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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SEPARATION AND ESTIMATION OF CHOLINE, GLYCINEBETAINE ALDEHYDE AND RELATED COMPOUNDS

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SUMMARY

Procedures are described for the separation and quantitative estimation of choline and glycinebetaine aldehyde in plant saps and extracts. Glycinebetaine aldehyde in the plant material, and a suitable internal standard, were converted to their *p*-nitrobenzyl oximes during extraction. The extract was purified on Sep-Pak C_{18} cartridges and on ion-exchange columns, after which the choline fraction was benzoylated to yield UV-absorbing derivatives. High-performance liquid chromatography was performed on an aliphatic carboxylic acid or an aromatic sulphonic acid cation-exchange column eluted with the phosphate salt of choline in aqueous acetonitrile. The detection limit for both compounds was less than 1 ng. This procedure may also be applied to the analysis of choline in hydrolysates of phospholipids.

INTRODUCTION

Choline is a major constituent of the polar lipid fraction of biological membranes and free choline is frequently present in plants and animals. The commonly used assay procedures which rely on the formation of insoluble periodides[1–4] or thin-layer scanning densitometry[5,6] are neither specific nor sensitive. The somewhat more sensitive gas chromatographic methods use either pyrolysis[7] or chemical demethylation[8–12] to generate volatile derivatives. Neither of these methods is entirely satisfactory since pyrolysers are not standard equipment in most laboratories and chemical demethylation with sodium benzenethiolate is technically demanding. Furthermore, both methods may give false results as a result of the breakdown of choline esters such as acetylcholine[13,14].

A number of enzymatic methods have been developed for the estimation of choline in relatively crude extracts using choline kinase[15–17] or choline oxidase[18]. Separation of choline and acetylcholine has been achieved by reversed-phase high-performance liquid chromatography (HPLC) followed by post-column hydrolysis with acetylcholine esterase and oxidation of choline with choline oxidase. The resulting hydrogen peroxide was detected electrochemically[19,20]. A simpler method is

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based on esterification of choline with 3,5-dinitrobenzoyl chloride and separation of the dinitrobenzoate on reversed-phase HPLC columns in the presence of an ion-pairing agent[21]. The present paper describes a more rapid and efficient method involving the formation of the benzoyl ester and its chromatography on cation-exchange materials. This work was stimulated by the need for a reliable method for the isolation and estimation of [^{14}C]choline during the course of radiotracer experiments into the biosynthesis of glycinebetaine in plants. A method was also developed for the separation and estimation of glycinebetaine aldehyde, an intermediate in the oxidation of choline to glycinebetaine, as a UV-absorbing oxime.

EXPERIMENTAL

Chemicals

Choline chloride and glycinebetaine aldehyde were purchased from Sigma (Poole, U.K.) and stored in a desiccator over phosphorus pentoxide. HPLC-grade acetonitrile, hydroxylamine hydrochloride, iodomethane and benzoyl chloride were obtained from BDH (Poole, U.K.), choline–methanol (45:55) and *p*-nitrobenzylhydroxylamine hydrochloride from Fluorochem (Glossop, U.K.), and dimethylaminoethanol, diethylaminoethanol, dimethylaminoacetone, dimethylamino-3-methyl-2-butanone, dimethylaminopropan-1-ol, dimethylaminopropan-2-ol, 4-nitrobenzoyl chloride, 3,5-dinitrobenzoyl chloride, Dowex 1X2-100, Dowex 50X8-100, 2-bromoethanol, dimethylaminoacetaldehyde dimethyl acetal and triethylamine from Aldrich (Gillingham, Dorset, U.K.). [1,2-^{14}C]choline chloride (7.2 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Glycinebetaine aldehyde (trimethylaminoacetaldehyde) was also prepared by the aqueous acidic hydrolysis of trimethylaminoacetaldehyde dimethyl acetal, which was synthesized from dimethylaminoacetaldehyde dimethyl acetal and iodomethane.

