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Short communication

Thin-layer chromatography of gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechol, resorcinol, hydroquinone, catechin, epicatechin, cinnamic acid, *p*-coumaric acid, ferulic acid and tannic acid

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Abstract

Six solvent systems of varying suitability are reported for the thin-layer chromatographic separation of simple phenolics and related compounds such as gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechol, resorcinol, hydroquinone, catechin, epicatechin, cinnamic acid, *p*-coumaric acid, ferulic acid and tannic acid. The solvent system chloroform–ethyl acetate–acetic acid (50:50:1) facilitated the separation of all the compounds except pyrogallol and ferulic acid; resorcinol and hydroquinone, which were in turn resolved in benzene–dioxane–acetic acid (85:15:1). Detection was carried out using iodine vapour, ferric chloride reagent, ferric chloride–ferricyanide reagent and vanillin–sulfuric acid reagent.

Keywords: Phenols; Tannic acid; Catechin; Epicatechin; Cinnamic acid; *p*-Coumaric acid; Ferulic acid

1. Introduction

The presence of polyphenolics such as tannins and lignin in fodder and feeds limits their digestibility [1,2]. Efforts are underway for the biodegradation of these substances using biological approaches [3–5]. During our studies on the biodegradation of tannins and lignin, a need arose for the quick monitoring of the putative metabolic products [6,7]. Tannic acid has been used as a model compound for the biodegradation of hydrolysable tannins and is known to be metabolised to gallic acid, pyrogallol and resorcinol [8,9] (Fig. 1). Likewise, the monomeric units of condensed tannins are catechin and epicatechin [10] (Fig. 2). The composition of lignin is very complex and some of its biodegradation products are *p*-coumaric acid, cinnamic acid and ferulic acid [3,11]. The separation of compounds on thin layers has the advantage that a variety of supporting media can be used and detection can even be carried out using corrosive reagents and high temperatures. In addition, thin-layer chromatographic (TLC) methods are very rapid and separations can usually be completed in under an hour [12]. No satisfactory thin-layer chromatographic procedures are available for the resolution of monomers and putative metabolites of tannins, lignin and related compounds such as catechol, phloroglucinol and hydroquinone. We report here the evaluation of various solvent systems using silica gel G thin layers for the resolution of

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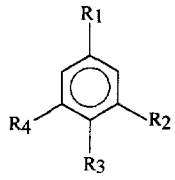

	R_1	R_2	R_3	R_4
Gallic acid	-COOH	-OH	-OH	-OH
Pyrogallol	-H	-OH	-OH	-OH
Phloroglucinol	-OH	-OH	-H	-OH
Catechol	-H	-H	-OH	-OH
Resorcinol	-H	-OH	-H	-OH
Hydroquinone	-OH	-H	-OH	-H
Methylgallate	-C(=O)-CH3	-OH	-OH	-OH
Cinnamic acid	-CH=CH-COOH	-H	-H	-H
Ferulic acid	-CH=CH-COOH	-O-CH3	-OH	-H
p-Coumaric acid	-CH=CH-COOH	-H	-OH	-H

Fig. 1. Chemical structures of the simple phenolics, cinnamic acid, ferulic acid and *p*-coumaric acid.

simple phenolics, catechin, epicatechin, cinnamic acid, *p*-coumaric acid and ferulic acid.

2. Experimental

Silica gel G was purchased from Sisco Research Laboratories (Mumbai, India). The solvents were of analytical grade and freshly distilled before use. Thin-layer glass plates (200×200 mm) were coated with silica gel G to a thickness of 0.2 mm, air-dried and activated at 110°C for 1 h [12]. Solutions of gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechin, epicatechin (Sigma, St. Louis, USA), hydroquinone, ferulic acid (Sisco Research Laboratories), resorcinol (Ranbaxy Fine Chemicals, New Delhi, India), catechol (S.D. Fine Chemicals, Mumbai, India), *p*-coumaric acid (Fluka Chemika Biochemika Buchs, Switzerland) and tannic acid (Qualigens Fine Chemicals, Mumbai, India) at a concentration of 4 mg/ml, and cinnamic acid (Loba Chemie, Mumbai, India) at a concentration of 8 mg/ml were prepared in methanol. Aliquots (10 μl) of each solution were applied to the plates. The following solvent systems were used:

(I) Chloroform–methanol–acetic acid (90:10:1)
(II) Petroleum ether (60–80°C)–ethyl acetate–formic acid (40:60:1)
(III) Benzene–dioxane–acetic acid (85:15:1)
(IV) Chloroform–ethyl acetate–acetic acid (50:50:1)
(V) Toluene–acetonitrile–formic acid (70:30:1)
(VI) Petroleum ether (60–80°C)–methanol–acetic acid (90:10:1).

The chromatograms were developed at room temperature (about 20°C), air-dried and the spots were detected as follows.

