THIN-LAYER CHROMATOGRAPHY OF PRESERVING AGENTS

J. W. COPIUS-PEEREBOOM AND HENNY W. BEEKES
*Government Dairy Station, Leiden (The Netherlands)**

(Received August 16th, 1963)

At the present time, the use of substances with preservative properties is wide-spread in food technology. In many countries the addition of preservatives is now regulated by food legislation. Benzoic acid, sorbic acid, the ethyl and propyl esters of *p*-hydroxybenzoic acid, and the corresponding sodium salts, are permitted in most European countries. Reliable methods for the analysis of these preserving agents, preferably by chromatography, are therefore of prime importance to laboratories working in this field.

Many paper chromatographic systems have been devised for the purpose. A suitable system was described by JARCZYNSKI[1], using the upper layer of the mixture *n*-butanol–35% ammonia–water (70:20:10), to which 2.5% of 96% ethanol is added. In the Netherlands many laboratories are using this procedure, *vide* CATS[2]. However, the time-consuming character of the paper chromatographic analysis may sometimes present difficulties. Furthermore, few colour reactions specific for the various types of preservatives have been described in the literature**.

The technique of thin-layer chromatography (T.L.C.) may be advantageous especially since more aggressive reagents can be applied on chromatoplates and the analysis time may be decreased considerably.

Separation of the methyl, ethyl, propyl, and *n*-butyl esters of *p*-hydroxybenzoic acid has been described by GÄNSHIRT[3]. Kieselgel G-coated chromatoplates were strongly activated (2 h at 160°) and developed in the solvent mixture pentane–acetic acid (8:2). The four esters are completely fractionated. However, we did not succeed in separating benzoic acid and sorbic acid either on normally or on strongly activated kieselgel G plates.

At the time of this investigation no other papers dealing with T.L.C. of preservatives had come to our notice.

CELLULOSE CHROMATOPLATES

To begin with, we investigated the migration rates of benzoic acid and sorbic acid in several polar solvent mixtures using chromatoplates coated with a cellulose layer (cellulose powder MN 300, gypsum-free). The best separation, comparatively, of

* Rijkszuivelstation, Vreewijkstraat 12 B, Leiden.

** In our laboratory we are accustomed to use a procedure of developing the spots of sorbic acid and benzoic acid on paper strips. Successive spraying with a bromophenol blue–methyl red mixture and with potassium permanganate yields yellow-green and purple spots respectively. The sorbic acid spot may be detected specifically by spraying first with a 10% potassium dichromate solution and afterwards with a saturated thiobarbituric acid solution. Sorbic acid is revealed by a bright red coloured spot.

these two preservatives was obtained in the *n*-butanol–ammonia–water (70:20:10) mixture described by JARCZYNSKI, but in this system the development procedure still takes about 5–6 h. The degree of separation of benzoic acid and sorbic acid is only slightly better than when paper strips are used (see Fig. 1). The R_F values of several preservatives in this system are given in Table I.

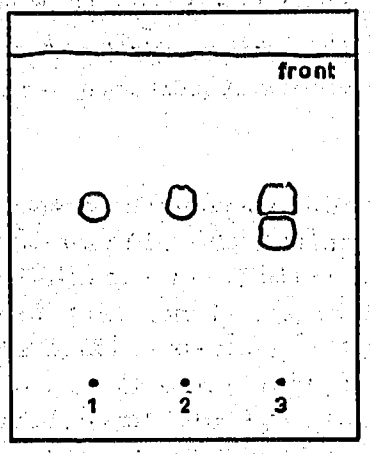

Fig. 1. Separation of preservatives on cellulose plates in *n*-butanol–35% ammonia–water (70:20 10). Spotted amount = 100 μg. Detection: bromophenol blue, followed by potassium permanganate. Spot 1 = salicylic acid. Spot 2 = sorbic acid. Spot 3 = a benzoic acid–sorbic acid mixture (1:1).

In this system the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid are completely separated.

