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Nonaqueous electrochromatography on continuous bed columns of sol–gel bonded large-pore C_{18} material: separation of retinyl esters

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Abstract

A nonaqueous electrochromatographic reversed-phase separation method for retinyl esters using continuous bed columns has been developed. The packing material 7 μm Nucleosil 4000 Å C_{18} was sol–gel bonded in 180 μm I.D. capillaries. The mobile phase used was 2.5 m*M* lithium acetate in N,N-dimethylformamide–acetonitrile–methanol (2+7+1, v/v). At 350 V/cm and 30°C, this mobile phase composition gave rise to an electroosmotic flow of 1 mm/s. No Joule heating nor bubble formation were observed even at 625 V/cm (17 μA). With a 36 cm L_{eff} column complete separation of the commercially available and synthesized standards (all-*trans*-retinyl acetate, palmitate, heptadecanoate, stearate, oleoate, and linoleoate) was obtained within 10 min. The within-day and between-day variations of retention times of all-*trans*-retinyl palmitate were <0.3% relative standard deviation (RSD) ($n=3$) and <2% RSD ($n=6$), respectively. The within-day and between-day variations of peak areas were both <2% (both $n=3$). The columns were used for more than 1 month without degradation. Liver extracts from arctic seal were analyzed.

Keywords: Electrochromatography; Stationary phases, CEC; Continuous bed column; Retinyl esters

1. Introduction

Capillary electrochromatography (CEC) generally offers high efficiencies [1]. Nonaqueous mobile phases are required for hydrophobic analytes, and the use of nonaqueous reversed-phase CEC has been demonstrated for large PAHs [2], fullerenes [2], triglycerides [3], and retinyl esters [4,5]. Retinyl esters (Fig. 1) are the major storage forms of vitamin A [6,7], and are found in, e.g. the liver of arctic species such as polar fox and seal. Non-aqueous CEC methods using columns packed with C_{18} and C_{30} materials have recently been reported [4,5].

The use of packed columns in CEC introduced the problem of bubble formation. The pressurization of both inlet and outlet ends solved this problem to some extent, and several commercial capillary electrophoresis instrument suppliers implemented the necessary extras to perform successful CEC runs. The source of bubble formation has been traced to the on-column frits and to the nonuniformity of the packed bed generated during frit formation [8].

Recently Tang et al. described a new method for preparing continuous bed columns based on sol–gel bonded packing material for both aqueous CEC and LC [9–11]. These continuous bed columns have a homogenous structure from the inlet to the detection point, thereby reducing the problem of bubble formation. They used a large-pore material to prepare

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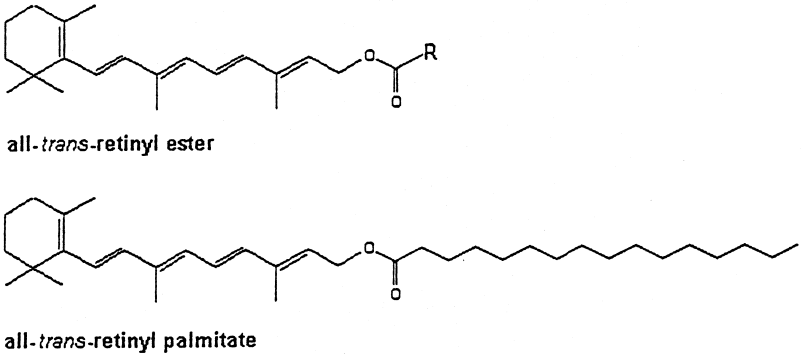

Fig. 1. Structures of all-*trans*-retinyl esters. R denotes a methyl group (all-*trans*-retinyl acetate) or a saturated or unsaturated hydrocarbon chain.

continuous bed columns with high permeability for CEC [10]. Stol et al. [12] demonstrated higher mobile phase velocities and higher separation efficiencies by using a large-pore (4000 Å) packing material for packed columns compared to 500 Å particles.

The object of the present work was to investigate the use of continuous bed columns of large-pore particles for nonaqueous reversed-phase CEC separation of retinyl esters. Different mobile phases were examined, and the separation system was investigated in terms of repeatability, intermediate precision, and robustness. The method has been applied for the separation of retinyl esters in liver extracts of seal.

