SOLID-PHASE IMMUNOEXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE SELECTIVE DETERMINATION OF THYROXINE IN HUMAN SERUM

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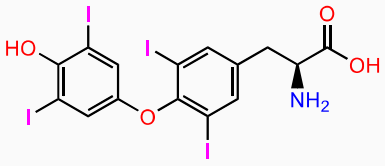
INTRODUCTION

Thyroxine (T4) is a tyrosine-based hormone produced by the thyroid gland, whose main function is to regulate biological processes in humans such as growth and development, basal metabolism, or reproduction [1]. The normal serum concentration of total T4 ranges from 60 to 160 nM (*i.e.*, 47 to 124 ng mL^{-1}) [2]. However, people suffering from thyroid-related diseases present abnormal levels of T4, causing hypo- or hyperthyroidism. Several health conditions can induce these disorders, such as autoimmune diseases (*e.g.*, Hashimoto or Grave's diseases) or thyroiditis. Therefore, the monitoring of T4 is important to show the proper functioning of the thyroid gland.

The **objectives** of this work are the synthesis and characterization of **two T4-specific immunosorbents (ISs)** by the immobilization of **two different monoclonal antibodies** on the surface of a CNBr-activated-Sepharose® 4B support and their application as selective extraction phase in **solid-phase extraction (SPE)**, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [3].

THYROXINE (T4)



LC-MS/MS CONDITIONS

- **Column**: Atlantis® dC18 (150 mm, 2.1 mm, 5 µm)
- **Mobile phase**: MeOH:H_2O 70:30 (0.1% formic acid)
- **Flow**: 0.25 mL min^{-1}
- **Retention time**: 3.7 min
- **Polarity**: ESI$^+$
- **MRM transitions**: 777.7 → 731.7 (20 V) Q
  777.7 → 633.6 (20 V) q
  777.7 → 604.9 (35 V) q

SYNTHESIS & GRAFTING YIELD

CNBr-Sepharose® 4B
35 mg

Anti T4 **Antibody**[a]
or Immunoglobulin G (IgG)[b]

Immunosorbent
1-mL SPE cartridge

[a] Two different purified monoclonal antibodies specific for T4 were tested since information about the epitopes used to produce each antibody was not provided by the supplier

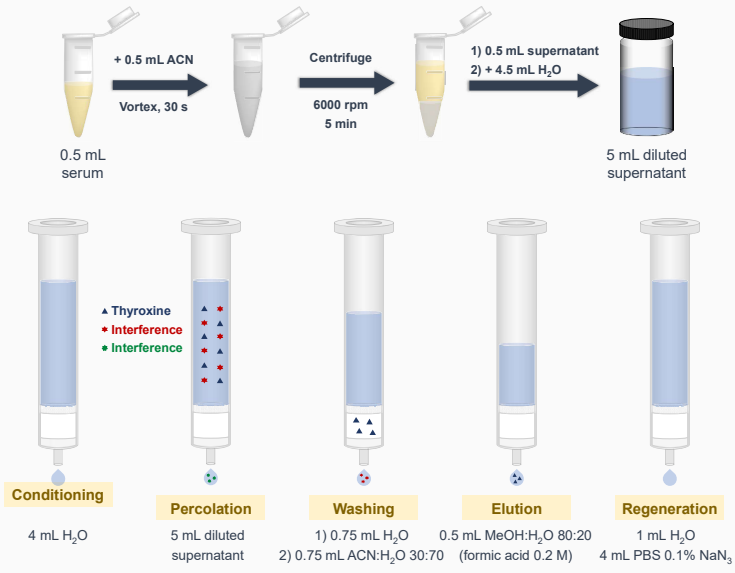
[b] The IgG IS was used as control in order to study the selectivity of the specific ISs

% Grafting yield (n=3): Bicinchoninic acid (BCA) assay

- Antibody 1: **94 ± 2%**
- Antibody 2: **91 ± 3%**
- Control (IgG): **91 ± 4%**

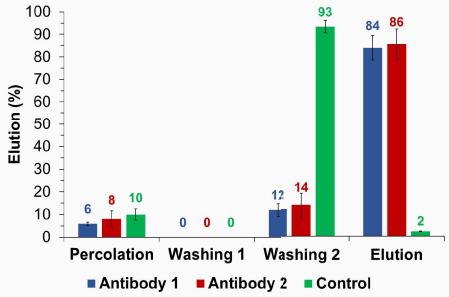
→ Successful covalet bonding of Antibodies to the CNBr-Sepharose® 4B solid support

