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the authority on seafood

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Review of polyphosphates as additives and testing methods for them in scallops and prawns

Campden BRI

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Executive summary

Phosphates, including polyphosphates, occur naturally in all forms of life and are therefore present in almost all food. Naturally occurring phosphates range from single phosphate groups (orthophosphates) to differing lengths of chains of phosphates; 2 phosphate groups (pyrophosphates), 3 phosphate groups (tripolyphosphates) and chains of 4 or more phosphate groups (polyphosphates). In cells phosphates are involved in metabolic functions, including acting as an energy source.

Phosphates can also be used as food additives; when added to seafood they have legitimate functional aims including retention of natural moisture, inhibition of flavour and lipid oxidation, aiding emulsification and removal of shell fish shells and offering cryoprotection. The actual mechanism of the action of added phosphates on proteins is not well understood. It is thought that one of the effects of adding phosphates is to alter the conformation (structural arrangement) of proteins, which increases the space between muscle fibres, creating more capacity for water holding. Phosphates have also been shown to stabilise the protein structure of meat, form a surface layer of coagulated (solid) protein around meat, swell muscle fibres and solubilise muscle proteins.

However, because phosphates can be used to retain moisture there are concerns that they could be misused to retain 'added water', thus increasing the size and weight of products resulting in unfair trade practices. Legislation pertaining to added phosphates differs around the world. In the EU, phosphates can be added to frozen and deep-frozen molluscs and crustaceans to a level of 5g/kg (0.5%), while the USA has no limit on their use. In the EU phosphates are not permitted in fresh/chilled molluscs and crustaceans; however those products which have previously been frozen but later defrosted and supplied chilled are permitted to contain phosphates.

The measurement of 'added' phosphates in seafood products is not straight-forward. There is the need to distinguish between naturally occurring levels of phosphates, which are not well defined, and the level of added phosphates. Attempts to use measurements of moisture content or phosphates (as phosphorus) have been found to be unsuitable to show that phosphates have been added to products, due to their large natural variation.
An additional complication is that added polyphosphates are broken down into pyrophosphates and eventually into orthophosphates in uncooked products due to the activity of naturally occurring enzymes called phosphatases. The rate of this break down increases with increasing temperature, but even occurs at 0˚C. In contrast, very little polyphosphate breakdown occurs in cooked products stored frozen even up to 11 months. This is thought to be due to phosphatases being inactivated during cooking.

Work has been conducted to show that ratios of moisture:protein and free-phosphate:protein-bound phosphate can provide valuable information on 'added' phosphates in seafood. These calculations however, rely on the use of interim nitrogen factors for the estimation of protein in scallops and prawns. More recently, chromatographic and capillary electrophoretic methods have been routinely used to determine levels of phosphates in seafoods; each method has inherent advantages and disadvantages, and different sensitivities. Although other methods are available to measure polyphosphates (nuclear magnetic resonance, thermo-differential-photometry and microwave dielectric spectroscopy) these are currently predominantly used in research settings rather than for routine analysis.

Contents

1. Introduction

Phosphates are a naturally occurring component of all living things and are therefore present in almost all food. Phosphates can also be used as food additives in foods such as processed cheese and meat, and may be added to seafood with the legitimate functional aim to;

- retain natural moisture, i.e. reduce drip losses through the freeze/thaw cycle during processing and storage
- inhibit fluid losses of fresh shipments
- inhibit the oxidation of flavours and lipids
- aid emulsification
- aid loosening the shell of shell fish ready for peeling
- offer cryoprotection

There are concerns though that as phosphates can be used to help retain moisture, then by application of excess phosphates the retention of "added water" can lead to unfair trade practices resulting in economic fraud.

1.1 The issue

Sea Fish Industry Authority is interested in the use of polyphosphates in scallops and prawns in particular, as excess levels of polyphosphates could be added to these products, which are difficult to detect. Further to this there are restrictions on what can be used in seafood products; the European Community Directive 95/2/EC "Food Additives Other Than Colours and Sweeteners" (which continues to apply, until 1 June 2013, under the transitional provisions of Regulation (EC) No 1333/2008) states in Annex IV that a maximum level of 5g/kg of additives E338-E452 (which includes phosphates, see Table 1) can be added to frozen and deep-frozen molluscs and crustaceans (please note the controls on phosphates in seafood are exactly the same in Directive 95/2/EC and Regulation 1333/2008). Food Standards Agency (FSA) guidance notes relating to the additives legislation state that "It is recognised that certain substances, for example phosphates and glutamates, are naturally present in certain foods. The quantitative limits set by these Regulations in nearly all cases relate only to the amount of additive *added*" (Food Standards Agency, 2002[a]). No information however is provided in the guidance on what those naturally present levels of phosphates are. Based on interpretation of the EC Directive, phosphates are not permitted in fresh/chilled molluscs and crustaceans; the FSA has however commented that "scallops which were previously frozen but later defrosted and supplied chilled are permitted to contain phosphates so long as the product is labelled as 'Previously frozen'" (Stephen Johnson, FSA Food Additives Branch, Email to stakeholders, 20/07/11). The legislation also differs around the world, for example in Japan only sodium pyrophosphate is allowed in scallops, in the United States phosphate compounds are "Generally Recognised as Safe " (GRAS) with no limitations for use, whilst in Canada phosphates are not permitted in raw scallops (Codex Alimentarius Commission, 2003).

To summarise, the main issues that the Sea Fish Industry Authority are interested in are:

1. Excess levels of phosphates in products that are added for fraudulent gain.
2. Phosphates that are used in products which are not permitted to contain them.
3. The lack of labelling on imported treated products.

Table 1: Permitted additives to frozen and deep-frozen crustacean products, as listed in Annex IV of Directive 95/2/EC (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1995:061:0001:0040:EN:PDF).
N.B. The maximum quantities of phosphoric acid and the phosphates E338, E339, E340, E341, E450, E451 and E452 may be added individually or in combination (expressed as P_2O_5).

E Number	Additive name
E338	Phosphoric acid
E339	Sodium phosphates (i) Monosodium phosphate (ii) Disodium phosphate (iii) Trisodium phosphate
E340	Potassium phosphates (i) Monopotassium phosphate (ii) Dipotassium phosphate (iii) Tripotassium phosphate
E341	Calcium phosphates (i) Monocalcium phosphate (ii) Dicalcium phosphate (iii) Tricalcium phosphate
E450	Diphosphates (i) Disodium diphosphate (ii) Trisodium diphosphate (iii) Tetrasodium diphosphate (iv) Dipotassium diphosphate (v) Tetrapotassium diphosphate (vi) Dicalcium diphosphate (vii) Calcium dihydrogen diphosphate
E451	Triphosphates (i) Pentasodium triphosphate (ii) Pentapotassium triphosphate
E452	Polyphosphates (i) Sodium polyphosphate (ii) Potassium polyphosphate (iii) Sodium calcium polyphosphate (iv) Calcium polyphosphates

1.2 Objective of this document

Sea Fish Industry Authority requested that Campden BRI conduct a desk-based literature review to provide information on the use of polyphosphates in seafood products, notably scallops and prawns. The document aims to fulfil this request and include the following information, as agreed with Sea Fish Industry Authority;

- **Review of polyphosphates** – To include details on the use of polyphosphates in the seafood industry in terms of what polyphosphates are, which polyphosphates are used and some background chemistry. Information on presence, composition, how polyphosphates interact with the product, whether/how they breakdown over time, breakdown products and naturally occurring levels.

- **Testing methods for polyphosphates –** To include what methods are available, what they involve, efficacy of testing methods, what to test for and issues with testing e.g. sensitivity.

2. Glossary

Throughout this document words with definitions included in the glossary are hyperlinked; click on the word of interest to go to the definition in the glossary.
Definitions adapted from http://www.thefreedictionary.com

Adenosine triphosphate	A nucleotide that occurs in muscle tissue; the major source of energy for cellular reactions.
Aerobic	Occurring in the presence of oxygen or requiring oxygen to live. In aerobic respiration, which is the process used by the cells of most organisms, the production of energy from glucose metabolism requires oxygen.
Anaerobic	Occurring in the absence of oxygen or not requiring oxygen to live.
Chelation	The combination of a metal ion with a chemical compound to form a ring, giving chemical compounds the ability to sequester metal ions.
Eukaryote	A single-celled or multicellular organism whose cells contain a distinct membrane-bound nucleus. All organisms except for bacteria and archaea (single-celled microorganisms that have evolved differently to bacteria) are eukaryotes.
Glycolysis	The metabolic breakdown of glucose and other sugars that releases energy in the form of ATP (adenosine triphosphate) and occurs in nearly all living cells.
Hydrolysis	The breaking down of a chemical compound into two or more simpler compounds by reacting with water.
Hydrophobic	Repelling, tending not to combine with, or incapable of dissolving in water
Mitochondria	A structure in nearly all eukaryotic cells in which food molecules (sugars, fatty acids, and amino acids) are broken down in the presence of oxygen and converted to energy in the form of ATP (adenosine triphosphate)
Oxidative phosphorylation	The process in cell metabolism in which enzymes in the mitochondria synthesize ATP (adenosine triphosphate) by oxidation of metabolites.
Proteolysis	The breaking down or hydrolysis of proteins into simpler compounds, as occurs during digestion.

3. Polyphosphates review of literature

A review of literature has been undertaken to provide information on polyphosphates. The review is presented as a list of references (in boxes), with relevant information extracted beneath. The list of publications is divided into sections according to area of interest;

- the chemistry of polyphosphates
- the role of naturally occurring polyphosphates
- presence and levels of naturally occurring polyphosphates in scallops and prawns
- how polyphosphates interact with the product
- how polyphosphates breakdown over time and their breakdown products

Each section also includes a summary, which draws together key information for each subject area.

A list of sources of additional information is provided at the end of the document.

3.1 The chemistry of polyphosphates

The terms phosphate and polyphosphate, used in this review, are defined in the following documents.

Torry Research Station (2001) *Polyphosphates in Fish Processing*. FAO Corporate Document Repository. (31).
URL: http://www.fao.org/wairdocs/tan/x5909E/x5909e01.htm
Accessed: January 2012.

