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Note

Simultaneous determination of monoethanolamine and glycine betaine in plants

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The association of monethanolamine (EA) and glycine betaine (GB) with drought resistance of plants requires the determination of these compounds in any study of stress physiology and of the alleviation of stress by bioregulators.

Methods used for the determination of EA and GB include thin-layer chromatography (TLC)[1–3], gas chromatography (GC)[4–7] and high-performance liquid chromatography (HPLC)[8–11]. In addition, GB was determined spectrometrically as its reineckate[12], periodide[13] or by means of an enzymatic method[15] in biological material.

Comparatively little is known about the quantitation of EA in plant material. Reissmann *et al.*[5] suggested a GC procedure for barley grains, but this method is laborious and needs a time-consuming derivatization step.

Since none of these methods allows a simultaneous determination of EA and GB and meets all requirements concerning specificity, sensitivity and rapidity, we developed a simple ion-exchange chromatography–TLC procedure which is especially suitable for a simultaneous determination of EA and GB in routine analysis.

MATERIALS AND METHODS

Plant material

Barley plants (*Hordeum vulgare* L., cv. Salome) were grown in Mitscherlich pots under well watered and drought stressed conditions as described previously[16].

Chemicals and radiochemicals

EA, reagent grade, was obtained from VEB Laborchemie (Apolda, G.D.R.). GB, reagent grade, and Dowex resins were obtained from Serva (Heidelberg, F.R.G.). All other chemicals used in extraction, purification and quantification were of reagent grade quality.

[1-^{14}C]EA used as the internal standard was from Isocommerz (Dresden, G.D.R.). [$^{14}CH_3$]GB was synthesized from [$^{14}CH_3$]choline (Isocommerz) according to Lintzel and Fomin[17]. Both labelled compounds were purified by the analytical procedure described below and their purity confirmed by comigration with authentic standard compounds in TLC and checked by means of a TLC scanner Berthold II. Radiochemical impurities were $\leqslant 0.5\%$.

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Extraction and precleaning

Fresh plant material (main shoots and tillers, respectively) was immediately homogenized with 200 ml methanol–chloroform–water (70:20:10, v/v/v) after harvesting. To this mixture a standard amount of [^{14}C]EA (43.40 kBq/μmol, 1.43 μg) and [^{14}C]GB (20.19 kBq/μmol, 4.10 μg) were added to each sample[a]. These samples were kept at room temperature in the dark for more than 24 h. Then, the solution was filtered and the residue reextracted with additional solvent until chlorophyll removal was complete. The pooled extracts were shaken with one-third volume of water. After separation the aqueous phase was evaporated to 10 ml under vacuum and for precleaning passed through a column of the strong cation exchanger Dowex 50W-X8(H^+) 8 ml in the case of 25 g fresh plant matter. After washing with water, EA and GB were eluted with 1 *M* ammonia.

Separation of EA from GB

After concentration of the 1 *M* ammonia eluate (to about 10 ml) it was passed through two columns (8 ml each) coupled in series and containing the strong anion-exchange resin Dowex 2-X8(OH^-) and the weak cation-exchange resin Dowex CCR-2(H^+), respectively. The effluent, including water for washing, contained GB and was evaporated to dryness and for quantification redissolved in 2 ml 80% methanol[a]. EA retained on the weak cation exchanger was eluted with 1 *M* ammonia. After removing the ammonia by concentrating to 10 ml under vacuum, 1 ml 1 *M* HCl was added and the solution evaporated to dryness. The residue was redissolved in 1 ml methanol for quantification.

Quantification of EA and GB

Determination of EA and GB was performed by TLC using precoated silica gel plates (Silufol, Prague, Czechoslovakia) without prior activation. Aliquots of the prepared solutions (1 μl in the case of GB and 2 μl in the case of EA) were applied to the plates. The developing solvent systems were: EA methanol–25% aqueous ammonia (8:2, v/v); GB methanol–25% aqueous ammonia–water (7:2:1, v/v). Plates were developed one-dimensionally at room temperature over a distance of 8 cm in a saturated chamber. After drying the plates at 110°C for 5 min, EA spots were visualized by spraying with 0.2% ninhydrin in ethanol and heating again at 110°C for 3 min (R_F = 0.3, limit of detection 10 ng). Visualization of GB was carried out by treating the developed and dried plates with hydrogen chloride vapour, removing surplus HCl at 110°C for 10 min and spraying with 0.05% aqueous methyl orange. GB showed deep red spots on a yellow background (R_F = 0.5, limit of detection 0.3 μg). Quantification of both compounds was performed by means of a scanning densitometer (FZB Müncheberg, Bereich Jena, G.D.R.). The calibration graph for each plate was obtained by spotting in 50-ng steps for EA and in 0.5-μg steps for GB. The response of the scanning densitometer was linear in the range 0–250 ng for EA (r = 0.9995, wavelength 570 nm) and 0–4 μg for GB (r = 0.991, wavelength 550 nm).

