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RESEARCH ARTICLE

Is deproteinization necessary in the determination of human plasmatic steroids by GC/IT-MS/MS analysis?

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Human plasma is composed on average of 7% of proteins, where the main one is albumin, with concentrations range of 3.2-4.8 g/dL. Many studies consider plasmatic proteins to be a problem in relation to plasma analysis, so they must be eliminated. In this work, we have performed several deproteinization tests with acetonitrile, methanol, ethanol, HCl 0.5 M/methanol (1:1) (v/v), trichloroacetic acid (10%), sulfosalicylic acid (20%) and perchloric acid, proteases and activated carbon before analyzing twelfe plasmatic steroids hormones using gas chromatography directly coupled ion-trap mass spectrometry. Signal/noise ratios of tests were determine to assess whether a previous deproteinization treatment is necessary or not prior to their analysis. The use of chemical deproteinization methods, proteases and active carbon did not improve the signal/noise ratio. Deproteinization is not required for the analysis of steroid hormones in plasma using gas chromatography-directly coupled ion-trap mass spectrometry.

Keywords: deproteinization, protease, activated carbon, human plasma, steroids.

Introduction

For many years concentrations of functional steroid hormones or their precursors have been determined mainly in plasma or serum samples of patients, occasionally in saliva or tissues, or as their metabolites, in urine or amniotic fluid [1]. Gas Chromatography/Mass Spectrometry (GC/MS) is well-suited for the identification of a large number of potential steroids and metabolites due to its high chromatographic resolution capacity and reproducible ionization efficiency [2]. Albumin is dominant in the binding, transport, and delivery of a range of endogenous ligands and pharmaceuticals because it accounts for approximately 60% of the total proteins in blood serum with a typical concentration of 3.4–5.0 g/dL [3]. Biblio graphy shows that protein precipitation for human plasma analysis performed by GC/MS can be performed successfully with methanol by centrifugation [4, 5, 6] with acetonitrile [7, 8, 9] with 70% perchloric acid and n-hexane [10], with 5% sulfosalicylic [11], with ethanol [12], with acetone [13], and with trichloroacetic acid [14]. Other methods for plasma deproteinization focus on physical methods as described [15]. These methods include ultrafiltration and centrifugation with varied molecular weight cut-offs, or using other simple methods such as heating the plasma. However, it has not been shown that these deproteinization methods interfere with gaschromatography-ion trap-tandem mass spectrometry (GC/IT-MS/MS) analysis of plasmatic steroids. Hence, the aim of this work was to identify if it is necessary and useful to apply a deproteinization treatment to human plasma before its analysis in order to determine steroids hormones using GC/IT-MS/MS.

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Materials and methods

Following human plasmatic steroids: testosterone, methyltestosterone, androsterone, 5β-androsterone, dihydrotestosterone (DHT), cortisone, cortisol, tetrahydro-

cortisone (THE), nandrolone, dehydroepiandrosterone (DHEA), tetrahydrocortisol (THF), estriol, and epiandrosterone were analyzed using GC/IT-MS/MS under different conditions: receiving and not receiving a previous treatment with commonly used deproteinization methods. The signal/noise (S/N) ratios of the analytes studied were assessed to determine which are the best pre-treatments for the analysis of human plasmatic steroid profile.

Chemicals and steroids

Testosterone, methyltestosterone, androsterone, 5β-androsterone, dihydrotestosterone (DHT), cortisone, cortisol, tetrahydrocortisone (THE), nandrolone, dehydroepiandrosterone (DHEA), estriol, epiandrosterone, N-methyl N-trimethylsilyl-trifluoroacetamide (MSTFA), proteinase N from Bacillus subtilis and protease from Aspergillus saitoi, were purchased from Sigma-Aldrich (Barcelona, Spain). Tetrahydrocortisol (THF) was supplied by Steraloids. β-glucoronidase (type Escherichia coli K 12) was obtained from Boeheringer Mannheim (Mannheim, Germany). Dithioerythritol was obtained from Serva (Heidelberg, Germany). Ammonium iodide (NH_4I) was supplied by Panreac. Human albumin at 20% was obtained from Grifols. Active carbon Norit A was obtained from Serva (Heidelberg, Germany). All other reagents and solvents were of analytical grade and were mainly supplied by Scharlau (Hamburg, Germany), JT Baker (Tarragona, Spain) and Panreac (Barcelona, Spain).

