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FULL REPORT

Preliminary Study of Phosphate Assay in Fresh Meat and Meat Products for Producing Phosphate Test Kit

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PREFACE

In Thailand, the consumption of meat products such as sausage, ham, and bacon have been increased because these products are ready-to-eat, or almost ready-to-eat, products. The danger of meat product consumption is to be aware of. In meat processing, many kinds of food additives are used. Most of them may cause bad effects to human if too much consumed. Phosphate is a food additives used for improve the meat texture. Phosphate has adverse effects if it was left too much in the meat products. The meat product manufacture must investigate the amount of phosphate in meat and meat products. To determine phosphate in the meat and meat products, the standard analytical method has been used. However, the standard method is too tedious for local manufacture. Thus a simple phosphate test kit for phosphate assay in meat and meat products is of need. This work attempted to preliminarily study the feasibility of the production of a simple phosphate test kit for this purpose. The authors modified the standard method to a simple method, and to validate the proposed method, we compared the results obtained from the proposed method with the standard method. This work could be useful to the further work that is interesting in producing the test kit.

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ABSTRACT

A study of simple analysis method for the determination of phosphate in meat product and fresh meat samples was performed. Analysis by colorimetric techniques based on molybdenum blue (MB) and vanadomolybdate (VM) methods were compared. The results showed that the VM method, based on the formation of a yellow complex of molybdenum, vanadium and phosphorus, was more practical and simpler, and the reagent used was more stable than the MB method. To reduce drawbacks of the traditional sample preparation method, a simple water extraction was proposed. Effect of method for increasing the sample surface area (grinding by a machine and chopping manually) and extraction time (0.5-5 min) was studied. The phosphate contents found in the ground and the chopped samples were not significantly different (tested by *t* test at 95% confidence). However, the precisions as percentage relative standard deviation (%RSD) obtained from the chopped samples (0.1-8%) were better than those obtained from the ground samples (2-26%). For extraction by shaking using a shaker, increasing the extraction time did not have much effect on the phosphate content. For extraction by shaking manually, increasing the extraction time increased the phosphate contents obtained from the chopped samples. However, the extraction time of 1.5 min and preparing the samples by chopping were employed. The accuracy of the proposed method was validated by determining recovery percentage and comparing the results with the standard method. The recovery percentage of 85-112% was found. The results indicated that the water-soluble phosphate contents obtained by the proposed method were approximately two times less than the total phosphate contents obtained by the standard method. Multiplying the water-soluble phosphate contents by two did not provide any significant difference from the total phosphate contents (tested by *t* test at 95% confidence). The results showed the feasibility on exploiting the proposed method to produce a phosphate test kit for the simple determination of phosphate in meat product and meat samples.

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาวิธีการวิเคราะห์ฟอสเฟตอย่างง่ายเพื่อหาปริมาณฟอสเฟตในตัวอย่างผลิตภัณฑ์เนื้อและเนื้อสด โดยมีการเปรียบเทียบวิธีวิเคราะห์แบบคัลเลอริเมตรี 2 วิธีคือ วิธีโมลิบดินัมบลู และวิธีวานาโดโมลิบเดต จากการทดลอง พบว่า วิธีวานาโดโมลิบเดตซึ่งอาศัยการเกิดสารประกอบเชิงซ้อนสีเหลืองของโมลิบดินัม วานาเดียม และฟอสฟอรัส เป็นวิธีที่ทำได้ง่ายกว่า และรีเอเจนต์ที่ใช้ยังเสถียรมากกว่าวิธีโมลิบดินัมบลูด้วย สำหรับการศึกษาวิธีการเตรียมตัวอย่างผลิตภัณฑ์เนื้อและเนื้อสดอย่างง่ายนั้น ใช้วิธีการสกัดตัวอย่างด้วยน้ำ ซึ่งมีการศึกษาถึงวิธีการเพิ่มพื้นที่ผิวสัมผัสของตัวอย่าง (วิธีบดด้วยเครื่องบด และวิธีสับ) และเวลาในการสกัด (0.5-5 นาที) พบว่า ปริมาณฟอสเฟตที่สกัดจากตัวอย่างซึ่งเตรียมโดยวิธีบดและสับไม่แตกต่างอย่างมีนัยสำคัญ (ทดสอบด้วย *t* test ที่ระดับความเชื่อมั่นร้อยละ 95) อย่างไรก็ตาม ความแม่นยำซึ่งประเมินโดยร้อยละค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ของการเตรียมตัวอย่างด้วยการสับ (ร้อยละ 0.1-8) น้อยกว่าของการเตรียมตัวอย่างด้วยการบด (ร้อยละ 2-26) เมื่อใช้วิธีการสกัดด้วยเครื่องเขย่านั้น พบว่า เวลาในการสกัดไม่มีผลกระทบต่อปริมาณฟอสเฟตที่สกัดได้ แต่เมื่อสกัดโดยการเขย่าด้วยมือนั้น พบว่าการเพิ่มเวลาในการสกัดทำให้ปริมาณฟอสเฟตที่สกัดได้เพิ่มขึ้นด้วย ในตัวอย่างที่เตรียมแบบสับ อย่างไรก็ตาม สภาวะที่เหมาะสมได้แก่ เวลาการสกัด 1.5 นาที และเตรียมตัวอย่างด้วยการสับ นอกจากนี้ยังได้ศึกษาความถูกต้องของวิธีโดยการหาร้อยละของการกลับคืน และเปรียบเทียบผลการวิเคราะห์จากวิธีที่เสนอและวิธีมาตรฐาน พบว่า ร้อยละของการกลับคืนเป็น 85-112 แต่ปริมาณฟอสเฟตที่ละลายน้ำได้ซึ่งหาได้จากวิธีที่เสนอนั้นน้อยกว่าปริมาณฟอสเฟตทั้งหมดที่หาได้จากวิธีมาตรฐานประมาณ 2 เท่า และเมื่อคูณจำนวน 2 เท่าดังกล่าวเข้ากับปริมาณฟอสเฟตที่ละลายน้ำได้ พบว่า ปริมาณฟอสเฟตที่ได้ไม่แตกต่างอย่างมีนัยสำคัญกับปริมาณฟอสเฟตทั้งหมด (ทดสอบด้วย *t* test ที่ระดับความเชื่อมั่นร้อยละ 95) ดังนั้น จึงมีความเป็นไปได้ในการนำวิธีการเตรียมตัวอย่างและวิธีวิเคราะห์ที่นำเสนอ ไปพัฒนาเป็นชุดตรวจสอบฟอสเฟตอย่างง่ายในผลิตภัณฑ์เนื้อและเนื้อสดต่อไป

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CHAPTER 1
INTRODUCTION

1.1 Statement and Significance of the Problem

A phosphate, an inorganic chemical, is a salt of phosphoric acid. The use of alkaline phosphates in meat curing is widely employed by the meat industry. Phosphates are added in meat to reduce cook losses, improve textural properties especially by increasing water holding capacity, retard oxidative rancidity, develop color and supply protection against microbial growth [Gonçalves, 2009]. However, adding too high amount of phosphates may cause adverse effects to customers due to over intake of phosphorus. The recommended dietary allowance (RDA) of phosphorus is 700 mg/day for adults 19-70 years old and 1.250 mg/day for children [Jastrzębska, 2003]. Some literature reported that the phosphate tolerance in human was 70 mg/kg per day [National Research Council, 2005]. Severe hyperphosphatemia can cause hypocalcemia (low calcium) severe enough to cause tetany and even death [Deshpande, 2002].

