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Journal of Trace Elements in Medicine and Biology, 2015; 29:75-82

Validation of an optimized method for the determination of iodine in human breast milk by inductively coupled plasma mass spectrometry (ICPMS) after tetramethylammonium hydroxide extraction

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Short title: Human milk iodine analysis by ICPMS

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SUMMARY

In this study a novel method to determine iodine concentrations in human breast milk was developed and validated. The iodine was analyzed by inductively coupled plasma mass spectrometry (ICPMS) following tetramethylammonium hydroxide (TMAH) extraction at 90^{o}C in disposable polypropylene tubes. While similar approaches have been used previously, this method adopted a shorter extraction time (1 hour vs. 3 hours) and used Antimony (Sb) as the internal standard, which exhibited greater stability in breast milk and milk powder matrices compared to Tellurium (Te). Method validation included: defining iodine linearity up to 200µg L^{-1}; confirming recovery of iodine from NIST 1549 milk powder. A recovery of 94 – 98 % was also achieved for the NIST 1549 milk powder and human breast milk samples spiked with sodium iodide and thyroxine (T4) solutions. The method quantitation limit (MQL) for human breast milk was 1.6 µg L^{-1}. The intra – assay and inter – assay coefficient of variation for the breast milk samples and NIST powder were <1% and <3.5% respectively. NIST 1549 milk powder, human breast milk samples and calibration standards spiked with the internal standard were all stable for at least 2.5 months after extraction. The results of the validation process confirmed that this newly developed method provides greater accuracy and precision in the assessment of iodine concentrations in human breast milk than previous methods and therefore offers a more reliable approach for assessing iodine concentrations in human breast milk.

INTRODUCTION

Iodine is a major constituent of thyroid hormones, and an adequate supply of iodine before birth and in early infancy is essential for achieving optimal physical growth and mental development [1]. Breast milk is the sole source of iodine for exclusively breast-fed infants, and it is therefore critical to ensure that the iodine content of the breast milk is sufficient to meet the nutritional needs of this infant population. However, relatively few studies have reported breast milk iodine concentrations, and this is largely due to the lack of a robustly validated method for assessing iodine concentrations in human breast milk.

Since breast milk is a complex matrix, consisting of a range of bioactive components and nutrients, existing studies which have measured iodine concentrations in breast milk have applied comparable methods to those used for assessing iodine content of food. A number of analytical methods have been used for the determination of iodine in foodstuffs including the classic Sandell and Kolthoff kinetic – catalytic method [2,3], ion chromatography [4,5], inductively coupled plasma mass spectrometry (ICPMS) [6-9], flame atomic absorbance spectrometry [10], high performance liquid chromatography [11] and ion – specific electrodes [9,11]. Of these methods, ICPMS is considered to be the gold standard, due to its high level of accuracy, precision and low detection limit, and is the most widely used approach for iodine quantification in foods. Moreover, ICPMS analysis following extraction by TMAH has been adopted by the European Committee for Standardization as the official method for the quantification of iodine concentrations in foodstuffs (EN 15111:2007) [12].

However, while the ICPMS method is routinely used for the assessment of iodine in foods [9,13-17] , its suitability for the assessment of iodine in human breast milk has not been systematically assessed, and various aspects of the method have not been

optimized. It is not clear, for example, whether TMAH is a strong enough digesting agent to liberate iodine from T4 in breast milk or whether the internal standard used in the assay is suitable for the human milk matrix. In addition, the limit of determination of the previously reported method is relatively high (30 µg kg^{-1}) [13], raising concerns about its sensitivity, and the extent of carryover between samples has not been thoroughly tested. The process of preparing the milk samples for analysis, in particular the procedure for homogenization of milk samples after thawing, has also not been clearly described.

Therefore, the aim of this study was to modify existing approaches for assessing iodine concentrations in breast milk to address these method – related issues and thereby develop an ICPMS method which was suitable for the accurate and reproducible determination of iodine in human breast milk.

MATERIALS AND METHODS

Reagents and Equipment

Two human milk samples were collected in 50 mL polypropylene (PP) tubes with screw caps (Cat no. 227261, Greiner Bio – One GmbH, Frickenhausen, Germany) and frozen at -20^0C until analysis for concentration, long term stability and spiked recovery.

Certified reference material (CRM) produced by the National Institute of Standard and Technology (NIST), NIST 1549 non – fat milk powder (Maryland, USA) with a certified iodine level of 3.38 ± 0.02 mg kg^{-1} was used to assess the accuracy of iodine determination.

Reagents for milk digestion included: high purity TMAH powder (Cat. No. T7505-100G) from Sigma – Aldrich (New South Wales, Australia), a commercial stock iodine

standard (1000 mg L^{-1}) from Australia Chemical Reagents (Queensland, Australia), two internal standard stock solutions, 1000 ± 3 mg L^{-1} Tellurium (Te) in 2% HNO_3 + 0.2% HF and 1000 ± 3 mg L^{-1} Antimony (Sb) in 5% HNO_3 + 0.1% HF from High – Purity Standards (South Carolina, USA). L – Thyroxine (T4) powder (Cat. No. T2376-1G) for recover tests was purchased from Sigma – Aldrich (New South Wales, Australia).

