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Separation of Pyrimidine Bases on a HPLC Stationary RP-18 Phase Coated with Calix[4]resorcinarene

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Abstract. Lipophilic calix[4]resorcinarenes, derived from lauryl aldehyde and resorcinol, are strongly adsorbed on the modified silica gel RP-18 for HPLC chromatography, while their solutions are passed through the column. The calix[4]resorcinarene-coated RP-18 phases were found to be useful beds for HPLC separation of uracil, thymine and cytosine.

Key words: HPLC chromatography, phenols, nucleic bases, RP-18 phase, calix[4]resorcinarene.

1. Introduction

Supramolecular chemistry provides novel possibilities in separating the mixtures of compounds difficult for resolution through standard methods, by means of macrocyclic compounds as modifiers of the chromatographic beds. The differences in complexing capabilities between macrocycles and compounds to be resolved cause profound changes in partition coefficients, compared to nonmodified beds. As a consequence, compounds unresolvable in the traditional way on a nonmodified bed are resolvable when the modifier is applied.

There are several classes of macrocyclic compounds used as modifiers for stationary chromatographic phases in HPLC and GLC, such as cyclodextrins [1–5], crown ethers [6–11], calixarenes derived from *tert*-butylcalix[8]arenes [12] and more recently calix[4]- and calix[6]arene esters [13]. In two recent papers we reported the use of calix[4]resorcinarenes as modifiers for the RP-18 phase [14, 15].

It was of interest to use the lipophilic calix[4]resorcinarene as a coating for RP-18 bed. The molecules of C-tetra-*n*-undecylcalix[4]resorcinarene (Figure 1) possess distinct hydrophilic properties at the upper rim and lipophilic properties at the lower rim.

It has been observed by Stirling *et al.* [16, 17] that lipophilic calix[4]resorcinarenes formed multilayers, where long *n*-alkyl chains form an

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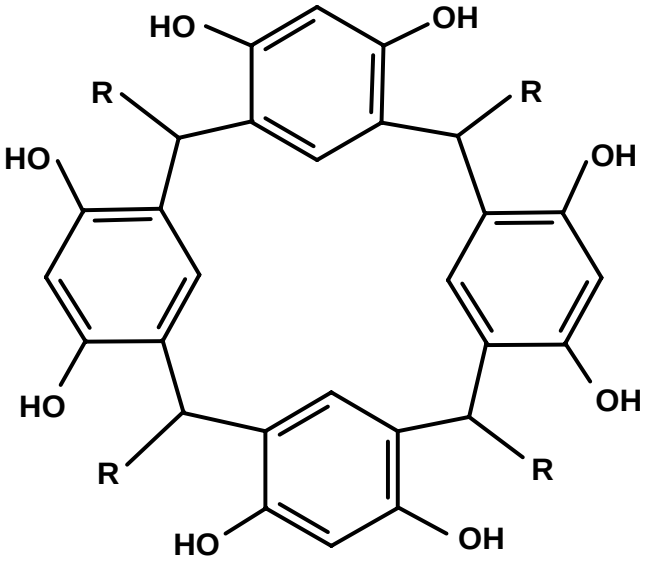

Figure 1. C-Tetra-*n*-undecylcalix[4]resorcinarene.

interpenetrating network. A similar mechanism was expected while coating the surface-modified RP-18 silica gel (Figure 2).

In the case of positive results it may be assumed that calix[4]resorcinarenes may appear to be an interesting class of modifiers for the RP-18 phase, because the lipophilic part will be strongly adsorbed on the bed and the chemical modification of the upper rim will allow for the fine adjustment of this molecule for particular recognition tasks.

In our earlier work we have used two calix[4]resorcinarenes for the RP-18 bed modification; the above-mentioned C-tetra-*n*-undecylcalix[4]resorcinarene and its octamethyl ether. It was shown that calix[4]resorcinarenes form very stable coatings for RP-18 phases. Five months of an intensive work showed the perfect stability of the columns [14].

We have investigated the separation of substituted phenols, as model compounds, on columns with a modified RP-18 phase. Comparing the retention factors for the phenols studied in the eluent consisting of 40% methanol in water we noted that for some pairs of compounds we have obtained much better separations on the column modified with calix[4]resorcinarene, than on the nonmodified column, or on the column coated with calix[4]resorcinarene methyl ether (Table I, Figure 3).

