การตกตะกอนโปรตีนเพื่อการเตรียมตัวอย่างสำหรับการตรวจวัดปริมาณไอโอดีนในไข่ด้วยวิธีโฟลว์อินเจกชันแซนเดลล์-คอลทอฟฟ์

นายทศพร ศรีวรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2558

PRECIPITATION OF PROTEINS AS SAMPLE PREPARATION FOR DETERMINATION OF IODINE CONTENT IN EGGS BY SANDELL-KOLTHOFF FLOW INJECTION METHOD

Mr. Todsaporn Srivorakul

A Thesis Submitted in Partial Fulfillment of the Requirements
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ทศพร ศรีวรกุล : การตกตะกอนโปรตีนเพื่อการเตรียมตัวอย่างสำหรับการตรวจวัดปริมาณไอโอดีนในไข่ด้วยวิธีโฟลว์อินเจกชันแซนเดลล์-คอลทอฟฟ์ (PRECIPITATION OF PROTEINS AS SAMPLE PREPARATION FOR DETERMINATION OF IODINE CONTENT IN EGGS BY SANDELL-KOLTHOFF FLOW INJECTION METHOD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ปกรณ์ วรานุศุภากุล, 63 หน้า.

งานวิจัยนี้เป็นการพัฒนาวิธีการเตรียมตัวอย่างสำหรับตรวจวัดปริมาณไอโอดีนในไข่โดยใช้วิธีการตกตะกอนโปรตีน วิธีการตกตะกอนโปรตีนที่ใช้ในการศึกษาในงานวิจัยนี้ได้แก่ การตกตะกอนโปรตีนที่พีเอชต่างๆ การตกตะกอนโปรตีนด้วยเกลือความเข้มข้นสูง การตกตะกอนโปรตีนด้วยโซเดียมโดเดซิลซัลเฟตและการตกตะกอนโปรตีนด้วยกรดไตรคลอโรแอซีติก หลังจากนั้นตรวจวัดไอโอดีนโดยใช้เทคนิคการตรวจวัดเชิงสีด้วยปฏิกิริยาแซนเดลล์-คอลทอฟฟ์ จากการสังเกตลักษณะทางกายภาพของตัวอย่างไข่หลังผ่านวิธีการเตรียมตัวอย่าง พบว่าตัวอย่างไข่ที่ผ่านวิธีการตกตะกอนโปรตีนด้วยโซเดียมโดเดซิลซัลเฟตและกรดไตรคลอโรแอซีติกได้สารละลายใสไม่มีสีและสามารถกรองผ่านกระดาษกรองที่มีเส้นผ่านศูนย์กลางเท่ากับ 0.45 ไมโครเมตรได้ ด้วยเหตุนี้ตัวอย่างที่ผ่านวิธีการเตรียมตัวอย่างทั้งสองวิธีจึงได้นำมาศึกษาปริมาณไอโอดีนโดยใช้เทคนิคการตรวจวัดเชิงสีด้วยปฏิกิริยาแซนเดลล์-คอลทอฟฟ์ พบว่าวิธีตกตะกอนโปรตีนด้วยกรดไตรคลอโรแอซีติก สามารถกำจัดตัวกลางที่รบกวนการวิเคราะห์ไอโอดีนในตัวอย่างได้ดีที่สุด จึงได้ศึกษาภาวะที่เหมาะสมในการเตรียมตัวอย่างด้วยกรดไตรคลอโรแอซีติกโดยศึกษาความเข้มข้นของกรดไตรคลอโรแอซีติก เวลาและอุณหภูมิ พบว่าที่ ปริมาตร 400 ไมโครลิตรของ 1 กรัมต่อมิลลิลิตรของกรดไตรคลอโรแอซีติก ต่อตัวอย่างไข่ 0.5 กรัม ที่อุณหภูมิห้องเป็นภาวะที่เหมาะสมที่สุดสำหรับตรวจวัดไอโอดีนในตัวอย่างไข่ โดยมีค่าร้อยละการคืนกลับอยู่ในช่วง 84-109 ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ ต่ำกว่า 6.4 เปอร์เซ็นต์ ขีดจำกัดการตรวจวัดของวิธีเท่ากับ 0.58 ไมโครกรัมไอโอดีนต่อกรัมไข่และขีดจำกัดการหาปริมาณของวิธีเท่ากับ 0.94 ไมโครกรัมไอโอดีนต่อกรัมไข่ ซึ่งวิธีนี้ถูกยืนยันให้มีความน่าเชื่อถือโดยการเปรียบเทียบกับการเตรียมตัวอย่างด้วยวิธีเผาให้เป็นขี้เถ้าที่อุณหภูมิสูง (Alkali dry ashing)

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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TODSAPORN SRIVORAKUL: PRECIPITATION OF PROTEINS AS SAMPLE PREPARATION FOR DETERMINATION OF IODINE CONTENT IN EGGS BY SANDELL-KOLTHOFF FLOW INJECTION METHOD. ADVISOR: ASST. PROF. PAKORN VARANUSUPAKUL, Ph.D., 63 pp.

In this research, the sample preparation methods based on protein precipitation were investigated and developed for determination of iodine in egg samples. The protein precipitation methods such as adjusting pH, salting out at high salt concentration, sodium dodecyl sulfate (SDS) and trichlroacitic acid (TCA) were studied. After treated, iodine was determined by colorimetric method based on Sandell and Kolthoff reaction. According to the physical observation of treated egg samples, treatment method using SDS and TCA could satisfactorily remove most of egg matrix components yielding colorless solution. According to the signal of iodine obtained by the colorimetric flow analysis method, the treatment method using TCA was the most effective egg matrix removal method for determination of iodine in egg samples. A 400 µg of 1 g/mL TCA at room temperature was used for 0.5 g egg sample. The concentration of TCA, incubation time and temperature for egg matrix removal were optimized. The recoveries of spiked iodine in egg samples after treated with TCA were in the range of 84-109%, relative standard deviation less than 6.4%, method detection limit was 0.58 µg iodine/g egg and method quantitation limit was 0.94 µg iodine/g egg. The method provided more reliable results compared to the conventional alkali dry ashing method.

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
Academic Year:	2015		

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
MDL	method detection limit
MQL	method quantitation limit
LOD	limit of detections
LOQ	limit of quantitation
M	molar
mg/L	milligram per liter
µg/L	microgram per liter
R^2	correlation coefficient
rpm	revolutions per minute
RSD.	relative standard deviation
UV-Vis	ultraviolet-visible
WHO	World Health Organization

CHAPTER I
INTRODUCTION

1.1 Problem definition

Iodine is an important element in human nutrition. It is a well known component of thyroid hormone that plays an important role for brain development, metabolic processes and several organ functions. Generally, the amount of iodine daily intake for adult is 150 μg of iodine per day recommended by WHO. People usually obtain iodine from iodine rich-food, especially sea food. Nevertheless, people in some regions in Thailand that are located far away from the sea such as the northern and north-eastern regions of Thailand, might not have efficient access to such foods. Consequently, those people might suffer from insufficient iodine intake or so called Iodine Deficiency Disorders (IDD) problem that leads to goiter, cretinism and hypothyroidism [1, 2].

In prevention of Iodine deficiency disorders, iodine fortified food products are available such as iodized table, milk and egg. Iodine fortified egg is interesting iodine fortified food because egg is a simple food, a source of protein and easily accessible to lots of people in all areas. In order to control the quality and provide information about iodine content in iodine fortified egg, development of a method for determination of iodine content in egg is important and necessary.

There are several analytical methods for determination of iodine such as ICP-MS, GC-MS including a colorimetric method that is simply measured by a spectrophotometer. Colorimetric methods based on Sandell and Kolthoff reaction is the most sensitive and simple method for determination of iodine, where the yellow color of Ce(VI) is reduced by As(III) to the colorless Ce(III) catalyzed by a quantity of iodide. Recently, the methods has been modified for being used in flow injection system [3]. The method is easy and provides high sample throughput.

Quantitative analysis of iodine in food requires sample preparation steps for destruction of organic matters and isolation of iodine into an appropriate solution prior to determination. Sample preparation is an important step for analytical process as well as bottleneck in several analytical methodologies. In addition, sample preparation is a directly effect to accuracy, precision analytical performance. Several sample preparation methods, have been utilized for determination of iodine in food samples such as alkali dry ashing and alkali extraction method using tetramethyl ammonium hydroxide (TMAH). These methods consist of several steps, use high temperature or harsh conditions and take a long preparation time. For these reasons, development of a simple, fast, inexpensive and environment friendly sample preparation method for determination of iodine in food is our interest.

Since the matrix of eggs mainly consists of proteins, a method of removal of proteins such as methods of protein precipitation have been explored. Protein precipitation methods have been normally used in proteomic methodologies, which can easily remove proteins in mild condition with fewer steps. Protein precipitation methods such as protein precipitation at isoelectric point, salting out at high salt concentration, alkaline lysis by sodium dodecyl sulfate (SDS) and trichloroacitic acid (TCA) are investigated for removal of egg matrices and keeping the iodine remaining in the solution for further analysis.

1.2 Literature review

The most widely used sample preparation technique for determination of iodine in food sample is alkali dry ashing [3-6]. In this technique, the egg matrices are incinerated into ashes while the iodine still remains in an alkali solution. Potassium carbonate and zinc sulfate as ashing agent, are added into the sample. The mixture is dried on hotplate and incinerated into ashes in a high temperature furnace oven. Then, the ashes are dissolved in deionized water and centrifuged for removal of suspensions.

The solution is determined for iodine content by a colorimetric methods. In this sample preparation technique, matrix in sample was destructed and iodine was liberated for analysis. Although this technique can remove most of organic matters but disadvantages of this technique are used of several steps, use of high temperature and taking a long preparation time. For these reasons, there are risks of losing iodine being contaminated during those sample preparation steps.

The method of alkali extraction has been used for extraction of iodine from food samples such as sea fish and milk [6-8]. Tetramethylammonium hydroxide (TMAH) was used as a strong alkali agent. TMAH was added to the sample and placed in a dry oven at 90 °C for 3 hours. Then the sample was extracted with deionized water. The solution was determined for iodine contents by inductively coupled plasma-mass spectrometry (ICP-MS). Although this technique is simple and fast but cannot ensure 100% iodine extraction.

SchÖnger combustion was another method for sample preparation for determination of iodine in food samples [4, 8]. The sample was combusted with an oxygen in a closed system at atmosphere. A volatile iodine was collected in sodium hydroxide. The solution was determined for iodine content with ICP-MS. The disadvantages of this method were several steps, using high temperature and taking a long preparation time. A 250 mL combustion flask could be used for a maximum of 50 mg of sample. Limit of detection is limited by sample size. Combustion of sample in an oxygen stream is the sample preparation for determination of total iodine in nutrition sample [7, 9]. This methods was used for solving these problem. The biological sample was pellet and placed on a sample holder, to be burned by infrared radiators in oxygen stream. The residue of non-volatile elements was remained in the chamber and volatile elements was condensed against the walls of the overlying cooling unit. Once the combustion was completed, the sample holder was turned upside down to lay at the bottom of the underlying test tube and a 2 mL of nitric acid was added. Infrared radiators were used to boil the reagent under the reflux for 30

min. After that, volatile and non-volatile elements were collected for determination by ICP-MS. The disadvantage of this technique is the use of unique device that is not available in common laboratory.

Microwave-assisted digestion by nitric acid and perchloric acid mixtures has been used for decomposition of sample matrices and oxidation of iodine to non-volatile iodate [10]. Sample was digested with HNO_3/$HClO_4$ mixture 45 min and diluted with deionized water. Sample was determined by ICP-MS. Although microwave-assisted digestion can completely decompose the sample matrices but the use of acid mixture and the microwave must be operated with cares.

