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Analysis of thyroid hormones in raw and treated waste water

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ABSTRACT

An analytical method for the quantification of thyroid hormones (3,5,3′,5′-tetraiodo-L-thyronine, 3,3′,5-triiodo-L-thyronine, 3,3′,5′-triiodothyronine, 3,5-diiodothyronine, 3,3′-diiodothyronine) in different water matrices has been developed. The method, consisting of solid phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS), was validated for tap and surface water as well as raw and treated waste water. The limits of quantifications (LOQs) were lowest in tap water, where they ranged from 1.1 to 13.3 ng L^{-1}, and highest in raw wastewater (10.5–84.9 ng L^{-1}). Of the target analytes 3,5,3′,5′-tetraiodo-L-thyronine (T_4) could be quantified in the influent and effluent of a waste water treatment plant (WWTP) in Finland. The study showed that despite a relatively high removal rate during treatment (66%), part of the incoming T_4 will reach the aquatic environment and, due to the high endocrine activity of this compound, further studies are needed in order to assess its environmental fate and impact on natural ecosystems.

1. Introduction

A vast number of studies worldwide have demonstrated that human active pharmaceutical ingredients (APIs) enter the aquatic environment predominantly via waste water treatment plant (WWTP) effluents [1,2]. Although the environmental concentrations normally are low (ng L^{-1}), some APIs such as the hormonal agent ethinylestradiol have been found to cause endocrine disruption at levels as low as 0.1 ng L^{-1} [3]. The occurrence of both thyroid and estrogen disrupting compounds in influents of several WWTPs has recently been reported [4]. Enhanced biological effects have also been observed when test organisms have been exposed to mixtures of APIs instead of single compounds [5]. As it is highly unlikely that APIs will reach concentrations in the environment at which they may pose an acute toxic threat to organisms, the major concern expressed over APIs relates to the potential hazardous effects caused by chronic exposure either to some specific compounds or to multi-component mixtures; subjects which are still largely uninvestigated. Therefore, there is an evident need both to monitor their occurrence in the environment, which requires development of analytical methods, and to assess the fate and potential ecological risks of these substances.

Thyroid hormones, which are found in all chordate animals, have important roles in many physiological processes e.g. embryonic development, cell differentiation, metabolism, and the regulation of cell proliferation [6]. Thyroxine (3,5,3′,5′-tetraiodo-L-thyronine, T_4) is the major thyroid hormone secreted from the thyroid gland. People who suffer from hypothyroidism are prescribed medicinal T_4 in order to maintain a normal plasma concentration. The synthetic form of T_4 (levothyroxine) has the same stereochemical configuration as the natural hormone, i.e. both adopt an S-configuration at the asymmetric carbon. Liothyronine (3,3′,5-triiodo-L-thyronine, T_3) is the more active of the thyroid hormones and is also used in the treatment of hypothyroidism. T_4 is converted to T_3 in the kidneys and liver through deiodination at the phenolic ring, although formation of 3,3′,5′-triiodothyronine (r-T_3) also occurs to some extent via deiodination at the alkyl substituted phenyl ring. Further deiodination of T_3 and r-T_3 yields 3,5-diiodothyronine (3,5-T_2) and 3,3′-diiodothyronine (3,3′-T_2). However, there are studies suggesting that the metabolic deiodination of T_4 and T_3 does not result in complete loss of biological activity. For example, 3,5-T_2 has been shown to affect mitochondria and bioenergetic mechanisms, ion-exchangers, enzymes, as well as the transcription of some genes [7]. In addition, both r-T_3 and 3,3′-T_2 have been demonstrated to have a stimulating effect on thyroid hormone regulated functions by acting via the same receptors that mediate T_3 actions [8].