The Food Control Division, Food and Drug Administration, Ministry of Public Health, Thailand, does not allow adding phosphates more than 3000 mg/ kg of meat and sets the maximum allowance in meat products at less than 0.5% by weight of meat products or less than 5000 mg/kg of product [Food Control Division, 2004]. Normally, meat contains phosphates of 2000 mg/kg [National Research Council, 2005]. Therefore, phosphate assay in meat and meat products is necessary for the meat industries. Beside, phosphate control benefits customers.

Several colorimetric methods have been widely used for the determination of total phosphates [Molins, 1991]. Advanced analysis techniques such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE), and X-ray fluorescence have also been used [Jastrzębvska *et al.*, 2003; Kavitha & Modi, 2007]. However, the main drawback in the total phosphate analysis in meat has been

the tedious and complex preparation method. To prepare a meat sample, the sample is ignited at 400-600 °C in a furnace or digested with concentrated acids.

In northern Thailand, like Chiang Rai province, meat products are produced by local manufactures. To control the quality of the meat products, these local manufactures should analyze their meat products for phosphates. However, they are not skilled and do not have much invested money to do the analysis by themselves. Thus, this work aimed to study the simpler sample preparation method and the analysis method. In addition, the possibility of producing the phosphate test kit was studied.

1.2 Objectives

1.2.1 To study the simple sample preparation method and analysis method for the determination of phosphates in fresh meat and meat product samples

1.2.2 To conduct a feasibility study for producing a simple phosphate test kit for phosphate analysis in fresh meat and meat products

1.3 Scope of Study

The analysis methods studied in this work were based on simple colorimetric methods, i.e. yellow vanado-molybdate and molybdenum blue methods. Accuracy and precision of the selected analysis method was studied. Potassium dihydrogen orthophosphate (KH_2PO_4) was used as a standard. Fresh meat and meat product samples were purchased from local Chiang Rai fresh markets and supermarkets. A simple sample preparation method was proposed. The results obtained by the proposed method were compared with those by a standard method (AOAC 986.24). This work also evaluated the possibility on producing a phosphate test kit for phosphate assay in fresh meat and meat products.

1.4 Benefits

1.4.1 A simple sample preparation method for the determination of phosphates in meat and meat products was developed.

1.4.2 A simple analysis method for the determination of phosphates in meat and meat products was studied and selected.

1.4.3 The possibility on producing phosphate test kit for the simple and rapid determination of phosphate in meat and meat products was studied.

1.4.4 This work could be published in an international journal and presented in conferences.

CHAPTER 2
LITERATURE REVIEW

2.1 Phosphate in Fresh Meat and Meat Products

2.1.1 Phosphates

A phosphate, an inorganic chemical, is a salt of phosphoric acid. Phosphates are widely used as food additives, in the form of phosphoric acid as acidulant, and as monophosphates and polyphosphates in a large number of foods and for a variety of purposes. Phosphates serve as buffering agents in dairy, meat and fish products. The largest group of phosphates and the most important in the food industry is the orthophosphate (PO_4^{3-}). Orthophosphate is the simplest in a series of phosphates, and is usually just called phosphate [deMan, 1999].

2.1.2 Functions of phosphates in meat products

Phosphates fulfill several functions in meat products. Firstly, it contributes greatly to the solubility of muscular protein. Through the addition of salt as well as phosphates at the same time to a meat product, the muscular protein becomes soluble and activated. The protein can then immobilize high levels of added water as well as emulsify a large amount of fat, given that activated meat protein is an excellent emulsifier of fat. Secondly, the addition of alkaline phosphates to slightly sour meat leads to a rise in pH inside the meat product and larger amounts of added water can be bound. Thirdly, phosphate increases the ionic strength of the meat and an increased ionic strength leads to a more severe degree of swelling from the muscle fibers and activation of protein. Enhanced level of activated and swollen protein support the immobilization of added water to meat products and emulsification of fat. Fourthly, phosphates are slightly bacteriostatic and growth of bacteria is marginally slowed down. Finally, phosphates can also slow down the process of rancidity because they can chelate heavy metal ions. [Feiner, 2006] In conclusions, phosphates are added in

meat to reduce cook losses, improve textural properties especially by increasing water holding capacity, retard oxidative rancidity, develop color and supply protection against microbial growth. [Branen, 2001; Gonçalves, 2009; Houben, 2005; Hui, 2001; Kormendy, 1992; Puolanne, 2001; Sheard, 2004; Yapar, 2006]

2.1.3 Effect of phosphates on human

Phosphorus is a key element in all known forms of life. Inorganic phosphorus in the form of the phosphate PO_4^{3-} plays a major role in biological molecules such as DNA and RNA where it forms part of the structural framework of these molecules. Living cells also use phosphate to transport cellular energy via adenosine triphosphate (ATP). Nearly every cellular process that uses energy obtains it in the form of ATP. Phospholipids are the main structural components of all cellular membranes. Every cell has a membrane that separates it from its surrounding environment. Biological membranes are made from a phospholipid matrix and proteins, typically in the form of a bilayer. [Goldfrank *et al.*, 2006; Hulse, 1995] Phosphates are important because they affect the absorption of calcium and other elements. Calcium phosphate salts assist in stiffening bones. The absorption of inorganic phosphorus depends on the amount of calcium, iron, strontium and aluminum present in the diet. [deMan, 1999]

An average adult human contains a little less than 1 kg of phosphorus, about 85% of which is present in bones and teeth in the form of apatite, and the remainder inside cells in soft tissues. A well-fed adult in the industrialized world consumes and excretes about 1-3 g of phosphorus per day, with consumption in the form of inorganic phosphate and phosphorus-containing biomolecules such as nucleic acids and phospholipids; and excretion almost exclusively in the form of urine phosphate ion. Only about 0.1% of body phosphate circulates in the blood, but this amount reflects the amount of phosphate available to soft tissue cells. [Goldfrank *et al.*, 2006]

In medicine, low phosphate syndromes are caused by malnutrition, by failure to absorb phosphate, and by metabolic syndromes which draw phosphate from the blood or pass too much of it into the urine. All are characterized by

hypophosphatemia, which is a condition of low levels of soluble phosphate levels in the blood serum, and therefore inside cells. Symptoms of hypophosphatemia include muscle and neurological dysfunction, and disruption of muscle and blood cells due to lack of ATP. Too much phosphate can lead to diarrhea and calcification (hardening) of organs and soft tissue, and can interfere with the body's ability to use iron, calcium, magnesium, and zinc. [Anderson, 1996] Severe hyperphosphatemia can cause hypocalcemia (low calcium) severe enough to cause tetany and even death [Deshpande, 2002].

