A Single-Step Purification for Glycine Betaine Determination in Plant Extracts by Isocratic HPLC

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The glycine betaine content of higher plants can be determined by rapid and simple isocratic high-performance liquid chromatography on a C_{18} column. The method described is particularly suitable for a large number of samples. Starting from a complex dry plant, it consists of a single aqueous extraction followed by a purification over a sole cationic ion-exchange resin (AG1, OH^-) before simple HPLC quantification. The single-step purification was allowed because the AG1, OH^- resin removed 80% of the total amino acids contained in the extracts. Proline, which interferes with GB HPLC quantification, was completly retained by this column. The optimized method for GB quantification was then validated by comparaison with an ^1H NMR method.

Keywords: *Halophytic plants; glycine betaine; ion exchange chromatography; ^1H NMR spectroscopy*

INTRODUCTION

Osmotic adjustment at the cell level in halophytic higher plants submitted to salt stress is known to be achieved by accumulating inorganic ions such as Na^+ or Cl^- mainly in the vacuole and compatible solutes in the cytosol (Storey and Wyn Jones, 1977; Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993). Quaternary ammonium compounds (QACs), tertiary sulfonium compounds, proline, and soluble carbohydrates such as sorbitol, sucrose, mannitol, and pinitol are the most common compatible solutes. All of these solutes have been assumed to act as osmoprotectants in higher plants as well as in bacteria (Czonka, 1989; Czonka and Hanson, 1991), algae (Blunden and Gordon, 1986), and animals (Anthoni et al., 1991).

A number of QACs are found in plants, but glycine betaine (GB) is the major cytoplasmic osmolyte in families of higher plants growing in salt marshes (Wyn Jones and Storey, 1981). The wide occurrence of these compounds in living organisms stimulated the development of analytical methods for their characterization and quantification. First analyses of QACs involved ion exchange chromatography or thin-layer chromatography for their purification and paper chromatography or high voltage electrophoresis for their separation (Rhodes and Hanson, 1993). In the case of betaine analyses, the Dragendorff reagent (Dawson et al., 1969) used for detection lacked sensitivity, with the colorimetric reaction being only semiquantitative. Other methods used for betaine estimation relied on the nonspecific precipitation of the QACs as periodides (Storey and Wyn Jones, 1977) or involved a modified Dragendorff reagent (Stumpf, 1984). However, these methods are nonspecific, not very sensitive, and nonreproducible. The introduction of a pyrolysis−gas chromatography technique (Hitz and Hanson, 1980) appeared to overcome all the problems met in QACs quantification techniques. Nevertheless, high-performance liquid chromatography (HPLC) separation of QACs or their esters was soon preferred because of the high cost of pyrolysis probes which required frequent replacement (Gorham et al., 1982; Rajakylä and Paloposki, 1983; Ford, 1984; Guy et al., 1984; Gorham, 1986; Al-Amoudi and Ali, 1989). More recent studies used spectrometric methods such as ^1H NMR (Jones et al., 1986), ^{13}C NMR (Larher, 1988), ^{15}N NMR (Lai et al., 1991), or fast atom bombardment mass spectrometry (FAB-MS) (Rhodes et al., 1987). However, these analytical methods require sophisticated and expensive facilities and preparation of samples is often tedious.

The aim of our work was to find an easy and suitable method for the analysis of betaine in a large number of samples obtained from halophytic plants collected on the salt marsh. A comparative study of the methods available showed that HPLC represented the most rapid, specific, and sensitive technique. Then, our aim was to optimize the different steps of the procedure from extraction to quantification of GB in order to make them rapid and more suitable.