The estimated normal daily metabolic turnover of T_4 is approximately 90 μg, of which ~80% is converted to T_3 and ~20% excreted through the bile, mainly as sulfate and glucuronide conjugates [9]. Following biliary excretion T_4 glucuronides may be hydrolyzed back to the aglycone by bacterial enzymes (β-glucuronidases) in the large intestine, which explains the presence of endogenous T_4 in feces [10]. T_4 concentrations in feces of normal humans have been reported to be 1.03 ± 0.64 nmol g^{-1} ($n = 6$) feces [9], while the reported mean daily urinary excretion of unconjugated T_4, T_3, r-T_3,

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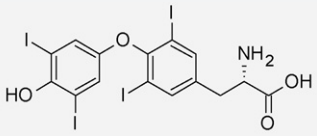
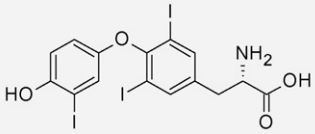
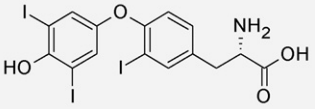
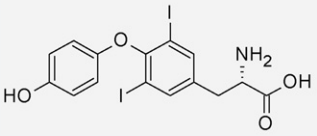
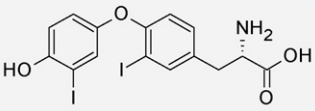
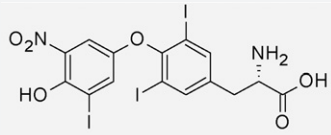
Table 1
Physico-chemical properties of the studied APIs.

Compound	MW (g mol^{-1})	Structure	pK$_a$	Consumption in Finland (kg year^{-1})	References
T_4	776.7		2.40 (COOH)	7.28	[18,19]
			6.87 (Ph–OH) 9.96 (NH_2)		[18]
T_3	650.8		8.4 (Ph–OH)	Not sold	[20]
r-T_3	650.8		n.a.	–	
3,5-T_2	524.9		n.a.	–	
3,3′-T_2	524.9		n.a.	–	
Nitro-T_3 (IS)	695.8		n.a.	–	

3,5-T_2 and 3,3′-T_2 by healthy humans is 1.41, 0.63, 0.06, and 0.52 μg, respectively [11]. Considering that a human on an ordinary diet produces on average 25 g of feces (dry weight) per day [12] the daily fecal excretion of unconjugated T_4 amounts to 20 ± 12 μg. Deconjugation of glucuronide conjugates of natural and synthetic estrogens has been reported to take place in raw waste water, probably due to the presence of significant amounts of the β-glucuronidase enzyme produced by fecal bacteria [13,14]. Consequently, deconjugation of glucuronides to the free hormones in raw waste water may affect the observed removal rate of the compounds during treatment [13]. Since β-glucuronidases are responsible for the *in vivo* deconjugation of T_4-conjugates, deconjugation to free T_4 may also be expected to take place in the waste water treatment facilities.

Despite a rather low predicted environmental concentration (PEC = 19 ppt) T_4 has been recognized as a potential waste water contaminant of considerable environmental concern [15]. The high potency of T_4 may be illustrated by comparing the therapeutic dose (defined daily dose, DDD) of a common anti-inflammatory agent such as ibuprofen (DDD = 1.2 g, ATC code: M01AE01) with that of T_4 (DDD = 0.15 mg, ATC code: H03AA01) [16]. Consequently, it is important to acknowledge that the environmental concentration-levels of potential concern may vary significantly between APIs, depending on the biological activity of the drugs.

The aim of this study was to develop a method for identification and quantification of the thyroid hormones T_4, T_3, r-T_3, 3,5-T_2 and 3,3′-T_2 in influents and effluents of WWTPs, surface water and tap water. The method is based on solid phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for separation and detection. Recently, the applicability of SPE and LC–MS/MS to the analysis of thyroid hormones in the thyroid gland was successfully demonstrated [17], however, to the best of our knowledge this is the first method for the simultaneous quantitative analysis of these compounds in the different water matrices.

