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Separation of hydroxylated polycyclic aromatic hydrocarbons by micellar electrokinetic capillary chromatography

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Abstract

A capillary electrophoretic method for effective separation of hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) is presented. The effect of urea as secondary modifier on the retention behaviour of 10 hydroxylated PAHs using cyclodextrin modified micellar electrokinetic capillary chromatography (CD-MECC) is investigated. The changes of analyte migration times have been attributed to a ternary analyte–cyclodextrin–urea complex. The separation of 10 common hydroxylated PAHs using the developed CD-MECC method is compared to the results gained by high-performance liquid chromatography (HPLC) separation using common reversed-phase conditions.

Keywords: Buffer composition; Cyclodextrin modified micellar electrokinetic capillary chromatography; Polynuclear aromatic hydrocarbons

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants present in all kinds of environmental matrices [1]. They are products of incomplete combustion of fossil fuels, major exhaust components of transportation, refuse, burning and industrial energy sources. Some PAHs have been proved to cause cancer in animals, and they are suspected to be human carcinogens as well [2,3]. Sources of population exposures to PAHs from various sources are e.g. smoking, food consumption and air pollution.

The determination of PAH metabolites in urine has been suggested as biological indicator of exposure to PAHs. Several industrial hygiene studies have shown that 1-hydroxypyrene in urine is a valid and sound biomarker for occupational exposure to PAHs. Furthermore the determination of the benzo[*a*]pyrene metabolite concentration in human urine can be used as indicator for a personal PAH contamination [4–6].

Several researchers used high-performance liquid chromatography (HPLC) for separation and identification of PAH metabolites [7,8]. However, separation of some of the structural isomers, enantiomers and closely related metabolites has been difficult to achieve. Viau et al. reported serious reproducibility problems which were overcome by adding ascorbic acid to the eluent [9]. Grimmer described a GC–MS method, including derivatisation with diazomethane and a multistage clean-up [10].

In recent years capillary electrophoresis (CE) with its high separation efficiency has found its place in various analytical fields among the conventional chromatographic techniques (HPLC, GC). While capillary zone electrophoresis has emerged as one of the most efficient methods available for the separation of components in mixtures, it is limited to the analysis of water soluble charged species. Therefore it is not applicable for the analysis of the neutral and

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water-insoluble PAHs and their metabolites. Modified CE methods for the separation of uncharged molecules have emerged, namely micellar electrokinetic capillary chromatography (MECC). The separation by MECC is based on partition of the analytes between the aqueous buffer and the charged and pseudostationary micellar phase [11,12]. However, the addition of a micellar phase provides separation of polar molecules as well as very non-polar substances (as for example PAHs) [13]. On the other hand the MECC separation of the hydroxylated PAHs is not satisfactory. Because of their structural similarity the different isomers show a nearly identical partition behaviour between micellar and aqueous phase and consequently show the same elution behaviour.

One possibility to achieve a separation of these substances is the addition of cyclodextrins [14–16]. Cyclodextrins (CDs) are water soluble cyclic oligosaccharides which consist of α-1,4-linked glucopyranose subunits. The most common forms are α-CD, β-CD, and γ-CD, with six, seven, and eight subunits. They possess a toroidal structure with a non-polar interior cavity and can form host–guest inclusion complexes with many hydrophobic compounds. Cyclodextrin modified MECC (CD-MECC) has been proved to be one of the most powerful separation techniques for neutral and hydrophobic compounds such as PAHs [14–16].

To the best of our knowledge this is the first report on the separation of hydroxylated PAHs with CD-MECC. In this work, the use of γ-CD as modifier in MECC of PAH metabolites was studied. Furthermore, the migration behaviour of the metabolites at different concentrations of sodium dodecyl sulphate (SDS) and urea in the electrophoretic media was investigated. The CD-MECC method is compared to a common RP-HPLC method in view of migration/elution order and separation efficiency.

