Aust. J. Plant Physiol., 1998, **25**, 793–800

Separation of sugars, polyols, proline analogues, and betaines in stressed plant extracts by high performance liquid chromatography and quantification by ultra violet detection

Bodapati P. Naidu

CSIRO Tropical Agriculture, Cunningham Laboratory,
306 Carmody Rd, St Lucia, Queensland 4067, Australia; email: naidu.bodapati@tag.csiro.au

Abstract. Osmoprotectants such as *(i)* sugars and sugar alcohols (polyols), *(ii)* proline and its analogues, and (iii) a number of quaternary ammonium compounds (betaines) play a significant role in plant adaptation to environmental stresses. Lack of a simple and rapid technique for the extraction and simultaneous determination of these osmoprotectant solutes from a large number of samples originating from plant breeding populations and agronomic trials, led to the development of this method.

Osmoprotectants were extracted using methanol-chloroform-water. Extracts were partially purified, where required, by treating with ion exchange resins. Solutes were separated using a high performance liquid chromatograph fitted with a Sugarpak-1 column and 5 mg L^{-1} Ca-EDTA solution as the mobile phase. The eluted solutes were quantified by ultra violet detection at 195 nm. This technique measures sugars/polyols, proline analogues, and betaines in a variety of plant species such as peanut, *Melaleuca uncinata*, and cotton, respectively. Pinitol levels in peanut estimated using this method correlated significantly with the determinations obtained by HPLC-refractive index determination ($R^2 = 0.983$, $P<0.001$). Glycinebetaine determinations in cotton using this method were significantly correlated with determinations achieved by ^{1}H NMR spectroscopy ($R^2 = 0.989$, $P<0.001$). Cotton leaf extracts spiked with a range of authentic glycinebetaine levels were also precisely measured using this technique (R^2=0.999, $P<0.001$). The described method is simple, rapid, sensitive, cost effective and simultaneously measures more than one class of osmoprotectants from a single chromatographic run.

Keywords: Osmoprotectants, solutes, cotton, peanut, *Melaleuca*.

Introduction

In response to stress, plants accumulate a variety of osmoprotectants such as *(i)* sugars and sugar alcohols (polyols; Yancy *et al.* 1982), *(ii)* proline (Aspinall and Paleg 1981) and its analogues (Naidu *et al.* 1987), and *(iii)* a number of quaternary ammonium compounds (betaines) and tertiary sulfonium compounds (Wyn Jones and Storey 1981; Rhodes and Hanson 1993). Plants accumulate these solutes as an adaptive mechanism to environmental stresses such as salinity (Hayashi *et al.* 1997), water deficit (Yancy *et al.* 1982; Monyo *et al.* 1992) and temperature extremes (Yang *et al.* 1996; Hayashi *et al.* 1997).

This fundamental understanding is currently being utilised to enhance plant tolerance to stress, and hence to increase plant yield under stress conditions. The two main avenues being investigated towards this goal are *(i)* the direct application of osmoprotectants by foliar application and to seed coating (Naidu 1995; Makela *et al.* 1998) and *(ii)* breeding plants which accumulate high levels of such compounds both by traditional breeding (Yang *et al.* 1996) and by genetic engineering to increase the levels of polyols (Thomas *et al.* 1995), proline (Kavi Kishore *et al.* 1995), and glycinebetaine (Hayashi *et al.* 1997).

Despite significant progress in the stress physiology and biochemistry of plants, a reliable analytical method to extract and simultaneously quantify the commonly occurring osmoprotectants is not available. Such a technique would greatly enhance the capacity of investigators to analyse the large number of samples from plant breeding populations and agronomic trials.

A number of separate techniques have been developed to quantify each class of osmoprotectants. The techniques used for the measurement of sugars and polyols require extensive derivatisation prior to gas chromatography (Lewis and Smith 1967) or high performance liquid chromatography (HPLC; Thomas *et al.* 1995). Proline is usually estimated by the ninhydrin-colorimetric method of Singh *et al.* (1973). Betaines have been measured using the periodide precipitate method (Storey and Wyn Jones 1977), thin-layer electrophoresis (Gorham *et al.* 1981), low temperature pyrolysis gas chromatography (Hitz and Hanson 1980), fast atom bombardment mass spectrometry (Rhodes *et al.* 1987), HPLC procedures involving ultraviolet absorbing esters (Gorham 1984) or straight ultra violet (UV) detection (Zamarreno *et al.* 1997), and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (Jones *et al.* 1986).

0310-7841/98/070793

Each of these protocols is limited in its ability to screen large sample numbers by the need for extensive sample preparation. Further complications involve low sensitivity and lack of specificity, high unit cost of determination, and the inability of most methods to simultaneously quantify different types of osmoprotectants. This paper describes a HPLC method for the separation and simultaneous estimation of sugars and polyols, proline and its analogues, and betaines which overcomes the problems associated with the previously described methods.

