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PERFORMANCE CHARACTERISTICS OF A RAPID METHOD FOR IODINE DETERMINATION IN MILK*

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Abstract

The repeatability and within-laboratory reproducibility were tested on 72 samples. The most important validation parameters were determined, including the components of uncertainty. The results of the analyses were compared with reference values. The repeatability and within-laboratory reproducibility of the method did not exceed 8% and 10%, respectively. The limit of quantification (LOQ) in the milk sample solution was 14 µg/L. The working range of the calibration curve was 7 to 200 µg/L. The uncertainty of the method (P≤0.05), which accounts for the errors of within-laboratory reproducibility, recovery and standard purity as well as the errors of volumetric glassware, was (%) 17.8 and 22.8 (n = 2 and n = 1), respectively. The coefficient of variation for repeatability should also be determined during routine analysis; it should not exceed the repeatability limit of 16%. The method has sufficient reliability, as confirmed by the validation results. The procedure is rapid, simple and has a low LOQ. The method was accredited according to ISO/IEC 17025:2005 international standard.

Key words: iodine in milk, validation, uncertainty, repeatability, within-laboratory reproducibility, accreditation, ISO/IEC 17025

Studies on the iodine content of foods are essential to ensure an adequate iodine supply at the population level. The daily iodine intake recommended by the WHO (WHO/UNICEF, 2007) is 120 µg for schoolchildren and 150 µg for adults, whereas pregnant and breast-feeding women should additionally receive 150–200 µg per day under medical supervision (Experts Group, 2011). Poland is an iodine-deficient country on account of its geophysical characteristics, which are particularly noticeable in the mountain areas. This is counteracted by effective iodine prophylaxis, which is

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based on mandatory iodization of table salt (20–40 mg KI/kg) and monitored under the Programme for Elimination of Iodine Deficiency, financed by the Ministry of Health (Szybiński and Lewiński, 1998). The latest WHO recommendations (WHO, 2007) to reduce the intake of salt because it raises blood pressure and contributes to atherosclerosis have limited the daily dose of iodine. In this situation, it has to be supplemented to human diets. Some mineral waters with known and controlled iodine concentrations as well as milk from cows receiving iodine-fortified feeds may become an additional source of this element (Szybiński et al., 2009). In particular, milk (and its products) is a major natural source of iodine, and can become the mainstay of iodine deficiency prevention. Because of feeding cattle forage brassicas, the iodine content of milk from Polish cows remained low until recently (Brzóska et al., 2009). Now that iodine is added to animal feeds, mainly salt licks (Brzóska, 2008), the situation has considerably improved, but the monitoring of milk iodine concentrations becomes a crucial issue.

Methods for determination of iodine in milk include neutron activation analysis (Hou et al., 1998), ICP-MS (Fecher et al., 1998; Mesko et al., 2010) and ion-exchange chromatography (Hurst et al., 1983). A well-tested and relatively simple method is the kinetic colorimetic method for determination of this element based on the Sandell-Kolthoff reaction catalysed by iodine (Górski and Bobek, 1960; Toledo et al., 2002). This method is easy, commonly used for the analysis of simple matrices such as urine and mineral water (Barikmo et al., 2011), and uses a simple spectrophotometric technique which does not require a complex equipment. Applying the method to more complex food matrices (‘dry’ furnace combustion in an alkaline environment) is much more difficult and considerably slows down the analysis. We used this method for the determination of iodine in the solid food material (Gąsior and Szczypuła, 2010). However, it can be greatly simplified for milk by using ‘wet’ digestion with ammonium persulfate. We have just developed and validated in this paper such a simpler and faster method for milk analysis, in collaboration with the Collegium Medicum of Jagiellonian University. Validation procedures have to be used to introduce a new laboratory method. They are essential in helping to recognize the limitations of a method and can be used to determine how to check the quality of results during routine analyses. Validation of different analytical methods in food was reported by some authors (Ake et al., 1998; de Souza et al., 2007). Additionally, the certified reference materials are recently increasingly used as components of the validation and quality assurance in the laboratory (Huang et al., 2010). These issues were also discussed in several other research works (Gąsior et al., 2005; Gąsior and Pieszka, 2006; Gąsior et al., 2009). The validation parameters are widely described in the literature (Ellison et al., 2000) and have the purpose of showing the correctness of the method in compliance with Good Laboratory Practice regulations and the ISO/IEC 17025 standard (Camino-Sánchez et al., 2011). However, there are no studies in the literature that describe a method for iodine determination in liquid milk with consideration of parameters such as recovery, limit of quantification (LOQ), limit of repeatability, repeatability, within-laboratory reproducibility and uncertainty. Gąsior and Szczypuła (2010) provide a similar description but it concerns the validation of a different, more complex and less rapid method for iodine determination in solid matrices.

