Determination of Mono-, Di-, and Triphosphates and Citrate in Shrimp by Ion Chromatography

Chanita Chantarasukon,[1] Suparerk Tukkeeree,[1] and Jeff Rohrer[2]
[1]Thermo Fisher Scientific, Bangkok, Thailand; [2]Thermo Fisher Scientific, Sunnyvale, CA, USA

Application Note 1007

Key Words
Reagent-Free IC (RFIC), Food, Dionex IonPac AS11 Column, Dionex InGuard HRP Cartridges, Seafood

Introduction

Polyphosphates are legally added to some food products such as meat, fish, and seafood. In the seafood industry, polyphosphates are used in both fresh and frozen products to increase their water-binding capacity. This improves the appearance and texture of the product and also increases the weight of the seafood. Polyphosphates are legal, but some countries require that polyphosphate usage be declared and may also limit the amount that can be added. For example, Switzerland has set the maximum concentration at 5 g per kg. The polyphosphates typically used in foods such as shrimp are small, containing two or three phosphate units, and they are often hydrolyzed to orthophosphate before the food is eaten. Citric acid is sometimes added to shrimp as a preservative to maintain an acidic environment.

Ion chromatography (IC) is commonly used to determine both large (>3 phosphates) and small polyphosphates, as well as citrate. An early study used a Thermo Scientific Dionex IonPac AS7 Anion-Exchange Column to determine triphosphosphate and its hydrolysis products in shrimp.[1] This method used a nitric acid eluent and a postcolumn reaction with ferric nitrate to produce UV-absorbing products for phosphate-containing analytes, rather than suppressed conductivity detection. Since then, Dionex (now part of Thermo Scientific) Application Note 71 demonstrated that polyphosphates can be separated using a hydroxide eluent gradient on the Dionex IonPac™ AS11 Hydroxide-Selective Anion-Exchange Column and detected by suppressed conductivity.[2] More recently, Dionex (now part of Thermo Scientific) Application Update (AU) 172 showed that the Dionex IonPac AS16 Hydroxide-Selective Anion-Exchange Column was a better choice for separating large polyphosphates. Furthermore, AU 172 demonstrated that if the sample did not have larger polyphosphates or they did not need to be measured, the method could be paired with an eluent generator (i.e., a Reagent-Free™ IC [RFIC™] system) to free the analyst from preparing hydroxide eluents and achieve consistent results.[3]

The introduction to AU 172 describes the application of IC to determining polyphosphates in a variety of samples, including fish and shell fish, using the Dionex IonPac AS11 and AS16 columns as well as the Dionex IonPac AS11-HC column. Two of those publications that focused on fish and shrimp were from Swiss government laboratories, each using the Dionex IonPac AS16 column and a hydroxide gradient with suppressed conductivity detection, and one using an eluent generator.[4,5]

Because the goal here was to measure only the small polyphosphates, an IC method was developed that used an eluent generator. Thus, the analyst must only add deionized water to the system to prepare the eluents necessary for accurately measuring small polyphosphates and citrate in shrimp. Additionally, the sample pretreatment required for this analysis to maintain good chromatography and extend column lifetime has been automated so that it is done on-line using a Thermo Scientific Dionex InGuard cartridge.

Goal

To determine small polyphosphates and citrate in shrimp using IC

Thermo SCIENTIFIC

Equipment

- Thermo Scientific Dionex ICS-3000 system* including:
  - DP Dual Pump
  - DC Detector Chromatography Compartment
  - EG Eluent Generator
  - AS Autosampler with Cooling Option
  - Dionex InGuard™ HRP Cartridges, package of 4 (P/N 074034)
  - EWP Electrolytic Water Purifier (P/N 071553)
- PC-100 Pump Controller (Trovion P/N 590100)
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 6.80, SR9 or above

*A Thermo Scientific Dionex ICS-5000 or any RFIC system may also be used.

