β-CYCLODEXTRIN AS A SELECTIVE AGENT FOR SEPARATION OF SELECTED AROMATIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

LUDĚK BAŽANT and MILAN WURST*

Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4 (Czechoslovakia)
and
EVA SMOLKOVÁ-KEULEMANSOVÁ

Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
(Received March 14th, 1988)

SUMMARY

β-Cyclodextrin, as a selective component of the mobile phase or as a chemically bonded stationary phase, was used as an alternative to isocratic high-performance liquid chromatographic separation of structural isomers of some biologically important hydroxy-, methoxy- and amino-substituted aromatic carboxylic acids. The effect of the composition and the pH of the mobile phase on the separation of aromatic carboxylic acids was investigated. The two systems were compared and their selectivity and separation mechanism are discussed.

INTRODUCTION

Hydroxy-, methoxy- and amino-substituted aromatic carboxylic acids play an important role in the metabolism of microorganisms and higher plants. They are also products of the metabolism of some chemotherapeutics. Reliable and sensitive analytical methods are necessary for the study of metabolic processes and for pharmacological studies. An high-performance liquid chromatographic (HPLC) separation of many phenolic compounds was described by Vande Casteele et al.[1]. Gradient elution with three- to four-component mobile phases in reversed-phase systems has been primarily used for separation of aromatic carboxylic acids in plant extracts[2-5]. For medical purposes, analyses under isocratic conditions are applicable only to mixtures containing a small number of acids[6].

Studies in which the inclusion properties of cyclodextrin (CD) have successfully been used for the separation of compounds that could not easily be separated by means of other chromatographic systems are becoming more frequent.

The formation of inclusion complexes is based on the ability of CD to incorporate hydrophobic molecules or their parts in its cavity and to the occurrence of hydrogen bonds between hydroxyl groups on the surface of the CD molecule and hydrophilic moieties of the included compound[7]. The ability to form inclusion

complexes depends on the size and polarity of the host molecule and its shape, which is manifested by an increased selectivity towards structural, geometric and occasionally even optical isomers.

The selective inclusion properties of CD can be applied in two ways in HPLC. First, CD can be used as a selective component of the mobile phase in a reversed-phase system. This procedure has been used to separate structural isomers of disubstituted benzene derivatives and some aromatic acids[8–11]. Secondly, CD bonded to silica gel in various ways can be employed. The properties of these stationary phases are influenced to a considerable extent by the type of bond between the CD and silica gel. The presence of nitrogen usually has an adverse effect on the inclusion separation mechanism[12–17], except for CD carbamate-bonded stationary phases[17,18]. The commercial Cyclobond phases in which α-, β- or γ-CD is bonded to silica gel by a nitrogen-free spacer have so far been applied most frequently[19–22].

Methanol is most often used as the organic modifier in the mobile phase, as the CD inclusion complexes have the greatest stability in methanolic solution, compared with ethanol, acetonitrile and other dipolar aprotic solvents[7,23].

The present work extends experiments[24] in which the above two methods were compared and conditions were optimized for the separation of structural isomers of biologically significant aromatic acids.

EXPERIMENTAL

Apparatus and procedure

The chromatographic system I consisted of a Model 8500 high-pressure pump (Varian, Walnut Creek, CA, U.S.A.), a Varian stop-flow injector and a stainless-steel column (250 mm × 2 mm I.D.) packed with the reversed-phase Separon Si C_{18}, 6 μm (Laboratorní Přístroje, Prague, Czechoslovakia). The column was water-jacketed. A Varian Model 4100 water thermostat, was used to maintain a constant temperature during analyses, and a Variscan UV–VIS detector was used.

The chromatographic system II was identical with system I, except for the injector and the chromatographic column. A Model 7010 valve was used with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.) and a Cyclobond I column (250 mm × 4.6 mm I.D.) packed with 5-μm silica gel with chemically bonded β-CD (Advanced Separation Technologies, Whippany, NJ, U.S.A.).

Mixtures of methanol with 0.01 M phosphate buffer pH 2.20, 4.20, 5.70 and 6.20 served as the mobile phases. The β-CD-containing phases were prepared by dissolving weighed amounts of β-CD in a 0.01 M phosphate buffer, with ultrasonic stirring and mixing with methanol. Before analysis, the mobile phases were degassed *in vacuo* and filtered. The flow-rates were 20 and 60 ml/h in the systems I and II, respectively.

The sample solutions of aromatic carboxylic acids were prepared by dissolving standard compounds in methanol to obtain concentrations of 1–0.1 mM. The sample volumes were 1 and 5 μl in the systems I and II, respectively. All the solutes were detected at 254 nm.

The dead volume of the column was determined in system I using potassium nitrate; methanol was used in system II as the retention standard. All the measurements were carried out at 25°C.

Chemicals

All aromatic carboxylic acids listed in Tables I and II were of the highest analytical purity. Acids 1–10 and 14–17 were obtained from Schuchardt (Munich, F.R.G.), 11–13 from the Research Institute of Organic Syntheses (Pardubice-Rybitví, Czechoslovakia), 18–20 from Fluka (Buchs, Switzerland) and 21–26 from Sigma (St. Louis, MO, U.S.A.). Orthophosphoric acid, the dodecahydrate of disodium mono-hydrogenphosphate, potassium dihydrogenphosphate and potassium nitrate, all of analytical grade, and methanol of HPLC grade were obtained from Lachema (Brno, Czechoslovakia). β-CD (Chinoin, Budapest, Hungary) was used without further purification.

