

Application of Flow Injection System to Ascorbic Acid Assay for Assessing Oxidative Potential of Metal Ions

by

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Abstract

Human exposure to fine particulate matter (PM_{2.5}) air pollution is known to cause adverse health outcomes. The mechanisms by which PM_{2.5} affects human health are not yet fully understood. However, accumulating evidence indicates that PM exposure induces oxidative stress via the generation of reactive oxygen species (ROS), a process often characterized by its oxidative potential (OP). Therefore, reliable tools are needed to estimate the oxidant-generating capacity of PM. One of the most widely reported methods for assessing PM oxidative potential is the ascorbic acid (AA) assay. This assay employs chemical analysis to determine the time-course of the aqueous AA concentration during its reaction with chemical species present in PM_{2.5}. The depletion rate of AA in solution is referred to as the OP of the test sample. The AA concentration is quantified by conventional spectrophotometry at a wavelength of 265 nm. However, determining the AA depletion rate requires multiple measurements on a single sample, making it a labor-intensive method when analyzing large numbers of samples. The purpose of this study is to develop a more simplified and user-friendly AA assay by applying a flow injection (FI) system to measure the oxidation rate of AA. The FI system was constructed based on an ultrafast liquid chromatography setup without a chromatographic column. Dilution series of Cu(II) and Fe(II) were prepared, and their OP values were investigated by absorptiometry at 265 nm using the constructed FI system. As a result, the AA depletion rate increased with rising concentrations of both metal ions in solution. The OP values were compared with those obtained via traditional spectrophotometry. The results indicated no statistically significant differences between OP values measured by the two methods, demonstrating the successful application of the FI system for OP measurement.

Keywords: PM_{2.5}, Oxidative potential, Ascorbic acid assay, Flow-injection system

1 INTRODUCTION

Many literatures indicate the association between airborne fine particulate matter ($\text{PM}_{2.5}$) and adverse human health effects [1–3]. Although the mechanisms underlying PM-related health effects are not completely understood, growing evidence from biochemical assessments — such as DNA methylation studies, animal models, and human biomarker analyses — indicates that exposure to PM can induce oxidative stress in the body [4]. This offers a potential mechanism for PM toxicity. In addition, identifying the specific particle properties responsible for these health effects, such as their physical and physicochemical characteristics as well as their chemical composition, remains a challenge [5]. One of the leading hypotheses for how particles cause harm is that they induce oxidative stress and inflammation, which can subsequently lead to disease — a process often described by the term oxidative potential (OP).

Over the past few years, the OP produced by $\text{PM}_{2.5}$ has attracted much attention, various research suggested that many adverse health endpoints are due to the OP produced by $\text{PM}_{2.5}$, resulting in the generation of reactive oxygen species (ROS), and the excessive ROS cannot be completely counteracted by the antioxidants in the body, leading to the beginning of inflammatory responses. ROS are molecules or ions that may be present on $\text{PM}_{2.5}$, originate from external sources, or be produced intracellularly as by-products of oxygen-related chemical processes. They are superoxide radical ($\text{O}_2 \bullet$), hydrogen per oxide (H_2O_2), hydroxyl radical ($\bullet\text{OH}$), alkoxyl radical ($\text{RO} \bullet$), carbon-centered radical ($\text{C} \bullet$) and singlet oxygen (O_2). An increase in intracellular ROS disrupts the existing balance between ROS and available antioxidants, which in turn leads to oxidative stress and various adverse health effects [6]. Consequently, OP has been widely recognized in multiple studies as a more suitable indicator than mass concentration alone for assessing the oxidative capacity of PM. Moreover, OP is considered one of the key mechanisms through which PM toxicity exerts its effects on human health.