Reagents and Standards

- Deionized (DI) water, Type I, reagent grade, 18 MΩ-cm resistivity or above
- Trisodium orthophosphate ($Na_3PO_4 \cdot 12H_2O$, 98.0–102%, AR grade, Ajax Finechem)
- Citric acid ($C_6H_8O_7 \cdot H_2O$, 99.0–102.0%, AR grade, Ajax Finechem)
- Tetrasodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$, 99.0–103%, AR grade, Ajax Finechem)
- Trisodium trimetaphosphate ($Na_3O_9P_3$, 100%, AR grade, Sigma-Aldrich®)
- Sodium triphosphate pentabasic ($Na_5O_{10}P_3$, ≥ 98.0%, AR grade, Fluka)
- Potassium hydroxide solution, 8 M (KOH, AR grade, Kanto)

Preparation of Solutions and Reagents

Eluent

The eluent generator (EG) produces the eluent using the Thermo Scientific Dionex EGC II KOH EluGen cartridge and DI water supplied by the pump. The eluent concentration is controlled by Chromeleon™ software. The EG degasser requires 14 MPa (2000 psi) system backpressure, which ensures optimum removal of electrolytic gas produced by the EG cartridge. For more information about adding system backpressure, refer to the ICS-3000 systems operator's manual, Document No. 065031, or any other RFIC system operator's manual.

Stock Standard Solutions, 1000 mg/L

Dissolve the appropriate weight of the salt listed in Table 1 into separate 100 mL volumetric flasks with DI water. Add 0.25 ml of 8 M KOH and bring to the volume with DI water.

Table 1. Preparation of 100 mL of 1000 mg/L standard

Standard	Salt	Weight (g)
Orthophosphate	Trisodium orthophosphate	0.400
Citrate	Citric acid	0.109
Pyrophosphate	Tetrasodium pyrophosphate	0.268
Trimetaphosphate	Trisodium trimetaphosphate	0.129
Triphosphate	Sodium	0.145

Table 2. Preparation of working standards

Level	Concentration (mg/L)	Volume of 1000 mg/L Stock Standard Solution for 100 mL Preparation (mL)
1	0.5	0.05
2	1.0	0.10
3	2.0	0.20
4	3.0	0.30

Working Standard Solutions

Add the appropriate volume of each 1000 mg/L stock standard solution into separate 100 mL volumetric flasks, add 0.25 mL of 8 M KOH, and bring to volume with DI water. Table 2 lists working standard concentrations and the volumes of 1000 mg/L stock standard solution used for each concentration level.

Sample Preparation

Puree the shrimp sample and weigh 0.2 g of the sample into a 100 mL bottle. Add 99.75 mL of DI water and 0.25 mL of 8 M KOH, shake, and place in ultrasonic bath for 5 min. Filter with a 0.45 µm syringe filter prior to injection.

Chromatographic Conditions	
Column:	Dionex IonPac AS11 Analytical, 4 × 250 mm (P/N 044076)
Guard:	Dionex IonPac AG11 Guard, 4 × 50 mm (P/N 044078)
Cartridge:	Dionex InGuard HRP, 9 × 24 mm (P/N 074034)
Trap Column:	Dionex IonPac UTAC-LP1 Ultra Trace Anion Concentrator - Low Pressure, 4 × 35 mm (P/N 063079)
Eluent Source:	Dionex EGC III KOH EluGen™ potassium hydroxide (P/N 074532) with Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
Gradient:	See Table 3
Flow Rate:	1.0 mL/min
Inj. Volume:	25 µL
Pressure:	~2000 psi
Sample Tray Temp.:	10 °C
Column Temp.:	35 °C
Detection:	Suppressed conductivity with Thermo Scientific Dionex ASRS 300 Anion Self-Regenerating Suppressor, 4 mm (P/N 064554), external water mode, current 130 mA

Results and Discussion

The on-line sample treatment is performed using a Dionex InGuard HRP cartridge. The RFIC system passes the prepared shrimp samples through the Dionex InGuard HRP cartridge to trap hydrophobic compound that can foul the analytical column. Orthophosphate, pyrophosphate, trimetaphosphate, triphosphate, and citrate are then separated on the Dionex IonPac AS11 column and detected by suppressed conductivity in 15 min. Overall, this is an efficient way to determine these compounds in shrimp as it eliminates time-consuming off-line sample preparation.

