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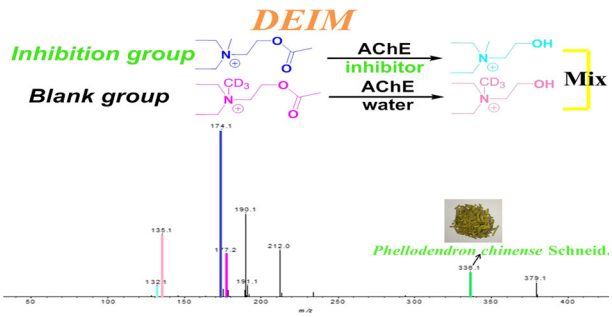
RESEARCH ARTICLE

Dual-Channel Enzymatic Inhibition Measurement (DEIM) Coupling Isotope Substrate via Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

Min Tao,[1,2] Li Zhang,[1] Yinlong Guo[1]

[1]State Key Laboratory of Organometallic Chemistry and National Center for Organic Mass Spectrometry in Shanghai, Shanghai Institute of Organic Chemistry, Center for Excellence in Molecular Synthesis, University of Chinese Academy of Sciences, Chinese Academy of Sciences, 345 Lingling Road, Shanghai, 200032, People's Republic of China
[2]Postdoctoral Programme, Mayinglong Pharmaceutical Group Co. Ltd., Wuhan, People's Republic of China



Abstract. A novel dual-channel enzymatic inhibition measurement (DEIM) method was developed to improve the repeatability with light/heavy isotope substrates, producing reliable relative standard deviations (< 3%) by employing acetylcholinesterase (AChE) as the model enzyme. The matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was adapted for enzyme-inhibited method due to its good salt-tolerance and high throughput; meanwhile, dual-channel enzymatic reactions were performed to improve the repeatability of each well. The acetylcholinesterase inhibition measurement was conducted by mixing the quenched enzyme reaction solution of blank group (with heavy isotope as substrate) and experimental group (with light isotope as substrate), of which the inhibition rate might be affected by isotope effects. Hence, inverse study and *Km* measurement were implemented to validate the method. The inverse study shows similar inhibition rate (68.9 and 70.3%) and the *Km* of isotope substrates are analogous (0.139 and 0.135 mM), which demonstrated that the novel method is feasible to AChE inhibition measurement. Finally, the method was applied to herb extracts, half of which exhibit inhibition to AChE. The precise dual-channel enzymatic inhibition measurement (DEIM) method could be regarded as a promising approach to potential enzyme inhibitor screening.

Keywords: Dual-channel enzymatic inhibition measurement, Isotope substrate, Repeatability, Enzyme kinetics

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Introduction

Almost all metabolic processes need enzyme catalysis. Due to their vital role in all aspects of life, the malfunction or lack of enzymes leads to severe pathologic conditions in organisms, and some clinical treatments act on enzyme, encompassing enzyme replacement therapy, enzyme inhibitor, enzyme prodrug therapy, etc. [1, 2]. As the most widely used therapeutic approaches, enzyme inhibitor has been under heated development for the treatment of various diseases, such as tumor, hepatitis, and HIV. In vitro tests are conducted to explore the influence of inhibitors on enzymes (e.g., inhibition type, enzymatic inhibition rate, and inhibitory concentration), paving the way for in vivo assays. Therefore, numerous analytical methods for the measurement of enzyme activity and inhibition thrived to meet the precise, sensitive, fast demand for inhibitor screening, comprising spectrophotometric method [3], electrochemical method [4], and mass spectrometric (MS) method [5–10].

Over the past decades, tremendous mass spectrometric methods have been used to measure enzyme activity and screen

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Correspondence to: Li Zhang; *e-mail:* zhangli7488@sioc.ac.cn, Yinlong Guo; *e-mail:* ylguo@sioc.ac.cn

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inhibitors [11–13]. As opposed to traditional spectrophotometric method requiring fluorescent labeling or chromogenic reagent, mass spectrometry shows *m/z* of substrate, product, and inhibitor directly offering more flexibility in assays. Moreover, mass spectrometry has a natural advantage that each *m/z* has a corresponded intensity or area, which is easy for quantitative calculation.

Since the invention of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), it was successfully applied not only to small or large molecule detection [14, 15] but also to enzymatic reaction monitoring [16, 17]. Particularly, MALDI-MS is most suitable to study the kinetics of enzyme reactions and for screening purposes because of its salt-tolerance, high throughput, and simultaneous detection of multiple assay components [18]. In assaying enzyme reactions, MALDI-FT-MS [19, 20], thin-layer chromatography/UV hyphenated with MALDI-TOF-MS [21], and intensity-fading MALDI-TOF-MS [22] were reported.

