Journal of Chromatography, 362 (1986) 243–253
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 681

REVERSED-PHASE, ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF AMINOETHANOL, CHOLINE AND RELATED COMPOUNDS, AND A DIRECT, QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR CHOLINE IN PLANTS

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(Received March 22nd, 1986)

SUMMARY

The separation of a wide range of aminoethanol and choline derivatives by ion-pair reversed-phase high-performance liquid chromatography on a styrene–divinylbenzene co-polymer stationary phase is described. Suppression of the conductivity of the eluent and electrical conductivity detection of the remaining cation provides a method for analyzing low concentrations of such compounds. Reineckate precipitation in conjunction with this method forms the basis of a rapid method for the quantitative analysis of choline in plant extracts. The method should also be useful for monitoring the concentrations of choline esters in pharmaceutical preparations, and for the purification of acetylcholine prior to analysis by more sensitive procedures.

INTRODUCTION

Various derivatives of aminoethanol and choline (trimethylaminoethanol) are of great importance in plant and animal biology. Phosphatidylaminoethanol (and its mono- and di-methyl derivatives) and phosphatidylcholine are important constituents of the phospholipid fractions of biological membranes, and choline esters are major neurotransmitters in animals. Several choline esters which are analogues of acetylcholine are of pharmaceutical interest. Aminoethanol and choline are precursors of the plant stress metabolite, glycinebetaine[1], and acetylcholine is also reported to occur in plants[2–5]. Chlorocholine chloride (CCC) is a synthetic plant growth retarder which has been reported to enhance salt tolerance[6,7].

Methods for the analysis of choline have been based on relatively non-specific precipitation of the periodide at high pH[8–11], scanning densitometry of thin-layer separations[12,13] or enzymatic reactions[14–16]. Gas–liquid chromatography (GLC) is not suitable for such highly polar compounds, but a number of methods have been developed for the analysis of choline and its esters which involve either chemical

0021-9673/86/$03.50

demethylation with benzenethiolate under strictly anhydrous conditions[17–27] or pyrolytic demethylation[28–32]. An alternative procedure for choline esters used potassium borohydride to hydrolyse the ester and reduce the liberated acid to an alcohol[33]. The resulting alcohols were separated by GLC on Carbowax 6000. A recent paper describes the use of a splitless capillary GC injector for the aminoethanol-catalysed demethylation of choline esters[34] and the separation of the products on bonded-phase fused-silica capillary columns. Direct high-performance liquid chromatographic (HPLC) separation of choline and acetylcholine have been used in conjunction with post-column enzymatic hydrolysis of the acetylcholine and oxidation of choline followed by electrochemical detection of the resulting hydrogen peroxide[35,36]. A more conventional approach to the chromatography of choline is the formation of UV-absorbing esters and their separation by ion-pair reversed-phase or cation-exchange HPLC[37,38].

The development of the method described in the present paper was stimulated by the need to separate metabolites of aminoethanol and choline in the course of radio-tracer experiments on the biosynthesis of glycinebetaine in plants. In particular, it was desirable to develop methods for the separation of the phosphorylated intermediates in the pathway between aminoethanol and glycinebetaine because of the number of different possible pathways between these two compounds[1,39]. Of the phosphorylated intermediates, phosphorylcholine presented the greatest problems because it cannot be analysed together with the other aminoethanols by derivatization of the amino group[40]. Another consideration was that the method should involve as few preliminary clean-up steps as possible, and preferably not require derivatization. Since few of the compounds in question possess any significant absorbance in the UV region, detection would be by refractive index or conductivity. The method described below uses ion-pair reversed-phase HPLC in conjunction with post-column supression of the conductivity of the eluent and detection of the cations in an electrical conductivity detector.