In vitro assessment of aerosol particle toxicity based on the determination of OP was first carried out from the early 2000s. Since then, a variety of methodologies have been optimized and applied to determine the OP of PM, generally including ascorbic acid (AA) assay (OP^{AA}) [7, 8], dithiothreitol (DTT) assay (OP^{DTT}) [9], glutathione (GSH) assay (OP^{GSH}) [10] and TPT assay (OP^{TPT}) [11]. Compared with the methods mentioned, the AA assay appears to be the most sensitive to transition metals [7, 8]. The AA assay is employed to monitor the oxidation rate of AA by measuring the remaining AA in the presence of metal species over a specific time interval. A conventional spectrophotometer and quartz cuvette have been used to measure the concentration of AA in solution. However, determining the depletion rate of AA requires multiple measurements on a single sample, making it a labor-intensive method when analyzing large numbers of samples.

This study aimed to develop a simplified and user-friendly AA assay by applying a flow injection (FI) system to measure the oxidation rate of AA for quantifying oxidative potential (OP). The OP values induced by metal species, including Cu(II) and Fe(II), were determined using the FI system and compared with those obtained via conventional spectrophotometry as a reference technique.

2 MATERIALS AND METHODS

2.1 Instruments and Apparatus

The flow injection (FI) system was adapted from an Ultrafast Liquid Chromatograph (UFLC), originally designed for rapid and high-resolution high-performance liquid chromatography (HPLC). The system was operated without a chromatographic column and was used to monitor absorbance at a wavelength of 265 nm using a UV/VIS detector connected to a data processor. The UFLC system consisted of an LC-20AD pump (Shimadzu, Kyoto, Japan), an SPD-20A UV/VIS detector (Shimadzu, Kyoto, Japan), an injection valve, and a Chromatopac C-R8A data processor (Shimadzu, Kyoto, Japan), as shown in Figure 1. Phosphate buffer solution at pH 7.4 was used as a carrier at a flow rate of 1.0 mL/min.

A spectrophotometer (AS ONE, model ASUV-3100PG) with a standard cuvette (Quartz, 10-mm optical pathlength) was employed to compare with the OP obtained from the FI system. Absorbance at 265 nm was measured using ultrapure water as reference.

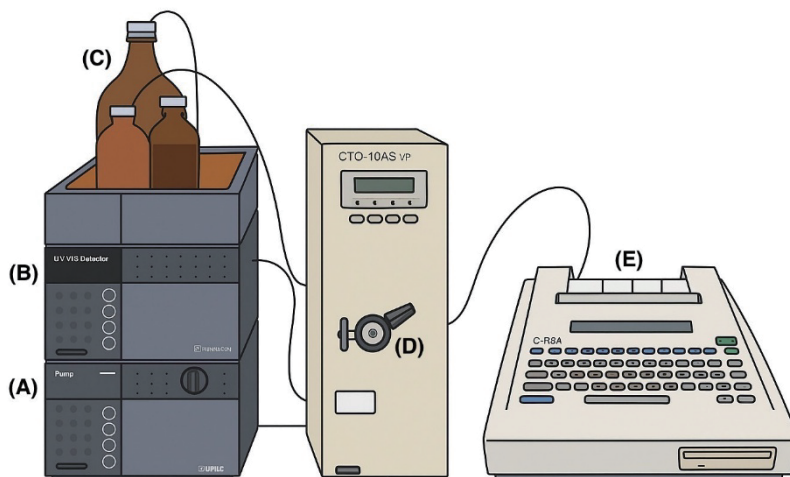


Fig. 1 Components of flow injection system for AA assay adapted from Shimadzu UFLC
 (A) LC-20AD pump (B) SPD-20A UV/VIS detector (C) Solvent container
 (D) Injection port (E) Chromatopac C-R8A data processor

2.2 Reagents

Copper (II) sulfate pentahydrate (FUJIFILM Wako Pure Chemical, Osaka, Japan, special grade) and iron (II) chloride tetrahydrate (Kanto Chemical, Tokyo, Japan, first grade) were dissolved and diluted with ultrapure water (Milli-Q ultrapure type1 water, 18.2 M Ω , Simplicity® water purification system, Merck KGaA, Darmstadt, Germany) to prepare stock aqueous solutions of 0.010 mM CuSO₄ and 1.0 mM FeCl₂ aqueous solutions, respectively. Since the ability of AA depletion differed between Cu(II) and Fe(II) [12], 0.010 mM CuSO₄ and 1.0 mM FeCl₂ solutions were used for the AA assay. L-ascorbic acid (FUJIFILM Wako Pure Chemical, Osaka, Japan; special grade) was dissolved in the ultrapure water to prepare the 10 mM solution used for the AA assay. Phosphate buffer system at pH 7.4 was prepared by mixing 0.10 M KH₂PO₄ (Kanto Chemical, Tokyo, Japan,

