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Research Article

Simultaneous determination of several forms of phosphate in food by ion exchange chromatography

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Abstract

The phosphate group is a group of food additives commonly used for moisturizing, improving the structure, and retaining the color and flavor of products prepared from meat, fish, milk, baked goods, and beverages. A reliable and fast ion exchange chromatography method developed for the simultaneous determination of several forms of phosphate (*ortho*-phosphoric acid; pyrophosphate, triphosphate, and hexametaphosphate) in food. Samples were extracted with deionized water at a temperature of 25 ± 5°C for 30 minutes. The extracts were determined by ion exchange chromatography under the conditions: Dionex IonPac™ AS11 column (4 x 250 mm, 9 µm) and Dionex IonPax™ AG11 column (4 x 50 mm, 9 µm) with the gradient program of KOH concentration from 20 mM to 80 mM. The flow rate is 1 mL/min. The method has good specificity, the calibration curves of the four substances have correlation coefficient values $R^2 > 0.9999$, repeatability and recovery meet the requirements of AOAC. The detection limits (LOD) and quantification limit (LOQ) of each substance in the phosphate group are 12 mg/kg and 40 mg/kg, respectively. The method was applied to simultaneously determine several forms of phosphate in 50 food samples: 15/50 samples detected pyrophosphate, 2/50 samples detected triphosphate, 18/50 samples detected hexametaphosphate and 44/50 samples detected *ortho*-phosphoric acid, 6/50 samples not detected any form of phosphate. The concentration of phosphate group in detected samples varied from 67.9 mg/kg to 2499 mg/kg.

Keywords: *polyphosphate, phosphate, ion exchange chromatography*.

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1. INTRODUCTION

The phosphate group is an essential component in body, regularly appearing in the daily diet. It plays a role in the structure of nucleic acids, adenosine triphosphate, and phospholipids of membranes; as well as in cell signaling through phosphorylation reactions. For these reasons, phosphate is essential for life, and the uptake phosphate-containing products is important for all animals and human [1].

The phosphate content in industrially processed foods is much higher than in natural foods since phosphate groups are often used as additives in food manufacturing for technical purposes. In the European Union, sodium phosphate (E339), potassium phosphate (E340), calcium phosphate (E341), and salts of ortho-phosphoric acid, diphosphate (E450), triphosphate (E451) and polyphosphate (E452) are legally added to foods in the form of preservatives, acidifiers, acid buffers, and emulsifiers. Phosphate salts are also added to many kinds of foods as stabilizers or taste enhancers. Due to the high demand for use and highly available of these compounds in food products, therefore, 4 compounds (acid ortho–phosphoric, pyrophosphate, sodium triphosphate, and sodium hexametaphosphate) was selected to analyze in this study. Structural formulas of four types of the phosphate group are shown in Figure 1.

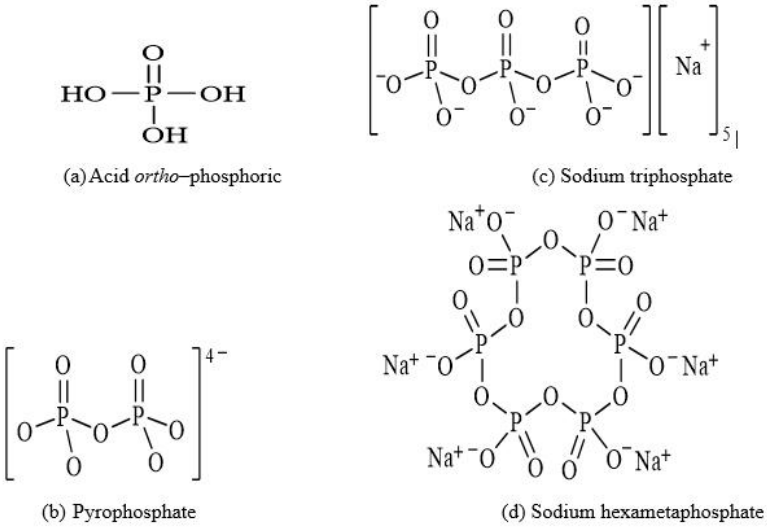

Figure 1. *Structural formulas of acid ortho–phosphoric (a), pyrophosphate (b), sodium triphosphate (c), and sodium hexametaphosphate (d)*

