CHROM. 6249

SEPARATION AND DETECTION OF WATER-SOLUBLE ACID DYES ON POLYAMIDE THIN LAYERS

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(Received May 15th, 1972)

SUMMARY

A method is described in which twenty water-soluble acid dyes, isolated from food products by means of DEAE-Sephadex, were detected by thin-layer chromatography using polyamide plates.

The isolation procedure involved shaking samples with the Sephadex and then eluting the dyes by column chromatography. In polyamide thin-layer chromatography, the solvent system suitable for the separation of the dyes was pyridine–methanol–28 % aqueous ammonia–water (5:6:1:16).

This is a simple and rapid method for the detection of acid dyes in cellulosic and proteinaceous samples, especially when the dyes are present in low concentrations.

INTRODUCTION

Recently there has been much discussion of possible harmful effects of some food additives on human beings. Many investigations of a long-term nature on food colors were in the past inadequate for carcinogenicity evaluation, the main emphasis being placed on studies of metabolism and chronic toxicity.

On the basis of evaluation of the toxicological data available, the Joint FAO/WHO Expert Committee on Food Additives has attempted to classify food colors and set acceptable limits for daily intakes of some colors in man[1]. However, there is the possibility that toxic colors are added to food stuffs, drugs or cosmetics erroneously, or illegally, because the addition of the colors has been carried out in accordance with past practice. Therefore, a useful method for separation and detection of food colors in food products has been urgently required.

As methods for the separation of water-soluble acid dyes from foods, the following procedures have been widely used: (a) solvent extraction procedures[2–4], (b) wool-dyeing methods[5,6], (c) absorption column chromatography[7–10] and (d) ion-exchange procedures[11–14]. Recently it has been reported that column chromatography using polyamide powder[7,8] gives results superior to those of solvent extraction and wool-dyeing procedures.

When any of the above four methods was used, the separated food colors have

generally been detected and identified by paper chromatography and thin-layer chromatography (TLC). For TLC, cellulose[15,16] and silica gel[17,18] are used as adsorbents, and polyamide[19–21] also has found to be a useful adsorbent in this field.

This paper deals with the detection of water-soluble acid dyes by TLC, using ready made polyamide plates, and a procedure for isolation of these dyes from food products.

EXPERIMENTAL

Thin-layer chromatography

Adsorbent. Ready made Polyamide UA plates, which are prepared by coating glass plates (5 × 10 cm) with Nylon 6 powder, were obtained from Wako Pure Chemical Ind., Ltd.

Reagents. Each of the test and standard solutions of water-soluble acid dyes listed in Table I was prepared by dissolving 10 mg of each in 10 ml of distilled water.

TABLE I

WATER-SOLUBLE ACID DYES USED IN THIS EXPERIMENT

Group	*Dye*	*Colour index (C.I.) No.*
Xanthene compounds	erythrosine	45 430
	eosine	45 380
	phloxine	45 410
	rose bengal	45 440
	acid red	45 100
Triphenyl-methane compounds	Guinea Green B	42 085
	Light Green SF (Yellowish)	42 095
	Fast Green FCF	42 053
	Brilliant Blue FCF	42 090
	Acid Violet 6B	42 640
Azosulphone compounds	Ponceau 3R	16 155
	Ponceau R	16 150
	Ponceau SX	14 700
	amaranthe	16 185
	new coccine	16 255
	tartrazine	19 140
	Sunset Yellow FCF	15 985
	Orange I	14 600
Naphthol compound	Naphthol Yellow S	10 316
Indigo compound	indigo carmine	73 015

The following chromatographic solvents were used: (1) Methanol–28% aqueous ammonia–water (3:0–24:16); (2) pyridine–methanol–28% aqueous ammonia–water (0–6:6:1:16).

Apparatus. The chromatographic chamber was a glass column of 27 cm in length and 9 cm in diameter) with a glass cover. The chamber was equipped with

a suspension unit devised for pre-equilibrating the plates with the solvent system, as previously reported in detail[22].

