

CHROM. 5712

AN APPARATUS FOR CONTINUOUS FLOW DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY*

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(First received June 28th, 1971; revised manuscript received September 16th, 1971)

SUMMARY

Examination of various methods of development showed that sorption of solvent vapour accelerated penetration of the solvent into the thin layer, and eliminated the solvent demixing that is often observed in the dry filling system.

A mechanical device was therefore constructed to provide a continuous supply of the solvent vapour to the cover plate of the sandwich-type developing chamber. This method was combined with overflow development, opening the tip of the developer to air, which resulted in an increased developing distance by continuous flow, and the sample was concentrated at the solvent front more easily.

The advantages of the present apparatus are simplicity of operation, rapid migration of samples owing to rapid saturation of vapour, and good reproducibility and separation.

INTRODUCTION

One of the greatest disadvantages of thin-layer chromatography (TLC) lies in its poor reproducibility, and one of the many factors responsible for this is incomplete saturation of the developing chamber with solvent vapour^{1,2}. In the box-type developing chamber, a long time is required to saturate the large volume of the box with the solvent vapour, and development is started before complete saturation of the chamber occurs, which leads to poor reproducibility.

To reduce the space surrounding the thin layer, the so-called sandwich-type developing chamber³⁻⁷ was devised. However, DE ZEEUW⁸ has pointed out that when a mixed solvent system is used, the results of separation with this sandwich-type chamber are often entirely different from those obtained with the box-type developing chamber. In addition to the results obtained during separation, there were many unsettled points concerning the correlation between these two types of developing method. Therefore, the present authors made a detailed comparative examination and then confirmed the differences between the two methods.

* This paper was read at the 4th International Symposium "Identification of Substances by PC and TLC" and a summary appeared in *J. Chromatogr.*, 48 (1970) 143.

In the sandwich-type developing chamber, penetration of the solvent into the thin layer is much slower than that in a saturated developing chamber, and this was considered to be due to evaporation of the solvent from the thin-layer surface. Consequently, in order to saturate the volume around the thin layer with the solvent vapour, a mechanism on the cover plate by which the solvent vapour is supplied continuously was devised. As expected, this device resulted in a better developing rate, with a separation that was the same as that obtained when the completely saturated box-type developing chamber was used, even when a mixed solvent system composed of polar solvents was used.

Moreover, the new method retained all the desirable features inherent in the sandwich-type chamber, and combination of this method with the continuous-flow technique^{9,10} was found to offer a system having many advantages. Fundamental experiments carried out to examine this newly developed apparatus and method are described in this paper.

EXPERIMENTAL

Thin layer

A thin layer prepared by the standard method¹¹ using Wakogel-B (silica gel for TLC, Wako Pure Chemical Co., Tokyo) and a thin-layer glass plate manufactured by the Merck Co. were used.

Sample

Nine kinds of the dyes of NEHER *et al.*¹² were used as 2% solutions in acetone, the F-62 being purified by preparative TLC¹⁰.

Sample applicator

The applicator used was the one reported by MONTEIRO¹³.

Development apparatus (Fig. 1)

The cover plate was a glass plate 3 mm thick, with a glass piece 0.5×3 mm in cross-section attached to both sides and the upper part of the plate by fusion, and glass spacers 2×6 mm in cross-section attached to both ends along the longer axis to make a groove, as shown in Fig. 2a.

The cover plates provided for sizes 20 cm in width with heights of 15, 13.5, 12, 10.5, 9, 7.5 and 6 cm. For the thick layer, a cover plate having a spacer 5×6 mm in cross-section, with another spacer 2×4 mm in cross-section attached to the top of the plate, was also used. These plates were also used in the seven sizes as noted above.

Other components of the apparatus were a stainless-steel spring, $0.2 \times 4 \times 195$ mm (Fig. 2a), a glass lifter consisting of a glass rod, $10 \times 10 \times 200$ mm, and clips, cast-iron stands, a brass support and a rectangular glass box for the solvent.

Method of development

The developing solvent is placed in the tank and the horizontal position of the tank and the height of the solvent (8 mm) are checked. About 10–15 mm of both sides of the thin layer are scraped off with a spatula (Fig. 2b). Sample solution is applied to the thin layer, 15 mm from the lower end of the adsorbent layer, as a spot or a line.