"A phosphate is a salt of phosphoric acid; when a number of simple phosphate units are linked to form a more complex structure, this is known as a polyphosphate. Phosphates fall into several categories depending on the length of the phosphate chain:

- Simple phosphates (orthophosphates) - 1 phosphate group
- Pyrophosphates - 2 phosphate groups
- Tripolyphosphates - 3 phosphate groups
- Polyphosphates - 4 or more phosphate groups

Phosphates are additives which are used to improve the quality of many foodstuffs, but specifically meat and seafood. In regards to seafood it is the longer polyphosphate chains, tripolyphosphates and polyphosphates, which prove useful in increasing the quality and shelf life of the product. Most processors use proprietary mixtures containing appropriate pyrophosphate, triplyphosphate and polyphosphate compounds".

Chen, K.Y. (1999). *Study of polyphosphate metabolism in intact cells by 31-P nuclear magnetic resonance spectroscopy.* Progress in Molecular and Subcellular Biology (23) p253-273.

Polyphosphates are naturally occurring polymers (chains) of orthophosphate monomers and are found in the majority of living things including microorganisms, animals and lower eukaryotes (e.g. yeast).

Phosphates have a tetrahedral structure; this is where four oxygen atoms are bonded to a central phosphorous atom. The remaining atoms involved in the overall structure depend on the type of phosphate. For example an orthophosphate molecule has 3 hydrogen atoms attached to three of the four oxygen atoms: one of the oxygen atoms has a double bond with the phosphorous and therefore cannot bond with any other atoms (Figure 1a). In comparison, sodium tripolyphosphate consists of 3 phosphate groups joined together to form a chain, by sharing an oxygen atom between different phosphate groups, and with sodium atoms bound to 5 oxygen atoms.

Figure 1a*:
A molecule of orthophosphate

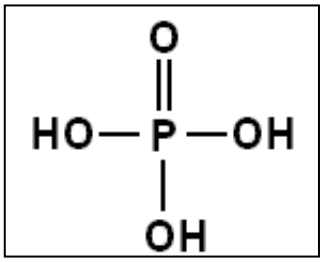

Figure 1b#:
A molecule of sodium tripolyphosphate

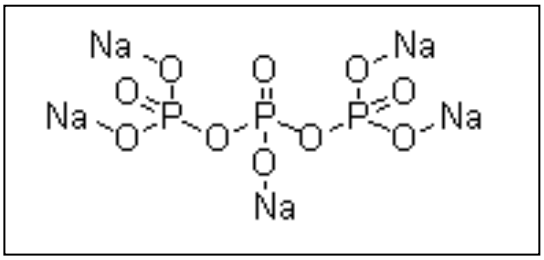

*****Figure 1a sourced from: http://www.guidechem.com/dictionary/14265-44-2.html
#Figure 1b sourced from: http://www.chemblink.com/products/7758-29-4.htm

Lampilla, L.E. (1993). *Polyphosphates: Rationale for use and functionality in seafood and seafood products.* Conference Proceedings from the Annual Tropical and Subtropical Fisheries Technological Conference of the Americas (August 29 - September 1, 1993, Williamsburg, Virginia, U.S.A.), p13-20

"Phosphates [used as additives] can either be refined from calcium phosphate (once this has been mined) or through varying degrees of neutralisation of phosphoric acid with alkali metal ions or alkaline earth metals, such as sodium or calcium respectively to produce salted polyphosphates. By undergoing various degrees of neutralisation, phosphoric acid can produce two general classes of phosphates; simple phosphates or condensed phosphates. Simple phosphates consist of a phosphorous atom surrounded by four oxygen atoms and [...] metal ions or hydrogen, some examples would be orthophosphate [see Figure 1a] or disodium orthophosphate. Condensed phosphates are considered to be short to long chains or cyclic forms: condensed phosphates have the broadest applications in the seafood industry. Orthophosphates have virtually no effect on water binding (Offer and Trinick, 1983)".

Gonçalves, A.A. and Ribiero, J.L.D. (2008). *Do phosphates improve the seafood quality? Reality and legislation.* Pan-American Journal of Aquatic Sciences. 3(3). p237-247.

Polyphosphates as additives help muscular protein solubilisation and increase the pH (reduce acidity). Phosphates commonly used as additives are sodium tripolyphosphate alone or in mixtures with sodium hexametaphosphate or sodium acid pyrophosphate and/or tetrasodium pyrophosphate. These phosphate compounds have a combination of properties, such as solubility, chelation of metal ions (which inhibits oxidative processes) and pH adjustment.

3.1.1 Summary

Phosphates occur naturally in all forms of life, and range from orthophosphates (with 1 phosphate group) to polyphosphates (consisting of chains of 4 or more phosphate groups). Polyphosphates may also include other elements, such as sodium, potassium and calcium to form compounds such as sodium polyphosphate, potassium polyphosphate and calcium polyphosphate.

Generally mixtures containing pyrophosphate, tripolyphosphate and polyphosphates are used as additives in the seafood industry.

3.2 The role of naturally occurring polyphosphates

Chen, K.Y. (1999). *Study of polyphosphate metabolism in intact cells by 31-P nuclear magnetic resonance spectroscopy.* Progress in Molecular and Subcellular Biology (23) p253-273.

"The ubiquitous occurrence, the wide range of content in different organisms, and the subcellular distribution of polyphosphates all suggest that polyphosphates may have diverse functions depending on the cell types, organisms, and environments." Polyphosphates have been reported to function as an energy storage source, a phosphate reserve, a substrate for enzymes, a buffer against pH stress, a counter ion to neutralize cationic species in the vacuole, a component of specific membrane channels for DNA entry and a regulator in response to environmental stress.

Polyphosphates also play an important role in the regulation of levels of adenosine triphosphate (ATP), which transports chemical energy within cells for metabolism. ATP cannot be stored but is continuously recycled (broken down and synthesised) in organisms. It has been difficult to study the metabolism, regulation and function of polyphosphates for several reasons, one of which is because they may exist as a mixture of polymers with varying chain lengths which are constantly broken down and produced during metabolism.

Wood, H.G. and Clark, J.E. (1988). *Biological aspects of inorganic polyphosphates. Annual Review of Biochemistry*. 57. p235-260.

A universal role for polyphosphates in all the organisms in which they are found (including bacteria, fungi, plants, insects and mammals) has not been demonstrated. Several biological functions have been proposed, although evidence is largely circumstantial. Part of the problem has been inadequate methods for studying these polymers, and poor knowledge of the enzymes involved in polyphosphate metabolism.

Huss, H.H. (1995). FAO Fisheries Technical Paper 348: *Quality changes in fresh fish*. Available at: http://www.fao.org/DOCREP/V7180E/V7180E00.HTM
Accessed: April 2012

Phosphates that are naturally present in muscle tissue play an important role in rigor mortis of meat and fish. Following death of an animal the degradation of ATP causes a contraction and stiffening of muscle tissues, which also results in the release of enclosed water from the muscle tissue.

Storey, K.B. (2004). *Oxygen Limitation and Metabolic Rate Depression.* Functional Metabolism: Regulation and Adaption. Wiley-Liss, New Jersey. p416-417.

During low level or sustained medium level intensity exercise ATP is made in the mitochondria by a process called oxidative phosphorylation. When high intensity or "burst" exercise is required e.g. when animals escape predators, then oxidative phosphorylation is not sufficient. There are 2 mechanisms that can be employed to produce enough energy for this high intensity activity;

- the rapid gathering of muscle phosphate reserves to produce an instant supply of ATP
- by increasing glycolysis, where glucose is broken down to produce ATP

Both of these routes produce sufficient energy to provide a short (usually only a few seconds) burst of energy which will allow the animal to evade capture. However, by performing these bursts of energy the amount of end product increases, in scallops the end-product is an opine called octopine. Octopine is formed from the breakdown of arginine phosphate. The resulting excess of octopine is not necessarily a bad thing, as an increased amount of octopine can cause the cellular balance to shift towards the production of ATP: hence giving more energy to the scallop.

Metabolism involving arginine phosphate degradation and octopine production is generally only seen in marine life, such as crustaceans and molluscs, while other forms of phosphate and opine metabolism are used by other species.

Bailey, D.M., Peck, L.S., Bock, C. and Pörtner, H-O. (2003). *High-Energy Phosphate Metabolism during Exercise and Recovery in Temperate and Antarctic Scallops: An In Vivo 31P-NMR Study*. Physiological and Biochemical Zoology. 76 (5) p622-633.

Scallops use ATP as an energy source, like all other living organisms. The breaking of bonds in ATP releases a large amount of energy. There are two different types of metabolism in scallops, anaerobic (not requiring oxygen) and aerobic (requiring oxygen). In scallops, the ATP used during muscular activity is initially regenerated by the breakdown of phospho-$_L$-arginine (PLA) followed by anaerobic and aerobic production of ATP. In anaerobic metabolism, octopine is formed from pyruvate, arginine and NADH; this is equivalent to the pyruvate to lactate pathway of vertebrates.

3.2.1 Summary

Polyphosphates play an active role in metabolism, in particular adenosine triphosphate (ATP) which is used as cellular energy, as a large amount of energy is released when it is broken down. In crustaceans and molluscs ATP can be generated in a pathway particular to these species, involving phospho-$_L$-arginine, octopine and pyruvate. The study of polyphosphates has been difficult as they may exist as a mixture of polymers with varying chain lengths which are constantly broken down and produced during metabolism.

3.3 Presence and levels of naturally occurring polyphosphates in scallops and prawns

Little information is available in the literature about the naturally occurring levels of phosphates in crustaceans and molluscs; this is because levels change rapidly depending on temperature, pH, storage conditions and/or enzyme activity. There are also differences between levels in different species, between individuals of the same species (Gibson and Murray, 1973) and between the same species but in different geographical locations. Differences can additionally occur depending on how the animals have been caught and handled.

Canadian Food Inspection Agency (2011). *Appendix 3 Canadian Guidelines for Chemical Contaminants and Toxins in Fish and Fish Products.* Product Inspection of Fish and Seafood. (online) Available at : http://www.inspection.gc.ca/english/fssa/fispoi/man/samnem/app3e.shtml Accessed: January 2012.

