

Introduction

Adequate supplies of thyroid hormones (THs) are essential for brain development. Abnormal serum THs is routinely used in regulatory toxicology to identify chemicals with the potential to induce a neurological deficit. Serum THs occupies a pivotal position in adverse outcome pathways (AOP) for developmental neurotoxicity. However, serum THs reductions may not correlate to changes in brain THs concentrations and/or lead to unfavorable downstream neurological deficits. Brain THs concentrations may be a more reliable predictor of adverse neurodevelopmental consequences than serum THs alone following exposure to thyroid-disrupting chemicals. Quantitation of brain THs following chemical exposures are rare and are mainly based on indirect antibody methodologies. This study presents optimized procedures for TH determination in the brain by mass spectrometry to advance the development of quantitative AOPs of thyroid disruption. **This work does not reflect EPA policy.**

Objectives

1. Optimize analytical procedures for quantifying THs in neonatal rat brain by streamlining sample preparation, reducing matrix interference, and enhancing recoveries.
2. Examine brain THs in neonatal rat brain as a function of age, sex and, examine the effects of blood contamination in the tissue.

Sample Extraction and Instrumental Analysis

Male and female offspring from Long-Evans pregnant rats (n=5) were euthanized by decapitation or an overdose of Euthasol™ followed by cardiac perfusion with phosphate buffered saline on postnatal day (PN) 0, 2, 6 and 14. The brain was removed, weighed, and flash-frozen in liquid nitrogen.

Samples were fortified with ¹³C₆ isotopically labeled surrogates and ¹³C₁₂ isotopically labeled T3 and T4 internal standards before extraction. Liquid-liquid extraction was performed using a series of chloroform and calcium chloride steps. The aqueous extracts were combined prior to anion exchange solid-phase extraction. Samples were analyzed by LC/MS/MS.

AB Sciex Exion AC UHPLC Parameters	
Column	Accucore RP-MS column, 100 mm x 2.1 mm
Column Temp (°C)	50 °C
Sample Temp (°C)	5 °C
Injection Volume (µL)	10 µL
Flow Rate	0.5 mL/min
Mobile Phase A	0.1% Acetic Acid in Water
Mobile Phase B	Methanol
Gradient	0.0 min- 75%A 25%B 4.0 min- 25%A 75%B 4.5 min- 5%A 95%B 5.0 min- 5%A 95%B 5.1 min- 75%A 25%B 6.0 min- 75%A 25%B

AB Sciex 6500+ QTRAP Scan Parameters			
Q1 Mass (Da)	Q2 Mass (Da)	Retention Time	ID
651.8	605.8	3.65	T3.1
651.8	478.9	3.65	T3.2
657.8	611.7	3.65	13C6-T3
663.8	617.8	3.65	13C12-T3.IS
651.8	605.8	3.99	rT3.1
651.8	478.9	3.99	rT3.2
657.8	611.7	3.99	13C6-rT3
777.6	731.7	4.14	T4.1
777.6	633.7	4.14	T4.2
783.5	737.7	4.14	13C6-T4
789.7	743.6	4.14	13C12-T4.IS



Table 1. HPLC and Mass Spectrometer Scan parameters for thyroid hormone analysis.

