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Phosphorylated Protein Levels in Animal-Sourced Food Muscles Based on Fe^{3+} and UV/Vis Spectrometry

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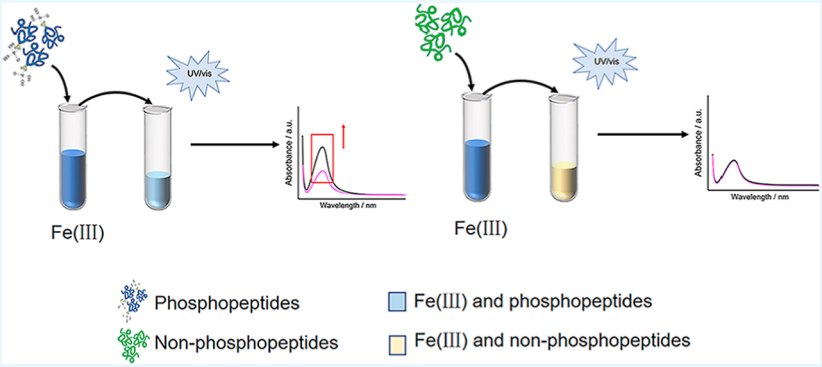

ABSTRACT: Protein phosphorylation, a post-translational modification of proteins, is important in biological regulation. The quantity of phosphorylated proteins is a key requirement for the quality change of animal muscle foods. In the present study, a new approach to quantify phosphorylated proteins and/or peptides was developed based on ferric ions (Fe^{3+}) and UV/vis spectrometry. This method is proved to be ultra-effective in discriminating phosphopeptides and non-phosphopeptides with the assistance of Fe^{3+}. The protocol of extracting proteins with 0.1% trifluoroacetic acid (TFA) solution from animal muscle samples coupled with Fe^{3+} was verified by using an artificial mixture of peptides with different phosphorylation sites and was successfully used to characterize the phosphorylation quantity in the samples via UV/vis spectrometry. A peptide with one phosphorylated site was taken as a reference standard and successfully utilized for the absolute quantification of phosphorylated proteins in caprine muscles during frozen storage and in fish muscle food samples. This present study paves a new way for the evaluation of phosphorylated protein quantitative levels in bio-samples.

INTRODUCTION

Phosphorylation is an essential post-translational modification (PTM) for regulating protein function and cellular signal transduction. Phosphorylation of proteins is a complex and highly dynamic process, and it is involved in numerous biological events.[1] Abnormal phosphorylation is one of the underlying mechanisms for the development of cancer and metabolic disorders.[1]

Numerous studies have utilized sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and western blotting techniques to measure changes in phosphorylation of the regulatory proteins.[2] Due to the limit of sensitivities, SDS−PAGE and western blotting approaches typically require a large amount of starting material to yield measurable assays of protein levels, phosphorylation, and protein−protein interactions.[3] Furthermore, the protein extraction procedures may result in the loss of functional compartmentalization and non-physiological aggregation and interactions.[4,5] Methods involved in immobilized metal affinity chromatography, chemical tagging techniques, mass mapping and precursor ion scans, the localization of phosphorylation sites by peptide sequencing, and the quantification of phosphorylation have been developed based on mass spectrometry (MS) to tackle the challenge of the analysis of phosphoproteins.[6] However, MS-based methods mostly involve relatively expensive reagents and instrumentation and the need of skilled operators.

Recently, spectroscopy-based methods have been developed for the study of phosphorylated proteins. A proof-of-concept study, based on surface-enhanced Raman scattering spectroscopy and an immobilized metal affinity strategy, was developed for the discrimination of Tau biomarkers in Alzheimer's disease.[7] An organic conjugated small molecule, WYF-1, with D−Pi−A structure was synthesized and applied for the detection of phosphorylated protein in placental tissue based

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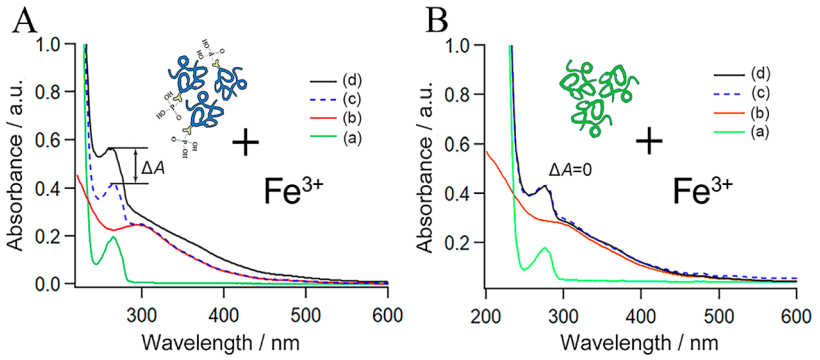

Figure 1. Proof-of-concept of (A) phosphopeptides and (B) non-phosphopeptides analyzed by UV/vis spectroscopy with the assistance of Fe^{3+}. The green solid curve is the UV/vis spectroscopic spectra of peptides (a). The red solid curve is the UV/vis spectroscopic spectra of ferric ion solution (b). The blue dashed curve is the algebraic addition of the UV/vis spectroscopic spectra of the solution of peptide and Fe^{3+} (c). The black solid curve is the UV/vis spectroscopic spectra of the resultant solution when mixing the solution of peptide and Fe^{3+} together (d). The detailed concentrations of the analyzed solutions are listed in Supporting Information S1.

on colorimetry and UV−vis spectroscopy.[8] Wang et al. developed a colorimetric sensor array for the quantification and identification of phosphorylated proteins by using a Zr-based metal−organic framework as a peroxidase mimic.[9]

Protein phosphorylation in food science has gained increasing attention. A gel-based phosphoproteomic analysis was performed to analyze the protein phosphorylation in sarcoplasmic proteins from three groups of pigs.[10] Sarcoplasmic proteins of postmortem ovine muscles were stained with Pro-Q Diamond and SYPRO Ruby after separating by SDS−PAGE to quantify protein phosphorylation by densitometric analysis.[11]

