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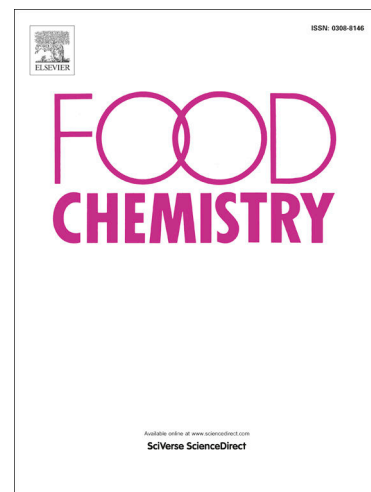
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## Detection of polyphosphates in seafood and its relevance toward food safety

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**Abstract**

Polyphosphates are permitted as food additives (Regulation EC No 1129/2011) but their undeclared utilisation is considered fraudulent. They improve water holding capacity of the seafood, preventing biochemical/physical changes during commercialization. The key objective of this study was the detection of polyphosphate in various seafood categories, by means of high-performance ion-exchange chromatography with suppressed conductometry (HPIEC-SCD) coupled to Q-Exactive Orbitrap high resolution mass spectrometry (HRMS-Orbitrap). Ten frozen cuttlefish samples did not reveal any treatment, while in ten frigate tunas, high concentration of orthophosphate was found. Unambiguous hexametaphosphate presence was demonstrated in four prawn samples, while triphosphate was quantified ( $11.2 \pm 4$  ug/g) in another four prawn samples that contained orthophosphate ( $10225 \pm 1102$  ug/g), as well. Other samples sporadically encompassed polyphosphates profiles that varied according species and processing type. This analytical approach provided sustenance in better understanding regarding utilization of polyphosphates through HRMS fingerprinting of anionic species that would be specific in food safety control.

**Keywords:** food safety, polyphosphates, fish and seafood, ion-exchange chromatography, high resolution mass spectrometry

## 1. Introduction

Polyphosphates belong to food additives that are legally added to different food items such as meat, dairy products, fish and seafood (Goncalves, 2012). Especially, in the fishery and processed marine products, polyphosphates are used to increase tissue water-binding ability slowing down the loss of moisture in tissues (Da Silva Oliveira & Goncalves, 2019). This improves the consistency of the final product, upholds the weight and preserves the sensory characteristics (Goncalves, Rech, Rodrigues, & Pucci, 2008; Goncalves and Ribeiro 2008; Campden, 2012). Generally, polyphosphates are acknowledged as nontoxic, but some authors have observed that a high dietary introduction of these additives can cause hypocalcemia, renal injury with calcium phosphate accumulation in the kidneys (Weiner et al., 2001; Cakar et al., 2012). Impairment of the calcium-phosphorus ratio leads to certain hormonal disorders, while excess phosphates species interferes with calcium absorption in the gut, which results in the release of calcium from bones (Weiner et al., 2001). For example, elderly population with compromised kidney function is particularly vulnerable to higher phosphate intake.

Because of all mentioned above, the increased utilization of polyphosphate in food processing have become a matter of concern of different governments authorities and this is reason why European Commission (EC) requested the European Food Safety Authority (EFSA) to re-evaluate the safety of food additives already permitted in the Union before 2009 (257/2010/EU), polyphosphates included. The EFSA scientific opinion published recently (EFSA, 2019) emphasized that (poly)phosphates are shown to be of low acute oral toxicity and there is no concern with respect to its genotoxicity and carcinogenicity, but its intake via processed food has to be highly controlled, as *per se* they could contain traces of some toxic metals (Pb, Cd, As and

Hg). The EFSA panel did not suggest any changes in the current legislation regarding the Maximum Permitted Level (MPL) for condensed phosphates in the different food items (Regulation EC No 1129/2011), but it clearly underlined that numerical MPL for (poly)phosphates must be define for food supplements as consumption of some of them exceeds proposed Average Daily Intake (ADI) value of 40 mg/kg body weight per day (bw/day).

Polyphosphates belong to a class of condensated phosphate anions, that are composed by orthophosphate residues linked by phosphoanhydride bonds to form linear chains or cyclic structures from two to several hundred residues (**Figure 1**). Typical ones used in the food industry are designated with codes E 338–341, E 343, E 450–452 and have been defined in Annex II to Regulation (EC) No 1333/2008 on food additives (Commission regulation EC No 1129/2011), as amended (231/2012). This document established MRLs for phosphoric acid, ortho-, pyro-, tri- and polyphosphates for the different categories of raw and processed food, including the fishery and marine products, have been established. The MPL of 5000 $\mu\text{g/g}$  (expressed as a total of all condensed phosphates and calculated as  $\text{P}_2\text{O}_5$ ) was fixed only for the unprocessed frozen and deep-frozen fish fillets, molluscs and crustaceans and for the fish/ crustacean paste, while for surimi and canned crustacean products from the category of processed seafood, a MPL of 1000 $\mu\text{g/g}$  was decided.

Specifically, polyphosphates are not permitted in raw seafood because their addition preserves the natural commodity color, misleadingly prolonging product shelf-life (Campden, 2012). Therefore, the real problem correlated to the use of polyphosphates in fishery products, is that of their fraudulent utilization in raw food matrices. When applied on raw fish or other unprocessed seafood, polyphosphates increase water retention in the tissues, which could

deceive the final consumer who can visually estimate as fresh a product that has instead already been on the market for a few days. Therefore, microbiological and chemical risk of ingesting such endangered fish products is extremely high and is to be considered of utmost importance.

In the past decade, some EU countries have reported to the European Commission, the detection of polyphosphates above the limits allowed in some animal products imported. However, while the IC-SCD method can successfully detect the ortho/pyro/tri(meta)/tetra phosphates it does not provide any structural elucidations of higher polymeric forms (EFSA, 2019). As a result of this notifications, the relevant EU authorities has urged the Control Organism to define and propose an appropriate analytical method for this type of investigation, bearing in mind that official AOAC method based on spectrophotometric monitoring has previously been deemed incomplete (McKie & McCleary, 2016). This technique, in fact, does not allow the identification of (poly)phosphates species, but only measurement of total phosphorus, which can lead to over-estimation if other additives containing phosphorus have been added to the products. As a matter of fact, the development of more specific analytical methods for the determination of phosphates was strongly encouraged by the EFSA Scientific Committee report (EFSA, 2019).

A promising analytical method based on ion-exchange chromatography (IEC) with suppressed conductometric detection (SCD) has been developed and validated for the separation and direct determination of polyphosphates in different matrices of animal origin, in which these additives are typically used. The most important literature data where IEC has been used for determination of polyphosphates in seafood are listed in the **Table 1S**. IC remains a priority choice for the analysis of (poly)phosphates because it was proven to be highly sensitive that is able to separate the main (poly)phosphates species (Cui, Cai, & Xu, 2000; Sekiguchi et al., 2000; Kafumann,

Maden, Leisser, Matera, & Gude, 2005; Iammarino & Di Taranto, 2012; Kim et al 2019). Nevertheless, IC methods have not yet been proposed as a reference protocol. This is due to the fact that some issues regarding the stability and fate of polyphosphates (especially E450 to E452) still remain to be resolved. IC-SCD methods have brought about a significant improvement in polyphosphate characterization as regards detection limits and analysis times allowing the efficient separation of different small polyphosphate oligomers present in a sample. However, while the IC-SCD method can successfully detect the ortho/pyro/tri(meta)/tetra phosphates it does not provide any structural elucidations when higher polymeric forms are present in the sample. For example, in the case of sodium hexametaphosphate (SHMP, E452) the IC-SCD chromatographic profile can be used just for screening purposes, but not for quantification or structural characterisation (Dionex 71 and 172, Kim et al., 2019).

Although recognised as a powerful analytical tool for bioorganic and environmental research, high-resolution mass spectrometry (HRMS) has not yet been applied for the characterisation of the polyphosphate profile in food and food products at risk. The only study that regards the electrospray (ESI)-MS characterisation of inorganic polyphosphates dates back to almost 20 years ago (Choi, Hercules & Houalla 2000).

