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Methodological Considerations for Hair Cortisol Measurements in Children

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Abstract

Background—Hair cortisol levels are used increasingly as a measure for chronic stress in young children. We propose modifications to the current methods used for hair cortisol analysis to more accurately determine reference ranges for hair cortisol across different populations and age groups.

Methods—The authors compared standard (finely cutting hair) vs. milled methods for hair processing (n=16), developed a 4-step extraction process for hair protein and cortisol (n=16), and compared liquid chromatography-mass spectrometry (LCMS) vs. ELISA assays for measuring hair cortisol (n=28). The extraction process included sequential incubations in methanol and acetone, repeated twice. Hair protein was measured via spectrophotometric ratios at 260/280 nm to indicate the hair dissolution state using a BioTek® plate reader and dedicated software. Hair cortisol was measured using an ELISA assay kit. Individual (n=13), pooled hair samples (n=12) with high, intermediate, and low cortisol values and the ELISA assay internal standards (n=3) were also evaluated by LCMS.

Results—Milled and standard methods showed highly correlated hair cortisol (r_s=0.951, p<0.0001) and protein values (r_s=0.902, p=0.0002), although higher yields of cortisol and protein were obtained from the standard method in 13/16 and 14/16 samples respectively (p<0.05). Four sequential extractions yielded additional amounts of protein (36.5%, 27.5%, 30.5%, 3.1%) and cortisol (45.4%, 31.1%, 15.1%, 0.04%) from hair samples. Cortisol values measured by LCMS and ELISA were correlated (r_s=0.737; p<0.0001), although cortisol levels (median [IQR]) detected in the same samples by LCMS (38.7 [14.4, 136] ng/ml) were lower than by ELISA (172.2 [67.9, 1051] ng/ml). LCMS also detected cortisone, which comprised 13.4% (3.7%, 25.9%) of the steroids detected.

Conclusion—Methodological studies suggest that finely cutting hair with sequential incubations in methanol and acetone, repeated twice, extracts greater yields of cortisol than does milled hair. Based on these findings, at least three incubations may be required to extract most of the cortisol

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in human hair samples. In addition, ELISA-based assays showed greater sensitivity for measuring hair cortisol levels than LCMS-based assays.

Keywords

stress; chronic stress; ELISA; assays; protein extraction

Established “gold standard” methods for measuring acute stress are used widely, although there is a paucity of methods for measuring chronic stress [1]. Measurements of cortisol from different biological sources (blood, saliva, urine) provide a measure of acute cortisol production, and thus may not reliably reflect chronic stress [2, 3]. On the other hand, hair cortisol is a good candidate for measuring chronic stress since the hair shaft grows at rates of 256±44 μm/day in African-Americans and 396±55 μm/day in Caucasians, averaging at rates of around 1 cm/month [4, 5]. Hair cortisol levels were originally measured in the hair of athletes thought to be abusing anabolic steroids and were later studied among humans and primates as a measure for chronic stress [6, 7]. Multiple studies showed positive correlation between subjective stress and hair cortisol levels [3, 8], further corroborated with serum and salivary cortisol in elementary school girls [9]. In earlier studies, liquid chromatography mass spectroscopy (LCMS) analysis was used more commonly to measure hair cortisol levels [6, 10, 11], however, since 2007 enzyme-linked immunosorbent assays (ELISA) [12] have been widely used for measuring hair cortisol [13].

Relatively few studies have examined hair cortisol as a marker for chronic stress in pediatric patients. Yamada et al. (2007) first reported hair cortisol levels in newborns receiving neonatal intensive care [14], showing that those requiring mechanical ventilation had higher hair cortisol levels than non-ventilated term infants. Palmer et al. (2013) found significantly higher hair cortisol levels in African American infants compared to Caucasian infants at 1 year of age [15], correlated with measures of maternal prenatal adversity, maternal postpartum depression, parenting stress and the child’s socioemotional development at age 1 year [15]. Among preschool children, hair cortisol levels were negatively correlated with the parent’s educational level, but not parental income [16]. Longitudinal studies found a natural decrease in hair cortisol levels with increasing age from 1 to 8 years [17]. Groneveld et al. (2013) reported that hair cortisol levels increased in children after starting school, with greater increases among the children who were fearful before starting school [18].

Despite these studies, the reported analytical methods and hair cortisol values vary significantly between laboratories [19] Thus, it is difficult to develop normative values for children across different ages or investigate hypotheses with long-term developmental effects. Factors that can influence hair cortisol levels include preterm birth and nutritional status [20] in addition to the frequency of hair washing, use of emollients and creams (which may contain steroids), race, socioeconomic status, and biological characteristics of the hair collected [3, 15, 21, 22].

We present three methodological variations in the ELISA-based measurement of hair cortisol. Specifically, our aims were to (1) compare hair cortisol and protein levels between finely cutting (standard) and milled methods for hair preparation (n=16), (2) investigate the fractions of hair protein and hair cortisol extracted by alternating incubations in methanol

and acetone, and (3) compare hair cortisol levels between ELISA and LCMS testing methods (n=28). We postulated that there would be no differences in the hair cortisol extracted and levels measured by these methodological variations. Hair cortisol data based on a single extraction may measure partial cortisol content. Although each laboratory can establish reference ranges based on populations they serve, however, similar amounts of cortisol may not be extracted from each sample because of differences between individual hair samples (such as hair texture, color, culturally-dependent cleaning practices, or other factors). Extracting all the cortisol content from each hair sample will generate more precise values, quantitative reference ranges, and may reveal the hair-related factors that lead to cortisol differences between hair samples. Previously used methods using a single extraction have greater time economy, but cannot guarantee accuracy. A lack of consistency in hair cortisol data from different laboratories using single extraction methods contributes to greater variability and inconsistency in the reported reference ranges, an inability to perform quantitative meta-analyses, or to examine age-related changes.

