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CHROMATOGRAPHY OF MICROBIAL METABOLITES OF AROMATIC AMINO ACIDS

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SUMMARY

A mixture of hydroxy-, methoxy- and aminobenzenecarboxylic acids (aromatic acids) was studied by gas (GC) and liquid (LC) chromatography. The gas and high-performance liquid chromatographs were attached to a computer. The GC method yielded elution data for trimethylsilyl derivatives of a large number of aromatic acids on four stationary silicone phases, OV-1, SE-52, OV-17 and 1,5-bis(*m*-phenoxyphenyl)-1,1,3,3,5,5-hexaphenyltrisiloxane. HPLC measurements afforded capacity factors, k, for these aromatic acids on a reversed stationary phase as a function of the mobile phase composition, which consisted of methanol (or ethanol), water and acetic acid. The GC and LC separation systems were described by an empirical function, embodying parameters such as the resolution, R, standard deviation, s, analysis time, t, and time for preparative work, t_p. Evaluation of the optimization function enabled assessment of the applicability of the chromatographic methods to the analysis and isolation of natural metabolites of aromatic amino acids from microbial material.

INTRODUCTION

Aromatic acids, *e.g.*, hydroxy-, methoxy- and aminobenzene carboxylic acids are important metabolites of aromatic amino acids in plants and microorganisms. Analysis of complex mixtures of these aromatic compounds is conveniently performed by chromatographic methods, including both gas (GC) and high-performance liquid chromatography (HPLC).

Aromatic acids have been separated by GC mostly as trimethylsilyl (TMS) derivatives[1–7], methyl[8,9], ethyl[10] or acetyl derivatives[11] on silicone stationary phases[1–6,9,10], isothermally[2,7,9] or with temperature programmes[1,3–7,9,10], and have been determined in a variety of biological materials[1–5,7,9,10].

The study of natural products in biology has recently witnessed an ever-increasing rôle of HPLC[12–16]. This efficient chromatographic method affords a direct separation and determination of aromatic acids on various stationary phases, such as silica gels[17,18], ion exchangers[16] and, especially, reversed phases[12–15,19–29]; the mobile phases used include mixtures of polar and less polar organic solvents[12–18,20–29], water[12–16,19–21,23–29], acids[12–16,19–23,25–29] and some buf-

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fers[12,16,24,25,29]. The separation of aromatic acids is carried out isocratically[13–16,19–22,28,29], often by gradient elution[12,13,15,20–28], with UV spectrophotometric[12–16,19–29], fluorescence[12,16] or voltammetric detection[27,29].

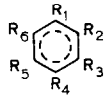
We have studied the separation of aromatic acids by chromatographic methods and compared the efficiency of separation and determination of these acids by GC and LC, with the aim of finding an efficient analytical method for mixtures of simple aromatic metabolites arising in the biosynthesis and metabolism of aromatic amino acids in microorganisms. This method will then be used in the study of natural aromatic products in microbiology.

EXPERIMENTAL

All the standard aromatic acids given below were of the maximum obtainable purity (Table I). Aromatic acids 1–11, 16–19, 35 and 36 were obtained from

TABLE I

AROMATIC CARBOXYLIC ACIDS



Acid	R_1	R_2	R_3	R_4	R_5	R_6
1 Benzoic acid	COOH					
2 Salicylic acid	COOH	OH				
3 3-Hydroxybenzoic acid	COOH		OH			
4 Catalpic acid	COOH			OH		
5 *o*-Pyrocatechuic acid	COOH	OH	OH			
6 *β*-Resorcylic acid	COOH	OH		OH		
7 Gentisic acid	COOH	OH			OH	
8 *γ*-Resorcylic acid	COOH	OH				OH
9 Protocatechuic acid	COOH		OH	OH		
10 *α*-Resorcylic acid	COOH		OH		OH	
11 2,3,4-Trihydroxybenzoic acid	COOH	OH	OH	OH		
12 Gallic acid	COOH		OH	OH	OH	
13 *o*-Anisic acid	COOH	OCH_3				
14 *m*-Anisic acid	COOH		OCH_3			
15 *p*-Anisic acid	COOH			OCH_3		
16 2,3-Dimethoxybenzoic acid	COOH	OCH_3	OCH_3			
17 2,6-Dimethoxybenzoic acid	COOH	OCH_3				OCH_3
18 Veratric acid	COOH		OCH_3	OCH_3		
19 3,5-Dimethoxybenzoic acid	COOH		OCH_3		OCH_3	
20 Vanillic acid	COOH		OCH_3	OH		
21 Syringic acid	COOH		OCH_3	OH	OCH_3	
22 Phenylacetic acid	CH_2COOH					
23 4-Hydroxyphenylacetic acid	CH_2COOH			OH		
24 Homovanillic acid	CH_2COOH		OCH_3	OH		
25 Homoveratric acid	CH_2COOH		OCH_3	OCH_3		
26 Mandelic acid	CHCOOH | OH					