Adding too high amount of phosphates in meat products may cause adverse effects to customers due to over intake of phosphorus. The recommended dietary allowance (RDA) of phosphorus is 700 mg/day for adults 19-70 years old and 1.250 mg/day for children [Jastrazębska, 2003]. Some literature reported that the phosphate tolerance in human was 70 mg/kg per day [National Research Council, 2005].

The Food Control Division, Food and Drug Administration, Ministry of Public Health, Thailand, does not allow adding phosphates more than 3000 mg/ kg of meat and sets the maximum allowance in meat products at less than 0.5% by weight of meat products or less than 5000 mg/kg of product [Food Control Division, 2004]. Normally, meat contains phosphates of 2000 mg/kg [National Research Council, 2005]. Therefore, phosphate assay in meat and meat products is necessary for the meat industries. Beside, phosphate control benefits customers.

2.2 Analytical Methods for Phosphate Determination

Many methods of phosphate analysis have relied on the conversion of long-chain compounds to the orthophosphate form. Those methods include traditional methods, e.g. titrimetry (volumetric titration) and gravimetry, and advanced methods, for examples; colorimetry, high performance liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance (NMR) spectroscopy, and X-ray fluorescence. [Jastrzębvska *et al.*, 2003; Kavitha & Modi, 2007; Molins, 1991] Traditional methods seemed to be quite tedious, and thus some drawbacks have been

found. Among these modern methods, several colorimetric methods have been usually used for the determination of total phosphates [Heimann & Jakobsen, 2007; Jeffery, 1989; Kirk, 1991; Korn *et al.*, 2002; Muñoz *et al.*, 1997; Neves *et al.*, 2008; Ünal *et al.*, 2004].

The standard colorimetric methods for phosphate determination widely used are molybdenum blue (MB) and vanadomolybdate (VM) methods. [Heimann and Jakobsen, 2007; Molins, 1991; Muñoz *et al.*, 1997; AOAC International, 2000] The basis of the MB method is the formation of an intense blue color when ammonium molybdate is reacted with an orthophosphate under acidic conditions, followed by the addition of a reducing agent. The resulting color, known as molybdenum blue, is measured spectrophometrically, and the phosphate content in the sample is calculated from a standard curve prepared with known amounts of an orthophosphate. Although the MB method is a rapid, accurate analytical tool in phosphate determinations, it has limitations. Acid digestion of the sample is required as a first step, which results in swift and inevitable hydrolysis of pyro- and other polyphosphates to orthophosphates. If the method is to be used to monitor orthophosphate concentration in a food product, the acid solubilization first step in the analysis would result in orthophosphate overestimation. This problem may be minimized by performing the analysis fast enough so that the blue color developed is read as soon as feasible after adding the final molybdate and reducing agent solutions (i.e., within 5-10 min). [Molins, 1991] The VM method is based on the action of molybdenum and vanadium by which a yellow complex is formed with phosphorus. The VM method is simple and the reagents used in this VM method are stable, but the method is troubled with spectrophotometric nonlinearity. [Baadenhuijsen *et al.*, 1977; Muñoz *et al.*, 1997; Neves *et al.*, 2008]

However, the main drawback in the total phosphate analysis in meat has been from the tedious and complex sample preparation method. To prepare a meat sample, the sample is ignited at 400-600 °C in a furnace or digested with concentrated acids. [Branen, 2001; Kirk, 1991; AOAC International, 2000]. It would be useful to modify

the traditional method or develop a new sample preparation method that is simpler and consumes less reagents, but is still accurate.

2.3 The Needs of a Phosphate Test Kit for Phosphate Assay in Fresh Meat and Meat Products

In northern Thailand, such as Chiang Rai province, meat products are produced by local manufactures. To control the quality of the meat products, these local manufactures should analyze their meat products for phosphates. However, they are not skilled and do not have much invested money to do the analysis by themselves. Thus, this work aimed to study the simpler sample preparation method and the analysis method. In addition, the possibility of producing the phosphate test kit was studied.

CHAPTER 3
METHODOLOGY

3.1 Chemicals

All chemicals used were of analytical grade. Phosphate standard stock solution (1000 ppm P) was prepared by dissolving 4.39 g of potassium dihydrogen orthophosphate (KH_2PO_4; Ajax, Australia) in 1 liter of pure water. The intermediate phosphate standard solution (10 or 100 ppm P) was freshly prepared by diluting the stock solution.

3.1.1 Reagents for the molybdenum blue method

Molybdate solution was prepared by dissolving 12.5 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$; Ajax, Australia) in 500 ml of 47%v/v sulfuric acid (H_2SO_4; Carlo Erba, Italy). Ascorbic acid (5%w/v; POCH, Poland) used as a reducing agent was prepared freshly. The mixture of molybdate solution (25 ml) and ascorbic acid (10 ml) was used as the coloring reagent.

3.1.2 Reagents for vanado-molybdate method

Vanado-molybdate reagent was prepared by mixing molybdate and vanadate solutions in acidic medium. Molybdate solution was prepared by dissolving 20 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; Ajax, Australia) in 400 ml warm water (app. 50°C). Vanadate solution was prepared by dissolving 1 g of ammonium metavanadate (NH_4VO_3; Ajax, Australia) in 300 ml hot water and then adding concentrated nitric acid (HNO_3; Carlo Erba, Italy). Then the molybdate solution was added gradually to the vanadate solution with stirring. Finally, the volume was adjusted to 1 liter with pure water.

3.2 Samples

Eleven meat product samples that were sausage, ham, and bolona samples produced by different suppliers were purchased from some local supermarkets in Chiang Rai. Each meat product sample was ground by two different methods: using a grinding machine and manual chopping. The samples were kept in a refrigerator (4°C).

Five fresh meat samples were purchased from some local fresh markets and supermarkets in Chiang Rai. Each fresh meat sample was ground by a grinding machine. The samples were kept in a refrigerator (4°C).

3.3 Analytical Methods

3.3.1 Molybdenum blue method

Working solutions (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ppm P) were prepared by pipetting 1.00, 2.00, 3.00, 4.00, 5.00, and 6.00 ml of 10 ppm P solution into volumetric flasks. Then 15 ml pure water and 20 ml molybdate-ascorbic acid solution were added, respectively. The mixture solutions were immersed in boiled water for exactly 15 min, and then cool to room temperature. The volume of each solution was adjusted to 50 ml with pure water. For sample solutions, 1 – 10 ml of sample was used. The other steps of preparation were same as preparation of the working solutions. The blue complex was measured spectrophotometrically at 823 nm.

