Identification and Quantification of Coumarin, Phthalide, and Sesquiterpene Compliance Markers in an Umbelliferous Vegetable Beverage

David S. Weinberg,* M. Lisa Manier, Mark D. Richardson, and Frederick G. Haibach

Analytical and Physical Chemistry Research Department, Southern Research Institute, Birmingham, Alabama 35205

Marker compounds are needed to determine dietary compliance in free-living human study populations participating in dietary intervention trials for cancer research. Three coumarin, two phthalide, and two sesquiterpene marker compounds were detected and identified in an umbelliferous vegetable beverage. A convenient method that involves solvent extraction and gas chromatographic/mass spectrometric analysis was developed to quantify the marker compounds in the umbelliferous vegetable beverage. The compounds and their concentrations are as follows: 5-methoxypsoralen (86.2 ng/g), 8-methoxypsoralen (194 ng/g), 5,8-dimethoxypsoralen (240 ng/g), butylidene phthalide (15.8 ng/g), 3-*n*-butyl phthalide (780 ng/g); β-caryophyllene (1560 ng/g), and α-humulene (146 ng/g).

INTRODUCTION

Southern Research Institute (SRI) undertook the identification and quantification of three coumarin, two phthalide, and two sesquiterpene marker compounds present in an anticarcinogenic umbelliferous vegetable beverage on a contract with the National Cancer Institute. [The function of marker compounds is discussed in the first paper in this series (Weinberg et al., 1993).] Coumarins occur in well over 100 plant families. It is not uncommon in some of these families, most notably the Umbelliferae, Guttiferae, and Rutaceae, to encounter species that elaborate 10, 20, or even more coumarins, and many species elaborate 4 or 5 coumarins (Thompson et al., 1984). Coumarins commonly found in umbelliferous vegetables include bergapten (Erdelmeier et al., 1985; Vo-Dinh et al., 1988; Glowniak et al., 1986; Spencer et al., 1987), xanthotoxin (Vo-Dinh et al., 1988; Glowniak et al., 1986; Spencer et al., 1987), isopimpinellin (Erdelmeier et al., 1985; Vo-Dinh et al., 1988; Glowniak, et al., 1986; Spencer et al., 1987), umbelliferone (Anderson et al., 1983), and psoralen (Beier et al., 1983; Erdelmeier et al., 1985; Spencer et al., 1987; Vo-Dinh et al., 1988). Phthalides found in umbelliferous vegetables include butylidene phthalide (Bohrmann et al., 1967; Gijbels et al., 1982), 3-*n*-butyl phthalide (Bohrmann et al., 1967; Wilson, 1970; Lund, 1978; Gijbels et al., 1982), sedanenolide (Wilson, 1970; Lund, 1978), senkyunolide (Gijbels et al., 1982), and 3-*n*-butyl hexahydrophthalide (Wilson, 1970). Sesquiterpenes such as β-caryophyllene and α-humulene commonly occur in umbelliferous vegetables.

MATERIALS, INSTRUMENTATION, AND METHODS

Materials. *Reagents.* Acetone and methylene chloride were obtained from Burdick and Jackson (McGaw Park, IL). Both were high-purity solvents. Pentane (GC^2 grade) was also obtained from Burdick and Jackson. Ethyl acetate (Nanograde) and potassium carbonate (AR grade) were obtained from Mallinckrodt Inc. (Paris, KY). Acetone-d_6 (minimum isotopic purity 96.96 atom %) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Phenylthiourea (BDA microanalytical reagent) was obtained from British Drug Houses Ltd. (Poole, England). Deionized water was prepared at the Institute using a Millipore Milli-Q water purification system.

Sample. Carrot juice concentrate, celery juice concentrate, and an umbelliferous vegetable beverage were provided to SRI by the National Cancer Institute. The mixture designated "umbelliferous vegetable beverage" by the National Cancer Institute was originally developed at the University of Arkansas and is composed of celery juice concentrate (10%), carrot juice concentrate (15%), a spice mix (35%), and tomato juice (40%).

