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Separation of water- and fat-soluble vitamins by micellar electrokinetic chromatography

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ABSTRACT

A mixture of seven water- and two fat-soluble vitamins was successfully separated simultaneously using micellar electrokinetic capillary chromatography. In addition to sodium dodecyl sulphate, modifiers such as γ-cyclodextrin, β-cyclodextrin, and isopropyl alcohol were introduced into the electrophoretic media to investigate their effect on the overall separation of the nine vitamins. Amongst these modifiers, the combination of γ-cyclodextrin with sodium dodecyl sulphate in the electrophoretic medium was found to provide the best selectivity for separating vitamins.

INTRODUCTION

The separation of vitamins by chromatographic methods has been known for many years [1,2]. Amongst the chromatographic techniques, high-performance liquid chromatography (HPLC) has been one of the most popular choices [3,4]. However, the use of HPLC for the separation of this group of compounds has been found to suffer from a number of problems. In the case of isocratic elution, the main problem is usually due to the broadening and tailing of some of the vitamin peaks [5,6]. Consequently it is often difficult to achieve complete separation of these compounds by this method [7]. Thus it is common to implement gradient elution to achieve satisfactory results [6]. Furthermore the analysis of the fat- and water-soluble vitamins can only be achieved with sequential elution employing mobile phases of different polarity. To date the simultaneous separation of both groups of vitamins in a single analysis has not been reported.

High-performance capillary electrophoresis (CE) is a fairly new techniques and interest in this area has been growing rapidly in recent years. One of the reasons for its popularity is the exceptionally high separation efficiency achievable with this techniques, which is based on a simple instrumental set-up. In the case of capillary zone electrophoresis, separation of charged compounds can be achieved relatively easily.

The introduction of micelles into the electrophoretic medium by Terabe *et al.* [8] in 1984 has given another new dimension to this mode of separation. Since then, there have been numerous papers reporting the usefulness of this new technique,

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referred to as micellar electrokinetic chromatography (MEKC) [9–17]. The success of the MEKC technique could largely be due to the additional partition mechanism between the solutes and the micellar pseuco-stationary phase. Consequently the selectivity and peak shape are considerably improved, especially for the separation of neutral species.

The separation of water-soluble vitamins has been previously investigated by MEKC [9,11]. In this work, besides the water-soluble vitamins, fat-soluble vitamins were also examined. One of the objectives of this investigation is to attempt to simultaneously separate the two groups of vitamins in a single analysis by MEKC. The migration behavior of the vitamins under different pH and sodium dodecyl sulphate (SDS) concentrations was studied. In addition, the effects of modifiers including γ-cyclodextrin, β-cyclodextrin and organic modifiers on the separation of the two groups of vitamins were examined.

EXPERIMENTAL

The experiments were performed using the instrumental set-up described elsewhere [12]. A Spellman (Plainview, NY, USA) Model RHR30AN10/RCA power supply, capable of delivering up to a maximum of 30 kV was used. A fused-silica capillary (50 cm effective length × 50 μm I.D.) obtained from Polymicro Technologies (AZ, USA) was used as the separation tube. The detection of peaks was carried out on a Micro-UVis detector (Carlo Erba, Milan, Italy) with wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused silica capillary. A Linear Instruments (Irvine, USA) Model 252A/MM chart recorder was used to record the chromatograms. Sample solution was introduced by gravity feed. An injection time of 5 s at a height difference of 5 cm between the reservoirs was used for sample introduction.

All chemicals used were of analytical grade or better. The buffer solution was prepared by dissolving sodium dihydrogen phosphate dihydrate and sodium tetraborate in Millipore water. The electrophoretic media containing SDS and the modifiers were prepared as previously described [8]. In this work, the seven water-soluble vitamins are vitamins B, B_1, B_2, B_6, B_{12}, C and H, and the two fat-soluble vitamins investigated are A and E. A standard solution containing the vitamins and Sudan III at a concentration of 1000 ppm for each species was prepared. All these chemicals are of the purest grade and were supplied by Fluka (Buchs, Switzerland).

