Thin-Layer (Planar) Chromatography

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Introduction

The thin-layer chromatography (TLC) of aliphatic and aromatic acids having a wide range of structures has proved to be of great practical value in the chemistry and biochemistry of this large group of organic compounds. This review of the TLC properties of acids is firstly conveniently divided into a discussion of qualitative aspects of the relative hR_F values of various classes of aliphatic and aromatic carboxylic acids having a wide range of structures on different layers and in different solvent systems. Some mention is made of compounds with other acidic functions. Secondly, there is a selective account of applications on the quantitative determination of acids in typical current synthetic and some natural sources.

Acyclic and Cycloaliphatic Compounds

Alkanoic, Alkanedioic, Hydroxy, Keto, Unsaturated, Arylalkanoic Acids and Other Related Acids of Biological Significance

***n*-Alkanoic acid** The separation of these acids by the technique of TLC with respect to the lower homologous fatty acids has a historic precedent in that their separation in the vapour phase on a column coated with a stationary phase was the first published example of gas chromatography.

Although it might be generally considered that gas chromatography is more suitable than TLC for the separation of alkanoic acids, **Table 1** shows some simple conditions that have been used in this series typical of a partition separation. Many of the values quoted in the ensuing tables have been adapted from extensive published information by Hanai (see Further Reading). For comparison, the hR_F values of the dibasic acids malonic, succinic, glutaric and adipic in solvent a are 9, 14, 18 and 22 respectively, and that of glycolic acid, 38. The first four acids in the table have also been examined on crystalline cellulose impregnated with sodium bicarbonate in ethanol–water (100 : 20) and detection by dicyclohexyl carbodiimide to separate formic acid, acetic, propionic and butanoic acids having the hR_F values 31, 37, 45 and 52 respectively.

***n*-Alkanedioic acids** The saturated dibasic acids have been more widely studied on a variety of layers and solvents, as illustrated in **Table 2** which again, as with the monobasic series, shows partition separations. In cases where a considerable number of solvents have been listed, the optimum conditions for the series of compounds have been given. For comparison, the hR_F value of glycolic acid under the conditions of g was 38. In another separation on silica gel (sil G25, Macherey Nagel) with the solvent *n*-pentyl formate–chloroform–formic acid (70 : 15 : 15) and detection by bromocresol green, nonlinearity was found in that malonic, succinic, glutaric and adipic acids had hR_F values of 40, 43, 54 and 48 respectively. Folic acid, which may be regarded as a 2-acylamino derivative of glutaric acid, had an hR_F value of 0 compared with 78 for nicotinic acid on silica gel G in water as developing solvent.

Hydroxy acids It is convenient to classify this group of saturated acids as monohydroxy, monohydroxy-

Table 1 hR_F values of homologous alkanoic acids on starch and on cellulose layers

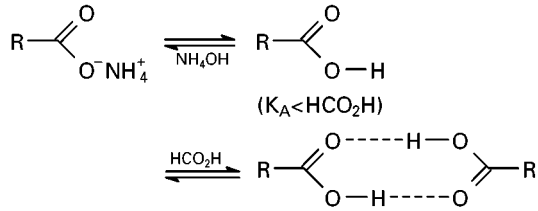
Alkanoic acid	*Conditions*		*Detection*	
	a	*b*	*a*	*b*
Formic	52	8	Fluorescein UV 254 nm	Methyl red
Acetic	56	19	Fluorescein UV 254 nm	Methyl red
Propionic	66	28	Fluorescein UV 254 nm	Methyl red
Butanoic	71	37	Fluorescein UV 254 nm	Methyl red
Pentanoic (valeric)	78	48	Fluorescein UV 254 nm	Methyl red
Hexanoic (caproic)	85	59	Fluorescein UV 254 nm	Methyl red

a, Ethanol–water–concentrated ammonia (78 : 20 : 13), rice starch; b, light petroleum (40–60°C)–acetone (2 : 1) 95% saturated with ethane–1.2-diol, cellulose and Dowex® 50 W. (With acknowledgement to Hanai, 1982.)

Table 2 hR_F values of *n*-alkane-α,ω-dioic acids (dibasic acids) on various layers

Dibasic acid	*Conditions*						
	a	*b*	*c*	*d*	*e*	*f*	*g*
Oxalic (C_2)					16	0	6
Malonic (C_3)	21	52			20	7.5	9
Succinic (C_4)	37	63	38	27	25	59	14
Gultaric (C_5)	46	71	47	32	31	74	18
Adipic (C_6)	55	82	55	37	38	84	22
Pimelic (C_7)					50	94	
Suberic (C_8)					58	100	
Azelaic (C_9)					67		
Sebacic (C_{10})					72		
Undecyl (C_{11})					82		

a, Ethanol–concentrated ammonia–water (150 : 8 : 40), cellulose (Merck 5552); b, 2-ethyl-1-butanol–formic acid–water (40 : 12 : 48); c, diethyl ether–light petroleum–CCl_4–water–formic acid (50 : 20 : 20 : 8 : 1); polyamide 6; d, ethanol–concentrated ammonia–water (100 : 16 : 12), cellulose MN300; e, di-*n*-butyl ether–formic acid–water (65 : 25 : 2.2), cellulose (Merck 5716); f, toluene–propionic acid–water (47 : 47 : 4.9), silica gel (Merck 5721); g, ethanol–concentrated ammonia–water (78 : 13 : 20), rice starch. (With acknowledgement to Hanai, 1982.) The use of formic acid diminishes streaking sometimes found in the TLC of acids in neutral solvents. It is thought that in acidic solvents the formation of a dimeric intermolecularly hydrogen-bonded species is then favoured in the equilibrium with the monomeric form, while in basic solvents the monomeric anion is largely present. Acidic adsorbents may likewise simulate acidic solvents.

