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Note

Estimation of quaternary ammonium and tertiary sulphonium compounds by thin-layer electrophoresis and scanning reflectance densitometry

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In recent years many laboratories have become interested in the quantitative estimation of quaternary ammonium and tertiary sulphonium compounds. In particular, the role of glycinebetaine in relation to salt and water stress has been extensively investigated, both in plants[1] and animals[2]. Unfortunately most of the analytical methods which are available for many quaternary ammonium compounds lack sensitivity, selectivity or reproducibility. One method which combines simplicity, tolerance of impurities and versatility is the combination of thin-layer electrophoresis (TLE) with scanning reflectance densitometry[1,3].

In this paper we describe the procedure which we have developed over the past few years for the estimation of glycinebetaine in particular, but which can also be used for a wide variety of other quaternary ammonium and tertiary sulphonium compounds.

EXPERIMENTAL

Chemicals

Unless otherwise stated, the chemicals used were commercially available. β-Alaninebetaine was synthesised from acrylic acid and trimethylamine as described by Le Berre and Delacroix[4], and stachydrine was produced by the methylation of proline using the method of Cornforth and Henry[5]. 3-(Dimethylsulphonio)propionate, 3-(dimethylsulphonio)-2-methyl-propionate and 5-(dimethylsulphonio)pentanoate were obtained from the reactions between dimethylsulphide and acrylic[6], methacrylic and 5-bromovaleric acids, respectively. Methyl and *n*-butyl esters were produced by treatment of the acids with freshly prepared, dry 4 *M* HCl in methanol or *n*-butanol.

Extraction of Suaeda maritima

Young shoots (1 g fresh weight) from *Suaeda maritima* seedlings grown in ½ strength Hoagland and Arnon's No. 1 solution[9] were extracted with methanol–chlo-

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roform–water (12:5:1) using the micro-method of Hanson *et al.*[7]. The aqueous phase was taken to dryness and redissolved in 1 ml of water, followed by 1 ml of methanol. After standing overnight at −4°C, the extract was centrifuged and 20-μl aliquots spotted onto a thin-layer chromatographic (TLC) plate.

Thin-layer electrophoresis

TLE and TLC were performed on 250-μm thick layers of silica gel G 60 (Merck, Darmstadt, G.F.R.) spread from a thin slurry on 200 × 200 mm glass plates. The buffers were: (a) 0.2 *M* citric acid (950 ml) plus 0.4 *M* disodium hydrogen phosphate (50 ml), adjusted to pH 2.6; (b) pyridine (10 ml), acetic acid (3.3 ml), water (1000 ml), adjusted to pH 5.5; (c) formic acid (25 ml, 90%), water (1000 ml). pH 2.2. A Shandon Southern U77/TLE thin-layer electrophoresis apparatus was used at 800 V (20–45 mA) for 30–60 min. Samples and standards in methanolic solution were applied either in small volumes (up to 10 μl) by hand with a SGE 10-μl syringe, or using an A.I.S. multispotter which can apply up to nine samples simultaneously and produce a uniform spot size. The buffer was sprayed onto the dry, loaded plates immediately before electrophoresis. After the electrophoretic separation, the plates were dried for 30 min in a fan-assisted oven at 80°C and cooled to 4°C before application of the spray reagent. The Dragendorff reagent was the main spray used in this investigation, together with cobalt chloride–potassium ferrocyanide[3]. Plates sprayed with Dragendorff reagent were kept in the dark at 4°C for at least 15 min to ensure full colour development before being scanned, and the analysis was completed within an hour of spraying the plate.

Scanning reflectance densitometry

The plates sprayed with Dragendorff reagent were scanned with a Zeiss PMQ II spectrophotometer fitted with attachments enabling it to scan thin-layer plates. The reflectance of the yellow background of the plates at 550 nm was examined and the quenching effect of the red or orange spots was measured. Quantitative estimation was greatly facilitated by the use of a Pye DP88 computing integrator placed between the galvanometer and a chart recorder. The spots were scanned in the direction of electrophoresis at a speed of 1 mm/sec. Two runs were made in a forward direction and two in reverse, so that each value represents the mean of four integrations.

RESULTS AND DISCUSSION

Chromatographic and electrophoretic data for a number of quaternary ammonium and tertiary sulphonium compounds are shown in Table I. In general, electrophoresis was preferred because it gave good separations and was less sensitive to impurities (especially inorganic salts) than TLC. All of the compounds tested gave a green colour with cobalt chloride–potassium ferrocyanide and most gave an orange spot with Dragendorff reagent. With this spray some spots took more than 15 min to develop (*e.g.* dimethylglycine), and some gave a purple colour (choline and β-alanine-betaine). However, the scanning reflectance densitometer was more sensitive to spot size than to colour. The pyridine–acetic acid buffer interfered with the Dragendorff reagent, giving a grey-brown background which could not be used in the reflectance densitometer. Furthermore, the other volatile buffer (formic acid) produced very

TABLE I

CHROMATOGRAPHIC AND ELECTROPHORETIC DATA FOR SOME QUATERNARY AMMONIUM AND TERTIARY SULPHONIUM COMPOUNDS

Solvent systems: 1, methanol–acetone–conc. HCl (90:10:4); 2, methanol–ammonium hydroxide (sp.gr. 0.88) (75:25). Electrophoretic mobilities are subject to variation depending on the thickness of the plate, the amount of the compound applied, the presence of impurities etc. The values given are only intended as an approximate guide.

