Analysis of Linear and Angular Furanocoumarins by Dual-Column High-Performance Liquid Chromatography

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Retention characteristics and response factors were determined for 14 linear and 6 angular furanocoumarins on both reversed-phase and conventional-phase HPLC. Detection was by UV at 254 nm with phenol as the internal standard. Response factors calculated from analyses of standards of known concentration ranged from 0.053 to 0.396 (phenol 1.000) and were dependent upon ring geometry and substitution. Most of the compounds were resolved by reversed-phase HPLC, but conventional-phase HPLC was useful for isolating certain isomers and for confirmation of identifications tentatively made from the reversed-phase system. The detection limit was 50–250 ng (depending upon the relative response), and analysis of solutions of known concentration gave results that agreed to within 10% of the known values.

Furanocoumarins are highly bioactive compounds that have received much attention because of their toxicity (Murray et al., 1982). Many are potent photosensitizers that can be moderately toxic to mammals (Ivie, 1978a), certain insects (Berenbaum, 1981), and man (Beier et al., 1983a). Methods for their analysis have usually been aimed at a particular biological matrix (e.g., one plant species), and therefore, more general approaches for identification and quantitation have not been advanced. HPLC is a logical choice for a chromatographic method because it combines speed, selectivity, and sensitivity with nondestructiveness. The last quality is important because certain furanocoumarins degrade under even mild heat, such as that found in a GC oven, yielding multiple peaks (Spencer, 1986).

In our search for natural germination inhibitors, we found that furanocoumarins have been identified as active in certain bioassays (Friedman et al., 1982; Shimomura et al., 1982; Sinha-Roy and Chakraborty, 1976; Kato et al., 1978), but no systematic examination of the wide range of substituted furanocoumarins has been made. While conducting this evaluation, we needed a method to assay both linear and angular compounds in seed or root extracts. Separations detailed in the literature either did not consider both types of furanocoumarins, did not consider many of the positional isomers, or did not provide quantitative results (Kubeczka and Rohde, 1984). The dual-column, internal standard approach described here gives reasonably fast, reliable identification and quantitation and is less prone to error in either dimension than are single-column methods. It is important to note that there are many components with UV absorption that occur in plants containing furanocoumarins (particularly other coumarins) that could interfere with this analysis.

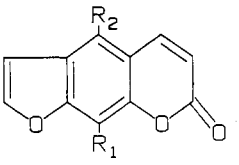
MATERIALS AND METHODS

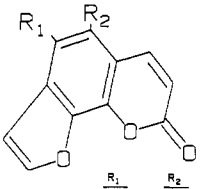
Apparatus. Two Waters M-6000A pumps were controlled by a Waters Model 660 solvent programmer at 2 mL/min and were programmed from 20% solvent B to 80% B over 30 min. For reversed phase, a Whatman ODS-2 column (250 × 4.6 mm) was eluted with solvent mixtures containing EtOH/CH_3CN/H_2O (solvent A, 1/1/6; solvent B, 1/1/1) by using Waters program curve 7. Conventional-phase chromatography employed a Whatman Partisil-10 PAC column (same dimensions) with solvent A (isooctane) and B (cyclohexane/EtOH, 10/1) with Waters program curve 5. Injection was through a Valco C6U valve with a 25-μL loop. Peaks were detected by a Schoeffel Model 770 Spectroflow UV monitor operated at 254 nm and 0.01–0.04 AUFS. Data acquisition was controlled by a computer system (Butterfield et al., 1978), and peak areas were integrated in either an automatic or a manual interactive (Payne-Wahl et al., 1981) mode.

Phenol was used as the internal standard. Responses were determined for each furanocoumarin by chromatography of five different solutions of known concentration plus phenol. Each solution was analyzed three times. Areas from these chromatograms were plotted against the known amounts in order to obtain slopes to be used as response factors (Payne-Wahl et al., 1981). All solutions were made in EtOH and were kept refrigerated after preparation to minimize concentration changes.

