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Analysis of polyphosphates in fish and shrimps tissues by two different ion chromatography methods: Implications on false-negative and -positive findings

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Abstract

Inorganic polyphosphates (di-, tri- and higher polyphosphates) can be used to treat fish, fish fillets and shrimps in order to improve their water-binding capacity. The practical relevance of this treatment is a significant gain of weight caused by the retention/uptake of water and natural juice into the fish tissues. This practice is legal; however, the use of phosphates has to be declared. The routine control testing of fish for the presence of polyphosphates, produced some results that were difficult to explain. One of the two analytical methods used determined low diphosphate concentrations in a number of untreated samples, while the other ion chromatography (IC) method did not detect them. This initiated a number of investigations: results showed that polyphosphates in fish and shrimps tissue undergo a rapid enzymatic degradation, producing the ubiquitous orthophosphate. This led to the conclusion that sensitive analytical methods are required in order to detect previous polyphosphate treatment of a sample. The polyphosphate concentrations detected by one of the analytical methods could not be explained by the degradation of endogenous high-energy nucleotides like ATP into diphosphate, but by a coeluting compound. Further investigations by LC-MS-MS proved that the substance responsible for the observed peak was inosine monophsosphate (IMP) and not as thought the inorganic diphosphate. The method producing the false-positive result was modified and both methods were ultimately able to detect polyphosphates well separated from natural nucleotides. Polyphosphates could no longer be detected ($<0.5\,mg\,kg^{-1}$) after modification of the analytical methodology. The relevance of these findings lies in the fact that similar analytical methods are employed in various control laboratories, which might lead to false interpretation of measurements.

Keywords: *Polyphosphates, fish, additives, ion chromatography*

Introduction

Organic phosphates play an important role in rigor mortis of meat and fish tissues (Wenzhi and Toyohide 1991; Huss 1995a,b; Veciana-Nouges et al. 1997). The degradation of endogenous adenosine triphosphate (ATP) causes a contraction and stiffening of animal muscle tissues. This process is mostly accompanied by the release of enclosed water from the muscle tissue (Huss 1995). Exogenous inorganic polyphosphates were observed to prevent the shortening of the muscle fibre. Hence, the treatment of tissues with such substances reduces the naturally occurring loss of water. Excessive addition of polyphosphates to intact fish even permits an uptake of foreign water into the tissues (Krzynowek and Panunzio 1995; Müller and Eber 1999). Polyphosphates are also thought to complex heavy metals, which otherwise might catalyse the oxidative degradation of fatty acids (Strack 1992). Besides, polyphosphates apparently increase the gloss (visual appearance) of fish or shrimps. However, the economic benefits created by introducing water into a high-price commodity seem to be the major driving force for the use of polyphosphates.

Polyphosphates are not known to be toxic, so their use is allowed in a number of countries, yet legislative bodies have defined maximal concentrations (e.g. $5000\,mg\,kg^{-1}$ in Switzerland) and require producers to declare such additives.

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Analytical methods to detect inorganic polyphosphates in meat or fish were based on thin layer chromatography. The more recent introduction of sensitive ion chromatography (IC) methods employing carbonate gradients (Kaufmann and Pacciarelli 2000) and hydroxide gradients (Cui 2000; Sekiguchi and Matsunaga 2000; Dafflon and Bosser 2003) significantly reduced the detection limits from 300 to 20 $mg\,kg^{-1}$, and even further to sub-mg kg^{-1} concentrations. Trichloroacetic acid was used as extraction agent (Cui 2000; Sekiguchi and Matsunaga 2000). Clean extracts were obtained, yet this acid causes severe system peaks in the IC chromatogram. The problem of false-negative results (caused by the ubiquitous presence of native phosphatase) was circumvented by a hot alkaline extraction (Kaufmann and Pacciarelli 2000) or by a pH neutral microwave shock treatment (Dafflon and Bosser 2003).

Polyphosphate concentrations—capable of increasing the water-binding capacity—are in the range of a few $g\,kg^{-1}$. Currently the maximum permitted level in Switzerland is 5 $g\,kg^{-1}$ (calculated as P_2O_5). Polyphosphates are expected to undergo chemical and enzymatic degradation in tissue, hence the proof that a particular sample has undergone a previous polyphosphate treatment requires analytical methods that are capable detecting even small fractions of this maximal permitted 5 $g\,kg^{-1}$.

A hydroxide gradient IC method used by the authors for the routine control of polyphosphates in fish quantified in a number of samples low levels of diphosphate <300 $mg\,kg^{-1}$. Doubts were raised whether the measurement of such concentrations could still be considered to be an unequivocal proof of a previous polyphosphate treatment.

The aim was to gain knowledge about the degradation of added inorganic polyphosphates in fish tissues, and the possible likelihood that traces of such substances are naturally present in such matrices. Furthermore, the origin of the divergent results produced by the two different IC methods was investigated. This process initiated methodical corrections that eliminated the problem.

Materials and methods

Samples

Various fish (tilapia, pangasius and trout), water and feed samples were taken from four different fish cultivators located in a variety of regions in Switzerland. Among the seven fish samples, four fish originated from closed and three from open breeding basins.