(a) Exposure of the developed plates to iodine vapour in an iodine-saturated chamber for about 10 min yielded yellow spots. The detection limit was 80 μg for cinnamic acid, 10 μg for catechol and *p*-coumaric acid, 5 μg for tannic acid and 2 μg for the remainder of the compounds.

(b) Ferric chloride reagent was prepared by dissolving 1 g anhydrous ferric chloride in 100 ml methanol [8]. Spots for gallic acid, methyl gallate, pyrogallol, catechol, catechin, epicatechin, cinnamic acid, *p*-coumaric acid, ferulic acid and tannic acid appeared immediately after spraying the plates. Spots for phloroglucinol, resorcinol and hydroquinone appeared after heating the plates at 110°C for 10 min. The detection limit was 10 μg for cinnamic acid and 2 μg for the remainder of the compounds.

(c) Ferric chloride–ferricyanide reagent was prepared by dissolving 1 g anhydrous ferric chloride, 1 g potassium ferricyanide and a few crystals of potassium permanganate in 100 ml water [13]. All the compounds except cinnamic acid gave blue spots immediately after spraying the plates. Cinnamic acid gave bluish green spots which turned blue on heating the plates at 110°C for 10 min. The detection limit for all the compounds was 2 μg.

(d) Vanillin–sulfuric acid reagent was prepared by

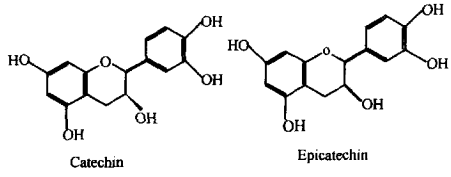

Fig. 2. Chemical structures of catechin and epicatechin.

dissolving 0.5 g vanillin in 100 ml sulfuric acid–ethanol (40:10) [12]. The plates were observed immediately after spraying and after heating at 120°C for 20 min. Cinnamic acid could not be detected using this reagent. The detection limit was 40 μg for resorcinol, 20 μg for *p*-coumaric acid, 10 μg for methyl gallate and ferulic acid and 2 μg for the remainder of the compounds.

2.1. Thin-layer chromatography of the biodegradation products of polyphenolics

Quebracho tannin (condensed tannin) was a gift from Trask Chemical Corporation, USA. Indulin (alkali lignin) was purchased from Sigma. Mildew test medium containing tannic acid, quebracho tannins or indulin as the sole carbon source (2%, w/v) was inoculated with *Aspergillus niger* van Tiegham 2425, a fungal isolate from hill cattle [6,7]. Samples of culture filtrate were obtained at 24, 48, 72, 96 and 120 h of incubation in an orbital shaker set at 120 rmp and 30°C. The filtrates were centrifuged at 5000*g* for 15 min. Aliquots (5 μl) of the supernatant were used for TLC using the solvent system chloroform–ethyl acetate–acetic acid (50:50:1) for monitoring the biodegradation products.

Table 2
hR_{st} values for different phenolics using catechol as the reference compound (the hR_{st} value for catechol was taken as 100)

Compound	hR_{st}					
	I	II	III	IV	V	VI
Gallic acid	10	57	8	40	27	22
Methyl gallate	52	74	35	71	64	58
Pyrogallol	48	87	46	78	76	56
Phloroglunicol	12	66	15	63	51	29
Resorcinol	64	94	75	95	90	76
Hydroquinone	62	91	69	95	88	76
Catechin	2	33	2	29	19	15
Epicatechin	2	30	2	21	19	11
Cinnamic acid	126	103	131	91	112	120
p-Coumaric acid	86	84	75	86	79	93
Ferulic acid	114	80	87	78	82	116

3. Results and discussion

The R_F and hR_{st} values of the simple phenols and related compounds, which are putative metabolites of polyphenols, in different solvent systems are given in Tables 1 and 2. Solvent systems II, III, IV and V were suitable for the resolution of tannic acid and methyl gallate in addition to gallic acid, pyrogallol and resorcinol which are the putative metabolites of tannic acid and methyl gallate in different biological

Table 1
R_F values for simple phenols and related compounds in different solvent systems

Compound	R_F						Detection	
	I	II	III	IV	V	VI	Ferric chloride reagent	Vanillin–sulfuric acid reagent
Gallic acid	0.04	0.40	0.04	0.26	0.18	0.12	Purple	Light purple[b]
Methyl gallate	0.22	0.52	0.17	0.46	0.43	0.32	Purple	Light purple[b]
Pyrogallol	0.20	0.61	0.22	0.51	0.51	0.31	Bluish-green	Pink
Phloroglucinol	0.05	0.46	0.07	0.41	0.34	0.16	Magenta[a]	Orange
Catechol	0.42	0.70	0.48	0.65	0.67	0.55	Blue	Purple[b]
Resorcinol	0.27	0.66	0.36	0.62	0.60	0.42	Magenta[a]	Pink
Hydroquinone	0.26	0.64	0.33	0.62	0.59	0.42	Magenta[a]	Light yellow[b]
Catechin	0.01	0.23	0.01	0.19	0.13	0.08	Bluish-green	Orange
Epicatechin	0.01	0.21	0.01	0.14	0.13	0.06	Bluish-green	Orange
Cinnamic acid	0.53	0.72	0.63	0.59	0.75	0.66	Yellow	N.D.
p-Coumaric acid	0.36	0.59	0.36	0.56	0.53	0.51	Orange	Light purple[b]
Ferulic acid	0.48	0.56	0.42	0.51	0.55	0.64	Orange	Purple[b]

