THIN LAYER CHROMATOGRAPHY OF CHLOROGENIC ACID ISOMERS

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In studies on the chemical composition of plants, chlorogenic acid is encountered with increasing frequency in nearly all plants. Although known for over 60 years[1], it is only recently that the structure of its three isomers—cryptochlorogenic, neochlorogenic and isochlorogenic acid—has been determined.

Chlorogenic acid is 3-caffeoylquinic acid[2]; cryptochlorogenic acid (known also as Band 510) is 4-caffeoylquinic acid[3]; and neochlorogenic acid is 5-caffeoylquinic acid[3]. The acid known until recently as isochlorogenic acid has proved to be a mixture of three dicaffeoylquinic acids[4].

These compounds take part in various biochemical processes, influence the growth of plants, their enzymes and defence mechanisms. In this study, trials were

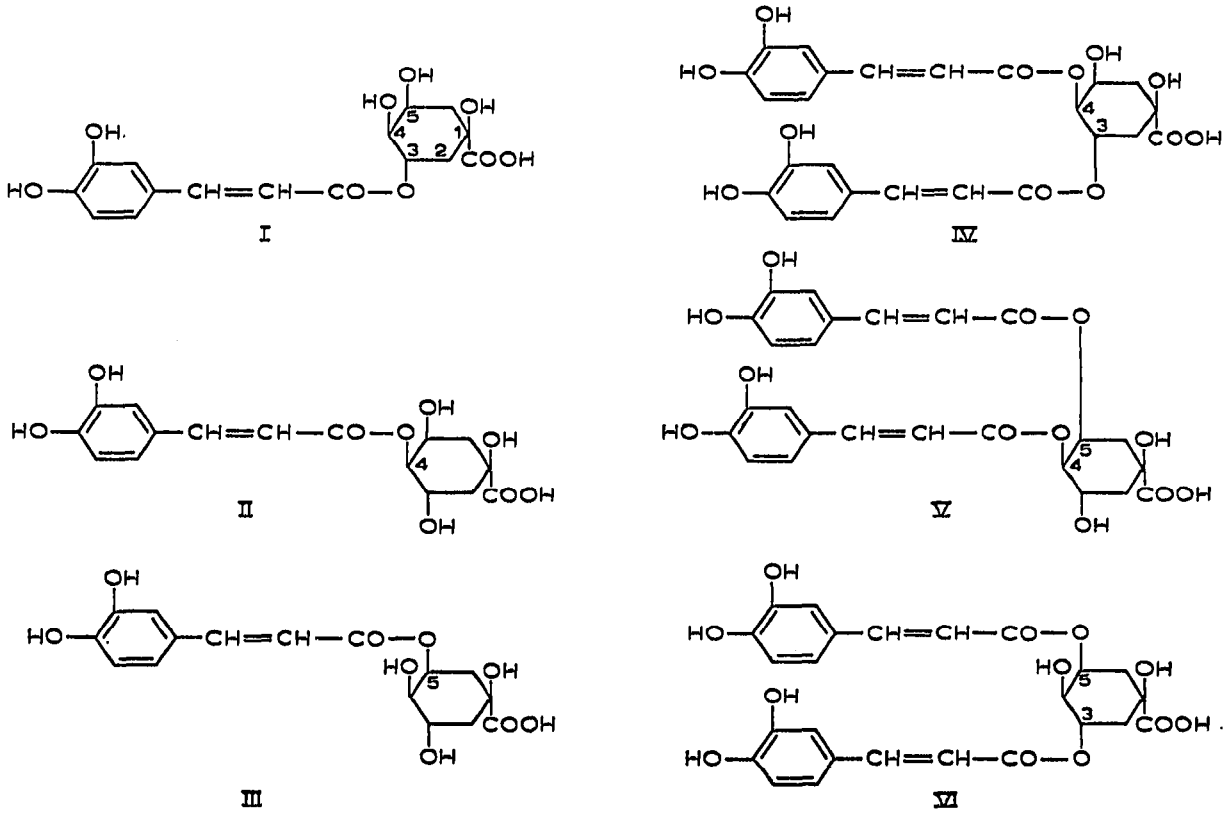

Fig. 1. Isomers of chlorogenic and isochlorogenic acid. I = Chlorogenic acid (3-caffeoylquinic acid); II = cryptochlorogenic acid (4-caffeoylquinic acid); III = neochlorogenic acid (5-caffeoylquinic acid); IV = 3,4-dicaffeoylquinic acid; V = 4,5-dicaffeoylquinic acid; VI = 3,5-dicaffeoylquinic acid.

undertaken to develop a rapid and convenient method of identifying these compounds. SCARPATI[5], in the course of studies on the structure of these compounds, employed thin layer chromatography and separated the isochlorogenic acids, but separation of chlorogenic and cryptochlorogenic acid gave unsatisfactory results. The main difficulty in the chromatographic separation of these isomers lies in the fact that, in various systems, neochlorogenic acid has an R_F value different from that of chlorogenic and cryptochlorogenic acid, while the two latter have very similar R_F values.

Attempts to separate chlorogenic acid from cryptochlorogenic and from neochlorogenic acid by means of thin layer chromatography using various solvent systems and a search for a specific colour reaction are reported here.

MATERIALS AND METHODS

Standard substances