Phosphate group is also present in many kinds of foods, especially animal products, whole grains, and nuts. However, consuming excessively high levels of phosphate can lead to reduce calcium absorption, increasing the risk of osteoporosis and fractures [2], increasing the risk of cardiovascular diseases including coronary heart disease and hypertension [2]. Besides, it can cause endocrine dysfunction, such as diabetes, obesity, and metabolic disorders. In particular, previous studies [3, 4] also reported the kidney abnormal cases including a reduction in ability of renal filtration, dehydration and electrolyte dysregulation. Therefore, it is essential to evaluate concentration of phosphate additives in food and beverages.

Currently, methods have been developed to determine the content of various types of phosphate additives in food and beverages such as thin-layer chromatography (TLC) [5], capillary electrophoresis (CE) [6, 7], nuclear magnetic resonance spectroscopy (NMR) [8, 9], liquid chromatography (HPLC) [10], ion exchange chromatography (IC) [2, 11, 12, 13]... Among these methods, ion chromatography (IC) is highly considered for analyzing phosphate group anions in food because of its sensitivity, accuracy, and high recovery efficiency. This study aims to develop an IC method for simultaneously determining several forms of phosphate in food to control food quality.

2. MATERIALS AND METHODS

2.1. Standard and materials

Standard substances: tetrabasic sodium pyrophosphate standards (Sigma Aldrich, purity > 99%), sodium tripolyphosphate standards (Sigma Aldrich, purity > 85%), sodium hexametaphosphate standards (Sigma Aldrich, purity > 95%), *ortho*-phosphoric acid standards (Sigma Aldrich, purity > 99%); chemicals: Dionex EGC 500 KOH solution wash, deionized water.

Samples: canned meat, banh pho (fresh noodles), sausages, soft drinks, instant noodles, cereals, … were randomly collected in Hanoi markets.

2.2. Equipment

The ion exchange chromatography tandem with a conductivity detector (Dionex ICS 5000, Thermo), using the Dionex IonPac^{TM} AS11 column (4 x 250 mm, 9 µm) and the Dionex IonPax^{TM} AG11 column (4 x 50 mm, 9 µm) and other auxiliary equipment of the laboratory.

2.3. Method

2.3.1. Sample preparation

Weigh exactly 0.1 – 5.0 g of homogenized sample into a 50 mL falcon tube, add 25 mL of deionized water, then shake to reach well dispersion. The sample was ultrasonic at room temperature (25 ± 5°C) for 30 min. Transfer quantitatively the extract into a 50 mL volumetric flask. Repeat the process with another 20 mL of deionized water if necessary for example solid sample matrices. The solution is filtered through a 0.2 µm membrane into a 5 mL vial tube before injection into the ion exchange chromatography system.

2.3.2. Ion exchange chromatography conditions

Conditions on the ion exchange chromatography system with conductivity detector: flow rate: 1.0 mL/min, injection volume: 2.5 mL; mobile phase of KOH solution with concentration gradient mode: from -5.0 to 0.2 min at 20.0 mM, from 0.2 min to 17.0 min increased from 20.0 mM to 80 mM and kept for 1 minute. The gradient time is 18 minutes.

2.3.3. Method validation

The method was validated according to AOAC requirements: the specificity (using blank sample), calibration curves, detection limits (LOD) and quantitative limits (LOQ) determined by standard deviation of the test samples (n=10) at low-level concentration (pork meat sample), the precision of the method (repeatability), the trueness of the method (recovery) on spiked blank sample at (200 – 1000 mg) for 4 matrices including canned meat, pho cakes, soft drinks, sausages.

2.3.4. Application in real samples

Applying the developed method to analyze 50 real samples collected in the Hanoi market. The results are described in each substance form and then calculated to phosphorus to compare regulations of Circular No. 24/2019/TT-BYT. The content of the analyzed sample is calculated as follows:

$X = C \times V \times K/m$

In there: X: analyte content in the sample (mg/kg, mg/L); V: volume of final extract (mL); C: concentrations calculated from the standard curve (µg/mL); K: dilution factor and m: sample mass (g; mL).

