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Determination of vitamins in food based on supercritical fluid extraction prior to micellar electrokinetic capillary chromatographic analyses of individual vitamins

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Abstract

The separation of 14 water-soluble vitamins and vitamin cofactors was investigated by micellar electrokinetic capillary chromatography and diode array detection using sodium cholate as the micellar phase. The method was optimised with respect to the effect of buffer composition, capillary temperature and applied voltage resulting in separation of all compounds in about 25 min. With the current method it is possible to predict the eluting order of the individual compounds from their net charges of each compound because of a low ion pairing between solutes and micelles. The linearities within concentration ranges of up to two-orders of magnitudes were good with correlation coefficients from 0.971 to 0.997. The separation efficiency was satisfactory with a good resolution ranging from 2 to 45 and a theoretical number of plates varying from 200 000 to 480 000. The repeatability of the developed method showed relative standard deviations on migration time in the range from 0.5% to 1.2% ($n=15$) and for normalised peak areas, relative standard deviations were approximately 6%.

Keywords: Food analysis; Vitamins

1. Introduction

Vitamins are a structurally heterogeneous group of essential food constituents that are required in small amounts for normal growth, maintenance and functioning of animal tissue. For classification purposes, vitamins are generally divided into the two main groups; lipid- and water-soluble vitamins. This classification is useful from a physiological point of view as it indicates the possible sources of vitamins. Furthermore, the classification is useful from an analytical point of view because it is necessary to group separate the individual vitamins prior to liquid chromatographic (LC) separation. For this purpose, attention should be paid to lipid extraction based on supercritical fluid extraction (SFE) [1] prior to LC separation of water-soluble vitamins because SFE is an attractive alternative to traditionally used fat extraction procedures.

The most promising methods of LC techniques previously used for determination of individual water-soluble vitamins are reversed-phase high-performance liquid chromatography (RP-HPLC) methods [2–4]. However, RP-HPLC may give serious problems with respect to column lifetime and simultaneous determination of all vitamins. Knowledge about the relative amounts of individual compounds in the various groups of water-soluble vitamins are, however, important for reliable studies of the vitamin's roles as precursors for enzyme cofactors. Such information is needed in order to determine inhibitors of such enzymes, and for correct

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evaluation of the bioavailability of e.g., vitamin C [5], vitamins B_1 or thiamin [6], vitamin B_2 (riboflavins) [7], vitamin B_6 [8], vitamin B_{12} [9], folates [10], biotin, pantothenic acid and niacin (NAD^+, $NADP^+$) [11].

Enzyme cofactors, that function as prosthetic groups, can only be extracted by use of methods comprising a hydrolytic step whereas coenzymes and non-covalently bound vitamins are more easily extracted. The two latter groups are also the most important to consider with respect to methods of analyses for water-soluble vitamins because the prosthetic groups only account for a minor part of the total amount of enzyme cofactors. In addition, vitamins are often relatively unstable and losses will occur under hydrolytic conditions, as the case will be for some types of food preparation, processing and storage, which give a need for gentle and efficient methods of analyses.

High-performance capillary electrophoresis (HPCE) is a relatively new separation technique, which has several advantages compared to RP-HPLC. These advantages comprise possibilities to obtain higher efficiency, higher resolution, method simplification in addition to the availability of various techniques developed for charged as well as uncharged analytes in free zone capillary electrophoresis (FZCE) and micellar electrokinetic capillary chromatography (MECC) [12]. As the heterogeneities of water-soluble vitamins both include variations in charges and hydrophobicities, FZCE may give limitations compared to MECC [13]. The MECC principle has previously been used for separation of water-soluble vitamins with sodium dodecyl sulphate (SDS) as the micellar phase [14–16].

The use of SDS as micellar phase results in negatively charged micelles that in addition to hydrophobic interactions are able also to display ionic interactions/ion pairing with positively charged solutes [15]. This dual interaction encompassing both hydrophobic and ionic interactions results in electropherograms that are fairly complex and it is difficult to predict the relative position or migration time for the analytes because analytes with similar charges may not be present as groups in the electropherograms. However, with the use of cholate as micellar phase, negatively charged micelles with hydrophobic surfaces are obtained, as cholate micelles are oriented with their negative charges at the centres of the micelles [17].

