

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Selection of optimum condition for RGB intensity

3.1.1 The measurement of background from different areas on the standard chart

The photograph was taken in the white box, then RGB intensity of background was measured near the standard chart of phosphate test for 3 points as shown in Fig. 3.1. The color intensity of background as shown in Tables 3.1, 3.2 and 3.3.

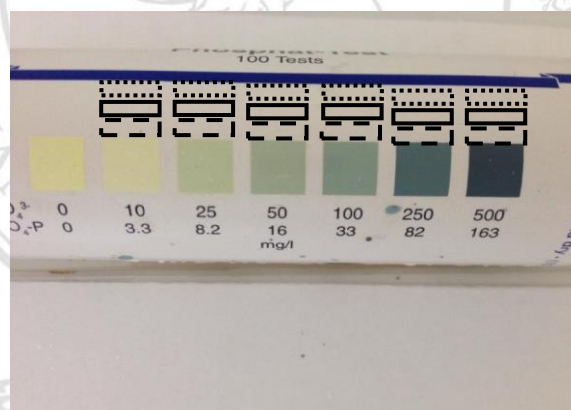


Figure 3.1 Image obtained from taking the standard chart of phosphate test in the white box with the background area that was chosen to measure RGB intensity of background (----), (—) and (.....)

Table 3.1 The effect of background area on R intensity measurement

PO ₄ ³⁻ concentration from standard chart (mgPO ₄ ³⁻ /L)	R intensity			Mean ± SD
	1	2	3	
10	192	195	195	194 ± 2
25	189	191	193	191 ± 2
50	189	189	191	190 ± 1
100	186	189	191	189 ± 2
250	186	189	192	189 ± 3
500	186	190	190	189 ± 3

Table 3.2 The effect of background area on G intensity measurement

PO ₄ ³⁻ concentration from standard chart (mgPO ₄ ³⁻ /L)	G intensity			Mean ± SD
	1	2	3	
10	186	188	188	187 ± 1
25	183	188	189	187 ± 3
50	183	187	187	186 ± 2
100	181	185	188	185 ± 4
250	179	183	186	183 ± 4
500	177	182	182	180 ± 3

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Table 3.3 The effect of background area on B intensity measurement

PO ₄ ³⁻ concentration from standard chart (mgPO ₄ ³⁻ /L)	B intensity			Mean ± SD
	1	2	3	
10	168	171	175	172 ± 4
25	165	173	177	172 ± 6
50	169	173	177	173 ± 4
100	164	171	175	170 ± 6
250	161	167	171	166 ± 5
500	157	164	167	163 ± 5

As presented in Table 3.1, 3.2 and 3.3, the SD values of intensity of background are less than 10. Shown in the Table 3.1, 3.2 and 3.3. These SD values indicated that there is no interference from environmental light. So the RGB intensity of background can be measured from any area on standard chart.

3.1.2 The measurement of background from different points around test strip

RGB intensity values around the test strip in the white box were measured using iPhone4S with colorsampler application. There were 6 selected points as shown in Fig. 3.2. The values of RGB intensity are displayed in Table 3.4, 3.5 and 3.6



Figure 3.2 Image of the standard chart and the test strips taken in the white box and 6 measure points of background of the strips

Table 3.4 R intensity from 6 points around the test strips

Images	R intensity from 6 point						Mean \pm SD
	1	2	3	4	5	6	
1	181	182	187	186	176	195	184 \pm 6
2	182	182	184	186	170	191	183 \pm 6
3	191	194	193	195	180	201	192 \pm 6
4	183	188	188	189	175	197	187 \pm 6
5	177	186	187	189	174	197	185 \pm 7
6	180	188	187	190	175	200	187 \pm 6
7	186	191	190	195	179	188	188 \pm 6
8	175	181	190	181	167	192	181 \pm 9
9	178	185	186	189	170	198	184 \pm 8
10	175	181	181	187	165	193	181 \pm 8

Table 3.5 G intensity from 6 points around the test strips

Images	G intensity from 6 point						Mean \pm SD
	1	2	3	4	5	6	
1	173	175	180	177	170	186	177 \pm 6
2	175	175	176	179	163	184	175 \pm 7
3	184	188	187	188	174	196	186 \pm 7
4	176	181	180	182	170	192	180 \pm 7
5	169	178	180	180	167	192	178 \pm 9
6	171	180	179	182	167	192	179 \pm 9
7	177	183	182	186	172	178	180 \pm 5
8	172	178	182	177	165	191	178 \pm 9
9	175	181	182	185	169	194	181 \pm 8
10	174	178	178	185	164	192	178 \pm 10

Table 3.6 B intensity from 6 points around the test strips

Images	B intensity from 6 point						Mean \pm SD
	1	2	3	4	5	6	
1	155	160	164	161	154	175	162 \pm 7
2	155	158	159	161	146	171	158 \pm 8
3	167	170	171	170	158	182	170 \pm 8
4	159	165	168	168	154	181	166 \pm 9
5	150	162	162	163	158	181	163 \pm 10
6	153	163	162	165	152	180	162 \pm 10
7	159	165	165	168	154	161	162 \pm 5
8	161	167	165	165	154	183	166 \pm 10
9	162	171	171	173	157	184	170 \pm 9
10	162	168	170	175	153	186	169 \pm 11

As presented in Table 3.4, 3.5 and 3.6, The SD values of intensity of background are less than 10. These SD values indicated that there is no interferent from

environmental light, so the RGB intensity of background can be measured from any points around the strip. In this work, point 3 was chose to measure the RGB intensity of background of test strip.

