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# Determination of natural corticosteroids in urine samples from sportsmen☆

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**Abstract**

A method for the determination of natural corticosteroids (cortisone, cortisol, 5β-dihydrocortisone, 5β-dihydrocortisol, tetrahydrocortisone and tetrahydrocortisol) found in the urine of sportsmen, was developed using a capillary gas chromatography–mass spectrometry ion trap system. 17α-Methyltestosterone was used as an internal standard. The different corticosteroids were determined from the peak area ratios of the [M]; [M−90] and [M−90−90] fragment ions of their methoxime-trimethylsiyl derivatives. Sensitivity (15 ppb), specificity, accuracy (96%) and reproducibility (RSD=4–10%) of the method were demonstrated to be satisfactory for measuring the urinary concentrations of the selected natural corticosteroids. 

*Keywords:* Corticosteroids

## 1. Introduction

Corticosteroids are steroid hormones synthesized and liberated in the suprarenal cortex. These have wide clinical use because of their anti-inflammatory effects [1]. Corticosteroids are excreted in urine basically as conjugated metabolites and in a non-metabolized form [2,3]. These compounds are detected and quantified in biological fluids for different purposes, e.g., calculation of pharmacokinetic parameters, development of new corticosteroids, monitoring of therapeutic doses, and recently in doping control.

This report details a method for the routine screening, confirmation and quantitation of cortisol (HC), cortisone (C), tetrahydrocortisol (THCol), tetrahydrocortisone (THC), 5β-dihydrocortisone (5β-DHC), and 5β-dihydrocortisol (5β-DHCol) in human urine using capillary gas chromatography–mass spectrometry (GC–MS) (ion trap). The method is simpler and faster than any other described in the literature [4–7] by doing away with the cleaning process of derivative samples, the role of which is to eliminate as much as possible the background and to

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protect the equipment from derivative excess. We demonstrate that this phase is useless because the derivative agent excess does not interfere with our compounds. With that, we obtain better recoveries and the detection limits are similar to those reported in the literature. There are few recent publications concerning GC–MS and GC–MS–MS techniques [8]. This communication is the first contribution from our present investigation, and shows the results derived from our studies on competitive stress as related to THC, THCol, C and HC, which are considered as the main measures of these aspects of sport.

## 2. Experimental

### *2.1. Urine collection*

The urine of five healthy sportsmen (two male marathon runners, one female marathon runner and two male basketball players) was analyzed. The urine sample obtained was the first urine of the day. The fraction volumes were measured and an aliquot was stored at −4°C until analysis.

### *2.2. Chemicals and reagents*

Cortisol (11β,17α,21-trihydroxypregnane-4-ene-3,20-dione), cortisone (4-pregnane-17α,21-diol-3,11,20-trione), tetrahydrocortisol (5β-pregnane-11β,20-dione-3α,17α,21-triol), tetrahydrocortisone (5β-pregnane-11,20-dione-3α,17α, 21-triol), 5β-dihydrocortisone (5β-pregnane-17α,21-diol-3,11,20-trione), 5β-dihydrocortisol (5β-pregnane-11β,17α,21-triol-3,20-dione), 17α-methyltestosterone as an internal standard, *N* - methyl - *N* - trimethylsilyl - trifluoroacetamide (MSTFA) and 1-(trimethylsilyl)-imidazole (TMS-imid), were purchased from Sigma–Aldrich (St. Louis, MO, USA) and β-glucuronidase from *Escherichia coli* enzyme came from Boehringer Mannheim (Mannheim, Germany). $Na_2HPO_4$, $KH_2PO_4$, $K_2CO_3$, $KHCO_3$ were products of Panreac (Barcelona, Spain). These reagents were used as supplied.

All corticosteroid stock solutions were prepared by dissolving the substances in methanol to give a concentration of 2000 ppm, standard working solutions were prepared monthly.

Methoxyamine hydrochloride and 8% (w/v) anhydrous pyridine were used, both purchased from Sigma–Aldrich, and equilibrated at room temperature. Diethyl ether and methanol were purchased from Panreac. These solvents used were of HPLC grade.

