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Development of a Capillary Electrophoresis-Based Immunoassay with Laser-Induced Fluorescence for the Detection of Carbaryl in Rice Samples

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A capillary electrophoresis-based competitive immunoassay (CEIA) with a laser-induced fluorescence (LIF) detector for the determination of carbaryl was developed. The method was based on the competitive reactions between fluorescently labeled carbaryl tracer (Ag*) and free carbaryl (Ag) with a limited amount of anticarbaryl antibody (Ab), and the relative amounts of each were separated and determined by capillary electrophoresis (CE) with an LIF detector. Using CEIA, equilibrium was reached in 30 min, and the analytical results were obtained within a further 8 min. The linear range and the detection limit for carbaryl were 0.16−50 ng/mL and 0.05 ng/mL, respectively. The sensitivity of this CEIA with an LIF detector was almost 14 times greater than that of ELISA, which used the same immuno-reagents. The method was also applied to the analysis of carbaryl in rice with rapid and simple sample pretreatment. The method is thus proposed as a fast and sensitive assay for the detection of carbaryl.

KEYWORDS: Capillary electrophoresis-based immunoassay; laser-induced fluorescence; carbaryl; fluorescently labeled carbaryl tracer

INTRODUCTION

Pesticides are widely used throughout the world to prevent crop losses. The toxicity of many pesticides has raised public awareness of the consequences of their presence in the environment upon the ecosystem and human health.

Carbaryl (1-naphthol-*N*-methylcarbamate) was the first successful carbamate insecticide used due to its broad-spectrum efficacy to control insecticide pests. It is widely used in chinese agricultural systems to combat a large number of pests. It affects the behavior of amphibian communities at sublethal levels (*1*) and has been reported as a neurotoxin with toxicity close to that of some chemical warfare agents (*2*). The presence of traces of carbaryl in agricultural products poses a potential hazard for consumers because of their bioaccumulation in food and the subsequent bioconcentration through the food chain.

Several methods have been used for carbaryl determination such as the chromatographic assay (*3, 4*), ELISA, or immunosensor formats (*5*−*9*). Capillary electrophoresis (CE) as a modern separation technique has emerged recently. The prospects for CE in pesticide analysis are very promising because of its advantages, such as higher separation efficiency, high analysis speed, and very small consumption of expensive reagents and toxic solvents. There are a few reports for the detection of carbaryl using CE. They are capillary zone electrophoresis with ultraviolet (UV) detection or amperometric detection and micellar electrokinetic chromatography with UV detection or on a microchip (*10*−*13*). However, the main drawback of these methods by CE for the analysis of carbaryl is the lack of sensitivity as compared with the immunoassay (IA). Three detectors (UV, laser-induced fluorescence (LIF), and mass spectrometric (MS)) are commonly combined with CE. UV detection is a standard detection method today but is less sensitive than LIF and MS. For MS detection, pretreatment and purification of samples are required. LIF is the most sensitive detection method for CE when the analyte is labeled with a fluorescent marker (*14*).

Combining the effective separation power of CE and the ligand specificity of IA, CEIA has proved to be a powerful technique for the separation and analysis of biological fluids and other complex matrices. It also shows some advantages such as high detection sensitivity and capability for simultaneous analysis of multicomponents. By CEIA, the assay time is faster because the immunoreaction occurs in solution, which allows rapid reaction by solution phase kinetics, and CEIA permits direct visualization of immunocomplex formation and dissociation and simplified the interpretation of the test results. So far, most of the CEIA methods rely on an LIF detector. It has been

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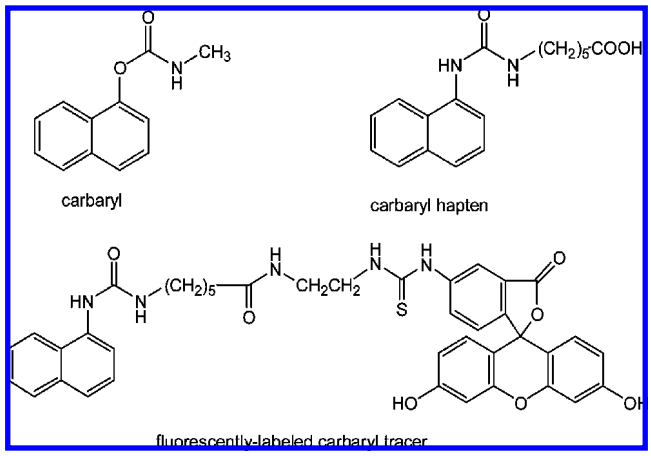

Figure 1. Structures of carbaryl, carbaryl hapten, and fluorescently labeled carbaryl tracer.

