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# SEPARATION AND QUANTITATIVE ESTIMATION OF BETAINE ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Procedures are described for the separation and quantitative estimation of betaines as their *p*-bromophenacyl, phenacyl, *p*-nitrobenzyl or methyl esters. The aromatic esters are separated on weak or strong cation-exchange columns eluted with a choline buffer in 5–30% aqueous acetonitrile and detected with a variable-wavelength UV absorbance detector. A Dionex ion chromatograph is used for the separation of methyl esters on a neutral column in the presence of hexanesulphonic acid. In this case a conductivity detector is used after suppression of the background conductivity of the eluant. Both methods offer improved sensitivity and reduced background noise when compared with the direct separation of betaines and their detection by UV absorbance at 200 nm.

## INTRODUCTION

The continuing interest in glycinebetaine, which accumulates in many plants and other organisms as a result of salt or water stress[1–3], has stimulated the search for improved methods of detection and quantitative estimation of this compound. Previous assays have been based on non-specific precipitation with either periodide[4–6] or reineckate[7] salts, scanning densitometry of thin-layer separations[8], gas–liquid chromatography of degradation products of either the hydroxide form or of the chloride salts of esters[2,9,10], or of an uncharacterised trimethylsilylation product[11], $^{1}$H NMR spectroscopy[12,13], enzymatic estimation[14] and purification followed by micro-Kjeldahl determination of the nitrogen content of the sample[15]. There have also been several reports of the separation of betaines by high-performance liquid chromatography (HPLC) and their detection using refractive-index[16–20], or either high-wavelength UV absorbance for the aromatic betaines such as trigonelline and homarine[21,22], or low-wavelength UV absorbance for the majority of betaines[20,23,24]. Neither the refractive-index nor low-wavelength UV detection methods are very sensitive or selective. Another assay was based on the formation of UV-absorbing esters of betaines and their estimation by direct UV absorbance spectrometry of the aqueous phase after the reaction mixture had been partitioned between

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chloroform and water[25]. This paper included a preliminary report of the separation of the reagent (*p*-bromophenacyl bromide) from trigonelline and its *p*-bromophenacyl ester on a strong cation-exchange column eluted with 200 mol $m^{-3}$ ammonium dihydrogen phosphate in 50% methanol. Details are given below of the separation of a range of betaine esters on both weak and strong cation-exchange materials using choline as the counter-ion, and of a new method for the separation of methyl esters of betaines and their sulphur analogues based on ion-pair chromatography.

## EXPERIMENTAL

### *Chemicals*

Choline was purchased as a 45% solution in methanol from Fluorochem (Glossop, U.K.). 2-Trimethylaminopropionate (α-alanine betaine), 2-triethylaminopropionate, 5-trimethylaminovalerate and 2-triethylaminoacetate were synthesized, as their bromide salts, from either trimethylamine or triethylamine and the appropriate brominated carboxylic acid. Stachydrine (2-carboxylato-N,N-dimethylpoyrrolidinium), 3-dimethylsulphoniopropionate and 2-dimethylsulphonioacetate were synthesized as previously described[8,23]. A sample of β-stachydrine (3-carboxylato-N,N-dimethylpyrrolidinium) was kindly supplied by Prof. G. Blunden, Porthsmouth Polytechnic. Other compounds were obtained from commercial sources.

### *Preparation of mobile phase for cation-exchange chromatography*

An appropriate amount of choline, as a solution of the free base in methanol, was dissolved in water and the pH adjusted to the desired value with dilute orthophosphoric acid. Acetonitrile was added and the resulting solution filtered through 0.22-μm GVHP Durapore filters using all-glass filtration equipment.

