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Development of USI-Kit for Evaluation of Iodine Content in Iodized Salt[†]

Sakaewan OUNJAIJEAN[1], Kongsak BOONYAPRANAI[1], Kanokwan KULPRACHAKARN[1] and Kittipan RERKASEM[1,2,*]

[1]*School of Health Science Research, Research Institute for Health Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[2]*Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

([*]Corresponding author's e-mail: rerkase@gmail.com)

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Abstract

Iodine deficiency has been considered as a serious public health problem for the past decades. Universal salt iodization program is introduced and implemented to address such problem. To encourage this program in an effective and sustainable way, it is essential to regularly monitor whether salt is adequately iodized at various points along the supply chain. The traditional iodometric titration method has problems related to accessibility, cost, and time. Colorimetric test kits have been used extensively to measure coverage of iodized salt in household surveys due to its expediency and affordability. In Thailand, "I-KIT" is the most widely used. The visualization of intensive color, however, is inconvenient for untrained-user in determining the adequacy of iodine content. Thus, an improvement to make testing more precise and affordable is still required. In this respect, a new test kit namely USI-Kit was developed to assess iodine quality and semi-quantity in edible salt. The kit was tested to evaluate its performance, by comparing the result with the I-KIT and with the spectrophotometric method. Compared with I-Kit, the USI-Kit exerted the relative accuracy, sensitivity, specificity, false positive rate, false negative rate and Kappa coefficient value of 74.0, 76.3, 72.6, 27.4, 23.7 and 0.47, respectively. Compared to the spectrophotometric method, USI-Kit exerted the relative accuracy, sensitivity, specificity, false positive rate, false negative rate and Kappa coefficient value of 85.4, 80.1, 89.3, 10.7, 19.9 and 0.70, respectively. The finding suggested that a newly developed iodine test kit holds promise to be used in field inspection of iodine content in salt.

Keywords: Iodized salt, Iodates, Determination kit, USI-Kit, Evaluation study

Introduction

Iodine deficiency is one of the major global public health problems since it affects mental development, intellectual capacity, and growth of a person. Universal salt iodization (USI) has evolved as a major cost-effective strategy to combat iodine deficiency disorders [1,2].

Over the past decades, the success of USI programs in many countries have raised the coverage of household iodized salt, and thereby, consistently attenuated iodine deficiency and its disorders [3]. Unconsciously, fortification of iodine into edible salt, along with the high rate of salt consumption cause excessive iodine intakes in some countries [4], which is responsible for an increased risk of occurring disorders such as hyperthyroidism and thyrotoxicosis.

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Monitoring of iodine content in iodized salt is obviously an important process indicating the efficacy of the national salt-iodization program. The rigorous assessment of the adequacy of iodine content in salt is vital, since it is insufficient to just verify the presence of iodine in the salt and how much iodine it contains [5]. In Thailand, salt iodization is a prescribed national policy. Since 2011, the setting of appropriate iodine content in edible salt has changed from not less than 30 ppm to between 20 - 40 ppm [2] according to WHO (World Health Organization) / UNICEF (The United Nations Children's Fund) / ICCIDD (International Council for the Control of Iodine Deficiency Disorders) recommendation standard [1].

Iodometric titration is the traditional method widely accepted as a standard tool for determining iodine content. Though it is highly accurate, the method is time-consuming, costly, which requires capital infrastructure and skilled personnel. Iodine spot-testing kits which rather simple, inexpensive, and give rapid acceptable semi-quantitative result have gained more consideration, especially for the use in field inspection of the fortifying agent.

A number of iodine-testing kits have been developed and distributed, either commercially or as a tool of the national survey of iodine coverage. In Thailand, I-Kit, developed by Mahidol University is the most widely used. The kit works by adding a few drops of an acidic-starch reagent onto the surface of the salt sample. An acidic buffer act as reducing agent to reduces the iodate to free iodine. Released iodine is then simultaneously reacting with starch solution, and forms a dark blue complex. The result is read by visually comparing the color developed with a representative calibration chart. The visualization of intensive color, however, makes it difficult to be interpreted, especially for the sample containing high iodine content.

Our new salt iodine (in form of iodate) testing kit which is USI-Kit, was developed to verify the potency of iodized salt, both as means of quality control at the manufacturing level and for field monitoring of delivered levels. A basic principle of the test of iodine content was applied, using acetate buffer pH 4.0 as reducing agent and p-aminophenol (PAP) as an iodine-forming ligand. Purple color is developed in proportionally with the amount of iodine in a salt sample.