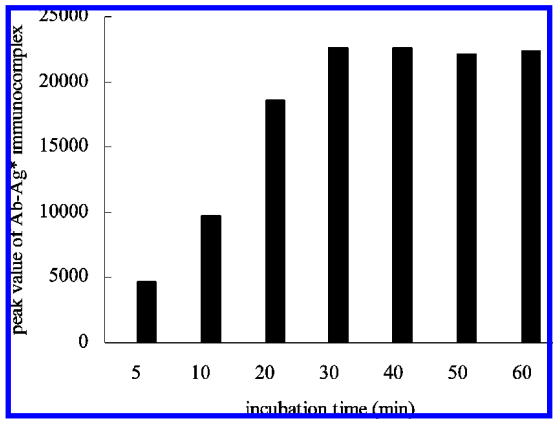

Figure 2. Equilibration study for carbaryl by CEIA with an LIF detector.

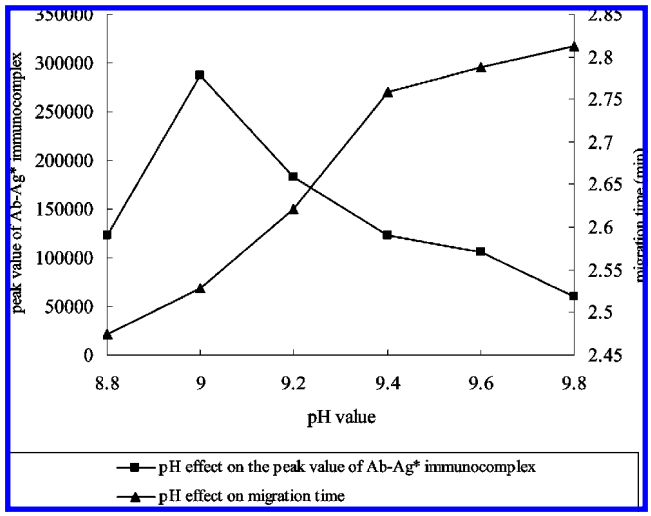

Figure 3. Effect of pH for running buffer on separation.

applied to a wide range of compounds including vasopressin (*15*), hirudin (*16*), estrone (*17*), and clenbuterol (*18*), and so forth. However, carbaryl detection using CEIA with an LIF detector has not been studied so far.

In this article, a method for the determination of carbaryl was developed by CEIA with an LIF detector based on the competitive reaction between a fluorescently labeled carbaryl tracer and free carbaryl with a limited amount of anticarbaryl antibody. The comparison for the detection of carbaryl using the two methods (both CEIA with an LIF detector and ELISA, which used the same immunogen) was also investigated. In China, rice is widely planted as the main agricultural product, and it is closely related to people's life as a daily staple food. Therefore, the developed method was applied for the analysis of carbaryl in rice samples.

MATERIALS AND METHODS

Instrumentation. All experiments were performed on an automated P/ACE5500 CE system (Beckman Instrument, Fullerton, CA, USA) fitted with an LIF detector (excitation at 488 nm and emission at 520 nm). Untreated fused-silica capillary (Yongnian Optical Conductive Fiber Ltd., Hebei, China) with an inner diameter of 75 μm and total length of 40.2 cm (30 cm to the detector) was used. The capillary column was preconditioned by successively flushing with methanol, 1 M NaOH, 1 M HCl, H_2O, and buffer for 10 min. Mass spectra were obtained using an electrospray ionization (ESI) source on an ion trap mass spectrometer (Finnigan LCQ Advantage MAX, San Jose, CA, USA).

