

[54] **THIN LAYER CHROMATOGRAPHIC METHOD**

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[51] Int. Cl. **B01d 17/06**

[58] Field of Search **210/31 C, 198 C**

[56] **References Cited**

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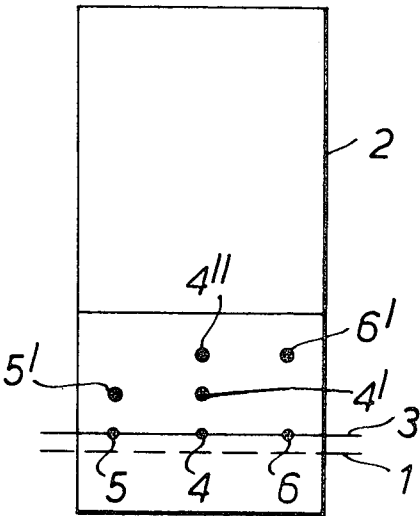
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Primary Examiner—Charles N. Hart
Attorney—Keith Misegades and George R. Douglas, Jr.

[57] **ABSTRACT**

A method for the chromatographic separation of substances, wherein the improvement comprises the steps dipping or wetting one edge of the surface of a non-porous solid constituting the stationary phase into or by a liquid constituting the mobile phase, and making said liquid rise by spreading on said surface as a continuous liquid film.

13 Claims, 4 Drawing Figures



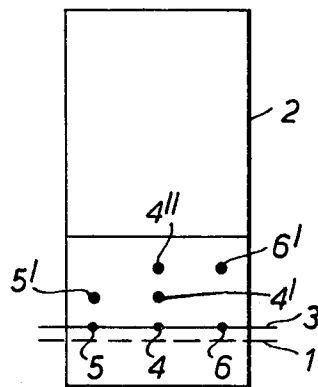


Fig. 1.

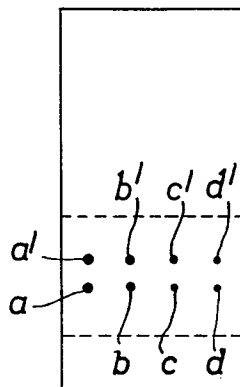


Fig. 2.

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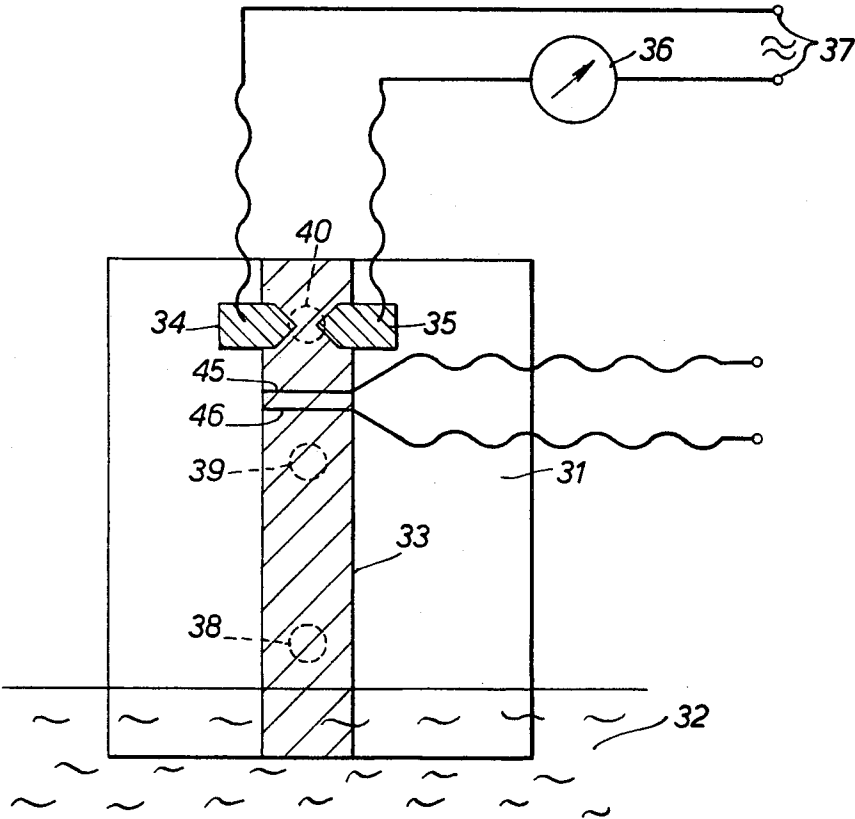