Protein precipitation methods have been normally used in proteomic methodologies. The quality of protein precipitation depends on the properties of samples. For this reason, various protein precipitation methods were compared for pre-treatment of sample. Organic solvent [11, 12], Salting out at high salt concentration [13, 14] and Trichloroacitc acid (TCA) protein precipitation [15-18] were studied. Proteins could be precipitated at low temperature (less than 4°C) and be removed by centrifugation at high round per minute (higher than 10,000 rpm for 10-30 min). The proteins were separated from other matrices and determined by electrophoresis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, alkaline lysis method using sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) solution has been used for cell debris precipitation in plasmid extraction. The SDS and sodium hydroxide (NaOH) solution can solubilize cell membrane and break down cell wall of bacterias. SDS can also denature most of proteins in the cells. Acetate buffer was used for neutralization charged proteins and genomic DNA by bringing the pH to the normal.

The methods of protein precipitation are interesting for sample preparation for determination of iodine in the egg because the matrix of eggs mainly consists of proteins [19-22].

1.3 Scope of this research

In this research, the sample preparation methods based on protein precipitation were developed for determination of iodine in egg samples. The methods such as adjusting pH, salting out at high salt concentration, sodium dodecyl sulfate (SDS) and trichlroacitic acid (TCA) were studied. After treated, iodine was determined by colorimetric method based on Sandell and Kolthoff reaction.

CHAPTER II
THEORY

2.1 Composition of chicken egg

Eggs laid by female animals of many different species, including birds and reptiles, have been eaten by humans for thousands of years. The most popular choice for egg consumption is chicken egg. The chicken egg consists of a protective eggshell, albumen (egg white) and vitellus (egg yolk) contained within a thin membranes. The egg compositions that are important for human consumption are egg yolk and egg white.

2.1.1 Egg yolk

The yolk makes up a 36% of the egg weight. The composition of egg yolk is shown in Table. 2.1: The main components are lipids (62.5% dry matter) and proteins (lipid to protein ratio is 2:1)

Table 2.1 Composition of chicken egg yolk [23]

	Dry yolk (%)
Lipids	62.5
Proteins	33.0
Carbohydrates	1.0
Minerals	3.5

Lipids of egg yolk are associated with lipoproteins, which are 62% triglycerides, 35% phospholipids and less than 5% cholesterol. Proteins are present as free proteins or apoproteins (proteins binding with lipids). Apoproteins are the main constituents of egg yolk, which are attributed to 68% low-density lipoproteins (LDL), 16% high-density lipoproteins (HDL), 10% globular proteins (livetins), 4% phosphoprotein (phosvitin) and 2% minor proteins.

2.1.2 Egg white

The egg white takes up a 58% of the egg weight. The egg white protects the yolk and provides additional nutrition to the growth embryo. Dry matter of egg white primarily consists of proteins. The composition of protein in eggs white is shown in Table. 2.2

Table 2.2 Composition of proteins in egg white [23]

Protein	Dry egg white (%)	Isoelectric point (pI)
Ovalbumin	54	4.5
Conalbumin	13	6.6
Ovomucoid	1.2	3.9-4.3
Lysosome	3.5	10.7

2.2 Techniques for protein precipitation

Generally, the surface of protein molecules consists of hydrophobic and hydrophilic patches as shown in Figure 2.1. These patches are important for solubility of protein. The solubility of protein is mainly based on protein-water interaction and protein-protein interaction. For this reason, conditions that increase protein-protein interaction or decrease protein-water interaction could cause protein to aggregate and precipitate. The techniques for protein precipitation can be divided into non-denature protein technique and denature protein technique.

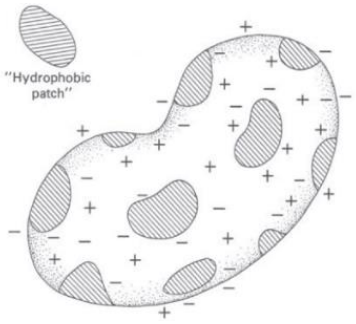

Figure 2.1 Distribution of charge and hydrophobic patches on protein molecule [12]

2.2.1 Non-denatured protein techniques

2.2.1.1 Protein precipitation by isoelectric point (I.E.P)

The isoelectric point, is the pH at which amino acid molecule net charge is zero. When amino acid are in solution at the pH corresponds to their isoelectric points, protein-protein interaction increases; the solubility of protein would be decreased; subsequently, proteins would be aggregated and precipitated out of the solution. Figure 2.2 shows protein precipitation when pH is close to its isoelectric point. Proteins are divided into 2 categories based on their isoelectric points that are basic proteins having isoelectric point higher than pH 7 and acidic proteins having isoelectric point lower than pH 7. For these reasons, information of protein components and their isoelectric points are important for protein precipitation.

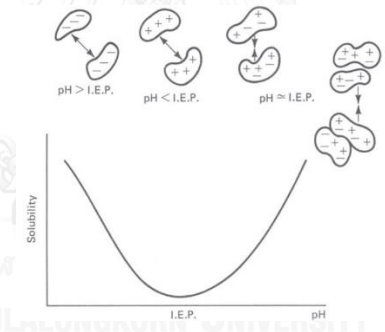

Figure 2.2 Solubility of protein at pH around isoelectric point [12]

2.2.1.2 Protein precipitation by salting out at high salt concentration

Figure 2.3 shows water molecule and hydrophobic patch on protein molecule in a normal condition where soluble proteins are surrounded by water molecules. Addition of salt at high concentration would pull off the water molecules around the protein molecules decreasing protein-water interaction resulting to that protein-protein interactions between hydrophobic patch on protein surfaces are increased.

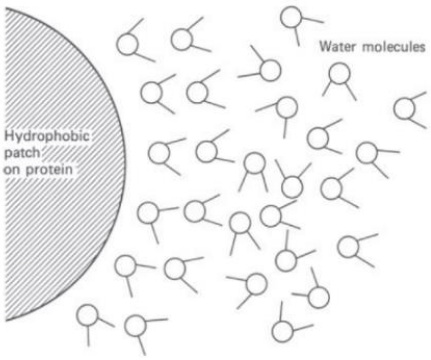

Figure 2.3 Water molecules around hydrophobic patch on the surface of a protein [12]

The important properties and characteristics of salts to be considered for salting out are the solubility of salts and the salting out ability of charged ions. The solubility of salt in water depends on number of molecule of salt ions and salting out ability of salt. This effect is described in Hofmeister series [14].

Hofmeister series for anions:

$$PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > ClO_4^- > I^- > SCN^-$$

Hofmeister series for cations:

$$NH_4^+ > Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$

For these conditions, ammonium sulfate ($(NH_4)_2SO_4$) is often used for salting out because of its high solubility and high salting out ability.

2.2.1.3 Protein precipitation by organic solvent

Addition of organic solvent such as ethanol and acetone is another way that leads to protein precipitation. Acetone and ethanol are widely used for protein precipitation because they are miscible with water. The organic solvent would reduce the dielectric constant of water so that the protein-water interactions decrease and the protein-protein interaction are increased. The effect of organic solvent for protein precipitation is shown in Figure 2.4

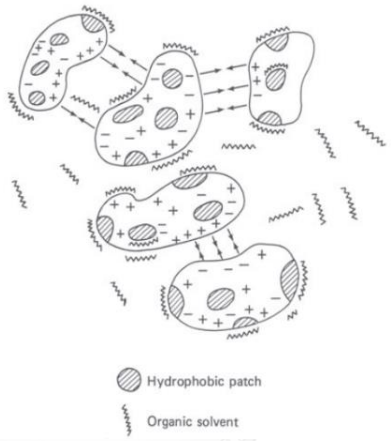

Figure 2.4 Aggregation of protein by protein precipitation by organic solvent [12]

2.2.2 Denatured protein techniques

2.2.2.1 Protein precipitation by sodium dodecyl sulfate (SDS) and acetate buffer

Protein precipitation by sodium dodecyl sulfate (SDS) and acetate buffer is a part of alkali lysis technique in plasmid isolation. SDS is anionic surfactant. When applied to proteins, SDS could unfold folding proteins making negatively charged unfold proteins as depicted in Figure 2.5. Acetate buffer is added to neutralize the charged proteins, causing them to aggregation and precipitation.

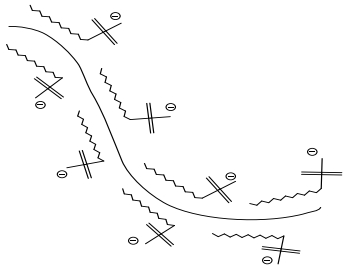

Figure 2.5 physical property of protein molecule after treated with SDS [22]

2.2.2.2 Protein precipitation by Trichloroacitic acid (TCA)

TCA has been widely used as protein precipitating agent in proteomic methodology. Despite this technique is widely used, the principle of TCA precipitation remains elusive and unclear. However, it might be simply described that the negatively charged trichloroacetate ions might have disrupted protein-protein interaction making folding proteins unfolded. Unfolded proteins can expose nonpolar surfaces and interact with other nonpolar surface proteins [15-18]. Resulting to aggregation and precipitation.

2.3 Colorimetric method for determination of iodine based on Sandell and Kolthoff reaction

There are several analytical methods for determination of iodine including a colorimetric method that is simply measured by a spectrophotometer. Colorimetric methods based on Sandell and Kolthoff reaction is the most sensitive and simple method for determination of iodine, where the yellow color of ceric ammonium sulfate (Ce(VI)) is reduced by arsenious acid (As(III)) to the colorless Ce(III) catalyzed by a quantity of iodide. This reaction has been described to be a psudo-first order reaction that depends on the concentration of Ce(VI) solution and iodine solution is catalyst [24]. For this reason, when increase the iodine concentration, reducing of yellow of

Ce(VI) to colorless of Ce(III) were increased [3]. This reaction was shown in Equation 2.1.

$$2Ce^{4+} + As^{3+} \xrightarrow{I^-} 2Ce^{3+} + As^{5+} \qquad \textbf{Equation 2.1}$$

$$2Ce^{4+} + 2I^- \longrightarrow 2Ce^{3+} + I_2$$
$$As^{3+} + I_2 \longrightarrow As^{5+} + 2I^-$$

(IO_3^- to I^- : $IO_3^- + 6H^+ + 3As^{3+} \longrightarrow 3As^{5+} + I^- + 3H_2O$)

A pseudo-first order reaction is reaction that depended on second order but rate of reaction can be approximated to first order. Because one of reactant is very large concentration. When concentration of Ce^{4+} is very higher than the concentration of As^{3+}. The rate of reaction is depend on concentration of Ce^{4+} and could be consider to be first order reaction [25].

2.4 Flow injection analysis

Flow injection analysis (FIA) is an automatic method of chemical analysis. The diagram of basic FIA is shown in Figure 2.6. The liquid sample is injected into a carrier stream and mixed with reagent and transported toward a detector and detected.

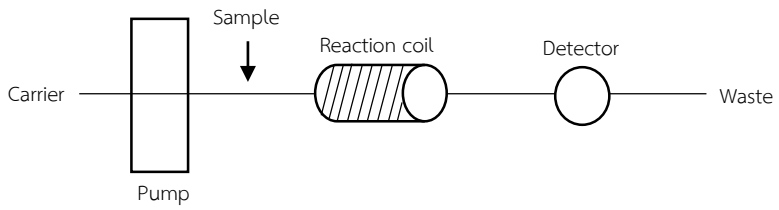

Figure 2.6 Diagram of basic FIA [26]

The typical recorded output is the form of peak height as shown in Figure 2.7. The height (H), width (W) and area (A) are related to the concentration of the analyte. The time between each sample injection and the peak maximum is the residence time during which the chemical reaction taking place. FIA is simple, can be operated automatically, provides high sampling rate and uses small amount of sample and reagent [26].

