Flow Injection Analysis of Oxalate in Foods Using Titanium(IV)-Porphyrin Reagent

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A rapid and sensitive flow injection analysis (FIA) with spectrophotometric detection by a Ti(IV)-porphyrin reagent is described for the determination of oxalate in foods using an immobilized oxalate oxidase reactor to yield hydrogen peroxide. The reagent is an aqueous solution of oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), $\text{TiO}(\text{tpypH}_4)^{4+}$, which reacts with hydrogen peroxide to form a monoperoxo complex, $\text{TiO}_2(\text{tpypH}_4)^{4+}$, having an absorption peak at 450 nm. The absorbance was proportional to the concentration of hydrogen peroxide. The flow-injection manifold comprised a two-channel system with an immobilized oxalate oxidase column. A linear relation was observed between the absorbance and the oxalate concentration ranging from 5×10^{-7} to 2.5×10^{-4} M ($r=0.999$), and 25 samples could be analyzed in one hour. The relative standard deviation for ten replicate measurements was 0.48% at 2.5×10^{-5} M (500 pmol/injection). The application of this system for the determination of oxalate in foods gave satisfactory results. A good correlation ($r=0.996$) was seen between the results obtained by the proposed method and by the conventional method using a commercial F-kit.

Keywords Oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), oxalate determination, immobilized oxalate oxidase, flow injection analysis, hydrogen peroxide, food, Ti(IV)-porphyrin reagent

Oxalate exerts undesirable effects in food-manufacturing processes, because it adversely affects the taste of food and forms insoluble salts with calcium ion present in water, which gives rise to turbidity in beer and fruit juices.¹ Oxalate is undesirable for health reasons, and also because it causes a renal calculus.² Although it is prescribed to be removed in food manufacturing, it may be used as a food additive.³ Therefore, the determination of oxalate is important in the field of food chemistry and a sensitive, simple and rapid method is required for measuring oxalate in foods.

The most common methods for the determination of oxalate in foods are titration by potassium permanganate⁴, ion chromatography⁵, gas chromatography⁶ and enzymatic analysis.⁷ Analytical methods using enzymes are also becoming popular because of their high selectivity. These methods are based on the detection of hydrogen peroxide or β -nicotinamide-adenine dinucleotide, reduced (NADH) produced in a reaction using the respective enzymes. Recently, a method for measuring the absorbance of NADH produced in a reaction by dehydrogenase has been marketed as a kit.⁸ However, the results from the NADH method are affected by reducible substances in food, and the assay procedure is complicated. On the other hand, in methods used to determine the hydrogen peroxide produced from oxalate, the chemiluminescence

of luminol or spectrophotometry with chromogens using peroxidase is generally used.⁹ However, the results from these methods are considerably affected by reducible substances, since they are based on oxidative reactions of the fluorophores or chromophores with hydrogen peroxide using peroxide, which has a low selectivity for hydrogen donors.

A Ti-TPyP (denoted as Ti(IV)-porphyrin) reagent was recently developed for the spectrophotometric determination of trace amounts of hydrogen peroxide.¹⁰ The Ti-TPyP reagent, an aqueous solution containing oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), and $\text{TiO}(\text{tpypH}_4)^{4+}$, reacts with hydrogen peroxide to form monoperoxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), $\text{TiO}_2(\text{tpypH}_4)^{4+}$, exhibiting a maximum absorbance at 450 nm, viz. the Soret band. A highly sensitive method using the Ti-TPyP reagent for the coloration of hydrogen peroxide yielded a 10-times larger absorbance than did that of peroxidase methods using an aminoantipyrin-phenol solution. Further, this reagent has the advantage that the reagent is almost unaffected by reducible substances, because of a coloration reaction based on complexation. Thus, a sensitive flow injection analysis (FIA) using the Ti-TPyP reagent was developed for the determination of trace amounts of hydrogen peroxide.¹¹

In this paper, a simple FIA incorporating spectro-

photometric detection using the Ti-TPyP reagent is described for the determination of oxalate in foods, using an immobilized oxalate oxidase reactor.