High purity water generated by a Sartorius Water Purification System (Sartorius Stedim Australia Pty. Ltd., Dandenong South Victoria, Australia) was used for the preparation of all reagents, standards and samples.

Graduated 50 mL PP digestion tubes with screw caps (Cat. No. SC475, Environmental Express South Carolina, USA) were used for digestion/extraction with TMAH.

Graduated 15 mL PP tubes with screw caps (Cat. No. 188261 Greiner Bio – One GmbH, Frickenhausen, Germany) purchased from Interpath Services Pty Ltd (South Australia, Australia) were used as the analysis tube for the ICPMS.

Millex HV disposable syringe filters (33 mm diam. 0.45 μm pore size; Millipore Corp, MA, USA) and Terumo 10 mL syringes (Binan Laguna, Philippines) were used to filter the digested breast milk samples.

An IKA T25 digital Ultra Turrax homogenizer (IKA Ltd, Germany) was used to macerate breast milk samples before analysis.

The digestion step was performed in a 54 well HotBlock™ heating block with digital temperature control (Environmental Express Cat. No. SC154) purchased from DKSH Pty. Ltd. Australia.

All reagent additions and dilutions were carried out using a semi – automated Gilson 402 diluter (John Morris Scientific, Keswick, South Australia). The dilutor tubing (FEP

– Fluorinated ethylene propylene) was soaked in 8% TMAH overnight and thoroughly rinsed with high purity water before use.

Pure water was dispensed with a Brand® bottle top dispenser (5–50 mL Dispensette® Organic, Digital Cat. No. 4730 360)

The TMAH powder and all containers and equipment used to prepare the samples or store/collect the breast milk were checked for iodine contamination before use.

Reagents and Standard Solution Preparation

To prepare the 25% TMAH and 8% TMAH solutions, 125 g or 40 g respectively of TMAH was dissolved in high purity water and made up to 500 mL. These solutions were stored at room temperature.

Internal standard stock solutions. 1.6 mL of Te stock standard, 2 mL of Sb stock standard and 40 mL of 25% TMAH were diluted in high purity water to a final volume of 1000 mL to make solutions of 1.6 mg L^{-1} Te and 2 mg L^{-1} Sb which were stored at room temperature. These solutions were further diluted during the calibration standard preparation and digestion process to yield 40 µg L^{-1} Te and 50 µg L^{-1} Sb in the final analyzed solutions.

Iodide spiked solutions. To prepare iodide spiked solutions, the 1000 mg L^{-1} iodide stock solution was diluted with high purity water to yield 50 mg L^{-1} iodide solution. This solution was further diluted in 1% TMAH to produce final concentrations of 0.2, 0.4 and 0.8 mg L^{-1}. These solutions in turn were used as spiked solutions in recovery tests, producing concentrations of 2.5, 5.0 and 10 µg L^{-1} in the final analyzed samples

Thyroxine spiked solutions. To prepare thyroxine (T4) spiked solutions, 0.1531 g of T4 was dissolved in 20 mL of 25% TMAH and diluted to 2 L with high purity water to

yield a 50 mg L^{-1} T4 iodine solution in 1% TMAH. This solution was further diluted in 1% TMAH to produce final concentrations of 0.2, 0.4 and 0.8 mg L^{-1}. These solutions in turn were used as spiked solutions for recovery tests producing concentrations of 2.5, 5.0 and 10 µg L^{-1} T4 in the final analyzed samples.

Iodine calibration solutions (0, 1, 2.5, 5, 10, 25, 50, 100 and 200 µg L^{-1}). The iodine stock solution (1000 mg L^{-1}) was diluted in high purity water to a concentration of 5 mg L^{-1} (intermediate stock standard). This solution was further diluted to prepare intermediate standard 1 and 2 with concentrations of 1.0 and 0.05 mg L^{-1} respectively and these 2 standards in turn were used to make the calibration solutions in 1% TMAH. 1 mL of internal standard solution was added to 40 mL of each these iodine calibration solutions. The calibration solutions were stored at room temperature and fresh solutions prepared every 4 months.

Iodine drift correction standard solutions (0 and 5 µg L^{-1}). The iodine intermediate standard 2 solution (0.05 mg L^{-1}) was used to make the drift correction standard. Both these standards were prepared in 1% TMAH. 1 mL of internal standard solution was added into 40 mL of each these iodine drift correction standard solutions.

Blanks. Four types of blanks were used; a calibration blank, a method digestion blank processed in exactly the same way as the samples and containing all the reagents used in the assay, a drift correction blank and a system blank. All blanks were prepared in 1% TMAH and also contained the internal standard mix. The calibration blank was used to establish the analytical calibration curve, the method digestion blank was used to account for batch to batch variation and to overcome system memory effects from the instrument by subtraction during result calculation, the drift correction blank was used to normalize the drift standard solution every 25 samples during each analytical run and

the system blank was used to monitor the overall system memory from the instrument during each analytical run.