Materials and methods

Preparation of plant material and extraction of solutes

Peanut (*Arachis hypogaea* L.), *Melaleuca uncinata* R.Br. and cotton (*Gossypium hirsutum* L.) plants were selected to develop this method as these species accumulate pinitol (polyol), proline analogues and glycinebetaine, respectively. Cotton and peanut were grown in pots in a glasshouse for 1 month and stressed by withholding water. Youngest fully expanded leaves were collected 10 times for cotton and nine times for peanut on alternate days. Only one collection of *M. uncinata* leaf tissue was used from an irrigated field trial in northern Queensland. Leaves of all three species were frozen in liquid nitrogen and lyophilised. Lyophilised leaf tissue (100–200 mg) was placed in a 10 mL plastic disposable centrifuge tube (Disposable Products, Adelaide) and 5 mL of ice cold methanol:chloroform:water (MCW; 60:25:15) was added to the plant material. The centrifuge tube was then maintained in a beaker containing ice and the contents were completely homogenised with Heidolph Diax600 (Germany) homogeniser fitted with a 10 mm grinding head at 20 000 rpm. The time required to break the plant material varied from 0.25 to 1 min depending on the strength and fibre content of the material. Homogenisation continued until fibrous plant material liberated most of the chlorophyll. At this point, cells would have broken down with the release of their contents including osmoprotectants. Cold MCW was added and the centrifuge tube was maintained in ice to reduce heat generation and possible break-down of solutes. The grinding head was washed with 5 mL distilled water and this was added to the MCW-plant homogenate in the centrifuge tube. This water addition also served to break the MCW emulsion. The contents of the tube were shaken on a test tube mixer for 10 s and the resulting homogenate was centrifuged at 60 *g* for 10 min at room temperature. The clear upper methanol-water (MW) phase was removed, its volume measured and stored below 4°C for the estimation of osmoprotectants. Re-extraction of the resulting pellet with further addition of MCW could only extract about an additional 2% of solute compared to the first extraction (data not presented).

Rapid purification of solutes using ion exchange resins

In some species amino acids or inorganic salts interfere with the measurement of osmoprotectants by co-eluting or eluting close to the peak of interest. To overcome this interference, about 1 mL of plant extract (MW phase) was treated with 1 mL of an equal mixture of strong anion exchange resin (quaternary ammonium type, OH^- form, e.g. IRN-78 supplied by Sigma) and weak cation exchange resin (Na^+ form, eg. Amberlite supplied by BDH). A large amount of both the resins was activated separately in a large glass column prior to use by treating with four volumes of 1 M NaOH and washing until the pH of the eluent was close to neutral. Activated resin was stored in a sealed container at 4°C. This resin kept for several months without any loss of activity.

One mL of the resin mixture was placed in a 5 mL plastic syringe and about 1 mL of the plant extract was drawn into the syringe. The contents of the syringe were shaken well. A 0.45 mm HA Millipore cellulose acetate membrane filter cartridge was attached to the syringe end and the contents of the syringe were pushed through the filter slowly. The filtrate was collected into an auto sampler vial.

HPLC Analysis

Instrumentation

The HPLC system (Waters Australia Pty Ltd) consisted of a 717 plus auto-sampler, in-line degasser, 600E pump, 996 photodiode array (PDA) detector and Millennium Chromatography Manager software (version 2.15). The absorption spectrum of the eluted compounds was scanned at every second from 190 to 400 nm at an interval of 1.2 nm. The sensitivity of the measurement of the absorption spectrum was optimum at 195 nm. UV absorption spectrum at 195 nm was used for the quantification because increasing the wavelength above 195 nm decreased peak areas; for example, peaks were three times larger at 195 nm than at 200 nm. The whole of the PDA absorption spectrum was used only for authentication of compounds.

Column

A Waters Sugar-Pak I HPLC column (part no. 85188) of 6.5 × 300 mm was used for the separation of osmoprotectants. The stationary phase was sulfonated styrene divinylbenzene resin in calcium form. The column was maintained at 90°C. Pre-column inserts were used to prevent damage to the column.

Mobile phase

The mobile phase was deionised, degassed (vacuum filtration through a Millipore HA 0.45 mm filter) and bacteria free Millipore water containing 5 mg L^{-1} Ca-EDTA (Aldrich Chemical Company). The mobile phase was passed through an in-line degasser to further ensure that the mobile phase was gas free. The flow rate was maintained at 0.6 mL min^{-1}. Prior to the initial use and after running about 200 plant samples, the column was reconditioned by passing 1 L of 500 mg L^{-1} Ca-EDTA solution. The column was then washed with the mobile phase (5 mg L^{-1} EDTA solution) until the base line was stable.

Comparison of the techniques

The reliability and accuracy of this technique was demonstrated by measuring pinitol in peanut and glycinebetaine in cotton.

Pinitol was measured using the HPLC-UV method described here and the HPLC-refractive index (RI) method (Campbell *et al.* 1998). The stationary phase in the column was 4 mm Nova-Pak spherical silica bonded with trifunctionalaminopropylsilane. The mobile phase was acetonitrile: water (75:25) at a flow rate of 1.4 mL min^{-1}. Separated sugars were detected and quantified with a differential refractometer.

Glycinebetaine levels in cotton were measured using the ^1H NMR method of Jones *et al.* (1986). A Bruker 500 MHz spectrometer was used with sodium trimethylsilylpropionate as an internal standard. In a separate experiment, cotton leaf extracted as described in this paper and containing 61.7 μmol g dry $weight^{-1}$ of glycinebetiane was spiked with 0 to 100 μmol g dry $weight^{-1}$ of glycinebetaine. Glycinebetaine was chromatographed and quantified as described earlier in this method.