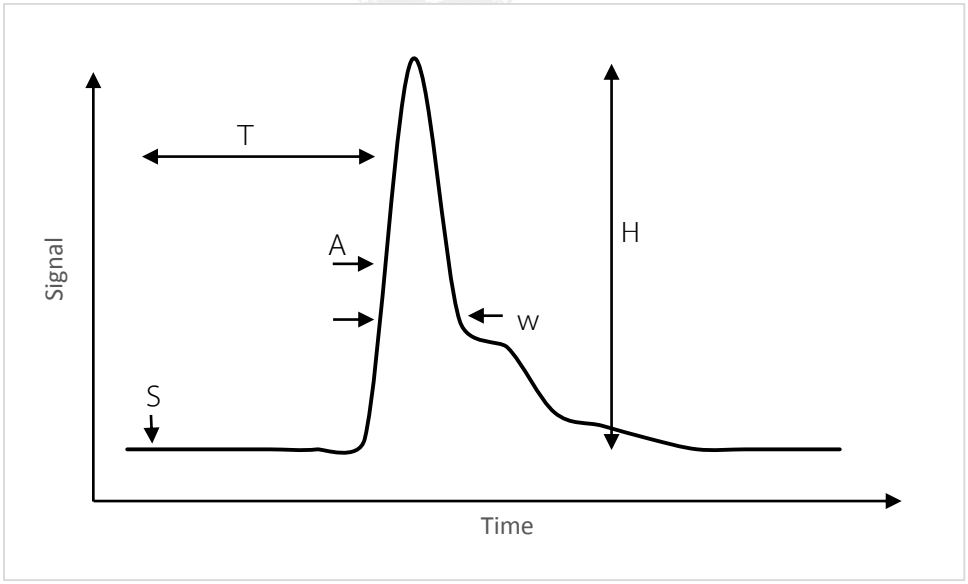

Figure 2.7 Typical output form of a peak, time of injection (T), peak height (H), peak width (W), peak area (A) [26]

CHAPTER III

EXPERIMENTAL

3.1 Instruments and Equipments

Fiber optic, UV-visible spectrophotometer, (Avantes)

Peristaltic pump (Ismatic)

Centrifuge (Hettich UNIVERSAL 320 R)

Furnace (Nabertherm)

3.2 Chemicals and Reagents

Trichloro acetic Acid (Carlo-Erba)

Ammonium Sulphate AR (Carlo-Erba)

Zinc sulphate heptahydrate (Carlo-Erba)

Potassium iodate (Carlo-Erba)

Sodium chloride (Carlo-Erba)

Potassium acetate (Carlo-Erba)

Hydrochloric acid (MERCK)

Sodium hydroxide (MERCK)

Acetic acid (MERCK)

Sulfuric acid (MERCK)

Potassium carbonate (Ajax)

Sodium dodecyl sulfate (SIGMA-ALDRICH)

Ceric sulfate (A&M)

Arsenious oxide (A&M)

Phenol (MERCK)

Chloroform (MERCK)

3.3 Egg samples

Eggs were purchased from a local supermarket and stored in a refrigerator. Five eggs were cracked and only yolk and white egg were homogenized and used as a composited egg sample.

3.4 Stock solution of standard Iodine

The stock standard iodine solution of 500 mg iodine/L was prepared by dissolving 0.2106 g of potassium iodate (KIO_3) and diluting the solution with deionized water in a 250 mL volumetric flask.

3.5 Flow analysis method for determination of iodine based on Sandell and Kolthoff reaction

3.5.1 Reagents for Sandell and Kolthoff reaction

3.5.1.1 0.008 M Ceric sulfate ($Ce(SO_4)_2$)

A 0.008 M $Ce(SO_4)_2$ was prepared by dissolving 5 g of $Ce(SO_4)_2$. H_2O in 1000 mL of 1.75 M Sulfuric acid (H_2SO_4).

3.5.1.2 0.1 M Arsenious acid (As_2O_3)

A 10 g As_2O_3 and 47 g NaCl were dissolved in 500 mL deionized water with heat in hot plate. After cooling to room temperature, 27.8 mL of concentrate H_2SO_4 was added to this solution followed by dilution with 1000 mL deionized water.

3.5.2 Set up of flow analysis method system

The configuration of the FIA system for determination of iodine based on Sandell and Kolthoff reaction has been modified and shown in Figure 3.1. A solution was injected into a continuous flowing stream of water carrier. The sample zone was mixed with 0.1 M As(III) and 0.008 M Ce(IV), respectively, The sample solution was carried to the reaction coil and to z-flow cell successively for detection at 420 nm.

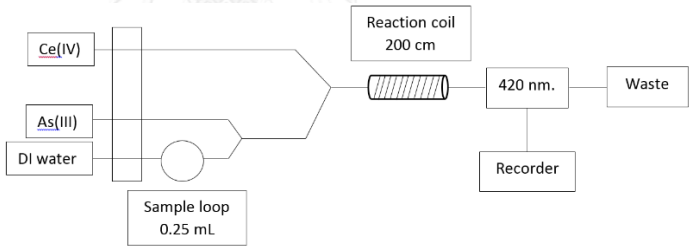

Figure 3.1 Flow analysis method based on Sandell and Kolthoff reaction system [3]

3.5.2.1 Calibration curve

A series of 25 mL of 100, 250, 500, 750, 1000 µg iodine/L standard iodine solution was used for calibration curve. The calibration curves were plotted between logarithmic absorbance and initial iodine concentrations. The linear regression method was used to obtain slope, intercept and R^2.

3.5.2.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) was defined as the concentration giving a signal of $Y_B + 3S_B$, where Y_B was blank signal and S_B was standard deviation of blank signal. The corresponding concentration was then calculated from the calibration equation. The limit of quantitation (LOQ) was the concentration giving a signal of $Y_B + 10S_B$.

3.6 Investigation of methods for egg matrix removal

3.6.1. Egg matrix removal by adjusting pH

3.6.1.1 Reagents

3.6.1.1.1 1 M Sodium hydroxide (NaOH) pH= 14

A 1 M NaOH (pH=14) was prepared by dissolving 4g of NaOH and diluting with deionized water in a 100 mL volumetric flask.

3.6.1.1.2 NaOH pH= 12, 10, 8

NaOH solution of 10^{-2}, 10^{-4}, 10^{-6} M NaOH (pH=12, 10, 8) were prepared by pipetting 1 mL of 1, 10^{-2}, 10^{-6} M NaOH and diluting with deionized water in 100 mL volumetric flasks, respectively as depicted in Figure 3.2.

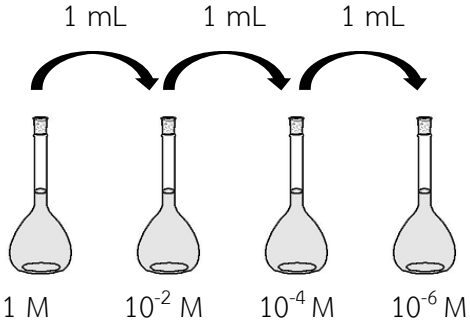

Figure 3.2 Preparation of NaOH pH 12, 10, 8

3.6.1.1.3 1M Hydrochloric acid (HCl)

A 1 M HCl was prepared by pipetting 8.84 mL of HCl and diluting with deionized water in 100 mL volumetric flask.

3.6.1.1.4 HCl pH = 2, 4, 6

HCl solution of 10^{-2}, 10^{-4}, 10^{-6} M HCl (pH=2, 4, 6) were prepared by pipetting 1 mL of 1, 10^{-2}, 10^{-6} M HCl and diluting with deionized water in 100 mL volumetric flasks respectively as describe in Figure 3.3.

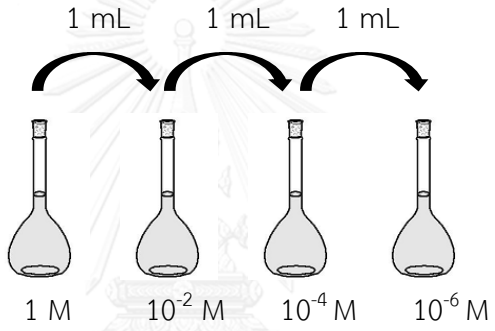

Figure 3.3 Preparation of HCl pH 2, 4, 6

3.6.1.2 Procedure

A 0.5 g of homogenized egg sample was treated with each of 10 mL of solutions with various pH. The samples were stirred and cooled down at 4 ºC for 0, 15, 30, 60, 120 min and 24 hour. The sample was centrifuged at 12,000 rpm at 4 ºC for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.2 Egg matrix removal by salting out at high salt concentration

3.6.2.1 Reagents

3.6.2.1.1 Saturated Sodium chloride (Sat. NaCl)

Sat. NaCl was prepared by dissolving 40 g of NaCl (solubility of NaCl at 25 ℃ = 35.9 g/100mL) and diluting with deionized water in a 100 mL.

3.6.2.1.2 Saturated Ammonium sulfate (Sat. $(NH_4)_2SO_4$)

Sat. $(NH_4)_2SO_4$ was prepared by dissolving 80 g of $(NH_4)_2SO_4$ (solubility of $(NH_4)_2SO_4$ at 25 ℃ = 74.4 g/100mL) and diluting with deionized water in a 100 mL.

3.6.2.2 Procedure

A 0.5 g of homogenized egg sample was treated with each of 0.5, 1, 2, 5 mL of Sat. NaCl and $(NH_4)_2SO_4$. The mixtures were agitated and cooled down at 4 ℃ for 0, 15, 30, 60, 120 min and 24 hour. The sample was diluted with 10 mL deionized water. The sample was centrifuged at 12,000 rpm at 4 ℃ for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.3 Egg matrix removal by SDS/Acetate buffer

3.6.3.1 Reagents

3.6.3.1.1 1% (w/v) Sodium dodecyl sulfate (SDS)

A 1% (w/v) SDS was prepared by dissolving 1 g of SDS and diluting with deionized water in a 100 mL volumetric flask.

3.6.3.1.2 0.2 M NaOH

A 0.2 M NaOH was prepared by dissolving 2 g of NaOH and diluting with deionized water in a 250 mL volumetric flask.

3.6.3.1.3 3 M acetate buffer

A 3 M acetate buffer was prepared by mixing 60 mL of 5 M potassium acetate and 11.5 mL of acetic acid and diluting in 100 mL of deionized water.

3.6.3.2 Procedure

A NaOH-SDS solution was freshly prepared by mixing 0.2 M NaOH with 1% (w/v) SDS (1:1). The NaOH-SDS solution was added to each of 0.5 g of homogenized egg sample in 400, 800, 1200, 1600 and 2000 mL respectively. Then, 3M acetate buffer was added in 3:4 of acetate buffer to NaOH-SDS solution. The sample was agitated and cooled down at 4 ºC for 0, 15, 30, 60, 120 min and 24 hour. The sample was diluted with 10 mL deionized water. The sample was centrifuged at 12,000 rpm at 4 ºC for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.4 Egg matrix removal by TCA

3.6.4.1 Reagent of egg matrix removal by TCA

A 1 g/mL TCA was prepared by dissolving 50 g of TCA and diluting with deionized water in 50 mL volumetric flask.

