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Separation and quantification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones in maize root extract by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatography method is described for the separation and quantification of 1,4-benzoxazin-3-ones and benxoxaxolin-Zones in maize root extract. The four compounds, 2,4-dihydroxy-7-methoxy-1,4-benxoxazin-3(4H)-one, 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3(4H)-one, 2-hydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one and 6-methoxybenzoxazolinone, were separated and identified within 30 min on a C_{18} reversed-phase column using a gradient of methanol and phosphoric acid. The technique has useful applications in studies of the chemical ecology of plant root and subterranean pest interactions.

INTRODUCTION

It is well known that 1,4-benzoxazin-3-ones are present in several species of Gramineae, such as maize, wheat [1] and rye [2]. Some of these compounds and their decomposition products have been found to play an important role in the resistance of plants to insect pests such as European corn borer, ***Ostrinia nubilalis*** (Hiibner) [3], cereal aphids, ***Rhopalosiphum maidis*** (Fitch) [4], ***Metopolophium graminum*** (Walker) [5] and to plant pathogenic fungi, ***Helminthosporium turcicum*** [6] and bacteria, ***Erwina carotovia*** [7]. We recently found 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a major 1,4-benzoxazin-3-one in maize, to be toxic to western corn rootworm, ***Diabrotica virgifera virgifera*** (LeConte) [8].

During the past three decades, several methods have been developed to separate

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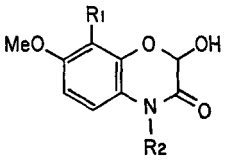
and to quantify 1,4-benzoxazin-3-ones and related compounds. The simplest method available is to measure total hydroxamic acid content by measuring the absorbance of the blue complex formed between hydroxamic acids and $FeCl_3$ [9–11]. This method can only estimate total cyclic hydroxamic acids as $FeCl_3$ does not react with benzoxazolinones. Another method is based on the assumption that 1 mole of hydroxamic acid (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one, DIMBOA) produces 1 mole of benzoxazolinone (6-methoxybenzoxazolinone, MBOA) after degradation, and measures hydroxamic acid content as benzoxazolinones. The benzoxazolinones were quantified by isotopic dilution [3], infrared spectrophotometry [12], fluorometry [13], gas chromatography (GC) [14] and by high-performance liquid chromatography (HPLC) [15]. However, it has been found that the amount of MBOA formed from DIMBOA varies with temperature, pH and composition of the reaction medium and always yields less than 75% of MBOA from DIMBOA [16]. Therefore, it is clear that these methods can lead to erroneous estimates of DIMBOA content in plant extracts. More recently, GC and HPLC methods have been developed which can separate and quantify individual cyclic hydroxamic acids and related compounds [14,17–20].

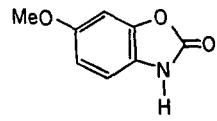
Most of the authors cited above used plant leaves (corn or wheat) as experimental materials to quantify the compounds concerned, although Klun and Robinson [21] have reported that corn root, compared with corn stalk, whorl and leaf, contains the highest concentration of 1,4-benzoxazinones. Corn roots have been used as experimental materials to quantify total cyclic hydroxamic acids [22,23], but none of these studies has quantified the individual hydroxamic acids and/or related compounds. Our previous work [8] has demonstrated that DIMBOA, the main hydroxamic acid in maize, plays an important role in the defence of maize to the western corn rootworm, a serious insect pest which damages the corn root system. To understand the chemical ecology of corn root subterranean pest interactions, it is necessary to develop a method for the quantitative determination of the content of individual hydroxamic acids and related compounds in corn root.

In this paper we report an accurate HPLC method for separation and quantification of two hydroxamic acids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one (DIMBOA), 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3(4H)-one (DIM_2BOA), one lactam, 2-hydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one (HMBOA) and one benzoxazolinone, 6-methoxybenzoxazolinone (MBOA) (Fig. 1) in corn root extracts.



1,4-benzoxazin-3-ones

R	R2	Abbreviation
H	OH	DIMBOA
MeO	OH	DIM_2BOA
H	H	HMBOA



benzoxazolin-2-one (MBOA)

Fig. 1. 1,4-Benzoxazin-3-ones and benzoxazolin-2-one found in maize root extracts. Me = Methyl.

It represents the first gradient HPLC method to separate a variety of hydroxamates and derivatives in maize root extracts.