3. RESULTS AND DISCUSSION

3.1. Ion exchange chromatography conditions optimization

The mobile phase is the decisive factor in the efficiency of separation. In general, the mobile phase can affect the selectivity, the retention time of the analyte, the width of the peaks, etc. Referring to reports [2, 11, 12, 13], some gradient programs were studied (Table 1).

The results are shown in Figure 2. When using the 1st gradient program, the peaks were not completely separated, and the peak of hexametaphosphate lengthen up to 50 minutes leading to time-consuming and solvent-wasting. When using the 2nd gradient program, the peaks could be well separated, but they were unsymmetric and jagged, while the baseline signal was quite high. When using the 3rd gradient program, the peaks were separated well, sharp, and low baseline signal. Therefore the 3rd gradient program was used for further investigation.

Table 1. *The investigated KOH concentration gradient programs*

Gradient program	Time (minutes)	Flow rate (mL/min)	C (mM)
1st gradient	-5.00	1.00	10.0
	0.00		10.0
	4.00		10.0
	6.00		20.0
	7.00		45.0
	16.00		45.0
	50.00		80.0
2nd gradient	-5.00	1.00	10.0
	0.00		10.0
	0.20		10.0
	20.0		80.0
	50.0		80.0
3rd gradient	-5.00	1.00	20.0
	0.00		20.0
	0.20		20.0
	17.0		80.0
	50.0		80.0

