Journal of Chromatography, 161 (1978) 271–278

CHROM. 11,191

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF THE NATURALLY OCCURRING TOXICANTS MYRISTICIN, RELATED AROMATIC ETHERS AND FALCARINOL*

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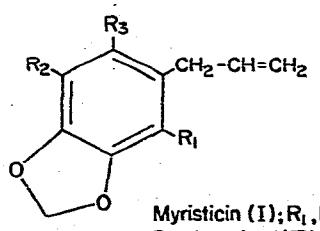
(First received February 13th, 1978; revised manuscript received May 16th, 1978)

SUMMARY

The naturally occurring toxicants myristicin, twelve related aromatic ethers and the toxic acetylenic alcohol, falcarinol, were separated from one another by high-pressure liquid chromatography (HPLC). The technique employed a microparticulate nitrile phase column and used heptane and tetrahydrofuran as the eluting solvents. Preparative HPLC with 5-μm silica allowed isolation of gram quantities of parsleyapiole and dillapiole from extracts of plain parsley seeds and dill seeds, respectively. Commercially available myristicin as well as other aromatic ethers were also purified in gram quantities with the preparative column.

INTRODUCTION

The natural occurrence of myristicin (I) and related aromatic ethers in foods has recently received attention because of physiological responses induced by these compounds. Myristicin (I) is recognized as the major hallucinogenic principle of nutmeg and mace[1] and may be a strong teratogen[2]. Other aromatic ethers such as



Myristicin (I); $R_1, R_3 = H, R_2 = OCH_3$
Parsleyapiole (II); $R_1, R_2 = OCH_3, R_3 = H$
Dillapiole (III); $R_1 = H, R_2, R_3 = OCH_3$
Safrole (IV); $R_1, R_2, R_3 = H$

* Scientific Paper No. 5029, College of Agriculture Research Center, Pullman, Wash., U.S.A., Project No. 4334.

parsleyapiole (II) and dillapiole (III) are also hallucinogenic[3] and safrole (IV) is metabolized to the hepatocarcinogen 1′-hydroxysafrole[4]. Myristicin is present in large quantities in nutmeg[5] and in the seeds of several plants in the *Umbelliferae*[6] including those of dill, parsley, fennel and parsnip. Harborne *et al.*[6] cite records of the occurrence of myristicin in the leaves of parsley, dill, celery and fennel and in the roots of parsnip. Myristicin is also a constituent of the carrot[7]. Other aromatic ethers present in the *Umbelliferae*[6] are dillapiole (III) in dill seeds and parsleyapiole (II) from parsley seeds and leaves.

The usual methods for the detection of myristicin and related compounds have been thin-layer chromatography[6,8] and gas chromatography[5,7]. The objectives of this study were to develop a high-pressure liquid chromatographic (HPLC) method for the separation of myristicin, related compounds and the toxic acetylenic alcohol, falcarinol, a constituent of carrots[7,9], and apply the method to the separation of such compounds in carrots.

EXPERIMENTAL

High-pressure liquid chromatography

The equipment used for HPLC consisted of two Waters Assoc. Model 6000A pumps, a Waters Assoc. Model 660 solvent programmer and U6K injector. Detection was with a Micromeritics Model 780 variable wavelength detector operated at 280 nm. Peak areas and retention times were measured with a Spectra Physics Minigrator. Analytical HPLC was performed on a 25 cm × 4.6 mm I.D. nitrile phase column, Partisil PAC (Whatman, Clifton, N.J., U.S.A.). Preparative HPLC was done on Partisil 5 silica (Whatman) which was packed as a slurry in carbon tetrachloride[10] into a 25 cm × 9.4 mm I.D. stainless-steel column (Whatman). The column was packed at 7000–8000 p.s.i.g. using an extended tube apparatus similar to that of Cassidy and LeGay[11] and Coq *et al.*[12].