Authentic osmoprotectants

All sugars, polyols, amino acids and glycinebetaine were obtained from Sigma Chemical Company. *N*-methyl-L-proline (MP), *trans*-4-hydroxy-*N*-methyl-L-proline (MHP), *trans*-4-hydroxy-*N*-dimethyl-L-proline (DHP), stachydrine (proline betaine), and trigonelline were synthesised and supplied by Dr G. P. Jones, the University of Adelaide. Methanol and chloroform used were of analytical reagent grade.

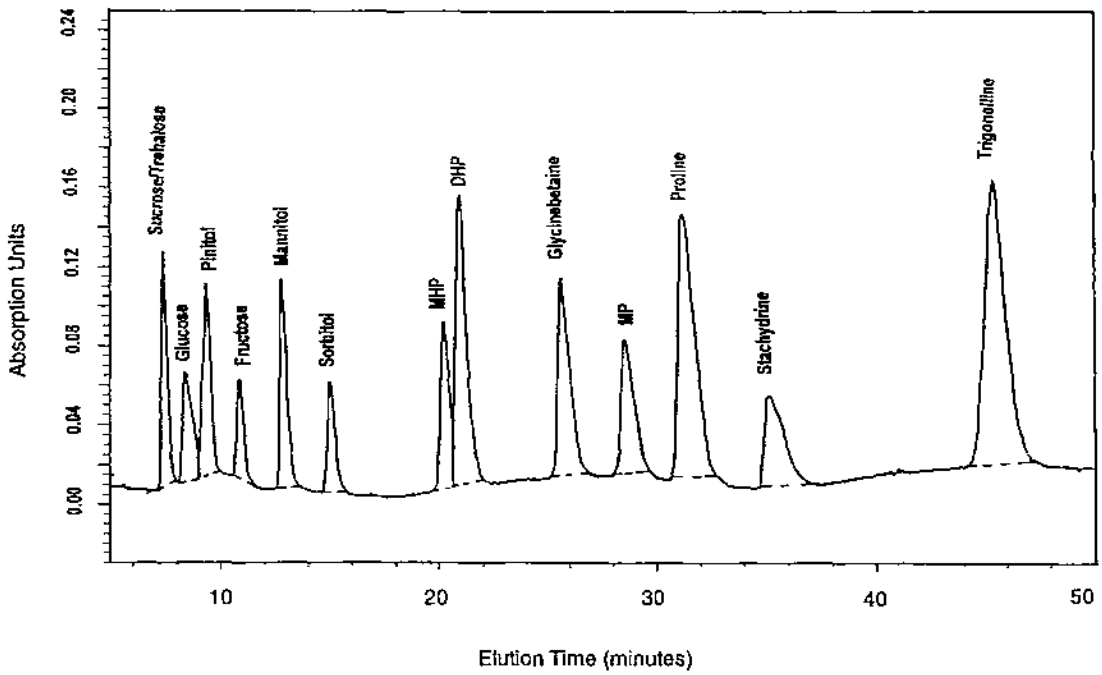

Fig. 1. Separation of commonly accumulated authentic osmoprotectants. The amount (μmol) of each authentic compound is shown in the parentheses: sucrose (0.5); glucose, fructose, and pinitol (0.1); mannitol or sorbitol (0.25); MHP, DHP, glycinebetaine, MP, or stachydrine (0.01); proline (0.02); and trigonelline (0.002).

Results

Separation of osmoprotectants

A mixture of 13 authentic osmoprotectants at concentrations typically found in stressed plants was injected into the HPLC system and all these compounds were resolved clearly (Fig. 1). This mixture contained a disaccharide (sucrose), polyols (mannitol and sorbitol), cyclitol (pinitol), proline analogues (proline, MHP, DHP, MP and stachydrine) and betaines (DHP, stachydrine, glycinebetaine and trigonelline), all dissolved in water. Methanol was not added to the standard mixture as it eluted close to mannitol. Sucrose and trehalose did not resolve from each other on this column.

The minimum practical detection limit of the peaks (25 000 $\mu V\ S^{-1}$) was determined by using the Millennium software based on noise level and peak width. Using the concentrations shown in Fig. 1, practical minimum detectable limits were calculated for the solutes (and shown in the parentheses in μmol) as follows: sucrose (0.025); glucose and fructose (0.05), pinnitol, mannitol or sorbitol (0.0025); MHP, DHP, glycinebetaine, MP or stachydrine (0.0005) proline (0.001); and trigonelline (0.00005).

Osmoprotectants in peanut leaf extract were separated using the method described in this paper, including the treatment with a weak cation exchange resin to remove amino acids interfering with proline (Fig. 2). This method enabled simultaneous estimation of the three classes of compounds: sugar related (pinitol), amino acids (proline) and quaternary ammonium compounds (trigonelline). Leaf extracts of *M. uncinata* (Fig. 3) and cotton (Fig. 4) were not treated with any resin as the interference with proline analogues (MHP and DHP) and glycinebetaine, respectively, in these species was negligible.