However, poor repeatability is an inevitable limitation for MALDI because of the non-uniform nature of the crystallization of the matrix/analyte complex. As a consequence, for a parallel of analyte/matrix sample dropped in different wells, the detector response of each well is remarkably variable, which is disadvantageous for quantification. To circumvent this problem, several sample drying methods have been developed to form uniform and fine crystals that provide superior signal repeatability, for instance, deposition of droplets by microdispenser [23, 24], preparation with sandwich methods [25], fast evaporation methods using highly volatile solvents [26, 27], and addition of glycerol in matrix [28]. Alternatively, sample preparation methods without conventional matrix were also reported to improve signal repeatability: solvent-free sample preparation [29, 30], ionic liquid matrix [31, 32], carbon nanotube matrix [33], and prestructured sample supports such as SALDI [34, 35] and DIOS [36–39]. For the reliable quantification of enzymatic reactions via MALDI-MS, application of an appropriate internal standard was developed [40, 41] and measurement of substrate-to-product ratio also had good results [42, 43]. In analytical chemistry, ratio metric method is widely used to obtain accuracy and precision, through which a broad range of substances can be detected. For instance, bisphenol A can be measured by dual-ratiometric fluorescent measurements with excellent repeatability between 1.09 and 7.38% (RSD, $n = 3$) [44]. However, enzymatic inhibition measurement through dual-ratio metric method has not been reported.

In this work, dual-channel enzymatic inhibition measurement (DEIM) method was employed to improve the well-to-well and sample-to-sample repeatability. Acetylcholinesterase (AChE) was selected as the model enzyme for inhibition measurement. Isotope substrates, ACh-N $(Et)_2$ (short for *N*,*N*-diethyl-*N*-methyl-aminoethyl acetate iodide) and ACh-N $(Et)_2$-d_3 (short for *N*,*N*-diethyl-*N*-methyl-d_3-aminoethyl acetate iodide), were synthesized by two steps to implement dual-channel enzymatic reaction. With low RSD (< 3%) of parallel wells, the novel method manifested great repeatability. The method was further validated by inverse study and enzyme kinetics assay. Moreover, dual-channel enzymatic inhibition measurement (DEIM) method was applied to measure the IC_{50} of donepezil hydrochloride and analyze the crude extraction of herbs for potential inhibitor screening. This method is anticipated to broaden perspectives for precise measurement of enzyme inhibition and inhibitor screening.

Experimental

Chemicals and Reagents

Acetylcholinesterase from *Electrophorus electricus* (Type VI-S, EC 3.1.1.7, 374 U/mg), α-cyano-hydroxycinnamic acid (CHCA), disodium hydrogenphosphate (Na_2HPO_4), and sodium dihydrogenphosphate (NaH_2PO_4) were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Potassium iodide (KI), potassium carbonate (K_2CO_3), dichloromethane, and chloroform were from Shanghai Chemical Reagent Corporation (Shanghai, China). Neostigmine bromide, donepezil hydrochloride, 2-bromoethyl acetate, diethylamine, iodomethane, and iodomethane-d_3 were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The ultrapure water used in this paper was purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Methanol, acetonitrile and ethanol were of HPLC-grade quality and were purchased from Merk (Darmstadt, Germany).

Synthesis of Light-Isotope/Heavy-Isotope Substrates

Synthesis of 2-(diethylamino) ethyl acetate: to a solution of diethylamine (8.78 g, 0.12 mol, 2.4 equiv), K_2CO_3 (8.3 g, 0.06 mol, 1.2 equiv), and KI (100 mg, 0.6 mmol) in CH_3CN, 2-bromoethyl acetate was added (8.35 g, 0.05 mol, 1 equiv). The mixture was refluxed for 4 h. The resulting mixture was filtered and concentrated. Vacuum distillation gave 3 (6.89 g, 0.043 mol, 86.6% yield) as yellow liquid.

Synthesis of *N*,*N*-diethyl-*N*-methyl-aminoethyl acetate iodide: 2-(diethylamino) ethyl acetate (500 mg, 3.14 mmol, 1 equiv) was dissolved in chloroform in a Schlenk tube. The CH_3I (0.5 mL, 8 mmol, 2.5 equiv) was added dropwise at 0 °C. The solution was stirred for 4 h at 50 °C. Removal of solvents and excess CH_3I by reduced evaporation gave *N*,*N*-diethyl-*N*-methyl-aminoethyl acetate iodide (0.95 mg, 3.14 mmol, 100% yield) as yellow liquid (see Supporting Information Fig. S3).

