USE OF THIN LAYER CHROMATOGRAPHY IN DETECTING THE ADDITION AND DEGRADATION OF SODIUM PHOSPHATES IN SEAFOOD PRODUCTS

By

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To my parents, Eric and Helen Holmberg, and to all who nurtured my intellectual curiosity, academic interests, and sense of scholarship throughout my lifetime, making this milestone possible

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
CFR	Code of Federal Regulations
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
G&H	Gutted and headed
GMP	Good manufacturing practices
GRAS	Generally recognized as safe
HPLC	High performance (or pressure) liquid chromatography
ICP-MS	Inductively coupled plasma - mass spectroscopy
Metol	4-methylaminophenolsulfate
MP	Monophosphate
OP	Orthophosphate
P	Phosphorus
PP	Pyrophosphate
RNA	Ribonucleic acid
SHMP	Sodium hexametaphosphate
STPP	Sodium tripolyphosphate
T	Temperature
TLC	Thin layer chromatography
TCA	Trichloracetic acid
TPP	Tripolyphosphate

Abstract of Thesis Presented to the Graduate School
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Sodium phosphates are added to many food products including seafood. Proper use of these additives can help improve the texture and flavor of products, as well as, reduce the drip loss during freezing, thawing, and cooking. Over-treatments can actually diminish the quality of seafood products and can even be considered adulteration due to excessive additions of water. Labeling issues have arisen and mislabeling continues due to the lack of testing for phosphate additives. Accurate detection methods are necessary to better direct phosphate use and to assure compliance with regulatory and customer expectations. Since seafood, such as shrimp and fish, naturally contain variable levels of phosphates in the form of monophosphate, detecting polyphosphates is necessary to positively conclude that phosphates have been added. High performance liquid chromatography (HPLC) can be used to identify and measure polyphosphates, however, this requires expensive equipment that may not be available or feasible in many cases. Thin layer chromatography (TLC) is a considerably more affordable and practical method that can clearly indicate whether a seafood product contains any added sodium polyphosphates, yet some previous studies found TLC lacks sensitivity to detect routine additions. Subsequent studies

showed TLC does have potential use for detecting phosphate additives in shrimp. The objectives for this study were to thoroughly test and describe a TLC method to be used as a practical detection device for the addition of polyphosphates in seafood. The main focus was to delineate the TLC methods so it can be easily replicated. Shrimp and Alaska pollock were treated in different commercial phosphate and non-phosphate blends, frozen, and tested with the TLC method every three months for one year. The TLC plates showed spots for polyphosphates in all samples treated with phosphates throughout the full year of frozen storage. Sensitivity testing found less than 0.05% added phosphate could be detected. Non-phosphate and control samples only showed monophosphate spots every time they were tested. It was concluded that the TLC method described in this work is a useful and practical option for routinely testing for phosphate additives in seafood. This method is now available for reference and practical utilization in commerce, regulation and research.

CHAPTER 1
LITERATURE REVIEW

Introduction

Sodium phosphates are added to many food products to improve and maintain favorable quality attributes. When properly used with seafood, they can help improve the texture and flavor of products, as well as reduce the drip loss during freezing, thawing, and cooking. Unfortunately, over-treatments can actually diminish the quality of seafood products and be considered adulterants due to excessive additions of water. The use of phosphate in seafood has always presented problems with regulation due to the history of misuse and lack of practical detection methods. Accurate detection methods are necessary to better direct phosphate use and to assure compliance with customer expectations. Since seafood such as shrimp and fish naturally contain variable levels of inherent phosphate, in the form of monophosphate, detecting added polyphosphates is necessary to positively conclude that phosphate additives have been used. High performance liquid chromatography (HPLC) can be used to identify and measure polyphosphates, however, this requires expensive equipment that may not be available or feasible in many cases. It is hypothesized that a more convenient thin layer chromatography (TLC) method can be developed and standardized for a considerably more affordable and practical application to indicate whether a seafood product contains any added sodium polyphosphates.

Phosphate

The element phosphorus (P) is essential for all living organisms and is almost exclusively utilized in the form phosphate anion (PO_4^{3-}). The various chemical functions of phosphate in biological systems are extensive and complex as it is present in DNA

(deoxyribonucleic acid), RNA (ribonucleic acid), ATP (adenosine triphosphate), phospholipids, nucleoproteins, phosphoproteins, and many other compounds essential for life. Phosphates also play key roles in many biological processes such as energy storage and exchange, as well as synthesis and breakdown of carbohydrates, proteins (including nucleic acids, hormones, and enzymes), and lipids (Molins 1991).

Much of the functionality of phosphates is based on the ability to form chains which is utilized in a variety of ways in biological systems. One of the most important functions of phosphate is its role in the energy transfer mechanisms as chemical bond energy is transformed into other forms of energy. The exchange of phosphate from ATP is the main source of energy transfer in most biological processes (Ellinger 1972). Since phosphate is necessary for muscle contraction, as well as playing a central role in the changes that occur during rigor mortis, phosphate is naturally present in all meats.

The phosphate molecule consists of one phosphorus atom covalently bonded to four oxygen atoms forming a tetrahedral shape in the case of monophosphate (orthophosphate). This basic structure can polymerize by dehydration reactions forming chains of polyphosphates such as pyrophosphate (PP) and tripolyphosphate (TPP). These polyphosphates can be separated and are typically crystallized into sodium salt forms. Sodium phosphate salts are commonly used in foods, in addition to other compounds that can be formed with potassium, calcium, ammonium, iron, and magnesium. Many of these compounds have several different names due to different methods of nomenclature (Molins 1991). To simplify, all phosphate molecules can be classified into four groups: orthophosphates which are not attached to any other phosphate tetrahedron; polyphosphates (including pyrophosphate) which are, at most,

linked to two other phosphate tetrahedra; metaphosphates in which the polyphosphate chain attaches to itself forming a ring; and ultraphosphates which contain at least one phosphate tetrahedron joined to three others (Branen 2002). The orthophosphates, polyphosphates, and metaphosphates will be discussed as ultraphosphates are not commonly used in foods.

Figure 1-1 shows the basic chemical structure of four phosphate ions that are commonly found and used in seafood products: orthophosphate, pyrophosphate, tripolyphosphate, and hexametaphosphate. These ions are especially important to this study as they are the four compounds detected in the TLC method being researched. Orthophosphate is naturally present in the muscle tissue of all seafood and is therefore found in both untreated and phosphate added products. Pyrophosphate and tripolyphosphate are the most common phosphates used for moisture retention in seafood products and some blends also contain hexametaphosphate. All of the polyphosphates are formed from orthophosphates by driving off water and are thus referred to as condensed phosphates. Each of the compounds has different properties and is used in various foods. Some examples of different phosphate compounds used in food and some of their functions are shown in Table 1-1. The natural levels of phosphorus in various foods are shown in Table 1-2.

Phosphates in Seafood

Virtually all foods contain phosphate and muscle foods such as meats and seafood naturally contain variable amounts of phosphorus in the form of the phosphate anion (Ellinger 1972). Much of the source of natural phosphorus in muscle foods is found in adenosine triphosphate (ATP) which is the immediate source of energy in these biological systems. The amount of phosphorus in muscle foods can be highly

variable. For example, the typical concentration of endogenous total phosphorus in white shrimp (*Penaeus setiferus*) when harvested from the water has been reported to range from about 187 to 233 mg per 100 grams (Sidwell 1981). Other studies have found common GMP's (good manufacturing practices) yield shrimp with total phosphorus contents ranging from 140 to 290 mg/100g depending on exposure time to ice slush (Garrido and others 1999). As the shrimp is processed, this level typically decreases due to the addition of water which dilutes the compounds.

Polyphosphates are used in seafood for their unique properties to protect quality which is mainly due to its ability to bind water. The mechanisms involved in the hydration of proteins and the effects of phosphate are complex. The main factors affecting protein solubility in water are pH, ionic strength, and temperature. The pH influences the net charge of the protein molecule which determines its solubility. In general, proteins are least soluble at the isoelectric point(s) where there is no net charge. Solubility increases as pH is raised or lowered away from the isolectric point and the net charge becomes more polar. Ionic strength is a measurement that includes the concentration and valence of ions, and is therefore increased with the addition of phosphates. As ionic strength is increased, the ions bind to oppositely charged ionized groups on protein molecules. This results in a decrease of electrostatic attraction between protein molecules, thus aggregation is reduced and protein solubility is enhanced. Furthermore, the ions attached to the proteins also increase attraction to water allowing for greater protein solubilization. This combined effect is referred to as “salting in.” Phosphates have a strong effect on the solubility of proteins because it

alters their net charge by affecting the pH and increasing the ionic strength of solutions (Molins 1991).

This water binding capacity of phosphates results in less weight loss during cooking and reduced thaw-drip losses of frozen products. Therefore when used properly, phosphate additives can maintain the yield and water content of the final product which can also enhance the textural quality. Phosphates affect other properties of seafood as well, including improved taste or desirable flavor, stabilizing proteins against denaturation, improving emulsification and buffering capacity, nutrient contribution, metal chelating and possible antioxidant activity (Molins 1991).

Phosphates can be added to seafood products in many different ways as there is no standard method for treatment. The most common and simple way of incorporating phosphate into a seafood product is referred to as a “static soak.” This basically involves submersing the seafood in a phosphate solution of a particular concentration for any amount of time (Shimp 1983). Absorption of the phosphate into the tissue can be adjusted by varying the concentration of the phosphate solution and/or by varying the soak time. Other methods of treatment can also be used to enhance or increase the rate of phosphate absorption. The product can be tumbled with the phosphate solution for more uniform coverage and increased absorption. A vacuum can also be pulled which forces the treatment solution into the tissue of the seafood product (Kin and others 2009).

The most thorough and rapid absorption would be accomplished by a combination of treatment methods, for example: placing the seafood in a container with the phosphate solution, pulling a vacuum, and then tumbling the vacuum sealed

container. One other incorporation method used is actually injecting the treatment solution directly into the tissue using needles (Swart 1971). This can be done on a large scale using machines that inject many needles at once into a product to incorporate the phosphate solution rapidly and automated for commercial processors. Phosphate salts can also be added as a dry mix in certain products such as sausages or surimi as extra ingredients in the mixture of meat and seasonings (Kanayama and others 1994).

In addition to the method used for treating the seafood, several other variables can affect the absorption of phosphate additives and water uptake including the permeability of the particular tissue, dip time, blend type and concentration, temperature and pH. Tenhet and others (1981b) investigated the penetration mechanism for white and brown shrimp dipped in different concentrations of polyphosphates for various times. It was found that at low treatment concentrations of 0.5% STPP (sodium tripolyphosphate), an apparent surface reaction forms a concentration equilibrium or barrier-type restriction to further STPP uptake. Treatment at higher concentrations (5% and 10% STPP) caused substantial uptake even in the interior fractions of the shrimp. Other treatment methods can also be used to enhance uptake such as injecting the phosphate solution directly into the tissue, tumbling the product with the solution, and pulling a partial vacuum on the product and phosphate solution mixture (Sturno and others 1986).

While proper use of phosphate additives in seafood can help improve the texture and flavor of products, as well as, reduce the drip loss during freezing, thawing, and cooking (Tenhet and others 1981a), over-treatments can actually diminish the quality of

seafood products. Excessive addition of phosphates can cause the seafood product to become slimy and glassy with higher water content. The water content can be increased with phosphates to a point where it can even be considered adulteration since the consumer is essentially being sold water (Sturno and others 1987). This improper use of phosphates is a potential problem with the lack of regulations on phosphates in the United States.

