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Surface Plasmon Resonance Sensor for Phosmet of Agricultural Products at the ppt Detection Level

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ABSTRACT: A surface plasmon resonance (SPR) immunoassay using a PH−BSA immobilized sensor chip was developed to measure phosmet in food samples. The in-competitive inhibition assay showed highly sensitive and good specificity of the cross-reactivity with analogue structure pesticides. The biosensor exhibited a linear detection range from 8.0 to 60.0 ng/L of phosmet with a lower detection limit of 1.6 ng/L (S/N = 3). The sensitivity obtained with the present SPR affinity biosensor was significantly higher than most of the sensors reported with different measurement methodologies for phosmet. A recovery test of pesticide quantification in peaches, apples, cabbages, and rapes was also studied. Good recoveries (86.4−102.8%) and coefficients of variation (CVs) (5.1−12.6%) were obtained in all cases. The SPR biosensor assay method was compared with cd-ELISA in terms of analysis time, antibody dosage, recoveries, precision, detection limit, pretreatment, and testing costs, and clear advantages could be seen over the traditional ELISA-based detection systems. The developed SPR method was suitable for the rapid quantitative or qualitative determination of phosmet in agricultural products.

KEYWORDS: *phosmet, surface plasmon resonance, cd-ELISA, cross-reactivity*

1. INTRODUCTION

People have shown more concern for the protection of the environment and the quality and safety of food products because of the widespread abuse of pesticides in recent years. It is crucial to control the pesticides by a rapid search for sensitive and reliable technology to detect and quantify pesticides and monitor the environment. In modern biomedical technology, the development of surface plasmon resonance (SPR) based immunosensors with high selectivity in complex matrixes is playing a key role in addressing the accurate characterization of emerging human health challenges and in accomplishing environmental analysis.[1,2]

Phosmet is a common organophosphorus insecticide used for pest control. Residues of this pesticide have been found in fruits and vegetables.[3] Since the 1970s, EPA has developed a special regulatory program to deal specifically with pesticide contamination of agricultural products, but most analytical methods for the determination of phosmet are instrumental methods, such as HPLC[4,5] and GC/MS[6−9] methods. The customary analytical methods provide sensitive and specific techniques; however, they are laborious, expensive, and need skilled personnel. In 2009, a competitive direct enzyme-linked immunosorbent assay (cd-ELISA) for the rapid detection of phosmet in fruits was developed.[10] Immunoassay methods based on a specific antigen−antibody biorecognition event have become a promising alternative for the specific determination of target analytes direct from complex sample matrices, because of the high affinity and selectivity of antibodies and of the technology to produce antibodies for any organic compound.[11]

In recent years, SPR immunosensors have been growing rapidly. Several SPR optical biosensor assays have been developed for detecting low levels of contaminant residues in food,[12,13] for it has a highly specific detection of small molecules with extraordinarily low detection limits for a wide variety of analytes in complex matrices.[14−19] SPR is advantageous because the labeling of antibodies or enzymes is not required. The SPR biosensor approach is a label-free technique which for detection relies on an increase in molecular mass due to antibody−analyte interactions on a chip surface. Furthermore, the assay performance is frequently enhanced through automation and real time analysis. The cost of SPR biosensors is higher than ELISA, but the savings can be seen through automation, which reduces labor costs and improves reliability of results; besides, the sensor chip can be tested several times continuously. A further advantage of the technique is the simplicity of assay transfer between laboratories through reduction of the operator effects through the elimination of plate washing and incubation steps.[20] Overall, SPR has inherent advantages over other types in its versatility and compatibility with portable instrumentation, miniaturization, and real-time analysis.

In this work, we devoted ourselves to develop an SPR-based immunosensor working with in-competitive reaction for selective and sensitive detection of phosmet in vegetables and fruits, which is also suitable for field tests. In addition, the developed detection methods were compared with cd-ELISA, clear advantages of SPR biosensor assay can be seen over traditional ELISA based detection systems, which are frequently

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dedicated to specific assays, require laborious sample preparation and take longer to setup prior to analysis.