Purification of solvents for HPLC

The non-volatile impurities in the solvents heptane, tetrahydrofuran, ethyl acetate and 1,2-dichloroethane were removed by distillation with a rotary evaporator. The heptane and tetrahydrofuran (THF) were further treated in order to reduce their ultraviolet (UV) absorbance so that the baseline drift during a gradient was minimal. The heptane was passed through 4 × 60 cm columns of 20–40 mesh activated silica and redistilled. The THF was passed through a 3 × 10 cm column of activated carbon, the first portion of eluent discarded because of high UV absorbance, and the remainder was distilled from a few lumps of sodium borohydride. In this manner, the UV absorbance of the heptane and THF was reduced to 0.1 and 0.4, respectively, at 220 nm against distilled water. The heptane eluent from both the preparative and analytical HPLC columns was reused as many as four times after extensive washing with water, distillation, passage through the silica columns and redistillation.

Conditions for HPLC separation

The separation of the aromatic ethers was investigated using heptane as the A solvent and mixtures of heptane with THF, 1,2-dichloroethane or ethyl acetate as the secondary or B solvent. The B solvents were 5% THF in heptane, 60% 1,2-

dichloroethane in heptane and 5% ethyl acetate in heptane. A mixture of 95% solvent A and 5% solvent B at 3.0 ml/min was used for initial elution. The linear solvent gradient (curve 6 of the Model 660 solvent programmer) to 95% of solvent B was started 5 min after injection. The solvent gradient was 45 min with the THF mixture, 25 min with the 1,2-dichloroethane mixture and 30 min with the ethyl acetate mixture.

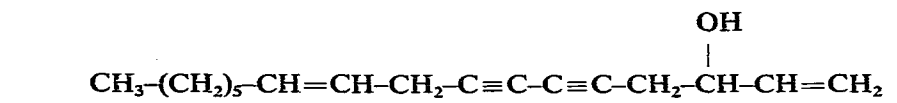
An accelerated gradient was used for analyzing carrot volatiles; solvent A was again heptane, but solvent B was 7% THF in heptane. The initial conditions of 8% B at 4.0 ml/min were held for 5 min and the linear gradient was to 80% B in 10 min. The detector was set at 260 nm.

Carrots

All carrots used were purchased from a local supermarket and were of the Imperator variety.

Chemicals

Myristicin was obtained from Saber Labs. (Morton Grove, Ill., U.S.A.). Isomyristicin was prepared by boiling 500 mg myristicin for 30 min in 10 ml of 5% KOH in ethanol, neutralizing with HCl, adding 5 ml of water and extracting twice with 20 ml of heptane. Asarone (2,4,5-trimethoxypropenylbenzene) was purchased from Sigma (St. Louis, Mo., U.S.A.). Safrole, methyl eugenol, methyl isoeugenol, eugenol and isoeugenol were products of Pfaltz and Bauer (Flushing, N.Y., U.S.A.). The compound, 2-methoxynapthalene was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Crude samples of elemicin (3,4,5-trimethoxyallylbenzene) and isoelemicin (3,4,5-trimethoxypropenylbenzene) were supplied by the Food and Drug Administration (Beltsville, Md., U.S.A.). Falcarinol, believed to be



based on work by Crosby and Aharonson[9], was isolated from carrots by Dr. Ron G. Butterey of the Western Regional Research Laboratory (USDA, Berkeley, Calif., U.S.A.). This was supplied to us as either a pure compound or a crude preparation.

Dr. Butterey established the identity of this isolate as falcarinol by nuclear magnetic resonance (NMR), infrared (IR) and UV spectra all of which compared excellently with the spectra originally established for falcarinol by Crosby and Aharonson[9].

