Extraction of α-Tocopherol from Serum Prior to Reversed-Phase Liquid Chromatography

Dirk Hoehler,* Andrew A. Frohlich, Ronald R. Marquardt, and Helena Stelsovsky

Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

A reversed-phase high-performance liquid chromatographic method for the determination of α-tocopherol in serum with ultraviolet (UV) absorbance as well as fluorescence detection is described. The fluorescence monitor was considerably more sensitive than the UV monitor. In addition, systematic studies on the influence of different extraction procedures on the recovery of vitamin E were performed. The extraction of vitamin E from the serum into hexane was markedly affected by the relative volume of ethanol and to a lesser degree by the relative volume of hexane in the mixture. The extracting time also affected the recovery of vitamin E, depending on the amount of ethanol added to the extracting solution. Vitamin E was relatively stable for a considerable length of time when both concentrated and diluted solutions of ethanol and serum were stored in the dark at −20 or 4 °C.

Keywords: *Vitamin E; α-tocopherol; serum; liquid chromatography; extraction*

INTRODUCTION

Quantitative determination of vitamin E is commonly performed by liquid chromatographic procedures with reversed-phase columns using either fluorometric (Lehmann and Martin, 1982; Burton et al., 1985; Clevidence and Ballard-Barbash, 1991; Kaneko et al., 1991), electrochemical (Ikenoya et al., 1979; Lang et al., 1986; MacCrehan, 1990), or spectrophotometric (Catignani and Bieri, 1983; Biesalski et al., 1986; Milne et al., 1986; Carcelain et al., 1992) detection. Liquid chromatographic procedures have been shown to be accurate and have high sensitivities, especially when a fluorescence monitor is used (Cort et al., 1983; Seta et al., 1990; Teissier et al., 1996). In the past, three basic extraction procedures have been used: a simple organic solvent extraction, organic extraction followed by saponification, and saponification followed by organic phase extraction (McMurray et al., 1980). However, saponification using ethanolic or methanolic solutions of potassium hydroxide to convert α-tocopherol esters to their free alcohol has been shown to be disadvantageous because it can cause a partial loss of tocopherols (Nierenberg and Nann, 1992).

Several different procedures have been used to extract vitamin E from tissue and serum or plasma samples. Sarzanini et al. (1993) extracted plasma by adding 1-butanol/ethyl acetate/acetonitrile (1:1:1, v/v/v) and injected a portion of the organic phase directly into the chromatograph after centrifugation. A direct extraction procedure was also applied by Woodall et al. (1996), by adding hexane/propan-2-ol (3:2, v/v) to plasma samples. The most common procedure is to "deproteinize" serum or plasma with ethanol (1:1, v/v) followed by extraction of vitamin E with hexane (Bieri et al., 1979; Howell and Wang, 1982; Catignani et al., 1983; MacCrehan, 1990; Nierenberg and Nann, 1992; Zaman et al., 1993; Sowell et al., 1994) or by extraction of vitamin E with butanol/ethyl acetate (1:1, v/v; Lee et al., 1992). Seta et al. (1990) extracted a mixture containing 100 μL of plasma, 1 mL of ethanol, and 1 mL of water with hexane. According to Catignani et al. (1983), the proportion of serum or plasma to ethanol should be maintained at a ratio of 1:1 (v/v). No studies, however, have been reported on the effects that different proportions of ethanol and hexane have on the recovery of vitamin E from serum or on other conditions that affect the extractability of vitamin E. Preliminary studies in our laboratory have indicated that variation of the proportion of the solvents used in the extraction of vitamin E from serum can have a dramatic effect on the recovery of vitamin E from serum.

The objective of this study was to systematically establish the effects that different proportions of ethanol and hexane have on the recovery of vitamin E from serum. In addition, the influence of other factors such as time of hexane extraction was determined.

MATERIALS AND METHODS

Reagents. Standards of DL-α-tocopherol and DL-α-tocopheryl acetate were obtained from Sigma Chemical Co., St. Louis, MO. HPLC grade methanol and *n*-hexane were from Anachemica, Winnipeg, MB, Canada, and were used without further purification. The 95% ethyl alcohol was from Commercial Alcohol Inc., Montreal, QC, Canada. Double-distilled deionized water was used for the preparation of aqueous solutions. All other chemicals were of reagent grade. Polypropylene Eppendorf microcentrifuge tubes (1.7 mL) with snap cap were obtained from VWR Scientific of Canada Ltd. Standard Reference Material 968b was obtained from the National Institute of Standards and Technology (NIST).

Preparation of Vitamin Standards and Serum Sample. Stock solutions of DL-α-tocopherol (20 mg mL^{-1}) and DL-α-tocopheryl acetate (20 mg mL^{-1}) were dissolved in ethanol and stored at −20 °C. These solutions could be stored indefinitely (>1 year) without loss of vitamin E. Working standards were

* Address correspondence to this author at the Institute of Animal Nutrition, Physiology and Metabolism, University of Kiel, Olshausenstr. 40, D-24098 Kiel, Germany (telephone +49-431-880-2078; fax +49-431-880-1528; e-mail hoehler@aninut.uni-kiel.de).

