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An automated sequential injection spectrophotometric method for evaluation of tyramine oxidase inhibitory activity of some flavonoids

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ABSTRACT

An automated sequential injection (SI) spectrophotometric system has been developed for evaluation of tyramine oxidase (TOD) inhibitory activity. The method is based on the inhibition of TOD that catalyzes the oxidation of tyramine substrate to produce aldehyde and hydrogen peroxide (H_2O_2). The produced H_2O_2 reacts with vanillic acid and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to form a quinoneimine dye, the absorbance of which is measured of absorbance at wavelength of 490 nm. The decrease of the quinoneimine dye is related to an increase of TOD inhibitory activity. Under the optimum conditions: 1.0 mM tyramine, 8 U mL^{-1} TOD, 1.0 mM vanillic acid, 1.0 mM 4-AA and delay time of 10 s, some flavonoid compounds were examined for the TOD inhibitory activity expressed as IC_{50} value. It was found that flavonols (quercetin and myricetin) and flavans (epicatechin gallate (ECG) and epigallocatechin (EGC)) showed higher TOD inhibitory activity than flavones and flavanones. The results of IC_{50} values obtained from the proposed method and a batch-wise method were not significantly different from each other. Moreover, the SI system enabled automation of the analysis, leading to more convenient, more sensitive and faster analysis than the batch-wise method. A precise timing of the system also improves precision and accuracy of the assay, especially when the measurement of absorbance at non-steady state condition is involved.

1. Introduction

Monoamine oxidase (flavin-containing; MAO; E.C. 1.4.3.4) is an enzyme that oxidizes various physiologically and pathologically important monoamine neurotransmitters and hormones. The reaction catalyzed by MAO is generally presented as

$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + NH_4^+ + H_2O_2$$

MAO is bound to the outer membrane of mitochondria in most cell types in the body. The enzyme was originally discovered by Mary Bemheim [1] in the liver and was named tyramine oxidase (TOD). It exists in two isoforms, namely MAO-A and MAO-B, which are the products of two distinct genes [2]. MAOs play a vital role in the inactivation of neurotransmitters and MAO dysfunction (too much or too little MAO activity) is related to a number of psychiatric and neurological disorders such as depression [3], schizophrenia [4], substance abuse, attention deficit disorder [5], migraines [6], and irregular sexual maturation. Monoamine oxidase inhibitors (MAOIs) can be used as drugs to regulate MAOs activities. The MAOI drugs are associated with numerous side effects that often limit their usefulness and tolerability. For example, the "cheese reaction" hypertensive crisis can occur when tyramine-rich foods are ingested in conjunction with MAOIs drugs [7,8]. In the presence of MAOIs, tyramine is not broken down and large amounts of it may get absorbed. High levels of tyramine can suddenly and dangerously elevate blood pressure.

Nowadays, substances derived from natural product are of interest because they usually have less side effects than synthesized drugs [9]. Flavonoid compounds have shown promising inhibitory activity on MAO [10–16]. In order to evaluate inhibitory activity of the potential substances, various methods have been developed for evaluation of MAO activity, as listed in Table 1. Different detection techniques such as colorimetry [17], spectrophotometry [17–22], capillary electrophoresis [23], bioluminescent [24], high performance liquid chromatography [25] and fluorometry [26–29] were employed. Spectrophotometry is often used in preference to the other techniques listed because the instrumentation is simpler and less expensive. It can be seen from Table 1 that the method based on the use of tyramine as a substrate and detection of H_2O_2 by forming

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Table 1
Various methods for assay of MAO inhibitory activity.