3.1.3 Study the relationship between the RGB intensity and phosphate concentration

The RGB intensity was obtained from the measurement of the standard chart image. It was taken by iphone4S and was measured for the intensity by icolorsampler application. The relationships between the RGB intensity and concentration of phosphate on standard chart present are in the Fig 3.3. The intensities of red, green and blue values increased with increasing phosphate concentrations from 10 to 100 mgPO₄³⁻/L. The linear ranges of this are 10 to 100 mgPO₄³⁻/L as presented in the Fig. 3.4.

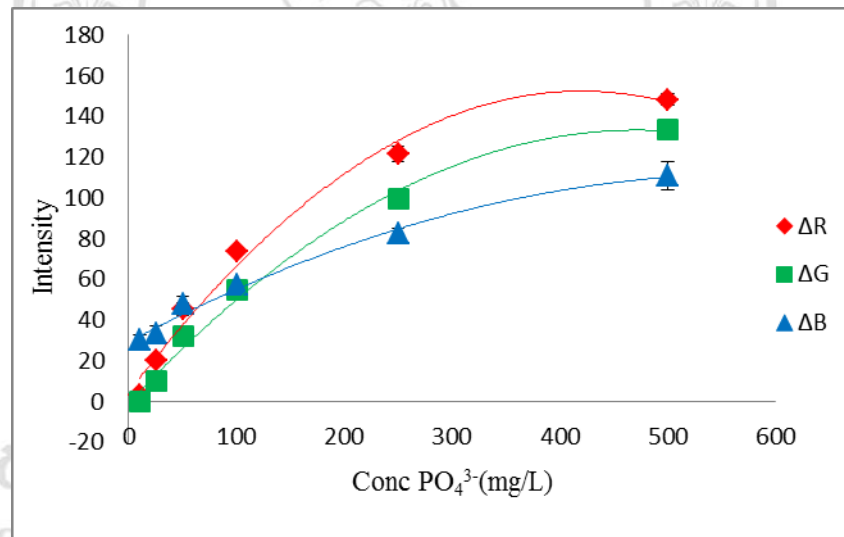


Figure 3.3 Relationship between R, G and B intensity and phosphate concentration from 10 to 500 mgPO₄³⁻/L

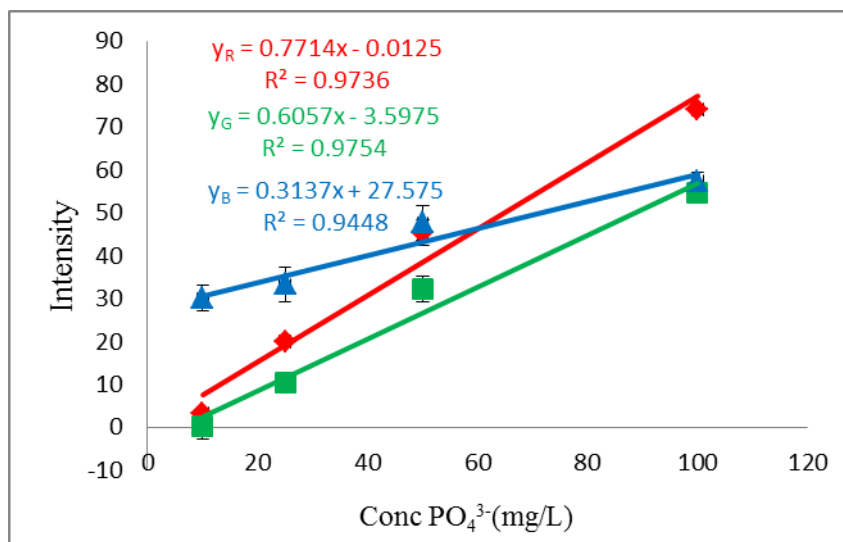


Figure 3.4 Relationship between RGB intensity and phosphate concentration from 10 to 100 mgPO₄³⁻/L

Fig 3.4 presents the relationship between the intensity of each color component and the concentration of phosphate. The ranges of this method are 10 to 100 mgPO₄³⁻/L. The slope from the calibration graph show the sensitivity value. The intensity of red has better sensitivity than that of green and blue intensity. Their equations: $y = 0.7714x - 0.0125$, $y = 0.6057x - 3.5975$ and $y = 0.3137x + 27.575$ respectively. Next study, the R intensity would be used for determination of phosphate in seafood and frozen seafood.

3.1.4 Comparison of the relationship between R intensity of phosphate concentration on standard chart and phosphate standard solution

The 10 concentrations of phosphate standard solutions were prepared in the ranges of concentration on standard chart (10 to 500 mgPO₄³⁻/L). The test strips test were dipped in the standard solution. The reagent was dropped on the reaction zone and left for 15 seconds. A drop of reagent was split from the reaction zone. It stood for color develop for 1 minute in the white box and a photo was taken by an Iphone4S. It was measured for the R intensity value by icolorsampler application. The relationship between R intensity of phosphate concentration from standard chart and phosphate standard solution was overlay as shown in Fig. 3.5. The calibration graphs of both as shown are represented in Fig. 3.6.