Canadian regulations do not allow the use of phosphates in scallops. Table 2 shows the background (naturally occurring) levels of phosphates, as disodium phosphate (Na_2HPO_4), as stated in the Canadian Guidelines for Chemical Contaminants and Toxins in Fish and Fish Products for several types of molluscs and crustaceans. Scallops would be rejected if the disodium phosphate level exceeds 1.47%, although the following caveat is stated "If a processor can provide reliable data for naturally occurring background levels that are higher than those shown [in Table 2], this may be considered before product action is taken".

Table 2: Background levels for non-permitted additives.

Background Levels for Non-permitted Additives		
Additive	**Product Type**	**Background Level**
Phosphates*	Shrimp (raw, cooked and canned)	1.60 %
	Scallops (raw)	1.47 %
	Crab (raw and cooked)	1.70 %
	Lobster (raw and cooked)	1.47 %
	Surf clams (raw and cooked)	1.00 %

*calculated as disodium phosphate (Na_2HPO_4)

Vovcsko, J. (1997). *When does the use of tripolyphosphates equal economic fraud?* Seafood Supplier. Available at: http://www.southeasternfish.org/Members/Documents/stpreg.htm Accessed: January 2012.

"The amount of polyphosphates present in the product can be estimated based on the amount of water that is present in the product: this is because the longer the product stays in contact with the polyphosphates, then the higher the water retention. For example, naturally sourced scallops would expect to have around 80% of its mass as water, whereas a scallop that has been in contact for an excess amount of time with a polyphosphate solution might contain greater than 85% of its mass as water. The latter would be deemed adulterated and it would be seen that the use of polyphosphates has been abused for financial gain".

According to this reference, in 1997 the American Shrimp Processors Association commissioned Dr Steve Otwell of the University of Florida to develop labelling guidelines for phosphate. The guidelines were at that time very preliminary, and no direct mention of them has been found in subsequent literature, so it is not clear what happened (though the use of phosphorus and water content to determine added phosphates has been subsequently opposed – see note below). For information the shrimp and scallop preliminary guidelines are provided below;

Shrimp containing up to 82 percent water and no more than 250mg of phosphorus per 100 grams of shrimp would be labelled "phosphate added".
Shrimp containing more than 85 percent water and over 250mg of phosphorus per 100 grams of shrimp would require labelling that states "phosphate and water added".

Scallops that are less than 80 percent water would be called scallops (or sea scallop, or some similar designation), while scallops containing between 80 and 84 percent water would be called "scallop product" (or water-added scallop product). Scallops with a water content above 85 percent would be considered adulterated.

National Oceanic and Atmospheric Administration. (2011). Inspection Manual 25: Seafood Inspection Programme, Chapter 4: Policies, Procedures, and Requirements for the Audit of Fisheries Products on a Lot by Lot Basis: Product (Lot) Inspection Procedures – Scallop moisture determination.
Available at:
http://www.seafood.nmfs.noaa.gov/NOAA%20Handbook25/Scallop_Moisture.html
Accessed: April 2012.

In 1992, the USA Food and Drug Administration (FDA) through the Office of Seafood issued a policy memo entitled 'Interim Labelling Policy Established for Scallops'. It was stated that "Scallops less than 80.0% total moisture, if not subjected to processing conditions utilizing excessive water and/or phosphate treatment, could be labelled simply as scallops. As opposed to scallop products whose total moisture analysis demonstrated a percentage of 80.0 % to 84.0% would have to be labelled " X % Water Added Scallop Product" appearing in the principal display panel of the label. The statement, "Processed with Sodium Tripolyphosphate," or any other polyphosphates used, is also to appear in the identity statement if the product has been processed with the ingredient. In addition, the ingredient statement on the labels for these products must include water and sodium tripolyphosphate

(or other phosphate, as appropriate). Products having a moisture content over 84.0 % were considered adulterated". In 2004 the FDA rescinded the Interim Labelling Policy of 1992, so the percentages used for defining labelling statements above are no longer being enforced.

The National Oceanic and Atmospheric Administration Inspection Manual (2011) also states that "because of the concern over improper labelling or misuse of the process, the Seafood Inspection Program will continue to require that all lots of scallops over 200 pounds destined for domestic use be tested for total moisture [...]. The results of the analysis will be noted on the certificate, score sheet or memorandum. If the inspector has definitive knowledge that the product has been treated in some way to add water to the product, the label must reflect that. Also if the product tests over 83.0 % for total moisture, the SIP will assume that the product has been treated and must be properly labelled. This assumption is based on studies and data collected by various governmental agencies, academia, and other organizations that have demonstrated total moisture content of scallops consistently less than 83%. At this time there is no upper limit for moisture content".

N.B. The use of water content alone to prove adulteration of scallops with polyphosphates has been opposed by other authors; due to great variations in moisture content in scallop meat depending on the species, seasonality, harvest practices and geographical location (Botta and Cahill, 1993).

Þórarinsdóttir, K.A., Arason, S. and Þorkelsson, G. (2010). *The role and fate of added phosphates in salted cod products.* Icelandic Food and Biotech R&D, Report Summary, Matís. July 2010. p1-28.

"Protein rich foods, such as seafood products, include phosphorus containing compounds such as nucleotides, phospholipids and naturally occurring orthophosphates. There is approximately 0.11-4.8% naturally occurring orthophosphates in seafood, dependant on their regional location and composition to name but a few: this makes detection of added phosphates difficult.

The quantification of phosphate alone cannot be used to verify the presence of added phosphates due to naturally occurring orthophosphates and other varying phosphorous compounds (e.g. phospholipids). There is also large variation in phosphorous content between individuals within a species. The level of phosphate compound added to the product can be estimated using the phosphate to protein ratio, which is used to calculate the difference between the total phosphorous and the phosphorous bound to proteins. The protein content depends upon the concentration of nitrogen in meat products".

The protein content of meat is calculated based on the nitrogen content (measured by Kjeldahl method) and using a conversion factor (typically 6.25 for the determination of crude protein in foods). Codex (2003) state that a specific nitrogen factor for scallops needs to be developed, and that this would require considerable time, labour and expense. However, a Code of Practice on the Declaration of Fish Content in Fish Products (1998) does provide interim nitrogen factors for several fish and shellfish species, including prawn, shrimp, scallops and queens. For more details on protein content determination and the use of nitrogen factors, see section 4.1.2.

3.3.1 Summary

All seafood contains naturally occurring levels of phosphates and therefore phosphorus. It is difficult to determine the level of added polyphosphates by measuring phosphate or phosphorus alone, due to the large variation in naturally present levels.

It has been proposed that a measure of moisture content could be used to determine when polyphosphates have been added to seafood products, but like phosphate, moisture content varies greatly making this method alone unsuitable.

An estimation of polyphosphate levels using the phosphate to protein ratio has also been proposed, but to calculate the protein content of scallops the interim nitrogen conversion factors for scallops may need to be examined, which could involve considerable time, labour and expense.

3.4 How polyphosphates interact with the product

Although much work has been conducted on the effects of polyphosphate treatment on food products including meat and seafood, the actual mechanism of the action of polyphosphates on proteins is not well understood. It is however known that the water holding capacity of a proteinaceous food involves interactions between the protein and water; increased water holding capacity is hypothesised to be due, in part, to increased space between muscle fibres, creating more capacity for water holding.

Work looking at the microstructure of whole-muscle processed meats, which have been tumbled with sodium chloride and polyphosphate solutions, has shown that a surface layer of coagulated (solid) protein forms. This helps to hold the water within the muscle (Velinov, Zhikov and Cassens, 1990). Sutton (1973), used phase contrast microscopy to show that tripolyphosphate disrupts the close-packed arrangement of the protein molecules in natural cod muscle, by altering the conformation of the proteins. Others have shown that addition of polyphosphates results in swelling of muscle fibres (Offer and Trinick, 1983) and solubilisation of muscle proteins which increases their dispersion (Lewis, Groves and Holgate, 1986). Polyphosphates have also been shown to sequester metal ions, which inhibits oxidative processes (Dziezak, 1990) and alter the pH of meat (Trout and Schmidt, 1984).

Gonçalves, A.A. and Ribiero, J.L.D. (2008). *Do phosphates improve the seafood quality? Reality and legislation.* Pan-American Journal of Aquatic Sciences. 3(3). p237-247.

When used as additives, polyphosphates interact with the surface of the product, due to the way the product is exposed to the polyphosphate solution. The exact concentration of polyphosphate solution and time of treatment depend on the seafood species, examples of how polyphosphates are added to products and concentrations commonly used by industry are shown below (Schnee, 2004):

- Ice making - 3%
- Dipping - 2-6% for 20 minutes
- Washing - 2-6% for 20 minutes
- Spraying - 5-10%
- Tumbling - 2-6%
- Injecting - 5-8%
- Glazing - 5%

Treating seafood with polyphosphate increases the amount of water that is retained by the product, therefore allowing the product to be sold at a higher weight. However if the product is exposed to too high a concentration or is exposed for too long then there is a risk of damage; a distinct soap like taste may be detected when eaten or the product may develop sliminess, translucency and decomposition, due to the increase in pH. In the worst scenario excess treatment can lead to proteolysis.

In terms of shrimp, the phosphates do not penetrate the shell and therefore to improve water holding capacity the shell would need to be removed before the polyphosphate solution was added. Indeed addition of polyphosphates aids removal of the shrimp exoskeleton and increases recovery of shrimp meat (Crawford, 1980, Henson and Kowalewski, 1992).

Scallops have been treated with sodium tripolyphosphate at concentrations of 10% or 4% for 1 and 20 minutes respectively and 2.5% until the scallops reach a level not exceeding 86% moisture. The polyphosphate solution was administered in the presence of low levels (1% concentration) of sodium chloride. These treatments were found to be efficient at reducing drip loss between freezing and defrosting, as well as improving the water holding capacity of the product (Rippen *et al*. 1993). The addition of sodium chloride along with phosphates has been shown to improve both the interaction with protein and the distribution of flavour (Lampilla, 1992).

Taylor, P.G. (1993). *The application of phosphates in the processing of the pacific shrimp, or what's so different about this use*? Conference Proceedings from the Annual Tropical and Subtropical Fisheries Technological Conference of the Americas (August 29 - September 1, 1993, Williamsburg, Virginia, U.S.A.), p.72-77.

Keeping pacific shrimp on ice for three to four days after catching improves the ability of mechanical peelers to remove the shell from the meat. This is due to the enzymatic action degrading the connective tissue between the musculature and the shell. This however can result in the loss of some solubilised tissues upon separation and washing.

