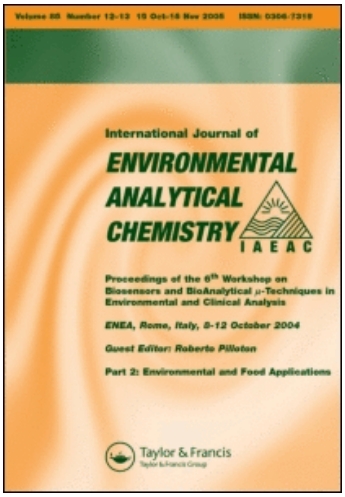

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Simultaneous enzymatic kinetic determination of carbamate pesticides with the aid of chemometrics

Yongnian Ni[ab]; Na Deng[b]; Serge Kokot[c]
[a] Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China [b] Department of Chemistry, Nanchang University, Nanchang, Jiangxi 330047, China [c] School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Queensland 4001, Australia

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Simultaneous enzymatic kinetic determination of carbamate pesticides with the aid of chemometrics

Yongnian Ni[ab]*, Na Deng[b] and Serge Kokot[c]

[a]*Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China;* [b]*Department of Chemistry, Nanchang University, Nanchang, Jiangxi 330047, China;* [c]*School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Queensland 4001, Australia*

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A method for the simultaneous enzymatic kinetic determination of the pesticides, oxamyl, aldicarb and aminocarb in fruit, vegetables and water samples, has been researched and developed. It was based on enzymatic reaction kinetics and spectrophotometric measurements, and results were interpreted with the aid of chemometrics. The analytical method relies on the inhibitory effect of the pesticides on acetylcholinesterase (AChE), and the use of 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB) as a chromogenic reagent for the thiocholine iodide (TChI) released from the acetylthiocholine iodide (ATChI) substrate. The complex rate equation for the formation of the chromogenic product, P, was solved under certain experimental conditions, and this enabled the absorbance (A_p, at $\lambda_{max} = 412$ nm) from the mixtures of the three pesticide inhibitors to be directly related to their concentrations. The detection limits of the enzymatic kinetic spectrophotometric procedures for the determination of the oxamyl, aldicarb and aminocarb were 0.81, 2.13 and 1.25 ng mL^{-1}, respectively. Calibration models were constructed for principal component regression (PCR), partial least squares (PLS), and radial basis function-artificial neural network (RBF-ANN), and verified with synthetic samples of the three pesticides. The prediction performance of these models showed generally satisfactory results, and the RBF-ANN one performed slightly better than the other two ($RPE_T = 7.59\%$ and average %recovery = 99%). This model was then successfully applied to estimate the amounts of the three compounds in fruit, vegetables and water with satisfactory results.

Keywords: enzymatic kinetic method; acetylcholinesterase; chemometrics; pesticides; carbamates

1. Introduction

Numerous chemical compounds, routinely applied in agriculture and chemical industry, can form persistent toxic residues in air, soil, water and foods. Pesticides are one of the principal classes of such environmental pollutants widely spread throughout the world, and millions of tons are consumed by the agriculture sector each year, which results in serious food safety problems.

*Corresponding author. Email: ynni@ncu.edu.cn

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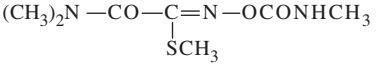
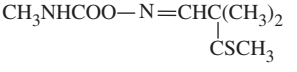
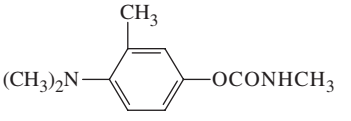
Carbamate pesticides show low environmental persistence but display high acute toxicity. Their presence in water and food poses a potential hazard to human health. Their structures (Table 1) are based on N-substituted carbamic acid esters ($R_1OCONHR_2R_3$). The R_1 group is typically a phenyl or a heterocyclic aromatic ring, the R_2 group is usually a methyl substituent, and the R_3 is either a hydrogen, methyl, or a more complex group. Fifteen *N*-methylcarbamates form an important family of insecticides, and are widely applied for the treatment of seeds, soil and crops [1]. Many methods have been proposed and developed for analysis of these residues with the aid of techniques such as thin layer chromatography (TLC) [2], gas chromatography (GC), high performance liquid chromatography (HPLC) [3,4], and gas chromatography-mass spectrometry (GC-MS) [5]. Since such carbamate pesticides are thermally labile and can be easily decomposed to methylisocyanate and phenol, especially at relatively high temperatures, it is difficult to analyse them and their derivatives by GC. For this reason, HPLC has become the method of choice for the determination of carbamates, because in this case the thermal lability problem is obviated. However, HPLC has to be commonly preceded by some preprocessing steps, derivatisation of compounds and post-column fluorimetric labelling [6].

In general, these carbamates inhibit the acetylcholinesterase (AChE) participating in nerve-impulse transmission. The inhibition mechanism is very specific and has led to the development of several analytical techniques for the identification and quantification of such pesticides on the basis of the inhibition of cholinesterases [7]. Compared with the traditional procedures based on gas chromatography (GC) and high-performance liquid chromatography (HPLC), which are reliable but expensive, complicated and time-consuming [8], these enzymatic analytical techniques are rapid, inexpensive and do not need any involved pretreatment.

The development of biosensors based on enzyme inhibition is a matter of considerable interest, and many kinds of biosensors have been reported in the past decade. They can be divided into three main types according to the transducers used: potentiometry [9], amperometry [10], and optical biosensors [11]. However, these biosensors often have

Table 1. Chemical structures of the pesticides.

