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SEPARATION OF PLANT BETAINES AND THEIR SULPHUR ANALOGUES BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Recent interest in stress-induced accumulation of glycinebetaine in plants has stimulated the search for improved methods of assaying betaines. A method based on high-performance liquid chromatographic separation on a strong cation-exchange material combined with low-wavelength ultraviolet absorbance detection is described. This forms the basis of a rapid assay procedure for glycinebetaine using plant saps as the starting material and avoiding the need for derivatization.

INTRODUCTION

The quaternary ammonium compound glycinebetaine (Fig. 1) has been shown to accumulate in some plants as a result of water or salt stress[1,2], and also in the halophilic bacterium *Ectothiorhodospira halochloris*[3] and the cyanobacterium *Synechocystis*[4]. It occurs in amounts varying from 1 to >500 mmol kg^{-1} fresh weight in members of the Chenopodiaceae[5–7] and has been used to detect the addition of beet sugar to wine[8–10]. The distribution of a number of betaines and 3-dimethylsulphoniopropionate (Fig. 1) may be of taxonomic significance, for example in marine algae[11]. There is a need for an improved analytical method, particularly for glycinebetaine, but also for other betaines and related compounds. Previous methods have relied on non-specific precipitation with periodide[12–14] or reineckate[15], scanning densitometry of thin-layer chromatographic or thin-layer electrophoresis separations[16], thermal degradation of the hydroxide form and gas–liquid chromatography (GLC) of the resulting trimethylamine[1], esterification and thermal degradation of the chloride salt followed by GLC of the dimethylamino acid ester[8,9], GLC of an unidentified

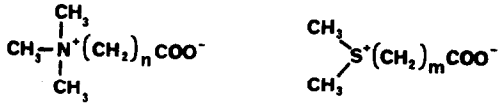

Fig. 1. Structure of simple betaines and their sulphur analogues: $n=1$, glycinebetaine; $n=2$, β-alaninebetaine; $m=1$, dimethylsulphonioacetate; $m=2$, 3-dimethylsulphoniopropionate.

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product of trimethylsilylation[17], purification followed by isotope dilution and micro-Kjeldahl determinations[18] or enzymatic estimation[19], NMR spectrometry[20] and esterification with α,*p*-dibromoacetophenone, partition between water and chloroform and UV spectral analysis of the aqueous phase[21]. None of these methods is sufficiently simple, specific or accurate for use with large numbers of samples, and they are not particularly suitable for automation. There have also been a number of reports of high-performance liquid chromatography (HPLC) separations of glycinebetaine from sugars on amino-bonded or carbohydrate columns[10,22,23], of homarine (isonicotinic acid betaine) on Corasil[24] and of trigonelline (nicotinic acid betaine) on anion or cation-exchange materials[25,26]. Unlike homarine and trigonelline, most betaines do not have significant UV absorbance above *ca.* 210 nm, so that refractive index or low UV absorbance detection must be used. The method described below combines rapid extraction and deionization techniques with the use of an internal standard and HPLC analysis on Partisil 10-SCX with detection at 190–200 nm.

EXPERIMENTAL

The betaines and tertiary sulphonium compounds were prepared as described previously[16]. Dimethylsulphonioacetate was synthesized from dimethylsulphide and bromoacetic acid. Two HPLC systems were used, the first consisting of an Applied Chromatography Systems Model 750/03 isocratic HPLC pump, a Rheodyne 7120 injection valve with a 20-μl loop, and a Cecil CE 212A variable-wavelength UV detector. The other system comprised a Spectra-Physics SP 8700 solvent delivery system fitted with a 10-μl loop, an Altex 165 variable-wavelength UV detector and a Spectra-Physics SP 4100 computing integrator. Buffers were filtered through Millipore 0.22-μm GVHP 04700 Durapore filters and kept saturated with helium. Separations were performed on a 250 × 5 mm I.D. stainless-steel column packed with Partisil 10-SCX and fitted with a Chrompack type B cation-exchange guard column.

