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SEPARATION AND DETECTION OF GALLIC ACID AND ITS ALKYL ESTERS BY POLYAMIDE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A procedure for preparing polyamide thin layers and chromatographic solvents necessary for a distinct separation of gallic acid and its alkyl esters has been described. A clean-up procedure necessary for the separation and identification of these compounds from fatty foods was investigated. All the procedures were very simple, convenient and feasible for the analysis of these compounds and will also be applicable to the separation and identification of other gallic acid esters in fatty foods.

INTRODUCTION

When fatty foods are oxidized or hydrolyzed under the influence of air, moisture, light and microorganisms during storage, they deteriorate because of the production of small amounts of hydroxy fatty acids, ketones, aldehydes and organic peroxides. Consequently, alteration in taste and odor of fatty foods, namely rancidity, occurs. This rancidity is primarily due to organic peroxides, hydroxy fatty acids and aldehydes. One effective and secure means of preserving fatty foods from deterioration is the addition of antioxidants in very low concentrations. Antioxidants generally available in most countries are nordihydroguaretic acid (NDGA), butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), some alkyl esters of gallic acid, *e.g.* propyl gallate (PG) and isoamyl gallate (IAG), and ethyl protocatechuate (EPC). The addition of these antioxidants to fatty foods has found increasing use in recent years. Therefore a simple, rapid and reliable method for the separation and detection of these antioxidants from fatty foods is strongly demanded today.

Although various methods for the separation and detection of these compounds from fatty foods have been reported by many investigators, generally antioxidants are separated from fatty foods by extraction with a suitable organic solvent and are detected by thin-layer, paper and gas chromatography with or without any purification procedure; of these methods thin-layer chromatography is widely applied since it is rapid, economical and convenient.

Since SEHER[1] first succeeded in separating some antioxidant mixtures on silica

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gel thin layers, some other investigators[2–4], testing the separation and detection of various mixtures of antioxidants on silica gel or acetylated cellulose, have not obtained satisfactory results. The use of polyamide powder in TLC has been introduced by DAVÍDEK *et al.*[5,6] and by EGGER[7], and the application of this stationary phase for the separation of various natural and synthetic products has been frequently reported to date (for a review see HÖRHAMMER *et al.*[8]). This chromatographic adsorbent has also been successfully applied to column chromatography for the analysis of many groups of natural phenolic[9–16] as well as other[17] products. DAVÍDEK[18] has used polyamide layers ("loose layers") to obtain a distinct separation of gallic acid from its alkyl esters and to shorten the long development time which is inevitably required in paper chromatography. Later COPIUS-PEEREBOOM[19] succeeded in preparing firmly bound layers of polyamide, containing such a binding agent as starch or polyvinyl acetate to improve the "loose layers". He has found these layers to be useful for the separation of fat antioxidants.

However, the authors were confronted with several problems when making use of the above experiences:

(1) Testing DAVÍDEK's method[18], it proved impossible to prepare a uniform firmly bound thin layer of polyamide.

(2) Using "loose layers" great care had to be taken to avoid disturbing or damaging the thin layer, especially during solvent development and during spraying with a visualizing reagent.

(3) A good distribution and sharpness of the spots of alkyl esters of gallic acid were observed neither on "loose layers" nor on polyamide layers containing a binding agent.

The present investigation was undertaken to eliminate the above-mentioned difficulties experienced when using polyamide layers and to propose a simple and reliable method for the separation and identification of antioxidants from fatty foods.

EXPERIMENTAL

Thin-layer chromatography

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

Reagents. Gallic acid and six of its alkyl esters (Table I) were obtained from Tokyo Kasei Kogyo Co., Ltd. Before use they were twice crystallized from dilute ethanol. Test solutions were prepared by dissolving 10 mg of each of the compounds in 20 ml of acetone. Aliquots (μl) of this solution were used for detection and separation.

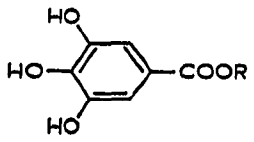
The visualizing reagent was prepared by dissolving 10 mg of analytical reagent grade 2,6-dichloroquinone-4-chlorimide (obtained from Tokyo Kasei Kogyo Co., Ltd.) in 100 ml of isopropanol.

The solvents used are listed in Table III. They were all of analytical reagent grade.