Preparation of mobile phase

For use with the carboxylic acid column, choline (9.5 ml of a 45% solution in methanol) was dissolved in 1 l of distilled water and the pH adjusted to the required value (normally 6.0) with orthophosphoric acid. After addition of acetonitrile to 10%, v/v the solution was filtered through 0.22-μm GVHP Durapore filters using all-glass filtration equipment. For elution of the Partisil 10-SCX column the acetonitrile content was increased to 20%.

High-performance liquid chromatography

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. Separations were performed on a 250 × 4.6 mm I.D. stainless-steel column packed with 5-μm spherical silica particles to which an aliphatic carboxylic acid had been bonded (Bakerbond column supplied by Linton products, Harlow, U.K.). A 250 × 5 mm I.D. stainless-steel column packed with Partisil 10-SCX (bonded aromatic sulphonic acid) and fitted with a guard column packed with

pellicular cation exchange material was also employed. Detection was by UV absorbance at 232 nm (benzoates) or 270 nm (*p*-nitrobenzyloximes).

Synthesis of internal standards

N,N,N-Trimethylaminopropan-1-ol, N,N,N-trimethylaminopropan-2-ol, N,N,N-trimethylaminoacetone and N,N,N-trimethylamino-3-methyl-2-butanone were synthesized from the corresponding N,N-dimethylamino compounds using iodomethane in ethanol, essentially as described by Buchanan *et al.*[21]. N,N-Diethyl-N-methylaminoethanol was similarly synthesized from N,N-diethylaminoethanol. N,N,N-Triethylaminoethanol was synthesized by refluxing 2-bromoethanol in a 2 *M* excess of triethylamine for 14 h, evaporating *in vacuo* and recrystallizing from ethanol. (For structure see Fig. 1). The internal standards were usually used as their

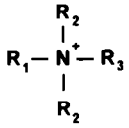

Fig. 1. Structures of the quaternary ammonium compounds used in this investigation. X = H or X = COC_6H_6 for benzoates; Y = O or Y = $NOCH_2C_6H_6NO_2$ for *p*-nitrobenzyloximes.

R_1	R_2	R_3	
CH_3	CH_3	CH_2CH_2X	Choline
H	CH_3	CH_2CH_2OX	Dimethylaminoethanol
CH_3	CH_3	$CH_2CH_2CH_2OX$	Trimethylaminopropan-1-ol
CH_3	CH_3	$CH_2CHOXCH_3$	Trimethylaminopropan-2-ol
CH_3	CH_3CH_2	CH_2CH_2OX	Diethylmethylaminoethanol
CH_3CH_2	CH_3CH_2	CH_2CH_2X	Triethylaminoethanol
CH_3	CH_3	CH_2CHY	Glycinebetaine aldehyde
CH_3	CH_3	CH_2CYCH_3	Trimethylaminoacetone
CH_3	CH_3	$CH_2CH(CH_3)CYCH_3$	Trimethylamino-3-methyl-2-butanone

iodides, recrystallized from ethanol, but chlorides were also produced by passing aqueous solutions of the iodides through columns of Dowex 1 (chloride form), evaporating and recrystallizing from ethanol.

Extraction and purification of choline and glycinebetaine aldehyde p-nitrobenzyl oxime from plant material

Extraction with methanol–chloroform–water has been reported to destroy glycinebetaine aldehyde[22,23], whereas glycinebetaine aldehyde *p*-nitrobenzyl oxime was found in the present investigation to be more stable. In methanolic extracts there is also the possibility of phospholipase D catalyzed transesterification of phospholipids and the release of free choline. Therefore plant material was frozen with liquid air and homogenized with isopropanol containing the internal standards (trimethylaminopropan-1-ol or diethylmethylaminoethanol and trimethylaminoacetone) and *p*-nitrobenzylhydroxylamine hydrochloride (1 mg ml^{-1}). The homogenate, in a screw-capped vial, was then heated rapidly to 100°C in a preheated aluminium heating block and kept at that temperature for 1 h. The extract was filtered and reduced in volume either *in vacuo* or under a stream of nitrogen and the aqueous residue par-

