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Polyphosphate Determination in Seafood and Processed Cheese using High-Performance Anion Exchange Chromatography after Phosphatase Inhibition using Microwave Heat Shock*

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Introduction

Polyphosphates are often used as additives in various foodstuffs such as fish, meat, and processed cheese for their sequestering (or chelating), dispersing or water binding properties. According to the current Swiss legislation (1), the highest amounts of phosphates allowed in fillets of unprocessed fish, frozen and deep frozen, fish paste and cheese products (processed cheese) are 5 g/kg (1 g/kg for Surimi) and 30 g/kg respectively expressed as P_2O_5. Although several analytical methods (2–5) have been developed for isolating these compounds from foods, difficulties may arise when applying them to biological materials or food matrices containing active phosphatase. In aqueous media, even at neutral pH-values, this enzyme 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 by means of a microwave oven has been tested, followed by a simple and rapid high-performance anion exchange chromatography (IEC) procedure. This analytical method makes it possible to separate linear sodium polyphosphates (food additive E452) (1), monophosphate, diphos-

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phate (pyrophosphate; food additive E450) (1), triphosphate (tripolyphosphate; food additive E451) (1) as well as cyclic polyphosphate. The objective of the present study is to test the efficiency of this heat treatment as well as to validate the analytical method proposed.

Principle of the method

Polyphosphates are soluble in water and are therefore easily extractable with water after homogenisation. The extraction process should be carried out with water only, i.e. without any trichloracetic acid. On the one hand such a simple, rapid and easy procedure avoids the large peak of trichloracetate in the IEC chromatogram which may overlap phosphate components, but on the other hand does not ensure the chemical denaturation of the phosphatase.

Different techniques have been compared for inhibiting this enzyme without introducing a new peak in the chromatogram. This could theoretically be done by treating the samples at a high pH-value or by using a hot water bath. The practical results obtained were however not satisfactory already indicating a hydrolysis of the polyphosphates a few minutes after the extraction with water. Moreover high temperature as well as low and high pH-values can also accelerate the hydrolysis of these components.

The proposed flash heat treatment using a microwave oven is a convenient solution to this problem because both the acidic and alkaline phosphatase of the extract are sufficiently rapidly inactivated. Thereafter, the (poly)phosphates can be extracted with water, separated from the matrix by centrifugation, filtered on a Millipore filter, and injected onto an ion exchange column. The quantification is performed by external standard. This method, which gives both qualitative and quantitative results, uses high performance ion exchange chromatography with conductometric detection.

Experimental

Chemicals and reagents

Deionised water with a resistivity $>15.0\ M\Omega \cdot cm$ from a Millipore (Bedford, MA, USA) Milli-Q-water purification system was used to prepare all reagents and standards. The following standards were used as references: sodium phosphate (tribasic), sodium diphosphate (pyrophosphate), sodium triphosphate (tripolyphosphate) and sodium trimetaphosphate (cyclic) from Plüss-Staufer, Oftringen (Switzerland).

Microwave oven

The microwave treatment was carried out with 20 % power in a MDS-2100 oven (CEM Corporation) which delivered approximately 950 W at 2450 MHz at full power.

Sample selection

Various samples of scampis, shrimps, calmars and processed cheeses were procured from a local market.

Sample treatment and extraction

After homogenisation of the whole sample (100–150 g) with a laboratory blender, an aliquot part of 3.0 g was weighted (±1 mg), 3.0 g water was added to the sample in a 100 ml glass centrifuge tube and the diluted sample was submitted to shock heat treatment at atmospheric pressure using the microwave oven for 1 min at 20% of its full power. The mixture was cooled in an ice bath. A volume of 44 ml of water was added. The mixture was homogenised using a Polytron tissue macerator running at 10000 rpm for 1 min and centrifuged at 5000 rpm for 5 min to separate the matrix. Finally 1 ml of supernatant was taken with a pasteur pipette and filtered through 0.20 µm Millex (supplied from Millipore) filter. The filtrate was diluted fivefold and injected within two hours.

