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Characterization of betaines using electrospray MS/MS

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Abstract

Betaines are an important class of naturally occurring compounds that function as compatible solutes or osmoprotectants. Because of the permanent positive charge on the quaternary ammonium moiety, mass spectrometric analysis has been approached by desorption methods, including fast atom bombardment and plasma desorption mass spectrometry. Here we show that electrospray ionization MS gives comparable results to plasma desorption MS for a range of authentic betaine standards and betaines purified from plant extracts by ion exchange chromatography. A distinct advantage of electrospray ionization MS over plasma desorption MS is the capability of obtaining product ion spectra via MS/MS of selected parent ions, and hence structural information to discriminate between ions of identical mass.

Keywords: Betaines; Electrospray ionization; MS/MS; *Lamium maculatum*; *Limonium latifolium*; *Physostegia virginiana*; *Achillea filipendulina*

1. Introduction

Betaines are a class of naturally occurring compounds that have an important role in osmotic stress resistance in a variety of organisms, including bacteria, algae, mammals and plants (Blunden and Gordon, 1986; Csonka and Hanson, 1991; Rhodes and Hanson, 1993; Wyn Jones and Storey, 1981). As the inner salts, they possess a permanently positively-charged quaternary ammonium group, and a negatively charged carboxyl group (Rhodes and Hanson, 1993; Wyn Jones and Storey, 1981). High concentrations of these solutes in the cytoplasm stabilize folded protein structures and membranes against the adverse/destabilizing effects of inorganic ions and high temperatures (Rhodes and Hanson, 1993). Common naturally occurring betaines in higher plants include glycine betaine, β-alanine betaine, proline betaine, hydroxyproline betaines, pipecolate betaine, hydroxypipecolate betaines, and trigonelline (nicotinic acid betaine) (Blunden et al., 1996; Bonham et al., 1995; Hanson et al., 1991, 1994; McLean et al., 1996; Rhodes and Hanson, 1993; Wood et al., 1991; Wyn Jones and Storey, 1981; Yuan et al., 1992). Many other betaines have been identified in marine algae (Blunden and Gordon, 1986; Blunden et al., 1982, 1988).

Betaines have been analyzed by a wide variety of desorption-type ionization techniques including fast atom bombardment (FAB) (Rhodes et al., 1987; Hanson and Gage, 1991; Hanson et al., 1991, 1994), desorption chemical ionization (DCI) (Wood et al., 1991) and plasma desorption (PD) (Bonham et al., 1995; Yang et al., 1995) mass spectrometry. FAB detection of betaines benefits from reverse derivatization, optimally as the *n*-butyl or *n*-propyl esters (Rhodes et al., 1987). This imparts increased surface activity to the betaine in a glycerol matrix, and produces intense molecular cations associated with the molecular weight of the betaine ester (Rhodes et al., 1987; Brunk et al., 1989; Hanson et al., 1991; Nolte et al., 1997). Fragment ions are typically associated with loss of the alcohol group in the FAB mass spectrum of a given betaine–alcohol ester (Rhodes et al., 1987). However, FAB has been successfully applied to the underivatized compounds, where typically the major ion observed is from the protonated

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species ($M+H^+$) or its sodium adduct ($M+Na^+$) (Blunden and Gordon, 1986; Hanson and Gage, 1991; Yuan et al., 1992). This is very similar to the PD mass spectrum of the underivatized betaines (Bonham et al., 1995; Yang et al., 1995). The DCI mass spectrum of underivatized betaines is dominated by the protonated species ($M+H^+$) and fragment ions resulting from thermal loss of one of the quaternary methyl groups (Wood et al., 1991). The obvious advantage of direct detection of betaines without prior derivatization is that this eliminates a time-consuming step in sample preparation. However, it has the drawbacks of reduced sensitivity, and incomplete resolution of complex mixtures of betaines, often found in plant tissues. For example, whereas trigonelline and hydroxyproline betaine can easily be resolved as their *n*-butyl esters, in the underivatized forms the sodium adduct of underivatized trigonelline has the same molecular weight as the $M+H^+$ ion of hydroxyproline betaine, rendering it difficult to quantify hydroxyproline betaine in the presence of an excess of trigonelline. In the underivatized form, glycine betaine yields a transmethylation product ($M+CH_3^+$) that has the same molecular mass as the $M+H^+$ ion of β-alanine betaine, rendering it difficult to quantify trace levels of β-alanine betaine in the presence of an excess of glycine betaine.