Separation

Citrate, phosphate, and polyphosphates are anions that are strongly retained on moderate or high-capacity anion-exchange columns. Thus, a high eluent concentration or long run time will be required to elute them from the column. For these analytes, it is often advantageous to use a low-capacity column designed for fast elution of species that are strongly retained on many anion-exchange columns, such as the Dionex IonPac AS11 column. Phosphate species and citrate are separated using a gradient of potassium hydroxide eluent with the highest eluent concentration, 50 mM, easily suppressed for conductivity detection. Common and other anions that are mono- and divalent elute before the phosphate species and citrate, and thus do not interfere with their determination. Figure 1 shows the separation of phosphate species and citrate.

Method Calibration

The method was calibrated before sample analysis using four different concentrations and three injections of each concentration. It is difficult to obtain polyphosphate in a pure form. Phosphate species that have lower numbers of phosphate units are typically present, so it is better to calibrate the method using a single-component working standard. For this application, working standards were prepared in 20 mM potassium hydroxide. This slows the hydrolysis of the polyphosphate species and also stops growth of microorganisms that can degrade the standards (because phosphate is a necessary nutrient for microorganisms). Table 4 shows the calibration results for the five standard analytes.

Table 3. Gradient program

Time (min)	KOH Conc. (mM)
-5.0	30
3.0	30
3.1	45
7.0	45
7.5	50
12.9	50
13.0	30

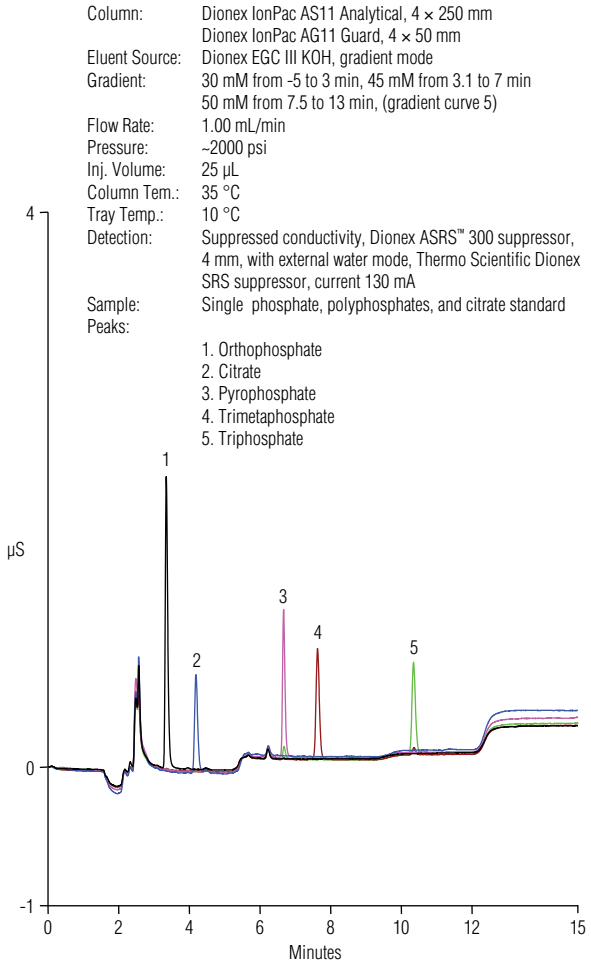


Figure 1. Overlay of chromatograms of single standard injections of a phosphate, three polyphosphates, and citrate