Standards. Xanthotoxin (3) was purchased from Sigma Chemical Co. and psoralen (1) from Automergic Chemetals Corp., Farmingdale, NY. All other compounds were isolated from plant extracts that were first fractionated by chromatography on a gravity column filled with 80 g of 60–100-mesh silica gel (Mallinckrodt) and eluted with 250-mL volumes of hexane/EtOAc in the following proportions: 100/0, 90/10, 80/20, 60/40, 0/100. The fura-

Chart I





		R_1	R_2
1.	psoralen	H	H
2.	bergapten	H	OCH_3
3.	xanthotoxin	OCH_3	H
4.	isopimpinellin	OCH_3	OCH_3
5.	isoimperatorin	H	
6.	imperatorin		H
7.	heraclenin		H
8.	oxypeucedanin	H	
9.	oxypeucedanin hydrate	H	
10.	heraclenol		H
11.	isovaleryl oxypeucedanin hydrate	H	
12.	saxalin	H	
13.	phellopterin		OCH_3
14.	byakangelicin		OCH_3

		R_1	R_2
15.	angelicin	H	H
16.	sphondin	OCH_3	H
17.	isobergapten	H	OCH_3
18.	pimpinellin	OCH_3	OCH_3
19.	heratomin		H
20.	8-isopentenyloxy isobergapten		OCH_3

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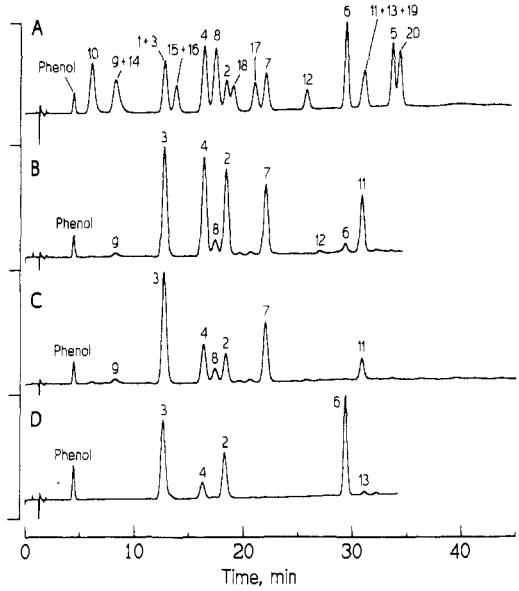

Figure 1. Reversed-phase HPLC. Column: Whatman ODS-2, 4.6 × 250 mm. Solvent: A, EtOH/CH_3CN/H_2O (1/1/6); B, EtOH/CH_3CN/H_2O (1/1/1). Programmed from 20% B to 80% B over 30 min, Waters curve 7 at 2 mL/min. Curves: A, standards; B, *A. majus* seed (Peoria greenhouse) hexane extract; C, *A. majus* seed (Texas field-collected) hexane extract; D, *P. sativa* seed hexane extract.

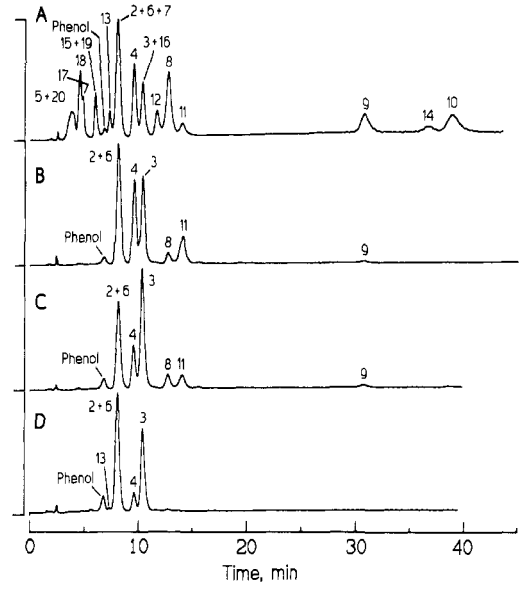

Figure 2. Conventional-phase HPLC. Column: Whatman Partisil 10-PAC. Solvent: A, isooctane; B, cyclohexane/EtOH (10/1). Programmed from 20% B to 80% B over 30 min, Waters curve 5 at 2 mL/min. Curves A–D same as Figure 1.

