

Uncertainty assessment

In this work, uncertainty assessment was carried out in terms of combined uncertainty (u_{comb}) of the method for each analyte, which can be obtained as:

$$u_{comb} = \sqrt{u_{SD}^2 + u_{bias}^2}$$

where u_{SD} is the mean standard deviation from the reproducibility experiment of both CRMs ($n=4$), and u_{bias} the uncertainty associated to any source of bias contributing to the method bias. This includes the uncertainty associated to certified concentration values of the CRMs (u_{ref}), obtained as the mean u_{comb} of the certified concentration values for each analyte, and the method and laboratory bias assessed through the root mean square (RMS):

$$RMS = \sqrt{\frac{\sum_i bias_i^2}{2}}$$

Then, we have that:

$$u_{bias} = \sqrt{RMS^2 + u_{ref}^2}$$

| Analyte | u(ref) | RMS | u_{bias} | u_{SD} | u_{comb}(%) |
|---------|--------|-------|-------------------------|-----------------------|----------------------------|
| T | 2.1% | 5.1% | 5.5% | 1.8% | 5.8% |
| EpiT | 3.0% | 3.1% | 4.3% | 1.8% | 4.7% |
| AN | 1.5% | 23.1% | 23.2% | 10.3% | 25.4% |
| Etio | 1.9% | 2.6% | 3.2% | 5.2% | 6.1% |

Similarly, in the case of ratios the same procedure is applied except for the calculation of u_{ref} , which is obtained from the u_{comb} calculated by error propagation theory equations for ratios:

$$u_{comb} \left(\frac{A}{B} \right) = \sqrt{\left(\frac{u_{comb}(A)}{[A]} \right)^2 + \left(\frac{[A]}{[B]^2} \right)^2 \cdot u_{comb}(B)^2}$$

Then, u_{ref} of each ratio is readily obtained as the mean u_{comb} of the two CRMs and the rest of steps are identical as in the case of concentration determination.

Table S.1. Certified concentrations of steroids and their u_{comb} in the two CRMs.

| Analyte | NMIA MX002 | | NMIA MX005 | |
|---------|------------|------------|------------|------------|
| | C (ng/mL) | u_{comb} | C (ng/mL) | u_{comb} |
| T | 16.6 | 0.322 | 40.2 | 0.878 |
| EpiT | 18.3 | 0.591 | 10.74 | 0.291 |
| AN | 1262 | 19.31 | 1184 | 17.41 |
| Etio | 814 | 17.22 | 1290 | 20.50 |

Table S.2. Steroid ratios and their u_{comb} in the two CRMs.

| Ratio | NMIA MX002 | | | NMIA MX005 | | |
|---------|------------|-------------------|------------------------|------------|-------------------|------------------------|
| | Value | u_{comb} | $u_{\text{comb}} (\%)$ | Value | u_{comb} | $u_{\text{comb}} (\%)$ |
| T/EpiT | 0.907 | 0.034 | 3.8% | 3.743 | 0.130 | 3.5% |
| AN/T | 76.024 | 1.877 | 2.5% | 29.453 | 0.776 | 2.6% |
| AN/Etio | 1.550 | 0.040 | 2.6% | 0.918 | 0.020 | 2.2% |

Table S.3. Calculation of total method u_{comb} of the ratios.

| Ratio | U_{ref} | RMS | U_{bias} | U_{SD} | $u_{\text{comb}}(\%)$ |
|---------|------------------|-------|-------------------|-----------------|-----------------------|
| T/EpiT | 3.6% | 7.8% | 8.6% | 2.7% | 9.0% |
| AN/T | 2.6% | 19.0% | 19.2% | 8.8% | 21.1% |
| AN/Etio | 2.4% | 21.3% | 21.5% | 5.6% | 22.2% |

Table S.4. Analysis of 9 female urine samples by IPD.