2. Experimental

2.1. Chemicals

T_4 (CAS: 6106-07-6), T_3 (CAS: 55-06-1), r-T_3 (CAS: 5817-39-0), 3,5-T_2 (CAS: 1041-01-6) and 3,3′-T_2 (CAS: 4604-41-5) were purchased from Sigma–Aldrich (Schnelldorf, Germany). The internal standard 2-amino-3-[4-(4-hydroxy-3-iodo-5-nitrophenoxy)-3,5-diiodophenyl]propanoic acid (IS) was synthesized according to the procedure described in Section 2.5. All standards were of ≥95% purity (Table 1). All organic solvents were of HPLC or LC–MS grade. Stock solutions of 0.1–1 g L^{-1} were prepared in methanol (MeOH) and stored at −18 °C. Working standard solutions were prepared by dilution of the stock solutions with MeOH. All glassware were washed with concentrated solutions of sodium hydroxide and sulfuric acid, hot water and rinsed with distilled water and acetone prior to use.

2.2. Solid phase extraction (SPE)

The analytes were extracted from water samples with Waters Oasis HLB extraction cartridges (0.2 g, 6 cc). The sample volume was 500 mL for tap water and surface water, and 250 and 100 mL for WWTP-effluent and WWTP-influent samples, respectively. Surface water, effluent and influent water were filtered through 0.45 μm glass fiber filters (GF 6, Whatman)-prewashed with methanol and distilled water-prior to extraction. Following adjustment of the water solutions to pH ~ 2.5, using concentrated HCl and 10% NaOH, 250 ng of the IS was added. The SPE cartridges were preconditioned with 6 mL of MeOH and 6 mL of distilled water (pH 2.5) before extraction. Extraction was carried out at an approximate flow rate of 5–10 mL min^{-1} through the PTFE tubes, depending on the sample. After drying the cartridges over a gentle stream of nitrogen gas the analytes were eluted with 4 mL × 1 mL of MeOH. A reactitherm heating module (50 °C) from Pierce (Rockford, IL, USA) was used for evaporation of the solvent. The analytes were redissolved in 150 μL MeOH and 150 μL 0.2% formic acid. All samples were analyzed immediately after preparation.

2.3. Liquid chromatography

The Agilent 1100 system (Agilent Technologies, Espoo, Finland) used consisted of a binary pump, a vacuum degasser, an autosampler, and a thermostated column oven (set to 30 °C). Chromatography was achieved on a reversed phase phenyl column (ACE 5 μm phenyl, 125 mm × 2.1 mm) from Advanced Chromatography Technologies (Aberdeen, Scotland). The analytical column was equipped with a guard column of the same material and from the same company. Sample aliquots of 30 μL were introduced into the column at a flow rate of 0.350 mL min^{-1}. The analytes were separated using aqueous (0.2%) formic acid (A) and 0.2% formic acid in a 1:1 mixture of MeOH:ACN (B). The initial condition (10% B) was kept for 1 min, after which the concentration of B was linearly increased to 85% over 15 min. The initial condition was then reestablished over 1 min and the column was conditioned for 6 min prior to the next injection.

2.4. Mass spectrometry

A Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ionization (ESI) source operating in positive ionization mode, was used for detection of the analytes. The instrument was set to operate in multiple reaction monitoring mode (MRM), recording parent–daughter transitions at a dwell time of 0.2 s and an inter-channel delay of 0.2 s. Argon was used as collision gas at a collision cell pressure of 4.98×10^{-3} mbar. Optimization of the MS and MS/MS dependent parameters (Table 2) was achieved by introducing pure standard solutions (5 μg mL^{-1}) of the analytes into the ESI at a flow rate of 10 μL min^{-1}. Nitrogen was used as desolvation and nebulizing gas at 700 and 20 L h^{-1}, respectively. The source temperature was 120 °C and the desolvation temperature was 325 °C.