nocoumarins (that were concentrated in the last three eluates) were further purified by HPLC on a 9.4 × 250 mm Zorbax ODS-2 column (Du Pont) with mixtures of CH_3CN and H_2O pumped at 5 mL/min. Solvent mixtures were adjusted to optimize separations and ranged from 9/1 to 1/1 CH_3CN/H_2O depending on the retention of the solutes. Peaks were detected by a differential refractometer. Each purified compound gave a single peak in the HPLC analytical systems detailed below and a single spot on TLC [silica gel 60 F-254 precoated plates, Brinkman, developed with hexane/EtOAc (70/30)]. Their structures were established by ^1H NMR (Bruker WM-300) in $CDCl_3$ and by mass spectra (Finnigan 4535/TSQ/MS/MS/DS). These spectra when compared to literature values (Steck and Mazurek, 1972; Ivie, 1978b) confirmed the identifications; the absence of extraneous signals in these spectra gave further evidence that the compounds were pure. Primary natural sources included the following: *Pastinaca sativa* L. roots and seeds, **15, 16**; *Angelica lucida* L. seeds, **2, 5–10**; *Ammi majus* L. seeds, **4, 9, 11, 12**; *Heracleum lanatum* Michx. seeds, **13, 14, 17–20**.

A. majus, collected in Texas (Ivie, 1978b), was grown in a greenhouse (20–24 °C) in 8-in. pots filled with Redi-Earth (W. R. Grace Inc.) under ambient light. Plants were treated once a week with Peters (W. R. Grace Inc.) 20/20/20 N/P/K fertilizer at 200 ppm in H_2O. The mixture included EDDHAFe^{3+}. Seed heads were harvested upon ripening and stored in a refrigerator. Wild *Pastinaca sativa* was harvested from roadside stands near Peoria, IL.

Procedure. Ground plant parts were extracted overnight in a fume hood in a Soxhlet apparatus with hexane or acetone as the extracting solvent. Extracts were evaporated under reduced pressure to an oily residue that was taken up in EtOH and transferred to a volumetric flask (flask size was dependent upon the amount of plant material extracted; for example, the hexane extract from 5 g of seed was diluted to 50 mL). One milliliter of the diluted sample was thoroughly mixed with 1 mL of a 2 mg/mL solution of phenol. Fifty microliters of this solution was injected into the loop and the chromatography begun. The loop was flushed between samples with EtOH.

RESULTS AND DISCUSSION

Identification by Relative Retention. Separations achieved by this chromatographic method are illustrated in Figures 1 and 2. Reversed-phase HPLC (Figure 1) resolves the compounds more completely than conventional phase (Figure 2) and, therefore, was the primary system used for quantitation. With this column and solvents, isovaleryl oxypeucedaninhydrate (**11**), phellopterin (**13**), and heratonin (**19**) were eluted as one peak. These three are quite nicely separated by conventional-phase chromatography, although in this system heratonin coelutes with angelicin (**15**). Of more serious consequence was the inability of either system to clearly distinguish sphondin (**16**). In reversed phase, sphondin is eluted with angelicin and in conventional phase with xanthotoxin. Because angelicin and xanthotoxin are much more prominent and abundant compounds in extracts that we have examined, it is easily possible to overlook sphondin (we detected it by TLC in a solvent system similar to Ivie's 5 (1978b). All of the natural mixtures surveyed present much less complicated chromatograms than those of the standards. This observation is also illustrated in Figures 1 and 2 where hexane extracts from *A. majus* and *P. sativa* seeds are shown. It is interesting to note that *A. majus* seed grown in a Peoria greenhouse is markedly different in its relative oxypenadanin **8** content from its progenitor, the wild Texas seed (Ivie, 1978b).