This paper describes the use of MECC with cholate as micellar phase for separation and quantitation of 14 water-soluble vitamins and vitamin cofactors with the aim of overcoming some of the above mentioned problems encountered in analyses of water-soluble vitamins. The method is optimised using a standard solution of commercially available vitamins. The evaluation of buffer composition on migration time and separation efficiency has been investigated. Theoretical plate numbers per meter of capillary, resolution, repeatability and linearity have been determined. The migration order in the system developed for vitamin analysis is discussed for easier identification of vitamins and vitamin cofactors in feed and food. In addition, preliminary work has been done for employing the optimised method for food and feed analysis. The method for vitamin analysis of food and feed comprises an initial separation of vitamins into the two groups: water- and lipid-soluble vitamins. Such a separation has been included in this work by use of SFE prior to MECC analysis of vitamin tablets and orange juice.

2. Experimental

2.1. Apparatus

MECC optimisation was done on a Hewlett-Packard HP^{3D} CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector and a 645 mm×0.05 mm I.D. fused-silica capillary. Detection was performed on-column at a position 560 mm from the inlet.

2.2. Samples and reagents

Thiamine hydrochloride, pyridoxine hydrochloride and D-biotin of analytical grade were obtained from Merck (Darmstadt, Germany). Riboflavin, nicotinamide, *N*-methylnicotinamide (trigonellinamide, I.S.), nicotinamide-adenine dinucleotide (NAD^+), nicotinamide-adenine dinucleotide phosphate ($NADP^+$), cyanocobalamine, sodium cholate and taurine, all analytical grade, were obtained from Sigma (St. Louis, MO, USA). Disodium

hydrogenphosphate-2-hydrate and 1-propanol of analytical-reagent grade were purchased from Riedel-de Haën (Seelze, Germany). Riboflavinphosphate (FMN), pyridoxamine dihydrochloride, ascorbic acid, pyridoxamine-5-phosphate (sodium salt) and folic acid were obtained from a local pharmacy. The names and structures of the vitamins and vitamin cofactors together with charges at the pH 7.4 of the run-buffer are presented in Fig. 1.

2.3. Sample preparation

Mixtures of vitamins from natural sources were obtained from freeze–dried orange juice. Fat and other lipid-soluble substances were removed by SFE on a Spe-ed SFE (Applied Separations, Allentown, PA, USA) at 62 MPa and 50°C using CO_2 as fluid (99.998% purity) (Hede Nielsen, Taastrup, Denmark) [1]. The residue was solubilised in water, centrifuged and analysed using the optimised conditions.

A vitamin tablet was ground to a homogeneous powder and dissolved in 2 ml water and 20 ml ethanol. The mixture was homogenised with an Ultra Turrax homogeniser, centrifuged and the supernatant evaporated to dryness in a rotary evaporator and then dissolved in an appropriate volume of water.

2.4. Procedure

The test mixture of vitamins was prepared by mixing the single vitamins to appropriate concentrations. The separation buffers for optimisation were prepared with variations in the concentration of taurine (200–500 m*M*), disodium hydrogenphosphate (20–100 m*M*), cholate (35–125 m*M*) and 1-propanol (2–8%). The pH of the buffers was unadjusted (about 7.4). Furthermore, the voltage (10–22 kV, anode at the injection end) and the capillary temperature (25–50°C) were varied.

All buffers were filtered through a 0.20-μm membrane filter before use. The capillary was preconditioned by flushing at a pressure of 4 kPa with 1.0 *M* NaOH for 2 min and with buffer for 5 min before each analysis. The linearity tests, repeatability and analyses of vitamin containing samples were performed using a run buffer containing 100 m*M* Na_2HPO_4, 500 m*M* taurine, 75 m*M* sodium cholate and 2% 1-propanol. New buffer was used for each analysis. The temperature was 30°C and the voltage 17 kV. Detection was performed at 214 nm and 255 nm. For identification of individual vitamins and impurities, spectra from 200 nm to 500 nm were recorded. Pressure raised injection was performed at 4 kPa for 1 s.

Calculation of relative migration times (RMTs), relative normalised peak areas (RNAs) (relative to internal standard, trigonellinamide) and normalised peak areas (NAs) were performed as described elsewhere [18]. Repeatabilities were estimated from calculated means and standard deviations (S.D.s) and linearity were determined from linear regression analysis. Relative response factors (RRFs) for the individual vitamins were calculated as the ratio between the slope of trigonellinamide and the slope of the vitamin, derived from the linearity test.