RESULTS AND DISCUSSION

The effect of the mobile phase pH and of the methanol and β-CD contents on the retention behaviour of the aromatic carboxylic acids was studied for individual groups of structural isomers. All the data obtained are summarized in Tables III–IX.

Effect of the mobile phase pH

The stability of the β-CD inclusion complexes as a function of the pH was described for a series of organic compounds[23,25]. The most stable complexes were obtained at pH values at which the molecules of the included compounds were uncharged or had the smallest charge.

Compounds that are relatively strong acids (except for methyltryptophans) were studied in the present work. Phosphate buffers were thus used as components of the mobile phase in order to suppress the dissociation.

The relationship between the capacity factors, k', of benzoic acid and mono-hydroxybenzoic acids (MHBAs), dihydroxybenzoic acids (DHBAs), monomethoxy-benzoic acids (MMBAs), dimethoxybenzoic acids (DMBAs), monomethoxymandelic acids (MMMAs), indolecarboxylic acids and methyltryptophans and the pH is shown in Fig. 1 for system I. Differences in the course of this relationship reflect the varying degrees of dissociation of the individual isomers in the given medium and the varying ability of the ions produced to interact with the β-CD molecule or with the reversed stationary phase. With the methyltryptophan isomers, this relationship passes through a minimum at a pH close to the pI value of these amino acids (see Fig. 1G).

The k' values and the resolution decreased with increasing pH in both systems. Only the conditions given in Fig. 1B for the separation of 3,4-DHBA from the other isomers were optimal at pH 4.20.

Effect of the methanol content in the mobile phase

A typical course of the relationship between the k' values and the methanol content in the mobile phase is given in Fig. 2A for the DMBA isomers. Changes in the elution order of the isomers occur in some cases with increasing methanol content (see Fig. 2B). A similar phenomenon was observed with a mixture of the MHBA isomers with benzoic acid in system II at pH 4.20 (see Table IV).

This behaviour is probably due to changes in the solvation of the individual isomers under the given experimental conditions. Solvation may lead to changes in both the interaction of the studied compounds with the CD cavity and in the hydrogen-bonding interaction with hydroxyl groups.

TABLE I

BENZENE-RING CONTAINING CARBOXYLIC ACIDS
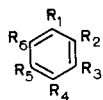
No.	Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Benzoic acid	COOH					
2	o-Hydroxybenzoic acid (salicylic acid)	COOH	OH				
3	m-Hydroxybenzoic acid	COOH		OH			
4	p-Hydroxybenzoic acid (catalpic acid)	COOH			OH		
5	2,3-Dihydroxybenzoic acid (o-pyrocatechuic acid)	COOH	OH	OH			
6	2,4-Dihydroxybenzoic acid (β-resorcylic acid)	COOH	OH		OH		
7	2,5-Dihydroxybenzoic acid (gentisic acid)	COOH	OH			OH	
8	2,6-Dihydroxybenzoic acid (γ-resorcylic acid)	COOH	OH				OH
9	3,4-Dihydroxybenzoic acid (protocatechuic acid)	COOH		OH	OH		
10	3,5-Dihydroxybenzoic acid (α-resorcylic acid)	COOH		OH		OH	
11	o-Methoxybenzoic acid (o-anisic acid)	COOH	OCH$_3$				
12	m-Methoxybenzoic acid (m-anisic acid)	COOH		OCH$_3$			
13	p-Methoxybenzoic acid (p-anisic acid)	COOH			OCH$_3$		
14	2,3-Dimethoxybenzoic acid	COOH	OCH$_3$	OCH$_3$			
15	2,6-Dimethoxybenzoic acid	COOH	OCH$_3$				OCH$_3$
16	3,4-Dimethoxybenzoic acid (veratric acid)	COOH		OCH$_3$	OCH$_3$		
17	3,5-Dimethoxybenzoic acid	COOH		OCH$_3$		OCH$_3$	
18	o-Methoxymandelic acid	CH–COOH | OH	OCH$_3$				
19	m-Methoxymandelic acid	CH–COOH | OH		OCH$_3$			
20	p-Methoxymandelic acid	CH–COOH | OH			OCH$_3$		

TABLE II

INDOLE-RING CONTAINING CARBOXYLIC ACIDS
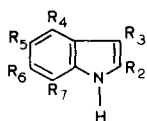
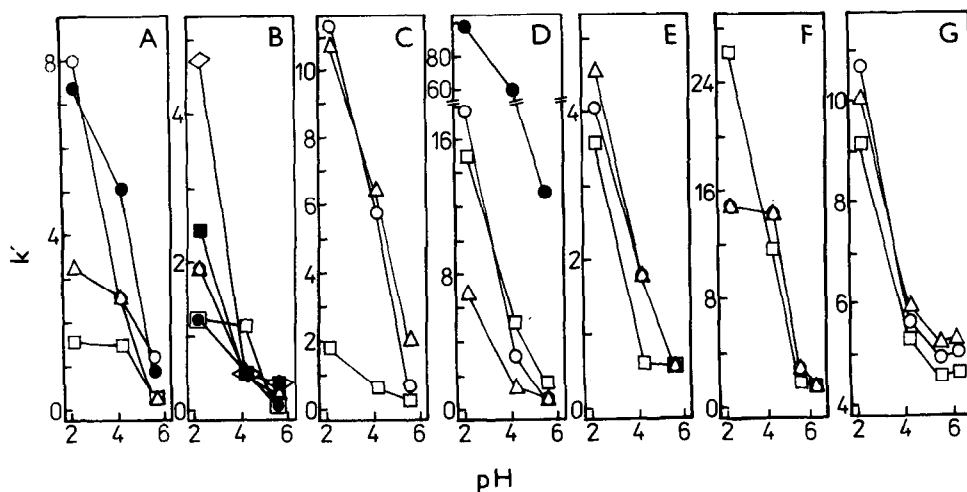| No.	Compound	R_2	R_3	R_4	R_5	R_6	R_7
21	Indole-2-carboxylic acid	COOH					
22	Indole-3-carboxylic acid		COOH				
23	Indole-5-carboxylic acid				COOH		
24	4-Methyltryptophan		CH$_2$CH–COOH $\mid$ NH$_2$	CH$_3$			
25	5-Methyltryptophan		CH$_2$CH–COOH $\mid$ NH$_2$		CH$_3$		
26	6-Methyltryptophan		CH$_2$CH–COOH $\mid$ NH$_2$			CH$_3$	

