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Separation of δ-, γ- and α-tocopherols by CEC

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Abstract

In this study capillary electrochromatography (CEC) was used for the separation of three tocopherols (TOHs), namely δ-, γ- and α-TOH and the antioxidant compound, butylated hydroxytoluene (BHT). The CEC experiments were carried out using an octadecylsilica (ODS) stationary phase packed, in our laboratory, in a fused-silica capillary (100 μm I.D., 365 μm O.D. × 33 cm of total length and 24.6 or 8.4 cm effective length). The mobile phase was composed by a mixture of methanol (MeOH) and acetonitrile (ACN), at different concentrations and 0.01% (w/v) of ammonium acetate. Retention time (t_R), retention factor (k), resolution (R_s) of the three TOHs were strongly influenced by the organic solvent composition of the run buffer and by the effective length of the capillary. Optimum experimental conditions were found even employing the short effective length of the capillary achieving the baseline separation of the studied analytes in a relatively short time (less than 5 min). The optimized method was applied to the qualitative analysis of vitamin E (α-TOH) present in a human serum extract.

Keywords: Capillary electrochromatography; Tocopherols; Vitamin; Capillary electrophoresis; Serum

1. Introduction

Capillary electrochromatography (CEC) is a modern and powerful electrophoretic technique recently used for the analysis of charged and/or uncharged compounds. The separation of analytes is based on the combination of both chromatographic and electrophoretic principles, e.g. high selectivity of high performance liquid chromatography (HPLC) and high efficiency of CZE.

A relatively high electroosmotic flow (EOF), generated at the surface of the stationary phase applying a relatively high electric field across the column, is the main driving force responsible for the transport of the mobile phase along the column containing the stationary phase either packed or bound to the capillary wall. The utility

Abbreviations: CEC, capillary electrochromatography; TOHs, tocopherols; BHT, butylated hydroxy toluene.

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of the EOF in transporting mobile phase and analytes along a chromatographic column was firstly shown by Pretorius et al. [1].

Several stationary phases, designed for HPLC, have been successfully employed in CEC and among them reversed phase RP_{18} silica particles are currently used mainly for the analysis of uncharged compounds associated with selection of the appropriate experimental conditions such as the organic modifier, the buffer type and its pH [2]. However, the above mentioned stationary phase was also used in CEC also for the separation of polar compounds. We recently separated several aryl propionic acids using a background electrolyte at pH 2.5 where the acidic analytes were protonated and moved through the column by a relatively strong EOF generated by the presence of free silanols in the modified silica phase [3].

In this study, we present our results on the separation of δ-, γ- and α-tocopherols (TOHs) by CEC in a packed capillary column with RP_{18} silica gel as the stationary phase.

The analytes investigated in this paper belong to a class of phenol derivative compounds named TOHs. TOHs bear shielding methyl groups in the proximity of the phenolic hydroxyl group of the chromane moiety, and their phytyl side chain favor the positioning into lipid phases. The main modality of vitamin E antioxidant activity relies on the ability of blocking the propagation of radical reactions triggered by the reactive oxygen species (ROS) [4]. However, vitamin E is a term that comprises several naturally occurring TOHs and tocotrienols which are ingested with the normal diet, and, in particular, α- and γ-TOHs are the predominant analogues in human blood and tissues.

Since α-TOH has a major role as antioxidant in this group of compounds, because it has been proven to have the highest activity against lipid peroxidation induced by peroxyl radical, its name is often interchangeable with that of vitamin E [5,6]. Moreover, it is known that α-TOH transfer protein binds α-TOH selectively and eliminates other forms of TOH, with the result that α-TOH is the most abundant TOH in vivo [6].

Even if the antioxidant activities of α-TOH have been studied extensively comparing to other TOHs, it has been highlighted preferential modality of action of the individual TOHs. In particular, γ-TOH possesses peculiar ability of interfering with the mutagenic action of NO-derived electrophiles, being a more efficient trap for nitric oxide than α-TOH. Moreover, enzymatic activities may be selectively inhibited by γ- than α-TOH [7,8]. On the other hand, it has been shown that α-TOH alone acted as potent antioxidant against lipid peroxidation induced by peroxynitrite [9].

