a false positive may result if a 6 ft long × 4 mm i.d. SE-30/OV-210 column operated at 165 °C is used to compare the retention time with that of a methylated 2,4,5-T standard.

Because of the data found, another usual method for determining human 2,4,5-T exposure through urine analysis was investigated. The so-called multiphenol method (Shafik et al., 1973) has been used in our laboratory for analysis of about 5000 human urine samples. One of the substituents determined is 2,4,5-T. Extraction of the acid-hydrolyzed sample into diethyl ether could solubilize some saccharin. However, the acid hydrolysis step in the multiphenol method is adequate to convert most of the saccharin to the ammonium *o*-carboxybenzenesulfonate (DeGarmo et al., 1952). Subsequent columning on silica gel retains saccharin and the ammonium *o*-carboxybenzenesulfonate. Thus, ethylation of an 80% benzene–20% hexane eluate does not contain *N*-ethylsaccharin and would not possibly be confused with ethylated 2,4,5-T.

We have noticed in chromatograms where *N*-methylsaccharin was observed that a second, late-emerging peak appears to be characteristic of urine excreted following saccharin exposure. The relative retention time to *N*-methylsaccharin is about 3.5–3.6. No attempt has been made to identify this peak.

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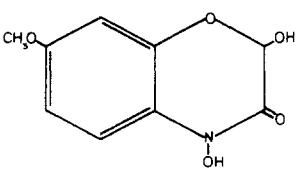
Received for review March 2, 1982. Accepted August 9, 1982. This work was performed as a result of investigations done on Cooperative Agreement CR806903 with the Pesticide Hazard Assessment Program, Health Effects Branch, Hazard Evaluation Division, Office of Toxic Substances and Pesticide Programs, U.S. Environmental Protection Agency, Washington, DC. The conclusions and suggestions are those of the authors and do not necessarily represent the viewpoint of the Environmental Protection Agency.

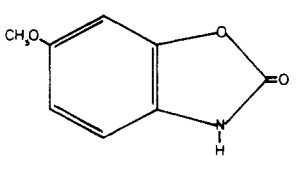
A High-Performance Liquid Chromatographic Method for Quantitation of DIMBOA and MBOA in Maize Plant Extract

A high-pressure liquid chromatographic (LC) method for quantitation of DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxacin-3(4*H*)-one] and its main degradation product MBOA [6-methoxy-2-(3*H*)-benzoxazolinone] in aqueous extract from corn (*Zea mays* L.) is described. After recovering DIMBOA and MBOA from the aqueous extract with ethyl acetate, underivatized samples were chromatographed on LiChrosorb SI 100 by using a hexane–ethanol gradient and monitored at 288 nm. A typical LC pattern shows five main peaks, two of them eluting respectively at the same position as the MBOA and DIMBOA standards and enhanced with the addition to the sample of these compounds. Variation coefficients of 1.11% and 1.07% were respectively obtained for the molar responses of MBOA and DIMBOA standards. For the retention times coefficients of variation of 0.011% for MBOA and 0.019% for DIMBOA were determined. The samples recoveries ranged from 104 ± 2% for DIMBOA to 106 ± 3% for MBOA.

DIMBOA (1) is a cyclic hydroxamic acid first isolated from wheat and corn as a β-D-glucopyranoside (Wahlroos and Virtanen, 1959). The glucoside is enzymatically transformed into the aglycon by the action of β-glucosidases upon crushing the plant tissue (Wahlroos and Virtanen, 1959). The aglycon is converted into MBOA (2), its corresponding benzoxazolinone, by heating in aqueous solutions (Virtanen and Wahlroos, 1963), although a 100% conversion rate varies with temperature, pH, and some unknown working conditions (Woodward et al., 1978).



1 DIMBOA



2 MBOA

The biological role played by DIMBOA is as yet little understood. Nevertheless, this compound has been considered by several authors to be directly implicated in resistance to some pests and diseases as well as in detoxification of triazine herbicides as an iron carrier [for a

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review, see Willard and Penner (1976)].