Apparatus. Thin-layer applicator and accessories were obtained from Yasawa Seisaku Co., Ltd. Glass plates were 20 cm × 5 cm. The chromatographic chamber had

TABLE I

STRUCTURAL FORMULAS OF GALLIC ACID AND ITS ALKYL ESTERS



Compound	*R*
Gallic acid	H
Methyl gallate	CH_3
Ethyl gallate	CH_2CH_3
Propyl gallate	$CH_2CH_2CH_3$
Isoamyl gallate	$CH_2CH_2CH(CH_3)_2$
Lauryl gallate	$CH_2(CH_2)_{10}CH_3$
Stearyl gallate	$CH_2(CH_2)_{16}CH_3$

a diameter of 9 cm and a height of 27 cm. The UV light source (3650 Å) was supplied by Manasulu Ultraviolet Kagaku Kogyo Co., Ltd.

Preparation of polyamide layers. According to the method generally used in the preparation of silica gel thin layers, glass plates were coated with a slurry composed of 15 g of polyamide powder and 50 ml of isopropanol. An applicator giving a thin layer approximately 250 μ thick was used. After drying the plates in air for about 15 min, they were further dried at 60° for 30 min and allowed to cool at room temperature. Then they were stored, until required, in a dessicator containing silica gel.

Development and detection. 0.5–1 μl of the test solution of gallic acid and its alkyl esters were spotted with a micropipette on the starting line 2 cm from the bottom of the plate. The plate was then placed inside a chamber containing the mobile phases to a depth of about 1 cm. Development was carried out by the ascending technique until the solvent front had travelled a distance of 10 cm from the starting line. After development the plate was dried in air, and the spots were observed under UV light before spraying with the visualizing reagent.

Column chromatography

Adsorbent. Polyamide powder (obtained from M. Woelm, Eschwege, G.F.R.) was used as the adsorbent. Before use it was treated in a manner similar to that described for TLC.

Reagents. Of the test compounds listed in Table I, gallic acid and stearyl gallate were used after two crystallizations from dilute ethanol.

The solvents *n*-hexane, benzene, acetonitrile, methanol and ethyl acetate were of analytical reagent grade.

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

Preparation of the polyamide column. A slurry of polyamide powder and *n*-hexane was poured into a glass tube to make a 10-cm high column bed. A round piece of filter paper was placed on the surface of the column bed.

Separation and detection. A sample of fatty food containing 1 mg of both gallic acid and its alkyl esters was weighed into a centrifuge tube, and 30 ml of acetonitrile were added. The mixture was shaken to extract the antioxidants and then centrifuged at 3000 r.p.m. The supernatant was then decanted into a 100-ml flask; an additional 20 ml of acetonitrile were added to the sediment and the same extraction procedure was repeated. All the supernatants were combined in the flask and evaporated to dryness at 40–45° under reduced pressure. After the residue had been dissolved in 20 ml of benzene, 30 ml of *n*-hexane and small amounts of anhydrous sodium sulfate were added to the solution. The mixture was allowed to stand for 30 min and was then transferred to the polyamide column by means of a pipette. The flask containing the anhydrous sodium sulfate was rinsed thrice with small portions of benzene, and the washings were also transferred onto the column. Before the benzene solution was eluted from the column, the residue in the flask was rinsed two to three times with small portions of ethyl acetate–methanol (1:4), and the washings were also applied on the column. The flow rate was maintained at approximately 2 ml/min. The effluent was collected upon application of the ethyl acetate–methanol mixture on the column. About 30 ml of effluent were collected in a 100-ml flask and evaporated to dryness at 40–45° under reduced pressure. The residue was dissolved in small amounts of methanol. The solution was applied to polyamide plates for TLC, as already described. An outline of the above purification procedure is given in Fig. 1.