Optimizing Phospholipid Removal

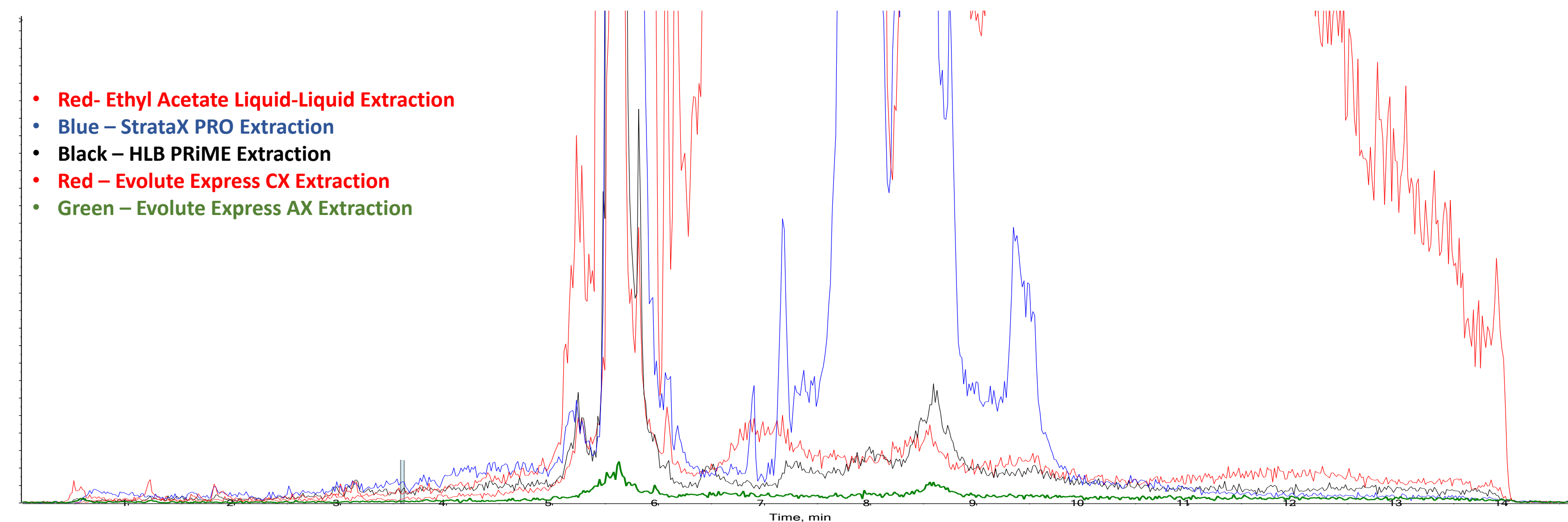


Figure 1. Investigation of phospholipids reduction from brain matrix using precursor ion scanning (184 m/z product ion) from several different extraction procedures. Solid-phase extraction with the Evolute Express AX cartridge (green) performed the best, reducing phospholipids with the addition of a 2% formic acid in dichloromethane wash.

Chromatogram and Calibration Curves

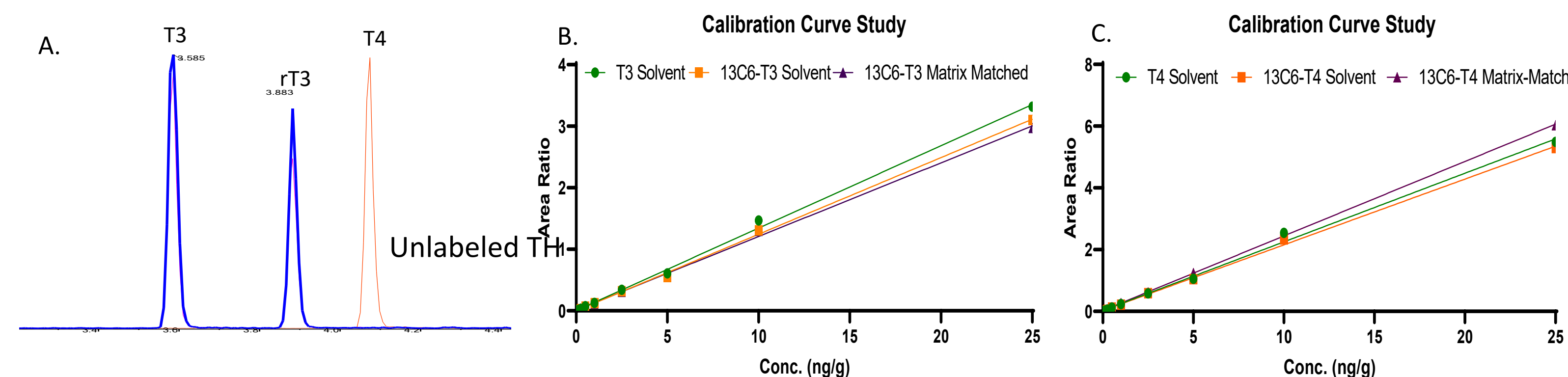


Figure 2. A. Representative chromatogram of unlabeled thyroid hormones. B. and C. Calibration curves of T3 and T4 respectively. Calibration range 0.05 ng/g- 25ng/g. Measured calibration points were within ± 20% of their predicted value. The coefficient of determination, r² for all curves was ≥ 0.995.

