CLLEVERT Technology as a novel extraction approach for the determination of estrogenic hormones in human plasma by LC/MS

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Introduction:

The quantitative determination of estrogenic hormones, particularly estrone (E1) and estradiol (E2), in human plasma poses a significant analytical challenge due to their very low physiological concentrations, typically in the range of several tens to hundreds of pg/mL. In addition, the complex composition of the biological matrix further complicates accurate quantification, as matrix components may interfere with analyte detection and ionization efficiency in mass spectrometry.

To achieve reliable results, efficient sample cleanup prior to LC–MS analysis is essential. Solid-phase extraction (SPE) is the most commonly employed approach for plasma purification; however, it is often time-consuming and requires relatively large volumes of organic solvents, which increases both the cost and environmental impact of the analysis.

The present study investigates the applicability of a novel technology, **CLLEVERT – Capillary Liquid-Liquid Extraction Insert (CLLEVERT insert)**, as a simple, rapid, and solvent-efficient alternative for plasma sample preparation prior to LC–MS determination of estrogenic hormones E1 and E2.

The **CLLEVERT insert** technique is based on liquid–liquid extraction (LLE), followed by phase separation using a patented insert specially designed for extraction of two immiscible phases. The insert (Figure 1, page 2) features a U-shaped bottom with an ultra-fine orifice and a capillary outlet engineered to be either hydrophobic or hydrophilic. This design enables instantaneous and precise separation of two immiscible liquid phases, offering a streamlined benchtop solution for efficient sample preparation.

Figure 1: CLLEVERT – Capillary Liquid-Liquid Extraction Insert

An important advantage of the **CLLEVERT insert** is its compatibility with standard 96-well microtube racks, allowing the inserts to be positioned above 96-well deep-well collection plates (Figures 2 and 3). This configuration enables simultaneous processing of multiple samples and direct collection of the lower phase into the wells. No additional equipment, such as vacuum manifolds or positive-pressure processors typically used for SPE methods, is required.

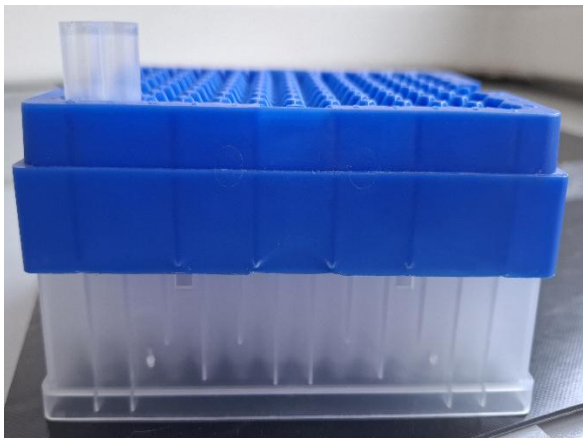

Figure 2: CLLEVERT insert in a 96-well deep-well plate.

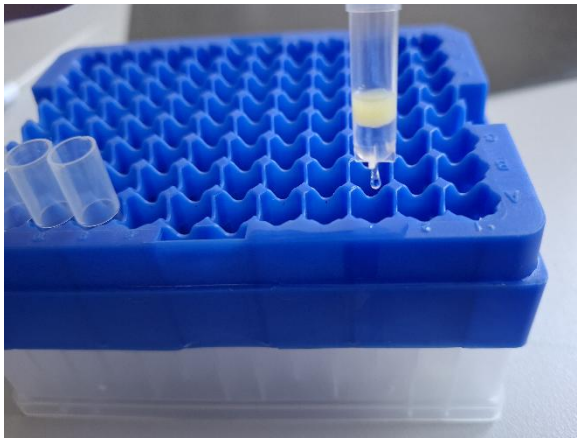

Figure 3: CLLEVERT insert - the separation of the plasma layer from the organic solvent containing the extracted analytes.

Materials and Method:

Two types of biological matrices were used for testing. The first matrix consisted of human plasma containing physiological endogenous levels of estrone (E1) and estradiol (E2), obtained from a healthy volunteer. The second matrix was commercially available purified human serum free of hormones (DC Mass Spect Gold® Serum 4000, Merck).

Both matrices were processed in two variants:

I) Spiked samples, prepared by adding a solution of E1 and E2 to achieve a final concentration of 200 pg/mL, along with isotopically labelled internal standards (cE1 and cE2).
II) Blank samples, processed without the addition of these solutions.

