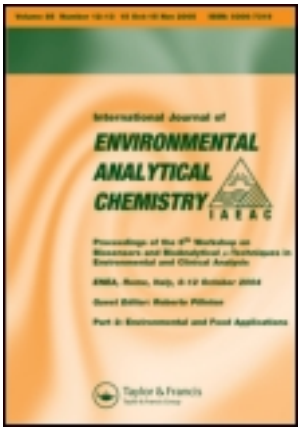

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/geac20

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Available online: 23 Mar 2011

To cite this article: Diyuan Yang, Jingqun Tang, Xiaolan Yang, Ping Deng, Yunsheng Zhao, Sha Zhu, Yanling Xie, Xinbi Dai, Hong Liao, Ming'an Yu, Juan Liao & Fei Liao (2011): An integration strategy to measure enzyme activities for detecting irreversible inhibitors with dimethoate on butyrylcholinesterase as a model, International Journal of Environmental Analytical Chemistry, 91:5, 431-439

To link to this article: http://dx.doi.org/10.1080/03067310902871315

Intern. J. Environ. Anal. Chem.
Vol. 91, No. 5, 20 April 2011, 431–439

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(*Received 3 November 2008; final version received 4 March 2009*)

An integration strategy was investigated to measure initial rates of horse butyrylcholinesterase (BChE) at 50.0 μmol L^{-1} butyrylthiocholine (BTCh) for detecting irreversible inhibitors as pollutants in environment and foods with dimethoate as a model. In this integration strategy: (a) if BTCh consumption within 5.0 min was >60%, BChE initial rates were derived from maximal reaction rates, estimated by an improved integrated method, according to Michaelies-Menten kinetics at 47.0 μmol L^{-1} BTCh and Michaelis-Menten constant at 94.0 μmol L^{-1}; (b) or else initial rates were determined by the classical initial rate method. Thus, the differences in BChE initial rates without and after dimethoate treatment indexed final dimethoate contents in reaction mixtures to treat BChE. Results supported that this integration strategy determined BChE activities with a linear range about two magnitudes and an upper limit about twice that by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh. The coefficient of variation with this integration strategy was below 5%. The difference in BChE initial rates before and after dimethoate treatment was proportional to final dimethoate contents in reaction mixtures. By enzymatic analyses, the molar contents of dimethoate extracted from polluted cabbages were consistent with the summed molar contents of dimethoate and dimethoxon by gas-chromatography. Therefore, this integration strategy was effective to detect irreversible inhibitors as pollutants in environment and foods.

Keywords: integration strategy; butyrylcholinesterase; enzymatic analysis; irreversible inhibitors; dimethoate

List of abbreviations: BChE: butyrylcholinesterase; BTCh: butyrylthiocholine; CV: coefficient of variation; GC: gas-chromatography; K_m: Michaelis-Menten constant; V_i: initial rate; V_m: maximal reaction rate

1. Introduction

The inhibition of some enzymes by irreversible inhibitors, for example, the inhibition of butyrylcholinesterase (E.C. 3.1.1.8) by organophosphate pesticides as pollutants in

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ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310902871315
http://www.informaworld.com

environment and foods, is effective to detect such inhibitors [1–6]. For detecting irreversible inhibitors, the methods to measure enzyme activities should have wide linear ranges, low costs and high efficiency. The classical initial rate method in common use has satisfactory efficiency, but needs substrate at concentrations much higher than Michaelis-Menten constants (K_m) and inevitably suffers from higher costs on substrates and the potential interference from substrate-activation [7,8] or substrate-inhibition [9]. Besides, it tolerates narrow linear ranges at relatively low substrate concentrations for costs [10]. Therefore, new methods are desired to measure enzyme activities.

