Investigation of iodine bioavailability from chicken eggs versus iodized kitchen salt with in vitro method

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Abstract The goal of the presented studies was to investigate speciation and bioavailability of iodine from chicken eggs *versus* iodized kitchen salt with an in vitro method. Determination of iodine total content in chicken eggs and iodized kitchen salt was carried out by inductively coupled plasma mass spectrometry (ICP MS). The majority of iodine was accumulated in the yolk—the concentration was even 37 times higher than in white. Chicken eggs were treated with buffer (Tris HCl pH = 7.5) and enzymatic extraction media and analyzed by size exclusion chromatography hyphenated with inductively coupled plasma mass spectrometry (SEC ICP MS). The enzymatic extraction being an in vitro bioavailability assessment method was based on two-stage digestion model simulating gastric (pepsin digestion) and intestinal (pancreatin digestion) juices. Speciation analyses along with bioavailability studies presented iodide as the major form in chicken eggs. The bioavailability was established as 33% from white and 10% from yolk and decreased with longer time of boiling. It allows to suggest that the majority of iodine remains in forms bound to non-digestible coagulated and water-insoluble proteins.

Keywords Food analysis · Iodine · Iodized kitchen salt · Chicken egg · Bioavailability · Speciation analysis

Introduction

Iodine plays an important role in human nutrition, and it is well known as an essential trace element for growth, development and human essential functions. Insufficient iodine intake can be responsible for the thyroid gland disease and fetus congenital anomalies [1, 2]. WHO recommendation for daily iodine intake is varies from 50 (for infants) to 170 µg (for women during lactation). This led to universal salt iodination process, which was considered to be the most efficient way to improve iodine intake. Unfortunately, the process appears to be insufficient to cover the daily doses of iodine, as global consumption of salt decreases [3]. This is the reason of growing attention of iodine supplemented food that could replace most commonly used iodide supplemented kitchen salt. In case of bioavailability studies, iodide is supposed to be completely absorbed from the gastrointestinal tract [4]. On the other hand, iodate and iodine bound to protein has to be reduced to iodide before absorption. That is why kitchen salt can be expected to offer best bioavailability—thanks to simple inorganic matrix. However, it has never been confirmed experimentally. Replacing of the supplemented salt in diet can be challenging due to loss of volatile iodine even up to 60% during processing steps (especially boiling) of iodized food [5]. According to Goindii et al. [6], for these losses are responsible mainly redox activity of iodate and iodide, being able to react liberating volatile iodine. To ensure good effectiveness of human nutrition, supplemented food should be as commonly used as salt. It is almost impossible to fulfill such a goal due to widespread food allergies caused by lactose, histamine or gluten intolerance, or by economic situation of different nationalities [7].

Saltwater fish and seafood are known and confirmed to be the main source of iodine in human diet [8].

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Additionally, the intake of iodine has also increased from cow milk, eggs and meat since year 2000 due to replacement of low iodine content meat and bone meals with commercially iodized mineral feed. However, Polish National Research Institute of Agricultural and Food Economics has reported that average consumption per year of iodine-rich food in Poland reaches only 6.5 kg of fish, 180 L of milk and 206 eggs, while in Western Europe exceeds 20 kg, 350 L and 250 pieces, respectively. Such disproportion was explained by low price of eggs in comparison to fish and very low consumption of milk by people older than 30. In such case, chicken eggs are considered as a promising additive to diet [9, 10] not only in case of iodine, but also selenium and vitamin B₁₂ [11, 12].

The earlier investigation about iodine forms and its combination with other micronutrients [13] showed that most of the products use potassium iodide. Few powders are fortified with potassium iodate and only one case with sodium iodide. But, a possible problem of consuming multiple commercial fortified foods could be the risk of overconsumption of micronutrients and iodine. Nevertheless, one is not likely to reach the toxic levels of iodine since the toxic symptoms appear at a level of consumption 20 times the RDA (1,100 µg day⁻¹) [2].

Redox properties of iodine species are source of many problems during sample preparation. At first, mineralization of samples was carried out at highly oxidizing conditions (HClO₄, H₂O₂) to ensure conversion of iodine species to non-volatile iodate [14, 15]. The iodide oxidation to iodate can be explained with the reaction studied by Bray and Liebhansky [16, 17]. H₂O₂ was found to be enough strong and fast oxidizing agent in acidic conditions (H⁺ > 0.2 M), which prevents loss of volatile iodine in case of standards mixture. Another approach was alkaline extraction (TMAH—tetramethylammonium hydroxide, NH_{3aq}, (NH₄)₂CO₃), which was found to provide the best stability of iodide and molecular iodine species in water solutions [18–20]. Unfortunately, both alkaline solubilization and acidic digestion methods are not fully reliable, as the digestion efficiency is strongly matrix dependent [21]. Furthermore, oxidizing potential of acidic methods might not be high enough to digest biological samples without iodine losses [18]. On the other hand, alkaline conditions are vulnerable to high concentrations of starch or calcium ions leading to less efficient digestion [21].