RESULTS AND DISCUSSION

In Fig. 1, the migration times for the vitamins obtained at different pH values are shown. From the results it can be seen that the migration time of each vitamin increased with increasing pH. This observation is consistent with previous investigation [9]. At the same time, it was noted that the migration order for all the vitamins remained unchanged throughout the range of pH values examined. This observation seems to suggest that there are no major changes in the extent of ionization for the two groups of vitamins at all the pH values investigated. The water-soluble vitamins were found to migrate much faster than the fat-soluble vitamins. This is in agreement

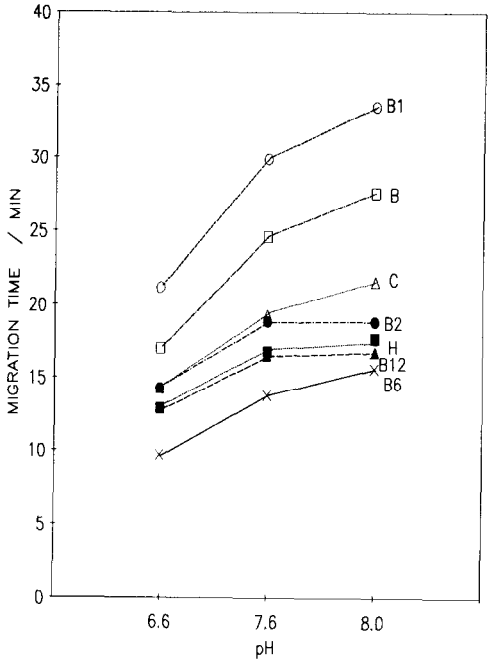

Fig. 1. Plot of migration times *versus* pH. All these experiments are conducted at a voltage of 15 kV across the 50 cm × 50 μm I.D. separating tube filled with 0.1 *M* borate–0.05 *M* phosphate buffer with 30 m*M* SDS. Note that the curves for the fat-soluble vitamins are not plotted since their capacity factors are very large. Vitamins: ○ = B_1; □ = B; △ = C; ● = B_2; ■ = H; ▲ = B_{12}; × = B_6.

with the fact that since the water-soluble vitamins are less lipophilic than the fat-soluble vitamins, they will not be influenced by the micelles and therefore would migrate earlier. On the other hand, the fat-soluble vitamins, A and E, co-eluted with Sudan III at lower pH. At a higher pH, vitamin A was found to migrate out faster than Sudan III. The main reason for these species having a similar migration time as that of Sudan III is that they are highly hydrophobic (*i.e.* they have very similar log P values to Sudan III). However at pH 8, the higher pH would have resulted in the dissociation of the carboxylate group of the vitamin A. Thus the decrease in the migration time in this case would presumably be due to the repulsive ionic interaction between the negatively-charged vitamin A and the anionic micelles.

The results obtained for the investigation on the effect of different SDS concentrations on the separation of the vitamins are illustrated in Fig. 2. From the figure, it can be seen that a similar migration pattern is observed, *i.e.* the water-soluble vitamins migrated out earlier and the two fat-soluble vitamins again have a similar migration time to that of Sudan III. It should be recognised that in the case of MEKC, when the solutes are electrically neutral the migration time is proportional to the SDS concentration. If the migration time is independent of the SDS concentration, one may conclude that the solute is totally exluded from the SDS micelles. On the other hand, if the migration of a solute is influenced by changes in the SDS concentration, then interaction between the solute and the micelles is expected. In the case of charged solutes, their migration due to electrophoretic interaction must be considered.