EXPERIMENTAL

Standard compounds

All standard compounds (DIMBOA, DIM_2BOA, HMBOA and MBOA) were synthesized in our laboratory, and the complete synthetic details were described by Atkinson [24].

Extract sample preparation

Seeds of maize (*Zea mays* L.) (hybrid 359GC3-6 × 46-43) were soaked in tap water for 24 h and planted in a plastic pot (15 cm x 15 cm) with growing medium of one part perlite and one part vermiculite. Greenhouse temperature was maintained at approximately 22/20°C day/night and the photoperiod was 12L: 12D (12 h light: 12 h dark; 12 h light provided by natural sunlight plus 3 h artificial light at the beginning and at the end of the photoperiod). After two weeks of growth under these conditions, corn roots were taken out and washed with tap water, then with distilled water. One gram of this fresh root material was cut in small pieces and homogenized with 2 x 5 ml distilled water using a mortar and pestle. The slurry was extracted by a modification of the method described by Gutierrez *et al.* [18].

Apparatus and HPLC procedures

The root extract in methanol was analyzed using a Perkin-Elmer HPLC system equipped with a Model 250 binary pump, and a Model LC-480 auto scan diode-array detector. A 20-μl volume of the extract sample in methanol was injected into the liquid chromatography system. The sample was run under the following conditions: Ultrasphere ODS 5 μm, 25 cm x 4.6 mm reversed-phase C_{18} column (Beckman); a flow-rate of 1 ml/min; detection wavelength of 265 nm; an online UV scan of 190–400 nm; and a two-solvent system, solvent A 100% methanol and solvent B 20 m*M* H_3PO_4 (pH 2.3), was used as a mobile phase. A gradient program was processed as follows: Solvent A at the initiation was 10%; then when sample was injected, 10% A was linearly altered to 56% A in 25 min, and then to 100% A in 2 min. This ratio was held for 4 min to clean the column, after which the mobile phase was returned to the starting concentration (10% A) in 2 min. This ratio was maintained for 2 min to equilibrate the column.

Identification and quantitation of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones

Identification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones in corn root extract was carried out by comparison of retention times, UV spectra and by peak enrichment of known standard compounds. Quantitation of related compounds was derived from standard curves created by a dilution series (O-20 μg) of different compounds. Three replicates were prepared for each concentration.

Recovery efficiencies

A triplicate of different concentrations of DIMBOA, DIM_2BOA, HMBOA and MBOA were separately added to an erlenmeyer (7 cm x 7 cm) containing 1 g of

homogenated tissue from corn root and the extraction was processed as described above. In order to detect how much of the reference compounds was lost by binding to the tissue, a triplicate of different concentrations of the reference compounds were added to an erlenmeyer containing solvent (ethyl acetate) alone and the same extraction procedure was carried out.

RESULTS AND DISCUSSION

The purity of DIMBOA, DIM_2BOA, HMBOA and MBOA standards were checked by HPLC. Under the HPLC procedures described above, each of the standards showed one peak. When a standard mixture of DIM_2BOA, HMBOA, DIMBOA and MBOA was injected into the HPLC system, four peaks were resolved within 25 min with a retention time of 18.95 min (DIM_2BOA), 20.10 min (HMBOA), 20.83 min (DIMBOA) and 24.32 min (MBOA), respectively. The retention times of the mixture of four standards were almost identical as when they were injected individually.

A typical chromatogram of a corn root sample extracted for 1,4-benzoxazin-3-ones and related compounds is shown in Fig. 2. DIM_2BOA, HMBOA, DIMBOA and MBOA were identified as the major compounds in corn root extracts based on comparison of retention times and spectra. Other minor hydroxamates appear to be present in the extract sample shown in Fig. 2, but we have, so far, no available data for the identification and quantification.

Fig. 3 shows typical UV spectra of 1,4-benzoxazin-3-ones and related compounds in corn root extracts which are useful in confirmation of the identification of the compounds concerned. DIM_2BOA, HMBOA and DIMBOA have similar but distinct UV spectra with absorbance maxima at wavelengths of 264 nm, 261 nm (shoulder 289 nm) and 264 nm (shoulder 292 nm), respectively. MBOA has two absorbance peaks with maxima of 230 nm and 292 nm.