TABLE I (*continued*)

Acid	R_1	R_2	R_3	R_4	R_5	R_6
27 *o*-Methoxymandelic acid	CHCOOH | OH	OCH_3				
28 *m*-Methoxymandelic acid	CHCOOH | OH		OCH_3			
29 *p*-Methoxymandelic acid	CHCOOH | OH			OCH_3		
30 *p*-Acetoxymandelic acid	CHCOOH | OH			$OCOCH_3$		
31 Cinnamic acid	CH=CHCOOH					
32 *o*-Coumaric acid	CH=CHCOOH	OH				
33 *m*-Coumaric acid	CH=CHCOOH		OH			
34 *p*-Coumaric acid	CH=CHCOOH			OH		
35 Caffeic acid	CH=CHCOOH		OH	OH		
36 Ferulic acid	CH=CHCOOH		OCH_3	OH		
37 Hydrocinnamic acid	CH_2CH_2COOH					
38 Melilotic acid	CH_2CH_2COOH	OH				
39 Phenylpyruvic acid	$CH_2COCOOH$					
40 Phenylalanine	$CH_2CHCOOH$ | NH_2					
41 Tyrosine	$CH_2CHCOOH$ | NH_2			OH		
42 Anthranilic acid	COOH	NH_2				
43 4-Aminobenzoic acid	COOH			NH_2		
44 *m*-Hydroxyanthranilic acid	COOH	NH_2	OH			

Schuchardt (Munich, F.R.G.), 12–15, 20, 21, 37, 39–41 from Aldrich, Europe (Beerse, Belgium), 22, 23, 25–34 from Fluka (Buchs, Switzerland) and 24 from Calbiochem (Luzerne, Switzerland). Acid 38 was prepared at the Institute of Microbiology (Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Acids 42–44 were obtained from the Research Institute of Organic Synthesis (Pardubice-Rybitví, Czechoslovakia).

Other chemicals were of reagent grade; organic solvents from Lachema (Brno, Czechoslovakia) were of UV spectroscopic purity.

Sample preparation

The fungus *Oudemansiella mucida* (Schrader ex Fr.) Höhnel from our laboratory collection was cultivated at 23°C in 500-ml flasks containing 80 ml complex medium with glucose and corn-steep on a reciprocal shaker (1.9 Hz, amplitude 7 cm).

An appropriate amount of mycelium was filtered by suction, washed with cold water and homogenized in a small volume of ice-cold water in a laboratory blender for

2 min. After centrifugation (10 000 *g*, 10 min, 0°C) the medium was desalinized on a Sephadex G-25 column (50 cm × 3 cm I.D.).

Medium or homogenized mycelium was acidified to pH 2.0 with 1 *M* hydrochloric acid and extracted with diethyl ether. The ether fraction was separated, dried with sodium sulphate and evaporated to dryness. Drying was completed over phosphorus pentoxide in a desiccator and the residue was used for preparing trimethylsilyl (TMS) derivatives of aromatic metabolites.

Derivatization

A silylation procedure was modified for derivatization of samples or standard aromatic acids[5]: a dried sample of a standard acid (about 1 mg) was placed in a glass ampoule, dissolved in a mixture of 0.4 ml bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 0.1 ml trimethylchlorosilane (TMCS) and heated in the sealed ampoule for 10 min at 125°C. The procedure yielded samples converted completely into TMS derivatives. After cooling, the samples were used directly for GC analysis.