Method Detection and Quantitation Limits

Method Detection Limit (MDL)	T3= 0.005 ng/g	T4 = 0.005 ng/g
Method Quantitation Limit (MQL)	T3 = 0.025 ng/g	T4 = 0.025 ng/g

Table 2.. Method detection limit was determined by EPA Method Validation Guidebook, 40 CFR 136, Appendix B.

Quality Control Data

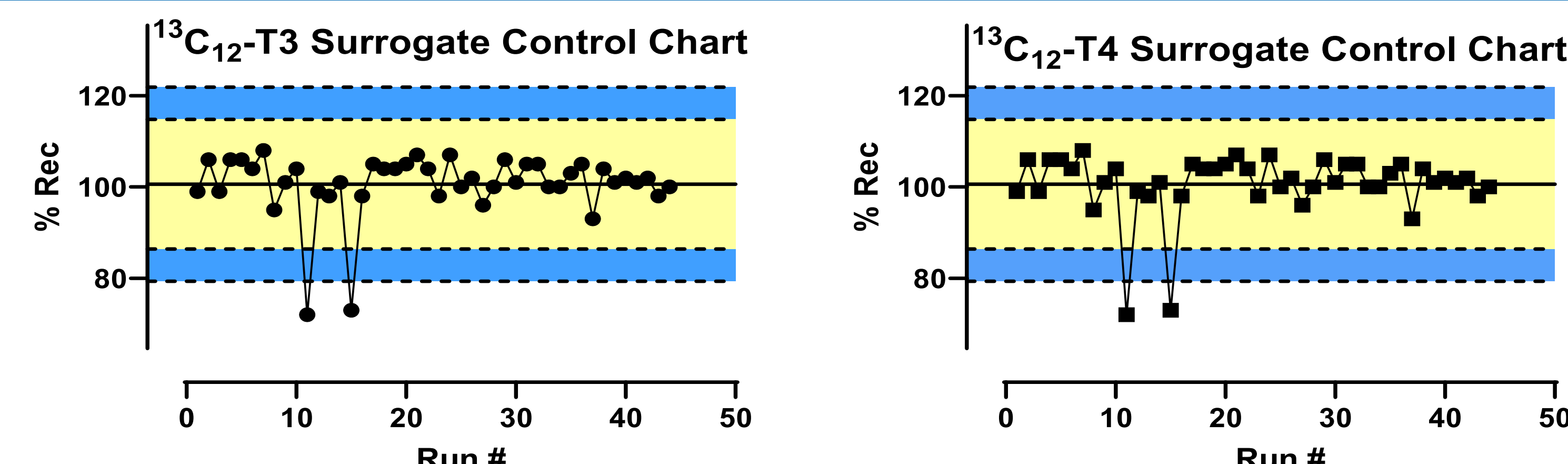


Figure 3. Quality controls charts showing recoveries of ¹³C₁₂-T3 and ¹³C₁₂-T4 during each run analytical run. In each analysis 2 out of 44 run (4.5%) were outside the lower control limits of 80%.

Does Serum in Brain Confound Brain TH Measures?

