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Effect of fluorescence intensifiers on the fluorodensitometric determination of flavones and flavonols after detection with diphenylboric acid 2-aminoethyl ester

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Abstract

The measurement of the fluorescence emitted by flavones and flavonols in situ on thin-layer chromatography plates can be improved by dipping the developed plates in appropriate solutions. The effect of different substances on the intensity and stability of the fluorescence of 14 flavone and 26 flavonol derivatives detected with diphenylboric acid 2-aminoethyl ester (Naturstoffreagens A, NA) is described. Silicone oil or paraffin is most appropriate for lipophilic flavonoids while the more hydrophilic polyethylene glycol 4000 is most appropriate for less lipophilic aglycones as well as glycosides. Authentic reference standards in appropriate concentrations must be measured on the same plate as the experimental sample since flavonoids demonstrate specific fluorescence behaviour and because in situ fluorodensitometry measurements are difficult to reproduce. The correlation between fluorescence and molecular structure is discussed briefly. The test conditions established are an improvement on the accepted in situ fluorodensitometry determination of flavonoids.

Keywords: Fluorescence detection; Detection, TLC; Flavones; Flavonols; Diphenylboric acid 2-aminoethyl ester

1. Introduction

Many substances, including flavonoids, fluoresce more strongly on cellulose, as a chromatographic material, than on silica gel [1–3]. Consequently, initial fluorescence enhancement is not necessary; however, after detection with diphenylboric acid 2-aminoethyl ester (Naturstoffreagens A, NA), the fluorescence emitted by flavonoids on cellulose decreases in intensity and stability. When $AlCl_3$ or $Al_2(SO_4)_3$ are used in a detection system, the already extended and blurred flavonoid bands enlarge. This unsatisfactory separation is further degraded by a dipping procedure. Poor separation coupled with the limited capacity of cellulose as a coating material, significantly limits the range of linear detectability. These weaknesses constitute a serious argument against the use of cellulose as a coating substance in the fluorodensitometry of flavonoids. Since silica gel is a suitable chromatographic material for the separation of flavonoids, we tried to improve the fluorodensitometric determination by applying various fluorescence intensifiers after detection with NA.

An exact quantification by fluorodensitometry is not possible for many flavonoids since the emission is not stable enough. While there are many publications dealing with the densitometric quantification

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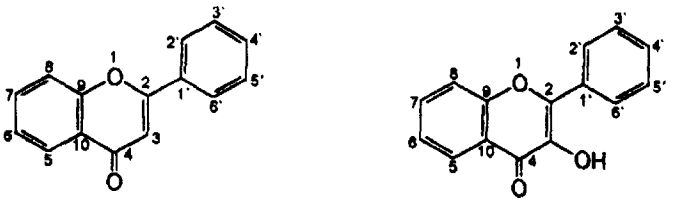

Fig. 1. Structures of flavone and flavonol.

of flavonoids after reaction with NA [4] describing various analytical procedures [5,6], the physicochemical investigations that are reported deal almost exclusively with measurements in solution [7,8]. Since we had experience in the fluorodensitometric determination of hypericin and pseudohypericin [9] we investigated the effect of fluorescence enhancement and stabilizing agents on the fluorodensitometric determination of flavones and flavonols (Fig. 1) after detection with NA (Fig. 2).