### *Cation-exchange high-performance liquid chromatography*

A simple isochratic system comprising an Applied Chromatography Systems Model 750/03 pump, a Rheodyne 7120 injection valve and a Cecil CE 212E variable-wavelength UV detector was used together with either a 250 × 4.6 mm I.D. stainless-steel column packed with 5-μm spherical silica to which had been bonded an aliphatic carboxylic acid (Bakerbond column supplied by Linton Products, Harlow, U.K.), or a 250 × 5 mm I.D. stainless-steel column packed with Partisil 10-SCX (bonded aromatic sulphonic acid). The UV detector was used at 270 nm for the *p*-nitrobenzyl esters, 262 nm for the *p*-bromophenacyl esters and 247 nm for the phenacyl esters.

### *Ion-pair chromatography*

Ion-pair chromatography was performed on a Dionex 2010i ion chromatograph fitted with a 200 × 4 mm I.D. MPIC-NS1 (10 μm, porous, highly cross-linked, neutral styrene–divinylbenzene copolymer) separator column preceded by a MPIC-NG1 guard column. The conductivity of the eluant was suppressed in a cation fibre suppressor regenerated with 40 m*M* barium hydroxide and the betaine esters were detected by a conductivity detector giving a background of 8–10 μS. The normal eluant was 2 m*M* hexanesulphonic acid (free acid supplied by Dionex) in 5% aqueous acetonitrile. Distilled, deionized and membrane (0.22 μm) filtered water was used to prepare the mobile phase. The injection volume was *ca.* 70 μl.

*Preparation of betaine esters*

Aromatic esters of the betaines were prepared essentially as previously described[25]. The betaines were first converted to their potassium salts by passage through a Dowex 50 cation-exchange column in the $K^+$ form. After evaporation to dryness in a 3.5-ml reaction vial under a stream of air, 10 $\mu$l of a solution containing 10 m*M* potassium dihydrogen phosphate, 10 m*M* potassium bicarbonate and 5 m*M* 18-crown-6 in 40% aqueous acetonitrile was added and the vial shaken. A 50-$\mu$l volume of a solution of 20 mg ml$^{-1}$ of phenacyl bromide, *p*-bromophenacyl bromide or *p*-nitrobenzyl bromide in acetonitrile was added and the vial capped. After incubation for 30 min at 80°C the vial was cooled and 250 $\mu$l each of water and chloroform added. The vial was shaken vigorously and the contents transferred to a 1.5-ml microcentrifuge tube for centrifugation at 9000 *g* for 1 min. Aliquots of the aqueous phase (20 or 100 $\mu$l) were injected into the chromatograph.

Methyl, ethyl and isopropyl esters were prepared by incubating dry samples of the betaines in an alcoholic solution of dry hydrochloric acid (5 *M*) in a sealed reaction vial at 70°C for 1 h. The reaction mixture was evaporated to dryness in a stream of nitrogen and dissolved in water prior to ion chromatography.

*Extraction and purification of glycinebetaine and 3-dimethylsulphonio propionate from plant material*

Leaves of the grass *Thinopyrum scirpeum* were frozen in 1.5-ml microcentrifuge tubes and the sap extracted as previously described[26]. A 200 $\mu$l volume of sap was mixed with 200 $\mu$l of isopropanol in a microcentrifuge tube which was shaken vigourously and 600 $\mu$l of water was added. A volume of 300 $\mu$l of the centrifuged (9000 *g* for 1 min), isopropanol-treated extract was applied to a small column (1.5 ml) of a mixture of one part Amberlite CG-50 ($H^+$ form) and two parts Dowex 1 ($OH^-$ form). The extract was washed onto a 1-ml column of Dowex 50 ($H^+$ form) with 2 ml of water and eluted from the Dowex 50 column with 5 ml of 2 *M* ammonium hydroxide. The ammonium hydroxide was evaporated to dryness under nitrogen and the residue redissolved in methanol, aliquots of which were used for the determination of glycinebetaine by three different methods (see below).