Fig. 1. Effect of the pH of the mobile phases on the capacity factors (A) of o- (○), m- (△) and p-hydroxybenzoic acid isomers (□) with benzoic acid (●), (B) 2,3- (◇), 2,4- (○), 2,5- (■), 2,6- (△), 3,4- (□) and 3,5-dihydroxybenzoic acid isomers (●), (C) o- (○), m- (△) and p-methoxybenzoic acid isomers (□), (D) 2,3- (○), 2,6- (△), 3,4- (□) and 3,5-dimethoxybenzoic acid isomers (●), (E) o- (○), m- (△) and p-methoxymandelic acid isomers (□), (F) indole-2- (□), -3- (△) and -5-carboxylic acid isomers (○) and (G) 4- (○), 5- (△) and 6-methyltryptophan isomers (□). Conditions: chromatographic system I; Separon Si C$_{18}$ packed column (250 mm × 2 mm I.D.); mobile phase, methanol–0.01 M phosphate buffer (20:80) containing $5 \cdot 10^{-3}$ M β-CD. For other conditions see Experimental section.

TABLE III

CAPACITY FACTORS OF BENZOIC ACID, MONOHYDROXYBENZOIC AND DIHYDROXYBENZOIC ACIDS IN CHROMATOGRAPHIC SYSTEM I

Conditions: Separon Si C_{18} packed column (250 mm × 2 mm I.D.); flow-rate, 20 ml/h; temperature, 25°C.

Benzoic acid compound	Methanol–0.01 M phosphate buffer*													
	40:60	30:70	30:70	30:70	30:70	20:80	30:70	30:70	30:70	20:80	20:80	10:90	10:90	10:90
pH														
	2.20	2.20	2.20	2.20	2.20	2.20	4.20	4.20	4.20	4.20	5.70	5.70	5.70	5.70
Concentration of β-CD (· 10^{-3} M)														
	5	0	2	5	10	5	0	5	10	5	5	0	5	10
Benzoic	3.55	9.01	—**	6.41	4.21***	7.38	5.86	3.34	3.12	5.05	0.88	2.48	2.11	1.79
o-Hydroxy-	3.81	10.02	—	7.30	4.87***	8.35	2.34	1.65	1.53	2.53	1.22	3.41	2.36	2.09
m-Hydroxy-	1.46	3.39	—	2.63	1.82***	3.23	2.34	1.65	1.53	2.53	0.22	1.05	0.52	0.44
p-Hydroxy-	0.91	2.33	—	1.41	0.87***	1.56	1.05	0.82	0.81	1.51	0.22	0.72	0.52	0.44
2,3-Dihydroxy-	1.89	4.41	3.68***	3.64	2.75	4.75	0.55	0.25	0.22	0.47	0.36	1.13	0.90	0.88
2,4-Dihydroxy-	1.24	4.41	2.48***	1.73	1.12	1.94	0.55	0.25	0.22	0.47	0.24	0.99	0.58	0.47
2,5-Dihydroxy-	1.24	2.71	2.02***	1.93	1.44	2.42	0.55	0.25	0.22	0.47	0.36	0.99	0.90	0.88
2,6-Dihydroxy-	1.24	4.41	2.48***	1.73	1.12	1.94	0.55	0.25	0.22	0.47	0.24	0.99	0.58	0.47
3,4-Dihydroxy-	0.61	1.44	1.07***	1.01	0.76	1.24	0.84	0.55	0.48	1.14	0.12	0.34	0.33	0.33
3,5-Dihydroxy-	0.61	1.44	1.07***	1.01	0.76	1.24	0.55	0.25	0.22	0.47	0.12	0.34	0.33	0.33

* Numbers represent the volume ratio of the solvent components.
** Not measured.
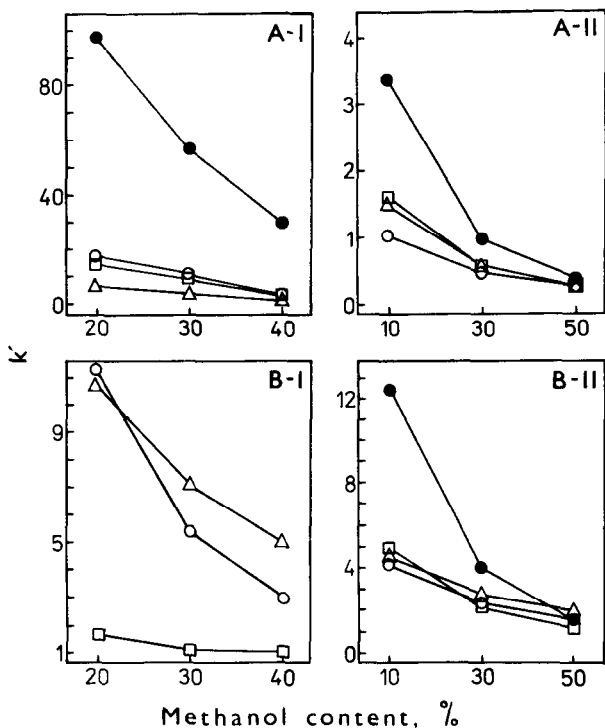*** Value obtained under optimum conditions.