2. Experimental

2.1. Chemicals

The sources of the flavonoids were as follows: primuletin (Roth, Karlsruhe, Germany, product No. 7103), 6-OH-flavone (Roth, product No. 6634), 7-OH-flavone (Roth, product No. 5266), chrysin (Roth, product No. 6475), apigenin (Roth, product No. 7002), apigenin-5-glucoside[1], apigenin-7-glucoside (Roth, product No. 6348), vitexin (Roth, product No. 5579), acacetin (Roth, product No. 6531), luteolin (Roth, product No. 4546), luteolin-5-glucoside (Roth, product No. 8960), luteolin-7-glucoside (Roth, product No. 4164), orientin (Roth, product No. 6151), chrysoeriol (Roth, product No. 6044), 3-OH-flavone (Roth, product No. 7659), fisetin (Roth, product No. 7457), galangin (Roth, product No. 7460), kaempferol (Roth, product No. 7503), robinin (Roth, product No. 7172), astragalin (Roth, product No. 9321), kaempferol-7-neohesperidoside (Roth, product No. 9467), isokaempferide[2], kaempferide (Roth, product No. 5845), rhamnacitrin[2], ermanin[2], kaempferol-,7,4′-dimethyl ether[2], kaempferol-3,7,4′-trimethyl ether[2], robinetin (Roth, product No. 7421), morin (Roth, product No. 6459), quercetin (Roth, product No. 7138)[3], isoquercitrin (Roth, product No. 7586), azaleatin[1], rhamnetin (Roth, product No. 7418), isorhamnetin (Roth, product No. 7589), tamarixetin (Roth, product No. 7425), quercetin-3,7-dimethyl ether (Roth, product No. 5157), quercetin-3,7,3′,4′-tetramethyl ether (Roth, product No. 7221), myricetin (Roth, product No. 4187), myricitrin (Roth, product No. 9391), quercetagetin (Roth, product No. 6962)

The other chemicals used were as follows: viscous

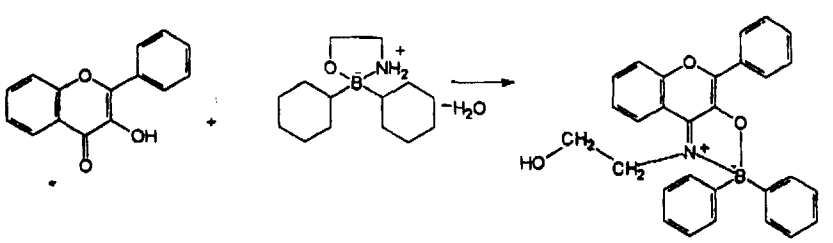

Fig. 2. Reaction between 3-hydroxy-flavone and NA [5].

[1]Generous gift of Prof. H. Geiger.

[2]Generous gift of Prof. E. Wollenweber.

[3]Purified by TLC.

paraffin (Merck, Darmstadt, Germany, product No. 7160), highly liquid paraffin (Merck, product No. 7174), liquid paraffin (Merck, product No. 7162), paraffin in block form (Merck, product No. 7150), poly(ethylene glycol) 4000 (Merck, product No. 807490), silicone oil DC 200, 110 mPa·s (polydimethylsiloxane) (Fluka, Buchs, Swiss, product No. 85414), silicone oil DC 200, 33.3 Pa·s (polydimethylsiloxane) (Fluka, product No. 85424), silicone oil DC 200, 375 mPa·s (polydimethylsiloxane) (Fluka, product No. 85416), silicone oil DC 200, 53 Pa·s (polydimethylsiloxane) (Fluka, product No. 85413), silicone oil DC 200, 66.9 Pa·s (polydimethylsiloxane) (Fluka, product No. 85425), silicone oil for oil baths (Merck, product No. 7742), Triton X-100 (Merck, product No. 8603), polyoxyethylene 100 stearate (Sigma, product No. P 3690), NA (Roth, product No. 9920.2).

2.2. Thin-layer chromatography

TLC aluminium sheets coated with silica gel 60 (without fluorescent indicator), 5×10 cm (Merck, product No. 16835), were developed in unsaturated tanks (Camag, Muttenz, Swiss, product No. 022.5155). A 3-μl aliquot of the test solutions (1 mg of flavonoid in 10 ml of methanol) were applied as 6-mm wide bands (micropipette by Haack, Vienna, Austria). The mobile phase for aglycones was toluene–ethyl formiate–formic acid (5:4:1) [10] and for glycosides ethyl acetate–formic acid–water (100:10:5) [11]. After development over 8 cm, solvent was evaporated in a warm airstream (ca. 10 min) and immediately afterwards the plate was prepared for fluorodensitometric determination.