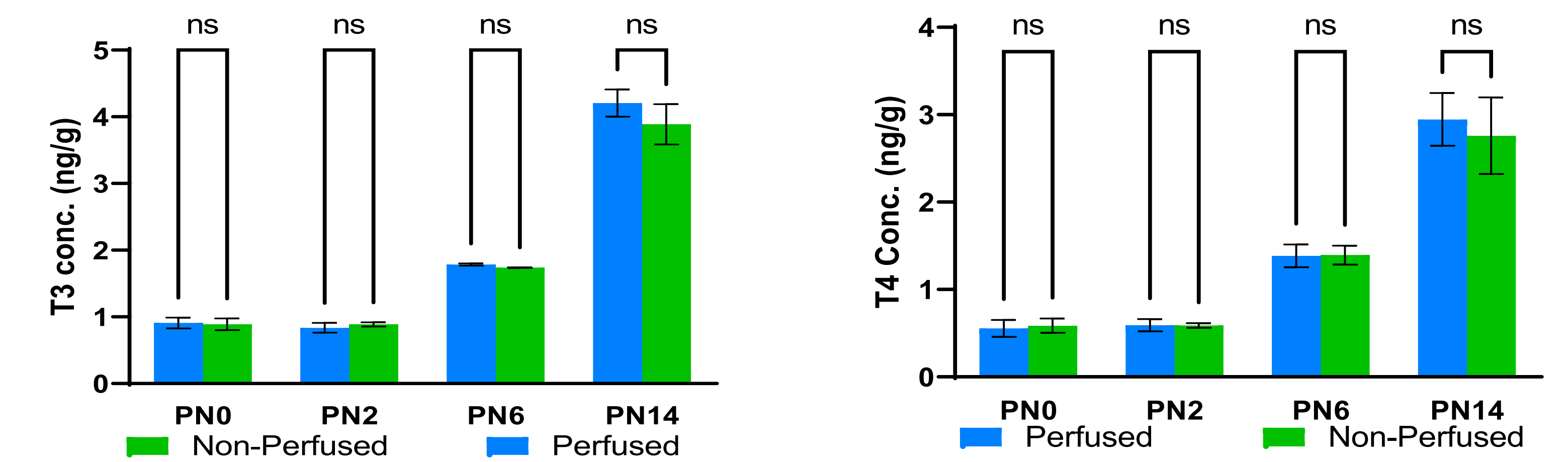


Figure 4. Removal of all blood by intracardiac perfusion prior to brain hormone analysis had no significant (ns) effect of brain T3 or T4 at any age. (n=5, mean ± SEM) P>0.999

Are There Sex Differences in Brain TH Measures?