Fig. 2. Effect of the methanol content in the mobile phase on the capacity factors (A-I, II and B-II) of 2,3- (○), 2,6- (△), 3,4- (□) and 3,5-dimethoxybenzoic acid isomers (●) and (B-I) o- (○), m- (△) and p-methoxybenzoic acid isomers (□). Conditions: (I), chromatographic system I, mobile phase, methanol–0.01 M phosphate buffer containing $5 \cdot 10^{-3}$ M β-CD, pH 2.20; (II) chromatographic system II, mobile phase, methanol–0.01 M phosphate buffer, (A) pH 5.70, (B) pH 4.20. For other conditions see Experimental section.

Effect of the β-CD concentration in the mobile phase

It follows from the relationship between k' and the β-CD concentration in the mobile phase given in Fig. 3A and B that the separation is generally improved with increasing β-CD concentration and/or the analysis time is reduced. In addition, changes in the elution order of the individual isomers occur in the example given in Fig. 3C.

On the basis of the decrease in k' with increasing β-CD concentration, it is possible to evaluate the ability of the individual isomers to form inclusion complexes under the given experimental conditions. However, the phenomena described here may be even more complex, as shown in Fig. 3D, indicating that the k' value of the *ortho* MMBA isomer slightly increases with increasing β-CD content during the initial phase and then decreases with a further increase in the β-CD concentration. The *ortho* MMMA isomer behaved similarly in methanol–phosphate buffer (20:80) at pH 2.20 (see Table VI). These isomers produce only weak inclusion complexes with β-CD due to steric hindrance, as indicated by their behaviour in system II in which they are least retained. The formation of inclusion complexes is characterized by an equilibrium between the inclusion complex and the guest and free β-CD molecules. At a low β-CD
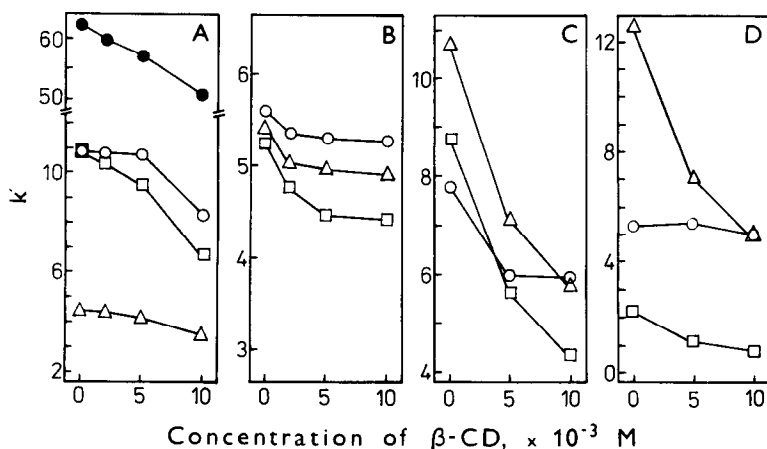
Fig. 3. Effect of the concentration of β-CD in the mobile phase on the capacity factors (A) of 2,3- ($\bigcirc$), 2,6-($\triangle$), 3,4- ($\square$) and 3,5-dimethoxybenzoic acid isomers ($\bullet$), (B) 4- ($\bigcirc$), 5- ($\triangle$) and 6-methyltryptophan isomers ($\square$), (C) o- ($\bigcirc$), m- ($\triangle$) and p-methoxymandelic acid isomers ($\square$) and (D) o- ($\bigcirc$), m- ($\triangle$) and p-methoxybenzoic acid isomers ($\square$). Conditions: chromatographic system I, Separon Si C$_{18}$ packed column (250 mm × 2 mm I.D.), mobile phase, methanol–0.01 M phosphate buffer (30:70) (A, B, D), (10:90) (C), pH 2.20. For other conditions see Experimental section.

TABLE IV

CAPACITY FACTORS OF BENZOIC ACID, MONOHYDROXYBENZOIC AND DIHYDROXY-BENZOIC ACIDS IN CHROMATOGRAPHIC SYSTEM II

Conditions: Cyclobond I column (250 mm × 4.6 mm I.D.); flow-rate, 60 ml/h; temperature, 25°C.

Benzoic acid compound	Methanol–0.01 M phosphate buffer					
	50:50	30:70	10:90	50:50	30:70	10:90
	pH					
	4.20	4.20	4.20	5.70	5.70	5.70
Benzoic	2.06	3.44	4.89	0.42	0.91*	1.81
o-Hydroxy-	3.12	5.26	8.80	0.42	1.43*	3.56
m-Hydroxy-	1.54	2.30	3.01	0.27	0.58*	1.05
p-Hydroxy-	1.54	3.44	4.89	0.42	0.91*	1.81
2,3-Dihydroxy-	2.64	4.93	—	0.63	1.06*	4.03
2,4-Dihydroxy-	2.64	4.93	—	0.63	1.06*	4.03
2,5-Dihydroxy-	2.03	2.83	—	0.46	0.62*	1.76
2,6-Dihydroxy-	3.58	8.26	—	0.83	1.78*	6.68
3,4-Dihydroxy-	2.64	4.93	—	0.63	1.06*	4.03
3,5-Dihydroxy-	1.25	1.81	—	0.28	0.39*	0.80

* Value obtained under optimum conditions.