System	Substrate	Reagent	Product	Inhibitor/sample	Ref.
Colo. (450 nm)	Tyramine	2,4-Dinitrophenyl- hydrazine	2,4-Dinitrophenyl-hydrazone	–	[17]
Spec. (360 nm)	Kynuramine	–	Aldehyde	BAPN	[18]
Spec. (242, 280 nm)	Serotonin, benzylamine	Butyl acetate, cycrohaxan	Product in organic phase	Antler velvet	[19]
Spec. (498 nm)	Tyramine	Vanallic acid/4-AA/HRP	H_2O_2/quinoneimine dye	Clorgyline, pargyline	[20,21]
Spec. (490 nm)	Tyramine, benzylamine	Vanallic acid/4-AA/HRP	H_2O_2/quinoneimine dye	Clorgyline, pargyline	[22]
Spec. (490 nm)	Tyramine	Vanallic acid/4-AA/HRP	H_2O_2/quinoneimine dye	Quercetin	[10]
Flu. (Ex:315, Em: 380 nm)	Tyramine	Vanallic acid/4-AA/HRP	H_2O_2/Fluorescent	Poppy seedlings	[26]
Flu. (Ex:545, Em: 590 nm)	Tyramine	Amplexâ Red MAO assay kit	H_2O_2/Fluorescent	3-Heteroaryl-coumarin derivatives	[37]
Flu. (Ex:320, Em: 380 nm)	Kynuramine	–	4-Hydroxyquinoline	Tobacco	[28]
CE (280 nm)	Dopamine	–	Homovaniuic acid	Natural extract	[23]
Biolumi.	Aminopropylether	Methyl ester luciferin	Luminescence	Clorgyline	[24]
RP-HPLC	Kynuramine	–	4-hydroxyquinoline	β−carbolin alkaloids	[25]
SI-Spec. (490 nm)	Tyramine	Vanallic acid/4-AA/HRP	H_2O_2/quinoneimine dye	Flavonoids	This work

4-AA, 4-aminoantipyrine; BAPN, lathyrogen-β-aminopropionitrile; Biolumi, bioluminescent assay; CE, capillary electrophoresis; Colo, colorimetry; Flu, spectrofluorimetry; HRP, horseradish peroxidase; RP-HPLC, reversed-phase high performance liquid chromatography; SI, sequential injection; and Spec, spectrophotometry.

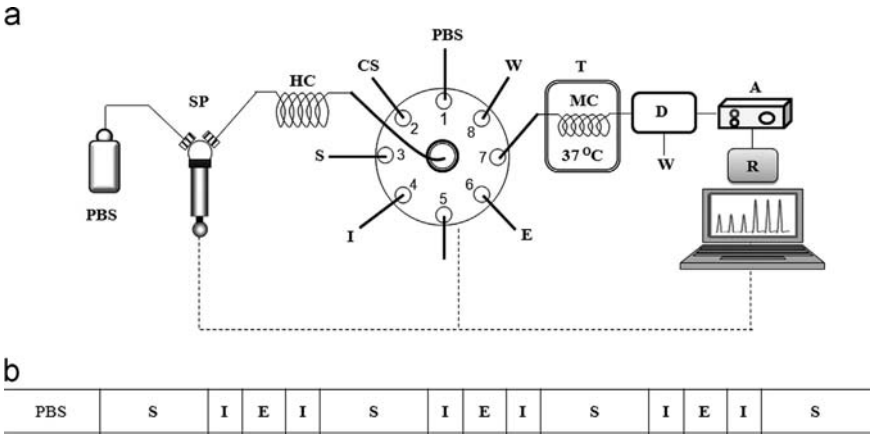


Fig. 1. (a) Manifold of sequential injection spectrophotometric system for evaluation of TOD-inhibitory activity; (b) sequence of solutions in a holding coil of the SI system; PBS: phosphate buffer solution, SP: syringe pump, HC: holding coil, E: TOD, S: tyramine, I: standard/sample (inhibitor), CS: chromogenic solution, MC: mixing coil, T: temperature controller, D: spectrophotometer, W: waste, A: home-made amplifier unit, and R: recorder.

a quinoneimine dye are widely used. However, when the assay is carried out manually, it is time consuming and susceptible to errors arising from inaccurate and imprecise timing of the various steps and final steady-state absorbance measurement.

Therefore, a sequential injection (SI) system was applied in this work in order to automate the assay of inhibitory activity on TOD. The same chemistry as is widely used in the batch-wise method was employed. It is based on measurement of the H_2O_2 formed during the oxidation of tyramine by the TOD, followed by reacting with vanillic acid and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to form a quinoneimine dye. The dye was then detected spectrophotometrically at wavelength of 490 nm. Some flavonoid compounds were identified for their IC_{50} values on TOD inhibition. Results obtained are compatible to those obtained by the batch-wise method [10]. Moreover, the SI system reduces analysis time and improves precision and accuracy of the assay due to its high degrees of automation.