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[a] In order to economize on reagents and ion exchangers it is possible to miniaturize the method up to 0.5 g plant fresh matter. However, from a plant physiological viewpoint, a representative sample size must be guaranteed. That means that the sample size can be miniaturized only if the plant material is sufficiently homogenized before extraction, *e.g.* freeze-dried and ground.

Isotope dilution analysis

After spotting on the TLC plate, 50-μl aliquots (in triplicate) of each sample were immediately taken for scintillation counting (Liquid Scintillation Counter Rackbeta 1219; LKB, Sweden) in a dioxane-based scintillator with the scintillators 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazolyl-2)benzene (POPOP) and quench correction with an external standard. Knowing the specific radioactivities of the authentic ^{14}C-labelled compounds added and of the EA and GB recovered, the endogenous levels of EA and GB can be calculated.

RESULTS AND DISCUSSION

As a result of its unique charge properties (a permanent positive charge on the quaternary ammonium group with a carboxyl group of low pK_a), GB is not retained on either strong anion or weak cation-exchange resin[6]. This allows an excellent separation from the cationic EA as well as from impurities by an exchanger system arranged in series (see Fig. 1). By means of the strong anionic resin [Dowex 2-X8(OH^-)] all anions were removed, especially amino acids which disturb the subsequent quantification, and on the other hand all cations were retained on the weak cation resin [Dowex CCR-2(H^+)]. The effluent of this column combination contains GB, whereas EA is retained on the weak cation exchanger. After separating the columns EA can be eluted with ammonia.

A precondition for a good separation and quantification is a precleaning step involving filtration of the crude extract through a column of a strong cation exchanger [Dowex 50W-X8(H^+)]. This resin suppresses the ionization of the carboxyl group of GB and retains GB as well as EA, whereas uncharged compounds and anions were removed by the effluent. In addition, this precleaning prevents an overcharge of the anion resin in the subsequent separation step, in particular when the sample size exceeds 0.5 g plant dry matter. After washing the column with water, EA and GB can be eluted with ammonia (see Fig. 1).

The separation efficiency achieved by the column combination together with the preceding purification step is shown in Table I. The results demonstrate that artefacts are not produced during the analytical procedure and that there is no overlap between EA and GB. However, the recovery data show that there are some losses of EA, which can be explained by the volatility of the basic EA during the evaporation of the ammonia eluate. Therefore, the analytical procedure was generally performed with an internal standard. We used the ^{14}C-labelled compounds of EA and GB, allowing quantification by an isotope dilution procedure. The good separation of EA and GB (see Table I) enables the addition of both compounds before starting the extraction procedure. Because of the high specific radioactivity of [^{14}C]EA and [^{14}C]GB used, the amount added was less than 1% of the EA and GB content found in the plant tissue (Table IV). The low radioactivity of about 1 kBq per sample does not require any special radiohygienic precautions, taking into account the low volatility of EA.

Even if the purity of the EA and GB fractions allows the derivatization and quantification by GC, it was found that TLC offers a more rapid and simpler method giving well reproducible results with an high sensitivity. For GB we developed a sensitive detection method because the usual Dragendorff reagent[18] was too insensi-

