Journal of Agricultural and Food Chemistry

Volume 30, Number 3 May/June 1982

Analysis of the Garden Carrot (*Daucus carota* L.) for Linear Furocoumarins (Psoralens) at the Sub Parts per Million Level

G. Wayne Ivie,* Ross C. Beier, and Douglas L. Holt

Extracts of fresh roots and foliage of the garden carrot (*Daucus carota* L.) were analyzed by high-performance liquid chromatography for the presence of linear furocoumarins (psoralens). Studies with samples fortified at sub-ppm levels with 12 psoralens of known structure, including psoralen, xanthotoxin, bergapten, isopimpinellin, heraclenin, and oxypeucedanin, showed that the procedure used was appropriate for the analysis of trace levels of psoralens in carrot. Results from studies with nonfortified samples showed that carrot does not contain these photosensitizing, photomutagenic, and photocarcinogenic chemicals or, if present, they occur at very low levels (≪0.5 ppm).

Linear furocourmarins (psoralens) occur widely in nature as constituents of hundreds of plant species, particularly in the families Umbelliferae, Rutaceae, Leguminosae, Moraceae, and Orchidaceae (Pathak et al., 1962; Scott et al., 1976). Many psoralens are potent photosensitizers when activated by long-wavelength ultraviolet light, and episodes of psoralen phototoxicity are well-known in both man (Pathak et al., 1962) and domestic animals (Ivie, 1978b). Psoralens are used medicinally as light-activated drugs for the treatment of certain skin disorders in man, including leukoderma (skin depigmentation) and psoriasis (Pathak et al., 1962; Scott et al., 1976).

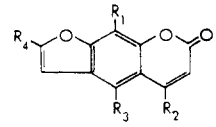
Psoralens readily intercalate into DNA strands where they form light-induced mono- or diadducts with pyrimidine bases (Scott et al., 1976), and they are therefore photomutagenic and photocytotoxic. These characteristics account for the desired medicinal properties of psoralens but also raise questions regarding potential toxicological problems, particularly carcinogenicity. There are, in fact, strong indications that the medicinal use of psoralens in man has resulted in photocarcinogenicity (Reed, 1976; Stern et al., 1979), and some investigators have concluded that the toxicological risks of psoralens to man are of sufficient magnitude that medicinally unnecessary exposures should be avoided (Ashwood-Smith et al., 1980).

Psoralens are known to occur in several vegetables of the family Umbelliferae, including celery (Scheel et al., 1963), parsley (Musajo et al., 1954), and parsnip (Fahmy, 1956; Steck, 1967). However, the toxicological implications to man of dietary exposure to psoralens has only recently received serious consideration (Ivie et al., 1981). It does seem apparent that the extent of human dietary exposure to psoralens should be more fully evaluated, and toward this end, we have undertaken a study of the garden carrot (*Daucus carota* L.), a widely consumed vegetable of the family Umbelliferae whose taxonomic relationship to known psoralen-containing plants makes it appropriate for detailed study of possible psoralen content. Although previous phytochemical studies with carrot have failed to show the presence of psoralens and carrot is generally considered to lack these chemicals (Berenbaum and Feeny, 1981; Ivie et al., 1981), the analytical techniques of the present study were developed to detect a number of carrot psoralens, if present, at trace (sub-ppm) levels.

MATERIALS AND METHODS

Chemicals. The structures of the authentic psoralens used in this study are indicated in Figure 1. Nine of the compounds were as previously obtained (Ivie, 1978a). Sources of the remaining three were as follows: xanthotoxin (Biochemical Laboratories, Redondo Beach, CA), trisoralen (Elder Pharmaceuticals, Bryan, OH), and psoralen (generously provided by M. A. Pathak, Harvard

Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas 77841.