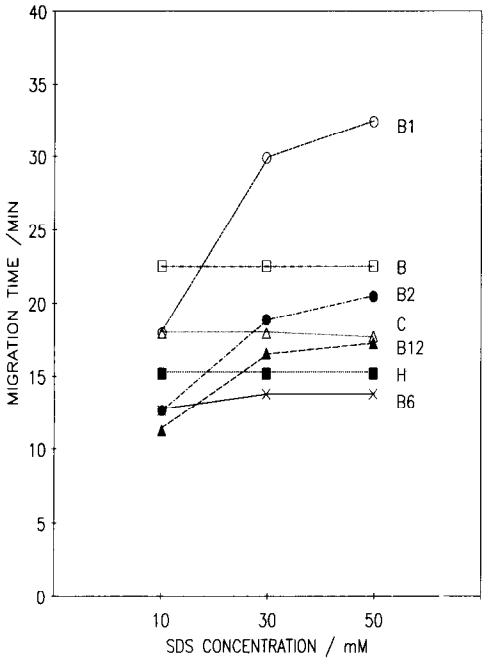

Fig. 2. Plot of migration times *versus* SDS concentration. All these experiments are conducted at a voltage of 15 kV across the 50 cm × 50 μm I.D. separating tube filled with 0.1 *M* borate–0.05 *M* phosphate buffer at pH 7.6. Note that the curves for the fat-soluble vitamins are not plotted since their capacity factors are very large. Symbols as in Fig. 1.

From the migration order observed, it seems that the vitamins can be categorised into the following groups:

(1) group I consisting of vitamins B_6, H, B and C;
(2) group II consisting of vitamins B_1 and B_{12}; and
(3) group III consisting of the vitamins B_2, A and E.

The vitamins in group I are generally the more hydrophilic species. Therefore they would be least solubilised by the micelles, and are expected to migrate out earlier than the vitamins in the other groups. Within this group, the migration times can be arranged in the following order: vitamins B_6 < H < C < B. The reason for this order observed can be explained mainly by the number of hydrophilic substituent groups present in each species. At the same time, the presence of any intra-molecular interaction (*i.e.* hydrogen bonding), which render the species less polar would also need to be considered. For example in the case of vitamin C, due to the orientation of the oxygen of the carbonyl group and hydroxyl groups, intra-molecular hydrogen bonding between these groups are very favourable. With the possibility of formation of this type of bonding, the hydrophobicity of vitamin C would be increased since the polar groups are now "caged up". Consequently, vitamin C, inspite of having more polar substituent grousp than most of the water-soluble vitamins, is less hydrophilic. Therefore because of these "caging" effect, vitamin C has a migration time longer than expected.

For vitamin B, the carboxylate hydrogen should be fairly acidic in the presence of the pyridine ring. Therefore, vitamin B would probably be assuming a negative charge at the pH investigated. Consequently vitamin B would migrate more slowly than the rest of the vitamins in this group because of the negative electrophoretic effect (ionic attraction to the positive electrode). In contrast to vitamin B, vitamin H has a small migration time despite the presence of a carboxylate group. This is because of the presence of the long alkyl chain which makes the carboxylate hydrogen less acidic to effect ionisation. Therefore, vitamin H is expected to be in the "neutral" form. At the same time, the presence of other polar groups in vitamin H would have hinder effective solubilisation by the micelles, hence resulting in the observed shorter migration time.

As for the group II vitamins, both vitamins B_1 and B_{12} are expected to assume positive charges (N^+ and Co^{2+} respectively). These vitamins are prone to the formation of an ion pair between its cationic group and the polar groups of the anionic micelles. This ionic interaction could have resulted in the large migration time for vitamin B_1 observed in Fig. 2. However for B_{12}, the migration times were much shorter. It seems that the electron-rich nitrogen atoms surrounding the metal centre could have neutralised the positive charge on the CO^{2+}. Consequently formation of the ion pair is more difficult. At the same time, its bulky substituent groups would have rendered effective solubilisation of the "neutral" B_{12} molecules by the SDS. Hence, vitamin B_{12} is found to migrate out much earlier than anticipated.

The migration order for the vitamins in group III is primarily governed by the extent of solubilisation by the micelles. In the case of vitamin B_2, despite the presence of the numerous polar substituent groups in this molecule, its apparent high hydrophilicity is somewhat reduced by its bulky size. Consequently the solubilisation of this molecules by the micelle was observed. As for the fat-soluble vitamins (A and E), since they are very lipophilic, they would be completely solubilised by the micelles. Hence they would migrate out together with the micelles.