FIG. 3.

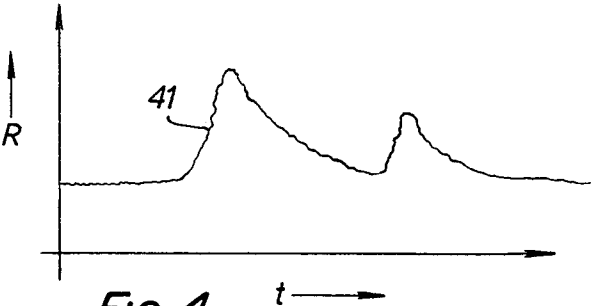


FIG. 4.

THIN LAYER CHROMATOGRAPHIC METHOD

BACKGROUND OF THE INVENTION

The present invention relates to a chromatographic method for the separation of substances which are soluble in a liquid serving as a transporting medium, the so-called "mobile phase," the absorbing power of the surface of solids being exploited past which the liquid is moved.

Thin-layer chromatography is a method generally used nowadays for the analytical detection of small amounts of chemical substances. In this chromatographic method a so-called "stationary phase" is deposited on a plate as a porous layer of a thickness of the order of magnitude of 1 mm. Occasionally layer thicknesses smaller than 1 mm have been used, but according to the view held generally (conf. e.g. E. STAHL: Thin-Layer Chromatography, 2nd Edition, Springer Verlag 1968) a limit is reached at 0.1 mm below which it does not make sense to go since then a marked deterioration in the dissolving capacity occurs.

Accordingly in this known thin-layer chromatography a thin layer of porous structure is needed, the capillaries of which play a similar part as the interstices between the individual grains of a chromatographic column.

BRIEF SUMMARY OF THE INVENTION

The present invention has the object of providing a new kind of chromatography which offers advantages as regards the minimum amounts required for obtaining a chromatography of the substances to be analyzed, and in many cases also as regards the separation effect by which substances of similar adsorption capacity can be distinguished by chromatography, as well as in regard of simplicity of the apparatus required for carrying out the method and of the reduction of the time required for obtaining a chromatograph to one tenth or even to one hundredth.

The method according to the present invention for the chromatographic separation of substances by letting a liquid flow as the mobile phase over a solid serving as the stationary phase is characterized in that by wetting the lower edge of the surface of a non-porous solid body the liquid is made to rise by spreading as a closed liquid film.

In particular, freshly prepared rough surfaces and the surfaces of thin layers produced by vapor deposition of a metal and subsequent oxidation at elevated temperatures in air have proved effective. Such surfaces show a so-called specific (true) surface exceeding considerably the macroscopic (apparent) surface.

An advantage of the use of vapor deposited layers in thin-layer chromatography consists in the complete homogeneity of the layers. By standardizing the vapor deposition process layers of identical properties can be produced, and thus accurately reproducible separation results can be attained. Such layers have great mechanical stability and can be used repeatedly since they can be regenerated after use simply by rinsing, boiling or annealing in air at 400°-700° C. They are exceptionally suited for the analysis of traces since very small amounts only of the substances are required for a faultless separation.

As further detailed investigations have shown the said layers have a very high Bodén-number of theoretical plates (which is known to constitute a theoretical parameter for the quality of the stationary phase).