3.6.4.2 Egg matrix removal by TCA methods

A 1 g/mL TCA was added to each of 0.5 g of homogenized egg sample in 100, 200, 400, 800 µL. The solution was agitated and cooled down at 4 ºC for 15 min. The sample was diluted with 10 mL deionized water. The sample was centrifuged at 12,000 rpm at 4 ºC for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.5 Methods optimization

3.6.5.1 Egg matrix removal by SDS/Acetate buffer

A NaOH-SDS solution was freshly prepared by mixing 0.2 M NaOH with 1% (w/v) SDS (1:1). The NaOH-SDS solution was added to 0.5 g of homogenized egg in 400, 800, 1200, 1600 and 2000 mL respectively. Then, 3 M acetate buffer was added in 3:4 of acetate buffer to NaOH-SDS solution. For studied optimization of concentration acetate buffer, the NaOH-SDS solution was added to 0.5 g of homogenized egg in 800 mL. Then, 3M acetate buffer was added in 1200 and 2400 µL of acetate buffer to NaOH-SDS solution. The sample was vortex and cooled down at 4 ºC for 0, 15, 30, 60, 120 min and 24 hour. After that, Sample was diluted with 10 mL deionized water. The sample was centrifuged at 12,000 rpm at 4 ºC for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.5.2 Egg matrix removal by TCA

1 g/mL TCA was added to 0.5 g of homogenized egg in 100, 200, 400, 800 µL. The solution was vortex and cooled down at room temperature and 4 ºC for 0 and 15 min. After that, Sample was diluted with 10 mL deionized water. The sample was centrifuged at 12,000 rpm at 4 ºC for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.6.6 Alkali dry ashing method

3.6.6.1 Reagents

3.6.6.1.1 10% (w/v) Zinc sulfate ($ZnSO_4$)

A 10% $ZnSO_4$ was prepared by dissolving 17.86 g of $ZnSO_4.7H_2O$ and diluting with deionized water in 100 mL volumetric flask.

3.6.6.1.2 30% (w/v) Potassium carbonate (K_2SO_4)

A 30% K_2SO_4 was prepared by dissolving 30 g of K_2SO_4 and diluting with deionized water in 100 mL volumetric flask.

3.6.6.2 Procedure

A 0.5 g of homogenized egg was mixed with 0.5 mL of 10% (w/v) zinc sulfate and 0.5 mL of 30% (w/v) potassium carbonate in a porcelain crucible. The sample was gently heated to dryness. Then, the sample was incinerated in the furnace at 550 ºC for 1 h. Another 0.5 mL of 10% (w/v) zinc sulfate was added to the sample and incinerated again for 2 h. The white residue was dissolved in deionized water. The solution was centrifuged at 5000 rpm at 4 °C for 20 min. The supernatant was filtered with 0.45 µm syringe filter.

3.7 Comparison of performances between egg matrix removal method and alkali dry ashing method for determination of iodine in egg samples

Five different egg samples were prepared by egg matrix removal method and alkali dry ashing method. The recovery of spiked iodine and relative standard deviation were determined and compared.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Methods for determination of iodine by flow analysis methods based on Sandell and Kolthoff reaction

4.1.1 Relationship between the absorbance of Ce(IV) and the concentration of iodine

Colorimetric methods based on Sandell and Kolthoff reaction is a simple method for determination of iodine. The yellow color of Ce(VI) is reduced by As(III) to the colorless Ce(III) catalyzed by a quantity of iodine. The signal of iodine are shown in Figure 4.1.

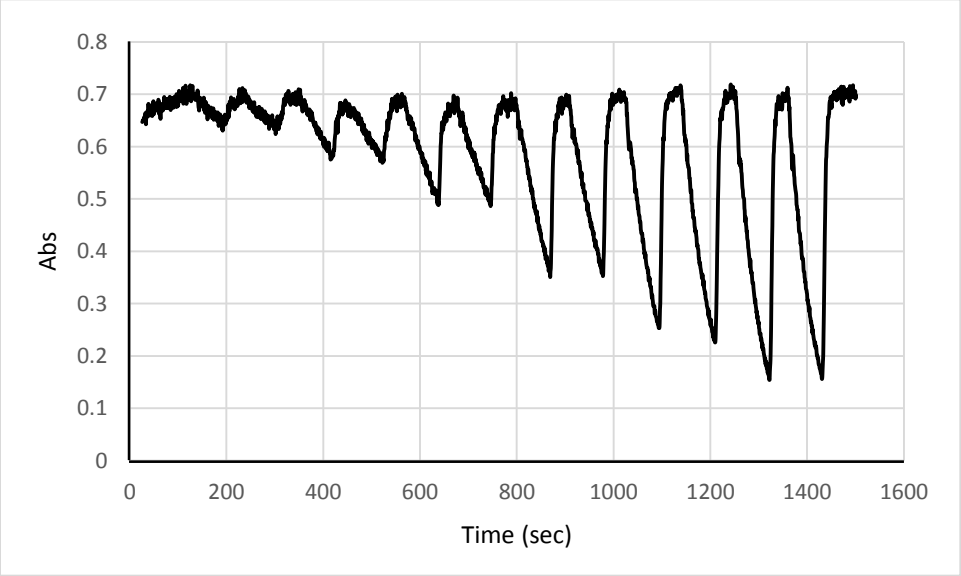

Figure 4.1 Signals of iodine obtained by flow analysis method based on Sandell and Kolthoff reaction

The relationship between the absorbance and the concentration of iodine based on Sandell and Kolthoff reaction has been described to be a pseudo-first order reaction. Since our method was modified from Nacapricha, D. et al in 2001, the

relationship between the absorbance and the concentration of iodine was verified as shown in Figure 4.2. The relationship was exponential, which confirmed that the reaction was pseudo-first order To obtain a linear calibration curve, natural logarithmic absorbance of Ce(IV) was applied. The linearity of this method was obtained for the concentration of iodine ranging from 0–1000 µgI/L shown in Figure 4.3. The coefficient of determination (R^2) > 0.999 was achieved.

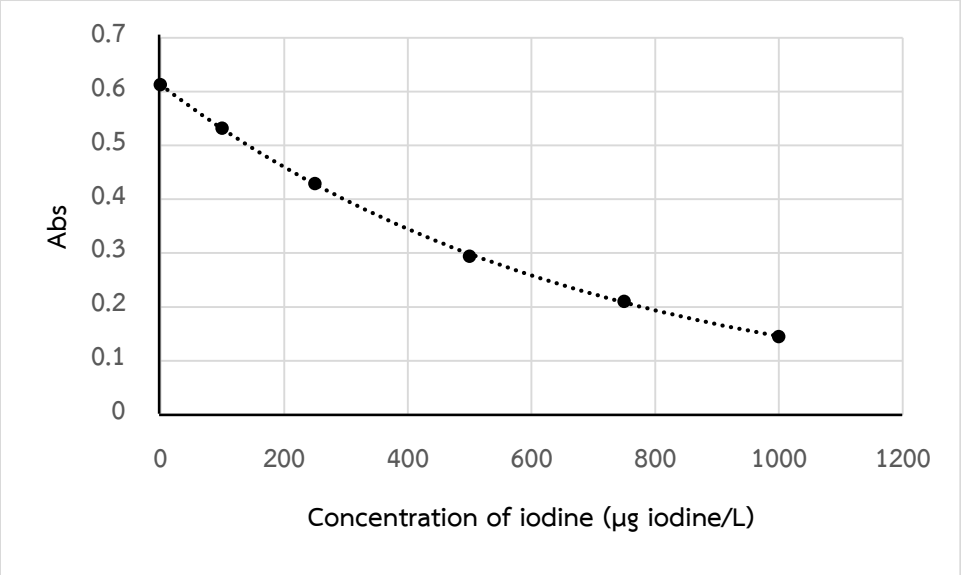

Figure 4.2 The relationship between the absorbance of Ce(IV) and the concentration of iodine obtained by flow analysis method based on Sandell and Kolthoff reaction

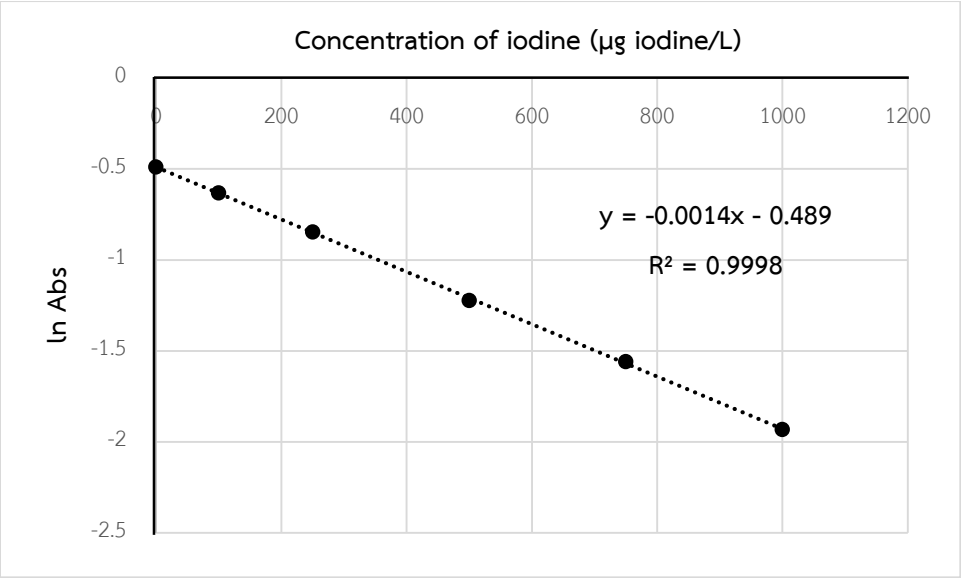

Figure 4.3 The relationship between the natural logarithmic absorbance of Ce(IV) and the concentration of iodine obtained by flow analysis method based on Sandell and Kolthoff reaction

4.1.2 Limit of detection (LOD) and limit of quantitation (LOQ) for the determination of iodine by flow analysis method based on Sandell and Kolthoff

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the concentration of iodine giving a signal of $Y_B + 3S_B$ and $Y_B + 10S_B$, respectively. The average of blank signal (Y_B) and standard deviations of blank signals (S_B) were obtained from 50 replicate analysis of deionized water blanks. The results showed that the limit of detection (LOD) was 29.2 µg iodine/L and limit of quantitation (LOQ) was 47.0 µg iodine/L.

4.2 Investigation of methods for egg matrix removal

4.2.1 Egg matrix removal by adjusting pH

In this study, 10 mL of acidic solutions of HCl and basic solutions of NaOH at various pH ranging from 1-14 were added into egg samples. The samples were

incubated at 4 ℃ for 15, 30, 45, 60, 120 min and 24 hour. After that, the samples were centrifuged at 12,000 rpm at 4 ℃ for 20 min and filtered with 0.45 µm syringe filter. The results are shown in Figure 4.4. The solutions were yellow and could not be filtrated through a 0.45 µm syringe filter. Therefore, adjusting pH was not suitable for egg matrix removal for determination of iodine by colorimetric flow analysis method.

