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APPLICATION OF COLUMN AND THIN-LAYER CHROMATOGRAPHY TO THE DETECTION OF ARTIFICIAL SWEETENERS IN FOODS

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SUMMARY

A method for the purification and detection of three artificial sweeteners, saccharin, cyclamate and dulcin, in food products is described. It was developed on the basis of the separation of the sweeteners from other impurities by extraction with ethyl acetate and by adsorption on and elution from silica gel and alumina columns, followed by thin-layer chromatography using polyamide. The sweeteners were detected on the chromatograms by observation with three chromogenic spray reagents having sensitivity ranges from 0.01 to 2 μg of the sweeteners. Various foods containing levels of 10–100 p.p.m. and artificially sweetened foods were successfully analysed for the sweeteners.

INTRODUCTION

Because artificial sweeteners have been widely used in food products in recent years, mainly for economic and also for other reasons, the toxicological evaluation of sweeteners as well as other food additives such as preservatives, antioxidants and colorants, is necessary in foods for human consumption. Of various artificial sweeteners, dulcin recently became known to have a toxic effect in humans, and the Joint FAO/WHO Expert Committee therefore recommended that this substance should not be used as a food additive[1]. Cyclohexanesulphamic acid (cyclamic acid)[2] and cyclohexylamine[3] (a raw material and a metabolite of cyclamic acid) have also been found to be toxic to some individuals. On the basis of the toxicological evaluation of the sweeteners the requirements for useful analytical methods for the compounds are discussed in this paper.

Many workers have reported that artificial sweeteners could be detected by thin-layer chromatography using silica gel[1–6] or a mixture of polyamide and acetylcellulose.[7] We were successful in the separation of three artificial sweeteners on alumina layers by using the solvent systems isopropanol–aqueous ammonia and amyl alcohol–pyridine–water[8].

In the detection of artificial sweeteners in food products by thin-layer chromatography, impurities in the extract obtained by a partition extraction often interfered with the detection on the chromatograms. In the detection of four artificial sweeteners in beverages, KORBELAK[6] succeeded in eliminating the co-extracted materials by pre-washing with light petroleum. Such a treatment, however, will not always give satisfactory detection results in certain forms of food preparations, such as fatty foods.

We succeeded in the quantitative separation of three artificial sweeteners, dulcin, saccharin and cyclamic acid, by column chromatography with Amberlite CG 4B (ref. 9). We also found that saccharin and cyclamic acid in food products were taken up easily by Amberlite CG 400 by applying a shaking procedure and could be detected by paper chromatography after elution with alcoholic diluted acids[10]. WOIDICH *et al.*[11], using the liquid anion exchanger Amberlite LA-2 for the same purpose, isolated acidic sweeteners applicable to acetylcellulose–polyamide layers by eluting them with aqueous ammonia from exchangers.

However, the application of such exchangers to the isolation of sweeteners from food products may not always be the most suitable clean-up procedure for detection by thin-layer chromatography, for the following reasons: (a) after elution of the sweeteners bound to exchangers, the concentration of the aqueous effluent for obtaining samples for thin-layer chromatography always delays the whole procedure; (b) because all the acids in food products, such as fatty acids with several carbon chains, citric acid, etc., are taken up by the exchangers, the sweeteners fraction obtained thereby may contain a large amount of the acids, which influences the distribution of the sweeteners on thin layers; and (c) when dulcin has to be detected in food products, the samples have to be transferred to an extraction procedure after treatment with the exchangers.

A main objective of this investigation was to find a simple and rapid clean-up procedure applicable to all food products. Three sweeteners, saccharin, cyclamic acid and dulcin, obtained by extraction with ethyl acetate from acidified samples, were purified on silica gel and alumina columns and separated on polyamide layers with a solvent system composed of benzene, ethyl acetate and formic acid. The detection system consisted of colouring spots with three chromogenic spray reagents after pre-observation under UV radiation. The detection limits were also investigated.