Experimental

Reagents

Oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), denoted as a TiO(tpyp) complex, was obtained from Tokyo Kasei Kogyo Co. The Ti-TPyP reagent (3×10^{-5} M) was prepared by dissolving 10.0 mg of the TiO(tpyp) complex in 2 ml of 5 M hydrochloric acid and adding 1 M sulfuric acid up to 500 ml. A standard oxalate solution (1×10^{-3} M) was prepared by dissolving 13.4 mg of sodium oxalate in 100 ml water. CNBr-activated Sepharose 4B was obtained from Pharmacia LKB. Oxalate oxidase (from barley seedlings) was obtained from Boehringer Mannheim. All other chemicals were of analytical grade. Distilled and deionized water was used for all of the procedures.

Preparation of the immobilized enzyme column

CNBr-activated Sepharose (0.2 g dry weight) was swollen in 1 mM HCl for 10 min and washed with a 0.1 M phosphate buffer (pH 7.0) on a sintered-glass filter (G-3). The gel was allowed to react with oxalate oxidase (7 U) in 1 ml of 0.1 M phosphate buffer (pH 7.0) overnight at 4°C, and then washed with a 0.1 M phosphate buffer (pH 7.0). The enzyme-bearing Sepharose was packed in a Teflon tube (3 cm long, 2 mm i.d.). The immobilized enzyme column could be used continually for more than 200 runs over a period of 8 h. When stored at 4°C in a refrigerator, no significant decrease in activity on the column was observed, even after 6 months.

Flow system

A schematic diagram of the flow system is shown in Fig. 1. The system comprised a pump (Sanuki Kogyo, DMX-2300T) with two channels, an injector (Rheodyne, Model 8125) equipped with a 20 µl sample loop, an immobilized oxalate oxidase column (EC: 3 cm long, 2 mm i.d.), a mixing coil (MC: 15 m long, 0.5 mm i.d.) and a spectrophotometer (D: Jasco Model UV 970) with a flow cell. The carrier solution (CS) was a 0.05 M succinate buffer (pH 3.0). The flow rates of the carrier solution and the Ti-TPyP reagent (RS) were both 0.4 ml/min. The volume of the sample loop was 20 µl, which was injected into the carrier stream through the sample injector. The sample solution, injected into the carrier solution, passed through the immobilized oxalate oxidase column to produce hydrogen peroxide. The produced hydrogen peroxide was reacted with the Ti-TPyP reagent to form a peroxo complex in the mixing coil. The absorbance of the peroxo complex was monitored at 450 nm in the spectrophotometer, and recorded as the peak height.

Fig. 1 Flow diagram of the FIA system for oxalate determination using an immobilized oxalate oxidase column reactor. CS, 0.05 M succinate buffer (pH 3.0); RS, 30 µM Ti-TPyP reagent; S, 6-way sampling valve (20 µl); T, thermostat bath (75°); MC, mixing coil (0.5 mm i.d., 15 m long); D, spectrophotometer (450 nm).

Preparation of sample

Working solutions were prepared by the following procedure.⁸ Approximately 20 g of homogenized fruits and vegetables were weighed out accurately, and 60 ml of distilled water was added. After adjusting to pH 2–3 with hydrochloric acid, the solutions were incubated at 50°C for 15 min with occasional shaking. After cooling to room temperature and adjusting to pH 3.0 with potassium hydroxide, the mixtures were transferred to 100 ml volumetric flasks, and distilled water was added to 100 ml. The mixtures were passed through membrane filters (0.45 µm) to form the working solutions. Samples containing carbonates, such as beer and so on, were filtrated using a membrane filter (0.45 µm), and then degassed to form working solutions. Millet jelly (10 g) was dissolved in hot distilled water, then transferred to a 25 ml volumetric flask. After filling up to the mark with distilled water, the solution was used for the assay.

