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Monitoring of vitamin C species in aqueous solution by flow injection analysis coupled with an on-line separation with reversed-phase column

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ABSTRACT

Two vitamin C species of ascorbic acid and dehydroascorbic acid in aqueous solution were monitored by flow injection analysis. Ascorbic acid and dehydroascorbic acid were resolved by a reversed-phase column, and dehydroascorbic acid was reduced to ascorbic acid by an on-line post-column reaction with dithiothreitol. Both natural and reduced ascorbic acids were photometrically detected at 260 nm, and the two vitamin C species were simultaneously determined. The determination range was from 0 to 8×10^{-5} M with a limit of detection of 1.7×10^{-6} M. The proposed method was applied to the conversion monitoring of ascorbic acid and dehydroascorbic acid in weakly acidic to weakly alkaline aqueous solutions, as well as to the determination of the vitamin C in some beverage samples.

1. Introduction

Ascorbic acid is widely known as vitamin C, and it has been studied in various fields including medical, pharmaceutical, and biochemistry. Ascorbic acid is generated in plants especially in vegetables and fruits, and is taken into human body. Ascorbic acid in various matrices was determined, and the analytical methods have been reviewed [1–3]. The vitamin C includes some chemical forms, and the major species are ascorbic acid (AA) and dehydroascorbic acid (DHAA). Ascorbic acid as its natural form is also known to be transformed to semi-dehydroascorbic acid as a reaction intermediate and to dehydroascorbic acid by stepwise oxidations, and further to 2,3-diketogulonic acid (DKGA) by hydrolysis [1]. The reaction between AA and DHAA is reversible by the oxidation–reduction reactions, and total amount of vitamin C is determined by reducing DHAA to AA.

Among the vitamin C species, ascorbic acid alone has been determined by means of its reducing ability. Mixed complex of AA with iron(III) and *p*-carboxyphenylfluorone was formed in cationic micellar solution, and it was photometrically detected [4]; flow injection analyses (FIA) provided reproducible and rapid analysis, and AA was determined. Solid $Fe(OH)_3$ was reduced and dissolved with AA as analyte, and the formed soluble Fe^{2+} was photometerically detected as 1,10-phenanthroline complex [5]. Kinetic phenomena were utilized on the reduction of Fe(III)-1,10-phenanthroline complex [6]. Photochemical reaction was also applied to the formation of Fe(II)-1,10-phenanthroline complex [7]; the sensitivity was increased but some interference occurred. Catalytic effect of ascorbic acid on the formation of Cu(II)–porphyrin complex was utilized coupled with flow injection-spectrophotometric detection [8]. On-line removal of interfering anions with amine-modified silica [9], adsorption of ascorbic acid on a flow-through solid phase [10], and amperometric detection with arrays of gold electrodes [11] have been proposed for the flow injection analysis of AA. Reversed-phase high performance liquid chromatography (HPLC) with dual electrode electrochemical detection was also proposed for the determination of AA [12].

Dehydroascorbic acid is also a component of vitamin C species, and it has also been detected in addition to AA at the same time. Simultaneous determination of AA and DHAA is classified into two major groups. One principle is that DHAA is reduced to AA and that the total AA is determined. The amount of the dehydroascorbic acid is calculated by subtracting the initial amount of AA. Dithiothreitol was firstly investigated as a reducing agent of DHAA [13]. Pre-column reduction was used for the reversed-phase HPLC with electrochemical detection [14], coulometric detection [15], and photometric detection [16–19]. Direct photometric detection of AA with or without the reduction of DHAA is more convenient on detection of AA and DHAA. The subtraction method was also applied in ion-pair reversed-phase HPLC [20–22], as well as microfabricated glass-channel [23]. Tris(2-carboxyethyl)phosphine hydrochloride [24] and 2,3-dimercapto-1-propanol [25] also showed the reducing potential of DHAA.