Pesticides	Molecular	Structure
Oxamyl	$C_7H_{13}N_3O_3S$	
Aldicarb	$C_7H_{14}N_2O_2S$	
Aminocarb	$C_{11}H_{16}N_2O_2$	

relatively short lives, and this greatly limits their uses in practice. In addition, the analyses often need more than half an hour and several milliliters of samples.

Enzyme inhibition procedures involve the measurement of uninhibited activity of an enzyme, which is followed by an incubation period for the reaction between the enzyme and an inhibitor. The measurement of the enzyme activity is made after the inhibition stage. Advantages of the enzyme inhibition methods are that they are simple, fast and do not require expensive apparatus. The effect of the pesticides on the inhibition of an enzyme of the reaction system is dependent on their concentration and their own chemical properties. Also, this method can only measure the total content of the pesticides in a sample, and it is unable to analyse individual pesticides. However, recently, Ni *et al.* [12] explored the application of a spectrophotometric method for the simultaneous kinetic determination of binary mixtures of carbaryl and phoxim. This method was based on the inhibitory effect of the pesticide analytes on acetylcholinesterase of AChE, and offered a possible new direction of analysis in this field.

In this paper, a method for the simultaneous determination of the pesticides, oxamyl, aminocarb and aldicarb, was developed. It is also based on their inhibitory effect of acetylcholinesterase, AChE, with the use of acetylthiocholine iodide (ATChI) as substrate, and 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB) as a chromogenic reagent. The levels of inhibition between oxamyl, aminocarb and aldicarb were found to be different, and this was reflected in their different reaction rates, which facilitated the development of the differential kinetic analysis model. In theory, some simple traditional models involving proportional equations and the logarithmic extrapolation based on well defined reaction mechanisms, can discriminate the analyte components. However, such models are dependent on the knowledge of the reaction orders and rate constants for the chemical system. These are often unavailable [13].

Consequently, several multivariate calibration methods, principal component regression (PCR), partial least squares (PLS), and radial basis function-artificial neural network (RBF-ANN), were used to resolve the kinetic data. The proposed method was then applied for determination of the three pesticide residues in vegetables, fruit and water samples.

2. Theoretical background

2.1 *Kinetic models*

One of the most used kinetic models to describe enzymatic catalysis was proposed by Michaelis and Menten [14]. The model includes two steps: (1) reversible fixation of the substrate to an enzyme so as to produce an activated complex or a transition state; (2) reaction and desorption of the final products. The enzyme is then regenerated. This model has been commonly applied on the assumption that the stoichiometric coefficients are equal under the conditions of free diffusion and thermodynamically-driven random collisions. Such conditions are common to the real world.

In this work, we consider the following catalysed reaction process:

$$\mathrm{E} + \mathrm{I} \rightarrow \mathrm{D} \quad \text{(slow)} \tag{1}$$

$$\mathrm{S} + \mathrm{R} \xrightarrow{\mathrm{E}} \mathrm{P} + \mathrm{Q} \quad \text{(slow)} \tag{2}$$

where reaction (1) represents the inhibitory effect of inhibitor, I (pesticide) on the catalyst, E (AChE) which accelerates the reaction (2) between the reactant, S (substrate, ATChI) and R – chromogenic reagent, DTNB. P and Q are the products.

If it is assumed that the inhibitor reaction (1) follows pseudo-first-order kinetics when the concentration of E is much higher than that of I, i.e. $c_E \gg c_I$, then its rate equation can be represented as:

$$-\frac{dc_I}{dt} = k_1 c_I \tag{3}$$

where c_I and k_1 represent the concentration of I, and the rate constant of the inhibitory reaction (1), respectively. The integration of Equation (3) yields:

$$c_{I,t} = c_{I,0} \exp(-k_1 t) \tag{4}$$

where $c_{I,0}$ is the initial concentration of the inhibitor and $c_{I,t}$ is its concentration at time t.

Furthermore, in terms of reaction (1), since the relationship between the concentrations of I and E is known, Equation (5) can be derived:

$$c_{E,t} = c_{E,0} - \Delta c_{I,t} = c_{E,0} - c_{I,0}(1 - \exp(-k_1 t)) \tag{5}$$

where $c_{E,0}$ is the initial concentration of the catalyst, E, $c_{E,t}$ is its concentration at time, t, and $\Delta c_{I,t}$ is the concentration decrease of inhibitor, I, from $t = 0$ to some time, t.

Reaction (2) can be regarded as a pseudo-zero-order reaction when the concentration of the reagents c_R and c_S are much higher than that of the catalyst c_E, i.e. $c_R \gg c_E$ and $c_S \gg c_E$. Thus, for reaction (2) in terms of E, the rate equation is:

$$\frac{dc_P}{dt} = k_0 + k_2 c_{E,t} \tag{6}$$

where k_0 and k_2 represent the uncatalysed and catalysed rate constants of the reaction (2), respectively. Consequently, for the enzyme-catalysed reaction, k_0 is much smaller than the k_2, and therefore, Equation (6) can be simplified to:

$$\frac{dc_P}{dt} = k_2 c_{E,t} \tag{7}$$

When Equations (5) and (7) are combined and rearranged, the following differential equation can be obtained:

$$\frac{dc_P}{dt} = k_2 c_{I,0}(1 - \exp(-k_1 t)) \tag{8}$$

The solution of Equation (8) is:

$$c_{P,t} = c_{I,0} k_2 \left[t - \frac{1}{k_1}(1 - \exp(-k_1 t)) \right] \tag{9}$$

Equation (9) can be simplified to:

$$c_{P,t} = K' c_{I,0} \tag{10}$$

where $K' = k_2[t - \frac{1}{k_1}(1 - \exp(-k_1 t))]$ and is the constant of proportionality at a given time, t.