EXPERIMENTAL

Chemicals

The normal ion-pair reagent, hexanesulphonic acid, was purchased as a 100 mol m^{-3} solution from Dionex U.K. Methanesulphonic acid was purchased from Aldrich and a 100 mol m^{-3} stock solution was prepared with deionized water. This solution was treated with Dowex 50 (H^+ form) to reduce background conductivity. Glycinebetaine aldehyde (trimethylaminoacetaldehyde), trimethylaminoacetone, trimethylamino-3-methyl-2-butanone, trimethylaminopropan-1-ol, trimethylaminopropan-2-ol, triethylaminoethanol, diethylmethylaminoethanol and trimethylaminoacetaldehyde dimethyl acetal were synthesized as previously described[38]. Sulphocholine was produced by the action of concentrated sulphuric acid on choline[41].

High-performance liquid chromatography

HPLC was performed on a 200 × 4 mm I.D. Dionex MPIC-NS1 column with a Dionex MPIC-NG1 guard column, mounted in a Dionex 2010i ion chromatograph and connected to a cation fibre suppressor which was regenerated with 40 mol m^{-3} barium hydroxide. The conductivity detector was routinely operated at 30 μS full

scale deflection and the background conductivity was 5–9 μS. For the detection of glycinebetaine and trigonelline a Cecil CE 212A variable-wavelength monitor was connected to the end of the MPIC-NS1 column and operated at 200 nm. For gradient elution and method development a Spectra-Physics SP 8700 solvent delivery system replaced the Dionex pump. Injections were made either automatically with a 50-μl loop in a Dionex injection valve loaded and activated by a Pye 4700 autoinjector or manually through a 20-μl loop mounted in the Rheodyne injection valve of the SP 8700. Peak heights were determined with a Pye DP 88 integrator.

Extraction and purification of choline from plant material

Either plant material was extracted with propan-2-ol[38] or plant saps were obtained from freeze-thawed samples as previously described[42] and treated with propan-2-ol to precipitate proteins and inhibit enzyme activity[43]. Initially an ion-exchange procedure was used to purify the choline[37,38]. This involved passage through an anion exchanger, loading onto a cation exchanger, washing the cation exchanger with ammonium hydroxide and finally eluting the choline with hydrochloric acid. The main disadvantage of this procedure is the large quantity of ammonium chloride in the final eluate. This has previously been removed by drying the eluate and dissolving the choline with either methanol or acetonitrile[37,38]. It was subsequently found that precipitation with reineckate after reduction in volume *in vacuo* was more effective. A volume of 0.5 ml of a saturated solution of ammonium reineckate was added to the concentrated eluate (1 ml) together with 50 μl of trimethylamine hydrochloride solution (0.5 mg ml^{-1} in water) to act as a co-precipitant. After standing at 4°C for at least 1 h (preferably overnight) the mixture was centrifuged (9000 *g* for 1 min) in a 1.5-ml polypropylene microcentrifuge tube and the supernatant discarded. The reineckate ions were removed with an anion-exchange resin in methanol in a procedure similar to that described for the purification of acetylcholine from brain tissue[27]. In the present case, however, it was not necessary to maintain completely anhydrous conditions. Methanol (1 ml) was added to the microcentrifuge tube together with *ca.* 0.5 ml of Dowex 1 (OH^--form, 100 mesh) dried on a sintered glass funnel to remove surface water. The tube was then shaken mechanically for 10 min, making sure that the reineckate pellet was thoroughly suspended in the methanol. A pin was used to make a small hole in the base of the microcentrifuge tube and the methanolic solution was collected in a small glass vial. Another 1 ml of methanol was added to the microcentrifuge tube to wash the Dowex 1 beads and recover all of the choline. After evaporation to dryness in a stream of air, the purified extract was dissolved in 100 μl of water and centrifuged at 9000 *g* for 1 min before being injected into the high-performance liquid chromatograph. In most cases it was found that the ion-exchange steps could be omitted and the extract (or sap) treated directly with ammonium reineckate. Trimethylaminopropan-2-ol was found to be a suitable internal standard for the quantitative estimation of choline. The same procedure can be used to simultaneously purify choline and glycinebetaine from plant saps, provided that the sap is first acidified to pH 4 or below.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC has previously been used to separate choline