Gas chromatography

The analyses were performed on a Sigma 3B gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) with a flame ionization detector. TMS derivatives of aromatic acids were separated on 2-m glass columns, 3.0 mm I.D., packed with Chromosorb G AW DMCS, particle size 0.15–0.17 mm (Johns Manville, Denver, CO, U.S.A.). The stationary phase was impregnated with 1% silicone polymer. The column packing weighed 13.6 g. Elution data for the TMS derivatives were determined on four polymethylphenylsiloxane phases, differing in the proportion of methyl and phenyl groups: OV-1 (100% polydimethylsiloxane), SE-52 (5% phenyl groups), OV-17 (50% phenyl groups) and MPTH with a defined structure, 1,5-bis(*m*-phenoxyphenyl)-1,1,3,3,5,5-hexaphenyltrisiloxane (100% phenyl groups)[7]. The efficiency of the chromatographic column packings, expressed as the number of theoretical plates, *n*, for benzoic acid, was: OV-1, $n = 2116$; SE-52, $n = 1546$; OV-17, $n = 2461$; MPTH, $n = 1527$. TMS derivatives of aromatic acids were separated either isothermally at 160°C or with a temperature programme from 100 to 250°C with a temperature gradient of 2°C/min. The carrier gas was nitrogen at a flow-rate of 20 ml/min. The detector temperature was 280°C, and the injector temperature was 250°C.

Individual compounds in mixtures were identified at 160°C by comparing their retention data on the above stationary phases with those of TMS derivatives of pure standard aromatic acids. Quantitative analysis was performed by the internal standard method. Detector signals were processed by an on-line computer (data system) Chromatographics 2 (Perkin-Elmer).

High-performance liquid chromatography

Aromatic acids in mixtures were analyzed on a Series 3B LC instrument with two pumps, which ensure the programming of mobile phase concentration and flow-rate, (Perkin-Elmer) and detected by an LC-75 variable wavelength spectrophotometric detector. Separation and determination of the acids was carried out on glass analytical columns SIX C_{18}, SGX C_{18} (150 mm × 3.2 mm I.D.), packed with a reversed phase (Laboratorní Přístroje, Prague, Czechoslovakia). Elution was performed with a methanol (or ethanol)–water–acetic acid mixture, having a variable content of the

organic solvent. The flow-rate of the mobile phase was 30 ml/h; the column temperature was maintained at 25°C. Samples of 1–5 μl were applied to the chromatographic column with an Hamilton syringe via a 7-105 Rheodyne valve.

Aromatic acids were identified and determined by methods analogous to those used in GC. Detector signals were processed in the Chromatographics 2 data system.

RESULTS AND DISCUSSION

Polymeric silicone phases, such as polydimethylsiloxanes (OV-1), polymethylphenylsiloxanes (SE-52, OV-17) and silicone phases with a defined structure, containing exclusively phenyl groups (MPHT), display widely different characteristics in GC separations of a number of TMS derivatives of aromatic acids (Table II).

The separation properties of silicon phases with an high or total content of phenyl groups (OV-17, MPHT) are more suitable for separating TMS derivatives of methoxy-, methoxyhydroxybenzene carboxylic and cinnamic acids than for distinguishing TMS derivatives of benzenecarboxylic acids, containing solely hydroxy groups. Sorption of TMS derivatives of aromatic acids with methoxy groups is stronger than that of TMS derivatives of aromatic acids with hydroxy groups, because the former type of groups exhibits less steric hindrance in interactions of π-electrons of the benzene ring of the analyte with the phenyl groups of the stationary phase as compared with the trimethylsilyl groups of hydroxybenzenecarboxylic acids.

Extension of the side chain by two carbon atoms and a double bond in TMS derivatives of cinnamic acids causes, during analysis, a marked interaction of π-electrons of the double bond and the aromatic ring of the analyte with the phenyl groups of the stationary phase. Retention of TMS derivatives of hydroxy- and aminobenzenecarboxylic acids is greater on a polydimethylsiloxane phase (OV-1) than on OV-17 and MPHT phases in view of the more pronounced interaction of trimethylsilyl groups of the analyte with the methyl groups of the stationary phase. Interaction of TMS derivatives of aromatic acids with the stationary silicon phases involves to a larger or smaller degree Van der Waal's forces in combination with weak polar forces involving π-electrons of the benzene ring[7].

Temperature programming of the chromatographic column from 100 to 250°C (2°C/min) reduces the analysis time and improves somewhat the separation of some TMS derivatives of positional isomers of the aromatic acids (Fig. 1). Fig. 1 shows a chromatogram of a mixture of TMS derivatives of selected aromatic acids under optimum working conditions. A better separation of positional isomers of aromatic acids can be achieved only on capillary columns.

The efficiency of separation and determination by GC and HPLC for aromatic acids was assessed by means of several parameters, such as the selectivity, α, the resolution, R, and standard deviation, s. Table III shows the evaluation of GC for a selected mixture of TMS derivatives of aromatic acids; the determination of individual components was verified by the internal standard technique, the relative standard deviation being wthin $\pm$ 2% (Table III).