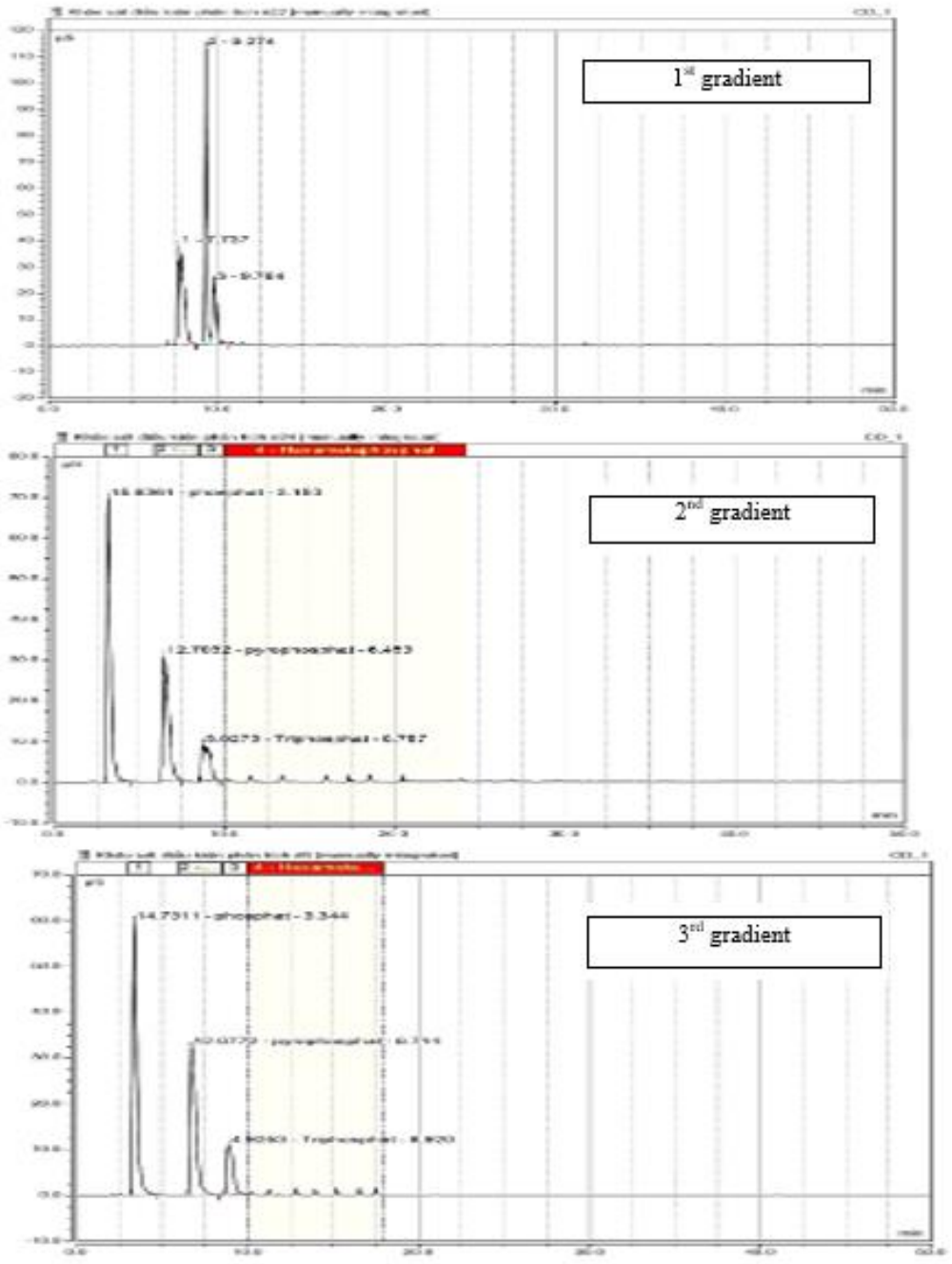

Figure 2. *Chromatography of standard solution*

3.2. Sample preparation optimization

3.2.1. Extraction technique

The extraction technique affects the ability to disperse and extract substances from the matrices. In this study, four different extraction methods: handshakers, horizontal shaker, vortex shaker, and ultrasonic extraction were examined at an extraction time of 30 minutes on raw meat samples. 2-3 g of pork sample were extracted in the difference extraction technique, other conditions were remained as described in 2.3.1 and compare the results. The results are shown in Figure 3.

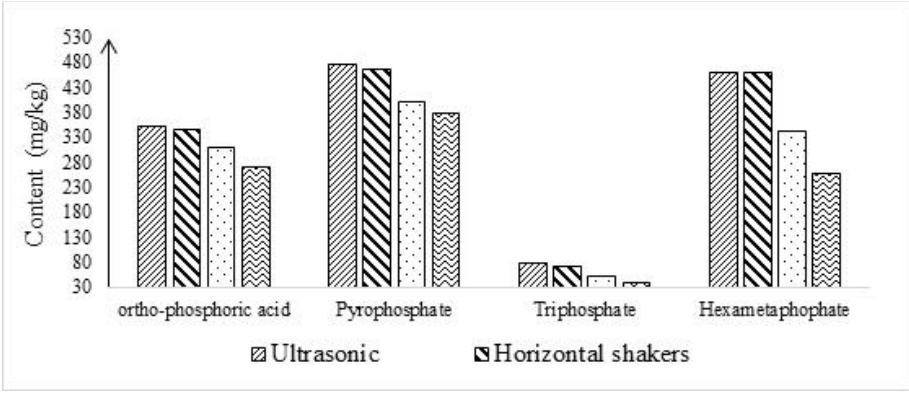

Figure 3. *Results of sample extraction technique investigation*

Based on the results obtained, when using the vortex shaking and handshaker, the content of the four phosphate substances were lower than that of horizontal shaking and ultrasonic extraction. The contents of hexametaphosphate when using horizontal shaker and ultrasonicator have no significant difference. However, the contents of *ortho*-phosphoric acid, diphosphate, and triphosphate when using ultrasonicator were higher than themselves when using a horizontal shaker. Therefore, the ultrasonic extraction was chosen in this study.

3.2.2. Sample extraction time

Raw meat samples were selected for this study. Sample extraction is conducted at different ultrasonic time levels: 15 min, 30 min, 45 min, and 60 min. The results are shown in Figure 4.