Also in the chromatographic method according to the present invention the rate of travelling of the front of the liquid medium obeys the square root law of the height reached by it, as known for paper and thin layer chromatography. Although it has been found that the total amount of the vapor deposited layer substance has a certain influence on the height of rising attainable, these factors can easily be kept within narrow limits so that layers are obtained which ensure accurately reproducible results.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 diagrammatically shows a sheet constituting the solid phase dipped in the mobile phase, and rise of the latter with spots of the substances to be separated, and spots of control materials.

FIG. 2 is a similar view showing spots of various amounts of substances to be separated.

FIG. 3 shows diagrammatically an arrangement for the electrical evaluation of the separation of the substances.

FIG. 4 is a graph of resistance value measured by the arrangement of FIG. 3 plotted against time.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLE 1

Hereinafter some examples of the invention will be described in more detail. By vapor deposition of indium on a glass plate firstly metal layers were produced which subsequently were tempered in a furnace in air at about 400° to 700° C. and thus converted into indium oxide. Layers of indium oxide of a thickness from 0.6 to 5 μ were obtained which in an electron microscope showed a rough, wart-like surface structure, the individual excrescences having dimensions in the order of magnitude of 10⁻⁶ cm.

When a glass plate with the indium oxide layer described hereinabove was dipped into methanol (as the mobile phase), a height of rise of the liquid film covering the glass plate of 1.5 cm resulted after 5 minutes. In spite of this comparatively low rise a faultless separation could be obtained of a mixture of bromine phenol blue and eosin with the use of methanol as the liquid medium.

The drawing (FIG. 1) shows the result. 1 denotes the level of liquid (methanol) contained in a trough into which the indium oxide layer 2 deposited on a glass carrier was dipped. 3 denotes the starting line on which small droplets (amount of the sample 10⁻⁷ grams) of a bromine phenol eosin mixture was placed, with, for control, a droplet 5 of pure eosin being placed on the left, and a droplet 6 of pure bromine phenol on the right thereof. After a period of 5 minutes the separation shown in FIG. 1 was reached. The pure eosin travelled up to the mark 5', the pure bromine phenol up to 6', while the mixture of the two substances produced the two marks 4' and 4'', the lower one of which indicates the eosin component, while the upper one corresponds to the bromine phenol component, as indicated by the conformity with the control mark 5' and 6'.

EXAMPLE 2

In a further example according to FIG. 2 four different amounts (a - d) of a 1:1 mixture of bromine phenol blue and eosin were placed on a layer of indium oxide of a thickness of 2 μ , produced in the same manner as in Example 1. The mobile phase was again methanol. The period of the tests amounted to 2 minutes each. The travel distance of the bromine phenol amounted to 1 cm.

It is a surprising fact that by the use of the indium oxide layer described on this short travel path the same separation effect was attained as hitherto in the thin-layer chromatography with a travel path of 10 cm. This shows clearly that with the new method considerable space-saving can be attained, and that substantially smaller amounts of substances are needed. FIG. 2 shows the chromatograph of the four different amounts of samples of the aforesaid mixture of dyes. It is surprising that even the amount of substance (d) of only 2.10⁻⁹ grams produces an unequivocal and clear separation.

Apart from layers of indium oxide also layers of bismuth oxide and tin oxide are well suited for carrying out the invention, which layers can be produced in the same way as described for the indium oxide layers by vapor deposition of the metal and subsequent oxidation. Similar although less pronounced effects can be attained with any other layers of rough surfaces. (Conf. Example 5).

The method according to the invention is naturally not confined to the separation of dyes. The examples of dyes have the

advantage that the separation effect can be observed without special aid. Obviously all the detection methods used in other thin-layer chromatography methods may be used, e.g. those based on ultra-violet absorption, fluorescence, variation of the refractive index or dyeing by chemical means.

A particularly favorable method of detection for chromatographs produced according to the invention will be explained in more detail with reference to FIG. 3.