Nowadays, many chemometric methods had been developed to measure enzyme initial rates [11–17], but they tolerated the susceptibility to common systematic errors, limited linear ranges and unfavourable costs. Recently, based on an improved integrated method to robustly estimate maximal reaction rates (V_m) [18] and related parameters [19–23], a new chemometric strategy was developed to measure enzyme initial rates by integrating the classical initial rate method for lower enzyme activities with this improved integrated method for higher enzyme activities [24,25]. This integration strategy at moderate substrate concentrations gave wider linear ranges, and had the efficiency comparable to the classical initial rate method. Therefore, this integration strategy was promising to measure enzyme activities for detecting irreversible inhibitors.

The pollution of environment and foods by organophosphate pesticides is a great concern and the inhibition of butyrylcholinesterase by organophosphate pesticides was already used for detecting such pollutants [1–6,26]. The classical initial rate method to measure BChE activities at millimolar levels of butyrylthiocholine (BTCh) tolerated the limited linear ranges and strong substrate-activation interference [7,8]. Herein, with the inhibition of BChE by dimethoate as the model, this integration strategy at BTCh lower than K_m was investigated to measure BChE activities with negligible substrate-activation for detecting irreversible inhibitors.

2. Experimental

2.1 *Materials and chemicals*

Recombinant horse serum butyrylcholinesterase (BChE), butyrylthiocholine iodide (BTCh) and dithiobis-(2-nitrobenzoic acid) (DTNB) were from Sigma. Dimethoate was from Chongqing Center for Disease Control and Prevention (Chongqing 400042, China). Other reagents were analytical grade reagents from Chongqing Beibei Chemical Factory (Chongqing 400708, China).

2.2 *Extract of residual dimethoate on vegetables*

Acetone solution of dimethoate, 1.0 mL in total at 0.5 mg mL^{-1}, was dropped on pieces of cabbages (~20 g in total). After the evaporation of acetone, the cabbages were homogenised with 20 mL dichloromethane and the dichloromethane phase was recovered by centrifugation in a sealed glass tube. After dehydration with anhydrous sodium sulfate, dichloromethane was removed under reduced pressure at room temperature within 30 min. Finally, the residuals were dissolved in 1.0 mL acetone and stored in a sealed glass tube in dark at 4°C for analyses within 6 h.

2.3 *Monitor of BChE reaction curves*

The buffer was 50.0 mmol L^{-1} sodium phosphate at pH 7.4, and was preheated to $(25 \pm 0.5)^{\circ}C$ before use. Each reaction mixture 1.20 mL contained 100 μL DTNB solution (12.0 mmol L^{-1} in the buffer), 100 μL BTCh solution in the buffer for an indicated final concentration, 50 μL BChE solution and an appropriate amount of the buffer. Before the addition of BChE to initiate reaction, other components were mixed and incubated at $(25.0 \pm 0.3)^{\circ}C$ for 10.0 min. Absorbance at 410 nm < 1.300 was recorded within 10 min at intervals from 1 to 10 s after a 30 s lag, with a Xinmao UV 7504 spectrophotometer linked to a compatible computer (Shanghai 200233, China) in a small isolated room air-conditioned at 25°C. The final concentration of BTCh was 50.0 μmol L^{-1} with the integration strategy, whereas it was 2.0 mmol L^{-1} with the classical initial rate method alone. All assays were performed independently in duplicate.

2.4 *Data processing with the improved integrated method*

When BTCh levels were low enough, BChE followed Michaelis-Menten kinetics with negligible substrate-activation [7,8]. Assign t_l to the lag time for steady-state reaction, t_i to any instantaneous reaction time, A_i to the instantaneous absorbance at t_i, A_m to the maximal absorbance after the complete hydrolysis of BTCh and A_b to background absorbance. Then, the integrated Michaelis-Menten rate equation for any single-substrate irreversible reaction with neither substrate-activation nor substrate-inhibition was Equation (1) [19,24].

$$(A_m - A_i)/(\varepsilon \times K_m) + \ln(A_m - A_i) = (A_m - A_b)/(\varepsilon \times K_m) + \ln(A_m - A_b) - (V_m/K_m) \times (t_i - t_l) \quad (1)$$