Another principle for the simultaneous determination of AA and DHAA is the on-line column separation. Weak anion-exchange HPLC was used for the separation between AA and DHAA, and the two analytes were detected photometrically or by refractive index [26]. Post-column reduction was done after the separation of AA

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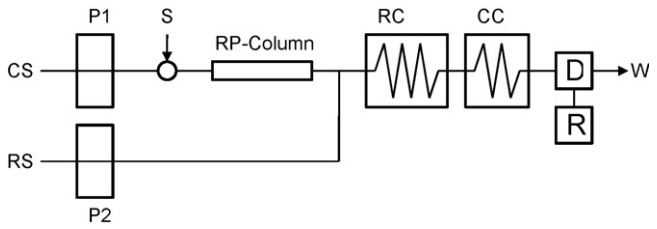

Fig. 1. Flow diagram for simultaneous determination of ascorbic acid and dehydroascorbic acid. CS: carrier solution, 0.025 M phosphate buffer (pH: 2.4). RS: reducing solution, 10 mM dithiothreitol in 1 M ammonium acetate buffer (pH: 8.5). P1 and P2: pumps, flow rate at 0.5 mL min^{-1} each. S: sample injection, 200 μL. RP-Column: Yokogawa, SIL-C18 5B, 150 mm × 4.6 mm i.d. RC: reaction coil, 2 m × 0.5 mm i.d. held in aluminum block bath controlled at 64 °C. CC: cooling coil, 1 m × 0.5 mm i.d. held in water bath controlled at ambient temperature. D: photometric detector (260 nm). R: recorder. W: waste.

and DHAA with a reversed-phase column, and the AA reduced from DHAA was determined as well as the initial AA [27]. The on-line column separation seems to be more convenient than the pre-column reduction, because single injection can offer the simultaneous determination of AA and DHAA.

In this study, we also aimed at developing the simultaneous determination of ascorbic acid and dehydroascorbic acid by a spectrophotometric FIA by means of a reversed-phase column separation coupled with a post-column reduction with dithiothreitol. The determination method was used for the validation of the standard solution of the dehydroascorbic acid. By oxidizing AA in an aqueous solution to form DHAA with activated charcoal, the calibration graphs for AA and DHAA agreed well with each other. Simultaneous determination of AA and DHAA was also utilized in the monitoring of reaction dynamics from AA to DHAA in an aqueous solution. The degradation of AA and DHAA in air-saturated water and in N_2 gas-purged water was monitored successfully.

2. Experimental

2.1. Apparatus

A spectrophotometric FIA system was constructed with two pumps of Tosoh (Tokyo, Japan) HLC-803D and Sanuki Kogyo (Tokyo, Japan) 1P2M, and a Tosoh UV-8000 photometric detector. All PTFE tubing used for the construction of the system was of 0.5 mm i.d. A TOA (Tokyo, Japan) flatbed recorder FBR-252A was used to record the FIA signals. Samples and standard solutions were introduced into the carrier stream via a six-way rotatory injection valve (Hamilton, USA) with a 200-μL sample loop. A reversed-phase column of Yokogawa (Hachioji, Japan) SIL-C18 5B (150 mm in length and 4.6 mm i.d.) was used to separate AA and DHAA. An Iuchi (Tokyo, Japan) hot dry bath SHD-1 was used to control the reaction temperature of the reduction tubing to reduce DHAA to AA with dithiothreitol. The flow diagram is shown in Fig. 1. A Shimadzu UV-2400PC spectrophotometer was used for the measurement of the absorption spectra. A Horiba (Kyoto, Japan) M-11 pH meter was used to measure and to adjust the pH of the solutions. Disposable membrane filter (Dismic-25AS, 45 μm; Advantec Toyo Kaisha, Tokyo, Japan) was used for the sample filtration.