Lundgren et al. (1981) point out the urgent need to develop methods of selection for biochemical characters of resistance. The implication of DIMBOA in plant resistance against pests and diseases suggests the utilization of this compound as a criterion of selection for plant breeders, providing that a rapid screening test is developed (Russell et al., 1975). Quantitation of DIMBOA has been done by isotopic dilution (Klun and Brindley, 1966), infrared spectrophotometry (Scism et al., 1974), fluorometry (Bowman et al., 1968), and gas–liquid chromatography of either free (Tang et al., 1975) or trimethylsilyl derivatives (Woodward et al., 1979). With the exception of an unspecific method based on the colorimetric quantification of the complex formed between hydroxamic acids and $FeCl_3$ (Long et al., 1974), the actual available procedures are too slow to be used in plant breeding programs where large numbers of samples have to be analyzed in a short period of time. We report here a rapid and precise LC method for quantitation of DIMBOA and MBOA in aqueous corn plant extracts.

EXPERIMENTAL SECTION

Apparatus. LC was performed in a Hewlett-Packard high-pressure liquid chromatograph equipped with an LC terminal (HP 79850 A) and a variable-wavelength UV–vis detector (HP 1030 B). For gas chromatography (GC), a 5830 A Hewlett-Packard gas chromatograph with dual flame ionization detector and a GC terminal (HP 1885 A) was used. Ultraviolet and infrared spectra were respectively recorded in a Variant 635 UV–vis spectrometer and in a Beckman IR-4240 spectrophotometer.

Purification of Standards. DIMBOA used as standard was isolated from fresh corn shoots (hybrid 2087 × 2128) by the method of Klun et al. (1967) and then purified by preparative thin-layer cromatography (TLC) using silica gel GF_{254} as the coating material and chloroform–methanol (9:1 v/v) as the mobile phase. The MBOA standard was prepared by the method of Klun and Brindley (1966). All reagents used in this and subsequents steps were analytical grade.

Sample Preparation. Seeds of corn (*Zea mays* L.) (hybrid 2087 × 2128) were disinfected by soaking during 10 min in a mixture of 0.1% $HgCl_2$ and 96% of ethanol (3:1 v/v) and washed several times with sterilized water. After 7 days of dark germination at 25 °C in a plastic tray lined with wet filter paper, shoots were hand removed, and 1 g of this fresh material was cut in small pieces, suspended in 4 mL of distilled water, and homogenized in a Sorvall Omni-Mixer for 1 min. After incubation at 25 °C overnight, the slurry was sonicated in a cleaning bath during three periods of 1 min and then filtered through four layers of cheesecloth, with a 10-mL Luer lock syringe and a Millipore filter holder. The filtrate was acidified to pH 2 with concentrated HCl, heated to 65 °C for 1 min, and cooled in an ice bath for 10 min. The resulting precipitate was discarded after filtration through Whatman No. 42 filter paper as described above. Then 6 mL of ethyl acetate was added to the resulting filtrate, and after the mixture was shaken, the organic phase was separated with a syringe and evaporated to dryness under vacuum in a desiccator at 40 °C. The residue was redisolved in 1 mL of ethyl acetate and procesed by LC.

LC Conditions. Ten microliters of the final ethyl acetate solution was injected by using an automatical system into the liquid chromatograph and processed under the following conditions: column, 4.6 mm × 30 cm LiChrosorb SI 100 (5 μm); mobile phase, a gradient of ethanol–hexane as indicated in Figure 1; temperatures, hexane 60 °C ethanol and column 70 °C; wavelength 288 nm; sensitivity, 0.02 aufs or other appropriate settings.