The spots are developed by spraying successively with a bromophenol blue–methyl red mixture and with potassium permanganate[1].

The cellulose plate is dried thorougly in air (with care on account of acid vapour). Bromophenol blue (120 mg) is dissolved in 100 ml of water and 60 mg of methyl red in 100 ml of 96% ethanol. The two solutions are mixed and 100 ml of phosphate buffer (pH = 7.17) is added. The plate is sprayed intensively with this mixture and

TABLE I

R_F AND R_S* VALUES OF PRESERVATIVES ON CELLULOSE CHROMATOPLATES IN *n*-BUTANOL–35% AMMONIA–WATER (70:20:10)

"Kammerübersättigung". Spotted amount = 100 μg.

	R_F value	R_S value
Benzoic acid	0.50	0.91
Sorbic acid	0.58	1.07
Salicylic acid	0.56	≡1.0
Dehydroacetic acid	0.09	0.16
p-Hydroxybenzoic acid	0.09	0.16
Methyl *p*-hydroxybenzoate	0.75	1.42
Ethyl *p*-hydroxybenzoate	0.86	1.62
Propyl *p*-hydroxybenzoate	0.90	1.70
o-Phenylphenol	0.95	1.61

* S = salicylic acid.

afterwards with a solution of potassium permanganate (0.5 %) and sodium carbonate (1 %) (see Table III, reagent 6).

At first the spot of benzoic acid attains a violet colour, but after some time it becomes quite colourless. Upon spraying, sorbic acid exhibits a green colour that changes quickly into a stable purple colour (on a light blue background).

KIESELGEL–KIESELGUR PLATES

Testing several types of adsorbents and mobile phases—among others the system of GÄNSHIRT[3] and the systems of PETROWITZ[4] and BRAUN[5] devised for the separation of polyfunctional acids—we were not able to obtain a separation of benzoic acid and sorbic acid. However, we found that a mixture of equal amounts of kieselgel G–kieselgur G (both of Merck) was suitable for the separation of these preservatives. Especially when using the solvent mixture hexane–acetic acid (96:4) under "Kammerübersättigung"-conditions a distinct separation of benzoic acid and sorbic acid was obtained. With this adsorbent mixture (15 g of adsorbent mixed with 32 ml of water) layers of about 0.25 mm are prepared. The amount of preservative used for spotting is 50 μg and the length of the run is about 20 cm (in 1.5 h). The R_F and R_S (S = salicylic acid) values of some preservatives in this system are given in Table II.

The esters of *p*-hydroxybenzoic acid were not resolved under these circumstances.

TABLE II

R_S (S = SALICYLIC ACID) VALUES OF PRESERVATIVES ON KIESELGEL G–KIESELGUR G (1:1) PLATES IN THE SOLVENT MIXTURES HEXANE–ACETIC ACID (96:4), A, AND PETROLEUM ETHER–ETHER–ACETIC ACID (80:20:1), B.

Spotted amount = 50 μg. Detection: Ultraphor W.T. and Rhodamine B.

	A	B
Benzoic acid	1.54	1.11
Sorbic acid	1.28	0.91
Salicylic acid	≡1.00	≡1.00
Dehydroacetic acid	0.60	0.88
p-Hydroxybenzoic acid	0.07	0.41
Methyl *p*-hydroxybenzoate	0.12	0.75
Ethyl *p*-hydroxybenzoate	0.16	0.79
Propyl *p*-hydroxybenzoate	0.18	0.84
o-Phenylphenol	1.13	1.36

SPOT DEVELOPMENT

The spots of the preservatives are developed by admixing 0.02 % of the fluorescence indicator Ultraphor W.T. (B.A.S.F.) to the adsorbent–water mixture and viewing the chromatoplate under 366 nm radiation (COPIUS-PEEREBOOM[6]). Alternatively, 2 % of "Leuchtpigment ZS-Super" (RIEDEL DE HAAN) is admixed to the adsorbent mixture and the plates are viewed in 254 nm radiation (GÄNSHIRT[3]).