It is assumed that the simpler and more reliable method of milk digestion will be developed, allowing for a faster determination of the iodine content.

The aim of the study is to validate a rapid method for iodine determination in milk after mineralization with ammonium persulfate using the kinetic colorimetric technique based on the Sandell–Kolthoff reaction catalysed by iodine.

Material and methods

The method validation procedure involves the use of the iodic ion (I-)-catalysed oxidation-reduction reaction between Ce and As ions, represented as:

$$2Ce^{+4} + As^{+3} \rightarrow 2\ Ce^{+3} + As^{+5}$$

followed by determination of the time-variable absorbance of the analysed solution at a wavelength of 420 nm.

Reagents and equipment

The following reagents and standards were used: sodium chloride (NaCl, POCH, Gliwice), sulfuric acid (H_2SO_4, Chempur, Piekary Śląskie), ammonium cerium (IV) sulfate ($(NH_4)_4Ce(SO_4)_4 \times 2\ H_2O$, Sigma-Aldrich, St. Louis, USA), ammonium persulfate ($(NH_4)_2S_2O_8$, Sigma-Aldrich, St. Louis, USA), arsenic (III) oxide (As_2O_3, VEB Jenapharm-Laborchemie Apolda, Germany), iodine standards – potassium iodate (KIO_3) and potassium iodide (KI) (Merck, Darmstadt, Germany). The reagent and standard solutions were prepared as follows:

a) 1.75 M solution of H_2SO_4 (97 ml of H_2SO_4 / 1000 ml H_2O),

b) 2.5 M solution of H_2SO_4 (140 ml of H_2SO_4 / 1000 ml H_2O),

c) 1 M solution of ammonium persulfate (228.2 g $(NH_4)_2S_2O_8$ / 1000 ml H_2O), when stored in the dark is stable for 6 months,

d) solution of arsenic acid (5 g As_2O_3 + 25 g NaCl + 200 ml 2.5M H_2SO_4 / 1000 ml H_2O),

e) solution of ammonium cerium (IV) sulfate (24 g $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ + 1 L 1.75 M H_2SO_4), prepared at least 24 h before use, when stored in the dark is stable for 6 months,

f) iodine standard solution A: 100 µg I/ml (0.168 g KIO_3 /1000 ml H_2O),

g) iodine standard solution B: 0.5 µg I/ml (0.5 ml of iodine standard solution A / 100 ml H_2O).

Extreme caution was exercised when using these reagents. Double-distilled water was used for the analyses. Standard solutions A and B, stored in the dark and in a refrigerator (+2ºC to +8ºC) were stable for 6 months and 1 month, respectively. For determination of recovery potassium iodate (f) and potassium iodide solutions were used. Potassium iodide solution was prepared analogously, taking into account the molar mass of potassium iodide, to iodine standard (f), and they were both used as standard addition for recovery tests (2.4).

Basic laboratory equipment was used in addition to a spectrophotometer (Beckman 640 DU, USA), a cuvette thermostat for spectrophotometric determinations, a digestion heating block with holes that fit 13×100 mm test tubes, with heating temperature adjustable up to 110ºC and temperature stability of about 0.1ºC, 13×100 mm screw-cap test tubes (Schott, Merck), a vortex shaker, a centrifuge (MPW 211, Poland) and centrifuge test tubes (10 ml). The sample of the material was stored in a freezer until analysis. Prior to analysis, the sample was thawed, brought to room temperature and thoroughly mixed to distribute fat uniformly throughout the sample.