Synthesis of *N*,*N*-diethyl-*N*-methyl-d_3-aminoethyl acetate iodide: 2-(diethylamino) ethyl acetate (500 mg, 3.14 mmol, 1 equiv) was dissolved in chloroform in a Schlenk tube. The CD_3I (0.5 mL, 8 mmol, 2.5 equiv) was added dropwise at 0 °C. The solution was stirred for 4 h at 50 °C. Removal of solvents and excess CH_3I by reduced evaporation gave *N*,*N*-diethyl-*N*-methyl-d_3-aminoethyl acetate iodide (0.96 mg, 3.14 mmol, 100% yield) as yellow liquid (see Supporting Information Fig. S4).

Materials Preparation

Stock solutions of neostigmine bromide, donepezil hydrochloride were prepared in deionized water at a concentration of 0.1 mg/mL. Stock solutions of AChE were prepared and diluted by 10 mmol/L Na_2HPO_4-NaH_2PO_4 buffer (pH = 7.4) to 20 U/mL, and were stored at − 20 °C in dark. Working solutions were obtained by appropriate mixture and dilution of the stock. The light-isotope/heavy-isotope substrates solutions (0.05 mg/mL) were prepared with Na_2HPO_4-NaH_2PO_4 buffer freshly. The matrix solution was prepared by adding 10 mg of CHCA to 1 mL of ethanol/water solution (V:V = 50:50).

The dry powder (1 g) of *Phellodendron chinense* Schneid. was extracted in ethanol (70%, 20 mL × 3) via ultrasonic for 30 min at 35 °C. The extracted solution were filtered, concentrated, and reconstituted in H_2O to give stock solution. The stock solution was diluted by 1000 times to afford final aqueous solution used in enzyme reaction for potential inhibitor screening.

The fresh leaves (0.2 g) of 21 different herbs were extracted in acetonitrile (50% in water, 20 mL × 3) via ultrasonic for 30 min at 35 °C. The extracted solution were filtered, concentrated, and reconstituted in H_2O to give stock solution. The stock solution was diluted by 10,000 times to afford final aqueous solution (0.1 mg/L) to be used in enzyme reaction for inhibition measurement.

Validation of Novel Method

Isotope Substrate Inverse Study All reactions were conducted simultaneously in Eppendorf plastic (EP) tubes (see Supporting Information Fig. S5). Each group was carried out in triplicate (in three EP tubes). In group A, AChE solution (50 μL, 0.1 U/mL) was incubated with water (50 μL) for 5 min at 36 °C, then light-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group B, AChE solution (50 μL, 0.1 U/mL) was incubated with neostigmine bromide (50 μL, 5 μg/L) for 5 min at 36 °C, then heavy-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group C, AChE solution (50 μL, 0.1 U/mL) was incubated with water for 5 min at 36 °C, and then heavy-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group D, AChE solution (50 μL, 0.1 U/mL) was incubated with neostigmine bromide (50 μL, 5 μg/L) for 5 min at 36 °C, then light-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. The reaction was quenched by acetonitrile (200 μL). For Mix 1, triplicate of group A was mixed to one EP tube, and then 50 μL of the mixture in the new-formed EP tube was blended with 50 μL of solution in each EP tube of group B to form H1, H2, and H3. For Mix 2, triplicate of group C was mixed to one EP tube, and then 50 μL of the mixture in the new-formed EP tube was blended with 50 μL of solution in each EP tube of group D to form H4, H5, and H6. Finally, each sample of H1, H2, H3, H4, H5, and H6 was dropped to 3 different wells and 18 (3 × 6) wells were formed. Before being dropped to well, all samples were mixed with CHCA matrix (sample:matrix = 1:5). The reason for dropping sample of one EP tube to three different wells is that the final value should be the average of different wells in order to decrease random errors.

Enzyme Kinetics Assay AChE solution (50 μL, 0.1 U/mL) was incubated with light-isotope substrate (50 μL, 0.2 mg/mL) at 36 °C. The mixture was quenched at 3, 6, 9, 12, 15, 20, 30, 40, 50, 60, and 75 min by the addition of acetonitrile (200 μL) to draft the progress curve. To fully demonstrate the accuracy of the linear correlation between conversion and time before 5 min, experiments of time data acquisitions are added below 5 min.

A series of light-isotope/heavy-isotope substrates in different concentrations (0.025, 0.05, 0.1, 0.2, 0.4 mg/mL, 50 μL) were incubated with AChE solution (0.1 U/mL, 50 μL) at 36 °C to calculate the Michaelis constants of different isotope substrates.

