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Comparison of the separation of fat-soluble vitamins using β-cyclodextrins in high-performance liquid chromatography and micellar electrokinetic chromatography

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Abstract

The use of various types of cyclodextrins (β-cyclodextrin, dimethyl-β-cyclodextrin, hydroxyethyl-β-cyclodextrin) as mobile phase additives were successful in the reversed-phase high-performance liquid chromatographic and micellar electrokinetic chromatographic (MEKC) separation of fat-soluble vitamins. The use of MEKC over HPLC gave either comparable or improved resolution of these hydrophobic solutes. Both techniques proved successful in the separation of vitamins D_2 and D_3. Dimethyl-β-cyclodextrin as mobile phase and buffer additive provided the best separation of these two compounds. Generally, separation increased in both techniques with increasing cyclodextrin concentration. The addition of various cyclodextrins to the mobile phase aided in the separation of vitamins E and E acetate by HPLC with a phenyl stationary phase. The addition of cyclodextrin to the running buffer in MEKC gave little improvement in the resolution of these two compounds. In the separation of α-, β-, γ-, δ-tocopherol, the use of various derivatized β-cyclodextrins as HPLC mobile phase additives was not able to separate β- and γ-tocopherol; however the use of dimethyl-β-cyclodextrin as a buffer additive in MEKC resolved all four isomers. © 1997 Elsevier Science B.V.

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1. Introduction

Vitamins are molecules essential for normal metabolic processes. They serve catalytic functions and serve as co-enzymes, prosthetic groups on proteins, and hormones. Vitamins can be divided into two categories: fat-soluble and water-soluble. This investigation focuses on the analysis of the fat-soluble variety. These are very hydrophobic compounds comprised, entirely or separately, of 5-carbon isoprenoid units derived initially from acetyl CoA in plant and animal species capable of their biosynthesis.

Fat-soluble vitamin analysis by HPLC is usually performed in the normal phase due to their considerable hydrophobic character [1–3], although the separation of vitamins D_2 and D_3 has been reported using reversed-phase methods [4]. Analysis by reversed-phased HPLC methods is not ideal as the elution of these vitamins typically suffers from excessive band broadening due to very high retention factors. However, the preference of reversed-phase techniques over normal-phase ones due to increased versatility in solute selectivities and the use of less hazardous solvents [5] demonstrates the need to develop re-

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versed-phase methods for analytes that are usually performed in the normal-phase.

Recent advances in capillary electrophoresis has resulted in the development of a new analytical technique that is complementary to HPLC. The innovation of micellar electrokinetic chromatography (MEKC) has allowed for the analysis of uncharged molecules using a charged micellar pseudo-phase.

Cyclodextrins are common additives in both HPLC and MEKC. They are toroidally shaped oligosaccharides made up of D-(+)-glycopyranose units joined by α-(1,4)-linkages. There is sufficient evidence that fat-soluble vitamins form inclusion complexes with β-cyclodextrin (β-CD), as a variety of vitamin–CD complexes have been synthesized and characterized [6–8]. To date, only a minimal amount of research has been published using cyclodextrins for vitamin analysis by HPLC or MEKC. In HPLC, El-Gizawy and co-workers [9] separated various water-soluble vitamins using a β-cyclodextrin stationary phase while Abidi and Mounts [10] separated α-, β-, γ-, and δ-tocopherol on β- and γ-cyclodextrin columns using a non-aqueous mobile phase. Capillary electrophoretic separation of vitamins has focused mainly on the water soluble variety. Jegle [11] separated water-soluble vitamins by capillary zone electrophoresis (CZE). CZE was used by Ma and co-workers [12] to look at retinol (vitamin A)–protein complexes and by Huopalahti and Sunell [13] to separate some B-series vitamins. Nishi and co-workers [14] separated 11 water-soluble vitamins using MEKC while Boonkerd and co-workers [15] used it to separate the vitamins of the B group. Yik and co-workers [16] used MEKC to analyze a mixture of B_6 vitamin isomers while using amperometric detection. Ong and co-workers [17] separated nine vitamins using MEKC with β- and γ-cyclodextrin in the buffer. The only two fat-soluble vitamins studied here were vitamin A and α-tocopherol.

