Extraction of Thyroid Hormones, T3, rT3 and T4 from Serum Using EVOLUTE® EXPRESS AX Plates Prior to LC-MS/MS Analysis

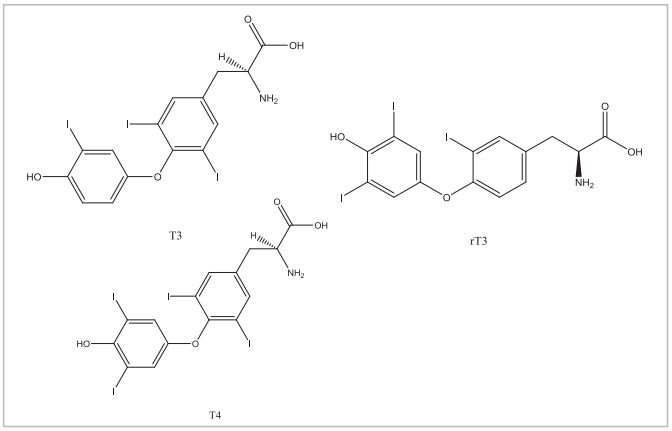

Figure 1. Structures of T3, rT3 and T4

Introduction

This application note describes a polymer based-based strong anion exchange mixed-mode SPE protocol for the extraction of T3, rT3 and T4 from serum prior to LC-MS/MS analysis.

The method described in this application note demonstrates selective extraction of the thyroid hormones T3, rT3 and T4 from serum. 500 µL of serum was extracted using the EVOLUTE EXPRESS AX 30mg fixed well plate format. High reproducible recoveries and extremely low phospholipid content were observed, demonstrating limits of quantitation of 50 pg/mL.

Analytes

Tri-iodothyronine (T3), reverse Tri-iodothyronine (rT3) and Thyroxine (T4).

Sample Preparation Procedure

Format:	**EVOLUTE® EXPRESS AX 30 mg Fixed Well Plate, part number 603-0030-PX01**
Serum Pre-treatment:	Take 500 µL of serum and spike with internal standard (10 µL of a 25 ng/mL solution). Add 500 µL of a mixture of 5% NH_4OH (aq) and acetonitrile (MeCN) (50/50, (v/v)) and vortex mix thoroughly.

Solid Phase Extraction

Plate Conditioning:	Condition each well with methanol (1 mL)
Plate Equilibration:	Equilibrate each well with water (1 mL)
Sample Loading:	Load pre-treated sample (1 mL) to the 96 well-plate
Wash 1:	Elute interferences with 5% NH_4OH (aq)/MeCN (50/50, (v/v), 1 mL)
Wash 2:	Elute interferences with 5% NH_4OH (aq) (1 mL)
Wash 3:	Elute interferences with Methanol (1 mL)
Wash 4:	Elute interferences with 2% formic acid in dichloromethane (1 mL)
Elution:	Elute analytes with 5% formic acid in methanol (500 µL) into a collection plate containing 2 µL of ethylene glycol in each well
Post Elution:	Evaporate at 40 °C in a stream of air or nitrogen using a SPE Dry. **Note:** The use of ethylene glycol helps to minimize non-specific binding to the plastic collection plate by avoiding complete drying during the evaporation procedure.
Reconstitution:	Reconstitute in 50/50 (v/v) UPLC Mobile Phase A/B (100 µL)

UPLC Conditions

Instrument: Waters ACQUITY UPLC

Column: Phenomenex Kinetex C18 UHPLC column (1.7 µ, 100 x 2.1 mm id)

Mobile Phase: A: 2 mM ammonium acetate/0.1% formic acid (aq)
B: 2 mM ammonium acetate/0.1% formic acid/MeOH

Flow Rate: 0.3 mL/min

Table 1. Gradient Conditions

Time	% A	% B	Curve
0	50	50	1
2.5	10	90	6
2.6	50	50	11

Curve 11: Conditions in line initiated immediately once time passed. i.e. 50:50 resumed at 2.6 minutes

Curve 6: Linear Gradient

Injection Volume: 15 µL (partial loop with overfill)

Sample Temperature: 20 °C

Columns Temperature: 40 °C

Mass Spectrometry Conditions

Ions were selected in order to achieve maximum sensitivity, and the MS operated in positive ion polarity mode, using multiple reaction monitoring.