Chlorogenic, cryptochlorogenic, neochlorogenic and isochlorogenic acids, received from Dr. E. SONDHEIMER, U.S.A., were used as standard substances in the experiments.

Adsorbent

Silica Gel G (Merck Co.) was used as adsorbent.

Preparation of plates

Three types of separation were carried out:

(1) Separation on ordinary gel using solvents containing CH_3COOH.

(2) Separation on gel impregnated with $KHSO_4$.

(3) Separation on gel impregnated with $KHSO_4$ and acidified with HCl vapour according to the method described by SCARPATI[5].

Thus, three types of plates were prepared, *viz.*: (1) plates 7 × 17 cm covered with 2 g of gel suspended in 4 ml H_2O; (2) plates 7 × 17 cm covered with 2 g of gel suspended in 5.6 ml of 2.5 % $KHSO_4$ solution; (3) plates 7 × 17 cm covered with 2 g of gel suspended in 5.6 ml of 2.5 % $KHSO_4$ solution and exposed to HCl vapour for 5 min.

Solvent systems

Of the numerous solvents that were investigated, forty, which proved of greatest interest, are listed in Tables I–III.

Colour reactions

(I) Chlorogenic acids

These are yellow at pH values above 7 and in air the colour turns brown[6]. The plates were therefore either exposed to NH_3 vapour or sprayed with 2 *N* KOH dissolved in CH_3OH.

(II) Reaction for phenols

Because of the phenolic character of the caffeic acid residue, chlorogenic acids give a colour reaction with reagents used to detect phenols, *viz.*:

(1) $FeCl_3$. Grey-green spots appear on plates sprayed with 2 % aqueous $FeCl_3$ solution.

(2) *Turnbull's blue reaction*[7]. After spraying the plates with 3 % $FeCl_3$, followed by 3 % $K_3[Fe(CN)_6]$, blue spots appear on a pale-blue background.

(3) *$KMnO_4$*[8]. After spraying the plates with 1 % $KMnO_4$ in 0.1 *N* H_2SO_4, yellow spots appear on a violet background.

(4) *Diazo-reaction*. Diazo-reaction is carried out with:

(a) Diazotized *p*-nitroaniline[8]. 5 ml of *p*-nitroaniline (0.5 % in 2 *N* HCl) is mixed with 0.5 ml of $NaNO_2$ (5 %), and 15 ml of sodium acetate (20 %) is then added. Spraying the plates with this mixture produces brown spots.

