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Microemulsion electrokinetic chromatography in suppressed electroosmotic flow environment Separation of fat-soluble vitamins

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Abstract

Microemulsion electrokinetic chromatography (MEEKC) was carried out in a pH 2.5 phosphate buffer to effectively suppress the electroosmotic flow (EOF). With 66.6% (w/w) 25 m*M* phosphate buffer pH 2.5, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) sodium lauryl sulphate (SDS), and 0.8% (w/w) *n*-octane as the separation medium, the fat-soluble vitamins A palmitate, E acetate, and D_3 were baseline separated within 11 min. With strongly suppressed EOF, the polarity of the separation voltage was reversed (positive electrode at the outlet); the *n*-octane micro droplets surrounded by negatively charged SDS molecules migrated towards the detector. The aqueous part of the microemulsion was modified with 20% (w/w) 2-propanol to improve partition between the *n*-octane phase and the surrounding aqueous medium. The fat-soluble vitamins were separated in order of decreasing hydrophobicity with a high migration time stability (repeatable within 0.1% RSD). Excellent accuracy and precision were obtained when the system was applied for the determination of vitamin E acetate in commercial vitamin tablets; quantitative data corresponded to 97.0% of label claim, intra-day results varied within 1.72% RSD ($n=6$), and inter-day results varied within 3.22% RSD ($n=5$).

Keywords: Microemulsion electrokinetic chromatography; Electroosmotic flow; Buffer composition; Vitamins

1. Introduction

For several years, pharmaceutical quality control has been accomplished by micellar electrokinetic chromatography (MEKC) [1], where neutral drug components are separated based on different partition between the aqueous background electrolyte and the pseudostationary phase of charged micelles. The neutral drug components migrate in a finite separation window in order of increasing hydrophobicity, providing a highly efficient separation method with a very low consumption of reagents and solvents. In addition to this, selectivity manipulation is easily accomplished utilizing different micelles or by modification of the aqueous background electrolyte [1].

Although MEKC is a very versatile separation technique for many neutral drug substances, hydrophobic compounds like fat-soluble vitamins may be difficult to analyze by MEKC. Owing to their highly hydrophobic nature, strong interactions with the micelles are evidenced resulting in relatively long separation times [2–6]. In addition, the most hydrophobic vitamins like vitamin A palmitate may precipitate during electrophoresis due to a very low

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solubility in aqueous MEKC buffers [7]. As an alternative to MEKC, fat-soluble vitamins have been separated by electrokinetic chromatography (EKC) with tetradecylammonium ions (TDA^+) as the pseudostationary phase [7–9]. In two of the papers [7,8], the EKC separation medium consisted of acetonitrile–water with 4 m*M* borate (80:20, v/v) to effectively dissolve the highly hydrophobic vitamins. TDA^+ at a concentration of 80 m*M* was added to the separation medium as pseudostationary phase, and the vitamins A palmitate, E acetate, and D_3 were separated prior to the electroosmotic flow with the cathode at the outlet based on different hydrophobic interactions to the TDA^+ ions. In the third paper [9], a separation system has been reported for vitamin K_1, where TDA^+ ions in pure propylene carbonate were utilized as the separation medium.

Although the TDA^+-based EKC systems have been utilized for the analysis of commercial vitamin formulations [2–4], the applicability may be limited in several cases owing to the background UV absorbance of the TDA^+ ions. Some of the fat-soluble vitamins are present at very low levels in commercial formulations (like vitamin D_3), and for these the signal-to-noise characteristics of TDA^+-based EKC systems may be insufficient. Therefore, alternative CE separation systems are of high interest for fat-soluble vitamins as well as for hydrophobic compounds in general. MEEKC has been utilized briefly as another alternative for the separation of fat-soluble vitamins [10,11]. In MEEKC, the vitamins were separated based on different partition with moving oil droplets present in a microemulsion buffer. The separation mechanism is similar to MEKC, but solubilization of the fat-soluble vitamins is significantly improved utilizing a microemulsion as the separation medium. The microemulsion buffers used in the two papers consisted of either *n*-hexane or *n*-octane as the oil droplet phase, sodium lauryl sulphate (SDS) as the surfactant stabilizing and charging the oil droplets, 1-butanol as co-surfactant, and an alkaline phosphate or borate buffer as the principal buffer medium. Both papers demonstrated promising separations of fat-soluble vitamins in alkaline buffer media. With the alkaline buffers, the electroosmotic flow (EOF) was greater in the direction of the detector than the electrophoretic mobility of the negatively charged microemulsion droplets in the opposite direction. Unfortunately, this resulted either in relatively long separation times or reduced separation efficiencies for fat-soluble vitamins.