The more distinct differences in the retention factors for the RP-18 phase modified with calix[4]resorcinarene can be rationalized in this way, that the free hydroxyl groups cause the change of the polarity of the RP-18 bed, more than in

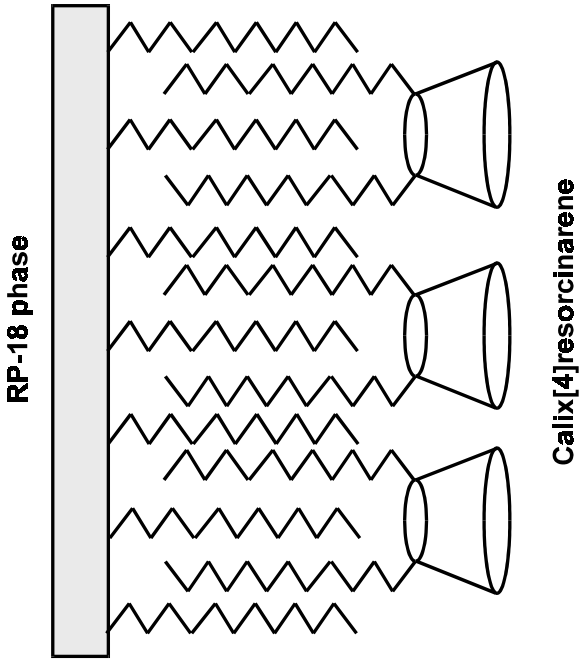

Figure 2. Schematic representation of coated RP-18 bed.

Table I. Retention characteristics of the selected pairs of phenols

Substituted phenols	RP-18		RP-18 + **1**[a]		RP-18 + **2**[b]	
	k	*α*	*k*	*α*	*k*	*α*
m-nitrophenol	11.8		8.0		11.2	
m-cresol	12.0	1.09	9.0	1.13	12.4	1.10
o-cresol	13.8		9.0		13.0	
o-nitrophenol	14.8	1.07	17.0	1.89	14.0	1.08
o-chlorophenol	14.7		9.7		14.2	1.01
o-nitrophenol	14.8	1.01	17.0	1.75	14.0	
p-chlorophenol	21.0		12.5		19.0	
3,4-dimethylphenol	21.5	1.02	15.6	1.25	20.0	1.05
m-chlorophenol	22.7		13.5		20.6	
2,6-dimethylphenol	23.5	1.04	17.1	1.27	22.3	1.08

[a] **1**, C-tetra-*n*-undecycalix[4]resorcinarene.
[b] **2**, C-tetra-*n*-undecylcalix[4]resorcinarene octamethyl ether.

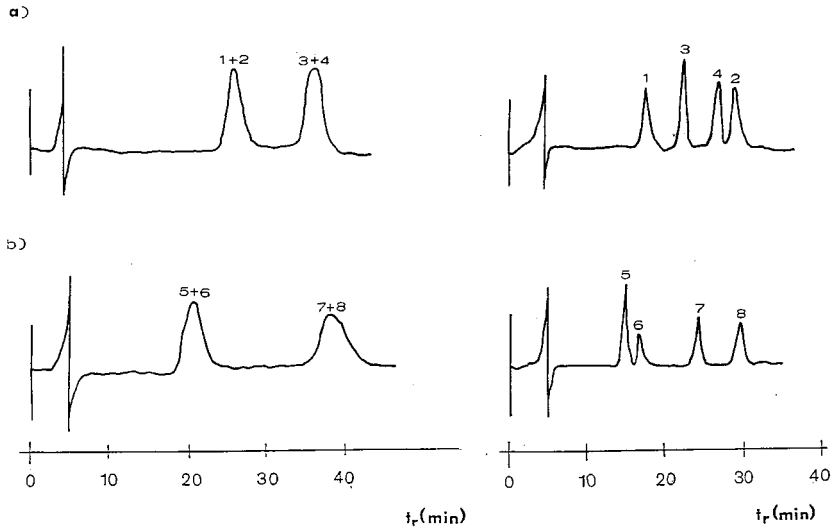

Figure 3. Chromatograms of selected phenols on nonmodified and modified RP-18 bed: (a) *o*-chlorophenol (1), *o*-nitrophenol (2), *p*-chlorophenol (3) and 3,4-dimethylphenol (4); (b) *m* -nitrophenol (5), *m*-cresol (6), *m*-chlorophenol (7) and 2,6-dimethylphenol (8); (40% methanol in water, the eluent flow, 40 μL/min, temperature 23 °C).

case of the methyl ether, and also a pronounced complexation of *o*-nitrophenol was observed (retention factor is higher than in the case of nonmodified column, and coated with methyl ether). This interaction of the coated bed with *o*-nitrophenol, indicating the close proximity of the functional groups that may form the hydrogen bonding with the receptor molecule, led to a favorable interaction with the hydroxyl groups of the calix[4]resorcinarene ring. Thus, it was conceivable to use compounds that might display suitable molecular interactions with calix[4]resorcinarenes.

