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Short Communication

Quantitation and Purification of Quaternary Ammonium Compounds From Halophyte Tissue

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ABSTRACT

A simple and sensitive spectrophotometric assay for quaternary ammonium compounds (QACs) based on Dragendorff's reagent is described. Although not specific for a particular QAC, the assay allows for rapid survey of tissue for QAC content. A separation method for QACs in halophyte tissue containing high levels of cations was developed using ion exchange resins and ninhydrin to remove the amino acid fraction.

A variety of methods exist in the literature for the analysis of QACs[1] in plant tissues. These methods vary from the time consuming spectrophotometric methods measuring reineckate or periodate salts (4, 11) or a cobalt chloride-ferrocyanide complex (1) to the use of the Dragendorff spray reagent used in conjunction with thin layer or paper electrophoretic methods (12). More recently, more rapid methods using HPLC (8) or gas pyrolysis (9) have been reported but both require sophisticated and expensive equipment. Methods for the purification of QACs using ion-exchange resins (3, 6, 9) have been developed using plant tissue from glycophytes. If the plant tissue to be assayed is a halophyte and separation of QACs from the crude sample is required, these methods are not adequate due to the high cation content, mainly Na^+, of the tissue.

We report a quick, convenient, and sensitive spectrophotometric assay for QACs using a modified Dragendorff reagent. The assay was developed in conjunction with a simple and rapid system for the separation and purification of glycinebetaine and other QACs from halophyte tissue extracts. The technique has been applied to the halophytes *Atriplex nummularia*, *Spergularia marina*, and *Salicornia europaea*.

MATERIALS AND METHODS

Dowex 50-x8 (H^+ form, 200–400 mesh), glycinebetaine, trigonelline hydrochloride, choline chloride, and ninhydrin were purchased from Sigma Chemical Company. All other chemicals were of analytical grade. TLC plates were purchased from American Scientific Products, Phoenix, AZ.

The standard curve for glycinebetaine was made by placing 1.0, 2.0, 4.0, 6.0, or 8.0 μl of a stock solution of glycinebetaine (0.500 g/ml) into plastic 1.5-ml microcentrifuge tubes. Addition of 100 μl of modified Dragendorff reagent (made by mixing equal volumes of 0.35 M bismuth nitrate in 20% acetic acid (v/v) and 2.45 M NaI (in distilled H_2O) causes precipitation of the QAC. Glycinebetaine gives a bright orange precipitate while trigonelline hydrochloride and choline chloride give a brick red precipitate. The sample was centrifuged for 1 min at 7,000 *g*. The supernatant must be completely removed or the remaining bismuth nitrate will cause an increased background (we routinely use a 250-μl syringe for supernatant removal). The pellet was dissolved in 1.0 ml of 2.45 M NaI solution. A 10-μl aliquot was then added to 1.0 ml of 0.49 M NaI solution and OD_{467} (the absorption maximum) recorded against a blank solution of 0.49 M NaI. Figure 1 shows a standard curve for glycinebetaine.

Plant tissue extracts were brought to dryness in microfuge tubes and then the modified Dragendorff reagent added if prior work has demonstrated the presence of only one QAC in the material. If the sample to be assayed is a halophyte and the separation of QACs is required, previously published methods are not adequate due to the high cation (mainly Na^+) content. We developed the method that follows in order to further validate the use of the spectrophotometric assay.

The ion-exchange columns are constructed from 6-ml disposable syringe barrels. A 14-mm frit of Whatman No. 3 filter paper is placed at the bottom of the syringe, a 1-ml resin bed is poured in a water slurry, and then a 14-mm frit is placed on top to protect the resin during sample application.

A 0.25- to 0.50-ml aqueous extract (equivalent to 0.5–1.0 g fresh weight) extract (plant tissue is extracted with boiling 95% ethanol for 20 min with a 20:1 ethanol to tissue ratio) was applied to the top of a 1-ml Dowex-50 (H^+ form, 200–400 mesh, × 8) column. The applied extract ws allowed to drain into the column and the eluent collected. A 1-ml wash with distilled H_2O follows and the two eluents combined. This fraction contains the anionic and neutral compounds while the Dowex-50 resin retains the amino acid and QACs. No ninhydrin or Dragendorff positive material was present in the combined washes (as determined by spot tests using TLC plates and Dragendorff reagent or 0.2% ninhydrin in acetone (w/v) for QACs and amino acid detection, respectively. Additionally, no organic acids remained on the Dowex-50 as determined using gas chromatography (10, 13).

Elution of the cation fraction was achieved by placing 3.5 ml of 4 N NH_4OH to the Dowex-50 resin, the eluent collected, and reduced to dryness under a stream of compressed air while being heated at 80°C in a sand bath. This residue contained the amino acids and QACs except choline which was irreversibly bound to the Dowex-50.