In the present work, attention was focused on the development of a MEEKC system in suppressed electroosmotic flow environment. This was accomplished by utilizing an acidic phosphate buffer as the principal microemulsion medium and by modification of the aqueous phase with organic solvents to obtain acceptable analyte resolution. A principal objective was to develop a rapid and highly efficient separation system for fat-soluble vitamins. In addition, although several MEEKC papers have emerged based on alkaline buffers [12–20], very little attention has been focused on the utility of acidic MEEKC systems in general [12,13,21,22]. Therefore, the present paper also served as a fundamental study on this promising type of separation system.

2. Experimental

2.1. Chemicals

Vitamin A palmitate, vitamin E acetate, and vitamin D_3 were all purchased from Sigma (St. Louis, MO, USA). Sodium lauryl sulphate (SDS) of 99% was supplied by Sigma, and 1-butanol (analytical grade) was obtained from Merck (Darmstadt, Germany). *n*-Octane (spectroscopy grade), *n*-hexane (analytical grade), and ethyl acetate (analytical grade) were purchased from Merck, 1-octanol (99%) was from Aldrich (Steinheim, Germany), 2-octanone (97%) was from Fluka (Buchs, Switzerland), and chloroform (99.99%) was from BDH (Poole, UK). Methanol (high-performance liquid chromatography (HPLC) grade), acetonitrile (HPLC grade), and tetrahydrofuran (HPLC grade) were all purchased from Merck, 2-propanol was from Rathburn (Walkerburn, UK), and ethanol (96%) was from Vinmonopolet (Oslo, Norway). Eighty-five percent orthophosphoric acid (analytical grade) was from Ferak Laborat (Berlin, Germany) and sodium dihydrogenphosphate (analytical grade) was from Merck. Vitamin E acetate tablets (50 mg per tablet) were obtained from Nycomed Pharma (Asker, Norway).

2.2. Preparation of standard solutions and vitamin extracts

A stock solution containing 20 mg/ml vitamin A palmitate, 10 mg/ml vitamin E acetate, and 10 mg/ml vitamin D_3 was prepared in 2-propanol and stored protected from light at −20°C. From this stock solution, a working standard solution was prepared daily by a 100-fold dilution with a microemulsion containing 66.6% (w/w) water, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) SDS, and 0.8% (w/w) *n*-octane. The final concentrations were 200 μg/ml for vitamin A palmitate, 100 μg/ml for vitamin E acetate, and 100 μg/ml for vitamin D_3.

An extract of commercial vitamin E acetate tablets (50 mg per tablet) were prepared in the following way; one tablet was pulverized and tablet granulate corresponding to approximately 1 mg E acetate was transferred to 10 ml microemulsion with 100 μg/ml vitamin D_3 as internal standard and with the following composition: 66.6% (w/w) water, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) SDS, and 0.8% (w/w) *n*-octane. This was ultrasonificated for 15 min and subsequently exposed to filtration (0.45 μm). The filtrate was transferred to sample-vials and subjected to automated injection on the CE instrument.

2.3. Preparation of microemulsions

The final microemulsion used for separation of the fat-soluble vitamins was prepared in the following way; 0.6 g SDS and 0.66 g 1-butanol was mixed, and subsequently 0.08 g *n*-octane, 2.0 g 2-propanol, and 6.66 g phosphate buffer pH 2.5 was added and the mixture was exposed to ultra-sonification for 30 min to obtain a clear and highly stable microemulsion. The other microemulsions used during the optimization studies were prepared in a similar manner.

2.4. Apparatus

The CE system was a P/ACE 5510 Series from Beckman (Fullerton, CA, USA) equipped with a diode array detector. Separations were performed inside 50 μm I.D. uncoated fused-silica capillaries from Beckman with an effective length of 30 cm (37 cm total length). The anode was placed at the outlet of the capillary. Sample introduction was accomplished by hydrodynamic injection with pressure (0.5 p.s.i., 5 s; 1 p.s.i.=6894.76 Pa). The compounds were detected on-column at 205 nm (unless stated otherwise) with an aperture of 100×800 μm. Electropherograms were recorded and processed with the capillary electrophoresis software for the P/ACE System 5000 Series (Beckman). The capillary was conditioned prior to use each day by rinsing for 5 min with ethanol, 5 min with water, and for 10 min with the microemulsion utilized as the separation medium.

3. Results and discussion

3.1. Basic principle

Separation in MEEKC is based on different partition of the sample constituents into moving oil droplets present in the microemulsion buffer. Traditionally, MEEKC is carried out with an alkaline separation medium providing a substantial electroosmotic flow in the direction of the detector [12–20]. In this type of separation system, where the negative electrode is placed at the capillary outlet, the electroosmotic flow towards the detector exceeds the electrophoretic mobility of the negatively charged microemulsion droplets in direction of the capillary inlet (up-stream system). Thus, with up-stream systems, compounds of low affinity towards the oil droplet phase rapidly pass through the effective length of the capillary to the detector, whereas compounds strongly partitioning into the oil droplet phase are significantly retained; different analytes are separated in order of increasing hydrophobicity in up-stream MEEKC.