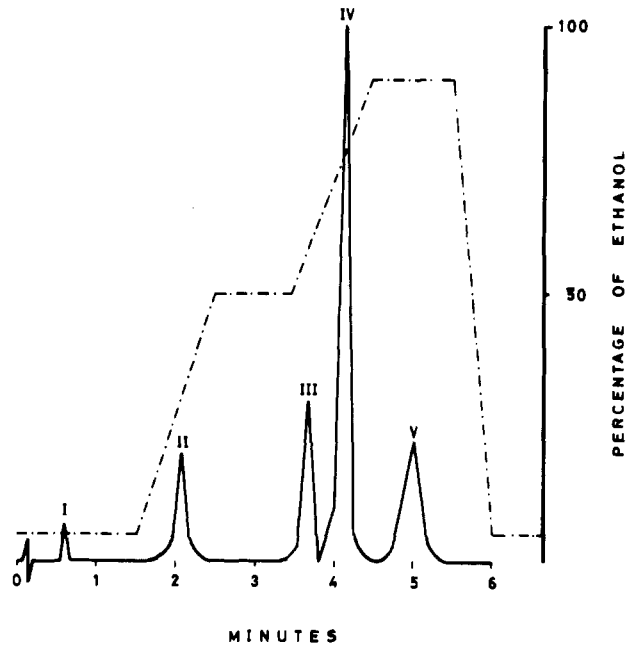

Figure 1. High-presure liquid chromatogram of an ethyl acetate extract from maize hybrid 2087 × 2128. (–·–) represents the percentage of ethanol in the gradient.

Identification and Quantitation of DIMBOA and MBOA in the Final Ethyl Acetate Extract. The identity of DIMBOA and MBOA in the final ethyl acetate extract was established by peak enrichment, showing coincident retention time, with the authentic standards in the LC system previously described. Quantitation has been done by refering to the molar responses of our standards.

Percentage of Recovery. A triplicate of different quantities of DIMBOA and MBOA were added to test tubes containing 1 g of homogenated tissue from maize hybrid 2087 × 2128 and processed as previously described.

RESULTS AND DISCUSSION

The identity of DIMBOA and MBOA standards has been stablished on the basis of UV and IR spectrum data. The UV spectrum in absolute ethanol of our DIMBOA preparation [λ_{max} 288 nm (shoulder) and 261 (ϵ 10660)] agrees with that described by Woodward et al. (1978) and the IR spectrum in a KBr disk [3360 (m), 3160 (m), 1670 (s), 1610 (m), 1520 (m), 1320 (m), 1285 (m), 1165 (m), 1030 (m), and 810 (m) cm^{-1}] is in concordance with the one reported by Klun et al. (1967). The UV spectrum in absolute ethanol of our isolated MBOA [λ_{max} 288 nm (5770) and 231 (10320)] and the IR spectrum in a KBr disk [3200 (m), 1790 (s), 1640 (w), 1500 (m), 1320 (m), 1210 (m), 1140 (m), 1100 (m), 1025 (m), and 970 (m) cm^{-1}] are in agreement with those published by Woodward et al. (1978).

The homogeneity of MBOA and DIMBOA standards were checked by LC and GC. In the LC conditions previously described our MBOA standard show a single peak with a retention time of 2.31 min, but the DIMBOA standard exhibited two peaks, a main one eluting at 4.26 min and accounting for a 93% of the total area and a minor one that eluted at the same position as MBOA. The small peak was enhanced with the addition of pure MBOA. The same results were obtained when isocratic LC runs were performed in a 4.6 mm × 30 cm packed with LiChrosorb

