Online Solid Phase Extraction and LC/MS Analysis of Thyroid Hormones in Human Serum

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Thyroid hormones play critical roles in the regulation of biological processes, such as growth, metabolism, protein synthesis, and brain development. Specifically, both 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine or T4) and 3,3′,5-triiodo-L-thyronine (T3), are essential for development and maintenance of normal physiological functions [1]. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3), are important for the diagnosis and monitoring of thyroid diseases. Most clinical laboratories measure thyroid hormones using immunoassays. The immunoassay-based methods offer a relatively rapid, high patient sample throughput that lends itself to automation, but are significantly compromised by problems with assay interference and are complicated by changes in protein levels that alter the free hormone availability [1,2]. These drawbacks lead to inaccuracies of immunoassays and can lead to false high or low results [3].

Liquid chromatography mass spectrometry (LC/MS) has been reported [1-3] to offer superior specificity and speed over the immunoassays for determination of thyroid hormones in biological matrices such as serum and tissues. Nevertheless, the reported sample preparation procedures, typically liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time-consuming steps, and are less compatible with automation [3,4]. The present work demonstrates successful on-line SPE with LC/MS for rapid determination of T4, T3, and 3,3′,5′-triiodo-L-thyronine (rT3) from biological matrices. The method development process included the use of 2 on-line SPE cartridge chemistries: C8 and RP-Amide. The serum samples underwent protein precipitation procedure to release the protein-bound thyroid hormones. The capture of analytes on the on-line SPE cartridges was confirmed by washing the cartridges directly into analytical HPLC column using higher concentrations of organic solvent and tandem mass spectrometry detection.

Experimental:

Materials: SupelTMGenie C8 and RP-Amide (RPA) on-line cartridges (2 cm length x 4.0 mm i.d.), human serum (MilliporeSigma Cat. H-1388), protein crashing solvent: methanol with 1% (w/v) ammonium formate.

