Determination of human serum semicarbazide-sensitive amine oxidase activity *via* flow injection analysis with fluorescence detection after online derivatization of the enzymatically produced benzaldehyde with 1,2-diaminoanthraquinone

Mahmoud H. El-Maghrabey[a,b], Naoya Kishikawa[a], Kaname Ohyama[a], Takahiro Imazato[c], Yukitaka Ueki[c], Naotaka Kuroda[a,*]

[a] Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[b] Analytical Chemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

[c] Sasebo Chuo Hospital, 15 Yamato-machi, Sasebo 857-1165, Japan

* Corresponding author. Tel.: +81 95 819 2894, fax: +81 95 819 2444, E-mail address: n-kuro@nagasaki-u.ac.jp (N. Kuroda)

Abstract

A fast, simple, and sensitive flow injection analysis method was developed for the measurement of semicarbazide-sensitive amine oxidase (SSAO) activity in human serum. Benzaldehyde, generated by the action of SSAO after incubation of serum with benzylamine, was derivatized with a novel aromatic aldehyde-specific reagent (1,2-diaminoanthraquinone) and the fluorescent product was measured by fluorescence detection at excitation and emission wavelengths of 390 and 570 nm, respectively. Serum SSAO activity was defined as benzaldehyde (nmol) formed per mL serum per hour. The method was linear over SSAO activity of 0.2-150.0 nmol mL^{-1} h^{-1} with a detection limit of 0.06 nmol mL^{-1} h^{-1}. The %RSD of intra-day and inter-day precision did not exceed 9.4% and the accuracy ranged from -6.5 to -0.6%. The method was applied for the determination of the serum SSAO activity in healthy controls (*C, n* = 24) and diabetes mellitus patients (*DM, n* = 18). It was demonstrated that the activity (mean ± SE) of SSAO in diabetics sera was significantly higher than that in healthy subjects' ones (DM; 73.3 ± 1.8 nmol mL^{-1} h^{-1} vs C; 58.9 ± 2.2 nmol mL^{-1} h^{-1}, $P<0.01$)

Keywords: Semicarbazide-sensitive amine oxidase activity; Benzaldehyde; 1,2-Diaminoanthraquinone; Flow injection analysis; Fluorescence detection

1. Introduction

Semicarbazide-sensitive amine oxidase (SSAO) is a family of heterogenous enzymes that can catalyze the deamination of various exogenous and endogenous mono-amines. It is a copper-containing glycoprotein possessing topa-quinone as a cofactor. These enzymes are found in most of the mammalians in two forms: tissue-bound and soluble isoforms (i.e., serum SSAO) [1, 2]. The SSAO enzymes are different from the monoamine oxidases (MAO-A and MAO-B) in their inhibition pattern: they have tolerance to MAO inhibitors like clorgiline, pargyline and deprenyl, but sensitive to semicarbazide and other hydrazines [3]. SSAO causes oxidative deamination to various amine compounds including dopamine, polyamines, tyramine, tryptamine, and benzylamine by converting them to the corresponding aldehydes with the generation of hydrogen peroxide and ammonia. Oxidation of methyl amine, allylamine, and aminoacetone by SSAO gives rise to formaldehyde, acrolein, and methylglyoxal, respectively [3]. These products are all potentially cytotoxic, e.g., formaldehyde is well known to be a major environmental risk factor, acrolein and methylglyoxal may disrupt the functions of proteins, lipids, and enzymes, and hydrogen peroxide is a major generator of oxidative stress which is commonly associated with numerous diseases [4]. The activity of serum SSAO has been reported to be increased in certain clinical conditions such as heart failure [5] and diabetes mellitus [6,7]. Also, when the activity of SSAO is high, the increased enzymatic-mediated deamination is proposed to be involved

in the pathogenesis of many vascular disorders [8]. Hence, the determination of SSAO might be a useful marker in the prognostic evaluation of diabetic angiopathy [9].

Several methods have been described for the measurement of SSAO activity, mostly using benzylamine as the preferred substrate. By the action of SSAO, benzylamine is deaminated and oxidized into benzaldehyde, hydrogen peroxide, and ammonia. These methods include HPLC-fluorescence detection after derivatization of benzaldehyde with dimedone [10], HPLC-UV detection [11] and light scattering measurement [12] after derivatization with 2,4-dinitrophenylhydrazine, and radiometric method using [^{14}C]-benzylamine as enzyme substrate [13]. Other methods depend on direct measurement of the enzyme using LC-MS [14], reverse transcription polymerase chain reaction (RT-PCR) [15], and Western blot and immunoassays [2, 16]. Although some of these methods are sensitive enough to give valuable data about the SSAO activity, most of them suffer from various drawbacks such as time-consumption, tedious extraction procedures and poor recoveries of benzaldehyde from biological fluids [11, 12], long derivatization reaction times [10-12], use of radioactive compounds [13], harsh condition (*e.g.*, 9 M sulfuric acid) [10], and the use of sophisticated and expensive instruments [2, 14-16]. Also, these methods that are based on the direct measurement of the SSAO enzyme [2, 14-16] do not reflect its activity inside the human body. In addition, the RT-PCR method [15] was not able to make a precise determination of the expression of

SSAO neither *in-vivo* nor *in-vitro*. Also, the Western blot and immunoassays [2, 16] need expensive antibodies. So, these methods are not suitable for routine analysis of a large number of samples and there is a need to develop a sensitive, fast, simple, and convenient method for the determination of SSAO activity in serum.

In the present study, we developed a flow injection analysis (FIA) method for the determination of SSAO activity in serum that allows the processing of a large number of samples within a short analysis time. The method is based on incubation of serum with benzylamine, an exogenous SSAO substrate. Then the benzaldehyde generated by the SSAO activity is derivatized online with the novel aromatic aldehyde-specific reagent 1,2-diaminoanthraquinone (DAAQ) and the formed imidazole derivative is determined by fluorescence detection at excitation and emission wavelengths of 390 and 570 nm, respectively. Serum SSAO activity is defined as benzaldehyde (nmol) formed per mL serum per hour.

DAAQ is a non-cytotoxic and non-fluorescent probe that was used previously for nitric oxide sensing through the formation of a fluorescent triazole derivative which is detectable by means of fluorescence microscopy [17]. The use of DAAQ as a derivatizing reagent for aldehydes has not been previously reported, which indicates the novelty of the proposed method. Also, the non-fluorescent property of DAAQ allows its use for online derivatization in FIA.