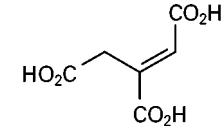


(Modified with permission from Hanai, 1982.)

dibasic, monohydroxytribasic, dihydroxydibasic and polyhydroxy types. **Table 3** lists the hR_F values of a number of acids with this functionality. For comparison, the hR_F value of malonic acid under condition f was 40 and in the aromatic series that of mandelic acid (α-hydroxyphenylacetic acid) was 57. In general, cellulose has been used as adsorbent in examples a to e and silica gel in f. In early work, silica gel G-kieselguhr (1 : 1), kieselguhr impregnated with polyethylene glycol and polyamide layers were also employed. It is possible that in acidic developing solvents certain of these acids are present as intramolecularly hydrogen-bonded structures and that five-membered are likely to be more stable than six-membered rings. Thus glycolic and lactic acids would be expected to have high hR_F values whereas acids having hydrogen-bonded rings and additional acidic groups would have lower values. Under basic conditions with ammonia the solutes are more polar and the polarity of the developing solvent has to be increased by the use of ethanol. The meso and DL forms of tartaric acid show a small difference of hR_F which can be enhanced by the use of silica gel impregnated with boric acid. It is also possible to separate the enantiomers of racemic hydroxy acids by the incorporation of a chiral additive in the adsorbent layer. The role of impregnated layers has been reviewed by Hauck *et al.* (see Further Reading).

Keto acids The hR_F values of a number of mono keto derivatives of monobasic and dibasic acids are given in **Table 4**. The compounds shown from top to bottom in the table are glyoxylic, pyruvic, 2-oxobutanoic, 2-oxovaleric, 2-oxoisocaproic, oxaloacetic and 2-oxoglutaric acid. The need of formic acid in high concentration to effect a separation is illustrated in d compared with f. For comparison, the hR_F values under conditions d of citric and malic acids were 44 and 56 respectively. Intramolecular hydrogen bonding may account for the higher hR_F values of the monobasic compounds. The *cis* and *trans* 2,4-dinitrophenylhydrazones of a range of keto acids have been examined.

Unsaturated monobasic dibasic and polybasic acids The unsaturated acids are a large group which have technical and medicinal uses. The majority are either di- or tribasic. **Table 5** summarizes the hR_F values of a selection of compounds. Extensive details of separations have been described by Hanai and also in early work a limited range of monobasic keto-, hydroxy acids and of dibasic acids was studied. The separation of *cis* and *trans* isomers, for example maleic and fumaric acids, appears to be generally straightforward and free of the requirement for argentation TLC, as in the case of unsaturated fatty acids. The stereochemistry of the glutaconic acid described in Table 5 was not stated. The formulae of (1) *trans*-aconitic acid, (2) itaconic acid, (3) *trans*-glutaconic acid, (4) mesaconic acid (*trans*) and (5) citraconic acid (*cis*) are depicted.



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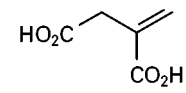

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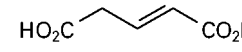

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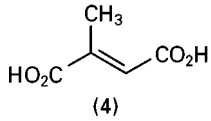
Table 3 hR_F values of hydroxyacids on various layers

Acid	*Conditions*					
	a	*b*	*c*	*d*	*e*	*f*
Glycolic, $HOCH_2CO_2H$		67	46	50		31
Lactic, $HOCH(CH_3)CO_2H$ (DL)	76	72	73	89		36
Malic, $HO_2CCH_2CH(OH)CO_2H$ (DL)	29	30	32	35	50	26
Citramalic, $HO_2CCH_2C(Me)(OH)CO_2H$					65	
Citric, $HO_2CCH_2C(CO_2H)(OH)CH_2CO_2H$	16	11	18	23	42	22
IsoCitric, $HO_2CCH(OH)CH(CO_2H)CH_2CO_2H$					40	
Glyceric, $HOCH_2CH(OH)CO_2H$		60	32	24	36	
Tartaric, $HO_2CCH(OH)CH(OH)CO_2H$ (DL)		24	19	18	31	19
Quinic, 1*R*,3*R*,4*R*,5*R*-Tetrahydroxycyclohexane carboxylic					18	
Ascorbic						15

a, Diisopropyl ether–formic acid, (3 : 1), cellulose MN 300HR, detection by UV; b, ethanol–concentrated ammonia–water, (150 : 8 : 40), cellulose, (Merck 5552), detection by bromocresol green or starch-iodine reagent; c, 2-ethyl-1-butanol–formic acid–water, (40 : 12 : 48), cellulose, (Merck 5552), detection as in b; d, diisopropyl ether–formic acid–water, (65 : 25 : 10), cellulose (Merck 5716), detection by aniline-xylose, furfural; e, propanol–methyl benzoate–90% formic acid–water, (7 : 3 : 2:1), cellulose, detection by Pásková and Munk reagent; f, *n*-pentyl formate–chloroform–formic acid, (70 : 15 : 15), silG25, detection by bromocresol green. (With acknowledgement to Hanai, 1982.)



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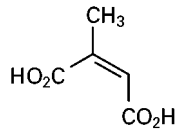

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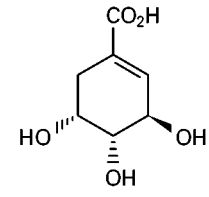

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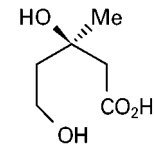

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Arylalkanoic acids Prior to an account of the TLC properties of aromatic acids it is of interest to note those of the semi-aromatic group typified by phenylacetic acid and its homologues and analogues. The hR_F values of a range of these compounds are shown in **Table 6**. The need to use the least polar combination of solvents is illustrated by the conditions with c and d where the latter is ineffective while the former affords a separation of homologous compounds. In the case of the unsaturated compound, the separation in conditions d would almost certainly be improved with argentated silica gel.

Acidic Compounds of Biosynthetic and Biological Importance

A number of polyfunctional cyclohexanyl derivatives classifiable in several of the above groups are (6) shikimic acid, (7) mevalonic acid and (8) abscisic acid, all of which have biological significance. Their TLC properties in a number of solvents have been described.