Ion exchange chromatography

The chromatographic system was a Dionex DX-320 EGC-OH cartridge, including an IC 20 conductivity detector, and an Anion Self-Regenerating Suppressor (ASRS) operated in the autosuppression recycle mode. The operating conditions were as follows: The suppressor current was set at 500 mA. A 25 µl loop was used. The initial pressure was 120 bar at room temperature. The separation column was an IonPac AS16 (4×250 mm I.D.) with 4 µm packing; the guard column was an IonPac AG16 (4×50 mm) and the trap column an ATC-1 (9×24 mm). The mobile phase was a potassium hydroxide solution used with an elution gradient (table 1) using an EG40 Eluent Generator. The eluent flow rate was set at 1 ml/min.

Table 1
Elution gradient

Time (min)	*Concentration of potassium hydroxide (mmol/l)*
Initial*	80.0
0.00	30.0
5.00	30.0
5.50	30.0
14.00	80.0
18.00	80.0

*) Remaining from the previous injection

The data acquisition and instrument control was performed with the Dionex Software PeakNet program.

Qualitative and quantitative analysis

The identification was carried out by comparing the retention times of the various phosphates analysed with those of standards. Quantitative determinations were made using an external standard curve. The calibration curves were obtained by injection of mixed standard solutions of sodium monophosphate (tribasic), diphosphate (pyrophosphate), triphosphate (tripolyphosphate) and trimetaphosphate (cyclic) in water at three concentrations (5.0, 10.0 and 20.0 mg/l) under the same conditions as those used for the samples analysed (fig. 1).