2. Experimental

2.1. Materials and reagents

Benzaldehyde, benzylamine, and glacial acetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium dihydrogenphosphate dihydrate (NaH_2PO_4:$2H_2O$) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Nacalai Tesque (Kyoto, Japan). DAAQ and clorgiline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Kanto Chemical Company (Tokyo, Japan). The water used was purified by a Simpli Lab UV (Millipore, Bedford, MA, USA). Phosphate buffer solution (PBS, 0.1 M, pH 7.8) was prepared by dissolving 0.13 g of Na_2HPO_4 and 0.14 g of NaH_2PO_4:$2H_2O$ in 100 mL distilled water and a Horiba F22 pH-meter was used to check the pH of the buffer. Stock solution of benzaldehyde (10 mM) was prepared in acetonitrile and stock solutions of clorgiline (0.2 mM) and benzylamine (5.6 mM) were prepared in PBS. 8.0 mM and 0.8 mM solutions of DAAQ were prepared in glacial acetic acid. Because of the expected photosensitivity of DAAQ, its solution was kept in amber-colored glass bottles. It was found to be stable for at least 2 weeks when kept at 4 °C in the refrigerator.

2.2. Confirmation of the identity of benzaldehyde-DAAQ derivative

The method of Huang *et al*. [18] was followed for the synthesis of benzaldehyde-DAAQ derivative. The obtained yellowish-brown compound was

subjected to electron impact mass spectrometry (EI-MS) to identify the molecular ion and ^{1}H NMR studies to prove the structure. The EI-MS spectra were recorded using JMS DX-303 mass spectrometer (Joel Ltd., Japan) and ^{1}H NMR spectra were recorded using Varian5-inova500 (500 Hz) spectrometer (Varian, CA, USA).

2.3. Fluorescence measurement of benzaldehyde after reaction with DAAQ

To a 100 µL portion of methanolic solution of 0.2 mM benzaldehyde in a screw capped vial, 100 µL of 8.0 mM DAAQ in acetic acid was added. After vortex mixing, the reaction mixture was heated at 100 °C for 30 min. The reaction mixture was diluted 10 times with methanol and the fluorescence spectra were recorded by a Shimadzu RF-1500 spectrofluorophotometer (Kyoto, Japan). A blank experiment was carried out simultaneously using 100 µL of methanol instead of benzaldehyde solution.

2.4. FIA System

A schematic flow diagram of the FIA system is shown in Fig. 1. The FIA system consisted of two Shimadzu LC-20AT pumps (Kyoto, Japan), a Rheodyne injector (Cotati, CA, USA) with a 20 µL sample loop, a Shimadzu RF-20AXS fluorescence detector, and an EZ Chrom Elite chromatography data acquisition system (Scientific software, Pleasanton, CA, USA). Polytetrafluoroethylene (PTFE) tubing (10 m x 0.5 mm i.d., GL Sciences,

Tokyo, Japan) was used as a reaction coil. Temperature of the reaction coil was kept at 100°C using a Shimadzu CTO-6A oven. Methanol and 0.8 mM DAAQ solution in glacial acetic acid were used as carrier and fluorogenic reagent streams, respectively. The flow rates of the carrier and fluorogenic reagent streams were set at 0.2 mL min^{-1}. Fluorescence detection was performed at 390 nm for excitation and 570 nm for emission.

2.5. Clinical samples

Serum samples of 24 healthy human subjects (10 females, 14 males; mean age 49.2 ± 6.6) and 18 diabetic ones (10 females, 8 males; mean age 59.0 ± 12) were supplied by Sasebo Chuo Hospital and stored at -80 °C until analyzed. The present experiments were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, and performed in accordance with the established guidelines.

2.6. Sample preparation procedure for SSAO enzyme activity

An aliquot of 50 µL human serum was pippeted into a 1.5 mL Eppendorf tube, to which 100 µL clorgiline (0.2 mM) was added to inhibit the MAO-A and B activity in serum. The tubes were vortexed for 1 min and incubated in a thermostatically controlled water bath at 37 °C for 30 min. An aliquot of 250 µL of 5.6 mM benzylamine was added to each tube giving a final concentration of 3.5 mM, then vortexed for 1 min and incubated at 37 °C for 1 h. An aliquot of

400 µL acetonitrile was immediately added to each tube, then was vortexed for 1 min and centrifuged at 1,200 × *g* for 5 min. The mixture was then kept at -30 °C for 20 min to separate the acetonitrile phase from the aqueous phase. An aliquot of 20 µL of the acetonitrile phase was injected into the FIA system.

2.7. Method validation

Method validation was performed in accordance with Guidance for Industry, Bioanalytical Method Validation (U.S. Department of Health and Human Services, Food and Drug Administration, FDA) [19]. The fundamental parameters for a Bioanalytical method validation are calibration curve, selectivity, accuracy, precision, and recovery.

The calibration curve was constructed by spiking seven samples covering the concentration range of 0.2-150.0 nmol mL^{-1} (including the limit of quantification, LOQ) to the enzymatic reaction mixture (using 250 µL of PBS instead of the SSAO's substrate, benzylamine). Each concentration was measured five times by applying the procedure described under section "2.6.". The LOQ was determined as the lowest concentration in the calibration curve where the analyte could be detected with acceptable accuracy (80-120%) and precision (relative standard deviation RSD ≤ 20%). The LOQ was established using five independent serum samples spiked with standard benzaldehyde [19]. In addition, the limit of detection (LOD) was determined as the concentration

corresponding to the peak area of the blank plus three times of its standard deviation (blank+3SD).

Selectivity of the proposed method, defined as its ability to differentiate and quantify the analyte in the presence of other components in the serum, was evaluated by the analysis of blank serum samples obtained from six sources (six human volunteers).

Accuracy and precision of the proposed method were also evaluated by applying the procedure described under section "2.6." for replicate analysis of serum samples containing known amounts of the analyte. Benzaldehyde was spiked to enzymatic reaction mixture at three different concentration levels: low, medium, and high (0.2, 5.0, and 100.0 nmol mL^{-1}, respectively) using 250 µL of PBS instead of the SSAO's substrate benzylamine. Each concentration was measured five times. The mean value was calculated and the deviation of the mean from the true value served as the measure of accuracy. Intra-day precision was investigated by calculating the %RSD for the peak areas for the five injections of benzaldehyde solution at these three concentrations; similarly, the inter-day precision (repeatability) was also investigated by calculating the %RSD for the peak areas of the three benzaldehyde concentrations on five successive days.

Recovery experiments were performed by comparing the analytical results for extracted enzymatic reaction mixtures (a) spiked at three concentrations of benzaldehyde (0.2, 5.0, and 100.0 nmol mL^{-1}) with un-

extracted standards (b) that represent 100% recovery. % Recovery was calculated according to (Eq. (1)):

$$\% \text{ Recovery} = (a/b) \times 100 \qquad (1)$$

2.8. Statistical analysis

In order to compare the levels of SSAO activity in the two studied groups (healthy and diabetic groups), variance ratio *F*-test was used to determine if there is a difference in their variances. Then, according to the result of the *F*-test we use either Student's *t*-test assuming equal variances or Student's *t*-test assuming unequal variances for comparing of the mean SSAO activity in each group [20]. Tests were two-sided and the significance was established at $P < 0.01$.