Materials and Methods

Testing Strategy

Analyses were conducted to test our hypotheses on two sets of samples. First, 16 hair samples from individual children were used to compare standard vs. milled methods for hair preparation and cortisol/protein extraction. The standard method involves finely cutting the hair to a powder consistency and the milled method includes mechanically grinding the hair to a powder. Protein and cortisol levels were detected in the reconstituted residue from each sample. Second, the ELISA vs. LCMS testing methods were compared for measuring hair cortisol in 28 samples, obtained from individual subjects (n=13), internal controls from the ELISA kit (n=3), and pooled hair samples (n=12) derived from the low, intermediate, or high ranges of cortisol levels (4 pooled samples from each range). Pooled samples, a common approach for assay validation with limited sample volumes [23], contained the hair residue extracts from 20 different subjects (remaining after the ELISA assay) that were combined for specific age groups if their cortisol values were within the low, intermediate, or high ranges defined *a priori*.

Human Subjects

After approval from the University of Tennessee Health Science Center's Institutional Review Board (IRB), hair samples were obtained from children enrolled in the **C**onditions **A**ffecting **N**eurocognitive **D**evelopment and **L**earning in **E**arly childhood (CANDLE) study. This includes children residing in urban and suburban areas of Shelby County, Tennessee born to women between 16–40 years of age enrolled during the second trimester of pregnancy. To obtain a healthy child cohort, maternal exclusion criteria included existing chronic maternal disease of any kind (such as hypertension, diabetes, sickle cell disease etc.) and known pregnancy complications (such as pre-eclempsia, placenta previa, oligohydramnios). More detailed descriptions of the CANDLE cohort [24] and race distribution [15] were published earlier. Informed consent was given by the mothers or by their legally authorized representatives. Characteristics of the study participants used for hair cortisol analyses are listed in Table 1.

Collection of Hair

Hair samples were cut as close to the scalp as possible from the posterior vertex of children (1–3 cm length), taped at the cut end, weighed in an analytical balance (Mettler-Toledo scale AL54, Greifensee, Switzerland), sealed in plastic bags and stored at room temperature (RT) until analysis. Only hair samples weighing 100 mg or more were used for the standard vs. milled comparisons. Each of 16 hair samples were divided into 2 equal parts with 50 mg reserved for each method.

Preparation of Hair

(a) Standard method—Pre-weighed hair was finely cut to a powder consistency using scissors (ROBOZ RS-5853; Gaithersburg, MD), then 4 successive extractions were performed on each hair sample (n=16): Hair was extracted alternating 1 mL of methanol incubated at 52°C for 15 hours, rotated at 200 rpm followed by 1 mL of acetone rotated at 200 rpm for 5 minutes at room temperature (RT). These extraction steps were repeated twice and the supernatants for each individual subject were pooled. Pooled supernatants from each sample were kept in an explosion-proof refrigerator (4°C) for air evaporation. The completely dried residue was reconstituted in phosphate-buffered saline (PBS) according the hair sample's weight (i.e., 350 μl for 50 mg hair).

(b) Milled method—Hair samples from the same subjects (n=16) were precut to approximately 0.5 cm, milled at 20,000 rpm with 0.2 mm zirconium beads for 10 minutes using a Bullet Blender (Next Advance Inc., Averill Park, NY), followed by the same 4-step extraction process as described above. Each of the four extracts was centrifuged at 10000 rpm for 10 minutes and supernatants were not pooled but collected in separate glass vials; hair protein and cortisol were measured separately in each of these four fractions. As with the standard method, supernatants from the milled samples were air evaporated at 4°C and the dried residues were reconstituted in PBS according to the hair weight and fraction (fraction 1 in 150 μl, fractions 2, 3, and 4 in 67 μl each; total 350 μl for 50 mg hair).

Protein and Cortisol Quantification

Measurements of the protein levels extracted from hair indicate the hair dissolution state for the release of cortisol. Therefore, total protein yield (mcg/ml) of supernatants isolated using the standard method was compared to the total protein levels from the milled method fractions. The Epoch BioTek® plate reader with Nanodrop attachment was used to read protein concentrations by calculating the ratio of spectrophotometric absorption at 260 nm and 280 nm. A calibration curve is not required for this method. We used the Take 3, Gen5 2.05 program (BioTeK plate reader software, Winooski, VT) for calculating protein concentrations (mcg/ml).

Total cortisol yield of standard method supernatants was compared to the individual and total cortisol levels from the milled samples. Hair cortisol was quantified with a salivary cortisol ELISA assay kit (ALPCO Diagnostics, Salem, NH), according to the manufacturer's instructions. The Epoch BioTek® plate reader (BioTeK Instruments, Winooski, VT) was used to quantify samples against a generated standard curve (ng/ml). Our intra- and inter-assay coefficients of variation were 6% and 7% respectively.

Mass Spectroscopy

A representative set of 28 samples was sent to the Wisconsin National Primate Center (University of Wisconsin, Madison) for confirmation of cortisol expression and evaluation of the presence of cortisone by LCMS, as reported previously [25]. The antibody in the ELISA cortisol kit (ALPCO Diagnostics, Inc.) reports a cross-reactivity of 6.2% with cortisone; thus, it was important to determine if the levels of detected cortisol may have been influenced by cortisone cross-reactivity. We evaluated hair cortisone since our goal was to establish accurate reference intervals of hair cortisol content for the age groups and ethnicities of the CANDLE subjects. All samples were first evaluated using the ELISA assay prior to evaluation by LCMS. The sample set included 13 individual hair samples, 3 positive controls from the ELISA kit at high (100 ng/ml, standard), intermediate (47.4 ng/ml quality control-1), and low (11.7 ng/ml, quality control-2) concentrations, and 12 pooled hair samples (each consisting of 20 individual samples with known cortisol ranges per pool; see Table 1 for details). Technicians at the Wisconsin National Primate Center were blinded to the sample type and reported cortisol and cortisone levels in ng/ml of the reconstituted hair extracts.

Statistical Analyses

GraphPad Prism version 6.0d (GraphPad Software, La Jolla, CA) was used to perform descriptive statistics, Bland-Altman plots and Spearman rank correlations (r_s). Non-parametric tests were used because the values for hair protein and hair cortisol did not satisfy the conditions of a normal distribution. Measurements of each milled hair fraction (1–4) were calculated for the percentage of protein and cortisol obtained from that fraction, compared to the total amounts obtained from standard assay of the same hair sample. Percent differences between the standard vs. milled method were plotted for paired cortisol and protein levels using the formula: (standard value *minus* milled value)/standard value) ×100. Similarly, the formula for the percent differences between the ELISA and LCMS measures of cortisol content was (ELISA value *minus* LSMS value)/ELISA value) × 100. Levels of cortisol and cortisone detected by LCMS in each sample were totaled to calculate the percentage of cortisone measured in each sample and descriptive statistics were obtained from these data.