The two ends of a filter-paper or glass-fibre paper are inserted into the grooves on both ends of the cover plate and secured in place with 2 or 3 springs also inserted into the same grooves at the upper and lower ends of the cover plate (Fig. 2a). Care should be taken to place the filter-paper flat on the plate. The cover plate is placed



Fig. 1. Front view of the assembled apparatus.

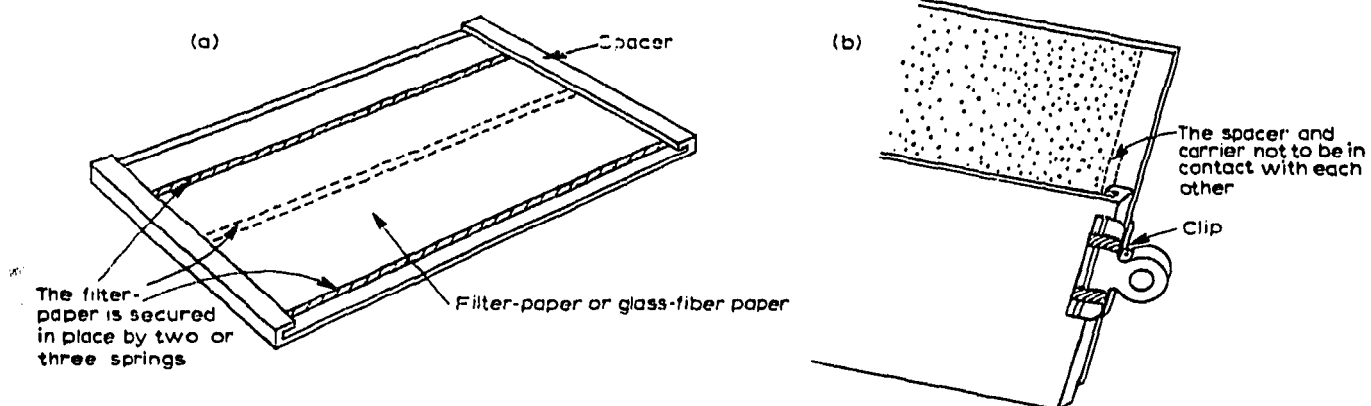


Fig. 2. Cover plate.

in a lower position, 12–15 mm from the thin-layer plate, and the two plates are secured in place by two clips at the edges (Fig. 2b). The clips should press the spacer.

The thin-layer plate with the cover plate attached is immersed carefully in the solvent. As the cover plate is lowered into the solvent, but not the thin layer, the filter-paper is soaked with the solvent. In this case, the cover plate may be on the upper or lower side, but it is better to put the cover plate below the thin layer because direct observation of the thin layer can then be made and contact of the thin layer with the filter-paper can be prevented.

It usually takes a few minutes for the solvent front to reach the top of the filter-paper, which is allowed to stand for a few minutes longer to ensure that the volume inside the cover plate is saturated with the solvent vapour. The clips are then removed carefully by holding the cover plate and thin-layer plate (taking care not to allow the vapour to escape) and the thin-layer plate is allowed to move down into the solvent. The two plates are again fixed with the clips and the development is started (Fig. 1).

In order to compare the results with those obtained by the classical Stahl method, the development is discontinued when the solvent front reaches 10 cm on the thin layer, and the R_F value is determined.

Further development will result in evaporation of the solvent from the top of the cover plate. In this case, care should be taken not to allow disturbed air to come into contact with the developing area. A stream of air should be avoided by using a screen. The sample band will reach the top of the cover plate and will be concentrated there as a line as the solvent evaporates. The solvent should be supplied continuously by careful addition along the inside wall of the tank so that the solvent surface will always be at a height of 8 mm.

In order to separate and collect the second and third components of a mixed sample, the cover plate should be slid down at fixed time intervals during which the thin-layer plate will move upwards and the solvent surface should be elevated a little (*ca.* 5 mm) by immersing a lifter (a glass rod for elevating the surface).

