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GLYCINEBETAINE IN OILSEED RAPE AND FLAX LEAVES: DETECTION BY LIQUID CHROMATOGRAPHY/CONTINUOUS FLOW SECONDARY ION-MASSSPECTROMETRY

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Key Word Index—*Brassica napus*; Cruciferae; *Kochia scoparia*; Chenopodiaceae; *Linum usitatissimum*; Linaceae; osmolyte; osmotic stress; betaine; *N*,*N*,*N*-trimethylglycine; quaternary ammonium compounds.

Abstract—Glycinebetaine [betaine; $(Me)_3-N^+-CH_2-COO^-$] is likely to contribute to osmoregulation in those plants that accumulate significant amounts of it, and hence the interest in engineering its synthesis in 'non-accumulators'. Although only large amounts of betaine, as found in the chenopods *Kochia scoparia* (298.6 μmol g^{-1} dry wt) and *Salicornia rubra* (162 μmol g^{-1} dry wt), would be physiologically significant, its presence in flax (*Linum usitatissimum*, 4 μmol g^{-1} dry wt) and rapeseed (*Brassica napus*; 4.9 μmol g^{-1} dry wt) indicates a genetic potential for betaine synthesis in these important plants. The liquid chromatography/continuous flow secondary ion-mass spectrometry method described here offers a 10-fold increase in sensitivity over other methods, and allows for detection of as little as 5 pmol μl^{-1} of underivatized betaine. The sensitivity of this method was further demonstrated by quantitation in the range of 50–1000 pmol μl^{-1}.

INTRODUCTION

Betaine is a non-perturbing solute accumulated by some bacteria, plants and animals [1, 2]. Identified first in 1863 in an arid zone shrub *Lycium barbarum*, its scattered presence has since then been demonstrated in plants of diverse taxa [2–4]. Significant quantities of it (> 100 μmol g^{-1} dry wt) are found in some salt- and drought-tolerant plants but much less (0.2 μmol g^{-1} dry wt) or no measurable amount in others. A physiological role for betaine as a biocompatible osmolyte has been proposed [4]. This hypothesis is based on and supported by observations pertaining to the presence of betaine vis-à-vis plant habitat, upward regulation of betaine synthesis in plants subjected to salt or drought stress, and the stabilizing effect of betaine on biomacromolecules against denaturing conditions. More recently, a genetic proof for osmoprotective function of betaine has been secured in bacterial systems [5–7]. Currently, there is substantial interest in genetic engineering of betaine synthesis as a means for ameliorating tolerance to osmotic stress in plants [8–10]. An important question in this regard—from both an evolutionary and engineering perspective—is whether the so-called non-accumulators are indeed genetically incompetent for betaine synthesis. Sensitive techniques for betaine estimation are necessary to address this question satisfactorily.

The methods for betaine analysis vary in sensitivity and specificity. These include the earlier and somewhat semi-quantitative or non-specific methods involving TLC [11] or spectrophotometry of periodide precipitates [12], and the more recent methods that make use of ^1H NMR [13] or mass spectrometry [2, 14]. Of all these, fast atom bombardment (FAB)-mass spectrometry or secondary ion (SIMS), where compounds are ionized (by neutral atoms or primary ion bombardment) and the mass-to-charge (m/z) signatures for the resultant protonated molecular ions ($[MH]^+$, secondary ions, hence SIMS) are generated, is the most sensitive. In mass spectral analysis of betaine, prior derivatization of betaine yielding *n*-butyl or *n*-propyl esters is employed to enhance the surface activity and consequently the sensitivity of detection [15]. We describe an LC-mass spectral method utilizing continuous-flow packed fused-silica capillary secondary ion mass spectrometry that allows for detection of 5 pmol μl^{-1} of betaine. This method does not require complex derivatization but offers a significant measure of sensitivity, specificity and ease required for analysing small amounts of samples. Furthermore, we show for the

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first time that the most widely cultivated varieties of the oilseed crop plants *Brassica napus* and *Linum usitatissimum* contain low but appreciable amounts of betaine.