The reversed stationary phase with methanol (or ethanol)–water–acetic acid as the mobile phase was highly suitable for HPLC analysis of aromatic acids (Table IV). A low pH (3.5) mobile phase solution suppressed the dissociation of active hydrogen

TABLE II

RETENTION VOLUMES OF TMS DERIVATIVES OF AROMATIC CARBOXYLIC ACIDS

Columns: 1% OV-1, 1% SE-52, 1% OV-17 and 1% MPHT on Chromosorb G AW DMCS. Column temperature: 160°C. Carrier gas: nitrogen at 20 ml/min.

Acid	*Relative retention, $r_{i,s}$, at 160°C*			
	OV-1	*SE-52*	*OV-17*	*MPHT*
Benzoic acid	1.00	1.00	1.00	1.00
Phenylacetic acid	1.26	1.24	1.43	1.52
p-Acetoxymandelic acid	1.61	3.43	2.74	3.64
Melilotic acid	2.37	2.85	5.82	9.06
3,5-Dimethoxybenzoic acid	2.57	2.62	2.71	2.67
Hydrocinnamic acid	2.57	2.50	3.23	1.15
Mandelic acid	2.84	2.78	2.93	2.57
Salicylic acid	3.46	3.53	3.30	3.32
Cinnamic acid	3.87	4.02	4.79	5.17
Phenylalanine	3.91	6.06	5.25	5.47
3-Hydroxybenzoic acid	4.29	4.33	4.05	3.52
2,3-Dimethoxybenzoic acid	5.09	5.72	7.69	9.05
Anthranilic acid	5.26	5.24	4.05	5.30
2,6-Dimethoxybenzoic acid	5.42	6.37	11.7	16.2
o-Methoxymandelic acid	5.74	5.78	7.37	7.49
Catalpic acid	5.97	5.94	5.45	4.52
4-Hydroxyphenylacetic acid	6.08	5.84	6.56	5.60
m-Methoxymandelic acid	7.00	7.44	8.54	8.30
p-Methoxymandelic acid	7.98	8.88	10.5	10.1
Veratric acid	8.00	8.90	13.8	16.2
Homoveratric acid	8.04	8.22	15.1	18.4
Phenylpyruvic acid	8.49	8.40	8.11	5.25
3-Hydroxyanthranilic acid	10.1	18.3	11.3	10.0
o-Pyrocatechuic acid	10.2	10.0	9.19	6.93
Vanillic acid	10.8	11.1	12.6	10.2
Homovanillic acid	10.8	11.5	13.9	12.6
γ-Resorcylic acid	11.3	11.4	11.3	8.88
Gentisic acid	12.5	12.3	10.9	8.13
o-Coumaric acid	13.0	12.9	14.9	13.0
4-Aminobenzoic acid	13.8	13.7	17.3	16.9
β-Resorcylic acid	14.3	14.0	12.4	9.21
α-Resorcylic acid	15.0	14.4	12.9	9.78
Protocatechuic acid	15.3	14.8	13.1	9.35
m-Coumaric acid	17.7	18.1	18.9	16.0
Tyrosine	18.1	18.7	5.14	20.3
Syringic acid	19.6	21.3	27.8	23.5
p-Coumaric acid	22.6	24.2	27.2	22.3
2,3,4-Trihydroxybenzoic acid	23.8	23.3	19.2	12.0
Gallic acid	31.3	29.5	26.6	15.8
Ferulic acid	43.7	47.9	62.7	27.2
Caffeic acid	58.9	55.9	55.3	38.1
V_g (benzoic acid) (ml/g)	185	285	134	126

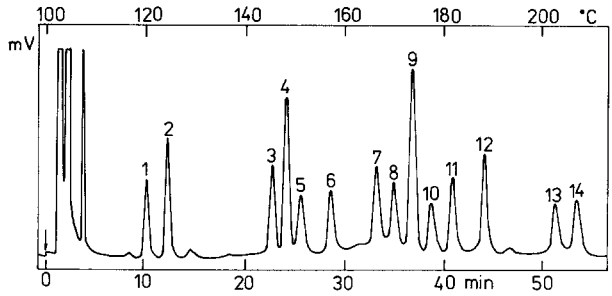


Fig. 1. Chromatogram of a mixture of TMS derivatives of some aromatic acids (GC). Column: 1% SE-52 on Chromosorb G AW DMCS. Temperature programme: linear from 100 to 250°C at 2°C/min. Carrier gas: nitrogen at 20 ml/min. Peaks: 1 = benzoic acid; 2 = phenylacetic acid; 3 = salicylic acid; 4 = cinnamic acid; 5 = 3-hydroxybenzoic acid; 6 = catalpic acid; 7 = veratric acid; 8 = *o*-pyrocatechuic acid; 9 = gentisic acid; 10 = β-resorcylic acid; 11 = *m*-coumaric acid; 12 = *p*-coumaric acid; 13 = ferulic acid; 14 = caffeic acid.

atoms of hydroxy-, methoxy- and aminobenzene carboxylic acids and activated the individual functional groups on the benzene ring; consequently, the aromatic character of the compounds analyzed became more prominent in their interactions with the stationary phase.

