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Note

Reversed-phase high-performance liquid chromatographic procedure for the determination of maysin in corn silks

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The resistance of certain corn (*Zea mays* L.) genotypes to the corn earworm, *Heliothis zea* (Boddie), has been associated with the toxicity of their cornsilks[1–3]. Elliger *et al.*[4] showed that the activity of "Zapalote Chico" cornsilks could be attributed to one major flavonoid called maysin identified as 2″-*O*-α-L-rhamnosyl-6-*C*-6-deoxy-*xylo*-hexos-4-ulosyl)luteolin (Fig. 1). Waiss *et al.*[5] and Wiseman and co-workers[6,7] demonstrated that the activity of maysin and of Zapalote Chico cornsilks was true antibiosis. Waiss *et al.*[5] developed a spectrophotometric method for the measurement of maysin in silk extracts. Widstrom *et al.* used this method of maysin analysis to determine genetic variability in maysin contents of various maize populations[8] and maize grown in diverse environments[9]. However, considerable variation in maysin content was found in these studies. Recently, Wiseman *et al.*[10] reported extremely variable levels of maysin from year to year in resistant corn lines subjected to laboratory feeding bioassay studies with corn earworm larvae. No correlation between maysin concentration and larval weights was apparent among lines with varying levels of maysin.

It appeared that the discrepancy was due to the maysin analysis, as it was initially recognized that only half the absorbance intensity at 352 nm for corn silk extracts was actually due to maysin[5]. Consequently, it was essential to develop a more specific chromatographic method for maysin in corn silks. As maysin is not commercially available, it was necessary to develop an isolation procedure to obtain (from Zapolote Chico silks) a pure maysin standard.

EXPERIMENTAL[a]

Materials

Solvents were Burdick & Jackson (Muskegon, MI, U.S.A.) "distilled-in-glass" grade. Chrysin (5,7-dihydroxyflavone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was recrystallized from methanol.

High-performance liquid chromatography (HPLC)

Reversed-phase HPLC was performed with a Hewlett-Packard 1084B liquid chromatograph, using an Altex Ultrasphere ODS column, 25 cm × 4.6 mm I.D. The solvent gradient was: linear from methanol–water (20:80) (solvent A) to methanol (solvent B) in 40 min, with a flow-rate of 1 ml/min and a 12-min recycle time. Both solvents contained 0.1% orthophosphoric acid. The column effluent was monitored at 340 nm (reference wavelength, 550 nm).

Sample collection and preparation

To obtain a representative sample, silks (one silk bundle from an individual ear) from five different plants were combined, weighed and placed in amber wide-mouth jars. The silks were covered with 500 ml methanol and the jars were sealed with PTFE-lined caps and kept frozen until analysis. A second five-silk composite was obtained for determination of moisture content of the silks, which were dried at 100°C to constant weight. The silk–methanol sample was warmed to room temperature and 20 ml of a solution of the internal standard, chrysin (17 mg/20 ml methanol), were added. After ultrasonication of the sample for 30 min, an aliquot was filtered through a Nylon 66 membrane filter (0.45 μm, Micron Separations, Westboro, MA, U.S.A.) into an autoinjector vial and a 20-μl aliquot was analyzed by HPLC.

Isolation of maysin

Solvent extraction. Maysin was isolated from Zapalote Chico [selection ZC 2451 No. (P)(C3)] grown at the facilities of the USDA-ARS Southern Grain Insects Lab., Tifton, GA, U.S.A. Approximately 3230 g of fresh Zapalote Chico silks (representing about 910 silks) were blended with 10 l of methanol in a Waring Blender and stored at 0°C, prior to transport to our laboratory. After warming to room temperature, the silk–methanol mixture was ultrasonicated for 30 min and then filtered through filter paper. The resulting methanol extract, now also containing the water from the fresh silks, was concentrated on a rotary evaporator to a final volume of approximately 1650 ml. The resulting aqueous solution was then extracted with methylene chloride (3 × 500 ml) to remove lipids. The remaining solution was divided into three equal parts for ease in handling. Each portion was extracted with ethyl acetate (3 × 500 ml) and the ethyl acetate extracts were pooled. The residual aqueous solutions were then mixed with equal volumes of acetonitrile, which gave a totally miscible mixture. The solutions were stored at 0°C overnight to effect separation of the

[a] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

water and acetonitrile layers. The acetonitrile layers were decanted, pooled and added to the previous ethyl acetate extracts. The combined ethyl acetate and acetonitrile extracts were concentrated on a rotary evaporator to give 25.68 g of a dark reddish, syrupy residue.