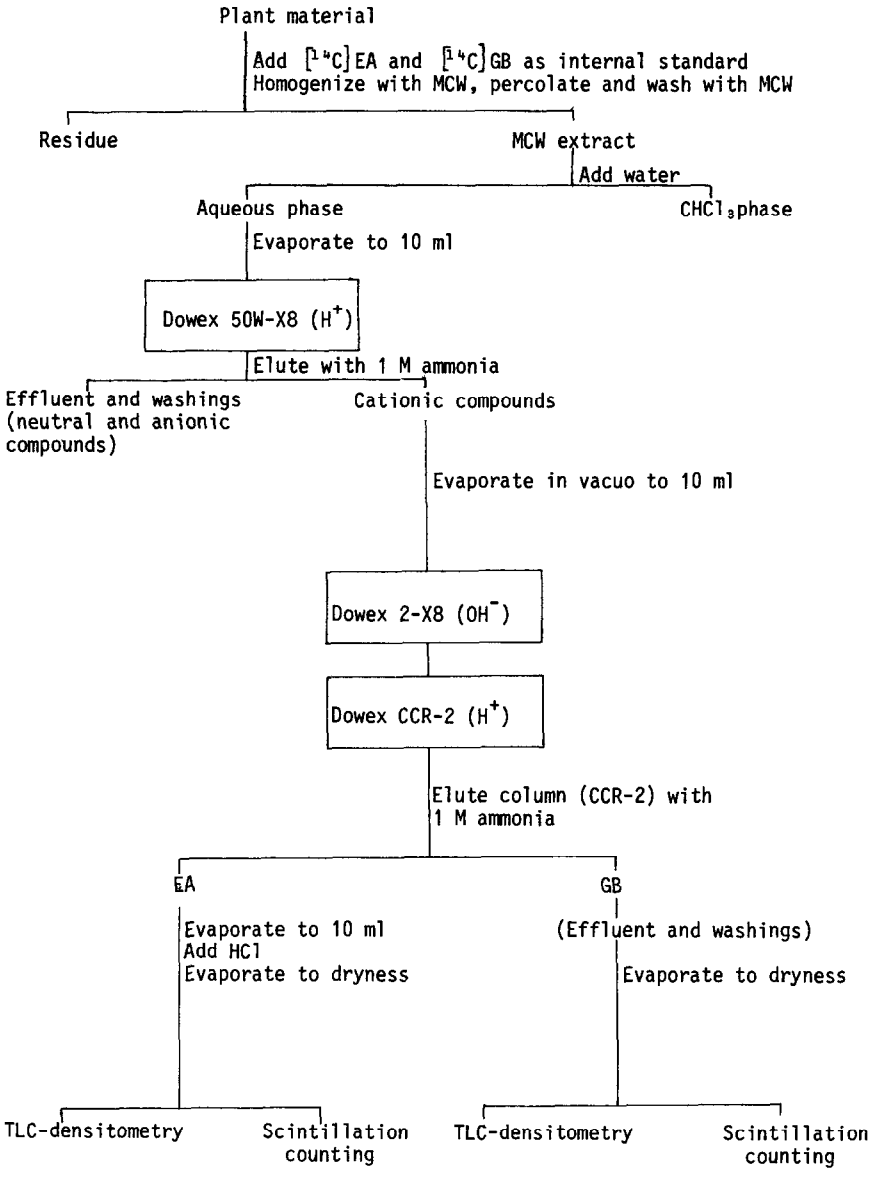

Fig. 1. Analytical procedure for determination of EA and GB in barley shoots. MCW = Methanol–chloroform–water (70:20:10, v/v/v).

tive (limit of detection $\approx$ 10 μg). With regard to the acidity of the GB hydrochloride, visualization with a methyl orange reagent proved to be suitable and allows a detection limit of 0.3 μg. EA was detected with the sensitive ninhydrin reagent[19] (detection limit 10 ng). The accuracy of the method was tested by analysing prepared mixtures of EA and GB (Table II). Good recoveries with low standard deviations were obtained. When the method was applied to prepared extracts of spring barley (previously ana-

TABLE I

SEPARATION EFFICIENCY OF THE ION-EXCHANGE PROCEDURE TESTED AFTER ADDITION OF [^{14}C]EA OR [^{14}C]GB TO 15 g PLANT FRESH MATTER (n = 3)

Analytical fraction	*[^{14}C]EA (0.5 mg, 59.8 kBq) = 100*	*[^{14}C]GB (15 mg, 64.9 kBq) = 100*
Radioactivity found (% of ^{14}C added):		
Extraction residue[a]	0.3	0.2
Chloroform phase[a]	0.6	0.2
Aqueous phase	98.2	98.7
Effluent (Dowex 50W-X8)	0.1	0.4
Eluate (1M ammonia)	96.8	97.6
GB fraction (effluent Dowex 2-X8/ Dowex CCR-2)	0.0	96.3
EA fraction (eluate Dowex CCR-2)	85.4[b]	0.6
Recovery ± S.D.	85.4 ± 4.9	96.9 ± 3.8

[a] After combustion in the Oxymat IN 4101.
[b] Losses of EA resulted from evaporation of the ammonia eluate.

lysed by GC[5,7]) to which definite amounts of EA and GB had been added, good recoveries were obtained too (Table III). Moreover, the standard deviations in Table II and III show that the precision of the method is satisfactory.

