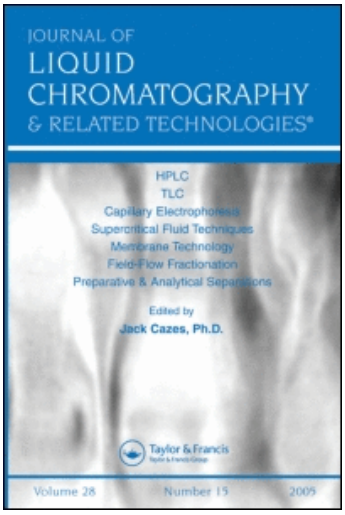

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A High Performance Liquid Chromatography Method for Quantification of Diboa, DIMBOA, and MBOA from Aqueous Extracts of Corn and Winter Cereal Plants

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTIFICATION OF DIBOA, DIMBOA, AND MBOA FROM AQUEOUS EXTRACTS OF CORN AND WINTER CEREAL PLANTS

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ABSTRACT

A new HPLC method is described for the quantification of aglycones derived from cyclic hydroxamic acids: DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), its degradation product MBOA (6-methoxybenzoxazolinone) and the demethoxy analogue DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) in crude aqueous solutions from homogenized cereal tissues. After hydrolysis of ß-glycosides to aglycones, extracts of cereal samples were chromatographed on a PRP-1 column using a gradient of tris/citric acid and methanol/acetonitrile, and monitored at 288 nm. The three compounds were separated within 26 min. The minimum detection limit for all of them was 50 pmol. The recovery percentage was 100% for DIBOA, 87% for DIMBOA and 96% for MBOA. The levels of aglycones were determined in aqueous extracts from corn and winter cereal seedlings (20-100 mg), ranging from 3.80 to 11.50 mg DIBOA/g dry weight and 4.75 to 100.43 mg DIMBOA/g dry weight.

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INTRODUCTION

Hydroxamic acids have been associated with a broad range of functions in cereal plants, including disease and insect resistance, detoxification of herbicides, allelopathic effects, mineral metabolism and growth regulation (1,2).

Cyclic hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) isolated from gramineous species are found as ß-glycosides (3). When plant tissues are damaged, ß-glycosides are enzymatically hydrolyzed to their corresponding aglycones (4). The main aglycone found in maize and wheat is DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-(4H)-one), while its demethoxy analogue, DIBOA, is mainly found in rye (3,5). The aglycones are converted to their corresponding benzoxazolinones MBOA and BOA when heated in aqueous solutions (6,7).

The naturally occurring glycosides showed little activity against insects (8), but the aglycones DIMBOA and DIBOA are known to mediate in the resistance of cereals to several insect pests (9). Furthermore, it has been suggested that these compounds could be used as a criterion of selection in cereal breeding programs to increase their pest and disease resistance, (10). Consequently, in order to screen large amounts of plant samples, a fast and accurate method to quantify these compounds is needed.

Several methods for qualitative and quantitative analysis of hydroxamic acids have been developed. Colorimetric methods based on quantification of a $FeCl_3$ complex are unspecific because they do not differentiate between the various hydroxamic acids species present in

an extract (11). More specific methods such as isotopic dilution (12), spectrofluorimetry (13), gas liquid chromatography (GLC) (14), high performance liquid chromatography (HPLC) (15) and thin layer chromatography (TLC) (16) measure hydroxamic acid concentrations after their conversion to benzoxazolinones, but the yields are not quantitative because this is not a stoichiometric reaction (17).

The quantitative GLC (18) and HPLC (19,20 and 21) methods previously reported are time consuming since they require complex sample extraction procedures and they are therefore not suitable to screen large amount of samples in breeding programs. On the other hand, another sample preparation methods (22,23) are fast and easy, but the HPLC quantification reported by these authors does not provide for the determination of the MBOA, the degradation product of DIMBOA, and the result is an underestimation of the total amount of DIMBOA.

Here we report on a simple and rapid quantitative extraction procedure and a sensitive HPLC analysis to quantify the most biologically active aglycones, DIMBOA and DIBOA, and the benzoxazolinone, MBOA, from corn and winter cereal aqueous extracts.

MATERIALS AND METHODS

Preparation of Standards

DIMBOA was isolated from 300 g of etiolated corn shoots of the hybrid EA2173 X Mo17 using Klun et al.'s method (24), MBOA was prepared by the Klun and Brindley method (12) using 500 g of fresh material. The identity of these compounds was confirmed by ultraviolet

and infrared spectra, as reported by Gutiérrez et al. (19). DIBOA was isolated from 800 g of etiolated rye seedlings (Cv. Petkus) by the Virtanen and Hietala method (8). The UV spectrum in absolute ethanol and the IR spectrum in a KBr pellet recorded in a Shimadzu 160 UV-VIS spectrophotometer and Perkin-Elmer IR 1420 spectrophotometer respectively, were in agreement with that obtained by Tipton et al. (25).