2. Experimental

2.1. Chemicals

Tyramine oxidase (EC 1.4.3.4, 4.6 U mg^{-1} solid), quercetin, morin, and catechin were purchased from Tokyo Chemical Industry Ltd. (Japan). 4-Aminoantipyrine, peroxidase (POD), naringenin, and epigallocatechin were purchased from Sigma Chemical Co. Ltd. (USA). Tyramine was purchased from Nacalai Tesque Inc. (Japan). Other chemicals (NaOH, $NaH_2PO_4 \cdot 2H_2O$, Na_2HPO_4, ethanol, vanillic acid, kaempferol, luteolin, apigenin, and myricetin) were purchased from Wako Pure Chemical Industries Ltd. (Japan). A Milli-Q water obtained from the water purification system (Millipore, Tokyo, Japan) was used to prepare solutions.

2.2. Instrument setup

The manifold of the sequential injection (SI) system employed in the present study was schematically depicted in Fig. 1(a). The system was produced by MGC JAPAN Co., Ltd., Japan. It consisted of a syringe pump, a multiposition valve, a temperature controller and a spectrophotometric detector (UV–vis detector S-3702, SOMA OPTICS, Co., Ltd., Japan). PTFE tube (i.d. 0.8 mm, o.d. 1.58 mm) was used for making a holding coil (HC) and a mixing coil (MC), and connecting various devices of the system. An output signal from the spectrophotometer was amplified by a home-made amplifier unit before being recorded by a recorder. The SIA system was controlled by a computer via a SI controller software (MGC JAPAN Co., Ltd. Japan).

2.3. Preparation of solutions

Phosphate buffer solution (PBS) of pH 7.6 (0.2 M total phosphate) was prepared from NaH_2PO_4–Na_2HPO_4 by dissolving them in water. A stock solution of 1.0 mM tyramine was prepared by

dissolving 4.34 mg tyramine hydrochloride with 25.0 mL PBS. The original TOD solution of initial activity of 4.6 U mg^{-1} solid was diluted with PBS to a concentration of 8 U mL^{-1}. Chromogenic solution (CS) was prepared by mixing vanillic acid, POD, and 4-AA in 0.2 M PBS, the final concentrations were 1.0 mM, 4 U mL^{-1}, and 1.0 mM, respectively. Stock solutions of quercetin, kaempferol, luteolin, myricetin, morin, catechin, epicatechin gallete, and epigallocatechin of 5.0 mM, apigenin of 10.0 mM, and naringenin of 15.0 mM were prepared in 5.0 mL ethanol. Working solutions of each chemical were freshly prepared by diluting the stock solution with 50% (v/v) ethanol.

2.4. Sequential injection procedure

The analytical steps of the SI spectrophotometric system as shown in Fig. 1(a) are summarized in Table 2. Briefly, the procedure started by filling all the lines with a carrier solution (PBS) and then dispensing the carrier solution to the flow-through cell while recording the baseline signal. Then, PBS, tyramine (S), TOD (E) and sample (I) were aspirated to a HC with solution sequence as shown in Fig. 1(b), the H_2O_2 being produced. Then half of the solution was dispensed to a MC. Next, a chromogenic solution (CS) was aspirated to the HC and another half of the solution in the MC was pulled back to the HC. Then all of the mixed solution was dispensed to the MC placing in a water bath, the temperature of which was controlled at 37 °C and stopped there for a delay time of 10 s. During this period, the H_2O_2 reacted with vanillic acid and 4-AA in the presence of POD to form quinoneimine dye. Finally, the reaction solution was dispensed to a flow-through cell to be detected by a spectrophotometer and recorded as SIA peak on a recorder. At the same time, the mixing coil and the flow cell were washed by the carrier. Peak height of SIAgram obtained was linearly proportional to the content of quinoneimine dye which was oppositely proportional to the TOD inhibitory activity.

The TOD inhibitory activity was expressed as percent inhibition of TOD, calculated as follow:

$$\%\text{Inhibition} = 100(A_0 - A)/A_0$$

where, A_0 is the signal of the assay without inhibitor and A is the signal of the assay with inhibitor. So, the %inhibition increased when the sample contained high concentration of TOD inhibitor.

IC_{50} value, the concentration of an inhibitor that give 50% inhibition, was then determined from the plot of %inhibition (y) versus an inhibitor concentration (x).