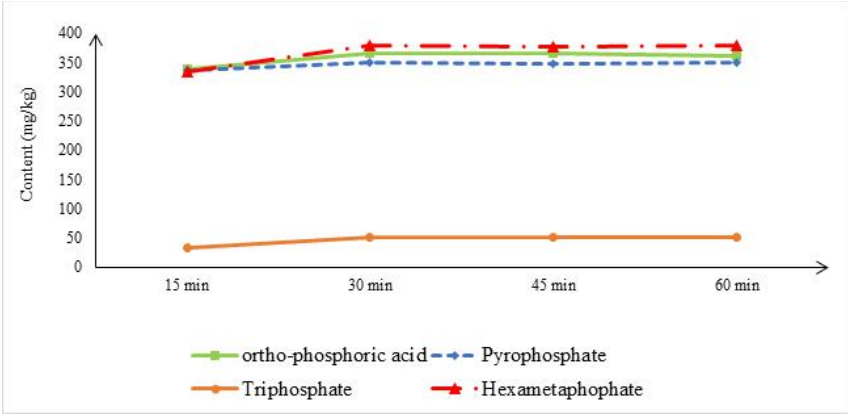

Figure 4. *Results of the sample extraction time investigation*

The results in Figure 4 show that there is no difference in the content of all four substances (*ortho*-phosphoric acid, diphosphate, triphosphate, and hexametaphosphate) at an extraction time of 30 minutes and the higher. To save time and energy while still ensuring efficiency, a sample extraction time of 30 minutes is selected in this study.

3.2.3. Sample extraction temperature

Since the analytes can be decomposed in the presence of high temperature. Using the same raw meat samples, sample extraction is carried out at different temperatures: room temperature (25±5°C), 40°C, and 50°C. The results are shown in Figure 5.

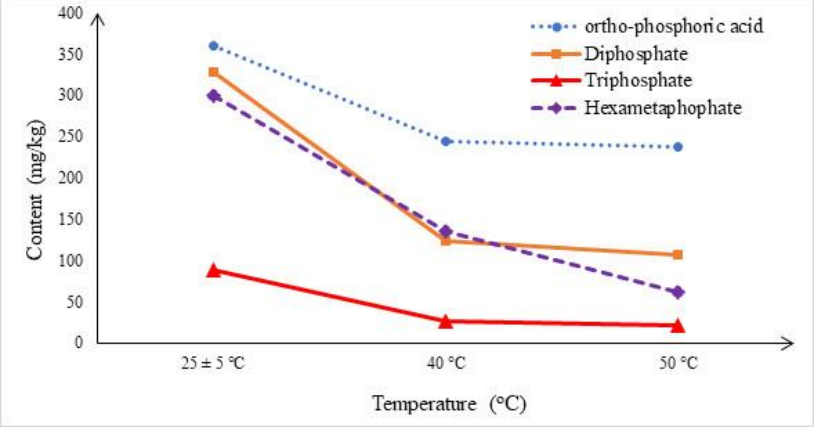

Figure 5. *Results of the sample extraction temperature investigation*

Based on the results obtained in Figure 5, the content of four substances *ortho*-phosphoric acid, pyrophosphate, triphosphate, and hexametaphosphate reached the highest in temperature of 25°C and decreased while increasing temperature to 40°C and 50°C. Therefore, the sample extraction temperature was chosen at 25°C for further studies.

3.2.4 Investigation of repeated extraction times

Repeated extraction is one way to ensure the analyte is maximally extracted from the sample. The number of extractions: 1 time, 2 times, 3 times was conducted in this study. The results are presented in Figure 6.

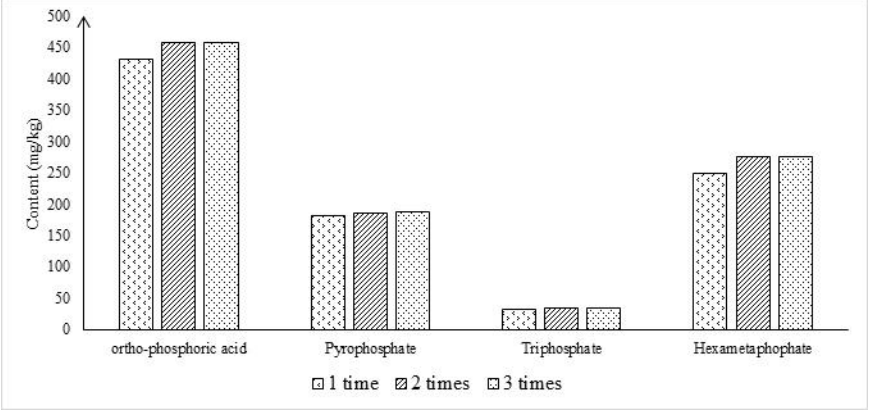

Figure 6. *Results of the investigation of the repeated sample extraction times*

The results shown in Figure 6 show that for all four *ortho*-phosphoric acids, diphosphate, triphosphate, and hexametaphosphate, the results of 2 times and 3 times repeated extraction were insignificantly different. Thus, the 2 times repeated extraction was selected for time and energy saving.