2. MATERIALS AND METHODS

2.1. Reagents. Phosmet was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A 1 mM stock solution of phosmet was prepared in dry *N*,*N*-dimethylformamide (DMF) and stored at −20 °C. Working standards were freshly prepared from concentrated stock by sequential dilution in PBS or PBST buffer (PBS 10 mM phosphate 135 mM NaCl with 0.05% of Tween 20, respectively). 11-Mercaptoundecanoic acid (MUA) was purchased from J&K Scientific Ltd. (Europe). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC&HCl) and *N*-hydroxysuccinimide (NHS) were obtained from Aladdin (Shanghai, China). Chlorpyrifos-methyl, parathion, azinphos-methyl, and azinphos-ethyl were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All other chemicals were analytical grade and purchased from Tianjin Chemical Co. Ltd. (Tianjin, China).

The antibody against phosmet used to develop the immunoassay has been described by Song et al.[10] The phosmet hapten was coupled to BSA as conjugated antigen. The method of conjugation was the active ester method, which has been described previously by Wang et al.[21]

2.2. Instrumentation. The SPR biosensor (Autolab ESPRIT) and gold-coated glass SPR sensor chips (Au thickness, 50 nm) were obtained from Eco Chemie B.V. (Holland). BIAcore 3000 instrument (Eco Chemie B.V.) is a system for real-time analysis of biomolecular interaction using SPR technology. Measurements were made under conditions of a continuous flow, where the detection surface consists of the four flow cells in a sequential array on the sensor chip. All solutions were prepared using ultrapure water (18 M/cm) obtained from a Milli-Q ultrapure water system. The temperature inside the apparatus was kept at 25 °C. All experiments were performed at a flow rate of 15 μL min^{-1}, unless mentioned otherwise. All SPR signals were represented in resonance value (m°), where the resonant angle change of 0.1° corresponds to the mass change of about 1 ng mm^{-2} of protein on the surface of the sensor chip. A scanning electron microscope (SU 1510) Japan was used for topographic imaging of conjugate immobilized gold surface. The HPLC (LC-10AT pump and SPD-10 AVP built-in UV−visible detector, Shimadzu, Japan) with a C_{18} reversed-phase column (15 cm × 4.6 mm i.d., 5 μm) was used for analyzing phosmet (at 218 nm).

2.3. Immobilization for the Preparation of Sensor Chip. Prior to gold coating, the glass substrates were carefully cleaned using "piranha" solution (3:1 mixture of H_2SO_4 and H_2O_2), and then the gold chips were washed using ethanol and distilled water. The gold chips were prepared by 50 nm of high-purity gold on the glass substrates. The Au surface of SPR chip was modified by coating with mercaptoundecanoic acid (11-MUA), and the self-assembly process was monitored by SPR angle measurements.

During the immobilization process, PH-BSA was immobilized onto a sensor chip by the amine coupling kit. pH 4.5 acetate buffer was used as a running buffer at a flow rate of 15 μL/min. The sensor chip was activated by injection of 50 μL of 0.1 mol/L NHS and 0.4 mol/L EDC (1:1, v/v). 20 mg/L PH−BSA diluted with acetate buffer was then loaded on the activated sensor chip. Finally, 1 mol/L ethanolamine solution (pH 8.5) was used as a blocking solution. Each step, including activation, immobilization, and blocking was carried out for 600 s. The sensor chip was regenerated by 0.1 mol/L HCl. (Figure 1)

2.4. Indirect Competitive Method for Phosmet Measurement. The incident wavelength of the ESPRIT SPR biosensor is 670 nm. The intensity of reflected light is measured over a range of 4000 mill degrees (m°). The SPR angle of a buffer solution can be fixed manually by a spindle with an offset SPR angle of 62−78°, which corresponds to a refractive index range of 1.33−1.43 of the sensor surface. The SPR angle resolution is less than 0.02 m°, and the average baseline fluctuation is 0.l m°. 180 Da is the minimum molecular weight that can be detected by the SPR sensor.[22] The reaction temperature is controlled at 25 ± 0.5 °C. The data acquisition software is Autolab

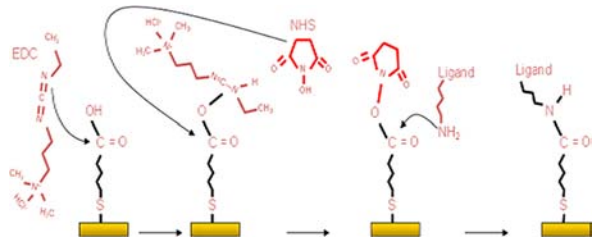

Figure 1. The principle of immobilization.