Hydrolysis of Polyphosphates

Polyphosphates in foods naturally separate into smaller phosphate molecules over time through hydrolysis reactions until, eventually, all of the phosphate is in the form of orthophosphate. This hydrolysis of the polyphosphates must be considered in food systems because of differences in chemical properties between different phosphate molecules. For example, the resulting orthophosphate products from hydrolysis do not have the same water binding properties as the tripolyphosphate molecule originally added to the food product (Crowther and Westman 1953). It is also very important for detection methods such as TLC which depend on the presence of polyphosphates to positively distinguish the presence of added phosphates.

The basic chemistry of the polyphosphate hydrolysis reaction generally involves cleavage at the terminal PO_4 groups rather than scission at a middle group. However, under very low pH conditions, long polyphosphate chains can rupture at random locations to form shorter chain polyphosphates. Hydrolysis of polyphosphates continues to occur until all phosphate is in the form of orthophosphate. For example, tripolyphosphate will cleave into a pyrophosphate and orthophosphate molecule. The resulting pyrophosphate will then cleave into two orthophosphate molecules. It is believed that one step cleavage of tripolyphosphate into three orthophosphate ions

generally never takes place (Molins 1991). The basic reaction equation for polyphosphate hydrolysis is shown in Figure 1-2 as it displays the cleavage of pyrophosphate into two orthophosphate molecules.

The polyphosphate hydrolysis reactions occur spontaneously and are temperature dependent. Hydrolysis can be slowed under low storage temperatures and the reactions increase with higher temperatures. Polyphosphates are also relatively stable in neutral solutions, however, lowering or raising pH levels away from neutral can also accelerate hydrolysis reactions (Molins 1991). Likewise, raw seafood products contain phosphatase enzymes which catalyze the hydrolysis reactions and rapidly increase the reaction rate at temperatures above freezing. These enzymes are endogenous in raw seafood tissues and rapidly increase polyphosphate degradation (Reddy and Finne 1986). When the seafood is cooked, however, the phophatase enzymes are denatured and are therefore no longer functional.

Reddy and Finne (1986) investigated the polyphosphate hydrolysis reactions in shrimp under various storage temperatures comparing the reaction rates with and without the phosphatase enzyme. It was found that presence of the enzyme resulted in a six-fold increase in the rate of hydrolysis of sodium tripolyphosphate at 5°C and a four-fold increase at 10°C. Sodium hexametaphosphate hydrolyzed ten times faster at 5°C and eight times faster at 10°C than the uncatalyzed reactions. It was also observed that tripolyphosphate completely broke down into monophosphate within 12 days at 5°C and within 15 days at 10°C in the presence of phosphatase (Reddy and Finne 1986). If samples are cooked prior to storage, the phosphatase enzymes can be denatured and are no longer available to catalyze the hydrolysis reactions. The inactivation of

phosphatase is reported to occur between 60°C and 85.6°C (Painter and Bradley 1997). Eckner (1992) reported the D-values for (alkaline phosphatase) ALP to range between 6.04 min at 63°C to 0.35 min at 71°C, with a Z-value of 6.7°C. Commercially cooked shrimp are typically steamed or boiled, which is 100°C, and certainly high enough temperature to inactivate phosphatase enzymes. Therefore, polyphosphates would remain intact in cooked products for significantly longer periods of time.

Phosphate Regulations

In the United States, common phosphate additives used in the food industry are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) as stated in the Federal Register (FDA 21 CFR 182.1810, 182.6760, 182.6787). Food grade phosphates for seafood can be used in United States commerce without restriction but are meant to be used in amounts to achieve the "intended effect" and are processed in accordance with the good manufacturing practices (GMP) found in 21 CFR 110. Prior use of phosphates must be declared in the ingredient statement on the product label. In contrast, there is a guideline or limit set in Europe of 0.5% (expressed as P_20_5 or phosphate) as the allowable level that can be added to filets of unprocessed, frozen and unfrozen fish; and unprocessed and processed mollusks and crustaceans, frozen and deep frozen (European Parliament and Council Directive 1995). This document also specifies that "the maximum levels of use indicated refer to foodstuffs ready for consumption prepared following manufacturers' instructions" (European Parliament and Council Directive 1995). The total phosphorus in the cooked seafood products are measured, then multiplied by 2.287 for conversion to phosphate (% P_20_5). The amount of added phosphate is then determined by subtracting the natural phosphate level, expected to be 0.5% phosphate (P_20_5), from the total amount found in

the cooked seafood. Theoretically, the total phosphate (P_20_5) level in most European seafood could range as high as 1.0% P_20_5 which represents the maximum amount allowed in treated seafood (Souci and others 2008).

The same level of 0.5% phosphate (P_20_5) as referred to in Europe, is also the limit set for use of phosphate additives in the processing of meat and poultry in the United States for the intended effect to “decrease the amount of cooked out juices” and “help protect flavor” (9 CFR Part 381.147 and 9CFR Part 318.7). The U.S. FDA proposed a regulation in 1979 for a 0.5% limit of added phosphates in seafood products, but it has never been approved to be enforced (Federal Register 18 Dec 1979: 74845).

There is additional confusion with the measurement of phosphate or phosphorus in food products. Some levels are reported as “total phosphorus” while others are reported as phosphate content expressed as P_20_5. This is a considerable difference as a reported phosphate content is more than double the value of phosphorus content. For example, phosphate content expressed as mg/100 g edible shrimp is equivalent to 2.286 times the corresponding phosphorus content (Otwell 1993). Since this significant difference is often overlooked or unrealized, there can be a large discrepancy between the understanding of reported phosphate levels. With the lack of regulation and enforcement, phosphate usage and proper labeling of phosphate additives has continued to be a controversial and problematic issue in the seafood industry. Accurate detection methods are necessary to better direct phosphate use and to assure compliance with customer expectations.

Methods of Detection

Since seafood such as shrimp and fish naturally contain variable levels of phosphate, in the form of monophosphate (Tenhet and others 1981a), the measurement of total phosphorus alone is not sufficient evidence of phosphate additives. Therefore, detecting polyphosphates (i.e. tripoly-, pyro-, hexametaphosphates) is necessary to positively conclude that phosphates have been added (Heitkemper and others 1993). An important limitation to consider for phosphate detection is the fact that polyphosphates hydrolyze into smaller phosphate molecules over time until all detectable phosphates are eventually in the form of monophosphate, which is indiscernible to the phosphate that is naturally present in the animal tissue.

One detection method which has been used for phosphate detection in seafood is high performance liquid chromatography (HPLC) (Sturno and others 1987). HPLC involves an instrument that pumps a prepared sample solution through a column which separates molecules based on the differences in affinities of the stationary phase. The separated compounds then exit the column at different times and are analyzed by a detector. The separated molecules can then be identified thus determining the presence and concentration of the components of the sample. The main disadvantage to this method, however, is that it requires expensive equipment that may not be available or feasible for practical, routine applications.

Thin layer chromatography (TLC), on the other hand, is a considerably more affordable and practical method that can clearly indicate whether a seafood product contains any added polyphosphates. TLC separates molecules based on their individual affinities to the mobile phase and stationary phase. Phosphate can be extracted from seafood products and run through a TLC plate. After separation, the

plate is treated with visualizing solutions and heat. Each phosphate compound can then be seen as a distinct spot. The presence and position of a spot is used to identify the compound such as tripolyphosphate or pyrophosphate. Therefore, a tripolyphosphate spot would indicate that the sample has been treated with polyphosphates.

Some initial applications of TLC to monitor for phosphates in seafood were presented by Tenhet and others (1981a), which were modified methods introduced by Gibson and Murray (1977) and Stahl (1969). Tenhet and others (1981a) used the TLC method to detect the presence of phosphate residuals in shrimp treated with solutions of sodium tripolyphosphate (STPP). The shrimp were treated in a variety of concentrations 0.5%, 1.0%, 5.0, and 12% with different dip times of 20 seconds, 1 minute, 5 minutes, and 20 minutes. Two extraction solutions were used, 7% trichloracetic acid (TCA) and distilled water, where 100 ml was blended with 50 g of the shrimp sample and then centrifuged at 5000 x G for 20 minutes at 4°C. They found that the samples extracted with TCA resulted in hydrolysis of polyphosphates as the reaction progresses under acidic conditions. Tenhet and others (1981a) favored TLC analysis using water extraction, but concluded that the method was not sensitive enough for analyzing added phosphates in treated shrimp (Tenhet 1981a). This lack of sensitivity may be attributed to several factors. First of all, 100 ml of extraction solution (TCA or water) was added to 50 g of the shrimp samples so the phosphate concentrations were diluted about three-fold. Also, the samples were centrifuged for a prolonged time (20 minutes) which would allow for hydrolysis of the polyphosphates. Furthermore, only STPP was used as a polyphosphate additive in this study. STPP alone does not absorb as quickly and

thoroughly as some of the current common commercial phosphate blends that can contain buffers and salts to aid penetration. The differences between these previous studies from this research are discussed in later sections and contribute to its higher detection sensitivity.

Another study by Reddy and Finne (1985) treated shrimp samples in STPP only and also performed extraction by blending with distilled water (1:2). There were several differences in the solvents used for the mobile phase and spraying solutions from those used in the current study. It was stated that hydrolysis of sodium triphosphate in treated shrimp will occur sometime after 5 weeks of frozen storage. This also differed from the current study which will be discussed in the results. This study did conclude that TLC is a useful method for determining whether or not a fish has been treated with polyphosphates.

Later work by Krzynowek and Panunzio (1995) used a TLC method to determine presence of polyphosphates in cod and scallops treated with 10% STPP at 1 minute and 10 minutes. Again, extracts were made blending a 1:2 sample to water ratio. In addition, the drip loss from the thawed samples was also used as a spotting solution on the TLC plates. They found that tripolyphosphate was detectable after 38 weeks of continuous frozen storage but evidence of its use disappeared from all samples after one year. Again hydrolysis was assumed to reduce the amount of detectable polyphosphate additions.

In Europe, the TLC method currently used to routinely detect the addition of polyphosphates in seafood is based on trichloracetic acid (TCA) extraction. This TLC method was described in German by Rössel and Kiesslich (1966) and another German

publication was found published by Chemische Fabrik Budenheim KG (1982) with no English translations. These methods were not readily available or known to the United States. The TLC procedures described by this study were initially adapted from this German company (Chemische Fabrik Budenheim KG). The objectives for this study were to thoroughly test and describe a TLC method to be used as a practical detection device for the addition of polyphosphates in seafood. A main focus will be on the detail in the description of the procedures to facilitate future duplication and widespread use of this TLC method.

