Journal of Chromatography, 258 (1983) 111–124
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,489

SEPARATION OF PHENOLICS (BENZOIC ACIDS, CINNAMIC ACIDS, PHENYLACETIC ACIDS, QUINIC ACID ESTERS, BENZALDEHYDES AND ACETOPHENONES, MISCELLANEOUS PHENOLICS) AND COUMARINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KAREL VANDE CASTEELE
Laboratory of Plant Biochemistry, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, 9000 Ghent (Belgium)
HANS GEIGER
Institut für Chemie, Universität Hohenheim, Garbenstrasse, 30, D-7000 Stuttgart 70 (G.F.R.)
and
CHRISTIAAN F. VAN SUMERE*
Laboratory of Plant Biochemistry, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, 9000 Ghent (Belgium)
(Received November 1st, 1982)

SUMMARY

The high-performance liquid chromatographic separation of a relative large series of simple phenolics and related substances by means of a reversed-phase system is described. The system consists of a LiChrosorb RP-18 (10 μm) Knauer column and a combination of isocratic and linear-gradient elution (solvent A, water–formic acid (95:5); solvent B, methanol; temperature, 35°C). The series of compounds studied embraces derivatives of benzoic acid (29 compounds), phenylacetic acid (11 compounds), cinnamic acid (26 compounds) and dihydrocinnamic acid (8 compounds), quinic acid esters of phenolic benzoic and cinnamic acid derivatives (9 compounds), benzaldehydes and acetophenones (25 compounds), miscellaneous phenolics (27 compounds) and coumarins (43 compounds). Amongst these substances, phenolic acids, lactones, aldehydes and ketones are represented.

The possible use of the system for the analysis of complex mixtures of natural products as well as for the elucidation of the structures of more complex natural compounds is discussed.

INTRODUCTION

In two previous papers[1,2], the separation of N-acylamino acids and flavonoids by high-performance liquid chromatography (HPLC), using an RP-18 column and a water–formic acid–methanol gradient, was described. The present paper, which deals with phenolic acids, aldehydes, ketones, lactones and related substances, complements these papers. There are two main reasons for this extension.

(1) Most of the phenolic compounds under discussion are ubiquitous in

0021-9673/83/0000–0000/$03.00

nature, in the free and/or bound form[1,3–6]; therefore, when biological extract or fluids have to be analysed it is a pre-requisite to possess good knowledge of the chromatographic behaviour of these compounds.

(2) Since several of the latter substances may be building stones of more complex natural products, which for final identification may have to be degraded to smaller molecules (*e.g.* hydrolysis of glycosides, esters and amides or alkaline cleavage of flavonoids), the separation of the simple phenolics and related substances, in a chromatographic system which is as universal as possible, must be known.

In addition, when complex products are converted into simple phenolics the reaction mixtures usually contain relatively large amounts of inorganic salts. These frequently prevent analysis by straightforward thin-layer chromatography (TLC) or normal HPLC. In such cases reversed-phase HPLC is useful as this method eliminates inorganic salts and other polar materials during the fore-run, thus preventing these substances from affecting the chromatographic processes. The HPLC separations of certain phenolic acids and derivatives[1,7–12] phenolic aldehydes[13,14], phenylacetic acids[15–17], quinic acid and glucose esters of phenolic acids[18–20], coumarins[21–23] and flavonoids[2] have already been studied. However, most studies from other laboratories have dealt with a limited number of compounds. Therefore, with the development of a suitable system[1,2] our aim has been the compilation of as many retention times (t_R) as possible for phenolics and related substances. Further, this work has led to a reference system that has proved to be of great value when plant extracts or fluids of biological origin have to be analysed because the reference system reduces a large series of possibilities to just a few. In addition, eluates containing critical pairs can be collected. These eluates, free from interfering substances (*e.g.* relatively large amounts of salts, sugars, etc.), can subsequently be further analysed by other suitable methods such as TLC, gas–liquid chromatography (GLC) and mass spectrometry (MS).

EXPERIMENTAL

Apparatus

A Hewlett-Packard 1084 liquid chromatograph equipped with a variable wavelength Pye–Unicam LC3 UV detector, set at 280 nm (optical bandwidth 8 nm) and a Knauer (D-6380 Bad Homburg, G.F.R.) prepacked analytical column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm) was employed throughout this work.

Elution

Two solvents were used: A, formic acid–water (5:95); B, methanol. The elution profile was: 0–2 min, 7% B in A (isocratic); 2–8 min, 7–15% B in A (linear gradient); 8–25 min, 15–75% B in A (linear gradient); 25–27 min, 75–80% B in A (linear gradient); 27–29 min, 80% B in A (isocratic).

The chromatographic conditions were as described earlier[1].

Detection

The UV detector was set at 280 nm (optical bandwidth 8 nm).