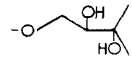
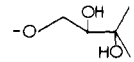
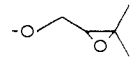
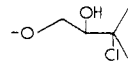
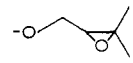
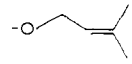
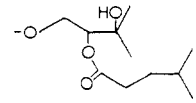
Compound	Number	HPLC retention, min	R_1	R_2	R_3	R_4
Heraclenol	I	1.8		H	H	H
Oxypeucedanin hydrate	II	2.1	H	H		H
Psoralen	III	3.7	H	H	H	H
Xanthotoxin	IV	4.1	$-OCH_3$	H	H	H
Bergapten	V	5.4	H	H	$-OCH_3$	H
Isopimpinellin	VI	5.4	$-OCH_3$	H	$-OCH_3$	H
Heraclenin	VII	6.3		H	H	H
——	VIII	8.2		H	H	H
Oxypeucedanin	IX	8.6	H	H		H
Trisoralen	X	15.8	$-CH_3$	$-CH_3$	H	$-CH_3$
Imperatorin	XI	21.0		H	H	H
——	XII	22.6	H	H		H

Figure 1. Structures of psoralens used in the analysis of carrot for the presence of these chemicals at sub-ppm levels. HPLC retention times on a Supelco LC-18 column, 15-cm length, preceded by a Whatman Co:Pell ODS guard column. Solvent system: acetonitrile–water (35:65 v/v); flow rate 2.0 mL/min.

Medical School, Boston, MA).

Plants. Carrot seed (Gold Pak variety, Hastings Co., Atlanta, GA), were planted in sandy garden soil in Brazos Co., TX, during Nov 1980, and the plants were harvested during April 1981. The plants were washed thoroughly in tap water, and the roots and green foliage were separated. The roots averaged about 18–20 cm in length and were about 2 cm in diameter near the crown. The fresh samples were immediately ground in a food processor, and 5.0-g subsamples were transferred to 40-mL Nalgene centrifuge tubes and frozen for later analysis.

Extraction and Cleanup. To triplicate 5.0-g samples of ground carrot root or foliage was added either 50 μL of acetonitrile or 50 μL of acetonitrile containing 2.5 μg each of the 12 psoralens indicated in Figure 1. The amount of psoralens added to the fortified carrot samples was equivalent to 0.5 ppm of each psoralen. To each sample was added 15 mL of glass-distilled water and 10 mL of diethyl ether, and then the samples were homogenized (Willems Polytron, PT-10 generator) and centrifuged to facilitate separation of phases. The ether was drawn off, and each sample was extracted 3 additional times as before with 10-mL volumes of ether. The combined ether extracts from each sample were dried over anhydrous sodium sulfate and transferred to a boiling flask, and the solvent was removed with a rotary evaporator. The residue was dissolved in 5.0 mL of acetone, half (2.5 mL) was transferred to a conical centrifuge tube, and the solvent reduced to ~0.25 mL under a gentle stream of nitrogen. This sample was then applied as a band (~15 cm) near the bottom of a silica gel TLC plate (Brinkmann Silplate F-22, 20 × 20 cm, 0.25-mm gel thickness, with fluorescent indicator) and the plate was developed in a solvent system consisting of a mixture of hexane, ethyl acetate, and methanol, 5:5:1. Preliminary TLC studies with the 12 psoralen standards had shown that the solvent system used resolved these compounds into two "groups", the diols I and II at R_f ~0.23–0.34 and the remaining 10 psoralens at R_f ~0.42–0.65. These two gel areas were scraped from a given sample plate and combined, and the components were eluted with ether. The eluate was transferred to a boiling flask and the solvent removed by rotary evaporation. For final cleanup, the residue was dissolved in 1.2 mL of acetonitrile, 0.8 mL of distilled water was added,