Crawford, D.L. (1980). *Meat yield and shell removal functions of shrimp processing*. Oregon State University Extension Marine Advisory Program. Land Grant/Sea Grant Cooperative Special Report 597. Available at: http://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/4804/SR%20no.%20597_ocr.pdf?sequence=1
Accessed: February 2012

Crawford (1980) found that a 6% phosphate solution could be used at low temperature to gelatinise the connective tissue between the musculature and the shell of shrimp. The 6% phosphate solution increased meat recovery by 10% compared to recoveries made when the phosphate solution was not used.

During these experiments the phosphorous content of untreated shrimp was measured following cooking, giving values in the range of 537 to 727mg total phosphate (P_2O_5)/100g of wet weight; therefore a variation of 190mg. The 6% polyphosphate solution increased the phosphate content of the cooked shrimp by as much as 110mg/100g over the control samples. Therefore, the quantity of phosphate added to the shrimp is within the range of phosphate levels naturally occurring in these animals.

Þórarinsdóttir, K.A., Arason, S. and Þorkelsson, G. (2010). *The role and fate of added phosphates in salted cod products.* Icelandic Food and Biotech R&D, Report Summary, Matís. July 2010. p1-28.

During initial immersion in a phosphate solution the total phosphate in the product may decrease before it increases. This is due to the natural orthophosphates diffusing out of the product in to the solution; referred to as a counter-current direction. The initial diffusion rate of orthophosphate from the muscle to solution occurs quickly, but the rate reduces once the protective phosphate barrier is formed around the product. During exposure of the product to the phosphate solution, the muscle fibres are thought to swell: this in turn reduces the extracellular space and causes a narrowing of the channels within the muscles, which prevents further loss of natural orthophosphates.

Ünal, S.B., Erdo, F., Ekiz, H.B. and Özdemir, Y. (2004). *Experimental theory, fundamentals and mathematical evaluation of phosphate diffusion in meat*. Journal of Food Engineering. 65(2). p263-272.

Polyphosphates control the loss of natural juices, reduce the susceptibility to freezer burn and cook losses by increasing water binding capacity of the products due to the increased pH and ionic strength.

Naturally occurring orthophosphates in meats and sodium tripolyphosphates in solutions in which meats were treated resulted in a counter-current diffusion. The phosphate content of the meats started to decrease until a certain time where they showed an increase. On the contrary, phosphate content of the solutions regularly increased during dipping due to the diffusion of orthophosphates from within the samples.

Lampilla, L.E. (1993). *Polyphosphates: Rationale for use and functionality in seafood and seafood products.* Conference Proceedings from the Annual Tropical and Subtropical Fisheries Technological Conference of the Americas (August 29 - September 1, 1993, Williamsburg, Virginia, U.S.A.), p13-20

Pyrophosphates have been shown to affect hydrophobic interactions in beef proteins, which stabilised the protein structure and therefore the thermal stability of the protein (Trout and Schmidt, 1984). Increasing the pH with 1M NaOH in combination with pyrophosphate increases the temperature for the protein to denature (irreversibly alter its 3 dimensional shape).

Water retention increases in the product if the pH is increased and a polyphosphate, such as sodium tripolyphosphate, is used. Orthophosphates have little to no effect on the water retaining properties of the product, whereas pyrophosphates are associated with improved protein solubility, especially for muscle fibres. Water binding is dependant on the type/blend of phosphate used and the type of product being treated.

Phosphated ice is a combination of polyphosphate solution along with ice, which could be used to store shrimp. Phosphated ice is proven to increase the weight and reduce the moisture loss in the shrimp. However storing shrimp in these conditions increases the risk of

the products being over exposed to polyphosphates, if they are treated again during further processing, which could result in a soapy flavour and/or physical quality defects such as sliminess or translucency.

Gonçalves, A.A and Ribiero, J.L.D. (2008). *Do phosphates improve the seafood quality? Reality and legislation.* Pan-American Journal of Aquatic Sciences. 3(3). p237-247

Due to the highly perishable nature of shrimp it is crucial that the additive is added quickly and at low temperatures. The low temperature should also help with the solubility of the polyphosphates.

Tenhet, V., Finne, G., Nickelson., R. and Toloday, D. (1981). *Penetration of sodium tripolyphosphate into fresh and prefrozen peeled and deveined shrimp*. Journal of Food Science, 46. p344-349

"The distribution of sodium tripolyphosphate (STP) in peeled and deveined shrimp tails after treatment was investigated using radioactive (P^{32}) labelled STP. When shrimp were treated in 0.5% and 1% STP solutions, a phosphate concentration gradient was evident in the shrimp muscle. After such treatments, STP was shown to accumulate on the surface of the muscle, preventing further STP uptake. At higher STP concentrations (5% and 10% solutions) prolonged time treatments (5 and 20 minutes) overcame the concentration gradient as STP became equally distributed through the shrimp muscle (4.8mm)".

So penetration of phosphate solution into shrimp muscle depends on the concentration of phosphate in the solution, time of application and the thickness of the muscle.

Torry Research Station (2001). *Catching and Processing Scallops and Queens.* FAO Corporate Document Repository. (46).
Available at: http://www.fao.org/wairdocs/tan/x5923E/x5923e01.htm
Accessed: February 2012.

Table 3 shows that dipping scallops in water alone (without addition of polyphosphate) will increase the weight of the scallops after dipping, after freezing and glazing, and after thawing. Differing concentrations of polyphosphate solutions also increase the weight of scallops, but to varying degrees; i.e. polyphosphate treatment for 1 minute increased the weight of scallops, but the increase is less than the controls treated with water. When scallops were dipped in polyphosphate solution for 2 minutes the weight increased after dipping, but was actually lower than scallops dipped for 1 minute. This is thought to be due to the counter current diffusion of the orthophosphates from the product into the polyphosphate solution. Both the 10 minute and 30 minute treatments show a significant percentage weight increase over all other treatments following thawing, however scallops treated with polyphosphate for 30 minutes had less weight increase after freezing and glazing than those treated with water. This may be due to proteolysis (breakdown of proteins) due to over exposure.

Table 3: Percent weight increases of scallop after various treatments

Dip treatment	Dipping time (minutes)	% Weight increase after dipping	% Weight increase after freezing and glazing	% Remaining weight gain after thawing
Water	30	3.0	7.3	2.0
Polyphosphate Solution	1	1.0	6.5	1.5
Polyphosphate Solution	2	0.4	7.7	1.5
Polyphosphate Solution	10	1.5	8.2	3.2
Polyphosphate Solution	30	2.0	6.6	3.4

3.4.1 Summary

The way polyphosphates interact with seafood products depends on how they are applied; in the majority of cases polyphosphates are applied to the surface of the product by dipping, washing, spraying, tumbling, glazing or holding products on phosphated ice. Depending on the concentration of polyphosphate applied, the application time and the size of the product, the polyphosphates diffuse through the meat to varying degrees.

Polyphosphates have been shown to stabilise the protein structure of meat, form a surface layer of coagulated (solid) protein, swell muscle fibres and solubilise muscle proteins.

3.5 How polyphosphates breakdown over time and their breakdown products

Torry Research Station (2001). *Polyphosphates in Fish Processing*. FAO Corporate Document Repository. (31).
Available at: http://www.fao.org/wairdocs/tan/x5909E/x5909e01.htm
Accessed: January 2012.

"Most polyphosphates added to food are broken down to single phosphate units (orthophosphates) in the stomach when the food is eaten; indeed, many are converted to single units in the food before it is eaten, for example in chill storage or during cooking."

Tenhet, V., Finne, G., Nickelson., R. and Toloday, D. (1981). *Penetration of sodium tripolyphosphate into fresh and prefrozen peeled and deveined shrimp*. Journal of Food Science, 46, p344-349.

Experiments showed that after two weeks of frozen storage only 12% of the total phosphorous in uncooked shrimp muscle corresponded to the originally added tripolyphosphate. By ten weeks of frozen storage the phosphorous levels corresponded to 45% orthophosphate. This is probably due to naturally occurring hydrolysis; there was no heat treatment to facilitate the hydrolysis of the tripolyphosphates.

Taylor, P.G. (1993). *The application of phosphates in the processing of the pacific shrimp, or what's so different about this use?* Conference Proceedings from the Annual Tropical and Subtropical Fisheries Technological Conference of the Americas (August 29 - September 1, 1993, Williamsburg, Virginia, U.S.A.), p.72-77.

At elevated temperatures, such as in steam cooking, sodium tripolyphosphates will be quickly and efficiently hydrolysed to orthophosphates, which are one of the natural phosphates found in muscle. Orthophosphates, unlike polyphosphates, are not involved in water binding and therefore do nothing to improve the water retention of meat.

Kaufmann, A., Maden, K., Leisser, W., Matera, M. & Gude, T. (2005). *Analysis of polyphosphates in fish and shrimps tissues by two different ion chromatography methods: Implications on false-negative and -positive findings.* Food Additives and Contaminants 22(11). p1073–1082.

Analysis using ion chromatography measured the stability of the polyphosphates in fish and shrimps under the following conditions; untreated, treated, 1 day after treatment, 2 days after treatment and 3 days after treatment. In raw shrimps kept at 4^{o}C the level of polyphosphate drops from approximately 1500mg P_2O_5/kg to zero after 4 days. However in previously cooked shrimp treated with polyphosphate after cooking, no degradation of polyphosphates was observed. Initial concentrations of polyphosphates measured in cooked, treated shrimps were higher than in raw, treated shrimps; 2600mg P_2O_5/kg and 1500mg P_2O_5/kg respectively.

This was considered to be due to rapid enzymatic degradation of polyphosphate in the raw shrimps by the action of the enzyme phosphatase. Whereas the lack of degradation in the cooked shrimp was thought to be due to the inactivation of phosphatase during the cooking procedure.

Heitkemper, D.T., Kaine, L. A., Jackson, D.S., and Wolnik. K. A. (1993). *Determination of tripolyphosphate and related hydrolysis products in processed shrimp*, as published in the Conference Proceedings from the Annual Conference Tropical and Subtropical Fisheries Technological Conference of the Americas (August 29 - September 1, 1993, Williamsburg, Virginia, U.S.A.), p92-101.