Table 4. Calibration standard concentrations and calibration results

Analyte	Concentration (mg/L)				Calibration Results			
	Level 1	Level 2	Level 3	Level 4	Points	r^2	Offset	Slope
Orthophosphate	0.5	1.0	2.0	3.0	12	0.9989	0.0091	0.0820
Citrate	0.5	1.0	2.0	3.0	12	0.9992	-0.0024	0.0725
Pyrophosphate	0.5	1.0	2.0	3.0	12	0.9998	-0.0100	0.0969
Trimetaphosphate	0.5	1.0	2.0	3.0	12	0.9999	-0.0079	0.0981
Triphosphate	0.5	1.0	2.0	3.0	12	0.9994	-0.0092	0.0883

Sample Analysis

A shrimp sample was purchased for analysis from a local supermarket in Bangkok, Thailand. The sample was prepared as described and injected five times into the IC system. The sample was loaded into the sample loop, then flushed through the Dionex InGuard HRP cartridge. Anionic species, including phosphate and citrate, were trapped on the concentrator. While the sample was flowing through the Dionex InGuard cartridge, fat, protein, and hydrophobic species were trapped on the cartridge.

Figure 2 shows the system configuration. The initial work did not use the Dionex InGuard cartridge and retention times were significantly reduced after 300 sample injections. Dionex InGuard cartridges can trap matrix compounds before they reach the column, preventing loss of column capacity. Figure 3 shows the overlay of chromatograms of the first spiked sample injection and 300th spiked sample injection without using the Dionex InGuard cartridge. Figure 4 shows the overlay of chromatograms of spiked sample injection with the same injection number using the Dionex InGuard cartridge. The chromatography results observed in Figures 3 and 4 are quantified in Table 5, which shows the retention times of the first and 300th sample injection with and without using the HRP cartridge. This demonstrates that use of the cartridge protected the column and preserved column capacity. Please note that with the Dionex InGuard cartridge in line, the starting retention time is longer due to the time required for the analytes to pass through the cartridge.