Still, the most problematic are bioavailability studies of iodine species as simulation of gastric digestions requires acidic pH values. The best method to resolve or at least to control the problem is mass balance test [22]. Nevertheless, determination of iodine traces in extracts and residue of digested biological sample is necessary. Inductively coupled plasma mass spectrometry (ICP MS) offers the most advantages with selectivity and low limits of detection as

primary features. It is used to determine total amounts of iodine [23] as well as to access bioavailability of iodine from foods [22, 24]. However, it has to be emphasized that analysis of iodine species by ICP MS is vulnerable to memory effects, instability of the signal and other problems due to possible adsorption of iodine species on plastic surfaces (Teflon[®], Rytan[®]) or its volatilization inside the mist chamber. To some degree, the problems can be resolved by glass or quartz mist chambers and polyether ether ketone (PEEK) tubings/connections as well as prolonged washing steps enforced by alkalic media [20, 25].

The objective of the presented study was a verification of the nutrition potential of eggs by establishing the bioavailability of iodine from technologically powdered eggs and shell eggs with the most common in vitro digestion method [26] in reference to iodized kitchen salt containing completely bioavailable iodine (Table 1).

Materials and methods

Standards and reagents

All reagents used were of analytical-reagent grade purchased from Sigma–Aldrich (Sigma–Aldrich, Buchs, Switzerland). Water (18 MΩ cm) prepared with a Milli-Q system (Millipore Elix 3, Millipore, Saint-Quentin, France) was used throughout.

Samples

The powdered egg white and yolk received from technological process based on ultrafiltration and drying as well

Table 1 Operational parameters for LC (liquid chromatography) and ICP MS (inductively coupled plasma mass spectrometry)

Parameter	Setting
ICP MS	Agilent 7500a
rf Power	1,210 W
Plasma, auxiliary and nebulizer gas flow	15.0, 1.0 and 1.05 L min ⁻¹
Cones	Sampler—Ni, Skimmer—Ni
Monitored isotopes	¹²⁷ I
Dwell time	0.1 ms
LC	Agilent 1100
Column	Superdex 75 (10 × 300 mm × 10 µm)—GE Healthcare Life Sciences
Mobile phase	30 mM Tris HCl buffer (pH 7.5)
Elution program	Isocratic
Flow	0.7 mL min ⁻¹
Sample volume	100 µL

as fresh chicken eggs from Polish market (also declared by producer as iodine enriched) were analyzed. Selected 6–9 eggs from batches originated from different Polish farms were boiled for 5, and 10 min as a one of the most common way of preparation at home; after that, they were easily fractionated into shell, white and yolk. White and yolk were ground with grater and placed in a wide-bed beaker. The height of sample layer was below 5 mm. Not more than 150 g of grated eggs was placed in beakers to ensure that process of freeze-drying at $-50\text{ }^{\circ}\text{C}$ will be completed in 3 h in lyophilizator (Alpha 1–4 Model, Christ, Osterose, Germany). Dried sample was ground using agate mortar and pestle until a homogenous powder was formed; the powder was stored at $4\text{ }^{\circ}\text{C}$. A certified reference material of whole egg powder was used—NIST (National Institute of Standards and Technology, Gaithersburg, USA) 8415 Whole Egg Powder, $1.97 \pm 0.46\text{ }\mu\text{g I g}^{-1}$. Iodized kitchen salt bought in retail market according to manufacturer note consisted $30 \pm 10\text{ mg kg}^{-1}$ of potassium iodide—the iodine content was confirmed by ICP MS.

Buffer extraction method

Homogenized samples of eggs and kitchen salt (0.6 g) were extracted with 5 mL of 30 mM Tris HCl ($\text{pH} = 7.5$) during 1 h in ultrasonic bath. The obtained solution was centrifuged for 15 min at 12,000 rpm at $22\text{ }^{\circ}\text{C}$. Fat from supernatant was extracted from sample with 500 μL of dichloromethane. Water phase was cooled in freezer for 10 min and centrifuged once again. The final supernatant was filtered with 0.45 μm syringe filter (Sigma–Aldrich, Bellefonte, PA, USA), two first drops were discarded, and only the remaining part of the filtrate was injected on the size exclusion column. Pre-concentration of samples by lyophilization was optional toward size exclusion chromatography hyphenated with inductively coupled plasma mass spectrometry (SEC ICP MS) analysis.