2.2. Reagents

Ascorbic acid and dehydroascorbic acid were purchased from Wako (Osaka, Japan) and Aldrich (Milwaukee, WI, USA), respectively; they were used without further purifications. Dithiothreitol was purchased from Fluka (Buchs, Switzerland). Other reagents used were of analytical reagent grade. Activated charcoal was from Wako. Water used was de-ionized and distilled one.

Stock solution of ascorbic acid was prepared daily by dissolving it in the purified water at the concentration of 1.0×10^{-3} M; it was used after dilution with the purified water. The carrier solution was prepared by mixing 0.025 M KH_2PO_4 and 0.025 M H_3PO_4 solutions to adjust the pH at 2.4. The reducing solution was prepared by dissolving dithiothreitol at the concentration of 10 mM in 1 M ammonium acetate buffer (pH: 8.5), just before use.

2.3. Standard procedure for the simultaneous determination of AA and DHAA

The carrier solution was propelled at a flow rate of 0.5 mL min^{-1}. The separation column was held in a thermostatic water bath kept at 25.0 °C. After the separation column to be equilibrated, a standard/sample solution was injected into the carrier stream using a six-way rotatory valve. After the analytes passing through the column, the stream was merged with the stream of the reducing solution at its flow rate of 0.5 mL min^{-1}. The stream merged was heated at 64 °C in a Teflon tubing (RC: 2 m in length and 0.5 mm i.d.) in a temperature controlled hot dry bath, where DHAA is reduced to AA. The stream was cooled to around ambient temperature in a cooling coil (CC: 1 m in length and 0.5 mm i.d., immersed in water), and the ascorbic acid was detected photometrically at 260 nm.

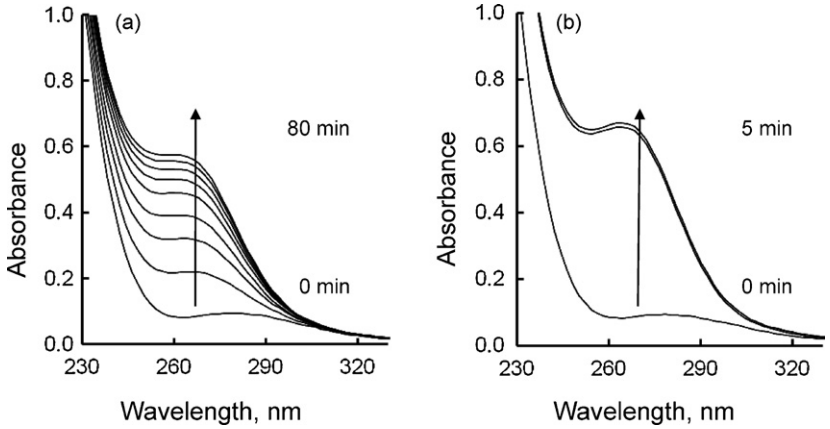

Fig. 2. UV spectra on monitoring of reduction of DHAA to AA with dithiothreitol. Initial concentration of DHAA: 8×10^{-5} M. [dithiothreitol] = 5 mM. Reaction temperature: (a) 20 °C and (b) 80 °C.