Table 4 hR_F values of keto acids on various layers

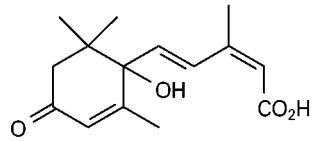
Keto acid	*Conditions*				
	a	*b*	*c*	*d*	*e*
$OHCCO_2H$	50		37	55	43
CH_3COCO_2H	68	25			60
$CH_3CH_2COCO_2H$	78				
$CH_3CH_2CH_2COCO_2H$		45			
$(CH_3)_2CHCH_2COCO_2H$		86			
$HO_2CCOCH_2CO_2H$	18		86	53	
$HO_2CCH_2CH_2COCO_2H$	36		74	50	45

a, Ethyl formate–light petroleum (60–80°C)–acetic acid (50 : 50 : 7), silica gel; b, ethanol–concentrated ammonia–water (78 : 13 : 20), rice starch, detection by fluorescein and UV; c, water-saturated diethyl ether–88% formic acid (7 : 1), silica gel G, aniline ribose reagent; d, chloroform–methanol–formic acid (80 : 20 : 1), silica gel G, aniline ribose; e, *n*-pentyl formate–chloroform–formic acid (70 : 15 : 15), sil G25, bromocresol green. (With acknowledgement to Hanai, 1982.)

Table 5 hR_F values of unsaturated di- and tribasic acids on various layers

Acid	*Conditions*								
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
Maleic	3				18	30		27	22
Fumaric	32	87	31	83	47	37	82	49	72
Itaconic	49					45	79	53	
Mesaconic (*trans*)						82	88	62	
Citraconic (*cis*)						36		39	
Glutaconic						44		56	
Hex-3-ene dicarboxylic	54								
cis-Aconitic	1					4	65		
trans-Aconitic	9	35	9	78			57		

a, Toulene–propionic acid–water, (47 : 47 : 4.9), cellulose (Merck 5716), detection by aniline-xylose, furfural; b, diisopropyl ether–formic acid (3 : 1), cellulose MN300HR, detection by dichlorofluorescein; c, diethyl ether–formic acid–water, (10 : 2 : 1), cellulose (DC Fertigplatten) detection by fluorescence; d, 95% ethanol–25% ammonia–water (8 : 2 : 1), same layer and detection as c; e, diisopropyl ether–light petroleum–carbon tetrachloride–water–formic acid (50 : 20 : 20 : 8 : 1), polyamide 6, detection by K ferricyanide, ferric ammonium sulfate; f, *n*-pentyl formate–chloroform–formic acid (20 : 70 : 10), sil G25, detection by bromocresol green; g, propanol–methyl benzoate–90% formic acid–water (7 : 3 : 2 : 1), layer not stated but probably cellulose, detection by Pásková and Munk reagent; h, butyl formate–ethyl acetate–formic acid (82 : 9 : 9), polyamide, bromocresol green; i, diisopropyl ether–formic acid–water, (90 : 7 : 3) silica gel, bromocresol green. (With acknowledgement to Hanai, 1982 and to Copius-Peereboom, 1969.)



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Shikimic acid (6), a hydroxy unsaturated cyclic compound, in the solvent g (Table 5) had an hR_F of 32. The hR_f value of the keto hydroxyacid, mevalonic acid, in diethyl ether–formic acid (7 : 1) on silica gel (Eastman) was 29 and that of abscisic acid in *n*-propanol–25% ammonia–water (80 : 10 : 10) on kieselgel (HF254) was 57. The rooting hormone, indole-3-acetic acid, under the same conditions was 45.

Table 6 hR_F values of derivatives and homologues of phenylacetic acid

Acid	*Conditions*			
	a	*b*	*c*	*d*
Phenylacetic	68	74	54	95
4-Phenylbutanoic			75	95
4-Phenylbut-3-enoic			71	95
Phenoxyacetic	64	63		
trans-Cinnamic			67	95

a, *n*-pentyl formate–chloroform–formic acid, (70 : 15 : 15), sil G25, bromocresol green; b, *n*-pentyl formate–chloroform–formic acid (20 : 70 : 10), sil G25; c, light petroleum–acetic acid, (49 : 1), silica (Eastman), bromocresol green; d, light petroleum–diethyl ether–formic acid, (45 : 5 : 1), silica (Eastman). (With acknowledgement to Hanai, 1982.)

Table 7 The hR_F values of cyclohexane- and dienecarboxylic acids (dihydro- and tetrahydro-derivatives of benzoic acid)

Compound	*Conditions*	
	a	*b*
Cyclohexanecarboxylic acid	83	92
Cyclohexa-1-enecarboxylic acid	91	95
Cyclohexa-3-enecarboxylic acid	91	95
Cyclohexa-1,4-dienecarboxylic acid	77	83
Cyclohexa-2,5-dienecarboxylic acid	77	82
Benzoic acid (cyclohexa-1,3,5-trienecarboxylic)	77	88
2-Hydroxycyclohexanecarboxylic acid	54	21

a, Benzene–dioxane–acetic acid (90 : 25: 4), kieselgel G, detection by autoradiography; b, light petroleum–diethyl ether–acetic acid (50 : 50 : 1), as before in a. (With acknowledgement to Hanai, 1982.)

The hR_F values of other cyclic compounds which are metabolites of benzoic acid and also structurally related to shikimic acid are given in **Table 7** alongside the reference compound benzoic acid. Isomeric compounds were not separable, although by the use of argentation TLC this may be possible.

Aromatic Acidic Compounds

Substituted Benzoic Acids

In this category the compounds under consideration are those in which the carboxyl group is directly attached to the aryl ring.

The isomeric hydroxybenzoic acids have been listed in the section on phenols. In **Table 8** the TLC properties of the aminobenzoic acids are given alongside the reference compounds benzoic acid, 2-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid (gallic acid) and phthalic acid.