In vitro gastrointestinal digestion method

The in vitro digestion method (Fig. 1) was based on Luten [26], modified to the eggs and iodized kitchen salt studies. 5 mL of gastric juice (6% w/v pepsin in 0.15 M NaCl, acidified with HCl to $\text{pH} = 1.8$) was added to 1.0 g of white, yolk and iodized kitchen salt and sonicated for 1 min in ultrasonic bath. The mixture was incubated in thermostatic water bath for 3.5 h at $37\text{ }^{\circ}\text{C}$. After 1 h of incubation, the pH was checked and adjusted by HCl to 3.0. After gastric digestion, the part of samples was centrifuged at 12,000 rps for 15 min. An aliquot of the supernatant, referred later on as “gastric extract,” was analyzed. The sodium bicarbonate was added to the other part of sample

to raise the pH to 7.5 to ensure good stability of iodine species [27]. After that, 3.5 mL of intestinal juice (1.15% w/v pancreatin in 0.15 M NaCl) was added, and the mixture was incubated in thermostatic water bath for 3.5 h at $37\text{ }^{\circ}\text{C}$. After gastrointestinal digestion, the sample was centrifuged at 12,000 rps for 15 min. The supernatant recovered after centrifugation was referred further on as “gastrointestinal extract.” To simulate gastrointestinal digestion of iodide and iodate, 85 μL of the standard solutions (100 ppm) were treated as white and yolk samples. Aliquots of the gastric and gastrointestinal extracts were filtered with 0.45 μm syringe filter and analyzed by size exclusion chromatography, performed using Agilent 1100 gradient HPLC pump (Agilent Technologies, Waldbronn, Germany) coupled to ICP MS. All connections were made of PEEK tubing (0.17 mm i.d.).

Iodine determination by inductively coupled plasma mass spectrometry (ICP MS)

Sample (0.5 g dry mass) was digested by microwave-assisted mineralization with a mixture of 5 mL of HNO_3 and 2 mL of H_2O_2 . After cooling down, the digest was diluted to final volume of 10 mL with water and further diluted before ICP MS analysis. A certified reference material of whole egg powder and a blank were analyzed along with each series of samples.

The determination of amount of iodine in chicken egg samples (after mineralization, buffer and enzymatic extraction) was carried out by Agilent 7500a ICP Mass Spectrometer (Agilent Technologies, Tokyo, Japan) using 10 ng mL^{-1} of indium (^{115}In) as an internal standard for analyzed samples and iodine standard solution used for instrument calibration. For determination of total amount of iodine in eggs after acidic mineralization, solutions of iodate, stable in acidic media, were prepared in 2% HNO_3 for external calibration curve. On the other hand, for determination of iodine in buffer and enzymatic extracts, the standard solutions of iodide, stable in alkaline media, were prepared. In aim to compensate matrix effect, solutions of iodide were prepared by 10 times dilution of extracting mixture with 10 mM of $\text{NH}_{3\text{aq}}$ to ensure good pH stability above 7.5. To prevent memory effect, sample introduction system was washed after each sample with 50 mM of $\text{NH}_{3\text{aq}}$ for 1 min and stabilized with an appropriate solvent for 40 s before next analysis. Curves were linear in the investigated range from 1.0 to 128 ng mL^{-1} with r^2 above 0.999. Limit of detection (LOD) was calculated as standard deviations (SD) of 10-times-measured blank solution, and it was found to be $0.14\text{--}0.70\text{ ng mL}^{-1}$. ICP MS measurement conditions (nebulizer gas flow, rf power and lens voltage) were optimized daily using a standard built-in procedure.

Fig. 1 Flow chart of the experimental procedure of the in vitro digestion method and mineralization method

Results and discussion

SEC ICP MS characteristic of iodine compounds extracted from eggs and salt

Extraction method, based on 30 mM Tris buffer solution (pH = 7.5), was developed to gently extract water soluble forms and analyze them with size exclusion chromatography supported by ICP MS detection. It is the simplest method to gather information about iodine speciation, with minimized risk of form transformation due to the presence of oxidizing or reducing agents. As the first step before the analysis of extracts of chicken egg white and yolk, the effect of boiling on iodine forms was investigated. The standard solutions containing iodate and iodide were boiled for 5, 10 and 30 min according to preparation of eggs. The SEC analysis presented two well-separated forms, indicating that both iodine species were stable regardless of duration of boiling (not shown). Noteworthy is the fact that separation of iodine forms was possible despite their molecular size was not within separation range of size exclusion column—Fig. 2a. The effect can be explained with the influence of ionic interactions as the secondary separation mechanism [28, 29].