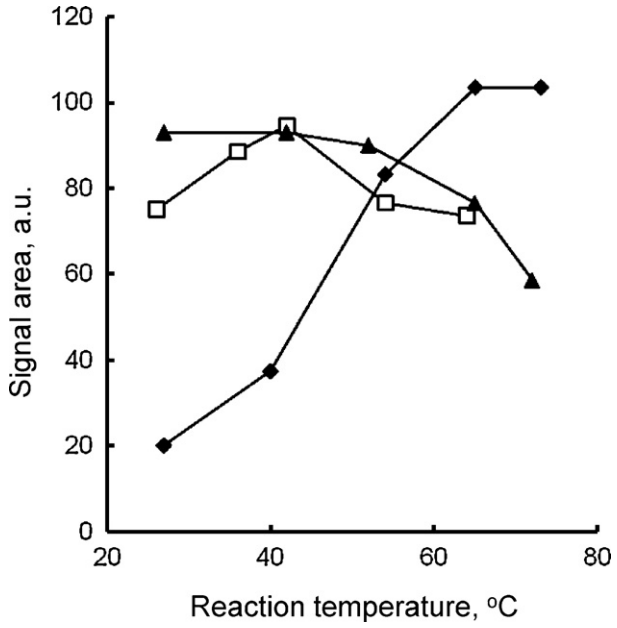

Fig. 3. Effect of reaction temperature on on-line reduction of DHAA to AA with dithiothreitol. FIA manifold shown in Fig. 1 without the RP-column was used. Coil length of the reaction tube: (■) 2 m; (□) 4 m; (▲) 8 m. Initial concentration of DHAA: 4.0×10^{-5} M. [dithiothreitol] = 5 mM. pH: 7.0.

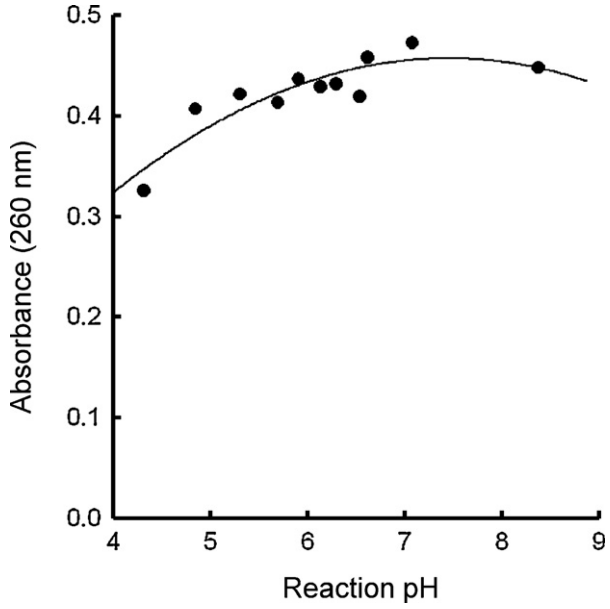

Fig. 4. Effect of pH on reduction of dehydroascorbic acid to ascorbic acid. Initial concentration of DHAA: 4.0×10^{-4} M. [dithiothreithol] = 5.0 mM. Temperature: 25 °C.

3. Results and discussion

3.1. Reduction of dehydroascorbic acid to ascorbic acid with dithiothreitol

When dehydroascorbic acid is to be detected photometrically, its weak light-absorptivity even at shorter UV region would be disadvantageous to realize sensitive detection. Reduction of dehydroascorbic acid to ascorbic acid helps the sensitive detection, and dithiothreitol (DTT) was used as a reducing agent in this study. Dithiothreitol was first developed as a reducing agent from dehydroascorbic acid to ascorbic acid by Okamura [13]. Since then, DTT has commonly used as a reducing agent of dehydroascorbic acid in pre-column and post-column reductions [14–23]. Okamura has shown the reduction conditions that DHAA is reduced to AA with DTT at the concentration of 10 mM within 10 min at room temperature [13]. To increase the sample throughput by the present FIA system, fast reduction of DHAA to AA was aimed in this study and the reaction temperature was examined in a volumetric flask. The phosphate buffer solution at neutral pH and the reducing solution containing DTT are mixed at 1:1 volume ratio, and dehydroascorbic acid was dissolved in it; the results are shown in Fig. 2. While it took longer reaction time at room temperature, the reaction can be completed within 5 min at 80 °C.

The effect of reaction temperature on the reduction of DHAA to AA was also examined in the flow injection analysis with different reaction tube length; the results are shown in Fig. 3. When the reaction tube is long, the reduction was completed at lower temperature, while higher temperature around 60–80 °C was required with 2 m reaction tube to complete the reduction from DHAA to AA. To realize short analysis time and high sample throughput by the present FIA, shorter tube length is desirable; the length of a reducing reaction coil was set at 2 m and the reaction temperature at 64 °C. A post-column reaction coil with its length of 2 m and cooling coil of 1 m was included in the flow injection manifold, as is shown in Fig. 1.