2. Experimental

2.1. Chemicals and solutions

N,N-dimethylformamide (DMF) for UV spectroscopy (Fluka, Buchs, Switzerland), HPLC-grade methanol (Labscan, Dublin, Ireland), and acetonitrile (HPLC-grade, SDS, Peypin, France) were used to prepare the mobile phases. Lithium acetate (LiA; Acros Organics, Geel, Belgium) was added to all mobile phases at 2.5 m*M*. All mobile phases were filtered (0.45 μm RC-25; Sartorius, Göttingen, Germany) and degassed by sonication.

All-*trans*-retinyl palmitate ($RC_{16:0}$) and all-*trans*-retinyl acetate (RC_2; both from Sigma, St. Louis, MO, USA) were used to prepare stock solutions in DMF. All-*trans*-retinyl heptadecanoate ($RC_{17:0}$), stearate ($RC_{18:0}$), oleoate ($RC_{18:1}$), and linoleoate ($RC_{18:2}$) were synthesized from heptadecanoyl, stearoyl, oleoyl, and linoleoyl chloride, respectively, and all-*trans*-retinol as described earlier for $RC_{17:0}$ [4]. All substrates were from Sigma. The synthesized products were purified [13] and redissolved in DMF to a concentration of 150–200 μg/ml.

KH_2PO_4 (Merck, Haar, Germany), K_2HPO_4 (Fluka), $Na_2EDTA.2H_2O$ (Fluka), and ascorbic acid (Riedel-de-Häen, Seelze, Germany), all analytical grades, were used to prepare a homogenization buffer for liver samples. For extraction, *n*-hexane (HPLC grade) and dichloromethane (HPLC glass distilled grade), both from Rathburn (Walkerburn, Scotland) were used.

Tetramethoxysilane (TMOS; 99+%) and ethyltrimethoxysilane (ETMOS; 97+%), used as precursors in the sol–gel process, were delivered by Aldrich (Milwaukee, WI, USA). An aqueous solution of trifluoroacetic acid (TFA) was prepared at pH 2. Formamide for the sol–gel solution was delivered by Rathburn.

2.2. CEC columns

Fused silica capillaries of 180 μm I.D./350 μm OD (Composite Metal Services, Hallow, Worcester, UK) were packed as earlier described [14], only with a reduced end-pressure of 3600 p.s.i. Stol et al. observed a decreased separation efficiency with large-pore packing material at inlet pressures exceed-

ing 250 bar [12]. The packing material in the present work was 7 μm endcapped C_{18} Nucleosil 4000 Å (Machery-Nagel, Düren, Germany). Before packing the capillary was washed with 1 *M* KOH and water, respectively, and dried with nitrogen at 120°C, according to Ref. [10]. A 15% sol solution (200 μl methanol, 30 μl TMOS, 30 μl ETMOS, 22 μl TFA, and 200 μl formamide) was used in the preparation of a continuous bed column according to Tang et al. [11]. The solution was manually pumped into the column using a syringe. After curing at ambient temperature for 24 h the columns were dried with supercritical CO_2 as detailed by others [10]. A detection window immediately after the packed bed was made using a scalpel. The polyimide at the inlet of the column was also removed.

2.3. Instrumentation and operating procedure

A Model 270A Capillary Electrophoresis System (Applied Biosystems, Santa Clara, CA, USA) connected to a Chromatopac C-R6 integrator (Shimadzu, Kyoto, Japan) was used for electrochromatography with columns of 22–36 cm effective length (L_{eff}) (41–55 cm total length (L_{tot})). The retinyl esters were detected at 325 nm.

The CEC columns were conditioned with 2.5 m*M* LiA in DMF–methanol (99:1, v/v) using a syringe when installed in the CEC system. Care was taken to avoid drying of the column ends. Columns were electroconditioned at 350 V/cm until a stable baseline was obtained (less than 1 h). The same procedure was used for subsequent changes to new mobile phase compositions. Samples were injected electrokinetically for 3 s at 100 V/cm.