Instrumentation

The GC-IT-MS/MS analysis of the samples was conducted on a Varian 3800 gas chromatograph directly coupled to a MS/MS Saturn 2000 ion-trap mass spectrometer and equipped with an auto-sampler Varian 8200 with a capacity for 48 samples. Separation was performed using a HP-5MS (crosslinked 5% Ph-Me-silicone) capillary column having 30 m x 0.25 mm i.d.; film thickness, 0.25µm. Helium was used as the carrier gas (1mL/min) and the column oven temperature was programmed to maintain at 100 ºC for 1.5 min, rise at 25 ºC per minute until it reached 190 ºC, increase of 3 ºC per minute until 280 ºC, maintaining the temperature for 5 minutes, the temperature rose 25 ºC per minute until 325 ºC and maintained this temperature for 3 minutes. Analysis was performed in splitless mode. The injection volumen was 3µL.

Sample preparation

In this article, the schema of procedure was:

A. For deproteinized plasma, the procedures were based in three steps:

- Deproteinization of human plasma with different methods of deproteinization.
- Steroids extraction with organic solvent mixture.
- Derivatization of extracted steroids.

B. For non-deproteinized plasma, the procedures were based in two steps [16]:

- Steroids extraction with organic solvent mixture.
- Derivatization of extracted steroids.

A. Deproteinization of human plasma

- Deproteinization by chemical deproteinizing agents.

To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added. The deproteinization was carried out with 1 mL of the following reagents; acetonitrile, methanol, ethanol, HCl 0.5 M/methanol (1:1) (v/v), trichloroacetic acid (10%), sulfosalicylic acid (20%) and perchloric acid after 30 minutes of shaking.

- *Deproteinization by heat*

To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added. The deproteinization was carried out by heat at 60ºC for 30 minutes.

- *Deproteinization by proteases*

Deproteinization was performed by using following proteases: Aspergillus saitoi and Bacillus subtilis.

- Deproteinization by Aspergillus saitoi

To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added and the pH was adjusted to 2.8 using HCl 0.1N. The deproteinization was carried out with 1 mL of the Aspergillus saitoi (1mg/mL) in a thermoblock for 24 hours at 36 ºC.

- Deproteinization by Bacillus subtilis

To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added and the pH was adjusted to 7 using acetic acid 1M (CH_3COOH) and a phosphate buffer (K_3PO_4/Na_2HPO_4) at pH 7. The deproteinization was carried out with 1 mL of the Bacillus subtilis (1mg/mL) in a thermoblock for 24 hours at 36 ºC.

Deproteinization by a mixture of Aspergillus saitoi and Bacillus subtilis without adjusting the pH

To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added. The deproteinization was carried out with 1 mL of a mixture of Aspergillus saitoi (1mg/mL) and the Bacillus subtilis (1mg/mL) in a thermoblock for 24 hours at 36 ºC.

- Deproteinization by a mixture of Aspergillus saitoi and Bacillus subtilis adjusting the pH to the optimal pH of Aspergillus saitoi
To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added and the pH was adjusted to 2.8 using HCl 0.1N. The deproteinization was carried out with 1 mL of a mixture of Aspergillus saitoi (1mg/mL) and the Bacillus subtilis (1mg/mL) in a thermoblock for 24 hours at 36 ºC.

- Deproteinization by a mixture of Aspergillus saitoi and Bacillus subtilis adjusting the pH to the optimal pH of Bacillus subtilis
To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added and the pH was adjusted to 7 using acetic acid 1M (CH_3COOH) and a phosphate buffer (K_3PO_4/Na_2HPO_4) pH 7. The deproteinization was carried out with 1 mL of a mixture of Aspergillus saitoi (1mg/mL) and the Bacillus subtilis (1mg/mL) in a thermoblock for 24 hours at 36 ºC.

- Deproteinization by successive addition of Aspergillus saitoi and Bacillus subtilis
To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added and the pH was adjusted to 2.8 using HCl 0.1N. It was added 1 mL of the Aspergillus saitoi (1mg/mL) in a thermoblock for 24 hours at 36 ºC. After the pH was adjusted to 7 using acetic acid 1M (CH_3COOH) and phosphate buffer (K_3PO_4/Na_2HPO_4)at pH 7 and 1 mL of the Bacillus subtilis (1mg/mL) was added keeping samples in a thermoblock for 24 hours at 36 ºC.

