Introduction

Organophosphorus pesticides (OPs) are widely used in agricultural fields because of their high-performance and moderate environmental persistence, hence the sensitive and specific detection of OPs is highly significant. Based on the inhibitory effect of acetylcholinesterase (AChE) induced by inhibitors, including OPs and carbamates, a colorimetric analysis was used for detection of OPs with computer image analysis of color density in CMYK (cyan, magenta, yellow and black) color space and non-linear modeling. The results showed that there was a gradually weakened trend of yellow intensity with the increase of the concentration of dichlorvos. The quantitative analysis of dichlorvos was achieved by Artificial Neural Network (ANN) modeling, and the results showed that the established model had a good predictive ability between training sets and predictive sets. Real cabbage samples containing dichlorvos were detected by colorimetry and gas chromatography (GC), respectively. The results showed that there was no significant difference between colorimetry and GC ($P > 0.05$). The experiments of accuracy, precision and repeatability revealed good performance for detection of OPs. AChE can also be inhibited by carbamates, and therefore this method has potential applications in real samples for OPs and carbamates because of high selectivity and sensitivity.

Keywords Organophosphorus pesticides, colorimetry, quantitative analysis, computer image analysis, ANN

(Received December 23, 2015; Accepted February 24, 2016; Published July 10, 2016)
the color fingerprint in a specific color space may be more suitable for qualitative analysis or classification of OPs because of the lack of a continuous range of color metrics.

A traditional spectroscopic method based on Ellman’s assay was used for the detection of OPs and carbamates in the 1950s, and now it has become a relatively mature and rapid detection method.31–33 Because color can be directly observed by the naked eye or image-forming system, some studies have recently focused on color analysis for detection of OPs on the basis of the inhibition reaction of AChE.34 OPs are currently the largest amount of pesticides in agriculture in China and the overuse of single OPs is quite common according to recent surveys, and therefore, a simple, rapid, inexpensive and on-site detection method for OPs is highly significant. In this work, after Ellman’s reaction, the yellow density in CMYK space was extracted and used for the quantitative analysis of OPs with computer image analysis and artificial neural network (ANN).

**Experimental**

**Reagents and chemicals**

Dichlorvos standard solution (100 μg/mL in acetone) was purchased from Aladdin Industrial Corporation (Shanghai, China). AChE stock solution, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide (ATCh) were obtained from Dayuan Oasis Food Safety Technology Co., Ltd. (Guangzhou, China). The Sylgard 184 Silicone Elastomer Kit was obtained from Dow Corning Corporation (Midland, Michigan, USA). Ultra-pure water was generated by a Millipore Direct-Q Water system (Molsheim, France). Other chemical reagents were analytical or chromatographic grade.

**Apparatus**

Poly(methyl methacrylate) (PMMA) plates (thickness of 1.2 mm) were obtained from Li Yang Co., Ltd. (Nantong, China). Pipettors were obtained from Eppendorf (Hamburg, Germany). CanoScan LiDE700F scanner was from Canon (Tokyo, Japan). GC7890A gas chromatography with flame photometric detection (FPD) and B1701 capillary column (30 m × 0.25 mm × 0.25 μm) was obtained from Agilent Technologies Inc. (California, USA).

**Fabrication of colorimetric plate**

The base plate (80 × 80 mm) was made of PMMA plate (thickness of 1.2 mm). The protective film of the base plate was removed carefully. A photoresist template was molded by polydimethylsiloxane (PDMS). After curing, the PDMS was removed from the template and combined with PMMA by hot pressing technology. The cover plate was also processed by PMMA with a thickness of 1.2 mm. To add sample and exhaust air, 36 circular holes were punched in the cover plate. Each hole was 6 mm in diameter and 3 mm in depth. A colorimetric plate was composed of base plate (upper PDMS layer and bottom PMMA layer) and cover plate (Fig. 1).

**Sample preparation**

Pesticide-free fresh Chinese cabbages were obtained from the Laboratory of Horticultural Crops, Chongqing Three Gorges University, Wanzhou, China. All the samples of cabbage were analyzed by GC to make sure there were no pesticides remaining at the beginning of treatment. Cabbage leaves were sprayed with dichlorvos in the concentrations of 0, 10, 20, 40, 60, 90 and 120 μg/mL, respectively, and then kept for 4 h at room temperature in a fume hood. After that, cabbage leaves were cut into small pieces. Next, 2.0 g pieces of each sample was weighed accurately and added into 10 mL of phosphate buffer. The mixture of cabbage samples was shaken for 1.5 min. Next, 2.0 g pieces of each sample was ultrasonically extracted for 5 min. After a 5-min centrifugation time at 12000 rpm, the supernatant of each cabbage sample was collected for simultaneous analysis of GC and colorimetry. Three replicates were performed separately.