3. Results and discussion

3.1. Fluorescence spectra and identification of benzaldehyde-DAAQ derivative

The fluorescence spectra obtained from the reaction mixture of benzaldehyde with DAAQ are shown in Fig. 2. They were recorded in methanol showing excitation and emission maxima of 390 and 545 nm, respectively. On the other hand, neither benzaldehyde nor DAAQ exhibited intrinsic fluorescence at the specified excitation and emission wavelengths. Since the

reaction is a fluorogenic one, it can be used for online derivatization in FIA determination of benzaldehyde formed by the action of serum SSAO.

In order to elucidate the structure of the fluorescent derivative, the yellowish-brown precipitate obtained from the reaction of benzaldehyde with DAAQ was analyzes by EI-MS and ^{1}H NMR. The most abundant ion peak was found at *m /z* 324 corresponding to the molecular formula of $C_{21}H_{12}N_2O_2$ and ^{1}H NMR (500 MHz, DMSO-d6) d (ppm): d7.56 (3H, d, *J* = 8.5 Hz), d7.87 (2H, t, *J* = 7.0 Hz), d8.04(2H,d, *J* = 7.0 Hz), d8.16(2H, m), d8.30 (2H, d, *J* = 4.0 Hz). These results conform to the results of Huang *et al*. [18] and the previous reports for the reaction of benzaldehyde with DAAQ or their analogues [21]. Thus, the derivatization reaction was suggested to proceed as shown in Fig. 3.

3.2. Optimization of FIA conditions

In order to obtain the highest fluorescence intensity, we optimized the conditions for the fluorogenic derivatizaion reaction including DAAQ concentration, catalyst type and concentration, reaction temperature, and carrier stream composition. The effect of DAAQ concentration on the fluorescence intensity was studied over the range of 0.1-1.0 mM. The fluorescence intensity increased with increasing the DAAQ concentration up to 0.4 mM then reached a plateau (Fig. 4A). 0.8 mM of DAAQ was selected as the optimum reagent concentration to ensure method robustness.

The influence of different catalysts on the fluorescence intensity was examined using acetic acid, acetic acid/sodium acetate, acetic acid/copper acetate, trifluoroacetic acid, sulfuric acid, acetic acid/Mn(III), and acetic acid/sodium metabisulfite as solvents for preparation of DAAQ. The study of these catalysts is based on previous reports for the synthesis of 2-arylbenzimidazoles from benzaldehyde and DAAQ or their analogues [21]. Among these catalysts, the highest fluorescence intensity was obtained with acetic acid, so it was selected for subsequent experiments (Fig. 4B). Subsequently, the effect of acetic acid concentration on the fluorescence intensity was studied over the concentration range of 10-100% using methanol as a diluent. The maximum fluorescence intensity was achieved using 100% acetic acid (Fig. 4C); hence, it was used as a solvent for preparation of DAAQ.

The effect of the reaction temperature was examined over the range of 40-100 °C. The fluorescence intensity increased with increasing the temperature up to 100 °C (Fig. 4D). As a result, 100 °C was selected as the optimum reaction temperature. Also, different solvents were tried as carrier streams including water, methanol, and acetonitrile. Methanol gave the highest fluorescence intensity, so it was chosen as the optimum carrier stream.

Successively, FIA parameters such as flow rate and reaction coil dimension were optimized. The effect of the flow rate of the two streams, the carrier and the fluorogenic reagent solutions, on the fluorescence signal and residence time was investigated by changing their flow rate from

0.3 to 1.0 mL min^{-1}. A total flow rate of 0.4 mL min^{-1} was selected as a compromise between sensitivity and analytical throughput (Fig. 4E) giving a reasonable residence time of 5 min. Then, the flow rate ratio (carrier stream: fluorogenic reagent stream) was investigated maintaining a total flow rate of 0.4 mL min^{-1}. The highest fluorescence intensity was obtained at a flow rate of 0.2 mL min^{-1} for the two streams.

Both PTFE and metal tubes were investigated as a reaction coil, and the PTFE one gave better fluorescence signal, so, it was selected as the optimum in subsequent experiments. The effect of the reaction coil dimension on the fluorescence intensity was investigated using different PTFE tubes while keeping a total residence time of 5 min by varying the flow rate. Three PTFE reaction coils were tested; the first one has dimensions of 10 m X 0.5 mm (coil volume 2 mL) and the used flow rate was 0.4 mL min^{-1}, the second one was 15 m X 0.5 mm in dimensions (coil volume 3 mL) and the used flow rate was 0.6 mL min^{-1}, and finally the third one was X 0.75 mm in dimensions (coil volume of 4.5 mL) and the used flow rate was 0.9 mL min^{-1}. The maximum fluorescence intensity was obtained using the first reaction coil (Fig. 4F).

An online study was conducted for selection of the optimum excitation and emission wavelengths of the reaction product. The highest fluorescence signal was obtained up on measuring the formed derivative at emission wavelength of 570 nm and excitation wavelength of 390 nm. A variation of the optimum emission wavelength of the product in the reaction medium from that

in the online system was observed (545 and 570 nm, respectively), which is probably attributed to the difference in medium polarity. So, for achieving maximum sensitivity under the operating conditions, detection was done at 390/570 nm (excitation/emission).

Fig. 5 illustrates a representative detector response for benzaldehyde in enzymatic reaction mixture under the optimum FIA conditions.

3.3. Pretreatment of serum samples for determination of SSAO activity and Michaelis-Menten constant (K_m)

To prevent possible participation of plasma MAO in the conversion of the substrate benzylamine to benzaldehyde, the reaction mixture was first incubated with 100 µL clorgiline solution (0.2 mM) at 37 °C for 30 min. Under these conditions, clorgiline makes a complete and irreversible inhibition of both MAO-A and B [3]; meanwhile, SSAO is insensitive to clorgiline [13]. Clorgiline acts by binding the MAO enzyme active sites stoicheometrically to form adduct with the flavine group in a 1:1 ratio [22]. After incubation with clorgiline, the reaction was started by the addition of 250 µL of benzylamine. Different benzylamine concentrations were tried and the reaction rate was calculated each time as the amount formed of benzaldehyde in (nmol mL^{-1} h^{-1}). The apparent K_m *(K')* and V_{max} *(V')* of serum SSAO were estimated by the nonlinear curve fitting (Fig. 6) of Michaelis–Menten equation (Eq. (2)) using GraphPad Prism trial version 6.05, (GraphPad Software, La Jolla, CA, USA).

$V = (V_{max} \, x \, [S]) / (K_m + [S])$ (2)

Where V is the initial rate of reaction, V_{max} is the maximum reaction velocity, $[S]$ is the substrate concentration, and K_m is the Micaelis-Menten constant.

K' was found to be 322.2 ± 23.2 nmol mL^{-1} where V' equals 46.1 ± 0.9, which compares well with previously reported values [10, 23]. Since it is usual to use a concentration of substrate about 10 folds higher than the K' value to determine the activity of an enzyme in a sample, a concentration of 3.5 µmol mL^{-1} of benzylamine was used as optimum substrate concentration in the incubation mixture.