Components. 5-Methoxypsoralen (4-methoxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one, bergapten, heraclin, majudin, 5-MOP, Psoraderm) and *8-methoxypsoralen* (9-methoxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one, 6-hydroxy-7-methoxy-5-benzofuranacrylic acid δ-lactone, 8-methoxy-4′,5′:6,7-furocoumarin, 8-methoxy[furano-3′,2′:6,7-coumarin], xanthotoxin, 9-methoxypsoralen, methoxsalen, ammoidin, 8-MOP) were obtained from Aldrich. The purity of each compound was listed as 99%. *5,8-Dimethoxypsoralen* (isopimpinellin; 5,8-MOP) was obtained from Indofine Chemical Co., Inc. (Somerville, NJ). The purity was not listed. *β-Caryophyllene* (*trans*-caryophyllene) and *α-humulene* were obtained from Fluka Chemical Corp. (Ronkonkoma, NY). The purity of each was listed as greater than 98%. *Butylidene phthalide* was obtained from Penta International Corp. (Caldwell, NJ). The purity was listed as 99%. Gas chromatographic/mass spectrometric analysis (GC/MS) showed one major gas chromatographic peak, and the mass spectrum of the component producing the peak exhibited the expected parent and fragment ions. The psoralens are described as light-sensitive and were stored at −24 °C in a freezer. The other compounds were stored at 2–6 °C in a refrigerator.

3-n-Butyl phthalide was obtained as a solution in methyl *tert*-butyl ether from Dr. Eric Lund of the South Atlantic Area Citrus and Subtropical Products Laboratory, Agricultural Research Laboratory, USDA (Winterhaven, FL). GC/MS showed one minor peak and one major peak, and the mass spectrum of the component producing the major peak showed the expected parent and fragment peaks. The solution of methyl *tert*-butyl ether containing 3-*n*-butyl phthalide was quantitatively transferred using acetone to a 10-mL pear-shaped flask. The solution was rotary evaporated to dryness using gentle heating and a water aspirator vacuum. The residue was dissolved in 1.00 mL of acetone-d_6 containing 0.0209 mmol of ethyl acetate. The molar ratio of 3-*n*-butyl phthalide and ethyl acetate was determined by proton NMR spectroscopy. The concentration of 3-*n*-butyl phthalide was then calculated to be 1.96 mg/mL of acetone-d_6 solution. (The concentration of ethyl acetate in acetone-d_6 was originally determined by NMR spectroscopy using phenylthiourea as a primary standard.)

Surrogates and Internal Standards. 4-Chlorobenzophenone, methyl 4-chlorobenzoate, and 1-chlorodecane were obtained from Aldrich. The purity listed was 98 or 99%. GC/MS showed one major peak, and the mass spectrum of the component produced the expected parent and fragment ions. Phenanthrene-d_{10} (98

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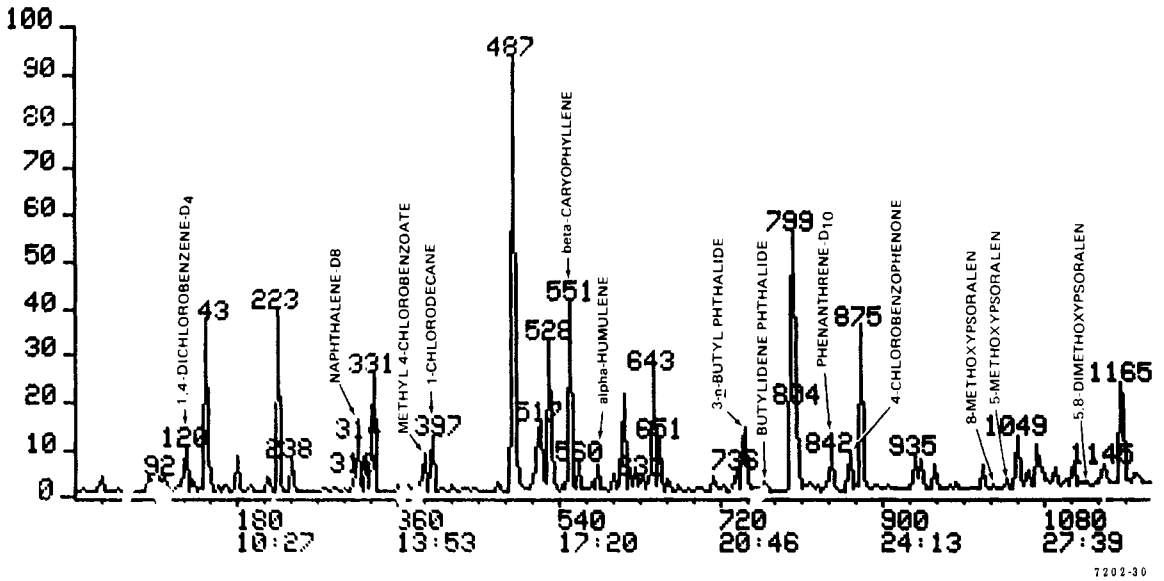