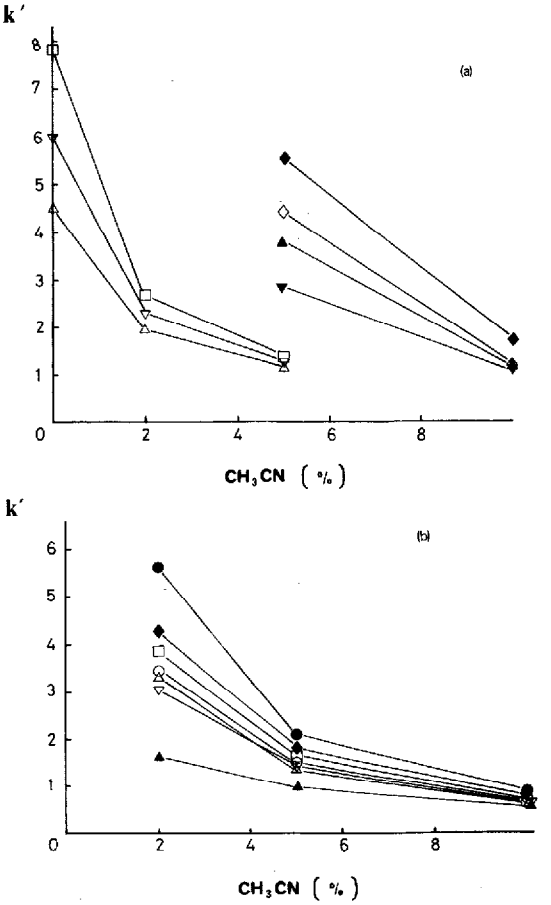

Fig. 1. Capacity factors (k') for a range of aminoethanol and choline analogues against acetonitrile concentration. (a) △ = Aminoethanol; ▽ = methylaminoethanol; □ = dimethylaminoethanol; ◆ = phosphorylcholine; ▲ = triethylaminoethanol; ▼ = acetylcholine; ◇ = glycinebetaine aldehyde. (b) ▲ = Ammonium; ▽ = choline; △ = trimethylaminopropan-1-ol; ◆ = trimethylaminopropan-2-ol; ● = diethylaminoethanol; ○ = dimethylaminopropan-1-ol; □ = dimethylaminopropan-2-ol.

esters on octadecylsilyl stationary phases bonded to silica[37,44]. A similar system (LiChrosorb 10 RP-18) gave broad peaks and poor resolution when used in conjunction with the reagents and detector used in the present investigation. Attempts to use a low-capacity strong cation-exchange column with the Dionex ion chromatograph were also unsuccessful. The Dionex MPIC-NS1 column used here is packed with a 10-μm, porous, neutral, highly cross-linked styrene–divinylbenzene co-polymer. This column has previously been used to separate methyl esters of betaines[45]. That an ion-pair reversed-phase partition mechanism is in operation is demonstrated by the low retention of the quaternary ammonium compounds in the absence of an ion-pair reagent, the increase in retention with increasing concentration or increasing chain length of the ion-pair reagent and the decrease in retention times with increasing concentrations of acetonitrile. The effect of acetonitrile on the ca-