This first section of this paper describes the use of cyclodextrins as mobile phase additives in the reversed-phase high performance liquid chromatographic separation of a series of fat-soluble vitamins. The second part investigates the separation of various fat-soluble vitamins using MEKC and several β-cyclodextrins as buffer additives. The vitamins under investigation include vitamins A, D_2, D_3, the four isomers of E and E acetate.

2. Experimental

2.1. Apparatus

Chromatography was performed using a liquid chromatographic system consisting of a Model M6000-A pump (Waters, Milford, MA, USA), a model 7125 injector containing a 10-μl loop (Rheodyne, Cotati, CA, USA), and a Model LC290 UV detector (Perkin–Elmer, Norwalk, CT, USA). The chromatograms were recorded on a Model DE-120 strip chart recorder (Goerz Electro, Austria). Separations were performed on a Zorbax ODS (150×4.6 mm I.D.) and a phenyl column (100×4.6 mm I.D.) purchased from Chromatographic Specialities (Brockville, Canada). Capillary electrophoresis was performed on a CE system comprising of a Bertan Model 230R power supply (Bertan Associates, Hicksville, NY, USA) and an Isco CV^4 (Isco, Lincoln, NE, USA) UV–Vis detector. The output of the power supply was connected to the buffer reservoir via platinum electrodes (Bioanalytical Systems, West Lafayette, IN, USA). Separations were performed on fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 375 μm O.D.. Electropherograms were acquired with the Waters System Interface Module (Millipore, Milford, MA, USA) and then processed on the Waters Maxima 820 Chromatography Workstation.

2.2. Chemicals

Ergocalciferol (D_2), cholecalciferol (D_3), α-tocopherol (E), β-cyclodextrin (β-CD), hydroxyethyl-β-cyclodextrin (HE-β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and sodium tetraborate (borax) were obtained from Aldrich (Milwaukee, WI, USA). δ-Tocopherol was purchased from Sigma (St. Louis, MO, USA). β-Tocopherol and γ-tocopherol were purchased from Matreya (Pleasant Gap, PA, USA). Ultra pure sodium dodecyl sulfate (SDS) was obtained from ICN Biochemicals (Montreal, Canada). Sodium hydroxide and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Methanol was purchased from Fisher (Montreal, Canada). Potassium phosphate, ethanol and HPLC grade acetonitrile were obtained

from A and C Chemicals (Montreal, Canada). Water was doubly distilled and deionized.

2.3. Procedures

The HPLC mobile phases were prepared by mixing methanol with water. Cyclodextrin was then dissolved and the mixture was degassed and filtered through a 0.45-μm membrane filter. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 1 μl. The flow-rate was 1.0 ml/min. The wavelength of detection was 280 nm.

The CE capillaries had a total length of 77 cm and a separation length of 55 cm and were conditioned prior to use with 1 *M* NaOH for 10 min. Capillaries were rinsed between runs with 0.1 *M* NaOH, distilled water and then the running buffer for two min each. The pH of the running buffer was adjusted by the addition of appropriate amounts of boric acid, hydrochloric acid or sodium hydroxide. All buffers were filtered through a 0.45-μm membrane and then degassed prior to use. Samples were dissolved in the running buffer, degassed and injected hydrodynamically at a height of 15 cm and the injection time was 8–10 s.

Retention and migration times for HPLC and CE were determined by averaging at least three separate determinations. A reproducibility study was conducted where five injections had a R.S.D. of less than 2.5% for the retention factor and of less than 7% for the peak resolution.