Procedure. Each 0.5–1 μl volume of test solutions of the water-soluble acid dyes was spotted with a micropipette on a starting line 1.5 cm from the lower edge of the plate. After being placed in the chamber without any previous immersion, the plates were equilibrated with the respective chromatographic solvent for 20 min and were then developed until the solvent front had travelled a distance of 8 cm from the starting line. After development, the plates were removed from the chamber and dried immediately by an air-drier. Each spot was detected under normal illumination.

Isolation and detection of water-soluble acid dyes in foods

Reagents. DEAE-Sephadex A-25, obtained from Pharmacia Fine Chemicals Co., Uppsala, Sweden, was allowed to swell with an appropriate volume of distilled water and poured into a column of 3.5 cm in diameter and 60 cm in length. The Sephadex was equilibrated with 0.5 *N* aqueous ammonia to obtain the free form and then washed with water until chloride ion in the effluent was barely detectable by means of a 1 % silver nitrate solution acidified with nitric acid. Subsequently the Sephadex was equilibrated with 1 *N* acetic acid in a beaker overnight. After a thorough washing with water, the Sephadex (acetate form) was stored in a refrigerator. A DEAE-Sephadex suspension was prepared by mixing one part of the DEAE-Sephadex thus obtained with one part of distilled water. 2 *N* hydrochloric acid (or 2 *N* sulfuric acid) -isopropyl alcohol (1:1) was used as solvent I for the elution. 4 *N* aqueous ammonia-isopropyl alcohol (1:1) was applied as solvent II for neutralizing the effluents. Each of the test and standard solutions of water-soluble acid dyes was prepared by the method as described in the section above on TLC.

Apparatus. A small glass column of 0.4 cm diameter and 3.5 cm length was equipped with a stopcock and a glass-wool plug (Fig. 1).

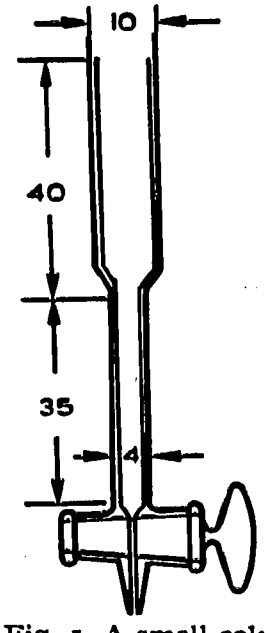

Fig. 1. A small column for separation of water-soluble acid dyes (dimensions are in mm).

Procedure. A 5–10 g quantity of the solid sample was mixed with 20–30 ml of distilled water and well ground in a mortar. For removal of larger particles, the mixture was filtered through a cloth (200–300 mesh) or, if necessary, homogenated. Liquid samples were treated without grinding in the mortar. Fatty samples were extracted several times with appropriate volumes of *n*-hexane for the elimination of fat and then processed according to the procedure described above The sample solution thus prepared was added little by little with stirring into a 50-ml volumetric centrifugal tube in which about 1 ml of the DEAE-Sephadex suspension had already been placed. The addition of the solution was continued until no more dye was adsorbed on the Sephadex and the non-adsorbed dyes began to color the supernatant. At the same time, if any Sephadex particles floated on the surface of the solution, a small volume of acetone was poured onto the surface, or the mixture was centrifuged at 1000 r.p.m. for 1 min. The Sephadex thus obtained was rinsed with 30 ml of distilled water in the tube and the washings thrown away. This treatment was repeated until any turbidity in the washings disappeared. To the Sephadex, 10 ml of 50 % isopropyl alcohol solution was added, and the slurry was transferred into the small column with a pipette, to make up a column bed of 1 cm. The solution on the column bed was sucked up with the pipette as completely as possible. A few milliliters of solvent I were applied to the column and the flow-rate was maintained at approx. 0.10–0.15 ml/min. As soon as the dyes began to elute at higher concentrations or the effluent had become acidic, a 0.5-ml volume of the effluent was collected and then neutralized with 0.25 ml of solvent II. A 0.5–2-μl volume of the solution obtained by the above procedure and a 0.5-μl volume of each standard solution were then spotted on the plates. According to the method described in the section above on TLC, the dyes in the sample were identified after development with pyridine–methanol–28 % aqueous ammonia–water (5:6:1:16).