Addition of 200 μl of 75 mM ninhydrin (13.5 mg/ml water) to the dried extract and then heating the mixture for 30 min at 80°C in a sand bath destroys the amino acid component of the cation fraction. The ninhydrin-reacted material was placed on a second 1.0-ml Dowex-50 column. Two ml of 95% ethanol was

[1] Abbreviation: QAC, quaternary ammonium compound.

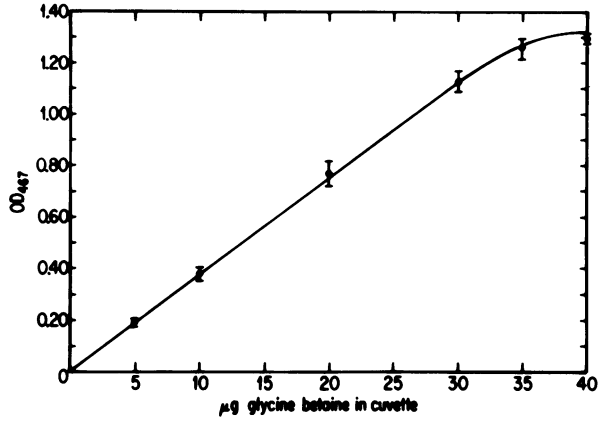

FIG. 1. Standard curve for glycinebetaine. Each point represents the mean ± SD of five separate determinations. Note that the curve is plotted as absorbance *versus* μg of glycinebetaine in the cuvette.

Table I. *Glycinebetaine Content of Atriplex nummularia, Spergularia marina, and Salicornia europaea Grown at 30 Parts per Thousand Total Salts*

Each value represents the mean ± SD of triplicate samples.

Sample	Glycinebetaine Content
	μmol g^{-1} *dry wt*
A. nummularia	585 ± 11
S. marina	13.9 ± 1.9
S. europaea	457 ± 20

washed through the column to remove the unreacted ninhydrin and the reaction by-products. Two ml of water was then washed through the column to remove the ethanol.

The QAC fraction was eluted from the column with 3.5 ml of 4 N NH_4OH, collected, and brought to dryness under a stream of compressed air in a 80°C sand bath. The residue remaining contained amino acid free QACs.

The complete separation and purification of individual QACs is accomplished using the TLC system of Gorham *et al.* (7). We have modified the Dragendorff spray reagent as follows. To 10 ml of 20% acetic acid (v/v), add 330 μl of 2.45 M NaI, mix thoroughly, and then add 110 μl of 0.35 M bismuth nitrate (in 20% acetic acid [v/v]). The mixture will turn black and then clear orange. Addition of the NaI and bismuth nitrate in reverse order will result in a black precipitate not suitable for use. The TLC plate is then sprayed and heated at 135°C for 2 min. The QACs will appear as orange (glycinebetaine, glycinebetaine aldehyde) or red (trigonelline and choline) spots against a yellow background. While the positive reaction of the QACs can be seen after the heat treatment, storage overnight in the dark will markedly enhance the contrast. We have also found that, with samples containing less than 50 μg of glycinebetaine, the spots are more easily seen under natural light than in the fluorescent lighting of the laboratory.

RESULTS AND DISCUSSION

The Dragendorff reagent for use with TLC systems is based on the precipitation of QACs with bismuth potassium or bismuth NaI giving a characteristic red-orange spot (3, 5, 7). In both the TLC reagent and the spectrophotometric assay, the formation of bismuth-triiode which precipitates as minute black crystals (14) is a competing reaction with the formation of the QAC-precipitate. Use of at least a 7-fold molar excess of NaI or KI will prevent bismuth triiode formation if the bismuth nitrate is added to the NaI or KI solution (even at this excess, the reverse sequence of addition will result in bismuth triiode formation and resultant precipitation). Since the absorption spectrum of the trigonelline and choline precipitates are identical to that of the glycinebetaine absorption spectrum (data not shown), the basis for the absorption at OD_{467} is most likely the bismuth-NaI complex that redissolves in the presence of the excess NaI reagent.

Exhaustion of the precipitatory capability of the reagent is readily apparent as the supernatant above the centrifuged QAC-bismuth-NaI precipitate will become light orange and the OD_{467} *versus* glycinebetaine will plateau as seen in Figure 1.

Routine recovery of glycinebetaine from plant samples using the described separation procedure is 90 to 95%. When a 0.50 mg 'spike' of glycinebetaine was added to a plant sample, 92% recovery of the added spike glycinebetaine was achieved (data not shown). We have used this assay with two known accumulators of glycinebetaine, *A. nummularia* and *S. europaea*, and one that has shown no accumulation, *S. marina* (15). Our results (Table I) agree with those previous reports.

The spectrophotometric technique described here allows for the rapid estimation of QAC in plant tissue. Although this assay is not specific for particular QAC, it is the simplest method available for QAC quantitation as a class of compounds. In particular, the use of ninhydrin to remove aminoacids from the cationic fraction greatly simplifies the analysis of QACs in halophytic plant tissue where the presence of large quantities of cations complicates the use of weak cation exchange resins such as Bio-Rex-70.

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