MATERIALS AND METHODS

Plant Material. Three halophytic higher plants, *Suaeda maritima* Dum. (annual sea-blite), *Atriplex portulacoides* L. (oraches), and *Puccinellia maritima* (Huds.) Parl. (common saltmarsh-grass) were collected in the middle part of the salt marsh at a site called "Ferme Foucault" in the Mont Saint Michel Bay (France). *S. maritima* and *A. portulacoides* are species known to accumulate GB as a compatible solute in response to salt stress (Wyn Jones and Storey, 1981; Stewart et al., 1979). *P. maritima* accumulates mainly proline and *δ*-acetylornithine (Stewart et al., 1979). Shoots were washed in distilled water, blotted dry, frozen in liquid

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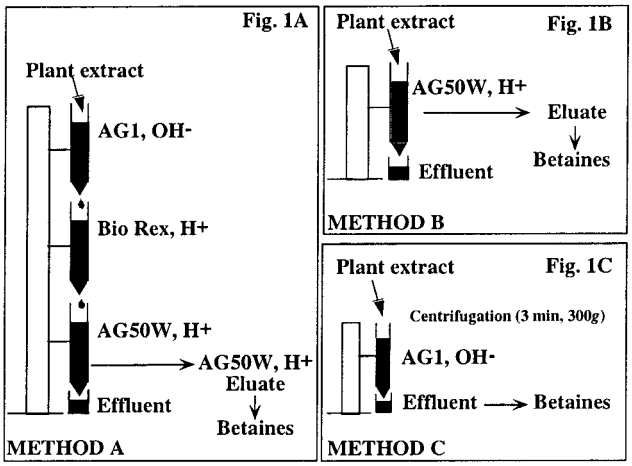

Figure 1. Schemes for plant crude extracts fractionation on various exchangers: (A) the three exchanger system (Hitz and Hanson, 1980); (B) AG 50W, H^+; (C) AG1, OH^-.

nitrogen, and stored at −20 °C. The samples were then freeze-dried, kept in a desiccator, and finally ground just before extraction.

Extraction of Osmoprotectants. Three methods of extractions were compared. The samples (200 mg) were mixed at 4 °C with 5 mL of distilled water (method 1), 5 mL of a mixture of methanol/chloroform/water 12:5:3 v/v (method 2), or 5 mL of a mixture of ethanol/water 90:10 v/v (method 3). Extracts were dried under reduced pressure at 35 °C. The residues were then solubilized in 1 mL of distilled water and stored at −20 °C before purification by ion exchange chromatography.

Fractionation of Crude Extracts by Ion Exchange Chromatography. Three procedures were compared.

Method A (Figure 1A). The three-exchanger system described by Hitz and Hanson (1980) and Guy et al. (1984) was used. The crude extract (500 μL) was first applied to a 4.5 mL column of AG1, X8 resin (200−400 mesh, OH^- form, Bio-Rad), the effluent of which flowed directly to a 4.5 mL Bio-Rex 70 resin (200−400 mesh, H^+ form, Bio-Rad) column and then onto a 4.5 mL AG 50W resin (200−400 mesh, H^+ form, Bio-Rad) column. The whole system was washed with 20 mL of distilled water. The adsorbed betaines were then eluted from the AG 50W, H^+ column with 10 mL of 4 M NH_4OH and 10 mL of distilled water. The eluates were dried at 35 °C under reduced pressure and then solubilized in 1 mL of deionized water for qualitative and quantitative determinations.

Method B (Figure 1B). The sole 4.5 mL column of AG 50W resin (200−400 mesh, H^+ form, Bio-Rad) was used. The crude extract (500 μL) was first applied, and the column was washed with 10 mL of distilled water. Then, the adsorbed betaines were eluted and concentrated as in method A.

Method C (Figure 1C). Only the AG1 resin (200−400 mesh, OH^- form, Bio-Rad) was used in a small column (1.6 mL). The column was dried down by centrifugation (3 min, 4 °C, 300*g*) before being loaded with 125 μL of crude extract. The column was then washed with 875 μL of distilled water. The whole effluent was collected by another centrifugation (3 min, 4 °C, 300*g*), frozen, and used for the assays.

Qualitative Determinations. The qualitative determination of GB and proline was made by high-voltage electrophoresis carried out on Whatman No. 3 paper, in 0.75 M formic acid (pH 1.9) at 40 V cm^{-1} during 90 min. Electrophoregrams were dried in an aerated oven at 40 °C for 2 h. Glycine betaine was revealed by spraying Dragendorff reagent as described by Dawson et al. (1969). Proline was revealed by spraying isatine (0.2% in acetone/acetic acid 50:1 v/v) and heating at 105 °C in an aerated oven for 30 min. Pure proline (Sigma) and GB (Sigma) were added or not to the samples as standards.