Figure 1. Total-ion chromatogram of extract of umbelliferous vegetable beverage containing surrogates and internal standards.

atom % D) was obtained from Cambridge Isotope Laboratories (Woburn, MA). 1,4-Dichlorobenzene-d_4 (99 atom % D, Cambridge Isotope Laboratories) and naphthalene-d_8 (98+ atom % D, Aldrich) were purchased and spiked into various samples (refer to Figure 1, for example). They were not used for calculations because phenanthracene-d_{10} was used instead as an internal standard.

Instrumentation. A Hewlett-Packard Co. (Atlanta, GA) Model 5890 gas chromatograph was coupled to a VG 70S high-resolution mass spectrometer by means of a direct inlet for capillary column gas chromatography. A Nicolet Co. (now General Electric NMR Instruments, Fremont, CA) NT 300B nuclear magnetic resonance spectrometer operated at 300.635 MHZ for observing protons. Data were stored on a magnetic disk, and hardcopy was produced using a Nicolet Zeta 8 digital printer.

Methods. *Large-Scale Extraction of Celery and Carrot Juice for the Identification of Components.* Celery or carrot juice concentrate (60.1 and 60.6 g, respectively) was shaken with 60 mL of deionized water and 60 mL of methylene chloride. The bulk of the methylene chloride phase was removed, and the process was repeated using first 60 and then 30 mL of methylene chloride. The methylene chloride extracts were combined and concentrated to 1 mL in a Kuderna-Danish apparatus.

Gas Chromatographic/Mass Spectrometric Analyses. A 25-m, 0.32-mm-i.d., HP-5 gas chromatographic capillary column coated with a 0.52-μm bonded-phase film of methyl silicone was used for quantitative analyses. The column was maintained at 45 °C for 3 min and then heated to 300 °C at 8 °C/min. The final temperature was held for up to 30 min. The solvent delay was 7 min. Splitless injections were made. The injection port was maintained at 275 °C, and the flow of helium carrier gas was maintained by a head pressure of 4.2 psig. One microliter of sample was injected. (In preliminary studies, similar 10- or 60-m columns were used.) The mass spectrometer transfer line was operated at 300 °C, the ion source was operated at 260 °C, and the electron voltage was 70 eV. The magnet was scanned from mass 300 to 35 at 1 s/decade and with a rest time of 0.5 s. The nominal resolution was 1000. The electron multiplier was set to the highest value that was practical to use.

Preparation of Standards. The surrogate solution contained the following components in ethyl acetate: methyl 4-chlorobenzoate (39.52 ng/μL), 1-chlorodecane (42.56 ng/μL), and 4-chlorobenzophenone (40.40 ng/μL). The internal standard solution contained phenanthrene-d_{10} (40.56 ng/μL) in ethyl acetate. An instrument calibration solution that we prepared in ethyl acetate contained β-caryophyllene (20000 ng/mL), α-humulene (2060 ng/mL), 3-*n*-butyl phthalide (7800 ng/mL), butylidene phthalide (606 ng/mL), 5-methoxypsoralen (800 ng/mL), 8-methoxypsoralen (2500 ng/mL), and 5,8-dimethoxypsoralen (1470 ng/mL). By serial dilution, solutions were prepared that contained $^1/_2$ and $^1/_4$ of those concentrations. Instrument calibration solutions were then prepared by adding 50 μL of the surrogate solution and 50 μL of the internal standard solutions described above to 1-mL portions of the calibration solution. Matrix spiking solutions were also prepared that contained all of the components at appropriate concentrations.

