Separation and Quantification of Cyclic Hydroxamic Acids and Related Compounds by High-Pressure Liquid Chromatography

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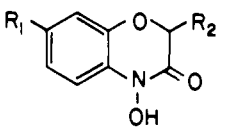
A procedure is described for the quantitative extraction and HPLC analysis of cyclic hydroxamic acids and related compounds including 2-(2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) β-D-glucopyranoside (DIMBOA-glc) and the demethoxy analogue DIBOA-glc; their respective aglycons, DIMBOA and DIBOA; and their respective 2(3*H*)-benzoxazolinone derivatives, MBOA and BOA. The six compounds were separated within 15 min on a C18 reversed-phase column using a linear gradient of acetic acid and methanol. The minimum detection limit for DIMBOA-glc and DIMBOA was 0.20 nmol. DIMBOA-glc concentrations, which ranged from 8 to 2600 μg/g fresh weight, were measured by this procedure in methanolic extracts of corn leaves and seedlings on samples of 0.05–0.10 g; DIBOA-glc was also detected in many of the samples. DIMBOA, DIBOA, MBOA, and BOA were detected in aqueous extracts of corn samples after hydrolysis of the glucosides by endogenous β-glucosidases.

Cyclic hydroxamic acids (1,4-benzoxazin-3-ones) occur in several gramineous species, including corn, wheat, and rye (Wahlroos and Virtanen, 1959). These compounds are purported to serve various functions in the plant, such as disease and insect resistance, herbicide tolerance, growth regulation, and mineral metabolism (Stoessl, 1983). Their actual roles in most of these functions have not been conclusively established, however, and there is evidence that cyclic hydroxamic acids often do not persist in mature plant tissues (Klun and Robinson, 1969; Baker and Smith, 1977).

In the undisturbed plant, cyclic hydroxamic acids exist as stable β-glucosides that, upon cell disruption, are enzymatically converted to the corresponding aglycons (Hofman and Hofmanova, 1971). In aqueous solutions, and in some organic solvents, the aglycons are converted to 2(3*H*)-benzoxazolinones. Both the aglycons and the benzoxazolinones exhibit biological activity, whereas the glucosides are essentially inactive (Baker and Smith, 1977; Wahlroos and Virtanen, 1959; Argandona et al., 1981).

The predominant cyclic hydroxamic acid in corn and wheat is 2-(2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one β-D-glucopyranoside (DIMBOA-glc, I) (Hofman and Hofmanova, 1969). DIMBOA-glc, the corresponding aglycon DIMBOA (II), and the benzoxazolinone derivative MBOA (III) are the best characterized members of this group of related compounds. The respective demethoxy analogues DIBOA-glc (IV), DIBOA (V), and BOA (VI) also occur. DIBOA-glc is commonly present in corn and wheat, but its concentration is usually severalfold lower than that of DIMBOA-glc; in rye the inverse situation is found (Hofman and Hofmanova, 1969). Several other cyclic hydroxamic acids have been isolated from various sources (Hofman and Hofmanova, 1971).

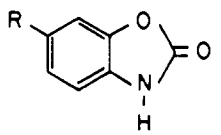


I. DIMBOA – glc : $R_1 = OCH_3$; R_2 = O – glucosyl

II. DIMBOA : $R_1 = OCH_3$; $R_2 = OH$

IV. DIBOA – glc : $R_1 = H$; R_2 = O – glucosyl

V. DIBOA : $R_1 = H$; $R_2 = OH$



III. MBOA : R = OCH_3

VI. BOA : R = H

Interest in these compounds has resulted in the development of several methods for quantifying them. Total hydroxamic acid content is estimated spectrophotometrically (Baker and Smith, 1977) and colorimetrically by measuring the absorbance of a blue complex with $FeCl_3$ (Argandona et al., 1981). In conjunction with paper or thin-layer chromatography these methods have been used to quantify specific cyclic hydroxamic acids (Knott and Kumar, 1972). This approach is laborious and is limited by the fact that $FeCl_3$ does not react with benzoxazolinones. Isotopic dilution (Klun and Brindley, 1966) and spectrofluorimetry (Bowman et al., 1968) have been used to measure specific cyclic hydroxamic acids indirectly, based on their conversion to the respective benzoxazolinone. The former method requires radioactively labeled substrates; both methods suffer from the fact that conversion of DIMBOA to MBOA is not stoichiometric (Woodward et al., 1978).

