Application of a New Iron Reagent, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine, to Spectrophotometric Determination of Tocopherols

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Tsen's modification of Emmerie-Engel method was further improved by applying 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT) in place of bathophenanthroline as iron reagent. The reaction mixture of PDT, ferric chloride and tocopherol gave a spectrum with the maximum absorption at 554 nm. The sensitivity of PDT was about 1.2-fold of bathophenanthroline for each tocopherol. The rate of color development of PDT was fast similarly to bathophenanthroline. The calibration curves obtained for α -, β -, γ - and δ -tocopherols by the method using PDT followed Beer's law. The present method was much more economical than the bathophenanthroline method.

The procedure for analysis of tocopherols in biological materials, foods, and feedstuffs includes generally four steps; extraction of the lipid containing tocopherols, removal of interfering substances, separation of individual tocopherols, and determination of the tocopherols separated. For the last step spectrophotometric measurement has been most widely used.

Emmerie-Engel method¹⁾ in which α,α' -dipyridyl and ferric chloride are used has been considered to be the most suitable for the spectrophotometric determination of purified tocopherols. The method is based on the determination of the color formed from α,α' -dipyridyl with the ferrous ions reduced by tocopherols. By substituting bathophenanthroline, 4,7-diphenyl-1, 10-phenanthroline, for α,α' -dipyridyl, the original method was successfully improved by Tsen.²⁾ Bathophenanthroline increased the sensitivity of the original method by approximately 2.5-fold and has been the best known iron reagent at the present time.

Recently, more excellent iron reagent, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT), has been synthesized by Case³⁾ and its sulfate, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), was applied to determine iron in water with a higher sensitivity and a lower cost than those of bathophenanthroline by Stookey.⁴⁾ We have tried to apply PDT

to the determination of tocopherols and established a method in which PDT is substituted for bathophenanthroline.

MATERIALS AND METHODS

Chemicals. Bathophenanthroline and α,α' -dipyridyl were purchased from the Wako Pure Chemical Co., Tokyo. Hydrazine and 2-cyanopyridine were purchased from the Tokyo Kasei Kogyo Co. Benzil was obtained from the Nakarai Chemical Ltd., Kyoto. DL- α -Tocopherol, DL- β -tocopherol, D- γ -tocopherol and D- δ -tocopherol were gifted from the Institute of Eisai Co., Tokyo. Their purities analyzed by the supplier were 98, 95, 93 and 96%, respectively. By the ferric chloride-bathophenanthroline reaction, the respective tocopherol was confirmed to give a monospot on thin-layer chromatography. Absolute ethanol was purified in an usual way.

PDT was synthesized and purified according to the method of Case³⁾. The result of the elemental analysis showed 18.14%, 77.36% and 4.50% for N, C and H, respectively, in agreement with the values calculated for $C_{20}H_{14}N_4$ (C, 77.42; N, 18.03; H, 4.55%). Melting point measured by a Micro Melting Point Apparatus (Yanagimoto Seisakusho, Tokyo) was within 189.5~190.5°C in concordance with that reported by Case.³⁾

Reagents. Solution of ferric chloride, $FeCl_3 \cdot 6H_2O$, (0.1%, w/v), was prepared in absolute ethanol in an amber bottle prior to use.

All iron reagents were dissolved in absolute ethanol and stored in a refrigerator. The concentration was 0.5% (w/v) for bathophenanthroline and α,α' -dipyridyl

and 0.1% (w/v) for PDT.

The standard tocopherol solutions were prepared by the appropriate dilution of the stock tocopherol solutions (0.1%, w/v) to the desired concentration with absolute ethanol and refrigerated.