The absorptivity (ε) for 2-nitro-4-mercapto-benzoic acid was 13.6 $mM^{-1}\,cm^{-1}$ at 410 nm. With known K_m and ε, there was Equation (2) to fit BChE reaction curves.

$$(A_m - A_i)/(\varepsilon \times K_m) + \ln(A_m - A_i) = (A_m - A_b)/(\varepsilon \times K_m) + \ln(A_m - A_b) - (V_m/K_m) \times t_i \quad (2)$$

Thus, V_m/K_m was estimated by weighted nonlinear fitting of Equation (2) to reaction curves based on *F*-test to judge the goodness of fit with A_m as a nonlinear parameter adjusted at the step of 0.001 [18]. Then V_m was derived from V_m/K_m with the known K_m. Equation (2) only applied to steady-state reaction data. Usually, BChE reaction achieved steady-state 40 s after reaction initiation. Therefore, the lag time for steady-state reaction was preset at 40 s. An executable program written in Visual Basic 6.0 was used on compatible computers to estimate V_m of BChE reactions [24,25].

2.5 *Estimation of initial rates*

One unit of BChE activity was its amount to hydrolyse one micromole BTCh per min. Classical initial rates were the averaged reaction rates from 50 to 90 s after reaction initiation. With the integration strategy, initial rates were determined as described below: (a) if BTCh consumption was above 60% within 5.0 min based on the absorbance at 410 nm minus A_b of 0.025, initial rates were derived from V_m, estimated by the improved integrated method (See above), according to Michaelis-Menten kinetics with K_m preset at

94.0 μmol L^{-1} and the preset substrate concentration (PSC) at 47.0 μmol L^{-1}, unless otherwise stated [24,25]; (b) if substrate consumption was equal or below 60% within 5.0 min, initial rates were the classical initial rates per se.

2.6 *Enzymatic analyses of dimethoate contents*

Reaction mixtures to treat BChE by dimethoate at 25.0°C contained 0.30 ml BChE solution (6.0 U mL^{-1} by the classicla initial rate method alone at 2.00 mmol L^{-1} BTCh), 0.30 mL dimethoate solution diluted in the phosphate buffer (final acetone was 8% for negligible intefrence with the assay of BChE). After treatment in duplicate for 2 h, residual BChE activities were immediately measured with 50 μL aliquots of each BChE sample, by both the integration strategy at 50.0 μmol L^{-1} BTCh and the classical initial rate method alone at 2.0 mmol L^{-1} BTCh, respectively. The differences between BChE initial rates before and after dimethoate treatment were checked with respect to final contents of dimethoate in reaction mixtures to treat BChE, respectively.

2.7 *Gas-chromatography analysis*

SC-2000 gas-chromatography (GC) from Chongqing Chuanyi General Factory (Beibei, Chongqing 400073, China) linked to a flaming photometry detector was used. The column was 30 m × 0.53 mm with the immobilised phase of 0.25 μm BPX. Nitrogen was flushed at 30 mL min^{-1}, mixed with 0.31 mPa hydrogen and 0.06 mPa air in the detector. The temperature gradient cycle was 2.0 min at 110°C, a linear increase to 180°C at 15° min^{-1}, to 230 at 15°C min^{-1} after being kept at 180°C for 1.0 min. The injector was kept at 250°C while the detector was maintained at 250°C. In this system, the retention time for dimethoate was 8.655 ± 0.033 min ($n = 5$) and that for dimethoxon was 7.132 ± 0.013 min ($n = 5$). Each sample was analysed twice.

2.8 *Statistical analysis*

The reliability of the improved integrated method was that to give results with deviation below 3% from that with reaction data of 90% substrate consumption for analyses, which was used to judge its detection limit [24]. With either the integration strategy or the classical initial rate method, the lower limit for linear response was three times the standard error of estimate. For either method, the upper limit was that with deviation below twice the standard error of estimate. Results were mean ± SD, and were compared by t-test with $P < 0.05$ as the confidence limit.

