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Short communication

HPLC–MS analysis of iodotyrosines produced by sample hydrolysis: A simple method for monitoring iodinated casein in feed premixes

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ABSTRACT

A new and simple HPLC–MS method was developed for monitoring iodinated casein in feed premixes. In this method, feed premixes were hydrolyzed, and the iodotyrosines thus released were analyzed. Sample pretreatment included precipitation of transition metals ions with Na_2S, hydrolysis with sodium hydroxide, and cleaning up with an Oasis SAX cartridge. Gradient elution was carried out on a C_{18} column with water (containing 0.1% formic acid) and acetonitrile. Ion detection was performed using ESI positive SIM at *m*/*z* 262, 308, 388, and 434. Iodinated casein levels were monitored by qualitative analysis of the iodotyrosines released upon sample hydrolysis and by quantifying the 3,5-diiodotyrosine released. The validation data demonstrated that the method was selective and sensitive ($\leq$0.2 mg g^{-1}) for iodinated casein and had acceptable accuracy (recoveries: 81.3–106.7%) and precision (RSD: 1.7–16.0%).

1. Introduction

Iodinated casein, a synthetic protein with high thyroid activity, has been used as a feed additive for more than 50 years, for example, to increase milk production in dairies [1] and reduce the abdominal fat of broilers [2]. However, since the impact of feed on food safety is an increasingly important issue [3], iodinated casein is now regarded as a potential risk to animal health and food safety, and its use as an additive in feed or drinkable water is forbidden in many countries. Feed premix is one of critical control points for iodinated casein supervision in livestock and aquaculture industries. However, there is no suitable method for monitoring the iodinated casein levels in these samples.

Since iodinated casein is not a pure compound, its direct analysis in feed samples is difficult. However, hydrolysis of iodinated casein can lead to the release of thyroid gland hormones and other iodoamino acids, and indirect monitoring of iodinated casein by analyzing these characteristic compounds is relatively easy. The first iodoamino acid separated from the hydrolysate of iodinated casein was the thyroid hormone thyroxine (T4) [4]. This was followed by 3,3′,5-triiodo-thyronine (T3), 3-monotyrosine (MIT) and 3,5-diiodotyrosine (DIT) [5], and 3,3′,5′-triiodo-thyronine (rT3) and a small amount of diiodo-thyronine (T2) [6]. In a recent study [7], it was found that the contents of MIT and DIT were relatively higher, and the hydrolytic stability of these compounds was greater than that of the other iodoamino acids released from iodinated casein. Therefore, MIT and DIT are suitable target analytes for monitoring iodinated casein levels.

Earlier, gas liquid chromatography was used for the analysis of MIT and DIT [8,9]; however, this method required derivatization of the analytes prior to detection. In recent years, high-performance liquid chromatography (HPLC) [10–12] and capillary electrophoresis [13] have been used for MIT and DIT detection, but the sensitivity and selectivity of these techniques are insufficient for monitoring iodinated casein in feed matrices. In this context, the objective of the present study was to establish a simple and new HPLC–MS method for monitoring the levels of iodinated casein in feed premixes. This was achieved by analyzing the MIT and DIT produced after sample hydrolysis.

2. Experimental

2.1. Chemicals and materials

MIT and DIT were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iodinated casein was obtained from the Huanyan Rongyao Chemical Factory (Zhejiang, China), and HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The other chemical reagents used in this experiment were of analytical grade (Beijing Chemical Reagent Co., Beijing, China). Distilled water was purified through a Milli-Q® system (Millipore, Bedford, MA, USA). Iodinated casein-free feed premixes were obtained from the pilot workshop of the Ministry of Agriculture Feed Industry

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Center (Beijing, China). The Oasis® MAX 3cc (60 mg) SPE cartridges were purchased from Waters China Limited (Beijing, China).

2.2. Preparation of standards and the iodinated casein solution

Stock solutions of MIT and DIT were prepared at concentrations of 1 mg mL^{-1} in methanol containing 0.1% (v/v) concentrated ammonia solution. These were stored at −12 to −18 °C. Working standard solutions were prepared by diluting the stock solution with a methanol–water–formic acid solution (25:24:1, v/v) according to requirement, and these were then stored at 4 °C.

A stock solution of iodinated casein (10 mg mL^{-1}) was prepared by dissolving 10 g iodinated casein in approximately 250 mL sodium bicarbonate solution (10 mg mL^{-1}) and heating it in a water bath at 70 °C for 30 min to assist complete dissolution. This was then further diluted to 1 L with the sodium bicarbonate solution. This solution was stored at 4 °C and was stable for at least 2 weeks. A working solution of iodinated casein was prepared by diluting the stock solution with the same sodium bicarbonate solution.