Chemicals and Reagents. Fluorescein isothiocyanate (isomerI) (FITC), ethylenediamine dihydrochloride, poly (*N*-isopropyl-acrylamide), *N*-hydroxysuccinimide, dicylohexylcarbodiimide, tetrahydrofuran, dimethylformamide, and carbaryl were purchased from Sigma (St. Louis. MO, USA). Protein A-Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). TLC was performed on silica gel G (Yumin Yuan Silica Gel Reagent Factory, QingDao, China). All other reagents were of the highest grade available and at least of analytical grade.

Synthesis of Immunoassay Reagent and Fluorescently Labeled Carbaryl Tracer. Fluorescein thiocarbamyl ethylenediamine (EDF) was synthesized as previously described by Pourfarzaneh (*19*). One milliliter of 30 μmol FITC (isomer I) dissolved in methanol containing 10 mL/L triethylamine was mixed with a 5 mL solution of 150 μmol ethylenediamine dihydrochloride in methanol containing 10 mL/L triethylamine. The developed orange precipitate was filtered and washed with 1 mL methanol, then dried in a vacuum oven overnight at room temperature, and used for further coupling to the carbaryl hapten.

The synthesis of carbaryl hapten was previously described by Marco et al. (*20*). The chemical structures of carbaryl, carbaryl hapten, and the fluorescently labeled carbaryl tracer are shown in **Figure 1**. Hapten was then coupled to keyhole limpet hemocyanin (KLH) for use as immunogens, or coupled to EDF as the fluorescently labeled tracer. Carbaryl hapten, which contained −COOH, was first converted to the form of active ester with the addition of *N*-hydroxysuccinimide (NHS) and dicylohexylcarbodiimide (DCC) in dired tetrahydrofuran. Then the active ester of carbaryl hapten was reacted with EDF at the molar ratio of 4:1 dissolved in dimethylformamide and stirred at room temperature overnight in the dark. The yellow solution that formed was filtered through a 0.45 μm nylon membrane. The product was purified by thin-layer chromatography (TLC) using CH_2Cl_2/CH_3OH (6:1, v/v) as the mobile phase. The three major bands observed under UV light on the TLC plate were collected (R_f = 0.10, 0.30, and 0.72) and were extracted with methanol for further testing (MS and CEIA).

Antibodies (Abs) were produced in rabbits as described by Wang et al. (*21*). Female New Zealand white rabbits were immunized by intradermal and intramuscular injections of carbaryl haptens conjugated to KLH. IgG from the antisera was purified by Protein A-Sepharose affinity chromatography. Anticarbaryl antibody (2.5 mg/mL) was 10-fold diluted with PBS before analysis.

Capillary Electrophoresis-Based Competitive Immunoassay. Fluorescently labeled carbaryl tracer (Ag*) competed with carbaryl (Ag) for binding to a limited amount of anticarbaryl antibody. Ab, Ag, and Ag* were mixed in solution. Conditions for CE-based competitive immunoassay were as follows: fluorescently labeled carbaryl tracer was diluted 1:200000. One hundred microliters of carbaryl standard (from 0.01 to100 ng/mL) prepared in pH 7.2 PBS buffer containing 10% methanol was mixed with 100 μL of fluorescently labeled carbaryl tracer. Then 2 μL of anticarbaryl antibody (0.25 mg/mL) was added to the mixture. After incubation to allow equilibration, the solution was injected into the capillary and analyzed. The CE conditions were as follows: the temperature was maintained at 20 °C, the applied voltage

