

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

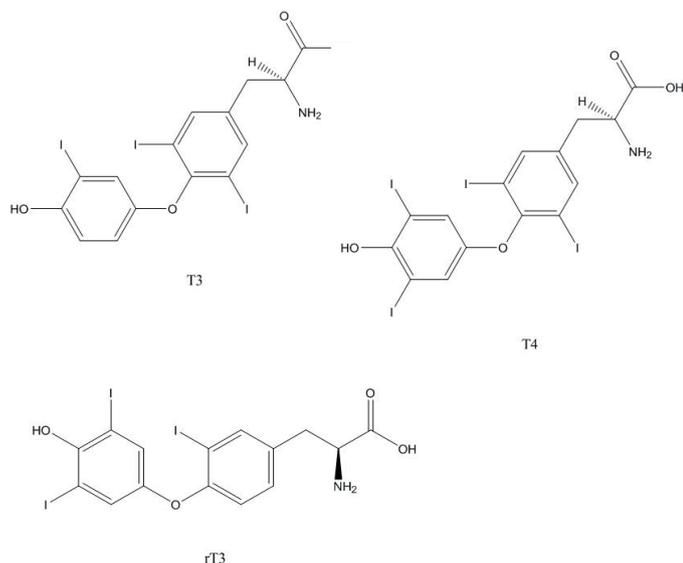


Figure 1. Structures of T₃, rT₃ and T₄.

Introduction

This application note describes a polymer-based 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. 200 µL of serum was extracted using the EVOLUTE® EXPRESS AX 30 mg fixed well plate format. High reproducible recoveries and low phospholipid content were observed, demonstrating limits of quantitation < 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

To serum (200 µL) add internal standard (D₆-T₃/rT₃, 10 µL of 25 ng/mL solution).

Add a further 100 µL of a mixture of citric acid, ascorbic acid and DL-dithiothreitol (25 mg/mL) and vortex mix thoroughly.

Note: the use of these stabilizers prevents conversion of T₄ to T₃ and rT₃ during extraction.

Solid Phase Extraction

Plate Conditioning

Condition each well with MeOH (1 mL).

Plate Equilibration

Equilibrate each well with H₂O (1 mL).

Sample Loading

Load the entire volume of pre-treated sample.

Wash 1

Elute interferences with 50 mM NH₄OAc buffer, pH 9 aq (1 mL)

Wash 2

Elute interferences with MeOH (1 mL).

Wash 3

Elute interferences with 2% formic acid in DCM (v/v) (1 mL).

Elution

Elute analytes with MeOH (500 µL).

(Optional) Add 2 µL of ethylene glycol to the collection plate and mix prior to evaporation.

Post Elution

Evaporate at 40 °C in a stream of air or nitrogen using a Biotage® 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 the sample with H₂O/MeOH (50/50 (v/v), 150 µL).

UPLC Conditions

Instrument

Waters ACQUITY I-Class

Column

ACE EXCEL2 C18-AR UHPLC column (2 μ, 100 x 2.1 mm id)

Mobile Phase

A: 2 mM ammonium acetate/o.1% formic acid (aq)

B: 2 mM ammonium acetate/o.1% formic acid/MeOH

Flow Rate

0.4 mL/min.

Table 1. Gradient Conditions.

Time	% A	% B	Curve
0	40	60	1
3	23	77	6
3.1	40	60	1

Curve 1: Conditions in subsequent line initiated immediately once time passed. i.e. 40:60 resumed at 3 minutes.

Curve 6: Linear Gradient

Injection Volume

10 μL (partial loop with overflow)

Sample Temperature

10 °C

Column Temperature

40 °C

Mass Spectrometry Conditions

Instrument

Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature

500 °C

Ion Source Temperature

150 °C

Collision Cell Pressure Temperature

3.7 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	50	22
	(651.8 > 507.8)		(22)
	(651.8 > 478.9)		(35)
rT3	651.8 > 605.8	50	22
	(651.8 > 507.8)		(22)
	(651.8 > 478.9)		(35)
T3/rT3-d6 ISTD	657.8 > 611.8	50	22
T4	777.7 > 731.7	50	25
	(777.7 > 351.0)		(45)
	(777.7 > 633.8)		(23)

Results

Good chromatographic separation of T₃/rT₃ and T₄ was achieved in less than 3 minutes, as shown in Figure 2