Simultaneous Enzymatic Catalysis Study A solution with the same concentration of light-isotope substrate (0.05 mg/mL) and heavy-isotope substrate (0.05 mg/mL) was prepared; 10 μL of the solution was mixed with 10 μL of enzyme solution (0.1 U/mL). The simultaneous enzymatic catalysis was quenched with acetonitrile. The quenched solution was mixed with matrix and dropped to wells.

Comparison Between Traditional Method and Novel Method

All reactions were conducted simultaneously in Eppendorf plastic (EP) tubes (see Supporting Information Fig. S6). In group A where reaction was carried out in one EP tube, AChE solution (50 μL, 0.1 U/mL) was incubated with water (50 μL) for 5 min at 36 °C, then light-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group B where nine parallel reactions (in nine EP tubes) were carried out, AChE solution (50 μL, 0.1 U/mL) was incubated with neostigmine bromide (50 μL, 5 μg/L or 2 μg/L or 1 μg/L) for 5 min at 36 °C, and then light-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group C where reaction was carried out in one EP tube, AChE solution (50 μL, 0.1 U/mL) was incubated with water (50 μL) for 5 min at 36 °C, and then heavy-isotope substrate (50 μL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. The reaction was quenched by acetonitrile (200 μL). For traditional method, on the one hand, 1 μL of solution in group A was dropped to nine different wells to explore the repeatability of well-to-well; on the other hand, 1 μL of solution in each EP tube of group B was dropped to three different wells, as a result, 27 (3 × 9) wells was detected in group B to explore the repeatability of sample-to-sample. For novel method which needs blending, 20 μL of solution in the EP tube of group C was mixed with 20 μL of solution in each EP tube of group B to form a new group M containing nine parallel EP tubes, and

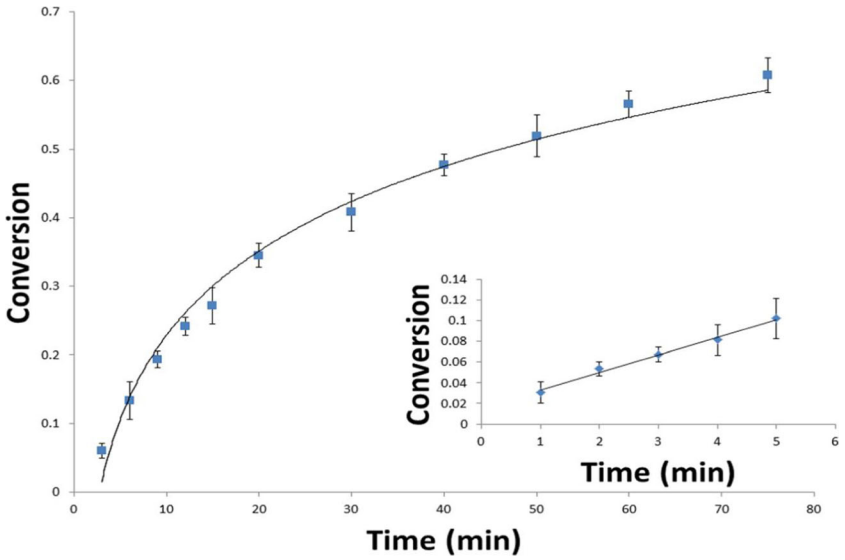

Figure 1. Progress curves of enzyme-catalyzed reaction. The initial substrate concentration was 0.2 mg/mL; the reaction was quenched at 3, 6, 9, 12, 15, 20, 30, 40, 50, 60, and 75 min by the addition of acetonitrile (200 µL); to fully demonstrate the accuracy of the linear correlation between conversion and time before 5 min, experiments of time data acquisitions are added below 5 min

then 1 µL of the mixture in each EP tube of group M was dropped to three different wells to explore the repeatability of sample-to-sample except that one tube in group M was dropped to nine different wells to explore the repeatability of well-to-well. Before being dropped to well, all samples were mixed with CHCA matrix (sample: matrix = 1:5).

Application

Various donepezil hydrochloride concentrations (0.1, 1, 2, 10, 50, 100, 500, 1000, and 2000 nmol/L) were prepared. Extraction of *Phellodendron chinense* Schneid. and 21 other herbs were described above. In group A, AChE solution (50 µL, 0.1 U/mL) was incubated with water (50 µL) for 5 min at 36 °C, and then heavy-isotope substrate (50 µL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. In group B, AChE solution (50 µL, 0.1 U/mL) was incubated with inhibitor (50 µL) for 5 min at 36 °C, and then light-isotope substrate (50 µL, 0.05 mg/mL) was added and incubated for 20 min at 36 °C. The reaction was quenched by 200 µL of acetonitrile; 50 µL of solution in each group was mixed and the mixture was dropped to three wells (see Supporting Information Fig. S7). Before being dropped to well, all samples were mixed with CHCA matrix (sample:matrix = 1:5). To fully

