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Highly efficient separation of amines by electrokinetic chromatography using resorcarene-octacarboxylic acids as pseudostationary phases

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Abstract

Resorcarene-octacarboxylic acids, macrocyclic molecules built up by four alkylidene-bridged resorcinol units, were synthesized and used as pseudostationary phases in electrokinetic chromatography (EKC). Resorcarenes provide a stable structure and good solubility in electrolytes even with organic modifiers. The high electrophoretic mobility of the resorcarene-octacarboxylic acids introduced here as pseudostationary phases is based on the eight partly deprotonated carboxylic groups. This offers a broad migration time window, which is the main parameter for the resolution of peaks. From three compounds with different alkyl chain lengths (C_1, C_5, C_{11}), the C_{11}-resorcarene-octa-acid possesses an extremely high selectivity for lipophilic compounds which is demonstrated by the efficient separation of thirteen homologous or isomeric amines derivatized with *o*-phthaldialdehyde and 2-mercaptoethanol. The order of peak elution is almost identical with that known in reversed-phase high performance liquid chromatography. Sensitive detection of amines is achieved using laser-induced fluorescence. Efficiencies up to 3 million plates/m were obtained resulting from the small detection window based on the intense focusing of the laser beam, a sample focusing effect in the sample zone and the absence of electrophoretic microheterogeneity of the pseudostationary phase.

Keywords: Pseudostationary phases; Amines; Resorcarene-octacarboxylic acids

1. Introduction

A number of approaches have been made to enhance the selectivity of capillary electrophoresis (CE) and its ability to analyze uncharged species. Since the early studies of Terabe et al. [1] with a micellar phase in 1984, various organic molecular aggregates, such as micelles in micellar electrokinetic chromatography (MEKC) [2–4] or microemulsions in microemulsion electrokinetic chromatography (MEEKC) [5–7] have been studied. But also discrete molecules like oligomerized sodium-10-undecylenate [8], cyclodextrins [9,10] and starbust dendrimers [11], ionic polymers [12,13] or chromatographic particles [14] have been used as pseudostationary phases in CE for the same purpose. All these systems offer the possibility for neutral species to be distributed between the added phase and the electrolyte with distinct partition coefficients.

The aim of developing new pseudostationary phases is to achieve a large variety of selectivities for various separation problems and to apply EKC with

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largely different electrolyte compositions (e.g. with a high content of organic modifier). Thus, problems like the lack of stability, solubility or mobility of pseudostationary phases in certain media, should be solved or minimized.

Due to their well defined structure and charge density, calixarenes and resorcarenes were recently shown to be highly promising pseudostationary phases. Shohat and Grushka [15] employed *p*-sulfonatocalix[6]arene to resolve chlorinated phenols and benzenediols. Very recently Sepaniak et al. [16] investigated the use of *p*-(carboxyethyl)calix-[*n*]arenes (with $n=4–8$) for the separation of small and polar-substituted polycyclic aromatic hydrocarbons (PAHs). Retention with these calixarenes is based on inclusion complexation of the analytes and best selectivities were observed with intermediate ring sizes ($n=5$ and 7).

In previous work we demonstrated the potential of resorcarenes **1** (see Fig. 1) as pseudostationary phases in EKC [17]. Resorcarenes are cyclic tetramers, which are easily obtained in one step through acid catalyzed condensation of resorcinol with a large variety of aldehydes R–CHO [18,19]. Often exclusively the all-*cis* stereoisomer is formed that adopts the cone conformation with an axial arrangement of the residues R.

Therefore, resorcarenes provide a well defined structure and conformation excluding microheterogeneity. Their selectivity can be modified by variation of the residues R. High electrophoretic mobility of resorcarenes is due to the easy formation of a tetra-anion stabilized by intramolecular hydrogen bonds and thus assuming a time-averaged C_{4v}-symmetry. The pK_a values for the first four protons have been shown to be lower than those of resorcinol by two units [20].

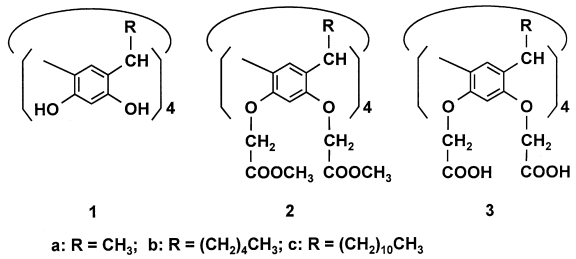

Fig. 1. Structures of the resorcarene-octacarboxylic acids investigated (**3a**–**c**) and their synthetic precursors (**1** and **2**).