Interfering amino acids

The retention times of 22 authentic amino acids, individually injected into the HPLC, are shown in Table 1. Of these, nine amino acids were either not detectable between 190 and 400 nm or not eluted under the present experimental conditions. The sugar region was clearly free of any amino acid interference. Amino acids and nitrogen containing osmoprotectants were only eluted after 18 min; after this time various amino acids may interfere (Table 1 and Fig. 1) with osmoprotectants of interest. These amino acids were completely removed by the use of strong anion exchange resin. On the other hand, weak cation exchange resin removed most of the interfering amino acids completely and altered the levels of proline analogues and betaines to a very small extent.

Comparison of HPLC-UV detection results with HPLC-RI detection and ^1H NMR

Measurement of pinitol using UV and RI detectors showed a highly significant correlation with an R^2 of 0.983 ($P<0.001$,

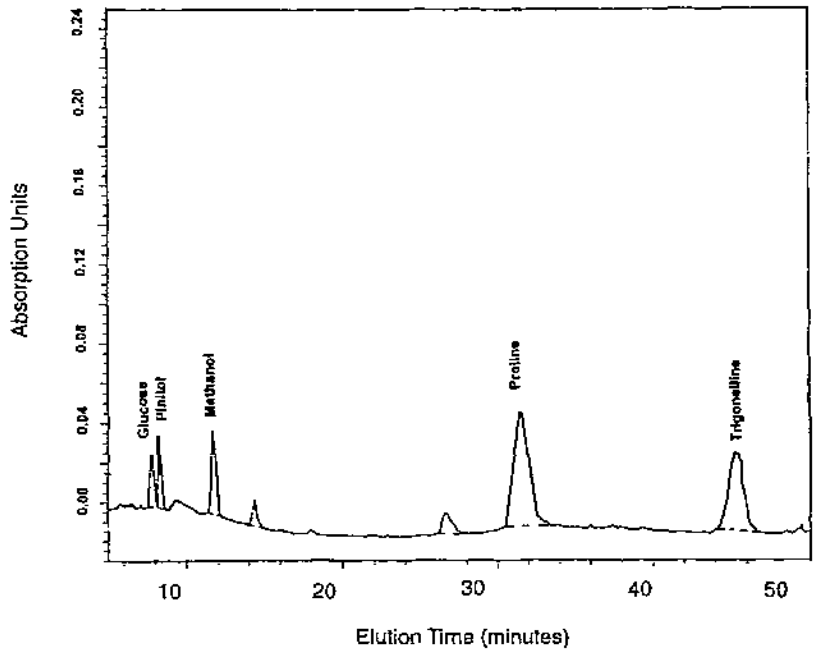

Fig. 2. Separation of pinitol, proline and trigonelline in peanut leaf sample after treatment with weak cation exchange resin.

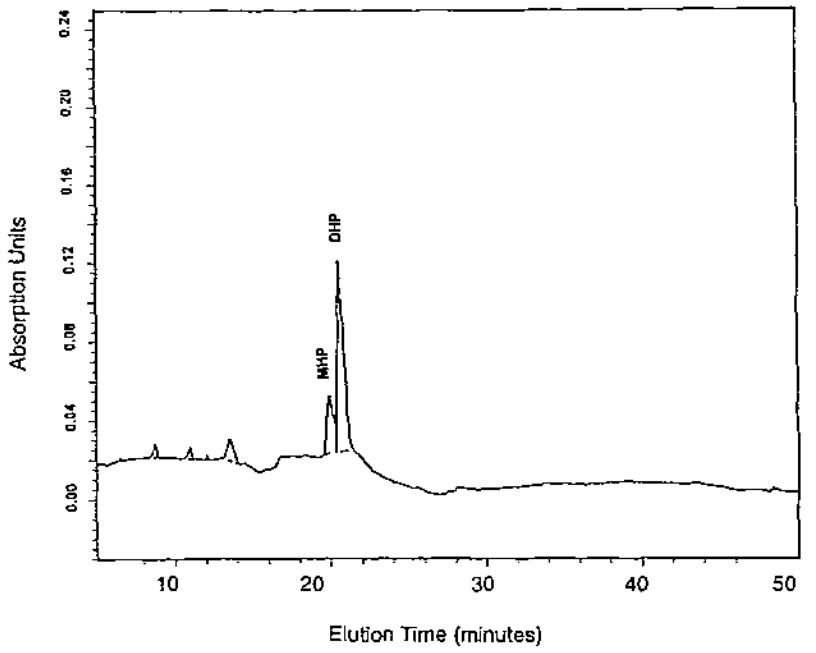

Fig. 3. Separation of proline analogues (MHP and DHP) from the crude extracts (without any ionic purification) of *Melaleuca uncinata* leaf.

Fig. 5). The peak area for a unit concentration of pinitol was larger with UV detection compared with the RI detection, implying a greater sensitivity of UV detection. However, pinitol values determined by the UV method were consistently about 20% higher than with RI detection. Glycinebetaine estimated using HPLC-UV detection and ^1H NMR measurements showed a significant correlation with an R^2 of 0.989 ($P<0.001$, Fig. 6). This technique estimated the