TABLE V

CAPACITY FACTORS OF MONOMETHOXYBENZOIC AND DIMETHOXYBENZOIC ACIDS IN CHROMATOGRAPHIC SYSTEM I

Conditions: Separon Si C_{18} packed column (250 mm × 2 mm I.D.); flow-rate, 20 ml/h; temperature, 25°C.

Benzoic acid compound	*Methanol-0.01 M phosphate buffer*														
	40:60	*30:70*	*30:70*	*30:70*	*20:80*	*30:70*	*30:70*	*30:70*	*20:80*	*20:80*	*20:80*	*20:80*	*10:90*	*10:90*	*10:90*
pH	*2.20*	*2.20*	*2.20*	*2.20*	*2.20*	*4.20*	*4.20*	*4.20*	*4.20*	*4.20*	*4.20*	*5.70*	*5.70*	*5.70*	*5.70*
Concentration of β-CD ($\cdot 10^{-3}$ M)	*5*	*0*	*5*	*10*	*5*	*0*	*5*	*10*	*0*	*5*	*10*	*5*	*0*	*5*	*10*
o-Methoxy-	3.02*	5.31	5.36	4.92	11.32	3.92	3.42	3.16	5.84	5.77	5.71	0.66	1.92	1.73	1.54
m-Methoxy-	4.95*	12.75	7.06	4.92	10.82	9.91	4.82	3.71	10.62	6.45	5.71	2.04	4.25	3.55	3.11
p-Methoxy-	0.93*	2.20	1.10	0.75	1.79	1.30	0.90	0.69	1.15	0.86	0.62	0.23	0.60	0.55	0.41
2,3-Dimethoxy-	4.00	10.90	10.81	8.30	17.78	2.18	1.97	1.79	—	3.15	—	0.66	2.21	1.84*	1.83
2,6-Dimethoxy-	1.84	4.46	4.39	3.47	6.90	0.78	0.74	0.72	—	1.32	—	0.66	1.58	1.27*	1.19
3,4-Dimethoxy-	3.69	10.90	9.55	6.79	14.98	4.12	3.57	3.34	—	5.12	—	1.57	4.18	3.55*	3.20
3,5-Dimethoxy-	29.79	62.15	57.25	50.65	97.55	35.11	31.97	28.48	—	58.73	—	12.79	25.43	23.85*	22.41

* Value obtained under optimum conditions.

TABLE VI

CAPACITY FACTORS OF MONOMETHOXYMANDELIC ACIDS IN CHROMATOGRAPHIC SYSTEM I

Conditions: Separon Si C_{18} packed column (250 mm × 2 mm I.D.); flow-rate, 20 ml/h; temperature, 25°C.

Mandelic acid compound	Methanol–0.01 M phosphate buffer														
	30:70	30:70	20:80	20:80	20:80	10:90	10:90	10:90	20:80	20:80	20:80	20:80	10:90	10:90	10:90
pH	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20	4.20	4.20	4.20	4.20	5.70	5.70	5.70
Concentration of β-CD (· 10^{-3} M)	0	5	0	5	10	0	5	10	0	5	10	5	0	5	10
o-Methoxy-	2.75	0.99	3.67	4.05*	3.33	7.76	5.98	5.97	2.24	1.80	1.28	0.58	1.00	0.92	0.81
m-Methoxy-	3.69	1.24	4.91	4.56*	3.63	10.70	7.13	5.73	2.24	1.80	1.28	0.58	1.36	1.28	1.07
p-Methoxy-	3.12	1.09	4.04	3.58*	2.75	8.71	5.61	4.33	0.64	0.60	0.56	0.58	1.00	0.92	0.81

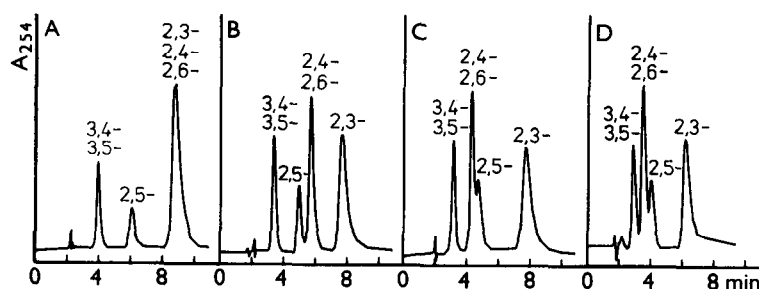* Value obtained under optimum conditions.