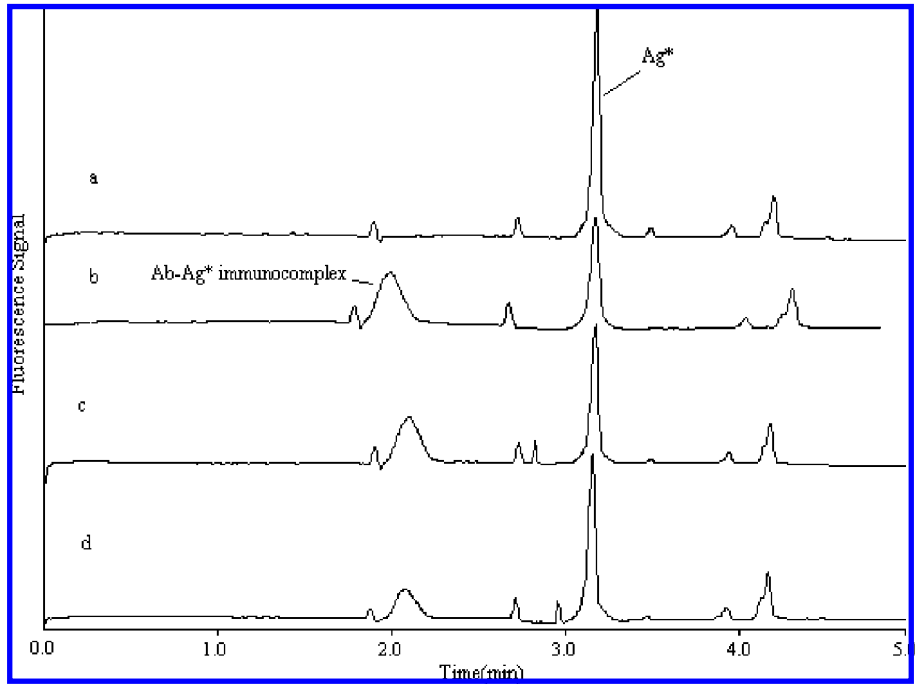

Figure 4. CEIA with an LIF detector for carbaryl detection. (**a**) Only free Ag* without carbaryl and anticarbaryl Ab; (**b**) control (free Ag*(1/200000) binding with 0.5 μg of anticarbaryl Ab without the addition of carbaryl); (**c**) 0.5 ng/mL carbaryl and free Ag*(1/200000) competed for binding with 0.5 μg of anticarbaryl Ab; (**d**) 5 ng/mL carbaryl and free Ag* (1/200000) competed for binding with 0.5 μg of anticarbaryl Ab.

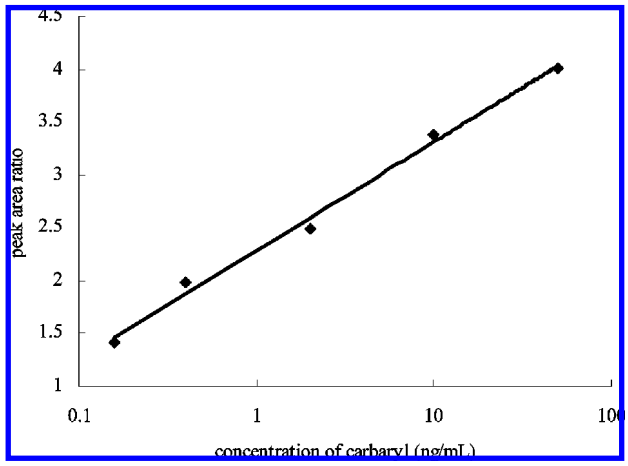

Figure 5. Calibration curve of carbaryl by CEIA with an LIF.

Table 1. Recovery Study of Carbaryl in Spiked Rice by CEIA ($n = 3$)

spiked level (μg/kg)	detected by CEIA (mean $\pm$ SD, μg/kg)	recovery (%) (mean $\pm$ RSD)
20	17.9 $\pm$ 1.2	89.5 $\pm$ 6.7
50	41.1 $\pm$ 2.1	82.2 $\pm$ 5.1
100	79.2 $\pm$ 4.5	79.2 $\pm$ 5.7

was 20 kV, the sample was injected at 0.5 p.s.i for 5 s, and the separation buffer consisted of 20 mM $Na_2B_4O_7$ and 10 mM Na_2HPO_4 (pH 9.0) containing 1 mg/mL poly-*N*-isopropylacrylamide (pNIPA). A thermally reversible hydrogel, pNIPA, was added to the buffer as dynamic modifier to improve the reproducibility and decrease the interaction between the protein and the capillary wall (*22*). The buffer was filtered through 0.45 μm membrane and was degassed by ultrasonication for approximately 15 min before use. Between consecutive analyses, the capillary was first flushed with 0.1 M sodium hydroxide (1 min), then with the running buffer (2 min). CEIA separation of the mixture produces two distinct fluorescent peaks corresponding to the free Ag* and the Ab−Ag* immunocomplex.