The aim of this present study is to evaluate the efficacy of USI-Kit for determination of iodine content in salt by comparing the accuracy, sensitivity, specificity, false positive rate, false negative rate and the Kappa co-efficiency (k) value of the kit with standard I-kit and spectrophotometric method.

Materials and methods

Chemicals and salt samples

Coloring agent, 4-Aminophenol hydrochloride (p-aminophenol, PAP), was purchased from Sigma-Aldrich, USA. All other chemicals including, potassium iodate (KIO_3), sodium chloride (NaCl), Sodium acetate (CH_3COONa), acetic acid glacial (CH_3COOH) were analytical GR grade, obtained from Merck, USA.

500 of un-iodized and iodized salts were randomly collected from 8 districts of Chiang Mai, Thailand including Meuang Chiang Mai, Hang Dong, San Pa Tong, Doi Lo, Chom Thong, Mae Ai, Chiang Dao, and Chai Prakan district during the year 2015 - 2016. Salt samples were kept in ziplock clear poly plastic bag in a light-avoiding container at room temperature throughout the study.

Preparation of standard iodized salt

Iodized salts of known iodine content were prepared in the Nutrition Analysis Laboratory, Research Institute for Health Sciences, Chiang Mai University. NaCl was completely dissolved in deionized (DI) water to obtained 20 g% NaCl solution. The NaCl solution was then added with various amounts of potassium iodate solution suitable to give final iodine concentration of 0, 10, 20, 30, 40, 50, and 100 ppm, respectively. Through the lyophilization process, iodized salts with fine and homogenous crystalized characteristic were gained. Quantitative analysis of iodine content was then performed using the spectrophotometric method to confirm its validity (20 samples of each concentration).

Spectrophotometric assay of iodine content in salt

Laboratory assessment of iodine in the form of iodate in salt samples was performed based on a simple colorimetric method explained by [6] with slight modification. Briefly, stock standard iodine solution of 1,000 ppm obtained by dissolving 0.1686 g KIO_3 in 100 mL DI water. Working standard was freshly prepared by serial dilution of the stock standard to the concentration of 10 - 40 ppm. Aqueous salt sample solutions were prepared in DI water into a final concentration of 20 g%. To each 25 µl of standard or sample, 75 µL of 20 g% NaCl solution and 100 µL of 0.5 g% PAP in 0.1 M acetate buffer pH 4.0 were added. After leaving at room temperature for 60 min, the absorbance of the obtained orange complex was read at wavelength 450 nm. All determinations were done in triplicate. The content of iodine in the salt was calculated with a standard calibration curve.

Determination of iodine content in salt using colorimetric test kit

Blind house-hold salt samples were collected from different areas of Chiang Mai Province. 100 out of 500 salt samples were randomly selected for the assessment of joint agreement of 2 readers, and a comparison of methods, according to the kit procedure explained below.

All 500 salt samples were used to test the performance of USI-Kit to determine the iodine in salt. This was performed by multiple untrained-users by following the kit manufacturer's instructions.

USI-Kit apparatus:

The field test apparatus consists of the following:

A. 2 of an 8-mL plastic bottle with red dropper cap containing the coloring agent (PAP, 30 mg)
B. 2 of an 8-mL plastic bottle with yellow dropper cap containing the diluent (0.1 M Acetate buffer pH 4.0, 6 mL)
C. 2 of a 2-mL plastic vial of standard iodized salts having iodine (in form of iodate) content of 20 and 40 ppm (1 g)
D. 1 plastic spoon (capable to transfer salt sample approximately 0.1 g)
E. 10 of 10-well, disposable flat bottom plastic plates (maximum volume 0.5 mL/well)
F. 1 iodized salt color standard chart, prepared by photographing a set of 5 standard salt tests (0, 20, 30, 40, 100 ppm) and making color-corrected photographs
G. 4 of 30-result reporting sheets
H. 1 of the kit procedure explanation sheet

USI-Kit procedure:

1. The working reagents must be freshly prepared by pouring 1 bottle of reagent B (acetate buffer) into 1 bottle of agent A (PAP) and mix gently.
2. To each well of plastic sampling plate, add one spoon of salt sample or standard (for granular salt, grounding is recommended).
3. Add 3 drops of the working reagent to each well and stir gently.
4. Reading the result by comparing the observed color with the standard color chart (or with the standard iodized salt, if necessary) in 1 - 5 min.