In the present work, rapid separations of fat-soluble vitamins were carried out by MEEKC in a down-stream mode where the electrophoretic mobility of the negatively charged microemulsion droplets exceeded the electroosmotic flow. In order to ensure short separation times, a phosphate buffer at pH 2.5 was selected as medium for the microemulsion. At this pH, the electroosmotic flow is strongly suppressed providing a maximum velocity for the negatively charged microemulsion droplets. The

polarity was reversed with the positive electrode placed at the outlet of the capillary. With the suppressed EOF environment, the negatively charged microemulsion droplets migrated towards the detector and different compounds were separated in order of decreasing hydrophobicity.

3.2. Optimization of the aqueous microemulsion medium

Initial experiments were carried out with a microemulsion buffer similar to an optimized system reported recently in the literature [11]; the only difference was that the alkaline buffer system was replaced with an acidic phosphate buffer at pH 2.5. Thus, 0.8% (w/w) *n*-octane was utilized as the oil droplet phase, 3.3% (w/w) SDS was added as surfactant to stabilize the microemulsion droplets, 6.6% (w/w) 1-butanol was added as co-surfactant, and 89.3% (w/w) 25 m*M* phosphate buffer pH 2.5 was utilized as the aqueous microemulsion medium. With this buffer and a 30 cm effective length capillary, no separation was observed between the vitamins A palmitate, E acetate, and D_3 selected as model compounds (Fig. 1). The short migration time combined with no separation suggested the three vitamins to be almost completely included in the oil droplet phase. In order to promote partition into the aqueous phase and consequently to obtain separation between the three vitamins, 2-propanol was added to the microemulsion. 2-propanol was selected owing to the high solubility in water and because 2-propanol readily dissolve the three fat-soluble vitamins of interest. As illustrated in Fig. 1, with an increasing level of 2-propanol, migration times increased and separation between the vitamins was achieved. Obviously, with addition of 2-propanol, the solubility of the vitamins increased providing some partition into the aqueous part of the microemulsion. The separation was gradually improved as the content of 2-propanol increased from 0 to 20% (w/w) (and with a corresponding reduction of the amount of phosphate buffer); the separation system was stable up to 20% (w/w) 2-propanol while baseline stability problems emerged above this level. In addition to improved separation, the current in the system was gradually reduced from 75 μA (0%) to 38 μA (20%) with increasing content of 2-propanol as a second beneficial effect.

In order to further study the possibility of adding organic solvents to the aqueous phase of the microemulsion, different organic solvents were added at the 20% (w/w) level including methanol, ethanol, 2-propanol, acetonitrile, and tetrahydrofuran. As illustrated in Fig. 2, 2-propanol and tetrahydrofuran were the two most effective modifiers for the separation of fat-soluble vitamins. This is in accordance with their solubilizing capacity for the vitamins; the vitamins are easily dissolved in both 2-propanol and tetrahydrofuran while especially methanol and acetonitrile are poor solvents for the three vitamins selected. With ethanol and 2-propanol, stable baselines were achieved indicating a high separation system stability, whereas methanol, acetonitrile, and tetrahydrofuran provided unstable baseline conditions at the 20% (w/w) level. The latter information may be of interest for future down-stream MEEKC of other hydrophobic compounds. Based on the experiments with modification of the MEEKC buffer, 20% (w/w) modification with 2-propanol was selected for the rest of the study. This buffer was found to be highly stable and no performance degradation was observed during storage for 1 month.

3.3. Optimization of the oil droplet phase of the microemulsion buffer

With an optimized aqueous phase, attention was secondly directed towards manipulation of the oil droplet phase. In a first experiment, the amount of SDS was varied from 3.3 to 6.0% (w/w) as illustrated in Fig. 3. With increasing concentration of SDS, the separation efficiency increased (number of theoretical plates) and migration times were reduced. The latter effect probably arose from an increased surface charge density of the oil droplets resulting in a higher electrophoretic mobility, while the former effect occurred owing to some type of improved analyte focusing during the initial electrophoresis (stacking process). SDS concentrations at and above 7.0% (w/w) were also tested but in these cases, baseline instability emerged indicating a reduced separation system stability. Consequently, 6.0% (w/w) was utilized during the rest of the study to