As part of physiological defence mechanisms against oxidative and radical injuries, TOHs can be consumed reaching levels below normal ranges. On the other hand, TOHs are assumed in variable quantity with the diet or can be administered in pills [10], and, as consequence, plasmatic levels of TOHs can be elevated.

Several animal studies and in vitro experiments provided evidences that TOHs prevent lipoproteins and cellular lipid components oxidation which is massively involved in atherosclerosis and longevity [11,12]. The role of vitamin E in infection and neoplasia has been also investigated [13,14]. The levels of γ- and α-TOHs are also monitored in clinical studies applied to type II diabetic patients [15]. As consequence, the plasma TOHs content may be influenced by a variety of physiological or pathological conditions and by habits like smoking as well [10].

Chromatographic separation methods of TOHs are based on either normal or reverse phase HPLC [16–19]. Moreover, capillary gas chromatographic methods have been applied to the analysis of TOHs [18–20]. Due to the recent establishment as analytical technique, the separation of TOHs by means of CEC is little explored [21]. The high efficiency and the rapidity of CEC analyses allow to separate compounds very close in structure in a short time with minimal solvent consumption. Such features help the assessment of plasma vitamin E content in high number of subjects in normal and pathological conditions. Moreover, the possibility to measure simultaneously the most diffuse TOHs, δ-, γ-, and α-analogues, allows to detect modifications of the individual TOHs.

2. Experimental

2.1. Reagents and chemical

Ammonium acetate was purchased from Carlo Erba (Milan, Italy). Methanol (MeOH) and acetonitrile (ACN) were from BDH (Poole, UK). δ-, γ- and α-TOH and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade and used without further purification. Run buffers were daily prepared by mixing the appropriate volumes of organic solvents and dissolving 0.01% of ammonium acetate.

Stock solutions of standard samples were prepared dissolving 10 mg of each compound in 10 ml of hexane containing 1 mg/ml of BHT as preservative.

2.2. Instrumentation

Electrochromatographic experiments were carried out in an automatic electrophoresis instrumentation (Hewlett-Packard, Waldbronn, Germany) equipped with an UV-visible diode array detector (DAD) and an air thermostating capillary cooling system. The vial carousel was at room temperature. Outlet and inlet vials were pressurized at 10 bar during CEC experiments in order to avoid bubble formation.

Fused silica capillaries (100 μm I.D., 375 μm O.D.) from Composite Metal Services (Hallow, Worcestershire, UK) were packed in our laboratory with a LiChrospher 100 RP_{18} (5 μm) particles (Merck, Darmstadt, Germany). The capillary was packed for the whole length and the modified silica particles were blocked by two retaining frits at the inlet and outlet ends of the capillary. The total length of the capillary was 33 cm while the effective length was either 24.6 or 8.4 cm.

The capillary was prepared as follows: a temporary frit was obtained by dipping one end of the capillary into a slurry of 2 μm silica particles in water and heating with a heated wire; the opposite end of the capillary was connected to a stainless steel HPLC pre-column filled with a slurry RP_{18} silica in MeOH (about 30 mg/ml) joined to a LC 10 HPLC pump (Perkin–Elmer). The pre-column and part of the capillary were dipped into an ultrasonic bath in order to keep an homogeneous slurry during the packing procedure. MeOH was pumped at ~2000 p.s.i. (1 p.s.i. = 6894.76 Pa) until the stationary phase filled the capillary at the desired length (about 35 cm). The slurry was removed from the pre-column and the capillary was pumped with distilled water for about 1 h at ~3000 p.s.i.

Inlet and outlet frits were prepared by using a heating coil (~600 °C for 60 s) disconnected from the pumping system and cut close to the two retaining frits. About 0.5 cm of polyimide layer was removed at 8.4 cm at ~300 °C for 30 s for the preparation of the detector window.

The capillary, prepared as described above, was equilibrated with the organic-aqueous mobile phase with the LC pump for ~15 min and positioned into a HP user assembler cartridge and inserted into the electrophoresis apparatus. Before the first run the capillary was equilibrated with the mobile phase pumping at 12 bar for 15 min and then applying a voltage of 25 kV and pressure at both ends (12 bar) for 15 min controlling the baseline and the current.