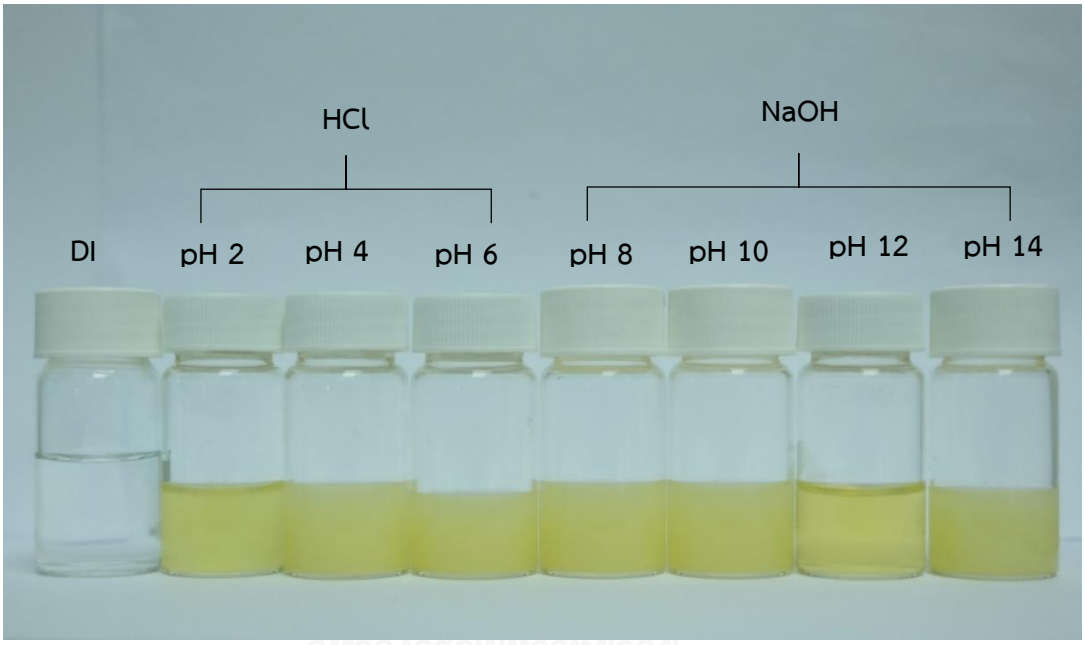

Figure 4.4 Physical observation of egg samples after treated by adjusting pH

4.2.2 Egg matrix removal by salting out at high salt concentration

In this study, saturated of ammonium sulfate ($(NH_4)_2SO_4$) and sodium chloride at various volumes ranging from 0.5-5 mL were added into egg samples. The samples were incubated at 4 ℃ for 15, 30, 45, 60, 120 min and 24 hour. After that, the samples were centrifuged at 12,000 rpm at 4 ℃ for 20 min and filtered with 0.45 µm syringe filter. The results are shown in Figure 4.5. The solutions were still yellow and could not be filtrated through a 0.45 µm syringe filter. Therefore, salting out at high salt concentration was not suitable for egg matrix removal for determination of iodine by colorimetric flow analysis method.

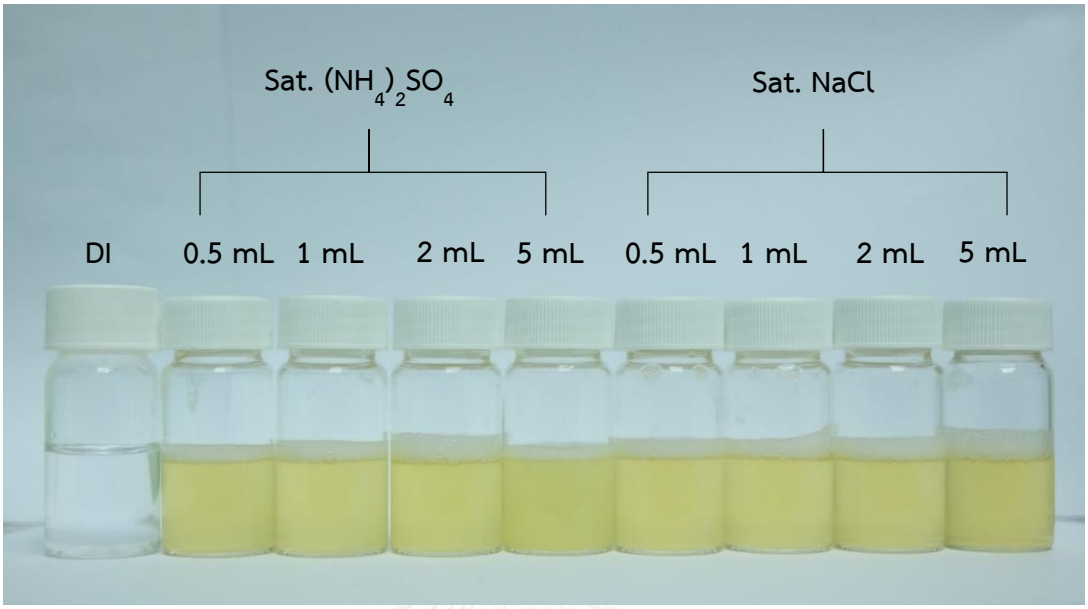

Figure 4.5 Physical observation of egg samples after treated by salting out at high salt concentration

4.2.3 Egg matrix removal by SDS/acetated buffer

In this study, the NaOH-SDS solution (1:1 ratio (v/v)) with various volumes of 400-2000 µL were added to 0.5 g of homogenized egg samples. The samples were incubated at 4 °C for 15, 30, 45, 60, 120 min and 24 hour. Then, 3M acetate buffer was added in 3:4 of acetate buffer to NaOH-SDS solution. After that, the samples were centrifuged at 12,000 rpm at 4 °C for 20 min and filtered with 0.45 µm syringe filter. As shown in Figure 4.6, the treated egg samples were colorless and filterable. Treatment with SDS could effectively remove most of matrix components so that it would be proceeded to the determination iodine by colorimetric flow analysis.

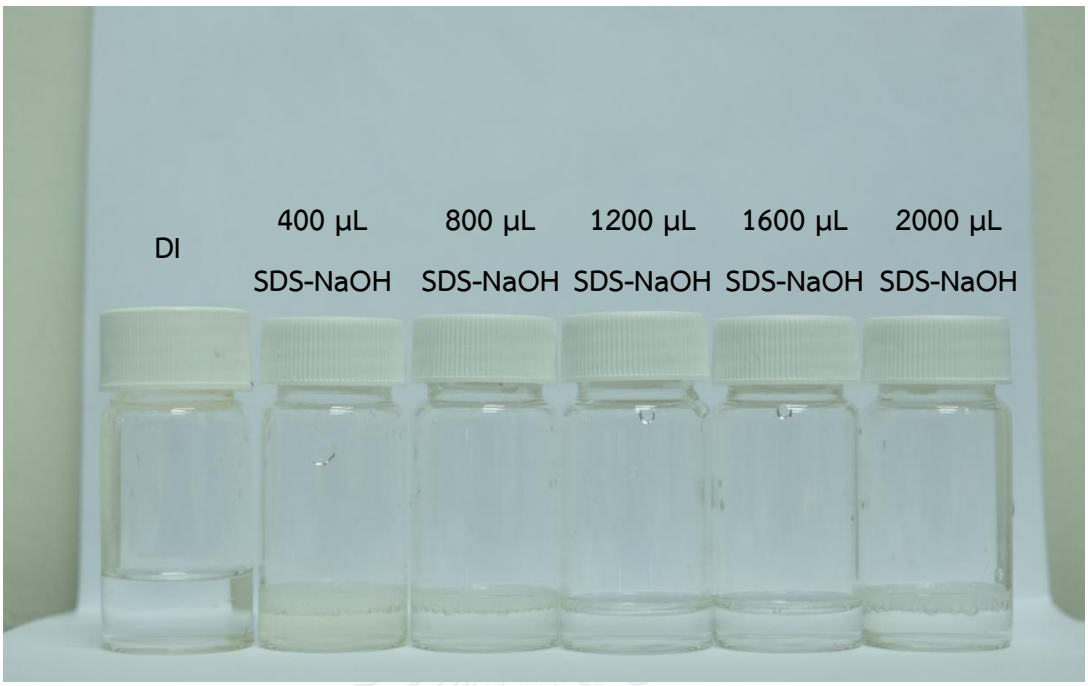

Figure 4.6 Physical observation of egg samples after treated by SDS/acetate buffer

4.2.4 Egg matrix removal by TCA

Various volumes of 1 g/mL TCA of 100-800 µL were added to 0.5 g of homogenized egg samples. The samples were incubated at 4 °C for 15, 30, 45, 60, 120 min and 24 hour After that, the samples were centrifuged at 12,000 rpm at 4 °C for 20 min and filtered with 0.45 µm syringe filter. As shown in Figure 4.7, the treated egg samples were colorless and filterable. Treatment with TCA could remove most of matrix components so that it would be proceeded to the determination iodine by colorimetric flow analysis.

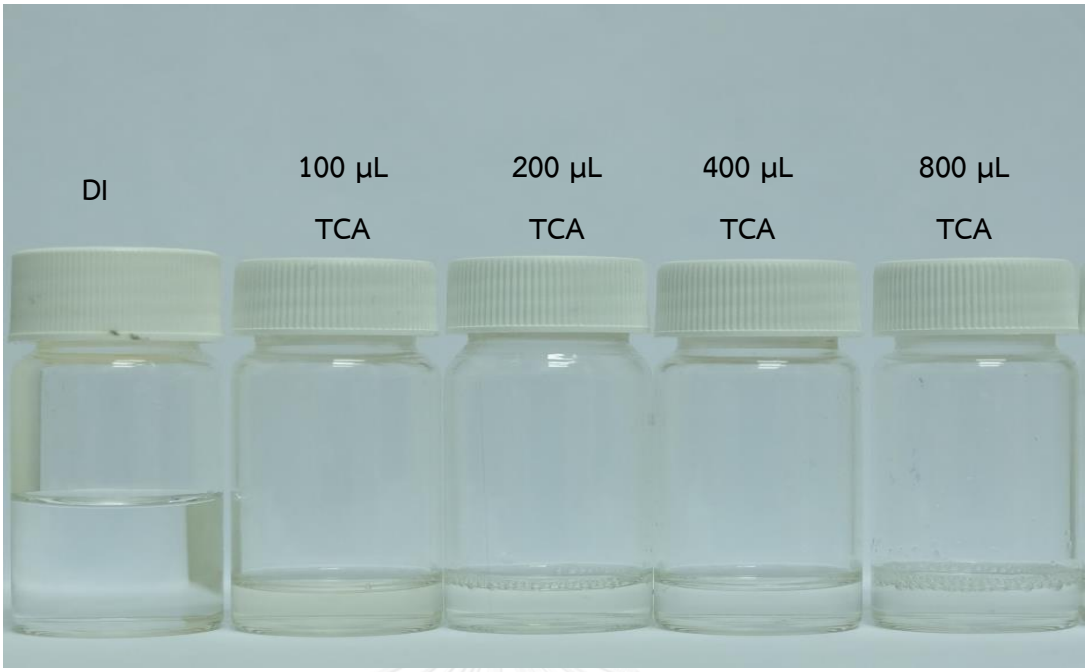

Figure 4.7 Physical observation of egg samples after treated by TCA

4.3 Determination of iodine in the treated egg samples

In this studied, eggs sample after treated with SDS and TCA were determined for iodine by flow analysis methods based on Sandell and Kolthoff reaction because these methods could effectively remove most of matrix components yielding colorless solution.

Figure 4.8 showed signals obtained from egg samples after treated by SDS. Signal (1) and (3) showed that there were no interferences from the reagents. It suggested that the treatment method with SDS/acetate buffer did not alter the signals of iodine or there was no loss of iodine during the treatment. But the shapes of signal (5) and (6) obtained from treated egg samples were not as good as the signals obtained from aqueous solution samples. It might suggest that there were interferences from egg matrices remaining in the solution.