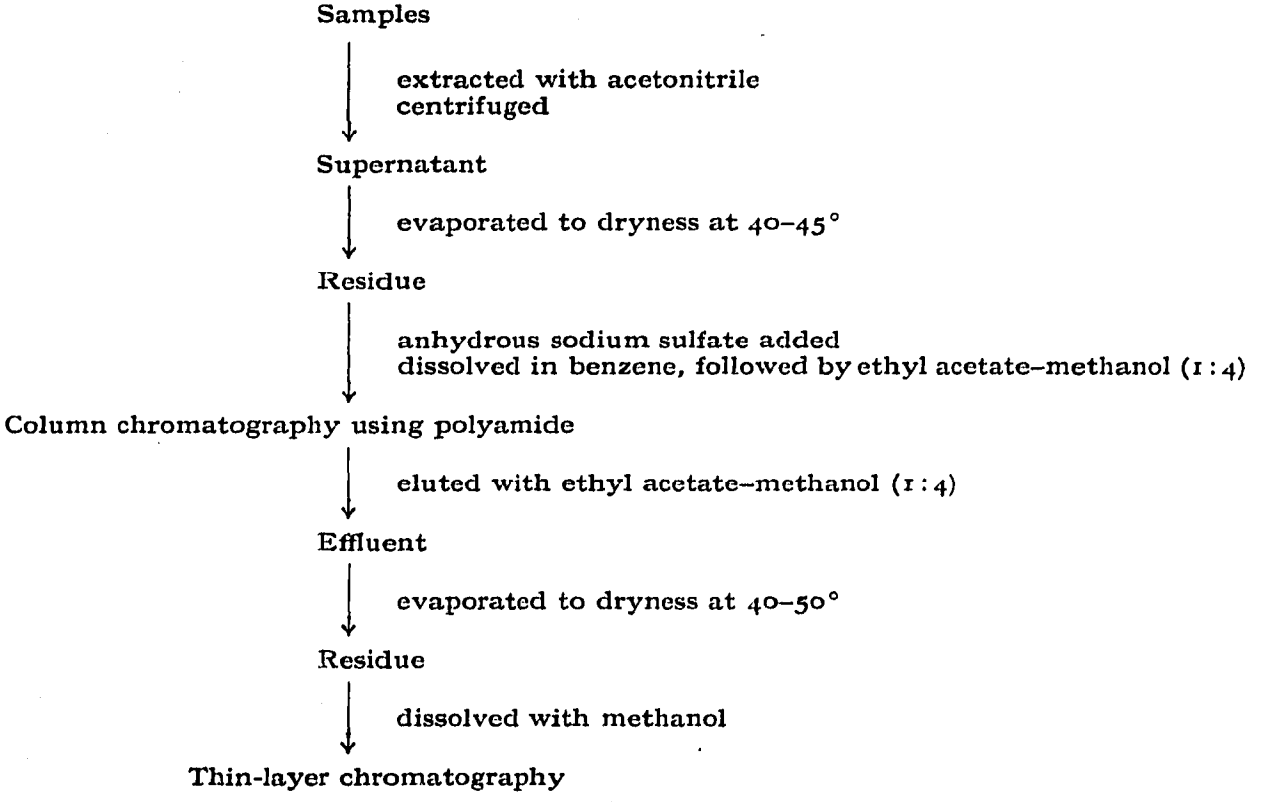


Fig. 1. Purification of gallic acid and its alkyl esters from fatty foods.

TABLE II

R_F VALUES OF GALLIC ACID AND ITS ALKYL ESTERS OBTAINED ON POLYAMIDE THIN-LAYER PLATES USING SOLVENT SYSTEMS 1–13

No.	*Solvent system*	*Gallic acid*	*Methyl gallate*	*Ethyl gallate*	*Propyl gallate*	*Isoamyl gallate*	*Lauryl gallate*	*Stearyl gallate*
1	Carbon tetrachloride	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	Ethyl acetate	0.0	0.03	0.03	0.04	0.06	0.05	0.06
3	Methanol	0.48	0.58	0.60	0.60	0.61	0.46	0.31
4	Ethanol	0.36	0.53	0.57	0.57	0.62	0.59	0.53
5	Isopropanol	0.12	0.20	0.31	0.33	0.39	0.41	0.40
6	Benzene–methanol (3:1)	0.13	0.45	0.53	0.56	0.67	0.84	0.89
7	Carbon tetrachloride–ethanol (3:2)	0.13	0.39	0.46	0.50	0.60	0.76	0.86
8	Carbon tetrachloride–isopropanol (3:2)	0.06	0.23	0.29	0.32	0.44	0.64	0.74
9	Carbon tetrachloride–ethyl acetate–acetic acid (25:5:1)	0.07	0.30	0.36	0.42	0.52	0.83	0.96
10	Carbon tetrachloride–ethanol–acetic acid (16:4:1)	0.01	0.36	0.41	0.54	0.67	0.87	0.96
11	Carbon tetrachloride–isopropanol–acetic acid (40:2:2)	0.04	0.17	0.25	0.30	0.43	0.72	0.90
12	Benzene–methanol–acetic acid (25:4:1)	0.05	0.23	0.29	0.40	0.52	0.71	0.84
13	Carbon tetrachloride–isopropanol–formic acid (40:6:2)	0.06	0.25	0.34	0.39	0.53	0.81	0.89

RESULTS AND DISCUSSION

Polyamide thin-layer plates

When the plates, coated with commercial polyamide powder, were used for development with solvent systems containing such a polar solvent as acetic acid or formic acid, the thin layers became so fragile that cracking and peeling appeared in all areas of the plates, particularly on the solvent front. In order to prevent these difficulties, commercial polyamide powder was thoroughly washed with both benzene and methanol before preparation of the thin-layer plates.