These resorcarenes **1** allowed the use of high contents of organic modifiers (up to 80% acetonitrile), and the efficient separation of 12 highly hydrophobic PAHs using a resorcarene with undecyl **1c** residues was demonstrated.

In the present paper we examine the suitability of resorcarene-octacarboxylic acids **3** (see Fig. 1) as pseudostationary phases. In contrast to the parent resorcarenes **1** investigated in the past, where highly alkaline conditions (pH>12) were required to form the tetra-anion, resorcarene-octacarboxylic acids **3** are easily deprotonated at lower pH values.

2. Experimental

2.1. Syntheses of resorcarenes

Resorcarenes **1a**–**c** [21–24] were completely O-alkylated through the reaction with methyl bromoacetate using suitably modified literature procedures [25,26], and the resulting octa-esters **2a**–**c** were hydrolysed under alkaline conditions.

*2.1.1. General procedure for the preparation of resorcarene-octa-ester **2a**–**c***

To a suspension of the resorcarene (4.5 mmol), potassium carbonate (48 mmol, 10.6 eq. rf. to **1**) and a catalytical amount of sodium iodide in acetonitrile (50 ml) was added methyl bromoacetate (3.9 ml, 36.7 mmol, 8.1 eq.). The suspension was refluxed with stirring under a nitrogen atmosphere for 48 h, during which time (after 24 h) another portion of methyl bromoacetate (3.9 ml) was added. After cooling to room temperature the mixture was filtered and the filter was washed twice with ether (40 ml). The filtrate was concentrated under reduced pressure to give the crude product which was subjected to recrystallization from dichloromethane–methanol. The precipitate was filtered off and washed with methanol. Compounds **2a**–**c** were obtained in >60% yield as colorless crystals which could be purified for analytical purpose by recrystallization from dichloromethane or acetone and methanol.

*2.1.2. General procedure for the preparation of the resorcarene-octacarboxylic acids **3a–c***

Resorcarene-octa-ester **2** (3.57 mmol) and potassium hydroxide (85.7 mmol, 25 eq.) were suspended in a mixture of 50 ml water and 100 ml ethanol and heated to reflux for 2 h under a nitrogen atmosphere. During this time a yellowish solution was formed. The potassium salt precipitating upon slow cooling was filtered, washed with ethanol, dissolved in ~160 ml hot water and dropped into 100 ml 0.5 *M* HCl. Extraction with three portions of 150 ml ether and standard workup (washing with water, drying over $MgSO_4$ and evaporation of the solvent) gave octa-acid **3** in >90% yield based on **2** (overall yield >53%).

Resorcarene-octa-acid **3c**: colorless solid, m.p. 180°C; δ_H(200 MHz, DMSO) 6.49, 6.34 (2 s, 4 H ea., ArH), 4.47 (br. s, 4 H, ArCH), 4.29 (d, 8 H, J=16.5 Hz, OCH_2), 4.20 (d, 8 H, J=16.3 Hz, OCH_2), 1.74 (br. s, 8 H, $ArCCH_2$), 1.18 (s, 72 H, $(CH_2)_{2-10}$), 0.81 (m, 12 H, CH_3), (Found: C, 66.0; H, 8.6; $C_{88}H_{128}O_{24}\cdot 2H_2O$ requires C, 65.8; H, 8.2%).

2.2. Apparatus

The electrokinetic experiments were carried out on a Spectraphoresis 100 (TSP, Egelsbach, Germany) with LIF detection using a KF-2 laser-induced fluorescence detector (Sopra, Büttelborn, Germany) with HeCd laser (excitation 325 nm, emission >389 nm cut off filter). Untreated fused-silica capillaries with 75 μm I.D. were employed (Chromatographie-Service, Langerwehe, Germany). The total length of the capillaries was 75 cm, the effective length was 50 cm.

2.3. Reagents

Chemicals used for the syntheses were analytically pure (Merck, Darmstadt, Germany; Aldrich, Steinheim, Germany; Fluka, Buchs, Switzerland). Ethanol and ether were of technical quality and distilled before use.

For the electrolytes p.a. acetonitrile, sodium tetraborate and sodium phosphate were purchased from Merck. Water de-ionized with a combination of Milli-RO plus 10 and Milli-Q systems (Millipore, Eschborn, Germany) was used. Amines in p.a. quality, *o*-phthaldialdehyde and 2-mercaptoethanol were purchased from Merck.