(b) Tetrazotized benzidine[7]. 1 g of benzidine is dissolved in 3 ml conc. HCl and diluted with H_2O to 200 ml. A 10 % aqueous solution of $NaNO_2$ is prepared separately. Before use, equal volumes of the two solutions are mixed. Spraying give rise to brown spots.

(c) Stabilized diazo salts[9]. An 0.05 % aqueous solution of the stabilized salts is used and coloured spots appear on sprayed plates.

(*III*) *Reactions for o-dihydroxyphenols*

(1) *Sodium molybdate*[10]. *o*-Dihydroxyphenols form complex red compounds with Mo. Orange-brown spots appear on plates sprayed with an 0.1 *M* aqueous solution of sodium molybdate.

(2) *Arnow's reaction*[11]. Arnow's reagent is prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water. After spraying the plates with this reagent orange-brown spots appear.

(3) *Phloroglucinol*[12]. On plates sprayed with 0.1 % solution of phloroglucinol in 1 *N* NaOH, *o*-dihydroxyphenols of the caffeic acid type give yellow spots which turn brown after heating at 80°.

RESULTS

The results of the experiments on the behaviour of chlorogenic acid, cryptochlorogenic acid and neochlorogenic acid under various condition of thin layer chromatography are summarized in Tables I, II and III.

The solvent systems used to develop the plates with ordinary gel are shown in Table I. The starting point of these experiments was the classic system of PARTRIDGE (*n*-butanol–glacial acetic acid–water, 4:1:5), in which these acids were only slightly separated. By increasing the amount of *n*-butanol in relation to water, and by increasing the acid in the organic phase, the following system was obtained: *n*-butanol–acetic acid–water (10:1.75:8). The organic phase was used, 4 ml of acetic acid per 100 ml of *n*-butanol being added. With this solvent system, the different isomers of chlorogenic acid gave markedly different R_F-values. However, in view of the long development time (140 min) and diffusion and superimposition of the spots, it was not considered entirely satisfactory.

The results of the separation of chlorogenic acid isomers on plates impregnated with $KHSO_4$ but not acidified with HCl are summarized in Table II. The best solvent system in this group was ethyl ether–acetic acid–water (50:12:50) (organic phase). Time of development was one hour. Differences between the R_F values were satisfactory, but the spots were somewhat diffuse and superimposed.

Table III lists the R_F values of chlorogenic acid isomers obtained on plates impregnated with $KHSO_4$ and acidified with HCl vapour. A system composed of aceto-

TABLE I

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with ordinary gel)

No.	Solvent	Time* needed to reach 13 cm	R_F values of acid			Remarks
			Neo.	Chlor.	Crypt.	
1	Water	30	—	—	—	Trails from the start to solvent front
2	*n*-Butanol	180	—	—	—	Spots at the start
3	Acetic acid, glacial	80	0.77	0.54	0.53	Similar R_F values of chlor. and crypt.
4	Acetic acid, 80%	100	0.72	0.67	0.61	Spots partly superimposed
5	Butanol–acetic acid–water (4:1:5)	150	0.49	0.44	0.39	Spots partly superimposed
6	Butanol–acetic acid–water (5:1.75:8)	130	0.55	0.55	0.55	Compact spots, but identical R_F
7	Butanol–acetic acid–water (6:1.75:8)	140	0.50	0.47	0.46	Poor separation
8	Butanol–acetic acid–water (8:1.75:8)	150	0.55	0.53	0.42	Better separation, spots superimposed
9	Butanol–acetic acid–water (10:1.75:8)	160	0.43	0.39	0.33	Better separation, spots diffuse
10	Butanol–acetic acid–water (15:1.75:8)	190	0.38	0.31	0.28	Worse separation
11	Butanol–acetic acid–water (20:1.75:8)	210	0.36	0.30	0.27	Worse separation
12	Butanol–acetic acid–water (10:1.75:8) + 1 ml**	140	0.45	0.38	0.32	Spots partly superimposed
13	Butanol–acetic acid–water (10:1.75:8) + 2 ml**	140	0.41	0.32	0.29	Better separation
14	Butanol–acetic acid–water (10:1.75:8) + 4 ml**	140	0.49	0.40	0.32	Best separation
15	Butanol–acetic acid–water (10:1.75:8) + 10 ml**	130	0.50	0.46	0.42	Worse separation

* Time in minutes.