EXAMPLE 3

By a method analogous to that of Example 1 a tin oxide layer of a thickness of some μ was produced by the vapor deposition of tin and subsequent annealing in air. When treating a mixture of ethyl acetate/methanol/ NH_3 of the ratio 50 : 10 : 4 as the mobile phase, which produced a height of rise of 6 mm in 3 minutes, one attained with such a layer a very good separation of eosine from butter yellow (p-dimethyl amino azobenzene).

For characterizing the effectiveness of the method according to the invention hereinafter the Rf-values will be stated. The Rf-value defines the ratio of travel path of one of the substances to be separated to the travel path of the mobile phase used. In the Example 3 an Rf value of 0.75 was attained for eosine, and of 0.45 for butter yellow.

EXAMPLE 4

Separation of $^{137}\text{Cs}^+$ from $^{60}\text{Co}^{2+}$

Separation was carried out on a layer of In_2O_3 (as in Example 1). The cations to be separated existed as sulphates in aqueous solutions, namely as Co^{2+} and Cs^+ in concentrations of about 10 ppm and 20 ppm, respectively. The aqueous solution was mixed with some wetting agent for improving the spreading when applying the sample. The sample was applied at a distance of 4 mm from the lower edge of the plate by the aid of a fine capillary. The amount applied amounted to about $1 \cdot 10^{-12}$ grams of Co and $2 \cdot 10^{-12}$ grams of Cs. The plate was placed into a small rack which assured a constant depth of dip, and was placed into the mobile phase.

The depth of dipping amounted to 2 mm, the mobile phase consisted of ethanol (CH_3OH) and ethyl acetate (CH_3COOH) at a ratio of 100 : 1. The height of rise amounted to 1 cm, the period to 2 minutes. Measuring of radio-active spots was made with a scanner at a rate of advance of 2 mm per minute and a time constant of 3 seconds. The recorder and advance feed ran synchronously. A Geiger-Muller counter tube served as the counting instrument; good separation resulted with the following Rf-values :

$$\text{Rf } ^{60}\text{Co}^{++} = 0.35$$

$$\text{Rf } ^{137}\text{Cs}^{+} = 0.65$$

EXAMPLE 5

An aluminum foil was used as a base the surface of which was covered by a layer of aluminum oxide, which could be reinforced in a known manner by anodizing or etching, whereby surfaces of good spreading characteristic are obtained. To such an aluminum oxide surface again a test mixture of eosine plus bromine phenol blue plus butter yellow was applied. With the base described serving as the stationary phase good separation could be attained with various solvents such as methanol.

EXAMPLE 6

In this case the method according to the invention was carried out on a surface produced in the following manner:

- An aluminum foil (of a thickness of 0.2 mm) was etched for 15 minutes in 5% NaOH whereby hydrogen was liberated and the formerly uniformly blanc surface was rendered slightly matt. Cooling was effected with flowing water.
- The foil thus pre-etched was anodized with direct current in a bath of 10 percent oxalic acid and 0.1 percent of chromic acid. The anode sheet metal is located in the middle between the two cathodes at a distance of 5 cm on each side. The voltage was 30 - 40 V direct current; the current density amounted to about 1 amp/(10cm)²; the period of electrolysis was 1 to 2 hours; rinsing was effected with flowing water.

- The oxidized layer was copper-plated in a solution of CuCl_2 in 5 percent hydrochloric acid. This layer was suspended as a cathode between two Cu anodes with a spacing of 5 cm on each side. The copper was deposited in the form of grains adhering to the layer. The voltage amounted to 5 to 20 volts direct current; current density 0.2 to 0.5 amps/(10cm)², corresponding to about 0.1 to 0.2 grams of Cu per (10cm)². Finally the foil was rinsed with water and acetone and dried at room temperature.

