Extraction and Determination of Glycinebetaine in Liquid Fertilizers

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Betaines and other quaternary ammonium compounds play an important role in the physiological response of plants to stress, especially during drought and salinity conditions. The analysis of glycinebetaine in plant tissues and fertilizer samples is usually carried out by methods which have poor selectivity or involve complex sample preparations. In this paper, we describe a fast and easy HPLC method for the selective and accurate determination of glycinebetaine in a complex mixture, such as a liquid fertilizer formulation, using UV detection at 195 nm and a cationic-exchange column. Sample treatment is reduced to acidification and charcoal purification, followed by filtration and direct chromatographic injection.

Keywords: *Glycinebetaine; liquid fertilizer; HPLC; UV-diode array*

INTRODUCTION

Betaines are quaternary ammonium compounds which can be considered as methylated derivatives of amino acids. They occur widely in nature, both in animal and plant tissues and fluids and are also found in a wide variety of algae (Blunden et al., 1992). A well-known betaine is carnitine, whose esters can be found in all animal tissues. Glycinebetaine, and probably other betaines such as stachridine (prolinebetaine), act as osmolytes involved in the drought resistance of plants (Jones and Storey, 1981) and are therefore the subject of a growing interest in agronomy.

The lack of an easy and reliable method of detection and quantification of betaines and related compounds is acutely felt at present. Traditional methods relied on nonspecific precipitation procedures, employing periodide (Barak and Tuma, 1979) or reineckate (Bao et al., 1989), and on chromatographic techniques, such as thin layer chromatography (Müller and Eckert, 1989), HPLC, gas−liquid chromatograpy (Dupuy, 1978), and various other techniques such as thin layer electrophoresis (Gorham et al., 1981), ^{1}H nuclear magnetic resonance (Wevers et al., 1994), and potentiometric titration (Plantinga et al., 1993).

Liquid chromatography, using reverse-phase, ion-exchange, or amino columns, is by far the most frequently used technique, but the lack of strong and long-wavelength chromophoric moieties on the betaine molecules (homarine and trigonelline being exceptions) makes detection by UV absorbance difficult, requiring the use of low-wavelength UV detection (below 220 nm) or refractive index (Kikuchi et al., 1993). Betaines do not show appreciable fluorescence, nor do their redox properties make them easily detectable using electrochemical detectors. There are certain precolumn derivatization procedures, all of which rely on the sterification of the betaine carboxylic group; the most frequently used reagent is *p*-bromphenacyl bromide, the *p*-bromophenacyl ester form being detected at 254 nm (Gorham, 1986). However, some authors have pointed out that this derivatization method is totally unsatisfactory because of the lack of reactivity toward common betaines and the thermal lability of betaines. These authors propose and test a new reagent, 4-bromophenacyl triflate (Lever et al., 1992), which is not yet commercially available.

Due to the lack of selectivity and/or propensity of interference of most of the techniques described above, extensive concentration and purification steps are compulsory. A variety of strong anion exchange resins are commonly employed, followed by exhaustive sample drying in precolum derivatization methods.

The method described below combines a rapid and extremely simple purification step with the use of a rapid HPLC analysis using standard UV detection. This method is suitable for the analysis of a liquid fertilizer (Fertiactyl Gz), a complex mixture of seaweed extracts, humic and fulvic acids, amino acids, low molecular weight organic acids, carbohydrates, and mineral nutrients, used as a bioestimulant and antistress agent in agricultural practice.

MATERIALS AND METHODS

Reagents. Standard betaine was obtained from the Sigma Chemical Co. (St. Louis, MO); methanol (LC gradient grade) and other reagents (GR grade) were from E. Merck (Darmstadt, Germany).

Apparatus. Chromatographic analysis was carried out by using a Waters Liquid Chromatograph (Waters Corp., Milford, MA) consisting of a Model 616 pump, a 717plus autosampler, a 996 photodiode array detector, and 2010 Millennium Chromatography Manager software, version 2.1.

Chromatographic Conditions. Isocratic elution was carried out in a Hypersil SCX, 5μm, 250 × 4.6 mm (Shandon HPLC), using a disodium phosphate buffer 0.05 M, pH 4.7 (95%)/methanol (5%) mixture as the mobile phase. Flow was set at 1 mL/min, oven temperature 30 °C, detection wavelength 195 nm, and injection volume 25 μL.

Preparation of Standard Glycinebetaine Solutions. Glycinebetaine was dissolved in distilled water to give a final concentration equivalent to 0.2 , 0.4 , 0.8, and 2% w/w, diluted 20 times as described in the sample preparation.

Sample Preparation and Glycinebetaine Purification. Liquid fertilizer (5 g) was diluted to 100 g with distilled water. HCl (35%) (2 mL) and 4 g of charcoal were added, and the mixture was magnetically stirred for 30 min. Following filtration through a double layer of standard filter paper, the filtrate was injected directly into the chromatograph.

