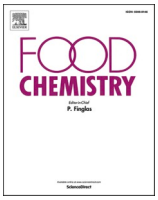
Food Chemistry 492 (2025) 145542

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Liquid chromatography (ion mobility) coupled to organic and inorganic mass spectrometry for determination of eight thyroid hormones in human milk with enantiomeric separation of thyroxine

Rafael de Fátima Vélez-Pérez [a], Ana Arias-Borrego [b,*], Inés Velasco [c], Berta Soldevila [d,e], Tamara García-Barrera [a,*]

[a] Research Centre of Natural Resources, Health and the Environment (RENSMA), Department of Chemistry, Faculty of Experimental Sciences, University of Huelva, Campus El Carmen, Fuerzas Armadas Ave., 21007 Huelva, Spain
[b] Department of Analytical Chemistry, Faculty of Chemistry, University of Seville, 41012, Spain
[c] Department of Gynaecology & Obstetrics, Hospital Universitari Germans Trias i Pujol, s/n Carretera del Canyet, 08916 Badalona, Spain
[d] Department of Endocrinology & Nutrition. Hospital Universitari & Research Institute Germans Trias i Pujol. s/n Carretera del Canyet, 08916 Badalona, Spain
[e] CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Barelona, Spain

ARTICLE INFO

Keywords:
Hollow-fiber liquid-phase microextraction
Ion mobility
Ultra-high-performance liquid chromatography
Iodine speciation
Quadrupole time of flight
Thyroid hormones
Human milk

ABSTRACT

Human milk is a crucial source of thyroid hormones, essential for neonatal development. We developed and validated a three-phase hollow-fiber liquid-phase microextraction method, coupled to ultra-high performance liquid chromatography and ion mobility mass spectrometry, to simultaneously quantify eight thyroid hormones, including the chiral forms D-thyroxine and L-thyroxine in human milk. The method showed excellent linearity ($R^2 > 0.999$), low limits of detection (0.7–19.8 μg L^{-1}), and high recoveries (83–114 %). The analysis of 30 human milk samples, revealed that D-thyroxine (115.1–157.5 μg L^{-1}) was more abundant than L-thyroxine (47.9–193.6 μg L^{-1}), and reversed triiodothyronine prevailed over triiodothyronine. This is the first report of chiral forms of thyroxine, diiodotirosine, and thyronine in human milk, with potential implications for lipid metabolism and infant thyroid regulation. These findings highlight the method sensitivity and biological relevance, offering a robust tool for future nutritional and endocrine studies.

1. Introduction

Human milk (HM) is the primary source of essential nutrients and bioactive compounds for infants during early life. Among its components, iodine plays a crucial role in neurological development and metabolic regulation, as it is a key element in the synthesis of thyroid hormones (THs). These hormones, particularly thyroxine (T_4) and triiodothyronine (T_3), are indispensable for brain maturation and energy metabolism during the first years of life (Dorea, 2002; Dror & Allen, 2018; Rohner et al., 2014; Zoeller & Rovet, 2004). Infants are especially vulnerable to iodine deficiency due to their high requirements relative to body weight and limited intrathyroidal iodine reserves (Etling, Padovani, Fouque, & Tato, 1986). Deficiency can impair carbohydrate metabolism, oxygen consumption, protein synthesis, and immune function (Reinhardt et al., 1998; Robison, Sylvester, Birkenfeld, Lang, & Bull, 1998) and may also alter the HM metabolome (Z.-M. Li, Albrecht, Fromme, Schramm, & De Angelis, 2020).

THs exhibit chirality, and their enantiomers show markedly different biological and pharmacological effects. L-thyroxine (L-T_4) is the naturally occurring form responsible for regulating metabolic rate and is widely used in hormone replacement therapy (Scanu, Toth, Edelstein, Koga, & Stiller, 1969). In contrast, D-thyroxine (D-T_4) does not affect basal metabolism, but it has been shown to influence lipid metabolism, suppress thyrotropin (TSH) secretion, and reduce serum cholesterol levels (Eisdorfer & Post, 1967; Gübitz & Juffmann, 1987). These differences highlight the importance of determining enantiomerically pure THs to better understand their physiological roles (Millan-Alanis et al., 2021). Other iodothyronines, such as reverse T_3 (RT_3) and diiodothyronine (T_2), have also been implicated in energy regulation and lipid metabolism (Goglia, 2005; Lanni et al., 2005; Lanni, Moreno, Cioffi, & Goglia, 1992). However, quantitative data on these analytes particularly in HM, are still limited.

* Corresponding authors.
E-mail addresses: aarias1@us.es (A. Arias-Borrego), tamara@dqcm.uhu.es (T. García-Barrera).

https://doi.org/10.1016/j.foodchem.2025.145542
Received 26 March 2025; Received in revised form 10 July 2025; Accepted 11 July 2025
Available online 16 July 2025

In recent years, microextraction techniques have gained significant attention due to their efficiency, low consumption and capability with modern analytical instrumentation. Conventional techniques such as solid-phase extraction (SPE) are labor-intensive, time-consuming, and require large sample volumes (Z.-M. Li et al., 2020). In contrast, solid-phase microextraction (SPME) has evolved with the development of novel coatings and fiber materials, enabling enhanced selectivity and sensibility for trace-level analyte detection in several complex matrices (Javanmardi, Roszkowska, & Pawliszyn, 2025; Yan et al., 2025). Similarly, liquid-phase microextraction (LPME), including hollow-fiber (HF) and dispersive formats, has improved extraction kinetics and analyte recoveries, making these approaches highly attractive for environmental and pharmaceutical analysis (Khan et al., 2025; López-Juan, Moreno-Calleja, Benedé, & Chisvert, 2025; Naccarato, Elliani, & Tagarelli, 2025; Zhao et al., 2025).

Numerous analytical methods have been developed for THs determination in serum, urine, and tissues, including capillary electrophoresis with UV detection (CE-UV) (P. Li, Hu, He, & Chen, 2014), high-performance liquid chromatography coupled to inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (Michalke, Schramel, & Witte, 2000), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kunisue, Eguchi, Iwata, Tanabe, & Kannan, 2011), and immunoassays (Van Wassenaer et al., 2002). In HM, T_3, T_4, and RT_3 have been reported (Z.-M. Li et al., 2020), but no method has yet addressed the quantification of chiral THs.

New findings in enantioselective separation have focused on the development of more efficient chiral selectors, improved stationary phases, and novel separation platforms. In particular, HPLC (Aydoğan, Çakan, & Ali, 2024; Lella, Nestor, De Bundel, Vander Heyden, & Van Eeckhaut, 2024; Papp, Szabó, Hancu, Farczádi, & Mircia, 2024) and CE (Hancu, Modroiu, Stroia, & Uilăcan, 2024; Peluso, Sechi, & Jibuti, 2024) have benefited from the incorporation of new stationary phases and ionic liquids, leading to improved enantiomeric resolution and shorter analysis times. The emergence of nanomaterials (Hao et al., 2025; Lucci et al., 2024) and molecularly imprinted polymers (Prasad, Tiwari, Madhuri, & Sharma, 2010) as chiral recognition elements has opened new avenues for selective enantiomer separation in complex samples. Additionally, coupling enantioselective techniques with advanced detector systems such as MS/MS (Lella et al., 2024), has significantly improved sensitivity and selectivity, enabling trace-level analysis. Recent advances in ultra-HPLC (UHPLC) with chiral stationary phases (Koidl et al., 2006; J. Lee, Adhikari, Lee, & Yoon, 2022) and ion mobility mass spectrometry (IM-MS) (Wainer, 1993) have significantly improved enantioselective detection and structural identification.

Herein, we propose a novel analytical workflow combining HF-LPME, UHPLC column-switching with chiral and reversed-phase columns, Q-TOF, ICP-QQQ-MS, and IM-MS for the simultaneous determination of eight TH, including D-T4 and L-T4, in HM samples. Under our knowledge, this is the first report of enantiomer-specific THs quantification in HM. This methodology aims to enhance understanding of the endocrine and nutritional roles of HM, with potential implications for maternal and infant health.