In the case of a colourless sample, a dye with a similar mobility should be placed on both edges of the thin layer as a marker. The mobility of the sample can also be measured by a preliminary experiment and the cover plate moved accordingly. The sample components can be concentrated in three different places and it is possible to obtain these three lines with about 15-mm intervals by adjustment of the cover plate. If even this is not sufficient, the cover plate can be replaced by one of a smaller size during the development. For this purpose, the new cover plate should be placed on the thin-layer plate immediately adjacent to the old plate, and the new plate moved into position by sliding, not lifting, the cover plate, so as to prevent the solvent from escaping during the exchange.

RESULTS AND DISCUSSION

Recently, DE ZEEUW¹⁴ and VAN DIJK AND MIJS¹⁵ reported that the separation effect on a silica gel thin layer is greatly affected by the adsorption of solvents, and it is natural that the sandwich-type developing tank, in which the air around the thin layer is devoid of solvent vapour at an early stage of development, will give different separation results from those obtained with the box-type saturated

tank because the adsorption of the solvent vapour will be insufficient. DE ZEEUW⁸ also confirmed this by experiments. However, these examples were limited to a small number of specific solvents, and it was desirable to make further comparative examinations on a wide range of solvent systems so as to clarify the differences between these two kinds of development.

In the present series of experiments, therefore, more detailed comparative experiments were carried out on the two methods of development by measuring the mobility on a silica gel thin layer, using a large number of mixed solvents used for the separation of steroids by the present authors^{10,17} and by NEHER¹⁸. For the samples, the dyes of NEHER *et al.*¹² were used, which possessed medium polarity and mobilities close to those of the steroids.

In order to ensure complete saturation with the solvent vapour, filter-paper was placed on three sides of the box-type developing tank and solvent vapour was allowed to saturate the tank for 20 min. In addition, the thin-layer plate was placed inside the tank and dipped into the solvent after vapour saturation, without taking the lid off¹⁷ (*B-V* type*). For the purpose of comparison, the standard procedure of inserting the thin-layer plate by taking the lid off after vapour saturation was also used^{9,11} (*B-V'* type). The sandwich-type developing tank³⁻⁷ is characterized by the small volume available for the vapour and it is desirable to use as small a spacer as possible. In the present experiment, a spacer 1 mm thick was used (*S-O* type).

Comparative examination of these methods revealed the following differences. When a mixture containing polar solvents, such as acetic acid, methanol and acetone, is used as the developer, a secondary solvent front (β -front¹⁰) appears in *S-O* type development. This is considered to be the de-mixing of solvents.

In accordance with the method of BRENNER *et al.*¹⁰, a mixed sample solution was spotted on the diagonal line on the thin layer and a secondary solvent front was detected. This result is summarized in Table I. The line connecting the same component often did not form a clear straight line and appeared to be more like an indented curve so that the solvent front was calculated not as a line but as a limited zone. This is probably due to the movement and adsorption of easily volatile solvent vapour through the small volume and a subsequent change in the solvent composition.

On the other hand, the phenomenon was quite different in development with the box-type tank (*B-V* and *B-V'*). With the exception of a few mixed-solvent systems, solvent de-mixing was not observed (Table I). This probably indicates that the vapourized solvent formed an adsorption equilibrium on the thin-layer surface.

During this experiment, it was found that there was a great difference in the rate of infiltration of the solvent into the thin layer in the box-type and sandwich-type developments. In the *S-O* type development, infiltration of the solvent was slow. For example, in a system in which a silica gel thin layer and benzene were used, 30 min were required for the solvent front to reach a distance of 10 cm, which was almost twice the time of 16 min required for the same development in the *B-V* type. A similar result was obtained with other solvent systems.

In order to remove these defects in the *S-O* type development, a device was

* Abbreviations used: *B* = box-type developing chamber; *V* = vapour saturated; *V'* = vapour incompletely equilibrated; *S* = sandwich-type developing chamber; *O* = without solvent vapour; *CF* = continuous development; *S-O-CF* = continuous development of the *S-O* type; and *S-V-CF* = continuous development of the *S-V* type.