Sample Preparation

Seeds of *Zea mays* (Pioneer Hybrid 3138), *Triticum aestivum* (Cv. Chinese spring), *Triticum durum* (Cv. Camacho), *Secale cereale* (Cv. Elbon), *Triticosecale* (Cv. Cachirulo) and *Hordeum vulgare* (Cv. Logra) were grown in 5x5 cm pots (one seed/pot) containing a potting soil mixture ("Floragard", Germany) at 22±1°C, 75±5% HR, 204 $\mu Em^{-2}\ s^{-1}$ of photon flux and 16 h light-8h dark photoperiod. Seedlings were harvested at the G.S. 11 (26). Plant tissues (20-100 mg) were homogenized with distilled water (4x0.04 mL/mg) in a potter homogenizer. Aqueous extracts were incubated for 15 min at 25°C, to hydrolyze the aglycones from glycosides (27). Sample aliquots (1 mL) were centrifugated at 12500 rpm for 10 min, and filtered through 0,22 µm cellulose membrane filters (Millipore).

HPLC Conditions

Samples were quantitatively analyzed on a Beckman System Gold equipped with a gradient controller Model 126, diode array detector Model 168 and an auto-sampler Model 502, with a 20 µL sample loop. A

250x4.1 mm PRP-1 column (10µm, polystyrene-divinylbenzene, Hamilton) protected by a 25x2.3 mm PRP-1 guard was used. Elution was carried out with a linear gradient of solvents A (49 mM tris, 16 mM citric acid, pH= 7.0) and B (methanol 80%-acetonitrile 20%): 8-80%B from 0 to 30 min, 80-8%B from 30 to 31 min. Sample injections were at 40 min intervals to allow equilibration of the column. The flow rate was 1 mL/min. Detection was at 288 nm and 0.025 AUFS, and the scanning range was from 220 to 400 nm. Calibration curves were obtained by injecting 20 µL of standard solution mixtures with seven concentration levels of DIBOA, DIMBOA and MBOA, ranging from 0.05 to 1.0 nM. Two determinations were performed for each concentration level.

Identification and Quantitation of DIBOA, DIMBOA and MBOA in Aqueous Extracts

The identity of benzoxazinones and benzoxazolinones in the aqueous extracts was determined by co-chromatography with the appropriate standards, and by comparing the UV spectrum of unknown peaks and the standard peaks. Quantification was made by referring to the molar responses of standards from standard curves.

Percent Recovery

Five replicates with different quantities for each standard were added to 1mL of homogenated tissue from *T. aestivum* (Cv. Chinese Spring) prior to incubation, and processed as previously described. The recovery of DIBOA, DIMBOA and MBOA was calculated after HPLC separation.

RESULTS AND DISCUSSION

Method Development

The HPLC analysis was carried out on a poly(Styrene-divinylbenzene)resin because this stationary phase is more stable at any pH that the silica-based packing materials (28). Figure 1 shows the separation of a standard mixture containing DIBOA, DIMBOA and MBOA with retention times of 15.10, 17.46 and 25.07 min, respectively Retention times were highly reproducible with standard errors lower than 0.02 min in 10 replicated runs injected at 40 min intervals. Diode array detection was employed since it allows for the identification of the peaks by comparing of their retention times and UV spectrum with those of standards, and also permits peak purity to be verified.

Peaks I, II, III were enhanced when DIBOA, DIMBOA and MBOA standards respectively, were added to the sample. On this basis, peak I was identified as DIBOA, peak II as DIMBOA and Peak III as MBOA. Due to the differences in the UV absorption spectra of the three compounds, a wavelength of 288 nm was chosen to allow their joint determination with the best quantitative results.

Seven different levels of each standard were analyzed to obtain the calibration data. The correlation coefficients (r) for DIBOA, DIMBOA and MBOA were r=0.998, r=0.993 and r=0.993 respectively, indicating a good linearity in the calibration curves. The minimum detection limit for these compounds was 50 pmol which improves the sensibility of the methods previously reported by Gutiérrez et al. (19), Lyons et al. (20) and Xie et al. (21). The recovery of DIBOA, DIMBOA and MBOA was