Table 1-1. Different phosphate compounds used in foods

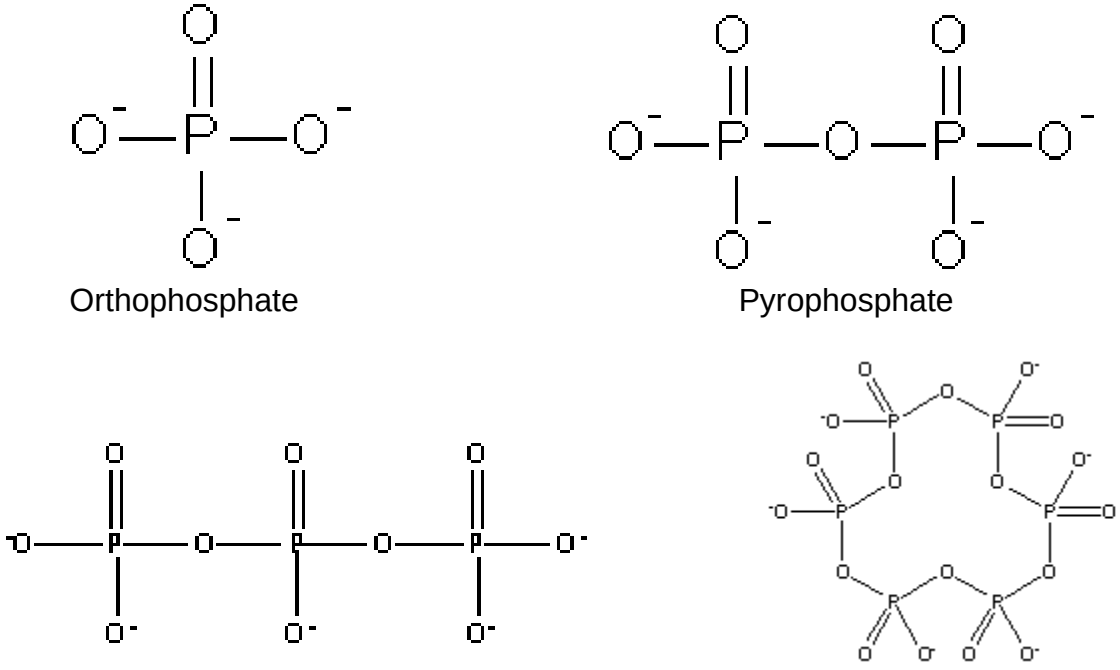
Common name	Abbr.	Chemical Formula	Use in Foods	Functions
		Orthophosphates (monomers)		
Phosphoric acid	PA	H_3PO_4	beverages, cheese, juices	acidulant, coagulant
Ammonium Phosphate	MAP	$NH_4H_2PO_4$	baked goods	buffering
Diammonium Phosphate	DAP	$(NH_4)HPO_4$	baked goods	buffering
Monocalcium phosphate	MCP	$Ca(H_2PO_4)_2$	baked goods, beverages, cheese, canned fruits, juices	acidulant, buffering, leavening agent, protein modifier
Dicalcium phosphate	DCP	$CaHPO_4$	baked goods	leavening agent
Tricalcium phosphate	TCP	$Ca_3(PO_4)_2$	beverages	adsorbent, flow conditioner
Monopotassium phosphate	MKP	KH_2PO_4	eggs	acidulant, buffering
Dipotassium phosphate	DKP	K_2HPO_4	cheese, dairy products	alkalnity
Monosodium phosphate	MSP	NaH_2PO_4*H2O	beverages, eggs	acidulant, buffering
Disodium phosphate	DSP	Na_2HPO_4	beverages, cereals, cheese, dairy	alkalnity, buffering, dispersing agent, emulsifier, protein modifier
Trisodium phosphate	TSP	Na_3PO_4	candy, cereals, cheese	alkalnity, buffering, emulsifier, protein modifier
		Pyrophosphates (dimers)		
Sodium acid pyrophosphate	SAPP	$Na_2H_2P_2O_7$	baked goods, candy	Acidulant, coagulant, dispersing agent, leavening agent, protein modifier, sequestrant
Tetrasodium pyrophosphate	TSPP	$Na_2P_2O_7$	beverages, candy, cheese, dairy	Alkalnity, coagulant, dispersing agent, protein modifier, sequestrant
		Polyphosphates (polymers with more than 2 P atoms)		
Sodium tripolyphosphate	STPP	$Na_5P_3O_{10}$	beverages, cheese, dairy, meats	Alkalnity, dispersing agent, protein modifier, sequestrant
Sodium hexametaphosphate	SHMP	$(NaPO_3)_n^h$	beverages, candy, cheese, dairy, eggs	anticoagulant, dispersing agent, emulsifier, sequestrant

Sources: Molins 1991 and Branen 2002

Table 1-2. Natural phosphorus content in various foods

Food Product	Phosphorus content (mg/g)
Ground Beef, 10% fat	2.31
Sirloin, lean and fat	2.45
Beef liver	4.76
Broiler Chicken	2.02
Turkey, flesh and skin	2.03
Duck, flesh and skin	2.70
Ham, lean and fat	2.47
Pork loin chop, lean	2.44
Rib Roast, lean	2.56
Veal Cutlet	2.48
Pork Sausage	1.85
Whole milk	1.97
Cheddar cheese	5.24
Whole egg	1.03
Wheat flour	1.01
Broccoli	0.72

Source: Molins 1991



Orthophosphate Pyrophosphate

Tripolyphosphate Hexametaphosphate

Figure 1-1. Chemical structures for various common phosphate molecules illustrating the polyvalent character

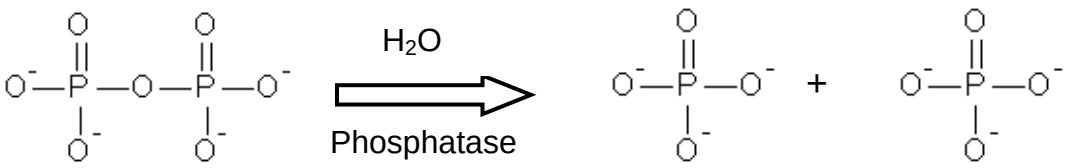


Figure 1-2. Reaction equation for the hydrolysis of pyrophosphate into two orthophosphate ions.

CHAPTER 2
MATERIALS AND METHODS

Two different types of seafood, penaeid shrimp and Alaska pollock, were used to test the utility of the TLC method. The samples were collected, processed and treated with a variety of phosphate and non-phosphate additives. Treatments were based on current commercial practice using existing blends and methods of application. The treated products were then kept frozen to be tested throughout one year of storage (-10°C).

The selection of these test species was based on their prominence in international commerce and the current trends in processing with a variety of phosphate compounds to retain moisture. Americans eat, on average, about 16 pounds of seafood per person per year. About 25% of this is shrimp, which is the most popular seafood consumed in the United States at 4.1 pounds per person per year (NOAA NMFS 2010), and penaeid shrimp represents one of the largest volumes of aquacultured product in the world. The Alaska pollock fishery is the largest U.S. fishery by volume. Annual catches from 2000-2009 have averaged 2.9 billion pounds (around 1.3 million metric tons) (NOAA NMFS Fish Watch 2009). Together these two products represent the leading seafood products both in terms of volume and value for consumption in the United States. They are primary candidates calling for routine monitoring techniques to assure proper use of phosphate treatments.

Sample Collection and Treatments

Shrimp

To ensure there were no prior treatments on the shrimp, white shrimp (*Penaeus setiferus*) were collected directly from an aquaculture pond operation in Champerico,

Guatemala. They were then deheaded, peeled, and deveined at a local processing plant within hours postharvest. The shrimp were then separated into seventeen groups for treating with different phosphate and non-phosphate solutions. The selection of treatments was based on current commercial practice, both proper and abusive. The intent was to impart a range of residual concentrations and compounds commonly used in commerce. The concentrations of the additive treatment solutions ranged from 0.5% to 5.0% and all (except three low sodium groups) contained 2.5% sodium chloride (NaCl) that is used to aid penetration. Two of the groups were not treated with additives to be used as controls, one with and one without NaCl. The details for the treatment solutions for the shrimp are organized in Table 2-1.

Each sample group was dipped for a static soak for two hours at 5°C to replicate a common method for addition of moisture retention agents in seafood. The conductivity, pH, and temperature of the treatment solutions were measured before the dipping, at one hour soak time, and at the end of the two hour treatment (Table 2-2). Half of each treated group was sent into a cooking line where they were thoroughly cooked by steam and then cooled. The other half remained raw. All of the samples were individually quick frozen (IQF), dipped in cold water to form a protective glaze, and then packaged into one pound bags and placed in frozen storage. The frozen samples were shipped to the University of Florida where they were held in frozen storage at -10°C.

The type of phosphate and non-phosphate additive blends used in the experimental design to treat the shrimp and pollock were chosen for several reasons. All of the additives used were chosen partially because of their use in current commercial practices. Several additive blends were selected from different producers

because it was hypothesized that they would differ in chemical compositions. It was necessary to use both phosphate and non-phosphate additives for comparison purposes. Therefore, the experiment would be testing a range of additives for variability and comparison providing more validity in the results.

Fish (Pollock)

The other series of seafood collection and treatment involved the acquisition of Alaska pollock (*Theragra chalcogramma*). The pollock were harvested on one vessel from one area in the Bering Sea in February 2009. They were gutted and headed (G&H) then frozen into blocks onboard the processing vessel. The fish blocks were shipped to a processing plant in Qingdao, China where they were held in frozen storage for four months prior to thawing for further processing. In the presence of University of Florida staff, the fish were gutted and headed pollock were skinned and filleted, and the fillets were separated into 18 groups. Each group was treated in a different combination of phosphate or non-phosphate additives and dip times, including an untreated control group (Table 2-3). All samples were then drained, weighed, packed and plate frozen in 16.5 pound (lb.) blocks. The frozen blocks were shipped to a processor in the USA where they were cut into 3.75”L x 2.5”W x 0.7” pieces about 100 grams each, before being shipped to the University of Florida and held in frozen storage at -10°C. A variety of phosphate and non-phosphate additives were chosen for the same reasons explained above for the shrimp treatments.

Table 2-1. Treatments used in preparation of 17 shrimp samples applied as static soaks

Sample	Treatment Additive	Primary Ingredients	Additive Conc.	NaCl Conc.	Exposure Time
1	A&B Nutriphos STPP	Sodium tripolyphosphate	3.0%	2.5%	2 hrs
2	STPP China	Sodium tripolyphosphate	3.0%	2.5%	2 hrs
3	BK 512	Sodium phosphates	3.0%	2.5%	2 hrs
4	BK 750 low sodium	Sodium and potassium phosphates	5.0%	N/A	2 hrs
5	Hasenosa AquaFish	Sodium phosphates and salt	3.0%	2.5%	2 hrs
6	Hasenosa Mez 1396	Sodium phosphates	3.0%	2.5%	2 hrs
7	Carnal 659s	Sodium pyrophosphate, tripolyphosphate and hexametaphosphate	3.0%	2.5%	2 hrs
8	Carnal 659s		2.5%	2.5%	2 hrs
9	Carnal 2110s (low Sodium)	Sodium Phosphates	4.0%	N/A	2 hrs
10	Carnal 2110s (low sodium)	Sodium Phosphates	5.0%	N/A	2 hrs
11	Budenheim SP 313(Natural)	No Phosphates	0.5%	2.5%	2 hrs
12	Budenheim SP 313(Natural)	No Phosphates	1.0%	2.5%	2 hrs
14	Sodium Citrate	Sodium citrate	1.5%	2.5%	2 hrs
15	Altesa PS	Sodium citrate, sodium carbonate	1.5%	2.5%	2 hrs
16	HA-5096 FOSFREE/CH	No Phosphates	1.5%	2.5%	2 hrs
17	Control (salt)	NaCl	N/A	2.5%	2 hrs
18	Control	N/A	N/A	N/A	N/A

Sample 13 was omitted because additive was not available

Table 2-2. Analysis to characterize the treatment solutions used to prepare the 17 shrimp samples during initial and subsequent exposure (1-2 hours post-application)