Plant material was extracted in one of two ways. Either hot methanol or methanol–chloroform–water (12:5:1) was used to extract dry material, the extract filtered and reduced to dryness *in vacuo* and the residue dissolved in water. Alternatively, fresh leaves or roots were placed in 1.5-ml polypropylene microcentrifuge tubes, frozen, thawed and crushed using a metal rod with a tapered end. Small holes were pierced in the cap and base of each tube and the sap centrifuged into a second microcentrifuge tube at about 6000 *g*. The collected sap was further centrifuged at 9000 *g* for 2 min, and the supernatant (diluted if necessary) was deionized in the same manner as the aqueous solutions of the methanolic extracts, *i.e.* by the addition of ion-exchange resins in the ratio of 1:2 cation to anion exchanger, and in a two to three fold excess of that required to remove inorganic ions, amino acids etc. After shaking for about 5 min. the ion-exchange beads were removed by centrifugation or by passage through a small Millipore GSWP 0.22-μm filter. The resulting deionized extract was used directly for HPLC or reduced in volume in a stream of filtered air.

The ion-exchange resins were prepared as described below. A strong anion-exchange resin, either Amberlite IRA-401 or Dowex 1X2-100 (depending on the volume to be deionized) was washed and regenerated in the OH^- form (or the acetate form in the cases of the alkali-labile compounds β-alaninebetaine and 3-dimethyl-

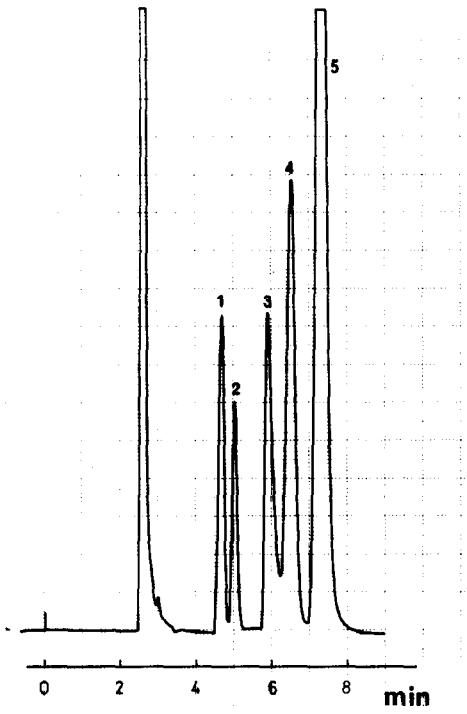

Fig. 2. Separation of betaine standards on Partisil 10-SCX eluted with 50 mol m^{-3} KH_2PO_4 with 5% methanol at 1.5 ml/min: 1 = dimethylglycine; 2 = dimethylsulphonioacetate; 3 = glycinebetaine; 4 = homarine; 5 = trigonelline.

sulphoniopropionate) on a sintered glass funnel. After washing, surplus water was removed by suction. Similarly, a weak cation-exchange resin, either Amberlite IRC-50 or Amberlite CG-50 100–200 mesh, was converted to the H^+ form.

RESULTS AND DISCUSSION

The separation of quaternary ammonium compounds by column chromatography on strong cation-ion exchange resins was reported in the 1960s[27]. Since then efficient ion-exchange materials have been developed for HPLC, but they have not been widely used for the separation of betaines. Fig. 2 illustrates that a number of common plant betaines can be separated on Partisil 10-SCX. Elution was achieved with very low concentrations of buffer (50 mol m^{-3} KH_2PO_4 plus 5% methanol, pH 4.6), and retention was not much greater with distilled water as eluent. Slightly longer retention times were achieved with the column in the protonated form. Insufficient separation of betaines from each other or from interfering substances was obtained on an anion-exchange column (Partisil 5-SAX). The effect of buffer strength on retention times of a range of quaternary ammonium and tertiary sulphonium compounds is illustrated in Fig. 3. Whilst the retention times of most of the early eluting peaks was unaffected by buffer strength, β-alaninebetaine, carnitine and 3-dimethylsulphoniopropionate all eluted earlier at higher buffer concentrations, although to