Silicic acid chromatography. This residue was dissolved in 400 ml of methanol–ethyl acetate–acetonitrile–isooctane (1:1:1:1) and mixed with 60 g of silicic acid (Mallinckrodt, 100 mesh, washed with methanol, dried at 150°C for 16 h). After evaporation of the solvent from the sample–silicic acid mixture, the coated silicic acid was slurried with methylene chloride and placed on top of a 300-g silicic acid column (4 × 70 cm), packed in methylene chloride. The column was eluted with the following solvents (%, v/v): 2 l methylene chloride, 1.5 l ethyl acetate–methylene chloride (25:75), 1 l ethyl acetate–methylene chloride (50:50), 1 l ethyl acetate–methylene chloride (75:25), 1 l ethyl acetate, 5.5 l acetone–ethyl acetate (10:90), 4.5 l acetone–ethyl acetate (20:80), 2 l acetone–ethyl acetate (40:60), 4 l acetone–ethyl acetate (50:50), 5 l acetone, 1 l acetone–methanol (50:50), 1 l methanol. The 10% and 20% acetone in ethyl acetate fractions were combined and on evaporation yielded 8.26 g of material that was approximately 80% pure maysin. The first 1.5 l of acetone–ethyl acetate (10:90) from the silicic acid column contained 89% pure maysin in 3.77 g of material.

Reversed-phase chromatography. The silicic acid–maysin fraction was then purified by C_{18} reversed-phase chromatography. The packing material from a Waters (Milford, MA, U.S.A.) PrepPak 500 C_{18} reversed-phase cartridge was repacked into a smaller glass Cheminert LC column (54 × 2.54 cm) (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The smaller diameter column afforded better resolution of the maysin and conserved solvents. The column was washed with methanol and then recycled to water as the initial solvent. Samples of 1 g of the silicic acid–maysin fraction were dissolved in 3 ml of methanol–water (25:75) and applied to the column with a 4-ml loop injection valve. The column was operated at a flow-rate of 2 ml/min. Gradient elution, utilizing two Altex Model 110 pumps and Model 420 programmer (Beckman Instruments, Altex Division, San Ramon, CA, U.S.A.), was employed. The column effluent was monitored at 340 nm (Altex Model 153 UV detector), and 8-ml fractions were collected (Mini-Fractionator, Gilson Medical Electronics, Middleton, WI, U.S.A.). Two different solvent programs were tested.

The first solvent program used a linear gradient: 100% water to 100% methanol over 24 h. A 1-g sample of the silicic acid–maysin yielded 372 mg of maysin of >98% purity and 220 mg of maysin of >95 98% purity.

The second solvent program utilized a linear gradient of methanol–water (50:50) to methanol–water (75:25) over 400 min, then a linear gradient of methanol–water (75:25) to 100% methanol over 200 min. A 1-g amount of the silicic acid–maysin fraction gave 700 mg of >80% maysin which, when rechromatographed under the same conditions, gave 600 mg of >95% pure maysin.

The isolated maysin was recrystallized from acetonitrile–methanol (approx. 1:1, v/v). It gave a decomposition point of 200–210°C (lit.[4], decomposition point of 225°C, crystallized from acetone–methanol). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Model AM spectrometer (250 MHz) in [$^{2}H_6$]dimethyl sulfoxide at ambient temperature.