The identity of EA and GB found in the extract was confirmed by GC after derivatizing the hydrochlorides. Although GC represents an alternative to TLC, the method is too laborious for routine analysis and does not provide a decisive improvement in sensitivity. GB was derivatized according to Ranfft and Gerstl[7] and EA

TABLE II

RECOVERY OF EA AND GB FROM SYNTHETIC MIXTURES

Mixture	*Concentration (ng µl^{-1})*		*Recovery (%)*	*S.D. (%)*
	Original	*Found*[a]		
(1) EA	25.0	25.8	103.2	2.9
GB	509	528	103.7	3.4
(2) EA	54.0	55.3	102.4	2.6
GB	1021	1016	99.5	1.6
(3) EA	78.2	77.5	99.1	1.8
GB	1492	1522	98.0	2.4
(4) EA	108.7	110.0	101.2	1.9
GB	2012	1996	100.8	2.1
(5) EA	129.4	133.1	102.9	3.1
GB	2586	2534	102.0	1.9

Mean recovery ± S.D.: EA, 101.8 ± 1.6%; GB, 100.8 ± 2.2%

[a] Mean of four replicates.

TABLE III

RECOVERY OF EA AND GB ADDED TO EXTRACTS OF MAIN SHOOTS OF BARLEY AFTER ION-EXCHANGE CHROMATOGRAPHY

Mixture	*Concentration (ng μl^{-1})*			*Recovery (%)*	*S.D. (%)*
	Original[a]	*Added*	*Found*[b]		
(1) EA	68.1	0	65.3	95.9	4.1
GB	1440.0	0	1492	103.6	6.5
(2) EA	68.1	10	82.1	105.1	5.0
GB	1440.0	600	2178	106.8	6.0
(3) EA	68.1	20	91.9	104.3	3.2
GB	1440.0	900	2306	98.5	1.6
(4) EA	68.1	30	103.4	105.4	5.7
GB	1440.0	1200	2531	95.9	3.5
(5) EA	68.1	40	112.4	104.0	6.4
GB	1440.0	1500	2910	99.0	3.0

Mean recovery ± S.D.: EA, 102.9 ± 3.9%; GB, 100.8 ± 4.2%

[a] Previously analysed by GC

[b] Mean of six analyses.

according to the method of Reissmann *et al.*[5]. The determination was performed with a gas chromatograph GCHF 18.3-4 (VEB Chromatron Berlin) on a glass column (1 m × 3 mm I.D., 3% SE-30 on Gas-Chrom Q, 100–120 mesh) by means of flame ionization detection (FID). The oven temperature was 120 (GB) or 100°C (EA) and the retention times 2.03 (GB) or 2.3 min (EA). Since EA and GB found in the plant material were eluted at retention times which correspond to those of authentic standard compounds and since moreover the concentrations determined by TLC can be confirmed by GC, the identity of the extracted EA and GB was considered to be established.

It should be noted that at least trigonelline and stachydrine cannot be separated from GB by the ion-exchange procedure or the TLC system used. However, preliminary checks with a multiple TLC development or changes of developing systems which allow a clear separation of the three betaines showed that trigonelline and stachydrine were absent or present only in traces in well watered and drought stressed barley plants, respectively. Likewise, it was not possible to separate basic amino acids, *e.g.*, arginine and lysine from the EA fraction by the ion-exchange procedure. However, applying the TLC system methanol–ammonia a clear separation was achievable. Any addition of water to this system deteriorates the separation and can lead to a misinterpretation of the EA content.

The advantages of the procedure described consist in the possibility of a simultaneous determination of both compounds and above all in the specific and sensitive detection as well as in the rapidity and simple applicability.

The procedure outlined above was used to determine the levels of endogenous EA and GB in well watered and drought stressed barley plants at the end of the shooting stage (Table IV). The results show that the GB content significantly increased under stress, whereas no change in the EA content was found. The GB levels

TABLE IV

CONTENTS OF EA AND GB IN MAIN SHOOTS AND TILLERS OF WELL WATERED AND DROUGHT STRESSED BARLEY PLANTS (μmol g^{-1} DRY MATTER)

Plant organ	*Water status*[a]	*Dry matter (g)*	*GB*[b]	*EA*[b]
Main shoots	+	1.59	34.4	2.9
	−	1.34	62.7[c]	2.6
Tillers	+	2.64	46.6	3.2
	−	1.77[c]	56.7[c]	3.7

[a] + Well watered; − drought stressed.
[b] Mean of four analyses.
[c] P = 5% significance between + and −

corresponded well with the results of other authors[20,21]. For EA, quantitative results in plant material have not been reported in the literature. Since, however, the extraction efficiency was high with about 90% (verified by feeding experiments with ^{14}C-labelled EA and GB, respectively) and no hydrolysis of phosphatidylethanolamine was detected (experiments with L-α-kephaline, dipalmitolyl), the EA levels found seem to be reliable.

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