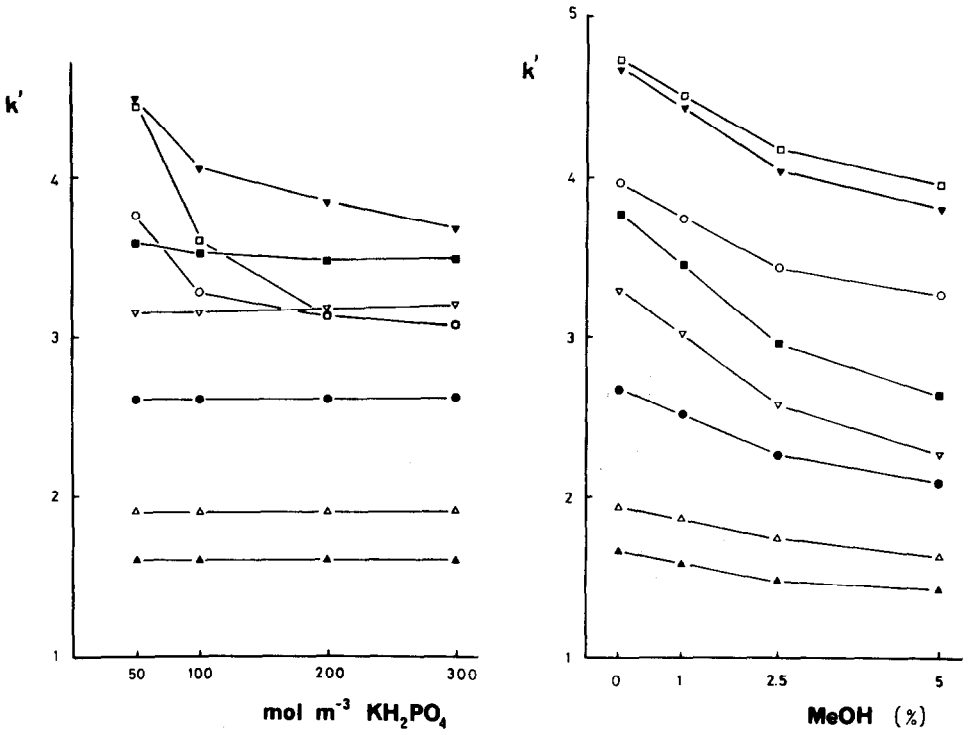

Fig. 3. Effect of buffer strength on capacity factors (k') for betaines and some sulphur analogues on Partisil 10-SCX: ▲ = dimethylglycine; △ = dimethylsulphonioacetate; ● = glycinebetaine; ▽ = homarine; ■ = trigonelline; ○ = 3-dimethylsulphoniopropionate; □ = carnitine; ▼ = β-alaninebetaine.

Fig. 4. Effect of methanol concentration on capacity factors (k') for betaines and some sulphur analogues on Partisil 10-SCX eluted with 50 mol m^{-3} KH_2PO_4. Symbols as in Fig. 3.

differing extents. This property is useful if, for example, 3-dimethylsulphoniopropionate and trigonelline need to be separated. Below about 20 mol m^{-3} KH_2PO_4 some compounds (notably 3-dimethylsulphoniopropionate) gave poor peak shapes. Increasing the pH of the mobile phase with K_2HPO_4 did not significantly alter retention times or affect resolution, and greatly increased the background absorbance at 190–200 nm. At lower pH values retention of some betaines was slightly increased, but again resolution was not improved.

