111. Z. H. Ahrens, Geochim. Cosmochim. Acta, 2, 155 (1952).
112. E. J. Wheelwright, F. H. Spedding, and G. Schwarzenbach, J. Amer. Chem. Soc., 75, 4196 (1953).
113. S. A. Shchukarev, I. V. Vasil'eva, M. K. Morozova, T. I. Lakhtin, T'ao Huang-chi, and K'ang Huo-ping, in "Referaty Dokladov VIII Mendeleevskogo S'ezda, Sektsiya Neorganicheskoi Khimii i Tekhnologii" (Abstracts of Papers at the Eighth Mendeleev Congress, Inorganic Chemistry and Technology Section), Moscow, 1959, p. 173.
114. D. I. Mendeleev, "Osnovy Khimii" (Principles of Chemistry).
115. B. V. Nekrasov, "Kurs Obshchei Khimii" (Textbook of General Chemistry), GKhCh, Moscow–Leningrad, 1948, p. 268.
116. G. Hevesy, "Die seltenen Erden vom Standpunkte des Atombaues", Berlin, 1927, p. 51.
117. C. Marigne, Ann. Chim. Phys., [3], 38, 148 (1853).
118. J. J. Berzelius, Pogg. Ann., 46, 648 (1839).
119. J. J. Berzelius, Pogg. Ann., 47, 207 (1839).
120. B. Brauner, Monatsh., 3, 486 (1882).
121. W. Prandtl and J. Rauchenberger, Ber., 53, 843 (1920).
122. M. Delafontaine, J. prakt. Chem., 94, 297 (1865).
123. G. Jantsch and H. Gawalowsiki, Z. analyt. Chem., 107, 389 (1936).
124. A. F. Kapustinskii, Zhur. Obshch. Khim., 12, 193 (1942).
125. C. Matignon, Ann. Chim. Phys., [8], 8, 243 (1906).
126. C. Matignon, Ann. Chim. Phys., [8], 8, 386 (1906).
127. C. Matignon, Ann. Chim. Phys., [8], 8, 402 (1906).
128. C. Matignon, Ann. Chim. Phys., [8], 8, 426 (1906).
129. C. Matignon, Ann. Chim. Phys., [8], 10, 204 (1907).
130. H. H. Willard and R. D. Fowler, J. Amer. Chem. Soc., 54, 496 (1932).
131. L. Wöhler and K. Flick, Ber., 67, 1679 (1934).
132. L. Wöhler and M. Grünzweig, Ber., 46, 1726 (1913).
133. H. F. V. Little, "A Textbook of Inorganic Chemistry", Vol. IV, London, 1917, p. 254.
134. J. Ant-Wuorinen, Teknillinem Aikakauslehti, 21, 365 (1931).
135. T. Curtius and A. Darapsky, J. prakt. Chem., 61, 408 (1900).
136. G. Kommpa and J. Wowrinenn, Acta chem. Fennika, 2, 60 (1929).
137. H. C. Holden and C. James, J. Amer. Chem. Soc., 36, 1418 (1914).
138. G. Hughes and B. C. Hopkins, J. Amer. Chem. Soc., 55, 3121 (1933).
139. G. T. Morgan and E. Cahen, J. Chem. Soc., 91, 475 (1907).
140. C. Baskerville and G. F. Catlett, J. Amer. Chem. Soc., 26, 75 (1901).
141. F. Zambonini and G. Carobbi, Gazzetta, 54, 46 (1924).
142. M. Wunder and A. Schapiro, Ann. Chim. analyt., 17, 323 (1912).
143. L. G. Beck, Angew Chem., 52, 536 (1939).
144. E. Zintl and W. Morawietz, Z. anorg. Chem., 245, 26 (1940).
145. I. G. Farbenindustrie, U. S. P. 395 657 (1938).
146. H. G. Grimm, Z. phys. Chem., 101, 403 (1922).
147. G. Hevesy, Z. anorg. Chem., 147, 217 (1925).
148. G. Hevesy, Z. anorg. Chem., 150, 68 (1925).
149. M. Stakelberg, Z. phys. Chem., 118, 342 (1925).
150. W. Klemm, Angew Chem., 151, 577 (1938).
151. G. H. Cartledge, J. Amer. Chem. Soc., 50, 2855 (1928).
152. G. H. Cartledge, J. Amer. Chem. Soc., 50, 2863 (1928).
153. Yu. V. Khodakov, "Elementy Elektrokhimii" (Fundamentals of Electrochemistry), Gostekhizdat, ONTI, 1934.
154. C. E. Sun, J. Chinese Chem. Soc. (Formosa), 5, 148 (1937).
155. C. F. Sun and S. L. Li, J. Chinese Chem. Soc. (Formosa), 7, 69 (1940).
156. R. Abegg and G. Bodländer, Z. anorg. Chem., 20, 458 (1899).
157. J. Heyrovský, Proc. Roy. Soc., 102, 628 (1923).
158. N. A. Tananaev, "Analiticheskaya Khimiya" (Analytical Chemistry), ONTI, 1934.
159. N. A. Tananaev, Uspekhi Khim., 10, 621 (1941).
160. N. V. Aksel'rud and Ya. A. Fialkov, Ukrain. Khim. Zhur., 16, 296 (1950).
161. A. Günther, A. Katowski, and H. Lehl, Z. anorg. Chem., 200, 287 (1931).
162. S. J. Levy, "The Rare Earths", London, 1915, p. 117.
163. R. Mare, Ber., 35, 2382 (1902).
164. S. H. Katz and C. James, J. Amer. Chem. Soc., 36, 779 (1914).
165. B. Braumer and E. Svagr, Coll. Czech. Chem. Comm., 4, 49 (1932).
166. B. Braumer and E. Švagr, Coll. Czech. Chem. Comm., 4, 239 (1932).
167. G. Hughes and B. S. Hopkins, J. Amer. Chem. Soc., 55, 3121 (1933).
168. J. H. Kleinheksel and H. G. Kramers, J. Amer. Chem. Soc., 50, 959 (1928).
169. A. C. Weish and J. W. Burns, Canad. Chem. Met., 5, 69 (1921).
170. E. W. Noddak and A. Brukl, Angew Chem., 50, 362 (1937).
171. J. F. Spenser, "The Metals of the Rare Earths", 1919, p. 34.
172. N. V. Aksel'rud and V. I. Ermolenko, Zhur. Neorg. Khim., 6, 777 (1961) [Russ. J. Inorg. Chem., 397 (1961)].

Institute of General and Inorganic Chemistry,
Academy of Sciences of the Ukrainian SSR

U.D.C. 543.544

THIN-LAYER CHROMATOGRAPHY

A. A. Akhrem and A. I. Kuznetsova

CONTENTS

I. Introduction 367

II. General section 367

1. Methods of separating mixtures of substances on a thin layer of adsorbent 367
2. Chromatography on a fixed layer of adsorbent 368
3. Chromatography on an unfixed layer of adsorbent 369
4. Application of substance 370
5. Choice of adsorbent and solvent 370
6. Detection of the spots 371
7. Preparative separation of mixtures of substances 371

III. Classified section 372

1. Mono-, sesqui-, di-, and tri-terpenes 372
2. 2,4-Dinitrophenylhydrazones 373
3. Steroids and cholesterol esters 373
4. Glycosides 375

5. Lipids 375
6. Carbohydrates and polyhydroxy-compounds 377
7. Phenols, phenolcarboxylic acids, and quinones 378
8. Coumarins and flavones 378
9. Carotenoids and vitamins 379
10. Antioxidants and plasticisers 379
11. Insecticides 380
12. Amino-acids, amines, peptides, and nucleotides 380
13. Alkaloids 382
14. Pteridines 383
15. Heterocyclic compounds 383
16. Other examples of the use of thin-layer chromatography 383

I. INTRODUCTION

The progress made in the chemistry of natural compounds in the last 10–15 years is to a considerable extent due to the extensive application of effective methods of separating mixtures of substances, particularly the method of adsorption chromatography discovered by Tsvet[1]. After the development of the method of partition chromatography, the new technique of paper chromatography was extensively applied. This makes it possible to solve a number of complex and difficult problems in the chemistry of natural compounds.

A new effective method of chromatography on plates in a thin layer of adsorbent has recently been developed and has been called thin-layer chromatography. This method originated in work by Izmailov and Shraiber[2], who first separated alkaloids present in extracts from medicinal herbs on an unfixed layer of aluminium oxide on glass. Later, Meinhard and Hall[3] used radial surface chromatography on a layer of aluminium oxide fixed with starch for the separation of a mixture of certain inorganic ions. On the basis of this work, Kirchner *et al.*[4] proposed a new method for the separation and identification of terpenes in the determination of the composition of essential oils. This method, developed by these and other workers, particularly Stahl[5-16], on layers of adsorbent fixed to a glass plate, was then used extensively for the separation of both weakly polar and strongly polar ("hydrophobic" ["hydrophilic"? (Ed. of Translation)]) substances.

In 1955, Mottier[17,18] separated various dyes on an unfixed layer of aluminium oxide. The rapid development of this procedure began only recently, and a number of papers[19-22] have appeared, describing the use of thin-layer chromatography on unfixed layers of aluminium oxide and silica gel. The simplicity and rapidity of the method using an unfixed layer of adsorbent is attracting the attention of many research workers and the number of works in which it is used is increasing continuously.

Recent experimental data[13,16,23-25] show that thin-layer chromatography is a very convenient method for separating small quantities of multicomponent mixtures of substances. The advantages of this method over paper chromatography are its rapidity, the stability of the layer to heat and to active developers, and the high sensitivity: by using several developing reagents, it is possible to detect extremely small quantities of substances (0.1–0.005 μg).

II. GENERAL SECTION

1. Methods of Separating Mixtures of Substances on a Thin Layer of Adsorbent

The essential features of the method of thin-layer chromatography are as follows. A thin layer of adsorbent is deposited on one side of a small glass plate (20 × 5 cm). The substances and their mixtures are applied to this layer on a starting line, as on the paper in paper chromatography, and the plate is placed in a vessel containing solvent. The separation of the mixtures of substances takes 30–90 min. The plate is removed from the vessel, dried, and sprayed, as in paper chromatography. The position of the test substances, which are found in the form of spots between the starting line and the liquid front (limit of ascent) is noted (Fig. 1), the distance from the centre of the spot to the starting line (length *OS*) and the distance from the liquid front to the starting line (length *OF*) are measured. The position of the spot is denoted by a constant characterising the position of the substance on the given chromatogram as the ratio of the distance between the starting line and the centre of the spot *OS* to the distance between the starting line and the liquid front *OF*, *i.e.* $Rf = OS/OF$. The Rf value is characteristic of the compound on a given adsorbent in a given system and depends on a number of conditions: the procedure, the purity of the adsorbent, the activity of the layer, the thickness of the layer, the purity of the solvent, the quantity of substances deposited, the distance moved by the solvent, and the position of the starting line, but is almost independent of temperature.

The separation of substances on a thin layer of adsorbent is carried out on layers of adsorbent fixed to the glass plate or on unfixed layers. The substance is applied to the side of the plate from which the solvent is supplied.

There are several methods of moving the solvents over the plate and adsorbent. Usually, the solvent moves only once over the plate and adsorbent to separate the substances, but a number of methods of repeated chromatography also exist. If a mixture of substances, of which some are separated better by one system of solvents and others by another system, is being separated, use is made of a stepwise chromatographic method[8]; the separation is first carried out with a system which separates the less polar

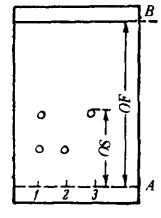


Fig. 1. Separation of a mixture of substances on a plate with a thin layer of adsorbent: *A*) starting line; *B*) liquid front. 1) Mixture of two substances 2 and 3; 2) and 3) individual substances.

substances. The solvent moves almost to the end of the plate. The chromatogram is then dried to remove the solvent and the chromatographic process is repeated with another system which moves over a shorter distance than the first system of solvents. This method often gives good results. Stepwise separation of mixtures using a third stage is also possible. In this case the third stage of the separation is carried out in a new solvent with the plate rotated through 180°. In this case the system moves in a direction opposite to that for the first two systems[26,27].

Two-dimensional chromatography is carried out on square plates (usually 20 × 20 cm) carrying adsorbent[10,28,29]. The mixture of substances to be analysed is applied at one corner of the plate and the separating liquids move first in one direction and then, after the chromatograms have been dried, in another system in a direction at right angles to the first. Two-dimensional chromatography is often used and gives particularly good results in the separation of multicomponent mixtures of substances. The two-dimensional method of separation using the same system of solvents in both directions, the so-called "separation, reaction, separation" method, is used to determine the homogeneity of a substance[10]. In some cases, two spots appear on the chromatogram and it is not clear whether the second spot is another form of the same substance or a product of a change which this substance undergoes during separation on the plate (for example, oxidation, isomerisation, etc.). If no such change takes place during the chromatographic process, the spots should lie on the plate diagonal. If, however, reaction products are formed, the spots of the new substance lie above or below the diagonal.

Sometimes, to improve the separation of substances, it is desirable to increase the length of the path of solvent flow. Use is then made of a second chromatographic process in the same system, or of a flow method of chromatography[30], based on the continuous supply of fresh solvent to the plate and adsorbent. A sheet of glass is placed above the plate, out of contact with the layer, to prevent volatilisation of solvent. The solvent reaching the end of the plate drains away or simply evaporates.

In work with an unfixed layer of adsorbent, the plate is placed at an angle of 15°–20°. In work with a fixed layer, however, any plate position is possible, and the vertical position is usually employed. The process is known as ascending chromatography when the solvent moves from below upwards under the influence of capillary forces. The height to which the liquid rises in this case has a definite limit. The process is known as descending chromatography when the solvent moves from above downwards, mainly under the influence of gravity[31–33].

A method involving horizontal movement of solvent is used in radial (circular) chromatography on plates. In this case the plate with a fixed layer of adsorbent is placed on a Petri dish with the layer facing downwards. The solvent is supplied from the dish by means of a wick in the centre of the plate. The substance to be separated is applied in a circle around the centre of the plate.

2. Chromatography on a Fixed Layer of Adsorbent

The chief adsorbent used for thin-layer chromatography on a fixed layer is silica gel, whose chromatographic properties enable it to be used for both adsorption and partition chromatography. Other adsorbents used include aluminium oxide, kieselguhr (for strongly polar compounds), magnesium silicate[34], calcium hydroxide, cellulose powder, polyamide resins, etc.

The silica gel should have a macroporous structure and suitable particle size (150–180 mesh). Its capacity with respect to water should be 80–100%. Good results are obtained with Soviet KSK grade silica gel[35–37], ground and passed through a 150 mesh sieve (hole size 0.1 mm). The silica gel should first be washed free from iron impurities, if these are present, by boiling with concentrated hydrochloric acid and washing free from chloride ions with distilled water. Fine suspension should then be removed by shaking and decantation and the product dried at 120° for 48 h.

The layer of adsorbent is usually fixed to the glass by means of a suitable fixing agent. Plaster[4], gypsum (medical)[35], and rice or maize starch† are used for this purpose.

As a result of the development of thin-layer chromatography with a layer fixed to glass, it has been found necessary to standardise the conditions of working in order to obtain reproducible results. For this purpose Stahl proposed the following conditions for standardising the layers[6]:

1. The layer should be even, smooth, 0.25–0.30 mm thick, and without cracks or bubbles. Such a layer is best obtained with a special apparatus. After deposition of the layer, the plate should be activated in a drying oven at 105° for 30 min and kept in a desiccator over blue gel (calcined silica gel impregnated with cobalt salts) or calcium chloride. This layer has an activity corresponding approximately to an aluminium oxide activity of II/III on the Brockmann scale. To obtain a less active layer, the plates are sometimes dried in air.

2. The layer should be sufficiently strong and resistant to active developing agents. These conditions are completely satisfied by KSK silica gel containing 5% medical gypsum or by silica gel G (Merck).