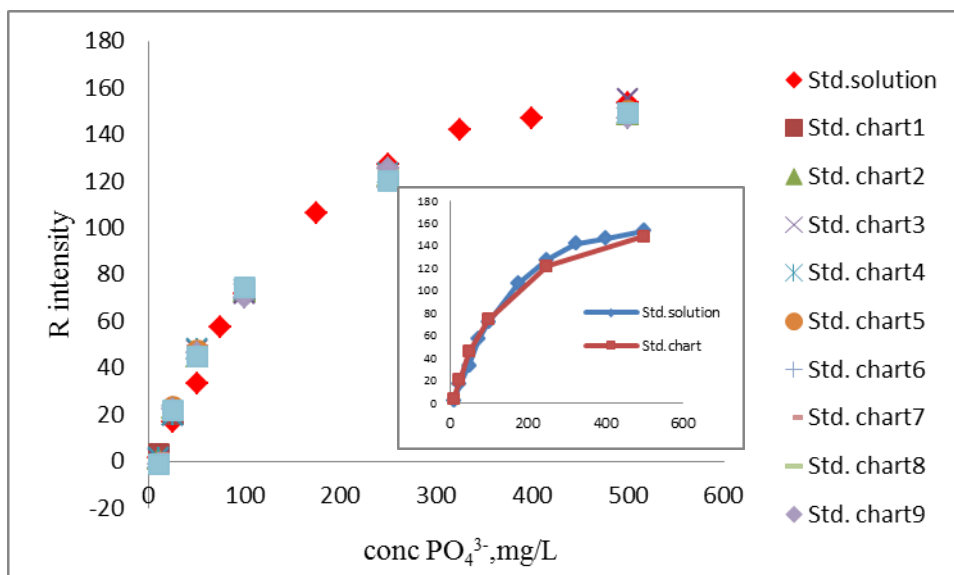


Figure 3.5 Relationship of R intensity of phosphate concentration from standard chart and phosphate standard solution

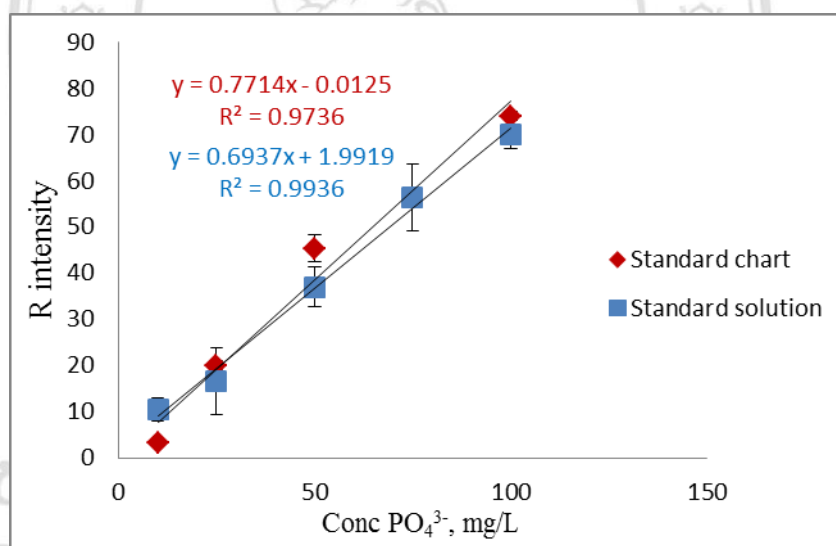


Figure 3.6 The relationship between R intensity and concentration of phosphate from standard solution range 10 to 100 mgPO₄³⁻/L

3.2 Optimization for the phosphate test strip use

The phosphate solution was extracted from frozen seafood by the DI water, which could contain the different fat amounts. After centrifugation the fat portion stayed as upper part over of the extract solution. When the strips were dipped into the extract solution for phosphate determination, the fat may interfere. The phosphate test strip method was studied including the reagent holding time, color developed time.

3.2.1 Using the phosphate test strip with standard solution

The phosphate test strip was dipped into the 50 mg/L of standard phosphate solution then the reagent solution was dropped on reaction zone and stood for 15 sec. A drop of reagent was split from the reaction zone. It stands for color develop for 1 min in the white box and took a photo by an Iphone4S. The R intensity was measured by icolorsampler application. Next, the standard solution dropped on the reaction zone was studied. The step of study was followed by dipping the test strip into the solution but the step was changes from dipping to dropping the 50 mg/L of phosphate standard solution on the reaction zone. The results are represented show in the Tables 3.7 and the Fig. 3.7.