Quantification of Target Compounds in the Umbelliferous Vegetable Beverage. To a disposable culture vial were added 10.0 g of the umbelliferous vegetable beverage, 100 μL of a surrogate solution, 10.0 g of potassium carbonate, 2 mL of ethyl acetate, and 100 μL of the internal standard solution. The suspension was vigorously shaken for up to an hour and then centrifuged at 3000 rpm for 10 min. Four phases formed. A small quantity of undissolved potassium carbonate remained in the bottom of the vial. Next was the slightly discolored, clarified beverage. On top of the beverage was a thick interface of semisolid material from the beverage, and on top of the interface was the discolored ethyl acetate phase. A small quantity of the top phase was removed, and an aliquot was analyzed by GC/MS using the conditions specified above. A typical total-ion chromatogram is shown in Figure 1.

Quantification of Target Compounds in the Spiked Umbelliferous Vegetable Beverage. The procedure was conducted as described above except 5 mL of deionized water was used in place of 5 of the 10 g of umbelliferous beverage. In addition, 1 mL of the previously described spiking solution was added in place of 1 of the 2 mL of ethyl acetate.

RESULTS AND DISCUSSION

Identification of Coumarins, Phthalides, and Sesquiterpenes in the Umbelliferous Vegetable Beverage. Large quantities of carrot juice and celery juice were separately extracted with methylene chloride, and the extracts were concentrated to a small volume. Each extract was analyzed using capillary column GC/MS. On the basis of their mass spectra, psoralen, 5-methoxypsoralen, 8-methoxypsoralen, 5,8-dimethoxypsoralen, umbelliferone, butylidene phthalide, 3-*n*-butyl phthalide, sedanenolide, β-caryophyllene, and α-humulene were tentatively identified.

The components tentatively identified as psoralen and umbelliferone were present at such low concentrations that no attempt was made to confirm their presence. A sample of authentic sedanenolide was not available, and so its presence was not confirmed. For the identification of a compound, the mass spectrum of the tentatively identified compound must be essentially identical to that of the mass spectrum of the authentic compound. Al-

Table I. Difference in GC/MS Retention Times of Components and Phenanthrene-d_{10} in Extracts from an Umbelliferous Vegetable Beverage and in Instrument Calibration Standards

component	relative GC/MS retention time difference (Δ scans)			
	sample run	calibration runs		
	1	1	2	3
phenanthrene-d_{10}[a]	0	0	0	0
β-caryophyllene	298	298	298	299
α-humulene	267	267	267	268
3-*n*-butyl phthalide	102	102	102	102
butylidene phthalide	85	85	84	84
8-methoxypsoralen	−182	−183	−185	−184
5-methoxypsoralen	−198	−198	−203	−199
5,8-dimethoxypsoralen	−309	−310	−311	−311

[a] Internal standard.

though some extraneous peaks from the matrix may be present in the mass spectrum of the tentatively identified component, especially when the component is present at low concentration, peaks such as the parent peak, base peak, and key fragment peaks should have similar m/z values and relative intensities. In addition, the GC/MS retention time of a tentatively identified component relative to that of an internal standard should be within 2 scans (i.e., about 2 s) of the GC/MS retention time of the authentic compound relative to that of the same internal standard when the samples are analyzed under the same conditions.

Authentic samples of 5-methoxypsoralen, 8-methoxypsoralen, 5,8-dimethoxypsoralen, butylidene phthalide, β-caryophyllene, and α-humulene were purchased. Comparison of the mass spectrum of each tentatively identified compound (Figures 2a, 3a, 4a, 5a, 6a, and 7a in the supplementary material) with the mass spectum of the corresponding authentic compound (respectively, Figures 2b, 3b, 4b, 5b, 6b, and 7b) shows that a good match is obtained in all cases. Also, the retention times of the components present in the umbelliferous vegetable beverage listed in Table I meet the requirement specified above.