Leaves of *Melanthera (Wedelia) biflora* (9.8 g) were homogenised in hot isopropanol using a Polytron homogeniser. The isopropanol extract was filtered and reduced to dryness *in vacuo*. The residue was partitioned between chloroform and water and an aliquot of the aqueous phase was applied to a 5-ml mixed bed column of Amberlite CG-50 ($H^+$ form) and Dowex 1 ($OH^-$ form). The eluate from this column was applied to a 1.5-ml Dowex 50 ($H^+$ form) column and the 3-dimethylsulphoniopropionate was eluted with 2 *M* ammonium hydroxide as above.

## RESULTS AND DISCUSSION

The *p*-bromophenacyl esters of a number of betaines were separated on the bonded carboxylic acid column eluted with 80 m*M* choline phosphate (pH 5) in 10% acetonitrile. Choline has previously been found to be a useful counterion for the elution of other quaternary ammonium compounds in cation-exchange HPLC[27]. The effect of pH on the capacity factors for a number of betaine ester in this system is shown in Fig. 1 (for details of structures and symbols used in the figures, see Table

TABLE I

STRUCTURES OF THE BETAINE ESTERS AND THE RETENTION TIMES OF METHYL ESTERS IN THE ION CHROMATOGRAPH ELUTED WITH 5% ACETONITRILE

HSA = Hexanesulphonic acid; ND = not determined.

| *Name of betaine* | *Trivial name* | *Structure of ester* | *Retention time (min)* | |
|---|---|---|---|---|
| | | | *2 mM HSA* | *4 mM HSA* |
| Dimethylaminoacetic acid | Dimethylglycine | $(CH_3)N^+CH_2COOR$ | 2.1 | 2.2 |
| Trimethylaminoacetic acid | Glycinebetaine | $(CH_3)N^+CH_2COOR$ | | |
| | | R = $CH_3$ | 6.4 | 10.2 |
| | | R = $CH_3CH_2$ | 11.4 | 20.1 |
| | | R = $CH(CH_3)_2$ | 21.6 | >30 |
| Triethylaminoacetic acid | | $(CH_3CH_2)_3N^+CH_2COOR$ | 9.1 | 13.8 |
| 2-Trimethylaminopropionic acid | α-Alaninebetaine | $(CH_3)_3N^+CH(CH_3)COOR$ | 4.7 | 5.6 |
| 3-Trimethylaminopropionic acid | β-Alaninebetaine | $(CH_3)_3N^+(CH_2)_2COOR$ | 8.0 | 11.5 |
| 2-Triethylaminopropionic acid | | $(CH_3CH_2)_3N^+CH(CH_3)COOR$ | 8.4 | 13.6 |
| 4-Trimethylaminobutyric acid | γ-Aminobutyric acid betaine | $(CH_3)_3N^+(CH_2)_3COOR$ | 12.3 | 20.8 |
| 3-Hydroxy-4-trimethylaminobutyric acid | Carnitine | $(CH_3)_3N^+CH_2CHOHCH_2COOR$ | 7.8 | 11.9 |
| 5-Trimethylaminovaleric acid | δ-Aminovaleric acid betaine | $(CH_3)_3N^+(CH_2)_4COOR$ | 6.7* | ND |
| 2-Dimethylsulphonic acetic acid | | $(CH_3)_2S^+CH_2COOR$ | 6.7 | 8.9 |
| 3-Dimethylsulphonicpropionic acid | Dimethylpropiothetin | $(CH_3)_2S^+(CH_2)_2COOR$ | 8.1 | 12.0 |
| 3-Carboxylato-N,N-dimethylpyridinium | Trigonelline (nicotinic acid betaine) | $N^+$ COOR | 9.7 | 15.3 |
| 2-Carboxylato-N,N-dimethylpyridinium | Homarine (isonicotinic acid betaine) | $N^+$ COOR | 9.5 | 16.2 |
| 2-Carboxylato-N,N-dimethylpyrrolidinium | Stachydrine (prolinebetaine) | $N^+$ COOR | 7.8 | ND |
| 3-Carboxylato-N,N-dimethylpyrrolidinium | β-Stachydrine | $N^+$ COOR | 8.2 | ND |

* 10% acetonitrile.