For efficient extraction of benzaldehyde from human serum, we first attempted a simple protein precipitation procedure with methanol or trichloroacetic acid for the pretreatment of serum samples. However, it was found that the presence of water in the reaction mixture interfered with the fluorogenic reaction. Therefore, in order to remove the water from extracted solution, a subzero-temperature liquid-liquid extraction based on the phenomenon that acetonitrile can be separated from the aqueous phase at a temperature below -20 °C [24, 25], was adopted. The application of this technique enabled water to be eliminated from the reaction mixture and the fluorogenic reaction then proceeded well. Moreover, the addition of acetonitrile terminated the action of SSAO on benzylamine *via* protein denaturation.

3.4. Validation study

To confirm the reliability of the proposed method, it was validated according to the Guidance for Industry on Bioanalytical Method Validation [19].

The calibration curve prepared from the analysis of enzymatic reaction mixture spiked with standard benzaldehyde solution (seven-points curve) showed excellent linear relationship (r = 0.9999) between the concentrations and the average peak area over the concentration range of 0.2-150.0 nmol mL^{-1} according to the following regression equation: $Y = 0.388x10^5 + 1.62x10^5 X$ (Y is the average peak area and X is benzaldehyde concentration, nmol mL^{-1}). The LOQ was found to be 0.2 nmol mL^{-1} with a %RSD of 9.4% and accuracy of -6.5%. The LOD (blank + 3 SD) was 0.06 nmol mL^{-1}.

To ensure selectivity of the proposed method and its ability to measure and differentiate benzaldehyde in the presence of components which may be expected to be present in the serum samples, serum samples from six healthy volunteers were analyzed and the response was compared to the signal of the analyte at the LOQ. Under these conditions, no interference from endogenous matrix components was observed indicating the selectivity of the proposed method for benzaldehyde. In addition an interference study has been carried out and will be discussed in details later.

The intra- and inter-day accuracy and precision of the proposed method were determined at 0.2, 5.0, and 100 nmol mL^{-1}. The results, expressed as the

deviation of mean value from the true one for accuracy and %RSD for precision, are shown in Table 1. The data showed good accuracy (intra-day -4.0 to -1.4 %; inter-day -6.5 to -0.6%) and adequate precision (intra-day 2.9 to 6.7%; inter-day 2.2 to 9.4%).

Recovery pertains to the extraction efficiency of the analytical method within the limits of variability. It was calculated by comparing the analytical response for extracted serum samples at three concentrations with un-extracted standards. The % recoveries ranged from 94 to 98% with a standard deviation did not exceed 8.5% proving the high efficiency, precision, and reproducibility of the subzero-temperature liquid-liquid extraction method. Results of recovery study are shown in Table 2.

The results of the validation procedure of the proposed FIA method agreed well with the requirements of the guidelines for Bioanalytical method validation [19].

3.5. Interference investigation

In order to apply the proposed method to the determination of benzaldehyde formed by the action of serum SSAO, the influence of some aldehydes typically found in human serum was investigated. The aldehydes and their concentration ranges shown in Table 3 were selected for investigation of the interferences with the present method. Firstly, a mixed solution containing benzaldehyde (100 nmol mL^{-1}) and aldehyde to be examined at constant

concentration of 100 nmol mL^{-1} was injected into the carrier stream. The error due to the potentially interfering substances in the determination of benzaldehyde was determined by comparing the signal obtained for the mixed solution with that obtained for the 100 nmol mL^{-1} benzaldehyde solution without the interfering aldehyde.

Secondly, the same experiment was repeated in the enzymatic reaction mixture by spiking it with benzaldehyde (100 nmol mL^{-1}) and the aldehyde to be examined at constant concentration of 100 nmol mL^{-1} following the procedure mentioned in section "**2.6.**" using 250 µL of PBS instead of the SSAO's substrate benzylamine. Furthermore, both previous experiments were repeated using a mixture of all these possibly interfering aldehydes (100 nmol mL^{-1} of each) and benzaldehyde (100 nmol mL^{-1}) and the error resulted from these aldehydes mixture was calculated.

The results of all experiments are illustrated in Table 3. It can be noted that the presence of glyoxal, methylglyoxal, acrolein, heptanal, or malondialdehyde causes negligible small interferences in the direct determination of benzaldehyde, proving specificity of the reagent for aromatic aldehydes. In addition, the % error measured following the subzero-temperature liquid-liquid extraction was obviously less than that measured upon direct injection of samples into the FIA system. A % error of +1.1 was obtained upon analysis of serum sample spiked with benzaldehyde and mixed aldehydes following the subzero-temperature liquid-liquid extraction *versus* +10.1% upon

injection of the mixture directly into the carrier stream. These results evidenced the selectivity of the extraction procedure for benzaldehyde in presence of other aliphatic aldehydes.

Interference that may be encountered from benzylamine, the exogenous substrate of SSAO, with the derivatization reaction of benzaldehyde with DAAQ was also tested. A mixed solution containing benzaldehyde (100 nmol mL^{-1}) and benzylamine (3.5 mM) in acetonitrile was injected into the carrier stream. Comparing the signal obtained for this solution with that obtained for a standard benzaldehyde (100 nmol mL^{-1}), revealed a negative interference (-46.0%). The reason for this interference is probably due to condensation reaction of benzaldehyde with benzylamine forming a Schiff's base [26], such reaction abstracted benzaldehyde from the medium causing a negative interference with the assay.

Indeed, to mimic the actual assay conditions in serum, the same experiment was repeated in PBS pH 7.8 (as a substitute for serum) then the solution was extracted by the subzero-temperature liquid-liquid extraction procedure. 20 µL of the acetonitrile phase was injected into the FIA system. Interestingly, a negligible positive deviation (+0.6%) from the actual value for benzaldehyde alone was observed. This indicates the high selectivity of the extraction procedure for benzaldehyde in the presence of benzylamine. This is because benzylamine has a distribution coefficient (log *D*) = -0.56 at pH 7.4 (the physiological pH of blood serum), while that of benzaldehyde at the same

pH is 1.61 [27], which indicates very high polarity of benzylamine relative to benzaldehyde. From this, we can conclude that under the working condition (at pH 7.8); benzylamine is not extractable in acetonitrile phase while benzaldehyde is readily extracted in it, which offers a high selectivity for the assay.

3.6. Application of the proposed method to determination of the SSAO activity in healthy and diabetic sera

The proposed FIA-fluorescence detection methodology was applied to determine the human serum SSAO activity of 24 healthy human subjects and 18 patients with diabetes mellitus. Serum SSAO activity is defined as benzaldehyde (nmol) formed per mL serum per hour. The average SSAO activity in healthy human subjects (mean ± SE) was found to be 58.9 ± 2.2 nmol mL^{-1} h^{-1} which agreed well with the previous results [11, 28, 29].