Table 3.7 The results of R intensity obtained from dipping or dropping the strips in the 50 ppm standard phosphate solution and treated with the sulfuric acid for different timing

How to use the strips	R intensity \pm SD	Substitute R intensity into the equation $y = 0.5438x + 17.833$ (conc. PO_4^{3-} , mg/L)
dipping	48 ± 3	49 ± 3
dropping	49 ± 3	50 ± 3

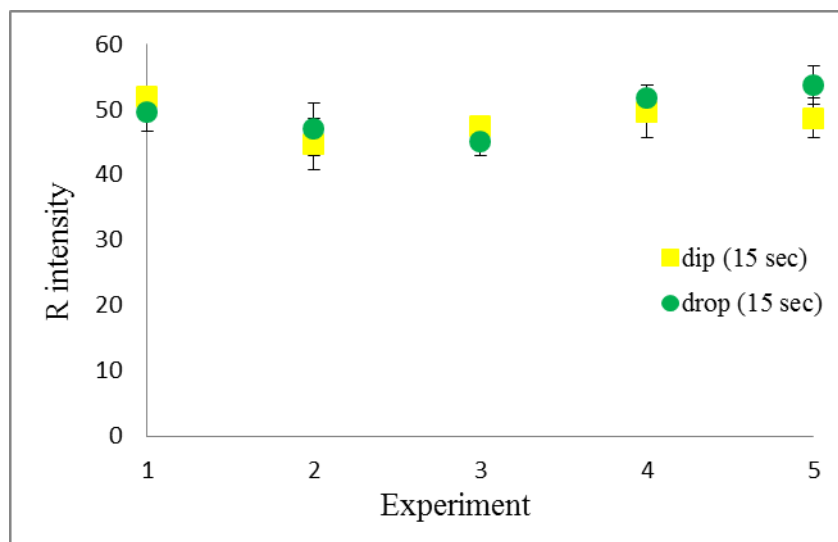


Figure 3.7 Overlay the R intensity of the strips that study repeat 5 times; (■) dipping the strip in the 50 ppm phosphate standard solution then drop the sulfuric acid solution on reaction zone stand for 15 sec and removing the solution; (●) dropping.

The results show no difference in the R intensity. Dipping should be more convenient than dropping the solutions on the reaction zone.

3.2.2 Holding time

Firstly, the test strip was dipped into the 50 ppm standard phosphate solution then dropping the reagent solution on reaction zone stand for 0 sec, the reagent was split from reaction zone, with standing for color develop for 1 min in the white box. Finally, taking photo by iphone4S and the R intensity was measured by icolorsampler application. The step of study was followed by the first step but the time for standing was for 5 and 15 sec, respectively. The results are represented in Table 3.8.

Table 3.8 R intensity obtained from the time for left the sulfuric acid on the reaction zone

The time for left the reagent solution reaction zone (sec.)	R intensity \pm SD	conc PO_4^{3-} , ppm by substitute R intensity into the equation $y = 0.5438x + 17.833$
0	50 \pm 2	56 \pm 5
5	42 \pm 1	44 \pm 3
15	50 \pm 2	46 \pm 3

3.2.3 The time for development of color of the test strips

The time for color development of the strip is also important. The color of the reaction zone after the reagent solution was removed could provide error, continued to change after the specific reaction time has elapsed in phosphate concentration. So the time for color development was studied by using the standard phosphate solutions by following the standard chart of phosphate test (10, 25, 50, 100, 250 and 500 mgPO₄³⁻/L). Immersing the reaction zone of the test strips in the phosphate standard solution, before dropping the reagent solution on the reaction zone holding for reaction for 15 sec and removing the solution out. Finally taking a photo after leaving for color developing (30, 50, 60, 70, 80, 100, 120 and 150 sec). The R intensity was measured by icolorsapler application of iphone4S. The relationship between R intensity with various the timing for color development are presented in Fig. 3.8.

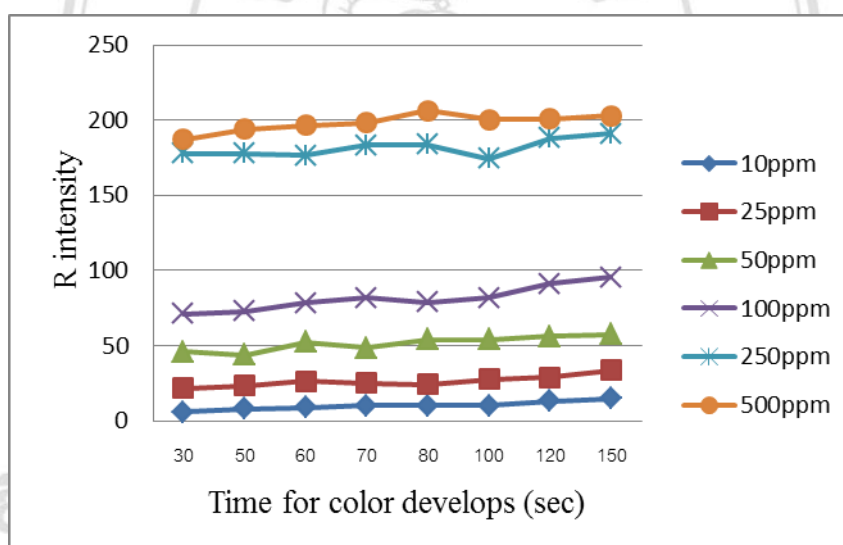


Figure 3.8 The relationship between R intensity with various timing for color develop