The hR_F values of a wide range of other phenolic acids have been recorded, as have those of more complex compounds, the polycyclic series of lichen acids. In the case of *cis* and *trans* isomers of aromatic acids having an unsaturated side chain, separations do not seem to be difficult. Thus, on silica gel 60 (F_{254}) with diethyl ether–hexane–chloroform–acetic acid (12 : 38 : 50 : 0.5) the hR_F values of *cis*- and *trans*-3,4-dihydroxycinnamic acid were 18 and 27 and of the corresponding isomers of

Table 8 hR_F values of aminobenzoic acids and some hydroxybenzoic acids

Acidic compound	*Conditions*					
	a	*b*	*c*	*d*	*e*	*f*
Benzoic	53	15	72	69	79	
2-Aminobenzoic	38					
3-Aminobenzoic	28					
4-Aminobenzoic	24					
2-Hydroxybenzoic	55			72	78	54
2,4-Dihydroxybenzoic	19					30
3,4,5-Trihydroxybenzoic	4					11
Phthalic	17			55	41	

a, Ethanol–butanol–water–cocentrated ammonia (40 : 30 : 15 : 15), rice starch, detection by UV; b, *n*-hexane–acetic acid (96 : 4), SIF silica gel sheet, UV; c, same as b, cellulose-TLC alumina, UV; d, *n*-pentyl formate–chloroform–formic acid (70 : 15 : 15), sil G25, detection by bromocresol green; e, *n*-pentyl formate–chloroform–formic acid (20 : 70 : 10), same as d, UV; f, 2-butanone–methyl phenyl ketone–50% acetic acid (5 : 5 : 4), poly-*N*-vinylpyrrolidone-gypsum, detection by molybdate, diazotized sulfanilic acid, phloroglucinol. (With acknowledgement to Hanai, 1982.)

4-hydroxy-3,5-dimethoxycinnamic acid, 43 and 55 respectively.

The effect of the aryl nucleus, Ar, on the hR_F value of the acid $ArCO_2H$ is seen with benzoic, naphthalene-2-carboxylic and diphenyl-2-carboxylic acids, which are 53, 70 and 75 respectively with the solvent ethanol–butanol–water–conc ammonia (40 : 30 : 15 : 15) and the adsorbent, rice starch. The water-soluble vitamin, nicotinic acid (pyridine-3-carboxylic acid) on sil G25 had an hR_F value of 5 in *n*-pentyl formate–chloroform–formic acid (70 : 15 : 15), while that of benzoic acid was 69.

The hR_F values of the isometric benzenedicarboxylic and certain polybasic reference acids were the subject of early studies under a variety of conditions and are shown in **Table 9**.

More recent experiments on the separation of the dicarboxylic acids have been carried out with chloroform–tetrahydrofuran (2 : 1) on silufol in the presence of an ion pair reagent but the separations described earlier were just as effective.

The influence of the substituent position on the hR_F values of substituted benzoic acids has been studied with reference to amino, nitro, chloro, hydroxy and carboxy compounds, although nothing appears to have been described on the separation of the isomeric toluic acids. However, there has been extensive work by Guinchard *et al*. (see Further Reading) on (1) benzoic acid compared with (2) 2-chloro, (3) 3-chloro, (4) 4-chloro, (5) 2-hydroxy, (6) 3-hydroxy, (7) 4-hydroxy, (8) 2-nitro, (9) 3-nitro, (10) 4-nitro, (11) 2-amino and (12) 4-aminobenzoic acid. **Figure 1** depicts the effect on the R_F of small changes in the aqueous formic acid concentration in benzene when this range of acids was chromatographed on silica gel G.

Figure 2 depicts the effect on the R_F value of the same series when run in benzene containing diethyleneglycol monoethyl ether with various concentrations of formic acid. Separation of all 12 acids can be achieved in either system with the appropriate formic acid concentration. The more polar compounds have lower R_F values than the less polar ones.

By contrast, under reversed-phase conditions with benzene as the developing solvent, **Figure 3** shows the separations of a number of 2-substituted benzoic acids on silanized silica gel (RP-8) with various aqueous organic solvents (organic solvent–water, 40 : 60, v/v) containing 0.1 mol L^{-1} tetramethylammonium bromide. With this system the more polar solutes have higher R_F values.

An extensive range of adsorbents and solvents for a variety of aromatic carboxylic acids have been summarized by Tyman (see Further Reading).

While carboxylic acids have been the main group of acidic compounds studied, by contrast sulfonic acids RSO_3H, of both aliphatic and aromatic origin, have received little attention. 1-*N*-Acylamino-8-hydroxynaphthalene-3,6-disulfonic acid derivatives of interest for anti-human immunodeficiency virus activity have been studied on S III Chromarods with methanol or methanol–chloroform–ammonia (35 : 55 : 10) as solvents.

Sulfuric esters, $ROSO_3H$ of substituted phenols, have been examined on silica gel G with benzene–butanone–ethanol–water (30 : 30 : 30 : 10). The less acidic group, for example the sulfonamides, $NH_2C_6H_4SO_2NHR$ (where R comprises a wide variety of

Table 9 The hR_F values of carboxy derivatives of benzoic acid

Acid	*Conditions*			
	a	*b*	*c*	*d*
Phthalic (1,2)	51	30	39	36
Isophthalic (1,3)	75	64	71	59
Terephthalic (1,4)	0	69	81	0
Trimellitic (1,2,4)	41	13	14	13
Pyromellitic (1,2,4,5)	0	2	2	0
Hexahydrophthalic	60	65	79	66

a, Diisopropyl ether–formic acid–water (90 : 7 : 3), silica gel; b, same as b but saturated with polyethylene glycol M 1000 kieselguhr impregnated with polyethylene glycol; c, diisopropyl ether–light petroleum–carbon tetrachloride–formic acid–water (50 : 20 : 20 : 8 : 1), polyamide; d, butyl formate–ethyl acetate–formic acid (82 : 9 : 9), same polyamide as c. (With acknowledgement to Copius-Peereboom, 1969.)