In view of these considerations, the pivotal objective of this study was to detect the presence of polyphosphate residues in various types of seafood by using the IC-SCD-HRMS platform as well as to investigate the feasibility of this approach as a tool to support inspective controls according to present regulations. This study should help to provide a better understanding of the following issues: 1) whether the food examined has been treated with polyphosphates 2) whether there are any traces of unambiguous SHMP treatment 3) the characterization of new polyphosphate

species that would be more specific in food safety control 4) distinguish variable presence of naturally occurring orthophosphate from added polyphosphates.

## 2. Material and methods

### 2.1. Chemicals and reagents

Sodium hydroxide was procured from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

While water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

### 2.2. Reference materials and standard solutions

Certificated orthophosphate standard solution (1 mg/mL), potassium pyrophosphate ( $K_4P_2O_7$ , 97 %), sodium triphosphate pentabasic ( $Na_5P_3O_{10}$ , 90–95%), trisodium trimetaphosphate ( $Na_3P_3O_9$ , 95 %) Sodium hexametaphosphate (65-70%  $P_2O_5$ ) were purchased from Sigma–Aldrich (Stenheim, Germany). The principal standard solutions of each compound (1 mg/mL) were prepared in water and stored at 4°C.

### 2.3. Sample collection

Different categories of fishery and processed marine food products, were collected from markets in Milan (IT). As far as unprocessed samples are concerned, 10 cuttlefish (*Sepia officinalis*) originated from different FAO zones and 10 frigate tuna (*Auxis thazard*) filets derived from same Mediterranean sea fishery zone were enrolled in this study. Regarding the processed seafood products, the following types were analysed: caramote prawn (*Melicertus kerathurus*), shrimp (*Solenocera membranacea*), squid (*Loligo vulgaris*), octopus (*Octopus vulgaris*), scallop (*Pectinidae*) and surimi (in total 24 samples). Except one surimi, all samples were frozen at arrival. All samples were analysed in duplicate.



#### **2.4. Sample preparation**

After being defrosted at room temperature, the samples were extracted as following: 2 g of previously homogenized edible part was transferred in 50 mL polypropylene conical centrifuge tubes and mixed with 40 mL of DI water. The resultant suspension was then centrifuged for 10 min at 5000 rpm on 4°C (Centrifuge SORVALL™ ST 8 SERIES, Thermo Fisher). The supernatant (~2 mL) was filtered on Anotop 10-LC filters (0.2  $\mu$ m, 10 mm, Whatman) diluted 1:10 with DI prior to chromatographic analysis. No further clean-up steps were implemented.

#### **2.5. High-performance ion-exchange chromatography coupled to Q-Exactive Orbitrap high resolution mass spectrometry (HPIEC-HRMS-Orbitrap) analysis**

The separation of polyphosphate was achieved by an IEC Dionex ICS-5000+ system (Sunnyvale, CA, USA) consist of a dual pump, a conductivity detector, a KOH eluent generator and an autosampler. The column was a Thermo Scientific Dionex IonPac AS11-2 $\mu$ m (2  $\times$  250 mm, 2  $\mu$ m particle size) with a guard column Dionex IonPac AS11-4  $\mu$ m (2  $\times$  50 mm) thermostated at 30 °C. The eluent flow rate was 0.30 mL/min with a gradient from 40 mM KOH (aq), increased to 60mM KOH (aq) in 5 min, followed by liner increase to 100mM KOH (aq) up to 28min, held in these conditions for 2 min and back to 40mM KOH (aq) at 30min, with a cycle time of 35 min. The KOH eluent was neutralized using Dionex anion self-regenerating suppressor set to 35 mA (ASRS II, 4 mm) Sample solution of 50 $\mu$ L was injected. The IC system was controlled by Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA), that was used for IC data elaboration, as well. Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI) source was used for detection of different polyphosphates species.

The temperatures of capillary and of vaporizer were set at 330°C and 280°C. The electrospray voltage operating in negative mode was adjusted at 3.50 kV. Sheath and auxiliary gas were set at 35 and 15 arbitrary units, with S lens RF level of 60. Regular instrument calibration was performed by a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA).

The high-resolution mass spectrometry acquisition method was adjusted according to our previous publications where operative parameters of Q-Exactive Orbitrap detector coupled to an ion-exchange chromatograph were set up (Chiesa, Nobile, Panseri, & Arioli, 2019a; Chiesa, Arioli, Pavlovic, & Panseri, 2019b). The full scan (FS, resolution - 70,000 FWHM) operating in negative mode was combined with a data-independent acquisition (DIA resolution - 35,000 FWHM, negative mode). By means of DIA it was possible to record MS<sup>2</sup> spectra for all polyphosphate anions. A scan range of 50–750 m/z was selected for FS, while the automatic gain control (AGC) was set at 1E<sup>6</sup>, with the maximum injection time of 100 ms. In the DIA segment the AGC was set to 2E<sup>5</sup>, with an auto-regulated injection time. The quadrupole that select the precursor ions operated at an isolation window of 1 m/z, while a collision energy was adjusted at 10 eV. The mass tolerance window was set to 0.4 ppm. The analytes was detected by their retention time (RT), by the exact mass of the parent anions, and by fragmentation spectra. The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition, used to confirm the various polyphosphates spices, are reported in **Table 1**. Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HRMS instrument.

## **2.6. Method validation**

Linearity, precision, recovery, specificity, selectivity, limit of detection (LOD) and limit of quantification (LOQ) were determined in order to accomplish the method validation. Linearity was calculated using squared correlation coefficients ( $r^2$ ) on five calibration curve points prepared in the solvent in the range 0.1-10  $\mu\text{g}/\text{mL}$ . Precision and recovery were established by adding two fortified levels to the blank cuttlefish sample. Precision was expressed as the coefficient of variability (CV) for the intra-day and inter-day repeatability, while recovery (indicated as %) was defined by comparing the response obtained for the same blank sample spiked before and after extraction. Detection (LOD) and quantification (LOQ) limits were calculated using following equations:  $\text{LOD} = 3.3\text{SD}/b$  and  $\text{LOQ} = 10\text{SD}/b$ , where SD is the standard deviation of the intercept and b is the slope of the regression line got from the principal calibration curve.

### 3. Results and discussion

#### 3.1. HPIEC-SCD-HRMS-Orbitrap characterisation of hexametaphosphate anion

SHMP, known as Graham's salt, by chemical definition is a hexamer with molecular formula  $\text{Na}_6(\text{PO}_3)_6$ . Nevertheless, commercially available SHMP that is used as a food additive (E452) is typically a mixture of polymeric metaphosphates, of which the hexamer is just one. Hence, this mixture is more correctly termed sodium polyphosphate. Due to its polymeric structure that is susceptible to the breakdown, its exact composition differs from lot to lot (Dionex, 2002) and the control of the final mixture during its manufacturing is "both an art and a science" (Baluyot & Hartford, 1996). Thus, SHMP used as food additive, does not exist as chemically pure product, but it is mixture of varying degrees of polyphosphates. As a consequence, the application of this additive could be sometimes wrongly identified as the treatment with short chain polyphosphates, for example trimetaphosphate, that are its main degradation products.

The presence of SHMP in real food samples was confirmed recently by Kim et al (2019), by means of IC-SCD, but to the best of our knowledge, HRMS has not yet been applied for the SHMP characterisation. IC-SCD-HRMS methodology has been confirmed as a useful tool in the analysis of various inorganic substances in a vast variety of matrices (Chiesa, Panseri, Pasquale, Malandra, Pavlovic & Arioli, 2018; Nobile et al., 2019) with the final objective of utilizing it in food safety and food control purposes, but has not yet been exploited in polyphosphate fingerprinting. "Fingerprinting" of complex polymeric linear and cyclic phosphates mixtures such SHMP can be accomplished by IC using 2-mm AS11 column with a KOH gradient (Dionex 2010, Kim et al 2019).