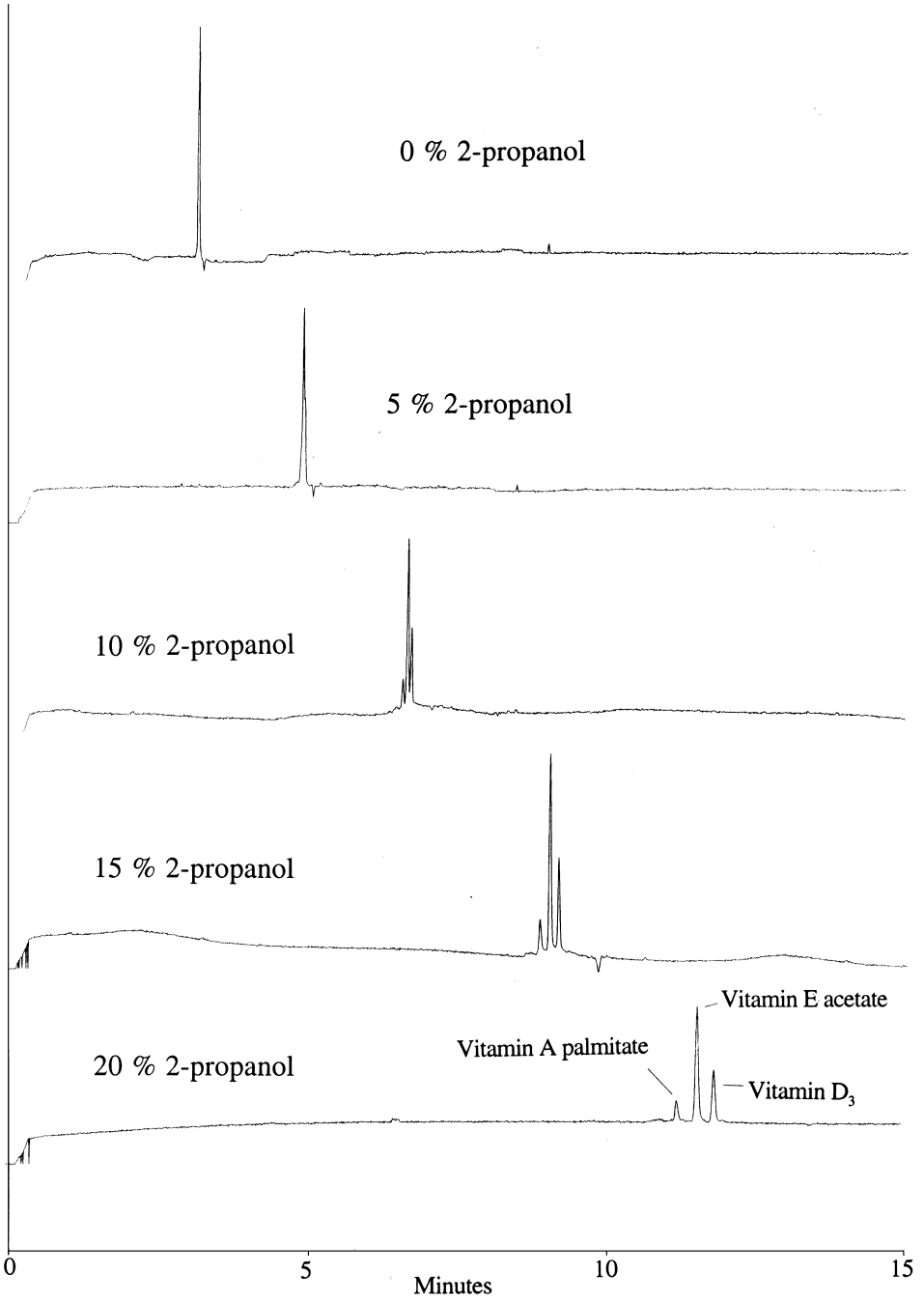

Fig. 1. Effect of 2-propanol in the microemulsion buffer on the separation of the vitamins A palmitate, E acetate, and D_3.

minimize separation times and to maximize the separation efficiency; at this SDS level, the current (which increased with the SDS concentration) was 60 μA.

In addition to the surfactant stabilizing the microemulsion, different oil phases were tested including *n*-octane, *n*-hexane, 1-octanol, 2-octanone, ethylacetate, and chloroform. With all of these solvents,

