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Short Communication

Diphenyltin dichloride as a chromogenic reagent for the detection of flavonoids on thin-layer plates

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Abstract

Among several complexation reagents, diphenyltin dichloride (DTC) proved to be a useful chromogenic compound for both the qualitative and quantitative analysis of flavones and flavonols on thin-layer plates by forming fluorescent complexes of different colour. For qualitative analysis, comparative detection with DTC and diphenylboric acid 2-aminoethyl ester can indicate the position of the glycosidated hydroxyl of kaempferol and luteolin glycosides. Fluorimetric densitometry showed high sensitivity to flavonols with a free 3-hydroxyl group and flavones containing two adjacent hydroxyl groups in ring B.

1. Introduction

Different chromogenic reagents such as NH_3, $AlCl_3$, $Al_2(SO_4)_3$, $ZrOCl_2$, diphenylboric acid 2-aminoethyl ester (Naturstoffreagens A, NA), 2,6-dichloroquinonechlorimide and EDTA are available for the detection of flavonoids on thin-layer plates in order to reveal the spots of these compounds and also to obtain information about their degree of oxidation and substitution patterns [1–8]. For example, when detected with NA on cellulose layers, 3,5-dihydroxyflavones fluoresce at 510–527 nm if two adjacent hydroxyl groups in ring B are missing, but at 560–567 nm with 3′,4′-dihydroxy groups [6].

We report here on investigations on further chromogenic compounds that are able to form fluorescent flavonoid complexes and provide structure information. Some chromogenic reagents were chosen as they were already known in flavonoid analysis [$FeCl_3$, $SbCl_3$, $Be(NO_3)_2$, $Na_2B_4O_7$] [2,6,9] but had not been thoroughly investigated for their ability to form complexes with different types of flavonoids. Additionally we took some similar reagents {$FeSO_4$, $Bi(NO_3)_3$, $SnCl_2$, Ph_2SnCl_2 ($SnCl_4$ has already been used for detection of flavonoids on thin-layer plates [10])}. First their complexes with flavonoids in methanolic solutions were investigated for stability and for the ability to show structure-dependent bathochromic shifts of the UV bands compared with pure methanolic flavonoid solutions. Fluorescence studies were also carried out. In the course of these investigations it turned out that these reagents caused bathochromic shifts of flavonoid UV bands and also caused fluorescence. Among those examined, diphenyltin dichloride (DTC) proved to be the most suitable. DTC also was adapted as a spray

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reagent for the TLC of flavonoid compounds. The best results were obtained with a 2% solution in acetone–methanol (1:1). Different flavonoids varying in degree of oxidation and substitution pattern (isoflavone, flavanone, flavone and flavonol aglycones and glycosides) were investigated on silica gel layers after detection with DTC by fluorimetric densitometry and compared with diphenylboric acid 2-aminoethyl ester as a chromogenic reagent. The fluorescent colours on polyamide and cellulose layers were also determined.

2. Experimental

2.1. Materials

All flavonoids except kaempferol-3-O-glycosides, kaempferol-3,7-O-dirhamnoside and kaempferol-7-O-rhamnoside were obtained from Carl Roth (Karlsruhe, Germany). Kaempferol-3-O-rhamnoside, kaempferol-3-O-glucoside and kaempferol-3-O-glucuronide were isolated from *Epilobium angustifolium* and kaempferol-7-O-rhamnoside and kaempferol-3,7-O-dirhamnoside from *Prunus spinosa*. The identity of the isolated compounds was confirmed by UV, ^{1}H NMR and mass spectrometry.

Organic solvents and inorganic reagents were of analytical-reagent grade from Merck (Darmstadt, Germany). Diphenyltin dichloride was of zur Synthese purity from Merck. Diphenylboric acid 2-aminoethyl ester was purchased from Carl Roth.

2.2. UV spectrophotometry

UV spectrophotometry was carried out on a Kontron Uvikon 810 spectrophotometer. The absorbances of 0.02 mmol l^{-1} solutions were determined between 220 and 500 nm in comparison with pure solvent (scan speed 100 nm min^{-1}). To 2.0 ml of methanolic flavonoid solution 0.05 ml of reagent solution [1% (w/v) in methanol] were added.