3.3.2 Vanado-molybdate method

A 100 ppm P solution was used to prepare the working solutions (5.0, 8.0, 10.0, 15.0, and 20.0 ppm P). To develop color solutions, 100 ppm P solution or 2.00 ml sample was pipetted into volumetric flasks, and 2.00 ml of vanado-molybdate reagent was added. The mixture was diluted to 10.00 ml with pure water. The color

solutions were let stand for at least 10 min, and then measured spectrophotometrically at 400 nm.

3.4 Preparation of Samples

Simple extraction method was studied. The ground meat and meat product samples (1.25 g) were extracted with pure water (25 ml). A shaker (KS 250 Basic Model, IKA Laborteclmik, Germany) was used for shaking the meat-water mixture. The extracted solution was filtered through 450 and 200 nm nylon syringe filter (FiltrEX, USA), respectively, before use. Effect of extraction time (0.5, 1.0, 2.0 and 3.0 min) was studied. Effect of meat grinding method (manual chopping and using a grinding machine) was also studied.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Phosphate Analysis Method

This work compared the results obtained from the molybdenum blue (MB) and the vanadomolybdate (VM) methods. **Figure 4.1** shows the calibration graphs obtained by the MB and VM methods. It indicates the linear graph in the different ranges of concentration. The MB method provided the linearity in a lower concentration range (0.2 – 1.2 ppm P) whereas the VM method gave the dynamic range in a higher concentration range (5 – 20 ppm P). This agreed with the concentration range stated in the reference standard methods [Jastrzębska, 2009; Neves, 2008].

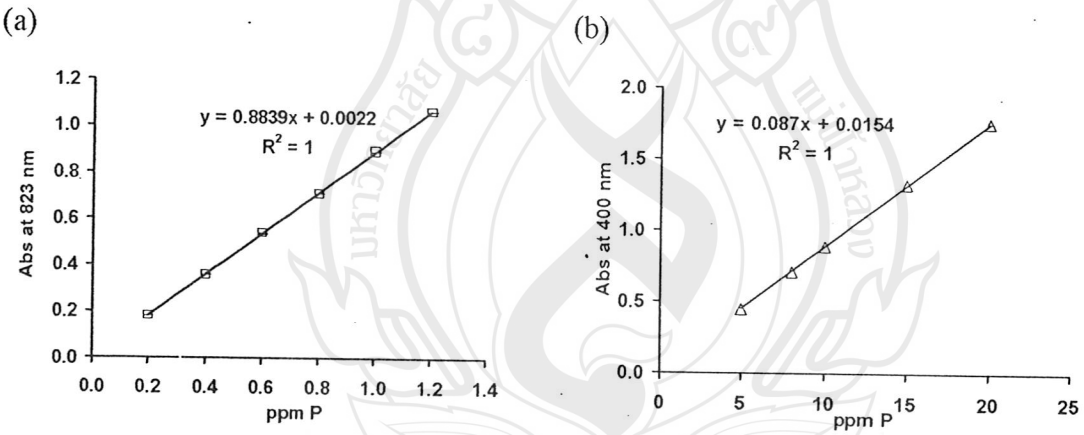

Figure 4.1 Calibration graphs of phosphate standards using (a) molybdenum blue and (b) vanadomolybdate methods

Some drawbacks of using the MB method were found. Fast performing the analysis was needed to avoid the hydrolysis of polyphosphates to orthophosphates. The blue complex should be measured within 5-10 min after adding the molybdate reagent and reducing agent [Molins, 1991]. Otherwise, the color intensity would be increased due to the orthophosphate increase from the mentioned hydrolysis. Moreover, it was found that the reaction was temperature-dependent. High

temperature could increase the speed of reaction so that it speeded up the color development. Unfortunately, using an available-in-lab hotplate, we could not control the temperature of water used for warming the solutions after adding the molybdate and reducing agent solutions. Thus, it was not practical and easy to exploit this MB method.

The yellow vanadomolybdate method, compared to the MB method, was simpler. To develop the yellow color, the vanadomolybdate reagent was added and the mixture was let stand for 10 min. The color was quite stable and more independent of time and temperature. This observation was also reported in literatures [Baadenhuijsen *et al.*, 1977; Muñoz *et al.*, 1997; Neves *et al.*, 2008]. Although yellow color is not as easy as blue color to be observed by eyes, we could still see the difference of the yellow color at the different concentration of phosphate. Therefore, the VM method was used for further study.

4.2 Sample Preparation Method

To reduce the tediousness of the traditional digestion method, a simple extraction was proposed. The phosphates in the meat product samples were extracted with pure water. The meat-water mixture was shaken horizontally by a shaker for a time. Then the solution was filtered through 450-nm filter to remove the meat and 200-nm filter to remove fat and oil in the solution. Finally, the clear solution was brought to react with the vanadomolybdate reagent to develop the yellow color.

The effect of the method for increasing the surface area (using a grinding machine and chopping manually) and the extraction time (0.5, 1, 2, 3, 4, and 5 min) was studied.

4.2.1 Method for increasing the surface area

In order to increase the extraction efficiency, the sample surface area was increased by grinding using a grinding machine and by chopping manually. The results obtained at the extraction time of 1 – 5 min showed that the obtained

phosphate contents in the meat product samples that were ground by a grinding machine and chopped manually were not significantly different (tested by *t* test at 95% confidence) in (**Figure 4.2**).