2. Materials and methods

2.1. Chemicals and reagent solutions

Ultrapure water (H_2O), employed for the preparation of all aqueous solutions, was procured from a Milli-Q system (Millipore Milli-Q, Watford, UK). Ammonium acetate (CH_3COONH_4), sodium hydroxide (NaOH), glacial acetic acid (CH_3COOH), formic acid (HCOOH), phosphoric acid (H_3PO_4), calcium chloride ($CaCl_2$), propylthiouracil, sodium dodecyl sulphate (SDS), ascorbic acid, citric acid, dithiothreitol (DTT), 1-octanol, anhydrous sodium sulphate (Na_2SO_4) and the standards L-T_4, D-T_4, RT_3, T_2, DIT, MIT, T_0 and $^{13}C_6$-L-T_4 (internal standard, IS) were purchased from Merck (Darmstadt, Germany), while T_3 was from MP Biomedicals (Irvin, CA, USA), ammonia (NH_3) and sodium carbonate (Na_2CO_3) were from Quality Chemicals (Badalona, Barcelona, Spain), and methanol (CH_3OH), acetonitrile (CH_3CN) and acetone (CH_3OCH_3) optima grade for LC/MS were purchased from Fisher Scientific (Waltham, MA, USA).

Pure standards were dissolved in 10 mM NaOH in a 1:1 (*v*/v) mixture of CH_3OH:H_2O (ultrapure) and subsequently stored at −20 °C. Calibration standard solutions (1–75 μg L^{-1} of MIT, DIT and T_0; 10–250 μg L^{-1} of T_2; 10–750 μg L^{-1} of T_3, RT_3, D-T_4 and L-T_4 and 100 μg L^{-1} of internal standard) were prepared from individual stock solutions by diluting them with 0.05 M CH_3COONH_4 in a 60:40 (v/v) mixture of ultrapure H_2O:CH_3CN and the pH was set at 4.5 with CH_3COOH for SPE extraction, and diluting them with 50 mM Na_2CO_3 for HF-LPME.

2.2. Sample collection and preparation

Thirty HM samples were collected during the first four months of infancy and subsequently stored at −80 °C until analysis. HM samples were collected during 2023 at the University Hospital Germans Trias I Pujol, Badalona, Spain. A local ethics committee approved the study and written informed consent was obtained from all participating women (Ethics Committee Reference: PI-23-042). Comprehensive oral and written information regarding the study was provided to all participants. Women with multiple gestations, diseases, or perinatal complications were excluded from the study. The exclusion criteria encompassed women who were unable to breastfeed due to severe symptomatology requiring intensive care unit support, women who required the use of medications with potential adverse effects on the infant, and those for whom obtaining HM was not feasible.

2.3. Three-phase hollow fiber liquid phase microextraction of human milk thyroid hormones

The sample preparation method was adapted and optimized for HM starting from a previously published method for serum (P. Li et al., 2014). The optimized sample preparation procedure is shown in Fig. S1 and comprises two clean-up steps: liquid-liquid extraction (LLE) for protein and lipid removal, and HF-LPME for extraction and preconcentration of TH.

A total volume of 500 μL of HM was mixed with 500 μL of CH_3COCH_3 and 62.5 μL of an antioxidant mixture containing 25 g L^{-1} citric acid, ascorbic acid and DTT in ultrapure H_2O. The mixture was centrifuged after mixing and equilibrating for 30 min (min). The procedure was repeated twice by mixing the supernatant with 250 μL of a 1:1 (*v*/v) mixture of ultrapure H_2O:CH_3COCH_3. After dilution up to 25 mL with ultrapure H_2O, 150 μL 0.1 mM of SDS were added as an ion pair reagent, and the pH was set to 2.8 with H_3PO_4.

HF segments were freshly prepared for each individual sample to avoid cross-contamination. No fiber segment was reused at any point in the study. Polypropylene HF (Accurel Q3/2, 600 μm i.d., 200 μm wall thickness, 0.2 μm pore size, 3 M Deutschland GmbH, Germany) were cut into 4.5 cm segments using a sterile blade under clean laboratory conditions. To remove any manufacturing residues, fibers were immersed in CH_3COCH_3 and sonicated for 5 min, then rinsed thoroughly with ultrapure H_2O and dried under nitrogen. To prepare the supported liquid membrane (SLM), each dried segment was immersed in 1-octanol for 25 s and immediately assembled on the tip of 1 mL micro syringes in a U-shaped configuration. The acceptor phase (20 μL of 50 mM Na_2CO_3) was preloaded into one end of the fiber using a calibrated syringe to avoid air bubbles. Fibers were then immersed in the donor phase (i.e., cleaned HM extract) and stirred at 1250 revolutions per min (rpm) for 15 min at 20 °C.

Under these conditions, analytes were extracted from the aqueous HM matrix into the organic phase (1-octanol) and then transferred into the aqueous acceptor phase inside the fiber lumen. After extraction, the acceptor phase was collected from each fiber segment and directly

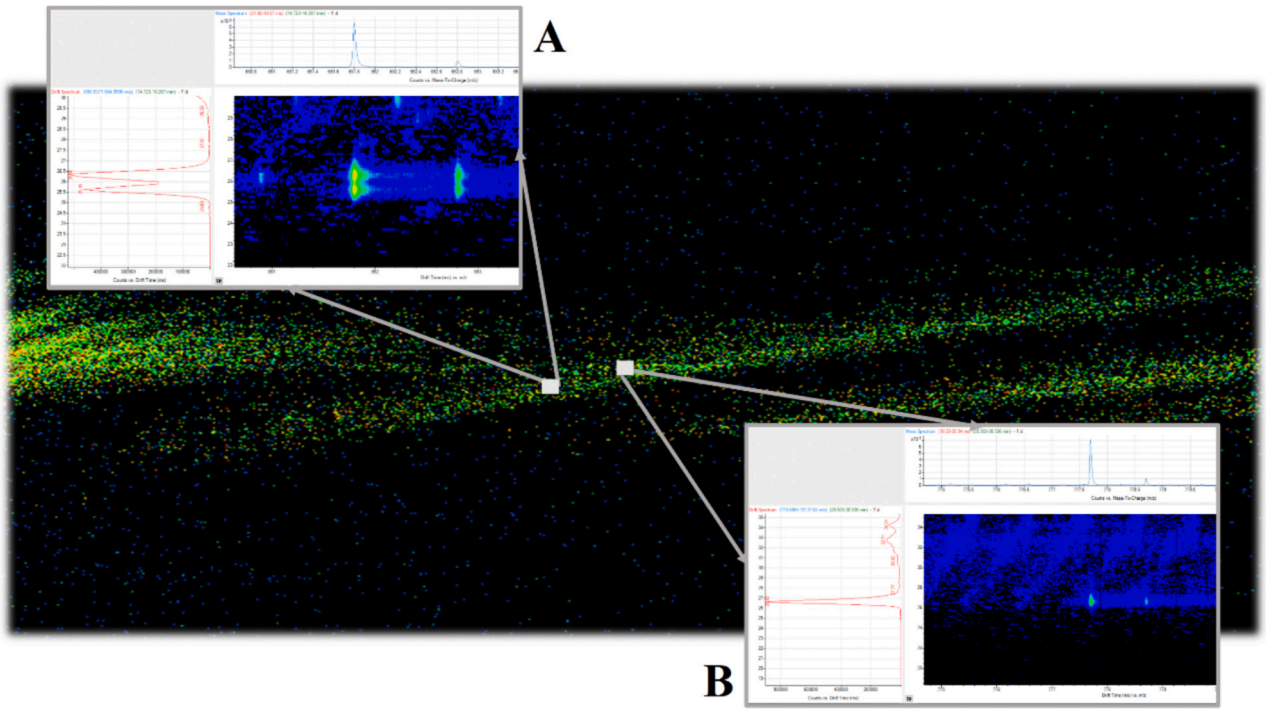


Fig. 1. 2D Spectrum of THs using IM-MS. (A) Separation of T_3 and RT_3 in DT spectrum and (B) no separation of stereoisomers of thyroxine.

analyzed. All fiber segments were prepared using the same batch of tubing and subjected to identical timing, solvent, and temperature conditions to ensure batch-to-batch reproducibility. Visual inspection was used to assess fiber integrity and completeness of filling. Method precision and reproducibility were validated using intra-day and inter-day tests. Extracts were subsequently analyzed using UHPLC-Q-TOF, HPLC-ICP-MS, and IM-MS.