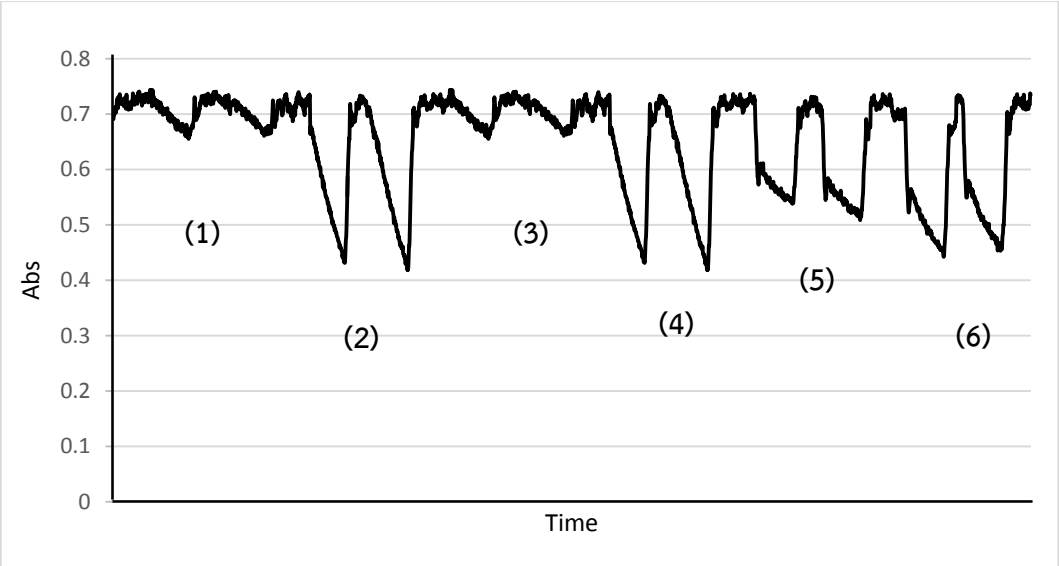

Figure 4.8 Signals of iodine obtained from (1) DI water, (2) 500 µgI/L KIO_3, (3) DI water after treated with SDS, (4) spiked 500 µgI/L KIO_3 after treated with SDS, (5) egg sample after treated with SDSr and (6) spiked egg sample (500 µgI/L KIO_3) after treated with SDS

In addition, the concentrations of acetate buffer were varied. The amounts of acetate buffer were varied from 600, 1200, 2400 µL (SDS-NaOH solution: acetate buffer is 4:3, 2:3, 1:3 ratio). The results showed that varied amounts of acetate buffer did not improve the quality of the signals obtained from treated egg samples.

Determination of iodine in the egg samples treated with TCA was shown in Figure 4.9. Signal (1) and (3) showed that there were no interferences from the reagents. It suggested that the treatment method with TCA did not alter the signals of iodine or there was no loss of iodine during the treatment. The shape of signal (5) and (6) obtained from treated egg samples were as good as the signals obtained from aqueous solution samples. The results show that TCA can effectively remove matrix component so that it is suitable for further determination of iodine.

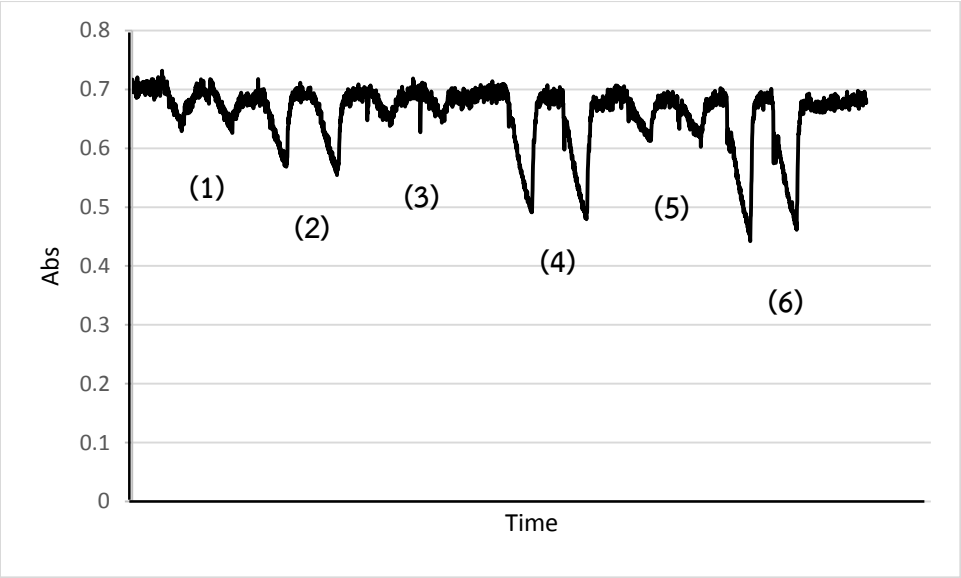

Figure 4.9 Signals of iodine obtained from (1) DI water, (2) 500 µgI/L KIO_3, (3) DI water after treated with TCA, (4) spiked 500 µgI/L KIO_3 after treated with TCA, (5) egg sample after treated with TCA and (6) spiked egg sample (500 µgI/L KIO_3) after treated with TCA

The concentration of TCA was varied. Additional volumes of 1g/mL of TCA were investigated from 100-800 µL. The sample was made up to 10 mL with deionized water. The recoveries of iodine spiked egg samples after using 1g/mL TCA 100-800 µL were shown in Figure 4.10. The results showed that addition of 400 µL 1g/mL TCA effectively remove egg matrices yielding the best %recovery, %RSD and iodine signal. This concentration is suitable for sample preparation for determined iodine in flow analysis method based on Sandell and Kolthoff reaction.

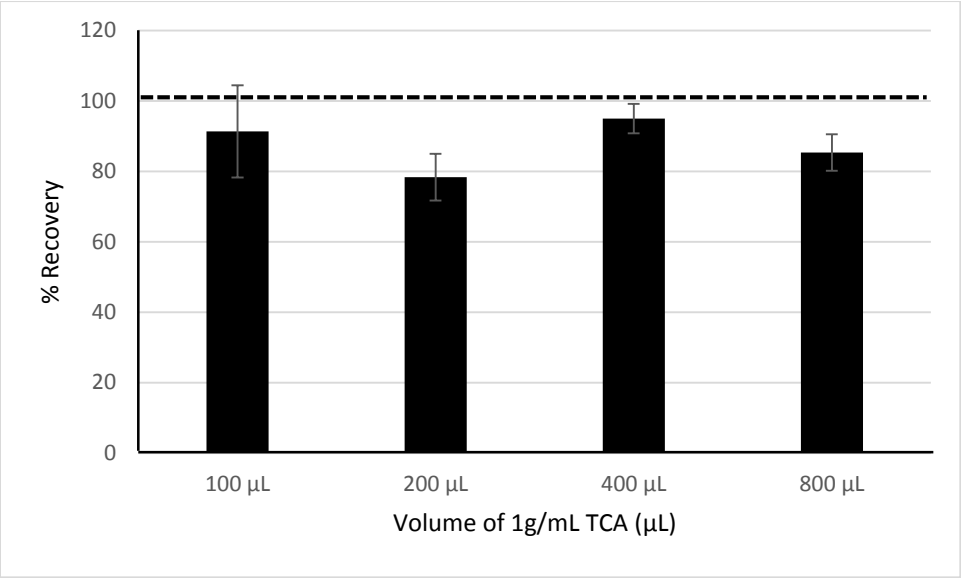

Figure 4.10 Recovery of iodine from egg samples treated with different amounts of TCA

The incubation temperatures were studied. At low temperature, the kinetic energy of protein molecules might be decreased so that the protein molecule may be aggregated better than at room temperature. A portion of 400 µL of 1g/mL of TCA was added to the egg samples and incubated at room temperature and 4 °C for 15 min. The sample was diluted with 10 mL deionized water. The results were shown in Figure 4.11. The incubation temperature at room temperature and 4 °C did not yield significantly different performance on egg matrix removal (paired-t test, Reliability<0.05). This parameter is not critical for determination of iodine by flow analysis method based on Sandell and Kolthoff reaction.

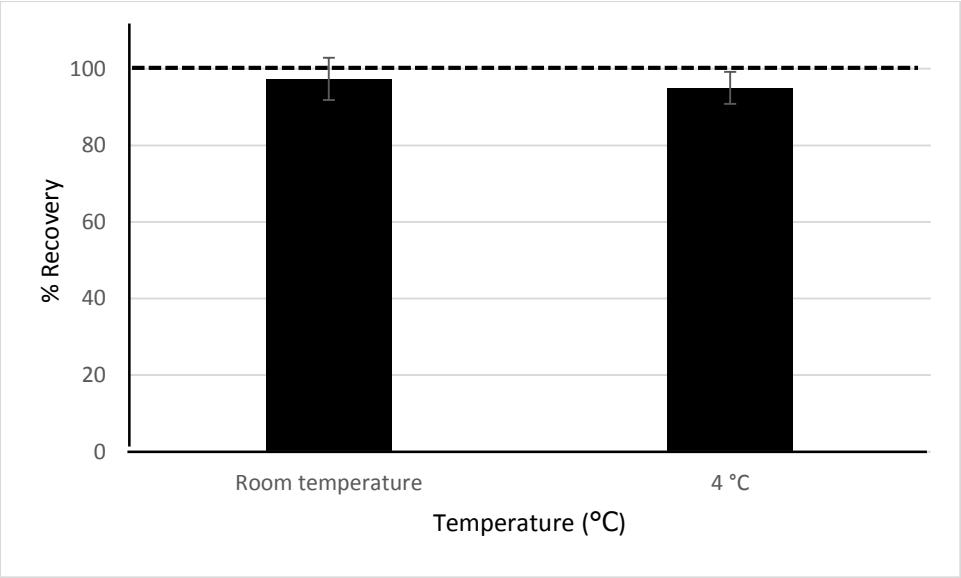

Figure 4.11 Recovery of iodine from egg samples treated with TCA at differents temperature

The incubation time for egg matrix with TCA was studied because the matrix in the egg may take time to aggregate and precipitate. A portion of 400 mL of 1g/mL of TCA was added to the egg sample and incubated at room temperature for 0 min and 15 min. The sample was diluted with 10 mL deionized water. The results were shown in Figure 4.12. The incubation time did not yield significantly different performance on egg matrix removal (paired-t test, Reliability<0.05). This parameter is not critical for determination of iodine by flow analysis method based on Sandell and Kolthoff reaction.

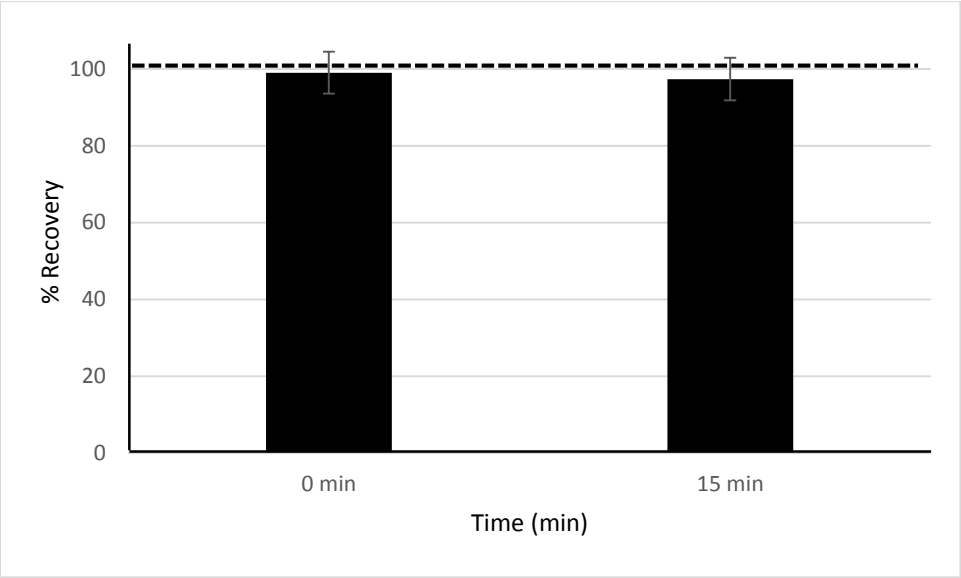

Figure 4.12 Recovery of iodine from egg samples treated TCA for different incubation time

This studied can conclude that, 400 µL of 1g/mL TCA for 0.5 g egg sample was effectively remove egg matrix components for determination of iodine in eggs. This method detection limit (MDL) was 0.58 µg iodine/g egg and method quantitation limit (MQL) was 0.94 µg iodine/g egg, %Recovery was in the range 84-109% and %RSD was less than 6.4%.