The optimum mobile phase composition for a given reversed phase was determined in order to ensure the highest possible differences in the capacity factors for individual compounds. We used ethanol or methanol as the organic solvents; an higher amount of a weakly polar solvent strongly suppresses ionogenic components of the solution and decreases the polarity of the phase, while increasing the solubility of the

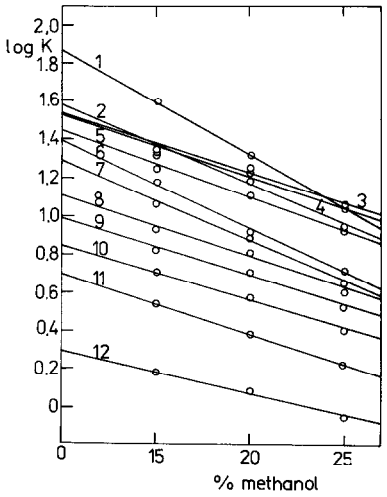


Fig. 2. Dependence of the capacity factors, *k*, of some aromatic acids on the methanol content in the mobile phase of methanol–1% acetic acid. 1, *m*-Coumaric acid; 2, *p*-coumaric acid; 3, benzoic acid; 4, salicylic acid; 5, phenylacetic acid; 6, syringic acid; 7, caffeic acid; 8, 3-hydroxybenzoic acid; 9, catalpic acid; 10, gentisic acid; 11, α-resorcylic acid; 12, gallic acid.

TABLE III

GAS CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF TMS DERIVATIVES OF SOME AROMATIC CARBOXYLIC ACIDS

Column: 1% SE-52 on Chromosorb G AW DMCS. Carrier gas: nitrogen at 20 ml/min.

Acid	*Relative retention, $r_{i,s}$*		*Retention temperature* (°C)*	α	*R*	*Amount (%)*		*Relative standard deviation (%)*
	Isothermal at 160°C	*Temperature programming**				*Theoretical*	*Found*	
Benzoic acid	1.00	1.00	120	1.20	2.36	4.75	4.29	1.65
Phenylacetic acid	1.24	1.20	124	1.87	10.2	7.36	7.18	1.23
Salicylic acid	3.53	2.29	145	1.06	1.17	7.86	7.41	1.20
Cinnamic acid	4.02	2.42	147	1.06	1.13	12.44	11.60	0.71
3-Hydroxybenzoic acid	4.33	2.58	150	1.13	2.74	3.93	4.39	1.41
Catalpic acid	5.94	2.90	156	1.17	3.54	4.66	4.75	0.95
Veratric acid	8.90	3.37	164	1.06	1.22	5.57	5.93	0.62
o-Pyrocatechuic acid	10.0	3.56	170	1.06	1.35	5.07	6.44	0.51
Gentisic acid	12.3	3.76	173	1.05	1.41	16.93	17.58	0.96
β-Resorcylic acid	14.0	3.95	177	1.05	1.58	4.09	5.07	0.99
m-Coumaric acid	18.1	4.17	181	1.08	2.67	6.22	6.77	1.32
p-Coumaric acid	24.2	4.49	187	1.16	5.45	8.51	8.47	0.83
Ferulic acid	47.9	5.25	202	1.04	1.65	6.71	5.10	1.90
Caffeic acid	55.9	5.47	207			5.89	5.02	1.79

* Temperature-programmed gas chromatography from 100 to 250°C at 2°C/min.

TABLE IV

RELATIVE RETENTION OF AROMATIC CARBOXYLIC ACID

Column: SIX C_{18}, particle size 5 μm; 150 mm × 3.2 mm I.D. Flow-rate: 30 ml/h. Pressure: 12.5–27.0 MPa. Mobile phase: ethanol–water–acetic acid. Detection: UV at 280 nm.

