# Simple Method for Large Scale Isolation of the Cyclic Arylhydroxamic Acid DIMBOA from Maize (*Zea mays* L.)

Erik Larsen and Lars P. Christensen*

Department of Fruit, Vegetable and Food Science, Danish Institute of Agricultural Sciences, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark

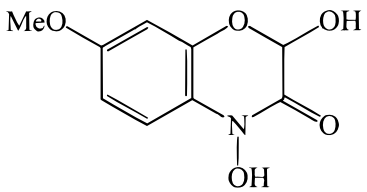
The 2-*β*-*O*-D-glucoside of the cyclic arylhydroxamic acid 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) that occurs in large amounts in young maize shoots (*Zea mays* L.) is converted enzymatically to its aglycone upon tissue damage. The aglycone DIMBOA possesses strong biologically activity toward various organisms whereas the glucoside is almost biologically inactive. A simple procedure yielding DIMBOA in gram quantities, from 7-day-old maize seedlings, was developed by using solid-phase extraction.

**Keywords:** *Zea mays; maize; Gramineae; DIMBOA; isolation; solid-phase extraction; Amberlite XAD-7*

## INTRODUCTION

Cyclic arylhydroxamic acids of the 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one type (benzoxazinoids) are produced in many wild and cultivated gramineous plant species, including rye, wheat, and maize (Niemeyer, 1988). The predominant benzoxazinoids in maize and wheat are 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA, Figure 1) and its demethoxy derivative (DIBOA) (Argandoña et al., 1981; Niemeyer, 1988; Massardo et al., 1994; Nakagawa et al., 1995; Cambier et al., 2000). In intact plants benzoxazinoids exist predominantly as the corresponding stable 2-*β*-*O*-D-glucosides. These glucosides are almost biologically inactive, but upon tissue damage they are enzymatically converted to the active aglycones by *β*-glucosidases (Argandoña et al., 1981; Massardo et al., 1994; Hashimoto and Shudo, 1996). DIMBOA is one of the best studied benzoxazinoid aglycones and its toxicity to a broad range of organisms has been described, including plant pathogens (Long et al., 1975; Corcurea et al., 1978; Niemeyer, 1988; Hashimoto and Shudo, 1996), the European corn borer (Campos et al., 1989), western corn rootworm (Xie et al., 1990), and aphids (Argandoña et al., 1980, 1981; Givovich et al., 1994; Niemeyer, 1988). Furthermore, it has been shown that DIMBOA and other benzoxazinoid aglycones are strong allelochemicals (Barnes and Putnam, 1987; Pérez, 1990), inhibit growth of algae (Bravo and Lazo, 1996), and possess mutagenic activity (Hashimoto and Shudo, 1996). These results clearly demonstrate that DIMBOA and related benzoxazinoid aglycones are of agricultural importance as chemical defense compounds and may have beneficial pharmacological activities.

Analytical methods for isolation and quantification of benzoxazinoids have been developed which primarily are based on different high-performance liquid chromatography techniques (Lyons et al., 1988; Givovich et al., 1994; Nakagawa et al., 1995). These methods are, however, not suitable for large scale isolation of these compounds. Due to the limited possibilities for obtaining DIMBOA and related benzoxazinoid aglycones in relatively large quantities by the extraction of plant material (Woodward et al., 1978), some efforts directed toward chemical syntheses of these compounds have been successful (Atkinson et al., 1991; Sicker and Hartenstein, 1993). Although it is possible to obtain large amounts of DIMBOA by chemical syntheses, these procedures are time-consuming, expensive, and troublesome.




**DIMBOA**

**Figure 1.** Structure of DIMBOA.

Maize produces large amounts of DIMBOA-glucoside and the purpose of the present study was to develop a simple method for isolating the aglycone DIMBOA in a pure state and in large quantities from this plant.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions.** Maize seeds (*Zea mays* L.) from the cultivar Apache were obtained from DLF Trifolium (Denmark). Seedlings were grown in the laboratory at approximately 20−25 °C in the dark. Seeds (525 g; approximately 1660 seeds) were sown in a plastic tray (46 cm × 27 cm × 8 cm: $w \times l \times h$) filled with potting soil. Aboveground parts were harvested 7 days after sowing (fresh weight 1470 g) and frozen (−20 °C) until use.

**Extraction, Isolation, and Identification of DIMBOA.** Frozen shoots from 7-day-old maize seedlings (1000 g) were defrosted and homogenized in a blender (Waring Blendor, Waring Commercial) with water (1 L). The homogenate was squeezed through one layer of cheesecloth and the filtrate allowed to stand for 1 h to allow enzymatic release of DIMBOA from DIMBOA-glucoside. Amberlite XAD-7 nonionic polymeric

* To whom correspondence should be addressed (telephone, +45 63 90 43 43; fax, +45 63 90 43 95; e-mail, larsp.christensen@agrsci.dk).