2.4. UHPLC-Q-TOF conditions

Quantification of THs was performed using an Agilent Technologies 6550 iFunnel Q-TOF LC/MS-MS system coupled to an Agilent 1290 Infinity II LC system. The injection volume was 20 µL. Data acquisition and analysis were performed using Agilent MassHunter Workstation software. The separation of THs were carried out using a column-switching system, which combined a reversed-phase column (KromaPhase, C18, Octadecylsililsilica, 5 µm particle size x 150 mm length x 4.6 mm inner diameter, Scharlab, Barcelona, Spain) and a chiral column (Chiralpak QN-AX, O-9-(tert-butylcarbamoyl) based on quinine immobilized on silica gel (5 µm particle size x 150 mm length x 4.6 mm inner diameter, Daicel, Osaka, Japan).

The mobile phases used in the UHPLC method were ultrapure H_2O (A) and CH_3CN (B) each containing 0.05 M CH_3COONH_4 and pH 4.5 with CH_3COOH. The column temperature was set at 30 °C. In position 1 of the column switching valve, the injected HM extract passes through the reversed-phase column and then to the Q-TOF, while in position 2, the sample passes for the chiral column also before the detector. The following gradient procedure was applied: Position 1: 5 % B remained for 3 min, increased linearly to 20 % B in 1 min and kept for 5 min, ramped to 40 % B gradually in 1 min and kept for 5 min, then increased to 80 % B in 1 min. Position 2: 80 % B kept for 17 min. Position 1: finally returned to initial conditions in 1 min and re-equilibration during 3 min. The reference masses *m/z* 121.0509 and 922.0098, were constantly introduced into the system for mass correction.

Targeted MS/MS mode was recorded in the 50–800 m/z range. Other parameters were set as follows: capillary voltage (3500 V), drying gas flow rate (13 L min^{-1}), temperature (200 °C), gas nebulizer pressure (35 psi), sheath gas (350 °C) and flow rate (11 L min^{-1}). The fragmentor voltage was adjusted at 175 V, nitrogen was used as collision gas and voltages were in the range of 14–24 V for the fragmentation of compounds. Data were acquired with a scan rate of 0.9 spectrum/s at centroid mode. Table S1 shows the MS/MS parameters for THs detection.

2.5. HPLC-ICP-MS conditions

The analysis was carried out in a triple quadrupole ICP-MS Model 8800 (Agilent Technologies) using helium as a collision gas at a flow rate of 4.5 mL min^{-1}. A mixture of H_2 (2 mL min^{-1}) and O_2 (40 %) was used in the MS/MS mode for iodine (127 → 143). A tuning solution containing of Li, Co, Y, and Tl at 1 µg L^{-1} was used to tune the ICP-MS. The calibration standard solutions containing THs were used to prepare internal calibration curves. Other operational parameters for ICP-MS, such as sampling depth, RF forward power, carrier gas flow rate, or type of nebulizer, are detailed in Table S2.

2.6. IM-MS conditions

The IM-MS (an Agilent Technologies 6560 Ion Mobility LC/Q-TOF coupled to an Agilent 1290 Infinity II LC system) conditions were similar to those described for the UHPLC-Q-TOF. The differences were the following: MS mode was recorded in the range of 100–1700 *m/z*; the parameters IM Transient Rate (18 IM transient/frame), Max Drift Time (60.02 ms), Trap Fill Time (20 ms) and Trap Release Time (0.15 ms) were selected for IM conditions, the drift gas used was nitrogen and the Multiplexing was disabled.

2.7. Validation of the methodologies based on HF-LPME followed by UHPLC-Q-TOF or HPLC-ICP-MS

To evaluate the practical reliability of the extraction method, the following figures of merit were determined, namely: linearity, limits of detection (LOD), limits of quantification (LOQ), repeatability (intra-day) and reproducibility (inter-day). The linearity was obtained by plotting

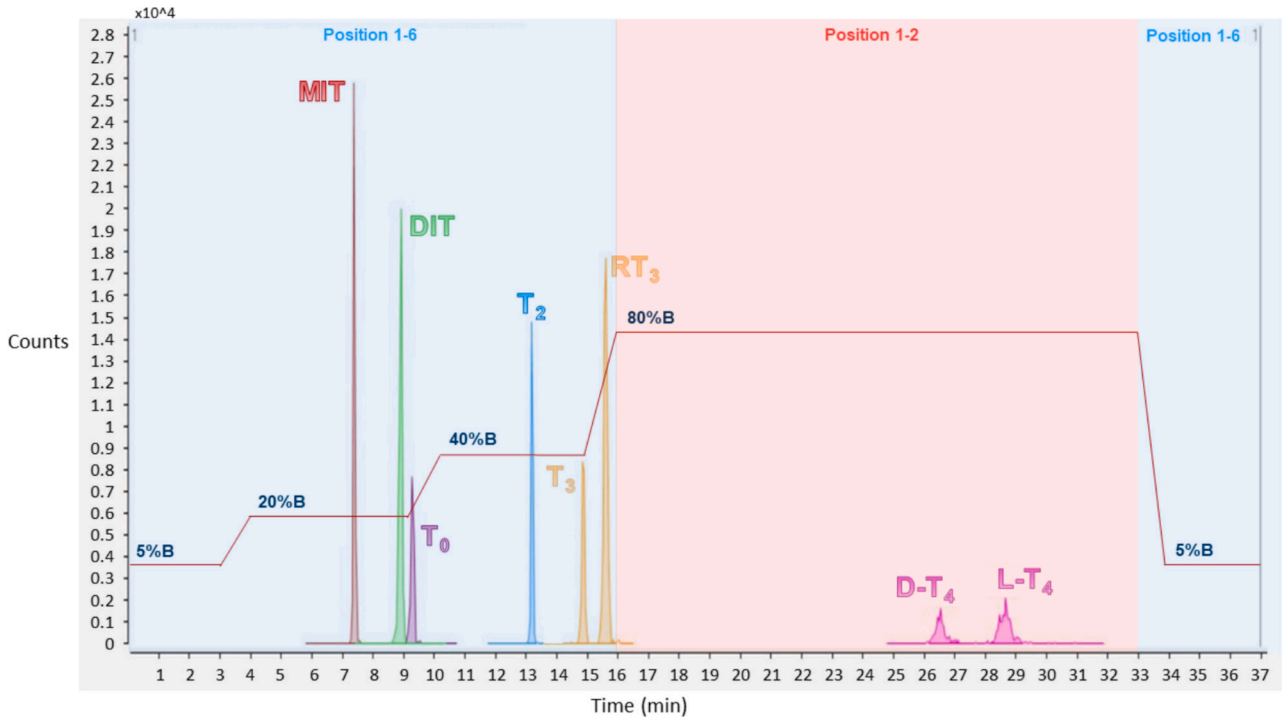


Fig. 2. Superimposed chromatographic gradient and UHPLC-Q-TOF chromatogram showing the separation of MIT, DIT and T_0 standards at 200 μg L^{-1} and T_3, RT_3 and T_4 at 2 mg L^{-1}.