In the sera of diabetic patients, activity of SSAO was significantly higher compared to control healthy subjects at $P < 0.01$ (Table 4). This finding is in accordance with previous reports [6, 7, 30-32]. Nunes *et al*. attributed the significant increase in SSAO activity in diabetic patients to the translocation of SSAO from the tissue-bound to plasma due to alterations in arterial permeability [33]. Thus, the measurement of serum SSAO activity is a valuable clinical biomarker for evaluation of the prognosis of diabetic complications [13].

3.7. Comparison of performance of the proposed method with that of reported literature for SSAO activity determination

Table 5 illustrates a comparison of the performance of the proposed method with the reported literature for the determination of SSAO activity. The proposed method is 50 and 2 times more sensitive for benzaldehdye than the reported HPLC methods for determination of SSAO activity [10, 11]. Even if the reported light scattering technique [12] exhibited higher sensitivity than the proposed method, it suffers from some drawbacks such as tedious extraction procedure, long drivatization time, and poor recovery.

Although the time required for sample pretreatment and incubation period is a common feature of the proposed method and reported literature, the proposed FIA method has the advantage of online derivatization reaction and short run time (5 min) which offers high sample throughput (27 h^{-1}). In addition, the proposed method uses small serum volume (50 µL), these attributes, together with the excellent recovery and selectivity obtained adopting the subzero-temperature liquid-liquid extraction procedure, make the proposed method suitable for the routine measurement and comparison of serum SSAO activity in healthy and diabetic subjects.

The results of the proposed FIA method were statistically compared with those obtained using the reported HPLC-UV detection method [11] by applying both methods for the analysis of serum samples from ten healthy volunteers (4 females, 6 males; mean age 51.2 ± 5.6) for determination of SSAO activity.

Statistical comparison of the results of the proposed and reported methods by two-sided paired Student *t*-test and variance ratio *F*-test revealed no significant difference between the performances of the two methods with regard to accuracy and precision [20]. Results of the comparison study are illustrated in Table 6.

4. Conclusion

A novel fluorogenic derivatizing agent (DAAQ) was used to convert benzaldehyde, generated by the action of SSAO on benzylamine, into a fluorescent derivative. DAAQ showed high selectivity for the aromatic aldehyde, benzaldehyde, and there was no significant interference from aliphatic biological aldehydes. Based on this finding, a well optimized and validated FIA-fluorescence detection method was developed for the assay of human serum SSAO activity. The proposed method provided a sensitive and accurate tool to monitor the difference in SSAO activities in sera of healthy and diabetic human subjects.

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Table 1

Accuracy and precision of the proposed method for the determination of benzaldehyde in the spiked enzymatic reaction mixtures.

Spiked concentration (nmol mL^{-1})	**Found Conc. (nmol mL^{-1})**	**% Accuracy**	**Precision RSD (%)**
Intra-day (n = 5)			
0.2	0.192	-4.0	6.7
5.0	4.85	-3.0	3.4
100.0	98.6	-1.4	2.9
Inter-day (n = 5)			
0.2	0.187	-6.5	9.4
5.0	4.72	-5.6	4.5
100.0	99.4	-0.6	2.2

Table 2

Results of recovery study of benzaldehyde from spiked enzymatic reaction mixtures

Spiked level (nmol mL^{-1})	**% Recovery**	**SD**
0.2	94	8.5
5.0	96	4.1
100.0	98	2.1

Table 3

Effects of some aldehydes normally exist in human serum on the determination of benzaldehyde (100 nmol mL^{-1}).

Aldehyde (100 nmol mL^{-1})	**Standard mixture**	**Enzymatic reaction mixture**
	% Error [a,b]	**% Error [a,c]**
glyoxal	+0.1	+0.2
methyl glyoxal	+0.7	-0.5
acrolein	+2.5	-1.1
heptanal	+1.1	-0.3
malondialdehyde	+5.2	-1.2
mixture of all previous aldehydes	+10.1	+1.1

[a] Calculated as $(A_{B+I} - A_B)/A_B$. Where, A_{B+I} and A_B denote the peak area obtained for benzaldehyde solution containing the interfering aldehyde and that obtained for benzaldehyde without aldehyde, respectively.

[b] % error calculated after direct injection of acetonitrile solutions containing benzaldehyde alone or with interfering aldehydes

[c] % error calculated after subzero temperature liquid-liquid extraction of serum samples spiked with benzaldehyde alone or with interfering aldehydes

Table 4

Statistical analysis of the results for determination of the SSAO activity in healthy and diabetics human subjects' sera.

Parameter	**Control healthy human subject**	**Diabetic patients**
Number of samples (n)	24	18
SSAO activity range (nmol mL^{-1} h^{-1})	29.7-79.3	59.9-84.4
SSAO activity mean ± SE (nmol mL^{-1} h^{-1})	58.9 ± 2.2	73.3 ± 1.8
Variance ratio *F*-test	$P = 0.09$ (NS)[a]	
Student's *t*-test	$P < 0.01$	

[a] NS: no significant difference

Table 5

Critical comparison of the performance of the proposed and reported methods for determination of SSAO activity.

Method	**Reagent**	**Reaction time (min)/ temperature (°C)**	**% Recovery**	**LOD (nmol mL^{-1} h^{-1})**	**Remarks**	**Ref.**
HPLC-FL	Dimedone	45/95	97	1.50	Derivatization required 9 M sulphuric acid	10
HPLC-UV	2,4-Dinitrophenylhydrazine	30/37	64- 68	0.100	Unstable, flammable, and irritant reagent	11
Light-Scattering	2,4-Dinitrophenylhydrazine	30/40	68	0.002		12
Radio-enzymatic assay	[^{14}C] Benzylamine	-----	N/A [a]	N/A [a]	Using radioactive compound	13
FIA-FL	1,2-Diaminoanthraquinone	5/100	94-98	0.060	Automated online reaction	Present method

[a] N/A: data is not available.

Table 6

Statistical comparison of the proposed FIA method and a reference method for the determination of SSAO activity in human sera.

Parameter	**Proposed FIA method**	**Comparison HPLC-UV method [11]**
Number of samples (n)	10	
SSAO activity range (nmol mL^{-1} h^{-1})	35.7-63.1	33.9-63.6
SSAO activity mean ± SE (nmol mL^{-1} h^{-1})	48.9 ± 3.4	47.6 ± 3.3
Variance ratio *F*-test	$P = 0.44$ (NS)[a]	
Paired student's *t*-test	$P = 0.19$ (NS)[a]	

[a] NS: no significant difference

Figure Captions:

Fig. 1 Fig. 1. Schematic diagram of the FIA system.

Fig. 2 Fluorescence spectra of benzaldehyde-DAQQ derivative and reagent blank. (A), (B): excitation and emission spectra of benzaldehyde-DAAQ derivative, respectively, (A'), (B'): excitation and emission spectra of reagent blank, respectively.

Fig. 3 Suggested mechanism for benzaldehyde reaction with DAAQ.