2.5. Synthesis of 2-amino-3-[4-(4-hydroxy-3-iodo-5-nitrophenoxy)-3,5-diiodophenyl]propanoic acid (IS)

To a stirred solution of T_4 (0.1 mmol, 90 mg) in 2.5 M H_2SO_4 and tetrahydrofuran (1:1, 8 mL) at 0 °C was added drop wise an aqueous solution (1 mL) of sodium nitrite (0.13 mmol, 9 mg). The reaction mixture was kept at 0 °C for 1 h, after which it was slowly brought to room temperature and allowed to react for an additional 4 h. The mixture was diluted with water and the precipitate was separated and dissolved in MeOH and water (~4:1). The product was purified with semi-preparative HPLC on a reversed-phase phenyl column (ACE 5 μm phenyl, 250 mm × 10 mm) from Advanced Chromatography Technologies. Finally, the pure product was recrystallized in MeOH/H_2O and dried. Yield = 43.8%, 30.5 mg. ^{1}H NMR (399.75 MHz, DMSO-d_6, 25 °C) δ = 7.87 (s, 2H, H-2 and H-6), 7.65 (d, ^3J = 3.1 Hz, 1H, H-5′), 7.21 (d, ^3J = 3.1 Hz, 1H, H-3′), 4.05 (dd, ^3J = 8.3, 5.5 Hz, 1H, CH), 3.18 (dd, 14.6, 5.5 Hz, 1H, CH_2), 2.95 (dd, 14.6, 8.3 Hz, 1H, CH_2), ^{13}C NMR (100.52 MHz, DMSO-d_6, 25 °C) δ = 170.00 (COOH), 151.71 (C-3, C-5), 148.05 (C-4′), 147.80 (C-1′), 141.15 (C-2, C-6), 137.55 (C-4), 135.28 (C-2′), 132.46 (C-5′), 110.48 (C-3′), 92.76 (C-6′), 92.01 (C-1), 53.17 (CH), 34.06 (CH_2). HRMS (ESI+): 696.7806; 696.7824 ($C_{15}H_{12}I_3N_2O_6$, calc. m/z); error 2.6 ppm.

2.6. Quantification and method validation

For verification of compound identities the retention times and the parent–daughter ion transitions obtained for the reference compounds were used. A calibration curve ranging from 5 to 1000 ng L^{-1} (7 points) for each analyte was obtained by spiking non-contaminated surface water (500 mL) with the analytes and the IS (250 ng) prior to SPE-extraction. The calibration curves were plotted as the ratios of the analyte to IS peak area (analyte/IS) vs. concentration. The instrumental quantification limit (IQL) and instrumental detection limit (IDL) were determined by analyzing pure standards of decreasing concentration with the LC–MS/MS method. A signal to noise ratio (S/N) of 10 and 3 was used to define the IQL and IDL, respectively. To evaluate the repeatability of the LC–MS/MS system a standard solution (c = 100 μg L^{-1}) was analyzed ten consecutive times. The limit of quantification (LOQ) for the complete method in the respective water matrix was calculated according to the equation:

$$\mathrm{LOQ} = \frac{\mathrm{IQL} \times 100}{Rec \times C} \quad (1)$$

where IQL is the instrumental limit of quantification (ng L^{-1}), Rec is the absolute recovery of the analyte (%), and C is the concentration factor (1667, 1667, 833, 333 for tap water, surface water, WWTP-effluent and WWTP-influent, respectively). For calculation of the LODs, the same equation as shown above was applied by inserting IDL instead of IQL. Absolute recoveries for all analytes were determined by dividing the peak areas of SPE-concentrated spiked water samples with those of reference samples prepared directly in the eluent. Similarly, relative recoveries were calculated by taking the relationship between the analyte to IS ratios of SPE-concentrated spiked samples and those of reference samples prepared by dilution in the eluent.

Table 2
LC–MS/MS parameters for each analyte.

