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Methodological Aspects of the Definition of Organic Iodine (Iodotyrosine) in Foods

AV Kulikovskii*, AB Lisitsyn, IF Gorlov and MI Slozhenkina

Volga Research Institute of Production and Processing of Meat and Dairy Products, Volgograd, Russia

***Corresponding Author:** AV Kulikovskii, Volga Research Institute of Production and Processing of Meat and Dairy Products, Volgograd, Russia.

Received: September 24, 2019; **Published:** December 10, 2019

Abstract

A procedure is developed for the determination of iodinated amino acid residues-iodotyrosines- in food. The procedure is based on the hydrolysis of proteins of a food sample (meat, dairy products, bakery products, canned juices, etc.) followed by purification by solid-phase extraction. Analytes 3-iodo-L-tyrosine and 3,5-diiodo-L-tyrosine were determined by HPLC with mass spectrometric detection. The parameters of the chromatographic separation of iodotyrosines and the conditions of electrospray ionization are presented.

Keywords: *Iodotyrosines; Organic Iodine; HPLC with Mass Spectrometric Detection; Food Products*

Introduction

Iodine occurs in humans and animals as inorganic iodides and organic (about 75% of the total iodine) covalently bound forms, like thyroglobulin and iodinated amino acids monoiodotyrosine and diiodotyrosine, as well as iodine-containing hormones thyroxine and triiodothyronine [1,2]. Covalently bound iodine (via amino acid residues, for example, tyrosine or histidine) is easily transferred into organic form and ingested by endocrine organs (a thyroid system) [3,4]. Iodotyrosines are synthesized in thyroid follicles as part of thyroglobulin protein. Organic iodine in the form of iodotyrosines is produced as biologically active supplements (BAS) to combat iodine-deficiency diseases. The production of an organic form of iodine for supplements is based on the enzymatic iodination of amino acid residues of the whey protein of cow milk [5].

The main criteria for proving the authentication of dietary supplements based on iodinated milk proteins is the confirmation of the presence of iodotyrosines and determination of the degree of their iodination [3]. Until now, the existing procedures were based on the voltammetric determination of inorganic forms of iodine. A unique procedure for determining the concentration of iodotyrosines in dietary supplements was developed in the Gorbatov All-Russian Research Institute of Meat Industry and certified *(MI* (Methodical Instructions) *103.5-132-2012)* [6]. Supplements based on iodotyrosines are used for the enrichment of foods, and their final concentration in the products is not controlled because of a lack of certified procedures for determining iodotyrosines.

The problem of creation, implementation, and industrial production of iodine-containing preparations is closely related to the development of documents, normalizing the composition and structure of the drug compound, the quality of substrates and auxiliary compounds, and periods and conditions of the storage of dosage forms. There is a need in the development of sensitive methods for the determination of organic polyhalides at various stages of their production and in dosage forms, BAS, and biological samples. The need in the control of the concentration of iodotyrosines is due to both technological factors in the production of dosage forms and the use of an increasing number of chemical compounds in the manufacture of food products, which may react to form new substances.

HPLC with mass spectrometry (HPLC-MS/MS), time-of-flight mass spectrometry, and matrix-assisted laser desorption/ionization mass spectrometry detection are most informative to confirm the authenticity of BAS and to control the concentration of iodotyrosine in enriched products. They enable the determination of structural fragments, amino acid sequence, post-translational modifications of proteins and amino acids, and molecular weights of a wide range of proteins with high accuracy [7].

Aim of the Study

The goal of the present work was to develop a procedure for determining iodotyrosines in foods.

Experimental

A key step in determining iodotyrosines in BAS is the splitting of the protein components of the sample with an enzyme (protease from *Streptomyces griseus,* Sigma Aldrich) and their subsequent purification by ultracentrifugation. Iodotyrosines are identified by reversed-phase chromatography with conventional UV detection. The identification of iodotyrosines in complex food matrices (meat or dairy products, bakery products, canned juices, etc.) and biological fluids requires the use of tandem liquid mass spectrometry, the concentration of iodotyrosines cannot be determined after the pre-enrichment of the product with an iodinated protein because of the low sensitivity of UV detectors [8]. As a result, we developed a procedure for the determination of iodotyrosines in foods by tandem liquid chromatography-mass spectrometry. The optimum conditions of enzymatic hydrolysis were found, the degree of hydrolysis and the stability of the analytical signal in complex matrices were studied and determined.