Table 2. Analysis of Carbaryl in Rice Samples by CEIA

samples	carbaryl found (mean $\pm$ SD, $n = 3$) (μg/kg)
no.1	26.3 $\pm$ 1.7
no.2	ND[a]
no.3	12.6 $\pm$ 0.8
no.4	76.2 $\pm$ 3.9
no.5	ND
no.6	ND
no.7	90.3 $\pm$ 5.1
no.8	ND
no.9	58.7 $\pm$ 3.2
no.10	ND

[a] ND, not detected, < 5μg/kg.

Spike and Recovery Study for Rice Samples by CEIA with an LIF Detector. Rice samples were obtained from the Tianjin Exit-Entry Inspection Bureau. No incurred residues were detected by HPLC analysis. They were spiked with cabaryl for screening and determination. It was reported by Wang et al. that methanol was an efficient extractant for carbaryl from food matrix (*23*). A rapid extraction method was developed: 2 g of spiked sample was treated by mixing 10 mL of methanol and was shaken at 200 rpm on the rotary shaker for 40 min. The supernatant was filtered through a 0.45 μm nylon membrane, and the resulting filtrate was diluted 10-fold with buffer (pH 7.2 PBS). One hundred microliters of sample extract, 100 μL of Ag*, and 2 μL of Ab were incubated for 30 min, then injected into the capillary for analysis.

RESULTS AND DISCUSSION

Purification and Characterization of Fluorescently Labeled Carbaryl Tracer. TLC is a sensitive, fast, simple, and inexpensive analytical technique. It was used to purify the small amount of fluorescently labeled carbaryl hapten. Three bands ($R_f = 0.10$, 0.30, and $R_f = 0.72$) from the TLC experiments were detected. They were isolated and extracted into methanol. The three extracts were analyzed by MS, and their fluorescent activity was determined using CEIA. The MS spectrum of R_f

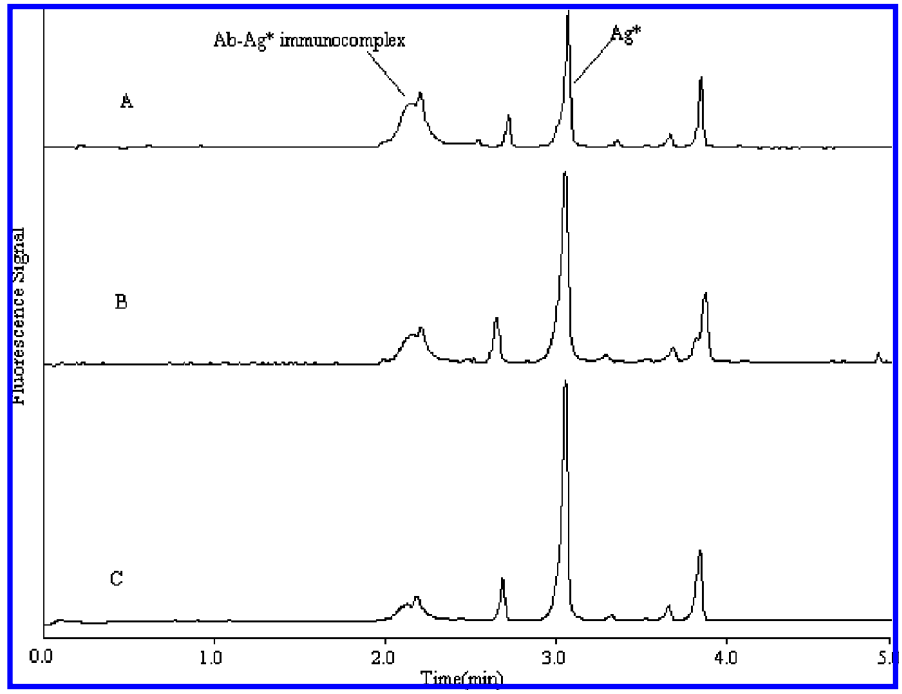

Figure 6. Electropherograms of spiked rice samples by CEIA with an LIF detector. **A**, rice sample with no carbaryl spiked; **B**, rice sample spiked with 0.5 mg/kg carbaryl; **C**, rice sample spiked with 2.0 mg/kg carbaryl.