ESPRIT Data Acquisition 4.3. The curet is separated into two areas (channel 1 and channel 2) to simultaneously monitor two macromolecular interactions. One of the channels is treated as the blank control in the experiments. Furthermore, the instrument is equipped with a continuously mixed cuvette but not a flowcell. After a certain sample volume was injected, the sample was mixed on the polymer-coated gold chip for reaction.

10 mmol/L PBS buffer was used as a running buffer at a rate of 15 μL/min, and 0.1 mol/L HCl was used for sensor chip regeneration. Contact times were 900 s for sample injection and 300 s for regeneration. Before injection, different standard concentrations of phosmet in 0, 0.5, 2, 8, 16, 32, 64, 256 ng/L were prepared by serial dilutions in PBS from a stock solution of 1 mM phosmet in methanol. 1:1 sample mixtures were obtained at concentration of 8 mg/L antibody solutions in PBS buffer were incubated for 10 min at room temperature (the amount of antibody was optimized; detailed data were not given). The binding between the immobilized hapten derivative and antibodies with free binding sites was monitored in real-time. After each affinity binding reaction, 0.1 mol/L HCl solution was used as regeneration reagent to break the antibody−analyte association. All measurements for phosmet concentration were repeated at least three times and good reproducibility was observed. The time for the affinity reaction was 30 min.

2.5. Sample Preparation. To validate the immunosensor performance, fruit and vegetable samples were analyzed by both the immunosensor and HPLC methods. Vegetable samples were bought from local markets. Before the spiking and recovery studies, each test sample was verified that it did not contain phosmet by HPLC.

2.6. Sample Extraction. *For SPR Analysis.* Two gram samples were spiked with phosmet at three different levels (0.5, 1, 2.5 μg/kg). The spiked samples were mixed with 10 mL of acetonitrile and then centrifuged at 3500 rpm at 4 °C for 5 min. The clear supernatant was diluted with PBS buffer 10-fold, and the solution was mixed with antibody equally and filtered using a 0.22 μm filter and finally injected into the SPR system.

For HPLC Analysis. The extraction procedure of phosmet for application to HPLC was same as that for SPR. The supernatant was cleaned up using solid phase extraction (SPE) cartridges (Strata C18-E, 55 μm, 70 Å, 1000 mg 6 mL^{-1}). SPE cartridges were first conditioned with 5 mL of methanol and 5 mL of water, then extracts were applied, followed by washing with 40% methanol in water, and phosmet was eluted from the column with 4 mL of methanol. The elution filtered using a 0.22 μm filter was analyzed by HPLC.

2.7. HPLC Determination. Assay validation was conducted using a Shimadzu HPLC. A C18 reversed-phase column (15 cm × 4.6 mm i.d., 5 μm) was used. Analyses were performed at 218 nm, the mobile phase was acetonitrile−water (45:55, v/v) at a flow rate of 1.0 mL min^{-1}. The temperature of the column oven was 35 °C.

3. RESULTS AND DISCUSSION

3.1. Characterization by Scanning Electron Microscope. In this study, we examined formation and characterization of the immobilized antigen by SEM. Figure 2a,b showed, with an SU1510 scanning electron microscope at 2000 times magnification, the whole antigen protein molecules at different observation positions, fixed with the chip surface. We can clearly see the macromolecular protein distribution in the

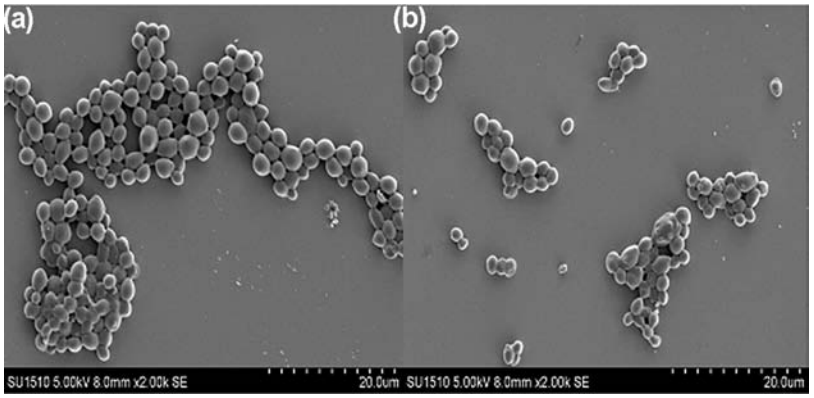

Figure 2. (a, b) Scanning electron microscopy of antigens in different areas.