**Procedure**

As shown in Fig. 1, the analysis system consisted of a colorimetric plate, an imaging system and a personal computer. The color intensity was obtained from enzyme-substrate reaction, which could be inhibited by OPs. An image was acquired by a scanner and analyzed in CYMK space by a personal computer. Then, 300 μL of varying final concentrations of dichlorvos solutions were respectively prepared and added into 32 Eppendorf tubes. The first seven final concentrations of dichlorvos were 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0, respectively, and the other 25 concentrations were in the range of 2.0 – 50.0 μg/mL at an interval of 2 μg/mL. Each tube was respectively added into enzyme reaction solution (including 20 μL DTNB with 8 g/L, and 20 μL AChE with 10.0 g/L) and incubated at 37°C for 15 min. Then each tubes was added into 20 μL of 8 μg/mL ATCh and shaken several times. Next, 50 μL of mixed solution was accurately measured and added into a hole of the plate. The parameter of the scanner was set to 2400 dots per inch (DPI) and a tagged Image File Format (TIFF)
image was obtained. Five replicates were performed separately, and therefore there were 160 samples in all.

To evaluate the feasibility of detecting the actual sample, the quantitative analysis based on colorimetry was compared with GC in this work. Seven kinds of supernatant solutions of real cabbage sample containing dichlorvos were mixed with DTNB, AChE and AChE in the ratio of 15:1:1:1 (by volume), respectively. Also, 50 μL of mixed solution was detected by colorimetry as described above. The operating conditions of GC were as follows: initial temperature of 60°C (2 min), increase to 210°C at 40°C min⁻¹ for 3 min, injector temperature 200°C, detector temperature 250°C, column flow rate of 2.5 mL min⁻¹ and 2 μL injection volume of real samples. Others were nitrogen (>99.999%) with the flow rate of 1.2 mL min⁻¹ and hydrogen (>99.999%) with the flow rate of 75 mL min⁻¹.

Data processing method and software

The TIFF images were acquired and converted from RGB to CMYK color space. The yellow color in CMYK space was extracted and used to explore the relationship between the concentration of OPs and the color density. All data processing and mapping were performed in Matlab 2013a (Mathworks, Natick, USA), Photoshop CS4 (Adobe, San Jose, California, USA), Origin 8.5 (Originlab, Northampton, USA) for Windows7.

Results and Discussion

Quantitative analysis of OPs

Traditional Ellman’s method, based on spectroscopic analysis at 412 nm, achieves the qualitative or quantitative analysis of OPs and carbamate.31,35 The principle of the Ellman’s method is shown in Fig. 2. Thioclinene is generated by ATCh via the catalysis of AChE, and can be converted into 2-nitro-5-thiobenzoic acid (TNB), a yellow colored compound. The activity of AChE can be inhibited by certain chemicals, such as OPs and carbamate pesticides, and therefore, the quantitative analysis can be achieved by inhibition rate. Recently, there has been a new approach to achieve the detection of chemical materials by color analysis in different color space, such as RGB, CMYK and L*a*b* space.36–38 The CMYK color model refers to cyan, magenta, yellow, and key (black). The K stands for key because cyan, magenta, and yellow printing plates are carefully aligned with the key of the black plate. Each color intensity ranges from 0 to 100% in CYMK space. In this work, the yellow color density in CMYK space was used for the quantitative analysis of OPs. Dichlorvos standard samples were analyzed by colorimetry. The results showed that yellow density in CMYK space gradually decreased with the increase of dichlorvos concentration (Fig. 3). In general, an ideal quantitative approach is achieved in accordance with linear relationships. However, there were no strong correlations or determination coefficients between the yellow density and the concentration of dichlorvos or their transformations (for example, logarithmic transformation) by variable-substitution, which might be related to the nonlinear system. In this paper, the relationship of yellow density and concentration was explored by Back Propagation Neural Network (BPNN), also known as a “feed forward back propagation network”. In the process of neural network training, the conjunction weights of the neural network are continuously modified layer by layer from output layer to input layer to reduce the errors between the anticipated and actual outputs until the predetermined network parameters are reached.39 According to the Kolmogorov theorem, a three-layer BPNN can approach any nonlinear continuous function in the closed interval.40 As a supervised method, BPNN has a powerfully nonlinear mapping ability to deal with nonlinear data. A back propagation neural network with the Levenberg-Marquardt training algorithm (LMBP) was used for predictive analysis. The network structure of LMBP

![Fig. 2 Principle of quantitative colorimetric detection for OPs. ATCh, Acetylthiocholine; AChE, acetylcholinesterase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid.](image)