More recently, GLC and HPLC methods have been used to quantify cyclic hydroxamic acids and related compounds (Gutierrez et al., 1982; Tang et al., 1975; Woodward et al., 1979). None of these methods, however, measure the cyclic hydroxamic acids directly as the parent glucosides. This capability is desirable since the glucoside is the form in which these compounds actually exist in the undisturbed plant. Differences have been reported in estimates of cyclic hydroxamic acid content when these compounds were measured spectrophotometrically both directly as glucosides and indirectly as aglycons (Baker and Smith, 1977). Thus, if enzymatic hydrolysis of the glucosides is prevented during extraction, direct measurement of cyclic hydroxamic acid glucosides can avoid errors that may result from indirect measurement. Furthermore, the combined capability for measurement of the glucosides, aglycons, and benzoxazolinones is useful for studies on the biosynthesis of these compounds and for studies on their fate under conditions of insect feeding, disease, or other stress. In this paper we present a simple and accurate method for the quantitative extraction and HPLC analysis of the principal cyclic hydroxamic acid glucosides of corn and of

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their respective aglycon and benzoxazolinone derivatives.

MATERIALS AND METHODS

Preparation of Standards. DIMBOA-glc, DIMBOA, and DIBOA-glc were isolated from etiolated corn seedlings of the hybrid $Mo17_{Ht} \times B73_{Ht}$, which were grown at 28 °C in continuous darkness for 5 days. DIMBOA-glc was purified from 200 g of seedlings according to the procedure of Wahlroos and Virtanen (1959) except that cellulose chromatography was omitted. DIMBOA was purified from 600 g of seedlings as described by Woodward et al. (1978). Both compounds were tentatively identified as cyclic hydroxamic acids by their positive reaction with $FeCl_3$. The putative DIMBOA-glc was stable to extensive heating in water, but upon acid hydrolysis (2 N HCl, 100 °C, 2 h) it yielded an ethyl acetate soluble compound that cochromatographed in 1-butanol/acetic acid/water (5:1:4, v/v/v, upper phase, BAW) on silica gel (silica gel 60, E. Merck) with the putative DIMBOA. The aqueous hydrolysate fraction contained glucose as determined by its positive reaction with glucose oxidase (Statzyme, Worthington Chemicals) and by cochromatography in BAW on 3MM Whatman paper. The identities of DIMBOA-glc and DIMBOA were confirmed by UV and mass spectra. The UV spectrum of DIMBOA-glc was λ_{max} 266 nm with a shoulder at 282 nm. This is in agreement with other reports in which λ_{max} ranges from 260 to 270 nm (Gahagan and Mumma, 1967; Knott and Kumar, 1972; Wahlroos and Virtanan, 1959). Mass spectrum, which agreed with that reported by Gahagan and Mumma (1967): *m/e* 373 (parent, 0%), 357 (8%), 195 (65%), 166 (base peak), 165 (11%), 138 (15%), 110 (20%). UV spectrum of DIMBOA, which agreed with that reported by Woodward et al. (1978): λ_{max} 262 nm, with a shoulder at 282 nm. Mass spectrum for DIMBOA: *m/e* (211 (parent, 9%), 195 (4%), 193 (11%), 166 (25%), 165 (base peak), 150 (50%), 138 (10%), 122 (15%), 110 (20%), 109 (20%), 106 (36%). This agrees very closely to that reported by Woodward et al. (1978).

MBOA was obtained by heating an aqueous solution of DIMBOA at 70 °C for 1 h (Woodward et al., 1978). MBOA was extracted from the aqueous solution into ethyl acetate and was purified by TLC in BAW on silica gel. The λ_{max} for MBOA was 288–290 nm, which agrees with reports in the literature ranging from 285 to 290 nm (Klun and Brindley, 1966; Gutierrez et al., 1982; Wahlroos and Virtanen, 1959; Woodward et al., 1978). Mass spectrum: *m/e* 165 (parent, base peak), 150 (45%), 136 (3%), 122 (15%), 109 (20%), 106 (36%). This agreed very closely with that reported by Woodward et al. (1978).

DIBOA-glc was extracted in methanol from 20 g of seedlings and purified by HPLC using the same procedures as described below for routine analyses of samples for DIMBOA-glc content. Eluant fractions containing DIBOA-glc corresponded to a peak eluting before DIMBOA-glc (Figure 1). Thin-layer chromatography of these combined fractions (BAW, silica gel) gave a single spot that reacted with $FeCl_3$. This compound was water soluble and resistant to extensive heating. Acid hydrolysis yielded an ethyl acetate soluble compound that also reacted with $FeCl_3$ but did not cochromatograph with DIBOA-glc. The UV spectra of the water-soluble and ethyl acetate soluble compounds agreed with those reported for DIBOA-glc and DIBOA (Hietala and Virtanen, 1960), respectively: DIBOA-glc, λ_{max} 255, 281 nm; DIBOA, λ_{max} 254, 282 nm. Their identities were confirmed by mass spectra that were previously unreported. Mass spectrum for DIBOA-glc: *m/e* 343 (parent, 0%), 327 (14%), 165 (46%), 163 (6%), 149 (35%), 136 (base peak), 135 (50%), 120 (40%), 109 (20%). Mass spectrum for DIBOA: *m/e* 181 (parent, 90%), 165 (15%), 152 (48%), 149 (67%), 136 (68%), 135 (base peak), 109 (33%), 108 (90%).