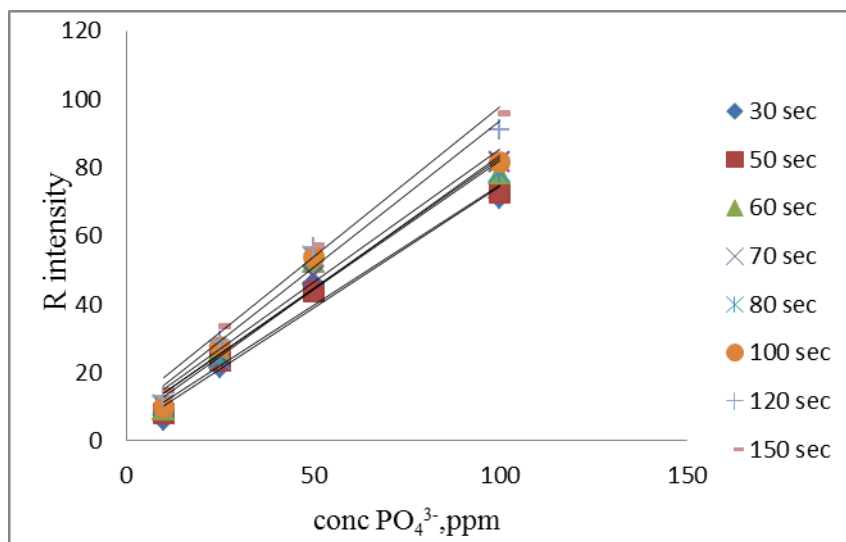


Figure 3.9 The relationship between the R intensity of the phosphate concentrations (10 to 100 mgPO₄³⁻/L) with various timing for color development

Table 3.9 Equations from the calibration graph (the relationship of the R intensity of the phosphate concentration (10-100 mgPO₄³⁻/L) with various the timing for color development

Times (sec)	Equation
30	y = 0.7091x + 3.3686, R ² = 0.9661
50	y = 0.7033x + 4.3869, R ² = 0.9852
60	y = 0.7541x + 6.6216, R ² = 0.9619
70	y = 0.785x + 5.0268, R ² = 0.9909
80	y = 0.7596x + 6.6198, R ² = 0.9569
100	y = 0.78x + 7.2579, R ² = 0.9662
120	y = 0.86x + 7.5596, R ² = 0.9858
150	y = 0.8803x + 9.6185, R ² = 0.9902

The results indicate that the R intensity increases with increase in timing from 30 sec to 60 sec and becoming constant until to 100 sec. The relationship between the R intensity of the phosphate concentrations of 10 to 100 mgPO₄³⁻/L with various timing for color development. Fig 3.9 and Table 3.9 summarize the results.

3.3 Limit of detection

The phosphate concentrations in the standard chart were in the range to 10 to 500, mgPO₄³⁻/L. It was found that the lowest concentration (10 mgPO₄³⁻/L) the blank solution showed similar color as showing in the Fig 3.10. So the limit of detection in this method was considered to be 10 mgPO₄³⁻/L. The test strips were dipped into the milli-Q water, then the reagent solution was dropped on the reaction zone following by holding for 15 sec and removing the liquid drop. Next, the test strips were put in the white box with standing for color measurement at 60 sec by taking photos was using iPhone4S camera.

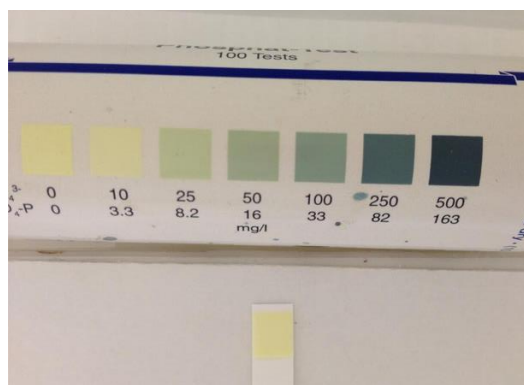


Figure 3.10 The test strip with blank solution

Table 3.10 RGB intensities of blank, 10 mg/L on the standard chart together with background

Suspect areas	Intensity		
	R	G	B
Blank	43±2	53±2	103±6
0 mg/L on standard chart	48±1	60±1	118±2
10 mg/L on standard chart	54±1	63±1	113±1
Background	57±1	68±1	85±1

3.4 Sample preparation

3.4.1 The ratio of the sample with water for extraction

The frozen murrell fish was chosen for study. The sample (1 pack) was blended until homogenous for sample portion of 5.xx, 10.xx and 20.xx g before into 100 mL plastic bottle. 100 ml of milli-Q water was added and shaken to become suspension. A 30 ml of the suspension was transferred into a 50 mL centrifuge tube for vortexing for 2 min. Then three portions of 10ml suspension each were three transferred into 15 mL centrifuge tubes for 10 min centrifuging. The supernatant solution was determined the phosphate concentration using the test strips with the iphone4S camera and measured the intensity by icolorsampler application. The linear range of calibration graph is 25 to 100 mgPO₄³⁻/L and equation is: $y = 0.7029x + 12$, $R^2 = 0.9842$. The results of the phosphate concentrations are summarized in Table 3.11.