The sampled fishes were caught directly from the ponds, killed on the spot, partly cleaned, cut in half, immediately frozen and transported in dry ice to the SQTS and the Kantonales Labor laboratories. The whole sampling process was performed under the presence and supervision of trained SQTS staff to ensure that no exogenous inorganic polyphosphates were used. All samples were separately analysed in both laboratories the day after sampling. Samples for the polyphosphate stability tests were frozen fish (cod) and shrimp (tiger prawn) obtained from a local supermarket.

Materials

The following chemicals were obtained commercially: sulfuric acid 98% p.a., tetra-sodium pyrophosphate decahydrate, penta-sodium triphosphate (Fluka, Buchs, Switzerland), di-sodium hydrogen phosphate dodeca-hydrate, sodium hydrogen carbonate, sodium carbonate (water free), sodium chloride, nitrate standard solution (1 $g\,l^{-1}$), nitrite standard solution (1 $g\,l^{-1}$) PO_4 solution 1000 $mg\,l^{-1}$ (Merck, Darmstadt, Germany), NaOH 50% (J. T. Baker, Deventer, the Netherlands), ADP (adenosine 5′-diphosphate) (Boeringer, Mannheim, Germany), and IMP inosin 5′-monophosphate disodium salt octahydrate (Fluka).

The materials used were as follows. Centrifuge tubes, Chromafil PES-20/25 microfilter. Homogenizer, Polytrone (Kinematica, Littau, Switzerland). Centrifuges (Sorvall RC 5C, Kendro, Zurich, Switzerland; and Heräus Megafuge, Switzerland), centrifuge tubes, Spartan 30/0, 45 RC fine filter.

Extraction procedure A (carbonate IC gradient)

Frozen fish (5 g) was weighed into a centrifuge tube (spike solution was added if recoveries were to be determined). A total of 150 ml extraction solution (60 ml mobile phase B l^{-1} water) were added. The centrifuge tube was capped and immediately placed into a bath of boiling water. Ten minutes later, the hot contents of the centrifuge tube were homogenized. Afterwards the vessel was placed for another 5 min into the hot water bath.

The mixture underwent cooling for 1 h in an ice bath and was centrifuged at 14 500 rpm (equivalent to 15 000 g) for 5 min. The supernatant was filtrated through a microfilter directly into an HPLC vial.

Extraction procedure B (hydroxide IC gradient)

Frozen fish (5 g) was weighed into a centrifuge tube. The sample was heated for 40 s in the microwave at maximum power (750 W) to denaturate any present phosphatases. After cooling to room temperature 250 μl ADP standard solution

Table I. Technical details of the two used chromatographical systems.

Parameter	Procedure A: Carbonate gradient	Procedure B: Hydroxide gradient
Pump	Quarternary pump (stainless steal) HPLC Model 1100 (Agilent, Waldbronn, Germany)	Dionex DX500 (Dionex, Sonnyvale, CA, USA)
Autosampler	Model 1100 (Agilent)	Dionex DX500 (Dionex)
Detector	IC 753 solid-phase suppressor module and an IC 732 IC conductivity detector (Metrohm, Herisau, Switzerland) UV variable detector: wavelength: 215 nm (Agilent)	ASRS Ultra 4 mm Supressor (Dionex)
Pre-column	Metrosep Anion Dual 1, 3.0 × 35 mm (Metrohm)	n.a.
Column	Anion Dual 1, 3.0 × 150 mm (Metrohm)	AS-16 (Dionex)
Post-column	Hypercarb Guard, 4.6 × 10 mm (Alltech, Lausanne, Switzerland)	n.a.
Mobile phase	(A) distilled water (B) 50 mmol sodium carbonate and 50 mmol sodium hydrogencarbonate in distilled water n.a.	(A) H_2O (Millipore (maximum 5 ppb TOC) (B) 5 mmol l^{-1} NaOH (C) 200 mmol l^{-1} NaOH 0–7 min 95/0/5% (A/B/C) 7–25 min 80/0/20% 25–50 min 40/0/60%
Gradient	0.0–0.1 min 94/6% (A/B) 0.1–14.0 min 80/20% 20.0–20.9 min 0/100% 21.0–29.0 min 94/6%	50–70 min 70/30/0%. modified: 0–5 min 50/50/0% 5–25 min 90/0/10% 25–35 min 44/0/56% 35–42 min 50/50/0%
Flow rate	0.5 ml min^{-1}	1.5 ml min^{-1}
Column temperature	20°C	35°C
Injection volume	10 µl	25 µl
General	chromatograms were subtracted with a blank before integration	n.a.
Recovery	95%	95%
Limit of detection, $s/n = 3:1$	5 mg kg^{-1}	0.5 mg kg^{-1}

(1 mg ml^{-1}) were added and filled with water to 35.0 g. Afterwards, the sample was homogenized using a polytron for about 1 min, centrifuged (4000 U min^{-1}) for 5 min and filtrated. The filtrate (0.5 ml) was diluted with 4.5 ml methanolic NaOH (85% 20 mmol l^{-1} NaOH + 15% MeOH) and injected into the HPLC system. Water samples were tenfold diluted and analysed. Feed samples were extracted as described for fish samples.

Ion chromatography

The instruments and parameters used for the two methods are listed in Table I. The carbonate IC gradient method (carbonate procedure) is described in more detail in Kaufmann and Pacciarelli (2000).