Increasing concentrations of organic modifiers such as methanol reduced the retention times of all of the compounds tested, although not to the same extent in each case (Fig. 4). Thus separation of 3-dimethylsulphoniopropionate from trigonelline was better with the addition of 5% methanol than with 50 mol m^{-3} KH_2PO_4 alone. Again, however, the peak shapes of some of the compounds deteriorated at higher levels of methanol and other organic modifiers such as acetonitrile.

The retention times and response factors relative to glycinebetaine of a number of betaines and their sulphur analogues are given in Table I. In the cases of glycinebetaine, β-alaninebetaine and their sulphur analogues (Fig. 1), retention time was determined more by carbon chain length than by the nature of the onium group.

TABLE I

RETENTION TIMES AND MOLAR RESPONSE FACTORS (BASED ON THE ANHYDROUS BETAINES) OF A NUMBER OF BETAINES AND BETAINE ANALOGUES

Conditions: Partisil 10-SCX eluted with 50 mol m^{-3} KH_2PO_4 at 1.5 ml/min. The void time was 1.5 min. ND = not determined.

Compound	*Retention time (min)*	*Molar response factor*	
		195 nm	*200 nm*
Dimethylglycine	3.97	0.90	0.76
Dimethylsulphonioacetate	4.42	1.38	1.60
Glycinebetaine	5.47	1.00	1.00
Homarine	6.43	40.49	61.60
Trigonelline	7.08	71.90	78.56
3-Dimethylsulphoniopropionate	7.17	1.19	1.30
β-Alaninebetaine	8.51	0.73	0.83
Carnitine	8.44	1.01	1.15
Stachydrine	8.97	ND	ND

Homarine and trigonelline have absorbance maxima at about 270 nm in addition to those at lower wavelengths, but the absorbance at 195 nm is several times greater than at the longer wavelengths. Thus 3-dimethylsulphoniopropionate and trigonelline or homarine may also be distinguished by dualwavelength monitoring or "in flight" wavelength scanning, as is possible with the Altex 165 detector for example. As is shown in Table I, homarine and trigonelline have response factors 40–80 times that of glycinebetaine at 195 or 200 nm, and the relative response factors of the other compounds were near unity, although varying with wavelength. At 195 nm the detector response for all peaks was about double that at 200 nm.

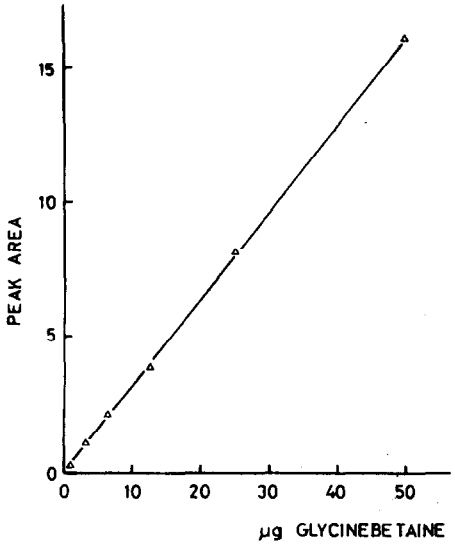

Fig. 5. Calibration curve for glycinebetaine measured at 195 nm. $r^2 = 0.999$.