3.3. Method validation

The specificity of the method is assessed through the analysis of blank samples, standard samples, and spiked samples. The results in Figure 7 showed that there is no analyte signal in the blank sample, the analyte signals of the standard, and the spiked samples were at the same retention time with deviation under 1%. Therefore, the method has good specificity.

The calibration curves were formulated with concentrations varying from 5 – 200 mg/L for sodium pyrophosphate and sodium tripolyphosphate solutions; from 5 – 400 mg/L for *ortho*-phosphoric acid and hexametaphosphate. This experiment was conducted in spiked blank sample of diphosphate, triphosphate, hexametaphosphate and sample containing analytes (applied with *ortho*-phosphoric acid). The accuracy of the method is evaluated by the recovery of adding standards (with 3 levels of added content at 50%, 100%, 150%) to a number of sample matrices containing the analyte and the recovery of adding standard (at 3 concentration levels: low, medium and high within the working linear range) for blank samples. The results of calibration curves, correlation coefficients, detection limits (LODs), and quantitative limits (LOQ) of analytes are presented in Table 2. The precision (RSD%) and trueness (R%) of the analytes on sample matrices are shown in Table 3.

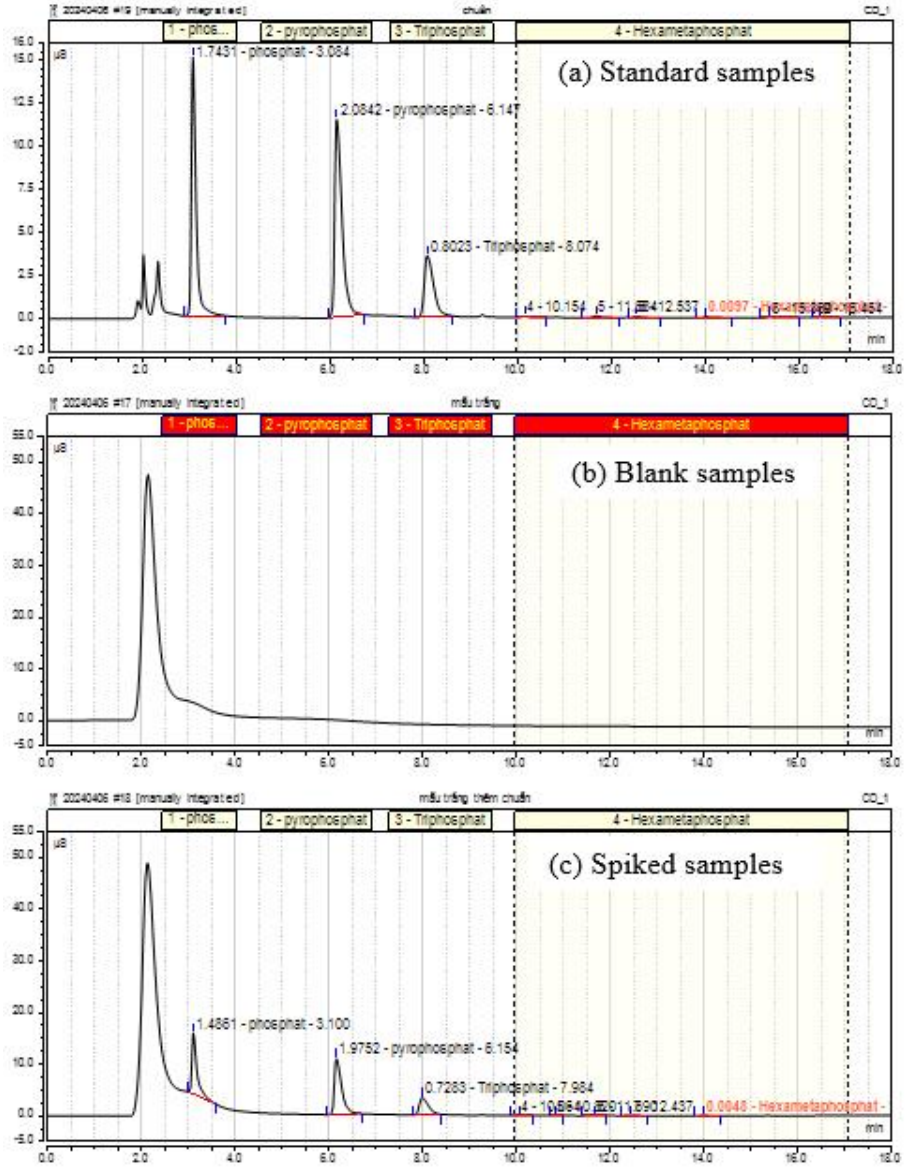

Figure 7. *Chromatography of specificity results*

Table 2. *Results of calibration curve, LOD, and LOQ*

Analyte	Calibration equation	Correlation coefficient, R^2	LOD (mg/kg)	LOQ (mg/kg)
Ortho–Phosphoric acid	y = 0.1546x – 03132	1	12.0	40
Pyrophosphate	y = 0.0681x – 0.1563	1		
Tripolyphosphate	y = 13.052x – 1.7095	1		