The AS11 resin is hydroxide-selective, meaning that polyphosphates, considered as highly

retained analytes, could be readily eluted by an appropriate hydroxide gradient. With instrumental conditions (2 mm ASRS at a flow rate of 0.25 mL/min, current of 35mA) applied herein gradient from 40mM to 100mM of KOH successfully separate the short chain polyphosphates (ortho-, pyro-, tri-, and trimetaphosphate) that can be quantified as their standards are commercially available. For these compounds HRMS DIA mass fragmentation is presented in **Figure 1S**. A typical HPIEC-SCD chromatogram that followed by HRMS profile of 15 main peaks found in SHMP solution (100 $\mu$ g/g) is showed in **Figure 2A**. Chromatogram obtain by SCD is in the line of recently published finding of Kim et al. (2019) where low-chained polyphosphates emerged as the main products. The peaks revealed by the means of HRMS corresponds to the polyphosphates listed in **Table 1** and are comparable with only one literature datum available regarding the electrospray mass spectrometry characterisation of polyphosphates (Choi, Hercules & Houalla 2000). The most important finding regards the characterization of penta- and hexa- polymers, both cyclic (meta) and open chain species (**Figure 2S**). Their pure individual standards are not commercially available which is why their HRMS profile could be useful only for screening purposes to disclose eventual undeclared treatment of food with SHMP. Also, the typical SHMP “fingerprint” is obtained by characterization of the polyphosphates that had been eluted with the high KOH gradient (up to 100mM), that we named “progressive polyphosphates”. Their final structure cannot be elucidated as signals of higher condensed polyphosphates had not been detected. We can speculate that this is due to their hydrolysis during the suppression event prior to conductometric detection when from extremely basic conditions the eluent reaches pH-7, and/or due to their breakdown in the ESI source. Anyway, the typical species that confirm the polyphosphates presence are well defined within

the IC peaks and regards the tetra-, tetrameta, penta-, pentameta-, hexa and hexameta-pseudomolecular anions (**Figure 2S**).

### **3.2. Method validation**

Since there are no detailed analytical procedures designed for polyphosphate determination in food, the method described herein was validated by an in-house validation protocol, in accordance with Decision 657/2002/EC and Regulation 882/2004/EC, which describe the analytical parameters to be assured to verify method reliability (Chiesa, Nobile, Panseri, & Arioli, 2019a).

The validation parameters for orthophosphate (certificated standard) and the commercially available standards (pyro/tri/trimeta) are reviewed in **Table 2S**. It is apparent that the HPIEC-HRMS method showed excellent accuracy, with appropriate CVs for intra- and inter-day precision indicators. Good linearity was confirmed within corresponding concentration ranges, with correlation coefficients ( $r^2$ ) higher than 0.990 for all four anions. LOD and LOQ are much lower than those described in the literature for IC-SCD (Iammarino & Di Taranto, 2012). The HPIEC-HRMS method, presented very high selectivity as can be realised from the ion-extracted chromatogram gotten for a real sample (**Figure 1S**). Selectivity presented a good agreement with relative retention times, which, in our case, were within 2.5% tolerance.

### **3.3. Detection of polyphosphate in real fish and marine products samples**

The cuttlefishes sample enrolled in this study that were collected from different FAO zones contained measurable levels only of orthophosphate anion (**table 2A**). Generally, information regarding naturally occurring levels of orthophosphate in processed seafood are rather

incomplete since its levels depend on phosphatase activity in the tissue matrix, pH, temperature and other storage conditions. There are also differences between levels in different species, between individuals of the same species and between the same species but in different geographical locations (Campden, 2012). Variations could also derive from the capture procedure and post-harvest handling. Although without statistical evaluation, there is evident differences between FAO zones, most probably provoked by native habitat/environmental situations which could be accompanied by storage/transport conditions. The high amount found in two samples from FAO 57 cannot rule out the treatment with polyphosphates that eventually by hydrolysis give the  $\text{PO}_4^{3-}$  species as predominant. Generally, it is more likely that orthophosphate derives from the degradation of adenosine triphosphate (ATP). Indeed, many marine edible products contain naturally occurring levels of orthophosphate (Kim et al., 2019). Literature do not give any information on the presence, composition, and way in which any of eventually added polyphosphates could interact with cuttlefish tissues, nor whether they breakdown over time results in producing  $\text{PO}_4^{3-}$ . Our results, showing a large variation in the endogenous amounts of orthophosphate in cuttlefish, were also similar for other seafood species (Kim et al, 2019, Campden, 2012). This poses the question of whether the current official AOAC method based on spectrophotometric measurement is appropriate for this type of food (McKie & McCleary, 2016). Moreover, a high concentration of orthophosphate was found in frozen frigate tuna filets, also in this case without the detection of any higher condensed phosphate forms. The mean concentration was  $8182 \pm 1010 \text{ug/g}$ , ( $n=10$ ), which corresponds to  $\text{P}_2\text{O}_5$  concentration of  $4745 \pm 585 \text{ug/g}$ . These findings are very close to the MRL imposed by EU regulation 1129/2011 set up at  $5000 \text{ug/g}$  for fresh filets. Current legislation specifies that MRLs refer to the  $\text{P}_2\text{O}_5$  equivalents

and include all polyphosphate forms, orthophosphate included. Therefore, some of our samples contained the calculated  $P_2O_5$  (irrespective of counter ion) above legally permitted levels and it derive only from the monomeric phosphate. Thus, as regards the presence and maximum levels of total phosphates, it would be highly recommendable to express analytical results in terms of the individual phosphate species (EFSA, 2019). The European legislation does not consider the natural levels of orthophosphate that usually significantly contributes to overall  $P_2O_5$  concentration while, for example, Canadian Guidelines for Chemical Contaminants and Toxins in Fish and Fish Products provides so called “background levels” calculated as  $Na_2HPO_4$  equivalents (Canadian Food Inspection Agency, 2011).

The importance of the application of an analytical method that would separate the various (poly)phosphates is evident also for the third group of samples that includes processed, exoskeleton free caramote prawn samples (**Table 2B**). The first 4 samples contained pyro-, trimeta- and triphosphate and at the same time demonstrated a characteristic chromatographic pattern with related mass fragmentation that confirmed SHMP presence. The SCD detector revealed only traces of higher condensed forms (**Figure 3A**) while in the HRMS they were very well defined and characterised. Among various species detected, three signals that correspond to pentaphosphate anion ( $m/z$ -416.83494) were the most stable for confirmation, both in FS and DIA scan modes (**Figure 3B**).

Another subgroup of caramote prawn (samples No 5-8, **Table 2B**) exhibited the typical profile of polyphosphates used in these processed foods. It consist mostly of three phosphate units, that are often hydrolyzed to orthophosphate before the food is eaten (Campden, 2012). As a matter of fact, in these samples measurable amounts of trimetaphosphate with a high amount of



orthophosphate were found. In this case, it can be speculated that orthophosphate accumulation is due to trimetaphosphate degradation.

Regarding the various type of processed seafood (**Table 2C**) that was randomly collected from the market polyphosphates were detected in processed shrimp (3), octopus (2) and surimi (2). Being different in species and in processed techniques, each sample from this group exhibited the characteristic (poly)phosphate profile. The most interesting results concern the confection of surimi in brine where very low amount of orthophosphate was revealed with simultaneous presence of traces of pyro- and triphosphates and higher condensed forms. This finding points out the necessity for a sensitive analytical method capable of detecting remaining traces of once applied polyphosphates. The interpretation of a positive result is always based on the hypothesis that an inorganic polyphosphate is neither an endogenous constituent of fish tissue nor a possible degradation product of natural high-energy polyphosphate (Kafumann, Maden, Leisser, Matera, & Gude, 2005). In one shelled cooked shrimp (Shrimp 4) notably high concentration of triphosphate was accompanied with appearance of SHMP fingerprint. The absence of SHMP degradation in the cooked shrimp is most probably due to the inactivation of phosphatase during the cooking (Carneiro et al., 2013).