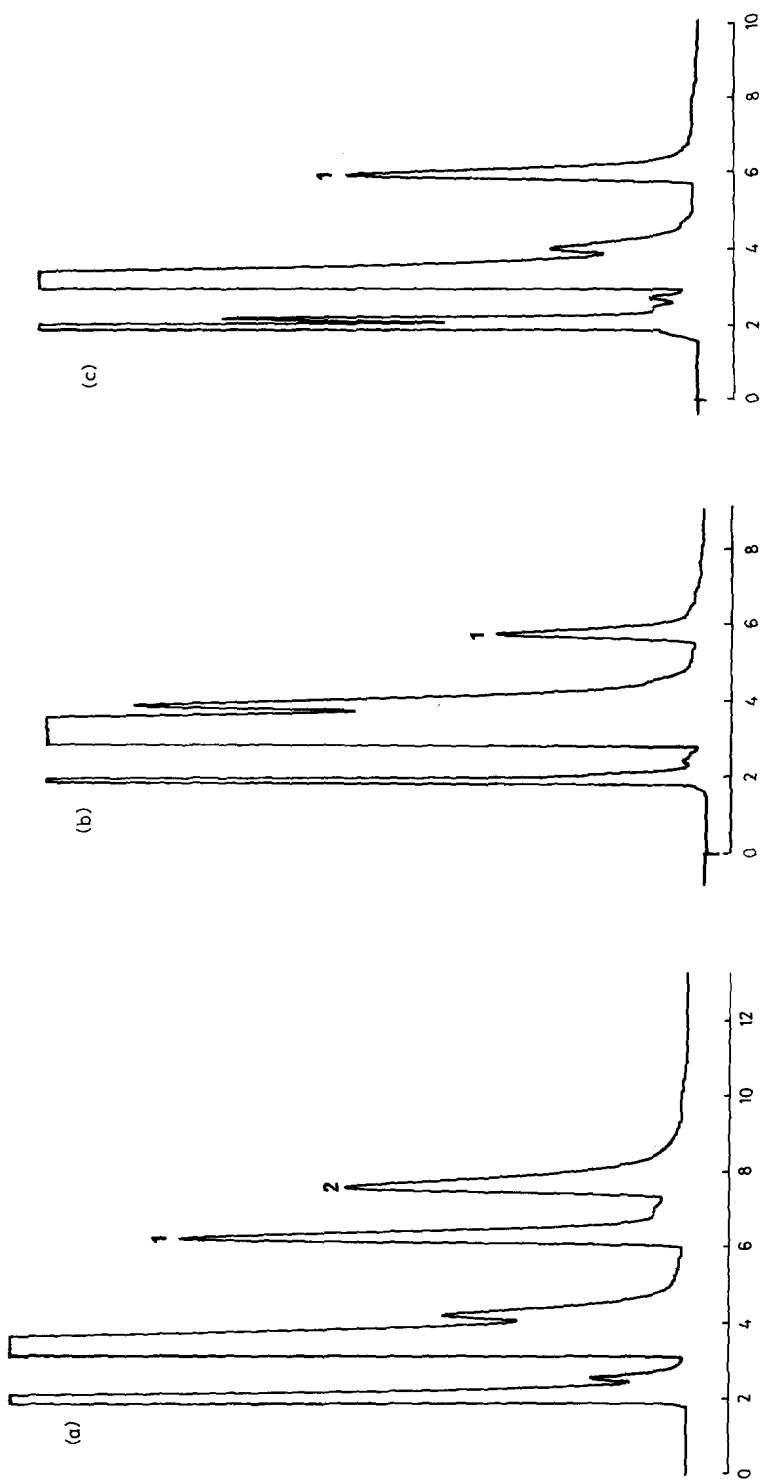

Fig. 2. Separation of benzoyl quaternary ammonium compounds on a 250 × 4.6 mm I.D. 5 μm Bakerbond aliphatic carboxylic acid column eluted with 35 m*M* choline phosphate (pH 6.0) in 10% acetonitrile (flow-rate, 1.5 ml min^{-1}). Detection, UV absorbance at 232 nm. The first two peaks are benzoic acid and pyridine. 1 = Benzoyl choline; 2 = benzoyl trimethylaminopropan-1-ol. (a) Standards (5 nmol each), (b) extract of spinach leaves (without internal standard), (c) hydrolysate of egg lecithin.

titioned against chloroform. The aqueous phase (or plant sap extracted as previously described[24]) was passed through a pre-wetted Sep-Pak C_{18} cartridge (Waters Assoc., Northwich, U.K.) which was washed with a further 1 ml of water. *p*-Nitrobenzyl oximes were eluted from the Sep-Pak cartridge with 2 ml of methanol. After reduction in volume, the oxime fraction was passed through Dowex 1X2-100 (OH^- form) and analysed by HPLC.