RESULTS AND DISCUSSION

Betaine profile in LC-MS and establishment of a calibration standard

The elution profile of betaine in LC was analysed by selected ion monitoring of the protonated molecular ion of betaine at m/z 118.087 with an input of 1 nmol μl^{-1} (Fig. 1A) or 5 pmol μl^{-1} (Fig. 1B) of betaine. Betaine was found to elute as a single peak at *ca* 5 min. The visible noise level (signal-to-noise of 4:1) in the 5-pmol sample indicates that this might be approaching the lower detection limit. Rhodes *et al.* [14] mentioned that the lowest detection limit for *n*-propyl betaine was 50 pmol μl^{-1} at a signal-to-noise ratio of 2:1. Thus, much lower amounts of betaine were detectable using the protocol described here.

An internal standard of deuterated betaine [$(CD_3)_3$–N^+–CH_2–COO^-; abbreviated as d_9] was used, negating any variability in sample extraction, recovery and instrument sensitivity. The mass spectral response ratio of different concentrations of undeuterated betaine (referred to as d_0; 50–1000 pmol μl^{-1}) to a constant amount of d_9 (890 pmol μl^{-1}) plotted as a function of the concentration ratio provided the response factor (Fig. 2). The calibration plot was linear for this range. The R_F was slightly more than 1.0 for an equimolar concentration, probably because of some differences in the fragmentation of the deuterated standard. As evident from the noise levels in Fig. 1B, quantitation at the lower detection limits would not be accurate. Although not attempted here, use of multiple samples and subtraction of the noise might offer a solution. Nonetheless, the sensitivity of detection of 5 pmol μl^{-1} compares very favourably with other methods. Rhodes *et al.* [14] found that their calibration standard for *n*-propyl betaine was linear in the range of 450–45 000 pmol μl^{-1} and further mentioned that the lowest detection limit was 50 pmol μl^{-1} with FAB-mass spectrometry. A higher value of 85 pmol μl^{-1} was reported for HPLC/UV detection [16].

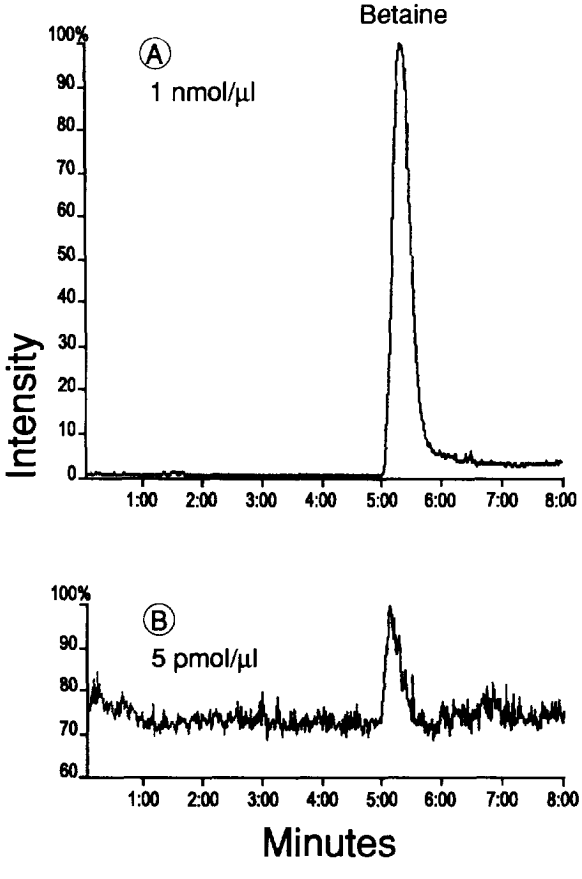

Fig. 1. LC-CFSI-MS of betaine showing the relative intensity of the sample contents at different retention times. Selected ion chromatograms for the protonated molecular ion of betaine (m/z 118.087) were obtained for 1 nmol μl^{-1} (A) and 5 pmol μl^{-1} (B) of betaine as described in Experimental.