2. Experimental

2.1. Micellar electrokinetic capillary chromatography

CD-MECC experiments were performed using a Dionex CES-1 (Dionex, Idstein, Germany). Separation was achieved at 10 to 15 kV (+polarity) using gravity injection (30 mm, 10 s) and UV-absorbance detection (270 nm). Bare fused-silica capillaries of 60 cm (55 cm effective length)×50 μm I.D. were used with N_2 forced cooling.

A detection wavelength of 270 nm was found optimal for all investigated components. The UV spectrum of benzo[*a*]pyrene-*trans*-4,5-dihydrol is shown in Fig. 1 as a typical example.

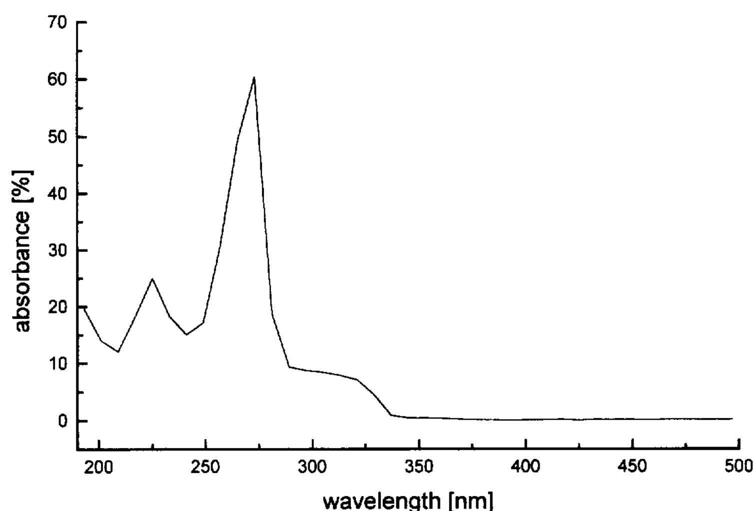

Fig. 1. UV spectrum of benzo[*a*]pyrene-*trans*-4,5-dihydrodiol in acetonitrile–water; wavelength: 190–500 nm.

2.2. High-performance liquid chromatography

HPLC separations were obtained on a modular system composed of a Hewlett–Packard 1050 pump and autosampler, combined with a HP 1040M diode-array detector (270 nm). The components were separated on a Bakerbond PAH 16-plus column (250 mm×3 mm) using a linear solvent gradient at a flow-rate of 0.5 ml min^{-1}. The solvent system was water–acetonitrile with a 30-min gradient from 50–80% acetonitrile. All hydroxylated PAHs were dissolved in acetonitrile.

2.3. Samples and solutions

All chemicals were analytical-reagent grade. Sodium tetraborate, boric acid, urea and SDS were obtained from Merck (Darmstadt, Germany). γ-CD was purchased from Wacker Chemie (Düsseldorf, Germany). Methanol and acetonitrile were obtained from Baker (Deventer, Netherlands).

The ten PAH metabolites of interest were obtained from Midwest Research Institute (Kansas City, USA). The isomers and their structures of these PAH metabolites are shown in Fig. 2.

All CE buffers were prepared using purified water (Seralpur 90 CN) and contained 2.5 m*M* sodium tetraborate, 12.0 m*M* boric acid and different concentrations of SDS and γ-CD. Buffer pH was adjusted to 9.0 with 0.1 *M* NaOH. Prior to use all buffer solutions were filtered through a 0.45-μm cellulose nitrate membrane filter (Schleicher and Schüll, Germany) and vacuum degassed.

3. Results and discussion

Separation in MECC is based on the partitioning of the analyte between the aqueous buffer and the charged pseudostationary micellar phase. Therefore retention of the solutes depends on their polarity.