Samples

Samples of 0.0025–0.025% solutions in aqueous methanol were applied to the column by means of a 20-μl loop valve. Most of the phenolic compounds, with the

exception of the majority of the coumarins, came from our own laboratories. They were either prepared or purchased. The quinic acid esters of *p*-hydroxybenzoic, cinnamic, *p*-coumaric, ferulic and sinapic acid were synthesized as described[24,25]. Most of the coumarins were kindly donated by colleagues. The authors are indebted to Dr. R. T. Williams (St. Mary's Hospital Medical School, London, Great Britain) for the hydroxycoumarins, to Dr. Baltzly (Welcome Research Laboratories, Tuckhahoe, NY, U.S.A.) for 7-hydroxycoumarin-3-carboxylic acid and to Dr. W. Steck (Prairie Regional Laboratory, Saskatoon, Canada) for most of the naturally occurring coumarins and furanocoumarins. The following coumarins were synthesized or isolated in our laboratories: daphnetin (Plant Biochemistry, Ghent) and fraxetin (Chemisches Institut Hohenheim, G.F.R.). Coumarin, umbelliferon and esculetin were purchased from Fluka (Buchs, Switzerland), coumarin-3-carboxylic acid from Aldrich Europe (Beerse, Belgium), herniarin from K & K Labs. (Plainview, NY, U.S.A.) and 4- and 6-methylcoumarin from L. Light and Co., (Bucks., Great Britain).

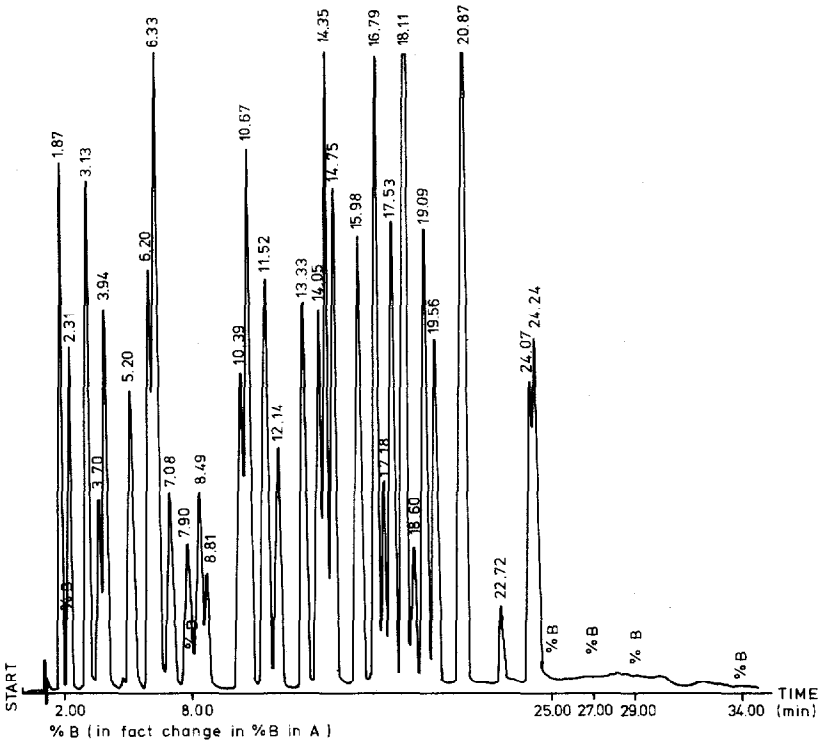

Fig. 1. Separation of phenolic compounds on a prepacked column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm). For the eluting system see Experimental section. Retention times (min): gallic acid, 1.87; homogentisic acid, 2.31; protocatechuic acid, 3.13; 2,3,4-trihydroxybenzoic acid, 3.70; 2,4,6-trihydroxybenzoic acid, 3.94; salicin, 5.20; *p*-coumaric acid-β-glucoside, 6.20; orcinol, 6.33; β-resorcylic acid, 7.08; *p*-hydroxybenzaldehyde, 7.90; ferulic acid β-glucoside, 8.49; coniferin, 8.81; ferulic amide, 10.38; dihydro-*p*-coumaric acid, 10.67; phloracetophenon, 11.52; 2,6-dimethoxybenzoic acid, 12.14; ferulic acid, 13.33; sinapic acid, 14.05; isoferulic acid, 14.35; benzoic acid, 14.75; 3-methoxybenzoic acid, 15.98; *o*-anisaldehyde, 16.79; 3,4-dimethoxycinnamic acid, 17.18; 3,4-dimethoxycinnamaldehyde, 17.53; 2,4-dimethoxybenzaldehyde, 18.11; cinnamic acid, 18.60; *p*-methoxycinnamic acid, 19.09; *o*-methoxycinnamic acid, 19.56; fagaramide, 20.87; piperinic acid isobutylamide, 22.72; piperin, 24.07; curcumin, 24.24.