On a surface produced like that, e.g., separation of a mixture of eosine, bromine phenol blue and butter yellow can be effected. For this purpose the mixture is applied with the aid of thin glass capillaries to the surface of the foil (a 0.1 percent solution, amount applied about 0.1/ul corresponding to about 10^{-7} grams). Separation took place with 95 percent methanol and 5 percent benzene as the solvent.

Height of rise:	2 cm in 2 minutes
Separation effect:	Eosine 1 mm
	Bromine phenol blue 4 mm (at the center of each spot)
	Butter yellow 18 mm

The spots were proved in spectrometer by extinction.

EXAMPLE 7

As in the Example 6, first an aluminum foil was oxidized and electro-plated with a copper layer. Subsequently a layer of indium was applied. The following electrolyte was used:

$\text{In}_2(\text{SO}_4)_3$	40 grams
Na_2SO_4	3 grams
NaCl	1.5 grams
H_2O	300 grams

After being rinsed with distilled water and ethanol this layer was dried at 150° C in a drying cabinet.

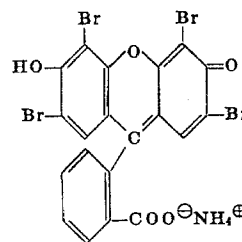
For the base with indium coating thus produced the following results were found:

With ethyl acetate as the solvent:			
Height of rise:	30 mm after 30 seconds		
Rf values:	Bromine phenol blue	0.05	
	Eosine	0.05	
	Butter yellow	0.95	
With ethyl acetate plus 2% acetic acid			
Height of rise:	30 mm after 30 seconds		
Rf values:	Bromine phenol blue	0.05	
	Eosine	0.75	
	Butter yellow	0.95	

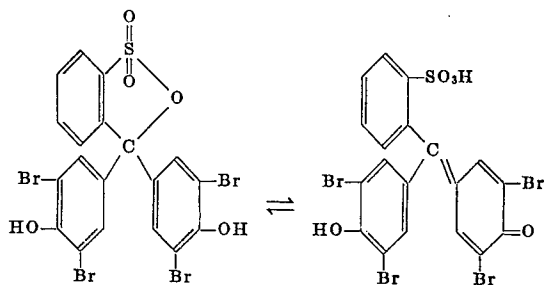
Hereinafter a number of further examples of the separation of food stuff dyes, indicator dyes and of some physiologically important substances will be compiled in Tables. Predominantly dyes have been used as examples since their separation can be proved in the simplest way. However, as states hereinabove the invention relates primarily not to the manner of detection of the separation, but permits the use of any known methods for detection after separation.

The following table shows firstly the substances used for tests and their chemical formulas:

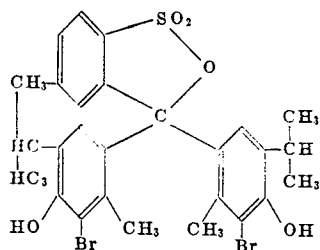
(1) Eosin- NH_4^+ :



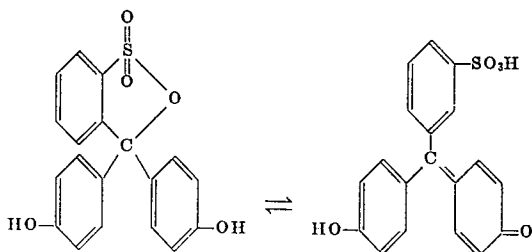
(2) Bromine phenol blue



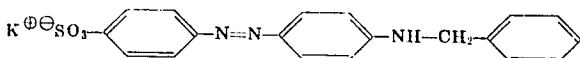
(3) Bromine thymol blue



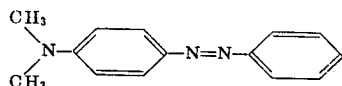
(4) Phenol red



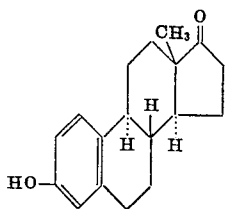
(5) Benzyl orange



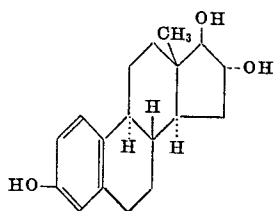
(6) Dimethyl yellow (butter yellow)