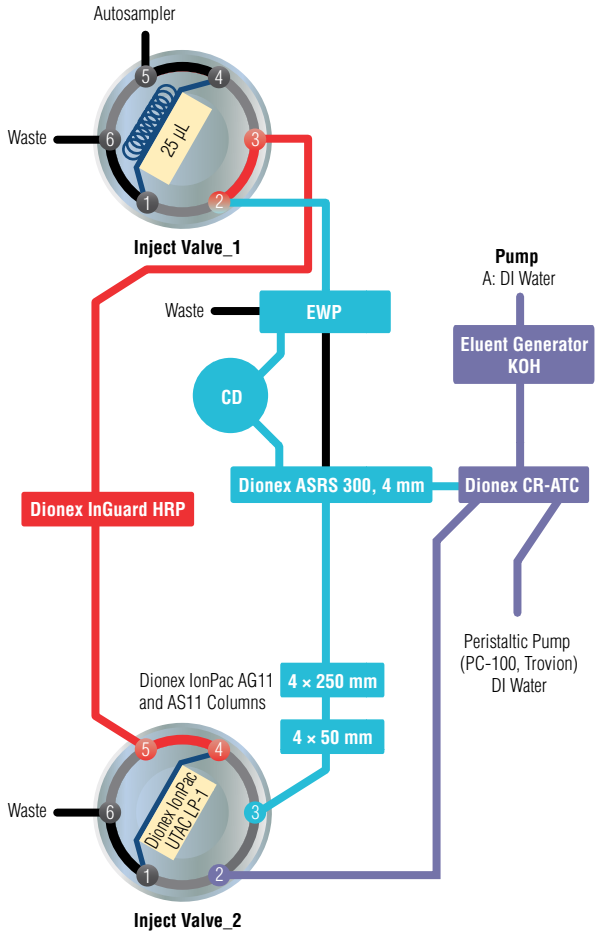
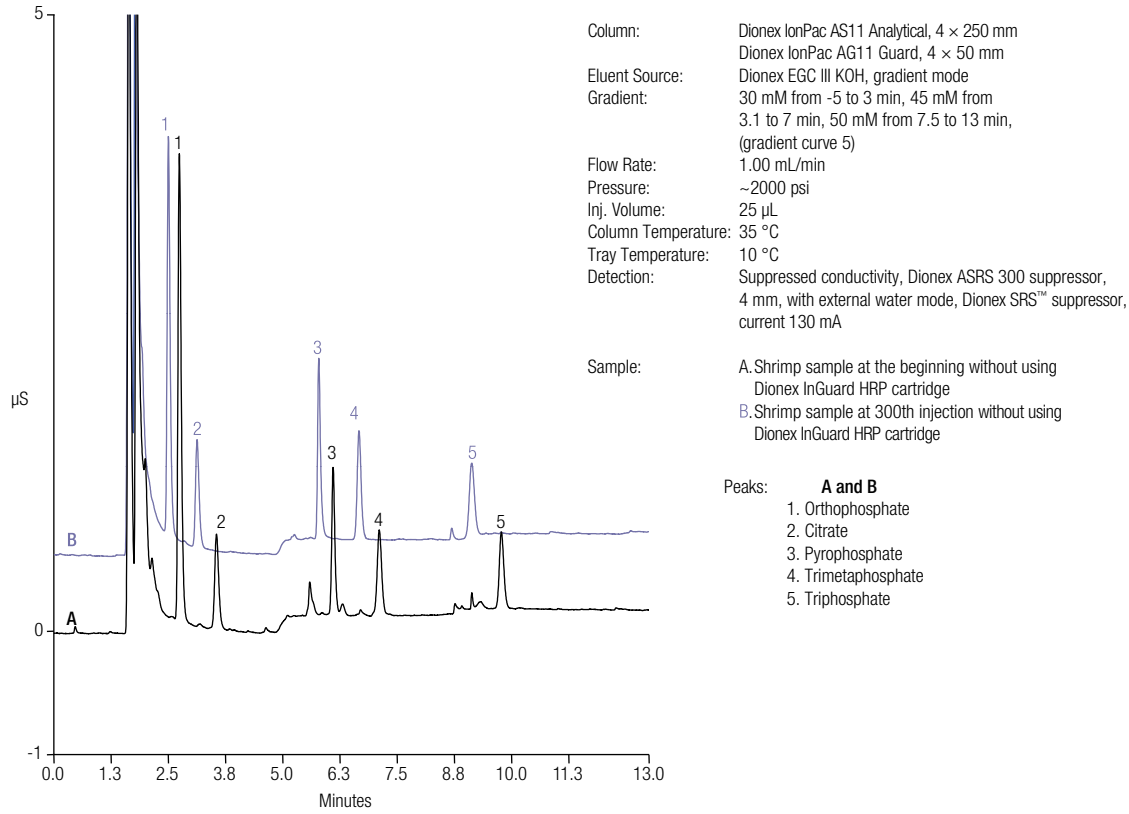