The molybdenum blue method was also used for phosphate determination. The mixed reagent was prepared by mixing 15 mL ammonium molybdate solution, 50 mL

sulfuric acid solution, 15 mL potassium antimonyl tartrate solution and 30 mL L-ascorbic acid solution. A calibration graph was constructed by using a single standard solution ($50 \mu\text{gPO}_4^{3-}/\text{mL}$). Different volumes (0.02, 0.1, 0.2 and 0.3 mL) of the standard solution and the extracted solution were taken into a series of 25 mL volumetric flasks before adding the mixture (0.8mL) of reagents and following by adjusting with milli-Q water to the mark. After standing for 10 min, absorbances at 880 nm were measured. The calibration graph was plotted for absorbances vs the amounts of PO_4^{3-} (μg). The linear range of standard calibration graph was found to be 1-15 μg with equation being $y = 0.0045x - 0.002$, $R^2 = 0.9971$. The result are displayed in Table 3.11

Table 3.11 The results of phosphate concentration from the test strips with the digital image analysis and the molybdenum blue method

Method	Weight of the sample (g)	Volume of water (mL)	Time for Vortex (min)	Time for Centrifuging (min)	mg $\text{PO}_4^{3-}/\text{kg}$	%RSD
Strip test + camera	5	100	2	10	4393 ± 323	6
	10	100	2	10	3527 ± 301	6
	20	100	2	10	3540 ± 198	4
Molybednum blue method	5	100	2	10	5296 ± 540	10
	10	100	2	10	4946 ± 0	0
	20	100	2	10	4242 ± 565	13

Sample extraction should be by taken 5.xx, 10.xx or 20.xx g of sample and extracted with water.

3.4.2 Sampling

One pack of frozen murrell fish was blended and weighed for 10.xx g into 100 mL plastic bottle. 100 mL of milli-Q water was added and shaken. After that, 30 mL of suspension was transferred into a 50 mL centrifuge tube and vortexed for 2 min. 10 ml of the suspension was transferred into a 15 mL centrifuge tube (3 tubes) and centrifuged for 10 min. The supernatant solution was determined for the phosphate concentration by using the test strips with taking photo by the iphone4S camera. The intensity data was measured by icolorsampler application. Table 12 and Figure 3.11, present the results.

Table 3.12 Phosphate contents ($\text{mgPO}_4^{3-}/\text{kg}$) in sampling study

Sampling (g)	Concentrations of phosphate $\text{mgPO}_4^{3-}/\text{kg}$				%RSD
	Tube 1	Tube 2	Tube 3	Mean \pm SD	
10.19	2889	2835	3053	2926 \pm 113	4
10.07	2562	2944	3053	2853 \pm 258	9
10.11	2453	3544	2726	2908 \pm 567	19

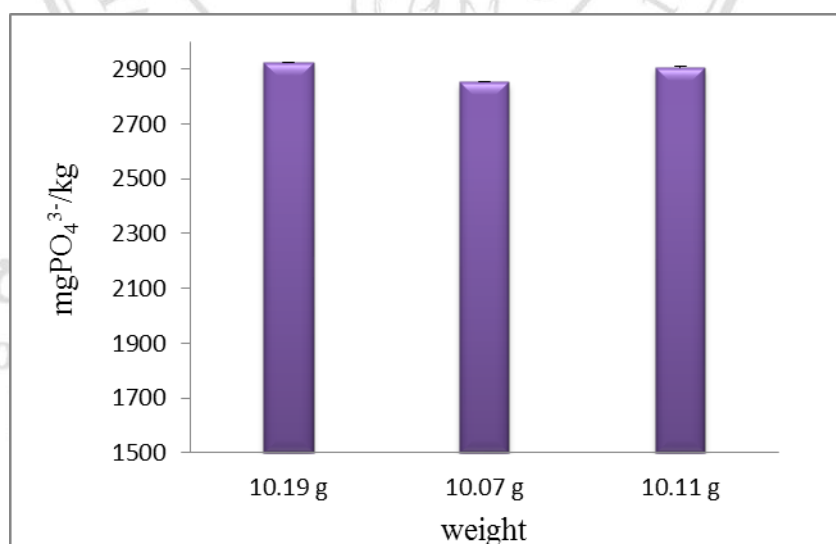


Figure 3.11 The relationship between the phosphate concentration ($\text{mgPO}_4^{3-}/\text{kg}$) with the sampling of various weights

3.4.3 Optimization of the vortex time

Vortex may be applied to increase the extraction efficiently. The time of phosphate extraction for frozen and sea food samples was studied. One pack of murrell fish frozen was blended and weighed for 10.xx g and putting into a 100 mL plastic bottle. 100 mL of milli-Q water was added and shanked until the suspension solution becoming. After that, a portion (30 mL) of the suspension was transferred into the 50 mL of centrifuge tube and vortexed for 1 min. One portion of the suspension (10 mL) was transferred into the 15 mL centrifuge tube and centrifuged for 10 min. The supernatant was determined for the phosphate concentration by the test strips with the iphone4S camera. The intensity was measured by icolorsampler application. The results are shown in Table 3.13. It was found that the vortex times didn't affect to the extracted phosphate samples. So, 2 min was chosen to ensure the extraction complete.

Table 3.13 The phosphate concentrations in the frozen murrell fish sample at different vortex times