Fig. 4. Separation of dihydroxybenzoic acid isomers in methanol–0.01 M phosphate buffer (30:70), pH 2.20, containing β-CD at concentrations of 0 (A), $2 \cdot 10^{-3}$ (B), $5 \cdot 10^{-3}$ (C) and $10 \cdot 10^{-3}$ M (D) in chromatographic system I. For other conditions see Experimental section.
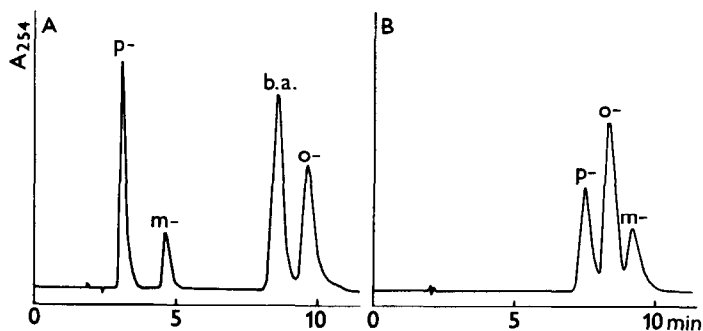

Fig. 5. Separation (A) of hydroxybenzoic acid isomers with benzoic acid (b.a.) in methanol–0.01 M phosphate buffer (30:70) containing $10 \cdot 10^{-3}$ M β-CD, pH 2.20 and (B) methoxymandelic acid isomers in methanol–0.01 M phosphate buffer (20:80) containing $5 \cdot 10^{-3}$ M β-CD, pH 2.20 in chromatographic system I. For other conditions see Experimental section.
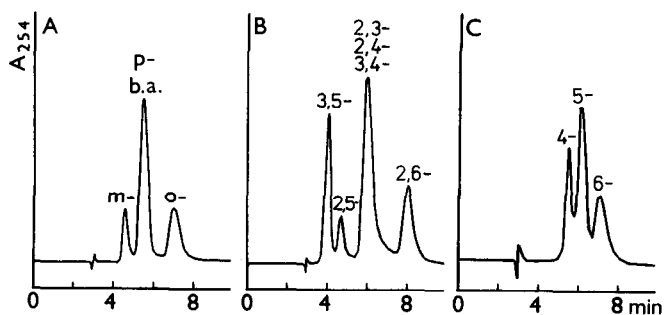

Fig. 6. Separation (A) of hydroxybenzoic acid isomers with benzoic acid (b.a.) and (B) dihydroxybenzoic acid isomers in methanol–0.01 M phosphate buffer (30:70), pH 5.70 and (C) methyltryptophan isomers in methanol–0.01 M phosphate buffer (10:90), pH 4.20 in chromatographic system II. For other conditions see Experimental section.

TABLE VII

CAPACITY FACTORS OF MONOMETHOXYBENZOIC, DIMETHOXYBENZOIC AND MONO-METHOXYMANDELIC ACIDS IN CHROMATOGRAPHIC SYSTEM II

Conditions: Cyclobond I column (250 mm × 4.6 mm I.D.); flow-rate, 60 ml/h; temperature, 25°C.

	Methanol–0.01 M phosphate buffer					
	50:50	30:70	10:90	50:50	30:70	10:90
	pH					
	4.20	4.20	4.20	5.70	5.70	5.70
Benzoic acid compound						
o-Methoxy-	1.43	2.30*	4.06	0.26	0.53	1.04
m-Methoxy-	2.03	4.33*	9.90	0.36	0.87	2.35
p-Methoxy-	1.53	3.85*	8.86	0.36	0.87	1.96
2,3-Dimethoxy-	1.60	2.33	4.20	0.27	0.47	1.05*
2,6-Dimethoxy-	2.10	2.81	4.54	0.27	0.56	1.51*
3,4-Dimethoxy-	1.23	2.23	4.94	0.27	0.56	1.60*
3,5-Dimethoxy-	1.60	4.15	12.50	0.35	1.01	3.38*
Mandelic acid compound						
o-Methoxy-	1.69	2.17*	2.82	0.41	0.53	0.84
m-Methoxy-	2.05	3.04*	4.36	0.41	0.89	1.43
p-Methoxy-	2.28	3.56*	5.46	0.41	0.89	1.67

* Value obtained under optimum conditions.

concentration, inclusion complexes are probably not formed at all with these isomers and the components are present as the free molecules. The polar surface of β-CD probably slightly increases the polarity of the mobile phase, resulting in a decreased elution strength in the reversed-phase system and thus in the observed increase in k'. Only a further increase in the β-CD concentration results in a shift of the equilibrium in favour of the inclusion complex formation and thus in a decrease in k'.

Also from Fig. 3C and D, it is seen that for the MMMA and MMBA isomers the selectivity decreases with increasing β-CD concentration in the mobile phase. Even in these cases the addition of β-CD may favourably influence the separation, particularly with respect to reduction of the analysis time. However, it is necessary to optimize the β-CD concentration, according to the relationships determined.

A typical example of the effect of β-CD on the retention behaviour of the compounds studied is given in Fig. 4.

Separation of isomers and comparison of the two systems

Ortho, meta and *para* isomers of MHBA and MMBA were completely separated in both systems. The isomers of MMMA were also satisfactorily separated (with resolution $R_s \geqslant 1.0$) in both systems. Chromatograms of MHBAs with benzoic acid and MMMAs obtained in system I are given in Fig. 5. The separation of MHBA isomers with benzoic acid, DHBAs and methyltryptophans in system II is illustrated in Fig. 6. In system II it was possible to separate the 2,4- and 2,6-DHBA isomers, similarly to 3,4- and 3,5-DHBA, which were not separated in system I (*cf.*, Fig. 4). The

TABLE VIII

CAPACITY FACTORS OF INDOLECARBOXYLIC ACIDS AND METHYLTRYPTOPHANS IN CHROMATOGRAPHIC SYSTEM I

Conditions: Separon Si C$_{18}$ packed column (250 mm × 2 mm I.D.), flow-rate, 20 ml/h; temperature, 25°C.