## Conclusions

In this study, the HPIEC-SCD-HRMS-Q-Exactive Orbitrap method for the analysis of polyphosphates in marine food products was set up and validated. This analytical approach enabled, for the first time, unequivocal detection of hexameric polyphosphate structures, both cyclic and open-chained forms. This finding is fundamental because the treatment with SHMP has not been explicitly demonstrated previously. The other important issue highlighted by the

results of this study is the highly variable and, in some species, extremely high concentration of orthophosphate. It is not still well established whether it is completely endogenous or does it derive from higher, polyphosphate formulations. For this purpose our method could be used as a crucial tool to distinguish artificial (poly)phosphates from those present naturally. Furthermore, this method can be applied not only to fishery and seafood commodities but also to other food typology such as dairy products and processed meat. Taken together, our findings could contribute to the revelation of the inadequate utilization of the condensed phosphates in the food industry.

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**Table 1.** HPIEC-HRMS data of the polyphosphates spices detected in Full Scan and Data Independent (MS/MS) negative ionisation mode: formulas, retention time (RT) exact mass of parent pseudomolecular anions

Compound	Formula of acidic form	R <sub>t</sub> (min)		Charge (-)	Precursor M-1 (m/z)
		HRMS	SCD		
orthophosphate	H <sub>3</sub> PO <sub>4</sub>	6.18	5.98	1	96.96962
pyrophosphate	H <sub>4</sub> P <sub>2</sub> O <sub>7</sub>	9.48	9.32	1	176.93595
triphosphate	H <sub>5</sub> P <sub>3</sub> O <sub>10</sub>	12.16	12.01	1	256.90228
				2	127.94750
trimetaphosphate	H <sub>3</sub> P <sub>3</sub> O <sub>9</sub>	13.49	13.21	1	238.89171
				2	118.94222
tetraphosphate	H <sub>6</sub> P <sub>4</sub> O <sub>13</sub>	15.60	15.23	1	336.86861
				2	167.93067
tetrametaphosphate	H <sub>4</sub> P <sub>4</sub> O <sub>12</sub>	16.77	16.27	1	318.85804
				2	158.92538
pentaphosphate	H <sub>7</sub> P <sub>5</sub> O <sub>16</sub>	17.95	17.58	1	416.83494
				2	207.91383
pentametaphosphate	H <sub>5</sub> P <sub>5</sub> O <sub>15</sub>	18.90	18.52	1	398.82438
				2	198.90855
hexametaphosphate	H <sub>6</sub> P <sub>6</sub> O <sub>18</sub>	18.72	18.24	1	478.79071
				2	238.89171
hexaphosphate	H <sub>8</sub> P <sub>6</sub> O <sub>19</sub>	21.69	21.12	1	496.80127
				2	247.89700
Internal standard	H <sup>[15]</sup> NO <sub>3</sub>	7.02	6.88	1	62.98540

**Table 2.** Orthophosphate and polyphosphates in in different type of seafood

<b>A) cuttlefish originated from different FAO</b>						
<i>FAO</i>	<i>Geografic area</i>	<i>Orthophosphate (µg/g)</i>				
FAO 34		159.1				
FAO 34		104.0				
FAO 34	ATLANTIC OCEAN	122.9				
FAO 34	CENTRAL EAST	261.7				
FAO 34		221.6				
FAO 34		135.2				
FAO 51	INDIAN OCEAN WESTERN	36.4				
FAO 57	INDIAN OCEAN EAST	1748.4				
FAO 57		2018.1				
FAO 61	PACIFIC OCEAN WESTERN	31.1				

<b>B) Processed, exoskeleton free prawn frozen samples</b>					
<i>Sample No.</i>	<i>Ortho-</i>	<i>Pyro-</i>	<i>Trimeta-</i>	<i>Tri-</i>	<i>Tetra/Penta/Hexa-peaks for SHMP screening*</i>
		<i>(µg/g)</i>			
1.	7351.1	1412.5	445.3	61.0	chromatographic fingerprint and MS/MS pattern of SHMP species
2.	13048.4	<LOQ	<LOQ	4.94	
3.	12236.5	165.2	16.4	1580.9	
4.	16345.6	112.1	16.3	1383.5	
5.	12645.3	<LOQ	7.3	ND	No peaks detected
6.	12722.6	<LOQ	14.1	ND	
7.	10579.4	ND	16.1	ND	
8.	9578.4	ND	8.9	ND	
9.	6759.9	ND	ND	ND	No peaks detected
10.	5792.4	ND	ND	ND	
11.	7058.5	ND	ND	ND	
12.	8312.5	ND	ND	ND	
13.	6885.5	ND	ND	ND	
14.	8599.1	ND	ND	ND	

<b>C) Various seafood samples</b>						
<i>Sample</i>	<i>Form and condition at arrival</i>	<i>Ortho-</i>	<i>Pyro-</i>	<i>Trimeta-</i>	<i>Tri-</i>	<i>Tetra/Penta/Hexa-peaks for SHMP screening</i>
		<i>(µg/g)</i>				
Shrimp 1	raw tails frozen	5014.1	ND	<LOQ	ND	yes
Shrimp 2	raw tails frozen	475.4	ND	ND	ND	no
Shrimp 3	raw entire frozen	9846.3	ND	3.1	ND	no
Scallop	raw shelled frozen	2027.4	ND	ND	ND	no
Octopus 1	raw entire frozen	64.68	29.29	ND	<LOQ	no
Squid	raw entire frozen	56.3	ND	ND	ND	no

Surimi 1	raw frozen	344.6	75.4	<LOQ	21.2	yes
Surimi 2	In brine non frozen	47.2	<LOQ	ND	<LOQ	yes
Shrimp 4	shelled cooked frozen	1245.4	399.1	ND	1793.0	yes
Octopus 2	tentacles cooked frozen	509.5	14.3	ND	38.0	yes

\* chromatographic fingerprint and MS/MS pattern of SHMP species

LOQ – limits of detection

ND - not detected

## Figures captions

**Figure 1.** Structures of the phosphates investigated in this study

**Figure 2A.** HPIEC–SCD and corresponding q-Exactive Orbitrap HRMS total ion chromatogram (TIC) of pure SHMP at concentration 100 µg/g

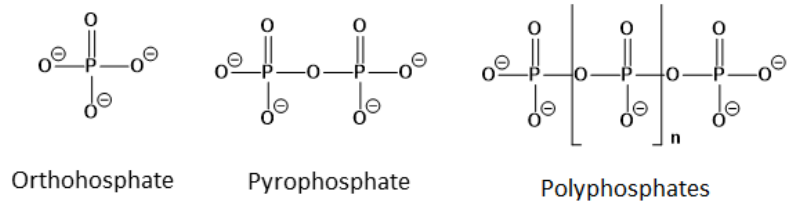
**Figure 2B.** q-Exactive Orbitrap data-independent mass fragmentation of 15 peaks that emerged in upper HPIEC chromatographic run of a pure SHMP sample (100 µg/g).

**Figure 3A.** HPIEC–SCD chromatogram of shrimp sample that contained scarcely SCD visible SHMP signals

**Figure 3B.** In-series HRMS-Q-Exactive Orbitrap characterisation of upper HPIEC–SCD run that revealed at least three chromatographic peaks that contained linear pentaphosphate anion ( $m/z$ -416.83494 with its fragmentation pattern



Figure 1.

**Linear (open chain) polyphosphates**

n = 1, Tripolyphosphate  
 n = 2, Tetrapolyphosphate  
 n = 3, Pentapolyphosphate  
 n = 4, Hexapolyphosphate

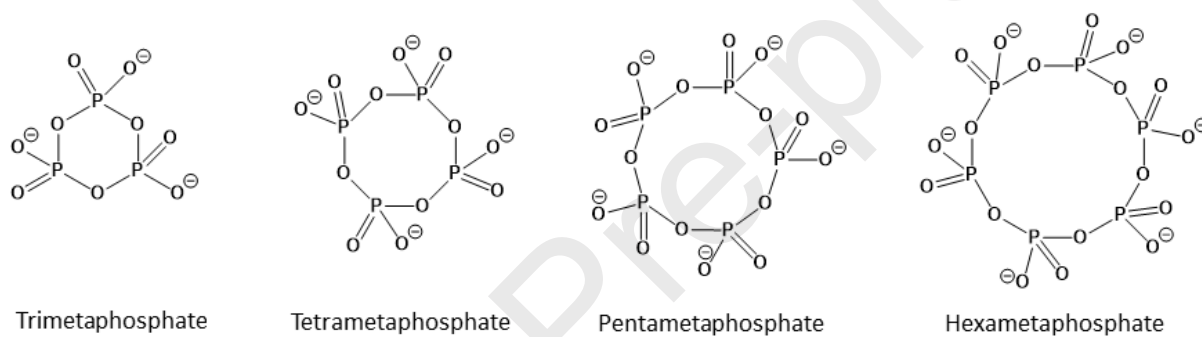
**Cyclic (meta) polyphosphates**

Figure 2A.