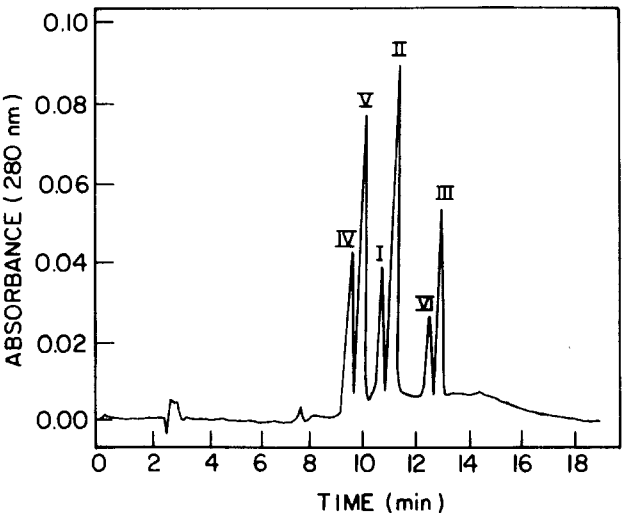

Figure 1. HPLC chromatogram of cyclic hydroxamic acid and benzoxazolinone standards separated by C18 reversed-phase chromatography: I, DIMBOA-glc; II, DIMBOA; III, MBOA; IV, DIBOA-glc; V, DIBOA; VI, BOA.

BOA was obtained from DIBOA by heating in water at 70 °C and purified in the same manner as MBOA. Its UV spectrum agreed with that reported by Hietala and Virtanen (1960): λ_{max} 270 nm. Its identity was confirmed by mass spectra that were previously unreported: *m/e* 135 (parent 70%), 52 (base peak), 51 (70%).

Mass Spectral Analysis. The mass spectra of the standard compounds were run with the direct-insertion probe of a Finnigan 4000 mass spectrometer. Both 70-eV electron impact (EI) and isobutane chemical ionization (CI) (0.5 Torr) were used. The data presented are for EI only.

Extraction of Corn Leaf and Seedling Samples. DIMBOA-glc and DIBOA-glc were extracted by placing each sample (usually 0.05–0.10 g) directly into 5 mL of boiling methanol (65–70 °C) in a test tube for 15 min. The boiled extract was removed and saved. The residue was homogenized for 20 s in 5 mL of methanol with a Polytron homogenizer and centrifuged (200*g*), and the two extracts were combined. The residue was vortexed with an additional 5 mL of methanol, and the rinse was combined with the first two extracts. Methanol was removed under vacuum, and the sample was quantitatively transferred to a test tube in 3 mL of water. After the pH was adjusted to 3 with 1 N HCl, the sample was extracted by vortexing three times with equal volumes of ethyl acetate and the ethyl acetate was discarded. The aqueous fraction was taken to dryness under vacuum, and the sample was dissolved in 1 mL of methanol for HPLC.

DIMBOA and DIBOA were obtained by homogenizing each sample (0.05–0.10 g) in 5 mL of water and allowing it to stand at room temperature (25–30 °C) for 30 min. This resulted in enzymatic conversion of the glucosides to the aglycons. After the pH was adjusted to 3 with 1 N HCl, the aqueous homogenate was vortexed twice with equal volumes of *n*-hexane, which were discarded. The compounds were then partitioned three times into equal volumes of ethyl acetate. The ethyl acetate fractions were combined and saved, and the aqueous homogenate was discarded. Ethyl acetate was removed under vacuum, and the sample was dissolved in 1 mL of methanol for HPLC.

HPLC Procedures. Samples were chromatographed on a Beckman Model 110A HPLC equipped with a sample injector and a 20-μL sample loop on a 4.5 mm × 25 cm C18

column (5 μm, Ultrasphere ODS, Altex). The column was protected by a 2-cm C18 pellicular guard column (Supelco).

