

Rapid Acid Digestion and Simple Microplate Method for Milk Iodine Determination

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Iodine deficiency leads to deficiency of thyroid hormones, which causes mental retardation in infant. Laboratory confirmation is important in its diagnosis. The major problems associated with the existing methods for iodine determination in milk samples are: 1) nonsafe alkaline solution; 2) harsh thermal condition; and 3) extra time required to complete various steps. In this study, a simple and rapid colorimetric method was investigated, which used acid digestion in combination with a rapid microplate reading format method to determine the total iodine content in milk. Sample digestion was done on 50 μ L milk in metavanadate/perchloric, at 230°C for 10 min. After digestion, iodine determination was based on the Sandell-Kolthoff reaction. The reaction results were read in 96-well microplates by an enzyme-linked immunosorbent assay (ELISA) reader. The

determination range of the assay was between 2 and 40 μ g/dL. The within-run coefficient of variation percent in three levels (3, 12, and 36 μ g/dL) ranged from 6.7 to 9.3 and between-run coefficients of variation ranged from 8.6 to 12.3%. The results obtained ($n = 70$) by the optimized method have good correlation with the results of alkaline incineration as a reference method ($n = 70$; $r^2 = 0.907$; $y = 0.952x + 1.77$). Recovery tests for accuracy assessment in six levels from 6.2 to 34.2 μ g/dL were between 91.3 and 113%. This method has enabled us to achieve 0.12 μ g/dL sensitivity. The results of this study show that a quick acid digestion combined with mild thermal and low sample volume with a quick reading of assay results were the main advantages of the acid digestion and microplate reading format. *J. Clin. Lab. Anal.* 21:286–292, 2007. © 2007 Wiley-Liss, Inc.

Key words: acid digestion; milk iodine; microplate reading

INTRODUCTION

Iodine is an essential trace element that is necessary for the synthesis of thyroid hormones. The low intake or deficiency of iodine leads to abnormal or deficiency of thyroid hormones, which causes poor mental and physical development in children and goiter in adults. Fortunately, iodine deficiency is not only the most preventable cause of mental retardation but its correction is technically simple (1). Laboratory confirmation is very important in diagnosis of iodine deficiency and also in monitoring of its treatment. There are reports for milk iodine determination (2–5). But after Sandell and Kolthoff's (6,7) catalytic reaction report, iodine

determination methods are technically very simple and many laboratories have been measuring iodine levels on the basis of iodine catalytic rule in CeIV and AsIII redox reaction for many years. Breast milk, cow's milk, and

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formula milk are exclusive source of iodine intake in breast-fed, cow's milk-fed, and formula-fed children, respectively, for the first months of life. Therefore, milk iodine concentration is a helpful index for infant iodine intake. Most iodine in biological media is covalently bonded and there are some substances that interfere with the determination reaction (8). The high lipid content of milk can potentially cause problems in spectrophotometric readings, so a mineralization step prior to analysis is essential. This is a critical step because during the mineralization step, iodine can easily be lost by volatilization (9). All reported methods have a digestion or ashing step that uses alkaline media and high temperature (10). Alkaline media are generally considered to be harsher media than acidic media for milk digestion. Therefore, the objective of this study was to design and optimize a new mild acidic media that uses a short amount of time for milk digestion and also develop a simple and rapid method for determination of its iodine content with low sample volume. Microplate reading format of the results by an enzyme-linked immunosorbent assay (ELISA) microplate reader to decrease assay time has been another objective of this study.

MATERIALS AND METHODS

Equipment and Apparatus

Sample digestion was performed in a tube Heating Block (Gebr. Liebisch GmbH & Co. KG, Bielefeld, Germany), colorimetric measurement was performed in a Microplate ELISA Reader (Sun Rise, TECAN A-5082, TECAN Groups Ltd., Salzburg, Austria).

Chemicals

All chemicals in analytical grade: ammonium metavanadate, potassium iodate, arsenic trioxide, potassium chlorate, perchloric acid (72%), tetraammonium cerium (IV) sulfate dihydrate, sodium chloride, and sulfuric acid were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH, Munich, Germany). Deionized glass double-distilled water was used for the preparation of the reagents.

Solutions

Acidic digestion reagent

Digestion reagent was prepared by dissolving 0.50 g ammonium metavanadate in 1,000 mL of 72% perchloric acid—slowly adding with constant stirring.