RESULTS AND DISCUSSION

Separation and identification of water-soluble acid dyes by polyamide thin-layer chromatography

Although the analysis of food dyes has been previously carried out by TLC using various adsorbents, it is very difficult to find conditions to separate the twenty water-soluble acid dyes listed in Table I in one run. In the present study, using ready made polyamide UA plates as the adsorbent, chromatographic solvents capable of separation of these dyes were investigated.

After an aliquot (μl) of each standard test solution of the dyes had been spotted on polyamide UA plates, their behaviors were examined by development with a series of chromatographic solvents. By development with an acidic solvent system, benzene–ethyl acetate–formic acid (5:10:2) (ref. 23), only the xanthene dyes traveled, with poor separation, and other dyes did not migrate at all. On the other hand, the behavior of five xanthene dyes (erythrosine, eosine, phloxine, rose bengal and acid red) and five azosulphone dyes (Ponceau R, Ponceau 3R, Ponceau SX, new coccine and amaranthe) were investigated on the plates using a basic solvent system, methanol–ammonia–water (15:5:80) which DAVÍDEK AND DAVÍDKOVÁ[21] had used in the separation of ten water-soluble dyes. This system did not give such good results, in that all the dyes tested were observed to tail considerably. In this con-

nection, the addition of a small amount of pyridine to the above system prevented the spots from tailing and gave R_F values of the dyes which were remarkably higher.

Firstly, in order to learn the effect of ammonia in the solvent system on the separation, the development was carried out with a series of solvent systems prepared by adding 28 % aqueous ammonia to a mixture of methanol–water (3:16) at various volume ratios (0, 1, 4, 8, 12, 16, 20 and 24). When the volume ratio of 28 % aqueous ammonia was between 4 and 8, all the dyes tested traveled on the plates with such strong tailing that R_F values could not entirely be measured. When the dyes were developed with solvent systems containing higher or lower concentrations of ammonia, all the spots showed lower R_F values with slight tailing.

Secondly, the effect of the concentration of methanol in the solvent systems on the distribution of the spots was determined. As each of the dye spots showed the lowest R_F value by development with the solvent system, methanol–28 % aqueous ammonia–water (3:1:16), two series of solvent systems were prepared by mixing methanol with the 28 % aqueous ammonia–water systems (1:16 and 24:16) at volume ratios of 6, 9 and 12. Figs. 2 and 3 show that the higher content of methanol in the systems leads to slightly increased R_F values only for the dyes belonging to the azosulfone group. This contrasts with the results of DAVÍDEK AND DAVÍDKOVÁ[21] on the effects of methanol on the distribution of ten water-soluble acid dyes.

