INTRODUCTION
Increased exposure to estrogens is associated with increased risk of breast cancer. Clinical researchers have postulated that certain estrogen metabolites are genotoxic, while other estrogen metabolites are anti-proliferative. For this reason, there is a growing interest in developing accurate and sensitive methods to enable the measurement of estrogens and their methoxy- and hydroxy- metabolites in serum. In this work we have developed an LC-MS/MS method for the measurement of Estrone (E1), Estradiol (E2), Estriol (E3), 2-Methoxyestrone (2ME1), 2-Methoxyestradiol (2ME2), 16α-Hydroxyestrone (16αHE1), 4-Methoxyestrone (4ME1), and 4-Methoxyestradiol (4ME2) in serum. The analysis of these compounds by LC-MS/MS is challenging due to the high number of observed interferences, and the need to chromatographically resolve the 2-methoxy and 4-methoxy metabolites.

MATERIALS AND METHODS
To measure unconjugated estrogens and estrogen metabolites, 400uL of serum was aliquoted into 1.5mL polypropylene microcentrifuge tubes, to which 10uL of internal standard solution was added. Liquid-liquid extraction of the estrogens and estrogen metabolites was accomplished by adding 1 mL of MTBE to each tube, and vortex mixing for 60 seconds. The samples were then centrifuged at 10,000 rpm for 10 minutes, and 900uL of the upper organic layer was removed and transferred into a clean 1.5 mL microcentrifuge tube. The samples were dried down under nitrogen gas at room temperature, and finally reconstituted using 100uL of 70:30 v/v water:methanol. 70uL of the prepared samples were injected onto the LC-MS/MS system.

HPLC method
- Phenomenex Kinetex Phenyl Hexyl column (50x3.0mm, 2.6um, 100A)
- Oven temperature: 50C
- Flow rate: 400uL/minute
- Mobile Phase A: Water, 0.2mM NH₄F
- Mobile Phase B: Methanol

MS/MS method
- SCIEX Triple Quad™ 6500+ system
- Negative electrospray ionization
- Multiple Reaction Monitoring (MRM) mode
- 2 x MRM’s per compound
- 1 x MRM per internal standard

RESULTS
Calibrators were prepared in double-charcoal stripped serum. Interferences were observed in the MRM chromatograms, however these were adequately separated chromatographically. The isomers 2-Methoxyestrone and 4-Methoxyestrone were not completely baseline separated, however they were sufficiently resolved to enable independent quantitation at the relevant concentration levels.

CONCLUSIONS
Without resorting to derivatization of the target analytes, we have developed a sensitive LC-MS/MS method for the analysis of a panel of estrogens and metabolites in serum: Estrone (E1), Estradiol (E2), Estriol (E3), 2-Methoxyestrone (2ME1), 2-Methoxyestradiol (2ME2), 16α-Hydroxyestrone (16αHE1), 4-Methoxyestrone (4ME1), and 4-Methoxyestradiol (4ME2). To evaluate the method, several anonymized serum samples were analyzed. The method enabled an LOQ of 1.0 pg/mL or better for all of the analytes. A run-time of 12 minutes was required in order to ensure adequate chromatographic separation of the 2-Methoxy and 4-Methoxy metabolites of estrone and estradiol.

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