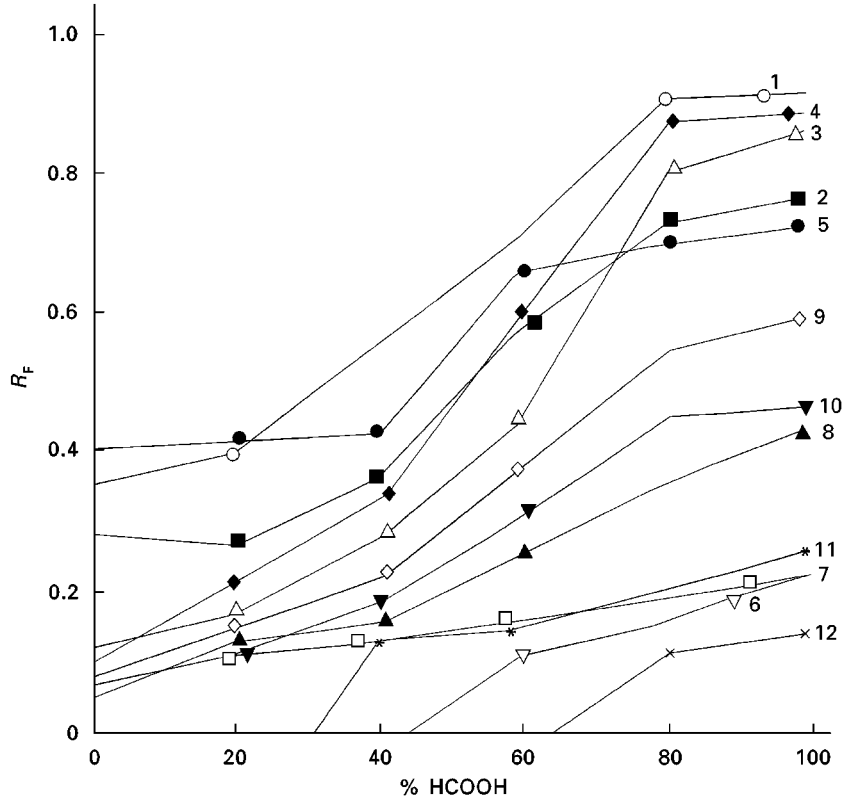


Figure 1 R_F values of aromatic carboxylic acids in benzene containing formic acid. 1, Benzoic; 2, 2-chloro; 3, 3-chloro; 4, 4-chloro; 5, 2-hydroxy; 6, 3-hydroxy; 7, 4-hydroxy; 8, 2-nitro; 9, 3-nitro; 10, 4-nitro; 11, 2-amino; 12, 4-aminobenzoic acids. (Reproduced with premission from Guinchard *et al.*, 1976.)

groups), has been examined in detail. Monoalkyl phosphate esters, $ROP(O)(OH)_2$, dialkyl esters, $(RO)_2P(O)OH$ and monoalkylphosphonic acids $RP(O)(OH)_2$ do not seem to have been examined by TLC.

Visualizing Agents for Aromatic Carboxylic Acids

In this article, reference has frequently been made to the detection of acids with bromocresol green and other systems. Some other reagents for aromatic carboxylic acids are hydrogen peroxide or alkaline potassium permanganate. Several new visualizing agents and sodium hydroxide (10% aqueous solution) were compared with respect to the minimum quantity of acid detectable (ug per spot) and the type of layer. Generally, of the three layers, silica gel 60 GF_{254}, silica gel–kieselguhr mixtures and polyamide, the first was preferred. Although the minimum detectable amount of solute varied with the 13 different solutes and the 12 different visualizing agents examined, thymol blue detected all the solutes while bromothymol blue and bromocresol green detected all but 4-hydroxybenzoic acid and 3-hydroxycinnamic acid respectively with silica gel as adsorbent.

Quantitative TLC Determination of Organic Acids in Synthetic and Natural Mixtures

Examples of the application of TLC for the quantitative determination of a variety of acids in edible, potable and polymeric products are discussed in this section. Many simple aliphatic acid aromatic acids, notably benzoic acid, citric and sorbic acids, are employed in edible materials such as preservatives while salicylic acid and its acetyl derivative appear in numerous pharmaceutical preparations. Accordingly, their quantitative determination is important and for such analyses planar methods have been widely used. Some typical quantitative applications are described in detail.

HPTLC Determination of Organic Acid Preservatives in Beverages

In a high performance TLC (HPTLC) method sorbic acid (2,4-hexadienoic acid) and benzoic acid were determined without preliminary extraction or clean-up by the chromatography of aliquots of samples and of standards on preadsorbent silica gel or C_{18}-bonded silica gel plates containing fluorescent indicator.