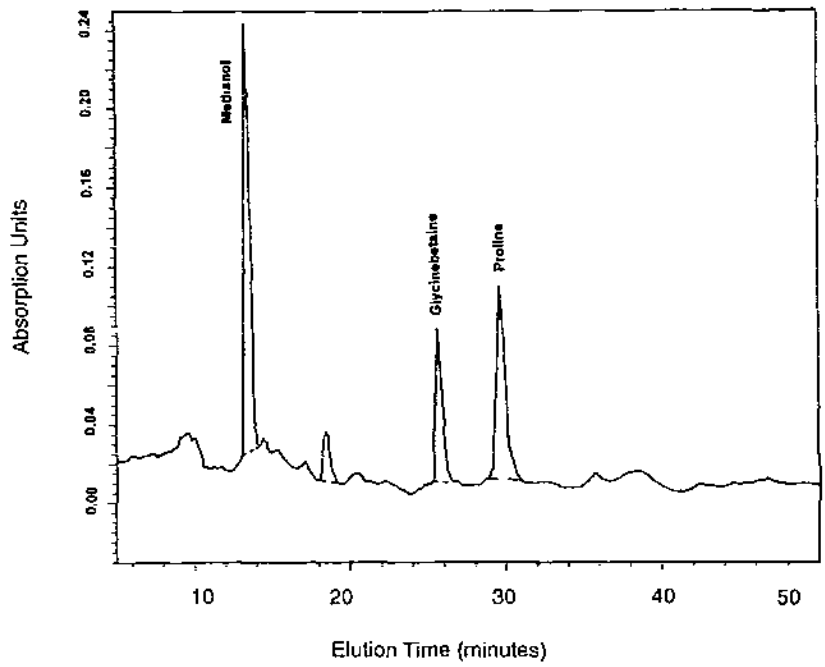

Fig. 4. Separation of glycinebetaine and proline from crude extract of cotton leaf.

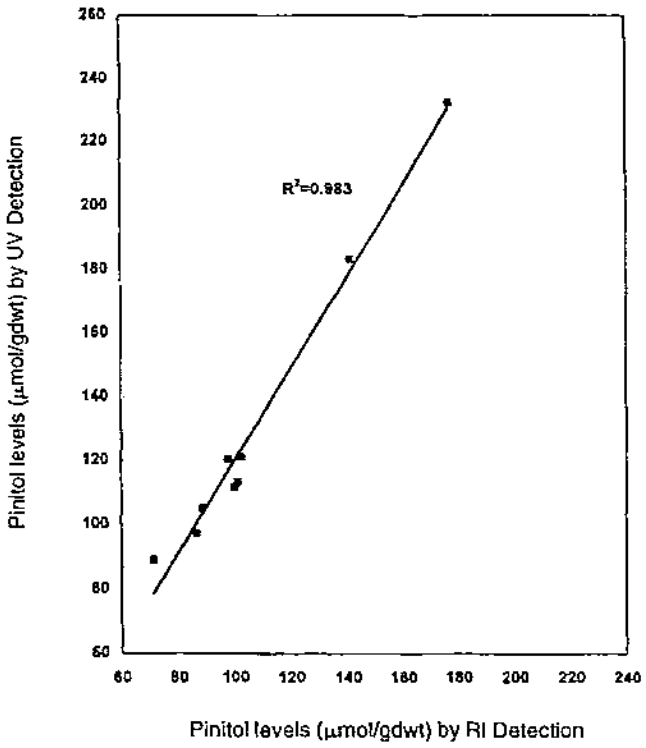

Fig. 5. The relationship between pinitol estimations by HPLC method described in this paper with UV detection and HPLC method involving RI detection (Campbell *et al.* 1998).

glycinebetaine added to the cotton extracts with a very high accuracy (Fig. 7, R^2=0.999, P<0.001) suggesting high degree of sensitivity.

Discussion

The technique described in this paper has the advantage of extraction and measurement of a variety of osmoprotectants such as sugars and sugar alcohols, proline analogues, and betaines from a single chromatographic run. No published method could simultaneously measure these compounds, except the method of Jones *et al.* (1986) which was modified by Erskine *et al.* (1996). However, only a high power NMR spectrometer as used in the work of Erskine *et al.* (1996) could resolve sugars and amino compounds. Such NMR spectrometers are often cost prohibitive and can be about 10 times more expensive than the HPLC system described in this work. Additionally, the method described in this paper minimises the sample preparation requirements such as derivatisation (Gorham *et al.* 1982) and purification of plant extracts (Jones *et al.* 1986).

Osmoprotectants in this study are separated on the principles of size exclusion and ligand exchange and/or weak cation exchange. Size exclusion principle allows larger molecules to elute earlier than smaller ones. The ligand exchange principle resolves sugars and polyols by coordination of their hydroxyl groups with Ca^{2+} ions on the resin. The overall effect is that all sugar related compounds elute within 15 min of injection because they interact with the resin very weakly. Failure to resolve sucrose and trehalose on this

Table 1. Removal of various osmoprotectants by pre-treatment with weak cation exchange resin (Na^+ type), and strong anion exchange resin (OH^- type)

Each standard solution (10 μL, 25 μg) of compound was injected with and without the resin treatment. ND, not detectable or not eluted under the experimental conditions