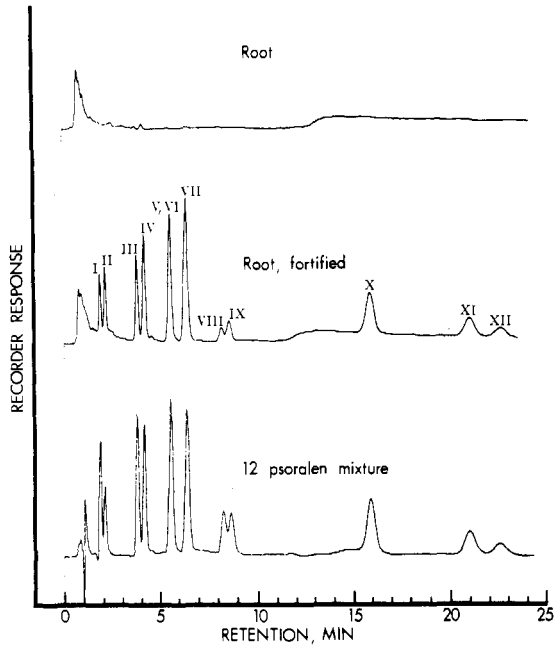


Figure 2. Reverse-phase HPLC analysis of carrot root extract for the presence of linear furocoumarins (psoralens). Top: whole carrot root. Middle: whole carrot root fortified prior to extraction with 12 psoralens at 0.5 ppm each. Bottom: mixture of 12 psoralen standards (50 ng each injected). See Figure 1 for structures of indicated compounds. Comparison of peak areas between the fortified carrot sample and the standard mixture is a direct indication of the recoveries obtained during extraction and cleanup of the fortified samples. See the text for analysis parameters.

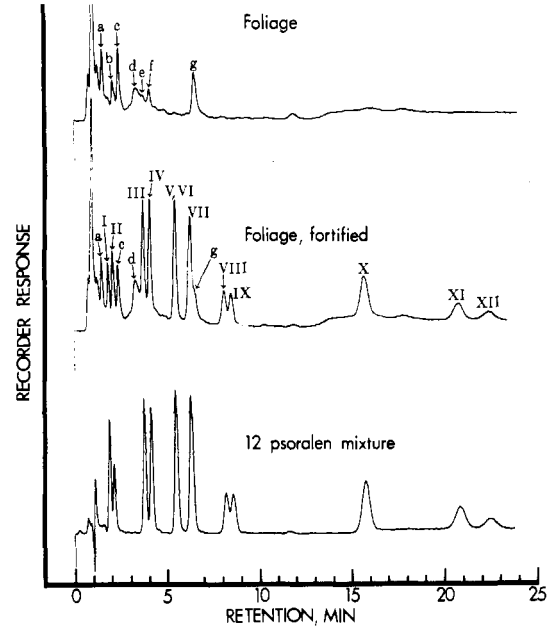


Figure 3. Reverse-phase HPLC analysis of carrot foliage extract for the presence of linear furocoumarins (psoralens). Top: carrot foliage. Middle: carrot foliage fortified prior to extraction with 12 psoralens at 0.5 ppm each. Bottom: mixture of 12 psoralen standards (50 ng each injected). See Figure 1 for structures of indicated compounds. Comparison of peak areas between the fortified carrot sample and the standard mixture is a direct indication of the recoveries obtained during extraction and cleanup of the fortified samples. See the text for analysis parameters. Components b, e, and f in the foliage extracts were chromatographically *not* the same as II, III, and IV, respectively, when examined by HPLC under different parameters (see the text).

this solution was applied to a Sep-PAK C_{18} cartridge (Waters, Milford, MA), and the eluate was collected. The cartridge was then flushed with 4.0 mL of acetonitrile–water (6:4 v/v), and the combined eluates were evaporated to dryness by rotary evaporation (anhydrous ethanol was added to facilitate removal of the water by azeotropic distillation). The residue was dissolved in 0.5 mL of the acetonitrile–water (6:4) mixture, filtered through a 0.45-μm Bioanalytical Systems MF-1 microfilter, and held for analysis.

