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Identification and quantitation of iodotyrosines and iodothyronines in hydrolysate of iodinated casein by capillary electrophoresis

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Abstract

In this paper, a new method for separation, identification and quantitation of iodotyrosines and iodothyronines [3-monoiodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT), L-thyronine (T_0), 3,5-diiodo-L-thyronine (T_2), 3,5,3′-triiodo-L-thyronine (T_3) and 3,3′,5,5′-tetraiodo-L-thyronine (T_4)] was described by using capillary electrophoresis with photodiode-array ultraviolet–visible detection (CE-UV). The certain influence factors were systematically investigated, including the type, concentration and pH of buffer, and additive. We found that 10 mM sodium borate running buffer (pH 8.5) containing 0.10 mM β-CD as additive reagent allowed the best instrumental conditions for the optimum separation of the iodotyrosines and iodothyronines. Under optimized conditions, the analytical time was within 6 min, using an uncoated fused-silica capillary of 75 μm inner diameter with an effective length of 30 cm. The reproducibility of the migration time and peak area was less than 0.6% and 6.8%, respectively. A linear range from 10–1000 μg/mL and low limits of detection from 1.3–3.4 μg/mL were obtained at the detection wavelength of 280 nm. Our preliminary results show that the method is well suitable for determination of the hydrolysate of iodinated casein.

Keywords: Iodotyrosine; Iodothyronine; Iodinated casein; Capillary electrophoresis; Ultraviolet–visible detection

1. Introduction

Iodinated casein is an active protein in thyroid gland. Reineke and Turner [1] reported its first synthesis in 1942. Today iodinated casein produced by industry is always used as additive in animal feedstuff. The effects of iodinated casein were proved by many researches [2–4]. When iodinated casein enters into the animal, it participates to basic metabolism and stimulates thyroid gland to excrete thyroxine. As a result, it leads to accelerate the growth of little animal or increase the outputs of poultry and livestock. Unfortunately, the scientists found that there was latent danger to human being when they were supplied with meat, egg or milk containing iodinated casein. It was confirmed that iodinated casein in those food came from feedstuff to animal. In China, iodinated casein is forbidden as additive in feedstuff or drinkable water. Therefore, it is essential and emergent to establish a quick, simple and accurate method to detect the iodinated casein in feedstuff for human being health.

Though it is very difficult to determine iodinated casein directly, several methods such as HPLC, GC-MS have been described to determine the iodoamino acids in iodinated casein, body fluid and human thyroglobulin [5–9] to evaluate iodinated casein level indirectly. These techniques have many useful chemical and biomedical applications, although they show a lack of sensitivity and required approximately 1–20 μg of substrate for reliable detection. Other indirect quantitation methods like isotopic equilibrium followed by thin-layer chromatography [10,11] or radioimmunoassay (RIA) have also been used to estimate biologically active thyroid hormones and their metabolites in body fluids and tissues [12]. Recently, isotope dilution-GC-MS, isotope dilution-HPLC-MS [13] and isotope dilution MS-MS [14] were reported to detect the T_3 and T_4 in the serum sample. However, these techniques require iodoamino acid being derivatized prior to detection, and the sample cannot be recovered for further analysis besides time-consuming and laborious.

Compared to these methods, capillary electrophoresis (CE) is being recognized as a powerful analysis tool offering high efficiency, large peak capacity and fast separation, and requiring only minute amount of sample. The potential of CE for com-