Instrument: Premier XE triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis

Desolvation Temperature: 450 °C

Ion Source Temperature: 150 °C

Collision Cell Pressure: 3.76 e^{-3} mbar

Positive ions acquired in the multiple reaction monitoring (MRM) mode:

Table 2. MRM Conditions (Qualifier ion details shown in parenthesis)

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
T3	651.8 > 605.8 (651.8 > 479.0)	35 (35)	21 (35)
rT3	651.8 > 605.8 (651.8 > 508.0)	35 (35)	21 (20)
T3/rT3-d6 ISTD	657.9 > 611.9	35	21
T4	777.8 > 731.8 (777.8 > 351.2)	40 (40)	25 (45)

Results

Good chromatographic separation of T3/rT3 and T4 was achieved in less than 2.5 minutes, as shown in **Figure 2.**

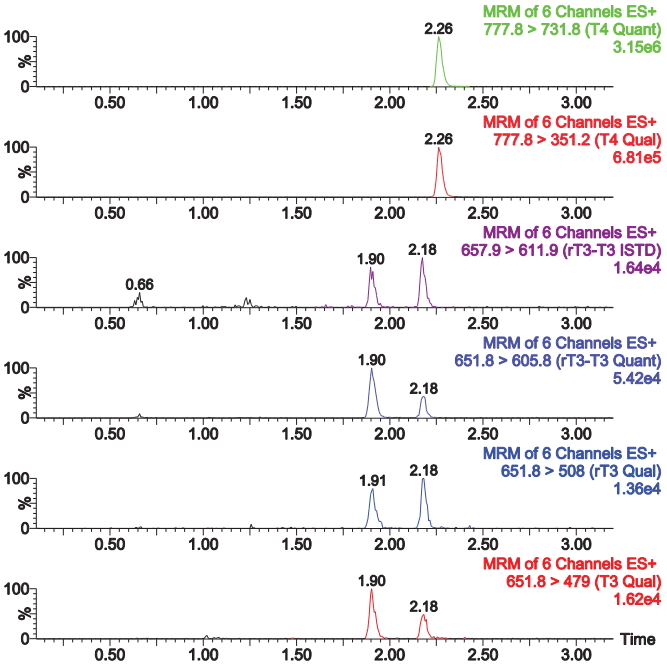

Figure 2. Chromatographic separation of T3, rT3 and T4 from spiked serum at 100 pg/mL

Recovery

Stripped serum was spiked at various levels from 50-5000 pg/mL. High reproducible recoveries › 80% with corresponding RSDs ‹ 10% were demonstrated. Typical recovery data is shown in **Figure 3**.

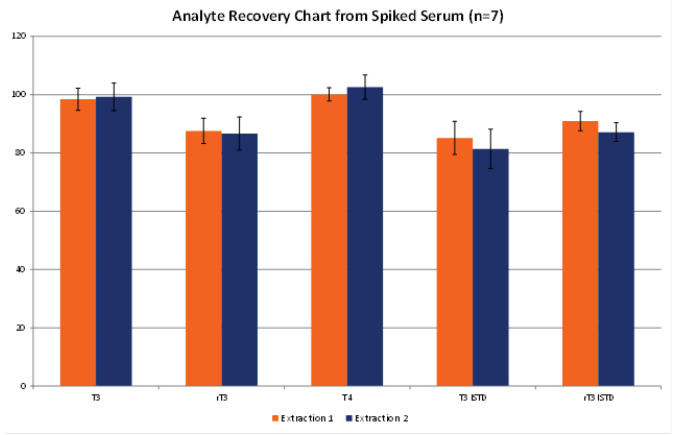

Figure 3. Recovery profile for Thyroid hormones extracted at 2 ng/mL

Calibration Curves

Calibration curves were generated using stripped serum spiked at concentrations from 50-5000 pg/mL. Good coefficients of determination were obtained for T3 and rT3 (r^2 › 0.99). Endogenous levels of T4 contributing towards a substantial intercept affected calibration curve performance, demonstrating r^2 values of only 0.97.