Methanol-0.01 M phosphate buffer

	30:70	30:70	30:70	30:70	20:80	30:70	30:70	30:70	20:80	20:80	10:90	10:90	10:90	30:70	20:80
pH	2.20	2.20	2.20	2.20	2.20	4.20	4.20	4.20	4.20	5.70	5.70	5.70	5.70	6.20	6.20
Concentration of β-CD ($\cdot 10^{-3}$ M)	0	2	5	10	5	0	5	10	5	5	0	5	10	5	5
Carboxylic acid															
Indole-2-	22.43	—	15.66	13.41	26.39	5.97	3.53	3.36	11.62	1.87*	7.22	5.49	4.70	0.99	1.64
Indole-3-	8.50	—	7.01	6.61	14.87	7.66	5.98	5.36	14.24	3.02*	10.06	9.12	8.43	0.99	1.64
Indole-5-	8.50	—	7.01	6.61	14.87	7.66	5.98	5.36	14.24	3.02*	10.06	9.12	8.43	0.99	1.64
Tryptophan compound															
4-Methyl-	5.59	5.37	5.30	5.28	2.91	10.69	2.70	2.41	5.69	4.96*	12.12	11.88	11.59	5.07	2.63
5-Methyl-	5.41	5.01	4.95	4.89	2.91	10.07	2.88	2.87	5.92	5.18*	12.12	12.06	11.59	5.28	2.82
6-Methyl-	5.24	4.76	4.46	4.43	2.91	9.18	2.45	2.24	5.28	4.61*	11.76	10.41	9.72	4.66	2.50

* Value obtained under optimum conditions.

TABLE IX

CAPACITY FACTORS OF INDOLECARBOXYLIC ACIDS AND METHYLTRYPTOPHANS IN CHROMATOGRAPHIC SYSTEM II

Conditions: Cyclobond I column (250 mm × 4.6 mm I.D.); flow-rate, 60 ml/h; temperature, 25°C.

	Methanol–0.01 M phosphate buffer					
	50:50	*30:70*	*10:90*	*50:50*	*30:70*	*10:90*
	pH					
	4.20	*4.20*	*4.20*	*5.70*	*5.70*	*5.70*
Carboxylic acid						
Indole-2-	4.73	11.60	35.44	0.99*	2.68	—
Indole-3-	0.47	2.22	6.19	0.54*	1.23	—
Indole-5-	0.47	2.22	6.19	0.54*	1.23	—
Tryptophan compound						
4-Methyl-	0.57	0.73	1.03*	0.55	0.68	0.94
5-Methyl-	0.63	0.86	1.24*	0.55	0.68	1.16
6-Methyl-	0.78	1.11	1.60*	0.55	0.88	1.57

* Value obtained under optimum conditions.

separation of methyltryptophan isomers in system I without β-CD in the mobile phase was very difficult compared with system II, and was incomplete with β-CD. On the other hand, a complete separation of all DMBA isomers was obtained only in system I, but with high k' values for 3,5-DMBA. Indole-3-carboxylic acid and indole-5-carboxylic acid could not be separated in these two systems.

The results obtained in both systems are qualitatively comparable and it is difficult to decide which system is more selective. It can be concluded only that system I is more suitable for isomers of MHBA with benzoic acid and system II is better suited for methyltryptophans.

The recommended optimum conditions for the separation of isomers of the acids studied are summarized in Tables III–IX. All the analyses required less than 20 min, except the separations of DMBA isomers in system I. According to the experimental values of k' for the individual groups of isomers, it is also possible to evaluate the separation of mixtures containing an higher number of compounds.

The retention data obtained in the two chromatographic systems show that β-CD influences the separation of the acids studied. Using indolecarboxylic acids, MMBAs and DMBAs, as examples, Figs. 7 and 8 show chromatograms obtained in the two systems under comparable conditions. However, the retention behaviour of the individual compounds cannot be explained by a simple relationship between the ability to form the inclusion complexes with β-CD based on the shape and size of the molecule of the included compound and the elution order and, hence, when comparing the two systems, an inverse elution order is not expected. The observed behaviour of the structural isomers of aromatic acids is a result of several factors, such as the different acidities of the individual isomers, varying affinity towards the non-polar stationary phase and the stability of the inclusion complexes which is determined by
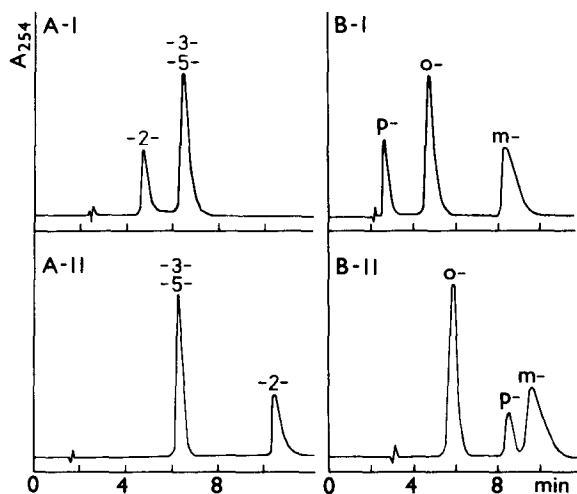
Fig. 7. (A) Separation of indolecarboxylic acid isomers: (I), chromatographic system I, mobile phase, methanol–0.01 M phosphate buffer (20:80) containing $5 \cdot 10^{-3}$ M β-CD, pH 5.70; (II), chromatographic system II, mobile phase, methanol–0.01 M phosphate buffer (30:70), pH 5.70. (B) Separation of methoxybenzoic acid isomers: mobile phase, methanol–0.01 M phosphate buffer (10:90), pH 5.70; (I), chromatographic system I, $5 \cdot 10^{-3}$ M β-CD; (II) chromatographic system II. For other conditions see Experimental section.
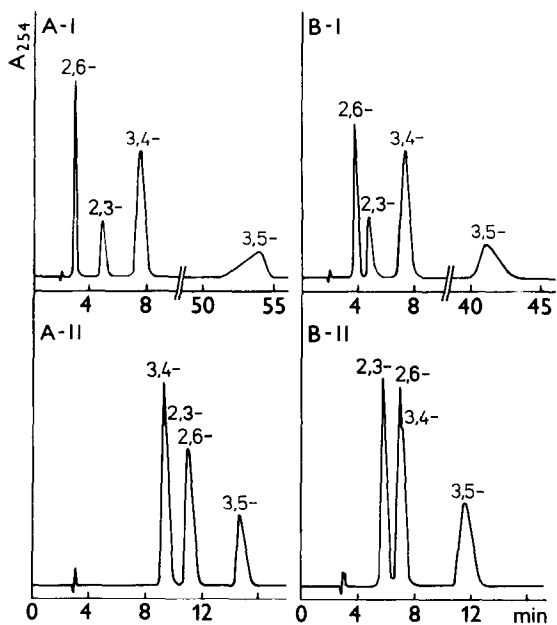