I-Kit procedure:

1. Put one spoonful of salt, using provided spoon, on the white plate provided.
2. Shake test-kit bottle and discharge 3 drops of solution on salt.
3. Mix by stirring with the end of spoon’s handle.
4. Observe the blue color of salt slurry in 1 - 5 min.
5. Read ppm of iodate in salt by comparing the observed color with the standard color value provided on the box, or values in between, i.e., 10 - 20, 20 - 30, 30 - 40 and 40 - 50 ppm.
6. Wash spoon and plastic plate and keep for further use.

Statistical analysis

Results were analyzed using Wilcox on signed rank test for Matched paired difference (Z). To validate the efficacy of the kit at 95 % confidence, reading results from USI-Kit were compared with I-Kit or spectrophotometric method using 2-way frequency table. The relative accuracy, sensitivity, specificity, false positive rate, false negative rate and Cohen's Kappa coefficient (κ) were calculated using the following standard formulas [7,8]:

with: TP = True positive, TN = True negative, FP = False positive, FN = False negative

$$\text{Relative accuracy} = \frac{(TP + TN) \times 100}{(TP + FP + FN + TN)}$$

$$\text{Relative sensitivity} = \frac{TP \times 100}{(TP + FN)}$$

$$\text{Relative specificity} = \frac{TN \times 100}{(TN + FN)}$$

$$\text{False positive rate} = \frac{FP \times 100}{(FN + TN)}$$

$$\text{False negative rate} = \frac{FN \times 100}{(TP + FN)}$$

$$\text{Total accuracy or overall agreement } (P_0) = \frac{(TP + TN)}{(TP + FP + FN + TN)}$$

$$\text{Positive agreement } (P_{pos}) = \frac{(TP + TP)}{(FP + TP) + (FN + TP)}$$

$$\text{Negative agreement } (P_{neg}) = \frac{(TN + TN)}{(FP + TN) + (FN + TN)}$$

$$\text{By chance agreement } (P_e) = \frac{(TP + FP) \times (TP + FN)}{(TP + FP + FN + TN)} + \frac{(FN + TN) - (FP + TN)}{(TP + FP + FN + TN)}$$

$$\text{Cohen's Kappa coefficient } (\kappa) = \frac{P_0 - P_e}{1 - P_e}$$

Results and discussion

Field test requirements

An understanding of the field conditions and the requirements is vital for the development of an effective field test. In most developing countries, iodine is fortified to salt in the form of potassium iodate rather than iodide [9]. Accordingly, test kits were designed to determine iodine in the form of iodate. During the process of field inspection, the tests were carried out by personnel with limited training, under conditions which generally lack equipment and supplies. For this reason, all of the apparatus needed for the test must be self-contained in a kit. The kit must be carefully designed to be simple easy-to-use, compact, long shelf-life period and inexpensive. In addition, the test must be capable to give rapid results with acceptable precision. The laboratory analysis of efficacy must be followed up to ensure that the developed kit meets the needs of the client group.

Quality control of standard iodized salt

Standard iodized salts with various concentration ranging from 0 to 100 ppm were prepared by the in-house method as explained in Materials and Methods. Quality control was performed by spectrophotometric analysis of iodine content in salt of 20 samples of each concentration. The acceptable

range was set at not more than ±2SD, at the precision of less than 5 % CV (Coefficient of Variation). Results of a single preparation lot are shown in **Table 1**.

Table 1 Assessment of iodine content in in-house prepared standard iodized salts by spectrophotometric analysis at 450 nm of the orange complex with PAP.

Calculated iodine content (ppm)	Actual iodine content (ppm)	
	Mean ± SD (n = 20)	**% CV**
0 ppm	-0.05 ± 1.02	0.00
10 ppm	10.04 ± 1.06	0.11
20 ppm	19.54 ± 1.56	0.30
30 ppm	27.51 ± 1.56	0.43
40 ppm	39.16 ± 2.55	1.00
50 ppm	49.61 ± 2.47	1.22
100 ppm	100.09 ± 2.85	2.86
Average %Yield	**98.56 %**	

Measurement of agreement of two readers

Comparison of performance between well-trained and untrained users in the intra-assay of the same blind salt samples found significantly different semi-quantitative reading results ($p < 0.05$). While well-trained user correctively reading iodine content in salt at the rate of 79 %, the untrained user could have achieved at 70 %. In the test of reading consider of the same 100 salt samples that iodine content is interpreted as either adequate (20 - 40 ppm) or inadequate (< 20 or > 40 ppm) by 2 readers. The results are presented in as a joint agreement by a 2-way frequency table. General indices of the agreement including overall proportion agreement (P_0), positive agreement (P_{pos}) and negative agreement (P_{neg}) were 0.77, 0.48 and 0.79, respectively. By means of these results, the explanation of the kit procedure may need to be more definite. The USI-Kit offered two different ways of interpretation of developed color, qualitative test: by compared color intensity with actual standard iodized salt (20 and 40 ppm), or semi-quantitative test: by compared with the standard color chart provided on the box (0, 20, 30, 40, 100 ppm). These although provided client a choice of application, it may cause confusion, especially to those with limited training.