2.3. Spectrofluorimetry

Measurements were carried out on a Perkin-Elmer LS-5 luminescence spectrometer between 200 and 900 nm with 0.02 mmol l^{-1} methanolic solutions. To 2.0 ml of flavonoid solution 0.05 ml of DTC solution [1% (w/v) in methanol] were added. Because of the very intense fluorescence, solutions of kaempferol, its 7-O-glycosides and luteolin had to be diluted tenfold.

2.4. Preparation of spray reagents for TLC

A 2% (w/v) solution of DTC in acetone–methanol (1:1, v/v) was used as a chromogenic reagent for TLC. Spraying was repeated twice with intermediate drying of the TLC plates in a cold stream of air. To enhance and stabilize the fluorescence, spraying with a 5% (w/v) ethanolic solution of polyethylene glycol (PEG) 4000 (Merck) and with "Paraffin dünnflüssig" (Merck) followed [11]. Detection with diphenylboric acid 2-aminoethyl ester (Naturstoffreagens A, NA) was carried out in the same way except that a 1% (w/v) methanolic solution of NA was used. Measurements were made 15 min after spraying.

2.5. TLC procedures

Volumes of 1 μl of 0.0035 mol l^{-1} methanolic flavonoid solutions were applied with Blaubrand intraEND single-use 1-μl micropipettes (Brand, Wertheim, Germany) to TLC plates: silica gel (precoated aluminium-backed TLC plates, Kieselgel 60 F_{254}, 20 × 20 cm; Merck); polyamide (precoated plastic-backed thin layer plates, Polygram polyamide 6-UV_{254}, 20 × 20 cm; Macherey–Nagel, Düren, Germany); and cellulose (precoated plastic-backed TLC plates, Polygram cel300; Macherey–Nagel).

Samples were applied as spots 3 mm in diameter at intervals of 10 mm and with a distance of 15 mm from the lower edge and 15 mm from the side edges. Chromatography was carried out over a distance of 8 cm in a 20 × 20 cm twin-trough chamber with a stainless-steel lid (Camag, Muttenz, Switzerland) under saturated conditions.

Flavonoid aglycones were separated on silica gel plates using (I) toluene–methanol–2-butanone–acetylacetone–cyclohexane (20:15:10:5:50, v/v) and flavonoid glycosides using (II) ethyl acetate–formic acid–water (68:8:8, v/v) as mobile phase. On polyamide layers flavonoids were separated with (III) toluene–methanol–2-butanone–acetylacetone (40:30:20:10, v/v) and (IV) water–2-butanone–acetone–formic acid (60:30:9:1, v/v). On cellulose layers, (V) 10%, (VI) 20% and (VII) 30% (v/v) acetic acid were used as mobile phases. When separation was complete the plates were dried in a warm stream of air and detected as described above.

2.6. Densitometric analysis

Measurements were carried out on a Shimadzu CS-9000 dual-wavelength flying spot scanner. TLC lanes were scanned at a wavelength of 436 nm in the fluorescence mode using filter 3 (split width 10 × 1 mm). During measurement the TLC plates were fixed to a 20 × 20 cm glass plate to the back of which a plastic tube (2 mm I.D.) was attached in several loops. The temperature of the TLC plates was controlled by the flow-rate of ice-cooled water which was pumped using a Minipuls 2 pump (Gilson, Villiers-le-Bel, France) through the plastic tube. The relative standard deviation (R.S.D.) range of the densitometric measurements was 4.3–7.1% (5.2% on average) ($n = 4$). A calibration plot for 0.02–0.4 μg of quercetin (3,5,7,3′,4′-pentahydroxyflavone) showed good linearity between 0.16 and 2 μg [method of linear regression, y (area units) = ax (μg) + b, $y = 102510x - 9156$, standard deviation of a (S.D.$_a$) = 1812, standard deviation of b (S.D.$_b$) = 1905, correlation coefficient (r) = 0.99887, nine calibration points, $n = 4$]. The limit of detection of quercetin (TLC system I) with DTC was 20 ng, which matched that with NA detection.

