CHROMATOGRAPHY OF FAT-SOLUBLE VITAMINS ON THIN LAYERS OF ALUMINA

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The chromatographic separation of fat-soluble vitamins constitutes a problem that has been extensively studied. This is due above all to the wide distribution of these substances in natural materials, in which they mostly occur in low concentrations accompanied by large amounts of ballast substances, some of which are chemically closely related to the vitamins (*e.g.* the vitamin D group occurs together with other steroids, the vitamin K group together with various substituted quinones, β-carotene together with other carotenoid pigments, etc.). These accompanying substances behave and react practically in the same manner as the vitamins, but in most cases without producing the effect of the related vitamins. Hence they interfere with the analytical determinations of the vitamin. That is why chromatography is so frequently used for the separation and purification of fat-soluble vitamins and has become almost indispensable, particularly for the estimation of these substances by chemical and physico-chemical methods.

For the chromatographic separation and purification of fat-soluble vitamins use has been made of adsorption chromatography on various adsorption materials (most commonly on alumina, magnesia and silicagel)[1], or partition paper chromatography with a stationary non-polar phase (*i.e.* on paper impregnated with a non-polar substance, mostly olive or paraffin oil)[2].

We have succeeded in achieving further progress in the chromatography of fat-soluble vitamins by applying the method described by MOTTIER AND PATTERAT[3] for the separation of some synthetic fat-soluble pigments. We were also able to recommend universal detection reagents for the vitamins separated by this method, which reagents react with all the fat-soluble vitamins[4].

EXPERIMENTAL PART

Materials and method

The following 0.5% solutions of the vitamins were prepared: crystalline vitamin A alcohol, acetate, palmitate, and oil concentrate, crystalline α-carotene and β-carotene, vitamin D_2, standard oil preparation of vitamin E (α-tocopheryl acetate), oil con-

centrate of vitamin K_1 and K_2 (in petroleum ether), and a crystalline preparation of vitamin K_3 (in ethyl alcohol).

The following solvents were used: anhydrous ethyl alcohol, methyl alcohol, *n*-butyl alcohol, benzyl alcohol, hexane, cyclohexane, petroleum ether, petrol, benzene, toluene, xylene, chloroform, carbon tetrachloride, acetone.

As the adsorption medium a special brand of aluminium oxide for chromatography (produced by Lachema, Inc., Brno), particle size up to 0.3 mm and with activity III–IV according to Brockmann, was used.

Detection was carried out: (1) by examination in day light, (2) by examination in U.V. light, (3) with a chloroform solution of antimony trichloride and pentachloride, (4) with an ethanolic solution of potassium hydroxide, (5) with 1 % ethanolic solution of 2,6-dichlorophenol-indophenol, (6) with perchloric acid 70 %, (7) and with sulphuric acid 98 %.

Preparation of chromatographic plates with a thin layer of alumina

For the preparation of chromatographic plates coated with a thin layer of alumina, sheet glass plates of 55 × 350 mm size were used. The alumina was applied in the dry state to the plates and smoothed by means of a roller made from a glass rod or tube, or a blade, over the ends of which small pieces of rubber tubing had been slid so that the distance between the pieces was about 50 mm (according to the width of

Fig. 1. Roller made from a glass rod. 1 = glass rod; 2 = rubber tube.

the glass plate used) (Fig. 1). The thickness of the layer depends on the thickness of the rubber bands on the roller or blade. The plate is prepared by pouring alumina of the required activity onto the glass plate and spreading it out and flattening with a spoon. The roughly prepared plate is then smoothed with the roller (or blade),