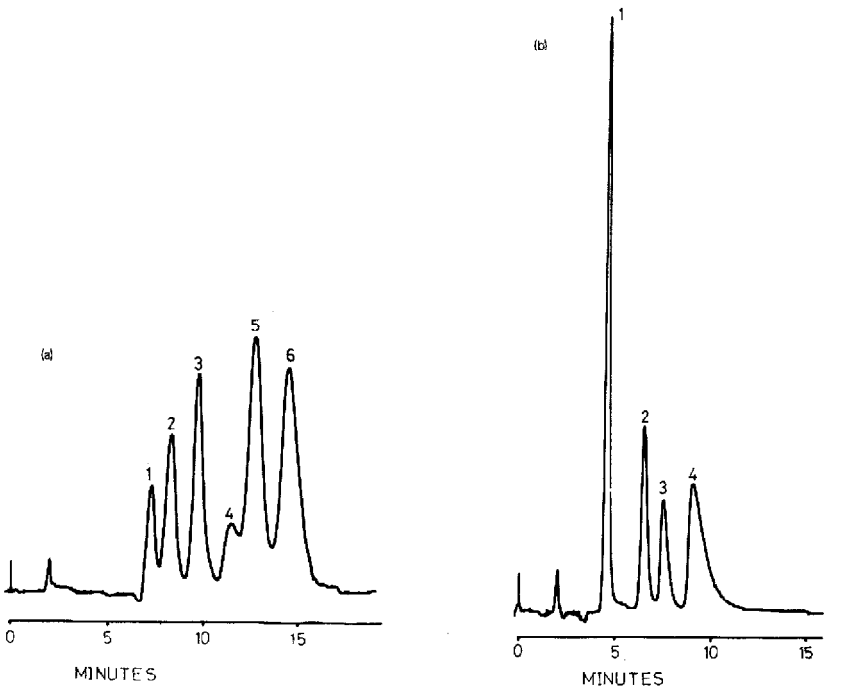

Fig. 2. Isocratic separation on Dionex MPIC-NS1 eluted with 2 mol m^{-3} hexanesulphonic acid. (a) 0.75% Acetonitrile; 1 = ammonium; 2 = aminoethanol; 3 = methylaminoethanol; 4 = dimethylaminoethanol; 5 = choline; 6 = trimethylaminopropan-1-ol. (b) 7% Acetonitrile; 1 = choline; 2 = acetylcholine; 3 = triethylaminoethanol; 4 = phosphorylcholine.

TABLE I

RETENTION TIMES (min) FOR AMINOETHANOL AND RELATED COMPOUNDS ON DIONEX MPIC-NS1 AND MPIC-NG1 COLUMNS ELUTED AT 1 ml min^{-1} WITH 2 mol m^{-3} HEXANESULPHONIC ACID CONTAINING VARIOUS AMOUNTS OF ACETONITRILE

Unretained compounds eluted after 2.5 min.

Compound	*Acetonitrile concentration*		
	0.6%	*2.1%*	*Gradient**
Aminoethanol	8.9	6.3	8.6
Methylaminoethanol	11.0	6.9	10.3
Dimethylaminoethanol	12.7	7.5	12.3
Diethylaminoethanol	>20.0	15.3	24.4
Dimethylaminopropan-1-ol	>15.0	9.3	15.4
Dimethylaminopropan-2-ol	>15.0	10.2	17.5

* Linear gradient between the following set points; 0 min, 0.6% acetonitrile; 9 min, 0.9% acetonitrile; 20 min, 3% acetonitrile; 30 min, 15% acetonitrile; 38 min, 30% acetonitrile.

TABLE II

RETENTION TIMES (min) FOR CHOLINE ANALOGUES

Conditions as in Table I

*Compound**	*Acetonitrile concentration*			
	0.6%	*2.1%*	*4.5%*	*Gradient*
Trimethylaminoethanol (choline)	13.1	7.9	5.1	13.4
Trimethylaminopropan-1-ol	15.9	9.1	5.5	15.4
Trimethylaminopropan-2-ol	21.2	10.9	6.0	19.4
Diethylmethylaminoethanol	>20.0	15.2	7.0	26.1
Triethylaminoethanol	>20.0	>20.0	9.5	29.5
(2-Chloroethyl)trimethylammonium (chlorocholine)	>20.0	16.6	7.8	26.1

* As the chloride or iodide salt.

pacity factors of a number of aminoethanol and choline derivatives is shown in Fig. 1. Addition of methanol or tetrahydrofuran similarly decreased retention times, but acetonitrile was found to give the best peak shape. An ion-pair reagent with a smaller hydrophobic side chain, methanesulphonic acid, was also used to decrease the retention times of the less polar compounds (Table VI).