To monitor the effective reduction of DHAA to AA with DTT by the FIA system, UV absorbance at 260 nm was measured by a spectrophotometer at the pH range from 4.3 to 8.4. The results are shown in Fig. 4. Maximum absorbance attributed to ascorbic acid was obtained at pH conditions around 7.0 with ammonium acetate buffer, and effective reduction was performed. The concentration of dithiothreitol was also examined at the concentration range between 0.5 and 10 mM. The concentration of DTT at 5 mM was found to be enough.

3.2. Column separation of AA and DHAA

Simultaneous determination of AA and DHAA has been developed by Ziegler et al. by post-column reduction with DTT [27]. We aimed at simultaneous determinations of AA and DHAA by an FIA system, and we also adopted a separation column to realize fast and simple analysis. A reversed-phase column with aqueous carrier solution containing 0.025 M phosphate buffer (pH: 2.4) resolved AA and DHAA when operated at the flow rate of 0.5 mL min^{-1}. A reagent stream of DTT at the flow rate of 0.5 mL min^{-1} was merged to the carrier stream just after the reversed-phase column. The pH of the carrier solution was adjusted at 2.4 with the phosphate buffer, and pH of the reducing solution was set at 8.5 with 1 M ammonium acetate buffer; the merging of the two streams resulted in the reaction pH at 7.0. The concentration of DTT in the reducing solution was set at 10 mM, which resulted in its concentration of 5 mM at the merged stream.

3.3. Preparation of the standard solution of dehydroascorbic acid

We firstly used commercially available solid DHAA crystal to prepare the standard DHAA solution. This system adopts the on-line reduction of DHAA to AA, and therefore, signal areas for DHAA and AA obtained in chromatogram should be identical for the same concentrations of DHAA and AA. However, the signal assigned to DHAA was about 40% area compared to the AA at the same concentrations. Koshiishi and Imanari have also experienced such concentration drop and pointed out that DHAA solid cannot be used as a standard [28]. Koshiishi and Imanari corrected the concentration of the standard DHAA solution by comparing the signal intensity. Zorzi et al. also pointed out the difficulty on using DHAA standard [29]. Doner

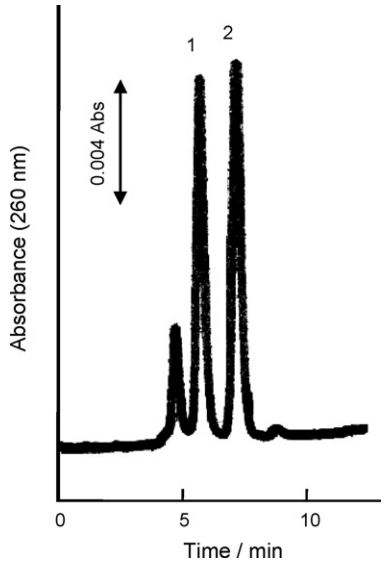

Fig. 5. Typical FIA signals for AA and DHAA. Sample solution: [DHAA] = 2.0×10^{-5} M. [AA] = 2.0×10^{-5} M. Signals: (1) DHAA (detected as AA) and (2) AA. Other conditions are shown in Fig. 1.

and Hicks [26], as well as Liu et al. [30], prepared DHAA standard solution from AA solution by oxidizing AA with activated charcoal. We also used activated charcoal to prepare standard DHAA solution from AA solution. Activated charcoal (Wako) was added to a known concentration of AA in an aqueous solution, and the solution was allowed to stand at room temperature. The oxidation of AA to DHAA was monitored by the present FIA system. Signal area corresponding to the AA got smaller and vanished along with the standing time, while the one corresponding to the DHAA increased. More than 97% of AA was converted to DHAA within 10 min, and the concentration of DHAA formed was almost identical to the initial AA concentration. The results suggested the complete oxidation to DHAA and adsorption of DHAA to the activated charcoal was very little. The DHAA concentration was kept constant in the reaction time of at least 40 min. Therefore, the reaction time with the activated charcoal was set at 20 min to prepare the standard DHAA solution.