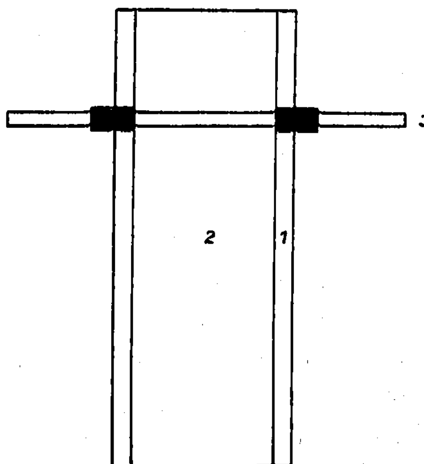

Fig. 2. Preparation of a chromatographic plate with a thin layer of alumina. 1 = glass plate; 2 = layer of alumina; 3 = glass roller.

which is carefully moved over the plate, with the rubber bands resting on the plate over a distance of about 3 mm from the edges (Fig. 2). In this way a smooth homogeneous layer of alumina of an equal thickness all over is obtained. The plates thus prepared are ready to be used directly for the chromatographic separations.

Experimental arrangement

The solution of the vitamin (in either petroleum ether or ethyl alcohol), or the extract of the mixture to be separated is applied at a distance of about 2–3 cm from the edge of the plate. After the solvent has dried, the plate is placed in an inclined position (of about 20–30°) in the chromatographic chamber, together with the solvent; it is developed with the same solvent by the ascending technique until the solvent has penetrated about 30 cm. The time of development depends on the grain size of the adsorption material and on the solvent used. In general it does not exceed 45–60 min.

Detection

The detection was first carried out by the usual method of spraying with the reagents; however, this had to be done very carefully and from a distance, to avoid the adsorbent layer being blown away by the aerosol stream. The reactions of the fat-soluble vitamins with the individual detection reagents are listed in Table I. Later 70 % perchloric acid and 98 % sulphuric acid were used for the detection. Both acids were found suitable for the detection of fat-soluble vitamins, since the latter all give colour reactions with these acids. The application of the acids is performed in such a way that the acid (or other detection reagent) is allowed to soak up in the alumina layer in a direction at right angles to that of the previous development (a technique similar to conventional chromatography). This method proved to be adequate, since no destruction of the alumina layer occurred as in the case of the spraying technique. This method is especially suitable for fat-soluble substances whose reaction with the detection reagents leads to products that are only slightly soluble.

TABLE I

COLOUR REACTIONS OF FAT-SOLUBLE VITAMINS WITH SOME ACIDS

Vitamin	*70 % perchloric acid*	*98 % sulphuric acid*
A	violet	blue-violet
D_2	orange-brown	orange-red
E	brown	brown
K_1	yellow-brown	yellow-brown
K_2	yellow-brown	yellow-brown
K_3	yellow-brown	yellow-brown
Provitamins A	blue	blue

DISCUSSION

Model experiments were performed with all the fat-soluble vitamins. The dependence of the R_F values of the individual substances on the solvent used (Table II), the sensitivities of the group representatives of the fat-soluble vitamins (Table III), the plate capacity, optimal grain size of alumina used, and various detection methods (Table I) were studied.

TABLE II

R_F VALUES OF FAT-SOLUBLE VITAMINS

Solvents	Carotene		Vitamin					
	α	β	A	D_2	E	K_1	K_2	K_3
Methyl alcohol	0.76		0.78	0.80	0.81	0.78	0.71	0.93
Anhydrous ethyl alcohol	0.87		0.71	0.91	0.98	0.89	0.84	0.91
n-Butyl alcohol	0.90	0.89	0.90	0.93	0.91	0.92	0.91	0.92
Benzyl alcohol	0.90	0.89	0.89	0.98	0.92	0.89	0.92	0.93
Hexane	0.93		0.90	0.79	0.90	0.85	0.85	0.80
Cyclohexane	0.92		0.88	0.98	1.00	0.90	0.94	0.98
Petroleum ether	0.75	0.64	0.24	0.05	0.05	0.31	0.21	0.29
Petrol	0.50	0.50	0.13	0.00	0.00	0.21	0.11	0.10
Benzene	0.87		0.91	0.24	0.93	0.94	0.88	0.74
Toluene	0.88		0.91	0.17	0.69	0.90	0.88	0.71
Xylene	0.89		0.91	0.12	0.72	0.91	0.87	0.72
Chloroform	0.94		0.93	0.58	0.87	0.94	0.95	0.92
Carbon tetrachloride	0.95	0.90	0.63	0.09	0.54	0.74	0.70	0.49
Acetone				R_F cca	0.90			