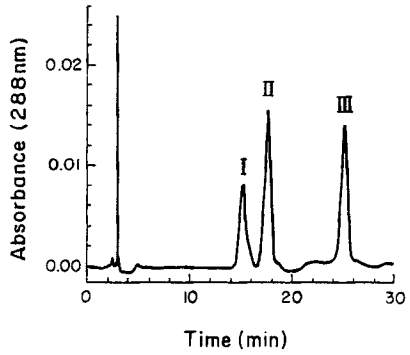

FIGURE 1. HPLC chromatogram of cyclic hydroxamic acids standards separated on a PRP-1 Column: I, DIBOA; II, DIMBOA; III, MBOA.

estimated to test the accuracy of the method. In order to avoid the differential distribution of hydroxamic acids in different tissues (27,29), five replicates of known amounts of DIBOA, DIMBOA and MBOA standards were added to 1 mL of homogenated seedlings of *T. aestivum* (Cv. Chinese Spring). Figure 2 shows the results obtained. The Y intersect values are the amounts of DIBOA, DIMBOA and MBOA present in the sample prior to the addition of standards. The percentages of recovery obtained by the slope of each regression were 100% for DIBOA, 87% for DIMBOA, and 96% for MBOA. These results point out the accuracy of this method in the quantification of DIBOA, DIMBOA and MBOA in plant extracts, and represent an improvement in respect to the previously reported DIMBOA recovery (20).

Sample Preparation

The hydroxamic acids naturally occur as glycosides, but in this work we quantified their aglycones for on the following reasons:

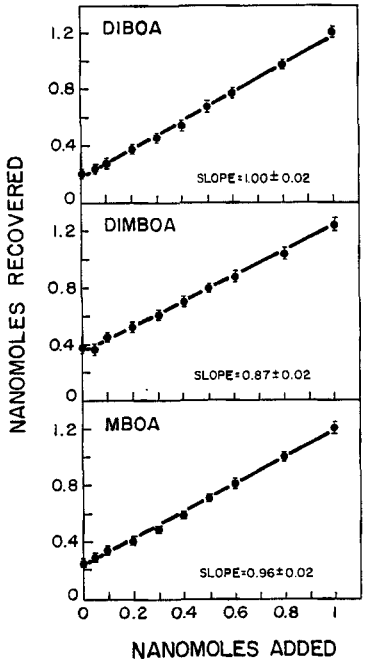

FIGURE 2. Recovery of DIBOA, DIMBOA and MBOA added to an aqueous extract from *T. aestivum* (Cv Chinese Spring). The slope of each regression show the amount of compound recovered. Values are means ± s.e. (5 replicates). Intersection represents concentrations obtained in vivo.

a) The isolation of the glycosides involves the inactivation of the hydrolytic enzymes present in the plant tissues before extraction since in damaged plant tissues a ß-glycosidase converts them into aglycons. Therefore the sample preparation for HPLC quantification of the glycosides is time consuming (20), and consequently it is not suitable for screening large amounts of samples in cereal breeding programs.

b) Corcuera et al. (8) showed that natural levels of glycosides in cereals were less active against insects than their corresponding

aglycones. Additionally, the incubation for 15 min at 25° C of aqueous cereal extracts hydrolyses their glycosides to aglycones (27) and the aqueous extraction of these compounds significantly reduces the sample preparation time when compared to their extraction with organic solvents as previously reported (19,20 and 21). Therefore we propose the quantification of these aglycones as a more suitable method to estimate the host-plant resistance.

Applications

To analyze the suitability of this method to screen samples in breeding programs, several species of cereal seedlings were processed under the conditions described above (see materials and methods).

The chromatograms obtained from the analysis of foliar corn seedling extracts and whole seedling extracts from other cereals are shown in Figure 3. DIBOA, DIMBOA and MBOA were detected in all cereals except barley. The presence of MBOA in the samples indicates that decomposition of DIMBOA took place during the extraction, pointing out the importance of MBOA identification in the samples to give an accurate quantification of DIMBOA. This identification is less sensible when samples are analyzed according to Niemeyer et al. (22) and Xie et al., (21); because these authors detected the compounds at 263 nm and 265 nm respectively, and under such conditions MBOA has a minimum of absorbance.

The DIMBOA level decreased when aqueous homogenates from *T. aestivum* (Cv. chinese Spring) were heated at 70°C. 45% DIMBOA was