3. Results and discussion

3.1. Separation of vitamins by HPLC

Reversed-phase HPLC analysis of fat-soluble vitamins is challenging as their hydrophobic nature makes them difficult to elute from the hydrophobic stationary phases used for this technique. The problem can be alleviated by the use of additives to the mobile phase that will complex with the vitamins and assist in their elution from the chromatographic column. Cyclodextrins are suitable for use as mobile phase additives. This part of the study will discuss the separation of fat-soluble vitamins by reversed-phase HPLC using β-cyclodextrin and derivatized β-cyclodextrins as mobile phase additives to increase the vitamins' affinity for the mobile phase thus reducing retention factors and band broadening in reversed-phase systems.

The use of cyclodextrins in the mobile phase can lead to the separation of different vitamins while maintaining a reasonable retention time. Fig. 1 shows the separation of vitamins A, D_2, D_3, and E (α-tocopherol) on a C_{18} column with 10 mg/ml dimethyl-β-cyclodextrin in the mobile phase. The addition of the cyclodextrin to the mobile phase reduced the retention time of the vitamins by approximately 50%. Table 1 shows the effect of varying the cyclodextrin concentration on the retention factor of these four compounds.

As the cyclodextrin concentration increased, the retention factor of vitamins D_2, D_3 and E decreased,

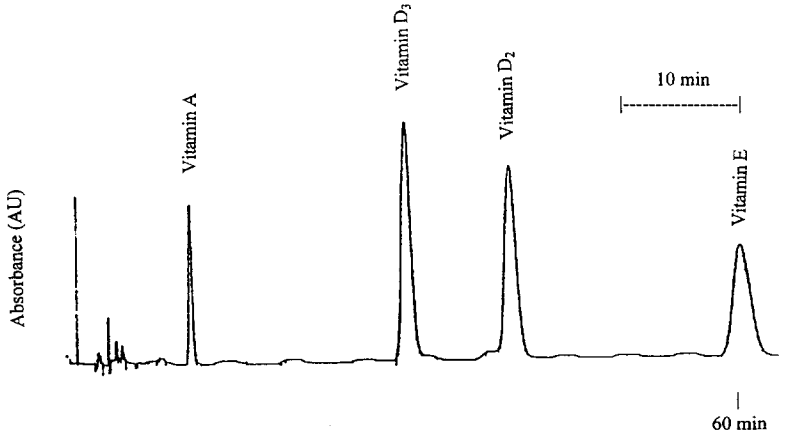

Fig. 1. Separation of vitamins A, D_2, D_3 and E with 10 mg/ml DM-β-CD in the mobile phase. Conditions: methanol–water (90:10, v/v).

Table 1
Effect of DM-β-CD concentration on retention factor

[DM-β-CD] (mg/ml)	k'			
	Vitamin A	Vitamin D_2	Vitamin D_3	Vitamin E
0	6.05	30.9	33.1	50.5
0.5	6.00	31.1	32.4	51.0
1.0	6.00	31.1	31.1	50.4
2.5	5.37	28.2	27.5	45.8
5.0	4.92	27.2	23.4	43.0
7.5	4.88	23.8	18.6	38.8
10.0	4.39	19.9	14.7	30.8
13.0	4.33	19.6	13.5	30.4

indicating the formation of inclusion complexes. The vitamin–cyclodextrin inclusion complexes acquire a greater affinity for the mobile phase and therefore have a shorter elution time. A significant decrease in retention factor was not observed for vitamin A. It was concluded that vitamin A does not form a strong inclusion complex with cyclodextrin in the mobile phase at these high organic concentrations. The small size of the cyclohexene and the relatively low polarity of the mobile phase would hinder strong inclusion complex formation.