Wash solutions. Three types of wash solutions were used. Two of the solutions, the auto-sampler wash station rinse solution and an extra clean wash solution, consisted of 1% TMAH and high purity water. Another pre – wash solution was prepared with 1% ammonia (NH_4) in high purity water.

Instrumentation

Iodine determination was carried out using an Agilent7500ce ICPMS system consisting of an Integrated Sample Introduction System (ISIS) unit plus a CETAC ASX-510 auto-sampler (Agilent Technologies Australia) equipped with a Ceramic VeeSpray nebulizer (Glass Expansion Pty. Ltd, Melbourne, Australia). The ISIS peristaltic pump program was used to reduce the washout time and speed up sample uptake to the instrument between samples to reduce analysis time per sample. Instrument performance optimization, including nebulizer gas flow rate, ion lens voltage and torch alignment, was set up following the manufacturer's instructions and optimized before each run. Operating conditions for the systems are shown in Table 1.

All raw concentration data from the ICPMS was exported to Microsoft Excel. Blank subtraction, drift correction and other data processing (mass and volume adjustments) were performed off – line, using custom – written macro programs operated within Excel.

Sample Digestion

1 mL of homogenized breast milk measured using the Gilson 402 diluter or 0.1 g of milk powder was placed into labeled 50 mL PP tubes, 5 mL of 8% TMAH plus 0.75 mL of pure water was then added to each of the tubes using the diluter and the tubes recapped. Samples were mixed by shaking/vortexing at low speed and allowed to stand overnight in a fumehood at room temperature. On the following day, samples were mixed again by shaking/vortexing and digested at 90^0C for 1 hour using the heating block system. Samples were mixed by shaking/vortexing at least twice during the incubation period to ensure complete digestion. The tubes were then removed from the heating block and cooled at room temperature. 1 mL of internal standard solution plus 2.25 mL of pure water was added to all tubes using the diluter and the volume made up to 40 mL by the addition of 30 mL high purity water using a Brand® bottle top dispenser. The tubes were tightly recapped, shaken/vortexed until thoroughly mixed and then 5 – 10 mL of each digested solution was filtered and transferred into graduated 15 mL PP tubes prior to ICPMS analysis.

Method Optimization

Optimal digestion time and temperature for iodine (iodide and T4) extraction from human breast milk

The efficiency of iodine extraction from breast milk and NIST 1549 milk powder by TMAH was tested under 3 different conditions: (1) 80^0C for 1 hour, (2) 90^0C for 1 hour and (3) 90^0C for 2.5 hours. The recovery of iodine from the NIST 1549 milk powder and 2 human milk samples were tested under each of these conditions.

Instrument/system memory effect

Depending on the prior use of the ICPMS instrument (nitric acid digests or TMAH digestions) the system showed various levels of iodine contamination. The most effective pre – wash solution was determined by comparing the time taken for the background count to decrease to an acceptable and stable level (600 - 1000 counts/second for iodine) following a continuous pre – wash with 1% TMAH or 1% ammonia.

The efficiency of cleaning up the ICPMS system with the 1% TMAH pre – wash solution was tested for 3 levels of iodine contamination, namely highly contaminated (0.46µg L^{-1}), slightly contaminated (0.3µg L^{-1}) and a clean system (0.06µg L^{-1}). The cleaning efficiency of the 1% NH_4 pre – wash solution was also tested on a highly contaminated system. Furthermore, the clean – up efficiency of the pre – wash solution was monitored in each analytical run by collecting iodine concentrations of all the blank solutions defined above to assess the instrument memory effect.

Sample – to – sample carryover

Previous studies in our group had indicated that 4 % perchloric acid digests and high salt matrices had the potential to cause precipitation within a range of nebulizers leading to significant drift throughout long analytical runs. These studies also suggested the use of wet argon in the nebulization gas could prevent significant drift and reduce sample – to – sample carry over. In the present study, sample carryover from a previous analytical sample was determined after washing the nebulizer with either dry or wet argon.

The sample carryover in these tests was determined by assessing iodine concentration in 15 successive replicate blanks following the measurement of iodine in a highly concentrated standard solution (200 µg L^{-1}).

*Stability of the iodine calibration standard*s

Three of the iodine standard solutions (2.5, 5 and 10µg L^{-1}) were analyzed on repeated occasions over a 2.5 month period to assess the stability of iodine standards during storage. The iodine concentrations measured at each of the time points across the 2.5 month period were compared to those in the equivalent standard immediately after preparation.

Stability and reliability of internal standards

The stability and reliability of two internal standards, Sb and Te, were tested in the human breast milk matrix in order to assess their suitability for routine analysis. Human breast milk samples, NIST non – fat milk powder reference material and standards were prepared as described earlier with the addition of a mixture of 2 internal standards (Sb and Te). The raw counts of iodine (I), Sb and Te were monitored to check for stability. The raw I counts were also normalized to both the raw Sb and Te counts to evaluate the stability and reliability of each internal standard across the analytical run.