It was noted that with the exception of B_1, B_2 and B_{12}, the migration times for the water-soluble vitamins remained fairly constant with the increase in the SDS concentration in the electrophoretic media. Although the trend was not observed in previous investigations [10, 12], this observation seems to be characteristic of compounds in Group I. A possible reason could be that the majority of the water-soluble vitamins, especially for those in group I, are highly hydrophilic. It is expected that their interaction with the micelles is minimum. Consequently, changes in the SDS concentration would not have caused any significant influence on the migration behaviour of these compounds. On the contrary, the other three water-soluble vitamins; vitamins B_1, B_2 and B_{12}, exhibit an entirely different trend. Because of stronger interaction with the micelles, the usual trend of increasing migration times with an increase in SDS concentration was observed. In other words, for these three species, the increase in SDS concentration would mean that there would be a higher probability of interaction with the micelles. Due to this increment in the concentration of micelles, a corresponding increase in the migration times would be observed. As a result of these two different trends, cross-over of peaks for the water-soluble vitamins are observed as shown in Fig. 2. Another point to note from Fig. 2 is that the increase in migration time is more pronounced in vitamin B_1 than in vitamins B_2 and B_{12}. The behaviour could be attributed to the fact that the ion-pair formation in B_1 would

result in stronger electrostatic attraction with the micelles compared to the mere Van der Waals type of interaction experienced by the other two vitamins. Furthermore, the bulky sizes of these two vitamins would prevent effective solubilisation into the micelles. Consequently, their migration times are much smaller than those of vitamin B_1.

So far our attempts to optimize the separation of both fat- and water-soluble vitamins by changing pH and SDS concentration in the electrophoretic media failed to achieve satisfactory results. The main difficulty lies in the separation of the two fat-soluble vitamins. This could be largely attributed to the fact that large molecules