Fig. 4 Effect of different reaction and flow conditions on the fluorescence intensity of the reaction product of DAAQ with benzaldehyde (100 nmol mL^{-1}). (A) effect of DAAQ concentration, (B) effect of type of catalyst, (C) effect of concentration of acetic acid, (D) effect of oven temperature, (E) effect of total flow rate of carrier and fluorogenic reagent streams, (F) effect of reaction coil volume and flow rate.

Fig. 5 FIA signals for the determination of benzaldehyde spiked to the enzymatic reaction mixture. Concentrations of benzaldehyde are: (a) 0 nmol mL^{-1} (blank), (b) 0.2 nmol mL^{-1}, (c) 5 nmol mL^{-1}, (d) 20 nmol mL^{-1}, (e) 50 nmol mL^{-1}, (f) 80 nmol mL^{-1}, (g) 100 nmol mL^{-1}, and (h) 150 nmol mL^{-1}.

Fig. 6 Nonlinear fitting of Michaelis–Menten plot of SSAO activity *vs* benzylamine concentration in the enzymatic reaction mixture using GraphPad Prism trial version 6.05.

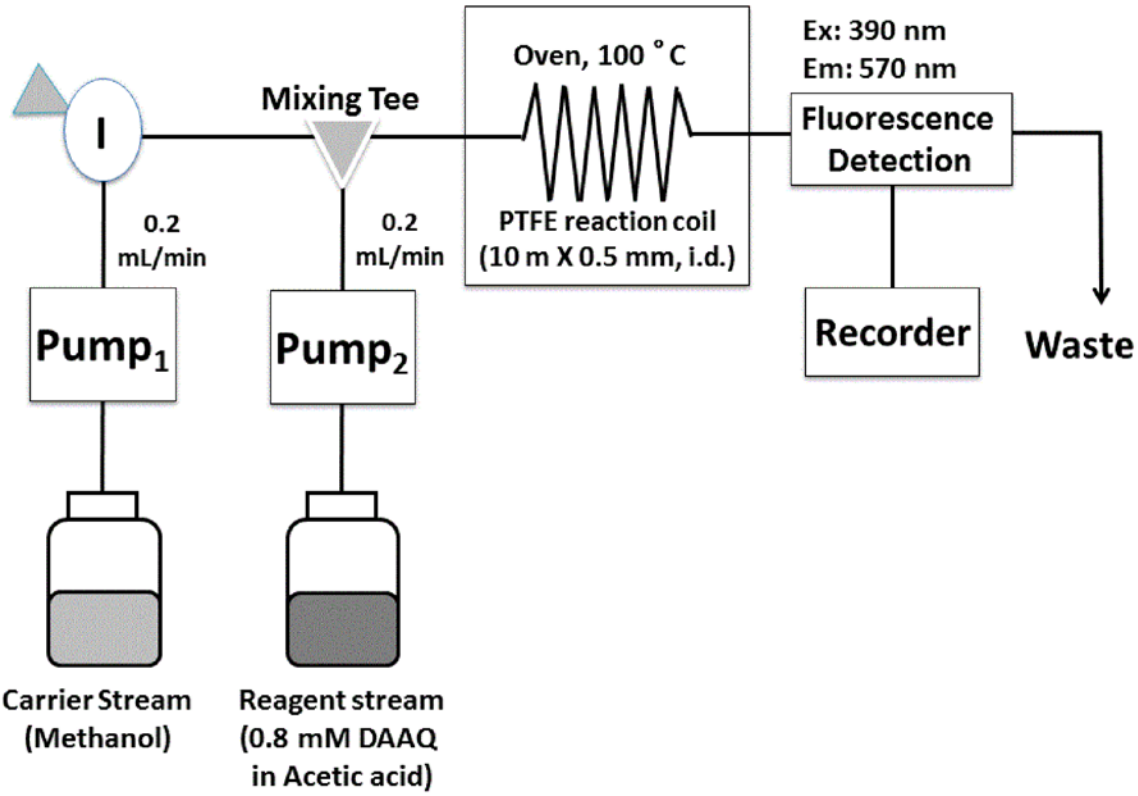

Oven, 100 °C
Ex: 390 nm
Em: 570 nm
Mixing Tee
Fluorescence Detection
0.2 mL/min
0.2 mL/min
PTFE reaction coil (10 m X 0.5 mm, i.d.)
Pump1
Pump2
Recorder
Waste
Carrier Stream (Methanol)
Reagent stream (0.8 mM DAAQ in Acetic acid)

Fig. 1.