Arsenious acid solution

For preparation of arsenious reagent, 1.8 g sodium chloride and 9.0 g arsenic trioxide were dissolved in a

mixture of 83 mL concentrated sulfuric acid and 906 mL distilled water.

Ceric ammonium sulfate solution

For preparation of the ceric reagent, 6.0 g ceric ammonium sulfate was dissolved in a mixture of 730 mL deionized double distilled water and 270 mL concentrated sulfuric acid.

Iodine calibrators

Standard iodine solutions (2, 5, 10, 20, 30, and 40 µg/dL) were prepared using serial dilution of 1,000 µg/mL stock solution. Stock solution was prepared by dissolving 1.6860 g potassium iodate in 1,000 mL deionized double distilled water.

Milk Samples

Human milk samples were collected from volunteer mothers who were referred to the Endocrine Research Center of Shaheed Beheshti University of Medical Sciences and Taleghani Hospital (Tehran, Iran) for the Neonatal Hypothyroidism Screening Program.

Optimizing of Acid Digestion

Optimum digestion conditions (temperature, time, and metavanadate concentration) were defined as having sufficient recovery of iodine added to four milk samples according to the method described below.

Procedure

Milk samples and calibrators (50 µL) were added to 16 × 160 mm test tubes, and 2000 µL of digestion acid solution was added. After being covered with plastic caps, the tubes were placed into the wells of the heating block for 10 min at 230°C in a fume hood. For safe cooling, the tubes were left under the hood for cooling to room temperature. Then 50 µL of the digested samples was transferred to the wells of a polystyrene 96-well microtiter plate (MicroWell; Nunc International, Roskilde, Denmark). Arsenious acid solution (100 µL) was added to the wells and mixed; 50 µL of ceric ammonium sulfate solution was then added quickly by using a multichannel pipette (Finnpipette, Labsystems, Helsinki, Finland). The absorbance was measured at 405 nm with a microplate reader just after 20 min

Assay Evaluation

Calibration curve

A calibration curve was prepared for each plate by plotting the logarithmic conversion of the means of

duplicate absorbance at 405 nm on the *y*-axis against the iodine (2, 5, 10, 15, 20, 30, and 40 µg/dL iodine) on the *x*-axis. The milk iodine concentration was determined using linear regression. Deionized distilled water was used as a zero calibrator.

Detection limit

On the basis of mean zero standard signals ($n = 10$) plus two standard deviations (2SD) method, the assay sensitivity was determined. Deionized glass double-distilled water was used for the zero calibrator. A serially diluted pooled sample was used for this purpose.

Precision

The determination for precision of the method was made by intraassay and interassay coefficient of variations of three pooled milk samples with low, medium, and high iodine content (3, 12, and 36 µg/dL). Replication number in intraassay was eight on the same plate, and in interassay it was 12.

Recovery

The recovery of the assay was determined by triplicate assaying of six different milk samples that were supplemented with standard solutions (6.2, 9.2, 14.2, 19.2, 24.2, and 34.2 µg/dL). Water-added samples were used as comparison.

Parallelism

Six pooled milk samples containing low, medium, and high concentrations of iodine were serially diluted with water. The testing was carried out in triplicate.

Method comparison

The comparison between the new mild acidic method and the common alkaline incineration method was performed using a total of 70 milk samples. The samples were collected from the Endocrine Research Center of Shaheed Beheshti University of Medical Sciences and Taleghani Hospital (Tehran, Iran) with iodine concentrations of 5.1–38.5 µg/dL. Pearson correlation was applied to the results.

Interfering substances

The effects of 15 mmol/L of the following interfering substances: L-ascorbic acid, potassium thiocyanate, and ferrous ammonium sulfate, as common interfering substances in the assay, were assessed. Standard solutions and milk samples with and without interfering substances were assayed for iodine content.

RESULTS

Optimizing of Acid Digestion

The recovery of iodine that was added to four milk samples was compared with various combinations of three variables including: heating block temperature, digestion time, and final concentration of ammonium metavanadate. Data analysis showed that the combination of 5% ammonium metavanadate in perchloric acid, a heating block temperature of 230°C, and only a 10 min incubation were the optimum conditions that gave the highest recovery (Fig. 1).