To enhance the solubility of the analytes in the buffer and to achieve separation of isomeric analytes cyclodextrins were added as modifier to the buffer. The elution order in CD-MECC strongly depends on the complex stability of the analyte–cyclodextrin complex. This complex stability is mainly influenced by the relationship between cavity size and analyte size. The larger the difference in size is, the weaker is the complex binding. Therefore a good fit into the CD cavity results in a short retention time. Even separation of isomeric substances with nearly the same polarity, as for example the three *trans*-dihydroxy-PAHs was possible. Their geometrical structure causes different complex constants for each enantiomer–CD complex.

Running buffer additives, such as cyclodextrins, are capable of changing the overall polarity of the mobile phase. Therefore they influence the hydrophobic interactions between the solute and the pseudostationary phase [17]. In summary, there is a competitive equilibrium for the non-polar solute between the pseudostationary phase (micelle) and the running buffer (cyclodextrin). We started our investigations with a CD-MECC buffer, containing SDS and γ-CD in a ratio of 3:2 with a low SDS-concentration. γ-CD was chosen as a result of the work of Li et al. who pointed out that γ-CDs provide a better separation of PAHs than β-CDs [18].

The electropherogram obtained with this buffer system is shown in Fig. 3. The ten analytes were eluted as four broad peaks within 10 min. The reason for the low separation efficiency was probably an insufficient concentration ratio between the micellar phase (SDS) and the CD in the buffer. The SDS/γ-CD ratio of 3:2 and the total SDS concentration proved to be too low and resulted in a shift of the analyte distribution equilibrium towards the analyte–CD complex.

Therefore optimization of the SDS/γ-CD ratio had to be performed in order to improve the separation. As shown in Fig. 4 the SDS concentration on the separation was investigated for a γ-CD amount of 20 m*M*. Five electropherograms using CD-MECC buffer with SDS concentrations from 30–70 m*M* are shown in Fig. 4.

As expected the elution time of the electroosmotic flow increases with higher concentrations of SDS. In this figure the change of the distribution ratio of each compound with changing SDS concentration can be seen. The best separation but the longest elution time was obtained when using 70 m*M* SDS. On the other hand complete separation of these five substances had been achieved using a SDS concentration of 40 m*M*. To improve our method a compromise between analysis time, cost and separation efficiency had to