In this study electrospray ionization (ESI) has been used to ionize both authentic underivatized betaines as well as underivatized ion-exchange purified betaine fractions from several different plant species. In addition MS/MS was used to obtain structural information, and in particular to test whether this could overcome some of the drawbacks of the methods described above. ESI has become an invaluable ionization technique for nonvolatile components (Gaskell, 1997; Yates, 1998). Samples can be analyzed by ESI using either direct injection or through liquid chromatographic introduction. Typically ESI forms protonated molecules with little or no fragmentation.

2. Results

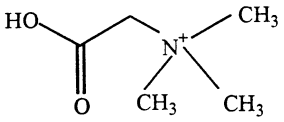
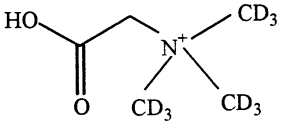
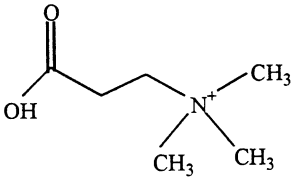
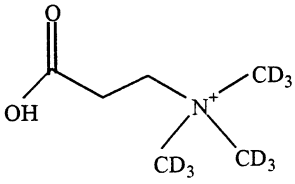
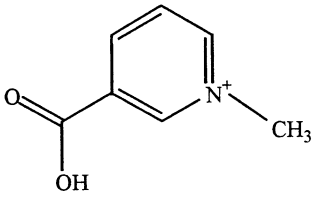
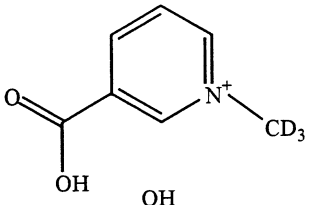
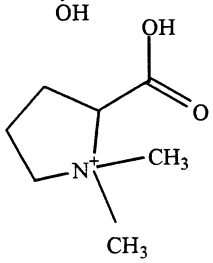
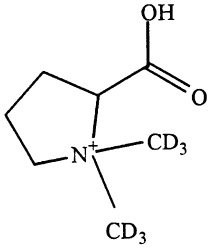
The standards, glycine betaine, d_9-glycine betaine, β-alanine betaine, d_9-β-alanine betaine, trigonelline, d_3-trigonelline, proline betaine and d_6-proline betaine all give good electrospray data. The spectra all show large peaks due to the protonated molecules ($M+H^+$) as well as peaks due to their sodium adducts ($M+Na^+$), and the proton- and sodium-bond dimers (M_2+H^+), and (M_2+Na^+).

The base peak in the ESI mass spectrum of proline betaine ($M+H^+ = m/z$ 144) is the proton-bound dimer (m/z 287), with the $M+H^+$ ion of proline betaine being the next most intense ion. This is similar to the PDMS spectrum, except that the dimers were less abundant in the PD spectrum (not shown). This difference, not investigated, may be related to the relative sensitivites of the two techniques.

As noted in the Introduction, one of the limitations of PDMS, in the analysis of underivatized betaine mixtures, is the fact that there are overlapping mass-to-charge ion ratios for certain species; e.g. the $M+Na^+$ ion of trigonelline has the same m/z ratio as the $M+H^+$ ion of hydroxyproline betaine; and the $M+CH_3^+$ transmethylation product of glycine betaine has the same m/z ratio as the $M+H^+$ ion of β-alanine betaine. This can pose problems when attempting to identify betaines in mixtures. For this reason the MS/MS spectra of a series of authentic betaines were analyzed in order to assess the structural information that could be obtained. These MS/MS spectra were produced by exciting the protonated molecule with an equivalent tickle voltage of 32.5% (see Table 1 and Experimental). The ion at m/z 59, corresponding to $(CH_3)_3N^+$, is the base peak in the MS/MS spectrum of glycine betaine ($M+H^+ = m/z$ 118). This is confirmed by the MS/MS spectrum of the d_9-analog ($M+H^+ = m/z$ 127) which produces a base peak, m/z 68, nine mass units higher than the unlabeled compound. For β-alanine betaine ($M+H^+ = m/z$ 132) the base peak in the MS/MS spectrum is m/z 60, consistent with $(CH_3)_3NH^+$. The corresponding d_9-β-alanine betaine gives a base peak at m/z 69, consistent with deuteration of the three methyl groups: $(CD_3)_3NH^+$. The MS/MS spectrum of trigonelline ($M+H^+ = m/z$ 138), gives a base peak at m/z 94, consistent with the loss of CO_2. d_3-Trigonelline has only one deuterium labeled methyl group and the base peak in the MS/MS spectrum shows an increase in mass of three Daltons to m/z 97, as expected. The base peak in the MS/MS spectrum of proline betaine ($M+H^+ = m/z$ 144) is m/z 84. The ion results from a rearrangement fragmentation process resulting in the loss of C_3H_6 followed by loss of water. The d_6 analog, m/z 150, produces a base peak at m/z 90, in the MS/MS spectrum. Of the four standard betaines analyzed, trigonelline is the only one whose base peak is not a result of the loss of C_xH_y–CO_2H. This is because trigonelline has an aromatic ring as its backbone. In all cases, however, charge retention in the MS/MS spectra of the base peak is with the quaternary ammonium ion. These results indicate that electrospray ionization MS/MS can be used to provide structural information for identifying unknown betaines, or for resolving complex mixtures.