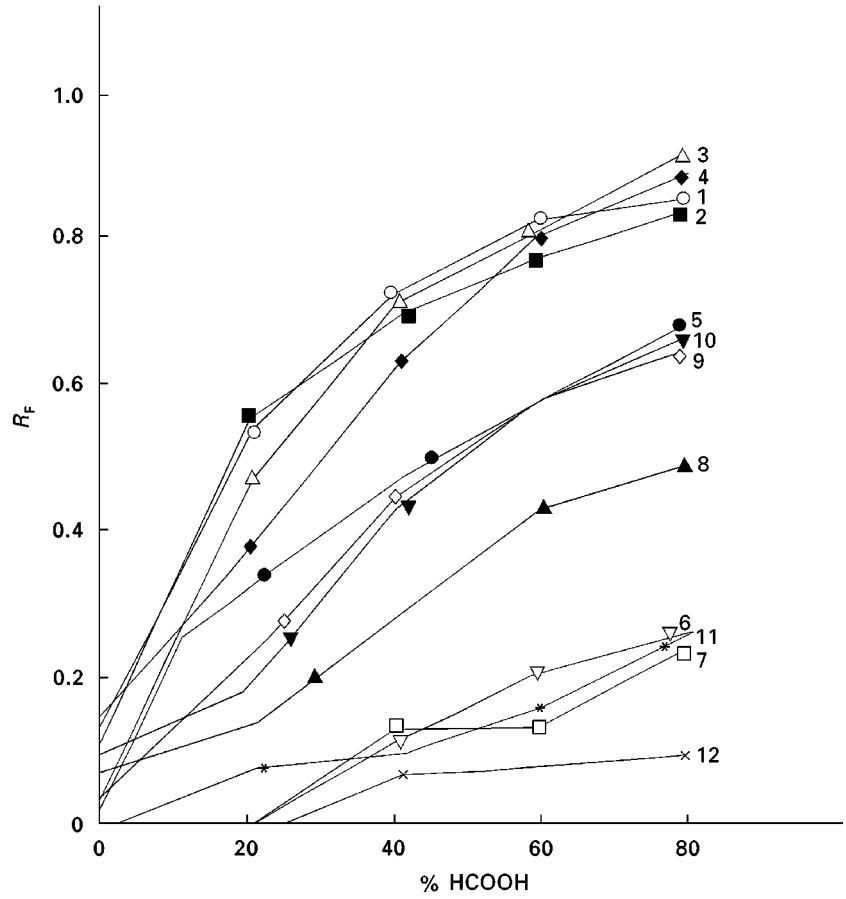


Figure 2 R_F values of aromatic carboxylic acids. 1, Benzoic; 2, 2-chloro; 3, 3-chloro; 4, 4-chloro; 5, 2-hydroxy; 6, 3-hydroxy; 7, 4-hydroxy; 8, 2-nitro; 9, 3-nitro; 10, 4-nitro; 11, 2-amino; 12, 4-aminobenzoic acid in benzene containing formic acid and diethylene glycol monoethyl ether. (Reproduced with premission from Guinchard *et al.*, 1976.)

The zones which quenched fluorescence upon UV irradiation at 254 nm were compared by scanning densitometry. This procedure was preferred to measurement of densitometry based on UV absorption.

Preadsorbent high-performance LHPKDF silica gel (Whatman) plates (20 × 10 mm) with 19 lanes were used for normal-phase experiments with the solvent *n*-pentyl formate–chloroform–formic acid (2 : 7 : 1) in which the hR_F values for sorbic acid and benzoic acid were 61 and 58. For reversed-phase TLC on (Whatman) C_{18} $LKC_{18}F$ plates (20 × 20 mm) with methanol–0.5 mol L^{-1} sodium chloride (1 : 1), the respective hR_F values for these two acids were 44 and 59. It was found necessary to apply a stream of warm air during spotting of samples with a 10 μL Drummond digital microdispenser and, after this stage, to dry the plates. Development was then effected in a Camag twin-trough chamber to 7 cm beyond the sorbent–preadsorbent interface with normal-phase plates and to 10 cm for C_{18} plates. The plates were then dried and the areas of the dark quenched zones against a fluorescent background were scanned at the predetermined maximum absorption (between 200 and 370 nm) with a Shimadzu Model 930 densitometer operated in the reflectance mode. From the chromatography of 0.50, 1.00, 2.00, 4.00, 6.00 and 8.00 μL of standards for sorbic and benzoic acids containing 125–2000 ng and 1.00–16.0 μg respectively, linear calibration plots of scan area/weight were obtained. For quantification, the sample scan area was compared with that of a closely matching standard within the linear calibration range and the corresponding weight found. Recovery analyses were carried out with beverage samples spiked with sorbic and benzoic acids, which were compared with the corresponding unfortified samples. They averaged at 98.0% for all analyses.

By the HPTLC method, sorbic and benzoic acids present separately in a variety of beverages have been directly quantified. The analysis of standards on the same TLC plate eliminates the requirement for an internal standard, as in high performance liquid chromatography (HPLC). By contrast with the HPTLC and HPLC methods, spectrophotometric