4.4 Comparison of sample preparation methods between egg matrix removal by TCA and alkali dry ashing

Egg matrix removal method by TCA was compared to the conventional alkali dry ashing method for the determination of iodine.

Egg samples from 5 different sources were prepared by alkali dry ashing and TCA and determined for iodine contents. The amounts of iodine obtained from those two methods were compared in Figure 4.13. %Recovery and %RSD obtained from both methods were shown in Table 4.3

The results show that the amounts of iodine from egg samples prepared by TCA were relatively higher than those prepared by alkali dry ashing method, which can be clearly seen from Iodine fortified egg sample (Dr. Henn). However, the recoveries of spiked iodine prepared by alkali dry ashing method were in the range 42-54% (Table 4.3). The low recovery of alkali dry ashing method could be attributed to the ashing process and the matrix effect that might have caused the loss of the amount of iodine. To verify the effect of ashing process, the standard iodine solutions were treated by alkali dry ashing method and compared to those without any treatment as shown in Figure 4.14. The slopes of standard iodine solutions with and without alkali dry ashing were the same. It means that the alkali dry ashing process does not cause any loss of iodine. Therefore, the low recovery obtained from egg samples treated by alkali dry ashing method could be attributed to the egg matrices that might affect the ashing efficiency resulting that the iodine might not be leached out effectively. The ashing conditions may have to be optimized for egg matrices in order to obtain the resonable comparison.

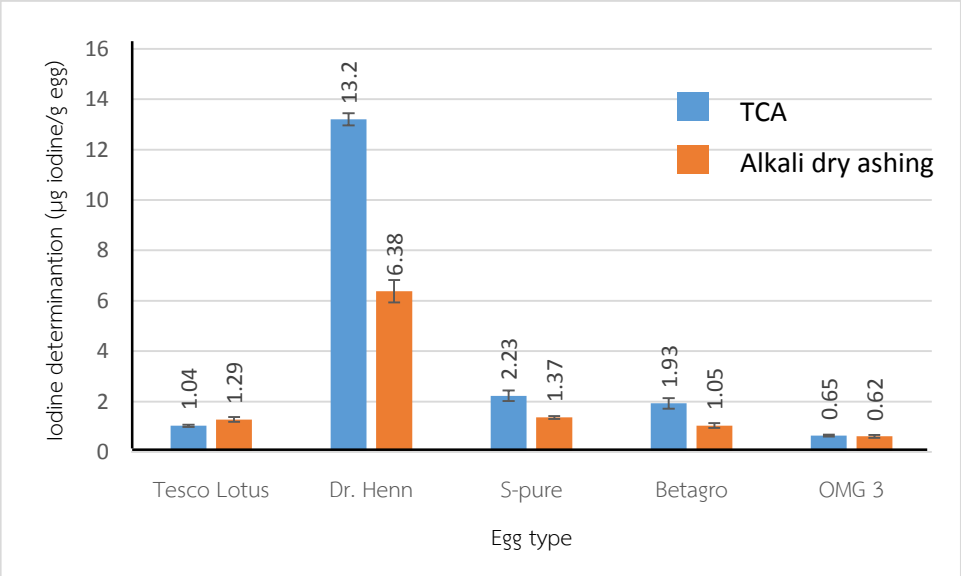

Figure 4.13 Iodine contents in egg samples after treated by TCA method and alkali dry ashing method

Table 4.3 %Recovery and %RSD of iodine spiked egg samples treated by alkali dry ashing and TCA method

Methods	%Recovery	%RSD (N=3)
Alkali dry ashing	42-54	10.4
TCA	103-106	1.10

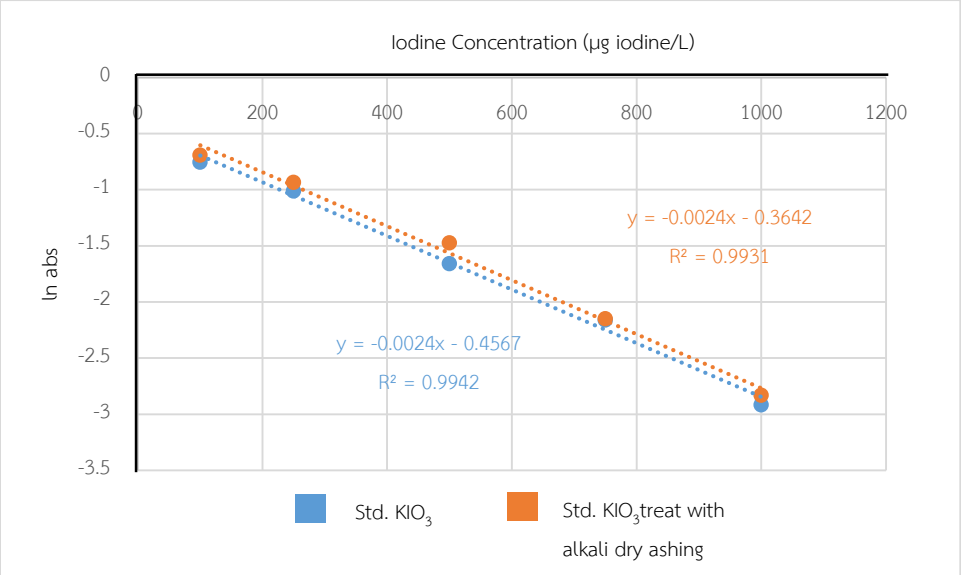

Figure 4.14 Comparison of relationship between the signals of standard iodine treated with and without alkali dry ashing method

CHAPTER V
CONCLUSION AND SUGGESTION OF FUTURE WORK

5.1 Conclusion

The sample preparation for egg matrix removal based on protein precipitation was developed for determination of iodine in the egg sample using colorimetric methods based on Sandell and Kolthoff reaction. The protein precipitation techniques such as isoelectric point precipitation, salting out at high salt concentration, using SDS and TCA protein precipitation agent were studied. According to the physical observation of treated egg samples, treatments with SDS and TCA could remove most of egg matrix components yielding colorless solution that would be suitable for determination iodine by colorimetric flow analysis. The signals obtained after treated with TCA were in the better shape than those obtained after treated with SDS. Using SDS might not be able to remove egg matrices effectively. The recovery of spiked egg samples treated with 400 µL of 100 g/mL of TCA at room temperature were in the range of 84-109% with RSD less than 6.4%. The egg sample preparation method using TCA was compared with alkali dry ashing.

In addition, egg matrix removal method by TCA was compared to the conventional alkali dry ashing method for the determination of iodine. The results show that the amounts of iodine from egg samples prepared by TCA were relatively higher than those prepared by alkali dry ashing method. Therefore, the low recovery obtained from egg samples treated by alkali dry ashing method could be attributed to the egg matrices that might affect the ashing efficiency resulting that the iodine might not be leached out effectively. The ashing conditions may have to be optimized for egg matrices in order to obtain the resonable comparison.

5.2 Suggestion in the future work

Since, the matrix removal by TCA has been successfully applied for determination iodine in the egg samples, this method maybe applied to other food sample mainly consisting of proteins such as milk. In addition, matrix removal by alkali lysis with SDS have tendency for protein precipitation couple with the other sample preparation technique.

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APPENDIX

Table A-1 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 100 µL of 1 g/mL TCA at 15 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.4774	0.5268	0.4823
250 µg iodine/L	0.4077	0.4180	0.4221
500 µg iodine/L	0.3289	0.2819	0.2795
750 µg iodine/L	0.2798	0.1783	0.2043
1000µg iodine/L	0.1900	0.1256	0.1437
Egg 1	0.4834	0.5062	0.4962
Egg 2	0.4830	0.5025	0.4928
Egg 3	0.4790	0.511	0.4952
Egg 4	0.4845	0.5041	0.4992
Egg5	0.4666	0.5125	0.4769
Egg spike 1	0.2669	0.2683	0.2384
Egg spike 2	0.2513	0.2603	0.2559
Egg spike 3	0.3226	0.2622	0.1666
Egg spike 4	0.2761	0.2491	0.2679
Egg spike 5	0.2968	0.2780	0.2725

Table A-2 %Recovery and %RSD of matrix removal by 100 µL of 1g/mL TCA

Amount of 1g/mL TCA	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
100 µL	1.20	0.4975	9.99	13.88	119	17.20
		0.4952		15.11	131	
		0.5041		9.82	78	
		0.5053		13.33	113	
		0.5016		11.57	96	
	2.58	0.4952	9.98	10.68	81	14.10
		0.5093		11.13	86	
		0.4990		8.228	57	
		0.5031		10.09	75	
		0.4977		9.296	67	
	1.96	0.4958	10.06	12.52	105	8.00 (N=4)
		0.4972		11.47	94	
		0.4992		-	-	
		0.4930		10.90	89	
		0.4990		10.52	85	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-3 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 200 µL of 1 g/mL TCA at 15 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.4732	0.5322	0.4686
250 µg iodine/L	0.4013	0.4291	0.3999
500 µg iodine/L	0.2862	0.2945	0.2872
750 µg iodine/L	0.2357	0.2104	0.2100
1000 µg iodine/L	0.1245	0.1450	0.1517
Egg 1	0.4724	0.524	0.4592
Egg 2	0.4853	0.5217	0.4791
Egg 3	0.4757	0.5251	0.4871
Egg 4	0.4797	0.5234	0.4915
Egg5	-	0.5482	0.4900
Egg spike 1	0.3071	0.297	0.2603
Egg spike 2	0.3144	0.2841	0.2601
Egg spike 3	0.2996	0.2828	0.2648
Egg spike 4	0.3207	0.2956	0.2718
Egg spike 5	0.3344	0.3024	0.2622

Table A-4 %Recovery and %RSD of matrix removal by 200 µL of 1g/mL TCA

TCA spike	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
200 µL	2.58	0.4992	9.99	8.92	63	11.90
		0.5047		8.49	59	
		0.4916		9.56	70	
		0.5042		7.82	62	
		0.5027		7.65	51	
	2.28	0.5059	10.02	10.24	79	4.60
		0.4954		11.09	88	
		0.5038		11.00	87	
		0.4947		10.54	82	
		0.4995		10.15	79	
	1.77	0.4991	10.05	11.23	94	3.30
		0.4939		11.36	96	
		0.5010		10.93	91	
		0.4980		10.59	88	
		0.4962		11.19	94	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-5 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 400 µL of 1 g/mL TCA at 15 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.5116	0.5276	0.4991
250 µg iodine/L	0.4252	0.4483	0.4172
500 µg iodine/L	0.2986	0.3056	0.3019
750 µg iodine/L	0.2095	0.2191	0.2202
1000 µg iodine/L	0.1345	0.1455	0.1566
Egg 1	0.5128	0.5160	0.4846
Egg 2	0.5086	0.5177	0.488
Egg 3	0.5089	0.5247	0.4876
Egg 4	0.5099	0.5153	0.4918
Egg5	0.5033	0.5156	0.4880
Egg spike 1	0.2540	0.2722	0.2554
Egg spike 2	0.2681	0.2522	0.2498
Egg spike 3	0.2689	0.2520	0.2490
Egg spike 4	0.2727	0.2666	0.2649
Egg spike 5	0.2803	0.2540	0.2570