### *2.3. Gas chromatography–mass spectrometry*

Electronic impact (EI) studies and MS–MS were performed in a Varian 3800 gas chromatograph and Saturn 2000 ion trap detector coupled to the Saturn GC–MS WorkStation data processing system (Table 1).

A TRB-1, capillary cross-linked methyl silicone (15 m×0.2 mm I.D., 0.1 μm film) column was used in the gas chromatograph. Ultra-high-purity helium at a flow-rate at 1 ml/min on-column was used as the carrier gas. The split injection mode was used with the injector set at 280°C, the split ratio was 40. The initial oven temperature was set at 120°C (for 2 min) and programmed to increase at 20°C/min to 250°C (for 10.75 min), then the temperature was increased at 20°C/min to 300°C and held for 5 min.

Table 1
GC–EI–MS and GC–MS–MS conditions

| Compound | EI | | MS–MS | | |
|---|---|---|---|---|---|
| | TR | Quantitation ions | Parent ion | Voltage (V) | RF (Hz) |
| THC | 12.335 | 579, 489 | 579 | 71 | 120 |
| THCol | 13.016 | 563, 473 | 563 | 78 | 130 |
| 5B-DHC | 13.633 | 534, 444 | 534 | 77 | 130 |
| 5B-DHCol | 14.683 | 608, 518 | 608 | 70 | 130 |
| C | 16.284 | 532, 441 | 532 | 67 | 120 |
| HC | 17.667 | 606, 516 | 606 | 64 | 115 |

Table 2
Linear regression equations and detection limits for studied corticosteroids

| Compound | Calibration curve | *R* | LD (ppb) |
|---|---|---|---|
| THC | $y=-0.012+0.020x$ | 0.945 | 13.1 |
| THCol | $y=0.112+0.011x$ | 0.930 | 15.3 |
| C | $y=-0.014+0.003x$ | 0.908 | 17.7 |
| HC | $y=0.198+0.015x$ | 0.952 | 12.4 |
| 5β-DHC | $y=0.068+0.004x$ | 0.950 | 12.7 |
| 5β-DHCol | $y=0.127+0.010x$ | 0.962 | 11.0 |

The detector temperatures were 200°C (trap), 50°C (manifold) and 280°C (transfer line).

### 2.4. Sample preparation

In accordance with Yap et al. [4], a 2-ml aliquot of human urine was centrifuged at 3000 rpm to eliminate solid residue, and then buffered to pH 7 with 250 μl of 0.2 *M* $Na_2HPO_4$–$KH_2PO_4$ solution. 17α-Methyltestosterone internal standard solution (600 ng) was added and the sample was mixed for 3 s. The hydrolysis was done with 50 μl of β-glucuronidase enzyme at 50°C for 1 h. After hydrolysis, we added 250 μl of $K_2CO_3$–$KHCO_3$ (7%), pH 9–10. The corticosteroids were extracted with 2 ml of diethyl ether for 15 min. After that, the organic phase was dried under $N_2$.

### 2.5. Derivatization of urine extract

The dry corticosteroid residue was treated [4] with 100 μl of methoxyamine hydrochloride solution in pyridine at 60°C for 30 min. After cooling, the excess pyridine was evaporated under $N_2$. The residue was reconstituted with 50 μl of the MSTFA–TMS-imidazole (0.2%) reagent, and the mixture reacted at 70°C for 50 min to make the MO-TMS ether derivatives. A 1-μl volume of the liquid residue was analyzed by GC–MS in the EI and MS–MS modes.

### 2.6. Calibration curve

Standard solutions (2 ml), were prepared from children's urine samples, cleaned by three extractions with diethyl ether, containing 50, 100, 200, 500 ppb of each compound, and also 1000 ppb for some compounds (THCol and THC), and were used to prepare the calibration graphs. Peak area response ratios were plotted against concentration ratios. The