Spearman rank correlations (r_s) with 99% confidence intervals (CI) were used to assess the relationships between hair protein and hair cortisol levels and between the ELISA and LCMS methods for measuring hair cortisol content. Bland-Altman plots with 95% limits of agreement were used to compare the standard (S) vs. milled (M) methods and the ELISA vs. LCMS testing methods for cortisol detection and to determine the mean bias from differences between the values measured by these two methods. Narrow limits (i.e., small biases) would indicate that the two methods used for detection of cortisol levels were equivalent. The level of significance was set at $p<0.05$.

Results

The percentages of cortisol and protein levels extracted by each step (Fractions 1–4) of the milled extraction in 16 hair samples are listed in Table 2. Highly significant correlations

occurred between the standard and milled methods measuring hair cortisol (ng/ml) and protein (mcg/ ml) content (Table 3). Higher cortisol yields occurred from the standard (103±98 ng/ml) vs. milled (68±94 ng/ml, p=0.0453) methods in 13/16 samples and higher protein yields occurred from the standard (5.98±5.54 mg/ml) vs. milled methods (4.36±4.41 mg/ml, p=0.0387) in 14/16 samples (Figure 1). Bland-Altman bias (mean±SD) are reported and plots were used to assess the level of agreement between the standard and milled methods for both cortisol (Figure 2; bias = 78±77) and protein (Figure 3; bias = 35.2±31.8). Between the two methods, the mean differences in cortisol values were 34.4±63.0 ng/ml and in protein values were 1.62±2.86 mcg/ml, with the standard method yielding higher values compared to the milled method.

ELISA cortisol values were correlated with LCMS cortisol (r_s=0.737; p<0.0001) and cortisone values (r_s=0.636; p=0.0003). The LCMS cortisol vs. cortisone values were also correlated (r_s=0.735; p<0.0001). Pooled hair samples showed the greatest correlation between the two testing methods (r_s=0.972, p<0.0001). In the positive controls provided by the ELISA assay kit, the LCMS method detected lower levels of cortisol (11.7 vs. 6.7, 47.6 vs. 16.7, 100 vs. 48.8 for ELISA vs. LCMS, respectively) and spuriously detected some amounts of cortisone (25%, 11%, 5%) in these samples. On average, lower cortisol levels (median [IQR]) were detected by LCMS (38.7 ng/ml [13.4–130.8]) than by ELISA (172.2 ng/ml [66.8–1034]) and 13.4% [3.7%–25.9%] of the steroids detected by LCMS were measured as cortisone across all the samples (N=28). Bland-Altman plots showed a large bias between the two methods (101.3±96.6 ng/ml, mean±SD) when percent-differences between the two values were plotted against the average of the two values (Figure 4), whereas absolute differences between the cortisol values reported by the two testing methods appeared to be related to the averages of both values (Figure 5).

Discussion

Identifying hair cortisol as a putative marker for chronic stress has led to several different laboratories measuring and reporting cortisol values from hair samples in children. A single-phase extraction procedure is commonly performed to capture hair cortisol content for quantification (Table 4). Our data suggest that repeated extractions with methanol and acetone are required to maximize the extraction of cortisol and protein from human hair. Caucasian and Asian hair mostly contains protein, whereas African hair contains significant amounts of lipid moieties as well [26]. Methanol extraction denatures the protein by breaking non-covalent bonds, thus allowing release of hair cortisol. When heated to 52°C in methanol, Africoid hair forms clumps, thus not allowing cortisol to be released into the supernatant. Acetone solubilizes the lipids in these hair clumps, by breaking annular and non-annular lipid-protein bonds. Hair dissolution is simply a marker for the amounts of cortisol released from the hair sample. A 5-minute acetone wash is the minimum time required to dissociate the hair, prevent further clumping, while also ensuring that acetone does not over-dry the precipitated protein. Alternating between methanol and acetone exposures improves the effectiveness of these chemical processes. Acetone also dissolves the lipid/protein film on the inner surface of the glass vial, speeds the evaporation time of supernatant and increases the yield of residue to be solubilized in PBS.

Further, we found that milling or grinding the hair does not extract more cortisol than by finely cutting the hair and that ELISA-based assays yielded higher cortisol values compared to LCMS, despite greater analytical specificity of the latter method. Before hair cortisol levels can be used as a biomarker for HPA axis development or chronic stress in early childhood, methodological considerations must be applied to ensure the accuracy and reproducibility of the reported data.

Despite meticulous transfers of supernatant, the milling process may lead to sample escaping from the sealed tubes, or the pitting, scratching, and plastic folding or rippling in the tube caused by zirconium beads may prevent exposure of hair particles to the methanol/acetone. Chemical degradation of hair protein and/or steroids may also occur during the milling process. These results imply that the standard method of finely cutting the hair in glass vials is more effective for extraction of protein and cortisol than the milled method. In previous pilot studies (unpublished) conducted within our laboratory, we continued hair sample extractions and analyzed each fraction until a zero level of detection for cortisol was reached, which typically occurred in fraction 3 or 4. Pilot studies showed that fractions 5 or 6 (using additional acetone incubations for 5 minutes at RT) yielded <5 mcg/ml of protein in Fraction 5 and none in Fraction 6 and yielded no detectable levels of cortisol.

Hair dissolution in other laboratories occurs by incubating hair in methanol at varying temperatures (RT or 52°C) [16–18], [19, 27]), although multiple variations in the methods for extraction and detection have been reported (see Table 4). Our data suggest that a single methanol extraction may yield on average 46.1% of cortisol or 38.7% of protein content. Many laboratories fail to adjust for inherent differences of protein/lipid bonding between different ethnicities that may contribute to varying ratios of cortisol extraction in single-phase extractions. A 4-step process to extract protein and cortisol from hair, modeled after standard methods for tissue protein/RNA extraction [28], results in higher cumulative extraction of protein and cortisol (98–100%), arguably leading to more accurate readings for total hair cortisol content. Increasing the accuracy of extraction and analysis methods for cortisol levels in hair is most important before we can establish reference ranges for hair cortisol in children.

