CHROMSYMP. 211

α- AND β-CYCLODEXTRIN COMPLEXATION AS A TOOL FOR THE SEPARATION OF o-, m- AND p-NITRO-cis- AND trans-CINNAMIC ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The capacity factors of o-, m- and p-nitro-cis- and trans-cinnamic acids on reversed-phase columns at various pH values of the mobile phase and concentrations of α- and/or β-cyclodextrin were determined. Attempts to calculate the stability constants, K° and K^-, as well as the capacity factors, k', of all individual species (neutral and anionic, free and bound to β-cyclodextrin) were made on the basis of experimental data. It was found that β-cyclodextrin enhances the selectivity of the system towards m- and p-nitrocinnamic acids, of whether cis- or trans-configuration[2]. Under optimum conditions, attempts were made to separate all the compounds investigated.

INTRODUCTION

Recently we have successfully applied α- and β-cyclodextrins (CDs) as mobile phases in reversed-phase high-performance liquid chromatography (RP-HPLC) for resolution of mandelic acid[1] and some its derivatives[2,3] into enantiomers, and for separation of o-, m- and p-nitrobenzoic acids[4].

These results encouraged us to undertake a systematic study on the use of CDs for separation of various types of isomers in RP-HPLC. The aim of this research project is to identify the true complexation and adsorption mechanisms including the selectivity rules governing the host–guest interactions.

The present paper reports on the separation of geometric cis-trans isomers as well as of substitution (ortho, meta, para) isomers of nitrocinnamic acid (NCAH).

EXPERIMENTAL

Reagents

α-Cyclodextrin was supplied by Janssen Chimica (Beerse, Belgium) and β-cyclodextrin by Chinoin (Budapest, Hungary). All other materials were of analytical or reagent grade and were used without further purification.

cis-o-NCAH   trans-o-NCAH

cis-m-NCAH   **trans-m-NCAH**

cis-p-NCAH   trans-p-NCAH
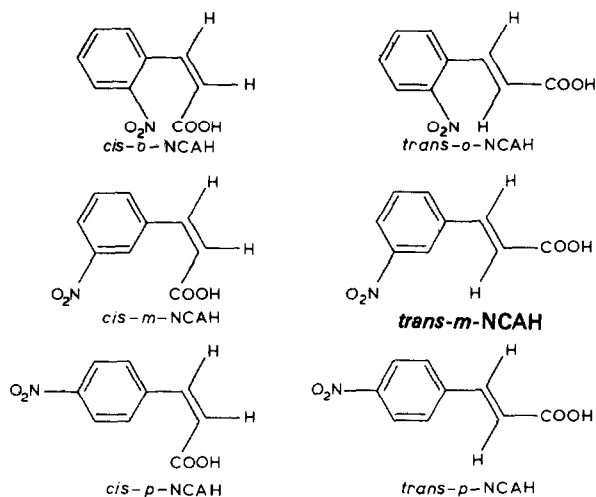
Apparatus and procedure

Chromatographic measurements were performed using a Type 302 apparatus (Institute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland) equipped with a 5-μl high-pressure injection valve and a spectrophotometric detector (254 nm) with Z-shaped passage (volume 8 μl).

pH measurements were performed using a Type N517 pH-meter (Mera-Tronic, Poland).

For HPLC, use was made of stainless-steel columns (100 × 4.6 mm I.D. and 30 × 3 mm I.D.) packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, F.R.G.).