	Before Treatment			1 hour			2 hours	
Sample	Conductivity	pH	T (°C)	Conductivity	pH	T (°C)	Conductivity	Change*
1	45.4	9	0.5	37.4	9	3.5	35.7	9.7
2	46.1	9	2.3	36.7	9	4.7	35.6	10.5
3	45.0	8	-1.0	36.8	8	2.7	34.7	10.3
4	33.0	9	0.5	30.3	9	2.7	27.5	5.5
5	52.2	9	-0.6	42.1	9	1.8	38.2	14.0
6	45.3	9	1.1	36.8	9	2.7	34.7	10.6
7	45.9	10	1.1	36.8	9	3.8	35.5	10.4
8	44.4	10	1.4	35.0	9	2.2	33.1	11.3
9	59.5	8	-0.8	44.1	9	2.8	41.5	18.0
10	76.0	10	1.6	52.9	8	3.5	39.7	36.3
11	33.8	10	9.1	29.5	9	10.4	29.4	4.4
12	32.6	11	8.1	30.1	10	9.2	29.2	3.4
14	41.8	7	0.8	32.9	8	3.5	32.3	9.5
15	49.1	9	1.4	37.5	9	3.4	34.8	14.3
16	47.3	10	-0.9	35.9	10	2.3	36.0	11.3
17	40.5	7	1.1	30.9	7	4.1	29.8	10.7
18	Not applicable - no treatment solution or addition of salt							

*Change in conductivity throughout treatment

Table 2-3. Treatments used to prepare 18 pollock samples applied as static soaks

Treatment Additive	Primary Ingredients	Additive Concentration	Exposure Time
1a) Untreated	N/A	N/A	N/A
1b) Innophos STPP	Sodium tripolyphosphate	3.00%	30 sec
1c) Innophos STPP		3.00%	30 s @ 2°C
1d) Innophos STPP		3.00%	5 min
1e) Innophos STPP		3.00%	60 min
1f) Water only	Water		60 min
2b) Innophos STPP + Salt	Sodium tripolyphosphate	3.0% + 1.0% NaCl	5 min
3a) Carnal 659s	Chemical blend of Sodium pyrophosphate, tripolyphosphate and hexametaphosphate	2.50%	30 sec
3b) Carnal 659s		2.50%	5 min
3c) Carnal 659s		2.50%	60 min
4a) Altesa ABC5	Trisodium citrate, Sodium carbonate, Sodium acid carbonate	2.00%	30 sec
4b) Altesa ABC5		2.00%	5 min
4c) Altesa ABC5		2.00%	60 min
5) Innophos STPP + MTR-79 *	STPP + Citric acid, Sodium bicarbonate, Salt	1.0% + 1.0%	60 min
6) Innophos STPP +ALTESA ABC2D *	Sodium tripolyphosphate	1.0% + 1.0%	60 min
8a) DPA Zefish	Citric acid, Sorbitol, Potassium carbonate, Salt	2.00%	30 sec
8b) DPA Zefish		2.00%	60 min
9) MTR-79	Citric acid, Sodium bicarbonate, Salt	2.00%	60 min

*Blends made by UF researchers to mimic common commercial practice

Thin Layer Chromatography

Detailed TLC Procedures

The shrimp and pollock were treated in different commercial phosphate and non-phosphate blends, then frozen prior to testing with the TLC method every three months for one year. This approach would account for any influence in detection relating to progressing hydrolysis of any phosphate residuals in the muscle samples. Detection was based on visualizing the separated residuals on the TLC plates. After the TLC plates were visualized, pictures were taken for permanent record. An example of a TLC plate, shown in Figure 3-1, indicates the spots which correlate to the orthophosphate, pyrophosphate and polyphosphate molecules.

The thin layer chromatography (TLC) method used in this study was adapted from a European company (Chemische Fabrik Budenheim KG, Budenheim, Germany), where it is currently used to detect phosphate additives in various foods. The researcher traveled to Budenheim, Germany to observe and practice the method on a variety of seafood products and treatments. Practice provided experience to improve and properly run the TLC methods. A descriptive yet concise (one page) method was delineated (Figure 2-1) so that the TLC method could be easily followed and duplicated. The TLC procedure was also summarized into a flow chart (Figure 2-2) so it could be easily understood and followed.

This method varied from the TLC methods used in previous studies (Gibson and Murray 1973; Tenhet 1981a; Reddy and Finne 1985). First of all, the extract in the old method was prepared by homogenizing 50 grams of shrimp with 100 grams of water for one minute and filtering. The new method, on the other hand, extracts liquid from the sample by adding 5 g of trichloracetic acid to 20 g of shrimp (or other seafood). The

TCA denatures the protein and extracts the liquid already in the muscle tissue which contains the phosphate molecules. This is advantageous over the old method which added a significant amount of water (50% of the weight of the sample), which certainly dilutes the concentration of the phosphates to be detected. Another difference found is the mobile phase in the old method consisted of 2 g ammonium carbonate, 160 ml acetonitrile, and 90 ml p-toluenesulfonic acid. The mobile phase of the new method has the following composition: 70 ml dioxane and 30 ml of (160 g trichloracetic acid, 8 ml ammonia solution 24%, completed to 1000 ml). The spraying reagents were also different, although they were both based on molybdate and sulfite solutions. The old method used 50 g ammonium sulfate, 75 mg ammonium molybdate, and 150 ml concentrated nitric acid completed to 1 liter with water. The new method uses 40 g sodium molybdate dehydrate and 50 g ammonium nitrate completed to 1 L with distilled water. The reducing spray used in the old method contained 288 g sodium pyrosulfite, 10.5 g sodium sulfite and 1 g methylaminophenol dissolved in 1 L of water. The new method uses 300 g sodium metabisulfite, 10 g sodium sulfite and 2 g metol (4-methylaminophenolsulfate) completed to 1 L with water.

The applications of the TLC method were tested using the shrimp and pollock samples described in the previous section. One bag of shrimp from each sample group (all 17 raw and cooked) was run under tap water to remove the glaze, allowed to drain for two minutes and then weighed to determine the net weight. About ten shrimp from each sample group were taken to be tested with the TLC method. The pollock were in 100 g pieces and could be thawed and ready for the following TLC method.

The thawed sample (shrimp or pollock) to be tested was blended in a food processor to homogenize and 20 g was weighed in a 100 ml glass beaker. Five grams of trichloracetic acid (TCA) was weighed and added to the sample, then thoroughly stirred and mashed with a glass stirring rod which causes liquid to extract from the sample as the acid denatures the proteins. The liquid was filtered through 589 blue ribbon ashless filter paper into a test tube. The TLC plate (Merck Cellulose 10x20 cm) was labeled and marked properly to separate samples by 1.5 cm apart and from the edges. The plate could then be spotted using a 1 µl capillary pipette by placing one microliter (µL) for each sample, allowing to dry, and spotting with one more microliter on the same spot. Each sample was spotted twice on the plate to provide duplication for stronger results. After the spots were dry, the plate was placed in a glass chamber containing 100 ml of the mobile phase solution [70 ml of 1,4 dioxane + 30 ml of (160g of TCA, 8 ml ammonia solution 24%, complete to 1000 ml with water)]. After about 90 minutes, when the mobile phase reaches 8 cm on the plate, the plate was removed and allowed to dry. The plate was then heated over a hot plate at 240°C for 3-5 minutes and then evenly sprayed with the molybdate solution (40 g Na_2MoO_4 x $2H_2O$ + 50 g NH_4NO_3 dissolved in water to make 1000 ml, and add to 80 ml concentrated HNO_3, filter) using a Kontes Reagent Sprayer and pressurized air. This heating and spraying step was repeated once and yellow spots appeared on the plate. After spraying the molybdate solution twice and heating over the hot plate, the plate was sprayed with a sulfite reducing agent [300g ($Na_2S_2O_5$) sodium metabisulfite, 10g (Na_2SO_3) sodium sulfite, 2g metol (4-methylaminophenolsulfate) complete to 1000 ml w/ dist. water and filter] and returned to the hot plate for another 3-5 minutes. Blue spots were then visible

corresponding to different phosphate molecules which qualitatively indicated whether or not a sample contained polyphosphate additives. Pictures were taken of all the finished plates and kept for records. This TLC method was repeated on all samples every three months for one year of frozen storage to ensure that the phosphates were still detectable throughout an extended storage period.

To determine the sensitivity of this TLC method, six different solutions of polyphosphate were prepared using pure sodium tripolyphosphate and Carnal 659s, each with concentrations ranging from 0.5%, 0.05, and 0.005%. These solutions were then spotted on a TLC plate, separated and visualized just as a solution extracted from a seafood sample would be used according to the described TLC method. This range was chosen because 0.5% is maximum residual phosphate content set by European regulations and has also been proposed as action levels by the U.S. FDA. The low end of the range was below the concentration of 0.05%, which represents minimal treatment with phosphate additives. Any lower concentrations would insignificantly affect the chemical and physical properties of the food.

Step by Step TLC Procedures

1. Grind shrimp or other seafood sample in a food processor to homogenize into a paste (15-30 seconds), do not overheat. Proceed immediately to next step or freeze sample.
2. Weigh exactly 20 g of the sample paste in a 50 ml glass beaker.
3. Add 5 g of trichloracetic acid (CCl_3COOH) - BE CAREFUL WITH THIS ACID! WEAR GLOVES AND GOGGLES THROUHOUT THE ENTIRE PROCEDURE
4. Stir and mash the shrimp and acid together well with a glass rod for at least 30 seconds until some liquid is visible.
5. When three samples are ready, filter the extracted liquid into small test tubes through filter paper (Whatman grade 589 ashless).

- Do not let these samples sit for too long as the phosphate will hydrolyze, transfer them to the plate as quickly as possible.

6. Prepare a plate (Merck cellulose TLC glass plate 10 x 20 cm) as shown in Figure 2-1:

7. After the samples have been filtered, place 1 microliter (1 µL) of each sample on the plate using a 1 µL capillary micropipette. Be sure to use a new micropipette for each sample to avoid cross-contamination. Allow the spots to dry (or dry with a fan) and then add a second µL of each sample. Make sure the second drop is placed in the same location as the first spot. Allow the spots to dry completely.

8. Place the plate standing up, with the spotted side on the bottom, in a glass chamber containing 100 ml of the following solution:
   70 ml - 1,4 Dioxane
   30 ml - [160g of trichloracetic acid, 8 ml ammonia solution 24%, complete to 1000 ml with water]
   (The solution must sit in the chamber for at least one hour to saturate before using. Replace the solution after 3-4 weeks).

9. Remove the plate from the chamber and allow it to dry.

10. Heat the TLC plate 2.5 cm over a hot plate (240^{o}C) for 3-5 min. Make sure the TLC plate is not in direct contact with the hot plate as it will burn.

11. In a fume hood, place the TLC plate on a side edge in a tilted position. Lightly and evenly spray the TLC plate with the molybdate solution:
    [40 g sodium molybdate dihydrate (Na_2MoO_4 x $2H_2O$) and 50 g ammonium nitrate (NH_4NO_3) dissolved in water to make 1000 ml, and add to 80 ml concentrated HNO_3, filter]. Heat on hot plate for 3 to 5 minutes.

12. Repeat step 11 once. The spots will turn yellow after first application.

13. Spray the TLC plate with the sulfite reducing solution:
    [300g sodium metabisulfite ($Na_2S_2O_5$), 10g sodium sulfite (Na_2SO_3), 2g Metol (4-Methylaminophenolsulfate) complete to 1000 ml with dist. water and filter]. Dry 3-5 min. on hot plate. The spots will turn blue.

Troubleshooting

- It is important to follow these methods continuously to limit hydrolysis of polyphosphates over time. Do not let the samples sit for extended periods of time

as polyphosphates will hydrolyze into monophosphate which is indiscernible from natural levels of monophosphate in the muscle tissue.

- When blending the sample, do not overheat which would increase the rate of hydrolysis. It should take no more than 30 seconds to homogenize into a paste.
- The TLC plate must be placed on a support (e.g. aluminum foil tray) to separate it from the hot plate surface so it does not burn.
- Avoid spraying too heavily to avoid streaking or development of droplets that will diminish the visual quality of the finished plate.