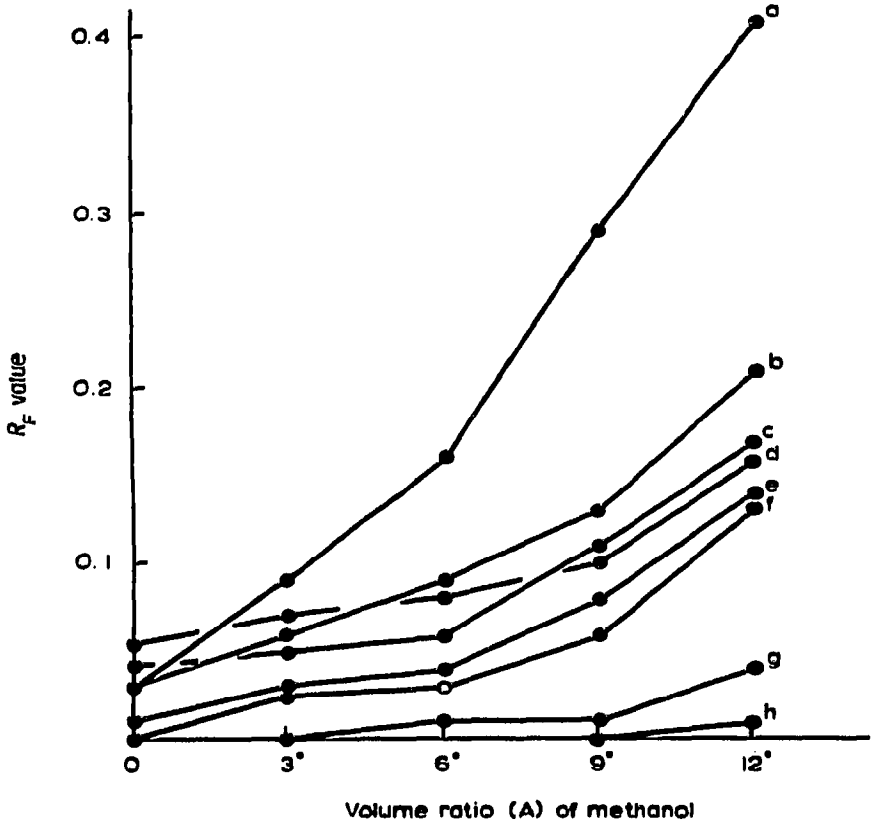

Fig. 2. Relationship of R_F values of dyes and volume ratio of methanol in the solvent system. Solvent system: methanol–28% aqueous ammonia–water (A:1:16). (a) Acid red; (b) amaranthe; (c) new coccine; (d) Ponceau SX; (e) Ponceau R; (f) Ponceau 3R; (g) eosine; (h) erythrosine, phloxine and rose bengal. At the volume ratio, •, of methanol in the solvent system, the spots of all the dyes tested showed slight tailing.

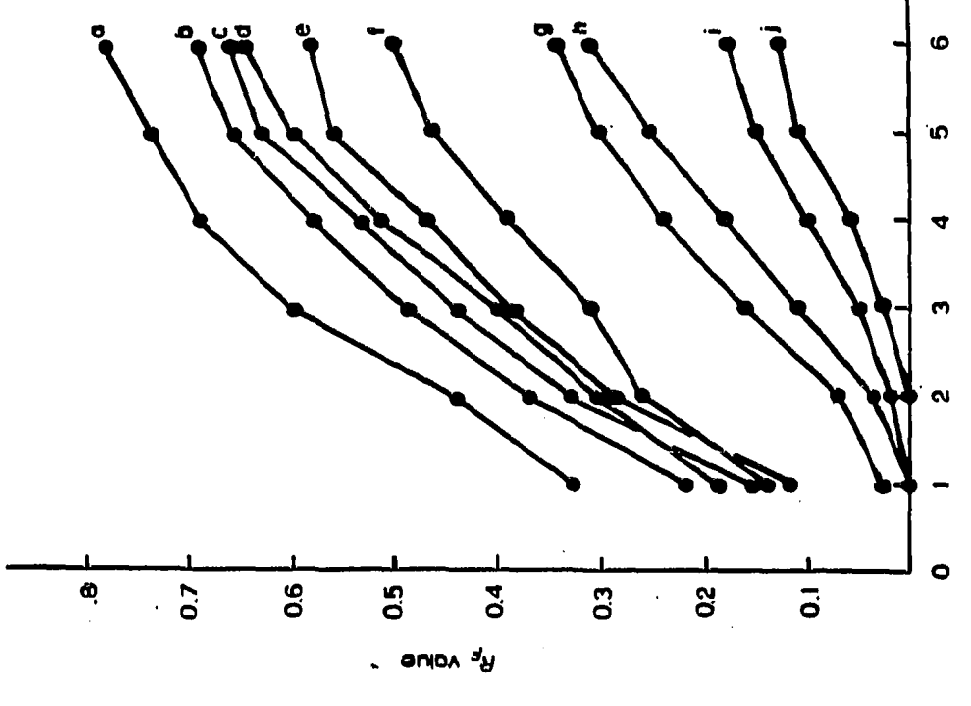

Fig. 4. Influence of additional volume ratio of pyridine in pyridine–methanol–28% aqueous ammonia–water (A:6:1:16) upon R_F values of dyes on polyamide layers. (a) Acid red; (b) amaranthe; (c) Ponceau R; (d) Ponceau 3R; (e) new coccine; (f) Ponceau SX; (g) eosine; (h) phloxine; (i) erythrosine; (j) rose bengal.

