Journal of Chromatography B, 878 (2010) 3156–3160

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid screening of monoamine oxidase B inhibitors in natural extracts by capillary electrophoresis after enzymatic reaction at capillary inlet

Kun Hu, Lichun Zhang, Xiangtang Li, Shulin Zhao*

The Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources (Ministry of Education), College of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 541004, China

ARTICLE INFO

Article history:
Received 2 August 2010
Accepted 27 September 2010
Available online 7 October 2010

Keywords:
Monoamine oxidase B
Capillary electrophoresis
Kinetic constants
Inhibitor screening

ABSTRACT

A facile capillary electrophoresis (CE) method was developed for the screening of monoamine oxidase B (MAO-B) inhibitors in natural extracts. In this method, the enzymatic reaction occurred at the capillary inlet during a predetermined waiting period, followed by the electrophoretic separation of the reaction compounds, and detected by their UV absorbance at 280 nm. Conditions for the separation of substrates, products and enzyme were optimized. The optimal buffer composition was 50 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethane sulphonic acid (HEPES) solution containing 10 mM SDS (pH = 7.4). Under the optimal condition, the baseline separation of substrates, products and enzyme was achieved within 2 min. The present method was used to determine MAO-B kinetic constants, K_i, K_m and IC_{50} based on quantitative of the substrate peak area compared with the reference electropherogram obtained from without the inhibitor. A validation study shows good reproducibility for both migration time (RSD = 1.8%) and peak area (RSD = 3.9%). Finally, the screening of 16 natural extracts was performed, and 2 natural extracts from *Fructus crataegi* and *Radix polygoni multiflori* were identified to be positive for MAO-B inhibition.

1. Introduction

Monoamine oxidase B (MAO-B) is one of the two flavin-adenine dinucleotide-depending isozymes playing a key role in the regulation of endogenous monoamine neurotransmitters as dopamine. MAO-B can catalyze dopamine to homovaniuic acid (HVA) and produce hydrogen peroxide in the brain, liver, intestinal mucosa and other organs, and induce oxidative stress and result in neuron death [1,2]. Inhibitors of MAO-B not only lead to enhanced dopaminergic neurotransmission, but also prevent activation of toxin and free radical formation, and then alleviated the process of neuron denaturalization. In clinical practice, MAO-B activity is detected to increase many neurodegenerative diseases. Inhibitors of MAO-B are used to relieve symptoms or slow the progression of Parkinson's disease (PD) [3–5], and used for the symptomatic treatment of PD [6]. Therefore, MAO-B is an important target for developing new drugs against PD. Corresponding inhibitors are currently being explored as potential drugs for the clinical treatment of PD, Alzheimer's disease and cerebral ischemia during strokes [7].

The availability of screening methods that allow the selection of drug candidates is a basic aspect of the drug discovery process. Traditional Chinese medicines (TCM) have been used to treat human diseases in china for centuries, which provide an essential source of compound for the discovery of new drugs. Therefore, it is absolutely necessary to develop rapid, low-cost, and effective method for MAO-B inhibitor screening in natural extracts from TCM.

Due to the importance of MAO-B inhibitor, several methods have been developed for monitoring MAO-B activity and its inhibitor-screening. These methods include the multi-dimensional chromatographic [8] and fluorometric assays [9–12]. Capillary electrophoresis (CE) is a powerful and relatively new analytical tool, characterized mainly by high resolution separations, short analysis times and low sample load. In recent years, many CE methods have been reported for the determination of the kinetic parameters and screening of enzyme inhibitors. For example, Krylov and co-workers developed a CE method named inject-mix-react-separate and quantitate for screening enzyme inhibitors [13]. Iqbal et al. reported a highly sensitive CE method applying dynamic coating and on-line stacking for monitoring of nucleotide pyrophosphatases/phosphodiesterases and screening of inhibitors [14]. Kang's group developed an electrophoretically mediated microanalysis (EMMA) based CE method for screening hexokinase and acetylcholinesterase inhibitors [15,16]. To the best of our knowledge, hitherto the CE method for monitoring MAO-B activity and its inhibitor-screening has not been reported.