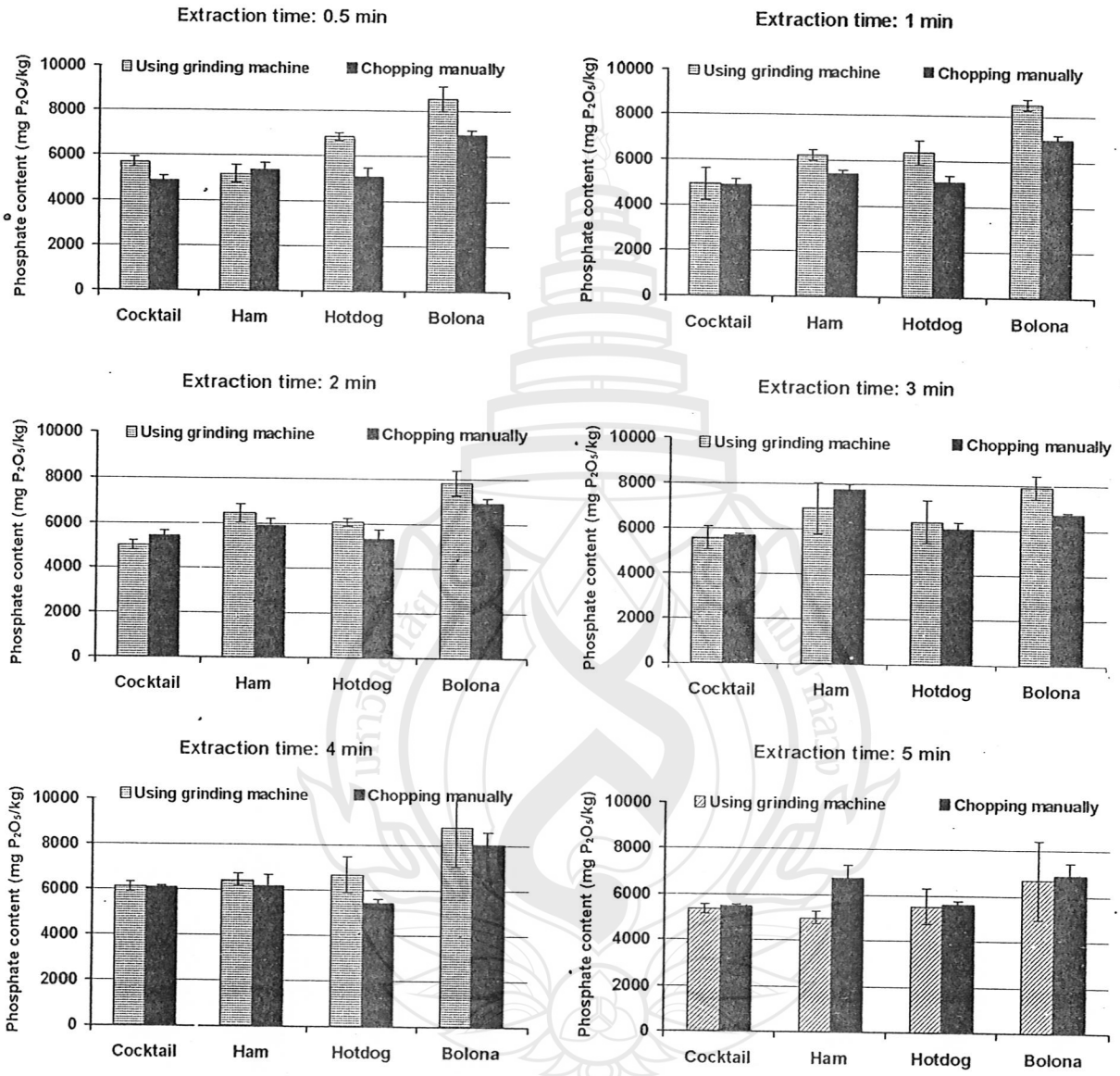

Figure 4.2 Effect of the method for increasing the surface area of the sample at the different extraction time

However, at the extraction time of 0.5 min, the phosphate contents in the samples that were ground by a grinding machine were found to be higher than those samples that were chopped manually. It could be explained that the surface area of the

ground meat product samples was higher than that of the chopped samples. When the samples were shaken in water, phosphates in the higher surface area samples could dissolve more than those in the lower surface area samples. When the extraction time was increased, the phosphates in both the ground and the chopped samples could dissolve at the similar degree due to the ordinary content of phosphates because all phosphates could dissolve when more time was applied.

Considering the precision of the methods, it was found that the relative standard deviations (RSD) of the phosphate contents extracted from the ground samples (2-26%RSD) were higher than those from the chopped samples (0.1-8%RSD). This was because shaking horizontally could not break up the cluster of the ground meat product although the speed of shaking was high. Thus, increasing the surface area of the samples by chopping manually would be used to analyze the samples.

4.2.2 Extraction time

To extract the phosphates from the meat product samples, the meat-water mixture was shaken by a shaker at the highest speed (600 rpm) at different extraction times. It was found that the extracted phosphate content was independent of the extraction time (**Figure 4.3**). The difference of the extracted phosphate contents at the different extraction times was not significant (tested by *t* test at 95% confidence).

However, since the purpose of this study was also to study the feasibility on producing a simple phosphate test kit for the determination of phosphate in meat product samples. the extraction time of 0.5 – 3 min was employed for the study of manually shaking.

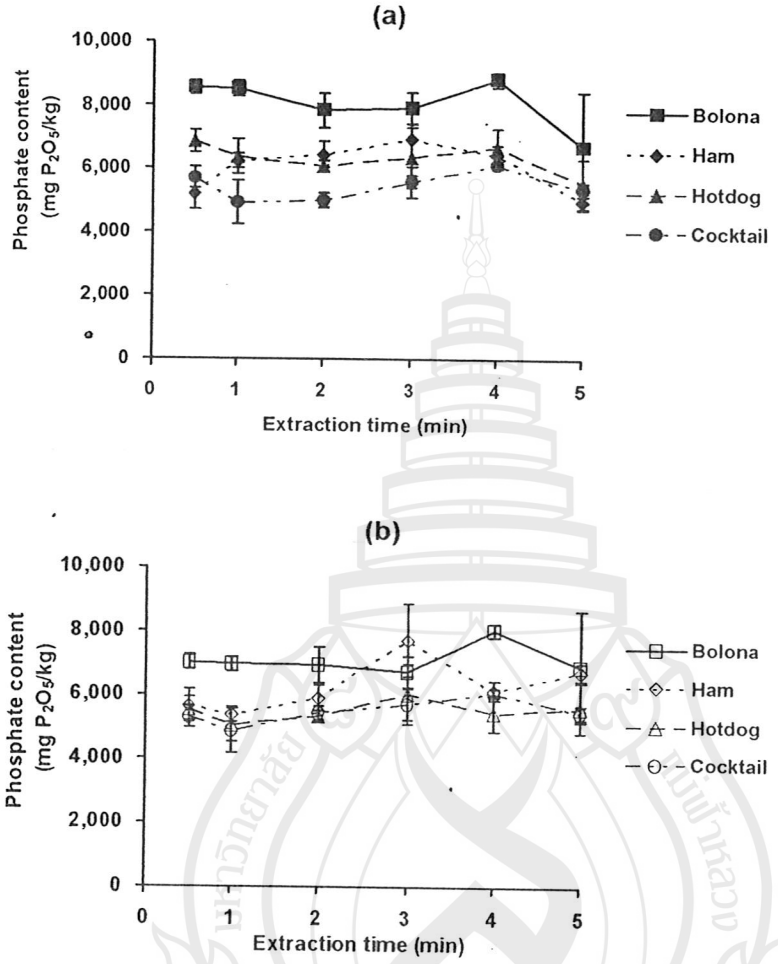

Figure 4.3 Effect of extraction time on the phosphate content in some meat product samples that were increased the surface area by (a) using grinding machine, (b) chopping manually

4.2.3 Extraction by manually shaking

In order to simplify the extraction method and reduce the cost of analysis, we attempted to study the extraction by manually shaking. Effect of the extraction time (0.5, 1, 2, and 3 min) and the method to increase the surface area of sample (using a grinding machine and chopping manually) was studied.