RESULTS AND DISCUSSION

With the reversed-phase system used, good separation with narrow (high plate number) and symmetrical peaks are obtained (Figs. 1–3). This demonstrates that the eluent, which contains formic acid, is sufficiently acidic to suppress the dissociation of the acids under investigation. This is certainly not the case with eluents containing acetic acid, as can be seen from recent papers by Villeneuve *et al.*[12] and Nagels *et al.*[20].

Table I shows the t_R values of 29 benzoic acid derivatives. Some of these are naturally occurring in the free state and all have been recognized as products of the chemical degradation of more complex natural substances. Most of the isomers are also well separated (*e.g.* out of six isomeric dimethoxybenzoic acids only two (2,4- and 2,6-dimethoxybenzoic acids) form a "critical pair") but different series of isomers are overlapping. It is therefore not possible to deduce the degree of substitution from the t_R values only. However, substitution patterns can easily be determined by MS; in this respect the new HPLC–MS combination promises to become very useful.

In Table II, t_R values of eleven phenylacetic acids are compiled. Some of these

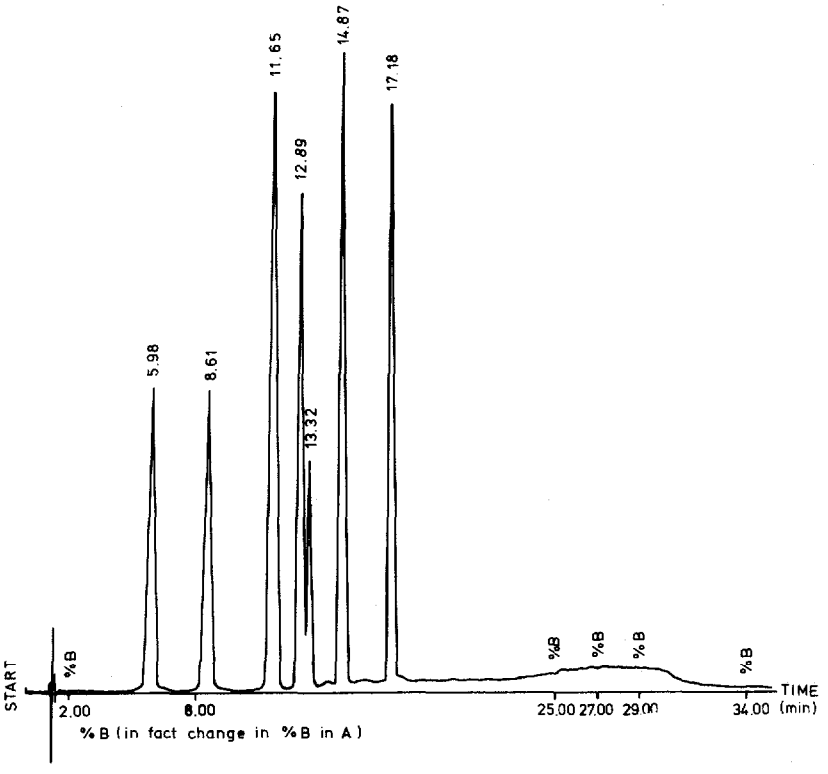

Fig. 2. Separation of quinic acid esters of phenolic benzoic and cinnamic acid derivatives on a prepacked column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm). For the eluting system see Experimental section. Retention times (min): 3-O-*p*-benzoyl-D-quinic acid, 5.98; chlorogenic acid, 8.61; 3-O-*p*-coumaroyl-D-quinic acid, 11.65; 3-O-feruloyl-D-quinic acid, 12.89; 3-O-sinapoyl-D-quinic acid, 13.32; 3-O-*o*-coumaroyl-D-quinic acid, 14.87; 3-O-cinnamoyl-D-quinic acid, 17.18.

