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Journal of Chromatography A, 985 (2003) 471–478
JOURNAL OF CHROMATOGRAPHY A
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Separation of conjugated trienoic fatty acid isomers by capillary electrophoresis

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Abstract

A method for direct resolution of conjugated trienoic fatty acid isomers by capillary electrophoresis has been developed. To obtain complete separation a dual cyclodextrin system was used. This contained heptakis-(6-sulfo)-β-cyclodextrin (charged), β-cyclodextrin (uncharged) and sodium dodecylsulfate. Under optimized conditions, all seven isomers were well separated. On average, separation efficiency was 2.9×10^5 plates/m.

Keywords: Geometrical isomers; Positional isomers; Fatty acids; Octadecatrienoic acids; Eleostearic acid

1. Introduction

Conjugated polyenoic acids do not occur to any significant extent in animal lipids but are found in certain seed oils mainly as C_{18} trienes or tetraenes [1]. Seven species of conjugated trienoic fatty acids (CTFAs) are known, which include punicic acid (18:3Δ9*Z*,11*E*,13*Z*), jacaric acid (18:3Δ8*Z*,10*E*,12*Z*) α-eleostearic acid (18:3Δ9*Z*,11*E*,13*E*), catalpic acid (18:3Δ9*E*,11*E*,13*Z*), α-calendic acid (18:3Δ8*E*, 10*E*,12*Z*), β-eleostearic acid (18:3Δ9*E*, 11*E*,13*E*) and β-calendic acid (18:3Δ8*E*, 10*E*,12*E*), Fig. 1. The only well-known conjugated tetraenoic acid in seeds is α-parinaric acid (18:4Δ9*Z*,11*E*,13*E*,15*Z*) and its geometrical isomers, which recently have been separated by means of capillary electrophoresis (CE) by Öhman et al. [2]. The conjugated triene and tetraene fatty acids are much more susceptible to oxidation and polymerization reactions compared to regular methylene-interrupted polyunsaturated fatty acids [3]. In biological contexts, the rapid conversion of α-parinaric acid into relatively oxidized products renders this

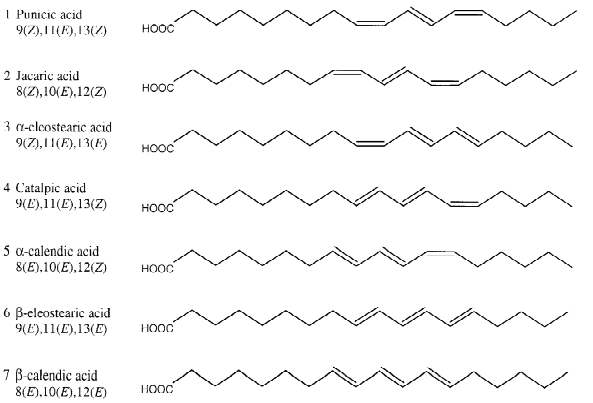

Fig. 1. Structures of CTFA isomers.

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01526-1

fatty acid cytotoxic, especially to malignant cells, and its use as chemotherapeutic agent for gliomas has been suggested. Also the conjugated trienoic acids under study in the present paper display cytotoxic effects [4,5]. In experimental work, α-parinaric acid finds use as a fluorescent membrane probe to monitor, e.g. lipid peroxidation [6] whereas the conjugated trienoic and tetraenoic fatty acids are potent inhibitors of prostaglandin biosynthesis [7]. Also in other areas, advantage is taken of the high oxidizability of conjugated trienoic and tetraenoic fatty acids. For example, supplementation of paints and varnishes with tung oil and other oils enriched with conjugated trienoic acids speeds up the drying process and produces more resistant films [8].

During recent years, CE has been demonstrated to be a quite powerful technique for the separation of isomers, in particular for relatively polar enantiomers. However, in a series of papers, we have shown also that isomers that are non-chiral and relatively non-polar can be efficiently resolved in CE [2,9,10]. Separation has thus been achieved according to position of functional groups and double bonds on the hydrocarbon chain as well as of *cis*/*trans* isomers. Some different classes of fatty acids were examined in that work. These included epoxy fatty acids (EFAs), divinyl ether fatty acids (DVEFAs), conjugated linoleic acids (CLAs) and parinaric acids (PAs). Our interest in these fatty acids emanates from their medical importance. In the present work, we have developed a method for the separation of CTFA isomers by CE.