Protein phosphorylation plays a regulatory role in the contraction of skeletal muscles and myofibrillar protein degradation. A positive correlation was found between the phosphorylation level of glycogen phosphorylase and the rate of glycolysis by studying postmortem ovine muscles.[11]

The effect of phosphorylation level of proteins in postmortem muscles on the quality attributes of muscles has gained attention. The change in protein phosphorylation levels with postmortem time between muscles with different tenderness levels has been studied to understand the correlation of postmortem meat quality with protein phosphorylation. Phosphorylation of myofibrillar proteins in postmortem ovine muscles was investigated to figure out the impact of phosphorylation of myofibrillar proteins on the tenderness of sheep muscle samples.[12] Quantitative phosphoproteomics was utilized to reveal that protein phosphorylation at early postmortem in ovine muscles may indirectly affect the glycolysis pathway through the regulation of proteins involved in glycolysis and muscle contraction.[13] Li et al. studied the effect of lairage after transport on postmortem muscle glycolysis, protein phosphorylation, and lamb meat quality[14] and found that the lairage after transport treatments did not affect ($P > 0.05$) global protein phosphorylation during 24 h postmortem.[14] Furthermore, it was found that protein phosphorylation may be involved in meat color development by regulating glycolysis and the redox stability of myoglobin.[15]

Seafood is one of the most important food sources worldwide. The differences in phosphorylated proteins of seafood have been explored to understand the effect of phosphorylation of protein on the muscle traits. Quantitative phosphoproteomic analysis of fish muscle samples was performed by taking soft and firm grass carp muscles as models to reveal the phosphoproteins related to the firmness of fish muscles.[16] Flow cytometric immunofluorescence assay and enzyme linked immunosorbent assay were utilized to examine the expression variation of tyrosine-phosphorylated proteins in hemocytes of shrimp (*Litopenaeus vannamei*) after white spot syndrome virus infection.[17] More methods with characteristics of being facile and efficient for the quantification of phosphoproteins in animal-sourced food matrix can assist to understand the correlation of phosphoproteins with food quality.

The present study aims to develop a rapid and facile protocol for the quantification of phosphoproteins in food matrices. Based on ferric ions (Fe^{3+}) and UV/vis spectrometry, this method was the first to show its ultra-effective capability for the discrimination of phosphopeptides and non-phosphopeptides. The extraction of proteins with 0.1% trifluoroacetic acid (TFA) (v/v) solution from animal muscle samples coupled with Fe^{3+} was used to characterize the phosphorylation quantity in muscle samples via UV/vis spectroscopy. The presently developed method was further utilized to detect phosphopeptides and characterize the protein phosphorylation level in muscle food samples based on the interaction between Fe^{3+} and phosphorylated peptides or/and proteins. The developed protocol was applied to study the phosphorylation levels in crude protein extracts of caprine muscle, and to compare the phosphorylation levels in protein extracts of muscle samples of large yellow croaker (*Larimichthys crocea*, LC) and small yellow croaker (*Larimichthys polyactis*, LP). A peptide with one phosphorylated site was taken as a reference standard to absolutely quantify the phosphorylated proteins in caprine muscle sample during frozen storage and in fish muscle food samples. The present protocol is facile and provides a new research approach for exploring the quantitative aspect of phosphorylated proteins in animal-sourced muscle foods.

RESULTS AND DISCUSSION

Proof-of-Concept of Sensing Phosphopeptides by Fe^{3+}Solution. The advantage of UV/vis spectroscopic method for quantitative analysis is that UV/vis spectroscopy is very straightforward. Herein, a strategy for sensing phosphopeptides from a solution was developed based on Fe^{3+} and UV/vis spectroscopy. During the experiment, the concentrations were controlled with caution to meet the needs of comparison between different solution systems and the

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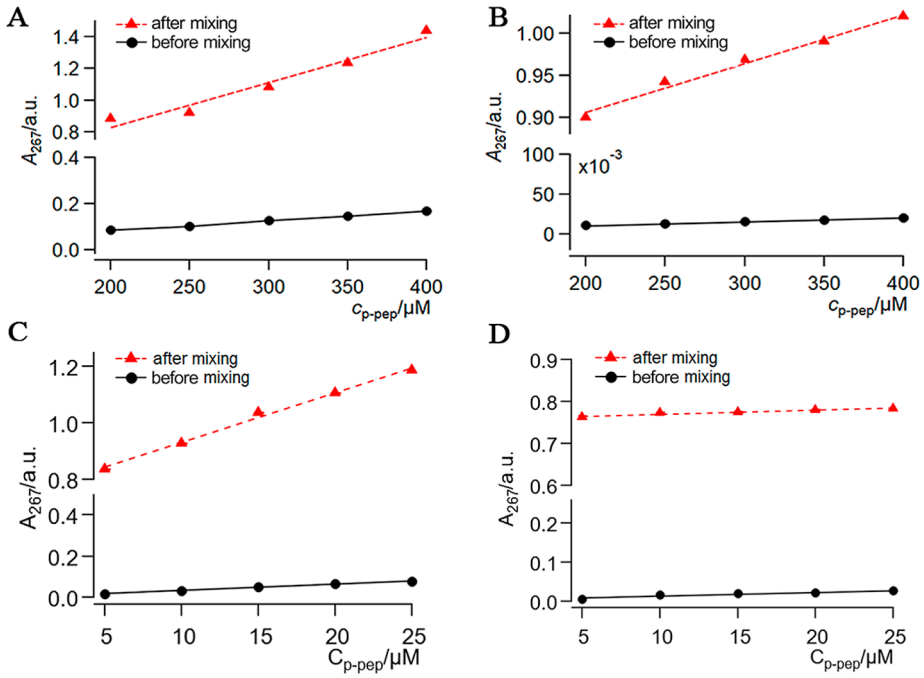

Figure 2. Plots of the absorbance at peptide absorbance wavelength (*ca.* 267 nm) *vs* the concentration of solution of phosphopeptides and non-phosphopeptides before (solid black dots) and after (solid red triangles) mixing with 1 mM of $FeCl_3$ solution at 267 nm. In A, B, and C, phosphopeptides were used and in D, non-phosphopeptides were used. (A) Single-site phosphorylated peptide; (B) double-site phosphorylated peptide; and (C) triple-site phosphorylated peptide. The concentrations listed on the *x*-axis are the final concentrations in the mixed solution. The change of concentration caused by solution mixing has been taken into account.

amount of Fe^{3+} was in excess during the interaction to keep its amount and absorbance as a pseudo-constant.