Tannic acid remained at the origin in all solvent systems. It gave a purple colour with ferric chloride reagent immediately after spraying. Tannic acid gave a purple colour with vanillin–sulfuric acid reagent on heating the plates at 120°C for 20 min. N.D., not detected.
[a]Colour appeared on heating the plates at 110°C for 10 min.
[b]Colour appeared on heating the plates at 120°C for 20 min.

systems [8,9,14]. The R_F values of methyl gallate and pyrogallol were similar in solvent systems I and VI. Solvent system IV was the most suitable for the resolution of catechin and epicatechin, the monomeric units of condensed tannins [10]. Resorcinol and hydroquinone had very similar mobility in all solvent systems except solvent system III which was found to be more suitable for the resolution of these compounds. Cinnamic acid, *p*-coumaric acid and ferulic acid, which are the putative biodegradation products of lignin, were resolved well in all the solvent systems except system V where *p*-coumaric acid and ferulic acid had similar R_F values.

All the compounds could be detected using iodine vapour, ferric chloride reagent and ferric chloride–ferricyanide reagent. The background of the plates sprayed with ferric chloride–ferricyanide reagent became deep green when the plates were maintained at room temperature and the spots lost contrast. On the other hand, the plates sprayed with ferric chloride reagent had a uniform light yellow background which provided excellent contrast to the spots of the different compounds and the plates could be stored without any significant change in the spots and the background. The reagent reacted rapidly and produced a purple or bluish-green colour with phenolics such as gallic acid, methyl gallate, pyrogallol, catechol, catechin and epicatechin, which have vicinal hydroxyl groups in their structure (Figs. 1 and 2). Cinnamic acid, *p*-coumaric acid and ferulic acid also had a very fast reaction with ferric chloride reagent but the chromogen formed was yellow or orange. Other phenolics and related compounds gave a rather weak reaction only after heating the plates at 110°C for 10 min (Table 1). Vanillin–sulfuric acid reagent was more suitable for the detection of compounds such as pyrogallol, phloroglucinol, resorcinol, catechin and epicatechin, which have at least two hydroxyl groups *para* to each other (Figs. 1 and 2) but without any substitution in the ring as in gallic acid or methyl gallate. The other compounds, except cinnamic acid, could be detected with vanillin–sulfuric acid reagent on heating the plates at 120°C for 20 min. Unlike some systems described previously [8–10], the TLC systems described here have the advantage of the use of corrosive reagents, provide a wide choice of systems for the resolution and detection of a large number of phenolics (Table 1) and the spots obtained are very compact [12].

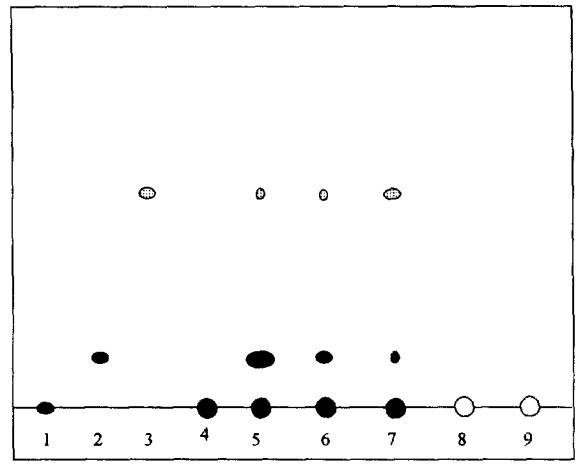

Fig. 3. Diagram of the TLC profile of the culture filtrates obtained on incubation of tannic acid with *Aspergillus niger* van Tiegham. Lanes: (1) tannic acid; (2) gallic acid; (3) pyrogallol; (4) 0 h sample; (5) 24 h sample; (6) 48 h sample; (7) 72 h sample; (8) 96 h sample; (9) 120 h sample.

Aspergillus niger van Tiegham utilized tannic acid as the sole carbon source and degraded it to gallic acid and pyrogallol. Tannic acid, as well as both of the metabolic products, disappeared by 96 h of incubation (Fig. 3). Other possible metabolic products of tannic acid, viz. phloroglucinol and resorcinol [8], could not be detected. Nelson et al. [9] detected only pyrogallol when tannic acid was incubated with a diplococoid anaerobic ruminal bacterium. *Aspergillus niger* van Tiegham did not elicit the biotransformation of quebracho tannin or indulin, as evidenced by the lack of fungal growth (data not shown) and the absence of any monomers or other metabolites on monitoring the culture filtrates by TLC.

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