If the absorbance of the product, P, is proportional to its amount, Equation (10) can be expressed by:

$$A_{\mathrm{I},t} = Kc_{\mathrm{I},0} \tag{11}$$

where $A_{\mathrm{I},t}$ is the absorbance of the product P, and K is the product of K' and the absorptivity for P.

If there are n analytes, I_i $(i=1,2,\ldots,n)$ in the reactions (1) and (2), and the absorbances of the products for each analyte are additive, then Equation (11) can be represented as:

$$A_t = \sum_{i=1}^{n} K_{\mathrm{I}i,t} c_{\mathrm{I}i,0} \quad (i = 1, 2, \ldots, n) \tag{12}$$

where A_t, $K_{\mathrm{I}i,t}$, and $c_{\mathrm{I}i,0}$ represent the total absorbance of the sample, the constant of proportionality for component, I_i at time, t, and the original concentration of I_i, respectively.

If m standard samples are prepared, Equation (12) can be extended and represented in matrix form:

$$\boldsymbol{A}_{m\times t} = \boldsymbol{C}_{m\times n}\boldsymbol{B}_{n\times t} \tag{13}$$

Thus, Equation (13) can be used to establish multivariate calibration models, which facilitate the prediction of individual analytes by suitable chemometrics methods in unknown samples.

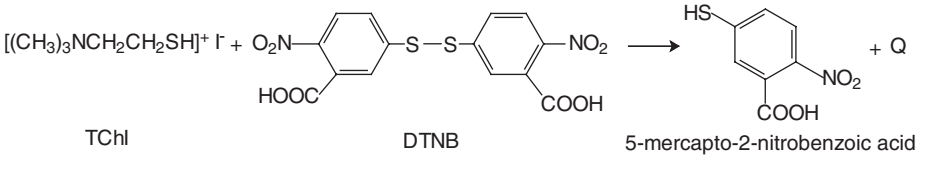
2.2 *Chemical reaction mechanism*

The common toxicological mechanism of organophosphorus and carbamate pesticides is based on the inhibition of acetylchlolinesterase activity (AChE), and the catalytic reaction of acetylcholine (ACh) by AChE is:

$$\underset{\text{Acetylcholine(ACh)}}{[(CH_3)_3NCH_2CH_2OOCCH_3]^+ \cdot OH^-} + H_2O \xrightarrow{\text{AChE}} \underset{\text{Choline(Ch)}}{[(CH_3)_3NCH_2CH_2OH]^+ \cdot OH^-} + CH_3COOH$$

The acetylcholine is accumulated when the AChE activity is inhibited by pesticides, and the normal nerve conduction is influenced as well. In order to detect the inhibited activity of AChE, the following chemical reaction was carried out:

$$\underset{\text{ATChI}}{[(CH_3)_3NCH_2CH_2SOCCH_3]^+ \cdot I^-} + H_2O \xrightarrow{\text{AChE}} \underset{\text{TChI}}{[(CH_3)_3NCH_2CH_2SH]^+ \cdot I^-} + CH_3COOH$$



According to the above method, the hydrolysis product of the substrate catalysed by AChE reacted with DTNB to give a coloured product with a λ_{max} at 412 nm. Thus, kinetic data could be obtained at this λ_{max}, and hence, the simultaneous determination of oxamyl, aldicarb and aminocarb could be investigated.

2.3 *Chemometrics methods*

2.3.1 *Multivariate calibration*

Partial least squares (PLS) and principal component regression (PCR) are two well-known multivariate calibration methods. Both are factor analysis based multivariate mathematical tools and have been successfully utilised for analysis of many multicomponent mixtures [15]. The two techniques are similar in many ways and the theoretical relationship between them has been discussed extensively in the literature [16,17]. PCR and PLS perform data decomposition extracting orthogonal factors which are characterised by variable loadings and object scores. Such factors can be used for building calibration models. In PCR, the data decomposition is performed with the use of only the signal information, while PLS employs signal and concentration data. In general, PLS is regarded as somewhat more robust than the PCR method. However, often the prediction results are not significantly different [18].

2.3.2 *Radial basis function-artificial neural networks method*

Artificial neural networks (ANNs) are among the best known methods for solving non-linear problems [19]. Their potential has been investigated with topics ranging from image processing and speech recognition to financial forecasting, as well as with analytical chemistry [20]. An ANN model consists of a series of interconnected nodes (neurons) that receive and/or send number values to other nodes. The network architecture generally has several layers: an input layer, in which each node represents an explanatory variable; an output layer, in which a node represents a dependent variable; and in between these two layers, there is one or more 'hidden' layers [21]. The value at each node in the hidden or output layer is the weighted sum of the inputs from the incoming nodes, transformed by a 'transfer function', e.g. commonly, a sigmoid function. Training of an ANN consists of presenting it with a series of known dependent and explanatory data, and iteratively modifying the weights, which are initially set to random values, in order to optimise the predictions of the dependent variables made at the output nodes. Training should be neither too short – when the performance of the network will be poor because of 'underfitting' – nor too long – when the network may start learning irrelevant information and noise from the training data. This generally leads to an 'overfitted' model, which is unable to generalise successfully to new, unseen observations.

