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GAS CHROMATOGRAPHIC DETERMINATION OF 2(3)-BENZOXAZOLINONES FROM CEREAL PLANTS*

CHUNG-SHIH TANG, SIEW HOONG CHANG,† DOLAN HOO and KARL H. YANAGIHARA

Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822

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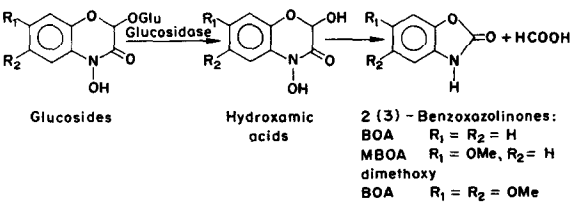
Key Word Index—*Zea mays*; *Triticum aestivum*; *Secale cereale*; *Coix lacryma jobi*; cereal plants; corn; wheat; rye; Job's tears; Gramineae; 2(3)-benzoxazolinones; GLC determination; GC–MS.

Abstract—2(3)-Benzoxazolinone (BOA), 6-methoxy-2(3)-benzoxazolinone (MBOA) and 6,7-dimethoxy-2(3)-benzoxazolinone (dimethoxy-BOA) could be separated by GC. The identity of these components was verified by combined GC–MS. BOA and MBOA were determined quantitatively in 0·1 g samples of corn seedling. The presence of analogs not previously reported was demonstrated in seedlings of wheat, rye and in leaves of Job's tears. Seedlings of rice, barley, oat and sorghum did not contain any detectable amount of benzoxazolinones.

INTRODUCTION

In 1955, Koyama [1] first reported the isolation and identification of 6-methoxy-2(3)-benzoxazolinone (MBOA) in the root of Job's tears (*Coix lacryma jobi* L.), an ornamental and medicinal plant of the family Gramineae [2]. In the same year, Virtanen and Hietala isolated 2(3)-benzoxazolinone (BOA) from rye (*Secale cereale*) [3]. MBOA was later reported in wheat (*Triticum aestivum*) [4]. Both BOA and MBOA were found in corn (*Zea mays*) [5], and more recently, 6, 7-dimethoxy-2(3)-benzoxazolinone (dimethoxy-BOA) was identified in the dried tissue of corn. This new analog may have biosynthetic and functional characteristics similar to those of BOA and MBOA [6].

Because of their germicidal and insecticidal activities, naturally occurring benzoxazolinones and their precursor hydroxamic acids have been recognized as plant resistance factors. The reactions responsible for the formation of benzoxazolinones in macerated corn seedlings can be summarized as follows:



Despite the general interest among biologists and chemists studying the resistance of benzoxazolinone-containing crops, ideal methods are not yet available for the analysis of the individual compounds. So far, four procedures have been adopted for the quantitative determination of hydroxamic acids and benzoxazolinones in plant materials. They are the isotope dilution technique [7]; the direct measurement of benzoxazolinones by A at 285 nm [8]; spectrofluorometry of benzoxazolinones [9]; and finally, the colorimetric procedure for the determination of hydroxamic acids using $FeCl_3$ as a chromogenic reagent [10]. The isotope dilution technique, besides being tedious and requiring the synthesis of C^{14}-benzoxazolinones, also needs a relatively large amount of plant material. The three spectrophotometric methods

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† Graduate Student and East–West Center grantee, Department of Horticulture.

are generally non-specific in nature and usually require clean-up of sample extracts prior to analysis. However, Hamilton [10] reported that the presence of interfering compounds in corn seedlings was insignificant when the $FeCl_3$-colorimetric method was used, and recently a rapid procedure for the estimation of hydroxamic acids in corn seedlings has been developed by Long *et al.* [11].

Bowman *et al.* worked on the GLC determination of BOA and MBOA, but were not able to obtain satisfactory results [9]. We wish to report a GLC method capable of determining the concentration of each benzoxazolinone analog in *ca* 0·1 g of fresh corn seedlings. Amounts of BOA and MBOA in etiolated corn seedlings were determined to demonstrate this procedure. Because of the high sensitivity and specificity of the present method, BOA was found in wheat, MBOA in rye and in the mature leaves of Job's tears. Presence of these benzoxazolinone analogs has not been reported previously in the above plants.