Procedure

Two ml to 5 ml of milk (V1, depending on expected iodine content) was pipetted to the centrifuge test tube (10 ml), 25 µl of 1.75 M H_2SO_4 (a) per ml of milk was added, vortexed and centrifuged at relative centrifuge force (RCF) ≈7500 g for 10 min. After the first portion of the supernatant was transferred to a volumetric flask (10 ml), the precipitate in the test tube was washed (2 ml water), ground with a glass rod, mixed and centrifuged at RCF ≈7500 g for 10 min, after which the supernatant was transferred again to the same flask as previously. The washing procedure was repeated, and after the solutions were combined the flask was made up with water to a volume of 10 ml (V2). In the case of the milk samples, which were difficult to centrifuge, the centrifuge tube contents, after casein precipitation, was filtered into a volumetric flask (10 ml). Then, 250 µl of the flask liquid was pipetted to a 13×100 mm test tube, 1 ml of ammonium persulfate solution (c) was added and mixed after the tube was screw-capped. After heating in a heating block at 91–95ºC and cooling to room temperature, 3.5 ml of arsenic acid (d) was added, mixed and left for 15 min. This was followed by the first measurement: at 60 sec intervals, 400 µl of the solution of ammonium cerium (IV) sulfate (e) was added to each tube, mixed again and after pouring into a measuring cuvette, initial extinction (E1) was measured at a wavelength of λ=420 nm, and the cuvette was then placed in a Peltier thermostat (25ºC). The second measurement (final extinction E2, λ=420 nm) was made exactly 30 min after the solution of ammonium cerium (IV) sulfate (e) was added to the first test tube. E2 extinction of the other samples was read successively every 60 sec. The standard solutions were prepared by pipetting 0, 5, 75 and 150 µl of the iodine standard solution B (g) in duplicate, into 8 tubes containing 250, 245, 175 and 100 µl water, respectively, to obtain a total solution volume of 250 µl in each tube. Iodine concentrations in standard solutions were 0, 10, 150 and 300 µg/L, respectively. They served as a basis for plotting the calibration curve (4 calibration points). To such prepared standard solutions 1 ml of ammonium persulfate solution (c) was added, and further procedure was the same as for the samples.

Calculations

The iodine (I) content of milk (µg/L) was calculated from formula 1:

$$I = [a(\log E1 - \log E2) + b] \times d \times \frac{100}{R} \quad (1)$$

where: *E1* and *E2* are initial and final absorbance of the sample, respectively, and a, b are coefficients of the calibration curve (slope and y-intercept, respectively), *d* is the *V2/V1* volume ratio (Procedure), and R is Recovery %.

Validation

Repeatability and within-laboratory reproducibility were tested on 56 and 16 samples (72 in total, with the content range from about 30 µg/L to 750 µg/L) of market cow milk, respectively, from different regions of Poland. Repeatability % was determined as being not less than the pooled coefficient of variation for individual determinations performed with the same method, using identical material, in the same laboratory, by the same laboratory assistant, during the same time period. Within-laboratory reproducibility % was determined as being not less than the pooled coefficient of variation for individual determinations performed with the same method, using identical material, in the same laboratory, by two laboratory assistants, at different times. The pooled coefficient of variation CV_{mn} for m samples analysed in n replications was calculated from formula 2, where CV_{n2} is the coefficient of variation for determination of a given sample in duplicate (n = 2):

$$CV_{mn} = \sqrt{\frac{\Sigma CV_{n2}{}^2}{m}} \tag{2}$$

Double the coefficient of variation for repeatability was accepted as the criterion for repetition of the determinations (limit of repeatability). The recovery was determined using the standard addition method. It was calculated based on two standards (KI and KIO_3), which were added to the samples prior to the mineralization. The limit of quantification was determined from the formula $LOQ=10 \times SD$, where *SD* is standard deviation of iodine content in a blank sample, corresponding to zero calibration point (Procedure). Linearity of the calibration curve was also tested and its working range was determined.