3. Results and discussion

3.1. Migration order

Names, structures and net charges at the selected buffer pH of the vitamins and vitamin cofactors used for method development are presented in Fig. 1. As shown, the vitamins comprise a complex group of structures with net charges from +1 to −3, resulting in very different electrophoretic mobilities in the capillary electrophoresis system. Cholate concentrations above the critical micelle concentration (CMC) have been suggested to result in rod-like or cylindrical micelles with the hydrophilic and negatively charged part turned inward and the hydrophobic part at the surface [17]. Therefore, the equally charged compounds are expected to be separated due to their hydrophobicity, i.e., their interaction with the micelles, which migrates toward the anodic end of the capillary and therefore retards the solutes. Groups of compounds with main differences related to differences in charges should separate due to their differences in electrophoretic mobilities caused by their different net charges. Therefore the migration order was expected to be monovalent cationic-, neutral-, monovalent anionic-, divalent anionic- and trivalent anionic compounds. Anions were also detectable in the detection window present at the cathode end of

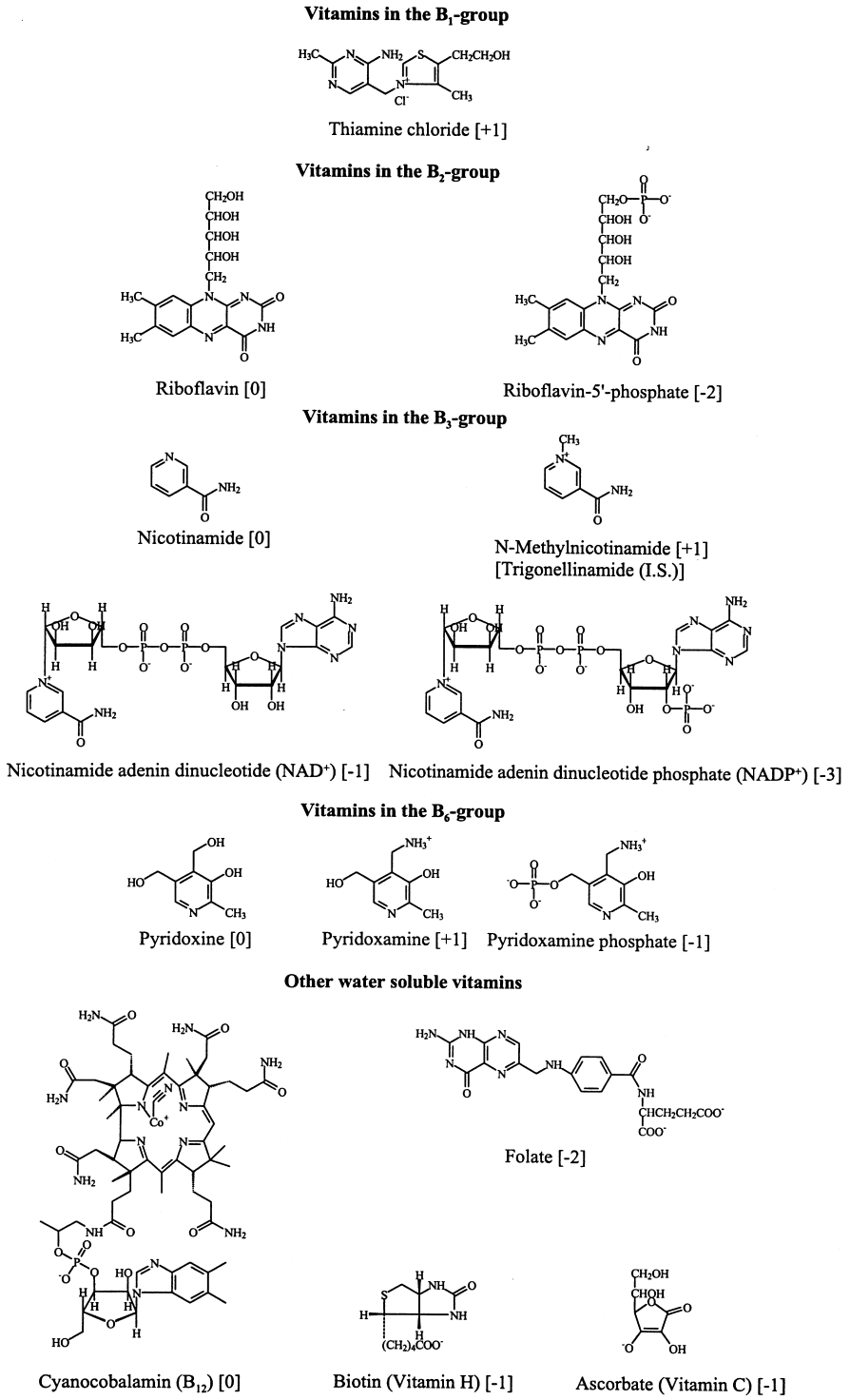

Fig. 1. Molecular structures, net charges at the used buffer pH (pH 7.4) and names of the vitamins and vitamin cofactors analysed with the developed MECC method.

the capillary because the electroosmotic flow (EOF) was much stronger than the electrophoretic mobility of ions [19].

The migration orders of the analysed compounds are shown in Fig. 2. As seen, the cationic compounds migrate faster than the solvent front, whereas the neutral compounds separate just after the solvent front due to different degrees of hydrophobic interaction with the micelles. The negatively charged vitamins, especially the activated phosphate esters, had considerable migration times because of the retarding effect of the negative charges. However, it was possible to separate most phosphate esters with net negative charges below three in an acceptable time of analysis.