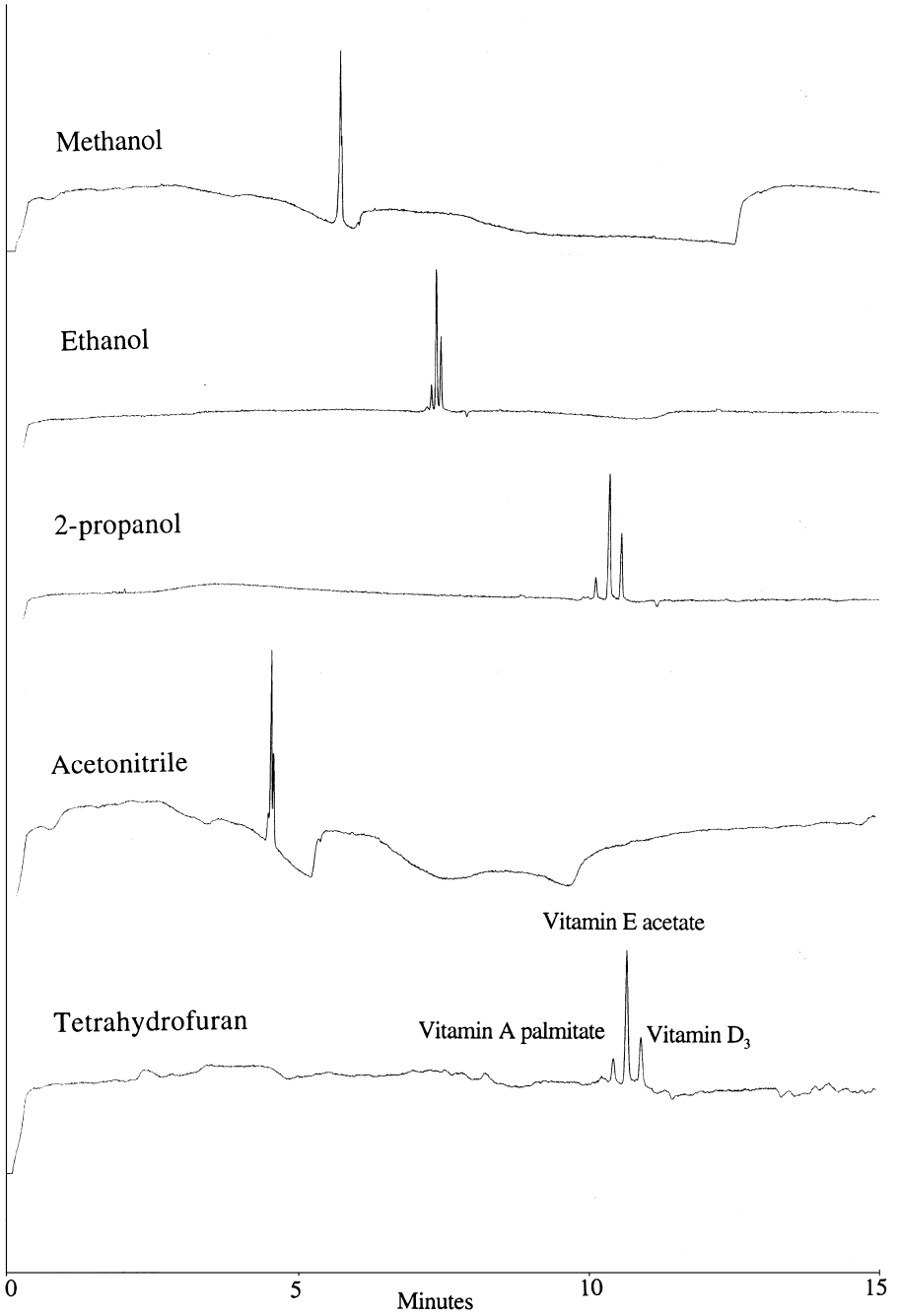

Fig. 2. Effect of different organic modifiers (at 20%, w/w level) in the microemulsion buffer on the separation of the vitamins A palmitate, E acetate, and D_3.