The mobile phase used was 100% polar organic solvent (MeOH–ACN at different concentrations) containing 0.01% of ammonium acetate. Experiments were carried out by applying 30 kV and both ends of the capillary were pressurized at 10 bar at 25 °C. A plug of sample was injected into the capillary at 12 bar for 0.2 min followed by a plug of mobile phase (12 bar for 0.2 min).

2.3. Standard and sample preparation

For the preparation of diluted solutions, exact volumes of the standard solutions were dried under argon and dissolved in the appropriate volume of mobile phase. TOHs were extracted according to an adapted method previously reported [12]. Briefly, to 2 ml of plasma were added 1 ml sodium dodecyl sulfate (SDS) 1% aqueous solution, and 3 ml ethanol for protein denaturation, and 200 μg of BHT to prevent sample autoxidation. The obtained emulsion was extracted three times with 3 ml of fresh hexane. The fractions of hexane were collected and dried under

argon. The extracted lipids were dissolved in 200 μl of mobile phase (ACN–MeOH, 70:30 v/v containing 0.01% w/v of ammonium acetate).

3. Results and discussion

TOHs belong to phenol class characterized by low solubility even in organic solvents due to their physico-chemical properties. δ-, γ- and α-TOHs differ from each other by the presence of the number of methyl groups on the aromatic ring (1, 2 and 3, respectively) (for their chemical structure see Fig. 1).

Based on published data and on the physico-chemical properties of the three studied compounds, we selected a RP_{18} packed silica capillary for the CEC experiment in order to separate the analytes. A polar organic solvent mixture, composed by MeOH–ACN at different concentrations, supplemented by 0.01% of ammonium acetate was used as the mobile phase.

The effect of organic solvent composition of the mobile phase on retention time (t_R), retention factor (k) and resolution (R_s) was studied in the range: 100% ACN–0% MeOH and 0% ACN–100% MeOH.

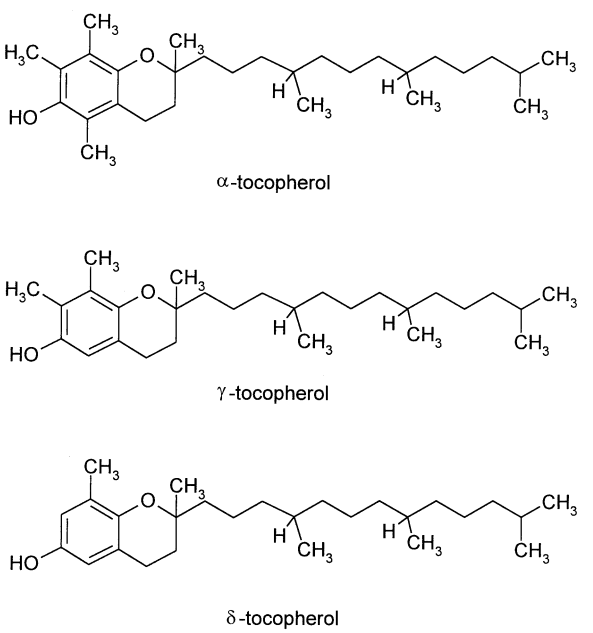

Fig. 1. Chemical structures of test compounds.

