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Analytical Methods Section

A spectrofluorimetric procedure for the determination of α-tocopherol in nutritional supplement products

Ibrahim B. Razagui, Philip J. Barlow & Keith D. A. Taylor

School of Food, Fisheries and Environmental Studies, Humberside Polytechnic, Nuns Corner, Grimsby, South Humberside, DN34 5BQ, UK

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A direct spectrofluorimetric method for the determination of vitamin E in nutritional supplement products is described. Samples analysed include single vitamin and multivitamin tablets and capsules as well as dietary formula preparations. The analytical procedure was carried out in a single reaction vessel and consisted of saponification of samples for 10 min in a water bath set at 85°C using absolute ethanol (5 ml) and 80% (w/v) potassium hydroxide solution (2 ml) as the saponification medium, with ascorbic acid (125 mg) as antioxidant. The analyte was extracted *in situ* with *n*-hexane (20 ml). Treatment of the *n*-hexane layer with 60% (v/v) sulphuric acid (2 ml) was sufficient to remove interference by retinol, and the n-hexane layer was then used for direct fluorescence measurement at 298 nm (excitation) and 330 nm (emission). The scanning of several excitation and emission spectra of sample extracts, showed no evidence of interference by any excipient matter or extraneous fluorescing lipid soluble components. Vitamin E values obtained using the described procedure showed good agreement with manufacturers' label expectations.

INTRODUCTION

The essentiality of vitamin E to human health is well recognized, and recommended dietary allowances (RDAs) for this nutrient are found in the dietary guideline publications of many countries (Truswell *et al.*, 1983). Although the RDA for vitamin E is currently set at 8 and 10 mg per day for adult women and men, respectively (NAS, 1989), there seems to be no universal agreement about the level of its physiological requirements for the maintenance of optimum health. Its use as a prophylactic and therapeutic agent for various health disorders has been the subject of discussion and reviews in the literature (Farrell, 1980; Horwitt, 1976, 1980). Epidemiological evidence of frank vitamin E deficiency is seldom encountered in adults; however in premature infants, diagnosable deficiency cases, manifested as haemolytic anaemia, have been reported (Pfeiffer, 1975; Phelps, 1979). Conditioned vitamin E deficiency in epileptic patients receiving long-term anticonvulsant therapy, has also been reported (Higashi *et al.*, 1980), and the use of vitamin E as co-medication, has been observed to result in significant reductions in epileptic seizures (Ogunmekan & Hwang, 1989).

The practice of self-administration of nutritional supplements, has become increasingly prevalent in industrialized countries, with vitamin E as one of the micronutrients that are available as single preparations or part of multinutrient preparations. The recent dietary and nutritional survey of British adults reported about 5% of those surveyed as taking regular vitamin E supplements (Gregory *et al.*, 1990). The health benefits associated with such a practice remain however largely unsubstantiated, but the general consensus is that large doses of vitamin E are unlikely to result in adverse health effects (Bieri, 1975; Witting, 1975).

In view of the continued and increasing interest in the importance of vitamin E as a nutrient as well as a potential pharmacological agent, there is also a continued interest in the development of sensitive and reproducible methods for its determination in various natural and synthetic products.

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The most widely used method for the determination of vitamin E in foods and other biological materials has been based on the Emmerie–Engle reaction, which involves reduction of ferric ions by α-tocopherol to ferrous ions which are then reacted with α,α'-dipyridyl resulting in the formation of a chromophoric red coloured complex, the concentrations of which can be measured colorimetrically and related to α-tocopherol concentration. The method is however susceptible to poor specificity as other lipid-soluble components such as sterols and vitamin A may reduce ferric iron, resulting in analytical bias. Although modifications to this assay have been introduced to produce a more stable and specific chromophore, the procedure remains long and tedious as it involves multiple extractions, drying and analyte concentration steps all of which increase the probability of sample and analyte loss. Subsequent improvements to vitamin E assays include the use of thin-layer and gas chromatography which offer the advantage of higher specificity. These methods are, however, also time consuming and subject to inaccuracies associated with oxidative loss.