Human breast milk homogeneity

After collection, the breast milk sample was mixed by shaking vigorously, split into 2 aliquots and then frozen at -20°C until analysis. After thawing, one aliquot was homogenized at 20,000 min^{-1} for 30 seconds while in the other the milk was allow to separate into two phases, the aqueous and fatty fractions. The iodine level of each fraction was measured and compared to the concentration measured in the homogenized sample.

Stability of extracted samples

To assess the stability of iodine in samples post – digestion, NIST 1549 milk powder and 2 human milk samples were digested by using the digestion procedure described above and iodine levels measured on the day of digestion and following 2.5 months storage at room temperature.

Contamination check of components used in the milk collection and analysis

A small amount of high purity water was placed into the various containers and devices used in the milk collection process. This included 2 types of collection containers, 2 types of breast pump systems and 2 types of storage tubes. The water was left to stabilize for periods varying from 3 hours to 2.5 weeks. Samples of this high purity water were then collected and tested for iodine contamination using the same method as for human milk samples. The contamination check was also conducted for all the disposable PP tubes used in the digestion and analysis steps.

Method Validation

Linearity

The iodine standards (0, 1, 2.5, 5, 10, 25, 50, 100, 200 µg L^{-1}) were used to establish the calibration curve. In order to ensure accuracy, the iodine concentrations of all samples analyzed are required to fall within the range of the calibration curve, otherwise the samples need to be diluted.

Recovery

A breast milk sample, NIST 1549 milk powder and a method digestion blank were used to determine the percent recovery of various levels of added iodine in the samples. The

breast milk and milk powder samples were spiked with 5 and 10 µg L^{-1} of iodide solution and also with 5µg L^{-1} and 10 µg L^{-1} of T4 iodide solution. The method digestion blank was spiked with 2.5 and 5.0 µg L^{-1} of iodide solution and also with 2.5µg L^{-1} and 5.0 µg L^{-1} of T4 iodide solution. The unspiked samples and all spiked samples were measured in quadruplicate. The measured iodine concentration was divided by the expected value in order to determine the percent recovery.

Precision

The intra – assay and inter – assay variation were determined by analyzing 2 breast milk samples and NIST 1549 milk powder either 4 times in a single run (intra – assay coefficient of variation) or on 4 different days in 4 separate assays (inter – assay coefficient of variation).

Accuracy

The method was validated for accuracy by comparing the iodine concentration obtained for 78 replicates of NIST CRM 1549 milk powder analysis carried out over a period of ~ 3 years. The % relative standard deviation (%RSD) of repeatability and 95 % confidence interval (CI) were calculated.

Limit of Detection (LOD) and Method Quantitation Limit (MQL)

LOD refers to the lowest concentration where we can just distinguish a signal from the background. For this publication we used the definition of the limit of detection set by the International Union of Pure and Applied Chemistry (IUPAC) [18] and the National Association of Testing Authorities, Australia (NATA) [19]

According to this definition, LOD was calculated as the mean concentration plus 3 X the standard deviation of the concentration of a calibration blank measured in the same assay at least 7 times.

The MQL is defined as the minimum concentration of an analyte that can be measured within specified limits of precision and accuracy and is calculated as 3 X LOD multiplied by the dilution factor. This takes into account any matrix related effects. Instrument Detection Limit (IDL) is equivalent to the LOD in our case. The Instrument Quantitation Limit (IQL) is calculated as 3 X LOD.

RESULTS

Method Optimization

Optimal digestion time and temperature for iodine extraction from human breast milk

It was critical to achieve a complete sample digestion to ensure the accuracy of iodine measurements conducted in downstream applications, including ICPMS. As illustrated in Table 2 the efficiency of extraction of iodine from human breast milk was similar under all tested conditions. The extraction efficiency for the NIST CRM 1549 milk powder also showed good agreement with the certified value (3.38 ± 0.02 mg kg^{-1}) for all 3 processes tested. We chose to use a digestion temperature of 90^0C for 1 hour for all subsequent experiments to reduce the overall time required for sample preparation.

Instrument/system memory effect

We found 2 significant areas within the ICPMS that showed memory effect; the auto-sampler wash station and the uptake tubing/nebuliser/spray chamber area. There was a marked difference in the time taken for the background counts to drop to acceptable levels between the 1% TMAH and 1% NH_4 pre – wash solutions. When using 1% TMAH to wash out the the 2 areas identified above, the background count levels decreased gradually and often did not reach an acceptable level even after ~ 2 - 3 hours from the start of the wash procedure or well into the actual analysis run. In contrast,

using a 1% NH_4 solution resulted in a sharp drop in the background counts to an acceptable level after only 10 minutes in the uptake area. The auto – sampler wash station also required extra soaking with 1% NH_4 to remove long term buildup (data not shown).