2. Experimental

2.1. Equipment

All separations were achieved on a Hewlett-Packard ^{3D}CE system (Waldbronn, Germany), equipped with a diode array detector and an air-cooled capillary cartridge. Detection was done with direct UV. The diode array detector was first scanned from 190 to 400 nm and the wavelength that gave maximum absorbance, 268 nm, was chosen as detection wavelength. Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). All new capillaries were conditioned for 20 min with 2 *M* NaOH, 20 min with 0.1 *M* NaOH and 10 min with Milli-Q water (Millipore, Molsheim, France). Between runs, the capillary was flushed for 2 min with 0.1 *M* NaOH, 2 min with organic modifier, 3 min with Milli-Q water and 3 min with running buffer. Injection of samples was carried out by applying a pressure of 50 mbar for 10 s. The temperature was set to 15 °C.

2.2. Chemicals

Sodium dodecyl sulfate (SDS) was obtained from Fluka (Steinheim, Germany); boric acid from Aldrich (Steinheim, Germany); (*R*)-*N*-dodecoxycarbonylvaline [(*R*)-*N*-DOCV] from Waters (Milford, MA, USA); β-CD from Sigma (St Louis, MO, USA); heptakis-(6-sulfo)-β-cyclodextrin (HS-β-CD) and heptakis-(2,3-dimethyl-6-sulfo)-β-CD (HDMS-β-CD) from Regis Technologies (Morton Grove, IL, USA), highly sulfated β-CD from Beckman Coulter (Fullerton, CA, USA). All other chemicals were of analytical grade.

2.3. Electrolyte preparation

Stock solutions of SDS, urea and boric acid were prepared by dissolving appropriate amounts in Milli-Q water. Boric acid was adjusted to pH 9.2 by adding 20 *M* NaOH as required. Prior to use the solutions were filtered through a 0.45-μm polypropylene filter. Fresh running buffers were prepared daily. Additives such as organic modifier and selectors were mixed with the buffer directly in the electrophoresis buffer vial and degassed in a sonication bath.

2.4. Sample preparation

The conjugated trienoic acids used in the present study were all purchased from Larodan (Malmö, Sweden). Stock solutions of CTFAs were prepared by dissolving appropriate amounts in 99.5% ethanol. The solutions were kept at −25 °C under argon. The samples were diluted in 99.5% (v/v) ethanol and running buffer was added. The final concentrations of CTFA isomers were 200 μ*M* and the concentration of the running buffer was 10% (v/v) in the sample vials. Finally, the mixtures were degassed

and mixed in a sonication bath. Samples were prepared daily.

3. Results and discussion

The fatty acids examined are very hydrophobic and differ only in position and geometry of double bonds. This makes the fatty acids difficult to separate without the presence of a pseudostationary phase or other types of selectors in the running buffer [2,9,10]. Different surfactants were tested as pseudostationary phase, SDS, sodium cholate (SC), (*R*)-*N*-DOCV and polyoxyethylene lauryl ether (Brij 35). The naturally occurring surfactant SC is useful for chiral separation of relatively hydrophobic compounds. Also the neutral surfactant Brij 35 has been found to separate hydrophobic compounds like unsaturated fatty acids and related isomeric hydroperoxides, either alone or together with SDS as mixed micelles [11–13]. However, Brij 35 has not improved selectivity for divinylether fatty acid (DVEFA) [9], conjugated linoleic acid (CLA) or parinaric acid (PA) [2] and did not provide separation of CTFA either. Like Brij 35, SC did not provide enhanced separation resolution or efficiency. Instead SDS gave some separation, which indicates that a negatively charged surfactant is required (Fig. 2). Several studies have shown that SDS is a good choice of surfactant when separating fatty acid isomers [2,9,10,14]. To enhance the solubility of the hydrophobic fatty acids, urea was added to the running buffer. Without urea, the fatty acids easily precipitated and the current was interrupted after a few runs.