Model peptides were used to demonstrate the responses of different peptides to Fe^{3+}. Figure 1 shows the proof-of-concept results where solutions of peptides with and without phosphorylated sites were mixed with the solution containing Fe^{3+}. It can be seen that when mixing a solution of peptide without a phosphorylation site with Fe^{3+} solution, the resultant UV/vis spectroscopic curve is the algebraic addition of the UV/vis spectroscopic curves of Fe^{3+} solution and peptide solution, illustrating that the sum of UV/vis absorbance does not change, and no interaction occurs between the mixed reagents. Contrarily, when mixing a phosphopeptide solution with Fe^{3+} solution, there is an enhancement of UV/vis spectroscopic signal at the peptide absorption wavelength. The results here show that peptides with phosphorylation sites can interact with Fe^{3+}. It is promising to sense phosphorylated peptides or proteins via Fe^{3+} and UV/vis spectroscopy.

Theoretical Aspect of Interaction of Fe^{3+} and Peptides. The theoretical aspect of interaction between Fe^{3+} and peptides in solution was considered based on the Lambert−Beer law and the reaction equations of peptides and Fe^{3+}. The theoretical aspect of interaction of Fe^{3+} and peptides with and without phosphorylation sites was considered as follows.

The chelation of Fe^{3+} with phosphopeptides can be expressed as

$$Fe^{3+} + \text{P-pep} \longrightarrow \text{P-pep-Fe}$$

$$t = 0, \quad y \quad x$$

$$\text{if } y \gg x, \quad \text{when } t = t_{eq}, \quad y - x \quad 0 \quad x$$

The chelation is a relatively quick reaction, and the mono-phosphopeptide dominates the reaction. During the experiment, the measurement was done after mixing the peptide solution and ferric chloride solution. The chelation ratio of Fe^{3+} and phosphopeptides was set as 1:1 to simulate the reaction of Fe^{3+} and mono-phosphopeptides. Assuming the concentration of Fe^{3+}, *y*, is much larger than the concentration of phosphopeptide, *x*, that is, $y \geq 20x$, when the reaction reaches an equilibrium, the concentration of phosphopeptides can be infinitely near to zero. Because both ferric chloride solution and phosphopeptides have UV/vis absorbance at 264 nm (267 nm is used in the main text), the absorbance at 264 nm after the complexion of Fe^{3+} and phosphopeptides, A_{264}, can be expressed as

$$\begin{aligned} A_{264} &= \varepsilon_{Fe^{3+},264} b(y - x) + \varepsilon_{Fe(III)P-pep-,264} bx \\ &= \varepsilon_{Fe^{3+},264} by + (\varepsilon_{Fe(III)-P-pep,264} - \varepsilon_{Fe^{3+},264}) bx \end{aligned} \quad (1)$$

The values of $\varepsilon_{Fe^{3+},264}$ and the initial concentration of Fe^{3+}, *y*, are constants. Thus, the UV/vis absorbance of the mixture of Fe^{3+} and phosphopeptide solution, A_{264}, is supposed to be proportional to the concentration of phosphopeptides, *x*.

The difference of absorbance at the peptide absorbance wavelength, here it is 264 nm, after mixing with Fe^{3+}, ΔA_{264nm}, can be written as

$$\Delta \sum A_{264} = (\varepsilon_{Fe(III)-P-pep,264} - \varepsilon_{Fe^{3+},264} - \varepsilon_{P-Pep,264}) bx \quad (2)$$

Because the chelation of Fe^{3+} and phosphopeptides is supposed to always occur, the value of $\varepsilon_{Fe(III)-P-pep,264} - \varepsilon_{Fe^{3+},264} - \varepsilon_{P-Pep,264}$ should be always larger than zero. That is, the value of $\Delta \sum A_{264}$ is always larger than zero when phosphopeptides exist in a sample.

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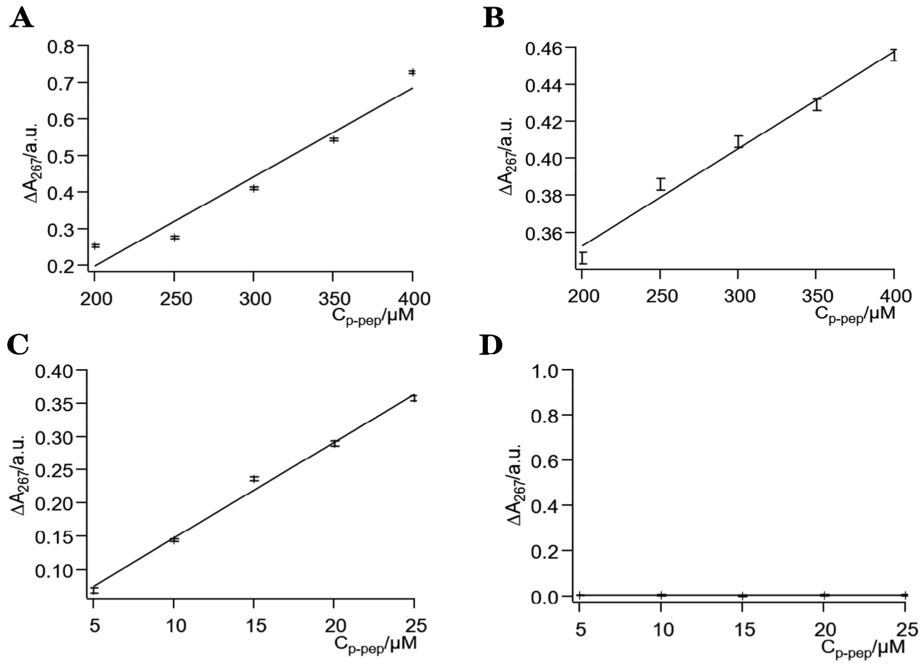

Figure 3. Plots of the enhancement of absorbance, ΔA, at 267 nm *vs* the concentration of different peptide solutions. In A, B, and C, phosphopeptides were analyzed and in D, non-phosphopeptides were analyzed. (A) Single-site phosphorylated peptide; (B) double-site phosphorylated peptide; and (C) triple-site phosphorylated peptide.