2.3. Sample preparation

2.3.1. Hydrolysis

Homogenized feed premix (1.0 g) was placed in a 50-mL screw-cap plastic digestion tube, and 4.0 mL of 0.5 mol L^{-1} Na_2S was added. The digestion tubes were vortexed to disperse the sample and left standing for several minutes. Subsequently, 4.0 mL of 10 mol L^{-1} sodium hydroxide and 2.0 mL of distilled water were added. After tightening the cap, the tube was placed in an oven at 110 °C for 16 h. Distilled water (20 mL) was added to the digestion tube, and after vortexing, the digestion tube was centrifuged at 4000 × *g* for 10 min, and the supernatant was transferred to a 100-mL volumetric flask. Another 20 mL of distilled water was added to the digestion tube, and it was shaken and then centrifuged. Finally, the supernatant was transferred to the same volumetric flask and then diluted to the required volume with water.

2.3.2. Solid-phase extraction (SPE) clean-up

Two milliliters of the above sample hydrolysate solution was applied to SPE cartridges for clean-up. The SPE clean-up steps were as follows: conditioning with 3 mL of methanol followed by 3 mL of water; loading; rinsing with 3 mL of methanol and aspirating to dryness under vacuum; and finally, elution with 2 mL of a methanol–water–formic acid solution (25:24:1, v/v) followed by complete collection of the eluent into an HPLC vial using a syringe. The eluent was then injected and analyzed.

2.4. HPLC–mass spectrometry (MS) analysis

The HPLC–MS system consisted of an Alliance 2690 Separation Module and ZQ mass spectrometer (Waters Corporation, Milford, MA, USA). A Waters XTerra® MS C_{18} analytical column (5 μm particle size, 2.1 mm × 150 mm) with a Phenomenex Security Guard guard cartridge (4 mm × 3 mm, C_{18}) (Phenomenex, Torrance, CA, USA) was used and run at room temperature. Gradient elution was performed with a solution of acetonitrile and 0.1% formic acid in water (0.1%, v/v) at a flow rate of 0.2 mL/min. The acetonitrile content was increased linearly from 10% (v/v) to 32% (v/v) within 10 min. An injection volume of 5 μL was used for all sample solutions.

The ESI positive SIM mode was used for MS. The *m*/*z* values of the monitored ions were 308 and 262 for MIT and 434 and 388 for DIT. The dwell time for each of the ions was 40 ms during detection. The other parameters were as follows: capillary voltage, 3.0 kV; cone voltage, 40 V; extraction voltage, 5 V; RF voltage, 0.5 V; source temperature, 90 °C; nitrogen gas flow for desolvation, 400 L/h; and temperature of the nitrogen gas for desolvation, 380 °C.

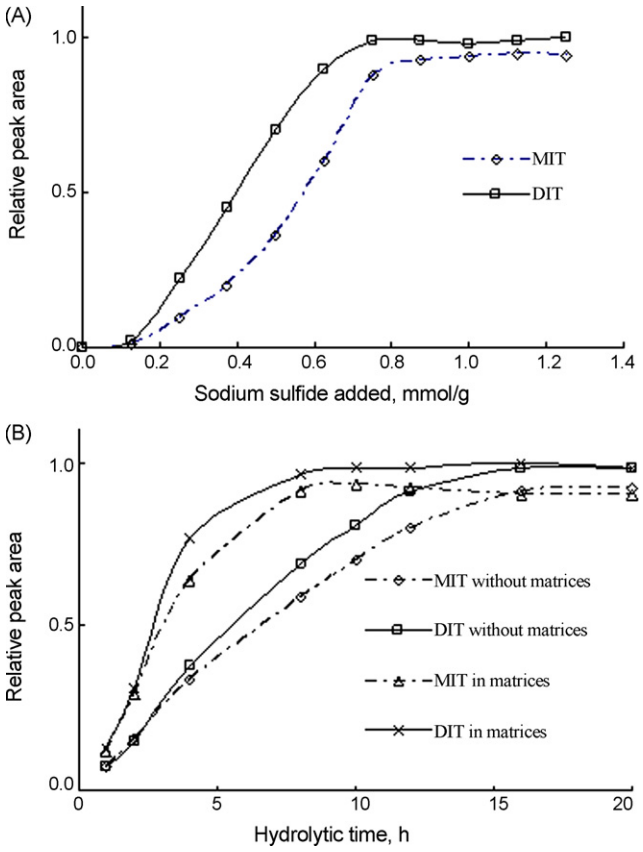

Fig. 1. Effect of feed premix matrixes on MIT and DIT during hydrolysis of a dairy feed premix. (A) Relationship between amount of Na_2S fortified and yields of MIT and DIT for a dairy feed premix; (B) relationship between hydrolysis time and yields of MIT and DIT with and without feed premix.