3. Results

3.1 *Substrate-activation of BTCh*

Initial rate reactions were observed within 90 s at 50.0 μmol L^{-1} BTCh if its consumption was less than 60% BTCh within 5.0 min. Lineweaver-Burk plot analysis of BChE initial rates versus BTCh concentrations from 0.010 to 2.0 mmol L^{-1} gave a downward curved line (Figure 1). K_m of BChE was 105 ± 20 μmol L^{-1} ($n = 5$) at BTCh < 0.12 mmol L^{-1} and 0.30 ± 0.06 mmol L^{-1} ($n = 3$) at BTCh concentrations from 0.30 to 2.0 mmol L^{-1}. The ratio

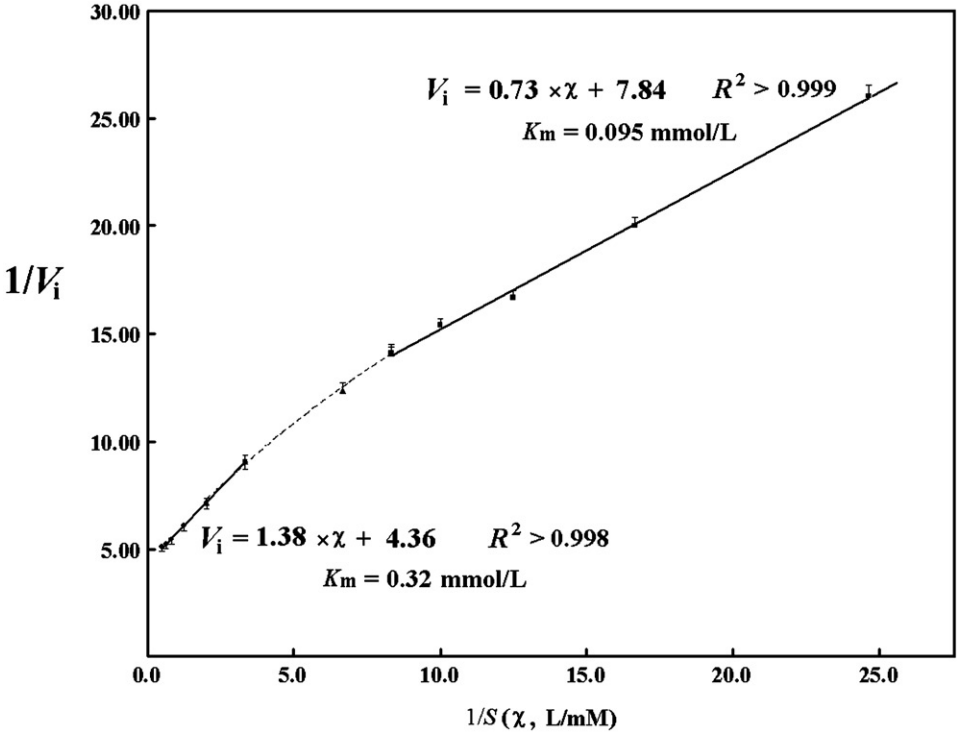

Figure 1. Lineweaver-Burk plot analysis of BTCh activation on recombinant horse BChE. Initial rate V_i was in arbitrary unit and BTCh concentration was in $\mu mol\,L^{-1}$.

of the resultant maximal reaction rate of BChE at BTCh above $0.30\,mmol\,L^{-1}$ to that at BTCh $< 0.12\,mmol\,L^{-1}$ was 1.3 ± 0.1 ($n = 2$). Besides, the linear response of BChE activities from $5\,U\,L^{-1}$ to $25\,U\,L^{-1}$ by the classical initial rate method alone at $2.0\,mmol\,L^{-1}$ BTCh to maximal reaction rates estimated by the improved integrated method at $50.0\,\mu mol\,L^{-1}$ BTCh gave the slope of 1.4 ± 0.1.