LC-MS-MS

LC-MS-MS was performed by means of a type Agilent Model 1100, binary pump, autosampler (Agilent, Waldbronn, Germany) with a tandem mass spectrometer with electrospray interface, Quattro LCZ and MassLynx software (Micromass, Manchester, UK).

Stability study of polyphosphates in fish and shrimps

Uncooked frozen cod fillets and black tiger shrimps were obtained from the local market. The skin of the shrimps was removed for further experiments. For practical reasons and to be able to repeat the experiment with the same material, frozen samples were used. The tissues were cut into cubes of approximately 1 cm^3. A total of 50 g cut tissues was covered with 200 ml 1% aqueous sodium triphosphate solution and left at 4°C in a refrigerator for 12 h. The solution was afterwards discarded and the cubes were carefully moped up by tissue papers. The semi-dried cubes were stored in dry beaker at 4°C in a refrigerator. Samples were taken and analysed for polyphosphates (procedure A: carbonate IC method) before, immediately after the treatment and then in consecutive 1-day intervals.

To investigate whether the degradation was caused by enzymatic action, one batch of shrimps was put into boiling water for 5 min. These cooked shrimps were afterwards treated with polyphosphate solution and analysed as described above.

Results and discussion

Stability study of polyphosphates in fish and shrimps

Figure 1 indicates the stability of triphosphate in fish and shrimps. The purchased samples were before the experiment, tested negative for polyphosphate by the use of method A.

The treatment of raw fish and shrimps and with triphosphate solution resulted in an initial concentration of some 1500 mg kg^{-1} (sum of di- and triphosphate). No polyphosphate was any longer detectable after 2 days (fish) and 4 days (shrimps) storage at 4°C. Previously cooked shrimps and afterwards treated with triphosphate solution behaved differently. No degradation of the polyphosphates was observed, which is probably due to the thermal deactivation of phosphatase. Furthermore, the concentrations measured (some 2600 mg kg^{-1}) were higher than initial concentrations measured in uncooked samples. We interpreted these lower initial concentrations in