General procedures. A desired amount of the standard tocopherol solution was added into a 10 ml test tube and the volume was made up to 3.0 ml with absolute ethanol. Then 0.4 ml of iron reagent was added and the content was gently mixed. Under dim artificial light in a darkened room, 0.4 ml of ferric chloride solution was added and the content was immediately gently swirled. At the end of a selected time, 0.2 ml of 0.2 M orthophosphoric acid in absolute ethanol was added and mixed. After allowance to stand for about 30 min in an ordinary laboratory, the absorbance of the solution was measured with a Hitachi-Perkin Elmer 139 UV-VIS spectrophotometer at the wavelength selected for each iron reagent against absolute ethanol, using 1 cm cells. The blank was prepared in the same manner except that absolute ethanol was used instead of the standard tocopherol solution.

RESULTS AND DISCUSSION

Sensitivity and maximum absorption

The sensitivities of iron reagents in determination of α -tocopherol are compared in Table I. Among iron reagents tested, PDT was more sensitive by 1.21 times and 3.0 times than bathophenanthroline and α, α' -dipyridyl, respectively. The absorption spectrum of the $\text{Fe}(\text{PDT})_3^{2+}$ complex⁴⁾ showed a single sharp peak with the maximum absorbance at 554 nm. Almost identical spectra with the same maximum absorbance were obtained for β -, γ - and δ -tocopherols.

TABLE I. COMPARISON OF SENSITIVITIES OF DIFFERENT IRON REAGENTS USED FOR α -TOCOPHEROL DETERMINATION

The concentration of α -tocopherol was 30.0 μg per 4 ml. α, α' -Dipyridyl and bathophenanthroline were applied to the original Emmerie-Engel method and its modified method, respectively.

Iron reagent	λ_{max} (nm)	$E_{1\text{cm}}^{1\%}$	Sensitivity
α, α' -Dipyridyl	520	392	0.40
Bathophenanthroline	534	977	1.00
PDT	554	1180	1.21

Optimum PDT concentration

The effect of concentration of PDT on the

FIG. 1. The Effect of PDT Concentration on the Color Development of α - and δ -Tocopherols.

The numerals in the figure indicate the concentration (μg per 4 ml) of tocopherols. The reaction was stopped by adding orthophosphoric acid at specified time after the addition of ferric chloride under dim artificial light in a darkened room.

maximum color intensity was measured with α - and δ -tocopherols. The optimal concentration of PDT changed with the concentration and the sort of tocopherol (Fig. 1). The minimum concentration of PDT necessary for the quantitative determination was approximately 7 times (at molar ratio) of the concentration of α -tocopherol present and 10 times of that of δ -tocopherol. From the results of Fig. 1, 0.1% was selected as the concentration of PDT suitable for the quantitative determination of tocopherols. This concentration is about one-fifth of that of bathophenanthroline.⁶⁾ PDT seems to have a more excellent combining activity with ferrous ions than bathophenanthroline.

Rate of color development

The rates of color development for α -, β -, γ - and δ -tocopherols are indicated in Fig. 2. The maximum color intensity was attained in 30 seconds for α - and β -tocopherols, while in 4 min for γ - and δ -tocopherols. The time reaching the maximum color intensity for γ - and δ -tocopherols depended on their concentration.

FIG. 2. The Rate of Color Development for α -, β -, γ - and δ -Tocopherols.

The numerals in the figure indicate the concentration (μg per 4 ml) of tocopherols used in these experiments.

In addition the color intensities of γ - and δ -tocopherols increased slightly as the reaction period was prolonged as found when bathophenanthroline was used.^{2,5)} Thus PDT has almost the same rate of color development as bathophenanthroline for each tocopherol.⁵⁾

The rate of color development changed with different tocopherols as found when bathophenanthroline was used.^{2,5)} The different tocopherol reduces ferric ions in different amount and rate. In particular, the reduction rate by α -tocopherol is faster than that by δ -tocopherol and the amount of ferrous ions reduced by α -tocopherol is less than that by δ -tocopherol. Such different reactivities of tocopherols in the presence of ferric ions and PDT seemed to be due to the structural variation; the number and the position of methyl group in the chroman ring of tocol.