10.1021/jf0000934 CCC: $19.00 © 2000 American Chemical Society
Published on Web 05/17/2000

adsorbent (100 g, Aldrich) was added and the mixture stirred for 1 h and filtered, and the Amberlite XAD-7 was washed with water (2 × 250 mL). The Amberlite XAD-7 was restored by washing with acetone (2 × 250 mL, HPLC grade, Aldrich), and the filtrate containing primarily DIMBOA was evaporated to dryness in vacuo. The residue was partially dissolved in methylene chloride (100 mL, HPLC grade, Aldrich) and stored overnight at −20 °C. The resulting precipitate was filtered and washed with 25 mL of ice-cold methylene chloride and 25 mL of hexane (HPLC grade, Aldrich) yielding pale yellow amorphous crystals, which consisted of pure DIMBOA (>98%). The isolation procedure for DIMBOA was made in two replicates and gave yields of 1.36 ± 0.27 g. The amounts of DIMBOA and DIMBOA-glucoside present in the homogenized shoots were determined by analytical HPLC according to the method of Lyons et al. (1988) and the isolated amounts of DIMBOA corresponded to 80 ± 6% of what is present in the shoots after enzymatic release of DIMBOA from its glucoside.

The purity of DIMBOA was determined by analytical HPLC (Lyons et al., 1988). NMR data ($^1$H and $^{13}$C), UV, melting point, and mass spectrum were in accordance with those previously reported for DIMBOA (Woodward et al., 1978; Lyons et al., 1988; Atkinson et al., 1991).

## RESULTS AND DISCUSSION

DIMBOA is mainly present in maize as its 2-*β*-*O*-D-glucoside and can be isolated from both roots and shoots immediately after germination (Argandoña and Corcuera, 1985; Ebisui et al., 1998). In light-grown maize shoots, the concentration of DIMBOA-glucoside is on the order of 4 mmol/kg fresh weight 7 days after germination, whereas the levels have declined to approximately one-third or less 20 days after germination (Argandoña and Corcuera, 1985; Cambier et al., 2000). Furthermore, the production of DIMBOA-glucoside is higher in seedlings of wheat grown at low light or in darkness compared to plants grown in high light intensity (Åhman and Johansson, 1994). This also appears to be true for maize as maize seedlings grown in the dark contained approximately 2 times as much DIMBOA-glucoside compared to maize seedlings grown in natural light, in preliminary experiments. Freezing and defrosting as well as homogenization of the plant material releases *β*-glucosidase that rapidly degrades DIMBOA-glucoside to DIMBOA (Massardo et al., 1994; Ebisui et al., 1998).

From frozen shoots of 7-day-old etiolated maize seedlings we obtained an aqueous extract with a high concentration of DIMBOA-glucoside that was completely converted to DIMBOA after 1 h, at room temperature, as shown by analytical HPLC. A method for obtaining pure DIMBOA from an aqueous extract by liquid extraction with diethyl ether has been described (Woodward et al., 1978), but the technique requires large amounts of solvent since DIMBOA is also soluble in water. For example, approximately 2 L of diethyl ether were used to isolate 150 mg of DIMBOA. Furthermore, this technique requires additional purification steps such as a rapid heating and cooling procedure resulting in relatively low yields (Woodward et al., 1978). Consequently large scale production of pure DIMBOA by liquid extraction is troublesome and time-consuming.

Solid-phase extraction of an aqueous solution has several advantages over liquid extraction, especially for compounds with some degree of solubility in water. The solid adsorbent material can be specified to allow maximum adsorption of the desired compound, is easy to handle on a large scale, and can be reused repeatedly. In the present procedure we have used Amberlite XAD-7 because of its high affinity to phenolic compounds. The concentration of DIMBOA in the aqueous phase after extraction with Amberlite XAD-7 was close to zero, as shown by analytical HPLC, proving the efficiency of this adsorbent material. Release of DIMBOA from Amberlite XAD-7 was easily accomplished by washing with acetone, and pure DIMBOA could then be isolated after crystallization in methylene chloride. Although some DIMBOA is present in the squeezed plant material and the compound is unstable in aqueous solution being degraded to MBOA (Woodward et al., 1978), DIMBOA was obtained in yields of approximately 80% of what was present in the shoots. From 1 kg of maize shoots, 1.36 g of DIMBOA was isolated and stored at −20 °C for several months without any sign of degradation.

The method described here for isolating DIMBOA from maize can easily be scaled up using larger amounts of plant and adsorbent material. This allows laboratories to prepare relatively large amounts of this valuable compound for use in biological experiments and for the synthesis of DIMBOA derivatives with very little effort compared to existing methods.

## LITERATURE CITED


Argandoña, V. H.; Luza, J. G.; Niemeyer, H. M.; Corcuera, L. J. Role of hydroxamic acids in the resistance of cereals to aphids. *Phytochemistry* **1980**, *19*, 1665−1668.