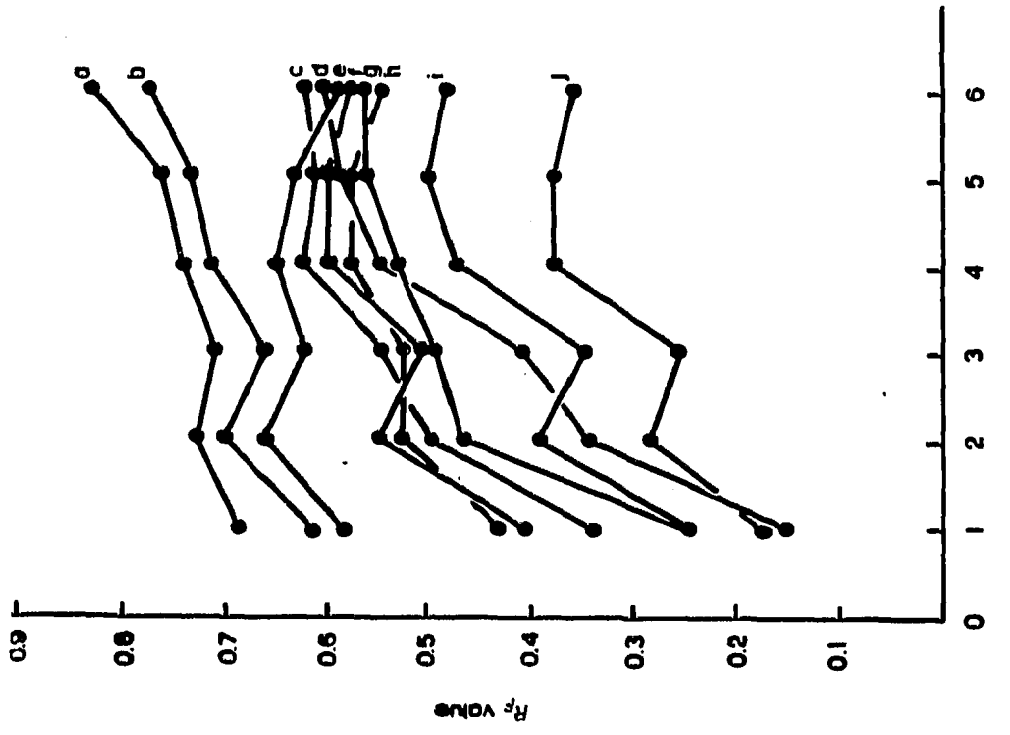

Fig. 5. Influence of additional volume ratio of pyridine in pyridine–methanol–28% aqueous ammonia–water (A:6:1:16) upon R_F values of dyes on polyamide layers. (a) Fast Green FCF; b) Brilliant Blue FCF; (c) Light Green SF; (d) Guinea Green B; (e) tartrazine; (f) Sunset Yellow FCF; (g) Acid Violet 6B; (h) indigo carmine; (i) Orange I; (j) Naphthol Yellow S.