Another micelle system using SDS has been applied to vitamin analysis by other groups [14–16]. In the SDS system, cations have a greater binding to the negatively charged SDS micelles than neutral and anionic compounds have because of the electric force of attraction between the cations and the negative charges on the surface of the micelles. Consequently, cations migrate slower than expected and therefore these compounds may co-migrate with neutral or anionic compounds. Ion-pair formation between cationic solutes and SDS micelles has in fact been suggested to be an important factor for the often long migration time of these compounds, especially at high SDS concentrations [15]. In contrast, the cholate system has a better predictability of migration order because binding of the solutes to the micelles is based mainly on a hydrophobic interaction.

3.2. Optimising separation conditions

Increasing the phosphate concentration from 20 m*M* to 100 m*M* increased the migration time of all compounds analysed, mainly because of the effect on the EOF. It has earlier been reported that increasing the ion strength of the run-buffer decreases EOF due to a lower zeta-potential on the capillary surface [20]. As seen in Fig. 3, however riboflavinphosphate (two net negative charges) migrates faster than ascorbic acid (one net negative charge) and $NADP^+$

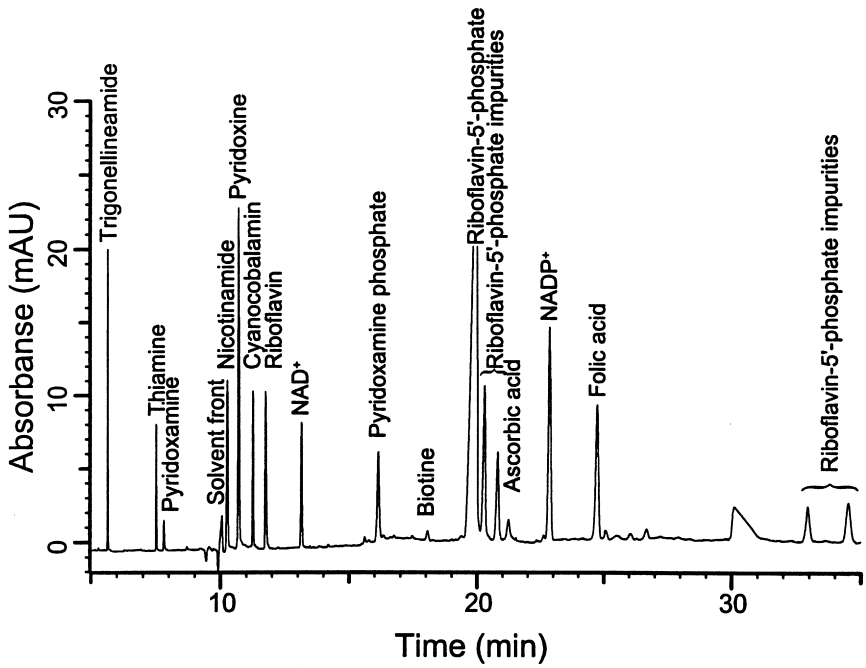

Fig. 2. Electropherogram of a test mixture of vitamins and vitamin cofactors. Separation was performed using a buffer consisting of 100 m*M* Na_2HPO_4, 500 m*M* taurine, 75 m*M* sodium cholate and 2% 1-propanol (pH unadjusted). Voltage: 17 kV; temperature: 30°C; UV detection was performed at 214 nm.