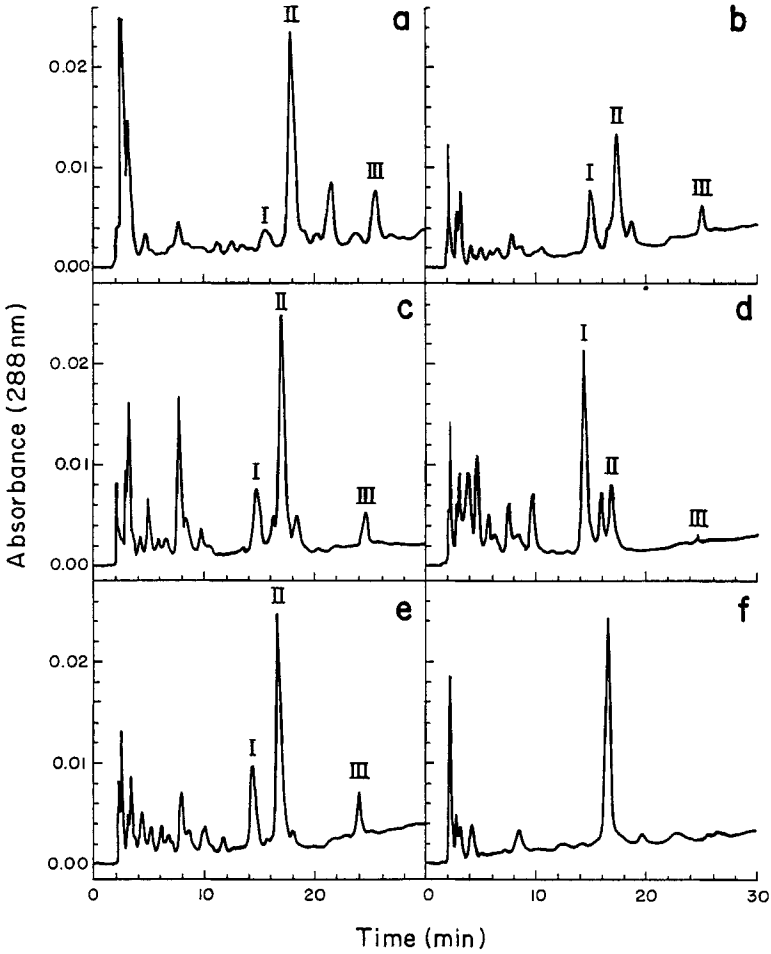

FIGURE 3. HPLC chromatograms of *Zea mays* leaf extracts (Pioneer hybrid 3138) (a) and seedlings from *Triticum aestivum* (Cv Chinese Spring) (b), *T. durum* (Cv Camacho) (c), *Secale cereale* (Cv Elbon) (d), *Triticosecale* (Cv Cachirulo) (e) and *Hordeum vulgare* (Cv Logra) (f); I, DIBOA; II, DIMBOA; III, MBOA.

degraded after 5 min and 65% after 10 min. Only 3% DIBOA was degraded when heated at 70°C for 10 min. Since DIBOA is more stable than DIMBOA (20), and no degradation to BOA was detected in our extraction conditions, we consider that its quantification should be unnecessary.

Table 1 shows our results from the quantification of DIMBOA and DIBOA in aqueous cereal extracts. The DIMBOA concentration was in most cases higher than DIBOA, and ranged from 4.75 to 100.43 mg/g dry weight, except for rye where the inverse situation was found. None of these aglycones were found in barley seedlings.

Under our experimental conditions, we have also found that the molar concentrations of DIBOA, DIMBOA and MBOA in standard methanolic solutions and cereal seedling aqueous extracts remained stable after several months of storage at -20°C.

We have described here a method for the quantification of the most biologically active hydroxamic acids: DIMBOA and DIBOA and the benzoxazolinone MBOA. This method improves other ones previously developed, since it provides a very simple and fast sample preparation procedure avoiding extraction with organic solvents. Additionally, the HPLC conditions described here give a good resolution of the sample components and a sensitive and accurate quantification of these compounds in aqueous extracts from winter cereals and grasses, which have lower concentrations of these chemical than corn.

Additionally, the great stability of these compounds in samples stored at -20°C will allow the harvesting, preparation and storage of

TABLE 1.

Levels of DIMBOA and DIBOA in Cereal Seedlings.

Species	Hydroxamic acids (mg/g dry wt.) * DIBOA	DIMBOA
Zea mays (Hybrid Pioneer 3138)	10.70 ± 0.34	100.43 ± 1.83
Triticum aestivum (Cv Chinese spring)	3.80 ± 0.45	12.63 ± 0.10
Triticum durum (Cv Camacho)	4.86 ± 0.18	24.47 ± 0.34
Secale cereale (Cv Elbon)	11.50 ± 0.28	4.75 ± 0.19
Triticosecale (Cv Cachirulo)	4.92 ± 0.24	19.57 ± 0.51
Hordeum vulgare (Cv Logra)	0.00	0.00

* Means and s.e. (n=5) are represented.

large amounts of plant material and aqueous extracts prior to their HPLC analysis. Therefore, this method is suitable for screening samples in plant breeding programs dealing with pests and disease resistance that involve the analysis of a large number of genotypes.

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