Other researchers have also compared ELISA and LCMS-based methods for measuring hair cortisol levels from humans and primates. In a round robin analysis, four different laboratories used specific ELISA-based assays and found high correlations in the measured cortisol values (R^2 = 0.91–0.98; all $p<0.0001$) [19]. The cortisol values measured by LCMS in two laboratories also showed high correlation (R^2 = 0.9829, $p<0.0001$), whereas the ELISA and LCMS values showed lower correlations (R^2 = 0.89–0.98; $p<0.0001$) [19]. Similar to our findings, the round robin reported greater sensitivity in ELISA-based assays than LCMS methods [19]. Pooled hair samples from our study showed greater correlation between the two testing methods ($r=0.972$, $p<0.0001$), whereas individual hair samples showed moderate correlation across the two methods ($r=0.665$, $p=0.016$). We hypothesize that variable rates of degradation may be associated with the protein content in the reconstituted hair extract. Indeed other labs have found that adding protein in their reconstitution cocktail favors cortisol detection (see Table 4, protein added as bovine serum albumin (BSA)). Thus, hair samples with higher protein content, irrelevant of the cortisol

level, may have a slower decay rate for loss of the cortisol signal. If this is true, then the law of averages would tend to protect the cortisol content of pooled samples more than that of individual samples, thus explaining the greater correlation between pooled vs. individual samples across the two testing methods in our study.

Our studies on hair cortisol methods have both advantages and limitations. One advantage is that single extractions using methanol for an overnight incubation may be insufficient to capture all the available cortisol in hair samples, with different assays detecting between 40% and 65% cortisol under these conditions. A second advantage is that pre-washing the hair sample with alcohol has little to marginal effect on hair cortisol content, thus eliminating an unnecessary extraction step. Other investigators reported that cortisol arising from sweat or cortisol solutions is rapidly absorbed into the hair shaft [1]. Therefore, hair samples should not be collected from children just after strenuous activity, when the release of exercise-induced cortisol and sweat may affect hair cortisol values. Finally, another advantage of this study is that the comparison of standard vs. milled methods included 9 African-Americans and 7 Caucasians, whereas the comparison of ELISA vs. LCMS methods included 6 African-Americans and 7 Caucasians, thus accounting for racially dependent variations in hair type in our methods [15].

One limitation in the ELISA-based assay has a known cross-reactivity of the antibody with progesterone (7.2%) and cortisone (6.2%) [29]. However, our hair samples came from children at 1–4 years of age, who were unlikely to have significant progesterone levels and thus would have minimal cross-reactivity at this age [30]. Another limitation is that some of the hair cortisol can be converted to cortisone. The enzyme 11-β-hydroxysteroid dehydrogenase (11β-HSD) metabolizes cortisol to cortisone and vice-versa [31]. The 11β-HSD1 isoform converts cortisone to cortisol, while 11β-HSD2 isoform converts cortisol to cortisone [31]. The 11β-HSD1 isoform is expressed in keratinocytes, dermal mesenchymal cells, and outer root sheath follicles while expression of the 11β-HSD2 gene remains at the background level according to one report [32], while it is detectable at the protein level according to another [33]. Thus inter-conversion of cortisol to cortisone possibly occurs in the skin. It is unlikely, however, that this conversion occurs in the cortisol bound to hair, since the hair shaft is a non-viable structure produced by the hair follicle. Furthermore, the LCMS analyses of our individual and pooled hair samples showed relatively low concentrations of cortisone (on average cortisone comprised 12% (median 13.4%) of the total steroids detected). If 6.2% of this cortisone cross-reacts with the cortisol antibody in the ELISA assay, then the measured cortisol levels would be increased by <1% because of cortisone cross-reactivity in the cortisol ELISA assay, which can be considered minimal.

Conclusion

Further refinements in the methods used for hair cortisol analysis may be required before the data reported in the clinical literature can be considered precise enough for clinical decision-making, or for establishing reference ranges for different age groups. These analyses will be useful for examining early HPA axis development or function, or for determining the long-term effects of chronic stress during early childhood in life-course studies. We propose methods that include finely cutting the hair for processing samples, a four-step extraction

procedure to maximize the amount of cortisol extracted, as well as using ELISA-based assays developed specifically for human hair. These and other methodological improvements would allow hair cortisol levels to be a reliable and reproducible measure of chronic stress in childhood.

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Abbreviations

LCMS	liquid chromatography mass spectroscopy
ELISA	enzyme-linked immunosorbent assay
RT	room temperature
PBS	phosphate-buffered saline
CANDLE	Conditions Affecting Neurocognitive Development and Learning in Early childhood).

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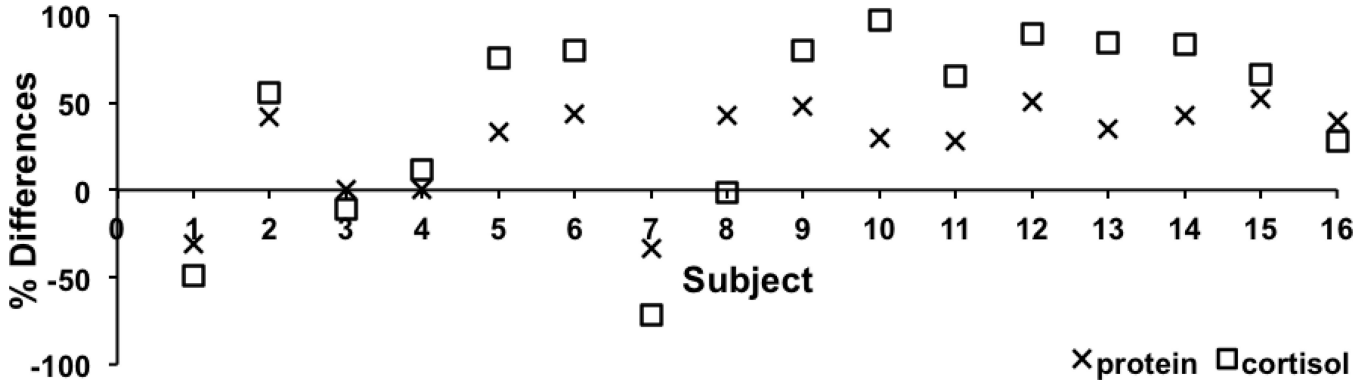

Figure 1.