3.2 *Estimation of BChE initial rates by the integration strategy*

With initial BTCh at $50.0\,\mu mol\,L^{-1}$, BTCh concentrations were $<47.0\,\mu mol\,L^{-1}$ at the time of 40 s after reaction initiation. The improved integrated method required BTCh consumptions >55% in reaction curves for analyses. At $50.0\,\mu mol\,L^{-1}$ BTCh, on the one hand, the classical initial rate method gave the upper limit for linear response about $4.5\,U\,L^{-1}$ while the lower limit was about $0.40\,U\,L^{-1}$. On the other hand, with data monitored within 5.0 min, the detection limit as the initial rate by the improved integrated method using a PSC at $47.0\,\mu mol\,L^{-1}$ and K_m at $94.0\,\mu mol\,L^{-1}$ was about $2.5\,U\,L^{-1}$ while the maximum was $>60\,U\,L^{-1}$ (limited by the available data for analyses).

When K_m was preset at $94.0\,\mu mol\,L^{-1}$ to derive initial rates from V_m with the PSC at $47.0\,\mu mol\,L^{-1}$, the slope for linear response of initial rates by the improved integrated method to amounts of BChE showed the deviation below 0.2% from that by the classical initial rate method under the same conditions, and there were the consistent initial rates

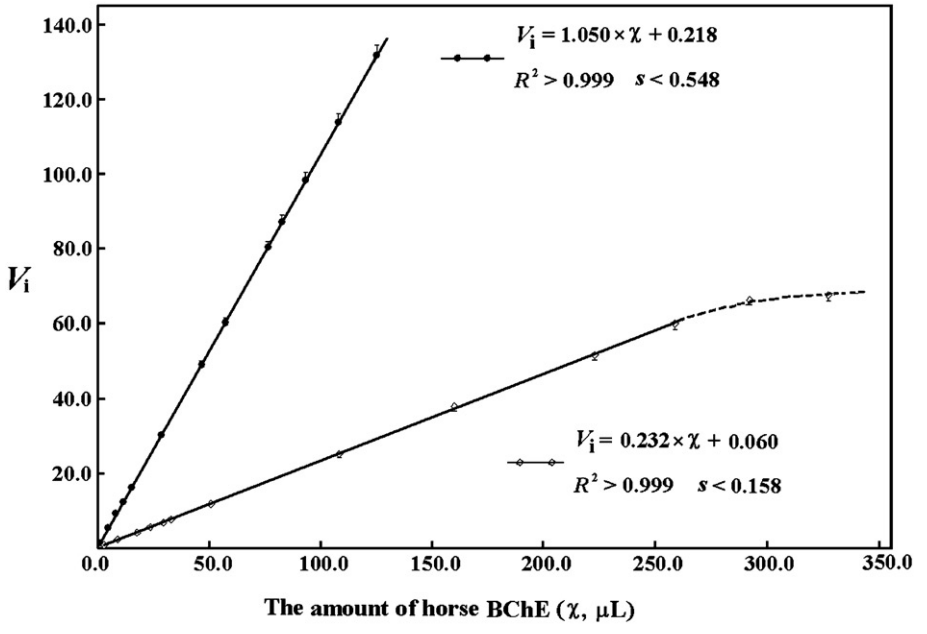

Figure 2. Responses of BChE initial rates to amounts of BChE. —●— the classical initial rate method at 2.0 mmol L^{-1} BTCh. —◇— the integration strategy at 50.0 μmol L^{-1} BTCh.

within the overlapped range. Nevertheless, when K_m was preset at 105.0 μmol L^{-1} to derive initial rates from V_m even with the PSC at 50.0 μmol L^{-1}, the slopes for linear responses by the two individual methods showed deviation above 1.5%. Therefore, K_m at 94.0 μmol L^{-1} and the PSC at 47.0 μmol L^{-1} were used throughout the following analyses to derive initial rates from V_m with the integration strategy.