In the present study, an EMMA based CE method was developed for activity study and inhibitor screening of MAO-B. Experimental conditions were optimized and the method was validated with

* Corresponding author. Tel.: +86 773 5849646; fax: +86 773 5832294.
E-mail address: zhaoshulin001@163.com (S. Zhao).

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doi:10.1016/j.jchromb.2010.09.030

a commercially available MAO-B inhibitor. This method allows measuring product and screening MAO-B inhibitor within a few minutes with high accuracy and reproducibility.

2. Experimental

2.1. Chemicals and reagents

MAO-B, dopamine (DA) and HVA were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was purchased from Shanghai Chemical Reagents Corporation (Shanghai, China). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained from Shanghai Qcbio Science & Technologies Co. Ltd. (Shanghai, China). Selegiline and borate were obtained from Guangzhou Chemical Reagents Corporation (Guangzhou, China). All other chemicals and organic solvents used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. All solutions were filtered through 0.45 μm membrane filter.

2.2. Instrumentation

All separations were performed on an HP^{3D} CE system (Hewlett Packard, Waldbronn, Germany) equipped with a diode array detector (190–600 nm). Data were processed on an HP chemstation. Fused-silica capillary with a dimensions of 50 μm i.d. × 360 μm o.d. × 34.5 cm (26 cm to the detection window) were purchased from Yongnian Optical Fiber Co. (Hebei, China). The capillary and carousel were thermostated at 37 °C. All analytes were detected at a UV wavelength of 280 nm.

2.3. Sample preparation

Radix polygoni multiflori and *Fructus crataegi* were obtained from Guilin Pharmaceuticals Group of China (Guilin, China). Air-dried *Radix polygoni multiflori* and *Fructus crataegi* were ground into a fine powder with a pulverizer. The heat reflux extraction was used to prepare the extracts from TCM. A 2.5 g dried powdered sample was extracted with 38 mL water in heat reflux water bath for 3.0 h, and this process was repeated three times. The extracted solutions were evaporated to 10 mL and diluted to 25 mL with water. Then 500 μL extracted solution was transferred into a 1.5 mL vial, and double amount of acetonitrile solution was added. The solution was vortexed and centrifuged (12,000 × *g* for 10 min). The supernatant was transferred into another 1.5 mL vial and dried with a N_2 stream. The residue was dissolved in 500 μL HEPES buffer (pH 7.4) containing 10 mM SDS. The solution was kept at 4 °C temperatures.

2.4. CE procedure

The new capillary was preconditioned by rinsing the capillary with 1.0 M HCl for 10 min, water for 5 min and 1.0 M NaOH for 20 min, respectively. Between two runs the capillary was rinsed sequentially with 0.1 M NaOH, water and running buffer for 2 min each. The EMMA experiment can be divided into three continuous steps: (1) 50 mM HEPES buffer was injected into the capillary by pressure at 20 mbar for 4 s. Then MAO-B and substrate solutions with or without inhibitor, as well as HEPES buffer, were successively injected into the capillary by pressure at 20 mbar for 8 s. It should be noted that before each injection, the inlet end of the capillary and the electrode were cleaned by dipping them in water in a vial to avoid contamination of each other. (2) A voltage of 1.0 kV was applied for 25 s to mix the enzyme solution with the substrate solution. (3) A voltage of 18 kV was applied to separate the product, unreacted substrate and enzyme. The activity of MAO-B was assayed by measuring the peak area of product HVA, and the percentage of inhibition was calculated according to the peak area of HVA compared with the reference electropherogram obtained without the inhibitor.

3. Results and discussion

3.1. Effect of incubation time on yield of reaction product

EMMA technique utilizes the different electrophoretic mobilities of enzyme and substrate to initiate the reaction inside the capillary [17]. The reaction time is an important influence factor for the enzymatic reaction. In this study, the effect of the incubation time on the yield of reaction product HVA was investigated by applying the mixing voltage of 1.0 kV for 0–30 s. Fig. 1 shows the relationship between mixing time and yield. As can be seen, the yield increased with increasing incubation time up to 25 s, where maximum yield was reached. After the incubation time was longer than 25 s, almost constant yield was obtained. Then a 25 s incubation time was selected for following experiments.