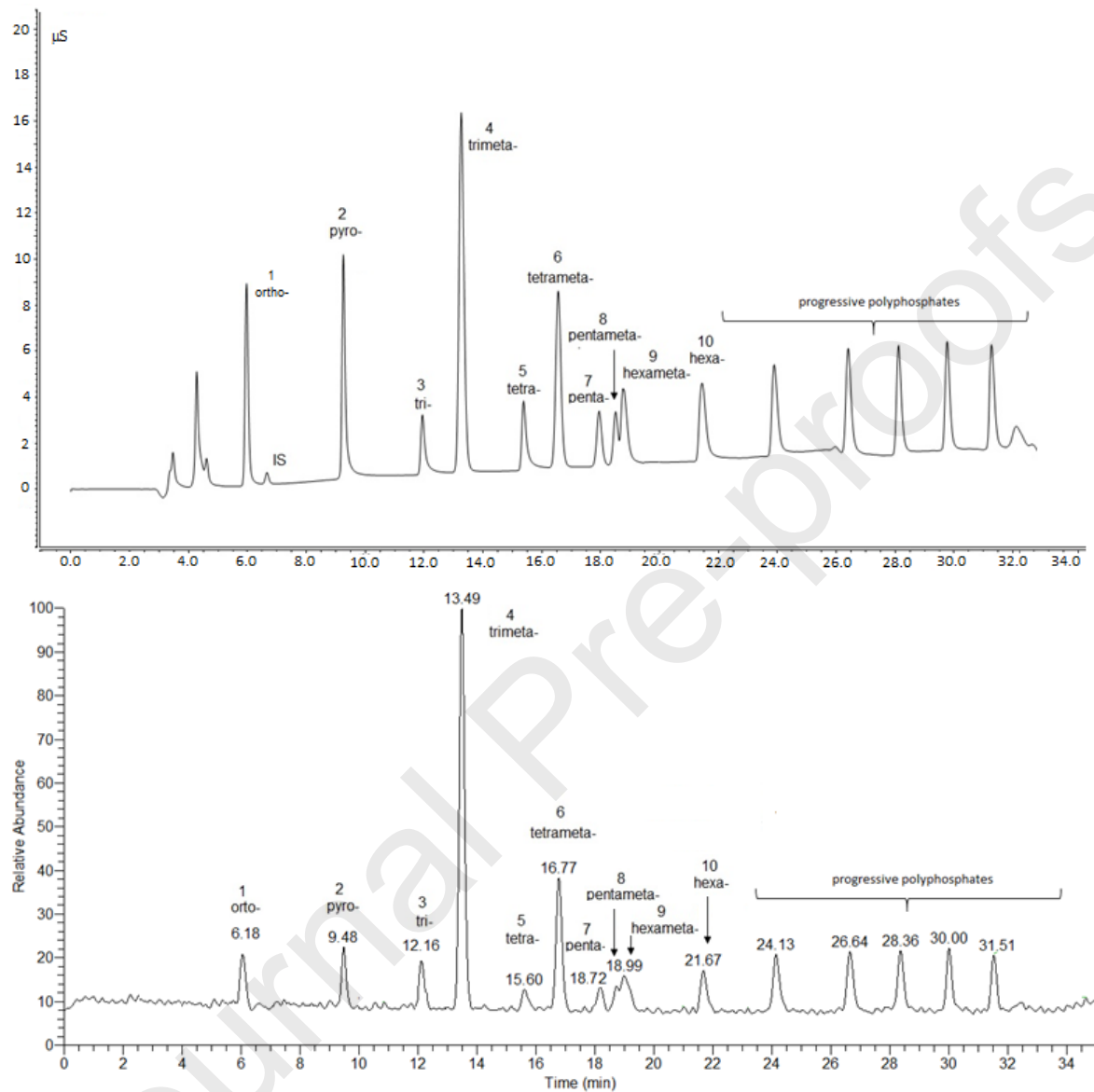


Figure 2B.

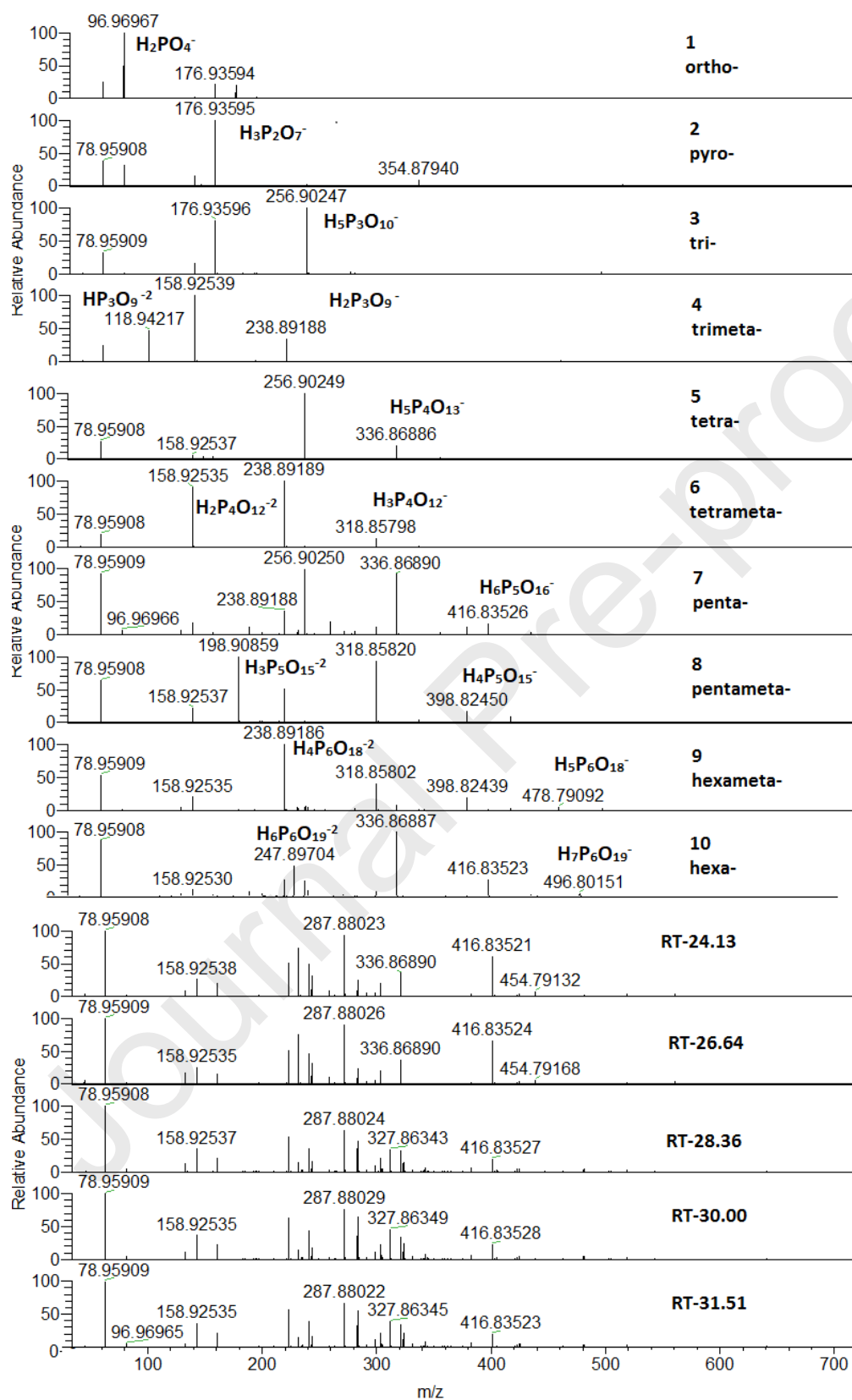


Figure 3A.