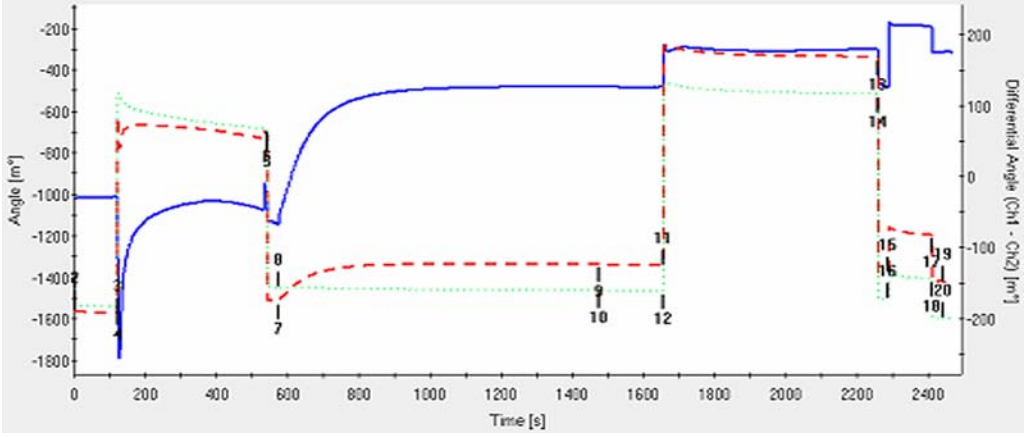

Figure 3. The results of the immobilization process.

gold surface; the protein molecule is positively sphere shaped, size is uniform, the surface is smooth, and tight and rare protein molecular overlap distribution, so that we determined the complete antigen was fixed to the chip surface.

3.2. Fabrication of the Biosensor Surface by Conjugate Immobilization. PH−BSA was immobilized using a conventional amine coupling method (Figure 1). After the immobilized step, amount of PH−BSA remained 540 m°. The interaction between antibody and immobilized PH−BSA on the sensor chip was determined by monitoring real-time response value changes in PBS buffer. The antibody after Reproducibility can be detected 50 times with an RSD of 0.93%. Figure 3 showed the result of the SPR immobilization process with the concentration of PH−BSA of 20 μg/mL. From the binding curve, we found that the immobilization reaction nearly saturated after 7 min. Accordingly, in the subsequent immobilization of the process, the reaction time is set at 7 min, in order to save time.

3.3. Surface Regeneration Research. The PH−pAb antibody bound on the PH−BSA sensor surface has to be detached without destroying the base conjugate layer for multiple use of the same sensor chip. The regeneration process determines the chip of reproducibility, stability, and accuracy, which further decided the practical life of the chip. The bond between the antigen−antibody with elution by a certain concentration of acid, alkali, or high ionic strength of electrolyte solution, and regeneration in conditions (such as acids, alkalis, etc.) easily led to the sensor chip substrate failure or fixed protein inactivation.[23] Regeneration is a noncovalent destruction of antigen−antibody binding. The process is to remove the antibody residues on the chip, to prevent follow-up experiments; keep the surface antigen of the chip, to maintain its activity; remove nonspecific adsorption on the chip, to prevent the accumulation of the chip. In the present work, different regeneration conditions including pH 2.5 glycine-HCl, 20% 0.1 mol/L NaOH +80% CH_3CN, 0.1 mol/L NaOH, and 0.1 mol/L HCl were studied by adjusting pH and ionic strength to optimize the most suitable experimental regeneration liquid. From Figure 4, pH 2.5 glycine-HCl after five times regeneration, the antigen−antibody cannot be separated; the cumulative response value of the baseline response therefore has a certain impact on regeneration, and the chip's life has