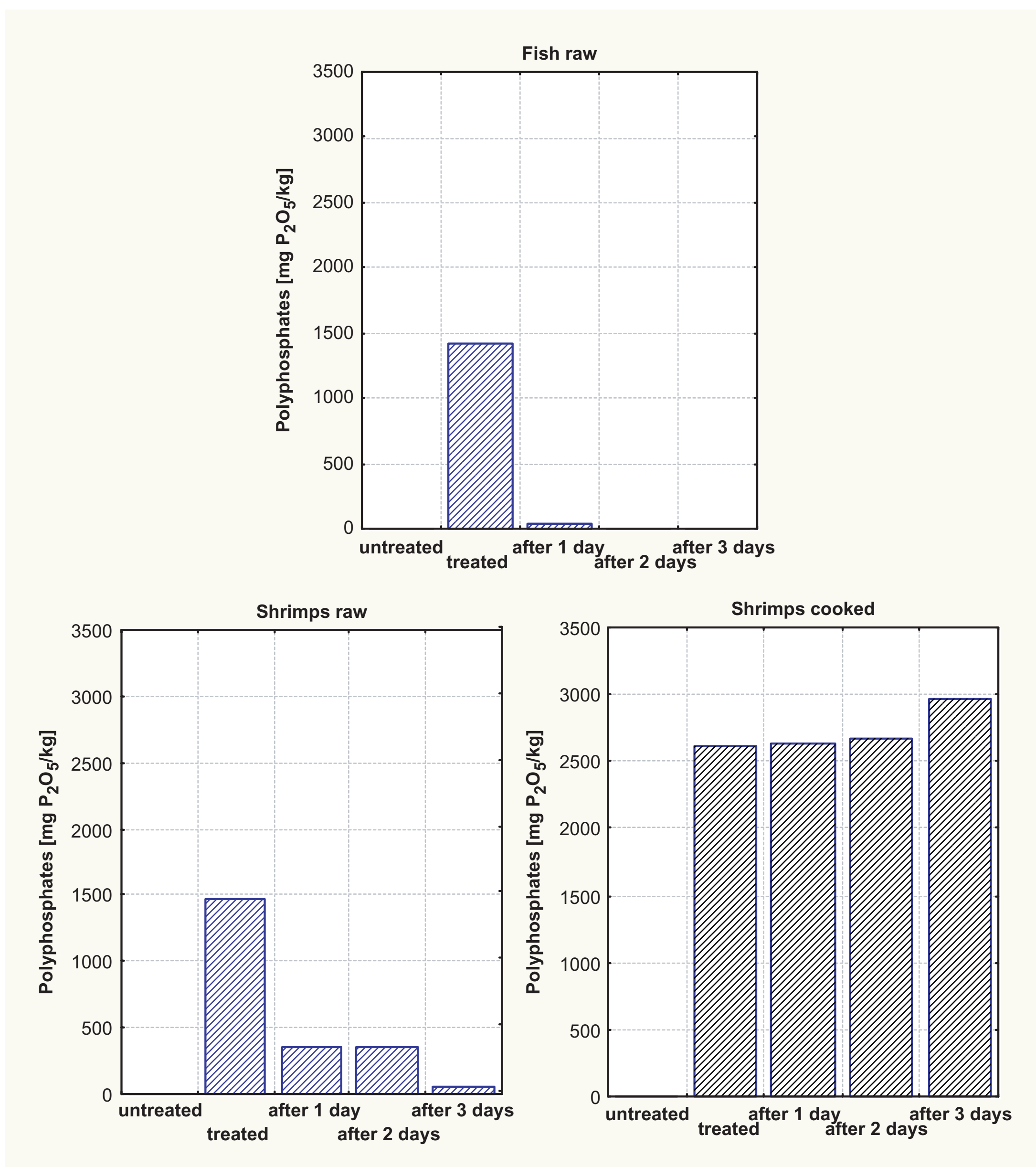

Figure 1. Stability of polyphosphates in raw fish and shrimps as compared with boiled shrimps. Concentrations refer to the sum of di- and triphosphate.

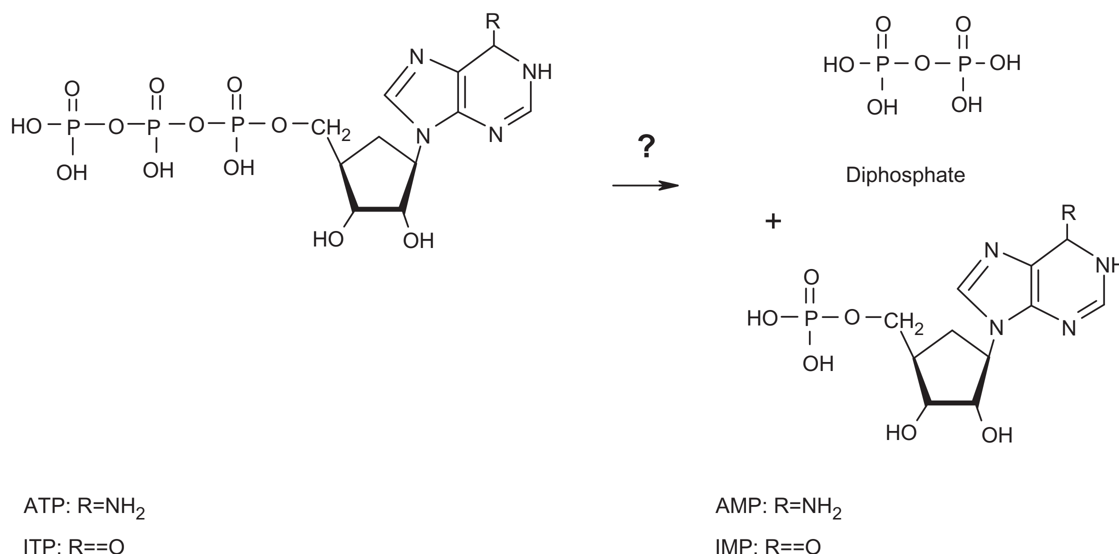

Figure 2. Possible degradation reaction of high-energy nucleotides.

uncooked fish and shrimps by the fast enzymatic degradation of polyphosphate.

The data confirm that a fast degradation of polyphosphates occurs in tissue and indicates the need for a sensitive analytical methods capable of detecting remaining traces of once applied polyphosphates. The interpretation of a positive finding is always based on the assumption that inorganic polyphosphates are neither a natural low concentration component in fish tissue nor a possible degradation product of endogenous high-energy polyphosphates. Such a hypothetical degradation reaction is shown in Figure 2.

Comparison of the results produced by the two different IC methods

Fish and corresponding feed and fish pond water samples were analysed (within 1 day after sampling) by both described methods. No inorganic polyphosphates in the water and feed samples could be detected by any of the two employed analytical methods. However, fish samples analysed with the carbonate and with the hydroxide gradient method produced significantly different results. The hydroxide method quantified in all the above-mentioned fish samples some 300 $mg\,kg^{-1}$ diphosphate, while the carbonate method did not detect any inorganic polyphosphates (limit of quantification 20 $mg\,kg^{-1}$).

Effect of different extraction procedures

To rule out the possibility of diphosphate being an artefact of ATP degradation, as postulated by Figure 2, a stability study was performed. ATP solutions were microwave treated (which is an integral component of the hydroxide method). Even prolonged heating did not produce any measurable levels of inorganic phosphate breakdown products. Hence, the positive diphosphate results could not be explained by a thermal breakdown of ATP related to the sample processing (e.g. microwave).

Extracts produced with the hydroxide method were analysed with the carbonate method and vice versa. Still, the hydroxide separation and detection approach found clearly measurable diphosphate concentrations, while the carbonate separation and detection did not detect any diphosphate. This led to the conclusion that the different extraction procedures cannot explain these diverging results.

Effects of different separation procedures

It was suspected that the graphitized carbon post-column used by the carbonate method removes possible interfering organic polyphosphates. The use of this column in the hydroxide gradient method could not be tested. The supplier of the IC column and suppressor advises against using any metal parts in the IC equipment. Yet, the graphitized carbon material is only available in a stainless steal column. Removal of this column from the carbonate gradient system caused the appearance of a new peak eluting 2 min ahead of the diphosphate. If this peak was quantified (extending the calibration window significantly) as if it was diphosphate, a concentration of some

300 mg kg^{-1} resulted. More significantly, this peak showed a much stronger ultraviolet light than conductivity response. Since this peak completely disappeared after treating the sample extract with phosphatase, it was concluded to be an organic polyphosphate.