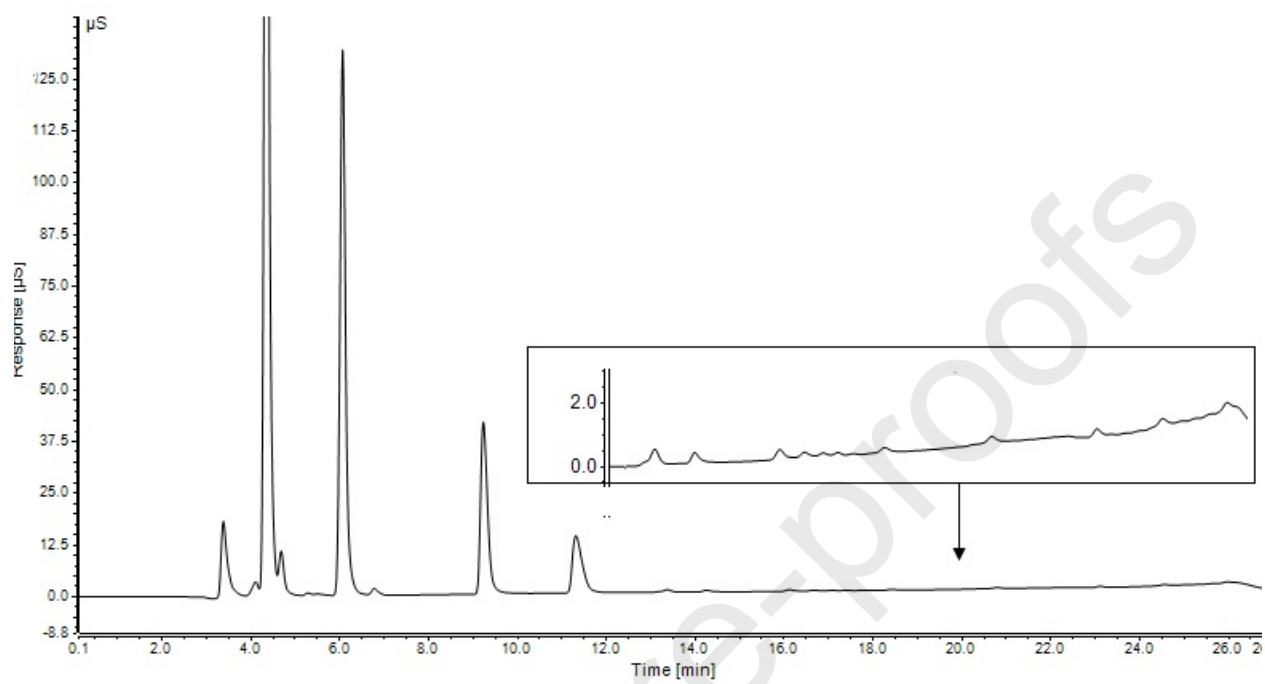
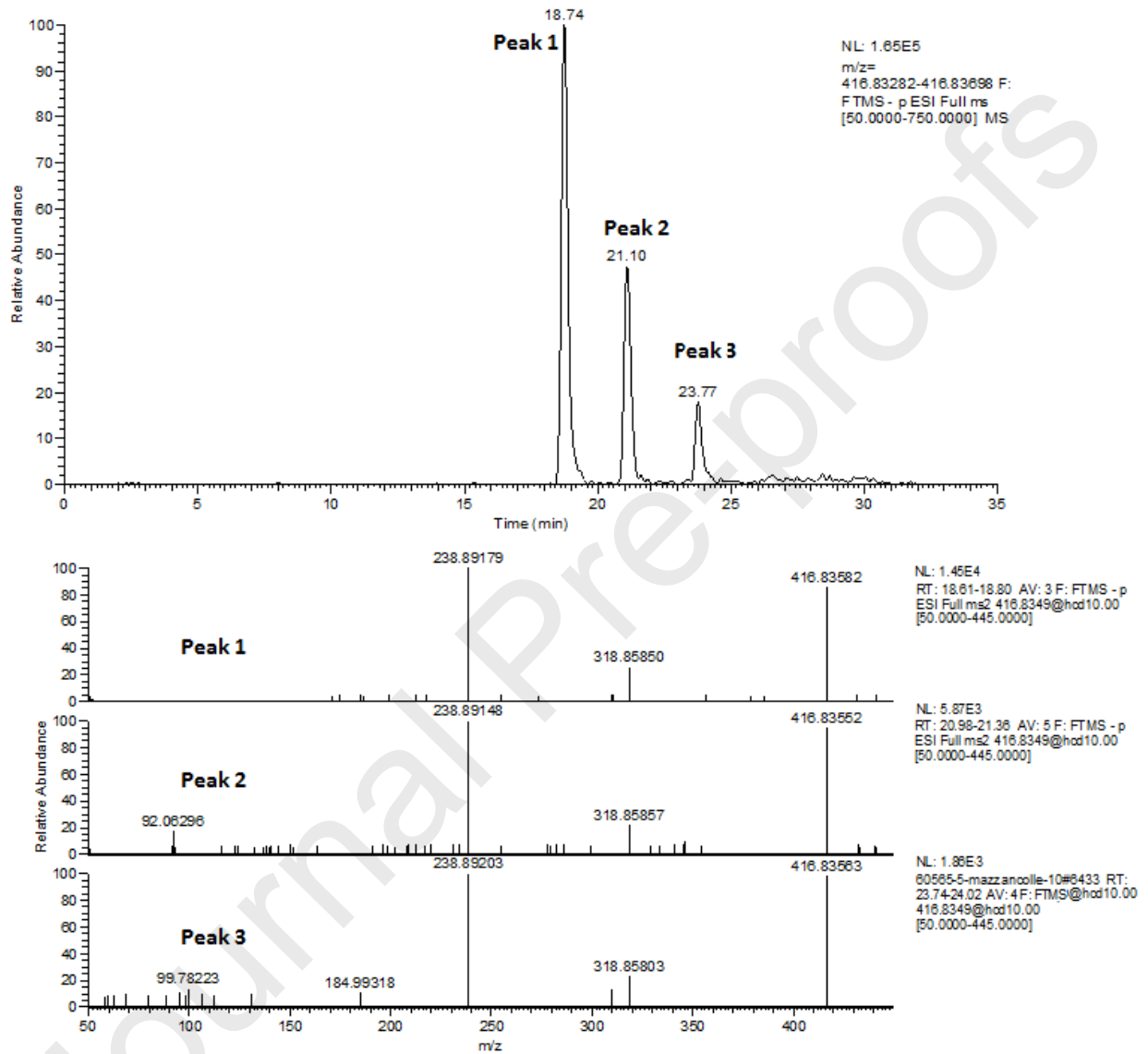
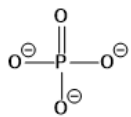
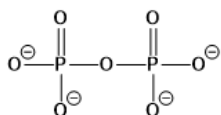


Figure 3B.