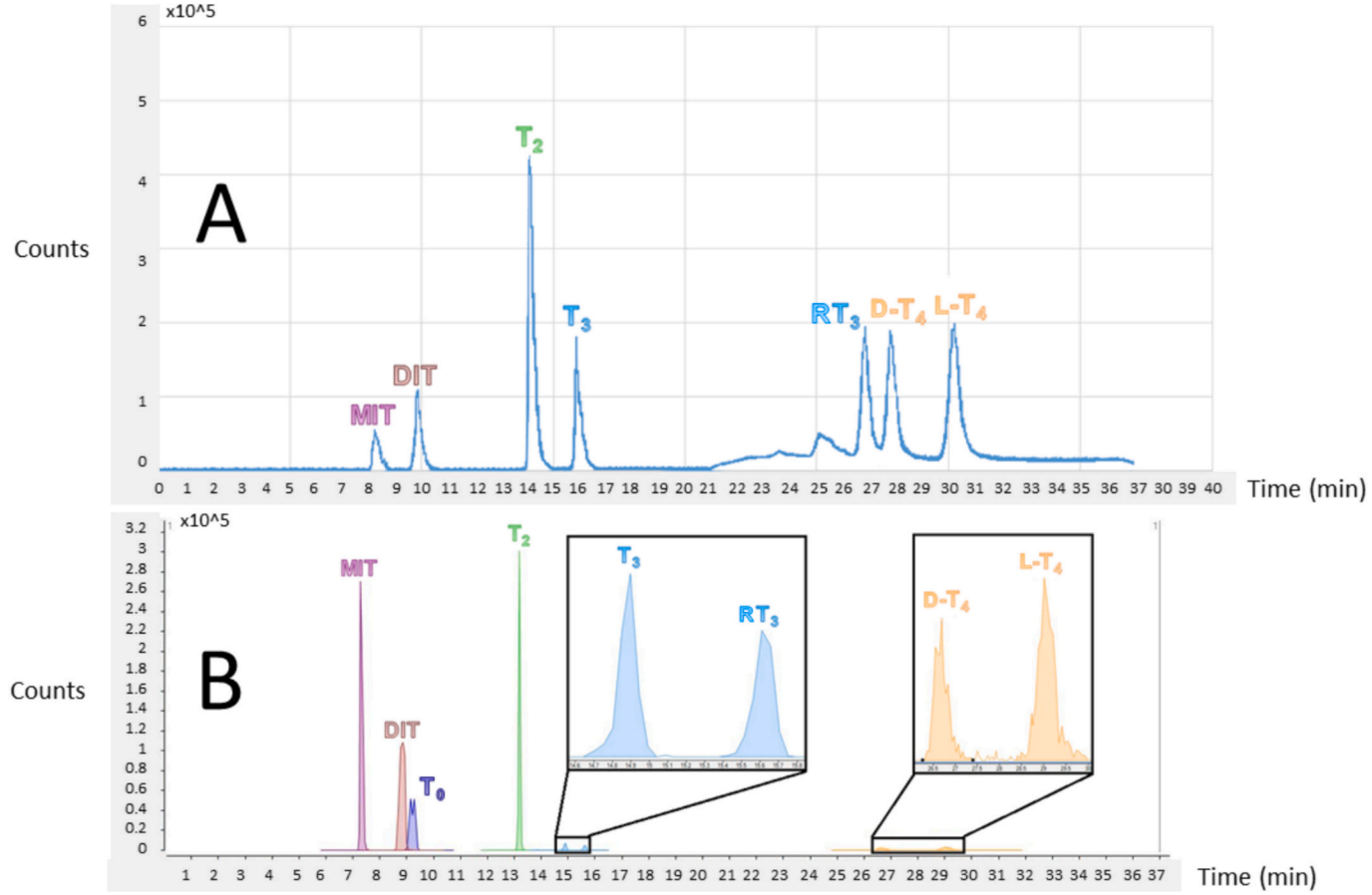


Fig. 3. Chromatograms showing the separation of THs standards, at 1 mg L^{-1} by (A) HPLC-ICP-MS and (B) UHPLC-Q-TOF. The insets of Fig. B show the magnified sections of the chromatogram for better visualization of T_3, RT_3, D-T_4 and L-T_4.

Table 1
Limits of detection and quantification, repeatability and reproducibility for THs using UHPLC-Q-TOF and HPLC-ICP-MS.

Thyroid Hormones		MIT	DIT	T_0	T_2	T_3	RT_3	D-T_4	L-T_4
UHPLC-Q-TOF	LOD (μg L^{-1})	0.7	1.5	1.5	5.6	6.6	2.9	19.8	10.7
	LOQ (μg L^{-1})	2.3	5.0	5.0	18.6	22.1	9.5	65.9	35.5
	%RSD intraday (n = 10)	8	4	7	5	9	6	8	5
	%RSD interday (n = 10)	9	5	7	9	9	9	9	6
HPLC-ICP-MS	LOD (μg L^{-1})	4.1	4.2	N.D.	4.5	5.7	2.7	7.0	7.6
	LOQ (μg L^{-1})	13.6	14.0	N.D.	14.9	19.0	9.1	23.5	25.5
	%RSD intraday (n = 10)	7	8	N.D.	5	6	8	9	8
	%RSD interday (n = 10)	8	9	N.D.	6	7	9	9	9

LOD: limits of detection; LOQ: limits of quantification; N.D.: not detectable.

calibration curves of the relative area (analyte peak area/internal standard peak area) versus the concentration of each analyte. The analytical curves were constructed first at ten levels from 1 to 1000 μg L^{-1} of all THs using both UHPLC-Q-TOF and HPLC-ICP-MS. LOD and LOQ were calculated as $a + 3S_{y/x}$ and $a + 10S_{y/x}$, respectively, where "a" is the origin ordinate and "$S_{y/x}$" is the random error in the slope and intercept values. After reshaping the calibration function, the concentrations at LOD and LOQ were obtained. THs standard solution at 1 mg L^{-1} each was measured ten times ($n = 10$) for the repeatability (intra-day) and reproducibility (inter-day). UHPLC-Q-TOF was finally selected as the methodology for the analysis of samples based on the first validation study. Thus, another calibration with eight levels was performed by UHPLC-Q-TOF at the following concentration ranges: 1–75 μg L^{-1} for MIT, DIT and T_0; 10–250 μg L^{-1} for T_2; 10–750 μg L^{-1} for T_3, RT_3, D-T_4 and L-T_4. Additionally, a pool of spiked samples was used. Thus, a volume of 500 μL of the HM pool spiked with the commercial standards at different concentrations were mixed and 50 μL of the final extract was analyzed. The spiked commercial standards used in both calibration and recovery experiments correspond to the free, non-conjugated forms of THs, which are the same structural forms detected in HM samples.

A IS ($^{13}C_6$-L-T_4) was added to all samples, including calibration standards and quality controls, at a final concentration of 100 μg L^{-1} before the HF-LPME extraction step. The internal standard was used to reduce errors in the analyte signals, and to control matrix effects and extraction variability. Calibration curves were constructed based on the analyte-to-IS response ratio. Blank samples (ultrapure H_2O) were included at the beginning and end of each sample batch to monitor carryover and background contamination. In addition, calibration verification standards were analyzed after every 10 injections to assess instrumental consistency. No significant carryover or drift was observed.

3. Results and discussion

3.1. Ion mobility mass spectrometry for thyroid hormones in human milk

IM-MS was used as another tool for the unequivocal identification of the THs using the drift times (DT) and cross collision section (CCS), which are unique for each one of them. A standard solution of analytes was measured by ion mobility (IM) coupled to Q-TOF. Table S3 shows the DT and CCS experimentally obtained, and those results were compared with databases (ccsbase.net, mcleanresearchgroup.shinyapps.io, brcwebportal.cos.ncsu.edu), and Fig. 1 corresponds to a two-dimension spectrum where it can be seen a mass spectrum at X-axis and a DT spectrum at Y-axis. Fig. 1 also shows two insets: Inset A highlights the separation of the position isomers T_3 and RT_3, while inset B shows no separation between D-T_4 and L-T_4. The separation was possible in 5 min. However, the separation of the stereoisomers (D-T_4 and L-T_4) was not possible as they have the same DT and CCS; otherwise, the position isomers and the other THs were separated successfully. In addition, the quantification of THs was not possible by using IM-MS as it is a qualitative technique.

3.2. Optimization of the column switching system combining chiral and reversed phase columns coupled to UHPLC-Q-TOF and HPLC-ICP-MS

Initially, only the chiral column was used, but the separation of the THs was not possible and only the chiral forms of T_4 were completely resolved. The separation of T_3 stereoisomers was not possible due to the absence of a commercial standard and the limitation of the authors for their synthesis. Then, using the reversed phase, the THs were separated, but not the chiral forms of T_4. Then the chromatographic separation of THs was carried out using a six-ways valve connected to both columns with chiral and reversed phase stationary phases and then to the UHPLC-Q-TOF or HPLC-ICP-MS. As can be seen in Fig. 2, the optimized gradient by increasing the proportion of the apolar mobile phase allowed the separation of eight THs in 37 min. MIT, DIT, T_0, T_2, T_3 and RT_3 were separated in 17 min with the reversed-phase column and then, the column switching valve changed to position 2 to separate T_4 into D-T_4 and L-T_4 with the chiral phase column. After that, the THs were detected in the Q-TOF system by MS/MS or into the ICP-MS using the iodine of the molecule as a "tag" in the atomic detector (Fig. 3). As shown in Fig. 3A, changing the column-switching system to position 2 in 16 min, as previously optimized for the UHPLC-Q-TOF, led to the elution of RT_3 besides T_4 into the chiral column. The reason is the short delay in the HPLC-ICP-MS system due to the path length from the HPLC to the detector, conditioned by the nebulizer, torch and plasma. When the position changed at 17 min, the T_4 eluted from the reverse phase and it was detected in the ICP-MS without chiral separation. Then, the position changes at the column switching valve was set at 16 min. As shown **in** Fig. 3**A,** the detection of T_0 was impossible in the ICP-MS because this THs does not contain iodine.