The mobile phases were aqueous phosphate buffer solutions of pH $\approx$ 2.2, 4.2, 5.2 and 6.4, containing Na_2SO_4 (0.013, 0.030, 0.046, 0.063 and 0.095 M) and α-CD at concentrations of $0.4 \cdot 10^{-4}$, $8 \cdot 10^{-4}$, $12 \cdot 10^{-4}$, $14 \cdot 10^{-4}$ and $18 \cdot 10^{-4}$ M or β-CD at concentrations of $0.4 \cdot 10^{-4}$, $8 \cdot 10^{-4}$, $16 \cdot 10^{-4}$, $24 \cdot 10^{-4}$, $4 \cdot 10^{-3}$, $6 \cdot 10^{-3}$, $9 \cdot 10^{-3}$ and $12 \cdot 10^{-3}$ M. Phosphate buffer solutions of concentration *ca.* $7 \cdot 10^{-3}$ M were prepared by titration of aqueous H_3PO_4 solutions with Na_2HPO_4 solution to a given pH (checked with the pH-meter). Insignificant changes of pH produced by addition of Na_2SO_4 were also controlled with a pH-meter.

The samples of *o-*, *m-* and *p-trans*-NCAH, dissolved in methanol at concentrations of $(2-8) \cdot 10^{-3}$ M were injected on the columns, both separately and as mixtures. *cis*-Isomers were generated photochemically by exposure to the light of methanolic solutions of the corresponding *trans*-isomers. Therefore, when injected on the columns, they were always accompanied by the *trans*-isomers.

All chromatographic experiments were performed at 25 ± 1°C. Flow-rates were 10, 15, 25, 35, 60 and 75 μl/sec.

Equilibria and equations

In the RP systems containing CD in the mobile phase, there are many neutral and ionic species, free and bound to one or more CD molecules. Consequently the equilibria of adsorption and complexation are very complex. Supposing that only

complexes of 1:1 stoichiometry are formed, we obtain the following scheme
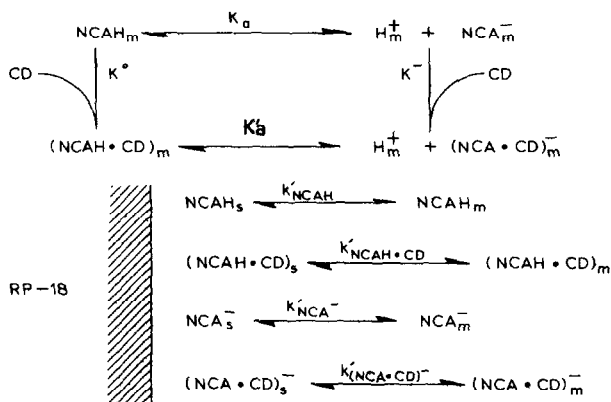
where the subscripts s and m denote the stationary and mobile phases, respectively, K_a is the acidity constant, K° and K^- are stability constants of CD complexes of neutral and anionic species, respectively, and k'_{NCAH}, $k'_{NCAH \cdot CD}$, k'_{NCA}^- and $k'_{(NCA \cdot CD)}^-$ are the capacity factors of the individual species.

For more simple systems containing only one form of the sorbate, Uekama *et al.*[5] derived an equation relating observed retention times, t_{obs}, of ionic species on an ion exchanger with the concentration of CD in the mobile phase

$$t_{obs} = \frac{t'_0 + t_c K_c [CD]_m}{1 + K_c [CD]_m} \tag{1}$$

where t'_0 is the retention time of the sorbate, t_c that of the sorbate–CD complex and K_c is the stability constant of the 1:1 complex.

Taking into account the acid–base equilibria and adsorption on a reversed phase of both neutral and ionic species, we have recently derived a more general equation[4] which relates observed retention times or observed capacity ratios, k'_{obs}, with pH and [CD]. Expanding the non-linear function $k'_{obs} = k([H^+], [CD])$ into a Taylor series and applying numerical procedures, we have calculated the parameters K°, K^- and the individual capacity factors for *o*-, *m*- and *p*-nitrobenzoic acids:

$$k'_{obs} = \frac{k'_{NCAH} + k'_{NCA}^- K_a/[H^+] + k'_{NCAH \cdot CD} K^\circ [CD] + k'_{(NCA \cdot CD)}^- K^- [CD] K_a/[H^+]}{1 + K_a/[H^+] + K^\circ [CD] + K^- [CD] K_a/[H^+]} \tag{2}$$

Both eqns. 1 and 2 were applied to the experimental data obtained in this work.

