Detection and Quantification of 3,5,3′-Triiodothyronine and 3,3′,5′-Triiodothyronine by Electrospray Ionization Tandem Mass Spectrometry

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A novel and rapid method for identifying and quantifying 3,5,3′-triiodothyronine (T3) and 3,3′,5′-triiodothyronine (rT3; reverse T3) has been introduced using electrospray ionization tandem mass spectrometry (ESI-MS/MS). MS^2 spectra in either negative ionization mode or positive ionization mode can be used to differentiate T3 and rT3. Quantification of the T3 and rT3 isomers under the negative ionization mode is also achieved without prior separation by HPLC. (J Am Soc Mass Spectrom 2005, 16, 1781–1786) © 2005 American Society for Mass Spectrometry

The thyroid hormones, 3,5,3′,5′-tetraiodothyronine (T4), 3,5,3′-triiodothyronine (T3), and 3,3′,5′- triiodothyronine (rT3; reverse T3) (Figure 1), are important in regulating a number of biological processes, including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis, and fetal neurodevelopment [1]. T4 is secreted by the thyroid gland. Both T3 and rT3, triiodo analogues of T4, are mainly produced by peripheral deiodination of T4 [2].

T3 is considered to be the most metabolically active thyroid hormone. Although some T3 is produced in the thyroid, 80–90% is generated outside the thyroid, primarily by removal of an iodine atom from the outer phenolic ring of T4 in the liver and kidneys [3, 4]. The pituitary and nervous systems are also capable of converting T4 to T3, and thus these tissues are not dependent on T3 produced in the liver or kidney. Alternatively, T4 can be converted to rT3 by removal of an iodine atom from the tyrosine ring of T4. Under normal conditions, 45–50% of the daily production of T4 is transformed into rT3 [3]. rT3 is currently thought not to have hormonal activity itself, but rather to act as a major competitive inhibitor of T3 activity at the cellular level [5]. There are three known deiodinases in vertebrates. D1, found mostly in thyroid, liver, and kidney but widely expressed in other tissues also, is selenium-sensitive and can catalyze both outer and inner ring deiodinations. Its preferred substrate is rT3, which it further deiodinates to 3,3′-diiodothyronine (3,3′-T2) by outer ring deiodination, but it can also convert T4 to T3 or to rT3 [6]. D2, found mainly in pituitary, brain, brown adipose tissue, and placenta, catalyzes only outer ring deiodinations, and its preferred substrate is T4. Thus, its main function is to produce T3 locally for use in specific tissues [6]. D3, found mainly in liver, brain, and placenta, is also selenium-dependent, but catalyzes only inner ring deiodinations. It primarily inactivates T4, converting it to rT3, and T3, converting it to 3,3′-T2 [6]. Sulfations of T4, T3, or rT3 can also affect their responsiveness to the various deiodinases [6]. In addition, experimental data suggest that rT3 may inhibit deiodinases, thereby directly interfering with the generation of T3 from T4, or with subsequent breakdown of T3 [5–7]. Thus, the molar ratio of T3 to rT3 is an important diagnostic marker for the metabolism and function of thyroid hormones in clinical chemistry.

Total serum T3 and rT3 measurements are routinely performed using methods based upon immunoassays, an approach with high sensitivity, but which lacks specificity for many analytes [8–11]. In recent years, mass spectrometry (MS) techniques have attracted attention for the analysis of T4 and T3 because they provide high mass accuracy, structural information, and the ability to quantify the hormones [12–19].

A recently developed methodology shows that electrospray ionization tandem mass spectrometry (ESI-MS/MS) can be used for identification and quantification of mixtures of isomers, such as chondroitin sulfate disaccharides, diastereomeric hexosamine monosaccharides, and diastereomeric N-acetylhexosamines isomers [20–22]. However, quantification of T3 and rT3 isomers in mixtures based on the relative abundance of fragment ions in MS^2 spectra has not been reported so far. In this study, we used ESI-MS/MS techniques to dis-