Table 1. Comparison of the Plots, Shown in Figure 3, of Absorbance of Peptide Solutions at 267 nm *Versus* the Concentration of Peptide Solutions[a]

sequence of peptides	presence of phosphorylated peptidesin a solution	$\varepsilon_{after}/\varepsilon_{before}$	ΔA is a	r^2 (ΔA vs c)
DSKRHESR	no	=1	constant	N/A
Ac-I(pY)GEF-NH_3	yes	>1	non-constant	0.9483
D(pS)KRHE(pS)R	yes	>1	non-constant	0.9832
(Yp)RGD(Yp)EKFH(Yp)	yes	>1	non-constant	0.9917

[a]The final concentration of $FeCl_3$ solution is 0.5 mM. ΔA was calculated by subtracting the absorbance of solution after mixing the solutions of peptides and Fe^{3+} from the sum absorbance of peptide solution and Fe^{3+} at 267 nm. ε_{after} and ε_{before} were calculated from Figure 2.

Contrarily, when Fe^{3+} do not react with peptides,

$$Fe^{3+} + Pep \not\rightarrow Pep\text{-}Fe(III)$$

Before mixing,	y	x
After mixing,	y	x

the absorbance at 264 nm, A_{264}, should be the sum of the absorbance of the two solutions at the corresponding wavelength. That is

$$A_{264} = \varepsilon_{Fe^{3+},264}\, by + \varepsilon_{pep,264}\, bx \quad (3)$$

If the concentration of Fe^{3+}, y, is fixed, but the concentration of peptides, x, is changed, A_{264} is proportional to the concentration of peptides.

For a solution with non-phosphopeptides, the enhancement of absorbance at 264 nm before and after mixing with Fe^{3+} does not change, that is

$$\Delta \sum A_{264} = 0 \quad (4)$$

When adding Fe^{3+}solution to a solution of non-phosphopeptides, for the peptide solution itself, the apparent absorbance difference can be calculated as

$$\Delta A_{264}(\text{apparent}) = \varepsilon_{Fe^{3+},264}\, by \quad (5)$$

It can be found that theoretically the enhancement of absorbance at a characteristic wavelength illustrates that an interaction exists between phosphopeptides and Fe^{3+}, and the absorptivity of the complex of Fe^{3+} and phosphopeptides is larger than the sum of absorptivity of Fe^{3+} and the absorptivity of phosphopeptides, respectively. That is, the difference of absorbance at the maximum absorbance of peptide, after interacting with Fe^{3+}, $\Delta\Sigma A_{264nm}$, is always larger than zero and proportional to the concentration of phosphopeptide solution, x. As a control, when Fe^{3+} do not interact with the peptides, $\Delta\Sigma A_{264nm} = 0$. These findings have been verified in the proof-of-concept results shown in Figure 1. Besides, $\varepsilon_{Fe(III)-P-pep,264}$ is larger than $\varepsilon_{Fe^{3+},264} + \varepsilon_{P-pep,264}$ as shown in eq 2.

Interaction of Fe^{3+} and Peptides. To further assess the interaction of Fe^{3+} with peptides, more peptide samples were investigated. The interactions between peptides and Fe^{3+} were revealed by studying the absorbance change of peptide solutions with and without adding Fe^{3+} solution.

Peptides, including CDPGYIGSR, Ac-I(p-Y)GEF-NH_2, D(pS)KRHE(pS)R, and Y(p)RGDY(p)EKFHY(p) were analyzed. During the experiment, solutions of phosphopeptides at different concentrations were mixed with freshly prepared ferric chloride solution. The interaction between peptides and Fe^{3+} was assessed by UV/vis spectrometry. The interaction of non-phosphopeptides and Fe^{3+} was studied as a control.

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Table 2. Absorbance Change (ΔA) Generated by Solutions with Different Peptides[a]

sample	concentration of peptide with one phosphorylation sites	concentration of peptide with two phosphorylation sites	concentration of peptide with three phosphorylation sites	equivalent concentration as peptide with one phosphorylation site	ΔA
1	43.8 μM	14.85 μM	~	ca. 74 μM	0.238 ± 0.003
2	~	29.25 μM	5.15 μM	ca. 74 μM	0.273 ± 0.153

[a] ΔA was calculated by subtracting the absorbance of solution after mixing the solutions of peptides and Fe^{3+} from the sum absorbance of peptide solution and Fe^{3+} at 267 nm. The concentrations were controlled with caution to make the comparison reasonable.