The chromatograms of buffer extracts of chicken egg white and yolk consists of the same main iodine peak at $t_r = 34$ min, which was identified as an iodide by measuring of retention times for external standards (Fig. 2c) and internal standard addition method. For samples pre-concentrated 10 times by means of lyophilization, high molecular weight (HMW) iodine compound (>70 kDa) was found (Fig. 2c). The presence of the HMW peak was recorded only for one batch of eggs with highest content of investigated element (6% of total peak areas for iodine); no correlation to the method of farming was possible to find. This is in agreement with other reports, showing that iodine can bind with HMW compounds [24].

The simulation of digestion process was carried out in two-step procedure consisting of the simulation of: (1) gastric and (2) intestinal digestions. Chromatograms obtained for enzymatic digestion of egg yolk and white were similar to those obtained for Tris extracts after lyophilization. However, additional preconcentration step to observe HMW fraction of iodine (5% of total peak area) was unnecessary. It can be suggested that there is no conversion of iodide during enzymatic hydrolysis, and it is the major compound present in the egg samples as well as in kitchen salt (data not shown).

Fig. 2 SEC ICP MS (size exclusion chromatography hyphenated with inductively coupled plasma mass spectrometry) chromatograms of **a** iodine standards, **b** samples of iodine standards (IO_3^- solid line, and I^- dotted line) after simulated gastric digestion, **c** buffer extract of chicken eggs white (dashed line) and yolk (solid line); Peak identification: 1 excluded species (>70 kDa), 2 iodate, 3 iodide

The stability of iodine species during digestion was checked for standard solutions. As the result of the first step, reduction of iodate to iodide was found, which was observed as an appearance of the third peak and the disappearance of the second one on the SEC ICP MS

chromatogram (Fig. 2). After the second step, iodine remained as an iodide. According to the literature, the pH below 7.0 and the presence of reducing compounds in the sample induces the reduction of iodate to iodide [30]. After complete simulation of gastrointestinal digestion of iodine standard solutions, the recovery was from 98 to 101% in reference to fresh standard solution analyzed by SEC ICP MS. The lack of HMW iodine compounds on the chromatograms indicates no affinity of iodide and iodate to enzymatic proteins used during gastric and intestinal digestions. However, ICP MS calibration for determination of iodine in Tris, gastric and gastrointestinal extracts of eggs and iodized kitchen salt samples should be carried out using iodide standards prepared in extracting media to compensate matrix effect; pH of the solution should be higher than 7.5 to ensure its good stability.

Determination and bioavailability of iodine

To estimate the bioavailability of iodine from chicken eggs, samples were treated with enzymatic solutions simulating human digestive juices. The mass balance of iodine was established by calculating the total amount of iodine in extract and sediment against total amount of iodine in mineralized sample (Fig. 1).

The mineralization method, based on HNO_3 with addition of H_2O_2 , was chosen due to the higher oxidation potential of H_2O_2 than HClO_4 ($E_{\text{H}_2\text{O}_2}^0 = +1.78$ V and $E_{\text{HClO}_4}^0 = +1.23$ V) [31]. Additionally, the degradation products of hydrogen peroxide are not considered as a troublesome during ICP MS measurements in contrast to chloride and chlorine generated from perchloric acid. The method was validated using reference material of chicken egg powder (Table 2). The presence of iodate during ICP MS analysis is favorable as it is more stable in acidic solutions of HNO_3 , which is most commonly used to prepare samples and to wash the apparatus. Contents of accumulated iodine in supplemented and regular chicken eggs are presented in Table 2 and allowed to conclude that concentration of iodine in yolk is usually higher than in white. This finding is in agreement with the literature [32]. The vast differences between total amounts of iodine in eggs allow to believe that chicken eggs have great supplementation potential.