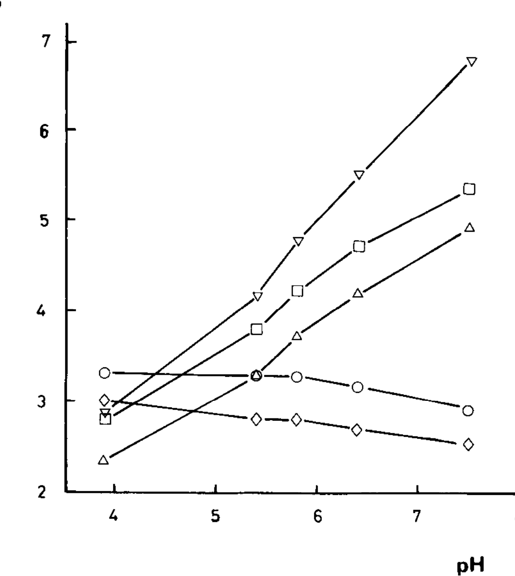


Fig. 1. The effect of pH on the capacity factors ($k'$) for $p$-bromophenacyl esters of betaines on a 5-$\mu$m bonded aliphatic carboxylic acid column eluted with 10% acetonitrile containing 100 m$M$ choline phosphate. ▽ = Trigonelline; □ = $\gamma$-aminobutyric acid betaine; △ = glycinebetaine; ○ = $\beta$-alaninebetaine; ◇ = homarine.

I). For most betaine esters the capacity factors were increased at higher pH values, but this was not the case for the $p$-bromophenacyl esters of homarine and $\beta$-alaninebetaine. Increasing the acetonitrile concentration resulted in earlier elution, as can be seen from the reduction in capacity factors for the $p$-bromophenacyl ester in Fig. 2a. The phenacyl esters of glycinebetaine and trigonelline eluted earlier than the $p$-bromophenacyl esters, and the $p$-nitrobenzyl esters eluted earlier still (Fig. 2b). These esters offered no particular advantage over the $p$-bromophenacyl esters, which had higher extinction coefficients. The 5-$\mu$m bonded carboxylic acid column was considerably more efficient than the Partisil 10-SCX column, but operated at considerably higher pump pressures (> 150 bar compared to 70 bar).

The effect of pH on capacity factors for $p$-bromophenacyl esters on the Partisil 10-SCX column can be seen in Fig. 3a. In this case the mobile phase contained 22 m$M$ choline phosphate in 25% acetonitrile. There was little change in the capacity factors below pH 5.5 for the $p$-bromophenacyl (Fig. 3a), phenacyl and $p$-nitrobenzyl (Fig. 3b) esters. Above pH 5.5 the capacity factor increased at higher pH values. Increasing the acetonitrile concentration considerably reduced the capacity factors for all esters eluted with 22 m$M$ choline phosphate at pH 5.4 (Fig. 4). At the highest acetonitrile concentration (40%) the order of elution of some of the esters was changed. The Partisil 10-SCX column did not provide sufficient resolution to completely separate some of the esters of the common betaines.

Unlike choline and similar quaternary ammonium compounds, most betaines cannot be detected with a conductivity detector. Formation of an ester, however, removed the acidic group and allowed the betaines to be detected. Separation of the