Calibration Curve

The milk-iodine concentration was linear for the logarithmic scale of the means of absorbance, at 405 nm between zero and 40 µg/dL. Pearson analysis showed an acceptable correlation coefficient for the linearity, which was >0.993 . A typical obtained calibration curve is shown in Fig. 2.

Detection Limit

The detection limit of milk iodine in this new mild acid method, on the basis of 2SD from the zero calibrator definition, was 0.12 µg/dL.

Precision

Intraassay coefficients of variation (CV%) at low, medium, and high level of iodine concentration were 8.6, 6.7 and 9.3%, respectively. Interassay CV% for the three levels were 9.8, 8.6, and 12.3%, respectively (Table 1).

Recovery

The recoveries of added iodine to six different milk samples were between 91.3 and 113% (Table 2).

Parallelism

The measured to expected ratio of iodine content after serial dilution was calculated. The ratio after dilution up to 1:32 was between 0.90 and 1.05 (Table 3).

Method Comparison

The mild acid digestion method was compared with the alkaline incineration method as a common method for milk iodine determination using the regression analysis. The correlation between the two methods was acceptable ($n = 70$; $r^2 = 0.907$; $y = 0.952x + 1.77$) (Fig. 3).

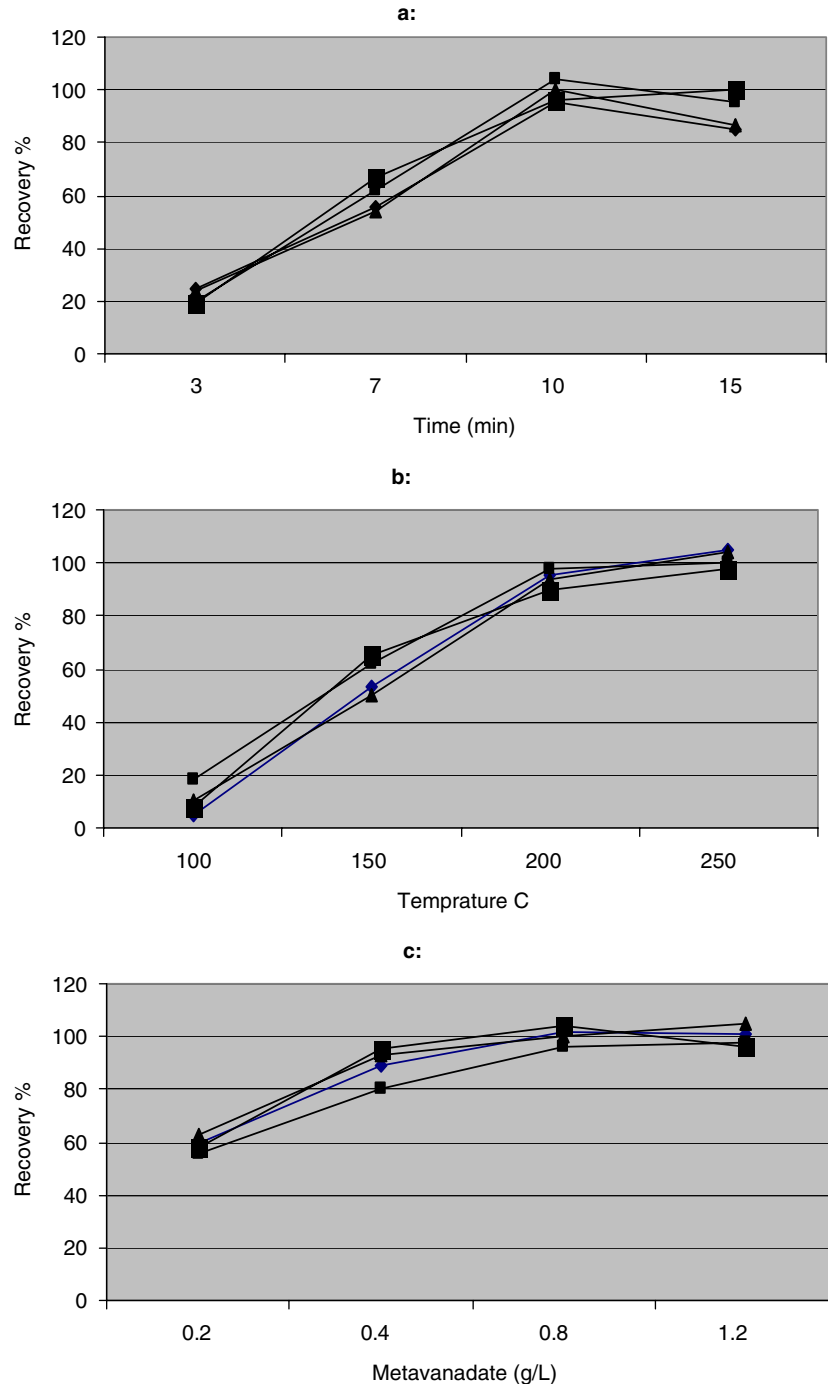


Fig. 1. Optimization of time (a), temperature (b), and metavanadate amount (c) in acid digestion step.