3.4. FIA signals and calibration graphs for AA and DHAA at optimized conditions

Typical FIA signals for AA and DHAA are shown in Fig. 5. Two analytes have been well resolved and detected within 10 min. An injection signal (4.7 min) was observed precedent to the AA and DHAA signals. Linear calibration graphs with negligible blank signal were obtained in the concentration range from 0 to 8×10^{-5} M. Slopes and correlation coefficients for AA and DHAA are almost identical with each other; 17.8 (arbitrary unit) and $r^2 = 0.9999$ for AA, and 17.4 (arbitrary unit) and $r^2 = 0.9995$ for DHAA. Limit of detection corresponding to the signal to noise ratio of three was 1.7×10^{-6} M for both AA and DHAA.

Effect of coexisting substances was also examined. The substances examined were added against 2×10^{-5} M AA. More than 1000 mole ratio of succinic acid, DL-malic acid, malonic acid, citric acid (monohydrate), L-glutamic acid, L-cysteine, L-phenylalanine, glycine, D-glucose, Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, SO_4^{2-}, and CO_3^{2-} did not interfere with the determination of AA; the peak area obtained for AA were in the ranges of 95–105%.

3.5. Application to the determination of vitamin C in beverage drinks

The proposed method was applied to the determination of AA and DHAA in beverage samples. The sample solutions were diluted with the purified water and filtered through a disposable membrane filter as the need arose. Analytical results are summarized in Table 1. Total concentrations of AA and DHAA were close to the indicated values, and AA and DHAA were preferably determined. The proposed system is proved to be useful for the analysis of practical samples.

3.6. Application to the analysis of reaction dynamics in aqueous solution

It has been pointed out that ascorbic acid is not stable in aqueous solution, especially in alkaline solutions. Niemela investigated the degradation products from AA in aqueous alkaline media (pH: 8–12) by GC–MS coupled with the trimethylsilylization [31]. Iwase and Ono showed the stability of AA in 1% metaphosphoric acid solution, as well as in 100 mM KH_2PO_4 + 1 mM ethylenediamine tetraacetic acid solution [32]. In the present study, the reaction dynamics of AA and DHAA have been investigated.

Ascorbic acid was dissolved in pH-controlled water at the concentration of 4×10^{-5} M, and the solution was stirred gently under air exposure throughout at 25 °C. The solution was continuously stirred, and the dissolved oxygen (DO) was considered to be saturated with oxygen in ambient air at the amount of ~8 mg/L. A small portion of the solution was sampled at arbitrary intervals, and the concentrations of AA and DHAA were monitored by the present FIA system. The time–course assays of AA and DHAA are shown in Fig. 6. While AA was stable in acidic solution, the degradation of AA was much faster at neutral to alkaline pH solutions (Fig. 6b–d), as expected. The result agrees with the reaction [1] that dehydroascorbic acid is generated from the anionic species of AA (acid dissociation constant of AA: $pK_a = 4.17$, Merck Index). It can also be noted in Fig. 6 that DHAA was gradually generated along with the degradation of AA, and that DHAA was not generated in an alkaline solution as in Fig. 6d. Total concentration of AA and DHAA also decreased along with the stirring time, which should be attributed to the further hydrolysis to 2,3-diketogulonic acid. The results can be explained that the hydrolysis of DHAA to DKGA is much predominant in alkaline solutions. When the solution was bubbled with nitrogen gas and placed in a glove compartment

Table 1
Analytical results for practical beverage samples.