The main components of method uncertainty (expressed in relative form, %) were determined, such as uncertainty of within-laboratory reproducibility (*u1%*), uncertainty of recovery (*u2%*), uncertainty of purchased standard purity (*u3%*) and uncertainty associated with lack of trueness of pipettes (*u4%*) and flasks (*u5%*) (trueness was defined in VIM (2008) and described by Hauck et al. (2008)). Before combining, uncertainties were expressed as standard uncertainties $u_i\%$ (68% confidence level, P≤0.32). The combined standard uncertainty of the $u_c\%$ method was calculated based on the law of propagation of uncertainty from formula 3:

$$u_c\% = \sqrt{u1\%^2 + u2\%^2 + u3\%^2 + u4\%^2 + u5\%^2} \tag{3}$$

The standard uncertainty of within-laboratory reproducibility (*u1%*), which includes most errors, including sample preparation errors, was defined as within-laboratory reproducibility % divided by the root of n analyses of a given sample (formula 4):

$$u1\% = \frac{Reproducibility\%}{\sqrt{n}} \quad (4)$$

The standard uncertainty of recovery was calculated as a coefficient of variation for the arithmetic mean of recovery values determined during the validation. The standard uncertainties concerning standard purity and the flasks and pipettes used (but only partly due to error of bias, which was not included in repeatability and within-laboratory reproducibility) were calculated based on certain values of limiting errors a_i, expressed in relative form (%). For flasks and pipettes, a_i values were estimated based on the calibration procedure accepted in the laboratory and the resulting assumptions. For standard purity, a_i values were estimated based on the manufacturer's specifications. Assuming a symmetric rectangular distribution of the means measured around the nominal value in the range determined by a_i, $ui\%$ uncertainties are calculated using the formula $ui\% = a_i / \sqrt{3}$ (Ellison et al., 2000). Because several pipettes and flasks were used during the analysis, the uncertainty factor associated with the lack of trueness of the pipettes and flasks was calculated by combining the individual components in accordance with the law of propagation. Method uncertainty $Uc\%$ (95% confidence level, $P \leq 0.05$) was computed by multiplying combined standard uncertainty of the $u_c\%$ method by the coverage factor k = 2. The uc% and $U_c\%$ uncertainties were determined for n = 2 and n = 1.

An additional component of the validation involved comparison of the results obtained in the analysis of three milk reference materials: SRM 1549, SRM 1849 (National Institute of Standards and Technology, USA) and BCR 063R (European Commission, Institute for Reference Materials and Measurements, Belgium), with the reference values ascribed to these materials (µg/g): 3.38, 1.37 and 0.81, and standard uncertainties (µg/g) of: 0.01, 0.20 and 0.025, respectively.

Results

The values of repeatability, repeatability limit, within-laboratory reproducibility and standard uncertainty of within-laboratory reproducibility are given in Table 1. Recovery, determined by adding the known amount of the standard to milk samples was 83.6% (n = 19). LOQ, corresponding to the iodine range that can be determined with sufficient certainty, equalled ten times the standard deviation value of the blank sample and was 14 µg/L milk. The calibration curve, plotted based on the standard iodine solutions, was a straight line that represents the linear relationship between iodine content of the analysed solution and the difference in logarithms of measurement extinction at the beginning and after a certain time of reaction, characterized by the coefficient of determination r^2 being not less than 0.99. The working range of the calibration curve ranged from 7 to 200 µg/L. The uncertainty budget, which includes all the identified factors of uncertainty, combined standard uncertainty, and combined expanded uncertainty (n = 2 and n = 1) is shown in Table 2. The results of analyses from the Central Laboratory are compared with the reference values in Fig. 1 and Table 3.

Table 1. Validation parameters of the method for iodine determination in liquid milk

Material analysed	Repeatability (as CV%) (%)	Repeatability limit (as CV%) (%)	Within-laboratory reproducibility (as CV%) (%)	Standard uncertainty (P≤0.32) of within-laboratory reproducibility (*u1*%), *n = 2/n* = 1* , (%)
Liquid milk	8.0	16.0	10.0	7.1/10.0

* n – number of analyses of one sample.

Table 2. Standard uncertainty budget, combined standard uncertainty u_c% (P≤0.32) and combined expanded uncertainty U_c% (P≤0.05, *k* = 2)

Material analysed	*u1%* * *n = 2/n = 1*	*u2%* *	*u3%* *	*u4%* *	*u5%* *	u_c % *n = 2/n = 1*	U_c % (*k* = 2), *n = 2/n = 1*
Liquid milk	7.1/10.0	2.9	3.6	2.8	0.5	8.9/11.4	17.8/22.8

* for explanations, see Validation in Material and methods section.