The retention data provide information that will allow the prediction of elution of compounds not studied here. Among linear furanocoumarins, substitution at R_1 gives increased retention on reversed phase and decreased retention on conventional phase. Thus **3, 6, 7,** and **10** are eluted before **2, 5, 8,** and **9**, respectively, on reversed phase, and the elution order of the respective compounds is inverted on conventional phase. The elution position of a compound such as the isovaleryl ester of heraclenol would therefore be predicted to be before 11 on reversed phase and after it on conventional phase. Only two isomeric angular compounds (**16, 17**) were analyzed; they were widely separated on both columns with the elution order

reversed. One would suppose that this reversal of retention might be found for other isomers.

Two pairs of isomers (1 and **15**, **4** and **18**) serve to demonstrate differences in retention between linear and angular compounds. Linear isomers are eluted earlier on reversed phase and are retained longer on conventional phase. Isomers **13** and **20** are chromatographed in a similar nature relative to each other.

Quantitation. The chromatograms illustrated in the figures are of hexane extracts only. The acetone extracts were similar qualitatively but usually contained greater proportions of the more highly oxygenated compounds. The magnitude of UV absorption of furanocoumarins is dependent on the geometry of the rings and on their substitution (Lee and Soine, 1969). Beier et al. (1983b) and Ivie et al. (1982) show great differences in peak sizes from equal amounts of furanocoumarins, and Enriquez et al. (1984) calculated response factors for four furanocoumarins. Since our survey entailed analyses of samples from widely diverse sources and various extraction solvents, it was desirable to be able to collect data on a common basis, and thus an internal standard method seemed appropriate. Phenol was selected because of its availability and its lack of retention in the reversed-phase system. Also, it was not eluted with any of the standards on conventional phase. Response factors obtained from linear regression of values from analysis of the standards of known composition ranged from 0.056 (xanthotoxin, phenol 1.000) to 0.396 (isopimpinellin) on reversed phase and 0.053 (isobergapten) to 0.281 (isopimpinellin) on conventional phase. Correlation coefficients from the regression equations were >0.995. Differences in response factors presumably reflect shifts in UV maxima due to the elution solvents. It is likely that these factors need to be derived for each chromatographic system, but we will make our values available upon request.

Several solutions containing the furanocoumarin standards in known concentration were prepared and analyzed. Resultant values were within at least 10% of the known ones, indicating that this method is reliable to that degree. These data can also be made available upon request.

In order to check the detection limit, a sample of xanthotoxin was successively diluted and analyzed. When 50 ng was injected, the signal-to-noise level had deteriorated such that the noise comprised 10–15% of the peak height. Therefore, 50–200 ng of material (depending on the relative response) is necessary in order to make a successful analysis. This concentration range is the same as that found by Ashwood-Smith et al. (1986) for UV detection of four linear furancoumarins.

ACKNOWLEDGMENT

We thank G. Wayne Ivie for supplying Texas *A. majus* seed, Karen Hall-Fenster for growing and harvesting *A. majus* in the greenhouse, and Carlotta D. Williams for technical support.

Registry No. **1**, 66-97-7; **2**, 484-20-8; **3**, 298-81-7; **4**, 482-27-9; **5**, 482-45-1; **6**, 482-44-0; **7**, 2880-49-1; **8**, 26091-73-6; **9**, 2643-85-8; **10**, 31575-93-6; **11**, 109151-07-7; **12**, 36150-06-8; **13**, 2543-94-4; **14**, 482-25-7; **15**, 523-50-2; **16**, 483-66-9; **17**, 482-48-4; **18**, 131-12-4; **19**, 61265-06-3; **20**, 24099-29-4.

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Received for review November 3, 1986. Accepted April 8, 1987. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.