2.5. MAO activity assay (batch-wise method)

MAO activities were measured using the coupled colorimetric method as described previously by Saaby et al. [10] with some modification. In brief, 50 μL of sample; 50 μL chromogenic solution (0.8 mM vanilic acid and 2.5 mM 4-AA in 0.2 M PBS, pH 7.6) containing 4 U mL^{-1} POD; 100 μL of 3 mM tyramine and 20 μL 8 U mL^{-1} TOD were added to each well of a 96-well microplate, which was incubated at 37 °C for 20 min and the absorbances at 490 nm were measured at the beginning and at 20 min. The TOD inhibitory activity was expressed as percent inhibition of TOD, calculated as follow:

$$\%\text{ Inhibition of TOD} = [((A_1 - A_0) - (B_1 - B_0))/(A_1 - A_0)]100$$

where, A_1 is the change in absorbance of the assay without inhibitor, A_0 is the change in absorbance of the assay without inhibitor and enzyme; B_1 is the change in absorbance of the assay with inhibitor, B_0 is the change in absorbance of the assay with inhibitor and without enzyme.

3. Results and discussion

The widely used chemistry for the MAO inhibitory assay as previously reported [10,20–22] was adapted for the development of the SI spectrophotometric method. The involved reactions were summarized in Fig. 2. According to the scheme, the increase of TOD inhibitory activity resulted in the decrease of H_2O_2 which could be spectrophotometrically measured as the quinoneimine dye. A common TOD inhibitor, quercetin was selected as a TOD inhibitor standard for optimization of the proposed system.

The SI system in Fig. 1(a) and an operational sequence in Table 2 were used to study the inhibitory effect of quercetin on the TOD. Total volumes of tyramine, TOD, CS and sample solution were fixed at 100, 30, 50 and 48 μL, respectively, with the zones of solutions as shown in Fig. 1(b). The following conditions: concentrations of tyramine, TOD, vanillic acid, 4-AA and POD of 3 mM, 8 U mL^{-1}, 0.8 mM, 2.5 mM, and 4 U mL^{-1}, respectively, and reaction temperature of 37 °C were used as a starting point for optimization. Further optimizations of other parameters were carried out as described in Sections 3.1–3.5.

3.1. Effect of tyramine concentration

Tyramine was used as a substrate in this experiment; the tyramine concentration was investigated in the range of 0.0–2.0 mM. Peak height obtained in the absence of inhibitor linearly increased with the

Table 2
Protocol sequence of the SI system for the evaluation of TOD inhibitory activity.

Step	Valve position	Operation time (s)	Flow rate (mL s^{-})	Volume (mL)	Description
1	–	5	400	2000	Filling syringe with a carrier solution
2	7	20	100	2000	Dispensing a carrier solution to detector for recording baseline signal
3	1	0.44	50	22	Aspiration of the PBS to HC
4	3	0.5	50	25 (× 4)	Aspiration of the tyramine to HC
5	4	0.8	10	8 (× 6)	Aspiration of the inhibitor sample to HC
6	6	1	10	10 (× 3)	Aspiration of the TOD to HC
				Sum=200	Steps 3–6 are followed by using solution sequence in Fig. 1(b)
7	7	2.5	50	100	Dispensing mixed solution to MC
8	2	1	50	50	Aspiration of the CS solution to HC
9	7	2	50	100	Reversing the mixed solution to HC
10	7	5	50	250	Dispensing the mixed solution to MC
11	–	10	–	–	Delayed time
12	–	7.5	400	3000	Filling syringe with a carrier solution
13	7	32.5	100	3250	Dispensing MC content towards the detector