Identification of possible co-eluting compounds by LC-MS-MS

Fractions of the carbonate eluate (not using the graphitized carbon column but passing the suppressor) were taken and flow injected into an LC-MS-MS system. Negative ESI produced a strong signal. The presence of a base peak with smaller mono- and di-sodium adducts corresponded to the idea of an organic polyphosphate. Collision experiments resulted in typical phosphate fragments. The measured $[M-H]^-$ mass (346.7) was tentatively identified as inosin-monophosphate. The purchased reference substance produced an identical fragmentation spectrum (Figure 3) and eluted at the same time as the unknown peak in the carbonate gradient and co-eluted with inorganic diphosphate when injected into a hydroxide gradient system (Figure 4).

The hydroxide gradient system was carefully adjusted to produce after these modifications well

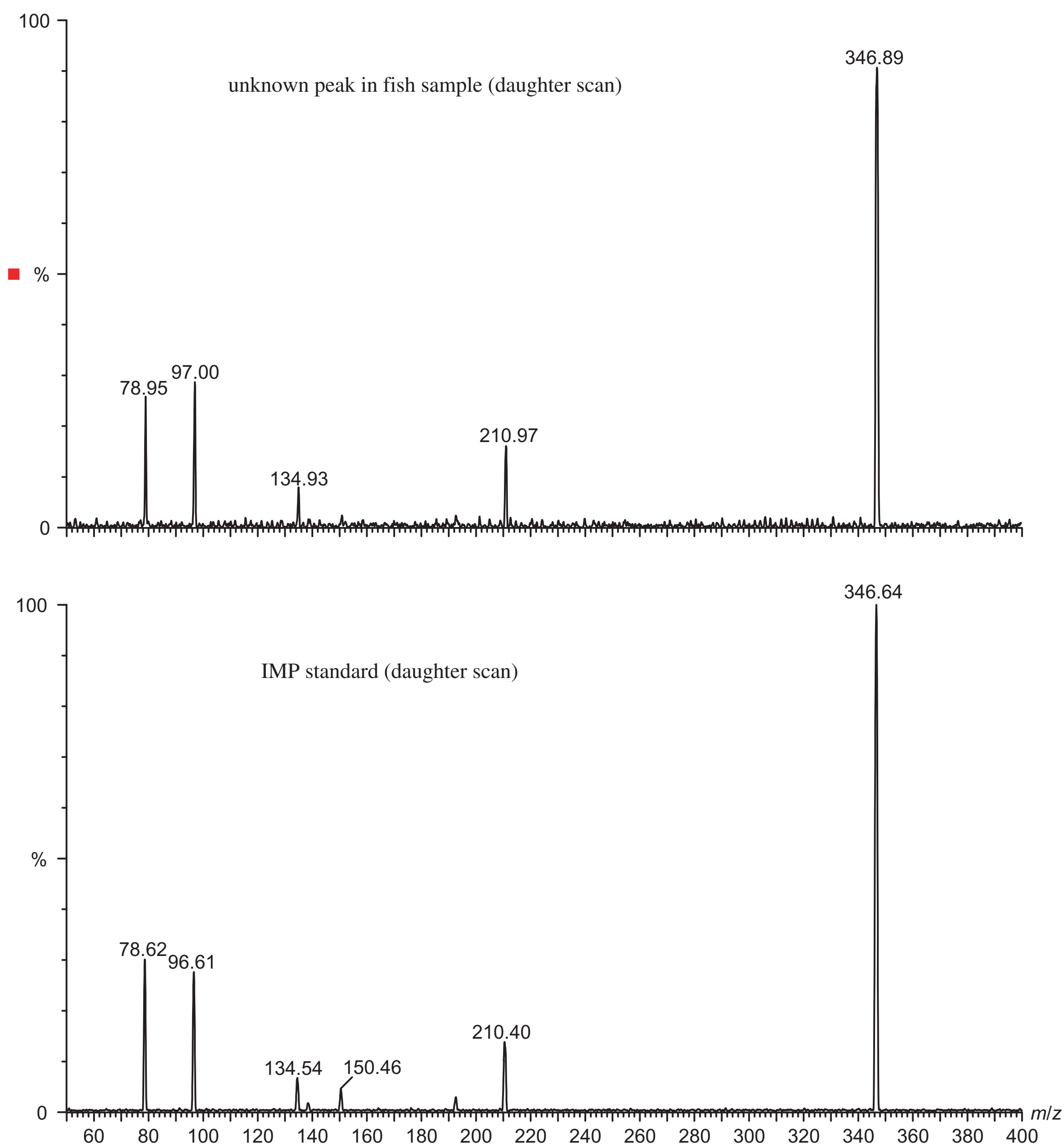

Figure 3. Fragmentation spectra of unknown peak (top) and of inosin-monophosphate (bottom).