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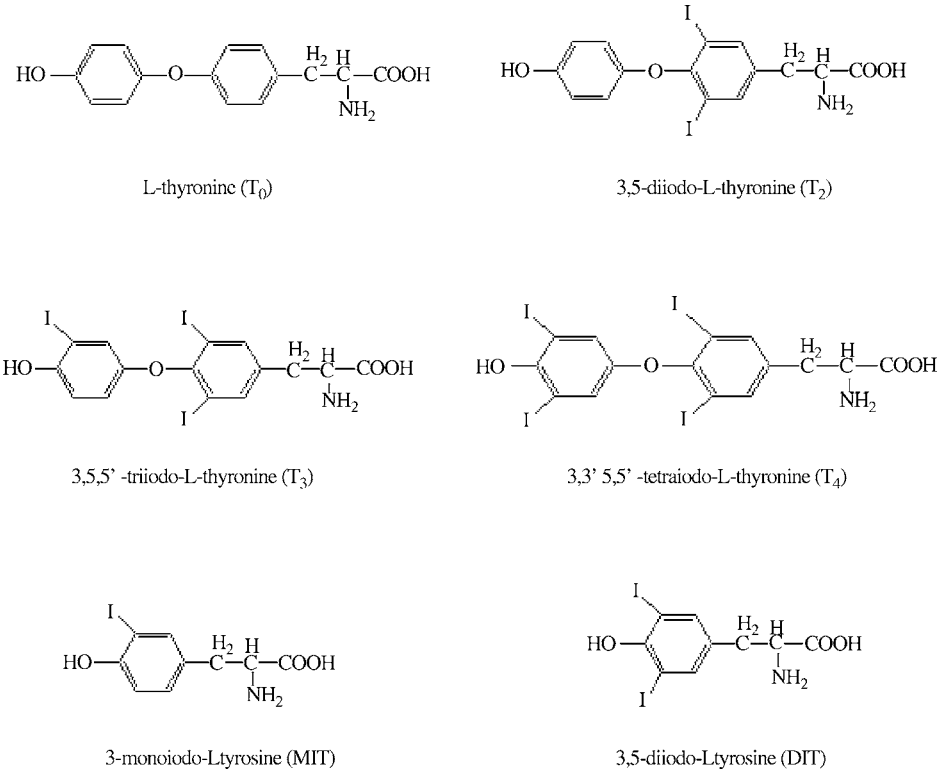

Fig. 1. Structure of iodotyrosines and iodothyronines.

pounds ranging from small ions to large biological molecules has been extensively demonstrated [15–22]. In this paper, we report a fast and simple CE method using PDA-UV–vis detection for identification and quantitation of iodinated casein hydrolysate, iodotyrosines and iodothyronines, and their structures are shown in Fig. 1.

2. Experimental

2.1. Instruments and materials

The identification and quantification were performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA) with PDA-UV–vis detection. Fused-silica capillaries with 75 μm internal diameter (ID) were purchased from Yongnian Optical Fiber Factory (Yongnian, Hebei, PR China). MIT, DIT, T_0, T_2, T_3 and T_4 were purchased from Sigma (St. Louis, MO, USA). The other reagents were of analytical grade and prepared with 18.2 MΩ/cm ultrapure water (Millipore, Bedford, MA, USA) and filtered through 0.22 μm membrane filters prior to use. The iodinated casein was generous gift of Huangyan Rongyao chemical factory (Zhejiang, China).

The six standard stock solutions of MIT, DIT, T_0, T_2, T_3 and T_4 were prepared by dissolving in ultrapure water and by adding 4–10 μL 0.1 M NaOH to assist dissolution of standard compounds. The concentrations of standard stock solutions were 2 mg/mL and they were stored in the refrigerator. The mixed standard solutions were prepared by diluting the standard stock solution 100 times before injection, and the final concentration of standard solutions was 20 μg/mL.

Sodium hydrogen carbonate, sodium dihydrogen phosphate and disodium tetraborate decahydrate were used to prepare background electrolytes (BGE) which were adjusted to the required pH value with 0.1 M NaOH.

2.2. Procedure for hydrolysis of iodinated casein

According to [23], the iodinated casein was hydrolyzed with microwave because of time saving. 100 mg of iodinated casein was put into the digestion tube. After adding 2 mL of 5 M NaOH, the sample was shaken gently in order to be soaked fully by NaOH solution. In order to avoiding hydrolysate oxidation in the process of digestion, N_2 was introduced into the digestion tube for half an hour to be full of the tube. Then the sample was placed in microwave oven for 2 h, where temperature was set at 110 °C. The hydrolysate solution was stored in 100 mL flask with adding ultrapure water to the scale. The sample solution was directly injected without any treatments, and then analyzed.

2.3. Procedure

Before analyses, the capillary was rinsed with 0.1 M NaOH for 10 min, ultrapure water and buffer for 5 min, respectively, and then balanced under the separation voltage for 10 min. The capillary was rinsed with buffer for 2 min between runs.