Prevention of photochemical reduction

In order to prevent the photochemical reduction of the residual ferric ions after completion of the quantitative reduction of ferric ions by tocopherol, orthophosphoric acid was used in our present method in accordance with the procedure of Tsen.²⁾

The net extinction of the color-developed solution without addition of orthophosphoric acid decreased markedly with the time exposed

in an ordinary laboratory, whereas photochemical reduction of the residual ferric ions was effectively prevented by addition of orthophosphoric acid (from 0.05 to 0.5 M), which forms $\text{Fe}(\text{PO}_4)_2^{3-}$ complex.²⁾ During time course of the color-developed solution exposed under an ordinary light condition, the addition of orthophosphoric acid less than 0.1 molar concentration increased gradually the net extinction, while strong orthophosphoric acid (0.5 M) decreased slightly it with the time exposed. The addition of 0.2 to 0.3 M orthophosphoric acid kept the net extinction almost constant for 120 min (Fig. 3) and therefore 0.2 M was selected.

FIG. 3. The Effect of Orthophosphoric Acid on Prevention of Photochemical Reduction of the Residual Ferric Ions.

The concentration of α -tocopherol was 40 μg per 4 ml. A: 0.2 ml of 0.2 M orthophosphoric acid was added after color development. B: 0.2 ml of absolute ethanol instead of orthophosphoric acid was added. Both were exposed under light condition in an ordinary laboratory. Almost the same results were obtained for β -, γ - and δ -tocopherols.

When orthophosphoric acid was added before the addition of ferric chloride, no color was produced, as pointed out by Tsen.²⁾

The recommended method.

The outline of the recommended method has been already described under the general procedure. Additional remarks which were not mentioned there are as follows.

The concentration of PDT, ferric chloride,

and orthophosphoric acid solutions were selected to be 0.1% (w/v), 0.1% (w/v), and 0.2 M, respectively.

The time for color development, that is, the interval between the additions of ferric chloride and orthophosphoric acid, was specified to be at least 30 seconds for α - and β -tocopherols and 4 min for γ - and δ -tocopherols. The color development should be carried out under dim artificial light in a darkened room. After the addition of orthophosphoric acid, the color-developed solutions were allowed to stand for 20 min under the light condition of an ordinary laboratory and the absorbance was measured at 554 nm.

According to the recommended method different concentrations of α -, β -, γ - and δ -tocopherols were determined and their calibration curves were prepared as shown in Fig. 4. The curve of each tocopherol followed Beer's law. The amounts of tocopherol determinable by the recommended method were less than about 40 μ g and more than about 2 μ g per 4 ml. From the calibration data, $E_{1\%}^{1\text{cm}}$ and $1/E$ for each tocopherol were calculated and are indicated in Table II.

FIG. 4. Standard Calibration Curves of α -, β -, γ - and δ -Tocopherols.

TABLE II. ABSORPTIVITY OF PDT FOR DETERMINATION OF INDIVIDUAL TOCOPHEROLS

	$E_{1\%}^{1\text{cm}}$	$1/E$
α -Tocopherol	1180	33.90
β -Tocopherol	1264	31.65
γ -Tocopherol	1435	27.86
δ -Tocopherol	1668	23.98

For the quantitative determination of tocopherols in biological materials, foods and feed-stuffs, the interfering substances in the unsaponifiable matter should be removed from the tocopherols by column and/or thin-layer chromatography and other methods.^{5,~7)} The color intensities developed with some of interfering substances were determined by the present method. It was found that β -carotene was most sensitive and 1 μ g of β -carotene had the reducing power equal to that of 2.5 μ g of α -tocopherol. Cholesterol, stigmasterol and squalene gave almost negative values, while β -sitosterol, lanosterol and vitamin A alcohol reduced ferric ions but not so strongly as β -carotene.

The great advantage of the use of PDT is a low cost of this reagent. PDT is now commercially available and its price is about one-third of that of bathophenanthroline. Furthermore its concentration required for determination of tocopherol can be reduced to one-fifth of that of bathophenanthroline. In addition, some increase of the sensitivity is also an advantage of using PDT.

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