Percent differences of Protein and Cortisol values per subject between Standard vs. Milled methods (calculated using the formula = (standard value *minus* milled value)/standard value) × 100) suggest that percent differences of hair protein and cortisol between the standard and milled methods both occurred in the same direction.

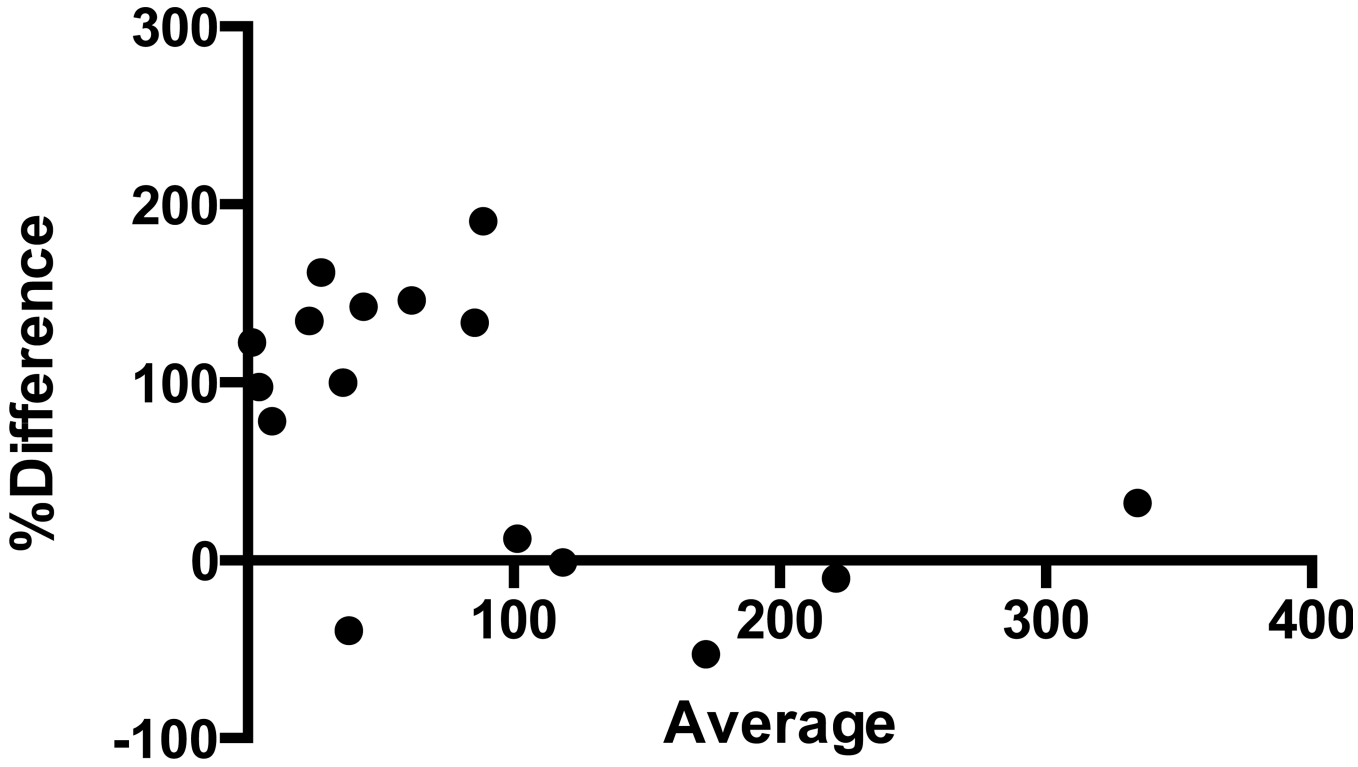

Figure 2.

The Bland-Altman plot for percent difference vs. average values of cortisol measured by standard and milled methods show that hair cortisol extracted by our standard method had greater yields in 13/16 samples (95% agreement limits −72.98, +228.9).