Compound	R_F		*Mobility (cm/min) in*	
	Solvent system 1	*Solvent system 2*	*Citrate/ phosphate, pH 5.5*	*Pyridine/ acetic acid, pH 5.5*
Glycinebetaine	0.46	0.56	0.53	0.33
β-Alaninebetaine	0.43	0.46	1.31	0.30
Trigonelline	0.34	0.43	0.56	–
Prolinebetaine (Stachydrine)	0.38	0.53	0.39	0.27
Choline	0.29	0.03	1.82	2.30
Acetylcholine	0.27	0.09	1.11	1.44
Phosphorylcholine	0.32	0.11	0.13	0.93
Glycinebetaine aldehyde	0.39	0.07	1.91	2.44
N,N-Dimethylglycine	0.61	0.68	0.55	–
3-(Dimethylsulphonio)propionate	0.39	0.35	1.42	0.18
5-(Dimethylsulphonio)pentanoate	0.31	0.32	1.04	0.33
3-(Dimethylsulphonio)-2-methylpropionate	0.44	0.46	0.80	1.44
Glycinebetaine methyl ester	0.31	0.10	1.25	1.76
Glycinebetaine *n*-butyl ester	0.43	0.11	0.53	1.04
β-Alaninebetaine methyl ester	0.35	0.42	0.71	1.36
β-Alaninebetaine *n*-butyl ester	0.38	0.37	0.53	0.91
N,N-Dimethylglycine methyl ester	0.49	0.77	1.27	2.22
N,N-Dimethylglycine *n*-butyl ester	0.62	0.81	1.09	1.60
3-(Dimethylsulphonio)propionate methyl ester	0.32	–	0.87	1.56
3-(Dimethylsulphonio)propionate *n*-butyl ester	0.35	0.16	0.56	1.22

diffuse spots with compounds which were at all mobile (data not shown). Thus the citrate–phosphate buffer was used routinely throughout these investigations. Peak areas in the scanning reflectance densitometer are influenced both by the nature of the reaction between the onium compound and the Dragendorff reagent, and by the extent of diffusion of the spot, which in part is related to the mobility of the compound.

This is illustrated in Fig. 1 where the standard curves for glycinebetaine, 3-(dimethylsulphonio)propionate and choline are compared. Although 3-(dimethylsulphonio)propionate · HCl and choline · HCl differ only slightly in mobility, choline produced a more diffuse spot with a stronger, purple colour. In contrast, glycinebetaine is less mobile (see Table I) and produced a smaller, more compact spot. With 40 μg of choline and of 3-(dimethylsulphonio)propionate the spots were sufficiently close for the peaks of the two spots to fuse at their bases when they were scanned. This did not seriously affect the integration of the peak areas.

Because of the large variation in peak areas between different plates for the same sample[3], it is necessary to run a standard curve on every plate. This means that

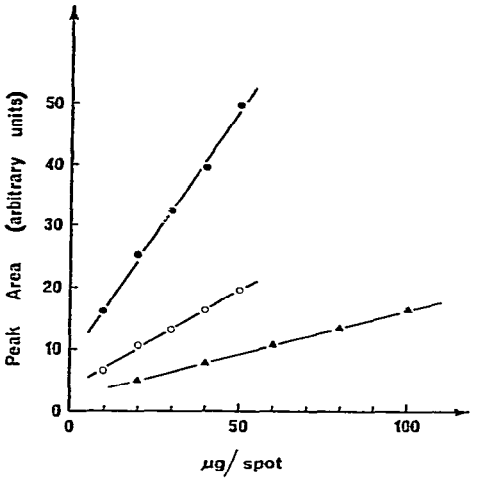

Fig. 1. Standard curves for onium compounds subjected to thin-layer electrophoresis in citrate–phosphate buffer (pH 2.6) for 30 min, sprayed with Dragendorff reagent and scanned at 550 nm in a reflectance densitometer. All the standards were run on the same plate. Regression equations and coefficients of determinations were: glycinebetaine (▲): $y = 0.144x + 1.983$, $r^2 = 0.99$; 3-(dimethylsulphonio)propionate·HCl (○): $y = 0.316x + 3.698$, $r^2 = 0.98$; choline chloride (●): $y = 0.811x + 8.272$, $r^2 = 0.99$.

only four or five samples may be run together. Five replicates of 20 μl of the *Suaeda* extract were run on a plate together with four different standards for glycinebetaine and choline. The choline in the extracts was below the range of the standard curve, but the glycinebetaine spots were estimated at 60.5, 62.3, 57.0, 62.1 and 68.0 μg, giving a mean of 61.98 μg with a standard error of 1.78 μg. For extracts of tissues containing very small quantities of onium compounds a purification step such as passage through ion-exchange resins[8] may be necessary, although for many tissues precipitation of the inorganic salts with cold methanol is sufficient. Since all the onium compounds tested here were highly soluble in methanol this technique can be used for all of them. The combination of strong cation-exchange materials with alcoholic solvents should be avoided because of the ease of esterification of the carboxylic acid groups of the betaines. Ion exchange is also not recommended for 3-(dimethylsulphonio)propionate or β-alaninebetaine because of the alkali-lability of these two compounds.

The technique described here allows the estimation of individual onium compounds with the minimum of preliminary purification. Whilst it is not particularly sensitive (limits are about 10 μg/spot for glycinebetaine and 5 μg/spot for choline and 3-(dimethylsulphonio)propionate), it is relatively simple and very adaptable. Work is in progress on the development of techniques for the estimation of very small amounts of onium compounds in plants.

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