![Fig. 3 Relationship between the concentration of dichlorvos and yellow color density.](image)
was a single hidden layer, and the number of neurons for input layer and output layer were 1 and 1, respectively. The number of hidden layer neurons has a great influence on the approximation ability of the network. In order to obtain the optimal number of hidden neurons, the LMBP was trained when the number of hidden layer nodes was 3 to 12 according to trial-and-error method. Learning rate, maximum epoch and mean square error (MSE) were set at 0.01, 2000 and 0.00001, respectively. As shown in Fig. 4, when the number of hidden layer neurons was 10, there was a relatively small MSE in the process of function approximation, and thus, the optimal number of neurons in the hidden layer was 10. The 160 samples were split into training sets (96 samples) for modeling and prediction sets (64 samples) for evaluation of model robustness. The process of nonlinear approximation based on LMBP consists of training, cross validation and testing. The correlation analysis of the actual concentration and predicted concentrations was analyzed by established LMBP network. As shown in Fig. 5(a), the fitting of the LMBP reached 0.9999, and the prediction ability of LMBP reached 0.9952 from Fig. 5(b). The results of the training sets and the prediction sets showed a high accuracy. It indicates that the established model has a good generalization ability when the concentration of dichlorvos is in the range of 100 ppb - 50 ppm. According to the National Food Safety Standard of the P. R. C.: Maximum Residue Limits for Pesticides (GB 2763-2014), the maximum residue limit of dichlorvos is 500 ppb in Chinese cabbages. The prediction interval of LMBP contains the limit. The established LMBP was saved for the analysis of real samples. Traditionally, linear regression, partial least squares regression and principal component regression have been widely used in the quantitative analysis because of good adaptability of the linear system. The accuracy of these prediction models is strongly influenced by outlier data, system properties, etc. In addition, the transformation of variables are mainly determined by conjecture and experience in a linear analysis system. Moreover transformed variables may also lead to a change of the random error of model. Through trial and exploration, LMBP was well fit for the modeling of experimental data because of powerful approximation ability and correction capability.

Detection of real samples and comparison with GC
To assess the reliability of the analysis of the method, real cabbage samples were sprayed with different concentrations of dichlorvos (10, 20, 40, 60, 90 and 120 μg/mL) and analyzed by GC and colorimetry. In addition, other cabbage samples were sprayed with equal volumes of ultra-pure water and used as a blank control. Each sample was analyzed separately by the colorimetric method and GC. The concentrations of dichlorvos in real samples were obtained by saved LMBP in colorimetric analysis. The difference of detecting results between the two methods was compared by t-test. The results showed that there was no statistically significant difference (P > 0.05) between GC and colorimetry (Fig. 6). In addition, the results also showed that the colorimetric analysis of OPs was not affected by the components of the cabbage. It indicates that the...
obtained by the same analyst in a short time interval with the same operating conditions) of the colorimetric method. The evaluation of the system precision was performed at four different concentration levels of 0.5, 1.0, 3.0, 6.0 μg/mL. There were six samples at each concentration level. In order to evaluate the repeatability, a sample of each concentration level was prepared and measured six times. The process of detection and computation was performed as described above. The results of precision and repeatability were presented by relative standard deviations (RSD). From Table 1, the recovery rate of dichlorvos at four different concentration levels were between 80 to 120%, and separate recovery values were 0.501 ± 0.013, 1.002 ± 0.022, 3.022 ± 0.094 and 6.058 ± 0.196 μg/mL when the spiked concentration of dichlorvos were 0.5, 1.0, 3.0, 6.0 μg/mL. For precision and repeatability, most of results were less than 3% at the different dichlorvos concentration levels (Table 1). The results show good accuracy, precision and repeatability. It indicates the colorimetry is accurate and reliable for detection of OPs.

Compared with the traditional methods (such as chromatography and ELISA), the colorimetric method based on yellow density analysis have many advantages, such as lower cost, speed, portability and simplicity. In addition, the colorimetric method also has the advantage of easy miniaturization, on-site analysis and easy of use in comparison with traditional methods. Although AChE can also be inhibited by carbamate pesticides or OPs other than dichlorvos, the determination of total OPs and carbamate pesticides can be achieved with reference to Pesticide Detection Cards. The cards consist of chromogenic reagent and cholinesterase and are used for rapid detection of pesticide residues based on blue color changes, which are generated as a result of indoxyl acetate hydrolysis catalyzed by AChE and the inhibition of AChE activity by pesticides.

### Conclusions

A simple, inexpensive and on-site approach for detection of OPs was developed by colorimetry and artificial neural network (ANN) in this work. Based on the non-linear relationship between yellow intensity and the concentration of OPs, the prediction model of LMBP was established, and showed good prediction accuracy, whether standard samples or actual samples. In addition, with the ANN model, the results showed that colorimetric analysis of yellow density had good practicability by comparing with gas chromatography. The analysis of accuracy, precision and repeatability indicated that the method was reliable and practical. Although there are currently many detection methods for OPs, the colorimetric method has potential applications for rapid detection of OPs and carbamate pesticides due to the characteristics of simplicity, speed, low cost and portability.

### Acknowledgements

This work was financially supported by the National Natural Science Foundation (31171684), Key Technologies R&D Program of China (2014BAD07B02), Liquor Making Biology Technology and Application of the Key Laboratory Program of Sichuan Province, China (No. NJ2014-03), Chongqing Graduate Student Research Innovation Project, China (CYB15026) and sharing fund of Chongqing University’s large equipment.

### References

13. Z. L. Xu, H. Deng, X. F. Deng, J. Y. Yang, Y. M. Jiang,