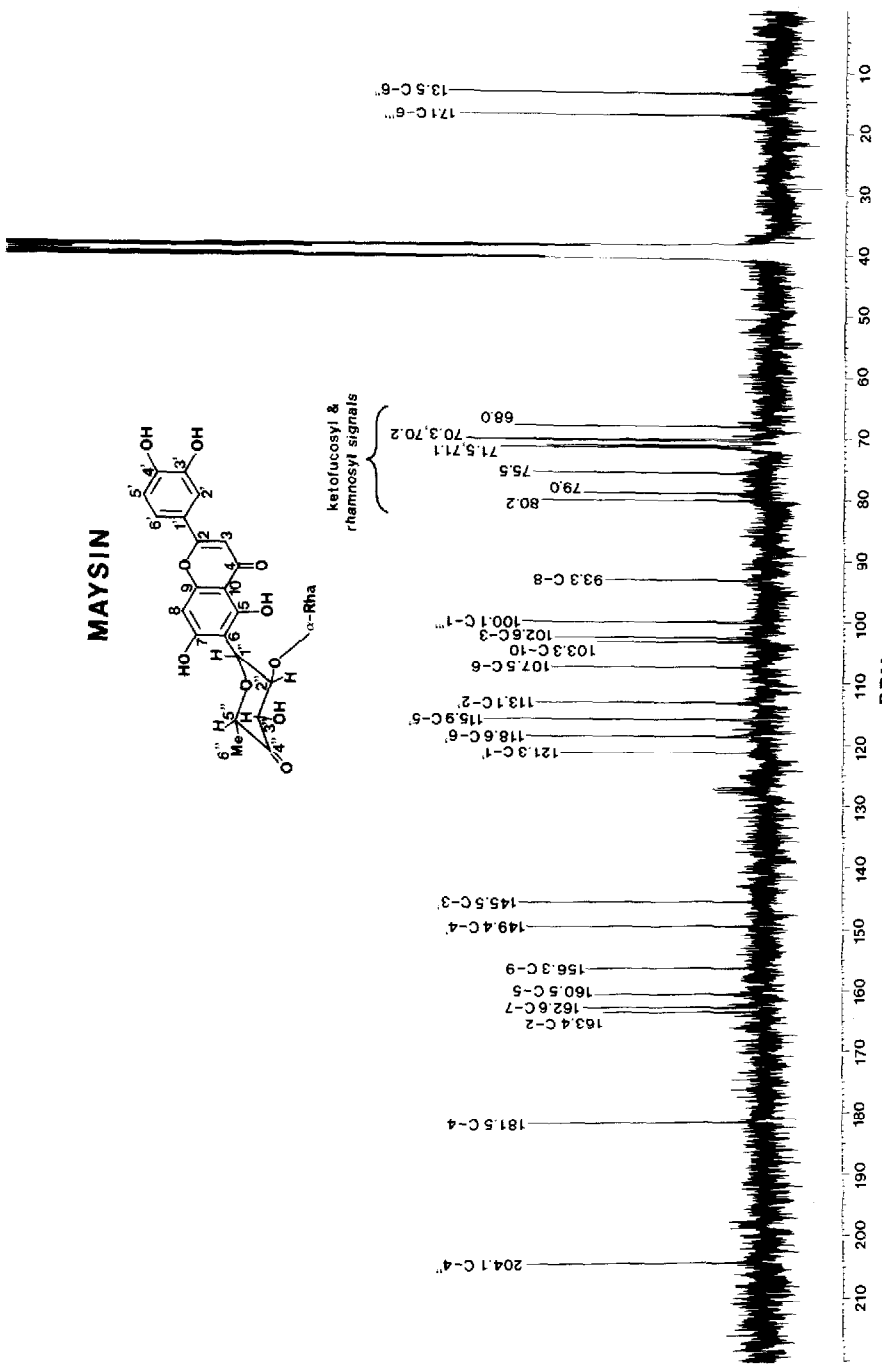

Fig. 1. ^{13}C NMR spectrum of isolated maysin ([$^2H_6$]dimethyl sulfoxide).

RESULTS AND DISCUSSION

Our initial screenings of methanol extracts of corn silks demonstrated the utility of reversed-phase HPLC for analyses of the flavonoids of silk. However, for quantitative analysis, we required a pure sample of maysin. Accordingly, pure maysin was isolated from silks of Zapalote Chico by a combination of solvent partitioning, silicic acid column chromatography, and preparative reversed-phase chromatography. The isolated maysin was shown to be pure by HPLC and its ^{1}H NMR spectrum matched that in the literature[4].

The ^{13}C NMR spectrum of isolated maysin is given in Fig. 1, while chemical shift assignments of maysin and related flavonols are given in Table I. Assignments were made by comparison with those of 6-*C*-glucosylluteolin and 2″-*O*-rhamnosyl-8-*C*-glucosylapigenin (2″-*O*-rhamnosylvitexin)[11]. These agreed with ^{13}C spectra of maysin assignments provided by Elliger[12]. The ^{13}C chemical shift at 100.1 ppm in the maysin spectrum (Fig. 1) was assigned to the C-1 carbon of rhamnose based on analogous chemical shifts of rhamnose in 2″-*O*-rhamnosylvitexin (Table I).

The commercially available aglycone, chrysin (5,7-dihydroxyflavone), was found to be an acceptable internal standard. Methanol was used to dilute concentrated silk samples to retain chrysin in solution. An HPLC chromatogram of the separation of chlorogenic acid, maysin and chrysin is given in Fig. 2. Excellent peak shapes were obtained with the Altex Ultrasphere ODS column and a linear gradient from methanol–water (20:80) to 100% methanol. In our system (HP 1084, UV monitor set at 340 nm), the response factor of maysin relative to chrysin was found to be 1.10. Although other HPLC UV detectors may give slightly different responses, HPLC quantitation of maysin, assuming a unitary UV response equal to chrysin, should be an acceptable method.