Chemical structures of the thyroid analytes

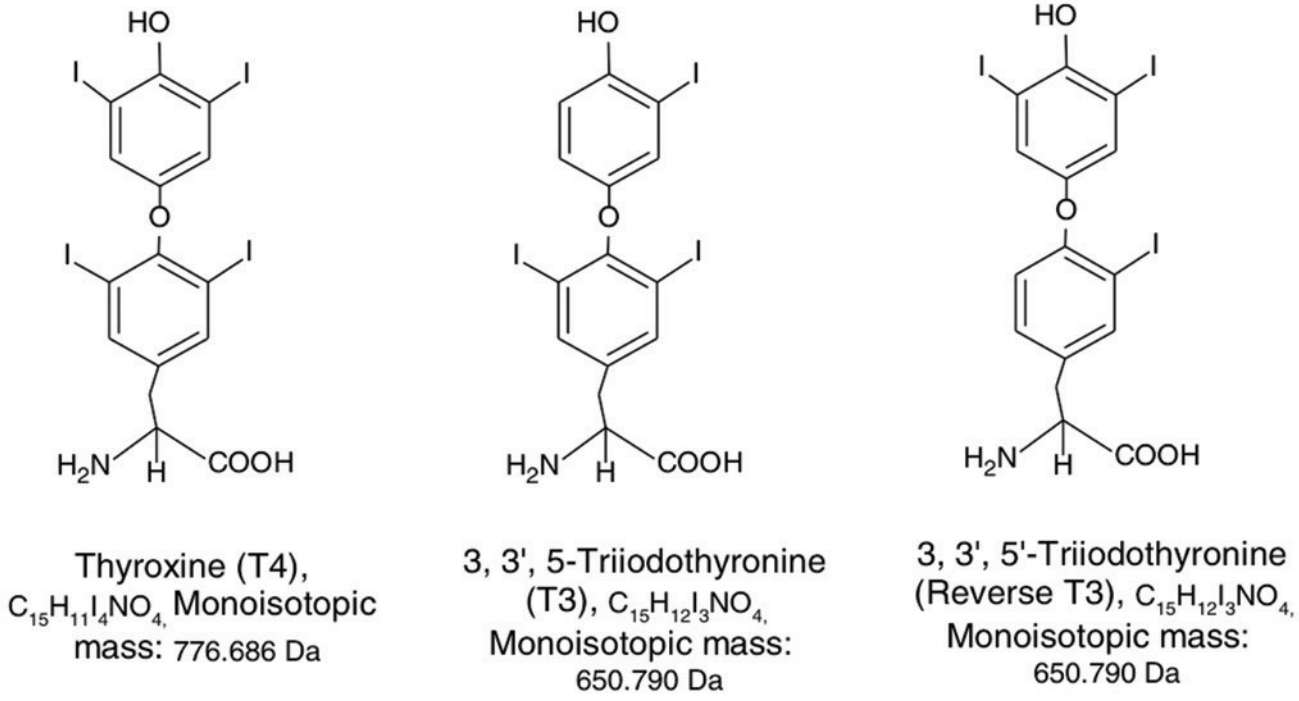


Figure 1: The chemical structures of the thyroid analytes. Note T3 and rT3 are isobaric.

Sample processing procedure: the human serum spiked with analytes was protein precipitated by vortex mixing with the crashing solvent at a 1:3 ratio. Then the mixture was centrifuged at 10,000 x g for 3 min and the resulting supernatant was collected and directly injected for LC/MS analysis.

On-line SPE-LC/MS setup

As shown in Figure 2, the on-line SPE-LC/MS setup consists of a 6-port switching valve and two pumps; one for sample loading and washing, the other for sample elution. To minimise the potential peak broadening from the cartridges, the flow of sample loading/washing and the subsequent elution are in reversed directions.

On-line SPE-LC/MS:

Instrument:	Shimadzu LCMS-8030 with 2DLC setup
HPLC column:	Ascentis Express Biphenyl 10 cm x 2.1 mm (MilliporeSigma Cat# 64065-U)
Mobile phase:	(A) Water; (B) MeOH, each with 0.1% acetic acid
Isocratic:	70% B% for 10 min
Flow:	0.3 mL/min
Column temperature:	35°C
Sample loading/ washing:	0.3 mL/min for 2 min, then the valve switches to in-line with HPLC column, before sample loading the cartridge is equilibrated with the loading solvent for 2.5 min.
Sample loading solvent:	10% methanol
Injection Vol:	2 µL injection
Detection:	MS, ESI(+), MRM mode

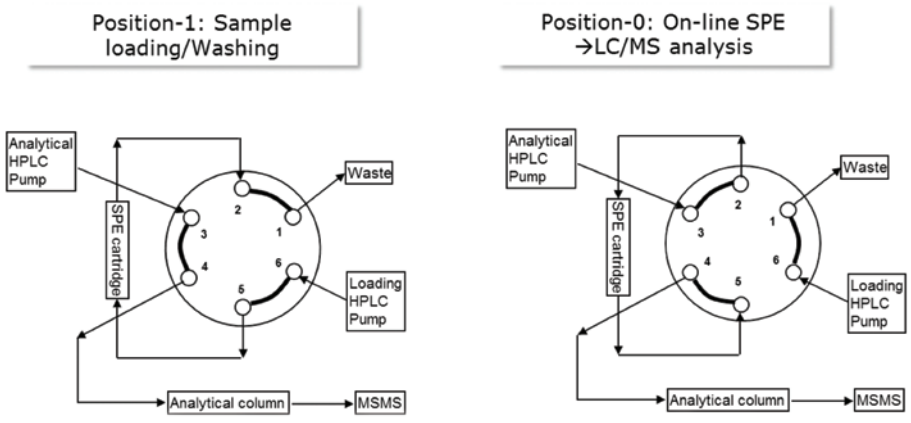
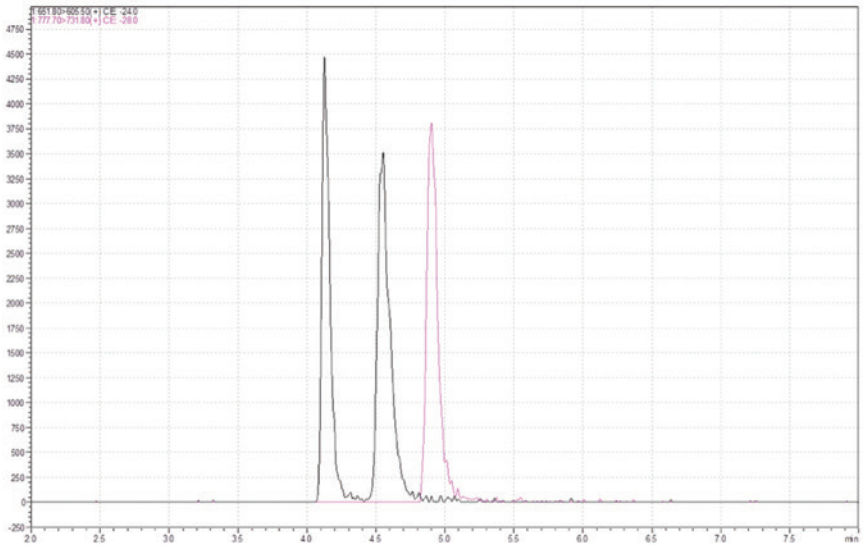