Compound	Retention time (min)	Parent ion (m/z)	Daughter ion (m/z)	Cone voltage (V)	Collision energy (eV)
T_4	15.0	777.4	732	28	21
T_3	13.9	651.7	606	28	23
r-T_3	14.4	651.7	606	28	23
3,5-T_2	12.2	525.7	480	28	20
3,3′-T_2	13.2	525.7	480	28	20
IS	15.8	696.5	650.9	31	23

2.7. Sample collection

The influent and effluent water were collected in February 2010 from a WWTP processing waste water from approximately 300,000 residents living in Turku and its environs. The incoming waste water is treated according to a five-step procedure: (1) screening (debris and sand removal); (2) primary clarification (sedimentation); (3) activated sludge process (biological treatment); (4) aeration (enhancement of microbial activity: ferrous sulfate addition, oxygenation); (5) secondary clarification (biomass removal, sand filtration). Samples from influent and effluent water were prepared (filtered and extracted) within 4 h upon collection and analyzed immediately after preparation. The surface water was collected from Lake Kakskerta, a secluded lake about 15 km south-southwest of central Turku, with 1390 permanent and about 4900 holiday residents living around the lake area. Waste water (black water) from these households is mostly collected in septic tanks, although outside lavatories may be commonly used by holiday residents.

3. Results and discussion

3.1. SPE

The retentive ability of Waters Oasis HLB, containing a polymeric sorbent made up of divinylbenzene and *N*-vinylpyrrolidone units, was evaluated for extraction of T_4 and T_3 at pH 2.5, 6 and 10. Distilled water (500 mL) at the respective pH was spiked with T_4 and T_3 (50 ng L^{-1}) and extracted. No retention was observed at pH 10 for neither of the analytes and the best results were observed at slightly acidic conditions (pH 2.5). Another stationary phase material, i.e. Waters Oasis MAX (mixed mode anion exchanger), was also tested for extraction of T_4 and T_3 from distilled water (500 mL). The MAX cartridges were treated according to a previously described procedure successfully employed for the analysis of beta-lactam antibiotics in waste water [21]. It was found that the HLB (at pH 2.5) provided significantly better recoveries (~80% for HLB vs. ~50% for MAX) for both compounds and was selected for use in the further work.

The absolute recoveries of T_4 and T_3 after extraction with HLB-cartridges were 75 ± 3% and 82 ± 8%, respectively (n = 3). Distilled water (DW) is virtually free from all impurities and thus the influence of coeluting matrix components on the LC–MS/MS analyte signal intensities should be minimal. Therefore, the SPE-recoveries from spiked DW may be considered representative of the ability of the solid phase material to retain the studied compounds. On the basis of the observed signal intensity, it could be concluded that about 20% of T_4 and T_3 was not recovered by the SPE procedure. At pH 2.5 the amino group will be protonated and hence positively charged (Table 1) and since charged molecules normally tend to adsorb less readily to the sorbent, this may be a plausible explanation for the losses during SPE. Poor SPE-recoveries of other amphoteric pharmaceuticals has also been reported previously [22].

3.2. LC–MS/MS

The ACE 5 μm phenyl column used provided excellent peak shapes and good separation of the analytes, despite their close structural resemblance (Fig. 1). Phenyl columns, although not being used as frequently as their aliphatic counterparts (e.g. C4, C8 and C18), offer an additional retention mechanism (aside from the hydrophobic interactions) by introducing the possibility of π–π interactions between the stationary phase and the unsaturated analyte molecule [23]. When a C18 column was used significant tailing of the analyte peaks was observed, however, changing the stationary phase to phenyl eliminated this problem. We found that by using acetonitrile (with 0.2% formic acid) as the organic solvent nice peak shapes could be achieved, but the separation of the analytes, especially of the two isomeric pairs (T_3/r-T_3 and 3,5-T_2/3,3′-T_2), was not satisfactory. Methanol, instead, gave better separation but slightly broader peaks. Thus the needed separation and sharpness of the peaks could be achieved with a 1/1 mixture of methanol and acetonitrile with an addition of 0.2% formic acid. Acetonitrile has previously been found to impede the selective π–π interactions between analytes and the phenyl groups in the stationary phase, whereas the use of methanol as an organic modifier has been shown to improve the selectivity based on π–π interactions [23].

