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A comparative analytical assessment of iodides in healthy and pathological human thyroids based on IC-PAD method preceded by microwave digestion

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ABSTRACT

The aim of the study was to examine correlations between the content of iodides in 66 nodular goiters and 100 healthy human thyroid tissues (50- frozen and 50 formalin-fixed). A fast, accurate and precise ion chromatography method on IonPac AS11 chromatographic column (Dionex, USA) with a pulsed amperometric detection (IC-PAD) followed by alkaline digestion with tetramethylammonium hydroxide (TMAH) in a closed system and with the assistance of microwaves was developed and used for the comparative analysis of two types of human thyroid samples. Statistical analysis revealed over eightfold reduction of iodine concentration in the pathological tissues (the mean value was 77.13 ± 14.02 ppm) in comparison with the control group (622.62 ± 187.11 ppm for frozen samples and 601.49 ± 192.11 ppm for formalin-fixed ones). A good correspondence (for 10 additional determinations) between the certified (3.38 ± 0.02 ppm with variation coefficient (V.C.) of 0.59% for Standard Reference Material (SRM) NIST 1549-non-fat milk powder) and the measured iodine concentrations (3.52 ± 0.29 ppm; V.C. = 10%) was achieved. It was pointed out that the way of tissue preservation (either in formalin or by freezing) had no significant effect on the iodine determination result ($\alpha = 0.1$). Significantly lower iodide content was found in nodular goiter thyroid samples. The applied conditions of digestion, reinforced by the action of microwaves, brought about a decidedly shorter (less than 20 min) sample preparation time. Suitability of the developed IC method was supported by validation results.

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1. Introduction

A nodular goiter is the most common pathology of the thyroid gland [1,2]. Numerous factors are involved in the etiology of a nodular goiter. Many disturbances in the synthesis and metabolism of thyroid hormones, so called enzymatic defects, have genetic background [3,4]. Biochemical abnormalities may be related to the iodine trap defect, iodotyrosine binding, mechanism responsible for iodine separation and loss, activity of peroxidase as well as impaired production of iodine–protein bindings. All the named disturbances result in decreased thyroxine and triiodothyronine levels and, in accordance with the feedback principle, increased thyrotropin (TSH) levels, which is a goitrogenic factor. Deficiency of thyroid hormones may result from the activities of natural chemical goitrogenic compounds contained in some food products. They are mainly 5-vinyl oxazolidine and rhodanates. Synthetic goitrogenic substances include agents of the tyreostatic group (imidazole and tiouracyl derivatives) as well as potassium perchlorate. Agents used in the treatment of other pathological conditions and capable of blocking tyrosine iodification are: sulfonamides, cobalt and lithium salts, paraaminosalicilic acid, butazolidine [5,6].

Morphology and functioning of the thyroid gland are largely affected by iodine supply [7,8]. Iodine deficiency may result in formation of an endemic goiter, even with the presence of clinical hypothyroidism [9]. A similar pathogenic mechanism is observed at those stages of goiter formation when the bodily demand for the thyroid hormone is increased, i.e. during puberty, gestation and lactation periods. International research on iodine deficiency in Europe has been in progress for many years. This project is monitored by the International Council for Iodine Deficiency Disorders (IDD) and works in cooperation with UNICEF and WHO. According to their reports only four European countries, i.e. Finland, Sweden, Norway and Iceland, were free of diseases resulting from iodine deficiency in the early 1990s. In a subsequent report, published in 2002, it was announced that despite iodine prophylaxis, only 17 countries, out of 31 in Western and Central Europe, had adequate iodine supply. According to these data Poland has recently been assumed to be free of IDD [10]. As a result of compulsory

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table salt iodification (a model of compulsory iodification of table salt at the level of 30 ± 10 mg KI/kg of salt has been operating in Poland since 1996), a marked decrease in the incidence of diseases connected with iodine deficiency, including a nodular goiter, has been observed in the recent years [11]. However, a substantial population of patients who had developed thyroid disorders before the compulsory table salt iodification was launched (long-standing nodular goiters) is still present. In the beginning the activities of the goitrogenic factors presented above bring about the thyroid gland enlargement and next, after a longer period of TSH stimulation, uneven hypertrophy of the glandular tissue develops (e.g. at the sites of richer vascularization), which results in the formation of nodules, also of adenocarcinomic nature [7,12]. In a long-standing goiter retrogressive changes appear (colloid cysts, calcifications, fibroses, hyalinizations and posthaemorrhagic cysts) [13,14]. A nodular goiter may be present for years with concomitant euthyreosis. However, more often than not the nodules become activated and a set of symptoms indicative of hyperthyroidism develops.