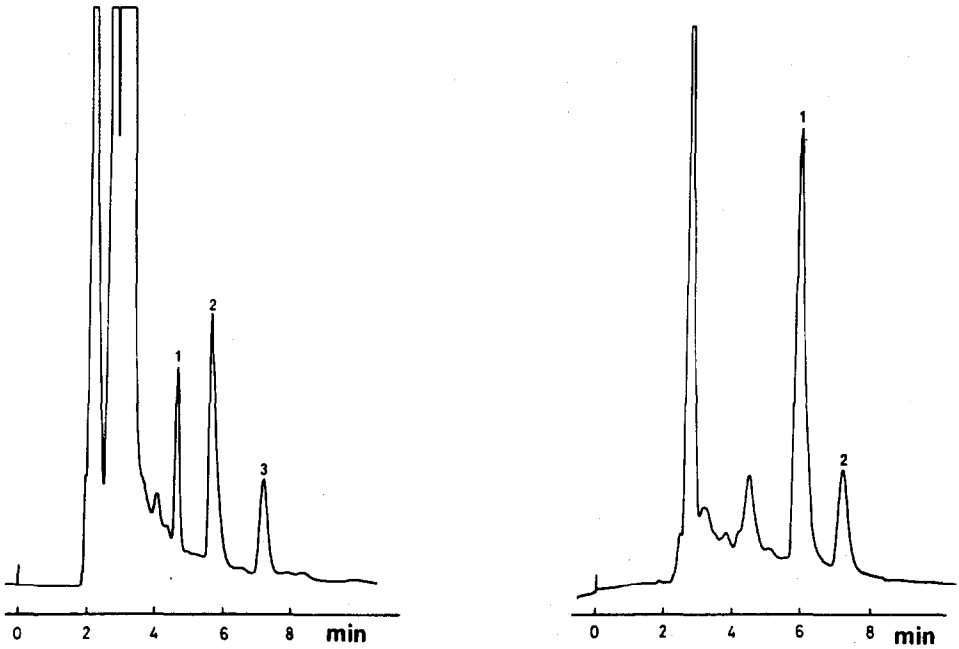

Fig. 6. Analysis of betaines in sap extracts of *Leymus sabulosus* on Partisil 10-SCX eluted with 50 mol m^{-3} KH_2PO_4 plus 5% methanol at 1.5 ml/min: 1 = dimethylsulphonioacetate (internal standard); 2 = glycinebetaine; 3 = trigonelline.

Fig. 7. Analysis of betaines from a methanolic extract of dried shoots of *Salicornia europaea* on Partisil 10-SCX eluted with 50 mol m^{-3} KH_2PO_4 plus 1.5% methanol at 1.25 ml/min: 1 = glycinebetaine; 2 = trigonelline.

The sensitivity of detection will be determined by a number of instrument-dependant factors, *i.e.* the lowest wavelength available and the performance of the detector at low wavelengths and high sensitivities. With the equipment used here the detection limits for quantitative work were < 100 ng. A calibration curve in the more generally useful range of 1–50 μg is shown in Fig. 5. For quantitative work it is useful to add an internal standard to the extract before deionization. The choice of internal standard will depend on the position of peaks from the extract, but dimethylsulphonioacetate (the sulphur analogue of glycinebetaine) is a good candidate and has been used with extracts of the grass *Leymus sabulosus* (Fig. 6). A chromatogram of an extract of the halophytic chenopod *Salicornia europaea* is shown in Fig. 7. In both plant extracts a small trigonelline peak corresponds to 0.01–0.005 times the amount of glycinebetaine.

The technique described above has been used to determine glycinebetaine levels in a number of plant saps and in methanolic extracts of dried plant material and *Aster tripolium* pollen, and 3-dimethylsulphoniopropionate in *Melanthera biflora* (data not shown). High performance cation-exchange chromatography should also be a useful adjunct to the methods currently employed for the identification and quantitation of 3-dimethylsulphoniopropionate in marine algae[28].

CONCLUSIONS

Some commonly occurring plant betaines and 3-dimethylsulphoniopropionate may be separated within 10 min on a column of Partisil 10-SCX eluted with 50–300 mol m^{-3} KH_2PO_4, with or without small amounts of methanol. Detection by UV absorbance at 190–200 nm is sufficiently sensitive for most applications and the process can, to some extent, be automated. Thus a technique is now available for screening reasonably large numbers of plant samples in studies of the relationship of betaine and 3-dimethylsulphoniopropionate contents to salinity or drought tolerance.

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