The data presented in Figure 1 supports these findings. . In the clean system, achieved after 1 day of running routine breast milk iodine analyses, the iodine concentration in the blank decreased only slightly at the start of the run before stabilizing, indicating that there was minimal iodine contamination prior to the start of the run. In contrast, in both the highly contaminated and slightly contaminated systems, the iodine concentrations in the blank solutions analyzed after the pre-wash with 1% TMAH dropped sharply at the beginning of the analysis run before declining to acceptable iodine levels for the blank sample, indicating that 1% TMAH wash – out was not effective at reducing the iodine concentrations to acceptable levels prior to the analytical run. In the highly contaminated system cleaned using the 1% NH4 pre – wash solution, however, we achieved a similar washout profile to the clean system washed with 1% TMAH. This suggested that a 1% NH_4 pre – wash solution could be used once before each run to achieve an acceptable background count at the beginning of the analytical run.

We also observed a substantial and variable drop in the baseline counts depending upon the extent of the system contamination, indicating that the way the method digestion blank is handled is critical with respect to the calculation of the final results. We therefore subtracted our method digestion blank value from each batch of samples as a part of calculation.

Sample – to – sample carryover

Figure 2 shows the result of the sample carryover experiment comparing the extent of carryover after using either wet or dry argon to rinse the nebulizer between samples.

There was no significant difference in the reduction of carry over effects between the wet and dry argon in this system.

*Stability of the iodine calibration standard*s

Over the 2.5 month period, the percentage error for the measurement of the 3 iodine calibration solutions ranged from -1.3 to +0.5% with respect to their actual concentration. Iodine standard solutions appeared to be very stable after preparation for a period of at least 6 months (data not shown).

Stability and reliability of internal standards

As expected with ICPMS the raw count stability over the various runs varied significantly for both internal standards as well as iodine (data not shown). When the raw iodine counts were normalized to the raw counts for the 2 internal standards in the drift check standard, the stability for iodine concentration measured across the run improved significantly for both Sb and Te. When Sb was used the internal standard, the values for the NIST milk powder certified reference material were consistent with the expected value. However, when the iodine counts were normalized to the Te standard counts, the calculated result for the NIST milk powder certified reference material exceeded the expected value by 10% of the expected value. Sb was therefore selected as the internal standard used to correct for the variability caused by matrix effects and instrument drift in this assay.

Human breast milk homogeneity

The iodine level in the fatty fraction (145 ± 48 μg L^{-1}) was considerably higher and more variable than in the aqueous fraction (88.5 ± 2.1 μg L^{-1}). The iodine concentration in the homogenized samples (105.7 ± 0.6 μg L^{-1}) were notably less variable, and were intermediate to that of the two separate fractions. These iodine concentrations were

within the expected range of breast milk iodine levels in breast-feeding women from iodine sufficient populations(unpublished data).

Stability of extracted samples

There was no difference in the iodine concentration measured in digested samples immediately after digestion or after 2.5 months of storage post-digestion (Table 3).

Contamination check of components used in milk collection and analysis.

No iodine contamination was detected in any of the equipment used to collect and analyze human milk. All results were below the MQL (1.6 µg L^{-1}) for all tested components.

Method Validation

Linearity

The standard curve was linear up to 200µg L^{-1} iodine and the slope and coefficient of correlation were 0.0162 and 0.9999 respectively.

Recovery

For the NIST SRM (n=4), recoveries between 95.5 % and 96.5 % were achieved for solution spiked with both low (5µg L^{-1}) and high (10 µg L^{-1}) amounts of iodine (Table 4). For human breast milk samples, the percentage recovery of iodine from all the spiked samples was between 96.5 % and 97.2 % (n=4 for each spiked concentration level). A recovery of ~ 96 % also found in breast milk samples and NIST milk powder spiked with the 2 different concentrations of T4. The percentage recoveries for all T4-spiked blanks were between 96.2 % and 98.2 % with the variation of 0.5 %.

Precision

409 The intra – assay CoVs for iodine concentration of 2 breast milk samples were 0.1%
410 and 1.8%, respectively whilst NIST 1549 milk powder were 0.97 %. The inter – assay
411 CoVs were 2.2%, 3.06% and 0.25 %, respectively

412

413 *Accuracy*

414 The results obtained for the NIST milk standard using this method was 3.38 ± 0.02 mg
415 kg^{-1} (N = 78) was in a close agreement with the certified value of 3.38 ± 0.02mg kg^{-1}.
416 The RSD was 0.5 % with a 95 % CI of 0.005.

417 *Detection limits*

418 The IDL for iodine in human milk was 0.013 µg L^{-1} if carryover and contamination
419 were eliminated as described in this paper. Assuming a dilution factor of 40, the MQL
420 was 1.6 µg L^{-1}.

421

422 **DISCUSSION**

423 This paper describes the development and validation of a method for the assessment of
424 iodine concentration in human breast milk which offers significant improvements over
425 existing methods in relation to detection/quantitation limit, choice of internal standard
426 and minimization of instrumental memory effect during analysis.

