to extract cortisol as described in the following section. Three duplicates (6 wells per kit) of each hair sample were used for kit 1 as well as for kit 2, for a total of 12 wells for original hair sample collected. The mean coefficient of variation (%CV) across duplicates of samples within the same kit (intra-assay variability) and between the two kits (inter-assay variability) was calculated. Coefficients of variation for intra and inter-assay were calculated using the mean of two wells per duplicate. Intra-assay %CVs lower than 10 and inter-assay %CVs lower than 15 were considered acceptable, in accordance with the instruction manual provided by the kit producer. For dog 1, cortisol concentration in undercoat hair was expected to be lower than in coat hair [68].

Linearity of the kit was assayed by spiking a series of 5 hair samples from a pool of coat hair of a dog, different from the one used in the precision test. During the cortisol extraction procedure, minced hair obtained was combined all in a single, homogeneous pool. In order to maintain as much homogeneity as possible, the pool was made in a sufficiently spacious and wide glass container, to avoid a possible deposit of the thinner minced hair parts on the bottom and the thickest ones on the surface. Five samples of different concentrations(0.5x, 0.75x, 1x, 1.5x, and 2x) were created, where 1*x* represents the regular amount of minced hair needed, i.e., 25 mg for 0.5x, 37.5 mg for 0.75x, 50 mg for 1x, 75 mg for 1.5x, and 100 mg for 2x). The coefficient of correlation (R^2), the ordinate at the origin, and the slope of the regression line formed by these 5 points were calculated.

2.3. Extraction of cortisol from hair samples

Cortisol extraction from hair samples was based on Bennett and Hayssen [35] with some slight modifications. The entire procedure was performed by the same person, maintaining samples as homogeneous as possible. Main steps were:

a) Washing: 200 mg of each sample were weighted. Three 3-min consecutive washes with methanol, manually shaking the tubes, were made for each sample using 40 µL of methanol per milligram of hair (i.e., 8 mL of methanol for 200 mg of hair). Instead of isopropanol [63], methanol was used; recent studies have reported no difference in HCC when the same samples were washed with methanol or isopropanol for polar bears, whilst higher HCC were found when using isopropanol for grizzly bears [100]. Then, liquid was discarded and samples were dried under laminar flow overnight.

b) Cut and shave: hair was firstly cut with scissors and then shaved with a razor (Rowenta Tn5140F0) by placing the hair sample on two sheets of paper with the aim of avoiding loss of material, and until the length of hair pieces was < 1 mm.

c) Extraction of cortisol: 50 mg of minced hair were transferred to Eppendorf tubes and 1ml of methanol was added for each sample. Samples were vortexed 20 sec and incubated in an orbital shaker (DLAB™ SK-

O180-E) for 24 h at room temperature (all tubes placed horizontally and firmly attached to the plate by elastic bands).For testing linearity, the amount of methanol used was 1 mL for samples 0.5x, 0.75x and x; 1.5 mL for sample 1.5x; and 2 mL for sample 2x.

d) Drying: samples were centrifuged (15 min at 9,000 rpm), supernatant (0.6 mL) was collected, dried under nitrogen flow (32 ºC, 90-120 min), and tubes were stored in the freezer (-20 ºC).

e) Cortisol determination.

2.4. EIA kit

The cortisol EIA kit used in this study is an expanded range-high sensitivity salivary cortisol enzyme competitive immunoassay kit (Salimetrics®,Carlsbad, USA). Two kits were used and all samples were measured in duplicates for each kit. Each hair sample was reconstituted with 200 µl of the Assay Diluent provided by the kit [35].

Optical density values were read on a standard plate reader (Thermo Fisher Scientific Multiskan™, Waltham, Massachusetts, USA) at 450 nm within 15 min.

3. Results

Both kits 1 and 2 obtained an excellent coefficient of correlation ($R^2 = 0.99$) and read the control between the acceptable range (1.036 ± 0.259µg/dl).

Mean cortisol concentrations for coat and undercoat samples were very similar one to the other in both kits.

In both kits, CVs were low, being <10% in most cases (9 out of 10, table 1). This is in accordance with a good intra-assay variability.

Table 1. Intra-assay and inter-assay test results.