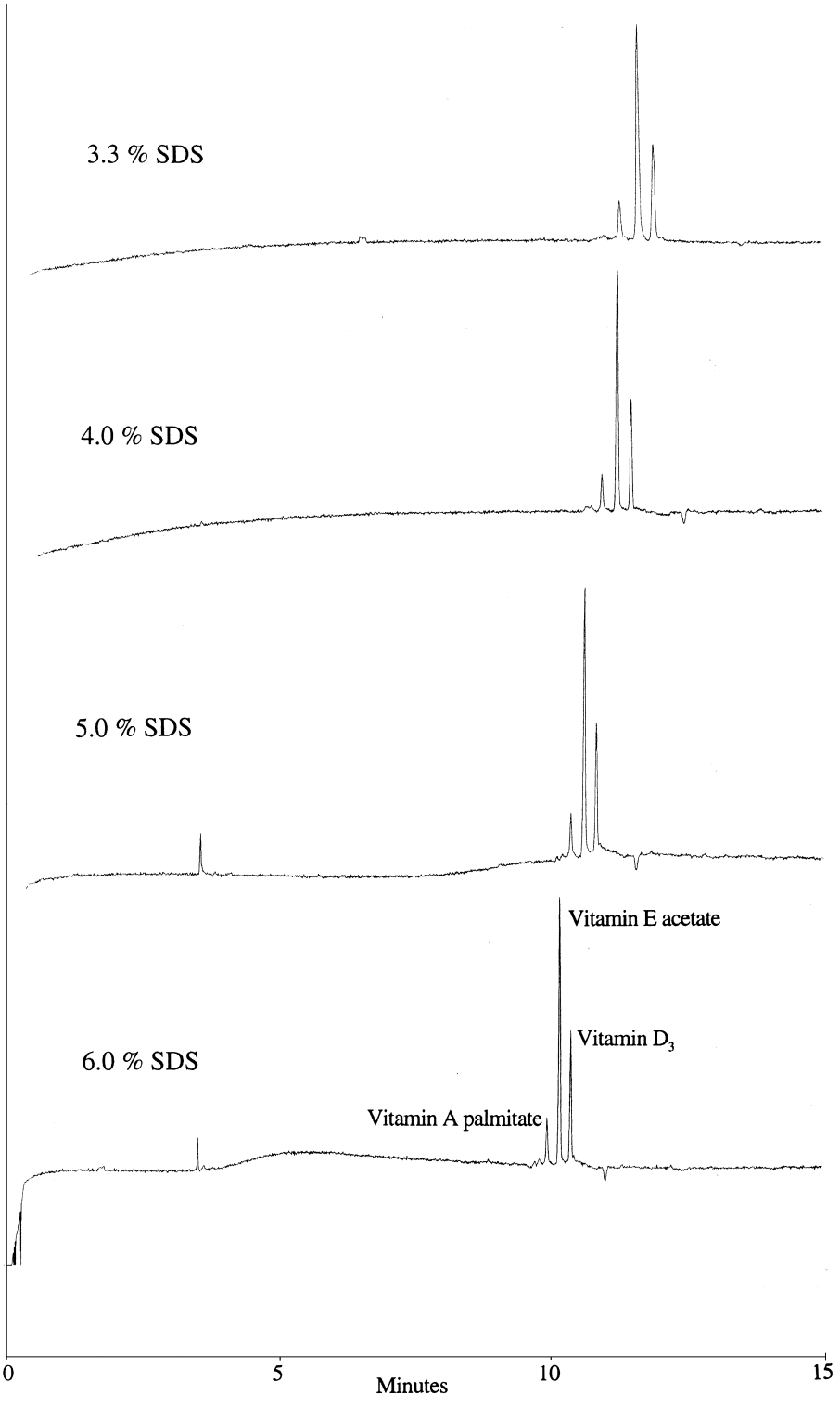

Fig. 3. Effect of the SDS concentration in the microemulsion buffer on the separation of the vitamins A palmitate, E acetate, and D_3.

no significant selectivity differences were observed for the vitamins A palmitate, E acetate, and D_3. This indicates that in the current rapid down-stream MEEKC system, modifications of the aqueous phase is the most effective parameter for changing the separation selectivity for very hydrophobic analytes. With all the different oil droplet phases tested, excellent baseline stability was obtained except for chloroform; the latter problem probably arose from the UV-background absorbance of chloroform. Thus, for future MEEKC of more hydrophilic analytes, a broad range of different oil phases may potentially be utilized for selectivity optimization. For the rest of this study however, *n*-octane was utilized as the oil droplet phase.

In a final optimization experiment, the amount of *n*-octane in the oil droplet phase was varied from 0.5 to 1.0% (w/w). Also in this case, no effects were observed on the separation of the vitamins A palmitate, E acetate, and D_3. Thus, 0.8% (w/w) *n*-octane was utilized as the oil droplet phase during the rest of the study. The possibility of increasing both the load and type of oil droplet phase may be of interest in future MEEKC-work if the solubilizing capacity of the separation system has to be increased.

3.4. System performance and stability

With a microemulsion buffer containing 66.6% (w/w) 25 m*M* phosphate buffer pH 2.5, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) SDS, and 0.8% (w/w) *n*-octane, with a 30 cm× 50 μm I.D. capillary, and with 20 kV as the separation voltage, the vitamins A palmitate, E acetate, and D_3 were baseline separated within 11 min as illustrated in Fig. 4. The current generated in the capillary was 60 μA which ensured repeatable separation conditions. The vitamins were dissolved in a similar microemulsion as the separation medium except that the phosphate buffer was replaced with pure water. This ensured a high sample compatibility and samples were injected at standard conditions for the instrumentation (0.5 p.s.i for 5 s) without affecting the system stability. The lower ionic strength of the dissolution medium served to promote analyte stacking resulting in a very high number of theoret-