TABLE I

SOLVENT DE-MIXING OF MULTICOMPONENT SOLVENT SYSTEMS

<i>Mobility phase</i>	<i>Relative value of β-front to the solvent front</i>		
	<i>S-O or S-O-CF developing method</i>	<i>B-V developing method</i>	<i>S-V or S-V-CF developing method</i>
Benzene-acetone (4:1)	0.70	1.0	1.0
Benzene-methanol (9:1)	0.35-0.49, 0.72	0.87 (0.30) ^a	0.82 (0.30-0.60) ^a
Chloroform-acetone (3:1)	0.86-0.92	1.00	1.00
Chloroform-methanol (97:3)	0.31	1.00	1.00 (0.30-0.60) ^a
Hexane-ethyl acetate (2:8)	0.96-0.98	1.00	1.00
Benzene-ethyl acetate (3:7)	1.00	1.00	1.00
Bush LB21/A85 ^b	0.35	1.00	1.00
Chloroform-acetic acid (3:1)	0.75	1.00	1.00

^a Figures in parentheses show the indistinct β -front zone.

^b In these experiments the upper non-polar phase was used for saturating the atmosphere as well as for development.

made to supply solvent vapour continuously into the cover plate (*S-V* type) and its function was examined. In this case, the system supplying the vapour must have a rapid rate of solvent infiltration and should not exhibit de-mixing of the solvent. Filter-paper and glass-fibre paper were tested and were found to be satisfactory for this purpose. An attempt had already been made⁷ to promote solvent vapour saturation by having two thin-layer plates facing each other and allowing one of the plates to adsorb the solvent, but infiltration of the solvent was slow and solvent de-mixing occurred. When the filter-paper was saturated with a solvent, the paper became bent owing to solvent infiltration and there was a chance that this filter-paper might disturb the thin layer. Therefore, a groove was made in the spacer into which a strong spring was placed to hold the filter-paper in place. When a 1-mm spacer was used, the solvent was adsorbed by capillary action to a certain height above the solvent level and the solvent front became non-linear. The spacer with a 2-mm slit was therefore used.

In order to determine the effect of pre-saturation time, the cover plate with the filter-paper was placed on the thin-layer plate with 15 mm extending from the latter, the cover plate was placed in the solvent and the thin-layer plate was allowed to dip into the solvent for 5, 10 or 15 min after dipping the filter-paper into the solvent (the solvent front reached the top of the filter-paper in about 2 min). The rate of solvent infiltration into the thin layer and the mobility of the sample were examined. There was no apparent difference between migration rates for the pre-saturation times described above.

The infiltration rate of the solvent is shown in Fig. 3. It is known that in infiltration of a solvent into a dry adsorbent system, there is a proportional relationship between the time of infiltration and the square of the distance¹⁰. These values were therefore plotted as shown. It is known from this graph that the infiltration rate is very slow in the *S-O* type but the rate is accelerated in the *S-V* type, the rate becoming close to that of the *B-V* type.

In liquid-phase chromatography, it is evident that a faster rate of developmen

does not necessarily increase the degree of separation of a sample, but it is desirable that the rate should be greater, if the separation is not disturbed, from the practical viewpoint.