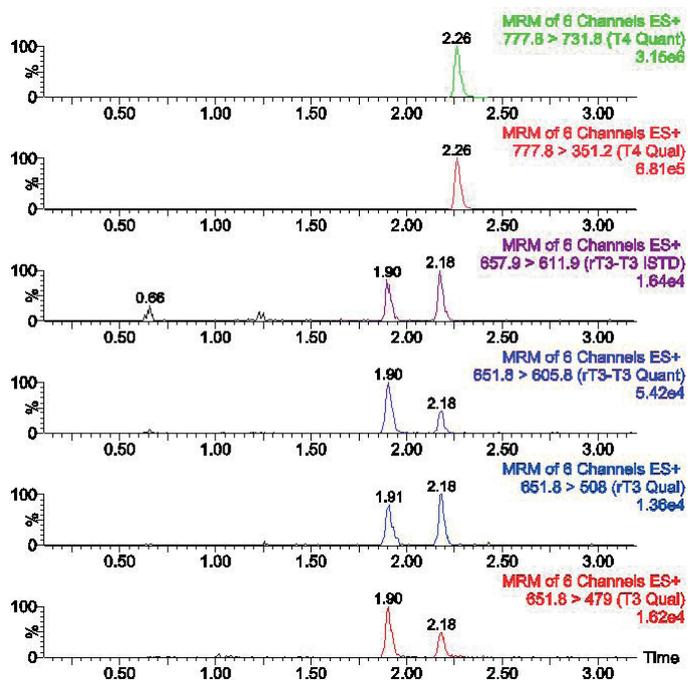


Figure 2. Chromatographic separation of T₃, rT₃ and T₄ from spiked serum at 50 pg/mL.

Recovery

Stripped serum was spiked at various levels from 25–2000 pg/mL. High reproducible recoveries > 85% with corresponding RSDs < 10% were demonstrated. Typical recovery data at 2 ng/mL is shown in Figure 3.

Calibration Curves

Calibration curves were generated using stripped serum spiked at concentrations from 25–1000 pg/mL. Good coefficients of determination were obtained for T3 and rT3 ($r^2 > 0.99$). Residual endogenous levels of T4 contributing towards a substantial intercept affected calibration curve performance.

Calibration curves were run before and after multiple serum samples to demonstrate robustness of quantification using this method.

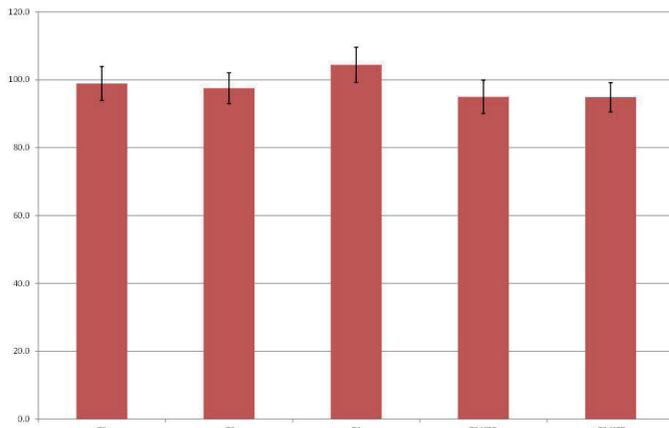


Figure 3. Recovery profile for thyroid hormones extracted at 2 ng/mL.

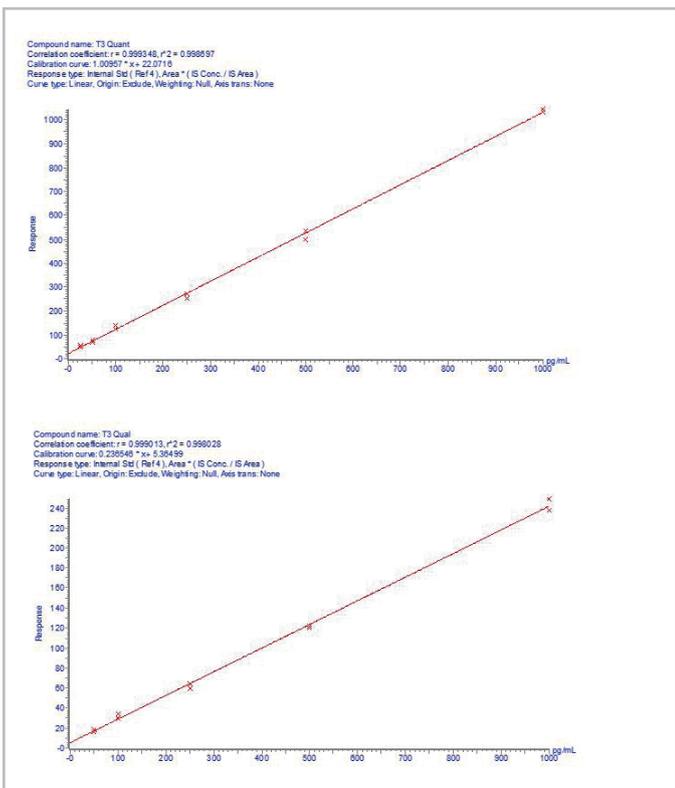


Figure 4. Spiked serum calibration curve demonstrating T3 linearity from 25–1000 pg/mL (curves run before and after real samples).

