Determination of Individual Tocopherols by Derivative Spectrophotometry

Gabriel J. Bukovits and **Alberto Lezerovich***
Molinos Rio de la Plata, Laboratorio de Investigaciones, Paseo Colon 746, 1323 Buenos Aires, Argentina

The second order derivative UV spectra of α-, β-, γ- and δ-tocopherols showed small differences that allowed development of a derivative spectrophotometric method for determining individual tocopherols in a mixture. The procedure consists of measuring the second derivative spectrum of the sample at four specified wavelengths and calculating each concentration by means of a method for multicomponent analysis that uses mixtures as standards. Vegetable oils and other fatty products usually require a previous clean-up process (under study) in order to remove interfering substances.

Tocopherols are substances widely distributed in nature. Four forms, α-, β-, γ-, and δ-tocopherols (α-T, β-T, γ-T, and δ-T) (Fig. 1) are found in vegetable matter.

Methods for the determination of tocopherols can be classified as spectrometric, electrochemical, or chromatographic. Among spectrometric methods, which determine only total tocopherols, the procedure of Emmerie and Engel (1) is the most widely used, although fluorometry also has been applied (2). Individual tocopherols can be determined by polarography (3,4), TLC (5-7), GLC (8,9), column liquid chromatography (10,11) and HPLC (12-15). No direct spectrophotometric method for individual tocopherols in their mixtures is found in the literature.

In the course of work on fractionation of deodorization condensates (scum oils), the need arose for a simpler and more rapid method to determine tocopherols. A derivative spectrophotometric procedure with those features, also applicable to vegetable oils and other fatty products, is being developed. With the method reported herein, the determination of individual tocopherols in a mixture with no interfering compounds can be achieved.

Tocopherols absorb in the UV giving wide bands centered at approximately the same wavelength, so the determination of each one in a mixture is not feasible by conventional spectrometry. Characteristics of derivative spectra have been discussed elsewhere (16). It is important to remember that the second order derivative of absorbance with respect to wavelength ($d^2A/d\lambda^2$) gives negative peaks at about the same wavelengths as absorbance maxima occur, and that an enhancement of resolution results in the process of differentiation. Another general effect is a discrimination in favor of sharper features against the background or broader bands. Discrimination

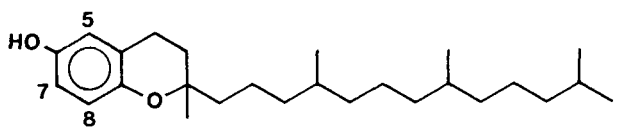

FIG. 1. Structure of tocopherols: 5,7,8-trimethyl (α-T); 5,8-dimethyl (β-T); 7,8-dimethyl (γ-T); 8-methyl (δ-T).

*To whom correspondence should be addressed.

TABLE 1

Calibration Mixtures of Tocopherols

Tocopherol	Standard mixtures[a] (Concentrations μg/ml)				
	1	2	3	4	5
α	39.38	8.31	2.64	72.19	0
β	0	0.76	2.25	20.81	11.62
γ	57.29	82.10	11.90	0	30.38
δ	0.68	26.07	32.80	0	17.06

[a]Standard mixtures 1 to 4 have relative compositions similar to those found in cottonseed, soybean, castor and wheat germ oils, respectively (9,13).

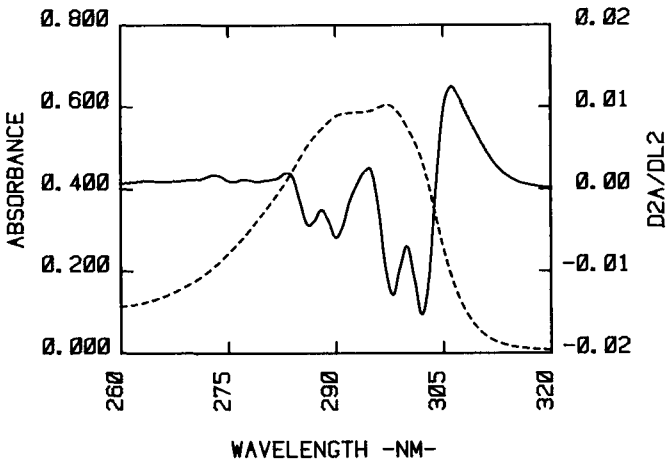

FIG. 2. Spectra of α-tocopherol in absorbance (– – –) and second derivative (——), in hexane.

