**OVERVIEW**

- A negative mode assay for estradiol quantification using the ionKey/MS™ system.
- Post-column addition of modifiers to facilitate deprotonation and enhance the ionization process.
- Positive-negative polarity switching allows analysis of estradiol and testosterone in a single run.

**INTRODUCTION**

Measurement of estradiol is critical in understanding human biology and health\(^1\). However, achieving the required level of sensitivity in positive ESI-MS is challenging due to the non-polar structure and low proton affinity of estradiol. Estradiol derivatization\(^2\) can compromise the assay specificity and the lengthy and delicate sample preparation is less suitable for large-scale studies.

There are numerous applications where a basic pH is necessary to promote ionization. However, while stationary phases specifically designed for higher pH operation are available, developing a suitable LC method may be inconvenient or even impossible. Using post-column addition, we can adjust the pH without affecting the chromatographic separation.

Estradiol is well suited for reversed phase chromatography without any mobile phase modifiers. However, its negative electrospray ionization is challenging and can be improved by addition of a suitable modifier. Since the pKa value of estradiol is 10.7, adjusting the eluent pH with a volatile base to facilitate deprotonation is advantageous. The ionization process can be further improved by post-column addition of isopropanol to lower the surface tension of the LC eluent.

**METHODS**

The experiments were performed using an ionKey/MS™ system comprised of an ACQUITY UPLC® M-Class system in combination with a Xevo® TQ-S mass spectrometer. Preliminary trapping was achieved using a Symmetry C8, 300 µm x 50 mm trap column. The post-column addition (PCA) iKey\(^3\) contains two channels. An empty channel used for modifier addition was tee in after the separation channel. The effluents from the separation channel and the post-column addition (PCA) channel are merged and collected at the inlet of the emitter.

**RESULTS**

In this study, we present a negative electrospray ionization method for detection and accurate quantification of estradiol that provides high reproducibility of analysis with enhanced sensitivity without the need for derivatization. The use of a 20 trap-and-elute configuration enabled the system to handle relatively large volume injections of 20 µl while maintaining excellent peak shape, further enhancing sensitivity and system flexibility.

In this method, isopropanol was introduced post-column to lower the surface tension of the LC eluent and enhance the ionization process and consequently the sensitivity. The ammonium hydroxide with its high gas-phase basicity facilitates deprotonation and is also known to be effective in improving sensitivity for small molecules in negative mode LC-MS.

The standard calibration curve was linear between 1 and 500 pg/ml with a R\(^2\) value of 0.99. The lower limit of quantitation was <1 pg/ml (CV <9%).

The same LC conditions were applied for the separation of a mixture of three estrogens: Estrone (E1), Estradiol (E2) and Estriol (E3). The selectivity of the HSS T3 material allowed an adequate resolution of the three analytes with a total run time of 12 minutes (Figure 5).

The method was also successfully tested for the simultaneous analysis of estradiol and testosterone using positive/negative ion switching.

**CONCLUSION**

- A highly sensitive and robust method for low-level quantitation of underivatized Estradiol has been developed.
- A PCA Key was used to assist the ionization process by post-column addition of ammonium hydroxide and isopropanol. The ammonium hydroxide facilitates deprotonation and the isopropanol lowers the surface tension of the LC eluent. The combination of these modifiers enhances the ionization process and consequently the sensitivity.

**REFERENCES**


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**Figure 1. Steroids structures.**

- Estradiol
- Estrone
- Estriol
- Testosterone

**Figure 2. Post-column addition PCA iKey.** The analytical channel is connected to the upper port and the post-column addition channel is connected to the lower port.

One concern over post-column addition of a modifier is the prospect of extra-column band broadening. The setup used in these experiments is design to minimize any dead volume. When compared with a regular iKey, the average peak widths at 10% for a 0.5uL/min post-column addition flow rate were similar: 3.96 sec for (the PCA iKey) versus 4.02 sec for (the regular iKey). (A gradient of 1.7% B/min)

**Figure 3. Quantification of various levels of estradiol by LC/MS**

**Figure 4. Standard curve of estradiol.**

**Figure 5. SRM chromatograms of Estrone (E1), Estradiol (E2) and Estriol (E3).** Standard solution of 50 pg/ml each in 25% methanol.

**Figure 6. SRM chromatograms of estradiol (-ve mode; 0.5 ng/ml) and testosterone (+ve mode; 5 ng/ml).**