Separation and detection of gallic acid and its alkyl esters on polyamide thin layers

As seen from the R_F values in Table II, when using solvent systems 1–5, gallic acid and its alkyl esters were not clearly separated. However, it was observed, when using solvents 3 and 4, that the R_F values of isoamyl, lauryl and stearyl gallate decreased as the length of the carbon chain increased. Such a tendency was also found by COPIUS-PEEREBOOM[19] when he analyzed some alkyl esters of gallic acid on a starch-bound polyamide layer using a solvent system consisting of methanol–acetone–water (60:20:20).

Gallic acid and its alkyl esters separated considerably better in solvent systems 6–8. The distribution and sharpness of spots on the thin-layer chromatograms were in general better than when using carbon tetrachloride–ethanol (7:3), which had been recommended by DAVÍDEK[18]. It was observed, however, that the spots of propyl and ethyl gallate did not separate from each other.

Solvent systems 9–13 contain formic or acetic acid. The distribution and sharpness of spots using solvent systems 9–11 were the best thus far obtained. It was found, however, that the separation between isoamyl and propyl gallate was not sufficient. On the other hand, as shown in Fig. 2, gallic acid and its alkyl esters were distinctly separated with round spots using solvent system 13. In addition, the distribution and sharpness of spots on the chromatogram using solvent system 12

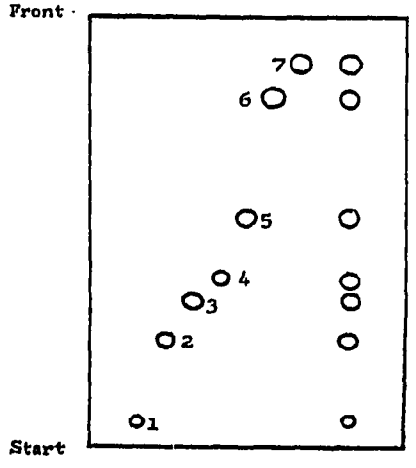


Fig. 2. Chromatogram of gallic acid and its alkyl esters and a mixture of them on polyamide thin layers. Solvent system: carbon tetrachloride–isopropanol–formic acid (40:6:2). Development time: 210 min. Temperature: 20 ± 1°. 1 = gallic acid; 2 = methyl gallate; 3 = ethyl gallate; 4 = propyl gallate; 5 = isoamyl gallate; 6 = lauryl gallate; 7 = stearyl gallate.

were similar to those using solvent system 13. Development time required 190 min in solvent system 12 and 210 min in solvent system 13 at 20 ± 1°.

Relationship between the R_F values of gallic acid and its alkyl esters and the alcohol content in solvent systems 12 and 13

As the best distribution and sharpness of spots was obtained using solvent systems 12 and 13, the question was posed whether any amount of methanol or isopropanol in two solvent systems is suitable for separation. It was found that the compounds tested were distinctly separated with round spots when the methanol content was about 13–19 vol.% in system 12 and when the isopropanol content was about 12–16 vol.% in system 13. In addition, the sharpness of all the spots was superior in solvent system 13 to that in solvent system 12.

Sensitivity test for the detection of gallic acid and its alkyl esters on polyamide thin layers

When spotting 0.1, 0.2, 0.3 and 0.5 μg of the compounds on a thin layer and then developing it with solvent system 12, each spot was detected both by spraying with the visualizing reagent and under UV light. UV light produced dark spots and the visualizing reagent produced grayish-green spots.

TABLE III

MINIMUM AMOUNTS OF GALLIC ACID AND ITS ALKYL ESTERS DETECTED ON POLYAMIDE THIN-LAYER PLATES UNDER UV LIGHT OR WITH A VISUALIZING REAGENT

Solvent system: benzene–methanol–acetic acid (25:4:1). The color observed with the visualizing reagent was grayish-green in all cases. Absorption was observed in all cases.

Compound	*R_F value*	*Amount detected (μg)*	
		Visualizing reagent	*UV light (3650 Å)*
Gallic acid	0.05	0.1	0.3
Methyl gallate	0.23	0.1	0.3
Ethyl gallate	0.29	0.1	0.3
Propyl gallate	0.34	0.1	0.3
Isoamyl gallate	0.43	0.1	0.3
Lauryl gallate	0.71	0.1	0.5
Stearyl gallate	0.84	0.1	0.5

The results, shown in Table III, indicate that detection using the visualizing reagent is more sensitive than under UV light. This is clearly attributable to the fact that the thin layer itself has a considerable UV absorption. The detection limit for the compounds was 0.1 μg with the visualizing reagent and under UV light it was 0.3 μg for gallic acid and methyl, ethyl, propyl and isoamyl gallate and 0.5 μg for stearyl and lauryl gallate. It is assumed that low sensitivities of stearyl and lauryl gallate might be dependent mainly upon any dispersion in their spots which might be brought about by the long development time and their higher R_F values.