Among potential candidates we found cytosine, uracil, and thymine, since Aoyama and coworkers demonstrated a remarkable versatility of the water-soluble and also hydrophobic calix[4]resorcinarenes in recognition of biologically relevant molecules (for instance riboflavin and cytosine) [18, 19].

The separations of these three pyrimidine bases have been reported in the following conditions: on Aminex-type anion exchangers with 0.13 M $(NH_4)_2SO_4$ as an eluent at 65 °C [20], or on reversed phase, TSKgel ODS-120T with gradient elution of methanol – 10 mM tetra-*n*-propylammonium phosphate (ion pairing agent) – 50 mM sodium phosphate – water [21]. For these relatively complicated separation conditions we have found the calix[4]resorcinarene-coated RP-18 phase as a good alternative to make the separation process easier.

2. Experimental

Methanol was purchased from Merck. Distilled water was purified by a Millipore system. Physical coating was achieved by passing the acetonitrile solution of calix[4]resorcinarene through the column filled with the RP-18 phase until saturation was reached. The kinetics of deposition of the calix[4]resorcinarenes was followed by monitoring the eluent leaving the column. 2 mL of the eluent were collected and the absorbance was measured at 280 nm on a Shimadzu UV-3100 spectrometer. The condition of modification of the stationary RP-18 phase was described in our former paper [14]. We have used the microbore chromatograph type 310, custom-made at the Institute of Physical Chemistry, for the HPLC work, equipped with a UV-254 detector. The column dimension was 1×250 mm and filled with Nucleosil 120-5C_{18} Marcherey–Nagel. The eluent flow was 30 μL/min. All experiments were run at 23 °C. For each compound injection was repeated 4 times. As a reference we have used also a nonmodified column. The eluents were: 20% methanol in water (v/v, eluent A), water adjusted to pH 2 and pH 4 with phosphoric acid (eluent B and C, respectively), water (eluent D), 18% methanol in water (eluent E).

3. Results

The calix[4]resorcinarenes proved to form coatings quite easily on the RP-18 solid supports. The kinetics of deposition of the calix[4]resorcinarenes on the RP-18 phase was verified by absorbance measurements of the eluent passed by.

Figure 4 shows the spectrum of undecylcalix[4]resorcinarene in acetonitrile (concentration 5×10^{-5} M).

A typical kinetic curve for the generation of the modified RP-18 phase is illustrated in Figure 5.

In this work we have studied the influence of the modification of the RP-18 phase on the partition and selectivity coefficients of pyrimidine bases in various eluents.

Table II collects the data for the chromatographic separations of three bases on nonmodified (column 1) and coated columns (column 2).

Figure 6 presents chromatograms of cytosine, uracil and thymine for both columns using different solvents.

The experimental data showed that no separation of the pyrimidine bases was achieved on both columns with the eluent A. In acidic condition (eluent B, pH 2), when cytosine exists in its protonated form, good separation was noted on both columns. In the case of eluents C and D much better results were achieved on the modified RP-18 phase. It is clear that water as the eluent had the strongest impact on the retention time for the cytosine, and this is not surprising, since cytosine is remarkably more basic than the other two remaining pyrimidine bases, and it has been observed already that calixarenes had a tendency to form defined molecular complexes with aliphatic amines, of acid-base type [22]. So the eluent consisting of

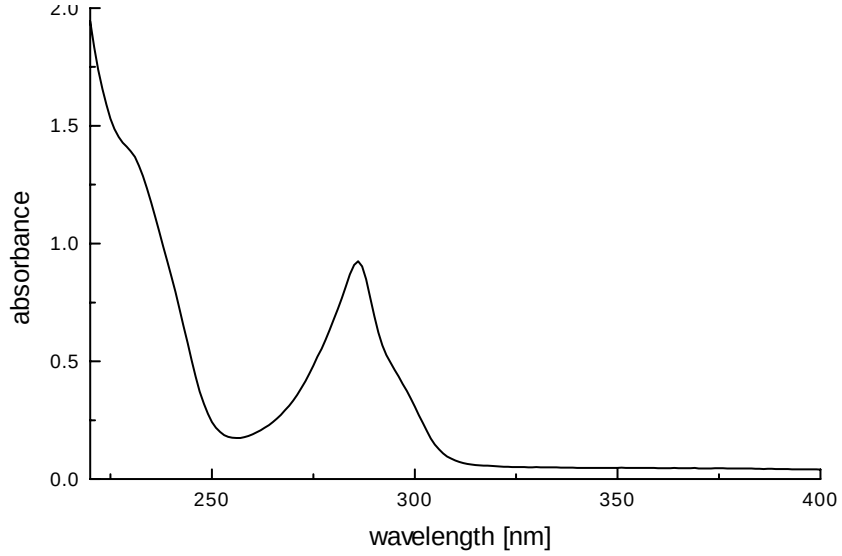

Figure 4. UV-vis spectra of calix[4]resorcinarene in MeCN, concentration 5×10^{-5} M.