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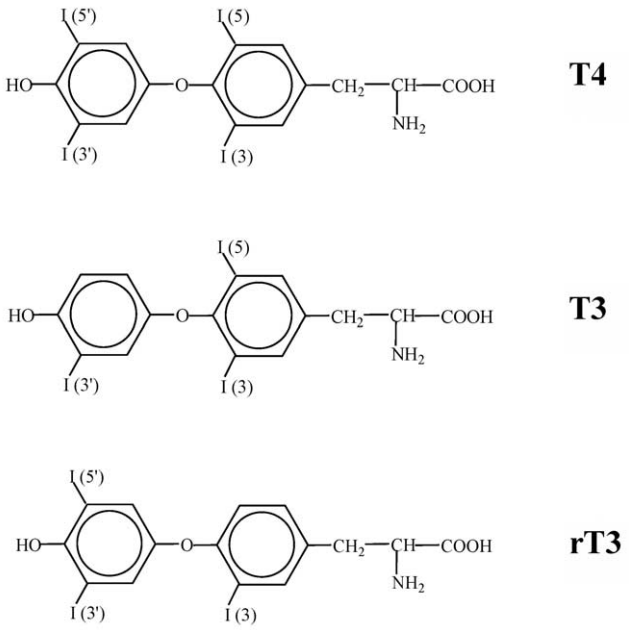

Figure 1. Chemical structures of T4, T3, and rT3.

tinguish T3 and rT3, and directly quantify these isomers without prior separation by HPLC.

Experimental

Materials

Standards of T3 and rT3 were purchased from Sigma (St. Louis, MO). Human serum containing natural levels of thyroxine was purchased from Sigma. Acetic acid and ammonium hydroxide were purchased from Fisher (Santa Clara, CA). Solvents used were of HPLC grade and purchased from Fisher. Oasis MAX SPE™ cartridges were purchased from Waters (Milford, MA). Ultra free-MC centrifugal filter units (5000 NMWL, Millipore) were purchased from Fisher (Pittsburgh, PA).

Preparation of T3 and rT3 Samples

Initially, stock solutions of T3 and rT3 were made in methanol to give a concentration of 1 mg/mL. Standard solutions were made by diluting stock solutions to 1 ng/μL in methanol. Standard solutions were stored at −20 °C and wrapped with aluminum foil.

For positive ionization mode analysis, the diluting solution consists of 2 mL/L acetic acid in methanol-water (55:45 by volume). For negative ionization mode analysis, the diluting solution consists of 2 mL/L ammonium hydroxide in methanol-water (55:45 by volume) [12, 13]. For qualitative analysis, 10 μL of each standard solution was diluted by adding 90 μL of the appropriate dilution solution.

Preparation of Human Serum Using an Oasis MAX SPE Cartridge

Human serum was thawed at room-temperature. 0.5 mL of the serum was made alkaline with 30 μL of 2% ammonium hydroxide, diluted to a total volume of 1 mL with water, and then applied to an Oasis MAX 3 cc (60 mg) SPE cartridge which had been preconditioned by washing consecutively with 3 mL of methanol and then 3 mL water. After the loading step, the first effluent from the cartridge was collected and reloaded to obtain high percentage recovery. The cartridge was then washed with 2 mL of a 2% aqueous solution of ammonium hydroxide followed by 2 mL of methanol. The cartridge was eluted with 2 mL of methanol containing 0.05% hydrochloric acid. The elute was collected and evaporated to dryness at 50 °C under a stream of nitrogen [13]. The evaporated residue was reconstituted in 50 μL of a solution consisting of 2 mL/L ammonium hydroxide in methanol-water (55:45 by volume). Before ESI-MS/MS measurement, the reconstituted sample was applied to an ultra free-MC centrifugal filter unit (3000 NMWL, Millipore) and centrifuged at 3800 *g* for 30 min at 4 °C.