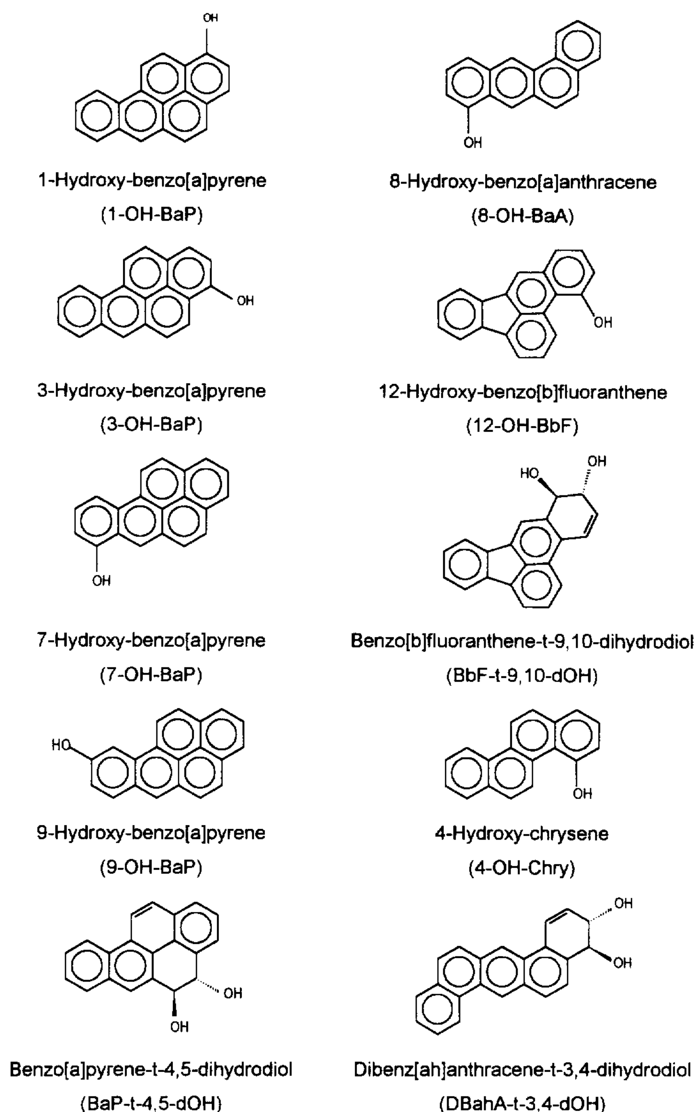

Fig. 2. Structures of the hydroxlated polycyclic aromatic hydrocarbons.

be made. Therefore we decided to use a concentration of 50 m*M* SDS in further experiments.

To achieve optimal conditions for adjustment of the complex equilibrium the ideal concentration of the chiral modifier was evaluated. The γ-CD concentration was raised stepwise from 2–50 m*M* while the SDS concentration was kept constant at 50 m*M*. Under these conditions the six electropherograms shown in Fig. 5 were obtained.

The plot indicates that the distribution ratio of the analytes shift from the micellar phase to the CD complex with increasing γ-CD concentration. At low CD concentrations the bulk of the analytes is located in the micelles, whereas at high CD concentrations they are preferably positioned in the CD complex. This leads to badly resolved broad peaks either at the beginning, or at the end of the retention window. Therefore, only a short range of the SDS/CD ratio is

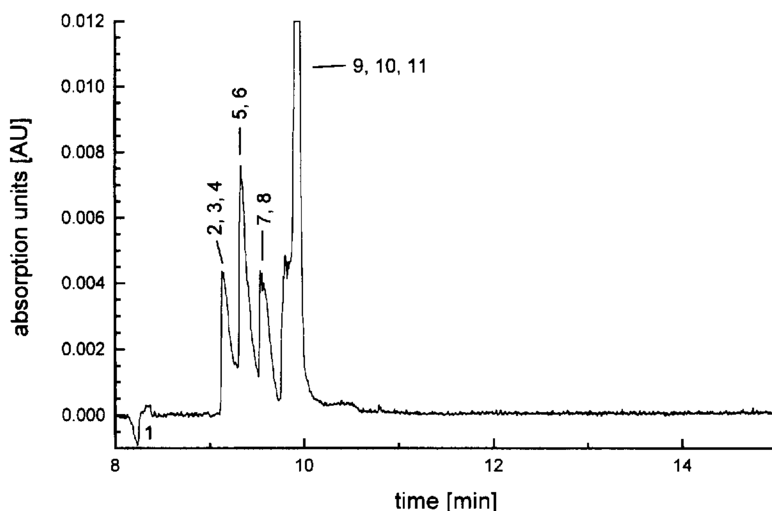

Fig. 3. CD-MECC separation of ten hydroxylated polycyclic aromatic hydrocarbons; analytes: 1=EOF, 2=7-OH-BaP, 3=3-OH-BaP, 4=8-OH-BaA, 5=9-OH-BaP, 6=4-OH-Chry, 7=DBahA-*t*-3,4-dOH, 8=1-OH-BaP, 9=BaP-*t*-4,5-dOH, 10=12-OH-BbF, 11=BbF-*t*-9,10-dOH; electrophoretic conditions: 12 m*M* $Na_2B_4O_7$, 2.5 m*M* H_3BO_4, 30 m*M* SDS, 20 m*M* γ-CD; UV detection at 270 nm, 10 kV (+).

