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A KINETIC METHOD FOR THE DETERMINATION OF ASCORBIC ACID WITH 2,6-DICHLOROPHENOLINDOPHENOL

Keitaro HIROMI, Haruko FUJIMORI, Junko YAMAGUCHI-ITO,
Hiroshi NAKATANI, Masatake OHNISHI, and Ben'ichiro TONOMURA
Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Kyoto, Kyoto 606

A kinetic method for the determination of ascorbic acid is described. The method is based on the linear relationship, in a wide range, between the apparent first-order rate constant, k_{app}, of the reduction of 2,6-dichlorophenolindophenol by ascorbic acid and the concentration of ascorbic acid. The method is sensitive, specific, and applicable to turbid or colored samples.

The titration of ascorbic acid with 2,6-dichlorophenolindophenol (DCIP) has been widely used as one of the standard methods for the determination of the reduced form of ascorbic acid because of its simpleness and convenience (1,2). Drawbacks of the conventional procedure, however, are its nonspecificity (other reducing substances interfere with the determination) and susceptibility to the interference of turbidity and color of samples.

We have been successfully using, as a test reaction for the performance of stopped-flow apparatuses, the reduction of DCIP by ascorbic acid (3), for which the apparent first-order rate constant, k_{app}, is proportional to the ascorbic acid concentration in a wide range. In this paper we aimed to make use of this linear relationship for the determination of ascorbic acid.

The reaction obeys apparently a second-order kinetics (3). Let A and D respectively represent ascorbic acid and DCIP, and k, the second-order rate constant. When ascorbic acid is in large excess of DCIP, we have:

$$-d[D]/dt = k[A][D] = k_{app}[D] \quad (1)$$

where k_{app} is the apparent first-order rate constant given by

$$k_{app} = k[A]_0 \quad (2)$$

where $[A]_0$ is the initial concentration of ascorbic acid, which is to be determined. Since the value of the second-order rate constant, k, has been determined at various pH's at 25°C (3), the measurement of k_{app} enables us to obtain the concentration of ascorbic acid, $[A]_0$. The initial

concentration of the dye, $[D]_0$, need not accurately be known, so far as it is sufficiently lower than $[A]_0$. This is in marked contrast to the conventional titration method, in which the accuracy of the dye concentration is of critical importance.

Our previous study on the pH dependence of the rate constant, k, of the same reaction (3) suggests that it is convenient to use pH plateaux in the acidic and the neutral region: pH 2-3 for fast reactions and pH 7.5-8.5 for slow reactions, k being about 180 times larger at the acidic pH than at the neutral. A stopped-flow apparatus is most suitable for this kinetic measurement, but an ordinary spectrophotometer can also be used at the neutral pH; under these conditions the half-time of the reaction, $t_{1/2}$, are 430 and 22 sec for ascorbic acid concentrations of 5 and 100 μM, respectively.

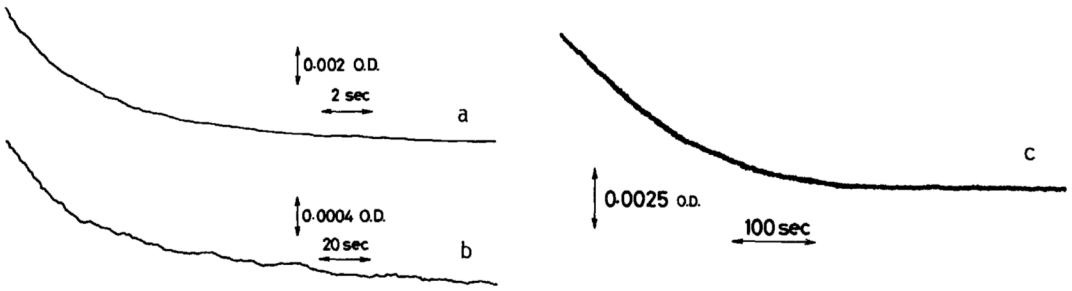


Fig. 1. Typical reaction curves of the reduction of DCIP by ascorbic acid.
(a) and (b): Obtained with a stopped-flow apparatus (Union Giken RA-401). 10 mm cell, 524 nm. pH 2.6, 25°C. (a): $[A]_0$ = 5 μM, $[D]_0$ = 1 μM. (b): $[A]_0$ = 0.5 μM, $[D]_0$ = 0.2 μM. (c): Obtained with an ordinary spectrophotometer (Union Giken SM-401), 10 mm cell, 524 nm. pH 7.2, 25°C. $[A]_0$ = 7.4 μM, $[D]_0$ = 1.24 μM.

Figure 1 shows typical examples of reaction curves obtained with a stopped-flow apparatus (Union Giken RA-401) at pH 2.6 and with a spectrophotometer (Union Giken SM-401) at pH 7.2. The proportionality between k_{app} and $[A]_0$ is excellent in the range of $[A]_0$ = 0.5 μM - 50 mM, as shown in Fig. 2. The lower limit, 0.5 μM, is about 1/10 of the lowest level usually obtained with the conventional methods, showing thus high sensitivity of the present method.