Chemical Analyses

Supplemental data was collected on the chemical composition of the samples as all of the shrimp and pollock were analyzed for total phosphorus, sodium, and moisture contents. The sodium and phosphorus contents were both measured using inductively coupled plasma mass spectroscopy (ICP-MS) at the ABC research corporation. The ICP-MS draws prepared solutions from the samples through a tube into a plasma flame which ionizes the sample to be read by a mass spectrometer to provide an accurate measurement of the concentration of the metal being tested for. These analyses were conducted once for each of the shrimp sample groups and ran three times for each of the pollock sample groups.

The moisture content was determined by spreading 10 grams of the homogenized sample on a tray and placed in an Ohaus MB200 moisture balance. This instrument dries the sample and uses the initial and final weights to derive a value and give a reading for percent moisture. Each sample group was measured in triplicates to provide data for the calculation of averages and standard deviations to increase statistical significance.

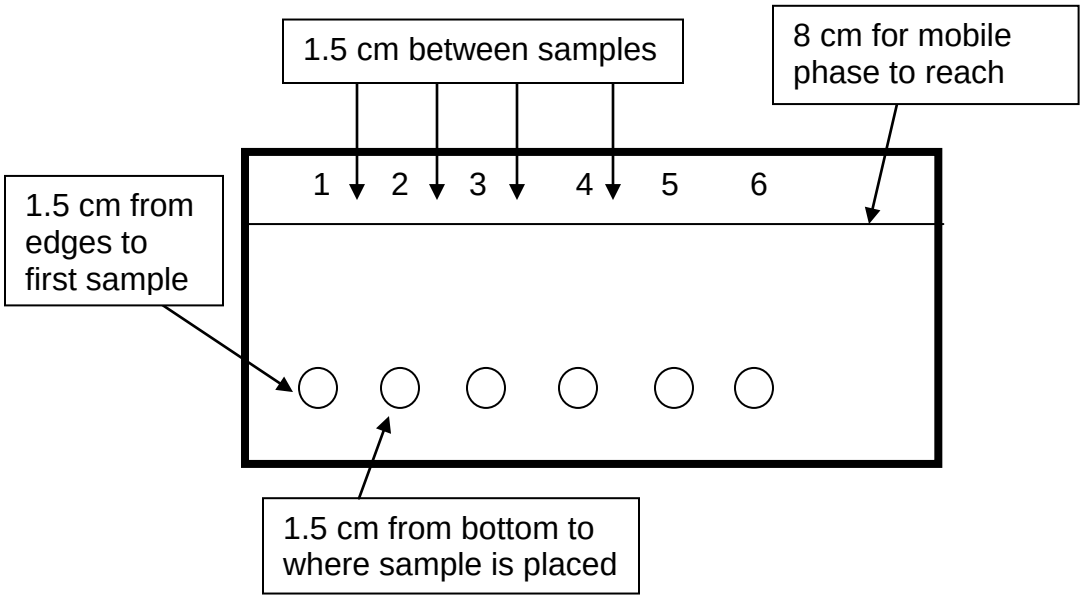


Figure 2-1. Diagram for the proper labeling and spotting of a TLC plate

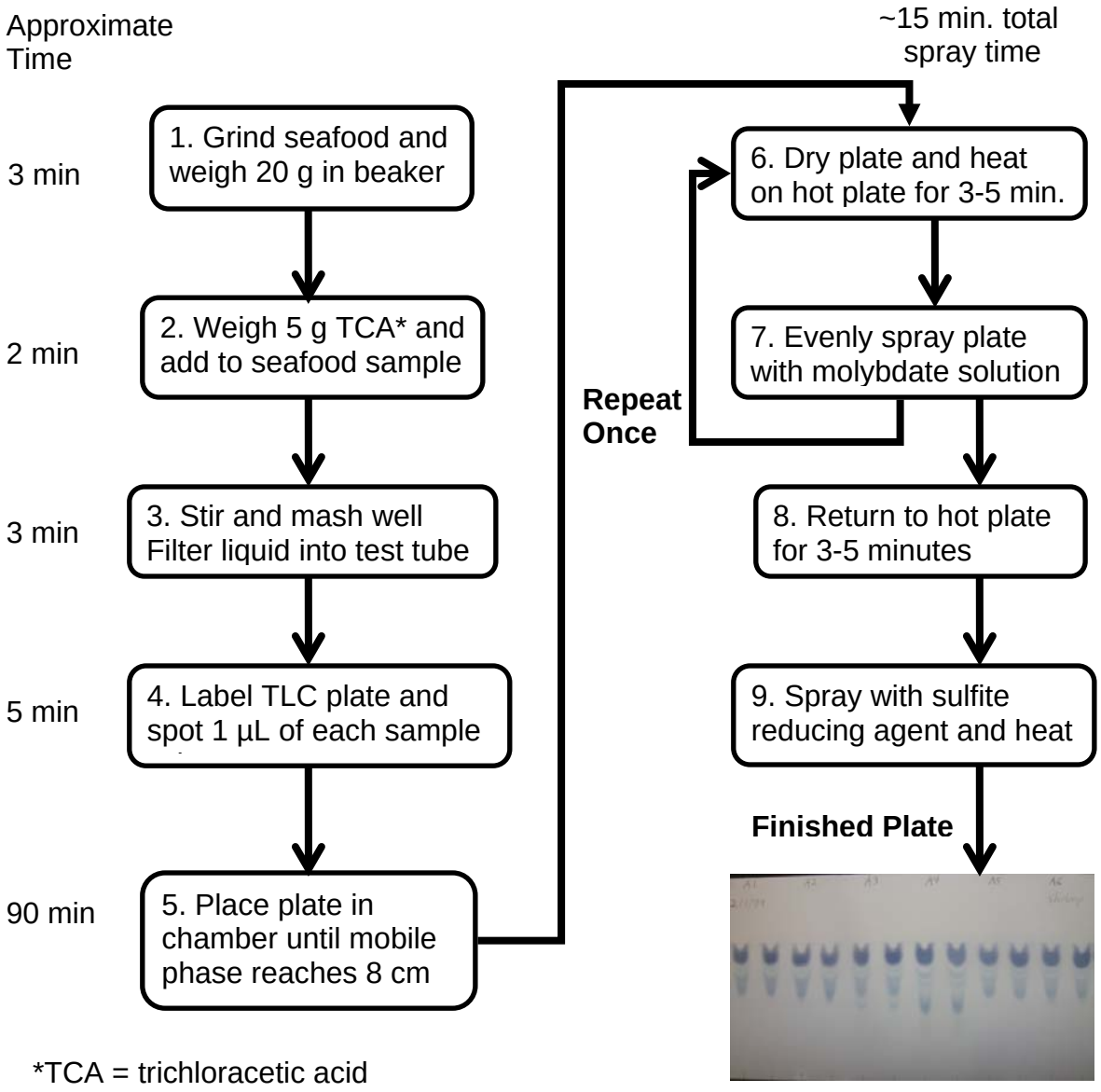


Figure 2-2. Flow chart of the thin layer chromatography method for detecting phosphates in seafood

CHAPTER 3
RESULTS AND DISCUSSION

Thin Layer Chromatography

All of the shrimp and pollock samples that were treated with phosphate additives showed spots on the TLC plates which correspond to polyphosphates, in addition to the orthophosphate spot. These results held true all four times (every three months for one year) samples were run with the TLC method throughout one year of frozen storage. This applies to all shrimp samples (raw and cooked), as well as all pollock samples. Every sample was also run in duplicates each time to provide stronger results. There were no significant differences between any of the duplicate samples. The resulting plates, one from each group in the study (raw shrimp, cooked shrimp, pollock) are shown in Figures 3-2 through 3-7.

This shows that the TLC method was 100% effective for indicating the presence of phosphate additives for this experiment. All of the samples that did not have phosphate additives, including controls and non-phosphate blends, only showed spots for orthophosphate. Therefore, the TLC method was also 100% effective in identifying samples (raw and cooked) that do not contain phosphate additives. A control sample (1A) with no additives is shown on the far left of the first pollock plate (Figure 3-6), revealing only a spot for orthophosphate.

The TLC plates showed spots for polyphosphates in all samples treated with phosphates throughout the entire year of testing. Therefore, there was no apparent degradation of the polyphosphates into orthophosphate to the extent that the additives could no longer be detected. All of the non-phosphate and control samples only showed orthophosphate spots every time they were tested (in duplicates every four

months for one year). The TLC method was just as effective on pollock as it was for shrimp. This shows that the applications for the TLC method are useful for a variety of seafood products. The reliability of the TLC method on the samples tested was fully confirmed for positively indicating the presence or absence of polyphosphate additives in shrimp (raw and cooked) and pollock through 12 months frozen storage after the initial treatments. These results differ from the previous studies (Tenhet and others 1981a; Krzynowek and Panunzio 1995) where they found the TLC method lacked sensitivity and could not detect polyphosphates after one year.

Some differences in phosphate levels between the different sample groups were evident based on different intensities of the spots on the TLC plates (Figures 3-6 and 3-7). Pollock samples 1B through 1E were all treated in the same concentration (3.0%) of STPP (sodium tripolyphosphate), but for different exposure times. Samples 1B and 1C were dipped for thirty seconds, 1D was dipped for five minutes, and sample 1E was dipped for one hour (Table 2-3). The STPP spots on the sample (1E) exposed for the longest time (1 hour) were clearly darker than the spots on 1B, 1C, and 1D (Figure 3-6). This can be explained by the difference in resulting phosphorus content from pollock samples exposed to 0.5 and 5 minutes (Table 2-3). Again, these results were previously discussed as 1E was exposed for the longest time (1 hour) resulting in “salting in” of the sodium tripolyphosphates to the highest residual level (302 mg/100g of fish) (Table 3-3).

Samples 3A, 3B, and 3C also displayed a similar variation in TLC spot color intensity due to prolonged exposure causing elevated phosphate levels (Figure 3-7). All three samples were soaked in the same concentration (2.5%) of Carnal 659 (commercial phosphate blend) but for different periods of time. Sample 3A was dipped

for thirty seconds, 3B for five minutes, and 3C for one hour (Table 2-3). The spots for sample 3C (291 mg P/100g) are noticeably darker than the other two (193 and 227 mg/100g) (Figure 3-7, Table 3-3). Although these observations are mainly qualitative, the implications for using this TLC method for quantification of phosphates have potential. Future studies could research possible colorimetry methods for quantifying the color intensities to correlate to a particular phosphate concentration. Even if exact measurements of phosphate concentration are not made, possibly a range could be established to place certain color intensities into categories that represent, for example, “high,” “medium” or “low” phosphate treatments.

The TLC plate prepared with STPP and Carnal concentrations ranging from 0.005% to 0.5%, provided an approximation of the sensitivity of the method. It was determined that the TLC method would show a spot for concentrations as low as 0.05% additive. Since STPP contains 25.26% phosphorus (Merck Index 1989), the TLC plate detected about 0.01% phosphorus or less than 0.03% phosphate (P_2O_5). Likewise, Carnal 659s is about 60% P_2O_5 (Certificate of Analysis 2004), so the TLC actually detected about 0.03% P_2O_5.

This sensitivity is well below (less than 10%) residual limits of 0.5% added phosphate that have been mandated in the European Union (European Parliament and Council Directive 1995) and proposed in the United States (Federal Register 18 Dec 1979: 74845). This means the TLC method is effective in detecting concentrations well below routine phosphate additions of about 0.5% added phosphate, which are more than ten times the concentration of the threshold of the TLC method. Seafood with phosphate concentrations below the threshold of detection for this TLC method (<0.05%

added phosphate) would likely have insignificant levels of added phosphates, related to moisture retention and nutritional content.