EXPERIMENTAL

Column chromatography

Adsorbents. Silica gel (0.05–0.20 mm) for chromatography and aluminium oxide (active I, basic) for chromatography, obtained from E. Merck AG, Darmstadt, G.F.R., were used as the adsorbents. Before use, the adsorbents were dried for 1 h at 110°.

Reagents. Dulcin and saccharin, obtained as reagent-grade materials, were recrystallized from diluted ethanol. Cyclamic acid was prepared as follows. 1 g of sodium cyclamate (obtained from Daiichi Seiyaku Co., Ltd., Tokyo) was dissolved in 5 ml of water, and the solution was acidified with 1 *N* hydrochloric acid, saturated with sodium chloride and then extracted twice with 30-ml volumes of ethyl acetate. After washing three times with small portions of water, the combined extract was

dried over a few grams of sodium sulphate and then evaporated to dryness under reduced pressure. Cyclamic acid thus obtained was recrystallized from a mixture of ethyl acetate and benzene.

The solvents were purified by conventional methods for use in extraction and chromatography. The other reagents were of analytical-reagent grade.

Apparatus. The column consisted of a glass tube, 1 cm in diameter and 20 cm in length, and was equipped with a stop cock.

Preparation of the silica gel and alumina columns. About 2 g of silica gel and 3.5 g of alumina were suspended separately in benzene, and each slurry was poured into the column to make a 5-cm column bed.

Column chromatography of the sweeteners on silica gel for a clean-up procedure. A 5-ml volume of the test solution, prepared by dissolving 1 mg of dulcin, 20 mg of saccharin and 200 mg of cyclamic acid in 50 ml of ethyl acetate, was loaded on the top of the silica gel column. As soon as the meniscus of the solution had reached the surface of the column, the inside of the tube was washed four times with 5-ml volumes of ethyl acetate. After the solvent had flowed out of the column, elution of the sweeteners was carried out with a mixture of ethyl acetate and methanol (2:1) at a flow-rate of 1.0–1.5 ml/min, and each 1-ml volume of the effluent was collected in a small test-tube immediately after the test solution had been loaded on to the column.

The existence of the sweeteners in each fraction was examined as follows. For the estimation of dulcin and saccharin, each sample was transferred to a 10-ml flask and evaporated to dryness at 50° under reduced pressure. After each residue had been dissolved in appropriately diluted ethanol, the absorbances were measured at $\lambda_{max.} = 238$ and 275 nm, respectively, using ethanol as a blank. For cyclamic acid, 1 ml each of 1% sodium nitrate solution (freshly prepared) and 5% hydrochloric acid solution were added to the samples. After the mixtures had been allowed to stand for 30 min, 2 ml of glycerin and 1 ml of 1% barium chloride solution were added successively to them. They were mixed by shaking the tubes for 1 min. The turbidity thus produced in each mixture was measured at 500 nm.

Column chromatography of dulcin on alumina for a clean-up procedure. A 5-ml volume of the test solution prepared by dissolving 1 mg of dulcin was loaded on the top of the alumina column. With the same procedure as for the silica gel column chromatography, the inside of the tube was washed with 10 ml of ethyl acetate and the elution of dulcin was carried out with a mixture of ethyl acetate and methanol (4:1). Each 2-ml volume of the effluent was collected and evaporated to dryness under reduced pressure. The concentration of dulcin in each fraction was measured by using the same procedure as described above.

Thin-layer chromatography

Adsorbent. Polyamide powder (obtained from E. Merck AG, Darmstadt, G.F.R.) was used as the adsorbent. Before use, it was washed twice with a volume of methanol equal to the volume of polyamide powder, and dried at 60° (ref. 12).

Reagents. Saccharin, dulcin, cyclamic acid and all the solvents were the same as those used in column chromatography. All other reagents were of analytical-reagent grade.

The test solutions used were 0.2% ethanolic solutions of saccharin and dulcin and a 1% ethanolic solution of cyclamic acid.

Food preservatives and dyes (see Tables I and II) were used in a test in which they might interfere with the detection of the sweeteners by thin-layer chromatography. Each solution of the preservatives and dyes for the test was prepared by dissolving 10 mg of each of the preservatives in 5 ml of ethanol and 5 mg of each of the dyes in 5 ml of water.