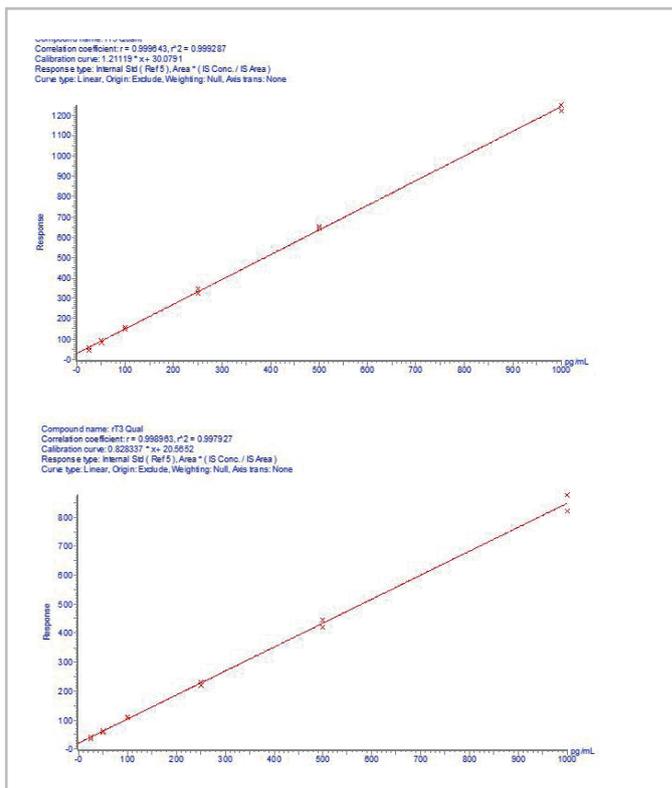


Figure 5. Spiked serum calibration curve demonstrating rT3 linearity from 25–1000 pg/mL (curves run before and after real samples).

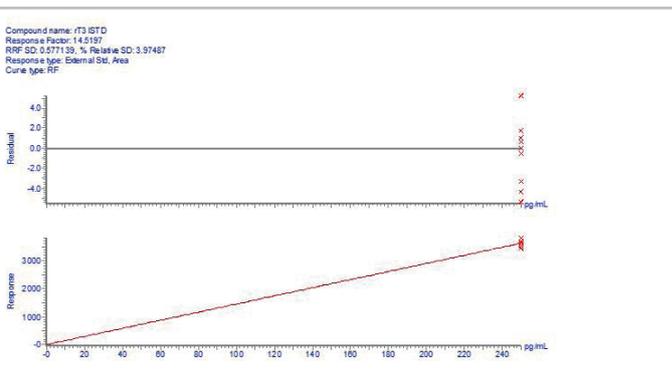
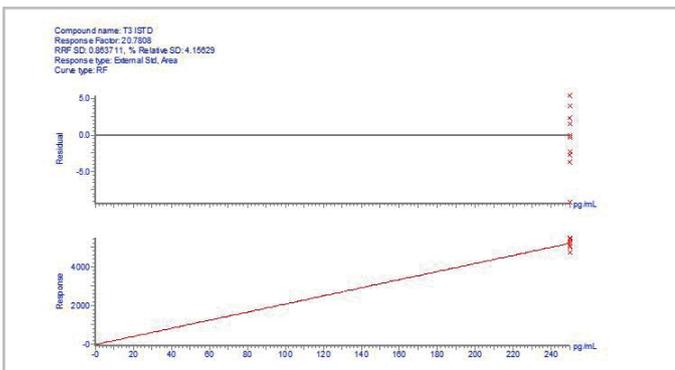


Figure 6. Plots demonstrating T3 and rT3 ISTD performance over calibration curves.

Phospholipid Removal

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 shows phospholipid content comparing protein precipitated serum, and serum extracted using the optimised extraction protocol.

The complete removal of matrix phospholipids improves assay robustness.