We prefer, however, to use more specific colour reactions which may provide some valuable information as to the identity of unknown preservatives, isolated from food products.

TABLE I

COLOUR REACTIONS OF SOME PRESERVATIVES (S
KIESELGEL G–KIESELGUR G

Compound	1	2	3	4	5	
	Rhodamine B–H_2O_2 366 nm	Bromocresol green	Thiobarbituric acid	Thymol	Successive H_2O_2, $FeCl_3$ sprays	
					A	B
Salicylic acid	purple	orange-yellow	faint purple	—	purple	purple
Benzoic acid	dark purple	yellow	—	—	faint purple (with yellow centre)	*dark brown*
Sorbic acid	pink	yellow	*bright red*	*purple*	faint yellow	—
o-Phenylphenol	purple	—	—	—	—	violet
Dehydroacetic acid	purple	yellow	—	—	yellow-brown	orange-brown
p-Hydroxybenzoic acid	blue	yellow	—	yellow	yellow-brown	green
Methyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray
Ethyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray
Propyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray

After the development procedure, the plate is dried and then intensively sprayed with a 0.05 % Rhodamine B solution. The spot of benzoic acid is coloured violet to purple, while the sorbic acid spot attains a more pink hue. The fluorescence of both spots under 366 nm radiation shows still greater differences. Benzoic acid exhibits a dark purple-blue fluorescence and sorbic acid only a light orange-pink one. These characteristic differences are enhanced by spraying afterwards with a 3 % H_2O_2 solution (Table III, reagent No. 1). The spots have to be marked directly after the spraying procedure, because they tend to diffuse after a time. Both detection procedures, *viz.* 0.02 % of Ultraphor in the adsorbent layer and spraying with the Rhodamine B–H_2O_2 solutions, can be combined advantageously, yielding a strong blue fluorescence of the benzoic acid and a pink one of the sorbic acid spot (366 nm radiation). Of course other acid–base indicators can be used in the same way, *e.g.* bromocresol green (see Fig. 2 and Table III, reagent No. 2).

The spots of benzoic acid and of sorbic acid are clearly separated after one single development. When Rhodamine B is used, the degree of separation of these preservatives is decreased after two or three developments, but when Ultraphor is added to the adsorbent layer a multiple development may be applied.

Analysis of extracts isolated from various types of food products by means of specific colour reagents should give valuable evidence concerning the identity of the preservatives present.

For that purpose we have elaborated several characteristic colour reactions (*vide*

III

(SPOTTED AMOUNTS 100 μg) ON G CHROMATOPLATES

6	7	8	9	10	11	12
Bromophenol blue–$KMnO_4$	*Bromophenol blue –methyl red + Pauly's reagent*	*D.Q.C.*	*p-Nitro-aniline*	*Ceric sulphate*	*$TiCl_3$*	*$FeCl_3$*
yellow	faint yellow	—	—	brown-gray	yellow	purple
faint yellow	—	—	—	—	—	—
yellow	faint yellow	—	—	brown	—	faint yellow
purple	*strong yellow*	*orange-brown*	*brown-red*	*violet*	—	—
yellow	—	gray	—	—	*blue-purple*	brown-yellow
yellow	yellow	yellow	—	faint purple	—	yellow-brown
yellow	—	faint yellow	—	faint yellow	—	—
yellow	—	faint yellow	—	faint yellow	—	—
yellow	—	faint yellow	—	faint yellow	—	—

Table III). Thus, *sorbic acid* is detected specifically by spraying with a saturated solution of thiobarbituric acid (Table III, reagent no. 3). The spot of sorbic acid is revealed by a bright red colour, while most of the other preservatives are not coloured at all. In another colour reaction the plate is sprayed with a 20% ethanolic thymol solution, heated 10 min at 90°, sprayed with 4*N* sulphuric acid, and finally heated 10 min at 120° (reagent No. 4). Sorbic acid is then revealed by a blue-purple spot.