2.4. Liver extracts

Liver samples of seal were kindly provided by the Institute of Nutrition Research, University of Oslo and stored at −20°C until further sample preparation and subsequent analysis. Liver samples (1–1.5 g) were thawed and homogenized in 15 ml buffer (2 m*M* KH_2PO_4, 2 m*M* K_2HPO_4, 0.7 m*M* $Na_2EDTA.2H_2O$, and 1.4 m*M* ascorbic acid, pH 7.3). The mixture was extracted with 30 ml of *n*-hexane–dichloromethane (5+1, v/v). The solvents were evaporated under a stream of N_2 (99.99%, AGA, Oslo, Norway) and the residue was redissolved in 1 ml DMF.

2.5. Calculation and data handling

Efficiency (plate number) was calculated using $N=5.54(t_R/w_{0.5})^2$, where t_R denotes the retention time and $w_{0.5}$ is the full width at half height. The electrophoretic mobility, μ_{EOF}, was calculated using $\mu_{EOF}=L_{eff}\cdot L_{tot}/(t_{EOF}\cdot V)$, where t_{EOF} is the elution time of an unretained compound and V is the applied voltage.

3. Results and discussion

High efficiencies have been reported with reversed-phase electrochromatography on continuous bed columns using aqueous mobile phases [9,10]. Also, no bubble formation has been observed, even at high field strengths. For continuous bed columns prepared with large-pore C_{18} material, even higher efficiencies and higher electroosmotic flow were reported [10]. In the present work, the use of nonaqueous mobile phases in a reversed-phase system employing continuous bed columns is explored.

3.1. Continuous bed columns

A new method for preparing continuous bed columns based on sol–gel bonded packing material for both CEC and LC was described by Tang et al. [9–11]. Most CEC workers have used packed columns with two on-column end frits. In contrast to the packed columns, the continuous bed columns have a homogenous structure from the inlet to the detection point, thereby reducing the problem of bubble formation.

In this work, the column preparation technique described by Tang et al. [9–11] was adapted for nonaqueous CEC with 180 μm I.D. columns and Nucleosil 4000 Å 7 μm C_{18} endcapped material. The columns were easily prepared, and could be used without the overpressure needed for packed columns with frits. No excessive Joule heating nor bubble formation were observed even at 625 V/cm (17 μA). With the investigated nonaqueous CEC method based on continuous bed columns with sol–gel

bonded material, a reduced plate height of 1.9 was demonstrated for $RC_{18:1}$ (75 000 plates/m). The columns showed no degradation after 1 month of continuous use.

The electroconditioning procedures were simpler with the continuous bed columns than the earlier reported packed column conditioning [4,5]. With the packed columns, a voltage program was necessary to obtain a stable current. In the present study, the continuous bed CEC system showed a stable current at the onset of the operating electric field strength. A stable baseline was achieved with the continuous bed columns after only minutes, in contrast to the earlier reported packed columns [4,5].

The electroosmotic flow-rate was higher with the continuous bed columns used in the present study compared to the packed columns reported earlier [4,5], even though an end-capped material was used in the present study. An electroosmotic flow of 1 mm/s was obtained at 350 V/cm as compared to 650 V/cm on packed C_{18} and C_{30} materials [4,5], corresponding to an increase of electrophoretic mobility close to 50% on the continuous bed columns. Hence, lower field strengths could be used for separation to provide similar flow-rates. The use of lower field strengths when employing continuous bed columns make possible the use of longer columns, providing better resolution. The higher permeability of the continuous bed columns are by large related to the use of a large-pore packing material. In addition a higher flow-rate is expected for continuous beds with sol–gel bonded particles due to charge contribution from dissociated silanol groups of the sol–gel matrix [10].

3.2. Separation of retinyl esters

Retinyl esters are hydrophobic compounds, with limited solubility even in methanol and acetonitrile. Nonaqueous CEC separation systems for these compounds using mobile phases based on DMF and columns packed with C_{18} and C_{30} materials have recently been described [4,5].