MIT and DIT were quantified based on the areas of the chromatographic peaks of *m*/*z* 262 and 388, respectively, using the external standard method.

3. Results and discussion

3.1. Sample preparation

3.1.1. Selection of the hydrolytic reagent

Barium hydroxide has been successfully used to hydrolyze iodinated casein with enhanced yields of T4 and good repeatability for all iodoamino acids [7]. However, the procedure is tedious since Ba^{2+} has to be removed with sulfuric acid after the hydrolysis. Recently, It was shown that in comparison to hydrolysis with barium hydroxide, hydrolysis with 4 mol L^{-1} sodium hydroxide had no effect on the yield and repeatability of MIT and DIT release. Since sodium hydroxide is much simpler to use, it was selected as the hydrolyzing agent in this study.

3.1.2. Effects of feed premix matrices on the yield of iodotyrosines

When feed premixes containing iodinated casein were hydrolyzed, adverse matrix effects were observed on the MIT and DIT yields, and no signals could be detected on the chromatogram. We found that some transition metals ions were responsible for this phenomenon. Therefore, an excess of Na_2S was added to precipitate these ions prior to hydrolysis, and this was found to be very effective for counteracting these adverse effects. Fig. 1A shows the relationship between the amount of Na_2S added and the yields of

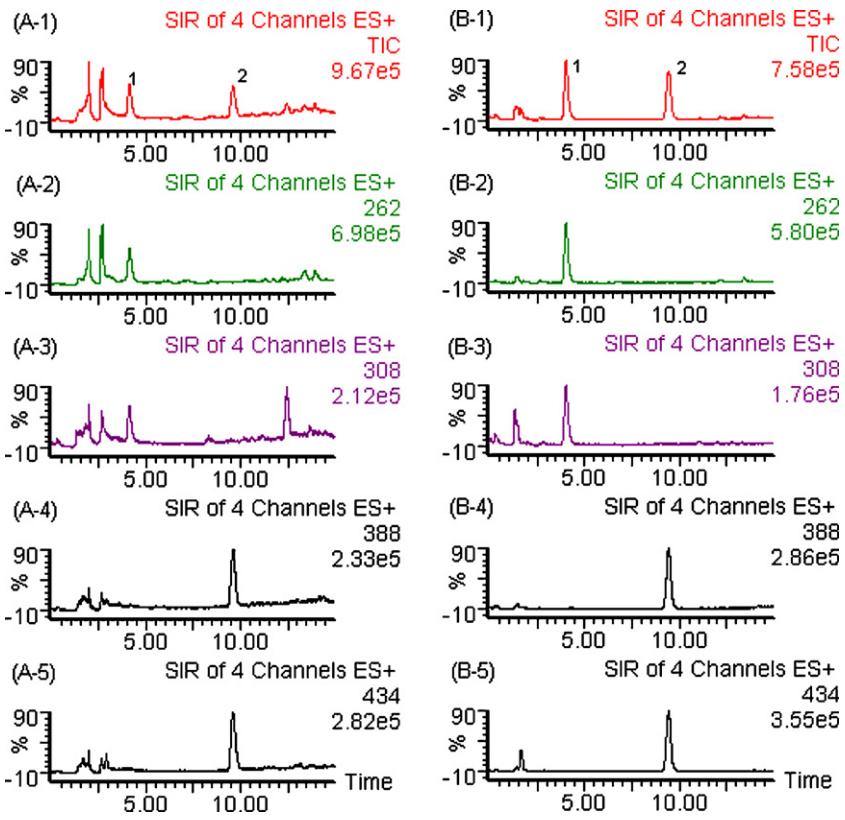

Fig. 2. Chromatograms of the analytes detected in the hydrolysate. (1) MIT and (2) DIT. (A-1)–(A-6) Total ion and extractive ion chromatograms of a duck feed premix containing 1.0 mg g^{-1} iodinated casein; (B-1)–(B-6) total ion and extractive ion chromatograms of an equivalent amount of iodinated casein in the absence of matrices.

analytes from a dairy cow feed premix. In general, 1 mmol g^{-1} Na_2S was sufficient for most samples.