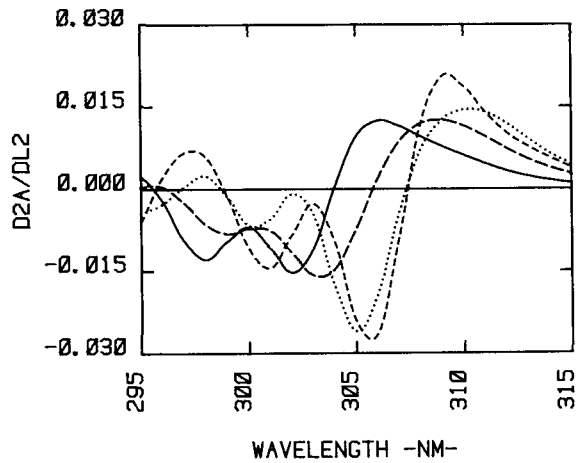

FIG. 3. Second derivative spectra of α (——), β (— — —), γ (- - -) and δ (. . .) tocopherols in hexane normalized to equal concentrations.

increases with the derivative order, and a point is reached where the background becomes negligible, allowing the determination of an otherwise difficult substance using direct absorbance data.

EXPERIMENTAL

Solvents. Spectroscopic grade hexane or isooctane were used. Other solvents were analytical grade.

Apparatus. A Hewlett-Packard 8450A UV-visible spectrophotometer, which outputs directly first and second derivative values of absorbance with respect to wavelength, was used for measurements of $d^2A/d\lambda^2$ values in 1 cm path quartz cells.

Standards. Tocopherol standards were assayed by UV or HPLC. α-T (100%) was purchased from Serva Feinbiochemica (Heidelberg, West Germany). γ-T and δ-T (both 99%) were a gift from F. Hoffmann-La Roche & Co.

TABLE 2

Determination of Individual Tocopherols

Sample[a]	Tocopherol analyte	Concentration (μg/ml)			
		Known		Found	
		Individual	Total	Individual	Total[b] (% error)
1	α	50.8	94.1	51	95 (1)
	β	0		0	
	γ	41.5		44	
	δ	1.8		0	
2	α	26.9	77.0	28	78 (1)
	β	0		−1	
	γ	50.1		51	
	δ	0		0	
3	α	90.1	96.5	90	98 (2)
	β	1.4		3	
	γ	4.7		7	
	δ	0.3		−2	
4	α	76.7	82.0	77	82 (0)
	β	0		1	
	γ	0		4	
	δ	5.3		0	
5	α	69.0	70.1	68	69 (2)
	β	0		1	
	γ	1.1		6	
	δ	0		−6	
6	α	21.6	97.8	19	95 (3)
	β	2.2		4	
	γ	67.0		66	
	δ	7.0		6	
7	α	47.5	54.5	46	51 (6)
	β	0		2	
	γ	7.0		8	
	δ	0		−5	
8	α	31.7	68.7	34	72 (5)
	β	0		−1	
	γ	19.9		22	
	δ	17.1		17	
9	α	24.1	92.8	27	98 (6)
	β	15.2		13	
	γ	26.6		28	
	δ	26.9		30	

[a]Samples 1 to 7 have relative compositions similar to those found in peanut, sesame, olive, safflower, sunflower, corn and olive oils, respectively (9,13).

[b]Calculated as the sum of individual values.

(Basel, Switzerland). β-T (83%) was isolated from wheat germ oil by the following sequence of operations: saponification, acetylation of the unsaponifiable fraction in pyridine with acetic anhydride, selective deacylation according to the procedure of Foster and Cross (17), ion exchange in absolute ethanol-isopropanol (3:2, v/v) with Merck Ion Exchanger III resin (strong-base anion exchanger), elution with absolute ethanol-isopropanol-acetic acid (54:36:10, v/v/v) and, finally, preparative silicagel TLC with cyclohexane-ethyl acetate (98:2, v/v) as elution solvent (two runs).