Enhanced resolution using negatively charged additives is mainly due to longer migration times due to their electrophoretic mobility opposite to the electroosmotic flow. Hence, in order to keep migration times reasonable, a high pH buffer was used. A high pH buffer (at least one pH unit higher than pK_a) also allows the acids to stay in their dissociated form. This will give the analytes negative electrophoretic mobility and thus provide further separation.

3.1. Influence of SDS concentration

The influence of SDS concentration on the separation was examined. It was found that an increase in SDS concentration from 24 to 35 m*M* resulted in improved resolution of peaks 3 and 4 but peaks 1 and 2 became fused. The result is expected. In the system used in this work, elution is according to hydrophobicity and the less hydrophobic isomers are eluted first. As a general rule, the more hydrophobic the analytes, the lower the SDS concentrations need to be for selectivity as well as efficiency [10].

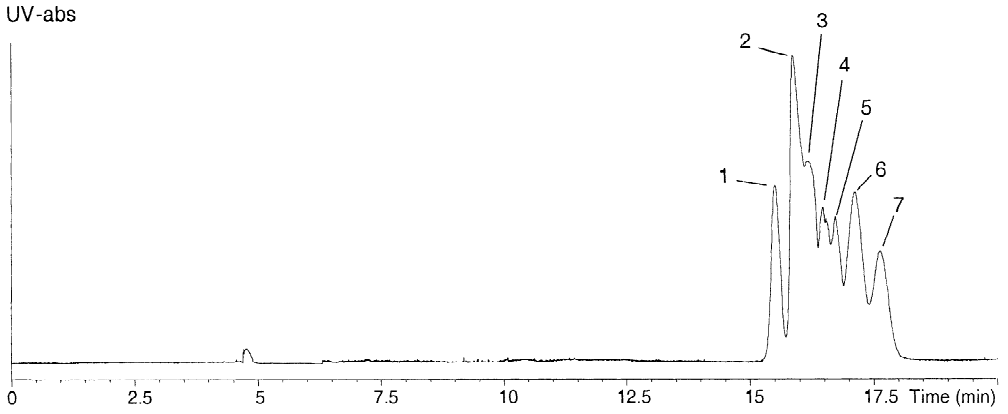

Fig. 2. Separation of CTFA isomers without selectors. Conditions: Capillary, fused-silica 58 cm (effective length 50 cm)×50 μm I.D.; 30 kV; 21 μA; 15 °C. Detection, UV at 268 nm. Background electrolyte, 24 m*M* SDS, 20% (v/v) acetonitrile, 40 m*M* borate (pH 9.2), 4 *M* urea.

3.2. (R)-N-DOCV and β-CD as selectors

The chiral negatively charged surfactant (*R*)-*N*-DOCV has been successfully used as an additive rather than as a pure micellar solution when separating different types of fatty acid isomers like DVEFA and CLA [2,9], in particular separation efficiencies have been improved with this selector. In an attempt to enhance separation for CTFAs, (*R*)-*N*-DOCV was added in sub-micellar concentration to the SDS buffer. However, separation became less complete. The migration times were largely the same but the peaks started to overlap. In addition, no efficiency improvement was seen compared to the simple SDS buffer. In order to improve selectivity, native β-CD was added to the SDS buffer with (*R*)-*N*-DOCV. The migration time and selectivity slightly increased, but the efficiency decreased (Fig. 3a,b). Better results concerning efficiency were found when only β-CD was used in the SDS buffer (Fig. 3c). This is mainly due to shorter migration time which leads to decreased band broadening. However, selectivity was somewhat decreased which indicates that the interactions with β-CD are too strong.