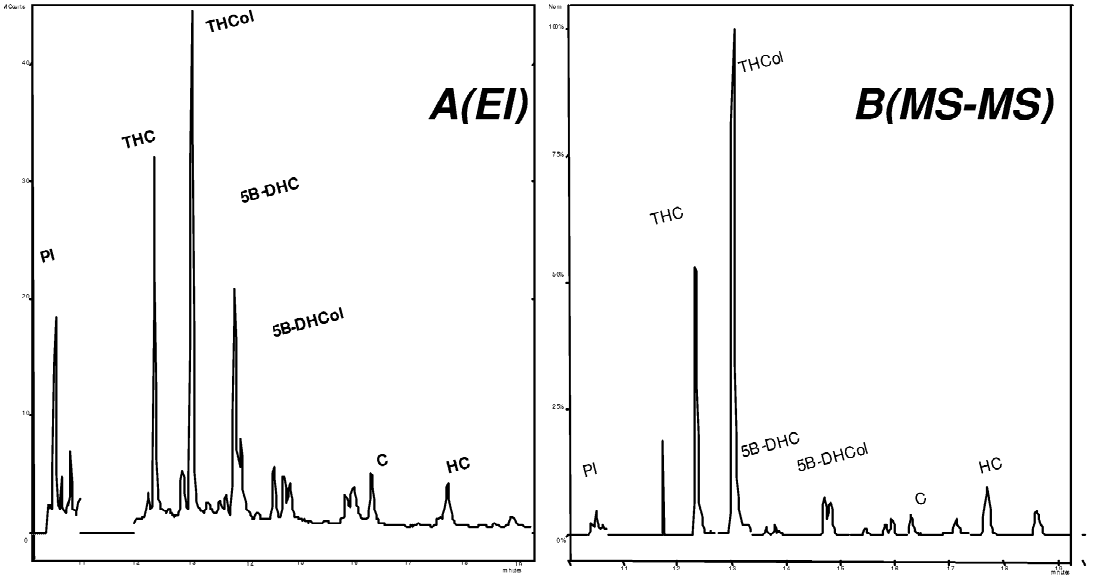


Fig. 1. EI and MS–MS chromatograms of multicomponent mix.