Mass Spectrometry

Mass spectra were obtained using an electrospray ionization source on a quadrupole ion trap instrument

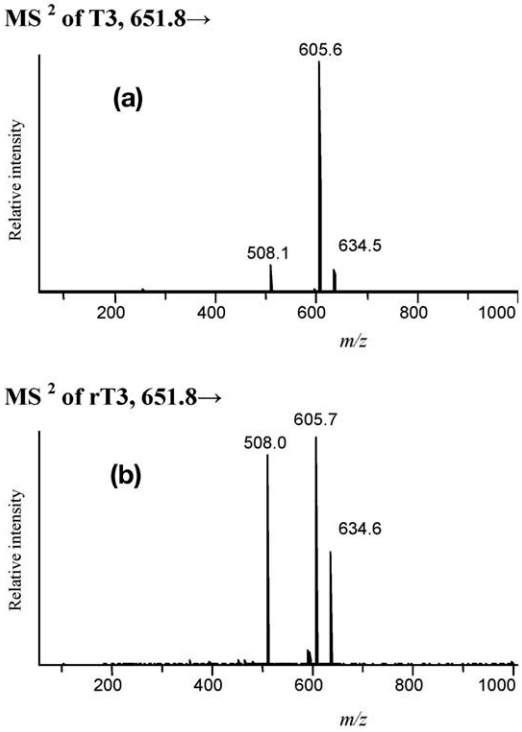

Figure 2. MS^2 spectra of T3 and rT3 under positive ionization. (**a**) For T3, using *m/z* 651.8 as a precursor ion, the fragment ions are observed at *m/z* 634.5, 605.6, and 508.1. (**b**) For rT3, using *m/z* 651.8 as a precursor ion, the fragment ions are observed at *m/z* 634.6, 605.7, and 508.0.

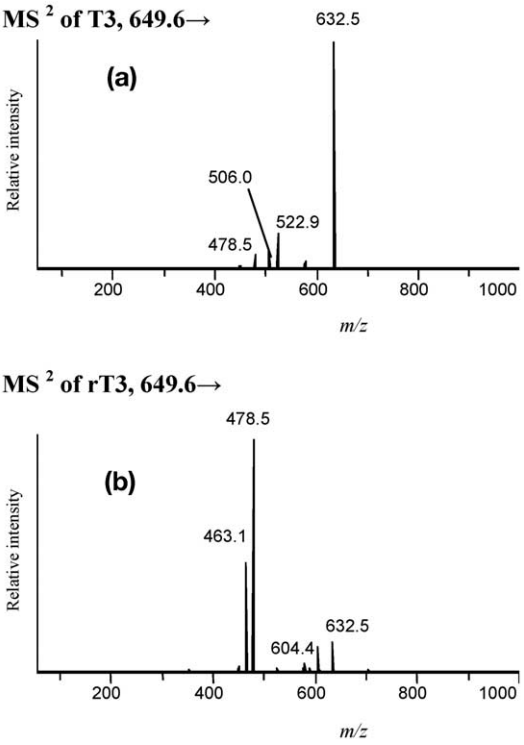

Figure 3. MS^2 spectra of T3 and rT3 under negative ionization. (**a**) For T3, using *m/z* 649.6 as a precursor ion, the fragment ions are observed at *m/z* 632.5, 522.9, 506.0, and 478.5. (**b**) For rT3, using *m/z* 649.6 as a precursor ion, the fragment ions are observed at *m/z* 632.5, 604.4, 478.5, and 463.1.

(Bruker Daltonics Esquire 3000, Bremen, Germany). The spray voltage was 3.5 kV. Dry gas (nitrogen) flowed at 5.0 L/min, and the drying temperature was 320 °C. Samples were introduced by flow injection at 2 μL/min. The mass range scanned was *m/z* 50–1000. For MS/MS experimentation, the fragmentation amplitude was set at 1.0 V, the isolation width was 4.0 *m/z*. Each mass spectrum consisted of 25 scans. These same instrument conditions were used for all standards, mixtures of T3 and rT3, and human serum samples. For quantification, each standard sample was analyzed a total of eight times over the period of one week. The mixture sample of T3 and rT3 was analyzed six times over the period of one week. Human serum sample was analyzed six times in one day. The data acquisition software used was Bruker Daltonics Data Analysis 3.0.