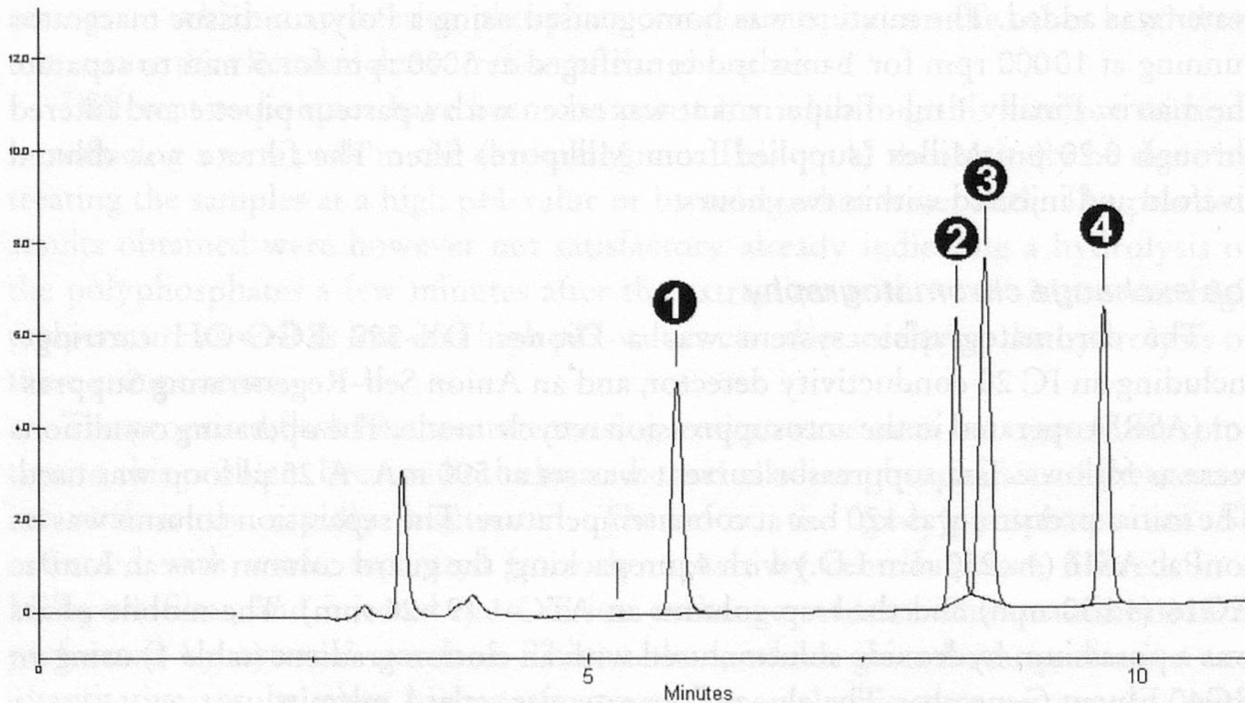

Figure 1 **IE-chromatogram of a standard solution containing 10 mg/l of sodium monophosphate (5.9 min; peak 1), diphosphate (8.4 min; peak 2), trimetaphosphate (8.7 min; peak 3) and triphosphate (9.7 min; peak 4)** (for working conditions see text)

Results and discussion

Inhibition of phosphatase

Without heat shock treatment an extensive hydrolysis of sodium diphosphate and sodium triphosphate was observed (sample with 1000 mg/kg polyphosphates added). The peak of sodium trimetaphosphate (cyclic) was not changed. On the other hand, experiments showed the inhibitory effect of the proposed heat shock on the enzyme and the corresponding stability of polyphosphates during the extraction and clean-up step (fig. 2).

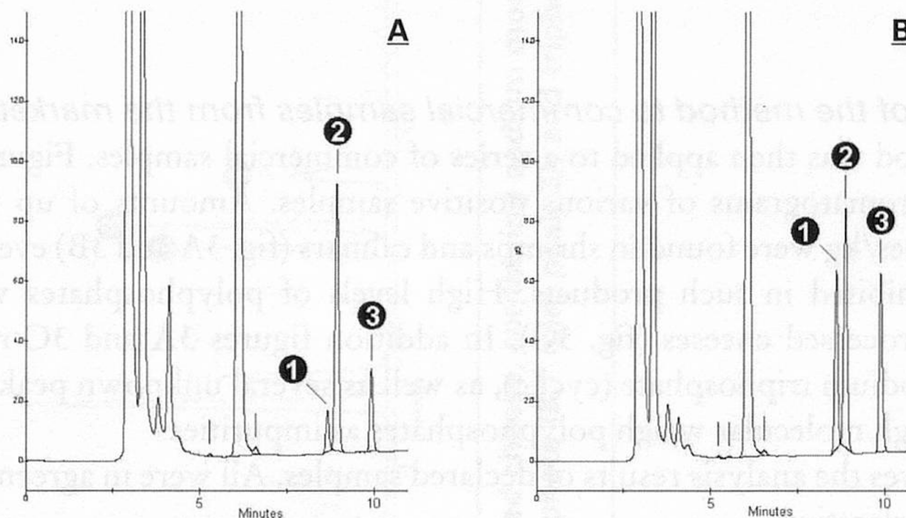

Figure 2 **Polyphosphate stability (sample fortified with 1000 mg/kg) and effect on sodium diphosphate (8.4 min; peak 1), trimetaphosphate (8.7 min; peak 2) and triphosphate (9.7 min; peak 3) without (A) and with (B) phosphatase inactivation with a microwave heat shock** (for working conditions see text)

Linearity of the response and detection limit

The linearity range was investigated using mixed standard solutions within a dilution series. The response of polyphosphates was linear at least from 0.5 to 25 mg/l with a correlation coefficient $r^2>0.99$ for a linear least square fit (linear regression) (data not shown).

The detection limit (signal/noise ration >3) was found at 50 mg/kg for every food investigated.

Recovery rate and repeatability

The recovery rate was determined with scampi samples to which three different concentrations of polyphosphates (500, 1000 and 2000 mg/kg) had been added in five replicates for each concentration level tested. Most recoveries are >80%. The higher the concentration, the better the extraction rate (table 2).