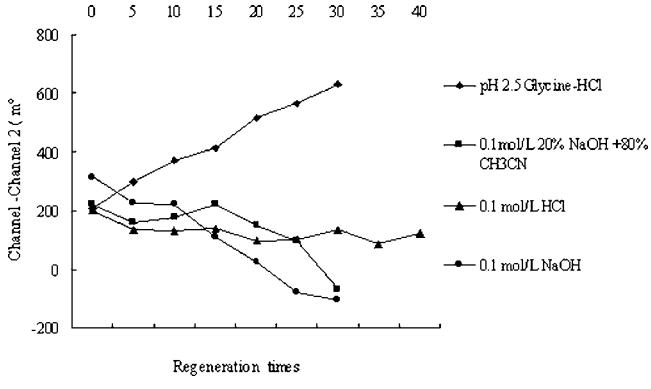

Figure 4. Baseline shift with different regeneration solutions.

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been shortened. For 0.1 mol/L of NaOH + CH_3CN, and 0.1 mol/L NaOH regeneration solution, the renewable baseline decreased significantly after 20 times. Because the strong elution ability of regeneration solution will change the activity of the chip surface antigen or part of their elution, the baseline signal decreased significantly. Ultimately, 0.1 mol/L HCl was chosen as the regeneration solution. Because after regeneration 40 times by 0.1 mol/L HCl solution, no significant changes appeared.

3.4. Detection of Phosmet by SPR Imaging. The detection principle of the present sensor is based on the indirect competitive immunoreaction of analyte with its antibody and has been described briefly. The antibody was first mixed with phosmet, and the mixture was injected into the sensor chip. The portion of antibody, which did not bind to phosmet but to the PH−BSA immobilized sensor chip, was detected by SPR. Response values obtained by SPR were correlated with the phosmet concentrations.

Here, equal volumes (16 μg/mL) of anti-PH were incubated with different concentrations of phosmet for 10 min and introduced over the PH−BSA immobilized gold-surface. The concentration of phosmet was 0.5, 2, 8, 16, 32, 64, 256 ng/L, respectively. Figure 5 shows an overlay plot of the sensograms

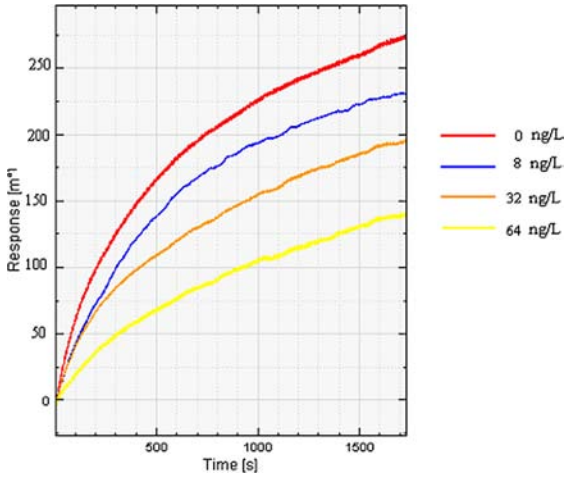


Figure 5. The SPR curves with different phosmet concentrations.

observed for the affinity reaction between immobilized PH−BSA conjugate and anti-PH in the absence of 0 ng/L and in the presence of 8, 32, 64 ng/L phosmet, respectively.

The dependence between the concentration of phosmet as a standard curve is shown in Figure 6. The sigmoidal calibration curve indicates clearly the inhibition of the binding interaction between anti-PH and PH−BSA conjugate in the presence of

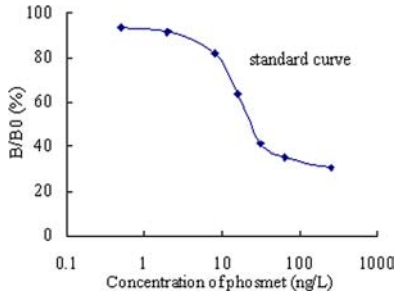
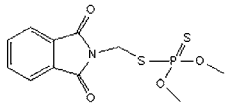
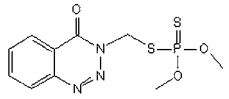
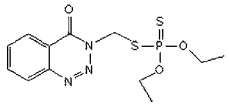
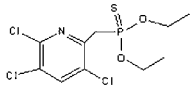
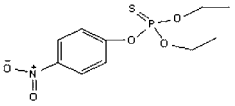