Identification and Quantification. The initial identity assignment of glycinebetaine was based on comparison retention data between standard and sample chromatograms and

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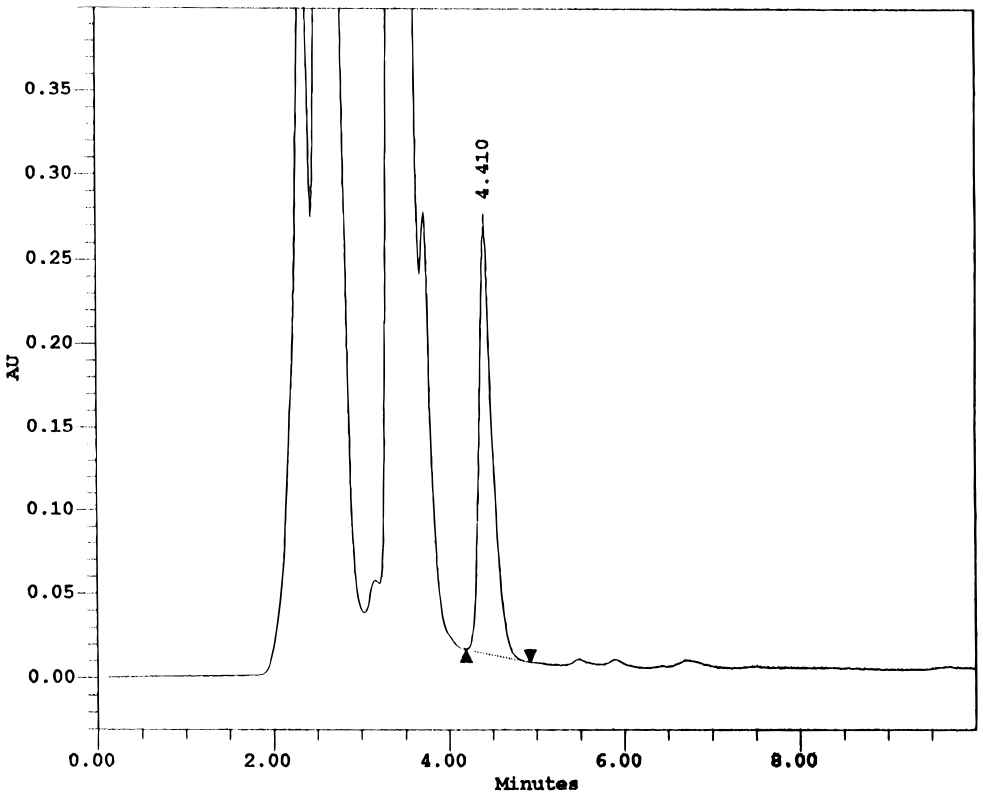

Figure 1. HPLC profile of glycinebetaine in liquid fertilizer sample.

Table 1. Repeatability and Accuracy of Sample Solutions (n = 4)

	curve 1		curve 2		curve 3	
	repeatability (%)	accuracy (%)	repeatability (%)	accuracy (%)	repeatability (%)	accuracy (%)
0,2	0.72	5.48	0.33	1.10	1.23	2.19
0,4	0.11	1.89	0.44	2.41	0.55	4.79
0,8	0.40	0.25	0.33	0.50	0.19	1.06
2,0	1.6	0.00	0.53	1.66	0.41	0.96

spectral comparison. Peak purity was checked and confirmed by the above-mentioned software.

Calculation of glycinebetaine concentration was based on the external standard method. The concentration was calculated from peak areas using the equation

$$C = ax + b$$

where a is the curve slope and b is the curve intercept.

The concentrations of glycinebetaine were determined in triplicate, and the average concentrations were expresed as percent w/w.

RESULTS AND DISCUSSION

Prior to sample analysis, the chromatographic technique was validated. Measurements for the construction of three calibration curves were made in three consecutive days, both for standards and samples, using four solutions for each concentration. The linearity of these curves was tested by comparing the correlation coefficients. The lack of statistically significative differences between the curves as well as repeatability, accuracy, reproducibility, and detection limits were checked for both standards and samples.

Standard solutions: the correlation coefficient for three curves was >0.998, without any significative differences among them. There were no statistically significative differences between the curves. Repeatability, accuracy, and reproducibility were entirely satisfactory, with values lower than 5%.

Table 2. Reproducibility of Sample Solutions (n = 12)

	reproducibility (%)
0,2	2.35
0,4	1.50
0,8	0.63
2,0	1.51

Sample solutions: as in standard solutions, the correlation coefficient for three curves was >0.998, without any significative differences among them. Repeatability and accuracy (Table 1) and reproducibility (Table 2) were again satisfactory. Recovery was calculated as the ratio between the slopes of sample solution and standard solution calibration curves. Under the above-mentioned conditions, glycinebetaine recovery was 84.3%.

The detection limit, calculated using the ordinate value at the origin expressed as concentration units (Castro et al., 1989) was 0.043%, corresponding to 21 μg g^{-1} in standard solutions without dilution.

A typical sample solution chromatogram profile was obtained (Figure 1). The peak corresponding to glycinebetaine was well separated with good peak resolution, sharpness, and symmetry and a typical retention time of 4.5 min.

The addition of HCl shifts the pH of the solution to a value near 1, allowing the precipitation of numerous substances, especially humic acids. The addition of charcoal permits the adsorption of numerous organic compounds such as phenols, polyphenols, etc., rendering a clear filtrate ready for immediate chromatographic

analysis. Other purification methods tested such as ionic exchange resins, insoluble polyvinylpyrrolidone, organic solvents extraction, and trichloroacetic acid precipitation gave unsatisfactory results for the complex matrix examined.

The sensitivity of this technique does not reach the limits published using refraction index detection or UV detection after derivatization, but it is good enough for the determination of glycinebetaine in the matrixes tested. The main virtue of this technique lies in its simplicity, being easy to carry out in any HPLC equipped laboratory. We have not tested this technique on other kinds of matrices such as seaweed or plant material, but it seems likely that the sensitivity of the method may be not enough, at least using UV detection without prior derivatization. Other detectors such as refraction index or light-scattering detectors, in combination with the present technique will probably be able to quantify glycinebetaine in the above-mentioned matrixes, but further studies are needed to check this hypothesis.

We conclude that this extraction and chromatographic technique is easy, accurate, precise, and sensitive enough for glycinebetaine determination in liquid fertilizer formulations.

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