Examples of isocratic separations of aminoethanol and choline derivatives are shown in Fig. 2. For choline eluted with 2 mol m^{-3} hexanesulphonic acid in 1.5% acetonitrile the efficiency of this system was *ca.* 5000 plates m^{-1}. Increasing the size of the hydroxy side-chain or of the substituents on the nitrogen atom increased retention times of both aminoethanol (Table I) and choline (Table II) analogues. Replacement of the hydroxy group with chlorine in chlorocholine also increased the retention time considerably (Table II).

The retention times of a number of choline esters are given in Table III. Increasing the size of the ester again increased the retention time for the organic esters. Phosphorylcholine eluted considerably later than sulphocholine. These two compounds have been implicated in phosphate and sulphate transport in plants[46,47] and

TABLE III

RETENTION TIMES (min) FOR CHOLINE ESTERS

Conditions as in Table I.

Compound	*Acetonitrile concentration*			
	4.5%	*10%*	*15%*	*Gradient*
Acetylcholine	7.5	4.1	3.1	27.0
Acetyl-B-methylcholine (methacholine)	11.2	4.8	3.2	30.5
Butyrylcholine	>20.0	9.4	4.2	35.0
Benzoylcholine	>20.0	>20.0	9.3	39.0
Phosphorylcholine	9.4	4.9	3.1	30.0
Choline-O-sulphate (sulphocholine)	8.0	4.2	3.1	25.0

TABLE IV

RETENTION TIMES (min) FOR QUATERNARY AMMONIUM ALDEHYDES

Conditions as in Table I.

Compound	*Acetonitrile concentration*	
	4.5%	*Gradient*
Trimethylaminoacetaldehyde (glycinebetaine aldehyde)	6.4	22.0
Trimethylaminoacetaldehyde dimethyl acetal	9.6	29.5
Trimethylaminoacetone	6.4	23.2
Trimethylamino-3-methyl-2-butanone	10.4	29.6

have been detected at high concentrations in the salt glands of *Avicennia*[48]. Table IV gives the retention times for a number of quaternary ammonium aldehydes including glycinebetaine aldehyde which is an intermediate in the biosynthesis of glycinebetaine from choline[1,39]. Data on the retention of a number of other compounds are shown in Table V. These data were obtained partly to provide information on possible interferences with the assays of choline and other compounds. The monovalent inorganic cations eluted before aminoethanol whereas the divalent inorganic cations were strongly retained, eluting between acetylcholine and phosphorylcholine. Amino acids and betaines gave very low responses in the conductivity detector. Since the aim of the present work was to provide a system for the analysis of intermediates in glycinebetaine biosynthesis, it was desirable to determine where glycinebetaine itself eluted. This was done by replacing the conductivity detector with a UV absorbance detector. Both glycinebetaine and trigonelline, the most common betaines in plants, eluted before aminoethanol and choline (Table V).

TABLE V

RETENTION TIMES (min) OF VARIOUS COMPOUNDS

Conditions as in Table I. ND = Not determined.

Compound	*Acetonitrile concentration*		
	0.6%	*4.5%*	*Gradient*
Sodium	6.2	3.5	6.2
Ammonium	7.3	4.4	7.3
Potassium	7.3	4.2	7.2
Calcium	ND	ND	29.2
Barium	ND	ND	29.0
Trimethylamine	7.5	5.0	11.7
Benzyltrimethylammonium	>20.0	>20.0	35.0
Methyl viologen	>20.0	16.5	32.3
Proline	ND	ND	–
Glycinebetaine*	ND	ND	3.6
Trigonelline*	ND	ND	7.4

* Detection by UV absorbance at 200 nm.