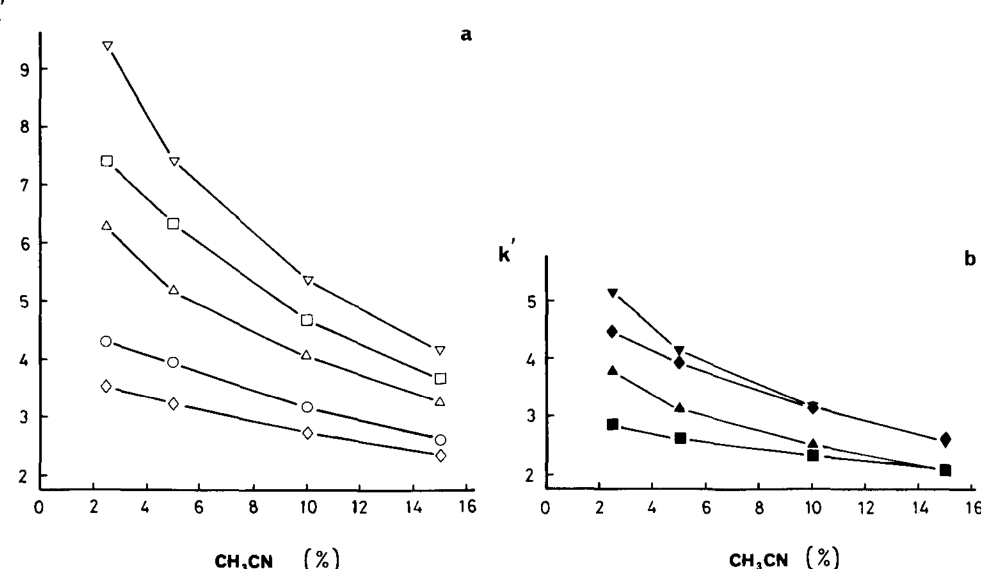


Fig. 2. (a) The effect of acetonitrile concentration on the capacity factors ($k'$) for $p$-bromophenacyl esters of betaines on a 5-$\mu$m bonded aliphatic carboxylic acid column eluted with 80 m$M$ choline phosphate (pH 6.2) at 1.5 ml min$^{-1}$. Symbols as in Fig. 1. (b) The effect of acetonitrile concentration on the capacity factors ($k'$ for phenacyl and $p$-nitrobenzyl esters of betaines on a 5-$\mu$m bonded aliphatic carboxylic acid column eluted with 80 m$M$ choline phosphate (pH 6.2) at 1.5 ml min$^{-1}$. ▲ = Glycinebetaine phenacyl ester; ■ = glycinebetaine $p$-nitrobenzyl ester; ▼ = trigonelline phenacyl ester; ◆ = trigonelline $p$-nitrobenzyl ester.

