

## HPLC WITH UV DETECTION FOR MEASUREMENT OF VITAMIN E IN HUMAN MILK

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### SUMMARY

Extraction of vitamin E from human milk, by use of hexane, with and without prior saponification, was compared. Analysis of milk extracts was performed by HPLC with a C<sub>18</sub> column and UV detection. With both methods a significant relationship ( $P < 0.01$ ) was obtained between levels of  $\alpha$ -tocopherol and  $\gamma$ -tocopherol in human milk, although saponification resulted in higher and more consistent recovery of the internal standard,  $\delta$ -tocopherol ( $99.6 \pm 4.0\%$ ), and significantly improved the reliability of the data. The detection limit of the method (including saponification, extraction, and HPLC analysis) was  $0.65 \mu\text{g mL}^{-1}$   $\delta$ -tocopherol in milk. The proposed saponification step was a simple modification of the method, because it was performed in the same tube as the extraction, took 30 min only, and did not involve use of an inert gas. The improved method was used to measure vitamin E in several milk samples.

### INTRODUCTION

The main biological function of vitamin E is as an antioxidant which protects the polyunsaturated fatty acids of cell membranes from free-radical damage. An adequate supply of vitamin E in milk to newborn and, especially, preterm babies is vital for their normal development. Several publications have reported use of a variety of different methods [1–3] for determination of levels of tocopherols in human milk at different stages of lactation. In this work tocopherols were measured after direct extraction with hexane, after disruption of milk fat globules with ethanol

or methanol, without saponification. This method was originally developed for extraction of tocopherols from human plasma and serum [1–3] and has been used for human milk without additional validation [4,5]. The milk matrix is, however, very different from that of plasma or serum and the efficiency of liquid–liquid extraction may be different. There is also a possibility that some of the vitamin E, as a result of its interaction with other lipid and non-lipid constituents of milk, will not be readily available for extraction with hexane. The official method of measurement of vitamin E in foods, oils, and a variety of lacteal matrices involves alkaline saponification and has been widely used [6–8]. Saponification is performed before extraction as a separate step and is designed to overcome interactions between lipids, lipid-soluble vitamins, and the matrix. It is regarded as essential for overcoming both hydrophobic and polar interactions. Saponification is often regarded as a lengthy and tedious procedure with the potential to cause degradation of vitamin E; this seems to be a possible reason why it has been avoided [9,10]. Sometimes, as a precaution against damage to vitamin E, it is performed under an inert gas [6] and always in the presence of antioxidants such as pyrogallol, BHT, and ascorbic acid [6–8]. Use of an internal standard is very important to ensure that this protection is adequate. In this work we have compared two hexane-based methods for extraction of vitamin E from human milk. One is liquid–liquid extraction with hexane after simple saponification, the other is liquid–liquid extraction with hexane directly after disruption of milk fat globules with alcohol.

## **EXPERIMENTAL**

All organic solvents were HPLC grade (HiPerSolv). KOH and HCl (Aristar grade) were purchased from VWR International (Poole, UK).  $\alpha$  and  $\gamma$ -tocopherol and tocopherol acetate were obtained from Sigma (Dorset, UK).  $\delta$ -Tocopherol was purchased from Supelco (Dorset, UK). Ultra-pure water was used throughout the study.

### **Collection of Milk Samples and Preparation of Standards**

Recruitment of mothers into the study was performed with the approval of The Local Research Ethics Committee. Informed consent was obtained. Twenty mothers with a plentiful supply of milk who had given birth prematurely were recruited into the study (Table I). Milk was expressed into sterile plastic containers using an electric pump that was used

routinely at the neonatal unit. Each milk specimen represented complete expression from one breast. After collection milk was immediately frozen and kept in the freezer at  $-40^{\circ}\text{C}$  until analysis. Analyses were performed within 10 days of collection.

**Table I**

Clinical data for milk donors ( $n = 20$ )

Data	Average $\pm$ SD	Range
Age (years)	$28 \pm 6$	20–41
Gestation (weeks)	$32 \pm 4.5$	24–38
Milk volume per breast (mL)	$86 \pm 32$	35–170
Days since delivery	$9 \pm 2.5$	3–13
Days expressing	$8 \pm 2.3$	3–12

Stock solutions of  $\delta$ -tocopherol standards ( $10 \text{ mg mL}^{-1}$ ) were prepared in ethanol and kept for one week at  $-20^{\circ}\text{C}$ . Spiking solutions of  $\delta$ -tocopherol standards were prepared on the day of analysis by appropriate dilution of stock solutions. Other tocopherol standards were prepared in the same way.  $\delta$ -Tocopherol was used as internal standard because its chemical structure was similar to those of the other forms of tocopherol assayed (i.e.  $\alpha$  and  $\gamma$ -tocopherol and tocopherol acetate).