3.2. Separation of vitamins D_2 and D_3 by HPLC

The addition of cyclodextrin to the mobile phase greatly improved the separation of vitamins D_2 and D_3 on a C_{18} stationary phase. Note in Table 1 that vitamin D_3 elutes before D_2 at a cyclodextrin concentration below 1 mg/ml. There is complete loss of resolution at a cyclodextrin concentration of 1 mg/ml. This was because vitamin D_3 formed a stronger inclusion complex with cyclodextrin than D_2 resulting in the co-elution of D_3 with D_2 at this cyclodextrin concentration. The elution order of these vitamins was reversed at higher cyclodextrin concentrations.

A comparative study on the effect of derivatization of cyclodextrin on the separation of vitamins D_2 and D_3 was carried out. Dimethyl-β-cyclodextrin, triacetyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin were investigated. Underivatized β-cyclodextrin, with a water solubility of 1.85 g/100 ml [18], was not soluble at these low mobile phase water concentrations. In all cases, the retention order of the two vitamins was reversed at a cyclodextrin concentration of 5 mg/ml when compared to no additive in the mobile phase. The DM-β-CD provided greater separation ($\alpha = 1.20$) than the other two derivatives ($\alpha = 1.05$ and 1.06 for HE- and TA-β-CD, respectively) as the methyl groups present on the cyclodextrin rim produce a more hydrophobic cavity than the hydroxyethyl or acetyl groups, thus allowing for the formation of a stronger inclusion complex resulting from greater interactions between the guest and cyclodextrin rim groups.

3.3. Separations of vitamin E isomers by HPLC

As was observed in Fig. 1, vitamin E was strongly retained on a C_{18} stationary phase, even with cyclodextrin present in the mobile phase. The severe band broadening associated with very large retention factors would make the separation of vitamin E isomers, namely α-, β-, γ-, δ-tocopherol and E-acetate, practically impossible with this stationary phase. The use of a stationary phase with phenyl functional groups proved more favourable in the separation of the vitamin E isomers. Phenyl stationary phases are less hydrophobic than C_{18} and have slightly different selectivities due to the electron density of the aromatic ring [19]. Hydrophobic solutes will be retained to a lesser degree on a phenyl than a C_{18} column thus reducing band broadening enough to make cyclodextrins useful as a mobile phase additive in the separation of vitamin E isomers.

The resolution of vitamin E from E acetate, its water soluble derivative, was not possible without the addition of cyclodextrins to the mobile phase. Fig. 2 shows the separation of these two vitamins

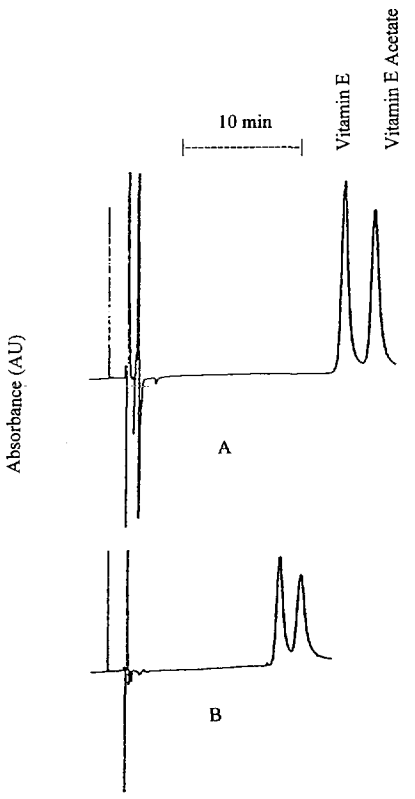

Fig. 2. Separation of Vitamins E and E acetate on a phenyl column with (A) β-CD; (B) DM-β-CD in the mobile phase. Mobile phase: 0.05 *M* KH_2PO_4–methanol (55:45, v/v) plus 5 mg/ml of the indicated cyclodextrin.

with β-cyclodextrin and dimethyl-β-cyclodextrin (DM-β-CD) as mobile phase. The underivatized β-cyclodextrin was a practical additive in this case as the aqueous content of the mobile phase was high enough for it to be soluble. Both cyclodextrins gave similar separations for the two vitamins. At a concentration of 5 mg/ml, both cyclodextrins gave a separation factor of 1.14.