2.3. Preparation of the plate for fluorodensitometric determination

The developed and dried plate was first dipped in one stroke (=1 s) in the direction of development into a solution of 1% (m/v) NA in diethyl ether–methanol (2:1) (solution A) in a dipping tank (Desaga, Heidelberg, Germany, product No. 12 41 52). After the solvent was evaporated in a warm airstream (ca. 10 min) the plate was dipped in one stroke (=1 s) in the direction of development into solution B also in a dipping tank. Solution B was 60% (v/v) silicone oil 110 mPa·s in diethyl ether, 10% (m/v) poly(ethylene glycol) 4000 (PEG) in diethyl ether–methanol (2:1) or 60% (v/v) viscous paraffin in diethyl ether.

Subsequently the plate was situated vertically on a flat, not absorbing surface for 45 min and the emitted fluorescence was then measured.

2.4. Equipment

A Shimadzu CS 9000 dual-wavelength flying-spot scanner was used with the following settings: photo mode: fluorescence normal; λ_{ex}=313, 366 or 436 nm; for λ_{ex}=313 nm, emission filter 1 was used for emission, for λ_{ex}=366 nm and 436 nm, emission filter 3 was used for emission. Zero set mode: At start; beam size 0.4×10.0; delta "y"=0.04; peak detection: pkf filter: 2; drift line: 0.0000; mini width: 2.0; mini area: 100. Signal process: b.c. accum: 8; accum No.: 1; linearizer: off; smoothing: 7 points. Output: area.

2.5. Comments on the procedure of the fluorodensitometric measurement

The development and preparation of the plate as well as the measurement of the fluorescence have to be done in one stroke. The times mentioned have to be observed exactly. For quantifying a flavonoid the substance has to be applied in various amounts (100 ng to 500 ng). To set up the calibration curve 100 ng and 500 ng of the authentic reference substance have to be applied to the same plate. The fluorodensitometric measurement of the prepared plate in the densitometer has to be finished within 30 to 35 min. The fluorescence intensity is expressed in peak-area units. All procedures have to be done at 22°C±2°C.

Detection limit: 20 ng to 50 ng, depending on the structure of the flavonoid. Quantitation range: 100 ng to 500 ng. Relative standard deviation ($R.S.D._{within}$) of fluorodensitometric measurement: 0.4% to 9.0%, depending on the structure of the flavonoid.

Table 1
Intensity and stability of the fluorescence of flavones and flavonols after detection with NA without and with fluorescence intensifier (FI)