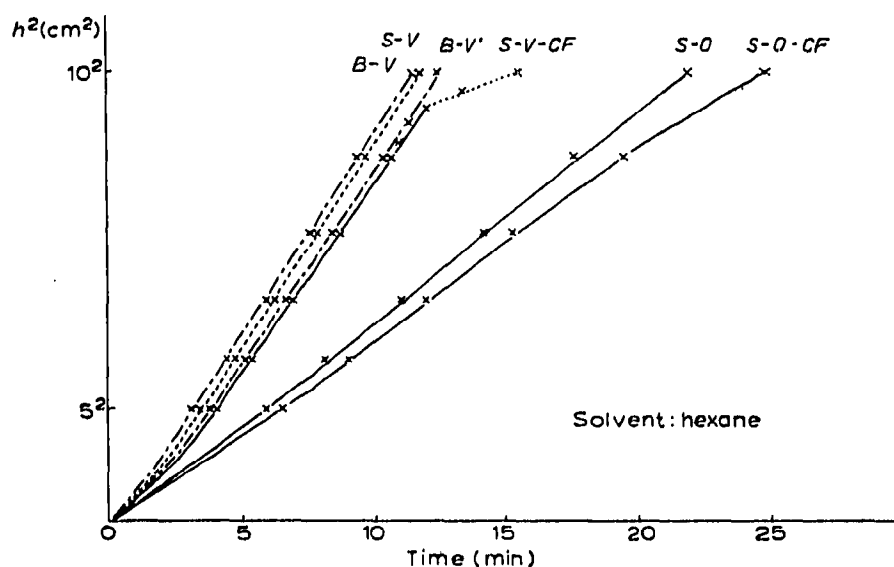


Fig. 3. Speed of migration of solvent front on silica gel layer. Pre-saturation times: *B-V* and *B-V'*, 20 min; *S-V* and *S-V-CF*, 10 min.

De-mixing of the developing solvent characteristic of the *S-O* type disappears in the *S-V* type, in which the result becomes approximately the same as that in the completely saturated *B-V* type (Table I). On the other hand, while the *B-V* type gives a good result, a long time is required for pre-treatment and the procedure is rather complicated. These disadvantages of the *B-V* type have been overcome in the *S-V* type, whose procedure is facile and rapid and which gives a good reproducible result.

Vapour-saturated continuous ascending development

An attempt was also made to utilize the foregoing observations in the continuous flow development reported previously¹⁰. The sandwich-type developing apparatus with an open-end cover plate (*S-O-CF* type) makes it possible to carry out continuous development. If the above function of solvent vapour saturation is added to this device (*S-V-CF* type), this method can be applied more widely. Therefore, the characteristics of these *S-O-CF* and *S-V-CF* types were examined by measuring the infiltration time of the solvent and the formation of a secondary solvent front.

Movement of the solvent front in the *S-V-CF* type development was similar to that of the *S-V* type soon after the start of the development, but the movement suddenly slowed down (dotted line in Fig. 3) when the solvent front reached the lower end of the evaporation-controlling spacer on the upper part of the cover plate. It took a few minutes for the solvent to travel through the length of about 3 mm of the spacer. However, as will be mentioned later, there seemed to be no great variation in the movement of the sample in this region (Fig. 5) and the movement

TABLE II

 R_F VALUES OF NEHER'S DYES

Mobile phase	Developing method	Pre-saturation time (min)	Time of development (min)	$R_F \times 100$ values of Neher's dyes ^a								
				F-52 (yellow)	F-59 (red)	F-63 (red)	F-11 (blue)	F-62 (brown)	F-22 (yellow)	F-9 (violet)	F-14 (blue)	F-5 (red)
Benzene-acetone (4:1)	<i>S-O-CF</i>	—	34	96	87	82	70	68	60	58	47	15
	<i>B-V'</i>	20	17	72	65	59	47	45	38	37	29	10
	<i>B-V</i>	20	19	58	52	48	38	38	31	29	22	7
	<i>S-V-CF</i>	10	20	62	57	53	42	42	34	31	24	8
Benzene-methanol (9:1)	<i>S-O-CF</i>	—	42	92	68	61	48	39	34	38	32	25
	<i>B-V'</i>	20	21	74	68	63	47	35	26	32	24	13
	<i>B-V</i>	20	24	62	57	53	42	33	25	26	20	8
	<i>S-V-CF</i>	10	18	62	57	52	43	37	34	31	26	24
Chloroform-methanol (97:3)	<i>S-O-CF</i>	—	33	95	68	62	40	32	29	34	32	18
	<i>B-V'</i>	20	20	77	73	68	54	28	23	37	30	10
	<i>B-V</i>	20	25	62	58	56	47	28	22	32	27	8
	<i>S-V-CF</i>	10	19	60	54	50	42	31	30	34	30	9
Chloroform-acetone (3:1)	<i>S-O-CF</i>	—	48	100	100	100	77	67	53	57	45	20
	<i>B-V'</i>	20	21	77	72	68	57	54	47	46	38	18
	<i>B-V</i>	20	24	60	55	52	43	42	35	34	27	12
	<i>S-V-CF</i>	10	20	65	60	56	48	45	40	38	33	15
Bush LB21/A85	<i>S-O-CF</i>	—	32	48	45	45	36	38	29	15	0	3
	<i>B-V'</i>	20	17	60	48	37	17	18	16	8	0	3
	<i>B-V</i>	3h	16	58	49	40	20	21	17	9	0	3
	<i>S-V-CF</i>	10	17	60	50	41	17	16	14	7	0	2
Hexane-ethyl acetate (2:8)	<i>S-O-CF</i>	—	28	100	100	95	89	99	90	82	78	22
	<i>B-V'</i>	20	18	75	69	63	54	58	52	48	47	10
	<i>B-V</i>	20	20	60	53	49	42	48	42	39	37	11
	<i>S-V-CF</i>	10	15	62	58	53	45	51	47	43	43	13
Benzene-ethyl acetate (3:7)	<i>S-O-CF</i>	—	40	100	100	95	89	80	80	73	73	20
	<i>B-V'</i>	20	16	73	66	62	53	55	51	48	45	10
	<i>B-V</i>	20	19	56	50	48	40	44	39	35	36	10
	<i>S-V-CF</i>	10	14	60	55	50	44	48	46	40	43	13