Table 1. Shoot GB Content of *Suaeda maritima* and *Atriplex portulacoides* Determined by HPLC after Three Methods of Extraction (Mean ± SD, $n = 3$)[a]

species	GB content (mmol g^{-1} dry weight)		
	method 1	method 2	method 3
S. maritima	93.4 ± 4.7	110.2 ± 6.0	105.2 ± 8.6
A. portulacoides	183.6 ± 12.4	206.0 ± 14.1	157.5 ± 11.1

[a] Purification was performed by ion exchange chromatography as described by Hitz and Hanson (1980) (method A). Method 1: distilled water extraction. Method 2: methanol/chloroform/water extraction. Method 3: ethanol/water extraction. Refer to Materials and Methods for details.

Quantitative Determinations. *Proline.* The proline amount was assessed by the method of Troll and Lindsley (1954) improved by Bates et al. (1973) and more recently by Magné and Larher (1992).

Betaine. The GB amount was determined by HPLC on a Spherisorb 5 ODS2 column (250 × 4.6 mm i.d.) preceded by a precolumn (10 × 1 mm) packed with the same phase. The mobile phase contained 13 mM sodium heptane sulfonic acid (Aldrich) and 5 mM Na_2SO_4 (Prolabo) in deionized water. The pH was adjusted to 3.7 with 1 N H_2SO_4. The degassed solvent was delivered by a LDC Analytical isocratic pump at a flow rate of 0.8 mL min^{-1}. The column effluent passed through a SM 4000 variable-wavelength UV detector (LDC Milton Roy), and the resolved compounds were detected at 200 nm. Quantitative determination of GB was achieved by comparing peak surface areas with those obtained with pure GB solutions in the range 0−5 mM.

Another analytical method involving ^1H NMR spectroscopy (Jones et al., 1986) was used to confirm the efficiency of our optimized GB quantification procedure. Accordingly, GB was extracted from *S. maritima* with a mixture of methanol/chloroform/water and purified on a AG 50W, H^+ exchanger. Then, ^1H NMR titration was realized on a Brüker spectrometer at a frequency of 500 MHz.

RESULTS AND DISCUSSION

GB Extraction. The efficiency of the three extracting methods was compared using crude extracts of two GB accumulators *S. maritima* and *A. portulacoides*. Subsequent purification was carried out by method A using the three-exchanger system (Hitz and Hanson, 1980).

For *S. maritima*, no significant difference was found among the three extraction procedures for GB content in the crude extracts (Table 1). For *A. portulacoides*, the amount of GB was slightly variable. In this latter species, the extraction with the ethanol/water mixture (method 3) yielded the lowest amount of GB. Among the two other extraction procedures, we did not find any significant variation. These results altogether led us to choose the simplest, the cheapest, and the equally efficient extractant as the others, i.e., cold water. In addition, this method is suitable for the titration of GB in large series of samples because numerous extracts