Gas–liquid chromatography (GLC)

A Varian Aerograph, Model 1740 dual column gas chromatograph equipped with flame ionization detectors was used for GLC. Glass columns (10 ft. × 2 mm I.D.) were packed with 7% Carbowax 20M on Gas-Chrom Q (80–100 mesh). The injector and detector temperatures were 250° and the column temperature was 220°. The flow-rate of the carrier gas (nitrogen) was 36 ml/min. Approximate retention times were safrole, 3.5 min; methyl eugenol, 4.5 min; methyl isoeugenol, 7.1 min; elemicin, 8.0 min; myristicin, 9.5 min; isoelemicin, 13.1 min; and asarone 15.5 min.

Thin-layer chromatography (TLC)

TLC of myristicin and related aromatic ethers was on 400-μm layers of silica gel G developed in a tank saturated with benzene[6,8]. The dried plate was sprayed with 1% vanillin in 1% ethanolic H_2SO_4 and heated 5 min at 120° (refs. 6 and 8). Markers were myristicin (brown, R_F 37), safrole (brown, R_F 53), eugenol (brown, R_F 18), asarone (purple, R_F 8) and thymol (red, R_F 22).

TLC of falcarinol on silica gel G utilized hexane–acetone (2.5:1) for development and a spray of 1% $KMnO_4$ in aqueous 2% Na_2CO_3 for detection[9]. Falcarinol gave an immediate yellow spot at R_F 45. The falcarinol purified by Dr. Butterey was run as a marker.

Spectral measurements

UV spectra were recorded with a Beckman DU equipped with Gilford accessories. The spectra of myristicin, parsleyapiole and dillapiole were measured in methanol while that of falcarinol was measured in heptane.

NMR spectra were recorded in CCl_4 with a Varian T-60 spectrophotometer.

Mass spectra were determined on a Hitachi Model RMU-6H mass spectrometer coupled by a Beaman separator to an Aerograph Model 600 gas chromatograph equipped with a flame ionization detector, splitter and an Aerograph Model 328 temperature controller. The GC column was 5 ft. × $\frac{1}{8}$ in. stainless-steel packed with 5% OV-1 on Gas-Chrom Q (80–100 mesh). The column temperature was 180°, detector temperature 230° and injector temperature 220°. Myristicin eluted at 3 min.

Preparation of carrot samples. Steam distillation

Carrots were steam distilled using the extraction head of Likens and Nickerson[13]. External steam was used in the steam distillation by using a Bunsen burner to boil water which was contained in a 1-l reagent bottle. Steam was delivered from the reagent bottle to the distillation pot (a three-neck, 1-l round bottom flask) with the appropriate ground glass tubes. The distillation pot and solvent reservoir of the Likens and Nickerson head were heated by heating mantles.

Carrots were prepared by maceration of 100 g of carrots with 100 ml of water and 4.275 g of sodium bisulphite in a blender for 10 sec. A 1-ml volume of a solution of internal standard (2-methoxynaphthalene, 20 μg/ml in 95% ethanol) was added and blended for an additional 60 sec. The puree was transferred to the distillation pot with not more than 150 ml of water and a drop of Dow Corning Antifoam A was added. A 600-ml volume of water was used in the external steam generator and 40 ml of redistilled hexane containing 100 ppm butylated hydroxytoluene were used as an antioxidant in the extraction head.

Distillation was stopped when 500 ml of water had been boiled from the external steam generator. The hexane solution was evaporated to near dryness on a rotary evaporator and made to 5 ml with heptane. For HPLC, 25 μl was injected.

Identification of myristicin and falcarinol in carrots

The hexane extract of the steam distillate from 100 g of carrots was reduced to less than 1 ml and the entire sample separated on Partisil PAC with the accelerated gradient. The peaks with the elution times of myristicin and falcarinol were collected and concentrated. The myristicin peak was analyzed by GLC and TLC and its UV

spectrum measured. The falcarinol peak was subjected to TLC and its UV spectrum measured.