Stock standard solutions of each tocopherol in hexane or isooctane containing a known amount of about 1 mg/ml were prepared. Standard solutions for measurements, with the composition shown in Table 1, were obtained by proper dilution and mixing of stock solutions.

Samples. Sample solutions were prepared by mixing adequate quantities of stock standard solutions of each tocopherol, in such a way that absorbance reading did not exceed 1.5.

Procedure. The second derivative values at 298, 302, 304 and 306 nm were measured on standards and samples, and concentrations of individual tocopherols were calculated by the procedure of Brown et al. (18). Measurements were done during several seconds to obtain values representing averages of more than 20 readings; in this way standard deviations of $d^2A/d\lambda^2$ values stayed in the order of $\pm 1.5 - 2 \times 10^{-4}$ for readings from about −0.03 to 0.03.

RESULTS AND DISCUSSION

The spectra of α-tocopherol in absorbance and its second derivative are shown in Figure 2. In the second derivative spectrum the absorbance band in the range 280–320 nm is resolved into four negative peaks, a common characteristic to many phenolic substances. The second derivative spectra of the other tocopherols (Fig. 3) are very similar, differing slightly in the wavelengths of maxima and minima, with shifts of at most 3–4 nm. It is worthwhile to mention that even γ-tocopherol and δ-tocopherol, which have zero order (absorbance) spectra with very close wavelength maxima and absorptivities, give different second derivative spectra.

Advantage of the small differences in the second derivative spectra was taken in developing a method to obtain quantitative results on concentrations from a mixture spectrum. Due to the strong peak overlapping, analytical conditions to solve the system are critical, and several procedures—including use of zero crossing points (19) and different multicomponent analysis—were tested. The best results were obtained with the method of Brown et al. (18), which uses a matrix representation for performing a least-squares regression analysis of calibration data for standard mixtures, instead of single components, in an over-determined multicomponent analysis. A square matrix (from the derivative data) was generated by using five mixtures with two to four tocopherols each, which allowed some mathematical simplifications, minimizing round-off errors.

Four mixtures of tocopherols resembling, according to literature data (9,13), the relative composition of natural oils (cottonseed, soybean, castor and wheat germ oils), and a fifth one with intermediate composition, were used as standards.

With such standards, the method was capable of handling samples with relative compositions varying within a wide range. However, if a series of samples with similar relative compositions were to be analyzed, the use of standard mixtures with compositions close to those of the samples would be preferred in order to obtain better results.

The most useful wavelengths for measurements, chosen by checking results, were 298, 302, 304 and 306 nm. Some of them are close to, but do not correspond with, the minima in the derivative spectra. The relationship between total concentration of standards and second derivative values was linear up to about 200 μg/ml. A computer program was developed to perform matrix operations and solve the equations for multicomponent analysis; otherwise, calculations are tedious.

The results obtained in the analysis of several samples prepared as described in the experimental section are summarized in Table 2, which includes only those cases with a relative composition different from that of the standard mixtures. When the relative compositions of the samples were similar to that of a standard mixture the results were, as expected, much closer to the known values. When the analyte concentrations are higher, errors are lower. Accuracy for total tocopherols was acceptable, with percentage errors less than 6%.

Deviations of found concentrations in relation to the known ones for α-T, β-T, γ-T, δ-T and total tocopherols were respectively ± (1.6, 1.6, 2.7, 3.4 and 2.4 μg/ml). They were calculated by $\sqrt{[\sum_i (f_i - k_i)^2/n]}$ where f_i, k_i and n represent the concentration values found and known and the number of samples respectively.

The method described has the advantage of determining not only total but also individual tocopherols. All of them are antioxidants and present vitamin E activity, although at different rates, α-T being the most important from the nutritional point of view. For this reason, methods determining only total tocopherols, like that of Emmerie and Engel, should be considered outdated.

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