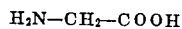
(7) Oestron



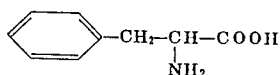
(8) Oestriol



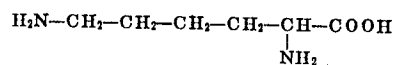
(9) Glycin:



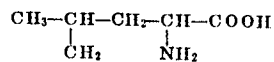
(10) Phenylalanin:



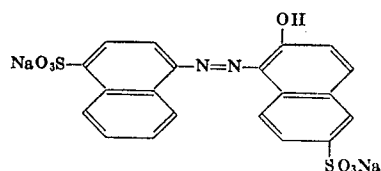
(11) Lysin:



5 (12) Leucin:

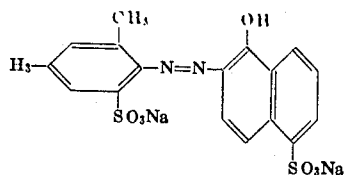


10 (13) Red No. 2 (True red E)



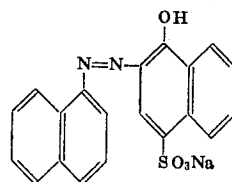
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20 (14) Red No. 6 (Scarlet GN) E 125



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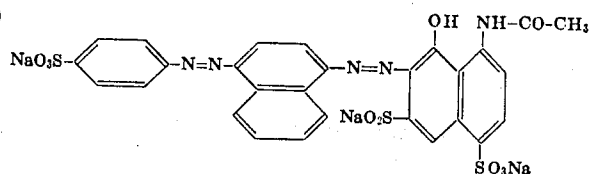
(15) Red No. 1 (Azo rubin S) E 122



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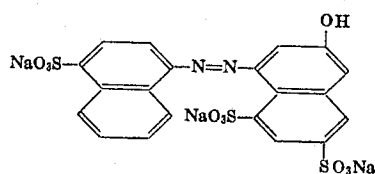
(16) Black No. 1 (Brilliant black BN) E 51



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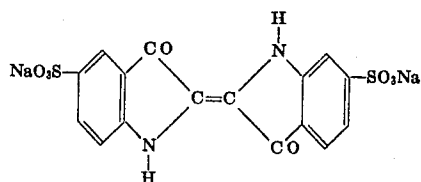
(17) Red No. 4 (Cochenille red A) E 124



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(18) Blue No. 2 (Indigotin I, Ia) E 132

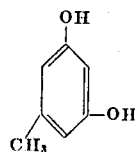


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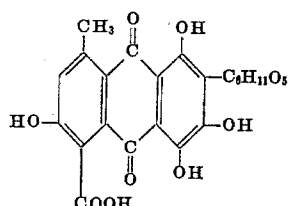
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(19) Orceinum $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_7$ (non-homogeneous lichen dye, main constituent=Orcein)

70



75

(20) *Acidum carminicum* (Carminic acid) Cochenille dye

The separations were carried out (unless otherwise stated) on In_2O_3 layers. For this purpose plates of the size 45×45 mm were provided on their sides with millimeter-scales in pencil. The origin (null point) was placed about 4 mm above the lower edge. The dyes were mostly in approximately 0.1 percent solutions in methanol or methanol-water 75 : 25. The application of the samples was made by means of a fine glass capillary at distances of about 3 mm to the right and left from the null point. The spots applied had diameters of 0.5 to 1 mm which corresponded to about 2 to $8 \cdot 10^{-9}$ grams of the substance.