The presence of an acid additive (0.2% formic acid) in the eluent was found to further improve the chromatographic behavior of the analytes. At the pH of the aqueous part of the eluent (~2.5) T_4 is quite hydrophobic [18], which enables good retention to the stationary phase upon introduction into the analytical column. In addition, the presence of an acidic buffer facilitated the formation of positive ions during ESI.

The MRM transitions for each compound were optimized upon introducing pure standards into the ESI-interface followed by fragmentation of the resulting $[M+H]^+$ ions in the collision cell of the mass spectrometer (Table 2). The most stable and intense daughter ion was chosen for monitoring. In all cases, a mass loss of 46 Da, corresponding to the elimination of water and carbon monoxide [$H_2O + CO$], was observed. α-Cleavage at the amino acid side chain with subsequent formation of the immonium ion $[RCH{=}NH_2]^+$ is a characteristic fragmentation behavior of protonated α-amino acids upon collision induced dissociation (CID) [24]. The $[RCH{=}NH_2]^+$ ions formed in this study were highly abundant in the spectrum and a further increase of the collision energy generally resulted in very unstable fragment ions, which were not considered suitable for monitoring. Both cone and collision energies were optimized separately for each compound (Table 2).

3.3. Method validation

The repeatability of the LC–MS/MS method was assessed by analyzing a standard solution (100 μg L^{-1}) 10 consecutive times. The relative standard deviations obtained ranged from 6 to 9% and the method was considered repeatable. The linearity ($R^2 > 0.99$) of the LC–MS/MS system was assessed over the concentration range 1–1000 μg L^{-1} by injection of pure standard solutions. All compounds were found to give a linear signal response over the investigated range except 3,3′-T_2 and r-T_3, which were linear from 7.5 to 1000 μg L^{-1}. The calibration curves for the off-line SPE LC–MS/MS method were prepared in unpolluted surface water and in this matrix all analytes displayed R^2-values > 0.999 over the concentration range 5–1000 ng L^{-1}. In order to attest method linearity a statistical F-test was applied to two series of measurements and for all analytes values close to one (≥0.95) were observed.

LOQs were calculated for the analytes in each of the different matrices (tap water, surface water, WWTP-effluent and WWTP-influent water) with Eq. (1) (Table 3). T_4 showed the lowest LOQs in all matrices except the influent water, where T_3 could be quantified at a slightly lower concentration (Table 3). The IQLs and IDLs, presented as injected amounts of each API, ranged from 20 to 186 pg and 6 to 56 pg, respectively (Table 3).

Absolute and relative recoveries of the analytes were determined by analyzing three spiked samples of each matrix with the off-line SPE LC–MS/MS method. The spiked amount was 50, 500, 500 and 1000 ng for tap water, surface water, influent water and effluent water, respectively (Table 4). For each water sample, three