Few retinyl esters are commercially available. Therefore several were synthesized, including $RC_{17:0}$, $RC_{18:0}$, $RC_{18:1}$, and $RC_{18:2}$. Commercially available RC_2 and $RC_{16:0}$ and the synthesized standards were used for evaluation of the CEC separation of retinyl esters on the continuous bed columns.

3.2.1. Mobile phase composition

Nonaqueous mobile phases consisting of DMF–methanol (99+1, v/v) have earlier been used in combination with columns packed with C_{18} and C_{30} materials [4,5]. On the continuous bed columns using Nucleosil 4000 Å C_{18} material, the retention of retinyl esters was too low for successful chromatography. Increasing acetonitrile content has earlier been shown to increase both EOF and retention [5]. Mobile phases consisting of 2.5 m*M* LiA in DMF–acetonitrile–methanol (10–29+80–70+10–1, v/v) were examined. Increasing the methanol content to 10% increased the solubility of LiA when acetonitrile was used. At 10% methanol, increasing the acetonitrile content beyond 70% resulted in precipitation of LiA.

To avoid band broadening, the injection solutions of the retinyl esters should match the composition of the mobile phase. The retinyl esters have a very low solubility in methanol and even acetonitrile, but are highly soluble in DMF. The analytes were therefore dissolved in DMF before addition of acetonitrile and subsequently methanol. At initial concentrations of 50 g/l in DMF, $RC_{16:0}$ precipitated instantaneously from solution upon adding acetonitrile. Reducing the initial concentration to 10 g/l resulted in no precipitation. Hence stock solutions were all prepared from initial concentrations of $\leq$ 10 g/l.

A separation of commercial available and synthesized retinyl esters on a 22 cm L_{eff} column using 2.5 m*M* LiA in DMF–acetonitrile–methanol (2+7+1, v/v) as mobile phase is demonstrated in Fig. 2a. As shown in Fig. 2b baseline separation of the critical pair $RC_{18:1}$ and $RC_{16:0}$ was obtained within 10 min by increasing the efficient column length to 36 cm. An electroosmotic flow of 1 mm/s was generated at a field strength of 350 V/cm.

The use of C_{30} material for the CEC separation of retinyl esters has shown higher degree of selectivity as compared to C_{18} material [5]. At present, however, commercially available large-pore silica materials are limited.

3.2.2. Field strength

The effect of the separation field strength on the

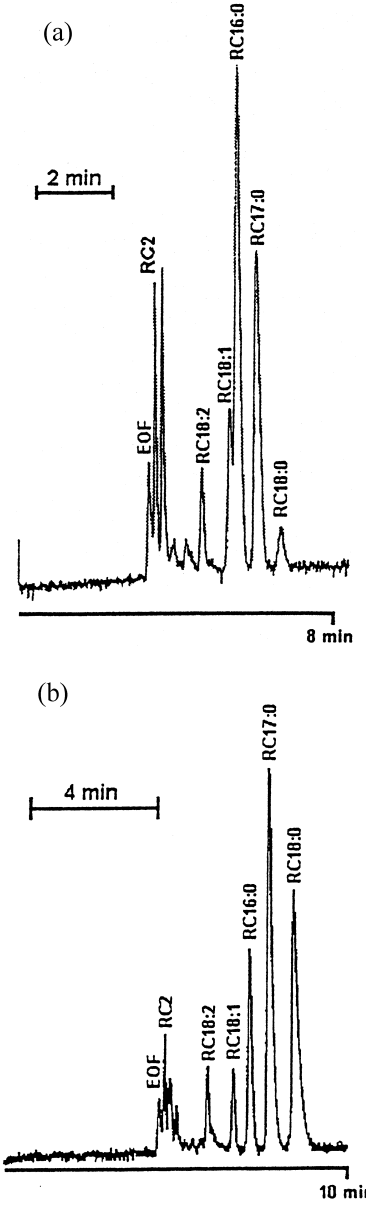

Fig. 2. Separation of retinyl esters in DMF–acetonitrile–methanol (2+7+1, v/v) with 2.5 m*M* LiA. The sample contained 60–200 μg/ml of each component and the injection time was 3 s at 100 V/cm. The separation was performed at 30°C and 350 V/cm using a 180 μm I.D. continuous bed column with sol–gel bonded Nucelosil 4000 Å 7 μm C_{18} material. Column lengths (L_{eff}) of (a) 22 cm and (b) 36 cm were used. Unlabeled peaks are degradation products of the synthesized retinyl esters.