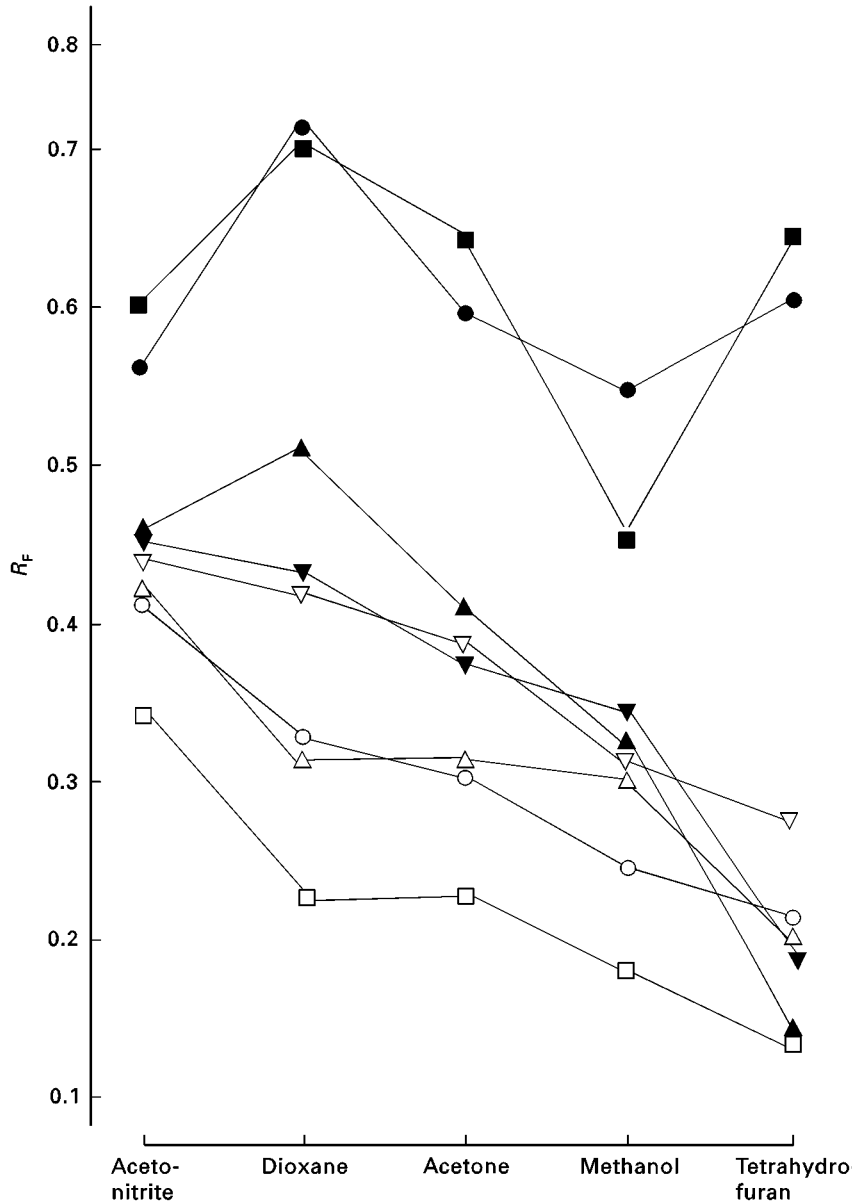


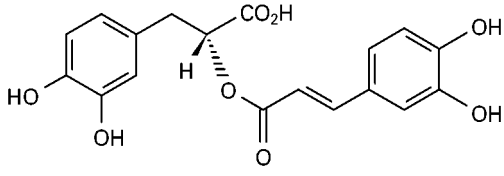
Figure 3 R_F values of 2-substituted benzoic acids in different solvents. The solvent composition, organic component–water (40:60 v/v) with addition of 0.1 mol L^{-1} tetramethylammonium bromide (pK value in parantheses): open circles, benzoic acid (4.19); filled triangles, 2-hydroxy (2.97); open squares, 2-acetoxy (3.5); filled squares, 2-carboxy (2.91/5.59); filled circles, 2-nitro (2.16); open squares, 2-methyl (3.91); open triangles, 2-amino (6.97); filled/inverted triangles, 2-chloro (2.92). (Reproduced with permission from Jost *et al.*, 1984.)

analysis requires a preliminary sample preparation by steam distillation. However, very low concentrations of benzoic acid are more amenable to HPLC analysis and when sorbic and benzoic acids are present together the method is less satisfactory due to sample streaking, even on a C_{18}-bonded silica gel layer (particularly at higher loads).

In view of these limitations, a modified method was adopted, involving solid-phase extraction (SPE) on a C_{18} cartridge followed by the preceding quantification method established on preadsorbent C_{18} plates. The extraction procedure was validated by spiking commercial samples with known amounts of the acids in turn and demonstrating the satisfactory recovery of each. With this total method, sample interferences were eliminated and samples too low for analysis by direct spotting could be analysed. The whole TLC methodology is considered to be applicable to a wide range of solid and syrupy-type samples containing either or both of the two preservatives at concentrations as low as those measurable by HPLC.

Quantitative Fluorescence Densitometry for the Analysis of Rosmarinic Acid

Rosmarinic acid (9), a useful natural antimicrobial compound of potential interest to the food industry, occurs in eel grass (*Zostera marina*) from which it is extractable together with a number of other phenolic acids. It has been directly quantitatively and rapidly analysed by an HPTLC densitometric method which utilized the fluorescence of the material upon excitation at 366 nm.



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Crushed leaves (200 mg) of the natural product were extracted with 5% acetic acid–methanol (1:2) accompanied by ultrasonication during 30 min. The extract was filtered and then employed for direct HPTLC on plates (10 × 20 cm) pre-coated with cellulose without fluorescent indicator. Samples and standard solutions (2 μL) were applied to plates as 7 mm wide bands with a Linomat IV applicator under a pressure of 2.5 bar; this was developed in a twin-trough chamber with 3% sodium chloride in 0.5% acetic acid–acetonitrile–tetrahydrofuran (100 : 24 : 1) until the solvent had migrated 4.5 cm. The dried plate was irradiated with a mercury vapour lamp and the resultant fluorescence emission measured through a cut-off filter (400 nm) by scanning with a TLC scanner II (Camag) equipped with CATS software (version 3.14).

Plots of either peak area or height/concentration were linear over concentration range 0.1–0.6 mg mL^{-1} (i.e. 0.2–1.2 μg) and the weight of rosmarinic acid in unknown samples was readily found.

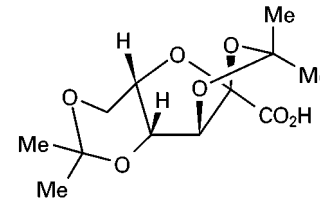
Densitometric Analysis of Gallic Acid in Fermentation Liquors

One of the ways used for obtaining gallic acid (3,4,5-trihydroxybenzoic acid), an important intermediate in synthesis for the pharmaceutical and food industries, is by the acid hydrolysis of natural gallotannins, for example from gall nuts, tara pods or sumac leaves. In an enzymatic procedure hydrolysis of these types of raw material with a fungal tannin acylhydrolase which cleaves depside bonds, the monitoring of a large number of samples by a simple and rapid TLC method was investigated as a potential alternative to HPLC analysis.

Crude samples from enzymatic solutions were diluted between one- and 100-fold with methanol and filtered through a Minisart NML 0.45 μm filter and gallic acid used at known concentrations as an internal standard. TLC analysis was performed on glass plates (5 × 20 cm), coated with a 0.25 mm layer of RP-18 F_{254} (Merck 15683); the glass plates were precleaned with a single development in methanol. Samples (6 μL) were applied with a Linomat IV spotter and then developed to a distance of 12 cm, with M aqueous acetic acid–methanol (1 : 1) for 2 h. Densitometry was effected by spectrophotometry and a mercury light source (254 nm) in the absorbance mode, to determine extinction of fluorescence, as an area measurement, with a TLC scanner II (Camag) controlled by CATS software. Calibration plots were found to be near to linearity with between 10 and 75 μg gallic acid on the plate when the ratios of the peak area of the acid to the internal standard were between 0.3 and 1.5, although in practice ratios of areas between 0.5 and 1.25 (corresponding to gallic acid between 25 and 62.5 μg) were adopted in the analytical method. An inherent difficulty was found to be slight inhomogeneity in the coating of the fluorescent indicator: to improve on this, the plate was scanned before an assay to determine any background fluorescence, which was then subtracted to 'zero' the plate. With this proviso and by the use of the strict linearity range, the values obtained for gallic acid were 98 ± 2.1% of those found by HPLC.