3. The capillary properties of the layer should be such that when a mixture of the three dyes Indophenol Blue, Sudan Red-IV, and *p*-dimethylazobenzene is deposited on the plate, it remains at the starting line when hexane is used as solvent (plate immersed in the liquid to a depth of 0.5 cm) and gives three spots with benzene. The rate of movement of the solvent depends on the quality of the layer, the solvent, and the degree of saturation in the vessel, and in the separation of the above dyes in benzene, the time taken for the liquid to rise to a height of 10 cm should be not more than 30–45 min. Under these conditions the variation in the R_f values is ±0.05. It is fairly difficult to obtain an even layer of this type without special apparatus, but results obtained on a layer prepared without this apparatus are quite suitable for characterising the compounds being studied if the R_f value obtained is related to the R_f value of a known compound subjected to the chromatographic process on the same plate.

Several pieces of apparatus have now been described for applying a thin fixed layer of substance. The best of these, which is used everywhere and is manufactured industrially, is Stahl's apparatus[9], designed to produce five 20 × 20 cm plates with a layer thickness of 0.27 mm (Fig. 2*a*). There are also several simpler models made of plastic, giving a layer of about 0.30 mm[38] or 0.25 mm[39], etc. (Fig. 2*b* and 2*c*).

† Some foreign firms prepare special silica gel G, kieselguhr G, and aluminium oxide G containing 5% gypsum for thin-layer chromatography by Stahl's method (Desaga of Heidelberg, or Merck of Darmstadt).

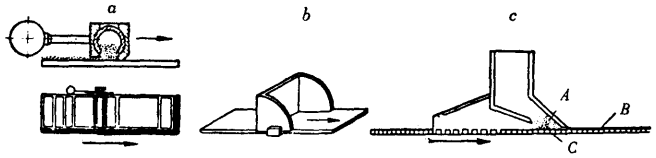


Fig. 2. Apparatus for obtaining a thin layer of sorbent. *a*) Stahl's apparatus, *b*) and *c*) plastic apparatus. The arrows indicate the direction of movement of the apparatus; *A*) adsorbent; *B*) thin layer of adsorbent; *C*) glass plate.

Glass of any quality can be used as a base in the preparation of the thin layers (window glass, photographic plates with the emulsion removed, mirror glass, and Pyrex). The dimensions usually employed are 20 × 20 cm and 5 × 20 cm.

To obtain a 0.27 mm thick layer on five 20 × 20 cm plates, 25 g of silica gel containing 5% gypsum are mixed thoroughly with 24 ml of water and the resulting mass is spread on the surface of the glass by means of Stahl's apparatus. The plates are then dried in air, activated, and stored as described above. An 11 × 17 cm plate with an approximately 0.3 mm thick layer is prepared, without apparatus, as follows: 6.9 g of silica gel and 0.35 g of gypsum are shaken thoroughly with 18 ml of distilled water in a 100–150 ml flask with ground stopper. The resulting thick uniform mass is poured on to the glass and spread evenly over its surface by means of a spatula with shaking. The plate is left for 20 min on a horizontal surface and then activated and stored in the usual way. The layer can also be fixed on the plate by means of starch[40], but this method is almost never used at the present time.

To obtain a cellulose layer, cellulose powder is shaken with water or acetone and applied to the plate using Stahl's apparatus.

Sometimes it is necessary to prepare so-called modified layers[8,13], for which solutions of acids, alkalis, buffers, etc. are used instead of water in the preparation of the layer. Acidic layers are obtained by adding 0.5 *N* oxalic acid[41] or 0.1 *N* boric acid[42]. Alkaline layers are prepared with 0.1–0.5 *N* potassium hydroxide[43] or sodium hydroxide[44]. Layers with Sörensen's phosphate buffer are prepared by adding equal volumes of 0.2 M potassium dihydrogen phosphate solution and 0.2 M disodium hydrogen phosphate solution[11]. A layer with acetate buffer is prepared with 0.02 M sodium acetate[11,13]. A cellulose layer can be impregnated with a 20% solution of formamide in acetone[45].

Use is also made of layers impregnated with hydrocarbons[46,47], liquid paraffin, tetradecane or the petroleum fraction with boiling point 240°, kerosene with boiling range 240°–250°, and silicone oils of low molecular weight (viscosity 50 cS) or high molecular weight (viscosity 1000 cS). The impregnation is usually carried out by carefully lowering the plate into a 5% solution of these substances in ether or petroleum ether, after which the plate is dried to remove the solvent.

In some cases, if the substance itself does not fluoresce in ultraviolet light, fluorescent layers are used, particularly for preparative separations. These are prepared by adding aqueous solutions of fluorescent substances which are firmly held by the adsorbent and are not wetted by the usual solvents. For a silica gel layer, Rhodamine G-6 is normally used for this purpose (0.0011 g in 54 ml water)[40].

In work with a fixed layer of silica gel, the ascending method is usually employed. The chromatographic separation is carried out in a flat-bottomed vessel, of any shape, with a size appropriate to the dimensions of the plate. The height of the vessel should be about 25 cm for work with 20 × 20 cm plates.

The quantity of solvent poured into the vessel is such that a plate with applied substance, placed almost vertically in the vessel, is immersed in the solvent to a depth of 5 mm. A sheet of filter paper moistened with solvent and reaching to the foot of the vessel is placed against one wall of the vessel to ensure supersaturation of the atmosphere inside the vessel. A ground lid is used to close the vessel. In a supersaturated atmosphere the spots are deeper and the duration of flow of liquid is reduced by one-third. The height to which the liquid rises should be not more than 10–11 cm, since when the distance moved by the solvent is high, diffusion of the spots and therefore greater variation in the *Rf* values are observed. With this method of working[48], the variations in the *Rf* values are of the same order of magnitude as those observed in paper chromatography.

3. Chromatography on an Unfixed Layer of Adsorbent

The chief adsorbents used for thin-layer chromatography on an unfixed layer are aluminium oxide and silica gel. The unfixed layer can be prepared using commercial "chromatographic" grade aluminium oxide with the required activity on the Brockmann scale[49].

The choice of an adsorbent with suitable quality and activity is of very great importance for an effective separation of mixtures, and it is necessary to remember that certain adsorbents, for example, aluminium oxide, may induce side reactions during the chromatographic process.

If the substance is not stable to alkalis, the aluminium oxide is washed until neutral, dried at 350° for 6–8 h, and deactivated by adding distilled water until the necessary activity on the Brockmann scale is reached[49].

The activity of aluminium oxide can be conveniently determined by thin-layer chromatography[50]. For this purpose, a standard mixture of azo-dyes (azobenzene, *p*-methoxyazobenzene, Sudan Yellow, Sudan Red, and *p*-aminoazobenzene) is separated by means of carbon tetrachloride on a plate with a thin layer of the test specimen of aluminium oxide and the activity is determined from the published Table[50].

An unfixed thin layer is prepared using KSK silica gel treated as described in Section II. 2. The silica gel prepared in this way is deactivated by adding 3–25% distilled water to give an adsorbent with the required activity.

The method of preparing an unfixed layer of adsorbent is very simple. The adsorbent is sprinkled on the glass (preferably matt) and spread evenly over the surface by means of a stainless steel roller thickened by 0.5 mm or 1 mm (±0.01–0.02 mm) at the ends, or by means of a glass tube with pieces of rubber tubing placed over the ends. The distance between the thickened ends of the roller or tube should be 10–12 mm less than the width of the plate used. The roller or tube is moved slowly by means of the thickened ends along the edges of the plate to give even layers

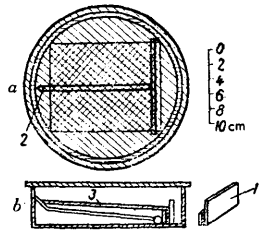


Fig. 3. Apparatus for the separation of a mixture of substances on an unfixed layer: 1) device for producing slit; 2) T-shaped glass rod; 3) glass plate with thin layer; *a*) view from above; *b*) side view.

of adsorbent. When the substance has been applied, the plate is placed in a glass crystallisation dish containing solvent, at an angle of 15°–20°, and one edge is lowered into the solvent[17,19,22]. Alternatively, the solvent may be supplied to the plate by means of a capillary slit[21], in which case a total of 25–30 ml of solvent is required for the separation. The top of the crystallisation vessel is covered with ground glass. Fig. 3 illustrates a vessel for work with an unfixed layer of adsorbent. The plate is placed in the vessel on the step of the device that forms the slit and on a bent glass rod with cross-piece. The device that forms the slit consists of two strips of glass 2 cm wide and 1 cm high. Pieces of Teflon [PTFE (Ed. of Translation)] or cover glass are placed between these strips along the edges. The strips are fastened to one another with Nichrome wire. The device is held in a vertical position by means of the cross-piece of the glass rod. With this method of working the distance travelled by the liquid has no influence on the R_f value. The variations in the R_f value from the mean value amount to not more than 2% of the distance between the starting line and the liquid front.

4. Application of Substance

Between 0.1 and 50 μg of the test substance is applied in a 0.1–0.5% solution, usually in a volatile solvent such as ether or chloroform. Drops of this solution are applied through a 0.1 ml pipette or a specially prepared capillary to a starting line at a distance of up to 1.5 cm from the edge of the plate. The distance between the points at which the specimens of substance are placed should be not less than 1 cm. The substances are applied carefully to avoid damage to the adsorbent layer. The quantity of compound or mixture applied plays a significant part in the separation of substances by thin-layer chromatography. The sensitivity of the detecting reagent is equally important. Very large quantities of compound may be difficult or impossible to detect if a reagent with low sensitivity is used. The R_f value depends on the quantity of substance applied to the plate. Very large quantities of substance often give spots which are far too large and of poor shape, and which merge with the spots given by compounds with similar R_f values.

Fig. 4 shows that in the identification of glycine, the best results are obtained with a quantity of substance equal to about 0.5–1.0 μg[51].

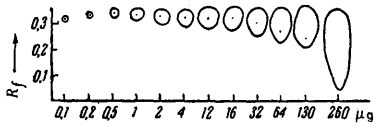


Fig. 4. Dependence of the R_f value on the quantity of substance (μg) taken for the separation, for the case of glycine[51].

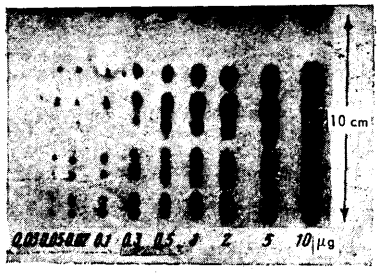


Fig. 5. Chromatogram for a mixture of sugars. The quantities of mixture applied increase from left (0.03 μg) to right (10 μg)[12].

Considerable attention is now being paid to the question of the sensitivity of the reagents used in thin-layer chromatography. Reagents are available for the detection of down to 0.005 μg of substance[15]. To obtain accurate results, therefore, it is necessary to know the sensitivity of the detecting reagent and to choose the correct quantity of compound or mixture of substances to be tested. In order to choose the necessary quantity of a mixture of substances, specimens of different quantities of the mixture, for example, from 1 to 10 μg[8] or from 0.03 to 10 μg[11], are applied to the plate, the mixture is separated in a suitable system, and detected with an appropriate reagent. The sensitivity of a reagent is verified in the same way, usually with the individual compounds. Fig. 5 illustrates the separation of a mixture of eight sugars[12]. The best separation is achieved with 0.5 μg of mixture, although separation is still observed with smaller quantities of substance.

5. Choice of Adsorbent and Solvent

The solvents used in thin-layer chromatography should be pure and thoroughly dry. The purification and drying of the solvents is carried out by the methods usually employed for other forms of chromatography.

The separation of mixtures of substances can be carried out with a single solvent, but systems consisting of two, three, and even four solvents are usually employed. A given portion of solvent or system cannot be used again for a second chromatographic separation.

In the choice of adsorbent, allowance must be made for the polarity and chemical stability of the substances to be

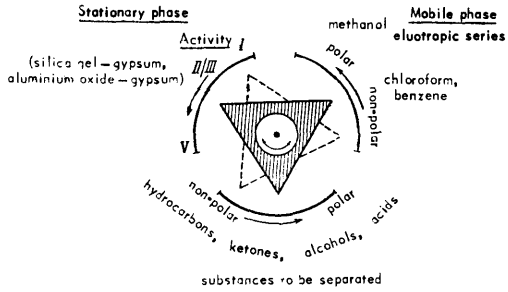


Fig. 6. Selection of conditions for chromatography on a thin layer of adsorbent. The Roman numerals indicate the activity on the Brockmann scale. The shaded triangle rotates around its centre. One angle of the triangle indicates the compounds to be separated, another indicates the necessary activity of the adsorbent, and the third indicates the solvent.

separated. Hydrophilic (polar) adsorbents (aluminium oxide, silica gel, kieselguhr) adsorb a substance more strongly, the greater the number of polar groups it contains and the greater its degree of unsaturation. Weakly polar compounds are usually chromatographed on strongly active adsorbents, and strongly polar compounds on weakly active adsorbents.

The adsorptive activity of compounds containing functional groups increases in order:

$$CH{=}CH < OCH_3 < COOR < C{=}O < CHO < SH < NH_2 < OH < COOH.$$

In the choice of solvent for adsorption thin-layer chromatography, use is made of the eluotropic series[16], starting from a non-polar solvent and finishing with a strongly polar solvent. For this purpose a microcirculation method is used, with the following essential features. Several specimens of substance are applied to a strip of glass with a layer of adsorbent in the usual way at a distance of 2 cm from one another. After a few minutes, the solvent or system being studied is applied by means of a thin capillary to the centre of each point where the substance or mixtures have been applied. The chromatogram is dried and developed to reveal the substances, separated more or less successfully, in the form of circular rings. A suitable solvent or system can then be selected.

On the basis of the above experiment, Stahl has proposed a simple graphical method of selecting conditions for chromatography on a thin layer of adsorbent[16]. This is illustrated in Fig. 6.

Recently, thin-layer chromatography has been used not only for adsorption chromatography, but also for partition chromatography. A recent paper[52] deals with the theoretical basis of the method. The use of Hecker's mixotropic series[53] for selecting a solvent for thin-layer partition chromatography is recommended. Initially, the mobile phase is always saturated with the stationary phase.

6. Detection of the Spots

In work with coloured substances, coloured spots of the separated components are observed. To detect colourless substances, it is first necessary to determine whether the substance fluoresces in ultraviolet light.

For substances which do not fluoresce, the chromatograms are sprayed from an atomiser with solutions of reagents which give colour reactions with the compounds being studied. The spraying is carried out with the smallest possible quantity of detecting agent to avoid damage to the layer and change in the shape of the spot. In some cases plates containing a fluorescent substance are used, or the plate may be sprayed with a solution of a fluorescent substance.

Most of the reagents used in paper chromatography can be used to detect the spots obtained in a thin layer of adsorbent. An advantage of thin-layer chromatography is the possibility of working with purely inorganic layers on which almost all substances can be detected by spraying with reactive detecting agents such as concentrated sulphuric acid, with or without aldehydes (vanillin, anisaldehyde, etc.), or a mixture of concentrated sulphuric and nitric acids. After spraying with these reagents, it is sometimes necessary to heat the plate at 80°–100°, or even to 400°. Good results are often obtained by spraying with $SbCl_3$ and $SbCl_5$ solutions and then heating to 80°–100°.

Compounds containing a double bond or capable of adding on iodine are detected with iodine vapour. When the plate is heated to 100°–150° after saturation with iodine vapour, compounds which undergo dehydration readily are revealed.

The carbonyl group is detected by spraying with 2,4-dinitrophenylhydrazine solution. Aldehydes are detected with a solution of *o*-dianisidine in acetic acid. Acids are detected with a weakly alkaline solution of Bromocresol Green, etc.

In the detection of the spots obtained on an unfixed layer, the spraying is carried out carefully with the atomiser at a distance of 1 m from the moist or dry plate. Under these conditions the compounds are detected without disturbing the layer. In an alternative method proposed for detecting the substances on an unfixed layer of aluminium oxide, the plate is dried in air after the substances have been separated, and the layer of aluminium oxide is impregnated with concentrated acids in a direction at right angles to that of the separation. In this case the colouring takes place immediately[54].