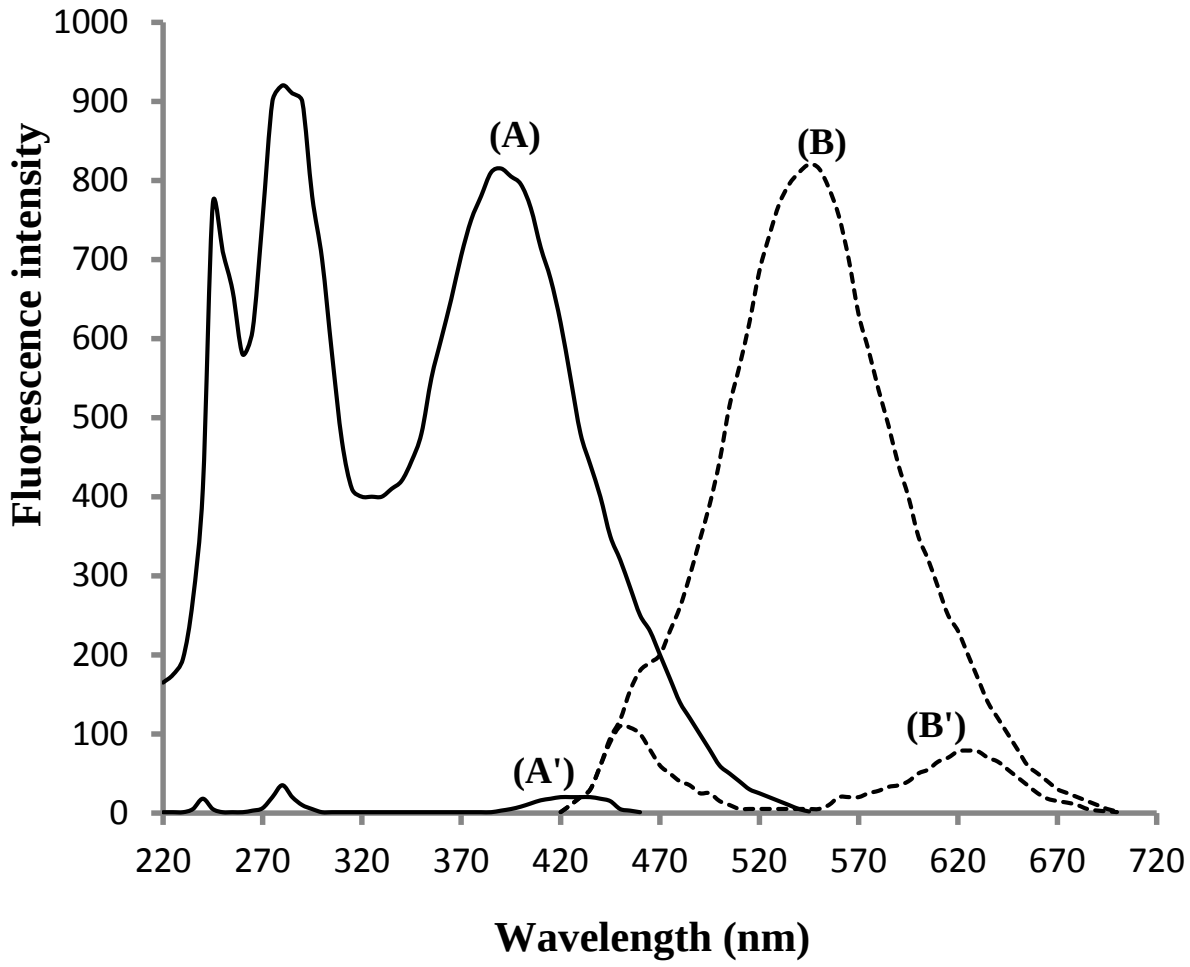
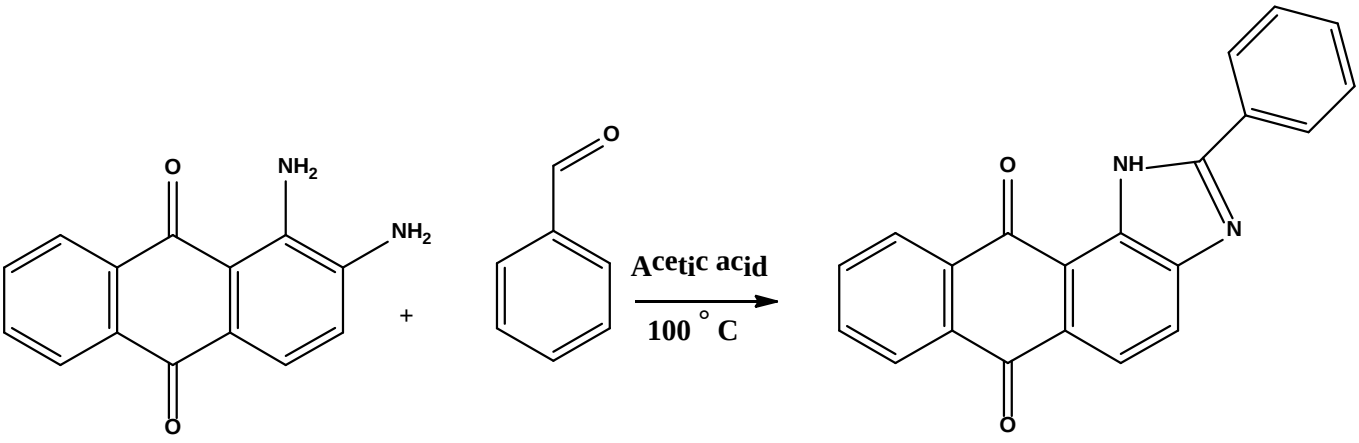

Fluorescence intensity
1000
900
800
700
600
500
400
300
200
100
0
(A)
(B)
(A')
(B')
220
270
320
370
420
470
520
570
620
670
720
Wavelength (nm)

Fig. 2.



1,2-Diaminoanthraquinone Benzaldehyde Imidazole derivative

Fig. 3.