3.2. Optimization of separation conditions

In order to achieve an efficient separation between the substrate (DA) and product (HVA), the separation conditions such as concentration of buffer and SDS, pH of electrolyte solution were investigated. Firstly, two separation modes, capillary zone electrophoresis and micellar electrokinetic chromatography (MEKC), were tested for the separation. The results show that the product and substrate could not been separated by capillary zone electrophoresis. So MEKC was selected for the separation.

SDS was used as a micellar solution added into running buffer in the MEKC, and the influence of the SDS concentration ranging from 5 mM to 20 mM on the separation was investigated. The results indicate that the resolution of the substrate with product increased with increasing SDS concentrations to 10 mM. With further increases in the SDS concentrations, almost constant resolutions were observed. So 10 mM SDS was used for further experiment.

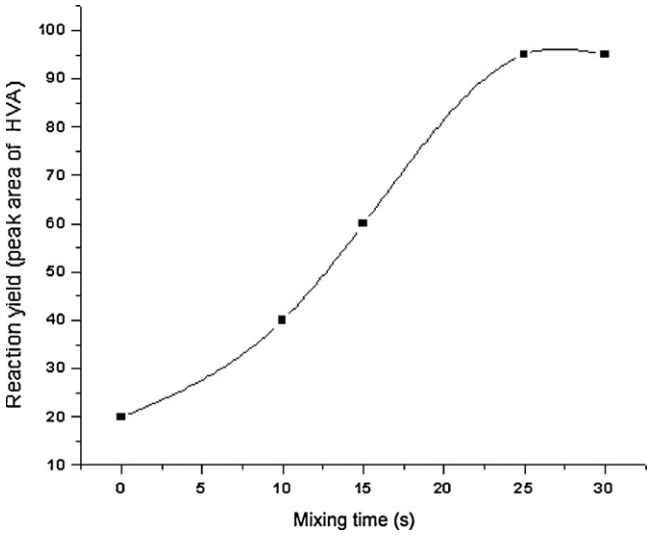


Fig. 1. Dependence of the yield of the reaction product HVA on incubation time. CE conditions: fused silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 50 mM HEPES buffer (pH 7.4) containing 10 mM SDS; sample injection, 20 mbar for 5 s; voltage for separation:18 kV; detection wavelength, 280 nm; column temperature, 37 °C; concentration of MAO-B: 0.6 U/mL; concentration of substrate: 1.0 mM.

Because the MAO-B activity at pH 7.4 is optimal, the effect of buffer pH in the range of 6.5–8.5 was tested. The result indicates that increasing buffer pH improve effectively the resolution between substrate with product. And a well separation was obtained after the pH value of buffer was higher than 7.0. Thus, pH 7.4 was chosen for further optimization.

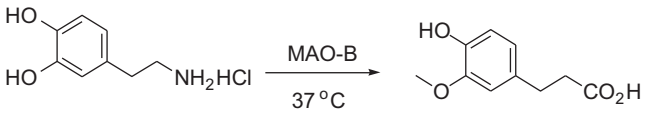
In this work, the HEPES buffer was used as background electrolyte for the separation, and the concentration of HEPES buffer was optimized. It was found that the migration times and the resolution of substrate and product increases with the increase of HEPES buffer concentration from 30 mM to 70 mM, and the resolution was more than 1.5 after the concentration was higher than 40 mM. Therefore, a concentration of 50 mM HEPES was chose as the optimization.

After a careful study, the separation conditions were selected as the following: 18 kV applied voltages and a running buffer containing 10 mM SDS and 50 mM HEPES at pH 7.4. The detection temperature was set at 37 °C, and 280 nm was used as the detection wavelength. Under the optimum separation conditions, the substrate and product were eluted in less than 2 min, and narrow peaks were obtained.

The method repeatability in terms of intraday ($n = 7$ times) was evaluated by analyzing a 1 mM DA solution and measuring the peak area and migration time of the product. The repeatability of the method was demonstrated by the mean relative standard deviation (RSD). The RSDs % for both the peak area and migration time of the product HVA were 3.9% and 1.8%, respectively.