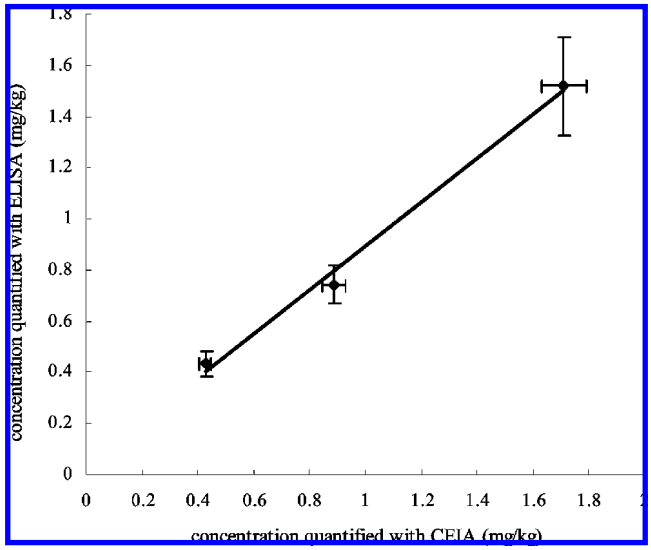

Figure 7. Samples analyzed by CEIA with an LIF detector vs ELISA ($n = 3$).

$= 0.72$ had a single charged base peak at m/z 730.63 corresponding to the $[M - 2H]^-$ ion of Ag*. Using the CEIA, we were able to show that only one band (with $R_f = 0.72$) was able to produce an immunocomplex (Ab−Ag*). The other two bands ($R_f = 0.10$ and 0.30) presumably had byproducts of the reaction and/or excess EDF. Given this, we used the initial methanol extract ($R_f = 0.72$) in the following test.

Effect of Incubation Time on Immunocomplex Formation. In order to determine the effect of incubation time on equilibrium, Ag* and Ab solutions were incubated at room temperature for 5, 10, 20, 30, 40, 50, and 60 min before injection into the capillary. As shown in **Figure 2**, the Ab−Ag* immunocomplex signal increased as the incubation time increased. The peak value of the immunocomplex did not change significantly at incubation times greater than 30 min, suggesting that the reaction had reached equilibrium by this time. Thirty minutes was thus chosen as the incubation time for this assay. This contrasts with a solid-phase IA, such as an ELISA, where only free Ags are in solution while the Ab−Ag immunocomplexes are in a solid-phase, the thermodynamics of solutions cannot be applied to the system and therefore needs a relatively long time to reach equilibrium.

pH Effect for the Separation Buffer. The buffer pH is also an important parameter in the CEIA. For solution-phase CEIA, the immunoassay is performed in solution, and the immunological species are separated via CE. A slightly basic running buffer was chosen for CE to ensure Ab−Ag binding and minimize the protein adsorption on the inner capillary wall. We determined the effect of pH values of 8.8, 9.0, 9.2, 9.4, 9.6, and 9.8 on the separation of the immunocomplex and Ag* (**Figure 3**). pH 9.0 produced the highest peak value for the immunocomplex, while above pH 9.2, the Ab−Ag* immunocomplex peak broadened, then obviously decreased, and the migration time was slightly prolonged. The pH change may induce conformational changes in the formation of the Ab−Ag* immunocomplex; therefore, the value and shape of immunocomplex peak was altered. This effect might also be due to the fact that electro-osmotic flow was reduced or eliminated, thereby prolonging the CE separation time and increasing the likelihood of immunocomplex dissociation. In extreme pH conditions, antibody protein could be denatured because the pH value could alter the charges of the analytes. This would cause the peak of Ab−Ag* to disappear and become immunologically unresponsive. Taking into consideration the effects on the immunocomplex peak, the optimal pH value for the separation buffer was chosen as 9.0.

Capillary Electrophoresis-Based Competitive Immunoassay for Carbaryl Detection. We used the competitive model in which the fluorescently labeled carbaryl tracer was first mixed with different concentrations of the carbaryl, which then competed for binding with the anticarbaryl antibody to form an immunological complex. The amount of fluorescent immunocomplex will be inversely proportional to the amount of immunocomplex formed by the antibody and unlabeled carbaryl. As shown in **Figure 4**, as the amount of carbaryl increased, more fluorescently labeled carbaryl tracer was replaced and

released from the immunocomplex through competitive reaction. Compared with a control (without the addition of carbaryl, **Figure 4b**), the peak area of Ab−Ag* immunocomplex gradually decreased, and the peak area of free Ag* correspondingly increased (**Figure 4** b−**d**).