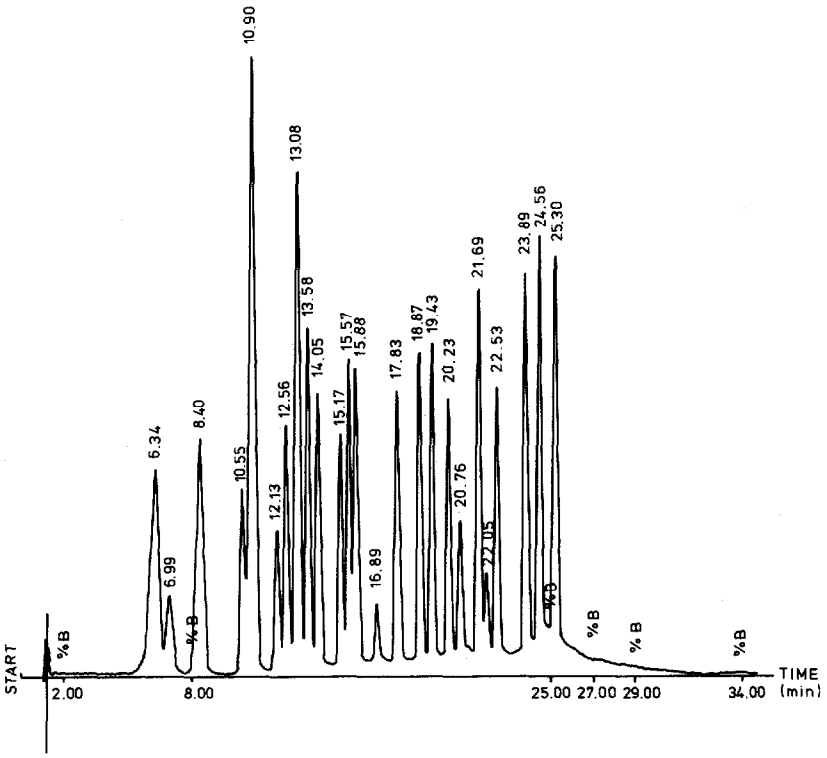

Fig. 3. Separation of coumarins, furanocoumarins and derivatives on a Knauer prepacked column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm). For the eluting system see Experimental section. Retention times: esculin, 6.34; 3,5,7-trihydroxycoumarin, 6.99; esculetin, 8.40; daphnetin, 10.55; 6-hydroxycoumarin, 10.90; fraxetin, 12.13; umbelliferon, 12.56; 8-hydroxycoumarin, 13.08; scopoletin, 13.58; 4-methyldaphnetin + coumarin-3-carboxylic acid, 14.05; 8-methoxycoumarin-3-carboxylic acid, 15.17; 4-methylumbelliferon, 15.57; coumarin + esculetindimethylether + 8-methoxycoumarin, 15 88; 4-hydroxycoumarin, 16.89; herniarin, 17.83; psoralen + xanthotoxin, 18.87; sphondin, 19.43; isopimpinellin, 20.23; bergapten, 20.76; osthenol, 21.69; 4-methyllimettin, 22.05; 4,6-dimethylherniarin, 22.53; imperatorin, 23.89; ostruthol, 24.56; osthol, 25.30.

acids are fairly widely distributed in the plant[3,26] and animal[27] world. Their biosynthesis from aromatic amino acids appears to involve catabolic processes. In addition, certain of these compounds show plant growth regulatory activity[28,29] while others occur in urine from humans suffering from alkaptonuria[30]. In the field of structure elucidation they are important because they have been recognized to arise during the alkaline degradation of isoflavonoids[31]. With the exception of phenylacetic acid itself (this compound has to be detected at 260 nm; in fact, 254 nm would be ideal but the gradient employed does not allow detection below 260 nm) all other derivatives represented in Table II can be detected at 280 nm.

Table III shows t_R values of 26 cinnamic acid derivatives. Cinnamic acids are widespread in nature[5,26,32,33]. They occur in both the free and bound forms[5,6,34]. Normally the *trans* form is present[35] but in UV light *trans–cis* isomerization can occur and it is now accepted that in plants such a reaction comprises the final step in the formation of bound coumarin[36]. Under the conditions used, *trans* and *cis* isomers

TABLE I

t_R VALUES OF BENZOIC ACID AND DERIVATIVES

Systematic name	*Trivial name*	t_R *(min)*
3,4,5-Trihydroxybenzoic acid*	Gallic acid	1.75
3,5-Dihydroxybenzoic acid	α-Resorcylic acid	3.00
3,4-Dihydroxybenzoic acid	Protocatechuic acid	3.21
2,3,4-Trihydroxybenzoic acid	Pyrogallolcarboxylic acid	3.84
2,4,6-Trihydroxybenzoic acid	Phloroglucinolcarboxylic acid	4.02
4-Hydroxybenzoic acid	*p*-Hydroxybenzoic acid	5.53
2,5-Dihydroxybenzoic acid	Gentisic acid	5.70
2,6-Dihydroxybenzoic acid	γ-Resorcylic acid	7.30
2,4-Dihydroxybenzoic acid	β-Resorcylic acid	7.31
3-Hydroxybenzoic acid	*m*-Hydroxybenzoic acid	7.77
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	8.53
2,3-Dihydroxybenzoic acid	–	9.25
3-Hydroxy-4-methoxybenzoic acid	Isovanillic acid	10.00
3,5-Dimethoxy-4-hydroxybenzoic acid	Syringic acid	10.82
2,6-Dimethoxybenzoic acid	–	12.27
2-Methoxybenzoic acid	*o*-Methoxybenzoic acid	13.29
2,4,5-Trimethoxybenzoic acid	Asaric acid	13.85
3,4-Dimethoxybenzoic acid	Veratric acid	14.38
3,4-Methylenedioxybenzoic acid	Piperonylic acid	14.60
2,3-Dimethoxybenzoic acid	–	14.75
2,5-Dimethoxybenzoic acid	–	14.79
Benzenecarboxylic acid	Benzoic acid	14.92
2,4-Dimethoxybenzoic acid	–	15.47
2-Hydroxy-3-methoxybenzoic acid	*o*-Vanillic acid	15.47
2-Hydroxybenzoic acid	Salicylic acid	15.81
3,4,5-Trimethoxybenzoic acid	Endesmic acid	16.05
3-Methoxybenzoic acid	–	16.13
4-Methoxybenzoic acid	Anisic acid	16.20
3,5-Dimethoxybenzoic acid	–	18.07