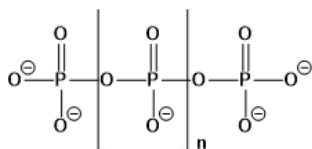


**Linear (open chain) polyphosphates**

Orthophosphate

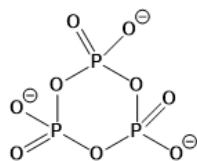


Pyrophosphate

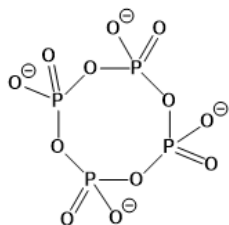


Polyphosphates

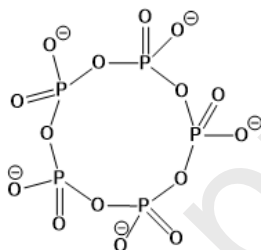
n = 1, Tripolyphosphate  
 n = 2, Tetrapolyphosphate  
 n = 3, Pentapolyphosphate  
 n = 4, Hexapolyphosphate

**Cyclic (meta) polyphosphates**

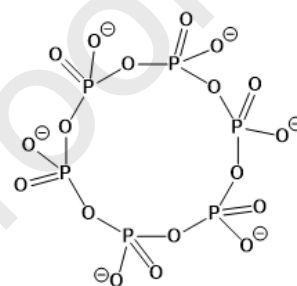
Trimetaphosphate



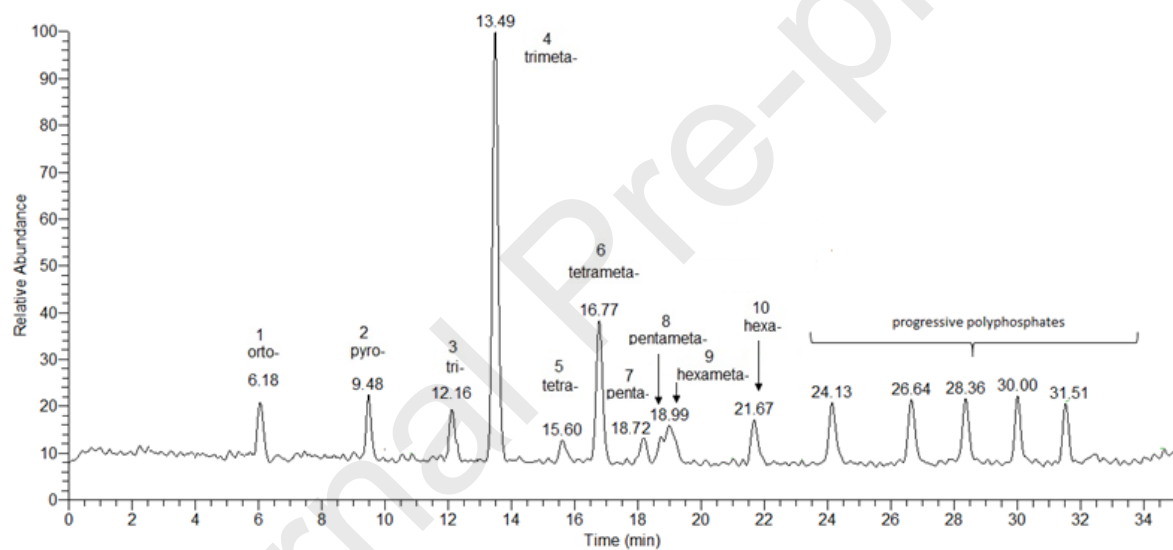
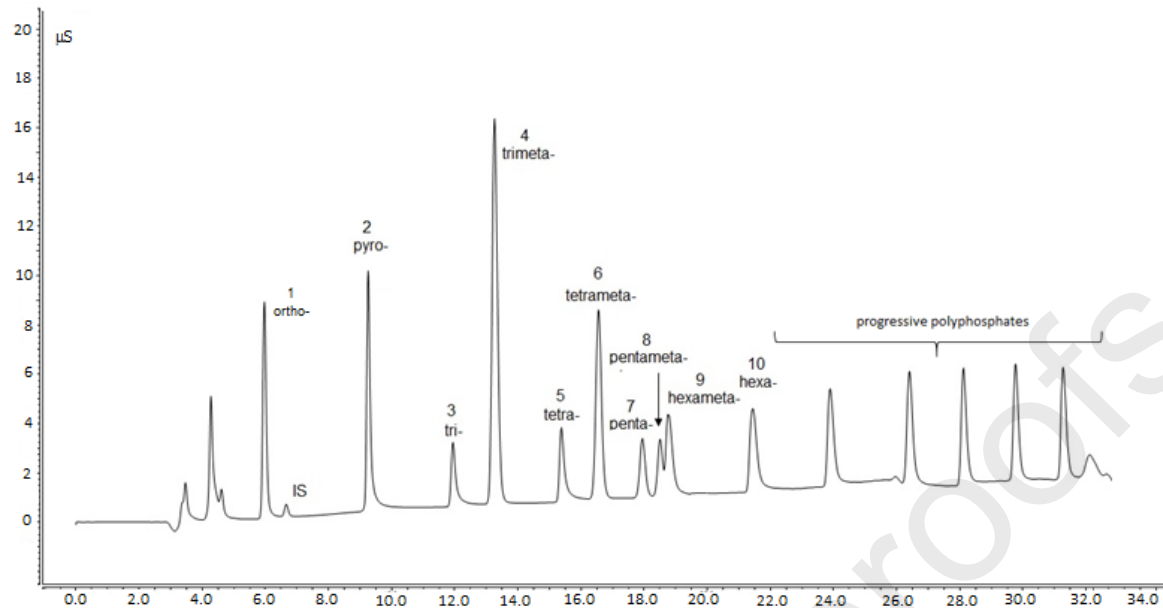
Tetrametaphosphate