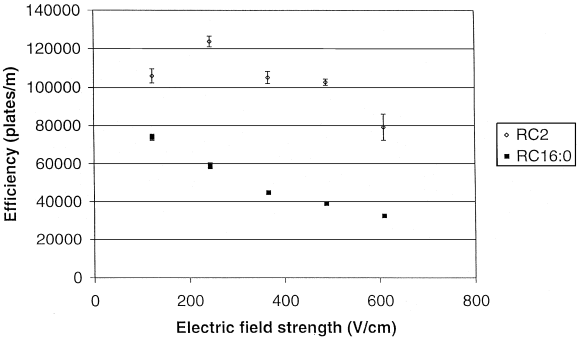

Fig. 3. Efficiencies of RC_2 and RC_{16} in the field strength range 120–610 V/cm. A column of 22 cm L_{eff} was used. Other conditions as in Fig. 2. The error bars reflect the standard deviation of the data ($n=3$).

efficiencies of RC_2 and $RC_{16:0}$ was investigated at 30°C. Fig. 3 shows the efficiency when increasing the electric field from 120 V/cm to 610 V/cm. Maximum efficiency for RC_2 was obtained at about 240 V/cm. The efficiency for $RC_{16:0}$ decreased continuously from 120 V/cm. This is comparable to earlier results obtained with nonaqueous packed column CEC [4,5]. In addition, the resolution of the critical pair $RC_{18:1}$ and $RC_{16:0}$ decreased. At 350 V/cm, a reasonable separation time was obtained, and the critical pair $RC_{18:1}$ and $RC_{16:0}$ was resolved.

3.2.3. Temperature

The lowest possible temperature setting with the instrument used was 10°C above room temperature. At ordinary room temperatures, this corresponds to 30°C. The maximum temperature setting was 60°C. Increasing the temperature from 30 to 60°C, as shown in Fig. 4, resulted in a small efficiency increase. A separation temperature of 30°C was nevertheless chosen, to resolve the critical pair $RC_{18:1}$ and $RC_{16:0}$. No stability problems were observed following the temperature changes.

3.3. System evaluation

Standard solutions of $RC_{16:0}$ were prepared from stock solutions by dilution (1+9, v/v) with mobile phase. Injection for 3 s at 100 V/cm was used for all experiments. The repeatability ($n=3$) of the retention time was <0.3% relative standard deviation (RSD). Correspondingly, the between-day variation was

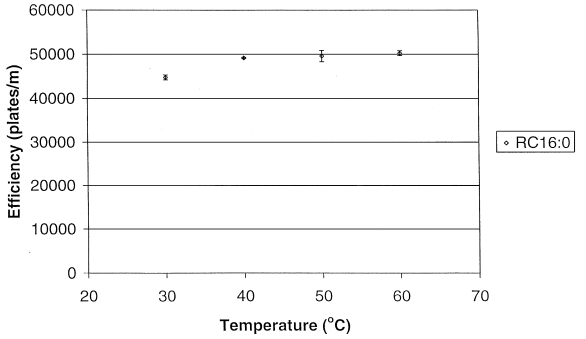

Fig. 4. Efficiencies of RC_{16} in the temperature range 30–60°C. A column of 22 cm L_{eff} was used. Other conditions as in Fig. 2. The error bars reflect the standard deviation of the data ($n=3$).

<2% RSD ($n=6$). This corresponded well with the results obtained with a packed column [4].

Without internal standard, both the repeatability and between-day variation of peak areas of $RC_{16:0}$ were <2% RSD (both $n=3$). In contrast, it was necessary to use an internal standard with a packed column to obtain acceptable repeatability and intermediate precision of the peak areas [4].