### Extraction of Tocopherols from Human Milk

After defrosting, milk specimens were warmed to  $38^{\circ}\text{C}$  and briefly sonicated to ensure sample homogeneity. For the same reason the sampling of milk was performed at  $38^{\circ}\text{C}$  with continuous stirring. All milk specimens were extracted using both methods, each in triplicate. To evaluate native  $\delta$ -tocopherol content, all milk specimens were also extracted by both methods without  $\delta$ -tocopherol spike.

#### *Method I*

Milk (1 mL) was spiked with  $10 \mu\text{L}$   $\delta$ -tocopherol standard (7–10  $\mu\text{g}$ ). Methanol (1 mL) containing pyrogallol (3%, w/v) then 1 mL aqueous KOH (10%, w/v) were added. Samples were vortex-mixed and placed in a water bath. Saponification was performed at  $70^{\circ}\text{C}$  for 30 min. After the first 15 min of saponification the tubes were briefly vortex-mixed. After cooling of the tubes on ice, samples were acidified to pH  $\sim 2$  with 6 M

HCl. Then 4 mL hexane was added. Tubes were vigorously vortex-mixed for 20 s, three times, and kept on ice between mixing. To separate the emulsion the tubes were centrifuged at room temperature at 1300g for 10 min. The top, organic, layer was carefully transferred to a clean Pyrex container and evaporated, under a gentle stream of nitrogen, on a warm plate at 40°C. The fatty residue was reconstituted in 0.5 mL methanol–propan-2-ol (1:1 v/v) with warming to 30°C.

#### *Method II*

Milk (1 mL) was spiked with 10 µL δ-tocopherol standard (7–10 µg). To disrupt milk fat globules 1 mL methanol containing pyrogallol (2%, w/v) was added. Tubes were kept on ice for 10 min. Then 3 mL hexane was added. After this step, extraction with hexane, separation, evaporation, and reconstitution of the samples were identical to the procedures used in Method I, described above.

To assess the linearity of the recovery by both methods we used a pooled milk sample ( $n = 18$ ). Pooled milk was sampled as described above, spiked with different amounts of δ-tocopherol (0.7, 1.4, 2.8, 7, 14, and 28 µg mL<sup>-1</sup> for Method I and 1.49, 4.66, 7.46, 18.5 and 37.3 µg mL<sup>-1</sup> for Method II;  $n = 4$  for each concentration) and extracted accordingly.

To protect vitamin E from possible degradation during extraction and saponification we used pyrogallol (1% final concentration).

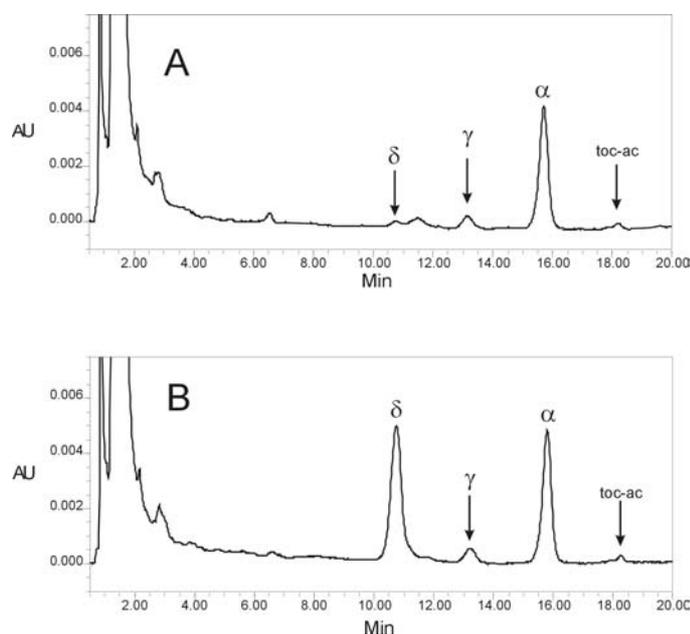
#### **Chromatographic System and Conditions**