Interfering Substances

Addition of 15 mmol/L of interfering substances (L-ascorbic acid, potassium thiocyanate, and ferrous ammonium sulfate) to the samples after acid digestion did not affect the results of the assay when compared to the samples with no interfering substances added. However, the above mentioned concentration of interfering

substances in samples without mild acid digestion increased the estimated iodine content of the standard solutions that were found to be statistically significant.

DISCUSSION

Iodine is an essential part of the structure of thyroid hormones. Thyroid hormones play an important role in

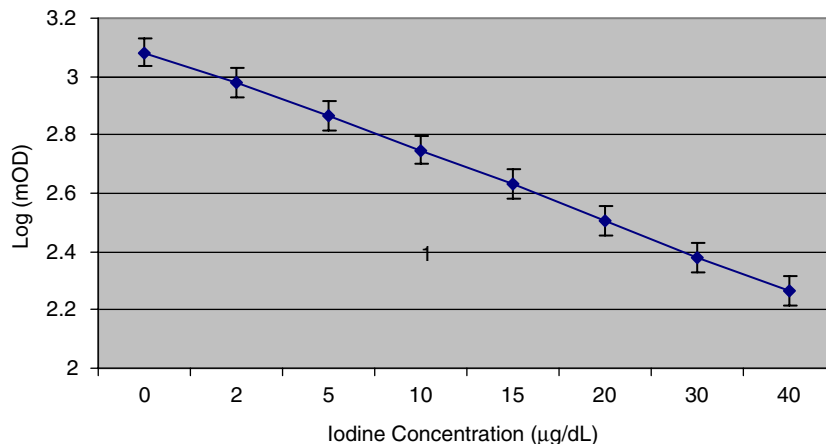


Fig. 2. Typical standard curve of the acid digestion method.

TABLE 1. Intraassay and interassay coefficients of variation of the acid digestion method

Level	Mean	No. of Replications	SD	CV%
Intraassay				
Low	3.5	8	0.3010	8.6
Medium	12.7	8	0.8502	6.7
High	36.2	8	3.3666	9.3
Interassay				
Low	3.3	12	0.3234	9.8
Medium	12.9	12	0.1109	8.6
High	35.7	12	0.4391	12.3

TABLE 2. The data from recovery test using the acid digestion method

Standard (µg/dL)	Measured	Expected	Sample	% Recovery
2	5.6	6.2	4.2	93.3
5	8.4	9.2	4.2	91.3
10	13.7	14.2	4.2	96.4
15	21.1	19.2	4.2	109
20	25.9	24.2	4.2	107
30	36.4	34.2	4.2	113

the development of brain function. Iodine deficiency causes serious delays in neurological development. Various kinds of milks (breast, cow, and formula) are the exclusive source of iodine intake in milk-fed children for the first months of life, so milk iodine concentration is a helpful index for infant iodine intake (11). Historically, iodine has been a difficult element to measure accurately at trace levels in biological and food samples for a number of reasons, including sample preparation, analyte volatility leading to losses, and contamination at trace levels in the laboratory.

There are two main methods for milk iodine determination. First, there is an instrumental method,

TABLE 3. The data from parallelism test using the acid digestion method

Ratio	Measured	Expected	Dilution
1.00	38.8	38.8	1
1.05	20.5	19.4	2
0.90	8.7	9.7	4
0.91	4.2	4.6	8
0.91	2.1	2.3	16
0.91	1.0	1.1	32

which includes instrumentation such as X-ray fluorescence (12), inductively coupled plasma mass spectrometry (13), polarography (14), and atomic absorption spectrometry (15). The second method includes colorimetric methods on the basis of Sandell and Kolthoff's reaction (6,7). The first method, which includes high-technology-based instrumentation has very high costs, is very time consuming, requires personnel with high expertise, and has a requirement for pre-concentration and/or separation steps. In all methods, the sample must be processed for iodine release from organic compounds and interference substances must be eliminated.