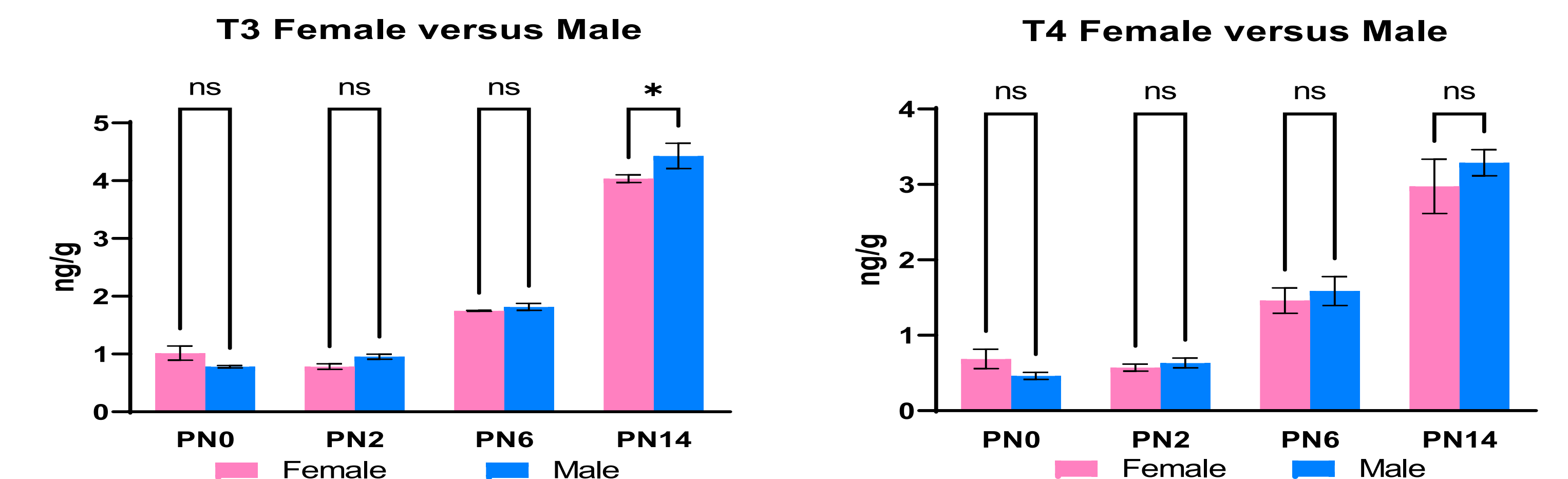


Figure 5. No significant (ns) differences in brain T3 or T4 were seen between male and female pups at any age, with one exception. T3 was slightly higher in male than female brain on PN14 (n=5, mean ± SEM) * P>0.034

Serum Hormones and Brain Hormone Ratios

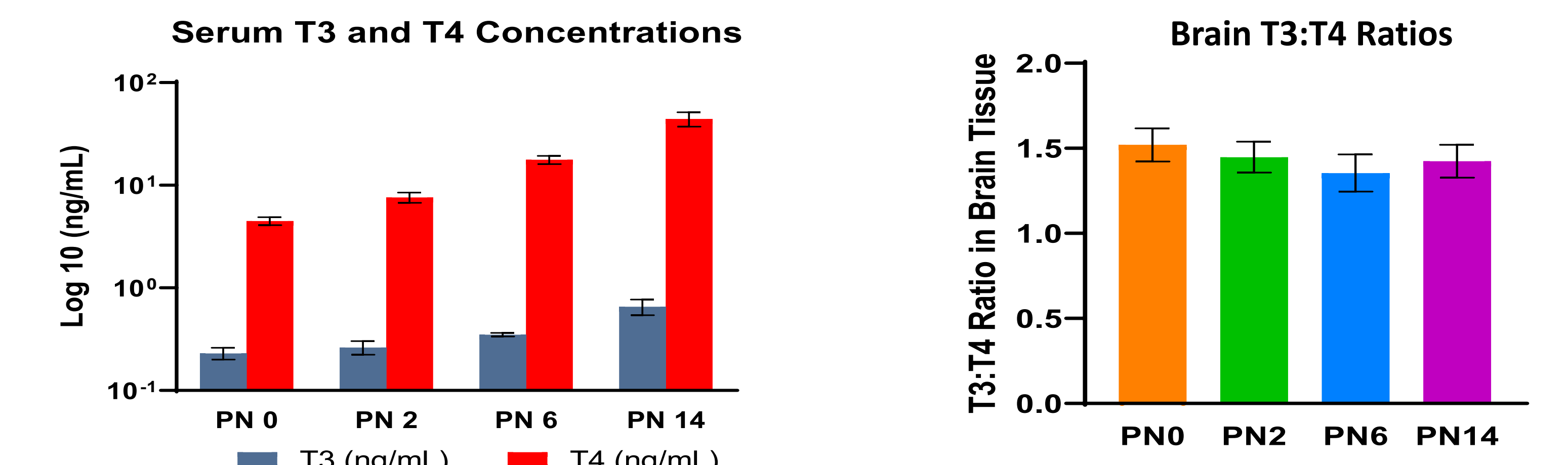


Figure 6. Serum T4 is greater than serum T3 and both increased with age (n=5, mean ± SEM).