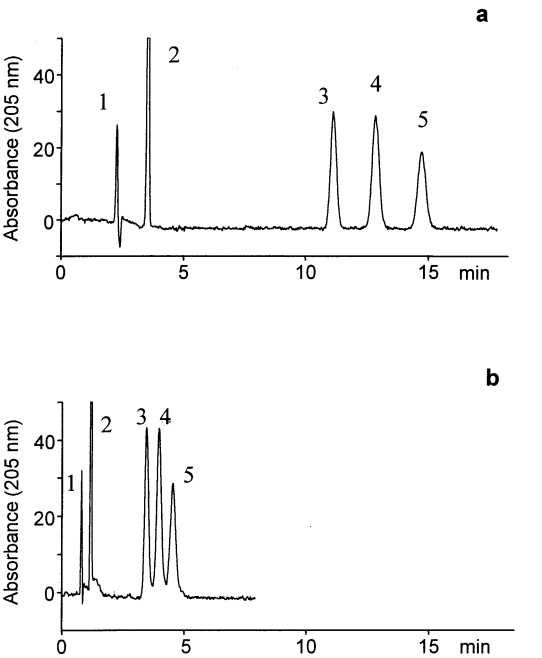

Fig. 2. (a and b) Separation of: (1) thiourea; (2) BHT; (3) δ-TOH; (4) γ-TOH; (5) α-TOH by CEC. Conditions: packed capillary for the whole length with RP_{18} silica 5, 100 μm I.D., total length 33.0 cm, effective length, (a) 24.6 cm; (b) 8.4 cm; mobile phase: ACN–MeOH 50:50% (v/v) and 0.01% ammonium acetate, pressurized at 10 bar both sides. Applied voltage, 30 kV, 25 °C. Injection, 12 bar for 0.2 min of 0.3 mg/ml of δ-, γ-, α-TOH, 0.05 mg/ml of thiourea; after the sample injection, a plug of mobile phase was introduced into the capillary applying 12 bar for 0.2 min.

Fig. 2a shows the electrochromatogram of the separation of the mixture of the three TOHs, the antioxidant BHT and thiourea (EOF marker). Besides, it was reported that the use of such compound could not be appropriate due to interactions with the RP_{18} stationary phase, we used this marker in order to measure the t_o because, in our experiments, we observed a reproducible negative peak moving close to that of thiourea due to the EOF [22,23]. Thus we supposed that thiourea was not interacting with the stationary phase eluting with the selected mobile phase.

As can be observed in the electrochromatogram, the separation of the four compounds was achieved in less than 15 min using the longest

effective length of the packed capillary. As expected the less polar analytes were more retained due to the strongest interaction with the stationary phase (α-TOH > γ- > δ-). Considering the very good resolution of the studied compounds achieved in Fig. 2a, we tried to find different conditions in order to shorten the analysis time. Thus the same sample mixture was analyzed running the experiments in the short end of the capillary using the same mobile phase and applied voltage. The separation of the four analyzed compounds was carried out in less than 5 min with almost baseline resolution for the three TOHs (see Fig. 2b).

The content of organic solvent in the mobile phase was changed, ACN–MeOH (100–0:0–100%) keeping constant the content of ammonium acetate (0.01% w/v) and CEC experiments of studied analytes performed.

As can be observed in Fig. 3 the EOF decreased by decreasing and increasing the ACN and MeOH concentration, respectively. This effect was not surprising because the presence of different organic solvents in the mobile phase is modifying the charge of the silica particles and the double layer responsible for the EOF.

After an increase of retention time of the three studied TOHs by decreasing the ACN concentration, retention times decreased finding a minimum

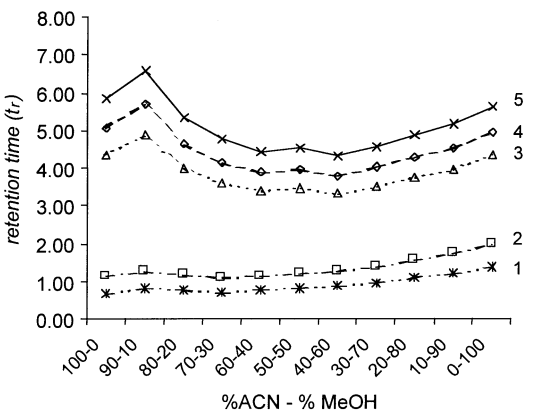

Fig. 3. Effect of the organic polar solvent composition in the mobile phase on retention time of studied compounds. Conditions: effective length, 8.4 cm; mobile phase 0.01% (w/v) ammonium acetate dissolved in ACN–MeOH (100:0–0:100%, v/v). For other experimental conditions see Fig. 2.