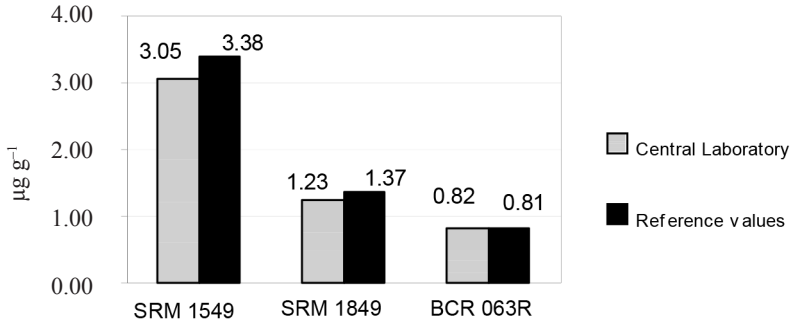

Figure 1. Comparison of Central Laboratory analyses results with reference values

Table 3. Compliance of analysis results of SRM 1549, SRM 1849 and BCR 063R with reference values. Comparison of absolute differences *I diff I* with limit criterion U_{max} (P≤0.05, *k* = 2)

Reference Material analysed	Result µg/g	Reference value, µg/g	I diff I µg/g	u_{ref} µg/g	u_{lab}* (n=2), µg/g	u_{max}* µg/g	U_{max}** µg/g, (k=2)	Compliance I diff I< U_{max}
SRM 1549	3.05	3.38	0.33	0.01	0.27	0.27	0.54	Yes
SRM 1849	1.23	1.37	0.14	0.20	0.11	0.23	0.46	Yes
BCR 063R	0.82	0.81	0.01	0.025	0.073	0.077	0.15	Yes

* uref and ulab – standard uncertainties of reference material and laboratory analysis (P≤0.32).
** $U_{max}=k \times (u_{ref}2 + u_{lab}2)^{1/2}$, k = 2.

Discussion

The method described and validated in the present work is repeatable and reproducible. This is confirmed by the Horrat value H = 0.58 located in the accepted range (0.5–2), calculated for the target repeatability (%) RSDr = 13.8 (Korol et al., 2011), according to the equation H = Repeatability/RSDr (8/13.8, %). RSDr was calculated from the equation: $0.67 \times 2C^{-0.1505}$, for the concentration $C = 1.88 \times 10^{-7}$ (the average iodine content in the samples was 188 µg/L, the content range was from about 30 µg/L to 750 µg/L). The method has a low LOQ value. It enables small amounts of iodine (even as low as 14 µg per 1 L liquid milk) to be quantified, but in practice, milk iodine content exceeded 30 µg/L. The recovery determined in our study confirmed our earlier results for the method of iodine determination in solid matrices (Gąsior and Szczypuła, 2010). In that study, repeatability for milk was indeed lower (5.8% compared to 8.0% in the present study) but important parameter values were less defined in reproducibility conditions, i.e. within-laboratory reproducibility (15.8% in the previous vs. 10.0% in the present study) and standard uncertainty of within-laboratory reproducibility (for $n = 2$, 11.3% vs. 7.1%). Thus, the use of wet mineralization has a positive effect on the analysis uncertainty. It seems also that 'wet' digestion with ammonium persulfate allows determining the total amount of iodine in milk. This is evidenced by the consistency of the results with the reference values (Table 3). The recovery less than 100%, as determined by standard addition method, implies some accepted component losses during sample preparation, rather than low iodine obtained from the matrix. The additional advantage is that the method is relatively simple and fast, and allows analysing about 40 samples (in duplicate) of liquid milk per week (compared to only 12 duplicate samples per week for solid matrices).