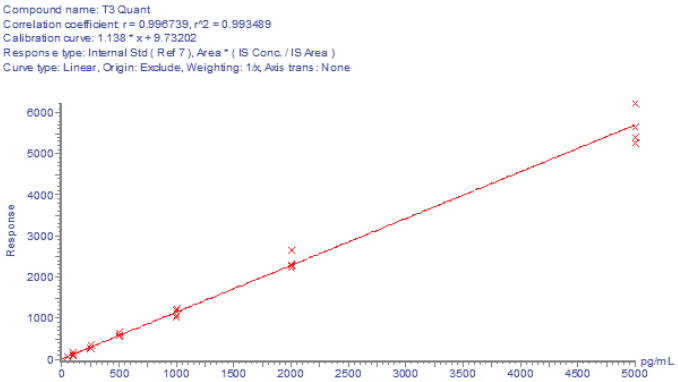

Figure 4. Spiked serum calibration curve demonstrating T3 linearity from 50-5000 pg/mL (n=4).

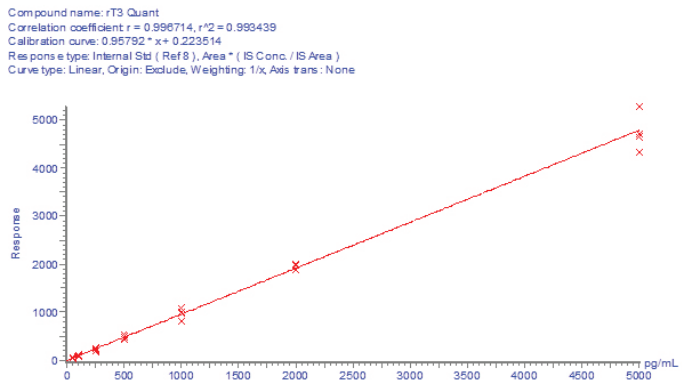

Figure 5. Spiked serum calibration curve demonstrating rT3 linearity from 50-5000 pg/mL (n=4).

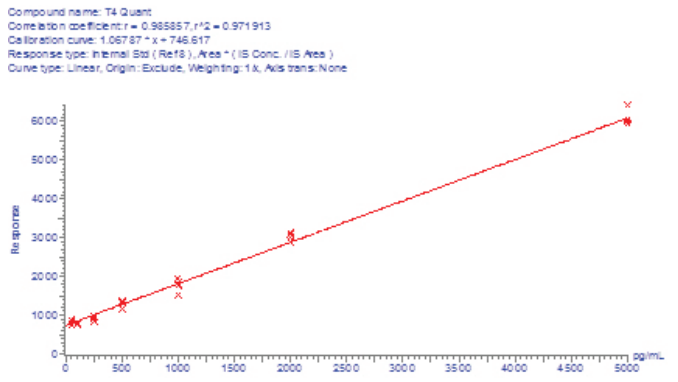

Figure 6. Calibration Curve demonstrating T4 linearity from 50-5000 pg/mL.

Phospholipid Removal

The robust washing protocols that are possible when using EVOLUTE® EXPRESS AX plates led to extremely clean extracts with very low matrix effects. Residual phospholipids were investigated to provide an indication of extract cleanliness. We investigated the most abundant phospholipids (selected from full scan, SIR and precursor ion scanning experiments) using MRM transitions monitoring the common 184 product ion. Figure 8 demonstrates phospholipid content comparing protein precipitated serum, solvent blank and the final extraction protocol.