In the classified section of this review, reagents used for detection purposes are described in each subsection.

7. Preparative Separation of Mixtures of Substances

Thin-layer chromatography can be used for the preparative simultaneous separation of mixtures of substances in quantities of 0.4–0.5 g or more. The yields are almost quantitative.

Preparative separation on a fixed layer is usually carried out on a fluorescent silica gel–gypsum layer. The spots of substance are detected with ultraviolet light, marked, scraped from the plate, and the substance is extracted from the adsorbent with a suitable solvent. The chromatographic process is carried out several times and the solutions obtained by extracting identical spots are combined. The individual compounds are isolated after removal of the solvent.

On an ordinary 20 × 20 cm plate with a fluorescent silica gel–gypsum layer, the mixture of substances can be applied in the form of a continuous line, in quantities of up to 1 mg.

In this case the individual compounds are detected with ultraviolet light in the form of bands which are marked and treated as described above.

The method of preparative separation on an unfixed layer of aluminium oxide and silica gel is particularly convenient[19]. The separation is carried out on plates up to 40 cm broad and up to 80 cm long with an adsorbent-layer thickness of 1.5–3 mm. For a layer thickness of 1.5 mm, the quantity of substance applied can amount to about 20–25 mg per 1 cm length of starting line.

The mixture of substances is dissolved in a suitable volatile solvent and applied to the plate in the form of a continuous line, after which the plate is impregnated with pure solvent to ensure deeper penetration of the substance into the layer. The solvent is evaporated and the mixture of substances is separated in a suitable system. If a substance does not fluoresce in ultraviolet light, the plate is sometimes sprayed with morin and the bands of separated substances are detected in ultraviolet light and their positions are marked. Alternatively, the chromatographic process can be carried out on a layer of aluminium oxide impregnated with morin[55]. The substance and adsorbent are removed from the plate by means of a simple apparatus (see Fig. 7). A stopper with bent tube is placed in a funnel with porous filter (No. 3 and No. 4). The lower end of the funnel is connected to a water pump. The adsorbent and substance are removed by suction from the plate through the tube by means of the water pump. The substance is extracted from the adsorbent by a suitable solvent in the same funnel.

The detection of a substance may be carried out with iodine vapour. For this purpose crystals of iodine are placed in the funnel and a current of air is blown carefully through the tube placed in the stopper. At the same time the end of the funnel is moved slowly over the layer of adsorbent along the two edges of the plate. The sections coloured by the iodine on both edges of the plate are marked and joined up to one another. The zones containing the substances, marked out on the plate, are collected in the funnel and extracted as described above.

Several examples of the quantitative separation of mixtures of substances on a thin layer of adsorbent have been described. These include a visual method, based on measurement of the size and intensity of the spot[32,41,56,57], and a photometric method[58–60].

Various techniques have been recommended for the documentation of chromatograms: drawing on oil-paper, black-and-white photocopying[6], storage on coloured film, fixation of the results with paraffin wax[17,18], and photocopying in ultraviolet light[30].

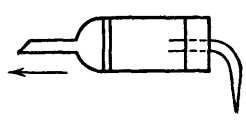


Fig. 7. Apparatus for removing substance and adsorbent from the plate.

III. CLASSIFIED SECTION

1. Mono-, Sesqui-, Di-, and Tri-terpenes

The development of thin-layer chromatography began with the study of the composition of essential oils[4]. It was found that on a thin layer of silica gel–starch in a hexane–ethyl acetate (85 : 15) system it is possible to separate terpene hydrocarbons, alcohols, esters, cinnamaldehyde, and normal caprylic acid. The common reagent used to detect the terpenes was concentrated sulphuric acid and its mixture with nitric acid (95 : 5), with subsequent heating to 500° and detection in ultraviolet light. Compounds capable of adding on bromine were detected by the fluorescein–bromine test, aldehydes were detected by spraying with a 1% solution of dianisidine in acetic acid, and acids were detected with Bromocresol Green. Use was also made of chromatography on a silica gel–gypsum layer with added fluorescent substances (zinc cadmium sulphate or zinc silicate) and irradiation with short-wave ultraviolet light.

The method of thin-layer chromatography on a layer of silica gel[61] has been used for the control of the fractions obtained in the separation of essential oils by column adsorption chromatography, and has been used successfully for the separation of many essential oils[62–64].

Various saturated and unsaturated aliphatic, aromatic, and terpene hydrocarbons have been examined systematically on a silica gel layer in hexane[65].

Detailed studies have also been made[66] of 32 examples of the separation of different terpene alcohols, aldehydes, ketones, ethers, and epoxides on a silica gel–gypsum layer, but for these compounds a number of other systems were used: (I) hexane–ethyl acetate (85 : 15); (II) chloroform–ethyl acetate (9 : 1); (III) benzene–ethyl acetate (85 : 15); (IV) chloroform–ethyl acetate (95 : 5); (V) hexane–nitropropane (1 : 1); (VI) hexane–ethyl acetate (70 : 30); (VII) chloroform–ethyl carbonate (85 : 15); (VIII) hexane–isopropyl formate (70 : 30); (IX) hexane–isopropyl ether (1 : 1); (X) hexane; (XI) chloroform.

Good results were obtained by spraying the chromatograms with concentrated sulphuric acid containing 1% vanillin[67–68]. This method has been used to determine *L*-menthol, *L*-menthene, *L*-menthyl acetate, and *L*-limonene in Japanese mint, on an SiO_2 layer in the hexane–ethyl acetate (85 : 15) system.

The preparation of a fluorescent layer by the addition of Rhodamine G-6, which does not give addition products with the substances being tested and is firmly held by silica gel, has also been suggested[40,69]. Good results were obtained with detection by ultraviolet light. Thus ketones were detected by spraying the plate with a solution of 2,4-dinitrophenylhydrazine (0.4 g DNPH in 100 ml 2 *N* HCl). The spots were marked in daylight and in ultraviolet light. The chromatogram was then heated at 105° for 10 min and re-examined in daylight and in ultraviolet light. This repeated examination made it possible to detect several substances which were previously not observed.

A layer of silica gel–starch in the hexane–ethyl acetate (85 : 15) system has been used to separate 12 aliphatic and terpene aldehydes and ketones[40,64], and, in the same system, diterpenes of the phytol class[55,70,71]. In the latter case, in addition to the usual methods, the ethers were detected by spraying with 0.25% aqueous potassium permanganate solution.

A number of essential oils have also been studied on a silica gel–gypsum layer in benzene. Borneol, cineol, bornyl acetate, thujone, linolyl acetate, and pinene were detected[72]. Menthol, cineol, menthone, menthyl acetate, menthofuran, pinene, and phellandrene were detected in the benzene–ethyl acetate (95 : 5) system[73].

A particularly interesting example is the separation of epimeric compounds, namely the isomeric γ-menthols, on a layer of silica gel–gypsum in the n-hexane–ethyl acetate (85 : 15) system[74] and later in the benzene–methanol (95 : 5) system[75].

The sesquiterpenes and their alcohols[76]: patchoulene, patchoulyl alcohol, and eugenol have been separated on a silica gel–starch layer in a n-hexane–ethyl acetate (85 : 5) system containing 5% chloroform, and detected with sulphuric acid.

Multicomponent mixtures of triterpenes have also been separated successfully on a silica gel–gypsum layer[77]. This method has been used for the separation of a number of triterpenic acids and also for neutral triterpenes (ethers, alcohols, and hydrocarbons). The compounds were detected by spraying with chlorosulphonic and acetic acids (1 : 2) and then drying at 130° for 5 min, and also by using ultraviolet light. The lower limit of detection with the above reagent is 0.02 μg in the case of oleanolic acid.

The following systems have been used for the separation of acids: (I) diisopropyl ether–acetone (5 : 2); (II) diisopropyl ether–acetone (5 : 2) containing 5% pyridine; (III) ethyl acetate–methanol–diethylamine (14 : 4 : 3); (IV) chlorobenzene–acetic acid (9 : 1); and (V) methylene chloride–pyridine (7 : 2).

Hydrocarbons and esters have been effectively separated in the following systems: diisopropyl ether–acetone (5 : 2); diisopropyl ether–cyclohexane; and benzene–methylene chloride.

The same layer has also been used to separate methyl bredemolate and the acetonide of methyl bredemolate in the diisopropyl ether–acetone (10 : 19) system[78] and to separate five new triterpenic acids, isolated from the resin of *Commiphora gladulosa*, in the chloroform–ethyl acetate (4 : 1) system[79].

The quantity of squalene and other terpenes and sesquiterpenes in essential oils has been determined on a silica gel–gypsum layer in benzene[6,7]. This method has also been used[80] for the semi-quantitative estimation of geranyl acetate, asarone, and isoeugenol methyl ether in certain essential oils.

2. 2,4-Dinitrophenylhydrazones

Aliphatic aldehydes and ketones are conveniently separated as their 2,4-dinitrophenylhydrazones. The best system for the separation of aliphatic aldehydes[81] in the form of these derivatives on a thin layer of silica gel–starch was found to be a saturated benzene–water solution (98:8.2).

A number of aldehydes and ketones present in essential oils: carvone, citral, menthone, cinnamaldehyde, benzaldehyde, fenchone, vanillin, salicylaldehyde, lauric aldehyde, furfural, and piperonal, have been identified as the 2,4-dinitrophenylhydrazones on a silica gel–starch layer in the ligroin–ether (2 : 8) system[82] and on a silica gel–gypsum layer in the hexane–ethyl acetate (17 : 3) system[83].

The 2,4-dinitrophenylhydrazones of a number of aromatic aldehydes and ketones have been identified with good reproducibility on a silica gel–gypsum layer in the benzene–petroleum ether (b. p. 60°–80°) (3 : 1) and benzene–ethyl acetate (95 : 5) systems[84].

3. Steroids and Cholesterol Esters

The first steroids to be separated on a thin layer of silica gel–gypsum were the lactones of Δ^5-3β, 20-dihydroxycholic acid and Δ^5-3β-acetoxy-20-hydroxycholic acid, cholesterol, its acetate, and the lipids from blood extract[9]. The separation was carried out in chloroform in a supersaturated vessel and the spots were detected by spraying with a saturated solution of antimony trichloride in chloroform, with heating at 110°.

The separation of cholesterol esters is very important in biological and medical chemistry and has recently aroused considerable attention. Work on the separation of the lipid extracts of blood on a silica gel–gypsum layer have been continued[26,27]. Good results have been obtained using a three-stage method of separation. The separation was first carried out in a propanol–ammonia (2 : 1) system. When the liquid front had moved a distance of 30 mm, the plate was removed, the solvent was evaporated, and the chromatographic process was repeated in a chloroform–benzene (3 : 2) system until the liquid had risen to a height of 100 mm. The plate was then removed, turned through 180°, and the chromatographic process was repeated by the ascending method in carbon tetrachloride (height of liquid ascent 40 mm). The chromatogram was sprayed with a solution of $SbCl_3$ in chloroform and examined in ultraviolet light. This method separated cholesterol and its esters, leucine, cephalin, and oleic, butyric, palmitic, and stearic acids.

The separation of cholesterol esters[85] and certain other weakly polar steroids was examined in detail on a thin layer of silica gel–gypsum in 13 different solvents and systems. It was found possible to separate, in 90 min, the neutral fraction of the products of the oxidation of the side chain of cholesterol, consisting of more than 20 compounds, and even, by a semi-quantitative method, to estimate the concentration of some of these in the total mixture.

Fig. 8 illustrates the semi-quantitative separation of the neutral fraction of the products of the side chain oxidation of cholesterol, on a silica gel–gypsum layer, by visual comparison of the spots with spots containing known quantities of these compounds.

In addition to the above systems, tetrachloromethane has been used to separate the cholesterol esters present in blood serum[86].

In a more detailed study of this problem[87], the following esters of cholesterol with saturated and unsaturated acids were specially synthesised and prepared in pure form: cholesterol formate, acetate, propionate, palmitate, stearate, oleate, linoleate, linolenate, and erucate. In the above systems, many of the cholesterol esters have the same R_f values. Best results in the separation of the esters were obtained in the tetralin–hexane (1 : 1) system[87]. The author was unable, however, to find a system which would give a sharp separation of the esters of the higher aliphatic saturated acids. For this purpose the separation was carried out on a silica gel–gypsum layer impregnated with liquid paraffin, in the ethyl methyl ketone–acetonitrile

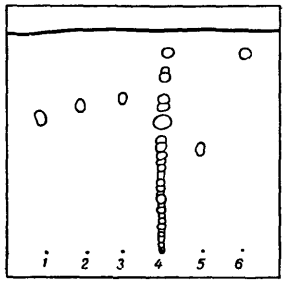


Fig. 8. Chromatogram of the neutral products of the side chain oxidation of cholesterol, obtained on a silica gel–gypsum layer:
1) 3β-acetoxyandrost-5-en-17-one;
2) 3β-acetoxypregn-5-en-20-one;
3) 3β-acetoxy-26-norcholest-5-en-25-one;
4) neutral fraction of the oxidation of cholesterol acetate dibromide;
5) 3β-acetoxy-chol-5-en-(24,20)-olide;
6) cholesteryl acetate.
The neutral fraction contains: 1) 4 ± 1%; 2) 3 ± 1%; 3) 3 ± 1%; 4) 22 ± 2%; 5) 12 ± ± 2%.

(7:3) system. This gave good separation of the cholesterol esters of unbranched higher aliphatic acids with short and long chains. Mixtures of saturated and unsaturated cholesterol esters were separated by a two-dimensional method in the tetralin–hexane (25:75) and ethyl methyl ketone–acetonitrile (7:3) systems using partial hydrophobisation of the layer with paraffin wax. The compounds were detected with molybdophosphoric acid.

Very good separations were obtained[38] on a silica gel–gypsum layer in ethyl acetate–cyclohexane (15:85 and 30:7) systems, for weakly polar steroids, chiefly esters of etianic acid, whose separation by paper chromatography was unsuccessful.

The more polar steroids cholic and deoxycholic acid have been separated in ethyl acetate on a plate with a silica gel–starch layer deactivated in air. The *Rf* values of the following compounds have been found for the systems indicated: 5β-cholestan-3-one 0.71, 5α-cholestan-3-one 0.86, and cholest-4-en-3-one 0.63 in the ethyl acetate–cyclohexane (15:85) system and 5β-cholestan-3β-ol 0.15 and 3α,4α-epoxycholestane 0.85 in the ethyl acetate–cyclohexane (30:70) system. The spots were detected by spraying the chromatograms with antimony trichloride solution. The carbonyl compounds were detected by spraying with 2,4-dinitrophenylhydrazine.

A number of steroids[35] were recently separated on a layer of KSK silica gel and medical gypsum using mixtures of cyclohexane and ethyl acetate in different proportions. The spots were detected with the above reagents and also with concentrated sulphuric acid and with ultraviolet light.

Mixtures of the epimeric 5,6-α- and 5,6-β-epoxides of epiandrosterone 3β-acetate and the 5,6-α- and 5,6-β-epoxides of ethynylandrostenediol 3β-acetate have been separated in the cyclohexane–ethyl acetate system[35]. Later, the epimeric cholest-4-en-7α- and -7β-ol-3-ones were separated in the benzene–ethyl acetate (2:1) system[88].

Recently, steroids have been separated on silica gel–gypsum and aluminium oxide–gypsum layers in the more polar systems: benzene–chloroform, benzene–acetone, chloroform–acetone, chloroform–ether, and butyl acetate–methanol in different proportions[89].

In the study of the Uzara root, 3β-hydroxypregn-5-en--20-one and 3β-hydroxy-5α-pregnan-20-one, present as the glycosides, and also β-sitosterol, were detected[90]. The products obtained were identified by oxidising with chromium trioxide and separating the oxidation products on silica gel–gypsum plates in diisopropyl ether or the diisopropyl ether–ethyl acetate (5:2) system.