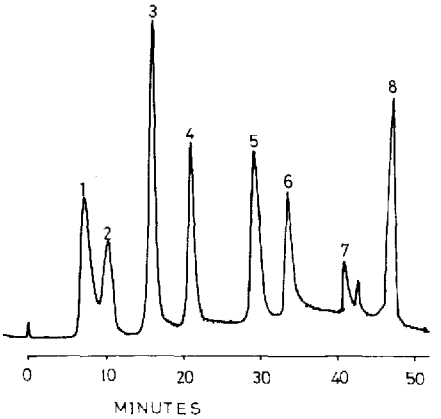

Fig. 3. Separation of choline and related compounds on Dionex MPIC-NS1 eluted with 2 mol m^{-3} hexanesulphonic acid and a gradient of acetonitrile concentration linear gradients between set points given in Table I. 1 = Ammonium; 2 = aminoethanol; 3 = choline; 4 = trimethylaminopropan-2-ol; 5 = acetylcholine; 6 = phosphorylcholine; 7 = butyrylcholine; 8 = benzoylcholine.

Because of the wide range of retention times displayed by the compounds used in this investigation the possibility of gradient elution was explored. Only a small rise in the background conductivity was observed when a gradient was run from 0.6 to 30% acetonitrile (Fig. 3). This gradient separated compounds ranging from ammonium to benzoyl choline in a single run. Retention data obtained with this gradient for most of the compounds investigated are shown in Tables I–V. Data obtained with 2 mol m^{-3} methanesulphonic acid and an acetonitrile gradient from 0 to 10% are given in Table VI. All compounds eluted much earlier with this ion-pair reagent, even when the concentration was increased to 5 mol m^{-3}.

The response of the conductivity detector for choline was linear between 0.5 and 20 nmol (regression coefficient, $r^2 = 0.9997$). Examples of choline separations in extracts of plant saps are shown in Fig. 4. The choline peak is well separated from the large peak of inorganic cations, and is the main peak eluting after 10 min. Trimethylaminopropan-2-ol, a possible internal standard for quantitative choline determinations, was completely separated from choline and did not coincide with any other peaks in the extract. Purification of choline by reineckate precipitation was

TABLE VI

RETENTION TIMES (min) FOR CHOLINE DERIVATIVES ELUTED WITH 2 mol m^{-3} METHANESULPHONIC ACID

Acetonitrile concentration gradient from 0 to 10% in 20 min.

Compound	*Retention time (min)*
Choline	2.6
Chlorocholine	2.8
Acetylcholine	3.1
Acetyl-β-methylcholine	4.5
Butyrylcholine	11.8

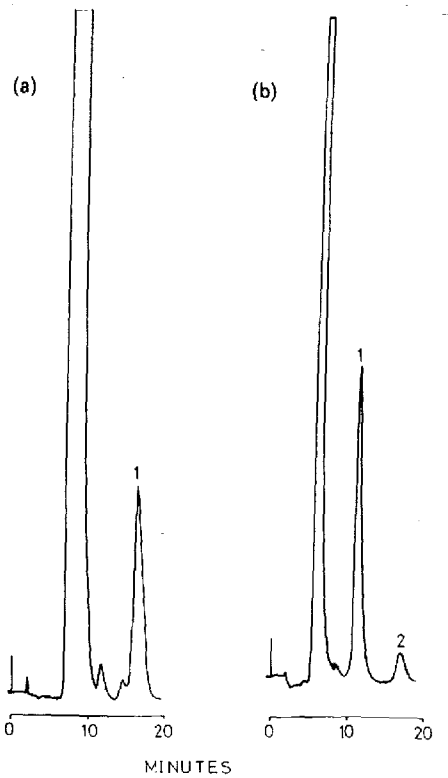

Fig. 4. Choline in plant saps treated with ammonium reineckate. (a) *Thinopyrum scirpeum*, 20-μl injection equivalent to 30 μl of sap. 30 μS full-scale deflection, 2 mol m^{-3} hexanesulphonic acid in 1% acetonitrile. (b) *Phaseolus aureus*, 20-μl injection equivalent to 10 μl of sap. 30 μS full-scale deflection, 2 mol m^{-3} hexanesulphonic acid in 1.2% acetonitrile. 1 = Choline; 2 = trimethylaminopropan-2-ol (internal standard).