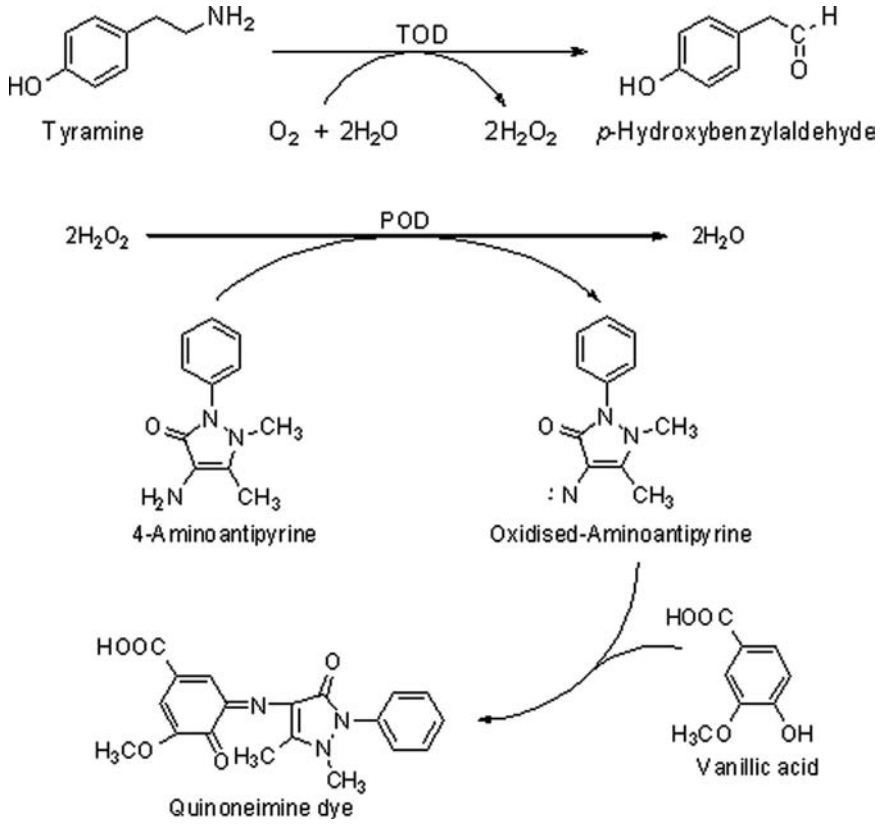


Fig. 2. Schematic of the TOD assay: determination of the H_2O_2 generated from the oxidation of tyramine by TOD. The POD-linked continuous assay for TOD enzymes, 4-AA acts as the proton donor in the POD reaction and then condenses with vanillic acid. The absorbance of quinoneimine dye was recorded at 490 nm.

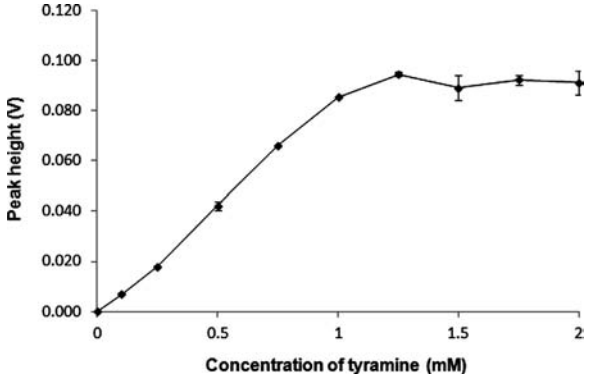


Fig. 3. Effect of the tyramine concentration; tyramine volume 100 μL, 8 U mL^{-1} TOD/volume 30 μL, sample volume 48 μL, CS (0.8 mM vanillic acid, 2.5 mM 4-AA and 4 U mL^{-1} POD)/volume 50 μL.

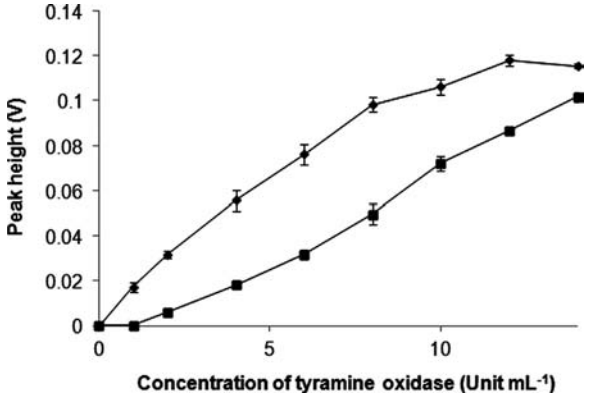


Fig. 4. Effect of the TOD concentration; ◇, 0.0 mM; ▯, 0.5 mM quercetin; 1.0 mM tyramine/volume 100 μL, TOD volume 30 μL, sample volume 48 μL, CS (0.8 mM vanillic acid, 2.5 mM 4-AA and 4 U mL^{-1} POD)/volume 50 μL.

increase of tyramine concentration and then level off at tyramine concentration higher than 1.0 mM, as shown in Fig. 3. This may be because the enzyme was saturated with the substrate at tyramine concentrations higher than 1.0 mM. Therefore, the 1.0 mM tyramine was chosen for further studies since this condition should give high sensitivity (large change in absorbance) when the inhibitor was presented.

3.2. Effect of the enzyme concentration on inhibitory activity

Effect of TOD concentration on the sensitivity of the measurement and inhibitory activity was studied without and with 0.5 mM quercetin in the sample zone. The TOD concentration varied from 0.0 to 14.0 U mL^{-1}. It was found that peak height of solution without the inhibitor increased with increasing TOD concentration up to 12.0 U mL^{-1} of TOD, over this concentration, a constant peak height was observed as shown in Fig. 4. Although, 10.0–14.0 U mL^{-1} of TOD provided higher sensitivity, they gave too low %inhibition (lower than 50%). Therefore, the 8 U mL^{-1} of TOD which gave the higher sensitivity and %inhibition was chosen for further experiments.