Fig. 8. Separation of dimethoxybenzoic acid isomers in methanol–0.01 M phosphate buffer (30:70), pH 4.20 (A) and (10:90), pH 5.70 (B) in (I) chromatographic system I and (II) chromatographic system II, respectively. Concentration of β-CD was $5 \cdot 10^{-3}$ M in chromatographic system I. For other conditions see Experimental section.

the size and shape and, in addition, by the degree of ionization of the molecule in a given medium.

In spite of the fact that these measurements do not clarify the separation mechanism for the tested compounds in more detail, they facilitate optimization of the conditions for analyses of more complex mixtures in biological materials. The results described indicate that CD can be used successfully for the separation of certain compounds that could be difficult to separate in other systems under isocratic conditions.

ACKNOWLEDGEMENT

REFERENCES

 1 K. Vande Casteele, H. Geiger and Ch. F. Van Sumere, *J. Chromatogr.*, 258 (1983) 111.
 2 J. B. Murphy and C. A. Stutte, *Anal. Biochem.*, 86 (1978) 220.
 3 J. M. Hardin and C. A. Stutte, *Anal. Biochem.*, 102 (1980) 171.
 4 F. Villeneuve, G. Abravanel, M. Moutounet and G. Alibert, *J. Chromatogr.*, 234 (1982) 131.
 5 J. Mollerup Andersen and W. Batsberg Pedersen, *J. Chromatogr.*, 259 (1983) 131.
 6 T. C. Kwong, *J. Liq. Chromatogr.*, 10 (1987) 305.
 7 M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer, New York, 1978.
 8 D. Sybilska, J. Lipkowski and J. Wójcikowski, *J. Chromatogr.*, 253 (1982) 95.
 9 D. Sybilska, J. Dębowski, J. Jurczak and J. Żukowski, *J. Chromatogr.*, 286 (1984) 163.
10 M. Tanaka, T. Miki and T. Shono, *J. Chromatogr.*, 330 (1985) 253.
11 K. Fujimura, T. Ueda, M. Kitagawa, H. Takayanagi and T. Ando, *Anal. Chem.*, 58 (1986) 2668.
12 K. Fujimura, T. Ueda and T. Ando, *Anal. Chem.*, 55 (1983) 446.
13 Y. Kawaguchi, M. Tanaka, M. Nakae, K. Funazo and T. Shono, *Anal. Chem.*, 55 (1983) 1852.
14 M. Tanaka, Y. Kawaguchi, M. Nakae, Y. Mizobuchi and T. Shono, *J. Chromatogr.*, 299 (1984) 341.
15 M. Tanaka, Y. Kawaguchi, T. Shono, M. Uebori and Y. Kuge, *J. Chromatogr.*, 301 (1984) 345.
16 K. G. Feitsma, J. Bosman, B. F. H. Drenth and R. A. De Zeeuw, *J. Chromatogr.*, 333 (1985) 59.
17 K. Fujimura, M. Kitagawa, H. Takayanagi and T. Ando, *J. Chromatogr.*, 350 (1985) 371.
18 K. Fujimura, M. Kitagawa, H. Takayanagi and T. Ando, *J. Liq. Chromatogr.*, 9 (1986) 607.
19 D. W. Armstrong and W. De Mond, *J. Chromatogr. Sci.*, 22 (1984) 411.
20 D. W. Armstrong, A. Alak, K. Bui, W. De Mond, T. Ward, T. E. Riehl and W. L. Hinze, *J. Incl. Phenomena*, 2 (1984) 533.
21 D. W. Armstrong, W. De Mond, A. Alak, W. L. Hinze, T. E. Riehl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234.
22 D. W. Armstrong, A. Alak, W. De Mond, W. L. Hinze and T. E. Riehl, *J. Liq. Chromatogr.*, 8 (1985) 261.
23 W. L. Hinze, *Sep. Purif. Methods*, 10 (1981) 159.
24 L. Bažant and E. Smolková-Keulemansová, presented at the *Fourth International Symposium on Inclusion Phenomena and the Third International Symposium on Cyclodextrins, Lancaster, July 20–25, 1986*.
25 K. Uekama, F. Hirayama, M. Otagiri, Y. Otagiri and K. Ikeda, *Chem. Pharm. Bull.*, 26 (1978) 1162.