RESULTS AND DISCUSSION

Influence of pH and salt concentration

The capacity factors, k', of all the investigated acids NCAH, observed at three

different pH values of the mobile phase, and the values of pK_a are collected in Table I. The approximate values of K_a were evaluated by simplifying (for [CD] = 0) the eqn. 2 to:

$$k'_{obs} = \frac{k'_{NCAH} + k'_{NCA}- K_a/[H^+]}{1 + K_a/[H^+]} \tag{3}$$

These K_a values possess some uncertainty resulting from the fact that adsorption of anionic species ($k'_{NCA}-$) is found to be not stable as was assumed in eqn. 2, but is dependent to some extent on the salt concentration (see Fig. 1). Nevertheless, there are no great divergences between literature data[6] determined conductometrically only for the compounds of *trans*-configuration and our data evaluated chromatographically.

From these K_a it can be concluded that nitrocinnamic acids of *cis*-configuration are stronger acids than the corresponding *trans* compounds: $pK_{a_{cis-NCAH}} < pK_{a_{trans-NCAH}}$. The same sequence was found earlier for non-substituted cinnamic acids[6].

Examination of the k' values (Table I) leads to the conclusion that the main analytical difficulties lie in the separation of two pairs of compounds, *i.e.*, the *para*- and *meta*-isomers of NCAH of both *cis*- and *trans*-configurations. Their k' values are similar and they also have similar K_a values.

Fig. 1 shows the dependence of the capacity factors of anionic species, $k'_{NCA}-$, on salt (Na_2SO_4) concentration in the mobile phase; the measurements were performed at pH 6.4. Analysis of these curves yields the equation

$$k'_{NCA}- = a + b\sqrt{[Na_2SO_4]}$$

where a and b are constants. Similar effects were observed at pH 6.4 in the solutions containing CDs. On the other hand, the adsorption of non-dissociated molecules (k'_{NCAH}) is not influenced by salt concentration in the mobile phase at pH 2.2.

TABLE I

VALUES OF OBSERVED CAPACITY FACTORS, k'_{obs}, AND pK_a VALUES OF ISOMERIC NCAH

Chromatographic measurements are made on 10-μm LiChrosorb RP-18 at different pH values of the mobile phase containing Na_2SO_4 (0.095 M).

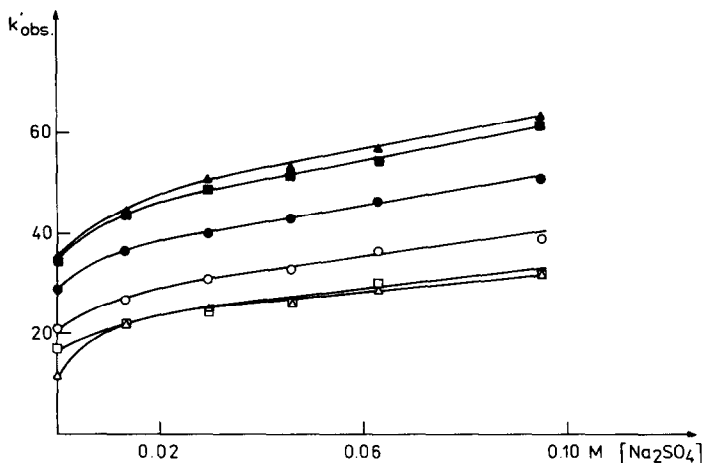| NCAH	k'_{obs} at pH			pK_a	Ref. 6
	2.28	5.18	6.39	Obs.	
cis–ortho	212	49	39	3.99	
trans–ortho	444	79	51	4.09	4.15
cis–meta	325	39	31	3.64	
trans–meta	644	109	64	4.13	4.12
cis–para	308	39	32	3.60	
trans–para	713	108	61	4.09	4.05