HPLC was performed with a Waters (USA) system incorporating an Alliance 2690 separations module and a 996 photodiode-array detector and operated by Millennium<sup>32</sup> software. Tocopherols were separated on a Waters Symmetry C<sub>18</sub> column (3.9 mm × 150 mm) with a Waters Symmetry C<sub>18</sub> (3.9 mm × 10 mm) guard column. The mobile phase was initially a linear gradient of acetonitrile in water (from 95 to 100% in 10 min) then 100% of acetonitrile for 10 min; the flow rate was 1 mL min<sup>-1</sup>. The column temperature was 45°C and the wavelength-range scanned was 275–350 nm. Samples were kept in an autosampler at 30°C and the injection volume was 25 µL. Peak areas of tocopherols were integrated at 295 nm. Tocopherols were identified by coelution after spiking the extracted samples with solutions of the tocopherols in ethanol. To ensure reproducible retention on the column, after each batch of 30–40 milk extracts the column was washed with propan-2-ol (1 mL min<sup>-1</sup> for 60 min at 45°C). To

evaluate the recovery of the  $\delta$ -tocopherol spike after extraction, 10  $\mu\text{L}$  spiking solution was diluted with 0.5 mL methanol–propan-2-ol (1:1, v/v) and 25  $\mu\text{L}$  of this solution was injected on the column. Peak areas of  $\delta$ -tocopherol spikes in milk extracts were expressed as percentages of the peak areas of  $\delta$ -tocopherol solutions prepared as described above. The amounts of native  $\alpha$  and  $\gamma$ -tocopherols were calculated on the basis of their peak areas and the percentage recovery. The latter was calculated for each sample using the peak area of  $\delta$ -tocopherol spike. Regression analysis was performed using Minitab software. The difference was considered significant if  $P < 0.05$ . Means and standard deviations are reported.

## RESULTS AND DISCUSSION

Typical chromatograms recorded at  $\lambda = 295$  nm showing the elution profile of  $\delta$ ,  $\gamma$ , and  $\alpha$ -tocopherol in spiked and unspiked human milk are shown in Fig. 1. In agreement with previous reports [4,5]  $\delta$ -tocopherol



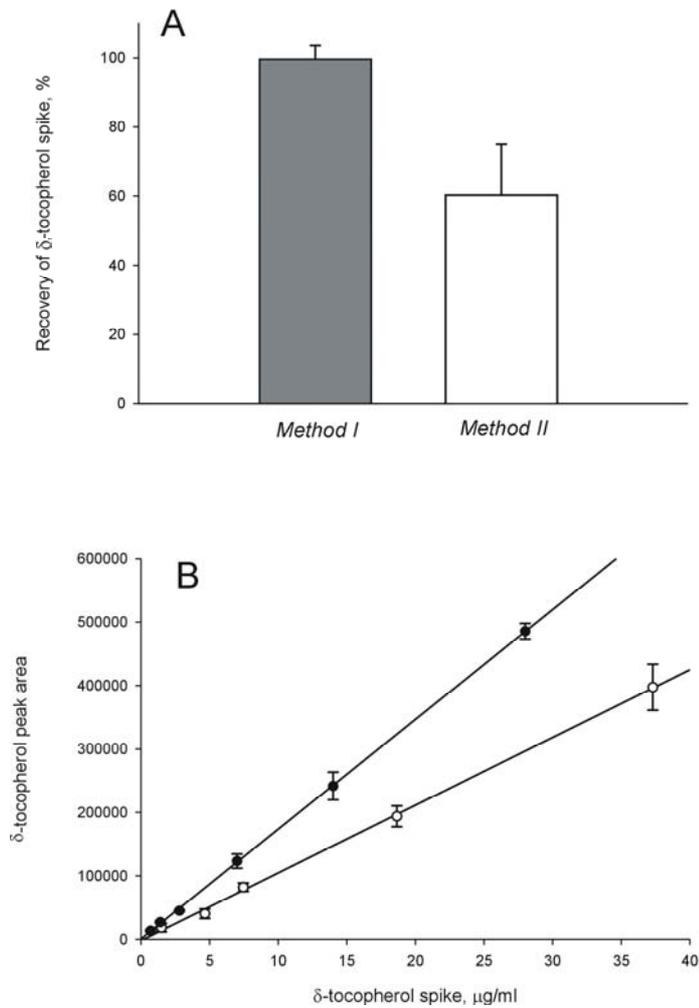
**Fig. 1**

Chromatograms at 295 nm of different forms of vitamin E extracted from human milk using Method I. Extraction conditions are as described in the experimental section. A. Unspiked milk sample. B. The same milk sample spiked with 7  $\mu\text{g mL}^{-1}$   $\delta$ -tocopherol. Arrows indicate the elution times for  $\delta$ ,  $\gamma$ , and  $\alpha$ -tocopherols and tocopherol acetate.