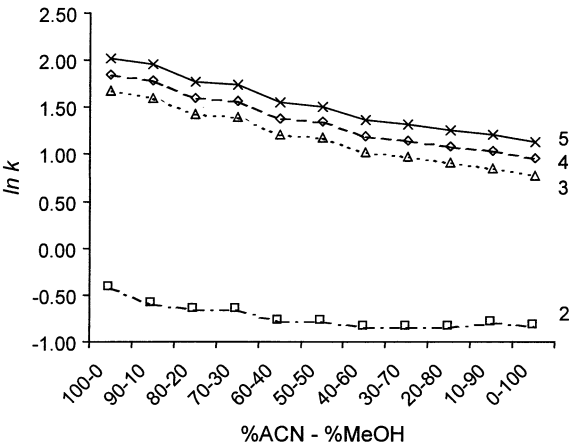

Fig. 4. Effect of percentage of ACN in the mobile phase containing ACN–MeOH and 0.01% ammonium acetate on the logarithm of retention factor ($\ln k$) of the studied compounds. Experimental conditions as reported in Fig. 3.

when the mobile phase contained 60–40 and 40–60% ACN–MeOH. Further decrease of ACN concentration caused an increase of migration time for all studied compounds. The reduction of analytes velocity by using ACN concentration lowers than 40% (v/v) is mainly due to the decrease of the EOF. This is also documented in Fig. 4 where an almost linear decrease of $\ln k$ by decreasing the ACN concentration (increasing content of MeOH) was observed.

The resolution factor (R_s) was measured for δ–γ and γ–α (R_{s1} R_{s2}, respectively) running the experiments with mobile phase containing different ratio of ACN–MeOH. Baseline resolution of the mixture $R_s > 1.5$ was recorded when the ACN concentration was higher than 60%. Experiments performed by using the longest effective length of the capillary revealed that baseline resolution of the three TOHs could be achieved at any ACN concentration.

Three capillary columns were packed with LiChrospher RP_{18} particles using the same procedure and tested injecting a mixture of thiourea and BHT and eluting with a polar organic mobile phase (ACN–MeOH–0.01% ammonium acetate) in order to verify the reproducibility. Satisfactory results were achieved: STD% of EOF = 1.5%, $t_{BHT} = 2$, peak $area_{BHT} = 2$. However, the three

columns did not exhibit the same efficiency probably due to the laboratory made packing.

The repeatability of the method was verified analyzing seven times the sample mixture and measuring the retention times and peak areas. Very good repeatability was observed for retention time of EOF and δ, γ, α-TOH ($< 1\%$) while for peak area we found STD% in the range 2.25–2.95%.

The satisfactory results achieved for peak area repeatability for the three studied TOHs document the stability of the analytes at least during the total analysis time and at ambient temperature. It is noteworthy to mention that the injected samples: (i) were daily prepared from stock solutions kept at -20 °C; and (ii) contained BHT as an antioxidant agent.

The limit of detection (LOD, S/N = 5) and limit of quantification (LOQ, S/N = 10) were calculated by analyzing the sample mixture at different dilutions using the mobile phase allowing to achieve baseline resolution at the shortest retention time (ACN–MeOH, 70:30 containing 0.01% ammonium acetate) finding LOD = 10 μg/ml and LOQ = 25 μg/ml for each analyte. Fig. 5 shows the electrochromatograms obtained for LOD and LOQ measurements.

An extracted sample of human plasma was injected and analyzed using the optimum experimental conditions for CEC analysis in order to show the applicability of the optimized method to the biomedical field. The results are reported in Fig. 6 where α-TOH is present (panel a); in panel b, the separation of the plasma extract spiked with the standard sample mixture is also shown.

4. Conclusions

From the above discussed results we can conclude that the separation of three TOHs compounds with very similar chemical structure can be achieved by using CEC employing a reversed phase stationary phase (RP_{18}) and a mobile phase composed by a mixture of organic polar solvents supplemented with 0.01% (w/v) ammonium acetate.