Chromogenic spray reagents were prepared as follows. (1) MR-BCG reagent: 3 parts of a 0.2% ethanolic solution of Methyl Red were mixed with 2 parts of a 0.1% ethanolic solution of Bromocresol Green; (2) Pinacryptol Yellow reagent[13]: a 0.2% methanolic solution; and (3) *p*-dimethylaminobenzaldehyde reagent: 95 parts of a 3% *p*-dimethylaminobenzaldehyde solution in isopropanol were mixed with 4 parts of 36.5% hydrochloric acid (sp. gr. 1.18).

Apparatus. A thin-layer applicator and other accessories were obtained from Yazawa Seisaku Co., Tokyo. Glass plates were 20 × 5 cm. A chromatographic chamber was equipped with a suspender for pre-equilibrating the thin layer with solvent systems that the author had devised[14]. The UV light sources (253.6 and 365.0 nm) were supplied by Manasulu Ultra Violet Kagaku Kogyo Co., Tokyo.

Preparation of polyamide thin-layer plates. According to the conventional method, glass plates were coated with a slurry composed of 20 g of the polyamide powder and 70 ml of isopropanol using the applicator to give a thin layer approximately 300 μm thick. After having been allowed to stand for 15 min at room temperature, the plates were dried for 30 min at 70°, and stored in a desiccator containing silica gel.

Solvent systems for thin-layer chromatography. The solvent systems used were prepared by mixing 99.5% formic acid at various ratios (A) to the mixture of benzene and ethyl acetate (5:10).

Application of samples and development of chromatographic plates. Volumes of 0.5 μl of each test solution were spotted with a micropipette on the starting line, 2 cm from the lower edge of the plate. In the chamber containing the solvent system, the inside of which had been partially lined with filter-paper soaked with the solvent, the plate was equilibrated with the solvent vapour for 20 min before commencing the development. The development was carried out at 20 ± 1° by immersing the plate in the solvent system to a depth of about 0.5 cm without opening the cover of the chamber, and was continued until the solvent front had travelled 10 cm from the starting line.

Detection of spots on the chromatographic plates. After the developed plates had been dried for 20 min at 70°, the spots were observed under UV light (253.6 nm) and then visualized by spraying with the chromogenic reagents. For the application of the Pinacryptol Yellow reagent, the plates were examined under UV light (365.0 nm) after spraying with the reagent.

Extraction, purification and detection of artificial sweeteners in foods

For solids, a 10–20-g sample was mixed or ground with 20–30 ml of water in a mortar. With liquids, 10–20 ml of the sample were used directly. The liquid sample thus obtained was acidified with 1 *N* hydrochloric acid, saturated with sodium chloride or magnesium sulphate, and transferred into a 100-ml separating funnel. The solution was shaken twice with 30-ml volumes of ethyl acetate, and the combined extract was washed three times with 4-ml volumes of a saturated solution of sodium chloride. When ethyl acetate formed an emulsion with the sample, the mixture was centrifuged. In fatty foods, the sample was shaken with 30-ml volumes of *n*-hexane

to eliminate most of the fat before the extraction with ethyl acetate. With or without concentrating the extract, a volume of benzene equal to that of the extract was added, and the mixture was dried with a small amount of anhydrous sodium sulphate for a period longer than 20 min.

The solution was loaded on to the silica gel column. The effluents obtained were successively loaded on to the alumina column until the washing of the silica gel column with ethyl acetate had been completed according to the procedure for the silica gel column chromatography described above. The elution of dulcin from the alumina column was carried out with 50 ml of a mixture of ethyl acetate and methanol (4:1) and those of saccharin and cyclamic acid from the silica gel column with 30 ml of a mixture of ethyl acetate and methanol (2:1), using the same procedure as described for column chromatography. The two fractions thus obtained were evaporated to dryness under reduced pressure, and each residue was dissolved in a small volume (0.2–0.5 ml) of ethyl alcohol. The solutions were subjected to thin-layer chromatography using polyamide.