The presence of *benzoic acid* on a chromatoplate is determined as follows: The dried chromatoplate is sprayed with a 3% H_2O_2 solution, heated 5 min at 90°, cooled and sprayed with a 2% ferric(III)chloride solution (reagent no. 5A). The spot of benzoic acid attains a faint purple hue, while sorbic acid is coloured faintly yellow. The plate is heated 5 min at 90°, sprayed again with 2% ferric(III)chloride and afterwards with 3% H_2O_2. The colour of the sorbic acid spot now fades away quickly, whereas benzoic acid is at first revealed as a white spot on a yellowish background. After some time (about 1 h) benzoic acid attains a dark brown colour, while the sorbic acid spot has become completely colourless (reagent No. 5B).

The spot of *o-phenylphenol* can be revealed by several specific reagents *viz.* (a) bromophenol blue–methyl red and PAULY's reagent, (b) dichlorobenzoquinone chlorimine (D.Q.C.), (c) *p*-nitroaniline, and (d) ceric(IV)sulphate.

(a) The plate is sprayed with the bromophenol blue–methyl red reagent mentioned above (see cellulose plates) followed by PAULY's reagent (diazotized sulphanilic acid). The spot of *o*-phenylphenol is coloured bright yellow (reagent No. 7).

(b) The plate is sprayed with a 1 % solution of 2,6-dichloro-*p*-benzoquinone-4-chlorimine (reagent No. 8), dried and sprayed with a 2 % borax solution (in 40 % ethanol). The *o*-phenylphenol spot is revealed by an orange-brown colour.

(c) The plate is sprayed with a solution of diazotized *p*-nitroaniline (reagent No. 9). The spot of *o*-phenylphenol has a brown-red colour.

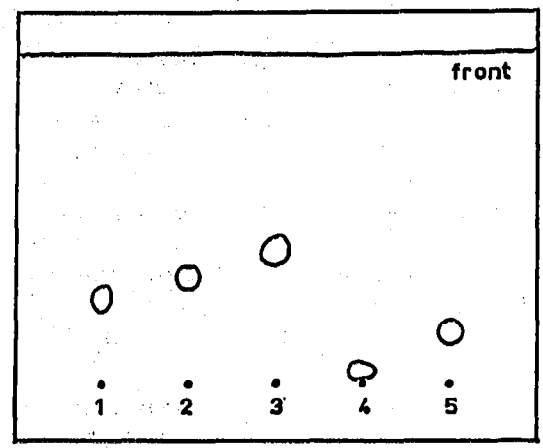

Fig. 2. T.L.C. of preservatives on kieselgel G–kieselgur G plates using the mobile phase hexane–acetic acid (96:4). Spotted amount = 100 μg. Detection: bromocresol green (0.04 % in 96 % ethanol with addition of potassium hydroxide till green, *vide* Table III, reagent No. 2). Spot 1 = salicylic acid. Spot 2 = sorbic acid. Spot 3 = benzoic acid. Spot 4 = *p*-hydroxybenzoic acid. Spot 5 = dehydroacetic acid.

(d) Trichloroacetic acid (1 g) is dissolved in 4 ml of a 2.5 % solution of ceric(IV) sulphate and heated. Concentrated sulphuric acid is added until the solution remains clear. When sprayed with this mixture (reagent No. 10), the *o*-phenylphenol attains a violet colour.

The presence of *dehydroacetic acid* can be detected by spraying the chromatoplate with a 3 % titanium(III)chloride solution (reagent No. 11). Salicylic acid attains a yellow colour, dehydroacetic acid a purple-blue one.

The spot of *salicylic acid* is revealed specifically on Ultraphor-free chromatoplates by a bright fluorescence under 366 nm radiation and by a purple colour when sprayed with a 2 % ferric(III) chloride solution (reagent No. 12).

The colours in the above reactions given by a number of preservatives are listed in Table III.