Table A-6 %Recovery and %RSD of matrix removal by 400 µL of 1g/mL TCA

Amount of 1g/mL TCA	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
400 µL	2.40	0.5041	10.01	11.59	92	4.80
		0.4979		11.01	86	
		0.4950		11.04	86	
		0.4973		10.80	84	
		0.5021		10.33	79	
	2.71	0.5020	9.97	11.86	92	4.20
		0.5005		12.98	103	
		0.5070		12.83	101	
		0.4945		12.34	97	
		0.5033		12.81	101	
	2.46	0.4983	10.02	12.47	100	3.60
		0.4985		12.80	103	
		0.5005		12.80	103	
		0.5021		11.81	93	
		0.4958		12.43	100	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-7 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 800 µL of 1 g/mL TCA at 15 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.5129	0.4978	0.4908
250 µg iodine/L	0.4434	0.4242	0.3582
500 µg iodine/L	0.3075	0.2677	0.2495
750 µg iodine/L	0.2363	0.2148	0.1637
1000 µg iodine/L	0.1867	0.1029	0.1270
Egg 1	0.5166	0.5125	0.4527
Egg 2	0.5222	0.5351	0.488
Egg 3	0.5292	0.5146	0.4919
Egg 4	0.5191	0.5056	0.4888
Egg5	0.5251	0.5140	0.5086
Egg spike 1	0.2991	0.2632	0.2405
Egg spike 2	0.2910	0.2579	0.2475
Egg spike 3	0.2977	0.2640	0.2669
Egg spike 4	-	0.252	0.2684
Egg spike 5	-	0.2398	0.2657

Table A-8 %Recovery and %RSD of matrix removal by 800 µL of 1g/mL TCA

Amount of 1g/mL TCA	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
800 µL	1.55	0.4975	10.02	10.74	92	2.56 (N=3)
		0.4952		11.33	983	
		0.5041		11.01	94	
		0.5053		-	-	
		0.5016		-	-	
	2.25	0.4912	10.01	9.917	77	5.46
		0.5041		9.888	76	
		0.5056		9.601	74	
		0.5015		10.20	79	
		0.4961		10.86	86	
	1.57	0.4960	9.99	10.86	93	7.62
		0.5022		10.35	88	
		0.5009		9.372	78	
		0.5008		9.299	77	
		0.5016		9.419	79	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-9 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 400 µL of 1 g/mL TCA at 0 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.4454	0.5124	0.5124
250 µg iodine/L	0.3347	0.3829	0.3829
500 µg iodine/L	0.2579	0.2946	0.2945
750 µg iodine/L	0.1803	0.2079	0.2079
1000 µg iodine/L	0.1341	0.1322	0.1322
Egg 1	0.4841	0.5038	0.5373
Egg 2	0.4899	0.5125	0.5377
Egg 3	0.4894	0.5090	0.5466
Egg 4	0.4754	0.5046	0.5524
Egg5	0.4785	0.5091	0.5475
Egg spike 1	0.2396	0.2735	0.2762
Egg spike 2	0.2427	0.2624	0.2746
Egg spike 3	0.2533	0.2519	0.2799
Egg spike 4	0.2541	0.2614	0.2771
Egg spike 5	0.2478	0.2578	0.2511

Table A-10%Recovery and %RSD of matrix removal by 400 µL of 1g/mL TCA at 0 min

Incubation time	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
0 min	0.17	0.4982	9.96	10.99	109	4.20
		0.5083		10.58	104	
		0.5086		9.92	98	
		0.5000		10.05	99	
		0.4982		10.56	104	
	1.97	0.5072	9.94	10.66	87	4.80
		0.5028		11.34	94	
		0.5051		11.87	100	
		0.4964		11.54	96	
		0.5039		11.57	97	
	0.97	0.5093	9.93	10.48	96	1.89 (N=4)
		0.5012		10.73	98	
		0.5087		10.30	94	
		0.5018		10.59	97	
		0.4971		-	-	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-11 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 400 µL of 1 g/mL TCA at 15 min

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.4454	0.5124	0.5124
250 µg iodine/L	0.3347	0.3829	0.3829
500 µg iodine/L	0.2579	0.2945	0.2945
750 µg iodine/L	0.1803	0.2079	0.2078
1000 µg iodine/L	0.1341	0.1322	0.1322
Egg 1	0.4816	0.5149	0.5149
Egg 2	0.4931	0.5214	0.5214
Egg 3	0.5055	0.5215	0.5215
Egg 4	0.494	0.5318	0.5318
Egg5	0.5042	0.5303	0.5303
Egg spike 1	0.2670	0.2710	0.2710
Egg spike 2	0.2557	0.2623	0.2623
Egg spike 3	0.2564	0.2707	0.2707
Egg spike 4	0.2539	0.2801	0.2801
Egg spike 5	0.2589	0.2810	0.2810

Table A-12%Recovery and %RSD of matrix removal by 400 µL of 1g/mL TCA at 15 min

Incubation time	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
15 min	0.21	0.4992	9.78	10.97	108	4.60
		0.4988		10.78	106	
		0.5088		9.921	97	
		0.5032		9.983	98	
		0.4961		10.51	103	
	1.52	0.4965	10.01	11.02	95	5.70
		0.4903		11.63	101	
		0.5040		10.87	93	
		0.5097		10.27	87	
		0.4976		10.47	90	
	0.70	0.4977	10.00	9.654	90	6.40
		0.5035		9.900	92	
		0.5018		10.19	95	
		0.4963		10.778	101	
		0.5006		11.12	104	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-13 Pair t test between 0 min and 15 min

	0 min	15 min
Mean	99	97.33333
Variance	16	26.33333
Observations	3	3
Pearson Correlation	0.974355	
Hypothesized Mean Difference	0	
df	2	
t Stat	1.889822	
P(T<=t) one-tail	0.09968	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.199359	
t Critical two-tail	4.302653	

Table A-14 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 400 µL of 1 g/mL TCA at room temperature

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.4454	0.5124	0.5124
250 µg iodine/L	0.3347	0.3829	0.3829
500 µg iodine/L	0.2579	0.2945	0.2946
750 µg iodine/L	0.1803	0.2079	0.2079
1000 µg iodine/L	0.1341	0.1322	0.1322
Egg 1	0.4816	0.5149	0.5373
Egg 2	0.4931	0.5214	0.5377
Egg 3	0.5055	0.5215	0.5466
Egg 4	0.494	0.5318	0.5524
Egg5	0.5042	0.5303	0.5475
Egg spike 1	0.2670	0.2710	0.2762
Egg spike 2	0.2557	0.2623	0.2746
Egg spike 3	0.2564	0.2707	0.2799
Egg spike 4	0.2539	0.2801	0.2771
Egg spike 5	0.2589	0.2810	0.2511

Table A-15%Recovery and %RSD of matrix removal by 400 µL of 1g/mL TCA at room temperature

Incubation temperature	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
Room temperature (25°C)	0.21	0.4992	9.78	10.97	108	4.60
		0.4988		10.78	106	
		0.5088		9.921	97	
		0.5032		9.983	98	
		0.4961		10.51	103	
	1.52	0.4965	10.01	11.02	95	5.70
		0.4903		11.63	101	
		0.5040		10.87	93	
		0.5097		10.27	87	
		0.4976		10.47	90	
	0.70	0.4977	10.00	9.654	90	6.40
		0.5035		9.900	92	
		0.5018		10.19	95	
		0.4963		10.778	101	
		0.5006		11.12	104	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-16 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with 400 µL of 1 g/mL TCA at 4°C

Sample	Peak height 1	Peak height 2	Peak height 3
100 µg iodine/L	0.5116	0.5276	0.4991
250 µg iodine/L	0.4252	0.4483	0.4172
500 µg iodine/L	0.2986	0.3056	0.3019
750 µg iodine/L	0.2095	0.2191	0.2202
1000 µg iodine/L	0.1345	0.1455	0.1566
Egg 1	0.5128	0.5160	0.4846
Egg 2	0.5086	0.5177	0.488
Egg 3	0.5089	0.5247	0.4876
Egg 4	0.5099	0.5153	0.4918
Egg5	0.5033	0.5156	0.488
Egg spike 1	0.2540	0.2722	0.2554
Egg spike 2	0.2681	0.2522	0.2498
Egg spike 3	0.2689	0.2520	0.2490
Egg spike 4	0.2727	0.2666	0.2649
Egg spike 5	0.2803	0.2540	0.2570

Table A-17%Recovery and %RSD of matrix removal by 400 µL of 1g/mL TCA at 4°C

Incubation temperature	Original iodine (µg/g)	Weight egg (g)	Spike iodine (µg/g)	Iodine found (µg/g)	%Recovery*	%RSD (N=5)
4 °C	2.40	0.5041	10.01	11.59	92	4.80
		0.4979		11.01	86	
		0.4950		11.04	86	
		0.4973		10.80	84	
		0.5021		10.33	79	
	2.71	0.5020	9.97	11.86	92	4.20
		0.5005		12.98	103	
		0.5070		12.83	101	
		0.4945		12.34	97	
		0.5033		12.81	101	
	2.46	0.4983	10.02	12.47	100	3.60
		0.4985		12.80	103	
		0.5005		12.80	103	
		0.5021		11.81	93	
		0.4958		12.43	100	

* % Recovery = [Iodine found – (weight egg * Original iodine)] / Iodine spike * 100

Table A-18 Pair t test between room temperature and 4ºC

	Room temperature	4 ºC
Mean	97.33333	95
Variance	26.33333	61
Observations	3	3
Pearson Correlation	-0.93565	
Hypothesized Mean Difference	0	
df	2	
t Stat	0.3172	
P(T<=t) one-tail	0.390572	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.781143	
t Critical two-tail	4.302653	

Table A-19 Peak height of standard KIO_3, egg and spiked egg sample (500 µg iodine/L KIO_3) after treated with TCA

	Peak height (Tesco lotus)	**Peak height** (Dr.Henn)	**Peak height** (S-pure)	**Peak height** (Betagro egg)	**Peak heigh (**OMG egg**)t**
DI	0.5542	0.5542	0.5542	0.5542	0.5542
100 µg iodine/L	0.4723	0.4723	0.4723	0.4723	0.4723
250 µg iodine/L	0.3569	0.3569	0.3569	0.3569	0.3569
500 µg iodine/L	0.2083	0.2083	0.2083	0.2083	0.2083
750 µg iodine/L	0.1369	0.1369	0.1369	0.1369	0.1369
1000 µg iodine/L	0.0615	0.0615	0.0615	0.0615	0.0615
Egg 1	0.5158	0.1480	0.4530	0.4645	0.5406
Egg 2	0.5218	0.1522	0.4597	0.4732	0.5349
Egg 3	0.5125	0.1406	0.4586	0.4741	0.5393
Egg spike 1	0.1810	-	-	-	-
Egg spike 2	0.1763	-	-	-	-
Egg spike 3	0.1714	-	-	-	-

Table A-20 Peak height of standard iodine and standard iodine treat with alkali dry ashing

	Peak height	**Peak height** (Alkali dry ashing)
100 µg iodine/L	0.47135	0.50165
250 µg iodine/L	0.36375	0.39365
500 µg iodine/L	0.1904	0.22975
750 µg iodine/L	0.1154	0.11675
1000 µg iodine/L	0.054215	0.059085

Table A-21 Iodine concentration (μg iodine/g) in eggs obtained after treatment by alkali dry ashing and TCA

Methods	Egg type	Iodine concentration (μg iodine/g egg)	% RSD (N=3)
Alkali dry ashing	Tesco lotus	1.31	3.50
	Dr. Hen	6.47	3.60
	S-pure	1.39	14.9
	Betagro	1.06	6.50
	OMG 3	0.630	5.30
TCA	Tesco lotus	1.05	8.80
	Dr. Hen	13.3	3.30
	S-pure	2.23	2.60
	Betagro	1.95	4.70
	OMG 3	0.660	8.90

VITA

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