50 milliliters of a suitable solvent were placed into a commercially available stand cylinder for this-layer chromatography, and left there standing for some hours for saturation. Then the plate with the samples applied was dipped in by means of a dipping device so far that the solvent level was about 2 mm below the null point. The rise of the mobile phase could be observed best obliquely from above, with the mobile phase serving as the background. The average periods of rise amounted to about 2 minutes, the heights of rise to 3 to 10 mm. After separation, the plate was taken out, and its lower margin freed from adhering drops of the solution.

For regeneration, the plates were firstly rinsed with hot water, then with alcohol and acetone, and finally tempered for 3 hours at 500°C . The results are compiled in the Tables 1, 2 and 3.

TABLE 1

Separation of Indicator Dyes	Mobile Phase	Test	R _f
Example Number	(Height of rise)		
8	Methanol (15 mm)	(1)	0.50
		(2)	0.69
9	Methanol p.a. (4 mm)	(1)	0
	Acetonitril 1:1 (4 mm)	(2)	0.6
		(3)	0.7
10	Methanol p.a. (3 mm)	(1)	0
		(2)	0.65
		(3)	0.75
		(4)	0.5
	Methanol: Acetonitril: (1)	(1)	0
	Methylenchloride: (2)	(2)	0.32
	Ammonia (3)	(3)	0.62
	20:20:20:0.2 (4)	(4)	0.75
	(3 mm)	(4)	0
		(4)	0.45
12	Methanol p.a. (30 mm)	(2)	
		(5)	
13	Ethyl acetate (1) stationary	(1)	0.5
	Methanol: (1) Phase	(1)	0.7
	Ammonia 50:10:0.2 (6) Bi_2O_3	(6)	0.9
	(10 mm)		

TABLE 2

Separation of Dyes for Food Stuff	Mobile Phase	Test	R _f
Example Number	(Height of rise)		
14	Acetone-Methanol (13)	(13)	0.3
	Water (14)	(14)	0.8
	80:20:10 (6 mm)		

15	Acetone-Methanol- (15)	0.5
	Water (16)	0.05
	80:10:10 (6 mm)	
16	Acetone- (17)	0.65
5	Benzene-Water (18)	0.15
	80:10:10	
17	Methanol p.a. (19)	0.6
	(6 mm) (20)	0.0
18	Methanol-water (19)	0.7
	(20)	0.05
10	65:15 (18)	0.5
	(6 mm)	
19	Methanol- (19)	0.92
	Ethyl acetate (18)	0.41
	Water (16)	0.25
	70:30:15 (20)	0.05
15	(6 mm)	

TABLE 3

Separation Example Number	n of Oestrogens and Amino Acids Mobile Phase (Height of rise)	Test	R _f
20	Acetone (3 mm)	(7)	0
		(8)	0.6
25	Acetone: Methanol 10:1 (5 mm)	(7)	0.55
		(8)	0.7
22	Acetone: Methanol 20:1 (5 mm)	(7)	0.33
		(8)	0.6
30	Ethyl acetate (3 mm)	(7)	0
		(8)	0.75
23	Methanol (5 mm)	(9)	0
		(10)	0.3
24	Methanol: Buthanol: Acetum glaciale 100:10:10.3 (5 mm)	(11)	0
		(12)	0.3

In the separation of oestrogens and amino acids the application and development of the chromatograph was carried out in the same manner as described for the dyes. The separated substances which are not colored may be made visible by being sprayed with a suitable reagent.

For this purpose the following reagents were used:

For the oestrogens: a 1:1 mixture of potassium hexacyanoferrate (III) with a 2% solution of iron (III)-chloride

For the amino acids: a 0.3% solution of ninhydrin in ethyl alcohol p.a. Subsequently were developed at 110°C .