The limits of detection and the relative standard deviation (%RSD intra- and interday) of both methodologies are shown in Table 1. As can be seen, the sensitivity, selectivity, reproducibility and repeatability were comparable using the UHPLC-Q-TOF or HPLC-ICP-MS as detector. However, the former allows determining T_0 that does not contain iodine. For this reason, UHPLC-Q-TOF was selected for further experiments.

3.3. Optimization of the three-phase-HF-LPME general configuration

A pretreatment procedure was optimized prior to HF-LPME due to the complexity of the HM samples besides the possibility of oxidation of THs during the sample treatment procedures and their common binding to proteins. The antioxidant mixture used was ascorbic acid, citric acid and DTT, whereas CH_3OCH_3 was added for the leaching of TH, as used by other authors for HS (P. Li et al., 2014). The volumes of HM samples and those reagents were carefully optimized for the minimum amount to miniaturize the method, which is important for biological samples. Thus, 500 μL of HM was enough to detect the THs in the Q-TOF and ICP-MS due to their sensitivity, against 2 mL used for HS (P. Li et al., 2014). After this pretreatment, SDS was added as an ion pair reagent and the variables of the HF-LPME were optimized. For the optimization of the HF-LPME method, the UHPLC-Q-TOF was used. An aqueous acceptor phase is recommendable due to the structure of the THs and the

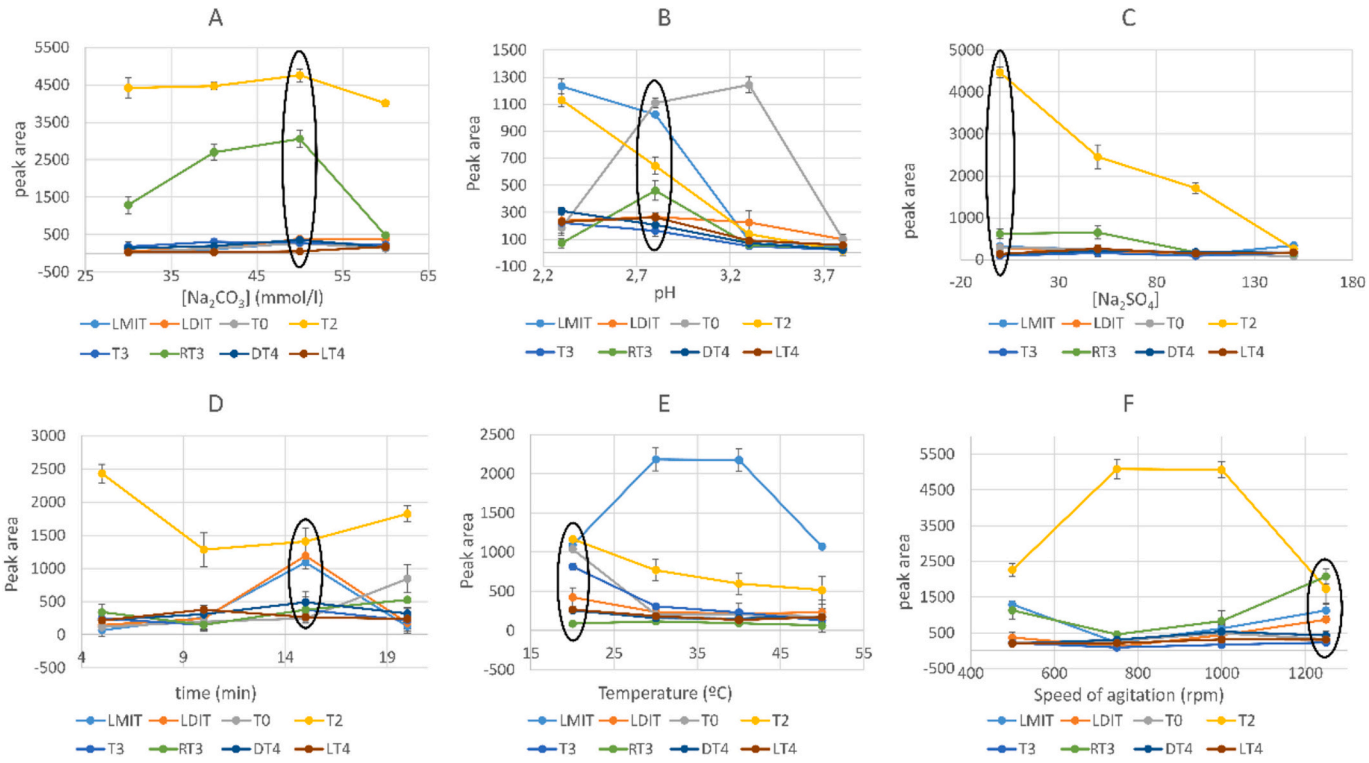


Fig. 4. Optimization of the following parameters of HF-LPME: (A) Concentration of acceptor phase, (B) pH, (C) ionic strength, (D) time of extraction, (E) agitation speed and (F) temperature of extraction.

Table 2
Validation parameters of the proposed method (HF-LPME).

Thyroid Hormones	MIT	DIT	T_0	T_2	T_3	RT_3	D-T_4	L-T_4
LOD (μg L^{-1})	0.7	1.5	1.5	5.6	6.6	2.9	19.8	10.7
LOQ (μg L^{-1})	2.3	5.0	5.0	18.6	22.1	9.5	65.9	35.5
%RSD intraday								
Spike 1 (n = 3)	<1	<1	3	<1	<1	2	1	<1
Spike 2 (n = 3)	2	2	4	<1	1	<1	6	6
Spike 3 (n = 3)	6	4	5	<1	4	<1	4	3
%RSD interday								
Spike 1 (n = 3)	8	6	5	4	8	7	9	9
Spike 2 (n = 3)	8	10	7	7	7	6	8	7
Recovery (%) (n = 3)	95	91	83	113	114	112	86	92
EF (%) (n = 3)	30	41	45	26	28	52	27	32
Linearity								
a	−1955.9	928.6	674.0	5840	−834	1387.2	−484.8	123.7
b	2101.5	1281	1040.3	437.3	56.2	186.8	28.8	55.2
R^2	0.9995	0.9996	0.9996	0.9993	0.9991	0.9993	0.9996	0.9993
Range (μg L^{-1})	2.3–75	5–75	5–75	18.6–250	22.1–750	9.5–750	65.9–750	35.5–750
Average concentration (μg L^{-1} ± SEM)	13.7 ± 5.6	15.5 ± 2.1	14.0 ± 2.6	37.4 ± 5.8	51.8 ± 10.3	65.0 ± 12.9	135.3 ± 4.7	94.0 ± 16.9

LOD: limits of detection; LOQ: limits of quantification; For the RSD, the spiked HM samples were the following: Spike 1: 5 μg L^{-1} of MIT, DIT and T_0; 50 μg L^{-1} of T_2, T_3, RT_3, D-T_4 and L-T_4; spike 2: 10 μg L^{-1} of MIT, DIT and T_0; 100 μg L^{-1} of T_2, T_3, RT_3, D-T_4 and L-T_4; and spike 3: 50 μg L^{-1} of MIT, DIT and T_0; 200 μg L^{-1} of T_2; 500 μg L^{-1} of T_3, RT_3, D-T_4 and L-T_4; EF: enrichment factor; a: y-intercept; b: slope; R^2: determination coefficient; SEM: standard error of mean. Validation parameters in LLE according to literature (P. Li et al., 2014).