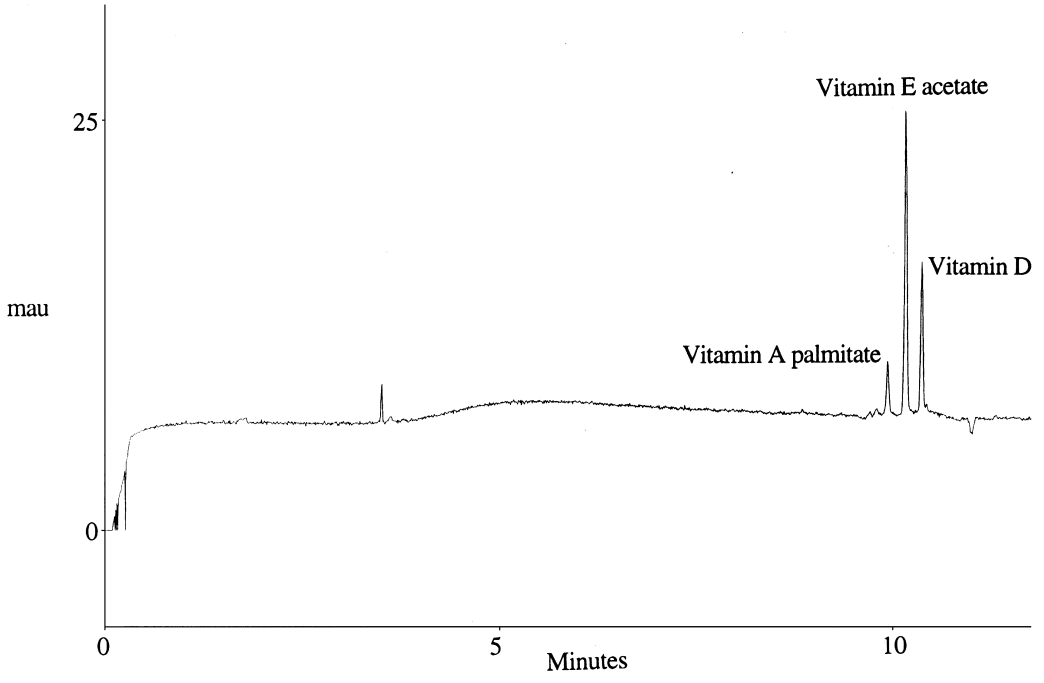

Fig. 4. Separation of the vitamins A palmitate, E acetate, and D_3 with a microemulsion consisting of 66.6% (w/w) 25 m*M* phosphate buffer pH 2.5, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) SDS, and 0.8% (w/w) *n*-octane. Effective capillary length, 30 cm; separation voltage, 20 kV; diode array detection (DAD) at 205 nm.

Table 1
Number of theoretical plates and detection limits for the vitamins A palmitate, E acetate, and D_3 obtained by MEEKC in suppressed electroosmotic flow environment

	Number of theoretical plates	Detection limit ($S/N=2$)
Vitamin A palmitate	452 000	6 μg/ml at 300 nm
Vitamin E acetate	448 000	2 μg/ml at 200 nm
Vitamin D_3	555 000	3 μg/ml at 260 nm

ical plates of each of the fat-soluble vitamins (Table 1). The high separation efficiencies combined the relatively low UV-background absorbance of the microemulsion enabled the vitamins to be detected down to the low μg/ml level in the standard solution used (Table 1), indicating very favorable signal-to-noise characteristics for practical applications.

In order to evaluate the stability of the MEEKC-system, both intra- and inter-day precisions were determined for migration times and raw peak areas (Table 2). For all of the three vitamins, migration times were highly repeatable and reproducible supporting that MEEKC carried out with suppressed electroosmotic flow provides very stable separation conditions. This was supported by the RSD data on peak areas; these may be further improved by utilizing an internal standard as demonstrated below.

3.5. Application to a commercial vitamin formulation

The separation system optimized for the three fat-soluble vitamins were briefly evaluated for the determination of vitamin E acetate in commercial vitamin tablets (50 mg per tablet). As discussed in the experimental section, the tablets were pulverized and subsequently extracted for 15 min in an ultrasonification bath with a microemulsion similar to the separation buffer prepared in pure water (not phosphate buffer at pH 2.5). This microemulsion contained vitamin D_3 as internal standard to improve the precision. As illustrated in Fig. 5, vitamin E acetate was effectively separated from both the internal standard and from other components of the tablets; within the separation window of interest for the vitamin assay, only a single peak emerged from the tablet matrix. As illustrated in Table 3, the content determined by MEEKC was in accordance with the label claim and the precision was acceptable. This supported that MEEKC carried out with suppressed EOF (down-stream mode) may be utilized for the determination of fat-soluble vitamins in commercial vitamin formulations.