Samples of different commercially available cooked shrimp, treated with tripolyphosphate, were stored frozen for 11 months. Levels of tripolyphosphate and polyphosphate were determined before and after storage. Table 4 shows the concentration of the total amounts of pyrophosphate and tripolyphosphate and the percentage of total polyphosphate (expressed as P_2O_5), both before and after storage. The total polyphosphate found after 11 months frozen storage in samples A, B and C were 87%, 89% and 103% of initial values respectively. These data indicate that very little hydrolysis occurred during frozen storage.

Table 4: Comparison of phosphate levels in commercially treated cooked shrimp samples, before and after 11 months of frozen storage.

Sample Type and Storage Time	Pyrophosphate Conc. (mg/kg)	Tripolyphosphate Conc. (mg/kg)	Total % polyphosphate (as P_2O_5)
(A) Small salad size			
0 Months	1410	4101	0.46
11 Months	2090	2740	0.40
(B) Medium 55-65/14 oz.			
0 Months	1340	3009	0.36
11 months	949	2840	0.32
(C) Large 30-40/14 oz.			
0 Months	1306	2933	0.35
11 Months	1218	3024	0.36

The same authors also report high levels of hydrolysis in uncooked products, due to enzymatic hydrolysis. For two uncooked samples, stored at refrigeration temperature, the tripolyphosphate concentration dropped from approximately 2500mg/kg to less than the detection limit (500mg/kg) in 3 days.

Sutton, A.H (1973). *The hydrolysis of sodium triphosphate in cod and beef muscle.* Journal of Food Technology. 8. p185-195.

Tripolyphosphates were shown to rapidly hydrolyse to pyrophosphate and then to orthophosphate in uncooked muscle due to the action of phosphatase at both 0 and 25^{o}C.

The rate at which tripolyphosphate breaks down is very rapid at 25^{o}C although it still degrades at 0^{o}C, though at a significantly reduced rate. In raw cod held at 0˚C, pyrophosphate was no longer detected after 30-40 hours, however orthophosphate remained detectable.

3.5.1 Summary

Polyphosphates are broken down into pyrophosphates and eventually into single phosphate units (orthophosphates). Hydrolysis (breakdown) of polyphosphates occurs in many products (including meat and seafood) due to the activity of naturally occurring enzymes called phosphatases.

In uncooked products (shrimp and other seafood) the rate of hydrolysis increases with increasing temperature. Tripolyphosphate levels have been shown to decrease below the limit of detection (500mg/kg) even at 0˚C within 2-3 days.

In cooked products, very little hydrolysis occurs in frozen storage even up to 11 months. This is thought to be due to phosphatases being inactivated during cooking.

4. Review of testing methods for polyphosphates

A review of literature has been undertaken to provide information on methods for detection of added phosphates in scallops and prawns. The review is presented in sections according to the type of method:

- Classical methods
  - Moisture content
  - Protein content
  - Quantification of phosphorus
  - Calculation of ratios
- Chromatographic methods
  - Thin layer chromatography
  - Ion chromatography
- Capillary electrophoresis
  - Capillary isotachophoresis
- Research methods
  - Nuclear magnetic resonance
  - Thermo-differential-photometry
  - Microwave dielectric spectra

Each section includes a summary of the theory behind the method and, where appropriate, a table describing use of the method for relevant sample types, showing limits, reporting units and references in which the method has been used. Information on the advantages and disadvantages of each method is also presented.

4.1 Classical methods

Classical methods, also known as wet chemistry methods, are generally conducted at the laboratory bench and do not require sophisticated analytical equipment. The following sections describe determination of moisture and protein contents, quantification of phosphorus (the use of a spectrophotometer for this method in recent time's means it is not strictly a 'classical method' nowadays) and the calculation of ratios to estimate the quantity of added phosphates based on results from these analyses.

4.1.1 Moisture content

The moisture content of foodstuffs (and many other sample types) is generally determined by gravimetric loss of water from the sample following drying in an oven (AOAC, 2011). Results are reported as a percentage by mass.

Codex Alimentarius Commission (2003) provides a summary of the issues with the use of moisture content to determine whether water has been added to scallops; the establishment of a moisture content limit is discussed. The idea is that above a particular moisture content it would be assumed that water had been added to the product, but there are significant problems with this concept. Firstly, it is pointed out that 'scientific research and studies have shown that a considerable range of moisture levels in scallop meats can be observed' and that 'species, seasonality, harvest practices (length of voyage) and geographical location (i.e. effect on water chemistry)' can all affect moisture content of scallops. In addition, the moisture content can be expressed in two ways; 'as a percentage calculated by proximate analysis (oven drying) or as a ratio of the moisture content to protein content calculated by

proximate analysis (oven drying and Kjeldahl)'. See Section 4.1.4 for further details on the use of the moisture:protein ratio.

4.1.2 Protein content

The protein content of foods is commonly derived by calculation, based on the measurement of nitrogen content using the Kjeldahl method (AOAC, 2011). The Kjeldahl method involves digestion of the sample with concentrated sulphuric acid, which converts organic nitrogen to ammonium ions. The resulting digest is made alkaline, distilled into excess boric acid and the ammonia trapped by the boric acid is titrated with hydrochloric acid. The nitrogen content is calculated from the amount of ammonia produced; standard factors are then used to convert total nitrogen to protein equivalent. A standard factor, termed nitrogen factor, of 6.25 is typically used in the determination of crude protein in foods, based on the assumption that the average nitrogen content of proteins is 16% (1/0.16 = 6.25). But, not all nitrogen in foods is found in proteins; it is also contained in variable quantities of other compounds, such as free amino acids and nucleotides. In addition, the nitrogen content of amino acids (the building blocks of proteins) is not always 16%, proteins can actually have nitrogen contents ranging from 13-19% depending on the different amino acids that are present; this would equate to nitrogen conversion factors ranging from 5.26 to 7.69. It has been suggested that N x 6.25 be abandoned and replaced by N x a factor specific for the food in question. Information above has been adapted from (Food and Agriculture Organisation of the United Nations, 2003).

Codex Alimentarius Commission (2003) have stated that 'the natural variability of the moisture content in scallops (species, seasonality, location of catch, etc), has an effect on protein content, so the development of a scallop nitrogen factor would need to consider these and other factors, requiring considerable time, labour and expense'. Interim nitrogen factors for several fish and shellfish species, including scallops, prawns and shrimps, have been provided in the Code of Practice (COP) on the Declaration of Fish Content in Fish Products (UKAFFP *et al*., 1998). The aforementioned COP also states that nitrogen factors will be reviewed periodically; indeed in 2001 the factor for scampi (washed and peeled) was altered from 2.33 to 2.45 based on data produced by the Analytical Methods Committee (2000). However, factors for prawn, shrimp and scallops remain as interim values (shown in Table 5), and in the absence of more up-to-date values continue to be used, for example by Public Analysts in the UK (Michaela Archer – Sea Fish Industry Authority (March 2012), personal communication) and in a Food Standards Agency survey (2002[b]) of added water in seafood, including scallops.

Table 5: Nitrogen factors for relevant shellfish

Species	Nitrogen factor	Reference
Prawn	2.62	Pearson (1976)[a]
Shrimp	2.73	Pearson (1976)[a]
Scallops (not specified)	2.64	MAFF, unpublished data[a]
Scallops (not specified)	2.55	[b]
Scallops (Queens)	2.55	MAFF, unpublished data[ab]
Scallops (King)	2.64	[b]

[a]As quoted in Code of Practice on the Declaration of Fish Content in Fish Products (1998)
[b]As quoted in Food Standards Agency survey (2002), where it is stated that "The factors for scallops were based on statutory limits of water content set by French authorities. These limits were based on data of scallop composition throughout the year".

4.1.3 Quantification of phosphorus

The determination of total phosphate content (P_2O_5) of meat and seafood is commonly derived by quantification of phosphorus by spectrophotometric analysis (the procedure of observing and measuring the wavelengths of light or other electromagnetic emissions). In the case of phosphorus quantification, spectroscopy is used to measure the colour resulting from the reaction of orthophosphates with chemicals in methods such as the molybdenum blue method, yellow vanadomolybdate complex method and malachite green method (Jastrzębska, 2009); the colour is proportional to the phosphorus concentration. It should be noted that quantification of phosphorus using spectroscopy requires decomposition of polyphosphates and other forms of phosphorus to orthophosphates, this is achieved in the presence of sulphuric or trichloroacetic acid (Jastrzębska *et al*., 2008).

Spectrophotometric methods for determination of total phosphorus have undergone modifications leading to higher sensitivity and precision, which have been summarised by Jastrzębska (2009).

The quantification of phosphorus alone cannot be used to definitively show that phosphates have been added to seafood, due to the presence of naturally occurring orthophosphates and other phosphorus containing compounds (Þórarinsdóttir *et al*., 2010). It has also been shown that the natural variation in total phosphorus content between individuals is large (Gibson and Murray, 1973). One way of estimating the quantity of added phosphates using the determination of total phosphorous is to calculate the ratio of free-phosphate:protein-bound phosphate (Dušek *et al*., 2003), see Section 4.1.4 for further details.

4.1.4 Calculation of ratios

The calculation of the ratio of total moisture to protein has been proposed to provide an indication of the presence of excess water in seafoods. It has been shown that when scallops are treated by soaking in water, the nitrogen content decreases as the water content increases (Kent and Anderson, 1996). During discussions presented by Codex (2003), the French delegation proposed use of a moisture:protein ratio of <5 to detect the presence of water added to scallops by processing rather than by natural causes. In scallops the moisture:protein ratio is considered to be between 4.0 to 4.9:1.0 (Lampilla, 1993), so if the ratio is above 5:1 then the product would be deemed to contain added water. Botta and Cahill (1993) have shown that use of a moisture:protein ratio of 5.0, rather than a moisture content

of 80% (see Section 3.3), to determine the presence of added water is more likely to detect added water in scallops that have a naturally low moisture content, and is more tolerant of water that has been added to scallops that naturally have a moisture content close to 80%.

