Title: **Development of a LC-MS method to evaluate reprotoxic effects in JEG-3 model cell line.**

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The human placental cells JEG-3 are suitable to evaluate the endocrine disrupting potential of contaminants because those compounds may enter inside the cells and interfere with the metabolic routes involved in hormone synthesis. This has been usually determined by assessing the activity of aromatase by measuring the amount of tritiated water (^3H_2O) formed during the aromatization of ^{3}H-androstenedione into estrone (E1). However, the analysis of tritium has associated health, safety and environmental risks. In this context, the present works aimed to develop a greener and safer methodology for this purpose. For this, liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) was explored to determine trace levels of steroids in culture medium with high selectivity and sensitivity.

After optimizing LC-MS conditions for the simultaneous determination in a single analytical run of the steroids of interest, namely estrone (E1), β-estradiol (E2), estriol (E3), testosterone (T) and androstenedione (A4), the performance of different extraction approaches (i.e., protein precipitation, liquid-liquid extraction, and TurboFlow™ clean-up) were also evaluated. TurboFlow™ technology provided the best results in terms of accuracy, precision and method sensitivity. Thus, this technology was fully validated for the analysis of target steroids in cell culture medium.

The fully automated method developed was linear (r^2 >0.999), accurate and precise, with relative recoveries between 77 and 98% and RSD values <5% (but in the case of E3 at the low concentration level an RSD value <16%). Trueness values were between 92 and 110% and matrix effects were not relevant (±25%). The methodology allowed detecting the steroids of interest at concentrations in the low pg/mL range (LOD range from 0.002 to 0.005 ng/mL). Additional advantages of this method are minimum sample manipulation (internal standard (IS)

addition), the requirement of low sample volumes (200 µL) and high sample throughput (20 min of analysis time). High accuracy and precision are essential to obtain a reliable detection of the small changes that chemical exposure may produce in steroid profile of JEG-3 cells (variations from -29 to 40% in the present study).

Application of the method confirmed that DHEA addition enhanced the metabolic pathways of steroids in JEG-3 cells, and that estrogenic and androgenic effects can be detected after evaluation of the estrogens and androgens balance. Overall, nonylphenol (NP) had an estrogenic effect (estrogens increase and androgens/estrogens decrease) on the metabolic pathways of JEG-3 cells and concentrations of bromoacetic acid (BAA), iodoacetic acid (IAA), and tribromoacetic acid (TBAA) at the low µM range had androgenic effects on the metabolic pathways of the JEG-3 cells (androgens/estrogens balance increased). Further experiments need to be conducted to confirm the effects observed.