S0021-8561(97)00596-7 CCC: $15.00
Published on Web 02/18/1998

prepared by dilution of the stock solutions with ethanol (800-fold) to yield a final concentration of 25 μg mL^{-1}. The working solution of DL-α-tocopherol was further diluted to obtain four concentrations as indicated in Figures 1 and 2. The standards were extracted using the same procedure as described for the standard extraction procedure except that vitamin E was prepared in ethanol and 0.1 M phosphate buffer (pH 7.2) was used as the diluent. The sample (50 μL) after extraction with hexane and reconstitution in methanol was injected into an HPLC column. The working standards were usually kept for 5 days or less at −20 °C in the absence of light. The solutions, however, were relatively stable when stored for much longer time periods.

For the standard curves two different ranges of α-tocopherol in ethanol were prepared, the first for a range from 0 to 1 μg mL^{-1} and the second for a range from 0 to 15 μg mL^{-1}. Triplicate extractions were made for each concentration. A typical standard sample contained 100 μL of α-tocopherol in ethanol (amount of tocopherol varies), 100 μL of ethanol, 200 μL of α-tocopheryl acetate in ethanol, and 200 μL of 0.1 M phosphate buffer (pH 7.2). This mixture was vortexed for 10 s followed by the addition of 300 μL of hexane and vortexing for 60 s. The sample was centrifuged at 15000*g* for 10 min to yield two distinct layers. A 200-μL aliquot of hexane (upper layer) containing the extracted vitamin E was withdrawn with the aid of a pipet and was transferred into a 1.7-mL polyethylene tube. The hexane was evaporated to dryness at room temperature under a stream of nitrogen, and the residue was reconstituted with 300 μL of HPLC grade methanol and injected into the HPLC column. In the case of 0−15 μg of vitamin E mL^{-1}, 50 μL of the sample was usually injected, and for the lower concentration range 100 μL was injected.

Blood was obtained from a total of 30 laying hens in the University Poultry Unit. The chickens had been fed diets containing 5.5 IU of vitamin E/kg. The serum for the analyses was pooled and generally used within 1 week of collection, although some samples were stored for up to 6 months at −20 or 4 °C.

Standard Extraction Procedure. The standard procedure, which was carried out at room temperature, was developed on the basis of studies reported in this paper. The method involved the addition of ethanol to serum or serum plus buffer, extraction of vitamin E into hexane, evaporation of hexane to dryness, and reconstitution of the sample in methanol for injection onto a C_{18} column. In this procedure 100 μL of 0.1 M phosphate buffer (pH 7.2) and 100 μL of serum were added to a 1.7-mL polyethylene Eppendorf tube with a snap cap followed by vortexing for 10 s. Four hundred microliters of 95% ethanol was then added to the vial. The mixture was vortexed for 10 s followed by the addition of 300 μL of hexane and vortexing for an additional 60 s. The sample was centrifuged at 15000*g* for 10 min to yield two distinct layers. A 200-μL aliquot of hexane (upper layer) containing the extracted vitamin E was withdrawn with the aid of a pipet and was transferred into a 1.7-mL polyethylene tube. The hexane was evaporated to dryness at 20 °C under a stream of nitrogen, and the residue was reconstituted with 300 μL of HPLC grade methanol followed by centrifugation at 15000*g* for 10 min. Part of the supernatant (usually 200 μL) was transferred into 250-μL conical microcentrifuge polyethylene vials used as inserts into the 2.7-mL glass HPLC vials. The amount of the sample that was usually injected into the column was 50 μL.

The recommended volume proportions of serum to buffer to ethanol to hexane are 1:1:4:3 (100/100/400/300 μL). In general, the proportion of serum plus buffer relative to that of ethanol should be 1:2 (i.e., 200:400 μL) or larger as discussed subsequently. The proportion of buffer water to serum, however, can be varied depending on the volume of serum available and the concentration of vitamin E in the serum. The volume of hexane relative to that of serum plus buffer can be varied without greatly affecting the recovery of vitamin E, although 1−2 volumes (200−400 μL) is most convenient. Standard Reference Material 968b (human serum) was extracted according to this procedure and analyzed for α-, γ-, and δ-tocopherol.

Liquid Chromatography. The HPLC system included an autoinjector (712 WISP from Waters, Milford, MA), an isocratic HPLC pump (110 A from Beckman Instruments Inc., Fullerton, CA), a C_{18} reversed-phase column (3.9 mm i.d. × 30 cm containing 10-μm particle size, μ-Bondapak, Waters), a fluorescence detector (RF-535, Shimadzu Corp. Analytical Instruments Division, Kyoto, Japan), a UV monitor (484, Waters), and an integrator (HP 3392 A, Hewlett-Packard, Avondale, PA). The mobile phase consisted of 95% HPLC grade methanol and 5% double-distilled water filtered through a 0.45-μm nylon filter (MSI Micron Separations Inc., from Fisher Scientific, Winnipeg, MB, Canada) and degassed in an ultrasonic bath. The flow rate was 2 mL min^{-1}, and the analysis was carried out at room temperature. The fluorescence monitor was set at excitation and emission wavelengths of 290 and 325 nm, respectively, while the UV monitor was set at 280 nm and 0.01 AUFS (full-scale absorbance units). See Figures 1 and 2 for further detail.