Table 2
Recovery rate and *coefficient of variation* (%) determined in scampis (*n*=5 replicates)

Polyphosphates in	*Recovery rate (%)*			*Coefficient of Variation (%)*		
fortified sample (mg/kg)	*500*	*1000*	*2000*	*500*	*1000*	*2000*
Diphosphate	71.6	81.5	88.1	5.2	5.5	3.6
Trimetaphosphate	93.4	97.3	96.2	5.1	3.8	3.1
Tripolyphosphate	82.7	90.4	88.2	1.9	4.2	1.2

The repeatability determined using the variation coefficient of five replicates was <6 %.

Application of the method to commercial samples from the market

The method was then applied to a series of commercial samples. Figure 3 presents some chromatograms of various positive samples. Amounts of up to 3.00 g polyphosphates/kg were found in shrimps and calmars (fig. 3A and 3B) even though they are prohibited in such products. High levels of polyphosphates were also detected in processed cheeses (fig. 3C). In addition figures 3A and 3C reveal the presence of sodium triphosphate (cyclic), as well as several unknown peaks, probably further high molecular weigh polyphosphates as impurities.

Table 3 gives the analysis results of declared samples. All were in agreement with the sample declaration.

Table 3
Results of analysis of declared samples (mg/kg)

Sample	*Declared*	*Origin*	*Di-phosphate*	*Trimeta-phosphate*	*Tri-phosphate*
Raw, whole, frozen shrimp	without polyphosphates	Madagascar	neg.	neg.	neg.
Raw, whole, frozen shrimp	without polyphosphates	Madagascar	neg.	neg.	neg.
Raw, whole, frozen shrimp	without polyphosphates	Nigeria	neg.	neg.	neg.
Whole, not washed calmar	without polyphosphates	Spain	neg.	neg.	neg.
Whole washed calmar	without polyphosphates	Spain	neg.	neg.	neg.
Rings of calmar	with polyphosphates	Spain	neg.	150	1029

neg. = negative sample

Conclusion

The combination of a rapid microwave oven heat treatment to inhibit phosphatase, a simple extraction and clean-up procedure, and ion exchange chromatography including an elution gradient, an anion self regenerating suppressor and conductivity detection was found to be a convenient tool for routine quality control of phosphates and polyphosphates in various food. The detection limit is approximately 50 mg/kg sample.

As regards the quality assurance of the laboratory, the analytical procedure was validated according to ISO 17025, where certified standards were included in each analytical run.