Application of Flow Injection System to Ascorbic Acid Assay for Assessing Oxidative Potential of Metal Ions special grade) and 0.10 M K_2HPO_4 (Kanto Chemical, Tokyo, Japan, special grade) solutions in a volume ratio of 1 : 4.

2.3 Measurement of AA depletion rate and OP

To investigate the Cu(II) and Fe(II) concentrations relating to the AA depletion rate, aqueous solutions of CuSO_4 (0, 0.10, 0.20, 0.30, and 0.50 μM) and FeCl_2 (0, 10, 20, and 100 μM) were prepared in ultrapure water, and these test samples were stored at 37°C in a thermostatic chamber before use. Subsequently, 0.1 ml of 10 mM ascorbic acid was added to 10 mL of test samples and mixed immediately to give test solutions. Twenty μL of the test solution was injected to the FI system, and absorbance at a wavelength of 265 nm were recorded at the start and at 1-minute intervals for 10 minutes. Note that each measurement required approximately 3.5 mL of sample solution by conventional spectrophotometry, whereas only 1 mL was sufficient for injection into the FI system.

Following the method reported by Souza *et al.* [13], the depletion rate of AA represents the consumption of ascorbic acid over a specific time range. It is determined by applying linear regression to the absorbance data (which are proportional to the loss of AA concentration) as a function of time. Using the slope and intercept derived from the linear relationship between absorbance (or instrumental response) and elapsed time, the AA depletion rate was calculated as equation (1)

$$\text{AA depletion rate } (\mu\text{M}/\text{min}) = -\frac{[\text{reductant}]_0}{p} \frac{dA_t}{dt} \quad (1)$$

where, p refers to the intercept of absorbance versus time, $[\text{reductant}]_0$ to the initial concentration of AA (= 99 μM), and $\frac{dA_t}{dt}$ to the slope. When using the Chromatopac C-R8A data processor, absorbance was recorded as the peak height of the voltage signal derived from the SPD-20A UV/VIS detector. Therefore, the AA depletion rate can be calculated using either the peak heights or the peak heights normalized to the initial value ($t = 0$), instead of absorbance.

Since the measured AA depletion rate tends to fluctuate depending on the experimental conditions of each day likely due to the spontaneous degradation of AA in solutions without metal ions, the blank measurements (i.e., 0 M Cu(II) or Fe(II)) were conducted under the same conditions. To assess the oxidative ability of the studied metal ions, the AA depletion rate of the blank was subtracted from that of the sample to obtain the OP, as shown in equation (2).

$$OP = \text{AA depletion rate of sample} - \text{AA depletion rate of blank} \quad (2)$$

2.4 Comparison of FI system with conventional spectrophotometric analysis

To demonstrate the validity of applying the FI system for assessing the OP of metal ions, the OP values obtained from the FI system were compared with those measured using the spectrophotometer at 1-minute intervals for 10 minutes. Statistical analyses, including the calculation of mean, standard deviation, and t-test, were performed for each method and between the two methods using Microsoft® Excel. Test samples were prepared for OP measurements by both techniques, including 0.1 μM Cu(II), 10 μM Fe(II) and a mixed solution of 0.1 μM Cu(II) and 10 μM Fe(II).