Four plant samples were analyzed by both ESI and PDMS. The results are summarized in Table 2, and are consistent with previous PDMS results (Bonham et al., 1995). As can be seen the same ions were observed in both sets of spectra except the electrospray spectra include significant ion intensity for the gas phase dimers—as pointed out when discussing the individual standard spectra. In addition, mixed dimers were also

Table 1
Structures and electrospray MS of betaine standards

Compound	Structure	Parent (m/z)	Product ions (m/z)
Glycine betaine		118	<u>59 (M-CH_2COOH)</u> 58 (M-CH_3COOH)
d_9-Glycine betaine		127	<u>68 (M-CH_2COOH)</u> 66 (M-CH_2DCOOH)
B-Alanine betaine		132	<u>60 (M-$CH_2CHCOOH$)</u> 74 (M-$N(CH_3)_3$)
d_9-*B*-Alanine betaine		141	<u>69 (M-$CH_2CHCOOH$)</u> 73 (M-$N(CH_3)_3$
Trigonelline		138	<u>94 (M-CO_2)</u> 110 (M-CO)
d_3-Trigonelline		141	<u>97 (M-CO_2)</u> 113 (M-CO)
Proline betaine		144	<u>84 (M-C_3H_6-H_2O)</u> 102 (M-C_3H_6) 98 (M-HCOOH) 58 ($CH_2N^+(CH_3)_2$
d_6-Proline betaine		150	<u>90 (M-C_3-H_6-H_2O)</u> 108 (M-C_3H_6) 104 (M-HCOOH) 64 ($CH_2N^+(CD_3)_2$

The underlined ions are the base peaks of the MS/MS spectra.

observed. A comparison of the PDMS and ESI spectra from the *Lamium maculatum* sample is given in Fig. 1. In *L. maculatum*, proline betaine ($M+H^+ = m/z$ 144), trigonelline ($M+H^+ = m/z$ 138), and hydroxyproline betaine ($M+H^+ = m/z$ 160) were the dominant betaines detected by both methods (Fig. 1). The ion at m/z 182, probably corresponds to the $M+Na^+$ ion of hydroxyproline betaine (Fig. 1).

When standards are available, the electrospray MS/MS spectra can be matched directly with the spectra of authentic betaine standards for confirmation. For example, the product ion spectrum of the $M+H^+$ ion of proline betaine (m/z 144) (Fig. 1) matches exactly with the authentic standard, giving diagnostic product ions at m/z 84, 102, 98 and 58 (cf. Table 1). The MS/MS spectra obtained for the m/z 138 ion matched exactly with the authentic trigonelline standard, with diagnostic product ions at m/z 94 and 110 (not shown). The major product ions observed for the *Lamium* m/z 160 peak were m/z 142, 98 and 88, consistent with the expected fragmentation behavior of hydroxyproline betaine. Thus, the product ion at m/z 142 results from loss of H_2O. However, an authentic standard of hydroxyproline betaine was not available for comparison of MS/MS spectra.