Amounts of betaine in plant samples

Brassica napus and *L. usitatissimum* are economically important oilseed crops. There have been no reports on the presence of betaine in these species. To assess the usefulness of this technique, and to test these plants for betaine accumulation, leaf extracts from oilseed rape and flax were analysed (Table 1). In addition, *Salicornia rubra*, a Chenopodiaceae from a local saline slough, was also included in this analysis. All other plants included were known to be either non-accumulators (tobacco; pea; [17]) or accumulators (kochia, an archetypal accumulator; [3, 18]). As expected, the halophytes contained much greater amounts (*ca* 100-fold) of betaine compared

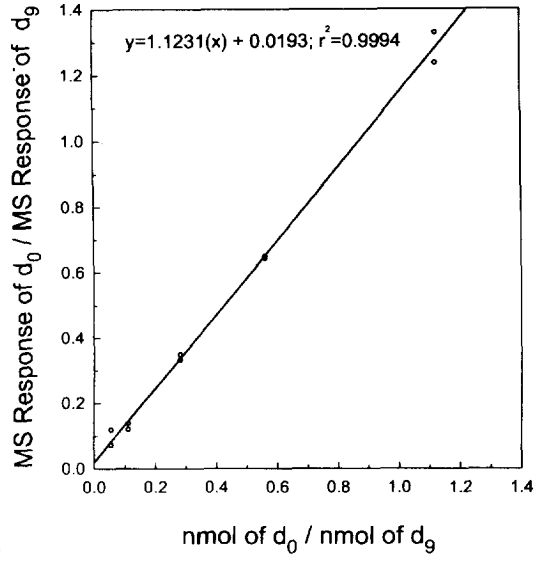

Fig. 2. Calibration curve for various amounts (50–1000 pmol μl^{-1}) of d_0 and a constant amount of d_9-betaine (890 pmol μl^{-1}) giving a concentration ratio (d_0/d_9) of 0.056:1.123.

Table 1. Amount of betaine in plant samples

Plant species	Betaine (μmol g^{-1}) Fresh wt	Dry wt
Brassica napus	0.4	4.9
Linum usitatissimum	0.3	4.0
Pisum sativum	0.3	1.9
Nicotiana tabacum	0.5	2.8
Kochia scoparia	50.8	298.6
Salicornia rubra	NA	162.0

With the exception of *S. rubra*, all other plants were greenhouse-grown in 2-1 pots containing vermiculite or Redi-earth (W. R. Grace Co., Saskatoon) and a standard nutrient/fertilizer solution (Plant Products Co. Ltd., Bramalea, ON). *Salicornia rubra* plants were from a saline slough, and the entire aerial part of the plants was used. Leaf samples collected from other plants were dried for 15 hr in a vacuum oven (60°) to obtain dry weights.

NA, Not applicable; dried samples were used for this plant.

The values are the average for two mass spectral analyses of single sample preparations.

with tobacco or pea. Both flax and oilseed rape were also found to contain betaine. The amounts were approximately twice as much as in pea, but given the small difference all these non-halophytes would be considered as non-accumulators. Interestingly, *N. tabacum* cv. Xanthi leaves were found to contain approximately 10 times more betaine than that previously reported for cv Samsum (0.3 μmol g^{-1} dry wt [17]). Such variations within a species have been observed, for example, in maize [19].

The betaine content in oilseed rape, flax, pea and tobacco would not be sufficient to combat osmotic stress. Nevertheless, its presence indicates that these plants have the genetic potential to produce betaine. Since choline oxidation via betaine aldehyde is the major pathway for biosynthesis of betaine, the low levels of betaine might suggest inefficient choline oxidation. This possibility aside, existence of other pathways (e.g, methylation of glycine as in *Methanohalophilus*; [20]) that might be less productive for betaine synthesis in plants has not been ruled out in non-accumulating plants. Alternatively, or additionally, betaine might be metabolized actively in the non-accumulating plants. In mammalian liver, for instance, betaine is considered to serve an important role as a methyl-donor [21]. Interestingly, Byerrum *et al.* [22] have reported methyl transfer from radiolabelled betaine in tobacco but these observations have neither been confirmed nor extended to other plant systems. These potential reasons for the apparent low levels of betaine are not mutually exclusive. But if inefficient synthesis were the cause, the prospects for engineering betaine synthesis in non-accumulators by installing the major pathway [8–10] would indeed be bright.