In the light of the recently changed iodine supply model in Poland, the effect of the new iodine supplementation conditions on the iodine content in the thyroid gland, morphologically changed into a goiter, appears to be an interesting issue. Approximately 15–20 mg of iodide is comprised in the adult human body. The vast majority of it is found in the thyroid gland where it amounts to 75–80% of the total bodily iodide content [15].

Several methods for measuring iodine are currently available, e.g. spectrophotometric methods based on catalytic reactions [16], X-ray fluorescence spectrometry [17], spectrophotometry [18], the use of ion-selective electrodes [19,20], inductively coupled plasma mass spectrometry (ICP-MS) [21–23] or neutron activation analysis (NAA) [24]. Most reports concerning thyroid analysis are grounded on research into the animal tissue and, unfortunately, scientific studies on the iodine content in human thyroids are scarce. Recently, electrospray ionization tandem mass spectrometry (ESI-MS–MS) has been developed for determination of iodides in human urine [25]. Unfortunately, most applied analytical methodologies require a time consuming sample preparation step or very often expensive detection systems. One of the exceptions is a simple capillary electrophoresis (CE) method with direct UV detection developed for determination of iodides in human plasma and urine [26]. Research work of Tadros et al. [27] proved the fluorescent X-ray scanning method to be useful for iodine measurements in both benign and malignant thyroid nodules. They expected the *in vivo* fluorescent technique to contribute to the pre-operative assessment of nodular goiters and help the clinician select those nodules which require surgical excision. Unfortunately, in spite of reported fairly good detection limit (20 μg/g of tissue) of the X-ray fluorescence method, their results were not confirmed by other methods.

Despite a wide choice of available analytical methodologies, determination of iodide in biological matrices remains a difficult problem. Possible reasons of such difficulties are related to preservation of the tissues, and inappropriate use of digestion reagents. Tissues must be preserved in a way that minimizes potential loss of analyte. Formalin is a routinely used tissue-fixing agent after surgical procedures. The other recommended agents for tissue preservation include, e.g. a mixture of glutaraldehyde and paraformaldehyde (i.e. Karnovsky fixative) followed by embedding in methacrylate.

Sample decomposition is a critical step in iodides' analysis as well. Ion chromatography method requires total digestion of the sample matrix. Some procedures, e.g. "Schoeniger Combustion", require a highly homogenous sample, which is sometimes difficult to obtain [23]. There are many options for biological sample preparations, among which alkaline digestion using tetramethylammonium hydroxide (TMAH) is the most common before the analysis of iodides [28,29]. Alkaline conditions during the extraction procedure have some advantages in comparison with acidic media where iodide may be oxidized into volatile forms (I_2 or HI). Firstly, mineralization in a closed system and with the assistance of microwaves significantly shortens the time of the sample preparation step (as reported in literature digestion of biological materials with TMAH usually requires up to 6 h [30]), and secondly, which has a crucial meaning in trace analysis, it significantly reduces the possibility of suffering loss of analyte. Since for all modern sample pretreatment methods time is a very important factor, fast and easy microwave-assisted alkaline digestion has been developed in our investigations.

The U.S. Department of Health and Human Services [31] presented NAA-MS as a method for determining iodide in the thyroid, however, the studies they cited were concerned with application of sulfuric acid for sample digestion. At present acidic digestion is considered to be highly inappropriate since no stable sample solution can be achieved.

Our study was also undertaken in order to compare fresh-frozen and formalin-fixed thyroid samples in context of iodine determination using ion chromatography method with pulsed amperometric detection. Due to its sensitivity (down to the ng/g concentration range), ion chromatography (IC) method requires a small amount of biological samples and appropriate conditions for chemical digestion. Therefore, digestion in a closed system, where the contamination problems are significantly reduced, can be recommended for this kind of samples. Additional assistance of microwaves was applied in the presented study.