3.3. MAO-B activity assay

In this study, MAO-B catalyze DA to generate HVA, the reaction process can be represented by following expression:



The MAO-B activity was detected simply by carrying out a CE separation. The MAO-B and substrate solutions were injected successively to the capillary inlet part by pressure at 20 mbar for 8 s and incubated for 25 s by applying a voltage of 1 kV, followed by applying a voltage of 18 kV to separate the product from the unreacted substrate. The activity of MAO-B was calculated by measuring the peak area of product HVA at substrate concentration from 1 mM to 5 mM. Linear relation between the reciprocal of peak area of HVA and the reciprocal of concentration of substrate was determined, and the correlation coefficients were higher than 0.9942. The result shows that the method can be used to determine the activity of MAO-B.

3.4. Inhibition kinetics

A commercially available MAO-B inhibitor, selegiline, was employed as a model compound for kinetic property study. Typical electropherograms for the enzymatic reaction without (a) and with (b and c) selegiline as inhibitor added into the plug of substrates are shown in Fig. 2. As can be seen, the peak area of HVA decreases with the increase in selegiline concentration. As the most important kinetic constant of the enzyme reaction, the Michaelis constant is determined conveniently by Lineweaver and Burk's plotting method [18]:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (1)$$

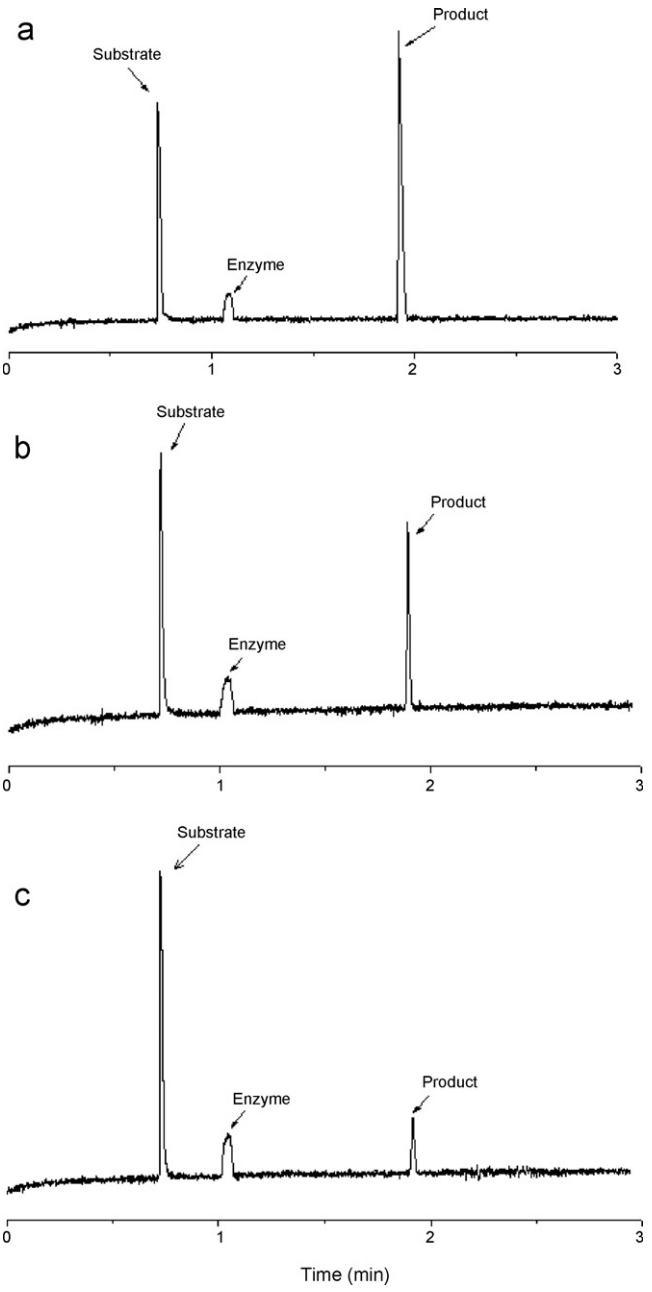


Fig. 2. Typical electropherograms obtained after on-line reaction at the capillary inlet, without (a) and with (b and c) selegiline added to the substrate plug. Concentration of MAO-B: 0.6 U/mL; concentration of DA: 1.0 mM; concentration of selegiline: 15 μM (b) and 30 μM (c). CE conditions were as in Fig. 1.

where v and V_{max} are initial and maximal reaction velocity, [S] is the substrate concentration, and K_m is the Michaelis constant.