Thin-layer chromatography has also been used to follow the course of the reactions of steroids involving microbiological methods (the introduction of hydroxyl groups, double bonds, etc.)[91].

A number of oestrogen hormones have been separated on silica gel–gypsum or silica gel–starch layers in pentane–ethyl acetate (9:1 and 3:2) systems[92] and in the benzene–ethanol (9:1) system[93]. The spots were detected with a solution of antimony trichloride in chloroform with heating at 200°–250°, and in ultraviolet light. In the case of the plates prepared with starch, the compounds were detected with iodine vapour or with 0.5% potassium permanganate solution followed by water. A method for the spectrophotometric determination of the composition of a mixture of these substances has also been described[93].

A new effective method for separating steroids[19] involves chromatography on a thin unfixed layer of aluminium oxide or silica gel. This method makes it possible to use adsorbents of any activity. The separation was carried out on a layer of aluminium oxide (activity V) in different simple systems and solvents (benzene, ether, and mixtures of hexane and benzene, benzene and ether, and ether and ethanol). A study was made of the dependence of the adsorptive properties[19] of different steroids, chiefly of the androstane and cholestane series, on their polarity and the position and configuration of the functional groups in the steriod skeleton. The compounds were detected with H_2SO_4.

Bases were detected with Dragendorf's reagent. The lower limit of detection with sulphuric acid varied from 5 to 100 μg for six compounds.

Detection has also been carried out by spraying with morin solution or by carrying out the chromatographic process on a layer of aluminium oxide impregnated with morin[55].

The preparative separation of the epimers 5α-cholestan-3α-ol and 5α-cholestan-3β-ol in the benzene–ether (7:3) system on a layer of neutral aluminium oxide impregnated with morin has been described[19]. The separation is quantitative. Good results have been obtained in the analysis of a number of epimeric 3α- and 3β-steroids. The isomers with a polar substituent in the equitorial position are generally more firmly held on aluminium oxide. The only exceptions are androst-5-en-3β-, 17α-en--3β-, 17β- doils.

Good results have also been obtained in the separation of steroids of the pregnane series on an unfixed silica gel layer in the benzene–ethyl acetate (1:1) system[19].

Steroids have also been separated on an unfixed layer of aluminium oxide with activity III, in mixtures of benzene and ethanol in different proportions[20]. 50 steroids containing from one to five functional groups have been separated. The influence of various substituents on the overall adsorptive properties of steroids has been discussed[20]. It has been shown that separations on commercial (alkaline) and specially purified (neutral) specimens of aluminium oxide give identical results. In the case of alkaline aluminium oxide, however, partial hydrolysis is observed in the case of very unstable compounds, and compounds such as steroid trichloro- and trifluoro-acetates are decomposed completely.

4. Glycosides

Thin-layer chromatography on a silica gel–gypsum layer has been used to confirm the structure of certain cardiac glycosides extracted from the Uzara root[94]. This method is particularly effective for the separation of allo-uzari-genins and other uzarigenins. The *Rf* values of a number of cardenolides have been determined in the diisopropyl ether–acetone (3 : 1) system and in ethyl acetate. The spots were detected by drying the plate, heating at 130° for 5–10 min, spraying with a solution of chlorosulphonic acid in acetic acid (1 : 2), heating for 5 min at 130°, and examining in ultraviolet light.

Separation on a thin layer of silica gel–gypsum has also been used for the rapid analysis of mixtures of glycosides from Digitalis and Podophyllum[12]. The Digitalis cardiac glycosides were separated in a methylene chloride–methanol–formamide (80 : 19 : 1) system. The compounds were detected with trichloroacetic acid and chloramine (16 : 1) with subsequent heating at 110° for 10 min and examination in ultraviolet light. This detected down to 0.1 μg of cardiac glycoside in daylight and down to 0.01 μg in ultraviolet light. Existing methods of paper chromatography can detect not less than 3–5 μg. This method has been used to separate secondary-glucosides, digilanides A, B, and C, desacetyldigilanides A, B, and C, and digitaloids.

The separation of the lignanglycosides and aglycones present in both forms of Podophyllum has been carried out by a two-stage process. The separation was first carried out in a chloroform–methanol (90 : 10) system, in which the glycosides were separated, and then in a chloroform–acetone (65 : 35) system, in which the aglycones were separated. The solvent was removed between the two stages with cold air for 5 min. The products were detected by spraying with a 1 : 1 mixture of sulphuric and acetic acids with subsequent heating at 100° for 10 min. The lower limit of detection was 0.3 μg. This method has been used to separate glucosides (α-peltatin-β-glycoside, podophyllotoxinglycoside, and β-peltatin-β-*D*-glycoside) and aglycones (4,4′-dimethylpodophyllotoxin, α-peltatin, podophyllotoxin, β-peltatin, and 1-desoxypodophyllotoxin).

A recent paper[95] described the separation, on a layer of polyamide powder, of 14 glycosides, whose aglycones were kaempferol, quercitin, and myricetin, and whose carbohydrate fraction included rhamnose, arabinose, glucose, and galactose. A sharp separation of 3-monosides, 3-biosides, and 3,7-glycosides was also achieved. The separation was carried out in the ethanol–water (3 : 2), water–ethanol–acetylacetone (4 : 2 : 1), and water–ethanol–ethyl methyl ketone–acetylacetone (13 : 3 : 3 : 1) systems. The spots were detected in ultraviolet light.

5. Lipids

In recent years, considerable attention has been paid in biochemical studies to the determination of the composition of natural lipids. Usually, the hydrolysis products are studied, and the latter are separated and identified by chromatographic methods.

In the first paper devoted to the separation of lipids by thin-layer chromatography[96] it was shown possible to separate various classes of compound present in lipids. A silica gel–gypsum layer in the petroleum ether (b.p. 60°–70°)–ether (92 : 8) system was used to separate lipid hydrolysis products consisting of higher aliphatic alcohols, esters, acetylglycerides, diacetylmonoglycerides, and diacetylglyceryl ethers. The compounds were detected by the action of iodine vapour on a plate sprayed with a 0.2% alcoholic solution of α-cyclodextrin, by Rhodamine B, or by spraying with concentrated sulphuric acid.

The separation and identification of the products of the hydrolysis of natural fats, oils, and waxes on a silica gel–gypsum layer in the hexane–ether (95 : 5) and petroleum ether–ether–acetic acid (70 : 30 : 1 and 70 : 30 : 2) systems has also been described[97]. The chromatogram was sprayed with a 0.2% alcoholic solution of 2′,7′-dichlorofluorescein.

In addition to the adsorption method, a partition method has also been used for the analysis of lipids by thin-layer chromatography[98].

An impregnated layer[98] in ether gives good separation of various classes of compound obtained in the hydrolysis of lipids, from hydrocarbons to phosphatides. The petroleum ether (60°–70°)–ether–acetic acid (90 : 10 : 1) system has been used to separate higher aliphatic hydrocarbons, alcohols, aldehydes, acids, and esters. The more polar compounds, the monoglycerides, glyceryl ethers, and phosphatides were separated in the petroleum ether–ether–acetic acid (70 : 30 : 1) system. The chromatogram was sprayed with a 0.2% alcoholic solution of 2′,7′-dichlorofluorescein. Unfortunately, the *Rf* values for homologous series of compounds with long hydrocarbon chains are the same. By analogy with the separation of homologous series on impregnated paper, a silica gel–gypsum layer impregnated with a 5% solution of silicone oil in ether was used. In the acetonitrile–acetic acid–water (70 : 10 : 25) system this method separated mixtures of the methyl esters of higher aliphatic acids.

The unsaturated compounds were detected with an α-cyclodextrin–iodate solution. Unsaturated compounds were detected with iodine vapour.

A number of recent papers[46,47,99,100] have been devoted to a detailed study of the separation of lipids by thin-layer chromatography. Model systems of lipid hydrolysis products and mixtures obtained from natural lipids have been studied.

Paper I[99] describes the separation of the following classes of compound: higher aliphatic aldehydes, acids, keto-acids (C_{14}, C_{16}, C_{18}, C_{20}), and monoglycerides, on a silica gel–gypsum layer in ether. The mono- di-, and tri-glycerides of the higher aliphatic acids were separated in isopropyl ether. The triglycerides of aliphatic acids, hydroxy-, dihydroxy-, epoxy-, and diepoxy-aliphatic acids were also separated in isopropyl ether +1.5% acetic acid.

Alcoholic molybdophosphoric acid solution and Rhodamine B solution were used for detection. 11 compounds

of various classes were separated by a two-dimensional method on the same layer (Fig. 9).

The same paper[99] describes the separation of homologous series of higher aliphatic alcohols and saturated and unsaturated acids in the acetonitrile–acetic acid (1 : 1) system on a silica gel–gypsum layer impregnated with undecane. Epoxy- and epithio-aliphatic acids were separated on the same layer in 80% acetic acid. The plates were then sprayed with molybdophosphoric acid. Diglycerides were separated with the chloroform–methanol–water (5 : 15 : 1) three-component system. The use of water is necessary to decrease the solubility of undecane in chloroform. Rhodamine B was used for detection. Other systems have been used, for example, the acetone–acetonitrile (7 : 3) system, in which both saturated and unsaturated triglycerides were separated. The use of a two-dimensional method with a layer impregnated with undecane gives better separation than that with a non-impregnated layer. A chromatogram illustrating the separation of di- and triglycerides and aliphatic acids is shown in Fig. 10.

Paper III‡ describes a new method for colouring thin-layer chromatograms[100], based on fixation of the layer with dichloromethylsilane. This makes it possible to immerse the plate in aqueous solutions and thus to use the specific methods of detection used in paper chromatography. Examples are given of the separation of higher aliphatic acids on a kieselguhr–gypsum layer in the acetic acid–acetonitrile (1 : 1) system, of phosphatides in the chloroform–methanol–water (11 : 5 : 11) system, and of triglycerides in the acetone–acetonitrile (8 : 2) system. The acids were detected with copper acetate and rubeanic acid. Phosphatides were detected with a 1% solution of molybdophosphoric acid in a 1 : 1 chloroform–ethanol mixture; after removal of the solvents the plates were washed in running water and immersed in a 1% solution of stannic chloride in 2 *N* hydrochloric acid. The triglycerides were detected after immersing the plate in 20% aqueous ethanol, by the transparency method[101].

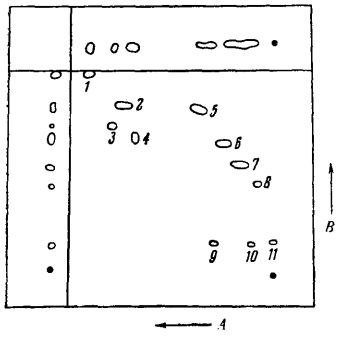


Fig. 9. Chromatogram for the separation of compounds of different classes by a two-dimensional method on a silica gel–gypsum layer. Systems: *A*) ether (30 min); *B*) isopropyl ether + 1.5% acetic acid (45 min). 1) Tristearin; 2) myristyl aldehyde; 3) distearin; 4) stearyl alcohol; 5) stearic acid; 6) stearic acid 9,10-epoxide; 7) 12-hydroxystearic acid; 8) stearic acid 9,10-12,13-diepoxide; 9) monostearin; 10) stearamide; 11) 9,10-dihydroxystearic acid. Compounds detected with molybdophosphoric acid.

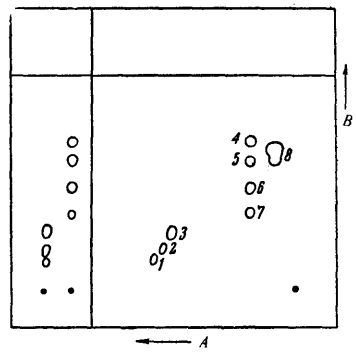


Fig. 10. Chromatogram for the separation of a mixture of di- and tri-glycerides and acids by a two-dimensional method on a silica gel–gypsum layer impregnated with undecane before separation in the *B* direction. Systems: *A*) dichloroethane; *B*) acetone–acetonitrile (7 : 3). 1) Triolein; 2) trimyristin; 3) trilaurin; 4) dilaurin; 5) dimyristin; 6) dipalmitin; 7) distearin; 8) aliphatic acids. Compounds detected with molybdophosphoric acid.

Paper IV by the same author and coworkers describes a more detailed study of the separation of triglycerides[46], including 20 synthetic and many natural triglyceride compounds, on a kieselguhr–gypsum layer impregnated with hydrocarbons or silicone oils. Several hydrocarbons were rested: undecane, liquid paraffin, dodecane, and tetradecane, or the petroleum fraction with b.p. 240° (the so-called Tetradecane Stand.). The last of these was found to be most convenient and was used for impregnating the layer. The same paper[46] describes the separation of saturated and unsaturated triglycerides and their mixtures on a kieselguhr–gypsum layer impregnated with tetradecane, in an acetone–acetonitrile (8 : 2) system 80% saturated with tetradecane. The compounds were detected with iodine vapour and by spraying with Rhodamine B solution.

The same paper gives examples of the separation of saturated synthetic triglycerides in the methanol–acetonitrile (5 : 4) system on a kieselguhr–gypsum layer impregnated with low-molecular-weight silicone oil with a viscosity of 50 cS. Unsaturated triglycerides were separated in the methanol–acetonitrile–propionitrile (5 : 4 : 1.5) system. Natural triglycerides were also separated on the same layer in a methanol–acetonitrile–propionitrile (5 : 4 : 1.5) system 95% saturated with high-molecular-weight silicone oil (1000 cS). The spots were detected with iodine vapour and molybdophosphoric acid.

Paper V[47] by the same author describes the separation of various synthetic keto-acids, 2-hydroxy-acids and lactones with long chains on an impregnated kieselguhr–gypsum layer. 14 synthetic aliphatic keto-acids and lactones were separated on a kieselguhr–gypsum layer impregnated with tetradecane in an acetic acid–water (80 : 20) system 80% saturated with tetradecane. The same acids and lactones in a mixture with aliphatic acids were separated on a layer impregnated with undecane, in an acetic acid–water (80 : 20) system 75% saturated with undecane.

The same paper gives an example of the separation of 2-hydroxy-acids and aliphatic acids and their mixtures on a layer impregnated with tetradecane, in acetic acid–water (75 : 25 and 90 : 70) systems 75% saturated with tetradecane. The compounds were detected with Rhodamine B solution.

‡ Paper II[87] describes the separation of cholesterol esters of aliphatic acids and is dealt with in the section "Steroids and Cholesterol Esters" (p. 373).

Thin-layer chromatography has been used[102,103] to separate various structural isomers of higher aliphatic acids and esters containing hydroxy- and chloro-groups. 9-Hydroxy--*trans*-10-*cis*-12-octadecadienoic and 13-hydroxy-*cis*-9--*trans*-11-octadecadienoic acids have been separated on a silica gel–gypsum layer in the petroleum ether–ether (8 : 2) system[102]. The same layer was used[103] to separate *threo*--12-chloro-13-hydroxyoleic and *threo*-13-chloro-12--hydroxyoleic acids and their esters, in the petroleum ether (b.p. 30°–45°)–ether system (9 : 1) in the case of the esters and in the same system with 1% acetic acid added in the case of the acids. The compounds were detected with sulphuric acid, 2′,7′-dichlorofluorescein, and ultraviolet light. The result indicates that in many cases thin-layer chromatography is more effective than paper chromatography, which has not yet given a separation of these compounds.