427

428 The performance of the method was evaluated with respect to linearity, recovery,
429 precision, accuracy and quantitation limit. The method exhibited strong linearity (R^2 >
430 0.999 and slope of 0.0162) and achieved high recovery (> 94%) of iodine from milk
431 samples spiked with either iodine or T4. The intra – and inter – assay coefficients of
432 variation of the breast milk and NIST milk powder samples were both <3.5% and the

iodine level measured in NIST 1549 milk powder using this method were in close agreement with the certified value, indicating a high degree of reliability and precision. The quantitation limit for this method of 1.6µg L^{-1}, was much lower than the quantitation limits which have been reported in previous methods, which have ranged from 14 – 30µg L^{-1} [3,13,14,20]. Importantly, the quantitation limit we achieved in our method is also well below the expected range of iodine concentrations in human breast milk, even in regions classified as iodine deficient (32 – 78µg L^{-1}) [21-23], suggesting that the method is appropriate for the assessment of iodine concentrations in breast milk across a broad range of populations.

Sample digestion is a critical step in ensuring accurate determination of the iodine content of samples by ICPMS, and establishing a method for the digestion of human milk samples which did not affect iodine concentrations in the sample was a critical component of this study. Previous studies had shown that extraction by acidic and ammonia media had the potential to affect the accuracy of the analysis, by inducing instrument memory effects [24] and variations in the efficiency of iodine extraction between samples [25,26]. As a result, we chose to use another widely applied digestion medium, TMAH, in this method. The use of TMAH in iodine extraction for ICPMS analysis has previously been applied to infant formula [9], milk powder [9,13,16,27] and herb milk [28], but this is the first report of its successful application in the assessment of iodine in human breast milk samples. In addition, we showed that we were able to obtain complete and reproducible digestion with considerably shorter heating times compared to previous studies (1 hour vs 2-3 hours) [7,13,16] thus reducing the total time required for the experimental procedure.

Although previous studies have suggested that iodine species in biological samples can be extracted by TMAH, there had been no reports of successful extraction of iodine from iodine – containing compounds, such as T4. The failure to liberate iodine from such compounds during the digestion process would result in underestimation of the iodine content of the sample, thus impacting on accuracy and reliability of the measurements. By assessing the recovery of iodine from human breast milk samples spiked with T4 in the present study, we confirmed that TMAH digestion was able to release iodine from this complex, and therefore provide an accurate measure of the total iodine content of the sample.

We did not find any difference between wet and dry argon in the efficacy of reducing carryover between samples in this study. Although not the focus of our study, flushing the system with wet argon is likely to be preferable in practice, since it is able to prevent the build of iodine contamination within the argon jet in the nebulizer, which has the potential to introduce random errors during large analytical runs (unpublished data). We identified the auto – sampler wash station as a major source of instrument carryover when the ICPMS machine had previously been utilized for the assessment of other elements and using other wash out solutions (e.g. nitric acid solutions). Thus, thoroughly flushing the system with a 1% NH_4 solution prior to commencing any new assay is necessary to avoid carryover effects due to previous analyses conducted on the same instrument.

Drift is an analytical error leading to the poor accuracy, and arises when instrument responses change through the run. The drift can be corrected by analyzing drift

standards after every four to five samples [29], however no previous studies involving assessment of iodine content in breast milk have included an approach for drift correction. Instrument performance in this method was monitored by including a high drift standard (5 μg L^{-1}) and drift correction blank (0μg L^{-1}) after every 25 samples, and we were able to use this to appropriately correct for the drift in the experimental procedure. Measurement of drift standards after 25 samples, instead of 4 – 5 samples reduces the cost and reading time in routine analysis. It was also noted that the largest difference between successive drift iodine levels occurred at the beginning of the run and this also needed to be taken into account during the drift correction to achieve the most accurate results.

Te has commonly been used as an internal standard in ICPMS methods and was selected due to the fact that its ionization status is closer to iodine than other elements [13]. However, the Te signal in the milk powder experiment was lower and more variable than expected, resulting in reduced accuracy in the assessment of iodine concentrations. This may be because Te precipitates with some components within the human milk matrix, and indicates that it is not the most appropriate internal standard for this method. However, Sb exhibited much greater stability in the human milk matrix in the present study and appears to be a more suitable internal standard for ICPMS assessment of human breast milk.

In a previous study reporting the analysis of iodine in food, high-cost tubes were used during sample digestion, which were washed and re-used for subsequent assays. Even with thorough washing, there is the potential for trace amounts of iodine to remain in

the tubes, which could produce carryover effects and introduce inaccuracies in the results [13]. In this method, we eliminated this source of contamination by using disposal screw cap polypropylene tubes [30]. Disposable auto – sampler tubes and pipette tips were also used to minimize the potential for contamination. Importantly, we confirmed that all the containers and equipment that were in contact with the sample at any stage in the process of collection, processing and analysis were free from iodine contamination, and can thus be confident that any iodine detected in this assay originated from the breast milk sample. Confirming the absence of contamination is critical to ensuring that the results of the assay are a true reflection of concentrations in the sample and should be standard practice in any application of this method.