3. Results and discussion

3.1. Selection of detection wavelength

There are three amino acids having certain absorbance in UV–vis spectrum range, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). According to [9], 280 nm was chosen as detection wavelength. It was beneficial for detecting the iodotyrosines and iodothyronines (Fig. 2). The spectra of Phe showed no absorption at 280 nm. Trp had the highest absorption in 280 nm and could be separated from iodotyrosines and iodothyronines using our experimental conditions (shown in Fig. 3). However, Tyr not only had absorption in 280 nm but also had the same migration time as T_0. No matter how we changed the electrophoresis conditions, Tyr could not be resolved from T_0. So we did not quantify T_0 in the real sample.

3.2. Optimization of the BGE

The analyte structures suggested that they could be changed into ions, and capillary zone electrophoresis was selected as the separation mode. In this work, three kinds of buffer solutions, carbonate, phosphate and borate buffer with pH 8.5 were used to examine the effect of buffer solution on separation efficiency. The results showed that the best sensitivity and resolution of six analytes were achieved when disodium tetraborate decahydrate was used as the running solution. Compared with borate solution, the peaks were wider in carbonate buffer, and became distortion in phosphate buffer. The capability of phosphate was lower than borate as well. Therefore, borate buffer was chosen as the BGE in subsequent experiments.

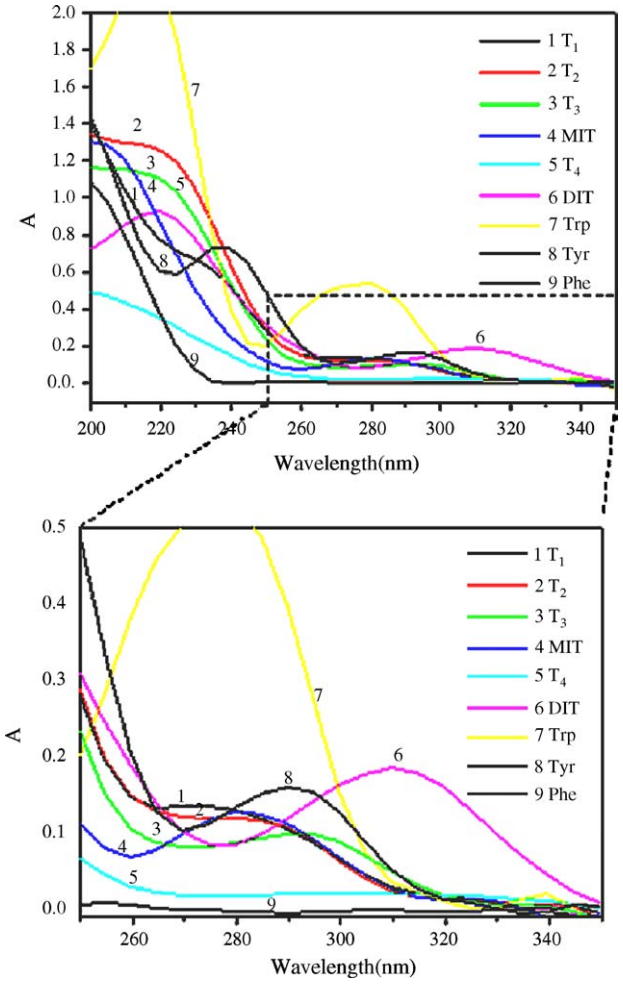

Fig. 2. The UV spectra of T_0, T_2, T_3, MIT, T_4, DIT, Trp, Tyr and Phe. The concentrations of nine analytes were all 20 μg/mL.

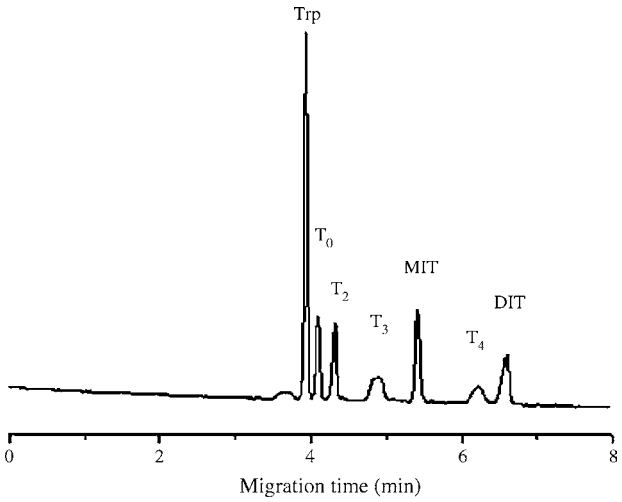

Fig. 3. The electropherograms of Trp, iodotyrosines and iodothyronines. 10.0 mM borate with 0.10 mM β-CD (pH 8.5) was used as electrophoresis buffer. Separation voltage was 8 kV and injection time was 5 s under pressure injection mode. Injection pressure was 0.5 psi. The concentrations of the seven analytes were all 20 μg/mL.