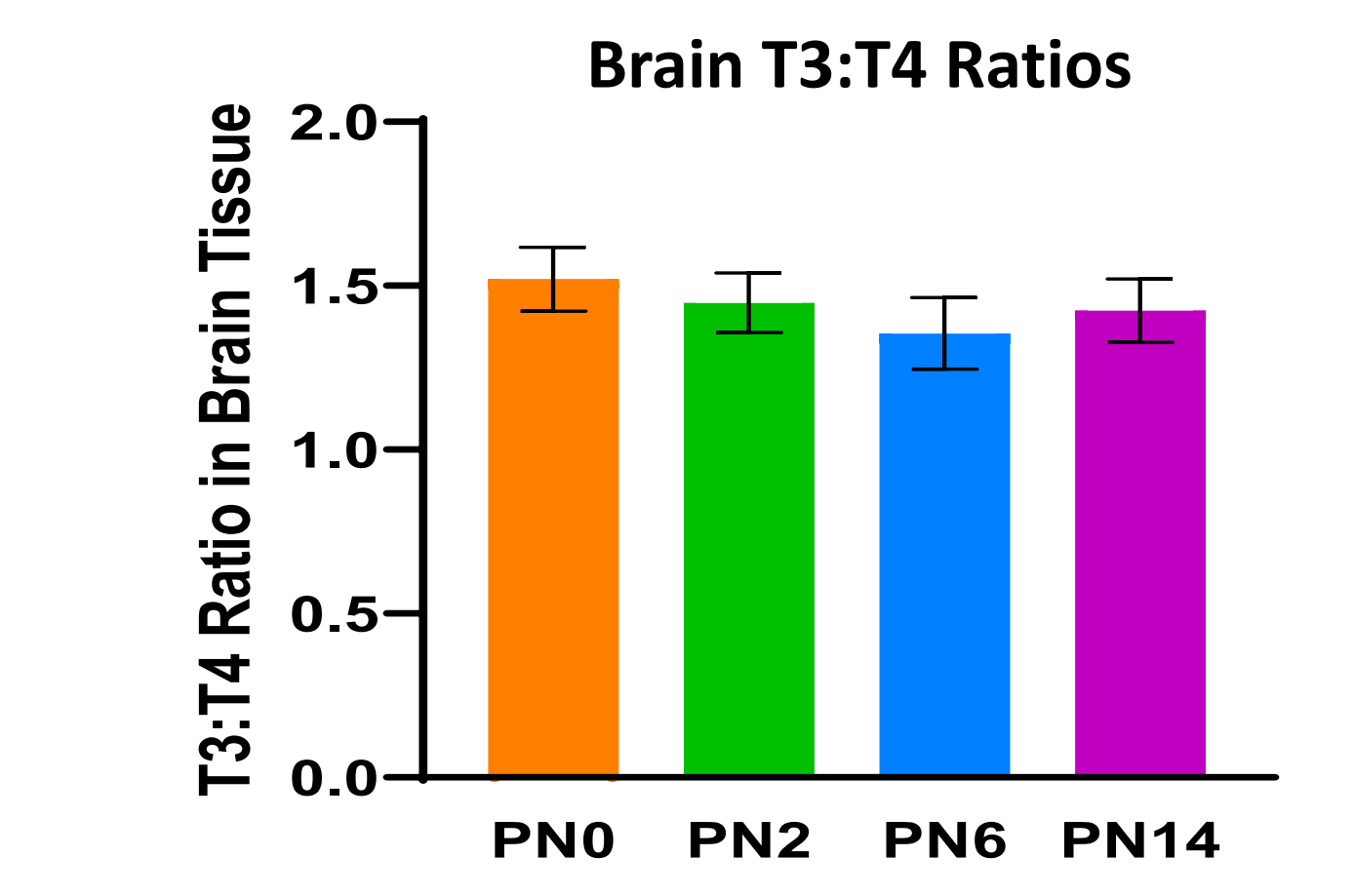


Figure 7. Brain T3 was consistently higher than brain T4 at all ages tested. (n=5, mean ± SEM).

Conclusions

- Optimized LCMS procedures produced reliable age-dependent measures of brain T3 and brain T4.
- MDLs and MQLs were determined with the optimized method using surrogate spiked brain matrix, increasing confidence in results.
- TH recovery was enhanced by reducing phospholipid breakthrough using liquid/liquid extraction procedures, an anion exchange column, a stringent column wash.
- Blood contamination does not have any significance on T3 and T4 levels in brain tissue.
- No sex differences in brain hormones were seen in neonatal rats.
- [T3] was consistently higher than [T4] in rat brain by ~50%.
- Reliable determinations of TH in fetal and neonatal brain will advance development of qAOP for TDCs.