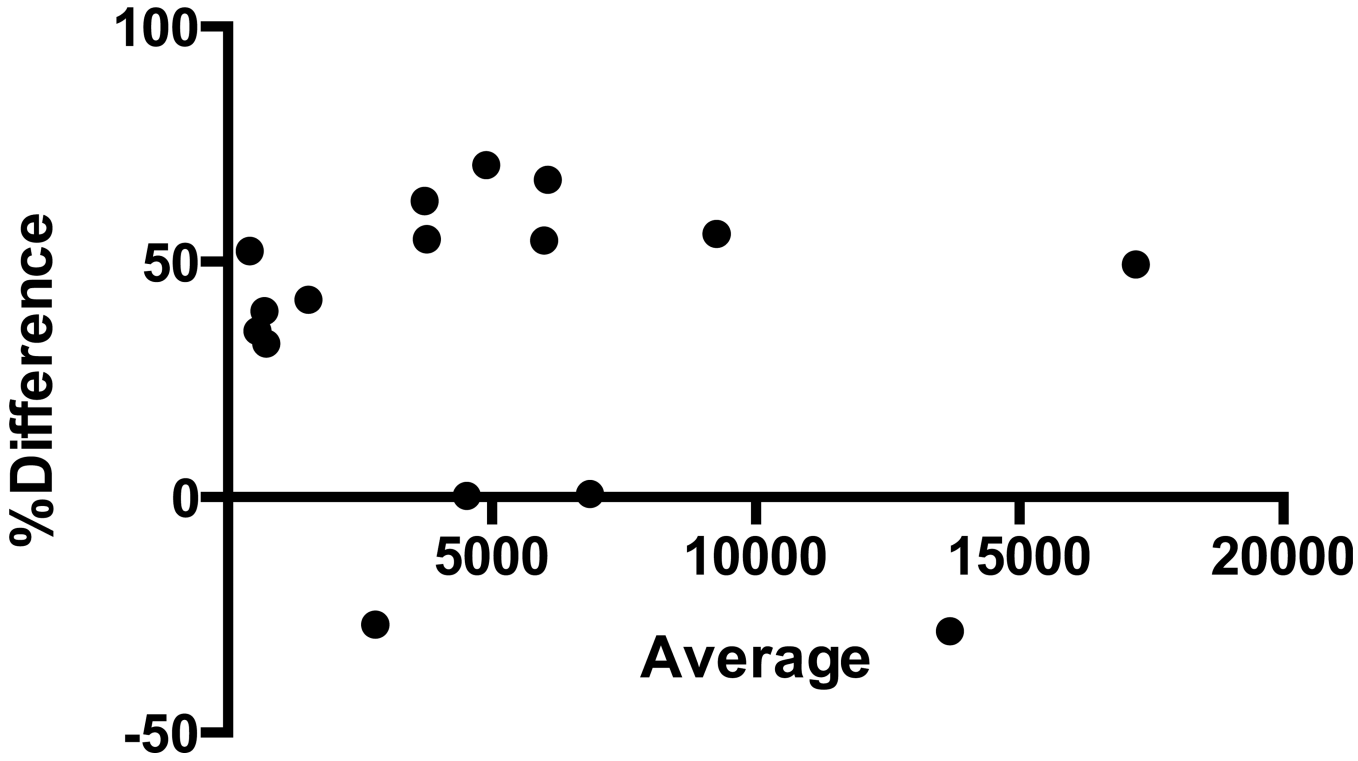

Figure 3.

The Bland-Altman plot for percent difference vs. average values of protein measured by standard and milled methods demonstrates that the hair protein extracted by our standard method had greater yields in 14/16 samples (95% agreement limits −27.07, +97.44).

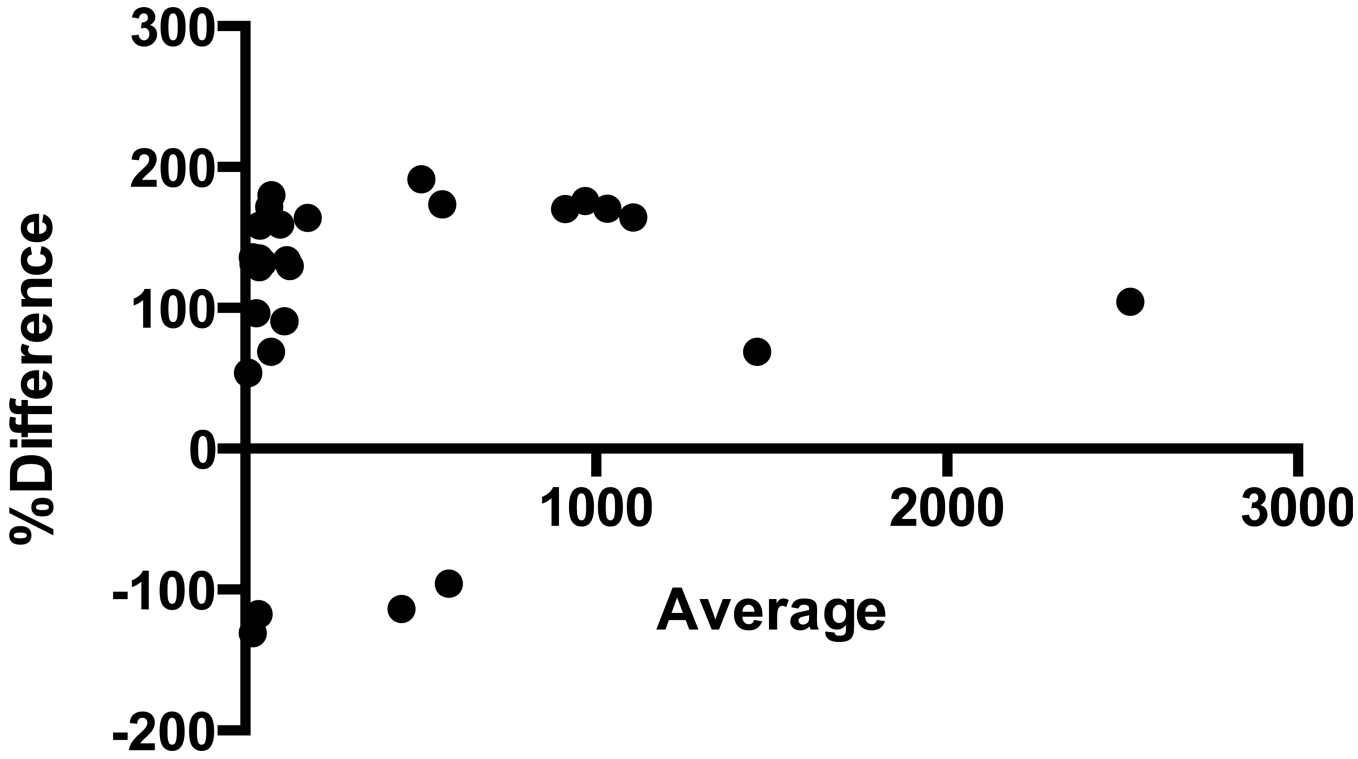

Figure 4.

Bland-Altman plot showing average values of cortisol derived from the ELISA and LCMS methods presented on the x-axis and percent differences between the two methods presented on the y-axis. The scatterplot shows no relationship between the percent differences and average cortisol values (95% agreement limits −88, +291).