Results and Discussion

Ti-TPyP reagent for determination of hydrogen peroxide

In a previous paper it was shown that the Ti-TPyP reagent could be used to detect trace amounts of hydrogen peroxide.¹⁰ The reagent is $\text{TiO}(\text{tpypH}_4)^{4+}$, which reacts with hydrogen peroxide to form a monoperoxo complex, $\text{TiO}_2(\text{tpypH}_4)^{4+}$, as follows:

After the addition of hydrogen peroxide to the Ti-TPyP reagent, the absorption spectrum of the acidic solution showed a sharp peak at 450 nm due to the formation of a monoperoxo complex. The absorbance at 450 nm was proportional to the concentration of hydrogen peroxide. The apparent molar absorptivity was $1.1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. Recently, a sensitive FIA using the Ti-TPyP reagent was developed for the determination of hydrogen peroxide, ranging from 0.5 to

1000 pmol/test.¹¹ The Ti-TPyP reagent also appeared to be useful in the FIA determination of trace amounts of oxalate, in combination with the oxalate oxidase column to form hydrogen peroxide, as follows:

Effect of acid on the formation of the peroxo complex

The use of a strong acid was found to enhance peroxo complex formation.¹⁰ Although in previous work a maximum absorbance was attained with 1.6 M perchloric acid in the reagent, the addition of high concentrations of acid caused a precipitation of the protein. Thus, in this work, sulfuric acid, which scarcely affects protein precipitation, was used to enhance the rate of formation of the peroxo complex by the Ti-TPyP reagent. The effect of the sulfuric acid concentration on the peak height was examined while maintaining constant concentrations of the Ti-TPyP reagent and hydrogen peroxide. As shown in Fig. 2, the peak height increased along with an increase in the concentration of sulfuric acid, ranging from 0.4 to 1.2 M. Because the instrument may be damaged by a high concentration of sulfuric acid, its concentration in the Ti-TPyP reagent was fixed at 1 M.

Optimization of the measurement conditions

To determine what conditions would be appropriate for the reagent to function in an FIA system equipped with an oxalate oxidase column, the effects of the pH, the length of the column, and the flow rate on the absorbance were examined.

As shown in Fig. 3, a constant peak height was obtained in the pH 2.8 to 3.6 range when using the succinate buffer, in which oxalate oxidase shows activity.¹² Thus, a 0.05 M succinate buffer (pH 3.0) was selected as the carrier solution.

The effect of the flow rate on the peak height was studied next. A decrease in the flow rate resulted in progressive increases in both the peak height and the width. Thus, a flow rate of 0.4 ml/min was selected in order to obtain a reasonable sampling number (25 samples) measured over a period of one hour.

The influence of the length of the column of immobilized oxalate oxidase was investigated at a flow rate of 0.4 ml/min. As shown in Fig. 4, the greatest and most constant peak heights were obtained with columns 3 cm in length or longer; the reactor was thus fitted with the shortest 3 cm column in order to avoid sample scattering.

The above mentioned method used a very small amount of sample because of the high sensitivity of the reaction with the Ti-TPyP reagent.

Under these conditions, the FIA signals obtained for oxalate standard solutions are shown in Fig. 5. An excellent linearity between the peak height and the concentration of oxalate was obtained, $y=0.224x+0.352$, $r=0.999$ (y and x being the peak height and molar concentration of oxalate, respectively), in the range 0.5 to

Fig. 2 Effect of the sulfuric acid concentration in the reagent solution on the formation of the peroxocomplex. The flow rate of each line in this system was set to be 0.4 ml/min. Hydrogen peroxide concentration, 100 μ M; carrier solution, distilled water; reagent solution, 30 μ M Ti-TPyP solution.

Fig. 3 Effect of carrier solution pH on peak height. The flow rate of each line in this system was set to be 0.4 ml/min. Oxalate concentration, 100 μ M; carrier solution, 0.05 M succinate buffer (pH 3.2); column length, 3 cm $\times$ 2 mm i.d.

250 μ M oxalate (10 – 5000 pmol per 20 μ l injection). The relative standard deviation for replicate runs ($n=10$) was 0.48% at a concentration of 25 μ M oxalate.

The effects of certain foreign substances present in foods were also examined; the results are summarized in Table 1. Inorganic ions, such as Na^+ , K^+ , and Mg^{2+} , had very little effect, even when present at several thousands times the concentration of oxalate in the sample. However, a decrease in the absorbance was found with the Fe^{3+} ion present at one part per forty parts of oxalate. The presence of the Fe^{3+} ion might possibly catalyze the decomposition of the produced hydrogen peroxide.