3.4. Liver extracts

Liver extracts of seal were analyzed. After evaporation of the extracting solvents, the residue was redissolved in DMF. Attempts to adjust the solvent strength to the mobile phase composition resulted in sample precipitation and decreased recovery. No precipitate was observed in the DMF solution. No loss in efficiency was observed, for all practical purposes, when the DMF solution was injected. The critical pair $RC_{16:0}$ and $RC_{18:1}$ was still resolved. No column degradation was observed following 2 weeks of continuous injections of liver extracts.

The seal liver retinyl ester profile using DMF–acetonitrile–methanol (2+7+1, v/v) with 2.5 m*M* LiA as mobile phase is presented in Fig. 5. $RC_{16:0}$ was the main component in the seal liver extract. All the retinyl esters RC_2, $RC_{16:0}$, $RC_{18:0}$, $RC_{18:1}$, and $RC_{18:2}$ were identified by spiking. In Fig. 5b the column length has been increased to 36 cm, thereby providing baseline resolution of the critical pair $RC_{18:1}$ and $RC_{16:0}$. The separation was completed within 10 min. Due to the absorbance at 325 nm, the unidentified components of the seal liver extracts in

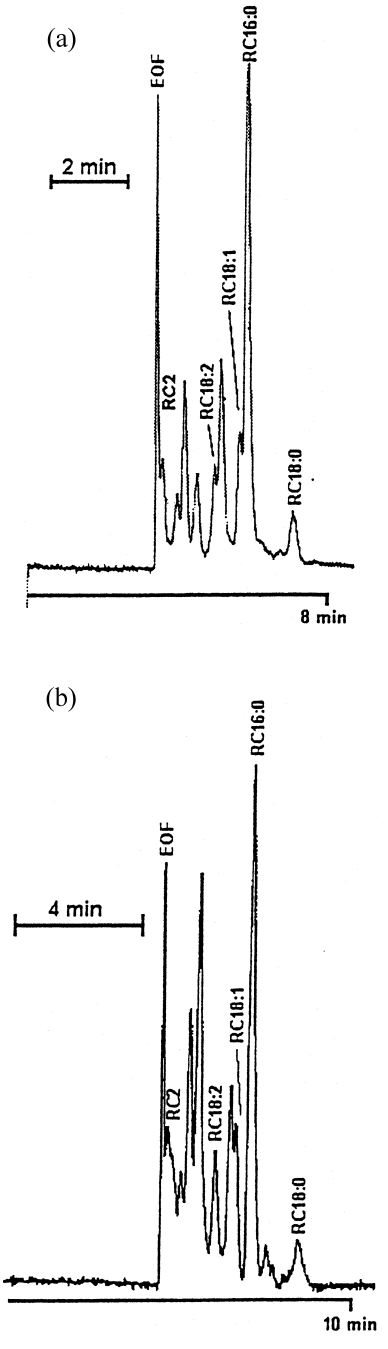

Fig. 5. Seal liver extract profiles using (a) 22 cm L_{eff} and (b) 36 cm L_{eff}. Other conditions as in Fig. 2.

Fig. 5 are expected to be isomeric and/or metabolic components of retinyl esters. With the longer column overall resolution with other unidentified components was not achieved. Further increase in bed length is expected to improve overall resolution.

The continuous bed C_{18} columns used in the present study show high resolution within a short analysis time. However, the use of C_{30} material has earlier been demonstrated to have a higher selectivity for retinyl esters as compared to C_{18} material [5]. The C_{30} material has also a documented shape selectivity [15]. Not being commercially available, large-pore C_{30} materials will have to be synthesized.

4. Conclusions

The newly described technique for preparing continuous bed columns based on sol–gel bonded packing materials [9–11] was simple and easily implemented. The columns were robust and stable, with long lifetimes. No bubble formation was observed, even at high current levels. This in turn makes possible the use of simpler CEC instrumentation. The columns prepared using commercially available large-pore C_{18} material demonstrated short analysis times and high resolving power for retinyl esters using a nonaqueous mobile phase. Synthesis of large-pore C_{30} material for continuous bed column purposes would be a natural next step to take, considering the C_{30} materials higher selectivity for these compounds as compared to C_{18} [5].

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