Figure 6. The standard curve of detection with phosmet by SPR.

phosmet. The percentage of inhibition with respect to the phosmet concentration is also shown in Figure 6. The extent of inhibition depends linearly with the concentration of free phosmet mixed with anti-PH. Under the optimal conditions, the biosensor exhibits a linear detection range from 8.0−60.0 ng/L of the phosmet with a lower detection limit of 1.6 ng/L (S/N = 3). The sensitivity obtained with the present SPR affinity biosensor is significantly higher than most of the sensors reported with different measurement methodologies for phosmet. The high sensitivity observed with the present biosensing system is possibly due to the combined advantages of the high affinity interaction between anti-PH and phosmet, the sensitivity of the SPR instrumentation, the unique surface chemistry of immobilized PH−BSA conjugate, and the indirect competitive inhibition assay principle.

3.5. Specificity Studies. Cross-reactivity of the fabricated SPR-based immunosensor to other possible interferents present is examined. For this experiment, four different aromatic compounds were chosen: chlorpyrifos-methyl, parathion, azinphos-methyl, and azinphos-ethyl, which have structural resemblance to phosmet. The chemical structure and the summarized analytical data are shown in Table 1. Azinphos-

Table 1. Cross-Reactivity of Phosmet with the Related Compounds in SPR

Compound	Chemical structure	IC_{50} (ng/L)	CR (%)
Phosmet		25	100
Azinphos-methyl		1000	2.5
Azinphos-ethyl		6000	0.4
Chlorpyrifos-methyl		>10000	<0.01
Parathion		>10000	<0.01

methyl and azinphos-ethyl which have similar aromatic structures to the hapten of interest were shown with a cross-reactivity of 2.5% and 0.4%, respectively. For chlorpyrifos-methyl and parathion which contain phosphate groups, their cross-reactivity was negligible (<0.01). This result indicates that the antibody has strong specificity between PH−BSA conjugate chips in SPR Biosensor to phosmet. Furthermore, the indirect competitive inhibition assay principle also plays an important role. Overall, the antigen coated gold chip exhibited high selectivity, again confirming that it is suitable for detecting phosmet in real samples.

3.6. Samples Detection on SPR and HPLC. Maximum residue levels (MRL) for phosmet have been fixed at 1 mg/kg for rape and cabbage products, and 0.5 mg/kg for apple and peach in Japan. SPR assays developed in this study have enough sensitivity for the rapid screening of phosmet in accordance with the MRL levels. Recovery test results for the detection

assay of phosmet at concentrations of 10, 20, and 50 ng/L are shown in Table 2. Recovery test of phosmet was performed.

Table 2. Recovery Studies of Four Samples at Three Levels by SPR (n = 3 Replicates)

sample	spiked (ng/kg)	average ± SD (ng/L)	recovery study (%)	CV (%)
cabbage	10	9.0 ± 1.0	86.4	11.6
	20	17.4 ± 2.2	87.1	12.6
	50	50.7 ± 8.0	101.4	8.4
rape	10	10.1 ± 1.2	100.1	11.9
	20	18.2 ± 2.0	91.0	11.0
	50	51.4 ± 8.7	102.8	8.2
apple	10	8.8 ± 0.6	87.8	6.8
	20	19.7 ± 1.1	98.5	5.6
	50	46.6 ± 4.2	93.2	9.0
peach	10	9.2 ± 0.9	91.9	9.8
	20	18.0 ± 1.2	90.1	6.7
	50	46.9 ± 2.4	93.9	5.1

Good recoveries (86.4−102.8%) and coefficients of variation (CVs) (5.1−12.6%) were obtained in all cases for each fortified sample determined three times. The correlation between the biosensor and HPLC methods was obtained from detecting treated apple samples spiked the phosmet concentrations of 10 ng/L, 20 ng/L, and 50 ng/L (n = 3). The good agreement (r^2 = 0.9976) we obtained is shown in Figure 7. The SPR methods were suitable for the accurate quantitative or qualitative determination of phosmet in food samples.