In order to examine the role of iodine in the development of thyroid pathology, it is necessary to carry out comparative studies in both healthy subjects and well-diagnosed patients. In our study we present an accurate assessment of the iodine content in the thyroids of patients with a nodular goiter as well as in the thyroids obtained at autopsy – considered here as a control group. To the best of our knowledge it is the first time ion chromatography method has been used to carry out iodide determination in healthy and pathological thyroid tissues in connection with the impact sample storage has on the results of the performed analysis. The applied method has been validated in terms of linearity, precision, accuracy, reliability and recovery.

2. Experimental

2.1. Instrumentation

Measurements were carried out on a Dionex model DX-500 ion chromatograph (Sunnyvale, CA, USA). The system was composed of a IP25 isocratic pump and ED40 electrochemical detector (Ag working electrode, Ag/AgCl reference electrode). The PAD waveform was as follows: (0 s, 0.1 V), (0.2 s, 0.1 V), (0.9 s, 0.1 V), (0.91 s, −0.8 V), (0.93 s, −0.3 V) and (1.00 s, −0.3 V). The current was integrated between 0.2 and 0.9 s and the collection of data was at 1 Hz. IonPac AS11 (Dionex Co., Sunnyvale, USA) was used as an analytical column (4 mm × 250 mm), while IonPac AG 11 (4 mm × 50 mm) was used as a guard column.

The column was equilibrated with the use of the nitric acid eluent. The eluent flow-rate was set at 1.5 mL/min. Injection valve was fitted with a 100-μL loop (injection volume was 50 μL). All measurements were taken at 25 ± 1 °C. The column backpressure was approximately 6.5 MPa (950 psi). All samples were injected at least in triplicate.

A Chromeleon (Dionex) chromatography workstation was used for instrument control and data acquisition.

2.2. Reagents

The mobile phase was 50 mM nitric acid solution (Sigma–Aldrich, Steinheim, Germany), which was obtained by adding 6.25 mL of concentrated HNO_3 to deionized water (18 MΩ cm) in a 2-L volumetric flask. Potassium iodide was obtained from Merck (Darmstadt, Germany), 25% water solution of tetramethylammonium hydroxide (TMAH) for sample digestion was obtained from Sigma–Aldrich, Steinheim, Germany.

The mobile phase, standards, and analyte solutions were made up in water purified with an Easypure system (Barnstead, USA). All reagents were of analytical grade. The eluent was filtered and degassed before use. All analyses were carried out using pre-washed polypropylene flasks and vials.

2.3. Patient population and samples

The studied material consisted of 166 human thyroids. Investigations were performed in 66 people who constituted the studied group (40 females and 26 males, the average age was 35) and had fragments of their thyroid glands removed during a surgical procedure (diagnosed nodular goiter). The patients underwent thyroidectomy in three hospitals in Lublin (a town of 380,000 population in the south-east of Poland, a region which suffered as a result of accident at the Chernobyl nuclear power plant in 1986). The diagnoses were confirmed by histopathological examination. All the patients diagnosed with a nodular goiter were in euthyreosis and did not suffer from hyperthyroidism. Neither did they take any iodine preparations or iodine contrast media in the period of 5 years before the thyroid operation. Authors followed procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983). The protocol was accepted by the local Ethics Committee. The samples obtained from the studied group were fixed in formalin (according to the standard procedure).

Healthy human thyroids were obtained from individuals (mainly from the south-east region of Poland) killed in accidents. The control group included 50 people (9 females and 41 males, the average age was 25 years). No morphological changes were detected in any of the removed tissues. In order to eliminate the methodological error, two specimens of the thyroid gland, were removed. One of them underwent the process of freezing (−25 °C), and the other was fixed in neutral formalin (so the total number of the control samples before sample digestion was 100). The frozen tissues were fixed in containers for at least 1 week prior to digestion and analysis. The formalin-fixed tissues were analyzed after 1–3 weeks of storage. Each sample of tissues was split with a quartz knife. All samples (both fixed in formalin and frozen) were transported and stored in polypropylene containers. Both kinds of thyroids (healthy and pathological) were treated in the same way. This uniform sample preparation allowed to perform a comparative analysis of the healthy and pathological thyroid tissues.