3 RESULTS AND DISCUSSION

3.1 Rate of AA depletion by metal ions measured by FI system

Figure 2 presents the time-course profiles of normalized peak heights for test solutions containing 0–0.5 μM Cu(II) and 0–100 μM Fe(II), as measured by the developed FI-AA assay. Each plot represents the arithmetic mean of three replicate measurements. In most cases, the normalized peak heights exhibited an approximately linear decrease over time, with the slope rising in terms of its absolute value in proportion to the metal ion concentration. However, for all test samples, the dependence deviation from linearity were observed with noticeable at higher concentrations than the lower ones. It was according to research reported by Sugimoto et al. [14]. This suggests that the reaction between ascorbic acid and metal ions may follow first-order kinetics in essential. Accordingly, when evaluating the oxidative potential (OP) of individual species such as Cu(II) or Fe(II), it would be appropriate to use the rate constant derived from a first-order reaction model. On the other hand, the AA assay was originally developed as a method for assessing the toxicity of complex mixtures such as $\text{PM}_{2.5}$. For the purpose of comparing OP across different samples, the current method — approximating the change in absorbance over a 10-minute reaction period with a linear fit — is considered suitable. Therefore, the AA depletion rates were calculated from the slope and intercept of the linear regression for each run based on Eq. (1).

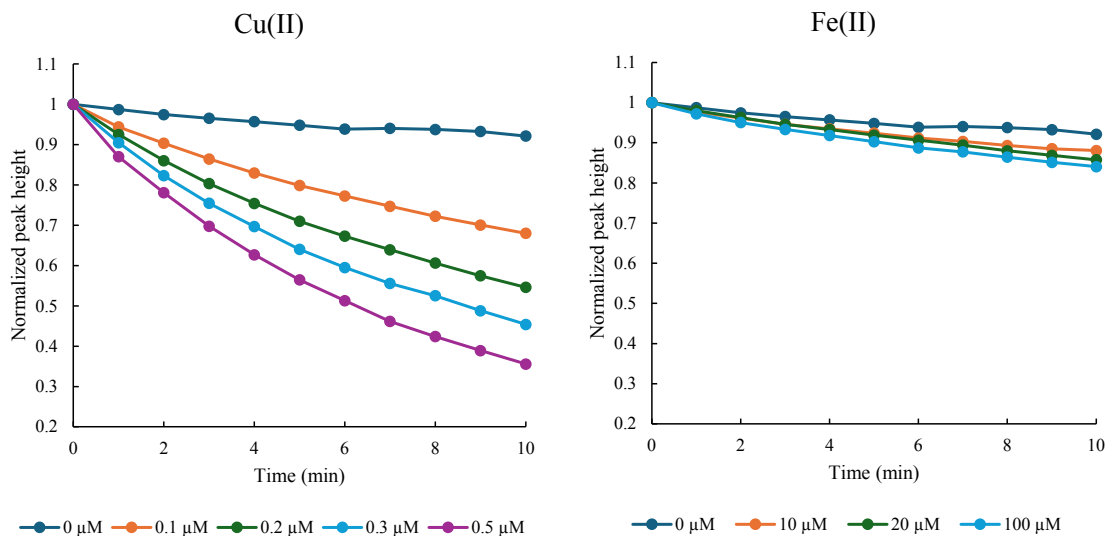


Fig. 2 Time-course of normalized peak heights of Cu(II) and Fe(II) solutions measured by the developed FI-AA assay. Each plot represents the arithmetic mean of three repeated runs ($n = 3$)

The results are shown in Figures 3 and 4. The X-axis shows the concentration of metal ions in the test sample and the Y-axis shows the AA depletion rate. Each test was conducted 3 times of measurement, and the error bars indicate the standard deviation. The AA depletion rate increased with increasing concentrations of both metal ions in the solutions. From the figures, it should be noted that at a 1/100 concentration ratio of Cu(II) to Fe(II), Cu(II) exhibited the AA depletion rate approximately three times higher than that of Fe(II). This trend is consistent with the study on effect of Werner-Type complex formation of Cu(II) and Fe(II) on OPs assessed using AA Assay