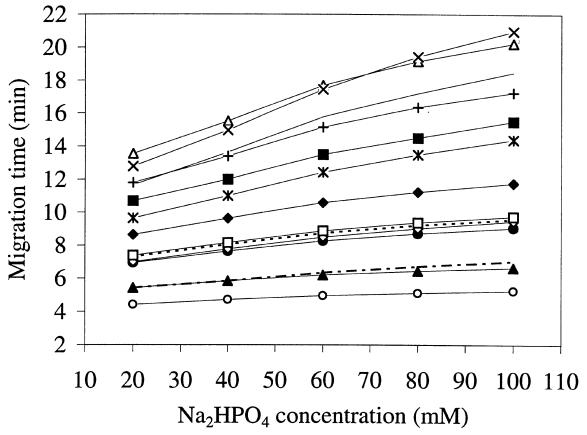

Fig. 3. Effect of disodium hydrogenphosphate concentration in the run buffer on the migration times of a test mixture of vitamins and vitamin cofactors. Other conditions were: buffer: 500 m*M* taurine, 75 m*M* sodium cholate and 2% 1-propanol (pH unadjusted). Voltage: 17 kV; temperature: 30°C; UV detection was performed at 214 nm. ×=Folic acid, △=$NADP^+$, —=ascorbic acid, +=riboflavinphosphate, ■=biotine, *=pyridoxamine-5-phosphate, ♦=NAD^+, □=riboflavin, - - -=cyanocobalamin, ◇=pyridoxine, ●=nicotinamide, – · –=pyridoxamine, ▲=thiamine, ○=trigonellinamide.

(three net negative charges) faster than folic acid (two net negative charges) at higher phosphate concentration, which may be explained by a changed interaction with the micelles.

Taurine did not affect the overall elution order of the vitamins in the concentration range from 200 m*M* to 500 m*M*. However, both migration times (t_m) and RMTs (calculated relative to trigonellinamide) were slightly decreased with increasing taurine concentration in the buffer. This effect was most pronounced for the anionic compounds, where a greater decrease in both t_m and RMT values was observed. The effect of adding taurine, a zwitterionic compound, to the buffer is complex. It has been mentioned that taurine decreases the association of cationic compounds to the negatively charged capillary surface thereby giving a faster migration and better separation of these compounds [21]. Moreover, by decreasing t_m and RMT for the anionic compounds at higher taurine concentrations, taurine seems to minimise the interaction of the solutes with the micelles, thereby giving a faster elution.

The concentration of cholate was found to be very important for efficient separation of all vitamins. Increasing the concentration gave slightly longer migration times of all compounds but also better separation of especially the compounds with zero net charge, which without micelles would be expected to co-migrate with the solvent front (Fig. 4). The better separation of the neutral compounds at higher micellar concentrations is a result of the increased capacity factor, defined as the molar ratio between micelle and water [22] and therefore an increased partition of the solute in direction of the micelle [23]. At 75 m*M* cholate, an efficient separation of all vitamins were found. When the cholate concentration was further increased, both cyanocobalamine and riboflavin migrate closer to the negatively charged NAD^+ because of a better/increased binding of these to the micelles than NAD^+.

Various types of modifiers can be used, but the use of 1-propanol with boiling point close to that of water reduces the risk of changed modifier concentration in the buffer due to evaporations (see below). It was furthermore found that 1-propanol was an acceptable and environmentally friendly modifier. 1-Propanol did not affect the relative migration times of the vitamins whereas the migration times increased for all compounds at increasing 1-propanol concentrations due to changes in the zeta potential, viscosity and dielectric constant and therefore reduced EOF [24]. At higher concentrations of