4. Conclusions

The present work has demonstrated that MEEKC may be carried out under suppressed EOF conditions with an acidic buffer providing a very high separation efficiency for fat-soluble compounds. In order to achieve analyte separation and to optimize the

Table 2
Migration time and peak area precision for the vitamins A palmitate, E acetate, and D_3 obtained by MEEKC in suppressed electroosmotic flow environment

	Intra-day precision (%RSD)[a]		Inter-day precision (%RSD)[b]	
	Migration times	Peak areas[c]	Migration times	Peak areas[c]
Vitamin A palmitate	0.09	4.85	1.13	3.00
Vitamin E acetate	0.10	3.09	1.14	4.66
Vitamin D_3	0.08	1.76	1.15	7.48

[a] RSD of six replicates.
[b] RSD of five different days.
[c] Raw peak areas at 300 nm for A palmitate, 200 nm for E acetate, and at 265 nm for D_3.

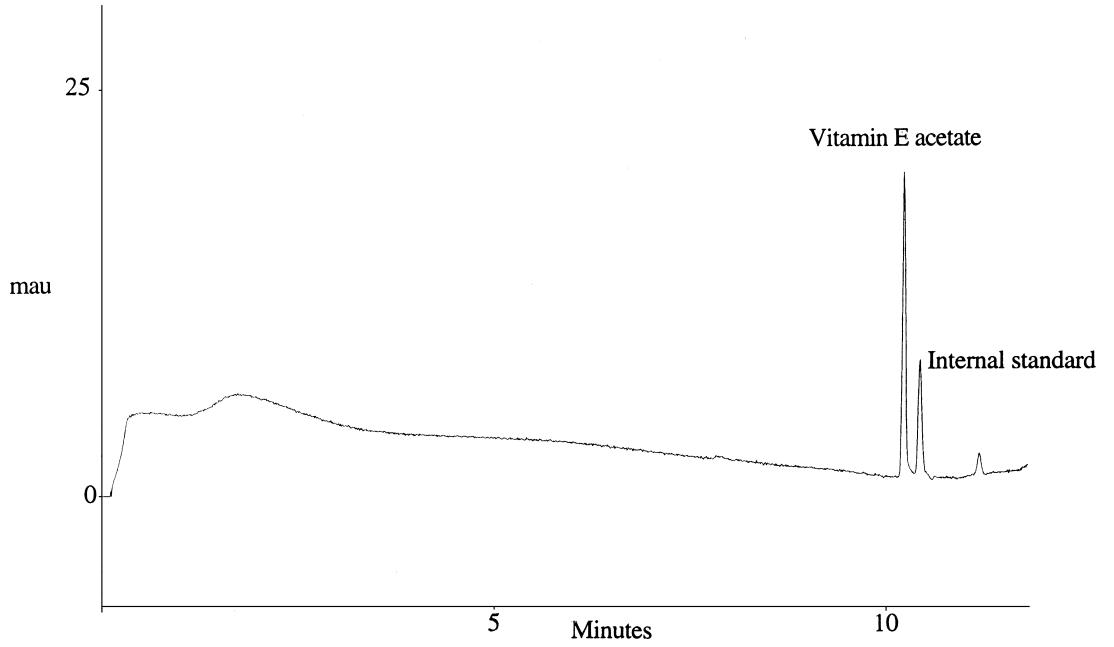

Fig. 5. Analysis of commercial vitamin E acetate tablets with a microemulsion consisting of 66.6% (w/w) 25 m*M* phosphate buffer pH 2.5, 20.0% (w/w) 2-propanol, 6.6% (w/w) 1-butanol, 6.0% (w/w) SDS, and 0.8% (w/w) *n*-octane. Effective capillary length, 30 cm; separation voltage, 20 kV; diode array detection, DAD at 205 nm.

selectivity, 2-propanol in the range 0–20% (w/w) may be added to the microemulsion without deteriorating the stability of the separation system. Ethanol may be utilized as an alternative modifier for the aqueous part of the microemulsion, and work is in progress to further evaluate organic modifiers for future MEEKC separations. Additionally, several organic solvents have been demonstrated for the oil droplet phase; with the current model compounds, these provided no differences in selectivity but this may be the case for more hydrophilic compounds in the future. The MEEKC system optimized for the separation of fat-soluble vitamins were highly stable and was found to provide reliable quantitative data. Work is in progress to further evaluate MEEKC under suppressed EOF conditions, which may be an interesting future technique for applications within pharmaceutical quality control and for other applications with samples of relatively low complexity.

Table 3
Comparison with label claim and precision for the determination of vitamin E acetate in commercial vitamin E tablets by MEEKC in suppressed electroosmotic flow environment

Label claim	MEEKC assay results	Precision (%RSD)	
		Intra-day[a]	Inter-day[b]
50 mg per tablet	48.5 mg per tablet	1.72	3.22

[a] RSD of six replicates.
[b] RSD of five different days.

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