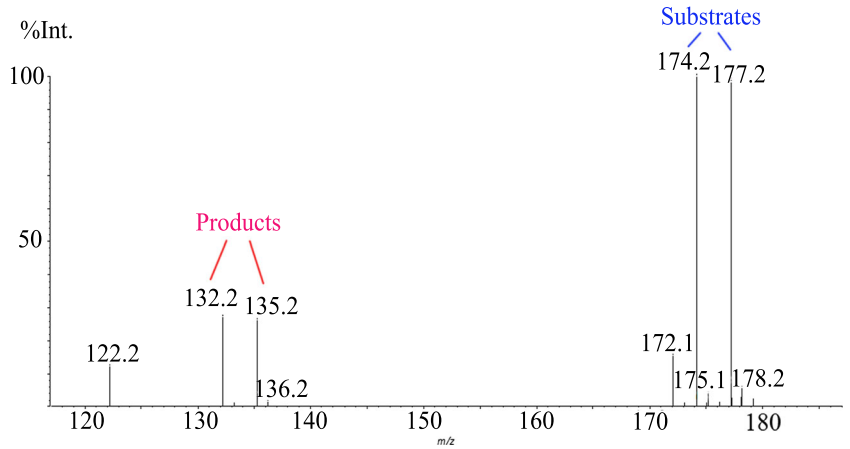

Figure 2. Spectrum of simultaneous enzymatic catalysis. Appearing simultaneously in one spectrum, the peaks of isotope substrates and isotope products show that isotope substrates have similar hydrolysis rate. (In the spectrum, *m/z* = 177.2 and 174.2 are peaks of isotope substrates; *m/z* = 135.2 and 132.2 are peaks of isotope products; *m/z* = 122.2 is peak of $[Tris+H]^+$ because the enzyme powder was lyophilized in Tris-HCl buffer before reconstitution)

Table 1. Repeatability of Conversion Rate/Inhibition Rate in Traditional Method

Neostigmine concentration (μg/L)	Statistic	Well-to-well[a]	Sample-to-sample[b]
5 μg/L	AVE (%)	78.3	68.0
	RSD (%)	4.5	5.6
2 μg/L	AVE (%)	49.0	49.3
	RSD (%)	6.1	7.1
1 μg/L	AVE (%)	52.1	29.8
	RSD (%)	5.0	6.5

[a]To explore the well-to-well repeatability of traditional method, the quenched solution of blank group was dropped to nine different wells, and the mean was calculated by averaging the conversion rates of nine wells. As a result, the RSD in the column of well-to-well stands for repeatability of conversion rate (n = 9)
[b]There are nine parallel EP tubes in the inhibition group, and then quenched solution of each EP tube was dropped to three different wells to calculate the average conversion rate of each EP tube. With the nine parallel average conversation rates of inhibition group and the blank group average conversation rate, nine parallel inhibition rates could be calculated. The mean and RSD was calculated by averaging the nine parallel inhibition rates. Hence, the RSD in the column of sample-to-sample stands for repeatability of inhibition rate (n = 9)

demonstrate the repeatability of the novel method in real-world samples, extraction of *Clerodendrum trichotomum.* Thunb. was adopted to perform method comparison study through the identical experimental procedure described in "Comparison between traditional method and novel method" section (see Supporting Information Fig. S6).

MALDI-TOF Analysis

All data were acquired via Axima performance, Shimadzu Biotech Lauchpad with an air-cooled Nd:YAG laser (337 nm). The spectrum of each well was accumulated in multiples of two laser shots and 199 dots in total with auto movement of the stainless steel target. The operation was conducted with reflection mode, 20 kV of accelerating voltage and 5 Hz of laser frequency. The TOF was calibrated with internal standard method by using the peaks derived from CHCA. The acquisition mass range was 50–500.

The abundance of peaks of substrates and products could be used to calculate the conversion rate (C) (see Supporting Information Fig. S1).

The inhibition rate (I, %) was calculated as follows:

$$I\,(\%) = [(Co - Ci)/Co] \times 100\%$$

(Co is the conversion rate of blank group and Ci is the conversion rate of inhibition group)

Sample of each EP tube was dropped to three different wells to calculate the average as the final value except that some sample was dropped to nine different wells to explore the repeatability of well-to-well.

The inhibition curve was plotted using GraphPad Prism 5 software (Mountain View, CA), and the IC_{50} value was calculated by the software.