Effect of Different Extraction Conditions on the Recovery of Vitamin E. A series of experiments were carried out to determine the effect of different extraction conditions on the recovery of vitamin E. The objective of experiment 1 was to determine the influence of ethanol in the extracting solution on the recovery of vitamin E. In experiment 1a, 100 μL of serum plus 100 μL of pH 7.2 buffer were diluted with different volumes of ethanol (0−1000 μL, see Figure 3) and extracted with hexane (300 μL). Experiment 1b was the same as experiment 1a except that a different serum sample was used. Other procedures were as described for the standard procedure.

The objective of the second experiment was to determine if both the proportion of ethanol and hexane affected the extraction of vitamin E. A mixture of 100 μL of serum, 100 μL of buffer, and 200 or 400 μL of ethanol was extracted with 300 or 600 μL of hexane. Other conditions were as described in the legend of Figure 4 and for the standard procedures.

The objective of the third experiment was also to determine the influence of the volume of hexane (200−600 μL) on the recovery of vitamin E. Conditions were as outlined in Figure 5 and for the standard procedures.

The objectives of the fourth experiment were to determine if time of extraction or type of extraction influenced the recovery of vitamin E. Conditions were as described for the standard procedure except the volume of ethanol was varied (200 and 400 μL) and the time that the complete mixture (serum, buffer, ethanol, and hexane) was vortexed varied from 45−180 s. Also see Figure 6 for further details. In a second study (experiment 4b) the final extract was mixed using a syringe. The polyethylene syringe (1 mL) was equipped with a 18 gauge needle with a 90° end. The extracting medium containing serum (100 μL), buffer (100 μL), ethanol (400 μL), and hexane (300 μL) was aspirated and evacuated back into the vial 2−18 times at room temperature. The results were compared with those obtained when the mixture was vortexed for 60 s.

The objective of the fifth experiment was to determine the recovery of vitamin E when serum was spiked with vitamin E. Serum (3 mL) from two different chickens was spiked with 100 μL of buffer, 100 μL ethanol, or 100 μL of ethanol plus 20 μg of vitamin E (3.1 mL final volume). The amount of vitamin E added to the spiked sample was 6.45 μg mL^{-1} serum (i.e., 20 μg of added vitamin E/3.1 mL). A slight precipitate formed in the serum samples spiked with ethanol, which was removed by centrifugation at 15000*g* for 10 min. The volumes of the different solutions in the extracting mixture were as follows: serum prepared as described above, 100 μL; buffer, 100 μL; ethanol, 400 μL; and 100, 200, or 300 μL of hexane. Other conditions were as described for the standard procedure. The final experiment (experiment 6) established the stability of vitamin E in serum when stored for different periods of time and under different conditions. In experiment 6a, serum was extracted in triplicate following the standard procedure, and the sample was stored for 5 h and 2, 3, and 4 days prior to

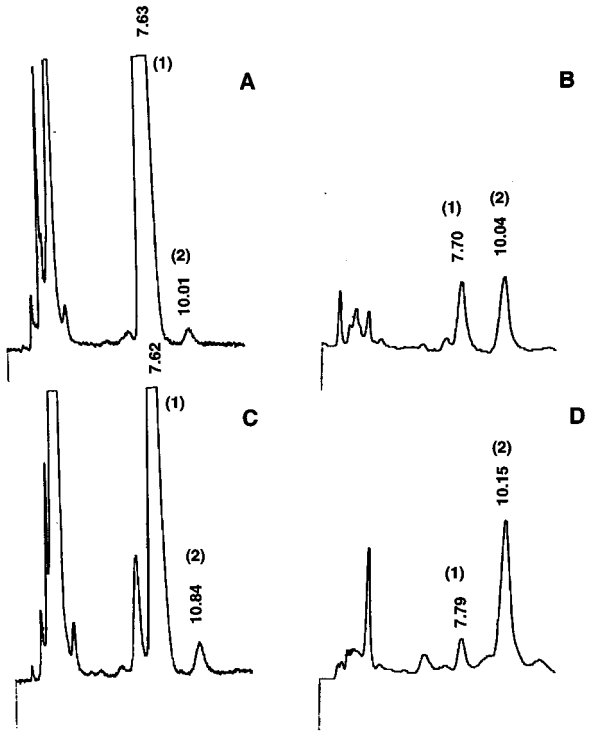

Figure 1. Typical chromatograms for the elution of a standard preparation of α-tocopherol (A and B) and a sample of serum spiked with α-tocopheryl acetate (C and D) as detected using a fluorescence (A and C) or an ultraviolet (B and D) monitor. Peak 1 is α-tocopherol, and peak 2 is α-tocopheryl acetate. The amounts of α-tocopherol and α-tocopheryl acetate added to the standards were 10 and 20.5 μg mL^{-1}, respectively. The amounts of sample injected into the column were 0.25 and 5 ng, respectively, with the injection volume being 50 μL. See Materials and Methods for further details.

HPLC analysis. In experiment 6b, serum was stored for 1 and 5 days prior to extraction, followed by analysis of vitamin E within 6 h of the preparation of the extract.