3.3. Dual CD systems

In order to balance the distribution between the buffer, micelle and CD phase, a second, negatively-charged β-CD was added to the SDS buffer. When separation selectivity is insufficient with one type of CD, a dual CD system can provide unique selectivity for structurally similar compounds [15]. A negatively charged CD acts as a counter-migrating phase and the neutral CD acts as a competing phase. The result is shown in Fig. 4a where a dual system with β-CD and HS-β-CD was used leading to increased selectivity and efficiency. The migration time was less than 14 min and the efficiency was high: 2.9×10^5 theoretical plates/m. Addition of HDMS-β-CD to the β-CD buffer (Fig. 4b) also offered improved selectivity and efficiency compared to the simple SDS buffer, but plate numbers were about four times lower than with HS-β-CD. When a dual CD system was used for separation of CLAs mainly the derivatized CD offered selectivity for the isomers [2]. For the CTFA isomers, the neutral CD gave selectivity. However, there was no separation using the dual CD system without SDS. A different type of derivatized CD, highly sulfated β-CD was obtained from another company. This CD is negatively charged and consist of a mixture where each CD has an average number of 12 sulfates per CD [16]. Since the product is a mixture, the molecular mass cannot be exactly calculated. A dual CD system with the highly sulfated β-CD and native β-CD was examined (Fig. 4c). Approximately the same concentration of the highly sulfated β-CD as that of the HS-β-CD (1 m*M* HS-β-CD ~1.85 mg/ml highly sulfated β-CD) was used. This gave an improved selectivity for the CTFA isomers compared to the SDS buffer in Fig. 2. As compared to HS-β-CD, resolution was slightly decreased and the efficiency was about 10 times lower.

As mentioned earlier, highly sulfated β-CD has an average number of sulfate groups. HS-β-CD and HDMS-β-CD are synthesized under precise control and are substituted with a sulfate group only at the sixth carbon in the glycopyranose ring. The degree of substitution, the exact position of substitution and purity of the product can have a large influence on the selectivity and reproducibility of the method. The fact that HS-β-CD offered both higher efficiency and better selectivity than highly sulfated β-CD indicates that a purer product and more precise derivatization of CDs are important for separating CTFA isomers. Different migration times were observed for the different types of CDs. This is simply explained by molecular mass and charge. HDMS-β-CD has the same number of charges as HS-β-CD but is heavier because of the dimethyl groups on the second and third carbon in the glycopyranose ring. Therefore it does not move as quickly as HS-β-CD. The highly sulfated β-CD has more negative charges and hence a higher electrophoretic mobility than HS-β-CD.

3.4. Capillary length

Since no baseline separation was achieved with the SDS buffer (Fig. 2), we tried a longer capillary (Fig. 5). As expected the selectivity was improved; however, peaks 2 and 3 coeluted. Further, the efficiency increased. Also for the highly sulfated β-CD buffer a longer capillary was used. Efficiency and selectivity was improved here as well; only peaks 3 and 4 were not fully separated. Theory