This reversed-phase system was used to attempt to separate the four E vitamin isomers, namely α-, β-, γ- and δ-tocopherol, with β-cyclodextrin and dimethyl-β-cyclodextrin as mobile phase additives. This was a very challenging separation due to the difficulty in separating β- and γ-tocopherol. This difficult separation was first performed by normal-phase HPLC [20] while little progress has been reported with reversed-phase methods. As was mentioned in the Introduction, Abidi and Mounts [10] separated them on β- and γ-cyclodextrin columns using a normal-phase mobile phase. Attempts at the resolution of all four E isomers on a phenyl stationary phase proved futile. Both types of cyclodextrins certainly improved the resolution of the α- and δ-isomers; however they failed to resolve β- and γ-tocopherol. Differentiation of the vitamin isomers by cyclodextrin is probably on the basis of inclusion complex strength alone as there are no differences in the segment of the molecules that protrudes from the torus; therefore there will not be any variation in interactions with the rim groups. It was found that the more hydrophobic isomers would form stronger inclusion complexes and therefore had shorter retention times. The successful separation of all four isomers by micellar electrokinetic chromatography will be discussed in Section 3.4.

3.4. Separation of vitamins by MEKC

The hydrophobic character of fat-soluble vitamins makes MEKC an obvious choice as a technique for their analysis. The vitamins will solubilize into the hydrophobic interior of an ionic micelle, such as SDS, and be capable of migrating electrophoretically. However, as will be shown, MEKC without the presence of cyclodextrins in the running buffer was not successful in separating most of the vitamins. Solute separation in micelle–cyclodextrin systems with anionic micelles are based on the neutral vitamins partitioning between the cyclodextrins, which travel with the electroosmotic flow towards the detector and the ionic micelles, which migrate electrophoretically in the opposite direction. As the electroosmotic flow is typically greater than the electrophoretic velocity, the net migration of the analyte is in the direction of the detector. Inclusion complexation will increase the migration velocity of the solutes through the capillary. This part of the study will discuss the use of CD-MEKC in the separation of fat-soluble vitamins, namely vitamins D_2, D_3, α-, β-, γ-, δ-tocopherol and E-acetate.

3.5. Separation of vitamins D_2 and D_3

Micellar electrokinetic chromatography, with various cyclodextrins in the buffer, was successful in the separation of vitamins D_2 and D_3. The separation

required the addition of 2% ethanol to the buffer to reduce the excessive band broadening due to resistance to mass transfer as the vitamins partition in and out of the micelle. The addition of a small amount of organic modifier is used to adjust the partition coefficient of the solutes in the micellar phase so that they are retained less by the micelles and are more available to interact with the cyclodextrins in the buffer. The addition of 2% ethanol to the buffer had a negligible effect on inclusion complexation. This had the consequence, however, of increasing migration times as the presence of ethanol reduced the electroosmotic flow.

The micellar system in the absence of cyclodextrin was not able to resolve vitamins D_2 and D_3; however resolution was achieved upon addition of cyclodextrin to the buffer. In the absence of cyclodextrins, the vitamins migrated at the same rate as the micellar flow, indicating that the solutes were not partitioning into the bulk solution. This is not surprising due to the vitamins' low water solubility. The micellar flow was estimated with Orange G as a marker. Fig. 3 shows the separation of vitamins D_2 and D_3 using β-cyclodextrin, dimethyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin as buffer additives.

The greatest separation was observed with dimethyl-β-cyclodextrin as the buffer additive. Resolution decreased when dimethyl-β-cyclodextrin was replaced with either underivatized β-cyclodextrin or hydroxyethyl-β-cyclodextrin. In the former case, some resolution was observed; however the limited water solubility of β-cyclodextrin limited its buffer concentration to no more than 14 m*M*. Comparing hydroxyethyl-β-cyclodextrin to dimethyl-β-cyclodextrin, the methylated derivative gave superior separation as the more hydrophobic cavity formed stronger inclusion complexes than the hydroxyethyl derivative thus allowing more time for separation.