In FIG. 3 a new arrangement is illustrated by means of which the separation of substances can be proved in a simple way. Therein 31 denotes a glass plate dipping into a solvent 32 for the substances to be ascertained, as shown. 33 denotes a strip of an indium oxide layer applied to the said glass plate, which upon the rise of the liquid prescribes the path thereof. 34 and 35 are two electrodes laterally contacting the strip of indium oxide, which may be produced by vacuum deposition of metal in vacuo. The two electrodes are connected via a current measuring instrument 36 to a source 37 of constant voltage, so that the value of the current constitutes a measure of the resistance between the electrodes 34 and 35.

When in performing the method according to the invention a sample is placed on the point 38 of the layer the substance components contained therein are chromatographically separated while the liquid 32 rises over the layer 33 by wetting the same, and one obtains e.g. the two bands 39 and 40 of two substances separated from each other. As soon as a certain band transported by the rising liquid film of a certain substance arrives between the electrodes, the resistance measured is varied. A chromatograph in the form of a resistance

curve 41 is obtained, as shown for example in FIG. 4; the abscissae 42 denote the time *t*, and the ordinates 43 the resistance *R*.

Instead of that arrangement of the electrodes 34, 35, in which the electric resistance in the direction transversely of the strip 33 of the chromatographic layer is measured, the electrodes could be arranged one behind the other along the strip, which electrodes would measure the resistance in the longitudinal direction. For this purpose metal strips 45 and 46 could be used for example, which consist of strips of metal of a width of a tenth of a millimeter vapor deposited on the layer of indium oxide, as indicated in FIG. 3, without disturbing the chromatograph. The spacing of these strips contributes to determining the dissolving power.

The arrangement illustrated shows the principle only. Obviously more complicated resistance measuring circuits may be used as required for measuring the sometimes very high electrical resistance of the layers adsorbed.

It is of advantage for the method of detection described last to use as the mobile phase an electrically non-conductive liquid, and as the stationary phase a solid having an insulating surface. The convenient shape of the electrodes depends on the dissolving power to be attained; the width of the electrode should be as small as possible as compared with the width of the bands occurring in a certain case measured in the direction of movement of the bands.

What we claim is:

1. A method for the chromatographic separation of substances, wherein the improvement comprises the steps of preparing a non-porous surface of micro roughness, and contacting said prepared surface with a liquid that spreads as a continuous thin film over the surface only, thus constituting the mobile phase of a chromatographic process.

2. A method as claimed in claim 1, comprising the preparation for use of a non-porous solid having a rough surface as a stationary phase.

3. A method as claimed in claim 1, comprising the step of

freshly preparing said surface before wetting the surface with said liquid.

4. A method as claimed in claim 1, comprising the step of producing a layer by vapor deposition in vacuo on a base to form said surface as a stationary phase.

5. A method as claimed in claim 4, wherein said layer has a maximum thickness of 10 μ .

6. A method as claimed in claim 4, wherein said layer has a surface roughness of the order of magnitude of 0.1 to 1 μ .

7. A method as claimed in claim 4, comprising the steps of producing said layer by vapor deposition of a metal, and subsequently oxidizing the same.

8. A method as claimed in claim 4, comprising the steps of vapor deposition of indium and subsequent oxidation thereof to form a layer of indium oxide.

9. A method as claimed in claim 4, comprising the steps of vapor deposition of bismuth and subsequent oxidation thereof to form a layer of bismuth oxide.

10. A method as claimed in claim 4, comprising the steps of vapor deposition of tin and subsequent oxidation thereof to form a layer of tin oxide.

11. A method for the chromatographic separation of substances wherein the improvement comprises the steps of preparing a non-porous surface of micro roughness, and contacting said prepared surface with a liquid that spreads as a continuous thin film over the surface only and past electrodes, and continuously measuring the electric resistance between said electrodes, said liquid constituting the mobile phase of a chromatographic process.

12. A method as claimed in claim 11, wherein an electrically non-conductive liquid is used as the mobile phase, and said surface is an electrically insulating surface used as a stationary phase.

13. A method as claimed in claim 11, comprising the step of forming said electrodes by vapor deposition of a metal on said solid.

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