Preparative HPLC

Preparative HPLC was done with the 9.4 mm I.D. Partisil 5 silica column at a solvent flow-rate of 8.0 ml/min. Myristicin, dillapiole and parsleyapiole were purified using a solvent composition of 1% ethyl acetate in heptane. The dillapiole and parsleyapiole were extracted from dill seeds and plain parsley seeds, respectively. Extraction of the apioles was performed by passing heptane–acetone (3:1) through a 30 × 3 cm column of seeds ground in a Wiley Mill to pass a 20-mesh screen. The concentrated extracts were vacuum distilled prior to preparative HPLC to remove the apioles from non-volatile materials which had been coextracted from the seeds. The distillation was stopped at 120° and 500 μmHg and the whole volatile fraction was used for preparative HPLC. Parsleyapiole was difficult to isolate in quantity by preparative HPLC because the parsley seeds contained huge amounts of myristicin. Therefore, the parsley seed extract was applied to a 3.5 × 30 cm column of TLC grade silica gel G and eluted with 10% chloroform in heptane. Fractions of 50 ml were collected and analyzed by HPLC. Fractions 7–13 were mostly parsleyapiole and therefore combined. Fractions 3–6 were rerun over the silica gel G column for further recovery of parsleyapiole. The appropriate fractions were combined with fractions 7–13 and concentrated.

Falcarinol was isolated from a steam distillate from 100 lbs. of carrots supplied to us by Dr. Butterey. Before preparative HPLC, low boiling volatiles were removed by vacuum distillation at 120° and 300 μmHg. The dark brown residue was dissolved in heptane and subjected to preparative HPLC using 6% ethyl acetate in heptane.

The falcarinol isolated from the Partisil 5 column was further purified using the Partisil PAC column, 7% THF in heptane at 3 ml/min and recycling. Elemicin, isoelemicin and methyl isoeugenol were purified on the Partisil 5 silica column with 3% ethyl acetate in heptane.

The purity of the compounds isolated by preparative HPLC was determined on the Partisil PAC column using 7% THF as the B solvent. The purity of the isolated compounds could only be assigned on an area percent basis. The actual purity would depend on the molar extinction coefficients of the compounds responsible for the HPLC peaks and whether any non-UV absorbing material was isolated with the desired components during preparative HPLC.

RESULTS AND DISCUSSION

Analytical HPLC separation

The HPLC separation of myristicin and twelve related aromatic ethers using 5% THF as the B solvent is illustrated in Fig. 1. The acetylenic alcohol, falcarinol, eluted under these conditions at 33 min.

All compounds with a propenyl-type side chain other than asarone and anethole (*e.g.*, isosafrole, isomyristicin, methyl isoeugenol, isoelemicin and isoeugenol) exhibited two peaks on the Partisil PAC column, irrespective of the B solvent. In each case, the larger, more retained peak was assumed to be the more stable *trans*-isomer. The small *cis*-isomer peaks were unresolved from the related compound with the allyl