The ability of α-tocopherol to exhibit measurable native state fluorescence has long been recognized as a useful characteristic in forming an analytical basis for its quantitation (Duggan *et al.*, 1957), and has subsequently been applied for its determination in biological matrices such as human plasma and serum (Duggan, 1959; Thompson *et al.*, 1973).

In foods, total vitamin E activity is related predominantly to the varying proportions of the vitamers α-, β-, γ- and δ-tocopherols. The molar fluorescence intensities of the four vitamers do however show variations that are of similar magnitude to their respective molar extinction coefficients, thus preventing adequate spectral resolution of their excitation and emission lines as well as their differential quantitation using direct fluorescence measurements.

This problem has largely been overcome by using open column chromatography to separate the vitamers before their fluorometric quantitation is made (Thompson *et al.*, 1972), and with the advent of modern laboratory instruments, the use of such techniques as HPLC incorporating fluorescence detection, have become widely adopted for the determination of total vitamin E activity in various foods and biological tissues (Thompson & Hatina, 1979; Desai & Machlin, 1985; Indyk, 1988; Hogarty *et al.*, 1989; Analytical Methods Committee, 1991).

Although semi-automated techniques such as HPLC and GLC are becoming more widely adopted and recommended for the determination of vitamin E in various natural and synthetic products, the availability of such facilities may be restricted in view of their cost and the degree of manipulative skill required, which may not always be justified unless the analysis is intended for the simultaneous determination of other fat-soluble vitamins or other tocopherol isomers. Nutritional supplements and dietary formula products contain vitamin E, predominantly as the acetate or hydrogen succinate ester of all-rac-α-tocopherol, which is a racemic mixture of the enantiomeric pairs of *d*-α-tocopherol and its epimer, *l*-α-tocopherol. The relative simplicity of such a matrix should allow for a direct analytical procedure that can readily be confined to determining the level of one vitamer, as the most nutritionally relevant index of vitamin E activity.

This paper reports a direct fluorimetric method for the determination of vitamin E in nutritional supplements and dietary formula products.

The method as described is based on direct saponification of the sample and extraction of the free tocopherol into an appropriate solvent, followed by direct fluorescence measurement of the extract. Manipulative steps are kept to a minimum since sample preparation is carried out in a single reaction vessel and the analyte is extracted *in situ*.

MATERIALS AND METHODS

All glassware was cleaned with sulphochromic acid, followed by repeated rinsing, first with tap water, and then with distilled water. All washed glassware was then rinsed with absolute ethanol and dried in an oven.

Subdued light was maintained throughout the period of sample preparation, extraction and measurement.

Reagents

dl-α-Tocopherol (95% pure), was obtained from Sigma (Dorset, UK). Purity was verified by spectrophotometric and absorptive measurements in absolute ethanol ($E^{1\%}_{1cm}$ = 71), using a Pye-Unicam UV/visible spectrophotometer, model SP900.

Preparation of standards

dl-α-Tocopherol standard solutions were prepared from a stock *dl*-α-tocopherol solution containing *dl*-α-tocopherol (0·95 g) in *n*-hexane (200 ml). From this solution a series of dilutions were made with the same solvent to obtain a concentration range between 0 and 5·5 μg ml^{-1}. These standard solutions were used to obtain a calibration graph of *dl*-α-tocopherol concentration versus fluorescence intensity.

Equipment

Spectral scanning and fluorescence measurements were carried out using a Shimadzu RF-540 recording spectrophotofluorimeter (Kyoto, Japan), coupled to a Shimadzu DR-3 data recorder. Instrumental parameters for spectral scanning and fluorescence measurements are listed in Table 1.

Table 1. Instrumental parameters used for fluorescence measurements and spectral scanning of *dl*-α-tocopherol in *n*-hexane

Excitation wavelength:	298 nm	Emission wavelength:	330 nm
Excitation start wavelength:	200 nm	Excitation end wavelength:	500 nm
Emission start wavelength:	200 nm	Emission end wavelength:	500 nm
Excitation slit	5 nm	Emission slit	10 nm
Scan speed:	Fast	Sensitivity:	High
Abscissa scale:	×4	Ordinate scale:	×3

Sample preparation

Tablets

For each sample, a total of five tablets were ground into a fine powder in a mortar and weighed.