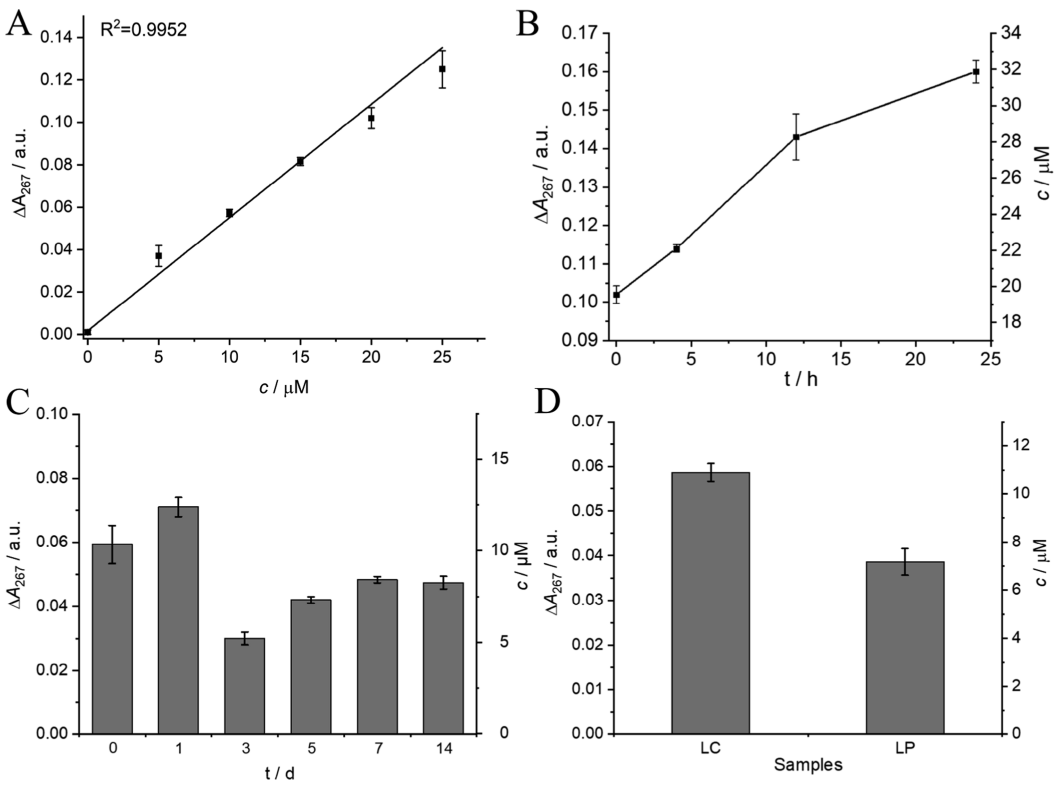

Figure 4. (A) Plot of the change of absorbance (ΔA) at 267 nm *vs* the concentration of a single-site phosphorylated peptide. The final concentration of Fe^{3+}in the mixture solution was 0.5 mM. The experiments were triplicated to obtain the means. The amino acid sequence of the detected peptide is Ac-I(p-Y)GEF-NH_2 (>98.58%, M_w 748.72). (B) Plot of phosphorylation levels in crude protein extracts of caprine muscles stored at −20 °C for different periods of time. *c* is the equivalent concentration as peptide with one phosphorylation site. Error bar is labeled at each point. The final concentration of protein sample was 50 μg/mL (BSA equivalent). The final concentration of Fe^{3+} in the mixture solution was 0.5 mM. The experiments were triplicated to obtain the mean values. (C) Bar chart/diagram of phosphorylation levels in crude protein extracts of caprine muscles at different frozen storage periods. The final concentration of protein sample was prepared as 50 μg/mL (BSA equivalent). The final concentration of Fe^{3+} in the mixture solution was 0.5 mM. The experiments were triplicated to obtain the mean values. (D) Phosphorylation levels of muscle protein extracts of two fish species. 3 g of muscle samples of *L. crocea* (LC) and *L. polyactis* (LP) were minced in 15 mL 0.1% TFA (v/v), homogenized for 2 min, heated at 80 °C for 2 min, and centrifuged at 20,000*g* for 5 min to obtain the supernatant for further analysis. LC and LP muscle samples were stored at −20 °C for 3 months. *c* is the equivalent concentration by taking peptide with one phosphorylation site as the reference standard.

Figure 2 shows the plots of the absorbance at 267 nm of the solution of these model peptides before and after interacting with Fe^{3+} *versus* the concentration. It can be observed that when peptide is not phosphorylated, the two obtained curves are parallel as shown in Figure 2D.

The absorbance enhancement, ΔA, that is, the difference between the absorbance of the mixed solutions of peptides and Fe^{3+} and the sum absorbance of peptide solution and Fe^{3+}, was calculated from the absorbance of the corresponding solutions at 267 nm. Figure 3 compares the plots of absorbance change of peptide samples at various concentrations. Table 1 lists the calculated ΔA of the peptides modified with different phosphorylation sites from zero to three. It can be seen that the calculated absorbance enhancement, ΔA, is zero when the analyzed peptide is not phosphorylated as shown in Figure 3D.

It can be observed that when the phosphorylation sites in a peptide are more than 0, the calculated ΔA is always non-constant and proportional to the concentration of peptide indicated by the correlation coefficient (r^2) whose value is close to 1.

The slopes of plots of peptide samples as models *versus* the concentration of different peptide solutions, as shown in Figure 2, with and without interaction with Fe^{3+} were calculated. It can be seen that when phosphorylated peptides are present in a sample, as listed in Table 1, the ratio of the absorptivities of solutions at 267 nm ($\varepsilon_{after}/\varepsilon_{before}$) is larger than 1, indicating that an interaction exists between them. The results further indicate that a chelation occurs between the phosphate group in phosphopeptides and Fe^{3+}. Fe^{3+} can be used to sense whether peptides contain phosphorylation sites or not.

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Characterization of Content of Phosphoproteins in Actual Samples by Fe^{3+}. To evaluate whether Fe^{3+} can be used to characterize the content of phosphoproteins in practical samples, two artificial solutions with an identical concentration equivalent to single-site phosphopeptide solution were prepared by using peptides with one, two, and three phosphorylation sites.

Table 2 lists the concentrations of peptides for preparing the mentioned peptide solutions. The obtained absorbance enhancement of the prepared artificial solutions, ΔA, is listed in Table 2. The T test can be used to determine whether there is a statistical significant difference between the means of the two groups of the obtained results.[18] Herein, ΔA, listed in Table 2, was compared by performing the T test. The result shows that the two sets of data have no significant difference, indicating that ΔA induced by the artificial solutions is very close, which may be induced by the close level of phosphorylated peptides.

The findings demonstrate that when the total number of phosphorylation sites contained by peptides in two different solution systems is in close range, the detected absorbance enhancement values are in close proximity. It is promising to apply the developed strategy to assess the amount/content of phosphproteins or/and phosphopeptides in a complex sample.