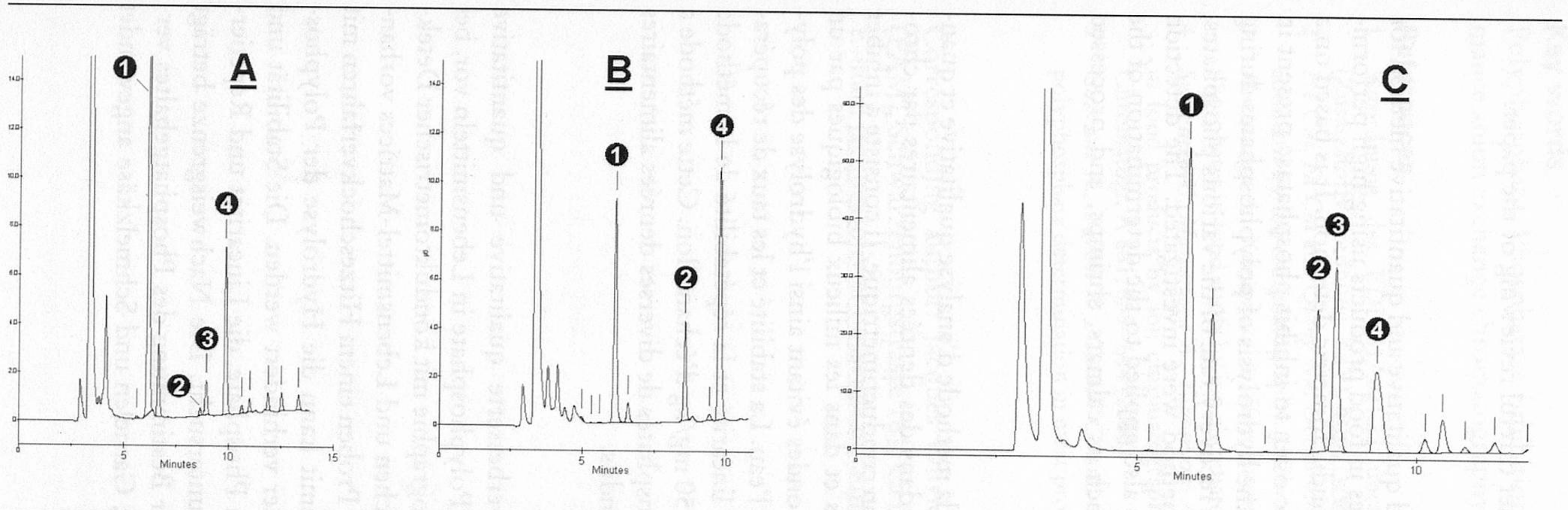

Figure 3 **IE-Chromatogram of positive samples: A) calmars, B) shrimps and C) processed cheese, containing sodium monophosphate (5.9 min; peak 1), diphosphate (8.4 min; peak 2) and trimetaphosphate (8.7 min; peak 3), and triphosphate (9.7 min; peak 4)** (for working conditions see text)

Acknowledgement

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Summary

The current report presents an improved qualitative and quantitative method for determining phosphates and polyphosphates in food products using high performance ion exchange chromatography with conductometric detection. It is based on a thermal shock treatment using a microwave oven to inhibit phosphatase present in biological and food matrices thus avoiding the hydrolysis of polyphosphates during their water extraction. The stability and the recovery rate of the various phosphates, the linearity and the repeatability of the method were investigated. The detection limit is 50 mg/kg sample. This method was also applied to the determination of the content of phosphates in several foods such as calmars, shrimps, and processed cheese.

Résumé

Ce travail présente une amélioration de la méthode d'analyse qualitative et quantitative des phosphates et polyphosphates dans des denrées alimentaires par chromatographie d'échange d'ions avec détection conductimétrique. Il consiste à inhiber la phosphatase présente dans ces dernières et dans les milieux biologiques par un choc thermique à l'aide d'un four à micro-ondes évitant ainsi l'hydrolyse des polyphosphates pendant la phase d'extraction à l'eau. La stabilité et les taux de récupération des divers phosphates, le domaine de linéarité et la répétabilité de la méthode ont été testés. La limite de détection est de 50 mg/kg d'échantillon. Cette méthode a été appliquée au dosage de la teneur en phosphates de diverses denrées alimentaires telles que calmars, crevettes et fromages fondus.

Zusammenfassung

Die vorliegende Arbeit stellt eine verbesserte qualitative und quantitative Bestimmungsmethode für Phosphate und Polyphosphate in Lebensmitteln vor, bei der Hochdruck-Ionenaustausch-Chromatographie mit konduktometrischer Detektion angewendet wird. Um die in biologischen und Lebensmittel-Matrices vorhandene Phosphatase zu hemmen, werden die Proben einem Hitzeschockverfahren mit einem Mikrowellenofen unterworfen. Damit kann die Hydrolyse der Polyphosphate während ihrer Extraktion mit Wasser verhindert werden. Die Stabilität und die Wiederfindungsrate der verschiedenen Phosphate, die Linearität und Repetierbarkeit der Methode wurden eingehend untersucht. Die Nachweisgrenze beträgt 50 mg/kg Probe. Diese Methode kann zur Bestimmung des Phosphatgehaltes verschiedener Lebensmittel wie Tintenfische, Garnelen und Schmelzkäse angewendet werden.

Key words
Polyphosphate, Phosphatase inhibition, Seafood, Processed cheese, High performance anion exchange chromatography

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