of the solvent seemed to be continuous. It was therefore considered that this specific change in the rate of movement of the solvent front is an apparent one that appears only at the front and there could not be any special change in the amount of flowing solvent.

This change can be interpreted as follows. As already pointed out by BRENNER *et al.*¹⁰, the amount of solvent retained (amount of flow) per unit area of the thin layer shows a sigmoid variation and the solvent front becomes concave as shown schematically in Fig. 4. When this front reaches this side of the upper edge of the spacer, the solvent front profile is disturbed owing to a decreased supply of the solvent vapour from the filter-paper and with the start of evaporation from the upper volume.

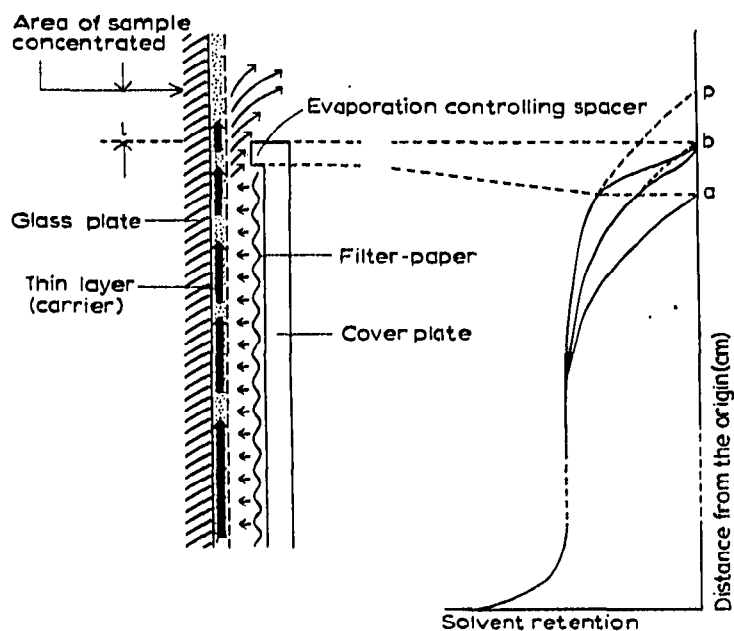


Fig. 4. Vertical section of the thin layer.

This results in a decrease in the solvent in the front and movement of the front becomes apparently slow, even when the solvent is supplied continuously. In Fig. 4, the parts marked a and b are due to the spacer area. When the apparent solvent front reaches the point b, the true front will have moved to point p and the R_F value read at the point b will be larger than the true R_F value for the hypothetical front at p. In the *CF* type development, therefore, the solvent front will be hidden by the spacer area but if the development is discontinued before the solvent front reaches the upper edge of the spacer, the R_F value measurement will not be disturbed by this phenomenon.

In order to find the effect of the variation of the R_F value between conventional and *CF* type developments, comparative examinations were made of the R_F values of the dyes of NEHER *et al.*¹² on a silica gel thin layer, using various solvent systems. Results of this experiment are summarized in Table II.