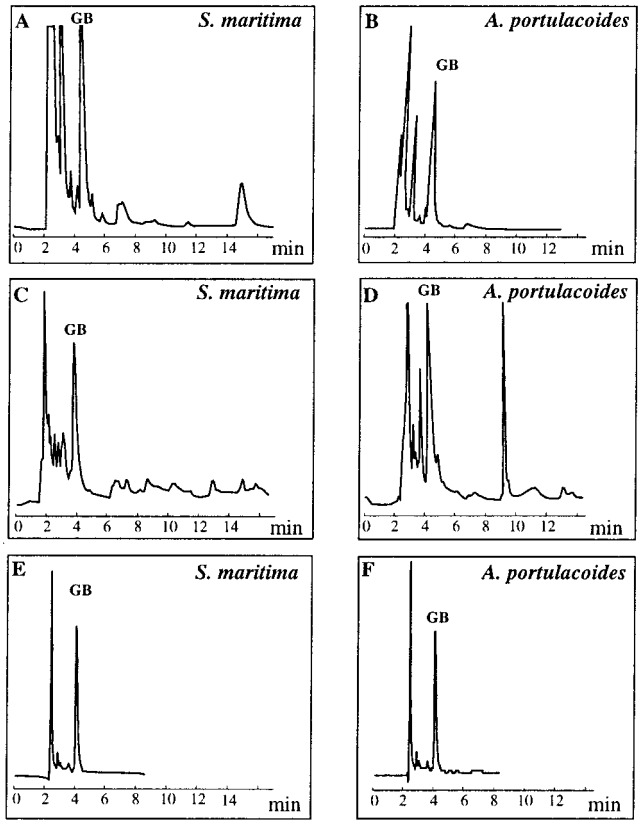

Figure 2. HPLC chromatograms obtained from water extracts of *S. maritima* (A, C, E) and *A. portulacoides* (B, D, F) purified by ion exchange chromatography using the three exchanger system (10) (A, B), a sole AG50W, H^+ column (C, D), or a small AG1, OH^- column (E, F).

can be freeze-dried easily instead of being concentrated under reduced pressure one after the other. Whatever the extraction procedure used, the HPLC profiles of the samples purified according to method A were quite similar (data not shown).

GB Purification. *Method A (Figure 1A).* While comparing the different methods of extraction, all chromatograms obtained either for *S. maritima* or *A. portulacoides* showed poor resolution for the GB signal. Indeed, while using a complex purification procedure with three types of resin, as described by Hitz and Hanson (1980), the GB signal for both species studied was always surrounded by various side signals (parts A and B of Figure 2). To avoid unidentified peaks interfering with that of GB in the HPLC chromatogram, we directed our efforts toward the research of an improved procedure of purification.

In a first step, the global effluent of the three-column system (method A) and the eluate of each exchanger were tested by high-voltage electrophoresis with a pure GB solution. After spraying Dragendorff reagent, we found a small spot corresponding to glycine betaine in Bio-Rex 70 eluate and a big one in AG 50W eluate but not in the global effluent. HPLC quantification of GB in both Bio-Rex 70 and AG 50W eluates showed that 94% of a pure sample of GB was adsorbed on the AG 50W exchanger and about 1% on the Bio-Rex 70. Similar results were obtained when a standard solution of GB was mixed with the plant crude extracts.

These results, together with the unsuitable HPLC patterns obtained by the method A purification (parts A and B of Figure 2), led us to test the AG 50W resin alone for the purification procedure of the aqueous extracts of *S. maritima* and *A. portulacoides* prior to HPLC quantification. Our interest focused on the effect of this simplification on the peak resolution by HPLC.

Table 2. Shoot GB Content of *Suaeda maritima* and *Atriplex portulacoides* Water Extracts Determined by HPLC after Three Methods of Fractionation (Mean ± SD, $n = 3$)[a]

species	procedure for fractionation	GB content (mmol g^{-1} dry weight)
S. maritima	method A	93.4 ± 4.7
	method B	95.1 ± 2.5
	method C	101.6 ± 3.0
A. portulacoides	method A	183.6 ± 12.4
	method B	220.2 ± 4.5
	method C	218.2 ± 6.9

[a] Method A: the three exchangers system. Method B: AG 50W, H^+ column. Method C: AG1, OH^- small column. Refer to Materials and Methods for details.

Method B (Figure 1B). When comparing the two methods of purification, the procedure using the single AG 50W resin gave a GB content similar to those obtained using method A for *S. maritima*, and 17% higher in the case of *A. portulacoides* (Table 2). Unfortunately, HPLC profiles of *S. maritima* and *A. portulacoides* water extracts still showed side peaks around the GB signal (parts C and D of Figure 2, respectively). In addition, it is likely that AG 50W resin alone used in method B did not allow separation of GB from compounds with similar retention times, unlike AG1 and/or Bio-Rex 70 resins used in method A. This could explain higher GB content in *A. portulacoides* compared with that found in method A.