Preparation of calibration solutions

3-Iodo-L- tyrosine (MIT) and 3,5-diiodo-L-tyrosine (DIT) from Sigma-Aldrich (United States) were used as reference samples. To prepare calibration solutions, MIT and DIT underwent derivatization. For this purpose, weighed portions of reference samples were dissolved in methanol in an ultrasonic bath (Branson 5510); the required portion of the resulting solution was collected and evaporated in a rotary evaporator (Heidolph Laborota 4003). The dry residue was dissolved in a mixture of butanol and acetyl chloride (4 : 1) and incubated in a water bath at 60°C for 15 minutes. The mixture was evaporated, and the dry residue was dissolved in a 20% acetonitrile solution in water.

Sample preparation

Sample preparation included the enzymatic hydrolysis of a food product and the extraction and purification of the target compounds by solid-phase extraction (SPE) followed by the derivatization of the extract. Before analyzing, the sample was predried in an oven at 65 - 70°C and degreased in a Soxhlet apparatus (Behr R 106 S). Milk and dairy products did not need preliminary treatment. A weighed portion of a sample was hydrolyzed in a buffer solution of Tris-HCl (tris(hydroxymethyl)aminomethane, Scharlau) at pH 8.0; protease was added in an amount of 1/10 of the protein weight, and the mixture was incubated for at least 16h at 37°C. Partial hydrolysates were obtained after enzymatic hydrolysis, which contained both small peptides and amino acids, including tryptophan, in different ratios. Papain, pancreatin, and protease were used in hydrolysis. The hydrolysis of proteins with proteolytic enzymes proceeds by no more than 85%; therefore, an empirical coefficient of 0.85 was introduced in calculations of the weight fraction of iodotyrosines, taking into account of the loss of iodotyrosines at the sample preparation stage. In acid hydrolysis, the sample was heated in the presence of conc. HCl at 110°C for 24h. Iodinated amino acids, particularly, iodotyrosines, are unstable to acid hydrolysis. In alkaline hydrolysis, the racemization of certain amino acids and the decomposition of arginine, lysine, cysteine, and cysteine occurred.

The hydrolysate was centrifuged, filtered, and then purified by SPE. An SPE cartridge was preconditioned successively with 2 mL methanol and 2 mL of twice- distilled water. A 0.4-mL portion of a hydrolysate sample was collected; 0.1 mL of acetonitrile was added, and the mixture was applied to a cartridge. The analytes were back extracted from the cartridge in two steps, using successive elutions with 1 mL of methanol and mL of a mixture of acetonitrile and 0.1 M HCl (4 : 1.8). The eluent was evaporated to dryness at 60°C; the analytes were then derived by a mixture of butanol- acetyl chloride (4 : 1), similar to reference samples.

These procedures for sample preparation gave a purified sample for reproducible analysis in complex matrices.

The conditions of HPLC-MS/MS analysis were as follows: Agilent Technologies 6410 Triple Quadrupole instrument; multiple reaction monitoring (MRM) mode after chromatographic separation under the optimized conditions using an Agilent Technologies 1200 Series HPLC system. The chromatographic separation of the hydrolysate mixture after derivatization was performed by reversed-phase chromatography using a C18 phase column (Agilent Eclipse XDB C18, 4.6 x 50 mm, 1.8 ^m) in the gradient elution mode (Table 1).

Time, min	Ratio of the mobile phase components		Flow rate, mL/min	Column temperature, °C
	Acetonitrile, %	1% HCOOH, %		
0.0	20	80		
2.0	90	10	0.6	30
5.0	90	10		

Table 1: *Conditions of chromatographic determination.*

The following parameters of mass-spectrometric detection were selected: source temperature was 100°C; desolvation gas temperature was 320°C; desolvation gas flow rate was 8 L/min; and spray needle pressure was 30 psi (2.06842 bar). The conditions for recording analytical signals in the MRM mode are summarized in table 2.

Analyte	Precursor ion, m/z	Product ion, m/z	Declustering potential (Frag), V	Dissociation energy (CE), V
3-Iodo-L-tyrosine (MIT)	364.0	134.9	112	30
	364.0	261.9	112	13
3,5-Diiodo-L-tyrosine (DIT)	489.9	387.8	116	17
	489.9	260.9	116	30

Table 2: *Parameters of impact on ions in the MRM mode and conditions of electrospray ionization in the positive ion mode.*

The prepared reference solutions were analyzed five times each. Then, a calibration curve was built using a Masshunter Quantitative Analysis software (Version Build 4.0.225.0) by the dependence of the peak area of product ions of reference samples of an analyte on the analyte concentration. The analytes were identified by the absolute retention time of product ions of target compounds. The detection limit for iodotyrosines (MIT and DIT) was 1 mkg/kg. The correlation coefficient was 0.9994 in the concentrations range of iodotyrosines 1 - 2000 ng/mL. The equation of the calibration curve for determining MIT was $y = 441.127x + 32.209$; for DIT, it was $y = 390.771x + 27.491$. In using columns packed with a fine-grained adsorbent (1.8 um), the duration of analysis did not exceed 5 min.