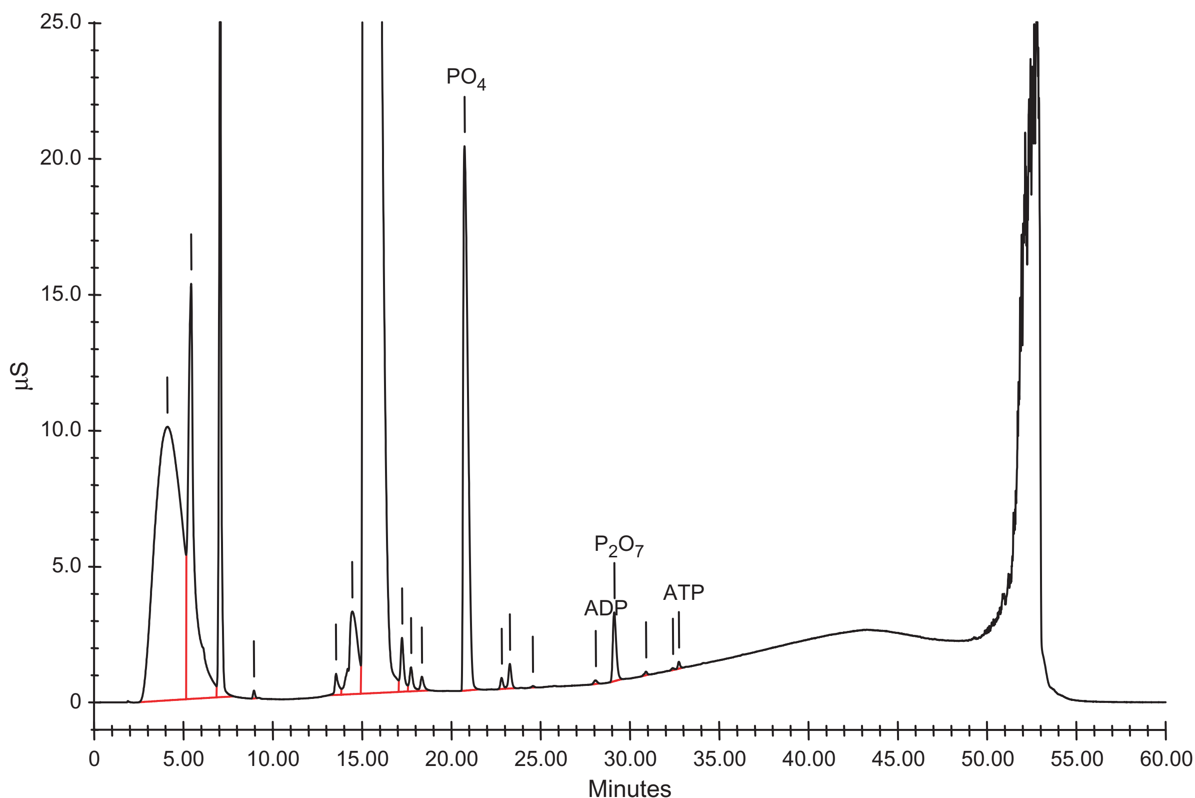

Figure 4. Pangasius sample analysed with the 'original' hydroxide gradient system. Note that IMP coelutes under the peak termed P_2O_7.

separated peaks (Figure 5). The re-analysed fish samples did not show any signal for diphosphate (Figure 6).

Analytical results obtained before and after methodical adjustments

The hydroxide method was used for the routine control of fish samples over a long period. A set of data is available for samples analysed before the methodical change ($n = 102$) and after the adjustments as described in the Materials and methods ($n = 36$). Figure 7 shows the relative distribution between di- and triphosphate results before and after the discussed methodical changes.

Results obtained before the methodical change show a number of samples where up to 300 mg kg^{-1} diphosphate but no triphosphate could be detected. This was no longer observed after the modified method was put into action. We attribute this difference to the interference of IMP. It is not known what kinds of polyphosphates are used for the treatment of fish in various parts of the world. Yet, we suppose that the quality employed consists of a mixture of poorly defined di-, tri- and higher polyphosphates. Hence, degradation would produce a mixture of mono-, di- and triphosphate. This assumption corresponds to the distribution depicted in the right graph of Figure 7.

Occurrence of IMP in fish tissue

IMP was only found in a minority of commercial fish samples available in Switzerland. The IMP containing fishes analysed were cultured species caught under strictly controlled conditions by applying a minimum of stress to the animals. Killing, processing and freezing were done as quickly as possible. This treatment might not be typical for fish caught on an industrial scale. We suspect that commercially caught fish experience a much higher stress level than the investigated animals. For example, netted fish undergo a severe struggle before death. Hence, their energy level (as indicated by ATP and related compounds concentrations) will have dropped significantly (Wenzhi and Toyohide 1991; Huss 1995; Veciana-Nouges et al. 1997). Nucleotide catabolism continues at typical storage and transport temperatures of about 3°C. IMP was reported to drop to one-tenth of the original concentration within 4 days (Huss 1995). Most likely no samples with high IMP concentrations were used/available for the validation of