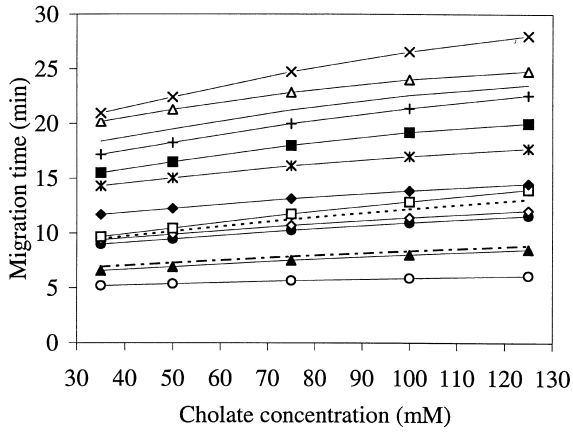

Fig. 4. Effect of sodium cholate concentration in the run buffer on the migration times of analysed vitamins. Other conditions were; buffer: 500 m*M* taurine, 100 m*M* Na_2HPO_4 and 2% 1-propanol (pH unadjusted). Voltage: 17 kV; temperature: 30°C; UV detection was performed at 214 nm. Symbols as in Fig. 3.

1-propanol (8% 1-propanol), it became difficult to keep the taurine in solution resulting in electropherograms with more baseline noise.

Neither temperature nor voltage affected the relative migration time of the vitamins, whereas migration times decreased non-linearly with increasing temperature or voltage. Increasing the capillary temperature reduced the viscosity of the buffer and therefore increased the EOF, resulting in overall faster migration times of the compounds analysed. When increasing the voltage of the system, this effect was enhanced. EOF was increased mainly because of a greater electric field strength in the capillary but also because of the greater Joule heating caused by too high current. This results in lower buffer viscosity and higher EOF [25].

3.3. Repeatability

A test mixture containing all vitamins in appropriate concentrations was used for determination of the repeatability of NA, RNA, t_m and RMT using the optimised buffer described in Section 2.4. The relative standard deviation (R.S.D.) of t_m and RMT was satisfactory for all analysed compounds with R.S.D. of t_m ranging from 0.5 to 1.2% and R.S.D. of RMT ranging from 0.1 to 0.8% calculated from 15 repetitions. These small variations in t_m and RMT were a result of buffer change between each analysis. R.S.D. of NAs ranged from 5 to 6%. However, R.S.D. could be decreased to between 1 to 5% by correcting for the area of the injected standard, trigonellinamide, resulting in RNAs. However, an internal standard with longer migration time (e.g., one net negative charge) would be more representative to use for correction of changes in EOF and other electrophoretic conditions caused by changes in capillary and buffer conditions.

3.4. Linearity and relative response factors

A test mixture was diluted to varying concentrations of vitamins and analysed using the optimised run buffer for determination of linearity. Correlation coefficients (R^2) calculated from linear regression analysis on normalised peak area (least squares method) for the varying vitamin concentrations ranged from 0.986 to 0.997 (Table 1). The linearity for ascorbic acid was not good, which was probably caused by its rather poor stability in the applied system, but antioxidants may be added to diminish the degradation [26]. RRFs for the individual vitamins were calculated from slopes of the linear regressions. Trigonellinamide was used as reference compound (Table 1). The relative response factors reflect the differences in extinction coefficients of the individual vitamins and this again is somewhat

Table 1
Correlation coefficients obtained from linearity tests of individual components in a test mixture of vitamins and vitamin cofactors

Vitamin	RRF (214 nm)	Concentration range (m*M*)	Correlation coefficient of linearity (R^2)
Trigonellinamide	1.00	0.03–4.01	0.9860
Thiamine	0.99	0.01–1.75	0.9915
Pyridoxamine	0.55	0.03–3.32	0.9951
Nicotinamide	0.79	0.17–2.65	0.9921
Biotin	7.70	0.31–4.91	0.9949
Pyridoxine	0.63	0.07–1.18	0.9948
Cyanocobalamine	0.14	0.0015–0.18	0.9943
Riboflavin	0.18	0.0018–0.22	0.9939
NAD^+	0.39	0.01–0.97	0.9943
Pyridoxamine-5-phosphate	0.52	0.01–1.76	0.9966
Riboflavinphosphate	0.56	0.02–2.58	0.9949
Ascorbate	Not calculated		0.9708
$NADP^+$	0.39	0.01–1.62	0.9970
Folate	0.37	0.03–3.42	0.9974

Relative response factors (RRFs) are calculated relative to trigonellinamide (internal standard).

reflected in the obtainable lower limits of concentration ranges, where compounds as cyanocobalamine and riboflavin with low RRFs also are detectable at low concentrations. Quantitative determination of the individual vitamins in samples using the relative response factors and known amounts of trigonellinamide as internal standard is preferable because improved accuracy can be obtained as found when using capillary zone electrophoresis (CZE) or MECC with SDS as micellar phase [16].