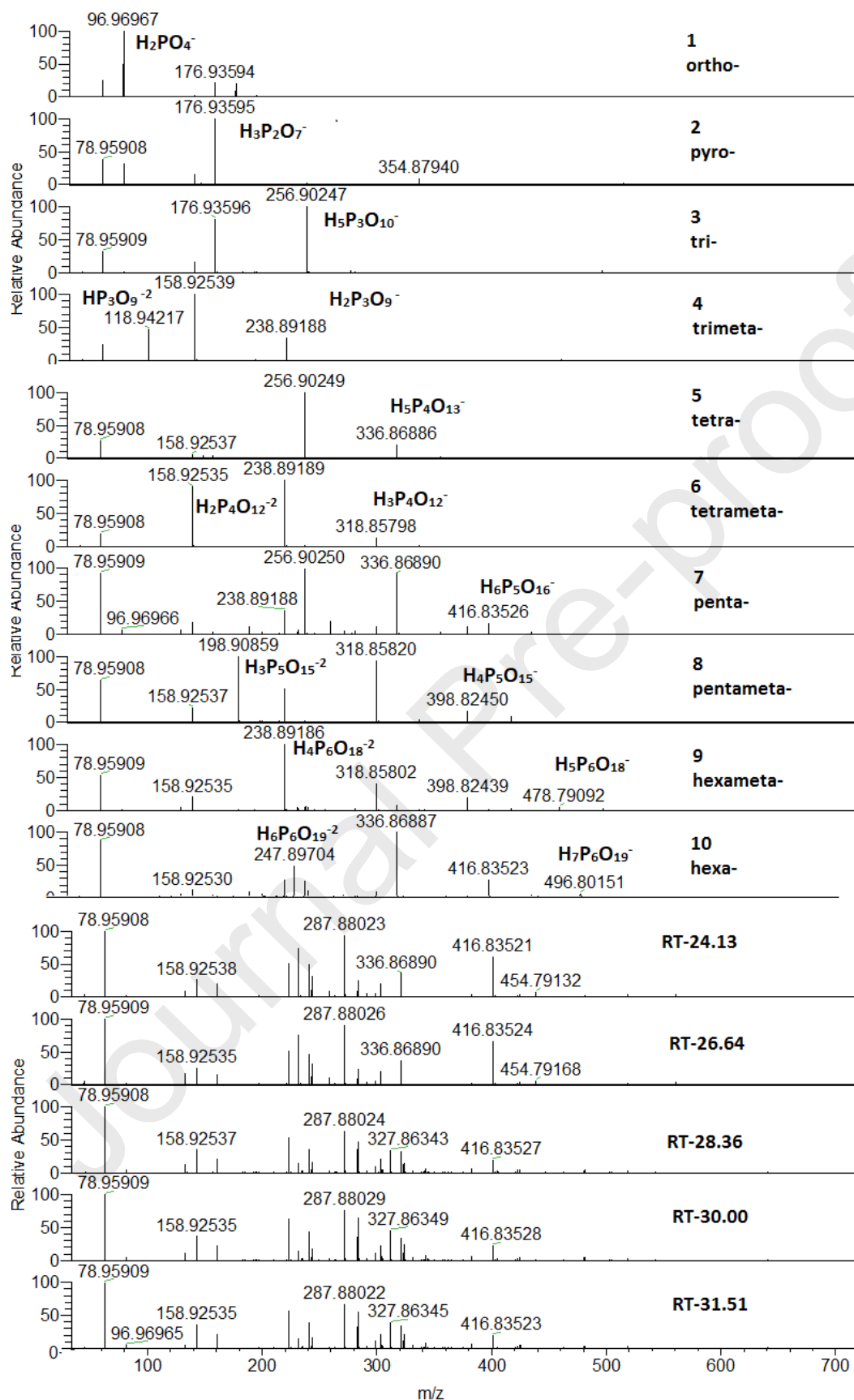


Pentametaphosphate

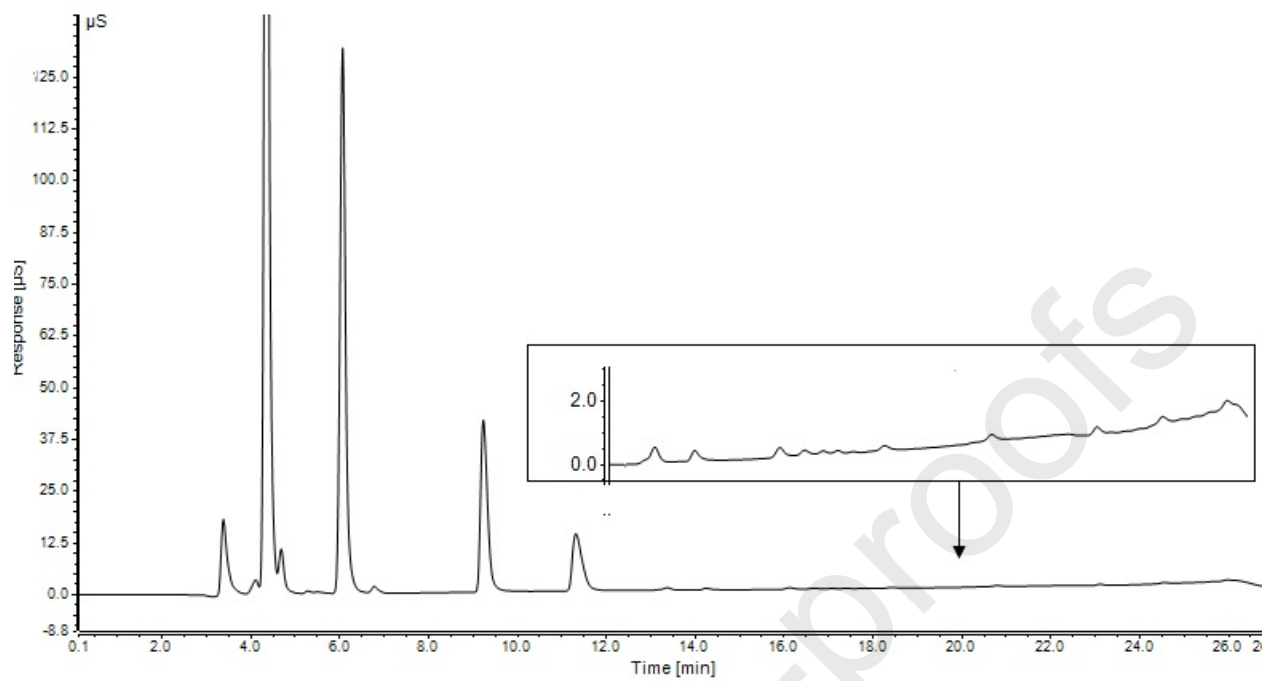


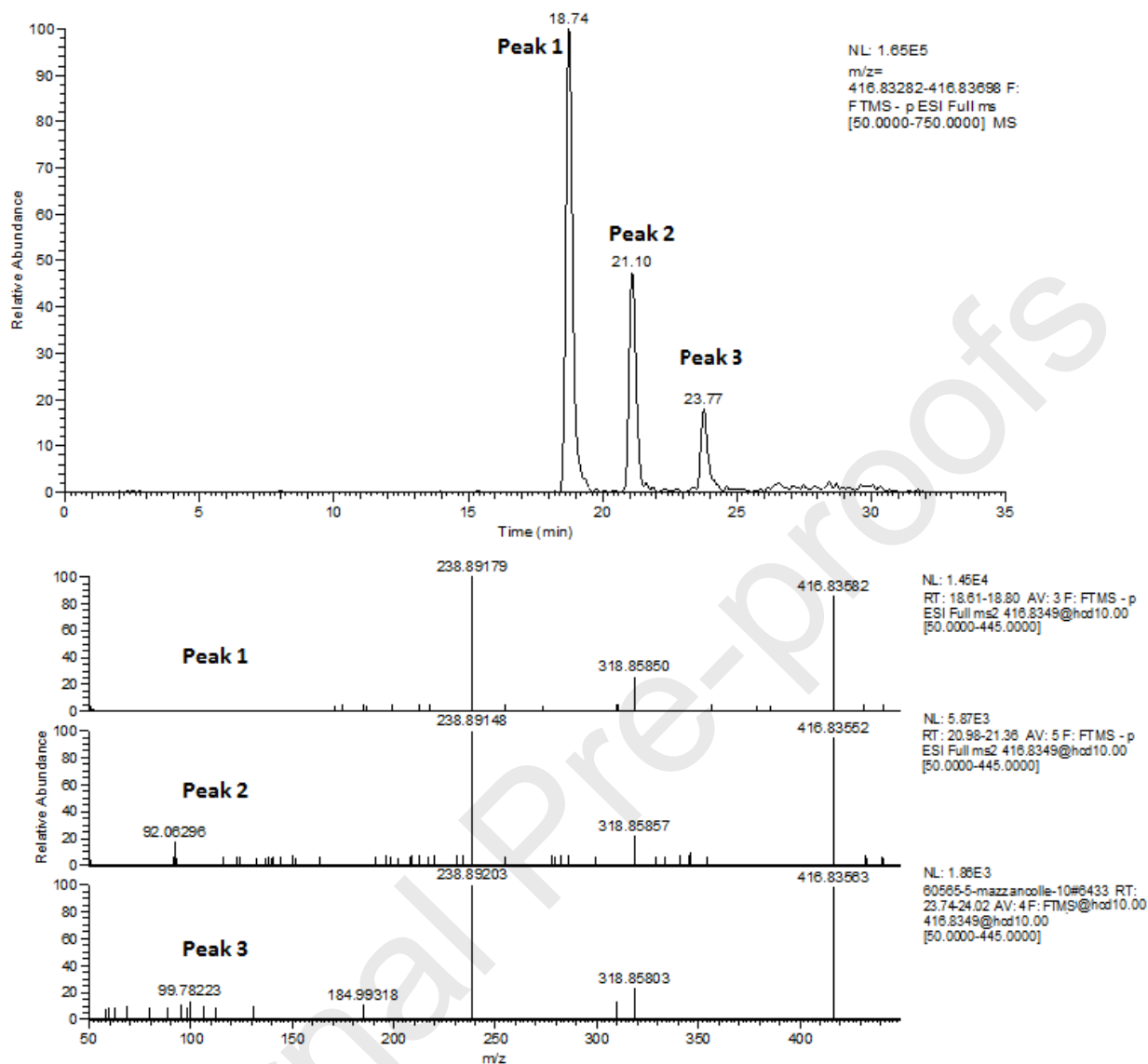
Hexametaphosphate











## Credit Author Statement

### Detection of polyphosphates in seafood and its relevance toward food safety

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- HPIEC-SCD- high-resolution mass spectrometry (HRMS) for polyphosphates profiling
- HRMS proposed new linear/cyclic polyphosphates forms specific for food control
- HPIEC-SCD-HRMS platform revealed hexametapolyphosphate in some fishery products

#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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