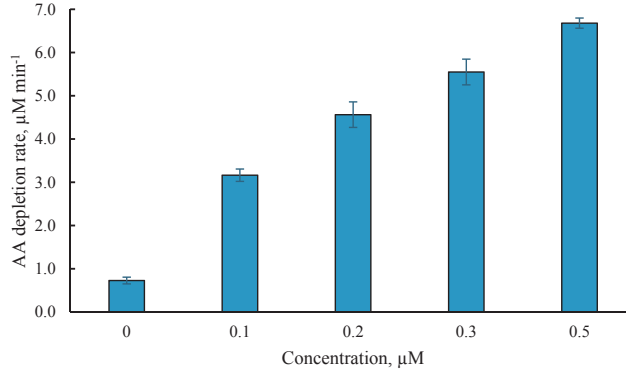


Fig. 3 Relationship between Cu(II) concentration and the rate of AA depletion measured by developed FI system. Error bars indicate the standard deviation of three repeated runs ($n = 3$)

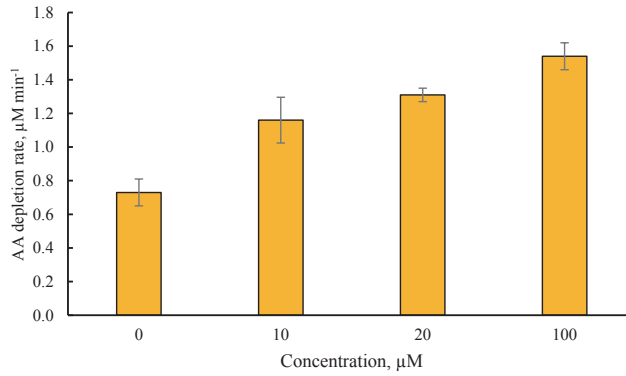


Fig. 4 Relationship between Fe(II) concentration and the rate of AA depletion measured by developed FI system. Error bars indicate the standard deviation of three repeated runs ($n = 3$)

reported by Sekine *et al.* [12] which OP measurements were carried out by spectrophotometer.

3.2 Comparative results of both techniques and statistical analysis

To avoid potential influences on the AA depletion rates of the blank measurements, the OP values were calculated based on Eq. (2) and compared between this FI system and conventional spectrophotometry, as shown in Table 1. As for 0.1 μM Cu(II), the OP measured by the FI system was $1.7 \mu\text{M min}^{-1}$, which was equivalent to the value obtained by spectrophotometry ($1.6 \mu\text{M min}^{-1}$). As for 10 μM Fe(II), the OP values were $0.34 \mu\text{M min}^{-1}$ (FI) and $0.31 \mu\text{M min}^{-1}$ (spectrophotometry). In the mixed solution of 0.1 μM Cu(II) and 10 μM Fe(II), the OP increased to $2.5 \mu\text{M min}^{-1}$ by FI and $2.3 \mu\text{M min}^{-1}$ by spectrophotometry. The OP values obtained from both techniques show good agreement for all samples, with p -values from the t-test exceeding 0.05, indicating no significant difference between the two approaches.

Overall, the results demonstrate that the FI system provides OP values comparable to those obtained by the conventional spectrophotometric technique, assuring the reliability and validity of the FI system for OP assessment.

Table 1 Comparison of OP values obtained by the two techniques over five repeated runs.

Test solution	OP in $\mu\text{M min}^{-1}$ measured by FI system	OP in $\mu\text{M min}^{-1}$ measured by Spectrophotometry	p -value from t-test
0.1 μM Cu(II)	1.7 ± 0.065	1.6 ± 0.149	0.13
10 μM Fe(II)	0.34 ± 0.06	0.31 ± 0.13	0.66
Mixed	2.5 ± 0.14	2.3 ± 0.10	0.14

4 CONCLUSION

The flow injection (FI) system was successfully applied to assess the oxidative potential (OP) driven by metal ions based on the rate of ascorbic acid (AA) depletion, showing results consistent with the OP values obtained by the conventional spectrophotometric technique. The AA depletion rate increased with increasing metal ion concentrations, and Cu(II) exhibited approximately three times more OP than Fe(II) at a 1/100 Cu-to-Fe concentration ratio. Statistical analyses indicated no significant difference ($p > 0.05$) between OP values measured by the two techniques. These findings shows that the FI system possibly provides reliable, accurate, and efficient determination of OP with reduced sample volume, and the simplified and user-friendly AA assay was realized.

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