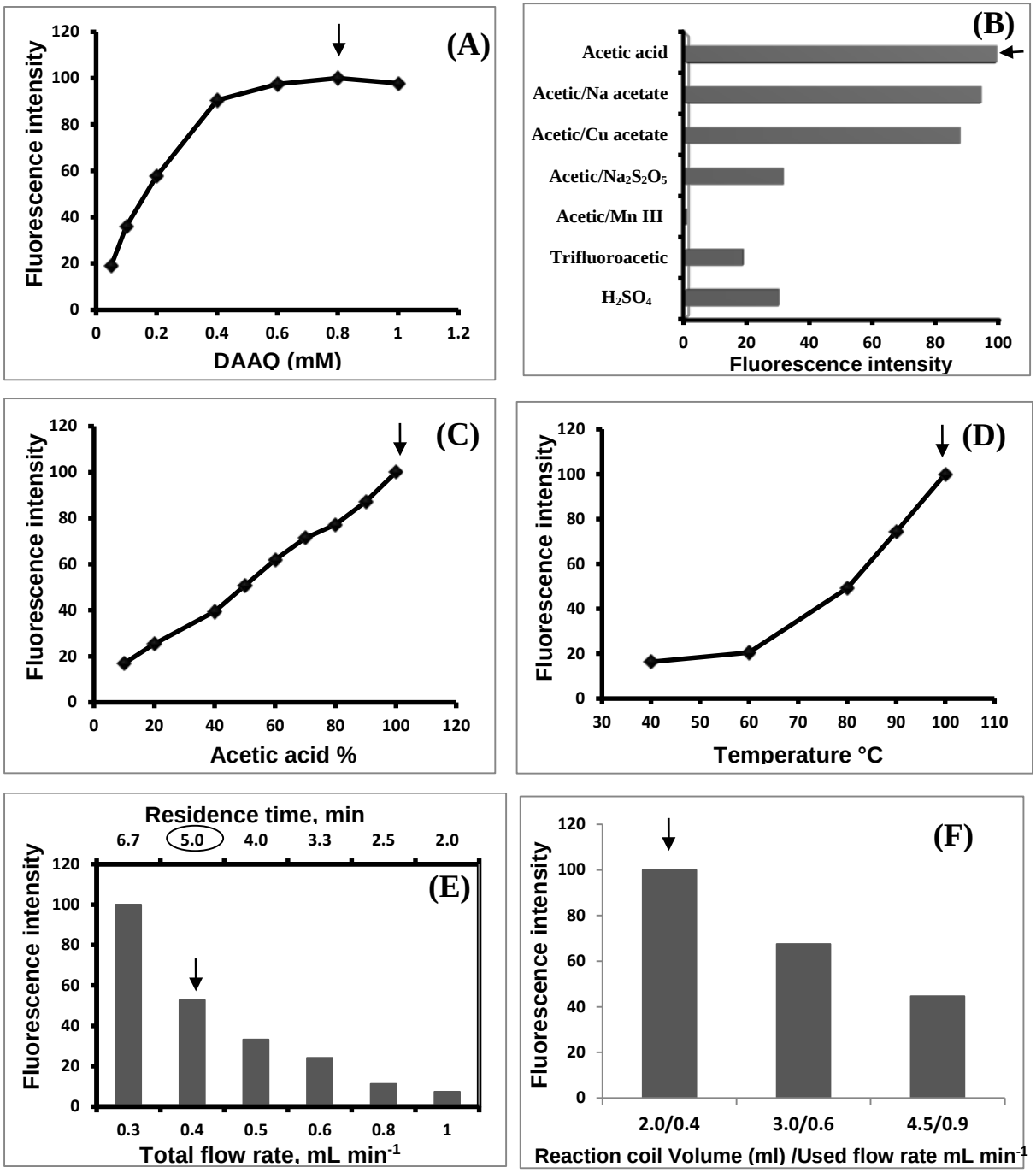

(A)
Fluorescence intensity
DAAO (mM)
(B)
Acetic acid
Acetic/Na acetate
Acetic/Cu acetate
Acetic/Na2S2O5
Acetic/Mn III
Trifluoroacetic
H2SO4
Fluorescence intensity
(C)
Fluorescence intensity
Acetic acid %
(D)
Fluorescence intensity
Temperature °C
(E)
Residence time, min
6.7
5.0
4.0
3.3
2.5
2.0
Fluorescence intensity
Total flow rate, mL min-1
(F)
Fluorescence intensity
2.0/0.4
3.0/0.6
4.5/0.9
Reaction coil Volume (ml) /Used flow rate mL min-1

Fig. 4.

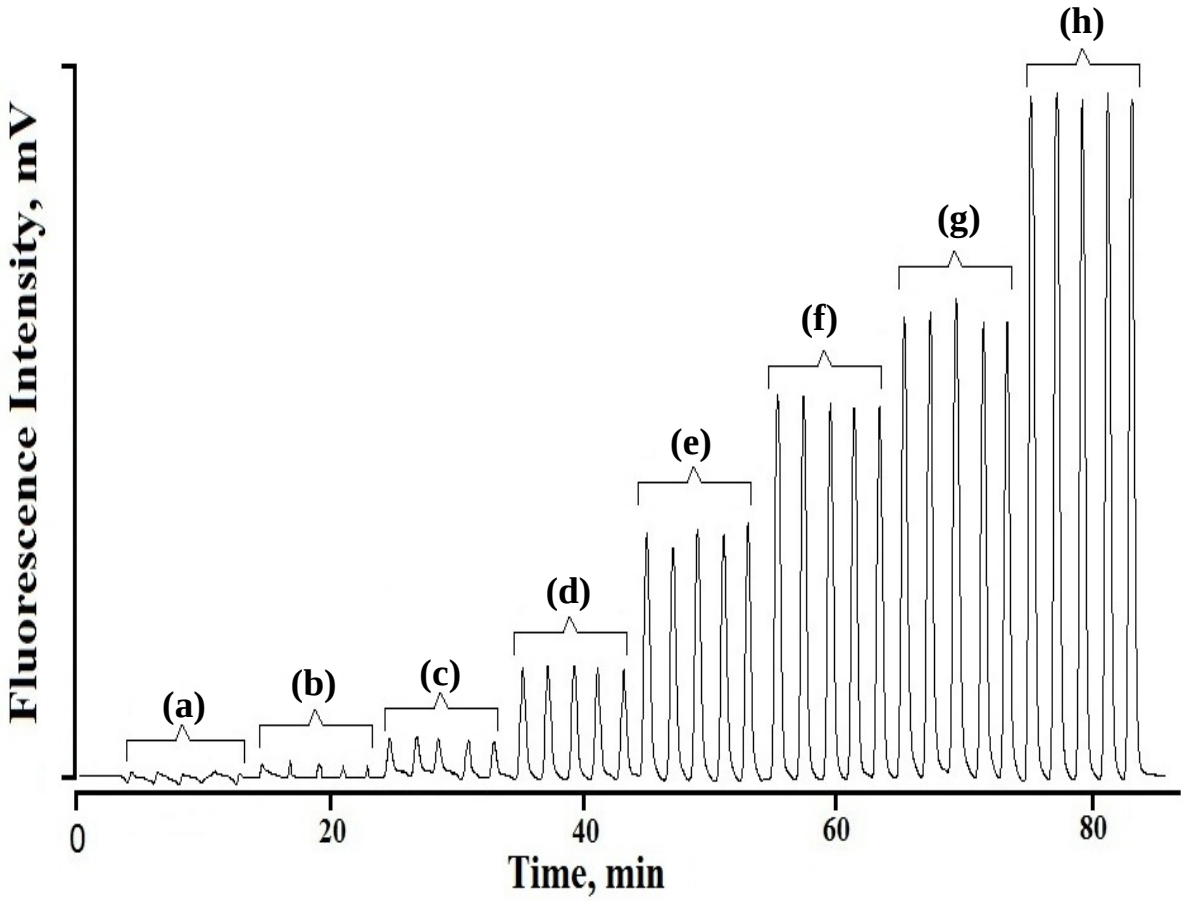


Fig. 5.

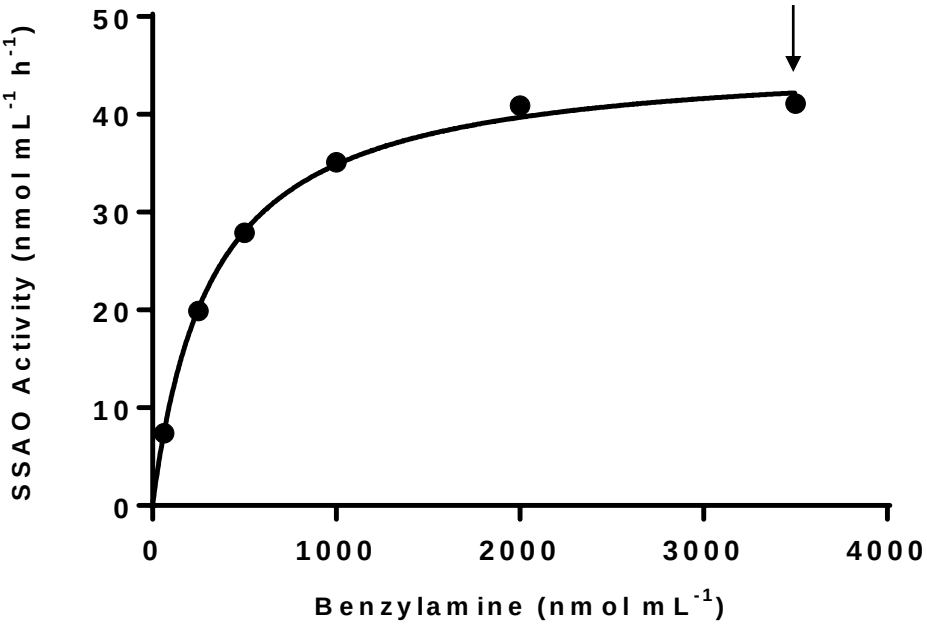

SSAO Activity (nmol mL-1 h-1)
50
40
30
20
10
0
0
1000
2000
3000
4000
Benzylamine (nmol mL-1)

Fig. 6.