Determination of Diacetonegulonic acid (DAG) in Water Samples

DAG (10) is the penultimate intermediate in the synthesis of ascorbic acid (vitamin C) and for many years was discharged in waste surface waters. This led to contamination of groundwaters and, although it is not toxic to humans, it has an inhibitory effect on the growth of grasses. Current European drinking water regulations restrict its concentration to 0.1 μg L^{-1}. A fast and efficient HPTLC method has been described.



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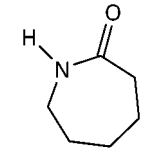

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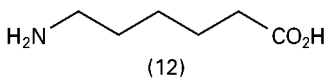
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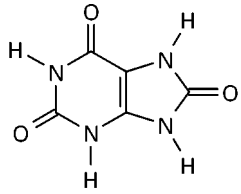
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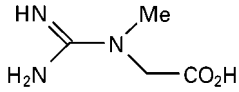
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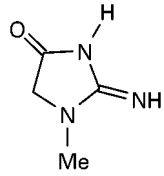
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Due to the low concentration of DAG, SPE is used for sample preparation. Because of the sensitivity of DAG to silica gel and, more particularly to acidic solutions, it was found necessary to adjust the water sample for analysis to no less than pH 4 and to effect SPE with Polyspher RP-18 (a 35 μm polystyrene-divinylbenzene polymer with C_{18} side chains) which gave a 100% recovery. For the extraction a cartridge (0.2 g) was first conditioned successively with ethyl acetate, methanol and water at pH 4 (1 cm^3 of each), after which the water sample for analysis adjusted to pH 4 (20 cm^3) was aspirated through the cartridge. The cartridge was dried in a stream of nitrogen and then eluted with ethyl acetate (2×1 cm^3) and the eluate after treatment with one drop of ammonia evaporated at less than 40°C to leave 0.5 cm^3, an aliquot of which was applied to an PTLC silica gel 60 F_{254} pre-coated plate (10×20 cm). In the case of original concentrations of less than 5 μg L^{-1}, the total eluate was used for TLC.

For analysis of sample volumes up to 20 μL, multiple development one-dimensionally with solvent A, chloroform–methanol (80 : 20) to 8 cm and then after drying, solvent B (chloroform–methanol–glacial acetic acid, 80 : 20 : 2) for 6.5 cm was carried out. Alternatively, two-dimensional development was carried out with the same two solvents, distances and drying. Spots or streaks were detected by immersion of the plate in an ethanolic solution of 4-methoxybenzaldehyde containing sulfuric acid, followed by drying and heating at 130°C for 2–3 min to form red fluorescent areas which were visible under UV light (366 nm) and quantified with a TLC scanner. Two-dimensional development was advocated for samples with less than 5 μg L^{-1} DAG, while for higher concentrations, one-dimensional development was adequate. The calibration of peak area/weight DAG was linear within the range 0.125–1.5 μg. It was found that for the determination of higher concentrations it was essential to apply DAG as streaks to preserve linearity over the range of concentrations and it was then established that from 0.25 to 250 μg could be analysed with consistent accuracy.

The SPE procedure followed by TLC appears to be superior to derivatization followed by GC-MS and it was considered that very small concentrations of DAG could even be estimated visually without any instrumentation, thus generally giving an inexpensive procedure. Other application of quantitative TLC to the analysis of humic acids in natural waters, 6-aminocaproic acid (12), ε-caprolactam in polyamide-6 (11) and to uric acid (13), creatine (14) and creatinine (15) mixtures in biological materials have been described.

Conclusions

Acids of simple and more complex structures are components of many edible, technical and medicinal products and TLC affords an ideal approach for their analysis because no derivatization is required and a wide variety of detection methods is applicable for their qualitative and quantitative determination. It can be envisaged that the use of HPTLC, of special layers and the employment of combined techniques will continue to extend and expand the planar approach to the analysis of acidic compounds.

See also: **II/Chromatography: Thin Layer (Planar):** Densitometry and Image Analysis; Ion Pair Thin-Layer (Planar) Chromatography; Spray Reagents. **III/Acids:** Gas Chromatography; Liquid Chromatography.

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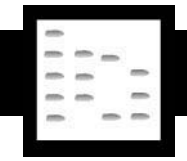
AFLATOXINS AND MYCOTOXINS



Chromatography

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Introduction

Mycotoxins have been defined as 'fungal metabolites which, when ingested, inhaled or absorbed through the skin, cause lowered performance, sickness or death in man or animals, including birds'.

Exposure to mycotoxins can produce both acute and chronic toxic effects ranging from death to deleterious effects on the central nervous, cardiovascular and pulmonary systems, and on the alimentary tract. Mycotoxins may be carcinogenic, mutagenic, teratogenic and immunosuppressive. The ability of some mycotoxins to compromise the immune system and, consequently, to reduce resistance to infectious disease, is now widely considered to be their most important effect.

The mycotoxins attract worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and both domestic and international trade. It has been estimated, for example, that annual losses in the USA and Canada arising from the impact of mycotoxins on the feed and livestock industries are in the order of US$5 billion. In developing countries where the food staples (e.g. maize and groundnuts) are susceptible to contamination, significant additional losses amongst the human population are likely, because of morbidity and premature death associated with the consumption of mycotoxins.

It is likely that mycotoxins have plagued mankind since the beginning of organized crop production. Ergotism (St Anthony's Fire), for example, which is caused by the consumption of rye contaminated with the 'ergot alkaloids', is discussed in the Old