Advantages of the method

As discussed above, the LC elution step affords a measure of specificity, and the method is more sensitive. There is no need for time-consuming sample derivatization steps, unless any interfering compounds persist through the analysis. Mass spectral analysis by selected ion recording (SIR) at a mass resolution of 5000 further mitigates chemical noise, allowing for more precision. Thus, this method would be useful for reliable analysis of large numbers of samples. Further, the sensitivity of this method should permit detection of minute amounts of betaine and betaine metabolites, as would be expected for samples from isolated cells, tissue and organelles. Metabolic engineering of betaine synthesis entails consideration of several factors, amongst which are precursor availability, timing and magnitude of synthesis, transport, spatial distribution and metabolic fate. This method would be useful to address some of these.

EXPERIMENTAL

Plants and chemicals. The initial seed lots for flax (*L. usitatissimum* cv McGregor), kochia (*K. scoparia*), oilseed rape (*B. napus* cv. Westar) pea (*P. sativum* cv. Homesteader), and tobacco (*N. tabacum* cv. Xanthi) were from laboratory stocks, *S. rubra* was from a saline slough. Non-isotopic betaine was purchased from Sigma and deuterated betaine was purchased from MSD Isotopes (Montreal, PQ). The chemicals used were of reagent or analytical grade. The ion-exchange resins were purchased from Bio-Rad (Mississauga, ON).

Preparation of plant extracts for betaine analysis. The following method is based on ref. [17]. Freshly harvested leaves from several plants were washed in large volumes of de-ionized water to remove any surface contaminants, shredded, and a 100-mg sample mixture was ground in 4 ml of MeOH–$CHCl_3$–H_2O (12:5:1) with white quartz sand. d_9-Betaine (445 nmol) was added to this mix as an internal standard, with the homogenate extracted with $CHCl_3$–H_2O (1:1.5). The aq. phase, sepd by centrifugation at 10000 *g* for 10 min, was collected and evapd under a stream of N_2 at 45°, and the sample was dissolved in 1 ml H_2O. It was passed through a 1.5-ml column of AG 1-X2 (converted to OH^- form by washing the original Cl^- form with 2 ml of 6M NaOH and 25 ml H_2O; mesh size 200–400) in series with a second column of AG 50W-X4 (H^+ form; 1.5 ml; mesh size 100–200). The columns were washed with 8 ml H_2O, and betaine was eluted from the second column with 8 ml of 6M NH_4OH. The eluate was dried under N_2 and taken up in 500 μl of a solution containing 0.1 % trifluoracetic acid (TFA) and 2 % glycerol in H_2O. The same solvent was used for dissolving the betaine standards.

Liquid chromatography/continuous flow secondary ion-mass spectrometry. Liquid chromatography (LC) was performed with an Applied Biosystems Model 140A syringe pump, modified to accommodate gradient analysis at low flow rates [23]. A Rheodyne Model 7520 injector was used to load the samples (1 μl for the

standards and plant extracts) on a 0.32 mm × 200 mm reverse phase fused silica column, packed in this laboratory with 3 micron Spherisorb C18 ODS2 packing material (Scientific Products and Equipment, Conrad, ON). Gradient LC analysis at a flow rate of 3 μl min^{-1} was used to elute the betaine. The mobile phase consisted of solvent A (0.1 % TFA and 2 % glycerol, in 80 % MeCN in H_2O) and solvent B (0.1% TFA, in 2%glycerol in H_2O). The proportion of solvent B was varied from 90 to 30% over 10 min, held at 30% for 2 min and decreased to 0% over 3 min. The LC was interfaced to a VG analytical 70-250 SEQ hybrid mass spectrometer equipped for continuous flow liquid secondary ion-mass spectrometry [23]. SIR analyses were done at a mass resolution of 5000. The molecular ion intensities of betaine and the internal standard d_9-betaine at m/z 118.087 and 127.143, respectively, were monitored at a dwell time of 80 ms.

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