Additional practicality of the TLC method is supported by its relative affordability. The cost of running samples using this TLC method was found to be approximately $20 per plate (including all chemicals and materials), and each plate holds up to 12 samples. Therefore, if 12 samples were run on one plate, it would only cost about $2 per sample.

Experience in refining this method provided recommendations to continuously follow the methods to minimize hydrolysis of polyphosphates over time. If the sample is not immediately used for the TLC method after receiving and blending, the sample should be frozen. When the sample is blended to a homogenized paste, it should take no more than 30 seconds. Over blending could heat up the sample and increase the rate of polyphosphate hydrolysis. When heating the TLC plate, it must be raised about 2.5 cm above the hot plate so it will not burn. A rack can be purchased to hold the plate or a simple tray can be formed with aluminum foil. When spraying the TLC plate, it should be done lightly and evenly with a mist. Spraying too heavily can result in streaking of the spots and/or development of droplets.

Chemical Analyses

Treatment Solutions

The pH, temperature and conductivity of the treatment solutions for the shrimp were measured to monitor the absorption of the additives (Table 2-2). The pH of the solutions remained relatively constant throughout the first hour of the treatment period. The pH of all of the solutions either remained the same or only changed by a value of one. This is most likely due to the buffering capacity of many of the additives. The

solutions included some ice slush to keep them near 0°C throughout the treatment period. There was a slight increase in temperature because no more ice could be added as it would dilute the concentration of the measured additives.

The conductivity of a solution roughly reflects the amount of ions in a solution; therefore, it can provide an estimation of the residual compounds remaining in the treatment solutions at a given time. A general trend in the data can be observed as most of the samples had a large drop in conductivity in the first hour of treatment and a much smaller drop between the first and second hour. This suggests that the additive absorption was most rapid in the first hour and began to slow over time. This is likely due to the shrimp approaching an equilibrium with the treatment solution. This has been commonly referred to as an "igloo" effect in that the surface portion of the treated product becomes saturated to a level that impedes penetration. The sample that had the largest change in conductivity of 36.3 during the static soak was sample 10 (5% Carnal 2110s). This was significantly higher than the other samples which ranged from drops of 3.4 to 18.0. An accurate prediction of the reason for this outlier is not possible as the exact chemical compositions of the treatment additives are unknown.

Shrimp Samples

The chemical analyses of phosphorus and sodium in the shrimp samples are summarized in Table 3-1 (raw) and Table 3-2 (cooked). The samples treated with phosphate (samples 1 through 10), as expected, had higher levels of total phosphorus than those treated in non-phosphate additives and the controls. The average total phosphorus content for the phosphate treated raw shrimp was 273 mg/100g and 240 mg/100g for the cooked shrimp. Compared to the total phosphorus levels of the shrimp not treated in phosphate, the raw shrimp contained an average of 145 mg/100g and the

cooked with 151 mg/100g. Sodium levels were lower in the cooked shrimp because sodium leeches out with water loss during cooking. The average sodium content of the raw shrimp was 502 mg/100g and the cooked shrimp contained an average sodium content of 368 mg/100g.

Pollock Samples

The chemical analysis results of the pollock samples, including the moisture content, are shown in Table 3-3. As expected, the untreated control (sample 1A) had the lowest moisture content of 82.7% because no moisture retention agents were used. The rest of the samples, which were treated with different additives, had moisture contents ranging from 83.0% to 88.4%. The sample with the highest moisture content was treated in the blend mixed by UF researchers, Innophos STPP and ALTESA ABC2D, for one hour. The lowest moisture of the treated groups was the sample treated in Innophos STPP for only 30 seconds (sample 1C), which does not allow for significant phosphate and water absorption. In contrast, sample 1E was treated in Innophos STPP for one hour and had the second highest moisture content of 88.0%. Therefore the longer treatment time in the same solution resulted in greater uptake and retention of moisture. In addition, sample 1D which was treated in the same 3.0% Innophos STPP solution but for 5 minutes had a moisture content of 85.4%, which falls in between the other two samples. Samples 3A, 3B, and 3C demonstrate a similar trend as increasing dip time (30 seconds, 5 minutes, and one hour) in 2.5% Carnal 659s resulted in increasing moisture in the pollock. Samples 3A, 3B, and 3C had moisture contents of 84.0%, 84.8%, and 87.0%, respectively. These results show a direct correlation between phosphate solution exposure time and moisture content. Samples 4A through 4C show similar results with the same variation of dip times in the 2.0% non-

phosphate commercial blend Altesa ABC5 solution. Samples 4A, 4B, and 4C had moisture contents of 83.3%, 84.8%, and 86.2%, respectively. This shows that there is also a positive correlation between the dip time in non-phosphate additives used in this study and moisture content of the pollock.

The phosphorus content, also in Table 3-3, shows high variability between samples. Sample 1A (untreated control) provides an approximate level to endogenous phosphorus levels in the pollock used for this study of 168 milligrams per 100 grams of fish (mg/100g). As expected, all of the samples treated in phosphate solutions contained higher levels of phosphorus. The phosphorus levels in the phosphate treated pollock ranged from 193 to 320 mg/100g. Sample 1E (3% Innophos STPP dipped for one hour) contained the highest phosphorus level of 320 mg/100g. As mentioned above, this sample also had nearly the highest moisture content, indicating that there was significant uptake of phosphate and water. All of the samples treated in non-phosphate solutions (including sample 1F dipped in water only) had phosphorus levels below the control (1A), ranging from 119 mg/100g to 153 mg/100g. This is likely mainly due to the adsorption of water causing the dilution of the endogenous phosphorus. This depression of phosphorus may also be caused by the leeching of phosphorus into the treatment solution during the static soak.

The sodium content of the pollock also shows large variation between samples, as they ranged from 50 mg/100g to 302 mg/100g. Sample 1A (control) had 72 mg/100g of sodium reflecting the approximate natural level of sodium in the fish tissue. The sample with the lowest sodium content was sample 1F which was soaked in water only. This was expected as soaking in water causes the endogenous sodium to diffuse out of

the fish tissue. Sample 1E had the highest sodium content as it was dipped in sodium tripolyphosphate for one hour, which allowed for significant absorption of sodium ions from the dissolved additive into the tissue.

Table 3-1. Chemical analysis of treated raw shrimp samples

Sample	Additive	Phosphorus (mg/100 g)	Sodium (mg/100 g)	% Moisture
A1	A&B Nutriphos STPP	296	668	80.80
A2	STPP China	287	675	81.20
A3	BK 512	274	551	81.70
A4	BK 750 low sodium	392	238	81.10
A5	Hasenosa AquaFish	264	511	80.60
A6	Hasenosa Mez 1396	287	532	80.60
A7	Carnal 659s (control)	282	539	79.30
A8	Carnal 659s (control)	261	645	81.30
A9	Carnal 2110s (low Sodium)	190	413	80.90
A10	Carnal 2110s (low sodium)	195	452	80.90
A11	Budenheim SP 313(Natural)	135	458	82.00
A12	Budenheim SP 313(Natural)	156	463	81.70
A14	Sodium Citrate	132	538	82.20
A15	Altesa PS	155	624	81.70
A16	HA-5096 FOSFREE/CH	149	591	82.40
A17	Control	151	459	81.40
A18	Control	163	178	81.10

Table 3-2. Chemical analysis of treated cooked shrimp samples

Sample	Additive	Phosphorus (mg/100 g)	Sodium (mg/100 g)	% Moisture
B1	A&B Nutriphos STPP	250	443	82.75
B2	STPP China	232	445	82.87
B3	BK 512	252	455	83.00
B4	BK 750 low sodium	331	228	82.07
B5	Hasenosa AquaFish	238	481	83.05
B6	Hasenosa Mez 1396	247	452	82.09
B7	Carnal 659s (control)	250	461	81.57
B8	Carnal 659s (control)	238	432	82.37
B9	Carnal 2110s (low Sodium)	178	255	82.42
B10	Carnal 2110s (low sodium)	185	338	81.03
B11	Budenheim SP 313(Natural)	165	322	81.86
B12	Budenheim SP 313(Natural)	145	272	81.88
B14	Sodium Citrate	133	381	82.22
B15	Altesa PS	140	385	82.34
B16	HA-5096 FOSFREE/CH	149	377	82.58
B17	Control	153	336	81.41
B18	Control	175	192	80.57

Table 3-3. Chemical analysis of 18 treated pollock samples

	Moisture		Phosphorus/Phosphate			Sodium	
Treatment additive	% Moisture*	Std. dev.	Phosphorus (mg/100g)*	Std. dev.	Phosphate (% P_2O_5)	Sodium (mg/100g)*	Std. dev.
1a) Untreated	82.7	0.25	162.0	1.15	0.37	72.1	1.15
1b) Innophos STPP	83.2	0.07	224.5	2.89	0.51	157.0	2.89
1c) Innophos STPP	83.0	0.11	213.0	2.08	0.48	145.5	2.08
1d) Innophos STPP	85.4	0.14	245.0	3.21	0.56	187.5	3.21
1e) Innophos STPP	88.0	0.05	320.2	2.89	0.73	302.0	2.89
1f) WATER ONLY	84.6	0.17	123.6	0.58	0.28	49.9	0.58
2b) Innophos STPP + salt	85.4	0.63	239.5	2.65	0.54	242.0	2.65
3a) Carnal 659s	84.0	0.06	192.5	1.53	0.44	130.5	1.53
3b) Carnal 659s	84.8	0.25	226.5	3.21	0.51	171.5	3.21
3c) Carnal 659s	87.0	0.20	291.0	2.08	0.66	253.0	2.08
4a) Altesa ABC5	83.3	0.05	146.5	7.77	0.33	121.5	7.77
4b) Altesa ABC5	84.8	0.17	149.5	0.58	0.34	179.0	0.58
4c) Altesa ABC5	86.2	0.26	125.5	1.53	0.28	275.0	1.53
5) Innophos STPP + MTR-79	86.5	0.34	201.5	6.08	0.46	251.5	6.08
6) Innophos STPP + ALTESA ABC2D	88.4	0.59	191.5	4.93	0.43	265.0	4.93
8a) DPA Zefish	83.5	0.45	152.5	0.58	0.35	99.8	0.58
8b) DPA Zefish	86.4	0.09	118.5	0.58	0.27	217.5	0.58
9) MTR-79	85.3	0.64	129.0	2.31	0.29	228.0	2.31

*Average of 3 samples

Table 3-4. Summary of TLC results for shrimp samples showing which phosphate molecules were visualized on the plates indicating its presence