Comparison of the mass spectrum of the tentatively identified compound (Figure 8a in the supplementary material) with that of 3-*n*-butyl phthalide obtained as a gift from the USDA (Figure 8b) shows that a good match is obtained. The retention time of 3-*n*-butyl phthalide that is present in the umbelliferous vegetable beverage meets the requirement specified above.

Quantification of Components in the Umbelliferous Vegetable Beverage. *Calibration of the Gas Chromatograph/Mass Spectrometer.* GC/MS was selected for the analysis procedure because it is a very sensitive technique. In addition, a low concentration of a target compound can be quantified in the presence of a high concentration of an interfering compound if the target compound generates a mass spectral quantification ion that is not produced by the interfering compound. Standard solutions containing the seven identified compounds, three surrogates, and an internal standard were used to calibrate the GC/MS. After an initial analysis of the umbelliferous vegetable extract, new standards were prepared that contained components whose concentrations bracketed the estimated concentrations of the components in the umbelliferous vegetable extract. A three-point calibration curve was then generated for each component. By linear regression analysis with a force of the curve through zero, the typical relative response factors and correlation coefficients shown in Table II were obtained.

Table II. Gas Chromatographic/Mass Spectrometric Relative Response Factors (RRF) for Identified Components in an Umbelliferous Vegetable Beverage

component	mass spectral quantifn ion, m/e	linear regression	
		RRF[a]	corr coeff
5-methoxypsoralen	216	0.239	0.998
8-methoxypsoralen	216	0.293	0.993
5,8-dimethoxypsoralen	246	0.147	0.955
butylidene phthalide	159	0.353	0.990
3-*n*-butyl phthalide	134	0.087	0.999
β-caryophyllene	133	0.104	0.995
α-humulene	93	0.043	0.997
4-chlorobenzophenone[b]	216	0.185	NA[c]
methyl 4-chlorobenzoate[b]	139	0.431	NA[c]
1-chlorodecane[b]	91	0.243	NA[c]

[a] Response factor relative to phenanthrene-d_{10} (m/z 188). [b] Surrogates. [c] A constant concentration of each surrogate was employed.

Table III. Measured Concentration of Identified Components in an Umbelliferous Vegetable Beverage

component	concn, ng/g	component	concn, ng/g
5-methoxypsoralen	86.2	β-caryophyllene	1560
8-methoxypsoralen	194	α-humulene	146
5,8-dimethoxypsoralen	240	4-chlorobenzophenone[a]	107
butylidene phthalide	15.8	methyl 4-chlorobenzoate[a]	189
3-*n*-butyl phthalide	780	1-chlorodecane[a]	120

[a] Surrogates (values represent % recovery).

4-Chlorobenzophenone, methyl 4-chlorobenzoate, and 1-chlorodecane, none of which was detected in the umbelliferous vegetable beverage, were selected as surrogate compounds. The extraction behavior of the surrogates should resemble that of the target compounds because of their structural similarity. Consequently, the recovery of the surrogates should be indicative of the recovery of the target compounds during the analytical procedure. Phenanthrene-d_{10}, which is very stable and produces a mass spectrum with only a few intense peaks, was used as the internal standard. The use of an internal standard generally increases the accuracy of an analysis because the internal standard responds to some extent to instrument fluctuations in the same manner as the target compounds and surrogates. In addition, with an internal standard present, it is not necessary to inject the identical volume of a sample during each GC/MS determination.

Extraction and Analysis of the Umbelliferous Vegetable Beverage. The goal was to generate a convenient method for rapidly but accurately determining the seven target compounds in an umbelliferous vegetable beverage. Two milliliters of ethyl acetate, 100 μL of a solution of the surrogates, and 100 μL of a solution of the internal standards were added to 10 g of the sample. Then 10 g of potassium carbonate was added to reduce the solubility of the target compounds in the umbelliferous vegetable beverage, and the suspension was vigorously shaken for 1 h to equilibrate the phases. Initially, the plan was to add surrogates to the sample prior to extraction and to add internal standards to the isolated extract for quantification. To calculate the concentration of the components in the beverage, the fraction of the organic phase, which presumably contains the components, must be known. However, the determination is complicated by the partial solubility of ethyl acetate in the beverage, the partial solubility of the beverage in ethyl acetate, and the presence of an emulsion that is only partially broken when the suspension is centrifuged. We decided to avoid these complications by adding the internal standard along with the surrogate to the vegetable beverage prior to extraction. That the components, surrogates, and internal standard