Figure 2: Configuration of the on-line SPE-LC/MS system.

Results and Discussion:

The conventional (off-line) sample preparation by SPE typically involves multiple labour-intensive and time-consuming steps, including: conditioning, sample loading, washing, elution, and finally evaporation and reconstitution of the sample in mobile phase. The on-line cartridges were developed to automate the sample preparation process, minimise hands on time and human error, and reduce overall sample processing time. The present work utilised the C8 and RPA on-line cartridges with LC/MS for the detection of thyroids from in human serum with C8 and RPA on-line cartridges, respectively. The human serum samples were simply protein precipitated with methanol containing ammonium formate and then directly injected for on-line SPE and LC/MS analysis. The sample loading/washing were carried out entirely by the instrument, without any hands-on effort. Additionally, the time-consuming solvent evaporation and reconstitution steps were eliminated. As can been seen from Figures 3 and 4, both C8 and RPA were capable of capturing a trace amount (100 ng/mL x 2 µL in this case) of thyroids from complicated human serum. All three thyroids are resolved from each other, with a peak width at half height <6s and tailing factor from 1.4-1.8. These indicate sharp and nice peak shapes with the on-line cartridges. The total run time is within 6 min.

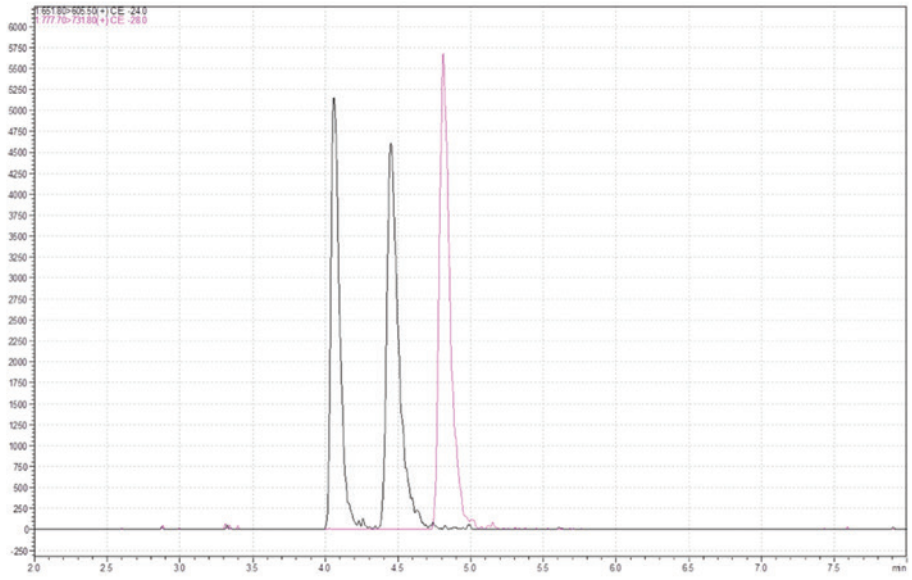
Table 1 and 2 shows the ruggedness of the on-line SPE-LC/MS with C8 and RPA cartridge, respectively, from 120 consecutive injections of the dirty human serum samples. As can be seen, the retention time of the thyroid analytes with C8 or RPA is very reproducible, with RSD's of 0.1%-0.2. The reproducibility (RSD%) of the peak area of the thyroid analytes with C8 and RPA cartridges is 6.2-7.0% and 5.1%-7.7%, respectively, which indicates great reproducibility.