Results and Discussion

Identification of T3 and rT3

Under positive ionization mode, both T3 and rT3 reveal a singly charged ion $[M + H]^+$ at *m/z* 651.8. Using the T3 MS^1 ion *m/z* 651.8 as a precursor ion, T3 MS^2 fragment ions are observed at *m/z* 634.5, 605.6, and 508.1 (Figure 2a). The product ion at *m/z* 634.5 corresponds to $[M + H - NH_3]^+$, and *m/z* 605.6 corresponds to $[M + H - HCOOH]^+$. For product ion *m/z* 508, the 144 Da loss could represent two possible compositional losses: a loss of HIO or H_3IN. Using the rT3 MS^1 ion *m/z* 651.8 as a precursor ion, rT3 MS^2 fragment ions are observed at *m/z* 634.5, 605.7, and 508.0 (Figure 2b). Although the composition of fragment ions in the MS^2 spectra generated from T3 and rT3 are the same, the percent ion contributions of each fragment ion are different. Thus, these MS^2 spectra can be used to identify a single unknown triiodothyronine, because in each case a "distinguishing product ion", which has significantly different abundance compared to other isomeric species [23], can be identified. The distinguishing product ion for T3 is *m/z* 605.7. Although *m/z* 605.7 is also the most abundant ion for rT3, the percent total ion for *m/z* 605.7 is only 42% for rT3, compared to 83% for T3. The distinguishing product ion for rT3 is *m/z* 508.0. The percent total ion for *m/z* 508.0 is 38% for rT3, compared to 9% for T3. This means that the MS^2 fragment ions at *m/z* 605.8 and 508.0 could be used to differentiate these two triiodothyronine isomers.

Under negative ionization mode, both T3 and rT3 reveal a singly charged ion $[M - H]^-$ at *m/z* 649.6. MS^2 spectra of *m/z* 649.6 for T3 and rT3 are shown in Figure 3a and b, respectively. It is obvious that T3 and rT3 each produce distinguishing product ions, which have substantially different abundances compared to each other. In its MS^2 spectrum, T3 has a distinguishing product ion at *m/z* 632.5, corresponding to $[M - H - NH_3]^-$, which is in much greater abundance (Figure 3a) than that of the rT3 (Figure 3b). Similarly, in its MS^2 spectrum, rT3 has a distinguishing product ion at *m/z* 478.5 (Figure 3b), which is in much greater abundance than

Table 1. Percentages calculated by solving equations 1 and 2 and normalizing the data, as described in the text

T3 : rT3	Calc. % 1	2	3	4	5	6	Avg. ± SD (n = 6)	Actual %
1:9	10.3	11.6	9.7	11.5	8.9	12.4	10.7 ± 1.3	10.0
	89.7	88.4	90.3	88.5	91.1	87.6	89.3 ± 1.3	90.0
3:7	28.9	27.8	30.8	28.2	29.3	31.1	29.4 ± 1.3	30.0
	71.1	72.2	69.2	71.8	70.7	68.9	70.6 ± 1.3	70.0
7:3	70.5	71.3	69.2	70.4	69.8	68.7	69.9 ± 0.9	70.0
	29.5	28.7	30.8	29.6	30.2	31.3	30.1 ± 0.9	30.0
9:1	87.4	88.7	89.9	87.2	86.7	90.6	88.4 ± 1.6	90.0
	12.6	11.3	10.1	12.8	13.3	9.4	11.6 ± 1.6	10.0