To identify the compounds possibly responsible for the overestimation of GB quantification in *A. portulacoides* extract, high-voltage electrophoresis was performed using pure GB and pure proline as references. Thus, it became clear that GB was adsorbed on the AG 50W resin. In addition, the isatine test performed on the electrophoregram demonstrated the presence of a typical blue spot easily attributed to proline. A colorimetric titration revealed indeed a proline level of 5 μmol g^{-1} dry weight. This might account for the increase of GB detected previously, as we showed that pure proline injected in the HPLC system had the same retention time (about 4 min).

Thus, the purification with either the AG1 or Bio-Rex 70 resin (method A) removed the proline interfering with the quantification. The tests made on the three-column system also showed that about 1% of the GB was retained on the Bio-Rex 70 resin.

Method C (Figure 1C). The behavior of proline was tested on the first ion-exchange resin AG1, OH^- used in method A for the purification of betaines (method C). Pure proline and that contained in the aqueous extracts were completely adsorbed on this resin, as shown by titration with ninhydrin, whereas GB went through completely. The latter was only adsorbed on the third resin of method A (AG 50W) as described by Hitz and Hanson (1980). Other assays also demonstrated that 80% of the total amino acids contained in crude extracts were retained on the anionic resin (AG1, OH^-).

Thus, it was suggested that a single-step purification procedure of the crude extracts of *S. maritima* and *A. portulacoides* allowed complete removal of proline, which was otherwise coeluted with GB in our HPLC method. Moreover, this single purification allowed the

removal of most of the amino acids which could also interfere with GB quantification. Furthermore, GB was detectable directly in the effluent.

The results of GB quantification are presented in Table 2. Method C yielded equal or slightly larger amounts of GB than those obtained with the two previous purification methods (A and B). Using only one-step purification minimized the losses due to the multiple filtrations in the case of the three-column system (method A). Moreover, recovering directly GB in the effluent (method C) avoided the losses related to the elution involved in the other methods and the contaminants arising from the elution of the column. These results could also be rationalized by observing the corresponding HPLC chromatograms. The absence of interfering signals close to that of the GB peak allowed a better measurement (parts E and F of Figure 2).

This method of purification using only AG1, OH^- (method C) was tested with extracts from *P. maritima*, another salt marsh plant known to accumulate proline but not GB in response to salt stress. To confirm the absence of GB and the presence of proline in the aqueous extract of *P. maritima*, a high-voltage electrophoresis was carried out. The electrophoregram developed with isatine indicated the presence of proline in the extract, but no spot corresponding to GB was found on the electrophoregram developed with Dragendorff reagent.

The HPLC profile of the aqueous extract of *P. maritima* purified following method C on this single anionic resin (AG1, OH^-) did not show any signal at 4 min (data not shown). This indicated that proline was well adsorbed on the resin as shown before and that no GB was detectable in this plant in our experimental conditions.

Optimized Protocol for GB Quantification in Crude Extracts from Halophytic Plants. Crude extract was obtained by mixing 50 mg of plant dry matter with 1 mL of distilled water at 4 °C for 20 min, followed by centifrugation at 4 °C and 3000*g* for 15 min. Supernatant was collected, freeze-dried, and then solubilized in 500 μL of deionized water.

For sample fractionation, the AG1, OH^- small resin column (1.6 mL) was first dried down by centrifugation at 4 °C and 3000*g* for 3 min before being loaded with 250 μL of crude extract. Tests were first performed in order to know the amount of water needed to recover all the GB applied on the column at concentrations ranging from 3.75 to 10 mM. For a 250 μL sample, when the anionic resin was washed by 500 μL of water, 90% of GB was recovered. With 750 μL of water, GB recovery went up to 98%. So, 750 μL of deionized water was used to wash the column and to recover all the GB present in the extract. The column was then centrifugated for 3 min at 4 °C and 300*g* for the total recovery of the effluent. The effluent was stored at −20 °C until further HPLC quantification.

The molecules adsorbed on the AG1,OH^- resin were eluted with 1.5 mL of 2 N formic acid and 3.5 mL of distilled water for a complete recovery of proline. The resin column was then recycled by washing with 1.5 mL of 2 N NaOH and rinsing with distilled water until the effluent pH reached neutrality. If plant extracts were rich in anion with higher affinity than OH^- for AG1 resin, 10 mL of 2 N NaOH would be necessary to wash the column completely before another purification cycle.