Osmoprotectant	Retention time (min)	% removed by ion exchange resins	
		Weak cation	Strong anion
Amino acids			
Alanine	22.68	100	100
Arginine	ND	ND	ND
Asparagine	22.03	94	100
Aspartic acid	ND	ND	ND
Cysteine	ND	ND	ND
Cystine	ND	ND	ND
Glutamic acid	ND	ND	ND
Glutamine	21.54	100	100
Glycine	25.04	100	100
Histidine	ND	ND	ND
Hydroxyproline	20.05	100	100
Isoleucine	27.68	100	100
Leucine	28.55	100	100
Lysine	ND	ND	ND
Methionine	25.86	98	100
Phenylalanine	ND	ND	ND
Serine	21.43	100	100
Threonine	19.84	98	100
Tryptophan	36.72	100	100
Tyrosine	ND	ND	ND
Valine	23.59	96	100
Methylated amino acids/betaines			
Proline	30.27	8	100
MP	26.00	9	100
MHP	18.07	7	100
DHP	19.17	0	0
Stachydrine	35.56	0	0
Glycinebetaine	23.09	0	0
Trigonelline	44.94	0	0

system may not pose a great problem for the application of this method to plant studies as trehalose accumulation is not common in higher plants with an exception in some resurrection plants (Goddijn and Smeekens 1998).

The charged amino acid based osmoprotectants are retained for a longer time on the weakly cationic column, thus getting a clear separation between sugar-related and amino-acid related osmoprotectants. However, the ligand exchange and/or cation exchange properties of the column should be used carefully to achieve separation of structurally closely related compounds such as MHP and DHP. To achieve optimum separation of these two compounds, the concentration of Ca-EDTA in the mobile phase should be 2.5 mg L^{-1}.

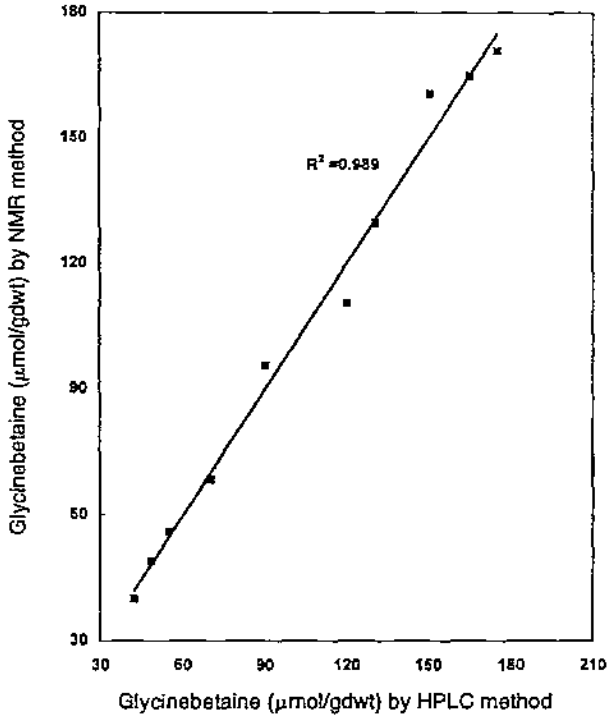

Fig. 6. The relationship between glycinebetaine estimations by the HPLC method described in this paper and ^1H NMR method (Jones *et al.* 1987).

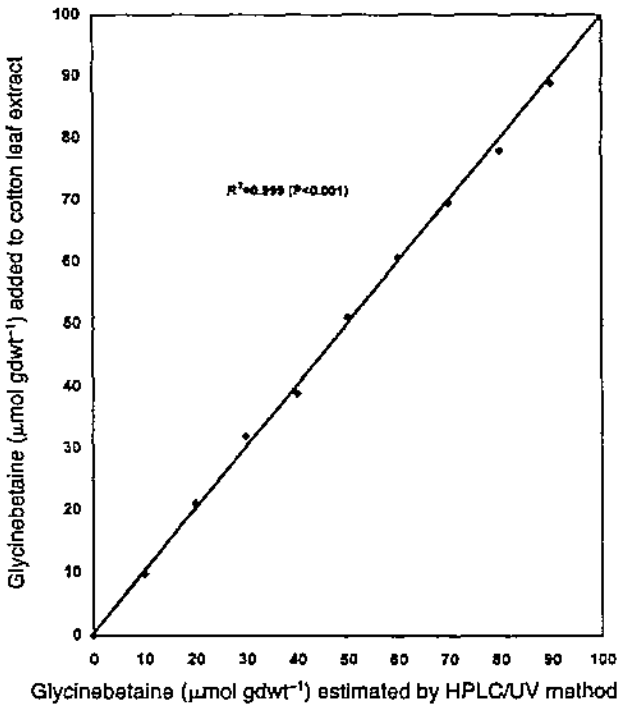

Fig. 7. Estimates of spiked glycinebetaine by the HPLC-UV method in cotton.

