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Note

High-performance liquid chromatography of naturally occurring benzoxazolinones

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Recently, 2-benzoxazolinone (BOA), 6-methoxy-2-benzoxazolinone (MBOA) and 6,7-dimethoxy-2-benzoxazolinone (DMBOA) have been identified as the active constituents of the naturally occurring "supernatant factor"[1], which modifies the auxin–receptor interaction in corn[2].

These compounds are formed, by enzymic hydrolysis and subsequent heating[3–7], from the naturally occurring glucosides of the corresponding benzoxazinones[8], which have been implicated in the resistance of certain species of *Gramineae* to fungi[9–14], 2-chloro-*s*-triazine herbicides[15,16] and insects[17–19]. Thus, qualitative and quantitative analysis of these biologically active compounds can be of interest in relation to several problems in plant physiology and pathology. Hitherto, some procedures have been reported for their quantitative determination in plant materials[18,20,21], and a gas chromatographic method[22] has also been described.

In this paper we report a rapid and simple high-performance liquid chromatographic (HPLC) method for the separation and quantitative analysis of the three benzoxazolinones and its application to their determination in etiolated maize seedlings.

EXPERIMENTAL

Apparatus

All separations were carried out with a Series 2/2 liquid chromatograph equipped with a LC-55 variable wavelength UV detector and a column (25 cm × 26 mm I.D.) packed with Silica A (all manufactured by Perkin-Elmer, Norwalk, Conn., U.S.A.). The use of a silica column was suggested by the good results previously obtained by Venis and Watson[1] in preparative chromatography of the same compounds.

Chemicals

All solvents were of analytical-reagent grade and were obtaided from E. Merck (Darmstadt, G.F.R.)

The standard mixture (BOA: 0.15 mg/ml; MBOA: 0.95 mg/ml; DMBOA: 0.60 mg/ml) was prepared from pure samples of benzoxazolinones provided through the kindness of Dr. M. A. Venis (Shell Biosciences Laboratory, Sittingbourne, Great Britain).

A crude mixture of the same compounds in chloroform was obtained from *Zea mays* (Dekalb XL 342) coleoptiles plus primary leaves (107 g) by following procedure 2 of Venis and Watson[1], omitting the silica column chromatographic step.

Quantitation

The operating conditions chosen for quantitative analysis were: flow-rate, 2.0 ml/min; pen range, 1 mV; attenuation, 0.1 a.u.f.s.; chart speed, 5 mm/min; wavelength 280 nm. The mobile phase was a linear gradient (from 10 to 40%) of chloroform in *n*-hexane, followed by 40% of chloroform in *n*-hexane. The working conditions were: pump A, chloroform–*n*-hexane (40:60); pump B, *n*-hexane; gradient rate, 4% A/min; starting point, 25% A/(A+B). These conditions were chosen in order to avoid the poor reproducibility and accuracy at the start of the gradient system formed when dual reciprocating pumps were used and which adversely affected the separation; resolution was unaffected by similar problems at the end of the gradient.

All separations were carried out at room temperature (26–28°). Standard curves were obtained by plotting peak area (peak height × peak width at half of the peak height) against the equivalent amount of compound injected; the plotting of peak height instead of peak area was less satisfactory.

RESULTS AND DISCUSSION

As shown in Fig. 1, the standard mixture of BOA, MBOA and DMBOA was satisfactorily resolved, giving three almost symmetrical peaks. The first two were separated by gradient elution, and the third by isocratic elution. As expected, retention times increased in the order BOA, MBOA, DMBOA (Table I).

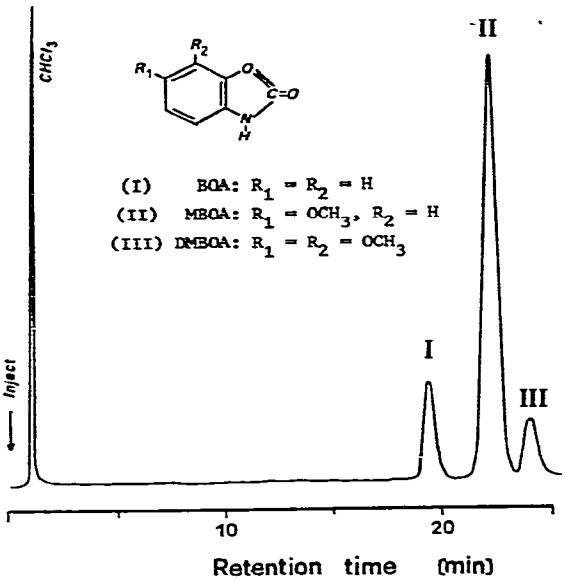

Fig. 1. Chromatogram of standard mixture of BOA (I), MBOA (II) and DMBOA (III); HPLC conditions as described in the text.

TABLE I

RETENTION TIMES FOR STANDARD AND NATURAL COMPOUNDS

Each value is the mean of nine determinations ± the standard deviation.

Compound	*Retention time (min)*
BOA	19.48 ± 0.22
MBOA	22.17 ± 0.30
DMBOA	24.44 ± 0.43

The chromatographic pattern was markedly affected by the operating temperature, resolution being severely impaired at temperatures lower than 26°.

Because of the differences in the UV absorption spectra of the three compounds, the chosen wavelength (280 nm) was a compromise that permitted their simultaneous determination with the best quantitative results. For all three compounds, there was a rectilinear relationship between peak area and amount applied to the column (Fig. 2), at least over the range 0.3–3.8 μg (the lowest figure is for BOA and the highest for MBOA).

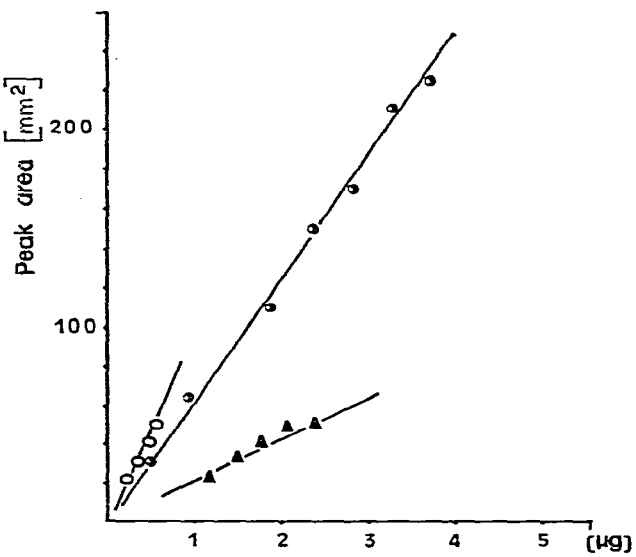

Fig. 2. Calibration graphs for benzoxazolinones: ○, BOA; ◑, MBOA; ▲, DMBOA. Each value represents the mean of three or four determinations.

When the method was applied to a crude extract of maize coleoptiles plus primary leaves, the chromatographic pattern was qualitatively identical with that given by the standard mixture. The nature of the compounds responsible for the individual peaks was confirmed by co-injection of the cited extract with each reference benzoxazolinone. The amounts of BOA, MBOA and DMBOA determined in maize seedlings were, respectively, 210, 1540 and 980 μg/g of fresh tissue.

These values are consistent with those determined by other procedures with different cultivars[1,7,22] and demonstrate the good sensitivity and specificity of the proposed method.

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