Table 2 Joint agreement of 2 readers about iodine content in 100 blind salt samples.

Untrained user	Well-trained user		Total
	Adequate (20 - 40 ppm)	**Inadequate** (< 20 or > 40 ppm)	
Adequate	33	10	43
Inadequate	13	44	57
Total	46	54	**100**

Comparison of the performance USI-Kit and I-Kit

Performance of newly developed, USI-Kit was compared with commercially available, I-Kit on an assessment of the same 100 salt samples by a single well-trained user. The difference in the percentage of correct reading between the two kits (79 % for USI-Kit, 64 % for I-Kit) was statistically significantly ($p < 0.05$). Interpretation of reading results is presented in **Table 3**. By calculation, USI-Kit gained the relative accuracy, sensitivity, specificity, false positive rate, false negative rate, and κ coefficient value of 74.0, 76.3, 72.6, 27.4, 23.7 and 0.47, respectively.

Table 3 Comparison of USI-Kit versus I-Kit for the determination of iodine content in salt by a single well-trained user.

USI-Kit	I-Kit		Total
	Adequate (20 - 40 ppm)	**Inadequate** (< 20 or > 40 ppm)	
Adequate	29	17	46
Inadequate	9	45	54
Total	38	62	**100**

Comparison of the performance USI-Kit and Spectrophotometric method

Testing of the capability of USI-Kit was further compared with the standard spectrophotometric method using similar coloring agent (4-aminophenol hydrochloride in acetate buffer pH 4.0). The results are presented in **Table 4**. Collectively, USI-Kit has a high level of accuracy (89.0 %), sensitivity (88.9 %) and specificity (89.1 %). The false positive rate and false negative rate of the kit found to be 10.9 and 11.1 %, respectively. Cohen's kappa coefficient value was 0.78. Regarding to this κ value, USI-Kit was considered as suitable iodine test kit with substantial strength of agreement to the spectrophotometric method [5,10,11].

Table 4 Comparison of USI-Kit versus the spectrophotometric method for the determination of iodine content in salt by a single observer.

USI-Kit	Spectrophotometric method		Total
	Adequate (20 - 40 ppm)	**Inadequate** (< 20 or > 40 ppm)	
Adequate	40	6	46
Inadequate	5	49	54
Total	45	55	**100**

To evaluate the feasibility of USI-Kit to be used in field inspection of iodine content in salt. 500 edible salt samples were randomly collected from 8 different districts of Chiang Mai Province, located either in an urban and rural area, and the test of iodine content was performed by multiple users. The same salt samples were brought back to the Nutrition Analysis Laboratory at Chiang Mai University to assessed iodine content using the spectrophotometric method as described in **Materials and methods**. The results showed in **Table 5** responsible for quite a similar level of efficacy of USI-Kit, expressed as the value of relative accuracy (85.4 %), relative sensitivity (80.1 %), relative specificity (89.3 %), false

positive rate (10.7 %) and false negative rate (19.9 %). The substantial strength of agreement to the spectrophotometric method indicated by means of Cohen's κ coefficient value of 0.70.

Table 5 Comparison of USI-Kit versus the spectrophotometric method for the determination of iodine content in salt by multiple-users.

USI-Kit	Spectrophotometric method		Total
	Adequate (20 - 40 ppm)	**Inadequate** (< 20 or > 40 ppm)	
Adequate	169	31	200
Inadequate	42	258	300
Total	211	289	**500**

Conclusions

Newly developed, USI-Kit is eligible to be used as a field test kit for determination of iodine content in salt. The kit gains acceptable accuracy, sensitivity, and specificity with a range of more than 80 %. The efficacy of the kit is comparable to the spectrophotometric method to identify "adequate" or "inadequate" iodine content in the salt. A false negative value, however, was rather high (about 20 %) that means USI-Kit possibly give underestimate iodine content in salt. Improvement of sensitivity may consecutively increase its accuracy and precision. Furthermore, errors may be introduced by salts of a large granular size that not completely dissolved in the reaction chamber. Thus, grounding the salt before the test should be recommended. More comprehensive field tests would be valuable before making the kit commercially available.

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