* Benzoic acid = benzenecarboxylic acid.

TABLE II

t_R VALUES OF PHENYLACETIC ACIDS

Systematic name	*Trivial name*	t_R *(min)*
2,5-Dihydroxyphenylacetic acid*	Homogentisic acid	2.42
3,4-Dihydroxyphenylacetic acid	Homoprotocatechuic acid	4.28
2,5-Dihydroxyphenylacetic acid lactone	Homogentisic acid lactone	6.95
4-Hydroxyphenylacetic acid	–	7.06
3-Hydroxyphenylacetic acid	–	8.23
2-Hydroxyphenylacetic acid	–	9.14
4-Hydroxy-3-methoxyphenylacetic acid	Homovanillic acid	9.87
3,4-Dimethoxyphenylacetic acid	3,4-Dimethylhomoprotocatechuic acid	14.36
4-Methoxyphenylacetic acid	–	15.27
3-Methoxyphenylacetic acid	–	15.41
2-Methoxyphenylacetic acid	–	15.81

* Phenylacetic acid = 2-benzeneethanoic acid.

TABLE III

t_R VALUES OF CINNAMIC ACIDS AND DERIVATIVES

Systematic name	*Trivial name*	t_R *(min)*
4-β-Glucosyloxycinnamic* acid	*p*-Coumaric acid β-glucoside	6.20
3,4-Dihydroxycinnamic acid	Caffeic acid	8.33
4-β-Glucosyloxy-3-methoxycinnamic acid	Ferulic acid β-glucoside	8.68
4-Hydroxy-3-methoxycinnamoylamide	Feruloylamide	10.20
4-Hydroxycinnamic acid	*p*-Coumaric acid	11.99
2-β-D-Glucosyloxycinnamic acid	*o*-Coumaric acid β-glucoside	12.65
4-Hydroxy-3-methoxycinnamic acid	Ferulic acid	13.69
3-Hydroxycinnamic acid	*m*-Coumaric acid	14.00
3,5-Dimethoxy-4-hydroxycinnamic acid	Sinapic acid	14.30
3-Hydroxy-4-methoxycinnamic acid	Isoferulic acid	14.68
2-Hydroxy-cinnamic acid	*o*-Coumaric acid	15.40
3,4-Dimethoxycinnamic acid	Dimethylcaffeic acid	17.14
2,4,5-Trimethoxycinnamic acid	–	17.72
3,4,5-Trimethoxycinnamic acid	–	17.80
4-Hydroxycinnamoylisobutylamide	*p*-Coumaric acid isobutylamide	17.89
Cinnamic acid	–	18.81
2,3-Dimethoxycinnamic acid	–	18.90
4-Hydroxycinnamic acid piperide	*p*-Coumaric acid piperide	18.99
4-Methoxycinnamic acid	Methyl-*p*-coumaric acid	19.18
3-Methoxycinnamic acid	Methyl-*m*-coumaric acid	19.45
2-Methoxycinnamic acid	Methyl-*o*-coumaric acid	19.67
2,4-Dimethoxycinnamic acid	–	19.97
3,5-Dimethoxycinnamic acid	–	20.14
3,4-Methylenedioxycinnamic acid isobutylamide	Fagaramide	20.91
4-Methylcinnamic acid	*p*-Methylcinnamic acid	21.53
3,4-Methylenedioxycinnamic acid piperidide	–	22.22

* Cinnamic acid = 3-phenyl-2-propenoic acid.