Figure 2. System configuration

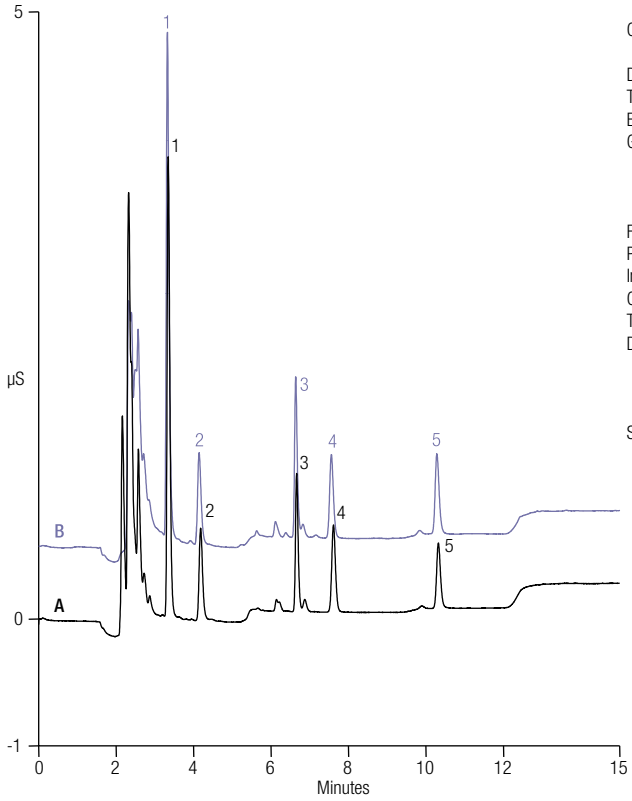


Column: Dionex IonPac AS11 Analytical, 4 × 250 mm
Dionex IonPac AG11 Guard, 4 × 50 mm
Eluent Source: Dionex EGC III KOH, gradient mode
Gradient: 30 mM from -5 to 3 min, 45 mM from 3.1 to 7 min, 50 mM from 7.5 to 13 min, (gradient curve 5)
Flow Rate: 1.00 mL/min
Pressure: ~2000 psi
Inj. Volume: 25 µL
Column Temperature: 35 °C
Tray Temperature: 10 °C
Detection: Suppressed conductivity, Dionex ASRS 300 suppressor, 4 mm, with external water mode, Dionex SRS™ suppressor, current 130 mA

Sample:
A. Shrimp sample at the beginning without using Dionex InGuard HRP cartridge
B. Shrimp sample at 300th injection without using Dionex InGuard HRP cartridge

Peaks: **A and B**
1. Orthophosphate
2. Citrate
3. Pyrophosphate
4. Trimetaphosphate
5. Triphosphate

Figure 3. Overlay of chromatograms of a shrimp sample at the first and 300th injection without using a Dionex InGuard HRP cartridge



Column: Dionex IonPac AS11 Analytical, 4 × 250 mm
Dionex IonPac AG11 Guard, 4 × 50 mm
Dionex InGuard Cartridge: HRP, 9 × 24 mm
Trap Column: Dionex IonPac UTAC-LP1, 4 × 35 mm
Eluent Source: Dionex EGC III KOH, gradient mode
Gradient: 30 mM from -5 to 3 min
45 mM from 3.1 to 7 min
50 mM from 7.5 to 9.9 min
80 mM from 10 to 15 min (gradient curve 5)
Flow Rate: 1.00 mL/min
Pressure: ~2300 psi
Inj. Volume: 25 µL
Column Temperature: 35 °C
Tray Temperature: 10 °C
Detection: Suppressed conductivity, Dionex ASRS 300 suppressor, 4 mm, with external water mode, Dionex SRS suppressor, current 130 mA

Sample:
A. Shrimp sample at the beginning using Dionex InGuard HRP cartridge
B. Shrimp sample at 300th injection using Dionex InGuard HRP cartridge

Peaks: **A and B**
1. Orthophosphate
2. Citrate
3. Pyrophosphate
4. Trimetaphosphate
5. Triphosphate

Figure 4. Overlay of chromatograms of a shrimp sample at the first and 300th injection using a Dionex InGuard HRP cartridge

Table 5. Retention time of the analytes at the first and 300th injection with and without using the Dionex InGuard cartridge

Analyte	Retention Time Without Cartridge (min)		Retention Time With Cartridge (min)	
	First Sample Injection	300th Sample Injection	First Sample Injection	300th Sample Injection
Orthophosphate	2.740	2.500	3.343	3.324
Citrate	3.553	3.127	4.180	4.140
Pyrophosphate	6.100	5.787	6.660	6.637
Trimetaphosphate	7.107	6.667	7.610	7.557
Triphosphate	9.777	9.130	10.320	10.280

The Dionex InGuard cartridge will not provide protection indefinitely, so when retention times start to drop, replace the cartridge column to avoid further capacity loss of the Dionex IonPac AS11 column. At this time, it is also advisable to perform a strong column wash, as instructed in the Dionex IonPac AS11 column manual. When not using an RFIC system, retention time loss will be difficult to judge because some differences in eluent concentration are unavoidable for hydroxide eluents, due to variable amounts of carbonate contamination.