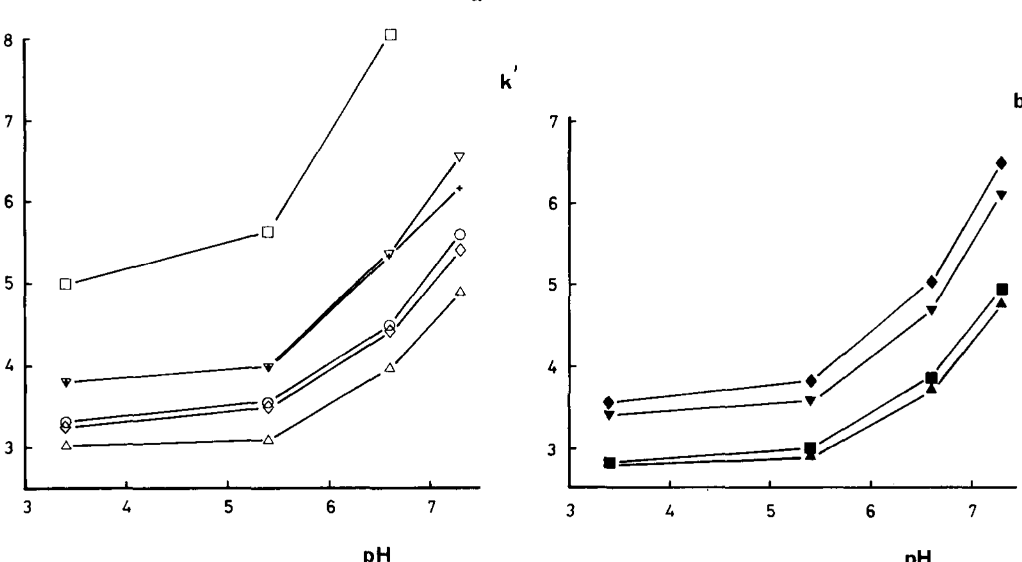


Fig. 3. (a) The effect of pH on the capacity factors ($k'$) for $p$-bromophenacyl esters of betaines on a Partisil 10-SCX column eluted with 25 m$M$ choline phosphate in 25% acetonitrile. + = Triethylaminoacetic acid; □ = $\gamma$-aminobutyric acid, other symbols as in Fig. 1. (b) The effect of pH on the capacity factors for phenacyl and $p$-nitrobenzyl esters of betaines on Partisil 10-SCX. Conditions as (a). Symbols as in Fig. 2b.

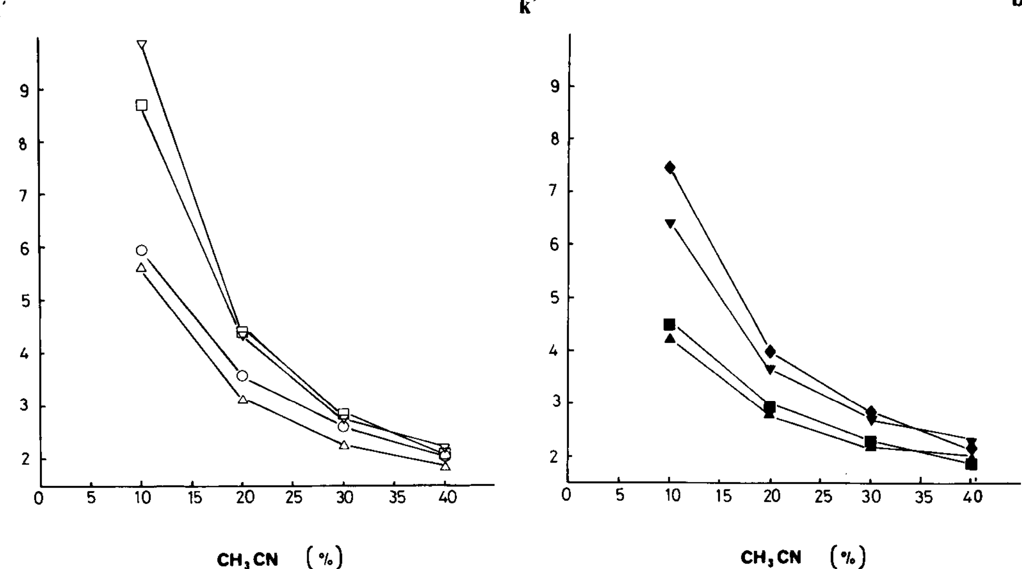


Fig. 4. (a) The effect of acetonitrile concentration on the capacity factors ($k'$ for *p*-bromophenacyl esters of betaines on Partisil 10-SCX eluted with 22 m*M* choline phosphate (pH 5.4). Symbols as in Fig. 1. (b) The effect of acetonitrile concentration on the capacity factors ($k'$) for phenacyl and *p*-nitrobenzyl esters of betaines. Symbols as in Fig. 2b.

esters was achieved in an ion-pairing system on a neutral Dionex column, and the background conductivity caused by the ion-pairing reagent (hexanesulphonic acid) was reduced in a cation fibre suppressor. It is necessary to use high-purity hexanesulphonic acid as the free acid in this system to avoid excessive background conductivities. Retention times for a number of betaine esters on this system are given in Table I. Also included are data for methyl esters of two related tertiary sulphonium compounds, 3-dimethylsulphoniopropionate and 2-dimethylsulphonioacetate. Increasing the concentration of hexanesulphonic acid increased the retention times, as can be seen from Table I. Increasing the size of the ester of glycinebetaine also increased retention time (Table I). Retention times were reduced with increasing acetonitrile concentrations, and the aromatic esters could be eluted with 30–40% acetonitrile. However, this system offered no improvement in resolution of the UV-absorbing esters compared with that obtained with the bonded carboxylic acid column. Fig. 5a illustrates the separation of the methyl esters of four betaines in this system, while Fig. 5b shows the analysis of 3-dimethylsulphoniopropionate in an extract of *Melanthera biflora*. Attempts to produce UV-absorbing esters of 3-dimethylsulphoniopropionate were unsuccessful.