Table IV. Recovery of Components Spiked into an Umbelliferous Vegetable Beverage

component	sample concn, ng/g	spiking mix, ng/g	spike A found concn, ng/g	spike A, % recovery	spike B found concn, ng/g	spike B, % recovery	RPD[a]
5-methoxypsoralen	86.2	80	146	118	151	89.9	27
8-methoxypsoralen	194	250	489	75.5	419	80.5	7
5,8-dimethoxypsoralen	240	147	445	140	416	119	15
butylidene phthalide	15.8	60.6	83.0	111	88.2	119	7
3-*n*-butyl phthalide	820	780	1631	104	1535	91.7	13
β-caryophyllene	1560	2000	3505	97.4	3708	107	10
α-humulene	146	206	346	97.2	333	90.8	7
4-chlorobenzophenone[b]	0	808	791	97.9	836	104	5.5
methyl 4-chlorobenzoate[b]	0	790	1327	168	1478	187	11
1-chlorodecane[b]	0	851	796	93.5	889	104	11

[a] RPD, relative percent difference. [b] Surrogates.

Table V. Concentration of Identified Components in an Umbelliferous Vegetable Beverage Measured over a Period of 12 Weeks

component	initial concn, ng/g	12-week av concn,[a] ng/g	% RSD
5-methoxypsoralen	86.2	104	14
8-methoxypsoralen	194	192	9
5,8-dimethoxypsoralen	240	180	11
3-*n*-butyl phthalide	15.8	22.1	8
butylidene phthalide	780	905	21
β-caryophyllene	1560	1513	8
α-humulene	146	177	9

[a] Average of 29 analyses. [b] % RSD, percent relative standard deviation.

are all similarly distributed among the various phases in the suspension and, hence, that accurate results are obtained is suggested by the excellent recovery of matrix spikes as mentioned below. An aliquot of the ethyl acetate top phase was then removed and analyzed by GC/MS.

Analysis of an aliquot of the umbelliferous vegetable beverage gave the results shown in Table III. The recovery of the surrogates is quite good. The results of the analysis of two matrix spikes are shown in Table IV. The recoveries of the target compounds and surrogates are quite good except for that of methyl 4-chlorobenzoate. The recovery of that compound is high because of matrix interference.

Over a period of 12 weeks, new cans of the umbelliferous vegetable beverage were opened and aliquots were analyzed in duplicate or triplicate. A three-point instrument calibration curve was generated each day that samples were analyzed. As shown in Table V, the average of the concentrations of each of the seven components measured during a 12-week period ranged from 75 to 140‰ of the originally determined concentration. Therefore, there is little change in concentration of the seven components during the 12-week period.

Conclusions. Although methodology was developed to detect, identify, and quantify 5-methoxypsoralen, 8-methoxypsoralen, 5,8-dimethoxypsoralen, butylidene phthalide, 3-*n*-butyl phthalide, β-caryophyllene, and α-humulene in an umbelliferous vegetable beverage and the components appear to be stable, the very low concentrations found for the components suggest that the utility of the compounds as compliance marker compounds would be limited.

ACKNOWLEDGMENT

We thank Dr. E. Lund, South Atlantic Area Citrus and Subtropical Products Laboratory, Agricultural Research Laboratory, USDA (Winterhaven, FL), for a solution of 3-*n*-butyl phthalide. Dr. W. C. Coburn, Jr., and Dr. J. M. Riordan, both at Southern Research Institute, performed the NMR analyses. This research was funded by National Cancer Institute Contract N01-CN-05276-01.

Supplementary Material Available: Figures 2–8 showing mass spectra of the compounds (7 pages). Ordering information is given on any current masthead page.

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Received for review April 16, 1992. Revised manuscript received July 15, 1992. Accepted October 6, 1992.