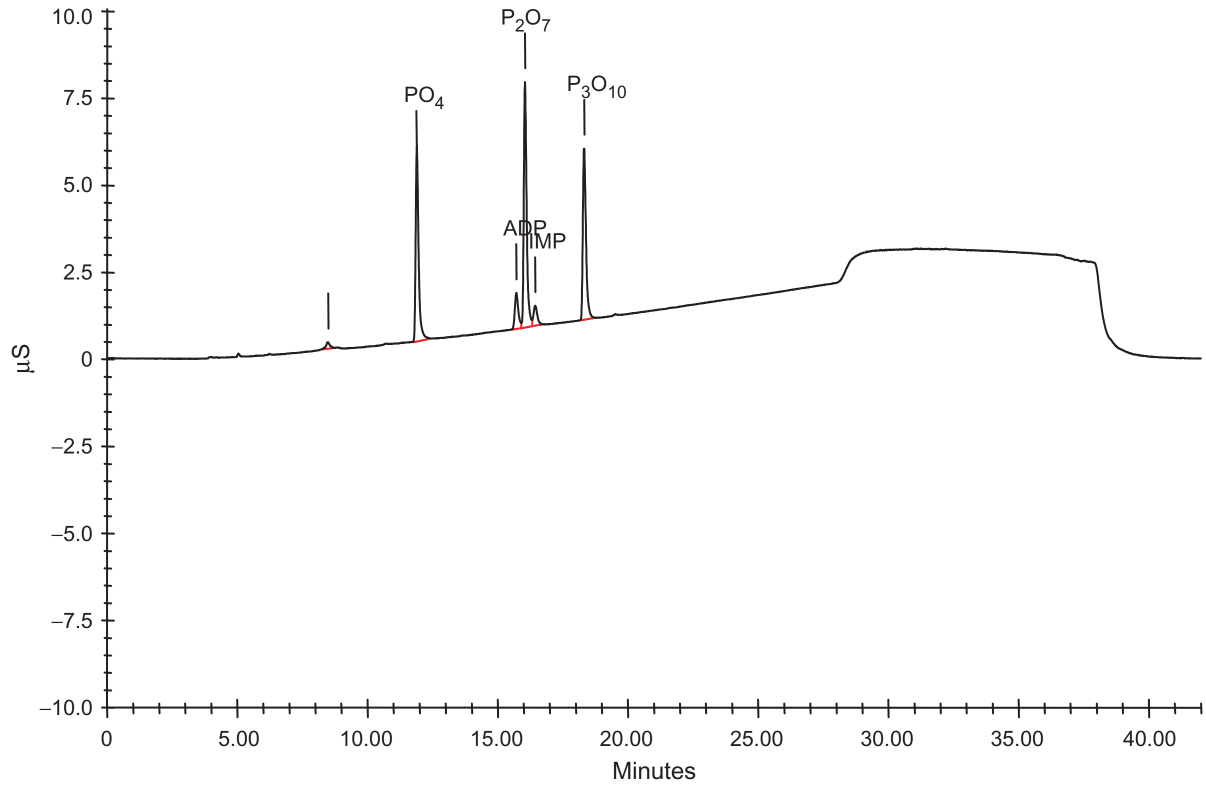

Figure 5. Standard solution analysed with the 'modified' hydroxide gradient system.