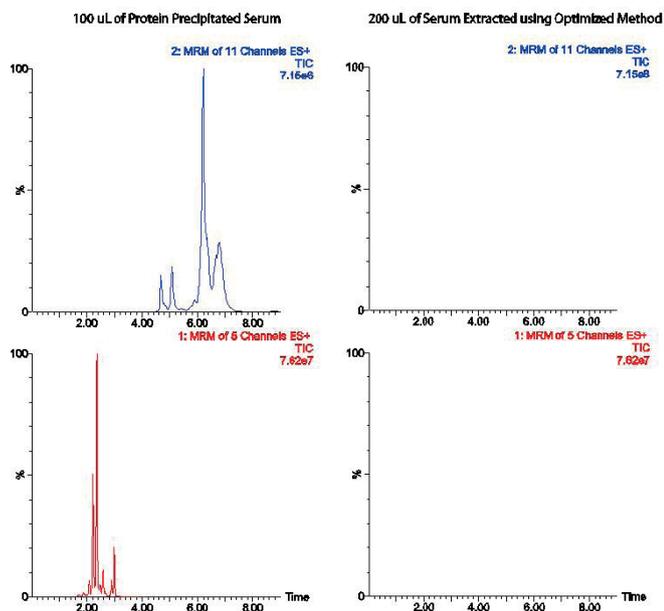


Figure 7. MRM TICs comparing phospholipid content (precipitated serum sample (L) and extracted serum sample (R))

Additional Notes

1. Stabilizers are added to the sample at the pre-treatment stage prior to extraction to prevent conversion of T₄ into T₃/rT₃ during the extraction procedure, which is otherwise possible under various pH conditions.
2. High pH conditions (pH 9) are used in wash 1 to ensure protonation of the carboxylic acid group (and suppress ionization of the amino group) during wash steps and therefore maintain full retention of the analytes on the EVOLUTE® EXPRESS AX strong anion exchange sorbent during the subsequent methanol wash.
3. An additional wash step using acidified dichloromethane (DCM) is incorporated to further clean up the extract, and improve robustness and reproducibility of the analytical method. Acid was required to give optimum matrix removal, and the use of DCM prevented analyte loss during this wash, despite the change in pH conditions deprotonating the carboxylic group and breaking anion exchange interaction. Analytes were not lost due to their low solubility in DCM.
4. Pure methanol can be used to elute the analytes following the rigorous wash protocol, due to the residual acidic conditions on the column. Analytes are eluted due to their high solubility in methanol.
5. T₄ is generally present at much higher levels than T₃/rT₃, and conversion of species can occur during sample storage, increasing the apparent levels of T₃/rT₃. In addition, conversion of T₄ to T₃/rT₃ can also occur in the MS source, potentially leading to inaccurate quantification and iodine deposits in the MS system, so a good chromatographic separation of the species is essential. If T₄ is not being analysed, it is good practice to divert T₄ to waste prior to the MS.

Buffer Preparation

1. Preservative mixture at 25 mg/mL: Citric acid, ascorbic acid and DL-dithiothreitol. Weigh 250 mg of each and dissolve in H₂O. Make up to 10 mL in H₂O.
2. 50 mM NH₄OAc buffer at pH 9 (aq): Weigh 3.854 g and dissolve in H₂O. Adjust pH to 9 with NH₄OH (28–30% concentration) and make up to 1 L.
3. 2% formic acid in DCM: Take 98 mL of DCM and add 2 mL of formic acid (98% stock).
4. 2 mM ammonium acetate/o.1% formic acid (aq): Weigh 0.15416 g and dissolve in H₂O. Add 1 mL of formic acid and make up to 1 L in H₂O.
5. 2 mM ammonium acetate/o.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.

Processing Conditions

Positive Pressure

Process at approximately 1–2 psi.

Vacuum Processing

Process at approximately -0.2 bar.

Conditioning and Equilibration Steps

EVOLUTE® EXPRESS 96-well plates may be used without conditioning and equilibration steps. Evaluate the requirement for these steps during method development.

Chromatographic Separation

- » It is important to get chromatographic separation of the T₄ from T₃/rT₃ peaks as ESI sources induce de-iodination of the T₄ forming both T₃/rT₃.
- » Good chromatographic separation may also be achieved using acetonitrile in place of methanol as the organic modifier in mobile phase B, along with alternative HPLC column chemistry.

Automated Extraction

The method described in this application note is compatible with automation using Biotage® Extrahera™. Contact Biotage for more information.

Ordering Information

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

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