RESULTS AND DISCUSSION

Sensitivity of HPLC Procedure. Results shown in Figure 1 compare the fluorescence and UV absorbance of a standard preparation of α-tocopherol and a serum sample spiked with α-tocopheryl acetate. Comparison of retention times and spectra with those of pure standards established the identities of the peaks. The two detectors were connected in series to the same HPLC unit. The order in which the vitamins were monitored in the two detectors did not affect the size (area) or shape of the peaks. The sensitivity of the two detectors was determined by comparing the height of the peak of α-tocopherol with that of the average for the baseline (signal-to-noise ratio). The signal-to-noise ratios when 10 ng of α-tocopherol was injected into the column were 39 (±3) for the fluorescence monitor and 4 (±0.1) for the UV monitor. The fluorescence:UV peak areas for α-, γ-, and δ-tocopherol (see below) were 141 (±11), 328 (±18), and 342 (±4), respectively. These results indicate that the fluorescence monitor was considerably more sensitive than the UV monitor for the detection of vitamin E. This is in agreement with the observation of other researchers (Cort et al., 1983; Seta et al., 1990; Teissier et al., 1996). The shape of the vitamin E peak obtained with the UV monitor following the analysis of blood was often nonsymmetrical, suggesting coelution of other UV-absorbing compounds, whereas the fluorescence monitor usually yielded symmetrical peaks that were similar in shape to the standard α-tocopherol peak. The fluorescence monitor was therefore used for all subsequent analyses. One disadvantage of using fluorescence to detect vitamin E is that it cannot be used to detect the ester form of vitamin E because it does not fluoresce. Therefore, both types of detection would be required if both an internal standard such as α-tocopheryl acetate and high sensitivity are required. The use of an internal standard would be most useful for samples for which the recovery of vitamin E was highly variable.

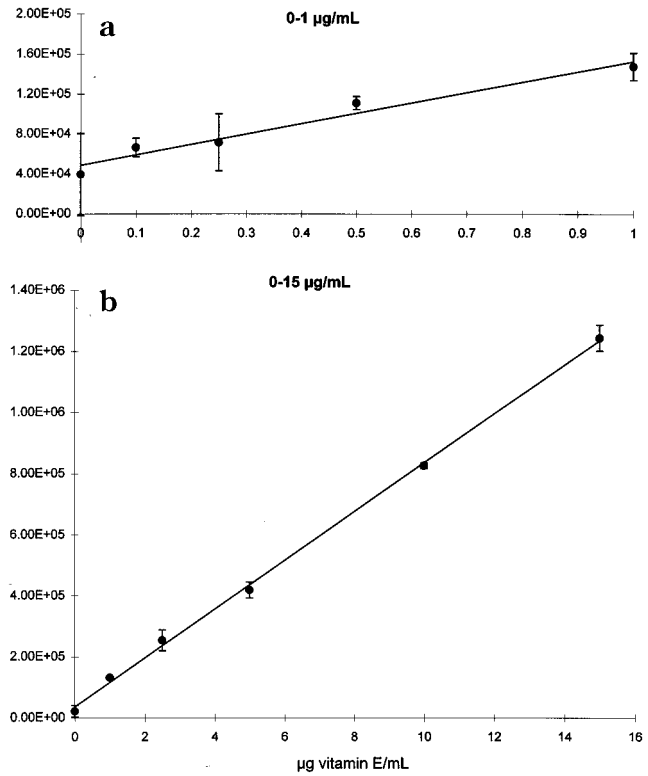

Figure 2. Typical standard curves for α-tocopherol. For the standard curves two different ranges of α-tocopherol in 95% ethanol were prepared, as indicated in the figure. Values represent means ± SD for triplicate analyses using a fluorescence monitor. See Materials and Methods for further details.

The standard curve was linear for concentrations of α-tocopherol between 0 and 15 μg mL^{-1}, with the overall coefficient of variation being ±5.4% (±SD). The coefficient of variation was also similar for the sample containing the low (1 μg mL^{-1}) and the high (15 μg mL^{-1}) concentration of vitamin E, with the values being 3.9% for 1 μg mL^{-1} and 2.9% for 15 μg mL^{-1} α-tocopherol. It is therefore possible to detect 1 ng μL $^{-1}$ of α-tocopherol in a sample, which is equivalent to a concentration of 0.45 μg mL^{-1} α-tocopherol in blood. A concentration of vitamin E lower than 0.45 μg mL^{-1} blood can be readily detected as reliable values can be obtained from the standard curve at concentrations of vitamin E as low as 0.5 μg mL^{-1} rather than 1 μg mL^{-1} as shown in Figure 2b. Further increases in sensitivity can also be obtained by increasing the volume of sample injected into the column from 50 to 100 μL and by omitting buffer from the serum (data not shown). Collectively, these factors should permit an 8-fold (2 × 2 × 2) increase in sensitivity of the assay to 0.06 μg mL^{-1} serum, compared with the 0.45 μg mL^{-1} serum indicated above. The detection limits for vitamin E in

Table 1. Concentrations of α-, γ-, and δ-Tocopherol in Standard Reference Material 968b (Lyophilized Human Serum, Three Concentration Levels) According to NIST and by Using the Standard Procedure As Described under Materials and Methods

	low level		middle level		high level	
tocopherol	NIST value	analyzed	NIST value	analyzed	NIST value	analyzed
α-,[a] μg/mL	7.07 ± 0.50	7.2 ± 0.3	10.11 ± 0.58	11.12 ± 0.6	17.8 ± 1.3	18.53 ± 0.4
γ-,[b] μg/mL	1.7	1.88	2.3	2.60	3.6	4.32
δ-,[b] μg/mL	0.09	0.11	0.1	0.15	0.2	0.20

[a] Certified concentration values. These values are equally weighted means of results from at least three analytical techniques of the NIST. [b] Noncertified concentration values. These values are not certified because either they are based on results from a limited number of analyses or the disagreement among methods is greater than expected for certified values.