2.4. Sample preparation before IC analysis

The washing and drying procedures of the thyroid tissues have already been described [32]. The samples (each time 0.5 g of dry tissue) were then mineralized using a microwave-assisted high pressure digestion system (UniClever BM-1, Plazmotronika, Poznań, Poland). Each time the alkaline digestion with 25% TMAH water solution was applied (2 mL of TMAH: 8 mL H_2O). A programme of microwave digestion consisted of the following four stages: 10–15 atm for 2 min (40% of power of microwave generator), 15–20 atm for 2 min (60% of power of microwave generator), 20–25 atm for 2 min (80% of power of microwave generator), and a cooling process for 10 min. These conditions of the digestion procedure were monitored each time in order to avoid the decomposition of TMAH (the temperature lower than 100 °C is recommended) and bursting of the closed vessels. After digestion, the Teflon vessels (with screw caps) were cleaned thoroughly twice with dilute digestion mixture in order to avoid memory effects (adsorption by the walls of containers). The solutions obtained were poured into volumetric flasks (PTFE) and when it was necessary they were tenfold diluted with deionized water before IC analysis.

2.5. Preparation of standard solutions

All standards, samples and reagents were stored in polyethylene bottles and kept in a dark place in order to minimize exposure to light. Instrument calibration was performed using iodide standard solutions (concentrations were in the range from 10 μg/L to 10 mg/L). Potassium iodide calibration standards were prepared using serial dilutions of a stock standard solution (a 100-mL aqueous stock solution with a concentration of 10 mg/L of KI was prepared by weighing out 1.307 g of KI, transferring the weighed reagent quantitatively to a 100 mL volumetric flask and dissolving it with high-purity water (18 MΩ cm)).

3. Results and discussion

3.1. Digestion method

It is well known that while performing iodide determination, destruction of the organic matrix in acidic environment is burdened with a big error [20,22]. This happens because of iodide's strong tendency to oxidize into volatile forms (I_2 or HI), thus no stable sample solution can be achieved. Therefore, alkaline digestion was used in the presented study.

In the majority of cases, sample preparation procedure (which directly affects the quality of the analytical result) takes up most of the expected analysis time and thus contributes substantially to the analysis cost. For the examinations performed, it is important that the number of reagents should be reduced to the absolute minimum (lower costs of the analysis). Relatively short times of digestion as well as simplicity of the procedure are of great importance as well. Thanks to a closed system of sample digestion the risk of a possible sample contamination was reduced to the minimum. However, the digestion of biological materials with TMAH is usually a time-consuming procedure since it normally takes several hours [30]. Descriptions of extraction processes using TMAH for iodide analysis in biological matrices in which decomposition procedure takes over 3 h at the shortest may be found in literature [23]. In comparison with the sample preparation methods presented in literature, in our study the sample decomposition time decreased substantially (only 16 min). Moreover, in the study cited above the solution was centrifuged and filtered after the extraction to separate the undissolved particles. In our study the solution after alkaline digestion was homogenous and after dilution with water it was injected directly into the chromatographic column (a 0.45 μm membrane filter is an integral part of the injection system of Dionex DX500 ion chromatograph).

Due to the applied technique of sample preparation, the problem connected with contamination and/or analyte loss has been significantly reduced. The procedure of mineralization is neither time nor reagent consuming and could be applied for a wide range of sample matrices.

3.2. IC analysis

Before injection, IonPac AS11 column was flushed with water for at least 30 min which was followed by equilibrating it with