3. Results and discussion

3.1. Absorbance measurements in solution

In preliminary studies, the influence of different complexation reagents [$Be(NO_3)_2$, $SnCl_2$, $Na_2B_4O_7$, $SbCl_3$, $Bi(NO_3)_3$, $FeSO_4$, $FeCl_3$, Ph_2SnCl_2] on the UV spectra of various flavo-

Table 1
UV absorbance and spectrofluorimetric measurements of different flavonoid–DTC complexes in methanolic solutions (0.02 mmol l^{-1})

Compound	UV [λ_{max} (nm)]		Fluorescence [λ_{max} (nm)]	
	Band I (MeOH)	Band I with DTC	Excitation	Emission
Aglycones				
Genistein (5,7,4′-OH-isoflavone)	327sh	327sh	420	475
Dihydrofisetin (3,7,3′,4′-OH-flavanone)	310	311sh	425	487
Apigenin (5,7,4′-OH-flavone)	335	337	390	491
Luteolin (5,7,3′,4′-OH-flavone)	349	366	420	505
Kaempferol (3,5,7,4′-OH-flavone)	367	424	424	474
Quercetin (3,5,7,3′,4′-OH-flavone)	370	433	436	496
Rhamnetin (3,5,3′,4′-OH-7-OMe-flavone)	369	432	443	504
Myricetin (3,5,7,3′,4′,5′-OH-flavone)	374	446	437	515
Glycosides				
Kaempferol-3-O-glucoside	351	352	422	484
Kaempferol-7-O-rhamnoside	366	423	424	474
Quercetin-3-O-rhamnoside	350	360	432	480
Myricetin-3-O-rhamnoside	352	362	438	490

noids in methanolic solutions was investigated in order to obtain information about the mechanism of complexation. Bathochromic shifts of band I were recognized with all flavonols with a free 3-OH group. Further investigations were carried out with DTC–flavonoid complexes and the results are given in Table 1.

The flavonols showed a marked shift of band I (from +54 to +72 nm) when DTC was added. 3′,4′-Dihydroxyflavones without a 3-OH group and also 3-O-glycosidated flavonols which contained two adjacent hydroxyl groups in ring B showed only smaller shifts (10–17 nm). Compounds that did not contain one of these structural elements did not show substantial shifts of band I. Hence complexation of DTC with 4-keto, 3-hydroxy (in the case of flavonols) and *o*-dihydroxy groups in ring B (in the case of flavones and flavonols) might be assumed. Detailed complexation studies with DTC and flavonoids have not yet been carried out. Measurements over a period of 20 min showed no decrease in intensity or bathochromic shift. None of the flavonoid–DTC complexes were acid resistant as the bathochromic shifts disappeared when 1 mol l^{-1} hydrochloric acid was added.

3.2. Spectrofluorimetric measurements in solution

Fluorescence was characterized by the determination of the λ_{max} of excitation and emission (Table 1). According to these results the intense spectral line of the mercury lamp at 436 nm was

Table 2
Visual analysis of flavonoids on silica gel plates after detection with DTC

Compound	Vis	UV (366 nm)
Aglycones		
Genistein (5,7,4′-OH-isoflavone)	Dark yellow	Olive
Dihydrofisetin (3,7,3′,4′-OH-flavanone)	Yellow[a]	Ochre[a]
3-OH-flavone	Green[a]	Blue
5-OH-flavone	Dark yellow[a]	Olive
7-OH-flavone	Not visible	Blue[a]
Apigenin (5,7,4′-OH-flavone)	Olive[a]	Light green[a]
Luteolin (5,7,3′,4′-OH-flavone)	Yellow	Yellow
Kaempferol (3,5,7,4′-OH-flavone)	Light yellow	Turquoise
Kaempferide (3,5,7-OH-4′-OMe-flavone)	Light yellow	Turquoise
Quercetin (3,5,7,3′,4′-OH-flavone)	Light orange	Light orange
Rhamnetin (3,5,3′,4′-OH-7-OMe-flavone)	Light orange	Light orange
Myricetin (3,5,7,3′,4′,5′-OH-flavone)	Light orange	Orange
Morin (3,5,7,2′,4′-OH-flavone)	Light yellow	Light green
Glycosides		
Luteolin-5-O-glucoside	Light yellow	Green
Luteolin-7-O-glucoside	Yellow	Yellow
Luteolin-7,3′-O-diglucoside	Light yellow[a]	Green[a]
Kaempferol-3-O-rhamnoside	Yellow[a]	Olive[a]
Kaempferol-3-O-glucoside	Yellow[a]	Olive[a]
Kaempferol-3-O-glucuronide	Yellow[a]	Olive[a]
Kaempferol-3,7-O-dirhamnoside	Yellow[a]	Olive[a]
Kaempferol-7-O-rhamnoside	Light yellow	Turquoise
Kaempferol-7-O-neohesperidoside	Light yellow	Turquoise
Quercetin-3-O-rhamnoside	Dark yellow	Yellow
Quercetin-3-O-glucoside	Dark yellow	Yellow
Myricetin-3-O-rhamnoside	Dark yellow	Yellow