Peak	Analyte	Peak width at 50% height (s)	Tailing factor
1	T3	3.7	1.6
2	rT3	5.2	1.5
3	T4	4.9	1.4

- Sample: 100 ng/mL spiked in human serum, 2 µL injection, the 120th injection.
- All peaks are narrow: <6s peak width at half height
- Peaks are all symmetric with a bit of tailing: tailing factors 1.4-1.6.
- Baseline is low and clean: no interference peaks

Figure 3: Representative LC/MS chromatogram of thyroids in human serum with C8 on-line cartridge.



Peak	Analyte	Peak width at 50% height (s)	Tailing factor
1	T3	4.4	1.5
2	rT3	5.6	1.8
3	T4	5.2	1.6

- Sample: 100 ng/mL spiked in human serum, 2 µL injection, the 100th injection.
- All peaks are narrow: <6s peak width at half height
- Peaks are all symmetric with a bit of tailing: tailing factors 1.5-1.8.
- Baseline is low and clean: no interference peaks

Figure 4: Representative LC/MS chromatogram of thyroids in human serum with RPA on-line cartridge.

Comparing the two types of on-line cartridges, RPA appears to deliver greater signals (peak height and area) for all three thyroid analytes compared to the C8 cartridge. The RPA phase has amide groups that are known to form hydrogen bonds and can provide better retention for analytes through hydrogen bonding interactions. Although the retention times and peak widths for thyroid hormones on the analytical column is very similar whether using the RPA or the C8 SPE cartridges, there appears to be a slight advantage of using RPA SPE with regards to the analyte's retention from the direct injection of protein precipitated serum samples.

Summary

An on-line SPE-LC/MS method has been developed for the rapid detection of thyroid hormones in human serum with minimal hands-on effort and time-consuming steps. Both C8 and RP-Amide on-line cartridges were shown to be capable of capturing trace amounts of thyroids from protein precipitated human serum samples. All three thyroid analytes, T3, rT3 and T4 were resolved on a Biphenyl column, with sharp and symmetrical peak shapes. In addition, reproducibility (RSD%) of the retention time of the thyroids from 120 consecutive injections is between 0.1% and 0.2%, with either C8 or RPA on-line cartridges, while the peak area reproducibility (RSD%) is between 5.1% and 7.7%. These RSD's indicate great ruggedness of the on-line SPE-LC/MS system.

References

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Table 1: Ruggedness of the system with C8 Cartridge.

Reproducibility of retention time and peak area

Analyte	MRM Quantifier	Retention time (min) (Avg. n = 120)	Retention time reproducibility (RSD%, n=120)	Peak area (Avg. n = 120)	Peak area reproducibility (RSD%, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.03	0.2	27046	5.1
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.43	0.2	33723	6.2
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.79	0.2	23766	7.7

Table 2: Ruggedness of the system with RPA Cartridge.

Reproducibility of retention time and peak area

Analyte	MRM Quantifier	Retention time (min) (Avg. n = 120)	Retention time reproducibility (RSD%, n=120)	Peak area (Avg. n = 120)	Peak area reproducibility (RSD%, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.13	0.1	17711	6.9
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.53	0.2	22081	7.0
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.89	0.1	22233	6.2