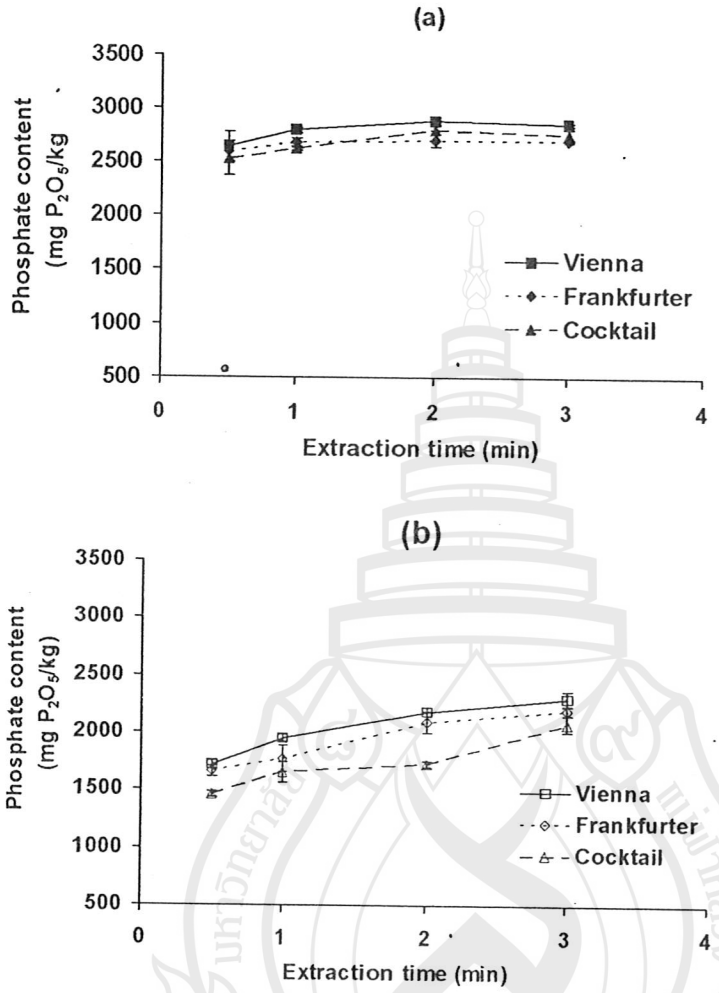

Figure 4.4 Effect of the extraction time and the method for increasing the surface area of the samples: (a) using a grinding machine and (b) chopping manually, that were extracted by shaking manually.

Each sample was shaken, triplicate by three experimenters. The phosphate contents extracted from the ground samples seemed to be independent of the extraction time while those from the chopped samples were dependent of the extraction time (**Figure 4.4**). This was contradictory to the results obtained by using a shaker (**Figure 4.3**) because the speed and, thus, the power of shaking by a man were obviously slower and less than those of the shaker. The phosphates in the samples with higher surface area (ground samples) were dissolved easier than those with lower surface area (chopped samples). Therefore, the phosphates extracted from the ground samples became constant at the extraction time of 1-3 min whereas those from the

chopped samples increased gradually. Nevertheless, the extracted solutions from the ground samples were highly fatty and turbid. It was very hard to filter the solutions through the 200-nm filter. We, thus, employed the chopping method for increasing the surface area of the sample. In term of the extraction time, 1.5 min was selected because the phosphate contents were increased slowly from 1-3 min and it was not too long for shaking manually.

Comparing shaking by a shaker and shaking manually, the range of the RSDs of the phosphate contents obtained by using a shaker (0.1-26%) was wider than those by shaking manually (0.4-6.5%) even when the longer extraction time (4-5 min) was used. It was because manually shaking was performed in all directions while the shaker performed horizontally move. Thus, by shaking manually, it was easier to split the cluster of the meat product sample into small pieces and gave the better precision. In addition, shaking manually costs nothing.

Therefore, we prepared the samples by chopping, extracted the phosphates from the samples by shaking manually, and used the extraction time of 1.5 min.

4.3 Validation of the Proposed Method

The validation of the proposed method was performed by employing the vanadomolybdate method, preparing the samples by chopping manually, extracting by shaking manually at the extraction time of 1.5 min. Percentage recovery was determined. The results obtained by the proposed method were compared statistically with those by the standard spectrophotometric method.

4.3.1 Percentage recovery

Percentage recovery could indicate the accuracy of the method. To determine the percentage recovery, certain amount of orthophosphate standard was added to seven meat product samples. Five replicates were performed. Percentage recovery of 85-115% was found (Table 4.1) and satisfactory.

Table 4.1 Percentage recovery of phosphates obtained by the proposed method

Sample No.	Phosphate content (mg P_2O_5/kg)*				%Recovery
	Sample	Sample + Standard	Recovered	Added	
1	2670	4861	2191	2290	96
2	2079	4713	2634	2290	115
3	2728	4772	2044	2290	89
4	2632	4589	1957	2290	85
5	2411	4885	2474	2290	108
6	2162	4730	2568	2290	112
7	2674	4711	2037	2290	89

*Average from five replicates

4.3.2 Comparison of the proposed and traditional methods

The traditional method for the determination of phosphate in meat samples is a spectrophotometric method based on vanadomolybdate reaction. The sample to be analyzed must be ashed at 600 °C and dissolve with concentrated acids (AOAC Official Method 986.24). The phosphate content was calculated from total phosphorus content. Thus, in this work, we compared the total phosphorus content calculated as mg P_2O_5/kg of sample with the water-soluble phosphate obtained by the proposed method.

Figure 4.5 shows that the total phosphate determined by the traditional method was higher than the water-soluble phosphate determined by the proposed method as expected. Interestingly, for all samples, the total phosphate was approximately two times higher than the water-soluble phosphate. We then analyzed more samples and used the factor two to multiply the water-soluble phosphate. It was found that the results obtained by the proposed method were not significantly different from those by the traditional method (tested by *t* test at 95% confidence) (**Figure 4.6**).