The usefulness of these two methods for estimating betaines in plant tissues was tested with an extract of *Thinopyrum scirpeum*. The extract was purified by passage through three ion-exchange resins, and portions of the purified extract were esterified with either *p*-bromophenacyl bromide or methanolic hydrochloric acid. A further portion of the purified extract was dissolved in water and analysed directly by cation-exchange HPLC with UV detection at 200 nm[23] (Fig. 6a). Standard curves

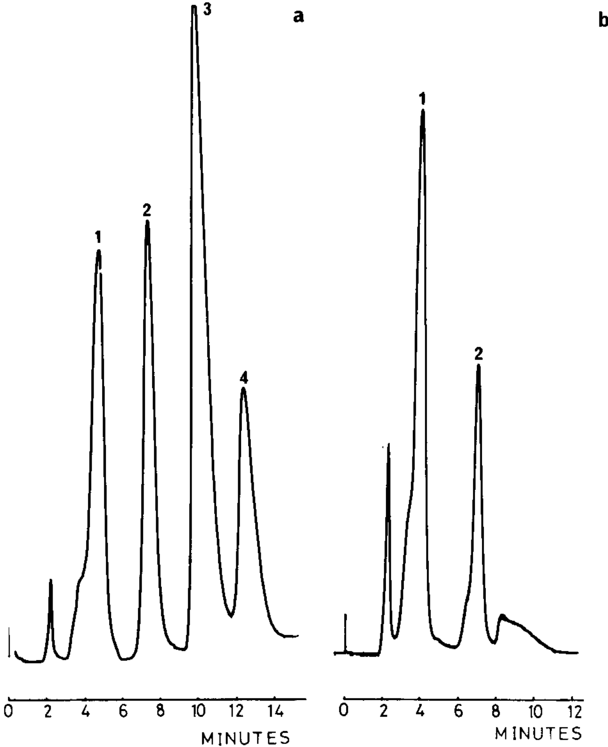


Fig. 5. (a) Separation of methyl esters of betaines by ion-pair chromatography. Dionex MPIC-NS1 column eluted with 2 m*M* hexanesulphonic acid in 5% acetonitrile at 1 ml $min^{-1}$. 1 = α-Alaninebetaine; 2 = glycinebetaine; 3 = trigonelline; 4 = γ-aminobutyric acid betaine. (b) Analysis of a methylated extract of *Melanthera biflora* by ion-pair chromatography. Conditions as in (a). 1 = Inorganic cations; 2 = 3-dimethylsulphoniopropionate methyl ester.

were produced for the analysis of the *p*-bromophenacyl esters of glycinebetaine on Partisil 10-SCX and for the analysis of glycinebetaine methyl ester on the Dionex ion chromatograph. Straight line calibrations were obtained with the former method for 1–25 μg of glycinebetaine (regression coefficient, $r^2 = 0.98$) and with the ion chromatograph 10–150 μg of glycinebetaine ($r^2 = 0.99$). These ranges do not represent the detection limits of the two systems, which were about 10 ng for the *p*-bromophenacyl esters and < 50 ng for the methyl ester of glycinebetaine. Fig. 6b shows the analysis of glycinebetaine in *T. scirpeum* sap as the *p*-bromophenacyl ester and Fig. 6c shows the analysis of the methyl ester. Quantitative results from these determinations are presented in Table II. Replication within each particular method was good, but there were differences between the different methods. The values obtained by direct, low-wavelength UV detection are the most unreliable because of the difficulty of interpreting the baseline in these chromatograms.