TABLE IV

t_R VALUES OF QUINIC ACID ESTERS OF PHENOLIC BENZOIC AND CINNAMIC ACID DERIVATIVES

Systematic name	*Trivial name*	t_R *(min)*
3-O-(4-Hydroxybenzoyl)-D-quinic acid*	*p*-Hydroxybenzoyl-D-quinic acid	5.98
3-O-(3,4-Dihydroxycinnamoyl)-D-quinic acid	Chlorogenic acid	8.61
1,3-Bis-O-(3,4-dihydroxycinnamoyl)-D-quinic acid	Cynarin	11.29
3-O-(4-Hydroxycinnamoyl)-D-quinic acid	3-O-*p*-Coumaroyl-D-quinic acid	11.65
3-O-(4-Hydroxy-3-methoxycinnamoyl)-D-quinic acid	3-O-Feruloyl-D-quinic acid	12.89
3-O-(4-Hydroxy-3,5-dimethoxycinnamoyl)-D-quinic acid	3-O-Sinapoyl-D-quinic acid	13.32
4-O-(2-Hydroxycinnamoyl)-D-quinic acid	4-O-*o*-Coumaroyl-D-quinic acid	13.49
3-O-(2-Hydroxycinnamoyl)-D-quinic acid	3-O-*o*-Coumaroyl-D-quinic acid	14.87
3-O-Cinnamoyl-D-quinic acid	–	17.18

* Quinic acid = D-(−)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid.

TABLE V

t_R VALUES OF COUMARINS, FURANOCOUMARINS AND DERIVATIVES

Systematic name	*Trivial name*	t_R *(min)*	*Source reference*
6-Hydroxy-7-glucosyloxycoumarin*	Cichoriin	6.04	W. Steck
7-Hydroxy-6-glucosyloxycoumarin	Esculin	6.34	Fluka
3,5,7-Trihydroxycoumarin	–	6.99	W. Steck
6,7-Dihydroxycoumarin	Esculetin	8.39	Fluka
7,8-Dihydroxycoumarin	Daphnetin	10.55	Synthesized
6-Hydroxycoumarin	–	10.90	R. T. Williams
7,8-Dihydroxy-6-methoxycoumarin	Fraxetin	12.13	Geiger
6,7-Dihydroxy-4-methylcoumarin	4-Methylesculetin	12.26	W. Steck
7-Hydroxycoumarin-3-carboxylic acid	–	12.43	Baltzly
7-Hydroxycoumarin	Umbelliferon	12.56	Fluka
8-Hydroxycoumarin	–	13.08	R. T. Williams
7-Hydroxy-6-methoxycoumarin	Scopoletin	13.58	Fluka
7,8-Dihydroxy-4-methylcoumarin	4-Methyldaphnetin	14.05	Aldrich
Coumarin-3-carboxylic acid	–	14.05	Aldrich
8-Methoxycoumarin-3-carboxylic acid	–	15.17	W. Steck
7-Hydroxy-4-methylcoumarin	4-Methylumbelliferon	15.57	W. Steck
Coumarin	Coumarin	15.88	Fluka
6,7-Dimethoxycoumarin	Esculetin dimethyl ether	15.88	W. Steck
8-Methoxycoumarin	–	15.88	W. Steck
4-Hydroxycoumarin	–	16.89	R. T. Williams
7-Hydroxy-8-allylcoumarin	8-Allylumbelliferon	17.35	W. Steck

7-Methoxycoumarin	Herniarin	17.83	K and K
3-Hydroxycoumarin	–	18.12	R. T. Williams
7-Hydroxy-4,5-dimethylcoumarin	4,5-Dimethylumbelliferon	18.79	W. Steck
6,7-Furanocoumarin	Psoralen	18.87	W. Steck
8-Methoxy-6,7-furanocoumarin	Xanthotoxin	18.87	W. Steck
7-Hydroxy-4,6-dimethylcoumarin	4,6-Dimethylumbelliferon	19.09	W. Steck
6,7-Dimethoxycoumarin-3-carboxylic acid	Esculetin dimethyl ether 3-carboxylic acid	19.30	Baltzly
6-Methoxy-7,8-furanocoumarin	Sphondin	19.34	W. Steck
6-Methylcoumarin	–	19.34	Light
4-Methylcoumarin	–	19.68	Light
5-Hydroxy-4-methylcoumarin-6-carboxylic acid	–	20.10	Aldrich
5,8-Dimethoxy-6,7-furanocoumarin	Isopimpinellin	20.23	W. Steck
5,7-Dimethoxycoumarin	Limettin	20.31	W. Steck
8-Methyl-7-methoxycoumarin	8-Methylherniarin	20.39	W. Steck
5-Methoxy-6,7-furanocoumarin	Bergapten	20.76	W. Steck
7-Hydroxy-8-isopent-2-enylcoumarin	Osthenol	21.69	W. Steck
4-Methyl-5,7-dimethoxycoumarin	4-Methyllimettin	22.05	W. Steck
7-Propoxycoumarin-3-carboxylic acid	–	22.05	Baltzly
7-Methoxy-4,6-dimethylcoumarin	4,6-Dimethylherniarin	22.53	W. Steck
8-Isopent-2-enyloxy-6,7-furanocoumarin	Imperatorin	23.89	Fluka
5-[3-Hydroxy-3-methyl-(2-methyl-2-butenoyloxy)-butyloxy]-6,7-furanocoumarin	Ostruthol	24.56	W. Steck
7-Methoxy-8-isopent-2-enylcoumarin	Osthol	25.30	W. Steck

* Coumarin = benzo-α-pyrone.