The radial basis function-artificial neural network (RBF-ANN) is a data processing method applied to problems such as modelling and classification [22]. In the RBF-ANN model, the input layer does not process information; it only distributes the input vectors to the hidden layer. The hidden layer consists of a number of RBF neurons (n_h) and a bias (b_k). Each neuron in the hidden layer employs a radial basis function as the non-linear transfer function to operate on the input data. A common RBF is the Gaussian function

that is characterised by the centre (c_j) and the width (r_j):

$$o_j(x) = \exp\left[-(||x_i - c_j||/r_j)^2\right] \tag{14}$$

In the iterative calculation, spread is the most important parameter of RBF-ANN networks, which controls the size of width (r_j). The larger the spread, the flatter and smoother will be the Gaussian function approximation. The output of these hidden nodes, o_j, is then forwarded to all output nodes through weighted connections. The output, y_i, of these nodes consists of a linear combination of the kernel function:

$$y_j = \sum_{i=1}^{n} w_{ji} o_j(x) \tag{15}$$

where w_{ji} represents the weights of the connections between the hidden layer i and output layer j.

The PCR and PLS methods were written in MATLAB 6.5 (Mathworks), and the data were pretreated by autoscaling. The RBF-ANN method was obtained from the ANN Toolbox of MATLAB 6.5, and the data was similarly pretreated.

3. Experimental

3.1 *Chemical reagents*

Analytical grade reagents were used and the solutions were prepared with doubly distilled water throughout the experiments. Stock solutions of each pesticide (100.0 mg L^{-1}, Shanghai Pesticide Research Institute) were prepared by dissolving 0.0100 g crystals of these compounds in methanol and diluted to 100 mL. Standard solutions (10 mg L^{-1}) of these pesticides or their mixtures were then diluted to the required concentrations with distilled water. Stock standard solutions of acetylcholinesterase (AChE, 500 U mg^{-1}), acetylthiocholine iodide (ATChI, 1.12 mol L^{-1}), and 5,5′-Dithio*bis*(2-nitrobenzoic) acid solution (DTNB, 4.0 mg mL^{-1}) were prepared and stored refrigerated at 5°C in the dark (AChE, ATChI and DTNB were purchased from Oudakeyi Inc. Co., Xiameng, China). Working standard solutions were prepared freshly from the stock standard solutions by diluting with water. Phosphate buffer solutions with different pH (5.5–9.0) were prepared with 0.1 mol L^{-1} sodium hydrogen phosphate and sodium dihydrogen phosphate, monitored by a pH meter.

3.2 *Apparatus and software*

Spectral and kinetic data were determined by using an HP 8453 spectrophotometer connected to a PC computer via a HP-IB interface. Thermostated, spectrophotometric cells (10 mm) were used throughout. The pH was measured with a Model SA-720 pH meter (Orion). The obtained data were processed by a Pentium computer with all the programs written in MATLAB 6.5 (Mathworks).

3.3 *Analytical procedure*

The reaction was carried out in a 10 mm cell. Taking into account that the total useful volume was 2.5 mL, 40 μL AChE solution were pipetted into the cell, followed by

appropriate amounts of the standard solution of oxamyl, aldicarb and aminocarb, 20 μL DTNB solution and appropriate amounts of pH 7.9 buffer to give a volume of 2.495 mL. After 10 min of equilibration at 40°C (see Section 4.2.3), 5 μL 1.12 mol L^{-1} ATChI was added to give a total volume of 2.5 mL. The absorbance of this reaction system was automatically recorded against a blank solution in the range of 230–600 nm every 5 s between 5–900 s.

3.4 *Procedure for the determination of oxamyl, aldicarb and aminocarb in commercial samples*

3.4.1 *Fruit and vegetables*

Samples of commercial vegetables and fruit were pulped and homogenised in a blender; then 10.0 g of this sample were transferred into a 100 mL Erlenmeyer flask (with a screw cap), and 20 mL dichloromethane were added. Because the concentration of pesticides in vegetable and fruit samples was too low to detect directly, 1.0 mL of each of the standard pesticide solutions was added. Also, 5.0 g anhydrous sodium sulfate was added to the flask to absorb any water in the sample. The sample was then stirred and stored overnight. Subsequently, the mixture was shaken in a laboratory shaker for 15 min (Model HY-4 oscillator), and filtered through a funnel. The collected residue was washed with 5 mL dichloromethane. The filtrate was treated with hexane-acetonitrile (1 : 1) in a separation funnel, and the carbamates were extracted into the acetonitrile phase because of their higher polarity, while the colorants and some impurities were extracted into the hexane layer. The acetonitrile phase was treated twice more with the hexane to extract any residual impurities. It was then collected in an evaporating dish, and evaporated to near dryness. Finally, the residue in the dish was dissolved in ethanol, transferred into a 10 mL volumetric flask and diluted to the mark with 50% ethanol.

3.4.2 *Water samples*

Three water samples were collected from the tap water, a pond in Nanchang University and Qianhu lake in Nanchang City, respectively. Each water sample (10 mL) containing 1.0 mL of stock solutions of oxamyl, aldicarb and aminocarb was transferred into a 100 mL Erlenmeyer flask (with a screw cap), and 1 g sodium sulfate and 25 mL toluene were added. The mixture was then shaken for 15 min (Model HY-4 oscillator). The collected aqueous phase was extracted with another 25 mL toluene. The extracts were combined and transferred into an evaporating dish, and evaporated to near dryness. Finally, the residue in this dish was dissolved in ethanol, transferred to a 10 mL volumetric flask, and diluted to the mark with distilled water.