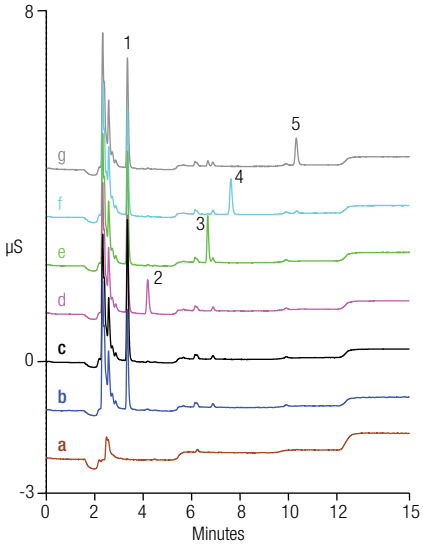
Table 6 shows that only orthophosphate was found in the sample at a concentration of 2.16 mg/L. The RSD of this determination was 0.51% (5 injections). The method accuracy was evaluated by preparing the sample with the addition of known amounts of each phosphate species and citrate. Then each sample was injected five times. The measured concentration of each analyte was compared to the added concentration to judge recovery. The recovery results were between 91.9 and 116%, with good reproducibility (Table 7). Figure 5 shows an overlay of chromatograms from this study, along with an injection of a 20 mM KOH blank.

Table 6. Amount of each phosphate species and citrate in a shrimp sample

Injection No.	Amount (mg/L)				
	Orthophosphate	Citrate	Pyrophosphate	Trimetaphosphate	Triphosphate
1	2.17	—	—	—	—
2	2.17	—	—	—	—
3	2.15	—	—	—	—
4	2.15	—	—	—	—
5	2.15	—	—	—	—
Average	**2.16**	—	—	—	—
RSD	**0.51**	—	—	—	—

Table 7. Recovery results for compounds spiked into sample shown in Table 6

Injection No.	Amount (mg/L)				
	Orthophosphate	Citrate	Pyrophosphate	Trimetaphosphate	Triphosphate
	(Spiked 1.0 mg/L)	(Spiked 1.0 mg/L)	(Spiked 1.0 mg/L)	(Spiked 1.0 mg/L)	(Spiked 1.0 mg/L)
1	3.14	1.16	0.967	1.01	0.917
2	3.13	1.16	0.966	1.00	0.919
3	3.14	1.16	0.966	1.00	0.921
4	3.14	1.16	0.967	1.00	0.919
5	3.14	1.16	0.967	1.00	0.919
Average	**3.14**	**1.16**	**0.967**	**1.00**	**0.919**
RSD	**0.12**	**0.09**	**0.050**	**0.21**	**0.160**
Recovery (%)	**98.10**	**116.00**	**96.70**	**100.00**	**91.90**



Column:	Dionex IonPac AS11 Analytical, 4 × 250 mm, Dionex IonPac AG11 Guard, 4 × 50 mm
Dionex InGuard Cartridge:	HRP, 9 × 24 mm
Trap Column:	Dionex IonPac UTAC-LP1, 4 × 35 mm
Eluent Source:	Dionex EGC III KOH, gradient mode
Gradient:	30 mM from -5 to 3 min, 45 mM from 3.1 to 7 min, 50 mM from 7.5 to 9.9 min 80 mM from 10 to 15 min, (gradient curve 5)
Flow Rate:	1.00 mL/min
Pressure:	~2300 psi
Inj. Volume:	25 µL
Column Temperature:	35 °C
Tray Temperature:	10 °C
Detection:	Suppressed conductivity, Dionex ASRS 300 suppressor, 4 mm, with external water mode, Dionex SRS suppressor, current 130 mA
Sample:	a. Blank b. Shrimp sample c. Spiked orthophosphate (1) in shrimp sample d. Spiked citrate (2) in shrimp sample e. Spiked pyrophosphate (3) in shrimp sample f. Spiked trimetaphosphate (4) in shrimp sample g. Spiked triphosphate (5) in shrimp sample