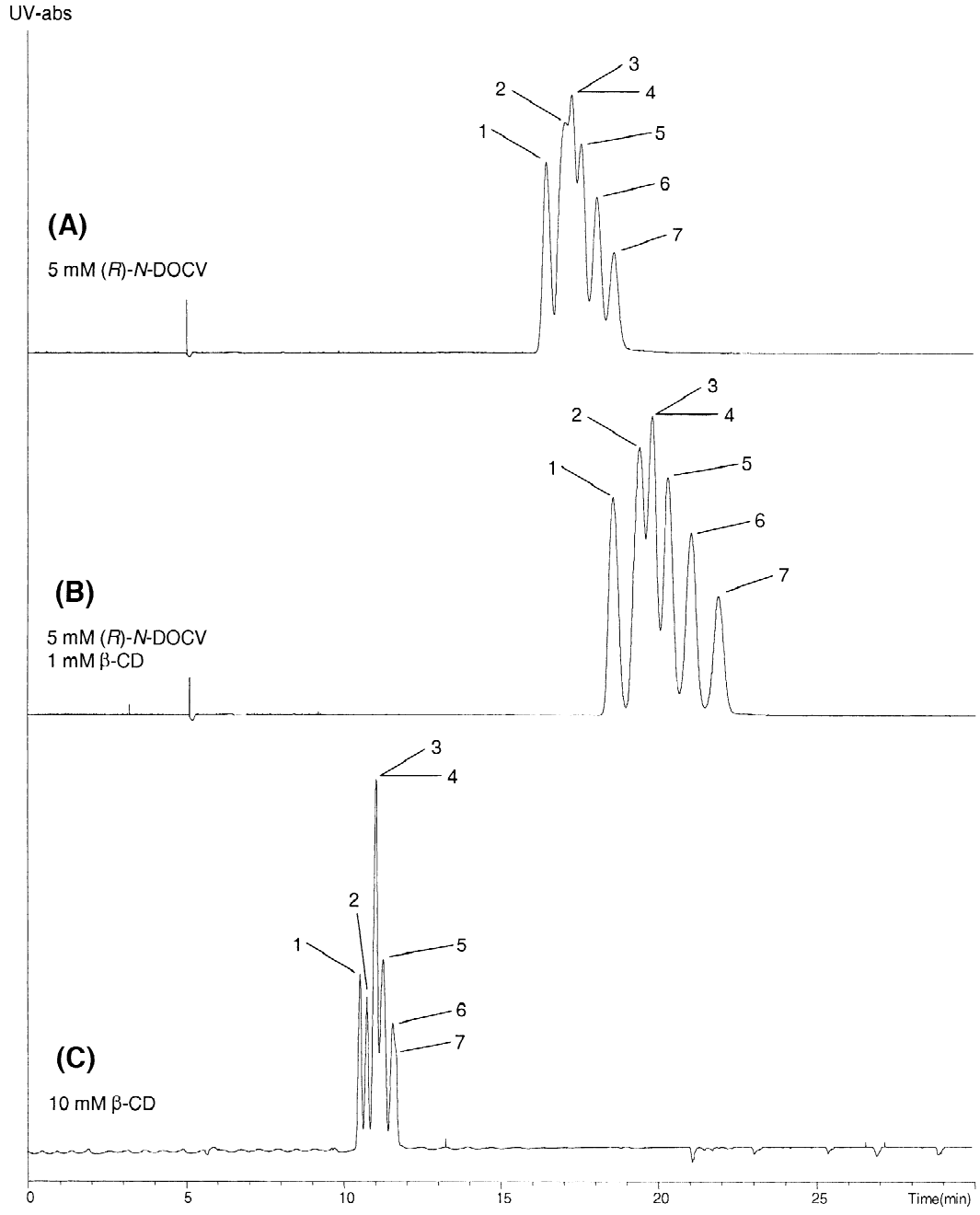

Fig. 3. Separation of CTFA isomers with different additives. Conditions as in Fig. 2. Background electrolyte, 24 m*M* SDS, 20% (v/v) acetonitrile, 40 m*M* borate (pH 9.2), 4 *M* urea, (A) 5 m*M* (*R*)-*N*-DOCV, (B) 5 m*M* (*R*)-*N*-DOCV, 1 m*M* β-CD, (C) 10 m*M* β-CD.

predicts that a longer capillary leads to longer migration times and hence band broadening due to longitudinal diffusion. Band broadening leads in turn to lower efficiency. In our CE system, the temperature on 8 cm of the capillary could not be controlled. With a longer capillary, effective cooling over a