Fig. 1. Observed capacity factors, k'_{obs}, of isomeric NCAH on 10-μm LiChrosorb RP-18 stationary phase as a function of Na$_2$SO$_4$ concentration at pH = 6.4. $\bigcirc$, *cis-ortho*; $\bullet$, *trans-ortho*; $\triangle$, *cis-meta*; $\blacktriangle$, *trans-meta*; $\square$, *cis-para*; $\blacksquare$, *trans-para*.

Influence of α- and β-CD

Figs. 2 and 3 show the k'_{obs} values as functions of α- or β-CD concentration respectively at pH 2.2(a) and 6.4(b). These pH values were chosen since in the range of pH < pK_a − 2 acidic dissociation may be neglected while in the range pH > pK_a + 2 this dissociation is complete.

Under these conditions it was possible to apply not only the general equation 2 (which is charged with the same error as eqn. 3) but also the equation of Uekama *et al.*[5] The latter was transformed for neutral (eqn. 4) and anionic species (eqn. 5):

$$k'_{obs} = \frac{k'_{NCAH} - k'_{obs}}{[CD]_m} \cdot \frac{1}{K^\circ} + k'_{NCAH \cdot CD} \qquad (4)$$

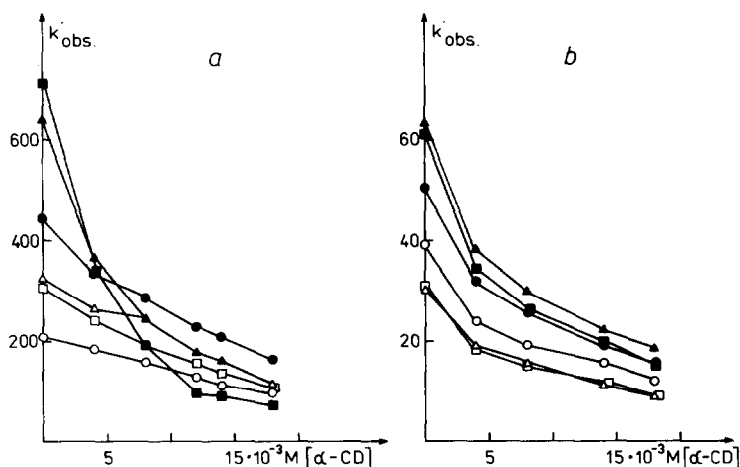Fig. 2. Observed capacity factors, k'_{obs}, of isomeric NCAH as a function of α-CD concentration ([Na$_2$SO$_4$] = 0.095 *M*) at pH = 2.2 (a) and pH = 6.4 (b). Symbols as in Fig. 1.
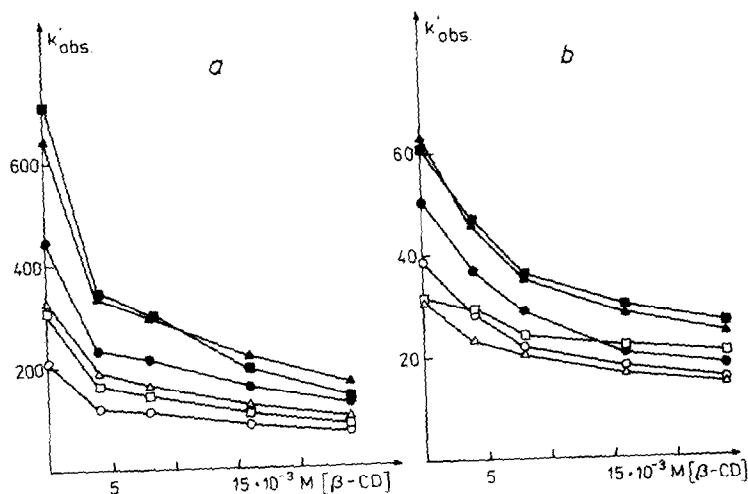
Fig. 3. Observed capacity factors, k'_{obs}, of isomeric NCAH as a function of β-CD concentration ([Na$_2$SO$_4$] = 0.095 *M*) at pH = 2.2 (a) and pH = 6.4 (b). Symbols as in Fig. 1.