Beverage sample	[AA] ($\times 10^{-3}$ M[a])	[DHAA] ($\times 10^{-3}$ M[a])	Indicated value ($\times 10^{-3}$ M)	pH
Drink A	13.8 ± 0.2	N.D.[b]	11.3	–
Drink B	1.86 ± 0.04	N.D.[b]	2.07	–
Drink C	1.32 ± 0.01	0.037 ± 0.006	1.13	2.71
Drink D	5.61 ± 0.03	0.108 ± 0.003	4.05	3.71
Drink E	0.311 ± 0.002	0.055 ± 0.004	0.36	4.23
Drink F	0.984 ± 0.004	0.049 ± 0.002	0.81	2.58

[a] Uncertainity range: standard deviation of 5 measurements.
[b] Not detected.

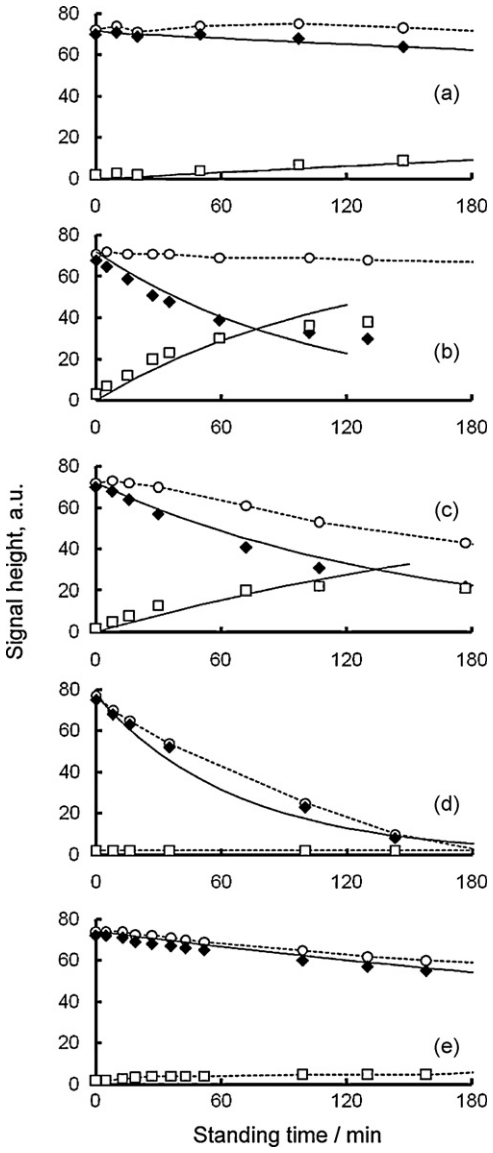

Fig. 6. Reaction dynamics of AA and DHAA in aqueous solution at several pH conditions.
Initial concentration of AA: 4×10^{-5} M. pH of the AA solution: (a) 3.40; (b) 5.27; (c) 7.00; (d) 8.90; (e) 8.99 (bubbled and purged with nitrogen gas).
(♦) AA; (□) DHAA; (○) AA + DHAA.

purged with nitrogen gas, the decomposition of AA was much suppressed (Fig. 6e). These results indicate that AA is oxidized by the dissolved oxygen. Slight decrease in AA at O_2-bubbled condition would be attributed to the radiation of light. On the other hand, the generation of DHAA was little, because the hydrolysis of DHAA to 2,3-diketogulonic acid would not have been suppressed at the similar pH conditions.

Rate constants of the decrease in AA and the increase in DHAA have been evaluated in this study. Simple reactions were assumed that DHAA is generated from AA and that DKGA is further generated from DHAA as in reaction (1). Since the reactions proceeded under oxidative conditions in this study, a reverse reaction from DHAA to AA with its rate constant, k_{-1}, is considered to be negligible.

$$\mathrm{AA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{DHAA} \xrightarrow{k_2} \mathrm{DKGA} \tag{1}$$

A first-order reaction rate of k_1 on the basis of the decrease in AA, k_{1AA}, is written in Eqs. (2) and (3), using initial concentration of AA, $[AA]_0$, and its concentration [AA] at the reaction time, t (s).

$$-\frac{\mathrm{d[AA]}}{\mathrm{d}t} = k_{1AA}\,[AA] \tag{2}$$

$$\ln \frac{[AA]}{[AA]_0} = -\,k_{1AA}\,t \tag{3}$$

By plotting left-hand member of Eq. (3) vs. t, k_{1AA} was determined from the slope by a linear analysis.