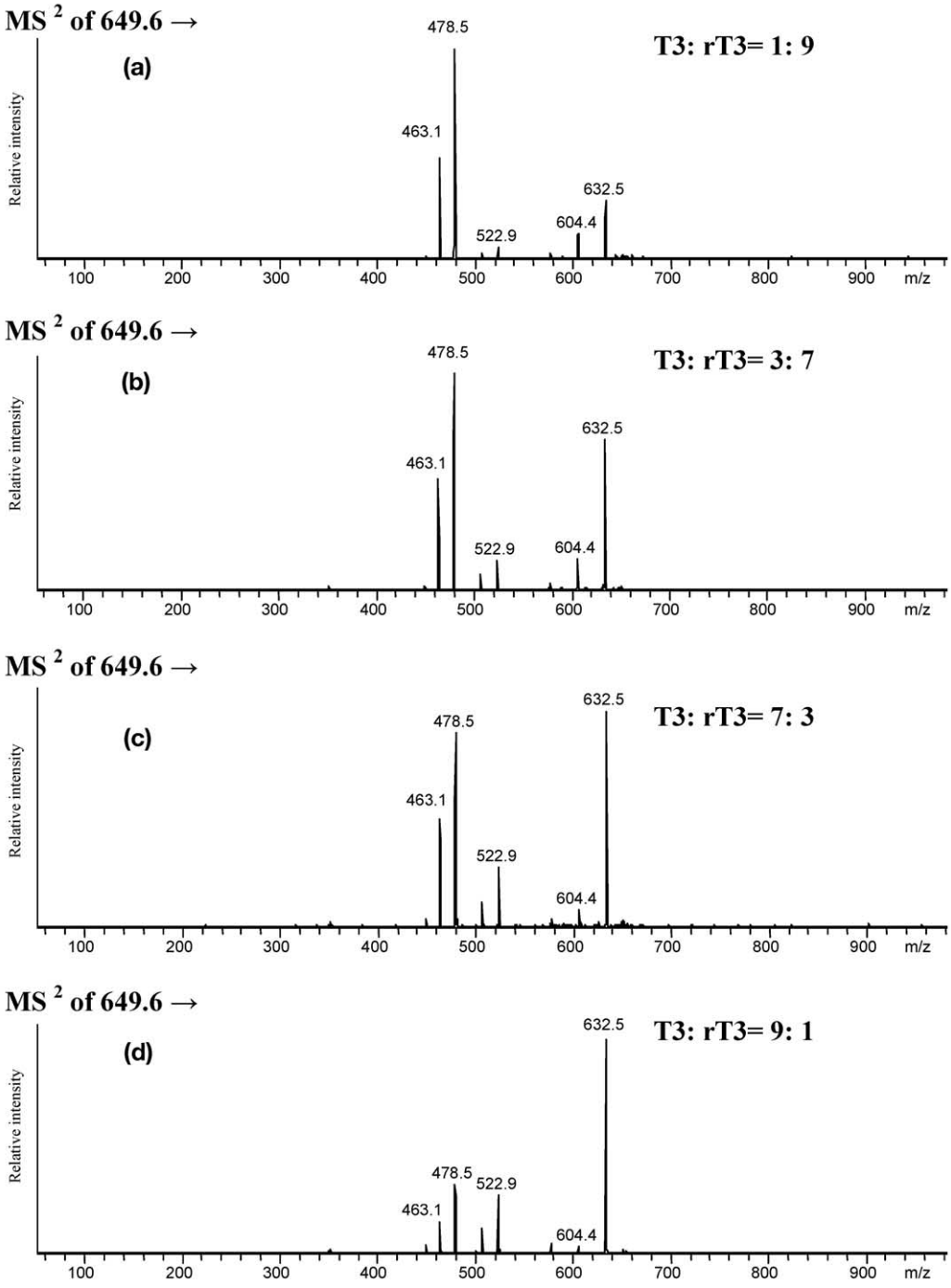

Figure 4. MS^2 spectra of the mock mixtures of T3 and rT3, under negative ionization mode. (**a**) T3:rT3 = 1:9; (**b**) T3:rT3 = 3:7; (**c**) T3:rT3 = 7:3; (**d**) T3:rT3 = 9:1.

that of the T3 (Figure 3a). For product ion *m/z* 478.5, the 171 Da loss could represent a possible compositional loss: a radical loss of ClO_2. However, without extensive labeling experiments or high-resolution measurements, this product ion loss cannot be unambiguously assigned. Nonetheless, the ion at *m/z* 478.5 is a predominant ion in the rT3 spectrum. MS^2 experiments reveal that in the negative ionization mode the distinguishing product ions for T3 and rT3 are also the most abundant ions in their respective spectra. Therefore, for further quantitative analysis, the ions at *m/z* 632.5 and 478.5 in MS^2 spectra in negative ionization mode were selected to represent T3 and rT3, respectively.

Quantification of T3 and rT3

The relative molar percentages of T3 and rT3 isomers in a mixture were determined using a system of two

equations, as described previously [20–22]. Briefly, each of the pure isomeric triiodothyronine standards, T3 and rT3, was analyzed eight times by MS^2 experiments. The percent ion contribution of each distinguishing product ion to the total ion current (TIC) was obtained by exporting the mass list of the spectrum of interest into ACD SpecManager. For T3 and rT3, excellent reproducibility for *m/z* 632.5 and *m/z* 478.5 is observed, with standard deviations of less than 1.5%, respectively.