In order to improve the resolution and solubility of analytes, alkaline borate buffer was employed in this study as iodotyrosine and iodothyronine assume negative charge in alkaline solution because the carboxyl p*K*a value of L-tyrosine is 2.2 and the p*I* value is 5.66. The acidity of the running buffer affects the ξ-potential, the electroosmotic flow (EOF) as well as the overall charges of the analytes, which determine the migration time and the separation. The running buffer was 10 mM borate BGE at five different pH values (8.0, 8.3, 8.5, 9.2 and 9.5). The resolution of T_0 and T_2 was poor at low pH. When the running buffer pH increased, the migration time increased and the resolution improved due to the dissociation of the carboxyl groups for all analytes. Meanwhile, the resolution of T_4 and DIT became poor at pH value above 8.5. At pH 9.5, only five peaks appeared in electropherogram. It is concluded that DIT possesses more negative charges than T_4 in the weak basic solution because its migration time is longer than T_4. When the solution pH values enhances, the two analytes have the similar negative charges. So in electropherogram, the two analytes have the same migration time as a result of one peak emergence. At pH 8.5, the six analytes can be well separated within a relatively short time. In this experiment, pH 8.5 was chosen as the running buffer considering resolution and the analytical time.

The concentration of buffer influences the viscosity of the running solution, the diffusion coefficient of analytes and the ξ-potential of the capillary inner surface, which further affects

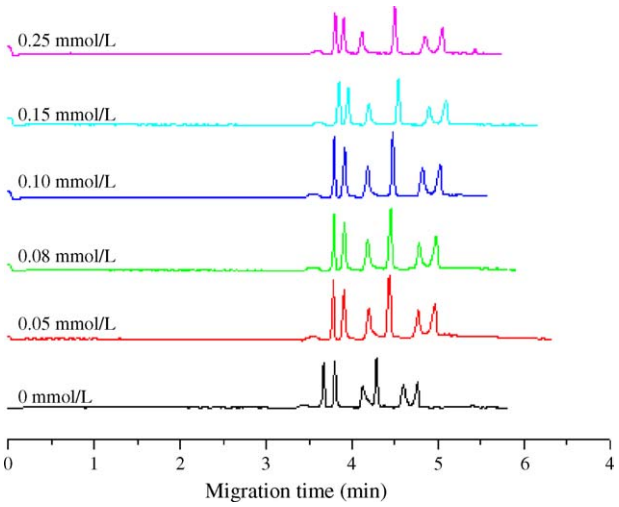

Fig. 4. The effects of β-CD concentration. Other conditions were as described in the legends to Fig. 3. The peaks orders were T_0, T_2, T_3, MIT, T_4 and DIT, respectively.

the resolution and migration time of the analytes. The effect of borate BGE (pH 8.5) concentration on migration time and separation efficiency has been examined. The results demonstrated that when the concentration of BGE was in the range of 6.25–12.5 mM, the migration time of the six analytes was increased with the concentration of BGE as well as the electrophoresis current. The resolution of the peaks became better with the buffer concentration increased, especially between T_0 and T_2. However, higher buffer concentration had a negative effect on the column efficiency due to high Joule heating. In order to obtain better resolution and shorten analytical time, 10 mM borate BGE at pH 8.5 was selected as the running buffer solution.

In capillary electrophoresis assays, additive reagent often was used to improve the separation efficiency and peak shape. In our experiment, β-CD was used to improve the peak shape of T_3 and resolution between T_3 and MIT (as shown in Fig. 4). When β-CD was up to 0.10 mM, the peak shape of T_3 became more symmetrical than lower concentration of β-CD. However, the peak height slightly decreased with the increase of β-CD concentration for the peaks became broader. 0.10 mM β-CD was used as additive reagent in the experiment as a result.

