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Short Communication

Adsorption losses during extraction and derivatization efficiency by benzoylation of plant putrescine for high-performance liquid chromatographic analysis

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ABSTRACT

The method for the determination analysis of plant polyamines through the reversed-phase HPLC separation of the benzamide derivatives was investigated for putrescine (1,4-diaminobutane) with the purpose of increasing reproducibility without losing speed. Putrescine was found not to adsorb on differential vial materials, but a significant binding to plant material in the extraction step was found. The overall benzoylation efficiency varied between 59 and 83%, and the use of 1,6-diaminohexane as an internal standard is suggested in order to obtain reproducible results.

INTRODUCTION

For the determination of aliphatic polyamines, Redmond and Tseng [I] introduced the reversed-phase HPLC separation of benzamide derivatives obtained through benzoylation by the Schotten–Baumann technique. Flores and Galston [2] developed it into a rapid and sensitive method for use on crude plant extracts, with subsequent modifications [3].

An investigation of the possible application of the free form of one of the polyamines, putrescine (1,4-diaminobutane), as a biomarker of pollution-induced stress in a higher plant test [4,5] created the necessity for a highly quantitative and reproducible determination of putrescine in plant material, to be used on large numbers of samples.

The method of Flores and Galston is a useful starting point, but it involves several steps with recoveries that vary and may be unsatisfactory. Furthermore, free polyamines are suspected to adsorb on glass surfaces [2,6], leading to possible losses of the amines before HPLC separation and detection. A thorough examination of the method of Flores and Galston was carried out for putrescine to optimize the different steps, to reveal operations leading to loss of this polyamine and, with this background, to improve the reproducibility of the benzoylation method for the determination of free putrescine in plant material.

EXPERIMENTAL

Chemicals

Putrescine (98% free base), 1,6-diaminohexane

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(crystalline) and benzoyl chloride (99%) were obtained from Sigma (Deisenhofen, Germany), and [^{14}C]putrescine (98.8% pure dihydrochloride, specific activity 3.3 GBq/mmol), labelled at C-l and C-4, from DuPont (Brondby, Denmark).

Cultivation of plants

Plant material (barley or rape) was cultivated as described elsewhere [4].

Determination of polyamines

The harvest and benzoylation procedure was adopted and modified from that of Flores and Galston [2].

Extraction of plant material. Approximately 200 mg (fresh mass) was macerated in 1 ml followed by washing in 1 ml of cold (4°C) protein denaturing acid extractant (a 5% solution of trichloroacetic acid or perchloric acid with 25 μM 1,6-diaminohexane added) in a blender (Ultra Turrax, 25 000 rpm for 60 s) and allowed to stand for 60 min on ice. The supernatant was isolated by centrifugation (45 000 g for 15 min), withdrawn and kept at − 18°C.

Benzoylation of plant extracts. To *500* μl of plant extract were added 1 ml of 2 M NaOH and 20 μl of benzoyl chloride. After 10 s of vortex mixing, 20 min at ambient temperature was allowed for reaction. A 2-ml volume of saturated NaCl was added followed by 2.0 ml of diethyl ether. The mixture was vortex mixed for exactly 10 s and allowed to separate for 10 min, then 1 .0 ml of the ether phase was transferred to an HPLC autosampler vial, from which the ether was allowed to evaporate during 4-5 h at ambient temperature. After addition of 250 μl of the HPLC eluent and vortex mixing for 10 s, the sample was analysed by HPLC or stored at − 18°C.

HPLC of benzoylated plant extracts. Separation of benzoylated amines was carried out by isocratic reversed-phase HPLC using a Waters Model 501 pump and a 200 × 3 mm I.D. C_{18} column, particle size 5 μm (ChromSpher B; Chrompack) with ethanol-water (3:7) as the eluent at a flow-rate of 0.3 ml/min. The amines were detected spectrophotometrically at 254 nm with a Waters Model 455 LC spectrophotometer, corrected with the internal standard and quantified against external standards.

Investigations using [^{14}C]putrescine

Adsorption of [^{14}C]putrescine on vial materials. The types of vials tested were (A) completely new 100 × 16 mm I.D. glass tube with virgin glass surfaces; (B) completely new 100 × 16 mm I.D. glass tube, surfaces exposed to contact with plant extract for 30 min; (C) old glass centrifugal tube, used and washed at least ten times; and (D) polycarbonate centrifuge tube. In the test, 1000 μl of $1 \cdot 10^{-3}$ A4 putrescine standard solution with a specific activity of *980* Bq/ml was vortex mixed in the vial and allowed to stand for 30 min before withdrawal of 500 μl for scintillation counting. Each type of tube was tested empty or containing 1 ml 2 M NaOH, and each test was replicated five times.