TABLE VI

t_R VALUES OF DIHYDROCINNAMIC ACIDS

Systematic name	*Trivial name*	t_R *(min)*
3-(3,4-Dihydroxyphenyl)propionic acid*	Dihydrocaffeic acid	7.02
3-(4-Hydroxyphenyl)propionic acid	Dihydro-*p*-coumaric acid	10.79
3-(2-β-D-Glucosyloxyphenyl)propionic acid	Melilotic acid β-glucoside	11.48
3-(4-Hydroxy-3-methoxyphenyl)propionic acid	Dihydroferulic acid	12.76
3-(3,5-Dimethoxy-4-hydroxyphenyl)propionic acid	Dihydrosinapic acid	13.69
3-(2-Hydroxyphenyl)propionic acid	Melilotic acid	14.39
3-(4-Methoxyphenyl)propionic acid	–	17.95
3-(2-Methoxyphenyl)propionic acid	–	19.18

* Propionic acid = propanoic acid.

TABLE VII

t_R VALUES OF BENZALDEHYDES AND ACETOPHENONES

Systematic name	*Trivial name*	t_R *(min)*
3,4-Dihydroxybenzaldehyde	Protocatechuylaldehyde	5.24
2,5-Dihydroxybenzaldehyde	Gentisic aldehyde	7.56
2,4,6-Trihydroxybenzaldehyde	–	7.73
4-Hydroxybenzaldehyde	*p*-Hydroxybenzaldehyde	8.11
3-Hydroxybenzaldehyde	*m*-Hydroxybenzaldehyde	9.65
2,4-Dihydroxybenzaldehyde	β-Resorcylaldehyde	9.91
4-Hydroxy-3-methoxybenzaldehyde	Vanilline	11.35
3-Hydroxy-4-methoxybenzaldehyde	Isovanilline	11.42
2,4,6-Trihydroxyacetophenone*	Phloracetophenone	11.81
2,4-Dihydroxyacetophenone	Resacetophenone	12.73
3,5-Dimethoxy-4-hydroxybenzaldehyde	Syringaldehyde	12.83
Benzaldehyde	–	14.04
3,4-Methylenedioxybenzaldehyde	Piperonal	14.54
2-Hydroxybenzaldehyde	Salicylaldehyde	14.99
3,4-Dimethoxybenzaldehyde	–	15.21
3,4,5-Trimethoxybenzaldehyde	–	16.77
4-Methoxybenzaldehyde	Anisaldehyde	16.77
2-Methoxybenzaldehyde	*o*-Anisaldehyde	16.97
2,6-Dihydroxy-4-methoxyacetophenone	–	17.15
2,4-Dihydroxy-6-methoxyacetophenone	–	17.20
2,3-Dimethoxybenzaldehyde	–	17.33
2,4-Dimethoxybenzaldehyde	–	18.21
2,5-Dimethoxybenzaldehyde	–	18.57
3,5-Dimethoxybenzaldehyde	–	18.97
2,4-Dimethoxy-6-hydroxyacetophenone	Xanthoxylin	21.50

* Acetophenone = methyl phenyl ketone.

of *p*-coumaric and ferulic acids could not be separated and most probably the same holds true for most of the isomers investigated. However, the system can be adapted to the separation of *cis* and *trans* cinnamic acids. In addition, other HPLC systems are available which allow separation of the *cis–trans* isomers[37,38]; GLC can also be employed for this purpose[39].

In Table IV, the t_R values of nine quinic acid esters of benzoic and cinnamic acids are shown. These compounds, especially chlorogenic acid (for which more information than for the related compounds of Table IV is available) are widely distributed throughout the plant kingdom. Chlorogenic acid has also been associated with the resistance of plants against disease[40], although, according to Kùc[41], the levels of chlorogenic and caffeic acids cannot necessarily account for all the inhibitory