Results and Discussion

Isotope Substrate Design and Analysis-Condition Optimization

In the beginning, *N*-(2-acetyloxyethyl)-pyridinium bromide was chosen as the substrate. However, the low hydrolysis rate made it difficult to conduct the catalytic process (see Supporting Information Fig. S1). In order to insure the affinity to AChE, the design of isotope substrates were based on the natural substrate. It is fortunate that the *Km* of ACh-N $(Et)_2$ is close to natural substrate [45]. On the other hand, the preparation of ACh-N $(Et)_2$ and ACh-N $(Et)_2$-d_3 was quite feasible with high purity and excellent yield. One challenge with MALDI-TOF for such assays with low MW substrates and products is the interference from matrix ions. By changing carbon chain from natural substrate, the novel isotope substrate can easily distinguish itself from CHCA matrix; the signals of the substrates and products are strong enough to outstrip those

Table 2. Repeatability of Inhibition Rate in Novel Method

Neostigmine concentration (μg/L)	Statistic	Well-to-well[a]	Sample-to-sample[b]
5 μg/L	AVE (%)	68.1	69.9
	RSD (%)	2.2	1.7
2 μg/L	AVE (%)	48.9	50.0
	RSD (%)	2.5	2.5
1 μg/L	AVE (%)	29.8	29.8
	RSD (%)	2.8	3.0

[a]To explore the well-to-well repeatability of novel method, one of the EP tube in group M (see Supporting Information Fig. S6) was dropped to nine wells. In the novel method, since the substrates and products appeared simultaneously in one spectrum, each spectrum has a correspondent inhibition rate. By averaging nine inhibition rates calculated with nine spectrums, the well-to-well RSD of inhibition rate could be determined (n = 9)
[b]There are nine parallel EP tubes in group M (see Supporting Information Fig. S6), and the mixture of each EP tube was dropped to three wells except one EP tube of which the mixture was dropped to nine wells to explore the well-to-well repeatability (see a). By averaging three inhibition rates of three wells, each EP tube has an average inhibition rate. Then the mean and RSD was calculated by averaging the nine parallel inhibition rates to exhibit the repeatability of sample-to-sample (n = 9)

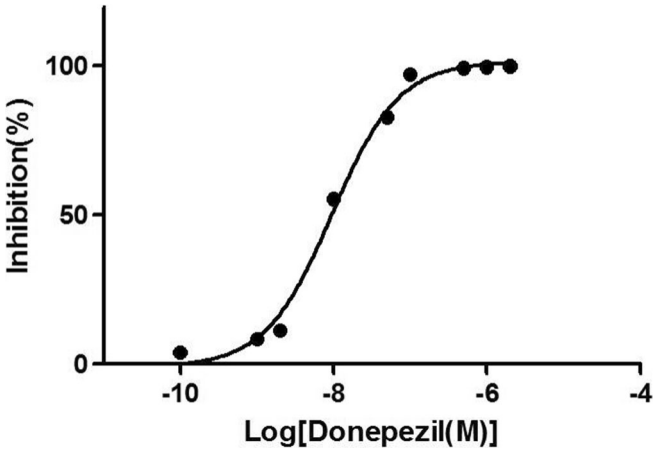

Figure 3. Plot of the inhibition efficiency of donepezil hydrochloride toward AChE. The concentration of light-isotope/heavy-isotope substrates were 0.05 mg/mL and AChE concentration was fixed at 0.1 U/mL

of matrix ions. Overall, we chose ACh-N $(Et)_2$ and ACh-N $(Et)_2$-d_3 as the isotope substrates. Generally speaking, with low MW substrates and products, there are many ways to eliminate the interference from matrix in the spectrum, such as altering carbon chain or isotope group to change the MW, adding groups which have higher responses in MALDI-TOFMS or simply changing the matrix.

To start our optimization, we should select an appropriate matrix. CHCA gave a more homogeneous crystallization and better quantitative results than DHB. The optimal ratio was determined as 5:1 (matrix:sample).

Validation of Novel Method

A chemical bond formed by a heavy isotope and another atom will be different from the same bond between the light isotope and that atom. Once breaking this chemical bond is the rate-limiting step in an enzyme-catalyzed reaction, the catalysis process of the molecule with the heavy isotope is slower than that of the molecule with the light isotope, which is known as the isotope effects [46]. On the other hand, even if the chemical bond including a heavy isotope is not involved in the rate-limiting step, it may affect the catalysis rate for the change of polarity, dipole moment, molar volume, electron donation, Van der Waal's forces, lipophilicity, and protein binding. In novel method where inhibition (I) was calculated by C_{light} (light-isotope substrate was added to inhibition group) and C_{heavy} (heavy-isotope substrate was added to blank group), the difference of enzyme catalysis rate between the heavy-isotope substrate and light-isotope substrate can certainly bias the calculation of inhibition. As a result, it is imperative to validate the novel method.