By using 60% consumption of BTCh within 5.0 min as the switch cutoff, initial rates via this integration strategy at 50.0 μmol L^{-1} BTCh linearly responded to amounts of BChE (Figure 2). And the ratio of the BChE amount for the upper limit to that for the lower limit was about 100-fold. Initial rates showed within-run CVs < 4%, and between-run CVs < 4.5% ($n = 4$, the same BChE was stored at 4°C within two days). Meanwhile, there was a linear response of classical initial rates at 2.0 mmol L^{-1} BTCh to amounts of BChE with a much larger standard error of estimate, and its upper limit was only 120 U L^{-1} due to the limited absorbance measurable by the spectrophotometer (Figure 2). However, the lower limits of BChE for linear responses by these two individual methods showed negligible difference. Nevertheless, the upper limit of BChE measurable by this integration strategy was about twice that by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh. And the ratio between the slopes of these two individual methods was consistent with that when only substrate-activation at 2.0 mmol L^{-1} BTCh was considered [6,7].

3.3 *Enzymatic analyses of dimethoate*

By both this integration strategy at 50.0 μmol L^{-1} BTCh and the classical initial rate method alone at 2.0 mmol L^{-1} BTCh, there were linear responses of inhibited BChE

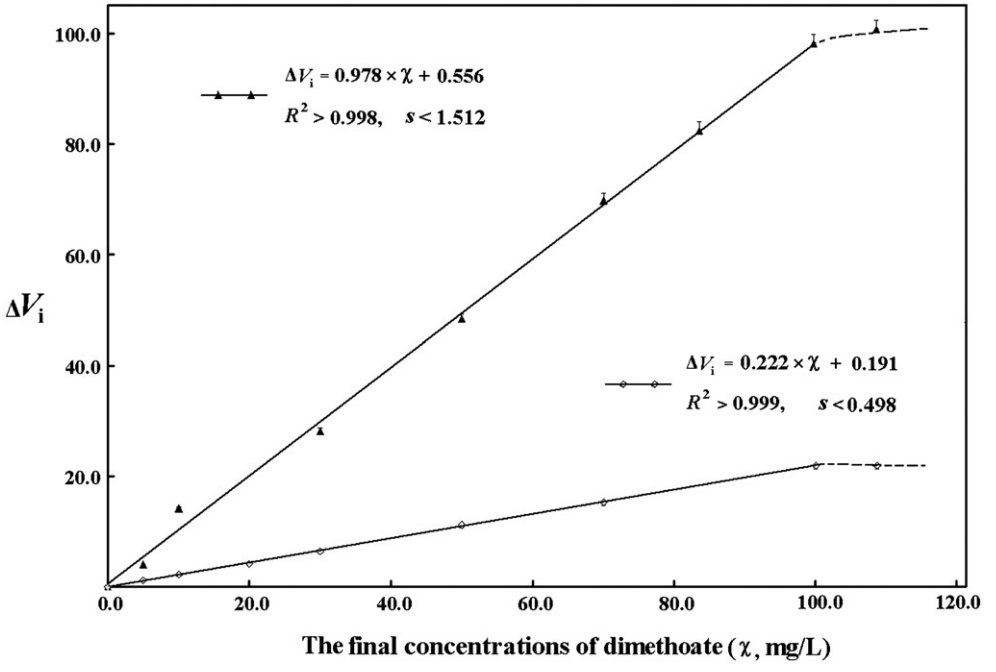

Figure 3. Responses of inhibited BChE activities to dimethoate contents in mixtures to treat BChE. —■—■— the classical initial rate method at 2.0 mmol L^{-1} BTCh. —◇—◇— the integration strategy at 50.0 μmol L^{-1} BTCh.

activities to dimethoate contents in reaction mixtures to treat BChE, respectively (Figure 3). The lower limit of dimethoate in reaction mixture for linear response was about 10.0 mg L^{-1} by the integration strategy, and 8.0 mg L^{-1} by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh. Besides, the upper limits of dimethoate contents in reaction mixtures by both enzymatic methods showed no difference.