The combined aqueous solutions from the Sep-Pak cartridge were then passed through 2 ml of Dowex 1X2-100 (OH^- form) and 1 ml of Dowex 50X8-100 (H^+ form) in series, and washed with 5 ml of water. Polypropylene microcentrifuge tubes (1.5 ml) with a small hole in the bottom and extended by the use of a truncated 5-ml plastic pipette tip were found to be convenient for making such small ion-exchange columns. The Dowex 50 column was eluted with 4 ml of 2 *M* ammonium hydroxide and washed again with 5 ml of water, after which choline and trimethylaminopropan-1-ol were eluted with 5 ml of 2 *M* hydrochloric acid in 50% methanol. After evaporation to dryness *in vacuo* the choline fraction was dissolved in a small amount (100 μl) of methanol, acetonitrile was added (2 ml), the solution centrifuged and the clear supernatant evaporated to dryness under nitrogen as described below.

Benzoylation of hydroxy-quaternary ammonium compounds

The choline fraction from an extract, or a standard solution of the quaternary ammonium compound in methanol, was evaporated to dryness in a 3.5-ml reaction vial at 80°C under nitrogen. To ensure that the sample was dry it was placed under high vacuum over phosphorus pentoxide for 1 h. To the dried sample was added 100 μl of a freshly prepared solution of benzoyl chloride (100 μl ml^{-1}) in pyridine (redistilled and stored over potassium hydroxide). After capping the reaction vial with a PTFE lined closure the mixture was heated to 80°C for 15 min with occasional shaking. Excess pyridine and benzoyl chloride were removed at 80°C under a stream of dry nitrogen and the residue partitioned between chloroform (1 ml) and water (0.5 ml). After thorough mixing the two phases were separated by centrifugation and the aqueous phase analysed by HPLC. If necessary, the aqueous phase could be reduced in volume before HPLC analysis.

Formation of oximes from quaternary ammonium aldehydes

Oximes of glycinebetaine aldehyde, trimethylaminoacetone and trimethylamino-3-methyl-2-butanone were formed by addition of excess hydroxylamine hydrochloride or *p*-nitrobenzylhydroxylamine hydrochloride in methanol (5 mg/ml) and heating for 15 min at 80°C. The reaction mixture was used directly for HPLC (*p*-nitrobenzyloximes) or evaporated to dryness under nitrogen and benzoylated (oximes).

Hydrolysis of lecithin

Egg lecithin (2 mg) was dissolved in a small amount of ethanol and hydrolysed with 2 *M* sulphuric acid (1 ml) at 100°C under nitrogen for 3 h. After dilution and partition against chloroform the hydrolysate was purified by ion-exchange chromatography as described above.

RESULTS AND DISCUSSION

Fig. 2a shows the separation of standard solutions of benzoyl choline from benzoyl trimethylaminopropan-1-ol on the aliphatic carboxylic acid column. The separations were influenced by both pH (Fig. 3a) and by acetonitrile concentration (Fig. 3b). Fig. 2b shows an example of the analysis of free choline in spinach, while