1 μl of 0.0035 mol l^{-1} methanolic flavonoid solutions; solvent systems I and II.
[a] Weak.

chosen for fluorimetric densitometry. Conspicuous was the intense fluorescence when DTC was added to solutions of kaempferol, its 7-O-glycosides and luteolin.

3.3 Measurements on TLC plates

Visual analysis showed above all intense fluorescing spots of 3′,4′-dihydroxy flavone and 3-hydroxy flavones with (yellow or orange fluorescence) or without (green or turquoise fluorescence) *o*-dihydroxy groups in ring B (Table 2).

In comparison with NA fluorescence, the colours with DTC were similar but generally tended to shorter wavelengths (*e.g.*, light orange instead of orange for quercetin; turquoise instead of green for kaempferol). Kaempferol, kaempferide and the 7-O-glycosides of kaempferol fluoresced very intensely turquoise, whereas the 3-O-glycosides of kaempferol showed a weaker greenish fluorescence. Obviously the hydroxyl at C-3 was necessary for complexation of flavonols lacking two adjacent hydroxyls in ring B. The fluorescent colours on polyamide and cellulose layers (TLC systems III to VII) were commensurate with those on silica gel.

In order to determine the fluorescence intensities, densitometric measurements on DTC-de-

Table 3
Fluorimetric densitometry of different flavonoids detected with DTC and NA on silica gel layers

Compound	R_F (I)	DTC	NA
Aglycones			
Genistein (5,7,4′-OH-isoflavone)	0.31	–[a]	0.6
Dihydrofisetin (3,7,3′,4′-OH-flavanone)	0.17	1.7	0.8
3-OH-flavone	0.63	57	52
5-OH-flavone	0.76	0.5	1.8
7-OH-flavone	0.37	–[a]	–[a]
Apigenin (5,7,4′-OH-flavone)	0.33	4.8	101
Luteolin (5,7,3′,4′-OH-flavone)	0.25	343	236
Kaempferol (3,5,7,4′-OH-flavone)	0.30	311	156
Kaempferide (3,5,7-OH-4′-OMe-flavone)	0.33	131	90
Quercetin (3,5,7,3′,4′-OH-flavone)	0.21	90	45
Isorhamnetin (3,5,7,4′-OH-3′-OMe-flavone)	0.30	98	24
Rhamnetin (3,5,3′,4′-OH-7-OMe-flavone)	0.31	70	23
Myricetin (3,5,7,3′,4′,5′-OH-flavone)	0.09	52	24
Morin (3,5,7,2′,4′-OH-flavone)	0.10	385	288
Glycosides	R_F (II)		
Luteolin-5-O-glucoside	0.30	231	222
Luteolin-7-O-glucoside	0.47	299	79
Luteolin-7,3′-O-diglucoside	0.10	1.2	64
Kaempferol-3-O-rhamnoside	0.73	5.5	64
Kaempferol-3-O-glucoside	0.55	13	69
Kaempferol-3-O-glucuronide	0.45	8.1	61
Kaempferol-3,7-O-dirhamnoside	0.44	5.8	85
Kaempferol-7-O-rhamnoside	0.79	309	266
Kaempferol-7-O-neohesperidoside	0.27	309	221
Quercetin-3-O-rhamnoside	0.66	55	50
Quercetin-3-O-glucoside	0.49	66	42
Myricetin-3-O-rhamnoside	0.53	50	54