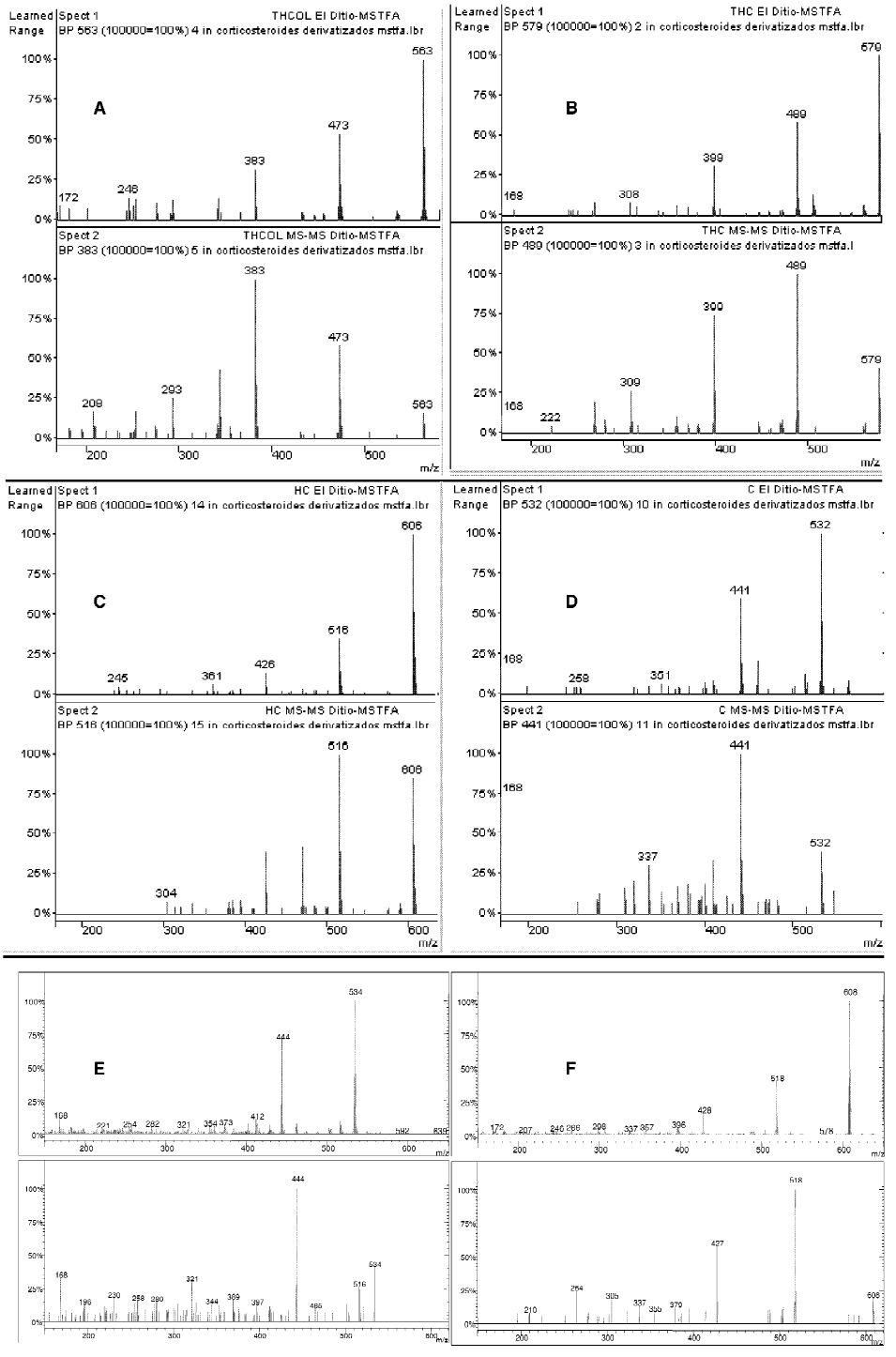


Fig. 2. EI and MS–MS spectra of the derivatives of corticosteroids and their metabolites: (A) MO-tetraTMS tetrahydrocortisone; (B) MO-tetraTMS tetrahidrocortisol; (C) di-MO-triTMS cortisone; (D) di-MO-triTMS cortisol; (E) tri-MO-di-TMS 5β-dihydrocortisone; (F) di-MO-tri-TMS 5β-dihydrocortisol.

Table 3
Extraction recoveries of corticosteroids

| | THC | THCOL | 5β-DHC | 5β-DHCol | C | HC |
|---|---|---|---|---|---|---|
| | 156.4 | 354.1 | 145.1 | 185.3 | 252.2 | 212.3 |
| | 131.5 | 427.1 | 170.3 | 197.8 | 255 | 243.6 |
| | 135.6 | 367.6 | 169.5 | 190.2 | 311 | 238.6 |
| | 147.6 | 382.5 | 145.3 | 198.7 | 279.4 | 207.8 |
| | 144.6 | 389.4 | 181.7 | 189.2 | 277.3 | 210.7 |
| | 169.5 | 434.6 | 174.5 | 187.4 | 352.4 | 246.4 |
| Real amount (ppb) | 150.0 | 400.0 | 175.0 | 200.0 | 300.0 | 250.0 |
| Mean (ppb) | 147.5 | 392.6 | 165.9 | 192.7 | 289.6 | 229.9 |
| RSD (%) | 9.44 | 8.19 | 8.86 | 3.12 | 12.09 | 8.15 |
| Recovery (%) | 98.36 | 98.14 | 94.81 | 96.33 | 96.54 | 91.97 |

calibration curve was made by plotting the peak area ratios of the analytes quantitation ions (Table 1), to each compound by EI and MS–MS techniques (Table 2), to that of the internal standard quantitation ions ($m/z$ 403+313). The plot was linear over the studied range with regression coefficients in the range from 0.906 to 0.968.

We have used injections in the EI mode for quantitative determination of samples and in the MS–MS mode for qualitative determination.