The effect of varying the concentration of all three types of cyclodextrins in the running buffer was also studied. Fig. 4 shows the effect of cyclodextrin concentration on the resolution (R_s) of vitamins D_2 and D_3. Increasing cyclodextrin concentration in the running buffer resulted in improved separation of vitamins D_2 and D_3 with all three types of cyclodextrins examined. As was mentioned previously, the two vitamins were not resolved in the absence of cyclodextrin. As the cyclodextrin concentration in-

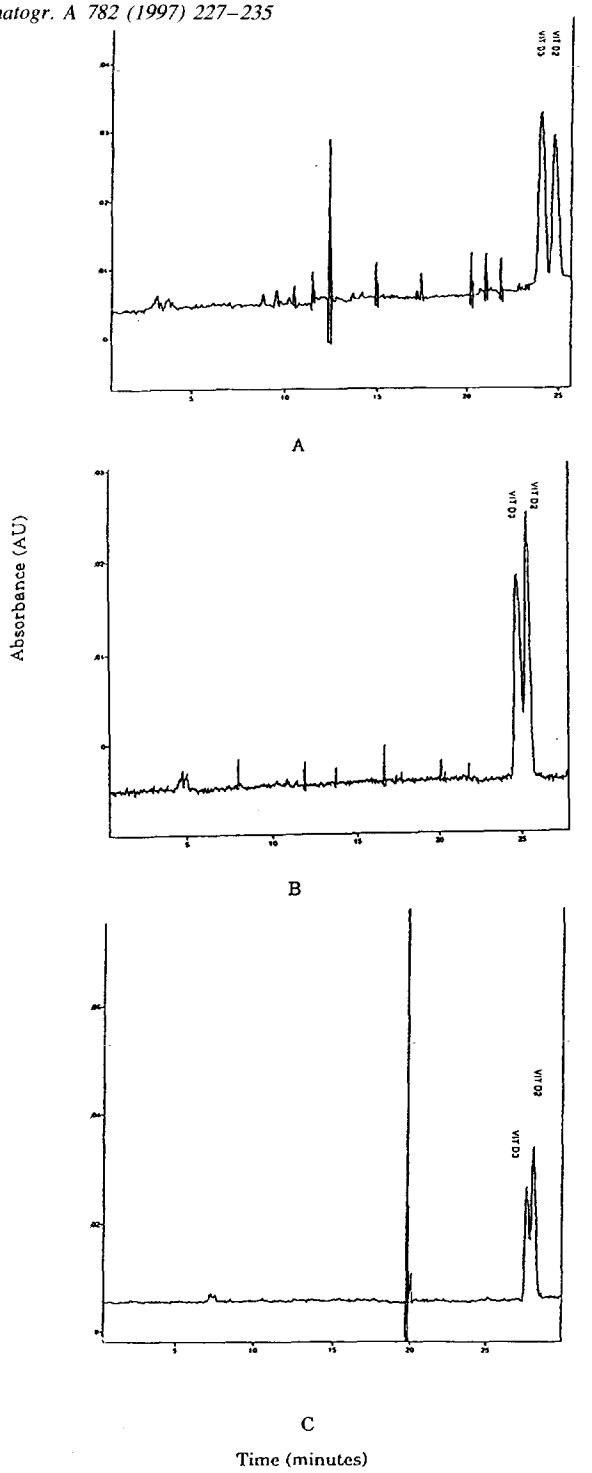

Fig. 3. Separation of vitamins D_2 and D_3 with various cyclodextrins in the buffer. Conditions: 15 m*M* phosphate, 20 m*M* SDS, 2% ethanol, pH 7.5, *V*=15 kV, plus (A) 40 m*M* DM-β-CD; (B) 14 m*M* β-CD; (C) 40 m*M* HE-β-CD.