An outline of the above extraction and purification procedures is given in Fig. 1.

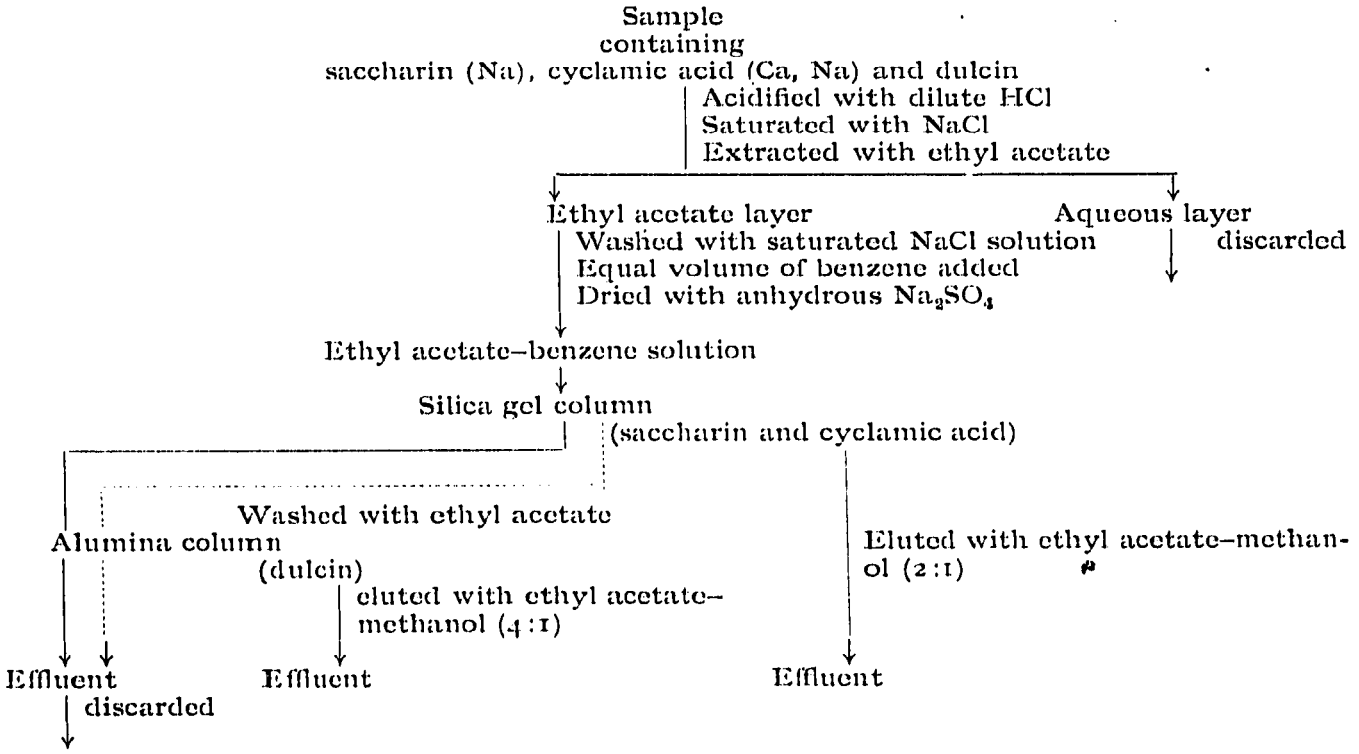

Fig. 1. Purification of artifical sweeteners from foods.

RESULTS AND DISCUSSION

Separation of artificial sweeteners on polyamide layers

Although SALO *et al.*[7] succeeded in separating artificial sweeteners on acetyl cellulose layers containing polyamide by using the solvent system Shell Sol A–propanol–acetic acid–formic acid (45:6:7:2), in this study we examined whether the compounds could be separated on polyamide layers only. When developed with neutral

solvent systems such as ethyl acetate and ethanol, saccharin and cyclamic acid did not travel on polyamide layers at all, because of their strong intramolecular interaction with polyamide. Even dulcin showed a tailing spot. It was found, however, that saccharin and cyclamic acid travelled on polyamide layers when they were developed with a solvent system containing formic acid.