Benzoylation of [^{14}C]putrescine. A 500-μl volume of [^{14}C]putrescine was benzoylated according to the procedure described above. After the diethyl ether extraction, the activities of the ether phase and the water phase were determined. The determination was replicated five times. The activity due to the content of [^{14}C]putrescine was determined by counting in a United Technologies Packard Model 2000 Tri-Carb liquid scintillation analyser. The counts obtained were corrected for background activity and quenching by the matrix.

Statistics

All statistical analysis was performed by using the PC-SAS statistics program pack, notably the procedures GLM (analysis of variance, Newman Keul range test) and CORR (correlation analysis) [7].

RESULTS AND DISCUSSION

The examination of the procedure for derivatization of free putrescine present in plant material suggested by Flores and Galston [2] was focused on the handling of plant extracts and the derivatization procedure.

Extraction of putrescine

Choice of extractant. Extraction of the plant material was carried out in 5% perchloric acid or 5% trichloroacetic acid, both of which are protein denaturing. No difference was found in the efficiency of these extractants (ANOVA, p = 0.05, n = 16).

Adsorption losses of polyamines. At physiological

TABLE I

RECOVERY OF [^{14}C]PUTRESCINE AFTER 30 min OF CONTACT WITH VIAL

Vial	Recovery (%)	
	Empty glass	1 ml of 2 *M* NaOH in glass
New glass vial, unused	99f0.9	100 ± 7.0
New glass vial, saturated	99 ± 0.4	101 ± 4.5
Old glass vial	99 ± 0.5	100 ± 1.9
Polycarbonate vial	104 ± 6.8	103 ± 2.8

[a] Each value is the mean of five replicates ± standardard deviation. None of the recoveries is significantly different (ANOVA and Newman Keul range test $p = 0.05$).

pH, the amino groups of the free polyamines carry positive charges, making them amenable to binding to negatively charged groups on the surfaces that they encounter. Following the rupture of plant cells in the extraction step, free polyamines may adsorb on glass surfaces by binding to free silanol groups. Such adsorption losses have been reported by Liang et *al.* [6], and caused Flores and Galston [2] to suggest that plant extracts and polyamine standards be handled and stored in plastic vials. The adsorption of putrescine on different vial materials was investigated in a tracer experiment with [^{14}C]putrescine. The vials were tested empty or containing 1 ml of 2 *M* NaOH (the benzoylation procedure starts with alkalinization of the acid plant extract in 1 ml of 2 *M* NaOH). The recoveries of the tracer are given in Table I.

The adsorption experiment revealed no significant difference between any of the vial materials tested (one-way ANOVA, $p = 0.05$, $n = 40$). Addition of plant extract to the putrescine standard produced similar results, and further studies comparing polyethylene and polycarbonate vials revealed no difference between the two plastic materials (data not shown). As no adsorption loss was observed for any of the materials tested, the investigation does not support the preference of plastic materials over glass materials for handling and storage of polyamine samples.

Although free putrescine does not adsorb on the vials, it may bind to the solid phase of the plant tissue slurry in the extraction step. In order to investigate the loss of polyamines from the liquid phase during the extraction of plant material, 1,6-diaminohexane [a diamine that is chemically closely related to putrescine (1 ,4-diaminobutane) but does not occur in plants] was added to the extractant prior to maceration in the extraction step. Table II gives the results obtained from the analysis of chromatographic data from experiments reported elsewhere [4].

The diaminohexane peak height shows a very strong negative correlation with the amount of plant material extracted, indicating a loss of diaminohexane from the liquid phase of the plant tissue slurry through adsorption on the plant tissue present. Similar adsorption must be expected for the endogenous plant amines, and should be corrected for in the calculation of free polyamine concentration in the plants.

Benzoylation of plant extract

Benzoylation takes place through the addition of a large excess of benzoyl chloride to the alkalinized acid extract. Contact between the hydrophobic benzoyl chloride and the hydrophilic amines is established by sonicating the mixture or shaking it with a vortex mixer. A comparison of different mixing methods and intensities (vortex mixing for 3, 10 or 30 s or sonication for 60 s) showed no significant difference in benzoylation efficiency ($p = 0.18$), indicating that this step is not particularly critical.

TABLE II

CORRELATION COEFFICIENTS BETWEEN PEAK HEIGHT OF 1,6-DIAMINOHEXANE ADDED PRIOR TO EXTRACTION, AND AMOUNT OF PLANT MATERIAL EXTRACTED

Results from individual polyamine analyses of 292 barley plants and 238 rape plants [4]. The range of the amount of plant material extracted for barley was 19–358 mg and for rape 75-598 mg.

Plant material	Correlation coefficient'
Barley	− 0.4977
Rape	-0.3219

[a] In both instances the correlation was significant at a level of $p = 0.0001$.