compatibility with the HPLC technique. However, the extraction of THs by HF-LPME using an aqueous acceptor phase was not possible because it is mixed with the donor phase (HM sample). Therefore, the HF-LPME for THs was configured in the three-phase mode using 1-octanol immobilized in the HF pores, thus providing a supported liquid membrane (SLM). The HF were cut into 4.5 cm segments, and they were fixed on the end needles of two commercial 1 mL microsyringes (U-shape configuration) as in a method previously described for THs in HS that also use HF-LPME (Z.-M. Li et al., 2020). An univariate optimization was carried out by varying one parameter as maintained constant the others using the following conditions: Na_2CO_3 30 mmol L^{-1} for donor phase, pH 3, no addition of salt for ionic strength, 20 min of extraction, 1000 rpm and 20 °C. Each parameter was varied in 4 levels and each level was analyzed in triplicate ($n = 3$). The acceptor phase and the SLM selected were Na_2CO_3 and 1-octanol, respectively, as used in a previously described method for THs in HM based on SPE (Z.-M. Li et al., 2020). The concentration of acceptor phase varied from 30 to 60 mmol L^{-1} and the optimum was 50 mmol L^{-1} (Fig. 4A). The pH varied from 2.3 to 3.8 using H_3PO_4 and the optimum was found at 2.8 (Fig. 4B), while the ionic strength varied from not salt addition to saturation with Na_2SO_4 and the

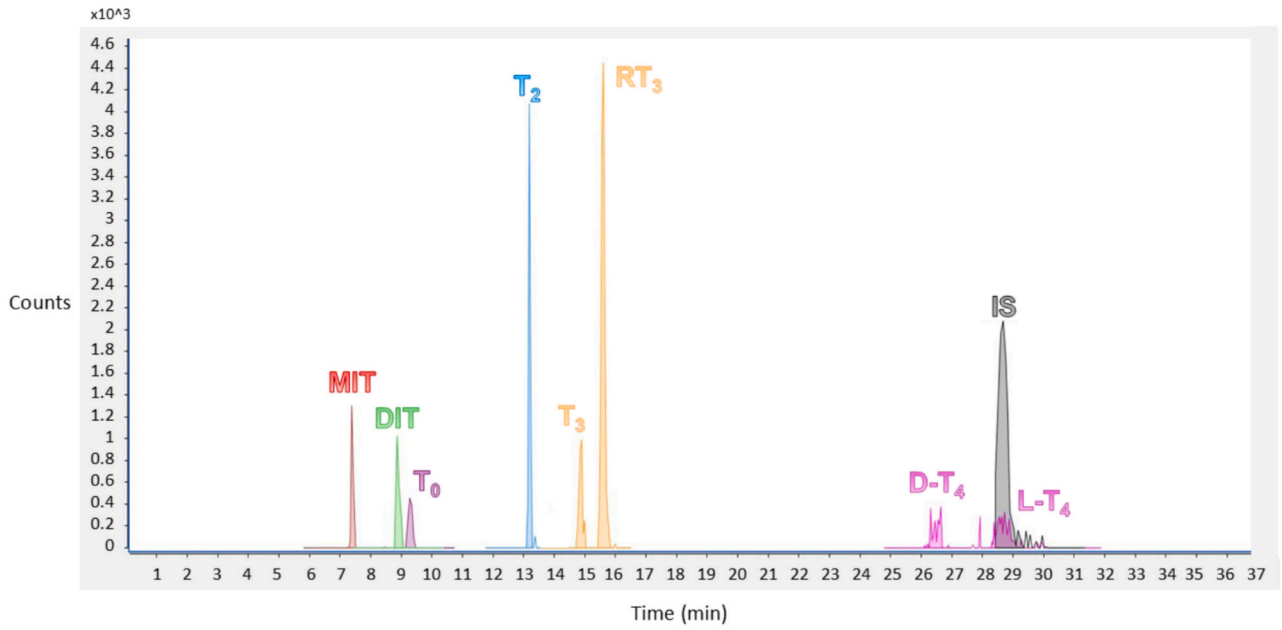


Fig. 5. Typical chromatogram of THs in HM obtained by HF-LPME followed by UHPLC-Q-TOF in MS/MS mode (extracted ion chromatogram). The $^{13}C_6$-L-T4 (IS) signal is also shown in the chromatogram and was used for normalization and quality control purposes.

optimum was not adding salt (Fig. 4C). The extraction time was optimized from 2 to 20 min (Fig. 4D) and the optimum was 15 min, while the temperature was optimized from 20 to 40 °C (Fig. 4E) and the optimum, as a compromise was 20 °C. Finally, the stirring speed was optimized from 750 to 1250 rpm (Fig. 4F) and the optimum value was 1250 rpm.

3.4. Validation of HF-LPME followed by UHPLC-Q-TOF

To evaluate the applicability of the proposed HF-LPME method, figures of merit were determined and compared with those reported for a previously published method based on SPE and HPLC-MS (Z.-M. Li et al., 2020). The use of $^{13}C_6$-L-T_4 as an IS significantly improved method precision and minimized ME, as reflected by the low %RSD values obtained in the validation experiments (Table 2).

Compared to SPE, the HF-LPME method offers several advantages. First, SPE is more time-consuming, involving a three-step LLE protocol, with two of those steps repeated three times. In contrast, HF-LPME consists of a single extraction step, repeated three times, which simplifies the workflow. Additionally, SPE requires a drying step using a vacuum concentrator and subsequent sample reconstitution, both of which are unnecessary in the HF-LPME protocol. As a result, SPE often takes more than one day to complete, potentially compromising sample stability, while HF-LPME allows analysis to be performed on the same day of extraction. Moreover, SPE is a volume-intensive technique that typically requires 2 mL of HM, whereas HF-LPME achieves effective extraction from only 500 µL.

A representative chromatogram of THs in HM extracted by HF-LPME and analyzed by UHPLC-Q-TOF is shown in Fig. 5.

Once the HF-LPME followed by UHPLC-Q-TOF method was optimized an exhaustive validation was performed with a pool of spiked HM samples as described in the Materials and Methods section (Table 2). Additionally, the matrix effect is a critical factor in LC-MS-based quantification, especially in complex biological fluids like HM. To evaluate the potential matrix effect, calibration curves were constructed in both solvent and matrix-matched conditions. The slopes of the calibration curves were similar for all the analytes. Additionally, the use of the isotopically labeled IS ($^{13}C_6$-L-T4) significantly enhanced the repeatability and reproducibility, as reflected in the low relative standard deviations (%RSD) reported in Table 2. This confirms the robustness of the method. Additionally, sample carryover was absent due to the fiber disposal after each extraction and instrumental drift were monitored and controlled throughout batch analysis.

3.5. Comparison with existing analytical methods

To contextualize the performance of the proposed HF-LPME-UHPLC-Q-TOF method, we compared it with previously reported approaches for THs analysis (**Table S4**), particularly those based on LC-MS/MS and SPE protocols. For instance, the method described by (Z.-M. Li et al., 2020) relies on SPE followed by LC-MS/MS, requiring 2 mL of HM, multi-step sample preparation, and overnight processing. In contrast, the proposed method utilizes only 500 µL of sample and enables analysis within the same day for moderate-sized batches due to the simplicity and rapidity of HF-LPME. Moreover, matrix effects and cross-contamination are also controlled due to the HF-LPME arrangement and HF disposal after each extraction, respectively.

Moreover, our method achieves comparable or lower LODs (0.7–19.8 µg L^{-1}) and intra-day precision (%RSD < 6.4), while avoiding drying and reconstitution steps commonly required in SPE workflows and achieving a reduction in total solvent and sample consumption. While previous methods rarely address chiral separation of L-T_4 and D-T_4, our protocol, combined with IM-MS, successfully allows detecting and quantifying both enantiomers. This chiral resolution is rarely achieved in conventional LC-MS/MS workflows and adds biological relevance to the analytical outcome.

These improvements demonstrate that the proposed method offers not only sensitivity and reproducibility, but also efficiency and a considerable reduction in sample and solvent consumption, making it a robust alternative for routine THs monitoring in biological matrices.

It should be noted that although the method allows for same-day analysis, this refers to small- to medium-scale batches. In our study, a total of 30 human milk samples were analyzed across several days, typically processing 8–10 samples per day. Each analytical day included the preparation of a fresh calibration curve. While the HF-LPME step was performed individually for each sample (15 min), multiple extractions (up to three) could be carried out simultaneously using independent HF systems. Conversely, the LLE pretreatment stage, which includes centrifugation and vortex agitation, was applied in parallel to all

Table 3
Concentration of THs in 30 HM samples by HF-LPME followed by UHPLC-Q-TOF.