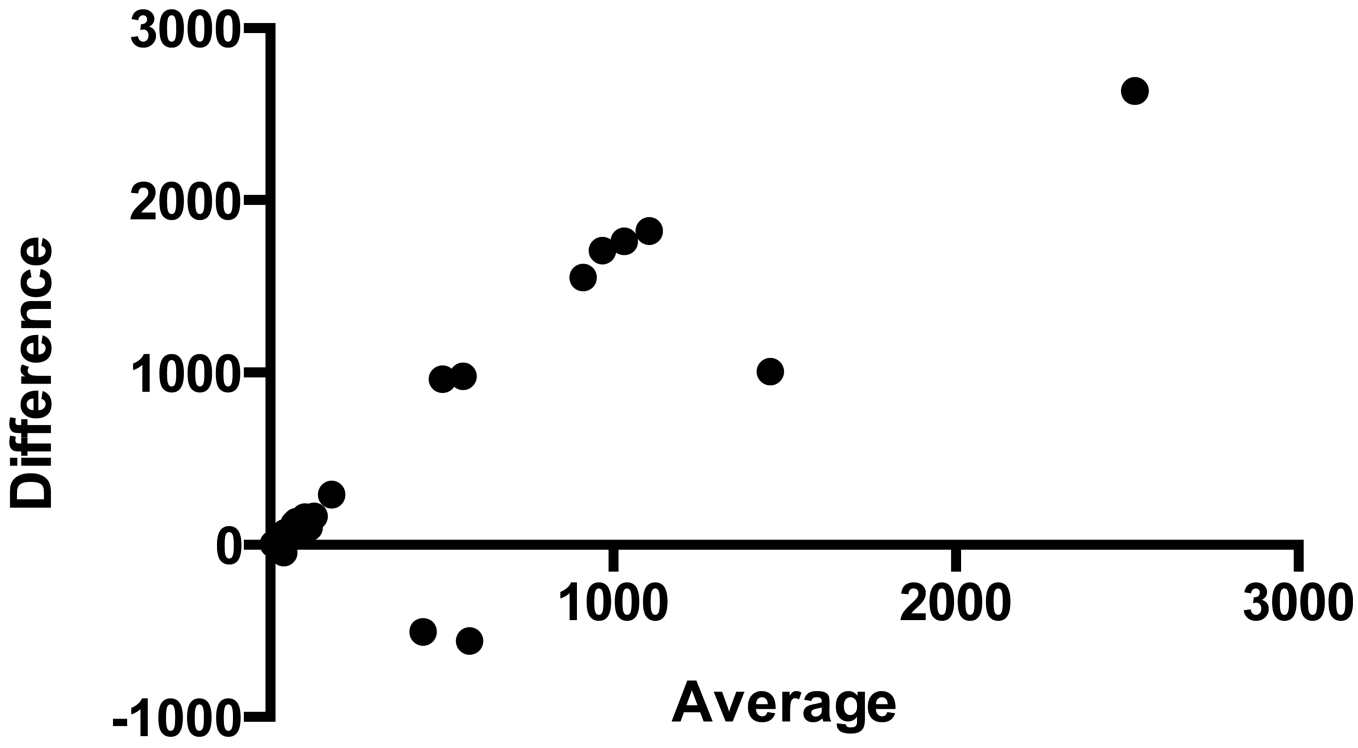

Figure 5.

Bland-Altman plot showing average values of cortisol derived from the ELISA and LCMS methods presented on the x-axis and absolute differences between the two methods presented on the y-axis. Scatterplot suggests a positive relationship between the absolute differences and average cortisol values (95% agreement limits −1075, +1991).

Table 1

Demographic Data related to Individual Samples

	Standard vs. Milled methods	ELISA vs. LC/MS methods*
Age groups		
12–17 months	-	3 (23.1%)
24–27 months	3 (19%)	3 (23.1%)
36–40 months	5 (31%)	4 (30.7%)
48–64 months	8 (50%)	3 (23.1%)
Sex		
male	3 (19%)	8 (61.5%)
females	13 (81%)	5 (38.5%)
Race		
African-Americans	9 (56%)	6 (46.2%)
Caucasians	7 (44%)	7 (53.8%)
Health Insurance		
Medicaid/Tenncare	7 (44%)	7 (53.8 %)
Other (private, employer, military, none)	9 (56%)	6 (46.2%)

* Data for individual hair samples only (N=13)

Table 2

Percent of Protein and Cortisol content extracted in each fraction

	Protein (n=16)		Cortisol (n=16)	
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**
Fraction 1	38.7 (11.6)	36.5 (32.5, 40.0)	46.1 (24.7)	45.4 (39.8, 65.1)
Fraction 2	26.0 (7.6)	27.5 (21.7, 31.4)	32.3 (19.3)	31.1 (21.0, 39.6)
Fraction 3	32.0 (13.3)	30.5 (25.5, 36.9)	21.0 (23.1)	15.1 (11.3, 28.0)
Fraction 4	3.3 (1.6)	3.1 (2.5, 5.3)	0.6 (0.9)	0.04 (0.0, 0.86)

Table 3

Correlation of Protein and Cortisol values from Standard (S) vs. Milled (M) methods

	M-Protein		M-Cortisol	
	r_s*	p-value	r_s*	p-value
M-Protein	-	-	0.699	0.0142
M-Cortisol	0.699	0.0142	-	-
S-Protein	0.902	0.0002	0.552	0.0667
S-Cortisol	0.755	0.0062	0.951	0.0000

Table 4

Analytical Methods used for the Measurement of Hair Cortisol: Summary of the Published Literature

Author Name(s)	Year	Journal Name	Extraction method	Purification method	Detection method	Comments
Russell, Kirschbaum, Laundenslager [19]	2014	Ther Drug Monit	**Washing:** isopropanol 2–3 times, 3 min-*Dresden*, *Colorado*, & *Western* NW- *Erasmus* **Cutting:** CH-*Western & Erasmus* MH-*Dresden & Colorado* **Extraction:** All groups-MeOH	n/a	**ELISA:** *Western*- (Alpco Salem, NH), *Colorado* (Salimetric, Pennsylvania), *Erasmus*- (DRG Instruments GmbH, Marburg, Germany). **Luminescence immunoassay:** *Dresden*- (IBL International, Hamburg, Germany) **LCMS:** *Dresden & Erasmus*	**Method comparison:** 4 labs **Unit:** ng/g **Subjects:** human hair-*Dresden*, *Western*, *Erasmus* vervet monkey- *Colorado* **Age:** not reported
Noppe, Van Rossum, Koper [27]	2014	Horm Res Paediatr	NW; CH, 25–40 mg; MeOH 16 hr, 52°C; evap, 37°C, N_2; 250 µl PBS	n/a	**ELISA** range, 2–80 ng/ml (DRG Instruments GmbH, Marburg, Germany)	**Unit:** pg/mg **Subjects:** children **Age:** 4–14 yr **Sex:** mixed **Race:** Caucasian
Simmons, Whittle, Patton [34]	2014	BMC Peds	Isopropanol wash, 3min, 2–3 times; MH, 50 mg; MeOH, 24 hr, 25°C; evap, 37°C N_2; 4 ml PBS	n/a	**ELISA** range, 0.012–3.000 µg/dL (Salimetric,Pennsylvania)	**Unit:** not reported **Subjects:** children **Age:** m=9.51yr **Sex:** mixed **Race:** not reported
Steude, Kirschbaum, Gao [35]	2013	Biol Psychiatry	Isopropanol wash; MH, 10 mg; MeOH 18 hr, 45°C; evap conditions not reported; 250 µl ddH_20	n/a	**LCMS**	**Unit:** pg/mg **Subjects:** adults **Age:** m=36.41 **Sex:** mixed **Race:** Caucasian
Palmer, Anand, Graff [15]	2013	J Peds	NW; CH, 5–50 mg; MeOH, 52°C, overnight (2–3 times); air evap 4°C; PBS (150 µl/10mg)	n/a	**ELISA** range, 0–100 ng/ml, (Alpco Diagnostics, Salem, NH)	**Unit:** ng/mg **Subjects:** Children **Age:** 1 yr **Sex:** mixed **Race:** African & Caucasian
Grunau, Cepada, Chau [36]	2013	PLOS One	Isopropanol wash (twice); CH, 10–15 mg; MeOH, 16 hr, 50°C; evap, hot plate N_2; 250 µl PBS	n/a	**ELISA** range, 0–100 ng/ml, (Alpco Salem, NH)	**Unit:** pg/mg **Subjects:** Children **Age:** mean= 7.8 yrs **Sex:** mixed **Race:** Caucasian & Asian
Hinkelmann Muhtz, Dettenborn [37]	2013	Biol Psychiatry	Isopropanol wash; 10 mg hair; MeOH, 18 hr, 45°C; 250 µl ddH_2O	n/a	**LCMS**	**Unit:** pg/mg **Subjects:** Humans **Age:** not reported **Sex:** not reported **Race:** not reported