Following a reaction period of 20 min (duration not critical), the benzamides are extracted from the mixture with diethyl ether. Intimate contact between the ether and water phases for the extraction is established through vortex mixing, the duration of which was found to be important. Equilibrium was not attained after 60 s of vortex mixing [ANOVA and the Newman Keul range test show significant differences ($p = 0.05$) between 10, 30 and 60 s of vortex mixing], and care should be taken to standardize this step of the benzoylation procedure.

The low surface tension of diethyl ether makes the addition and withdrawal of exact volumes difficult. However, the operation is facilitated by wetting the inside of the pipette tip with diethyl ether just prior to aspiration of the ether sample.

The ether and water phases are allowed to separate for 10 min (centrifugation is superfluous) before a well defined and fixed fraction (e.g., 50%) is withdrawn from the ether phase and evaporated to dryness. Drying is rapid under a flow of nitrogen. However, for the concurrent benzoylation of a large number of samples, passive ether evaporation at room temperature under a dust-preventing shelter for several hours may be preferable, and does not influence the final results (data not shown).

Overall benzoylation efficiency. The overall efficiency of the benzoylation procedure was investigated in a tracer experiment with [^{14}C]putrescine. An investigation of the distribution of [^{14}C]putrescine after a diethyl ether-water equilibration showed that only about 1% of the free putrescine is found in the ether phase (data not shown). Adsorption of putrescine on glass was found to be insignificant. The activity found in the ether phase following benzoylation is therefore ascribed to benzoylated [^{14}C]putrescine alone. The overall benzoylation efficiency, defined as activity of benzoylated putrescine in the ether phase after benzoylation divided by total activity of putrescine in mixture after benzoylation, can be calculated as activity of ether phase after benzoylation divided by total activity in sample prior to benzoylation.

The benzoylation efficiency was found to be 75%, varying between 59 and 83% ($n = 5$) with a standard error of 5%. The overall recovery of the tracer after benzoylation was 99%. Roberts *et al.* [8] found a benzoylation efficiency of 94% with [^{14}C]putrescine added to tissue slurries before benzoylation, and Lauren et *al.* [9] found an efficiency of 79% with a range of 68–97%.

Internal standard

The variation in benzoylation efficiency justifies the addition of an internal standard to the sample to ensure quantitative determination of the polyamines. Redmond and Tseng [1] suggested 1,6-diaminohexane. With no natural occurrence in plants, and close chemical similarity lo putrescine, it is an obvious choice as an internal standard for the determination of putrescine.

By addition of the internal standard to the solvent used for extracting the plant material, the internal standard will experience exactly the same conditions as the polyamines from the plant during benzoylation. Roberts *et al.* [8] found nearly identical recoveries of diaminohexane and putrescine after addition to plant extract and subsequent benzoylation. Variations in benzoylation efficiency can thus be corrected by the use of an internal standard. It is unclear, however, whether externally added diaminohexane and endogenous free plant polyamines will behave alike during the extraction of plant material, in which process diaminohexane starts outside the plant cells and the endogenous polyamines inside. Table III summarizes information obtained from analysis of chromatographic data from experiments reported elsewhere [4].

The plants were grown under identical conditions

TABLE III

CORRELATION COEFFICIENTS BETWEEN PEAK HEIGHT OF ADDED 1,6-DIAMINOHEXANE AND PEAK HEIGHTS OF PUTRESCINE AND SPERMIDINE AFTER HPLC ANALYSIS

Plants were grown under identical conditions: 53 barley plants for 2–4 weeks and 51 rape plants for 3-5 weeks (control plants from experiments reported elsewhere [4]). Peak heights of putrescine and spermidine were calculated for a fixed amount of plant material.

Compound	Correlation coefficient[a]	
	Barley	Rape
Putrescine	0.5192	0.7145
Spermidine	0.692 1	0.7205

[a] In all instances the correlation was significant at a level of $p = 0.000$ 1.

and were expected to show similar contents of putrescine and of spermidine. For a fixed amount of plant material extracted, the concentrations of each of the two polyamines would therefore be expected to be at the same level in all the samples, as is the case for the diaminohexane that was added together with the extractant.

The positive correlation between the peak heights of diaminohexane and putrescine or spermidine obtained after HPLC of the benzoylated plant extracts is very strong. This indicates that the overall behaviour of diaminohexane, putrescine and spermidine is similar, not only during the derivatization procedure but also in the plant tissue slurry. The endogenous plant polyamines therefore seem to suffer adsorption losses similar to the externally added diaminohexane during extraction of the plant material.

The addition of diaminohexane as an internal standard at the start of the polyamine analysis through the extractant used for extracting polyamines from the plant material thus permits the correction of errors and variations in efficiencies of the entire procedure.

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