Even gas–liquid chromatography, which provides the most effective method of separating the aliphatic acids as their methyl esters, cannot give complete separation of very complex mixtures. For example, the esters of saturated normal and branched acids overlap the esters of highly unsaturated acids with shorter chains, and this makes the quantitative analysis of lipids by this method difficult or impossible. A combination of the methods of gas–liquid and thin-layer chromatography makes this task easier. For this purpose[104], the esters of the unsaturated acids were converted into the acetoxymercury derivatives and the latter were separated from the esters of the saturated acids on a silica gel–gypsum layer by a two-stage process. The saturated esters (R_f 0.9) were separated from the mercury derivatives of the unsaturated esters (R_f 0.1) in the petroleum ether (b.p. 60°–70°)–ether (4 : 1) system (distance travelled 15–18 cm). The mercury derivatives were then separated in the same direction in the propanol–acetic acid (100 : 1) system (distance moved 12–14 cm; time 3–4 h). The mercury derivatives of acids containing one double bond (R_f 0.85), two double bonds (two spots with R_f 0.55 and 0.45), three double bonds (R_f 0.15), and more highly unsaturated acids (R_f 0.0) were then separated. The plate was dried and sprayed with a 1% alcoholic solution of diphenylthiocarbazone. The spots corresponding to the saturated esters (R_f 0.9) were then removed from the plate, the substance was extracted, and the saturated acids isolated were analysed by gas–liquid chromatography.

Thin-layer chromatography on a silica gel–gypsum layer in the ether–petroleum ether (b.p. 66°–67°) (8 : 92) has been used to follow the course of the acetylation of monoglycerides isolated from various fats[105], and the same layer in the petroleum ether (b.p. 30°–60°)–ether (95 : 5) system has been used to control the synthesis of linolenic aldehyde[106].

The separation of aliphatic acids and α-glyceryl ethers present in the alkoxyglycerides isolated from fish liver oil[107] and the use of thin-layer chromatography to determine the composition of extracts from faeces and faecaliths[108] have also been described.

In the study of lipids obtained by decomposing human brain, rapid identification of the main fractions obtained by column chromatography is necessary. Thin-layer chromatography on a silica gel–gypsum layer has been used for this purpose[109]. The method gives better separation than paper chromatography, thanks to the sharper separation of the zones. The time required for the separation is only 60–90 min, the distance moved being 10–15 cm. The universal reagent proposed[109] for the detection makes it possible to detect certain lipid components present to the extent of only 0.2% in the mixtures. The lower limit of detection varies from 0.1 to 1 μg. 30 specimens of lipids and their derivatives, some of which were specially synthesised, were examined in 12 different systems and solvents and a method for their separation was described.

The following two solutions were proposed as a universal reagent for lipids: (1) 50 g Bromothymol Blue, 1.25 g boric acid, 8 ml 1.0 *N* NaOH, and 112 ml water, and (2) 40 g Bromothymol Blue and 100 ml 0.01 *N* NaOH. The lower limits of detection lie between 0.1 μg (for example, sphingomyelin) and 1 μg (for example, cerebron sulphate).

A number of special reagents have been given for determining cholesterol and its esters (lower limit 2–4 μg), saturated and unsaturated α-monoglycerides (lower limit 1–3 μg), and phospho- and glyco-lipids (lower limit for cerebon 4 μg and for cerebron sulphate 10 μg). The same author[110] used this method to study the composition of the sphingo-lipid fraction of normal and pathologically altered human brain on the same layer in the n-propanol–12% ammonia (8 : 2) system.

The separation of the cerebroside fraction of the sphingo--lipids of cattle brain on a thin layer of KSK silica gel–gypsum was recently studied[36]. Four individual cerebrosides: cerebron, kerasin, nervon, and hydroxynervon, were detected in the chloroform–methanol (8 : 2) system. Concentrated sulphuric acid was used for detection. The results obtained on the plate were used for the separation of these compounds on a silica gel column.

Thin-layer chromatography has also been used to separate glyco-lipids and phosphatides[111] on a silica gel–gypsum layer in the chloroform–methanol–water (60 : 35 : 8) system. This method detected cerebroside and cerebroside sulphate. Lysolecithin, sphingomyelin, lecithin, colaminecephalin, cerebroside, and cardiolipin were separated in the same system with the ration 65 : 25 : 4. The compounds were detected with Rhodamine B and Dragendorf's reagent.

The same layer was used to separate some products of the ganglioside fraction of human brain in the n-butanol-pyridine–water (3 : 2 : 1) system[112]. The quantitative determination of colaminecephalin and lecithin in medicinal plants by thin-layer chromatography has also been described[60].

6. Carbohydrates and Polyhydroxy-Compounds

The separation of hydrophilic compounds such as the carbohydrates by thin-layer chromatography has been possible only by using modified layers of adsorbent. A number of sugars have been separated on a kieselguhr–gypsum layer[11,13] impregnated with 0.02 M sodium acetate solution, using the ethyl acetate–65% isopropanol (65 : 35) system in a supersaturated vessel. The sugars were applied to the plate in pyridine solution and were detected by spraying with a freshly prepared solution of concentrated sulphuric acid and anisaldehyde in ethanol§. This method was found to be 20 times more sensitive than previously-known methods of analysis on paper. The optimum quantity of a mixture of sugars for separation on paper is 100–500 μg and the lower limit of detection is not less than 5 μg. For analysis on a kieselguhr–gypsum layer using anisaldehyde–sulphuric acid to detect the compounds, the

§ 0.5 ml concentrated sulphuric acid and 0.5 ml anisaldehyde in 9 ml 95% ethanol.



Fig. 11. Chromatogram for a mixture of sugars on a buffered kieselguhr–gypsum layer. The figure shows the sugars: 1) lactose; 2) saccharose; 3) glucose; 4) fructose; 5) *D*(+)-xylose; 6) *D*(−)-ribose; 7) *L*(+)-rhamnose; 8) *D*(+)-digitose; 9) *L*(+)-arabinose; 10) *D*(+)-mannose; 11) *D*(+)-galactose; 12) maltose; 13) *L*(−)-sorbose; CI) Mixture containing 1–8; CII) mixture containing 9–12; CIII) mixture containing 5–9.

lower limit of detection for the sugars is 0.05 μg. The best separation is achieved with 0.5 μg of substance and the maximum quantity of sugar which can be applied is 5 μg. A chromatogram for a mixture of sugars on a buffer kieselguhr–gypsum layer[11,13] is shown in Fig. 11.

The qualitative and quantitative separation of sugars has also been carried out on a silica gel–gypsum layer impregnated with boric acid (6 ml 0.1 *N* boric acid to 4 g silica gel)[42]. In this case the separation was carried out in the acidic benzene–methanol–acetic acid (1 : 3 : 1) and ethyl methyl ketone–methanol–acetic acid (3 : 1 : 1) systems and the compounds were detected by spraying with a mixture of 20% sulphuric acid and a 0.2% solution of naphthoresorcinol (1,3-dihydroxynaphthalene) in ethanol (1 : 1) with drying at 105°. Mono- and di-saccharides and uronic acids were detected by this method. The method for the quantitative separation of sugars, described in the same paper, is based on the elution of the sugars from the spots after removal of the latter from the plate, and transformation of the compounds isolated.

In connection with the study of the chemistry of macrolide antibiotics[37], the possibility of detecting polyhydroxy-compounds on a thin layer of silica gel–gypsum has been examined. Studies have been made of carbohydrates and related substances (glucose, lactose, glucosaccharin), polyhydric alcohols (ethylene and propylene glycols, glycerol, 3-methylhex-1-yn-3,4-diol), and polyhydroxy-acids (tartaric and 2,3-dihydroxy-2-methylpentanoic acids). The compounds were detected with the reagents for the α-glycol group usually employed in paper chromatography: a mixture of 5% aqueous silver nitrate solution and ammonia, an alkaline solution of sodium periodate and potassium permanganate, lead tetraacetate with subsequent spraying with rosaniline, and potassium periodate with benzidine. Most of the substances studied were detected only in quantities of 20–50 μg. The sensitivity of all the reagents in this case was found to be less than that on paper by a factor of 2–5.

7. Phenols, Phenolcarboxylic Acids, and Quinones

There have been several examples of the separation of volatile phenols in different systems on a thin layer of silica gel fixed to the plate with starch[4] or gypsum[7,8]. A detailed description has been given[113] of the analysis of phenol, *o*-, *m*-, and *p*-cresols, guaiacol, eugenol, and thymol in chloroform, xylene, and their mixtures (75 : 25, 50 : 50, and 25 : 75) on a thin layer of silica gel–kieselguhr–starch (1 : 1 : 1). In all cases the compounds were detected by spraying with diazotised sulphanilic acid, and also with an alcoholic $FeCl_3$ solution and with concentrated sulphuric acid.

This method has also been used[114] to follow the stepwise methylation of β-resorcylic acid. Study of the methylation products during the reaction, by thin-layer chromatography on a fluorescent silica gel layer, made it possible to direct the reaction as required. The petroleum ether (b.p. 66°–67°)–ether (3 : 7) system was used. This method was also used to study a number of aromatic polyhydroxy-compounds.

The separation of a number of phenols and phenolcarboxylic acids on a silica gel–gypsum layer in the acidic benzene–dioxan–acetic acid (90 : 25 : 4) and benzene–methanol–acetic acid (45 : 9 : 4) systems has recently been described[115]. The compounds were detected by means of the following reagents, which were first diazotised: benzidine, sulphanilic acid, 5-nitro-2-aminoanisole, and 1-amino-4-benzoylamino-2,5-diethoxybenzene, and also with tetrazotised di-*o*-anisidine.

Some aromatic hydroxy-acids[13] and polyphenols have also been separated in the benzene–methyl acetate–80% formic acid (4 : 4 : 2) system. Natural benzoquinones have been successfully separated in the hexane–ethyl acetate (85 : 15) system on silica gel–starch plates deactivated in air.

The separation of a mixture of 2,2′-methylene-bis-(4-chlorophenol) and 2,2′-methylene-bis-(3,4,6-trichlorophenol) on a silica gel–starch layer in hexane saturated with acetic acid has also been described[117]. The compounds were detected with solutions of ferric chloride and potassium cyanoferrate(III).

8. Coumarins and Flavones

Several substituted coumarins have been isolated and characterised in a study of lemon extract by adsorption column chromatography and chromatography on thin fluorescent silica gel–gypsum[118] or on starch[119] layers in the hexane–ethyl acetate (75 : 25) system. This method was later used[57] for the quantitative photometric determination of some of the above coumarins and also for their determination in certain foodstuffs[120] and medicinal products[121].

Acidic systems of the toluene–ethyl formate–formic acid (5 : 4 : 1) type[13,14] have recently been used to separate multicomponent mixtures of α- and β-pyrones, coumarins, flavones, and hydroquinones by thin-layer chromatography. The method was found to be much more sensitive than paper chromatography. With detection by long-wave ultraviolet light the sensitivity for coumarins was 0.1 μg. Thin-layer chromatography has been used to separate the following coumarins: esculin, esculetin, scopoletin, 4-methylumbelliferone, xanthotoxin, bergenin, imperatorin, and atamanine.

The flavones robinetin, morin, toxipholin, quercetin, kaempferol, naringenin, and chrysin, separated by this method, were detected with a 1% solution of β-aminoethyl

diphenylborate in methanol and examination in ultraviolet light. The sensitivity of the method is 0.1 μg. Hydroquinone and its esters and glycosides were detected with Millon's reagent, prepared by dissolving 5 g mercury in 10 g fuming sulphuric acid and adding 10 ml water. The lower limit of detection for arbutin and methylarbutin is 5 μg, that for hydroquinone and its methyl ether 1 μg, and that for its diethyl ether 10 μg.

Flavone glycosides have been separated using the ethyl acetate–ethyl methyl ketone–formic acid–water (5 : 3 : 1 : 1) system. This paper also described the separation of aromatic hydroxy-acids, certain tannins, and phenolglucosides in the toluene–ethyl formate–formic acid (5 : 4 : 1) system and also the separation of substances present in lichens.

Flavones have also been separated on a thin layer (0.1–0.2 mm) of Silon (Kapron) [a type of Nylon (Ed. of Translation)] polyamide powder[122].

9. Carotenoids and Vitamins

Certain carotenoids were separated on a silica gel–starch plate in the hexane–ether (3 : 7) system as early as 1958.[23] Pigments of the tetrahydropyrrole series were separated on the same layer in the benzene–ethyl acetate–ethanol (90 : 20 : 7.5) system[23].

In the last two years thin-layer chromatography has been used extensively[123] to study different classes of carotenoids, particularly carotenoid aldehydes, which occur in very small quantities in natural products. A new sensitive reagent has been proposed for detecting these compounds[124,125]. The plate is sprayed with an alcoholic solution of Rhodanine and then with a concentrated aqueous solution of ammonia or sodium hydroxide. This reagent makes it possible to detect down to 0.03 μg of a polyene aldehyde. Retinene (vitamin A) and β-apo-8-carotenal were recently separated on a silica gel–gypsum layer impregnated with liquid paraffin, in methanol saturated with liquid paraffin[125].

Carotenoid aldehydes have been separated with both impregnated and non-impregnated layers[126]. A silica gel–gypsum–$Ca(OH)_2$ (5 : 20) layer impregnated with liquid paraffin in the petroleum ether–benzene (1 : 1) system or, in some cases, the same system with 1% methanol added, has been used to separate carotenoid aldehydes with the following R_f values:

C_{40}	C_{37}	C_{35}	C_{32}	C_{30}	C_{27}	C_{25}	C_{20}
0.28	0.34	0.377	0.473	0.488	0.633	0.588	0.855

A non-impregnated layer shows clearly two series with R_f values corresponding to C_{40}, C_{35}, C_{30}, and C_{25} aldehydes on the one hand and to C_{37}, C_{32}, and C_{27} aldehydes on the other.

Seven synthetic β-apocarotenals of the C_{25}–C_{40} series have also been separated on a calcium hydroxide–silica gel–gypsum (6 : 1) layer in the petroleum ether–benzene (2 : 3) system, and six methyl esters of β-apocarotenoic acids (C_{27}–C_{30}) have been separated in the same system[127].

This method was used to detect fat-soluble vitamins of α-tocopherol and vitamin A in the separation of lipids on a silica gel–gypsum layer in the ethyl acetate–petroleum ether (2 : 8) system[128]. The compounds were detected with sulphuric acid.

Thin-layer chromatography has also been used to separate vitamins of the B group (B_1, B_2, B_6, nicotinamide, biotin, and calcium pantothenate) and vitamin C.[129] The separation was carried out on a silica gel–gypsum layer containing 2% of a fluorescent substance (ZS-Super), in the acetic acid–acetone–methanol–benzene (5 : 5 : 20 : 70) system. In this system vitamin B_1 remains at the starting line. In the detection of the spots, the plate was first examined in ultraviolet light with a wavelength of 366 mμ, in which the fluorescent layer remains dark and the vitamin B_2 (R_f 0.35) shines brightly on a dark background. The plate was then illuminated with ultraviolet light with a wavelength of 254 mμ, in which dark spots of vitamin B_1 (R_f 0.0), vitamin C (R_f 0.30), and nicotinamide (R_f 0.65) are observed against a light background. Biotin was detected by spraying another plate with potassium iodoplatinate, which revealed biotin (R_f 0.80), vitamin B_1, nicotinamide, and vitamin C in the form of spots of different colours. To detect vitamin B_6 and calcium pantothenate, the lower part of another plate was sprayed with a 0.1% solution of dichlorobenzoquinone chlorimide in ethanol saturated with ammonia vapour. This revealed vitamin B_6 (R_f 0.15). The plate was then heated to 160° and the upper part of the plate was sprayed with a 0.5% solution of ninhydrin in ethanol. The plate was then reheated to 160° to reveal the calcium pantothenate. The sensitivity of the method is 1–10 μg for vitamins of the B group and 5–30 μg for vitamin C.

In the separation of A vitamins and provitamins and vitamins K_1, K_2, and K_3 [54] on an unfixed layer of aluminium oxide, the use of 70% perchloric acid and 98% sulphuric acid to detect these compounds is recommended. After the separation, the plate is dried in air and the aluminium oxide layer is impregnated with the above acids in a direction at right angles to the direction of separation.