RESULTS

In a typical gas chromatogram of the CH_2Cl_2 extract of macerated corn seedling 3 peaks were obtained with R_t's of 1 min, 3 min and 6 min. MS obtained from the first 2 peaks were identical to those obtained from the standard BOA and MBOA, respectively. The MS of GLC peak 3 (R_t 6 min) has a peak at *m/e* 195 (M^+, 100%) and a characteristic peak at 180 ($M^+ - Me$, 82%), corresponding to the reported values for dimethoxy-BOA [6]. Differences of 30 amu (OMe − H) are observed for M^+ of peaks 1, 2 and 2, 3, suggesting that they are analogs with different numbers of methoxy substitutions.

The amounts of BOA and MBOA determined from seedling samples of corn, wheat, rye and mature plants of Job's tears are summarized in Table 1. Dimethoxy-BOA was only found in samples of corn seedling. We have also examined the etiolated seedlings of rice, barley, oat and sorghum with the present procedure. None of these contained any detectable amount of benzoxazolinones.

Table 1. Concentration of BOA and MBOA from benzoxazolinone-containing plants*

Sample		Concentration mg/g fr. wt† BOA	MBOA
Corn (CM150)	shoot	0·22	2·55
(CI 21E)	shoot	0·36	2·61
Wheat	shoot	>0·01	0·56
	root	0·02	0·41
Rye	shoot	0·71	0·12
	root	0·09	0·31
Job's tears	leaf	ND‡	0·21
	root	ND	2·09

* 7-day-old etiolated seedlings except Job's tears, for which mature plant was used. † Average of three determinations for corn seedling samples. Standard deviation did not exceed 0·04. The rest are the average of 2 determinations. ‡ ND = not detected.

DISCUSSION

2(3)-Benzoxazolinones are naturally occurring cyclic carbamates. GLC determination of synthetic carbamate insecticide residues in plant material has been reviewed by Williams [12]. It is generally known that many of these compounds are readily decomposed on GLC columns, and care must be exercised in column preparation. We found that columns prepared according to Leibrand and Dunham [13] were consistently satisfactory. This could be attributed to the elimination of active sites on the supporting material by effective coating with the procedure they described. It is important, also, to use an all-glass GLC system to avoid catalytic decomposition of the benzoxazolinones by any metal surface.

In order to prove that the benzoxazolinone peaks observed on the gas chromatograms were resolved from other possible interfering volatile compounds, MS were taken at the front, apex and the rear portions of each peak. Identical fragmentation patterns were obtained indicating they were free from other compounds. Linear response with respect to peak height and concentration was obtained from standard MBOA and BOA solutions. As a further test, the percentage of recovery of MBOA from corn seedling was examined by adding a known quantity of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one(DIMBOA), the hydroxamic acid precursor of MBOA, to the homogenate of mutant corn seedling bxbx, which contains only a trace amount of DIMBOA [14]. Results from GLC analysis of the bxbx samples indicated almost complete recovery of the DIMBOA added. Based on the above evidence, the present procedure appears to be suitable for the assay for samples that are free of interfering volatile components.

Among the benzoxazolinones determined, BOA has not been reported in wheat and MBOA has not been reported in rye previously. Also, MBOA was reported to be absent in the leaves of Job's tears [15]. With the more sensitive and specific GLC method, however, their presence can be easily established. Dimethoxy-BOA is a relatively minor component among the benzoxazolinones in corn and it was not detected in the above plant samples.

Two types of standards were used in the present experiment. For BOA determination, standard solutions were prepared directly from a commercial sample. For MBOA, they were obtained by converting a known quantity of DIMBOA in boiling water [16] followed by CH_2Cl_2 extraction. Since the latter underwent the same preparative steps as those of the seedling samples, losses occurring during the conversion and extraction would be compensated for; and consequently, in Table 1, the values reported for MBOA would be more accurate than those for BOA.

The present procedure, unlike the other quantitative methods, is capable of determining the individual concentration of 2(3)-benzoxazolinone analogs in plant material. Data in Table 1 were obtained from less than 0·2 g of fresh sample, which suggests that our method may be adopted as a non-destructive assay procedure for plant breeding. Moreover, out of the vast number of plant species, benzoxazolinones have so far only been reported in corn, wheat, rye and Job's tears. This simple yet specific procedure should contribute to the further understanding of the distribution of these unique plant disease resistance factors.