The effect of separation voltage on the migration time of the mentioned above analytes has been investigated. With the separation voltage increment, the migration time reduced. It was obvious that the electrophoresis current was increasing with the increasing of voltage and the resolution of T_0 and T_2 became worse with the separation voltage enhancement. Moreover, the migration time was within 6 min even using 8 kV separation voltage, and both higher efficiency and better peak shape could be obtained.

We also examined the effects of sampling time in the range of 3–10 s at 0.5 psi pressure to obtain the highest sensitivity and the best selectivity. The results indicated that peak height increased with the sampling time. However, peak broadening appeared obviously for injection time longer than 5 s. In this study, 5 s was selected as the optimum injection time by considering the peak broadening and the sensitivity.

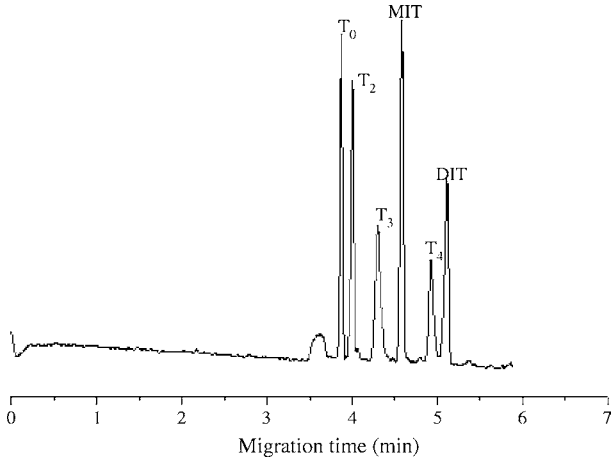

Fig. 5. Electropherogram of the standard mixture solution of T_0, T_2, T_3, MIT, T_4 and DIT. Other conditions were as described in the legends to Fig. 3.

To sum up, the optimum conditions for separation and detection of above mentioned compounds could be described as follows: 10 mM borate (pH 8.5) as the running buffer solution, 0.10 mM β-CD, as additive reagent, 8 kV as separation voltage, 5 s as injection time under pressure injection mode. Under the optimum conditions, a typical electropherogram for a standard mixture solution was shown in Fig. 5. The concentration of analytes was of 20 μg/mL. It was clear that the six compounds could be completely separated within 6 min. The results showed the method was rapid and effective for separation of iodotyrosines and iodothyronines.

3.3. Reproducibility, linear response range and detection limit

A series of the standard solutions of T_0, T_2, T_3, T_4, MIT, DIT were tested to determine the linearity for the analytes in this method. The linear relationships between the concentration of the six compounds and the corresponding peak area were obtained, and the regression equations and correlation coefficients were shown in Table 1. It can be known from Table 1 that the linear response ranges were 5.0–1000 μg/ml for T_0 and MIT, 10–1000 μg/ml for T_2, T_3, T_4 and DIT, respectively, and the detection limits were less than 3.4 μg/ml for iodotyrosines and iodothyronines. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3.

The same standard mixture solutions were analyzed to determine the reproducibility of the peak height, peak area and migration time under the optimum conditions. The reproducibility of migration time, peak height and peak area was shown in Table 2.

3.4. Analysis of hydrolysate solution of iodinated casein

The hydrolysates of iodinated casein were analyzed according to the procedure described in experimental section. When the real sample was analyzed, some coexistent substances affected the reproducibility of migration time, peak areas and separation of analytes. In order to improve the reproducibility of this method, when the capillary was used for analysis of real

Table 1
Linear response range of analytes

Analyte	Linear range (μg/ml)	Regress equation, C (μg/ml)	R	Detection limit (μg/ml) $S/N = 3$
T_0	5–1000	$A = -1659.1 + 140.4C$	0.9982	2.0
T_2	10–1000	$A = 791.0 + 151.2C$	0.9978	2.5
T_3	10–1000	$A = 837.3 + 97.4C$	0.9990	3.4
MIT	5–1000	$A = -372.7 + 245.8C$	0.9999	1.3
T_4	10–1000	$A = -1958.0 + 237.7C$	0.9998	1.4
DIT	10–1000	$A = -754.2 + 225.8C$	0.9995	1.5

Electrophoresis conditions are same as in Fig. 3.