3.3. Effect of concentration of vanillic acid in the CS

A chromogenic solution (CS) was prepared by mixing of vanillic acid, 4-AA, and POD in PBS. In this experiment, concentrations of 4-AA and POD were fixed at 2.5 mM and 4 U mL^{-1}, respectively, while the concentration of vanillic acid varied within the range of 0.0–1.4 mM. As shown in Fig. 5, peak height dramatically increased

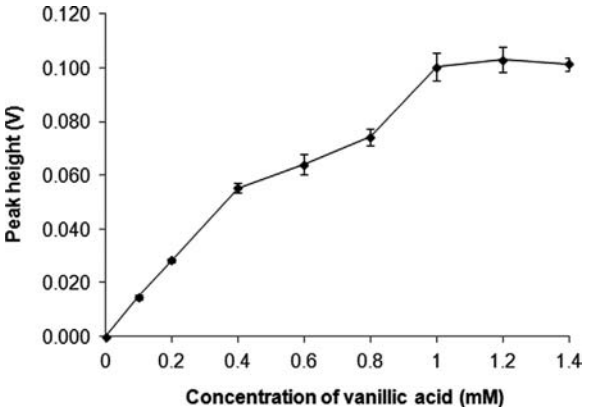


Fig. 5. Effect of the vanillic acid concentration; 1.0 mM tyramine/volume 100 μL, 8 U mL^{-1} TOD/volume 30 μL, sample volume 48 μL, CS (2.5 mM 4-AA and 4 U mL^{-1} POD)/volume 50 μL.

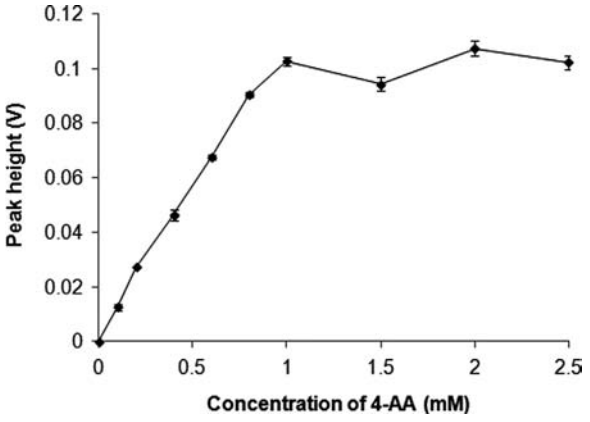


Fig. 6. Effect of the 4-AA concentration; 1.0 mM tyramine/volume 100 μL, 8 U mL^{-1} TOD/volume 30 μL, sample volume 48 μL, CS (1.0 mM vanillic acid and 4 U mL^{-} POD)/volume 50 μL.

up to 1.0 mM vanillic acid and then level off. Therefore, 1.0 mM of vanillic acid was selected as an optimum concentration.

3.4. Effect of the concentration of 4-AA in the CS

In this experiment, concentrations of the vanillic acid and POD were fixed at 1.0 mM and 4 U mL^{-1}, respectively. The concentration of 4-AA was studied in the range of 0.0–2.5 mM. Peak height linearly increased with the increase in 4-AA concentration up to 1.0 mM and then it reached a plateau, as shown in Fig. 6. Thus 1.0 mM of 4-AA was selected for further studies.

3.5. Effect of delay time

The effect of delay time or reaction time was investigated in the range of 0–20 s. The result was illustrated in Fig. 7. Peak height of the solution without an inhibitor increased when the delay time increased. However, at the delay time higher than 10 s the % inhibition of quercetin (0.5 mM) decreased and that of apigenin (3.0 mM) was level off. So, the delay time of 10 s was chosen as a proper time to study TOD inhibitory activity.