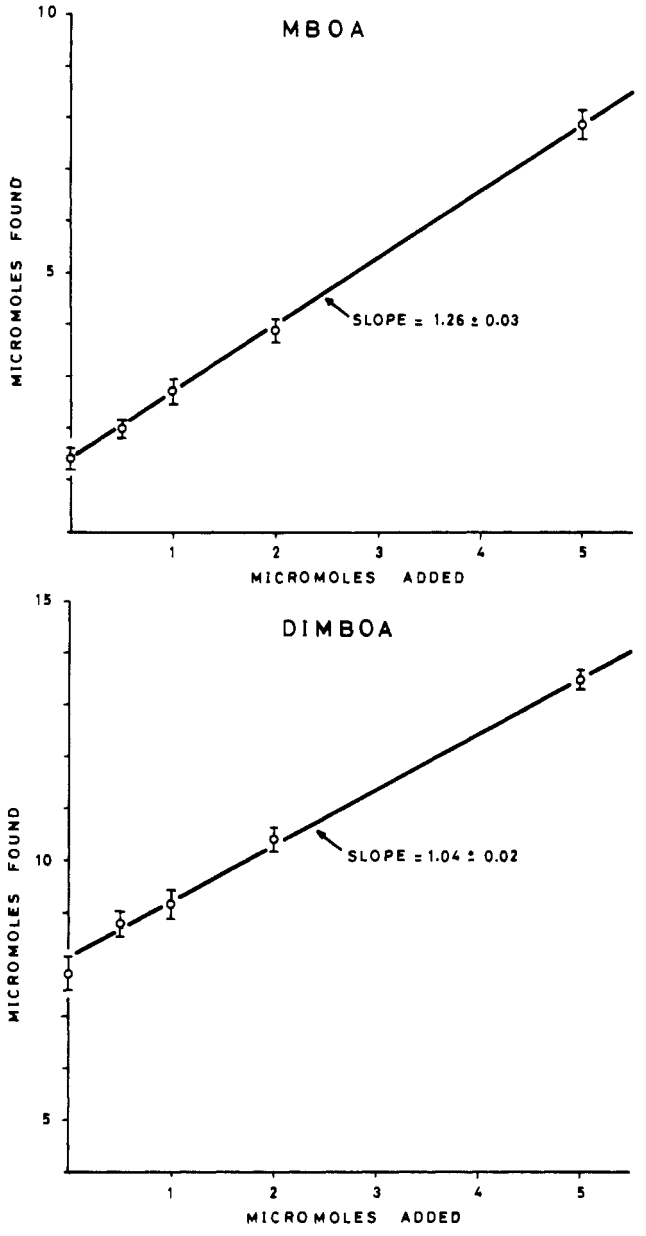

Figure 2. Recovery of DIMBOA and MBOA added to an ethyl acetate extract from maize hybrid 2087 × 2128.

RP-8 (10 μm) and ethanol–water (1:1 v/v) or water-saturated butanol–acetic acid (9:1 v/v) was used as the mobile phase. When our standards were tested by GC using the method described by Tang et al. (1975), the MBOA appeared as a single peak with a retention time of 6.35 min, but DIMBOA, however, decomposed under these analytical conditions.

The molar responses (expressed in units of area per mole) and the retention times of the standards remained unaltered over 1 year of storage at −20 °C under N_2.

The variation coefficients for retention times and molar responses in 10 replicated standard samples were used to test the precission of the method. Variation coefficients of 1.11% and 1.07% were respectively obtained for the molar responses of the standards MBOA and DIMBOA. The retention time coefficients of variation determined for MBOA and DIMBOA were respectively 0.011% and 0.019%. Therefore, this method has good precision.

A typical chromatogram from corn extract obtained under the LC conditions described is shown in Figure 1. Peaks II and IV are highly symmetrical, and their retention times of 2.29 and 4.26 min agree with our standards of MBOA and DIMBOA, respectively. Peaks II and IV were respectively enhanced when the MBOA and DIMBOA standards were added to the sample. On these basis peak II has been tentatively identified as MBOA and peak IV as DIMBOA.

We have no data for structural assignements of compounds I, III, and V. Neither extra peaks nor significant differences in retention times were observed, providing that the column was cleaned with acetonitrile at 70 °C every 100 runs.

So that the accuracy of the present method could be tested, triplicated aliquots of DIMBOA and MBOA were added to tubes containing 1 g of homogenated shoots from maize and processed as described under in Experimental Section. A plot of added vs. found micromoles in a homogenated mayze sample is shown in Figure 2. In such a plot, intercept values with ordinates axes represent the original amounts of MBOA and DIMBOA present in the sample. A 104 ± 2% recovery of the added DIMBOA is indicated by the slope of 1.04 ± 0.002 obtained in the plot of added vs. found micromoles for this compound (Figure 2). The experimentally determined slope of 1.26 for the MBOA recovery is converted into 1.06 if corrections are made to account for the 7% of MBOA contamination in the standard DIMBOA. Then, a 106 ± 3% recovery for MBOA is obtained (Figure 2). Hence, this is an accurate method for the quantitation of these compounds.

The LC method reported here, besides being quicker than the GC methods, saves the derivatitation step and the losses involved on it. Furthemore, its precission and accuracy make it appropriate for quantitation of MBOA and DIMBOA in plant materials. We have found that two operators with this procedure can process about 400 samples in a normal working week. Therefore, this method could be an useful tool in plant breeding programs dealing with pest and disease resistance or with triazine detoxification ability, where a large number of genotypes must be screened in a short period of time.

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Received for review February 26, 1982. Accepted July 27, 1982.