- Deproteinization by activated carbon
To 1 mL of plasma sample, 2 ng of methyltestosterone as internal standard were added. After we added 1 mL of a mix of activated carbon (1%) in buffer (K_3PO_4/Na_2HPO_4) at pH 7 samle was shaken during 20 minutes, centrifugated at 8000 rpm during 10 minutes, the supernatant was collected.

B. Steroids extraction
The pH was adjusted to 9.5 using NaOH 1M and buffer (HCO_3^- 1.34M /CO_3^{2-} 0.2M) at pH 9.5. The extraction was carried out with 2 mL of n-hexane/ethyl acetate (70/30%, v/v). After 30 minutes of shaking, the mixture was centrifuged for 5 minutes and the organic phase was dried under a stream of nitrogen.

- Derivatization procedure
To the dried plasma extracts 50 µL of a mixture of MSTFA:NH_4I:dithioerythritol (1000:2:4) (v/w/w) was added. The reaction mixture is heated in a thermoblock for 30 minutes at 60 ºC to perform the reaction of derivatization, and after, it was encapsulated and injected into the chromatograph.

Measurement of proteins contents
In order to verify the effectiveness of the different deproteinization treatments, total proteins and albumin were determined in the extraction solvent and in the plasma after its deproteinization. The analysis of proteins and albumin contents was as follows. 1 mL of plasma was added to three sample tubes:

- Test tube number 1
One extraction was performed with 2 mL of extraction solvent based in our method (N-hexane/ethyl acetate 70:30% (v/v). Organic layer, middle layer and aqueous layer were separated. The contents of total proteins and albumin were measured.

- Test tube number 2
Two extraction were performed with 2 mL of extraction solvent based in our method (N-hexane/ethyl acetate 70:30% (v/v). Organic layer, middle layer and aqueous layer were separated. The contents of total proteins and albumin were measured.

-Test tube number 3
Three extraction were performed with 2 mL of extraction solvent based in our method (N-hexane/ethyl acetate 70:30% (v/v). Organic layer, middle layer and aqueous layer were separated. The contents of total proteins and albumin were measured. To identify the deproteinization capacity, the assessment of plasmatic total protein and albumin were performed once the proteases had acted.

Results and discussion
The characteristics of the analytes are reflected in **Table 1 & Figure 1**, in which we described retention time of analytes, retention time relative to internal standard (IS) methyltestosterone, qualifications ions , quantification ion of each studied compound and the limits of detection (LOD) of our method so for androsterone and 5β-androsterone 0.20 ng/mL, for DHEA 0.05 ng/mL, for DHT and cortisol 0.02 ng/mL, for epiandrosterone 0.36 ng/mL, for nandrolone 0.33 ng/mL, for testosterone 0,48 ng/mL, for estriol 0.06 ng/mL, for tetrahydrocortisone (THE) 0.18 ng/mL, for tetrahydrocortisol (THF) 0.04 ng/mL and for cortisone 0.29 ng/mL.
The signal/noise values for GC/IT-MS/MS detection of plasmatic steroids after performing different chemical deproteinization treatments are shown in **Table 2.** In

Table 1. Quantification- and qualification ions of different steroids

Compound	Retention time (min.)	Rel. Retention time (min.)*	Parent Ion *(m/z)*	Qualifier Ions *(m/z)*	Quantifier Ion *(m/z)*	LOD (ng/mL)
Androsterone	21.942	0.776	420	420-329-239	329	0.20
5β-androsterone (etiocholanolone)	22.207	0.785	420	420-329-239	329	0.20
Dehydroepiandrosterone (DHEA)	23.530	0.832	418	418-327-237	327	0.05
Epiandrosterone	23.786	0.841	420	420-329-239	329	0.36
Nandrolone	24.491	0.866	418	418-403	403	0.33
Dihydrotestosterone (DHT)	24.491	0.866	434	434-419-405-377	419	0.02
Testosterone	25.311	0.895	432	432-417-327-301	417	0.48
Estriol	29.752	1.052	504	504-311-295-281-269	311	0.06
Tetrahyidrocortisone (THE)	33.035	1.168	635	635-619-530	619	0.18
Tetrahydrocortisol (THF)	34.232	1.210	636	636-531-430	531	0.04
Cortisone	36.009	1.273	617	617-525-435	525	0.29
Cortisol	38.155	1.349	632	632-543-437	543	0.02

* Relative retention time is refered to internal standard (IS) methyltestosterone; LOD: limit of detection.

general, the signal/noise ratio decreased preventing most of the analytes from beying analyzed qualitatively.