3. Results and discussion

Three resorcarene octa-acids **3a–c** with different alkyl chain lengths (C_1, C_5, C_{11}) were studied. A homologous series of ten amines (C_1–C_{10}) and additionally three partly isomeric amines were used as test analytes. As resorcarenes show high UV absorptivity due to the aromatic rings, fluorescence detection methods are preferable to UV detection. Fig. 2 shows the UV spectrum of resorcarene **3c** in the electrolyte used for the separations (compare Fig. 3). The resorcarene shows no considerable absorption above 300 nm. Therefore, the amines had to be derivatized to obtain fluorescent products which could be excited at higher wavelengths, in order to avoid any effect of the resorcarene upon the fluorescence intensity of the derivatives. The well-known OPA–2-mercaptoethanol reaction [27–29] was used for this purpose. The fluorescence detection of the formed isoindole compounds by laser-induced fluorescence using the 325 nm line of a HeCd laser allows the highly sensitive determination of the analytes.

The selectivity for these analytes increased with the increased length of the alkyl residues R of the resorcarenes. This result complies with former studies [17], in which the alkyl chains of the resorcarenes were found to be the interaction sites

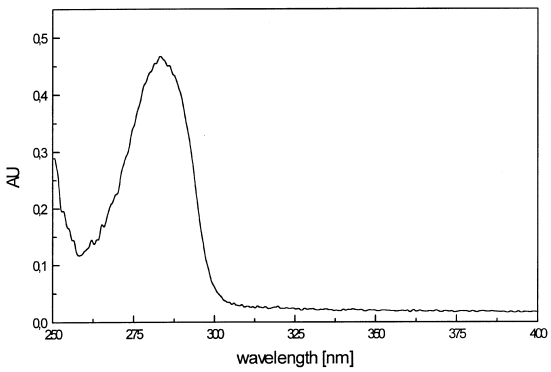

Fig. 2. UV spectrum of resorcarene **3c**.

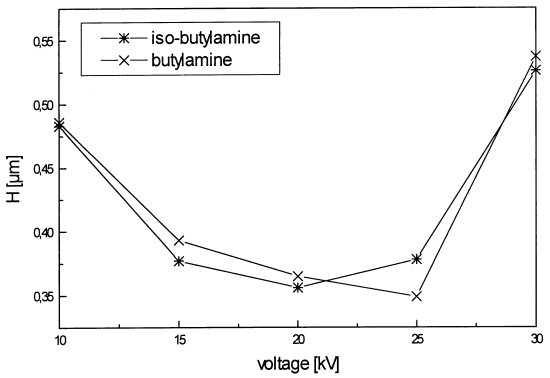

Fig. 3. Dependence of the plate height H of two amines on the applied voltage; Electrolyte: 0.75 mM resorcarene **3c**, 5 mM sodium phosphate, 10 mM sodium tetraborate, 10% acetonitrile (v/v), (pH 9.5); Capillary: fused silica, length 75 cm, 50 cm to detector, 75 μm I.D. Conditions: varying voltages. Detection: LIF, HeCd laser, ex. 325 nm, em. >389 nm (cut off filter).

with the analytes rather than the macrocycle, which does not contribute to retention. With resorcarene **3a** no separation of amines was achieved, whereas resorcarene **3b** allowed the separation of some homologs. The highest selectivity was observed with resorcarene **3c**.

Therefore, only the results with this undecyl-resorcarene octacarboxylic acid **3c** as pseudostationary phase are described.

*3.1. Electrophoretic characterization of resorcarene **3c***

According to Terabe et al. [30] resolution in EKC depends on the ratio of electrolyte migration t_0 to migration of the pseudostationary phase t_{pp} (Eq. (1)).

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2'}{1+k_2'}\right)\left(\frac{1-(t_0/t_{pp})}{1+(t_0/t_{pp})k_1'}\right) \quad (1)$$

For efficient separations of uncharged analytes, a high electrophoretic mobility of the employed pseudostationary phase is required, to provide a large migration time window. Consequently, the complete characterization of a new pseudostationary phase should include the determination of the mobility of the pseudostationary phase and the electroosmotic flow under the used conditions.

As no suitable markers for pseudostationary phases in hydroorganic media exist, the mobilities of the resorcarenes t_{pp} were determined using the reiterative procedure first described by Bushey and Jorgenson for MEKC [31]. This procedure is based on the retention data of homologous series and was thus directly transferable to the homologous amines employed in this work. The baseline disturbance which occurred at the migration time of the EOF was used as t_0-marker. The calculated values for t_0 and t_{pp} for resorcarene **3c** were found to be 7.2 min and 18.4 min respectively, corresponding to a migration time window of 11.2 min.