Table 3. GB Content of Young Seedlings and Plant Parts of *Suaeda maritima* Collected on the Salt Marsh Determined by HPLC[a] or by ^{1}H NMR[b] (Values are Means of Three Determinations ± SD)

		GB content (mmol g^{-1} dry weight)	
	organ	HPLC	^{1}H NMR
seedlings		343 ± 5	343 ± 10
	roots	109 ± 5	108 ± 2
mature plants	young twigs	332 ± 25	330 ± 15
	mature twigs	241 ± 19	270 ± 4
	caulinary leaves	202 ± 4	218 ± 9

[a] GB content was determined by our optimized procedure. [b] GB content was assessed by Jones' NMR method (17).

Finally, the quality of the AG1,OH^- resin column was checked every 10 purification runs using pure glycine betaine test solutions at a concentration of 5 mM. It was shown that after 50 runs using the same resin, GB recovery was still higher than 92% and the chromatograms of the test solutions exhibited only the GB signal.

To validate our optimized procedure for GB quantification in crude extracts of halophytic higher plants, comparison was performed with an ^{1}H NMR technique (Jones et al., 1986). The GB contents of young seedlings and plant parts of *S. maritima* collected on the salt marsh were determined either by ^{1}H NMR or HPLC. The results, summarized in Table 3, established that our procedure gave similar values to those obtained with ^{1}H NMR. However, the NMR method (Jones et al., 1986) suffered from time-consuming and fastidious sample preparation and required expensive facilities. Consequently, our optimized procedure appears to be suitable for analysis of GB in large series of halophytic higher plant samples as it is as reliable, cheaper, and more rapid.

CONCLUSION

Among the extraction procedures tested, we showed that the simplest and the cheapest one, i.e., water extraction, was as efficient as the others.

For GB purification, Hitz and Hanson (1980) used a three-exchanger system while Guy et al. (1984) proposed a simplified one consisting of the two resins AG1, OH^- and Bio Rex 70, H^+ when no interferences with sugars were expected. Our results indicate that a simple purification of an aqueous plant extract over the sole anionic ion-exchange resin (AG1, OH^-) gives direct access to GB in the collected effluent. Furthermore, proline, 80% of amino acids, and soluble sugars, including glucose, fructose, and sucrose were removed by this procedure. Sugar analysis peformed with pure glucose, fructose, and saccharose showed that 100% of glucose or fructose, and 92% of sucrose, were retained on the AG1 small-column system.

The following HPLC titration took 5 min at ambient temperature, as compared to the 25 min at 60 °C needed by the HPLC method of Guy et al. (1984). Additional HPLC advantages are also numerous: low cost of the solvents, clear GB signal on the chromatograms, fast analysis, and accuracy and reproducibility of the results.

From all the different extraction and purification trials performed, we were able to describe a new simple, accurate, and fast method for GB determination, particularly suited for a large number of samples. Starting from a complex dry plant sample, it consists of a single aqueous extraction, purification over a sole cationic ion-

exchange resin, and an easy isocratic HPLC quantification on a C_{18} column. It has been shown elsewhere that the described procedure could also be of interest for GB determination in nonhalophytic higher plants induced to accumulate this compound through its uptake from the external medium (Gibon et al., 1997).

ABBREVIATIONS USED

QACs, quaternary ammonium compounds; GB, glycine betaine.

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LITERATURE CITED

Al-Amoudi, O. A.; Ali, A. Y. Some practical aspects of measurements of betaines and their sulphur analogues by the use of HPLC. *J. Microbiol. Methods* **1989**, *10*, 289−296.

Anthoni, U.; Christophersen, C.; Hougaard, L.; Nielsen, P. H. Quaternary ammonium compounds in the biosphere—an example of a versatile adaptative strategy. *Comp. Biochem. Physiol.* **1991**, *99B* (1), 1−18.

Bates, L. S.; Waldren, R. P.; Teare, I. D. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205−207.