3.5. Separation efficiency

The resolution was generally high, ranging from about 2 to 45 showing a good separation of the analysed compounds. The theoretical number of plates per meter of capillary (N/m) for the individual peaks was satisfactory, varying from about 200 000 to 480 000 with the highest number of plates obtained for thiamine and cyanocobalamine. A previous system using CZE gave similar number of theoretical plates, with 180 000 N/m for thiamine, 370 000 N/m for nicotinamide and 470 000 N/m for nicotinic acid [13]. Thus, the use of micelles in the system does not cause a loss of obtainable theoretical plates per meter compared to CZE.

3.6. MECC on samples

Analysis of the vitamin tablet extract was done in about 25 min. Both thiamine, nicotinamide, pyridoxine, cyanocobalamine, riboflavin, ascorbic acid and folic acid were detected (Fig. 5). The concentration of biotin in the vitamin extract was below the detection limit for biotin. The use of recorded UV spectra for identification of individual peaks in the electropherograms were valuable because most of them had characteristic absorption spectra, which has also been noted in an earlier experiment [13,27].

Direct analysis of the defatted orange juice resulted in an electropherogram with many peaks. Therefore MECC analysis of original extracts needs further purification before quantitative and qualitative determination of most vitamins. Thiamine, pyridoxine and ascorbic acid were, however, identifiable in the electropherogram (Fig. 5) and these compounds will therefore be possible to quantitate from direct analysis of defatted orange juice. However, for many food extracts, these limits are too high to determine the vitamin levels without previous purification and concentration steps. Procedures for such vitamin quantification in different food and feed matrices are currently being investigated in our laboratories, and with initial group separations of the vitamins, the currently developed MECC method will be applicable for further separation of the individual vitamins present in the different groups obtained.

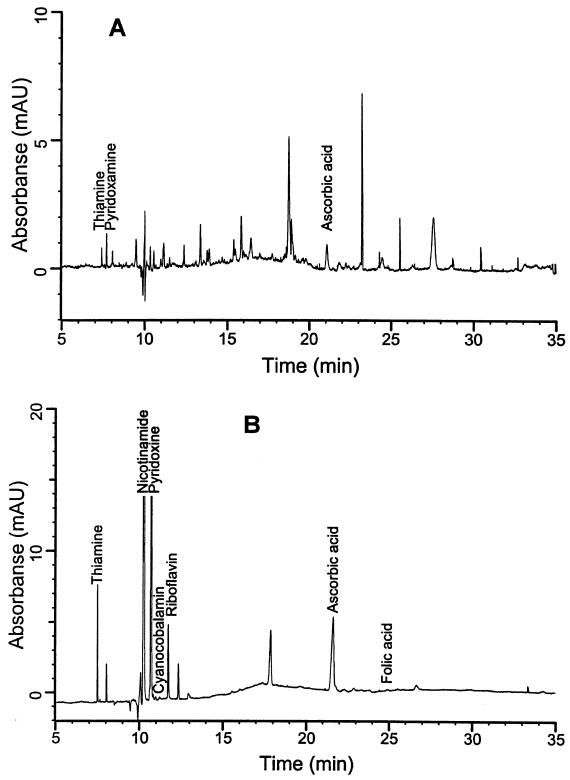

Fig. 5. Electropherograms from MECC analysis of defatted orange juice (A) and a prepared vitamin tablet extract (B). The extracts were separated using the MECC buffer consisting of 100 mM Na_2HPO_4, 500 mM taurine, 75 mM sodium cholate and 2% 1-propanol (pH unadjusted). Voltage: 17 kV; temperature: 30°C; UV detection was performed at 214 nm.

4. Conclusions

In this paper, MECC with cholate as the micellar phase was successfully applied for separation of a

range of water-soluble vitamins and vitamin cofactors. The migration time of the analysed compounds were charge dependent, with cations eluting first, then neutral compounds followed by the anionic compounds with one to three negative charges. The theoretical number of plates was high and the resolution satisfying. Good repeatability and linearity were obtained with the developed MECC method and the method could therefore very well be used as method of analysis for water-soluble vitamins. The method was successfully applied for analysis of a vitamin tablet extract whereas analysis of juice extracts needs further purification before good electropherograms can be obtained.

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