Argandoña, V. H.; Niemeyer, H. M.; Corcuera, L. J. Effect of content and distribution of hydroxamic acids in wheat on infestation by the aphid *Schizaphis graminum*. *Phytochemistry* **1981**, *20*, 673−676.

Argandoña, V. H.; Corcuera, L. J. Distribution of hydroxamic acids in *Zea mays* tissues. *Phytochemistry* **1985**, *24*, 177−178.

Atkinson, J.; Morand, P.; Arnason, J. T.; Niemeyer, H. M.; Bravo, H. R. Analogues of the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one: Decomposition to benzoxazolinones and reaction with *β*-mercaptoethanol. *J. Org. Chem.* **1991**, *56*, 1788−1800.

Barnes, J. P.; Putnam, A. R. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* **1987**, *13*, 889−906.

Bravo, H. R.; Lazo, W. Antialgal and antifungal activity of natural hydroxamic acids and related compounds. *J. Agric. Food Chem.* **1996**, *44*, 1569−1571.

Cambier, V.; Hance, T.; de Hoffmann, E. Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry* **2000**, *53*, 223−229.

Campos, F.; Atkinson, J.; Arnason, J. T.; Philogène, B. J. R.; Morand, P.; Werstiuk, N. H.; Timmins, G. Toxicokinetics of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in the European corn borer, *Ostrinia nubilalis* (Hübner). *J. Chem. Ecol.* **1989**, *15*, 1989−2001.

Corcuera, L. J.; Woodward, M. D.; Helgeson, J. P.; Kelman, A.; Upper, C. D. 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, an inhibitor from *Zea mays* with differential activity against soft rotting *Erwinia* species. *Plant Physiol.* **1978**, *61*, 791−795.

Ebisui, K.; Ishihara, A.; Hirai, N.; Iwamura, H. Occurrence of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and a *β*-glucosidase specific for its glucoside in maize seedlings. *Z. Naturforsch.* **1998**, *53C*, 793−798.

Givovich, A.; Sandström, J.; Niemeyer, H. M.; Pettersson, J. Presence of a hydroxamic acid glucoside in wheat phloem sap, and its consequences for performance of *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *J. Chem. Ecol.* **1994**, *20*, 1923−1930.

Hashimoto, Y.; Shudo, K. Chemistry of biologically active benzoxazinoids. *Phytochemistry* **1996**, *43*, 551−559.

Long, B. J.; Dunn, G. M.; Routley, D. G. Relationship of hydroxamic acid content in maize and resistance to northern corn leaf blight. *Crop Sci.* **1975**, *15*, 333−335.

Lyons, P. C.; Hipskind, J. D.; Wood, K. V.; Nicholson, R. L. Separation and quantification of cyclic hydroxamic acids and related compounds by high-pressure liquid chromatography. *J. Agric. Food Chem.* **1988**, *36*, 57−60.

Massardo, F.; Zuñiga, G. E.; Pérez, L. M.; Corcuera, L. J. Effects of hydroxamic acids on electron transport and their cellular location in corn. *Phytochemistry* **1994**, *35*, 873−876.

Nakagawa, E.; Amano, T.; Hirai, N.; Iwamura, H. Noninduced cyclic hydroxamic acids in wheat during juvenile stage of growth. *Phytochemistry* **1995**, *38*, 1349−1354.

Niemeyer, H. M. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* **1988**, *27*, 3349−3358.

Pérez, F. J. Allelopathic effect of hydroxamic acids from cereals on *Avena sativa* and *A. fatua*. *Phytochemistry* **1990**, *29*, 773−776.

Sicker, D.; Hartenstein, H. A new general approach to the 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one skeleton via diisobutylaluminum hydride reduction of 2,3-dioxo-1,4-benzoxazines. *Synthesis* **1993**, 771−772.

Woodward, M. D.; Corcuera, L. J.; Helgeson, J. P.; Upper, C. D. Decomposition of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one in aqueous solutions. *Plant Physiol.* **1978**, *61*, 796−802.

Xie, Y. S.; Arnason, J. T.; Philogène, B. J. R.; Lambert, J. D. H.; Atkinson, J.; Morand, P. Role of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in the resistance of maize to western corn rootworm, *Diabrotica virgifera virgifera* (Leconte) (Coleoptera: Chrysomelidae). *Can. Entomol.* **1990**, *122*, 1177−1186.

Åhman, I.; Johansson, M. Effect of light on DIMBOA-glucoside concentration in wheat (*Triticum aestivum* L.). *Ann. Appl. Biol.* **1994**, *124*, 569−574.

Received for review January 24, 2000. Revised manuscript received March 29, 2000. Accepted March 30, 2000. The research was part of a project funded by the Danish Research Centre for Organic Agriculture (DARCOF).

JF0000934