Absolute Quantification of Phosphorylated Proteins in Bio-Samples. For the purpose of absolute quantification, a peptide with one phosphorylated site can theoretically be utilized as a reference standard. The quantities of all the studied bio-samples can be equivalent to the amount of peptide with one phosphorylated site.

Herein, Ac-I(p-Y)GEF-NH_2 (>98.58%, M_w 748.72) was taken as the reference standard. The calibration curve of the reference standard with one phosphorylated site at low concentration range was established. Experimentally, aqueous solutions of Ac-I(p-Y)GEF-NH_2 were prepared for UV/vis spectroscopic measurement. Freshly prepared 1 mM Fe^{3+} solution was recommended to interact with the peptide samples. The interaction between peptides and Fe^{3+} was monitored by a UV/vis spectrophotometer. The enhancement of absorbance, ΔA, that is, the difference between the absorbance of the mixed solution of peptide and Fe^{3+} and the sum of peptide solution and Fe^{3+}, was calculated from the absorbance of the corresponding solutions at 267 nm.

Figure 4A shows the plot of the enhancement of absorbance (ΔA) at 267 nm *versus* the concentration of a single-site phosphorylated peptide with the regression equation of $\Delta A = 0.0048c + 0.007$ where ΔA is the measured absorbance enhancement and c is the concentration of peptide with unit μM. It is possible to calculate the content/level of phosphoproteins/peptides in a sample by taking the UV/vis absorbance enhancement of the standard peptide as a reference.

Comparison of Levels/Content of Proteins with Phospho-PTMs in Sheep Muscle Samples. Phosphorylation levels in animal muscles may impact the quality traits of muscle samples.[12] To verify whether the developed strategy can be used for evaluating the level/content of proteins with phosphorylated PTMs (phospho-PTMs), caprine muscle sample was used as a model. The UV/vis spectra of the protein extracts from immediately obtained caprine muscle samples were measured, and the extracted crude protein was calculated with a concentration of 90.35 ± 6.88 mg/g (n = 4) (bovine serum albumin (BSA) equivalent). During the experiment, the caprine muscle samples were stored at −20 °C for different periods of time to prepare the crude protein extracts. A combination of extracting proteins in 0.1% TFA solution with UV/vis spectrometry and Fe^{3+} was utilized to characterize the phosphorylated protein level in caprine muscle samples.

Figure 4B plots the changes of phosphorylation levels in extracts of the crude proteins of caprine muscle tissue samples at early postmortem by illustrating the absorbance enhancement and the levels of phosphorylated proteins equivalent as the reference peptide. It can be observed that there is an increase of phosphorylated proteins in the muscle samples. Analysis of variance (ANOVA), a statistical method, can be used in the testing of hypothesis for comparison of means among groups.[18,19] A significant P value of the ANOVA test indicates that the difference of means among the detected ΔA in the four groups is statistically significant ($P < 0.05$). The results show that the studied caprine muscles at early postmortem followed a similar tendency as the one in a previous research where the protein phosphorylation level at early postmortem ovine muscles was obtained by calculating the P/T ratio, where P is the intensity of phosphoprotein in the band of gel image and T is intensity of total protein.[11] The findings illustrate that the present method can be used to absolutely quantify the change of protein phosphorylation levels in muscle samples.

The changes of phosphorylation levels in extracts of the crude proteins of caprine muscle tissue samples at different periods of storage time were investigated. As shown in Figure 4C, it can be observed that there is an increase of phosphorylated proteins in the muscle samples at frozen storage time of 0 d and 1 d. At frozen storage time of 1 d and 3 d, it can be noted that there is a decrease of phosphorylated proteins in the muscle samples. However, in the 3 d, 5 d, and 7 d of frozen storage times, it can be detected that there is an increase of phosphorylated proteins in the muscle samples. A significant P value of the ANOVA test indicates that the difference of means among the detected ΔA and c in the six groups is statistically significant ($P < 0.05$), showing the difference of phosphorylated proteins during the storage time.

Comparison of Phosphorylated Protein Content in Different Fish Muscle Foods. Phosphorylation level/content of proteins in postmortem muscles of farm animals can impact quality attributes such as the tenderness and color of muscle foods. Fish samples of *L. crocea* (LC) and *L. polyactis* (LP) were taken as models to assess whether the presently developed strategy can be used to compare the level of phosphorylated protein in different animal muscle samples. The extraction of crude protein was conducted following the protocol described in the subsection of extraction of proteins from animal muscle samples. The UV/vis spectra of solutions of the protein extracts from LC and LP muscle samples, respectively, were measured, and the obtained crude protein contents of LC and LP were calculated with 1.98 ± 0.00(4) and 1.32 ± 0.01 mg/mL (BSA equivalent), respectively. The corresponding protein contents in LC and LP muscle are 79.2 ± 0.0(1) mg/g and 52.8 ± 0.0(02) mg/g (BSA equivalent), respectively.

To sense the protein phosphorylation levels in muscle samples, the protein extracts of fish muscles of LC and LP were prepared with a final protein content of 80 μg/mL (equivalent to BSA) and mixed with 1 mM Fe^{3+} aqueous solution. ΔA was calculated by subtracting the absorbance of solution after

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mixing the solutions of peptides and Fe^{3+} from the sum absorbance of peptide solution and Fe^{3+} at 267 nm. As shown in Figure 4D, the absorbance change for LC and LP are 0.059 ± 0.002 and 0.039 ± 0.003, respectively. The phosphorylation levels in LC and LP muscles are 10.764 ± 0.547 and 6.597 ± 0.597 μM (equivalent to the peptide standard), respectively. The T test applied to the detected c generated by LC and LP protein samples shows that a significant difference ($P < 0.05$) exists between the protein phosphorylation levels of the two groups. The results show that the present protocol can be used to compare the content of phosphoprotein in muscle samples during storage and evaluate protein phosphorylation levels in different animal muscle foods.