3.4.3 *Chromatographic procedure*

The determination of the three pesticides in commercial products and water samples was verified by HPLC. It was done with an Agilent 1100 Series HPLC-DAD system a vacuum degasser, quaternary pump, autosampler, injector with a 100 μL loop, an Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm) with an Agilent Zorbax high pressure reliance cartridge guard-column (C18, 12.5 mm × 4.6 mm, 5 μm) and a variable wavelength UV visible detector. The optimised experimental parameters were shown as follows: mobile phase, 70% methanol (A) and 30% water (B) during 0–10 min; flow rate, 1 mL min^{-1},

temperature, 25°C; detector wavelength, 240 nm for three carbamate pesticides; retention times, 3.54 min for oxamyl, 3.86 min for aldicarb and 7.29 min for aminocarb.

4. Results and discussion

4.1 *Spectroscopic and kinetic studies of oxamyl, aldicarb and aminocarb*

Spectra of the coloured products obtained from the enzyme catalytic reaction under the conditions described (Section 3.3) were measured in the range of 230–600 nm after 900 s reaction time (Figure 1). The absorbance maximum of the product, P, was at 412 nm, and in general, the absorbance value in the presence of pesticides was lower than that without them. This indicated that the enzyme activity was inhibited by the pesticides. Kinetic curves for oxamyl, aldicarb, aminocarb, and those in the absence of the pesticides were plotted from the absorbance values at the above λ_{max} (Figure 2). These plots indicate that there are differences between the reaction rates of oxamyl, aldicarb and aminocarb, and all of these rates are lower than that for the case without the pesticides (kinetic rate constants were calculated by a well known procedure [23] (Table 2)). Thus, based on these reaction rate differences, and the Equations (11) and (13), Section 2, it should be possible to resolve the mixtures of oxamyl, aldicarb and aminocarb with the use of multivariate, chemometrics methods, such as PCR, PLS, and RBF-ANN.

4.2 *Optimisation of the reaction conditions*

In the development of the method, it was noted that the absorbance of the kinetic system was influenced by: the enzyme concentration, substrate concentration, pH and temperature. Therefore, the influence of these variables was investigated.

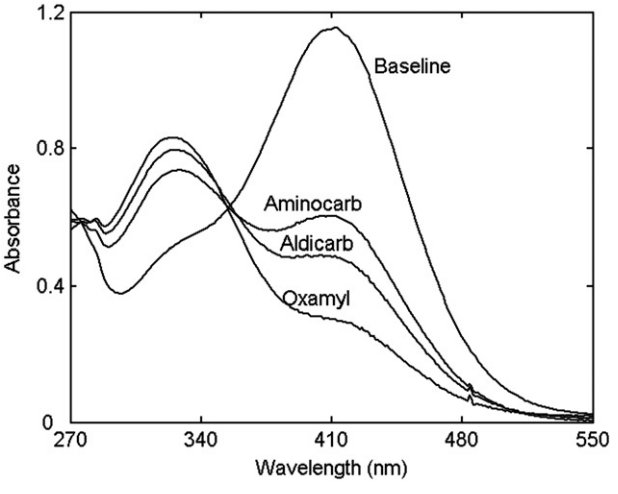


Figure 1. Spectra of the baseline (enzymatic reaction without pesticides), oxamyl ($60\,ng\,mL^{-1}$), aldicarb ($60\,ng\,mL^{-1}$) and aminocarb ($80\,ng\,mL^{-1}$) samples. $c_{AChE} = 1.2\,g\,L^{-1}$, $c_{ATChI} = 2.24\,mmol\,L^{-1}$, pH = 7.9, $t = 900\,s$, $T = 40°C$.

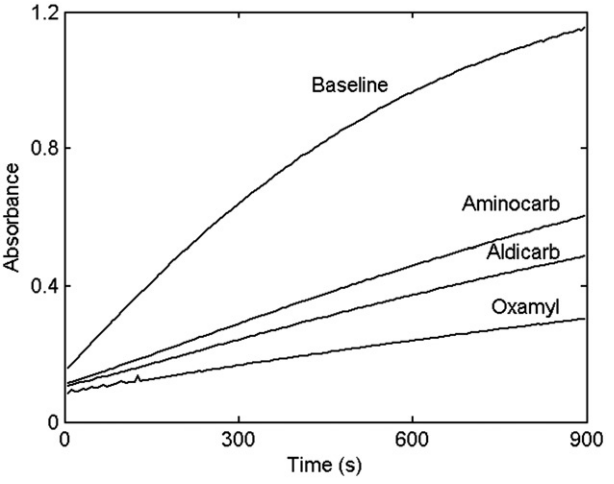


Figure 2. Plot of absorbance vs. time (measured at 412 nm) of: baseline, oxamyl, aldicarb and aminocarb analytes. Experimental conditions are the same as Figure 1.

Table 2. Kinetic rate constants of the inhibitory reaction with oxamyl, aldicarb and aminocarb.

Pesticide	Kinetic rate constant (s^{-1})
Oxamyl	0.0030
Aldicarb	0.0022
Aminocarb	0.0012
None[a]	0.0296

[a]No pesticides present in the inhibitory reaction.

4.2.1 *Optimisation of enzyme concentration*

The effect of various AChE concentrations (0.6–1.65 g L^{-1}) on the measured absorbance showed that its values increased considerably with increasing concentration of AChE. In general, the reaction of the AChE inhibition in the presence of carbamate pesticides is slow – at least 30 min to several hours is often required for analysis [24]. However, the use of low enzyme concentrations, showed that the enzyme inhibitor contact time could be reduced, and also, the detection of very low concentrations of the inhibiting compounds was possible [25]. On the other hand, lower concentrations of the enzyme gave narrower linear detection range of pesticides. Furthermore, the concentration of the enzyme had to be sufficiently high to give peaks of analytically useful intensity. Considering the above factors for the AChE analysis, a concentration of 1.2 g L^{-1} was chosen.