SAMPLE PREPARATION



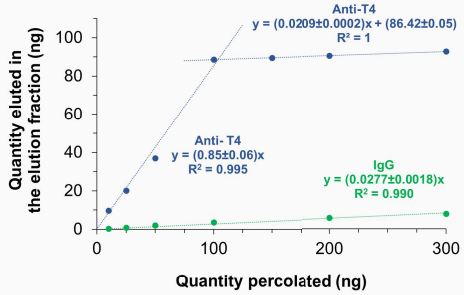
RESULTS AND DISCUSSION

Elution profile



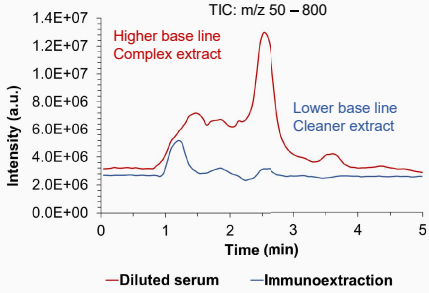
Extraction repeatability (n=3): **RSD < 8%**
Comparable performances for both ISs

Capacity of the IS



Estimated capacity of T4: **104 ng per 35 mg (3 µg g^{-1})**

Blank serum LC/MS chromatograms



Efficient clean-up of the sample by selective extraction of T4

Validation of the proposed SPE method

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Accuracy*			Precision (% RSD)
			Added (ng mL^{-1})	Found (ng mL^{-1})	Relative recovery (%)	
Thyroxine (T4)	0.15	0.5	0	68 ± 3	-	4
			5	72 ± 1	81 ± 7	9
			30	97 ± 2	96 ± 5	5
			60	132 ± 2	107 ± 3	3

* Mean of three replicates ± standard deviation

CONCLUSIONS

- The **successful immobilization of the antibodies** on the CNBr-activated-Sepharose® 4B has been demonstrated by means of the **high grafting yields** obtained (*i.e.*, **> 90%**)
- The **immunosorbent** has been **fully characterized** in terms of **extraction and synthesis repeatability, and capacity**
- The **need of the immunosorbent** was evidenced by comparing the LC-MS chromatograms of both eluate after the SPE method and filtered supernatant after protein precipitation without applying the immunoextraction method
- The optimized immunoextraction method was applied to a **pooled human serum sample** in order to evaluate its performance, showing **accurate results**

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[3] V. Vállez-Gomis, J.L. Benedé, A. Combès, A. Chisvert, V. Pichon, Talanta 265 (2023) 124864

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Solid-phase immunoextraction followed by liquid chromatography-tandem mass spectrometry for the selective determination of thyroxine in human serum

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ABSTRACT

In this work, an analytical method based on solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) has been developed for the selective determination of thyroxine (T4) in human serum. For this purpose, two immunosorbents (ISs) specific to T4 were synthesized by grafting two different T4-specific monoclonal antibodies on a cyanogen bromide (CNBr)-activated-Sepharose® 4B solid support. The grafting yields obtained from the immobilization of each antibody on the CNBr-activated-Sepharose® 4B were over 90%, demonstrating that most of the antibodies were covalently bound to the solid support. The SPE procedure was optimized by studying the retention capability and selectivity of the two ISs in pure media fortified with T4. Under the optimized conditions, high elution efficiencies were achieved in the elution fraction for both specific ISs (*i.e.*, 85%), whereas low ones were obtained in the control ISs (*ca.* 2%), showing the selectivity of the specific ISs. The ISs were also characterized by studying extraction and synthesis repeatability (RSD <8%), and capacity (104 ng of T4 per 35 mg of ISs, *i.e.*, 3 μg g^{-1}). Finally, the methodology was applied to a pooled human serum sample in order to study its analytical utility and accuracy. Relative recovery (RR) values between 81 and 107% were obtained, showing no matrix effects during the global methodology. Furthermore, the need to perform the immunoextraction was evidenced by comparing the LC-MS scan chromatograms and RR values with and without applying the immunoextraction procedure on a serum sample submitted to protein precipitation. This works exploits, for the first time, the use of an IS on the selective determination of T4 in human serum samples.

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Other communications presented by GICAPC Research Group at **25th International Symposium on Advances in Extraction Technologies (ExTech 2023):**

YP-51 *High-throughput determination of oxidative stress biomarkers in saliva by solvent-assisted dispersive solid-phase extraction for clinical analysis.* G. Peris-Pastor, S. Alonso-Rodríguez, J. L. Benedé and A. Chisvert.
See communication.

KN-02 *New miniaturized approaches for the analysis of low-availability samples*. A. Chisvert, J. L. Benedé, J. Grau, V. Vállez-Gomis, C. Azorín and G. Peris-Pastor.
July 19th, 10:40h, Auditorium.

YO-02 *Miniaturized magnetic-pipette tip microextraction: A new tool for microsample analysis*. J. Grau, M. Moreno-Guzmán, L. Arruza, M. Á. López, A. Escarpa and A. Chisvert.
July 19th, 12:35h, Auditorium.

YO-28 *Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability samples*. C. Azorín, J. L. Benedé and A. Chisvert.
July 20th, 17:40h, Atenas room.