It can be seen from Table II that the R_F values of the dyes used as samples decrease in the order of *S-O* (*S-O-CF*), *B-V'*, *S-V* (*S-V-CF*, and *B-V*) types with all the solvent systems used except the Bush system. The rate of mobility is dependent on the degree of vapour saturation and the rate tends to increase with incomplete

saturation. The above order of R_F values is entirely reversed with the Bush system solvent. However, in all the solvent systems used, including the Bush system, the specification of the $S-O-CF$ type is similar to that of the $S-O$ type, and that of the $S-V-CF$ type to that of $S-V$ type. The results obtained with the latter types were the same as those obtained with the $B-V$ type. The $B-V'$ type is a standard procedure now widely used and the R_F value obtained by this procedure lies between those for the $B-V$ and $S-O$ types, which show poor reproducibility.

The best feature of the $S-V-CF$ type is the acceleration of developing speed after the start of continuous flow. For example, movement of STAHL's standard dyes¹¹ using benzene as solvent on a silica gel thin layer gave the results shown in Fig. 5. In the $S-V$ type development, the curve became linear until the solvent front reached the top of the thin layer and the movement then stopped. In the $S-V-CF$ type, on the other hand, the curve became concave during development, with the flow point (10 cm) as the node, and the developing rate was accelerated compared with that of the usual ascending method; the rate became much faster than in any other existing method of development. In the standard method proposed by Stahl, the developing distance was limited to 10 cm but it was desirable to continue the development further. In the $S-V-CF$ type development described here, development can be extended to any desired distance as long as the solvent is supplied continuously, so that the method is very effective when a volatile solvent is used. By placing a heating wire on the back of the plate at the position where the solvent front rises or by blowing warmed air on to the gel layer, solvent systems with unsuitable volatilities, such as the Bush solvent systems, butanol, acetic acid and water, were readily evaporated to give favourable chromatograms.

Recently, DE ZEEUW²⁰ has reported a new type of developing apparatus which is characterized by a "vapour-programming chamber" as well as "continuous flowing" of the solvent, and its separability is thought not to be due to "vapour-programming", but to "continuous flowing". For instance, he reported the first