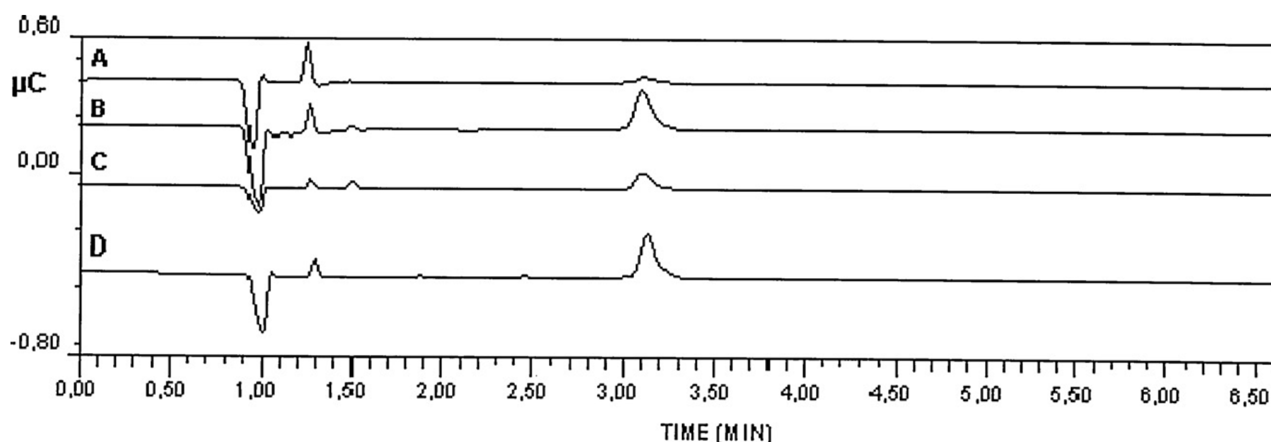

Fig. 1. Ion chromatograms of iodides in the studied samples (operating conditions and description of the types of samples are included in the text).

Table 1
Recoveries for iodide determination in human thyroids with the use of IC-PAD method.

Iodide determined in thyroid samples (μg/g of dry tissue)	Spike amount of iodide (ppm)	Recovery (%)
419.63	0.25	92.0
	1.00	94.0
637.99	1	104.0
960.45	10	90.5
Average		95.1

a nitric acid eluent. After equilibration of the analytical column, three calibration blank standards were analyzed in order to establish a representative blank level. Then, the prepared calibration standards were analyzed. Iodide elutes at approximately 3.15 min. Since PAD has high specificity for this ion, other halides, which are detected less efficiently, do not interfere with chromatographic analysis (chloride ions elutes at approximately 1.3 min). After injection of 50 μg/L sulfate, sulfide, thiosulfate, cyanate and bromide, no peaks were observed on chromatograms.

Since validation of any bioanalytical method is a process used to verify if the analytical performance parameters are adequate for their intended use, the precision and accuracy were determined in this study as well. Linearity of the method was established by analyzing calibration curves which had been prepared earlier for previous standards. A good linearity was found between 25 μg/L and 10 mg/L with the correlation coefficient of 0.9998 (calculated as peak height due to a slight amount of peak tailing). The peak height RSD of three sample injections was 1.5%, and the retention time RSD was 1.0%. The limit of determination (9 times the standard deviation of the blank) was 0.5 μg/L.

Precision of the method was determined in terms of repeatability (intra-assay precision) and quantified by the relative standard deviations (RSD) of the replicate measurements. We followed the suggested approach for a new bioanalytical method [33], i.e. precision was assessed at four unique concentrations in replicates of six, on four separate occasions (i.e. $4 \times 6 \times 4$). RSD was determined to be 2.15% for the studied ion. Accuracy of the proposed method was checked by adding known amounts of iodide ions to the digested samples of human thyroids. The measured concentrations were those obtained during precision assessment (i.e. from $4 \times 6 \times 4$ experiment).

Recovery values for iodide determination in thyroids are summarized in Table 1.

In order to check reliability of the developed digestion method, the results obtained by IC method were compared with the certified values. A good correspondence between the certified (3.38 ± 0.02 ppm with variation coefficient (V.C.) of 0.59% for Standard Reference Material (SRM) NIST 1549-non-fat milk powder) and the measured iodine concentrations (3.52 ± 0.29; V.C. = 10%) was achieved (for 10 additional determinations).

Fig. 1 presents chromatograms for iodide analysis in the following samples: A, blank sample; B, standard solution; C, nodular goiter; and D, control group (healthy thyroid).

Table 2 presents a comparison of results obtained using IC-PAD method, also in relation to the type of the tissue storage.

It is worth noting that approximately eightfold lower mean iodine concentration was observed in the group of the studied patients in comparison with the control group. The lowest concentration levels in the control groups are significantly higher than in the group of the studied patients. Parametric statistical tests of two arithmetic means' significance was performed for three cases presented in Table 3. Arithmetic means for both control groups were the first to be compared (A – fresh-frozen samples and B – samples fixed in formalin).