Alkaline ashing, alkaline digestion (16–18), and acid digestion (19,20) are three sample preparation methods. Ashing sample preparation needs high temperature, harsh condition, and potentially false-negative results that are caused by iodine volatility. The high lipid content of milk allows it to dissolve in hot alkaline media, but alkaline media is considered very harsh and even dangerous, mainly due to its low safety and quick destruction of biological membranes upon contact. Therefore, a mild acid digestion method is superior to alkaline solution. There have been some acid digestion methods reported for milk iodine determination, but the

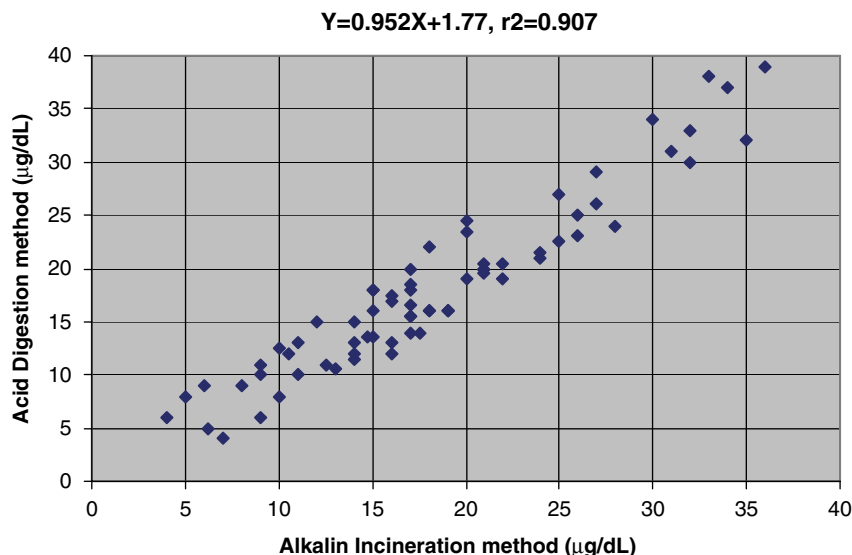


Fig. 3. Acid digestion and alkaline incineration method comparison (n = 70).

assay times are long and the digestion steps are harsh (19,20). The aims of this study were to use a new mild acidic media condition in combination with a quick assay time for digestion and to increase the sensitivity of assay reading results by microplate reading format through the use of a microplate ELISA reader.

In previously reported acid digestion methods, a mixture of concentrated nitric acid, concentrated sulfuric acid, and 70% perchloric acid (5,20) was used or digestion was performed using dangerous alkaline media (21). However, in this study, for the first time, perchloric acid containing 0.05% metavanadate was used as the acid digestion media. The digestion time in previous reports was around 60 min (20,21) but, in our mild acid method, digestion was completed in only 10 min. Minimum sample volume needed in previous methods was 500 µL (21); however, in the present study a sample volume of 50 µL was adequate for quantitative determination. The digestion temperature in previously reported methods was above 400°C, but in our method a short time in 230°C was acceptable for complete digestion of the milk sample. Compare to other reported methods, our mild acid digestion method had good precision, and intra- and interassay CV% of less than 9.3 and 12.3%, respectively. Recovery test results between 91.3 and 113% also indicated acceptable accuracy rates. The mild acid digestion method reported in this study can measure as low as 0.12 µg/dL iodine in milk samples, which clinically satisfies the aim of milk iodine content determination. Finally, the optimized method has acceptable validity, assay condition, and assay time for the milk iodine determination.

CONCLUSION

The results of the validation of the acid digestion method show that the method is sufficiently precise and accurate for the determination of iodine in milk. The main advantages that this method introduces are: quick sample digestion time (only 10 min), low thermal conditions (only 230°C), low sample volume (only 50 µL), and rapid assay results reading by a microplate reader (less than 1 min). The acid digestion has good correlation with the alkaline incineration method (n = 70; $r^2 = 0.907$; $y = 0.952x + 1.77$). Finally, this method is a simple, quick, practical, and economic method that can be put to use as a routine laboratory test for detection of iodine in milk in any laboratory equipped with standard reagents and instruments.

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