TABLE III

SENSITIVITY OF THE REACTION OF SOME FAT-SOLUBLE VITAMINS WITH PERCHLORIC ACID

Vitamin	Limit of detection in mg
A	$2 \cdot 10^{-3}$
D_2	$3 \cdot 10^{-3}$
E	$4 \cdot 10^{-1}$
K_3	$1.4 \cdot 10^{-1}$
Provitamin β-carotene	$2 \cdot 10^{-3}$

When establishing the optimal conditions for chromatographic separation of fat-soluble vitamins, it was found that the separating ability depends to a great extent on the grain size of the alumina. The finer the grains, the more perfect is the separation of the fat-soluble vitamins. When coarser grains are used the solvent flows through too quickly, which causes imperfect equilibrium conditions and hence un-

satisfactory separation. A grain size of 0.075 up to 0.30 mm proved optimal. The thickness of the layer has practically no influence on the separating ability of alumina, but it determines the adsorption capacity of the chromatographic plate. Thus, the method described makes it possible to separate 0.02 mg vitamin D from 1 mg vitamin A, using a layer thickness of 2 mm, a spot diameter of 2 cm, and alumina grains of 0.3 mm. Other vitamins can likewise be separated from each other, even in the case of relatively high concentrations. The time of development depends on the type of solvent and the grain size of the alumina used; at most it is 60 min.

Table II shows that some alcohols (methyl, ethyl, butyl alcohol), chlorinated hydrocarbons (particularly chloroform and carbon tetrachloride), and the aromatic solvents, benzene, toluene and xylene, proved most useful for the development of the chromatogram. Of the other solvents petroleum ether and petrol were also satisfactory. Acetone was found to be unsuitable. The choice of solvent is made according to the individual fat-soluble vitamins that are likely to be present, *i.e.* according to the nature of the material tested. Thus for instance, for the separation of vitamins A from vitamin D_2 the most suitable solvents are benzene, toluene, xylene, chloroform, and carbon tetrachloride respectively, for the separation of vitamin A alcohol or acetate from β-carotene petroleum ether is efficient; the same solvent is also very suitable for the separation of a natural mixture of vitamin K_1, vitamin E, and β-carotene (Figs. 3–5). For the separation of a mixture of several fat-soluble vitamins, the two-dimensional technique may be used together with chromatographic plates.

The method elaborated for the separation of fat-soluble vitamins on thin alumina layers has many advantages compared with column chromatography, especially when a rapid and simple detection is desired. In comparison with the separation of fat-soluble vitamins by paper chromatography the present method has still greater advantages. In the first place this is due to the rapidity of plate preparation and of the development of the chromatogram itself.

Furthermore, the adsorption capacity of the alumina in plate chromatography is substantially higher than that of the paper in paper chromatography, a fact that is especially important in cases where the separated components have to be isolated, either for preparative or analytical purposes. Studies of the potential application of these separations, *e.g.* in the analysis of fat-soluble vitamins, will be reported elsewhere. As regards the choice of detection reagents plate chromatography also offers more possibilities than paper chromatography. For the detection of fat-soluble vitamins after their separation on paper, antimony trichloride, 2,6-dichlorophenol-indophenol, dilute potassium hydroxide, etc., are mostly used. Some reagents, such as concentrated perchloric or sulphuric acid cannot be employed in paper chromatography because of their destructive effect on the paper, whereas they serve well in chromatography on alumina plates. The advantage of the colour reactions that the fat-soluble vitamins give with perchloric and sulphuric acid lies on the one hand in their universality, and on the other in the fact that the reaction products are relatively stable. Since alumina itself gives no coloration with the above-mentioned reagents it does not affect the actual colour reaction, and thus the coloured spots can easily be seen. The