Acid	*Solvent system: ethanol–1% acetic acid*					
	5:95		*10:90*		*20:80*	
	$r_{i,s}$	k	$r_{i,s}$	k	$r_{i,s}$	k
Tyrosine	0.17	0.73	0.20	0.35	–	–
Gallic acid	0.19	0.91	0.22	0.46	–	–
Phenylalanine	0.30	2.02	0.30	1.05	–	–
4-Aminobenzoic acid	0.32	2.16	0.31	1.12	–	–
α-Resorcylic acid	0.34	2.39	0.33	1.22	–	–
2,3,4-Trihydroxybenzoic acid	0.35	2.49	0.36	1.46	–	–
m-Hydroxyanthranilic acid	0.35	2.47	0.37	1.50	–	–
Protocatechuic acid	0.36	2.56	0.37	1.50	–	–
γ-Resorcylic acid	0.44	3.38	0.43	1.88	–	–
Mandelic acid	0.53	4.24	0.53	2.61	–	–
p-Acetoxymandelic acid	0.54	4.40	0.54	2.65	–	–
o-Methoxymandelic acid	0.62	5.16	0.54	2.65	–	–
p-Anisic acid	0.63	5.25	–	–	2.83	9.1
Gentisic acid	0.54	4.40	0.55	2.72	–	–
p-Methoxymandelic acid	0.69	5.84	0.59	2.99	–	–
4-Hydroxyphenylacetic acid	0.79	6.86	0.60	3.03	–	–
Catalpic acid	0.66	5.60	0.66	3.48	–	–
m-Methoxymandelic acid	0.82	7.16	0.70	3.72	–	–
Homovanillic acid	1.13	10.2	0.72	3.86	–	–
o-Anisic acid	2.29	21.7	–	–	1.32	3.69
m-Anisic acid	5.75	56.1	–	–	3.29	10.7
Vanillic acid	–	–	0.78	4.23	0.72	1.57
Syringic acid	–	–	0.82	4.55	0.63	1.26
Caffeic acid	–	–	0.85	4.76	0.76	1.69
o-Pyrocatechuic acid	–	–	0.86	4.83	0.85	2.04
Anthranilic acid	–	–	0.92	5.21	1.12	2.99
3-Hydroxybenzoic acid	1.00	8.93	1.00	5.74	1.00	2.56
β-Resorcylic acid	–	–	1.00	5.77	0.90	2.39
2,6-Dimethoxybenzoic acid	–	–	1.03	5.94	0.84	1.98
p-Coumaric acid	–	–	1.70	10.5	1.37	3.88
Homoveratric acid	–	–	1.75	10.8	1.16	3.13
Veratric acid	–	–	1.94	12.1	1.36	3.83
Ferulic acid	–	–	2.02	12.6	1.32	3.69
Phenylacetic acid	–	–	2.08	13.1	2.10	6.46
2,3-Dimethoxybenzoic acid	–	–	2.42	15.3	1.65	4.87
m-Coumaric acid	–	–	2.60	16.5	1.89	5.72
Benzoic acid	–	–	2.98	19.1	2.68	8.55
o-Coumaric acid	–	–	3.98	25.9	2.94	9.45
Salicylic acid	–	–	4.17	27.1	2.78	8.90
Cinnamic acid	–	–	11.9	79.1	6.83	23.3
Retention volume of 3-hydroxybenzoic acid (ml)	9.55		6.47		3.42	

TABLE V

CAPACITY FACTORS, k, OF SOME AROMATIC CARBOXYLIC ACIDS

Column: SGX C_{18}, particle size 5 μm, 150 mm × 3.2 mm I.D. Flow-rate: 30 ml/h. Pressure:9–12 (methanol); 12–25 MPa (ethanol). Detection: UV at 280 nm.

Acid	*Ethanol–1% acetic acid*			*Methanol–1% acetic acid*		
	5:95	*10:90*	*15:85*	*15:85*	*20:80*	*25:75*
Gallic acid	2.1	1.4	0.80	1.5	1.2	0.9
α-Resorcylic acid	5.1	2.9	1.8	3.5	2.4	1.7
Gentisic acid	6.4	4.2	2.9	5.0	3.7	2.6
Catalpic acid	8.7	5.8	4.3	6.6	5.1	3.4
3-Hydroxybenzoic acid	11.8	7.6	5.4	8.5	6.4	4.1
Caffeic acid	18.6	9.8	5.1	11.7	7.7	4.6
Syringic acid	23.8	10.5	5.7	14.8	8.4	5.3
Phenylacetic acid	22.8	14.5	10.0	17.9	12.8	8.6
Salicylic acid	26.0	18.8	13.8	21.3	17.0	11.0
p-Coumaric acid	34.4	18.9	11.5	22.6	15.4	8.9
Benzoic acid	29.4	20.3	14.5	23.0	17.6	11.4
Veratric acid	52.8	24.1	12.8	–	–	–
m-Coumaric acid	46.8	25.8	15.6	38.9	20.8	11.2

compounds, and as a secondary effect, affecting the pH of the solution. These effects enhance the similarity between the mobile and the stationary phase and speed up the elution.