Representative HPLC chromatograms of high-, medium- and low-maysin-containing corn silks are given in Fig. 3. Adequate separation of maysin from other flavonoids was obtained. As some variability in silk maysin levels was found among individual plants of a variety, it was decided to produce a representative sample by combining five silk bundles for analysis. Quantitative analyses of corn silks of varying maysin content (Table II) showed quite a large variation in maysin content among the various entries. Zapalote Chico, as expected, contained the highest level of maysin in its silks. Annual teosinte, (*Zea mays* L., ssp. *mexicana*) an insect-resistant primitive corn, also contained substantial quantities of maysin. However, a number of entries, that had been selected for corn earworm resistance in field trials, were found to contain very low levels of maysin. Thus, entries 3 and 14 were resistant to corn earworm attack, but were found to have widely different levels of maysin. Therefore, the resistance of entries 10–15 and 17 (Table II) on field plants may involve other chemical or physical factors, as opposed to the true antibiosis exhibited by Zapalote Chico (entry 1).

Maysin analyses data by the previously reported spectrophotometric method (UV analysis) did not correlate well with observed corn earworm resistance. Maysin levels by the reported HPLC method were often found to be contrary to the previous UV method values. However, the HPLC values correlated much more closely with the observed corn earworm resistance. It is thought that interfering compounds in the UV method resulted in erroneously high maysin levels of certain genotypes. For

TABLE I

^{13}C NMR CHEMICAL SHIFT ASSIGNMENTS OF MAYSIN AND RELATED FLAVONOLS ([$^2H_6$]DIMETHYL SULFOXIDE)

Carbon assignment[a]	*Maysin*	*Isoorientin*[b] *(6-C-glucosylluteolin)*	*2″-O-Rhamnosyl-8-C-glucosyl-apigenin*[b]
C-4″	204.1		
C-4	181.5	181.7	181.9
C-2	163.4	163.5	163.9
C-7	162.6	163.0	162.1
C-5	160.5	160.5	160.5
C-9	156.3	156.0	155.7
C-4′	149.4	149.5	161.0
C-3′	145.5	145.6	115.8 (C-3′,5′)
C-1′	121.3	121.3	121.5
C-6′	118.6	118.8	128.9 (C-2′,6′)
C-5′	115.9	115.9	
C-2′	113.1	113.2	
C-6	107.5	108.7	98.2
C-10	103.3	103.3	104.1
C-3	102.6	102.7	102.3
C-1‴	100.1		100.2
C-8	93.3	93.4	104.3
C*-5″	80.2	81.3	81.7
C*-3″	79.0	78.8	79.8
C*-2″	75.5	70.4	75.0
C*-1″,3‴	{ 71.5 71.1	72.9 (C-1″)	71.4 (C-1″,3‴)
C*-2‴,4‴	{ 70.3 70.2	70.1 (C-4″)	70.3 (C-4″,2‴,4‴)
C-5‴	68.0		68.1
C-6‴	17.1		17.6
C-6″	13.5	61.3	

[a] See Fig. 1; * indicate tentative assignments.

[b] From ref. 11.

example, Coe G12, apparently high in UV-interfering compounds, gave UV maysin values more than twice as large as Zapalote Chico and about fourteen times that of Stowell's Evergreen. The HPLC values (Table II) indicate that Zapalote Chico has about three times the maysin content of Stowell's Evergreen and Coe G12 which are similar in maysin content. However, relative maysin contents of Zapalote Chico and Stowell's Evergreen (genotypes with negligible UV-interfering compounds) by UV analysis indicate that the maysin content of Zapalote Chico was 2.5 to 6 times larger than for Stowell's Evergreen. The values for HPLC in Table II indicated 3.4 times as much maysin in Zapalote Chico silks as those for Stowell's Evergreen, a value not drastically different from the UV analyses.