Table 2
The reproducibility of the migration time, peak area and peak height ($n = 7$)

Analyte	Mean Migration time (min)	R.S.D. (%)	Mean Peak height	R.S.D. (%)	Mean Peak area	R.S.D. (%)
T_0	3.84	0.5	5514	2.8	10091	3.9
T_2	4.03	0.5	5228	1.7	10969	4.2
T_3	4.35	0.6	3807	3.8	10674	5.7
MIT	4.54	0.5	5521	3.4	11413	6.4
T_4	4.91	0.5	2993	4.2	10240	6.8
DIT	5.13	0.6	3929	5.0	11104	5.3

Electrophoresis conditions were same as in Fig. 3.

samples, it should be flushed sequentially with water for 1 min, 0.1 mol/l NaOH for 2 min, and water for 1 min and finally equilibrated with background electrolyte solution for 5 min before each injection. Under the optimum conditions, the hydrolysate was analyzed and six analytes were successfully separated. As shown in Fig. 6, peaks were identified by addition of standard substance of T_0, T_2, T_3, MIT, T_4 and DIT. Because the contents of T_2, T_3 and T_4 were low in this sample, only MIT and DIT were quantified in this paper. The detection results for the samples were shown in Table 3.

In order to examine the reliability of the method, the recoveries of MIT and DIT were investigated. The recoveries were determined by addition of standard solution of MIT and DIT into the hydrolysate sample under the same conditions stated above.

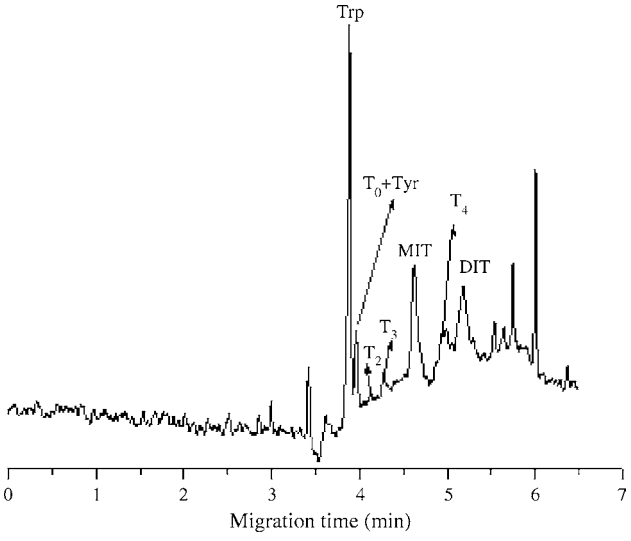

Fig. 6. Typical electropherogram of the hydrolysate of iodinated casein. Other conditions were as described in the legends to Fig. 3.

Table 3
Content of MIT and DIT in the hydrolysate iodonate casein

Component	Determined (mg/100 mg)	R.S.D. (%)
MIT	1.33	3.0
DIT	1.48	4.3

Electrophoresis conditions are same as in Fig. 3.

Table 4
Determination of recovery for this method ($n = 3$)

Compound	Added amount (mg)	Found amount (mg)	Recovery (%)	R.S.D. (%)
MIT	0.80	0.85	106	3.0
	1.20	1.27	106	5.2
	1.60	1.85	116	2.2
DIT	0.80	0.71	88.8	4.0
	1.20	1.17	97.5	1.8
	1.60	1.58	98.8	6.2

Electrophoresis conditions are same as in Fig. 3.

The recoveries of these two compounds were found to be in the range of 88.8–116% (see Table 4). The above results demonstrated that CE is a rapid, sensitive and reproducible method for determination of iodotyrosines and iodothyronines. This method promises to be applicable to qualitative analysis of iodinate casein.

4. Conclusions

In this paper, we have developed a CE method for the analysis of iodotyrosines and iodothyronines. The compounds were separated in their anionic forms using alkaline borate buffer, and this method was successfully used for analysis of the hydrolysate from iodinated casein sample. The results showed that this

method is rapid, simple and effective technique, and well suitable for identification, separation and determination of T_0, T_2, T_3, MIT, T_4 and DIT. In comparison to the chromatographic methods, the proposed method is a good alternative for simultaneous analysis of iodotyrosines and iodothyronines. Moreover, the method is also possible to be applicable to indicate the existence of iodinate casein in feedstuff through separating and identifying MIT and DIT in the hydrolysate sample with T_2, T_3 and T_4 as circumstantial evidence. It is profitable to control the feedstuff quality.

Acknowledgements

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