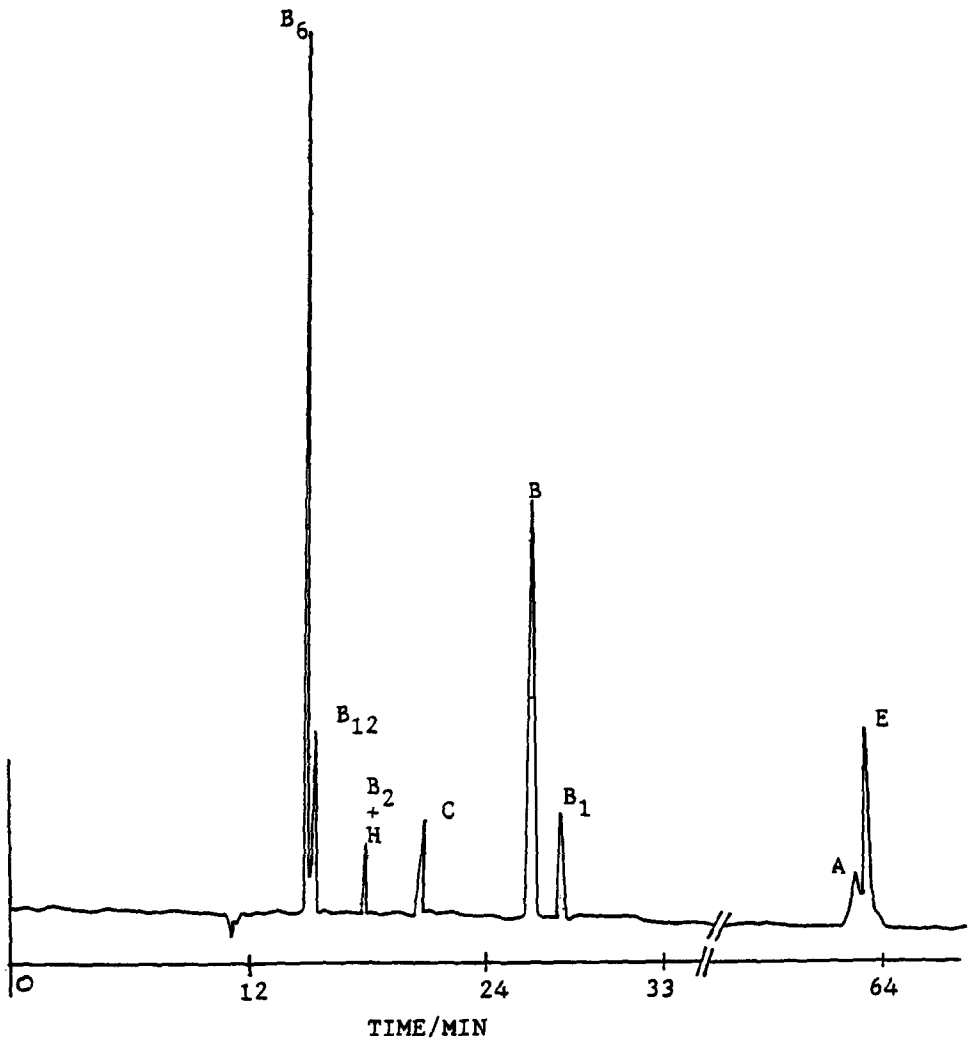

Fig. 3. Electrokinetic chromatogram of the vitamins with IPA. Electrophoretic solution: 30 m*M* SDS in 0.1 *M* borate–0.05 *M* phosphate with 1% IPA; pH 7.6; separation tube: 50 cm × 50 μm I.D. fused-silica capillary; voltage 15 kV; amount injected: 0.75 nl.

like vitamins A and E have strong hydrophobic interaction which would result in these molecules migrating together with the micelles.

It has been shown that a small percentage of organic modifiers can improve the separation efficiency in MEKC [14,15]. Upon addition of isopropanol (IPA) to the electrophoretic media, a marked improvement in the resolution of some peaks was observed, especially for the fat-soluble vitamins as these two compounds are no longer co-eluting. Typical chromatograms obtained using IPA as an organic modifier are given in Figs. 3 and 4. The improvement in the separation of some of the vitamins can be explained by the increase in the elution range provided as a result of the addition of