Noticing the generation of DHAA, a first-order reaction rate on the basis of the increase in DHAA, k_{1DHAA}, is written in Eqs. (4) and (5), using initial concentration of AA, $[AA]_0$, and the concentration of DHAA, [DHAA], at the reaction time, t (s). In Eq. (4), formation of DKGA is omitted for the simplification of the analysis.

$$\frac{\mathrm{d[DHAA]}}{\mathrm{d}t} = k_{1DHAA}([AA]_0 - [DHAA]) \tag{4}$$

$$\ln\left(1 - \frac{[DHAA]}{[AA]_0}\right) = k_{1DHAA}\,t \tag{5}$$

A rate constant, k_{1DHAA}, was also determined through a linear analysis as the same manner in the determination of k_{1AA}. The rate constants, k_{1AA} and k_{1DHAA}, have been determined at several pH conditions, and they are summarized in Table 2. Solid curves in Fig. 6 are drawn using the rate constants. At weakly acidic pH conditions (Fig. 6a and b), the total concentration of AA and DHAA are almost identical in the time range examined, and therefore, the decomposition rate of AA (k_{1AA}) is almost equal to the formation rate of DHAA (k_{1DHAA}). The rate constant, k_1, is smaller at much acidic solutions, because of the small fraction of the anionic ascorbate ion [1]. Hughes has already evaluated the irreversible oxidation kinetics and determined the oxidation rate constant from AA to DHAA at higher temperature [33]. The rate constants determined by Hughes are smaller than our study; it would be attributed to the acidic condition at pH 2.6.

The rate constants between k_{1AA} and k_{1DHAA}, however, do not agree with each other at more alkaline pH conditions. The result is attributed to the formation of DKGA at alkaline conditions, and the hydrolysis should be considered on the analysis of (k_{1DHAA}). Unfortunately, generation of DKGA was not monitored in this study, and the contribution of the hydrolysis reaction from DHAA to DKGA is difficult to evaluate simultaneously.

Table 2
Reaction kinetics for ascorbic acid in an aqueous solution.

pH condition	Rate constants[a]	
	k_{1AA} ($\times 10^{-4}$ s^{-1})	k_{1DHAA} ($\times 10^{-4}$ s^{-1})
3.40	0.12 ± 0.01	0.12 ± 0.01
5.27	1.60 ± 0.05	1.42 ± 0.09
7.00	1.08 ± 0.03	(0.68 ± 0.07)[b]
8.90	2.48 ± 0.21	–[c]
8.99[d]	0.29 ± 0.01	–[c]

[a] Uncertainty range: standard error.
[b] Reference value because the hydrolysis reaction of DHAA accompanied.
[c] The formation of DHAA was too little to determine.
[d] The solution was bubbled and purged with nitrogen gas.

4. Conclusions

Simultaneous determination of ascorbic acid and dehydroascorbic acid has been optimized in this study by using a reversed-phase column and on-line reduction of DHAA with dithiothreitol. Standard solution of DHAA was prepared from AA with the activated charcoal, and almost identical sensitivity was obtained for DHAA and AA. Ascorbic acid and dehydroascorbic acid in beverage samples have been determined with satisfactory results. The reaction dynamics of AA and DHAA in aqueous solution was also moni-

tored at several pH conditions, and the conditional rate constants have been determined. The proposed system would be useful for the analysis of total dynamics of AA and DHAA in solutions and in biological samples.

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