A sample of the powered tablets estimated to contain approximately 0·05 mg of vitamin E was placed in a 50 ml glass-stoppered tube into which was pipetted absolute ethanol (5 ml). An air condenser was fitted to the tube and the combination placed on a water bath maintained at 85°C. The sample solution was allowed to reflux for 5 min before 80% potassium hydroxide solution (2 ml) and ascorbic acid crystals (125 mg) were added. Refluxing continued for a further 10 min with occasional swirling. After the sample solution was allowed to cool to room temperature, water (5 ml) and *n*-hexane (20 ml) were pipetted into the tube. The tube was stoppered and shaken vigorously a few times, then allowed to stand until the organic top layer separated. This was followed by the addition of 60% sulphuric acid (2 ml) and mixing by shaking and inversion of the tube. Finally water (5 ml) was added followed by shaking and inversion for 1 min. A clear *n*-hexane layer was obtained after centrifugation at 1000 rpm for 5 min. The *n*-hexane layer was then used for direct fluorescence measurements at 298 nm excitation and 330 nm emission. For each set of samples analysed, a blanck was also run in the same manner.

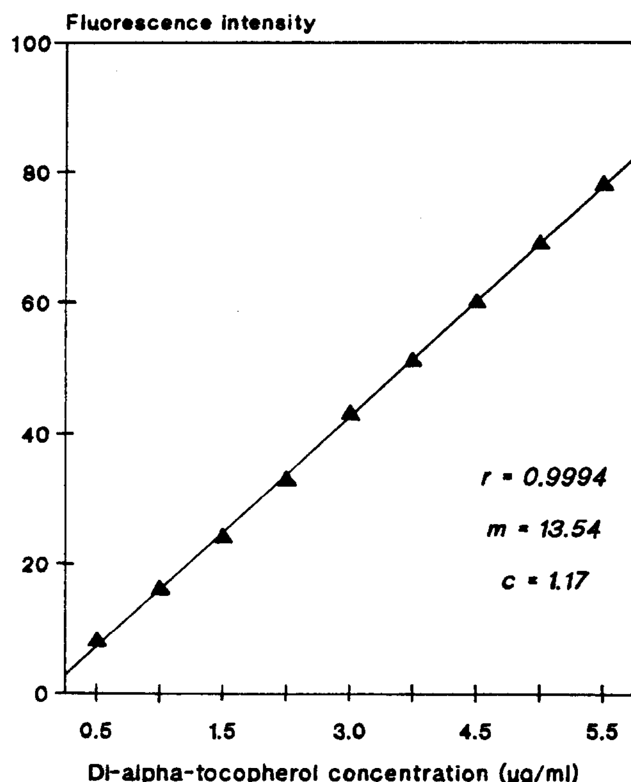

Fig. 1. Calibration curve of *dl*-α-tocopherol concentration in *n*-hexane versus fluorescence intensity. Data plotted are means of triplicate measurements. Instrumental parameters used are shown in Table 1.

Capsules

For each sample, 1 to 3 capsules were individually cut open with a scalpel and placed into a wide-necked glass-stoppered bottle containing 20 ml of absolute ethanol. To maximize expression of each capsule's content, the bottle was swirled and shaken a few times. Finally, the opened capsule shell was held with forceps and jet-rinsed with ethanol.

The ethanol solution was then transferred into a volumetric flask and the final volume made up to 50 ml with ethanol. A volume of this solution, estimated to contain approximately 0·05 mg of vitamin E, was pipetted into a 50 ml glass-stoppered tube, and when necessary the final volume made up to 5 ml with ethanol. The saponification, extraction and fluorescence measurement procedures were the same as for the tablet samples.

Dietary formula products

All dietary formula products were in a powdered form. A composite sample was made from three samples of the same brand product. An accurately weighed amount estimated to contain about 0·05 mg of vitamin E was treated in a similar manner as the tablet samples.