The amount of added phosphate in meat has also been determined by calculating the ratio of free (non-protein) phosphate and protein-bound phosphate (Dušek *et al*., 2003). Total phosphate content is determined by spectroscopy, then the amount of bound protein phosphate is calculated from the concentration of nitrogen (Kjeldahl method) and from the known ratio (0.0106g phosphorus (P)/g protein) of phosphate to protein; the difference between the total phosphate content and the protein-bound phosphate is calculated as the free-phosphate (Dušek *et al*., 2003).

Calculation of both the moisture:protein and free-phosphate:protein-bound phosphate ratios described above requires specific nitrogen factors for the determination of protein content (see Section 4.1.2).

4.2 Chromatographic methods

Chromatographic methods can be used to detect, and in some instances quantify, added phosphate in food products. Chromatographic methods use chromatography, in which mixtures of substances are separated into their components. All forms of chromatography work on the same principle and involve a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components of the mixture travel at different rates and are therefore separated from one another.

4.2.1 Thin layer chromatography (TLC)

In thin layer chromatography (TLC) the stationary phase (TLC plate) is usually a thin layer of an adsorbent substance such as silica, aluminium oxide or cellulose, which is dried onto an aluminum, plastic or glass plate. The mobile phase is usually a liquid solvent or mixture of solvents. A spot of sample is placed on to the bottom of the TLC plate, and allowed to dry. The TLC plate is placed into a closed tank with a small amount of mobile phase at the bottom. The mobile phase moves up the TLC plate bringing the sample with it. Each component in the sample travels at a different rate, so at the end of the TLC process several spots relating to the different components remain on the plate. If the sample components being separated are colourless the TLC plate can be sprayed with or dipped into other chemicals (for example iodine, bromine or ninhydrin) so the spots can be visualised. Measurements of the distance travelled by individual spots, are compared with the total distance travelled by the mobile phase to determine the R_f (retention factor) for each spot (R_f = distance travelled by component (spot) ÷ distance travelled by mobile phase). Under the same conditions (temperature, mobile phase composition etc) the R_f value for specific components should remain the same, therefore spots can be identified by comparison with standards containing known components. Information above has been adapted from Clark (2012). With regards to the use of TLC to detect polyphosphates; ortho -, pyro-, tri- and polyphosphates can all be separated from the same sample. See Table 6 for example studies where TLC has been used to detect phosphates in foodstuffs.

Table 6: Example studies where thin layer chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Commercially available proprietary mixtures of phosphates used to treat fish. - *Preparation of samples*: 1% solutions of proprietary mixtures of phosphates. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: isopropanol, n-propanol, trichloroacetic acid, ammonia solution. - *Detection*: ammonium molybdate, then stannous chloride in hydrochloric acid. - *Additional analysis*: concentration of phosphates in spots was determined by spectrophotometric comparison with known concentrations of standard phosphates run on the TLC plate at the same time. N.B. Under the low pH conditions of the TLC analysis some hydrolysis of the condensed phosphates occurs giving multiple spots.	Limits: TLC used to determine composition of commercially available proprietary mixtures of phosphates – no limits given. Units: - R_f values reported for TLC. - Phosphate content of spots reported as % composition of proprietary brands.	Gibson and Murray (1973)
- *Sample type*: Brown shrimp (*Penaeus aztecus*), treated with radioactive labelled sodium tripolyphosphate (STP) and unlabelled STP. - *Preparation of extract*: Comparison of extraction with trichloroacetic acid solution or distilled water. The slurries were centrifuged and the supernatants analysed by TLC. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: isopropanol, ethanol, n-propanol, n-butanol, trichloroacetic acid, ammonia solution. - *Detection*: Comparison of ammonium molybdate-stannous chloride method of Gibson and Murray (1973) and phosphate stain suggested by Kates (1972). N.B. For the pyrophosphate standard a faint spot could be seen at the R_f value of orthophosphate and for the tripolyphosphate faint pyrophosphate and orthophosphate spots were evident. This indicates that some hydrolysis of the condensed phosphates will occur during the TLC analysis. For the trichloroacetic acid extracted samples two distinct R_f values indicate complete hydrolysis of the sodium tripolyphosphate to pyro- and orthophosphate. This was not unexpected since polyphosphates undergo hydrolysis under acid conditions.	Limits: Only faint coloured spots seen after TLC analysis of shrimp treated with 0.5% and 1.0% unlabelled sodium tripolyphosphate. Added phosphate detected as distribution of radioactivity on the TLC plates. Units: - R_f values reported for TLC.	Tenhet *et al*. (1980)

Table 6 continued: Example studies where thin layer chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Meat and meat products. - *Preparation of extract*: Sample homogenised with trichloroacetic acid solution; resulting slurry then filtered using filter paper. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: isopropyl alcohol, trichloroacetic acid, ammonium hydroxide solution. - *Detection*: ammonium molybdate tetrahydrate, nitric acid and tartaric acid solution, followed by 1-amino-2-naphthol-4-sulphonic acid with sodium disulphite, sodium sulphite and sodium acetate trihydrate solution. N.B. Since polyphosphates are gradually hydrolysed by enzymes present in meat or meat products and during heat treatment of the meat or meat product, this International Standard only applies to the detection of polyphosphates that are still present in the sample at the time of investigation.	Units: - R_f values reported for TLC.	British Standard Method (1981)
- *Sample type*: Meat and meat products. - *Preparation of extract*: Sample (40-50g) homogenised with same weight of trichloroacetic acid solution; resulting slurry then filtered using filter paper. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: 2-propanol, tricholoracetic acid, ammonia solution. - *Detection*: ammonium heptamolybdate, hydrochloric acid, perchloric acid solution produces yellow spots; tin chloride, hydrochloric acid solution alters the yellow spots to blue to aid detection. N.B. Since the phosphates to be detected are generally hydrolysed by enzymes already contained in the meat, or because of the heat treatment of the meat products, only the condensed phosphates still present at the time of testing can be detected by this method.	Units: Results of TLC reported as presence or absence of ortho-, pyro-, or triphosphate.	German Official Standard (1982)

Table 6 continued: Example studies where thin layer chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: White shrimp (*Penaeus setiferus*). - *Preparation of extract*: Shrimp (50g) homogenised with distilled water (1:2 w/v); resulting slurry then filtered using filter paper. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: ammonium carbonate, acetonitrile and p-toluenesulfonic acid. - *Detection:* ammonium sulphate, ammonium molybdate and nitric acid solution produces yellow spots; sodium pyrosulphate, sodium sulphite and methylaminophenol solution alters the yellow spots to blue to aid detection. - *Additional analysis*: the amount of phosphorus in each spot was determined by colourimetric (spectrophotometric) method (Crowther, 1955). N.B. Results suggest that some hydrolysis of triphosphate takes place during TLC analysis. The amount of phosphorus in each spot on the TLC plate was determined by colourimetric method suggested by Crowther (1955).	Limits: Faint spots reported for phosphates added at the lowest treatment level (0.08% sodium triphosphate, w/w) Units: - R_f values reported for TLC. - Phosphate content of spots reported as % measured by comparison with % added.	Reddy and Finne (1985)
- *Sample type*: Cod (*Gadus morhua*), sea scallops (*Placopecten magellanicus*). - *Preparation of extract*: Cod was homogenised in distilled water at a 1:2 ratio, then filtered using filter paper. - *TLC stationary phase*: glass plates coated with cellulose. - *TLC mobile phase*: isopropyl alcohol, n-propyl alcohol, trichloroacetic acid, ammonia solution. - *Detection*: molybdenum blue spray reagent to produce light blue spots, followed by sodium pyrosulphate, sodium sulphite, methylaminophenol solution to produce darker blue spots. N.B. The sodium tripolyphosphate (STP) standard always had an additional faint spot at the R_f value of pyrophosphate. This spot indicates STP hydrolysis, probably due to the acidic TLC conditions. There are no false positives, but there can be false negatives. TLC spots were not analysed for phosphorus content. No spots were seen on the TLC plate for untreated samples (no STP added) in the region of the tripoly- and pyrophosphates.	Limits: The standard solutions containing 20µg of tripoly- and pyrophosphates and 100µg of ortho- and pyro-phosphates were clearly visible. Units: Rf values reported for TLC.	Krzynowek and Panunzio (1995)

The advantages of TLC include the relative simplicity and speed of the analysis; results are generated within a few hours. The low cost of equipment required also makes TLC an inexpensive assay to perform.

The main disadvantage of TLC is that hydrolysis of phosphates during sample preparation and analysis may influence the results obtained (see comments in Table 6). One mole of tripolyphosphate hydrolyses to one mole of orthophosphate and one mole of pyrophosphate, while one mole of pyrophosphate hydrolyses to two moles of orthophosphate (Heitkemper *et al*. 1993).

Without additional analysis, TLC is a qualitative technique, providing a positive or negative result. Presence of a spot on the TLC plate in the region of polyphosphate (by comparison with a standard) is taken to infer that polyphosphate has been added to the sample. However, it has been shown that TLC detection of added phosphates can give false negative results. The absence of a spot in the region of polyphosphate on a TLC plate means that the sample either has not been treated with added phosphate or has been treated, but the polyphosphates have completely hydrolysed to orthophosphates and are no longer detectable above the natural background phosphate content (Krzynowek and Panunzio, 1995).

4.2.2 Ion chromatography (IC)

Ion chromatography is a form of high-performance liquid chromatography (HPLC). The stationary phase is a column packed with ion exchange resin; the surface of the resin is coated with a thin layer of active material. The ions (negative or positive charged particles) in the sample are carried through the column by the mobile phase, which is an ionic solution (a solution that dissociates into ions and is therefore capable of conducting electricity, such as an acid, carbonate or hydroxide solution) termed the eluent. As the sample passes through the column, individual ions attach and detach from the active material layer on the resin, resulting in different retention times for different components within the sample. Therefore, the ions of particular components exit, or elute, from the column at a specific time. The detector at the end of the column measures either the conductivity (ability to conduct electricity) or spectroscopy (wavelength of light or other electromagnetic emissions) of the eluent. A chromatogram is generated, which is a plot of ion abundance measured by the detector versus retention time i.e. the time the ion takes to pass along the column. The position of a peak in the chromatogram (i.e. the retention time) is characteristic of a specific ion, and the peak size is a function of the concentration of the ion represented by that peak. Information above has been adapted from Materials Evaluation and Engineering (2010). For further details on the theory of IC see Eith *et al*. (2001).