The preparation of the capillary in our laboratory (completely packed with the retaining frits at the inlet and outlet ends) allowed to run CEC experiment in either the longest or the shortest effective length. Excellent resolution of the three TOHs in presence of the antioxidant (BHT) was achieved when the longest effective length of the capillary was used ($R = 3.5–3.8$), however employing the opposite side of the packed capillary and optimizing the content of the organic solvent in the mobile phase we achieved good resolution ($R = 1.8$) in shorter retention time (less than 5 min). The repeatability of retention time was very good for the analyzed compounds and the EOF that was measured by analyzing thiourea (RSD% in the range 0.25–0.85) while the repeatability of peak area was satisfactory (2–2.9%). The CEC method exhibited good LOD and LOQ. The optimum experimental conditions were found by using a mobile phase containing ammonium acetate dissolved in the mixture ACN–MeOH (70:30)

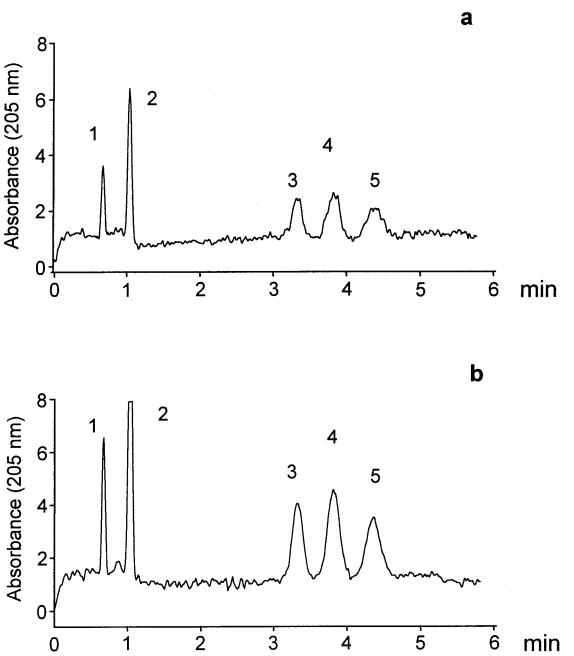

Fig. 5. (a and b) Electrochromatograms of the separation of TOHs: (a) LOD; and (b) LOQ. Conditions: mobile phase, ACN–MeOH (70:30 v/v) and 0.01% w/v ammonium acetate; injection (a) 10 μg/ml; (b) 25 μg/ml of each TOH. For other experimental conditions see Fig. 3.

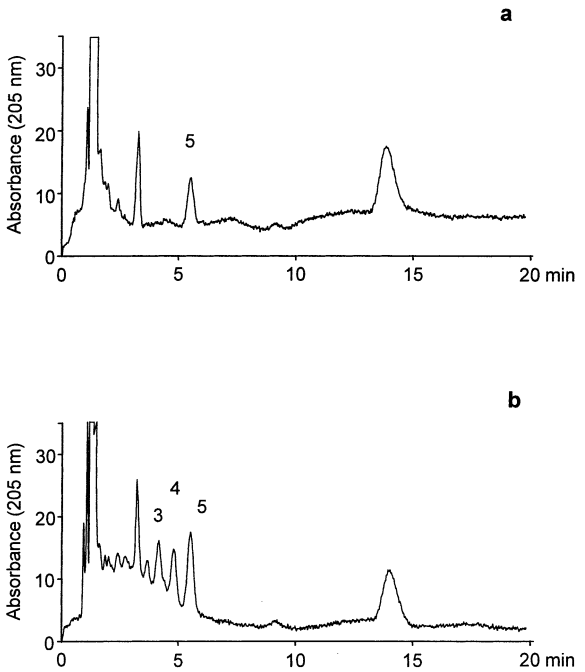

Fig. 6. (A and B) Analysis of: (A) extracted human plasma sample; (B) extracted human plasma sample spiked with δ-, γ-, α-TOH (0.1 mg/ml). Other experimental conditions as stated in Fig. 5 and in the text.

that allowed to achieve baseline resolution of the three TOHs in presence of the antioxidant BHT.

Compared to other analytical methods used for the separation of TOHs such as HPLC [16–19], CEC offers the advantages of using minute volumes of mobile phases as well as samples, small amount of stationary phases for laboratory made packed capillaries, achievement of high efficiency separations in short time etc.

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