Using the percent contributions for T3 and rT3, a system of equations was developed as follows:

$$70.205A + 4.09B = C_{632.5} \quad (1)$$

$$8.851A + 74.14B = C_{478.5} \quad (2)$$

$C_{632.5}$ and $C_{478.5}$ are the contributions of the ions *m/z* 632.5 and 478.5 in any given mixture of T3 and rT3. A and B represent the ratio of T3 and rT3 in the mixture. To determine the normalization factors, a mixture of T3 and rT3 (1:1) was also analyzed six times in one week. The percent contribution for each of the product ions, *m/z* 632.5 and 478.5 for the 1:1 mixture, is inserted into the system of equations, solving for A and B (24.165 and 76.92). 50/24.165 = 2.068 = Norm 1 for *m/z* 632.5; 50/76.92 = 0.65 = Norm 1 for *m/z* 478.5. This normalization accounts for various factors, including differences in the isomer's ionization efficiency [24]. After normalizing, these values are converted to the calculated percentages by dividing each value by the sum of the two values. Thus, the molar percent of T3 and rT3 in the mixture is obtained.

To demonstrate the utility of the above equations for quantitative analysis of T3 and rT3, a series of two component mixtures were prepared with variable concentrations of each of the isomers. These were analyzed, and the percent of each of the isomers in the mixture was calculated (Table 1).) Figure 4a–d show the MS^2 spectra of T3 and rT3 mixtures with various molar ratios. Clearly, the relative abundance of ion at *m/z* 632.5 increases with increasing T3 concentration. Conversely, the relative abundance of ion at *m/z* 478.5 decreases with decreasing rT3 concentration. The results reveal that the quantification procedure works quite effectively for any ratio of T3 and rT3. The error for all of the samples was very small (< 1.6%), establishing the utility of this method for quantitative analysis of T3 and rT3 mixtures.

Application to the Quantitative Analysis of Human Serum Samples

Having demonstrated the effectiveness of this quantitative analysis method on for T3 and rT3 mixtures, we applied this method to the analysis of human serum containing natural levels of thyroxine. Due to the combined reversed-phase and strong anion-exchange character of the Oasis MAX SPE cartridge, it is well suited for isolation of acidic substances from serum, such as T4 [13]. Because the acidity of T3 and rT3 are very similar to that of T4, we used Oasis MAX SPE cartridge to extract T3 and rT3 for direct analysis by ESI-MS/MS. The results indicate that the present method shows good reproducibility and precision (Table 2), and that the molar percents of T3 and rT3 in human serum are 81.5 ± 2.4 and 18.5 ± 2.4, respectively, with a T3/rT3 ratio of 4.5 ± 0.7. This ratio agrees with the ratio in normal human serum obtained by Santini et al. [25].

Table 2. Results of the T3 and rT3 in human serum

Sample	Molar percent of T3 (%)	Molar percent of rT3 (%)	Molar ratio (T3/rT3)
1	79.6	20.4	3.9
2	82.3	17.7	4.6
3	84.8	15.2	5.6
4	78.1	21.9	3.6
5	82.7	17.3	4.8
6	81.2	18.8	4.3
Avg. ± SD	81.5 ± 2.4	18.5 ± 2.4	4.5 ± 0.7

Conclusions

Quantification of the molar ratio of T3 to rT3 is an important initial step toward increased understanding of the relation between the metabolism and biological functions of the thyroid hormones. In the present study, MS^2 spectra in either negative ionization mode or positive ionization mode can be used to differentiate T3 and rT3. MS^2 experiments reveal that in the negative ionization mode the distinguishing product ions for T3 and rT3 are also the most abundant ions in their respective spectra. Therefore, the ions at *m/z* 632.5 and 478.5 in MS^2 spectra in negative ionization mode were selected to establish a protocol for quantification of the T3 and rT3 mixtures. Good precision and accuracy was demonstrated, and then the protocol was applied to human serum. The results suggest that the described method can be used to analyze T3 and rT3 from other animal sera as well as from human serum.

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