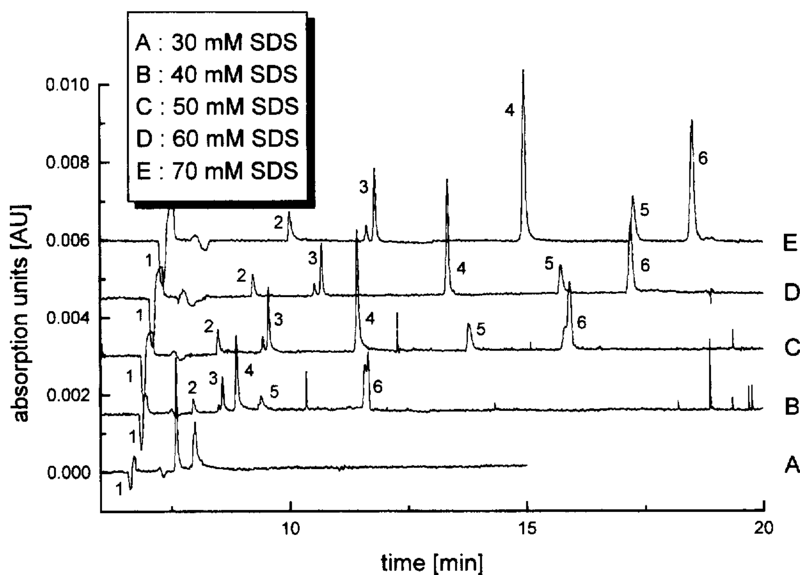

Fig. 4. CD-MECC separations of five hydroxylated PAH; analytes: 1=EOF, 2=3-OH-BaP, 3=8-OH-BaA, 4=4-OH-Chry, 5=12-OH-BbF, 6=BaP-*t*-4,5-dOH electrophoretic conditions: 12 m*M* $Na_2B_4O_7$, 2.5 m*M* H_3BO_4, 30 m*M* γ-CD, SDS concentrations from 30–70 m*M*, UV detection at 270 nm, 15 kV (+).

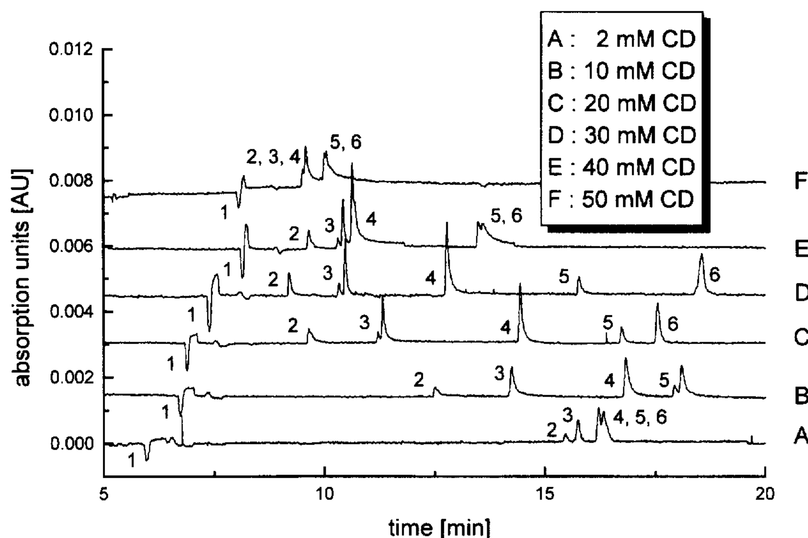

Fig. 5. CD-MECC separations of five hydroxylated PAH; analytes: 1=EOF, 2=3-OH-BaP, 3=8-OH-BaA, 4=4-OH-Chry, 5=12-OH-BbF, 6=BaP-*t*-4,5-dOH electrophoretic conditions: 12 m*M* $Na_2B_4O_7$, 2.5 m*M* H_3BO_4, 50 m*M* SDS, γ-CD concentrations from 2–50 m*M*; UV detection at 270 nm, 15 kV (+).

suitable for electrophoretic analysis. A separation with an enlarged elution range of the analytes took place with a SDS/CD ratio of 5:2 up to 5:3. The best separation efficiency with an constant SDS concentration of 50 m*M* was achieved at a SDS/CD ratio of 5:3. In all further experiments the concentrations were set to 50 m*M* SDS and 30 m*M* γ-CD. Fig. 5 additionally indicates a shift in the electroosmotic flow (EOF). This is presumably caused by changes of the buffer viscosity and the zeta-potential.