Blunden, G.; Gordon, S. M. Betaines and their sulphonio analogues in marine algae. *Prog. Phycol. Res.* **1986**, *4*, 39−80.

Czonka, L. N. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **1989**, *53*, 121−147.

Czonka, L. N.; Hanson, A. D. Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* **1991**, *45*, 569−606.

Dawson, R. M. C.; Elliot, D. C.; Elliot, W. H.; Jones, K. M. Methods for the detection of biochemical compounds on paper and thin layer chromatograms, with some note on separation. In *Data for Biochemical Research*; Dawson, R. M. C., Ed.; University Press: Oxford, 1969.

Ford, C. W. Simultaneous determination of proline and betaines by high performance liquid chromatography. *J. Sci. Food Agric.* **1984**, *35*, 881−886.

Gibon, Y.; Bessieres, M. A.; Larher, F. Is glycine betaine a noncompatible solute in higher plants that do not accumulate it? *Plant Cell Environ.* **1997**, *20*, 329−340.

Gorham, J. Separation and quantitative estimation of betaine esters by high-performance liquid chromatography. *J. Chromatogr.* **1986**, *361*, 301−310.

Gorham, J.; McDonnell, E.; Wyn Jones, R. G. Determination of betaines as ultraviolet-absorbing esters. *Anal. Chim. Acta* **1982**, *138*, 277−283.

Guy, R. D.; Warme, P. G.; Reid, D. M. Glycinebetaine content of halophytes: Improved analysis by liquid chromatography and interpretations of results. *Physiol. Plant.* **1984**, *61*, 195−202.

Hitz, W.; Hanson, A. D. Determination of glycine betaine by pyrolysis-gaz chromatography in cereals and grasses. *Phytochemistry* **1980**, *19*, 2371−2374.

Jones, G. P.; Naidu, B. P.; Starr, R. K.; Paleg, L. G. Estimates of solutes accumulating in plants by ^{1}H nuclear resonance spectroscopy. *Aust. J. Plant Physiol.* **1986**, *13*, 649−658.

Lai, M. C.; Sower, K. R.; Robertson, D. E.; Roberts, M. F.; Gunsalus, R. P. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. *J. Bacteriol.* **1991**, *173*, 5352−5358.

Larher, F. Natural abundance 13C-nuclear magnetic resonance studies on the compatible solutes of halophytic higher plants. *Plant Physiol. Biochem.* **1988**, *26*, 35−45.

Magné, C.; Larher, F. High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Anal. Biochem.* **1992**, *200*, 115−118.

Rajakylä, E.; Paloposki, M. Determination of sugars (and betaine) in molasses by high-performance liquid chromatography: Comparison of the results with those obtained by the classical lane-eynon method. *J. Chromatogr.* **1983**, *282*, 595−602.

Rhodes, D.; Hanson, A. D. Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 357−384.

Rhodes, D.; Rich, P. J.; Myers, A. C.; Reuter, C. C.; Jamieson, G. C. Determination of betaines by fast atom bombardment mass spectrometry I: Identification of glycine betaine deficient genotypes of *Zea mays*. *Plant Physiol.* **1987**, *84*, 781−788.

Stewart, G. R.; Larher, F.; Ahmad, I.; Lee, J. A. Nitrogen metabolism and salt-tolerance in higher plant halophytes. In *Ecological process in coastal environments*; Jefferies, R. L., Davy, A. J., Eds.; Blackwell Scientific Publication: London, 1979.

Storey, R.; Wyn Jones, G. Quaternary ammonium compounds in plants in relation to salt resistance. *Phytochemistry* **1977**, *16*, 447−453.

Stumpf, D. K. Quantification and purification of quaternary ammonium compounds from halophyte tissue. *Plant Physiol.* **1984**, *75*, 273−274.

Troll, W.; Lindsley, J. A photometric method for the determination of proline. *J. Biol. Chem.* **1954**, *215*, 655−660.

Wyn Jones, R. G.; Storey, R. Betaines. In *Physiology and Biochemistry of Drought Resistance in Plants*; Paleg, L. G., Aspinall, D., Eds.; Academic Press: Sydney, 1981.

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