In order to find the most appropriate content of formic acid in the solvent system for distribution and separation of the sweeteners, they were developed with solvent systems containing formic acid at volume ratios (A) of 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 in the mixture with benzene and ethyl acetate (5:10). When developed with a solvent system in which the A was between 1.5 and 2.5, they were separated with good distribution as shown in Fig. 2, and the circular spots showed consistent R_F values.

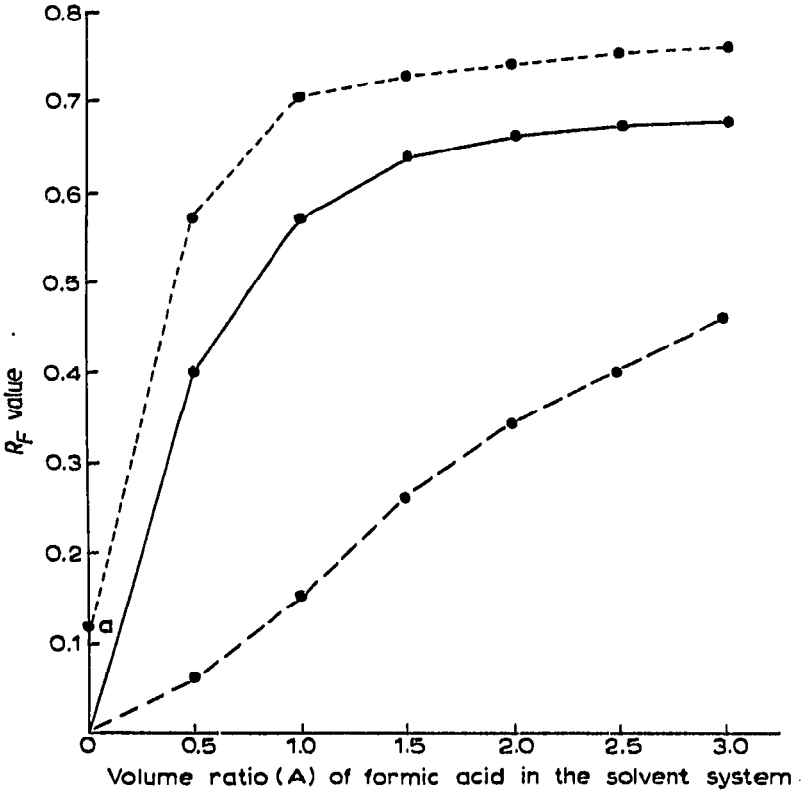

Fig. 2. Relationship of R_F values of artificial sweeteners and volume ratios of formic acid in the solvent system. Solvent system: benzene–ethyl acetate–formic acid (5:10:A). Point a = the spot was tailing.

Detection of the spots on chromatographic plates

When the plates thus developed were examined under UV light (253.6 nm), saccharin appeared as a blue fluorescent spot and dulcin as a dark spot. The fluorescent spot of saccharin especially was critical for the detection of the compound in the extract separated from foods.

Indicators such that spots of acidic compounds may be plainly distinguished from the background were selected: a chromogenic reagent composed of Methyl Red and Bromocresol Green was used for visualizing the acidic sweeteners. Thus saccharin

TABLE I

R_F VALUES OF FOOD PRESERVATIVES AND THEIR COLOURS OBSERVED ON POLYAMIDE LAYERS UNDER UV LIGHT AFTER SPRAYING PINACRYPTOL YELLOW REAGENT

Solvent system: benzene–ethyl acetate–formic acid (5:10:2).