When using acetonitrile as deproteinization method, androstenedione and progesterone were detected, whereas the other steroid hormones failed to improve their signal/noise ratio. The use of ethanol for deproteinization allowed detection of progesterone, whereas for the other steroid hormones, the signal/noise ratio decreased. The heat treatment allowed deproteinized progesterone to be detected, but the signal/noise ratio for the rest of detecting steroid hormones impaired. The use of 0.5 M HCl/MeOH (1:1) (v/v) improved the detection of progesterone but the signal/noise ratio for other steroid hormones got worse. The detection got MeOH progesterone, but does not improve the signal/noise ratio of the other steroid hormones. The TCA (10%) and sulfosalicylic acid (20%) impaired the signal/noise ratio of all steroid hormones. The use of perchloric acid caused all analytes under study to remain undetectable.

In **Table 3** are shown the results of using proteases as a deproteinization method. The use of a mixture of Aspergillus and Bacillus without pH adjustment improved the signal/noise ratio of epiandrosterone. Digestion with Aspergillus for 24 hours followed by another digestion with Bacillus during another 24 hours and the use of Aspergillus, allowed detection of progesterone. The signal/noise ratios of the other compounds under study did not improve.

The use of activated carbon as deproteinization method (**Table 4)** decreased all the signal/noise ratios of the compounds analyzed in this study. Most of them could hardly be detected. In relation to the analysis of the extraction solvent, neither proteins nor albumin appeared there, however in the middle layer, some proteins appeared as aqueous layer fractions (**Table 5**). The results presented in **Table 6** show how total protein values and albumin values in human plasma decreased after a treatment with proteases. According to the results of this study, using any standard deproteinization method to remove the protein content from human plasma did not improve the signal/noise ratio for the detection of a range of steroids by GC/IT-MS/MS. Furthermore, the investigated deproteinization methods caused a loss of detection in many of the tested compounds, maybe due to coagulation-flocculation produced by the addition of these solvents. In relation to the use of proteases, proteins were degraded into their corresponding amino acids, but it is not effective to liberate the steroids under study. This might be due to the residual amino acid digestion products attaching to the studied compound, preventing a free hydroxyl group for subsequent derivatization reaction, and thus preventing analysis by GC/IT-MS/MS. The use of activated carbon for deproteinization of plasma emerged as an idea of their use in radioim munoassay; however the results have not been good enough for the detection of human plasmatic steroid hormones of steroids by GC/IT-MS/MS. Its use decreased the signal/noise ratio for all compounds under study, maybe because although the carbon absorbs the steroids, our solvent extractor was not effective enough to remove them. When studied compounds were extracted, all proteins remained in the aqueous phase, so the protein doesn't pass to organic phase.This