Substance	OH	OMe	OGlyc	CGlyc	λ_{ex}	$\bar{x}$	Without FI					With FI				
							R.S.D.$_b$	% Relint	R.S.D.$_w$	Intf	$\bar{x}$	R.S.D.$_b$	% Relint	R.S.D.$_w$	f	Δs
Flavones																
Primuletin	5				313, 366, 436	–	–	–	–	Si	–	–	–	–	–	–
6-Hydroxy flavone	6				313	19 200	24	13.2	3.6	Si	53 300	15	10.4	0.7	5.6	s.
7-Hydroxy flavone	7				313	20 000	40	13.7	2.4	Si	63 700	36	12.4	0.5	3.2	s.
Chrysin	5,7				313, 366, 436	–	–	–	–	Si	–	–	–	–	–	–
Apigenin	5,7,4′				366	320	66	0.2	17.3	Si	6250	41	1.2	7.3	19.8	s.
Apigenin-5-glucoside	7,4′		5		313	110 000	22	76.3	11.0	PEG	117 500	24	22.9	5.0	1.1	s.
Apigenin-7-glucoside	5,4′		7		313, 366, 436	–	–	–	–	PEG	3000	33	0.6	7.4	–	–
Vitexin	5,7,4′			8	313, 366, 436	–	–	–	–	PEG	3170	21	0.6	8.2	–	–
Acacetin	5,7	4′			436	1390	24	1.0	48.6	Si	2780	18	0.5	9.0	2.0	s.
Luteolin	5,7,3′,4′				436	11 700	28	8.1	19.5	PEG	132 600	8	25.9	1.9	11.3	s.
Luteolin-5-glucoside	7,3′,4′		5		436	2200	19	1.5	16.7	PEG	136 600	30	26.7	1.4	62.1	s.
Luteolin-7-glucoside	5,3′,4′		7		436	17 200	29	11.9	5.3	PEG	64 500	28	12.6	0.9	2.0	s.
Orientin	5,7,3′,4′			8	436	70 000	34	48.1	7.9	PEG	103 600	21	20.2	3.5	1.5	s.
Chrysoeriol	5,7,4′	3′			436	900	51	0.6	70.5	PEG	16 000	37	3.1	4.3	17.5	s.
Flavonols																
3-Hydroxy flavone	3				436	6900	22	4.8	5.9	Si	33 600	(20)	6.6	4.0	4.9	s.
Fisetin	3,7,3′,4′				436	2600	28	1.8	5.2	Pa	45 800	(28)	8.9	6.4	17.8	–
Galangin	3,5,7				436	15 400	17	10.6	9.4	PEG	100 000	15	19.5	1.5	6.6	s.
Kaempferol	3,5,7,4′				436	35 000	32	24.1	6.3	PEG	105 500		20.6	0.5	3.0	s.
Robinin	5,4′		3,7		436	4600	41	3.2	17.3	PEG	19 500	36	3.8	3.7	4.2	s.
Astragalin	5,7,4′		3		313, 366, 436	–	–	–	–	PEG	–	–	–	–	–	–
Kaempferol-7-neohesperidoside	3,5,4′		7		436	19 900	18	13.7	6.7	PEG	198 000	(12)	38.6	1.5	9.9	s.
Isokaempferide	5,7,4′	3			313, 366, 436	–	–	–	–	PEG	–	–	–	–	–	–

Kaempferide	3,5,7	4′		436	38 500	21	26.6	6.9	PEG	136 000	14	26.6	2.8	3.5	s.
Rhamnacitrin	3,5,4′	7		436	24 500	31	16.9	2.6	PEG	96 600	30	18.9	1.0	3.9	s.
Ermanin	5,7	3,4′		313	1660	34	1.1	7.2	Pa	4400	35	0.9	7.2	2.6	n.s.
Kaempferol-,7,4′-dimethyl ether	3,5	7,4′		313, 366, 436	–	–	–	–	Pa	5200	21	–	7.7	–	–
Kaempferol-3,7,4′-trimethyl ether	5	3,7,4′		313	1800	24	1.2	16.5	Si	4800	28	0.9	6.4	2.8	n.s.
Robinetin	3,7,3′,4′,5′			436	12 100	14	8.4	6.3	PEG	26 200	21	5.1	3.7	2.2	s.
Morin	3,5,7,2′,4′			436	145 000	23	100.0	40.9	PEG	512 000	23	100.0	1.7	3.5	s.
Quercetin	3,5,7,3′,4′			436	18 600	17	12.8	9.8	PEG	27 400	27	5.3	2.7	1.5	s.
Isoquercitrin	5,7,3′,4′		3	436	1350	33	0.9	5.7	PEG	24 700	37	4.8	2.7	18.2	s.
Azaleatin	3,7,3′4′	5		436	78 900	15	54.4	1.2	PEG	111 000	11	21.8	0.4	1.4	s.
Rhamnetin	3,5,3′4′	7		436	1430	48	1.0	4.0	PEG	5100	36	1.0	5.8	3.5	s.
Isorhamnetin	3,5,7,4′	3′		436	34 400	38	23.7	4.3	PEG	95 400	18	18.6	2.3	2.8	s.
Tamarixetin	3,5,7,3′	4′		436	17 300	26	11.9	3.6	PEG	90 600	10	17.7	2.2	5.2	n.s.
Quercetin-3,7-dimethyl ether	5,3′,4′	3,7		436	6300	34	4.3	1.3	PEG	15 000	21	2.9	1.2	2.4	n.s.
Quercetin-3,7,3′,4′-tetramethyl ether	5	3,7,3′,4′		436	3550	12	2.5	9.7	Pa	17 500	18	3.4	2.5	4.9	s.
Myricetin	3,5,7,3′,4′,5′			436	13 300	29	9.2	7.1	PEG	21700	26	4.2	3.8	1.6	s.
Myricitrin	5,7,3′,4′,5′		3	436	4700	31	3.2	12.6	PEG	10 900	39	2.1	7.3	2.3	n.s.
Quercetagetin	3,5,6,7,3′,4′			436	17 300	40	11.9	3.6	PEG	18 700	28	3.7	4.5	2.7	n.s.