In *Achillea filipendulina*, the dominant ions in the ESI spectrum were m/z 160, 158, 144, 174 and 142 (Table 2). The MS/MS spectra obtained for the m/z 160 and 174 ions appear consistent with hydroxyproline betaine and

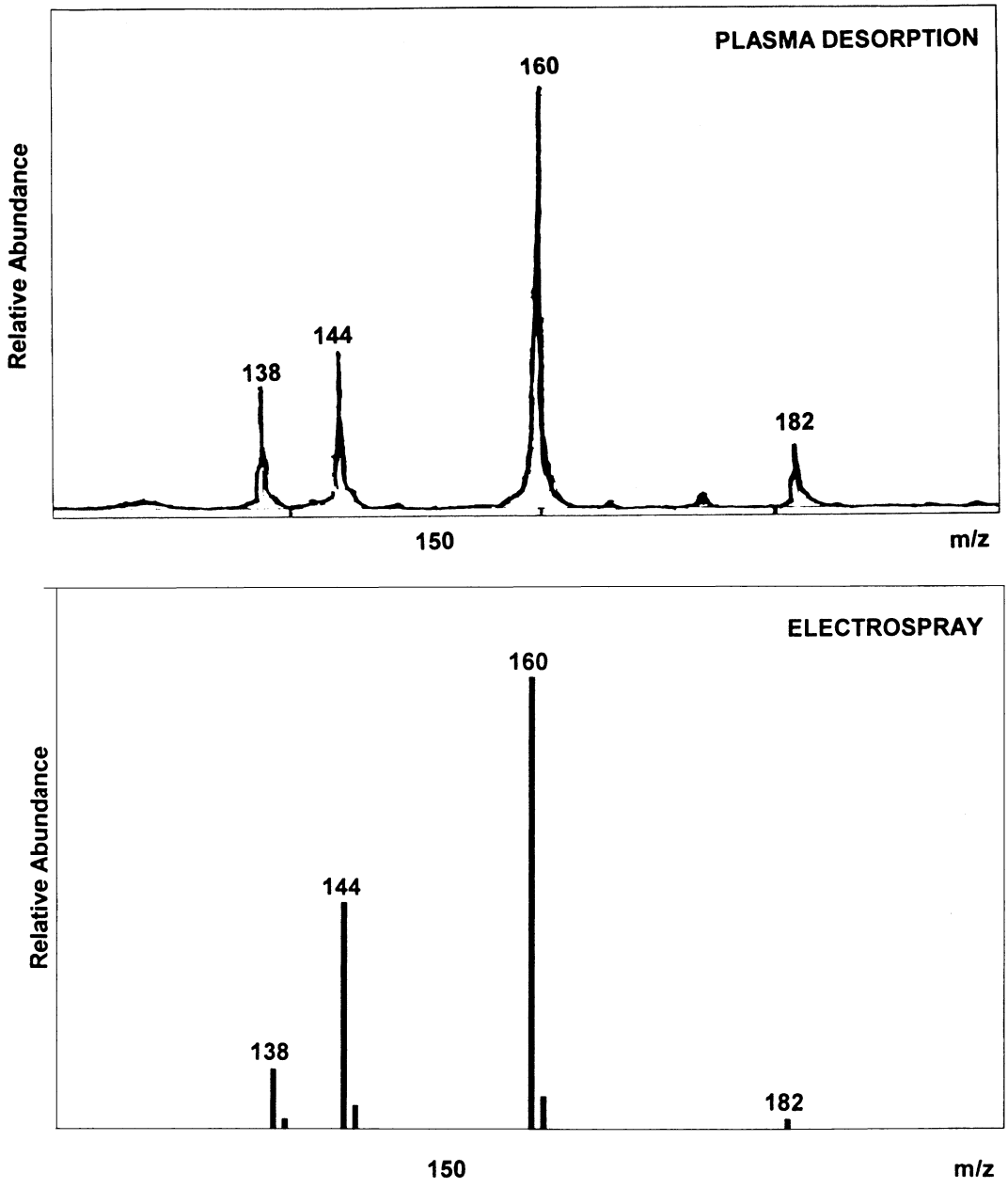


Fig. 1. Comparison of the PDMS and ESI mass spectra from the *Lamium maculatum* sample.

Table 2
PDMS and electrospray MS of betaines from four plant species[a]

Plant sample	PDMS ions m/z (intensity)	Electrospray ions m/z (intensity)
Lamium maculatum	138 (28%), 144 (32%), 160 (100%)	138 (13%), 144 (51%), 160 (100%)
Limonium latifolium	132 (100%)	132 (100%)
Physostegia virginiana	118 (8%), 138 (100%), 160 (13%)	118 (5%), 138 (100%), 160 (6%)
Achillea filipendulina	130 (6%), 142 (5%), 144 (41%), 158 (31%), 160 (100%), 174 (10%)	142 (5%), 144 (61%), 158 (83%), 160 (100%), 174 (10%)

[a] The underlined ions (m/z) are the base peak of the given mass spectrum; the intensities are relative to the base peak.