$$k'_{obs} = \frac{k'_{NCA^-} - k'_{obs}}{[CD]_m} \cdot \frac{1}{K^-} + k'_{(NCA \cdot CD)^-} \tag{5}$$

It was found that all investigated β-CD solutions, both at pH 2.2 and 6.6, yielded straight line dependences of k'_{obs} *vs.* $(k'_{NCAH} - k'_{obs})/[CD]$ and of k'_{obs} *vs.* $(k'_{NCA^-} - k'_{obs})/[CD]$. Some examples are illustrated in Fig. 4.

In Table II are given the values of parameters $k°$ and K^- and of individual capacity factors, k', calculated from eqns. 4 and 5. The corresponding values obtained by use of eqn. 2 are given in parentheses.

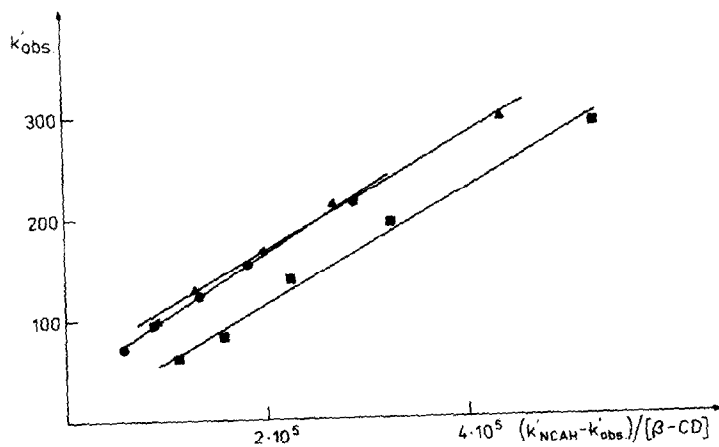In contrast, such straight line relations were not found with α-CD solutions.

Fig. 4. Relationship between observed capacity factors, k'_{obs} of *trans*-NCAH (●, *ortho*; ▲, *meta*; ■, *para*) and $(k'_{NCAH} - k'_{obs}) / [\beta$-CD] at pH = 2.2 ([Na$_2SO_4$] = 0.095 *M*).

TABLE II

STABILITY CONSTANTS, $K°$, K^-, AND CAPACITY FACTOR, k', FOR β-CD COMPLEXES OF ISOMERIC NCAH (AT 0.095 M Na$_2$SO$_4$), CALCULATED FROM EQNS. 4 AND 5 AND (IN PARENTHESES) FROM EQN. 2

NCAH	k'_{NCAH}	$k'_{NCA}{}^-$	$k'_{NCAH \cdot CD}$	$k'_{(NCA \cdot CD)}{}^-$	$K°$	K^-
cis-ortho	212.3	38.2	21.0	10.1	1453	1603
	(214.6)	(38.2)	(14.4)	(9.5)	(1226)	(1722)
trans-ortho	443.2	48.2	38.5	6.6	1621	1200
	(444.8)	(48.9)	(29.3)	(7.0)	(1431)	(1326)
cis-meta	325.2	30.1	36.4	10.0	1608	1364
	(323.5)	(30.0)	(24.3)	(9.3)	(1222)	(1114)
trans-meta	644.3	60.2	50.6	12.1	1723	1395
	(647.5)	(60.4)	(30.3)	(15.4)	(1383)	(1768)
cis-para	308.0	30.4	28.5	14.4	1779	795
	(312.5)	(30.4)	(26.7)	(13.4)	(1753)	(701)
trans-para	713.1	57.8	0	10.6	1749	984
	(719.2)	(58.6)	(−22.2)	(15.7)	(1487)	(1485)

This may suggest that some of our basic assumptions are not valid in this case; further studies on this matter are in progress.