10. Antioxidants and Plasticisers

Thin-layer chromatography on a silica gel–gypsum layer has also been used to separate and identify antioxidants in the food industry[130], and has made their study much easier. The best system was found to be a chloroform–methyl cellosolve (80 : 20) mixture. This method was used successfully to separate 24 different antioxidants. The compounds were detected by spraying the plate with a 2% solution of molybdophosphoric acid in methanol and treating with ammonia vapour after 1–2 min. The plate was then heated for 10 min at 120° to reveal some additional spots. It is also possible to spray with a 1% solution of 2,6-dichlorobenzoquinone chlorimide in ethanol and then with a 2% solution of borax in 40% ethanol. Antioxidants have also been separated by two-dimensional chromatography. The separation was carried out in one direction in benzene and in the other direction in chloroform.

Good results in the analysis of α-, β-, γ-, and δ-tocopherols and their mixtures have been obtained[58] by separation on a silica gel–gypsum layer in chloroform and on an aluminium oxide–gypsum layer in benzene. In both cases, the β- and γ-tocopherols, differing in the position of the hydroxyl groups, were found to have identical R_f values when molybdophosphoric acid solution was used for detection. Spraying with cerium(IV) sulphate reagent[131], however, gives spots of different colours, making identification easy. The quantitative separation of tocopherols has also been described.

The use of a modified silica gel–kieselguhr layer containing added oxalic acid[41], in the benzene–ethyl acetate (6 : 4) system, makes possible the qualitative and quantitative (with an accuracy of ±5%) determination of norhydroguaiaretic and ascorbic acids in a mixture of gallates. 2,6-Dichlorobenzoquinone chlorimide can be used instead of the cerium(IV) sulphate reagent for detection, in which gase the silica gel–kieselguhr layer should be impregnated with borax in the ratio 25 : 4.5. The quantitative separation of antioxidants is based on measurement of the size of the spots and by graphical interpolation.

Mixtures of plasticisers[132] have been separated on a silica gel–gypsum layer containing 0.05% Ultraphor, in the following systems: isooctane–ethyl acetate (9 : 1); benzene–ethyl acetate (95 : 5), and dibutyl ether–hexane (9 : 1). The compounds were detected in ultraviolet light with a wavelength of 365 mμ. This method achieved separation of the following compounds: glyceryl triacetate, ethyl *o*-ethoxycarbonylbenzoylglycolate, triethyl acetylcitrate, triethyl phosphate, diethyl phthalate, butyl *o*-butoxycarbonylbenzoylglycolate, 2-ethylhexyl diphenyl phosphate, tributylacetylcitrate, di-n-butyl phthalate, diisobutyl adipate, dibutyl sebacate, dinonyl phthalate, di-2-ethylhexyl phthalate, and butyl stearate. A number of specific colour reactions for detecting plasticisers have been given.

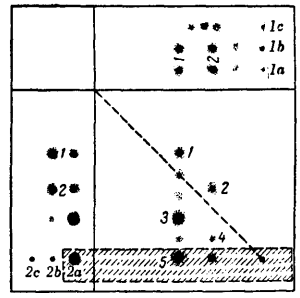


Fig. 12. Chromatogram for a pyrethrum concentrate on a silica gel–gypsum layer. 1*a*, 1*b*, 2*a*, 2*b*) Pyrethrum concentrate; 1*c*, 2*c*) mixture of dyes; 1) pyrethrin I; 2) pyrethrin II; 3) pyrethrin I peroxide; 4) pyrethrin II peroxide; 5) lumipyrethrin. After separation in the first direction the hatched part of the plate was exposed to ultraviolet light.

11. Insecticides

It has been reported[133] that a mixture of pyrethrin I (R_f 0.42) and pyrethrin II (R_f 0.21) can be separated on a silica gel–gypsum layer in the hexane–ethyl acetate (80 : 20) system. This system was then used to analyse pyrethrum extract on a silica gel column.

Under the influence of light and air, pyrethrins are rapidly converted into lumipyrethrins *via* their peroxides. Thin-layer chromatography has been used in the study of this process[10]. A particularly interesting feature of this work is the successful use of the "separation–reaction–separation" technique. By this method, changes in the polarity of the individual components of a mixture of organic compounds as a result of the action of, for example, γ-rays, X-rays, ultraviolet light, increased temperature, and various gases, on the mixture are readily detected. In the study of the oxidation of pyrethrins in this work, a two-dimensional method of separation was employed, using the hexane–ethyl acetate (75 : 25) system in both directions. The compounds applied to the plate were exposed to sunlight or ultraviolet light and heated. If no oxidation had taken place, the spots would have lain almost on the diagonal of the chromatogram, but since oxidation takes place, substances with different R_f values are produced (see chromatogram in Fig. 12), so that the spots for the reaction products (pyrethrin I and II peroxides and lumipyrethrins) lie below the diagonal.

Thin-layer chromatography has recently been used[134] to separate various synthetic insecticides. Thus a silica gel–gypsum layer in the hexane–acetone (4 : 1) system has been used to separate thiophosphates with the following R_f values: Diazinon 0.76–0.82, parathion 0.65–0.68, Meta-Systox 0.62–0.64, malathion 0.52–0.54, chlorthion 0.43–0.45, Fac 0.20–0.26, and Rogor 0.04–0.07. The compounds were detected by means of readily-oxidised 0.5% palladium(II) chloride solution.

The following chlorine-containing insecticides were separated on an aluminium oxide–gypsum layer with activity II on the Brockmann scale, in hexane in a supersaturated vessel: aldrin 0.78–0.82, DDT 0.59–0.62, Perthane 0.48–0.50, hexachlorocyclohexane 0.39–0.41, dieldrin 0.17–0.19, and methoxychlor 0.10–0.12. The compounds were detected by spraying with a solution of 0.5 g *NN*-dimethyl-*p*-phenylenediammonium in sodium ethoxide solution (1 g Na in 100 ml ethanol). The plate was then sprayed with water and heated by means of a quartz lamp for 1 min. The sensitivity of the method is < 5 μg.

Thin-layer chromatography has also been used to separate certain thiophosphate esters[16] on a silica gel–gypsum layer in the methylene chloride–methanol–10% ammonia (8 : 2 : 3) system. When iodine–azide reagent is used for detection, the lower limit is 1–5 μg.

12. Amino-Acids, Amines, Peptides, and Nucleotides

The first separation of amino-acids[135] in the form of their sodium salts was carried out in 1958 on a thin unfixed layer of aluminium oxide on glass. Four systems were used, the best being the n-butanol–ethanol–water (60 : 40 : : 40) system.

One year later, good results were obtained in the separation of the sodium salts of a number of amino-acids on a silica gel–gypsum layer by a two-dimensional method using the propanol–water (1 : 1) system in one direction and the phenol–water (10 : 4) system in the other[29]. The amino-acids were detected with ninhydrin solution.

Almost simultaneously, amino-acids were separated on a silica gel–gypsum layer with phosphate buffer[28]. In the preparation of the layer, a solution of 0.2 M KH_2PO_4 and 0.2 M Na_2HPO_4 (1 : 1) was used instead of water. The separation was carried out in 96% ethanol and in the 96% ethanol–25% ammonia (4 : 1) and 96% ethanol–25% ammonia–water (7 : 1 : 2) systems. In the two-dimensional separation, the first two systems were used. The compounds were detected with the reagents usually employed for this purpose in paper chromatography.

Finally, a silica gel–gypsum layer has recently given excellent results[136], thanks to a careful and thorough study of the technique of separating amino-acids by thin-layer chromatography. It was found that on a silica gel–gypsum layer, the spots obtained are smaller and of better shape, as a result of the less marked diffusion, than those obtained on paper, and that to obtain good results in the preparation of the layer, the plate should not be activated by heating, but simply by drying in air. The height to which the liquid rises should not be more than 10 cm from the starting line. The quantities of acids applied to the plate should be not more than 1–5 μg, since the size of the spot depends on the quantity of substance taken. When these conditions are observed, reproducible results are obtained and the variation in the R_f values is ±0.05. Five systems were examined. The best results were obtained by separation in the n-butanol–acetic acid–water (60 : 20 : 20) and phenol–water (75 : 25) systems, the results being particularly good when two-dimensional separation in these systems was used. The time required for the separation is 1.5–2 h.

In the detection of the compounds, the plate is dried for 10 min at 100° and sprayed with a freshly prepared 50 : 3 mixture of two solutions: (1) a solution of 0.2% ninhydrin in 50 ml absolute alcohol, 10 ml acetic acid, and 2 ml 2,4,6-collidine; and (2) a 1% solution of $Cu(NO_3)_2.3H_2O$ in absolute alcohol. The sensitivity of the method is 10 times greater than that of paper chromatography.

The results of the two-dimensional separation of amino-acids by paper chromatography and by thin-layer chromatography on a silica gel–gypsum layer in the same systems are compared in Fig. 13. Ten amino-acids on paper are shown on the left, and fourteen amino-acids on a silica gel–gypsum layer are shown on the right.

The separation of the same amino-acids, with even better results, on a silica gel–gypsum layer by a two-dimensional method using the chloroform–methanol–17% ammonia (2 : 2 : 2) system in the first direction and the previously used phenol–water (75 : 25) system in the other, has recently been described[137].

In the separation and identification of amino-acids, *N*-(2,4-dinitrophenyl)amino-acids, and their 3-phenyl-2-thiohydantoins[51,30], thin-layer chromatography has shown its advantages over paper chromatography. There is a saving in time, the time required for the determination being 1–3 h, and an increase in sensitivity by a factor of at least 10.

A silica gel–gypsum layer can be used to separate the dinitrophenyl (DNP) derivatives of almost all the protein amino-acids, soluble or insoluble in water and acids.

Dinitrophenylamino-acids soluble in water and acids have been identified in the n-propanol–34% ammonia (7 : 3) system[51]. Five systems have been used to separate the water-insoluble derivatives: (I) toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonia (100 : 30 : 60 : 60); (II) chloroform–benzyl alcohol–acetic acid (70 : 30 : 3); (III) chloroform–t-pentyl alcohol–acetic acid (70 : 30 : 3); (IV) benzene–pyridine–acetic acid (80 : 20 : 2); and (V) chloroform–methanol–acetic acid (95 : 5 : 1).

The best results were obtained by two-dimensional separation in these systems. The same paper also describes the results of a flow method of separation[30], which was used for the complete separation of various pairs of compounds, requiring an increase in the distance moved by the separating system. In this case good results were obtained in the separation of: (1) leucine and isoleucine; (2) *N*-(2,4-dinitrophenyl)leucine, *N*-(2,4-dinitrophenyl)isoleucine, and *N*-(2,4-dinitrophenyl)norleucine; (3) *N*-(2,4-dinitrophenyl)valine and *N*-(2,4-dinitrophenyl)-norvaline; and (4) *NN*-di-(2,4-dinitrophenyl)tyrosine and *NN*-di-(2,4-dinitrophenyl)lysine.

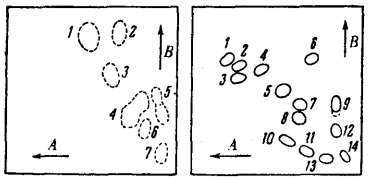


Fig. 13. Comparison of chromatograms for amino-acids on paper and on a silica gel–gypsum layer, obtained under identical conditions. Systems: *A*) n-butanol–acetic acid–water (6 : 2 : 2); *B*) phenol–water (75 : 25).
Paper chromatogram (*left*): 1) phenylalanine + isoleucine; 2) proline; 3) tyrosine; 4) glycine + glutamic acid; 5) arginine + lysine; 6) aspartic acid; 7) cysteine.
Thin-layer chromatogram (*right*): 1) phenylalanine; 2) isoleucine; 3) tyrosine; 4) valine; 5) alanine; 6) proline; 7) glycine; 8) serine; 9) histidine; 10) glutamic acid; 11) asparagine; 12) arginine; 13) cysteine; 14) cystine.

One-dimensional chromatography[51] makes it possible to detect down to 0.1 μg, and two-dimensional chromatography down to 0.5 μg of substance in daylight. Detection by photocopying in ultraviolet light is particularly sensitive. In this case 0.1–0.2 μg of amino-acid in a mixture of amino-acids is sufficient. The sensitivity for a single acid is 2×10^{-3} or 10^{-4} μmole (for example 0.02 μg for DNP-serine). With the application of ~100 μg of one DNP-amino-acid, it has been possible to detect the presence of ~0.05% of another DNP-amino-acid.

Mixtures of 3-phenyl-2-thiohydantoins (PTH) of amino-acids can readily be separated[51] on a silica gel–gypsum layer by one- or two-dimensional chromatography. 14 of the 19 amino-acid PTH studied were separated. The remainder are readily separated by a simple oxidation with hydrogen peroxide or by acid hydrolysis followed by separation of the free amino-acids on a thin layer of silica gel.

The amino-acid PTH were detected using a modified chlorotoluidine test[133]. The sensitivity of detection is 3×10^{-4} μmole, which corresponds, for example, to 0.05 μg for proline PTH.

The separation of amines, amino-acids, and adrenaline–noradrenalines on neutral and buffer silica gel–gypsum layers and on cellulose powder in the n-pentyl alcohol–acetic acid–water (4 : 1 : 5) system has been studied in detail[45]. The same paper also describes the separation, on neutral, phosphate-buffer, or acetate-buffer silica gel–gypsum layers, of the following amines: methylamine, ethylamine, n-propylamine, isopentylamine, cadaverine, putrescine, ethanolamine, histamine, tyrosine, phenylethylamine, benzylamine, and tryptamine. For the neutral layers, the

96% ethanol–25% ammonia (4 : 1), phenol–water (8 : 3), or butanol–acetic acid–water (4 : 1 : 5) systems were used. On the plates with Sörensen's phosphate buffer (pH 6.8), the separation was carried out in 10% aqueous ethanol, and on the plates with acetate buffer the separation was carried out in the butanol–acetic acid–water (4 : 1 : 5) system. On the neutral plates, the primary and secondary amines were best identified as the 3,5-dinitrobenzamides in the chloroform–ethanol (99 : 1) system.

The compounds were detected with a solution of iodine in chloroform, with α-naphthylamine solution, or with light from a quartz lamp.

An interesting example of the application of this method is provided by the separation of adrenaline and noradrenaline. Decomposition of these amines by oxidation during the chromatographic process is possible, but is prevented by the addition of a small quantity of sodium hydrogen sulphite as an antioxidant during the preparation of the phosphate-buffer layer. The separation was carried out in 70% ethanol. The spots were detected by spraying with a solution of iodine in chloroform and a solution of potassium cyanoferrate(III) in phosphate buffer and examining in ultraviolet light. The same amines were separated on a plate with a thin layer of cellulose powder in the n-pentyl alcohol–acetic acid–water (4 : 1 : 5) system.

A method for thin-layer ionophoresis and two-dimensional combined thin-layer ionophoresis and chromatography on a glass plate has recently been described[139]. Silica gel–gypsum, kieselguhr–gypsum, and aluminium oxide–gypsum layers were used for the ionophoretic separation of the amino-acids and a silica gel–gypsum layer was used for the combined ionophoretic and chromatographic separation.

In the determination of amino-acids and polypeptides by paper chromatography[140], quantities of substance of about 1 μmole (sometimes 0.1–0.2 mole) are required. The use of thin-layer chromatography on a thin layer of silica gel–gypsum for this purpose makes it possible to work with quantities of ~0.01 μmole. Glycyl-*L*-proline dipeptide thiohydantoin (R_f 0.36) and *L*-prolyl-*L*-leucylglycine tripeptide thiohydantoin (R_f 0.42) were identified in the heptane–pyridine–ethyl acetate (5 : 3 : 2) system[140]. To obtain good results, it was necessary to ensure that the layer was not saturated with atmospheric moisture, which has a marked effect on the R_f values. The compounds were detected with a starch–iodine–azide reagent: the plate was sprayed first with a 0.5% aqueous starch solution, then with iodine solution, and finally with sodium azide solution. The lower limit of detection is 0.01 μg of the peptide thiohydantoin.