** Systems 12, 13, 14 and 15 were prepared by shaking 100 ml of *n*-butanol with 1.75 ml of glacial acetic acid and 80 ml of water. After 24 h the layers were separated and 1, 2, 4 or 10 ml of acetic acid was added to the butanolic layers.

TABLE II

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with gel impregnated with $KHSO_4$, not acidified with HCl)

No.	Solvent	Time* needed to reach 13 cm	R_F values of acid			Remarks
			Neo.	Chlor.	Crypt.	
1	Ethyl ether–glacial acetic acid (50:5)	30	0.09	0.15	0.17	Compact spots, but poor separation
2	Ethyl ether–glacial acetic acid (50:10)	50	0.49	0.58	0.69	Spots diffuse
3	Ethyl ether–acetic acid–water (50:4:50) (organic layer)	60	0.03	0.07	0.06	Compact spots, chlor. and crypt. not separated
4	Ethyl ether–acetic acid–water (50:12:50)	60	0.36	0.51	0.57	Good separation, but spots superimposed
5	Ethyl ether–acetic acid–water (50:15:50)	60	0.51	0.58	0.61	Spots superimposed partly

* Time in minutes.

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TABLE III

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with gel impregnated with $KHSO_4$, acidified with HCl)

No.	*Solvent*	*Time** (*to reach 13 cm*)	*R_F values of acid*			*Remarks*
			Neo.	*Chlor.*	*Crypt.*	
1	Non-polar solvents: *e.g.* cyclohexane, benzene	different	—	—	—	At the start
2	Ethyl ether	15	0.00	0.03	0.02	Compact spots, but poor separation
3	Polar solvents: *e.g.* methanol, ethanol, acetone	60	—	—	—	Spots diffuse, in solvent front
4	Water	30	—	—	—	Trails from start to solvent front
5	*n*-Propanol	80	0.41	0.55	0.53	Compact spots, but poor separation
6	Ethyl acetate	30	0.20	0.32	0.34	Chlor. and crypto. not separated
7	Ethyl formate	40	0.27	0.46	0.49	Chlor. and crypto. not separated
8	Dioxan	40	0.44	0.54	0.55	Chlor. and crypto. not separated
9	Methyl ethyl ketone	60	0.84	0.87	0.90	Compact spots, but poor separation
10	Acetophenone	60	0.39	0.47	0.54	Very good separation but spots diffuse
11	Acetylacetone	100	0.32	0.48	0.48	Chlor. and crypto. not separated
12	Acetic acid 80%	70	0.80	0.80	0.80	
13	Ethyl ether–dioxan (1:1)	25	0.29	0.34	0.34	As in dioxan
14	Ethyl ether–ethyl acetate (1:9)	20	0.18	0.28	0.30	As in ethyl acetate
15	Ethyl acetate–acetone (8:2)	20	0.19	0.28	0.29	Chlor. and crypto. are superimposed
16	Chloroform–dioxan (7:3)	20	0.02	0.06	0.04	Compact spots, but poor separation
17	Dioxan–ethyl acetate (1:1)	20	0.24	0.35	0.38	Better separation than in ethyl acetate
18	Acetophenone–ethyl ether (1:1)	60	0.16	0.26	0.34	Good separation, but spots are superimposed
19	Acetophenone–ethyl acetate (2:1)	60	0.27	0.37	0.45	Good separation, but spots are superimposed
20	Acetophenone–methyl ethyl ketone (1:1)	60	0.41	0.50	0.58	Excellent separation

* Time in minutes.

phenone–methyl ethyl ketone (1:1) proved excellent for the separation of the chlorogenic acid isomers. The time of development was 1 h, the R_F values of the different acids differed distinctly, and the spots were compact, giving a clear picture of the separation of the studied compounds.