RESULTS AND DISCUSSION

The values of α-tocopherol content in the commercial samples analysed using the method described, are given in Table 2, and were obtained by relating fluorescence intensity to tocopherol concentration using a calibration curve (Fig. 1) of *dl*-α-tocopherol concentration versus fluorescence intensity over a linear concentration range between 0 and 5·5 μg ml^{-1}. The difference

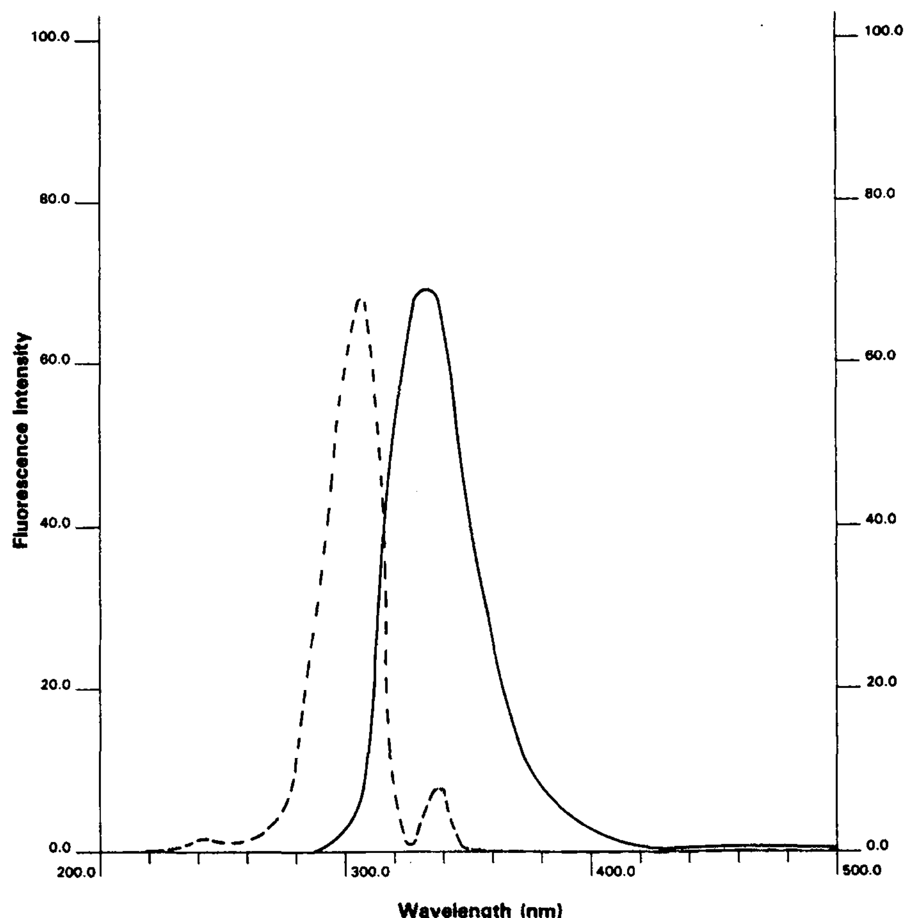

Fig. 2. Excitation and emission spectra of dietary formula sample extract in *n*-hexane using the described procedure. ———, Emission spectrum; - - - - - excitation spectrum.

between the label claim values and the ones obtained in this study varies but on the whole show a high degree of agreement.

The most common disadvantage encountered when employing optical methods, including fluorimetry, for tocopherol determination, has been interference by other lipid and/or lipid-soluble components present in the extract. In fluorimetry, quenching by retinol, which is extracted into the non-saponifiable fraction, appears to be the most common source of interference. The relative stability of tocopherols to acid attack, allows the treatment of the extract with acid which can selectively remove retinol, and to a large extent reduce interference during fluorescence measurements. In this study, it was found that 60% sulphuric acid (2 ml) was sufficient to remove any measurable interference from retinol without affecting the recovery of tocopherol.