The HPLC-determined maysin values are, therefore, expected to relate much better to the antibiosis type of resistance than the previously used UV-absorbance values. The primary reason for this expectation is that problems encountered with silks having other compounds, that interfere with the UV absorbance of maysin, have

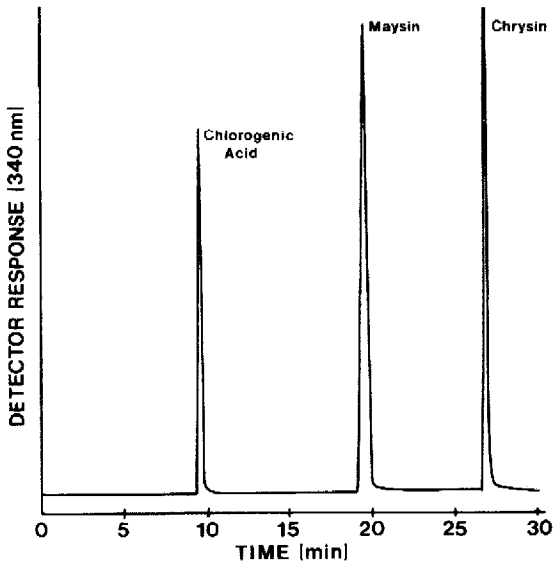

Fig. 2. HPLC of standards: chlorogenic acid, maysin and chrysin (internal standard).

TABLE II

MAYSIN CONTENT OF THE SILKS OF SEVERAL CORN ENTRIES AS DETERMINED BY HPLC ANALYSIS

Corn entry	*Maysin level (% of silks)*[a]	*Relative maysin content*
1 Zapalote Chico 2451	6.30	100.0
2 Annual teosinte	5.49	87.1
3 MAS	3.72	59.0
4 RFC-RI(C7)	3.66	58.1
5 SGP-M10	2.15	34.1
6 Coe G12	2.14	34.0
7 CC-M10	2.05	32.5
8 GT115	1.94	30.8
9 Stowell's Evergreen	1.84	29.2
10 10LDD Sel Rec	1.13	17.9
11 SwtCD Sel Rec-RM1	0.20	3.2
12 RFC-RM1-D1 Sel	0.17	2.7
13 GT119	0.15	2.4
14 DARS	0.10	1.6
15 DDSB	0.08	1.3
16 Coe G10	0.03	0.4
17 DDSA	0.01	0.2

[a] Percent dry weight.

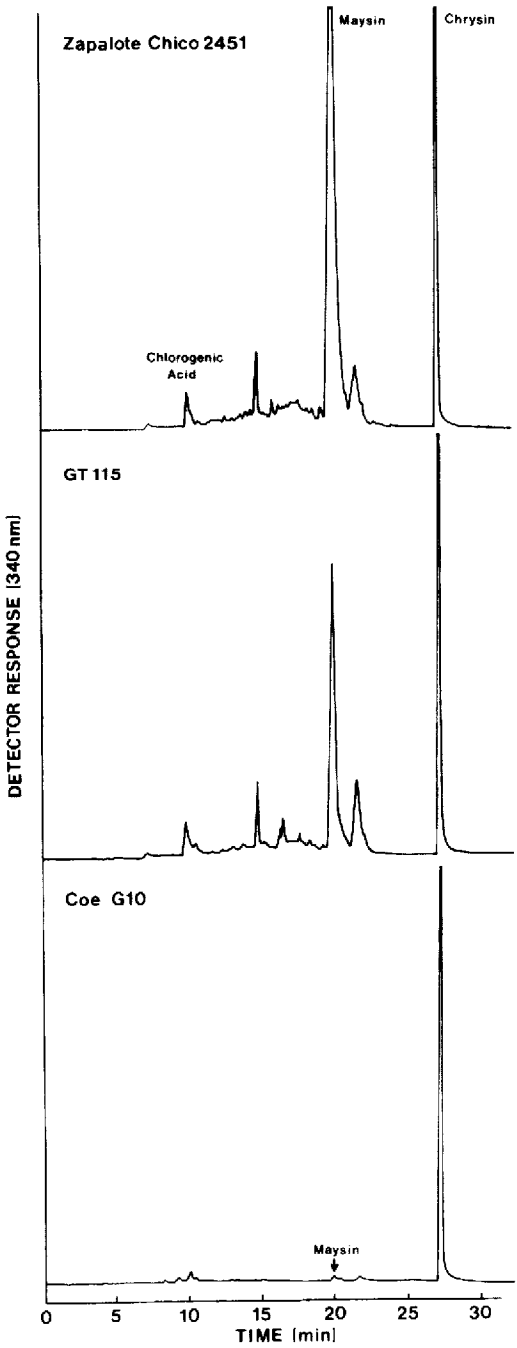

Fig. 3. HPLC of selected corn silk extracts.

been eliminated. Since the corn entry Stowell's Evergreen (Table II) is not considered a very high corn earworm-resistant line, it appears that in order to impart corn earworm resistance due to maysin content, silk levels of maysin above 2% dry weight may be needed.

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