A zero-hypothesis, assuming equality of both means, was formulated versus an alternative hypothesis which assumed that both means differed significantly. A bilateral parametric statistical test was performed. The obtained value of the test function was much lower than the critical value, which gives no ground for H_0 rejection and at the same time proves that a small difference in iodine concentration obtained in the control groups (A) and (B) is statistically insignificant (Table 3). Comparing arithmetic means of iodine concentrations in the group of the studied patients (C) and in the control group (B) was carried out according to the zero-hypothesis which assumed equality of both means vs. the alternative hypothesis which assumed that the mean in the group (B) was significantly higher than in the group of the studied patients. A right-hand side parametric test of significance was performed. At the significance level of $\alpha = 0.01$ the obtained value of the test function was much higher than the critical value, which proves beyond any doubt that the mean iodine concentration in the control group (B) is statistically significantly higher than in the group of the studied patients. Comparing arithmetic means of iodine concentrations in the group of the studied patients (C) and in the control group (A) was performed following the zero-hypothesis which assumed equality of both arithmetic means and vs. the alternative hypothesis assuming that the mean in the group (A) was significantly higher than in the group of the studied patients. A right-hand side parametric test was performed. At the significance level of $\alpha = 0.01$ the obtained value

Table 2
Comparison of the results of iodide analysis in thyroids of the patients with a nodular goiter and in the control group.

	Samples of studied thyroids	Number of samples before digestion	Number of samples after digestion	Number of injections	Total number of IC analyses	Concentration range (ppm)	Mean concentration (ppm)	Standard deviation SD (ppm)	Variance $(ppm)^2$
Studied group (nodular goiter)	Fixed in formalin	66	198	3	594	41.13–103.63	77.13	14.02	196.60
Control group (healthy thyroids)	Frozen	50	150	3	450	210.36–1000.32	622.62	187.11	35010.23
	Fixed in formalin	50	150	3	450	156.45–1005.36	601.49	192.11	36905.29

Table 3
Statistical analysis parameters of the obtained results.

	Object of analysis[a]	Zero-hypothesis (H_0)	Alternative hypothesis	t-Value[b]	Critical value (t_{α}[c])	Result of the test	Level of significance (α)
1	Comparison of the iodide content of (A) and (B) thyroid samples	$[I^-]_A = [I^-]_B$	$[I^-]_A \neq [I^-]_B$	0.552	1.663	No grounds for H_0 rejection	0.1
2	Comparison of the iodide content of (B) and (C) thyroid samples	$[I^-]_B = [I^-]_C$	$[I^-]_B > [I^-]_C$	21.739	2.362	H_0 rejection	0.01
3	Comparison of the iodide content of (A) and (C) thyroid samples	$[I^-]_A = [I^-]_C$	$[I^-]_A > [I^-]_C$	23.215	2.362	H_0 rejection	0.01

[a] (A) – frozen samples; (B) – samples fixed in formalin; and (C) – pathological thyroids samples.
[b] Value of the test function t.
[c] Value read from t-student distribution table.

of the test function was much higher than the critical value, which proves explicitly that the mean iodine concentration in the control group (A) is statistically significantly higher than in the studied group of patients (Table 3).

4. Conclusions

Statistical analysis showed approximately eightfold reduction of iodine concentration in the pathological tissues in comparison with the control group. It was also pointed out that the way of tissue preservation (either in formalin or by freezing) had no substantial effect on the iodine determination result. This finding has an enormous practical value for the future research into the effect of any studied chemical element on the development of various thyroid diseases. So far the tissues' preservation procedure in hospitals and clinics has relied mainly on fixing them in formalin. Investigations presented here will help carry out a thorough analysis of correlations between other chemical elements and iodine in pathologically changed thyroids. It is very difficult to compare our results with previously published studies involving ion chromatography technique because according to the authors' best knowledge there are no such data available. Despite the lack of such data, our results are similar to the literature data reportedly obtained by a different method (involving X-ray fluorescence) [27]. Our findings have shown that the IC-PAD method can be used for accurate, precise and fast determination of iodides in biological matrices at low ppb levels. Suitability of the developed method was supported by validation results. The main asset of the described procedure of iodide analysis in human thyroids lies in its simplicity and possibility of applying samples of various origin.

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