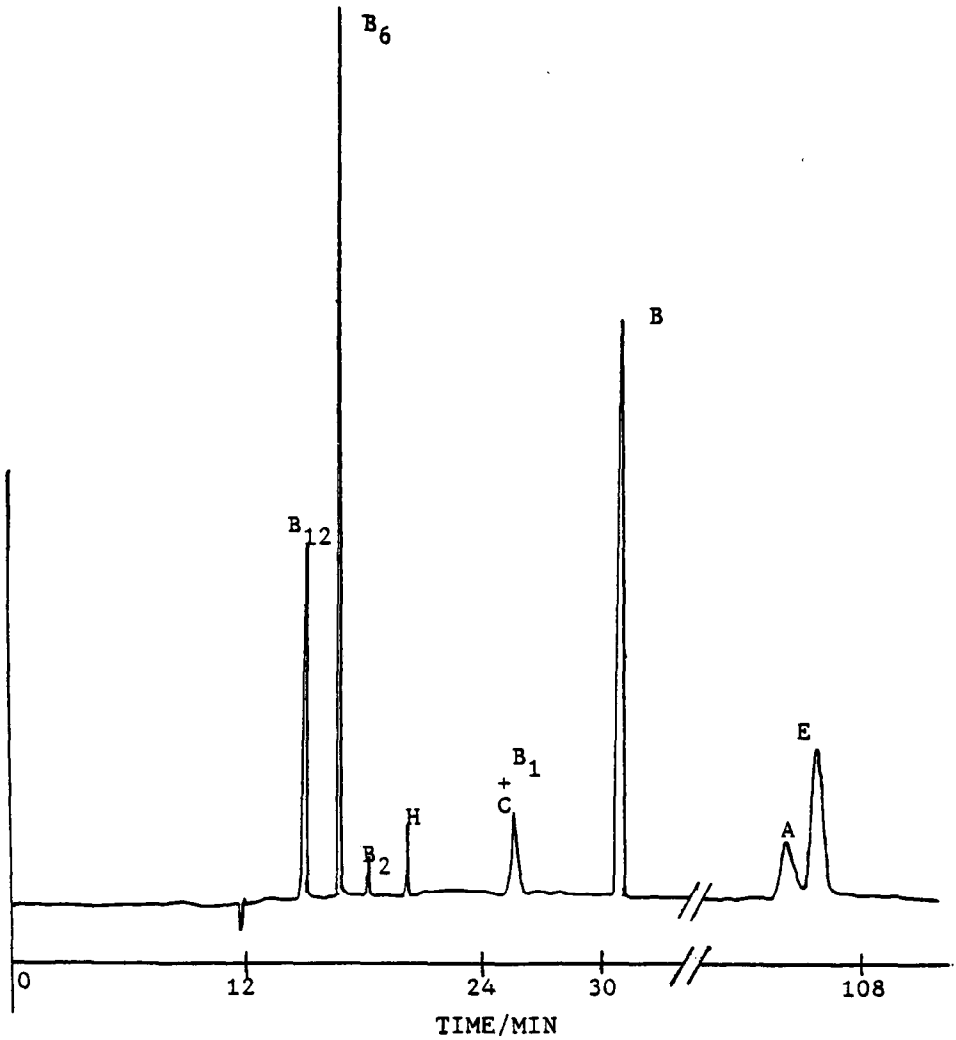

Fig. 4. Electrokinetic chromatogram of the vitamins with IPA. Electrophoretic solution: 30 *M* SDS in 0.1 *M* borate–0.05 *M* phosphate with 3% IPA; pH 7.6; separation tube: 50 cm × 50 μm I.D. fused-silica capillary; voltage 15 kV; amount injected: 0.75 nl.

the modifiers [14]. However, at the same time, it was noted that some of the water-soluble vitamin peaks are found to have overlapped with each other. Even though the two fat-soluble vitamin peaks are resolved to a certain extent, however, vitamin E is till migrating out together with Sudan III. Furthermore, it was found that after prolonged analysis using IPA, regeneration of the surface of the capillary tubing is required to give reproducible migration times. Taking into account these negative effects resulting from the use of IPA, it was considered an unsuitable modifier for the separation of the vitamins.

In previous investigations on the analysis of some polyaromatic hydrocarbons (PAH), γ-cyclodextrin was successfully adopted to separate this group of neutral compounds [16,17]. From the encouraging results obtained using cyclodextrin as modifiers, β-cyclodextrin was employed in this investigation to study its effect on the separation of the vitamins. The results obtained are shown in Fig. 5A en B. An important observation was that in all three sets of experiments, all the vitamins, including the two fat-soluble vitamins, are resolved. The migration times for the water-soluble vitamins, B_6, H, B_2, C and B are not affected by β-cyclodextrin. Their migration times remained fairly constant with varying β-cyclodextrin concentration. However for vitamins B_1, B_2 and B_{12}, there was a reduction in migration times. With