A silica gel–gypsum layer has been used for the qualitative and quantitative separation of the natural bile acids: lithocholic, cholic, desoxycholic, and chenodesoxycholic acids, and the synthesised peptide derivatives formed with glycine (glyco-conjugates) and taurine (tauro-conjugated)[141]. The separation was carried out in two stages. The free acids moved with the upper phase of a toluene–acetic acid–water (5 : 5 : 1) mixture, and the conjugates remained close to the starting line. The glyco- and tauro-conjugates were then separated in the butanol–acetic acid–water (10 : 1 : 1) system. In this case the free acids moved with the liquid front. The compounds were detected with molybdophosphoric acid solution and heating for 5 min at 110°. The spectrophotometric determinations were carried out using 65% sulphuric acid, which was used to elute the substances from the silica gel. The quantity of substance in the mixture was determined by comparing the extinction coefficients.

Derivatives of the nucleic acids have been separated on a thin layer of cellulose[142]. Nucleic bases and nucleotides were separated with distilled water. The nucleotides were separated in the n-butanol–acetone–acetic acid–5% ammonia–water (4.5 : 1.5 : 1 : 1 : 2) system. The compounds were detected in ultraviolet light. The sensitivity of the method is ~10^{-3} μmole for the adenine compounds and slightly more for the cytosine compounds.

Chromatography on a silica gel–gypsum layer has also been used to characterise certain intermediate products in the synthesis of (N-methyltyrosine)$_2$-oxytocin[143]. The same layer has been used to follow the synthesis of models of aminoacylribonucleic acids[144].

13. Alkaloids

The use of thin-layer chromatography for the rapid analysis of small quantities of alkaloids is particularly important in the study of medicinal products and in toxicology.

In 1960, a silica gel–gypsum layer with Sörensen's phosphate buffer[45] in the chloroform–ethanol (8 : 1) system was used to separate alkaloids containing different purine groups: caffeine, theobromine, and theophylline. The chromatogram was sprayed with an alcoholic solution of iodine and potassium iodide (1 g I and 1 g KI in 100 ml ethanol) and then with a solution of 25% HCl in 96% ethanol (1 : 1). The same paper describes the separation of ergoalkaloids on cellulose powder impregnated with a 20% solution of formamide in acetone. The results were better than those obtained by paper chromatography. In this case a two-stage separation was used in the benzene–heptane–chloroform (6 : 5 : 3) and benzene–heptane (6 : 5) systems.

The same authors separated several other groups of alkaloids: the Belladonna, Rauwolfia, opium, and other alkaloid groups[43]. The Belladonna alkaloids were separated on a neutral or alkaline silica gel–gypsum layer. In the case of the neutral layer, a dimethylformamide–diethylamine–ethanol–ethyl acetate (1 : 1 : 6 : 12) system was used and in the case of the alkaline layer (2 N KOH) the 70% ethanol–25% ammonia (99 : 1) system was used. The same alkaloids were also separated by a two-stage method on a cellulose layer impregnated with formamide, in the heptane–diethylamine (500 : 1) (distance moved 15 cm) and benzene–heptane–chloroform–diethylamine (6 : 5 : 1 : 0.02) (distance moved 10 cm) systems.

The Rauwolfia alkaloids were separated on a silica gel–gypsum layer in the chloroform–96% alcohol (9 : 1) or methanol–ethyl methyl ketone–heptane (8.4 : 33.6 : 58.0) systems. The same alkaloids were also separated on cellulose powder impregnated with a 20% solution of formamide in acetone, in the heptane–ethyl methyl ketone (1 : 1) system in an atmosphere of ammonia. The spots were detected in ultraviolet light or with trichloroacetic acid and ferric chloride.

The opium alkaloids were separated on neutral and alkaline silica gel–gypsum layers. The chloroform–ethanol (9 : 1) system was used in the case of the neutral layer, and the chloroform–ethanol (8 : 2) system in the case of the alkaline layer. These alkaloids were also analysed on a cellulose layer impregnated with formamide, in the benzene–heptane–

chloroform–diethylamine (6 : 5 : 1 : 0.02) system. The compounds were detected with Dragendorf's reagent.

Lobelia alkaloids were separated by chromatography on cellulose impregnated with formamide, in the benzene–heptane–diethylamine (1 : 6 : 0.02) system, and Helidonia alkaloids were separated on a silica gel–gypsum layer in the chloroform–ethanol (3 : 1) system. The alkaloids coniine, anabasine, nicotine, and arecoline were isolated by steam distillation and separated on an alkaline (2 *N* KOH) silica gel–gypsum layer in the chloroform–ethanol (8 : 2) system.

Thus thin-layer chromatography provides the most rapid method for the analysis of alkaloids and has been used successfully[44] for the systematic analysis of these compounds. As a result of the study of more than 50 alkaloids in seven different systems and solvents, it has been shown from their behaviour in these systems that the alkaloids can be separated into two groups. When the group to which the alkaloids being studied belong has been established, they are analysed in the systems cited and can be identified from a table of R_f values and colour reactions[44]. The separation was carried out on silica gel–gypsum, aluminium oxide–gypsum, and alkaline silica gel–gypsum layers in seven systems.

The alkaloids were detected by intense spraying with iodoplatinate reagent prepared from equal volumes of 0.3% aqueous chloroplatinic acid solution and 6% aqueous potassium iodide solution.

Thin-layer chromatography on a silica gel–gypsum layer in the methanol–ethyl methyl ketone–heptane (84 : 33.6 : 58) system has been used to separate the indole alkaloids reserpine and rescinnamine[145]. These alkaloids were determined quantitatively by spectrophotometry.

The separation of the opium alkaloids[146] on a thin layer of alkaline aluminium oxide (pH 5) in mixtures of ethanol, n-butanol, and water in different proportions has been described. These alkaloids have also been separated on a silica gel–gypsum layer in methanol[39]. This method has also been used to detect the alkaloids ergine and isoergine in the alkaloid fraction from *Rivea corymbosa* seeds[147].

14. Pteridines

The pteridines, representatives of the folic acid group, are of considerable physiological importance and play an important part in the exchange of substances in the organism. In papers on the synthesis of these compounds, the R_f value is often quoted as an important physical constant. Many pteridines are difficult to separate on paper. The use of thin-layer chromatography for this purpose has given good results[148] which could not previously be obtained on paper. The separation was carried out on a silica gel–gypsum or aluminium oxide–gypsum layer. A total of 24 neutral, alkaline, and acidic systems and solvents were studied. R_f values have been given for 14 pteridines[148].

The separation was carried out in the systems: dimethylformamide–water (190 : 10); 1, 5, and 10% citric acid; 5% malic acid; and 5% tartaric acid. In the case of leucopterin, 6-methylisoxanthopterin, 7-methylxanthopterin, isoxanthopterin, xanthopterincarboxylic acid, and a number of aminohydroxypteridines, the separation was carried out in ammonia solutions of different concentrations (1–25%) and certain other systems. The compounds were detected with ultraviolet light. The lower limit of sensitivity of this method is 1 μg.

15. Heterocyclic Compounds

The separation of small quantities of mixtures of indole derivatives on a silica gel–gypsum layer in a supersaturated vessel at 20° in the dark has recently been described[15]. Two reagents were used for detection: (1) Ehrlich's reagent, a solution of *p*-dimethylaminobenzaldehyde in a mixture of 50 ml 25% hydrochloric acid and 50 ml 96% ethanol, and (2) Procházka's reagent, a solution of 10 ml 35% formaldehyde, 10 ml 25% hydrochloric acid, and 20 ml 96% ethanol, with subsequent heating at 100° and examination in ultraviolet light. The latter reagent is very sensitive and in some cases can detect 0.005 μg of substance.

An unfixed layer of aluminium oxide with activity II/III, in the benzene–acetone (1 : 1) system, has been used to separate the following heterocyclic compounds[21]: the isomeric *N*-benzoyl- and *N*-acetyl-decahydroquinolinols (four isomers), the different 4-phenylpiperidin-4-ols, *NO*-diacetyldecahydroquinolin-4-ols, and cyanoethylcyclohexane-1,3-diones. The isomeric *O*-acetyldecahydroquinolin-4-ols were separated on the same layer in the acetone–methanol (3 : 1) system. The compounds were detected with iodine vapour and ultraviolet light.

16. Other Examples of the Use of Thin-Layer Chromatography

A number of other examples of the use of thin-layer chromatography to separate and identify various compounds and their mixtures have been described. The limits of applicability of this method are gradually being extended, and there are examples of the analysis of various hydrocarbons, alcohols, acids, organic peroxides, nitro-compounds, dyes, inorganic compounds, etc.

A thin layer of silica gel–gypsum in petroleum ether has been used to separate azulene and cadalene[5,7] and the same layer in the hexane–ethyl acetate (9 : 1) system has been used to separate chamazulene, chamazulenyl alcohol, and the acids of azulenic sesquiterpene and proazulene.

Anthracene derivatives[13] can be readily separated in the benzene–methyl acetate–80% formic acid (4 : 4 : 2) system or, more effectively, in the benzene–ethyl formate–formic acid (75 : 24 : 1) system[14]. The compounds are detected with a 2.5% solution of KOH in methanol and in long-wave ultraviolet light.

An unfixed aluminium oxide layer has been used to separate a number of acetylenic carbinols and the corresponding glycols in the benzene–ether (2 : 3) system. The spots were detected with iodine vapour and ultraviolet light[149].

Silica gel–gypsum layers in the benzene–methanol–acetic acid (45 : 8 : 4) and benzene–dioxan–acetic acid (90 : 25 : 4) systems[150] have been used to identify the following dibasic acids: oxalic, malonic, succinic, glutaric, adipic, pimelic, suberic, azelaic, and sebacic acids. The compounds were detected by drying the plate for 1 h at 120° and then spraying with bromophenol solution acidified with citric acid. The same layer in the first system was used to separate fumaric (Rf 0.23) and maleic (Rf 0.07) acids. The compounds were detected with permanganate solution made alkaline with sodium carbonate.

The same series of acids from oxalic to sebacic acid, were separated on a silica gel–gypsum layer in the 96% alcohol–water–25% ammonia (100 : 12 : 16) system[151]. The same paper also describes the separation of various aliphatic and aromatic acids: citric, tartaric, phthalic, terephthalic, benzoic, and *p*-toulic acids, and also phosphoric acid. The compounds were detected with Bromocresol Green.

Organic peroxides and acid peroxides have been separated[152] on silica gel–starch plates in the carbon tetrachloride–acetone (2 : 1) system. The compounds were detected with potassium iodide solution or a mixture of $Fe(NH_4)_2(SO_4)_2$ and KSCN.

A number of β-dicarbonyl compounds have been separated and identified on a silica gel–starch layer in the benzene–ethyl acetate (7 : 3) system[153]. The spots were detected with iodine vapour.

Various natural vegetable dyes[17,18] have been separated on an unfixed layer of aluminium oxide in mixtures of n-butanol, ethanol, and water in different proportions. Dyes of the Sudan Green series have been separated on a silica gel–gypsum layer[7].

A silica gel–gypsum layer in the hexane–ethyl acetate (85 : 15 and 1 : 1), benzene–ether (17 : 3), benzene–pyridine (1 : 1), and benzene systems has been used to separate various aromatic nitro-compounds and amines, azo-, azoxy- and hydrazo-compounds[154]. The spots were detected by spraying the plate with a mixture of 5% KOH solution and acetone (2 : 1) or with a 10% solution of $SnCl_2$ in concentrated hydrochloric acid. The nitro-compounds are usually detected after spraying and heating or after heating and subsequent spraying with conc. H_2SO_4 and HNO_3 (1 : 1). In the latter case, nitroamines are also detected.

Thin-layer chromatography on a silica gel–starch layer has been used[155] for the detection of blood meprobamate (2-methyl-2-n-propyl-propan-1,3-diol dicarbamate) in the absolute ethanol–cyclohexane (15 : 85) system. The compounds were detected with concentrated sulphuric acid.

Thin-layer chromatography has also been used to study medicinal products[29,156–162] and in clinical analysis[163].

The separation and identification of inorganic compounds of mercury, bismuth, cadmium, lead, copper, iron, zinc, cobalt, manganese, chromium, nickel, and aluminium on a thin layer of silica gel–gypsum has been described[164]. This method has been used for the separation and identification of U^{VI} and Ga^{III} in a mixture of cations[165]. The chromatographic separation and identification of the cations of the alkali-group Li^+, Mg^{2+}, Na^+, and K^+,[166] and of the halide anions F^-, Cl^-, Br^-, and I^-[167] has also been described. The analysis of orthophosphoric, pyrophosphoric, phosphorous, and hypophosphorous acids has been described[168].

This review has shown the development of the method of thin-layer chromatography on fixed and unfixed layers of adsorbent from 1938 to the present time. Chromatography on a thin layer of adsorbent is a simple and rapid method which rivals the method of paper chromatography and in a number of cases is more sensitive than the latter by factors of 10–20. The method is extending rapidly and is widely used in almost all fields of organic chemistry. It can be used not only for the separation of mixtures of substances and the identification of individual compounds, but also to examine their purity. The use of thin-layer chromatography is very convenient for the analysis of the fractions obtained in column chromatography and for following the course of chemical reactions in organic synthesis. Combined with gas–liquid chromatography, this method sometimes makes it possible to solve problems which cannot be solved by other methods of separation. Thin-layer chromatography on an unfixed layer of adsorbent will undoubtedly find extensive application for preparative purposes.

1. M. Tsvet, Ber. deut. bot. Ges., 24, 316, 384 (1906).
2. N. A. Izmailov and M. S. Shraiber, Farmatsiya, No. 3, 1 (1938).
3. J. E. Meinhard and N. F. Hall, Analyt. Chem., 21, 185 (1949).
4. J. G. Kirchner, J. Millere, and G. J. Keller, Analyt. Chem., 23, 420 (1951).
5. E. Stahl, Pharmazie, 11, 632 (1956).
6. E. Stahl, Chem.-Ztg., 82, 323 (1958).
7. E. Stahl, Parfümerie u. Kosmetik, 39, 564 (1958).
8. E. Stahl, Arch. Pharm., 292, 411 (1959).
9. E. Stahl, Pharm. Rdsch., 1, 1 (1959).
10. E. Stahl, Arch. Pharm., 293, 531 (1960).
11. E. Stahl and U. Kaltenbach, J. Chromatog., 5, 351 (1961).
12. E. Stahl and U. Kaltenbach, J. Chromatog., 5, 458 (1961).
13. E. Stahl, Z. analyt. Chem., 181, 303 (1961).
14. E. Stahl and P. J. Schorn, Z. physiol. Chem., 325, 263 (1961).
15. E. Stahl and H. Kaldewey, Z. physiol. Chem., 323, 182 (1961).
16. E. Stahl, Angew. Chem., 73, 646 (1961).
17. M. Mottier and M. Potterat, Analyt. Chim. Acta, 13, 46 (1955).
18. M. Mottier, Mitt. Gebiete Lebensmitt u. Hyg., 47, 372 (1956).
19. V. Černý, J. Joska, and L. Lábler, Coll. Czech. Chem. Comm., 26, 1658 (1961).
20. S. Heřmánek, V. Schwartz, and Z. Čekan, Coll. Czech. Chem. Comm., 26, 1669 (1961).
21. E. A. Mistryukow, Coll. Czech. Chem. Comm., 26, 2071 (1961).
22. Z. Procházka, Chem. Listy, 55, 975 (1961).
23. E. Demole, J. Chromatog., 1, 24 (1958).
24. E. Demole, J. Chromatog., 6, 2 (1961).
25. Z. Michalec, Chem. Listy, 55, 953 (1961).
26. H. Weicker, Klin. Wochschr., 37, 763 (1959).
27. K. Huhnstock and H. Weicker, Klin. Wochschr., 38, 1249 (1960).
28. E. Mutschler and H. Rochelmeyer, Arch. Pharm., 292, 449 (1959).
29. E. Nürnberg, Arch. Pharm., 292, 610 (1959).
30. M. Brenner and A. Niederwieser, Experientia, 17, 237 (1961).
31. W. L. Stanley and S. H. Vannier, J. Assoc. Offic. Agric. Chemists, 40, 582 (1957).
32. S. H. Vannier and W. L. Stanley, J. Assoc. Offic. Agric. Chemists, 41, 432 (1958).
33. W. L. Stanley, J. Assoc. Offic. Agric. Chemists, 42, 643 (1959).
34. L. H. Bryant, Nature, 175, 556 (1955).
35. A. A. Akhrem and A. I. Kuznetsova, Dokl. Akad. Nauk SSSR, 138, 591 (1961).