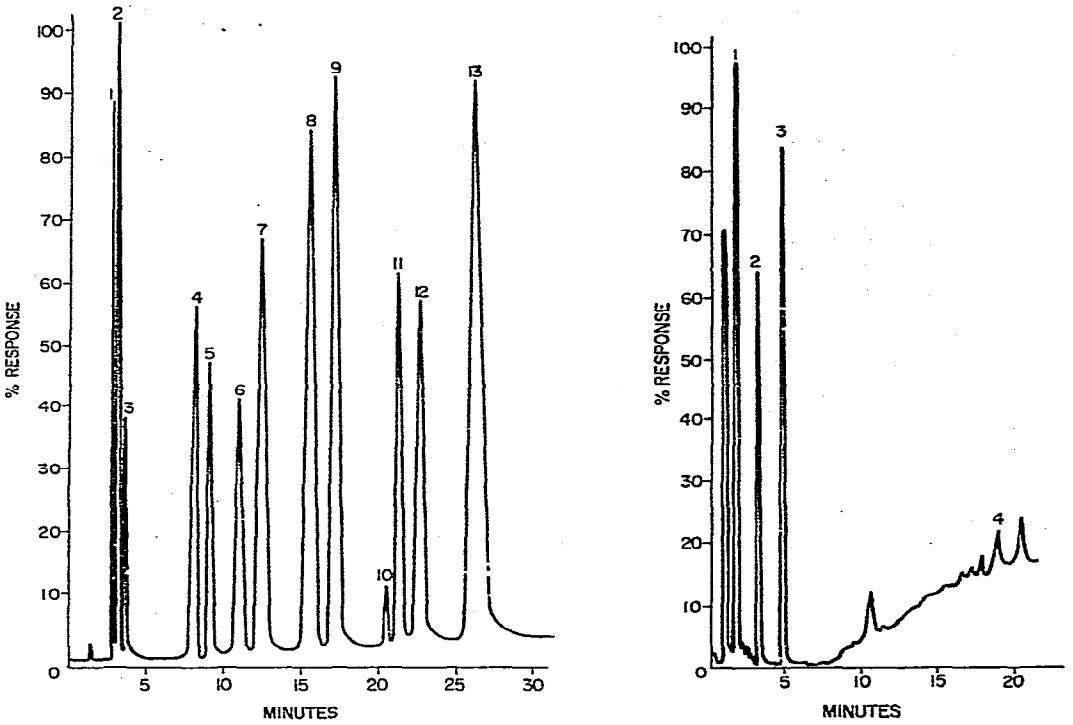


Fig. 1. Separation of myristicin and twelve structurally related compounds by HPLC on Partisil PAC. 1 = Safrole; 2 = *trans*-isosafrole; 3 = anethole; 4 = myristicin; 5 = *trans*-isomyristicin; 6 = dillapiole; 7 = parsleyapiole; 8 = methyl eugenol; 9 = *trans*-methyl isoeugenol; 10 = *cis*-isoelemicin; 11 = elemicin; 12 = *trans*-isoelemicin; 13 = asarone.

Fig. 2. Separation of a steam distillate of carrots by HPLC on Partisil PAC. 1 = Butylated hydroxytoluene; 2 = 2-methoxynaphthalene; 3 = myristicin; 4 = falcarinol.

side chain. In other words, *cis*-isosafrole and safrole were unresolved as were *cis*-methyl isoeugenol and methyl eugenol.

The elution times and peak shapes of eugenol and the two isomers of isoeugenol were quite variable and believed to be controlled by the amount of water absorbed onto the column. Flushing the column with anhydrous ethanol followed by anhydrous ethyl acetate (both dried with silica and stored over molecular sieves) and elution with 5% THF as the B solvent resulted in symmetrical peaks with eugenol eluting just beyond *trans*-isoelemicin and both isoeugenol isomers eluting beyond asarone. After extended use, however, the back sides of the peaks of compounds with a phenolic grouping became badly skewed and eugenol and *trans*-isoelemicin became inseparable as did *cis*-isoeugenol and asarone.

The 1,2-dichloroethane-heptane mixture did not separate eugenol, *trans*-methyl isoeugenol and *cis*-isoeugenol. Also, elemicin and both isomers of isoelemicin eluted after asarone, but otherwise the elution order with 1,2-dichloroethane in the eluent was the same as with THF–heptane. With ethyl acetate–heptane, *cis*-isoelemicin, elemicin, *trans*-isoelemicin and asarone were just resolved in that order but eugenol

eluted with *cis*-isoelemicin, *cis*-isoeugenol with *trans*-isoelemicin and *trans*-isoeugenol was only partially resolved from asarone. Excessive baseline drift was encountered during gradient elution with the ethyl acetate solvent.

Preparative HPLC

Myristicin from Saber Labs. was a light brown oil which by HPLC was 97 area percent pure and contained methyl eugenol and elemicin as impurities. Myristicin was purified to a colorless oil of 99.8 area percent purity by preparative HPLC. Four injections of 1.5 ml were required to purify 1 g of myristicin.