Appropriate sample preparation is essential to achieve reliable results. Despite the fact that human milk is known to separate into 2 distinct layers after thawing or during extended periods of standing, no previous method has provided detailed information regarding sample preparation. This study confirmed that iodine concentration differs markedly between the fatty and aqueous fractions of human milk and that complete homogenization of the samples is required prior to digestion in order to obtain reliable results.

The stability of extracted human milk samples had not been tested in previous methods. In this modified method we found that iodine level in extracted human milk also containing the internal standard was stable for at least 2.5 months when stored at room temperature. The finding increases the potential for this method to be utilized in large-scale clinical trials and population screening programs, since it makes it possible for samples collected at different times to be analyzed in the same ICPMS run.

In conclusion, we have successfully validated a method for the assessment of iodine in human breast milk which overcomes the limitations of previous approaches and highly accurate, reproducible and precise. The modified method is able to recover over 95% of iodine from spiked solutions, has a lower quantitation limit than previous method and has inter – and intra – assay coefficients of variation well below 5%. This method represents a significant advance in the assessment of iodine concentrations in human breast milk and its application will enable us to gain new insights into the iodine status of lactating women. This assay is currently being applied for routine assessment of iodine concentration in breast milk samples in our laboratory.

Acknowledgments

We are grateful to Waite Analytical Service, School of Agriculture, Food and Wine, University of Adelaide for assistance of method development and validation. Beverly Muhlhausler is supported by a Career Development Award from the National Health and Medical Research Council of Australia (NHMRC). Robert Gibson is supported by a NHMRC Senior Research Fellowship. Dao Huynh is sponsored by Vietnamese Ministry of Education and Training (project 322) and the University of Adelaide. This method was developed for the assessment of iodine concentrations in breast milk in a cohort study supported by a NHMRC project grant (ID 626800).

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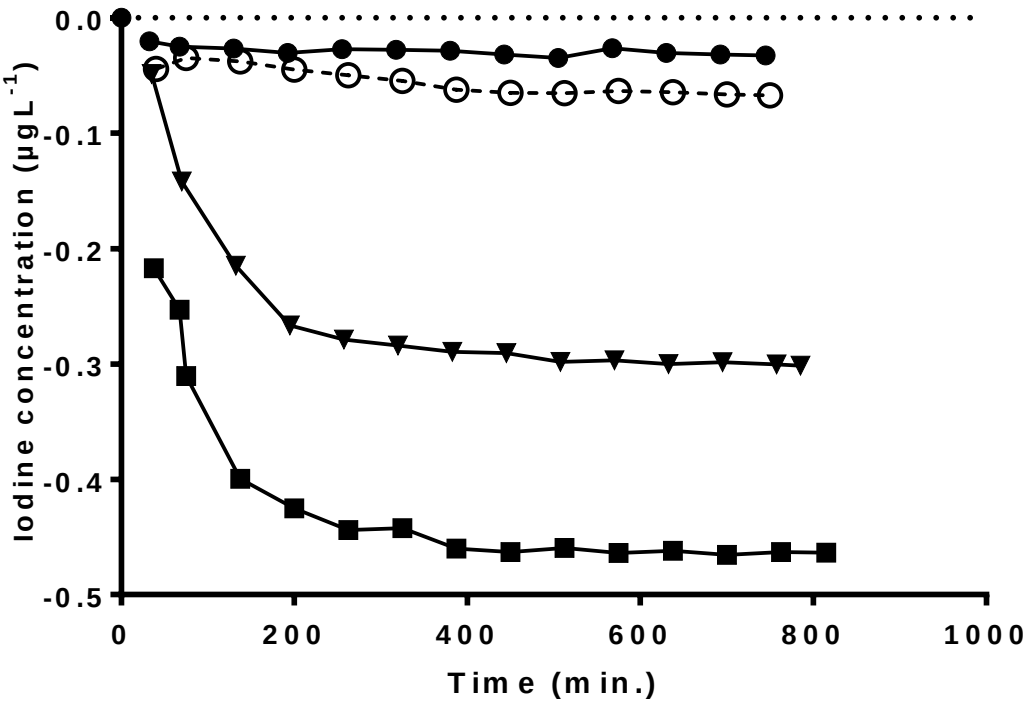
Figure 1: *Instrument/system memory effect*: Iodine concentration of the drift correction blank (0µg L^{-1}) over the course of an ICPMS run at 3 levels of system contamination; Contaminated system after 1% TMAH pre – wash solution (), slightly contaminated system after 1% TMAH pre – wash solution () and clean system after 1% TMAH pre – wash solution (), contaminated system after 1% NH_4 pre – wash solution (). The iodine concentration at t=0 represents the iodine calibration blank concentration. The negative iodine concentrations indicate the extent of wash – out during each analytical run.