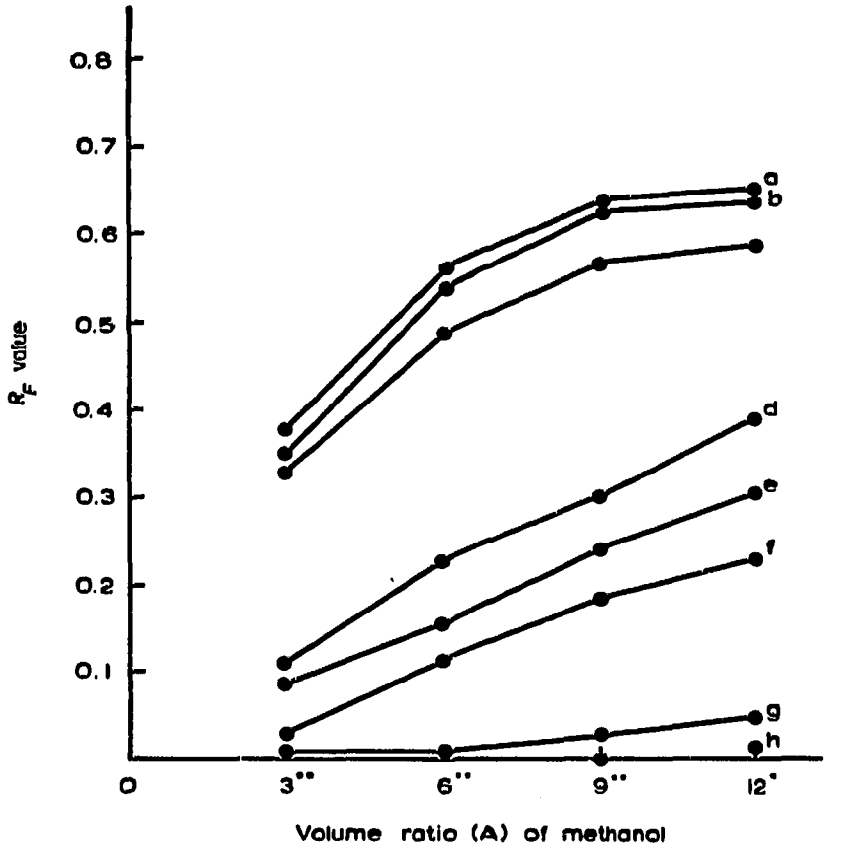

Fig. 3. Relationship of R_F values of dyes and volume ratio of methanol in the solvent system. Solvent system: methanol–28% aqueous ammonia–water (A:24:16). (a) Ponceau SX; (b) amaranthe; (c) new coccine; (d) acid red; (e) Ponceau R; (f) Ponceau 3R; (g) eosine; (h) erythrosine, phloxine and rose bengal. At the volume ratio, • and ••, of methanol in the solvent system, the spots of all the dyes tested showed slight or strong tailing, respectively.

As it was found at the beginning of this experiment that the addition of pyridine to a solvent system composed of methanol–28% aqueous ammonia–water was remarkably effective in making the spots circular, the following experiment was carried out to determine the additional amount of pyridine necessary in the above solvent systems to obtain the most suitable separation and spot sharpness. As shown in Fig. 4, the dyes showed higher R_F values with circular spots by an increase of the pyridine content, and the best distribution and sharpness of spots were obtained when the dyes were developed with pyridine–methanol–28% aqueous ammonia–water (5:6:1:16).

On the application of this solvent system to the separation of a further ten food dyes, comprising one naphthol, one indigo, three azosulphone and five triphenylmethane compounds, they were also separated well and distributed as small and circular spots, as shown in Fig. 5. However, it was difficult to separate the dyes completely from each other when they had the same number of substituent functional groups.

Separation of water-soluble acid dyes by column chromatography using DEAE-Sephadex

It was found in our previous investigations[11,12] that DEAE-Sephadex had such a large capacity for acid dyes that a 0.1-ml volume of the Sephadex adsorbed

over 0.25 mg of each of the dyes by a simple shaking procedure, and that by using the micro-chromatographic tube all the dyes were completely eluted by a small volume of 2 *N* hydrochloric acid (or 2 *N* sulfuric acid)–isopropyl alcohol (1:1), so that the effluent obtained could be subjected to TLC without concentration. There was, however, the problem of making a sharp tip on the micro-chromatographic tube, so that the flow-rate of the effluent might be naturally controlled.

The column used in this experiment had the same size as used in our previous studies[11,12] and was equipped with a stopcock (Fig. 1). Amaranthe is the most suitable dye in studying elution patterns, since it shows the longest retention on the column in the elution step (because it has the most functional groups in the molecule). The dye-binding DEAE-Sephadex prepared by shaking a mixture of 1 mg of amaranthe with a 1-ml portion of the DEAE-Sephadex suspension was poured into the column to make a column bed of 1 cm, and the dye was eluted with solvent I according to the method described in the EXPERIMENTAL section. The dye began to appear after the effluent had reached *ca.* 0.25 ml, and about 1 mg of the dye was eluted completely from the column in a 1-ml volume of the effluent. Even after neutralization with solvent II, the concentration of the dye in the solution was high enough for it to be directly subjected to TLC.