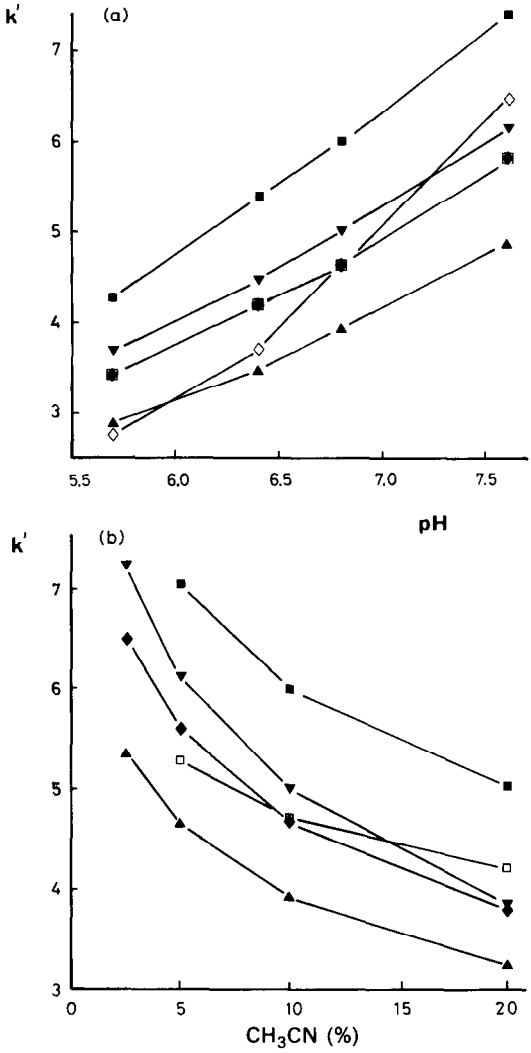

Fig. 3. (a) Effect of pH on the capacity factors (k') of benzoyl quaternary ammonium compounds on a 5-μm aliphatic carboxylic acid column eluted with 35 mM choline phosphate in 10% acetonitrile at 1.5 ml min^{-1}. Key to symbols: ▲ = benzoyl choline; ◇ = benzoyl dimethylaminoethanol; ▼ = benzoyl trimethylaminopropan-1-ol; ◆ = benzoyl trimethylaminopropan-2-ol; □ = glycinebetaine aldehyde p-nitrobenzyloxime; ■ = trimethylaminoacetone p-nitrobenzyloxime. (b) Effect of increasing acetonitrile concentration on the capacity factors (k') of benzoyl quaternary ammonium compounds. Conditions and symbols as in a, pH 6.8. Benzoyl dimethylaminoethanol and benzoyl trimethylaminopropan-2-ol co-eluted in this experiment.

the detection of choline released from phosphatidylcholine (lecithin) is shown in Fig. 2c. Peak area response was linear in the range 0.1–15 nmol per injection at least (regression coefficient, $r^2 = 0.996$), as was the ratio of sample peak area to internal standard peak area for both choline and glycinebetaine aldehyde. Free choline levels in seedlings of *Phaseolus aureus* were found to be 221.9 ± 0.6 nmol (g fresh weight)$^{-1}$ (mean ± standard deviation, $n = 4$) for dark-grown seedlings and 241.6 ± 0.7 nmol (g fresh weight)$^{-1}$ for seedlings grown in the light. These values are somewhat lower than those given by Miura and Shih[12], but were consistent with values obtained by ion chromatography for these tissues[25]. The discrepancies may reflect varietal differences in the plant material, but differences in extraction technique cannot be discounted. Rather lower amounts of free choline were found in mature leaves of *Thinopyrum bessarabicum* [46.4 ± 0.3 nmol (g fresh weight)$^{-1}$]. The level of free glycinebetaine aldehyde was also estimated in this material, and found to be 77 ± 15 pmol (g fresh weight)$^{-1}$. This is consistent with previous reports of low levels of free glycinebetaine aldehyde in plants[22,23].

The benzoates were also separated on a Partisil 10-SCX column, which had the advantage of operating at a much lower backpressure (about 70 bar compared to 150 bar for the 5-μm carboxylic acid column). Retention was again affected by both pH (Fig. 4a) and acetonitrile concentration (Fig. 4b). None of the conditions examined here successfully separated benzoyl choline from benzoyl trimethylaminopropan-1-ol or -2-ol on Partisil 10SCX. With both columns, increasing the proportion of acetonitrile above 40% did not further decrease retention times. The effect of choline phosphate concentration in the eluent on capacity factors is shown in Fig. 5. Increasing choline concentrations up to 15 m*M* greatly reduced retention times, whereas increases above 15 m*M* had little effect. The effect of choline on retention times was independent of the salt used; the chloride or sulphate could substitute for the phosphate.