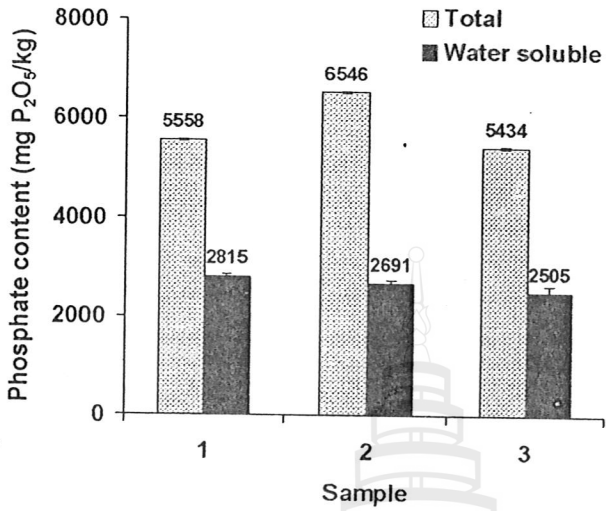

Figure 4.5 Comparison of the total phosphate determined by the traditional method and the water-soluble phosphate determined by the proposed method in three samples

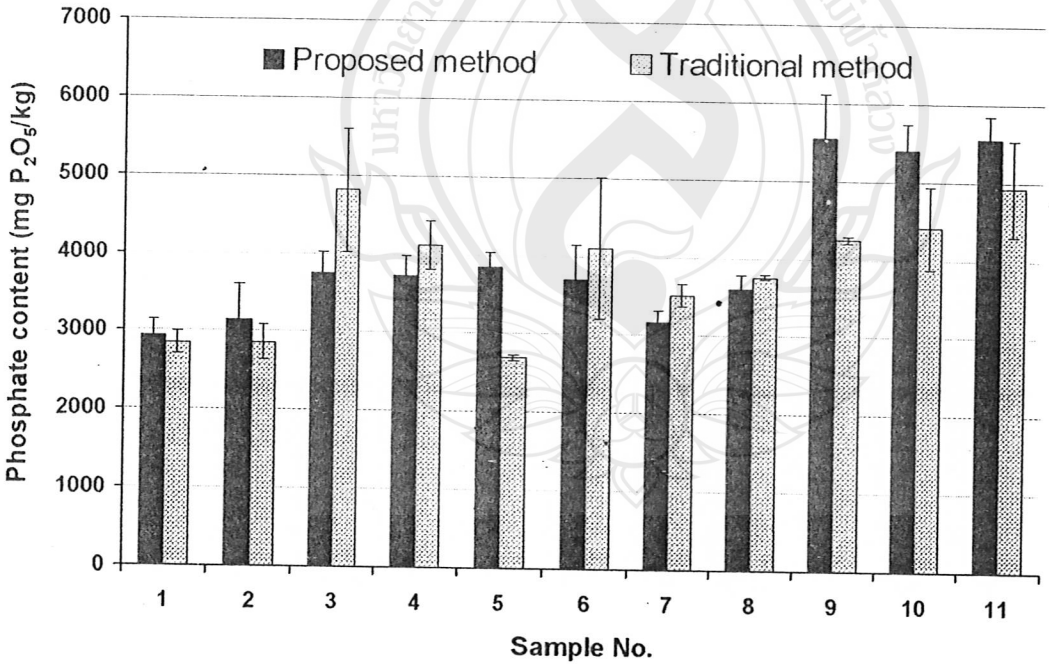

Figure 4.6 Comparison of the two times phosphate content determined by the proposed and the total phosphate determined by the traditional methods

This proposed method was also applied to determine phosphate content in fresh meat samples. **Figure 4.7** compares the phosphate contents obtained by the proposed method with those obtained by the standard method. The results showed the significant difference in the precision of the two methods (tested by F test). This could be due to the complex system in the fresh meat samples. Acid digestion might results in hydrolysis of pyro- and other polyphosphates to orthophosphates [Molins, 1991]. Thus the precision of the standard method was found to be quite dissatisfied. However, the analysis of fresh meat samples should be done again in order to be able to compare with the results obtained by the proposed method.

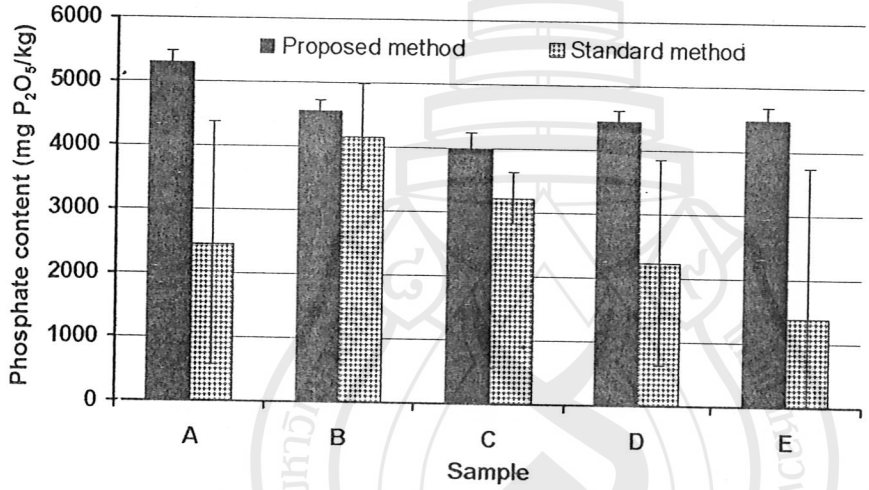

Figure 4.7 Comparison of the phosphate contents in some fresh meat samples determined by the proposed and standard methods

4.4 Possibility on Producing Phosphate Test Kit for Fresh Meat and Meat Products

From the study of the simple method for the determination of phosphate in meat product and fresh meat samples, it was feasible to develop a test kit for this purpose. The simple phosphate analysis method was the vanadomolybdate method. The method used only one reagent, and was somewhat time-independent. The method of preparing the samples was chopping manually, which was unproblematic for customers. Extracting water-soluble phosphates with pure water was highly safe and reproducible. The extraction time of 1.5 min was practical. Moreover, using the factor

of two to compensate the phosphate content provided no significant difference compared to the total phosphate content determined by acid digestion and the standard method. The proposed method could be applied to real samples. Therefore, it is highly possible to develop a test kit using this method for the determination of phosphate in meat product and fresh meat samples.

CHAPTER 5
CONCLUSIONS

The study of the simple method for phosphate analysis in meat and meat product samples was performed. The phosphate analysis methods based on spectrophotometry were studied. There were two standard spectrophotometric methods widely used for the determination of phosphates: (i) molybdenum blue (MB) and (ii) vanadomolybdate (VM) methods. It was found that the MB method provided the linearity in a lower concentration range (0.2 – 1.2 ppm P) whereas the VM method gave the dynamic range in a higher concentration range (5 – 20 ppm P). Phosphate contents in meat products and meat samples were found in the higher concentration range (5-20 ppm P). Therefore, the VM method provided the suitable dynamic rage for our purpose. In addition, although the MB method was a rapid and the developed blue color was observed easily, the reaction was rather time- and temperature-dependent. On the other hand, the VM reaction that produced a yellow complex was independent of temperature and simpler due to less steps of analysis. Thus, the VM method was selected.