	Intra-assay variability				**Inter-assay variability**	
	Kit 1		**Kit 2**		**Kit 1 + Kit 2**	
Sample	**HCC (pg/mg ± SD)**	**CV (%)**	**HCC (pg/mg ± SD)**	**CV (%)**	**HCC (pg/mg ± SD)**	**CV (%)**
Dog 1 (Coat)	11.22 ± 0.56	3.0%	10.87 ± 0.98	5.3%	11.05 ± 0.78	4.2%
Dog 1 (Undercoat)	11.57 ± 0.98	8.3%	11.25 ± 0.72	6.3%	11.41 ± 0.83	6.8%
Dog 2	16.27 ± 1.11	6.6%	13.28 ± 1.23	2.8%	14.77 ± 1.93	12.1%
Dog 3	5.54 ± 0.84	9.9%	4.98 ± 0.53	8.9%	5.25 ± 0.73	10.4%
Dog 4	4.86 ± 0.64	5.6%	4.37 ± 0.71	13.8%	4.62 ± 0.70	10.9%

For each hair sample, 6 different samples were obtained, each analysed in duplicate: 3 duplicates in kit 1 and 3 duplicates in kit 2, for a total of 12 wells per original hair sample collected. Mean cortisol concentrations (pg/mg ± SD) and coefficients of variation (CV)are shown. CVs were calculated by dividing the standard deviation of the data repetitions for each kit (separately for intra-assay variability; of both kits together for inter-assay variability) by the mean of the same data.

For linearity, values followed a good but not optimal proportion. In fact, the initial values obtained good linearity ($y = 0{,}151x + 0{,}016$; $R^2 = 0.95$); results were also good when transforming them in units of pg/mg (considering the amount of minced hair used), for which all values should be as similar as possible and a horizontal line was expected ($y = -0{,}849x + 12{,}25$; $R^2 = 0.16$) (Table 2).

Table 2. Linearity results for HCC.

Sample	**Initial HCC ± SD (µg/dl)**	**Final HCC ± SD (pg/mg)**	**CV (%)**

0.5x	0.08 ± 0.01	11.08 ±1.23	11.12
0.75x	0.14 ± 0.00	12.14 ± 0.11	0.93
1x	0.19 ± 0.1	12.67 ± 0.42	3.28
1.5x	0.21 ± 0.01	9.35 ±0.32	3.42
2x	0.33 ± 0.08	11.13 ±2.65	23.79

Results of cortisol initial values(µg/dl), final HCC concentrations(pg/mg) and %CVs for each linearity test sample (0.5x, 0.75x, 1x, 1.5x and 2x) are shown.

4. Discussion

In any clinical or experimental procedure in which analytical methods are used for the quantification of analytes, such as cortisol in a given biological matrix, it is necessary to know whether the assay results are reliable; this is the case of samples from domestic animals, before diagnostic use is made of any commercial kit designed for human samples [101]. Because values differ among species and they are often different from those for humans, assays should be validated for the singular species before application in veterinary diagnostics[102]. In addition, a partial validation is needed when a matrix other than that specified by the manufacturer is used [103].

Previous studies have investigated the possibility to measure cortisol concentrations in hair of different species, including dogs [45,81]. However, the reliability of EIA kits for such use has not been carefully investigated yet. For instance, Bennett & Hayssen [63] reported a slightly high inter-assay coefficient of variation, and samples with high %CVs were re-run until CV<10% was obtained. Considering that different EIA kits can cause differences in HCC results, some authors have already suggested the need to develop a reliable protocol using the same batch of immunoassay, and obtaining assays with low inter-assay variability [6]. Bryan et al. [41] have also validated the quantification of hair cortisol in dogs. Authors calculated intra-assay and inter-assay CVs using the standards (ranging 2.0% to 11.5%), and then on one pooled sample. We instead calculated intra and inter-assay variability calculated in multiple samples, thus increasing the accuracy of the measurement.

In the current study, CVs obtained in both intra and inter-assays were lower than 11% (except in a case, in which it was 12%), in agreement with previous studies on validation of an EIA method to quantify different opioid drugs [80,96,97], psychostimulants [95] and cortisol [3,64,66,75,81,89,98] in hair samples. However, it must be also highlighted that there was one CV that slightly exceed an acceptable threshold, i.e. that of 2x in linearity. Good results for precision were obtained for both intra and inter-assay tests, thus increasing the reliability of using an expanded range high sensitivity EIA kit validated for saliva (Salimetrics®, Carlsbad, USA) to measure cortisol levels in dog hair.