Warner et al. reported that the PAHs are encapsulated within the cyclodextrin cavity in a ratio of 1:1 (CD:PAH), 2:1, 1:2 or other [19]. Secondary modifiers that contain amine groups can stabilize these complexes by forming a ternary complex. The structure of this complex is unknown in detail. Warner tried to explain this effect by the formation of hydrogen bonds between the amine group of the secondary modifier and the peripheral hydroxyl functions of the cyclodextrin. An increasing stability of the ternary complex with an increasing amount of the secondary modifier was predicted.

Therefore we investigated the influence of urea as a secondary modifier in the buffer on the electrophoretic resolution of the PAH metabolites (Fig. 6). The increase of the urea concentration from 0–6.0 *M* resulted in an increase of the EOF retention by the value of 1.4 cm min^{-1}. In contrast to this the elution times of 3-OH-BaP, 8-OH-BaA and 4-OH-Chry (2, 3 and 4) increased more slowly than the shift in the EOF elution time. The migration velocity of BaP-*t*-3,4-dOH (peak 6) decreased with almost the same value as the EOF. Contrary to this the elution time of 12-OH-BbF increased significantly with the addition of urea. Therefore a higher concentration of urea influenced the stability of the analyte–CD complex in different ways: 3-OH-BaP-, 8-OH-BaA- and 4-OH-Chry complexes become stronger, the stability of BaP-*t*-3,4-dOH–CD complex is not affected by the addition of urea, and the 12-OH-BbF–CD complex is weakened.

A possible explanation of these facts may be given with reference to Warner et al. The formation of hydrogen bonds between the amine group of the secondary modifier and the peripheral hydroxyls of the CD changes the polarity and the inner diameter of the CD cavity. Due to the increased hydrophobic surrounding in the cavity some substances build stronger complexes with the cyclodextrin and elute faster (for example 3-OH-BaP, 8-OH-BaA and 4-OH-Chry). On the other hand, reduction of the inner

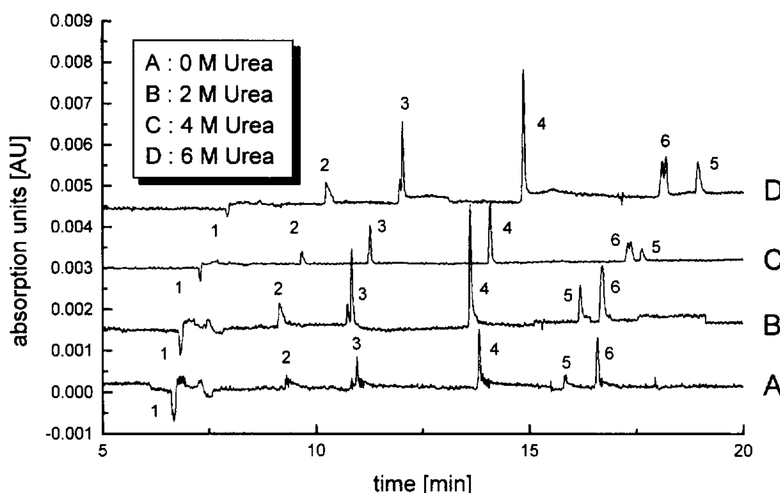

Fig. 6. CD-MECC separations of five PAH; analytes: 1=EOF, 2=3-OH-BaP, 3=8-OH-BaA, 4=4-OH-Chry, 5=12-OH-BbF, 6=BaP-*t*-4,5-dOH, electrophoretic conditions: 12 m*M* $Na_2B_4O_7$, 2.5 m*M* H_3BO_4, 50 m*M* SDS, 30 m*M* γ-CD, 0–6 *M* urea; UV detection at 270 nm, 15 kV (+).