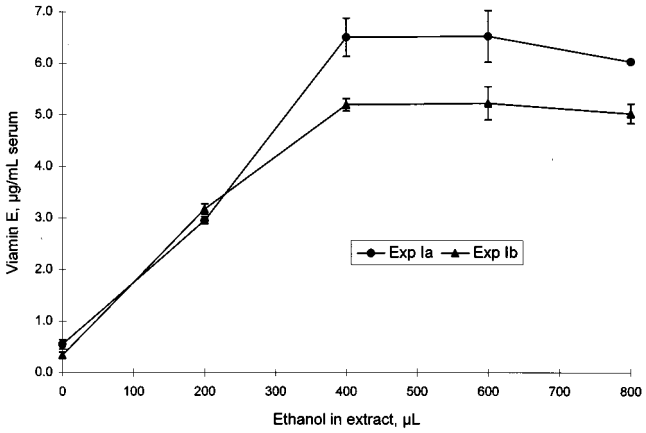

Figure 3. Effect of volume of methanol in extraction solution on the recovery of vitamin E in serum (experiment 1a,b). Two different serum samples (100 μL) plus 100 μL of buffer were mixed with variable volumes of 95% ethanol (0, 100, 200, 400, 600, 800, and 1000 μL), and the vitamin E was extracted from the mixtures with 300 μL of hexane. Other procedures were as given under Materials and Methods. Values represent means ± SD for experiments 1a (●) and 1b (▲) with the number of replicates per time period being 2 and 3, respectively.

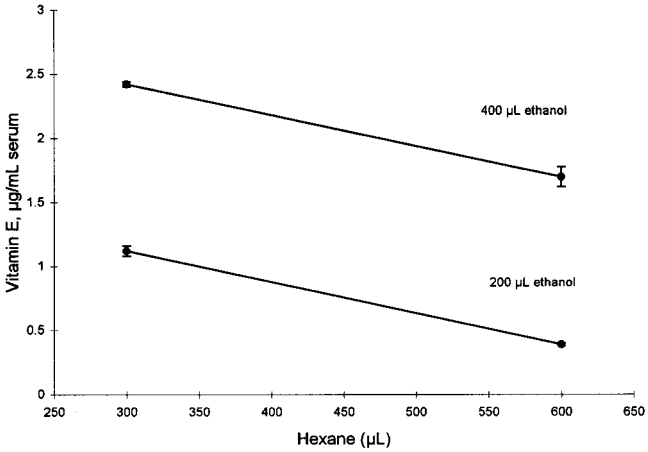

Figure 4. Effect of different concentrations of ethanol and hexane on the recovery of vitamin E from serum (experiment 2). Extraction conditions were as described under Materials and Methods and as given in the figure. Values represent means ± SD of four replicate analyses.

plasma or serum reported in the literature are 0.8 μg mL^{-1} (Catignani and Bieri, 1983), 0.5 μg mL^{-1} (Lee et al., 1992), 2.5 μg mL^{-1} (Zaman et al., 1993), and 0.2 μg mL^{-1} (Sowell et al., 1994) when a UV detector was used. Sarzanini et al. (1993) found a considerably lower detection limit of 3 ng mL^{-1} by using very sensitive liquid chromatography with coulometric detection. The frequently observed concentration of vitamin E in the serum of chickens fed a diet containing 5−10 IU (= 5−10 mg of DL-α-tocopheryl acetate) of vitamin E/kg of diet is around 4−7 μg mL^{-1} (data from this study). The current assay therefore is of sufficient sensitivity to detect vitamin E in the serum of chickens and other animals fed diets containing a wide concentration range of vitamin E.

For the validation of the method, this extraction procedure was carried out using Standard Reference Material 968b (human serum) from NIST. As shown in Table 1, the obtained results for α-, γ-, and δ-tocopherol at three different concentration levels are almost identical with the NIST values.

Effect of Ethanol. The results from experiment 1 demonstrated that the proportion of ethanol in the extracting buffer had a dramatic influence on the amount of vitamin E that was recovered in hexane. As indicated in Figure 3 there was a near linear increase in recovery of vitamin E as the amount of ethanol increased from 0 to 400 μL, after which the values tended to be the same up to a concentration of 800 μL. A volume of 1000 μL of ethanol could not be used as the water−ethanol phase did not readily separate from hexane. These results demonstrate that the extraction of vitamin E from the serum plus buffer solution into hexane is markedly affected by the proportion of ethanol in the mixture. As indicated above, the minimal volume of ethanol should be 400 μL. Under these conditions the ratio of serum plus buffer to ethanol is 1:2. The critical factor is that the ratio of aqueous solution to ethanol must be 1:2 or greater. Interestingly, essentially no vitamin E was extracted into hexane from the aqueous phase in the absence of ethanol. Increasing the amount of hexane in the extracting solution tended to have an effect opposite of that of ethanol (Figures 4 and 5, experiments 2 and 3) as the amount of vitamin E extracted per milliliter of serum tended to decrease as its volume increased from 300 to 600 μL, with the percent decrease in extracted vitamin E being 22% (4.43 vs 3.45 μg of vitamin E mL^{-1} of serum, Figure 5).