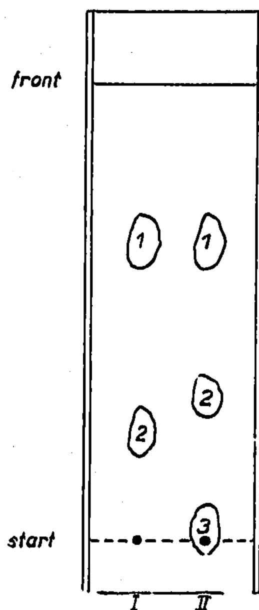

Fig. 3. Chromatograms of model solutions of vitamins. I: 1 = β-Carotene: 2 = Vitamin A acetate. II: 1 = β-Carotene; 2 = Vitamin K_1; 3 = α-Tocopheryl acetate. Solvent for I and II: petroleum ether.

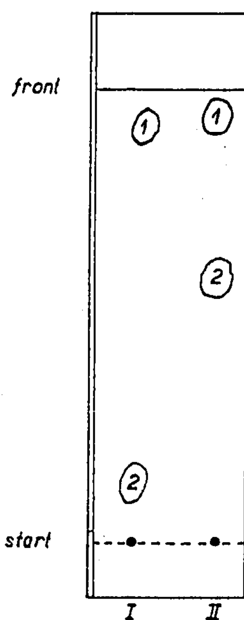

Fig. 4. Chromatograms of model solutions of vitamins. Solvent for I: xylene, for II: chloroform. 1 = Vitamin A acetate; 2 = Vitamin D_2.

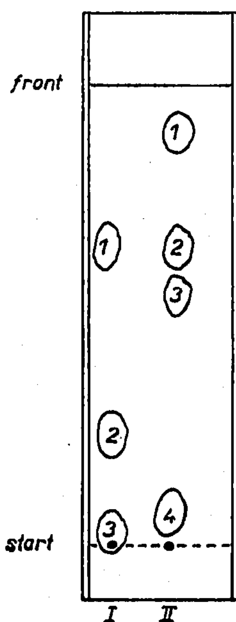

Fig. 5. Chromatograms of model solutions of vitamins. I: 1 = β-Carotene; 2 = Vitamin A acetate; 3 = Vitamin D_2. Solvent petroleum ether. II: 1 = β-Carotene; 2 = Vitamin A acetate; 3 = α-Tocopheryl acetate; 4 = Vitamin D_2. Solvent carbon tetrachloride.

actual detection was performed according to the procedure elaborated by us, which has great advantages compared to the methods used up to now. The detection is achieved directly on the plates in such a way that the acid used is allowed to soak into the alumina in a direction at right angles to that of the previous solvent development (a development similar to chromatography). In this way destruction of the alumina layer, which would occur in application by spraying, is avoided. A great advantage of this procedure lies also in its simplicity and in the uniformity of coloration.

All the equipment required for the plate chromatography is easily available, even in the average laboratory. The present method as elaborated here may prove very useful in projects involving the separation and estimation of fat-soluble vitamins, particularly in the pharmaceutical and food industry. It may also find application in the control of synthesis of fat-soluble vitamins, and in the determination of their specificity of action and their purity.

SUMMARY

A chromatographic separation and detection of fat-soluble vitamins on plates coated with alumina is described. The most suitable procedure of preparing the chromatographic plates in a dry state has been established, as well as the optimal thickness of the layer, the optimal grain size of the alumina, and the most suitable solvents for development. A procedure for separating nearly all the fat-soluble vitamins has been elaborated and a new procedure for detecting the fat-soluble vitamins on alumina plates is recommended. This method is also applicable for the detection of other fat-soluble substances, particularly those giving coloured reaction products that are insoluble in the detection reagent. The application of perchloric and sulphuric acid in this procedure is an innovation. Both reagents react with all the fat-soluble vitamins, and thus have a universal character. The method described for the separation and detection of the fat-soluble vitamins is simple and can be easily carried out in the average laboratory.

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