Thyroid hormone	MIT	DIT	T_0	T_2	T_3	RT_3	D-T_4	L-T_4
Minimum	2.4	5.0	5.6	20.2	32.1	21.3	115.1	47.9
Maximum	62.0	24.8	34.4	96.5	109.0	179.7	157.5	193.6
Mean	13.7	15.5	14.0	37.4	51.8	65.0	135.3	94.0
Standard Error of Mean	5.6	2.1	2.6	5.8	10.3	12.9	4.7	16.9

samples from the same batch, which significantly optimized handling time.

3.6. Biological significance of D-T4 and L-T4 detection in human milk

The simultaneous detection and quantification of both D-T_4 and L-T_4 enantiomers in HM represents a novel and biologically relevant advancement. While their physiological functions have been extensively characterized elsewhere, the specific occurrence of D-T_4 in HM has not been previously reported, to our knowledge. This observation raises new questions about its origin (whether it reflects passive transfer from maternal circulation, local mammary metabolism, or a stereoselective transport mechanism).

Given that the neonatal period is marked by an intense demand for THs to support growth and neurodevelopment, the enantiomeric composition of HM may influence the pool of bioavailable THs to the infant. Although the extent to which D-T_4 is absorbed or metabolized by the neonatal intestine remains unclear, its presence in HM suggests a potential role (either direct or modulatory) in shaping early endocrine or metabolic responses. Future studies addressing enantiomer-specific uptake and functional outcomes in breastfed infants are needed to clarify its physiological significance.

3.7. Application to human milk samples

As proof of concept, 30 samples of HM were determined by HF-LPME followed by UHPLC-Q-TOF (Table 3). Among the 30 samples, only 5 showed all THs above their LOQ, including T_4 stereoisomers. The order of abundance of the THs was variable but mostly was as follows: D-T_4 > L-T_4 > RT_3 > T_3 > T_2 > DIT>T_0 > MIT. It is important to highlight that the concentration of D-T_4 was always higher than the concentration of L-T_4, and the same happened between RT_3 and T_3 respectively. Furthermore, the levels of T_4 were higher than T_3 levels. Other THs were variable depending on the samples (MIT usually was the lowest TH). L-T_4 and D-T_4 have not been previously determined in HM samples, neither DIT nor T_0.

4. Conclusion

This study presents a robust and efficient method based on HF-LPME combined with UHPLC-Q-TOF for the extraction and quantification of eight THs in HM, including the enantiomers of thyroxine. The proposed approach offers several advantages over conventional analytical techniques, including reduced sample and solvent consumption, simplified preparation steps, and the possibility to quantify eight different THs in the same run. Method validation confirmed excellent precision, recovery and sensitivity.

Notably, the simultaneous detection of both D-T_4 and L-T_4 enantiomers in all analyzed HM samples constitutes a novel finding with potential biological relevance. While L-T_4 is well established as a key hormone involved in neonatal development, particularly in brain maturation, growth, and metabolic regulation, D-T_4 appears to exert more limited and selective effects. This enantiomer-specific activity suggests distinct physiological roles, with L-T_4 being essential for early developmental processes, and D-T_4 potentially contributing to the fine-tuning of metabolic functions during the neonatal period.

Future research should explore the stereoselective transport and metabolism of THs within maternal–infant physiology, identify potential sources of D-T_4, and assess its biological activity and clinical relevance during early developmental stages.

CRediT authorship contribution statement

Rafael de Fátima Vélez-Pérez: Writing – review & editing, Investigation, Formal analysis, Data curation. **Ana Arias-Borrego:** Writing – review & editing, Supervision, Data curation. **Inés Velasco:** Writing – review & editing, Resources, Investigation. **Berta Soldevila:** Writing – review & editing, Resources, Investigation. **Tamara García-Barrera:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the projects PID2021-123073NB-C21 (Spanish Ministry of Science and Innovation (MCIN). Generación del Conocimiento. MCIN/AEI /10.13039/501100011033/FEDER "Una manera de hacer Europa"), UHU-1256905 and UHU-202009 from the FEDER Andalusian operative program (Ministry of Economic Transformation, Industry, Knowledge and Universities, Andalusia, Spain). This study has been also funded by Instituto de Salud Carlos III (ISCIII) through the project PI22/01350, and co-funded by the European Union. The authors are grateful to FEDER (European Community) for financial support through grants UNHU13-1E-1611 and UNHU15-CE-3140. RFVP gratefully acknowledges the support of the Ministry of Science and Innovation (MCIN) for a FPI predoctoral fellowship (PRE2021-102165). Funding for open access charge: Universidad de Huelva / CBUA.

Appendix A. Supplementary data

Supplementary data includes details about the material and method, results and discussion sections. One supplementary figure and four supplementary tables are included in this file. The Supplementary data is available free of charge at https://doi.org/. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.foodchem.2025.145542].

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145542.

Data availability

Data will be made available on request.

References

Aydoğan, C., Çakan, B. B., & Ali, A. (2024). A review on the analysis of chiral molecules as disease biomarkers by LC/MS. *Biomedical Chromatography, 39*(1), Article e6044. https://doi.org/10.1002/bmc.6044

Dorea, J. G. (2002). Selenium and breast-feeding. *British Journal of Nutrition, 88*(5), 443–461. https://doi.org/10.1079/BJN2002692

Dror, D. K., & Allen, L. H. (2018). Iodine in human Milk: A systematic review. *Advances in Nutrition, 9*(1), 347S–357S. https://doi.org/10.1093/advances/nmy020

Eisdorfer, I. B., & Post, A. (1967). Method to estimate small amounts of l-triiodothyronine in d-triiodothyronine. *Journal of Pharmaceutical Sciences, 56*(9), 1092–1097. https://doi.org/10.1002/jps.2600560908

Etling, N., Padovani, E., Fouque, F., & Tato, L. (1986). First-month variations in total iodine content of human breast milks. *Early Human Development, 13*(1), 81–85. https://doi.org/10.1016/0378-3782(86)90101-5

Goglia, F. (2005). Biological effects of 3,5-diiodothyronine (T2). *Biochemistry (Moscow), 70*(2), 164–172. https://doi.org/10.1007/s10541-005-0097-0

Gübitz, G., & Juffmann, F. (1987). Resolution of the enantiomers of thyroid hormones by high-performance lingad-exchange chromatography using a chemically bonded chiral stationary phase. *Journal of Chromatography A, 404*, 391–393. https://doi.org/10.1016/S0021-9673(01)86882-5

Hancu, G., Modroiu, A., Stroia, D. G., & Uiläcan, A. (2024). Analyzing the chiral purity of pharmaceuticals: The application of Cyclodextrin-based chiral selectors in capillary electrophoresis. *Symmetry, 16*(10). https://doi.org/10.3390/sym16101354

Hao, C., Meng, D., Shi, W., Xu, C., Wang, Q., & Kuang, H. (2025). Chiral gold nanostructure monolayers as SERS substrates for ultrasensitive detection of enantiomer biomarkers of Alzheimer's disease. *Angewandte Chemie, International Edition, 64*(21). https://doi.org/10.1002/anie.202502115

Javanmardi, H., Roszkowska, A., & Pawliszyn, J. (2025). New microextraction techniques in exposome research: Bridging environmental exposures and human health. *Trends in Analytical Chemistry, 190*. https://doi.org/10.1016/j.trac.2025.118284

Khan, W. A., Hossein Fallah, S. A., Moltajihagh, S., Hamdi, F., Alipour, F., Mollahosseini, A., & Boczkaj, G. (2025). Automation and high throughput sample analysis with various platforms in microextraction techniques: A need for ecofriendly, green, and cost-effective sample preparation approaches – A review. *Trends in Analytical Chemistry, 189*, Article 118247. https://doi.org/10.1016/j.trac.2025.118247

Koidl, J., Hödl, H., Schmid, M. G., Konrad, M., Petschauer, S., Kostner, G. M., & Gübitz, G. (2006). Chiral separation of T3 enantiomers using stereoselective antibodies as a selector in micro-HPLC. *Journal of Biochemical and Biophysical Methods, 69*(1), 33–42. https://doi.org/10.1016/j.jbbm.2006.03.015