3.2. Efficiency

Another important resolution parameter is efficiency, which is usually described in terms of plate numbers N. Table 1 shows the plate numbers of five homologous amines separated with resorcarene **3c**. Efficiencies up to 3 million plates/m were reached.

To discuss these extremely high plate numbers, extra column and in column band broadening have to be distinguished. According to Terabe et al. [32], the total variance σ^2_{tot} is the sum of variances due to the column σ^2_{col}, injection σ^2_{inj} and detection σ^2_{det}, with σ^2_{inj} and σ^2_{det} corresponding to extra column effects.

The used LIF detection provided an improved focusing of the laser beam (about 5 μm spatial beam width) resulting in a detection volume in the pl-range. Hence, σ^2_{det} does not contribute to band broadening any more.

The width of the initially injected sample zone was about 1% of the effective capillary length. The sample zone width was further reduced using a focusing step at the beginning of the separation. This focusing is based on a phase ratio effect. Initially the analytes migrate with the velocity of the EOF, as no

Table 1
Plate numbers of five homologous amines separated with resorcarene **3c**

Compound	Plate number/m
Methylamine	$6.4\cdot10^5$
Ethylamine	$10.0\cdot10^5$
Propylamine	$13.6\cdot10^5$
Butylamine	$16.6\cdot10^5$
Pentylamine	$27.8\cdot10^5$

resorcarene is present in the sample zone ($k' = 0$). When the resorcarene migrates into the front of the sample zone, analytes are retarded according to their retention factors, whereas rear analytes move on with the EOF. The resulting focusing effect is stronger for analytes with high retention factors. Therefore, efficiency increases with the alkyl chain length of the amine (see Table 1). This assumption was confirmed when the analyte solution was prepared in a resorcarene-containing electrolyte. In this case, no focusing occurred and efficiency generally decreased and the effect increased with increasing carbon number of the amines.

Consequently, extra column band broadening was largely reduced due to the low detection volume of LIF and an initial sample focusing effect.

Terabe et al. [32] also investigated the mechanisms of in column band broadening in MEKC. Microheterogeneity was found to be an important factor. Zone spreading results from different electrophoretic mobilities of the molecules or molecular aggregates of which the pseudostationary phase consists. They may be due to inhomogeneous structures or charge densities. As resorcarenes possess a well-defined structure, conformation and charge, microheterogeneity should be excluded. In order to examine this, a solution of resorcarene **3c** was injected into a buffer not containing any resorcarene and CZE with UV detection was performed. As expected the resulting electropherogram showed only one distinct and sharp resorcarene signal.

In Fig. 3 the dependence of the plate height H on the applied voltage is shown for butylamine and isobutylamine. Optimum results were obtained for voltages between 20 kV and 25 kV corresponding to plate heights of about 0.36 μm. At lower voltages diffusion seems to be the dominating band broadening factor. Higher voltages lead to peak broadening due to increased joule heating.

3.3. Selectivity of resorcarenes

Fig. 4 shows the separation of 13 amines with resorcarene **3c**. Complete separation of the homologous amines (methylamine to decylamine), the isomers isopropylamine and isobutylamine, and the more polar 1-amino-2-propanol was achieved within 15 min. The order of peak elution is identical with

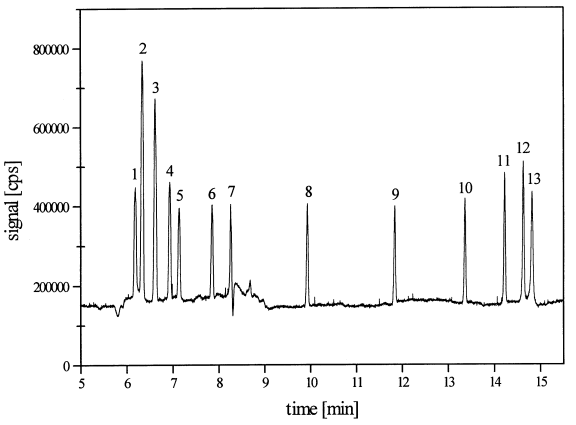

Fig. 4. EKC-separation of 13 amines using resorcarene **3c** as pseudostationary phase; experimental conditions as in Fig. 3, except voltage 20 kV, current 46 μA. Peak identities: 1 = 1-amino-2-propanol, 2 = methylamine, 3 = ethylamine, 4 = isopropylamine, 5 = propylamine, 6 = isobutylamine, 7 = butylamine, 8 = pentylamine, 9 = hexylamine, 10 = heptylamine, 11 = octylamine, 12 = nonylamine, 13 = decylamine; sample: each amine 10 μ*M*, derivatized with OPA–2-mercaptoethanol.