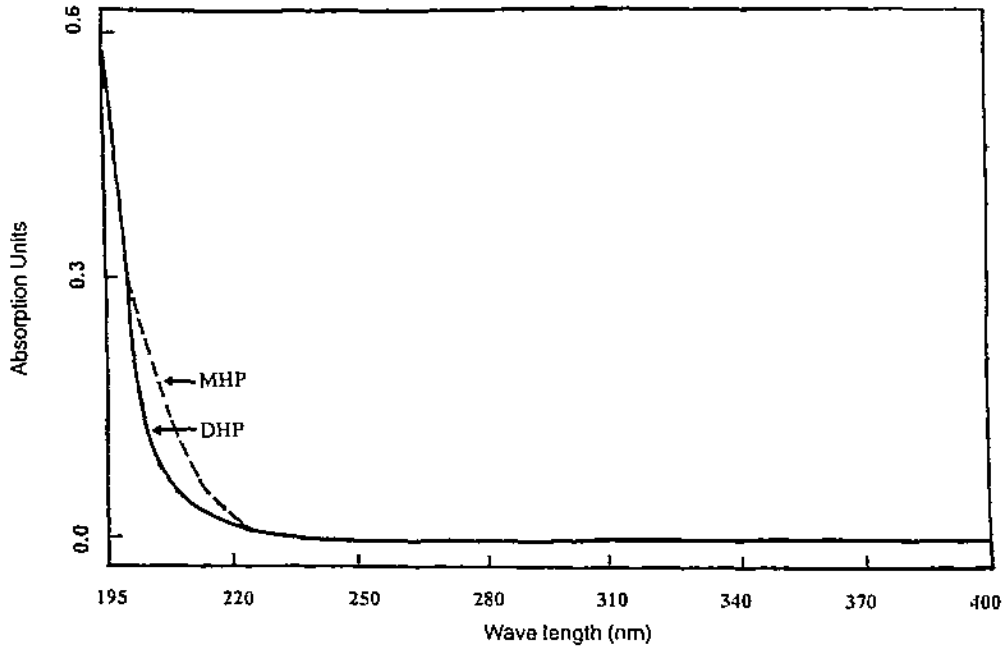

Fig. 8. UV spectra of authentic MHP and DHP.

High concentrations of Ca reduce the retention time of MHP and DHP by competing for cationic sites on the column. This results in the loss of resolution between MHP and DHP.

Crude MCW extracts (MW phase) could be utilised for the measurement of glycinebetaine in plant extracts (Figs 3 and 4). However, methanol and mannitol elute 12.5 and 13.1 min after injection. Although these two peaks separate from each other, to increase accuracy of mannitol measurement, the MW phase may be dried on a rotary eppendorf desiccator and the residue re-dissolved in water prior to chromatography.

The MW phase of the extracts or samples re-constituted in water may be directly chromatographed without any purification as in the case of cotton (Fig. 4). However, the chromatography of amino acid standards revealed that alanine elutes 0.4 min earlier and valine eluted 0.5 min later than glycinebetaine (Table 1). Alanine and valine levels in plants are usually low (Naidu *et al.* 1990) and if significant interference of these or other amino acids is suspected in any species, the following simple ion exchange method may be used for accurate detection of glycinebetaine.

Sugars (possessing no charge) and betaines (zwitterionic) are not retained by either strong anion or weak cation exchange resins. The presence of a permanent positive charge on the quaternary ammonium group, in combination with a carboxyl group of relatively low pKa, gives betaines unique charge properties which allow their isolation from amino acids. A strong anion exchange resin removes all anions except OH^- and all zwitterions other than betaines (Table 1). Weak cation exchange resin removes all cations other than sugars, betaines, proline and its analogues (Table 1). Thus, if proline and its analogues (MP and MHP) are to be assayed, then only weak cation exchange resin should be used. If sugars and betaines are of interest, then a mixture of strong anion and weak cation resins may be used to remove interfering organic or inorganic substances.

Osmoprotectants in plant extracts are mainly identified by comparing the elution times of appropriate standards. However, the identity of compounds with close elution times such as MHP and DHP could be further confirmed using a PDA detector (where available) and UV absorption spectra (Fig. 8), which is more reliable than retention times alone. However, PDA is not an essential requirement for routine quantification of the osmoprotectants described here; a UV spectrophotometer set at 195 nm is adequate.

This method has been in regular use in my laboratory for the last 4 years for the estimation of endogenous or externally applied osmoprotectants in several tropical pasture legumes (e.g. *Stylosanthes* and *Lablab*, Naidu and Harwood 1997), grass species (Harwood 1998) and crops such as cotton, peanut, rice and sugarcane (Naidu 1995).

In conclusion, the HPLC method described here simultaneously estimates various classes of osmoprotectants accumulating in stressed plants. This method is rapid, does not require extensive sample purification or derivatisation, and running costs are low.

Acknowledgments

I thank the Rural Industries Research and Development Corporation (RIRDC) of Australia for the financial support

(project no.CSC-55A), BHP Australia Coal for the funding to purchase the HPLC system, Mr Ross Hansen for the use of the RI detection facility, Dr Ian Brereton for running the NMR system, and Dr Graham P. Jones for the supply of various betaines. Preliminary work conducted at the Department of Primary Industries and Fisheries, Mt Pleasant Laboratory, Launceston, Tasmania, was partly funded by Finnsugar Bioproducts, Helsinki.

References

Aspinall, D., and Paleg, L.G. (1981). Proline accumulation: physiological aspects. In 'The Physiology and Biochemistry of Drought Resistance in Plants'. (Eds L.G. Paleg and D. Aspinall.) pp. 205–241. (Academic Press: Sydney.)

Campbell, J. A., Hansen, R. A., and Wilson, J. R. (1998). Cost effective colorimetric microtitre plate enzymatic assays for sucrose, glucose and fructose in sugarcane tissue extracts. *Journal of the Science of Food and Agriculture* (in press).

Erskine, P., Stewart, G. R., Schmidt, S., Turnbull, M. H., Unkovich, M., and Pate, J. S. (1996). Water availability—a physiological constraint on nitrate utilisation in plants of Australian semi-arid mulga woodlands. *Plant, Cell and Environment* **19**, 1149–1159.