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Author Name(s)	Year	Journal Name	Extraction method	Purification method	Detection method	Comments
Karlen, Frostell, Theodorsson [17]	2013	Peds	NW; MH, at least >5mg hair; N_2 frozen; MeOH overnight, 25°C (once); evap, vac; 150 µl 0.1 mol/L PBS, 0.02% BS	n/a	**RIA**	**Unit:** pg/mg **Subjects:** Children **Age:** 1,3,5,8 yr **Sex:** mixed **Race:** Caucasian
Vaghri, Guhn, Weinburg [16]	2013	Psychoneuroendocrino	Isopropanol wash (twice); MH, 30 mg hair; MeOH 24 hr, 25°C (twice); evap, air dried; ELISA kit dil	n/a	**ELISA** range, 0.012–3.000 µg/dL (Salimetric, Pennsylvania)	**Unit:** ng/mg **Subjects:** children **Age:** 4–6 yr **Sex:** mixed **Race:** mixed
Groeneveld, Vermeer, Linting [18]	2013	Stress	NW; CH, 20–30 mg; MeOH, 16 hr, 52°C; evap N_2; 250 µl PBS	n/a	**ELISA** range, 2–80 ng/ml (DRG Instruments GmbH, Marburg, Germany)	**Unit:** pg/mg **Subjects:** Children **Age:** 4–5 yr **Sex:** mixed **Race:** Caucasian
Manenschijn, Koper, van den Akker [38]	2012	J Clin Endocrinol Metab	NW; CH, 10 mg; MeOH, 16 hr, 52°C; evap; PBS dil (volume not reported)	n/a	**ELISA** range, 2–80 ng/ml (DRG GmbH, Marburg, Germany)	**Unit:** pg/mg **Subjects:** children & adults **Age:** 10–76 yr **Sex:** mixed **Race:** Caucasian
Li, Xie, Gao [39]	2012	Clinica Chimica Acta	MeOH wash, then either: a) UV irradiation @ 254 nm; b) in hot water (40, 65, 80°C), or c) immersed in 10% shampoo solution, 4 hr; 20 mg hair; MeOH, 24 hr, 40°C; N_2 dried; 50µl MeOH & 1ml H20	MeOH elution	**HPLC/MS**	**Unit:** pg/mg **Subjects:** Adolescents **Age:** 14–18 yr **Sex:** mixed **Race:** Asian
D'Anna – Hernandezez, Ross, Natvig [40]	2011	Physiol Behav	Isopropanol wash; MH, 50 mg; MeOH 24 hr, 25°C; N_2 dried; 400 µl assay buffer	n/a	**ELISA** range 0.003–3.0 µg/dl (Salimetric, Pennsylvania)	**Unit:** pg/mg **Subjects:** Pregnant females **Age:** 18–45 yr **Sex:** female **Race:** not reported
Karlen, Ludvigsson, Frostell [41]	2011	BMC Clin Path	NW, 5–17 mg; N2 frozen, MH; MeOH, 10 hr, 25°C (once); evap, N_2; PBS, 0.02% BSA (150 µl)	n/a	**RIA**	**Unit:** pg/mg **Subjects:** Humans **Age:** not reported **Sex:** mixed **Race:** not reported
Gao, Xie, Jin [42]	2010	Clin Biochem	MeOH wash, 2 min; MH, 50 mg; Soerensen buffer or 0.1 M NaCl or 0.1M NaOH); 16 hr, 40°C; N_2 evap; 50 µl MeOH	MeOH	**HPLC**	**Unit:** pg/mg **Subjects:** adults **Age:** not reported **Sex:** mixed **Race:** Asian
Raul, Cirmele, Ludes [43]	2004	Clin Biochem	Dichloromethane wash, 2 min (twice); MH, 30–100 mg; Sorensen buffer, 16 hr, 40°C; 5 ml NaOH 0.2M	column eluted	**HPLC/MS**	**Unit:** pg/mg **Subjects:** Humans **Age:** not reported **Sex:** mixed **Race:** not reported

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Abbreviations: Not applicable (n/a) Milled Hair (MH); Cut Hair (CH); No Wash (NW); Methanol (MeOH); Methylene Chloride (CH_2Cl_2); hours (hr); minutes (min); Room Temperature (RT); Liquid (LQ), Evaporation (evap); Diluent (dil); Phosphate Buffer Saline (PBS); High Performance Liquid Chromatography (HPLC); Liquid Chromatography (LC) Mass Spectrometer (MS); Radioimmunoassay (RIA); Nitrogen (N_2); Bovine Serum (BS); Bovine Serum Albumin (BSA); double-distilled (dd) water (H_20); Mean (m); Molar (M); Vacuum (vac)