that of reversed-phase high-performance liquid chromatography (RP-HPLC). Moreover, as in chromatography, the plot of log k' against the carbon number of the homologous compounds yields a straight line. This is shown in Fig. 5 for the ten homologous amines. The RP analogous retention behaviour indicates similar interactions of the resorcarene alkyl residues and the RP-alkyl chains with the hydrophobic analytes. This corresponds to previous results with resorcarenes and polycyclic aromatic

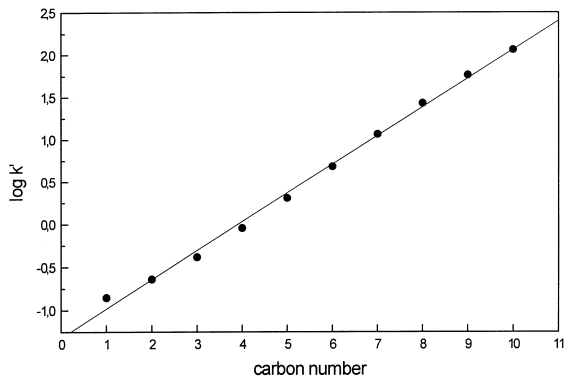

Fig. 5. Plot log k' for the homologous amines C_1–C_{10} against the carbon number of the compounds; regression coefficient is 0.9981; experimental conditions as in Fig. 3.

Table 2
Retention factors of the separated amines

Compound	k'	Compound	k'
1-Amino-2-propanol	0.09	Pentylamine	2.1
Methylamine	0.1	Hexylamine	4.8
Ethylamine	0.2	Heptylamine	11.6
Isopropylamine	0.3	Octylamine	27.1
Propylamine	0.4	Nonylamine	58.4
Isobutylamine	0.7	Decylamine	115.8
Butylamine	0.9		

hydrocarbons [17], where the same similarity with RP-HPLC was found.

In Table 2 the calculated retention factors of the compounds are listed, which cover a wide range. For more lipophilic compounds, retention factors become very large and these substances coelute at the migration time of the resorcarene t_{pp}.

According to Eq. (2) the retention factor for a given compound depends on the distribution equilibrium between pseudostationary phase and electrolyte and on the phase ratio ($V_{pseud.}/V_{mob.}$).

$$k' = K_{eq}\frac{V_{pseud.}}{V_{mob.}} \quad (2)$$

Increasing the organic modifier content leads to decreased retention factors and consequently the separation of highly hydrophobic compounds is achieved. However, the decreased electrolyte polarity can lead to bad resolution of the short chain amines which show only weak or even no interaction with the resorcarenes under these conditions. Another way to reduce retention factors efficiently is decreasing the concentration of the pseudostationary phase and thus the phase ratio in Eq. (2). In Fig. 6 the dependence of the capacity factors of some compounds on the concentration of resorcarene **3c** in the electrolyte is demonstrated. As for other pseudostationary phases, a linear dependence is observed. As shown in Fig. 4, for methyl- to decylamine a resorcarene concentration of 0.75 m*M* leads to complete resolution of all compounds.

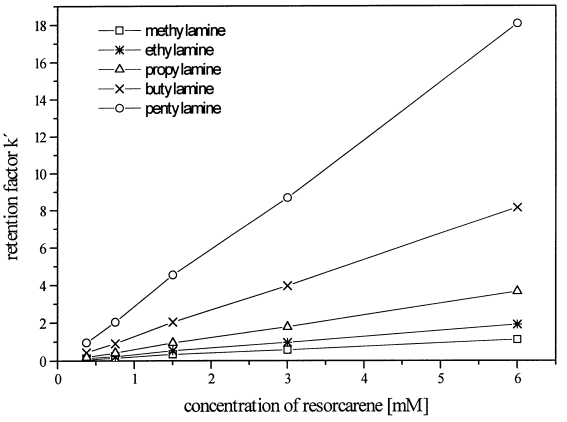

Fig. 6. Dependence of the retention factors of five amines on the concentration of resorcarene **3c**; experimental conditions as in Figure 3, except varying resorcarene concentration.

4. Conclusion

Resorcarene-octacarboxylic acids represent an interesting new pseudostationary phase for EKC. The RP analogous selectivity of the C_{11}-octacarboxylic acid **3c** together with the extremely high efficiency based on the largely reduced extracolumn band broadening and the absence of microheterogeneity allowed the successful separation of 13 amines.

Acknowledgements

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