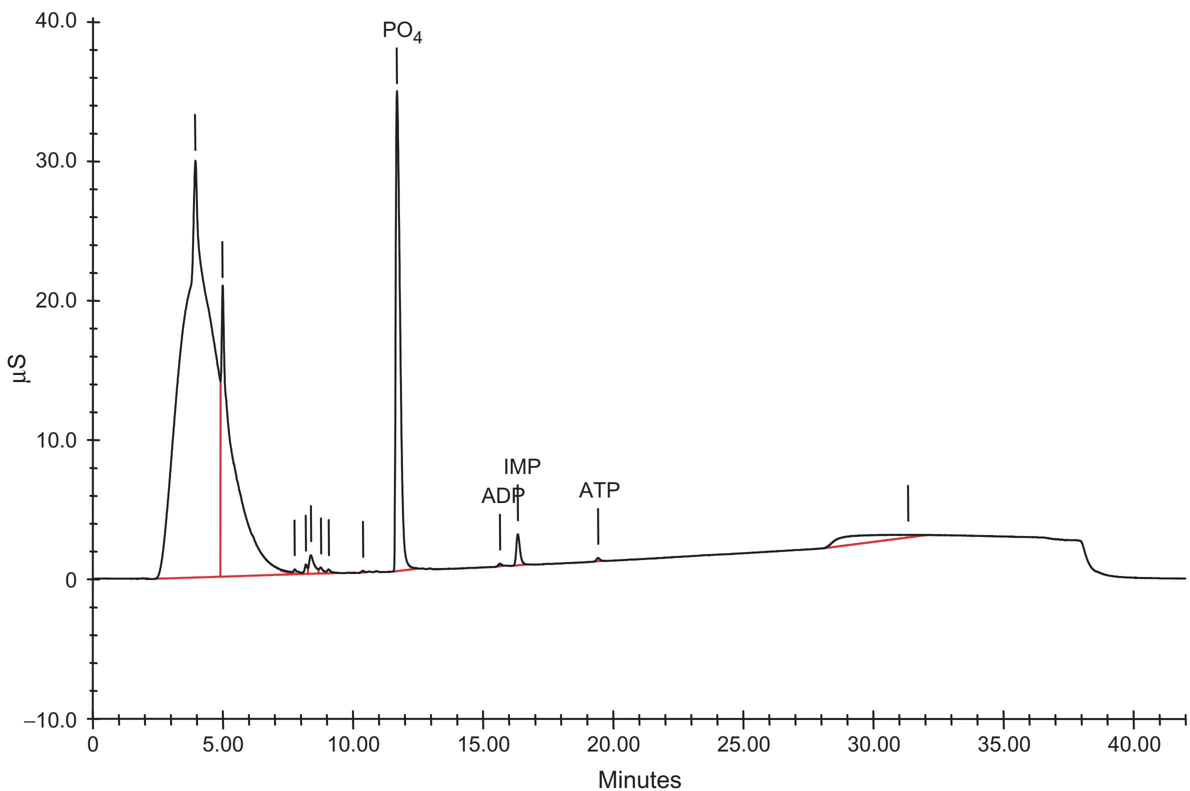

Figure 6. Pangasius sample analysed with the 'modified' hydroxide gradient system.

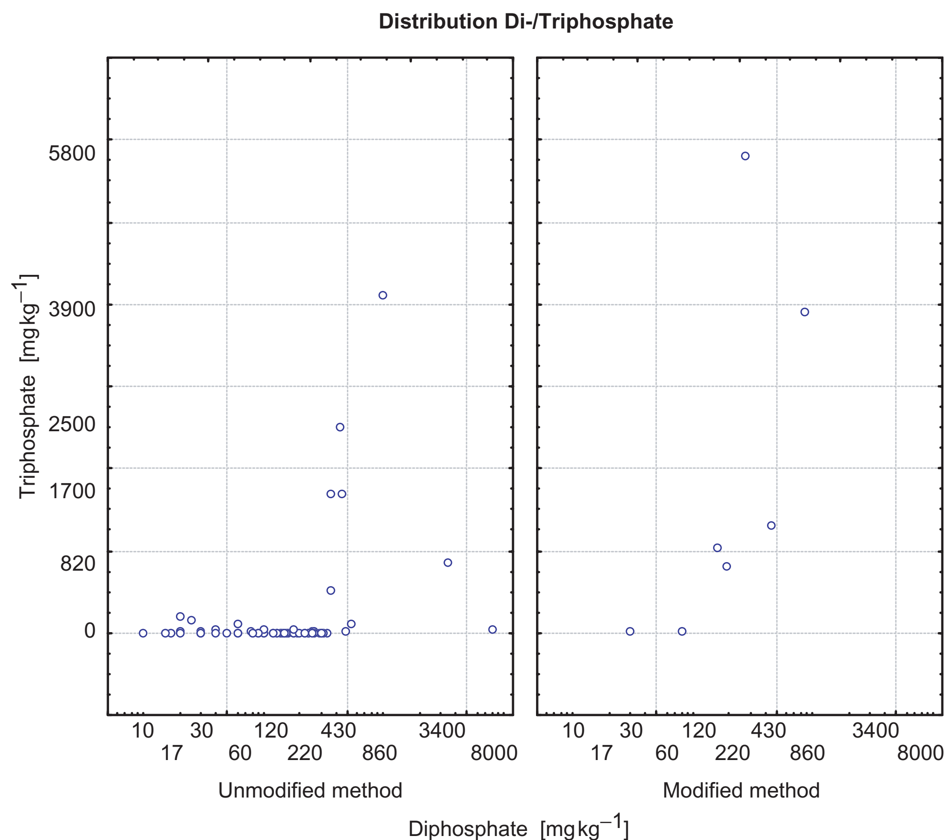

Figure 7. Relationship between concentrations of di- and triphosphate before and after modification of the analytical method (only samples with measurable di- and/or triphosphate levels are shown).

some commonly used polyphosphate IC methods. Hence, this potential problem might have escaped attention.

Conclusions

There is a risk of producing false-negative and -positive results when inorganic polyphosphates are to be quantified in fish and meat. The ubiquitous phosphatase has to be deactivated either by thermal or chemical means. Otherwise, fast degradation of polyphosphates occurs, i.e. false-negative results are produced. Many gradient IC systems do not separate inorganic polyphosphates from nucleotides occurring in some samples. If detection only employs a conductivity, but not a UV detector, such possible co-eluting peaks might be wrongly quantified as inorganic polyphosphates. If the used IC system permits the use of metal equipment, a graphitized carbon post-column is suggested in order to trap otherwise eluting nucleotides. Alternatively, the used hydroxide gradients can be carefully optimized to permit a baseline separation between diphosphate and IMP. Yet, a co-elution of inorganic polyphosphates with other nucleotides still cannot be completely ruled out. Hence, positive results should be confirmed by monitoring the peak ratio between the conductivity and the UV signal in order to detect possible co-eluting nucleotides.

The available data permit the conclusion that inorganic di- and triphosphates (limit of detection $< 0.5\,\mathrm{mg\,kg^{-1}}$) are neither naturally present in fish nor the product of a degradation reaction during storing/transporting of the animal/tissue or a processing of the sample. Traces of inorganic polyphosphates are to be considered as an indication that the sample has undergone a previous treatment with polyphosphates.

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