diameter of the cavity results in decreased complex stabilities of larger solutes, for example 12-OH-BbF. In case of BaP-*t*-3,4-dOH these effects seem to compensate each other. These different complex stabilities of 12-OH-BbF and BaP-*t*-3,4-dOH seem to cause the change in the elution order of the two substances that appeared with high urea concentration. At this stage of research we are unable to provide evidence to support this theory.

Following these optimisation experiments the method was applied to the separation of ten common PAH metabolites (Fig. 6). According to Fig. 5 the best compromise between separation efficiency, costs and separation time was obtained with an urea concentration of 4 *M*. However, baseline resolution of all analytes except 7-OH-BaP and 3-OH-BaA was achieved under these conditions in less than 23 min (Fig. 7).

Under these conditions (50 m*M* SDS, 30 m*M* γ-CD, pH 9.0 and addition of 4 *M* urea) sharp peaks of the hydroxylated PAHs were obtained. Therefore the adjustment of the distribution ratio of the analytes between the micellar and cyclodextrin phases has been completed successfully. In addition to baseline resolution of eight analytes the separation of the enantiomeric dihydroxy analytes was achieved. The separation of these enantiomeric substances successfully demonstrates the high potential of CD-MECC.

4. HPLC separation

Fig. 8 shows a HPLC separation of the ten hydroxylated PAHs on a C_{18} stationary phase with an acetonitrile–water gradient. For the separation of hydroxylated PAHs a HPLC method was developed, based on a common gradient program for determination of PAHs [20,21]. In contrast to CD-MECC separation the order of elution of the analytes does not appear to depend on the analyte structure. Consequently the diol-PAHs elute faster than the monohydroxy-PAHs with their lower hydrophobicity. Furthermore the position of the hydroxy function seems to influence the interaction between the stationary phase and the molecule and leads to a separation of the isomers.

In order to achieve comparable analysis times a relatively high slope of the gradient was applied. Unfortunately only five substances could be separated under these conditions. This shows the superior separation efficiency of CD-MECC compared to this HPLC method. Possibly a more moderate gradient