In the inverse study, the inhibition of Mix 1 is 68.9% and the inhibition of Mix 2 is 70.3%. The similar inhibitions of two mixtures indicate that the novel method can be an alternative of traditional method. A further validation was conducted by measuring the Michaelis constant of light-isotope/heavy-isotope substrates.

Firstly, a full progress curve was drawn to select quench time at which enzyme velocity is constant by monitoring the conversion rate as a function of time (Figure 1). The curve fits linear correlation well before 5 min and the time point was determined as 5 min. It is noteworthy that taking conversion rate as the ordinate rather than product concentration is not only convenient but also accurate [35].

Different initial enzyme velocities were detected at a wide range of substrate concentrations (from 0.14 to 2.30 mM). By transforming enzyme velocity (V) and substrate concentration (S) to the reciprocal, these data was fitted to Lineweaver-Burk plot to calculate *Km* (see Supporting Information Figs. S8 and S9). The *Km* of light-isotope substrate is 0.139 mM and the *Km* of heavy-isotope substrate is 0.135 mM. The difference of *Km* between light-isotope and heavy-isotope substrates was so little that there was no problem of calculating inhibition with C_{light} and C_{heavy}.

The result of simultaneous enzymatic catalysis indicated that AChE gave no discrimination to light-isotope substrate

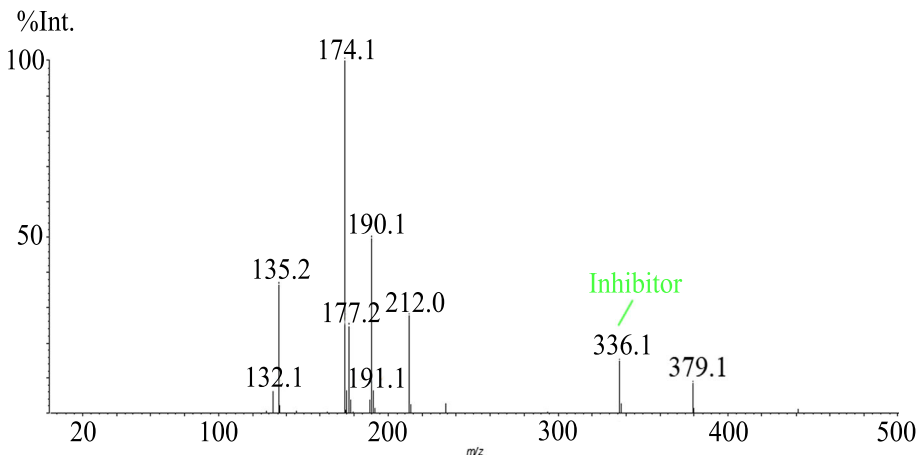

Figure 4. The mass spectrum for screening inhibitors from crude *Phellodendron chinense* Schneid. extracts. The active ingredient, Majarine (exact mass = 336.1230), can be shown in the spectrum. (In the spectrum, *m/z* = 177.2 and 174.2 are peaks of isotope substrates; *m/z* = 135.2 and 132.2 are peaks of isotope products; *m/z* = 190.1, 212.0, and 379.1 are peaks of CHCA matrix)

Table 3. Inhibition Activity of Crude Plant Extracts

Number	Plant	Part	Inhibition rate (%)
Control[a]	*Phellodendron chinense* Schneid.	Bark	9.98
1	*Podocarpus macrophyllus var. maki*	Leaf	0.63
2	*Sambucus williamsii* Hance	Leaf	1.98
3	*Podocarpus nagi* (Thunb.) Zoll. et Mor. ex Zoll.	Leaf	10.86
4	*Pittosporum tobira* (Thunb.) Ait	Leaf	1.56
5	*Pittosporum tobira* (Thunb.) Ait	Fruit	8.00
6	*Vitex negundo* L.var. Cannabifolia (Sieb. et Zucc.) Hand.-Mazz.	Leaf	8.08
7	*Flueggea suffruticosa* (Pall.) Baill.	Leaf	9.86
8	*Boehmeria nivea* (L.) Gaud.	Leaf	0.24
9	*Clerodendrum trichotomum.* Thunb.	Leaf	33.91
10	*Yulania denudata* (Desr.) D. L. Fu	Flower	2.31
11	*Euonymus maackii* Rupr.	Leaf	12.49
12	*Euonymus maackii* Rupr.	Fruit	16.69
13	*Fagopyrum dibotrys* (D.Don) Hara	Leaf	4.92
14	*Fagopyrum dibotrys* (D.Don) Hara	Flower	5.87
15	*Carpesium abrotanoides* Linn.	Leaf	1.69
16	*Carpesium abrotanoides* Linn.	Flower	12.87
17	*Rubus corchorifolius* L. f.	Leaf	44.70
18	*Eriobotrya japonica* (Thunb.) Lindl.	Leaf	0.20
19	*Morus alba* L.	Leaf	17.27
20	*Osmanthus fragrans* Lour	Leaf	3.78
21	*Ginkgo biloba* L.	Leaf	30.16
22	*Saxifraga stolonifera* (L.) Meerb.	Leaf	13.14
23	*Astragalus membranaceus* (Fisch.) Bge.	Root	17.00
24	*Polygonatum odoratum* (Mill.) Druce	Root	11.63
25	*Forsythia suspensa* (Thunb.) Vahl	Fruit	6.61