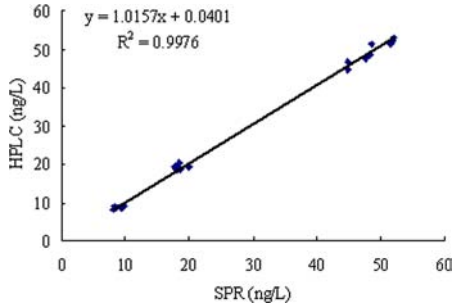


Figure 7. Correlation between SPR and HPLC results for apple samples spiked with phosmet.

3.7. Comparison with SPR and cd-ELISA. In this paper, the same sample preparation process was used for phosmet analysis using SPR biosensor and cd-ELISA. The two analytical procedures were evaluated for analysis time, antibody dosage, recoveries, precision, detection limit, pretreatment and testing costs. Specific data are as shown in Table 3.

In SPR experiments, the amount of antibody is 8 μg/mL less than the amount of ELISA experiments. It reduced antibody consumption. For sensitivity, connected to the sensor chips of the antigen, the detection range and LOD were 8.0−60.0 ng/L and 1.6 ng/L of phosmet, while the scope of testing using the cd-ELISA in 1−100 ng/L with the IC_{15} and IC_{50} was 0.6 and 20.0 ng/L. Therefore, it is clear that the SPR method has much a higher sensitivity to cd-ELISA, a but slightly narrower linear range may increase the difficulty for the sample pretreatment. The mean recovery of 20 ng/L was chosen to compare accuracy and precision of two analytical procedures. Accuracy and precision in spiked apple samples, SPR biosensor, and cd-ELISA were 98.5% and 78.4%, 5.6% and 8.0%, respectively. This result indicated that strong specificity between antibody and derivative in SPR biosensor reduced sample matrix interference. Two methods were both satisfactory while the matrix effect of cd-ELISA was more significant than the SPR biosensor.

Table 3. Comparison of SPR Biosensor and cd-ELISA[a]

	SPR	ELISA
antibody dosage	8 μg/mL	10 μg/mL
LOD (S/N = 3)	1.6 ng/L	
IC_{15}		0.6 μg/L
IC_{50}	25 ng/L^n	20.0 μg/L
linear range	8.0−60.0 ng/L	1−100 μg/L
detection time	30 min	2.5 h
testing costs	the same chip can be detected in 30 times	one-time use
pretreatment	solid extracted with acetonitrile	solid extracted with methanol
recoveries study (%)	86.0−103.0	78.4−102.3
precision (%)	<12.6	<18.0

[a]IC_{50} used to indicate the sensitivity in immunological methods, "n" in the table represents the sensitivity of SPR method to compare with the ELISA parameter.

A complete assay cycle, including regeneration, is accomplished in 30 min, while cd-ELISA required 2.5 h for detection. The chip can be repeatedly tested 30 times with the appropriate regeneration solution. The results are stable with the coefficient of variation below 5.0%. Therefore, in the SPR experiments, the service life of the chip changed the reaction 30 times, compared to single-use microtiter plates in the ELISA assay, with a significant savings in cost.

3.8. Conclusion. An indirect format where a PH−BSA conjugated onto a gold surface with self-assembled monolayer was shown with high sensitivity, selectivity, and good stability. The calibration curve of the SPR chips demonstrated good linearity range of 8.0−60.0 ng/L and a detection limit of 1.6 ng/L (S/N = 3), which were comparable to the values obtained by conventional cd-ELISA. The suitability of SPR for pesticide quantification in peach, apple, rape, and cabbage was developed with a satisfactory recoveries of 86.4−102.8% and CVs of 5.1−12.6% (n = 3). The SPR complete assay cycle including regeneration was accomplished in 30 min, and the binding of bioactive surface and antibody remained stable for 30 cycles. The described methodology allows an overall fast and selective detection of phosmet in food samples and had a good agreement with HPLC methods. It can be used as an alternative method for determining phosmet in food samples.

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Notes

The authors declare no competing financial interest.

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