36. N. K. Kochetkov, I. G. Zhukova, and I. S. Glukhoded, Dokl. Akad. Nauk SSSR, 139, 608 (1961).
37. L. D. Bergel'son, E. V. Dyatlovitskaya, and V. V. Voronkova, Dokl. Akad. Nauk SSSR, 141, 84 (1961).
38. M. Barbier, H. Jäger, H. Tobias, and E. Wyss, Helv. Chim. Acta, 42, 2440 (1959).
39. G. Machata, Microchem. Acta, 79 (1960).
40. R. H. Reitsema, Analyt. Chem., 26, 960 (1954).
41. A. Seher, Die Nahrung, 4, 466 (1960).
42. G. Pastuska, Z. analyt. Chem., 179, 427 (1961).
43. K. T. Teichert, E. Mutschler, and H. Rochelmeyer, Deut. Apoth. Ztg., 100, 477 (1960).
44. D. Waldi, K. Schnackerz, and F. Munter, J. Chromatog., 6, 61 (1961).
45. K. T. Teichert, E. Mutschler, and H. Rochelmeyer, Deut. Apoth. Ztg., 100, 283 (1960).
46. H. P. Kaufmann, Z. Makus, and B. Das, Fette u. Seifen, 63, 807 (1961).
47. H. P. Kaufmann, Fette u. Seifen, 63, 828 (1961).
48. M. Brenner, A. Niederwieser, G. Pataki, and A. R. Fahmy, Experientia, 18, 101 (1962).
49. H. Brockman and H. Schodder, Ber., 74, 73 (1941).
50. S. Heřmánek, V. Schwarz, and Z. Čekan, Coll. Czech. Chem. Comm., 26, 3170 (1961).
51. M. Brenner, A. Niederwieser, and G. Pataki, Experientia, 17, 145 (1961).
52. M. Brenner and G. Pataki, Helv. Chim. Acta, 44, 1420 (1961).
53. E. Hecker, Chimia (Switz.), 8, 229 (1954).
54. J. Blattná and J. Davidek, Experientia, 17, 473 (1961).
55. H. Brockmann, Chem. Ber., 80, 81 (1947).
56. J. G. Kirchner, J. M. Miller, and R. G. Rice, J. Agric. Food Chem., 2, 1031 (1954).
57. A. Seher, Mikrochim. Acta, 308 (1961).
58. J. S. Matthews, A. L. Perada V., and A. Aguilera P., J. Chromatog., 9, 331 (1962).
59. W. L. Stanley, S. H. Vannier, and B. Gentili, J. Assoc. Offic. Agric. Chemists, 40, 282 (1957).
60. H. Wagner, Fette u. Seifen, 63, 1119 (1961).
61. J. M. Miller and J. G. Kirchner, Analyt. Chem., 24, 1480 (1952).
62. W. Winkler and E. Lunau, Pharm. Ztg., 104, 1407 (1959).
63. C. H. Brieskorn and E. Wenger, Arch. Pharm., 293, 26 (1960).
64. E. Stahl and L. Trennheuser, Arch. Pharm., 293, 826 (1960).
65. J. G. Kirchner and J. M. Miller, Ind. Eng. Chem., 44, 318 (1952).
66. J. M. Miller and J. G. Kirchner, Analyt. Chem., 25, 1107 (1953).
67. M. Ito, S. Wakamatsu, and H. Kawahara, J. Chem. Soc. Japan, Pure Chem. Sect., 74, 699 (1953).
68. M. Ito, S. Wakamatsu, and H. Kawahara, J. Chem. Soc. Japan, Pure Chem. Sect., 75, 413 (1954).
69. R. H. Reitsema, J. Amer. Pharmaceut. Assoc., (Pract. Pharmaceut. Edn.), 43, 414 (1954).
70. E. Demole, Compt. rend., 243, 1883 (1956).
71. E. Demole and E. Lederer, Bull. Soc. chim. France, 1128 (1958).
72. C. H. Brieskorn and E. Wenger, Arch. Pharm., 293, 21(1960).
73. R. Jaspersen-Schib, Pharm. Acta Helv., 36, 141 (1961).
74. M. Ito, J. Chem. Soc. Japan, 78, 172 (1957).
75. H. J. Petrowitz, Angew. Chem., 72, 921 (1960).
76. G. Gogröf, Pharmazie, 12, 38 (1957).
77. R. Tschesche, F. Lampert, and G. Snatzke, J. Chromatog., 5, 217 (1961).
78. R. Tschesche, A. Kumar, and Sen Gurta, Chem. Ber., 93. 1903 (1960).
79. A. F. Thomas and J. M. Müller, Experientia, 16, 62 (1960).
80. E. Stahl and H. D. Wulff, Naturwiss., 47, 114 (1960).
81. K. Onoe, J. Chem. Soc. Japan, Pure Chem. Sect., 73, 377 (1952).
82. J. Labat and A. L. Montes, Anales Asoc. quim. argentina, 41, 166 (1953).
83. T. Furukawa, J. Chem. Soc. Japan, Pure Chem. Sect., 80, 45 (1959).
84. J. H. Dhont and C. de Rooy, Analyst, 86, 74 (1961).
85. M. J. D. van Dam, G. J. de Kleuver, and J. G. de Heus, J. Chromatog., 4, 26 (1960).
86. M. J. D. van Dam, Bull. Soc. chim. belges, 70, 122 (1961).
87. H. P. Kaufmann, Z. Makus, and F. Doine, Fette u. Seifen, 63, 235 (1961).
88. H. Danielsson, Acta Chem. Scand., 15, 245 (1961).
89. R. Neher and A. Wettstein, Helv. Chim. Acta, 43, 1628 (1960).
90. R. Tschesche and G. Snatzke, Annalen, 636, 105 (1960).
91. H. Metz, Naturwiss., 48, 569 (1961).
92. M. Barb'e and S. I. Zav'yalov, Izv. Akad. Nauk SSSR, Otd. Khim. Nauk, 1309 (1960).
93. H. Struck, Mikrochem. Acta, 634 (1961).
94. R. Tschesche, W. Freytag, and G. Snatzke, Chem. Ber., 92, 3053 (1959).
95. K. Egger, Z. analyt. Chem., 182, 161 (1961).
96. H. K. Mangold, Fette u. Seifen, 61, 877 (1959).
97. H. K. Mangold and D. C. Malins, J. Amer. Oil Chemists' Soc., 37, 383 (1960).
98. D. C. Malins, J. Amer. Oil Chemists' Soc., 37, 576 (1960).
99. H. P. Kaufmann and Z. Makus, Fette u. Seifen, 62, 1014 (1960).
100. H. P. Kaufmann, Z. Makus, and T. H. Khoe, Fette u. Seifen, 63, 689 (1961).
101. H. P. Kaufmann and G. Walter, Fette u. Seifen, 61, 782 (1959).
102. L. J. Morris, R. T. Holman, and K. Fontell, J. Amer. Oil Chemists' Soc., 37, 323 (1960).
103. L. J. Morris, R. T. Holman, and K. Fontell, J. Lipid Res., 2, 68 (1961).
104. H. K. Mangold and R. Kammereck, Chem. and Ind., 1032 (1961).
105. E. H. Gruger Jnr., D. C. Malins, and E. J. Gauglitz, J. Amer. Oil Chemists' Soc., 37, 214 (1960).
106. E. J. Gauglitz and D. C. Malins, J. Amer. Oil Chemists' Soc., 37, 425 (1960).
107. D. C. Malins, Chem. and Ind., 1359 (1960).
108. J. A. Williams, A. Sharma, L. J. Morris, and R. T. Holman, Proc. Soc. Exp. Biol. Med., 105, 192 (1960).
109. H. Jatzkewitz and E. Mehl, Z. physiol. Chem., 320, 251 (1960).
110. H. Jatzkewitz, Z. physiol. Chem., 320, 134 (1960).
111. H. Wagner, Fette u. Seifen, 62, 1115 (1960).
112. E. Klenk and W. Geilin, Z. physiol. Chem., 323, 126 (1961).
113. G. Wagner, Pharmazie, 10, 302 (1955).
114. R. L. Lyman, A. L. Livinston, E. M. Bickoff, and A. N. Booth, J. Org. Chem., 23, 756 (1958).
115. G. Pastuska, Z. analyt. Chem., 179, 355 (1961).
116. M. Barbier, J. Chromatog., 2, 649 (1959).
117. R. Bravo and F. Hernadez, J. Chromatog., 7, 60 (1962).
118. W. L. Stanley and S. H. Vannier, J. Amer. Chem. Soc., 79, 3488 (1957).
119. R. A. Bernhard, Nature, 182, 1171 (1958).
120. T. Akarawa and I. Uritani, Arch. Biochem. Biophys., 88, 150 (1960).
121. L. Hörhammer, H. Wagner, and B. Lay, Pharmazie, 15, 645 (1960).
122. J. Davidek and Z. Procházka, Coll. Czech. Chem. Comm., 26, 2947 (1961).
123. A. Winterstein, Angew. Chem., 72, 902 (1960).
124. A. Winterstein and B. Hegedüs, Chimia (Switz.), 14, 18 (1960).

125. A. Winterstein and B. Hegedüs, Z. physiol. Chem., 321, 97 (1960).
126. A. Winterstein, A. Studer, and R. Rüegg, Chem. Ber., 93, 2951 (1960).
127. O. Isler, R. Ruegg, and P. Schudel, Chimia (Switz.), 15, 208 (1961).
128. K. Fontell, R. T. Holmann, and G. Lambertsen, J. Lipid Res., 1, 391 (1960).
129. H. Gänshirt and M. Malzacher, Naturwiss., 47, 279 (1960).
130. A. Seher, Fette u. Seifen, 61, 345 (1959).
131. O. E. Schultz and D. Straus, Arzneimittel-Forsch., 5, 342 (1958).
132. J. W. C. Peereboom, J. Chromatog., 4, 323 (1960).
133. R. G. W. Spickett, Chem. and Ind., 561 (1957).
134. J. Bäumler and S. Rippstein, Helv. Chim. Acta, 44, 1163 (1961).
135. M. Mottier, Mitt. Gebiete Lebensm. Hyg., 49, 454 (1958).
136. M. Brenner and A. Niederwieser, Experientia, 16, 378 (1960).
137. A. H. Fahmy, A. Niederwieser, G. Pataki, and M. Brenner, Helv. Chim. Acta, 44, 2022 (1961).
138. M. Brenner, J. P. Zimmermann, J. Wehrmüller, P. Quitt, A. Hartmann, W. Schneider, and V. Beglinger, Helv. Chim. Chim. Acta, 40, 1497 (1957).
139. C. G. Honegger, Helv. Chim. Acta, 44, 173 (1961).
140. E. Charbulierz, B. Baether, and J. Rabinowitz, Helv. Chim. Acta, 43, 1871 (1960).
141. H. Gänshirt, F. W. Koss, and K. Morianz, Arzneimittel-Forsch., 10, 943 (1960).
142. K. Randerath and H. Stuck, J. Chromatog., 6, 365 (1961).
143. R. L. Hugnenin and R. A. Boissanas, Helv. Chim. Acta, 44, 213 (1961).
144. H. G. Zachaus and W. Karaus, Chem. Ber., 93, 1630 (1960).
145. F. Schlemmer and E. Link, Pharm. Ztg., 104, 1349 (1959).
146. A. Mariani and O. Mariani-Marelli, Rend. Ist. Super. sanita, 22, 759 (1959); Ref. Zhur. Biol., N16, 22200 (1960).
147. A. Hofman and H. Tscherter, Experientia, 16, 414 (1960).
148. B. J. R. Nicolaus, J. Chromatog., 4, 384 (1960).
149. A. A. Akhrem, A. I. Kuznetsova, Yu. A. Titov, and I. S. Levina, Izv. Akad. Nauk SSSR, Otd. Khim. Nauk, 657, (1962).
150. H. J. Petrowitz and G. Pastuska, J. Chromatog., 7, 128 (1962).
151. D. Braun and H. Greenen, J. Chromatog., 7, 56 (1962).
152. K. Marugama, J. Chem. Soc. Japan, Pure Chem. Sect., 77, 1496 (1956).
153. M. Barb'e, L. P. Vinogradova, and S. I. Zav'yalov, Izv. Akad. Nauk SSSR, Otd. Khim. Nauk, 162 (1961).
154. F. Furukawa, Chem. Abs., 52, 13364 (1958).
155. A. Fiori and M. Marigo, Nature, 182, 943 (1958).
156. S. Grüner and W. Spaich, Arch. Pharm., 287, 243 (1954).
157. H. Liebich, Deut. Apoth. Ztg., 99, 1246 (1959).
158. H. Gänshirt and A. Malzacher, Arch. Pharm., 293, 925 (1960).
159. H. Ganshirt and K. Morianz, Arch. Pharm., 293, 1065 (1960).
160. H. Liebich, Deut. Apoth. Ztg., 100, 393 (1960).
161. R. Fischer and H. Lauther, Arch. Pharm., 294, 1 (1961).
162. M. Frahm, A. Gottesleben, and K. Soehring, Arzneimittel-Forsch., 11, 1008 (1961).
163. G. Machata, Wien Klin. Wochschr., 71, 301 (1959).
164. H. Seiler and M. Seiler, Helv. Chim. Acta, 43, 1939 (1960).
165. H. Seiler and M. Seiler, Helv. Chim. Acta, 44, 939 (1961).
166. H. Seiler and W. Rothweiler, Helv. Chim. Acta, 44, 941 (1961).
167. H. Seiler and T. Kaffenberger, Helv. Chim. Acta, 44, 1282 (1961).
168. H. Seiler, Helv. Chim. Acta, 44, 1753 (1961).

Zelinskii Institute of Organic Chemistry,
Academy of Sciences of the USSR

U.D.C. 541.182:541.183

INFLUENCE OF FINE GRINDING ON THE PHYSICOCHEMICAL PROPERTIES OF SOLIDS

G. S. Khodakov

CONTENTS

1. Introduction 386
2. Decrease in particle size 387
3. Change in structure of solids 388
4. Exposure of clean surfaces and formation of free radicals 390
5. Increase in adsorption capacity 392
6. Increase in solubility 393
7. Some aspects of the practical application of finely ground materials; rational degree of grinding 395

1. INTRODUCTION

An increase in the degree of dispersion of solids produces an increase in the rate of heterogeneous chemical processes which is proportional to the specific surface of the species involved, and it also results in activation and the more effective utilisation of many materials. Dispersion raises the activity of several catalysts, improves the covering power of pigments and the quality of fillers for rubbers and plastics, lowers the temperature for sintering articles in powder metallurgy, etc. One of the most universal and widely used methods for the dispersion of solids is their mechanical disintegration — grinding — which is applied to very many materials employed in various branches of industry. Among them are cements, ores, metals, fuels, and paints, the total annual production of which is counted in hundreds of millions of tons. The degree of dispersion of solids which have undergone grinding is continually being raised, and the corresponding range of production extended.

With the modern level of development in grinding and mixing techniques the velocity of heterogeneous processes can be increased tens of thousands of times by grinding. However, this is not the sole result of the mechanical disintegration of solids. At the present time a considerable body of experimental data has accumulated showing that fine grinding produces also a considerable increase in the specific reactivity (calculated per unit surface) of substances in the solid state. Several other of their physical and physicochemical properties are also changed, such as density, phase transformation points, and adsorption capacity.

The study of the physicochemical properties of disperse systems forms part of a new field of science, physicochemical mechanics, developed by P. A. Rebinder and his