hydroxypipecolate betaine, respectively. Thus, the major product ions observed for the *Achillea* m/z 160 peak were m/z 142, 98 and 88; and the major product ion observed for the *Achillea* m/z 174 peak was m/z 156. The ion at m/z 158 in the betaine spectrum of *Achillea* has previously been attributed to pipecolate betaine (Bonham et al., 1995). However, the major product ions observed for the *Achillea* m/z 158 peak were m/z 140, 112, 102 and 72. The occurrence of a 140 product ion in the MS/MS spectrum of the *Achillea* m/z 158 peak, suggests loss of H_2O from the molecule. Such fragmentation behavior would not be expected of pipecolate betaine. Analysis of the MS/MS spectrum of the m/z 142 ion of the *Achillea* electrospray mass spectrum showed that this gave a major product ion at m/z 98, consistent with this compound being a dehydroproline betaine, as initially proposed by Bonham et al. (1995). It is conceivable that the ion at m/z 158 represents a hydroxy-derivative of dehydroproline betaine, and not pipecolate betaine. These results suggest the need for further syntheses and MS/MS analyses of authentic betaine standards, with particular emphasis on comparison of daughter spectra of pipecolate, hydroxypipecolate, dihydroxypipecolate, hydroxyproline, dehydroproline, and hydroxydehydroproline betaines.

Limonium latifolium was found to contain β-alanine betaine [as previously reported (Hanson and Gage, 1991; Bonham et al., 1995)]. *Physostegia*, was found to contain trigonelline (m/z 138), as previously documented (Bonham et al., 1995). The ion at m/z 160 in the *Physostegia* betaine fraction (Table 2) could correspond to either the $M+Na^+$ adduct of trigonelline or the $M+H^+$ ion of hydroxyproline betaine. Because the product ion spectrum of the *Physostegia* m/z 160 ion revealed a spectrum very similar to that of authentic trigonelline (with a base peak at m/z 116, loss of CO_2) (cf. Table 1), we conclude that this ion is the $M+Na^+$ adduct of trigonelline. The product ion spectrum of the ion at m/z 118 in the *Physostegia* sample (Table 2) matches the MS/MS spectrum of authentic glycine betaine, confirming that this ion is glycine betaine (not shown).

To further explore this capability of MS/MS to discriminate between ions of the same mass we applied electrospray MS/MS to the m/z 132 ion that is invariably found in samples that are rich in glycine betaine (Bonham et al., 1995). This ion is thought to arise by transmethylation during ionization, because it is seen when authentic glycine betaine is analyzed (Bonham et al., 1995). However, in samples of plant materials it is essential to prove that this ion is not from β-alanine betaine. In this case, the m/z 132 ion in the betaine fraction of maize, which contains glycine betaine and trigonelline (Bonham et al., 1995; Yang et al., 1995), was examined using MS/MS. The product ion spectrum is dominated by the ions at m/z 58 and 59 confirming that the ion at m/z 132 is the transmethylation product of glycine betaine and not β-alanine betaine.

3. Concluding remarks

Whereas both PDMS and electrospray give good spectra of the betaines, electrospray is more helpful in identifying betaines in a mixture due to its MS/MS capability. In this way a positive identification of the betaines present in several plant samples could be made, and the product ion spectra permit unambiguous discrimination between betaine ions of identical mass. Even more important, however, these results demonstrate the potential for identifying unknown betaines using the fragmentation pattern obtained in ESI MS/MS. The present results specifically lead us to question whether the compound ($M+H^+ = m/z$ 158) previously suggested to be pipecolate betaine in *Achillea filipendulina* (Bonham et al., 1995), may be a hydroxy derivative of dehydroprolinebetaine. In principle, this technique could be of great benefit in stable isotope tracer studies on betaine biosynthesis pathways because the MS/MS spectra provide information about the location of labeled atoms. We are particularly interested in following stable isotope incorporation into betaine methyl groups [e.g. from methyl deuterium (CD_3S-) labeled methionine], in order to estimate betaine synthesis rate and to quantify betaine synthesis demands on plant one-carbon metabolism.