Figure 2: *Sample-to-sample carryover:* iodine washout with dry argon (—) and wet (---) argon after analyzing 200 µg L^{-1} iodide. IQL, instrument quantitative limit; IDL, instrument detection limit.

Figure 1


0.0
-0.1
-0.2
-0.3
-0.4
-0.5
Iodine concentration (µgL-1)
0
200
400
600
800
1000
Time (min.)

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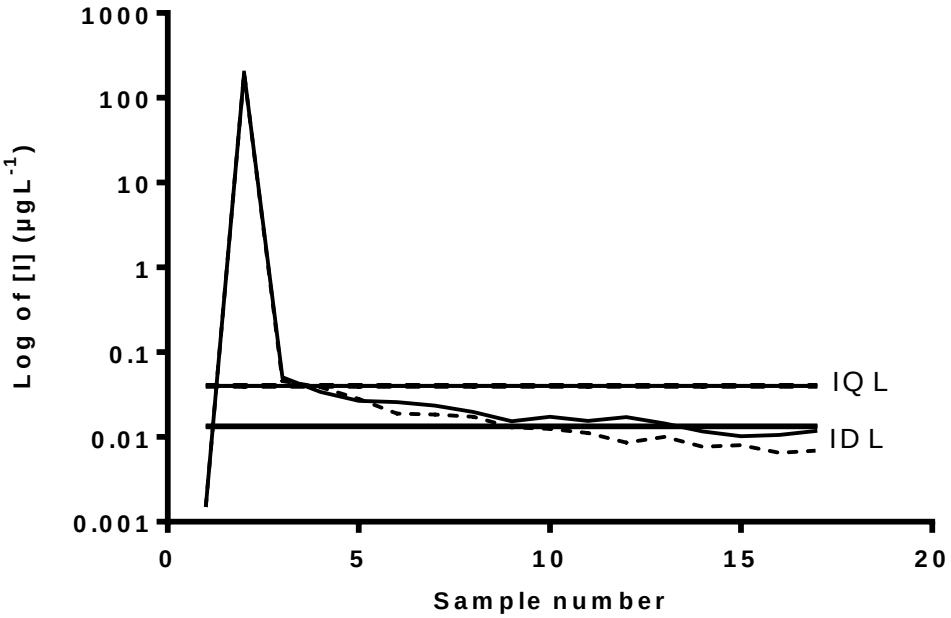
683 **Figure 2**



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686 **Table 1: Agilent** ICPMS operating conditions

RF power (W)	1500
RF matching (W)	1.66
Frequency (MHz, free running)	27
Sampling depth (mm)	0.8
Carrier gas (L/min)	1.00
Makeup gas (L/min)	0.20
Nebulizer	Ceramic VeeSpray
Spray chamber	Double Pass
Nebulizer pump (rps)	0.20
Lens Settings	Optimized with each run
Iodine (I) - Mass	127
Antimony (Sb) - Mass	121
Tellurium (Te) - Mass	128
Scanning mode	Peak hoping
Points / peak	3
Number of replicates	3

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689 **Table 2**: Comparison of iodine concentration determined in the sample NIST 1549 milk
690 powder and human breast milk samples for each of the 3 digestion conditions

Materials	**80^{0}C / 2.5h**	**90^{0}C / 1h**	**90^{0}C / 2.5h**
NIST 1549 milk powder (mg kg^{-1}) (N = 3)	3.39 ± 0.03	3.37 ± 0.01	3.38 ± 0.01
Human milk 1 (µg L^{-1}) (N = 2)	88.7 ± 0.5	89.7 ± 2.8	88.0 ± 0.02
Human milk 2 (µg L^{-1}) (N = 2)	74.3 ± 0.2	74.2 ± 0.1	74.4 ± 0.06

691 Values were expressed as mean ± SD.

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706 **Table 3**: The stability of extracted samples

Materials	0 month	2.5 months
NIST 1549 milk powder (mg kg^{-1}) (N = 2)	3.39 ± 0.01	3.39 ± 0.01
Human milk 1 (μg L^{-1}) (N = 3)	106 ± 1.0	105 ± 1.0
Human milk 2 (μg L^{-1}) (N = 4)	79.8 ± 1.7	81.0 ± 1.4

707 Values were expressed as mean ± SD.

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722 **Table 4**: Iodine recovery percentage for samples spiked with iodide and T4.

Sample	Iodine concentration			T4 concentration		
	2.5 µg L^{-1}	5 µg L^{-1}	10 µg L^{-1}	2.5µg L^{-1}	5.0 µg L^{-1}	10 µg L^{-1}
Blank	93.8 ±0.5	97.2±0.5	-	96.2±0.5	98.2±0.5	-
NIST milk powder	-	96.0±1.6	95.5±0.8	-	96.0±1.6	96.5±1.3
Breast milk	-	97.2±0.5	96.5±0.6	-	96.5±1.3	96.8±1.0

723 Values expressed as mean % ± SD. (N = 4)

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