Compound	*R_F value*	*Colour*
Dehydroacetic acid	0.82	Yellow
Butyl *p*-hydroxybenzoate	0.76	Dark blue
Propyl *p*-hydroxybenzoate	0.75	Dark blue
Ethyl *p*-hydroxybenzoate	0.74	Dark blue
Sorbic acid	0.74	Blue
Benzoic acid	0.72	Light blue
Salicylic acid	0.67	Purple
p-Hydroxybenzoic acid	0.56	Dark blue

TABLE II

R_F VALUES OF DYES ON POLYAMIDE LAYERS

Solvent system: benzene–ethyl acetate–formic acid (5:10:2).

Dye	*R_F value*
Phloxine	0.69
Rose Bengal	0.67
Eosine	0.65
Erythrosine	0.60
Acid Red	0
Amaranth	0
Ponceau SX	0
Ponceau 3R	0
Ponceau R	0
New Coccine	0
Naphthol Yellow S	0
Tartrazine	0
Sunset Yellow FCF	0
Orange I	0
Guinea Green B	0
Light Green SF	0
Fast Green FCF	0
Brilliant Blue FCF	0
Indigo Carmine	0
Acid Violet 6B	0

TABLE III

R_F VALUES AND DETECTION LIMITS OF ARTIFICIAL SWEETENERS ON POLYAMIDE LAYERS

Solvent system: benzene–ethyl acetate–formic acid (5:10:2). N.D. = no spot was detected.

Compound	*R_F value*	*Amount detected (µg)*			
		Chromogenic reagent			*UV light (253.6 nm)*
		1	*2*	*3*	
Saccharin	0.66	2.0	2.0	N.D.	0.2
Cyclamic acid	0.34	2.0	3.0	N.D.	N.D.
Dulcin	0.74	N.D.	0.2	0.05	0.05

and cyclamic acid appeared as orange-red spots against a greyish-green background. Residues of formic acid on the developed plates interfered with the visualization.

The Pinacryptol Yellow reagent was convenient for the detection of the sweeteners[10]. When the developed plates were observed under UV light (365.0 nm) after spraying with the reagents, dulcin appeared as a dark spot, while saccharin and cyclamic acid appeared as a pale blue fluorescence and yellow fluorescent spot, respectively, against a bright background. The combination of two visualizing systems using the MR-BCG and Pinacryptol Yellow reagents after pre-observation under UV light (253.6 nm) will make it easy to detect saccharin and cyclamic acid in foods.

Dulcin appeared as an orange spot by spraying with the *p*-dimethylaminobenzaldehyde reagent, which had been used for detection in paper and thin-layer chromatography. The detection by observation under UV light (253.6 nm) and visualization with this reagent is more specific for the identification than that with the Pinacryptol Yellow reagent.

Detection limits of the sweeteners by all the above methods are shown in Table III. Especially in the detection of cyclamic acid by spraying with the Pinacryptol Yellow reagent, it is necessary for the compound to eliminate formic acid on the developed plates before the visualization in order to obtain high sensitivity.

Influence of food preservatives and dyes on the detection of the sweeteners

After 0.5 μg each of eight food preservatives and twenty dyes had been spotted on polyamide layers, the chromatographic plates were developed with benzene–ethyl acetate–formic acid (5:10:2). The R_F values obtained are shown in Tables I and II.

It was found that of the spots of the compounds that were visualized by means of the MR-BCG reagent, the spot of *p*-hydroxybenzoic acid, which is the hydrolysate of *p*-hydroxybenzoates, showed an R_F value slightly higher than that of cyclamic acid. However, the former spots are clearly distinguished from the latter as there is a difference between the behaviours of the compounds under UV light (253.6 nm) and the Pinacryptol Yellow reagent on polyamide layers.

That only salicylic acid of the food preservatives tested interferes with the detection of saccharin on the layers is explained from the facts that the spot of salicylic acid showed almost the same R_F value as that of saccharin, and emitted a fluorescence under UV light (253.6 nm) similar to that from saccharin. It is therefore difficult to detect saccharin in the presence of salicyclic acid on polyamide layers using the solvent system described. As it is rare in practice for a food product to contain both compounds, the spot of saccharin can be distinguished from that of salicylic acid by observation under UV light (365.5 nm) after spraying with the Pinacryptol Yellow reagent.