No evidence of interference by retinol or other lipid-soluble components was found when the excitation and emission spectra of a standard *dl*-α-tocopherol solution in *n*-hexane were compared to those of an extract obtained after the saponification of a standard oil-based mixture containing retinol, retinol acetate and palmitate, cholecalciferol, calciferol, *dl*-α-tocopherol,

Table 2. Analytical results obtained using the fluorimetric procedure described

Sample description	α-Tocopherol equivalents		RSD(%)
	Label expectation	Found	
Selenium tablets with vitamins E, A & C.	30·2 mg per tablet	29·8 mg per tablet	4·2 (n = 5)
Vitamin E capsules	13·2 mg per capsule	13·9 mg per capsule	3·9 (n = 5)
Multivitamin tablets	10·0 mg per tablet	9·4 mg per tablet	3·1 (n = 7)
Multivitamin tablets	5·0 mg per tablet	5·8 mg per tablet	2·7 (n = 7)
Cod-liver oil capsules	0·55 mg per capsule	0·62 mg per capsule	4·6 (n = 7)
Infant milk formula	5·7 mg per 100 g	6·2 mg per 100 g	3·7 (n = 5)
Infant milk formula	9·0 mg per 100 g	8·7 mg per 100 g	3·4 (n = 5)
Fortified milk powder (adult-formula)	10·0 mg per 100 g	10·7 mg per 100 g	2·2 (n = 5)

Table 3. The effect of KOH concentration used during saponification on α-tocopherol recovery from the commercial samples analysed using the described procedure

KOH (%, w/v)	α-Tocopherol recovery (%)		
	Capsules	Tablets	Dietary formulae
40	65	98	96
45	68	89	97
50	70	98	90
60	67	97	98
70	75	98	96
75	82	92	97
80	101	95	98

Results obtained represent the mean values from triplicate analysis.

dl-α-tocopherol acetate and hydrogen-succinate. Furthermore, the excitation and emission spectra of an extract from a dietary formula preparation containing several lipid- and water-soluble nutrients were also similar to those of a standard *dl*-α-tocopherol (Fig. 2).

Since free tocopherols exhibit considerably stronger fluorescence than their corresponding bound forms, alkaline hydrolysis is required to release and extract the free tocopherol before fluorescence measurements are made. The use of 80% potassium hydroxide (KOH) for alkaline hydrolysis, was found to yield optimum analyte recovery for oil-based capsules, whilst a 40% KOH concentration was found to yield equally good analyte recoveries from powdered products. The reason for the higher KOH concentration required for oil-based samples was not investigated, although tentatively, it may be suggested that the comparatively higher lipid content of the oil-based capsules would require a stronger alkaline medium to maximize release of tocopherol and to attain maximal lipid removal from the sample matrix during saponification. Table 3 shows analyte recovery yields using different KOH concentrations.

The use of *n*-hexane for analyte extraction was justified in view of its known lipid extraction capabilities as well as its low background fluorescence and Raman scatter peak. Multiple extraction with *n*-hexane did not yield analyte recoveries higher than those obtained using single extraction, inferring a favourable partition coefficient between the *n*-hexane phase and the aqueous ethanolic phase, thus obviating the need for multiple extraction.

The susceptibility of tocopherols to oxidation by atmospheric oxygen in an alkaline medium necessitates the use of antioxidants to minimize oxidative losses during saponification. Pyrogallol has often been one of the antioxidants used in vitamin E assays. However, its conversion to pyrogallin during saponification does not make it an antioxidant of choice in fluorometric assays because pyrogallin is extractable into the *n*-hexane layer, resulting in increased background fluorescence and reduction in the sensitivity of the assay.

Although some multinutrient preparations analysed contained ascorbic acid as part of their formulation, the use of additional ascorbic acid during saponification at a level of 125 mg per saponification was considered as a precautionary measure to ensure effective protection against oxidation.

Furthermore, there appeared to be no need to use a nitrogen atmosphere during saponification. The refluxing of the sample prior to addition of potassium hydroxide, and during saponification allowed for the continuous presence of an ethanol vapour layer, which effectively protected the saponification mixture from atmospheric oxygen.

In conclusion, the analytical results obtained in this study suggest that the method offers a simple and sensitive analytical procedure for the determination of α-tocopherol in nutritional supplement products at a relatively low cost and short operational time. Confining sample preparation and analyte extraction into a single reaction tube, gives the method the advantage of keeping manipulative steps to a minimum thereby minimizing sample loss and allowing for optimum analyte recovery. The specificity and accuracy of the described method is supported by the characteristic excitation and emission spectra recorded from various sample extracts.

The method is to be recommended in routine analytical work especially as an alternative in the absence of more cost- and skill-demanding instrumental techniques such as GLC or HPLC.

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