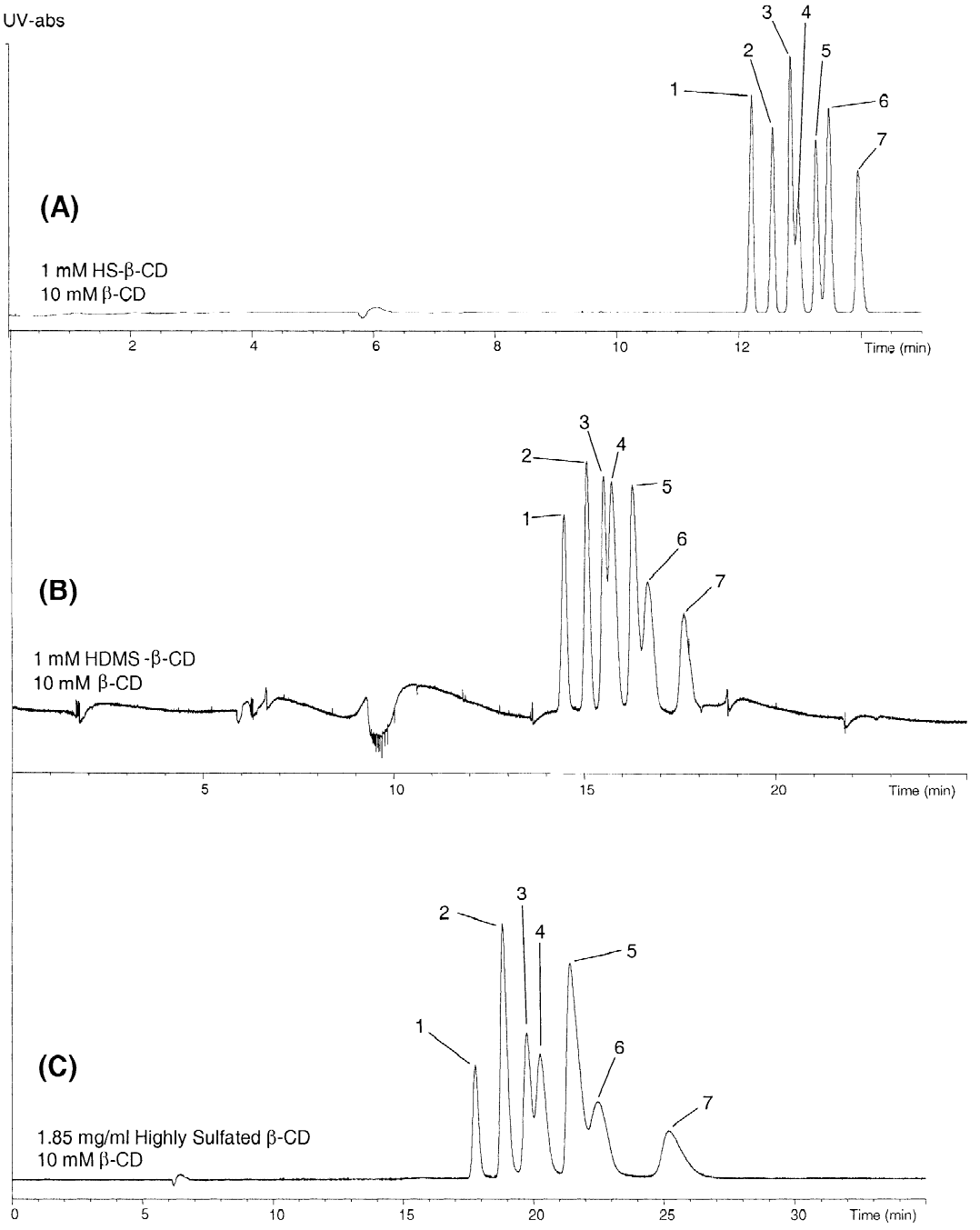

Fig. 4. Separation of CTFA isomers with mixed CDs. Conditions as in Fig. 2. Background electrolyte, 24 m*M* SDS, 20% (v/v) acetonitrile, 40 m*M* borate (pH 9.2), 4 *M* urea, (A) 1 m*M* HS-β-CD, 10 m*M* β-CD, (B) 1 m*M* HDMS-β-CD, 10 m*M* β-CD, (C) 1.85 mg/ml highly sulfated β-CD 10 m*M* β-CD.