was either absent from milk or native trace amounts were usually below the limits of UV quantification (i.e.  $S < 3 \times LD$ , where  $LD$  is the limit of UV detection).  $\delta$ -Tocopherol was therefore used in the study as an internal standard for quantification of recovery and levels of other tocopherols. Obviously, the native amount of  $\delta$ -tocopherol in human milk is negligible in comparison with the amount of  $\delta$ -tocopherol added as the internal standard. The major forms of vitamin E found in milk were  $\alpha$  and  $\gamma$ -tocopherol, and tocopherol acetate was present in minor amounts in some milk samples.

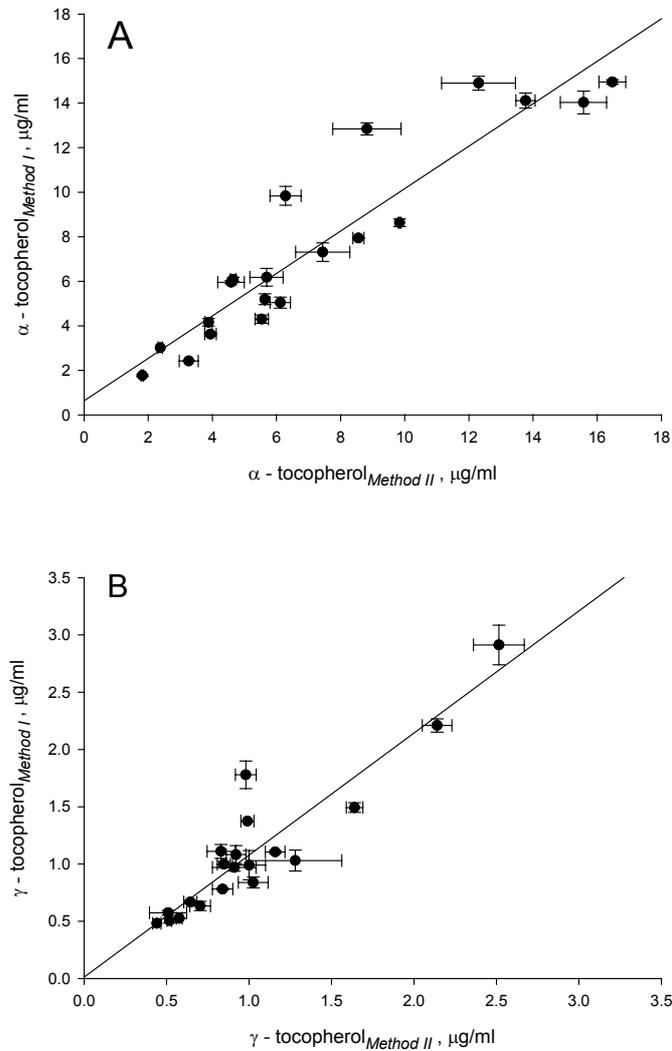
As is apparent from Fig. 2, the ranges of the amount of  $\delta$ -tocopherol spike used in both methods are reasonably comparable (Method I, 0.7–28  $\mu\text{g mL}^{-1}$ , and Method II, 1.49–37.3  $\mu\text{g mL}^{-1}$ ). Recovery of the  $\delta$ -tocopherol spike from pooled milk sample was satisfactory within the physiological range of concentrations for both extraction procedures. The correlation coefficients ( $r$ ) obtained for assay of  $\delta$ -tocopherol standard in milk samples by use of both methods were indicative of very good linearity of recovery of  $\delta$ -tocopherol standard in Methods I and II ( $r_I = 0.9995$  and  $r_{II} = 0.9995$ , respectively). The ratio of the slope coefficients of the regression lines for Methods I and II was 1.6342, which was in good agreement with the value of the ratio for average  $\delta$ -tocopherol recovery in Methods I and II (100 and 60%, respectively; Figs. 2A and 2B). For  $S/N > 3$  the overall limit of detection of Method I (including saponification, extraction, and HPLC analysis) was 0.65  $\mu\text{g mL}^{-1}$   $\delta$ -tocopherol in milk. Percentage recovery of the internal standard for the two methods of extraction are shown in Fig. 2A. When extraction was performed directly, without saponification, recovery was approximately 60% and variable (range 19–82%) depending on the milk sample. Recovery after saponification, on the other hand, was nearly 100% within a more reproducible range (94–107%). There are two possible reasons for this improvement. First, after saponification the milk matrix does not affect the recovery. Second, the conditions of liquid–liquid extraction with hexane from saponified milk are better optimised than in direct extraction. Reduced recovery of the internal standard leads to less accurate measurement.