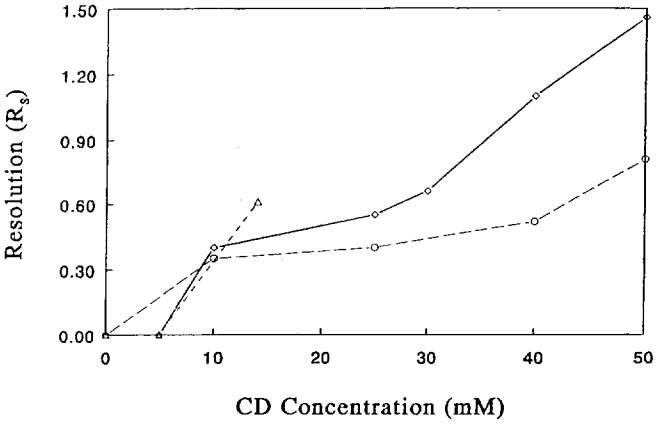

Fig. 4. Effect of cyclodextrin concentration on resolution of vitamins D_2 and D_3. Conditions: 15 m*M* phosphate, 20 m*M* SDS, 2% ethanol, pH 7.5, *V*=15 kV, plus the indicated amounts of cyclodextrin. ◊=DM-β-CD; △=β-CD; ○=HE-β-CD.

creased, separation improved remarkably with the greatest initial improvement occurring with the underivatized β-cyclodextrin. The underivatized β-cyclodextrin was not studied above 14 m*M* due to its limited water solubility. Dimethyl-β-cyclodextrin gave better results than the hydroxyethyl derivative as the stronger inclusion complex formed by DM-β-CD allowed for better resolution of vitamins D_2 and D_3 than HE-β-CD.

3.6. Separation of E vitamins

As was mentioned previously, the separation of α-, β-, γ- and δ-tocopherol is regarded as difficult due to their similar structures. As was mentioned earlier, cyclodextrins were successful in the partial separation of three of the four isomers by HPLC. Despite many attempts, resolution of β- and γ-tocopherol was not possible by HPLC; however, a CD-MEKC system was able to resolve all four isomers with DM-β-cyclodextrin in the buffer. Resolution without cyclodextrin in the buffer was not possible, as the micellar system alone resulted in only two peaks for the four components. The addition of DM-β-CD to the buffer succeeded in separating all four isomers. Fig. 5 shows the separation of α-, β-, γ- and δ-tocopherol by CD-MEKC. The improvement in the separation of the four vitamin E isomers by MEKC over HPLC was due to the greater efficiency observed in capillary electrophoretic techniques.

The separation of vitamin E (α-tocopherol) from vitamin E acetate was considerably easier by capillary electrophoresis than it was by high-performance liquid chromatography. There was good resolution with no cyclodextrin in the buffer, probably due to differences in the partition coefficient with the micellar phase. There was only a negligible improvement in separation with 25 m*M* dimethyl-β-cyclodextrin in the buffer, indicating that the cyclodextrin does not play a important role in the separation but rather differences in micelle solvation was a more important consideration in their resolution than inclusion complexation.

4. Conclusions

It has been demonstrated that using cyclodextrins in reversed-phase HPLC and MEKC proved to be useful in the separation of fat-soluble vitamins. The use of MEKC over HPLC gave either comparable or improved separation of these extremely hydrophobic solutes. Both techniques were successful in the separation of vitamins D_2 and D_3. Dimethyl-β-cyclodextrin provided the best separation of these two compounds. Generally, separation increased in both techniques with increasing cyclodextrin concentration.