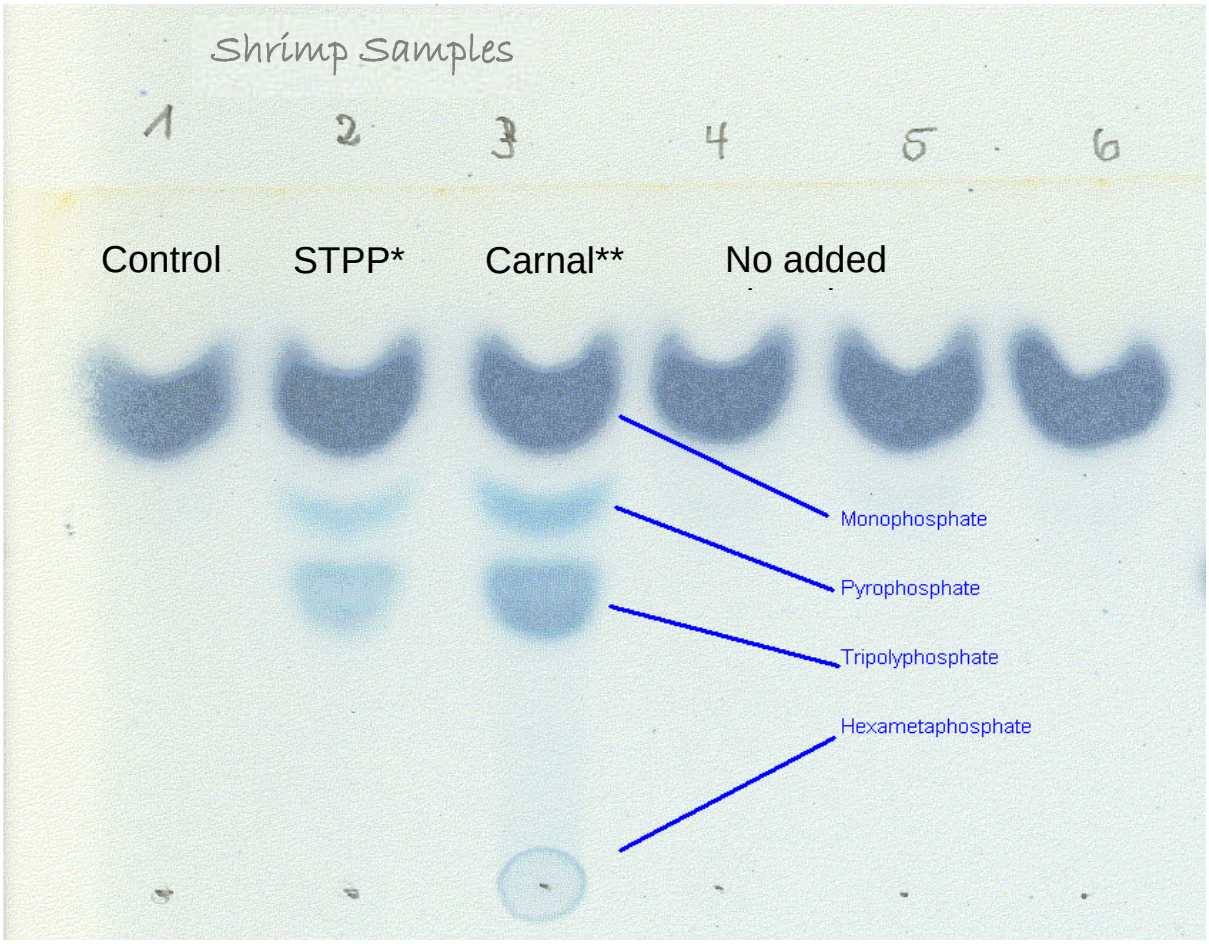
			Phosphate Molecule Visualized on TLC Plate			
Sample	Treatment Additive	Primary Ingredients	Ortho-	Pyro-	Tripoly-	Hexameta-
1	A&B Nutriphos STPP	Sodium tripolyphosphate	X	X	X	
2	STPP China	Sodium tripolyphosphate	X	X	X	
3	BK 512	Sodium phosphates	X	X	X	
4	BK 750 low sodium	Sodium and potassium phosphates	X	X	X	
5	Hasenosa AquaFish	Sodium phosphates and salt	X	X	X	
6	Hasenosa Mez 1396	Sodium phosphates	X	X	X	
7	Carnal 659s	Sodium pyrophosphate,	X	X	X	X
8	Carnal 659s	tripolyphosphate and hexametaphosphate	X	X	X	X
9	Carnal 2110s (low Sodium)	Sodium phosphates	X	X	X	
10	Carnal 2110s (low sodium)	Sodium phosphates	X	X	X	
11	Budenheim SP 313(Natural)	No phosphates	X			
12	Budenheim SP 313(Natural)	No phosphates	X			
14	Sodium Citrate	Sodium citrate	X			
15	Altesa PS	Sodium citrate, sodium carbonate	X			
16	HA-5096 FOSFREE/CH	No phosphates	X			
17	Control (salt)	NaCl	X			
18	Control	N/A	X			

X indicates visual appearance of molecule on TLC plate

Table 3-5. Summary of TLC results for pollock samples showing which phosphate molecules were visualized on the plates indicating its presence

			Phosphate Molecules Visualized on TLC Plate			
Sample	Treatment additive	Primary Ingredients	Ortho-	Pyro-	Tripoly-	Hexameta-
1A	Untreated	N/A	X			
1B	Innophos STPP	Sodium tripolyphosphate	X	X	X	
1C	Innophos STPP		X	X	X	
1D	Innophos STPP		X	X	X	
1E	Innophos STPP		X	X	X	
1F	Water only	Water	X			
2B	Innophos STPP + salt	Sodium tripolyphosphate, salt	X	X	X	
3A	Carnal 659s	Chemical blend of Sodium pyrophosphate, tripolyphosphate, hexametaphosphate	X	X	X	X
3B	Carnal 659s		X	X	X	X
3C	Carnal 659s		X	X	X	X
4A	Altesa ABC5	Trisodium citrate, Sodium carbonate, Sodium acid carbonate	X			
4B	Altesa ABC6		X			
4C	Altesa ABC7		X			
5	Innophos STPP + MTR-79	STPP, citric acid, sodium bicarbonate, salt	X	X	X	
6	Innophos STPP + ALTESA ABC2D	STPP, trisodium citrate, sodium carbonate, sodium acid carbonate	X	X	X	
8A	DPA Zefish	Citric acid, Sorbitol, Potassium carbonate, Salt	X			
8B	DPA Zefish		X			
9	MTR-79	Citric acid, Sodium bicarbonate, salt	X			

X indicates visual appearance of molecule on TLC plate



*STPP - Sodium tripolyphosphate
**Carnal - Commercial blend of sodium diphosphate, tripolyphosphate and hexametaphosphate

Figure 3-1. Example of typical TLC plate for shrimp samples revealing monophosphate in all samples and polyphosphates in samples 1 and 2 which were treated with polyphosphate additives

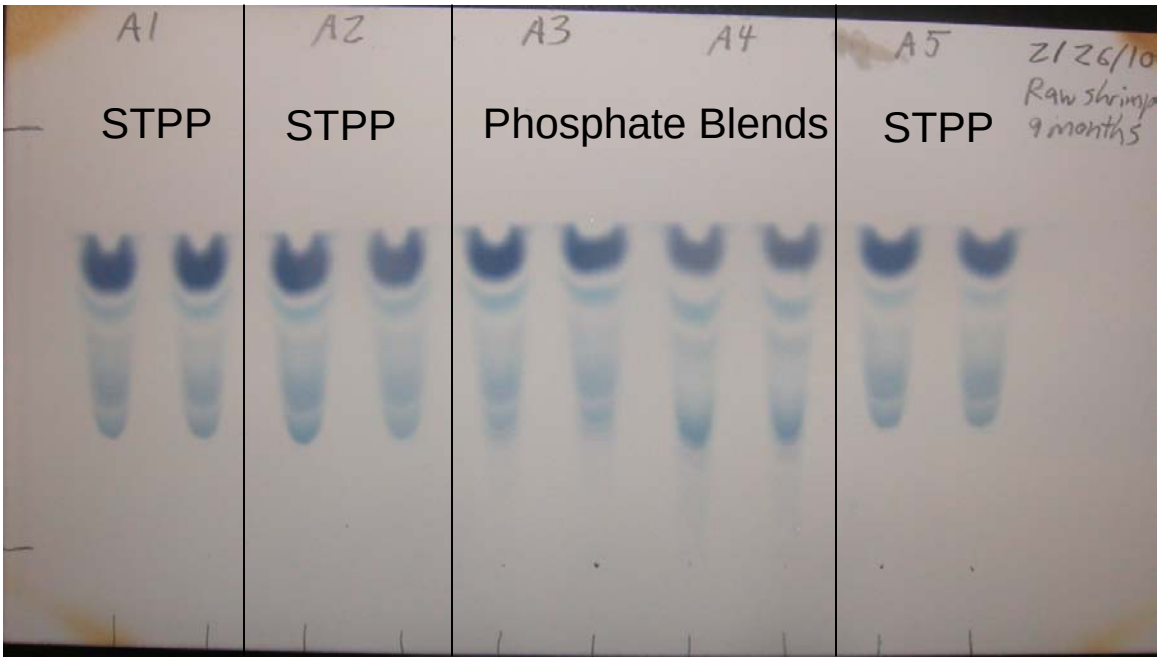


Figure 3-2. TLC results at 9 months frozen storage for raw shrimp samples A1-5 which all contained polyphosphates

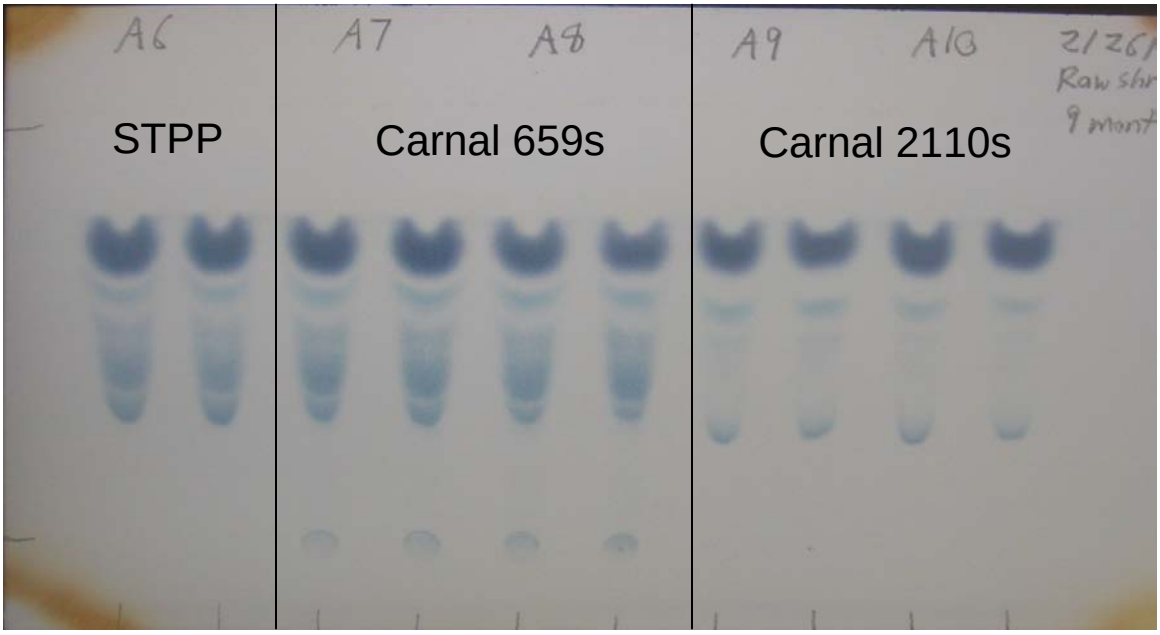


Figure 3-3. TLC results at 9 months frozen storage for raw shrimp samples A6-10 which all contained polyphosphates