Influence of hydrochloric acid, sulfuric acid or acetic acid in the effluent upon detection of water-soluble acid dyes on ready-made polyamide UA plates

It has already been reported[12] that in order to eliminate the problem that hydrochloric acid in the effluent has an undesirable influence on the distribution of some of the water-soluble acid dyes on silica gel plates, sulfuric acid should be used instead, since this acid remains as the ammonium salt at the starting line.

In this paper, in order to investigate the influences of hydrochloric acid, sulfuric acid and acetic acid (which might depend on elution of acetic acid with the eluant from the starting Sephadex) on the chromatographic patterns of the dyes, the solutions of the dyes obtained from each dye-binding Sephadex (by the procedure described in the above section) and the standard test solutions of the dyes were spotted on the plates and were developed with the solvent system pyridine–methanol–28% aqueous ammonia–water (5:6:1:16). After development, the migration of the acids on the plates was examined by spraying with 0.2% methyl red ethanolic solution–0.1% bromocresol green ethanolic solution (3:2). Any acid was visualized along the solvent front as a red diffusing band against a green background. In spite of the effects of the acids on the plates, the R_F values obtained for all the dyes corresponded well with those of the standard substances.

Separation and detection of water-soluble acid dyes in foods

In separation of the acid dyes from foods, many advantages are obtained by binding the dyes with basic materials and then eluting or extracting them. Although wool fibers, alumina, liquid ion exchanger, quinoline[24], quaternary ammonium compounds[25] and polyamide are generally used as the basic materials, there is no good material known which can be recommended for the separation of the acid dyes from foods. Recently a method for the separation of acid dyes using polyamide[14] was reported as a method that does not have the disadvantages of the wool dyeing procedure. These disadvantages are that (1) the dyes are removed very slowly from

hot acidic samples; (2) carbohydrates in the samples hinder adsorption of the dyes by wool and (3) protein precipitated on acidification in the dyeing procedure adsorbs dyes strongly and consequently renders wool adsorption of the dye impossible. However, even when the separation of the dyes is carried out according to the polyamide method, a large quantity of alkaline methanol is necessary for eluting dyes from the dye-binding polyamide, and also the effluent obtained must be concentrated before TLC. Consequently, the method is not efficient, since the concentration step induces the irreversible decomposition of dyes and moreover delays the whole procedures.

In this study, the dyes were taken up very easily from the samples, (candies, orange and tomato juices, fruit extract powders, fermented milk products, cookies rich in fat and protein by DEAE-Sephadex, and by subsequent elution of dyes from the dye-binding Sephadex with acidic solvents, a solution containing the dyes in high concentration was obtained. When the solution was subjected to polyamide TLC, the dyes were successfully detected and corresponded to the relative standard substances on the plates.

CONCLUSION

When developed with solvent systems composed of methanol–28% aqueous ammonia–water on ready-made Polyamide UA plates, the water-soluble acid dyes were only separated with tailing. However, the addition of a small amount of pyridine to these systems prevented all the dye spots from tailing. Thus the solvent system giving the best distribution and spot sharpness was pyridine–methanol–28% aqueous ammonia–water (5:6:1:16). As the chromatographic pattern of the dyes obtained under such conditions is relatively different from that obtained by development with a basic solvent, ethyl acetate–methanol–28% aqueous ammonia (3.3:1:1)[12] on silica gel layers, the data will be very useful for the identification of dyes.

The application of DEAE-Sephadex to the separation of food dyes has several advantages over other methods, such as wool-dyeing, polyamide-dyeing, and so on. These advantages are: (1) When food samples are shaken with DEAE-Sephadex, the Sephadex takes up the dyes very well, even those which had been firmly bound to cellulosic substrates or present in low concentration; (2) by eluting the dyes from the dye-binding DEAE-Sephadex with a mixture of 2 *N* hydrochloric acid (or 2 *N* sulfuric acid)–isopropyl alcohol (1:1), the concentration of dyes in an aliquot (μl) of the effluent was sufficient for direct application to plates for detection purposes.

In subsequent polyamide TLC, any acids in the effluent developed behind the solvent front as a widely diffusing band, which did not disturb the distribution of dyes at all. Thus the dyes can be effectively isolated from foods without heating and concentration steps, and then subsequently be detected by polyamide TLC. This is recommended as a rapid and reliable method for the detection of these dyes.

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