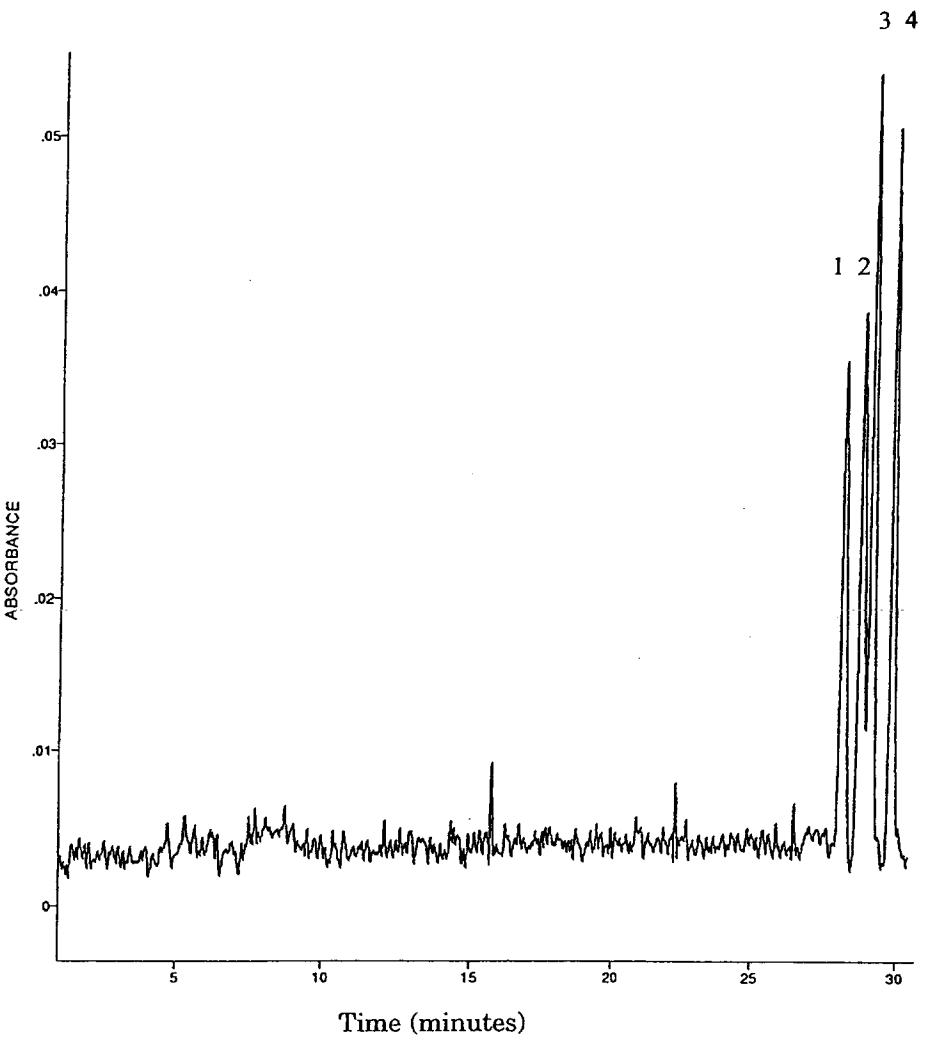

Fig. 5. Separation of α-, β-, γ- and δ-tocopherol by CD-MEKC. Conditions: 20 m*M* phosphate, 50 m*M* borate, 30 m*M* SDS, 25 m*M* DM-β-CD, pH 8.0, *V*=22 kV.

The addition of cyclodextrin to the mobile phase aided in the separation of vitamins E and E acetate by HPLC with a phenyl column. The addition of cyclodextrin to the running buffer was not necessary in their separation by MEKC. The addition of cyclodextrin to the buffer offered little improvement in separation. In the separation of α-, β-, γ-, δ-tocopherol, cyclodextrins were not able to separate β- and γ- tocopherol by HPLC; however they were successful in separating the other isomers. The use of cyclodextrin in MEKC procured the resolution of all four isomers.

Acknowledgments

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