Benzoylation is much more rapid (< 15 min) than the dinitrobenzoylation reaction used by Buchanan *et al.*[21], and can be carried out under milder conditions. The higher extinction coefficient of the dinitrobenzoate may, however, be an advantage for trace analysis of choline. Problems of solubility of quaternary ammonium compounds limited the reaction rate with 3,5-dinitrobenzoyl chloride and 4-nitrobenzoyl chloride.

Choline cannot be eluted from cation-exchange columns with NH_4^+ at high pH. It is, however, eluted with NH_4^+ at lower pH values, and with hydrochloric acid. The cation-exchange column may thus be washed with ammonium hydroxide after loading the sample, and the choline eluted with hydrochloric acid or sodium chloride[14]. Most of the salts in such eluates may be removed by first extracting the choline from the dry residue with a small amount of methanol and precipitating the dissolved salts with acetonitrile. Recovery of choline in this system was checked using [1,2-^{14}C]choline chloride and was found to be more than 95%.

Partitioning the products of benzoylation between water and chloroform removes both excess benzoic acid and also some of the products of reactions between pyridine and impurities in the sample, resulting in better separation of benzoyl choline from benzoic acid and pyridine in the HPLC. No reaction was observed between the reagents and choline in the eluting buffer.

Four compounds, N,N,N-trimethylaminopropan-1-ol, N,N,N-trimethylami-

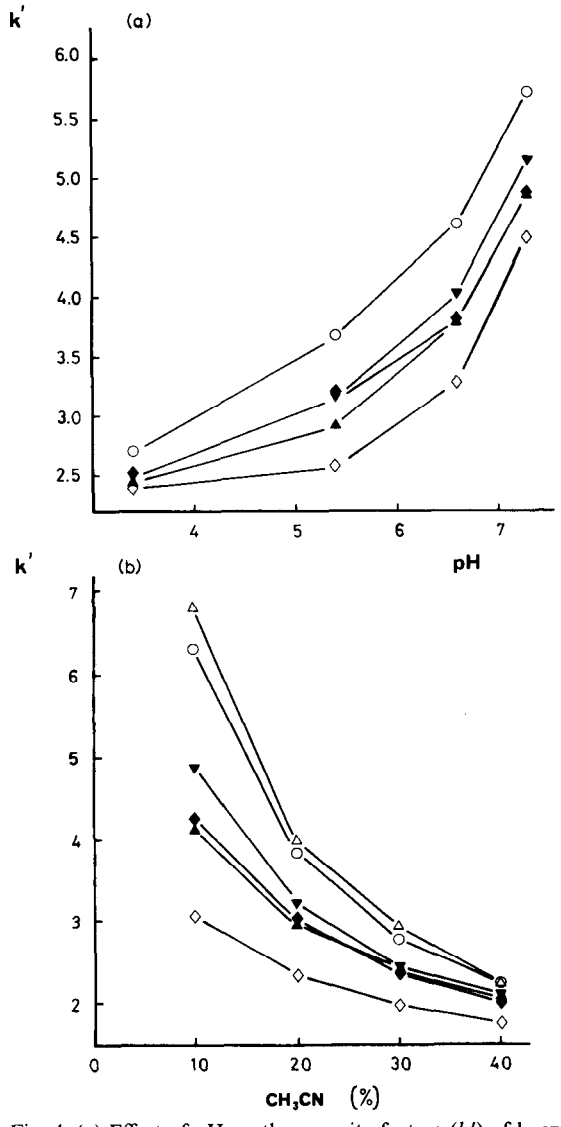

Fig. 4. (a) Effect of pH on the capacity factors (k') of benzoyl quaternary ammonium compounds on a 250 × 5 mm I.D. Partisil 10-SCX column (plus guard column) eluted with 22.5 mM choline phosphate in 25% acetonitrile at 2 ml min^{-1}. Symbols as in Fig. 3. ○ = Benzoyl diethylmethylaminoethanol. (b) Effect of increasing acetonitrile concentration on the capacity factors (k') of benzoyl quaternary ammonium compounds on Partisil 10-SCX eluted with 22.5 mM choline phosphate, pH 5.4, at 2 ml min^{-1}. Symbols as in Fig. 3. ○ = Benzoyl diethylmethylaminoethanol; △ = benzoyl triethylaminoethanol.