Fig. 4 Effect of enzyme column length on peak height. Oxalate concentration, 100 μ M; carrier solution, 0.05 M succinate buffer (pH 3.0); other conditions as in Fig. 3.

Fig. 5 Typical response curve for oxalate. The injection volume of each sample was 20 μ l. a, 5 μ M; b, 10 μ M; c, 20 μ M; d, 50 μ M; e, 100 μ M.

This interference was avoided by including a precolumn containing a cation-exchanging resin. Various organic acids (except for ascorbate) had no significant effects on the oxalate assay. Ascorbate present below one part per one hundred parts oxalate caused an increase in the absorbance, since ascorbate was oxidized by the catalytic action of the Ti-TPyP reagent at 75°C to produce hydrogen peroxide. However, ascorbate was removed by the simple addition of ascorbate oxidase to the sample prior to injection.

Table 1 Effects of foreign substances on the determination of oxalate

Compound	Conc. added in sample/M	Oxalate found ^a , %
NaCl	1.0×10^{-1}	96.1
CaCl ₂	5.0×10^{-4}	103.9
FeCl ₃	2.5×10^{-6}	97.2
Glucose	5.0×10^{-1}	102.6
Fructose	2.5×10^{-1}	101.8
Maltose	5.0×10^{-4}	102.6
Sucrose	2.5×10^{-1}	100.2
Malic acid	1.0×10^{-1}	101.4
Citric acid	2.5×10^{-2}	98.6
Formic acid	1.0×10^{-1}	102.1
Tartaric acid	5.0×10^{-1}	98.5
Malonic acid	1.0×10^{-2}	97.3
Ascorbic acid	1.0×10^{-6}	104.4
Riboflavin	5.0×10^{-5}	104.3

a. Oxalate added, 1.00×10^{-4} M.

Determination of oxalate in food samples

The proposed method was used to determine the oxalate content of various foods, such as vegetables, beverages, and fruits. For the calculation of the percentage recovery of oxalate, spiked food sample solutions were prepared as follows: a standard solution containing 0.9 mg oxalate was added to 100 g or 100 ml of food samples prepared by the procedure described above, and the absorbances of the solutions were measured as peak height. As shown in Tables 2 and 3, the recovery of oxalate was in the range 97.7 to 103.0%. A good correlation was obtained between the results of food oxalate determination by the proposed method and by a conventional method with an F-kit currently on the market (Tables 2 and 3).

The selectivity, precision and rapidity in our oxalate assay method could be improved compared to other methods.⁸

In this study, a detection method using the Ti-TPyP reagent to determine hydrogen peroxide proved to be useful for a flow injection analysis of the oxalate content of food.

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Table 2 Oxalate concentration in vegetables and fruits and recovery of added oxalate by the present method and the F-kit

Sample	Proposed method		F-kit
	Oxalate (mg/100 g)	Recovery ^a (%)	Oxalate (mg/100 g)
Spinach	515	101.9	540
Japanese radish	1.46	103.0	
Ginger	203	99.3	177
Japanese ginger	225	101.8	206
Kiwi fruit	4.50	100.0	
Persimmon	2.61	99.0	
Millet jelly	4.54	101.3	

a. Oxalate added 9.00×10^{-4} g/l.

Table 3 Oxalate concentration in beverages and recovery of added oxalate by the present method and the F-kit

Sample	Proposed method		F-kit
	Oxalate (mg/l)	Recovery ^a (%)	Oxalate (mg/l)
Beer 1	10.4	97.7	
Beer 2	3.29	97.7	
Beer 3	7.56	100.6	
Beer 4	18.0	102.0	
Beer 5	9.72	103.0	
Beer 6	18.9	104.0	20.8
Apple juice	4.77	100.8	
Orange juice	1.80	101.5	
Tomato juice	42.3	97.7	
Carrot juice	21.9	98.6	15.1

a. Oxalate added 9.00×10^{-4} g/l.

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