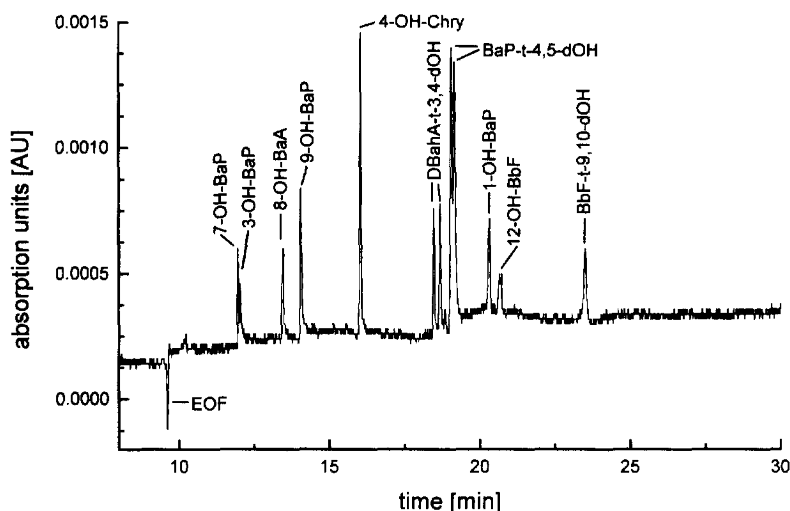

Fig. 7. CD-MECC separation of ten hydroxylated polycyclic aromatic hydrocarbons; analytes: 1-OH-BaP, 3-OH-BaP, 7-OH-BaP, 9-OH-BaP, 8-OH-BaA, 4-OH-Chry, 12-OH-BbF, BaP-*t*-4,5-dOH, BbF-*t*-9,10-dOH and DBahA-*t*-3,4-dOH; electrophoretic conditions: 12 m*M* $Na_2B_4O_7$, 2.5 m*M* H_3BO_4, 50 m*M* SDS, 30 m*M* γ-CD, 4 *M* urea; UV detection at 270 nm, 10 kV (+).

would improve the resolution, but it would also increase the analysis time. The use of chiral stationary phases or chiral additives (e.g. cyclodextrin) in the eluent may be advantageous but unfortunately only standard RP columns could be used for the comparison. However, HPLC methods suitable for PAHs are not necessarily adaptable to their hydroxylated species.

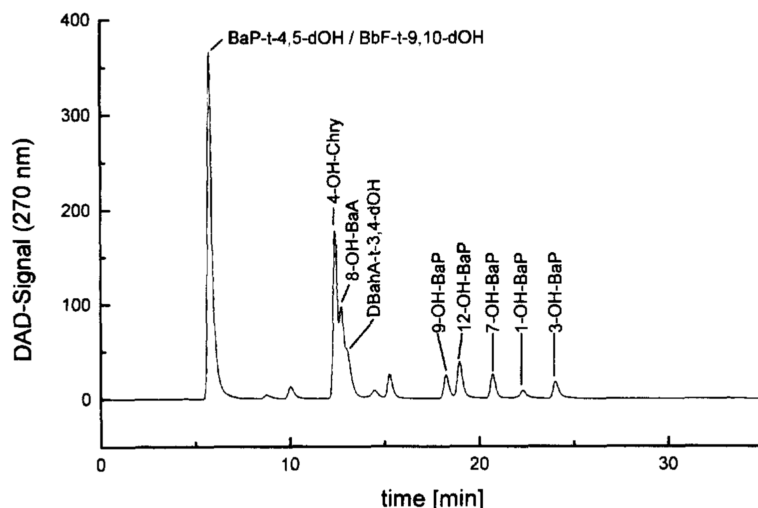

Fig. 8. HPLC separation of ten hydroxylated polycyclic aromatic hydrocarbons; analytes: 1-OH-BaP, 3-OH-BaP, 7-OH-BaP, 9-OH-BaP, 8-OH-BaA, 4-OH-Chry, 12-OH-BbF, BaP-*t*-4,5-dOH, BbF-*t*-9,10-dOH and DBahA-*t*-3,4-dOH; chromatographic conditions: acetonitrile–water gradient, 50–80% acetonitrile in 30 min, 0.5 ml min^{-1}, UV detection at 270 nm.

5. Conclusion

The authors developed a method for the separation of hydroxylated PAHs using CD-MECC with γ-CDs as chiral selector and urea as secondary modifier. Baseline resolution of eight hydroxylated PAHs could be achieved within 23 min. Additionally separation of enantiomeric dihydroxylated PAHs could be obtained.

The secondary modifier appears to strengthen and stabilize the complex formation of analytes and cyclodextrin, resulting in higher separation efficiency. In addition this modifier amplifies size-selective separation of the hydroxylated PAHs using CD-MECC.

The authors showed that a HPLC method suitable for the separation of PAHs that was modified for the separation of PAH metabolites cannot compete with the CD-MECC method in terms of separation efficiency and separation time. Differences in the separation mechanism between HPLC, which depends mainly on the analyte polarity, and CD-MECC with its size-selective separation mechanism could be shown. In future investigations the CD-MECC must be applied to actual samples like urine, blood and serum and to be compared to optimised HPLC and GC methods. When employed in the medical sector it could serve for fast and reliable monitoring of human exposure to PAHs.

Acknowledgments

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