nopropan-2-ol, N,N-diethyl-N-methylaminoethanol and triethylaminoethanol were examined as possible internal standards for choline. Benzoyl trimethylaminopropan-2-ol eluted slightly before benzoyl trimethylaminopropan-1-ol and was not completely resolved from benzoyl choline on the aliphatic carboxylic acid column. These three compounds were not fully resolved on the Partisil 10-SCX column. Benzoyl

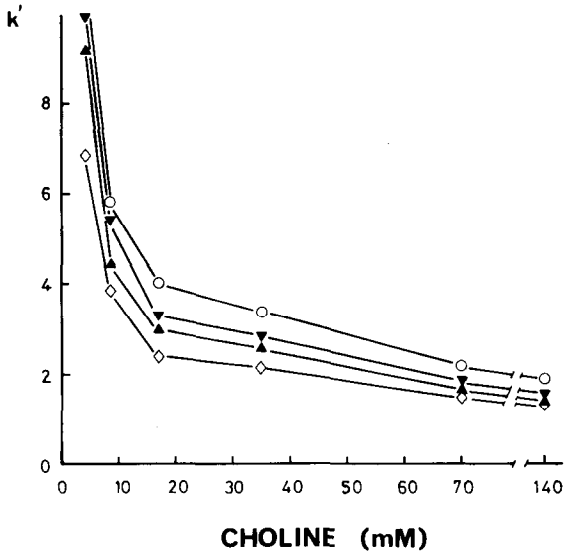

Fig. 5. Effect of increasing concentrations of choline phosphate (pH 6.2) on the capacity factors (k') of benzoyl quaternary ammonium compounds on Partisil 10-SCX eluted with 20% acetonitrile at 1.5 ml min^{-1}. (Symbols as in Figs. 3 and 4).

diethylmethylaminoethanol and benzoyl triethylaminoethanol eluted somewhat later than the other compounds, but could be useful if biological samples display peaks near to the retention time of benzoyl trimethylaminopropan-1-ol on the carboxylic acid column. They were also useful as internal standards on the Partisil 10-SCX column. Benzoyl dimethylaminoethanol was not completely separated from benzoyl choline and benzoyl trimethylaminopropan-1-ol in the HPLC systems used here.

Several possibilities were examined for the production of UV-absorbing derivatives of glycinebetaine aldehyde. The hydrazone, 2,4-dinitrophenylhydrazone and dansylhydrazone were all synthesized in acidic methanol and showed similar retention to the *p*-nitrobenzyl oxime on HPLC. The benzoyloxime resulting from benzoylation of glycinebetaine aldehyde oxime had a similar retention time to benzoyl trimethylaminopropan-1-ol. The *p*-nitrobenzyl oxime was chosen because of its ease of formation and greater stability than the other derivatives tested here. It was, however, difficult to elute from the cation-exchange column, but was retained on a Sep-Pak C_{18} cartridge eluted with water. Of the possible internal standards for glycinebetaine aldehyde, N,N,N-trimethylaminoacetone *p*-nitrobenzyloxime had a retention time sufficiently near to, but resolved from, glycinebetaine *p*-nitrobenzyloxime to be the most suitable of the two. N,N,N-trimethylamino-3-methyl-2-butanone *p*-nitrobenzyloxime eluted rather later and the peak shape was not as good as that of the former compound.

Ion exchange clearly accounts for only part of the separation mechanism of quaternary ammonium compounds on ionic bonded HPLC stationary phase. Results similar to those obtained on the weak cation-exchange aliphatic carboxylic acid column could also be achieved on the strong cation exchanger Partisil 10-SCX at only slightly higher concentrations of choline phosphate in the eluant, although the effi-

ciency was less than with the former column. A cation-exchange contribution from residual silanol groups on the silica may also contribute to the separation[26]. In the absence of an organic solvent retention times were considerably increased and peak shape was poor even when the retention time was reduced by increasing the concentration of choline phosphate. The presence of choline was also necessary to obtain reasonably short retention times, although above 15 m*M* choline no further reduction was obtained.