Hexametaphosphate	y = 0.0163x – 0.0478	0.9999		

Table 3. *The precision and trueness results*

Analyte	Parameter	Sample matrix			
		Canned pork meat	Banh pho	Orange juice	Sausage
Ortho–Phosphoric acid	RSDr %	-	-	3.22	3.61
Pyrophosphate		1.39	1.10	-	-
Tripolyphosphate		1.93	1.56	-	-
Hexametaphosphate		1.18	1.46	-	-
Ortho–Phosphoric acid	R %	-	-	95.2 – 102.6	95.5 – 102.7
Pyrophosphate		95.1 – 104.8	95.8 – 104.9	-	-
Tripolyphosphate		91.1 – 95.2	90.2 – 98.7	-	-
Hexametaphosphate		90.0 – 99.8	91.5 – 103.2	-	-

The method has good specificity, the reliable calibration curves of 4 substances with correlation coefficients $R^2 > 0.999$. Repeatability (RSDr: 1.10 – 3.61%) and recovery (R: 90.0-104.9%) meet AOAC requirements. The detection limits (LOD) and quantitative limits (LOQ) of phosphate groups are 12 mg/kg and 40 mg/kg, respectively. Thus, the method has good sensitivity, specificity, and accuracy for simultaneous analysis of forms of phosphate content in food.

3.4. Simultaneous analysis of forms of phosphate content in several food samples

Applying the optimized analysis conditions above to determine the content of the forms of phosphate by ion exchange chromatography in food samples collected randomly on the Hanoi markets, the results are obtained in Table 4.

Table 4. *Content of phosphates in some food samples*

No.	*Sample code*	*Content (mg/kg)*				*Total P content (mg/kg)*	*ML (mg/kg)*
		A	*B*	*C*	*D*		
1	Dumplings - 1	-	-	-	613.8	194.2	9300
2	Dumplings - 2	55.7	-	71.4	932.2	330.5	9300
3	Green sticky rice cake - 3	41.7	-	3547.1	286.7	1099.3	9300
4	Mung bean cake - 1	43.6	-	2901.2	358.1	940.9	9300
5	Mung bean pie - 2	-	-	6667.6	512.8	2036.2	9300
6	Bread - 1	-	-	-	672.4	212.7	9300
7	Bread - 2	-	-	-	283.6	89.7	9300