Four DA solutions ranging from 0.08 mM to 0.45 mM at different inhibitor concentrations were injected into capillary following MAO-B solution. Each of four solutions was analyzed three times, and the peak area of HVA was used to express initial reaction velocity. The Lineweaver–Burk plot of MAO-B in the presence of inhibitor selegiline is shown in Fig. 3. In this experiment, the apparent K_m was estimated to be 1.6 mM, the K_i value of selegiline was determined as 13 μM. The inhibition plot of selegiline is shown in Fig. 4. The IC_{50} value was determined as 0.91 μM at different inhibitor concentrations varied in the range from 0.01 μM to 100 mM and keeping the substrate concentration at 1.0 mM.

Table 1
Compound library for inhibitor screening.

Extracts	Percent of inhibition (%, $n = 3$)	Extracts	Percent of inhibition (%, $n = 3$)
Fructus crataegi	56.1 ± 1	*Radix glycyrrhizae*	0
Spina gleditsiae	0	*Fructus schisandare chillensis*	0
Radix polygoni multiflori	69.2 ± 1	*Fructus forsythiae*	0
Radix astragali	0	*Radix rehmanniae*	0
Plos carthami	0	*Radix bupleuri*	0
Radix notoginseng	0	*Lignum dalbergiae odoriferae*	0
Plos lonicerae	0	*Radix angelicae sinensis*	0
Radix aurantii	0	*Cortex cinnamomi*	0

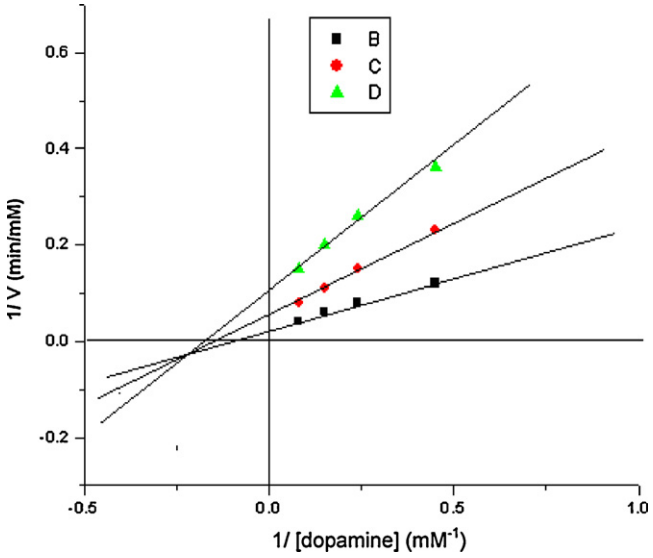


Fig. 3. Lineweaver–Burk plot of MAO-B in the presence of inhibitor selegiline. Concentrations of selegiline in the substrate solution were 0 (B), 15 μM (C) and 30 μM (D). CE conditions were as in Fig. 1.

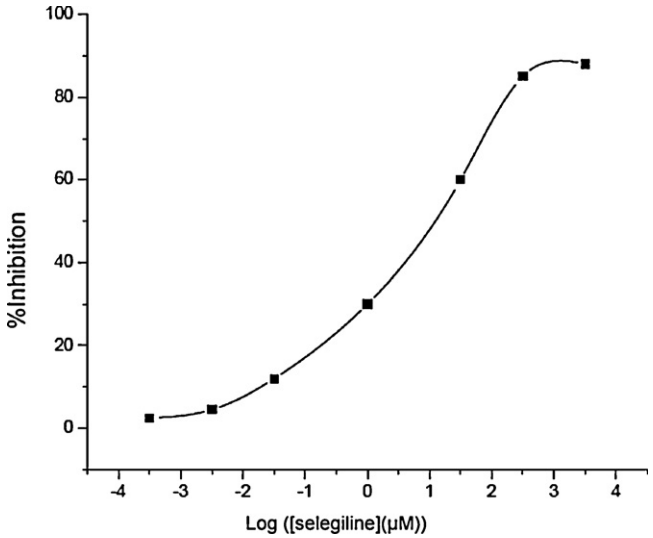


Fig. 4. Inhibition plot for selegiline. CE conditions were as in Fig. 1.