IC has been shown to be a useful method in determining individual polyphosphates in a sample. See Table 7 for example studies where IC has been used to detect and measure phosphates in foodstuffs.

Table 7: Example studies where ion chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Shrimp. - *Preparation of extract*: Samples shaken with deionised distilled water for 30 minutes, and centrifuged to separate heavier particulates. The extract was filtered through 0.2 or 0.45µm Nylon syringe filters then tested. - *IC apparatus*: Dionex system. - *IC stationary phase*: Dionex IonPac AS7 anion separator column (plus NG1 guard column) - *IC mobile phase*: Nitric acid solution. - *Detection*: Post-column reaction with ferric nitrate and perchloric acid, detection of reaction products by UV spectroscopy. N.B. Method is well suited for determination of tripolyphosphate and pyrophosphate: however determination of orthophosphate is susceptible to interference from other sample components. Method is unable to distinguish between additional orthophosphate (from hydrolysed polyphosphate) and naturally occurring orthophosphate.	Linear range[a]: - 10-100µg orthophosphate/g - 0.5-50µg pyrophosphate/g - 10-500µg tripolyphosphate/g (N.B. µg/g is equal to parts per million or PPM) Units: Quantitative results should be reported as total concentration of pyrophosphate + tripolyphosphate (expressed as % P_2O_5 or %P)	Heitkemper *et al*. (1993)
- *Sample type*: Cod and scallop adductor. - *Preparation of extract*: Samples extracted in deionised water for 10 minutes with sonication[b]. Sample was then filtered and trichloroacetic acid added to precipitate proteins, which were removed by filtering. The solution was adjusted to pH>8, diluted and filtered (0.45µm). - *IC apparatus*: Dionex Model 500 ion chromatograph with GP 40 gradient pump. - *IC stationary phase*: Dionex IonPac AG11-HC and IonPac AS11-HC columns. - *IC mobile phase*: Sodium hydroxide gradient. - *Detection*: Conductivity detector. N.B. Tripolyphosphate only investigated. Tripolyphosphate contents >5mg/kg in cod or scallop adductor can be detected.	Linear range: of a serially diluted stock solution, 1-100 mg tripolyphosphate/L Limit of detection: 5mg tripolyphosphate/kg sample (N.B. mg/kg and mg/L are equal to parts per million or PPM)	Cui, Cai and Xu (2000)

[a]Linear range: Concentration range over which the intensity of the signal obtained is directly proportional to the concentration of the species producing the signal (IUPAC, 1997).
[b]Sonication: Use of ultra high-frequency sound waves to disrupt cellular structure.

Table 7 continued: Example studies where ion chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Ham, fish paste, cheese. - *Preparation of extract*: Samples homogenised with cooled trichloroacetic acid solution, centrifuged, diluted and filtered (0.45µm). - *IC apparatus*: Dionex DX-500 ion chromatography system. Addition of a Dionex EG40 eluent generator equipped with an EGC-KOH cartridge led to greater retention time reproducibility and better method precision. - *IC stationary phase*: Dionex IonPac AS11 and IonPac AG11 columns. - *IC mobile phase*: Potassium hydroxide gradient. - *Detection*: Conductivity detector.	Linear range: 0.5-500µM for orthophosphate, pyrophosphate, tripolyphosphate and tetrapolyphosphate Limits of detection: 0.5µM orthophosphate 0.16µM pyrophosphate and tripolyphosphate	Sekiguchi *et al*. (2000)
- *Sample type*: Scampi, shrimp, squid. - *Preparation of extract*: Homogenised sample was mixed with water and heat shocked in a microwave for 1 minute. The suspension was cooled, further homogenised and centrifuged. The supernatant (liquid layer) was filtered (0.2µm) and tested. - *IC apparatus*: Dionex DX-320 EGC-OH cartridge. - *IC stationary phase*: Dionex IonPac AS16 and IonPac AG16 columns. - *IC mobile phase*: Potassium hydroxide gradient. - *Detection*: IC 20 conductivity detector. N.B. In aqueous (water-based) media, even at neutral pH values, phosphatase can hydrolyse linear polyphosphates into monophosphate during the extraction step leading to significant losses of these compounds and production of the corresponding hydrolysis products. To avoid such analytical problems, a short but intensive heat treatment of such samples using a microwave oven was tested.	Linear range: 0.5-25mg/L for polyphosphates Limit of detection: 50mg/kg sample	Dafflon *et al*. (2003)

Table 7 continued: Example studies where ion chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Fish (tilapia, pangasius, trout, cod), shrimp. - *Preparation of extract*: Two methods used: 1) Sample plus sodium carbonate solution in a tube, placed into boiling water for 10 minutes, then homogenised. Placed into boiling water for 5 minutes, cooled, centrifuged and filtered. 2) Frozen samples were heated in microwave for 40 seconds, cooled, homogenised in water, centrifuged and filtered. - *IC apparatus*: Two chromatographical systems were used: 1) Dionex DX500 and 2) Agilent Model 1100. - *IC stationary phase*: 1) Dionex AS-16 and 2) Metrohm Anion Duel 1. - *IC mobile phase*: 1) sodium hydroxide gradient and 2) sodium carbonate gradient. - *Detection*: 1) Dionex ASRS and 2) Metrohm conductivity detector and Agilent UV detector. N.B. False negative results are generated when ubiquitous phosphatase causes degradation of polyphosphates. Phosphatases can be deactivated by thermal or chemical means. False positive results may be generated when nucleotides co-elute with polyphosphates, this can be overcome by use of a column to trap nucleotides, or by optimising the hydroxide gradient to permit separation.	Limit of detection: <0.5mg/kg for pyro- and triphosphates	Kaufmann *et al*. (2005)
- *Sample type*: Fish, shrimp. - *Preparation of extract*: Samples extracted using sodium hydroxide. - *IC apparatus*: Dionex. - *IC stationary phase*: IonPac AS11-HC and IonPac AG11-HC columns. - *IC mobile phase*: Potassium hydroxide gradient. - *Detection*: Suppressor-type conductivity detector.	Linear range: 0.3 -60 mg/L for pyrophosphate and polyphosphate Limit of detection: 2.1 mg/kg to 2.3 mg/kg for pyrophosphate and polyphosphate	Zhong and Li (2009)

Table 7 continued: Example studies where ion chromatography has been used to detect phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Sausage. - *Preparation of extract*: Sample homogenised with water and placed into an ultrasonic bath for 15 minutes, then filtered prior to analysis. - *IC apparatus*: Dionex ICS-3000 system. - *IC stationary phase*: IonPac AS16-HC and IonPac AG16 columns. - *IC mobile phase*: Sodium hydroxide gradient - *Detection*: Suppressed conductivity detector.	Linear range: 1.25-80mg/L Limit of detection: 2.07µg/L for orthophosphate 16.39ug/L for pyrophosphate 43.56ug/L for tripolyphosphate	Dionex (2010)

IC is able to distinguish between ortho-, pyro-, tri- and polyphosphates; results are quantitative. Data has been shown to be reproducible and can be generated relatively quickly (within hours). Recent studies have overcome initial problems with the methodology (see Table 7), for example the use of chemical or heating steps to deactivate phosphatases and the use of hydroxide or carbonate gradients to significantly reduce the detection limits. The use of trichloroacetic acid in sample extraction was found to cause severe peaks in the chromatogram, but this has been alleviated with the use of water or ionic solutions.

Although the equipment required is initially expensive and a certain amount of expertise is necessary, the analysis of samples using IC is common in most food testing laboratories. With the inclusion of an autosampler, several samples can be analysed at the same time.

Issues with false positive and negative results have recently been elucidated and resolved, making IC one of the most commonly used methods for routine screening of food samples for added phosphates.

4.3 Capillary electrophoresis

Capillary electrophoresis (CE) is a family of related techniques used to separate charged particles based on their size to charge ratio. Separations are conducted in narrow-bore (20-200µm internal diameter) capillaries, through which a buffer and the sample extract flow under high voltage. The capillary is generally made of fused silica, with surface active groups. The most commonly used technique in CE is capillary zone electrophoresis (CZE), where separation is based on differences in solute size and charge at a given pH. Unlike chromatographic techniques nothing is retained in the capillary, so rather than using the term retention time, in CE the fundamental term is migration time. The migration time is the time it takes a solute to move from the beginning of the capillary to the detector. Detection in CE is generally by conductivity, fluorescence or UV. Information above is adapted from Li (1993).

4.3.1 Capillary isotachophoresis (CITP)

Capillary isotachophoresis is an analytical technique used to separate charged particles. Extracted samples are loaded into a capillary between two electrolytes (substances that

dissociate into ions in solution and acquire the capacity to conduct electricity); a leading electrolyte and a terminating electrolyte. An electric current is applied, which makes the sample components separate and form discrete zones between the electrolytes in order of their electrophoretic mobility. An equilibrium is established when all the ions are migrating at a constant rate and the bands can be easily identified (Cui and Ivory, 2011).

CITP has been used to determine addition of phosphates in meat and seafood. See Table 8 for example studies where CITP has been used to detect added phosphates in foodstuffs

Table 8: Example studies where capillary isotachophoresis has been used to detect added phosphates in foodstuffs.

Sample type and method details	**Limits and units**	**Reference**
- *Sample type*: Meat; ham, sausages, pate. - *Preparation of extract*: Sample homogenised with water in an ultrasonic bath, cooled and filtered (0.45µm). - *CITP apparatus*: Labeco ZKI 02 isotachophoresis analyser - *Leading electrolyte*: Hydrochloric acid, glycylglycine and hydroxypropylmethylcelullose solution. - *Terminating electrolyte*: Citric acid. - *Detection*: Zones detected by conductivity. N.B. CITP method is simple, quick and allows the determination of polyphosphates of different chain lengths. The main advantage [according to the authors] is the possibility to determine total soluble phosphates. Amount of added phosphates was calculated from the level of soluble phosphate (determined by CITP) and the protein content (determined by Kjeldahl method).	Limit of detection: 2µM for phosphate Limit of quantification: 5µM for phosphate	Dušek *et al*. (2003)
- *Sample type*: Pork and products thereof. - *Preparation of extract*: Sample extracted with sodium hydroxide solution, centrifuged and filtered. - *CITP apparatus*: Labeco EA 100\101 isotachophoretic analyser. - *Leading electrolyte*: hydrochloric acid, bis-tris-propane, hydroxyethylcellulose, β-alanine solution. - *Terminating electrolyte*: Citric acid solution. - *Detection*: Conductivity detector. N.B. This study involves a combination of CZE and CITP. The authors later state that CITP-CZE is more complicated than CITP alone (Jastrzębska *et al*., 2008). The concentration of sodium tripolyphosphate in pork and products thereof was directly measured using CITP and found to be below the legal requirements.	Limit of detection: 0.79mg P_2O_5/dm^3 (dm^3 is equivalent to L)	Jastrzębska (2006)

Table 8 continued: Example studies where capillary isotachophoresis has been used to detect added phosphates in foodstuffs.