TABLE VIII

t_R VALUES OF MISCELLANEOUS PHENOLICS

Systematic name	*Trivial name*	t_R *(min)*
1-O-β-D-Glucosyloxy-4-hydroxybenzene	Arbutin	1.33
3,4-Dihydroxymandelic acid*	–	1.33
1,3,5-Trihydroxybenzene	Phloroglucinol	1.53
1,4-Dihydroxybenzene	Hydroquinone	1.94
1,2,3-Trihydroxybenzene	Pyrogallol	2.06
4-Hydroxy-3-methoxymandelic acid	Vanillyl mandelic acid	2.26
3-Hydroxymandelic acid	–	2.45
1,3-Dihydroxybenzene	Resorcinol	3.07
1,2-Dihydroxybenzene	Pyrocatechol	4.44
1-β-D-Glucosyloxy-2-hydroxymethylbenzene	Salicin	5.47
1,3-Dihydroxy-5-methylbenzene	Orcinol	6.65
2-Methoxymandelic acid	–	7.60
4-Methoxymandelic acid	–	7.63
3-Methoxymandelic acid	–	8.64
3-(4-D-Glucosyloxy-3-methoxyphenyl)-allyl alcohol	Coniferin	8.92
Hydroxybenzene	Phenol	9.65
2-(4-Hydroxy-3-methoxyphenyl)ethanol	Homovanillyl alcohol	9.76
3-(4-Hydroxy-3-methoxyphenyl)allyl alcohol	Coniferyl alcohol	11.35
4-Hydroxyphenylpyruvic acid**	–	13.57
3-(4-Hydroxy-3-methoxyphenyl)acrolein***	Coniferylaldehyde	14.81
3,4-Dimethoxycinnamaldehyde §	–	17.65
1-Allyl-4-hydroxy-3-methoxybenzene	Eugenol	20.71
5-(3,4-Methylenedioxyphenyl)-2,4-pentadienoic acid	Piperinic acid	21.30
5-(3,4-Methylenedioxyphenyl)-2,4-pentadienoic acid isobutylamide	Piperinic acid-isobutylamide	22.86
5-(3,4-Methylenedioxyphenyl)-2,4-pentadienoic acid piperidide	Piperin	24.21
1,7-bis-(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione	Curcumin	24.51
β-phenylcinnamaldehyde	–	24.99

* Mandelic acid = 2-benzene-2-hydroxyethanoic acid.
** Pyruvic acid = 2-oxopropenoic acid.
*** Acrolein = propenal.
§ Cinnamaldehyde = 3-benzene-2-propenal.

activity against a range of fungi acting on potato tuber tissues. In addition, cinnamic acids and their quinic acid esters may also produce quite considerable effects in a given ecosystem[42].

The reversed-phase HPLC separation of some quinic acid esters has already been published[20]. However, the system proposed in the present paper is superior to the foregoing system, *e.g.* feruloyl- and sinapoyl-D-quinic acid, which were not resolved by Nagels and co-workers, are now separated (Fig. 2).

In Table V the t_R values of 43 coumarins and furanocoumarins are compiled and Fig. 3 shows the separation of 25 of these compounds. Several of these coumarins are naturally occurring and some show physiological and/or toxic effects[36,40,43]. The compounds listed in Tables VI (eight dihydrocinnamic acids) and VII (25 benzaldehydes and acetophenones) are not only naturally occurring substances but also important products of the chemical degradations of more complex natural compounds, especially flavonoids[32,44] or, as in the case of 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (isovanillin) and 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde), lignins[45].

Finally, Table VIII shows the t_R values of 27 miscellaneous compounds related to those shown in Tables 1–7. These additional t_R values are useful in interpreting chromatograms of extracts or mixtures containing these substances. Also, some of the compounds studied (*e.g.* coniferyl alcohol and coniferin) are interesting precursors of lignin[46] while the more simple phenolics and their glycosides (catechol, hydroquinone, arbutin, *etc.*[4,40,47,48]) may play a role in the resistance of plants against disease.

CONCLUSIONS

As demonstrated by Figs. 1–3 and refs. 1 and 2 the described reversed-phase HPLC system is well suited for the separation of various types of phenolic compounds, which occupy nearly the whole range of the gradient. As may be expected, several substances show identical or nearly identical t_R values. This should not necessarily be a limiting factor: indeed, during structural determinations, one is normally dealing with a rather limited number of compounds and, in addition, HPLC–MS combinations permit the proper identification of any of the substances studied.

However, when complex mixtures of natural origin are qualitatively or quantitatively analysed it may often be necessary to use a complementary HPLC technique (see ref. 1). Nevertheless, the reversed-phase HPLC system described is certainly one of the best available. It also allows the elimination of the bulk of interfering (and possibly interfering) highly polar materials (salts and sugars) and in this way it renders the collected fractions suitable for analysis using additional techniques.

ACKNOWLEDGEMENTS

We are greatly indebted to all those who generously furnished us with samples. H.G. thanks the "Fonds der Chemischen Industrie" for financial support and C.V.S. is grateful for a grant from the Belgian "Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw". The technical assistance of Mrs. G. Persoon-Saey, Mrs. H. Goetgeluck-Vermeulen and Mr. W. Hutsebaut is gratefully acknowledged.

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