CONCLUSIONS

Protein phosphorylation is a dynamic process throughout life's activity. The changes of protein phosphorylation in postmortem animal muscle foods can affect quality attributes such as the tenderness and color of postmortem muscles of farm animals. A new strategy of extracting proteins with 0.1% (v/v) TFA solution coupled with Fe^{3+} was proposed to quantify protein phosphorylation via UV/vis spectrometry. The effect from the adsorption of the complex of phosphopeptides after interacting with Fe^{3+} was responsible for the distinct non-phosphopeptide and phosphopeptide responses. Phosphorylation levels in crude protein extracts of caprine muscles during storage were evaluated, and the obtained results are consistent with a previous study. A peptide with one phosphorylated site can be taken as the reference standard and successfully correlated the detected UV/vis absorbance enhancement at characteristic wavelength with the absolute quantification of the phosphorylated proteins in caprine muscle samples during frozen storage and in fish muscle food samples. The results show that the protein extract of muscle samples of large yellow croaker (*L. crocea*, LC) contains more quantity of phosphorylated proteins than in small yellow croaker (*L. polyactis*, LP) under the utilized conditions. The developed method is facile, straightforward, and can be used to sense the presence of phosphopeptides and characterize the phosphorylation levels in postmortem muscles of farm animals, and can potentially be applied in nutrition evaluation of postmortem muscle samples of farm animals.

MATERIALS AND METHODS

Chemicals, Muscle Food Samples, and Setups. BSA (96%) was bought from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Iron chloride ($FeCl_3$, 99%) was obtained from Titan Scientific Co., Ltd. (Shanghai, China). TFA (99%, HPLC grade) was purchased from Fisher Scientific (Loughborough, UK).

The peptides, Ac-IY(p)GEF-NH_2 ammonium salt (>97%, M_w 748.73) and angiotensin I trifluoroacetate salt (>97%, M_w1296.5), were bought from Bachem (Bubendorf, Switzerland). Ac-I(p-Y)GEF-NH_2 (>98.58%, M_w 748.72) was bought from Suzhou ChinaPeptides Co., Ltd. (Suzhou, China). D(pS)KRHE(pS)R(>98%, M_w 1174.01) was bought from Suzhou PremierBiochem Co., Ltd. (Suzhou, China). CDPGYIGSR (>98%, M_w 967.06) and Y(p)RGDY(p)-EKFHY(p) (>95%, M_w 1617.49) were obtained from Shanghai Apeptide Co., Ltd. (Shanghai, China). All the reagents were used as received without further purification. Deionized (DI) water was produced by purifying ultrapure water with a Milli-Q DI water system (0.22 μm, Millipak Express 40, Darmstadt, Germany) and used in all aqueous solutions.

Caprine (*Capra aegagrus hircus*, shortened as CAH in the present study) muscle samples were purchased from a local grocery market (Guzong Road, Pudong New Area, Shanghai, China) and brought to the laboratory within 0.5 h, washed with DI water, and cut into ca. 1 × 0.5 × 0.5 cm^3 of pieces, and stored at −20 °C until assay. CAH samples were analyzed at 4, 12, and 24 h of frozen storage. Large yellow croaker (*L. crocea*, LC) and small yellow croaker (*L. polyactis*, LP) were purchased in August 2020 with the size, weight, and probable origins listed in Supporting Information S2. Six samples of each fish species were used as biological replicates. More CAH muscle samples were purchased from Dingdong Maicai App (Shanghai, China) in November 2021. Caprine muscle samples at frozen storage periods of 0 d, 1 d, 3 d, 5 d, 7 d, and 14 d were taken for phosphorylation quantification studies. All the animal muscle samples were stored at −20 °C until analysis. The frozen muscle samples were removed from the storage freezer and thawed at room temperature for 30 min prior to further analysis.

A vortex mixer (Vortex-5) was purchased from Haimen Kylin-Bell Lab Instrument Co., Ltd. (Jiangsu, China). A handheld homogenizer (F6/10) was purchased from Shanghai Jingxin Technology Co., Ltd. (Shanghai, China). A UV−vis−NIR spectrophotometer (UV-1900) was purchased from Shimazu Manufacturing Co., Ltd. (Kyoto, Japan). A hot plate (RH digital) was obtained from IKA (Staufen, Germany). A centrifuge system (5810 R) was purchased from Eppendorf AG (Hamburg, Germany).

Extraction of Proteins from Animal Muscle Samples. 3 g of frozen muscle tissue sample of CAH was minced in 15 mL of TFA solution (0.1%, v/v) and homogenated for 2 min, and then heated at 80 °C for 2 min on a hot plate. The resultant mixture was centrifuged at 20,000g for 5 min, and the supernatant was collected for further analysis. Similarly, 9 g of fish muscle samples were obtained from six fish samples (ca. 1.5 g/fish sample) and put into a beaker with 45 mL of TFA (0.1%, v/v) solution and then homogenized for 2 min. The obtained mixture was heated at 80 °C for 2 min and centrifuged for 5 min. The supernatant was collected and diluted 10-fold with DI water for UV/vis spectroscopic measurement. The equivalent content of crude protein extracted from muscles was calculated by taking BSA as the reference with an equation of $A = 0.4759\rho_B - 0.0057$ where A is the measured absorbance and ρ_B is the concentration of protein with unit mg/mL.

Interaction of Peptides with Fe^{3+}. Peptide solution was prepared with DI water at different concentrations for UV/vis spectroscopic measurement. Freshly prepared Fe^{3+} solution was recommended to interact with the peptide samples because Fe^{3+} easily hydrolyze in water. The interaction between peptides and Fe^{3+} was monitored by the UV/vis spectrophotometer. The change of absorbance ΔA, that is, the difference between the absorbance of the mixed solution of peptide and Fe^{3+} and the sum of peptide solution and Fe^{3+}, was calculated from the absorbance of the corresponding solutions at 267 nm.

Characterization of the Content of Phosphorylation in Muscle Samples by Fe^{3+}. Caprine muscle samples, at frozen storage times of 0, 4, 12, and 24 h, were taken for phosphorylation quantification studies. Caprine muscle protein solution at 100 μg/mL (equivalent to BSA) was interacted

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with 1 mM of iron chloride ($FeCl_3$) aqueous solution. 160 μg/mL (equivalent to BSA) of fish muscle extracted protein of large yellow croaker (LC) and small yellow croaker (LP) were mixed with 1 mM of $FeCl_3$ solution for phosphorylation quantification, respectively. The UV/vis absorbance values of the related solutions at 267 nm were measured. All the experiments were at least triplicated.