High-Performance Liquid Chromatography. Psoralens were resolved by reverse-phase HPLC, using a Waters Model M-6000 pump and a Tracor Model 970A variable-wavelength detector set at 250 nm. Sample peaks were recorded and integrated with a Hewlett-Packard Model 3390A integrator. The reverse phase column was a Supelco LC-18, 15-cm length, preceded by a Whatman Co:Pell ODS guard column. The solvent system was acetonitrile–water (35:65) at a flow rate of 2.0 mL/min. Samples of the extracts (20 μL) were injected with a Rheodyne 7125 injector equipped with a 20-μL loop.

RESULTS

As indicated in Figures 2 and 3, HPLC of the 12 psoralens studied resolved the compounds into 11 distinct components—only bergapten and isopimpinellin were not resolved from each other. Data from analysis of carrot root and foliage samples fortified at 0.5 ppm with each of the psoralen standards showed that recoveries were generally good in each case (Figures 2 and 3). Recoveries of the 12 compounds averaged 72% in fortified carrot root samples and 87% in fortified foliage samples. With carrot root samples not fortified with the psoralen mixture (Figure 2), the cleanup procedure was such that essentially no detectable components were seen at retention times corresponding to any of the psoralen standards—clearly carrot root does not contain any of the psoralens studied, or if present, the compounds occur at exceedingly low levels ($\ll$0.5 ppm).

Cleanup of carrot foilage extracts was somewhat less effective in removing detectable components eluting in the range of the added psoralen standards, and the initial analyses (Figure 3) were not definitive in ruling out the presence of oxypeucedanin hydrate (II), psoralen (III), and xanthotoxin (IV). However, additional HPLC studies utilizing coinjection of samples and standards with a solvent mixture of acetonitrile–water (3:7) demonstrated that, in fact, these three psoralens are not present in detectable quantities in carrot foliage. Under these conditions, component b in foliage extracts (Figure 3) eluted significantly earlier than II (2.8 vs. 3.1 min). Similarly, when eluted with acetonitrile–water (3:7) at a flow rate of 1.0 mL/min, no significant peaks in the d–e complex (Figure 3) corresponded with psoralen, and peak f eluted significantly earlier than did xanthotoxin (11.6 vs. 12.0 min). Thus, it is clear that carrot foilage likewise does not contain any of the 12 psoralens studied in detectable quantities.

Peak g in the carrot foliage extracts (Figure 3) appeared to be a relatively significant and homogeneous component, and we considered the possibility that it might be a substituted psoralen. However, HPLC isolation of the compound in near microgram amounts, followed by direct insertion probe electron impact mass spectrometry (Ivie, 1978a), showed that the compound did not exhibit fragmentation patterns consistent with its assignment as a linear furocoumarin.

DISCUSSION

The current studies with carrot represent the most definitive efforts yet undertaken in the analysis of plants

for trace levels of psoralens. We have shown that root and foliage samples of the carrot variety studied here contain no or, at most, very low (≪0.5 ppm) quantities of any of a number of psoralens, including several of simple biogenetic complexity. Thus, our studies suggest that carrot, unlike many related plants (including some edible vegetables) does not possess biosynthetic pathways for the production of linear furocoumarins.

Psoralens are highly biologically active compounds with much medicinal value. As a result of their biochemical modes of action, particularly their photomutagenic properties which may lead to photocarcinogenicity and possibly other detrimental effects in man, it seems prudent to carefully evaluate the potential for exposure of man to psoralens through foodstuffs. The procedures described in this report for the analysis of trace levels of psoralens in plants should prove useful in such investigations.