Figure 5. Overlay of chromatograms of blank; shrimp sample; and the shrimp sample spiked with orthophosphate, citrate, pyrophosphate, trimetaphosphate, and triphosphate, respectively

Conclusion

This work shows an accurate IC method for determination of mono-, di-, and triphosphate species and citrate in shrimp. The sample is treated on-line using the Dionex InGuard HRP cartridge to reduce sample preparation time, error, and contamination. The eluent used in this application is produced by an eluent generator to preclude labor or error associated with eluent preparation. Overall, the method is labor efficient and accurate.

References

1. Heitkemper, D.T.; Kaine, L.A.; Jackson, D.S.; and Wolnik, K.A. In *Determination of Tripolyphosphate and Related Hydrolysis Products in Processed Shrimp*, Proceedings of the 18th Annual Tropical and Subtropical Fisheries Technology Conference, Williamsburg, Virginia, USA, Aug 29–Sept 1, 1993; publication number SGR114, 1994. [Online] www.dionex.com/en-us/webdocs/4166-AN71_LPN034629-03.pdf (accessed Mar 23, 2012).
2. Dionex (now part of Thermo Scientific) Application Note 71: Determination of Polyphosphates Using Ion Chromatography with Suppressed Conductivity Detection. LPN 034629-03, Sunnyvale, CA, 2002. [Online] www. dionex.com/en-us/webdocs/4166-AN71_V18.pdf (accessed Mar. 23, 2012).
3. Dionex (now part of Thermo Scientific) Application Update 172: Determination of Polyphosphates Using Ion Chromatography. LPN 2496, Sunnyvale, CA, 2010. [Online] www.dionex.com/en-us/webdocs/86232-AU172-IC-Polyphosphates-Food-13April2010-LPN2496.pdf (accessed Mar. 23, 2012).
4. Dafflon, O.; Scheurer, L.; Gobet, H.; Bosset, J. O. Polyphosphate Determination in Seafood and Processed Cheese using High-Performance Anion-Exchange Chromatography after Phosphatase Inhibition using Microwave Heat Shock. *Mitt. Lebensm. Hyg*. **2003**, *94*, 127–135.
5. Kaufmann, A.; Maden, K.; Leisser, W.; Matera, M.; Gude, T. Analysis of Polyphosphates in Fish and Shrimp Tissues by Two Different Ion Chromatography Methods: Implications on False-Negative and -Positive Findings. *Food Addit. Contam*. **2005**, *22*, 1073–1082.

U.S./Canada	(847) 295 7500	**Brazil**	(55) 11 3731 5140	**India**	(91) 22 2764 2735	**Singapore**	(65) 6289 1190
Australia	(61) 2 9420 5233	**China**	(852) 2428 3282	**Ireland**	(353) 1 644 0064	**Sweden**	(46) 8 473 3380
Austria	(43) 1 616 51 25	**Denmark**	(45) 36 36 90 90	**Italy**	(39) 02 51 62 1267	**Switzerland**	(41) 62 205 9966
Benelux	(31) 20 683 9768	**France**	(33) 1 39 30 01 10	**Japan**	(81) 6 6885 1213	**Taiwan**	(886) 2 8751 6655
	(32) 3 353 42 94	**Germany**	(49) 6126 991 0	**Korea**	(82) 2 3420 8600	**United Kingdom**	(44) 1276 691722

Thermo SCIENTIFIC

Part of Thermo Fisher Scientific

AN70068_E 05/12S