found to be more convenient and more rapid than the ion-exchange procedures. The choline content was determined quantitatively in four samples of leaf sap from *Thinopyrum scirpeum* and four samples of sap from mung bean (*Phaseolus aureus*) cotyledons (Table VII). The variation within three replicate determinations was quite low and much less than the variation between the different samples. The results confirm the previous reports of high contents of choline in germinating mung beans[2].

The same purification technique was also used to determine simultaneously the contents of glycinebetaine and choline in control and salt-stressed plants of the hybrid wheat *Triticum aestivum* cv. Chinese Spring × *Thinopyrum bessarabicum*[49]. The isopropanol-treated expressed sap from the leaves was acidified with 50 μl of 1 mol m^{-3} hydrochloric acid and treated with ammonium reineckate and trimethylamine hydrochloride as described above. Purified extract corresponding to 200 μl of sap was dissolved in 200 μl of water and 50-μl aliquots used for the determination of choline. Glycinebetaine contents were determined in 20-μl aliquots by cation-exchange HPLC as previously described[42]. Reineckate precipitation greatly reduced the interferences with the detection of glycinebetaine by this method compared with ion-exchange purification. The results of these analyses are presented in Table VIII

TABLE VII

QUANTITATIVE ESTIMATION OF CHOLINE IN SAPS OF *PHASEOLUS AUREUS* SEEDLINGS AND MATURE LEAVES OF *THINOPYRUM SCIRPEUM*

Values are means of three replicates ± standard errors.

Sample	*Choline (mmol m^{-3} sap)*	
	Phaseolus aureus	*Thinopyrum scirpeum*
1	1387 ± 8	563 ± 5
2	1907 ± 16	554 ± 15
3	1630 ± 15	547 ± 8
4	1600 ± 18	663 ± 5
Overall mean	1631 ± 107	582 ± 27

TABLE VIII

CHOLINE AND GLYCINEBETAINE IN SAP OF CONTROL AND SALT STRESSED HYBRID WHEAT (*TRITICUM AESTIVUM* cv. CHINESE SPRING × *THINOPYRUM BESSARABICUM*)

Values are means of four replicates ± standard errors.

Growth conditions	*Choline (mol m^{-3})*	*Glycinebetaine (mol m^{-3})*
Control (no salt)	0.727 ± 0.050	2.89 ± 0.22
200 mol m^{-3} sodium chloride + 10 mol m^{-3} calcium chloride	1.140 ± 0.080	9.75 ± 0.61
t-Test probability	0.007	0.000

and show the expected increase in glycinebetaine in salt-stressed plants. They also show a slight increase in the choline content of the plants grown at 200 mol m^{-3} sodium chloride + 10 mol m^{-3} calcium chloride.

The method described above should be useful not only for the analysis of choline itself but also for monitoring the concentrations of choline esters in pharmaceutical preparations[44,50]. While it is not sensitive or efficient enough for the determination of trace amounts of acetylcholine, it may be useful for preliminary purification prior to analysis by GLC. It also has potential uses in the separation of ^{14}C-labelled metabolites of aminoethanol, and in the analysis of the products of phospholipase *c* action on phospholipids. Divalent metal ions may, however, interfere with the determination of some choline esters, but can be removed by precipitating the quaternary ammonium compounds with ammonium reineckate. The main advantage of the method described above is that it can be used for the analysis of aqueous solutions without the need for derivatization.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the financial support of the Overseas Development Administration of the United Kingdom, and the technical advice of M. Masters (Dionex U.K.).

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