As a result of the above results we attempted to establish optimal conditions for separations of all the investigated compounds. As already mentioned, the main difficulty lies not in the separation of cis- from trans-isomers, but in the separation of m- from p-substituted compounds whether in cis- or trans-configuration. To optimize the separation conditions, eqn. 2 was used to examine the relationship between the selectivity factor, $\alpha_{p/m}$, and pH as well as [CD] of the mobile phase. A typical
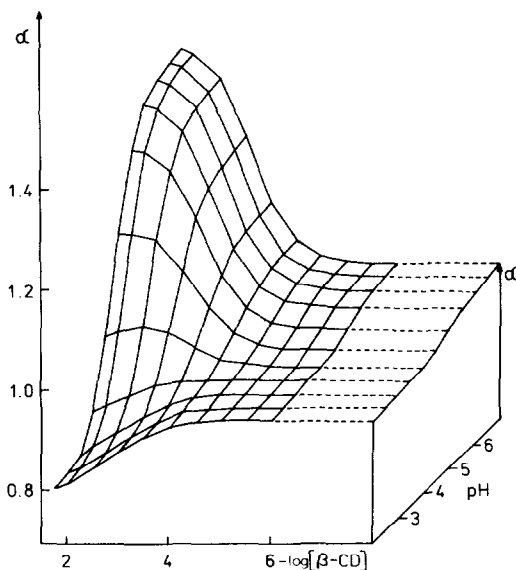

Fig. 5. Selectivity factor, $\alpha_{cis\text{-}p\text{-}NCAH/cis\text{-}m\text{-}NCAH}$, as a function of pH and log[β-CD], calculated from eqn. 2 and the data given in Tables I and II.
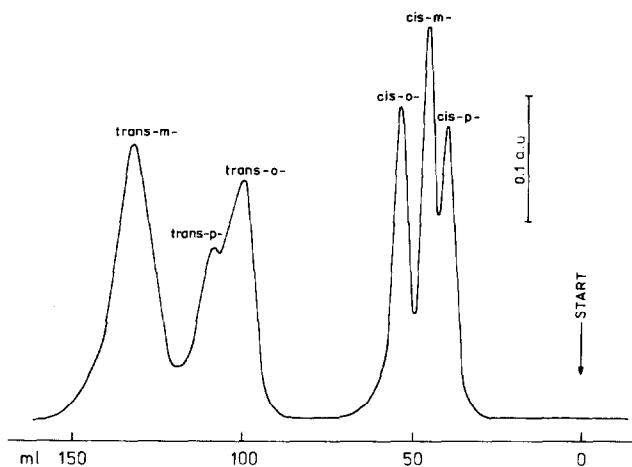
Fig. 6. Chromatogram of a *cis-trans* mixture of *o-*, *m-* and *p*-NCAH with an aqueous mobile phase of pH 2 and $[\beta\text{-CD}] = 4 \cdot 10^{-3} \, M([\text{Na}_2\text{SO}_4] = 0.095 \, M)$. Column: 100 × 4.6 mm I.D. LiChrosorb RP-18, 10 µm. Flow-rate: 25 µl/sec.

analysis of these relationships is given in Fig. 5.

Fig. 6 shows a chromatogram of a mixture of all six isomers investigated, performed under optimal conditions. Although the selectivities ($\alpha_{cis\text{-}m/cis\text{-}p} = 1.13$, $\alpha_{trans\text{-}m/trans\text{-}p} = 1.20$) are not small, the separation is not complete because of the low efficiency of the column; further studies to solve this problem are being undertaken.

ACKNOWLEDGEMENTS

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