Vortex times (min)	mgPO ₄ ³⁻ /kg
1	4378 ± 398
2	3880 ± 173
3	3880 ± 456

3.4.4 %Recovery study

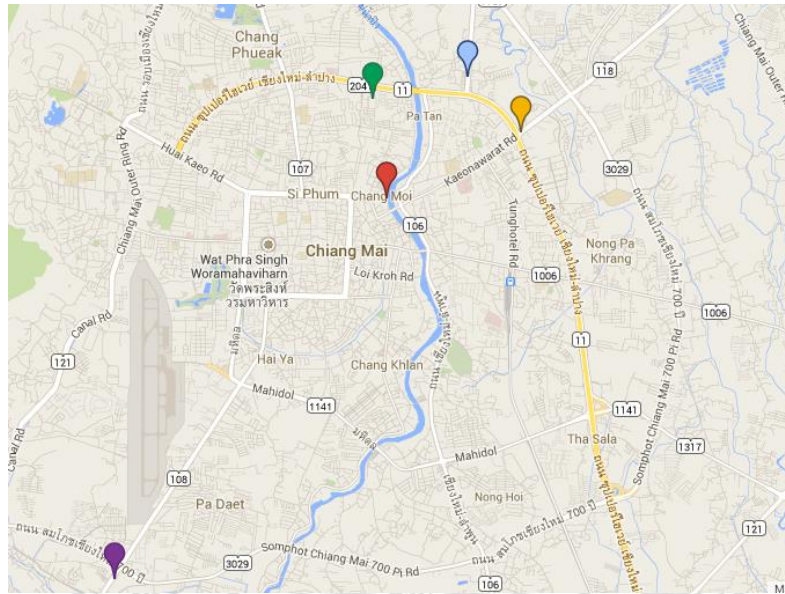
One pack of murrell fish frozen sample was blended and weighed for 10.xx g and put into 100 mL plastic bottle. 3 mL of phosphate standard solution (10000 mgPO₄³⁻/L) and 97 mL of milli-Q water were added. After that, the suspension was shaken. A portion (30 mL) was transferred of solution into the 50 mL centrifuge tube for 2 min vortex. The 10 mL sample suspension was transferred into 15 mL centrifuge tube for 10 min centrifuging. The supernatant was assayed for phosphate concentration using the test strips with the iphone4S camera. The intensity was measured by icolorsampler application. The results are presented in the Table 3.14. Recoveries were found to be 126-143%.

Table 3.14 Recoveries of the method

Experiments	Add PO ₄ ³⁻ (mg)	Found (mg PO ₄ ³⁻ /kg, n=3)	%Recovery
1	0	2795 ± 430	126 ± 5
	30	6553 ± 433	
2	0	2942 ± 157	148 ± 14
	30	7350 ± 393	
3	0	3071 ± 157	143 ± 27
	30	7332 ± 1062	

3.5 Application to on-site analysis assay

The phosphate test strip with the digital image analysis was applied to the phosphate contents in frozen and sea food samples in markets around Chiang Mai. The locations are shown in the Fig. 3.12. First day, 6 samples were analyzed. The calibration graph was established for the phosphate determination in concentration ranging from 25 to 100 mg PO₄³⁻/L (Fig.3.13). The results are shown in the Table 3.15. Second day, 6 samples were analyzed similarly to the first day, with the results in Fig. 3.14 and Table 3.16. The results are put onto Google map application. The lowest phosphate content found in the squid and the highest contents was higher than the value announced by Thai Industrial Standard Institute (5000 mgPO₄³⁻/kg in the fish product).



- ▼ Muk-tong Mahachai-Chiang Mai food
- ▼ Tesco Lotus, Kad Kamtiang
- ▼ Mueang Mai Market
- ▼ J 1 seafood (San Dek Intersection)
- ▼ Big C, Hang Dong

Figure 3.12 The locations of seafood markets around Chiang Mai

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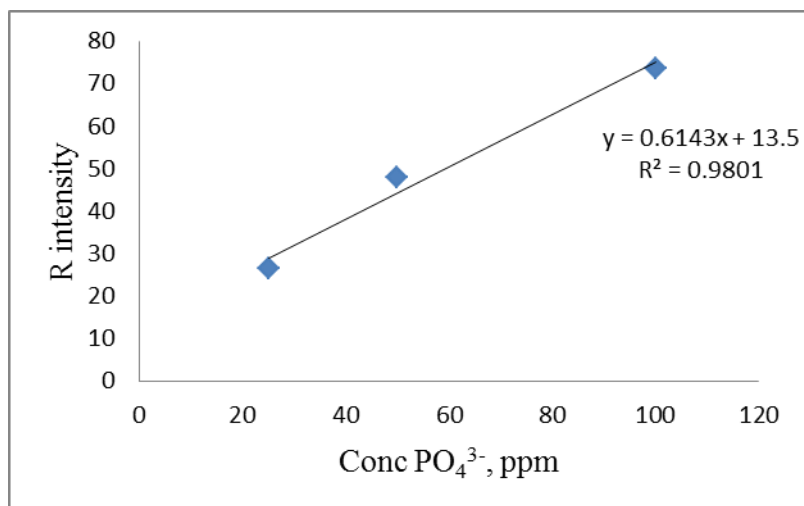


Figure 3.13 The calibration graph (R intensity with phosphate concentration (mgPO₄³⁻/L)) for day 1

Table 3.15 Phosphate contents found in Day 1

Markets	Samples	mg PO ₄ ³⁻ /kg (Net weight)	%RSD
		Mean ± SD	
Mueang Mai Market	Shrimp (frozen)	2080 ± 279	13
	Squid (frozen)	467 ± 42	9
	Dory fish (frozen)	1748 ± 256	15
Tesco Lotus Kad Kamtiang	Raw butterfly white shrimp (frozen)	927 ± 66	1
	Red snapper slice (frozen)	7046 ± 626	11
	Mixed seafood (frozen)	927 ± 6	9