Each solution of twenty water-soluble dyes was treated according to the procedure for the detection of artificial sweeteners in food products. In dyes such as Erythrosine, Eosine, Phloxine, Rose Bengal, Acid Red, Naphthol Yellow S, Orange I and Guinea Green B, some of the amount contained in the solution was distributed to the layer of ethyl acetate, and the first five dyes could not be eliminated from the fraction containing saccharin and cyclamic acid even by column chromatography using silica gel. The R_F values of the spots of Eosine and Rose Bengal dyes on polyamide layers were almost the same as that of saccharin. It was therefore assumed that the

presence of these two dyes in food products would interfere only with the detection of saccharin.

Behaviour of artificial sweeteners on the silica gel and alumina columns

It was found that 500 μg of dulcin loaded on the silica gel column were easily eluted in the step of washing the column with ethyl acetate, as it began to be eluted rapidly and smoothly from the column as soon as the eluant of the outer volume of the column had flowed out. When the effluent was successively loaded on to the alumina column, the dulcin was completely adsorbed on the column. The elution pattern obtained when dulcin was eluted from the column with the mixture of ethyl acetate and methanol (4:1) is shown in Fig. 3.

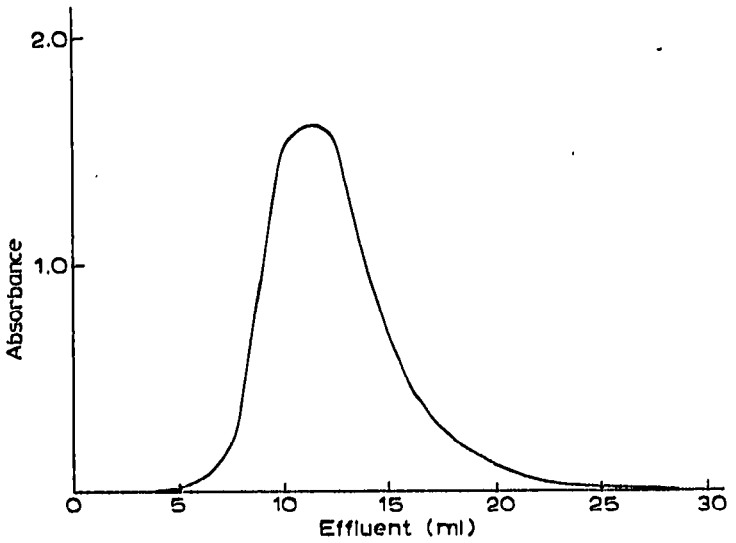

Fig. 3. Elution pattern of dulcin from an alumina column with ethyl acetate–methanol (4:1) as the eluting solvent. Alumina column: 1 × 5 cm; amount of dulcin loaded = 500 μg.

On the other hand, when 10 mg of cyclamic acid and 5 mg of saccharin in ethyl acetate were subjected to silica gel column chromatography, both compounds were perfectly adsorbed on the upper part of the column, and were not eluted in the step of washing the column with 20 ml of ethyl acetate. This was confirmed by subjecting the concentrated residue of the effluent to thin-layer chromatography. This step of washing the column was useful in cleaning up extracts for the detection of the sweeteners. The actual elution of the sweeteners from the column was carried out with the mixture of ethyl acetate and methanol (2:1). The elution patterns shown in Fig. 4 were obtained by the procedure described in the EXPERIMENTAL section. Although the turbidity of the barium sulphate formed by the reaction between barium chloride and sulphuric acid, which had been formed by the decomposition of cyclamic acid with nitrate and hydrochloric acid, did not always show the concentration of cyclamic acid in every fraction quantitatively, the pattern gave enough data to show how the compound would be eluted from the column.