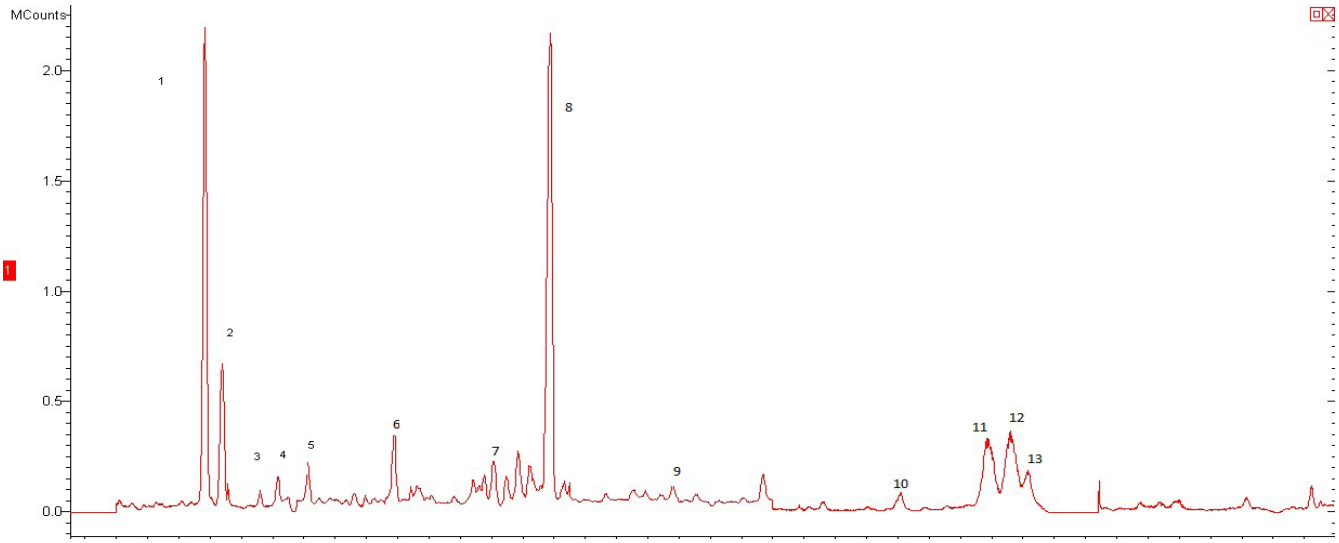

Figure 1. Steroids in human plasma. (1) Androsterone; (2) Etiocolanolone; (3) DHEA; (4) Epiandrosterone; (5) Nandrolone; (6) DHT; (7) Testosterone; (8) Methyltestosterone (IS); (9) Estriol; (10) THE; (11) THF; (12) cortisone; (13) Cortisol.

Table 2. Signal/noise values for GC-IT-MS/MS detection of plasmatic steroids performing chemical deproteinization treatments

Compound	ACN	EtOH	Heat	HCl/MeOH	MeOH	TCA (10%)	SS (20%)	$HClO_4$	No deproteinization
Androsterone	5	20	7	7	26	ND	ND	ND	1184
5β-androsterone	9	14	ND	12	18	ND	13	ND	523
Dehydroepiandrosterone	1	5	5	2	1	ND	5	ND	315
Epiandrosterone	2	4	ND	ND	ND	ND	ND	ND	4
Nandrolone	ND	ND	ND	ND	ND	ND	ND	ND	56
Dihydrotestosterone	ND	7	ND	17	10	3	ND	ND	24
Testosterone	6	6	ND	ND	ND	ND	ND	ND	74
Methyltestosterone (IS)	34	593	3	7	14	2	8	ND	195
Estriol	ND	ND	ND	ND	ND	ND	ND	ND	69
Tetrahydrocortisone	3	1	1	3	1	ND	7	ND	105
Tetrahydrocortisol	ND	ND	ND	ND	ND	ND	ND	ND	47
Cortisone	4	46	4	17	ND	ND	ND	ND	137
Cortisol	20	36	41	10	4	1	ND	ND	484

ND=signal/noise< 3; ACN:acetonitrile; EtOH:ethanol; MeOH: methanol; TCA: trichloroacetic acid; SS: sulfosalicylic acid; $HClO_4$: perchloric acid

Table 3. Signal/noise values for GC/IT-MS/MS detection of plasmatic steroids after using proteases.

Compound	Protease(s) from						Healthy subject
	Aspergillus & Bacillus[a]	Aspergillus	Bacillus	Aspergillus & Bacillus[b]	Aspergillus & Bacillus[c]	Aspergillus & Bacillus[d]	
Androsterone	12	10	12	10	2	13	1184
5β-androsterone	15	44	17	9	3	79	523
Dehydroepiandrosterone	ND	1	1	3	ND	4	315
Epiandrosterone	ND	ND	2	3	3	13	4
Nandrolone	ND	ND	ND	ND	ND	ND	56
Dihydrotestosterone	12	40	66	ND	ND	12	24
Testosterone	ND	ND	ND	ND	22	ND	74
Methyltestosterone (IS)	3	29	15	3	2	8	195
Estriol	ND	10	ND	ND	ND	ND	69
Tetrahydrocortisone	2	ND	3	ND	ND	ND	105
Tetrahydrocortisol	ND	ND	ND	ND	2	ND	47
Cortisone	16	ND	5	17	ND	ND	137
Cortisol	11	24	18	37	22	22	484

[a]24h; [b]at pH Aspergillus; [c]at pH Bacillus; [d]without pH adjustment; ND: Signal/Noise <3

Table 4. Signal/noise values for GC/IT-MS/MS detection of plasmatic steroids after using activate carbon.