In experiment 4, the effect of extracting time (vortexing or mixing of sample with a syringe) on the recovery of vitamin E in the hexane fraction was determined. The result depicted in Figure 6 demonstrates that the vortexing time had a marked effect on the recovery of vitamin E when 200 μL of ethanol was added to the extracting solution but not when 400 μL of ethanol was used. The recovery of vitamin E with the sample containing 200 μL of ethanol decreased by 82% (1.23 vs 0.22 μg of vitamin E mL^{-1} of serum) as the vortexing time was increased from 45 to 180 s, whereas the corresponding decrease with 400 μL of ethanol was only 6% (4.52 vs 4.25 μg of vitamin E mL^{-1} of serum). These results indicate that longer mixing times with the higher amounts of ethanol in the mixture did not greatly affect the recovery of vitamin E in

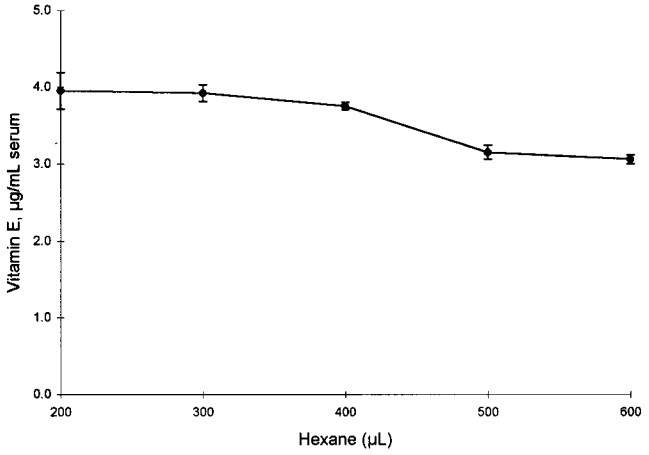

Figure 5. Effect of volume of ethanol and hexane on the recovery of vitamin E in serum (experiment 3). Serum (100 μL) plus 100 μL of buffer and 400 μL of 95% ethanol was extracted with different volumes of hexane (200, 300, 400, 500, and 600 μL). Other procedures were as given under Materials and Methods. Values represent means ± SD of triplicate extractions.

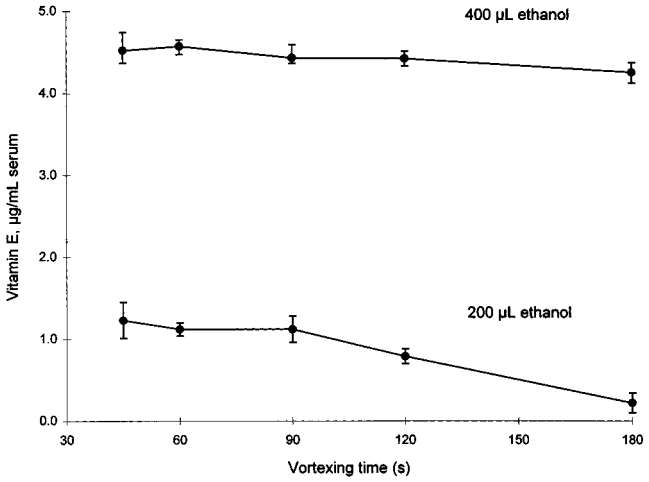

Figure 6. Effect of vortexing the final extraction mixture for different periods of time on the recovery of vitamin E (experiment 4). The final extraction mixture (100 μL serum, 100 μL buffer, 200 or 400 μL of ethanol, and 300 μL of hexane) was vortexed for different periods of time as indicated in the figure. The values represent means ± SD of three analyses.

hexane but that they dramatically reduced its recovery when the lower concentration of ethanol was present in the sample. It is quite possible that a substantial amount of emulsion is formed under the latter conditions, with part of vitamin E remaining in the aqueous fraction. The exact reason for this effect is not known; however, it suggests that mixing times of up to 1 min with optimal amounts of ethanol will yield maximal recoveries of vitamin E in hexane.

In another study (experiment 4b) it was shown that the calculated concentrations of vitamin E per milliliter of serum were 5.6 ± 0.7, 5.9 ± 0.3, 5.6 ± 0.5, 5.4 ± 0.1, 5.1 ± 0.1, and 4.8 ± 0.2 μg when a mixture containing 100 μL of serum, 100 μL of buffer, 400 μL of ethanol, and 300 μL of hexane was subjected to 2, 4, 6, 8, 12, and 18 mixing cycles using a syringe, with the value being 5.0 ± 0.15 when the same mixture was vortexed for 60 s. The results, which are consistent with the results obtained from the vortex mixer, demonstrate that mixing for as few as 2 to as many as 8 cycles did not affect the values but that mixing for 18 cycles compared with 6 resulted in a 14% decrease (5.9 vs 5.1 μg of vitamin E/mL of serum) in the recovery of vitamin E.