The efficiency of Tris buffer (pH = 7.5) extraction method was established following ICP MS calibration in 10-times-diluted extraction media and established against total amount of iodine in egg white and yolk as $22 \pm 2\%$ and $9 \pm 1\%$, respectively. The low efficiency was probably caused by the presence of water-insoluble proteins fraction that settled out of a suspension to the bottom of the liquid. The sediment was subsequently removed during centrifugation. Additionally, after longer time of egg boiling

Table 2 Total iodine content in the studied egg samples (average results for 3 samples)

Sample	Content in white, $\mu\text{g I g}^{-1}$ (dry weight) $\pm$ SD	HSD** ($Q_{0.05;5;20}$)	Content in yolk, $\mu\text{g I g}^{-1}$ (dry weight) $\pm$ SD	HSD** ($Q_{0.05;5;20}$)
Iodine-enriched shell eggs (1*)	0.98 ± 0.04	0.08	10.92 ± 0.11	0.22
Regular shell eggs (2)	3.24 ± 0.11	0.21	4.91 ± 0.06	0.11
Regular shell eggs (3)	1.42 ± 0.02	0.04	2.23 ± 0.03	0.06
Regular shell eggs (4)	1.13 ± 0.02	0.03	1.23 ± 0.01	0.02
Powder of chicken egg from technological process (5)	0.20 ± 0.01	0.01	7.44 ± 0.16	0.31
Content, $\mu\text{g I g}^{-1}$ (dry weight) $\pm$ SD				
Reference material (NIST 8415)	1.93 ± 0.06 (certified 1.97 ± 0.46)			
Iodized kitchen salt	24.13 ± 1.21 (declared 22.95 ± 7.65)			

(1–5)—eggs offered by different Polish farms

* Iodine-enriched eggs from Polish supermarkets described by the producers to be fortified with health-promoting additives including iodine

** Honestly significant difference obtained by Tukey's test for five observations in each group (different samples), 20 degrees of freedom, significance level 0.05 and studentized range distribution, $Q_{4.24}$. Limit of detection— $0.003 \mu\text{g I g}^{-1}$ (dry weight)

(30 min), iodine was not detected in the extracts, probably due to more advanced protein denaturation. In case of inorganic character of kitchen salt, the concentration of iodine in kitchen salt dissolved in Tris buffer was considered as a total amount of iodine in this sample (Table 2).

The recovery of iodine was also established for simulated gastrointestinal extraction of chicken egg white ($33 \pm 5\%$) and yolk ($10 \pm 3\%$). In case of egg boiled for 30 min, the recovery was about twice lower, indicating the problem of proteins temperature denaturation. This is in agreement with resistance of HMW iodine compound to enzymatic digestion noticed by SEC ICP MS. It should be pointed out that recovery for iodine from iodized kitchen salt was established as a $100 \pm 5\%$, which indicates the significant influence of matrix and form of iodine on its bioaccessibility.

The comparison of iodine amount in each fraction versus total amount in egg yolk and white allowed to conclude that iodine loss was below 5%, which is in the range of the uncertainty. Additionally, Tukey's test was applied for multiple comparisons of the results obtained for different groups of eggs. The level of significance was 0.05, and honestly significant difference (HSD) was obtained for each group of eggs (Table 2). The level of HSD is low enough to show that in each case, the difference of iodine concentration in different type of egg sample is greater than the standard error and that various ways of feeding carried at different farms considerably influence the level of iodine in eggs.

In typical 60 g egg, water content in yolk and white was assayed as 50 and 88%, respectively, by comparison of weight of both fractions of egg ($n = 5$) before and after lyophilization (until stable mass). Recalculated (from dry

Table 3 Iodine content recalculated per whole egg (60 g weight) containing 22 g of yolk

Sample	Amount of I (μg)	Bioaccessible amount of I (μg)
Iodine-enriched shell eggs (1)	122	39
Regular shell eggs (2)	68	19
Regular shell eggs (3)	31	9
Regular shell eggs (4)	18	5
Powder of chicken egg from technological process (5)	81	27

mass to real material) element contents are presented in Table 3. Assuming bioaccessibility of it from yolk as 33%, it was found that only about 40 μg was bioaccessible from the iodine-enriched egg after 10 min of boiling and the level decreased after 30 min to $<20 \mu\text{g}$.

Conclusions

The results obtained by SEC ICP MS method allowed to conclude that about 30% of iodine from eggs is bioavailable mainly in the form of iodide, while iodine in the iodized kitchen salt is present as iodide and is 100% bioavailable. However, the role of the protein-bound iodine in case of eggs should be clarified in future studies, and the obtained results should be verified with respect to different experimental conditions during enzymatic digestion or cell line Caco 2. The size exclusion method also allowed to separate main inorganic species (iodate and iodide), which was helpful to confirm good stability of both species in

typical extraction media (Tris HCl, pH = 7.5). Lower extraction recovery in case of egg boiled for 30 min, instead of 10 min, indicates that changes in egg proteins structures enforced by high temperature are of crucial importance for bioavailability of iodine.

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