No.	Sample code	Content (mg/kg)				Total P content (mg/kg)	ML (mg/kg)
		A	B	C	D		
8	Pastries - 1	1360.4	-	1238.8	1769.2	1287.9	9300
9	Pastries - 2	-	-	-	743.9	235.3	9300
10	Pastries - 3	-	-	-	2998.6	948.6	9300
11	Traditional product- 1	-	-	-	885.2	280.0	9300
12	Traditional product - 2	-	-	-	1121.0	354.6	9300
13	Traditional product - 3	-	-	-	817.7	258.7	9300
14	Traditional product - 4	-	-	-	220.2	69.7	9300
15	Traditional product - 5	-	-	-	914.2	289.2	9300
16	Traditional product - 6	-	-	-	549.5	173.8	9300
17	Traditional product - 7	-	-	1133.2	173.8	373.5	9300
18	Traditional product - 8	-	-	238,7	265.8	151.2	9300
19	Traditional product - 9	-	-	-	-	-	9300
20	Traditional product - 10	-	-	-	323.1	102,2	9300
21	Traditional product - 11	-	-	-	1492.4	472,1	9300
22	Traditional product - 12	-	-	-	-	-	9300
23	Traditional product - 13	1051.5	-	228.1	621.4	554,4	9300
24	Snack - 1	-	-	1695.5	201.3	540,2	9300
25	Snack - 2	-	-	2543.2	291.8	807,1	9300
26	Instant vermicelli - 1	-	-	-	514.4	162,7	2500
27	Instant vermicelli - 2	-	-	-	360.6	114,1	2500
28	Fresh vermicelli	-	-	-	-	-	2500
29	Instant porridge - 1	-	-	-	1125.5	356.1	2500
30	Instant porridge - 2	-	-	-	788.5	249.5	2500
31	Fried rice - 1	-	-	-	214.5	67.9	900
32	Fried rice - 2	-	-	-	323.6	102.4	900
33	Shrimp noodles - 1	286.3	-	1819.8	1539.5	1078.5	2500
34	Shrimp noodles - 2	-	-	3204.9	1938.2	1513.9	2500
35	Shrimp noodles - 3	188.7	-	-	1321.8	470.9	2500
36	Shrimp noodles - 4	217.0	-	1769.9	1268.5	959.3	2500
37	Shrimp noodles - 5	109.9	-	439.3	611.4	347.6	2500
38	Instant vermicelli - 1	-	-	-	-	-	2500
39	Instant vermicelli - 2	-	-	515.7	679.5	359.9	2500
40	dried vermicelli - 1	-	-	-	-	-	900
41	Dried vermicelli - 2	-	-	-	-	-	900
42	Cereals - 1	204.2	-	1257.3	839.0	675.8	2200
43	Cereals - 2	309.8	392.8	3676.5	4045.3	2498.9	2200
44	Cereals - 3	-	-	-	529.8	167.6	2200
45	Instant noodle soup - 1	109.9	-	439.3	611.4	347.6	2500

No.	Sample code	Content (mg/kg)				Total P content (mg/kg)	ML (mg/kg)
		A	B	C	D		
46	Instant noodle soup - 2	-	-	-	839.2	265.5	2500
47	Instant noodle soup - 3	-	-	-	509.7	161.2	2500
48	Meat -1	1700.8	-	-	1223.1	862.1	2200
49	Meat -2	770.2	-	2283.2	1381.4	1293.9	2200
50	Meat -3	253.3	539.8	715.7	2989.2	1354.0	2200

Note: A - Sodium pyrophosphate; B - Sodium tripolyphosphate; C – Hexametaphosphate; D - Acid ortho-phosphoric, ML – Maximum limit.

The results in Table 4 showed that 15/50 samples detected pyrophosphate, 2/50 samples detected triphosphate, 18/50 samples detected hexametaphosphate, and 44/50 samples detected *ortho*-phosphoric acid. Among 50 samples, samples in 6 out of 50 were not detected all four substances. Products appearing phosphate substances have phosphorus concentration ranging from 67.9 mg/kg to 2499 mg/kg.

According to Circular No. 24/2019/TT-BYT [14], phosphate groups are calculated according to phosphorus with different maximum limits for certain food groups. Comparing the results of Table 4 to the MLs in the circular shows that 01/50 samples exceeded the threshold (Cereals – 2), and the remaining products met the regulation in the circular. Thus, the products on the markets mostly ensure the limits of the circular, but there is still one disqualified sample. Therefore, authorities still need to plan and executive effective food safety inspection.

4. CONCLUSION

The study succeeded in developing a method for simultaneously determination 4 forms of phosphate including *ortho*-phosphoric acids, diphosphate, triphosphate, and hexametaphosphate in food by ion exchange chromatography with a conductivity detector. The analysis process is optimized for 4 food sample matrices. The method was validated for specificity, calibration curves, detection limits, quantitative limits, repeatability, and recovery. The aspects were met to AOAC requirements. The method was used to analyze 50 different food samples showing the ability of extending simultaneously determination the 4 types of phosphate in other food products.

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