The amount of dimethoate in the extract from the polluted cabbages was (0.41 ± 0.04) g L^{-1} ($n = 2$), equal to (1.79 ± 0.18) mmol L^{-1} by this integration strategy at 50.0 μmol L^{-1} BTCh. It was (0.43 ± 0.03) g L^{-1} ($n = 2$), equal to (1.88 ± 0.14) mmol L^{-1}, by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh. By GC analyses, the content of dimethoate in the extract was 0.35 ± 0.01 g L^{-1} ($n = 2$), equal to (1.53 ± 0.05) mmol L^{-1}, which was somewhat lower than that by either enzymatic method ($P < 0.066$ by one-way t-test). However, the molar content of dimethoxon in the extract accounted for about 19% that of dimethoate. When molar contents of dimethoxon and dimethoate in the extract were summed, GC analyses gave dimethoate equivalence of (1.81 ± 0.05) mmol L^{-1}, which was exactly consistent with that by either enzymatic method.

4. Discussion

Besides the validity of the improved integrated method itself [18–23], this integration strategy for enzyme initial rate assay required the simultaneous satisfaction to the following additional prerequisites [24,25]: (a) an overlapped region of initial rates

measurable by the two individual methods, which could be obtained after the optimisation of reaction duration to monitor reaction data for analyses with practical efficiency [18–23]; (b) a PSC optimised to derive initial rates from V_m for the consistent linear response slopes by the two individual methods and the consistent results within this overlapped region; (c) a switch cutoff to change from the classical initial rate method to the improved integrated method at high enzyme activities, which could be the substrate concentration due to its uniqueness [24].

To validate Equation (2), BTCh levels should be low enough to neglect substrate-activation interference [7,8,25]. The substrate-activation effect of BTCh on horse BChE at 2.0 mmol L^{-1} BTCh was about 1.4 if there was no substrate-activation at 50.0 μmol L^{-1} BTCh [7,8], and thus experimental results supported the validity of Equation (2). For practical efficiency, reaction duration was preset within 5.0 min. In this case, an overlapped region of initial rates measurable by the two individual methods, consistent slopes for linear response, and consistent initial rates within the overlapped region were simultaneously achieved with the PSC preset at 47.0 μmol L^{-1} and K_m preset at 94.0 μmol L^{-1}. And the upper limit with this integration strategy was much higher than that by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh. Therefore, this integration strategy was effective and advantageous [11–17]. Moreover, the PSC at 47.0 μmol L^{-1} accounted for about 93% of the initial BTCh concentration, which indicated that the PSC at 93% of the initial substrate concentration may be a universally optimal one for this integration strategy [24,25].

Surely, the upper limit of dimethoate contents in reaction mixtures detectable by either enzymatic method was restricted by the total amount of BChE in reaction mixtures. The integration strategy with the upper limit for linear response close to twice that by the classical initial rate method alone at 2.0 mmol L^{-1} BTCh could have expanded linear ranges to detect organophosphate pesticides with increased amounts of BChE in reaction mixtures. Enzymatic methods via the inhibition on BChE gave the sum of molar equivalence of diverse irreversible inhibitors on BChE. Dimethoxon inhibited BChE with potency stronger than that of dimethoate [27–29]. Thus, the presence of dimethoxon in the extract by GC analyses, the discrepancy between the dimethoate content by GC analyses and that by either enzymatic method, and the consistency between the sum of molar contents of dimethoate plus dimethoxon by GC analyses and the equivalence content of dimethoate by either enzymatic method, all clearly supported the reliability of this integration strategy for detecting irreversible inhibitors of enzymes.

In conclusion, this integration strategy exhibited much reduced costs on substrates, wide linear ranges, practical efficiency and negligible interference from substrate-activation or substrate-inhibition, and it was promising to measure enzyme activities for detecting irreversible inhibitors in environment and foods.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Nos. 30200266, 30472139, 30672009). This integration strategy was under evaluation for invention patent in China (Application No: 200710093081.4).

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