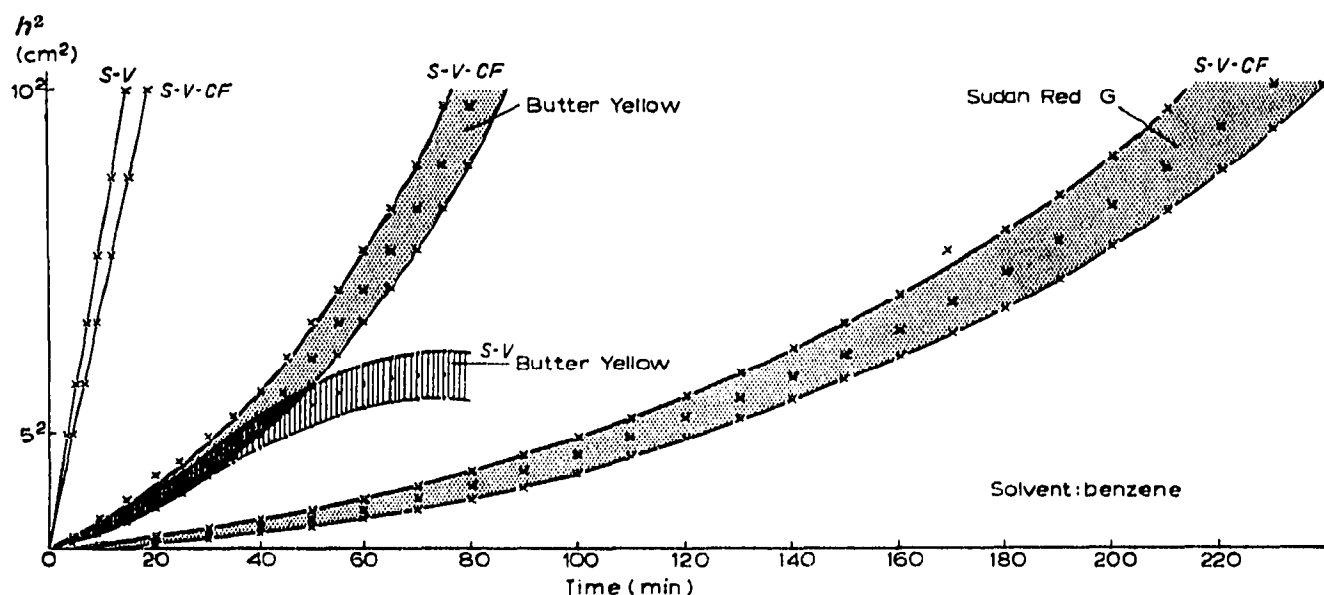


Fig. 5. Speed of migration of dyes on silica gel layer. Pre-saturation time of $S-V$ and $S-V-CF$ to min. Solvent: benzene.

separation of a mixture of indophenol, 4-nitroaniline and Sudan Red G, which cannot be resolved by the usual techniques, by using this continuous flow development system with a benzene-chloroform mixture as the solvent. However, we obtained better resolution of the three components with our apparatus, *i.e.*, *saturated continuous flow* type or *S-V-CF* type developing apparatus, using the same sample and developing time (110 min) as DE ZEEUW had reported. Moreover, DE ZEEUW's apparatus is a horizontal type and the procedure is rather complicated, while our ascending method is more simple and reliable.

As has already been reported¹⁰, the concentration of a sample component can easily be achieved with the *CF* type, and the *S-V-CF* type development can be used as a useful method for fractionation and purification of sample components. Actually, this method was used for the successful quantitative separation of steroid samples.

It was found that in the *S-V-CF* type, concentration of the sample components occurred in a position a few mm above the upper edge of the cover plate. The position of the concentration is determined by the equilibrium between the solvent ascending by capillary action of the carrier and the solvent evaporating from the carrier, and retardation of evaporation is probably responsible for the position of the concentration observed in the *S-V-CF* type. In the lower part of the thin-layer plate where solvent retention is great, *i.e.*, when a short cover plate is used, evaporation of the solvent becomes relatively slower and the position of the concentration becomes higher. For example, the distance between the edge of the cover plate and the position of the concentration found in the hexane-silica gel system is listed in Table III.

As stated above, the *S-V-CF* type development method has many favourable features not found in existing methods, and these points can be summarized as follows.

(1) The *S-V-CF* procedure is simple and reliable and gives highly reproducible results.

(2) In the new apparatus, a special cover plate provided with a filter-paper or glass-fibre paper on the inside surface is used and a long pre-saturation with the solvent vapour is not required. At the same time, development speed has been increased and a good separation is effected.

TABLE III

HEIGHT OF THE SAMPLE RETENTION FROM THE TOP EDGE OF THE COVER PLATE
Solvent: hexane.

Depth of immersion (mm)	Time after the solvent front reaches the top edge (min)	Height of the sample retention from the top edge of the cover plate (mm)				
		Distance from origin to top edge of the cover plate (cm)				
		10.0	8.5	7.0	5.5	4.0
6	20	2.5	5.0	5.5	7.0	11.0
	30	2.5	5.0	6.0	7.5	11.0
8	20	2.5	5.0	6.0	6.0	11.0
	30	2.5	4.0	6.0	6.5	11.0
10	20	2.5	5.0	7.0	7.0	11.0
	30	2.5	5.0	7.0	7.0	11.0

(3) In the existing method, the developing distance is limited to 10 cm, but in the present continuous flow system, the development can be carried out to an unlimited extent when a volatile solvent is used, as long as the solvent is supplied continuously. The separation is more efficient.

(4) In this apparatus, the once-diffused zone is concentrated and narrowed into a line for effective separation. This makes extraction and recovery of the sample easier, by simply scraping out the carrier.

ACKNOWLEDGEMENTS

The authors acknowledge helpful discussions with Dr. SABURO YAMASHITA, Hoshi College of Pharmacy. They express their gratitude to Messrs. GORO OIKAWA and KEI HASE of Toyo Roshi K.K., to Mr. SAKAE KUSANO of the Kusano Kagaku K.K., and to Messrs. YOSHIO FUWA and YOSHIHARU FUWA, for their co-operation and assistance in the manufacture of the apparatus, and to Miss HIDEKO SUZUKI for technical assistance.

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