Sample type and method details	Limits and units	Reference
- *Sample type*: Pork. - *Preparation of extract*: Sample extracted with sodium hydroxide solution, centrifuged and filtered. - *CITP apparatus*: Labeco EA 100\101 isotachophoretic analyser. - *Leading electrolyte*: Hydrochloric acid and β-alanine solution. - *Terminating electrolyte*: Glutamic acid. - *Detection*: Conductivity detector. N.B. The concentration of ortho-, pyro- and tripolyphosphate in pork was directly measured using CITP	Limit of detection: 0.4-1.1mg P/L Quantification limit: 1.2-3.7mg P/L	Jastrzębska, Hol and Szlyk (2008)
- *Sample type*: Meat (canned meat products, ham, smoked ham), seafood (prawns, squid and seafood mixes). - *Preparation of extract*: Samples were homogenised, extracted in redistilled water using an orbital shaker, centrifuged, double filtered and diluted prior to analysis. - *CITP apparatus*: Labeco EA 100/101 analyser. - *Leading electrolyte*: Hydrochloric acid, hydroxyethylcellulose, glycine solution. - *Terminating electrolyte*: Phosphoric acid solution. - *Detection*: Conductivity detector. N.B. Added phosphorus calculated as the difference between total phosphorus and protein-bound phosphate.	Limit of detection: 0.64mg P/L for pyrophosphate 0.27mg P/L for tripolyphosphate Limit of quantification: 2.12mg P/L for pyrophosphate 0.91mg P/L for tripolyphosphate	Jastrzębska (2011)

There are several advantages to the use of CITP for determination of added phosphates in foodstuffs: for example orthophosphates, pyrophosphates and tripolyphosphates (as well as nitrites and nitrates) can be simultaneously identified and quantified (Jastrzębska, 2011). The method is said to be environmentally friendly, as it involves a small volume of diluted electrolytes and no harsh chemical extraction. The separation time is in the region of 15 minutes, so results can be generated quickly. The amount of sample required for analysis is small, approximately 5g. Results have been shown to be precise and accurate, with low detection and quantification limits (Jastrzębska, 2011).

As with many other methods for food analysis, samples need to be homogeneous prior to extraction, and complicated matrices, variable texture, structure, presence of protein and fat decrease the precision of the method (Jastrzębska, 2011).

4.4 Research methods

The following methods have been used to detect added phosphates in foodstuffs. Due to the sophisticated equipment required and/or the stage of development of these methods they have been classified for the purposes of this document as 'Research methods', as they are currently predominantly used in research settings rather than for routine analysis for detection of phosphates.

4.4.1 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance is a phenomenon which occurs when the nuclei (the positively charged central region of an atom) of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. The positively charged subatomic particles of the nuclei spin in the magnetic field and generate a resonance signal. Some nuclei experience spin, whilst others do not. The resonance spectrum generated is therefore characteristic of the sample and reveals the chemical nature and abundance of the compounds containing the particular nuclei. An isotope, or form, of the element phosphorus that experiences spin in a magnetic field, and has therefore been studied using NMR, is the phosphorus-31 (or ^{31}P) isotope (Hornack, 1999).

As ^{31}P NMR is a noninvasive and nondestructive technique it has been applied to the study of phosphate metabolism in intact cells of bacteria, yeast and fungi (Chen, 1999); to investigate post-mortem changes in meat and the metabolic reaction of fish to environmental stress (for a review of ^{31}P NMR in food analysis see Spyros and Dias, 2009); and to elucidate phosphate metabolism during exercise and recovery in scallops (Bailey *et al*. 2003).

^{31}P NMR was first used to detect whether added polyphosphates were present in frozen foodstuffs, including chicken, fish and ground beef, by O'Neill and Richards (1978). Since this time the technique has mainly been applied to the detection and study of added phosphates in meat, for example Li *et al*., (2001) used ^{31}P NMR to study the hydrolysis of commercially used phosphates in chicken breast meat; samples were comminuted with EDTA (Ethylenediaminetetraacetic acid) immediately prior to analysis to limit phosphatase activity, no other sample preparation was required.

The use of ^{31}P NMR for qualitative and quantitative determination of added phosphorus compounds in pork has been studied (Jastrzębska and Szlyk, 2009); the detection limit was 0.0018mol/L for sodium tripolyphosphate and the authors suggest ^{31}P NMR as an alternative method of phosphorus determination in food analysis.

Szlyk and Hrynczyszyn (2011) prepared extracts from pork, prawns and squid, by shaking samples with boric acid solution, triple filtering and centrifugation, prior to ^{31}P NMR analysis. Results for levels of phosphates in foodstuffs ranged from 33mg P/kg for pyrophosphate to 1271mg P/kg for orthophosphate. Spectra though lacked signals for tripolyphosphate, despite the manufacturer declaration that it had been added, suggesting decomposition of tripolyphosphates in meat products during storage.

Advantages of ^{31}P NMR over chromatographic methods include, simultaneous observation of all phosphorus-containing species and only the phosphorus-containing species (meaning that resulting spectra are relatively simple), structural information, and quantification with or

without standards (Gard *et al*., 1992). A notable disadvantage of the technique is that although it is able to measure total phosphates or polyphosphates it cannot distinguish between natural and added compounds. There are also issues with quantification of longer chain (>tripolyphosphates) phosphates (Belloque *et al*., 2000).

4.4.2 Thermo-differential-photometry (TDF)

Thermo-differential-photometry can be used to quantify phosphate compounds. The method relies on the reaction of different phosphates to form the yellow molybdic vanadic heteropolyc acid. Orthophosphates react quickly, whereas pyro-, tri- and polyphosphates react much slower. The difference in the kinetics (speed) of the reaction (colour generation) can be used for quantification of polyphosphates. Results are measured using a spectrophotometer with a temperature controlled cuvette holder, readings are taken 15 and 90 minutes after initiation of the reaction (Þórarinsdóttir *et al.*, 2010). TDF has been tested on more than 1000 samples of fish products, for example salted, rehydrated cod. Results have been verified by comparison with ion chromatography and the method has been described by the authors as reliable and useful as a routine control method for products of animal origin (Kruse and Bartelt, 2009).

A major drawback though is that TDF cannot be used to differentiate between pyro-, tri- or other polyphosphates (Þórarinsdóttir *et al*. 2010).

4.4.3 Microwave dielectric spectroscopy

The technique 'microwave dielectric spectroscopy' measures the electromagnetic properties of a sample in the microwave region of the electromagnetic spectrum, which are dependent on its molecular structure. The theory behind the technique is based on the permittivity of a substance; a measure of the ability of a material to resist the formation of an electric field within it. The technique is non-destructive; the sample is placed into an electromagnetic field, which results in polarization (or orientation) of positive and negative charged particles (ions) in the sample. This happens to varying degrees depending upon the sample. If the sample is altered, for instance by the addition of water, then the spectrum that is obtained will be different to that of the untreated sample. By analysing the data using a statistical technique called principle component analysis, it is possible to separate treated and untreated samples based on the differences in their microwave dielectric spectra (Agilent, 2004; Kent *et al*., 2000).

Work using microwave dielectric spectroscopy to study seafood with added water and polyphosphates is based on the principle that as water is added, other constituents, such as naturally occurring salts, are diluted and can diffuse into the exterior water. Also, the addition of polyphosphates adds ions as well as water, resulting in an altered spectrum (Kent *et al.*, 2000)

Microwave dielectric spectroscopy has been used to measure the composition, including added water, of foods such as pork, poultry and prawns (Kent *et al*., 2001). It has also been used to estimate the quality of seafood, by distinguishing whether raw materials had been frozen and how well they had been stored (Kent *et al*., 2004). This work, conducted as part of the EU 5th Framework project 'SEQUID' (Seafood Quality Identification), has resulted in formation of a company, Sequid Gmbh, specialising in high frequency testing and analysis of

food and environmental samples. Sequid have also developed a measuring device, the RFQ-scan, based on the principle of dielectric spectroscopy, which detects added water independently of the used additive (for further details see http://www.sequid.de/index_en.html).

The main disadvantages of microwave dielectric spectroscopy are that it can only detect added polyphosphates if there is an untreated sample to compare against, and some of the calculations involved to determine differences between samples are complex. However, with the automation of the measurement procedure and provision of software to handle data, the issue of complex calculations may be negated.

4.5 Summary

A variety of testing methods for detection of added phosphates in seafood have been used. Classical methods, such as measurement of moisture, protein and phosphorus content have been used to calculate ratios, which can show when phosphates and/or water have been added to seafood. Levels of protein however, are currently determined using interim nitrogen factors for prawns and scallops.

Some methods measure phosphates directly, such as ion chromatography and capillary isotachophoresis; but may not be able to differentiate between added and naturally occurring phosphates. These methods suffer from the lack of background information on the natural variation in levels of phosphates and moisture content of crustaceans and molluscs. Other methods, such as nuclear magnetic resonance, thermo-differential-photometry and microwave dielectric spectroscopy, have not to date been used for routine determination of added phosphates.

Throughout the course of this review no official methods for measurement of 'added' phosphates or for total phosphate determination were found. There is a spectrophotometric AOAC method for phosphorus in meat and meat products (AOAC, 1996). There is also a British Standard method for the detection of polyphosphates in meat and meat products (British Standard Method, 1981), which uses thin layer chromatography. Due to the high variability in natural levels of phosphates and moisture in scallops and prawns, testing for 'added' phosphates may need to rely on calculation of ratios of constituents measured using robust, sensitive methods.

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