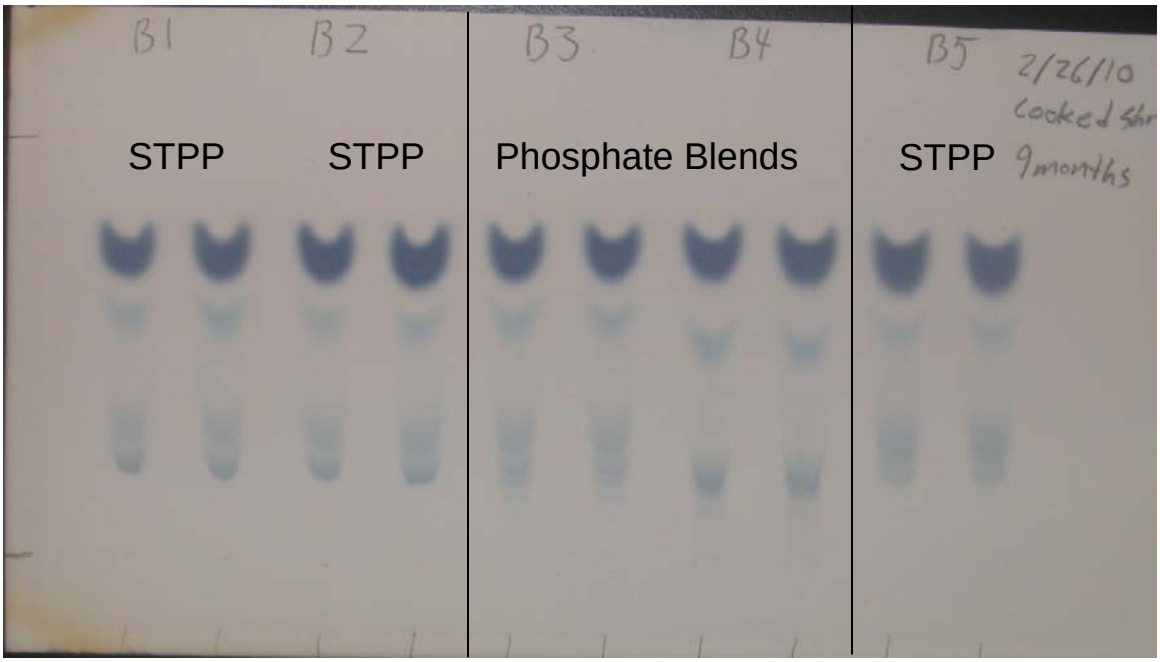


Figure 3-4. TLC results at 9 months frozen storage for cooked shrimp samples B1-5 which all contained polyphosphates

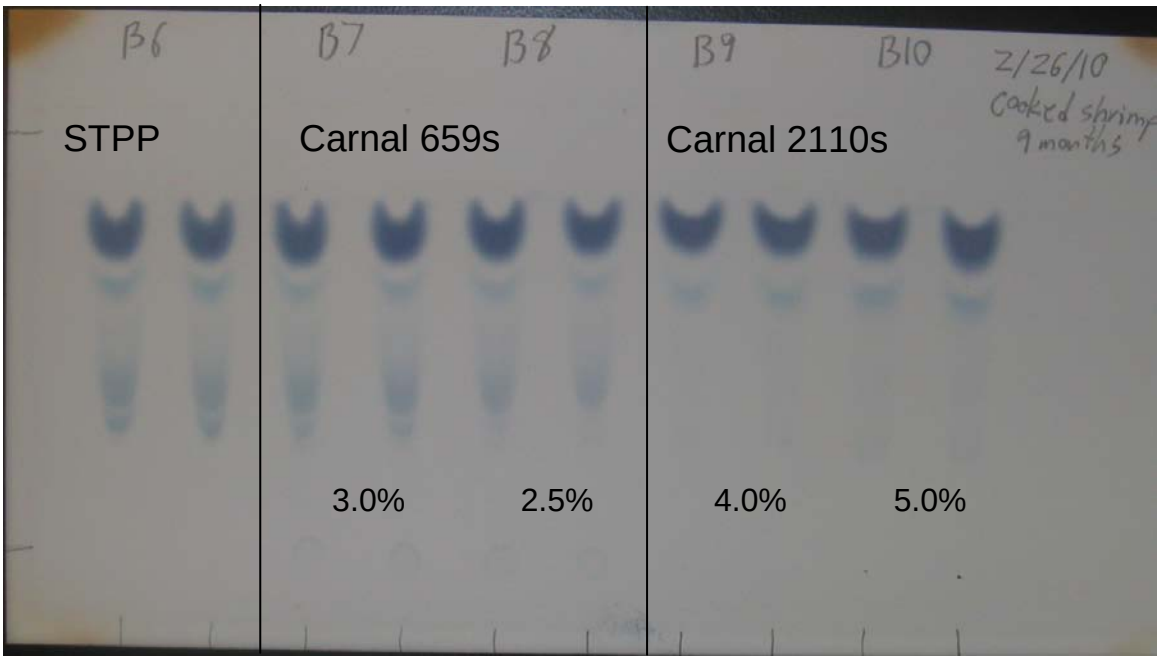


Figure 3-5. TLC results at 9 months frozen storage for cooked shrimp samples B6-10 which all contained polyphosphates

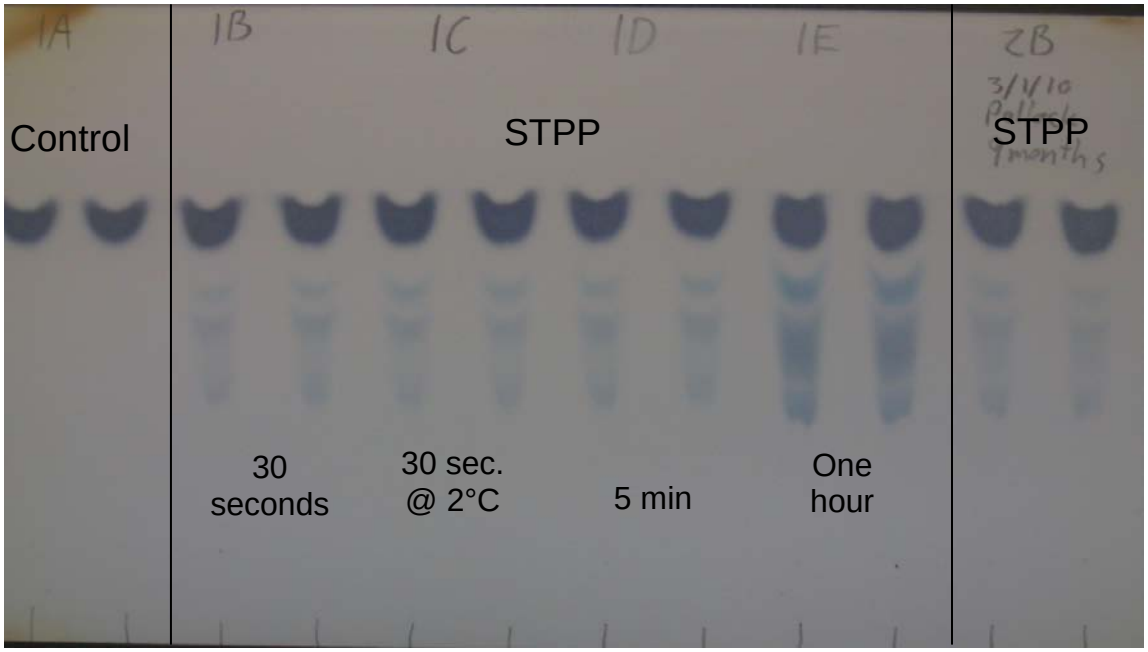


Figure 3-6. TLC results at 9 months frozen storage for pollock samples 1A-2B which all contained polyphosphates (except 1A control)

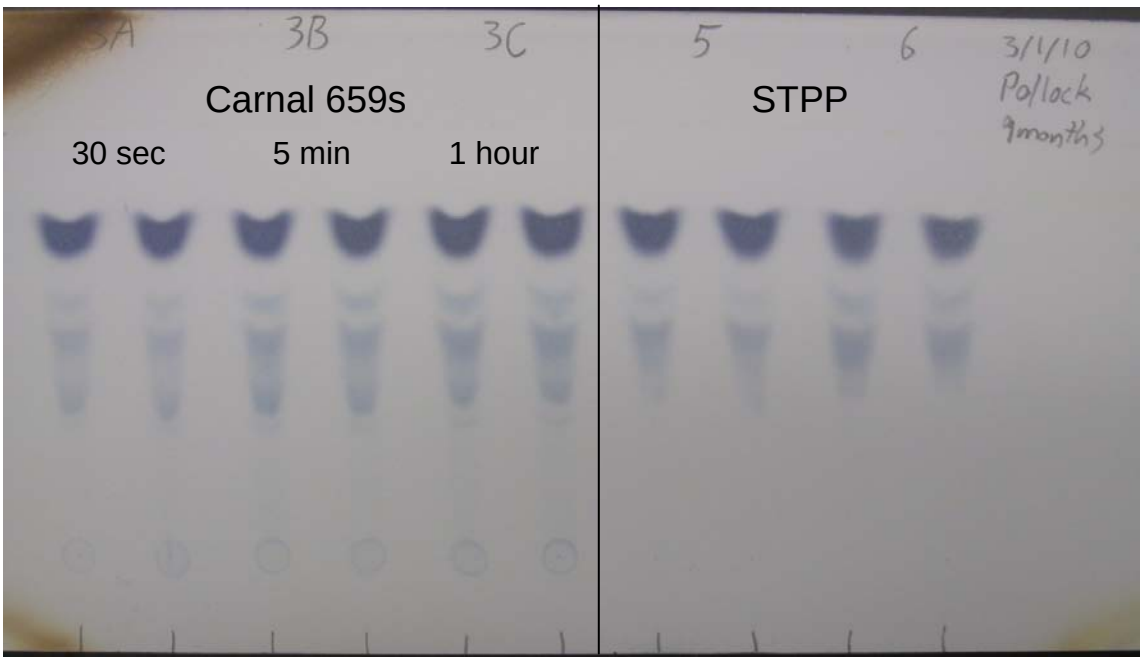


Figure 3-7. TLC results at 9 months frozen storage for pollock samples 3A-6 which all contained polyphosphates

CHAPTER 4
CONCLUSIONS

In conclusion, thin layer chromatography is a useful method for positively identifying the addition of polyphosphates in seafood products. This TLC method is effective for both raw and cooked seafood products. All of the phosphate treated shrimp and pollock samples were clearly and positively identified to contain polyphosphates by the TLC method. Furthermore, all of the samples that did not contain polyphosphate additives only showed spots for natural orthophosphate. Since there were no false positives or false negatives for the presence of polyphosphates, it was concluded that the method is 100% effective in identifying addition of polyphosphates in this study.

The degradation of polyphosphates was not significant in the frozen seafood as they were still detectable in shrimp and fish held in frozen storage for over one year. The high sensitivity of the TLC method allows for identity of prior phosphate treatment even at low concentrations (<0.05% added phosphates), which is well below ($1/10^{th}$) the proposed guidelines (0.5% added phosphates). The low cost (~$20 per plate) and convenience of the TLC method makes it suitable for routine use in regulatory and commercial sectors.

The chemical analyses of the samples used in the study provided supporting data which showed variations in phosphorus, sodium, and moisture contents between sample groups. Practical applications of the TLC method may supplement results with similar chemical analyses. This supporting data was of secondary importance to the development of the TLC method which was the main purpose of this study. Future studies could possibly correlate total phosphorus contents with color intensities of

corresponding spots on TLC plates to attempt to define ranges of phosphate concentrations.

The detailed description and simplicity of the procedures provide the ability for duplication and widespread use of this method. This TLC method can provide a much desired and needed test for better use, regulation, and labeling issues involved with phosphate additives and seafood products.

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BIOGRAPHICAL SKETCH

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