## 3. Results and discussion

### *3.1. Ion chromatograms and mass spectra*

Table 1 shows the EI and MS–MS conditions selected for this work. Fig. 1A and B show the ion chromatograms of a multicomponent mix from standards by EI and MS–MS, respectively, whereas Fig. 2A–F shows the comparative spectra from EI and MS–MS modes of THCol, THC, HC, C, 5β-DHC and 5β-DHCol, respectively, corresponding to MO-TMS derivatives. The results show chromatograms, in the MS–MS mode are cleaner than those in the EI mode, improving the signal/noise ratio ($S/N$). There are some significant fragments of these derivative compounds; [M−30], [M−30−90], [M−30−90−90] and [M−30−90−90−90]. The loss of 30 u corresponds to a methoxyamine derivative ($-OCH_3$) fragment and 90 u corresponds to a trimethylsilyl derivative ($-OSiC_3H_9$).

### *3.2. Detection limit*

The detection limit was calculated from four injections in the EI mode at four concentration levels (25, 50, 100 and 200 ppb) by the linear calibration curves method [7]. The results are shown in Table 2 and were calculated by introducing the result of the intercept from 95% upper security level with the Y axis in the calibration curve. The signal-to-noise

Table 4
Results of reproducibility studies

| | THC | THCOL | 5B-DHC | 5B-DHCOL | C | HC |
|---|---|---|---|---|---|---|
| | 1119 | 3011 | 310 | 501 | 833 | 900 |
| | 1153 | 3096 | 365 | 541 | 985 | 1111 |
| | 1250 | 3496 | 395 | 637 | 1060 | 1176 |
| | 1211 | 3121 | 400 | 636 | 875 | 1086 |
| | 1194 | 3091 | 383 | 591 | 1061 | 1056 |
| | 1155 | 3183 | 345 | 640 | 1061 | 1233 |
| Mean | 1180 | 3166 | 366 | 591 | 979 | 1094 |
| RSD (%) | 4.00 | 5.39 | 9.35 | 9.91 | 10.43 | 10.46 |