Choline has a number of advantages as a counter-ion for quaternary ammonium and other compounds in cation-exchange HPLC. It is non-UV-absorbing even at low wavelengths, is completely soluble in methanol and high concentrations of acetonitrile, and is a stronger counter-ion than monovalent inorganic cations. It has previously been used to elute [^{14}C]choline from small columns of Dowex 50 (ref. 27).

In comparison with the method of Buchanan *et al.*[21], the present derivatisation technique has the advantage of being more rapid and proceeding under milder conditions. The higher extinction coefficient of the dinitrobenzoate should, however, reduce the detection limits for trace analysis. Ion-exchange chromatography also allows more rapid separations to be carried out using cheaper eluants than are required for ion-pair HPLC.

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REFERENCES

1 D. Speed and M. Richardson, *J. Chromatogr.*, 35 (1968) 497.
2 J. S. Wall, D. D. Christianson, R. J. Dimler and F. R. Senti, *Anal. Chem.*, 32 (1960) 870.
3 R. Storey and R. G. Wyn Jones, *Phytochemistry*, 16 (1977) 447.
4 C. M. Grieve and S. R. Grattan, *Plant Soil*, 70 (1983) 303.
5 C. Radecka, K. Genest and D. W. Hughes, *Arzneim-Forsch.*, 21 (1971) 548.
6 J. Gorham, S. J. Coughlan, R. Storey and R. G. Wyn Jones, *J. Chromatogr.*, 210 (1981) 550.
7 P. I. A. Szilagyi, D. E. Schmidt and J. P. Green, *Anal. Chem.*, 40 (1968) 2009.
8 D. J. Jenden, I. Hanin and S. I. Lamb, *Anal. Chem.*, 40 (1968) 125.
9 N. R. Zahniser, D. Chou and I. Hanin, *J. Pharmacol. Exp. Ther.*, 200 (1977) 545.
10 D. J. Jenden, M. Roch and F. Fainman, *Life Sci.*, 23 (1978) 291.
11 I. Hanin and J. Scherberth, *J. Neurochem.*, 23 (1974) 819.
12 G. A. Miura and T.-M. Shih, *Physiol. Plant.*, 61 (1984) 417.
13 J. C. Sheenan and G. D. Davis, Jr., *J. Org. Chem.*, 29 (1964) 2006.
14 G. H. Posner and J.-S. Ting, *Synth. Commun.*, 4 (1974) 355.
15 M. Acara, B. Rennick, S. Lagraff and E. T. Schroeder, *Nephron*, 35 (1983) 241.
16 G. Von Puchwein and E. Mayr, *Landwirtsch. Forsch.*, 37 (1984) 53.
17 M. Gilberstadt and J. A. Russell, *Anal. Biochem.*, 138 (1984) 78.
18 M. K. Hise and C. M. Mansbach, *Anal. Biochem.*, 135 (1984) 78.
19 P. E. Potter, J. L. Meek and N. H. Neff, *J. Neurochem.*, 41 (1983) 188.
20 Y. Ikarashi, T. Sasahara and Y. Maruyama, *Folia Pharmacol. Jpn.*, 84 (1984) 529.
21 D. N. Buchanan, F. R. Fucek and E. F. Domino, *J. Chromatogr.*, 181 (1980) 329.
22 A. D. Hanson and N. A. Scott, *Plant Physiol.*, 66 (1980) 342.
23 S. J. Coughlan and R. G. Wyn Jones, *Planta*, 154 (1982) 6.
24 J. Gorham, *J. Chromatogr.*, 287 (1984) 345.
25 J. Gorham, in preparation.
26 D. L. Reynolds, A. J. Repta and L. A. Sternson, *J. Pharm. Biomed. Anal.*, 1 (1983) 339.
27 G. Mazzola and C. Kent, *Anal. Biochem.*, 141 (1984) 137.