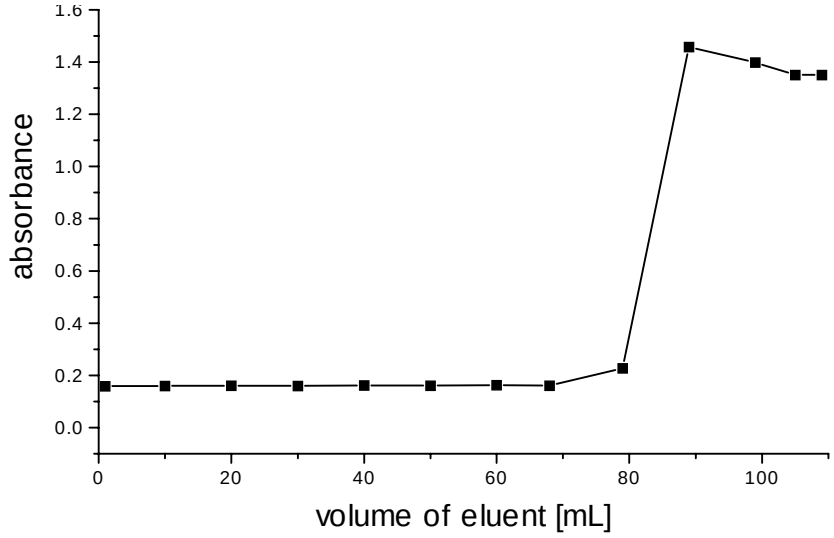

Figure 5. Kinetics of the generation of calix[4]resorcinarene modified stationary phase.

proper amounts of water and methanol can effect the separation of the three bases on the modified column in a shorter time than in pure water.

We noted that the use of the eluent composed of 18% of methanol in water (eluent E) was the best among the investigated eluents, because of the shorter time of analysis and satisfactory separations. The separation data are collected in Table III, whereas the chromatogram is shown in Figure 7.

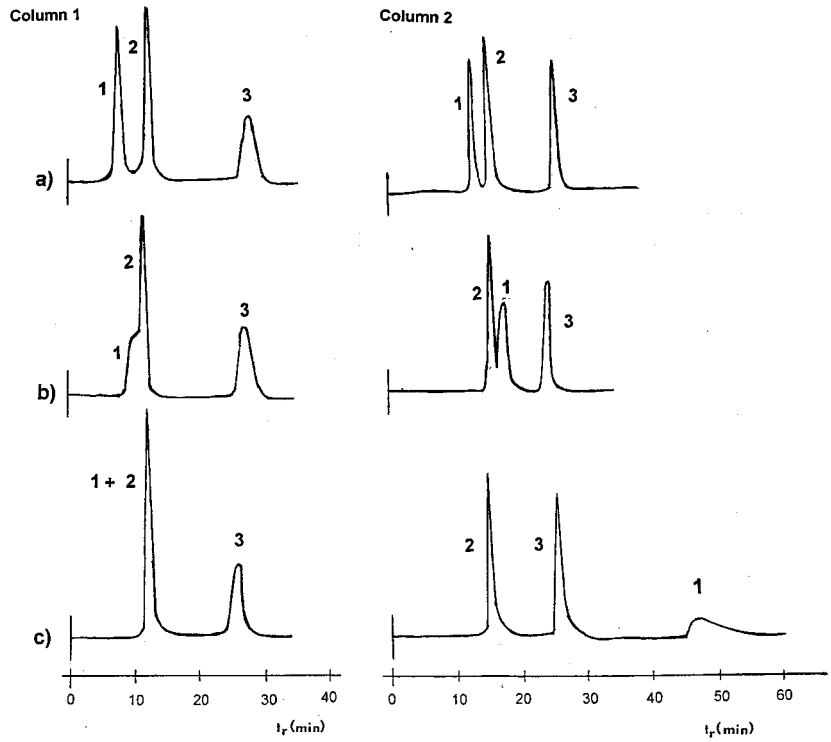

Figure 6. Separation of three pyrimidine bases on nonmodified and coated columns: (a) eluent B, (b) eluent C, (c) eluent D. Cytosine: 1, Uracil: 2, Thymine: 3.