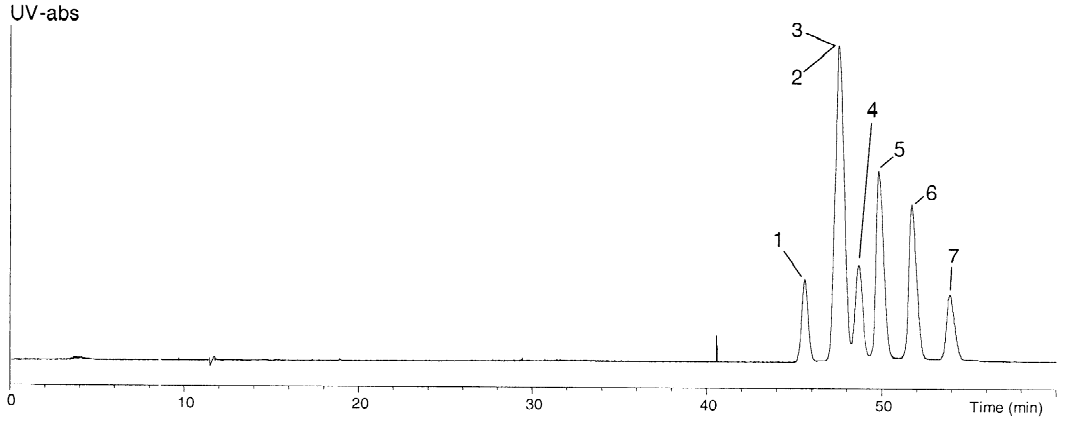

Fig. 5. Influence of capillary length on separation of CTFA isomers without selectors. Conditions as in Fig. 2. Capillary, 83 cm (effective length 75 cm); background electrolyte, 24 m*M* SDS, 20% (v/v) acetonitrile, 40 m*M* borate (pH 9.2), 4 *M* urea.

relatively longer length could be maintained and, in spite of an increase in longitudinal diffusion, efficiency was improved. This indicates that Joule heating gives a relatively large contribution to band broadening in the present micellar electrokinetic chromatography (MEKC) system [9,10]. Further, due to lower field strength the current, and thereby the heating, was much lower since the voltage was the same for both capillary lengths.

3.5. Elution order

Despite the fact that the CTFA isomers are structurally very similar it is possible to point out some general principles for elution order in an MEKC system. *cis* Isomers elute before *trans* isomers and the more *trans* bonds in the isomer, the longer the migration times. Regioisomers (1 and 2, 4 and 5, 6 and 7) are separated depending on the location of the conjugated triene structure in the carbon chain: closer to the carboxyl function means a longer retention time. Molecules with *trans* double bonds seem to be somewhat more hydrophobic than molecules with *cis* double bonds, which may be explained by the small dipole moment that is created at the *cis* bond. As mentioned above, the acids are being separated according to their difference in hydrophobicity; the more hydrophobic acids elute last. This is reasonable since SDS offers a hydrophobic environment. When methanol, which is more polar than acetonitrile, is used as the organic modifier, isomer number 5 elutes before number 3 (not shown). Methanol thus influences the hydrophobic environment and changes the elution order. Another explanation for the elution order could be that molecules with *cis* bonds sprawl more than molecules with *trans* bonds. Therefore the molecules with more *cis* bonds cannot access the interior of the micelles as easily as molecules with more *trans* bonds. The *cis* molecule therefore spends more time in the bulk solution than the *trans* molecule, hence it elutes earlier. Isomer numbers 3 and 4 were the most difficult to separate, and their peaks overlap to some extent in all electropherograms. These isomers are very much alike; it is only the position of the *cis* double bond that is different and this may explain the fact that they are more difficult to separate than the other isomers.

4. Conclusions

Capillary electrophoresis can be successfully used to separate all conjugated trienoic fatty acid isomers. This was achieved using an SDS buffer in combination with a dual CD system containing native and charged β-CDs. The performance in such a dual CD system of two types of charged β-CDs was compared. One charged CD had been synthesized under controlled conditions and it consisted, in principle, of a pure chemical compound. The other charged CD had been randomly synthesized and it consisted of a

mixture. The highest plate numbers and the best separations were obtained with the pure selector. Further, some general principles for elution order of CTFA isomers in the present MEKC buffer can be pointed out. *cis* Isomers elute before *trans* isomers and the more *trans* bonds in the isomer, the longer the migration times. The shorter the distance to the carboxyl group, the longer the retention time. Molecules with *trans* double bonds seem to be somewhat more hydrophobic than molecules with *cis* double bonds. This difference is crucial for separation. Moreover, changing the type of organic modifier can influence the hydrophobic environment and may alter the elution order.

Acknowledgements

This work was supported by the Swedish Research Council and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas).

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