Purification of gallic acid and its alkyl esters from fatty foods

When gallic acid and its alkyl esters, which were separated from fatty foods by extraction with acetonitrile only, were detected on polyamide plates, the R_F values

were considerably variable because of such interfering materials in the extract as lipids, tocopherols and fat-soluble pigments. Thus polyamide column chromatography, previously reported by us[20], was applied to remove these extraneous substances.

Judging from the R_F values of the compounds on polyamide thin layers, elution of gallic acid and stearyl gallate on the polyamide column was examined by the following process. One milliliter of a test solution, prepared by dissolving 10 mg of stearyl gallate in 10 ml of benzene, was applied to a polyamide column and eluted with a mixture of ethyl acetate–methanol (1:4). Every 2 ml of the effluent were collected in a small test tube immediately after loading the solvent onto the column, and each sample was transferred to a 10-ml flask for evaporation at 40–45° under reduced pressure. Each residue was dissolved in 10 ml of methanol to measure the absorbance of stearyl gallate of $\lambda_{max} = 275$ mμ with 1-cm quartz cells using methanol as a blank.

The behavior of gallic acid on a polyamide column was examined in a procedure similar to that used for stearyl gallate. However, the test solution for the column was prepared by dissolving gallic acid in ethyl acetate, and the absorbance of each effluent fraction was measured at $\lambda_{max} = 270$ mμ.

Each elution pattern of the two compounds obtained by this procedure is shown in Fig. 3. It may be observed from this figure that each 1 mg of gallic acid and stearyl gallate on the polyamide column is completely eluted when the effluents have reached about 20 ml.

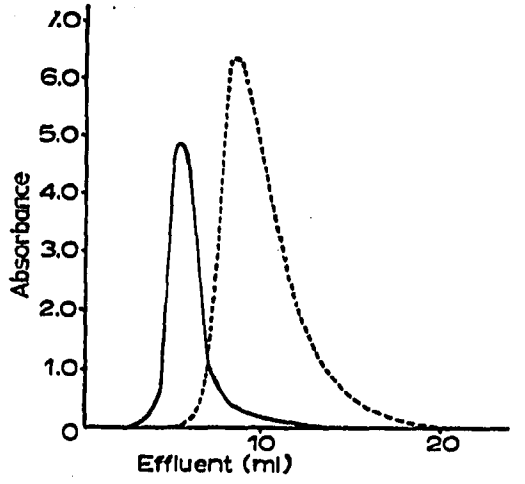


Fig. 3. Elution patterns on a polyamide column of stearyl gallate and gallic acid with methanol–ethyl acetate (4:1) as the solvent system.

Separation and identification of gallic acid and its alkyl esters from fatty foods

The separation and identification of gallic acid and its alkyl esters from beef tallow, lard or olive oil, in which each compound was added at a concentration of 100 p.p.m., were carried out according to the procedure described in EXPERIMENTAL. It was found that the R_F values of these compounds on polyamide thin layers were in good agreement with those of the standard substances.

Such a method is also applicable to the analysis of gallic acid and its alkyl esters in any other fat-rich foods.

CONCLUSIONS

The uniform and firmly bound thin layers of polyamide powder were easily prepared by using the polyamide powder treated with both benzene and methanol. It was found in this investigation that the polyamide thin layers were suitable for the analysis of gallic acid and its alkyl esters.

By the ascending development with one of two solvent systems containing an organic acid, solvent systems 12 and 13, gallic acid and its alkyl esters were distinctly separated with round spots on polyamide thin layers. In particular, it is worth insisting that some alkyl esters of gallic acid, carbon chains of which differ by one carbon atom, were separated without overlapping.

In the comparative sensibility test for the detection of gallic acid and its alkyl esters on polyamide thin layers under UV light and by spraying with the visualizing reagent, the detection limit under UV light was 0.3 μg for gallic acid and methyl, ethyl, propyl and isoamyl gallate and 0.5 μg for stearyl and lauryl gallate. By spraying with the visualizing reagent, 0.1 μg of these compounds was detected.

The simultaneous identification of gallic acid and its alkyl esters from some fatty foods was successfully carried out by extraction with acetonitrile and purification of the extract on the polyamide column and then detection on polyamide thin layers.

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