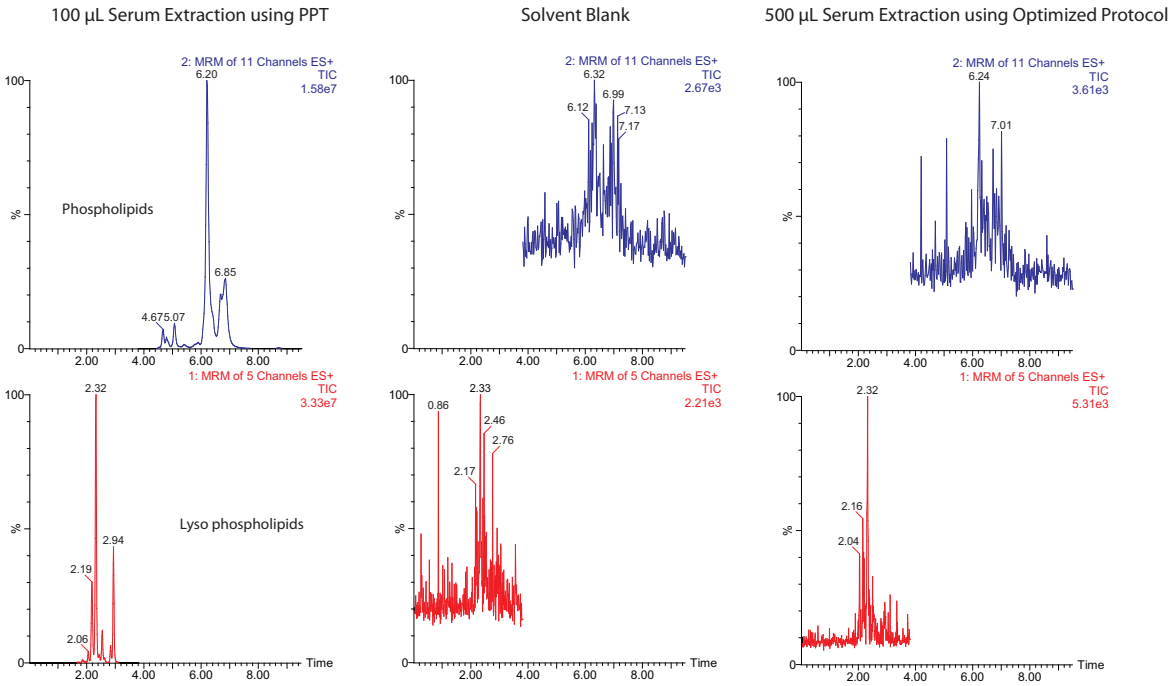

Figure 7. MRM TICs comparing phospholipid content

Additional Notes

1. **Buffer Preparation**
   a. 5% NH_4OH (aq): Take 95 mL of H_2O and add 5 mL of NH_4OH (28–30% concentration).
   b. 5% NH_4OH (aq)/MeCN (50/50 (v/v)): Take 50 mL of 5% NH_4OH (aq) and add 50 mL of MeCN.
   c. 2 % formic acid in dichloromethane: Take 98 mL of DCM and add 2 mL of formic acid (98% concentration).
   d. 5 % formic acid in methanol: Take 95 mL of MeOH and add 5 mL of formic acid (98% concentration).
   e. 2 mM ammonium acetate/0.1% formic acid (aq): Weigh 0.15416 g and dissolve in H_2O. Add 1 mL of formic acid and make up to 1 L in H2O.
   f. 2 mM ammonium acetate/0.1% formic acid/MeOH: Weigh 0.15416 g and dissolve in MeOH. Add 1 mL of formic acid and make up to 1 L in MeOH.
2. **Processing Conditions**
   a. Positive Pressure: Process at approximately 2–3 psi.
   b. Vacuum Processing: Process at approximately -0.2 bar.
3. **Conditioning and equilibration steps**
   a. EVOLUTE EXPRESS 96-well plates may be used without conditioning and equilibration steps. Evaluate the requirement for these steps during method development.

Ordering Information

Part Number	Description	Quantity
603-0030-PX01	EVOLUTE® EXPRESS AX 30 mg Fixed Well Plate	1
121-9600	Biotage® VacMaster™-96 Sample Processing Manifold	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold 96 Well	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1

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