To prepare a meat product or meat sample, digesting the sample with acids or ashing with acids was suggested by the traditional standard methods for phosphate assay in food samples. In order to reduce the tediousness of the sample preparation in the traditional method, a simple solvent extraction method was studied. The solvent used was pure water. The method for increasing the surface area of the sample (grinding and chopping) and the effect of extraction time were studied. There was no significant difference between the phosphate contents obtained from the ground and chopped samples. However, the chopping method provided better reproducibility (0.1-8 %RSD). Thus, the chopping method was employed. Extraction time of 0.5-5 min was studied. It was found that the extracted phosphate content was independent of the extraction time. However, extraction time of 0.5-3 min was used for the study of extracting manually. The extraction time of 1.5 min was found to be suitable for extracting manually because it was practical, and provided satisfactory precision.

The accuracy of the proposed method was studied by determining the percentage recovery and comparing the results obtained by the traditional standard method and the proposed method. The percentage recovery of 85-112% was achieved. The comparison of the phosphate contents in both meat product and fresh meat samples obtained by the standard method and that obtained by the proposed method showed that the phosphate contents obtained by the former method was approximately two times higher than those obtained by the later method. Interestingly, multiplying the phosphate contents found by the proposed method by two did not produce the significant difference from those found by the standard method.

Therefore, it was feasible to produce a relatively low-cost, practical and simple test kit for phosphate determination in meat product and fresh meat samples. The meat sample was simply extracted using pure water. The extracted solution was filtered and was added with the VM reagent to develop a yellow complex. Then comparing the sample solution with the known-concentration standard solution had to be done.

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APPENDICES

APPENDIX A

Calculation of Phosphate Content as P_2O_5

The concentrations of phosphorus in mg/l or ppm in the diluted samples were calculated from the obtained calibration graph. (Suppose that the concentration of phosphorus in a diluted sample obtained from the calibration graph was X mg/l) Then, to express the actual phosphate content of a phosphate without the mineral part such as sodium, potassium or calcium attached to it [Feiner, 2006], the phosphate content as P_2O_5 was calculated as follows:

Step 1: Calculate the concentration of phosphorus in the sample

The sample (2.00 ml) was diluted to 10.00 ml by adding 2.00 ml of reagent and water. The concentration of phosphorus in the extracted sample was 5 times more concentrated. Thus the concentration of phosphorus in the sample was 5X mg/l.

Step 2: Calculate the milligram of phosphorus in 25 ml of the sample solution

Solution 1000 ml contains phosphorus 5X mg

Thus solution 25 ml contains phosphorus [(5X mg) x (25 ml)]/1000 ml

= 0.125X mg

Because the sample solution (25 ml) was prepared from 1.25 g of sample, 0.125X mg is in 1.25 g of sample.

Step 3: Calculate mg P/g sample

mg P/g sample = 0.125X mg/1.25 g

= 0.1X mg/g

Step 4: Calculate mg P/kg sample

mg P/kg sample = (0.1X mg/g) (1000 g/kg)

= 100X mg/kg

Step 5: Calculate mg P_2O_5/kg sample

P_2O_5 1 mole (142 g) contains P 2 moles (62 g).

Thus P 100X mg is in P_2O_5 [(142 g/mol)/(62 g/mol)]*100X = 229X mg

Therefore, if there was X mg/l phosphorus in the measured sample, the phosphate content as P_2O_5 was 229X mg/kg sample.

APPENDIX B

The statistical *F* test

The *F* test is used to determine if there is a significant difference between two methods based on their standard deviations. *F* is defined in terms of the variances of the two methods, where the **variance** is the square of the standard deviation:

$$F = \frac{s_1^2}{s_2^2}$$

where $s_1^2 > s_2^2$. There are two different degrees of freedom, ν_1 and ν_2 , where degree of freedom is defined as *N*-1 for each case.

If the calculated *F* value from the above equation exceeds a tabulated *F* value at the selected confidence level, then there is a significant difference between the variances of the two methods. A list of *F* values at the 95% confidence level is given in **Table B1**.

Table B1 Values of *F* at the 95% confidence level

	ν_1=2	3	4	5	6	7	8	9	10	15	20	30
ν_2=2	19.0	19.2	19.2	19.3	19.3	19.4	19.4	19.4	19.4	19.4	19.4	19.5
3	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.70	8.66	8.62
4	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.86	5.80	5.75
5	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.62	4.56	4.50
6	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	3.94	3.87	3.81
7	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.51	3.44	3.38
8	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35	3.22	3.15	3.08
9	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	3.01	2.94	2.86
10	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.85	2.77	2.70
15	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54	2.40	2.33	2.25
20	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.20	2.12	2.04
30	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.01	1.93	1.84

APPENDIX C

The student *t* test

The *t* test is used to determine if two sets of measurements are statistically different. In this method, comparison is made between two sets of replicate measurements made by two different methods; one of them will be the test method, and the other will be an accepted method. A statistical *t* value is calculated and compared with a tabulated value for the given number of tests at the desired confidence level (**Table C1**). If the calculated *t* value exceeds the tabulated *t* value, then there is a significant difference between the results by the two methods at that confidence level. If it does not exceed the tabulated value, then we can predict that there is no significant difference between the methods. This in no way implies that the two results are identical.

Table C1 Values of *t* for *v* degrees of freedom for various confidence levels

v	Confidence Level			
	90%	95%	99%	99.5%
1	6.314	12.706	63.657	127.32
2	2.920	4.303	9.925	14.089
3	2.353	3.182	5.841	7.453
4	2.132	2.776	4.604	5.598
5	2.015	2.571	4.032	4.773
6	1.943	2.447	3.707	4.317
7	1.895	2.365	3.500	4.029
8	1.860	2.306	3.355	3.832
9	1.833	2.262	3.250	3.690
10	1.812	2.228	3.169	3.581
15	1.753	2.131	2.947	3.252
20	1.725	2.086	2.845	3.153
25	1.708	2.060	2.787	3.078
∞	1.645	1.960	2.576	2.807

Three ways in which a t test can be used are defined. If an accepted value of μ is available (from other measurements), then the test can be used to determine if a particular analysis method gives results statistically equal to μ at a given confidence level. If an accepted value is not available, then a series of replicate analyses on a single sample may be performed using two methods, or a series of analyses may be performed on a set of different samples by the two methods. One method should be an accepted method. In this work, the method of paired t test was employed.

Paired *t* test

In the chemistry laboratory, a new method is frequently tested against an accepted method by analyzing several different samples of slightly varying composition (within physiological range). In this case, the t value is calculated in a slightly different form. The difference between each of the paired measurements on each sample is computed. An average difference $\overline{D}$ is calculated and the individual deviations of each from $\overline{D}$ are used to compute a standard deviation s_d. The t value is calculated from

$$t = \frac{\overline{D}}{s_d}\sqrt{N}$$

$$s_d = \sqrt{\frac{\sum\left(D_i - \overline{D}\right)^2}{N-1}}$$

where D_i is the individual difference between the two methods for each sample, with regard to sign; and $\overline{D}$ is the mean of all the individual differences.