LITERATURE CITED

Ashwood-Smith, M.; Poulton, G.; Barker M.; Mildenberger, M. *Nature (London)* **1980**, *285*, 407.
Berenbaum, M.; Feeny, P. *Science (Washington, D.C.)* **1981**, *212*, 927.
Fahmy, I. *J. Pharm. Pharmacol.* **1956**, *8*, 653.
Ivie, G. *J. Agric. Food Chem.* **1978a**, *26*, 1394.
Ivie, G. In "Effects of Poisonous Plants on Livestock"; Keeler, R.; Van Kampen, K.; James, L., Eds.; Academic Press: New York, 1978b; p 475.
Ivie, G.; Holt, D.; Ivey, M. *Science (Washington, D.C.)* **1981**, *213*, 909.
Musajo, L.; Caporale, G.; Rodighiero, G. *Gazz. Chim. Ital.* **1954**, *84*, 870.
Pathak, M.; Daniels, F., Jr.; Fitzpatrick, T. *J. Invest. Dermatol.* **1962**, *39*, 225.
Reed, B. *Acta Derm.-Venereol.* **1976**, *56*, 315.
Scheel, L.; Perone, V.; Larkin, R.; Kupel, R. *Biochemistry* **1963**, *2*, 1127.
Scott, B.; Pathak, M.; Mohn G. *Mutat. Res.* **1976**, *39*, 29.
Steck, W. *Can. J. Chem.* **1967**, *47*, 3577.
Stern, R.; Thibodeau, L.; Kleinerman, R.; Parrish, J.; Fitzpatrick, T. *N. Engl. J. Med.* **1979**, *300*, 809.

Received for review August 24, 1981. Accepted February 5, 1982. This paper reports the results of research only. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

A Sensitive Colorimetric Procedure for Nitrogen Determination in Micro-Kjeldahl Digests

Charles Nkonge and G. Murray Ballance*

The need for a sensitive and accurate method for ammonia estimation in micro-Kjeldahl digests led to the reexamination of the optimum conditions of a colorimetric assay based on the Berthelot reaction. The specific procedure involves the reaction of salicylate and hypochlorite with ammonia. A minimum 40% improvement in sensitivity over other colorimetric procedures was achieved by optimization of reagent concentrations, temperature, pH, and incubation time. Comparison of values obtained by this procedure and by titration is in excellent agreement. A scaled-down procedure agreed well with the standard procedure.

The aim of our work was to measure lysine, tryptophan, and protein from single distal half-seeds. Such measurements should allow identification (without destruction) of genotypes with desirable protein quality characteristics. The main difficulty in achieving this goal was finding or developing assays which were sufficiently sensitive to allow the three analyses to be carried out on single half-kernels of common cereals.

For estimation of protein in cereals, the Kjeldahl method has been widely used. This is due to (1) the ability of the Kjeldahl method to quantitate nitrogen from either soluble or insoluble samples, (2) the nitrogen from cereal samples being largely derived from protein, and (3) the amino acid composition of endosperm protein being sufficiently constant so as to have a relatively fixed nitrogen to protein ratio within a given cereal. Thus the protein can be estimated from the nitrogen value.

The classical Kjeldahl procedure involves two steps: (1) digestion of the sample in concentrated acid and (2) distillation and titration of the liberated nitrogen as ammonia. The second step requires special equipment for distillation, and where large numbers of samples are to be analyzed, this step is relatively slow. Thus, alternative methods for estimation of liberated ammonia directly from the digest have been examined. These include spectrophotometric estimation of volatilized ammonia (Muroski and Syty, 1980), colorimetric methods (Wall and Gehrke, 1975; Mitcheson and Stowell, 1970), and quantitation using an ammonia specific electrode (Eastin, 1976).

A colorimetric method based on the Berthelot reaction was chosen because of its sensitivity, the availability of equipment, and the potential to automate the method at a later date. Various combinations of different phenol reagents and sources of available chlorine have been tested in the Berthelot reaction of ammonia estimation (Patton and Crouch, 1977; Yamaguchi et al., 1970; Reardon et al., 1966). Because of conflicting reports in the literature regarding optimum conditions, we have reexamined many of the parameters not only to obtain the optimized conditions but also to determine how a change in any one parameter affects the overall sensitivity of the reaction.

Our nitrogen assay procedure was developed to be used in conjunction with two other assays, so that a single half-kernel would provide adequate sample for all three analyses. Thus, the need for sensitivity in the nitrogen assay lies (1) in the desire to use the minimum amount of sample for digestion and (2) the requirement that the ammonia can be accurately quantitated after the digest

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.

0021-8561/82/1430-0416$01.25/0