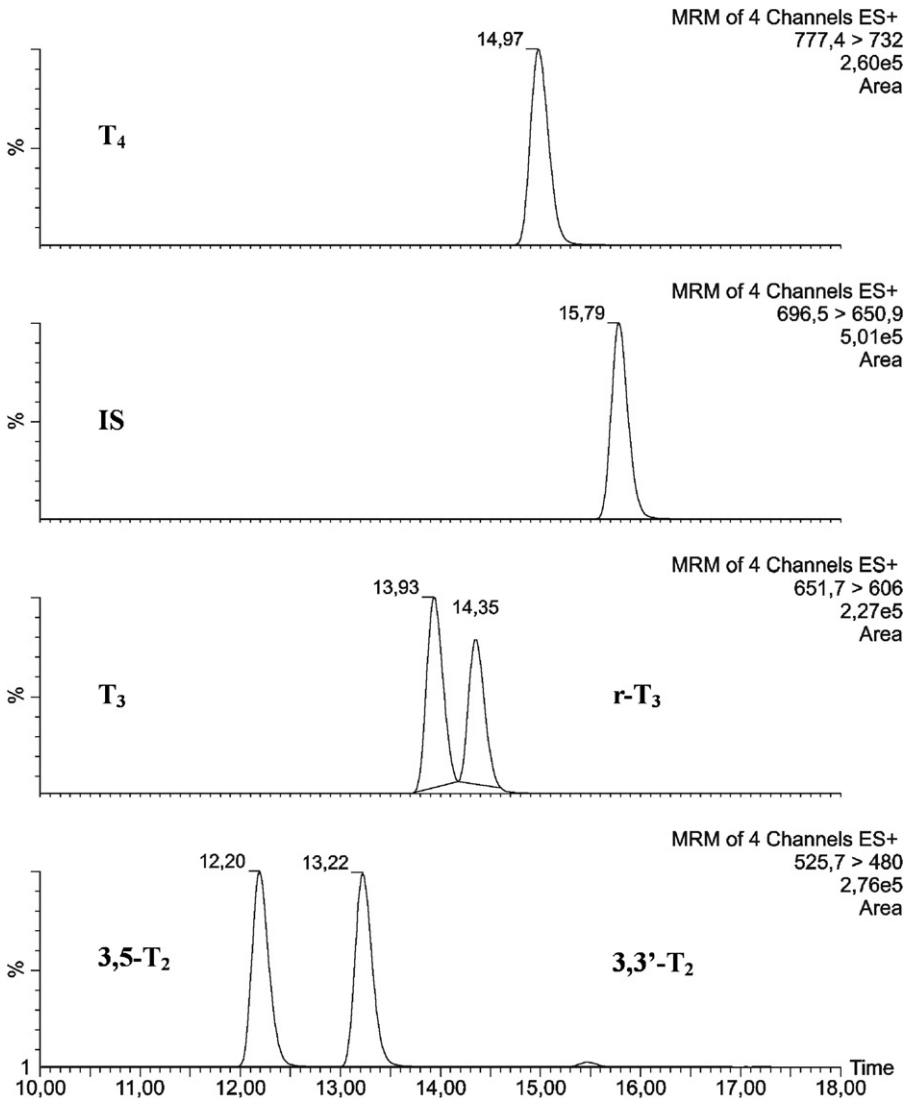


Fig. 1. LC–MS/MS chromatogram of a SPE-extracted spiked WWTP effluent sample.

Table 3
Method validation parameters.

Compound	IDL (pg injected)	IQL (pg injected)	LOQ/LOD (ng L^{-1})			
			TW	SW	WWTP effluent	WWTP influent
T_4	6	20	1.1/0.3	1.9/0.6	5.5/1.6	16.4/4.9
T_3	8	26	1.4/0.4	2.8/0.9	6.1/1.9	12.9/4.0
r-T_3	38	127	9.9/3.0	10.8/3.2	25.1/7.5	67.6/20.2
3,5-T_2	7	22	2.0/0.6	3.0/1.0	6.3/2.0	10.5/3.3
3,3′-T_2	56	186	13.3/3.9	18.9/5.7	48.3/14.5	84.9/25.6

Table 4
SPE-recovery data ($n = 3$) for the selected compounds and the dissolved organic carbon (DOC) content of each matrix.

Matrix	DOC (mg L^{-1})	Recovery (%)					
			T_4	T_3	r-T_3	3,5-T_2	3,3′-T_2
TW	1.93	AR	38 ± 9	36 ± 5	26 ± 4	22 ± 10	29 ± 7
		RR	95 ± 8	90 ± 3	64 ± 2	54 ± 9	71 ± 5
SW	229.9	AR	21 ± 6	19 ± 1	23 ± 2	15 ± 6	20 ± 5
		RR	67 ± 5	62 ± 7	77 ± 6	48 ± 8	65 ± 11
Effluent	130.5	AR	15 ± 1	17 ± 1	20 ± 3	14 ± 1	15 ± 4
		RR	50 ± 8	59 ± 10	69 ± 11	47 ± 11	53 ± 13
Influent	60.1	AR	12 ± 2	20 ± 1	19 ± 1	21 ± 2	22 ± 1
		RR	76 ± 6	127 ± 2	117 ± 1	132 ± 7	137 ± 5