Feed premix matrices also affected the liberation rate of MIT and DIT during hydrolysis, as shown in Fig. 1B. It was observed that stable levels of MIT and DIT were achieved in a shorter hydrolysis time than when hydrolysis was performed in the absence of a feed premix. In this study, the hydrolysis time was 16 h.

3.1.3. SPE clean-up

The C18 cartridge has been successfully used for the efficient clean-up of urine and serum samples and for quantifying iodoamino acids [12]. Here, a simple SPE clean-up method was developed using the Oasis MAX cartridge (Section 2.3.2). First, the hydrolysate, a strong basic solution, was loaded directly. Second, after the washing procedure, the analytes were eluted with a quantitative volume of eluent, and the eluent from the cartridge was directly injected for running analysis. Both steps simplify the method, making it easier to operate.

3.2. Chromatographic separation and MS detection

Since the only target analytes in this study were MIT and DIT, gradient elution was carried out for a short time. For mass detection, the quasi-molecular ions $[M+H]^+$ and segmental ions $[M-CO_2H]^+$ were used as the selective ions (Section 2.4), due to which could give much higher abundance easily. Under optimized conditions of chromatographic separation and mass detection, the analytes were well separated and easily detected. Typical total ion and selective ion chromatograms are shown in Fig. 2.

3.3. Method validation

3.3.1. Selectivity

Four representative free iodinated casein feed premixes were investigated. These included feed premixes for ducks (poultry), fish (aquatic animal), swine (single-stomach animals), and dairy cows (ruminant animals). It was noticed that there was not any interference at the retention times of the analytes.

3.3.2. Linearity, limit of detection (LOD), and limit of quantification (LOQ)

To determine the linearity, a series of working standards were prepared using serial twofold dilution of a working solution of concentration 5000 ng mL^{-1}. Two injections were performed at each concentration. The least-square regression between the response and concentration of each analyte was established. The LOD and LOQ were estimated as the amount of compound injected that produces a signal-to-noise ratio (peak-to-peak) of no less than 3 and 10, respectively, when the hydrolysates were spiked with MIT and DIT. The results of linearity, LOD, and LOQ are shown in Table 1. Good linearity was observed in the studied range, with sufficient sensitivity for both analytes.

Table 1
Linearity and values estimated of LOD and LOQ for MIT and DIT.

Analyte	Range investigated (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Calibration curve	R^2
MIT	20–5000	≤10	≤20	$y = 771.23x + 84661$	0.9998
DIT	20–5000	≤10	≤20	$y = 263.60x + 29308$	0.9994

Table 2
Accuracy and precision data for the analysis of MIT and DIT in hydrolysates of premix feed matrices ($n = 8$).

Analyte	Spiked level (ng mL^{-1})	Duck feed premix		Fish feed premix		Swine feed premix		Dairy cow feed premix	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
MIT	30	68.8	7.4	60.8	2.4	89.0	5.9	74.6	9.2
	1000	47.3	11.7	54.2	3.1	75.4	7.5	51.4	1.7
	5000	23.1	5.0	34.0	8.0	42.4	3.0	45.8	6.5
DIT	30	93.9	8.7	78.7	3.8	95.4	9.9	91.4	8.5
	1000	96.7	4.0	81.3	1.2	94.8	0.6	89.3	7.5
	5000	93.7	2.9	90.1	3.7	97.1	0.8	104.0	4.2

3.3.3. Accuracy, precision, and stability

The analytes were added at three different concentrations (30, 1000, and 5000 ng mL^{-1}) to the hydrolysates of free iodinated casein feed premixes after hydrolysis and before SPE clean-up. Recoveries and relative standard deviations (RSD) were calculated to evaluate the suitability of MS in terms of accuracy and precision. The results are shown in Table 2. Acceptable RSD values in the range of 0.6–11.7% were obtained for both the analytes, and good recoveries in the range of 78.7–104.0% for DIT were obtained at all concentrations. Although low recoveries of MIT were observed at high concentrations, which were attributed to matrix effects, there was no hinder as one of target analytes to identification of iodinated casein. Use of MS with the simple external sample method was found to be both appropriate and easy for quantifying DIT under the optimized analytical conditions.