Compound	Activated carbon	Healthy subject
Androsterone	13	1184
5β-androsterone	ND	523
Dehydroepiandrosterone	4	315
Epiandrosterone	ND	4
Nandrolone	ND	56
Dihydrotestosterone	ND	24
Testosterone	ND	74
Methyltestosterone (IS)	1	195
Estriol	ND	69
Tetrahydrocortisone	ND	105
Tetrahydrocortisol	ND	47
Cortisone	ND	137
Cortisol	ND	484

might be due to a greater polarity of the aqueous compared with the organic phase, causing the proteins, which have a polar character, to remain in the aqueous phase.

Conclusions

Even though the use of a mixture of proteases (Aspergillus saitoni & Bacillus subtilis 1 mg/mL) reached a 100% elimination of plasma albumin and 95% of plasma total protein, the signal/noise ratio for GC/IT-MS/MS detection of plasmatic steroids did not improve, or was impaired. This was true for the performance of the other deproteinization meth-

Table 5. Total protein and albumin values in different layers

Layer	Total protein (g/dL)	Reference values (g/dL)	Albumin (g/dL)	Reference values (g/dL)
Plasma	6.9	6.4 – 8.2	4.0	3.7-5.2
Plasma after 1 extraction	6.6	6.4 – 8.2	3.8	3.7-5.2
Plasma after 2 extractions	7.1	6.4 – 8.2	4.0	3.7-5.2
Plasma after 3 extractions	7.1	6.4 – 8.2	4.1	3.7-5.2
Middle layer after 1 extraction	6.4	6.4 – 8.2	3.4	3.7-5.2
Middle layer after 2 extractions	6.9	6.4 – 8.2	3.4	3.7-5.2
Middle layer after 3 extractions	1.1	6.4 – 8.2	-----	3.7-5.2
Extract after 1 extraction	0.0	6.4 – 8.2	-----	3.7-5.2
Extract after 2 extractions	0.0	6.4 – 8.2	-----	3.7-5.2
Extract after 3 extractions	0.0	6.4 – 8.2	-----	3.7-5.2

Table 6. Total protein and albumin values treated with different proteases.

Sample	Total proteins	Total protein elimination	Reference values	Albumin	Albumin elimination	Reference values
	(g/dL)	(%)	(g/dL)	(g/dL)	(%)	(g/dL)
Plasma	8.0	-	6.4-8.2	5.2	-	3.2-4.8
Plasma + 10mg/10mL Protease	2.1	73.8	6.4-8.2	< 0.1	100	3.2-4.8
Mixture of Proteases (10mg/10mL)	0.4	95.0	6.4-8.2	< 0.1	100	3.2-4.8
Plasma + (Aspergillus/ Bacillus 24 h)	0.6	92.5	6.4-8.2	< 0.1	100	3.2-4.8

ods, i.e. temperature treatments or activated carbon. The analysis of plasmatic steroid hormones in this study do not carry any problem because of there are not any proteins in the extraction solvent. None of the deproteinization methods tested in this paper effectively improve the signal/noise ratio of steroid hormones in plasma detected by GC/IT-MS/MS, and furthermore, these methods increase the pre-treatment time of the samples.

Abbreviations

GC/IT-MS/MS: Gas chromatograph directly coupled ion-trap mass spectrometer.
GC-MS: Gas Chromatography-Mass Spectrometry.
LOD: Limit of Detection.
ND: Not Detected.
S/N: Signal-to-noise ratio.
IS: Internal Standard.
THE : Tetrahydrocortisone.
DHT: Dihydrotestosterone
DHEA: Dehydroepiandrosterone.
THF: Tetrahydrocortisol.
MSTFA: N-methyl N-trimethylsilyl-trifluoroacetamide.

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