Kunisue, T., Eguchi, A., Iwata, H., Tanabe, S., & Kannan, K. (2011). Analysis of thyroid hormones in serum of Baikal seals and humans by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and immunoassay methods: Application of the LC-MS/MS method to wildlife tissues. *Environmental Science & Technology, 45*(23), 10140–10147. https://doi.org/10.1021/es203002a

Lanni, A., Moreno, M., Cioffi, M., & Goglia, F. (1992). Effect of 3,3′-diiodothyronine and 3,5-diiodothyronine on rat liver oxidative capacity. *Molecular and Cellular Endocrinology, 86*(3), 143–148. https://doi.org/10.1016/0303-7207(92)90138-V

Lanni, A., Moreno, M., Lombardi, A., De Lange, P., Silvestri, E., Ragni, M., … Goglia, F. (2005). 3,5-Diiodo-L-thyronine powerfully reduces adiposity in rats by increasing the burning of fats. *The FASEB Journal, 19*(11), 1552–1554. https://doi.org/10.1096/fj.05-3977fje

Lee, J., Adhikari, S., Lee, W., & Yoon, H.-R. (2022). *Development of ultra high-performance liquid chromatography-tandem mass spectrometry method for enantiomer resolution of thyroxine on a chiral crown ether derived chiral stationary phase*. https://doi.org/10.21203/RS.3.RS-1957831/V1

Lella, C., Nestor, L., De Bundel, D., Vander Heyden, Y., & Van Eeckhaut, A. (2024). Targeted chiral metabolomics of D-Amino Acids: their emerging role as potential biomarkers in neurological diseases with a focus on their liquid chromatography–mass spectrometry analysis upon chiral derivatization. *International Journal of Molecular Sciences, 25*(22), Article 12410. https://doi.org/10.3390/ijms252212410

Li, P., Hu, B., He, M., & Chen, B. (2014). Ion pair hollow fiber liquid–liquid–liquid microextraction combined with capillary electrophoresis-ultraviolet detection for the determination of thyroid hormones in human serum. *Journal of Chromatography A, 1356*, 23–31. https://doi.org/10.1016/j.chroma.2014.06.046

Li, Z.-M., Albrecht, M., Fromme, H., Schramm, K.-W., & De Angelis, M. (2020). Persistent organic pollutants in human breast Milk and associations with maternal thyroid hormone homeostasis. *Environmental Science & Technology, 54*(2), 1111–1119. https://doi.org/10.1021/acs.est.9b06054

López-Juan, A. L., Moreno-Calleja, L. M., Benedé, J. L., & Chisvert, A. (2025). Dispersive microextraction techniques as efficient strategies for the analysis of saliva: A comprehensive review. *Journal of Pharmaceutical and Biomedical Analysis, 255*, Article 116644. https://doi.org/10.1016/j.jpba.2024.116644

Lucci, E., Falcinelli, G., Antonelli, L., Dal Bosco, C., Felli, N., De Cesaris, M. G., & Gentili, A. (2024). Hydrophobic deep eutectic solvent-ferrofluid microextraction followed by liquid chromatography-mass spectrometry for the enantioselective determination of chiral agrochemicals in natural waters. *Analytical and Bioanalytical Chemistry, 417*, 1341–1357. https://doi.org/10.1007/s00216-024-05619-9

Michalke, B., Schramel, P., & Witte, H. (2000). Iodine speciation in human serum by reversed-phase liquid chromatography-ICP-mass spectrometry. *Biological Trace Element Research, 78*(1), 81–91. https://doi.org/10.1385/BTER:78:1-3:81

Millan-Alanis, J. M., González-González, J. G., Flores-Rodríguez, A., Singh Ospina, N., Maraka, S., Moreno-Peña, P. J., … Rodríguez-Gutiérrez, R. (2021). Benefits and harms of levothyroxine/L-triiodothyronine versus levothyroxine monotherapy for adult patients with hypothyroidism: Systematic Review and Meta-Analysis. *Thyroid®, 31*(11), 1613–1625. https://doi.org/10.1089/thy.2021.0270

Naccarato, A., Elliani, R., & Tagarelli, A. (2025). Microextraction and eco-friendly techniques applied to solid matrices followed by chromatographic analysis. *Separations, 12*(5), 124–147. https://doi.org/10.3390/separations12050124

Papp, L. A., Szabó, Z. I., Hancu, G., Farczádi, L., & Mircia, E. (2024). Comprehensive review on chiral stationary phases in single-column simultaneous Chiral–Achiral HPLC separation methods. *Molecules, 29*(6), 1346–1374. https://doi.org/10.3390/molecules29061346

Peluso, P., Sechi, B., & Jibuti, G. (2024). Enantioseparation of organometallic compounds by electromigration techniques. *Electrophoresis, 45*(11–12), 1018–1032. https://doi.org/10.1002/elps.202300231

Prasad, B. B., Tiwari, M. P., Madhuri, R., & Sharma, P. S. (2010). Enatioselective quantitative separation of d- and l-thyroxine by molecularly imprinted micro-solid phase extraction silver fiber coupled with complementary molecularly imprinted polymer-sensor. *Journal of Chromatography A, 1217*(26), 4255–4266. https://doi.org/10.1016/j.chroma.2010.04.055

Reinhardt, W., Luster, M., Rudorff, K. H., Heckmann, C., Petrasch, S., Lederbogen, S., … Mann, K. (1998). Effect of small doses of iodine on thyroid function in patients with Hashimoto's thyroiditis residing in an area of mild iodine deficiency. *European journal of endocrinology, 139*(1), 23–28. https://doi.org/10.1530/eje.0.1390023

Robison, L. M., Sylvester, P. W., Birkenfeld, P., Lang, J. P., & Bull, R. J. (1998). Comparison of the effects of iodine and iodide on thyroid function in humans. *Journal of toxicology and environmental health. Part A, 55*(2), 93–106. https://doi.org/10.1080/009841098158539

Rohner, F., Zimmermann, M., Jooste, P., Pandav, C., Caldwell, K., Raghavan, R., & Raiten, D. J. (2014). Biomarkers of nutrition for development—Iodine review. *The Journal of Nutrition, 144*(8), 1322S–1342S. https://doi.org/10.3945/jn.113.181974

Scanu, A., Toth, J., Edelstein, C., Koga, S., & Stiller, E. (1969). Fractionation of human serum high density lipoprotein in urea solutions. *Evidence for polypeptide heterogeneity. Biochemistry, 8*(8), 3309–3316. https://doi.org/10.1021/bi00836a027

Van Wassenaer, A. G., Stulp, M. R., Valianpour, F., Tamminga, P., Ris Stalpers, C., De Randamie, J. S. E., … De Vijlder, J. J. M. (2002). The quantity of thyroid hormone in human milk is too low to influence plasma thyroid hormone levels in the very preterm infant. *Clinical Endocrinology, 56*(5), 621–627. https://doi.org/10.1046/j.1365-2265.2002.01526.x

Wainer, I. W. (1993). The impact of new liquid chromatography chiral stationary phase technology on the study of stereoselective pharmacokinetics. *Trends in Analytical Chemistry, 12*(4), 153–158. https://doi.org/10.1016/0165-9936(93)87017-R

Yan, J., Huang, J., Peng, S., Sun, D., Lu, W., Song, Z., Ma, J., You, J., Fan, H., Chen, L., & Li, J. (2025). Recent advances in molecular-imprinting-based solid-phase microextraction for determination of pharmaceutical residues. *Journal of Chromatography A, 1754*, Article 466106. https://doi.org/10.1016/j.chroma.2025.466016

Zhao, L. Z., Cao, H. L., He, Z. Q., Sun, Y., Fang, L. L., & Li, W. L. (2025). Recent advances in green solvents-based liquid-phase microextraction techniques for chromatographic analysis of active components in traditional Chinese medicine. *Journal of Chromatography A, 1741*, Article 465604. https://doi.org/10.1016/j.chroma.2024.465604

Zoeller, R. T., & Rovet, J. (2004). Timing of thyroid hormone action in the developing brain: Clinical observations and experimental findings. *Journal of Neuroendocrinology, 16*(10), 809–818. https://doi.org/10.1111/J.1365-2826.2004.01243.X