The recovery of vitamin E from serum spiked with vitamin E was slightly >100% when the volume of hexane was 100 or 200 μL and ≈90% (94 and 83%) when the volume of hexane was 300 μL (Table 2).

In a final series of studies the effect of storage time on serum vitamin E concentration was determined. The concentrations of vitamin E ± SD in serum when analyzed ($n = 3$ samples per time period) on day 1 (16 h after preparation) through day 4 were 6.0 ± 0.1, 6.0 ± 0.2, 5.9 ± 0.2, and 5.6 ± 0.4 μg mL^{-1}, respectively (experiment 6a). These data indicate that vitamin E in extracts of serum is stable for up to 4 days at 4 °C. Serum that was stored for 1 and 5 days at 4 °C followed by extraction yielded vitamin E values of 5.9 ± 0.26 and 5.9 ± 0.8 μg mL^{-1}, respectively (experiment 6b). Likewise, serum that was stored for 6 months yielded values of 5.19 ± 0.12 μg mL^{-1} at the time of analysis and 5.30 ± 0.69 μg mL^{-1} after 6 months. The higher value may have been attributable to removal of a precipitate that formed after 6 months. These data indicate that vitamin in serum, in extracts of serum, and in standard solutions are relatively stable over several days provided the samples are kept refrigerated and in the dark.

It was also found by others (Catignani and Bieri, 1983; Lee et al., 1992) that standard solutions were stable for up to 1 year at −20 or −70 °C without significant losses of vitamin E. In another study, vitamin E did not show any significant degradation after repeated freezing and thawing or storage at room temperature for 7 days (Zaman et al., 1993) or when plasma was frozen at −20 °C and analyzed weekly over a 2-month period (Sarzanini et al., 1993). In contrast to the above results, Gunter et al. (1988) and Lee et al. (1992) reported significant losses of vitamin E of up to 66% when plasma or serum samples were thawed and

Table 2. Recovery of α-Tocopherol from Serum When Extracted with Various Volumes of Hexane[a]

sample no.	hexane, μL	n	vitamin E,[b] μg mL^{-1} of serum: buffer (A)	ethanol (B)	ethanol + vitamin E (C)	calcd B + 6.45 (D)	recovery, % (C/D × 100)
I	100	6	4.8 ± 0.7	5.1 ± 0.5	12.9 ± 0.2	11.6	111
I	200	6	4.2 ± 0.4	4.8 ± 0.3	12.5 ± 0.3	11.2	112
I	300	6	4.5 ± 0.4	4.1 ± 0.4	10.0 ± 0.1	10.6	94
II	100	6	5.7 ± 0.7	6.5 ± 0.4	16.8 ± 1.6	13.0	129
II	200	6	6.1 ± 0.5	6.7 ± 0.5	13.2 ± 1.2	13.2	100
II	300	6	5.3 ± 0.7	6.5 ± 0.3	10.8 ± 1.2	13.0	83

[a] Values represent means ±SD. [b] Serum (3 mL) was mixed with 100 μL of buffer (A), 100 μL of ethanol (B), or 100 μL of ethanol containing 20 μg of vitamin E (C). A precipitate formed in serum treated with ethanol. This was removed by centrifugation and was probably responsible for higher values in samples B compared with samples A. Vitamin E was extracted using the standard procedure except for volume of hexane, which was as indicated in the table.

refrozen up to five times. In addition to that, serum samples stored at −20 °C were relatively unstable, whereas degradation was diminished at −70 °C (Gunter et al., 1988). The reasons for the great variation in the apparent stability of vitamin E in stored plasma or serum samples are not known, but it might be possible that the degree of degradation is a function of the concentration of lipid peroxides or other pro-oxidant blood constituents in the samples.

Overall, the results of this study demonstrate that the amount of vitamin E extracted into hexane from a serum sample is influenced to a very great degree by the relative volume of ethanol in the sample and to a lesser degree by the volume of hexane. The optimal proportions of the aqueous phase:ethanol:hexane were found to be 2 (1 serum + 1 buffer):4:3. The ratio of serum to buffer can be varied from all serum to higher amount of buffer. The relative volume of ethanol can be increased up to 8 without affecting the recovery of vitamin E. The proportion of hexane can be increased from 3 to 6 volumes with only a small decrease in percent recovery of vitamin E. Vitamin E, when stored in the dark at −20 or 4 °C, is stable in both concentrated and diluted solutions of ethanol and in serum for a considerable length of time.

LITERATURE CITED

Bisalski, H.; Greif, H.; Brodda, K.; Hafner, G.; Bassler, K. H. Rapid determination of vitamin A (retinol) and vitamin E (α-tocopherol) in human serum by isocratic adsorption HPLC. *Int. J. Vitam. Nutr. Res.* **1986**, *56*, 319−327.

Burton, G. W.; Webb, A.; Ingold, K. U. A mild, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* **1985**, *20*, 29−39.