Falcarinol eluted in 11 min from the Partisil silica column and was 90 area percent pure with one major contaminant eluting just beyond falcarinol and two minor components eluting just before falcarinol. After purification with the Partisil PAC column, a colorless oil of 99 area percent purity was obtained. The material isolated had an HPLC retention time and UV spectrum identical to that of the pure falcarinol obtained from Butterey.

The purified elemicin contained 2.6 area percent isoelemicin. Isoelemicin and methyl isoeugenol both contained their *cis* and *trans* forms with the *cis*-isomers amounting to approximately 13 area percent in both compounds. The *trans*-isomers of both methyl isoeugenol and isoelemicin were purified by recycling until the *cis*-isomer was reduced to less than 0.7 area percent for isoelemicin and 0.2 area percent for methyl isoeugenol.

Identification of parsleyapiole and dillapiole

The materials isolated by preparative HPLC from parsley seeds and dill seeds were identified as parsleyapiole and dillapiole, respectively. Both compounds gave the expected brown spot at an R_F above that of thymol[6] at R_F 26. NMR spectra of both compounds were nearly identical to the data of Lichtenstein *et al.*[14]. Parsleyapiole exhibited two methoxy groups (δ 3.77, 3.81), a methylene dioxy function (δ 5.83), one aromatic proton (δ 6.17) and the allyl side chain resonances (δ 3.15, 3.26, 4.83, 5.07, 5.85).

The spectrum of dillapiole was almost identical to that of parsleyapiole with the exception of the shifts of the protons of the methoxy groups as also noted by Lichtenstein *et al.*[14]. The resonances of the two methoxy groups in dillapiole were at δ 3.70 and 3.93. Several peaks were present in the NMR spectrum below δ 1.5 in both compounds, but this was assigned to incomplete evaporation of heptane from the samples and/or contamination from solvent residues.

The mass spectra of the isolated compounds gave ions and intensities nearly identical to the data of Lichtenstein *et al.*[14] for parsleyapiole and dillapiole. The M^+ ion occurred at *m/e* 222 with both compounds and significant ions (>10% of the M^+ intensity) occurred at *m/e* 223, 207, 177, 149 and 121).

The UV maxima of parsleyapiole and dillapiole were 276 and 280 nm, respectively. These values are both 3 nm smaller than the maxima obtained by Lichtenstein *et al.* Myristicin, whether obtained from Saber Labs. or isolated by us from carrots, also exhibited a UV maximum 3 nm less than the maximum reported by Lichtenstein *et al.*

Approximately 2 g of parsleyapiole were isolated which analyzed at 99.2 area percent purity. The dillapiole contained some parsleyapiole as a contaminant which

could not be removed with the Partisil 5 silica column. The parsleyapiole content of the purified dillapiole amounted to 8 area percent.

Identification of myristicin and falcarinol in carrots

Fig. 2 illustrates the HPLC separation of a steam distillate of carrots with the accelerated gradient. Peaks 3 and 4 in Fig. 2 were identified, respectively, as myristicin and falcarinol by chromatographic and spectral means. Peak 3 had the same retention time by GLC and HPLC as myristicin obtained from Saber Labs. TLC of peak 3 yielded one spot with the same coloration and R_F as myristicin run as a marker. The UV spectra of peak 3 and myristicin were identical with a maximum at 274 nm (ref. 14).

Peak 4 had the same retention time by HPLC as falcarinol and exhibited the same R_F and coloration by TLC as falcarinol run as a marker. The UV spectra of peak 4 and falcarinol were identical with maxima at 231, 243 and 257 nm (ref. 9).

Another paper will be published which will examine the efficiency of steam distillation for recovering myristicin and falcarinol from carrots and will report the quantities of these two compounds in different varieties of carrots.

ACKNOWLEDGEMENT

This research was supported in part by Research Contract No. 223-76-2174 from the Food and Drug Administration, Beltsville, Md., U.S.A.

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