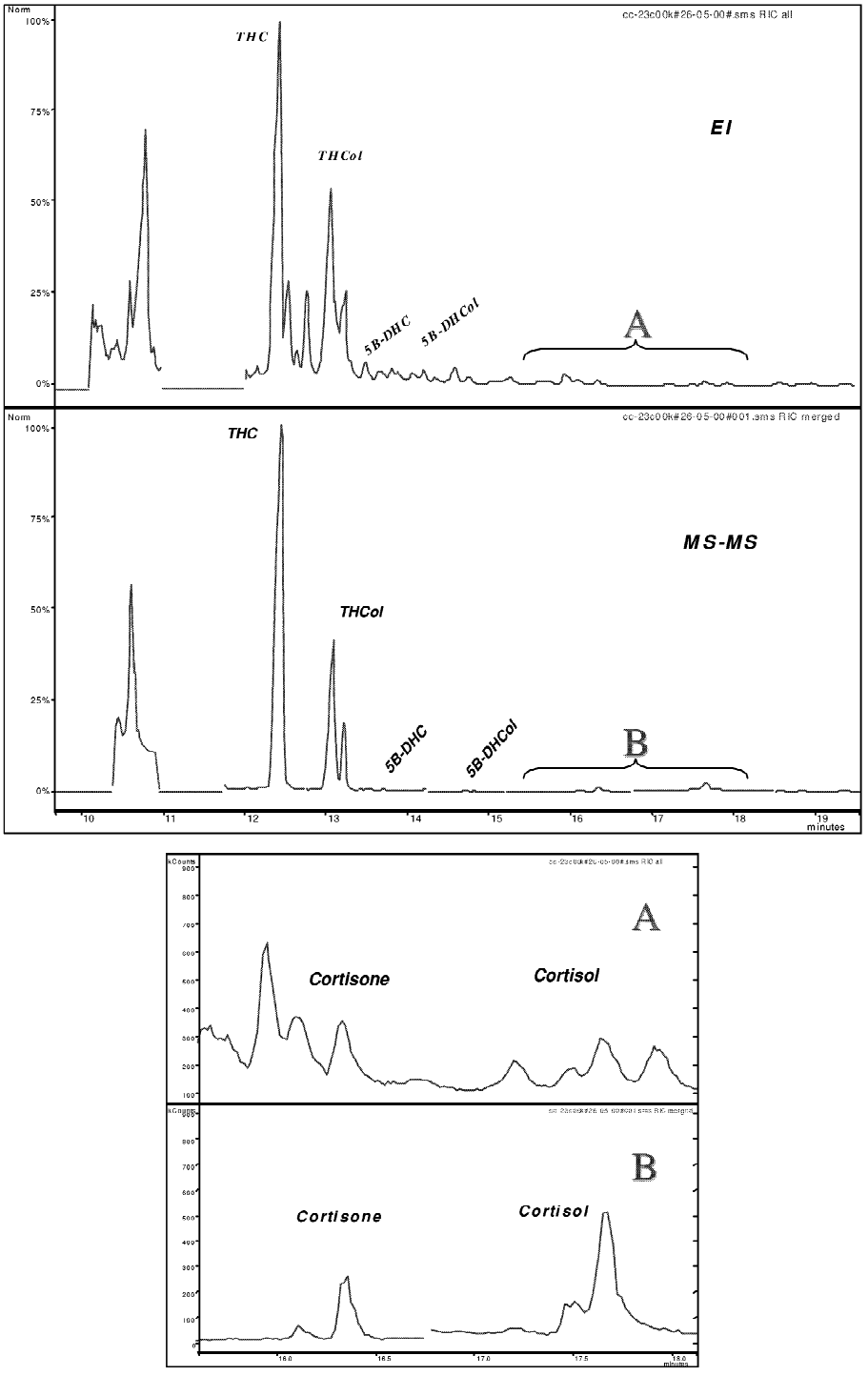


Fig. 3. Chromatogram of urine from a basketball player. Cortisone and cortisol detail in EI (A) and MS–MS (B).

ratio was higher than 3:1 for all compounds. These detection limits are in the same order as any others found in the literature [4,9].

### 3.3. Recovery and reproducibility

Known amounts of each compound were added to 2 ml of blank solution of human urine, the absolute recoveries for each one were estimated from a minimum of six replicated analyses of corticosteroids. Table 3 shows higher recoveries than other previously published values [4,9].

In order to determine the reproducibility of our method the same urine sample was injected several times (Table 4). The results show the high reproducibility of our method with RSDs between 4 and 10%.

### 3.4. Corticosteroid profile of urine

The method was applied to different urine samples of sportsmen from different sports. Fig. 3 shows the chromatographic typical profile of the corticosteroids in EI and MS–MS mode acquisition, corresponding to an athlete with a high responsibility in a basketball team. The high levels of metabolites from cortisone and cortisol is translated as a high level of stress.

The application of this method was extended so as to included different kinds of sportsmen of both sexes to determine the main metabolite levels of natural corticosteroids (Table 5).

In Table 5, we can see the levels of corticosteroids of two basketball players. Number 1 is a player who has not played regularly for his team (less stress), and number 2 who has played regularly (more stress).

Similar results were observed in the case of marathon runners. We observed that number 1, with the higher results, corresponds to a person who had run a marathon recently, just 3 weeks before (more stress). Number 2 was a runner in training and number 3 was an injured sportswoman not undergoing intensive training.

These results demonstrate that with the method applied we can study the increase corticosteroid levels produced in stressful situations (competition).

Work is in progress to apply said method to sedentary persons.

## 4. Conclusions

Sensitivity, specificity, accuracy and reproducibility of the method proved to be satisfactory for measuring the urinary concentrations of selected natural corticosteroids, which enable us to detect changes of endogenous levels of corticosteroids in urine under different stress situations due to competition (fatigue, tension, physical exertion, etc.) see example in Table 5 between numbers 1 and 2 marathon runners.

## Acknowledgements

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Table 5
Corticosteroid profile of urine

| | Basketball | | Marathon | | |
|---|---|---|---|---|---|
| | 1 (ppb) | 2 (ppb) | 1 (ppb) | 2 (ppb) | 3* (ppb) |
| THC | 2863 | 4936 | 2439 | 1544 | 289 |
| THCol | 4459 | 4913 | 2354 | 830 | 60 |
| C | 166 | 902 | 163 | 120 | 139 |
| HC | 76 | 432 | 98 | 35 | 42 |

*Woman. Note: The other compounds are lower than the quantitation limit (QL).

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