As for the linearity test, results followed a good, but not optimal, proportion: in fact, 1.5x resulted in a value lower than expected. However, linearity test does not only take into account the possible variability generated by running the EIA kit, but also that of the entire 3-days cortisol extraction procedure. In addition, it must be noticed that, due to the low dilutions and the relatively low values of the samples, the range between the minimum and maximum value obtained (9.35-12.14) was low and consequently the error. Therefore, the coefficient of correlation obtained in the current study (R^2= 0.95) can be considered good and the test valid. In further repetitions of the analysis, increasing the amount of methanol for extraction for each point instead of varying the amount of methanol by the amount of hair may be a possible solution to avoid outlying in the curve.

Hair cortisol samples from the current study came from pet family dogs, so HCC levels were expected to be lower than those of sheltered dogs [104], dogs experiencing some stressful stimulus [105] or suffering from some disease [49,81,82]. In fact, mean HCC value calculated on all the samples used for intra-assay plus the point 1x used for linearity test was 9.7 pg/mg, and values ranged 4.37 – 16.27 pg/mg. Such mean value, as expected, was lower than hair cortisol in a population of pet dogs, for whom hair cortisol has been reported to be positively correlated with the length of time left alone for both healthy (17.48 ± 8.95 pg/mg, ranging 1.70 – 28.79) and chronically ill dogs (15.22 ± 10.52 pg/mg, ranging 1.77 – 25.22) [106]. Our mean was also lower than the mean for dogs with atopic dermatitis (16.25 ± 2.86 pg/mg) [82]. Mean HCC value of the present study was instead higher than mean HCC reported for healthy pet dogs (1.5 pg/mg) [81], and dogs suffering from hypercortisolism (5.6 pg/mg) [81], although it must be noticed that specifically, the reported range (0.32–74.62 pg/mg) for dogs suffering from spontaneous hypercortisolism was quite wide in another study [49]. Our results were within range (3.42 – 27.09 pg/mg) previously reported for healthy pet and working dogs by other authors

using both EIA [63,82] and LC-MS/MS [41] methods. They were instead higher than values obtained using RIA in healthy dogs samples [45,49]. This brief overview of HCC values found in the scientific literature attest the need for more research, aimed at standardizing methods and reporting values that can be properly compared.

The discrepancy found with RIA might be due to the different analytical method, as well as the different extraction method, and needs further investigation. For instance, Accorsi et al. [45]minced the hair just until reaching 1-3 mm of length, whereas in the current study hair was minced until obtaining <1 mm of length. We can hypothesize that a shorter length of hair would increase the breakage and would allow a greater amount of cortisol to be extracted.Based on previous literature [68], cortisol concentration in undercoat was expected to be lower than in coat. This difference was not obtained for the current study. One possible explanation is that coat hair is more exposed to radiation than undercoat hair. Considering that undercoat hair segments would be closer to the scalp, being less exposed to environmental factors than coat hair segments, it could be speculated that undercoat hair would be less damaged due to UV irradiation and frequent washing, as Manenschijn et al. [107] discussed for old versus new hair. Consequently, cortisol could escape less from the undercoat hair, leading to a higher amount of the hormone. Another possible explanation is thatsince coat and undercoat were manually separated, some coat and undercoat hair segment might have mixed, producing that undercoat HCC to be higher than coat HCC. Future studies should deepen the knowledge about factors affecting the amount of HCC measured, including the kind of hair sampled.

In summary, the results of the partial analytical validation of the current study showed that the used expanded range – high sensitivity EIA kit can reliably quantify cortisol in dog hair. Future research, comparing results obtained through different methods (e.g. EIA versus RIA) may provide further information about its reliability. An evaluation of the recovery (extraction efficiency) would be also highly recommended and would aid in the evaluation of the linearity testing.

This study contributes to the evaluation of the use of EIA to reliably detect and quantify HCC in dogs. Research in these terms would be a great advance in the evaluation and development of non-invasive measures of welfare, this being of special relevance in a species as studied as the domestic dog. Considering that it has been reported that more than 70% of diseases are believed to be stress-related, a reliable and non-invasive assessment of chronic stress is essential to reduce welfare problems in the domestic dog and the incidence of related illnesses [108].

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