The dependence of the capacity factors, k, of selected aromatic acids on the ethanol or methanol content of the mobile phase is shown in Fig. 2, while values of k in given organic solvents are listed in Table V. The values of log k, and thus the analysis time are seen to decrease with increasing content of polar solvents in the mobile phase (Fig. 2, Table V). These results were used for selecting the optimum amount of ethanol or methanol in the mobile phase.

Table VI surveys the values of the qualitative and quantitative parameters for an HPLC analysis of a selected mixture of aromatic acids under optimum conditions. The

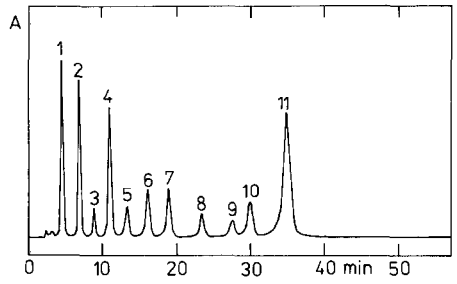


Fig. 3. HPLC of a mixture of aromatic acids. Column: SGX C_{18}; particle size 5 μm. Mobile phase: methanol–1% acetic acid (2:8). Flow-rate: 30 ml/h. Pressure: 9–12 MPa. Detection: UV at 280 nm. Peaks: 1 = gallic acid; 2 = α-resorcylic acid; 3 = gentisic acid; 4 = catalpic acid; 5 = 3-hydroxybenzoic acid; 6 = caffeic acid; 7 = syringic acid; 8 = phenylacetic acid; 9 = *p*-coumaric acid; 10 = benzoic acid; 11 = *m*-coumaric acid.

TABLE VI

SEPARATION AND DETERMINATION OF SOME AROMATIC CARBOXYLIC ACIDS BY HPLC

Column: SGX C_{18}, particle size 5 μm. Flow-rate: 30 ml/h. Pressure: 9–12 (methanol); 12–25 MPa (ethanol). Detection: UV at 280 nm.

Acid	*Ethanol–1% acetic acid (1:9)*			*Methanol–1% acetic acid (2:8)*			*Amount (ng)*		*Relative standard deviation (%)*
	k	α	*R*	*k*	α	*R*	*Theoretical*	*Found*	
Gallic acid	1.4			1.2			147.3	144.3	3.25
α-Resorcylic acid	2.9	2.07	2.05	2.4	2.00	2.93	142.2	127.9	3.13
Gentisic acid	4.2	1.45	3.25	3.7	1.54	2.65	413.1	396.2	3.23
Catalpic acid	5.8	1.38	3.08	5.1	1.38	2.37	28.1	26.2	3.20
3-Hydroxybenzoic acid	7.6	1.31	2.90	6.4	1.25	2.32	228.1	261.5	5.40
Caffeic acid	9.8	1.29	3.33	7.7	1.20	2.28	92.5	72.1	2.61
Syringic acid	10.5	1.07	1.00	8.4	1.09	1.03	94.6	93.5	2.92
Phenylacetic acid	14.5	1.38	4.65	12.8	1.52	5.30	1352.7	1360.6	2.97
p-Coumaric acid	18.5	1.30	3.79	15.4	1.20	2.55	129.6	117.6	3.77
Benzoic acid	20.3	1.07	1.07	17.6	1.14	2.00	586.8	488.4	4.24
m-Coumaric acid	25.8	1.27	3.79	20.8	1.18	2.56	145.1	120.0	3.20