Abbreviations: TW: tap water, SW: surface water, AR: absolute recovery, RR: relative recovery.

independent treatments were carried out. Although the absolute recoveries for all analytes were quite poor, the relative recoveries ranged from good to excellent (Table 4), indicating a good ability of the IS in compensating for analyte losses during SPE and signal intensity losses during LC–MS/MS analysis. In order to estimate the influence of the matrix on the loss of signal intensity during LC–MS/MS, three effluent samples (250 mL) were SPE-extracted according to the described procedure. Effluent water was selected due to the fact that the worst analyte recoveries were observed in this matrix. Two of these samples were spiked with IS (250 ng), whereas the remaining sample was extracted without added IS. One of the samples containing IS was prepared as normally (reference), while the extracts of the other two were combined, evaporated and diluted to the same volume as the reference. Finally, the two samples were analyzed by the LC–MS/MS system and the signals of the IS were compared. It was concluded that 50% of the signal response was lost for the sample containing twice the amount of extract. As there was no distinguishable decrease in the solubility of the extract (no precipitate), ion suppression during ESI, caused by components of the SPE-extract, is believed to be a major cause of the observed signal-intensity losses. Consequently, ion suppression during LC–MS/MS may significantly affect the calculation of SPE recoveries.

3.4. Samples

Of the studied thyroid hormones only T_4 could be found in the influent and effluent of the WWTP. The measured concentration of T_4 in the influent was 64 ng L^{-1} and in the effluent 22 ng L^{-1}, which gives a removal rate of approximately 66%. Theoretically, influent concentrations of APIs may be roughly estimated by the following equation [25]:

$$C = \frac{A \times P \times e}{365 \times Q}$$

where C is the theoretically calculated concentration of an API in the WWTP influent (g m^{-3}), A is the amount of API used per year and per capita (g inh^{-1} $year^{-1}$), P is the number of inhabitants serviced by the WWTP, 365 is the number of days per year, e is the fraction of the API excreted unchanged and Q is the influent flow (m^3 d^{-1}). Calculated values obtained using the above equation for several common APIs have previously shown good agreement with measured concentrations [25]. It is possible to conduct a similar calculation for unconjugated T_4, providing a few assumptions are made: (1) no net contribution from medicinal T_4, i.e. medicinal T_4 is assumed to induce normal T_4-levels in hypothyroid patients and; (2) the daily average (dry) weight of feces is 25 g [12]. The calculation is made on the basis of available data on daily excretions of unconjugated T_4 through feces and urine of normal, healthy individuals [10,11]. The following values were inserted into the equation: $A = 7.82 \times 10^{-3} \pm 4.84 \times 10^{-3}$ g inh^{-1} $year^{-1}$; $P = 300{,}000$ inh; $e = 1$; $Q = 100{,}000$ m^3 d^{-1}. Performing the calculation for the studied WWTP a value of 64 ± 40 ng L^{-1} is obtained. Despite the large deviation, it may be concluded that the measured and calculated concentrations are similar in magnitude and, consequently, provides further assurance of the reliability of the method. Also, the measured effluent concentration of T_4 is comparable to those reported for other natural hormones [13,26].

4. Conclusion

A method consisting of off-line SPE and LC–MS/MS has been developed for quantitative analysis of thyroidal hormones in tap water, surface water and in WWTP influents and effluents.

Of the target analytes only T_4 could be detected and quantified in the raw and treated waste water. The measured effluent concentration of T_4 can be argued to be low, but the compound may yet be of environmental significance due to its exceptional high biological activity and to its steady occurrence in waste water effluents. Therefore, the potential hazards to aquatic organisms and the environmental fate of T_4 need to be assessed.

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