3.6. Performance of the developed SI system

Under the optimum conditions obtained above, precision of the system was studied at different concentrations of quercetin and it was expressed as a relative standard deviation (RSD) of 7 replicated injections. The RSDs of 2.39%, 2.17%, and 2.55% were obtained for

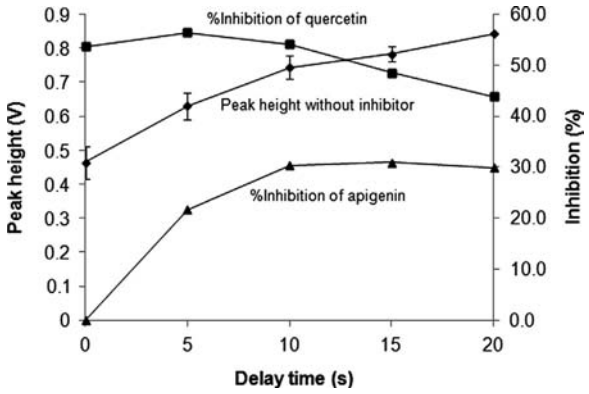


Fig. 7. Effect of the delay time; 0–20 s; ◇, 0.0 mM; ▯, 0.5 mM quercetin; Δ, 3.0 mM apigenin; 1.0 mM tyramine/volume 100 μL, 8 U mL^{-1} TOD volume 30 μL, sample volume 48 μL, CS (1.0 mM vanillic acid, 1.0 mM 4-AA and 4 U mL^{-1} POD)/volume 50 μL.

0.0, 0.3, and 0.6 mM quercetin, respectively. Precision of the SI method is better than the batch-wise well plate method, which had RSD in the range of 7–28%, $n=3$. This lack of precision may occur if the absorbance values are measured before a steady-state is reached. It is difficult to control the exact time in the batch assay (without expensive robotic system), so we noticed that only about 25 samples could be accurately analyzed at a time although the well plate has capability to accommodate many samples. The SI method had a short incubation time, leading to throughput of 40 injections h^{-1}. However, its drawback is only one sample can be analyzed at a time, whereas the well plate batch method, while needing a 20 min incubation time, permits analysis of several samples in parallel. Consequently there is no significant difference in sample throughput for the two methods. For the analysis of few samples, the SI method should be more suitable. Although the SI instrumentation and software program are quite complicated, this method is more precise, automated, and hence does not required a skilled analyst.

The sensitivity of the SI method was higher than the batch-wise method because the pathlength of the flow cell was 10 mm whereas that of the well plate was about 6.9 mm. An amplification unit was also used to improve the sensitivity of the SI method.

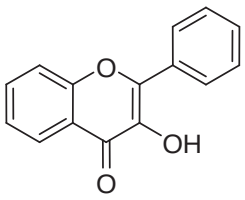
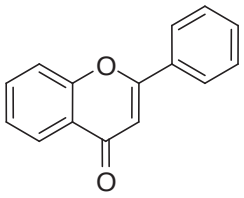
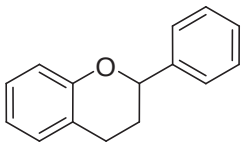
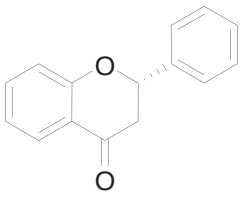
The SI method consumed 3.022, 0.100, 0.030, 0.050, and 0.048 mL of PBS, tyramine, TOD, CS, and sample solutions, respectively, per analysis, while the batch-wise method consumed 0.030, 0.100, 0.020, 0.050, and 0.050 mL of PBS, tyramine, TOD, CS, and sample solutions, respectively, per analysis. The higher consumption of buffer carrier solution used in the SIA procedure is necessary for flushing the coil and flow cell after each assay. Cleaning of the well plate was done later using detergent and deionized water.

Previously reported batch-wise assay of IC_{50} values based on inhibition of TOD by quercetin cover a wide ranges, i.e., 0.01 μM [11], 18 μM [10], 31.6 μM [12], and 50 μM [14]. We expect that the more automatic method should provide more consistent results.

3.7. Investigation of TOD inhibitory activity of some flavonoid compounds

Ten common flavonoids: quercetin, kaempferol, myricetin, morin, luteolin, apigenin, catechin, epicatechin gallate (ECG), epigallocatechin (EGC), and naringenin were investigated on TOD inhibitory activity by using the developed SI-spectrophotometric method and the batch MAO activity assay (as a comparison method). The inhibition of TOD by those compounds was expressed as the concentration that resulted in half maximal enzyme velocity (IC_{50}), the results were presented in Table 3. The effect of dispersion of sample zone in the SI system on the dilution of sample was investigated and thus IC_{50} values in Table 3

Table 3
The IC_{50} values of flavonoid compounds on inhibition of TOD, as determined by the proposed SI method compared with the batch method.