Goddijn, O., and Smeekens, S. (1998). Sensing trehalose biosynthesis in plants. *The Plant Journal* **14**, 143–146.

Gorham, J., Coughlan, S. J., Storey, R., and Wyn Jones, R.G. (1981). Estimation of quaternary ammonium and tertiary sulfonium compounds by thin-layer electrophoresis and scanning reflectance densitometry. *Journal of Chromatography* **210**, 550–554.

Gorham, J., McDonell, E., and Wyn Jones, R.G. (1982). Determination of betaines as ultraviolet absorbing esters. *Analytical Chemica Acta* **138**, 277–283.

Gorham, J. (1984). Separation of plant betaines and their sulphur analogues by cation-exchange high performance liquid chromatography. *Journal of Chromatography* **287**, 345–351.

Harwood, M. R. (1998). Selection of pasture grasses for revegetation of open-cut coal mines in central Queensland. PhD Thesis, The University of Queensland.

Hayashi, H., Alia, Mustardy, L., Deshnium, P., Ida, M., and Murata, N. (1997). Transformation of *Arabidopsis thaliana* with the *codeA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. *Plant Journal* **12**, 133–142.

Hitz, W. D., and Hanson, A. D. (1980). Determination of glycinebetaine by pyrolysis-gas chromatography in cereals and grasses. *Phytochemistry* **19**, 2371–2374.

Jones, G. P., Naidu, B. P., Starr, R. K., and Paleg, L. G. (1986). Estimates of solutes accumulating in plants by ^{1}H nuclear magnetic resonance spectroscopy. *Australian Journal of Plant Physiology* **13**, 649–658.

Kavi Kishore, P. B., Hong, Z., Mio, G., Hu, C. A., and Verma, D. P. S. (1995). Over expression of Δ^1-pyrroline-5-carboxylase synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **108**, 1387–1394.

Lewis, D. H., and Smith, D. C. (1967). Sugar alcohols (polyols) in fungi and green plants. *New Phytologist* **66**, 143–184.

Makela, P., Jokinen, K., Kontturi, M., Peltonen-Sainio, P., Pehu, E., and Somersalo, S. (1998). Foliar application of glycinebetaine—a novel product from sugarbeet—as an approach to increase tomato yield. *Industrial Crops and Products* **7**, 139–148.

Monyo, E. S., Ejeta, G., and Rhodes, D. (1992). Genotypic variation for glycinebetaine in sorghum and its relationship to agronomic and morphological traits. *Maydica* **37**, 283–286.

Naidu, B. P. (1995). Method for the treatment of seeds with betaines. Australian Patent No. 683177 (CSIRO Tropical Agriculture: Brisbane.)

Naidu, B. P., and Harwood, M. R. (1997). Opportunities for landscape stabilisation and revegetating lands with exotic or native forages. *Tropical Grasslands* **31**, 364–369.

Naidu, B.P., Jones, G.P., Paleg, L.G., and Poljakoff-Mayber, A. (1987). Proline analogues in *Melaleuca* species: response of *Melaleuca lanceolata* and *M. uncinata* to salinity and water stress. *Australian Journal of Plant Physiology* **14**, 669–677.

Naidu, B. P., Paleg, L. G., Aspinall, D., Jennings, A. C., and Jones, G. P. (1990). Rate of imposition of water stress alters the accumulations of nitrogen-containing solutes by wheat seedlings. *Australian Journal of Plant Physiology* **17**, 653–664.

Rhodes, D., and Hanson, A. D. (1993). Quaternary ammonium and tertiary sulfonium in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 357–384.

Rhodes, D., Rich, P. J., Myers, A. C., Reuter, C. C., and Jamieson, G. C. (1987). Determination of betaines by fast atom bombardment mass spectrometry. *Plant Physiology* **84**, 781–788.

Singh, T. N., Paleg, L. G., and Aspinall, D. (1973). Stress metabolism. I. Nitrogen metabolism and growth in barley plants during water stress. *Australian Journal of Biological Sciences* **26**, 45–56.

Storey, R., and Wyn Jones, G. R. (1977). Quaternary ammonium compounds in relation to salt resistance. *Phytochemistry* **16**, 447–453.

Thomas, J. C., Sepahi, M., Arendall, B., and Bohnert, H. J. (1995). Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. *Plant, Cell and Environment* **18**, 801–806.

Wyn Jones, R.G., and Storey, R. (1981). Betaines. In 'The Physiology and Biochemistry of Drought Resistance in Plants'. (Eds L.G.Paleg and D. Aspinall.) pp.172–204. (Academic Press: Sydney.)

Yancy, P.H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982). Living with water stress: evolution of osmolyte system. *Science* **217**, 1214–1222.

Yang, G., Rhodes, D., and Joly, R.J. (1996). Effects of high temperature on membrane stability and chlorophyll fluorescence in glycinebetaine-containing maize lines. *Australian Journal of Plant Physiology* **23**, 437–443.

Zamarreno, A., Cantera, R. G., and Garcia-Mina, J. M. (1997). Extraction and determination of glycinebetaine in liquid fertilisers. *Journal of Agricultural and Food Chemistry* **45**, 774–776.

Manuscript received 9 December 1997, accepted 6 August 1998