λ_{ex}=Excitation wavelength.

$\bar{x}$=Means of peak areas (corresponding to intensity).

R.S.D.$_b$=R.S.D.$_{between}$ (obtained by calculating the variation between the mean values of the fluorescence of each chromatogram, $n=5$).

% Relint=% of the fluorescence intensity of morin.

R.S.D.$_w$=R.S.D.$_{within}$ (obtained by pooling the R.S.D.s for each chromatogram, $n=10$).

Intf=Fluorescence intensifier.

f=Factor of increase in intensity.

Δs=Difference of relative standard deviations.

s.=The difference of relative standard deviations is significant. ($\alpha=0.05$).

n.s.=The difference of relative standard deviations is not significant. ($\alpha=0.05$).

Si=Silicone oil 110 mPa·s, 60% (m/v) in diethyl ether.

PEG=Poly(ethylene glycol) 4000, 10% (m/v) in diethyl ether–methanol (2:1).

Pa=Viscous paraffin, 60% in diethyl ether.

2.6. Establishing the values of Table 1 and Table 2

For each flavonoid, five separate chromatograms were performed. The spot of every chromatogram was measured ten times and mean values and $R.S.D._{within}$ were calculated. In addition to the mean value, the fluorescence percentage in relation to that of morin (100%) was provided in both tables to facilitate better understanding.

Whilst the mean values in some cases differed widely, the $R.S.D._{within}$ of the five chromatograms were quite similar. This seems to prove that statements about the stability of the fluorescence of flavonoids are reliable, the fluorescence intensity on the other hand may differ from plate to plate.

3. Results and discussion

3.1. Intensity and stability of fluorescence

To find out the effect of fluorescence intensifiers initially, fluorescence intensity and stability of the differently substituted flavones and flavonols were measured after reaction with NA without using a fluorescence-enhancing agent.

It is evident that the emission of seven flavonoids is too weak for quantification. The emissions of the remaining flavonoids differed considerably. The stability of the emission ($R.S.D._{within}<3.0$) seems only to be sufficient for the quantification of 7-hydroxyflavone, rhamnacitrin, azaleatin and quercetin-3,7-dimethyl ether but not for the remaining compounds.

As PEG, which usually is used as a fluorescence intensifier [5,6], only insufficiently enhances the emission and stability of some flavonoid chelates, further agents were tried out for the fluorodensitometric determination of flavones and flavonols. Different concentrations of agents, that were already known to have some fluorescence and stability enhancing properties, were employed as dipping solutions after detection with NA. Mean values and relative standard deviations were determined as described (see Table 1). The intensity factor f shows the enhancement in intensity after application of the fluorescence intensifier. By comparing the R.S.D.s using the F-test ($\alpha=0.05$), the significance of a stabilizing effect was evaluated. The effects obtained with different enhancers are demonstrated using isoquercitrin as an example (see Table 2).

With all flavonoids we found that the nature and concentration of the fluorescence enhancing agents may affect emission enhancement to a very different degree. In some cases, a reduction of fluorescence could even be seen. PEG, silicone oil, paraffin and the mixture of PEG and silicone oil are suitable agents. Applying these agents again and again the agents in Table 1 effected the best enhancement of emission and stability of the flavonoids in question. It became evident that, for the most lipophilic flavonoids silicone oil or paraffin and for the less lipophilic aglycones as well as for the flavonoid glycosides, the more hydrophilic PEG is most appropriate. This observation allows the conclusion, that fluorescence enhancement of flavonoid–NA chelates and of non-complex forming flavonoids on silica gel is based predominantly on the solubility effects, discussed by Jork et al. [5] as well as by Poole et al. [1].