4.2.2 *Optimisation of substrate concentration*

In order to provide an accurate measure of the enzyme activity, the concentration of the substrate (ATChI) should be high enough so that the hydrolysis rate of the substrate is only

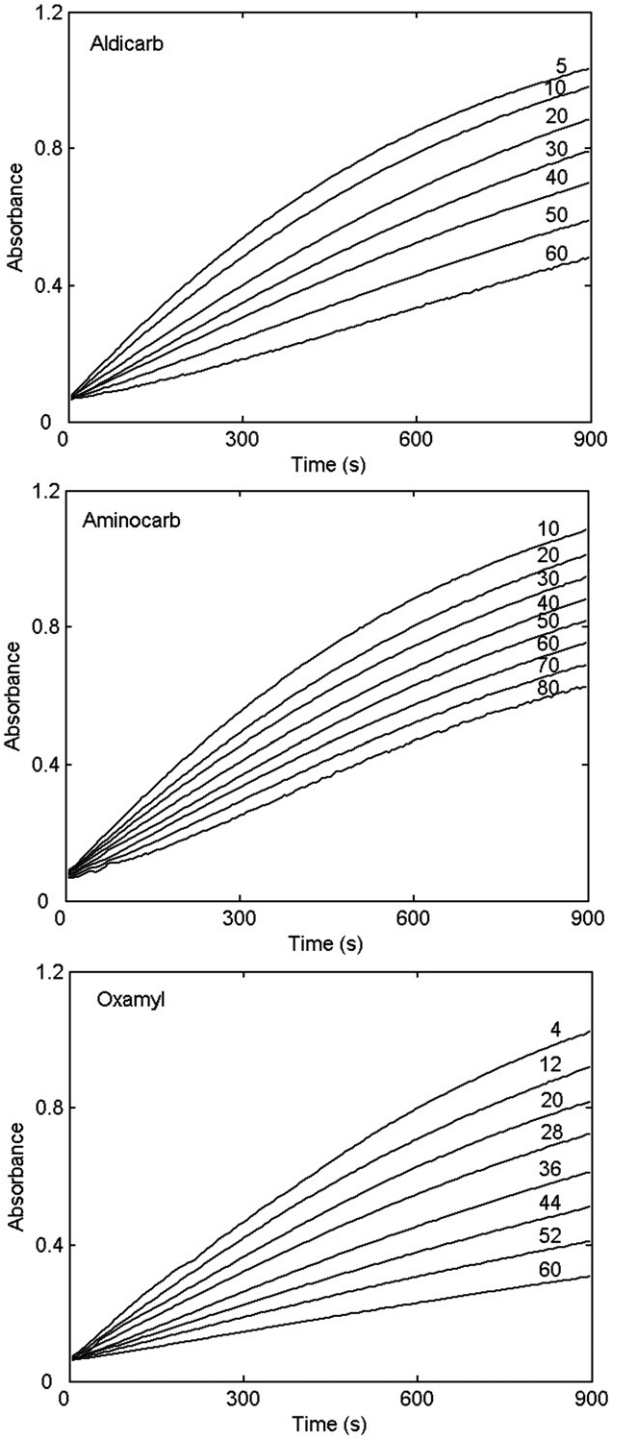


Figure 3. Kinetic spectral data for each pesticide at different concentrations ($ng\,mL^{-1}$). Experimental conditions are the same as Figure 1.

related to the enzyme activity. The reaction rates of AChE at various concentrations of ATChI (0.32–2.88 mmol L^{-1}) was studied. The results showed that the absorbance values increased continuously as the concentration of the substrate (ATChI) was increased to 1.6 mmol L^{-1}. There was no improvement in the absorbance values at higher concentrations of ATChI. Thus, the maximum reaction rate of AChE has been reached at this concentration of ATChI, and presumably, the active sites on the enzyme have been saturated with the substrate. Hence, a substrate concentration of 2.24 mmol L^{-1} was chosen.

4.2.3 *pH and temperature effect on the activity of the enzyme*

The activity of the enzyme also depends on pH and temperature of the working medium. Generally, the optimal working pH for AChE is about 7.5 [26–28], although other values between 8 and 9 have also been reported [29]. Calibrations in a thiocholine solution buffered with phosphate were performed within a pH range of 4.3–9.1. The enzyme activity was very sensitive to pH changes, and was almost undetectable at pHs <6.1. The highest enzyme activity was noted with the phosphate buffer in the range 7.0 to 9.0. Consequently, a pH of 7.9 was selected.

An investigation of effect of temperature on the absorbance intensity (range: 20–55°C) showed that the maximum enzyme activity occurred at the bath temperature of 40–45°C. Higher temperatures resulted in enzyme deactivation. Thus, the temperature of the water bath was maintained at 40°C.

4.3 *Interferences*

Various organic pesticides, such as triazophos, methylparathion, chlorpyrifos, isoprocarb, propoxur, isocarbophos, diethion, dipterex, and parathion, which may be present in food samples, were tested for interference during the enzymatic kinetic measurements under the same experimental conditions as before. It was found that chlorpyrifos and diethion did not interfere with the carbamate analysis because they were not enzymatic inhibitors. Triazophos and dipterex produced only a small interference because they inhibited the enzymatic kinetic reaction only to a small extent. However, some pesticides did interfere, and their degree of interference was estimated by determining how much of the interferant was required to give a 10% error. This analysis was carried out on 50 ng mL^{-1} of oxamyl, aldicarb and aminocarb in presence of each of the interfering pesticide, respectively, and the level of interference was expressed as a tolerance ratio i.e., a ratio of 10 : 1 (or simply 10) indicates that 10ng of inteferant to 1ng analyte pesticide produced a 10% error in the estimate of that analyte. The tolerance ratios for the determination of oxamyl, aldicarb and aminocarb were: 100 – parathion; 50 – methyl-parathion, propoxur and isoprocarb; 20 – isocarbophos. These results provide a guide for the effects of common pesticide interferences. The exact quantitative effects will have to be checked if the method is applied specifically for analysis of lower concentrations of the two analytes.