1 μl of 0.0035 mol l^{-1} methanolic solutions; solvent systems I and II; λ_{ex} = 436 nm; AUC (peak area) × 10^{-3}; R.S.D. = 5.2% (on average) (n = 4).
[a] Not quantifiable.

tected flavonoids were made on silica gel layers and compared with those for NA as a chromogenic reagent. Before *in situ* measurements the fluorescence was enhanced and stabilized by spraying with PEG 4000 (5% ethanolic solution) and liquid paraffin according to ref. 11. Preliminary investigations showed that additionally a constant temperature was necessary to obtain sufficient fluorescence stability. Therefore, all measurements were carried out at 20°C (temperature of TLC plates), which decreased the R.S.D. to 5.2% (on average) ($n = 4$). Measurements at the "normal" instrument temperature (varying from 25 to 32°C) gave R.S.D.s up to 10.4% ($n = 4$).

Flavones with two adjacent hydroxyl groups in ring B (luteolin) and flavonols (especially kaempferol and morin) formed the most intensely fluorescent complexes (Table 3).

In comparison with NA detection, the peak areas of DTC complexes of these compounds were up to four times larger. When detected with DTC glycosidation in position 7 or 5 had no marked influence on the fluorescence intensity compared with the corresponding aglycones (see, *e.g.*, kaempferol, kaempferol-7-O-rhamnoside and kaempferol-7-O-neohesperidoside). Methylation of the hydroxyl at the 4′-position (compare kaempferol and kaempferide) reduced the fluorescence but it still was higher than that of NA-detected kaempferide. On the other hand, kaempferol-3-O-glycosides and luteolin-7,3′-diglucoside showed a weak fluorescence. Glycosidation in these positions did not have such an influence when detected with NA. Hence for qualitative analysis comparative detection with DTC and NA can indicate the position of the glycosidated hydroxyl groups of kaempferol and luteolin glycosides.

Quantitative measurements of quercetin–DTC and –NA complexes every 5 min over a period of 40 min indicated a decrease in the DTC peak area of 3.8% within 15 min and a further 3.5% in the following 25 min. The decreases with NA detection were 13.8% and 3.1%, respectively. The R.S.D. range of densitometric measurements was 4.3–7.1% (5.2% on average) ($n = 4$). Densitometric measurements were best made at least 15 min after spraying.

Our investigations showed that DTC is suitable for both the qualitative and quantitative analysis of 3′,4′-dihydroxy flavones and of flavonols, especially kaempferol and its glycosides with a free 3-OH group.

References

[1] T.A. Geissmann, *The Chemistry of Flavonoid Compounds*, Pergamon Press, Oxford, 1962.
[2] J.B. Harborne, T.J. Mabry and H. Mabry, *The Flavonoids*, Chapman & Hall, London, 1975.
[3] P.P.S. Schmidt, *J. Chromatogr.*, 157 (1978) 217.
[4] R. Neu, *Fresenius' Z. Anal. Chem.*, 142 (1954) 335.
[5] R. Neu, *Fresenius' Z. Anal. Chem.*, 151 (1956) 328.
[6] H. Homberg and H. Geiger, *Phytochemistry*, 19 (1980) 2443.
[7] I.M. Hais and K. Macek, *Handbuch der Papierchromatographie*, Gustav Fischer, Jena, 1958.
[8] M. Billeter, B. Meier and O. Sticher, *J. Planar Chromatogr.*, 3 (1990) 370.
[9] T. Hayashi, K. Hara, S. Kawai and T. Ohno, *Chem. Pharm. Bull.*, 18 (1970) 1112.
[10] H. Jork, W. Funk, W. Fischer and H. Wimmer, *Dünnschichtchromatographie*, Vol. 1b, VCH, Weinheim, 1993.
[11] H. Jork, W. Funk, W. Fischer and H. Wimmer, *Dünnschichtchromatographie*, Vol. 1a, VCH, Weinheim, 1989.