Carcelain, G.; David, F.; Lepage, S.; Bonnefont-Rousselot, D.; Delattre, J.; Legrand, A. Simple method for quantifying α-tocopherol in low-density + very-low-density lipoproteins and in high-density lipoproteins. *Clin. Chem.* **1992**, *38*, 1792−1795.

Catignani, G. L.; Bieri, J. G. Simultaneous determination of retinol and α-tocopherol in serum or plasma by liquid chromatography. *Clin. Chem.* **1983**, *29*, 708−712.

Clevidence, B. A.; Ballard-Barbash, R. Tocopherol contents of lipoproteins from frozen plasma separated by affinity chromatography. *Lipids* **1991**, *26*, 723−727.

Cort, W. M.; Vicente, T. S.; Waysek, E. H.; Williams, B. D. Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. *J. Agric. Food Chem.* **1983**, *31*, 1330−1333.

Gunter, E. W.; Driskell, W. J.; Yeager, P. R. Stability of vitamin E in long-term stored serum. *Clin. Chim. Acta* **1988**, *175*, 329−336.

Howell, S. K.; Wang, Y. M. Quantitation of physiological α-tocopherol, metabolites, and related compounds by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1982**, *227*, 174−180.

Ikenoya, S.; Abe, K.; Tsuda, T.; Yamano, Y.; Hiroshima, O.; Ohmae, M.; Kawabe, K. Electrochemical detector for high-performance liquid chromatography. II. Determination of tocopherols, ubiquinones and phylloquinone in blood. *Chem. Pharm. Bull.* **1979**, *27*, 1237−1244.

Kaneko, T.; Nakano, S. I.; Matsuo, M. Protective effect of vitamin E on linoleic acid hydroperoxide-induced injury to human endothelial cells. *Lipids* **1991**, *26*, 345- 348.

Lang, J.; Gohil, K.; Packer, L. Simultaneous determination of tocopherols, ubiquinols and ubiquinones in blood, plasma, tissue homogenates and subcellular fractions. *Anal. Biochem.* **1986**, *157*, 106−116.

Lee, B. L.; Chua, S. C.; Ong, H. Y.; Ong, C. N. High-performance liquid chromatographic method for routine determination of vitamins A and E and β-carotene in plasma. *J. Chromatogr.* **1992**, *581*, 41−47.

Lehmann, J.; Martin, H. L. Improved direct determination of alpha- and gammatocopherols in plasma and platelets by liquid chromatography, with fluorescence detection. *Clin. Chem.* **1982**, *28*, 1784−1787.

MacCrehan, W. A. Determination of retinol, α-tocopherol, and β-carotene in serum by liquid chromatography. *Methods Enzymol.* **1990**, *189*, 172−181.

McMurray, C. H.; Blanchflower, W. J.; Rice, D. A. Influence of extraction techniques on determination of α-tocopherol in animal feedstuffs. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1258−1261.

Milne, D. B.; Botnen, J. Retinol, α-tocopherol, lycopene, and α- and β-carotene simultaneously determined in plasma by isocratic liquid chromatography. *Clin. Chem.* **1986**, *32*, 874−876.

Nierenberg, D. W.; Nann, S. L. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. *Am. J. Clin. Nutr.* **1992**, *56*, 417−426.

Sarzanini, C.; Mentasti, E.; Vincenti, M.; Nerva, M.; Gaido, F. Determination of plasma tocopherols by high-performance liquid chromatography with coulometric detection. *J. Chromatogr.* **1993**, *620*, 268−272.

Seta, K.; Nakamura, H.; Okuyama, T. Determination of α-tocopherol, free cholesterol, esterified cholesterols and triacylglycerols in human lipoproteins by high-performance liquid chromatography. *J. Chromatogr.* **1990**, *515*, 585−595.

Sowell, A. L.; Huff, D. L.; Yeager, P. R.; Caudill, S. P.; Gunter, E. W. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, *trans*-β-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. *Clin. Chem.* **1994**, *40*, 411−416.

Teissier, E.; Walters-Laporte, E.; Duhem, C.; Luc, G.; Fruchart, J.-C.; Duriez, P. Rapid quantification of α-tocopherol in plasma and low- and high-density lipoproteins. *Clin. Chem.* **1996**, *42*, 430−435.

Woodall, A. A.; Britton, G.; Jackson, M. J. Dietary supplementation with carotenoids: effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. *Br. J. Nutr.* **1996**, *76*, 307−317.

Zaman, Z.; Flieden, P.; Frost, P. G. Simultaneous determination of vitamins A and E and carotenoids in plasma by reversed-phase HPLC in elderly and younger subjects. *Clin. Chem.* **1993**, *39*, 2229−2234.

Received for review July 11, 1997. Revised manuscript received December 29, 1997. Accepted December 30, 1997. This research was supported in part by BASF, Mount Olive, NJ; Hoffmann La-Roche Ltd., Mississauga, ON; The Natural Science and Engineering Research Council of Canada (NSERC); The University of Manitoba; and the Deutsche Forschungsgemeinschaft (DFG, Habilitandenstipendium HO 1631/1-1), Germany. This paper was presented in part at the Conference of the Society of Nutrition Physiology, March 4−6, 1997, and was published in abstract form (*Proc. Soc. Nutr. Physiol.* **1997**, *6*, 153).

JF970596I