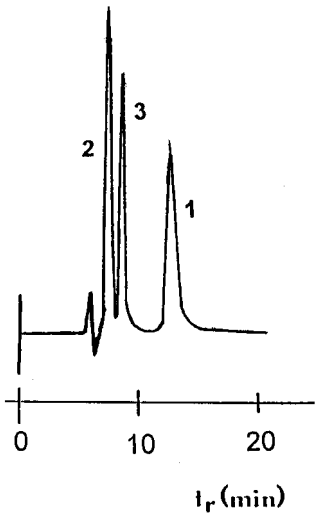

Figure 7. Separation on the coated column with the eluent E.

Table II. Separation and selectivity factors for pyrimidine bases

Column	Analyte	eluent A		Eluent B		Eluent C		Eluent D	
		k	α	k	α	k	α	k	α
1	Cytosine	3.25		3.50		5.25		6.00	
	Uracil	3.25	1.00	6.25	1.79	6.0	1.14	6.00	1.00
	Thymine	4.42	1.36	16.25	2.60	16.00	2.67	14.75	2.46
2	Cytosine	4.75	1.06	7.00		10.25	1.14	27.75	1.82
	Uracil	3.50		8.50	1.21	9.00		8.50	
	Thymine	4.50	1.29	15.00	1.75	14.75	1.44	15.25	1.79

Table III. Retention characteristics for pyrimidine bases in eluent E

column	Analyte	k	α
2	Cytosine	7.00	1.56
	Uracil	3.50	
	Thymine	4.50	1.28

4. Discussion

The application of eluent A (20% methanol in water) on both columns did not lead to the separation of the bases studied. The retention factors for the cytosine and uracil were the same on column 1, and very similar values for cytosine and thymine were observed for column 2. Although the separations were not achieved, the k values were not the same, indicating the interactions of the calix[4]resorcinarene coating with the analytes. At pH 2 we have observed satisfactory separations on both columns 1 and 2 (Figure 6a), whereas at pH 4 (eluent C, Figure 6b) the separation of cytosine and uracil was not satisfactory on column 1, but much better on the modified column 2, and the following order of elution was observed: uracil, cytosine, thymine. When pure water was applied as an eluent, cytosine and uracil were not separated on column 1, whereas remarkable separation was achieved for the three analytes on column 2 (Figure 6c). This shows that modification of the stationary phase with calix[4]resorcinarene led to observable molecular interactions in water solution with calix[4]resorcinarene that differentiated the analytes significantly during the chromatographic process. In this case the analytes were separated in the following order: uracil, thymine and cytosine.

It is interesting to note that at neutral conditions the longest retention time on the coated column was observed for cytosine. Cytosine is more basic than the two other analytes, thus its interaction with the calix[4]resorcinarene may be two fold:

the imide group ($NH_2C—NH—CO$) may interact with the hydroxyl groups of the calix[4]resorcinarene, as postulated by Aoyama and coworkers [23], but also it seems to be conceivable that protonation of the cytosine by calix[4]resorcinarene might contribute to the overall binding strength. The formation of salts between calixarenes and aliphatic amines was observed by Danil de Namor and coworkers [22]. Thus the interaction between the calix[4]resorcinarene and nucleobases may be of the surface recognition type involving the hydroxyl groups of the calix[4]resorcinarene and the imide fragments of the analytes, and the formation of hydrogen bonds between them. This type of surface recognition was observed recently by Atwood in his studies of the complexes between calix[4]resorcinarenes and heterocyclic bases in the solid state [24].

It is interesting to compare the retention factors for the bases studied on both columns. It is clear that the retention factors on the modified column are higher, which can be interpreted in terms of pronounced interaction of the analytes with the calix[4]resorcinarene immobilised on the surface of the RP-18 phase.

5. Conclusion

Lipophilic calix[4]resorcinarenes allowed for an easy and stable coating of the solid support RP-18, which resulted in new stationary phases for HPLC. The kinetics of calix[4]resorcinarene deposition suggest that other, variously substituted calix[4]resorcinarenes can be used for a variety of coatings. Thus, a range of coated silica gel RP-18 with desired properties can be prepared. A very good separation was achieved for uracil, thymine, and cytosine in a relatively short time (ca. 12 min.), using 18% methanol in water as an eluent. Optimisation of the chromatographic process (faster eluent flow and increased contents of methanol in water 18.5–19%) will allow for further shortening of the time of the analysis.

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