Statistical Analysis. Statistical analysis on the obtained UV/vis spectrometric data was performed by using IBM SPSS Statistics 25 Software (IBM Institute Inc., Armonk, New York, USA). ANOVA was used to compare the means between groups. The T test was performed to evaluate whether the difference of means between two groups is statistically significant.

Safety Considerations. TFA is highly corrosive and irritating and is harmful to the body if it is inhaled, taken orally, or absorbed through the skin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05641.

Concentrations of solutions; information of fish samples analyzed in the present study (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Batalha, I. L.; Lowe, C. R.; Roque, A. C. A. Platforms for enrichment of phosphorylated proteins and peptides in proteomics. *Trends Biotechnol.* **2012**, *30*, 100−110.

(2) Xie, Y.; Perrino, B. A. Quantitative in situ proximity ligation assays examining protein interactions and phosphorylation during smooth muscle contractions. *Anal. Biochem.* **2019**, *577*, 1−13.

(3) Jarvius, M.; Paulsson, J.; Weibrecht, I.; Leuchowius, K.-J.; Andersson, A.-C.; Wählby, C.; Gullberg, M.; Botling, J.; Sjöblom, T.; Markova, B.; Östman, A.; Landegren, U.; Söderberg, O. In Situ Detection of Phosphorylated Platelet-derived Growth Factor Receptor β Using a Generalized Proximity Ligation Method. *Mol. Cell. Proteomics* **2007**, *6*, 1500−1509.

(4) Feist, P.; Hummon, A. Proteomic Challenges: Sample Preparation Techniques for Microgram-Quantity Protein Analysis from Biological Samples. *Int. J. Mol. Sci.* **2015**, *16*, 3537−3563.

(5) Huber, L. A.; Pfaller, K.; Vietor, I. Organelle Proteomics. *Circ. Res.* **2003**, *92*, 962−968.

(6) McLachlin, D. T.; Chait, B. T. Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* **2001**, *5*, 591−602.

(7) Ma, H.; Liu, S.; Liu, Y.; Zhu, J.; Han, X. X.; Ozaki, Y.; Zhao, B. In-situ fingerprinting phosphorylated proteins via surface-enhanced Raman spectroscopy: Single-site discrimination of Tau biomarkers in Alzheimer's disease. *Biosens. Bioelectron.* **2021**, *171*, 112748.

(8) Wang, Y.; Yang, N.; Liu, Y. Organic conjugated small molecule materials based optical probe for rapid, colorimetric and UV-vis spectral detection of phosphorylated protein in placental tissue. *Spectrochim. Acta, Part A* **2018**, *194*, 45−49.

(9) Wang, L.; Hu, Z.; Wu, S.; Pan, J.; Xu, X.; Niu, X. A peroxidase-mimicking Zr-based MOF colorimetric sensing array to quantify and discriminate phosphorylated proteins. *Anal. Chim. Acta* **2020**, *1121*, 26−34.

(10) Huang, H.; Larsen, M. R.; Karlsson, A. H.; Pomponio, L.; Costa, L. N.; Lametsch, R. Gel-based phosphoproteomics analysis of sarcoplasmic proteins in postmortem porcine muscle with pH decline rate and time differences. *Proteomics* **2011**, *11*, 4063−4076.

(11) Chen, L.; Li, Z.; Li, X.; Chen, J.; Everaert, N.; Zhang, D. The effect of sarcoplasmic protein phosphorylation on glycolysis in postmortem ovine muscle. *Int. J. Food Sci. Technol.* **2018**, *53*, 2714−2722.

(12) Chen, L.; Li, X.; Ni, N.; Liu, Y.; Chen, L.; Wang, Z.; Shen, Q. W.; Zhang, D. Phosphorylation of myofibrillar proteins in post-mortem ovine muscle with different tenderness. *J. Sci. Food Agric.* **2016**, *96*, 1474−1483.

(13) Chen, L.; Li, Z.; Everaert, N.; Lametsch, R.; Zhang, D. Quantitative phosphoproteomic analysis of ovine muscle with different postmortem glycolytic rates. *Food Chem.* **2019**, *280*, 203−209.

(14) Li, X.; Xia, A.-q.; Chen, L.-j.; Du, M.-t.; Chen, L.; Kang, N.; Zhang, D.-q. Effects of lairage after transport on post mortem muscle glycolysis, protein phosphorylation and lamb meat quality. *J. Integr. Agric.* **2018**, *17*, 2336−2344.

(15) Li, M.; Li, X.; Xin, J.; Li, Z.; Li, G.; Zhang, Y.; Du, M.; Shen, Q. W.; Zhang, D. Effects of protein phosphorylation on color stability of ground meat. *Food Chem.* **2017**, *219*, 304−310.

(16) Chen, L.; Liu, J.; Kaneko, G.; Xie, J.; Wang, G.; Yu, D.; Li, Z.; Ma, L.; Qi, D.; Tian, J.; Gong, W.; Zhang, K.; Yu, E. Quantitative phosphoproteomic analysis of soft and firm grass carp muscle. *Food Chem.* **2020**, *303*, 125367.

(17) Tang, X.; Li, X.; Zhai, F.; Xing, J.; Sheng, X.; Zhan, W. Analysis and identification of tyrosine phosphorylated proteins in hemocytes of Litopenaeus vannamei infected with WSSV. *Fish Shellfish Immunol.* **2018**, *82*, 84−91.

(18) Mishra, P.; Singh, U.; Pandey, C.; Mishra, P.; Pandey, G. Application of student's t-test, analysis of variance, and covariance. *Ann. Card Anaesth.* **2019**, *22*, 407−411.

(19) Anders, K. Resolution of Students t-tests, ANOVA and analysis of variance components from intermediary data. *Biochem. Med.* **2017**, *27*, 253−258.