4.4 *Calibration models for single component analysis*

Calibration models were established as previously discussed for oxamyl, aldicarb and aminocarb analytes (Table 3). The correlation coefficients suggest good linearity over the concentration range of about 4–60 ng mL^{-1} for oxamyl, 5–60 ng mL^{-1} for aldicarb and

Table 3. Parameters of the linear models for each pesticide.

Parameters	Oxamyl	Aldicarb	Aminocarb
Sample number (n)	8	7	8
Linear range ($ng\,mL^{-1}$)	4–60	5–60	10–80
Correlation coefficient	−0.9999	−0.9995	−0.9999
Intercept, calibration	1.08	1.08	1.14
Slope, calibration ($mL\,ng^{-1}$)	−0.013	−0.0099	−0.0065
S_I ($\times 10^{-3}$)[a]	2.5	5.1	2.1
S_S ($\times 10^{-4}$)[a]	0.67	1.40	0.42
S_R ($\times 10^{-3}$)[a]	3.5	7.0	2.7
LOD ($ng\,mL^{-1}$)[a]	0.81	2.13	1.25

[a]S_I, S_S, S_R and LOD are the standard deviation of the intercept, slope and regression as well as the detection limits, respectively. They are calculated according to Miller's method [30].

10–80 $ng\,mL^{-1}$ for aminocarb (see Figure 3). The detection limits were 0.81, 2.13 and 1.25 $ng\,mL^{-1}$ for oxamyl, aldicarb and aminocarb, respectively. It is much better than the ones obtained by another kinetic method [31], which had, 0.12–0.26 $\mu g\,mL^{-1}$ as its detection limits.

4.5 *Prediction of oxamyl, aldicarb and aminocarb in a synthetic mixture*

Several different chemometrics models were investigated to study their effect on the prediction of individual pesticides found in synthetic and real pesticide mixtures.

In order to obtain maximum quantitative information with the use of minimum experimental trials, a training set of 16 standard samples, which were prepared according to a four-level orthogonal array design, denoted by $OA_{16}(4^3)$ [32], was taken from different ternary mixtures. The concentration levels of pesticides were selected to allow for a wide distribution of concentrations. Another set of 16 samples consisting of 16 synthetic mixtures was then used to evaluate the prediction ability of the calibration models. In this work, PLS, PCR, and RBF-ANN models were established for all the analytes simultaneously to simplify the calibration procedures. The prediction ability was expressed in terms of the root mean square error of prediction (RMSEP), relative prediction errors-RPE_S for individual compounds, and RPE_T for the overall error (Table 4).

$$\mathrm{RMSEP} = 100 \times \left[\frac{\sum_{i=1}^{n} \left(c_{ij(\text{found})} - c_{ij(\text{added})}\right)^2}{n} \right]^{0.5} \tag{16}$$

$$\mathrm{RPE_S} = 100 \times \left[\frac{\sum_{i=1}^{n} \left(c_{ij(\text{found})} - c_{ij(\text{added})}\right)^2}{\sum_{i=1}^{n} \left(c_{ij(\text{added})}\right)^2} \right]^{0.5} \tag{17}$$

$$\mathrm{RPE_T} = 100 \times \left[\frac{\sum_{i=1}^{n} \sum_{j=1}^{m} \left(c_{ij(\text{found})} - c_{ij(\text{added})}\right)^2}{\sum_{i=1}^{n} \sum_{j=1}^{m} \left(c_{ij(\text{added})}\right)^2} \right]^{0.5} \tag{18}$$

Table 4. Prediction performance for the different chemometrics calibration models obtained on Oxamyl, Aldicarb and Aminocarb analytes in synthetic mixtures ($ng\,mL^{-1}$).

	PCR (5)[a]			PLS (5)[a]			RBF-ANN (8, 6, 50)[b]		
Parameter	OXA[d]	ALD	AMI	OXA	ALD	AMI	OXA	ALD	AMI
Recovery[c]	106	98	95	105	98	95	104	99	95
RMSEP	0.48	0.70	0.73	0.48	0.69	0.72	0.53	0.71	0.68
RPE_S	5.91	8.38	8.17	5.86	8.29	8.16	6.53	8.51	7.69
RPE_T		7.68			7.63			7.59	

The values in the parentheses are:
[a]the number of factors used;
[b]the epochs, nodes in the hidden layer and the spread coefficient (sc), respectively.
[c]Recovery $(\%) = 100 \times \frac{\sum_{i=1}^{n} (c_{i(\text{pred})}/c_{i(\text{real})})}{n}$, where n is the number of samples.
[d]OXA, ALD and AMI represent oxamyl, aldicarb and aminocarb, respectively.

where $c_{ij,\text{added}}$ is the concentration of jth component in ith mixtures, and $c_{ij,\text{found}}$ is its estimation found by the above mentioned chemometric methods [33].