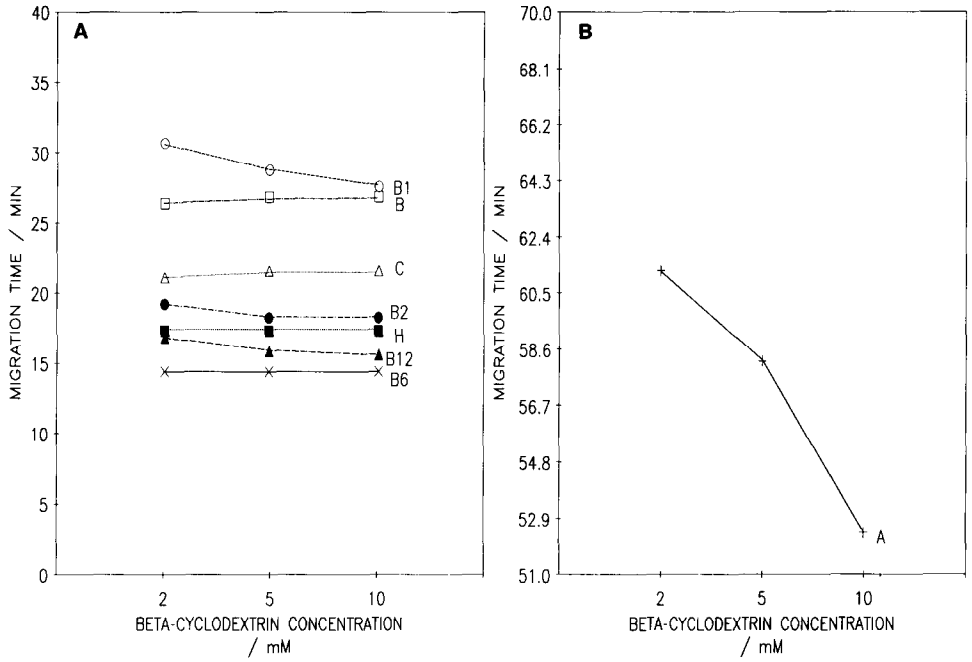

Fig. 5. (A) Plot of migration times *versus* β-cyclodextrin concentration. All these experiments are conducted at a voltage of 15 kV across the 50 cm × 50 μm I.D. separating tube filled with 0.1 *M* borate–0.05 *M* phosphate buffer at pH 7.6 with 30 m*M* SDS. Symbols as in Fig. 1. (B) Plot of migration times *versus* β-cyclodextrin concentration. All these experiments were conducted at a voltage of 15 kV across the 50 cm × 50 μm I.D. separating tube filled with 0.1 *M* borate–0.05 *M* phosphate buffer at pH 7.6 with 30 m*M* SDS. Note that the curve for the vitamin E is not plotted since its capacity factors are very large. + = Vitamin A.

further increment in the β-cyclodextrin concentration, the decrease in the migration times was even more apparent. This observation seems to suggest that there was keen competition between the micelles and β-cyclodextrin for these species. It is expected that for B_1 and B_{12}, unlike B_2, there would not be actual solubilisation of these positively charged vitamins into the neutral cavity of β-cyclodextrin. However, the presence of the electrically neutral β-cyclodextrin in the electrophoretic media seems to be able to lessen the interaction (ion-pair formation) between the positively charged solutes and the micelles. Consequently, a decrease in the migration times is observed for B_1 and B_{12}.