Procedure:

1. For each extraction, 200 µL of the biological matrix was mixed with 300 µL of chloroform in an Eppendorf microtube.
2. The mixture was briefly vortexed and then transferred into the **CLLEVERT insert** cat. nm. CLLEVERT HPB A2-900-10 (Medirekt Partner, Koprivnice, Czechia) (Figures 2 and 3).
3. Within the insert, the plasma (upper layer) was efficiently separated from the organic solvent containing the extracted analytes (bottom layer).
4. After elution of the organic layer into a 96-well plate, the eluate was evaporated to dryness in a water bath at 45 °C.
5. The residue was then dissolved in a 25 % acetonitrile solution and subsequently derivatized by the addition of a pyridine-3-sulfonyl chloride solution in acetonitrile. The total volume of the dissolved and derivatized residue was 200µL.
6. The prepared samples were analysed using a two-dimensional liquid chromatography–triple quadrupole tandem mass spectrometer (Quantiva, Thermo Fisher Scientific).

Chromatographic conditions:

- HPLC column 1: Kinetex Evo C18, 50 x 4.6 mm, 5 µm, Phenomenex
- HPLC column 2: Kinetex Biphenyl, 100 x 3 mm, 5 µm, Phenomenex
- Mobile phase: methanol / 50 mM NH_4HCO_3 / H_2O
- Injection: 40 µL

Mass spectrometer setup:

Analyte	Parent mass	Center mass	Peak width Q1	Peak width Q3	CE
E1	412	348	0.4	0.7	30
cE1	415	351	0.4	0.7	30
E2	414	350	0.4	0.7	32
cE2	417	353	0.4	0.7	32

Parameters of the H-ESI source:

Polarity: positive, Spray voltage: 2400 V, Collision gas pressure: 1.5 mTorr, Capillary temperature: 400 °C, Vaporizer temperature: 500 °C, Sheath gas: 50 units, Aux gas: 10 units

Results and Discussion:

The conducted experiments successfully confirmed the feasibility of employing **CLLEVERT inserts** for sample preparation in the determination of estrone (E1) and estradiol (E2) using an LC/MS system.

- No interfering substances originating from the sample matrix were detected in the chromatograms, thereby demonstrating the high selectivity of the method.
- Moreover, no extractable compounds leaching from the **CLLEVERT insert** material were observed, indicating the chemical compatibility and inertness of the inserts with respect to the method used.The retention times of estrone and estradiol obtained in the chromatograms were consistent with those observed when applying the same LC/MS method routinely used in conjunction with solid-phase extraction (SPE) at Quinta Analytica.