A two-solvent system was used to generate the mobile phase: solvent A was 1% acetic acid; solvent B was 100% methanol. The flow rate was 1.0 mL/min. The mobile phase at the initiation of each run was a 9:1 ratio (A to B). Upon injection of the sample, a 3-min linear gradient began which altered the mobile phase ratio to 1:1 (A to B). This ratio was maintained for 8 min, after which the mobile phase was returned in 0.5 min to the initial conditions. To allow complete equilibration of the column, sample injections were made at 20-min intervals.

Sample elution was monitored at 280 nm with a Beckman Model 160 fixed-wavelength detector at 0.10 AUFS. Peaks were recorded and peak areas were measured with a Hewlett-Packard Model 3390A recording integrator. Standard curves of peak area vs concentration were prepared for DIMBOA-glc and DIMBOA with use of a series of standard methanolic solutions of these compounds.

Recovery Efficiencies and Experimental Error. Recovery efficiencies were determined for DIMBOA-glc and DIMBOA by adding known amounts of each to 5 mL of methanol and 5 mL of water, respectively. Samples from mature leaves (which contain very low concentrations of cyclic hydroxamic acids) were then extracted in the solutions according to the procedures described above. The samples were chromatographed and recovery efficiencies determined for both compounds.

The experimental error in determining DIMBOA-glc in leaf samples was estimated by subjecting five identical samples to the extraction procedure. Identical samples were obtained by successively punching disks from the same area of a leaf directly into six test tubes containing 5 mL of boiling methanol. The samples were then extracted and chromatographed, and the mean concentration and standard error were determined. The experimental error for DIMBOA was similarly determined except that samples were punched into water and carried through the appropriate extraction procedure described above for this compound.

RESULTS AND DISCUSSION

A standard mixture of the glucosides, DIMBOA-glc and DIBOA-glc, the aglycons, DIMBOA and DIBOA, and the benzoxazolinone derivatives, MBOA and BOA, was resolved into six peaks within 15 min by this procedure (Figure 1). Retention times of the six components were consistently reproducible with standard errors of less than 0.02 min when samples were injected at 20-min intervals (Figure 1).

The minimum detection limit for DIMBOA-glc and DIMBOA in standard methanolic solutions with a 20-μL sample injection volume and a 280-nm filter was 0.2 nmol. Greater sensitivities should be possible with a variable-wavelength detector set to the maximum absorbance of each compound and by using larger sample injection volumes.

The sensitivity of the procedure made the use of small sample sizes feasible, minimizing tissue destruction. For leaves we typically used 0.05–0.10 g, and samples of this size were easily obtained by using a paper punch. This approach was especially useful for measuring glucoside content since the sample disks could be punched directly into boiling methanol, thus minimizing enzymatic degradation.

DIMBOA-glc content was measured in corn leaves of various ages. The concentration in leaves ranged from 2600 μg in young leaves to 8 μg/g fresh weight in older leaves that had collared. The principal cyclic hydroxamic acid of leaf tissue was DIMBOA-glc. DIBOA-glc was less frequently detected, and when present, its concentration was usually severalfold lower than DIMBOA-glc. A typical chromatogram of a leaf sample extracted for glucosides is shown in Figure 2a.

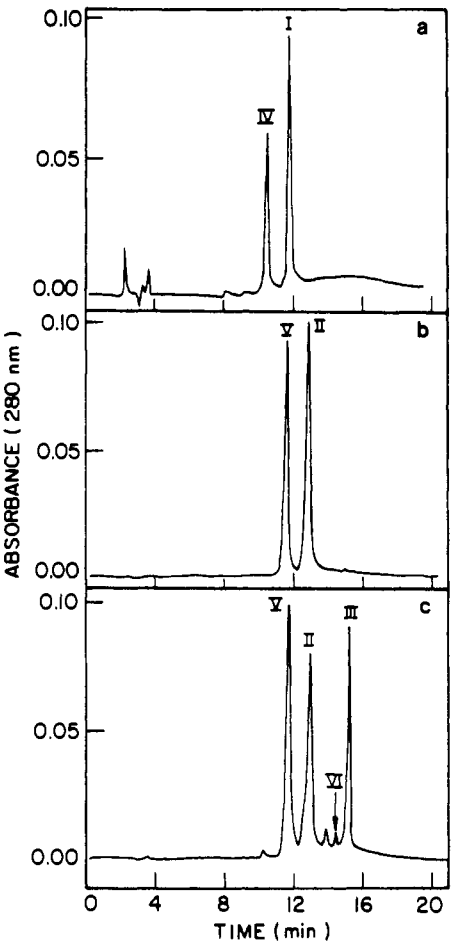

Figure 2. HPLC chromatograms of corn leaf extracts containing cyclic hydroxamic acids: (a) DIMBOA-glc (I) and DIBOA-glc (IV) extracted in boiling methanol; (b) DIMBOA (II) and DIBOA (V) extracted in methanol after allowing for enzymatic hydrolysis of I and IV in water; (c) sample in (b) after brief heating at 70 °C to partially convert DIMBOA (II) and DIBOA (V) to MBOA (III) and BOA (VI), respectively.