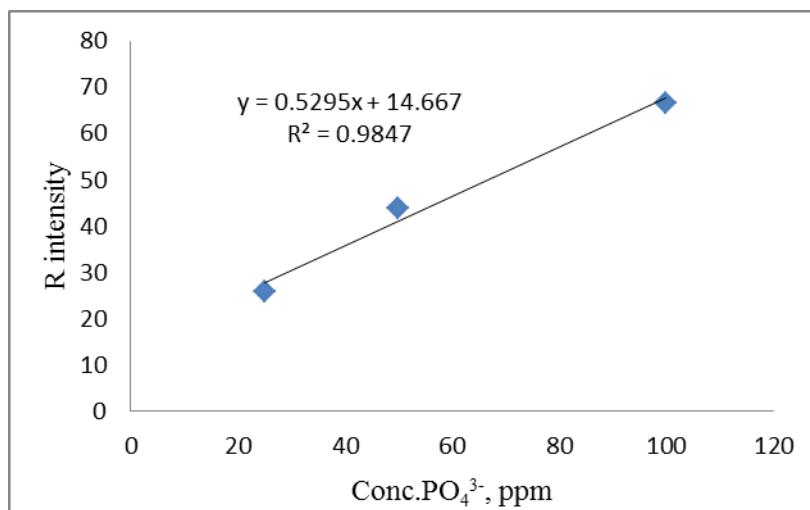


Figure 3.14 The calibration graph (R intensity with phosphate concentration (mgPO₄³⁻/L)) for day 2

Table 3.16 Phosphate contents found in Day 2

Markets	Samples	mg PO ₄ ³⁻ /kg (Net weight)	%RSD
		Mean ± SD	
Muk-tong Mahachai-Chiang Mai food	Squid (fresh)	6040 ± 475	8
	Octopus (frozen)	2126 ± 161	8
J-1 food	Red snapper	4956 ± 613	12
Tesco lotus, Hang Dong	Squid meat & Tentacle	2875 ± 183	6
	Flounder Filler (frozen)	6191 ± 197	3
	Dory fish (frozen)	3252 ± 170	5

3.5.1 The new equipment for on-site analysis

The equipments for sample preparation was found to the phosphate on-site determination because the power source required more than 1200 watts but it is most easy to have a mobile unit for such purpose inappropriate. The blender and the vortex mixture were replaced by to hand centrifuge and mixer cordless respectively.

A pack of sample was weighed on the mixer cordless bowl. The sample was cut into small pieces by using scissors and blended until becoming homogenous. 10.xx g of sample was weighed into a plastic cup and 100 mL of milli-Q water was added and mixed by whisk instead of vortex mixer for 2 min. The 10 ml sample solution was transfered into the 15 mL of centrifuge tube for 10 min hand centrifugtion. The clear solution was transfered into a small cup. The test strips was used for phosphate determination in this extract and taking photo by iphone4S camera. The R intensity was measured by iphone4S application. The results are presented in Table 3.17. It was found that equipment is appropriate.

Table 3.17 Phosphate contents when using new equipment

Sample	* Net weight (g)	Sampling (g)	ΔR	Substitute R intensity into the equation $y = 0.6286x + 12.333$	Calculation from 100 mL (mgPO_4^{3-})	dilute 10 fold (1 mL adjust volume to 10 mL)	mg PO_4^{3-} /kg (Net weight)
frozen murrell fish	130.81	10.11	38	41	4.1	41	4091
		10.05	41	45	4.5	45	4485
		10.07	37	40	4.0	40	3949
Averang							4175
SD							277
%RSD							7

* The weight of the contents and ice, not including materials packaging

3.5.2 Comparison of phosphate concentration obtained between digital image analysis and necked eye by test strips

The contents of phosphate samples obtained from substitution of R intensity into the equation and read by the naked eye of 10 people were compared. The results are in the Table 3.18 (day 1) and 3.19 (day 2), it was found that the contents from using the equation correspond to that obtained involve in reading from the naked eye of 10 people. However, using the naked eye may not thoroughly. It is to report in range of contents but the test strips with evaluation of R intensity by an iphone4S application. Should be more accurate than the naked eye.

Table 3.18 Comparison the phosphate concentration between digital image analysis and necked eye 10 people (day 1)

Markets	Samples	Phosphate concentration (mg/L)	
		Substitute R intensity into the equation $y = 0.6143x + 13.5$	Naked eye from 10 people
Mueang Mai Market	Shrimp (frozen)	43±6	44±6
	Squid (frozen)	48±4	55±2
	Dory fish (frozen)	37±5	38±0
Tesco Lotus Kad Kamtiang	Raw butterfly white shrimp (frozen)	49±2	66±14
	Red snapper slice (frozen)	73±7	83±14
	Mixed seafood (frozen)	49±2	75±0

Table 3.19 Comparison the phosphate concentration between digital image analysis and necked eye 10 people (day 2)

Markets	Samples	Phosphate concentration (mg/L)	
		Substitute R intensity into the equation $y = 0.6143x + 13.5$	Naked eye from 10 people
Muk-tong Mahachai-Chiang Mai food	Squid (fresh)	63±4	58±7
	Octopus (frozen)	111±5	103±5
J-1 food	Red snapper	51±7	54±18
Tesco lotus, Hang Dong	Squid meat & Tentacle	62±4	71±7
	Flounder Filler (frozen)	65±3	54±7
	Dory fish (frozen)	84±3	75±0

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