Results and Discussion

In the development of a procedure for determining organic iodine, we have tested *MUK* (Methodological Guidelines) *4.1.1187-03: Voltammetric Determination of Iodine in Food.* The method is based on the ability of iodide ions to accumulate on the surface of a mercury film electrode as a poorly soluble compound with mercury at a certain potential, followed by the cathode reduction of the deposit under varied potential. To decompose the organic form of iodine, samples were subjected to dry mineralization in a PM-16P-1200 muffle furnace; a KNO_3 solution was used as a stabilizer. The amount of generated iodide ions was determined using an ABC 1.1 Volt polarograph. Bread, milk, and milk products enriched with the iodocasein preparation were test objects. Enrichment was carried out in accordance with the recommendations of *MP* (Methodological Guidelines) *2.3.7.1916-04: Application of Iodocasein for Preventing Iodine-Deficiency Diseases as a Means for Population, Group, and Individual Prevention of Iodine Deficiency.* The desired concentrations were in the range 80 - 120 mkg/kg (in terms of iodine). We did not obtain converging results in the analysis, which may be related to possible losses during sample preparation. In the case of enrichment with an inorganic form of iodine, the error of this method in the concentration range 80 - 120 mkg/

kg did not exceed 20%. The titrimetric method is not applicable to foodstuffs, as its determination limit does not exceed 50 mg/kg, which is two to three orders of magnitude higher than the concentration of iodine in the enriched products.

Thus, the identification of iodotyrosine in complex food matrices enriched with organic iodine (meat and dairy products, bakery products, canned juices, etc.) required the use of HPLC-MS/MS equipment.

To identify and assess the site of iodine inclusion into proteins, we used mild enzymatic hydrolysis of iodinated proteins, followed by analysis by chromatography and mass spectrometry. It was shown that the hydrolysis of proteins by proteolytic enzymes had no drawbacks of acidic hydrolysis; there were no pathological changes in the hydrolysis products. As a result, the recovery of iodotyrosines from the food matrix was 85% or higher. The correlation coefficient in the measurement range 1.0 - 2000.0 ng/mL was 0.9994; the detection limit for iodotyrosines was no higher than 1 mkg/kg. Chromato- grams of a food product enriched with iodotyrosine are presented as an example in figure 1 and 2.

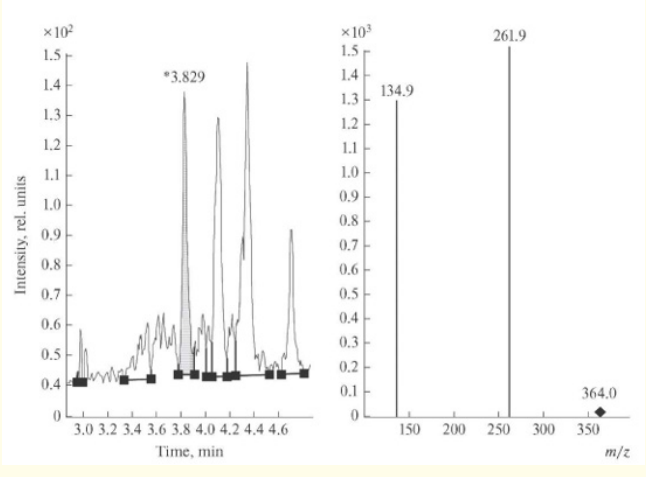

Figure 1: *Total ion current chromatogram of a sample of food enriched with iodotyrosines (MRM transitions of MIT).*

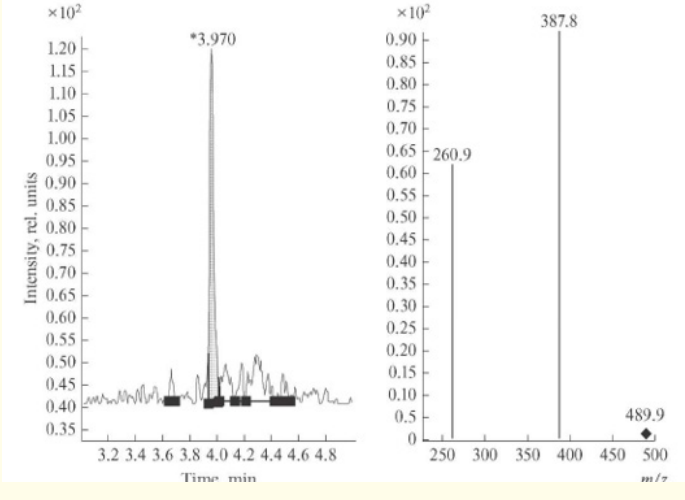

Figure 2: *Total ion current chromatogram of a sample of food enriched with iodotyrosines (MRM transitions of DIT).*