Quantitative estimates of the identified compounds were derived from standard curves for DIM_2BOA, HMBOA, DIMBOA and MBOA. Regression formulas for the quantitation of different compounds in practical samples were: y (μg DIM_2BOA/ml sample) = 31.99 + 7548.50. (absorbance units) ($r = 0.99$, $P < 0.001$); y (μg

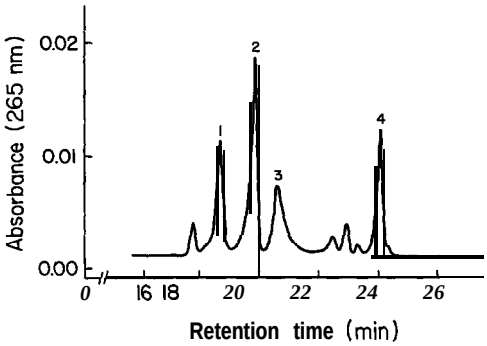


Fig. 2. Chromatogram of maize root extracts separated by the conditions as described in the text. 1 = DIM_2BOA; 2 = HMBOA; 3 = DIMBOA; 4 = MBOA.

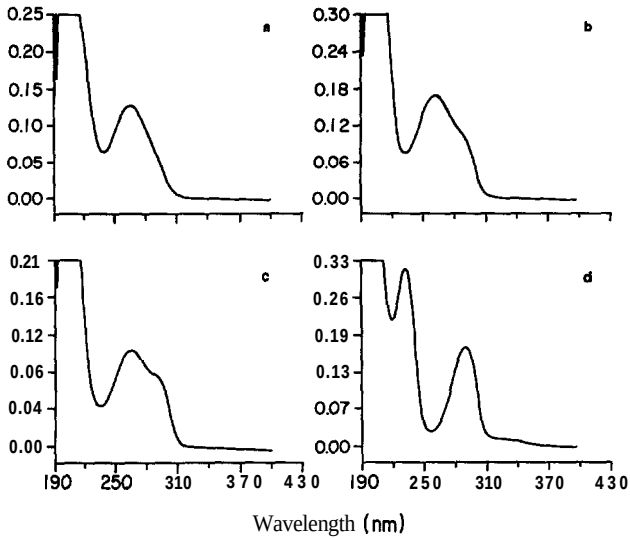


Fig. 3. LJV spectra of 1,4-benzoxazin-3-ones and benzoxazolin-2-one in a maize root extract. (a) DIM_2BOA; (b) HMBOA; (c) DIMBOA; (d) MBOA.

HMBOA/ml sample) = 11.42 + 2627.00. (absorbance units) (r = 0.98, $P <$ 0.0001); y (μg DIMBOA/ml sample) = 96.29 + 10898.50. (absorbance units) (r = 0.99, $P <$ 0.0001; y (μg MBOA/ml sample) = 63.79 + 12360.50 · (absorbance units) (r = 0.99, $P < 0.0001$). By using these formulas, concentrations of compounds in practical samples were calculated, and Table 1 shows the analysis of two different corn lines which were used for the study of corn resistance to western corn rootworm [8]. ITR 3872 contains considerably more of all the hydroxamates, which we believe explains its demonstrated resistance to rootworm [8].

The limits of detection of the present method were determined from a dilution series. It appears that this method can detect 1 μg/ml for DIM_2BOA, DIMBOA and MBOA, and 0.5 μg/ml for HMBOA. The accuracy of the present method was evaluated by a recovery test. Triplicate known amounts of each compound were individually added to an erlenmeyer containing 1 g of homogenated root sample and processed according to the extraction procedure. The results are shown in Fig. 4. The intercept values in these plots represent the original amounts of each known compound found in the root sample without adding any known compounds. The recoveries are represented by the slopes of the regressions. The recoveries of

TABLE I

CONTENTS OF 1,4-BENZOXAZIN-3-ONES AND BENZOXAZOLIN-ZONES IN TWO DIFFERENT MAIZE LINES (μg/g FRESH ROOT)

Maize line	DIM_2BOA	HMBOA	DIMBOA	MBOA
ITR 3872	120.95	86.96	718.48	214.14
NTR-2 Ger. 4042	2.72	9.78	36.20	69.64