The method uncertainty is comprised of the main factors of uncertainty such as uncertainty of within-laboratory reproducibility, uncertainty of recovery, uncertainty of purchased standard purity, as well as uncertainty associated with lack of trueness (i.e. bias understood as the difference between actual value and nominal value) of pipettes and volumetric flasks. The components mentioned above can be regarded as separate factors of uncertainty, which affect the combined uncertainty of the method. The other components of uncertainty, associated with precision of pipettes and volumetric flasks as well as precision of weighing were automatically accounted for in within-laboratory reproducibility and for this reason they are not included in the uncertainty budget as separate components (Gąsior et al., 2009). This procedure conforms with the notes of Ellison et al. (2000) to avoid double counting of uncertainty components. What is more, uncertainty of the calibration curve is also not listed in the uncertainty budget. This is because each series of analyses is made using a separate curve, which causes the associated errors to become included in the within-laboratory reproducibility. The uncertainty of this reproducibility contains most of the errors associated with sample preparation and spectrophotometric measurement itself. Significantly, however, these errors are automatically included in uncertainty only when the results calculated from two duplicates concern iodine determinations in two samples weighed in parallel. If the sample was weighed without duplicates and the solution for spectrophotometric measurement obtained was analysed twice,

the uncertainty of within-laboratory reproducibility would only include the error in instrumental determination (Gąsior et al., 2007). The factors of uncertainty noted above are the most important and, in accordance with the Gauss law of propagation, they make the greatest contribution to the method uncertainty value for analyses performed in one laboratory. The uncertainty of the method ($P \leq 0.05$) and the result (mean from measurements) has practical relevance during its interpretation and determines the tolerance interval in which the actual value of the determination result should fall with 95% probability. Uncertainty should be controlled in the analysis of every sample by checking, under repeatability conditions, the variation coefficient for individual determinations, which should not exceed the limit of repeatability determined during the validation.

Criterion for determining the compatibility between the average results of the analysis (n = 2) of reference materials: SRM 1549, SRM 1849, BCR 063R and the reference values assigned to them, is twice the combined standard uncertainty ($k = 2$, 95% confidence level), from the reference material standard uncertainty (u_{ref}) and the laboratory analysis standard uncertainty (u_{lab} – calculated from the relative standard uncertainty *uc%*, n = 2, Table 2), in accordance with the law of propagation. Central Laboratory results fall within the permissible limits, so they are consistent with the reference values (Table 3). It is worth noting that the described procedure of sample preparation and spectrophotometric iodine determination offers the possibility of changing reaction time (between spectrophotometric measurements) and reaction temperature according to the iodine content of the sample (the higher the iodine content, the lower the reaction time and/or temperature).

In conclusion, the validated method has sufficient reliability, as confirmed by the validation results. It is worth noting the low limit of quantification. It is essential that the procedure is less expensive, simple and fast, and has a lower uncertainty, compared to the procedure used for iodine determination in solid matrices. It must be added that method uncertainty can be decreased by increasing the number of determinations per sample ($n \geq 2$). The method was accredited according to ISO/IEC 17025:2005 international standard.

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Szybka metoda oznaczania jodu w mleku – walidacja i akredytacja zgodnie z normą ISO/IEC 17025:2005

STRESZCZENIE

Badania powtarzalności i odtwarzalności przeprowadzono na 72 próbkach. Określono najważniejsze parametry walidacji, w tym składowe niepewności. Wyniki analiz porównano z wartościami referencyjnymi. Powtarzalność i odtwarzalność metody nie przekraczały 8 i 10%, odpowiednio. LOQ w oznaczanym roztworze próbki mleka wynosił 14 µg/L. Zakres roboczy krzywej kalibracji wynosił od 7 do 200 µg/L. Niepewność metody ($P \leq 0,05$) uwzględniająca błędy odtwarzalności wewnątrzlaboratoryjnej, odzysku, czystości wzorca oraz błędy szkła miarowego wynosiła (%) 17.8 i 22.8 ($n = 2$ i $n = 1$). Podczas wykonywania rutynowych analiz powinien być sprawdzany współczynnik zmienności dla powtarzalności, który nie powinien przekraczać granicy powtarzalności wynoszącej 16%. Metoda cechuje się wystarczającą wiarygodnością, co zostało potwierdzone wynikami walidacji. Procedura jest szybka, prosta i ma niską wartość LOQ. Metodę akredytowano zgodnie z normą ISO/IEC 17025:2005.