According to the results of these studies, we developed *GOST* (State Standard) *33422-2015: Meat and Meat Products. Determination of Iodotyrosine Using High Performance Liquid Chromatography with Mass Spectrometric Detection.* All-Russia Research Institute of Metrological Service carried out a metrological examination and assessed the accuracy of the procedure in accordance with *GOST* (State Standard) *R ISO 5725-2002.* If all of the regulated conditions we met in strict accordance with this procedure, the error (and its components) of the results of measurements did not exceed the values presented in table 3.

	Relative	Repeatability	Reproducibility
Analyte	Error ± 5, %	Limit *r*, mkg/kg	Limit R, mkg/kg
3-Iodotyrosine (MIT)	20	$0.15x_{av}$	$0.25X_{av}$
3,5-Diiodotyrosine (DIT)	12	$0.10x_{av}$	$0.15X_{av}$

Table 3: *Performance characteristics of the procedure.*
x_{av} is the arithmetic mean of the results of two parallel measurements; X_{av} is the arithmetic mean of the two measurements performed in different laboratories.

Conclusion

The accuracy of the developed procedure was evaluated based on data obtained in the analysis of a mixture of reference samples of iodotyrosines by two independent operators under intermediate precision. The effect of sample preparation stage on the accuracy of the determination of iodotyrosines was evaluated by the standard addition method, by introducing a mixture of iodotyrosines into the food matrix (meat products). The data were obtained in analyzing 25 samples in terms of repeatability with the concentration of iodotyrosines 1, 10, 50, 100, and 200 mkg/kg. To confirm the specificity of the procedure, we tested ten samples of meat products free of iodotyrosines. For all ten samples, chromatographic peaks interfering with the determination of iodotyrosines were not found. The procedure enables the reliable determination of iodotyrosines in the presence of impurities and system peaks of the mobile phase.

It is found that, to control the quality of enriched products (after the introduction of functional additives containing iodinated milk proteins), tandem gas chromatography-mass spectrometry ensures the reliable identification of the form of organic iodine and help to control its amount (as iodotyrosines) in food products in a wide concentration range.

Acknowledgments

The part of the work performed in the Volga Research Institute of Production and Processing of Meat and Dairy Products was supported by the Russian Science Foundation, project no. 15-16-10000.

Bibliography

1. Gerasimov GA. "Iododefitsitnye zabolevaniya (IDZ) v Rossiiskoi Federatsii: politika v oblasti profilaktiki i ten- dentsii v epidemiologicheskoi situatsii (1950-2002g.) (Iodine Deficiency Disorders (DHS) in the Russian Federation: Policy in the Field of Prevention and Trends in the Epidemiological Situation (1950-2002))". Moscow (2003).

2. Mokhnach VO. "Teoreticheskie osnovy biologicheskogo deistviya galoidnykh soedinenii (Theoretical Basis of the Biological Effect of Halides)". Leningrad: Nauka (1968).

3. Savchik SA., *et al*. *Voprosy Pitaniia* 4 (2005): 3.

4. Kulikovskii AV., *et al*. "Methodology of the Determination of Polycyclic Aromatic Hydrocarbons in Foods". *Journal of Analytical Chemistry* 69.2 (2014): 205-209.

5. Tomchani OV. Cand. Sci. (Tech.) Dissertation, Obninsk (2002).

6. MI (Methodological Instruction) 103.5-132¬2012/01.00225-2011. Determination of Iodotyrosines in Iodinated Milk Proteins and Food Supplements Based on Them by High Performance Liquid Chromatography, Moscow (2012).

7. Vostrikova NL., *et al*. "Determination of Muscular Tissue Proteins by 2D Electrophoresis and Time-of-Flight Mass Spectrometry". *Journal of Analytical Chemistry* 72.10 (2017): 1102-1112.

8. Isaksson M., *et al*. "Determination of iodine in vivo and in vitro by X-ray fluorescence analysis: methodology and applications". In Comprehensive Handbook of Iodine: Nutritional, Biochemical, Pathological, and Therapeutic Aspects, Preedy, V.R., Burrow, G.N., and Watson, R.R., Eds., Elsevier (2009): 29.

Volume 15 Issue 1 January 2019