As Table 2 shows, the concentration of the fluorescence intensifier in the dipping solution and consequently the amount of the agent applied to the plate greatly influences the intensity and stability of the emission. In addition the agent has to be applied to the plate as homogeneously as possible. Of all the described application methods [1,5,12] dipping is most appropriate [5], not least because of the large molecular mass of most fluorescence enhancing and stabilizing agents and the viscosity of their solutions. The best results are obtained by dipping the chromatogram at one stroke in the direction of development. Subsequently the surplus solution is allowed to drain by placing the plate vertically on a flat, non-absorbing support. It is inevitable that the upper and lower edges of the plate remain in the dipping solution for different times. Additionally during draining, the solvent evaporates partly and the viscosity increases. Thus during this procedure the fluorescence intensifier is not spread homogeneously over the plate but only transverse to the direction of development. Consequently only the fluorescence intensity of substances with the same R_F-values can be compared.

Table 2
Effects of various substances on the intensity and stability of the fluorescence of isoquercitrin after a basic treatment with NA

Substance	Concentration (%)	Solvent	$R.S.D._{within}$	f	Δs
Highly liquid paraffin	20	Diethyl ether	2.7	1.7	s.
	50		2.7	2.0	s.
	70		3.1	1.9	s.
Liquid paraffin	20	Diethyl ether	2.7	1.3	s.
	50		3.6	1.2	n.s.
	70		3.4	1.9	n.s.
Viscous paraffin	20	Diethyl ether	3.3	2.0	n.s.
	50		3.0	1.1	s.
	70		5.5	1.1	n.s.
Block form paraffin	4	Toluene	–	–	–
Silicone oil for oil baths	20	Diethyl ether	1.8	3.1	s.
	45		1.5	5.2	s.
	50		0.8	4.4	s.
	55		1.3	5.8	s.
	60		0.8	4.7	s.
	65		1.1	4.4	s.
Silicone oil 53 mPa·s	45	Diethyl ether	1.5	4.1	s.
	50		1.9	4.9	s.
	55		1.1	5.3	s.
	60		1.5	5.1	s.
	65		2.1	4.3	s.
	70		2.4	4.7	s.
Silicone oil 110 mPa·s	45	Diethyl ether	1.6	4.5	s.
	50		1.0	5.5	s.
	55		0.9	5.6	s.
	60		1.1	5.8	s.
	65		1.3	4.8	s.
	70		2.6	3.1	s.
Silicone oil 375 mPa·s	20	Diethyl ether	3.2	3.3	n.s.
	25		1.6	4.8	s.
	30		1.9	3.5	s.
	35		1.1	4.5	s.
	40		1.4	5.4	s.
	45		1.2	5.6	s.
	50		1.9	3.6	s.
Silicone oil 33.3 Pa·s	1	Chloroform	2.9	1.7	s.
	3		2.1	1.9	s.
	5		1.4	2.6	s.
	10		4.8	1.0	n.s.
Silicone oil 66.9 Pa·s	1	Chloroform	4.5	1.3	n.s.
	3		3.0	1.9	s.
	5		1.0	3.6	s.
	10		3.2	1.0	n.s.
Triton X-100	1	Chloroform	12.9	0.8	s.
Stearic acid	1	Diethyl ether	9.7	1.0	n.s.
	2		4.7	1.0	n.s.
Glycerol	30	Chloroform–methanol (1:1)	2.6	2.0	s.
PEG 4000	5	Chloroform	2.3	4.3	s.
	10		0.9	5.6	s.
	15		4.8	5.4	n.s.

Table 2 (Continued)

Substance	Concentration (%)	Solvent	$R.S.D._{within}$	f	Δs
PEG + Silicone oil for oil baths	10+15	Chloroform	1.0	7.5	s.
	10+10		1.1	7.4	s.
	10+5		1.1	5.8	s.
	3+50		0.8	6.0	s.
PEG + Silicone oil 33.3 Pa.s	7+12	Chloroform	1.0	5.4	s.
PEG + Silicone oil 33.3 Pa.s	6+9	Chloroform	1.8	4.4	s.
Polyoxyethylene 100 stearate	1		0.8	4.0	s.
	5		3.1	2.6	s.
	10		3.6	2.6	n.s.