Correlation of levels of  $\alpha$  and  $\gamma$ -tocopherol in milk samples measured after direct extraction and extraction after saponification is shown in Fig. 3. There was a significant relationship between the two methods of extraction for both  $\alpha$ -tocopherol ( $r^2 = 0.868$ ,  $P < 0.001$ ) and  $\gamma$ -tocopherol ( $r^2 = 0.851$ ,  $P < 0.001$ ). Levels of the major form of vitamin E,  $\alpha$ -tocopherol, in milk samples were variable within the range 2.8–15  $\mu\text{g mL}^{-1}$  ( $7.2 \pm 4.2 \mu\text{g mL}^{-1}$  for Method I compared with  $7.0 \pm 4.1 \mu\text{g mL}^{-1}$  for Method II).



**Fig. 2**

A. Recovery of the internal standard ( $\delta$ -tocopherol spike) from human milk using the two methods of extraction (extraction conditions are as described in the experimental section). Means and standard deviations are shown ( $n = 20$ ). Recovery using Methods I and II is 100 and 60%, respectively. B. Recovery of  $\delta$ -tocopherol spike from pooled milk sample using two methods of extraction (extraction conditions are as described in the experimental section). Regression analysis (Method I,  $y(x) = 17311x + 255$ ,  $r^2 = 0.999$ ; Method II,  $y(x) = 10593x$ ,  $r^2 = 0.998$ ) was performed by plotting the integrated peak areas for  $\delta$ -tocopherol against the concentration of the spike. The value of the ratio of the slope coefficient of the regression line for Methods I and II is 1.6342; the value of ratio of the recovery for Method I/Method II is 1.6667. Key: closed circles indicate Method I, open circles indicate Method II. Means and standard deviations are shown,  $n = 4$ .



**Fig. 3.**

Correlation of the levels of native  $\alpha$  and  $\gamma$ -tocopherol in human milk samples, measured after use of the two methods of extraction. A. Regression analysis ( $y(x) = 0.063 + 0.952x$ ;  $r^2 = 0.868$ ;  $P < 0.001$ ) was performed by plotting integrated peak areas for native  $\alpha$ -tocopherol in twenty milk samples, measured after extraction using Method I, against integrated peak areas for native  $\alpha$ -tocopherol in the same samples measured after extraction using Method II. B. Regression analysis for  $\gamma$ -tocopherol ( $y(x) = 0.01 + 1.07x$ ;  $r^2 = 0.851$ ,  $P < 0.01$ ) was performed in the same way as for  $\alpha$ -tocopherol. Average and standard deviation of three measurements for the same milk specimen with the two methods are shown.

For determination of native concentration of  $\alpha$ -tocopherol in human milk by Methods I and II,  $\delta$ -tocopherol was used as the internal standard. Although we were able to demonstrate a significant correlation between results obtained with the two methods, the method including saponification gave higher and more consistent recoveries for the internal spike and, therefore, more reliable data. The proposed saponification step was a simple addition to the method, because it was performed in the same tube as the extraction, took 30 min only, and did not involve use of an inert gas.

## CONCLUSIONS

Two extraction methods for the evaluation of vitamin E in human milk were compared. Direct extraction of tocopherols with hexane resulted in  $60 \pm 15\%$  recovery of the internal standard,  $\delta$ -tocopherol. Application of the improved method, which included a simple saponification step, resulted in  $99.6 \pm 4.0\%$  recovery for the same internal standard. There was a significant relationship between tocopherol concentrations in milk specimens measured using the two extraction procedures ( $P < 0.01$ ). This implies that both methods of extraction can be used for determination of vitamin E in human milk, although the evidence presented herein suggests that extraction after saponification may significantly improve the reliability of such data.

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