| Sample | [T] | | | [EpiT] | | | [AN] | | | [Etio] | | |
|--------|-------------------|-------|------|-------------------|-------|------|-------------------|----|------|-------------------|-----|------|
| | Mean ¹ | SD | RSD | Mean ¹ | SD | RSD | Mean ¹ | SD | RSD | Mean ¹ | SD | RSD |
| 1 | 3.28 | 0.11 | 3% | 3.386 | 0.015 | 0.4% | 2090 | 41 | 2.0% | 1861 | 8 | 0.4% |
| 2 | 3.55 | 0.17 | 5% | 3.40 | 0.08 | 2.2% | 1760 | 27 | 1.5% | 2067 | 76 | 4% |
| 3 | 4.7 | 0.3 | 7% | 8.55 | 0.14 | 1.6% | 2204 | 72 | 3% | 3193 | 7 | 0.2% |
| 4 | 0.520 | 0.024 | 5% | 3.21 | 0.03 | 0.9% | 1736 | 54 | 3% | 1818 | 56 | 3% |
| 5 | 2.520 | 0.018 | 0.7% | 9.4 | 0.8 | 8% | 954 | 13 | 1.4% | 933 | 58 | 6% |
| 6 | 4.56 | 0.13 | 3% | 7.9 | 0.6 | 8% | 1422 | 48 | 3% | 1604 | 124 | 8% |
| 7 | 5.34 | 0.19 | 4% | 7.9 | 0.4 | 5% | 1203 | 46 | 4% | 2356 | 169 | 7% |
| 8 | 2.49 | 0.11 | 5% | 1.48 | 0.09 | 6% | 301 | 5 | 1.8% | 587 | 15 | 3% |
| 9 | 9.0 | 0.3 | 3% | 24.0 | 0.5 | 2.1% | 3363 | 85 | 3% | 2734 | 115 | 4% |

¹ ng/mL

Table S.5. Summary of figures of merit for some selected methods and steroids. All works make use of deuterated analogs as internal standard. Only the present work develops the isotope pattern deconvolution (IPD) mathematical tool to calculate concentration, thus providing one result per sample injection. The rest need to prepare calibration curve.

| Method and Matrix | Analytes ^a : concentration (ng/mL) | Recovery (%) | Intra-day CV (%) | Inter-day CV (%) | U _c (%) ^b | Calibration | LOQ (ng/mL) | Ref |
|-----------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|----------------------------------------------------------------------------------|-----------------------------------------------------------------------|---------------------------------|-------------|----------------------------|-----------|
| LC-(ID)MS/MS, enzymatic hydrolysis. Primate urine | T: 71.5; 135.6; 313.1 E: 10.8; 35.8; 101.2 A: 56.4; 96.5; 204.8 Etio: 142.4; 237.2; 418.7 | 77.4 – 84.6 88.6 – 103.0 93.7 – 98 89.8 – 91.7 | 8.0;6.5;5.9 8.7; 6.0; 5.6 9.4; 7.4; 7.4 8.5; 6.7; 6.0 | 9.9; 8.0; 10.7 11.5; 8.6; 10.8 8.3; 5.8; 10.1 8.9; 7.0; 10.9 | - | Needed | 0.3 0.3 1 1 | 25 |
| LC-(ID)MS/MS, enzymatic hydrolysis. Bovine urine | T: 1 | 90.7 | 6.6 | 14.7 | 27 | Needed | - | 29 |
| LC-(ID)MS/MS. Human urine | TG ^c : 1.25; 12; 100 EG ^c : 1.25; 12; 100 AG ^c : 25; 250; 2000 EtioG ^c : 25; 250; 2000 | 90 - 100 | 3; 3; 1 9; 1; 4 4; 4; 2 4; 4; 2 | - - - - | - | Needed | - - - - | 30 |
| GC-(ID)MS/MS, enzymatic hydrolysis, derivatization. Human urine | T: 250 E: 250 A: 5000 Etio: 5000 | 99 – 102 99 – 106 106 – 108 94 - 95 | 10 10 3 2 | 9 10 9 4 | - | Needed | 1 1 20 20 | 31 |
| CG-(ID)MS, enzymatic hydrolysis, derivatization. Human urine | T: 48; 128; 176 E: 6; 16; 22 | 84 – 99 ^d 82 – 99 ^d | 2.3-7.2; 2.7-3.8; 1.2-2.8 ^e 2.3-5.6; 1.5-6.1; 3.6-5.4 ^e | 7.2; 4.5; 4.3 5.7; 5.8; 6.7 | - | Needed | 5.3 1.1 | 32 |
| LC-(ID)MS/MS – IPD, enzymatic hydrolysis. Human urine-CRM | T: 16.6; 40.2 E: 18.3; 10.74 A: 1262; 1184 Etio: 814; 1290 | 93 – 98 102 – 108 75 – 79 95 - 103 | 1.8; 2.0 1.8; 3.0 1.4; 5 1.8; 2.4 | 2.4; 1.8 3.0; 3.0 8.0; 9.0 5.0; 4.0 | 5.8 4.7 25.4 6.1 | Not needed | 0.7 1.7 24.5 95.4 | This work |

^a Only results for T, E, A, and Etio (when available) are shown.

^b Total combined uncertainty

^c concentration expressed as free steroid although glucuronide metabolites are determined.

^d Calculated from relative error in reference 32

^e as stated in reference 32