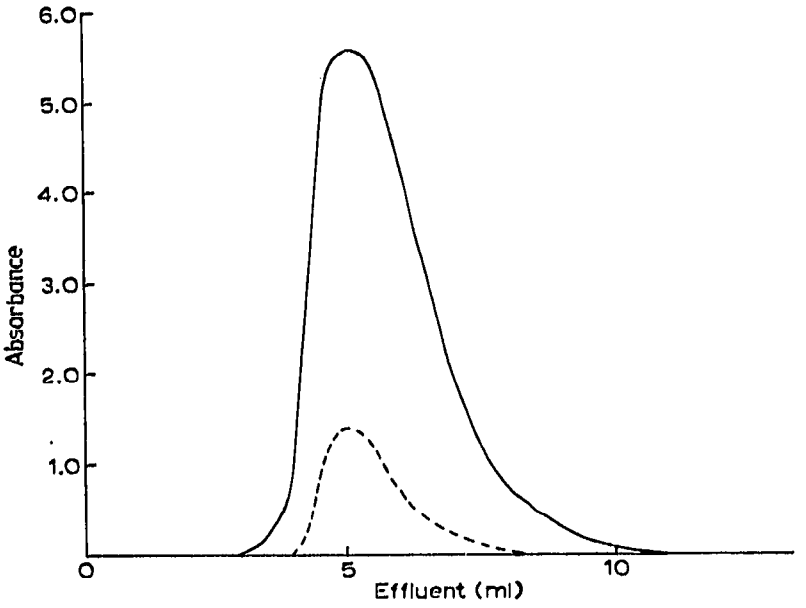

Fig. 4. Elution patterns of saccharin and cyclamic acid from a silica gel column with ethyl acetate–methanol (2:1) as the eluting solvent. Silica gel column: 1 × 5 cm. ———, 2.0 mg of saccharin; ———, 20 mg of cyclamic acid.

Separation and detection of the artificial sweeteners from food products

Saccharin or its sodium salt, sodium cyclamate and dulcin were added at levels of 10–50, 20–100 and 5–20 p.p.m., respectively, to each of tomato and orange juices, milk, ice-cream, canned fruits and biscuits, in some of which preservatives and dyes had been found by the methods reported earlier. After being separated from food products by extraction with ethyl acetate only or by extraction with ethyl acetate after the prior removal of fat with *n*-hexane, the sweeteners were detected by polyamide thin-layer chromatography. The R_F values obtained were sometimes considerably different from those of authentic substances because of contamination by such interfering materials in extracts as lipids and fat-soluble natural pigments.

When the detection of the sweeteners was carried out by the method described in the EXPERIMENTAL section, the R_F values obtained were in accordance with those of the authentic substances. Such application of double column chromatography using silica gel and alumina for the clean-up procedure was extremely effective in the elimination of extraneous materials from the extracts.

CONCLUSIONS

For the separation of saccharin, cyclamic acid and dulcin from food products, ethyl acetate is generally used as a solvent for the extraction. Although the sweeteners were easily extracted from several food products with ethyl acetate after the prior removal of fat with *n*-hexane, such procedures were inadequate for purifying the sweeteners for detection by thin-layer chromatography. One of the most important problems in the detection was the removal of interfering materials from the extracts. By passing the extract through silica gel and alumina columns, saccharin and cyclamic acid were adsorbed on silica gel, and cyclamic acid and dulcin on alumina. Subse-

quently they were separated by selective elution from both columns with mixtures of ethyl acetate and methanol (2:1 and 4:1, respectively) as the eluting solvents. Thus the sweeteners were almost completely freed from the co-extracted materials such as fat and natural pigments.

In thin-layer chromatography using only polyamide, the sweeteners were distinctly separated with a solvent system benzene–ethyl acetate–formic acid (5:10:2), and detected with high sensitivity by spraying with one of MR-BCG, Pinacryptol Yellow or *p*-dimethylaminobenzaldehyde reagents after pre-observation under UV light. In order to detect the sweeteners in food products containing other additives, the influence of eight food preservatives and twenty dyes on the detection was investigated. Among these compounds, it was found that only salicylic acid, Eosine and Rose Bengal interfered with the detection of saccharin.

The application of such double column chromatography using silica gel and alumina columns, and thin-layer chromatography using polyamide, was therefore useful in detecting sweeteners in various food products.

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