In samples extracted for indirectly measuring cyclic hydroxamic acid content as the aglycons, the DIMBOA to DIBOA ratios were similar to those found in glycoside extracts (Figure 2b). Regardless of the tissue extracted, DIMBOA (II) content was always significantly greater than DIBOA (V) content. Traces of MBOA were sometimes observed in these samples, indicating that conversion of the aglycon occurred during the extraction; BOA (VI) was rarely observed (Figure 2c). Benzoxazolinone levels increased if the incubation time of the aqueous extract was lengthened or if the extract was briefly heated at 70 °C (Figure 2c).

The experimental error in measuring the glucoside and aglycon content of corn leaves was estimated in two separate experiments. In one experiment five identical replicate samples were assayed for DIMBOA-glc; in another experiment a second group of five identical replicate samples was assayed for DIMBOA content. The mean concentrations and standard errors for DIMBOA-glc and DIMBOA in these experiments were, respectively, 434 (±13) and 1066 (±19) μg/g fresh weight.

Recoveries of DIMBOA-glc and DIMBOA were also determined in separate experiments by adding known

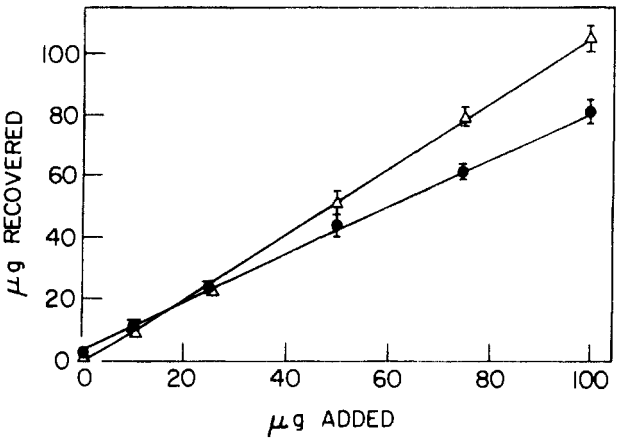

Figure 3. Amount of DIMBOA-glc (△) and DIMBOA (●) recovered vs amount of compound added to samples prior to extraction and HPLC analysis. The slope of each regression is equal to the fraction of the compound recovered. The y intercept is the amount of each compound present in the leaf samples prior to addition of DIMBOA-glc or DIMBOA. Individual points represent the mean (±SE) of four replicate samples.

amounts of each compound to methanol and water, respectively, before extracting leaf samples. The results are shown in Figure 3. Recoveries, represented by the slopes of the regressions, were 106% for DIMBOA-glc and 78% for DIMBOA. Thus, indirect measurement as the aglycon underestimated the DIMBOA-glc concentration in the plant. Others have reported DIMBOA recoveries of 100% by HPLC (Gutierrez et al., 1982) and by GLC (Woodward et al., 1979); however, these determinations were made by adding known amounts of DIMBOA to sample extracts just prior to chromatography. Results obtained in that manner are indicative of the efficiency of the chromatographic step, but not of the efficiency of the entire analytical procedure. The more efficient recovery of DIMBOA-glc by our procedure, compared to the recovery of DIMBOA, indicates that a more accurate estimate of actual cyclic hydroxamic acid content in unwounded plants can be obtained by measuring the glucosides directly.

The HPLC procedure that we have described represents a fast and accurate quantitative method for the direct determination of the two major cyclic hydroxamic acid glucosides in corn and for the measurement of the corresponding aglycon and benzoxazolinone derivatives. This combined capability should be useful for studies on the biosynthesis of these compounds and their fate in plants subjected to various stresses, such as insect damage and disease. Although our study was limited to corn, the procedure should also be applicable to other grasses in which these compounds occur, such as wheat and rye. We also found that, in methanolic solutions, the cyclic hydroxamic acid glucosides were stable for periods of several days to weeks. Thus, large numbers of samples could be collected, extracted, and stored for subsequent analysis. In fact, samples could simply be extracted in boiling methanol and stored for several days before homogenization without affecting the results. The ability to store samples in this manner without degradation is advantageous in studies in which large numbers of samples must be collected for analysis of cyclic hydroxamic acid content.

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