The results are summarised in Table 4. The prediction performances of the three models are satisfactory but no method stands out. It can be seen that both the results in the form of RPE_S and recovery for each carbamate pesticide given by PCR and PLS are very similar. And it can be seen that the total prediction error of RPE_T given by the RBF-ANN model was slightly better than those obtained by the PCR and PLS. Over the last few years we have been particularly interested in the research and development of relatively uncomplicated analytical methods for the simultaneous determination of analytes of industrial [34], environmental [35] and pharmaceutical [36] importance in real mixtures. Chemometrics methods have played a central role in facilitating the simultaneous analysis, and we have compared the performance of many chemometrics methods for prediction of analytes from responses obtained by voltammetric or spectrophotometric means. The above results reflect the general findings i.e., PCR is usually slightly worse than PLS, and RBF-ANN is rather better, especially for application to real samples [37]. It is well known that the ANN models need a big number of samples to develop a robust calibration model. However, in this work only a relatively simple quantitative analytical problem was performed, so RBF-ANN, as well as PCR and PLS, were used for the resolution of the spectral data for vegetable, fruit and water samples.

4.6 *Analysis of pesticides in real samples*

4.6.1 *Analysis of pesticides in vegetable and fruit samples*

Four common types of sample: pears, apples, cucumbers and tomatoes, were chosen for analysis. Each sample was treated as described in Section 3.4.1, and then 0.1 ml of the extract was transferred to the cell for analysis. The RBF-ANN method was applied for the analysis of the vegetable and fruit extracts because this method although marginally superior in this work, has been found to be quite better in other studies [12] on the basis of the $\%RPE_T$ criterion. The results (Table 5) showed that the procedure was further validated by standard addition of the three pesticides. This showed good %recovery values in the range of 98–105%. The % recoveries are uniformly consistent, which indicates that multivariate

Table 5. Determination of three carbamates in fruit and vegetable samples by RBF-ANN.

	Added			Measured			Recovery (%)		
Sample	OXA[a]	ALD	AMI	OXA	ALD	AMI	OXA	ALD	AMI
Pond water	0.250	0.250	0.500	0.259	0.260	0.510	102	103	102
($\mu g\,mL^{-1}$)	0.750	0.750	1.000	0.762	0.770	1.016	101	102	101
	1.250	1.250	1.500	1.248	1.270	1.503	99	101	100
Qianhu lake	0.250	0.250	0.500	0.253	0.254	0.483	101	101	97
($\mu g\,mL^{-1}$)	0.750	0.750	1.000	0.757	0.752	0.975	100.	100	97
	1.250	1.250	1.500	1.258	1.261	1.484	100	97	98
Tap water	0.250	0.250	0.500	0.252	0.244	0.493	100	97	98
($\mu g\,mL^{-1}$)	0.750	0.750	1.000	0.757	0.740	0.991	100	98	99
	1.250	1.250	1.500	1.246	1.246	1.494	99	99	99
Pear[b]	0.250	0.250	0.500	0.253	0.245	0.488	101	98	97
($\mu g\,g^{-1}$)	0.750	0.750	1.000	0.769	0.740	0.971	102	98	97
	1.250	1.250	1.500	1.304	1.233	1.499	103	98	99
Apple	0.250	0.250	0.500	0.245	0.250	0.493	97	99	98
($\mu g\,g^{-1}$)	0.750	0.750	1.000	0.732	0.757	0.987	97	100	98
	1.250	1.250	1.500	1.226	1.271	1.482	98	101	98
Cucumber	0.250	0.250	0.500	0.255	0.255	0.488	101	102	97
($\mu g\,g^{-1}$)	0.750	0.750	1.000	0.760	0.765	1.021	101	102	102
	1.250	1.250	1.500	1.307	1.299	1.519	104	103	101
Tomato	0.250	0.250	0.500	0.249	0.253	0.504	99	101	101
($\mu g\,g^{-1}$)	0.750	0.750	1.000	0.757	0.725	1.015	100	99	100
	1.250	1.250	1.500	1.270	1.291	1.557	101	100	100

[a]OXA, ALD and AMI represent oxamyl, aldicarb and aminocarb, respectively.
[b]All the vegetable samples were purchased from a supermarket in Nanchang city.

prediction models can produce satisfactory results for the simultaneous determination of oxamyl, aldicarb and aminocarb in complex vegetable and fruit samples.

4.6.2 *Analysis of pesticides in water samples*

Three samples, lake water, pond water and tap water obtained from different places in Nanchang, were chosen for analysis. Each sample was treated as described in Section 3.4.2, and then 0.1 mL of the extract was transferred to the cell for analysis. The set of calibration standards for these water samples was the same as for the synthetic samples in Section 4.5. The RBF-ANN method was also applied for the analysis of the water extracts, and the results (Table 5) showed that the usefulness of the procedure was further confirmed by the %recovery values (97–103%). The results obtained by the proposed method were close to those given by the reference method, HPLC (%recovery was in the range of 97–105%, results of HPLC not listed). This showed that the simultaneous determination of the three pesticides in real samples by the present method was an adequate alternative when compared to the HPLC method.

5. Conclusion

An analytical spectrophotometric method has been researched and developed for the simultaneous enzymatic kinetic determination of oxamyl, aldicarb and aminocarb

pesticides in mixtures. The outcomes were:

(1) the complex rate equation for the formation of the chromogenic product, P, was developed under certain experimental conditions, and this enabled the absorbance from the mixtures of the three pesticide inhibitors to be directly related to their concentrations.
(2) Calibration models were constructed for the principal component regression (PCR), partial least squares (PLS), and radial basis function-artificial neural network (RBF-ANN), and the RBF-ANN model performed slightly better than the other two ($RPE_T = 7.59\%$ and average %recovery = 99%).
(3) The RBF-ANN calibration was then successfully applied to estimate the amounts of the three compounds in fruit, vegetable and water samples.

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