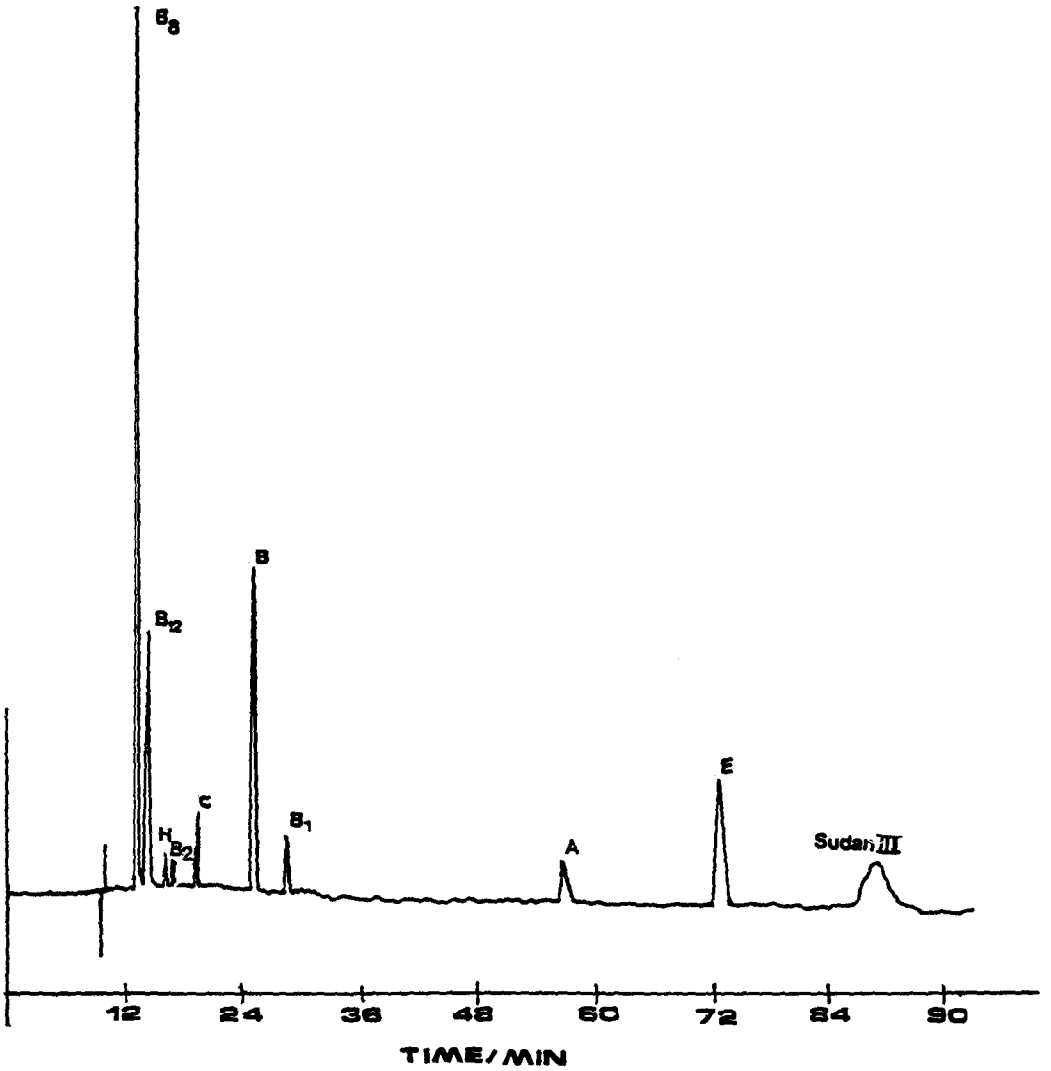

Fig. 6. Electrokinetic chromatogram of the vitamins with γ-cyclodextrin. Electrophoretic solution: 3 m*M* γ-cyclodextrin and 30 m*M* SDS in 0.1 *M* borate–0.05 *M* phosphate; pH 7.6; separation tube: 50 cm × 50 μm I.D. fused-silica capillary; Voltage 20 kV; amount injected: 0.75 nl.

Amongst the fat-soluble vitamins, only vitamin A is affected by the addition of β-cyclodextrin. It seems that the neutral vitamin A molecules can be solubilised into the β-cyclodextrin cavity much more readily than vitamin E. Since these neutral β-cyclodextrin molecules are not influenced by the electrophoretic attraction as much as the anionic micelles, they are expected to migrate faster than Sudan III. Therefore with the increment in the amount of β-cyclodextrin in the electrophoretic media, a corresponding decrease in the migration times for the affected species would be observed. On the other hand, the migration of vitamin E did not seem to be significantly influenced by β-cyclodextrin. This can be accounted for by the fact that its bulky size could have prevented its entry into the cavity of β-cyclodextrin. To demonstrate this fact, an additional experiment was carried out by replacing β-cyclodextrin with γ-cyclodextrin. With the increase in the size of the cavity due to the addition of another pyranose ring provided by γ-cyclodextrin, vitamin E can now be incorporated into the cavity. In this case, vitamin E is no longer eluting out together with the micelles, as shown in the chromatogram given in Fig. 6. From the chromatogram, it can be noted that all the peaks are satisfactorily separated. The usual tailing and broadening of peaks observed during analysis by HPLC were not observed in the present study.

This investigation is, to our best knowledge, the first ever reported separation of both fat- and water-soluble vitamins in a single analysis by CE. By the use of a simple instrumental set-up, separation of complex mixtures can be readily achieved by optimizing parameters such as pH and the concentration of SDS in the electrophoretic media or by the addition of modifiers such as β- and γ-cyclodextrins. The MEKC technique offers great flexibility and further investigations on its potential for the separation of other types of compounds should be considered.

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