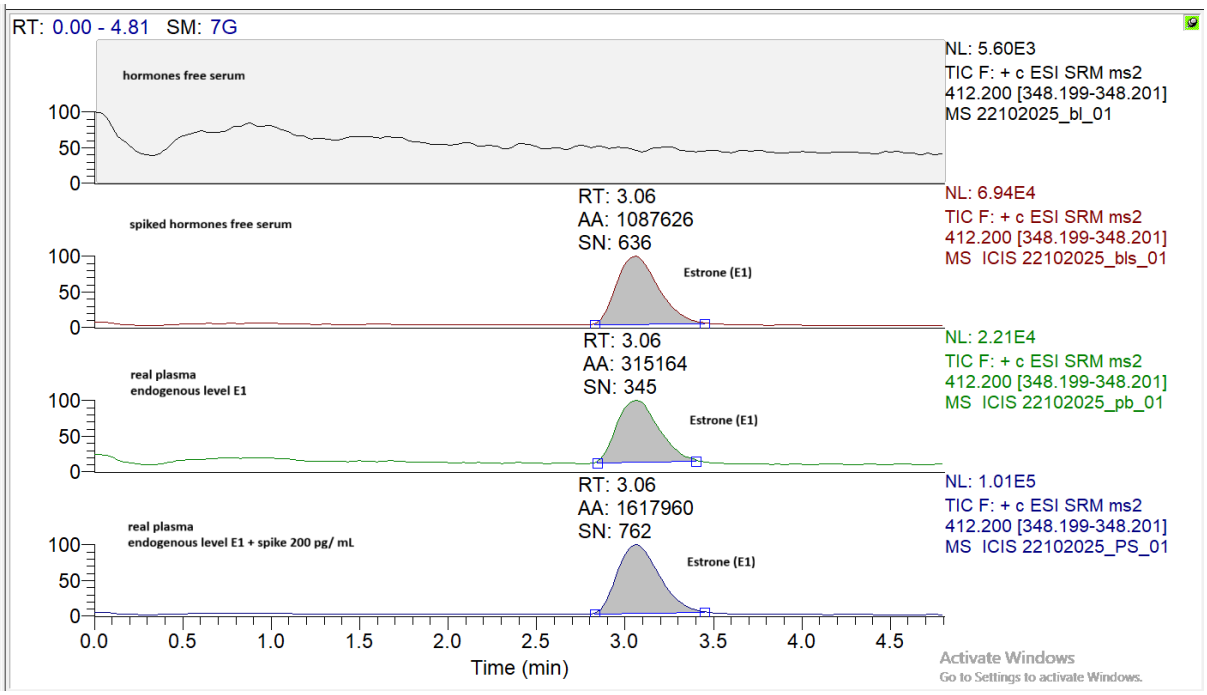

Figure 4: LC/MS chromatograms of estrone (E1) in purified human serum, serum spiked with E1, plasma with endogenous E1, and plasma with endogenous E1 spiked.

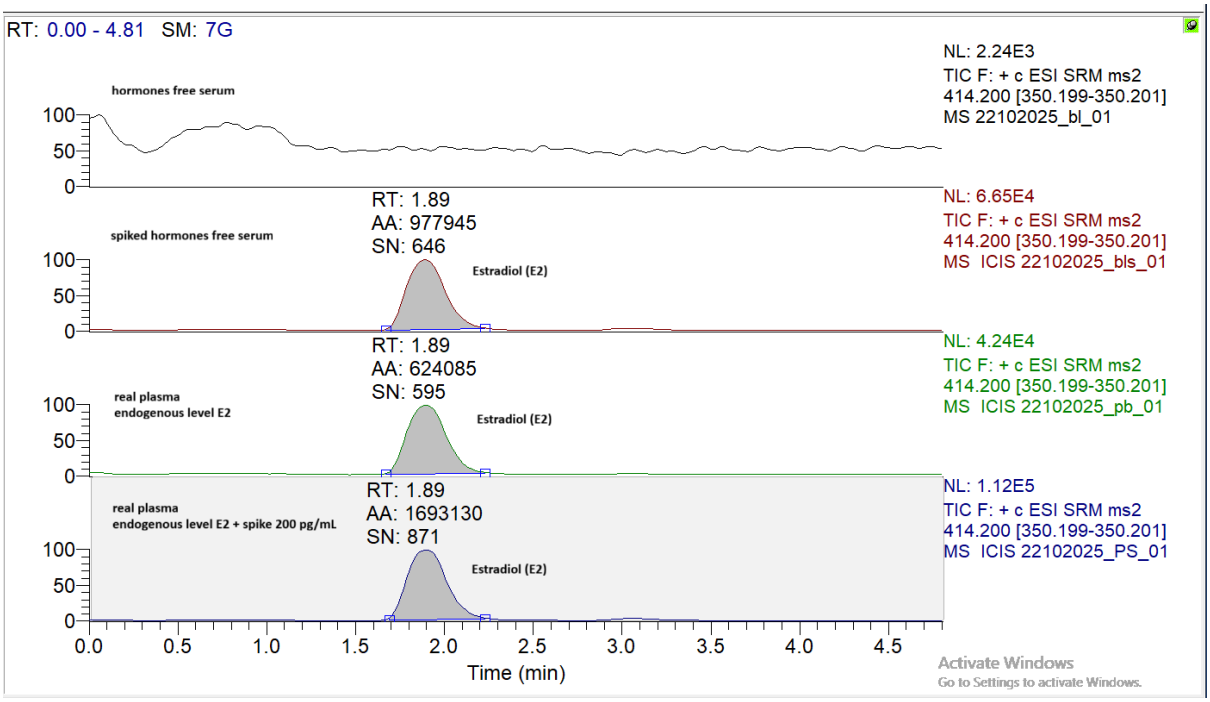

Figure 5: LC/MS chromatograms of estradiol (E2) in purified human serum, serum spiked with E2, plasma with endogenous E2, and plasma with endogenous E2 spiked.

Conclusion:

The results of the present experiment confirm applicability of **CLLEVERT inserts (CLLEVERT HPB A2-900-10)** for sample preparation in the determination of estrogenic hormones in plasma. The use of **CLLEVERT inserts** represents a novel and promising extraction approach, which, in many applications, has the potential to replace traditional SPE methods due to its simplicity, efficiency, and compatibility with LC/MS analysis.

All experiments were performed at **Quinta Analytica s.r.o., Bioanalytical Department, Pražská 1486/18c, Prague 102 00, Czech Republic**.

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