The relative values of k were determined for some reducing substances that often coexist with ascorbic acid: D-araboascorbic acid, triose reductone, cysteine, and glutathione (Table 1). D-araboascorbic acid can not be distinguished from L-ascorbic acid under the conditions tested. However, triose reductone, which is difficult to be determined separately from ascorbic acid with the conventional methods, can readily be discriminated through its lower value of k (about 1/20 of that of ascorbic acid). This difference in k values should effectively be used for the separate determination of the two compounds that frequently coexist in foods. Further studies on this line are now in progress. The separate determination of ascorbic acid from cysteine and glutathione

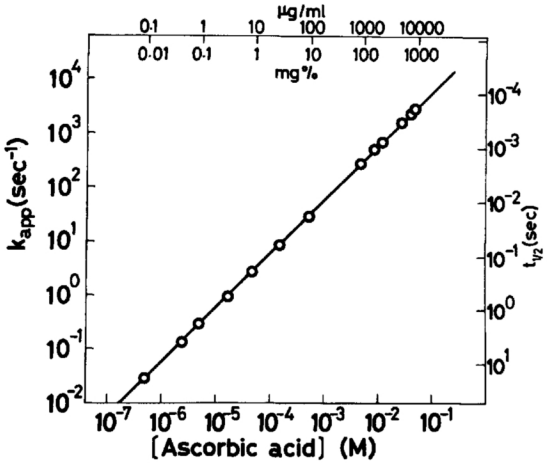


Fig. 2. Proportionality between k_{app} and ascorbic acid concentration.
The rate constant, k_{app}, was obtained with a stopped-flow apparatus (Union Giken RA-401). pH, 2.6, 25°C.

should be easily done even with the conventional static titration method, since the k values for the latters are much smaller.

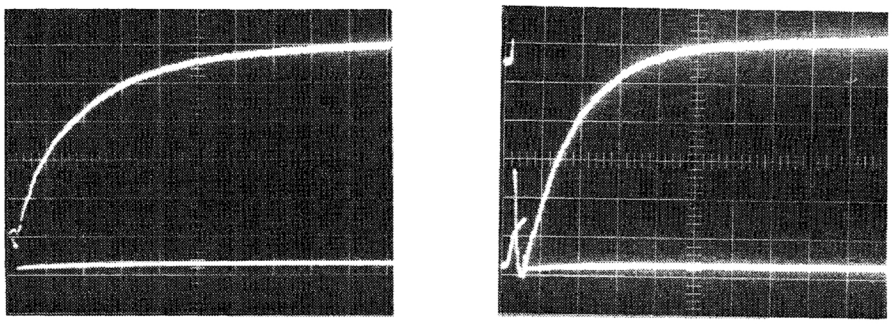
Table 1. Relative Rate Constants for DCIP Reduction

Compound	Relative Rate Constant
L-Ascorbic acid	1
D-Araboascorbic acid	1.0
Triose reductone	0.042
Cysteine	0.001
Glutathione	0.0003

pH 2.6, 25°C.

Figure 3 shows typical examples, obtained with a stopped-flow apparatus (Union Giken SF-70), of the time courses of the reduction of DCIP by ascorbic acid in food materials. Table 2 includes the rate constant, k_{app}, and the concentration of ascorbic acid of turbid and colored samples determined based on the relationship of Eq. 2 with $k = 5.2 \times 10^4$ $M^{-1}sec^{-1}$ at pH 2.6, 25°C (3).

The characteristics of the present kinetic method for the determination of ascorbic acid with DCIP are as follows: 1) The procedure is simple and rapid. 2) High sensitivity, the detection limit is about 0.5 μM with a stopped-flow apparatus and 5 μM with an ordinary spectrophotometer. 3) Applicable to a wide range of ascorbic acid concentration (0.5 μM - 50 mM). 4) More specific than the conventional static method, especially useful for discrimination between ascorbic acid and triose reductone. 5) The interference by turbidity and other color is much less than with the conventional static method.



(a) (b)

Fig. 3. Stopped-flow traces obtained with food samples.

pH 2.6, 25°C. 10 mm cell, 524 nm. Union Giken SF-70. (a): Beer. $[D]_0$ = 5 μM. Vertical scale, 0.0056 O.D. per major division. Horizontal scale, 200 msec per major division. (b): Tomato juice. $[D]_0$ = 5 μM. Vertical scale, 0.0056 O.D. per major division. Horizontal scale, 100 msec per major division. Refer also to Table 2.

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Table 2. Ascorbic Acid Determination[a] in Colored and Turbid Samples

Sample[b]	k_{app} (sec^{-1})	$[A]_0$ (before mix.) (μM)	$[A]_0$ (before mix.) (μg/ml)
Orange juice[c]	21.4	822	145
Green tea[d]	10.4	399	70.2
Tomato juice[e]	8.4	232	40.8
Beer[f]	3.8	146	25.7

a) Stopped-flow method. pH 2.6, 25°C. k = 52000 $M^{-1}sec^{-1}$. $[DCIP]_0$ = 5 μM (after mix.) (OD_{524nm} = 0.0335). b) Samples were filtered through filter paper, and adjusted to pH 2.6 with HCl. Apparent $OD_{524nm} \simeq 0.1$. c) Commercial, canned. d) 6g/200ml water. Percolated after keeping at 80°C for 90sec. e) Commercial, canned. f) Degassed before mixing.

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