TABLE VII

EVALUATION OF GC AND HPLC METHODS FOR ANALYSIS OF AROMATIC ACIDS WITH THE EMPIRICAL FUNCTION COF

Group of acids	*Method*	*Term in equation*				*COF*
		A	*B*	*C*	*D*	
I Benzoic acid Phenylacetic acid Cinnamic acid	GC HPLC	12.8 11.2	20.7 −10.7	−10 0	−3.6 −9.7	19.9 −9.2
II Benzoic acid Phenylacetic acid Catalpic acid *p*-Coumaric acid	 GC HPLC	 30.4 14.4	 −68.7 28.5	 −10 0	 −4.6 −14.2	 −52.9 28.7
III Benzoic acid Phenylacetic acid 3-Hydroxybenzoic acid *m*-Coumaric acid	 GC HPLC	 26.6 16.2	 32.1 17.4	 −10 0	 −5.6 −15.8	 43.1 17.8
IV Benzoic acid Phenylacetic acid Salicylic acid 3-Hydroxybenzoic acid Catalpic acid Gentisic acid *m*-Coumaric acid *p*-Coumaric acid Caffeic acid	 GC HPLC	 36.6 20.0	 −3.7 77.6	 −10 0	 −11.3 −30.7	 11.6 66.9

values of the standard deviation, s, are within the range commonly found in liquid chromatography. Differences between the theoretical and found values for aromatic acids are probably caused by the different purities of the standard compounds . Fig. 3

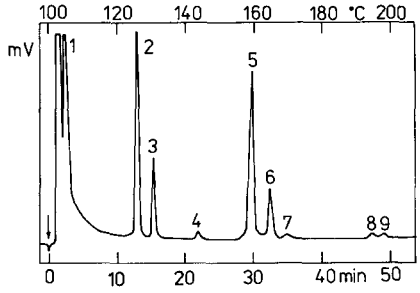


Fig. 4. Gas chromatogram of an extract from the glucose-containing cultivation medium with phenylalanine of the fungus *O. mucida* (13th day). Column: 1% SE-52 on Chromosorb G AW DMCS. Temperature programme: linear from 100 to 250°C at 2°C/min. Carrier gas: nitrogen at 20 ml/min. Peaks: 1 = solvent; 2 = benzoic acid; 3 = phenylacetic acid; 4 = unknown compound; 5 = cinnamic acid; 6 = phenylalanine; 7 = catalpic acid; 8 = α-, β-resorcylic acid and protocatechuic acid; 9 = *m*-coumaric acid.

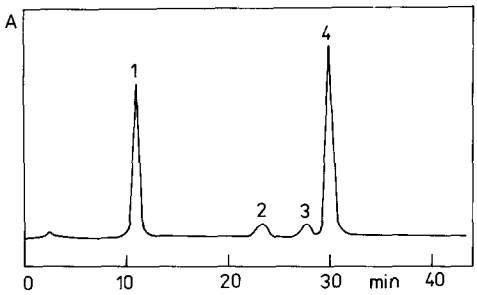


Fig. 5. Content of aromatic acids in the medium of the fungus *O. mucida*. The medium was enriched by benzoic acid. Column: SGX C_{18}; particle size 5 μm. Mobile phase: methanol–1% acetic acid (2:8). Flow-rate: 30 ml/h. Pressure: 12 MPa. Detection: UV at 280 nm. Peaks: 1 = catalpic acid; 2 = phenylacetic acid; 3 = *p*-coumaric acid; 4 = benzoic acid.

illustrates the HPLC of a mixture of aromatic acids under optimum isocratic conditions.

We found experimentally suitable working conditions for the qualitative and quantitative analysis of a large number of aromatic acids. However, in practice, biological materials are usually mixtures of fewer compounds; also, the origin of the sample may give hints as to the compounds expected in it. These facts may aid in finding an optimum separation system, ensuring the most rapid analysis.

We introduced an empirical function (COF), representing a simple chromatographic, mathematical and temporal parameter, which can be evaluated by a chromatographic method

$$\mathrm{COF} = V_1 \sum_{i=1}^{n-1} R_{S_{i,i+1}} + V_2 \sum_{i=1}^{n} (t_0 - t_1) + V_3 \sum_{i=1}^{k} t_{p_i} + V_4 \sum_{i=1}^{n} S_{i_{rel.}}$$

where R_S = resolution of two neighbouring peaks, s_{rel} = relative standard deviation, $t_0 = 30$ min, t_1 = duration of chromatographic analysis, t_p = duration of preparatory work, V_1, $V_2 = 1$, V_3, $V_4 = -1$. This empirical function was verified with several mixtures of aromatic acids containing a different number of components, which were analyzed by GC and HPLC under optimum working conditions. Table VII shows the values of individual terms of the equation and the resulting COF values for both GC and HPLC. An higher positive value of COF indicates a greater suitability of the given method. Both methods were used to study phenylalanine metabolism in the fungus *Oudemansiella mucida* (*O. mucida*). The spectrum of aromatic metabolites detected during the feeding experiments with phenylalanine and benzoic acid is demonstrated in Figs. 4 and 5.

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