4. Experimental

The synthetic betaines used in this study were prepared essentially as described by Chen and Benoiton

(1976). The starting materials for betaine synthesis were the corresponding amino acids (i.e. glycine for glycine betaine; L-proline for proline betaine; β-alanine for β-alanine betaine; and nicotinic acid for trigonelline). Samples of amino acids of ~100 mg (all from Sigma Chemical Co., St Louis, MO) were dissolved in 10 ml MeOH, to which was added 200 mg $NaHCO_3$, and either 3 ml methyl iodide (CH_3I) (for synthesis of unlabeled betaines) or 3 ml 99% deuterated methyl iodide (CD_3I) (Sigma Chemical Co., St Louis, MO) (for synthesis of deuterated betaines). Mixtures were stirred at room temperature in the dark in screw-capped vials with Teflon coated septa, for 48 h. Samples were then filtered through Whatman No. 1 filter paper, the filtrate was dried under a stream of air at room temperature, redissolved in 5 ml water and applied to a 10 cm column of a mixed-bed resin of Dowex-1-OH^- and Biorex-70-H^+ (1:1 v/v). This mixed-bed resin retains any unreacted amino acid but not betaines. The aqueous wash from this column (40 ml) was then applied to a 10×2 cm column of Dowex-50-H^+. The aqueous wash from this column (60 ml) was discarded and betaines were eluted with 30 ml 6 M NH_4OH and concentrated to dryness under a stream of air.

Plant samples [approx. 1 g fresh weight each of leaf tissue of *Lamium maculatum* cv. 'Beacon Silver' (Lamiaceae), *Limonium latifolium* (Plumbaginaceae), *Physostegia virginiana* cv. 'Vivid' (Lamiaceae) and *Achillea filipendulina* cv. 'Moonshine' (Asteraceae)] were collected from the Department of Horticulture and Landscape Architecture Garden, Purdue University in late June. These species were chosen because they have previously been found to contain trigonelline (*Lamium maculatum*, *Physostegia virginiana*, *Achillea filipendulina*), proline betaine (*Lamium maculatum*, *Achillea filipendulina*), hydroxyproline betaine (*Lamium maculatum*, *Achillea filipendulina*), pipecolate betaine (*Achillea filipendulina*), hydroxypipecolate betaine (*Achillea filipendulina*; *Lamium maculatum)* and β-alanine betaine (*Limonium latifolium*) (Bonham et al., 1995; Hanson et al., 1991; Yuan et al., 1992). Plant tissue was extracted in 10 ml MeOH at 4 °C for at least 48 h, and the extracts were then phase separated with 5 ml $CHCl_3$ and 6 ml water. The upper aqueous phase was removed and dried under a stream of air, redissolved in 2 ml water, and applied to a 4×1 cm column of a mixed-bed resin of Dowex-1-OH^- and Biorex-70-H^+ (1:1 v/v). The aqueous wash from this column (8 ml) was then applied to a 4×1 cm column of Dowex-50-H^+. The aqueous wash from this column was discarded and betaines were eluted with 6 ml 6 M NH_4OH and concentrated to dryness under a stream of air.

Maize leaf betaine extracts were from the glycine betaine-accumulating genotype PUD7, grown under salinized conditions (150 mM NaCl for 1 week) in the greenhouse, essentially as described by Yang et al. (1995). Betaines were extracted and purified from maize leaf tissue as described for other plant leaf samples, above.

The electrospray ionization analyses were carried out on a FinniganMAT LCQ (Thermoquest Corp, San Jose, CA) mass spectrometer system. The source voltage was set at 3.5 kV, the capillary voltage is typically set to (30 V) and the capillary temperature 225 °C. Typical background source pressure was 1.5×10^{-5} Torr as read by an ion gauge. The sample flow rate was 10 μl per min. The drying gas was nitrogen. The LCQ was scanned to 2000 amu for these experiments. The samples were dissolved in water and prior to injecting into the mass spectrometer a drop of MeOH and a drop of acetonitrile was added.

Helium was introduced into the system to an estimated pressure of 1 mTorr to improve trapping efficiency and also acted as the collision gas during the collisionally activated decomposition (CAD) experiments. The collision energy was set to 32.5% of the maximum available from the 5 V tickle voltage.

The PD mass spectrometric results were obtained using a Bioion 20R (BIOION KB; Uppsala, Sweden) plasma desorption mass spectrometer (PDMS). This instrument utilizes a ^{252}Cf ionizing source which produces MeV fission fragments. The interaction of the fission fragments with the sample produces ions which are mass analyzed with a time-of-flight mass spectrometer (Roepstorff, 1989). The betaines (redissolved in 50 μl of MeOH) were applied (2 μl) to a nitrocellulose-coated mylar target, and allowed to absorb and dry prior to being put into the mass spectrometer. The accelerating potential was set at 17,000 kV, with data being collected for 15 min.

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