Many other solvent systems were tested, but in none of them could benzoic acid and sorbic acid be separated distinctly. A comparatively good separation was also achieved using the solvent mixture petroleum ether–ether–acetic acid (80:20:1), as is shown in Table II. In that system the spots of benzoic acid and sorbic acid were separated after three successive developments (detection: Ultraphor W.T.). Salicylic acid and benzoic acid could not, however, be resolved under these conditions.

Applying the kieselgel G–kieselgur G/hexane–acetic acid (96:4) system and selecting the most appropriate colour reactions, the identity of a preservative can be definitely determined. The procedures described above were amply tested in practice, especially in the analysis of preservatives isolated from food products such as margarines, packaging materials and the plastic dispersions used as cheese coating.

KIESELGEL PAPER

We have succeeded in performing analogous separations on commercial Schleicher and Schüll, no. 289 kieselgel papers. The *p*-hydroxybenzoic acid esters are separated in the solvent mixture hexane–acetic acid (96:4), whereas the separation of benzoic acid and sorbic acid can be accomplished with hexane–acetic acid (99.8:0.2). The preservative spots are developed on the kieselgel paper by spraying with 0.05 % Rhodamine B or with 0.5 % quinine bisulphate (*vide* Fig. 3).

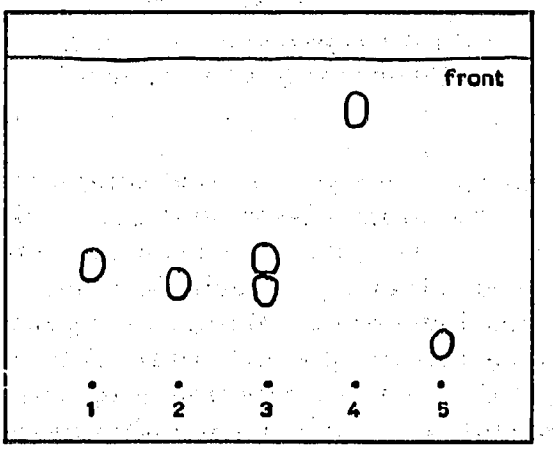

Fig. 3. Separation of preservatives on Schleicher and Schüll, No. 289 kieselgel paper with the mixture hexane–acetic acid (99.8:0.2). Spotted amounts = 80 μg. Detection: 0.05 % of Rhodamine B. Spot 1 = benzoic acid. Spot 2 = sorbic acid. Spot 3 = mixture 1 + 2. Spot 4 = *o*-phenylphenol. Spot 5 = dehydroacetic acid.

ACKNOWLEDGEMENT

The authors would like to express their gratitude to Dr. J. G. VAN GINKEL, Director of the Rijkszuivelstation (Government Dairy Station) for his permission to develop these methods and to publish the results in this paper.

SUMMARY

The separation of preserving agents and especially of benzoic acid and sorbic acid by thin-layer chromatography was studied. Both preservatives can be separated on cellulose chromatoplates using the upper phase of the solvent mixture *n*-butanol–35 % ammonia–water (70:20:10). Still better and much more rapid separations are obtained on chromatoplates coated with a kieselgel G–kieselgur G (1:1) adsorbent mixture, especially when developing the plate with the mobile phase hexane–acetic acid (96:4).

Several specific colour reactions are described which can be applied in the analysis of the preservatives isolated from food products, packaging material etc.

REFERENCES

1 R. JARCZYNSKI AND F. KIERMEIER, *Z. Lebensm. Untersuch. -Forsch.*, 99 (1954) 91.
2 H. CATS AND H. ONRUST, *Chem. Weekblad*, 54 (1958) 456.
3 H. GÄNSHIRT AND K. MORIANZ, *Arch. Pharm.*, 293 (1960) 1065.
4 H. J. PETROWITZ AND G. PASTUSKA, *J. Chromatog.*, 7 (1962) 128.
5 D. BRAUN AND H. GEENEN, *J. Chromatog.*, 7 (1962) 56.
6 J. W. COPIUS-PEEREBOOM, *J. Chromatog.*, 4 (1960) 323.