EXPERIMENTAL

Gas chromatography was performed on 1 m × 2 mm id glass column packed with Silar-5CP on 80–100 mesh Chromosorb W—HP prepared according to the procedure of ref. [9]. A 0·5% soln of Silar-5CP in $CHCl_3$ with a 3-vol excess of the solid support was used for coating. Flow rates were: N_2, 25 ml/min; H_2, 25 ml/min; air, 300 ml/min. The temps were: injector, 200°; dual F.I.D. detectors, 210°; column, 175° for BOA and 200° for MBOA analyses. Quantitative measurements were determined by comparison of the peak heights of standard solns and those of the samples. For GC–MS, a time of flight instrument was used with a sensitivity of 10^{-6} A/V, electron multiplier high voltage −2.00 kV and electron energy −69·5V; MS were usually taken at the apex of the peaks.

Sample preparation Seeds of corn (*Zea mays*, CM105 and CI 21E) were germinated in the dark for 7 days in a beaker lined with wet filter paper. Young shoots (*ca* 0·1 g) were weighed, homogenized in an all glass Ten Broeck homogenizer with *ca* 1 ml of H_2O, the homogenate was transferred to a centrifuge tube. The final vol was made up to 10 ml with H_2O. After incubation at room temp for 1 hr to allow complete enzymatic hydrolysis of the glucosides, hydroxamic acid aglucones were converted to benzoxazolinones by heating at 100° for 30 min. [16]. The aq. suspension was then centrifuged and supernatant extracted 2× with 15 ml of redist. CH_2Cl_2. Organic phases were combined, dried and evaporated at 100° under N_2 to a concn suitable for GLC analysis.

Viable seeds of wheat (*Titicum aestivum*, I.N.I.A. 66R), rye (*Secale cereale*, Merced) and several other cereal crops were obtained from the California Crop Improvement Association, University of California at Davis. Mature plants of Job's tears (*Coix lacryma jobi* L.) were collected from Lyon Arboretum, Honolulu, Hawaii. The seeds were germinated and the samples prepared as for the corn, except *ca* 0·2 g of shoot or root samples were used. The fresh leaf and root samples of mature Job's tears were also prepared from *ca* 0·2 g of tissue with the same procedure. Standard soln of DIMBOA was prepared from corn seedlings according to ref [16]. The MS of the isolated crystals, using a probe, was identical to that of a standard sample obtained from Dr. C. L. Tipton of Iowa State University and to the reported data in ref. [6]. A standard MBOA soln was prepared from a 0·2 mM aq. DIMBOA soln using a procedure similar to that described in the sample preparation section. Standard BOA soln was prepared from a commercial product.

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REFERENCES

1. Koyama, T. (1955) *J. Pharm. Assoc.* (Japan) **75,** 702.
2. Neal, M. C. (1965) in *In Gardens of Hawaii*, p. 80, Lancaster Press, Lancaster, Pa.
3. Virtanen, A. I. and Hietala, P. K. (1955) *Acta Chem. Scand.* **9,** 1543.
4. Virtanen, A. I. and Wahlroos, O. (1958) *Suomen Kemistrilehti* **B31,** 402.
5. Loomis, R. S., Beck, S. D. and Stauffer, J. F. (1957) *Plant Physiol.* **32,** 379.
6. Klun, J. A., Tipton, C. L., Robinson, J. R., Ostrem, D. L. and Benoza, M. (1970) *J. Agr. Food Chem.* **18,** 663.
7. Klun, J. A. and Brindley, T. A. (1966) *J. Econ. Ent.* **59,** 711.
8. Beck, S. D., Kaske, E. T. and Smissman, E. E. (1957) *J. Agr. Food Chem.* **5,** 933.
9. Bowman, M. C., Beroza, M. and Klun, J. A. (1968) *J. Econ. Ent.* **61,** 120.
10. Hamilton, R. H. (1964) *J. Agr. Food Chem.* **12,** 14.
11. Long, B. J., Dunn, G. M. and Routley, D. G. (1974) *Crop Sci.* **14,** 601.
12. Williams, I. H. (1971) *Residue Rev.* **38,** 1.
13. Leibrand, R. J. and Dunham, L. L. (1973) *Res. Develop.* **24,** 32.
14. Couture, R. M., Routley, D. G. and Dunn, G. M. (1971) *Physiol. Pl. Path.* **1,** 515.
15. Koyama, T., Yamato, M. Kubota, K. (1956) *J. Pharm. Assoc.* (Japan) **76,** 1077.
16. Wahlroos, O. and Virtanen, A. I. (1959) *Acta Chem. Scand.* **13,** 1906.