[a]*Phellodendron chinense* Schneid. was chosen as the positive control because it contains a well-known AChE inhibitor, Majarine, of which the content is not less than 3.0% stipulated by Pharmacopoeia of the People's Republic of China. The process of extracting *Phellodendron chinense* Schneid. and enzyme inhibition experiment was conducted simultaneously with other plants, forming final aqueous solution (0.1 mg/L), in which the amount of Majarine was estimated to be close to 3 μg/L

and heavy-isotope substrate because the reduction of substrate and generation of product are equal (Figure 2).

Comparison Between Traditional/Novel Method

Even though the repeatability remains to be improved, traditional method is widely used in MALDI enzyme inhibition study where the blank group and inhibition group was dropped to different wells (Table 1). In order to improve the repeatability, a novel method was developed. In this method, the blank group and inhibition group were mixed together and dropped to one well so that four peaks appeared in one spectrum (Table 2). It is apparent that the repeatability of novel method was improved dramatically since the RSD was reduced by half.

Application of Novel Method

The capability of novel method was evaluated with pure chemical compound of donepezil hydrochloride (Figure 3.) and *Phellodendron chinense* Schneid. (Figure 4.), both of which are reported to exhibit AChE inhibition [47, 48] and serve as positive control in the research. The IC_{50} of donepezil hydrochloride calculated by novel method was 8.88 nM, which is similar to 6.7 nM [47]. Therefore, the novel method is applicable for complex inhibition experiment. The extract of traditional Chinese medicine affects the activity of acetylcholinesterase with an inhibition of 89.6% with a conspicuous peak of Majarine (exact mass = 336.1230) (Figure 4), the representative active ingredient in *Phellodendron chinense* Schneid. [48], which exhibited that the novel method can be applied to the screening of potential inhibitor in herbs.

It is noteworthy that many clinical available drugs have come or been derived from natural molecules [49], because of the prominent chemical and functional diversity of biomolecules existing in organisms. In this work, as many as 25 herb extracts were tested via dual-channel enzymatic inhibition measurement (DEIM) with positive control *Phellodendron*

Table 4. Repeatability Comparison of *Clerodendrum trichotomum.* Thunb.

Method	Statistic	Well-to-well[a]	Sample-to-sample[b]
DEIM	AVE (%)	34.4	34.5
	RSD (%)	3.0	2.9
Traditional method	AVE (%)	53.1	33.5
	RSD (%)	5.6	7.0

[a]The well-to-well RSD in DEIM stands for repeatability of inhibition rate ($n = 9$), while the well-to-well RSD in traditional method stands for repeatability of conversion rate ($n = 9$)
[b]The sample-to-sample RSD in both DEIM and traditional method stand for repeatability of inhibition rate ($n = 9$)

chinense Schneid. in the same concentration, about half of which showed inhibition activity (Table 3). To fully demonstrate the repeatability of the novel method in real-world samples, extraction of *Clerodendrum trichotomum.* Thunb. was adopted to perform method comparison study and the result shows great priority to DEIM (Table 4).

Conclusions

In this study, a dual-channel enzymatic inhibition measurement (DEIM) coupling isotope substrate method was developed via MALDI-TOF-MS by using AChE as model enzyme. The novel method had solved the problem of poor repeatability of MALDI, with RSD below 3%. A two-step synthesis of light and heavy isotope substrates was designed, which is similar in ionization process and easy to distinguish in spectrum. The enzyme kinetics of light-isotope substrate and heavy-isotope substrate are almost equivalent, insuring the accuracy of inhibition calculation. This method was successfully applied to IC_{50} measurement of donepezil hydrochloride. We employed this method to crude herb extracts and half of which exhibit inhibition to AChE. Overall, dual-channel enzymatic inhibition measurement (DEIM) is a precise, sensitive and straightforward method for fast screening of biological molecules even in crude extracts without the need for complicated sample preparation. Importantly, while AChE was used in this work, this method can be extended to other types of enzymatic assays with well-designed substrates.

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