Flavonoid	Base structure	Compounds	IC_{50} values (mM)	
			SI	Batch
Flavonols		Quercetin	0.061 ± 0.004	0.08 ± 0.02
		Kaempferol	0.11 ± 0.01	0.14 ± 0.01
		Myricetin	0.041 ± 0.003	0.04 ± 0.01
		Morin	0.11 ± 0.01	0.07 ± 0.01
Flavones		Apigenin	1.4 ± 0.1	1.3 ± 0.1
		Luteolin	0.37 ± 0.01	0.40 ± 0.07
Flavans		Catechin	0.12 ± 0.01	0.13 ± 0.02
		ECG	0.039 ± 0.001	0.04 ± 0.01
		EGC	0.065 ± 0.002	0.12 ± 0.02
Flavanone		Naringenin	1.7 ± 0.1	1.8 ± 0.5

Values are means ± SD, $n=3$.

were examined with correction of dilution factor in the calculation to the final concentration of the sample being mixed with the enzyme.

It was found that, flavonol compounds, myricetin, quercetin, kaempferol and morin were inhibited TOD with IC_{50} values of 0.041, 0.061, 0.11, and 0.11 mM, respectively. According to the literature, the quercetin had previously been identified as MAO inhibitor contained in various natural products with the IC_{50} values lower than 0.1 mM [10–12]. It showed the inhibitory effect on the mitochondrial MAO-A reaction in the mouse brain, which was evaluated by Yoshino [13].

Flavone compounds, apigenin and luteolin (IC_{50}: 1.4 and 0.37 mM) showed lower TOD inhibitory activity than flavonol compounds. This may be due to the absence of hydroxy group in C-ring of the flavones (see structures of flavonoid compounds in Table 3).

In the case of flavanone, naringenin (IC_{50}: 1.7 mM), there is no double bond at the C2–C3 position, so that resulted in a significant decrease in TOD inhibitory activity [14]. Although the naringenin had the lowest TOD inhibitory activity (the highest IC_{50} value), some studies showed that it might be useful as a traditional medicine. Naringenin has been shown to pass the blood–brain barrier [29]. This fact means that it can exercise an effect on the central nervous system (CNS). It has been suggested that long-term use of low doses of herbal remedies might work well through an adaptive response in the CNS. This behavior explained why naringenin was interested as TOD inhibitor in the clinical studies [15].

In the case of flavan compounds, it was indicated that ECG (IC_{50}: 0.039 mM) presented higher TOD inhibitory activity than catechin and EGC (IC_{50}: 0.12 and 0.065 mM). As a possible reason, the ECG has many hydroxy groups in the C-ring, which may involve with more potent inhibition of TOD. In another research, the catechin isolated from *Uncaria rhynchophylla* was found to inhibit MAO-B with IC_{50} of 0.074 mM [16].

Several studies reported different IC_{50} values for MAO inhibition by some natural flavonoids. This discrepancy in the inhibitory effects might be due to the differences in the experimental conditions, type of substrate and source of the MAO enzyme. Therefore, we selected the widely used chemistry for the assay of TOD inhibitory activity to develop the proposed SI method, with the aims to increasing degree of automation and reducing variation in the experimental conditions, in order to obtain consistent and precise assay results. The proposed method was carefully compared with the batch-wise method, the results obtained by the both methods correlated well with correlation coefficient (r) of 0.981 ($y=0.9997x+0.011$). The experimental t-value between both the methods was 0.33, which

was less than the critical *t*-values 2.31 for 8 degrees of freedom at 95% confidence level, indicating that both the results were not significantly different. Moreover, higher automation and more precise control of the experimental condition by the developed SI method would reduce variation in the results of the assay (see Table 3).

4. Conclusions

In this work, we developed an automated, reliable and rapid method based on a SI-spectrophotometric system for the evaluation of TOD inhibitory activity. The reaction commonly used in the batch-wise assay was adapted in the SIA system in order to improve precision and accuracy of the assay by increasing automation and precise control of reaction time. The method was applied to the evaluation of IC_{50} value of some flavonoids on the inhibition of TOD. The IC_{50} values obtained by the developed SI method are comparable to those obtained by the batch method. In addition, the SI method is more precise, more automatic, better in sensitivity, and does not require highly skilled operator. The developed SI method would be an alternative method for the convenient evaluation of TOD inhibitory activity of various compounds from agricultural and natural products.

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