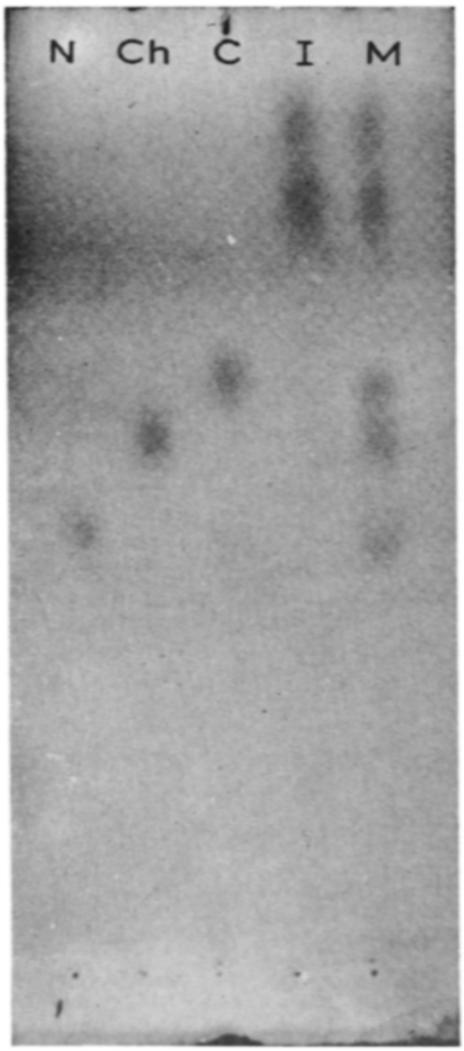

Fig. 2. Thin layer chromatography of chlorogenic acid isomers. Adsorbent: Silica Gel G impregnated with $KHSO_4$. System solvent: acetophenone–methyl ethyl ketone (1:1). Detection: NH_3 vapour. Substances separated: N = neochlorogenic acid; Ch = chlorogenic acid; C = cryptochlorogenic acid; I = isochlorogenic acid; M = mixture of the acids.

Table IV shows the reactions which served to detect the chlorogenic acids on silica gel plates. All the chlorogenic acids gave similar colour reactions with the reagents described above. Arnow's reaction proved to be the best test, giving very distinct orange-brown spots on a white background. In addition, this reaction is highly specific, giving positive reactions only with *o*-dihydroxyphenols. The reactions with ammonia vapour and diazo salts were also useful. The very sensitive but unspecific reactions with $KMnO_4$ and Turnbull's reagent can be used only for detecting plates developed in solvent systems with nonreducing properties.

TABLE IV

COLOUR REACTIONS FOR CHLOROGENIC ACIDS

No.	*Reagent*	*Colour reaction*
1	NH_3 vapour	Brown spots
2	KOH	Brown spots
3	$FeCl_3$	Grey-green spots
4	Turnbull's reagent	Blue spots on a pale-blue background
5	$KMnO_4$	Yellow spots on a violet background
6	Diazotized *p*-nitroaniline	Brown spots
7	Tetrazotized benzidine	Brown spots
8	Fast black salt K	Brown-red spots
9	Sodium molybdate	Orange-brown spots on a white background
10	Arnow's reagent	Orange-brown spots on a white background
11	Phloroglucinol	Yellow spots

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SUMMARY

Chlorogenic, neochlorogenic and cryptochlorogenic acids have been separated by the use of thin layer chromatography. Silica Gel G (Merck) plates impregnated with $KHSO_4$ were used. Forty chromatographic solvent systems used to develop the acids and eleven colour reactions are listed. A solvent system composed of methyl ethyl ketone–acetophenone (1:1) and the colour test with Arnow's reagent proved best.

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