3.5. Inhibitor screening

To evaluate whether the method was effective for the screening of MAO-B inhibitors in real sample, the screening of 16 natural extracts which were usually used as traditional Chinese medicine was performed. The screening results in terms of percentage of inhibition for each sample are summarized in Table 1. The percentage of inhibition was determined according to Eq. (2):

$$\text{Inhibition}\,\% = 100 - \left(\frac{x}{\text{blank}} \times 100\right) \quad (2)$$

where x represents the peak area of HVA determined in the presence of the inhibitor or the natural extracts, and the blank value is the peak area of HVA determined with the pure substrate. Four electropherograms for screening inhibitors are shown in Fig. 5. The electropherogram (a) represents a control assay in which only the substrate solution was injected. The electropherogram (b) represents a control assay obtained from the solution of substrate with the selegiline at 15 μM. The electropherograms (c) and (d) represent assays for screening inhibitors in natural extract of *Radix polygoni multiflori* and *Fructus crataegi*. Each sample contains 0.5 mg/mL total natural extract. The screening results indicate that *Fructus crataegi* and *Radix polygoni multiflori* were identified to be positive for MAO-B inhibition in 16 natural extracts tested.

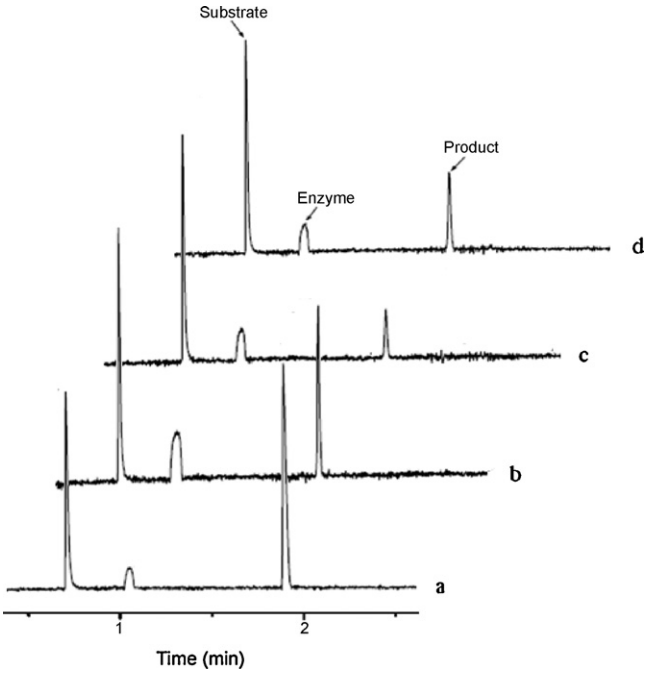


Fig. 5. Typical electropherograms for screening MAO-B inhibitors. Samples: (a) blank; (b) selegiline at 15 μM; (c) extract of *Radix polygoni multiflori*; (d) extract of *Fructus crataegi*. Each sample contains 0.5 mg/mL total natural extract. CE conditions were as in Fig. 1.

4. Conclusions

A CE method for screening MAO-B inhibitor was developed. This method was proved to be simple, rapid and low-cost. The analysis time took only 3 min for one assay. By reading out the reduction in

the peak area of HAV compared with the reference electropherogram obtained in the absence of inhibitors, the K_i and IC_{50} can be easily determined, and the inhibitors of MAO-B in natural extracts were easily found by carrying out a CE separation. Therefore, the CE method could be potentially used for screening inhibitor of MAO-B in complex TCM. In present work, 16 natural extracts were tried to screen MAO-B inhibitors. *Fructus crataegi* and *Radix polygoni multiflori* were found to be positive for MAO-B inhibition. So, the ability of this method for screening MAO-B inhibitors in TCM can obviously improve the assay throughput.

Acknowledgements

The authors gratefully acknowledge the financial support provided by National Natural Science Foundation of China (No. 20875019) and Guangxi Science Foundation of China (No. 0832004).

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