The estimation of betaines as either the methyl or *p*-bromophenacyl esters has three main advantages over direct separation and low-wavelength UV detection. The first is the greater sensitivity of the ester methods, by about an order of magnitude. This combines with the second advantage, that of reduced background signal, to

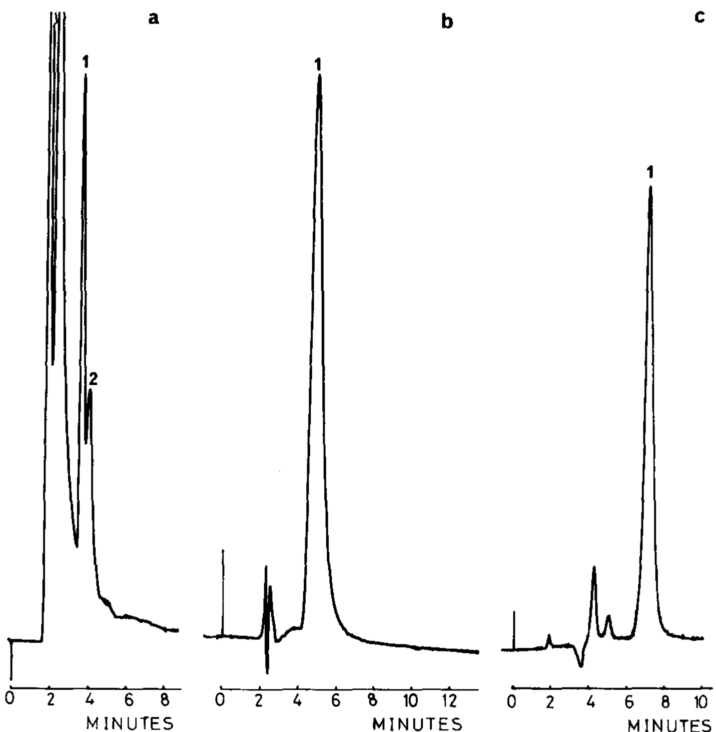


Fig. 6. Analysis of glycinebetaine in an extract of *Thinopyrum scirpeum*. (a) Direct cation-exchange and low-wavelength UV detection, full scale deflection (F.S.D.) = 0.1 A. (b) Separation of the *p*-bromophenacyl ester on Partisil 10-SCX, F.S.D. = 0.5 A. (c) Separation of the methyl ester by ion-pair chromatography on a Dionex MPIC-NS1 column, F.S.D. = 30 $\mu$S. 1 = Glycinebetaine; 2 = trigonelline.

enable the ester methods to be used to detect smaller concentrations of betaines in plant tissues with greater reliability. The third advantage is that the molar response factors for different betaines are very similar. This is not the case with the low-wavelength UV method since the nicotinic acid betaines, trigonelline and homarine, have considerable higher extinction coefficients than the aliphatic betaines[23]. Most plant tissue contains a small amount of trigonelline, and this interferes to some extent with

TABLE II

QUANTITATIVE ANALYSIS OF THE GLYCINEBETAINE CONTENT OF *THINOPYRUM SCIRPEUM* LEAVES

| *Sample* | *Glycinebetaine (mM in leaf sap)** | | |
|---|---|---|---|
| | *Direct UV* | *Methyl ester* | *p-Bromophenacyl ester* |
| 1 | 49.55 ± 0.17 | 52.70 ± 0.81 | 50.55 ± 0.61 |
| 2 | 45.96 ± 0.09 | 51.77 ± 0.47 | 49.43 ± 1.41 |
| 3 | 35.61 ± 0.28 | 48.90 ± 0.50 | 44.70 ± 0.30 |

* Mean of four replicates ± standard errors.

the determination of glycinebetaine contents by the low-wavelength UV method (see Fig. 6a). In contrast, this small amount of trigonelline was not detectable by the ester methods (Fig. 6b and c).

## CONCLUSIONS

The methods described above form the basis of assays of betaines and their tertiary sulphonium analogues as either their methyl or *p*-bromophenacyl esters. In combination with suitable purification techniques, the ester methods can detect low concentrations of these compounds in plant tissues.

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