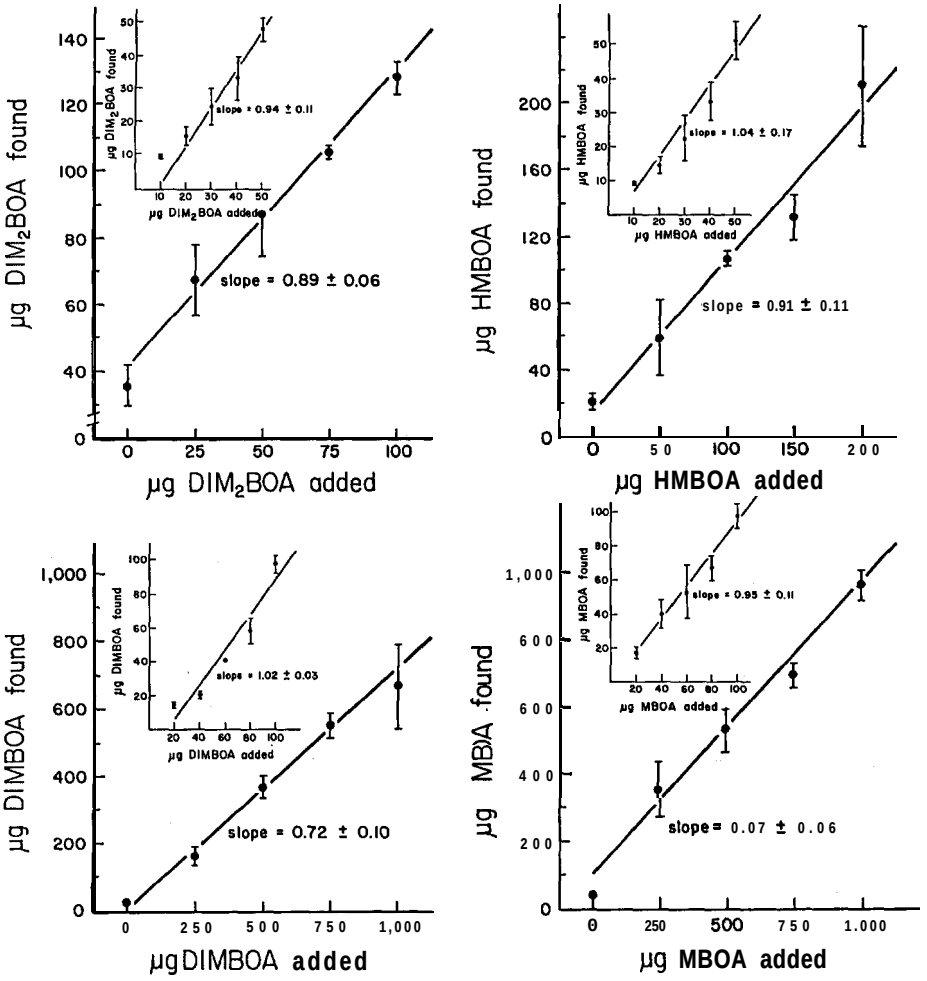


Fig. 4. Recovery of DIM_2BOA, HMBOA, DIMBOA and MBOA added to maize root samples prior to extraction and HPLC analysis. In each plot, the intercept values withy axes represent the original amount of the compounds present in the samples; the slope of each regression is equal to the fraction of the compounds recovered. The figures in the comers show the recovery of the compounds added to solvent (ethyl acetate) alone prior to extraction and HPLC analysis. Bars indicate standard deviation.

DIM_2BOA, HMBOA, DIMBOA and MBOA were 89 ± 4%, 91 ± 11%, 72 ± 10% and 87 ± 8% (mean ± S.D.). The recovery of DIMBOA was relatively low, due to decomposition of DIMBOA to MBOA. When the extraction was carried out with solvent (ethyl acetate) alone (no added plant tissue), the recoveries of DIM_2BOA, HMBOA, DIMBOA and MBOA were obviously increased (Fig. 4). This may be the result of cyclic hydroxamic acids reacting with plant tissue. It has been reported that cyclic hydroxamic acids react with protein thiol groups, which is one possible cause of yield loss and one of the unavoidable problems in quantification of cyclic hydroxamic acids [25.26].

The standard deviations in Fig. 4 are large for most data points. In comparison with the standard deviation in standard curves (> OS%), it indicates that the main source of error appeared in the extraction procedure rather than in the separation method used.

The HPLC procedure reported here is a rapid and accurate method for separation and quantification of individual 1,4-benzoxazin-3-ones and related compounds in corn root extracts. Compared with GC, the present HPLC method eliminates the derivatization step and can separate and quantify at least four 1,4-benzoxazin-3-ones and related compounds within 30 min.

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