Different concentrations of carbaryl (from 0.01 to100 ng/mL) were analyzed under the optimized CEIA conditions. The calibration curve for carbaryl (**Figure 5**, $R^2 = 0.9928$) was acquired by plotting the relative area ratio against the logarithm of the concentration of carbaryl. The linear range and LOD for carbaryl were 0.16−50 ng/mL and 0.05 ng/mL, respectively.

Spike and Recovery Study for Rice Samples. We investigated the efficiency of CEIA with an LIF detector by testing the recovery of carbaryl from spiked rice samples. The interference caused by matrix effects in an immunoassay can be reduced by manipulating the solvent and dilution range (*24*). For the rice methanol extract, the matrix effect could be removed by 10-fold dilution with buffer. The LOD of carbaryl in rice samples could reach 5 μg/kg. The application of the present CEIA method was tested in experiments with spiked rice at three different concentrations of carbaryl: 20, 50, and 100 μg/kg. The recoveries are shown in **Table 1**. Our results show that recovery was satisfactory with RSD values lower than 6.7%.

The method was also applied for the screening and determination of 10 samples, which were spiked with carbaryl at random between 0 and 100 μg/kg. The results (**Table 2**) were as follows: < 5 μg/kg in 5 samples, between 5−50 μg/kg in 2 samples, and > 50 μg/kg in 3 samples. These results demonstrate that the optimized method could be used as a sensitive, rapid, and reliable means of detecting carbaryl with low concentration in rice matrices.

Comparison of CEIA with an LIF Detector and ELISA for the Detection of Carbaryl in Rice Samples. The detection of carbaryl using ELISA has been described previously (*25*), and the LOD was obtained at 0.7 ng/mL. The antibody applied for analysis by ELISA is 1.0 μg. However, by CEIA with an LIF detector, the LOD was 0.05 ng/mL, and only 0.5 μg of antibody was used for analysis. The sensitivity of this CEIA with an LIF detector was almost 14 times greater than that of ELISA, which used the same immuno-reagents. Using the same extract method for the analysis of carbaryl in rice, the matrix effect was removed by 20-fold dilution for ELISA analysis. However, for CEIA with an LIF detector, 10-fold dilution was sufficient to manage interferences. The two methods were also applied for the analysis of carbaryl in rice samples (**Figure 6**), which were spiked at 0.5, 1.0, and 2.0 mg/kg. The correlation between the two methods was excellent (**Figure 7**, $R^2 = 0.99$). The assay time per sample for CEIA-LIF is faster because the immunoreaction occurs in solution, which allows rapid reaction by solution-phase kinetics. Problems associated with immobilization steps and nonspecific adsorption such as those encountered in solid-phase methods such as ELISA are avoided. CEIA with an LIF detector is more preferable, with higher sensitivity and rapid analysis time with simple sample pretreatment.

In conclusion, CEIA combines the advantages of CE and IA. It was proved to be a rapid and sensitive assay with high efficiency combined with an LIF detector. In this article, the method based on a competitive format was developed for the determination of carbaryl. Under optimal conditions, the linear range and the detection limit for carbaryl were 0.16−50 ng/mL and 0.05 ng/mL, respectively. Quantitative measurements of carbaryl in spiked rice samples have been demonstrated with simple pretreatment. The LOD in real samples could reach 5 μg/kg. Compared to conventional screening methods such as ELISA, CEIA with an LIF detector is more sensitive, uses less Ab, and reaches equilibrium faster. The developed analytical method could be used as a screening tool for the trace determination of carbaryl in rice samples.

ABBREVIATIONS USED

CE, capillary electrophoresis; CEIA, capillary electrophoresis-based immunoassay; ELISA, enzyme-linked immunosorbent assay; IA, immunoassay; KLH, keyhole limpet hemocyanin; LIF, laser-induced fluorescence; LOD, limit of detection; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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