Acid hydrolysis of iodinated proteins can destroy iodotyrosines [14]. Since these analytes are eluted from the SPE cartridge in an acidified solution and are placed in the same solution for injection, their stability is an important consideration. To evaluate the stability, the three concentrations of MIT and DIT in the acid solution were stored under laboratory bench conditions (20–28 °C) for 0 (samples taken immediately), 3, 6, and 9 days. The analytes were found to be stable through examination of changes in their concentration.

3.3.4. Identification of iodinated casein and determination of its levels

Iodinated casein present in feed premixes can be identified by analyzing the MIT and DIT content after hydrolysis. In this study, we set a criterion to confirm the presence of iodinated casein in a sample—if both MIT and DIT were detected in the hydrolyzed sample, the presence of iodinated casein was confirmed. Thus, sufficient information was obtained for identification even with single quad-MS experiment. The analytical results of MIT and DIT release in feed premixes spiked with different levels of iodinated casein are shown in Table 3. Based on the criterion defined above, we found that the level of iodinated casein in feed premixes was not higher than 0.2 mg g^{-1} at the LOD of MIT or DIT. Generally, the effective dose of iodinated casein required in premixes is not less than 1 mg g^{-1}, and the dose that is practically used is not more than 20 mg g^{-1}. Therefore, the sensitivity achieved by this method is sufficient for monitoring iodinated casein in these samples.

Stable MIT and DIT release from iodinated casein was achieved under the same hydrolytic conditions for the same kind of feed matrices, and it was expected to be in direct proportion to the quantity of iodinated casein present. Therefore, the level of iodinated casein could be evaluated based on the concentrations of MIT or DIT detected in the sample hydrolysates. DIT was selected for further study since it was suitable for MS analysis under the optimized analytical conditions. The following simple formula was devised to determine the level of iodinated casein present in the sample: $Y = kx$. In this case, Y is the level (mg g^{-1}) of iodinated casein in the sample, x is the concentration (ng mL^{-1}) of DIT detected in the sample hydrolysate, and k is a constant. The concentration of added iodinated casein and that of DIT detected in the sample hydrolysate were correlated by the least-square regression method and the intercept zero value. The k value (slope) was 0.0086, and the R^2 value was 0.99. Based on the k value and the quantitative results of DIT, the levels of iodinated casein were determined. The recoveries ranged from 81.3% to 110.5%, and the precision was the same as that of DIT (Table 3).

Table 3
Analytical results of MIT and DIT release in feed premixes spiked with different levels of iodinated casein ($n = 8$).

Sample	IC[a] spiked level (mg g^{-1})	MIT analyzed (ng mL^{-1})	RSD (%)	DIT analyzed (ng mL^{-1})	RSD (%)	IC recovery[b] (%)
Duck feed premix	0.2	17.2	13.5	24.8	16.0	106.7
	1.0	74.8	6.1	116.5	7.0	100.2
	5.0	323.0	10.9	598.2	8.2	102.9
	20.0	1269.3	9.6	2535.9	5.0	109.0
Fish feed premix	0.2	12.4	5.4	18.9	6.1	81.3
	1.0	69.5	4.8	106.6	4.7	91.7
	5.0	306.4	5.9	506.9	7.1	87.2
	20.0	1168.5	10.3	2006.6	7.5	86.3
Swine feed premix	0.2	10.6	12.6	23.7	1.7	101.8
	1.0	58.6	13.2	97.6	3.1	84.0
	5.0	349.8	6.6	552.3	2.0	95.0
	20.0	1261.7	2.5	2471.3	7.0	106.3
Dairy cow feed premix	0.2	14.5	5.4	23.9	1.9	102.9
	1.0	77.7	2.3	127.4	1.9	109.6
	5.0	349.1	10.0	636.1	3.1	109.4
	20.0	1460.8	2.7	2570.8	4.9	110.5

[a] IC: Iodinated casein.
[b] IC recovery was calculated based on the quantitative analytical results of DIT and the k (= 0.0086) value.

4. Conclusion

In summary, a sensitive and selective LC–MS method was established for determining MIT and DIT release in hydrolysate samples of feed premixes. The hydrolysis step for micro-iodinated casein in feed premix matrices was optimized. Iodinated casein was identified by qualitative analysis of two target analytes, and its level was determined on the basis of the quantitative results of DIT and the constant k value. By integrating the two abovementioned steps, a simple and practical method was developed for monitoring iodinated casein. The validation data demonstrated that the method was reliable and easy to use. It can be to apply this method for the quality control of animal feed.

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