Conc. (%)=% of fluorescence intensifier in solution.
$R.S.D._{within}$=relative standard deviation (%) (corresponding to stability).
f=Factor of increase in intensity.
Δs=Difference of relative standard deviations.
s.=The difference of relative standard deviations is significant. ($\alpha=0.05$).
n.s.=The difference of relative standard deviations is not significant. ($\alpha=0.05$).

The time the chromatogram spends in the dipping solution is of great importance. While Billeter et al. [6] keep the plates for 6 s in the dipping solution we do so for only 1 s. This short dipping time hinders the diffusion of the bands but requires dipping solutions at higher concentrations.

3.2. Fluorescence and structure

The correlation between structure and fluorescence of flavonoids is described in detail by Homberg and Geiger [13]. As the results of the present study show, the fluorescence enhancement after the application of NA and fluorescence intensifiers is obviously only determined by the nature, number and position of the substituents of the flavonoids. The different behaviour in fluorescence of flavonoids with OH and OCH_3 groups at position 4′ as the only substituents in ring B, like chrysin and apigenin, kaempferide and kaempferol etc., cannot be stated clearly after our investigations.

An explanation for this different behaviour could be the intra- and intermolecular excited-state proton-transfers (ESPT) observed in dissolved flavonoids with free OH groups which lead to tautomers with changed quantitative and qualitative fluorescence [7,8,14–17]. The observations in our study seem to indicate that these ESPT also occur on silica gel and are influenced by the presence of further substituents.

As Table 1 demonstrates the sugar component is important for the fluorescence behaviour of flavonoid glycosides. Even when the sugars do not prevent – like in 3- and 5-O-glycosides – the chelate formation, they influence the fluorescence behaviour. A comparison between apigenin and vitexin (see Table 1), which differ in the C-glycosidic bound sugar in position 8, shows a distinct difference in intensity of fluorescence, that cannot be explained by the different molecular weights only. Orientin, a luteolin–8-C-glycoside, emits without fluorescence enhancing about six times stronger than the aglycone itself. After treatment with PEG dipping solution (see Table 1) this difference is equalised to a dimension approximately that of the difference in the molecular masses.

The explanation of this difference in enhancement might be the following: a glycoside has, because of its sugar component, a different affinity than the aglycone to the coating substance and this influences the fluorescence behaviour. (Reciprocal actions between coating material and the substance, i.e. adsorbent and adsorbate, are able to decrease the fluorescence significantly [1–3,12,18–26]). An appropriate fluorescence intensifier, which does not intensify the emission in fact but abolishes the disturbing effects of the coating material, can equalise the difference in the affinities. A further attempt at explanation is given by Billeter et al. [6], after which OH groups in the *ortho*-position of the sugar

component form complexes with NA and that influences the fluorescence.

The differing fluorescence behaviour of aglycones and glycosides leads one to expect that also the flavonoids, that are glycosidized in the same position, e.g. quercitrin, isoquercitrin and hyperoside, by means of their various sugar components, to exhibit differing fluorescence intensities. Therefore because of the above limitations encountered with the determination of flavonoid glycosides, which differ only in the nature, but not in the position of the sugar component, one must prepare individual calibration curves for each substance.

4. Conclusions

The test conditions established by our investigations represent an amendment of the fluorodensitometric determination of flavones and flavonols. As the tested flavonoids show very different fluorescence behaviour and as fluorodensitometric measurements are difficult to reproduce (see Table 1, $R.S.D._{within}$), authentic reference substances in appropriate concentrations have to be applied to the chromatogram for all flavonoids. A further reason for this precept is the narrow range of concentrations which produce linear changes in fluorescence. Fluorodensitometry is not applicable to all flavones and flavonols without reservation because of the frequently high relative standard deviation (0.4 to 9.0%).

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